Bioaccumulation assessment of air-breathing mammals: a discussion paper

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Content

1. Background

In October 2019, ECHA's Persistence, Bioaccumulation and Toxicity Expert Group (PBT EG) agreed that a working group should be set up to discuss the use of toxicokinetic data for the prioritisation of substances which may bioaccumulate in terrestrial organisms. This group is led by Eric Verbruggen (RIVM/the Netherlands), Caren Rauert (UBA/Germany) and Doris Hirmann (ECHA, Finland) and includes members from academia, industry, and government. The group first convened in the beginning of 2020.

Bioaccumulation is the net result of competing rates of chemical uptake and elimination in an organism (Arnot and Gobas 2006). Bioaccumulation can be evaluated based on referencing chemical concentrations in an organism to its surrounding environment or its diet in a few different contexts:

(1) *bioaccumulation (quantified as bioaccumulation factor; BAF)*; the uptake of chemicals from environmental media including dietary uptake, followed by an increase in concentration in the organism compared to the environmental media

(2) *bioconcentration (quantified as bioconcentration factor; BCF)*; for the accumulated contaminant resulting solely from uptake through respiratory surfaces (body surface, tracheal tubes, gills, lungs), and

(3) *biomagnification (quantified as biomagnification factor; BMF);* the transfer and accumulation (in the sense of increased chemical activity or concentration) of chemicals along trophic levels in a food web (Connolly and Pedersen 1988, van Leeuwen et al., 2007, Radomyski et al., 2018).

The internal concentration level reached in (aquatic or terrestrial) organisms over long-term exposures may cause adverse effects, after reaching a critical threshold. Therefore, the capacity of chemicals to bioaccumulate in biota is recognised as a critical hazard property that contributes to a chemical's risk. The degree to which bioaccumulation occurs can be expressed through different bioaccumulation or biomagnification metrics, obtained from measurements in the environment, *in vivo* laboratory tests (usually fish exposed either via the aqueous or dietary path), *in vitro* laboratory tests (e.g., primary hepatocytes or liver S9 sub-cellular fractions from rainbow trout) coupled with *in vitro-in vivo* extrapolation (IVIVE) *in silico* models, or directly from *in silico* methods (applying mechanistic mass balance toxicokinetics (TK) models and quantitative structure-activity relationship (QSAR) predictions).

Historically, bioaccumulation assessment has focused mainly on aquatic (water-breathing) species. The fish bioconcentration factor (BCF), usually determined in a fish bioaccumulation test according to Organisation for Economic Co-operation and Development (OECD) test guideline (TG) 305 (OECD, 2012), has been a widely accepted endpoint. Internationally, the BCF is often used as a criterion in both PBT and Persistent Organic Pollutant (POP) assessment. Field measurements (Kelly and Gobas, 2001) and theoretical mathematical models (Kelly and Gobas, 2003, Czub and McLachlan, 2004) have indicated that some chemicals that may not be considered bioaccumulative using the aquatic-based BCF and associated criteria are bioaccumulative in air-breathing organisms, e.g., endosulfan, beta-hexachlorocyclohexane and many perfluorinated alkyl substances (Kelly et al., 2007). Nevertheless, the use of the aquatic BCF has previously been assumed to be sufficiently protective of both aquatic and terrestrial organisms (for example, fish BCF has been the standard information to address bioaccumulation under the REACH Regulation), but this needs to be re-addressed in view of newer data and improved understanding.

Under REACH, besides results from a bioconcentration or bioaccumulation study in aquatic species, other information on the bioaccumulation potential or information on the

ability of the substance to biomagnify in the food chain can be used to assess bioaccumulative (B) or very bioaccumulative (vB) properties (REACH Annex XIII, 3.2.2).

Such information could be derived from *in vitro* tests of mammalian primary hepatocytes, or liver sub-cellular fractions (i.e., microsomes or S9 fractions), monitoring on the occurrence and accumulation of chemicals in air-breathing animals, or toxicokinetic data from human health assessment (usually derived from rats).

Methods for collecting, generating, evaluating, and integrating various lines of evidence (*in vivo*, *in vitro*, *in silico* bioaccumulation and TK data) to assess the bioaccumulation of organic chemicals in aquatic and air-breathing organisms have been developed (Arnot et al., 2022). The methods and guidance have been formally implemented in the Bioaccumulation Assessment Tool (BAT) providing an operational framework to conduct and inform bioaccumulation assessment decision-making in a consistent and transparent manner (Arnot et al., 2022). The data reliability scoring methods for *in vitro*, *in vivo* and *in silico* bioaccumulation and TK data for the BAT were developed from OECD testing guidance (e.g., OECD 305 (2012), OECD 319A (2018), OECD 319B (2018), OECD 417 (2010)). The methods and the BAT are aligned with the stated objectives in REACH Annex XIII for definitive decision-making using a Weight of Evidence (WoE) approach and with OECD recommendations for a WoE approach for chemical assessments (OECD, 2019).

The objective of this working group was the formal development of an assessment scheme to account for chemical bioaccumulation in air-breathing organisms for chemical assessments, for example for the assessment of bioaccumulation under REACH. This discussion paper presents the scientific background and current knowledge on bioaccumulation in air-breathing organisms, and gives general guidance for a possible assessment scheme, its applicability domain, and what data is needed.

2. Scope

The focus is on accumulation processes in air breathing organisms, especially mammals, including marine mammals. For mammals, bioaccumulation essentially occurs through the dietary route, associated with poor elimination via urinary and faecal egestion, biotransformation (metabolism), and exhalation.

In the following sections, we have outlined the different concepts and approaches to assess bioaccumulation in air-breathers following a tiered approach. The tiered approach is somewhat analogous to the assessment of aquatic bioaccumulation that progresses from an initial screening assessment based on physical-chemical properties to a definitive assessment in the final tier.

3. Concepts

3.1. Bioaccumulation Theory and Models

Bioaccumulation is generally referred to as a process in which the chemical concentration in an organism achieves a level that exceeds that in the respiratory medium (e.g., water for a fish or air for a mammal), the diet, or both (OECD, 2012). The regulatory concern includes both bioaccumulation of chemicals in organisms and accumulation of chemicals through the food chain.

For the past 20 years, approaches for assessing and quantifying bioaccumulation in air-breathing organisms have been increasingly discussed. This is because the bioconcentration factor (BCF) in water-breathing organisms such as fish is not a suitable metric for bioaccumulation in air-breathing organisms. The BCF is defined in terms of

aqueous exposure of organisms to chemicals, described by the ratio of the concentration of the chemical in fish to the concentration of the chemical in water in absence of dietary exposure to the chemical. For air-breathing organisms, however, chemical uptake via the diet is often the most important exposure route in nature. Also, dietary exposure can cause certain chemicals to be absorbed by organisms to a greater extent than would be expected from equilibrium partitioning of the chemical between the ingested food and the organism. This phenomenon is usually referred to as biomagnification. It occurs because of food-digestion, where food components are assimilated (leading to a change in composition and volume of the ingested food in the gastro-intestinal tract) which causes the sorptive capacity of the food for the chemical to reduce in combination with a slow excretion of the chemical from the organism once taken up from the food (Gobas et al. 1999). Biomagnification represents uptake of the chemical against the apparent thermodynamic gradient (i.e., from a low fugacity or chemical activity in prey to a higher fugacity or chemical in the predator) over different trophic levels in a food web (e.g., Connolly et al., 1988). Substances that have the capacity to biomagnify tend to cause the greatest exposure in organisms at highest trophic levels (e.g., polar bears, peregrine falcons, eagles).

Biomagnification can be defined in chemical terms as the increase in the chemical potential, fugacity or chemical activity that occurs to the chemical when transferred from prey to predator (in nature) or from diet to organism (in laboratory tests). Of those fundamental thermodynamic quantities, fugacity (f; Pa) and chemical activity (a; unitless) are the most practical and are regularly used to describe the environmental fate of substances including bioaccumulation (Mackay 2001). Biomagnification can be calculated and expressed by the biomagnification factor (BMF), which is the ratio of the chemical fugacities or chemical activities in predator and prey:

$$BMF = \frac{f_{predator}}{f_{prey}} = \frac{a_{predator}}{a_{prey}} \quad (1)$$

Substances that exhibit a BMF greater than 1 biomagnify in food-chains, causing the chemical's fugacity and chemical activity to increase with trophic level. Substances that exhibit a BMF less than 1 biodilute in food-chains, causing the chemical's fugacity and chemical activity to decrease with trophic level.

Fugacities and chemical activities are closely related as the chemical activity (a) is defined as the ratio of the fugacity (f) of the chemical and fugacity of that chemical at a standard or reference (R) state f^R (i.e., $a = f/f^R$). A standard reference state fugacity that has been used is the vapour pressure for liquids and the sub-cooled liquid vapour pressure for solids at system temperature (Mackay 2001). Since the fugacity at the reference state is usually the same for predator and prey, it cancels out in the ratio, causing the predator-prey fugacity and chemical activity ratios to be the same. Equation 1 is advantageous as it provides two approaches for determining the BMF. For simple neutral hydrophobic organic substances, the fugacity ratio is often the simplest and most practical way to determine the BMF, while for ionising substances (e.g., perfluorinated alkyl acids) the chemical activity approach may be the most practical.

One of the useful features of defining biomagnification in terms of a predator-prey or organism-diet fugacity and chemical activity ratios is that it does not require making any assumptions on which medium or media in the organisms (e.g., neutral lipids, proteins, polar lipids) the substance predominantly bioaccumulates in. This means that it can be applied to a wide variety of chemical substances.

Because fugacity and chemical activity are abstract quantities, it is important to relate them to measurable quantities that can be determined in field studies and laboratory tests. Two general types of real-world data can be used for this purpose. They are (i) concentrations of chemicals in predator and prey (or organism and diet), often

determined in either field studies or laboratory tests, and (ii) kinetic parameters such as uptake and elimination rate constants, usually determined in laboratory tests.

Using concentration data:

Fugacities (f) and chemical activities (a) are directly related to concentrations C since f is defined as C/Z where Z is the chemical fugacity capacity ($mol.m^{-3}.Pa^{-1}$). Likewise, chemical activities can often be expressed as C/S where S is the chemical's solubility in units of $mol.m^{-3}$. This means that the BMF in equation 1 can be expressed as a simple predator-prey concentration ratio as long as the concentrations of the chemical in predator and prey refer to the same medium (with the same Z or S for the chemical). In that case, Z and S are the same for predator and prey and cancel out, giving:

$$BMF = \frac{f_{predator}}{f_{prey}} = \frac{a_{predator}}{a_{prey}} = \frac{C_{predator}}{C_{prey}} \quad (2)$$

However, concentrations of chemicals in predator and prey (or organism and diet) determined in field studies or laboratory tests refer to different media (e.g., cow and grass) and can be viewed as "apples and oranges" and therefore cannot be used to determine the BMF as defined by a unitless fugacity or chemical activity ratio. In order for measured concentrations in predator and prey to be used to determine the BMF according to equation 1, they have to be expressed in similar quantities or "normalised" to the relative solubility of the chemical in both the predator and prey. This can be mathematically described as:

$$BMF = \frac{f_{predator}}{f_{prey}} = \frac{a_{predator}}{a_{prey}} = \frac{C_{predator}}{C_{prey}} = \frac{(C^*_{predator}/\phi_{M,predator})}{(C^*_{prey}/\phi_{M,prey})} \quad (3)$$

Where:

$C^*_{predator}$ is the concentration of the chemical in the predator in units of g/kg predator,
C^*_{prey} is the concentration of the chemical in the prey in units of g/kg prey,
$\varphi_{M,predator}$ and $\varphi_{M,prey}$ represent the fractions of the organisms consisting of medium M in units of kg M.kg $predator^{-1}$ and kg M.kg $prey^{-1}$.

Which medium M (e.g., neutral lipids, proteins, polar lipids) is selected is not that important as long as it is the "same" or sufficiently similar in predator and prey. However, it is often practical to choose the medium M to be the medium into which the substance primarily bioaccumulates.

The "lipid normalisation" approach is often practised when assessing the biomagnification potential of simple neutral hydrophobic substances that preferentially accumulate in neutral (or storage) lipids. In those cases, fugacities of the chemical in predator and prey, and hence the BMF, are approximated from lipid-normalised or lipid-equivalent-normalised (i.e., for organism with very low lipid content) concentrations (Mackintosh et al., 2004; Kelly et al., 2007). In this case, the medium M is lipid L, and equation 1 becomes:

$$BMF_L = \frac{f_{predator}}{f_{prey}} = \frac{a_{predator}}{a_{prey}} = \frac{C_{predator}}{C_{prey}} = \frac{(C^*_{predator}/\phi_{L,predator})}{(C^*_{prey}/\phi_{L,prey})} \quad (4)$$

Where:

$C^*_{predator}$ and C^*_{prey} are the chemical concentrations measured in predator and prey in units of g/kg predator and g/kg prey respectively and
$\varphi_{L,predator}$ and $\varphi_{L,prey}$ are the neutral lipid contents of the predator and prey or samples from the predator and prey in units of kg lipid/kg predator and kg lipid/kg prey.

This normalisation approach is well accepted in field-based food-web bioaccumulation studies, and the OECD TG 305 (OECD 2012) specify methods for deriving lipid normalised BMFs in laboratory bioaccumulation tests in units of kg-lipid.kg-lipid^{-1}, which approximate the unitless quantities in equation 1.

For substances that do not preferentially bioaccumulate in neutral lipids (e.g., certain perfluorinated alkyl acids), other normalisation approaches such as protein normalisation and/or normalisation to the polar lipid content of organisms are required. For example, the BMF for a substance that preferentially binds to proteins can be derived from concentration measurements as:

$$BMF_P = \frac{f_{predator}}{f_{prey}} = \frac{a_{predator}}{a_{prey}} = \frac{C_{predator}}{C_{prey}} = \frac{(C^*_{predator}/\phi_{P,predator})}{(C^*_{prey}/\phi_{P,prey})} \quad (5)$$

Where $\varphi_{P,predator}$ and $\varphi_{P,prey}$ are the protein contents of the predator and prey or samples from the predator and prey in units of kg protein/kg predator and kg protein/kg prey.

