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Revisiting elimination half live as an indicator for bioaccumulation in fish and terrestrial mammals

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Abstract:

Current bioaccumulation regulation is focused on bioconcentration in fish. An extension to terrestrial mammals, e.g. rat, is urgently needed but will have to use a different metric, most likely the BMF. While both metrics are thermodynamically not equivalent the regulative testing requirements for both might be reduced to the investigation of the respective elimination rate constants k_2 for fish or rat. These k_2 values could be derived from animal tests or from *in vitro - in vivo* extrapolation and could be combined with estimated uptake rate constants to yield either a BCF or a BMF value. The possibility to use *in vitro* methods for k_2 has the advantage that animal tests can be avoided and it bears the chance to experimentally cover species differences which are currently ignored in bioaccumulation regulation. Existing data for BCF and the respective k_2 values for fish - either from feeding studies or from BCF studies themselves- indicate that this approach works. For terrestrial bioaccumulation this approach still needs further experimental support.

Keywords: bioconcentration, biomagnification, chemical risk assessment, up-take rate constant, elimination rate constant.

1. Introduction

Current regulation on bioaccumulation focuses on the bioconcentration factor (BCF) for fish. However, systematic bioaccumulation assessment should be extended to air-breathing organisms, in particular mammals. The BCF approach itself cannot be extended to terrestrial vertebrates due to the different prevalent uptake pathways and the little value of water as reference phase (Gobas et al., 2009). Instead, the biomagnification factor is often seen as a suitable metric for terrestrial vertebrates. A comprehensive bioaccumulation assessment will need to consider both, the aquatic and terrestrial organisms, which means: a chemical is classified as non-bioaccumulative if bioaccumulation is excluded in both cases. A few years ago, the use of elimination half-life as an indicator for biomagnification in air-breathing organisms was suggested (Goss et al., 2013). A comparable approach is also conceivable for fish and would reduce the regulative testing requirements to the investigation of the elimination rate constant k_2 which is already determined in BCF studies following OECD TG 305 (OECD, 2012).

The BCF is defined as the steady state concentration of a chemical *i* in fish divided by the aqueous concentration in the water that the fish is exposed to (while the fish is feeding uncontaminated food).

(1)

In the OECD TG 305 (OECD, 2012a) this definition of the BCF is complemented by a kinetic definition which can be derived mathematically from the steady-state approach if one assumes the fish to be a single, well-stirred compartment with instantaneous equilibrium partitioning within the fish and with all uptake and elimination processes following first order kinetics. According to the kinetic approach the BCF equals the first order uptake rate constant divided by the first-order elimination rate constant covering all elimination processes for the considered chemical.

(2)

Under REACH, a chemical is considered as bioaccumulative if the BCF exceeds a value of 2000 (L/kg) for a standardized fish with 5% lipid content. Both, steady-state measurements as well as kinetic measurements are accepted by the authorities. For fish growing substantially during the duration of the test, a growth correction of the experimental data is needed (Brooke and Crookes, 2012).

It has been suggested that existing kinetic BCF experiments could be simplified by just measuring the elimination rate while the uptake rate is estimated (Brooke and Crookes, 2012; OECD, 2012b; Goss et al., 2013). The reasoning behind this suggestion is that the uptake rate constant, k_1, contains mostly information that we are able to estimate rather reliably and that is not chemical specific (Brooke et al., 2012). In their report Brooke and Crookes (Brooke and Crookes, 2012) investigated this approach using a dataset from Jon Arnot (http://www.arnotresearch.com) with 169 BCF data points covering 108 chemicals and 14 fish species. They plotted these BCF data versus measured elimination rate constants, k_2, from the same experiments in a double logarithmic plot and found a linear correlation with a slope close to unity. This is what one would expect when the concept of using estimated k_1 works and if all fish had a similar size (which was not the case). But for unknown reasons Brooke and Crookes did not go the next step to really estimate BCF values based on this approach and based on actual fish sizes as required by the allometric formula for estimating k_1. Instead Brooke and Crookes (Brooke and Crookes, 2012) came to a rather negative conclusion about this approach apparently because of the rather high scatter in their plot. Interestingly, though, the authors did not consider that part of this scatter came from ignoring the size dependence and another part must have come from uncertainties in the experimental BCF values.

