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GUIDANCE

Guidance on Information Requirements and Chemical Safety Assessment

Chapter R.7c: Endpoint specific guidance

Version 4.0

December 2023

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Guidance on Information Requirements and Chemical Safety Assessment
Chapter R.7c: Endpoint specific guidance

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Preface

This document describes the information requirements under the REACH Regulation with regard to substance properties, exposure, uses and risk management measures, and the chemical safety assessment. It is part of a series of guidance documents that are aimed to help all stakeholders with their preparation for fulfilling their obligations under the REACH Regulation. These documents cover detailed guidance for a range of essential REACH processes as well as for some specific scientific and/or technical methods that industry or authorities need to make use of under the REACH Regulation.

The original versions of the guidance documents were drafted and discussed within the REACH Implementation Projects (RIPs) led by the European Commission services, involving stakeholders from Member States, industry and non-governmental organisations. After acceptance by the Member States competent authorities the guidance documents had been handed over to ECHA for publication and further maintenance. Any updates of the guidance are drafted by ECHA and are then subject to a consultation procedure, involving stakeholders from Member States, industry and non-governmental organisations. For details of the consultation procedure, please see:

https://echa.europa.eu/support/guidance/consultation-procedure/ongoing-reach/

Consultation procedure for Guidance [PDF]

The guidance documents can be obtained via the website of the European Chemicals Agency at:

http://echa.europa.eu/web/guest/guidance-documents/guidance-on-reach

Further guidance documents will be published on this website when they are finalised or updated.

This document relates to the REACH Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006[1].

[1] Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No 793/93 and Commission Regulation (EC) No 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC (OJ L 396, 30.12.2006, p.1; corrected by OJ L 136, 29.5.2007, p.3).

Document history

Version	Changes	Date
Version 1	First edition	July 2008
Version 1.1	Corrigendum: (i) replacing references to DSD/DPD by references to CLP (ii) further minor editorial changes/corrections	November 2012
Version 2.0	Second edition. Partial revision of this document was necessary to take into account the revised version (2.0) of Chapter R.11 of the Guidance on IR&CSA following amendment of Annex XIII to REACH (according to Commission Regulation (EU) No 253/2011 of 15 March 2011, OJ L 69 7 16.3.2011). Main changes in the guidance document include the following: • References to the updated Chapter R.11 have been added and the corresponding text updated; The document has been re-formatted to ECHA new corporate identity.	November 2014
Version 3.0	Partial revision of the document with respect to PBT/vPvB aspects to take into account the updated version of Chapter R.11 (v 3.0). Main changes in the guidance document include the following: • Update to Sections R.7.10.1 to R.7.10.8 on aquatic bioaccumulation; • Update to Section R.7.10.8 to R.7.10.14 on terrestrial bioaccumulation • New Section R.7.10.15 on Mammalian toxicokinetic data in bioaccumulation assessment; • Update of cross-references and links to the revised sections of Chapter R.11.	June 2017
Version 4.0	Partial revision of the document with respect to PBT/vPvB aspects to take into account the updated version of Chapter R.11 (v 4.0) and recent changes in the legal text in Annexes VII-X (REACH review, Action 2). Main changes in the guidance are listed below. The update Sections include:	December 2023

	• R.7.10.2 "Information requirements for aquatic bioaccumulation". • R.7.10.3 "Available information on aquatic bioaccumulation" regarding Hyalella azteca Bioconcentration Test (HYBIT), *in vitro data* on aquatic bioaccumulation and *in vitro-in vivo* extrapolation (IVIVE), use of field data. • R.7.10.4. "Evaluation of available information on aquatic bioaccumulation" regarding the data interpretation from fish dietary studies and the to include the Hyalella azteca Bioconcentration Test (HYBIT). • R.7.10.5 regarding the use of the Hyalella azteca Bioconcentration Test (HYBIT) in Step 4b in Weight-of-Evidence for concluding for aquatic bioaccumulation. • R.7.10.8 "Terrestrial bioaccumulation" regarding in air breathing species. • R.7.11.5.3, R.7.11.6.3 and the Table R.7.11—2 regarding the screening assessment based on EPM. • Appendix R.7.10-3 "Considerations for difficult substances" on ionisable and surface active substances. • Former Appendix R.7.10-2 In vitro methods for aquatic bioaccumulation removed • Former Appendix R.7.10-4 Quality criteria for data reliability of a (flow-through) fish bioaccumulation study removed • Update of cross-references and links to the revised sections of Chapter R.11.	

Convention for citing the REACH regulation

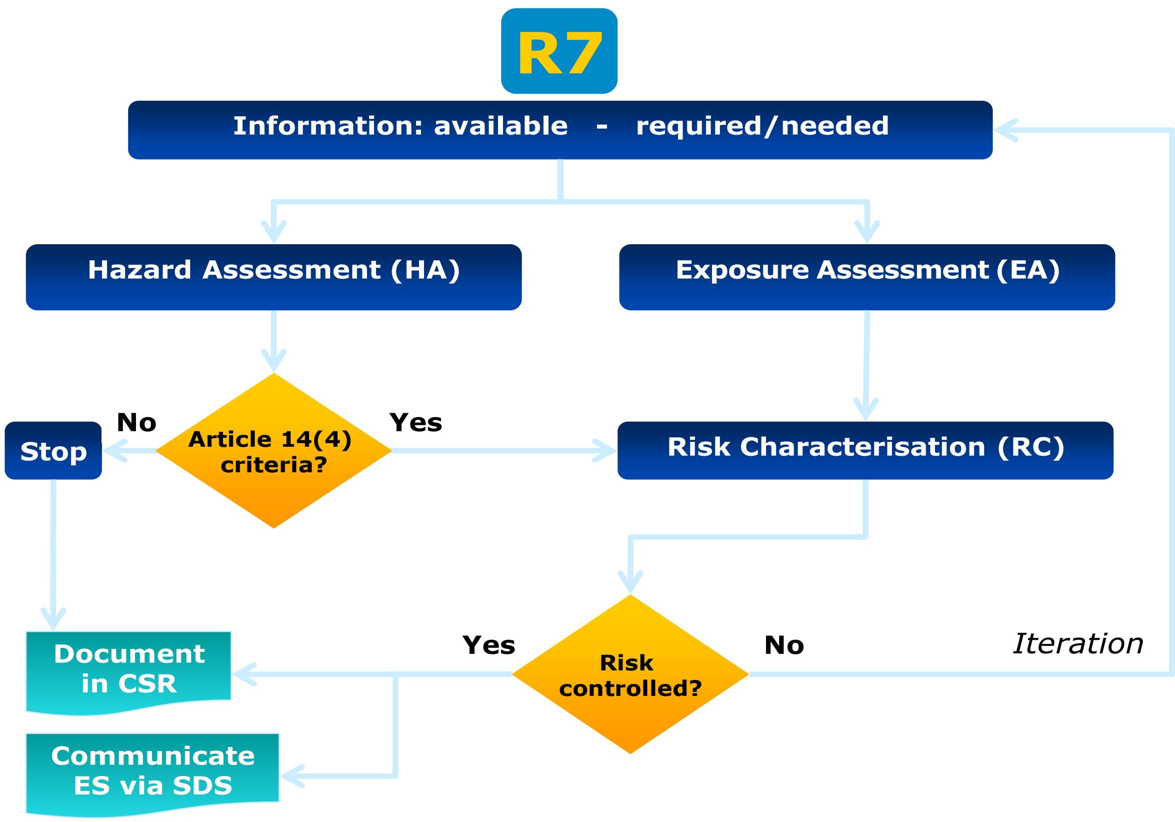
Where the REACH regulation is cited literally, this is indicated by text in italics between quotes.

Table of Terms and Abbreviations

See Chapter R.20.

Pathfinder

The figure below indicates the location of chapter R.7(c) within the Guidance Document:



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R.7.10 Bioconcentration and bioaccumulation; long-term toxicity to birds

R.7.10.1 Aquatic bioaccumulation

Information on accumulation in aquatic organisms is vital for understanding the environmental behaviour of a substance, and is a relevant consideration at all supply levels, even when it is not a specified requirement. The information is used for hazard classification and PBT assessment as well as wildlife and human food chain exposure modelling for the chemical safety assessment. It is also a factor in deciding whether long-term ecotoxicity testing might be necessary. This is because chemical accumulation may result in internal concentrations of a substance in an organism that cause toxic effects over long-term exposures even when external concentrations are very small. Highly bioaccumulative substances may also transfer through the food web, which in some cases may lead to biomagnification.

R.7.10.1.1 Definitions of aquatic bioaccumulation

Several terms have been used to describe chemical accumulation in biota, and slightly different definitions of these (all of equal validity) may be found in the literature. For the purposes of this document the following definitions have been used:

Accumulation is a general term for the net result of absorption (uptake), distribution, metabolism and excretion (ADME) of a substance in an organism. These processes are discussed in detail in the mammalian toxicokinetics guidance document (see Section R.7.10.15). In aquatic organisms, the main removal processes – referred to as elimination or depuration – is diffusive transfer across gill surfaces and intestinal walls, and biotransformation to metabolites that are more easily excreted than the parent compound. Further discussion of aquatic bioaccumulation processes may be found in other reference sources such as ECETOC (1996) and Boethling and Mackay (2000). Maternal transfer to eggs may add to depuration and can sometimes be significant, while growth may affect the concentration in an organism in the case when the rate of other excretion processes is in the same order of magnitude as the growth (dilution) rate.

Bioconcentration refers to the accumulation of a substance dissolved in water by an aquatic organism. Annex 1 of OECD test guideline (TG) 305 contains definitions for BCF. The steady-state *bioconcentration factor* (BCF_{SS}) is the ratio of the concentration of a substance in an organism to the concentration in water once a steady state has been achieved:

$$BCF_{SS} = C_o/C_w$$

where BCF is the bioconcentration factor (L/kg)

C_o is the substance concentration in the whole organism (mg/kg, wet weight)

C_w is the substance concentration in water (mg/L)

Please note that corrections for growth and/or a standard lipid content are not accounted for in this definition of the BCF.

The steady-state bioconcentration factor (BCF_{SS}) does not change significantly over a prolonged period of time, the concentration of the test substance in the surrounding medium being constant during this period.

Assuming that the organism can be mathematically represented as a homogeneously mixed single compartment (Sijm, 1991), and that first order kinetics applies, a BCF can also be expressed on a kinetic (i.e. non-equilibrium) basis as the quotient of the uptake and depuration rate constants:

$$\text{(Kinetic) } BCF_K = k_1/k_2$$

where k_1 is the uptake clearance [rate constant] from water (L/kg/day)

k_2 is the elimination rate constant (day^{-1}).

In principle the value of the BCF_{SS} and the BCF_K for a particular substance should be comparable, but deviations may occur if steady-state was uncertain or if corrections for growth have been applied to the kinetic BCF.

Bioaccumulation refers to uptake from all environmental sources including water, food and sediment. The *bioaccumulation factor* (BAF) can be expressed for simplicity as the steady-state (equilibrium) ratio of the substance concentration in an organism to the concentration in the surrounding medium (e.g. water in natural ecosystems).

For sediment dwellers, the bioaccumulation factor BAF is the ratio of the concentrations in the organism and the sediment, as defined by OECD TG 315. This may be normalised by multiplication with the quotient of the fraction of organic carbon of the sediment and the fraction of lipid in the invertebrate (f_{oc}/f_{lip}), in which case the term is referred to as the biota-sediment accumulation factor (BSAF).

Biomagnification refers to accumulation via the food chain. It may be defined as an increase in the (fat-adjusted) internal concentration of a substance in organisms at succeeding trophic levels in a food chain. The biomagnification potential can be expressed as either:

a *trophic magnification factor* (TMF), which is the concentration increase in organisms with an increase of one trophic level (Fisk *et al.*, 2001); or

a *biomagnification factor* (BMF), which is the ratio of the concentration in the predator and the concentration in the prey:

$$BMF = C_o/C_d$$

where BMF is the biomagnification factor (dimensionless)

C_o is the steady-state substance concentration in the organism (mg/kg)

C_d is the steady-state substance concentration in the diet (mg/kg).

Whereas BMFs describe the increase in concentrations from prey to predator, TMFs describe the average increase in concentration per trophic level.

Trophic dilution occurs when the concentration of a substance in a predator is lower than that in its prey (due to greater metabolic capacity and increased compartmentalisation of higher trophic level species, etc.).

Secondary poisoning refers to the toxic effects in the higher members of a food chain that result from ingestion of organisms from lower trophic levels that contain accumulated substances (and/or related metabolites).

In all of the above equations, the concentration in the organism should be expressed on a wet (rather than dry) weight basis. In addition, it is important to consider lipid normalisation and growth correction in some circumstances and these are considered further in Section R.7.10.4 and R.7.10.5.

R.7.10.1.2 Objective of the guidance on aquatic bioaccumulation

The aim of this document is to provide guidance to registrants on the assessment of all available data on a substance related to aquatic bioaccumulation, to allow a decision to be made on the need for further testing.

R.7.10.2 Information requirements for aquatic bioaccumulation

Annex VIII, Section 9.3., Column 2 specifies that "*Further information on bioaccumulation shall be generated if additional information on bioaccumulation as set out in Annex XIII, point 3.2.2, is required to assess PBT or vPvB properties of the substance in accordance with subsection 2.1 of that Annex.*

In case the generation of additional information requires further testing in accordance with Annex IX or Annex X, the registrant shall propose or the Agency may require such testing."

If a registrant, while conducting a CSA, cannot derive a definitive conclusion (i) "The substance does not fulfil the PBT and vPvB criteria" or (ii) "The substance fulfils the PBT or vPvB criteria" in the PBT/vPvB assessment using the relevant available information, he must, based on section 2.1 of Annex XIII to REACH, generate the necessary information. In such a case, the only possibility to refrain from testing or generating other necessary information is to treat the substance "as if it is a PBT or vPvB" (for further details, see Chapter R.11 of the Guidance on IR&CSA).

Annex IX, Section 9.3.2 to REACH indicates that information on bioaccumulation in aquatic – preferably fish – species is required for substances manufactured or imported in quantities of 100 t/y or more. In general, this means the establishment of a fish bioconcentration factor, although a biomagnification factor may also be appropriate in some circumstances. In column 2 of this section it is noted that "*the study does not need to be conducted if:*

- *the substance has a low potential for bioaccumulation (for instance a log Kow ≤ 3) and/or a low potential to cross biological membranes, or*
- *direct and indirect exposure of the aquatic compartment is unlikely.*

The study may not be waived on the basis of low octanol-water partition coefficient alone, unless the potential for bioaccumulation of the substance is solely driven by lipophilicity. For instance, the study may not be waived on the basis of low octanol-water *partition coefficient alone if the substance is surface active or ionisable at environmental pH (pH 4 – 9).*

For nanoforms, use of any physicochemical property (e.g. octanol water partition coefficient, dissolution rate, dispersion stability) as a reason for waiving the study shall include adequate justification of its relevance to low potential for bioaccumulation or unlikely direct and indirect exposure of the aquatic compartment." Further below in this Guidance it is explained when a bioaccumulation study may or may not be waived on the basis of low octanol-water partition coefficient alone and what may be considered and recommended to be done in such cases.

Reliable measured data are preferred if available (see Section R.7.10.5), but Annex XI to REACH also applies, encouraging the use of alternative information at all supply levels before a new vertebrate test is conducted. A number of alternative methods have been developed, such as the freshwater amphipod Hyalella azteca bioconctration test (HYBIT) (OECD draft TG under revision; OECD, 2023), which delivers an aquatic BCF value, or estimation of intrinsic hepatic clearance from *in vitro* assays according to OECD 319 A and B, which can be extrapolated to a BCF using *in vitro-in vivo* extrapolation (IVIVE) methods. A number of QSARs are also available, the applicability of which depends on the reliability and adequacy of the prediction for each specific substance. The OECD QSAR Assessment Framework provides guidance on how to support prediction from QSAR models appropriately.Prediction techniques are well developed for many classes of organic substance (see Section R.7.10.3), and surrogate information (e.g. the octanol-water partition coefficient or K_{ow}) may sometimes suffice on its own or as part of a *Weight-of-Evidence* approach. The methods to determine aquatic bioaccumulation are summarised in Section R.7.10.3.

R.7.10.3 Available information on aquatic bioaccumulation

The following sections summarise the types of relevant data that may be available from laboratory tests or other sources. It should be noted that most of the methods were developed for neutral (i.e. non-ionised) organic substances, and there may be problems applying some of the concepts to other substances – further guidance is provided in Section R.7.10.4.

Several databases exist that summarise such information on a large number of substances, and the more important ones are described in Appendix R.7.10-1.

R.7.10.3.1 Laboratory data on aquatic bioaccumulation

In vivo tests for aquatic bioaccumulation

Fish bioconcentration test

Traditionally, bioconcentration potential has been assessed using laboratory experiments that expose fish to the substance dissolved in water. A number of standardised test guidelines are available. The current EU C.13 method is based on the OECD test guideline (TG) 305, 1996, which was updated in October 2012 and is briefly described below. The OECD TG 305 (OECD, 2012a) is the most widely used test guideline. Other guidelines such as ASTM E1022-94 (ASTM, 2003) and the public draft guideline OPPTS 850.1730 (US EPA, 1996a) are very similar[2].

The revised OECD TG 305 (OECD, 2012a) provides guidance for the following three tests with different exposure methods and sampling schemes:

- OECD TG 305-I: Aqueous Exposure Bioaccumulation Fish Test
- OECD TG 305-II: Minimised Aqueous Exposure Fish Test
- OECD TG 305-III: Dietary Exposure Bioaccumulation Fish Test

The main changes in the revised test guideline compared to the previous version of OECD TG 305 from 1996 are the following:

- The testing of only one test concentration can be considered sufficient, when it is likely that the bioconcentration factor (BCF) is independent of the test concentration.
- A minimised aqueous exposure test design in which a reduced number of sample points is possible, if specific criteria are met.
- Fish lipid content should be measured so that BCF can be expressed on a lipid-normalised basis, as well as normalised to a 5% lipid content to allow comparison with other studies.
- Greater emphasis on kinetic BCF estimation (when possible) next to estimating the BCF at steady state.
- For certain groups of substances, a dietary exposure test will be proposed, where this is considered more suitable than an aqueous exposure test.
- Fish weight should be measured at least at the start and end of the study so that BCF_K can be corrected for growth dilution.

During aqueous bioconcentration testing, a sufficient number of fish are exposed to one or two sub-lethal concentrations of the test substance dissolved in water. Both fish and water are sampled at regular time-intervals and the concentration of test substance measured. Tests are generally conducted using a flow-through system, although a renewal system is allowed if the requirement of constant aqueous concentration is met (flow-through methods are preferred for hydrophobic substances (i.e. log K_{ow} >3)). After reaching an apparent steady-state tissue concentration (or after 28 days, whichever is sooner), the remaining fish are transferred to clean water and the depuration is followed. If a steady-state is not achieved within 28 days, either the BCF is calculated using the kinetic approach or the uptake phase can be extended. Further guidance on the duration of the uptake and depuration phases is included in paragraphs 17 and 18 of OECD TG 305.

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[2] The main differences concern the: (a) method of test water supply (static, semi-static or flow through); (b) requirement for carrying out a depuration study; (c) mathematical method for calculating BCF; (d) sampling frequency; (e) number of measurements in water and number of samples of fish; (f) requirement for measuring the lipid content of the fish; and (g) minimum duration of the uptake phase.

Paragraphs 49-51 of the OECD TG 305 explain the conditions under which use of a single exposure concentration is possible and further guidance is available in the OECD Guidance Document on aspects of OECD TG 305 on fish bioaccumulation (OECD, 2017). The main benefit of the single concentration bioconcentration test is that it uses fewer fish than the two concentrations test. Therefore, there are animal welfare benefits in performing the single concentration test.

The aim of the aqueous bioconcentration testing is to produce a reliable estimate of how much substance could concentrate from the aquatic compartment (C_w) to fish (C_f) so that a bioconcentration factor (BCF_{SS}) can be calculated by using the ratio C_f/C_w at steady-state. However, a BCF_K value is preferred, and it may also be calculated as the ratio of the uptake rate constant (k_1) and the depuration rate constant (k_2). OECD TG 305 (OECD, 2012a) contains a procedure for growth correction. To avoid uncertainty caused by growth correction, non-growing adult fish are preferred for testing. Aqueous exposure tests (i.e. OECD TG 305-I and 305-II) are most validly applied to substances with log K_{ow} values between 1.5 and 6. Practical experience suggests that if the aqueous solubility of the substance is low (i.e. below ~0.01 to 0.1 mg/L), this test might not provide a reliable BCF because it is very difficult to maintain exposure concentrations (Verhaar *et al.*, 1999). Volatile and degradable substances are also difficult to test with this method for similar reasons andflow-through testing is thus recommended.

<u>Previous OECD TG 305 (OECD, 1996)</u>

The 1996 OECD guideline consolidates five earlier guidelines (A-E) (OECD, 1981) into a single revised method. If data have been obtained with one of these earlier guidelines, the method should be compared to the consolidated version to determine if any significant differences exist (e.g. the 1996 and 2012 OECD guidelines no longer recommend the enhancement of solubility by using dispersants).

A related approach is the *Banerjee method* (Banerjee et. al., 1984), which assumes that the decline in measured aqueous concentrations of a test substance in a static exposure test system is due to accumulation by fish (the estimated increase in fish tissue concentrations being calculated as a mass-balance). An adaptation called the *adjusted Banerjee method* includes monitoring of fish concentrations as well (de Maagd, 1996).

<u>Fish dietary bioaccumulation test</u>

In fish dietary exposure tests, a sufficient number of fish are usually exposed to one sub-lethal concentration of the test substance spiked on fish food. Both fish and experimental diet are sampled at regular time intervals and the concentration of test substance measured. It is recommended to conduct the test using a flow-through system in order to limit potential exposure of the test substance via water as a result of any desorption from spiked food or faeces. However, semi-static conditions are also allowed. An uptake phase of 7-14 days is recommended but it can be extended if necessary. As fish may not reach steady-state during the uptake phase, the data treatment and results are usually based on a kinetic analysis of tissue residues. This lack of steady state may also apply to the BMF measured for any reference substances used in the test. The depuration phase begins when the fish are fed for the first time with unspiked food and usually lasts for up to 28 days or until the test substance can no longer be quantified in whole fish, whichever is sooner. It is important to remove any uneaten food and faeces shortly after feeding to avoid the test substance partitioning to the water leading to exposure via the water.

A dietary exposure test (OECD TG 305-III: Dietary Exposure Bioaccumulation Fish Test) should be considered for substances for which it is not possible to maintain and measure aqueous concentrations reliably and/or potential bioaccumulation may be predominantly expected from uptake via feed. As indicated in the OECD TG 305, for strongly hydrophobic substances (log K_{ow} > 5 and a water solubility below ~ 0.01-0.1 mg/L), testing via aqueous exposure may become increasingly difficult. However, an aqueous exposure test is preferred for substances that have a high log K_{ow} and a water solubility level that allow determination by available analytical techniques, and for which the maintenance of the aqueous concentration as well as the analysis of these concentrations do not pose any constraints.Also, if the expected fish concentration (body burden) *via* water exposure within 60 days is expected to be below the detection limit, the dietary test may provide an option to achieve body burdens that exceed the detection limits for the substance. As such, the principle idea of the dietary test is to obtain a depuration rate constant for substances for which this is impossible via the aqueous exposure route. However, an improved exposure method (e.g. column generated concentrations) and a refined analytical technique, e.g. solid phase microextraction (SPME) and the use of a radiolabelled substance could be considered first to improve the application and detection limit in the aqueous test as a preferable alternative to a dietary study. The endpoint for a dietary study is a dietary biomagnification factor (dietary BMF), which is the concentration of a substance in predator (i.e. fish) relative to the concentration in the prey (i.e. food) at steady state. The dietary test also provides valuable toxicokinetic data including the chemical assimilation efficiency (α, absorption of test substance across the gut) and the whole body elimination rate constant (k_2). Once the assimilation efficiency has been obtained, a kinetic BMF can be calculated by multiplying it with the feeding rate constant (I) and dividing the product by the overall depuration rate constant k_2. However, the preferred endpoint from the OECD TG 305 dietary exposure test is the BCF value estimated from a predicted uptake rate constant and the experimentally determined depuration rate using the Dietary Exposure Test Spreadsheet of OECD 305 TG[3], unless it can be demonstrated that the uptake rate constant (k1) cannot be reliably estimated with the available methods. Detailed description of the methods to estimate a BCF from a dietary study can be found in Annex 8 of OECD TG 305 (OECD, 2012a) and the Guidance Document on Aspects of OECD TG 305 (OECD 2017) in chapter 4.6.3, comprising 1) Uptake rate constant estimation method, 2) Relating depuration rate constant directly to BCF and 3) Correlating dietary BMF with BCF.

More information on the fish dietary bioaccumulation test and the use of the results from it in the PBT assessment can be found in the Chapter R.11 of the *Guidance on IR&CSA*. Further information about interpretation of these studies is available in Section R.7.10.4.1 and in OECD (2017).

[3] Accessible at https://www.oecd.org/chemicalsafety/testing/section-3-environmental-fate-behaviour-software-tg-305.htm (last accessed: October 2022)

Invertebrate tests: *Hyalella azteca* bioconcentration test (HYBIT)

Hyalella azteca is an epibenthic amphipod which is widespread in North and Central America and commonly used for ecotoxicity studies (Environment Canada 2013; US EPA 2000c; ASTM International 2020). The freshwater amphipods can be easily cultured in the laboratory and are available during the entire year. Due to their high reproduction rate and fast growth, experimental organisms can be raised within a few weeks to adult size to meet the need for a high amount of large organisms required for bioaccumulation testing (Schlechtriem *et al.* 2019).

A draft OECD TG for the Hyalella azteca bioconcentration test (HYBIT) is under revision (OECD draft TG under revision; OECD, 2023. It is discussed further in Section R.11.4.1.2.2 in Chapter R.11 of the *Guidance on IR&CSA.* This TG provides a non-vertebrate test to estimate the bioconcentration potential of substances.

The TG has been developed in such a way that it is as close as possible to the concept described in OECD TG 305. However, a minimised exposure design and a protocol for the performance of biomagnification experiments are not available in this TG.

Apart from the established flow-through regime commonly applied in fish bioconcentration studies, semi-static regimes are permissible as exposure scenarios in studies carried out according to this TG. Both regimes have been validated as part of an international ring trial. The aqueous exposure test is most appropriately applied to stable organic chemicals with log K_{OW} values between 1.5 and 6.0, but may still be applied to strongly hydrophobic substances (having log K_{OW} > 6.0), if a stable and fully dissolved concentration of the test substance in water can be demonstrated.

The decision on whether to conduct a flow-through or semi-static exposure experiment, should be based on the opportunity to maintain stable exposure concentrations in the water phase during uptake. Parameters derived from the test which characterise the bioaccumulation potential of chemicals include the uptake rate constant (k_1), the depuration rate constant (k_2), the steady-state bioconcentration factor (BCF_{SS}) and the kinetic bioconcentration factor (BCF_K).

Radio-labelled test substances can facilitate the analysis of water and tissue samples, and may be used to determine whether identification and quantification of metabolites will be necessary.

Invertebrate tests: others

Several other standardised guidelines for bioconcentration in invertebrates exist or are in development:

OECD TG 315 Bioaccumulation in Sediment-dwelling Benthic Oligochaetes is a further method for generating bioaccumulation information in aquatic invertebrates. The recommended oligochaeta species are Tubifex tubifex (Tubificidae) and Lumbriculus variegatus (Lumbriculidae). The species Branchiura sowerbyi (Tubificidae) is also indicated but it should be noted that it has not been validated in ring tests at the time of writing. The bioaccumulation factor (expressed in kg wet (or dry) sediment·kg^{-1} wet (or dry) worm) is the main relevant outcome and can be reported as a steady state bioaccumulation factor BAF_{SS} or as the kinetic bioaccumulation factor (BAF_K). In both cases the sediment uptake rate constant k_s (expressed in kg wet (or dry) sediment·kg^{-1}

of wet (or dry) worm d^{-1}), and elimination rate constant k_e (expressed in d^{-1}) should be reported as well. The biota-sediment accumulation factor (BSAF) is the lipid-normalised steady state concentration of test substance in/on the test organism divided by the organic carbon-normalised concentration of the substance in the sediment at steady state. To reduce variability in test results for organic substances with high lipophilicity, the BSAF should be reported (OECD, 2008). It should be noted that the term biota-sediment accumulation factor (BSAF) has been used in the literature to refer to bioaccumulation factors in sediment which have not been normalised to organism lipid and sediment total organic carbon content. Care should be taken to ensure it is clear what the reported value refers to.

OECD TG 315 recommends the use of artificial sediment. If natural sediments are used, the sediment characteristics should be specifically reported. For lipophilic substances, BSAFs often vary with the organic carbon (OC) content of the sediment. Typically a substance will have greater availability to the organism when the sediment OC content is low, compared to a higher OC content. It should be considered to test at least two natural sediments with different organic matter content, the characteristics of the organic matter, in particular the content of black carbon, should be reported. To ensure comparability of results between different sediments, BSAF normalised to organism lipid and sediment total organic carbon content is used. This allows tests on the same substance and tests on different substances to be comparable. The load rate should be as low as possible and well below the expected toxicity, however it should be sufficient to ensure that the concentrations in the sediment and in the organisms are above the detection limit throughout the test. The relevance of bioavailability of the substance for the test organism should also be considered. In (normal) cases, when accumulation from the porewater is expected to dominate, bioaccumulation could be expressed as a BCF between organism and dissolved pore water concentrations. It is important to consider the implications of the worm gut contents when interpreting the study results (Mount et al, 1999; OECD TG 315).

ASTM E1022-94 (replaced by ASTM E1022-22) describes a method for measuring bioconcentration in saltwater bivalve molluscs using the flow-through technique (ASTM, 2003). It is similar to the OECD TG 305, with modifications for molluscs (such as size, handling and feeding regime). Consequently it has similar applicability. Results should be reported in terms of total soft tissue as well as edible portion, especially if ingestion of the test material by humans is a major concern. For tests on organic and organometallic substances, the percentage of lipids in the tissue should be reported. Recommended species are Blue Mussel (*Mytilus edulis*), Scallop (*Pecten* spp.) and Oyster (*Crassostrea gigas* or *C. virginica*). A similar test is described in OPPTS 850.1710 (US-EPA, 1996b).

ASTM E1688-00a (ASTM, 2000) describes several bioaccumulation tests with spiked sediment using a variety of organisms (some of these are also covered by US-EPA guidelines), including: freshwater amphipods (*Diporeia sp*.), midge larvae (*Chironomus tentans*) and mayflies (*Hexagenia sp*.). Many of these are based on techniques used in successful studies and expert opinion rather than a specific standard method. The small size of many of these organisms sometimes means that large numbers of individuals are required for chemical analyses. Further useful information on sediment testing can be found in US-EPA (2000a).

In addition, non-standard tests may be encountered in the scientific literature, involving many species. Some information on uptake may also be available from sediment organism toxicity tests if tissue analysis is performed. However, a test specifically designed to measure uptake is preferable.

In vitro data on aquatic bioaccumulation

Procedures used to estimate intrinsic hepatic clearance from *in vitro* assay data were originally developed by the pharmaceutical industry to support preclinical screening of drug candidates (Rodrigues, 1997). These procedures have been used for several decades (Rane *et al.*, 1977), and significant progress has been made in refining the methods and applying them to a broad range of substrates (Riley *et al.*, 2005; Hallifax *et al.*, 2010). Most of this work has been performed using mammalian (rat, mouse, human) tissue preparations (liver microsomes, primary hepatocytes, and liver slices).

Fish *in vitro* methods have the potential to provide important data for bioaccumulation assessments, and although many require sacrifice of live animals, they may contribute to a reduction in (or refinement of) animal testing. In 2018, *in vitro* methods to measure intrinsic clearance of a test chemical have been adopted into OECD test guidelines, using either fish hepatocytes (OECD TG 319A, OECD 2018b) or liver S9 subcellular fractions (OECD TG 319B, OECD 2018c), and an accompanying guidance document (OECD, 2018a) together with excel spreadsheets for IVIVE calculations[4] has been published.

The use of *in vitro* data for bioaccumulation assessment requires a strategy for *in vitro-in vivo* extrapolation (IVIVE) of measured biotransformation rates and incorporation of estimated hepatic clearance into appropriate computational models. The *in vitro* assays are generally performed using a substrate depletion approach, wherein the goal is to measure loss of a test substance (parent compound) added to the biological matrix. This information is then converted to a whole-body biotransformation rate constant using several extrapolation factors and combined with estimates for uptake across the gills and all non-metabolic routes of elimination to predict an *in vitro* BCF. Uncertainties of the conversion in a whole body biotransformation rate constant concern the IVIVE models, the consideration of extrahepatic transformations, protein binding, and possible enzymatic induction of biotransformation enzymes that may bias the results (Laue et al 2020). For ionisable compounds, OECD TG 319 may apply, however, the currently available *in vitro-in vivo* extrapolation models may not always apply to all (types of) ionisable substances and adaptation may be needed (Regnery *et al.* 2022, chapter 3.5).

Over the past years, several computational models integrating the IVIVE approach have become available; the model complexities range from simple one-compartment models (Krause and Goss 2020, Nichols *et al.* 2013, Trowell *et al.* 2018) to more complex multi-compartment models (Krause and Goss 2020, Nichols *et al.*, 1990; Stadnicka *et al.*, 2012; Stadnicka-Michalak *et al.*, 2014). In most cases the use of a very simple approach (one-compartment model) may suffice (Krause and Goss 2020). Recent refinements that

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[4] OECD Guidance Document No 280: https://www.oecd.org/chemicalsafety/testing/series-testing-assessment-publications-number.htm; Hepatocytes: https://www.oecd.org/env/ehs/testing/HEPspreadsheet.xlsx ; S9-mix: https://www.oecd.org/env/ehs/testing/S9spreadsheet.xlsx

concern all models regardless of their complexity are the use of the revised *in vitro-in vivo* extrapolation formalism (Krause and Goss, 2018) and the use of composition-based binding algorithms (Krause and Goss 2021; Lee *et al.* 2017; Saunders *et al.* 2020), rejecting the assumption that binding *in vitro* and *in vivo* is the same (ratio of unbound fractions fu < 1), which is is especially important in case of hydrophobic organic chemcials. The development of integrated testing strategies in combination with data from different modeling approaches could lead to a more holistic insight into the bioconcentration mechanisms in future applications.

In vitro methods employing tissues other than liver, including gill and gastrointestinal tract, are in the earlier stages of development, as are assays using cell lines derived from these tissues. *In vitro* data from these extrahepatic systems may be of particular importance when substances are metabolised in the gills or gut, or when dietary uptake is the primary route of exposure. Although these methods have not been used as broadly as the liver S9 and primary hepatocyte assays, they are promising approaches that could also address the role of metabolism in bioaccumulation assessment once they are further developed, standardised and validated. Suitable computational models that allow the consideration of *in vitro* data in gill and/or GIT are already available (Krause and Goss, 2020; Stadnicka-Michalak *et al.*, 2018).

It should be noted that the presence/absence and activities of different metabolising enzymes varies among species, and quantitative correlations with fish have not yet been established. Moreover, the presence of measurable metabolism does not necessarily correspond to a decrease in risk. Although in general the products of biotransformation are eliminated more rapidly than the parent compound from which they derive, this is not always the case. This is also a relevant consideration for biotransformation which occurs *in vivo*.

Technical challenges associated with *in vitro* measurement of biotransformation include the limited working lifetime of these preparations and difficulties associated with the use of very hydrophobic (high log Kow) test substances. Liver spheroids remain viable for long periods of time and may be particularly well suited for low clearance compounds (Baron *et al.*, 2012), although this remains to be determined. Alternatively, it may be possible to employ existing S9 and hepatocyte assays using a relay approach, or some type of hepatic co-culture system (Di *et al.*, 2012; Hutzler *et al.*, 2015). Lee *et al.* (2012, 2014) demonstrated the use of a sorbent-phase dosing approach for very hydrophobic compounds. Research is needed to compare results obtained using this and similar methods to rates measured using conventional solvent dosing procedures.

Results of such studies can support the bioaccumulation assessment and can be considered as part of a Weight-of-Evidence approach. When comparing *in vitro* fish metabolism data with measured fish BCF data, only data for the same fish species should be compared. Currently, further experience is needed in performing *in vitro* fish metabolism studies on substances with log Kow values >7-8. Whilst such studies may help to explain the proportion of depuration attributable to metabolism it does not mean that a substance cannot reach high body burdens.

Biomimetic techniques

Biomimetic extraction systems try to mimic the way organisms extract substances from water. There are three main types:

- *semi-permeable membrane devices (SPMD)*, which are usually either a bag or tube made of a permeable membrane (e.g. low density polyethylene) containing an organic phase (e.g. hexane, natural lipids or the model lipid triolein) (Södergren, 1987; Huckins *et al.*, 1990). SPMDs have been used to assess effluents (Södergren, 1987), contaminated waters (Petty *et al.*, 1998) and sediments (Booij *et al.*, 1998) as animal replacements for assessing potentially bioaccumulative substances.

- *solid phase micro extraction (SPME)*, consisting of a thin polymer coating on a fused silica fibre (Arthur and Pawliszyn, 1990). Equilibrium may be achieved in hours to days, due to the high surface area to volume ratio (Arthur and Pawliszyn, 1990; Vaes *et al,* 1996 and 1997).

- *artificial membranes*, prepared from phospholipids that form small unilamellar vesicles in water (Gobas *et al.*, 1988; Dulfer and Govers, 1995; Van Wezel *et al.* 1996; Vaes *et al.*, 1997; Vaes *et al.*, 1998a). These vesicles are thought to resemble the lipid bilayers of natural membranes, and they have mainly been used to study toxicity (e.g. Vaes *et al.*, 1998b).

All three methods will extract only the freely dissolved (i.e. bioavailable) fraction of substances from water samples, in proportion to their partitioning coefficient, which is mainly related to the hydrophobicity of the substance and molecular size. In this way they simulate the potential for aquatic organisms to bioconcentrate organic substances by passive diffusion into storage lipids and cell membranes. Both SPMD and SPME are relatively easy to use. Due to the small size of the organic phase, SPME has a much shorter equilibration time than SPMD and relatively small sizes of water samples can be used without depleting the aqueous phase. SPMD is more suitable than SPME to assess the bioaccumulation potential in the field from prolonged exposure with fluctuating concentrations of contaminants.

Techniques like SPMD and SPME cannot account for metabolism by fish or invertebrates. It should also be noted that the partition coefficient measured with a particular device has to be translated to a BCF for organisms using an appropriate conversion factor. For example, a number of studies have established relationships between SPME partition coefficients, log K_{ow} and invertebrate BCFs for a variety of compounds (Verbruggen, 1999; Verbruggen *et al.*, 2000; Leslie *et al.*, 2002).

Biomimetic extractions are very useful for measuring the bioavailability of non-dissociating organic substances in the water phase, or to measure an average exposure over time in a specific system. However, when interpreting the results from such methods in the context of bioaccumulation, the following points need to be considered:

- The data produced are simple measures of substance bioavailability, and uptake rates will differ from uptake rates in organisms. Equations are needed to translate between the two. They therefore provide a maximum BCF value for most substances, linked to the potential passive diffusive uptake into an organism and distribution into the lipid.

- They do not simulate the ability of fish or other aquatic organisms to actively transport substances, nor mimic other methods of uptake and storage (e.g. protein binding), which can be important for some substances. They also neglect mechanisms of elimination, such as metabolism and excretion.
- The time to equilibration with water samples can be very long for some types of device. For example, Booij *et al.* (1998) suggested that results from SPMDs exposed for less than 2 months should be treated with caution.

Bioconcentration can therefore be either overestimated (for readily metabolised and actively excreted substances) or underestimated (e.g. in the case of active uptake of a substance that is poorly metabolised or when bioaccumulation is not governed by lipophilicity). In addition, since biomimetic methods are only capable of reaching equilibrium with freely dissolved substances they cannot be used to address the potential uptake *via* the gut. They are therefore of limited usefulness in the assessment of bioaccumulation.

R.7.10.3.2 Non-testing data aquatic bioaccumulation

Non-testing data can generally be provided by:

- Quantitative structure-activity relationships (QSARs);
- Expert systems; and
- Grouping approaches (including read-across, structure-activity relationships (SARs) and chemical categories).

These methods can be used for the assessment of bioaccumulation if they provide relevant and reliable data on the substance of interest.

(Q)SAR models

DISCLAIMER: this section does not include the latest information on the use of (Q)SAR models as it has not been updated since publication of the first version of this document.

(Q)SAR models for predicting fish BCFs have been extensively reviewed in the literature (e.g. Boethling and Mackay, 2000; Dearden, 2004; Pavan *et al.*, 2006). ECHA's *Practical Guide 5: How to use and report (Q)SARs* provides guidance on how to use and report (Q)SAR predictions under REACH. The Practical Guide also includes a list of QSAR models suitable for predicting bioaccumulation in aquatic species (Table R.7.10—1).

Table R.7.10—1 QSAR models suitable for predicting bioaccumulation in aquatic species

Software tool	Models/Modules	Free or Commercial
EPI Suite (US EPA)	BCF BAF	Free
T.E.S.T. (US EPA)	Bioaccumulation factor	Free
VEGA (IRFMN)	CAESAR, Meylan and KNN/Read-Across models	Free
CASE Ultra (MultiCASE)	EcoTox model bundle	Commercial
CATALOGIC (LMC)	Two BCF base-line models	Commercial

The most important approaches for aquatic bioaccumulation (Q)SAR models are presented below.

Some examples are given to illustrate each model type and the techniques used to develop them. This overview *is not intended to be an exhaustive list of models*: other methods and models should be considered if relevant. Not all the models were developed with European regulatory purposes in mind, and so it is important to assess in each case whether the predicted endpoint corresponds with the regulatory endpoint of interest.

BCF models based on log Kow

The most common and simplest QSAR models are based on correlations between BCF and chemical hydrophobicity (as modelled by log K_{ow}). The mechanistic basis for this relationship is the analogy of the partitioning process between lipid-rich tissues and water to that between *n*-octanol and water (whereby *n*-octanol acts as a lipid surrogate). In this model, uptake is considered to be a result of passive diffusion through gill membranes.

Several log BCF/log K_{ow} relationships for non-polar, hydrophobic organic substances have been proposed and used in the regulatory applications. Some were derived for specific chemical classes, like chlorinated polycyclic hydrocarbons (Schüürmann *et al.*, 1988) and anilines (Zok *et al.*, 1991), but several include diverse sets of substances (e.g. Neely *et al.*, 1974; Veith *et al.*, 1979; Ellgenhausen *et al.*, 1980; Könemann and van Leeuwen, 1980; Geyer *et al.*, 1982; Mackay, 1982; Veith and Kosian, 1983; Geyer *et al.*, 1984; Hawker and Connell, 1986; Connell and Hawker, 1988; Geyer *et al*, 1991; Bintein *et al.* 1993; Gobas, 1993; Lu *et al.*, 1999; Escuder-Gilabert *et al.*, 2001; Dimitrov *et al.*, 2002a). For example, Veith *et al.* (1979) developed the following QSAR for a set of 55 diverse substances:

$\log BCF = 0.85 \times \log K_{ow} - 0.70$ $\qquad$ $R^2 = 0.897$, log K_{ow} range = 1-5.5

where R^2 is the correlation coefficient.

The differences between the various correlations are probably due to variations in test conditions used for the substances in the training sets (Nendza, 1988). The range of log K_{ow} values of the substances under study may also be too broad.

Linear correlations give a good approximation of the BCF for non-ionic, slowly metabolised substances with log K_{ow} values in the range of 1 to 6. However, the relationship breaks down with more hydrophobic substances, which have lower BCFs than would be predicted with such methods. Several possible reasons for this have been identified (e.g. Gobas *et al.*, 1987; Nendza, 1988; Banerjee and Baughman, 1991), including:

- reduced bioavailability and difficulties in measuring exposure concentrations (due to the low aqueous solubility),
- failure to reach steady state because of slow membrane passage of large molecules, and
- influence of biological processes within the organism (growth dilution, metabolism), or the test system (degradation), etc.