For substances that bioaccumulate in several media to different degrees (e.g., polar lipids and proteins), water can be a useful medium for normalisation because of the current practice of measuring chemical partitioning properties in different media by partition coefficients relative to water. In this case, equation 3 can be presented as:

$$BMF_W = \frac{f_{predator}}{f_{prey}} = \frac{a_{predator}}{a_{prey}} = \frac{C_{predator}}{C_{prey}} = \frac{\left(\frac{C^*_{predator}}{(\phi_{W,predator}+\sum_{n=1}^{m}(\phi_n \cdot D_{nW})}\right)}{\left(\frac{C^*_{prey}}{(\phi_{W,prey}+\sum_{n=1}^{m}(\phi_n \cdot D_{nW})}\right)} \quad (6)$$

Where:

$\varphi_{W,predator}$ and $\varphi_{W,prey}$ are the water contents of the predator and prey or samples in unit of kg water/kg predator and kg water/kg prey and
D_{nW} is the chemical's distribution coefficient (similar to a partition coefficient) between the medium n and water, and
n refers each of the media included in the normalisation (e.g., neutral lipid, polar lipids, albumin, structural proteins) and m is the total number of media (i.e., 4 in this example) considered.

Burkhard et al. (2012) investigated the potential of the fugacity approach to interpret bioaccumulation information from different field and laboratory studies for 15 non-ionic organic chemicals, using 2393 measured data points from 171 reports. The authors concluded that fugacity ratios derived from the various bioaccumulation metrics were generally consistent in categorising substances as either biomagnifying or biodiluting and could therefore be considered for bioaccumulation assessment purposes.

<u>Using kinetic data from a laboratory experiment</u>

An alternative approach to deriving the fugacity or chemical activity ratios that make up the BMF is a kinetic approach that views the concentration of the chemical in organism as a result of competing rates of chemical uptake and elimination in an organism, which at steady-state is:

$$\frac{C^*_{predator}}{C^*_{prey}} = \frac{k_{diet}}{k_{elimination}} \quad (7)$$

Where:

k_{diet} is the dietary clearance rate (kg prey.kg predator^{-1}.day^{-1}), which is often measured as the product of the dietary uptake efficiency E_D and the proportional feeding rate G_{diet} (kg prey.kg predator^{-1}.day^{-1}), and
$k_{elimination}$ is the organism's elimination or depuration rate constant (day^{-1}).

Hence, the BMF can be expressed in both thermodynamic and measurable quantities as:

$$BMF_M = \frac{f_{predator}}{f_{prey}} = \frac{a_{predator}}{a_{prey}} = \frac{C_{predator}}{C_{prey}} = \frac{(C^*_{predator}/\phi_{M,predator})}{(C^*_{prey}/\phi_{M,prey})} = \frac{E_D \cdot G_{diet} \cdot \phi_{M,prey}}{k_{elimination} \cdot \phi_{M,predator}} \quad (8)$$

This equation provides an approach to interpret kinetic data in terms of a BMF.

Measurement of E_D and $k_{elimination}$ in a test and knowledge of the feeding rate G_{diet} and the relevant normalisation parameters (such as lipid or protein content) $\varphi_{M,predator}$ and $\varphi_{M,prey}$ provides a simple way to determine the BMF.

Equation 8 can also be used for identifying an elimination rate constant or the corresponding first order half-life time HL_T that results in a particular degree of biomagnification:

$$k_{elimination} = \frac{E_D \cdot G_{diet} \cdot \phi_{M,prey}}{BMF \cdot \phi_{M,predator}} \quad (9)$$

or

$$HL_T = \frac{BMF \cdot \phi_{M,predator} \cdot ln\ 2}{E_D \cdot G_{diet} \cdot \phi_{M,prey}} \quad (10)$$

Equations 9 and 10 can be used to determine whether a substance has the potential to biomagnify based on the elimination rate constant or corresponding first order half-life time. For example, for a chemical to biomagnify, i.e., BMF>1, $k_{elimination}$ or HL_T need to be:

$$k_{elimination} < \frac{E_D \cdot G_{diet} \cdot \phi_{M,prey}}{1 \cdot \phi_{M,predator}} \quad (11)$$

or

$$HL_T > \frac{1 \cdot \phi_{M,predator} ln\ 2}{E_D \cdot G_{diet} \cdot \phi_{M,prey}} \quad (12)$$

These equations show that it is challenging to propose a single elimination rate constant or half-life time threshold value that indicates whether a substance biomagnifies or not because $k_{elimination}$ and HL_T are dependent on several experimental and biological parameters that are difficult to control or standardise in experiments. However, there is often little need for such kinetic threshold values since the parameters in equations 11 and 12 are typically known from the test conditions or can be estimated and hence entered in equations 11 and 12 to determine what $k_{elimination}$ or HL_T indicates biomagnification or not.

If the measured $k_{elimination}$ is less than that determined using equation 11, then the substance can be deemed bioaccumulative, having a biomagnification factor greater than 1. Likewise, a measured half-life time greater than that calculated from equation 12 using the experimental conditions of the test used to derive the half-life time indicates a substance that biomagnifies. In a similar fashion, depletion rate constants or half-life times in *in vitro* tests can be interpreted in terms of biomagnification or no-biomagnification. The latter is described in more detail in section 6.

By defining biomagnification in terms of generally applicable thermodynamically relevant quantities, i.e., a fugacity or chemical activity ratio greater than 1, it becomes possible to use several methods to test whether experimental data indicate biomagnification or not. Some of these methods are already available. For example, measured or calculated

concentrations can be converted to fugacities and activities using measured or predicted chemical properties (e.g., vapour pressure, solubilities). While it is recognised that more research is required to address uncertainty in measuring quantities and properties as well as predicting properties and extrapolating measurements to calculate BMFs, this conceptual approach that forms the basis for bioaccumulation assessment is well-grounded with current knowledge relating to chemical thermodynamics and the parameters and processes resulting in biomagnification.

3.2. Biokinetic Models for Integrating Data and Simulating Biomagnification in Air-Breathing Organisms

For bioaccumulation in the environment key routes of chemical exposure and uptake include respiration, and ingestion of food and water. Key processes of chemical elimination from an organism include respiration, urinary excretion (or renal clearance), faecal egestion, and biotransformation (metabolism). The theoretical concepts outlined in Section 3.1 and knowledge relating to the processes of chemical biomagnification can be used to develop physiologically based biokinetic (PBK) mass balance models to integrate data and to calculate BMFs in air-breathing organisms. PBK models incorporate key physiological processes relating to chemical uptake and elimination including respiration rates, feeding rates, growth rates, digestion efficiencies, egestion rates and excretion rates. Generalised PBK models can be parameterised using only a few biological parameters (e.g., body mass, lipid contents, water content, protein contents) and a few physicochemical properties (e.g., molar mass, K_{OW} and K_{OA}) as well as biotransformation rate constants ($k_{biotransformation}$) or corresponding half-lives ($HL_{biotransformation}$), e.g., Czub and McLachlan (2004), Kelly et al., (2007), Arnot et al., (2022).

In terms of spatial representation within the organism, PBK models can consider the organism at a whole-body level or with more explicit spatial representation within the body. One compartment PBK (1Co-PBK) models assume the chemical is well-mixed throughout the organism. Following this assumption internal equilibrium is established (equi-fugacity between compartments in steady-state), but chemical concentrations between compartments and tissues are not necessarily equal. Multi-compartment PBK (MCo-PBK) models explicitly consider chemical distribution between different compartments in the organism (e.g., tissues/organs, blood) and range in complexity from a few compartments (e.g., blood, liver, lung, richly perfused tissues, poorly perfused tissues) to several compartments considering all major organs. Comparisons of 1Co-PBK and MCo-PBK models for humans show that the simpler 1Co-PBK models can be sufficient for certain purposes (Armitage et al., 2021).

General concepts of a 1Co-PBK model to calculate chemical uptake and elimination processes in mammals are outlined here based on various publications, (e.g., Kelly and Gobas, 2003, Czub and McLachlan, 2004, Kelly et al., 2007, Arnot et al., 2014, Arnot et al., 2022). The general 1Co-PBK mass balance model is:

$$dC_{organism}/dt = k_{respiration}C_{air} + k_{diet}C_{diet} + k_{water}C_{water} - (k_{respiration} + k_{egestion} + k_{urination} + k_{biotransformation} + k_{reproduction})C_{organism} \quad (13)$$

where:

$dC_{organism}/dt$ is the net change in concentration in the organism (g/kg) over time t (day), $C_{organism}$ is the chemical concentration in the organism,
$k_{respiration}$ is the respiration clearance rate $(L(kg \cdot day)^{-1})$,
C_{air} is the gaseous concentration $(g \cdot L^{-1})$,
k_{diet} is the dietary clearance rate $(kg(kg \cdot day)^{-1})$,
C_{diet} is the chemical concentration in the diet (g/kg food), k_{water} is the water clearance rate $(L(kg \cdot day)^{-1})$, and

C_{water} is the chemical concentration in drinking water (g/L).

The rate constants (day^{-1}) corresponding to chemical elimination from the organism via respiratory elimination, faecal egestion, urinary excretion, biotransformation, reproductive losses are $k_{respiration}$, $k_{egestion}$, $k_{urination}$, $k_{biotransformation}$, $k_{reproduction}$, respectively.

The first order total (or terminal) elimination rate constant ($k_{elimination}$) is the sum of the individual elimination rates processes. Reproductive losses are considered negligible for males and for bioaccumulation assessment can be ignored. The $k_{elimination}$ can be converted to a total (or terminal) elimination half-life as $HL_T = \ln 2/k_{elimination}$. Likewise, $k_{biotransformation}$ can be converted to a biotransformation half-life as $HL_{biotransformation} = \ln 2/k_{biotransformation}$. Following this first order approach, exposure concentrations are assumed to be high enough and exposure durations long enough so that enzymes are induced, and concentrations are assumed to be low enough that enzyme systems are not saturated. Michaelis-Menten kinetics can be considered for non-first order biotransformation conditions, but such calculations require tissue and chemical specific information on the Michaelis constant (k_M) and the maximum velocity (V_{MAX}).

At steady-state ($dC_{organism}/dt = 0$), Equation 13 becomes:

$$k_{respiration}C_{air} + k_{diet}C_{diet} + k_{water}C_{water} = (k_{respiration} + k_{egestion} + k_{urination} + k_{biotransformation} + k_{reproduction})C_{organism} \quad (14)$$

where the left side of equation quantifies chemical uptake into the organism through exposures to chemical in air, food and water and the right side of the equation quantifies parent chemical elimination from the organism.

As noted in Equation 8 the BMF can be calculated using steady-state ratios of chemical fugacities, activities, and concentration as well as kinetically using rate constants (OECD 2012). If there are significant changes to body mass a growth rate, k_{growth} (day^{-1}), can be included in the steady-state solution (Equation 14).

4. Proposed Approach

We propose a tiered approach for assessing chemical bioaccumulation in air-breathing organisms. The tiered approach is analogous to the approach used in REACH for the assessment of bioaccumulation in aquatic organisms because it progresses from an initial physical-chemical property-based "Screening Assessment" in the first tier to a "Definitive Assessment" in the final tier.

The second tier addresses the need to reduce unnecessary animal testing and incorporates alternative testing methods to inform decision-making, including priority setting.

The third tier is more resource intensive and uses whole animal testing or field data. The relevant assessment endpoint is the BMF; however, it is recognised that biotransformation and total elimination half-lives are closely related to the BMF.

Tier 1: Screening Assessment. Compare estimated or measured physical-chemical properties of a chemical with threshold values to separate chemicals with bioaccumulation potential in air-breathing animals from those that clearly do not have bioaccumulation potential. Suitable properties to consider at Tier 1 are the octanol-water partition ratio (K_{OW}) and the octanol-air partition ratio (K_{OA}). Tier 1 screening criteria for log K_{OW} and log K_{OA} have been developed based on available measurements of bioaccumulation (notably biomagnification and food web biomagnification) and bioaccumulation model calculations that specifically neglect key TK processes that can mitigate biomagnification potential, i.e. biotransformation.

Tier 2: Refined Screening Assessment using Alternative Testing Methods. Refine Tier 1 results by using alternative testing methods to reduce unnecessary animal testing. The same physiologically-based mass balance bioaccumulation models used to derive Tier 1 screening criteria can be parameterised with key TK parameters (i.e., biotransformation rates and absorption efficiencies) obtained from reliable quality data obtained from *in vitro* and *in silico* methods.

Tier 3: Refined Assessment using *In Vivo* Testing Methods. *In vivo* testing is required when no definitive conclusion on bioaccumulation can be drawn from the previous tiers (possibly in rat, but other mammals would also be possible). While there are no standardised BMF tests for air-breathing species, there are OECD tests that can be used (or possibly modified) to obtain *in vivo* TK parameters, i.e., HL_T, that can then be used to parameterise the same physiologically-based mass balance bioaccumulation models used in Tiers 1 and 2. Reliable quality *in vivo* field data, in particular high quality trophic magnification factors (TMF) may also be considered if they are available.

5. Tier 1: Screening Assessment for Bioaccumulation in Air-Breathing Organisms

A tier 1 assessment for bioaccumulation assessment in air-breathing organisms will most likely involve the comparison of the estimated or measured properties of a chemical with threshold values that are deemed to separate chemicals that are potentially bioaccumulative from those that clearly will not be able to bioaccumulate. Such threshold may refer to a chemical's:

- Volatility; chemicals that are sufficiently volatile will be readily eliminated by exhalation,

- Hydrophilicity; chemicals that are sufficiently water soluble will be readily eliminated by urinary excretion.

Susceptibility to biotransformation as a tier 1 parameter has been explored (Wania et al., 2022). However, biotransformation property information is suggested to be used only in the second tier to refine the screening outcome, by using alternative testing methods to reduce unnecessary animal testing (see 5.4 Unresolved issues related to tier 1 bioaccumulation assessment in air-breathing organisms).

