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# Evaluation of bioaccumulation thresholds for *Hyalella azteca* and fish relative to different environmental protection goals

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**Data Availability Statement:** A Microsoft® Excel spreadsheet in the Supporting Information contains the entire data set of 26 chemicals, their measured BCFs, and chemical property information. The model parameters and outputs are also provided in the Supporting Information. Data, associated metadata, and calculation tools are also available from the authors (environment@concawe.eu).

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# Evaluation of bioaccumulation thresholds for *Hyalella azteca* and fish relative to different environmental protection goals


## ABSTRACT

Bioaccumulation assessment is a key component of chemical regulations, with fish-based bioconcentration factors (BCFs) serving as the standard metric. Because conventional fish BCF tests (OECD TG 305) require many vertebrates, alternative tests such as the amphipod *Hyalella azteca* BCF test (OECD TG 321, HYBIT) have been developed. However, physiological and ecological differences between fish and aquatic invertebrates affect chemical uptake and elimination, raising uncertainty when applying fish-based BCF thresholds directly to invertebrate data. In this study, empirical and model-based approaches were combined to compare bioaccumulation potential in *H. azteca* and fish. An evaluation of empirical BCFs showed consistent categorizations between *H. azteca* and fish for non-bioaccumulative chemicals (log $K_{OW} \leq 4.5$) and for recalcitrant organochlorines categorized as (very) bioaccumulative. In contrast, for biotransformed chemicals, *H. azteca* BCFs frequently exceeded fish BCFs and often surpassed regulatory thresholds, reflecting the amphipod's lower metabolic capacity. A food-chain bioaccumulation model was used to assess the influence of chemical (e.g., $K_{OW}$, biotransformation rate constant) and biological (e.g., metabolic capacity) parameters. At log $K_{OW}$ 5, amphipod BCFs were lower than fish BCFs for poorly biotransformed chemicals. However, at log $K_{OW} \geq 5$, moderately to highly biotransformed chemicals showed higher BCFs in amphipods than both fish BCFs and biomagnification metrics (BMF, TMF), indicating potential overestimation of bioaccumulation potential. These findings highlight potential misalignment in bioaccumulation assessment outcomes when amphipod BCFs substitute fish data. Applying tiered, weight-of-evidence assessment frameworks that integrate multiple bioaccumulation metrics with biotransformation information can strengthen the scientific robustness of assessments and contextualize HYBIT results.


KEYWORDS: Bioaccumulation; bioconcentration; biomagnification; biotransformation; food chain

## INTRODUCTION

Bioaccumulative chemicals concentrate in organisms, and can increase in concentration along the food chain, particularly in higher trophic organisms, which can increase the risks of toxicological effects. The assessment of chemical bioaccumulation potential is a critical component of established chemical regulatory programs, including the Toxic Substance Control Act (TSCA) in the United States, the Canadian Environmental Protection Act (CEPA), and the European Union's Registration, Evaluation, Authorisation, and Restriction of Chemicals (REACH). The generally accepted protection goal of bioaccumulation assessment is to prevent chemical accumulation in higher trophic level organisms (Gobas et al. 2009;

Hommen et al. 2010; Matthies et al. 2016; Weisbrod et al. 2009). Comprehensive reviews of bioaccumulation assessments and associated metrics can be found elsewhere (Arnot and Gobas 2006; Gobas et al. 2009). Three common metrics for assessing bioaccumulation include the trophic magnification factor (TMF), the biomagnification factor (BMF), and the bioconcentration factor (BCF). The TMF characterizes the change in chemical concentrations in organisms through an entire food chain/web, and the BMF quantifies the extent to which a chemical accumulates in an organism relative to its diet (i.e., predator relative to prey). For both the BMF and TMF, values above one generally indicate that a chemical is bioaccumulative. The BCF describes aqueous accumulation and is defined as the ratio of the chemical concentration in (aquatic) organisms to the concentration in surrounding water. The BCF is often generated in fish using standardized test protocols (Organisation for Economic Co-operation and Development [OECD] Test Guideline [TG] 305; OECD 2012). Under REACH, the criteria for bioaccumulative ("B") and very bioaccumulative ("vB") substances are BCF=2,000 and BCF=5,000, respectively. Studies also indicate that a BCF > 5,000 in fish generally corresponds to a BMF > 1 (Gobas et al. 2020; 2025). As of the writing of this paper, an assessment of bioaccumulation in aquatic species, preferably fish, is a standard information requirement under REACH Annex IX (European Union, 2006). However, BCF data generated in either fish or aquatic invertebrates may be used for bioaccumulation assessments in Europe (European Chemicals Agency [ECHA], 2023).

Generating experimental BCF data according to the OECD 305 test guideline (OECD, 2012) is labour-intensive, technically challenging, and requires the use of many fish, which is inconsistent with the animal welfare principles of replacement, reduction, and refinement (i.e., 3Rs; ECHA 2017). In response, a range of new approach methods (NAMs) have been developed to generate BCF data with a reduced reliance on vertebrate animals. Such NAMs include quantitative structure activity relationships (QSAR) and mechanistic models for

estimating bioaccumulation potential, in vitro biotransformation assays, and non-vertebrate in vivo tests. In vitro tests using hepatocytes (OECD TG 319a; OECD 2018a) or hepatic subcellular fractions (OECD TG 319b; OECD 2018b) can be combined with in vitro–in vivo extrapolation (IVIVE; Saunders et al., 2019) to generate bioaccumulation data. In vivo NAMs include standard tests with invertebrate animals using benthic (OECD TG 315; OECD 2008) and terrestrial oligochaetes (OECD TG 317; OECD 2010), oysters (OCSPP 850.1710; U. S. Environmental Protection Agency [USEPA] 2016) or the freshwater amphipod *Hyalella azteca* (OECD TG 321; OECD 2024a). The *Hyalella azteca* bioconcentration test (HYBIT) enables BCF determination in a setup comparable to the aqueous-exposure fish bioconcentration test defined in OECD TG 305 (OECD 2012).

In 2024, The European Chemicals Agency's (ECHA) Member State Committee unanimously approved the HYBIT to fulfill the bioaccumulation Standard Information Requirement under REACH Annexes VIII and IX, endorsing it as a preferred alternative to vertebrate testing to fulfill REACH standard information requirements where an aqueous BCF is required (ECHA 2024). In cases where aqueous exposure is not feasible, dietary exposure studies following the fish OECD TG 305 protocol may still be considered on a case-by-case basis. Ultimately, the adoption of the HYBIT supports the European Commission's goals to use vertebrate testing only as a last resort, in line with the 3Rs principle of animal replacement (Cronin et al. 2025; Streck 2023).

Scientists have raised concerns with the exclusive use of HYBIT-derived BCF values for comparison against the fish-derived BCF regulatory criteria in chemical assessment frameworks, mainly due to a potential misalignment with the generally accepted bioaccumulation protection goal described above. These concerns reflect the fact that the regulatory framework for bioaccumulation and its associated criteria were largely developed using data derived from fish for neutral organic substances, and that misalignment may arise

when HYBIT data are treated as direct substitutes for fish data without additional examination of the underlying assumptions. In particular, Brinkmann et al. (2025) warn that the HYBIT generates bioaccumulation data only for the invertebrate *H. azteca*, which cannot be readily generalized to vertebrates without complementary information on biotransformation or biomagnification in fish or other higher-trophic level organisms that underpin regulatory bioaccumulation assessments.

Using the HYBIT as a single default test raises similar conceptual issues to those long associated with fish-based BCF tests, as both rely on a single organism to represent broader accumulation processes in food webs. Fish have historically been used in bioaccumulation assessments because they may occupy higher trophic levels and integrate both aqueous and dietary exposure pathways. In contrast, relying solely on a lower-trophic-level invertebrate such as *H. azteca* may not capture the full functionality of processes in higher trophic level organisms, potentially leading to different regulatory outcomes and levels of protection. While neither approach represents a full food-web assessment, the trophic position of the test organism should remain an important consideration when interpreting bioaccumulation test results.

These concerns are based on the fundamental physiological differences between fish and aquatic invertebrates. The HYBIT is designed specifically for aqueous bioconcentration testing and, unlike the OECD TG 305 with fish, is not validated for assessing dietary uptake. This may limit the HYBIT's relevance for assessing other bioaccumulation processes. The HYBIT also focuses on a short-lived, lower-trophic-level test organism, whereas bioaccumulation criteria have historically aimed to prevent bioaccumulation through food webs and in longer-lived higher trophic level species. Fish generally possess more efficient enzymatic detoxification mechanisms than invertebrates (Bleeker and Verbruggen 2009;

Kosfeld et al. 2020), so differences in metabolic capacity could complicate comparisons of bioaccumulation potential (Glüge et al. 2023). Consequently, for biotransformed chemicals, bioaccumulation assessments based on invertebrate data may yield more conservative conclusions compared to those based on fish. Although fish have a greater capacity to metabolize chemicals than *H. azteca* (Kosfeld et al. 2020), the extent of these differences is not extensively described or characterized, nor have these differences been explored in a broader bioaccumulation assessment context.