More complicated types of relationship have been developed to overcome this problem. Hansch (cited in Devillers and Lipnick, 1990) proposed a simple parabolic model; Kubinyi (1976, 1977 and 1979) and Kubinyi *et al.* (1978) subsequently proposed a bilinear model, successfully used in many drug design and environmental QSAR studies. Linear, parabolic and bilinear models were developed and compared by Bintein *et al.* (1983) on a dataset of 154 diverse substances with a log K_{ow} range from 1.12 to 8.60, highlighting the better performance of the bilinear relationship:

$$\log BCF = (0.910 \times \log K_{ow}) - (1.975 \times \log (6.8E\text{-}7 \times K_{ow} + 1)) - 0.786$$

$R^2 = 0.865$ $s = 0.347$ $F = 463.51$

Where R^2 is the multiple correlation coefficient, s is the standard error of the estimate and F is the Fisher test value.

Connell and Hawker (1988) proposed a 4th order polynomial relationship generated in such a way that the influence of non-equilibrium conditions was eliminated. The curve, based on data on 43 substances, resembles a parabola with a maximum log BCF value at a log K_{ow} of 6.7, and decreasing log BCF values for substances with higher log K_{ow} values. This relationship was recalculated and recommended for use (as the "modified Connell equation") in the risk assessment of new and existing substances (EC, 2003):

$$\log BCF = -0.2 \log K_{ow}^2 + 2.74 \log K_{ow} - 4.72 \qquad R^2 = 0.78$$

Meylan *et al.* (1999) proposed a suite of log BCF/log K_{ow} models based on a fragment approach from the analysis of a large data set of 694 substances. Measured BCFs and other experimental details were collected in the Syracuse BCFWIN database (SRC Bioconcentration Factor Data Base) and used to support the BCFWIN software (Syracuse Research Corporation, Bioconcentration Factor Program BCFWIN). Substances with significant deviations from the line of best fit were analysed carefully dividing them into subsets of data on non-ionic, ionic, aromatic and azo compounds, tin and mercury compounds. Because of the deviation from rectilinearity, different models were developed for different log K_{ow} ranges, and a set of 12 correction factors and rules were introduced to improve the accuracy of the BCF predictions. On average, the goodness of fit of the derived methodology is within one-half log unit for the compounds under study.

A single non-linear empirical model between log BCF and log K_{ow} was derived by Dimitrov *et al.* (2002a) for 443 polar and non-polar narcotic substances with log K_{ow} range from –5 to 15 extracted from the Meylan *et al.* (1999) data set. Hydrophobicity was found to explain more than 70% of the variation of the bioconcentration potential. A linear relationship was identified in the range for log K_{ow} 1 to 6. The compounds were widely dispersed around and beyond the maximum of the log BCF/log K_{ow} curve. This QSAR gives a Gaussian-type correlation to account for the log BCF approximating to 0.5 at low and high log K_{ow} values. The continuous aspect of the proposed model was considered more realistic than the broken line model of Meylan *et al.* (1999). The main originality of this model, compared to other non-linear QSARs, is its asymptotic trend for extremely hydrophilic and hydrophobic substances.

Overall, it can be concluded that:

- linear equations are applicable in the log K_{ow} range of 1-6; and
- non-linear equations show better performance above a log K_{ow} of 6.

A log K_{ow} of 6 can therefore be used as the switch point between the two types, based on the fact they cross at a log K_{ow} value just above 6.

BCF models based on other experimentally derived descriptors

Although not as extensively used as log K_{ow}, correlations of BCF with aqueous solubility (S) have been developed (e.g. Chiou *et al.*, 1977; Kenaga and Goring, 1980; Davies and Dobbs, 1984; Jørgensen *et al.*, 1998). It should be noted that a strong (inverse) relationship exists between log K_{ow} and aqueous solubility for liquids. However, aqueous solubility is not a good estimate of hydrophobicity for solids (since the melting point also has an influence), and instead the solubility of the supercooled liquid should be used (if this can be estimated, e.g. see Yalkowsky *et al.*, 1979).

As an example, Isnard and Lambert (1988) developed the following BCF model for 107 substances (both solids and liquids) where aqueous solubility is in mol/m^3:

$$\log BCF = -0.47 \times \log S + 2.02 \quad R^2 = 0.76$$

It should be noted that both the slope and regression correlation coefficient are relatively low. This is a common problem for such QSARs that include both solids and liquids in their training set. Predictions may therefore be prone to significant error. Consequently, specific justification should be made for applying QSARs based on aqueous solubility.

BCF models based on theoretical molecular descriptors

The mechanistic basis of the majority of BCF QSAR models based on either log K_{ow} or aqueous solubility was determined prior to modelling by ensuring that the initial set of training structures and/or descriptors were selected to fit a pre-defined mechanism of action. However, the empirical input parameter data might not always be available for every substance (e.g. there may be technical difficulties in performing a test), or the substance could be outside the domain of predictive models. Consequently, other models have been proposed in the literature following statistical studies based on theoretical descriptors. Examples include methods based on:

- **molecular connectivity indices** (MCI) (Sabljic and Protic, 1982; Sabljic, 1987; Lu *et al.*, 1999; Lu *et al.*, 2000),
- solvatochromic or linear solvation energy relationship (LSER) descriptors (Kamlet *et al.*, 1983; Park and Lee, 1993),
- **fragment constants**, based on substance fragmentation according to rules developed by Leo (1975) (Tao *et al.*, 2000 and 2001; Hu *et al.*, 2005),
- quantum chemical descriptors (Wei *et al.*, 2001), and
- **diverse theoretical molecular descriptors** selected by genetic algorithm (Gramatica and Papa, 2003 and 2005).

Theoretical descriptors do not suffer from variability, but are difficult to determine by the non-expert. In addition, such models are perceived by the developers to be capable of providing predictions for a wider set of substances than is normally the case. However, whilst the domain of these types of model is occasionally well described, most require a certain degree of competence to determine whether the training set of the model is relevant for the substance of interest. Since the mechanistic basis of these models is determined post-modelling, by interpretation of the final set of training structures and/or descriptors, they are often criticised for their lack of mechanistic interpretability. The use of this type of model should therefore be thoroughly described and justified if a registrant chooses to predict a BCF this way.

QSAR model for identifying "B-profile"

A base-line modelling concept was proposed by Dimitrov *et al.* (2005a), specifically for PBT assessment. It is based on the assumption of a maximum bioconcentration factor (BCF_{max}) (Dimitrov *et al.*, 2003) with a set of mitigating factors used to reduce this maximum, such as molecular size, maximum diameter (Dimitrov *et al.*, 2002b), ionisation and potential metabolism by fish (as extrapolated from rodent metabolic pathways). Substances in the training set were divided into groups based on log K_{ow} intervals of 0.5, and the five highest BCFs in each group were used to fit a curve of maximum uptake (via passive diffusion). The model therefore predicts a maximum BCF (BCF_{max}) for a substance, which may be higher than BCFs estimated using other techniques, especially for small non-ionised poorly metabolised substances.

For the training set used, the most important mitigating factor to obtain a predicted BCF closest to the actual measured BCF was metabolism. The derived model was demonstrated to perform very well in terms of sensitivity and specificity. In addition, the measured BCF data used for the training set are provided together with a general description of the applicability domain of the model.

Food web bioaccumulation models

While many QSARs have been proposed to model the BCF, fewer models are available for the bioaccumulation factor (BAF) (e.g. Barber *et al.*, 1991; Thomann *et al.*, 1992; Gobas, 1993; Campfens and Mackay, 1997; Morrison *et al.*, 1997).

Food chain or food web models can be used to predict bioaccumulation in aquatic (and terrestrial) organisms (Hendriks and Heikens, 2001; Traas *et al.*, 2004) as well as humans (e.g. Kelly *et al.*, 2004). These models integrate uptake from water, air and dietary sources such as detritus (water or sediment), plants or animals. Concentrations in organisms in a food chain can be modelled by linking a set of equations for each trophic level to describe uptake from water and consecutive food sources.

If species have several dietary sources, a more complex food web exists where fluxes between different species can occur simultaneously. Such a model is mathematically very similar to multimedia models to describe environmental fate. The great advantage of these models is that food webs of any dimension can be described, with as many food sources as needed, and concentrations in all species can be calculated simultaneously (Sharpe and Mackay, 2000).

In general, food web models successfully predict steady-state concentrations of persistent halogenated organic pollutants which are slowly metabolised (Arnot and Gobas, 2004; Traas *et al.*, 2004). However, these mass-balance models are often computationally intensive and typically require site-specific information, so are not readily applicable to screen large numbers of substances.

A different, simpler approach can be taken by estimating the BAF of species at different trophic levels that account for both water and food uptake with empirical regressions (Voutsas *et al.*, 2002) or a semi-empirical BAF model (Arnot and Gobas, 2003). These are calibrated on measured field BAF data and calculate a maximum BAF for organic substances in selected generic trophic levels (algae, invertebrates and fish). The Arnot and Gobas (2003) food web bioaccumulation model is a simple, single mass-balance equation that has been used extensively by Environment Canada for categorising organic substances on the Canadian Domestic Substances List. The model requires few input parameters (i.e. only K_{ow} and metabolic transformation rate, if available – the default is zero), and derives the BAF as the ratio of the substance concentration in an upper trophic level organism and the total substance concentration in unfiltered water (it also estimates an overall biomagnification factor for the food web). It accounts for the rates of substance uptake and elimination (a number of simple relationships have been developed to estimate the rate constants for organic substances in fish from Gobas, 1993), and specifically includes bioavailability considerations.

The main discrepancies between model predictions and measured BAF values are often due to biotransformation of a substance by the organism and to an overestimation of bioavailable concentrations in the water column and sediment. Other important sources of discrepancies relate to differences in site-specific food chain parameters versus generic assumptions (e.g. growth rates, lipid contents, food chain structure, spatial and temporal variation in exposure concentrations, sediment-water disequilibrium, etc.).

Read-across and categories

See also Sections R.6.1 and R.6.2 in Chapter R.6 of the Guidance on IR&CSA.

If a substance belongs to a class of chemicals that are known to accumulate in living organisms, it may have a potential to bioaccumulate. If a valid BCF for a structurally closely related substance is available, read-across can be applied. When applying read-across two generally important aspects have to be considered in addition to the normal criteria of read-across: hydrophobicity and the likelihood for metabolisation of both substances (see Section R.7.10.4.2).

R.7.10.3.3 Field data on aquatic bioaccumulation

Studies on bioaccumulation generally fall into one of the following categories: ecosystem monitoring using various biota species (hereafter called "field" or "monitoring data"), laboratory tests under controlled conditions, mass balance modelling, and *in vivo* and *in vitro* ADME studies (Mackay *et al.*, 2018). Although interpretation is often difficult, the results of field measurements from wildlife can be used to support the bioaccumulation assessment within a *Weight-of-Evidence* approach and the assessment of risks due to secondary poisoning (Ma, 1994). The following study types can provide information on the potential of a substance to bioaccumulate in wildlife based on bioconcentration and biomagnification processes:

Types of field studies

- **Monitoring or field data**: Detection of a substance in the tissue of an organism provides a clear indication that it has been taken up by that organism, but does not by itself indicate that significant bioconcentration or bioaccumulation has occurred. For that, the sources and contemporary exposure levels (for example through water as well as food) should be known or reasonably estimated.

- **Field measurements of specific food chains/webs**: Measurement of concentrations in organisms at various trophic levels in defined food chains or food webs can be used to evaluate biomagnification. However, as dietary and trophic biomagnification represent different processes than bioconcentration in aquatic organisms, BMF and/or TMF values <1 cannot be directly used to disregard valid BCF data > 2000 or BCF > 5000, but these data are separate lines of evidence and need to be considered together with other relevant available data in a *weight-of-evidence approach* for deriving conclusions.

- **Outdoor mesocosms**: Outdoor meso- or microcosm studies can be performed with artificial tanks or ponds or by enclosing parts of existing ecosystems (guidance is provided in OECD, 2006). Although the focus of such studies is usually on environmental effects, they can provide information on bioaccumulation in the system provided that adequate measurements of concentration are made.

- ***In situ* bioaccumulation tests using caged organisms**: Sibley *et al.* (1999) constructed a simple, inexpensive bioassay chamber for testing sediment toxicity and bioaccumulation under field conditions using the midge *Chironomus tentans* and the oligochaete *Lumbriculus variegatus*. They concluded that the *in situ* bioassay could be successfully used to assess bioaccumulation in contaminated sediments. These studies can bypass problems caused by sediment manipulation during collection for laboratory tests (disruption of the physical integrity of a sediment can change the bioavailability of contaminants). Organisms in *in situ* tests are exposed to contaminants via water and/or food. The tests cannot make a distinction between these routes. Also, environmental factors potentially modifying the bioaccumulation process are not controlled. These factors include (but are not limited to) lack of knowledge or control of exposure concentrations and bioavailability aspects. Temperature or water oxygen content may also impact the physiological status of the organism, and consequently influence the uptake rate. However, such studies are rarely conducted.

Field studies can be used to derive several bioaccumulation metrics. The **bioaccumulation factor (field BAF)** represents environmental exposure in the field to an aquatic organism from all routes and is referenced to the substance concentration in water (Arnot and Gobas, 2004; Burkhard *et al.*, 2012b). Field measured **biota-sediment accumulation factors (BSAF)** are derived by the concentration of a substance in biota divided by the concentration in the sediment (Burkhard *et al.*, 2010). Relationships between dietary exposures and bioaccumulation can be quantified by field BMFs (Burkhard *et al.*, 2012a), and trophic magnification factors (Borgå *et al.*, 2012). Laboratory biomagnification factors (laboratory BMFs; OECD, 2012) also derive a BMF. It has to be noted that a direct comparison of the different metrics is difficult. One of the current difficulties in comparing BCF and BAF data to other bioaccumulation metrics is the difference in numerical scale and reference media to which substance concentrations in organisms are compared (Burkhard *et al.*, 2012a). BCFs and BAFs express ratios of chemical concentrations in biota to water, while BMFs and TMFs reflect ratios of chemical concentrations in predator–prey relationships (Burkhard *et al.*, 2012a). Field measured BAFs, BMFs and TMF values can provide supplementary information indicating that the substance does or does not have bioaccumulation potential.

If field data indicate that a substance is effectively transferred in the food chain or leads to increased concentration in the predators, this is a strong indication that it is taken up from food in an efficient way and that the substance is not easily eliminated (e.g. excreted and/or metabolised) by the organism (this principle is also used in the fish feeding test for bioaccumulation) which will lead to biomagnification from predator to prey and trophic magnification.

Concerning field data as an indicator of bioaccumulation, generally, a high frequency of occurrence (measured concentrations) of chemicals in wildlife with increasing trends in monitoring studies, particularly in apex species over time can indicate an increased potential for bioaccumulation. To this end, top predators, like birds of prey, marine and terrestrial mammals, are valuable indicator species to monitor persistent bioaccumulative contaminants because (i) they integrate chemical signatures across space and time, including entire biological communities, (ii) have relatively high and easily measured contaminant concentrations and (iii) are consumed by humans or represent levels in human consumers of wild foods (Burger and Gochfeld, 2004; Elliott and Elliott, 2013).

If field BAF values (based on reliable information) are above the criteria for B or vB it should be considered whether this information is sufficient to conclude that the substance meets the B or vB criteria as part of the Weight-of-Evidence approach. For comparison of a fish field BAF with the Annex XIII criteria, BAF values should be on wet weight basis and for whole body and also lipid normalised to 5%. Care should be taken that the exposures from all relevant routes and compartments are considered when field BAF values are evaluated. Furthermore, a reliable field BMF or TMF value significantly higher than 1 (see also Section R.11.4.1.2.6 field data and biomagnification in Chapter

R.11 of *the Guidance on IR&CSA*) can be considered an indication of very high bioaccumulation. For aquatic organisms, this value indicates an enhanced accumulation due to additional uptake of a substance from food along with direct accumulation from water. However, as dietary and trophic biomagnification represent different processes than bioconcentration in aquatic organisms, field BMF and/or TMF values <1 cannot be directly used to disregard a valid assessment based on reliable BCF data fulfilling the numerical B/vB criteria in Annex XIII to the REACH Regulation.

To be able to compare field BMF values in a direct and objective manner, they should, as far as possible, be lipid normalised for the assessment of substances that partition into lipids in order to account for differences in lipid content between prey and predator. It should however be noted that non-lipophilic substances as e.g. PFAS may bioaccumulate by other mechanisms than partitioning/binding to lipids such as protein binding. In such a case, another reference parameter than lipid content may be considered for normalisation, e.g. protein content. In principle, field BMF values are not directly related to the lab BCF or BAF values, and in fact field BMFs and lab BCFs represent complementary bioaccumulation pathways.

It should also be noted that substantial variation can be found both within and between studies reporting field-derived BAFs for zooplankton (Borgå *et al.*, 2005), and this variability should not be overlooked when relating field BAFs to K_{ow} or other descriptors. The authors attribute the variability to difficulties with measurements of the substance in the water phase, additional dietary uptake and the possibility that substances partition into other organic phases than lipids. Field studies can be also used to derive biota–sediment accumulation factors (BSAFs). Both, BAFs and BSAFs , are simple ratios - neither definition includes any statement about ecosystem conditions, intake routes and relationships between the concentrations of substances in the organism and exposure media (see Ankley *et al.,* 1992; Thomann *et al.,* 1992). Both field derived endpoints are affected by ecosystem variables like the natural temporal and spatial variability in exposure, sediment-water column chemical relationships, changing temperatures, simultaneous exposure to mixtures of substances and nutrients, and variable exposures due to past and current loadings. In general, data obtained under steady-state like conditions are strongly preferred.

The quantity and quality of field data may be limited and their interpretation difficult. This is especially true for TMFs, which describe the accumulation throughout the whole food chain. The validity of a TMF value is strongly dependent on the spatial and time scales over which the related field samples were retrieved. See also publications from Borgå *et al.* (2012), Kidd *et al.* (2019), Kosfeld *et al.* (2021), Rüdel *et al.* (2020), and ECETOC (2014) for discussion on uncertainties.The uncertainty of using biota monitoring data in support of bioaccumulation assessment is discussed further in Section R.11.4.1.2 in Chapter R.11 of the *Guidance on IR&CSA*. Respective guidance documents and recommendations for assessing the quality of biomonitoring data, i.e. sampling, storage, chemical analysis and interpretation of wildlife biomonitoring have been elaborated by the EU LIFE APEX project and are available online[5].

[5] https://www.norman-network.com/apex/; last accessed: October 2022

R.7.10.3.4 Other indications of bioaccumulation potential

The following factors will be relevant for many substances as part of a *Weight-of-Evidence* approach, especially in the absence of a fully valid fish BCF test result.

n-Octanol/water partition coefficient

As a screening approach, the potential for bioaccumulation can be estimated from the value of the n-octanol/water partition coefficient (K_{ow}) (see Section R.7.1 in Chapter R.7a of the *Guidance on IR&CSA*). It is accepted that log K_{ow} values greater than or equal to 3 indicate that the substance may bioaccumulate to a significant degree. For certain types of substances (e.g. surface-active agents and those which ionise in water), the log K_{ow} might not be suitable for calculation of a BCF value (see Appendix R.7.10-3). There are, however, a number of factors that are not taken into consideration when the BCF is estimated only on the basis of log K_{ow}, namely:

- active transport phenomena;
- metabolism in organisms and the accumulation potential of any metabolites;
- affinity due to specific interactions with tissue components;
- special structural properties (e.g. amphiphilic substances or dissociating substances that may lead to multiple equilibrium processes); and
- uptake and depuration kinetics (leading for instance to a remaining concentration plateau in the organism after depuration).

In addition, n-octanol only simulates the lipid fraction and therefore does not simulate other storage sites (e.g. protein).

It should be noted that although log K_{ow} values above about eight can be calculated, they can not usually be measured reliably (see Section R.7.1 in Chapter R.7a of the *Guidance on IR&CSA*). Such values should therefore be considered in qualitative terms only. It has also been assessed whether an upper log K_{ow} limit value should be introduced based on the lack of experimental log K_{ow} and BCF values above such a value. Based on current knowledge, for PBT assessments, a calculated log K_{ow} of 10 or above is taken as an indicator of reduced bioconcentration. The use of this and other such indicators (such as large molecular size) is discussed further in Chapter R.11 of the *Guidance on IR&CSA*.

Adsorption

Adsorption onto biological surfaces, such as gills or skin, may also lead to bioaccumulation and an uptake via the food chain. Hence, high adsorptive properties may indicate a potential for both bioaccumulation and biomagnification. For certain substances, for which the octanol/water partition coefficient cannot be measured properly, a high adsorptive capacity (of which log K_p >3 may be an indication) can be additional evidence of bioaccumulation potential.

Hydrolysis and other abiotic degradation/transformation phenomena taking place in the exposure medium

The effect of hydrolysis may be a significant factor for substances discharged mainly to the aquatic environment: if the substance is sufficiently hydrophilic, its concentration in water may be reduced by hydrolysis so the extent of bioconcentration in aquatic organisms would also be reduced. However, for substances which are highly adsorptive to organic matter and/or lipids, the adsorption rate is, in most cases, faster than the hydrolysis rate. Therefore, hydrolysis rate should normally not intervene with assessment of bioaccumulation potential. In case a substance has a fast hydrolysis rate, the degradation potential of the substance in sediment and/or soil needs to be evaluated/tested first and if the substance is stable enough in sediment and/or soil from the perspective of quantitative risk assessment and/or PBT/vPvB assessment, the bioaccumulation potential of the substance itself needs to be evaluated/tested in conditions ensuring a stable exposure concentration despite fast hydrolysis. Where the hydrolysis half-life, at environmentally relevant pH values (4-9) and temperature, is less than 12 hours, and in cases where the above-described scenario does not apply, it may be appropriate to perform an exposure assessment, a hazard assessment and, if necessary, a bioaccumulation test on the relevant hydrolysis products instead of the parent substance. It should be noted that, in many cases, hydrolysis products are more hydrophilic and as a consequence will have a lower potential for bioaccumulation than the (registered) substance itself. This also applies by analogy to other abiotic degradation and transformation routes, such as complex dissolution/transformation processes.

Biodegradation

Biodegradation may lead to relatively low concentrations of a substance in the aquatic environment and thus to low concentrations in aquatic organisms. In addition, readily biodegradable substances are likely to be rapidly metabolised in organisms. However, the uptake rate may still be greater than the rate of the degradation processes, leading to high BCF values even for readily biodegradable substances. Therefore ready biodegradability does not preclude a bioaccumulation potential. The ultimate concentration in biota (and hence bioaccumulation factors) will also depend on environmental releases and dissipation, and also on the uptake and metabolism and depuration rate of the organism. Readily biodegradable substances will generally have a higher probability of being metabolised in exposed organisms to a significant extent than less biodegradable substances. Thus in general terms (depending on exposure and uptake), concentrations of most readily biodegradable substances will be low in aquatic organisms and evidence of ready biodegradability may provide useful information in a *Weight-of-Evidence* approach for bioaccumulation assessment. Information on degradation kinetics will usually be missing for most substances.

If persistent metabolites are formed in substantial amounts the bioaccumulation potential of these substances should also be assessed. However, for most substances information will be scarce (see Section R.7.9 in Chapter R.7b of the of the *Guidance on IR&CSA*). Information on possible formation of degradation products may also be obtained by use of expert systems such as METABOL and CATALOGIC, which is the successor of CATABOL which can predict biodegradation pathways and metabolites (see Section R.7.9 in Chapter R.7b of the of the *Guidance on IR&CSA*). Information on the formation of metabolites may be obtained from experiments with mammals, although extrapolation of results should be treated with care, because the correlation between mammalian metabolism and environmental transformation is not straightforward (see below). Predictions of possible metabolites in mammalian species (primarily rodents) may be obtained by use of expert systems such as Multicase and DEREK (see Sections R.7.9.6 in Chapter R.7b and R.6.1 in Chapter R.6 of the *Guidance on IR&CSA*), offering predictions of metabolic pathways and metabolites as well as their biological significance.

Interpretation of expert systems predicting formation of possible degradation products or metabolites like those referred to above require expert judgement. This applies for example in relation to identification of the likelihood and possible biological significance of the predicted transformation/degradation products, even though some of the systems do offer some information or guidance in this regard.

Molecular size

Information on molecular size can be an indicator to strengthen the evidence for a limited bioaccumulation potential of a substance. See Chapter R.11 of the *Guidance on IR&CSA* for further discussion.

Additional considerations

For air-breathing organisms, respiratory elimination occurs via lipid-air exchange, and such exchange declines as the octanol-air partition coefficient (K_{oa}) increases, with biomagnification predicted to occur in many mammals at a log K_{oa} above 5 (Kelly *et al.*, 2004). Such biomagnification does not occur if the substance and its metabolites are rapidly eliminated in urine (i.e. have a log K_{ow} of around 2 or less). Thus the bioaccumulation potential in air-breathing organisms is a function of both log K_{ow} and log K_{oa}. In contrast, respiratory elimination in non-mammalian aquatic organisms occurs via gill ventilation to water, and this process is known to be inversely related to the log K_{ow} (hence an increase in log K_{ow} results in a decrease in the rate of elimination and hence increase in the accumulation potential) (Gobas *et al.* (2003)).

Based on these findings, Kelly *et al.* (2004) proposed that substances could be classified into four groups based on their potential to bioaccumulate in air-breathing organisms. These groups are summarised below.

- Polar volatiles (low log K_{ow} and low log K_{oa}). These substances have low potential for bioaccumulation in air-breathing organisms or aquatic organisms.
- Non-polar volatiles (high log K_{ow} and low log K_{oa}). These substances are predicted to have a high accumulation potential in aquatic organisms but a low accumulation potential in air-breathing mammals.
- Non-polar non-volatiles (high log K_{ow} and high log K_{oa}). These substances have a high bioaccumulation potential in both air-breathing organisms and aquatic organisms.
- Polar non-volatiles (low log K_{ow} and high log K_{oa}). This group of substances has a low bioaccumulation potential in aquatic organisms but a high bioaccumulation potential in air-breathing organisms (unless they are rapidly metabolised).

These findings may be a relevant consideration for accumulation in top predators for some substances whose bioaccumulation potential in aquatic systems appears to be limited.

R.7.10.4 Evaluation of available information on aquatic bioaccumulation

R.7.10.4.1 Laboratory data on aquatic bioaccumulation

In vivo data on aquatic bioaccumulation

Fish bioconcentration test

In principle, studies that have been performed using standard test guidelines, such as OECD TG 305, should provide fully valid data. For this, certain aspects must be fulfilled:

- the test substance properties lie within the recommended range stipulated by the test guideline,
- concentrations are quantified with an appropriate analytical technique, and
- the data are reported in sufficient detail to verify that the validity criteria are fulfilled.

The results should be presented in unambiguously specified units as well as tissue type (e.g. whole body, muscle, fillet, liver, fat). Whole body measurements are preferred and the normalisation for lipid content and growth dilution is recommended (see section below on correction factors).

Detailed guidance on interpretation of OECD TG 305 fish bioaccumulation test data is provided in the related OECD Guidance Document (2017). However, the rules principly apply also to other aquatic bioaccumulation tests.

Test substance information

- The identity of the test substance must be specified, including the chemical name, CAS/EC number and purity (the latter particularly for radiolabelled test substances).
- Key physico-chemical properties (e.g. water solubility and K_{ow}) need to be considered in assessing data quality. The water solubility can be used to evaluate whether the dissolved substance concentration available to the organism may have been overestimated, leading to an underestimate of the BCF. The K_{ow} value can provide an indication of whether sufficient exposure time has been provided for achieving steady-state conditions (in small fish for non-polar organic substances assuming worst case conditions, i.e. no metabolism) (see OECD TG 305 for further details).

Test species information

- The test species must be identified, and ideally, test organisms should be of a specified gender, life stage and age/size (since these may account for differences in metabolic transformation potential or growth). A steady-state condition is reached faster in smaller organisms than in larger ones due to their higher respiratory surface-to-weight ratio. Fish size is therefore an important consideration for assessing whether the exposure duration is sufficient.

- Whole body lipid content is also a key organism parameter (although this is sometimes not reported), since this variable controls the degree of partitioning between the water and the organism for many organic substances.

<u>Analytical measurements</u>

- Studies that involve only nominal exposure concentrations are unreliable unless adequate evidence is available from other studies to suggest that concentrations would have been well maintained.

- A reliable study should use a parent substance-specific analytical method in both exposure medium and fish tissue. Studies that describe the use of accepted and sensitive substance-specific methods but fail to document (or give further reference to) analytical method validation (e.g. linearity, precision, accuracy, recoveries and blanks) should be assessed on a case-by-case – they might best be designated as *reliable with restrictions*. Studies that do not describe the analytical methods should be designated as not assignable, even if they are claimed to provide substance-specific measurements.

- Radiolabelled test substance can be useful to detect organ specific enrichment or in cases where there are analytical difficulties. However, total radioactivity measurements alone can lead to an overestimation of the parent substance concentration due to:

- small amounts of radiolabelled impurities that may be present in the test substance, and/or

- biodegradation and biotransformation processes in the exposure medium and fish tissue (i.e. the measurements may relate to parent substance plus metabolites (if the radiolabel is placed in a stable part of the molecule) and even carbon that has been incorporated in the fish tissue).
  A parent compound-specific chemical analytical technique or selective clean-up procedure should therefore preferably be used at the end of the exposure period. If the parent substance is stable in water and an enrichment of impurities is not likely from the preparation of the test solution, the BCF based on total radioactivity alone can generally be considered a conservative value. It is also important to evaluate the feeding regime as well, since high concentrations of (usually more polar) metabolites may build up in the gall bladder if the fish are not fed, which may lead to an overestimate of whole body levels (OECD, 2001). For example, Jimenez *et al.* (1987) measured a BCF of 608 for benzo[*a*]pyrene (based on total radioactivity) when fish were fed during the experiment, but a BCF of 3,208 when they were not. Decreased

respiration and metabolism as well as a decreased release of bile from the gall bladder in the intestinal tract are mentioned as possible explanations.

Exposure conditions

- Exposure concentrations should not exceed the aqueous solubility of the test substance. In cases where test exposures significantly exceed aqueous solubility (e.g. due to the use of dispersants), and the analytical method does not distinguish between dissolved and non-dissolved substance, the study data should generally be considered unreliable. An indication of the BCF might be given by assuming that the organisms were exposed at the water solubility limit.

- Aqueous exposure concentrations must be below concentrations that pose a toxicity concern. Generally, as explained in OECD TG 305, the concentration(s) of the test substance should be selected to be below its chronic effect level or 1% of its acute asymptotic LC50. The highest permissible test concentration can also be determined by dividing the acute 96 h LC50 by an appropriate acute/ chronic ratio (e.g. appropriate ratios for some chemicals are about three, but a few are above 100).

- Aqueous exposure concentrations should be kept relatively constant during the uptake phase. In the case of the OECD test guideline, the concentration of test substance in the exposure chambers must be maintained within ±20% of the mean measured value. In the case of the ASTM guideline, the highest measured concentration should be no greater than a factor of two from the lowest measured concentration in the exposure chamber.

Other test conditions

- While criteria vary, fish mortality less than 10-20% in treated and control groups is generally acceptable (e.g. according to OECD TG 305 mortality or other adverse effects/disease in both control and test group fish should be ≤10% at the end of the test). In cases where >30% mortality is reported, the study should be considered not reliable. If no mortality information is provided, one option is to designate the study as 'reliable with restrictions' if the exposure concentration used is at least a factor of 10 below the known or predicted fish LC_{50}.

- Standard guidelines require >60% oxygen saturation to be maintained in test chambers throughout the study. It is suggested that as long as unacceptable mortality does not occur, studies that deviate in this requirement could also be considered *reliable with restrictions*.

- Total organic carbon (TOC) in dilution water is also an important water quality parameter for some substances (especially for highly hydrophobic substances), since excess organic colloids can complex the test substance and reduce the bioavailability of aqueous exposure concentrations (e.g. Muir *et al.*, 1994). OECD and ASTM guidelines indicate that TOC should be below 2 and 5 mg/l, respectively. It is, therefore, suggested that studies with such substances that report TOC above 5 mg/l be considered not reliable (since this can result in an underestimation of the BCF). If no information is available on

TOC, a study may be considered reliable with restriction provided that it was conducted under flow-through conditions and that analysis of the substance was for the dissolved concentration. Further support for reliability may be provided where information on TOC can be derived from other sources (e.g. where the test water is from a natural source that is characterised elsewhere).

- The test endpoint should reflect steady-state conditions. When three successive analyses of concentration in fish made on samples taken at intervals of at least two days are within ± 20% of each other, and there is no significant increase of concentration in fish in time between the first and last successive analysis, the steady-state BCF can be calculated (see OECD, 2012a;). Alternatively, the BCF is derived using kinetic models. If neither of these approaches is used, the study should be considered unreliable (or at best reliable with restrictions) unless a case can be made that the exposure duration was sufficiently long to provide or allow correction to reflect steady-state conditions.

<u>Steady-state vs kinetic BCF</u>

The kinetic BCF (BCF_K) is preferred for regulatory purposes since for bioaccumulative substances a real steady state is often not attained during the uptake phase, and the conclusion of steady-state from the concentrations in fish at three consecutive time points could be erroneous.

This approach is especially useful in those cases in which steady-state is not reached during the uptake phase, as BCF_K in these cases will generally provide a statistically more robust value. If uptake follows first order kinetics and the BCF_{SS} was really based on steady state data, both methods should in principle lead to the same result. If the BCF_K is significantly different from the BCF_{SS}, this is a clear indication that steady-state has not been attained in the uptake phase. Besides that, the BCF_{SS} cannot be corrected for the growth of fish as no agreed method is available to correct BCF_{SS} for growth. The increase in fish mass during the test results in a decrease of the test substance concentration in growing fish (= growth dilution) and thus the BCF may be underestimated if no correction is made. Growth dilution may affect both BCF_{SS} and BCF_K and therefore the BCF_K should be calculated and corrected for growth dilution, BCF_{kg}, if growth of fish is significant during the test (this is especially important for fast growing juvenile fish, such as juvenile rainbow trout). In case the uptake and/or elimination phases appear as non-first order/biphasic, specific attention should be paid to whether the results can be considered as reliable and/or whether, on a case-by-case basis, any part(s) of the test results can still be used for chemical safety assessment or whether a new test should be carried out.

<u>Correction factors</u>

The accumulation of hydrophobic substances is often strongly influenced by the lipid content of the organism. Fish lipid content varies according to species, season, location and age, and it can range from around 0.5 to 20% w/w or more in the wild (e.g. Hendriks and Pieters, 1993). Normalisation to lipid content is therefore one way to reduce variability[6] when comparing measured BCFs for different species, or converting BCF values for specific organs to whole body BCFs, or for higher tier modelling.

The first step is to calculate the BCF on a per cent lipid basis using the relative lipid content in the fish, and then to calculate the whole body BCF for a fish assuming a fixed whole body lipid content. However, if the lipid content of individual fish are reported or lipid contents are reported for several phases of the study, it is more appropriate to perform the lipid normalisation to the default lipid content before a BCF is calculated (e.g. the steady state or kinetic parameters are determined from the normalised data).

A default value of 5% is most commonly used as this represents the average lipid content of the small fish used in OECD TG 305 (Pedersen *et al.*, 1995; Tolls *et al.*, 2000). Generally, the highest valid wet weight BCF value expressed on this default lipid basis is used for the hazard and risk assessment. In cases where BCFs are specified on tissue types other than whole body (e.g. liver), the results cannot be used unless tissue-specific BCF values can be normalised to lipid content and converted to a whole body BCF based on pharmacokinetic considerations.

Lipid normalisation should be done where data are available, except for cases where lipid is not the main compartment of accumulation (e.g. inorganic substances, certain perfluorinated compounds, etc.). Both OECD TG 305 and ASTM E1022-94 require determination of the lipid content in the test fish used. If fish lipid content data are not provided in the test report, relevant information may be available separately (e.g. in the test guideline or other literature although this bears considerable uncertainty with it, because lipid contents can vary for the selected species and even between individuals of the same species from the same laboratory). If no information is available about the fish lipid content, the BCF has to be used directly based on available wet weight data, recognising the large uncertainty this implies.

It should be noted that QSARs generally predict BCFs on a wet weight basis only. An exception to this is the Arnot-Gobas method included in BCFBAF of EPIWIN, which specifically calculates BAFs for different trophic levels and BCFs, where relevant (lipid content 10.7%, 6.85% and 5.98% for the upper, middle and lower trophic level, respectively). When using results from this model, there is a need to normalise the results to the standard 5% lipid content. Further work would be needed to determine whether any lipid correction is necessary for predicted values with other QSARs.

Growth dilution refers to the decline in internal test substance concentration that can occur due to the growth of an organism (which may lead to an underestimation of the BCF that would result from a situation in which the fish are not growing; OECD (2017)). It is especially important for small (juvenile) fish (e.g. rainbow trout, bluegill sunfish and carp) that have the capacity for growth during the duration of a test with substances that have a slow elimination kinetics (e.g. Hendriks *et al.*, 2001). Growth dilution can be taken into account by measuring growth rate during the elimination phase (e.g. by

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[6] Some residual variation will remain due to the way the lipid is extracted (e.g. extraction using chloroform gives different amounts for aliquots from the same sample than if hexane were used as the solvent) and measured (e.g. colometric versus gravimetric procedures). Also, it makes a difference whether lipids are determined on a sub-sample of the test population, or for an aliquot from each fish. Hence, it can be important to know which lipid determination method was used.

monitoring the weight of the test organisms over time). An exponential growth rate constant (k_g) can usually be derived from a plot of natural log(weight) against time. A growth-corrected elimination rate constant can then be calculated by subtracting the growth rate constant from the overall elimination rate constant (k_2). Hence:

$$\text{growth-corrected BCF} = k_1/(k_2 - k_g)$$

where k_1 is the uptake clearance [rate constant] from water (L/kg/day)

k_2 is the elimination rate constant (day^{-1})

k_g is the growth rate constant (day^{-1})

Clearly, the influence of growth correction will be significant if k_g is a similar order of magnitude to k_2.

For older fish bioaccumulation studies, information on growth may not be available. In this case, an assessment of the likely significance of growth on the results should be made to determine what weight should be given to the study in the Weight-of-Evidence assessment. As noted in the OECD 305 TG (paragraph 32) juvenile fish may be fast growing at the life-stage (and size) they are tested in the OECD TG 305. Small rainbow trout (*Oncorhynchus mykiss*) are an example of this. In contrast, fish such as zebrafish (*Danio rerio*) are usually adults and therefore significantly slower growing (for example see an analysis in Brooke and Crookes, 2012). In the absence of growth data, the uncertainty in a BCF value derived from a fast-growing fish will be greater than that for a slow growing fish, which is important for results near a regulatory threshold. Overall, any approach to using fish bioaccumulation data where growth data are not available needs to be considered on a case-by-case basis with justification for the conclusion drawn. It should be noted that apart from growth dilution, several other factors have been suggested to potentially influence test results, for example water-to-fish-ratios, temperature, sex differences, feeding procedure and slight variances in water chemistry and dissolved oxygen concentrations (Wassenaar *et al.*, 2019). Most of these, and other variables can influence the metabolic capacity of the test animals and/or are directly related to changes in activity or oxygen consumption. For relevance and scientific justification of correction for growth dilution when deriving BCF see R.11, Appendix R.11-6 of the *Guidance on IR&CSA*.

Fish dietary studies

Dietary studies (OECD TG 305-III) require careful evaluation and in particular the following points should be considered in assessing the data from such a study:

- Was a positive control used and were the data acceptable?
- Were the guts of the fish excised before analysis? The guts can sometimes contain undigested food and thus also test substance, which, for poorly assimilated or highly metabolised substances, leads to the generation of erroneous (though precautionary) values.
- Is there any evidence to suggest the food was not palatable due to use of extremely high substance concentrations in the food? This may be assessed by examining the growth of the fish during the course of the study.
- Was there homogeneity of the test substance in the spiked food? Further criteria for this are given in paragraph 113 of OECD TG 305.

The dietary study yields a number of data that allow to assess the biomagnification potential of chemicals, including the dietary chemical absorption efficiency (α) and the whole body elimination rate constant (k_2) and half-life for substances for which this is impossible via the aqueous exposure route.

The dietary bioaccumulation approach results a BMF rather than a BCF, which is commonly used for bioaccumulation assessment. However, Annex 8 of the OECD TG 305 summarises approaches currently available to estimate tentative BCFs from data collected in the dietary exposure study. Further detailed information is provided in the OECD guidance document on OECD TG 305 (OECD, 2017).

The calculation for the uptake rate constant estimation method (Method 1) is based on a model predicted uptake rate constant (k_1) and the depuration rate constant (k_2) determined from the dietary bioaccumulation study. In this way, it is possible to use the dietary experimental data to estimate BCFs, which allow for a comparison against the BCF criteria for PBT assessment outlined in Annex XIII. It should be noted that these calculated BCFs may be more uncertain than experimental BCFs due to the uncertainty in the k1 prediction. In particular, k1 is a function of chemical properties relating to the chemical transfer efficiency from water (e.g., membrane permeation or absorption efficiency), the physiology of the fish (body size, respiration rate), the experimental conditions (e.g., dissolved oxygen concentrations, water temperature, gill water pH for ionic substances) and the interdependence of these parameters. Several models are available to estimate a k1 value needed to calculate an aqueous BCF from a dietary bioaccumulation study (OECD, 2017). Results for k_1 must be used with reference to the models' assumed applicability domains (e.g. mostly restricted to neutral organic substances with log Kow above 3.5 but including some weakly acidic or basic substances as well). Uptake and elimination processes are different for ions compared to neutral chemicals (e.g. Rendal *et al.*, 2011) and ionic substances thus need to be discussed separately. For poorly soluble non-polar organic substances, first order uptake and depuration kinetics is assumed. More complex kinetic models should be used for substances that do not follow first order kinetics. Generally, estimates of k_1 should be derived according to all the models available to give a range of BCFs. These results should be used in a *Weight-of-Evidence* approach for the assessment of bioaccumulation, possibly together with other information on bioaccumulation. The estimation of k_1 may be less reliable for large or bulky molecules, log Kow above ca 9 and/or low assimilation efficiency (see paragraph 253 of OECD, 2017). Taking the uncertainties into account, and assuming that k_1 is accurately and appropriately predicted for the substance, the estimated BCF values derived from a dietary test can be directly compared to the B/vB criteria. For very hydrophobic substances, k1 estimates may become increasingly uncertain.

A field BMF > 1 indicates that biomagnification of a substance occurs. The dietary BMF however differs from the field BMF, because exposure is through a combination of water and food in the field situation, while in the dietary exposure study, the exposure through the water phase is excluded under strictly controlled conditions. This leads to dietary BMF values that are generally lower than field BMF values. For very bioaccumulative substances such as the often used reference compound hexachlorobenzene, the BMF values sometimes have been even below one (e.g. Hashizume et al 2018). In a study by Inoue *et al.* (2012) with carp, only two of the five substances that had a BCF value higher than 5000 L/kg, had a BMF value in excess of 1. In a study by Martin *et al.* (2003 a,b) with perfluorinated compounds, one of the three substances with a BCF > 2000 had a BMF of 1.0, while the two others had substantially lower BMF values. Therefore, a dietary BMF below 1 cannot be used to conclude on no B concern and it should be first assessed if the bioaccumulation potential can be concluded based on the estimated BCF, which can be directly compared to the criteria.

The dietary BMF derived from the OECD TG 305-III test can be compared with BMF values for substances with known bioaccumulation potential in a benchmarking exercise (see also Method 3 in OECD, 2017). For example, such an approach has been described for dietary bioaccumulation studies with carp (Inoue *et al.* 2012). Based on a regression between BCF_L and BMF_{kgL} for nine compounds tested in this set-up, it was shown that a BCF_L value of 5000 L/kg, normalised to a lipid content of 5%, corresponds to a lipid corrected BMF_{kgL} from the dietary test of 0.31 kg food lipids/kg fish lipids, and a BCF_L of 2000 L/kg corresponds to a BMF_{kgL} of 0.10 kg food lipids/kg fish lipids. A different benchmarking could be obtained from aqueous and dietary bioaccumulation studies for perfluorinated compounds with rainbow trout (Martin *et al.*, 2003a, b). These studies emphasise the fact that even if a BMF from an OECD 305 dietary bioaccumulation test is found to be <1, it cannot be considered as a good discriminator for concluding substances not to be (very) bioaccumulative according to the BCF criteria of Annex XIII. If benchmarking is used for comparing dietary BMF values with BMF values for substances with a known bioaccumulation potential, it must be ensured that these BMF values were obtained under (or normalised to) similar conditions (i.e. fish species, fish weight/size, diet lipid content and feeding rate).