Here we describe in turn:

- How one can derive thresholds for efficient loss by respiration and urination that are consistent (i) with each other and (ii) when applied to different air-breathing organisms, by using simple equations that relate the thresholds to an easily agreed upon B criterion, e.g. a BMF of 1. Incidentally, thresholds for efficient loss by biotransformation (Tier 2) could similarly be tied to that B criterion to achieve consistency among the thresholds.

- How the properties of a chemical to be assessed could be compared to those thresholds.

- How one could obtain the properties for a chemical and their uncertainties.

5.1. Chemical Property Thresholds for Bioaccumulation Assessment in Air-Breathing Organisms

5.1.1. Determination of Thresholds for Bioaccumulation Assessment in Air-breathing Organisms

It clearly would be desirable, if chemical property thresholds chosen for the three chemical characteristics of susceptibility to exhalation, urination and biotransformation were internally consistent. This can be achieved by deriving the volatility, hydrophilicity and susceptibility to biotransformation that would lead to a *BMF* of 1, if exhalation, urinary excretion and biotransformation were the only elimination processes, respectively.
We can formulate a mass balance for an organism that takes up a contaminant from the diet and eliminates it by various processes:

$$\frac{dC_{body}}{dt} = E_D G_{diet} C_{diet} - k_{elimination} C_{organism} \quad (15)$$

Where:
-E_D is the dietary uptake efficiency,
-G_{diet} is the body mass-normalised dietary intake rate (kg diet kg^{-1} organism day^{-1}),
-$k_{elimination}$ is the whole-body elimination rate (in day^{-1}) and
-$C_{organism}$ and C_{diet} are the chemical concentrations in the organism and its diet.

This formulation neglects the possibility of chemical uptake by breathing air and consuming drinking water. At steady-state:

$$E_D G_{diet} C_{diet} = k_{elimination} C_{organism} \quad (16)$$

To obtain lipid normalised concentrations we need to introduce variables $f_{lipid\text{-}diet}$ and $f_{lipid\text{-}body}$ describing the dietary lipid content and the lipid content of the organism (kg lipid · kg diet^{-1} and kg lipid · kg body mass^{-1}) respectively:

$$E_D G_{diet} \frac{C_{diet}}{f_{lipid-diet}} f_{lipid-diet} = k_{elimination} \frac{C_{organism}}{f_{lipid-organism}} f_{lipid-organism} \quad (17)$$

The *BMF*, when defined based on lipid-normalised concentration and applying to a steady-state situation, therefore can be derived as follows:

$$BMF = \frac{\frac{C_{organism}}{f_{lipid-organism}}}{\frac{C_{diet}}{f_{lipid-diet}}} = \frac{E_D \cdot G_{Diet}}{k_T} \cdot \frac{f_{lipid-diet}}{f_{lipid-organism}} \quad (18)$$

The elimination rate $k_{elimination}$ and therefore total whole-body elimination half-life HL_T corresponding to a *BMF* of 1, depends on the values of E_D, G_{diet} and $f_{lipid\text{-}organism}$ for a particular organism.

For example, assuming an E_D of 100 %, a $f_{lipid\text{-}diet}$ equal to $f_{lipid\text{-}organism}$, and a G_{diet} of 0.01 kg diet · kg^{-1} body mass day^{-1}, Goss et al. (2013) derived a $k_{elimination}$ of 0.01 day^{-1} for humans, corresponding to a HL_T of 70 days. However, because organisms and even humans differ in terms of their body lipid content, the lipid content of their diet and their dietary intake, the actual $k_{elimination}$ corresponding to a *BMF* of 1 is variable. The HL_T corresponding to a *BMF* of 1 is shorter for lean organisms with high intakes of a lipid-rich diet. For typical American adults, the exposure factor handbook of the US EPA recommends a G_{diet} of 0.029 kg diet kg^{-1} body mass day^{-1}, a $f_{lipid\text{-}diet}$ of 0.1 and a $f_{lipid\text{-}body}$

of 0.2[1], which corresponds to a HL_T of 50 days. A *BMF* of 1 plausibly corresponds to a HL_T in an air-breathing organism with the dietary characteristics of a human of ~50 days.

Clearly, a HL_T by biotransformation $HL_{biotransformation}$ of 50 days would lead to an HL_T of 50 days if biotransformation would be the only elimination process. But how volatile and hydrophilic does a compound need to be to achieve HL_T of 50 days, if exhalation and urination were the only elimination processes?

If we assume that a chemical is in equilibrium between an organism's body and the exhaled air, the HL_T due to respiration $HL_{respiration}$ (day) alone can be estimated as:

$$HL_{respiration} = \frac{ln2}{k_{respiration}} = \frac{ln2 \cdot K_{organism/air}}{G_{respiration}} \quad (19)$$

Where:

$k_{respiration}$ is the whole-body elimination rate by respiration (L · kg^{-1} ·day^{-1}),
$K_{organism/air}$ is the equilibrium partition ratio between organism and air at the temperature of exhaled air (L air kg^{-1} organism), and
$G_{respiration}$ is the body mass normalised respiration rate (L air · kg^{-1} body mass day^{-1}).

This equation may underestimate the $HL_{respiration}$ if there are kinetic limitations to the transfer of chemical from body to exhaled air (e.g., blood flow limitations to the lung, permeation limitations through the lung lining, etc.). Rearranging equation (19) we can obtain an expression that indicates what $K_{organism/air}$ corresponds to a particular HL_T due to respiration alone as:

$$K_{organism/air} = \frac{G_{respiration}\, HL_{respiration}}{ln2} \quad (20)$$

Equivalent equations for elimination for urinary excretion can be formulated. If we assume that a chemical is in equilibrium between an organism's body and its urine, the HL_T due to urination $HL_{urination}$ (day) is:

$$HL_{urination} = \frac{ln2}{k_{urination}} = \frac{ln2 \cdot K_{organism/water}}{G_{urination}} \quad (21)$$

$$K_{organism/water} = \frac{G_{urination}\, HL_{urination}}{ln2} \quad (22)$$

Where:

$k_{urination}$ is the elimination rate by urination (day^{-1}),
$K_{organism/water}$ is the equilibrium partition ratio between organism and water (L air kg^{-1} organism), and
$G_{urination}$ is the body mass normalised urination rate (L urine kg^{-1} body mass day^{-1}).

This equation may again underestimate the $HL_{urination}$. Kinetic limitations to the transfer of a chemical from body to urine are less likely because the time available for equilibration in the kidney is much longer than in the lung. However, active renal secretion or renal reabsorption processes, e.g., through organic anion transporters, may prevent equilibration between body and urine.

We can use equations (20) and (22) and values of $G_{respiration}$ of 250 L kg^{-1} day^{-1} and $G_{urination}$ of 0.017 L kg^{-1} day^{-1} appropriate for humans to estimate that a thresholds of log $K_{organism/water}$ of 0.09 and log $K_{organism/air}$ of 4.26 would lead to a HL_T of 50 days if respiration and urination were the only elimination processes. In other words, these four

[1] See https://www.epa.gov/expobox/about-exposure-factors-handbook#about

thresholds are internally consistent when applied to humans:

BMF = 1 HL_B = 50 days log $K_{organism/water}$ = 0.09 Log $K_{organism/air}$ = 4.26

Please note that the partition ratios $K_{organism/air}$ and $K_{organism/water}$ are those at the temperature of exhaled air and excreted urine, respectively.

For a different organism, a different set of thresholds would apply, because (i) a different HL_T would correspond to a *BMF* of 1, because of differences in E_D, G_{diet}, $f_{lipid\text{-}diet}$, $f_{lipid\text{-}organism}$, and (ii) the body-normalised respiration and urination rates $G_{urination}$ and $G_{respiration}$ differ between organisms. If all thresholds are referenced to a *BMF* of 1, the assessment would be consistent between different organisms.

As an example, we can perform a calculation for a rat. Assuming a feeding rate of 0.05 kg diet kg^{-1} body weight day^{-1} for a 300 g rat (John Hopkins, animal care protocol) lipid content of the rat (~5%) that is half of that of its diet (i.e. $\frac{f_{lipid-diet}}{f_{lipid-organsim}}$=2) and that the uptake efficiency from food is 100 %, we estimate that a *BMF* of 1 in a rat corresponds to a total elimination rate of 0.1 day^{-1} or a total elimination half-life HL_T of approximately 7 days. Using allometric scaling we estimate a $G_{respiration}$ of 650 L kg^{-1} day^{-1} (Mordenti, 1986) and a $G_{urination}$ of 0.033 L kg^{-1} day^{-1} (John Hopkins, animal care protocol), i.e., when normalised to body mass, a rat has an approximately two times higher urination rate and a 2.5 times higher breathing rate. When combined with equations (20) and (22), the elimination half-life of 7 days corresponds to a log $K_{organism/water}$ of -0.48 and a log $K_{organism/air}$ of 3.81, i.e., partitioning ratio thresholds that are quite a bit lower than those for the human. This can be explained by the rat eating a diet of higher fat content relative to its body. In brief, internally consistent thresholds for rats are:

BMF = 1 HL_B = 7 days log $K_{organism/water}$ = -0.48 log $K_{organism/air}$ = 3.81

Please note, that not only are the half-life thresholds specific for species and testing conditions (i.e. feeding rate, diet lipid content), but also the volatility and hydrophilicity thresholds.

We caution that equations (21) and (22) are not valid for extremely water-soluble chemicals, because several of the assumptions made in its derivation are not appropriate. In particular, the mass balance equation (15) ignores the possibility of uptake with water and the lipid-normalisation applied in equation (17) is neither valid. The threshold values for log $K_{organism/water}$ estimated above for humans and rat are exceptionally low and probably fall in a range where equations (21) and (22) are no longer applicable. We conclude that urination alone is very unlikely to be sufficiently efficient to prevent biomagnification of any organic chemical in air breathing organisms.

5.1.2. Expressing Thresholds in Terms of K_{OW} and K_{OA}

Goss et al. (2013) suggest the use of polyparameter linear free energy relationships (ppLFERs) for partitioning into different tissues in combination with information on the composition of the human body to estimate $K_{organism/air}$ and $K_{organism/water}$ for a chemical (Endo et al., 2013). Because existing thresholds are not formulated in terms of $K_{organism/air}$ and $K_{organism/water}$, it is also useful to express any thresholds for $K_{organism/air}$ and $K_{organism/water}$ in terms of octanol/air and octanol/water equilibrium partition ratios K_{OA} and K_{OW}.

By (i) using information on body composition given in Goss et al. (2013) (i.e., a 70 kg human is assumed to contain 12 kg of storage fat, 1.5 kg of membrane lipids and 10.6 kg of protein), (ii) assuming that both storage fats and membrane lipids have the same sorptive capacity as octanol, and proteins have 5 % of the sorptive capacity of octanol

(assessed for neutral organic chemicals by deBruyn and Gobas, 2007), and (iii) that the density of lipid is 0.92 kg L^{-1}, we derive a volume of "octanol-equivalent" in the human body f_O of 0.22 L octanol kg^{-1} body mass. This allows us to relate partition ratios to an organism to partitioning ratios to octanol as follows:

$$K_{organism/air} = f_O \cdot K_{OA} \quad (23)$$

$$K_{organism/water} = f_O \cdot K_{OW} \quad (24)$$

Using a f_O of 0.22, we deduce that a threshold of log $K_{organism/water}$ of 0.09 corresponds to a log K_{OW} threshold of 0.75 and a threshold of log $K_{organism/air}$ of 4.26 corresponds to a log K_{OA} threshold of 4.92. Equation (24) assumes that the water in the organism's body does not contribute to its uptake capacity for the chemical, which is not necessarily correct for chemicals with very small K_{OW} values. In such cases it is preferable to estimate $K_{organism/water}$ using an approach not based on the surrogate octanol (Endo et al., 2013).

Alternatively and analogous to equations (23) and (24), we could also formulate equations to directly calculate the K_{OA} and K_{OW} threshold values that are consistent with a *BMF* of 1, if one assumes that respiration or urination are the only elimination mechanisms and equilibrium between an organism's body and the exhaled air and the excreted urine is established:

$$K_{oa}(T_{org}) = \frac{G_{respiration}}{E_D \cdot G_{Diet} \cdot f_{lipid-diet}} \quad (25)$$

$$K_{ow}(T_{org}) = \frac{G_{urination}}{E_D \cdot G_{Diet} \cdot f_{lipid-diet}} \quad (26)$$

These are the partition ratios at the temperature of exhalation and urination and need to be temperature adjusted if the assessment procedure involves the comparison of K_{OA} and K_{OW} values at 25 °C with the threshold values. While the temperature dependence of $K_{organism/water}$ and K_{OW} is likely to be very minor and can be neglected, this is not necessarily the case for $K_{organism/air}$ and K_{OA}. If we apply the van't Hoff equation and assume a generic internal energy of octanol to gas phase transfer ΔU_{OA} of 50 kJ mol^{-1}, a log K_{OA} threshold of 4.92 at the human body temperature of 37 °C corresponds to a log K_{OA} at 25 °C of 5.26.

In summary, a consistent set of thresholds for bioaccumulation assessment in humans could consist of:

BMF = 1 HL_B = 50 days log K_{OW} = 0.75 Log $K_{OA@25\,°C}$ = 5.26

Similarly, for a 300 g rat we estimate a volume of "octanol-equivalent" f_O of 0.06 L kg^{-1} body mass (Lindstedt and Schaeffer, 2002), which is four times smaller than the sorptive capacity of the human body for chemicals. Accordingly, a set of consistent thresholds in the rat would be:

BMF = 1 HL_B = 7 days log K_{OW} = 0.74 Log $K_{OA@25\,°C}$ = 5.37

We can see that the $K_{organism/air}$ and $K_{organism/water}$ thresholds that were considerably lower in the rat than in the human are compensated by the lower sorptive capacity of the rat's body, which is relatively much leaner than the human, so that the partitioning ratio thresholds are quite similar in rat and human when expressed in terms of K_{OA} and K_{OW}. Please note, that all of these thresholds are variable and uncertain, because of the considerable variability between individual humans and individual rats, e.g. in terms of dietary and body lipid content.

