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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* (Hybit).

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Abstract: Bioaccumulation assessment predominantly relies on the bioconcentration factor (BCF) as sole decisive metric. The OECD Test Guideline number 305 (TG 305) provides the standard procedure for deriving this in vivo fish BCF, which requires a lot of animals, is expensive, and labor intensive. Accordingly, there is a lot of need and interest for alternative methods that can help to reduce, replace, and refine vertebrate tests as described in the 3R principles. Two alternative approaches have been developed in the past years: The bioconcentration test with the freshwater amphipod *Hyalella azteca* (HYBIT), and the OECD TG 319, 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 five substances of different physicochemical characteristics. The obtained 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 dataset suggests that both alternative methods pose promising alternatives to

predict bioaccumulation in fish, although, defined applicability domains have yet to be determined.

Keywords: *Hyalella azteca*, in vitro hepatocyte assay, biotransformation, alternative method, bioconcentration, risk assessment

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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, that 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 OECD Test Guideline (TG) 305 (OECD 2012). However, the TG 305 BCF test is time consuming, expensive, and requires a high number of laboratory animals (>108 fish per test) (de Wolf et al. 2007).

In vitro metabolism assays using isolated primary hepatocytes or liver S9 sub-cellular fractions from fish have been introduced as a promising and reliable tool to generate

hepatic biotransformation rates of xenobiotics, which can be used for in vitro - in vivo extrapolation (IVIVE) of BCFs (Fay, Mingoia, et al. 2014; Fay et al. 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, Fitzsimmons, et al. 2014) and new OECD test guidelines for the performance of the in vitro assays have just recently become available (OECD 2018a; OECD 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; Cowan-Ellsberry et al. 2008; Nichols et al. 2013; Fay, Fitzsimmons, et al. 2014). 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 also 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 to use invertebrate species as test organisms. Invertebrates provide some conceptual advantages, since 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 fourteen substances of different hydrophobicity (log K_{OW} 2.4 to 7.6) under flow-through conditions to determine steady-state and kinetic bioconcentration factors (BCF_{SS} and BCF_k). Bioconcentration studies with the freshwater amphipod *H. azteca* (HYBIT) resulted in BCF estimates which showed a good correlation with fish BCF values (R^2=0.69) (Schlechtriem et al. 2019). HYBIT BCF values can be assessed in accordance with the standard B criterion (BCF > 2000) and thereby enable the prediction of B or non-B classification in the standard fish test as part of PBT/vPvB assessment and CLH (harmonized classification and labelling) evaluations (European Commission 2006).

The HYBIT and IVIVE approach have a high potential to be used as alternative test approaches to reduce and replace the 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 due to potential differences in the metabolism of xenobiotics in fish and crustaceans. BCF values 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 both 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 bioaccumulation of five compounds with different chemical structures, hydrophobicity, and speciation using the IVIVE approach and the HYBIT test. The BCF values obtained with both 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 this 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 this study for media preparation, sample processing and analytics, and the respective sources of supply are available in SI-A.

Depletion assay (IVIVE), OECD TG 319A

Immature specimens of rainbow trout (*Oncorhynchus mykiss*) with an age of 8 to 11 months and an average body weight of 277 ± 27 g, showing normal behavior were fasted for 24 hours prior to hepatocyte isolation. Primary cells (RT-HEPs) were prepared and cryopreserved according to Bischof et al. 2016 (c.f. SI-B, Text S1). Two hepatocyte lots were prepared for the present study, each of them contained cells originating from four 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 two vials (one vial per lot) were pooled during the thawing procedure, generating a hepatocyte pool of eight 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 to display the depletion kinetics. First order kinetics were assumed, when linear regression showed a high degree of correlation for the logarithmized concentration values (OECD 2018a). The combination of start concentration and time period with the highest first order depletion rates were 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 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 minutes. 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 (c.f. SI-B, Table S 2 and Table S 4). 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 minutes) and centrifuged (20,000 g, 10 minutes, 4°C) and a 250 µl aliquot of the supernatant transferred to an HPLC vial with insert. Storage was at -20°C until

analysis. Each of the five chemicals were tested in three different runs carried out on three different days. Samples for identification of metabolite pattern were prepared in a similar way, with the following adaptions. 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 hours were applied to the assays in accordance to Bischof et al. 2016. No intermediate samples were taken during the incubation period. The entire assay was stopped after 4 hours 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.

Hyalella azteca bioconcentration test (HYBIT)

Aqueous exposure bioconcentration tests with *Hyalella azteca* were carried out to estimate the bioaccumulation potential of the five tested chemicals. *H. azteca* were obtained from the in-house culture. Animals were raised as described by Schlechtriem et al. 2019. Only male amphipods with an age > 2 months were used, approximately 1200 amphipods per test (c.f. 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 SI-D, Text S7) and consisted of two phases namely the exposure (uptake) and post-exposure (depuration) phases. The flow-through system was equilibrated for 2 to 3 days 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 semi-static approach for comparative reasons, details are given in SI-D, Text S8. In both exposure systems the water was continuously aerated via a glass capillary. A light/dark regime of 16/8 hours was applied and the water temperature kept in a range of 25 ± 2°C in accordance to the rearing conditions. Meshed steel shelters were placed into the aquarium to reduce stress of the amphipods. *H. azteca* were fed on a daily basis with DECOTABs (Kampfraath et al. 2012), which were prepared according to a slightly modified protocol (see SI-D, Text S6). The suitability of the modified DECOTABs was tested beforehand in a short preliminary study (c.f. SI-C, Text S3).