The integration of invertebrate bioaccumulation tests like the HYBIT into chemical assessment frameworks requires rigorous examination to ensure that BCF testing outcomes are applied appropriately and remain consistent with regulatory protection goals. Historically, the regulation of bioaccumulative substances was motivated by the protection of entire food webs (Matthies et al. 2016). Therefore, if this original motivation is retained, then it is important to assess whether accumulation observed in lower-trophic-level invertebrates is indicative of bioaccumulation potential in top predators. In this context, it remains important to determine how HYBIT BCF values compare to those measured in fish to establish their relevance and consistency. It is also equally necessary to consider whether BCFs, including HYBIT results, provide ecologically relevant insights into bioaccumulation and biomagnification processes in higher trophic level species, or whether additional metrics and lines of evidence are required for a more holistic assessment.

To reflect on the alignment of test methods with current protection goals, this study investigates potential differences in bioaccumulation assessment using metrics derived for fish and *H. azteca*, with a focus on the influence of biotransformation. Here, we employ both empirical and model-based assessments to compare BCFs derived in *H. azteca* to several bioaccumulation metrics derived in fish. We investigate biological and chemical factors that

may drive differences in accumulation between fish and amphipods and evaluate the relevance of current regulatory B/vB BCF thresholds when applied to *H. azteca*.

## METHODS

*Comparison of empirical BCFs derived for fish and H. azteca*

An empirical relationship between fish BCFs ($BCF_F$) and *H. azteca* BCFs ($BCF_H$) was reported previously by Schlechtriem et al. (2019):

$$\log BCF_{F,5\%} = (0.792 \times logBCF_{H,5\%}) + 0.251 \qquad (R^2 = 0.687) \quad \text{(Equation 1)}$$

Equation 1 describes the BCF relationship between fish and *H. azteca* for BCFs expressed on a 5% lipid-basis (denoted by subscript 5%). European Chemicals Agency guidance states that *H. azteca* BCF data should be expressed on a 3% lipid basis due to the typically lower lipid content of amphipods (ECHA 2023). This can be applied by multiplying $BCF_{H,5\%}$ (Equation 1) by the ratio of the target (0.03 kg lipid kg organism$^{-1}$) and original (0.05 kg lipid kg organism$^{-1}$) lipid contents:

$$BCF_{H,\,3\%} = BCF_{H,5\%} \times (0.03/0.05) \qquad \text{(Equation 2)}$$

To expand upon the comparison done by Schlechtriem et al. (2019), we assembled an empirical data set containing $BCF_H$ data for neutral (non-ionizable) organic chemicals from published literature (see online supplementary material, Supporting Information S2, Table S1). Several of these empirical BCF values were previously compiled and summarized by Ebert et al. (2023), and more recently reported in the HYBIT ring trial document (OECD 2024b). Empirical $BCF_H$ data were standardized to 3% total lipid (Equation 2) and compared to 5% total lipid-adjusted fish BCFs collected from available literature and databases (Arnot and Gobas 2006) for the same test chemicals (see online supplementary material, Supporting Information S2, Table S2). No additional correction of the BCF values was made to account for inter-study variability. BCF values for fish and *H. azteca* used in this analysis were sourced from peer-reviewed publications and regulatory or pre-regulatory datasets, with a range of

experimental conditions. No further evaluation of the robustness or reliability of the BCF studies was undertaken in this study.

For this comparison, we assigned chemicals into different groups on the basis of their expected level of accumulation. Chemicals with measured logarithmic octanol-water partition ratios (log $K_{OW}$) less than or equal to 4.5 ($n$=11) were grouped together because they are expected to be screened as non-bioaccumulative in regulatory assessment scenarios. Chemicals with log $K_{OW}$ values exceeding 4.5 were grouped based on their ability to be biotransformed in fish. Specifically, chemicals with known bioaccumulation potential included recalcitrant organochlorines (e.g., DDT, chlorobenzenes; $n$=8) that have negligible to slow rates of biotransformation (i.e., biotransformation rate constants [$k_B$] between 0.002 and 0.031 day$^{-1}$ [see online supplementary material, Supporting Information S2, Table S3]). Chemicals exceeding log $K_{OW}$ 4.5 with moderate rates of biotransformation (i.e., $k_B$ between 0.10 and 0.75 day$^{-1}$ [see online supplementary material, Supporting Information S2, Table S3]) were also assigned to separate groups (e.g., hydrocarbons [$n$=4], pesticides [$n$=1], organic ultraviolet filters [$n$=2]) to evaluate differences in bioconcentration between *H. azteca* and fish.

*Model-based assessment of invertebrate and fish bioaccumulation potential*

We amended an existing food web bioaccumulation model (Arnot and Gobas 2004) to develop an evaluative mass-balance food chain model to investigate factors influencing chemical bioaccumulation in aquatic invertebrates and in fish. The term "evaluative" means that the model is generic and not tied to a specific food chain or environment. Here, we used the model to expand traditional BCF assessments to include BMFs and TMFs to enable evaluation of bioaccumulation across multiple trophic levels (Mackay et al. 2016). Specifically, our aim was to evaluate the conditions under which a chemical may be considered "bioaccumulative" (B; BCF $\geq$ 2,000 L $kg^{-1}$) or "very bioaccumulative" (vB; BCF $\geq$ 5,000 L $kg^{-1}$) on the basis of a BCF derived in an aquatic invertebrate relative to bioaccumulation metrics derived in higher trophic level fish species (i.e., BCF or BMF) or through the evaluative food chain (i.e., TMF). To our knowledge, other published aquatic food chain and food web bioaccumulation models do not include invertebrate organisms explicitly parameterized for *H. azteca*. However, some models similar include copepod or amphipod species (e.g., Arnot and Gobas 2004) or generic invertebrate organisms (USEPA 2009) of comparable sizes and feeding habits. We therefore parameterized a generic amphipod using physiological descriptors for *H. azteca* (Ebert et al. 2023; see online supplementary material, Table S4).

*H. azteca* typically ingest organic detritus and filamentous algae (Hargrave 1970) and their growth is well supported by unicellular algae/diatoms (Kennedy et al. 2016; Trochine et al. 2020). In the laboratory, *H. azteca* are cultured for both water and sediment toxicity tests (Soucek et al. 2016) and, reflecting the aqueous exposure conditions described in OECD TG 321, we assumed the model amphipod to be in the water column and feeding exclusively on unicellular algae and diatoms (i.e., phytoplankton). Accordingly, the food chain model includes a water column compartment with constant, homogeneous chemical concentrations and excludes benthic (sediment) compartments. This assumption therefore omits descriptions of

sediment-associated exposure pathways despite the epibenthic nature of *H. azteca*, which can influence chemical uptake and transfer in food chains.

In addition to phytoplankton and amphipod, the modelled aquatic ecosystem includes three fish species of varying size and diet, which were assumed for modelling purposes (see online supplementary material, Table S4), to span different trophic levels within this simple pelagic food chain. Trophic levels (TLs) were assigned, with phytoplankton at the base of the model food chain designated as TL 1 (Mackay et al. 2016). For consumers, TL was defined as (Vander Zanden and Rasmussen, 1996):

$$TL_j = \sum(P_i \times TL_i) + 1 \qquad \text{(Equation 3)}$$

where $TL_j$ is the trophic level of consumer *j*, $P_i$ is the fraction of the diet consisting of prey item *i*, and $TL_i$ is the trophic level of that prey. In this evaluative model, we assumed that each consumer fed exclusively on a single prey item in the TL below it (i.e., $P_i$=1.0) so TLs reduced to whole numbers that increased by one unit at each step of the food chain. Model organisms, their respective diets, and resulting TLs are summarized in Table 1.

For each organism *j*, we determined total whole-body chemical concentration at steady-state ($C_{Bj}$; mg chemical kg organism$^{-1}$) as:

$$C_{Bj} = \left([k_1 \times C_W \times \varphi_{FD}] + [k_D \times \sum P_i \times C_{D,i}]\right)/(k_2 + k_E + k_G + k_B) \qquad \text{(Equation 4)}$$

where $k_1$ (L water per kg organism$^{-1}$ day$^{-1}$) and $k_D$ (kg food kg organism$^{-1}$ day$^{-1}$) are rate constants that describe respiratory and dietary chemical uptake, respectively; $C_W$ and $C_D$ are the total chemical concentrations in water (mg chemical L water$^{-1}$) and the diet (mg chemical kg food$^{-1}$), respectively; $\varphi_{FD}$ is the fraction of freely dissolved chemical available for respiratory uptake (unitless). Multiplication of $C_W$ by $\varphi_{FD}$ estimates the freely dissolved water concentration in the model ecosystem. The first order rate constants (day$^{-1}$) $k_2$, $k_E$, $k_G$, and $k_B$

describe chemical elimination from the organism by respiration, fecal egestion, growth dilution, and biotransformation, respectively. For each organism, the terms $k_1$, $k_D$, $k_2$, $k_G$, $k_E$, and $\varphi_{FD}$ were calculated according to Arnot and Gobas (2004) and are summarized in online supplementary material, Table S4.