We caution again, that a compound with a log K_{OW} as low as these threshold values is only ~5 times more soluble in octanol than in water, i.e., the assumptions we applied in the derivation of the threshold values are no longer valid. In other words, it may not be possible to indicate a K_{OW} threshold, below which organic chemicals can no longer biomagnify if they are not subject to biotransformation by only considering lipophilic partitioning.

5.1.3. Alternative approach for deriving Regulatory Thresholds for half-life, K_{OA} and K_{OW} based on accumulation in top predators

As described above (section 3.1) derived thresholds are still dependent on the chosen test conditions, i.e. species, food type. Therefore, there is a need to define standardised conditions that meet the concern for bioaccumulation of substances in air-breathing organisms. In general, this is the concern for biomagnification along the food chain and more specific biomagnification in top predators. Air-breathing top predators considered in generic risk assessments are mammalian and avian predators. These species usually feed on smaller mammalian and avian prey species and sometimes fish. Biomagnification occurs if the fugacity/activity in the top predator becomes higher than that in the prey species. If both top predator and prey species are mammals/birds, it can be assumed that the tissues of both prey and predator are rather similar in composition. This implies that the fugacity capacity in prey and predator is similar too, not only with regard to lipophilic substances, but also for other types of partitioning such as protein binding. Thus, the concentration ratio is a good measure of biomagnification in the top of the food web in terms of fugacity/activity, provided that tissue composition of prey and predator are similar.

The energy that organisms require from their food on a daily basis is denoted as the daily energy expenditure (DEE). For both mammals and birds strong allometric relationships exist that relate the DEE to the body weight (BW) of the species (DEFRA (2007), which is an update of Crocker et al. (2002). These relationships are also used in the Water Framework Directive (WFD) guidance for deriving quality standards for secondary poisoning of substances throughout the food chain (Verbruggen, 2014; EC, 2018). These relationships are for non-marine and non-desert eutherian mammalian species and for terrestrial non-passerine avian species, respectively:

$$log\ \ DEE\ [kJ\ d^{-1}] = 0.7149\ log\ \ BW\ [g] + 0.8136$$

$$log\ \ DEE\ [kJ\ d^{-1}] = 0.6694\ log\ \ BW\ [g] + 0.8387$$

Also, the energy contents for different food types are tabulated. For small birds and mammals that serve as food for top predators the value reported is 7331 $kJ/kg_{wet\ weight}$. (Smit, 2005; EFSA, 2009). Similar values for small mammals and birds and mammalian carrion are reported by Crocker et al. (2002).

G_{diet}, the body mass-normalised dietary intake rate [$kg_{diet}\ kg^{-1}_{organism}\ d^{-1}$] can then be estimated for terrestrial top predators from the ratio of DEE and BW, the assimilation efficiency of the food (AE) and the energy content of the food ($Energy_{food}$) as (Crocker et al., 2002):

$$G_{diet} = \frac{DEE}{AE \cdot Energy_{food} \cdot BW}$$

The absorption efficiency of the chemical (E_D) is assumed to be high and almost equal to the assimilation efficiency of the food (AE). Thus, if the assimilation efficiency from the food is less due to lower digestibility of the food, also the absorption efficiency of the chemical is assumed to be accordingly lower (see e.g. Hendriks et al., 2001). It is assumed that if the assimilation efficiency of the food and the absorption efficiency of

the chemical are similar, these two parameters cancel each other. In that case, the half-life of a substance from the food that leads to a higher concentration in predators than in their prey, is determined by the daily energy expenditure and the energy content of the food. In equilibrium the uptake and elimination of the chemical are equal:

$$E_D G_{diet} C_{diet} = \frac{DEE}{Energy_{food} \cdot BW} C_{diet} = k_{elimination} C_{organism}$$

It follows that biomagnification occurs if:

$$C_{diet} > C_{organism} \rightarrow k_{elimination} < \frac{DEE}{Energy_{food} \cdot BW} \; or \; HLT > \frac{ln2 \cdot Energy_{food} \cdot BW}{DEE}$$

Substituting the energy content for birds and mammals and using the equation for mammals, this leads to the following equation for biomagnification in mammalian top predators:

$$HLT[d] > 0.78 \cdot (BW[g])^{0.2815}$$

In this equation 0.78 is ln(2)·7.3312/$10^{0.8136}$ and 0.2815 is 1-0.7149 (using values from the equation for non-marine and non-desert eutherian mammalian species shown above).

This equation is developed for mammalian top predators that eat other mammalian or avian prey species, because the energy content of the diet has been set at that for mammalian and avian prey species. However, the daily energy expenditure is not specific for terrestrial top predators. The correlation between daily energy expenditure and body weight is applicable to all non-marine and non-desert eutherian mammalian species. Therefore, regularly tested laboratory species could be used to represent a mammalian terrestrial top predator with regard to metabolism. A half-life of 4 days would correspond to a body weight of 308 g, i.e. similar to that of rats, while a half-life of 2 days would correspond to a body weight of 27 g, i.e. similar to that of mice. With equation (20) and (22) and a body weight of 300 g for the rat, the following values are derived:

BMF = 1 HL_B = 4 days log $K_{organism/water}$ = -0.72 log $K_{organism/air}$ = 3.57

With an assumed octanol equivalent of 6% for the rat, and recalculating to 25 °C for log K_{oa} the following thresholds can be derived for log K_{ow} and log K_{oa}:

BMF = 1 HL_B = 4 days log K_{OW} = 0.50 Log $K_{OA@25\,°C}$ = 5.13

Similar exercises can be performed with data for birds. The equations for non-passerine birds can be applied to commonly used species in laboratory tests such as chickens, ducks and doves for example.

This approach gives a very simplified calculation of the half-life and screening thresholds for log Koa and log Kow. The advantage of such an approach is that the choices for the input parameters are very transparent. However, in the future such an approach could be further validated and/or refined by including more sophisticated PBK models in combination with food web modelling to identify relevant conditions for the concern of bioaccumulation in air-breathing organisms.

5.1.4. Comparison with existing Regulatory Thresholds for K_{OA} and K_{OW} for Screening for Chemicals with Potential for Bioaccumulation in Air-breathing Organisms

ECHA (2017) recommends thresholds for chemicals potentially bioaccumulating in air-breathing organisms of log K_{OW} > 2 and log K_{OA} > 5. While a log K_{OA} threshold of 5 is consistent with, although somewhat stricter than, the above derived thresholds for human and rat, the log K_{OW} threshold of 2 could be seen as too high, i.e., could categorise chemicals with a log K_{OW} between 1 and 2 as being of no bioaccumulation concern, even though they might be B. Using equation (26) we estimate that a log K_{OW} threshold of 2 corresponds to a $HL_{urination}$ in humans and rats in excess of 2 years, and 4 months, respectively, i.e., well in excess of the HL_T of 50 and 7 days corresponding to a *BMF* of 1.

It is therefore instructive to revisit the origins of the threshold values recommended by ECHA (2017), which are based on the food-web bioaccumulation simulations by Czub and McLachlan (2004) and Kelly et al. (2007) that assume there is no biotransformation occurring and also on the work of Armitage and Gobas (2007). The results of those simulations, presented as model-derived bioaccumulation metrics as a function of the chemical partitioning space defined by log K_{OW} and log K_{OA}, are displayed in Figure 1.

Both research groups used colour within the partitioning space to separate chemicals with elevated bioaccumulation potential from those that are unlikely to bioaccumulate. Czub and McLachlan (2004) used a threshold of 10 % of the maximum environmental bioaccumulation potential (EBAP) of a human eating a mixed marine and agricultural diet, whereby the EBAP is defined as the quotient of the chemical quantity in a human divided by the quantity of chemical in the whole environment. They refer to this choice of threshold as "reasonable, but not especially conservative". Kelly et al. (2007) used a threshold of 1 for the quotient of the lipid-normalised concentrations in the top predator and the primary producers of a food web.

Despite the completely different bioaccumulation metrics and the consideration of different types of food webs, the results by the two studies are stunningly consistent in that the thresholds separating coloured area, representing potentially bioaccumulating compounds, from grey or dark blue areas, representing chemicals unlikely to be bioaccumulative, correspond to a log K_{OA} of 6 and a log K_{OW} of 2 (Figure 1).

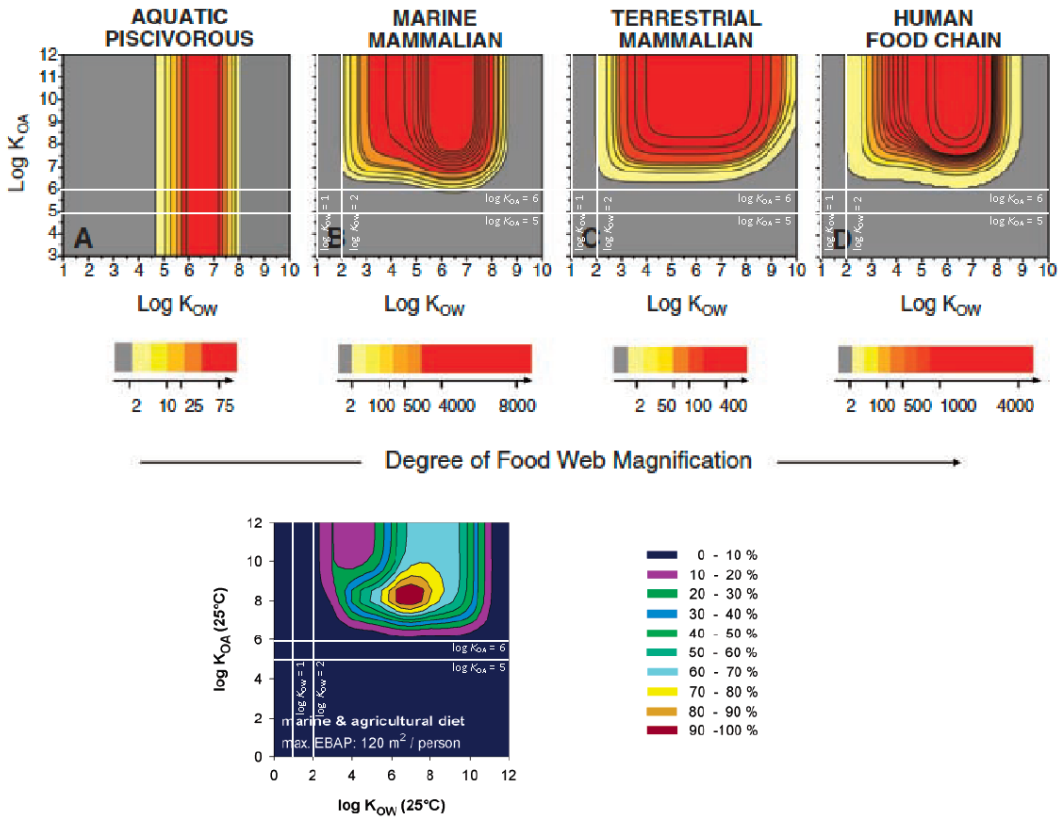

Figure 1 Chemical partitioning space plots displaying the food web bioaccumulation potential of persistent organic chemicals as a function of log K_{OW} and log K_{OA}. Modified from Kelly et al., (2007) at the top, and Czub and McLachlan (2004) at the bottom. White lines designate log K_{OW} values of 1 and 2 and log K_{OA} values of 5 and 6.

It may not be entirely valid to contrast thresholds for bioaccumulation in single organisms as derived above with thresholds proposed for entire food webs. However, it is noteworthy that the top predator in all investigated food chains in Kelly et al. (2007) and Czub and McLachlan (2004) is a warm-blooded, air-breathing, carnivorous mammal of fairly large size (polar bear, Arctic wolf, human), which means that many of the physiological rates and characteristics involved in the elimination threshold estimation above are likely to be similar. It is also plausible that bioaccumulation in the top predator strongly impacts the bioaccumulation metrics displayed as a function of the partitioning space in Figure 1. If the top predator efficiently eliminates a compound, the entire food web leading up to this top predator will also show a small bioaccumulation potential for this compound, if this potential is expressed with a metric involving the concentration in that top predator.

In other words, the four terrestrial food-webs in Figure 1 all have similar top predators, and the bioaccumulation behaviour of that top predator is decisive for the boundaries displayed in the partitioning space plots. Plotting the bioaccumulation metric for a food-web topped with a different type of predator (e.g., a reptile or a bird) as a function of the partitioning space might yield different thresholds.

It is not described why ECHA (2017) has adopted the value of log K_{OW} 2 but lowered the log K_{OA} threshold by an order of magnitude from 6 to 5. A log K_{OA} threshold of 5 is presented in earlier modelling studies (Kelly and Gobas, 2003, Kelly et al., 2003) and during the guidance adoption process for ECHA (2017) it was decided that a log K_{OA} of 5 would be more precautionary. It is clear, however, both from the calculations for

individual air-breathing organisms presented above and the model results by Czub and McLachlan (2004) and Kelly et al. (2007) that a log K_{OA} threshold of 5 is not consistent with a log K_{OW} threshold of 2 in the context of bioaccumulation assessment. The calculations above suggest that the threshold log K_{OW} < 2 to indicate no biomagnification potential may be too high. It may not be possible to indicate a K_{OW} threshold, below which neutral organic chemicals which do not biotransform can no longer biomagnify.

5.2. Comparing Chemical Properties with Threshold Values

5.2.1. Considerations to include Biotransformation in the Screening Assessment

A screening assessment for bioaccumulation in aquatic organisms is usually restricted to the comparison of a log K_{OW} value for a chemical with a threshold value, e.g. 4.5 (ECHA, 2017b) or 5 (UNEP, 2017). Similar to the derivation of thresholds for rapid elimination above, one could rationalise such a K_{OW} threshold as being related to the potential for rapid respiratory elimination from aquatic organisms (e.g., through the gills of a fish).

Assessing the potential for bioaccumulation in air-breathing organisms based only on partitioning properties (i.e., based on rapid elimination by respiration and urination), is considerably less efficient than the K_{OW} threshold for aquatic bioaccumulation, because of the relatively large number of chemicals with a moderate hydrophobicity (log K_{OW} between 2 (or lower) and 5). McLachlan et al. (2011) had observed that a compound's susceptibility to biotransformation is most likely to decide whether it will bioaccumulate or not. A procedure that includes additionally biotransformation half-life HL_B below which elimination is judged to be sufficiently rapid to prevent bioaccumulation has been explored by Wania et al. (2022). In the following sections it is explored how biotransformation prediction could be included in the screening assessment.

