

Hazard/Risk Assessment

Comparison of Alternative Methods for Bioaccumulation Assessment: Scope and Limitations of In Vitro Depletion Assays with Rainbow Trout and Bioconcentration Tests in the Freshwater Amphipod *Hyalella azteca*

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Abstract: Bioaccumulation assessment predominantly relies on the bioconcentration factor (BCF) as the sole decisive metric. The test guideline 305 by the Organisation for Economic Co-operation and Development (OECD) provides the standard procedure for deriving this in vivo fish BCF, which is not only expensive and labor-intensive, but also requires many animals. Accordingly, there is a great need for and interest in alternative methods that can help to reduce, replace, and refine vertebrate tests, as described in the 3R principles. Two alternative approaches have been developed: the bioconcentration test with the freshwater amphipod *Hyalella azteca* and the OECD test guideline 319 which provides a method to determine experimentally derived in vitro metabolism rates that can then be incorporated into in silico prediction models for rainbow trout BCF calculation. In the present study both alternative methods were applied to 5 substances of different physicochemical characteristics. The results were compared with literature values of fish in vivo BCFs and additional BCFs obtained with the alternative methods, if available. Potential differences between the results of the test methods are discussed utilizing information such as in vivo metabolism rates. The currently available data set suggests that these 2 alternative methods pose promising alternatives to predict bioaccumulation in fish, although defined applicability domains have yet to be determined. *Environ Toxicol Chem* 2020;39:1813–1825. © 2020 The Authors. *Environmental Toxicology and Chemistry* published by Wiley Periodicals LLC on behalf of SETAC

Keywords: *Hyalella azteca*; In vitro hepatocyte assay; Biotransformation; Alternative method; Bioconcentration; Risk assessment

INTRODUCTION

The evaluation of the potential for bioaccumulation of chemicals in aquatic organisms is an important component of chemical hazard assessment (Gobas et al. 2009). In a regulatory context, fish is the organism of choice for assessing the

bioaccumulation potential of chemicals in aquatic organisms (de Wolf et al. 2007). The most commonly used parameter to estimate the bioaccumulation potential of chemicals in fish is the bioconcentration factor (BCF). The BCF represents the ratio of the steady-state chemical concentration in the organism and the chemical concentration in the respiratory medium, which in this case is water (Gobas et al. 2009). The standard procedure to determine the BCF for regulatory applications is the flow-through bioconcentration fish test according to the Organisation for Economic Co-operation and Development (OECD) test guideline 305 by the Organisation for Economic Co-operation and Development (OECD 2012). However, this test is not only time-consuming and expensive, but also requires a high number of laboratory animals (>108 fish per test; de Wolf et al. 2007).

This article includes online-only Supplemental Data.

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In vitro metabolism assays using isolated primary hepatocytes or liver S9 subcellular fractions from fish have been introduced as promising and reliable tools to generate hepatic biotransformation rates of xenobiotics, which can be used for in vitro–in vivo extrapolation (IVIVE) of BCFs (Fay et al. 2014b, 2017; Nichols et al. 2018). Standard protocols for the isolation of hepatocytes and S9 fractions from rainbow trout have been developed (Han et al. 2007; Johanning et al. 2012; Fay et al. 2014a), and new OECD test guidelines for the performance of in vitro assays have recently become available (Organisation for Economic Co-operation and Development 2018a, 2018b). Using a substrate depletion approach, in vitro metabolism assays determine the depletion of a test chemical over time. The resulting intrinsic clearance rate values can then be extrapolated to the in vivo whole-body biotransformation rate constant (k_{MET}) of the test compound as part of an IVIVE approach (Nichols et al. 2006, 2013; Cowan-Ellsberry et al. 2008; Fay et al. 2014a). Incorporating such information into established bioaccumulation models for fish was shown to substantially improve their performance, leading to predicted BCF values that are generally closer to measured values from in vivo studies than in silico-based predictions obtained assuming no metabolism (Han et al. 2007; Cowan-Ellsberry et al. 2008; Nichols et al. 2018). In addition to the determination of metabolic rates, the in vitro hepatocyte assay may provide important information on the metabolite patterns of xenobiotics in fish. It has been suggested that the xenobiotic metabolite patterns produced by in vitro fish hepatocyte approaches are generally similar to those observed in vivo (Segner and Cravedi 2000). This was confirmed by Bischof et al. (2016) in a study on rainbow trout and common carp.

An alternative approach to replace fish in bioaccumulation testing would be the use of invertebrate species as test organisms. Invertebrates provide some conceptual advantages because they need less space, have shorter generation times, and may require smaller test setups, which again allows smaller-scale testing with lower substance usage. The freshwater amphipod *Hyalella azteca* was recently suggested as an alternative test organism for bioconcentration studies (Schlechtriem et al. 2019). The authors tested 14 substances of different hydrophobicity (log octanol–water partitioning coefficient [K_{OW}] 2.4–7.6) under flow-through conditions to determine steady-state and kinetic BCFs (BCF_{SS} and BCF_k). Bioconcentration studies with the freshwater amphipod *H. azteca* (*H. azteca* bioconcentration test [HYBIT]) resulted in BCF estimates which showed a good correlation with fish BCF values ($R^2 = 0.69$; Schlechtriem et al. 2019). The BCF values determined with the HYBIT can be used with the standard B criterion ($\text{BCF} > 2000$) and thereby enable the B or non-B classification similarly to the fish test as part of the PBT/vPvB assessment and allow for evaluations regarding the harmonized classification and labeling evaluation (European Commission 2006).

The HYBIT and IVIVE approaches have a high potential to be used as alternative tests to reduce and replace fish in bioconcentration studies. However, biotransformation processes (generally classified as phase I and phase II reactions) can be a key factor affecting bioconcentration. A general comparison of

the methods is thus still possible, although with caution because of potential differences in the metabolism of xenobiotics in fish and crustaceans. Values of BCF calculated for *H. azteca* tended to be higher compared to fish, which might be explained by the limited biotransformation capacity of the amphipods (Schlechtriem et al. 2019). Comparing the metabolite patterns and metabolism rates of the 2 test systems could shed some light on this theory and help to assess the impact of biotransformation processes on the outcome of bioconcentration studies.

The aim of the present study was to determine the bioconcentration of 5 compounds with different chemical structures, hydrophobicity, and speciation using the IVIVE approach and HYBIT. The BCF values obtained with the 2 approaches were compared with corresponding in silico and in vivo fish BCF values from the literature. Investigations on biotransformation in *H. azteca* and rainbow trout hepatocytes were carried out to explain potential differences in the bioconcentration kinetics of the different test compounds. The scope and limitations of the alternative methods, HYBIT and IVIVE, for regulatory bioaccumulation assessment are discussed.

MATERIAL AND METHODS

Chemicals

The test compounds were selected from a set of substances for which a decent set of metabolites has been identified and the respective analytical procedures have been established to facilitate the metabolite analysis as part of the present study. Care was taken to select substances with varying physicochemical characteristics. All test substances (azoxystrobin, terbutryn, prochloraz, diclofenac, trifloxystrobin) were purchased from Sigma-Aldrich. Deuterated internal standards were obtained from Sigma-Aldrich and TRC Canada. A detailed list of the chemicals used in the present study for media preparation, sample processing and analytics, and the respective sources of supply are available in Supplemental Data, SI-A.