Another endpoint from the dietary OECD 305 test is the elimination rate constant. The elimination rate constant has been proposed as endpoint for the bioaccumulation assessment (e.g. Brooke and Crookes, 2012; Goss *et al.* 2013, Goss *et al.* 2018). For example, Brookes and Crooke (2012) presented lipid normalised depuration rate constants of 0.181 and 0.085 d^{-1} as critical values for lipid normalised BCF values of 2000 and 5000 (see also Section R.11.4.1.2.3 of Chapter 11 of the of the [Guidance on IR&CSA](#)). Relating depuration rate constant directly to BCF is described as Method 2 in Guidance document on aspects of OECD TG 305 (OECD, 2017). The depuration rate constant is a useful metric for assessing bioaccumulation. However, it should be noted that the kinetics of uptake and depuration are still dependent on other factors, for example the size of the fish (e.g. Barber 2008; Brooke and Crookes, 2012). Thus, one criterion for all size of fish seems not justified. Indeed, from the analysis from Brooke and Crookes (2012) there is considerable scatter around the regression line between log BCF_L and log k_2 (lipid normalised), which may be caused by the variability in fish weight used in the underlying studies, at least partly.

In conclusion, the preferred endpoint from the OECD TG 305 III: Dietary Exposure Bioaccumulation Fish Test is the BCF value estimated from the experimentally derived elimination rate constant, which can be directly compared to the criteria, unless it can be demonstrated that the uptake rate constant cannot be reliably estimated with the available methods (see paragraphs 234-259 of OECD, 2017 for further information on the different estimation techniques for k_1 and their limitations). This would also be consistent with the data treatment of the OECD 319A/B *in vitro* tests, in which experimental data are only available for the depuration rate constant. In both cases (OECD 305-III dietary test and OECD TG 319 A/B *in vitro* tests) the estimation of the BCF from the depuration rate constant follows the same calculation procedure.

Additional information on the interpretation of the results can be found in an OECD guidance document that accompanies the OECD TG 305 fish bioaccumulation test guideline (OECD, 2017), and in section R.11.4.1.2.3 of Chapter R.11 of the *Guidance on IR&CSA*.

Invertebrate tests: *Hyalella azteca* bioconcentration test (HYBIT)

For detailed information on the *Hyalella azteca* bioconcentration test (HYBIT), see Section R.11.4.1.2.4 of the *Guidance on IR&CSA*. The draft OECD TG, which is currently under revision, has been developed in such a way that it is as close as possible to the concept described in OECD TG 305-I (OECD draft TG under revision; OECD, 2023). The HYBIT results in a BCF that allows comparison against the BCF criteria for PBT assessment outlined in Annex XIII.

Small organisms, such as *H. azteca*, have a larger surface/volume ratio compared with larger organisms such as fish. This can theoretically lead to higher estimates of bioconcentration in the small organisms due to adsorption of chemicals to their body surfaces. However, an apparent deviation from first order kinetics as a result of potential adsorption processes have not been observed for hydrophobic organic compounds. Nevertheless, according to available data, *Hyalella* BCF correlate well with fish BCF estimates when normalised to a common tissue lipid content of 5 % (w/w), but tend to be higher. This was explained by the limited biotransformation capacity of the amphipods (Schlechtriem *et al.* 2019).

Annex XIII criteria on B and vB properties refer to bioconcentration factor in aquatic species, not limiting the species to fish. Normalisation to a realistic lipid content for *H. azteca* rather than normalisation to the standard lipid content of fish avoids to be overly conservative regarding the resulting aquatic BCF (see the discussion in Section R.11.4.1.2.4 of the *Guidance on IR&CSA*).

The lipid content of *H. azteca* varies depending on the size and age of the amphipods and should therefore be normalised to an average maximum lipid content of 3 % observed for lab-raised and field-caught *H. azteca* (Schlechtriem *et al.* 2019; Kosfeld *et al.* 2020; Arts *et al.* 1995; Huff Hartz *et al.* 2021).

H. azteca BCF results converted to 3 % lipid (BCF_{KL}, 3%) are preferred for a comparison against the REACH Annex XIII criteria on B and vB properties, and deviations should be justified. If a substance has a valid and plausible *H. azteca* BCF >2000 or >5000 (indicating a significant accumulation in the test organism), the substance is defined as 'B' or 'vB', respectively. A *H. azteca* BCF (3%, w/w) <1200 and <3000 indicates 'not B' and 'not vB' for the aquatic compartment, because even with a lipid normalisation to 5 % (w/w) as applied for fish, the threshold values of 2000 and 5000 for 'B' and 'vB' would not be passed, respectively. A 'not B' and 'not vB' conclusion for the aquatic compartment can only be drawn if there is no other relevant and reliable information indicating the contrary. For lipid normalised *H. azteca* BCF values between 1200 and 2000 and between 3000 and 5000, it cannot be excluded that due to the lower lipid content of the amphipods (3 %, w/w) the bioaccumulation potential of a substance may be underestimated compared to fish (5 %, w/w) and further investigations are thus required to allow a clear "B" and "vB" conclusion. For further information, please refer to R.11.4.1.2.4 of the *Guidance on IR&CSA*.

The use of *H. azteca* to assess bioaccumulation is based on current knowledge and experience. Registrants are advised to follow-up recent and future developments in the field, e.g. via the ECHA website.

Invertebrate tests: others

Data obtained using standard methods are preferred. Further standard tests using invertebrate organisms are available (e.g. OECD TG 315, ASTM E1022-94, and ASTM E1688-00a). They are supplemented by several non-standard tests described in the literature. Generally, similar principles apply as for the evaluation of fish bioaccumulation data (e.g. the test concentration should not cause significant effects; steady-state conditions should be used, the aqueous concentration in the exposure vessels should be maintained, and should be below the water solubility of the substance; if radioanalysis is used it should be supported by parent compound analysis so that the contribution of metabolites can be assessed, etc.).

Additional factors to consider include:

- In general, estimated endpoints, e.g. BCF, BAF or BSAF, are expressed on a whole body wet weight basis. A measurement of tissue lipid contents should be made to allow lipid normalisation of the derived endpoints.
- For tests with marine species, the solubility of the test substance may be significantly different in salt water than in pure water, especially if it is ionised (for neutral organic substances the difference is only a factor of about 1.3).
- Bivalves stop feeding in the presence of toxins (e.g. mussels may remain closed for up to three weeks before they resume feeding (Claudi and Mackie, 1993)). Therefore, the acute toxicity of the substance should be known, and the test report should indicate whether closure has occurred.
- Since most test species tend to feed on particulates (including micro-organisms) or whole sediment, the assessment of exposure concentrations may need careful consideration if the test system is not in equilibrium, especially for hydrophobic substances. Tissue concentrations may also be overestimated if the gut is not allowed to clear.
- Whole sediment tests with benthic organisms tend to provide a B(S)AF, which can be a misleading indicator of bioaccumulation potential since it reflects sorption behaviour as well. A better indicator would be the BCF based on the freely dissolved (bioavailable) sediment pore water concentration. Ideally, this should be done using direct analytical measurement (which may involve sampling devices such as SPME fibres). If no analytical data are available, the pore water concentration may be estimated using suitable partition coefficients, although it should be noted that this might introduce additional uncertainty to the result.
- Many studies have shown that black carbon can substantially affect the strength of particle sorption and hence the bioavailability of a substance (Cornelissen *et al.*, 2005). Observed black carbon partition coefficients exceed organic carbon partition coefficients by up to two orders of magnitude. When interpreting data where the exposure system includes natural sediments it is

therefore important to account for the possible influence of black carbon partitioning to avoid underestimation of the substance's bioaccumulation potential from the freely dissolved phase.

- As described above, data on apparent accumulation in small organisms, such as unicellular algae, *Daphnia* and micro-organisms, can be confounded by adsorption to cell or body surfaces leading to higher estimates of bioconcentration than is in fact the case (e.g. cationic substances may adsorb to negatively charged algal cells). Adsorption may also result in apparent deviation from first order kinetics and may be significant for small organisms because of their considerably larger surface/volume ratio compared with that for larger organisms.

The validity of bioaccumulation data obtained from sediment organism toxicity tests must be considered on a case-by-case basis, because the duration of the test might not be sufficient to achieve a steady-state (especially for hydrophobic substances). Also, any observed toxicity (e.g. mortality) may limit the usefulness of the results.

In vitro data

Information from *in vitro* studies might be considered in a *Weight-of-Evidence* approach provided that they fulfil certain data quality aspects and comply with the Annex XI criteria.

There are OECD test guidelines 319 A/B using rainbow trout cryopreserved hepatocytes and liver S9 sub-cellular fractions for determination of *in vitro* intrinsic clearance (OECD, 2018b,c) and an accompanying guidance document (OECD, 2018a) providing information on how to best perform studies by these methods.

As explained in the guidance document (OECD, 2018a), there are significant differences between the two *in vitro* systems which should be considered before justifying choice of one specific. Hepatocytes contain the whole set of metabolic enzymes and cofactors at physiological levels. Liver S9 sub-cellular fractions are cell-free systems containing cytosolic and microsomal enzymes, but require the addition of cofactors. However, rate-limiting factors specifically associated with hepatocytes may include cofactor depletion and / or restricted chemical diffusion across the cell membrane. If uptake is rate-limiting on biotransformation, hepatocytes may be closer to the *in vivo* situation and a more appropriate choice for the *in vitro* system.

Both *in vitro* systems are considered to have a limited working lifetime due to a progressive loss of enzymatic activity. Hepatocytes are thought to maintain their biotransformational integrity longer, so they may be preferred for assessing slowly metabolized chemicals. The activity of a trout liver S9 substrate depletion assay has been shown to decline over time, presumably due to proteolytic degradation of biotransformation enzymes. To address this problem, protease inhibitors (i.e., phenylmethylsulfonyl fluoride) have been added to homogenization buffers and/or reaction mixtures which can increase the working lifetime of these assays and therefore could improve the detection of slow in vitro clearance rates (Nichols *et al.* 2021).

There have been few direct comparisons of the hepatocyte and liver S9 sub-cellular fraction assays (Han *et al.*, 2009; Fay et al, 2016; OECD, 2018d). Overall, available data suggests that there is no preference for one *in vitro* system or the other.

When evaluating data quality and adequacy of the test results for the bioaccumulation assessment, validity of the test should be confirmed on the basis of following:

- a validated analytical method to quantify test chemical is available;
- *in vitro* activity of the test system was confirmed during incubation time, taking account of validity criteria listed in OECD test guidelines 319 A/B;
- since biotransformation rates are temperature sensitive, the test temperature has been maintained within ranges indicated in the test guideline;
- the starting test substance concentration should be substantially lower than the Michaelis-Menten affinity constant (K_M) for the reaction in order to result in first-order depletion kinetics;
- to take account of potential losses of the substance due to other than metabolisation processes (e.g. due to volatilisation, adsorption, abiotic degradation etc.);
- that at least six time points were used to determine the clearance rate and two independent runs were performed;

If BCF is estimated by application of IVIVE, test substance should be within applicability domain of IVIVE (see R.7.10.3.1, *In vitro* data on aquatic bioaccumulation). Kosfeld, *et al.* (2020) have shown that IVIVE BCF estimation via rainbow trout hepatocytes delivers plausible result ranges for lipophilic organic substances, but recommend further investigations with a broader range of compounds. Experience with *in vitro* data and IVIVE is still limited, and therefore a resulting BCF estimate should be used with caution. See Section R.11.4.1.2.4 of Chapter R.11 of the *Guidance on IR&CSA*) for more information.

R.7.10.4.2 Non-testing data on aquatic bioaccumulation

In silico and (Q)SAR models

DISCLAIMER: this section does not include the latest information on the use of (Q)SAR models as it has not been fully updated since publication of the first version of this document.

The evaluation of the appropriateness of QSAR results should be based on an overall evaluation of different QSAR methods and models. The assessment of the adequacy of a single QSAR requires two main steps, as described below. These concepts are also considered generically in Section R.6.1.

Evaluation of model validity

A number of studies have evaluated the validity of various BCF (Q)SAR models. Important parameters are the correlation coefficient (R^2 value), standard deviation (*SD*) and mean error (*ME*). *SD* and *ME* are better descriptors of method accuracy than the R^2 value.

Among the QSAR models based on the correlation between BCF and K_{ow}, Meylan *et al.* (1999) compared their proposed fragment-based approach with a linear (Veith and Kosian, 1983) and bilinear (Bintein *et al.*, 1993) model, using a data set of 610 non-ionic compounds. The fragment method provided a considerably better fit to the data set of recommended BCF values than the other two methods, as shown by the higher R^2 value, but more importantly, a much lower *SD* and *ME*.

Some studies have also compared the performance of models based on molecular connectivity indices, K_{ow} and fragments (e.g. Lu *et al.*, 2000, Hu. *et al.*, 2005). Gramatica and Papa (2003) compared their BCF model based on theoretical molecular descriptors selected by Genetic Algorithm with the molecular connectivity index approach and the BCFWIN model. The use of apparently more complex descriptors was demonstrated to be a valuable alternative to the traditional log K_{ow} approach.

<u>Assessment of the reliability of the individual model prediction</u>

Evaluation of the reliability of a model prediction for a single substance is a crucial step in the analysis of the adequacy of a QSAR result. Several methods are currently available but none of these provide a measure of overall reliability. It is important to avoid the pitfall of simply assuming that a model is appropriate for a substance just because the descriptor(s) fall with the applicability domain. Several aspects should be considered and the overall conclusion should be documented (e.g. Dimitrov *et al.*, 2005b):

- Preliminary analysis of physico-chemical properties that may affect the quality of the measured endpoint significantly, such as molecular weight, water solubility, volatility, and ionic dissociation.
- Molecular structural domain (e.g. are each of the fragments and structural groups of the substance well enough represented in the QSAR training set?).
- Mechanistic domain (e.g. does the substance fit in the mechanistic domain of the model?).
- Metabolic domain (relating to information on likely metabolic pathways within the training set, identification of metabolites that might need to be analysed in addition to the parent compound).

Some of the steps for defining the model domain can be skipped depending on the availability and quality of the experimental data used to derive the model, its specificity and its ultimate application.

It should also be noted that BCF models tend to have large uncertainty ranges, and the potential range of a predicted value should be reported. Predictions for substances with log K_{ow} >6 need careful consideration, especially if they deviate significantly from linearity (see Section R.7.10.5).

Table R.7.10—2 lists some commonly used models that can be used to help make decisions for testing or regulatory purposes if a chemical category-specific QSAR is not available. The registrant may also choose other models if they are believed to be more appropriate. The table indicates some of the important considerations that need to be taken into account when comparing predictions between the models.

Table R.7.10—2 Commonly used in silico/QSAR models for predicting fish BCFs

DISCLAIMER: this table does not include the latest information on the use of (Q)SAR models as it has not been fully updated since publication of the first version of this document.

Model	Training set log K_{ow}	Chemical domain	Comments	Reference
Veith *et al.*, 1979	1 to 5.5	Based on neutral, non-ionised substances (total of 55 substances).	Not applicable to ionic or partly ionised substances, and organometallics.	Veith *et al.*, 1979; EC, 2003
Modified Connell	6 to ~9.8	Based mainly on non-metabolisable chlorinated hydrocarbons (total of 43 substances).	Claimed log K_{ow} range should be taken with caution: the model accounts for non-linearity above log K_{ow} 6, but is unreliable at log K_{ow} >8. Used historically for substances with a log K_{ow} > 6, but other models are now more appropriate (see below).	EC, 2003
EPIWIN©	1 to ~8	Wide range of classes included; 694 substances in data set used.	Carefully check any automatic assignment of chemical class. Assess if sub-structures of substance are adequately represented in the training set. May be unreliable above log K_{ow} of ~6.	Meylan *et al.*, 1999
BCF_{max}	1 to ~8	Wide range of classes covered; includes BCF data from dietary tests on hydrocarbons (log K_{ow} <7 only).	Preferred model for highly hydrophobic (log K_{ow} > 6) substances (due to conservatism). Can account for factors that can reduce BCF (e.g. metabolism, ionisation and molecular size).	Dimitrov *et al.*, 2005a

BIONIC	Evaluated dataset of -2 to ~8 (estimated range log D at pH 7 is ~ -4.0 – 5.0)	Neutral and ionisable organic chemicals	Mechanistic mass balance model, evaluated (validated) against independent empirical BCF data.	Armitage *et al.*, 2013

ECHA's *Practical Guide 5: How to use and report (Q)SARs* provides guidance on how to use and report (Q)SAR predictions under REACH. The Practical Guide also includes a list of QSAR models suitable for predicting bioaccumulation in aquatic species (Table R.7.10—1).

Read-across and categories

When applying read-across based on BCF two important aspects have to be considered, i.e. the lipophilicity and the centre of metabolic action for both the source and target substances.

The BCF value of a substance is generally positively correlated with its hydrophobicity which is determined by Kow. Therefore, when bioaccumulation is solely or partiatially driven by hydrophobicity, if the substance to be evaluated has a higher log K_{ow} than an analogue substance for which a BCF is available, the BCF value has to be corrected unless justified why it is not necessary. The use of the same factor of difference as for K_{ow} will be a reasonable worst-case estimate, because generally the relationship between BCF and K_{ow} is slightly less than unity. For example, if the substance to be evaluated has one methyl group more than the compound for which a BCF value is available, the log K_{ow} will be 0.5 higher and the estimated BCF from read-across is derived from the known BCF multiplied by a factor of $10^{0.5}$. In principle, this correction should give reasonable estimates as long as the difference in log K_{ow} is limited. However, the addition of one ethyl group already leads to a difference in log K_{ow} of more than one log unit or a factor of 10 on the BCF value. If the substance to be evaluated has a *lower* log K_{ow} than the substance for which a BCF value is available, care must be taken not to adjust the value too far downwards.

If the substance has such a large molecular size (see Section R.7.10.3.4) that the uptake of the substance by an organism might be hindered, a different approach should be followed. The addition of an extra substituent that leads to an increase of the log K_{ow} value does not necessarily lead to a higher BCF value in this case. On the contrary, such an addition may cause the substance to be less easily taken up by the organism, which may result in a lower instead of a higher BCF value. In such cases the ideal compound for read-across is a structurally similar compound with a slightly smaller molecular size.

Another important aspect is the capability of fish to metabolise substances to more polar compounds, leading to a lower BCF value (in some circumstances metabolism could lead to the formation of more bioaccumulative substances). Small changes to molecular structure can be significant. For example, metabolism may be inhibited if a substituent is placed on the centre of metabolic action. If read-across is applied, it must be recognised that the presence of such a substituent on the substance to be evaluated may lead to a strongly reduced metabolism in comparison with the substance for which the BCF is known. As a consequence, the BCF value may be underestimated. If there are indications of metabolism for the analogue substance for which a BCF value is available, it must be examined if the same potential for metabolism is present in the substance and the species to be evaluated.

An indication of metabolism can be obtained by comparing measured BCF values with predicted values from QSARs based on log K_{ow}. These QSARs are based on neutral organic compounds that are not metabolised strongly. If it appears that the BCF of a substance lies significantly below the estimate from the QSAR (e.g. more than one log unit), this is a strong indication for metabolism of the compound. Further indications of metabolism may be provided by *in vitro* methods (see Section R.7.10.3.1) and inferences from mammals (see Section R.7.10.3.4).

R.7.10.4.3 Field data on aquatic bioaccumulation

Bioaccumulation data obtained from field studies can differ from those measured in laboratory tests with fish or aquatic invertebrates. This is because the latter are designed to provide data under steady-state conditions, and generally involve water-only exposures, little or no growth of the test species, a consistent lipid content in the organism and its food, constant substance concentrations, and constant temperature. These conditions are not achievable in field settings, where there are also additional influences such as differences in food diversity and availability, competition, migration, etc. Field biomonitoring data may sometimes be available. This is discussed further in Section R.11.4.1.2 in Chapter R.11 of the *Guidance on IR&CSA.*

Caution should be used when interpreting bioaccumulation factors measured in studies with mesocosms or caged animals, because key environmental processes that occur in larger systems might not have been known or reported. For example, it should be confirmed whether exposure concentrations in a mesocosm were stable throughout the observation or if bioaccumulation may have taken place before the start of the observation period. Furthermore, sediment-water disequilibrium can be influenced by water column depth and primary production, which will influence substance bioavailability and uptake in the organisms sampled. Similarly, caged animals may not have the same interactions in the environment as wild animals, leading to differential uptake of the test substance in food or water. It is also imperative for caged animal studies that sufficient duration be allowed so that the organisms can approach a steady state (e.g. Burkhard *et al.*, 2003 and 2005).

The precision or uncertainty of a field B(S)AF determination is defined largely by the total number of samples collected and analysed. For practical reasons, precision of the measurements may be balanced against the costs associated with sample collection and analysis, and in many cases, pooling of samples is required to limit costs associated with the analytical analyses. Gathering and reporting too little information is far worse than providing too much information. The adequacy of the data on the intended purpose depends on their quality, and data from a field study that will be used to quantify bioaccumulation should ideally report the following:

- sampling design (site selection, spatial resolution, frequency of determination, etc.) and details of the sampling methodology, sample handling, sample storage and delivery conditions and stability, steps taken to reduce contamination, and of all equipment being used;

- description of analytical methods (including use of field blanks, procedural and instrumental blanks in analysis, laboratory pre-treatment, standard reference materials, etc.), as well as evidence of quality control procedures;
- spatial and temporal gradients in substance concentrations – in particular, care should be taken that the samples used to derive bioaccumulation factors are collected at the same time from the same location, and sufficient details provided to relocate the sampled site. Samples grabbed randomly without consideration of the organism's home range will, in high likelihood, have poor predictive ability for substance residues in the organisms because the water (and/or sediment) data will not be representative of the organism's actual exposure (Burkhard, 2003);
- physical details of the site, including temperature, salinity, direction and velocity of water flow, water/sediment depth and physico-chemical properties (e.g. particulate organic carbon and dissolved organic carbon levels);
- details of the organisms being analysed, including species, sex, size, weight, lipid content and life history pattern (e.g. migration, diet, and food web structure (which may be determined using measurements on nitrogen or carbon isotopes (Kiriluk *et al.*, 1995)) and composition). For resident species, the sample collection should be fairly straightforward. Migratory species may present special challenges in determining which food, sediment, or water sample should be used to calculate the BAF;
- information enabling an assessment of the magnitude of sorption coefficients to particulate matter, e.g. whether sorption is controlled by organic carbon or black carbon;
- details of data handling, statistical analysis and presentation; and
- any other detailed information that is important for understanding or interpreting the field data.

The Arctic Monitoring and Assessment Programme (AMAP, 2001) has published recommendations with regard to assessing the quality of monitoring data, suggesting that only data from studies with documented quality assurance for all or some stages of the data gathering process should be used for determining spatial and temporal trends and other types of data interpretations. If no information is available on quality assurance procedures, but the results are consistent with other reports concerning the same sample types, the data can be used to show relative trends (assuming that they are internally consistent). If there is no evidence of quality assurance or if the data are incompatible with other studies, the results should not be used. In addition, expert judgement will usually be required on a case-by-case basis.

Burkhard (2003) performed a series of modelling simulations to evaluate the underlying factors and principles that drive the uncertainty in measured B(S)AFs for fish, and to determine which sampling designs minimise those uncertainties. Temporal variability of substance concentrations in the water column, and the metabolism rate and K_{ow} for the substance appear to be dominant factors in the field-sampling design. The importance of temporal variability of concentrations of substances in water increases with increasing rate of metabolism. This is due to the fact that the rate of substance uptake from water (which is independent of the rate of substance metabolism) becomes more important in controlling the total substance residue in the fish with increasing rate of metabolism. Spatial variability of the substance concentrations, food web structure, and the sediment-water column concentration quotient had a lesser importance upon the overall design. The simulations also demonstrated that collection of composite water samples in comparison to grab water samples resulted in reductions in the uncertainties associated with measured BAFs for higher K_{ow} substances, whereas for lower K_{ow} substances the uncertainty in the BAF measurement increases.

Data on biomagnification (TMF, BMF or BAF-values) should be calculated based on lipid-normalised concentrations (unless lipid is not important in the partitioning process, e.g. for many inorganic compounds).

Substance concentrations from migratory populations of fish, marine mammals and birds may be available. Because sampling of satellite- or radio-tagged populations is extremely rare, noting the known migration routes and when sampling occurred along those historical timelines can be important for identifying trends in contaminant exposure and cycles of bioaccumulation and release of contaminants from fat stores (Weisbrod *et al.*, 2000 and 2001). If the migratory history of the sampled population is unknown, as is frequently the case for fish and invertebrates, stating what is known about the animals' expected duration at the site of collection can be insightful when comparing BAF values from multiple populations or sites.

The trophic magnification factor (TMF) is a metric that describes the average trophic magnification of a chemical through a food web. TMFs may be used for the risk assessment of chemicals, although TMFs for single compounds can vary considerably between studies despite thorough guidance available in the literature to eliminate potential sources of error. The practical realisation of a TMF investigation is quite complex and often only a few chemicals can be investigated due to low sample masses (Kosfeld *et al.* 2021). A study funded by Umweltbundesamt/Germany, evaluated whether a pragmatic approach involving the large-scale cryogenic sample preparation practices of the German Environmental Specimen Bank (ESB) is feasible. It was shown that food web samples derived from Lake Templin (Potsdam, Germany) allow an on-demand analysis and are ready-to-use for additional investigations. Since substances with non-lipophilic accumulation properties were also included in the list of analysed substances, it was concluded that the 'Food web on ice' approach provides samples which could be used to characterise the trophic magnification potential of substances with unknown bioaccumulation properties in the future which in return could be compared directly to defined benchmarking patterns. This approach could provide sufficient sample masses for a reduced set of samples allowing screening for a broad spectrum of substances and by that enabling a systematic comparison of derived TMFs (Kosfeld *et al.* 2021).

R.7.10.4.4 Other indications of bioaccumulation potential

High-quality experimentally derived K_{ow} values are preferred for organic substances. When HPLC generated estimates of log Kow are available, especially if the HPLC generated estimate of log Kow is in the range of one log unit below or above the screening value of log Kow = 4.5, it is advised to always generate QSAR estimations of log Kow together with it (see Chapter R.11 of the *Guidance on IR&CSA*, Appendix R.11-5 for a comparison between HPLC and KOWWIN QSAR generated logKow values. Alternative descriptors to log K_{ow} such as the membrane lipid-water partition (K_{MLW}/D_{MLW}) or membrane lipid -water distribution coefficient (D_{mw}) may be relevant for ionisable and surface-active substances and appropriate for use in some in-silico bioaccumulation approaches (further details are provided in Appendix R.7.10-3). If this is not possible (e.g. because the substance does not fall within the model domain), an estimate based on individual *n*-octanol and water solubilities may be possible. If multiple log K_{ow} data are available for the same substance, the reasons for any differences should be assessed before selecting a value. Generally, the highest valid value should take precedence. Further details are provided in Section R.7.1 in Chapter R.7a of the *Guidance on IR&CSA*.

Further guidance on the evaluation of mammalian toxicokinetic data is provided in Sections R.7.10.15 and R.7.12.

R.7.10.4.5 Exposure considerations for aquatic bioaccumulation

Column 2 of Annex IX to REACH states that a study is not necessary if direct and indirect exposure of the aquatic compartment is unlikely (implying a low probability of – rather than low extent of – exposure). Opportunities for exposure-based waiving are therefore limited. Furthermore, it should be noted, that if the registrant cannot derive a definitive conclusion (i) "The substance does not fulfil the PBT and vPvB criteria" or (ii) "The substance fulfils the PBT or vPvB criteria" in the PBT/vPvB assessment using the relevant available information, the only possibility to refrain from testing (or generating other necessary information) is to treat the substance "*as if it is a PBT or vPvB*" (see Guidance in IR&CSA, Chapter R.11 for details). Since bioaccumulation is such a fundamental part of the assessment of the hazard and fate of a substance, it may be omitted from further consideration on exposure grounds only under exceptional circumstances. This might include, for example, cases where it can be reliably demonstrated (by measurement or other evidence) that there is no release to the environment at any stage in the life cycle. An example might be a site-limited chemical intermediate that is handled under rigorous containment, with incineration of any process waste. The product does not contain the substance as an impurity, and is not converted back to the substance in the environment. Potential losses only occur from the clean-down of the process equipment, and the frequency and efficiency of cleaning (and disposal of the waste) should be considered.

It should be noted that if bioaccumulation data are only needed to refine the risk assessment (i.e. they will not affect the classification or PBT assessment), other exposure factors should be considered before deciding on the need to collect further data from a vertebrate test. For example, further information on releases or environmental fate (such as persistence) may be useful.

R.7.10.4.6 Remaining uncertainty for aquatic bioaccumulation

Both the BCF and BMF should ideally be based on measured data. In situations where multiple BCF data are available for the same substance, organism, life stage, test duration and condition, the possibility of conflicting results might arise (e.g. due to differing lipid contents, ratio of biomass/water volume, ratio of biomass/concentration of substance, timing of sampling, feeding of test fish, etc.). In general, BCF data from the highest quality tests with appropriate documentation should be used in preference, and the highest valid value (following lipid normalisation, except for cases where lipid is not the main compartment of accumulation) should be used as the basis for the assessment. When more reliable BCF values are available for the same species and life stage etc., the geometric mean (of the lipid normalised values, where appropriate) may be used as the representative BCF value for that species for bioaccumulation-- and risk assessment. The GHS criteria guidance mention that this is applicable in relation to chronic aquatic hazard classification when four or more such data are available (OECD, 2001).

If measured BCF values are not available, the BCF can be predicted using QSAR relationships for many organic substances. However, consideration should be given to uncertainties in the input parameters. For example, due to experimental difficulties in determining both K_{ow} and BCF values for substances with a log K_{ow} above six, QSAR predictions for such substances will have a higher degree of uncertainty than less hydrophobic substances. Any uncertainty in the derived BCF may be taken into account in a sensitivity analysis.

The availability of measured BMF data on predatory organisms is very limited at present. The default values given in Table R.7.10—3 should be used as a screening approach designed to identify substances for which it may be necessary to obtain more detailed information on variables influencing the secondary poisoning assessment. These are based on data published by Rasmussen *et al.* (1990), Clark and Mackay (1991), Evans *et al.* (1991) and Fisk *et al.* (1998), with the assumption of a relationship between the magnitude of the field-BMF, the BCF and the log K_{ow}. It is recognised that the available data are only indicative, and that other more complex intrinsic properties of a substance may be important as well as the species under consideration (e.g. its biology in relation to uptake, metabolism, etc.). It is recognised that, for the purpose of secondary poisoning assessment, the BMF to be used should be a value representing biomagnification in field conditions. A BMF resulting directly from a dietary fish bioaccumulation test (OECD TG 305) cannot be used without modifications as a BMF for secondary poisoning assessment.

When a BMF for secondary poisoning assessment cannot be derived on the basis of experimental or field data, a BMF may be estimated using log Kow data as described in Table R.7.10—3. The second column of this table shows (ranges of) BCF values. These values are meant to help select default BMF values if experimental BCF data are available. The programme BCFBAF within the EPISuite could also be used to estimate BMF/TMF values for hydrophobic substances in the pelagic environment. This could be done by comparing the BAF values calculated at different trophic levels after lipid normalisation of the BAF (lipid contents are 10.7%, 6.85% and 5.98% in the model for the upper, middle and lower trophic levels, respectively).

Table R.7.10—3 Default BMF values for organic substances for secondary poisoning assessment (not applicable for PBT/vPvB assessment)

log K_{ow} of substance	Measured BCF (fish)	BMF
<4.5	< 2,000	1
4.5 - <5	2,000-5,000	2
5 – 8	> 5,000	10
>8 – 9	2,000-5,000	3
>9	< 2,000	1

The recommended BCF triggers are less conservative than the log K_{ow} triggers because they more realistically take the potential for metabolism in biota (i.e. fish) into account. Due to this increased relevance, the use of measured BCF values as a trigger would take precedence over a trigger based on log K_{ow}.

If no BCF or log K_{ow} data are available, the potential for bioconcentration in the aquatic environment may be assessed by expert judgement (e.g. based on a comparison of the structure of the molecule with the structure of other substances for which bioconcentration data are available).

R.7.10.5 Conclusions for aquatic bioaccumulation

In view of the importance of this endpoint in the assessment of a substance, a cautious approach is needed. All types of relevant data as described in the previous sections should be considered together in a weight-of-evidence approach in order to derive a conclusion.

If the different lines of evidence coherently point to the same direction, or it is possible to plausibly explain the discrepancies between different data types, it may be possible to draw a conclusion on the bioaccumulation potential for PBT/vPvB assessment and/or to derive a BCF and BMF for secondary poisoning assessment without generating new information.

Reliable measured fish BCF data on the substance itself, if such data are available, are normally considered the most representative information on the bioaccumulation potential. The fish BCF is widely used as a surrogate measure for bioaccumulation potential in a wide range of gill-breathing aquatic species (e.g. crustacea). It should be noted that:

- Experimental BCF data on highly lipophilic/hydrophobic substances (e.g. with log K_{ow} above 6) will have a much higher level of uncertainty than BCF values determined for less lipophilic/hydrophobic substances. In the absence of data on other uptake routes, it is assumed that direct uptake from water accounts for the entire intake for substances with a log K_{ow} below ~4.5 (EC, 2003). For substances with a log K_{ow} ≥4.5, other uptake routes such as intake of contaminated food or sediment may become increasingly important.

- The BCF gives a partial picture of accumulation (especially for very hydrophobic substances), and additional data on uptake and depuration kinetics, metabolism, organ specific accumulation and the level of bound residues are also useful. Such data will not be available for most substances (OECD, 2001).

Furthermore, OECD TG 305 III: Dietary Exposure Bioaccumulation Fish Test provides a range of valuable experimental information which can be considered for the bioaccumulation assessment. Paragraph 167 of the test guideline lists all the relevant measured and calculated data from the study which should be reported and considered for the bioaccumulation assessment, including the BMF values, substance assimilation efficiency and overall depuration rate constant (k_2) which allows to calculate BCF values using modelled k_1 estimates. Further guidance on the OECD TG 305 is available (OECD, 2017). Reliable measured BCF/BAF data from aquatic invertebrates can be used, if available, in a Weight-of-Evidence assessment. As described in Sections R.7.10.3/R.7.10.4 and section R.7.10.6, existing information on field studies, *in vitro* fish metabolism studies and information on toxicokinetics should be considered as part of a weight-of-evidence approach as well. *In vitro* fish metabolism studies can provide useful evidence of the potential for metabolism. If the metabolism of a substance is shown to be high, this may indicate that the bioaccumulation potential is lower than predicted by its Log Kow.

Another line of evidence concerns predicted BCF/BAF/BMF values from validated QSAR models. Models that use measured data as input terms may be preferable to those that require calculated theoretical descriptors. Data from analogue substances can also be considered where relevant.

A further line of evidence concerns indications and rules based on physico-chemical properties. The log K_{ow} is a useful screening tool for many substances, and it is generally assumed that non-ionised organic substances with a log K_{ow} below 3 (log Kow below 4 for aquatic chronic classification categories) are not significantly bioaccumulative.

These lines of evidence can be assessed together as part of an overall *Weight-of-Evidence* to decide on the need for additional testing when a fully valid fish test is unavailable. In principal, the available information from testing and non-testing approaches, together with other indications such as physico-chemical properties, must be integrated to reach a conclusion that is fit for the regulatory purpose regarding the bioaccumulation of a substance. The following scheme presents the thought processes that must be considered for substances produced or imported at 100 t/y or above.

If conclusions on bioaccumulation potential cannot be drawn for the purpose of PBT/vPvB assessment (when relevant) and/or a BCF and a BMF cannot be derived for the purpose of secondary poisoning assessment based on available data, further data generation is necessary. The type of additional data to be generated depends on the available dataset and animal data should be generated as a last resort. If (new) animal data are needed, a flow-through bioaccumulation test according to OECD 305 TG is the preferred option. Where it is not technically feasible to perfom an aquatic fish bioaccumulation study under flow-through conditions, next preference is to generate new data with a fish dietary study. Also, measurements of existing specimen bank samples may be used for measuring field bioaccumulation. However, such alternative to experimental *in vivo* testing may only serve data generation in specific, well justified cases due to many uncertainties regarding field data. The possibility of generating new high quality field data with new samples is not excluded, in case animal use cannot be avoided. However, such new animal studies should only be considered in specific cases where other types of experimental studies are expected not to provide additional information on bioaccumulation.

It should also be noted that substances with a combination of log K_{ow} >2 and log K_{oa} > 5 have the potential to accumulate more preferably into air-breathing organisms than aquatic organisms. Therefore, a justification should be provided if such accumulation path into air-breathing organisms is not relevant for the assessment or, if relevant, a case-by-case assessment of risks in air-breathing organisms should be carried out (see Sections R.7.10.8 to R.7.10.15).

It should be noted, that currently no generic guidance on a systematic weight-of-evidence approach can be provided but basic principles are available for reference in a *Practical Guide on How to use alternatives to animal testing to fulfil your information requirements for REACH registration*.

Step 1 – Characterisation of the substance

Verification of the structure:

This information is essential for the potential use of non-testing techniques (e.g. (Q)SAR models). In the case of multi-constituent substances, it may be necessary to consider two or more structures, if a single representative structure is not considered sufficient (see Appendix R.7.10-3). LogK_{MLW} or logD_{MLW}[7] also may be appropriate and relevant for use in some in some circumstances (see Appendix R.7.10-3).

Physico-chemical properties of the substance:

Gather information on the physico-chemical properties relevant for assessment of bioaccumulation (see Section R.7.10.3), i.e. vapour pressure, water solubility and log K_{ow} (and, if available, octanol solubility, molecular weight (including size and maximum diameter, if relevant), Henry's law constant, adsorption (Koc/K_p) and pKa).

Information about degradation of the substance:

Gather information on degradation (including chemical reactivity, if available) and degradation products formed in environment (see Section R.7.10.3). This may include possible metabolites formed due to metabolism in organisms (e.g. based on available toxicokinetic data in fish or mammalian species, if available). Based on this information, conclude whether degradation products/metabolites should be included in the evaluation of the parent substance or not.

Preliminary analysis of bioaccumulation potential:

Based on the above considerations, make a preliminary analysis of the bioaccumulation potential of the substance (and degradation products/metabolites, if relevant):

[7] Membrane lipid-water partition/distribution

- Examine information on log K_{ow}. Does this suggest a potential for bioaccumulation at environmentally relevant pH (i.e. log K_{ow} > 3)? If so, then:
  - If log K_{ow} <6, estimate a preliminary BCF according to a linear model (e.g. Veith *et al.* (1979) and Meylan *et al.* (1999)).
  - If log Kow >6, the quantitative relationships between BCF and Kow are uncertain. A preliminary BCF of 25,000 (corresponding to a log Kow of 6) should be assumed in the absence of better information (see below).
  - Guidance on ionisable substances is given in Appendix R.7.10-3.
  - A series of molecular and physico-chemical properties can be used as indicators for a reduced uptake in relation to the PBT assessment (see Chapter R11 for further guidance). If it is concluded that the B criterion will not be met, a preliminary BCF of 2,000 may be assumed as a worst case (e.g. for the Chemical Safety Assessment).
  - Substance characterisation may highlight that the substance is 'difficult' (e.g. it may have a high adsorptive capacity (e.g. log Kp >3), or it might not be possible to measure or predict a Kow value); further guidance on some common problems is given in Appendix R.7.10-3.
  - Identify relevant exposure routes: only via water or by water and oral exposure (e.g. for substances with log K_{ow} >4.5).

Step 2 – Identification of possible analogues

Search for experimental bioaccumulation data on chemical analogues, as part of a group approach if relevant (see Section R.7.10.3.2). Justify why the chosen analogues are considered similar (as regards bioconcentration potential). Supplementary questions to be asked at this stage include:

- Does the substance belong to a group of substances that are known to have a potential to accumulate in living organisms (e.g. organotin compounds, highly chlorinated organic substances, etc.)?
- Is log K_{ow} a relevant predictor for bioaccumulation (i.e. based on expected accumulation in lipid)? Experimental evidence or other indications of sorption mechanisms other than partitioning into lipids (e.g. metals, perfluorinated compounds) should be thoroughly evaluated. In case there are reasons to believe that the substance may bioaccumulate but not in fat, a BCF study should be performed since there are currently no non-testing methods available to estimate bioaccumulation potential quantitatively for such compounds.

Step 3a – Evaluation of existing *in vivo* data

Available *in vivo* data may include invertebrate (including algal) BCFs, fish BCFs, BMFs for fish from dietary studies (which can be converted to a BCF), BSAFs for invertebrates, BMFs for predators from field studies, and toxicokinetic data from mammals (and birds if available). Assess all available results (including guideline and non-guideline tests) for their reliability according to the criteria provided in Section R.7.10.4.1. If data from one or several standard tests are available continue with the evaluation of this type of data in step 4b (below).

Other indications of the substance's biomagnification potential in the field should also be considered. For example, results from field studies (including monitoring data) may be used to support the assessment of risks due to secondary poisoning and PBT assessment. Reliable field data indicating biomagnification may indicate that the BCF of the substance is approximately equal to or greater than the BCF estimated from the K_{ow}.

Step 3b – Evaluation of non-testing data

(Q)SARs based on K_{ow} are generally recommended if K_{ow} is a good predictor of bioconcentration. Use of (Q)SARs based on water solubility or molecular descriptors may also be considered, although these may be associated with higher uncertainty. The selection of a particular QSAR should always be justified. If several generally reliable QSAR predictions are available, the reason for the difference should be considered. Expert judgement should be used, following the approach outlined in Section R.6.1 in Chapter R.6 of the *Guidance on IR&CSA*. In general, a cautious conclusion should be drawn, using the upper range of the predicted BCF values of the most relevant and reliable QSAR model(s).

If analogues with experimental BCF data are available, an indication of the predictability of the selected (Q)SAR(s) for the substance can be achieved by comparing the predicted and experimental results for the analogues. Good correlation for the analogues increases the confidence in the BCF prediction for the substance (the reverse is true when the correlation is not good). When read-across is done it is always necessary to explain and justify why the analogue is assumed to be relevant for the substance under assessment (including how closely related the analogue is in relation to the bioaccumulation endpoint).

See Section R.7.10.4 and the chapter for grouping of substances (Section R.6.2 in Chapter R.6 of the *Guidance on IR&CSA*) for further guidance.

Step 3c – Evaluation of *in vitro* data

If reliable *in vitro* metabolism data are available (see Section R.7.10.4, In vitro data), and the substance is within the applicability domain of IVIVE, then they may be used as supporting information within a Weight of Evidence approach to produce an estimated BCF or a qualitative indication for a reduced BCF due to metabolism. Further information is available in Section R.7.10.3.1.

Step 4a – *Weight-of-Evidence* assessment

Section 4.1 of the ECHA Practical guide on "How to use alternatives to animal testing to fulfil your information requirements for REACH registration" (ECHA, 2016)) provides a general scheme for building a Weight-of-Evidence approach. A tiered assessment strategy for fish bioaccumulation assessment has been proposed, but this strategy has not yet been tested in a regulatory context (Lillicrap *et al.*, 2016). Further discussion of how to use the Weight-of-Evidence approach in PBT assessment is available in Chapter R.11 of the *Guidance on IR&CSA*.

Step 4b – Weight-of-Evidence for multiple experimental BCF data

Studies that do not match evaluation criteria in Section R.7.10.4.1 should be considered of lower reliability and should normally be assigned a lower weight.

If several reliable fish data exist, reasons for any differences should be sought (e.g. different species, sizes, etc. – see Section R.7.10.4.1). Data should be lipid-normalised and corrected for growth dilution where possible (and appropriate) to reduce inter-method variability. Particular scrutiny should be given if results from the tests are close to the B or vB thresholds. If differences still remain (e.g. high quality BCF values for different fish species are available), the highest reliable lipid-normalised BCF value should normally be selected. Alternatively, the approach indicated by Section 4.1.3.2.4.3 of the Guidance on the application of the CLP criteria could be considered. This suggests using a geometric mean where four or more equivalent ecotoxicity tests are available. Overall, the approach used should be justified, and be supported by the Weight-of-Evidence available.