Equation 4 is used to model chemical bioaccumulation resulting from both dietary and respiratory exposure, thereby calculating chemical concentrations in each organism ($C_{Bj}$). The $C_{Bj}$ can be expressed on a lipid-equivalent basis ($C_{Bj,LE}$; mg chemical kg lipid-equivalent$^{-1}$) as $C_{Bj,LE} = C_{Bj}/LE_{Bj}$, where $LE_{Bj}$ is the whole body lipid equivalent content (kg lipid-equivalent kg organism$^{-1}$), and was previously described by Gobas et al. (2020) as:

$$LE_{Bj} = \varphi_{L,Bj} + \varphi_{P,Bj} \times \beta + \varphi_{NLOM,Bj} \times \theta + \varphi_{W,Bj}/K_{OW} \quad \text{(Equation 5)}$$

The composition ($\varphi$; kg component kg organism$^{-1}$) of total lipid (L), total protein (P), total non-lipid, non-protein organic matter (OM), and water (W) in each organism are displayed in Table 1. Proportionality constants $\beta$ and $\theta$ reflect the sorptive capacity of protein (0.05; deBruyn and Gobas 2007) and OM (0.035; Arnot and Gobas 2004), respectively, relative to that of lipid.

The logarithm of the $C_{Bj,LE}$ is regressed against organism trophic level (i.e., $TL_a$ [Equation 3]), and the trophic magnification factor (TMF) is derived from the slope of this relationship as $TMF = 10^{(slope)}$. A TMF > 1.0 is indicative of food web biomagnification, whereas a TMF < 1.0 is indicative of biodilution (Borgå et al. 2012). Biomagnification factors expressed on a lipid-equivalent normalized basis ($BMF_L$; kg lipid-equivalent food kg lipid-equivalent organism$^{-1}$) are derived from $C_{B,LE}$ in organism *j* relative to $C_{B,LE}$ in prey *i* as:

$$BMF_{Lj} = C_{Bj,LE}/C_{Bi,LE} \quad \text{(Equation 6)}$$

A $BMF_L > 1.0$ is indicative of biomagnification.

To isolate and assess bioconcentration processes within each organism in the model food chain, Equation 4 was arranged, omitting the term "($k_D \times [\sum P_i \times C_{D,i}]$)". This modification restricts chemical uptake to the aqueous route only and thereby yields the bioconcentration factor (BCF; L water kg organism$^{-1}$) consistent with the conditions of an experimental aqueous BCF test. The BCF is expressed on a freely dissolved basis as:

$$BCF = C_B/(C_W \times \varphi_{FD}) = k_1/(k_2 + k_E + k_G + k_B) \quad \text{(Equation 7)}$$

The food chain model was run for a set of hypothetical neutral organic chemicals with log $K_{OW}$ values ranging from 4 to 8. Whole-body biotransformation rate constants ($k_B$) were defined relative to a reference biotransformation rate constant ($k_{B,REF}$) assumed for a standard organism. Specifically, $k_{B,REF}$ was varied from 0 to 10 day$^{-1}$, where this range was assumed to apply to a 10 g fish at 15 °C, consistent with the default assumptions used in EPI Suite [USEPA 2012]).

To parametrize biotransformation across organisms within the simplified food chain, the $k_{B,REF}$ was scaled to organism-specific values based on body mass ($k_{B,N}$) according to Arnot et al. (2008) as:

$$k_{B,N} = k_{B,REF} \times \left(W_{B,N}/W_{B,REF}\right)^{-0.25} \quad \text{(Equation 8)}$$

where $W_{B,N}$ is the body mass of the organism $N$ (kg) and $W_{B,REF}$ is the reference body mass (0.01 kg, corresponding to a 10 g fish).

Due to the generally lower biotransformation capacity of invertebrates relative to fish observed for many organic chemicals (Bleeker and Verbruggen 2009; Kosfeld et al. 2020), fish-to-invertebrate $k_B$ scaling factors (denoted as "$k_B$ SF") ranging from 1 to 50 were applied to the assumed $k_B$ values of 0 to 10 day$^{-1}$ to generate a range of $k_B$ in the model amphipod. This method implies that the $k_B$ for a given chemical in an invertebrate may be up to 50 times lower

(indicating slower biotransformation) than in a fish of the same mass and temperature (OECD 2024). The $k_B$ SF values applied here are consistent with those used in model-based assessments (e.g., 3 [Arnot et al. 2023], 10 [OECD 2024]) and are within the reported range of biotransformation capacity differences between fish and *H. azteca* (i.e., 5 to 70; Kosfeld et al. 2020). These scaling factors were applied to evaluate how differences in the metabolic capacity of amphipods relative to fish can influence bioaccumulation dynamics in individual organisms and through food chains.

## RESULTS AND DISCUSSION

### *Comparison of empirical BCFs derived for fish and H. azteca reveals a non-linear relationship for biotransformed chemicals*

Schlechtriem et al. (2019) reported that bioconcentration tests with *H. azteca* yield higher BCFs than BCFs derived for fish when BCFs in both test organisms are expressed on a 5% lipid basis. Using the empirical relationship reported by Schlechtriem et al. 2019 (Equation 1), we derived BCFs for *H. azteca* from fish BCFs, and then applied Equation 2 to adjust the $BCF_H$ lipid content to 3% to be in line with ECHA R.11 Guidance (ECHA 2023). The resulting range of estimated $BCF_{H,3\%}$ values based on assumed $BCF_{F,5\%}$ values are provided in the Supporting Information (see online supplementary material, Supporting Information S2, Table S5). Conversion to a lower lipid content of 3% still yielded $BCF_{H,3\%}$ values that were approximately 1.4- to 3.3-fold greater than $BCF_{F,5\%}$ values (see online supplementary material, Table S5). Chemicals with a $BCF_{F,5\%}$ equal to the ECHA B (2,000 L $kg^{-1}$) or vB (5,000 L $kg^{-1}$) criteria corresponded to higher $BCF_{H,3\%}$ values of 4,306 and 13,714 L $kg^{-1}$, respectively (see online supplementary material, Table S5).

Since the Schlechtriem et al. (2019) relationship is based on a limited dataset (13 chemicals, log $K_{OW}$ 2.4–7.6), we expanded the analysis using all available empirical *H. azteca* BCF data for neutral organic chemicals (26 chemicals, log $K_{OW}$ 2.0–7.7; see online

supplementary material, Supporting Information S2, Table S1). Bioconcentration factors in *H. azteca* were expressed on a 3% lipid basis (Equation 2) and we compared them to 5% lipid-adjusted fish BCFs (see online supplementary material, Supporting Information S2, Table S2). Geometric mean BCFs for each test chemical in fish and *H. azteca* were then calculated from all available data for comparison (see online supplementary material, Supporting Information S2, Table S6). Variability in BCFs is expected due to differences in experimental design and study conditions, and this is partially reflected in the standard deviations generated for the geometric mean BCFs (see online supplementary material, Table S6).

As shown in Figure 1, the linear regression of the log-transformed BCFs for *H. azteca* and fish across all 26 chemicals resulted in a slope close to 1.0, indicating near equivalence ($\log BCF_{H,3\%} = 0.967 \times \log BCF_{F,5\%} + 0.213$; $R^2 = 0.76$ [see online supplementary material, Supporting Information S1, Figure S1A]). However, this correlation appears to be largely driven by the chemical groups at the upper and lower ends of the empirical BCF range. Lower hydrophobicity chemicals (i.e., $\log K_{OW} \leq 4.5$; purple data points in Figure 1) exhibit low BCFs both in *H. azteca* and fish (i.e., BCF < 2,000 L $kg^{-1}$) due to more rapid respiratory chemical elimination, and resulting in their categorization as non-bioaccumulative (nB) in both fish and *H. azteca*. Accordingly, BCF-based categorization is consistent (i.e., green shaded area; Figure 1), as these chemicals fall below the REACH $K_{OW}$ criterion of 4.5. At the upper extremes of the empirical BCF range, organochlorines with well-established bioaccumulation potential (red squares; Figure 1) show elevated BCFs in both test organisms, due to limited chemical biotransformation (see online supplementary material, Table S3) and thereby slow rates of elimination in aquatic organisms.