The aim of this study was to elucidate whether k_2 values (or elimination half-lives which is equivalent) can be used as an indicator for bioaccumulation in fish. Experimental BCF values from the literature were compared with BCF values calculated for given chemicals using experimental k_2 from the BCF studies and k_1 values estimated according to an allometric scaling formula. Experimental BCF data were further compared with BCF data which were calculated using experimental k_2 from fish feeding studies and estimated k_1 values. Following theory, the uptake path should not matter for the elimination process as long as the well-mixed compartment assumption holds. Therefore, it should be possible to derive BCF values also based on k_2 values from feeding studies. Indeed, this is suggested in the OECD 305 guideline from 2012 (OECD, 2012a) for those chemicals that are so hydrophobic that controlled aqueous exposure is difficult (see also (Gobas and Lo, 2016) (Schlechtriem et al., 2017). Interestingly, a validation of this approach has so far not been available.

Finally we discuss the possibility of also using elimination half-lives for the bioaccumulation assessment of terrestrial organisms

2. Methods

2.1. Literature search

BCF experiments have been performed for decades and thus many data are available in the published literature. However, in earlier times almost no standardization took place and important experimental parameters were not reported. Hence, there are still data around that are not standardized with respect to lipid content although a standard lipid content of 5% as a reference has been agreed on for a long time. Another important standardization – growth correction- has in fact only become commonly accepted since the latest revision of OECD guideline 305 in 2012. For our first goal, the validation of estimating BCF from a measured k_2

and an estimated k_1, lipid and growth corrected data would have been ideal but this could not be accomplished. The missing lipid correction was less of an issue because both BCF and k_2 had been measured for the same fish but in most cases experimental BCF values from the literature have also been reported without any information on fish weight, Hence, we eventually ended up with rather few data that would allow the calculation of k_1 from the allometric formula based on fish weight (see below). Data collection for our second goal, the comparison of k_2 from BCF experiments and from fish feeding experiments was even more difficult. Our first demand was that both data for a given chemical should have been measured for the same fish species because metabolism is known to be species dependent (Schultz and Hayton, 1999; Bischof et al., 2016). In addition data for similar fish size, normalised to lipid content and corrected for growth would have been desirable. The latter demands could not be fulfilled though.

2.2. BCF calculation with experimental k_2 and estimated k_1

A kinetic BCF can be calculated from an experimental k_2 (taken from the BCF experiment itself) and an estimated k_1. The uptake rate constant, k_1, is a function of the ventilation rate of the fish and the uptake efficiency of the chemical which is defined as the amount of chemical taken up into the circulatory system of the fish divided by the amount of chemical that was dissolved in the ventilated water. Data measured by (McKim et al., 1985) suggest that the uptake efficiency of rather hydrophobic chemicals (i.e. log $K_{ow} > 3.5$) is around 60% without much variance between different chemicals. In a recent physiologically based modeling approach (Larisch et al., 2016) we could confirm this by mechanistic reasoning and show that uptake of these hydrophobic chemicals from ventilated water in the gills is independent of the chemical´s properties and only a function of the ventilation rate and the fraction of ventilated water that can equilibrate with well perfused lamellae during the rather short residence time in the gills. This fraction of ventilated water volume is called the respiratory volume and amounts to about 60% of the ventilated water volume as determined in a study on rainbow trout (McKim et al., 1985). For less hydrophobic chemicals uptake efficiency is lower because of blood flow limitation (Larisch et al., 2016). Sijm et al. came to very similar results (Sijm et al., 1994; Sijm et al., 1995) in their studies with isolated perfused gills of rainbow trout. These authors suggested an allometric scaling formula with which the uptake rate constants of rather hydrophobic chemicals in fish of various weight can be predicted (Sijm et al., 1995):

$$k_1 = 520\ W^{-0.32} \qquad (3)$$

where k_1 is the uptake rate constant in (L/kg/day) and W is the fish fresh weight in g. This formula is already implemented in the OECD TG 305 on bioaccumulation. Brooke et al. (Brooke et al., 2012) published an overview of a large number of methods for estimating k_1 values for uptake via gills in fish and found the method of Sijm et al. to belong to the best performing models in their comparison by (Brooke et al., 2012).. One should note that Brooke et al. concluded that even the best performing methods show a relatively large uncertainty regarding the estimation of k_1 when compared to experimental data which is, according to our impression, at least partly due to a missing standardization of the experimental determination of k_1.