The duration of the exposure and depuration phases for each of the substances were estimated based on results obtained from previous studies with substances of similar log K_{OW} and sampling points were scheduled accordingly (SI-D; Table S12 – 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 (c.f. SI-D, Table S 15 & Table S 15). 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, as 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 (c.f. SI-D; Table S12 – S13). Animals which were sampled for tissue analysis during the semi-static experiments were also used for metabolite identification followed by the calculation of *H. azteca* biotransformation rates as described below in '*H. azteca* biotransformation rates'.

During the flow-through and semi-static 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 SI-K.

HYBIT - Effect of biomagnification processes

As mentioned above in '*Hyalella azteca* bioconcentration test (HYBIT), *H. azteca* is fed during the experiments. In theory it could be 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_{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 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 ultra-pure water was used.

H. azteca samples were processed by solid/liquid extraction using methanol to determine the concentration of the accumulated test substances. *H. azteca* samples were spiked with 25 µL of the respective internal standard solution (400 µg/L in methanol) and 4 mL pure methanol. Homogenization was performed using an Ultra-Turrax for 30 seconds, then by placing the samples in an ultrasonic bath for 10 minutes, and finally via vortexing for 30 seconds. The sample extracts were obtained by centrifugation of the samples for 6 minutes 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 LC-MS/MS in positive electrospray ionization mode (ESI +). The LC-system (Waters Acquity UPLC system) used was coupled to a Waters TQD triple quadruple mass spectrometer. Chromatographic separation of the samples was done on a Waters Acquity UPLC BEH C18 column in the dimensions 1.7 µm, 100 mm x 2.1 mm. Stable isotope labelled internal standards of the test substances were used as described in the chemicals section. The injection volume of each sample was 10 µL. Instrument settings applied for each substance are available in SI-I, Text S14.

Metabolite identification

Hepatocyte supernatants for metabolite identification were produced in the same manner as described above in 'Depletion assay (IVIVE), OECD TG 319A' for the depletion assay. The *H. azteca* samples collected at the end of the bioconcentration studies for metabolite identification were mixed with 100 µL of methanol containing an isotopic labeled internal standard (100 µg/L) corresponding to the respective test compound, 500 µL of pure methanol and 300 mg of 1-mm zirconia/silica beads (BioSpec Products, Inc.). The samples were homogenized with a FastPrep bead beater (MP Biomedicals) in two cycles of 15 s at 6 m/ s (cooling on ice in between). The homogenate was centrifuged (10 000 rpm × 6 min, 20 °C) and filtered through 0.45 µm regenerated cellulose filters (BGB Analytic AG). The filters were washed with 400 µL methanol and the filtrate combined with the wash solution.

Supernatants of fish hepatocytes and *H. azteca* samples were analyzed by online solid phase extraction (online SPE) coupled to reversed phase liquid chromatography high resolution tandem mass spectrometry (online SPE-LC-HRMS/MS) (Q Exactive, Thermo Fisher Scientific Inc.) 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 five (positive mode) and two (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 literature and in silico prediction. More details about the analytical procedure are provided in SI-I, Text S16.

To identify BTP candidates, suspect and non-target screening was performed by analyzing the acquired HRMS/MS raw data using Compound Discoverer software 2.1 (CD2.1, 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 estimates

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 (OECD 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 (OECD 2018c). Log K_{OW} values were obtained from the EPI Suite™ with the exception of diclofenac, since the EpiSuite™ value is not based on the anionic state.

HYBIT – Calculation of bioconcentration factors

All BCF calculations were performed in accordance with the OECD TG 305, Annex 5 (OECD 2012). Calculated metrics comprise the steady state BCF (BCF_{SS}) the kinetic BCF (BCF_k), the time weighted average of the water concentration during the uptake phase (TWA), and the average lipid content of *H. azteca* in the experiments (c.f. SI-D, Table S14 & Table S 15). Latter was used to normalize the BCFs to a 5% body lipid content, as done in the OECD TG 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 considerations of covariances (Mandel 1984; Schlechtriem et al. 2019). In order to standardize the calculation of bioconcentration factors, the guidance document for the OECD TG 305 suggests the application of the R package bcmfR (OECD 2016). The HYBIT data was evaluated with the bcmfR package in addition to the sequential method as described in the OECD TG 305. In this way it was evaluated whether the R package is suitable for the HYBIT test as well.