Organ-specific BCF data may be used on a case-by-case basis if adequate pharmacokinetic information is available (see Section R.7.10.4.1).

Against the background of the need to reduce vertebrate studies, it is the aim to use data from alternative experimental studies which can be assessed according to the BCF criteria of Annex XIII. BCFs of invertebrate studies (e.g. HYBIT, molluscs) may be used directly for bioaccumulation assessment, provided that valid studies following standard TGs are available. Reliable *H. azteca* BCF values from standard tests and converted to 3% lipid may be used to conclude on B and vB, if BCF is above 2000 L/kg and 5000 L/kg, respectively. In case bioaccumulation potential is indicated by *H. azteca* BCF 3%, but not reaching BCF criteria, further data are needed to avoid underestimation of the bioaccumulation potential due to the lower lipid content of the amphipods compared to standard fish (see Section R.11.4.1.2. in Chapter R.11 of the *Guidance on IR&CSA*). BCF values determined for other aquatic invertebrates (e.g. algae) should not be used, since they are prone to high uncertainty (see Section R.7.10.4.1).

The ITS presented in Section R.7.10.6. builds on these principles. Further discussion of how to use the Weight-of-Evidence approach in PBT assessment is available in Chapter R.11 of the *Guidance on IR&CSA*.

R.7.10.5.1 Concluding on suitability for Classification and Labelling [8]

All substances should be assessed for environmental hazard classification. Bioaccumulation potential is one aspect that needs to be considered in relation to long-term effects. For the majority of non-ionised organic substances, classification may be based initially on the log K_{ow} (estimated if necessary) as a surrogate, if no reliable measured fish BCF is available. Predicted BCFs are not relevant for classification

[8] The section on suitability of bioaccumulation data for classification and labelling refers to aquatic classification only (Guidance on the Application of the CLP criteria, Section 4.1). It does not address the new Classification criteria for PBT in Annex I (Part 4) to CLP Regulation (EC) No 1272/2008 (https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=OJ:L:2023:093:TOC).

purposes because the criteria for long-term aquatic hazard employ a cut off relating to log Kow, when the preferred type of information, measured BCF on an aquatic organism is not available. In cases where the K_{ow} is not a good indicator of accumulation potential (see Appendix R.7.10-3), an *in vivo* test would usually be needed if a case for limited bioaccumulation cannot be presented based on other evidence (e.g. metabolism, etc.). High quality BCFs determined for non-fish species (e.g. blue mussel, oyster and/or scallop) may be used directly for classification purposes if no fish BCF is available.

R.7.10.5.2 Concluding on suitability for PBT/vPvB assessment

Guidance on the suitability for PBT/vPvB assessment is provided in Chapter R.11 of the *Guidance on IR&CSA*.

R.7.10.5.3 Concluding on suitability for use in Chemical Safety Assessment

Fish BCF and BMF values are used to calculate concentrations in fish as part of the secondary poisoning assessment for wildlife, as well as for human dietary exposure. A BMF for birds and mammals may also be relevant for marine scenarios (in the absence of actual data, a fish BMF measured in a dietary test can be used as a surrogate provided it is higher than the default). An invertebrate BCF may also be used to model a food chain based on consumption of sediment worms or shellfish. An assessment of secondary poisoning or human exposure via the environment will not always be necessary for every substance; triggering conditions are provided in Chapter R.16 of the *Guidance on IR&CSA*.

In the first instance, a predicted BCF may be used for first tier risk assessment. If the PEC/PNEC ratio based on worst case BCF or default BMF values indicates potential risks at any trophic level, it should first be considered whether the PEC can be refined with other data (which may include the adoption of specific risk management measures) before pursuing further fish tests. Such data may include:

- release information,
- fate-related parameters such as determination of more reliable log K_{ow} or degradation half-life (any uncertainty in the derived values should be taken into account in a sensitivity analysis).

In some circumstances, evidence from *in vitro* or mammalian tests may be used as part of a *Weight-of-Evidence* argument that metabolism in fish will with a high probability be substantial. This could remove the concern case-by-case, especially if a worst case PEC/PNEC ratio is only just above one. Such evaluations will require expert judgement.

Other issues may be relevant to consider and use in a refinement of secondary poisoning assessment is required. Experience relating to risk assessment of certain data rich substances indicate that such issues could relate to bioavailability of the substance in prey consumed by predators, feeding preference of predator in relation to selection of type of prey (e.g. fish, bivalves etc.), feeding range of predators etc. If possible more complex food web models and specific assessment types may be employed if scientifically justified. The inclusion of such considerations may provide a more robust basis for performing secondary poisoning assessment.

Depending on the magnitude of the PEC/PNEC ratio and the uncertainty in the $PNEC_{oral}$, it might also be appropriate in special circumstances to derive a more realistic $NOEC_{oral}$ value from a long-term feeding study with laboratory mammals or birds before considering a new fish BCF test. If further mammalian or avian toxicity testing is performed, consideration could also be given to extend such studies to include satellite groups for determination of the concentration of the substance in the animals during exposure (i.e. to measure BMF values for top predators).

If further data on fish bioaccumulation are considered essential, it may be appropriate in special cases to start with fish dietary studies to determine the assimilation coefficient and the biological half-life of the substance prior to estimating or determining the BCF.

Although field studies can give valuable 'real world' data on bioaccumulation assessments, they are resource intensive, retrospective and have many interpretation problems. Therefore, field monitoring as an alternative or supplementary course of action to laboratory testing is only likely to be necessary in exceptional cases, Active sampling of (top) predators should generally be avoided on ethical grounds. Instead, studies are likely to require non-lethal sampling methods (e.g. collection of animals that are found dead, droppings, infertile birds' eggs or biopsies of mammalian skin or blubber). Consequently, they will need careful design, and the sampled environment must be appropriate to the assessment.

R.7.10.6 Integrated Testing Strategy (ITS) for aquatic bioaccumulation

R.7.10.6.1 Objective / General principles

The objective of the testing strategy is therefore to provide information on aquatic bioaccumulation in the most efficient manner so that animal usage and costs are minimised. In general, more information is needed when the available data suggest that the BCF value is close to a regulatory criterion (i.e. for classification and labelling, PBT assessment, and the BCF that may lead to a risk being identified in the chemical safety assessment).

R.7.10.6.2 Preliminary considerations

The first consideration should be the substance composition, the chief questions being: is the substance a non-ionised organic compound, and does it have well defined representative constituents? If the answer to these is no, then the use of K_{ow}- or QSAR-based estimation methods will be of limited help (see Appendix R.7.10-3). It is also important to have sufficient information on physico-chemical properties (such as vapour pressure, water solubility and K_{ow}), since these will have a significant impact on test design as well as the potential for aquatic organisms to be exposed (e.g. a poorly soluble gas might not need to be considered further). It may be possible at this stage to decide whether the substance is unlikely to be significantly bioaccumulative (i.e. log K_{ow} <3). Finally, if there is substantiated evidence that direct and indirect exposure of the aquatic compartment is unlikely, then this should be recorded as the reason why further investigation is not necessary.

R.7.10.6.3 Testing strategy for aquatic bioaccumulation

A strategy is presented in Figure R.7.10—1 for substances made or supplied at 100 t/y. References are made to the main text for further information. The collection of bioaccumulation data might be required below 100 t/y to clarify a hazard classification or PBT properties in some cases. Collection and/or generation of additional bioaccumulation data is required for the PBT/vPvB assessment in case a registrant carrying out the CSA cannot draw an unequivocal conclusion either (i) ("The substance does not fulfil the PBT and vPvB criteria") or (ii)("The substance fulfils the PBT or vPvB criteria") on whether the bioaccumulation criteria in Annex XIII to REACH are met or not (see Chapter R.11 of the *Guidance on IR&CSA* for further details) and the PBT/vPvB assessment shows that additional information on bioaccumulation is needed for deriving one of these two conclusions.

It should be noted that in some cases risk management measures could be modified to remove the concern identified following a preliminary assessment with an estimated BCF (in case the substance is potentially PBT/vPvB, see Chapter R.11 of the *Guidance on IR&CSA* for further details). Alternatively, it may be possible to collect other data to refine the assessment (e.g. further information on releases, non-vertebrate toxicity (which could be combined with an accumulation test) or environmental fate). In such cases a tiered strategy could place the further investigation of aquatic bioaccumulation with fish in a subsequent step.

It should also be considered whether a standard aquatic invertebrate test is a technically feasible and cost-effective alternative approach to estimating BCF for aquatic organisms. If refinement of the BCF is still needed following the performance of such a test, a fish study may still be required.

It should be noted that the ITS does not include requirements to collect *in vitro* or field data. The use of *in vitro* data will continue to be a case-by-case decision until such time that these techniques receive regulatory acceptance. Field data might possibly be of relevance if further information needs to be collected on the biomagnification factor. Related to this is the need to consider the K_{oa} value for high log K_{ow} substances (see Section R.7.10.3.4).

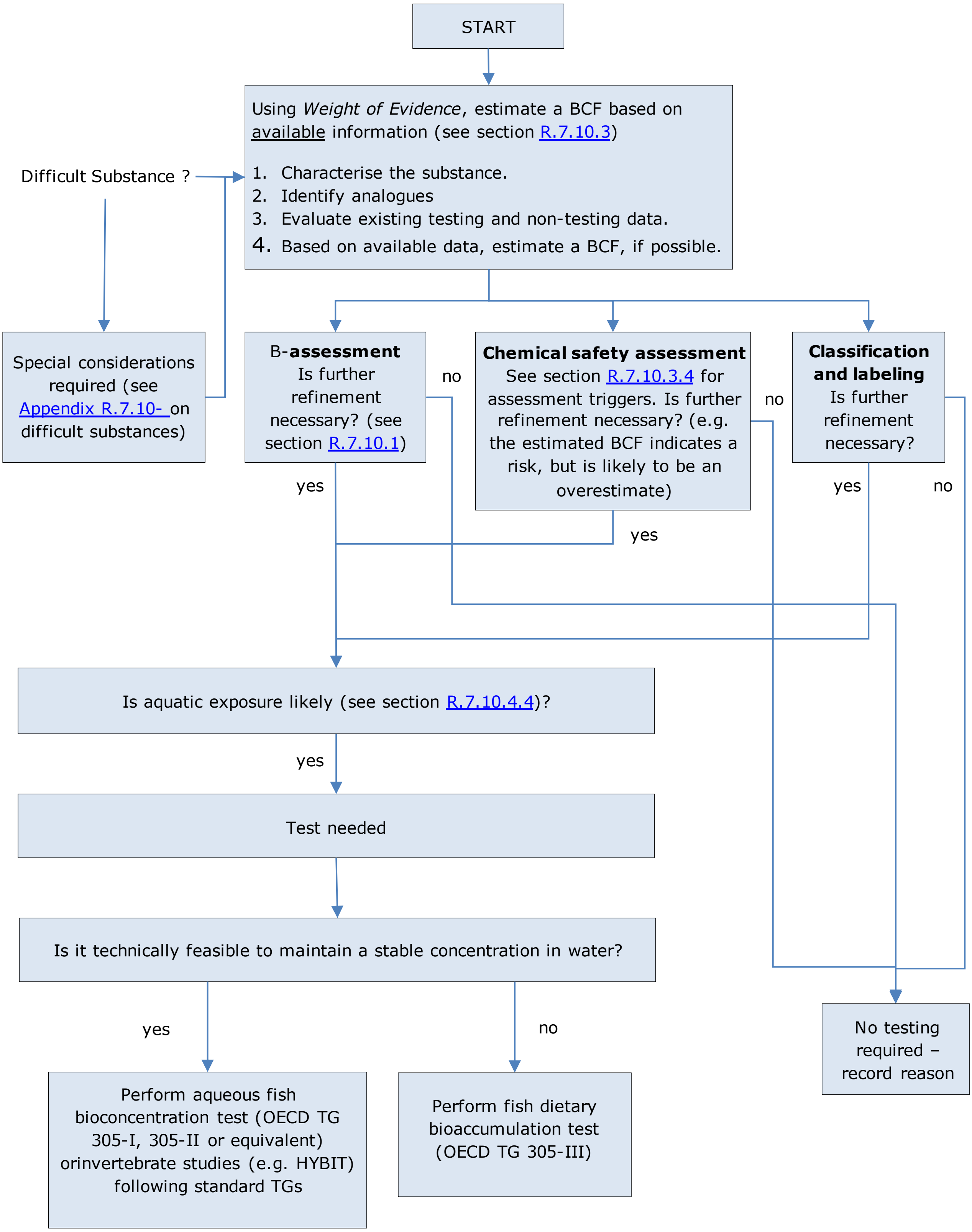

Figure R.7.10—1 ITS for aquatic bioaccumulation

R.7.10.7 References for aquatic bioaccumulation

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R.7.10.8 Terrestrial Bioaccumulation

Information on substance accumulation in terrestrial organisms is important for wildlife and human food chain exposure modelling and PBT assessment as part of the chemical safety assessment. This section addresses mainly terrestrial bioaccumulation as input to the assessment of secondary poisoning. For assessment of bioaccumulation in terrestrial mammals and other air-breathing organisms to address a B or vB concern, see R.11.4.1.2.8 "Bioaccumulation in air-breathing organisms and approaches" of the Chapter R.11 of the *Guidance on IR&CSA.*

This guidance considers the data that can be gathered from test and non-test methods for earthworms and plants, since these can be related to a clear strategy and standardised test guidelines. Further, the accumulation in terrestrial food chains is addressed briefly. Information on accumulation in earthworms is used for the assessment of secondary poisoning, and it can also be a factor in decisions on long-term soil organism toxicity testing. Information on plant uptake is used to estimate concentrations in human food crops and fodder for cattle. For substances used in down the drain products, assessment of indirect exposure of the soil via sewage sludge is important.

Accumulation in other relevant media (e.g. transfer of a substance from crops to cattle to milk) is considered in Chapter R.16 of the *Guidance on IR&CSA* whereas accumulation in air-breathing species is also addressed in Section R.7.10.15 "Mammalian toxicokinetic data in bioaccumulation assessment". Section R.11.4.1.2.8 of Chapter R.11 of the *Guidance on IR&CSA* describes a tiered approach to assess the bioaccumulation potential in air breathing species such as terrestrial mammals starting with physicochemical screening criteria at the lowest tier, the assessment of the biotransformation potential as refinement of the screening, and *in vivo* testing according to e.g. OECD TG 417 as last tier.

It is further noted that the concept of terrestrial bioaccumulation builds where relevant on the same one for the aquatic compartment, but the database underpinning the former is much smaller. Bioaccumulation assessments in the terrestrial compartment are more uncertain than similar ones for the aquatic compartment.

R.7.10.8.1 Definitions and metrics used in terrestrial bioaccumulation

Uptake of a substance by a soil-dwelling organism is a complex process determined by the properties of both the substance and the soil, the biology of the organism and climatic factors (UBA, 2003). For risk assessment, this complexity tends to be ignored, and the process is expressed in terms of simple ratios.

The bioaccumulation from soil to terrestrial species is expressed by the bioaccumulation factor, defined in OECD TG 317 as:

$$BAF = \frac{C_o}{C_s}$$

where BAF is the bioaccumulation factor (dimensionless), C_o is the substance concentration in the whole organism (mg/kg dry (or wet) weight), C_s is the substance concentration in whole soil (mg/kg dry (or wet) weight). Often the BSAF values normalised to the lipid content of the organisms and the organic carbon content of the soil are used to obtain more informative results.

Alternatively, the concentration in the organism may be related to the concentration in soil pore water. The resulting ratio is a bioconcentration factor and is defined as:

$$BCF = \frac{C_o}{C_{pw}}$$

where BCF is the bioconcentration factor (L/kg), C_o is the substance concentration in the whole organism (mg/kg wet weight), C_{pw} is the substance concentration in soil pore water (mg/L). Measurement of BCF is relevant only for certain cases, when accumulation from the porewater is expected to dominate over accumulation from ingestion of soil.

These partition coefficients can be used to estimate the concentration of a substance in an organism living in contaminated soil.

The biomagnification factor (BMF) and the trophic magnification factor (TMF) are factors that are used to express the transfer of a substance in the terrestrial food chain. The biomagnification factor is defined as:

$$BMF = \frac{C_{predator}}{C_{prey}}$$

where BMF is the biomagnification factor and $C_{predator}$ and C_{prey} are the substance concentration in the whole organism (mg/kg wet weight) of a predator and its prey. To obain comparable results, the BMF is often normalised to the lipid content of both predator and prey.

The trophic magnification factor is obtained from the slope of the log-transformed normalised concentrations of organisms in the entire food chain as a function of trophic level of those organisms. The TMF is calculated as:

$$TMF = 10^{Slope}$$

R.7.10.8.2 Objective of the guidance on terrestrial bioaccumulation

The aim of this document is to provide guidance to registrants on the assessment of all available data on a substance related to terrestrial bioaccumulation, to allow a decision to be made on the need for further testing (with earthworms or, where appropriate, plants).

R.7.10.9 Information requirements for terrestrial bioaccumulation

Data on terrestrial bioaccumulation are not explicitly referred to in REACH as a standard information requirement in Annexes VII-X, but an exposure assessment for secondary poisoning and indirect exposure to humans via the environment is a standard element of the chemical safety assessment at the level of 10 t/y or higher, according to Annex I to the REACH Regulation. The need to perform such an assessment will depend on a) substance properties (including PBT/vPvB properties) and b) relevant emission and exposure (see Chapter R.16 of the *Guidance on IR&CSA* for more details). If an assessment is required, this will involve an estimate of accumulation in earthworms and plants.

Section 9.3.4 of Annex X to REACH indicates that further information on environmental fate and behaviour may be needed for substances manufactured or imported in quantities of 1,000 t/y or higher, depending on the outcome of the chemical safety assessment. This may include a test for earthworm and/or plant accumulation.

Furthermore, if a registrant carrying out the chemical safety assessment (CSA) identifies in the PBT/vPvB assessment that a definitive conclusion cannot be derived, and the PBT/vPvB assessment shows that additional information on bioaccumulation is needed for deriving a conclusion, the necessary additional information must be provided by the registrant. This obligation applies for all ≥ 10 t/y registrations (see Chapter R.11 of the *Guidance on IR&CSA* for further details). In such a case, the only possibility to refrain from testing or generating other necessary information is to treat the substance "*as if it is a PBT or vPvB*" (see Chapter R.11 of the *Guidance on IR&CSA* for details).

R.7.10.10 Available information on terrestrial bioaccumulation

Earthworm bioaccumulation test

OECD TG 317 (OECD, 2010) is a standard test guideline for earthworms, which is applicable to stable neutral organic substances, metallo-organics, metals, and other trace elements. In principle, worms (e.g. *Eisenia fetida*) are exposed to the test substance in a well-defined artificial soil substrate or natural soil at a single test concentration that is shown to be non-toxic to the worms. After 21 days' (earthworms) or 14 days' (enchytraeids) exposure, the worms are transferred to a clean soil for a further 21 days (earthworms) or 14 days (enchytraeids). In both the uptake and elimination phases the concentration of the test substance in the worms is monitored at several time points.

When steady state is reached, the steady state bioaccumulation factor (BAF_{ss}) is calculated, while the kinetic bioaccumulation factor (BAF_k) is calculated from the uptake and depuration rate constants.

The biota-soil accumulation factor (BSAF) is the lipid-normalised concentration of the test substance in/on the test organism divided by the organic carbon-normalised concentration of the test substance in the soil at steady state. To ensure comparability of results between different soils, BSAF normalised to organism lipid and soil total organic carbon content is used. This is particularly important for organic substances with high lipophilicity (OECD, 2010).

It should be noted that the term biota-soil accumulation factor (BSAF) has been used in the literature to refer to bioaccumulation factors in soil which have not been normalised to organism lipid and soil total organic carbon content. Care should be taken to ensure it is clear what the reported value refers to.

The contribution of the gut contents to the total amount of substance accumulated by the worms may be significant, especially for substances that are not easily taken up in tissues but strongly adsorb to soil. The worms are therefore allowed to defecate before analysis, which gives more information on the real uptake of the substance (although trace amounts sorbed to soil may still remain in the worms even after defecation). This is to obtain a measure of real uptake of the substance by the worms, which is important for a bioaccumulation assessement. However, if secondary poisoning is considered worms are ingested with gut content and this should be accounted for in the exposure assessment. For the secondary poisoning assessment, it should be considered whether the test concentration used in the study was environmentally relevant. If a higher test concentration was used, it may be over-conservative to use the BSAF which includes the gut contents with contaminated soil.

This is especially important for worms sampled during the uptake phase, which have contaminated soil as gut contents. As soon as the contaminated gut contents are replaced by clean soil in the depuration phase, defecation is no longer necessary before chemical analysis (in that case, the weight of the gut contents is estimated to account for dilution of the test item concentration by uncontaminated soil).

ASTM E1676-04 describes a similar method for bioaccumulation testing with the annelids *Eisenia fetida* and *Enchytraeus albidus* over periods up to 42 days (ASTM, 2004).

Relevant data might also be available from field studies or earthworm toxicity studies (e.g. if tissue concentrations are measured). The suitability of data derived from such studies to provide meaningful information on a substance's bioaccumulation potential, has to be assessed on a case-by-case basis.

(Q)SAR models for earthworms

The model of Jager (1998) is recommended as a reasonable worst case for an initial assessment of the earthworm bioconcentration factor, and provides a description of this tool. The only input term required is the octanol-water partition coefficient (K_{ow}), and an application range of log K_{ow} 0-8 is advised. It was developed from a data set containing chlorobenzenes, pesticides, PCBs, PAHs, and chlorophenols. The model is limited to mostly neutral organic compounds and does not explicitly consider biomagnification or biotransformation. With due consideration it may be applicable to certain ionisable organics. Due to the narrow range of chemical groups within the model, it should be recognised that the model predictions have some limitations.

In cases where the K_{ow} is not a good indicator of bioconcentration (e.g. for ionic organic substances, metals or other substances that do not preferentially partition to lipids), either an alternative model for that specific substance or class of substances should be used, or an empirical BCF estimated from structural analogues. For example, Smit *et al.* (2000) provide a review of different equations for a limited number of metals.

Comparison of earthworms with benthic organisms

The results of bioaccumulation tests with suitable sediment-dwelling invertebrate species (e.g. the oligochaete *Lumbriculus variegatus*) may provide useful comparative information that can be used in a *Weight-of-Evidence* approach, if available. Further information on this test is given in the aquatic accumulation chapter. However, caution is warranted as a thorough comparison of bioaccumulation data for terrestrial and benthic species is currently lacking.

Terrestrial plants

Plants and crops can be contaminated by the transfer of substances from:

- soil (including solids and pore water) via the roots and translocation,
- air via the gas phase or particle deposition, and
- soil particles that splatter and stick on the foliage.

The need to assess these routes is determined by the approach adopted for the chemical safety assessment (see Chapter R.16 of the *Guidance on IR&CSA*).

Plant uptake test

Currently, no standardised test guidelines are specifically designed to develop bioaccumulation metrics (e.g., BCF, BAF) in plants (Gobas *et al.*, 2016; Doucette *et al.*, 2018). For simplicity in the discussion that follows, the term BAF will be used as a surrogate to represent all potential measures of bioaccumulation that have been used with plants.

A guideline that addresses plant uptake, translocation, and metabolism of substances (e.g. US-EPA 2012) could provide data useful in determining whether a substance accumulates in plants. The USEPA test guideline (2012) OCSPP 850.4800 outlines procedures for conducting a mass balance study of the distribution of a substance in environmental matrices and different components of the plant under root or foliar exposure for use in determining human and livestock food safety. Although these guidelines were not specifically designed to assess bioaccumulation in plants, they do evaluate the ability of pesticides to be taken up by and translocate throughout plants, using a maximum exposure scenario, or characterise metabolic or degradation pathways to identify residues of concern.

The data collected could allow for the calculation of a bioaccumulation metric(s) based on the ratio of the concentration of the substance in the plant relative to the concentration in the relevant environmental matrices, provided steady-state conditions are approximated. During the conducting of the test, the method of exposure (i.e. spraying, dusting, biosolids-amended soil, soil spiking), route of exposure (i.e. leaf and/or root), quantification of exposure, and characteristics of plant growth matrices would need to be considered carefully for the determination of a realistic bioaccumulation metric.

The guideline permits exposure via foliage as well as roots (and consequently provides advice on how to handle gaseous and volatile substances). Three test concentrations are recommended, with the number of replicates depending on the method of chemical analysis (fewer being required if radioanalysis is used). The test duration and number of plants selected are not specified, but should provide sufficient biomass for chemical analysis. Several species are suggested, including food crops and perennial ryegrass.

In principle, in case the test substance concentrations are measured in the environmental matrices, the collected data could allow for the calculation of a bioaccumulation metric(s). In order for this metric to be realistic, the method, route and quantification of exposure as well as characteristics of plant growth matrices have to be considered carefully.

Relevant data might also be available from non-guideline studies, field studies or plant toxicity studies (e.g. if tissue concentrations are measured), as well as from guideline toxicity studies with terrestrial plants, for which additional chemical analysis in the plants has been performed, e.g. according to OECD TG 208 (OECD, 2006).

(Q)SAR models for plants

Several models are possibly useful for estimating substance accumulation in plants. A review of these models has been made. The validation of all models is hampered by the lack of experimental standardised data in plants (Gobas *et al.*, 2016).

For most of the models, the only input required is the K_{ow}, but additional simple physico-chemical properties (e.g. molecular weight, vapour pressure and water solubility) are needed for some. As discussed in Gobas *et al.* (2016) and elsewhere (Doucette *et al.*, 2018), the applicability domain of the current plant models may be limited due to insufficient test data for a broad range of chemistry (i.e. range of K_{OW}, pKa, MW) and non-standardised testing. Plant uptake models are also discussed by Legind and Trapp, 2009 and Trapp, 2015.

Biomagnification in the terrestrial food chain

The default terrestrial food chain for secondary poisoning assessment is defined as soil - earthworm - earthworm eating bird/mammals (See Section R.16.6.7.2 in Chapter R.16 of the *Guidance on IR&CSA*).

Similarly to the aquatic food chain, in the terrestrial food chain, accumulation in higher trophic levels may occur as well, where small birds and mammals serve as prey for terrestrial predators, such as raptors and mustelids (Jongbloed *et al.*, 1994, Armitage and Gobas, 2007). This would lead to a default example terrestrial food chain that is defined as:

soil → earthworm/plant → worm or plant-eating birds or mammal → predator

Usually, to assess this type of information, modelling data are available that assess the accumulation in birds and mammals in the terrestrial environment. Furthermore, field data and/or toxicokinetic data in mammals may be available and should be addressed. More information on the interpretation of field data, modelling data and toxicokinetic data is given below.

QSARs for terrestrial food chain

Several models exist to estimate the biomagnification in terrestrial avian and mammalian species and food webs. Models have been developed for neutral, nonionic substances undergoing passive transport. These models are based on the K_{ow} and K_{oa} of the substance. Depending on the food web modelled, substances have the potential to biomagnify if the log K_{oa} > ~5-6 in combination with a log K_{ow} > ~2. Models for ionogenic substances and substances that are not accumulating by hydrophobic partitioning are lacking. There is further need to develop estimation methods for the rate of biotransformation and dietary assimilation efficiencies for all levels of the terrestrial food web (Gobas *et al.*, 2016).

Guidance on assessing the bioaccumulation potential in air-breathing species such as terrestrial mammals is described in Section R.11.4.1.2.8 of Chapter R.11 of the *Guidance on IR&CSA*. A detailed discussion of the scientific background and recommendations for future work is provided in the discussion paper "Bioaccumulation assessment of air-breathing mammals" (ECHA Working group on Toxicokinetics, 2022) available at the ECHA website.

Toxicokinetic data

Toxicokinetic studies in air-breathing organisms may provide useful information on bioaccumulation in particular for substances with a combination of log K_{ow} >2 and log K_{oa} >5. For further information, see Section R.7.10.15, Section R.7.12 and Section R.11.4.1.2.8 of the Chapter R.7 and R.11 respectively of the *Guidance on IR&CSA*.

R.7.10.11 Evaluation of available information on terrestrial bioaccumulation

Test data on terrestrial bioaccumulation

Experience with the evaluation of specific earthworm and plant bioaccumulation tests is limited, since they are rarely requested for industrial and consumer chemicals. Jager *et al.* (2005) provide some information on earthworm bioassays. Data obtained using standard methods are preferred. Non-guideline studies in particular need to be evaluated with care. Factors to be considered in general include:

- Where possible, the exposure duration should be sufficient to enable steady state to be achieved, in particular for highly hydrophobic substances (e.g. log K_{ow} >6). However, for most root crops, and most hydrophobic compounds, it may take much longer than the growth period to reach steady state. In such cases, crops should be monitored over their entire growing season.

- The test concentration should be ecologically relevant and should not cause significant toxic effects on the organism, while it also needs to be above the limits of quantification.

- Tissue sampling for plants should be relevant for the substance of interest (in terms of its expected distribution in root, foliage, etc.), and the requirement of the exposure assessment (e.g. vegetables should be considered whole rather than peeled, etc.).

- If plant root is the tissue of interest, there are several factors to consider. Pot sizes should not restrict root development. The test species should be a relevant food crop with a lipid-rich surface layer. The surface area-volume ratio may be important (i.e. is the surface area large in relation to the volume of the root?) The use of fast-growing miniature varieties may lead to bias, since transfer from the peel to the core of the root tends to be a slow process (Trapp, 2002).

- Sometimes plants are grown hydroponically to allow for simplified uptake and elimination phase logistics. However, this is not an environmentally relevant mode of exposure and a substance's ability to bioaccumulate can vary significantly as compared with a natural growth substrate (Hoke *et al.*, 2016; Karnjanapiboonwong *et al.*, 2011).

- In addition to organic carbon content, pH and soil texture are additional parameters that have been shown to cause variability in bioaccumulation in plants. As such, these have to be taken into account when selecting the type and number of test soils (Hoke *et al.*, 2016).

- Bioaccumulation also varies across plant species (Huelster *et al.*, 1994) and plant cultivars (Inui *et al.*, 2008).

- It is important to ensure that the organism is cleaned and (for worms) allowed to void its gut contents prior to analysis (since small amounts of retained contaminated soil could give false results). The inclusion of a elimination phase with clean soil as prescribed in OECD TG 317 will help to assess the influence of gut content on the organism's concentration.

- Analytical methods should be sensitive enough to detect the substance in both the soil and the organism tissue, and may require radiolabelled substances. It should be noted that radioanalysis does not by itself give information about the amount of intact substance within the organism, and preferably it should be supported by parent compound analysis so that the contribution of metabolites can be assessed.

- Whole soil tests tend to provide a BSAF, which is not very informative as indicator of bioaccumulation potential since it also reflects sorption behaviour. A better indicator would be the BCF based on the freely dissolved (bioavailable) soil pore water concentration. Ideally, this should be done using direct analytical measurement (which may involve sampling devices such as SPME fibres (Van der Wal *et al.*, 2004)). If no analytical data are available, the pore water concentration may be estimated using suitable partition coefficients, although it should be noted that this might introduce additional uncertainty to the result.

- The data may need to be transformed for use in a standardised way in the exposure assessment. For example:

  - Where possible, accumulation data should be normalised to the default lipid content of the organism. If lipid is not expected to play an important role in partitioning behaviour, such normalisation might not be appropriate. If applicable a different kind of normalisation could be considered (e.g. on dry weight or protein content).

  - If data are available regarding the variation in accumulation with soil type, etc., this should be described. If the organic carbon content of the test soil differs from the default soil used to derive the PEC (e.g. if the soil has been amended with sewage sludge), data should be normalised to the default organic matter/carbon content, if valid. This is relevant for neutral organic compounds; for metals and ionic or polar organic substances, soil parameters other than organic carbon may be more important and the validity of normalisation should be investigated first.

In the case of worms, the total amount of the substance present in the worm (i.e. tissue plus gut contents) is still a relevant parameter for secondary poisoning, because a predator will consume the whole worm. The fraction of the substance that is sorbed to the gut content can be estimated by assuming a fixed weight percentage of the gut content. The fraction of the gut content is by default set to 0.1 $kg_{dry\ weight\ soil}/kg_{wet\ weight\ worm}$ (Jager *et al.*, 2003; Jager, 2004).

An ILSI/HESI terrestrial bioaccumulation workshop was held in January 2013 and a publication by Hoke *et al.* (2016) presents a review of the application of laboratory-based approaches for terrestrial bioaccumulation assessment of organic substances.

Evaluation of toxicokinetic data for the purpose of bioaccumulation assessment is further explained in Section R.7.10.15 and Section R.7.12.

Non-testing data on terrestrial bioaccumulation

The use of QSARs will be mainly determined by the guidance for the chemical safety assessment as described by the report on exposure tools, which provides an evaluation of the recommended models, including their applicability domain. If a substance is outside of the applicability domain, then the results should be used with caution in the assessment. The use of any model should be justified on a case-by-case basis.

The 2013 ILSI/HESI terrestrial bioaccumulation workshop resulted in a publication by Gobas *et al.* (2016) which presents a review of the current terrestrial bioaccumulation models and their merits and limitations. In this review models for accumulation in terrestrial food chains are presented next to the above mentioned models for terrestrial invertebrates and plants. It should be noted that also the models for assessing accumulation through the terrestrial food chain are mainly restricted to neutral, nonionic organic substances. In addition to K_{ow} another important physico-chemical property for terrestrial bioaccumulation in air-breathing organisms is the octanol-air partition coefficient (K_{oa}).

General guidance on read-across and categories is provided in the section on aquatic bioaccumulation (see Section R.7.10.3.2).

R.7.10.11.1 Field data

General guidance for the evaluation of data from field studies is provided in the section on aquatic bioaccumulation (see Section R.7.10.3.3) and in Section R.11.4.1.2 of the *Guidance on IR&CSA*. The exposure scenario for the chemical safety assessment considers spreading of sewage sludge to land over a 10-year period, and consequently the exposure history of the soil should be described. Some of the factors described in Section R.7.10.4.3 are also relevant.

As noted previously, a terrestrial bioaccumulation workshop was sponsored by ILSI/HESI in 2013 and a publication by van den Brink *et al.* (2016) discusses the use of field studies to examine the potential bioaccumulation of substances in terrestrial organisms. In this review a comparison with aquatic bioaccumulation is made. The differences with the aquatic environment and the special points of attention for the terrestrial environment with regard to the derivation and use of experimentally derived endpoints from field data are highlighted.

R.7.10.11.2 Exposure considerations for terrestrial bioaccumulation

An assessment of secondary poisoning or human exposure via the environment is part of the chemical safety assessment. Triggering conditions are provided in Chapter R.16 of the *Guidance on IR&CSA*.

R.7.10.12 Conclusions for terrestrial bioaccumulation

There is a hierarchy of preferred data sources to describe the potential of a substance to bioaccumulate in terrestrial species for the assessment of secondary poisoning, as follows:

- In general, reliable measured BCF data on the substance itself in terrestrial plants or earthworms are considered as having the biggest weight among the different data types on bioaccumulation. It should be noted that experimental data on highly lipophilic substances (e.g. with log K_{ow} above 6) will have a much higher level of uncertainty than BCF values determined for less lipophilic substances. A BSAF might be an alternative measure.

- Next in order of preference comes reliable measured BCF data from the sediment worm *Lumbriculus variegatus* as a surrogate for earthworm data. Although differences are not expected to be large in principle, comparative information is lacking. Read-across on BCF data from a sediment organism to a terrestrial organism should therefore be made on a case-by-case basis, taking account of any differences in organic carbon and pore water contents between sediment and soil.

- Field data might also be useful at this *stage* as part of a *Weight-of-Evidence* argument (these require careful evaluation and will not be available for the majority of substances). Apart from field data on accumulation in terrestrial plants and invertebrates also data on biomagnification in terrestrial food chains should be taken into account.

- Toxicokinetic data may also be utilised, case-by-case, in the bioaccumulation assessment and should be addressed in the assessment when accumulation in air-breathing organisms is likely to be more pronounced than in water breathing organisms. See further details in Sections R.7.10.15 and R.11.4.1.2.8 of the Chapter R.7 and R.11 respectively of the *Guidance on IR&CSA*..

- The next line of evidence concerns data from non-testing methods.

- Other lines of evidence concern indications and rules based on physico-chemical properties. Nevertheless, the log K_{ow} is a useful screening tool for many substances, and it is generally assumed that non-ionised organic substances with a log K_{ow} below 3 (log Kow below 4 for aquatic chronic classification categories) are not significantly bioaccumulative for the aquatic environment. No such triggers can be given for the terrestrial environment. In additition, log K_{oa} >5 is a useful trigger to assess whether biomagnification in the terrestrial food chain might occur.

In principle, the available information from testing and non-testing approaches, together with other indications such as physico-chemical properties, must be integrated to reach a conclusion that is fit for the regulatory purpose regarding the bioaccumulation of a substance. A scheme is presented in the report for aquatic accumulation, and the broad principles are the same for terrestrial species. In summary:

- Make a preliminary analysis of bioaccumulation potential based on the structure and physico-chemical properties of the substance, as well as information about its degradation products in the environment. It may be possible at this stage to decide that the substance is unlikely to be significantly bioaccumulated.
- Evaluate any existing *in vivo* data, including field data if available.
- Identify possible analogues, as part of a group approach if relevant.
- Evaluate non-testing data (e.g. QSARs, including whether K_{ow} and K_{ow}-based models are relevant, and read-across, etc.).
- Weigh the different types of evidence and examine whether it is possible to reach a conclusion on terrestrial bioaccumulation. Difficulties in reaching a conclusion on the BAF, and/or BMF may indicate the need for further testing. If different data sources do not provide a coherent picture of the bioaccumulation potential of a substance, the reasons for such inconsistency should be addressed.

It should be noted that if a substance has a measured fish BCF that is significantly lower than predicted by QSAR, it cannot be concluded that the earthworm BCF will also be lower than the predicted fish value. This is because biotransformation processes in particular are more extensive in fish than earthworms (few compounds are appreciably biotransformed by earthworms).

R.7.10.12.1 Concluding on suitability for Classification and Labelling

Data on accumulation in earthworms and plants are not used for classification and labelling.

R.7.10.12.2 Concluding on suitability for PBT/vPvB assessment

For judging the suitability of the information for PBT/vPvB assessment, see guidance in Chapter R.11 of the Guidance on IR&CSA.

R.7.10.12.3 Concluding on suitability for use in Chemical Safety Assessment

In general, predicted BSAF (or pore water BCF) and BMF values (whether from QSAR or read-across) can be used for the initial assessment of secondary poisoning and human dietary exposure. If a prediction is not possible, measured BSAF (e.g. OECD TG 317) data will be necessary at the 1,000 t/y level.

R.7.10.13 Integrated testing strategy (ITS) for terrestrial bioaccumulation

R.7.10.13.1 Objective / General principles

The objective of the testing strategy is to provide information on terrestrial bioaccumulation in the most efficient manner so that costs are minimised. In general, test data will only be needed at the 1,000 t/y level, if the chemical safety assessment identifies the need for further terrestrial bioaccumulation information. Furthermore, collection and/or generation of additional terrestrial bioaccumulation data are required for the PBT/vPvB assessment in all cases where a registrant carrying out the CSA cannot derive a definitive conclusion based on aquatic accumulation data, either (i) ("The substance does not fulfil the PBT and vPvB criteria") or (ii) ("The substance fulfils the PBT or vPvB criteria") in the PBT/vPvB assessment, and the PBT/vPvB assessment shows that additional information on terrestrial bioaccumulation would be needed for deriving one of these two conclusions. This obligation applies for all ≥ 10 t/y registrations (see Chapter R.11 of the *Guidance on IR&CSA* for further details).

R.7.10.13.2 Preliminary considerations

If predicted BSAF and BMF values indicate potential risks for either wildlife or humans, the need for further terrestrial bioaccumulation testing should be considered as part of an overall strategy to refine the PEC with better data, including:

- more realistic release information (including risk management considerations);
- other fate-related parameters such as determination of more reliable soil partition coefficients (which may allow a better estimate of the soil pore water concentration) or degradation half-life.

These data might also be needed to clarify risks for other compartments, and a sensitivity analysis may help to identify the most relevant data to collect first.

In addition, if further sediment organism bioaccumulation or soil organism toxicity tests are required, it may be possible to gather relevant data from those studies.

Depending on the magnitude of the risk ratio and the uncertainty in the effects data, it might also be appropriate in some circumstances to derive a more realistic NOAEL value from a long-term feeding study with laboratory mammals or birds, although this would not usually be the preferred option.

R.7.10.13.3 Testing strategy for terrestrial bioaccumulation

In general, the octanol-air partition coefficient (K_{oa}) and octanol-water partition coefficient (K_{ow}) can be used as the initial input for terrestrial bioaccumulation models at a screening level for most neutral organic substances.

If the substance is outside the domain of the models, and a BSAF and BMF cannot be established by other methods (such as analogue read-across or derived from field data), a test may be needed at the 1,000 t/y level. Similarly, if a risk is identified that is not refinable with other information, a test will usually be necessary.

Standard test guideline studies are preferred. The choice of test will depend on the scenario that leads to a risk, and the test species should reflect the specific route of uptake that may be expected from the properties of the individual substance under consideration. For example, where a model predicts the highest concentration to be in roots, the test species would be a relevant food crop.

Field monitoring might be an alternative or supplementary course of action to laboratory testing in special cases, especially for more hydrophobic substances that may take a long time to reach steady state. This will not be a routine consideration, because of the difficulty in finding soils that may have had an adequate exposure history.

R.7.10.14 References for terrestrial bioaccumulation

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R.7.10.15 Mammalian toxicokinetic data in bioaccumulation assessment

Mammalian toxicokinetic studies may provide useful information in a *Weight-of-Evidence* approach for bioaccumulation assessment. Metrics to consider include:

- metabolic capacity/rate constants
- affinity for lipid or blood-rich tissues, which could include the volume of distribution, V_D (a parameter that quantifies the distribution of a substance throughout the body after oral dosing; it is defined as the volume in which a substance would need to be homogeneously distributed to produce an observed blood concentration. If there is significant distribution into lipids the V_D will be increased (although this may also be caused by renal and liver failure).
- the time taken to reach a steady-state (plateau) concentration in tissues, and
- uptake efficiency and clearance, and elimination rates/half-lives.

Standardised test methods (e.g. OECD TG 417 Toxicokinetics) are not widely used for deriving toxicokinetic data and therefore particular attention needs to be paid in the evaluation of such data to the sources of variation and their impact on the results.

Physiologically-based pharmacokinetic/toxicokinetic models (PBPK/PBTK) may support or expand the understanding of the toxicokinetic behaviour of a substance and their use should be considered, where a model applicable for the substance is available. For further information, see the IPCS/WHO project document on the PBPK models in risk assessment (2010).

Principles presented in OECD TG 417 Toxicokinetics should be as far as possible applied where relevant. When using elimination information the following aspects should be addressed as minimum:

- Species, age and gender of a test subject. Elimination rates/half-lives can vary between age and gender causing the need for half-life values to be determined for subgroups in the same species (Ng and Hungerbuhler, 2014).
- Sample type. Conventional practice to retrieve elimination data is to measure the concentration of a substance in serum, plasma or whole blood. In addition, urine, faeces, various tissue and organ specific data, and combination of such samples are frequently available.
- Study approach. Tests are usually conducted either using experimental (e.g. laboratory animal tests) or observational (e.g. human biomonitoring) approaches.
- Exposure aspects and dosing scheme. Exposure route(s), level, duration (short term/long term) and dosing scheme (single, episodic or continuous) should be addressed to define the overall scenario of a study. Results from studies conducted using ongoing exposure (intentional or unintentional) and single or repeated doses should all be reported and interpreted in a differentiating manner. Biomonitoring studies without or with only very limited and/or uncertain exposure information might call for estimation of likely exposure levels, routes,

duration/frequency and may due to high uncertainty not be particular useful as a single decision element in bioaccumulation assessments. A prerequisite for calculation of an elimination half-life is that the elimination pattern is shown to obey first-order kinetics or at least not deviate significantly from first order kinetics (pseudo-first-order kinetics). In case an elimination rate has been obtained from a study where exposure cannot be excluded, presentation of elimination half-lives needs to be coupled with explanation of the influence of continuing exposure to the results and a justification of why it can be assumed that the elimination follows (approximately) first-order kinetics.