Depletion assay (IVIVE), OECD test guideline 319A

Immature specimens of rainbow trout (*Oncorhynchus mykiss*) with an age of 8 to 11 mo and an average body weight of 277 ± 27 g, showing normal behavior, were fasted for 24 h prior to hepatocyte isolation. Primary cells (rainbow trout hepatocytes) were prepared and cryopreserved according to Bischof et al. (2016; see Supplemental Data, SI-B, Text S1). Two hepatocyte lots were prepared for the present study; each contained cells originating from 4 individual fish. One vial of each lot was used in the experiments to exclude variability effects between individual fish with respect to their biotransformation potential. Therefore, the hepatocytes of the 2 vials (one vial per lot) were pooled during the thawing procedure, generating a hepatocyte pool of 8 trout for the working solution.

Preliminary experiments were conducted to optimize the assay conditions. Starting concentrations, solvents for spiking and stopping solutions, and assay durations were optimized to obtain conditions that allow the display of depletion kinetics. First-order kinetics were assumed, when linear regression showed a high degree of correlation for the log concentration values (Organisation for Economic Co-operation and Development 2018a). The combination of start concentration and time period with the highest first-order depletion rates was selected for the main test. The general experimental procedure of the preliminary experiments and the following main tests were almost identical. Experimental conditions including starting concentrations and incubation time were identified in the preliminary tests as described in Supplemental Data, SI-B, Text S2. In each depletion assay 1 mL of a prepared working solution was transferred into a loosely capped 7-mL glass scintillation vial. Cells were adapted to the incubation conditions in a climate-controlled rotary shaker (Thermo Scientific, MaxQ™ 4000) for 10 min. The shaker was set to the fish rearing temperature of 11 °C and a gentle shaking speed (100 rpm). At the onset of the incubation period, the working solution was spiked with 5 µL of the solvent stock containing the test substance dissolved in acetonitrile or methanol, resulting in 0.5% solvent in the assay (Supplemental Data, SI-B, Tables S2 and S4). Throughout the incubation period, 100-µL samples were taken from the vial and transferred into 400 µL ice-cold methanolic stopping solution containing 12.5 µg/L internal standard. Eight samplings were carried out during one incubation period. Samples were vortexed (2300 rpm, 10 min) and centrifuged (20 000 g, 10 min, 4 °C), and a 250-µL aliquot of the supernatant was transferred to a high-performance liquid chromatography vial with insert. Storage was at –20 °C until analysis. Each of the 5 chemicals was tested in 3 different runs carried out on 3 different days. Samples for the identification of the metabolite patterns were prepared in a similar way, with the following adaptations: A starting concentration of 2 µM (azoxystrobin = 806.8 µg/L, diclofenac = 592.3 µg/L, prochloraz = 753.3 µg/L, terbutryn = 482.7 µg/L, trifloxystrobin = 816.7 µg/L) and the maximum recommended incubation period of 4 h were applied to the assays in accordance with Bischof et al. (2016). No intermediate samples were taken during the incubation period. The entire assay was stopped after 4 h by the addition of 4 mL stopping solution. This increased the resulting sample mass and the amount of metabolites for detection. In all assays, negative controls were run in parallel using heat-inactivated hepatocytes at a concentration of 2×10^6 cells/mL. In this way abiotic reduction of the test substance could be monitored.

HYBIT

Aqueous exposure bioconcentration tests with *H. azteca* were carried out to estimate the bioaccumulation potential of the 5 tested chemicals. All *H. azteca* were obtained from an in-house culture. Animals were raised as described by Schlechtriem et al. (2019). Only male amphipods with an age >2 mo were used, approximately 1200 amphipods were needed per test (Supplemental Data, SI-D, Text S5).

Substance toxicity was evaluated based on published data on previously conducted bioaccumulation studies or on data from chronic toxicity tests on *H. azteca* or other aquatic invertebrates (e.g., Morrison et al. 2013; Fu et al. 2018). All tests were conducted under flow-through conditions (apparatus details in Supplemental Data, SI-D, Text S7) and consisted of 2 phases: the exposure (uptake) and postexposure (depuration) phases. The flow-through system was equilibrated for 2 to 3 d prior to the test start to ensure stable exposure conditions, supported by analytical controls. Bioconcentration of prochloraz, terbutryn, and trifloxystrobin was additionally assessed using a semistatic approach for comparative reasons; details are given in Supplemental Data, SI-D, Text S8. In both exposure systems the water was continuously aerated via a glass capillary. A 16:8-h light:dark regime was applied, and the water temperature was kept in a range of 25 ± 2 °C in accordance with the rearing conditions. Meshed steel shelters were placed into the aquarium to reduce stress on the amphipods. The *H. azteca* were fed on a daily basis with decomposition and consumption tablets (DECOTABs; Kampfraath et al. 2012), which were prepared according to a slightly modified protocol (see Supplemental Data, SI-D, Text S6). The suitability of the modified DECOTABs was tested beforehand in a short preliminary study (Supplemental Data, SI-C, Text S3).

The duration of the exposure and depuration phases for each of the substances was estimated based on results obtained from previous studies with substances of similar log K_{OW} , and sampling points were scheduled accordingly (Supplemental Data, SI-D, Tables S12 and S13). At every sampling point triplicate samples were collected using a small dip net, each containing 20 amphipods. Amphipods were rinsed with purified tap water, shortly blotted on lint-free laboratory paper (Kimtech), weighed (Shimadzu AUW220D), and frozen at –20 °C. Additional triplicate samples for lipid analysis, each replicate consisting of 10 amphipods, were taken at the onset and end of the exposure phase, as well as at the end of the depuration phase. Lipid content determination was carried out as described by Schlechtriem et al. (2019; Supplemental Data, SI-D, Tables S14 and S15). For metabolite analyses an additional triplicate sample was collected at the end of the exposure phase. In this case, each replicate consisted of 30 amphipods to generate more biomass because some metabolites were suspected to occur in low concentrations. With the onset of the depuration phase, remaining amphipods were transferred into a new aquarium filled with purified tap water instead of test solution, and sampling was continued as described before (Supplemental Data, SI-D, Tables S12 and S13). Animals which were sampled for tissue analysis during the semistatic experiments were also used for metabolite identification, followed by the calculation of *H. azteca* biotransformation rates as described below (see *H. azteca* biotransformation rates).

During the flow-through and semistatic approach, experimental conditions (temperature, pH, and concentration of dissolved oxygen) were checked daily. Water quality parameters (nitrite, nitrate, ammonium) were measured at the onset and at the end of the uptake and depuration phases. During

the exposure phase, water samples (10 mL) were collected daily to measure the substance concentration of the test solution. An additional water sample was collected at the onset of the depuration phase to confirm that no major substance carryover into the clean vessel occurred. Details on water concentrations for all experiments are collected in Supplemental Data, SI-K.

HYBIT—Effect of biomagnification processes

As mentioned previously (see *HYBIT* section), *H. azteca* were fed during the experiments. In theory it is possible that the dissolved test substance in the water adsorbs to the administered food. Food contaminated in this way could lead to biomagnification of the chemical, elevating the body burden of the test substance in the amphipods, which could explain the overestimation of *HYBIT* BCFs compared to fish data. To exclude this possibility, supplementary investigations were conducted with ^{14}C -radiolabeled methoxychlor, which is a very hydrophobic compound ($\log K_{\text{OW}}$ of 5.08). In short, different food options were equilibrated in the test solution and fed to *H. azteca*, and the results were compared to data from a previous *H. azteca* bioconcentration study with ^{14}C -methoxychlor (Schlechtriem et al. 2019). Analysis of tissue and food was based on total radioactivity determination to provide a worst-case scenario point of view. Details are provided in Supplemental Data, SI-C, Text S4.

Chemical analysis

Water samples collected during the studies were analyzed without further sample processing. Each water sample (10 mL) was added to 2 mL of methanol in a glass vial and shaken by hand. Samples were measured immediately or stored at -20°C until analysis. If dilution was necessary, a 5 + 1 v/v solution of methanol and ultrapure water was used.