H. azteca biotransformation rates

H. azteca biotransformation rates for the different test compounds were determined based on metabolite concentrations measured in *H. azteca* samples collected during the semi-static bioconcentration studies. Concentration data for parent substance, and the sum of 1st phase and the sum of 2nd phase metabolites were each fitted to a 1st 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 1st 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 1st 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 bioconcentration factors, preferably from in vivo fish studies that were conducted in accordance with the OECD TG 305. Keywords were 'bioconcentration' 'fish' 'BCF' 'OECD' and the respective substance names and 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 (c.f. SI-G). Additionally, BCFs for all test substances were retrieved from the EPI Suite™ software (USEPA 2012) to provide a dataset of in silico BCF values. In a further step, the HYBIT and fish BCF dataset 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 (c.f. SI-H). Similar to Schlechtriem et al. 2019, fish and HYBIT BCFs were evaluated to establish any 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 SI-B, Table S 4. Average cell viability and cell concentration in the assays were monitored

and averaged at ≥ 80% and about 2×10^6 cells/mL, respectively (c.f. SI-B Table S 3). The resulting kinetics are presented in Figure 1. Terbutryn was readily degraded by 90 to 95% within 45 minutes (first order kinetics), and 63 to 70% of azoxystrobin were depleted within the same period. Trifloxystrobin was degraded by 70 to 75% within one hour of incubation. Although almost 50% of the initial amount of diclofenac were depleted in the preliminary tests after 4 hours, unexpectedly only 21 to 23% were 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 hours 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 that 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 et al. 2014; OECD 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 results are presented in Table 1 (Nichols et al. 2013; OECD 2018c). IVIVE BCFs for B[a]P, methoxychlor, PCB 153, and pyrene were obtained from the literature. B[a]P 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 TG 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 (Nichols et al. 2013, Han et al. 2007, Han et al. 2008, 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 (OECD 2018c). The extrapolation process as a whole starts to receive 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 modelling 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 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 (OECD 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, due to 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 mL/h/ g liver basis and assuming a hepatocellularity number of 510×10^6 cells/ g liver (Nichols et al. 2013; OECD 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 are 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. In order 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 1st order characteristic, which is needed for the extrapolation model (OECD 2018c). The results of the BCF extrapolation showed the broadest range of predicted BCFs (366 – 1140) of all substances tested in this study. The $CL_{IN\ VIVO,\ INT}$ rates extrapolated from the hepatocyte depletion rates were compared to modeled in vivo 1st phase metabolism rates of *H. azteca*. The extrapolated rates from the IVIVE system are

approximately 50-fold higher than the in vivo 1st 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 due to the atypical linear depletion kinetics observed for prochloraz. Since the linear depletion took place independent of the starting concentration and incubation duration the possibility of a saturated enzyme system as the cause for the linear depletion can be excluded (OECD 2018c). Generally, further investigations with a broader range of compounds are required in order 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 ± 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 (c.f. Figure 2), as confirmed by similar kinetic and steady-state BCFs (c.f. 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 (c.f. 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 SI-D, Table S14 & Table S15. For diclofenac a BCF_k of 1.36 was obtained. Since 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 TG 305 data (OECD 2016) to test the applicability of this tool for HYBIT studies. The obtained BCFs are similar to the ones calculated in the sequential method (c.f. SI-D, Text S 11). The fitting of the flow-through data for azoxystrobin and diclofenac was not as good on a visual basis as for the other three substances and a two-compartment depuration model was thus applied resulting in a visually better fit, but still producing comparable BCF results (c.f. SI-D, Text S 10).

Comparison of HYBIT BCFs of semi-static and flow-through exposures

Compared to their flow-through counterpart, the concentration profile in the semi-static experiment for prochloraz was almost identical. However, in case of terbutryn and trifloxystrobin, differences were visible in their respective concentration profiles. The calculated BCF_{kL} values are around half of the values derived under flow-through conditions. Interestingly, the uptake rate of terbutryn measured for the semi-static 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 semi-static and flow-through approach. In case of trifloxystrobin the water concentration was 3-times higher in the semi-static 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 semi-static 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 semi-static approach corresponds to only 15% of the 96h LC_{10}, 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 SI-D, Table S 16.

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 of the test animals with DECOTABs during the bioconcentration studies or the use of meshed steel shelters in the aquarium to reduce stress of 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 towards over- or under prediction 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 this 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 the data available (Figure 4). However, due to the fact that 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 Figure 4 and Figure 5. In Schlechtriem et al. 2019 it was concluded that *H. azteca* BCFs pose a promising alternative for obtaining fish BCFs, stating: "*BCF values calculated for 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 sufficiently conservative prediction for bioaccumulation in fish (Schlechtriem et al. 2019) was confirmed by the BCF data obtained in the present study. In order 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 higer BCF_{kL} values are potential biomagnification processes. *H. azteca* is fed with an uncontaminated diet during the bioconcentration test, 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, the 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 differences in the metabolite patterns and in the metabolic rates of *H. azteca* (in vivo) and rainbow trout (in vitro). The analysis of the metabolic patterns of *H. azteca* samples collected from in vivo studies and in vitro rainbow trout hepatocyte samples were able to detect between 2 and 30 BTPs in *H. azteca* and hepatocyte samples for each compound. 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 lead 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 confidently be assigned. 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 are reported, the full lists are available in 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).