- Descriptors of elimination half-life. The terminology used in the currently available studies is unfortunately not fully standardised. Applied toxicokinetic models and terminology (e.g. description of what is meant in a particular study by "half-life", "apparent half-life" or "intrinsic half-life") should be reported in detail. For the appropriate use of terminology, see Nordberg *et al.*, (2004).

- Analytical methods for detection and quantification (including sampling and extraction methods when relevant) of the substance concerned. Indicate whether direct detection or indirect detection by means of isotopic labels (e.g. radiocarbon C-14) was used. Report statistical methods applied for data analysis. Elimination half-lives are usually presented as arithmetic or geometric means, medians or ranges. All reported values, including the ranges, should be presented.

Guidance on assessing the bioaccumulation potential in air-breathing species such as terrestrial mammals is described in Section R.11.4.1.2.8 of Chapter R.11 of the *Guidance on IR&CSA*. A detailed discussion of the scientific background and recommendations for future work is provided in the discussion paper "Bioaccumulation assessment of air-breathing mammals" (ECHA Working group on Toxicokinetics, 2022) available at the ECHA website.

R.7.10.16 Avian Toxicity

Information on (long-term) avian toxicity only needs to be considered for substances supplied at 1,000 t/y or more (Section 9.6.1 of Annex X to REACH). The data are used to assess the secondary poisoning risks to predators following chronic exposure to a substance via the fish and earthworm food chains[9]. Given that mammalian toxicity is considered in detail for human health protection, the need for additional data for birds must be considered very carefully – new tests are a last resort in the data collection process. However, birds are fundamentally different from mammals in certain aspects of their physiology (e.g. the control of sexual differentiation, egg laying, etc.), and so mammalian toxicity data are of limited predictive value for birds. This document describes how to assess information that already exists, and the considerations that might trigger new testing with birds.

It should be emphasised that there is a marked lack of relevant data available for industrial and consumer chemicals, and further research could be performed to:

- establish relative sensitivities of birds and mammals following chronic exposures,
- establish the validity of read-across arguments between structurally related substances,
- investigate *in vitro* approaches for birds; for instance, Ball and Lavado (2021) examined the use, limitations, and published applications of avian cell-based models in an ecotoxicological context to understand the current state of these models, and
- identify structural alerts for chronic avian toxicity.

The guidance should therefore be reviewed as more experience is gained.

Readers should also refer to guidance related to the mammalian toxicokinetics (see Section R.7.12), repeated dose toxicity (see Section R.7.5 in Chapter R.7a of the *Guidance on IR&CSA*) and reproductive toxicity (see Section R.7.6 in Chapter R.7a of the *Guidance on IR&CSA*) endpoints for further relevant information.

R.7.10.16.1 Definition of avian toxicity

The aim of an avian toxicity test is to provide data on the nature, magnitude, frequency and temporal pattern of effects resulting from a defined exposure regime (Hart *et al.*, 2001). The three standard avian tests typically measure:

- lethal and delayed effects of short-term oral exposures (lasting minutes to hours, representing gorging behaviour, diurnal peaks in feeding (e.g. dawn and dusk) and products which depurate or dissipate very rapidly);

[9] Inhalation tests with birds are not considered necessary for industrial and consumer chemicals, since outdoor air concentrations are unlikely to exceed limits that will be set to protect human health (and other vertebrates by assumption). Dermal toxicity tests do not need to be considered for similar reasons.

- lethal effects of medium-term dietary exposures (lasting hours to days, representing scenarios with relatively high exposures over several days); or
- chronic lethal and reproductive effects of long-term dietary exposures (lasting up to 20 weeks).

Exposures are expressed in terms of either a:

- *concentration* of the substance in the food consumed by the birds (e.g. milligrams (mg) of test substance per kilogram (kg) of food[10]), or
- *dose* expressed relative to body weight (e.g. mg test substance/kg body weight (per day, if more than a single exposure)).

The main results from an avian toxicity study include:

- the limit dose at which no mortality occurs (LD_0);
- a median lethal dose or concentration, at which 50% of birds die ($LD(C)_{50}$);
- a 'no observed effect' level, at which no effects of specified type occur, or a concentration at which either a defined level of effect is seen in x% of tested individuals, or an average deviation of x% is seen when compared to the untreated control (EC_x); and
- a statement of the type and frequency of effects observed in a specified exposure scenario (e.g. in a field study).

Other types of information may include the slope of a dose-response relationship, 95% confidence limits for the median lethal level and/or slope, and the time at which effects appear.

R.7.10.16.2 Objective of the guidance on avian toxicity

Avian toxicity data are used in the assessment of secondary poisoning[11] risks for the aquatic and terrestrial food chains in the CSA. In the context of PBT/vPvB assessment (see Section R.7.10.20), avian toxicity data cannot be directly (numerically) compared with the T criterion (see Section 1.1.3 of Annex XIII to REACH). However, reprotoxicity studies or other chronic data on birds, if they exist, should be used in conjunction with other evidence of toxicity as part of a weight-of-evidence determination to conclude on substance toxicity (a NOEC ≤ 30 mg/kg food in a long term bird study should in this context be considered as a strong indicator of fulfilment of the T criterion).

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[10] Units of mg/kg may also be expressed as parts per million (ppm).

[11] Secondary poisoning concerns the potential toxic impact of a substance on a predatory bird or mammal following ingestion of prey items (i.e. fish and earthworms) that contain the substance. Accumulation of substances through the food chain may follow many different pathways along different trophic levels. This assessment is required for substances for which there is an indication for bioaccumulation potential (Appendix R.7.10-3).

R.7.10.17 Information requirements for avian toxicity

Annex X to REACH indicates that information on long-term or reproductive toxicity to birds should be considered for all substances manufactured or imported in quantities of 1,000 t/y or more. Since this endpoint concerns vertebrate testing, Annex XI to REACH also applies, encouraging the use of alternative information. Although not listed in column 2 of Annex X to REACH, there are also exposure considerations (see Section R.7.10.19.4).

Although not specified at lower tonnages, existing data may be available for some substances. These are most frequently from acute studies, and this document provides guidance on their interpretation and use. Nevertheless, data from long-term dietary studies are the most relevant because:

- Few (if any) scenarios are likely to lead to acute poisoning risks for birds, and
- Evidence from pesticides suggests that chronic effects cannot be reliably extrapolated or inferred from acute toxicity data (Sell, undated).

PBT/vPvB assessment:

In the context of the PBT/vPvB assessment, if the registrant cannot derive a definitive conclusion (i) ("The substance does not fulfil the PBT and vPvB criteria") or (ii) ("The substance fulfils the PBT or vPvB criteria") in the PBT/vPvB assessment using the relevant available information, he must, based on Section 2.1 of Annex XIII to REACH, generate the necessary information, regardless of his tonnage band (for further details, see Chapter R.11 of the *Guidance on IR&CSA*).

The general presumption is that avian toxicity testing will not normally be necessary. At the same time, care must be taken not to underestimate the potential hazard to birds. New studies should only be proposed following careful consideration of all the available evidence.

R.7.10.18 Available information on avian toxicity

The following sections summarise the types of data that may be available from laboratory tests.

Avian toxicity tests are often carried out for substances with intentional biological activity as a result of regulatory approval requirements (especially active substances used in plant protection products, but also veterinary medicines and biocides). They are rarely performed for most other substances. Although REACH does not apply to such products, they are relevant in this context as a source of analogue data.

There are currently no European databases for pesticides, biocides or veterinary medicines, although some are in development (e.g. the Statistical Evaluation of available Ecotoxicology data on plant protection products and their Metabolites (SEEM) database). Current pesticide data sources include:

- the British Crop Protection Council Pesticide Manual (BCPC, 2003),
- the German Federal Biological Research Centre for Agriculture and Forestry (BBA) database (http://www.bba.de/english/bbaeng.htm),

- the Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail (Anses) AGRITOX database (http://www.agritox.anses.fr/index2.php),
- the footprint database (http://sitem.herts.ac.uk/aeru/iupac/, and
- several US-EPA databases (http://www.epa.gov/pesticides/).

General searches might retrieve documents from regulatory agencies or the EXTOXNET project (a co-operative project by the University of California-Davis, Oregon State University, Michigan State University, Cornell University, and the University of Idaho, http://extoxnet.orst.edu/). Finally, IUCLID contains unvalidated data sheets for high production volume substances, a few of which might include data on avian toxicity (http://esis.jrc.ec.europa.eu/).

R.7.10.18.1 Laboratory data on avian toxicity

Testing data on avian toxicity

In vitro data

No specific avian *in vitro* methods are currently available or under development. A number of *in vitro* tests for assessing embryotoxic potential and endocrine disrupting properties in mammals have become available in recent years, and these are discussed in the specific guidance on reproductive and developmental toxicity (see Section R.7.6).

In vivo data

Table R.7.10—4 summarises the main existing test methods, as well as those proposed as draft OECD test guidelines. The guidelines for all three principal avian tests – acute, dietary and reproduction – are currently under review. Further details can be found in a Detailed Review Paper for Avian Two Generation Tests (OECD 2006a). It should be noted that acute tests will not be relevant to exposure scenarios normally considered for industrial and consumer chemicals, but they are included since the data might already be available for some substances.

A number of reviews of avian toxicity testing issues have been produced over the last decade, and these should be consulted if further details are required. All have a pesticide focus. The most up-to-date reviews are Hart *et al.* (2001), Mineau (2005), Bennett *et al.* (2005) and Bennett and Etterson (2006). Other useful sources of information include US-EPA (1982a, 1982b and 1982c), SETAC (1995), OECD (1996), EC (2002a and 2002b) and EPPO (2003).

Non-guideline toxicity studies may be encountered occasionally (e.g. egg exposure studies involving either injection or dipping). Such studies can be difficult to interpret due to the lack of standardised and calibrated response variables with which to compare the results. In addition, the exposure route will usually be of limited relevance to the dietary exposure route considered in the CSA. Metabolism in eggs may also be very different to that in the body. Such studies are therefore unlikely to provide information on use in quantitative risk assessment, although they might provide evidence of toxicity that requires further investigation.

Non-testing data on avian toxicity

(Q)SAR models

Toxicity to Bobwhite Quail following both 14-day oral and 8-day dietary exposure can be predicted for pesticides and their metabolites using a free web-based modelling tool called "DEMETRA" (Development of Environmental Modules for Evaluation of Toxicity of pesticide Residues in Agriculture) (Benfenati, 2007). The model was developed using experimental data produced according to official guidelines, and validated using external test sets. A number of quality criteria have been addressed according to the OECD guidelines[12]. It is unclear at the moment whether this model will be useful for other types of substance.

No other Q(SAR) models are currently available.

[12] The ECB may wish to produce a QRF to provide details on domain, no. of substances in training set, etc.

Table R.7.10—4 Summary of existing and proposed standardised avian toxicity tests

Test	Guideline	Summary of the test	Information derived
Acute oral toxicity[13]	Draft OECD TG 223 (OECD, 2002) USEPA/ OPPTS 850.2100 (US-EPA, 1996a)	The test involves direct exposure of birds to measured single oral doses of the test substance, followed by observation for a number of days. Administration is by gavage either in a suitable solvent vehicle or in gelatine capsules. The highest dose need not exceed 2,000 mg/kg bw. Regurgitation should be avoided because it compromises the evaluation of toxicity. Lowering dose volume or changing carriers may reduce the incidence of regurgitation.	The test provides a quantitative measurement of mortality (LD_{50} value), which acts as a standard index of inherent toxicity, since bird behaviour (i.e. dietary consumption) cannot influence the dose received. It is therefore useful as a general guide for range finding for other studies, and for comparative studies. The results are relevant to very short timescale exposures, and cannot be used to indicate chronic toxicity. This test is therefore of low relevance for the assessment of food chain risks.
Dietary toxicity	OECD TG 205 (1984a) USEPA/ OPPTS 850.2200 (US-EPA, 1996b)	This is a short-term test, in which groups of 10-day old birds are exposed to graduated concentrations (determined in a range-finding test) of the test substance in their diet for a period of 5 days, followed by a recovery period. Multiple oral dosing may be necessary for very volatile or unstable compounds. The test is not designed to simulate realistic field conditions, or provide a good characterisation of sub-lethal effects. Other drawbacks include: food avoidance[14], and lack of replication (which limits the power of the test to detect effects).	The test provides a quantitative measurement of mortality (e.g. 5-day LC_{50} value) and can act as a range-finder for the chronic reproduction test (a full test is not necessary if the range-finding test shows that the LC_{50} is above 5,000 mg/kg diet).

[13] Efforts to combine these two test methods into one internationally harmonised test guideline are currently ongoing in the OECD Test Guideline Programme

[14] Food avoidance responses can influence a substance’s hazard and also risk potential by restricting exposure, although this will vary between species. A draft OECD Guidance Document on Testing Avian Avoidance Behaviour is under development (OECD 2003). In the current revision of TG 205 the method will be revised to generate information that also can be used for the assessment of avoidance behaviour. There are no international protocols on avian repellency yet available. However a purpose of such a test i.e. the screening of repellent substances could be achieved by using the results of a revised dietary guideline (OECD, 2006b). Repellency is of limited relevance for long-term endpoints involving only low concentrations of test substance. Further guidance, if needed, can be found in the references cited in the main text.

Test	Guideline	Summary of the test	Information derived
Reproduction[15]	OECD TG 206 (1984b) USEPA/ OPPTS 850.2300 (US-EPA, 1996c)	Breeding birds are exposed via the diet over a long-term (sub-chronic) period to at least three concentrations of the test substance. The highest concentration should be approximately one half of the acute dietary LC_{10}; lower concentrations should be geometrically spaced at fractions of the highest dose. An upper dose limit should be set at 1,000 ppm (unless this would cause severe parental toxicity). The test substance should possess characteristics that allow uniform mixing in the diet. The test guideline cannot be used for highly volatile or unstable substances.	The test enables the identification of adverse effects on reproductive performance linked to gonadal functionality at exposure levels lower than those that cause serious parental toxicity. The most important endpoint is the production of chicks that have the potential to mature into sexually viable adults. Other intermediate parameters are also measured (e.g. mortality of adults, onset of lay, numbers of eggs produced, eggshell parameters, fertility, egg hatchability and effects on young birds). These can give information on the mechanisms of toxicity that contributes to overall breeding success. The test should provide a NOEC value (i.e. the concentration in adult diet that shows no reduction in the production of viable chicks) along with the statistical power of the test. It is critical that all endpoints be taken into account when using the results from the test for risk assessment. The weight given to intermediate endpoints in the absence of a problem in overall chick production is a case-by-case decision which must be made after consideration of the possible or likely consequences in the wild. The ecological significance of effects on each of the parameters measured may differ.

[15] Some work has been done to develop a one-gen test OECD draft TG (2000) Avian Reproduction Toxicity Test in the Japanese Quail or Northern Bobwhite) but this is not yet at a suitable stage to be discussed further.

Test	Guideline	Summary of the test	Information derived
OECD TG 206 was not designed to accurately reflect a bird's full breeding cycle, and some ecologically important endpoints are not covered (e.g. the onset of laying, parental competence in incubation, and feeding of young birds). Although these might not always be significant gaps, further work is underway to develop a test that will be able to detect all the potential effects of endocrine disrupting chemicals, and this is described briefly below.			
Two-generation avian reproduction toxicity	Draft OECD TG proposal (OECD, 2007)	The proposed guideline aims to examine the effects of a chemical on a broad set of reproductive fitness and physiological endpoints in a quail species over two generations. However, several research areas have been identified, and an agreed test guideline is unlikely to be available for some time.	The test is designed to determine whether effects are a primary disturbance (with direct impacts on the endocrine system) or a secondary disturbance (with impacts on other target organs that cause endocrine effects) of endocrine function. Currently, endpoints to be covered include egg production and viability, hatching success, survival of chicks to 14 days of age, genetic sex, onset of sexual maturation, body weight, and male copulatory behaviour, gross morphology and histology of specific organs, as well as levels of sex hormones, corticosterone, and thyroid hormones.

<u>Read-across and categories</u>

Experience of read-across approaches for avian toxicity is very limited for industrial and consumer chemicals. The same approach should therefore be adopted as for mammalian tests (see Section R.7.6 for specific guidance on reproductive and developmental toxicity).

In addition, it should be considered whether the substance has any structural similarity to other substances to which birds are known to be especially sensitive, such as organophosphates, certain metals and their compounds (e.g. cadmium, lead, selenium) and certain pesticide or veterinary medicine active substances (e.g. DDT). Further research is needed to identify structural alerts for chronic avian toxicity.

R.7.10.18.2 Field data on avian toxicity

Field data will not usually be available, and it is unlikely that a registrant will ever need to conduct a specific field study to look for bird effects (as sometimes required for pesticides). Recommendations on methodology are given in EC (2002a) and further discussion is provided in Hart *et al.* (2001) and SETAC (2005). The kind of data that result from such studies varies according to the test design, although they tend to focus on short-term impacts and are therefore of limited use for risk assessment of long-term effects.

Wildlife incident investigation or other monitoring schemes might rarely provide some evidence that birds are being affected by exposure to a specific substance. Interpretation is often complicated and it may be difficult to attribute the observed effects to a specific cause. However, such data can be used to support the assessment of risks due to secondary poisoning on a case-by-case basis.

R.7.10.19 Evaluation of available information on avian toxicity

R.7.10.19.1 Laboratory data on avian toxicity

Testing data on avian toxicity

In vitro data

No specific avian methods are currently available. The specific guidance on reproductive and developmental toxicity (see Section R.7.6) provides guidance on evaluation of some types of test that are relevant to mammalian reproduction. It should be noted that these are only relevant for one – albeit very important – aspect of long-term toxicity. In addition, these tests do not take metabolism into account, and metabolic rates and pathways may differ significantly between birds and mammals.

In vivo data

Ideally, test results will be available from studies conducted to standard guidelines with appropriate quality assurance, reported in sufficient detail to include the raw data. Data from other studies should be considered on a case-by-case basis. For example, expert judgement is needed to identify any deviations from modern standards and assess their influence on the credibility of the outcome. A non-standard test might provide an indication of possible effects that are not identified in other studies or evidence of very low or high toxicity. If the data are used, this must be scientifically justified.

For tests involving dietary exposure, stability and homogeneity of the substance in the food must be maintained. Results of studies involving highly volatile or unstable substances therefore need careful consideration, and it might not be possible to adequately test such substances or those that otherwise cannot be administered in a suitable form in the diet. In such cases, it is unlikely that birds would be exposed to the substance in the diet either, for similar reasons. If a vehicle is used, this must be of low toxicity, and must not interfere with the toxicity of the test substance. Validity criteria are given in the OECD guidelines.

Acute/short-term tests

Existing acute test data can be useful if no other avian data are available, although they are not preferred. Regurgitation/emesis can substantially reduce the dose absorbed in acute oral toxicity tests, and therefore affect the interpretation of the test results. Similarly, food avoidance in dietary tests may lead to effects related to starvation rather than chemical toxicity. Tests should therefore be interpreted carefully for any evidence of such responses - the test may not be valid if regurgitation occurs at all doses.

Long-term tests

A number of issues should be considered in the evaluation of long-term tests, as listed in Table R.7.10—5. In principle, only endpoints related to survival rate, reproduction rate and development of individuals are ecotoxicologically relevant.

Table R.7.10—5 Summary of interpretational issues for long-term toxicity tests

Long-term testing issue	Comment
Category of endpoint	Reproduction tests include parental and reproductive endpoints. An endpoint relating to overall reproductive success should normally be selected to define the long-term NOEC. Depending on the individual case and the availability of data, this could be the reproduction rate, the survival or growth rate of the offspring, or behavioural parameters in adults or young. In some cases, other endpoints (e.g. certain biochemical responses) may be more sensitive, although they might not be ecologically relevant. Guidance on interpretation of such data, if they are available, is provided in OECD (1996). In summary, any conclusions of biological significance must be based on changes that: Occur in a dose-response fashion (i.e. more abundant or pronounced in higher exposure groups); Are accompanied by confirmatory changes (i.e. differences in a biochemical parameter or organ weight, or histologically observable changes in tissue structure); and, Most importantly, are related to an adverse condition that would compromise the ability of the animal to survive, grow or reproduce in the wild (e.g. pronounced effects on body weight and food consumption (if this is a toxic response and not caused by avoidance)).
Statistical power	The NOEC is based on the most sensitive endpoint of the test as determined by the lack of statistical significance compared with the control. This does not necessarily equate to biological significance. For example, in a high quality (low variation coefficient, high power) avian reproduction test it may be possible to prove that a 5% deviation in hatchling weight is statistically significant, although this would not be detectable in normal tests. If the chick weight at day 14 is normal, such an effect should not be considered as biologically relevant. The NOEC may therefore be used as a worst case value for risk assessment, but it may be possible to refine this if necessary by considering the ecological relevance of the effects seen at doses above the NOEC (e.g. see Bennett *et al.*, 2005).
Time course of effects	Sublethal effects that are transient or reversible after termination of exposure are less relevant than continuous or irreversible effects (this may depend on how fast the reversal takes place). If reproductive effects in a multigeneration study are more pronounced in the second generation whereas in practice exposure will be restricted to a short time period then the reproductive NOEC after the first generation should be used as a possible refinement step (unless

Long-term testing issue	Comment
	in exceptional cases, e.g. with suspected endocrine disrupters, where effects in the second generation may be attributable to a brief exposure period in the first generation).
Parental toxicity	Parental toxicity should be avoided if possible. Effects that are only observed in the concentration range that leads to clear parental toxicity need careful consideration. For example, a decline in egg laying may be the result of reduced feeding by the adult birds, and would therefore not be a reproductive effect.
Exposure considerations	For highly hydrophobic substances, or substances that are otherwise expected to be significantly accumulative, measurements of the substance in tissues should be considered as an additional endpoint to determine whether concentrations have reached a plateau before the end of the exposure period.

Non-test data on avian toxicity

(Q)SAR models

If QSAR models that have been developed for pesticides are used, their relevance for a particular substance should be considered and explained (especially in relation to the applicability domain). It is likely that QSAR approaches will not be suitable for the majority of substances for the foreseeable future, in terms of both the endpoints covered (i.e. acute effects only) and the chemical domain.

Read-across and categories

The same principles apply as for mammalian acute toxicity (see Section R.7.4), repeated dose toxicity (Section R.7.5) and reproductive toxicity studies (Section R.7.6). Ideally, the substances should have similar physico-chemical properties and toxicokinetic profiles, and information will be available about which functional groups are implicated in any observed avian toxicity. The comparison should take account of reproductive or other chronic effects observed in fish and mammals as well as birds. The absolute toxicity of a substance cannot be directly extrapolated from fish or mammals to birds, but relative sensitivities might provide enough evidence in some circumstances.

R.7.10.19.2 Field data on avian toxicity

It will be very unusual for field studies to indicate chronic effects in wild birds, and these need to be considered case-by-case. Results should be interpreted with caution, and confounding factors addressed before deciding what level of any particular substance is linked to the observed effect. The relevance and statistical power of the study should also be assessed. Further discussion is provided in Hart *et al.* (2001) and OECD (1996).

R.7.10.19.3Remaining uncertainty for avian toxicity

Avian toxicity data are not available for the majority of substances. Assessments of secondary poisoning are therefore usually reliant on mammalian toxicity data. The relative sensitivities of birds and mammals following chronic exposures require further research. For example, there is some evidence from pesticide data that birds may be an order of magnitude more sensitive in some cases. The validity of read-across between analogue substances is also untested.

Even when studies are available, there are still many sources of uncertainty that need to be taken into account in the assessment of avian effects. Only a very few species are tested in the laboratory, and it is important to be aware that there is significant variation in response between species and individuals, and differences between laboratory and field situations (e.g. diet quality, stressors, differing exposures over time). Further details are provided in Hart *et al.* (2001). These issues are assumed by convention to be accounted for collectively using an extrapolation or assessment factor (see Section R.7.10.20). It should be noted that these factors have not been calibrated against the uncertainties.

In addition, it should be remembered that the model food chain for the screening assessment of secondary poisoning risks is relatively simplistic and reliant on a number of assumptions (see Section R.7.10.8 for further details). It may often be possible to refine the exposure scenario (e.g. by more sophisticated modelling, including use of more specific information about the most significant prey and predator organisms of the food chain considered concerning for example bioavailability of the substance in food and feeding habits and/or gathering better exposure information such as emission, degradation or monitoring data). Regardless of the calculations that are performed, it is always useful to perform a sensitivity analysis, i.e. list those items that have some associated uncertainty, and discuss whether these uncertainties can be quantified together with their overall impact on the conclusions of the assessment.

For complex mixtures, the toxicity test result is likely to be expressed in terms of the whole substance. However, the exposure concentration may be derived for different representative components, in which case the PEC/PNEC comparison will require expert judgement to decide if the toxicity data are appropriate for all components, and whether further toxicity data are needed for any specific component.

R.7.10.19.4 Exposure considerations for avian toxicity

No specific exposure-related exclusion criteria are provided in column 2 of Annex X.

In pesticide risk assessment, decisions on the need for reproduction tests may depend on whether adult birds are exposed during the breeding season (EC, 2002a). However, it is highly unlikely that the use of an industrial or consumer chemical would be so restricted as to exclude breeding season exposure. In some cases, the use pattern might limit exposure to birds. For example, production and use might only take place at a small number of industrial sites with very low releases, with low probability of any significant release from products (an example might be a sealant additive). In cases where the exposure is considered negligible, an appropriate justification should be given, taking care that this covers all stages of the substance’s life cycle.

If releases to air, water and/or soil can occur, then the need to perform a new avian toxicity test at the 1,000 t/y level should be decided following a risk assessment for secondary poisoning. It should be noted that the exposure of birds is generally only considered for the fish and earthworm food chains following the release of a substance via a sewage treatment works[16]. The need to conduct a secondary poisoning assessment is triggered by a number of factors (see Section R.16.4.3.5 of the *Guidance on IR&CSA* for further guidance). If these criteria are not met, then further investigation of chronic avian toxicity is unnecessary. For example, it is unlikely that a secondary poisoning risk will be identified for substances that:

- are readily biodegradable, and
- have a low potential for bioaccumulation in fish and earthworms (e.g. a fish BCF below 100, or in the absence of such data on neutral organic substances a log K_{ow} below 3).

These properties may therefore be used as part of an argument for demonstrating low exposure potential for birds, although care may be needed (e.g. high local concentrations could still be reached in some circumstances, for example due to widespread continuous releases).

R.7.10.20 Conclusions for avian toxicity

The aim is to derive a PNEC for birds based on the available data. Given the absence of reliable QSARs and *in vitro* methods, in most cases it is expected that an initial attempt to estimate avian toxicity can be made by read-across from suitable analogue substances (possibly as part of a category). The preferred value must be scientifically justified for use in the assessment.

R.7.10.20.1 Concluding on suitability for PBT/vPvB assessment

In the context of PBT/vPvB assessment, avian toxicity data should be used in conjunction with other evidence of toxicity as part of a weight-of-evidence determination to conclude on substance toxicity. If the existing avian toxicity study is of poor quality, or the effect is unclear or based on only minor symptoms, an additional study might be needed if the decision is critical to the overall assessment, in which case a limit test would be preferred. The ecological significance of the effect should also be considered (e.g. how important is a sub-lethal effect compared to those of natural stressors, and what would be their effect on population stability or ecosystem function?). Further guidance is provided in Bennett *et al.* (2005).

Further guidance on criteria is provided in Chapter R.11 of the *Guidance on IR&CSA*.

R.7.10.20.2 Concluding on suitability for use in chemical safety assessment

Data obtained from species used in standard test methods are assumed to be representative of all species (including marine). Since the scenario under consideration concerns the effects of a substance on birds via their diet, only toxicity studies using oral exposure are relevant. Dietary studies are preferred, since these are most relevant to the exposure route under investigation. Oral gavage studies might provide some evidence of very high or low acute toxicity in some cases, which could be used as part of

[16] It may sometimes be appropriate to model exposure of marine predators, in which case the scenario might not involve a sewage treatment stage.

a *Weight-of-Evidence* argument provided that a reasoned case is made. Egg dipping studies are not relevant, although they might indicate an effect that requires further investigation.

R.7.10.21 Integrated testing strategy (ITS) for avian toxicity

R.7.10.21.1 Objective / General principles

In general, a test strategy is only relevant for substances made or supplied at levels of 1,000 t/y or higher (although there may be a need for further investigation if a risk is identified at lower tonnage based on existing acute data). Furthermore, collection and/or generation of additional avian toxicity data are required for the PBT/vPvB assessment in all cases where a registrant, while carrying out the CSA, has identified is substance as P and B but cannot draw an unequivocal conclusion on whether the T criterion in Annex XIII to REACH is met or not and avian toxicity testing would be needed to draw a definitive conclusion on T. This obligation applies for all ≥ 10 tpa registrations (see Chapter R.11 of the *Guidance on IR&CSA* for further details).

The general presumption is that avian toxicity testing will not normally be necessary. At the same time, care must be taken not to underestimate the potential risks faced by birds. New studies should only be proposed following careful consideration of all the available evidence, and the objective of the testing strategy is therefore to ensure that only *relevant* information is gathered.

R.7.10.21.2 Preliminary considerations

The need for chronic avian toxicity testing is explicitly linked to the secondary poisoning assessment. A decision on the need to conduct avian testing may be postponed if other actions are likely to result from the rest of the environmental (or human health) assessment. For example:

- No further testing on birds is necessary if the substance is a potential PBT or vPvB substance on the basis of other data (the relevant PBT test strategy should be followed first). If such properties were confirmed, then further animal testing would be unnecessary since long-term effects can be anticipated.

- The exposure assessment may need to be refined if risks are initially identified for the aquatic or terrestrial environments. This may include the recommendation of improved risk management measures.

- A test with birds can await the outcome of any further chronic mammalian testing proposed for the human health assessment (unless it is already suspected that birds may be more sensitive, e.g. because of evidence from analogue substances).

Three main cases can be distinguished where further testing may be an option:

- **Only acute avian toxicity data are available**. A decision on the need for further chronic testing will depend on the outcome of the risk assessment using a PNEC based on these data, in comparison to the conclusions for mammalian predators. For example, if a risk is identified for birds but not mammals, a chronic test will allow the $PNEC_{bird}$ to be refined.

- **Only a poor quality chronic study is available**. If the risk is borderline (e.g. the PEC is only just greater or less than the resulting PNEC), a replacement study might be necessary to provide more confidence in the conclusion.

- **No avian toxicity data are available**. A decision must be made as to whether this represents a significant data gap or not. It is assumed that a risk characterisation based on the available mammalian toxicity data set will give an indication of the possible risks of the substance to higher organisms in the environment (care should be taken to consider any effects that have been excluded as irrelevant for human health). However, given the lack of information on relative sensitivities between birds and mammals, avian testing may be required if:

  - the substance has a potential for contaminating food chains – for example, because it is not readily biodegradable and is accumulative (e.g. fish BCF above 100, or other indications of bioaccumulation from mammalian tests such as low metabolic rate, high affinity for fat tissues, long period to reach a plateau concentration in tissues, or slow elimination rate), and

  - there is evidence of toxicity in mammalian repeat dose or reproduction tests.
    As a toxicity testing trigger *only*, it is suggested that the $PNEC_{mammal}$ is reduced by a factor of 10 to derive a *screening* $PNEC_{bird}$: if the subsequent risk characterisation ratio is above 1, and the exposure assessment cannot be refined further, then avian toxicity data should be sought (see Section R.7.10.21.3).

In all cases before a new toxicity test is performed, efforts should first be made to refine the PEC (including consideration of risk management measures) because the exposure scenario is based on a number of conservative assumptions. If avian testing is necessary, a limit test might be appropriate.

R.7.10.21.3 Testing strategy for avian toxicity

This assumes that chronic avian toxicity needs to be addressed. If no suitable analogue data exist (which will often be the case), or there is some doubt about the validity of the read-across, further testing is required on the substance itself. This may also be the case if the substance is part of a larger category for which avian toxicity data are limited (in which case it might be possible to develop a strategy to provide data on several related substances, based on a single (or few) test(s). The substance that appears the most toxic to mammals and fish should be selected for further testing with birds in the first instance).

The avian reproduction test (OECD TG 206) should be conducted to provide a reliable chronic NOEC. It may be possible to conduct a limit test (based on the highest PEC multiplied by 30): if no effects are observed at this limit concentration then no further investigation is necessary. A judgment will be needed as to whether this approach is likely to offer any disadvantage compared to a full test (e.g. the substance may be part of a category, where further information on dose-response may be needed). Exceptions to this test may be as follows:

- In some cases, it might be appropriate to conduct an acute test to provide a preliminary indication of avian toxicity. For example, this could be useful if several related substances have no avian toxicity data, and some comparative data are needed to test the appropriateness of a read-across argument when only one is subject to a reproduction test. This could be a limit test in the first instance, since it is not necessary to establish a full dose-response relationship. A tentative $PNEC_{oral}$ can be derived from the result of a dietary test (OECD TG 205), in which case the limit could be either 5,000 mg/kg diet or the highest PEC multiplied by 3,000 (whichever is the lowest). However, given the uncertainties in extrapolating from acute to chronic effects, a chronic test will usually be preferred.

- If the substance clearly shows an endocrine disrupting effect in mammals with a high potency (i.e. acting at doses well below the threshold for other endpoints), it may be appropriate to conduct a multi-generation test instead. Since the protocols for such tests have not been internationally agreed, these would need to be discussed with the relevant regulatory bodies before embarking on a study. In addition, it is likely that such substances would be authorised and so the sacrifice or more vertebrates might not be justified.

It should be noted that this scheme does not include requirements to collect field data. This should only be considered in exceptional circumstances.

The ITS is presented as a flow chart in **Error! Reference source not found.**.

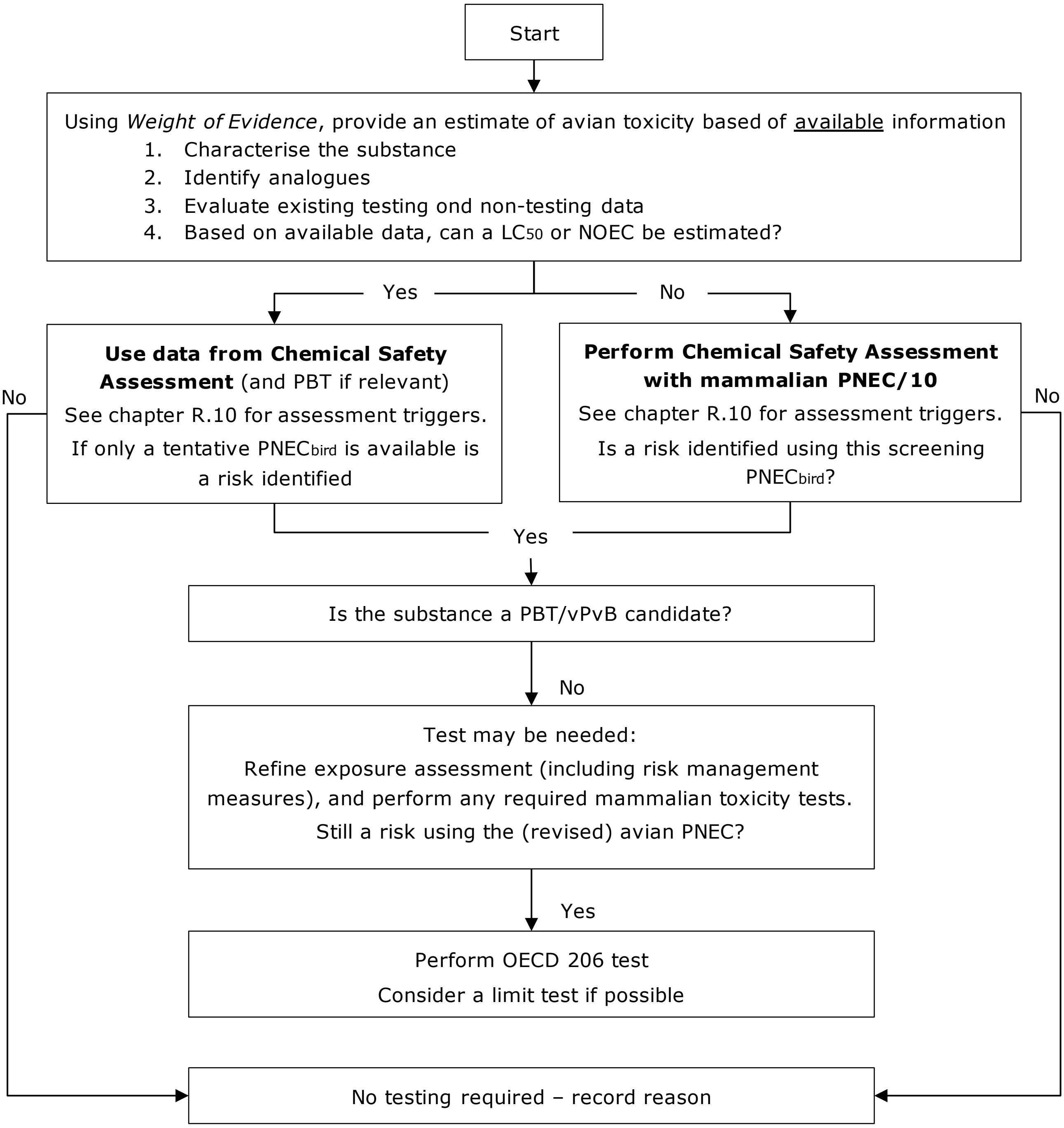

Figure R.7.10—2 ITS for avian toxicity[17]

[17] In the figure the reference to Chapter R10 corresponds to Section R.7.10.8 on secondary poisoning

R.7.10.22 References for avian toxicity

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Appendices to Section R.7.10

Appendix R.7.10-1	Databases
Appendix R.7.10 2:	In vitro methods for aquatic bioaccumulation (Deleted)
Appendix R.7.10-3	Considerations for difficult substances
Appendix R.7.10-4	Quality criteria for data reliability of a (flow-through) fish bioaccumulation study (Deleted)

Appendix R.7.10-1 Databases

DISCLAIMER: this section does not include the latest information on available databases.

Several BCF databases are available and the most widely used are described in this appendix (see Weisbrod *et al.* (2006) for additional details. Many of the earlier studies recorded in databases suffer from a number of potentially serious flaws, which are gradually being better understood. For example, the methodology may not always be consistent with the current OECD 305 test guideline. It is therefore important that the version of the database being interrogated is recorded, because the content may change over time. For example, following a quality control of the Syracuse database, a number of values were amended or removed. In a number of cases, the data quality might not have been checked, and in these circumstances the original source should also be sought so that the quality can be confirmed.

AQUIRE / ECOTOX Database

A very well known and widely used database is the AQUatic toxicity Information REtrieval (AQUIRE) (US-EPA, 1995) system, which is a part of the United States Environmental Protection Agency's ECOTOX Database (US-EPA ECOTOX Database). In 2005 more than 480,000 test records, covering 6,000 aquatic and terrestrial species and 10,000 chemicals, were included. The primary source of ECOTOX data is the peer-reviewed literature, with test results identified through comprehensive searches of the open literature. The bioconcentration factor sub-file includes 13,356 aquatic chemical records and 19 terrestrial chemical records, collected from over 1,100 publications, and encompassing approximately 700 distinct chemicals. The use of the on-line database is free and can be accessed through the Internet at http://cfpub.epa.gov/ecotox/.

Japan METI – NITE Database

The METI database is a collection of around 800 BCF values collected by the Japanese National Institute of Technology and Evaluation (NITE). The database collects bioconcentration values obtained according to the OECD TG 305C method (older data) as well as the more recent version of the OECD TG. The test fish (carp) is exposed to two concentrations of the test chemical substance in water under flow-through conditions. All tests are conducted by Good Laboratory Practice (GLP) laboratories and their test results are reviewed by the joint council of 3 ministries (METI: Ministry of Economy, Trade and Industry; MHLW: Ministry of Health, Labour and Welfare; MoE: Ministry of the Environment). The BCF data on about 800 existing chemicals are available at the Chemical Risk Information Platform (CHRIP) of the NITE's web site (http://www.nite.go.jp/en/chem/index.html). Maximum and minimum BCFs at two different exposure concentrations for the test species (Carp, *Cyprinus carpio*) are reported. The duration of exposure and exposure method (usually flow through) and lipid content are usually provided and occasionally the analytical method (e.g. gas chromatography) is included. However, it has to be highlighted that earlier studies were not conducted in accordance with the current OECD TG 305 method. Some used high levels of solvents/dispersants (which may give unreliable BCF values) and others were conducted far in excess of the test substance's water solubility limit (which may produce an underestimate of the BCF value).

US National Library of Medicine's Hazardous Substances Database

The Hazardous Substances Database (HSDB) is a toxicology database on the National Library of Medicine's (NLM) Toxicology Data Network (TOXNET®). HSDB focuses on the toxicology of potentially hazardous chemicals. It includes over 4800 chemical records. All data are referenced and peer-reviewed by a Scientific Review Panel composed of expert toxicologists and other scientists (U.S. NLM 1999). Although the data are primary source referenced there is little information about the details of the experiments used o measure BCF. The Hazardous Substances Database is accessible, free of charge, via TOXNET at: http://toxnet.nlm.nih.gov.

Environmental Fate Database

The Environmental Fate Database (EFDB) database (Howard *et al.*, 1982, Howard *et al.*, 1986) was developed by the Syracuse Research Corporation (SRC) under the sponsorship of the US-EPA. This computerised database includes several interconnected files, DATALOG, CHEMFATE, BIOLOG, and BIODEG. DATALOG is the largest file and it contains over 325,000 records on over 16,000 chemicals derived from the literature. The bioaccumulation and bioconcentration information is available only for a small fraction of the chemicals in the database. The database does not differentiate between BCF values that are derived experimentally based on testing the substance in question in a bioconcentration test or mathematically without such testing. A large number of reported BCF data is based on calculated values. The database can be accessed via the Internet at http://www.srcinc.com/what-we-do/efdb.aspx and is free of charge.

Syracuse BCFWIN Database and BCFBAF Database

The Syracuse BCFWIN database was developed by Meylan and co-workers to support the BCFWIN program (Syracuse Research Corporation, Bioconcentration Factor Program BCFWIN). The database development is described in Meylan *et al.* (1999). Experimental details captured in the database included fish species, exposure concentration of test compound, percent lipid of the test organism, test method (equilibrium exposure *versus* kinetic method), test duration if equilibrium method, and tissue analysed for test compound (whole body, muscle fillet, or edible tissue). Data obtained by the kinetic method were preferred to data from the equilibrium method, especially for compounds with high log K_{ow} values, which are less likely to have reached equilibrium in standard tests. Where BCF data were derived from the equilibrium method, and steady state may not have been reached, especially for chemicals with high log K_{ow} values, the data chosen was in the middle of the range of values with the longest exposure times. Low exposure concentrations of test compound were favoured in order to minimise the potential for toxic effects and maximise the likelihood that the total concentration of the substance in water was equivalent to the bioavailable fraction. Warm-water fish were preferred to cold-water fish because more data were available for warm-water species. Fish species were preferred in the order fathead minnow > goldfish > sunfish > carp > marine species (this list is not all inclusive). Fathead minnow data were generally selected over data from other species because such data were available for a large number of chemicals, and because they have been used to develop log K_{ow}-based BCF estimation methods. The database contains 694 discrete compounds. BCFWIN database was updated (Stewart *et al.*, 2005) to improve prediction for hydrocarbons. The current BCFWIN hydrocarbons database contains BCF data on 83 hydrocarbons.

The BCFWIN™ model has now been updated and replaced by the BCFBAF™ model. The model is available from the US EPA website https://www.epa.gov/tsca-screening-tools/epi-suitetm-estimation-program-interface

BCFBAF™ estimates fish bioconcentration factors and its logarithm using two different methods. The first is the traditional regression based on log KOW plus any applicable correction factors, and is analogous to the WSKOWWIN™ method. The second is the Arnot-Gobas method, which calculates BCF from mechanistic first principles. BCFBAF also incorporates prediction of apparent metabolism half-life in fish, and estimates BCF and BAF for three trophic levels (Arnot and Gobas, 2003).

Handbook of Physico-chemical Properties and Environmental Fate

The Handbook of Physico-chemical Properties and Environmental Fate (Mackay *et al.*, 2000), published by CRC, consists of several volumes, each covering a set of related organic chemical substances. It is available in book form and in a CD ROM format. The database provided in the book includes data on bioconcentration factors, octanol-water partition coefficient and several other physical chemical properties relevant for environmental fate assessments. Details about the BCF data have not been retrieved.