3. Results & discussion

3.1 BCF calculation: Experimental k_2 from fish BCF studies combined with estimated k_1

Fig. 1 shows the results of the comparison between experimental BCF values and BCF values estimated with eq. 2 from k_2 values taken from the depuration phase of the very same BCF experiments and estimated k_1 values according to eq. 3. The data represent 10 different fish species and 17 different compounds.

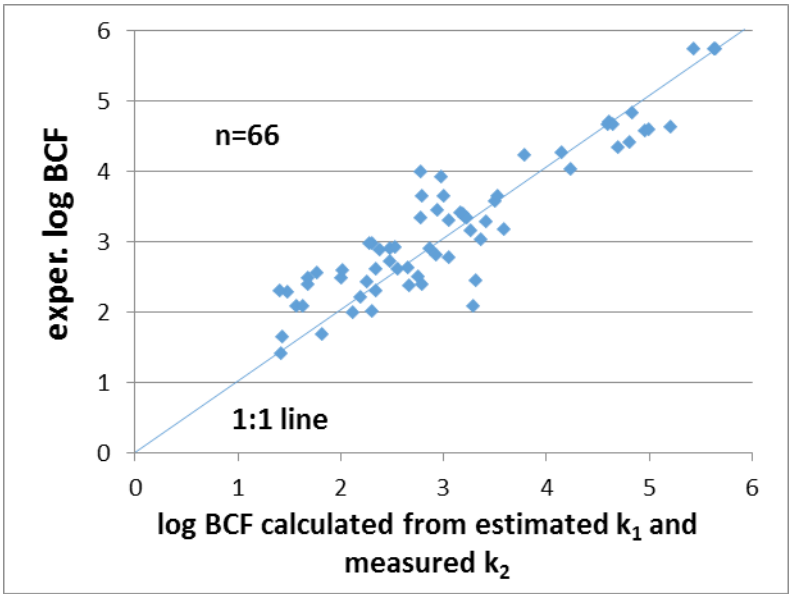

Fig. 1: Experimental BCF values from literature (SI Table S1) plotted versus BCF values calculated with eq. 2 using experimental k_2 from the BCF study and estimated k_1 according to eq. 3 for various chemicals and fish species in a double logarithmic plot (rmse= 0.47 log units)

We conclude that the method of combining a measured k_2 with an estimated k_1 value gives good results despite some scatter around the ideal 1:1 line. The scatter is due to the inevitable experimental scatter in both methods (in vivo BCF and k_2 based BCF) and cannot be taken as an argument against one of the methods. In fact, the scatter in experimental BCF data themselves is an inherent problem of the BCF regulation and not a specific problem of the use of k_2 values as an indicator of bioaccumulation. Evaluation of the Arnot data collection from (Brooke and Crookes, 2012) shows that BCF data from a single research group on a given chemical and a given fish species often vary by a factor 2, sometimes more. Data from different research groups and for different fish species can easily vary up to a factor 10 and in one case it was a factor 56 (see Table S2 in the Supporting information). This scatter comes from the use of different species (with possibly different metabolism rate constants), different fish sizes, missing growth correction and other factors that are not strictly standardized in BCF tests. The effect of fish size can be exemplified by eq. 3 for the uptake rate constant via gills. According to the allometric formula a 500 g fish will have an uptake rate constant of 70 (L/kg/day) compared to 1400 (L/kg/day) for a fish of 0.05 g. The authors of the recent OECD draft guidance document on *in vitro – in vivo* extrapolation (OECD, 2017a) came up with a very similar conclusion: “When used to evaluate the validity of *in vitro-in vivo* metabolism extrapolation efforts, it should also be kept in mind that even high quality experimental BCF data differ by >0.5 log units for at least 35% of chemicals tested and >1 log unit for at least 10% of chemicals (Nendza et al., 2010) which may result in BCFs values which are below and above a certain B threshold, e.g., as described for lindane (log BCF ranging from 2.16-3.32) (Arnot & Gobas,2006). “

3.2. BCF calculation: Experimental k_2 from fish feeding studies combined with estimated k_1

We have collected literature data for k_2 values from both, fish feeding studies and BCF studies with only aqueous exposure, for a given chemical and a given fish species and compared them in Figure 2A. Note that these data are neither growth corrected nor lipid standardized because in most cases the necessary information was missing. Also in some cases the fish size differed substantially. Data area averaged from literature data collected in SI Table S3.