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) for example. It is possible that differences in the metabolite pattern could be due to the differences between the two test systems (e.g. in vivo exposure at 25°C for up to 3 days and in vitro exposure at 11°C for up to 4 hours). Furthermore, the in vitro system could potentially be limited in co-factors 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 semi-static experiments, whereas the kinetic metabolite modeling data for prochloraz and azoxystrobin were taken from Fu et al. 2018 (c.f. SI-F). Kinetic metabolite data for diclofenac was not collated. The comparison of metabolism rates confirms that fish tends to have a higher metabolic activity rate than *H. azteca*. The only substance that deviated from this pattern is prochloraz, but as discussed above in 'Depletion assay (IVIVE)'this could be due to the fact that its depletion kinetic was not in agreement with 1st 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 metabolic rates should be viewed with caution, it still provides 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; Schlechtriem et al. 2019). PCB 153 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 to deduce 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 under predict them. In contrast, the in silico predictions using EPI Suite™ showed a tendency to over predict the fish BCF values. Accordingly, both test systems appear to show comparable predictive capacities for BCFs of organic, lipophilic chemicals.

Future research should focus on the sharpening the prediction range of the in vitro approach. Furthermore, the development of extrapolation models for other fish species suggested in the OECD TG 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 this 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 the flow-through and semi-static test designs.

Validation is needed to confirm the transferability of the test protocols and to prove the reproducibility of the results obtained in order to support the development of a new OECD TG. For this purpose, an international multi-laboratory ring trial involving the HYBIT is currently 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 considering 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, superhydrophobic or ionizable chemicals, need to be addressed. Both alternative methods for bioaccumulation assessment compared in this study have a potential to be used for regulatory purposes, for example as a first tier 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 DOI: 10.1002/etc.xxxx

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Data Availability Statement--The authors clarify that data and calculation tools are available on request from the corresponding author. For data requests please contact

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Author Contributions Statement-- V. Kosfeld, I. Bischof and C. Schlechtriem conceived and designed experiments. V. Kosfeld, D. Esser, and A. Schauerte performed experiments. Q. Fu and I. Ebersbach performed chemical analysis. V. Kosfeld, Q. Fu, J. Hollender, and I. Ebersbach performed data analyses. V. Kosfeld, and Q. Fu wrote the manuscript. C. Schlechtriem and J. Hollender conceptualized and supervised the project and provided editorial assistance.

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Figure 1: Kinetics of the *in vitro* depletion assays with rainbow trout hepatocytes. Each datapoint reflects the mean of three runs, the error bars display the standard deviation. Data of the active treatments was obtained using living hepatocytes at a concentration of 10^6 cells/mL, inactive runs were conducted using heat inactivated hepatocytes material and served as negative control to monitor abioticdisappearance of the test substance.