The *H. azteca* samples were processed by solid/liquid extraction using methanol to determine the concentration of the accumulated test substances. Samples were spiked with 25 μL of the respective internal standard solution (400 $\mu\text{g/L}$ in methanol) and 4 mL pure methanol. Homogenization was performed using an Ultra-Turrax for 30 s, then by placing the samples in an ultrasonic bath for 10 min, and finally via vortexing for 30 s. Sample extracts were obtained by centrifugation of the samples for 6 min at 4700 g (Heraeus Megafuge 16R). Hepatocyte supernatants collected during the *in vitro* depletion assays were ready to be measured without further processing.

Aqueous samples, hepatocyte supernatants, and *H. azteca* extracts were analyzed for substance concentrations. All test substances were analyzed via liquid chromatography tandem mass spectrometry (LC-MS/MS) in positive electrospray ionization mode. The LC system (Waters Acquity UPLC system) used was coupled to a Waters TQD triple quadrupole mass spectrometer. Chromatographic separation of the samples was done on a Waters Acquity UPLC BEH C18 column with the dimensions 1.7 μm , 100 mm \times 2.1 mm. Stable isotope-labeled internal standards of the test substances were used as described

previously (see *Chemicals* section). The injection volume of each sample was 10 μL . Instrument settings applied for each substance are available in Supplemental Data, SI-I, Text S14.

Metabolite identification

Hepatocyte supernatants for metabolite identification were produced in the same manner as described previously (see *Depletion assay [IVIVE]*, *OECD test guideline 319A* section) for the depletion assay. The *H. azteca* samples collected at the end of the flow-through bioconcentration studies for metabolite pattern identification were mixed with 100 μL of methanol containing an isotope-labeled internal standard (100 $\mu\text{g/L}$) corresponding to the respective test compound, 500 μL of pure methanol, and 300 mg of 1-mm zirconia/silica beads (BioSpec Products). Samples were homogenized with a FastPrep bead beater (MP Biomedicals) in 2 cycles of 15 s at 6 m/s (cooling on ice in between). The homogenate was centrifuged (10 000 rpm \times 6 min, 20°C) and filtered through 0.45- μm regenerated cellulose filters (BGB Analytic). Filters were washed with 400 μL methanol, and the filtrate was combined with the wash solution.

Supernatants of fish hepatocytes and *H. azteca* samples were analyzed by online solid phase extraction coupled to reversed-phase LC high-resolution MS/MS (LC-HRMS/MS; Q Exactive; Thermo Fisher Scientific) as described by Fu et al. (2018). Detection was full scan acquisition with a resolution of 70 000 (at m/z 200) in polarity switching mode (electrospray ionization) followed by 5 (positive mode) and 2 (negative mode) data-dependent MS/MS scans with a resolution of 17 500 (at m/z 200) with an isolation window of 1 m/z . The mass lists of potential biotransformation products (BTPs) used for triggering data-dependent MS/MS scans were obtained from the literature and *in silico* prediction. More details about the analytical procedure are provided in Supplemental Data, SI-I, Text S16.

To identify BTP candidates, suspect and nontarget screening was performed by analyzing the acquired HRMS/MS raw data using Compound Discoverer software 2.1 (Thermo Scientific). Only peaks with a 3-fold higher peak intensity in the samples with substance compared to the control without substance were further evaluated. Structure elucidation was based on the interpretation of the exact mass (± 5 ppm) and the isotopic pattern to assign molecular formulas and of MS/MS spectra to identify diagnostic fragments or losses characteristic for one specific structure or for several positional isomers. Reference compounds were only available for a few BTPs; therefore, the identification of all other BTPs remains tentative with a confidence level of 2 (diagnostic fragments point to one distinct structure) or 3 (several positional isomers possible) according to the classification by Schymanski et al. (2014).

IVIVE—Estimation of *in vitro* BCF

Measured concentrations in primary hepatocyte suspensions collected during the depletion assays were log-transformed and plotted against time. A linear regression was performed to describe the linear relationship between the

log-transformed concentration and time to derive the depletion rate (slope of regression line). The depletion rate was multiplied by 2.3 and corrected for the applied cell concentration, which was determined before each experiment (Organisation for Economic Co-operation and Development 2018c). The resulting in vitro intrinsic clearance rate ($CL_{INT, IN VITRO}$) was used for IVIVE calculations to derive in vitro BCF estimates, which were carried out according to the guidance document associated with OECD 319A/B (Organisation for Economic Co-operation and Development 2018c). Log K_{OW} values were obtained from EPI Suite™ with the exception of diclofenac because the EpiSuite value is not based on the anionic state.

HYBIT—Calculation of BCFs

All BCF calculations were performed in accordance with OECD test guideline 305, Annex 5 (Organisation for Economic Co-operation and Development 2012). Calculated metrics comprise BCF_{SS} , BCF_k , the time-weighted average (TWA) of the water concentration during the uptake phase, and the average lipid content of *H. azteca* in the experiments (Supplemental Data, SI-D, Tables S14 and S15). The latter was used to normalize the BCFs to a 5% body lipid content, as done in the OECD test guideline 305 for the fish BCFs to facilitate comparison across experiments. Uncertainties of the calculated BCFs were calculated applying the general law of propagation of errors without consideration of covariances (Mandel 1984; Schlechtriem et al. 2019). To standardize the calculation of BCFs, the guidance document for OECD test guideline 305 suggests application of the R package *bcmfR* (Organisation for Economic Co-operation and Development 2016). The HYBIT data were evaluated with the *bcmfR* package in addition to the sequential method as described in OECD test guideline 305. In this way it was evaluated whether the R package is suitable for the HYBIT as well.

H. azteca biotransformation rates

Biotransformation rates of *H. azteca* for the different test compounds were determined based on metabolite concentrations measured in *H. azteca* samples collected during the semistatic bioconcentration studies. Concentration data for the parent substance and the sum of first-phase and the sum of second-phase metabolites were each fitted to a first-order one-compartment model, as described in Fu et al. (2018). The *H. azteca* in vivo metabolic rates were compared to the extrapolated metabolic rates for fish, derived in the IVIVE procedure. The *H. azteca* in vivo metabolic rate most suitable for comparison with hepatocyte depletion data is the rate of first-phase metabolism, reflecting the degradation rate of the parent substance. The in vivo intrinsic clearance rate ($CL_{IN VIVO, INT}$) generated in the IVIVE process was compared with the *H. azteca* in vivo rate for first-phase metabolism. A quotient of both rates was formed.

Literature research and comparison of BCF data

A literature search utilizing search engines such as Google Scholar and Web of Science was conducted to compile a set of BCFs, preferably from in vivo fish studies that were conducted in accordance with OECD test guideline 305. Keywords were “bioconcentration,” “fish,” “BCF,” and “OECD” and the respective substance names and Chemical Abstracts Service (CAS) numbers. A regular Google search with the mentioned keywords was performed as well to cover additional sources of information. This set of data served as a comparative basis for both the BCFs derived via the HYBIT and the fish in vitro hepatocyte approach (Supplemental Data, SI-G and SI-H). In addition, BCFs for all test substances were retrieved from the EPI Suite software (US Environmental Protection Agency 2012) to provide a data set of in silico BCF values. In a further step, the HYBIT and fish BCF data set from Schlechtriem et al. (2019) was taken and added to the data generated in the present study. To enhance the collection of IVIVE BCF values, the literature was scanned for in vitro-based BCF estimations of substances that have already been tested for their bioaccumulation behavior in *H. azteca*, to complement the available data (Supplemental Data, SI-H). Similar to Schlechtriem et al. (2019), fish and HYBIT BCFs were evaluated to establish a correlation using a linear regression analysis (Origin 2018; OriginLabs).