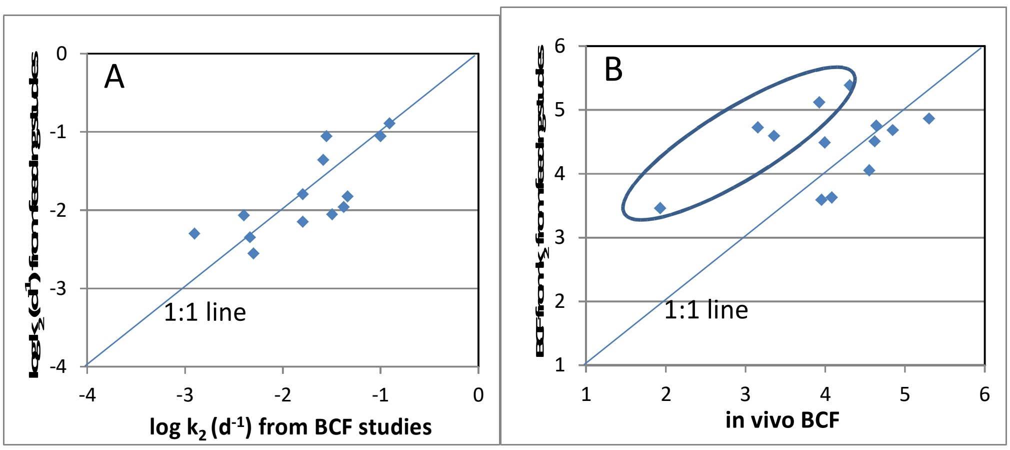

Fig. 2 Comparison of k_2 data for given chemicals and fish species but different exposure scenarios (aqueous exposure and feeding study) in Fig. 2A (rmse = 0.38 log units). In Fig. 2B

BCF data from the same aqueous exposure studies are compared with BCF data predicted from k_2 from the feeding studies and k_1 from eq. 3. The outliers are very hydrophobic chemicals (rmse = 0.37 log units without outliers).

It appears that these data support the idea that the type of exposure does not matter for experimental k_2 values. In a next step we have used these k_2 values, combined them with estimated k_1 values according to eq. 3 and plotted the resulting BCF values against those from the aqueous exposure BCF studies (Fig. 2B). Interestingly, in this plot 5 outliers appear. A closer look at the outliers reveals that they are extremely hydrophobic compounds (log K_{ow} > 8.0, except for Mirex (log K_{ow} =7.5)). For the outliers the experimental k_1 is much smaller than expected from eq.3. It has been argued that up-take of such super hydrophobic chemicals in organisms is indeed hindered but we believe that experimental artifacts are a much more likely explanation (see discussion in (Larisch and Goss, 2018) which is in fact the reason why the classical aqueous exposure BCF studies were supplemented by feeding studies for very hydrophobic chemicals, in the first place (OECD, 2012a).

3.3 Advantages of using experimental k_2 values for estimating BCF

Various efforts have been undertaken in the past to reduce the experimental effort needed for a BCF determination (Springer et al., 2008; Adolfsson-Erici et al., 2012; Carter et al., 2014). Using k_2 as the experimental test criterion for BCF in fish is an additional option for simplifying current regulation, because: a) No uptake curve has to be measured. b) Various means of contaminating the fish can be used for a k_2 experiment (provided that the fish is homogenously contaminated at the start of the clearance experiment) with no need to change the way in which the test results are used or interpreted (see also (OECD, 2012b; Gobas and Lo, 2016)). This is an important feature that has led to the addendum of OECD 305 that allows a fish feeding test for chemicals that are not well water soluble (see above). Note that such fish feeding experiments would result in a bioaccumulation classification inconsistent with the current BCF regulation if they were not evaluated in terms of k_2 as proposed in