The majority of the organochlorines are considered as vB in *H. azteca* and fish and are therefore aligned in terms of their bioaccumulation categorization (i.e., blue shaded area; Figure 1). Two chemicals in this group (methoxychlor [MXC] and pentachlorobenzene [PeCB]

were categorized as B in one species but vB in the other. These differences in accumulation, and thus B/vB categorization, could reflect different chemical toxicokinetics in the two test organisms. For MXC, the 3.8-fold lower BCF in fish (2,593 L water $kg^{-1}$) relative to *H. azteca* (9,895 L water $kg^{-1}$) could be explained by biotransformation differences. Although MXC is biotransformed slowly ($k_B$ 0.026 $day^{-1}$; see online supplementary material, Supporting Information S2, Table S3), this could still reduce bioconcentration potential in fish relative to *H. azteca* if fish have a higher capacity for biotransformation. The 1.7-fold lower BCF for pentachlorobenzene in *H. azteca* (4,229 L water $kg^{-1}$) relative to fish (6,394 L water $kg^{-1}$) likely reflects the lower body lipid content of *H. azteca* (3%) relative to fish (5%). PeCB has a log $K_{OW}$ of 5.03 and is not significantly biotransformed by fish ($k_B$ 0.009 $day^{-1}$; see online supplementary material, Supporting Information S2, Table S3) so its accumulation in either organism would be dominated by organism-water equilibrium partitioning. This is supported with equilibrium BCFs (i.e., $BCF_E=[\varphi_L \times K_{OW}] + [\varphi_P \times \beta \times K_{OW}]$) derived for PeCB in *H. azteca* and fish (4,554 L water $kg^{-1}$ [*H. azteca*]; 6,429 L water $kg^{-1}$ [fish]) which are very similar to the geometric mean of empirical BCF values for PeCB (see online supplementary material, Table S6).

Chemicals outside of the "log $K_{OW} \leq 4.5$" and "organochlorine" chemical groups included four hydrocarbons (green triangles), a pesticide (blue square), and two organic ultraviolet filters (orange circles; Figure 1) that are all moderately biotransformed by fish (i.e., $k_B$ 0.10–0.75 $day^{-1}$; see online supplementary material, Supporting Information S2, Table S3). For these biotransformed chemicals, there was a poor correlation in log BCFs between *H. azteca* and fish ($R^2$=0.16; n=7; see online supplementary material, Supporting Information S1, Figure S1C), as compared to the $R^2 \geq 0.76$ when the correlation is driven by the inclusion of the non-bioaccumulative and very bioaccumulative chemicals (see online supplementary material, Supporting Information S1, Figure S1AB). For six of these seven biotransformed

chemicals, reported BCFs in fish were below 2,000 L $kg^{-1}$, while *H. azteca* BCFs exceeded regulatory criteria for B (2,000 L $kg^{-1}$; orange shaded area [Figure 1]) or vB (5,000 L $kg^{-1}$; red shaded area [Figure 1]). Higher BCFs in *H. azteca* relative to fish for these biotransformed chemicals are likely due to differences in metabolic capacity. Available d4ata for biotransformed neutral organic chemicals show different assessment outcomes, with empirical BCFs in *H. azteca* tending to be higher than those derived for fish.

Although enzymatic pathways may be conserved between invertebrates and vertebrates (Kosfeld et al. 2020), this does not necessarily imply equal capacities for biotransformation. Aquatic invertebrates are reported to have lower cytochrome P450 enzyme activity and biotransformation capacity than fish (Kane Driscoll et al. 1997; Landrum and Scavia 1983; Lotufo et al. 2000; Sims and Steevens 2008). This would reduce an invertebrate's ability to metabolize chemicals, like PAHs and other hydrocarbons (Bleeker and Verbruggen 2009). For example, pyrene is well-metabolized by fish (DiMauro 2018; Nichols et al. 2023) but is reported to have slower metabolism in aquatic invertebrates (van Hattum and Montañés 1999). The 6.5-fold lower BCF in fish (922 L $kg^{-1}$) relative to *H. azteca* (5,970 L $kg^{-1}$) could therefore be explained by biotransformation differences. Several other hydrocarbons also showed *H. azteca* BCF values 1.4–5.0 times higher than fish BCF values. Additional moderately biotransformed chemicals included one pesticide (chlorpyrifos [CPY; log $K_{OW}$ 4.66]) and two organic ultraviolet filers (UV-329 [log $K_{OW}$ 6.21] and UV-234 [log $K_{OW}$ 7.67]). Empirical BCFs for CPY and UV-329 were higher in *H. azteca* relative to fish. CPY is readily biotransformed in fish, supported by in vitro hepatic biotransformation studies (Cowan-Ellsberry et al. 2008) and extensive fish BCF data ($n$=75; see online supplementary material, Supporting Information S2, Table S2). For UV-234 and UV-329, empirical in vivo $k_B$ values in rainbow trout are 0.06 and 0.17 $d^{-1}$, respectively, and BCFs reported in the same study were 1,063 L $kg^{-1}$ (UV-234) and 361 L $kg^{-1}$ (UV-329; Leubner et al. 2023).

Currently, the data set for neutral organic chemicals is limited ($n = 26$), making it difficult to determine when and to what extent HYBIT-derived BCFs are comparable to BCFs measured in fish. Although the ability of aquatic invertebrates to metabolize chemicals is generally assumed to be lower than that of fish, the exact magnitude and chemical specificity of these differences are uncertain. In this context, we use modelling to examine mechanistically how differences in biotransformation may translate into different bioaccumulation outcomes between fish and *H. azteca*.

*Comparison of modelled fish BCFs to amphipod BCFs indicates potential misalignment in bioaccumulation assessments across test species*

We applied the food chain bioaccumulation model to assess chemical (i.e., $K_{OW}$, $k_B$) and biological (i.e., fish-to-invertebrate $k_B$ scaling factor [$k_B$ SF]) parameters that can influence bioaccumulation in fish and *H. azteca* for hypothetical chemicals ranging in log $K_{OW}$ between 4 and 8. Although food chain models are typically used to assess dietary uptake and biomagnification, they also provide a framework for isolating individual uptake pathways. Here the model was used to isolate aqueous exposure (Equation 7) to derive BCFs comparable to those derived in experimental BCF tests. How these BCFs relate to dietary uptake and biomagnification is examined in the subsequent sections.

Modelled BCFs for small fish are presented in the main text because they best reflect the size of fish typically used in regulatory BCF studies (i.e., 10 g; Arnot and Gobas, 2006). Modelled outputs for all organisms are provided in Supporting Information (see online supplementary material, Supporting Information S2, Table S7). At log $K_{OW}$ 4, modelled BCFs in amphipod and fish at each trophic level fell below the BCF criterion of 2,000 L kg$^{-1}$ (see online supplementary material, Supporting Information S1, Figure S2). This outcome is consistent with the empirical dataset (Figure 1) and reflects more rapid elimination via the gills for lower hydrophobicity chemicals.

At log $K_{OW}$ 5 under negligible or slow biotransformation ($k_B \leq 0.01$ day$^{-1}$) and assuming equal metabolic capacity (1:1) in fish and invertebrate ($k_B$ SF = 1), modelled amphipod BCFs were approximately 4,000 L kg$^{-1}$, whereas values for fish exceeded 5,000 L kg$^{-1}$ (e.g., 5,110 L kg$^{-1}$ for small fish at $k_B = 0$ day$^{-1}$ [see online supplementary material, Table S7]). These differences reflect the lower lipid-equivalent content of amphipods compared to fish (Table 1). Under these "low" biotransformation conditions, bioconcentration is largely controlled by organism-water partitioning and BCFs scale proportionally (1:1) with organism lipid content (Armitage et al. 2021). Additionally, amphipod BCFs are only weakly sensitive to assumed reductions in metabolic capacity. For example, increasing the $k_B$ SF from 1 to 50 (at $k_B = 0.01$ day$^{-1}$) produced only a modest rise in amphipod BCF values (i.e., from 3,978 to 4,158 L kg$^{-1}$).

At log $K_{OW}$ 5, increasing $k_B$ reduces fish BCFs to values well below regulatory thresholds, whereas in amphipods the extent of BCF reduction depended strongly on the assumed $k_B$ SF. When amphipod biotransformation is ≥ 5-fold slower than in fish (i.e., $k_B$ SF ≥ 5), BCFs remain above 2,000 L kg$^{-1}$, while fish BCFs fall well below this threshold. Under these conditions, amphipod BCFs exceed those in fish by up to an order of magnitude. For example, at $k_B$ 1.0 day$^{-1}$ and $k_B$ SF ≥ 5 amphipod BCFs are 5.8- to 10.3-fold higher than BCFs for small fish (Figure 2).