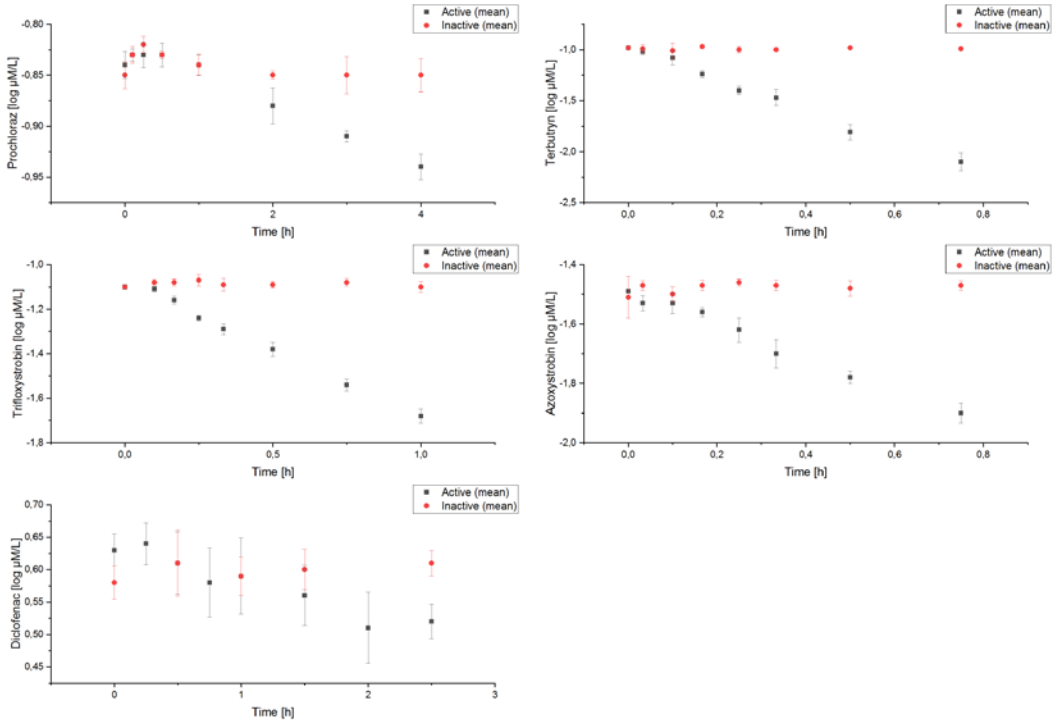

Figure 2: Determined concentrations in the flow-through HYBIT experiments: Left hand side: Uptake and depuration kinetics for the five test substances in *Hyalella azteca* under flow through (black squares and solid black lines) and semi-static (open circles and dashed lines) exposure conditions. Right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

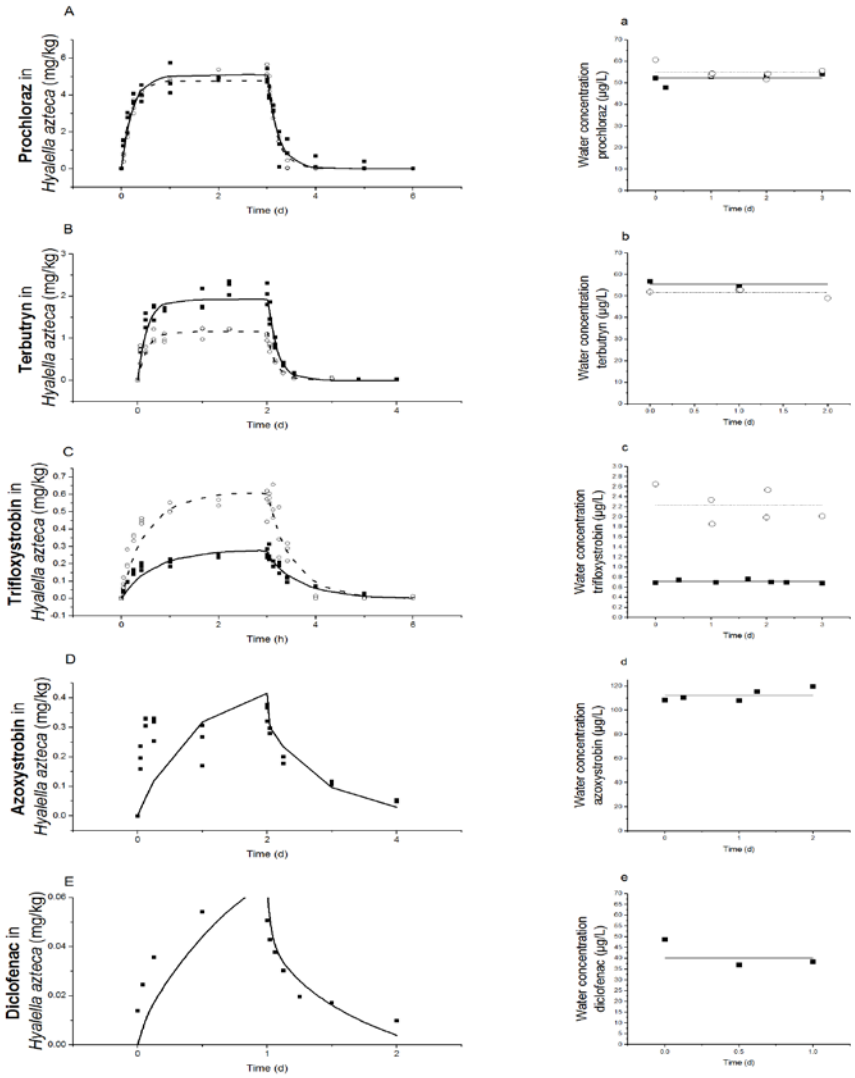

Figure 3: Comparison of log Hyalella BCFs and log in vivo fish BCFs found in the literature: Solid black circles represent data taken from Schlechtriem et al. 2019, open circles show data collated in the present study. All in vivo fish BCF values were taken from Schlechtriem et al. 2019. A linear regression was performed, the resulting equation y = 0.809x+0.19373 with a R^2 of 0.7215 is displayed by a solid black line. The dotted lines frame the 95% confidence interval, the dashed lines the 95% prediction band. A central grey cross marks the regulatory threshhold BCF =2000.