Annex 8 of OECD 305 but with a dietary biomagnification factor as endpoint (as suggested in an earlier version of OECD 305). See SI-2 for a back of the envelope calculation and (Gobas and Lo, 2016) for additional arguments. Gobas and Lo (Gobas and Lo, 2016) also support the idea of using the k_2 value from a feeding study to estimate a BCF value. However, rather than using an empirical estimation of k_1 they prefer to derive k_1 from the measurement of non-metabolizing reference chemicals. c) The use of k_2 allows further reduction of the experimental effort and the number of test animals if the metabolism rate constant is estimated from *in vitro* tests with hepatocytes or liver S9 fraction (see new OECD draft guideline (OECD, 2017b, c)): The total k_2 is then received by combining the extrapolated metabolic rate constant with estimated rate constants for clearance via gill and feces. Currently, all chemicals that exceed a certain level of hydrophobicity are suspected to be bioconcentrating in fish until animal tests have proven the opposite. In practice, many hydrophobic chemicals turn out to be not bioconcentrating because the chemicals are sufficiently metabolized in fish. If this can be proven reliably by an *in vitro* test, then most animal tests on BCF could probably be avoided in the future. These *in vitro* tests could also present an opportunity to learn more about interspecies variability in BCF (something that is currently not considered in the mandatory animal tests) by using hepatocytes from different fish species. A recent ring test on *in vitro* metabolism studies for 6 chemicals gave promising results in terms of reproducibility of the *in vitro* rate constants (OECD, 2017b, c). We see this as a proof of principle that should now be followed by more studies on the natural variability of *in vitro* data as a function of fish size, maturity, sex and species. Data from Gobas and coworkers (Lo et al., 2015; Gobas and Lo, 2016) indicate that it will also be necessary to account for biotransformation in gastro-intestinal cells in addition to hepatic metabolism if the in vitro –in vivo extrapolation (IVIVE) method is to become a valid replacement of animal tests.

3.4. Bioaccumulation in terrestrial mammals

Bioaccumulation regulation for mammals should be based on exposure via food which is typically the dominating uptake pathway. Bioaccumulation cannot occur from air (if we neglect transport by particles) because exhaled air has a greater transport capacity for chemicals (because it is warmer) than inhaled air. Accumulation via drinking water is also not relevant due to the small water volume that mammals consume and the low transport capacity of water for not very water soluble and potentially bioaccumulative chemicals. Thus, food typically is the major transport pathway for bioaccumulation in mammals and so it makes sense to use the biomagnification factor (BMF) as the relevant endpoint, which is defined as the steady state fugacity of a chemical in an organism divided by the steady state fugacity of the chemical in its food (Equation 4).

(4)

Similar to the BCF, the BMF of a chemical i can be described by a simple kinetic approach if the organism is treated as a single, well-stirred compartment and if uptake and elimination kinetics are first order processes.

Fish are rather efficient in clearing themselves via the ventilated water (roughly 1000 L water per kg fish and per day (Klyszeijko et al., 2003)) based on the physico-chemically driven equilibrium. In contrast, mammals cannot clear themselves effectively from chemicals via physico-chemical partitioning into exhaled air, or excreted urine and feces because the respective sorption capacities of these media are small and their excreted volumes are insufficient for clearance of hydrophobic chemicals. Hence, it has been concluded that chemicals with a log $K_{ow} > 2$ and a log $K_{oa} > 5$ would typically exceed the BMF > 1 threshold in mammals if no metabolism occurs (Gobas et al., 2003; Czub and McLachlan, 2004; Armitage and Gobas, 2007; Kelly et al., 2007; Goss et al., 2013). A physico-chemical screening of chemicals based on these screening criteria will leave many more suspect chemicals for terrestrial bioaccumulation than it does for aquatic bioconcentration (Gobas et al., 2003). Fig. 3 shows the screening results for some 10 000 neutral chemicals from the Canadian Domestic Substances list (note: a very similar figure has been presented in (Gobas et al., 2003)). All chemicals (roughly 5000) in the upper right box would be classified as potentially bioaccumulative in mammals and would have to undergo some kind of experimental testing.