5.2.2. How should Chemical Properties be compared with Thresholds?

One can imagine different approaches to comparing the properties X, i.e., the chemical's K_{OW}, K_{OA} and HL_B, to threshold values (TH_X) as identified in section 5.1.1, above. If the purpose of the tier 1 assessment is the identification of chemicals that are not bioaccumulative, as opposed to identifying substances that are bioaccumulative, the comparison will seek out substances with properties below the thresholds. Because meeting any one of these three conditions should be sufficient to prevent a chemical's bioaccumulation, any one of the three properties would need to be below one of the three thresholds for the chemical to be considered of no bioaccumulation concern:

$K_{OA} < TH_{KOA}$ or, $K_{OW} < TH_{KOW}$ or, $HL_B < TH_{HLB}$ (27)

If the goal is to identify substances that are likely to be B, the properties of the chemical have to exceed all three thresholds:

$K_{OA} > TH_{KOA}$ and, $K_{OW} > TH_{KOW}$ and, $HL_B > TH_{HLB}$ (28)

One could also estimate an overall whole-body elimination rate $k_{elimination}$ from a chemical's properties and compare it with a threshold value for that rate. For example, when identifying chemicals that are not bioaccumulative:

$$k_{elimination} = \frac{G_{urination}}{f_{lipid-organism} \cdot K_{OW}(T_{org})} + \frac{G_{respiration}}{f_{lipid-organism} \cdot K_{OA}(T_{org})} + \frac{ln2}{HL_{biotransformation}} > TH_{kelimination} = \frac{E_D \cdot G_{Diet} \cdot f_{lipid-diet}}{f_{lipid-organism}} \quad (29)$$

By using species-specific values when deriving $k_{elimination}$ and $TH_{kelimination}$ in equations (29), one would automatically account for differences in the thresholds for different organisms that are made explicit in equations (25) and (26). The advantage of equation (29) over approach (27) is that it allows for the identification of chemicals that are efficiently eliminated by a combination of more than one elimination mechanism, but not by any one individually. Using (27) would slightly increase the potential for false positive categorisations (i.e., chemical categorised as potentially bioaccumulative, though it is not bioaccumulative) relative to equation (29). Considering K_{OA} and K_{OW} at the same time could be beneficial for the assessment.

Finally, for neutral hydrophobic chemicals one could directly estimate the steady-state lipid-normalised *BMF* from the chemical properties and compare it with the threshold of 1:

$$BMF_L = \frac{E_D \cdot G_{Diet}}{\frac{G_{urination}}{f_{lipid} \cdot K_{OW}(T_{org})} + \frac{G_{respiration}}{f_{lipid} \cdot K_{OA}(T_{org})} + \frac{ln2}{HL_{biotransformation}}} \cdot \frac{f_{lipid-diet}}{f_{lipid-organism}} < 1 \qquad (30)$$

Applying equation (30) is equivalent to using simple biomagnification models for a particular animal species.

5.2.3. How to account for Uncertainty when comparing Chemical Properties with Thresholds?

Every measured or predicted chemical property value is uncertain. When comparing a property with a threshold value, this uncertainty should be taken into consideration. Ideally, a property value should be reported with a numerical confidence range, into which the true value is likely to fall with a certain probability. For example, if a measured property is normally distributed, the true value falls with 68.2% probability within one standard error of the reported measured value. Similarly, 19 out of 20 times a predicted property will fall within the 95% prediction interval.

When the comparison seeks to establish that the property value is smaller than a threshold, i.e., if chemicals that are not bioaccumulative are being identified (see equation 27), the upper end of the confidence range should be compared against the threshold.

If (X_i + PE_i) < T_X, then the chemical is not bioaccumulative. (31)

where Xi is a predicted property of chemical i, PEi is the prediction error of Xi and T is a threshold.

Vice versa, if one aims to identify potentially bioaccumulative substances (see equation 28), the lower end of that range should be compared:

If (X_i - PE_i) > T_X, then chemical may be bioaccumulative. (32)

What confidence range to use (e.g., standard error or 95% confidence interval) depends on the tolerance for false negative decisions. The more important it is to avoid false negatives (e.g., declaring a chemical not bioaccumulative even though it is bioaccumulative), the more stringent the selected confidence limit needs to be. For example, with a standard error, a false negative decision occurs at most one out six times, whereas with a 95% confidence limit, a false negative would at most occur in 2.5% of the decisions. By ignoring the uncertainty of the property value, one risks being wrong 50 % of the time, when the value being compared is very close to the threshold (Wania et al., 2022).

When the chemical properties themselves are not compared with a threshold, but

variables calculated from those properties (such as $k_{elimination}$ and *BMF* in equation (29) and (30) the uncertainty of the chemical properties should be propagated to the variables being used in the comparison, e.g., with the help of Monte Carlo simulation.

5.3. Obtaining Chemical Properties for Comparison with Threshold Values

The chemical property values required for a tier 1 assessment are either measured or predicted. When selecting a value for comparison with a threshold, preference should be given to values that have a reported uncertainty in order to allow for the consideration of that uncertainty (see 5.2.3 above). Many prediction methods now report prediction uncertainty or at least provide information that allows the estimation of such uncertainty, e.g., by estimating the fit with applicability domains or by using average discrepancies from evaluation data sets.

One could imagine tier 1 assessments where $K_{organism/air}$ and $K_{organism/water}$ partition ratios are estimated and compared with thresholds formulated in those terms (see section 5.1.1. above). In particular, the approach to estimating partition ratios to organisms and their tissues using biological composition and ppLFERs as described by Endo et al. (2013) should lend itself well to this purpose, also because it should be possible to estimate the uncertainty of ppLFER predictions and it may be feasible to make estimates of those properties at the body temperatures of the species of interest. This approach is clearly preferable for substances for which partitioning into octanol is unlikely to be a suitable surrogate for partitioning into organisms and their tissues.

In most cases, however, the tier 1 assessments will rely on partitioning ratios involving octanol as a surrogate for organisms and their tissues, i.e., on K_{OA} and K_{OW}. Numerous K_{OW} values and a smaller number of K_{OA} values have been measured and are accessible in databases. In some cases, those measured values are reported with an estimate of their error. There are also many methods, including ppLFERs, available for predicting K_{OA} and K_{OW} and again many of those provide estimates of the prediction uncertainty.

5.4. Unresolved Issues related to Tier 1 Bioaccumulation Assessment in Air-breathing Organisms

The selection of log K_{OW} and log K_{OA} as screening data is useful for lipophilic substances, but not so useful for substances which do not bioaccumulate in lipids and/or for which organism-water partitioning cannot be represented by K_{OW} and/or do not have measurable vapour pressures (e.g., ionic and/or surface-active substances). Substances like PFOS would be missed in Tier 1 screening, because the approach would judge them to be sufficiently water soluble to allow for rapid elimination by urination. The proposed approach cannot account for the process of renal re-absorption, which prevents this elimination from actually taking place. It may be possible to develop some structural alert models to minimise these types of potential errors in practical applications. The approach would be applicable to all chemicals if only their organism/air and organism/water partition coefficients were known.

Chemical property data should not be limited to K_{OW} and K_{OA} only but other metrics need to be considered as well, e.g. polar-lipid/water partition coefficients (e.g., membrane-water) for ionic substances, protein water partition coefficients or even going a step further and consider referring to organism/water and organism/air partition coefficients, rather than K_{OW} and K_{OA} to avoid having to presume the mechanism of bioaccumulation is partitioning in lipids that can be represented by octanol.

Screening assessment in a first tier aims at identifying those substances which have no potential for bioaccumulation, and the assessment can stop there. The substances which

fulfil the screening criteria, however, need further assessment.

In this discussion paper, biotransformation information is placed in tier 2 to refine the screening outcome, by using alternative testing methods to reduce unnecessary animal testing. In addition, chemicals that are rapidly biotransformed to transformation products/metabolites that are themselves not readily eliminated (e.g., because they are persistent) are a bioaccumulation concern. They would not be identified for further assessment if only the biotransformation of the parent molecule is considered.

6. Tier 2: Refined Screening Assessment using Alternative Methods

While physicochemical properties provide an indication of bioaccumulation potential, actual bioaccumulation is the net result of competing rates of chemical uptake and elimination in an organism. Bioaccumulation includes toxicokinetic (TK) processes such as Absorption, Distribution, Metabolism (Biotransformation), and Elimination (so called "ADME", Paini et al., 2019). It is well recognised that biotransformation rates are a key process mitigating biomagnification and bioaccumulation potential in air-breathing organisms (Arnot et al., 2014, Tonnelier et al., 2012). The Bioaccumulation Estimation Tool (BET) [2] and the BAT provide models for incorporating *in silico*, *in vitro* and *in vivo* TK data into BMF model calculations for a range of representative field and laboratory air-breathing organisms (e.g., lab rat, shrew, caribou, wolf, marine mammal).)

Before performing animal testing, a search for existing reliable quality measured *in vivo* data should be conducted. Relevant databases of measured TK data in air-breathing organisms include the OECD QSAR Toolbox[3], EAS-E Suite[2] and MamTKDB[4]. The OECD QSAR Toolbox presently contains HL_T for several substances and will soon be expanded with elimination half-lives from animals through addition of MamTKDB data. It is also possible to search publications for TK data for structurally similar substances. Existence of rat or other mammalian ADME/TK data should be investigated and evaluated. For example, more than 8,000 entries for more than 4,800 chemicals with *in vitro* biotransformation rate data and > 4,000 entries for > 600 chemicals from rodent studies have been critically evaluated and are publicly available[2]. The Arnot et al. 2014 HL_T dataset for humans is composed of 1105 heterogeneous organic chemicals. These data were collected from peer-reviewed sources that include some data quality assurance methods. MAMTKDB v1.0 includes 3927 elimination half-lives for 1407 xenobiotics in various species (rat, human, mouse, dog, monkey, rabbit, cattle, pig, sheep, guinea pig, hamster, horse and goat) with specification of compartment studied (whole body, organ/tissue, cell type, medium)(Hofer et al., 2021).

Biotransformation rates can be included in a PBK model at different levels of biological organisation, depending on the details of the model. For example, 1Co-PBK models like that shown in Equation (22) can be parameterised with estimates of whole-body biotransformation rate constants or half-lives (i.e., $HL_{biotransformation}$), whereas MCo-PBK require compartment specific clearance rates, e.g., for the hepatic clearance (CL_H). If biotransformation rate data are available for other tissues, e.g., lung or kidney or GIT, then MCo-PBK models can be parameterised accordingly. It is noted that $HL_{biotransformation}$ considers the potential for extrahepatic biotransformation as well as hepatic clearance. Biotransformation rates can be estimated from *in vivo* studies, *in vitro* bioassays and by QSARs. There are few studies that measure biotransformation rates *in vivo* directly; however, an approach to estimate whole body i*n vivo* biotransformation half-lives (HL_B)

[2] www.eas-e-suite.com, last accessed: September 2022
[3] www.qsartoolbox.org, last accessed: September 2022
[4] https://data.europa.eu/data/datasets/mammalian-toxicokinetic-database-mamtkdb-1-0?locale=en, last accessed: September 2022

from measured *in vivo* total elimination half-lives (HL_T) in human adults has been developed, tested, and applied (Arnot et al. 2014). The HL_T dataset is composed of 1105 heterogeneous organic chemicals. Four *in vivo* HL_B datasets were derived from different parameter assumptions of a 1-compartment mass balance model and uncertainty analysis was also included in the HL_B estimates in this study (Arnot et al. 2014).

If reliable quality *in vivo* TK data are not available to parameterise the BMF models, the possibility of predicting bioaccumulation metrics (e.g., elimination half-life) using read-across methods, QSARs or *in vitro* methods coupled with IVIVE models should then be investigated. The following sections summarise alternative methods that can be used to parameterise BMF models in Tier 2.

6.1. QSARs for Predicting TK parameters

Various QSARs for predicting HL_T and HL_B from chemical structure (Arnot et al., 2014; Papa et al., 2018) have been developed and validated from *in vivo* human data following OECD guidance for QSAR development and applications to support regulatory decision making (OECD, 2004 and OECD, 2007). The QSARs are readily available for parameterising the general BMF mass balance models in stand-alone software and EAS-E Suite (www.eas-e-suite.com). The Iterative Fragment Selection (IFS) method developed using molecular fragments (Arnot et al., 2014) has been applied to validate HL_T and HL_B QSARs. Another QSAR method that has been applied to validate HL_T and HL_B QSARs is based on holistic molecular descriptors selected by a Genetic Algorithm in the University of Insubria software QSARINS (Papa et al., 2018). Five externally validated HL_B-QSARs were developed using the IFS QSAR approach and multiple linear regression. Satisfactory values of $R2>0.70$ were reported for all the models with ranges of RMSE from 0.45 to 0.49 in the training sets and from 0.70 to 0.75 in the prediction sets. The external predictivity of the QSARINS HL_B-QSARs show slight improvement over the fragment based QSARs derived with the same training sets (RMSE Prediction range from 0.66 to 0.69). According to an analysis of the HL_B-QSARs (Papa et al. 2018), biotransformation potential is reduced mainly by the presence of halogen atoms covalently bonded to carbon atoms, as well as by the presence of polar atoms on large molecules (e.g., polybrominated diphenyl ethers, polychlorinated biphenyls, polychlorinated dibenzodioxins and polychlorinated dibenzofurans).

Both the IFS and QSARSINS methods include Applicability Domain (AD) information. The AD of the IFS method is determined by the similarity of a predicted chemical to those in the training dataset and how well the model fits those chemicals in the training dataset in terms of uncertainty levels (UL). The AD for the QSARINS models is estimated based on the range of the experimental response and of the molecular descriptors of chemicals in the training sets. The QSARINS AD also accounts for the leverage, which is a metric for chemical similarity with the training set and accounts for extrapolations. These two types of QSARs have been applied using a consensus approach as a case study to 12,123 organic chemicals showing that 96% of the HL_T and 98% of the HL_B predictions respectively are within the applicability domain of the models (Sangion et al., 2021).