RESULTS AND DISCUSSION

Depletion assay (IVIVE)

The hepatocyte depletion assays were conducted with the experimental conditions that have been established in the preliminary experiments that are summarized in Supplemental Data, SI-B, Table S4. Average cell viability and cell concentration in the assays were monitored and averaged at $\geq 80\%$ and approximately 2×10^6 cells/mL, respectively (Supplemental Data, SI-B, Table S3). The resulting kinetics are presented in Figure 1. Terbutryn was readily degraded by 90 to 95% within 45 min (first-order kinetics), and 63 to 70% of azoxystrobin was depleted within the same period. Trifloxystrobin was degraded by 70 to 75% within 1 h of incubation. Although almost 50% of the initial amount of diclofenac was depleted in the preliminary tests after 4 h, unexpectedly only 21 to 23% was depleted under identical conditions during the main tests. Prochloraz produced a slow and unusual linear depletion characteristic, leading to 25 to 40% reduction of the initially dosed substance over 4 h of incubation. With the current knowledge it can only be speculated why the observed differences occurred.

IVIVE BCF extrapolation

The spreadsheet provided with the draft OECD 319A guidance document was used to extrapolate BCFs from the depletion data gathered in the in vitro experiments. Two different settings are available concerning the binding term f_u , which corrects for binding effects in vitro and in plasma. This value can either be modeled or set to 1, the latter resulting in empirically more realistic results in case of slowly metabolized chemicals (Cowan-Ellsberry et al. 2008; Escher et al. 2011; Laue

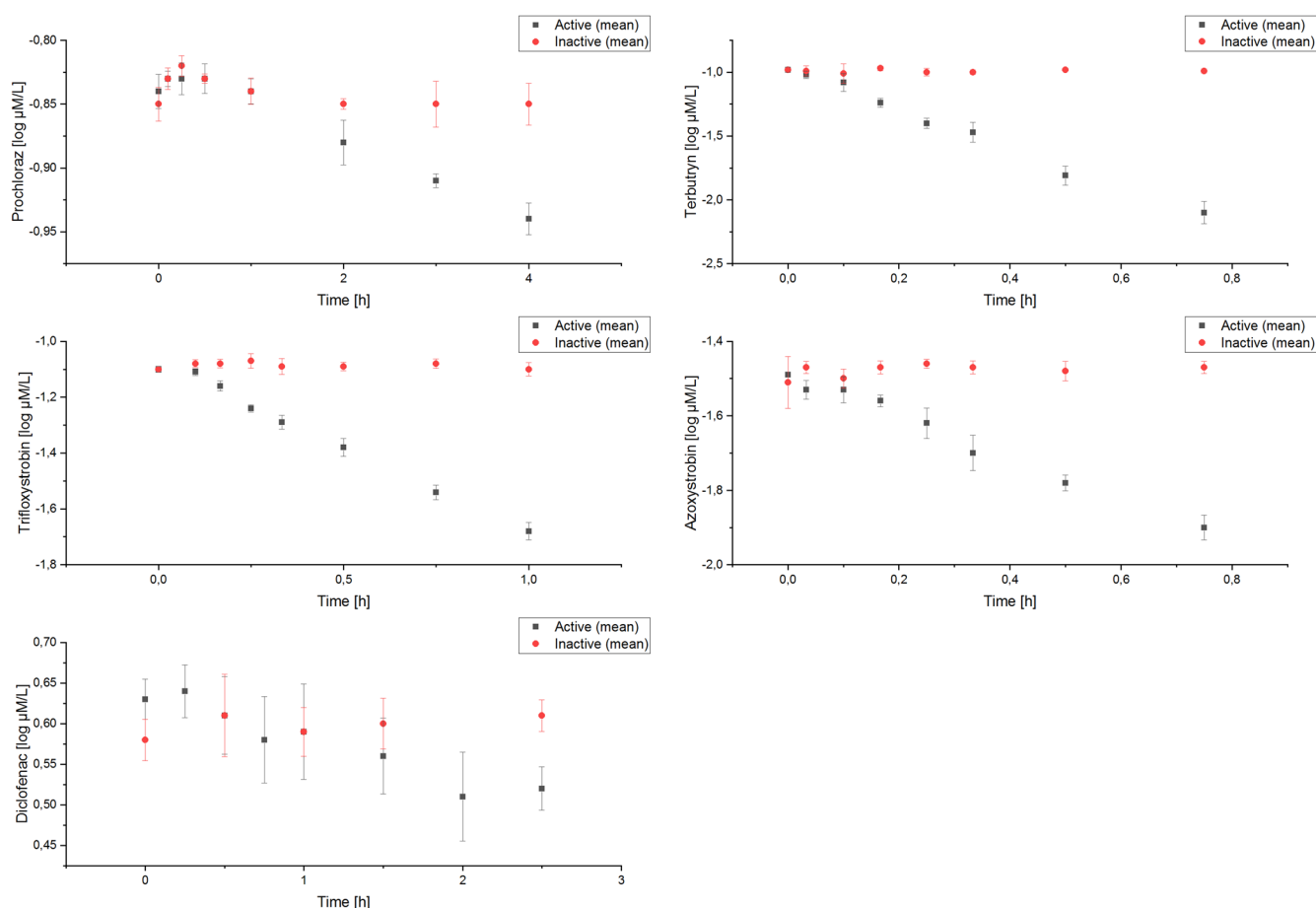


FIGURE 1: Kinetics of the in vitro depletion assays with rainbow trout hepatocytes. Each data point reflects the mean of 3 runs; error bars display the standard deviation. Data of the active treatments were obtained using living hepatocytes at a concentration of 10^6 cells/mL; inactive runs were conducted using heat-inactivated hepatocyte material and served as a negative control to monitor the abiotic disappearance of the test substance.

et al. 2014; Organisation for Economic Co-operation and Development 2018c). It is suggested that both settings are applied during the extrapolation procedure to receive upper and lower limits based on hepatic clearance; this was done, and the results are presented in Table 1 (Nichols et al. 2013; Organisation for Economic Co-operation and Development 2018c). Additional IVIVE BCFs for BaP, methoxychlor, PCB 153, and pyrene were obtained from the literature (see Supplemental Data, SI-H). The BaP and PCB IVIVE BCFs were calculated with extrapolation models that are described in Han et al. (2007) and Trowell et al. (2018). These can differ from the one in the OECD test guideline 319, which is based on the model described in Nichols et al. (2013). The resulting BCF extrapolations correlate with the $\log K_{OW}$ of the substances, which was expected with respect to the applied extrapolation model assuming K_{OW} -based partitioning as the primary driving force of the accumulation process (Han et al. 2008; Nichols et al. 2013; Trowell et al. 2018).