For higher hydrophobicity (log $K_{OW} \geq 6$) chemicals, modelled BCFs in both amphipod and fish exceed 5,000 L kg$^{-1}$ at negligible levels of biotransformation ($k_B \leq 0.001$ day$^{-1}$). At $k_B$ 0 day$^{-1}$, modelled BCFs in amphipod are 38,158 L kg$^{-1}$ (log $K_{OW}$ 6), 244,967 L kg$^{-1}$ (log $K_{OW}$ 7) and 779,391 L kg$^{-1}$ (log $K_{OW}$ 8) and exceed values derived for fish. Modelled amphipod BCFs exceed those derived for fish at log $K_{OW} \geq 6$ because the model estimates that fish have a higher capacity for faecal elimination relative to gill elimination compared to amphipods (see online supplementary material, Supporting Information S1, Figure S3). At log $K_{OW} \geq 6$,

elimination via faeces dominates over gill exchange, and BCFs no longer scale proportionally (1:1) with organism lipid content.

The influence of $k_B$ on modelled amphipod and fish BCF values becomes more apparent at log $K_{OW} \geq 6$. Higher assumed biotransformation rates greatly reduce modelled bioconcentration, as rates of elimination via other pathways, particularly respiratory elimination, are much slower by comparison. Furthermore, reducing amphipod metabolic capacity at $k_B \geq 0.10$ $d^{-1}$ to levels that are 3- to 50-fold slower than fish (i.e., $k_B$ SF 3 to 50) drastically increases predicted BCFs (see online supplementary material, Supporting Information S2, Table S7). Several of these modelled amphipod BCF values would screen as B or vB in regulatory assessment scenarios while they would be screened as nB in fish, showing potential for misalignment in bioaccumulation assessment outcomes (shaded orange and red areas; Figure 2). For example, at log $K_{OW} \geq 6$ when $k_B$ SF is set to 10 at $k_B$ 1.0 $day^{-1}$, modelled BCFs in amphipods are 7,337 L $kg^{-1}$ (log $K_{OW}$ 6), 8,760 L $kg^{-1}$ (log $K_{OW}$ 7), and 8,980 L $kg^{-1}$ (log $K_{OW}$ 8). At log $K_{OW} \geq 6$ and $k_B$ 1.0 $day^{-1}$, modelled BCFs for small fish are 398 L $kg^{-1}$ (log $K_{OW}$ 6), 402 L $kg^{-1}$ (log $K_{OW}$ 7), and 403 L $kg^{-1}$ (log $K_{OW}$ 8) and are each approximately 20-fold lower than the amphipod BCF values (Figure 2). Bioconcentration differences between amphipod and fish at $k_B$ SF 10 are slightly more pronounced in larger, higher trophic level fish (e.g., between 30 to 50-fold lower in medium and large fish at log $K_{OW} \geq 6$ and $k_B$ 1.0 $day^{-1}$ [see online supplementary material, Supporting Information S2, Table S7]).

Overall, for hydrophobic chemicals with moderate to high biotransformation rates ($k_B \geq$ 0.1 $day^{-1}$), amphipod BCFs exceed regulatory B and vB criteria, whereas fish BCFs are comparably lower and fall into different B categories (typically nB). This divergence is both $K_{OW}$ and $k_B$-dependent, and becomes more pronounced with increasing biotransformation scaling factors (i.e., $k_B$ SF $\geq 5$). Additionally, at log $K_{OW}$ 5, amphipod BCFs were lower than fish BCFs for poorly biotransformed chemicals. These findings highlight potential

misalignment in bioaccumulation assessment outcomes when amphipod BCFs substitute fish data.

*Dynamics of bioconcentration and biomagnification in model aquatic organisms*

The bioaccumulation model employed here considers aqueous and dietary chemical uptake into organisms (Equation 4). To determine whether bioconcentration or biomagnification is the dominant process controlling chemical accumulation in each organism, we calculated the relative contributions of respiratory (i.e., $k_1 \times C_W \times \varphi_{FD}$) and dietary (i.e., $k_D \times \sum P_i \times C_{D,i}$) uptake on the chemical body burden (i.e., $C_{Bj}$; Equation 4) in amphipods and fish (see online supplementary material, Supporting Information S1, Text S1). Figure 3 uses stacked bar plots to illustrate, that for each organism, the relative contributions of bioconcentration (blue) and biomagnification (green) to $C_{Bj}$, under the assumption of no biotransformation (i.e., $k_B = 0\ d^{-1}$).

At log $K_{OW}$ 4, chemical uptake and loss is primarily by respiration. At this hydrophobicity, accumulation within aquatic species is driven largely by bioconcentration, which accounts for the majority of the resulting $C_B$ in each organism (73 to 100%; Figure 3). When dietary uptake is taken into account at log $K_{OW}$ 4, its impact on overall body burden ($C_B$) is minimal, producing less than a 1.4-fold difference compared to when only respiratory uptake is considered (see online supplementary material, Table S8).

With increasing hydrophobicity and trophic level, the dominant uptake pathway shifts from respiratory to dietary uptake (Figure 3) and biomagnification becomes the main determinant of $C_{Bj}$. For example, at log $K_{OW}$ 6, dietary uptake contributed to 25 % of the $C_B$ in amphipods, which was substantially lower than its contribution in fish (i.e., 86%, 98%, 100% in small, medium, and large fish, respectively). At log $K_{OW}$ 7, the maximum contribution of dietary uptake to $C_B$ in amphipods was 49% whereas in fish the maximum contributions were above 95%. At log $K_{OW}$ 8, the contribution of dietary uptake on $C_B$ declined in all species

(although only by a small amount in medium and large fish) due to the decline in dietary chemical absorption efficiencies with increasing $K_{OW}$ (Arnot and Gobas 2004). These results align with well-established knowledge of the processes governing chemical accumulation in aquatic organisms (Thomann 1989, Arnot and Gobas 2003; 2004; Mackay et al. 2013), highlighting the relevance of dietary uptake for chemicals with log $K_{OW}$ values greater than 5. Accordingly, in our evaluation of modelled BMFs and TMFs, we focused our assessment on simulations conducted at log $K_{OW}$ between 5 and 8.

*Comparison of modelled fish BMFs to aquatic BCFs shows amphipod-based bioconcentration assessments may not reflect potential for biomagnification*

Lipid-equivalent normalized biomagnification factors ($BMF_L$; Equation 6) for modelled amphipod and fish are summarized in online supplementary material, Table S9. When biotransformation is negligible (i.e., $k_B \leq 0.001$ day$^{-1}$), $BMF_L$ values exceeded 1.0 at log $K_{OW}$ between 5 and 8 across all organisms, indicating biomagnification via dietary uptake. As biotransformation is increased, $BMF_L$ values fall below 1.0 at $k_B \geq 0.1$ day$^{-1}$ at log $K_{OW}$ between 5 and 7, and at $k_B \geq 0.01$ day$^{-1}$ at log $K_{OW}$ 8, reflecting the increasing dominance of biotransformation over other elimination pathways (i.e., fecal egestion, gill elimination). Under these conditions, $BMF_L$ values were below 1.0 in all fish (see online supplementary material, Table S9). Values were marginally higher in medium (1.7-fold on average) and large fish (2.1-fold on average) than in small fish (see online supplementary material, Table S9), reflecting their greater lipid-equivalent content and body size (Table 1), which slows chemical elimination (Arnot and Gobas 2004).

Modelled BCFs for amphipods and fish were compared with fish $BMF_L$ values to evaluate how BCF-based assessment outcomes align with biomagnification potential (Figure 4). Although the BCF is a measure of direct uptake from water rather than biomagnification,

BCF thresholds continue to serve as a regulatory trigger for bioaccumulation assessments, making this comparison relevant.

At log $K_{OW}$ 5 and $k_B \leq 0.01$ day$^{-1}$, modelled $BMF_L$ and BCF values in small fish were at or above the regulatory thresholds (i.e., $BMF_L$ 1.42–1.32; BCF 4,571–5,155), whereas, amphipod BCFs were lower (BCF 4,013–4,198; $k_B$ SF $\geq 1$) reflecting their lower lipid-equivalent content and the dominance of respiratory exchange in controlling accumulation. At log $K_{OW} \geq 6$, amphipod BCFs values exceed those for fish under low biotransformation conditions (and $k_B \leq 0.01$ day$^{-1}$; Figure 4), consistent with the BCF patterns described above. Under moderate biotransformation conditions ($k_B \geq 0.1$ day$^{-1}$), $BMF_L$ values in small fish were below 1.0, indicating low biomagnification potential, whereas BCFs for amphipods frequently exceeded regulatory REACH B and vB criteria, particularly when metabolic capacity was reduced ($k_B$ SF $\geq 1$).