Other predictive methods for estimating whole body clearance have also been developed, e.g., Lombardo et al. (2014). Furthermore, hepatic clearance (CL_H) QSARs have been developed, and proprietary software for predicting biotransformation is also available. QSARs for *in vitro* intrinsic clearance rates have also been developed, e.g., Obach (2011), Pirovano et al. (2016) and Dawson et al (2021). Recently as part of the CEFIC LRI ECO.44 project, *in vitro* intrinsic clearance rate QSARs have been developed at the University of Insubria, Italy, by Papa and colleagues. These new QSARs were developed using critically evaluated *in vitro* biotransformation rate assays derived from hepatocytes, and microsomal and S9 fractions of hepatic tissues from mammalian species (i.e., human, rat, and mouse). More than 100 new QSARs for predicting *in vitro* intrinsic clearance from chemical structure have been developed and these QSARs have

been implemented in two separate software packages[5]. Reliable predictions from *in vitro* intrinsic clearance rate QSARs can be used to parameterise mass balance BMF models using the IVIVE methods used for *in vitro* measurements as described below.

6.2. *In Vitro* Assays to determine Biotransformation Rates

One of the methods that can improve bioaccumulation assessment by including biological factors that affect bioaccumulation are *in vitro* biotransformation assays. These assays can provide information on the biotransformation rate and bioaccumulation potential of chemicals in organisms without *in vivo* experimentation and with minimal use of animals. In comparison to *in vivo* studies, *in vitro* tests are relatively simple and are conducted at less cost and effort and in less time, allowing for greater efficiency in chemical testing. Uncertainty exists in measured *in vitro* biotransformation rate data and methods to critically evaluate and score such data were developed (Arnot et al., 2022) based on OECD 319A (for hepatocytes) and 319B (for S9) Testing Guidelines for fish *in vitro* metabolism. Databases of critically evaluated *in vitro* biotransformation rates in mammals for thousands of chemicals exist[6].

There are many potential assays that can be used. However, there are two *in vitro* assays, i.e., the primary hepatocyte assay and the liver S9 assay, that are particularly promising for bioaccumulation assessment in terrestrial organisms because of their decades long use in predicting the clearance of pharmaceutical drugs. Assays using liver microsomes are also commonly used for evaluating the biotransformation of pharmaceuticals (Obach, 1999) and are generally more frequently used than assays using liver S9 subcellular fractions (Richardson et al., 2016). However, for bioaccumulation assessment, *in vitro* assays with liver S9 fractions are preferred over assays using microsomes, because microsomes contain mainly phase I enzymes (cytochrome P450 monooxygenases; CYP), while liver S9 subcellular fractions contain all major enzymes responsible for both phase I (e.g., CYP) and phase II biotransformation (e.g., sulfotransferases, uridine 5'-diphospho-glucuronosyltransferases, glutathione transferases) (Ekins et al., 1999). While *in vitro* assays can use hepatic materials from various species (e.g. human, rat, mouse, dogs), the rat may be a valuable animal model for bioaccumulation assessment of chemicals in air-breathing organisms because of the availability of large amounts of *in vivo* data that can be used to verify the *in vitro* approach and determine the uncertainty of the *in vitro* approach.

In vitro substrate depletion assays have been around for many years and have become a valuable tool for evaluating the behaviour of pharmaceuticals in organisms. However, their application for assessing the bioaccumulation potential of organic substances in air-breathing organisms is relatively new.

Many industrial organic chemicals including some pesticides are more difficult to test than pharmaceuticals due to their hydrophobicity, volatility, and poor solubility in aqueous and biological media (Black et al. 2021). As part of CEFIC-LRI ECO 41 project, Lee et al. (2017, 2022) developed a protocol for conducting rat liver S9 *in vitro* bioassays that is specifically designed for bioaccumulation screening of neutral hydrophobic organic chemicals with bioaccumulation potential in air-breathing organisms with the rat as the model organism. This protocol includes substrate depletion experiments that can be conducted in less than 2 hours and applies an experimental design that allows for test results to be compared or tested to regulatory criteria in a statistically valid fashion with specific confidence levels. The rat liver S9 protocol is a variation of the OECD TG 319B (rainbow trout liver S9 fractions) with some minor modifications such as a higher incubation temperature (37°C instead of 11°C), lower pH (pH 7.4 instead of pH 7.8), test optimisation procedures and an experimental design that

[5] https://dunant.dista.uninsubria.it/qsar/, last accessed: September 2022

facilitates statistical testing of the assay data to criterion values that might be used in a screening approach.

In the rat S9 *in vitro* protocol, the test chemical is incubated in potassium phosphate buffer (pH 7.4), rat liver S9 subcellular fractions (usually 1 mg/mL), major phase I (NADPH) and II cofactors (UDPGA, GSH and PAPS), and alamethicin for up to 2 hours. Alamethicin is a pore-forming peptide which enhances UGT activity by increasing the permeability of the ER (endoplasmatic reticulum) membrane that enhances the transport of the cofactor UDPGA across the ER membrane (Ladd et al. 2016). The incubation reaction is terminated at various time points using an appropriate solvent (e.g., methanol, acetonitrile) as stopping solution. The decrease of the test chemical concentration from the incubation vial is measured at the different time points with a validated analytical method (e.g., GC-MS, LC-MS). Enzymatically inactive S9 fractions (e.g., heat inactivated S9) are used as a negative control to evaluate potential abiotic losses due to e.g., adsorption or volatility. In addition, it is recommended to use an appropriate reference chemical like pyrene (Nichols 2018, Lee 2022) to check the enzymatic activity of subcellular fraction and the performance of the system. The *in vitro* biotransformation rate constant (h^{-1}) is determined from the regression slope of the log linear depletion curve taking into consideration the difference between the active S9 and the enzymatically inactive control (Lee et al. 2022).

Although assays using either cryopreserved or freshly prepared primary hepatocytes have been used in the pharmaceutical sector for many years, many different protocols are applied and no standardised method has yet been developed for *in vitro* assays with hepatocytes. A systematic review of published *in vitro* hepatic metabolic clearance studies by Louisse et al. 2020 revealed that hepatocyte concentrations, species (rat or human) and culture medium (in particular presence of serum) have the largest impact on the *in vitro* intrinsic clearance. Harmonisation of protocols is preferable and should produce a protocol that provides robust and reproducible *in vitro* clearance rates (Louisse et al. 2020).

In a recent study by Black et al. (2021), *in vitro* intrinsic clearance rates were compared in primary hepatocytes from humans, rats and rainbow trout for 54 industrial chemicals and pesticides using standardised methods employed to evaluate drug clearance (Wetmore et al. 2012, 2013). In contrast to the rainbow trout hepatocyte assay (OECD TG 319A), chemicals performed poorly in both the human and rat assay resulting in recoveries <80% for the heat inactivated controls. These mammalian assays are usually conducted in multi-well polypropylene plates, whereas the trout assay is done in glass vials. Therefore, *in vitro* assays with rat (or human) hepatocytes may need to be adapted to ensure the selected test system is appropriate for the range of test substances that may be employed in these assays for the purpose of assessing their bioaccumulation potential in air-breathing organisms. For example, capped glass vials may be a preferred test system over multi-well polypropylene plates for substances that are moderately volatile and/or hydrophobic. Alternative (passive) dosing approaches may be appropriate for chemicals that are poorly soluble in the assay system (Lee et al. 2012). When such protocols become available, they can be added to an *in vitro* approach to bioaccumulation assessment.

Rat hepatocyte assays can be expected to produce results similar to those of rat liver S9 *in vitro* bioassays for neutral hydrophobic chemicals. S9 subcellular fractions are technically easier to use for substrate depletion experiments compared to cryopreserved hepatocytes (OECD, 2018c). Studies with rainbow trout did not indicate a preference for one *in vitro* system or the other (OECD, 2018c, Fay et al., 2017, Nichols et al., 2018, Kropf et al., 2020).

6.3. *In vitro - In vivo* Extrapolation for Bioaccumulation Assessment

An important component of an *in vitro* approach to bioaccumulation assessment is the *in vitro-in vivo* extrapolation (IVIVE) of intrinsic clearance rates measured in *in vitro* bioassays to relevant bioaccumulation metrics. The BCF, which is today's preferred metric for regulatory bioaccumulation assessment is not an appropriate or practical metric for extrapolating *in vitro* bioassay data for terrestrial biota because many terrestrial organisms respire air instead of water. The biomagnification factor (BMF), however, is a useful and appropriate metric that can be used for bioaccumulation assessment in air-breathing organisms of terrestrial food-chains. For this reason, Lee et al. (2017, 2022) developed methods for the extrapolation of rat liver S9 *in vitro* bioassay data to the BMF. IVIVE methods for dietary uptake of pharmaceutical drugs in rats are well established and only minor modifications to existing IVIVE methods are required to derive relevant bioaccumulation metrics.

6.4. Performance Evaluation of the *In Vitro* Approach for Bioaccumulation Assessment

Lee et al. (2022) tested the ability of *in vitro* rat liver S9 assays to assess the elimination rates of 14 hydrophobic organic chemicals with terrestrial bioaccumulation potential by comparing elimination rates predicted by the *in vitro* assay data to observed elimination rates measured in rat *in vivo* experiments obtained from the literature. The elimination rate was used for evaluation instead of the BMF because (i) the *in vitro* assay informs on the elimination rate and the elimination rate is a key component of the BMF (together with the uptake rate); and (ii) literature studies that could be used for the performance evaluation reported elimination rates but not the BMF. *In vitro* predicted depuration rate constants correlated well with *in vivo* derived depuration rate constants and were very close (on average only 12% lower) to the *in vivo* measured depuration rate constants. While testing was limited to the chemicals studied, the results of the test are encouraging for the potential application of an *in vitro* approach to bioaccumulation screening.

6.5. Species Variability in Clearance Rates

Gobas et al. (2022) explored the extent of species-to-species variation in intrinsic clearance rates for 12 pharmaceutical substances for which inter-species variation in intrinsic clearance rates were studied using the same bioassay. The study found that geometric mean species-to-rat intrinsic clearance rate ratios for individual substances varied between 0.45 for humans to 2.2 for the horse and 1.06 (0.53 SD) on average (ideal is 1.0), suggesting good general agreement among intrinsic clearance rates measured in rats, humans, and other tested air-breathing species. However, the standard deviations of the geometric mean species-to-rat intrinsic clearance rate ratios were found to be equivalent to a factor ranging between 1.1 and 8.0, indicating there can be considerable inter-species variability in intrinsic clearance rates among substances tested. The combination of all *in vitro* species-to-rat intrinsic clearance rate ratios generated a geometric mean ratio of 0.82, which is close to 1, and suggests that the rat is perhaps a reasonable model for estimating hepatic intrinsic clearance rates in a range of species. The 95% confidence limits of the geometric mean ratio of 0.82 were equivalent to a factor of 9.3, indicating that 95% of all intrinsic clearance rates that were included in the analysis were within a factor of 9.3 of those in the rat. It can be concluded from the results of pharmacological studies that inter-species differences in *in vitro* biotransformation rates of chemical substances in terrestrial organisms can be substantial and may need to be considered in an *in vitro* approach to bioaccumulation assessment.

6.6. Application of *In Vitro* Assays for Bioaccumulation Screening

Lee et al. (2017, 2022) outlines methods for incorporating *in vitro* rat liver S9 bioassay data into a regulatory framework for bioaccumulation assessment. Specifically, the studies showed that in bioassays following the Lee et al. (2022) protocol (which uses a 1 mg/ml protein concentration), hydrophobic organic substances that exhibit a rat liver S9 depletion rate constant greater than 0.3 h^{-1} (which corresponds to a half-life time of 2.3 h) are not expected to biomagnify in rats because they are quickly biotransformed in the liver of the rat. This critical depletion rate constant combined with information on the species-to-species variation in depletion ratios may provide a useful benchmark for bioaccumulation screening. Given that the 95% confidence limits of the geometric mean species-to-rat intrinsic clearance rate ratios of 0.82 are approximately a factor of 9.3, a factor of approximately 10 was suggested as a margin of caution that can be applied to the benchmark *in vitro* depletion rate constant of 0.3 hr^{-1} for the rat proposed by Lee et al. (2022). An *in vitro* depletion rate constant threshold value of 10 x 0.3 h^{-1} or 3 h^{-1} (corresponding to an *in vitro* half-life time of 0.23 h) was suggested as a possible conservative screening value for rat liver *in vitro* S9 depletion tests. In other words, if a chemical exhibits a depletion rate constant in a rat liver S9 assay conducted following Lee et al. (2022) greater than 3 h^{-1} (or an *in vitro* half-life time less than 0.23 h), then the chemical could be expected not to biomagnify in air-breathing organisms. While this threshold value may be considered conservative, it can play a useful tool in terrestrial bioaccumulation screening of chemicals as many chemicals biotransform very quickly.

6.7. IVIVE

The approach of calculating the BMF from an uptake rate constant and an elimination rate constant requires quantitative information on biotransformation in the organism. The biotransformation rate can be measured in *in vitro* studies with hepatocytes or liver S9 subcellular fractions, assuming that the liver is the major organ for biotransformation as described above. The outcome of such *in vitro* studies is a biotransformation rate for the tested chemical normalised to the amount of S9 or the number of hepatocytes used in the assay. The extrapolation procedure that follows to transfer the quantitative information from the assay (*in vitro*) to the organism (*in vivo*) is based on the basic assumption that the hepatocytes or the S9 will have the same transformation capacity *in vivo* as *in vitro*.