Research needs of the IVIVE method

The results obtained in the present study confirm that, depending on the test substance, the setting of the f_u factor can

have a significant influence on the extrapolated BCF value. With the applied f_u settings BCF predictions are in the range of the fish BCF values or tend to over predict them. The proper settings for the binding factor f_u are currently under discussion as a known source of uncertainty in the IVIVE process, which is mentioned in the guidance document (Organisation for Economic Co-operation and Development 2018c). The extrapolation process as a whole has received much recognition, and different improvements are being proposed (Lee et al. 2017; Krause and Goss 2018; Trowell et al. 2018; Saunders et al. 2019). Furthermore, some alternative approaches are in development that, for example, take the substance's sorption to different biological matrices into consideration (Krause and Goss 2018). A combination of information from different modeling approaches could lead to a more holistic insight into the bioconcentration mechanisms in future applications. The use of benchmarking substances is a proposed way to monitor the differences obtained when altered modeling settings are applied and to control the quality of the depletion assay. It should be kept in mind, however, that a benchmark substance can only represent the metabolic pathways involved in its own degradation. Another source of uncertainty in the currently applied extrapolation method is the neglect of metabolically important processes, such as

TABLE 1: Bioconcentration factor (BCF) values determined for azoxystrobin, prochloraz, terbutryn, diclofenac, and trifloxystrobin utilizing 2 alternative bioconcentration test setups, HYBIT and IVIVE^a

Substance	Normalization	Log K_{OW}	IVIVE BCF		HYBIT (flow-through)		Fish BCF (literature)	QSAR EPI Suite
			$f_u = 1$	$f_u = \text{mod.}$	BCF_k	BCF_{SS}		
Azoxystrobin	Non-normalized	2.5 ^b	N/A	N/A	4	3	N/A	21
	Lipid-normalized		12	14	9	6		
Prochloraz	Non-normalized	4.1 ^b	N/A	N/A	97	94	196–371 ^c	236
	Lipid-normalized		366	1140	308	299		
Terbutryn	Non-normalized	3.74 ^b	N/A	N/A	37	37	13.3 ^d	41
	Lipid-normalized		73	133	78	76		
Diclofenac	Non-normalized	0.7 ^e	N/A	N/A	1.36	N/A	2–5 ^f 3–9 ^f	3
	Lipid-normalized		0.19	0.2	3	N/A		
Trifloxystrobin	Non-normalized	4.5 ^b	N/A	N/A	393	354	370–542 ^g	727
	Lipid-normalized		175	725	947	852		

^aReference BCF values in fish were retrieved from the literature, QSAR values are based on EPI Suite predictions.^bEPI Suite experimental value.^cEuropean Food Safety Authority (2011).^dTarja et al. (2003).^eChemistry Dashboard entry for diclofenac sodium salt, experimental value.^fMemmert et al. (2013).^gJackson et al. (2009).HYBIT = *Hyalella azteca* bioconcentration test; IVIVE = in vitro–in vivo extrapolation; K_{OW} = octanol–water partitioning coefficient; mod. = modeled; N/A = not available; QSAR = quantitative structure–activity relationship.

extrahepatic metabolism at the gills or in the digestive system (Pedersen and Hill 2000; Nichols et al. 2007).

The in vitro assay used to derive depletion data incorporates strict validity criteria to ensure high-quality input data for the extrapolation process (Organisation for Economic Co-operation and Development 2018a). Two of the substances tested in the present study, prochloraz and diclofenac, expressed an unusual depletion behavior; and the experiments would not be considered valid according to the standards set in the OECD guideline. However, because of its use as a pharmaceutical, diclofenac has been tested in different in vitro studies including assays using rainbow trout S9 fractions (Connors et al. 2013) and hepatocyte spheroids (Baron et al. 2017). Converting our $CL_{INT, IN VITRO}$ rate for diclofenac to be expressed on a milliliters per hour per gram liver basis and assuming a hepatocellularity number of 510×10^6 cells/g liver (Nichols et al. 2013; Organisation for Economic Co-operation and Development 2018c), a depletion rate of 31.11 is derived. This is higher than the one obtained from trout S9 fractions of 9.5 (Connors et al. 2013) but lower than the one obtained from trout hepatocyte spheroids of 49.8 (Baron et al. 2017). This implies that the depletion rate calculated in the present study for diclofenac in rainbow trout hepatocytes is comparable to the rates for diclofenac determined in other hepatic trout in vitro systems (Connors

et al. 2013; Baron et al. 2017), even though it would not have been considered valid in the guideline. To estimate IVIVE BCFs for ionic compounds such as diclofenac, updated extrapolation models which are not based on the hydrophobicity of the tested chemicals will be necessary.

In the case of prochloraz, the observed depletion kinetic was not in agreement with the first-order characteristic, which is needed for the extrapolation model (Organisation for Economic Co-operation and Development 2018c). The results of the BCF extrapolation for prochloraz showed the broadest range of predicted BCFs (366–1140) of all substances tested in the present study. All $CL_{IN VIVO, INT}$ rates extrapolated from the hepatocyte depletion rates were compared to modeled in vivo first-phase metabolism rates of *H. azteca*. The extrapolated rates from the IVIVE system are approximately 50-fold higher than the in vivo first-phase metabolism rates of *H. azteca*, as shown in Table 2. However, the $CL_{IN VIVO, INT}$ rate of prochloraz indicated a comparatively low capacity for fish to metabolize this substance. It is possible that the extrapolation procedure delivered unrealistic results because of the atypical linear depletion kinetics observed for prochloraz. Because the linear depletion took place independently of the starting concentration and incubation duration, the possibility of a saturated enzyme system as the cause for the linear depletion can be excluded (Organisation for Economic Co-operation and Development 2018c). Generally,

TABLE 2: Metabolism rates determined for *Hyalella azteca* and extrapolated as part of the IVIVE process for the substances azoxystrobin, prochloraz, terbutryn, and trifloxystrobin^a

Rate type	Units	Azoxystrobin	Prochloraz	Terbutryn	Trifloxystrobin
In vitro intrinsic clearance ($CL_{INT, IN VITRO}$)	d^{-1}	0.589	0.045	1.641	0.689
In vivo intrinsic clearance ($CL_{INT, IN VIVO}$)	l/d/kg fish (or mL/d/g fish)	107	8	297	126
<i>H. azteca</i> first-phase metabolism rate ($k_{m, 1st \text{ total}}$)	L/d/kg _{ww}	1.8 (1.4–2.1)	1.6 (0.5–4.4)	6.1 (5.3–7.1)	1.8 (1.4–2.2)
Metabolism rate ratio fish/ <i>H. azteca</i>	Unitless	59	5	49	69

^aThe *H. azteca* data for azoxystrobin and prochloraz were taken from Fu et al. (2018), and kinetic metabolite data for diclofenac were not retrieved. IVIVE = in vitro–in vivo extrapolation.

further investigations with a broader range of compounds are required to explore the options and limits of the IVIVE concept in a more conclusive manner. Nevertheless, the IVIVE BCF estimation via rainbow trout hepatocytes delivers plausible result ranges for lipophilic organic substances, as shown in the present study.

Bioconcentration in *H. azteca* (HYBIT)

All HYBIT experiments provided clear uptake and depuration kinetics and low standard deviations in the triplicate tissue samples measured at the different sampling points, as depicted in Figure 2. Nominal concentrations in the exposure media could be maintained during the exposure phases within a $\pm 20\%$ range of the TWA. Uptake and depuration behavior of the test substances were different, as expected in view of their specific hydrophobicity ($\log K_{OW}$ range). Steady-state conditions were reached in all experiments but diclofenac (Figure 2), as confirmed by similar kinetic and steady-state BCFs (Table 1). In the case of diclofenac, the uptake phase was obviously too short to reach stable steady-state conditions. In all experiments, the determined water quality parameters did not reveal any deviations from the ideal range, indicating that the experimental conditions were acceptable (Supplemental Data, SI-K).