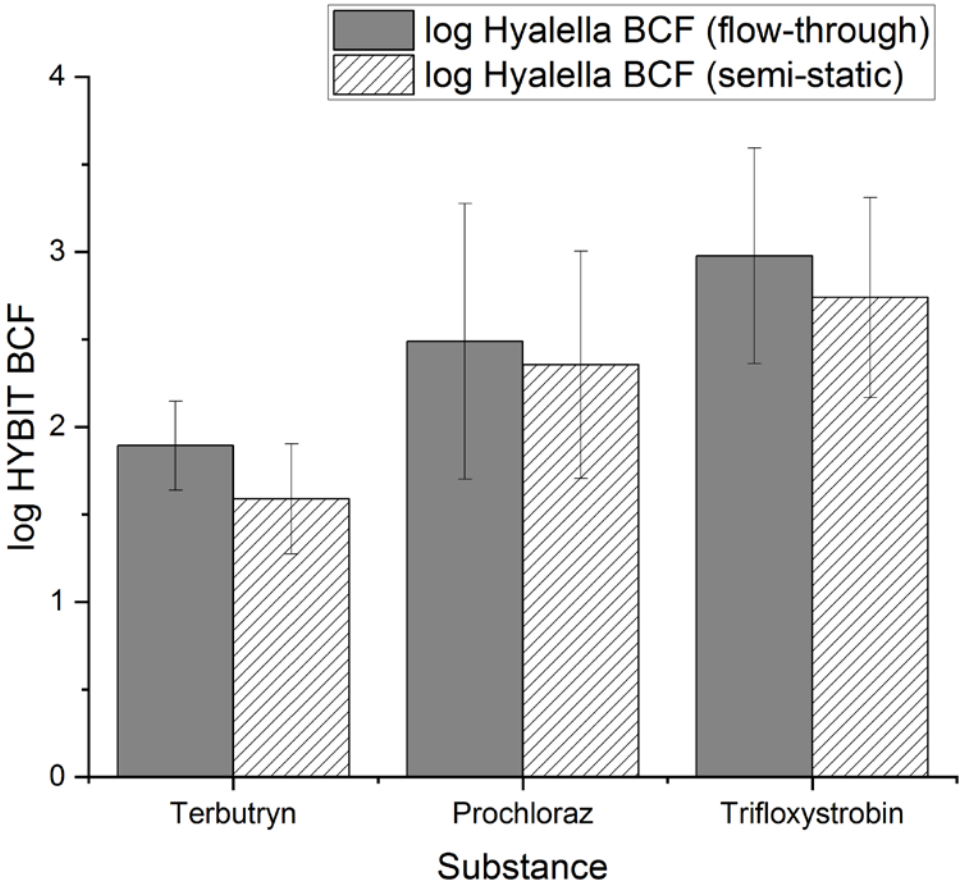

Figure 4: Comparison of log BCF values derived from HYBIT tests with terbutryn, prochloraz, and trifloxystrobin under flow-through and semi-static exposure scenarios: Error bar represent BCF errors determined using the general law of propagation of errors.

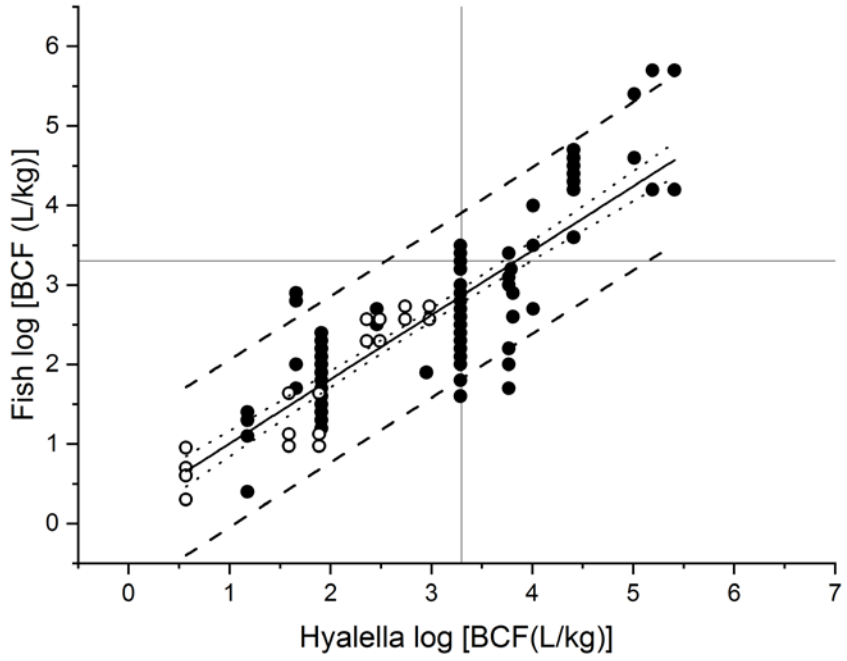

Figure 5: BCF comparison: HYBIT vs. IVIVE. BCF values retrieved from the two alternative test systems HYBIT (*Hyalella azteca* bioconcentration test) and IVIVE from rainbow trout hepatocyte depletion assays (OECD TG 319A). Log K_{OW} values of the substances are: Diclofenac = 0.7, azoxystrobin = 2.5, terbutryn = 3.66, prochloraz = 4.1, trifloxystrobin = 4.5. Additional in vitro BCF extrapolation were found in the literature for the following substances (log K_{OW} value in brackets): 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. 2018. *In vitro* BCF extrapolations for diclofenac and prochloraz should be taken with caution, since the assays showed deviations from the guideline demands.