Canadian database

Environment Canada has developed an empirical database of bioconcentration factor (BCF) and bioaccumulation factor (BAF) values to assess the bioaccumulation potential of approximately 11,700 organic chemicals included on Canada's Domestic Substances List (DSL) as promulgated by The Canadian Environmental Protection Act 1999 (Government of Canada, 1999). These data were collected for non-mammalian aquatic organisms, i.e. algae, invertebrates and fish, from approximately October 1999 until October 2005. The BCF data were compiled from a Canadian in-house database, the peer-reviewed literature and the above mentioned databases. Dietary feeding studies were not included in the data compilation. Values were compiled only if the test chemical and test organism could clearly be identified. BCF data were evaluated for quality according to a developed set of criteria based on standard test protocols (e.g. OECD TG 305E). The database includes approximately 5,200 BCF and 1,300 BAF values for approximately 800 and 110 chemicals, respectively. A data confidence evaluation is included based on the data quality criteria and methods. The database is available on request through the Environment Canada-Existing Substances branch.

CEFIC – LRI bio-concentration factor (BCF) Gold Standard Database

A research project has been funded by the CEFIC-LRI (www.cefic-lri.org/) to establish a BCF Gold Standard Database. The development of a database holding peer reviewed high quality BCF is considered a valuable resource for future development of alternative tests. In addition, having such a database – into which new data points could also be added – would considerably ease the potential to develop and begin the process for validation of alternative BCF studies. For example the database could act as a validation set of chemicals, for alternatives. The project will develop quality criteria, gather fish bioconcentration data, and critically review them. To prevent duplication of work, close contacts are held with other related projects, the HESI-ILSI bioaccumulation group, the SETAC advisory group and other interested parties.

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Appendix R.7.10-2 *In vitro* methods for aquatic bioaccumulation (Deleted)

In vitro methods for aquatic bioaccumulation is addressed in the OECD test guidelines 319 A and 319 B. Therefore the Appendix R.7.10-2 has been deleted.

Appendix R.7.10-3 Considerations for difficult substances

The estimation methods for aquatic bioaccumulation presented in Section R.7.10.3.2 were generally derived for non-ionised organic substances. They are therefore of limited usefulness for a large number of other substances, including complex mixtures and substances that are charged at environmental pH (such as inorganic compounds). These may be collectively termed *difficult substances*, and this appendix provides guidance on their assessment.

Inorganic substances

The availability of inorganic substances for uptake may vary depending on factors such as pH, hardness, temperature and redox conditions, all of which may affect speciation. BCF values will therefore be influenced by water chemistry. In general, only dissolved ions are potentially available for direct uptake.

Whilst some organo-metallic substances (e.g. methyl-mercury) behave like non-polar organics and are taken up across cell membranes by passive diffusion, the uptake of many types of dissolved inorganic ions (particularly metals) largely depend on the presence of specific active transport systems (e.g. copper ATPases regulate the uptake and excretion of copper in cells, and occur in a wide range of species from bacteria to humans (Peña *et al.*, 1999; Rae *et al.*, 1999). These systems are regulated by saturable kinetics, and the degree of uptake of a particular ion will also be strongly influenced by ligand binding and competitive interactions at the receptor site (e.g. Campbell, 1995; Mason and Jenkins, 1995). Once in the organism, the internal ion concentration may be maintained through a combination of active regulation and storage, which generally involves proteins or specific tissues rather than lipid (Adams, *et al.*, 2000; McGeer, *et al.*, 2003). Such homeostatic mechanisms allow the maintenance of total body levels of substances such as essential metals within certain limits over a range of varying external concentrations.

As a result of these processes, organisms may actively accumulate some inorganic substances to meet their metabolic requirements if environmental concentrations are low (leading to a high BCF). At higher concentrations, organisms with active regulation mechanisms may even limit their intake and increase elimination and/or storage of excess substance (leading to lower BCFs). There may therefore be an inverse relationship within a certain exposure concentration interval between exposure concentration and BCF value (McGeer, *et al.*, 2003). Active body burden regulation has been shown to occur in many aquatic species. Other species will, however, tend to accumulate metals and store these in detoxified forms (e.g. calcium or phosphate based granules, metallothionein-like protein binding, etc.), thereby homeostatically regulating the toxic body burdens (Rainbow, 2002; Giguère *et al.*, 2003). It must be recognised[18] however that in some cases the homeostatic regulation capacity may be exceeded at a given external concentration beyond which the substance will accumulate and become toxic. The relationship between accumulation and toxic effects for inorganic substances

[18] For some metals evidence indicates variation in BCF of around one order of magnitude when the water concentration varies over three orders of magnitude. The highest BCF values occur at the lowest exposure concentrations and generally BCF values at environmentally realistic concentrations should be used.

is complex, but is determined by the relative balance between the rates of uptake and depuration/detoxification (Rainbow, 2002).

The observed variability in bioaccumulation and bioconcentration data due to speciation and especially homeostatic regulation can therefore complicate the evaluation of data (Adams and Chapman, 2006). The data may be used for assessments of secondary poisoning and human dietary exposure. However, special guidance is required for classification of metals and inorganic substances are currently outside the scope of PBT assessments.

The octanol-water partition coefficient (K_{ow}) is not a useful predictive tool to assess the bioaccumulation potential for inorganic substances. Some indication may be given by read-across of bioaccumulation and toxicokinetic information from similar elements or chemical species of the same element. Factors such as ionic size, metabolism, oxidation state, etc., should be taken into account if sufficient data exist. This may limit the potential for read-across between different chemical species.

The OECD TG 305 is generally appropriate for determining a fish BCF, provided that the exposures are carried out under relevant environmental conditions and concentrations. Experimental bioaccumulation data should be assessed carefully on a case-by-case basis, paying particular attention to the dissolved exposure concentration. Based on the assessment of available data using expert judgement, there are two possibilities:

- A case may be made that the substance is unlikely to pose a risk to predatory organisms or humans exposed via the environment either:
  - based on the absence of food web biomagnification and information showing that organisms in higher trophic levels are not more sensitive than those in lower trophic levels after long-term exposure, or
  - because it is an essential element and internal concentrations will be well-regulated at the exposure concentrations anticipated.

Any such claims should be made on a case-by-case basis and substantiated with evidence (e.g. from field studies). It should be remembered that while a substance may be essential for a particular organism, it might not be essential for others.

- In the absence of the information mentioned above, bioconcentration factors for fish and other aquatic organisms are derived from the available data and taken into account in the CSA in the usual way. In the absence of suitable data, new studies must be performed. Considering the issues discussed above, an approach that allows the straightforward interpretation of BCF/BAF values has not been developed yet. Biomagnification factors may be more useful, although care must be taken in assessing trophic transfer potential. For example, the bioavailability of an inorganic substance to a bird or mammal may vary from that in aquatic species because of differences in detoxification mechanisms and digestive physiology, and this should be taken into account. Information may be obtained from field studies, although data may also be obtained from aquatic or terrestrial laboratory food chain transfer experiments.

Complex mixtures (including petroleum substances)

Complex mixtures pose a special challenge to bioaccumulation assessment, because of the range of individual substances that may be present, and the variation in their physico-chemical and toxicological properties. It is generally not recommended to estimate an average or weighted BCF value because:

- the composition of the constituents in the aqueous phase may vary in a non-linear fashion with substance loading rate, so that the BCF will also vary as a function of loading;
- differences in analytical methods used to quantify the total substance may introduce significant uncertainties in interpreting results; and
- this approach fails to identify specific constituents that could exhibit a much higher bioconcentration potential than the overall mixture.

In principle, therefore, it is preferable to identify one or more constituents for further consideration that can be considered representative of other constituents in the mixture in terms of bioaccumulation potential (acting as a worst case in terms of read-across between the constituents – see Section R.7.10.3.2 in the main text for further guidance). This could include the establishment of *blocks* of related constituents (e.g. for hydrocarbon mixtures). The BCF would be established for each selected constituent in the usual way (whether by prediction or measurement), and these data can then be used to evaluate the likely range of BCF values for the constituents of a given mixture. The OECD TG 305 method should be used if possible (i.e. provided that the constituents can be monitored for separately). Alternatively, the *Hyalella azteca* bioconcentration test can be applied which allows to apply water accommodated fractions of complex mixtures in a scaled down test system which is much smaller compared to the fish flow-through test. If a further confirmatory step is needed following the bioconcentration test, the most highly bioaccumulative constituent(s) should be selected for further bioaccumulation testing (assuming this can be extracted or synthesised).

It should be noted that branching or alkyl substitution sometimes enhances bioconcentration potential (e.g. due to a reduction in the biotransformation rate and/or an increase in the uptake clearance). Care should be taken to consider such factors when choosing a representative constituent. A form of *sensitivity analysis* may be useful in confirming the selection of constituents to represent a particular complex mixture. The logic/relevance behind selection of certain constituents for further testing may also depend on regulatory needs (e.g. for hazard classification the particular % cut off values for classification).

If it is not possible to identify representative constituents, then only a broad indication of bioaccumulation potential can be obtained. For example, it might be possible to derive a range of K_{ow} values from a HPLC method, or a biomimetic approach could be used (based on measurement of total organic carbon). If a potential concern is triggered for bioaccumulation potential, expert advice will be needed to refine the results.

Ionisable substances

In general, ionised organic substances do not readily diffuse across respiratory surfaces, although other processes may play a role in uptake (e.g. complex permeation, carrier-mediated processes, ion channels, or ATPases). Dissociated and neutral chemical species can therefore have markedly different bioavailabilities. It is therefore essential to know or estimate the pKa to evaluate the degree of ionisation in surface waters at enviromentally relevant pH (pH 4-9) and under physiological conditions (pH 3-9) (see Section R.7.1. of the *Guidance on IR&CSA* for further details of the pKa and how to predict log K_{ow} at different pH).

Escher *et al.* (2002) showed that the K_{ow} is not always a good indicator of biological membrane-water partitioning for ionised organic substances when there is reactivity with cell constituents. Armitage *et al.* (2017) summarised that aspects of the bioaccumulation potential of ionisable substances in fish that can be characterised relatively well include the pH dependence of gill uptake and elimination, uptake in the gut, and sorption to phospholipids (membrane–water partitioning). Key challenges include the limited empirical data for biotransformation and binding in plasma where fish possess a diverse array of proteins that may transport ionised substances across cell membranes. Furthermore, the general phenomenon known as the "ion trap" effect due to the large pH gradient between lysosomes and cytoplasm may result in the preferential concentration of the charged form in the lysosomal compartment, with differences of about 2-3 orders of magnitude, compared to the cytosol.

It can be concluded that assumptions about the bioaccumulation behaviour of ionised substances may lead to underestimates of the BCF. Where this is likely to be a significant issue in an assessment, a bioconcentration test with fish (or a suitable alternative assay where sufficient evidence for justification is provided) will likely be needed. This should preferably be carried out at an ecologically relevant pH at which the substance is at its most hydrophobic form (i.e. non-ionised, as either the free acid or free base) using an appropriate buffer (e.g. this would correspond to a pH below its pKa for an acid and above its pKa for a base).

However, prior to *in vivo* data generation, an argument for a tiered modelling approach (such as that outlined by Armitage *et al.* (2013, 2017) and using models such as BIONIC[19] therein described), supported by suitable and sufficient input values, may be appropriate to support an assessment of bioaccumulation if sufficient evidence of applicability and suitable justification can be provided. For ionisable compounds, OECD TG 319 may apply, however, the currently available in vitro-in vivo extrapolation models may not always apply to all (types of) ionisable substances and adaption may be needed (Regnery *et al.*, 2022).

There is continuous work and development on understanding the partitioning and bioaccumulation mechanisms of ionisable substances. This includes identification of parameters to predict bioaccumulation potential of such substances, similar to the log K_{ow} which is used to predict bioaccumulation potential of neutral organic substances when it is solely driven by the hydrophobicity (Rendal, 2013; Guidance document on

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[19] Accessible under https://arnotresearch.com/bionic/; last accessed: October 2022

aspects of OECD TG 305 (OECD, 2017), Armitage *et al.*, 2017; Droge *et al.*, 2021a; Kierkegaard *et al.*, 2021; Ribbenstedt *et al.*, 2022).

Based on the current knowledge there are two scenarios when bioaccumulation potential of an ionisable substance could be predicted on the basis of log Kow of the neutral form if properly justified (for instance, in line with requirements of Column 2, Section 9.3.2. of REACH Annex IX and/or under weight-of-evidence requirements of Annex XIII). First, when the extent of ionisation is always below 90% at pH 4-9. In this case, models that consider only the hydrophobicity of the neutral form may be sufficient to describe bioaccumulation (for instance, low potential for bioaccumulation could be predicted if log K_{ow} of the neutral form of such substance is ≤ 3). Second, when it can be justified that the charge is highly delocalised on the molecule. Similarly to the former case, the log K_{ow} of the neutral form may be used to predict the potential for bioaccumulation for such substances (Rendal, 2013). However, these two scenarios are not applicable to permanently charged substances and to ionised surface active substances (see section on Surface active substances (surfactants) below).

Data from fish feeding studies examined in the work of Arnot and Quinn (2015) indicate that ionic organic substances do not necessarily show a lower uptake from dietary ingestion than neutral compounds with similar properties, and the charge may have no decisive influence on the intake in the gastrointestinal tract (GIT) (Armitage *et al.* 2017). Due to the lower membrane permeability of ionic organic substances and the higher transepithelial resistance of the gills compared to the GIT, it is likely that ionic organic substances are better received via the GIT. The associated greater permeability of the GIT and the longer residence time in the GIT support this assumption. The bioaccumulation potential of selected ionic organic substances was evaluated in a dietary uptake study carried out according to OECD TG 305 combined with organ-specifc analysis (Mueller *et al.* 2020). The suspected dietary bioaccumulation potential of the selected ionic organic substances could not be confirmed in the feeding studies with rainbow trout. The results corroborate earlier findings that ionisation lowers the tendency of a chemical for dietary bioaccumulation, compared to non-ionised chemicals. In addition to the lipophobicity of ionic molecule moieties, fast depuration seems to be a major reason for the observed low dietary bioaccumulation of ionic compounds, in particular anions. Fast depuration may happen due to rapid metabolism of charged compounds which needs to be further elucidated for instance by determination of in vitro intrinsic clearance using cryopreserved rainbow trout hepatocytes or rainbow trout liver S9 sub-cellular fraction (RT-S9) (OECD, 2018a,b).

The following information may be used in a weight-of-evidence approach to justify that the ionisable substance has a low potential for bioaccumulation:

- Information on the toxicokinetics in aquatic organisms (as for any other substance type).
- Fish-water partitioning coefficient which addresses partitioning to lipids, phospholipids and proteins (UBA 2021).
- Membrane lipid -water partition/distribution coefficient (K_{MLW}/D_{MLW}) for ionisable surfactants (Droge *et al.*, 2021b) (see section on Surface active substances (surfactants) below).

Surface active substances (surfactants)

A substance is *surface active* when it is enriched at the interface of a solution with adjacent phases (e.g. air) and when it lowers the surface tension of the medium/phase in which it is dissolved. In general, surfactants consist of an apolar and a polar moiety, which are commonly referred to as the hydrophobic tail and the hydrophilic headgroup, respectively. According to the charge of the headgroup, surfactants can be categorised as anionic, cationic, non-ionic or amphoteric (Tolls and Sijm, 2000). This structural diversity means that bioaccumulation potential should be considered in relation to these subcategories rather than the group as a whole (see Tolls *et al.* (1994) for a critical review).

It is well established that BCFs for neutral organic chemicals are positively correlated with the $K_{ow.}$ However, K_{ow} is not a reliable parameter for predicting the BCFs of surfactants. Due to their amphiphilic properties, surfactants form aggregates in solution and have a tendency to accumulate at the interface of hydrophobic and hydrophilic phases. Surfactants can also emulsify the n-octanol/water system, making the measurement of $\log K_{ow}$ technically extremely challenging (Hodges *et al.*, 2019). See Section R.7.1 of the *Guidance on IR&CSA* for further details of how the K_{ow} can be measured or estimated.

Log K_{ow} determination is further complicated by the fact that surfactants may form micelles in water (i.e. not dissolving exclusively as single molecules), so their 'solubility' cannot be properly defined and is hard to measure. The maximum monomolecular solubility is defined as the critical micelle concentration (CMC), with formation of micelles occurring above this concentration. Although CMC is a commonly used surrogate for water solubility, CMC is not an appropriate solubility threshold, as micelles themselves are water-soluble (Hodges *et al.*, 2019). This can cause data interpretation problems for fish BCF tests, since the actual dissolved concentration of surfactant that the fish were exposed to may be uncertain.

Indicators of bioaccumulation potential of surfactants

Instead of log K_{ow}, other properties such as the length of the alkyl chains (Kierkegaard *et al.*, 2021) and the number of oxyethylene units are thought to be more indicative of uptake and bioaccumulation potential (Schlechtriem *et al.*, 2015). Other measures of hydrophobicity such as the critical micelle concentration (CMC) might be more appropriate in some cases (Roberts and Marshall, 1995; Tolls and Sijm, 1995). However, recent work shows that there is no simple general linear relationship between BCF and CMC of surfactants (Schlechtriem *et al.*, 2015), so its use requires sufficient evidence of applicability and suitable justification.

Due to their amphiphilic nature, the distribution and accumulation of surfactants in the organism depends on their interaction with biological interfaces such as membrane phospholipids, where they tend to absorb (Kierkegaard *et al.*, 2021; Schlechtriem *et al.*, 2015). Measured membrane lipid-water partitioning/distribution ratios, K_{MLW}/D_{MLW} (or K_{mw}), could thus be suitable as a first step to predict the bioaccumulation potential of surfactants. (Droge, *et al.*, 2021b). The phospholipid fraction of total fat in the whole body of fish is estimated to be approximately 25% (Armitage et al, 2013).

Ionised surfactants show low affinity for octanol (i.e. neutral storage lipids) and higher affinity for membrane phospholipids due to favourable electrostatic interactions with zwitterionic head groups (Droge, 2019; Droge *et al.*, 2021b; Kierkegaard *et al.*, 2021; Ribbenstedt *et al.*, 2022). Membrane (phospho)lipids are the driving component of the sorption of ionised surfactants to tissues (Droge *et al.*, 2021b). Ionised forms of organic molecules in general show much slower membrane permeation (by passive diffusion) than their neutral counterparts and their uptake is pH-dependent, in some cases leading to ion trapping (Escher *et al.*, 2020; Ribbenstedt *et al.*, 2022).

There is currently no standardised test guideline for the experimental determination of K_{MLW}/D_{MLW}. The three most commonly employed experimental methods are: 1) dissolved unilamellar liposomes, 2) lipid bilayers non-covalently coated on microporous silica and 3) covalently linked phospholipid monolayers on HPLC grade silica (Droge *et al.*, 2021b). For some strongly sorbing surfactants D_{MLW} may be difficult to derive experimentally (Timmer and Droge, 2017).

K_{MLW}/D_{MLW} can also be predicted using both atomistic (Yordanova *et al.*, 2017) and coarse-grained molecular dynamics simulation methods (Potter *et al.*, 2021). Commercial software packages can also predict the K_{MLW}/D_{MLW} (Droge, 2019; Klamt *et al.*, 2008).

Perfluoroalkyl surfactants have a specific affinity for certain proteins (e.g., serum albumin, human pregnane X receptor), can interact with membrane transporters and have a low biotransformation rate (Lai *et al.*, 2020, Droge, 2019; Droge *et al.*, 2021b). This results in higher bioaccumulation than anticipated by prediction from $\log K_{ow}$ or $\log K_{MLW}/\log D_{MLW}$ alone (Droge, 2019; Droge *et al.*, 2021b; Schlechtriem *et al.*, 2015). Therefore, BCFs estimated on the basis of K_{MLW}/D_{MLW} predictions alone for perfluoroalkyl surfactants must be treated with caution.

Droge *et al.*, (2021b) showed that a BCF can be estimated for ionic surfactants by multiplying D_{MLW} by the phospholipid fraction in tissue, and for non-ionic surfactants by multiplying D_{MLW} by the total lipid fraction. This simple correlation can be useful for screening purposes but not for definitive BCF determination since it does not consider biotransformation or binding to protein or muscle. Many straight alkyl chain surfactants are readily metabolised in fish (Tolls and Sijm, 1999; Tolls and Sijm , 2000; Comber *et al.*, 2003; Droge, *et al.*, 2021a; Dyer *et al.*, 2009) and so these regression models can overestimate the BCFs. A few models such as BIONIC (Armitage *et al*, 2013) are now available which can be applied to surfactants. Although there are some limitations, this model has the ability to integrate some of this additional information (e.g. biotransformation rates) into a refined in silico assessment of BCF. These can be used as part of a tiered modelling approach, if sufficient evidence of applicability and suitable justification can be provided. The following information may be used in a weight-of-evidence approach to justify that the surface active substance has a low potential for bioaccumulation:

- Information on the toxicokinetics in aquatic organisms (as for any other substance type), applied as part of modelling approach such as that outlined by Armitage *et al.* (2013, 2017).
- Membrane lipid-water partition/distribution coefficient (K_{MLW}/D_{MLW}) (Droge *et al.*, 2021b).

Where a low potential for bioaccumulation cannot be sufficiently demonstrated, an experimental study with aquatic organisms should be considered as a last resort. It should be considered whether an aqueous exposure test is feasible or whether a dietary study is more appropriate (OECD, 2017) and if invertebrate tests such as the Hyalella bioconcentration test could be performed instead of fish. Generally, the use of the kinetic approach for PFASs bioaccumulation was critically discussed by Liu *et al.*, (2011). They criticise the fact that the previous assumption of "first order uptake reaction" is inappropriate in the case of PFAS bioaccumulation which appears to follow an adsorption rather than a partitioning model.

An additional factor to consider is that commercial surfactants tend to be mixtures of chain lengths, each with its own BCF (Tolls, *et al.*, 1997 and 2000). The guidance for complex mixtures (see R.7.13.2 and R.11.14.2.2 of Chapter R.11 of the *Guidance on IR&CSA*) is therefore also applicable for commercial surfactants. If tests are needed it is recommended that they should be done with a single chain length where possible.

Organic substances that do not partition to lipid

Bioconcentration is generally considered as a partitioning process between water and lipid, and other distribution compartments in the organism can usually be neglected (the water fraction may play a role for water-soluble substances (de Wolf *et al.*, 1994)). However, proteins have been postulated as a third distribution compartment contributing to bioconcentration (SCHER, 2005), and may be important for certain types of substances (e.g. perfluorosulphonates, organometallic compounds such as alkyl- or glutathione-compounds, for instance methyl mercury, methyl arsenic, etc.). Evidence for such a role may be available from mammalian toxicokinetics studies.

Protein binding in biological systems performs a number of functions (e.g. receptor binding to activate and/or provoke an effect; binding for a catalytical reaction with enzymes; binding to carrier-proteins to make transport possible; binding to obtain/sustain high local concentrations above water solubility, such as oxygen binding to haemoglobin, etc.). In some circumstances, binding may lead to much higher local concentrations of the ligand than in the surrounding environment.

Nevertheless, the picture may be complicated because the process is not necessarily driven purely by partitioning (binding sites may become saturated and binding could be either reversible or irreversible). Indeed, it has been postulated that measured BCFs may be concentration dependant due to protein binding (SCHER, 2005). In other words, bioconcentration is limited by the number of protein binding sites rather than by lipid solubility and partitioning. Further work is needed to conceptualise how protein binding might give rise to food chain transfer across trophic levels, and assess its relative contribution compared with other (lipids and water) distribution mechanisms.

In the absence of such studies, elimination studies can be useful for comparing half-lives of substances that may accumulate via proteins with those for other substances that are known to be bioaccumulative.

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Appendix R.7.10-4 Quality criteria for data reliability of a (flow-through) fish bioaccumulation study (Deleted)

OECD test guideline 305 I, II and III gives clear instructions on how a fish bioaccumulation study should be conducted. Therefore the information in this appendix has been deleted.

R.7.11 Effects on terrestrial organisms

R.7.11.1 Introduction

Substances introduced into the environment may pose a hazard to terrestrial organisms and as such potentially have deleterious effects on ecological processes within natural and anthropogenic ecosystems. Due to the complexity and diversity of the terrestrial environment, a comprehensive effect assessment for the whole compartment can only be achieved by a set of assessment endpoints covering (i) the different routes by which terrestrial organisms may be exposed to substances (i.e. air, food, pore water, bulk-soil) and (ii) the most relevant taxonomic and functional groups of terrestrial organisms (micro-organism, plants, invertebrates, vertebrates) being potentially affected (CSTEE, 2000). The scope of the terrestrial effect assessment under the adopted REACH regulation is restricted to soil organisms in a narrow sense, i.e. on non-vertebrate organisms living the majority of their lifetime within the soil and being exposed to substances via the soil pathway and in line with the previous practice in the environmental risk assessment of new and existing substances in the EU. The actual scoping of the effect assessment for the terrestrial environment does not include (EU, 2003):

- terrestrial invertebrates living above-ground (e.g. ground dwelling beetles),
- terrestrial vertebrates living a part of their lifetime in soils (e.g. mice),
- groundwater organism (invertebrates and micro-organism), and
- adverse effects on soil functions that are only indirectly linked to the biota in soils (e.g. buffering capacity, formation of soil structure, water cycle etc.) It should be stressed however that by addressing direct effects on soil biota, potential effects on these soil functions indirectly addressed (see below).

As for terrestrial vertebrates living above-ground reference is made to the relevant sections for mammals (Sections R.7.2 to R.7.7) and birds (Section R.7.10.16).

The importance of assessing the potential adverse effects on soil organisms within the environmental risk assessment of substances is at least two-fold:

First, there is a general concern with regard to the exposure of soil organisms, as soils are a major sink for anthropogenic substances emitted into the environment. This is especially pivotal for persistent substances with an inherent toxic potential, which may accumulate in soils and thereby posing a long-term risk to soil organisms. Second, protection of specific soil organisms is critical due to their role in maintaining soil functions, e.g. the breakdown of organic matter, formation of soil structure and cycling of nutrients. In view of the latter, protection goals for soil can both relate to structure (diversity and structure of soil organisms communities) and functions (ecosystem functions provided by soil organism communities) of soil biota.

Valuable contributions for assessing the effect of a specific substance on soil organisms may be obtained from endpoints such as physico-chemical properties (Section R.7.1) and (bio-) degradation (Section R.7.9) providing information on the fate of the substance. In the absence of experimental data on soil organisms data can be used that were generated on aquatic organisms (Equilibrium Partitioning Method, EPM);

information requirements for aquatic organisms under REACH are addressed in Section R.7.8. However, due to the high level of uncertainty regarding the area of validity of the EPM, this approach should be limited to screening purposes only.

The complexity, heterogeneity and diversity of soil ecosystems are the major challenge when assessing potential adverse effects of substances on soil organisms. This holds true both regarding soil as substrate, and thus exposure medium, and the biota communities living in the soil. Spatial and temporal fluctuations in environmental conditions, i.e. climate increase the complexity of assessing potential effects in soil.

Soil

If considered as an exposure medium soil is characterised by a highly complex, three-phase system consisting of non-organic and dead organic matter, soil pore water and pore space (soil air). Substances released to the soil system are exposed to different physical, chemical and biological processes that may influence their fate (e.g. distribution, sorption/ de-sorption, transformation, binding and breakdown) and as such their bioavailability (see below) and effects on soil organisms. Moreover, structure, texture and biological activity greatly varies between different soil types and sites, respectively and soil properties even may alter due to changing environmental conditions (e.g. changes in organic matter content or amount of soil pores). As a consequence, the comparability of fate and effect data between different soils is limited, making extrapolations cumbersome. Hence, the selection of appropriate soils for biological testing or monitoring procedures is a crucial step when assessing the effects on soil organisms. Furthermore, standardisation of soil effect data to a given soil parameter (e.g. organic matter content or clay content) is common practice.

Soil organisms

Typical soil organism communities in the field are highly diverse regarding their taxonomic composition and structured by complex inter-relationships (e.g. food-webs). Due to the diversity of species, a multitude of potential receptors for adverse effects of toxic substances exist in soils differing in size, soil micro-habitat, physiology and life-history. Consequently, a set of indicators representing three soil organism groups of major ecological importance and covering all relevant soil exposure pathways is required for a comprehensive effect assessment of substances in soils (see Table R.7.11—1).

Table R.7.11—1 Major groups of soil organisms to be considered in effect assessment

Organism group	Ecological process	Soil exposure pathway	Important taxa
Plants	Primary production	Mainly soil pore water (by root uptake)	All higher plants
Invertebrates	Breakdown of organic matter Formation of soil structure	Diverse and multiple uptake routes (soil pore water, ingestion of soil material, soil air, secondary poisoning)	Earthworms, springtails, mites
Micro-organisms	Re-cycling of nutrients	Mainly soil pore water	Bacteria, protozoa, fungi

Soil bioassay

Soil bioassays are at present the most important method to generate empirical information on the toxicity of substances to soil organisms. Such bioassays are conducted by exposing test organisms to increasing concentrations of the test substance in soil, under controlled laboratory conditions. Short-term (e.g. mortality) or long-term (e.g. inhibition of growth or reproduction) toxic effects are measured. Ideally, toxicity testing results reveal information on the concentration-effect relationship and allow for the statistical derivation of defined Effect Concentrations (EC_x, i.e. effective concentration resulting in x % effect) and/ or No Observed Effect Concentrations (NOEC). By convention, EC_x and NOEC values generated by internationally standardised test guidelines (OECD, ISO) offer the most reliable toxicity data. However, only a limited number of standard test guidelines for soil organism are at present available, a fact that mirrors the generally limited data-base on the toxicity of substances towards soil organisms.

Bioavailability

By addressing bioavailability of substances in soil, a potential method to deal with the diversity and complexity of soils is provided. Bioavailability considers the processes of mass transfer and uptake of substances into soil-living organisms which are determined by substance properties (key parameter: water solubility, K_{OC}, vapour pressure), soil properties (with key parameter: clay content, organic matter content, pH-value, cation exchange capacity) and the biology of soil organisms (key parameter: micro-habitat, morphology, physiology, life-span). The practical meaning for effect assessment of both organic substances and metals is the observation that not the total loading rate, but only the bioavailable fraction of a substance in soil is decisive for the observed toxicity. Although being subject to extensive research activities in the past decade, there is actually no general approach for assessing the bioavailability of substances in soils. Major difficulties are the differences and the restricted knowledge about exposure pathways relevant for soil organisms and the fact that bioavailability is time-dependent. The latter phenomenon is commonly described as a process of "ageing" of substances in soil: Due to increasing sorption, binding and incorporation into the soil matrix, bioavailability and consequently toxicity changes (mostly decreases) with time. Additional factors like climate conditions and land use may also influence bioavailability. Nonetheless, bioavailability should be critically considered when interpreting existing soil toxicity data as well as during the design of new studies.

R.7.11.1.1 Objective

The overall objective of the effect assessment scheme proposed in this section is to gather adequate (i.e. reliable and relevant) information on the inherent toxic potential of specific substances to soil living organisms in order to:

- Identify if, and if so, which of the most relevant groups of soil organisms may potentially be adversely affected by a specific substance when emitted into the soil compartment, and to
- Derive a definite, scientifically reliable soil upper threshold concentration of no concern (Predicted No Effect Concentration for soil - PNECsoil) for those substances, for which adverse effects on soil organisms are to be expected.

Based on the information and relevant toxicity data gathered during effect assessment, the derivation of the $PNEC_{soil}$ for a specific substance follows the general hazard assessment schemes as presented in a flow-chart of Section R.7.11.6.3. Comparison of the $PNEC_{soil}$ with the respective Predicted Environmental Concentration expected for soil (PEC_{soil}) from relevant emission scenarios will finally lead to a conclusion concerning the risk to organisms living in the soil compartment (risk characterisation). A risk identified on the basis of a PEC/PNEC comparison can demonstrate the need for a more refined risk-assessment (either on the PEC or PNEC side), or – in cases where there are no options for further refinement - to risk management decisions.

R.7.11.2 Information requirements

R.7.11.2.1 Standard information requirements

Article 10 of REACH presents the information that should be submitted for registration and evaluation of substances. In Article 12 the dependence of the information requirements on production volume (tonnage) is established in a tiered system, reflecting that potential exposure increases with volume.

Annexes VII-X to REACH specify the standard information requirements (presented in column 1). In addition, specific rules for their adaptation (presented in column 2) are included. These annexes set out the standard information requirements, but must be considered in conjunction with Annex XI to REACH, which allows variation from the standard approach. Annex XI to REACH contains general rules for adaptations of the standard information requirements that are established in Annexes VII to X.

Furthermore, generation of data for the PBT/vPvB assessment is required, where a registrant, while carrying out the CSA, cannot draw an unequivocal conclusion on whether the criteria in Annex XIII to REACH are met or not and identifies that terrestrial (soil) toxicity data would take the PBT/vPvB assessment further. This obligation applies for all ≥ 10 tpa registrations (see Chapter R.11 of the *Guidance on IR&CSA* for further details).

The following represent the specific requirements related to terrestrial (soil) toxicity testing:

Information requirements (column 1) and rules for adaptation of the standard information requirements (column 2) of the Annexes VII-X)

a) Annex VII (Registration tonnage >1 t/y -<10 t/y)

No terrestrial effects testing is required at this registration tonnage

b) Annex VIII (Registration tonnage >10 t/y)

No terrestrial effects testing is required at this registration tonnage

c) Annex IX (Registration tonnage >100 t/y)

Column 1 of this Annex establishes the standard information required for all substances manufactured or imported in quantities of 100 tonnes or more in accordance with Article 12 (1) (d).

Column 1 **Standard Information Required**	**Column 2** **Specific rules for adaptation from Column 1**
9.2.3. Identification of degradation products	Unless the substance is readily biodegradable
9.4. Effects on terrestrial organisms	9.4. These studies do not need to be conducted if direct and indirect exposure of the soil compartment is unlikely. In the absence of toxicity data for soil organisms, the equilibrium partitioning method may be applied to assess the hazard to soil organisms. Where the equilibrium partitioning method is applied to nanoforms, this shall be scientifically justified. The choice of the appropriate test(s) shall be made on the basis of the results of the chemical safety assessment. In particular for substances that have a high potential to adsorb to soil or that are very persistent, the registrant shall propose or the Agency may require long-term toxicity testing as referred to in Annex X instead of short-term toxicity testing.
9.4.1. Short-term toxicity to invertebrates	
9.4.2. Effects on soil micro-organisms	
9.4.3. Short-term toxicity to plants	

Identification and/or assessment of degradation products

These data are only required if information on the degradation products following primary degradation is required in order to complete the Chemical Safety Assessment.

Column 2: "Unless the substance is readily degradable"

In these circumstances, it may be considered that any degradation products formed during such degradation would themselves be sufficiently rapidly degraded as not to require further assessment.

Effects on terrestrial organisms

Column 2: "these tests do not need to be conducted if direct and indirect exposure of soil compartment is unlikely."

If there is no exposure of the soil, or the exposure is so low that no refinement of the PEC_{local} or $PEC_{regional}$, or $PNEC_{soil\ organisms}$ is required, then this test may not be necessary. In general, it is assumed that soil exposure will occur unless it can be shown that there is no sludge application to land from exposed STPs and that aerial deposition are negligible and the relevance of other exposure pathways such as irrigation and/or contact with contaminated waste is unlikely.

In the case of readily biodegradable substances which are not directly applied to soil it is generally assumed that the substance will not enter the terrestrial environment and as such there is no need for testing of soil organisms is required. Furthermore, other parameters (e.g. low log K_{oc}/P_{ow}) should be considered regarding the exposure pathway via STP sludge. In case of aerial deposition, other aspects such as photostability, vapour pressure, volatility, hydrolysis etc, should be taken into consideration.

Column 2: "In the absence of toxicity data for soil organisms, the Equilibrium Partitioning Method may be applied to assess the hazard to soil organisms. The choice of the appropriate tests depends on the outcome of the Chemical Safety Assessment."

In the first instance, before new terrestrial effects testing is conducted, a $PNEC_{soil}$ may be calculated from the $PNEC_{water}$ using Equilibrium Partitioning. The results of this comparison can be incorporated into the Chemical Safety Assessment and may help determine which, if any of the terrestrial organisms detailed in the standard information requirements should be tested.

Column 2: "In particular for substances that have a high potential to adsorb to soil or that are very persistent, the registrant shall consider long-term toxicity testing instead of short-term."

Some substances present a particular concern for soil, such as those substances that show a high potential to partition to soil, and hence may reach high concentrations, or those that are persistent. In both cases long-term exposure of terrestrial organisms is possible and the registrant should consider whether the long-term terrestrial effects testing identified in Annex X may be more appropriate. This is addressed in more detail in the integrated testing strategy in Section R.7.11.6.

d) Annex X (Registration tonnage >1000 t/y)

Column 1 of this Annex establishes the standard information required for all substances manufactured or imported in quantities of 1000 tonnes or more in accordance with Article 12(1)(e). Accordingly, the information required in column 1 of this Annex is additional to that required in column 1 of Annex IX.

Column 1 Standard Information Required	Column 2 Specific rules for adaptation from Column 1
9.4. Effects on terrestrial organisms	9.4. Long-term toxicity testing shall be proposed by the registrant or may be required by the Agency if the results of the chemical safety assessment performed in accordance with Annex I indicates that it is needed to further investigate the effects of the substance or of transformation and degradation products on terrestrial organisms. The choice of the appropriate test(s) shall be made on the basis of the outcome of the chemical safety assessment. These studies do not need to be conducted if direct and indirect exposure of the soil compartment is unlikely.
9.4.4. Long-term toxicity testing on invertebrates, unless already provided as part of Annex IX requirements.	
9.4.6. Long-term toxicity testing on plants, unless already provided as part of Annex IX requirements.	

Effects on terrestrial organisms

Column 2: "These tests need not be conducted if direct and indirect exposure of soil compartment is unlikely."

If there is no exposure of the soil, or the exposure is so low that no refinement of the PEC_{local} or $PEC_{regional}$, or $PNEC_{soil\ organisms}$ is required, then this test may not be necessary. In general, it is assumed that soil exposure will occur unless it can be shown that there is no sludge application to land from exposed STPs and that aerial deposition are negligible and the relevance of other exposure pathways such as irrigation and/or contact with contaminated waste is unlikely.

In the case of readily biodegradable substances which are not directly applied to soil it is generally assumed that the substance will not enter the terrestrial environment and as such there is no need for testing of soil organisms is required.

Column 2: "Long-term toxicity testing shall be proposed by the registrant if the results of the chemical safety assessment according to Annex I indicate the need to investigate further the effects of the substance and/or degradation products on soil organisms. The choice of the appropriate test(s) depends on the outcome of the chemical safety assessment"

These tests need not be proposed if there is no risk to the soil compartment identified in the chemical safety assessment such that a revision of the $PNEC_{soil}$ is not required. Where further information on terrestrial organism toxicity is required, either on the substance or on any degradation products, the number and type of testing will be determined by the chemical safety assessment and the extent of the revision to the $PNEC_{soil}$ required.

PBT/vPvB assessment

In the context of PBT/vPvB assessment, if the registrant cannot derive a definitive conclusion (i) ("The substance does not fulfil the PBT and vPvB criteria") or (ii) ("The substance fulfils the PBT or vPvB criteria") in the PBT/vPvB assessment using the relevant available information, he must, based on Section 2.1 of Annex XIII to REACH, generate the necessary information for deriving one of these conclusions, regardless of his tonnage band (for further details, see Chapter R.11 of the *Guidance on IR&CSA*). In such a case, the only possibility to refrain from testing or generating other necessary information is to treat the substance "*as if it is a PBT or vPvB*" (see Chapter R.11 of the *Guidance on IR&CSA* for details).

R.7.11.3 Information and its sources

Different types of information are relevant when assessing terrestrial exposure and subsequent toxicity to soil organisms. Useful information includes chemical and physical properties of substances and test systems as well as available testing data (*in vitro* and *in vivo*) and results from non-testing methods, such as the Equilibrium Partitioning Method. Sources of ecotoxicity data including terrestrial data have been listed in Chapter R3. Additional useful databases include US EPA ECOTOX database (http://cfpub.epa.gov/ecotox/) and OECD Screening Information DataSet (SIDS) for high volume chemicals (http://www.chem.unep.ch/irptc/sids/oecdsids/indexchemic.htm).

Physical and chemical data on the test substance can assist with experimental design and provide information on the endpoint of interest. The following information is useful for designing the soil test and identifying the expected route of exposure to the substance: structural formula, purity, water solubility, n-octanol/water partition coefficient (log K_{ow}), soil sorption behaviour, vapour pressure, chemical stability in water and light and biodegradability.

R.7.11.3.1 Laboratory data

Non-testing data

There is limited terrestrial toxicity data available for most substances. In the absence of terrestrial data, one option is to generate Q(SAR) predictions. General guidance on the use of (Q)SAR is provided in Section R.4.3.2.1 and specifically for aquatic (pelagic) toxicity in Section R.7.8. However at present there are no Q(SAR)s for soil ecotoxicology that have been well characterised. For example there are a few Q(SAR)s for earthworms, but these have not been fully validated (van Gestel *et al.*, 1990). Therefore terrestrial endpoint predictions using Q(SAR)s should be carefully evaluated, and only used as part of a *Weight-of-Evidence* approach (see Figure R.7.11—1).

Grouping of substances with similar chemical structures on the hypothesis that they will have a similar mode of action is a method which has been used in the past to provide non-testing data. The underlying idea is that when (testing-) effect-data are available for a substance within the (structural similar) group, these can be used to "predict" the toxicity of other substances in the same group. This method has been successfully used for PCBs and PAHs.

Another option is to estimate concentrations causing terrestrial effects from those causing effects on aquatic organisms. Equilibrium partitioning theory is based on the assumption that soil toxicity expressed in terms of the freely-dissolved substance concentration in the pore water is the same as aquatic toxicity. Further guidance on how to use the equilibrium partitioning method is provided in Section R.10.6.1 of the *Guidance on IR&CSA* as well as in the ITS in Section R.7.11.6.

Testing data

In vitro data

There are no standardised test methods available at present, however there are a range of *in vitro* soil tests that may have been used to generate terrestrial endpoint data, and this information could be used as part of a *Weight-of-Evidence* approach (see Figure R.7.11—1). A useful review of *in vitro* techniques is provided in the CEH report, 'Review of sublethal ecotoxicological tests for measuring harm in terrestrial ecosystems' (Spurgeon *et al.*, 2004).

In vivo data

The officially adopted OECD and ISO test guidelines are internationally agreed testing methods, and therefore should ideally be followed to generate data for risk assessments. Further details have been provided in this section on the OECD and ISO standard test guidelines which are recommended to test the toxicity of substances to soil organisms. However, there are a range of other standard and non-standard tests available, which can also be used to generate terrestrial endpoint data. Appendix R.7.11-1 includes a detailed list of terrestrial test methodologies, including several test methods that are currently under development. The data from non-standard methodologies will need to be assessed for their reliability, adequacy, relevance and completeness.

OECD and ISO Test Guidelines

i) Microbial Assays

Microorganisms play an important role in the break-down and transformation of organic matter in fertile soils with many species contributing to different aspects of soil fertility. Therefore, any long-term interference with these biochemical processes could potentially disrupt nutrient cycling and this could alter soil fertility. A NOEC/ECx from these tests can be considered as a long-term result for microbial populations.

Soil Micro-organisms, Nitrogen Transformation Test – OECD 216 (OECD, 2000a); ISO 14238 (ISO, 1997a)

Soil Micro-organisms, Carbon Transformation Test – OECD 217 (OECD, 2000b) ; ISO 14239(ISO, 1997b)

The carbon and nitrogen transformation tests are both designed to detect long-term adverse effects of a substance on the process of carbon or nitrogen transformation in aerobic soils over at least 28 days.

For most non-agrochemicals the nitrogen transformation test is considered sufficient as nitrate transformation takes place subsequent to the degradation of carbon-nitrogen bonds. Therefore, if equal rates of nitrate production are found in treated and control soils, it is highly probable that the major carbon degradation pathways are intact and functional.

Further ISO-standard methodologies are available, however since no corresponding OECD guideline exists, these methods are less commonly used than the 2 microbial assays mentioned above.