The extrapolation of data derived from *in vitro* assays with S9 isolated from liver is performed in two steps (Krause and Goss 2018). The first step is the extrapolation to blood clearance without consideration of flow limitation $CL_{blood\ w/o\ flow\ lim}$ ($mL_{blood}/h/g_{organism}$):

$$CL_{blood\ w/o\ flow\ lim} = \frac{f_{blood}^{unbound}}{f_{assay}^{unbound}} * k_{in\ vitro} * \frac{C_{S9\ in\ organism}}{C_{S9\ in\ assay}} * \frac{w_{assay}}{w_{blood}} \quad (33)$$

The second step extrapolates to the whole-body biotransformation rate constants (1/h):

$$k_{biotransformation,LIVER} = CL_{blood\ w/o\ flow\ lim} * \frac{K_{blood/water}}{K_{organism/water}} \quad (34)$$

Where:

$CL_{blood\ w/o\ flow\ lim}$ ($mL_{blood}/h/g_{organism}$) corresponds to the bodyweight-normalised blood clearance due to biotransformation in liver without flow limitation,

$\frac{f_{blood}^{unbound}}{f_{assay}^{unbound}}$ is the ratio of unbound fractions in blood and in *in vitro* assay (unitless),

$k_{in\ vitro}$ is the rate constant derived from the *in vitro* assay (1/h),

$C_{S9\ in\ assay}$ is the used S9 concentration in the *in vitro* assay (mg_{S9}/mL_{assay}),

$C_{S9\ in\ organism}$ is the S9 concentration in the whole organism in $mg_{S9}/g_{organism}$ (given by the S9 content of the respective tissue and the respective fractional tissue weight),

$\frac{w_{assay}}{w_{blood}}$ is the ratio of water contents in assay and blood $\left(\frac{mL_{water}/mL_{assay}}{mL_{water}/mL_{blood}}\right)$,
$k_{biotransformtion,LIVER}$ corresponds to the whole-body biotransformation rate constant (1/h) based on the transformations that occur in liver.

$K_{blood/water}$ and $K_{organism/water}$ are the blood-water and organism-water partition coefficients of the chemical (mL_{water}/mL_{blood} and $mL_{water}/g_{organism}$).

Blood flow limitation is not considered in the above equations, because blood flow limitation becomes particularly important for chemicals with very fast biotransformation and those tend to be not relevant in the context of bioaccumulation. For extrapolation of *in vitro* data derived from assays with microsomes or hepatocytes, analogous equations apply.

Instead of the one compartment approach that we have followed so far, one can also apply a multi-compartment approach with the same *in vitro* information (but with additional and more detailed physiological information on the rat). This would account for possible blood flow limitation as well as other kinetic limitations such as a hindered membrane permeability, active transport and so on. The more complex multi-compartment approach would also allow for considering first pass effects. However, based on previous investigations this additional complexity may not provide a surplus in accuracy in the outcome that would justify the effort.

6.8. Considerations for Alternative Testing Methods

Rather than an explicit tiered approach for parameterising PBK models for biotransformation rates using *in vitro* or *in vivo* or *in silico* data, it may be better to recognise there can be uncertainties with all estimates and a weight of evidence approach may be warranted in some cases. While tier 2 serves as a way to reduce unnecessary animal testing, long-term integrated testing strategies to address uncertainty in estimating biotransformation rates from alternative methods is strongly encouraged.

Other key factors to consider at tier 2 are reduced dietary absorption efficiency of the chemical from the lumen of the gastrointestinal tract (GIT), the potential for significant first pass-effects, significant active processes that may occur for some chemicals, e.g., chemical binding, resorption at the kidney. It may be possible to develop QSARs and structural alerts to identify which chemicals would require these additional considerations.

7. Tier 3: Definitive Assessment using *In Vivo* Testing Methods

7.1. Availability and Applicability of *In Vivo* Data

The availability of ADME (absorption, distribution, metabolism and excretion) parameters can be useful to assess bioaccumulation properties *in vivo* in air breathing terrestrial mammals. While in the pharmaceutical industry pharmacokinetic/toxicokinetic (TK)

information is obtained early in the development phase by including different species including humans, the TK data availability is limited for other chemicals. Generally, chemicals can be divided into i) TK data rich chemicals (e.g. pesticides regulated under EU 1107/2009 and pharmaceuticals) and ii) TK data poor chemicals (e.g., chemicals regulated under REACH).

Data poor chemicals might obtain TK data from read across, QSARs, screening studies (e.g. OECD TG 422 'Combined repeated dose toxicity study with the reproduction/developmental toxicity screening test') and OECD TGs focussing on toxicodynamic effects (e.g. TG 407 'repeated 28 d'/TG 408 'repeated 90 d'/TG 409 'repeated 90 d in non-rodents') with optional parallel evaluation of TK data.

TK data rich chemicals have TK data that has been obtained from radiolabelled test substance-based OECD TGs like TG 417 'Toxicokinetics' mainly for rats and OECD TG 503 'Metabolism in Livestock' in goats/hens. Studies with unlabelled test item (OECD TG not available) can be performed separately or combined with toxicodynamic studies.

The rat is the commonly chosen species for toxicological studies and most ADME data are available for rats. For read-across purposes, it can be good to continue using rats as the standard model. However, for some groups of chemicals (e.g., negatively charged perfluorinated alkyl acids (PFAAs)), the rat may not always be the best model, and then other species (e.g., minipigs, dogs, sheep, goats, mice) might be chosen.

In general, the purpose of ADME studies is to obtain estimates of basic TK parameters such as C_{max}, T_{max}, rate and extent of absorption, bioavailability (F), area under the plasma concentration-time curve (AUC), volume of distribution (V_d), blood plasma or tissue clearance/depuration rates (and reciprocal elimination half-lives) as well as excretion (urine, faeces, air) rates, parameters of which some can be used to estimate bioaccumulation potential, e.g., tissue and whole body elimination half-lives (also called HL_T when for the whole body). Major metabolites in excreta and urine shall also be identified.

The absorption of a substance is often rapid in comparison to its elimination. Despite a long elimination half-life or high V_d, bioaccumulation may not be occurring if the exposure or absorption/bioavailability (F) is low. Most commonly, formed metabolites have shorter elimination half-lives than parent molecules but there can be exceptions (e.g. DDE formed from DDT) and the distribution may differ. The BMF is another relevant bioaccumulation metric determined at steady state or kinetically using rate constants. However, to date BMFs are reported for air breathing animals in just a few studies (Huwe 2007 and 2008 reported BCFs for rat organs/carcass but may have meant BMFs), no relevant OECD TG exists for the determination of BMF in air breathing animals, and it is difficult to determine this metric in humans, especially due to the diverse and variable diet composition.

As mentioned in previous sections, neutral hydrophobic chemicals tend to bioaccumulate more than hydrophilic ones since they often distribute into fatty tissues such as white (also called visceral adipose tissue) and brown fat, brain, and skin (skin is the largest organ and can contain a considerable amount of dermal adipose tissue depending on species). For retention of neutral hydrophobic substances, the animal's body content plays a role. For hydrophobic substances, excretion through faeces may be the only available route (through bile or direct secretion through intestinal membranes), and the intestinal lipid content can affect the excretion rate (fugacity capacity increases with dietary lipid content).

Moreover, data from OECD TG 305 (bioaccumulation in fish) studies can support the assessment of the chemicals. Beyond the obtained BCF, information about the formation of metabolites is rarely available. These data can be compared with the data obtained in

the OECD TG 503 (metabolism in livestock) studies or fish metabolism studies (Schlechtriem et al., 2016) to improve the assessment of the bioaccumulative properties of a chemical. Field BMF and TMF data can also provide support for bioaccumulation.

In the future it is expected that more human biomonitoring data will be available. These data can improve the data availability and also support PBPK modelling data (Pletz et al., 2020).

7.2. Strength and Limitations of OECD TG 417

OECD TG 417 'Toxicokinetics' (2010) focuses on the investigation of the biological fate of a chemical including the formation of metabolites (Phase I and II metabolites). The complex study is commonly performed with a ^{14}C radiolabelled test substance and is first administered as single (high and low) dose. Repeated (low) dose studies with ^{14}C substance are also possible and are commonly performed for at least 14 days (Hofer, 2021). What is considered a low or high dose will depend on the properties of the substance. In addition, so-called preconditioning repeated dose studies are a possibility (14 days unlabelled test substance plus one day ^{14}C radiolabelled test substance, 14+1 day study (OECD TG 417 §57)) to investigate if metabolism is induced or inhibited after repeated exposure. However, preconditioning appears not appropriate for bioaccumulation assessment since the last administered radiolabelled dose will not be present at steady state conditions (Hofer et al, 2021).

The aim of an OECD TG 417 study is to investigate mass balance, major TK parameters, the distribution within the body, and the detailed presence of metabolites in plasma, urine, bile, and faeces. In contrast to unlabelled test substances, the parent molecule and the respective metabolites are measured combinedly (using liquid scintillation counting, LSC) or separately (chromatographic separation) using HPLC-MS and/or thin layer chromatography (TLC) technology (or radio-HPLC or radio-TLC). The information gained on tissue concentrations might support the relationship of induction of toxicity in a specific organ. The duration of a single dose study is normally 7 days. Animals are young adults (6-12 weeks) and fed ad libitum with standard food. Notably, to obtain whole body (plasma/serum and urine/faeces/air excrete) elimination half-life, the animals are often kept alive, whereas to obtain tissue elimination half-lives the animals must be sacrificed. This often results in two cohorts given the same doses.

Several factors will influence the depuration rate (or the corresponding elimination half-life). It is not a fixed value but relates to the test conditions, food composition, rat strain, animal age (lipid content), etc. In repeated daily administration studies, it is preferable to measure depuration rates after steady state conditions have been reached. The time to establish steady state will differ depending on substance and dose; steady state concentration is determined by the dosage, dosing interval, absorption and clearance rate. It is not practically possible to check substance and metabolite depuration rates (or elimination half-life) in all ~50 organs/tissue types (some are also tough to homogenise), so prioritisation is needed. TG 417 specifies that tissues that should be analysed include liver, fat, GI tract, kidney, spleen, whole blood, residual carcass, target organ tissues and any other tissues (e.g., thyroid, erythrocytes, reproductive organs, skin, eye (particularly in pigmented animals)) of potential significance in the toxicological evaluation of the test substance. Bioaccumulation related parameters such as blood plasma elimination half-life shall be calculated. For major tissues/carcass, remaining total (radioactive) dose/concentration shall be presented/listed, but there is no requirement to present rates or elimination half-life values. The seldomly used method "quantitative whole-body autoradiography" can be used to check remaining radioactivity in minor organs (OECD TG 417 §39). The current version of OECD TG 417 lacks detail on how to include a steady-state phase and investigate the depuration period to assess accumulation properties of a chemical.

Chemicals may be retained in the body due to different reasons: i) enterohepatic circulation, ii) tubular reabsorption in the kidney, iii) affinity to biomolecules, and iv) retained in a compartment due to binding to specific transporters. The properties of the parent molecule can differ from the formed metabolites. Both could possibly also react covalently with biomolecules, e.g., to haemoglobin in red blood cells (increases the elimination half-life if following radioactivity only).

Hofer et al. (2021) scrutinised ADME study summaries in over 300 pesticide EU Draft Assessment Reports (DARs) and found a large report variation. The challenge is that the reported (commonly OECD TG 417 and GLP) studies sometimes had only performed single (not repeated) administration TK studies, or that they contained TK data (e.g., tissue concentrations in table form) but no elimination half-life value had been calculated. Moreover, the number of tissue types presented with elimination half-life values varied strongly among studies: some studies presented elimination half-lives for multiple compartments, others just a single value for one compartment. Moreover, analysing target tissues/organs for lipophilic substances such as white fat, brain and skin was often omitted and autoradiography checking was rarely performed. There were sometimes also unclarities regarding which blood parameter (whole blood, serum, plasma, blood cells or else) the data was for.

Repeated (compared to single) dosing should better ascertain a high radiolabeled substance load into peripheral organ/tissue compartments and establishment of steady state. A recent study that collected and analysed reported elimination half-lives in animal studies (Hofer *et al*., 2021) suggests that single dose administration can be sufficient to conclude on bioaccumulation potential in cases when elimination half-lives are very long (e.g., as for liver accumulating rodenticides). Repeated dose studies are required for most substances having short or medium elimination half-lives in single administration studies to generate representative elimination half-life data. The definition of "long", "medium" and "short" is still under discussion. Repeated dose studies may not be necessary if the HL_T > 50 days (or some conservative cut-off to be determined) after a single dose since this provides support for B/vB. Likewise, if HL_T <1 day (to be determined) after single administration and it is demonstrated that all substance is rapidly and completely eliminated from all organs, there is likely no need to do repeated studies (data supports a not B).

7.3. Strength and Limitations of unlabelled TK Studies

OECD TG 417 theoretically allows the use of unlabelled test item, if the sensitivity and specificity of the analytical methods is sufficient to identify the metabolites, which is a huge technical hurdle. Other guidelines and guidance documents on the performance of TK studies with unlabelled test item are currently not available.

In the pharmaceutical industry these studies are performed in early preclinical development and completed with human data in clinical trials.

In the chemical industry (except pesticides) the focus is on unlabelled TK/ADME studies. Depending on specific questions, tailor-made studies are conducted in e.g. rats or mice. The parallel blood sampling in OECD TG studies evaluating the toxicodynamic properties of a chemical is done very carefully. It must be avoided that the sampling procedure affects the animals, as toxicodynamic effects might be misinterpreted.

The advantage of unlabelled TK/ADME studies is that i) the safety requirements are lower because there is no handling of a radioactive substance, ii) no extra challenging and cost intensive synthesis of radiolabelled test item is required. It must be considered that even for complex test items (e.g. polymers) radiosynthesis, radiolabeled studies might not be appropriate. The technical equipment for analysis of non-labelled samples might be finally less complex (e.g. LC-MS/MS technology is often sufficient).

Furthermore, dietary exposure to an unlabelled substance is possible since mixing radiolabeled substances into food is not always feasible due to safety concerns. The readout of the study is the fate of the parent molecule (not metabolites), ignoring the existing metabolism and formation of Phase I and Phase II metabolites of the chemical. Unfortunately, evaluation of an exact mass balance is challenging and often impossible.

Finally, it must be considered that radiolabeled and unlabelled ADME studies cannot replace each other due to their different approaches and aims. Unlabelled TK/ADME studies should be considered as relevant for chemicals for which radiosynthesis is not applicable or appropriate.