* IVIVE extrapolation range determined with non-OECD TG 319A model. Cefer to SI H, Table S 40 for details

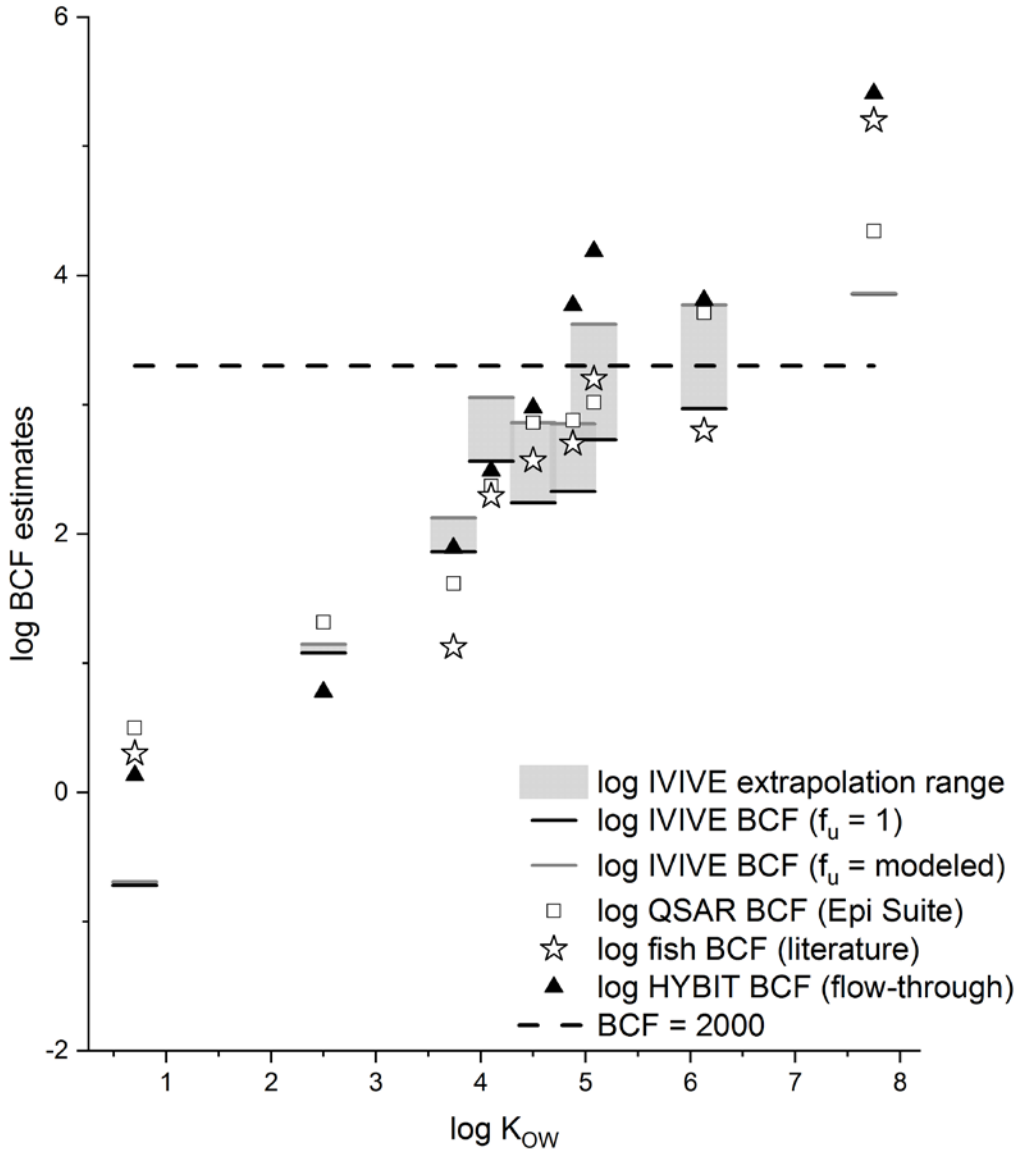

Figure 6: Metabolite classes of the five test substances detected in either *H. azteca* only, rainbow trout hepatocytes only, or both sample types.