Determination of potential nitrification, a rapid test by ammonium oxidation – ISO 5685 (ISO, 2004a)

Ammonium oxidation is the first step in autotrophic nitrification in soil. The method is based on measurement of the potential activity of the nitrifying population as assessed by the accumulation of nitrite over a short incubation period of 6 hours. The method does not assess growth of the nitrifying population. Inhibitory doses are calculated.

Determination of abundance and activity of the soil micro-flora using respiration curves – ISO 17155 (ISO, 2002)

This method is used to assess the effect of substances on the soil microbial activity by measuring the respiration rate (CO_2 production or O_2 consumption). The substance may kill the micro-flora, reduce their activity, enhance their vitality or have no effect (either because the toxicity of the substances is low or some species are replaced by more resistant ones). EC10/NOEC and EC50 are determined when toxicity is observed.

ii) Invertebrate Assays

Earthworm acute toxicity test – OECD 207 (OECD, 1984); ISO 11268-1 (ISO, 1993)

The test is designed to assess the effect of substances on the survival of the earthworms *Eisenia* spp. Although the OECD guideline provides details of a filter paper contact test, this should only be used as a screening test, as the artificial soil method gives data far more representative of natural exposure of earthworms to substances without requiring significantly more resources to conduct. Mortality and the effects on biomass are determined after 2 weeks exposure, and these data are used to determine the median lethal concentration (LC50). Although *Eisenia* spp. are not typical soil species, as they tend to occur in soil rich in organic matter, its susceptibility to substances is considered to be representative of soil fauna and earthworm species. *Eisenia* spp. is also relatively easy to culture in lab conditions, with a short life cycle, and can be purchased commercially.

Earthworm reproduction test – OECD 222 (OECD, 2004a); ISO 11268-2 (ISO, 1998)

The effects of substances on the reproduction of adult compost worms, *Eisenia* spp. is assessed over a period of 8 weeks. Adult worms are exposed to a range of concentrations of the test substance mixed into the soil. The range of test concentrations is selected to encompass those likely to cause both sub-lethal and lethal effects. Mortality and growth effects on the adult worms are determined after 4 weeks of exposure, and the effects on reproduction assessed after a further 4 weeks by counting the number of offspring present in the soil. The NOEC/ECx is determined by comparing the reproductive output of the worms exposed to the test substance to that of the control.

Enchytraeid reproduction test – OECD 220 (OECD, 2004b) ; ISO 16387 (ISO, 2004b)

Enchytraeids are soil dwelling organisms that occur in a wide range of soils, and can be used in laboratory tests are well as semi-field and field studies. The OECD guideline recommends the use of *Enchytraeus albidus*, which is easy to handle and breed and their generation time is significantly shorter than that of earthworms. The principle of the test is the same as for the earthworm reproduction test: adult worms are exposed to a range of concentrations of the test substance mixed into the soil. The duration of the reproductive test is 6 weeks, and mortality and morphological changes in the adults are determined after 3 weeks exposure. The adults are then removed and the number of offspring, hatched from the cocoons in the soil is counted after an additional 3 weeks exposure. The NOEC/ECx is determined by comparing the reproductive output of the worms exposed to the test substance, to the reproductive output of the control worms.

Inhibition of reproduction of Collembola (Folsomia candida) – ISO 11267(ISO, 1999a)

Collembolans are the most numerous and widely occurring insects in terrestrial ecosystems. This is one of the main reasons for why they have been widely used as bioindicators and test organisms for detecting the effects of environmental pollutants. The ISO guideline recommends the use of *Folsomia candida*, which reproduces by asexual reproduction and resides primarily in habitats rich in organic matter such as pot plants and compost heaps. A treated artificial soil is used as the exposure medium and a NOEC/ECx for survival and off-spring production is determined after 21 days.

iii) Plant Assays

The most suitable standard methodology for plants to be used for industrial substances that are likely to be applied via sewage sludge is OECD 208 (OECD, 2006a) guideline, which assesses seedling emergence and seedling growth. The second standard method OECD 227 (OECD, 2006b) is more suitable for substances that are likely to deposit on the leaves and above-ground portions of plants and through aerial deposition. There is also a recent ISO test guideline ISO 22030 (ISO, 2005a)), which assesses the chronic toxicity of higher plants.

Terrestrial Plant Test: Seedling emergence and seedling growth test – OECD 208 (OECD 2006a); ISO 11269-2(ISO, 2005b)

The updated OECD guideline is designed to assess the potential effects of substances on seedling emergence and growth. Therefore, it is specific to a part of the plants life-cycle and does not cover chronic effects or effects on reproduction, however it is assumed to cover a sensitive stage in the life-cycle of a plant and therefore data obtained form this study have been used as estimates of chronic toxicity. Seeds are placed in contact with soil treated with the test substance and evaluated for effects following usually 14 to 21 days after 50% emergence of the seedlings in the control group. Endpoints measured are visual assessment of seedling emergence, dry shoot weight (alternatively wet shoot weight) and in certain cases shoot height, as well as an assessment of visible detrimental effects on different parts of the plant. These measurements and observations are compared to those of untreated control plants, to determine the EC50 and NOEC/EC10.

Terrestrial plant test: Vegetative vigour test – OECD 227 (OEC, 2006b)

This guideline is designed to assess the potential effects on plants following deposition of the test substance on the leaves and above-ground portions of plants. Plants are grown from seed usually to the 2-4 true leaf stage. Test substance is then sprayed on the plant and leaf surfaces at an appropriate rate. After application, the plants are then evaluated against untreated control plants for effects on vigour and growth at various time intervals through 21-28 days after treatment. Endpoints are dry or wet shoot weight, in certain cases shoot height, as well as an assessment of visible detrimental effects on different parts of the plant. These measurements are compared to those of untreated control plants.

Soil Quality –Biological Methods – Chronic toxicity in higher plants – ISO 22030 (ISO, 2005a)

This ISO test guideline describes a method for determining the inhibition of the growth and reproductive capability of higher plants by soils under controlled conditions. Two species are recommended, a rapid cycling variant of turnip rape (*Brassica rapa*) and oat (*Avena sativa*). The duration of the tests has been designed to be sufficient to include chronic endpoints that describe the reproductive capability of test plants compared to a control group. The chronic toxicity of substances can be measured by preparing a dilution series of the test substance in standard control soils.

R.7.11.3.2 (semi-) Field data

Field tests are higher tier studies which provide an element of realism but also add complexity in interpretation. There are very few standardised methods for evaluating the ecotoxicological hazard potential of substances in terrestrial field ecosystems. An example of such guidance which has frequently been used is the ISO guideline 11268-3 for the determination of effects of pollutants on earthworms in field situations (ISO, 1999b) This approach aims to assess effects on population size and biomass for a particular species or group of species and there is guidance summarising the conduct of such studies (de Jong *et. al.* 2006).

<u>Gnotobiotic laboratory tests</u>

Gnotobiotic laboratory tests are relatively similar to single-species test and are run under controlled conditions. Usually a few species (2-5), either from laboratory cultures or caught in the field are exposed together in an artificial or (often sieved) field soil. Recently much work has been done with a gnotobiotic system called the Ohio type microcosm (Edwards *et al.*, 1997), which ranges in complexity between laboratory tests and terrestrial model ecosystems (CSTEE, 2000).

Terrestrial microcosms/mesocosms

Terrestrial microcosms/mesocosms can be used as integrative test methods in which fate and effect parameters are investigated at the same time and under more realistic field conditions. The Terrestrial Model Ecosystem (TME) is the only multi-species test that has a standardised guideline (ASTM, 1993). TMEs are small enough to be replicated but large enough to sustain soil organisms for a long period of time (Römbke *et al.*, 1994). TMEs can be used to address the effects on ecosystem structure and function which is not usually possible with single species tests. When TME's studies are conducted in the laboratory, they use intact soil cores extracted from a field site and therefore contain native soil communities. The degree of environmental relevance of these indoor TME's is therefore intermediate between laboratory and field studies.

Typically, in TME's after an acclimatisation period, 4-8 replicates are treated with increasing concentrations of the test-substance or left untreated as controls. They are then sampled at intervals for structural (plant biomass, invertebrate populations) or functional (litter decomposition, microbial activity) parameters. Such an approach may provide a link to effects to the field but under more controlled conditions (Knacker *et al.*, 2004). The statistical analysis of TME data is dependent on the number and inter-relatedness of the endpoints measured. If there are many endpoints measured a multivariate analysis to derive a single effect threshold for the whole system may be appropriate. Due to the complexity of the data obtained in a TME, a standard "one-suits-all" statistical method to generate end-points from these studies cannot be provided. Expert judgement is required.

Field Studies

At present there are no standardised test methods for designing field studies to assess the hazard potential of substances for multiple species. As such field study methodology tends to be specifically designed tests for a particular substance and is difficult to reproduce. Dose response relationships are often lacking (CSTEE, 2000). However, field studies are the most accurate assessment of the impact of a substance on soil function and structure under natural climatic conditions.

R.7.11.4 Evaluation of available information for a given substance

Existing relevant soil organism data may be derived from a variety of sources. Data used in the risk assessments according to Council Directive 91/414/EEC and Council Regulation (EEC) No. 93/793 are considered to be of high quality and preferred over data available from other sources. The next highest quality category is well founded and documented data. These data should compromise a conclusive description of e.g. test conditions, tested species, test duration, examined endpoint(s), references, preferably be conducted according to the principles of Good Laboratory Practice, as well as a justification why the provided data should be used. Further data of lower priority may be provided from publishes literature, and data retrieved from public databases.

R.7.11.4.1 Evaluation of laboratory data

Non-testing data

Preferably PNEC values should be derived using testing for the substance under evaluation but such data are not always available. If data can be derived via extrapolation based on information from similar substances, e.g. using QSAR or SAR models, then these may be used as supportive evidence and to advice on how to proceed with further testing. For the terrestrial ecosystems there are no OECD or ISO guidelines on (Q)SAR models, although some simple models have been published in the open literature e.g. van Gestel and Ma (1993), Xu *et al.* (2000), Wang *et al.* (2000) and Sverdrup *et al.* (2002). In general, if the models indicate little toxicity for a substance based on information from similar substances, this can imply reduced testing; expert judgement is required in these cases.

If no terrestrial data exist, read-across from available aquatic toxicity data, using the EPM method can be considered, as supportive evidence. If there is an indication that a specific group of aquatic organism is more sensitive then other groups e.g. if aquatic plants display a lower EC50 than Daphnia, then further testing of terrestrial plants may be most appropriate. Care should be taken as the aquatic test does not cover the same species groups as in the terrestrial system.

For more extensive modelling the guidance described in Sections R.6.1 and R.6.2 should be followed.

Testing data

<u>Test organisms</u>

In general priority is given to test organisms specified in the OECD and ISO guidelines. Species tested under other official and peer-reviewed guidelines e.g. ASTM can also be employed, but their relevance should be examined.

Non-standard species can also be accepted. However, when employing these in deriving PNEC in the absence of standard studies, it should be ascertained that the test-species is properly identified and characterised, and that the test method is suitable and complies with the standard guidelines in critical points. For example, recovery of the control animals or survival in the control, maximum level of variability in test results, exposure duration, endpoints studied should comply with those specified in the official test guideline. In general the same criteria as described for test species selected according the official guidelines should be applied.

The test species should ideally cover different habitats and feeding modes in the soil as well as different taxonomic groups. For strongly adsorbing or binding substances soil-dwelling organisms that feed on soil particles (e.g. earthworms) are most relevant. However, also a specific mode-of-action that is known for a given substance may influence the choice of the test species (e.g. for substances suspected of having specific effects on arthropods a test with springtails is more appropriate than tests on other taxonomic groups).

If a concern is raised on the relevance of a species then an expert should be consulted.

Endpoints

In general priority is given to test endpoints specified in the OECD and ISO guidelines, unless a special mode-of-action is known. Endpoints under other official and peer-reviewed guidelines e.g. ASTM can also be employed, but their relevance should be considered.

Non-standard endpoints can also be accepted. However, these should be evaluated in relation to ecological relevance and must be properly identified and characterised in order to ensure that the endpoint is suitable and complies with the guidelines in critical points. For example, if the guideline requires sub-lethal endpoints for a species after long-term exposure then the corresponding non-standard endpoint should be sub-lethal and comply with the general outlines specified in the standard test guideline. If non-standard endpoints are very different from the standard endpoints then these must be scientifically justified. For example, an endpoint can be particular sensitive or targeted to the mode-of-action for the substance in question. Screening endpoints such as behavioural responses, i.e. avoidance testing should not be interpreted in isolation. The criteria for reliability, e.g. uncertainty of non-standard endpoints should comply with those of standard endpoints.

If a concern is raised on the relevance of a species then an expert should be consulted.

Exposure pathways

In general, exposure pathway should be as specified in the OECD and ISO guidelines, unless special pathways should be considered.

Non-standard test can also be accepted. If non-standard data are available then it should be considered whether the characteristics of the test substance scientifically justify the chosen exposure pathway. The exposure route is partly dependent on the physico-chemical nature of the substance and also influenced by species-specific life-strategy of the test organism. For strongly adsorbing or binding substances, preference should be given to test designs and test organisms that cover the exposure via ingestion or strong soil particle contact, as this is likely the most relevant exposure route for such substances. As mentioned in Section R.7.11.3. some standard test methodologies include species with food exposure (earthworm reproduction, Enchytraeids and Collembola) while others have contact exposure only.

If a concern is raised on the relevance of the exposure regime then an expert should be consulted.

Composition of soils and artificial-soils

In general, soils in effect testing should be chosen as specified in the OECD and ISO guidelines, unless special conditions are considered.

Non-standard soils can also be accepted. For soils the composition and the choice of soil type have a very large influence on the toxicity of many substances. Hence, if non-standard soils are used it should be considered whether the soil chosen represent a realistic worst-case-scenario for the tested substance. For most substances there is a lack of detailed knowledge about how the toxicity depends of the soil parameters; as such there is little reason to judge the reliability of available data solely based on the site of origin/geography. In general the main parameters driving the bioavailability of substances in soils are clay and organic matter (OM) content, Cation Exchange Capacity (CEC) and pH. For many metals CEC and pH have been shown to be main drivers, whereas for non-polar organics OM has been shown important. For non-standard artificial soil the source of organic matter can also heavily influence the result. Hence, if one of the soil parameters e.g. CEC or pH is very different from those outlined in the guideline or the habitat in question, then a scientific justification of the importance of this derivation should be presented. Residual contaminants are generally not present in artificial substrates, but can be a potential confounding factor if natural soils are used for testing. This affects exposure considerations and is further described in Section R.7.11.4.2.

If a concern is raised on the relevance of a species then an expert should be consulted.

Method of spiking

In general soil tested should be as spiked as specified in the standard OECD and ISO guidelines, unless special conditions are considered.

If non-standard spiking methods are used, these should be scientifically justified. In general there are a variety of spiking methods including direct addition of the substance to soil, using water or a solvent carrier, application via sludge or direct spraying. Spiking soils tends to be problematic for poorly soluble substances (see also Aquatic Toxicity Section R.7.8.7.). The standard approach is to dissolve the test substance in a solvent and then to spike sand, blow-off the solvent and mix the sand into soil using different ratios of sand/soil to derive various test concentrations. The drawback with this technique is that even after hours/days of mixing, the substance may not be homogeneously mixed to the soil, but merely present as solid particles on the original sand. In some cases studies will have been carried out with the use of solubilisers. In these circumstances it is important to consider the change in bioavailability of the test-substance and also the potential impact of the solubiliser. Studies performed without solvents/solubilisers are preferred over studies with solvents/solubilisers. Solvent/solubiliser concentrations should be the same in all treatments and controls.

Bio-availability of substances in soil is known to change over time, aging of the substance in the soil after spiking (with or without solvents) is therefore to be considered. The appropriateness of the aging in studies to derive effect-endpoints depends on the use scenario and the type of risk assessment conducted with this endpoint. Expert judgement is as such required here. For metals and inorganic metal substances both short aging/equilibration times and high spiked metal concentrations in soils will accentuate partitioning of metals to the dissolved phase and increase the probability of exposure and/or toxicity via dissolved metals (Oorts *et al.*, 2006). Simulated aging and weathering processes may be desirable to take account of, but currently this is not included in standard test protocols.

Where a reasonable estimation of the exposure concentration cannot be determined then the test result should be considered with caution unless as part of a *Weight-of-Evidence* approach (see Section R.7.11.5).

Duration of exposure

In general, the test duration should be as specified in the standard OECD and ISO guidelines, unless special conditions are considered.

For non-standard test methodologies it is important to ensure that the duration of exposure in the test is long enough for the test substance to be taken up by the test organisms. In chronic tests the duration should cover a considerable part of the lifecycle. Especially for strongly adsorbing substances it may take some time to reach equilibrium between the soil concentration in the test system and in the test organisms. If the duration of the exposure is different from those in the corresponding guidelines, a scientific justification for the importance of this should provided or the study can be used in the *Weight of Evidence*.

If a concern is raised on the relevance of a species then an expert should be consulted.

Feeding

In general the soil type and soil conditions used for the test should be chosen as specified in the OECD and ISO guidelines, unless special conditions are required.

In long-term tests, especially with reproduction or growth as endpoint, feeding of the test organisms is necessary. Generally the tests are designed in such a way that the food necessary for the test organisms during the study is added to the soil after spiking with the test substance. In standard test methodology, the food is not spiked with the test substance. For non-standard methods the food type depends on the test species. It has to be considered that any food added to the test system either periodically during the test period or only at test initiation may influence outcome of the study and as such the reliability of the data obtained.

Ad-libitum feeding, or the lack of such may influence the state of health of the test organisms and as such their ability to cope with (chemical-) stress. Different feeding regimes are therefore a source of variation on the expression of the effect parameter.

Test design

In general the test-design should be as specified in the standard OECD and ISO guidelines, unless special conditions are required.

For standard test methodologies details of test design are normally well documented. To ensure the validity non-standard test methodology, these should to a large extend follow the specifications outlined in the standard guideline tests e.g. including sufficient concentrations and replications and positive and negative controls. For a proper statistical evaluation of the test results, the number of test concentrations and replicates per concentration are critical factors. If a solvent is used for the application of the test substance, an additional solvent control is necessary. The appropriate number of replicates to be included in a test is dependent on the statistical power required for the test. More guidance on statistical design is provided in the OECD (2006c). It is not a priori possible, to advice on what test design details are of key importance and which can be allowed to be missing before validity of the results becomes equivocal. If relevant information on test design is missing in non-standard test then they can only be used in a *Weight-of-Evidence* approach.

R.7.11.4.2 Field data and model ecosystems

Multi-species test

There are no OECD or ISO guideline on terrestrial multi-species test systems.

Since not standardised and given their complexity multi-species test should be judged on a case-by-case basis and expert judgement is necessary to fully interpret the results. Several test-designs and evaluation of these have been published, ranging from standardised gnotobiotic systems (Cortet *et al.*, 2003) to tests including indigenous soils and soil populations (Parmelee *et al.*, 1997, Knacker and van Gestel 2004). Fixed trigger values for acceptability of effects are not recommended as the impact of treatments can be significantly different depending on the test design. However, laboratory based multi-species studies should in general be given the same general consideration as the single species test, e.g. with regard to reliability and relevance. For terrestrial model ecosystems there may be a large natural variation inherent in the test systems compared to single species test. To address diversity and species interaction the multi-test systems should contain sufficient complex assemblages of species with diverse life strategies. In assessing the reliability of results from a model-ecosystems special attention should be given to the statistical evaluation and the capability of the test design to identify possible impact. Effects observed through time, whether permanent or transitory should be explored. Combinations of both univariate and multivariate analyses are preferred; guidance can be obtained from Morgan and Knäcker (1994), van den Brink and Braak (1999), Scott-Fordsmand and Damgaard (2006).

Field testing

In field trials, population level effects as opposed to effects on individuals are the desired goal or endpoint of the studies. The population effect on a species or group of species including time to recover should be analysed in comparison to control plots. Fixed trigger values for acceptability of effects are not recommended, as the impact of treatments can be significantly different for different organisms. Biological characteristics such as development stage, mobility of species and reproduction time can influence the severity of effects. Thus acceptability should be judged on a case-by-case basis and expert judgement is necessary to fully interpret field study results. Where significant effects are detected the duration of effects and range of taxa affected should be taken into consideration (Candolfi *et al.*, 2000).

R.7.11.4.3 Exposure considerations for terrestrial toxicity

Before their use the exposure data should be validated in respect of their completeness, relevance and reliability. Guidance on how to evaluate exposure data will be developed in Section R.5.1. Consideration should be given to whether the substance being assessed can be degraded, biotically or abiotically, to give stable and/or toxic degradation products. Where such degradation can occur the assessment should give due consideration to the properties (including toxic effects) of the products that might arise.

R.7.11.4.4 Remaining uncertainty

Soil is a very heterogeneous environment compartment where abiotic parameters and soil structural conditions can vary within very short distances; these introduce an extra dimension of variability into soil test. Therefore it is important to have a good characterisation of the media chosen in the test. In addition there is usually a larger variation around the individual results than from other media. For non-standard tests the variation in the toxicity results should be comparable to the one required in standard tests.

The available standardised test methods only deal with a few taxa of soil invertebrates. Therefore, not all specific effects of substances on the wide range of organisms normally present in soil may be covered by the available test methods. As these organisms may play an important role in the soil community, it may be relevant to consider results from non-standard test designs in completing Chemical Safety Assessment. Further standard test methods may be developed and a need may exist to revise the soil safety assessment concept accordingly in future.

R.7.11.5 Conclusions on "Effects on Terrestrial Organisms"

R.7.11.5.1 Concluding on suitability for Classification and Labelling

Soil toxicity data are generally not used for classification and labelling as hazardous to the aquatic environment (Annex I to the Regulation (EC) No 1272/2008). However, with the amendment of CLP Regulation (Commission Delegated Regulation 2023/707, entered into force in April 2023), results from long-term toxicity testing on terrestrial organisms, invertebrates and plants, are considered for the assessment of T properties (as part of Persistent, Bioaccumulative and Toxic properties, or Persistent, Mobile and Toxic properties).

R.7.11.5.2 Concluding on suitability for PBT/vPvB assessment

There is a potential use for both short-term and long-term soil toxicity data in determining the Toxicity component of PBT. However, there are currently no criteria included in Section 1.1.3 of Annex XIII to REACH for soil toxicity and thus no specific data requirements.

Where data exist showing short or long-term toxicity to soil organisms using standard tests on soil invertebrates or plants, these should be considered along with other data in a *Weight-of-Evidence* approach to the toxicity criteria (Section 3.2.3 of Annex XIII to REACH).

R.7.11.5.3 Concluding on suitability for use in Chemical Safety Assessment

Soil toxicity data are used in the chemical safety assessment to establish a $PNEC_{soil}$ as part of a quantitative assessment of risk to the soil compartment. Ideally, this will be calculated based on good quality data from long-term toxicity studies on soil organisms covering plants, invertebrates and micro-organisms. Where such data exist from studies conducted to standardised internationally accepted guidelines, these may be used directly to establish the $PNEC_{soil}$.

It must be recognised, however, that these type of data are rarely available, and may not be needed to characterise the risk for soil. In defining what can be considered as sufficiency of information, it is also necessary to have all available information on water solubility, octanol/water partitioning (log K_{ow}), vapour pressure, and biotic and abiotic degradation, and the potential for exposure

When soil exposure is considered negligible, i.e. where there is low likelihood of land spreading of sewage sludge, or aerial deposition of the substance and other pathways such as irrigation or contact with contaminated waste are equally unlikely, then neither a PEC, nor PNEC can or need be calculated and no soil toxicity data are necessary.

In general, the data available will be less than that required to derive a definitive PNEC for soil organisms. The following sections, nevertheless describe the circumstances where data-sets of differing quality and completeness can be considered 'fit for the purpose' of calculating a PNEC for the purposes of the chemical safety assessment.

Furthermore, a section on the *Weight-of-Evidence* approach is included at the end of this chapter, and guidance on testing strategies is presented in Figure R.7.11—2 and Figure R.7.11—3 and a Table R.7.11—2 in Section R.7.11.6 (integrated testing strategy) of this report.

Where no soil toxicity data are available

There will be circumstances where no soil organism toxicity data are available. In making a judgment on whether soil organism toxicity data should be generated, and if so which these should be, all available data including those available on aquatic organisms should first be examined as part of a stepwise approach. Where the data available are sufficient to derive a PNEC for aquatic organisms, this PNEC can be used in a screening assessment for soil risks through the use of the EPM approach. If comparison of a $PNEC_{soil}$ derived by EPM from the aquatic PNEC, shows a PEC/PNEC ratio <1, then the information available may be sufficient to conclude the soil assessment. Where the adsorption is likely to be high, i.e. where the log K_{ow} or Log K_{oc} >5, the PEC/PNEC ratio is multiplied by 10. The use of the EPM method, however, provides only an uncertain assessment of risk and, while it can be used to modify the standard data-set requirements of Annex IX and X, it cannot alone be used to obviate the need for further information under this Annex. This will be further elaborated on in Section R.7.11.6 and portrayed in tabular format in Table R.7.11—2 of Section R.7.11.6.

Where the PEC/PNEC ratio >1, then the information based on aquatic toxicity data alone (i.e. $PEC/PNEC_{screen}$) is insufficient and soil toxicity data will need to be generated.

When the substance is also readily degradable, biotically or abiotically, however, and has a log K_{ow} <5, this screening assessment showing no risk using aquatic toxicity data is sufficient to obviate the need for further information under Annex IX. In other circumstances, the derivation of a $PNEC_{screen}$ derived from aquatic toxicity data alone would be insufficient to derogate from Annex IX or X testing.

As is stated above, it will normally not be possible to derive a robust PNEC for the purposes of a soil screening assessment from acute aquatic toxicity testing showing no effect. This is, particularly true for poorly soluble substances. Where the water solubility is <1 mg/l, the absence of acute toxicity can be discounted as reliable indicator for potential effects on soil organism due to the low exposures in the test. The absence of chronic or long-term effects in aquatic organisms up to the substance solubility limit, or of acute effects within the solubility range above 10 mg/l can be used as part of a *Weight-of-Evidence* argument to modify/waive the data requirements of Annex IX and X.

Except in the specific situation described above, soil organism toxicity data are required as defined in Annex IX and X in order to derive or confirm a PNEC for the soil.

Normally, three $L(E)C_{50}$ values from standard, internationally accepted guidelines are required in order to derive a $PNEC_{soil}$. The species tested should cover three taxonomic groups, and include plants, invertebrates and micro-organisms as defined in Annex IX. Normally, when new testing is required, these tests would be the OECD Guidelines Tests 207 (Earthworm acute Toxicity), 208 (Higher Plant Toxicity) and 216 (Nitrogen Transformation). The PNEC can be derived by applying an assessment factor to the lowest $L(E)C_{50}$ from these test.

Before new testing is conducted, however, all available existing information should be gathered to determine whether the requirements of the Annexes are met. In general, the data required should cover not just different taxa but also different pathways of exposure (e.g. feeding, surface contact), and this should be taken into account when deciding on the adequacy and relevance of the data. Thus earthworm testing allows potential uptake via each of surface contact, soil particle ingestion and porewater, while plant exposure will be largely via porewater.

In considering all the data available, expert judgment should be used in deciding whether the *Weight-of-Evidence* (see below) will allow specific testing to be omitted.

In general, where there is no toxicity L(E)C50 in the standard acute toxicity tests at >10 mg/l, or no effects in chronic toxicity at the limit of water solubility, or the screening assessment based on EPM shows no concern, then a single short-term soil test on a suitable species would be adequate to meet the requirements of Annex IX. The soil PNEC would be derived by application of appropriate assessment factors to the aquatic data, and the soil short-term data, and the lowest value taken. Where the substance is highly adsorptive, e.g. where the log K_{ow}/K_{oc} >5, and/or the substance is very persistent in soil, this single test should be a long-term test. Substances with a half-life >180 days are considered to be very persistent in soil. This persistence would be assumed in the absence of specific soil data, unless the substance is readily degradable. The choice of test (invertebrate / plant / micro-organism) would be based on all the information available, but in the absence of a clear indication of selective toxicity, an invertebrate (earthworm or collembolan) test is preferred.

Acute or short-term soil organism toxicity data

If data on soil toxicity are already available, this should be examined with respect to its adequacy (reliability and relevance). Normally, micro-organism or plant testing alone would not be considered sufficient, but would be considered as part of a *Weight-of-Evidence* approach. In circumstances where less than a full soil toxicity data-set is available, both the available soil data and the EPM modified aquatic toxicity data should be used in deriving the $PNEC_{soil}$, as further detailed in Table R.7.11—2. In such circumstances, where the subsequent PEC/PNEC <1, this would constitute an adequate data-set and no further testing would be required to study effects on trophic levels which are part of the aquatic toxicity data set (invertebrates, plants). In all other circumstances, three short-term soil toxicity tests are needed to meet the requirements of Annex IX.

Intrinsic properties of chemicals on soil microbial communities are not addressed through the EPM extrapolation method because the standard aquatic toxicity data set (i.e. studies on fish, invertebrates and algae) used for derivation of PNEC for aquatic organisms does not include information on toxicity to microbial communities. Therefore, the EPM (outlined in column 2 of Annex IX, Section 9.4.) is not sufficient to derive the $PNEC_{soil}$ on its own and a toxicity study on soil microorganisms is required.

It may be possible to show by *Weight-of-Evidence* from other tests, that no further specific test is needed. Where such an argument is made, it must be clearly documented in the chemical safety assessment.

The L(E)C50s are used to derive a PNEC using assessment factors.

Chronic or long-term soil organism toxicity data

Chronic or long-term toxicity tests on plants and/or soil invertebrates conducted according to established guidelines can be used to derive a $PNEC_{soil}$. The NOEC or appropriate EC_x may be used with an appropriate assessment factor. Where such data from chronic or long-term tests are available, they should be used in preference to short-term tests to derive the PNEC. In general, three long-term NOECs/EC_xs are required, although the PNEC can be derived on two or one value with appropriate adjustment of the assessment factor. The tests should include an invertebrate (preferably earthworm reproduction test), a higher plant study and a study on micro-organisms (preferably on the nitrogen cycle). Other long-term tests can also be used if conducted to acceptable standard guidelines (see Section R.7.11.4).

Where adequate long-term data are available, it would generally not be necessary to conduct further testing on short-term or acute effects.

Where long-term toxicity data are not available, all the other data available should be examined to determine whether the data needs of the chemical safety assessment are met. The adequacy and relevance of these data are described above. Only where the data on aquatic effects, and/or short-term toxicity are insufficient to complete the chemical safety assessment, i.e. risks have been identified based on these data, new long-term testing need to be conducted. Where the substance is highly adsorptive or very persistent as described above, the effect of long-term exposures should be estimated. Hence at least the invertebrate data should be derived from a long-term toxicity test, although other long-term toxicity data may be considered.

Step 1 – Characterisation of the substance

- Verification of the identity/structure
- Physico-chemical properties of the substance
- Information about degradation of the substance in soil
- Identification of potential metabolites
- Preliminary analysis of uptake and fate

↓

Step 2 – Identification of possible analogues

- Collection of possible analogues
- Identification of new substance categories
- QSARs

↓

Step 3 – Collating of both testing and non-testing data

- *In vitro* methods
- EPM
- Non-standard *in vivo* soil testing
- Standard *in vivo* soil testing data

↓

Step 4 – *Weight-of-Evidence* assessment

- Quality assessment of existing data
- Identification of data-gaps
- Consider information relevant for waiving
- Summary of remaining uncertainty

Figure R.7.11—1 *Weight-of-Evidence* approach

The flow diagram above outlines a systematic approach how to use all available data in a *Weight-of-Evidence* decision. It provides a step-wise procedure for the assessment of different types of information, which might be helpful to come to an overall conclusion. The scheme proposes a flexible sequence of steps, the order of which depends on the quality and quantity of data: When for any given substance *in vivo* soil data of adequate quality are available (step 3) performance of step 2 may not be necessary to derive a $PNEC_{soil}$. However, it is deemed that even when in-vivo data are available, a *Weight-of-Evidence* assessment with other types of data may be useful to increase the confidence with the derived $PNEC_{soil}$ and reduce the remaining uncertainty.

Step 1 – Characterisation of the substance

Since there are no current requirements for soil testing to provide hazard data for classification and labelling (Section R.7.11.5.1) nor for PBT assessment (Section R.7.11.5.2) the need for any effect data on soil organisms should be steered by the need to develop the chemical safety assessment and in particular by the environmental exposure, fate and behaviour of the substance. The starting point of any assessment within the soil area should therefore be to gather key parameters that provide insight to fate and behaviour of the substance:

Physico-chemical properties. Water solubility, Kow, Koc, Henry's constant etc. will provide information about the distribution in soil, water and air after deposition in/on soil.

Data on degradation (in soil) will provide information as to whether the substance is likely to disappear from the soil after deposition, or alternatively remain in the soil or even accumulate over time which may indicate a potential to cause long-term effects. Any (major) metabolites being formed should be considered to provide a comprehensive safety assessment of a substance after deposition on/in soil

Step 2 – Identification of possible analogues and alternative data

The effort to identify chemical analogues (read-across) which may take away/modify the need to search/generate substance-specific data is often the more resource-effective way to proceed in the assessment. Fate data on an analogue may allow effect-testing of the substance to become more focused. Effect data on an analogue substance may potentially be used to waive certain substance-specific testing requirements. It is however important to understand the limitations of assessing a substance by surrogate data from analogues, therefore the assessment of remaining uncertainty (see also step 4) is of primary importance here.

Where non-testing data (QSARs) are available, these may also be used for a first screening assessment and to waive certain substance-specific soil-testing requirements (see Section R.7.11.5.3).

Step 3 – Collating of both testing and non-testing data

Highest priority is given to *in vivo* data which fulfil the data requirements specified in Annex IX and X. Where such data are available, they are subjected to a careful check of their quality and relevancy. Good quality data can be used to derive a quantitative conclusion on the endpoint.

Step 4. *Weight-of-Evidence* assessment

The principle of any comprehensive assessment is to gather all available and potentially relevant information on a substance, regardless whether these are non-testing (QSARs), EPM, or soil specific testing (*in vitro* or *in vivo*) data. Any source of information can potentially be used to focus an assessment or limit uncertainties that remain after derivation of the endpoint. Even when standard effect data on all 3 taxonomic groups are available for a substance, further non-standard or non-testing data can be useful in refining the assessment. Rather than a sequential gathering of data, a single step collating all the available information is the way into a *Weight-of-Evidence* assessment for soil organisms

<u>Standard studies available (no data-gap)</u>

The *Weight-of-Evidence* approach normally starts with an evaluation of the quality of available data. Standard effects data, using standard species, performed according to internationally harmonised guidelines (OECD/ISO) and generated under quality criteria (GLP) clearly represent the highest quality category of data, followed by secondary sources; non-standard *in vivo* test, *invitro* test and non-testing data. However, even when standard-tests are available for a substance, further secondary sources of information (non-standard testing or non-testing) can be used to gain confidence in the assessment. Supporting evidence from secondary sources reduce the remaining uncertainty associated with any assessment. Contradictory information between primary and secondary sources indicate the need to perform a thorough uncertainty analysis.

In the event that more than a single standard study is available for the same species and same endpoint, and there are no obvious quality differences between the studies a geometric mean value can be derived to be used in assessing the endpoint if the data are obtained in soils in which the bioavailability of the substance is expected to be similar. Even in case where data are obtained in soils in which the bioavailability of the substance is significantly different, a geometric mean can still be used when the data can be normalised to a given standard condition. If normalisation of the data is not possible, the value obtained in the soil with the highest bioavailability is to be taken to derive the PNEC.

If multiple data are available for the same species but different endpoints, in principle the most sensitive endpoint is to be taken to derive the PNEC. Prior to this step however, the relevance of all endpoints to describe the state of the ecosystem is to be considered.

If more than a single species was tested in any given organisms group (plant, invertebrate, micro-organism), allowance should be made for the reduction of the uncertainty that the availability of such data may provide. Species Sensitivity Distribution curves (SSD) and Hazard Concentration (HCx) approaches have been used successfully in Chemical Safety Assessments.

<u>Missing standard studies (data-gaps)</u>

A full set of standard (GLP) effect test is only infrequently available. There may therefore be a potential data gaps for substances reaching production volumes > 100 t/y (Annex IX and X). In this case secondary source data should be used to study whether there is a need for generating such data to complete the assessment of the end-point, e.g.:

If testing data on non-standard species is available, and these studies were carried out according to a high scientific quality, one may consider to waive the requirement for a standard test, e.g. a reliable NOEC for a soil-insect other than collembolan may be used as surrogate data for the group of soil invertebrates, especially when this test indicates that soil invertebrates are not particularly sensitive to the substance that is assessed.

The availability of a study on a standard species which does not completely follow OECD or ISO guidelines can be used to waive the requirement to run a new study on this standard species, if the data are scientifically sound, and indicate that this group of organisms is not critical in the safety assessment.

A further use of secondary source effect data is to steer testing requirements, especially in higher tiers. The identification of a particular sensitive group of organisms in literature, may lead to the need to extend the scope of higher tier/multi-species studies to include this group of organisms. For example information from secondary sources may show that the molecule has specific activity against a certain group of organism (e.g. plants) and this may allow the assessor to conclude on the end-point based on standard testing for plants only, and waive the invertebrate and micro-organism testing requirements in Annex IX and X.

If there are several secondary sources data available for the same species, data can be combined to increase either the statistical power of the conclusion, or the confidence that the assessor can have in deriving a (screening-) endpoint based on the secondary data.

At the end of any assessment - derivation of the endpoint (PNEC) and assessment of the remaining uncertainty associated with the assessment/endpoint is required. The TGD explicitly deals with uncertainties by using assessment factors in the derivation of PNEC's, but does so merely based on the amount of information available. It does provide little guidance on how to modify the assessment based on the specific profile of a substance, nor on the quality of the individual toxicological values (NOEC, ECx) derived from the studies. The confidence-level associated with any endpoint from an individual study is largely disregarded. Therefore, in parallel to the quantitative assessment of the endpoint some estimate on how much confidence the assessor has in this end-point should ideally be expressed by means of an uncertainty analysis.

R.7.11.6 Integrated testing strategy (ITS) for Effects on Terrestrial Organisms.

Fundamentally based on a *Weight-of-Evidence* approach, the integrated testing strategy (ITS) should be developed with the aim of generating sufficient data for a substance to support its classification (or exclusion from classification), PBT/vPvB assessment and risk assessment. For the soil compartment there are currently no criteria for classification and PBT assessment, therefore the ITS for soil is especially focussed on generating data for the chemical safety assessment.

R.7.11.6.1 Objective / General principles

The main objective for this testing strategy is to provide guidance on a stepwise approach to hazard identification with regard to the endpoint. A key principle of the strategy is that the results of one study are evaluated before another is initiated. The strategy should seek to ensure that the data requirements are met in the most efficient manner so that animal usage and costs are minimised.

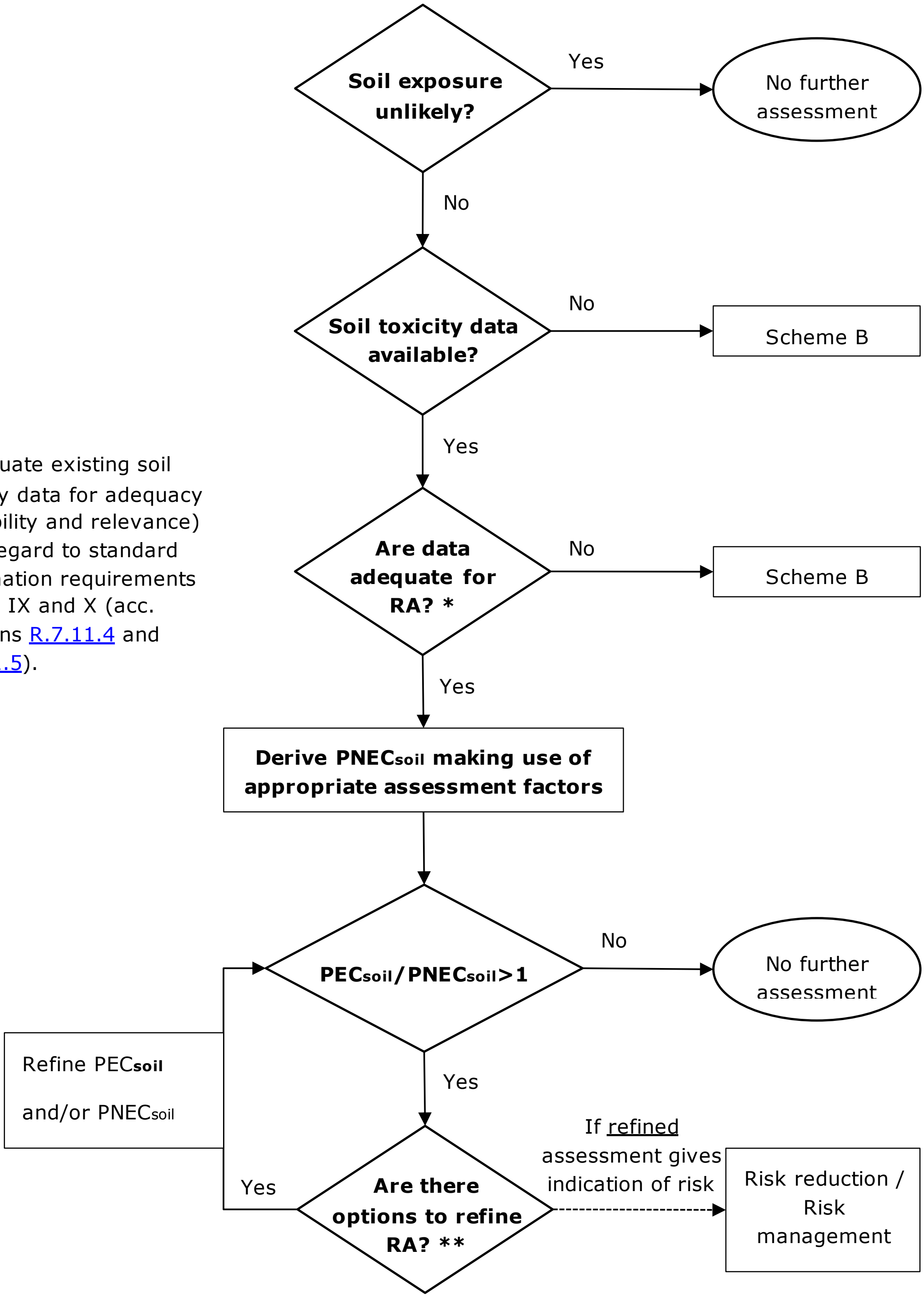
R.7.11.6.2 Preliminary considerations

The guidance given in Section R.7.11.2 to R.7.11.4 above will enable the identification of the data that are needed to meet the requirements of REACH as defined in Annexes VII to X. Careful consideration of existing environmental data, exposure characteristics and current risk management procedures is recommended to ascertain whether the fundamental objectives of the ITS have already been met. Guidance has been provided on other factors that might mitigate data requirements, e.g. the possession of other toxic properties, characteristics that make testing technically not possible – for more guidance, see Section R.5.2.

R.7.11.6.3 Testing strategy

The general risk assessment approach is given in Figure R.7.11—2 and the ITS in Figure R.7.11—3.

A testing strategy has been developed for the endpoint to take account of existing environmental data, exposure characteristics as well as the specific rules for adaptation from standard information requirements, as described in column 2 of Annexes IX and X, together with some general rules for adaptation from standard information requirements in Annex IX.



* Evaluate existing soil toxicity data for adequacy (reliability and relevance) with regard to standard information requirements Annex IX and X (acc. Sections R.7.11.4 and R.7.11.5).