Lipid-corrected kinetic BCFs (BCF_{KL}) for azoxystrobin, terbutryn, prochloraz, and trifloxystrobin are 6, 78, 308, and 947, respectively. Lipid contents average at 2.2% (1.6–2.7%); details are listed in Supplemental Data, SI-D, Tables S14 and S15. For diclofenac, a BCF_K of 1.36 was obtained. Because diclofenac is anionic at the pH in the test solution, a lipid correction is not an appropriate normalization procedure. An evaluation of the HYBIT data was also performed with the *bcmfR* package, a standardized tool for the evaluation of OECD test guideline 305 data (Organisation for Economic Co-operation and Development 2016) to test the applicability of this tool for HYBIT studies. The obtained BCFs are similar to the ones calculated in the sequential method (Supplemental Data, SI-D, Text S11). The fitting of the flow-through data for azoxystrobin and diclofenac was not as good on a visual basis as for the other 3 substances, and a 2-compartment depuration model was thus applied, resulting in a visually better fit but still producing comparable BCF results (Supplemental Data, SI-D, Text S10).

Comparison of HYBIT BCFs of semistatic and flow-through exposures

Compared to their flow-through counterpart, the concentration profile in the semistatic experiment for prochloraz was almost identical. However, in the case of terbutryn and trifloxystrobin, differences were visible in their respective concentration profiles. The calculated BCF_{KL} values are approximately half of the values derived under flow-through conditions. Interestingly, the uptake rate of terbutryn measured in the semistatic approach was approximately halved, whereas the depuration rate was almost identical to the one obtained in

the flow-through experiment. Therefore, the reduced uptake rate was obviously the sole source of the observed deviation. Further investigations are required to elucidate potential differences between the semistatic and flow-through approaches. In the case of trifloxystrobin, the water concentration was 3 times higher in the semistatic approach compared to the flow-through test, which might explain the observed differences. The measured trifloxystrobin body burden in *H. azteca* at steady state under semistatic conditions was 2 to 3 times higher in comparison to the flow-through approach. Trifloxystrobin is known to have a high toxicity in *H. azteca* (Morrison et al. 2013), and although the exposure concentration in the semistatic approach corresponds to only 15% of the 96-h 10% lethal concentration (LC10), it cannot be excluded that subtoxic effects might have altered the uptake and depuration kinetics. When errors are considered in the comparison of BCFs from both exposure scenarios, the resulting $\log BCF_{KL}$ values do not differ considerably, as shown in Figure 3; the detailed metrics of all HYBIT BCFs can be found in Supplemental Data, SI-D, Table S16.

The flow-through BCF studies carried out as part of the present study showed that the HYBIT test system is robust. A few modifications, such as feeding the test animals with DECOTABs during the bioconcentration studies or the use of meshed steel shelters in the aquarium to reduce stress on the amphipods, have been applied to further improve the test procedure described by Schlechtriem et al. (2019).

Comparison of BCFs

The currently applied IVIVE methods do not deliver BCFs with a distinct tendency toward over- or underprediction of BCFs compared to in vivo fish values. Comparing the IVIVE extrapolations with the in silico predictions of the BCFBAF™ model (EPI Suite), it can be seen that the EPI Suite predictions tended to be higher or at the upper prediction limit of the IVIVE methods, with the exceptions of terbutryn and prochloraz. This supports the statement that the incorporation of experimentally derived metabolism rates leads to more realistic BCF estimations as shown for the substances evaluated in the present study. However, it needs to be considered that also fish in vivo BCF values are always subject to variation, and different experiments can result in varying BCFs, especially where different test species are used (Schlechtriem et al. 2019).

The HYBIT BCF_{KL} values are in good agreement with the respective fish BCF values, as demonstrated by a good correlation ($R^2 = 0.7215$) of all of the data available (Figure 4). However, because it is not always clear whether the BCFs are lipid-normalized or not or whether they are based on total radioactivity measurements, this comparison could have some flaws. The lipid-corrected HYBIT BCF_{KL} values tend to be higher in comparison to the fish BCFs, especially in the case of more hydrophobic chemicals, where this also applies in comparison with the IVIVE, as shown in Figures 4 and 5. Schlechtriem et al. (2019) concluded that *H. azteca* BCFs pose a promising alternative for obtaining fish BCFs: "BCF values calculated for

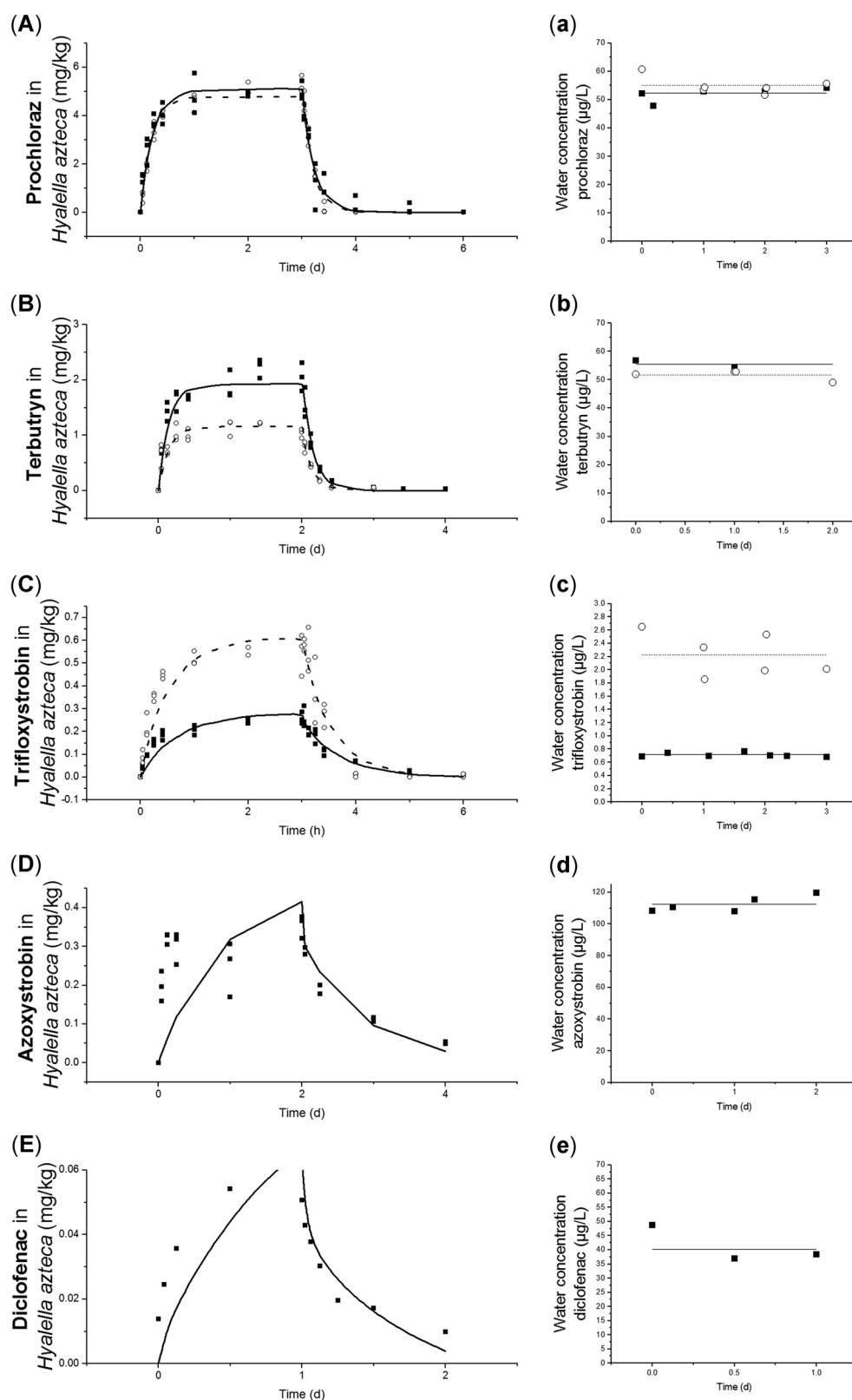


FIGURE 2: Determined concentrations in the flow-through *Hyalella azteca* bioconcentration test experiments. (Left) Uptake and depuration kinetics for the 5 test substances in *H. azteca* under flow-through (black squares and solid black lines) and semistatic (open circles and dashed lines) exposure conditions. (Right) Water concentration during the uptake phases of the 5 bioconcentration tests with *H. azteca* under flow-through (black squares and solid black lines) and semistatic (open circles and dashed lines) exposure conditions.