Gobas et al. (2025) report that substances with $BMF_L > 1.0$ tend to show BCF > 5,000, which applied here in all cases for fish (Figure 4). For amphipods, this practical guideline did not hold. At log $K_{OW}$ 5 and $k_B \leq 0.01$ day$^{-1}$, amphipod BCFs remained below 5,000 and did not adequately screen for biomagnification in fish. Conversely, at log $K_{OW}$ between 5 and 8 and $k_B \geq 0.1$ day$^{-1}$, fish $BMF_L$ values fell below 1.0, whereas amphipod BCFs often exceeded the vB threshold. Overall, amphipod BCFs did not consistently reflect biomagnification behaviour in fish, tending to overestimate bioaccumulation potential for highly hydrophobic biotransformed chemicals but underestimate it for poorly biotransformed chemicals of moderate hydrophobicity (i.e., log $K_{OW}$ 5).

*Comparison of modelled TMFs to modelled aquatic BCFs shows amphipod-based bioconcentration assessments may not reflect potential for trophic magnification*

Trophic magnification factors (TMF) derived using the evaluative food chain model show the same overall patterns as those described for the $BMF_L$ values. When

biotransformation is minimal (i.e., $k_B \leq 0.001$ day$^{-1}$), TMF values exceeded 1.0 at log $K_{OW} \geq 5$ (Figure 5), indicating magnification through the food chain. As biotransformation rates increased, TMF values declined in a manner consistent with the $BMF_L$ results described above. At log $K_{OW}$ between 5 and 7 and $k_B \geq 0.1$ day$^{-1}$, TMFs fall below 1.0 indicating trophic dilution rather than magnification. At log $K_{OW}$ 8, slower rates of biotransformation ($k_B \geq 0.01$ day$^{-1}$) are sufficient to bring TMFs below 1.0.

Comparison of modelled TMF values to BCFs for amphipods and fish reveals a systemic misalignment for amphipods (Figure 5). At log $K_{OW}$ 5 and $k_B \leq 0.01$ day$^{-1}$, amphipod BCFs remain below 5,000 even though TMF values suggest biomagnification through the food chain (TMF > 1.0). Under these conditions, amphipod-based BCF screening would not indicate bioaccumulation, even though trophic magnification is evident in the modelled food chain. In contrast, at log $K_{OW} \geq 5.0$ and $k_B \geq 0.1$ day$^{-1}$, amphipod BCFs frequently exceed the BCF 5,000 threshold, even though TMF values indicate trophic dilution (TMF < 1.0). This pattern is consistent with the divergence observed between amphipod BCFs and the fish-based BCF (Figure 2) and $BMF_L$ (Figure 4) metrics. The misalignment between amphipod BCFs and food chain TMFs becomes more pronounced when $k_B$ SF increases from 1 to 50.

Across the evaluated chemical space, BCFs for small fish were generally consistent with TMF outcomes, with high fish BCFs corresponding to TMF > 1.0. In contrast, amphipod-derived BCFs showed limited correspondence with TMF-based categorization, tending to underestimate bioaccumulation of poorly biotransformed substances at log $K_{OW}$ 5, or overestimate bioaccumulation for biotransformed substances that do not biomagnify.

While the food chain model applied here is evaluative and not parameterized to a specific ecosystem, the generated TMF patterns are consistent with broad empirical observations of chemical trophodynamics in aquatic food webs. For example, a large-scale meta-analysis of empirical TMF values indicates that biomagnification predominantly occurs for very slowly or

negligibly for biotransformed hydrophobic compounds, whereas more rapidly biotransformed chemicals generally exhibit biodilution (Walters et al., 2016). Furthermore, field (Mackintosh et al., 2004) and modelling studies (Arnot and Gobas 2003; Webster and Ellis 2012) of food webs demonstrate that greater biotransformation capacity in predators can result in concentrations lower than those in their prey. This general consistency of these trends to those observed in the present model supports its use for comparative evaluation of bioaccumulation outcomes.

We also note that derived TMFs can be sensitive to the model configuration. To align with the HYBIT conditions, we assumed an "aqueous-only" exposure for a pelagic food chain and excluded sediment-associated exposure. For superhydrophobic chemicals, sediment-associated exposure can contribute substantially to total dietary uptake and trophic magnification. However, in natural systems, chemical disequilibrium between sediment and water may reduce uptake into benthic organisms and thereby lower TMFs (Mackay et al. 2016). These processes can contribute to variability in field-derived TMFs (Gobas and MacLean 2003; McLeod et al. 2014), and it has been suggested that modelled TMFs may, in some cases, be more robust when based on pelagic species only (Mackay et al. 2016). Taken together, these considerations highlight the need for careful interpretation when applying results from simplified food chain representations to more complex ecosystems.

In this context, the TMF analysis indicates that BCFs derived from *H. azteca* may not consistently reflect food web bioaccumulation behaviour across different hydrophobicity and biotransformation scenarios. These results highlight the importance of considering trophic level-integrated metrics when interpreting amphipod-based BCFs in the context of bioaccumulation protection goals. Because TMFs quantify bioaccumulation through entire food webs, they are widely regarded to provide the most conclusive evidence of a chemical's bioaccumulation potential. Consistent with this, Gobas et al. (2009) proposed incorporating

food web bioaccumulation models into regulatory assessment frameworks, to enable a holistic assessment of bioaccumulation at multiple trophic levels. Although illustrated here using a simplified evaluative food chain, the same concepts can be extended to more complex food webs or tailored to specific organisms or ecosystems, provided that key processes, such as exposure pathways and trophic interactions, are appropriately represented.

*Alignment of the HYBIT-derived BCFs with bioaccumulation protection goals*

Our assessment indicates that amphipod (*H. azteca*) BCF data can inform evaluations of chemical bioaccumulation potential. However, both the empirical and model-based assessments indicate that amphipod BCF data applied according to REACH BCF criteria may overestimate the bioaccumulation potential of biotransformed chemicals compared with metrics derived for higher trophic level fish (i.e., BCFs, BMFs) and through food chains (TMFs). This misalignment conflicts with certain scientific definitions of bioaccumulation, which consider a substance as bioaccumulative if it biomagnifies in food chains, a concept believed to have guided the development of the bioaccumulation criteria under the Stockholm Convention (UNEP 2001) on persistent organic pollutants (Gobas et al. 2009).

Notably, within the evaluated chemical space of the model assessment (i.e., neutral organics of varying hydrophobicity), the opposite case arises at log $K_{OW}$ 5 and $k_B \leq 0.01$ day$^{-1}$ where amphipod BCFs remain below 5,000 even though TMF and $BMF_L$ values suggest biomagnification in fish. This was also observed in the empirical dataset for pentachlorobenzene (log $K_{OW}$ 5.03) where the BCF in *H. azteca* (4,229 L water kg$^{-1}$) was 1.70-fold lower than in fish (6,394 L water kg$^{-1}$). At this $K_{OW}$, bioconcentration in *H. azteca* and fish is dominated by organism-water equilibrium partitioning and BCFs scale proportionally (1:1) with organism lipid content. However, at log $K_{OW} \geq 6$, when passive elimination no longer controls bioaccumulation processes, the model predicts higher BCFs in amphipods than in fish. In this higher hydrophobicity range, fecal egestion becomes the primary elimination route (see

online supplementary material, Figure S3), and BCFs no longer scale 1:1 with organism lipid content.

The European Chemicals Agency R.11 Guidance (ECHA 2023) adjusts fish BCF thresholds of 2,000 and 5,000 (for 5% total lipid) to 1,200 and 3,000 for *H. azteca* (3% total lipid), but our assessment indicates that this proportional scaling cannot be applied broadly across chemical space and warrants consideration. Beyond differences in total lipid, differences in lipid (e.g., membrane) and protein (e.g., serum albumin, structural proteins) composition may influence the bioaccumulation of certain chemical classes. Extending this evaluation to other chemistries (e.g., ionizable chemicals) could provide additional insights. Furthermore, systematic comparisons of biotransformation characteristics (e.g., enzyme activities, biotransformation pathways, biotransformation rate constants) in *H. azteca* and fish would further clarify of how differences in metabolic capacity influence bioaccumulation potential in these test organisms.

The European Chemicals Agency currently accepts fish and invertebrate BCF data for regulatory bioaccumulation assessments in Europe (ECHA 2023). Reducing vertebrate testing is an important ethical and policy objective, however alternative approaches must still deliver reliable and relevant estimates of bioaccumulation. Balancing these two objectives requires explicit recognition of the value judgements in both regulatory and scientific assessments. To minimize inconsistent assessment outcomes, test selection should be informed by chemical property information (i.e., $K_{OW}$, biotransformation potential) and environmental behaviour (i.e., food web trophodynamics, environmental mass balance modelling considerations). Within this context, regulatory frameworks could benefit from greater flexibility in test selection or the adoption of tiered assessment approaches to ensure that the use of invertebrate alternatives supports policy goals without undermining the scientific robustness of bioaccumulation assessments.