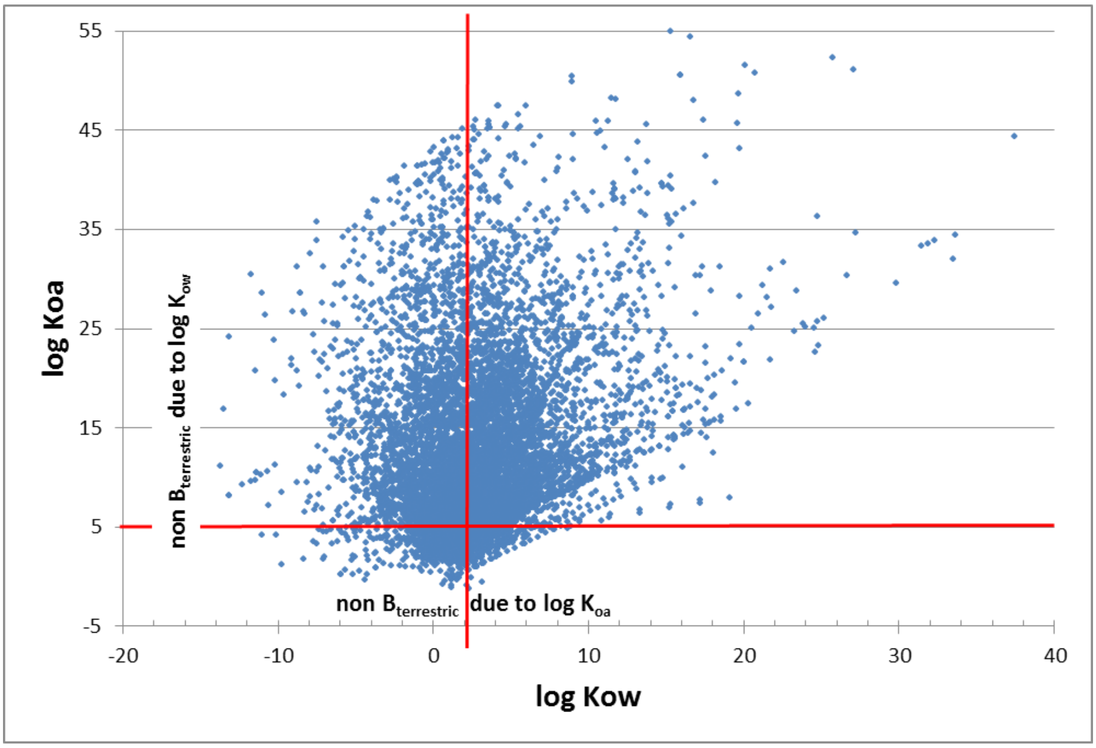

Figure 3: log K_{ow} and log K_{oa} for all neutral chemicals from the Canadian Domestic Substances List calculated with EPISuite (based on data reported in Wittekindt & Goss, 2009)

3.5. Testing bioaccumulation in terrestrial mammals

Currently there is no test guideline on mammalian bioaccumulation. In fact, any new guideline that would involve additional animal tests with mammals would be highly controversial for ethical reasons and also cost-prohibitive. For chemicals in commerce, field studies may be helpful, but they cannot easily be standardized and interpreted (van den Brink et al., 2016). For new registrations “Repeated Dose Oral Toxicity Study in Rodents” are mandatory under the current REACH regulation. These tests last 28 days (tonnages > 10 t / year) or 90 days (tonnages > 100 t / year) (OECD guidelines 407, 452 or 453). These studies could also be utilized to investigate the bioaccumulation behavior of the studied chemicals. While these studies are currently not designed to provide steady-state concentrations in rat, they could certainly be extended in this direction. This would require additional analytical efforts to analyse the sacrificed animals at the end of the experiment and it would require additional analytical effort to document the time course of the internal concentration of the chemicals during the experiment. For the latter one would preferably use blood samples from the test animals without sacrificing additional animals. While an adjustment of the “Repeated Dose Oral Toxicity Study in Rodents” might provide helpful bioaccumulation data for selected chemicals on the long run (when all stakeholders have agreed on and validated an updated guideline) it is hard to see how this could quickly help to assess the mammalian bioaccumulation potential of thousands of chemicals.

3.6. In vitro-in vivo approach for estimating BMF in rat

Similar to the BCF one can estimate the uptake rate constant of a chemical from food in a mammal fairly well by combining the known feeding rate with an up-take efficiency of 100%. This is a worst case assumption but also pretty close to realistic values (Moser and McLachlan, 2001; Kelly et al., 2004; Thomas et al., 2005). Hence, the main unknown quantity that remains to be tested experimentally in order to derive a kinetic BMF value is the clearance rate constant k_2. Note that due to the different allometric scaling of the rates of feeding, urination, fecal excretion etc. a BMF <1 threshold would actually translate into different k_2-values for different animals. Therefore, a k_2 value as such is not suitable as a bioaccumulation threshold. However, the BMF itself depends less on allometric effects because these allometric effects influence uptake and elimination rate constants in a similar way so that their influence on the BMF value of a chemical tends to cancel (Goss et al., 2013). We can thus expect that BMF values of a given chemical in different species tend to be the same as long as their metabolisation capability is not fundamentally different. This is supported by experimental data that were assembled in (Goss et al., 2013).