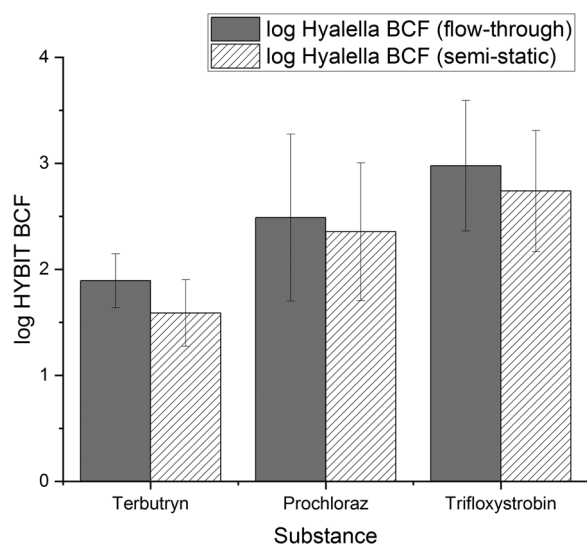


FIGURE 3: Comparison of log bioconcentration factor values derived from *Hyalella azteca* bioconcentration tests with terbutryn, prochloraz, and trifloxystrobin under flow-through and semistatic exposure scenarios. Error bar represents BCF errors determined using the general law of propagation of errors. BCF = bioconcentration factor; HYBIT = *Hyalella azteca* bioconcentration test.

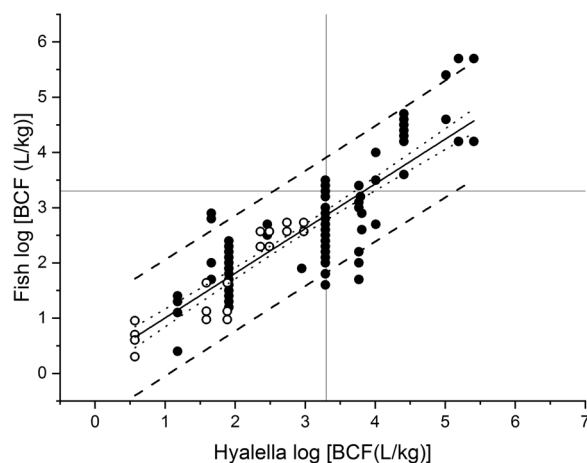


FIGURE 4: Comparison of log *Hyalella* bioconcentration factors (BCFs) and log in vivo fish BCFs found in the literature. Solid black circles represent data taken from Schlechtriem et al. (2019); open circles show data collated in the present study. All in vivo fish BCF values were taken from Schlechtriem et al. (2019). A linear regression was performed; the resulting equation $y = 0.809x + 0.19373$ with an R^2 of 0.7215 is displayed by a solid black line. The dotted lines frame the 95% confidence interval; the dashed lines frame the 95% prediction band. A central gray cross marks the regulatory threshold, BCF = 2000.

H. azteca tend to be higher compared to fish leading to a type I error falsely inferring the existence of a high bioaccumulation potential for a chemical in fish (BCF > 2000) that is not there. False positive findings are of minor concern from a regulatory perspective but should still allow for an appropriate assessment based on predicted fish BCF estimates." The results of the present study add information for less hydrophobic chemicals to the data pool and extend the basis for further comparisons. The statement that lipid-normalized *H. azteca* BCFs provide a

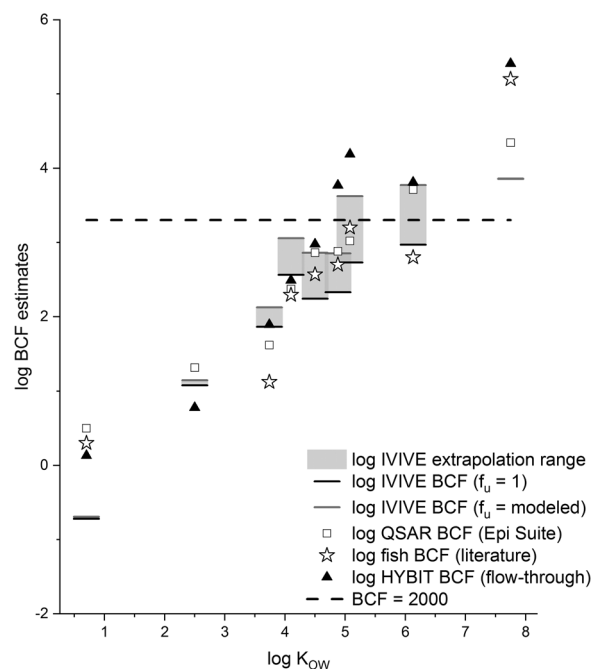


FIGURE 5: Bioconcentration factor (BCF) comparison: *Hyalella azteca* bioconcentration test (HYBIT) versus in vitro–in vivo extrapolation (IVIVE). The BCF values were retrieved from the 2 alternative test systems, HYBIT and IVIVE, using rainbow trout hepatocyte depletion assays (Organisation for Economic Co-operation and Development test guideline [OECD TG] 319A). Log octanol–water partitioning coefficient (K_{OW}) values of the substances are diclofenac = 0.7, azoxystrobin = 2.5, terbutryn = 3.66, prochloraz = 4.1, trifloxystrobin = 4.5. Additional in vitro BCF extrapolations were found in the literature for the following substances (log K_{OW} value in parentheses): pyrene (4.93), methoxychlor (5.67), benzo[a]pyrene* (6.11), PCB 153* (7.75). Corresponding fish and HYBIT BCF values were taken from Schlechtriem et al. (2019). In vitro BCF extrapolations for diclofenac and prochloraz should be taken with caution because the assays showed deviations from the guideline demands. (*The IVIVE extrapolation range was determined with a non-OECD test guideline 319A model. See Supplemental Data SI-H, Table S42 for details.) QSAR = quantitative structure–activity relationship.

sufficiently conservative prediction for bioaccumulation in fish (Schlechtriem et al. 2019) was confirmed by the BCF data obtained in the present study. To address the source of the higher observed values that are present in higher-log K_{OW} substances, different aspects were analyzed in further detail.

Investigation of sources of BCF differences: Effect of food contamination on BCF estimates

One explanation for the higher BCF_{KL} values is potential biomagnification processes. During the bioconcentration test *H. azteca* is fed with an uncontaminated diet, raising the concern that the hydrophobic test substance adheres to the food and is then ingested by dietary uptake. This could potentially elevate the body burden and thus lead to higher BCFs as a result of combined bioconcentration and biomagnification processes. However, supplementary investigations with ^{14}C -methoxychlor demonstrated that this concern is negligible. Even the worst-case perspective utilizing the ^{14}C -radiolabel

could not identify any meaningful transfer of substance into the amphipods. Accordingly, we can rule out that obtained HYBIT BCFs are influenced by biomagnification processes.