A more integrated approach of multiple types of test data could better reflect chemical behaviour in the environment and thereby enable more robust chemical bioaccumulation assessments (van Wijk et al. 2009). Because aquatic invertebrates may lack some metabolic pathways present in fish and wildlife (Glüge et al. 2023; Schlechtriem et al., 2019), amphipod BCF data could be combined with, or trigger the collection of, additional biotransformation and bioaccumulation information from higher trophic level organisms. *In silico* models (Arnot et al. 2009; Brown et al. 2012) and in vitro assays (Laue et al. 2023; Saunders et al. 2019) are examples of NAMs that enable both rapid and cost-effective means of generating biotransformation data for bioaccumulation assessments. This biotransformation information can be combined with organismal or food web bioaccumulation models to assess a chemical's bioaccumulation potential beyond single-species BCF tests. Finally, where uncertainties remain, in vivo studies in vertebrates (i.e., fish) may serve as a higher-tier option to confirm bioaccumulation estimates.

To make the best use of all available evidence, we support comprehensive weight-of-evidence frameworks that integrate diverse bioaccumulation data types (i.e., empirical and modelled, vertebrate and invertebrate) across different organisms and ecosystems (Arnot et al. 2023; OECD 2023). Such frameworks enable critical evaluation of the integrated data and can support clear and transparent decision making in bioaccumulation assessment schemes.

*Conclusions*

This study combined empirical data analysis with an evaluative food-chain bioaccumulation model to compare BCFs derived in *H. azteca* to bioaccumulation metrics derived for fish, showing how chemical biotransformation controls bioaccumulation in both test organisms. Empirical comparisons showed that BCFs in *H. azteca* and fish are broadly

aligned for non-bioaccumulative substances and for highly hydrophobic, slowly metabolized organochlorines. However, for moderately to rapidly biotransformed chemicals, *H. azteca* BCFs were frequently substantially higher than corresponding fish BCFs and often exceeded regulatory bioaccumulation thresholds. In contrast, *H. azteca* BCFs underestimated bioaccumulation potential in fish for moderately hydrophobic, poorly biotransformed chemicals. Model simulations demonstrated that discrepancies between BCFs may arise from fundamental differences in metabolic capacity between invertebrates and fish. Evaluation against BMFs and TMFs further showed that amphipod-derived aqueous BCFs do not consistently reflect bioaccumulation behaviour at the food-web level, particularly for biotransformed substances and for chemicals whose accumulation is dominated by dietary uptake.

While *H. azteca* BCF data can provide useful screening information, exclusive reliance on such data for regulatory classification may lead to assessment outcomes that are misaligned with bioaccumulation protection goals centered on trophic magnification. These findings support the use of tiered, weight-of-evidence bioaccumulation frameworks in which invertebrate BCFs should be interpreted alongside biotransformation data, dietary bioaccumulation metrics, and food-web modelling. Such integrated approaches can help reconcile efforts to reduce animal use in chemical assessments while reliably assessing bioaccumulation potential.

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**Figure 1.** The left panel compares *H. azteca* (3% lipid) and fish (5% lipid) geometric mean (± SD) bioconcentration factors (BCF) for 26 chemicals. Data points represent chemicals with log $K_{OW} \leq 4.5$ (purple diamonds), hydrocarbons (green triangles), organochlorines (red squares), and pesticides (blue squares), and organic ultraviolet filters (yellow circles). The solid black line is the line of identity; dotted-dashed lines correspond to a 3.1-fold variation (0.5 log units). Red dashed and solid lines represent regulatory BCF criteria of 2,000 and 5,000 L $kg^{-1}$, respectively. Shaded areas indicate different BCF categorization scenarios for fish and *H. azteca* (defined in the figure). The grey boxed area (asterisked) emphasizes BCF data for biotransformed chemicals and for the organochlorines methoxychlor, and pentachlorobenzene shown in the right panel. Abbreviated chemical names: BAP = benzo[a]pyrene, CPY = chlorpyrifos, FLA = fluoranthene, MXC = methoxychlor, PeCB = pentachlorobenzene, PYR = pyrene, o-TER = o-terphenyl, UV-234 = (2-(2H-Benzotriazol-2-yl)-4,6-bis(1-methyl-1-phenylethyl)phenol), UV-329 = 2-(2'-hydroxy-5'-teroctyl)phenylbenzotriazole. Other abbreviations: vB = very bioaccumulative, B = bioaccumulative, nB = non-bioaccumulative

**Figure 1 alt text:** Two-panel scatter plot comparing geometric mean bioconcentration factors (BCF) in fish (x-axis, log scale) and *Hyalella azteca* (y-axis, log scale) for 26 chemicals. Points are colour- and shape-coded by chemical class (log $K_{OW} \leq 4.5$, hydrocarbons, organochlorines, pesticides, and UV filters). Horizontal and vertical lines mark regulatory thresholds at 2,000 and 5,000 L $kg^{-1}$, defining regions of non-bioaccumulative (nB), bioaccumulative (B), and very bioaccumulative (vB). Shaded areas highlight differing classification outcomes between

species. The right panel zooms into selected compounds (e.g. hydrocarbons), showing deviations from the identity line.

**Figure 2.** Modelled bioconcentration factors (BCF) expressed on a freely dissolved basis in amphipod relative to modelled BCFs in small fish. BCFs were modelled at different assumed levels of biotransformation in fish ($k_B$; represented by the shapes of the data points) and different assumed fish-to-invertebrate scaling factors ($k_B$ SF; represented by different data point and line colours). The red lines represent regulatory BCF criteria of 2,000 L kg$^{-1}$ (dashed red line) and 5,000 L kg$^{-1}$ (solid red line). The red, orange, yellow, green, and blue shaded areas denote different bioaccumulation categorization scenarios based on bioaccumulation regulatory criteria for fish and invertebrates (defined in Figure 1).

**Figure 2 alt text:** Four-panel scatter plots compare modelled bioconcentration factors (BCF) in small fish (x-axis) and amphipods (y-axis) across increasing hydrophobicity (log $K_{OW}$ 5–8). Points and lines show different assumptions about biotransformation and scaling. Amphipod BCFs generally increases with fish BCF, with values shifting depending on assumptions. Red lines mark regulatory thresholds (2,000 and 5,000 L kg$^{-1}$), and shaded areas denote different bioaccumulation categorization scenarios based on regulatory BCF criteria.

**Figure 3.** Estimated proportional contributions of bioconcentration (Equation S7) and biomagnification (Equation S8) to chemical concentrations ($C_B$; Equation 4) in model amphipod and fish relative to log $K_{OW}$ (assuming no biotransformation and no scaling for invertebrate metabolic capacity, $k_B$=0 day$^{-1}$; $k_B$ SF=1). The proportional influence of bioconcentration (blue) and biomagnification (green) on $C_B$ is described in Supporting Information S1, Text S1.

**Figure 3 alt text:** Four-panel stacked bar charts show the relative contributions of bioconcentration (blue) and biomagnification (green) to chemical concentrations in amphipods and fish across increasing log $K_{OW}$ (4–8). Bioconcentration dominates at low log $K_{OW}$, while biomagnification becomes more important at higher log $K_{OW}$, especially in fish**.**

**Figure 4.** Modelled bioconcentration factors (BCF) in amphipod and small fish (purple data points) relative to biomagnification factors (BMF) modelled for small fish. BCFs and BMFs were modelled at different assumed levels of biotransformation in fish ($k_B$) and different assumed fish-to-invertebrate scaling factors ($k_B$ SF). The lines represent bioaccumulation criteria of BCF 2,000 L kg$^{-1}$ (dashed red line), BCF 5,000 L kg$^{-1}$ (solid red line), and BMF 1 (solid black line). The red, orange, yellow, green, and blue shaded areas denote different bioaccumulation categorization scenarios based on regulatory bioaccumulation criteria for fish and invertebrates (defined in Figure 1).

**Figure 4 alt text:** Four-panel scatter plots compare modelled bioconcentration factors (BCF) in amphipods and small fish (y-axis) with biomagnification factors (BMF) in small fish (x-axis) across increasing log KOW (5–8). Points show different model assumptions. BCF generally increases with BMF, with many values near or above regulatory thresholds. Red lines mark BCF criteria (2,000 and 5,000 L kg$^{-1}$) and a black line marks BMF = 1; shaded areas denote different bioaccumulation categorization scenarios based on regulatory bioaccumulation criteria.

**Figure 5.** Modelled bioconcentration factors (BCF) in amphipod and small fish (purple data points) relative to trophic magnification factors (TMF) modelled the evaluative food chain. BCFs and TMFs were modelled at different assumed levels of biotransformation in fish ($k_B$) and different assumed fish-to-invertebrate scaling factors ($k_B$ SF). The lines represent bioaccumulation criteria of BCF 2,000 L kg$^{-1}$ (dashed red line), BCF 5,000 L kg$^{-1}$ (solid red line), and BMF 1 (solid black line). The red, orange, yellow, green, and blue shaded areas denote different bioaccumulation categorization scenarios based on regulatory bioaccumulation criteria for fish and invertebrates (defined in Figure 1).