Also in this case it is appealing to think of an *in vitro- in vivo* extrapolation approach in order to derive experimental information on metabolism that is missing in the Tier 1 assessment. *In vitro* metabolic information from fish has been shown to be different from that for mammals and can therefore not be used for mammals (Han et al., 2007; Weisbrod et al., 2009). But in pharmaceutical science the use of *in vitro* assays with mammalian hepatocytes is a standard procedure (Pelkonen et al., 2009; Dvorak, 2016) and we expect that this could also become part of a BMF assessment. The half-lives that need to be covered by such *in vitro* tests are different though between fish and rat. For fish the critical elimination half-life is around 3.3 days (see section 2.2). Based on *in vitro-in vivo* extrapolation we can conclude that the *in-vitro* test for fish should cover half-lives of 1 - 2 hours at the most in a typical assay set up with 2 10^6 hepatocytes/ml assay in order to account for metabolic rates that can efficiently reduce bioconcentration. For a rat a rough calculation gives a different result: if we assume that a rat would typically have a daily feeding rate of 4 % of its body weight and that its food has the same fugacity capacity for the investigated chemical as the rat itself, and if we further assume an uptake efficiency of 100 % from the food then we can estimate an uptake rate constant k_1 for a rat of 0.04 kg_{food}/kg_{rat}/day. From this uptake rate constant we can conclude that an elimination half-life of 17 days would still suffice to keep the corresponding BMF below the threshold of 1 in rat (Goss et al., 2013). This would mean that an *in vitro* assay with rat hepatocytes should cover half-lives up to 8 h because these would still be relevant for the BMF assessment, rather than 1-2 hours for fish. Such long half-lives cannot be covered by typical hepatocyte assays. Hence, additional in vitro methods for measuring longer half-lives may be needed. Another difference in applying the *in vitro – in vivo* method to terrestrial organisms is that first pass effects can become important for oral uptake. For a worst-case assessment such effects can be ignored because they lower bioaccumulation. For a more precise assessment, data evaluation based on the simple one-compartment models that are currently used in the IVIVE scheme for BCF (Weisbrod et al., 2009) is not sufficient. Instead, multi-compartment modelling should become the standard for assessing terrestrial bioaccumulation from *in vitro* methods. And similar to fish it might improve the overall accuracy of this approach if the *in vitro* method would also cover biotransformation in other tissues than the liver.

A direct validation of such an *in vitro-in vivo* approach for estimating BMF in rat will not be possible due to missing BMF experiments with rat so far. However, a comparison between modelled and experimental toxicokinetic data in rat for chemicals with known *in vitro* metabolism rate constants might show whether our toxicokinetic understanding is sufficient to also predict the steady-state situation described by the BMF.

4. Conclusions

The quality and comparability of experimental BCF values from the literature may be by far not as good as implied in many discussions. Therefore, care must be taken not to use (low quality) BCF values from the literature as "the gold standard" against which every innovation suggested to further improve bioaccumulation assessment is compared.

Experimental k_2 values from aquatic or terrestrial animal tests can be combined with estimated uptake rate constants from water and the diet to yield BCF or BMF estimates, respectively. This is irrespective of the exposure scenario that led to the starting point of the depuration study. Ionic species were not considered in this study and need to be discussed separately. While the general requirements from the BCF or BMF regulation should also hold for ionic chemicals, the details of uptake and elimination processes are different for ions than for neutral chemicals (e.g.(Rendal et al., 2011)).

Standardized experiments for BMF in mammals do not exist yet. The “Repeated Dose Oral Toxicity Study in Rodents” which is already required under REACH could be adjusted and complemented by some chemical analysis in order to fulfill this purpose. *In vitro* generated metabolic rate constants may have the potential to reliably indicate whether metabolism of a chemical may be efficient enough to prevent bioaccumulation and they may also provide us with a better understanding of the effect of biological diversity on bioaccumulation when assays use hepatocytes from different animals belonging to different age groups, sex, size, species and so on. In fact, if one combines such diverse *in vitro* information not only with a simple 1-compartment model for an organism but with a multi-compartment PBTK model for various exposure scenarios then one has the chance to generate much more relevant bioaccumulation information than any single standardized animal test ever could.

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