Investigation of metabolite patterns of *H. azteca* (in vivo) and rainbow trout (in vitro)

The most prominent explanation for the conservative nature of *H. azteca* BCF predictions in comparison to fish is the assumption that aquatic invertebrates express a lower metabolic capacity than fish. The results of the present study allow us to elucidate both, the species specific differences in the metabolite patterns and in the metabolic rates of *H. azteca* (in vivo) and rainbow trout (in vitro). Different number of BTPs (between 2 and 30) for each compound was detected in both *H. azteca* samples in vivo and in vitro rainbow trout. The main biotransformation reactions of the tested substances in fish hepatocytes and *H. azteca* occurred at the biological activity sites of the molecules and therefore probably led to their detoxification. For example, many changes occurred at the (E)-methyl β -methoxyacrylate group of azoxystrobin and the imidazole ring of prochloraz. In most cases the transformation reactions could be assigned confidently. There were only a few cases where no plausible molecular formula or transformation reaction could be assigned. In these cases only the exact mass or the change in the molecular formula is reported; the full lists are available in Supplemental Data, SI-E.

In general, the main biotransformation reactions were similar in *H. azteca* and hepatocytes, including hydroxylation, demethylation (phase I reactions), as well as glutathione and sulfate conjugation (phase II reactions), summarized in Figure 6. The main differences are only present for phase II conjugated metabolites, which was expected. Glucuronide conjugates were only identified in fish hepatocytes, whereas glucose conjugates were only identified in *H. azteca*. This is in agreement with previous observations that glucuronide conjugation is mainly found in fish, whereas glucoside conjugation is more common in invertebrates (Livingstone 1998; Ikenaka et al. 2006; Katagi 2010).

<i>Hyaella</i> (in vivo)	Both	Fish hepatocytes (in vitro)
Conjugations: - Glucose - Malonyl-glucose - Taurine	Hydroxylation Hydrolysis Reduction Demethylation Deethylation Methylation Dehydrogenation Losses: - Imidazole ring - Propyl side chain Conjugations: - Sulfate - Glutathione	Conjugations: - Glucuronide

FIGURE 6: Metabolite classes of the 5 test substances detected in either *Hyaella azteca* only, rainbow trout hepatocytes only, or both sample types.

Some conjugates were identified to be present exclusively in *H. azteca* samples, namely taurine- and malonyl-glucose conjugates. Taurine conjugates are known to be formed in many vertebrates as well as fish (James 1987; Zamek-Gliszczynski et al. 2006), whereas malonyl conjugates have been found in soil invertebrates and plants (Stroomberg et al. 2004; Taguchi et al. 2010). It is possible that differences in the metabolite pattern could be due to the differences between the 2 test systems (e.g., in vivo exposure at 25 °C for up to 3 d and in vitro exposure at 11 °C for up to 4 h). Furthermore, the in vitro system could potentially be limited in cofactors to provide for conjugation as well, explaining why the metabolite pattern of the hepatocytes could be less diverse for some substances. In other studies, a larger number of metabolites could be identified in both *H. azteca* and hepatocyte samples (Fu et al. 2020). A more detailed metabolite pattern might have been detected in case a radiolabel had been used. Nonetheless, the acquired data for the metabolite patterns in *H. azteca* and rainbow trout hepatocytes do not indicate that *H. azteca* produces a lower range of metabolites compared to fish, indicating that this is an unlikely reason for the higher BCF values obtained by HYBIT studies.

Comparison of metabolism rates determined for *H. azteca* (in vivo) and rainbow trout (in vitro)

The metabolic rates of *H. azteca* and fish were compared. Kinetic metabolite data for terbutryn and trifloxystrobin were obtained from the semistatic experiments, whereas the kinetic metabolite modeling data for prochloraz and azoxystrobin were taken from Fu et al. (2018; Supplemental Data, SI-F). Kinetic metabolite data for diclofenac were not collated. Comparison of the metabolism rates confirms that fish tend to have higher metabolic activity rates than *H. azteca*. The only substance that deviated from this pattern is prochloraz, but as discussed in the section *Depletion assay (IVIVE)*, this could be due to the fact that its depletion kinetic was not in agreement with first-order characteristics. Depending on the applied “goodness-of-fit determinations” during the kinetic modeling approach, it is possible to obtain differing rate values for the metabolic activity of *H. azteca*. Accordingly, the fish in vitro/*H. azteca* in vivo comparison should be viewed with caution. Furthermore, the fish metabolism rates were derived from an in silico extrapolation using the depletion rates obtained from the hepatocyte assays. In vivo fish data derived under identical experimental conditions as for *H. azteca* would greatly improve the quality of the comparison. Unfortunately, no data on fish in vivo metabolism rates for the test chemicals are available. Such data would also enhance the understanding of fish metabolism required for improved IVIVE extrapolations. Although the results of the comparison of the metabolic rates should be viewed with caution, they still provide clear indications that *H. azteca* has a slower metabolism than fish, resulting in higher bioaccumulation. Similar in vivo BCFs were observed in fish and *H. azteca* for PCB 153 (Schlechtriem et al. 2017, 2019), a substance which is known to be inert and undergoes almost no metabolism (Trowell et al. 2018). Although BCFs of hydrophobic substances tend to

be overestimated in the HYBIT compared to the fish test, PCB 153 BCFs are almost equal, confirming that metabolism might be the key factor leading to the differences observed between *H. azteca* and fish BCF values.

SUMMARY AND OUTLOOK

Overall, the obtained BCFs for the substances showed an apparent dependence on their respective log K_{OW} value. The HYBIT BCFs were among the most conservative BCF values, especially in the case of substances with an increasing log K_{OW} value. However, so far only single data are available that do not allow us to deduce the potential variance of HYBIT BCFs. The extrapolation range of the results obtained with the IVIVE methods covered the fish BCF values without any clear trend to generally over- or underpredict them. In contrast, the in silico predictions using EPI Suite showed a tendency to overpredict the fish BCF values. Accordingly, the 2 alternative test systems appear to show comparable predictive capacities for BCFs of organic, lipophilic chemicals.

Future research should focus on sharpening the prediction range of the in vitro approach. Furthermore, the development of extrapolation models for other fish species suggested in the OECD test guideline 305 for BCF testing is recommended. However, testing with different fish species may lead to a range of different BCFs, a commonly known problem in fish BCF testing. Benchmarking the different test systems could pose a solution, but this has not been done systematically in fish in vivo studies so far; therefore, a reliable comparison basis to benchmark against is missing.

Based on the results of the present study, a protocol for carrying out bioconcentration tests with the aquatic invertebrate species *H. azteca* under standardized conditions has been developed. This protocol includes both flow-through and semistatic test designs. Validation is needed to confirm the transferability of the test protocols and to prove the reproducibility of the results obtained to support the development of a new OECD test guideline. For this purpose, an international multilaboratory ring trial involving the HYBIT is being carried out and aims to finish by the end of 2020. The different test systems need to be integrated into a coherent testing and assessment strategy that considers the specific regulatory requirements, such as in cosmetics assessment tests where vertebrates cannot be used. Also, substance-specific testing conditions, as required for compounds such as surfactants and superhydrophobic or ionizable chemicals, need to be addressed. Both alternative methods for bioaccumulation assessment compared in the present study have the potential to be used for regulatory purposes, for example, as a first tier prior to in vivo testing or as part of a weight-of-evidence approach. However, more data will be necessary to further identify the degree of variance and the most suitable applicability domain of the BCFs obtained with both methods.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at <https://doi.org/10.1002/etc.4791>.

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Data Availability Statement—Data and calculation tools are available on request from the corresponding author. For data requests please contact either verena.kosfeld@ime.fraunhofer.de or christian.schlechtriem@ime.fraunhofer.de

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