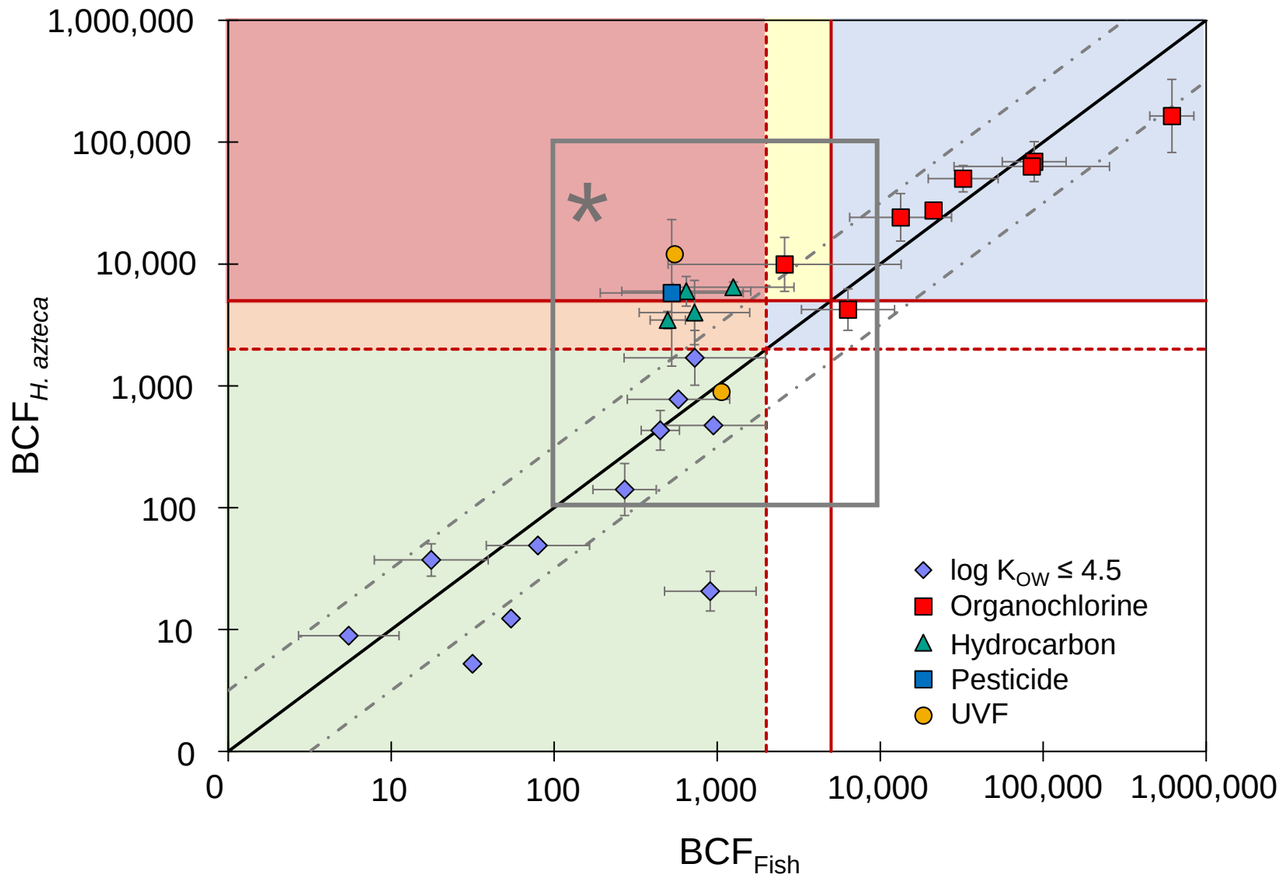
**Figure 5 alt text:** Four-panel scatter plots compare modelled bioconcentration factors (BCF) in amphipods and small fish (y-axis) with trophic magnification factors (TMF; x-axis) across log KOW 5–8. BCF generally increases with TMF, with results varying by model assumptions. Red lines show BCF thresholds (2,000 and 5,000 L $kg^{-1}$) and shaded areas denote different bioaccumulation categorization scenarios based on regulatory bioaccumulation criteria.

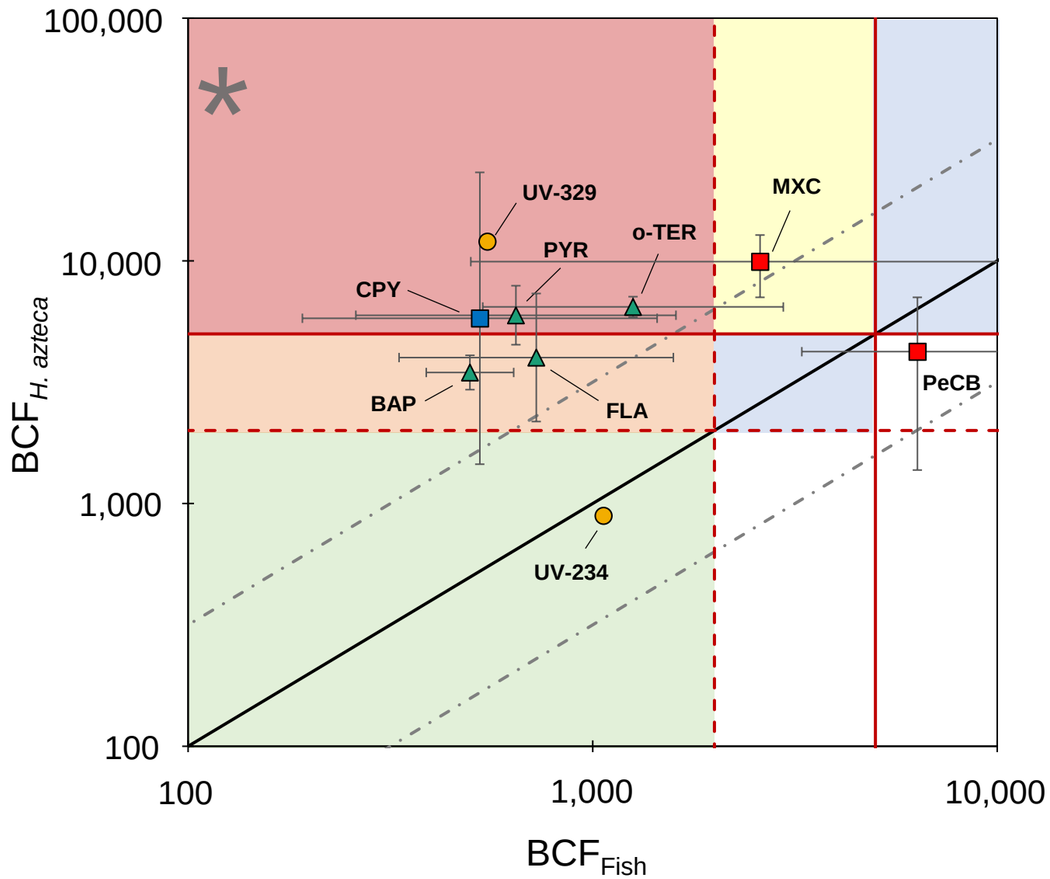
## TABLES

Table 1. Characteristics of evaluative food chain model species.

| Species | Phytoplankton | Amphipod | Small fish | Medium fish | Large fish |
|---|---|---|---|---|---|
| Weight ($W_B$; g) | 0.00007 | 0.003 | 10 | 250 | 2500 |
| Diet | Not applicable | 100% PH | 100% AM | 100% SF | 100% MF |
| Trophic Level (TL) | 1 | 2 | 3 | 4 | 5 |
| Lipid content ($\varphi_L$; kg kg$^{-1}$) | 0.010 | 0.030 | 0.050 | 0.075 | 0.100 |
| Protein content ($\varphi_P$; kg kg$^{-1}$) | 0.000 | 0.250 | 0.150 | 0.150 | 0.150 |
| OM content ($\varphi_{OM}$; kg kg$^{-1}$) | 0.060 | 0.000 | 0.000 | 0.000 | 0.000 |
| Water content ($\varphi_W$; kg kg$^{-1}$) | 0.930 | 0.730 | 0.800 | 0.775 | 0.750 |

Abbreviations: OM = total non-lipid, non-protein organic matter; PH = phytoplankton; AM = amphipod; SF = small fish; MF = medium fish




| vB in *H. azteca* nB in fish | B in *H. azteca* nB in fish | vB in *H. azteca* B in fish | nB in both *H. azteca* and fish | B or vB in both *H. azteca* and fish |
|---|---|---|---|---|