7.4. Expansion of TK Data Availability in the Future

Physiological based pharmaco/toxicokinetic modelling (PBPK) is a method which can be used to examine in which situations biomagnification could occur in field situations.. Weijs et al. (2012) already used PBPK modelling to address bioaccumulation properties in marine mammals. It must be considered that the applicability and performance of the PBPK data depends on the availability of input parameters like hepatic clearance and absorption properties (apparent permeability coefficient, Papp-value) and the applicability to the chemical domain. Thompson et al. (2021) mentioned in their scientific review paper, that "future effort is required to curate available models, identify appropriate similarity metrics to assist in the identification of PBPK models for analogues to chemicals of interest, and, ideally, make such resources widely available, for example, by incorporation into free webtools". Further investigations and generation of input parameters are needed to address bioaccumulation properties of a wide chemical class by PBPK modelling in the future.

Changing an OECD TG is a time and effort consuming process. In the case that the OECD TG 417 is evaluated in the future for adaptation, the inclusion of more detail on how to obtain relevant parameters and test conditions for bioaccumulation assessment is recommended. It may also be possible to write an OECD TG 417 B addendum for cases when bioaccumulation related TK information is requested. A proper study design with focus on bioaccumulation properties will ensure a high quality of the study. A more systematic calculation (and reporting) of compartmental elimination half-lives in TK studies will help build the database may allow better predictions of bioaccumulation potential of untested substances so that animal testing may not always be required. It is recommended to investigate/specify body fat (age dependent in rats) content as this will influence derived bioaccumulation parameters. The seldomly used method "whole body autoradiography" should be emphasised as a useful approach to determine chemical distribution and whole body concentrations in rat, while providing a visualisation of chemical accumulation in specific organs (e.g., small organs, eyes, etc.).

For repeated administration, dosing (preferably oral) shall be done daily all days of the week (not just regular working days). For animal welfare reasons, when long repeated administration is needed dosage through food is recommended over gavage. However, due to radiation safety rules in different countries, dosage via food is not always possible. During repeated administration studies, a small amount of blood should be collected (in one cohort) on an even basis, to check (blood plasma/serum analysis) if steady state has been established. To avoid excessively long experiments, repeated dosing may not need to be continued for more than 5x the elimination half-life for blood plasma determined in single dose administration studies (normally, 97% of the substance is eliminated after five half-lives).

The study report shall contain detailed information. Clearly state which compartment, tissue/organ, or cell type the presented value is for and unit used. Mention if tissues/organs were perfused to rinse/remove blood from blood vessels or not. Particularly for blood, mention experimental procedures (e.g., use of blood clotting

agent, centrifugation steps, etc.) supporting the presented data. Specify if data are for total radioactivity, parent substance, or a specific metabolite (metabolites are both formed and removed, complicating determination of their elimination half-lives).

When presenting rate (or half-life) data as a calculated average for a group of animals, state how the mean (e.g., arithmetic, geometric, harmonic, or weighed) was calculated. Median values are also of interest. For calculation of average depuration rates e.g. for a group of animals, harmonic means can be suitable (Martinez and Bartholomew, 2017).

7.5. Remaining open Questions:

Since bioaccumulation properties are not the main focus in OECD TG 417 or in unlabelled TK studies, there are several open questions, which should be considered and further investigated in the future:

1) Applicability of unlabelled OECD TG studies

Applicability of unlabelled OECD TG studies (e.g. OECD TG 422, repeated 28/90 day studies, and others) to derive toxicodynamic endpoints. Inclusion of new increasingly sensitive analytical technologies (e.g. MALDI-TOF) to support the identification of parent and its metabolites in blood as well as in tissues should be considered. For determination of elimination half-lives, inclusion of a depuration phase after ended administration is necessary, for tissues often N=4 animals/time-point, not to influence the main toxicodynamic readout (with time, e.g. tissue effects may vanish). In OECD TG 422, studying males may be more appropriate than females (pregnant).

2) Availability of other data sets according to OECD

Availability of OECD TG 503 (metabolism in livestock), OECD TG 505 (residues in livestock), and OECD TG 305 (radiolabelled fish metabolism studies) studies might provide useful data as those are performed at steady state and relevant matrices are assessed (e.g., fat, muscle, liver).

3) Adaption of the OECD TG 417

Specifying/adaption of the OECD TG 417 with respect to age, fat content of the animals and characterization of the food item (protein/fibres, fat) is necessary. The following questions should be addressed: i) what kind of PK parameters should be generated in the study (absorption phase $t_{1/2}$, V_d, standardised calculation of AUC, etc.) and ii) how all PK parameters can be assessed. The procedure of tissue perfusion, blood sampling, selection of the most relevant tissues and derivation of elimination half-lives in the context of bioaccumulation should be clearly defined.

4) Overall assessment strategy

Finally, a strategy is needed to assess the B properties of a chemical based on all compiled information (e.g. weight of evidence approach).

7.6. A Weight of Evidence Approach for Definitive assessments

Annex XIII of REACH (EU Regulation 1907/2006) includes general statements for using multiple Lines of Evidence (LoE) such as the *in silico*, *in vitro* and *in vivo* data sources described in previous sections, that can be used in a Weight of Evidence (WoE) Approach for "B" assessment.

In the ECHA guidance documents, Chapter R.4: "Evaluation of available information" deals with the considerations needed for a WoE approach that should consider all

available data, considering the relevance, reliability and adequacy of the information. A template and instructions on problem formulation, collection and documentation of information, reporting of evidence, assessment of the quality of individual evidence, integration and weighing of evidence, and uncertainty analysis is available under https://echa.europa.eu/support/guidance-on-reach-and-clp-implementation/formats.

The Organisation for Economic Cooperation and Development (OECD) recently formalised recommendations for a WoE approach for chemical evaluations (OECD 2019).

The Society of Environmental Toxicology and Chemistry (SETAC) has held various workshops in recent years which have led to the development of a framework for the practical application of WoE approaches to chemical assessment and management.

A formal WoE approach for bioaccumulation assessment has been developed following OECD guidelines for WoE approaches (Arnot et al., 2022). The new methods are also well aligned with Annex XIII of REACH. The primary objective of the published methods and guidance (Arnot et al., 2022) is to guide a person through various steps of conducting a bioaccumulation assessment for water respiring and/or air-breathing organisms. The methods include problem formulation, data gathering, data generation, data reliability evaluation, and data integration. The methods and organisation framework were formalised into the Bioaccumulation Assessment Tool (BAT, available since 2018) providing evaluations to be conducted following the guidance, i.e., including and evaluating multiple types of B and TK data and criteria and different B assessment objectives. The methods can consider virtually all current LoE from *in silico*, *in vitro* and *in vivo* sources for aquatic organisms and air-breathing organisms including those discussed in previous Sections of this report. The data reliability scoring methods developed for *in vitro*, *in vivo* and *in silico* LoE are based on OECD testing guidance. Users can select any criteria they want for categorisation. The BAT provides transparency and consistency as is often required for scientific decision-making and communication with stakeholders. Throughout the development of these methods, and the BAT itself, stakeholders from regulatory agencies were engaged for comments and feedback.

This discussion paper was developed by the ECHA working group on toxicokinetics to develop a scientific assessment scheme for bioaccumulation in air-breathing organisms. The members of the working group are listed at the top of the paper. As such, this paper has not been used for regulatory substance assessment yet. To that end, please refer to the current REACH guidance or the guidance or legislation relevant to the chemical to be assessed.

8. Key Terminology/Acronyms/Definitions

1Co-PBK	one compartment PBK models
a	chemical activity
$a_{predator}$	chemical activity in a predator
a_{prey}	chemical activity in a prey
AE	chemical absorption efficiency from the gastro-intestinal tract (GIT) into the organism (a unitless fraction with a maximum value of 1, or 100%)
AUC	area under the plasma concentration-time curve
BAF	bioaccumulation factor (L/kg) = $C_{Biota} \cdot C_{Water}^{-1}$
BCF	bioconcentration factor (L/kg) = $C_{Biota} \cdot C_{Water}^{-1}$
BMF	biomagnification factor (kg/kg) = $C_{Biota} \cdot C_{Diet}^{-1}$
BMF_L	lipid-normalised biomagnification factor
BMF_M	biomagnification factor normalised to a medium M (e.g., neutral lipids, polar lipids (L), proteins (P), water (W))
BMFmax	theoretical maximum BMF (excluding biotransformation)
C	concentration ($g \cdot kg^{-1}$)
C_{air}	gaseous concentration ($g \cdot L^{-1}$)
C_{diet}	chemical concentration in the diet ($g \cdot kg^{-1}$ diet)
$CL_{blood\ w/o\ flow\ lim}$	bodyweight-normalised blood clearance due to biotransformation in liver, lung, GIT ($mL_{blood}/h/g_{organism}$) or kidney without flow limitation
CL_H	hepatic clearance (accounting for possible blood flow limitation)
C_{lipid}	lipid concentration (mg/kg)
C_{max}	maximum or "peak" concentration
$C_{organism}$	chemical concentration in an organism ($g \cdot kg^{-1}$)
$C^*_{predator}$	chemical concentration in a predator ($g \cdot kg^{-1}$)
C^*_{prey}	chemical concentration in a prey ($g \cdot kg^{-1}$)
C_{water}	chemical concentration in the water ($g \cdot L^{-1}$)
EBAP	quotient of the chemical quantity in a human divided by the quantity of chemical in the whole environment
E_D	dietary uptake efficiency

f	fugacity (Pa)
$f_{predator}$	chemical fugacity in a predator (Pa)
f_{prey}	chemical fugacity in a prey (Pa)
$f_{lipid\text{-}diet}$	dietary lipid content (kg lipid · kg diet^{-1})
$f_{lipid\text{-}organism}$	lipid content of the organism (kg lipid · kg body mass^{-1})
f_O	volume of "octanol-equivalent" in body (L octanol · kg^{-1} body mass)
$f_{unbound\ assay}$	unbound fractions in *in vitro* assay (unitless)
$f_{unbound\ blood}$	unbound fractions in blood (unitless)
F	bioavailability (%)
G_{diet}	feeding rate (kg prey ·kg predator^{-1} · day^{-1}; kg diet · kg organism^{-1} · day^{-1})
GIT	gastro-intestinal tract
$G_{respiration}$	body mass normalised respiration rate (L air · kg^{-1} · body mass day^{-1})
GSH	L-glutathione
$G_{urination}$	body mass normalised urination rate (L urine · kg^{-1} · body mass day^{-1})
$HL_{biotransformation}$	whole-body elimination half-life by biotransformation (day)
$HL_{respiration}$	whole-body elimination half-life due to respiration (day)
HL_T	whole body terminal elimination half-life
$HL_{urination}$	whole-body elimination half-life due to urination (day)
IVIVE	*in vitro in vivo* extrapolation
$k_{biotransformation}$	biotransformation rate constant (day^{-1})
$K_{biotransformation,LIVER\ or\ LUNG\ or\ GIT\ or\ KIDNEY}$	whole-body biotransformation rate constant (hour^{-1}) based on the transformations that occur in liver, lung, GIT or kidney, respectively.
$K_{blood/water}$	blood-water partition coefficient of the chemical (mL water · mL blood^{-1})
k_{diet}	dietary uptake rate constant (kg prey.kg predator^{-1}.day^{-1}; kg food · kg organism^{-1}.day^{-1})
$k_{egestion}$	faecal egestion rate constant (day^{-1})
$k_{elimination}$	first-order whole body total terminal elimination rate constant, also referred to as k_T (day^{-1})
k_{growth}	growth rate constant (day^{-1})

$k_{in\ vitro}$	rate constant derived from the *in vitro* assay ($1 \cdot h^{-1}$)
$k_{reproduction}$	reproductive losses rate constant (day^{-1})
$K_{blood/water}$	blood-water partition coefficient of the chemical (mL water · ml^{-1} blood)
$K_{organism/air}$	equilibrium partition ratio between organism and air at the temperature of exhaled air ($L\ air \cdot kg^{-1}$ organism)
$K_{organism/water}$	equilibrium partition ratio between organism and water (L air · kg^{-1} organism)
$K_{respiration}$	respiration clearance rate ($L \cdot kg^{-1} \cdot day^{-1}$),
K_{OA}	octanol-air partition coefficient (unitless)
K_{OW}	octanol-water partition coefficient (unitless)
k_{uptake}	uptake rate (day^{-1})
$k_{urination}$	urinary excretion rate constant (day^{-1})
k_{water}	water clearance rate ($L \cdot kg^{-1} \cdot hour^{-1}$)
Log D	Logarithm of the octanol-water partitioning coefficient taking into account ionisation at different pH
MamTKDB	mammalian toxicokinetic database
MCo-PBK	multi-compartment PBK models
NADPH	β-Nicotineamide adenine dinucleotide 2'-phosphate
$\varphi_{M,predator}$	fraction of the organisms consisting of medium M (e.g., neutral lipids, polar lipids (L), proteins (P), water (W)) in units of kg M.kg $predator^{-1}$
$\varphi_{M,prey}$	fraction of the organisms consisting of medium M (e.g., neutral lipids, proteins, polar lipids) in units of kg M.kg $prey^{-1}$
PAPS	adenosine 3'-phosphate 5'-phosphosulfate
PBK	physiologically based biokinetic
PFOS	perfluorooctanesulfonic acid
pKa	negative base-10 logarithm of the acid dissociation constant (K a) of a solution
ppLFER	polyparameter linear free energy relationship
QSAR	Quantitative Structure-Activity Relationship
TH_X	threshold value for chemical property X
TK	toxicokinetic

T_{max}	extent of absorption
TMF	trophic magnification factor
UDPGA	Uridine 5’-diphosphoglucuronic acid
S	chemical’s solubility in an actual or subcooled liquid state (mol · m^{-3})
V_d	volume of distribution (L)
w_{assay}	water content in assay (ml water ml^{-1} assay)
w_{blood}	water content in blood (ml water ml^{-1} blood)
Z	chemical fugacity capacity ($mol.m^{-3}.Pa^{-1}$)

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