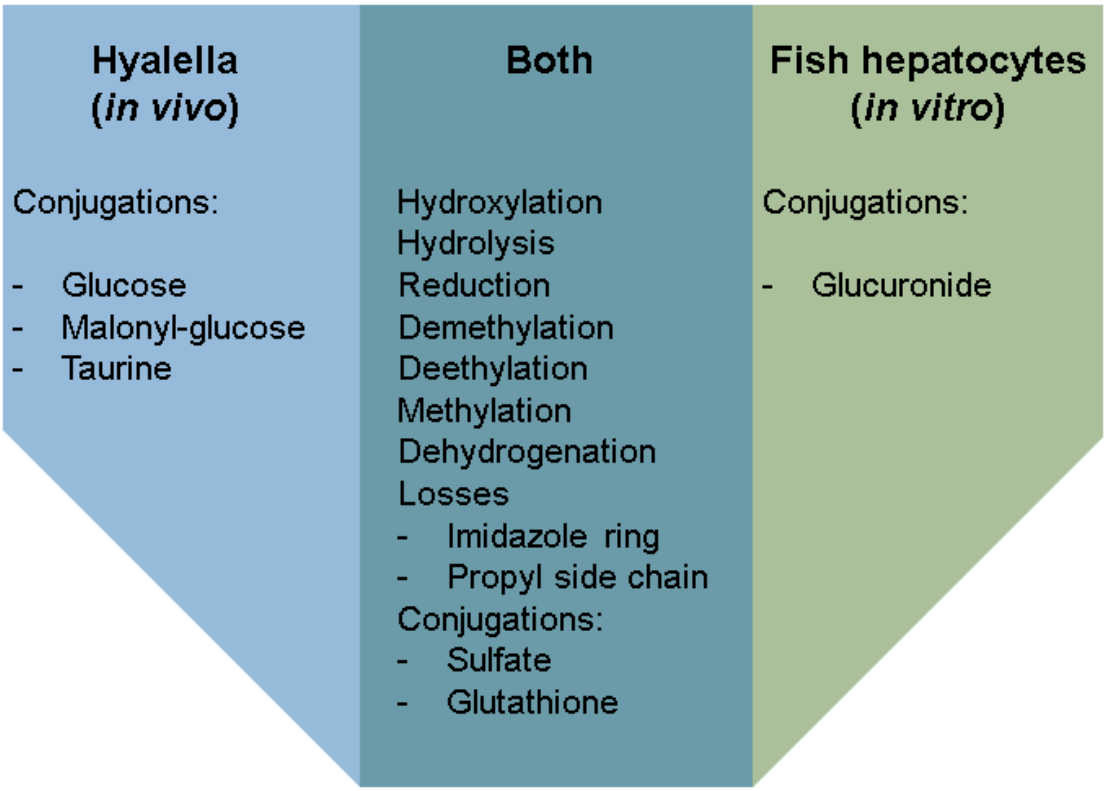

Table 1: BCF values determined for azoxystrobin, prochloraz, terbutryn, diclofenac and trifloxystrobin utilizing two alternative bioconcentration test setups: HYBIT and IVIVE. Reference BCF values in fish were retrieved from the literature, QSAR values are based on EPI Suite™ predictions.

Substance	Normalization	Log K_{OW}	IVIVE BCF		HYBIT (flow-through)		Fish	QSAR
			f_u = 1	f_u = mod.	BCF_k	BCF_{SS}	BCF (Literature)	EPI Suite™
Azoxystrobin	non-normalized	2.5[a]	N/A	N/A	4	3	N/A	21
	lipid-normalized		12	14	9	6		
Prochloraz	non-normalized	4.1[a]	N/A	N/A	97	94	196 – 371[b]	236
	lipid-normalized		366	1140	308	299		
Terbutryn	non-normalized	3.74[a]	N/A	N/A	37	37	13.3[c]	41

	lipid-normalized		73	133	78	76		
Diclofenac	non-normalized	0.7[d]	N/A	N/A	1.36	N/A	2 – 5[e]	3
	lipid-normalized		0.19	0.2	3	N/A	3 – 9[e]	
Trifloxystrobin	non-normalized	4.5[a]	N/A	N/A	393	354	370 – 542[f]	727
	lipid-normalized		175	725	947	852		

[a] = EPI Suite™ experimental value

[b] = EFSA 2011

[c]= Tarja et al. 2003

[d] = Chemistry Dashboard entry for diclofenac sodium salt, experimental value

[e]= Memmert et al. 2013

[f]= Jackson et al. 2009

IVIVE = In vitro to in vivo extrapolation; BCF = bioconcentration factor; HYBIT = *Hyalella azteca* bioconetration test; QSAR = Quantitative structure–activity relationship; mod. = modeled

Table 2: Metabolism rates determined for H. azteca and extrapolated as part of the IVIVE process for the substances azoxystrobin, prochloraz, terbutryn, and trifloxystrobin. The H. azteca data for azoxystrobin and prochloraz were taken from Fu et al. 2018. Kinetic metabolite data for diclofenac was not retrieved.

Rate type	Units	Azoxystrobin	Prochloraz	Terbutryn	Trifloxystrobin
In vitro intrinsic clearance ($CL_{IN\ VITRO,INT}$)	d^{-1}	0.589	0.045	1.641	0.689
In vivo intrinsic clearance ($CL_{IN\ VIVO,INT}$)	l/d/kg fish (or ml/d/g fish)	107	8	297	126
***H. azteca* 1st phase metabolism rate ($k_{m,\ 1st\ total}$)**	$L/d/kg_{ww}$	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/ *H. azteca*	unitless	59	5	49	69