** Consider both options: Refinement of $PNEC_{soil}$ and/ or refinement of PEC_{soil}.

Figure R.7.11—2 Scheme A: General risk assessment scheme

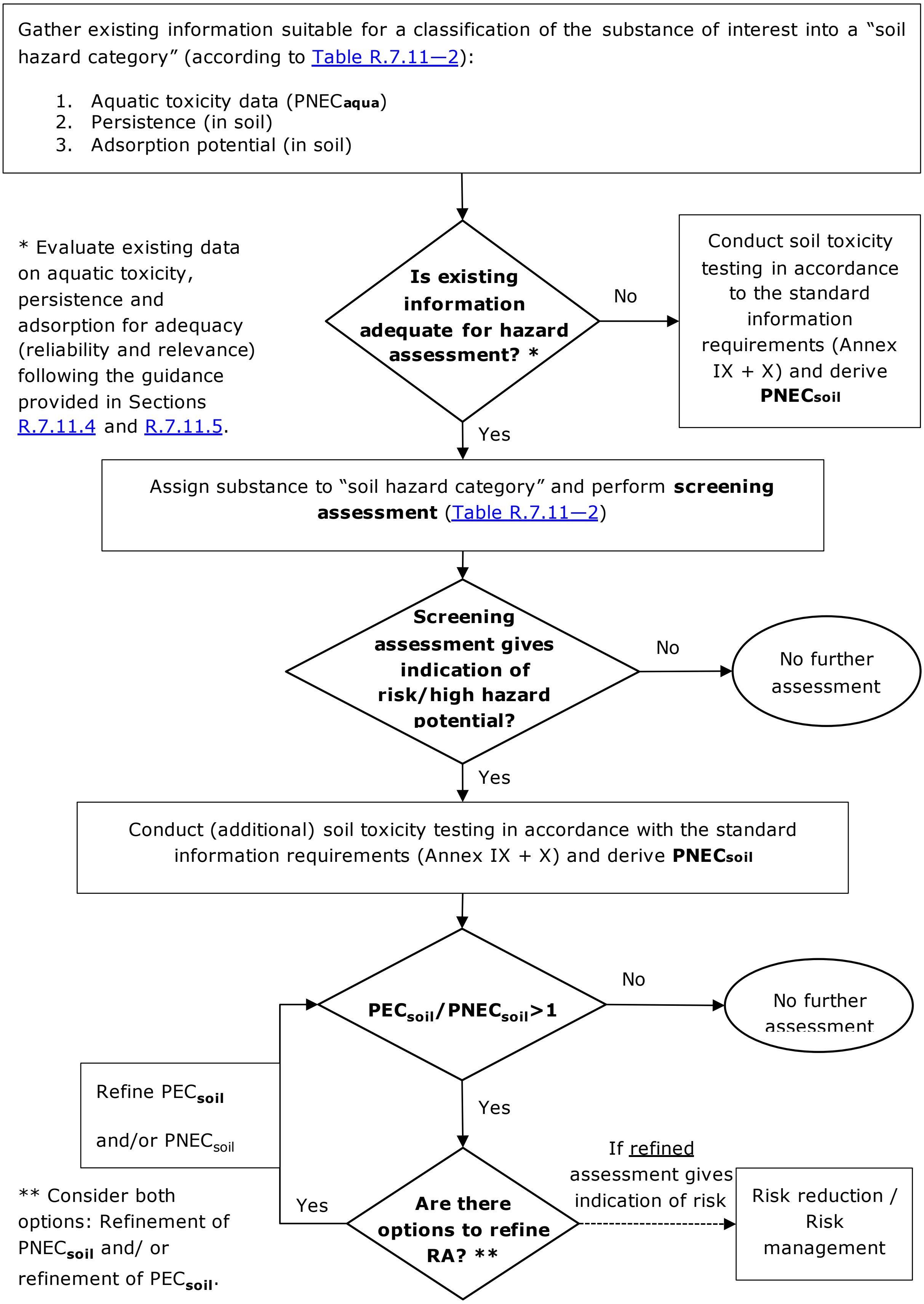


Figure R.7.11—3 Scheme B: Integrated testing strategy (Annex IX and Annex X substances)

Table R.7.11—2 Soil hazard categories and integrated testing strategy (for waiving standard information requirements according to Annex IX and X)

	Hazard category 1	Hazard category 2	Hazard category 3	Hazard category 4
<u>Assign substance to "soil hazard category":</u> Is there indication for **high adsorption**[20] **OR high persistence**[21] of the substance in soil?	No	No	Yes	Yes
Is there indication that the substance is **very toxic**[22] to aquatic organisms?	No	Yes	No	Yes
Screening assessment: Minimum information required to derive $PNEC_{soil}$	$PNEC_{screen}$ (based on EPM[23])	$PNEC_{screen}$ (based on EPM) AND one short-term soil toxicity testing according to the standard information requirements Annex IX (invertebrates, plants) (e.g. one limit test with the most sensitive organism group as indicated from aquatic toxicity data) AND toxicity testing on soil microorganisms	$PNEC_{screen}$ (based on EPM) AND one long-term soil toxicity testing according to the standard information requirements Annex X (invertebrates and plants) (e.g. one limit test with the most sensitive organism group as indicated from aquatic toxicity data) AND toxicity testing on soil microorganisms	Screening assessment based on EPM not recommended, intrinsic properties indicate a high hazard potential to soil organisms

[20] log K_{OW} > 5 or a ionisable substance

[21] DT50 > 180 days (default setting, unless classified as readily biodegradable)

[22] EC/LC50 < 1 mg/L for algae, daphnia or fish

[23] EPM: Equilibrium Partitioning Method

	Hazard category 1	Hazard category 2	Hazard category 3	Hazard category 4
Consequences from screening assessment: 1) Waiving of some standard information requirements possible based on EPM specified in the second column of Annex IX, Section 9.4, OR 2) Conduct (additional) toxicity testing with soil organisms according to the standard information requirements to derive $PNEC_{soil}$	If $PEC/PNEC_{screen} < 1$: No toxicity testing for soil organisms need to be done If $PEC/PNEC_{screen} > 1$: Conduct short-term toxicity tests according to the standard information requirements Annex IX (invertebrates, micro-organisms and plants), choose lowest value for derivation of $PNEC_{soil}$	If $PEC/PNEC_{screen} < 1$ and no indication of risk from soil toxicity testing: No further toxicity testing for soil organisms need to be done If $PEC/PNEC_{screen} > 1$ or indication of risk from soil toxicity testing: Conduct short-term toxicity tests according to the standard information requirements Annex IX (invertebrates, plants), choose lowest value for derivation of $PNEC_{soil}$	If PEC x $10/PNEC_{screen} < 1$ and no indication of risk from soil toxicity testing: No further toxicity testing for soil organisms need to be done If $PEC/PNEC_{screen} > 1$ or indication of risk from soil toxicity testing: Conduct long-term toxicity tests according to the standard information requirements Annex X (invertebrates and plants), choose lowest value for derivation of $PNEC_{soil}$	Not applicable Conduct long-term toxicity tests according to the standard information requirements Annex X (invertebrates and plants), AND toxicity testing on soil microorganisms (Annex IX). Choose the lowest value for derivation of $PNEC_{soil}$
Consequences from (additional) toxicity testing:	If PEC_{soil} / $PNEC_{soil} < 1$: No additional long-term toxicity testing for soil organisms need to be done If PEC_{soil} / $PNEC_{soil} > 1$: Conduct additional or higher tier test or refine PEC_{soil}	If PEC_{soil} / $PNEC_{soil} < 1$: No additional long-term toxicity testing for soil organisms need to be done If PEC_{soil} / $PNEC_{soil} > 1$: Conduct additional or higher Tier test or refine PEC_{soil}	If PEC_{soil} / $PNEC_{soil} < 1$: No additional long-term toxicity testing for soil organisms need to be done If PEC_{soil} / $PNEC_{soil} > 1$: Conduct additional or higher Tier test or refine PEC_{soil}	If PEC_{soil} / $PNEC_{soil} < 1$: No additional long-term toxicity testing for soil organisms need to be done If PEC_{soil} / $PNEC_{soil} > 1$: Conduct additional or higher Tier test or refine PEC_{soil}

R.7.11.7 References for effects on terrestrial organisms

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Appendix to Section R.7.11

Appendix R.7.11-1 Selected Soil Test Methodologies

Table R.7.11—3 Selected Soil Test Methodologies

Test Organism	Duration	End points	Reference/Source	Comments
Microbial Processes				
Microbial Processes N-Transformation	≥28 d	M	(i) OECD 216 Soil Microorganisms, Nitrogen Transformation Test (2000). (ii) ISO 14238 Soil quality – Biological methods: Determination of nitrogen mineralisation and nitrification in soils and the influence of chemicals on these processes (1997).	Based on soil microflora nitrate production. Bacteria are present at up to 10 million per cm^2 in soils. This corresponds to several tonnes per hectare.
Microbial Processes C-Transformation	≥28 d	M	(i) OECD 217 Soil Microorganisms, Carbon Transformation Test (2000). (ii) ISO 14239 Soil quality – Laboratory incubations systems for measuring the mineralisation of organic chemicals in soil under aerobic conditions (1997).	Based on soil microflora respiration rate. Bacteria are present at up to 10 million per cm^2 in soils. This corresponds to several tonnes per hectare.
Invertebrate Fauna				

Test Organism	Duration	End points	Reference/Source	Comments
Eisenia fetida/andrei (Oligochaeta)	7-14 d	S	(i) OECD 207 Earthworm acute toxicity tests (1984). (ii) ISO 11268-1 Soil Quality – Effects of pollutants on earthworms (*Eisenia fetida*). Part 1: Determination of acute toxicity using artificial soil substrate (1993). (iii) EEC (1985) 79/831. (iv) ASTM E1676-97 Standard guide for conducting laboratory soil toxicity or bioaccumulation tests with the Lumbricid earthworm *Eisenia fetida* (1997).	Adult survival assessed after 1 – 2 weeks. Important ecological function (enhance decomposition and mineralisation via incorporation of matter into soil). Important food source and potential route of bioaccumulation by higher organisms. Large size/ease of handling. Readily cultured/maintained in the laboratory. Litter-dwelling epigeic species. Standard test organism for terrestrial ecotoxicology. The Lumbricidae account for 12% of the edaphon (soil biota) by biomass and are therefore important prey species.

Test Organism	Duration	End points	Reference/Source	Comments
Eisenia fetida/andrei (Oligochaeta)	28d + 28d	S/G/R	(i) OECD (2004). Earthworm Reproduction Test. (ii) ISO 11268-2 Soil Quality – Effects of Pollutants on Earthworms (*Eisenia fetida*). Part 2: Determination of Effects on Reproduction (1998). (iii) EPA (1996). Ecological Effects Test Guidelines. OPPTS 850.6200 Earthworm Subchronic Toxicity Test. US EPA, Prevention, Pesticides and Toxic Substances (7104). EPA712-C-96-167, April 1996. (iv) Kula and Larink (1998). Tests on the earthworms *Eisenia fetida* and *Aporrectodea caliginosa*. In "*Handbook of Soil Invertebrates*" (Eds. Hans Løkke and Cornelis A.M. Van Gestel). John Wiley and Sons: Chichester, UK.	Adult growth and survival assessed after 4 weeks. Reproduction (juvenile number) assessed after a further 4 weeks (8 weeks total). Relatively long generation time (8 wks). Important ecological function (enhance decomposition and mineralisation via incorporation of matter into soil). Important food source and potential route of bioaccumulation by higher organisms. Large size/ease of handling. Readily cultured/maintained in the laboratory. Litter-dwelling epigeic species. Standard test organism for terrestrial ecotoxicology. The Lumbricidae account for 12% of the edaphon (soil biota) by biomass and are therefore important prey species.

Test Organism	Duration	End points	Reference/Source	Comments
Aporrectodea caliginosa (Oligochaeta)		S/G/R	Kula and Larink (1998). Tests on the earthworms *Eisenia fetida* and *Aporrectodea caliginosa*. In "*Handbook of Soil Invertebrates*" (Eds. Hans Løkke and Cornelis A.M. Van Gestel). John Wiley and Sons: Chichester, UK.	Mortality, growth and cocoon number assessed after 4 weeks. Relatively slow reproductive cycle. Cultures difficult to maintain. Horizontal burrowing (endogeic) mineral soil species. Selective feeders digesting fungi, bacteria and algae. Dominant in agro-ecosystems. Present at 10 – 250 per m^2.
Enchytraeus albidus (Oligochaeta)	21 - 42d	S/R	(i) OECD (2004). OECD 220 *Enchytraeidae* Reproduction Test. (ii) ISO 16387 Soil quality - Effects of soil pollutants on enchytraeids: Determination of effects on reproduction and survival (2004).	Adult mortality is assessed after 3 weeks. Reproduction (juvenile number) is assessed after a further 3 weeks (6 weeks total). Shorter generation time than earthworms. Ease of handling/culture. Enchytraeidae feed on decomposing plant material and associated micro-organisms i.e., fungi, bacteria and algae. Enchytraeids are abundant in many soil types including those from which earthworms are often absent. They account for approximately 0.5% of the edaphon (soil biota) by mass (up to 50 g per m^2). This corresponds to approximately 100,000 per m^2.

Test Organism	Duration	End points	Reference/Source	Comments
Cognettia sphagnetorum (Oligochaeta)	70 d	G/R	Rundgren and Augustsson (1998). Test on the Enchytraeid *Cognettia sphagnetorum*. In "*Handbook of Soil Invertebrates*" (Eds. Hans Løkke and Cornelis A.M. Van Gestel). John Wiley and Sons: Chichester, UK.	Mortality and asexual reproduction (fragmentation rate of adults) determined weekly over 10 weeks. Easy to culture. Enchytraeidae feed on decomposing plant material and associated micro-organisms i.e., fungi, bacteria and algae. *C. spagnetorum* is common in bogs, forests and other highly organic habitats. They are present at 10,000 – 25,000 per m^2.
Folsomia candida (Collembola)	28d	S/R	ISO 11267 Soil Quality – Inhibition of reproduction of Collembola (*Folsomia candida*) (1984).	Survival and reproduction after 4 weeks. Short generation time. Ease of culture. Springtails are important soil litter arthropods playing a role in soil organic matter breakdown and nutrients recycling. Feed on bacteria and fungi. Collembola are the most abundant soil fauna present at 40,000 to 70,000 per m^2. Prey for epigeic invertebrates such as mites, centipedes, spiders and carabid beetles.

Test Organism	Duration	End points	Reference/Source	Comments
Isomtoma viridis, Folsomia candida and *Folsomia fimetaria* (Collembola)	28 - 56 d	S/G/R	Willes and Krogh (1998). Tests with the Collembolans *Isomtoma viridis*, *Folsomia candida* and *Folsomia fimetaria*. In "*Handbook of Soil Invertebrates*" (Eds. Hans Løkke and Cornelis A.M. Van Gestel). John Wiley and Sons: Chichester, UK.	Survival and reproduction assessed weekly (cf. ISO protocol). Dermal and alimentary uptake. Springtails are important soil litter arthropods playing q role in soil organic matter breakdown and nutrients recycling. Feed on bacteria and fungi. The most abundant soil fauna present at 10,000 to 50,000 per m^2. Prey for epigeic invertebrates such as mites, centipedes, spiders and carabid beetles.
Hypoaspis Aculieifer (Gamasid mite) preying on *Folsomia Fimetaria* (Collembola)	21 d	S/G/R	Krogh and Axelson (1998). Test on the predatory mite *Hypoaspis Aculieifer* preying on the Collembolan *Folsomia Fimetaria*. In "*Handbook of Soil Invertebrates*" (Eds. Hans Løkke and Cornelis A.M. Van Gestel). John Wiley and Sons: Chichester, UK.	Mortality, growth and offspring number assessed after three weeks. Natural prey-predator relationship. Predacious species feeding on enchytraeids, nematodes and micro-arthropods. Important role in control of parasitic nematodes. Gamasioda mites are present at 5 - 10,000 per m^2.

Test Organism	Duration	End points	Reference/Source	Comments
Porcellio scaber (Isopoda)	28 – 70 d	S/G/R	Hornung *et al.* (1998). Tests on the Isopod *Porcellio scaber*. In "*Handbook of Soil Invertebrates*" (Eds. Hans Løkke and Cornelis A.M. Van Gestel). John Wiley and Sons: Chichester, UK.	Survival and biomass determined after 4 weeks (weekly measurements). Reproduction (oocyte number, % gravid females, % females releasing juveniles, number offspring) determined after 10 weeks. Alimentary uptake via dosed food or soil. Isopods woodlouse species. Macro-decomposers important part of detritus food chain. Important prey species for centipedes. Estimated population density of isopods is 500 – 1500 per m^2.

Test Organism	Duration	End points	Reference/Source	Comments
Brachydesmus superus (Diplopoda)	70 d	S/R	Tajovsky (1998). Test on the Millipede *Brachydesmus superus*. In "*Handbook of Soil Invertebrates*" (Eds. Hans Løkke and Cornelis A.M. Van Gestel). John Wiley and Sons: Chichester, UK.	Animal number, nest number, egg number and offspring number determined weekly. Difficult to maintain culture throughout year. Alimentary uptake via dosed food or soil. Millipedes are important primary decomposers of leaf litter and organic detritus. Their faecal pellets provide a micro-environment for micro-organisms such as fungi and micro-arthropods. Important prey for carabid beetles, centipedes and spiders and insectivorous birds and mammals. Diplopoda are present at 10 – 100 per m^2.
Lithobius mutabilis (Chilopoda)	28 – 84 d	S/G/L/M	Laskowski *et al.* (1998). Test on the Centipede *Lithobius mutabilis*. In "*Handbook of Soil Invertebrates*" (Eds. Hans Løkke and Cornelis A.M. Van Gestel). John Wiley and Sons: Chichester, UK.	Mortality, biomass, respiration rate and locomotor activity determined after 4 weeks (degradable substances) to 12 weeks (persistent substances). Food chain effect measured via use of dosed prey (fly larvae). Centipedes are important carnivorous arthropods feeding on small earthworms, millipedes, woodlice and springtails. They are in turn prey for birds and mammals. Chilopoda are present up to 100 per m^2.

Test Organism	Duration	End points	Reference/Source	Comments
Philonthus cognatus (Coleoptera)	42 – 70 d	S/R	Metge and Heimbach (1998). Test on the Staphylinid *Philonthus cognatus*. In "*Handbook of Soil Invertebrates*" (Eds. Hans Løkke and Cornelis A.M. Van Gestel). John Wiley and Sons: Chichester, UK.	Beetles exposed for one week to determine subsequent effect on egg production and hatching rate over 6 – 10 weeks. Mortality may also be assessed. Predators of springtails, aphids, dipterans and coleopteran larvae. Prey to birds, mice and large arthropods. Estimated densities of 1 adult per 2 – 5 m^2.
Competition between *Plectus acuminatus* (Nematoda) and *Heterocephalobus pauciannulatus* (Nematoda)	14 d	S/R	Kammenga and Riksen (1998). Test on the competition between the nematodes *Plectus acuminatus* and *Heterocephalobus pauciannulatus*. In "*Handbook of Soil Invertebrates*" (Eds. Hans Løkke and Cornelis A.M. Van Gestel). John Wiley and Sons: Chichester, UK.	Competition between two bacterivorous nematode species. Ratio determined after two weeks. Nematodes are important in decomposition and cycling of organic materials. Abundant and readily retrieved from soil and cultured. Nematodes are the most abundant element of the mesofauna and account for 2% by mass of the edaphon (soil biomass). This corresponds to approximately 10 million per m^2.

Test Organism	Duration	End points	Reference/Source	Comments
Caenorhabditis elegans (Nematoda)	1 d	S	(i) Donkin and Dusenbury (1993). A soil toxicity test using the nematode *Caenorhabditis elegans* and an effective method of recovery. *Arch. Environ. Contam. Toxicol.* 25, 145-151. (ii) Freeman *et al.* (1999). A soil bioassay using the nematode *Caenorhabditis elegans*. ASTM STP 1364. (iii) Peredney and Williams (2000). Utility of *Caenorhabditis elegans* for assessing heavy metal contamination in artificial soil. *Arch. Environ. Contam. Toxicol.* 39, 113-118.	Mortality assessed after 1 d. Important in decomposition and cycling of organic materials. Abundant and readily retrieved from soil and cultured. Nematodes are the most abundant element of the mesofauna and account for 2% by mass of the edaphon (soil biomass). This corresponds to approximately 10 million per m^2 or 1 g per m^2.
Caenorhabditis elegans (Nematoda)	3d	G/R	(i) Neumann-Hensel and Ahlf (1998). Deutsche Bundesstiftung Umwelt Report Number 05446. (ii) Höss (2001). Bestimmung der Wirkung von Sediment- und Bodenproben auf Wachstum und Fruchtbarkeit von *Caenorhabditis elegans* (Nematoda). Draft DIN standard.	Growth and reproduction assessed after 3 days. Abundant and readily retrieved from soil and cultured. Sublethal bioassay (high survival is a pre-requisite for test validity). Nematodes are the most abundant element of the mesofauna and account for 2% by mass of the edaphon (soil biomass). This corresponds to approximately 10 million per m^2 or 1 g per m^2.
Primary Producers				

Test Organism	Duration	End points	Reference/Source	Comments
Many test speciesincluding grass crops (monocotyledonae - Gramineae), *Brassica* spp. (Dicotyledonae – Cruciferae) and bean crops (Dicotyledonae – Leguminosae)	5d, 14 – 21 d	E/G	(i) OECD (2006). OECD 208 Seedling emergence and seedling growth test and OECD 227: Vegetative vigour test. (ii) ISO 11269-1: Soil quality – Determination of the effects of pollutants on soil flora – Part 1: Method for the measurement of inhibition of root growth (1993). (iii) ISO 11269-2 Soil quality – Determination of the effects of pollutants on soil flora – Part 2: Effects of chemicals on the emergence and growth of higher plants (1995). (iv) ASTM E1963-98 Standard guide for conducting terrestrial plant toxicity tests (1998). ISO 22030: Soil quality – Biological methods – Chronic toxicity in higher plants (2005).	Seed emergence (E) and early life stages of growth (G) in treated soils (208) Vegetative vigour (G) following foliar application (227). Root growth of pre-germinated seeds (ISO 11269-1). Minimum of three test species: one monocotyledon and two dicotyledon (OECD 208)

Key: S = survival; E = emergence; G = growth; R = reproduction; M = metabolism; L = locomotory activity

R.7.12 Guidance on Toxicokinetics

R.7.12.1 Upfront information you need to be aware of

The expression of toxicity arising from exposure to a substance is a consequence of a chain of events that results in the affected tissues of an organism receiving the ultimate toxicant in amounts that cause an adverse effect. The factors that confer susceptibility to certain species, and lead to major differences between animals and humans in their response to such chemical insults is based either on the nature and quantity of the ultimate toxicant that is presented to the sensitive tissue (toxicokinetics, TK) or in the sensitivity of those tissues to the ultimate toxicant, i.e. the toxicodynamic (TD) response (ECETOC, 2006).

There is no specific requirement to generate TK information in REACH. Annex I, Section 1.0.2 states that "the human health hazard assessment shall consider the toxicokinetic profile (i.e. absorption, metabolism, distribution and elimination) of the substance". Furthermore, REACH announces in Annex VIII (Section 8.8.1) that one should perform "assessment of the toxicokinetic behaviour of the substance to the extent that can be derived from the relevant available information".

Even though TK is not a toxicological endpoint and is not specifically required by REACH, the generation of TK information can be encouraged as a means to interpret data, assist testing strategy and study design, as well as category development, thus helping to optimise test designs: Prior to any animal study, it is crucial to identify the benefits that will be gained from conducting such a study. Applicability of physiologically based pharmacokinetic/toxicokinetic (PBPK/PBTK) models should also be considered to support or expand understanding of the TK behaviour of a substance (IPCS, 2010). The TK behaviour derived from available data might make further testing unnecessary in terms of predictability of other properties. The definition of actual TK studies on a case-by-case basis might further improve the knowledge about substance properties in terms of expanding knowledge on properties sufficiently to enable risk assessment. Overall the formation of data that are unlikely to be used and that constitute an unnecessary effort of animals, time, and resources shall be avoided using any supporting data to do so. Moreover, it can provide important information for the design of (subsequent) toxicity studies, for the application of read-across and building of categories. Taken together, Along with other approaches, TK can contribute to reduction of animal use under REACH.

The aim of this document is to provide a general overview on the main principles of TK and to give guidance on the generation / use of TK information in the human health risk assessment of substances, and to make use of this information to support testing strategies to become more intelligent (Integrated Testing Strategy, ITS).

The TK phase begins with exposure and results in a certain concentration of the ultimate toxicant at the target site (tissue dose). This concentration is dependent on the absorption, distribution, metabolism and excretion (ADME) of the substance (ECETOC, 2006). ADME describes the uptake of a substance into the body and its lifecycle within the body, (including excretion) (compare EU B.36[24], OECD TG 417):

[24] See Test Methods Regulation (Council Regulation (EC) No 440/2008).

ABSORPTION: how, how much, and how fast the substance enters the body;

DISTRIBUTION: reversible transfer of substances between various parts of the organism, i.e. body fluids or tissues;

METABOLISM: the enzymatic or non-enzymatic transformation of the substance of interest into a structurally different substance (metabolite);

EXCRETION: the physical loss of the parent substance and/or its metabolite(s); the principal routes of excretion are via the urine, bile (faeces), and exhaled air[25].

Metabolism and excretion are the two components of **ELIMINATION**, which describe the loss of substance by the organism, either by physical departure or by chemical transformation. For consistency, and unless otherwise specified, metabolism does not include largely reversible chemical transformations resulting in an observable equilibrium between two chemical species. This latter phenomenon is termed inter-conversion.

The sum of processes following absorption of a substance into the circulatory systems, distribution throughout the body, biotransformation, and excretion is called **DISPOSITION**.

R.7.12.1.1 Absorption

The major routes by which toxicants enter the body are via the lungs, the gastrointestinal tract (both being absorption surfaces by nature), and the skin. To be absorbed, substances must transverse across biological membranes. Mostly this occurs by passive diffusion. As biological membranes are built as layers consisting of lipid as well as aqueous phases a process like this requires a substance to be soluble both in lipid and water. For substances that do not meet these criteria, absorption may occur via facilitated diffusion, active transport or pinocytosis, processes that are more actively directed and therefore require energy).

R.7.12.1.2 Distribution

Once the substance has entered the blood stream, it may exert its toxic action directly in the blood or in any target tissue or organ to which the circulatory system transports or distributes it. It is the blood flow through the organ, the ability of the substance to cross membranes and capillaries, and its relative affinity for the various tissues that determine the rate of distribution and the target tissues. Regarding the cross-membrane transfer not only passive mechanisms but also active transport by transport proteins (e.g. p-glycoprotein) shall be taken into consideration, as this is of particular importance for crossing the blood-brain-barrier but also elsewhere (e.g. in the intestine).

Distribution is in fact a dynamic process involving multiple equilibria: Only the circulatory system is a distinct, closed *compartment* where substances are distributed rapidly. Distribution to the various tissues and organs is usually delayed. However, often compounds distribute so rapidly into the highly perfused tissues, such as liver, kidney and lungs, that kinetics cannot be distinguished from events in the blood; at that point, such organs are classed as being part of the initial, *central* compartment, and *peripheral compartment* is reserved for slowly equilibrating tissues e.g. muscle, skin and adipose.

[25] Breast milk is a minor but potentially important route of excretion.

There is equilibrium of the free substance between the so-called rapid, or central, and the slow or peripheral compartment. As the free substance is eliminated, the substance from the peripheral compartment is slowly released back into the circulation (rapid or central compartment).

This thinking in subdividing the body into different *compartments* is what is made use of in physiologically based kinetic (PBK) modelling. Based on data of available toxicological studies, tissue distribution is mathematically calculated using partition coefficients between blood or plasma and the tissue considered.

R.7.12.1.3 Metabolism or Biotransformation

Biotransformation is one of the main factors, which influence the fate of a substance in the body, its toxicity, and its rate and route of elimination. Traditionally biotransformation is divided into two main phases, phase I and phase II. Phase I, the so-called functionalisation phase, has a major impact on lipophilic molecules, rendering them more polar and more readily excretable. In phase II, often referred to as detoxicification, such functionalised moieties are subsequently conjugated with highly polar molecules before they are excreted. Both phases are catalysed by specific enzymes which are either membrane-bound (microsomal proteins) or present in the cytosol (cytosolic or soluble enzymes). Furthermore, it has been suggested that a phase III relates to the excretion of conjugates and involves ATP[26]-dependent plasma membrane transporters.

Most substances are potentially susceptible to biotransformation of some sort, and all cells and tissues are potentially capable of biotransforming compounds. However, the major sites of such biotransformation are substrate- and route-dependent; generally, the liver and the entry portals of the body are the main biotransformation sites to be considered. Notably, variations occur in the presence of metabolising enzymes in different tissues, and also between different cells in the same organ. Another aspect is the existence of marked differences between and within various animal species and humans in the expression and catalytic activities of many biotransforming enzymes. Any knowledge concerning metabolic differences may provide crucial insight in characterising the potential risk of substances to humans.

R.7.12.1.4 Excretion

As substances are absorbed at different entry portals, they can be excreted via various routes and mechanisms. The relative importance of the excretion processes depends on the physical and chemical properties of the compound and its various metabolites.

Besides passive transportation (diffusion or filtration) there are carrier-mediated mechanisms to shuttle a substance through a biological membrane. It is well known that there are a variety of pumps responsible for transportation of specific types of substances (e.g. sodium, potassium, magnesium, organic acids, and organic bases). Related compounds may compete for the same transport mechanism. Additional transport systems, phagocytosis and pinocytosis, can also be of importance (e.g. in the removal of particulate matter from the alveoli by alveolar phagocytes, and the removal

[26] Adenosine-tri-phosphate.

of some large molecules (Pritchard, 1981) from the body by the reticulo-endothelial system in the liver and spleen (Klaassen, 1986)).

R.7.12.1.5 Bioavailability, saturation vs. non-linearity and Accumulation

The most critical factor influencing toxicity is the concentration of the ultimate toxicant at the actual target site (tissue dose). In this context bioavailability is a relevant parameter for the assessment of the toxicity profile of a test substance. It links dose and concentration of a substance with the mode of action, which covers the key events within a complete sequence of events leading to toxicity.

Bioavailability

Bioavailability usually describes the passage of a substance from the site of absorption into the blood of the general (systemic) circulation, thus meaning systemic bioavailability (Nordberg *et al.*, 2004). The fact that at least some of the substance considered is systemically bioavailable is often referred to as systemic exposure.

Systemic bioavailability is not necessarily equivalent to the amount of a substance absorbed, because in many cases parts of that amount may be excreted or metabolised before reaching the systemic circulation. This may occur, for instance, for substances metabolised in the gut after oral exposure before any absorption has taken place. Conversely, substances absorbed from the intestine can be partly eliminated by the liver at their first passage through that organ (so-called first-pass effect).

Linearity vs. non-linearity and Saturation

When all transfer rates between the different compartments of the body are proportional to the amounts or concentrations present (this is also called a process of first order), a process is called linear. This implies that the amounts of a substance cleared and distributed as well as half-lives are constant and the concentrations are proportional to the dosing rate (exposure). Such linear kinetics display the respective dose-toxicity-relationships.

Once a kinetic process is saturated (e.g. by high level dosing/exposure) by the fact that enzymes involved in biotransformation processes, or transporters involved in distribution or elimination, or binding proteins (i.e. receptors) are inhibited or reaching their maximum activity, a process might become non-linear. This may result in concentration or dose-dependency, or time-dependency of some of the kinetic characteristics. In some cases this can lead to a change in biotransformation products or the metabolic capacity. It is advised to consider systematically the possible sources for non-linear kinetics, especially for repeated dose testing.

Accumulation (Kroes *et al.*, 2004)

Everything in a biological system has a biological half-life, that is, a measure of how long it will stay in that system until it is lost by mainly excretion, degradation, or metabolism. To put it in different words, the amount of a substance eliminated from the blood in unit time, is the product of clearance (the volume of blood cleared per unit time) and concentration (the amount of a compound per unit volume). For first order reactions, clearance is a constant value that is a characteristic of a substance. If the input of a substance to an organism is greater than the rate at which the substance is lost, the organism is said to be accumulating that substance. When the concentration has increased such that the amount eliminated equals the amount of substance-input there will be a constant concentration, a steady-state. The extent of accumulation reflects the relationship between the body-burden compared with the steady-state condition. Species differences in clearance will determine the difference in steady-state body-burden between experimental animals and humans.

R.7.12.2 Toxicokinetics in practice – derivation and generation of information

In general, testing a substance for its toxicological profile is performed in laboratory animals exposed to a range of dosages or concentrations by the most appropriate route of administration derived from the most likely human exposure scenario. In assessing gained information in terms of human relevance, the conservative approach of applying an *assessment factor* (default approach) is used for taking into account uncertainties over interspecies and intraspecies differences in sensitivity to a specific test substance.

In situations, e.g. where humans are demonstrably much less sensitive than the test species or, indeed, where it is known that the effects seen in the test animal would under no circumstances be manifested in humans, such conservatism can be considered inappropriate (ECETOC, 2006). The mode of action (key events in the manifestation of toxicity) underlying the effect can justify departure from the default approach and enable a more realistic risk assessment by the arguments even to the point of irrelevance for the human situation.

A tiered approach has been proposed by SANCO (EC, 2007) for the risk assessment of a substance. In alignment with this, a strategy can be derived on how much effort on TK evaluation for different levels of importance of a substance is appropriate. Considerations on the possible activity profile of a substance derived from physico-chemical and other data, as well as structurally related substances should be taken into account as a minimum request. This might help in the argumentation on waiving or triggering further testing and could provide a first impression of the mode of action of a substance. Subsequent toxicokinetic data needs to be focussed on which studies are needed to interpret and direct any additional toxicity studies that may be conducted. The advantage of such effort is that the results enable the refinement of the knowledge of the activity of a substance by elucidating step by step the mode of action. In this cascade, the application of assessment factors changes from overall default values to chemical specific adjustment factors (CSAFs).

R.7.12.2.1 Derivation of toxicokinetic information taking into account a Basic Data Set

The standard information requirements of REACH for substances manufactured or imported in quantities of ≥1 ton (see Annex VII of the respective regulation), include mainly physico-chemical (PC) data, and data like skin irritation/corrosion, eye irritation, skin sensitisation, *in vitro* mutagenicity, acute oral toxicity, short-term aquatic toxicity on invertebrates, growth inhibition of algae. Therefore, these data will be available for the majority of substances. This data will enable qualitative judgments of the TK behaviour. However, the physico-chemical characteristics of the substance will change if the substance undergoes metabolic transformation and the physico-chemical characteristics of the parent substance may not provide any clues as to the identity, distribution, retention and elimination of its metabolites. These are important factors to consider.

Absorption

Absorption is a function of the potential for a substance to diffuse across biological membranes. In addition to molecular weight the most useful parameters providing information on this potential are the octanol/water partition coefficient (log P) value and the water solubility. The log P value provides information on the relative solubility of the substance in water and the hydrophobic solvent octanol (used as a surrogate for lipid) and is a measure of lipophilicity. Log P values above 0 indicate that the substance is more soluble in octanol than water i.e. lipophilic and negative values indicate that the substance is more soluble in water than octanol i.e. hydrophilic. In general, log P values between -1 and 4 are favourable for absorption. Nevertheless, a substance with such a log P value can be poorly soluble in lipids and hence not readily absorbed when its water solubility is very low. It is therefore important to consider both, the water solubility of a substance and its log P value, when assessing the potential of that substance to be absorbed.

Oral / GI absorption

When assessing the potential of a substance to be absorbed in the gastrointestinal (GI) tract it should be noted that substances could undergo chemical changes in the GI fluids as a result of metabolism by GI flora, by enzymes released into the GI tract or by hydrolysis. These changes will alter the physico-chemical characteristics of the substance and hence predictions based upon the physico-chemical characteristics of the parent substance may no longer apply (see

Appendix R.7.12-1 for a detailed listing of *physiological factors*, data on stomach and intestine pH, data on transit time in the intestine and Table R.7.12—1).

One consideration that could influence the absorption of ionic substances (i.e. acids and bases) is the varying pH of the GI tract. It is generally thought that ionised substances do not readily diffuse across biological membranes. Therefore, when assessing the potential for an acid or base to be absorbed, knowledge of its pKa (pH at which 50% of the substance is in ionised and 50% in non-ionised form) is advantageous. Absorption of acids is favoured at pHs below their pKa whereas absorption of bases is favoured at pHs above their pKa.

Other mechanisms by which substances can be absorbed in the GI tract include the passage of small water-soluble molecules (molecular weight up to around 200) through aqueous pores or carriage of such molecules across membranes with the bulk passage of water (Renwick, 1994). The absorption of highly lipophilic substances (log P of 4 or above) may be limited by the inability of such substances to dissolve into GI fluids and hence make contact with the mucosal surface. However, the absorption of such substances will be enhanced if they undergo micellular solubilisation by bile salts (Aungst and Shen, 1986). Substances absorbed as micelles (aggregate of surfactant molecules, lowering surface tension) enter the circulation via the lymphatic system, bypassing the liver. Although particles and large molecules (with molecular weights in the 1000's[27]) would normally be considered too large to cross biological membranes, small amounts of such substances may be transported into epithelial cells by pinocytosis or persorption (passage through gaps in membranes left when the tips of villi are sloughed off) (Aungst and Shen, 1986). Absorption of surfactants or irritants may be enhanced because of damage to cell membranes.

Absorption can occur at different sites and with different mechanisms along the GI tract. In the *mouth* absorption is minimal and if at all, occurs by passive diffusion. Therefore, substances enter directly the systemic circulation, however, some enzymatic degradation may occur. Like in the mouth, absorption in the *stomach* is minimal and occurs only by passive diffusion - the acidic environment favours uptake of weak acids. There is a potential for hydrolysis and, very rarely, metabolism (by endogenous enzymes) prior to uptake. Once absorbed at this point, substances will go to the liver before entering the systemic circulation - first pass metabolism may then limit the systemic bioavailability of the parent compound. The *small intestine* has a very large surface area and the transit time through this section is the longest, making this the predominant site of absorption within the GI tract. Most substances will be absorbed by passive diffusion. However, lipophilic compounds may form micelles and be absorbed into the lymphatic system and larger molecules/particles may be taken up by pinocytosis. Metabolism prior to absorption may occur by gut microflora or enzymes in the GI mucosa. Since substances that enter the blood at this point pass through the liver before entering the systemic circulation, hepatic first pass metabolism may limit the amount of parent compound that enters the systemic circulation. In the *large intestine*, absorption occurs mainly by passive diffusion. But active transport mechanisms for electrolytes are present, too. Compared to the small intestine, the rate and extent of absorption within the large intestine is low. Most blood flow from the large intestine passes through the liver first.

Table R.7.12—1 Interpretation of data regarding oral/GI absorption

Data source	What it tells us
Structure	It may be possible to identify ionisable groups within the structure of the molecule. Groups containing oxygen, sulphur or nitrogen atoms e.g. thiol (SH), sulphonate (SO3H), hydroxyl (OH), carboxyl (COOH) or amine (NH2) groups are all potentially ionisable.
Molecular Weight[27]	Generally the smaller the molecule the more easily it may be taken up. Molecular weights below 500 are favourable for absorption; molecular weights above 1000 do not favour absorption.
Particle size	Generally solids have to dissolve before they can be absorbed. It may be possible for particles in the nanometer size range to be taken up by pinocytosis. The absorption of very large particles, several hundreds of micrometers in diameter, that were administered dry (e.g. in the diet) or in a suspension may be reduced because of the time taken for the particle to

[27] In the Chapter R.11 of the Guidance on IR&CSA (2023), the molecular weight parameter has been removed as indicator of limited uptake.

Data source	What it tells us
	dissolve. This would be particularly relevant for poorly water-soluble substances.
Water Solubility	Water-soluble substances will readily dissolve into the gastrointestinal fluids. Absorption of very hydrophilic substances by passive diffusion may be limited by the rate at which the substance partitions out of the gastrointestinal fluid. However, if the molecular weight is low (less than 200) the substance may pass through aqueous pores or be carried through the epithelial barrier by the bulk passage of water.
Log P	Moderate log P values (between -1 and 4) are favourable for absorption by passive diffusion. Any lipophilic compound may be taken up by micellular solubilisation but this mechanism may be of particular importance for highly lipophilic compounds (log P >4), particularly those that are poorly soluble in water (1 mg/l or less) that would otherwise be poorly absorbed.
Dosing Vehicle	If the substance has been dosed using a vehicle, the water solubility of the vehicle and the vehicle/water partition coefficient of the substance may affect the rate of uptake. Compounds delivered in aqueous media are likely absorbed more rapidly than those delivered in oils, and compounds delivered in oils that can be emulsified and digested e.g. corn oil or arachis oil are likely to be absorbed to a greater degree than those delivered in non-digestible mineral oil (liquid petrolatum) (d'Souza, 1990) or in soil, the latter being an important vehicle for children.
Oral toxicity data	If signs of systemic toxicity are present, then absorption has occurred[28]. Also colored urine and/or internal organs can provide evidence that a colored substance has been absorbed. This information will give no indication of the amount of substance that has been absorbed. Also some clinical signs such as hunched posture could be due to discomfort caused by irritation or simply the presence of a large volume of test substance in the stomach and reduced feed intake could be due to an unpalatable test substance. It must therefore be clear that the effects that are being cited as evidence of systemic absorption are genuinely due to absorbed test substance and not to local effects at the site of contact effects.
Hydrolysis Test	Hydrolysis data are not always available. The hydrolysis test (EU C.7[29]; OECD TG 111) conducted for >10 tons substances notified under REACH (Annex VIII) provides information on the half-life of the substance in water at 50°C and pH values of 4.0, 7.0 and 9.0. The test is conducted using a low concentration, 0.01 M or half the concentration of a saturated aqueous solution (whichever is lower). Since the temperature at which this test is conducted is much higher than that in the GI tract, this test will not provide an estimate of the actual hydrolysis half-life of the substance in the GI tract. However, it may give an indication that the parent compound may only be present in the GI tract for a limited period of time. Hence, toxicokinetic

[28] Ensure that systemic effects do not occur secondary to local effects!

[29] See Test Methods Regulation (Council Regulation (EC) No 440/2008).

Data source	What it tells us
	predictions based on the characteristics of the parent compound may be of limited relevance.

Respiratory absorption – Inhalation

For inhaled substances the processes of deposition of the substance on the surface of the respiratory tract and the actual absorption have to be differentiated. Both processes are influenced by the physico-chemical characteristics of a substance (Table R.7.12—2).

Substances that can be inhaled include gases, vapours, liquid aerosols (both liquid substances and solid substances in solution) and finely divided powders/dusts. Substances may be absorbed directly from the respiratory tract or, through the action of clearance mechanisms, may be transported out of the respiratory tract and swallowed. This means that absorption from the GI tract will contribute to the total systemic burden of substances that are inhaled.

To be readily soluble in blood, a gas or vapour must be soluble in water and increasing water solubility would increase the amount absorbed per breath. However, the gas or vapour must also be sufficiently lipophilic to cross the alveolar and capillary membranes. Therefore, a moderate log P value (between -1 and 4) would be favourable for absorption. For vapours, the deposition pattern of readily soluble substances differs from lipophilic substances in that the hydrophilic are effectively removed from the air in the upper respiratory tract, whereas the lipophilic reach the deep lung and thus absorption through the huge gas exchange region may occur. The rate of systemic uptake of very hydrophilic gases or vapours may be limited by the rate at which they partition out of the aqueous fluids (mucus) lining the respiratory tract and into the blood. Such substances may be transported out of the deposition region with the mucus and swallowed or may pass across the respiratory epithelium via aqueous membrane pores. Highly reactive gases or vapours can react at the site of contact thereby reducing the amount available for absorption. Besides the physico-chemical properties of the compound physical activity (such as exercise, heavy work, etc.) has a great impact on absorption rate and must also be addressed (Csanady and Filser, 2001).

Precise deposition patterns for dusts will depend not only on the particle size of the dust but also the hygroscopicity, electrostatic properties and shape of the particles and the respiratory dynamics of the individual. As a rough guide, particles with aerodynamic diameters below 100 μm have the potential to be inspired. Particles with aerodynamic diameters below 50 μm may reach the thoracic region and those below 15 μm the alveolar region of the respiratory tract. These values are lower for experimental animals with smaller dimensions of the structures of the respiratory tract. Particles with aerodynamic diameters of above 1-5 μm have the greatest probability of settling in the nasopharyngeal region whereas particles with aerodynamic diameters below 1-5 μm are most likely to settle in the tracheo-bronchial or pulmonary regions (Velasquez, 2006). Thus the quantitative deposition pattern of particles in the respiratory tract varies. Nonetheless general deposition patterns may be derived (Snipes, 1989). Several models exist to predict the particle size deposition patterns in the respiratory tract (US EPA, 1994).

Generally, liquids, solids in solution and water-soluble dusts would readily diffuse/dissolve into the mucus lining the respiratory tract. Lipophilic substances (log P >0) would then have the potential to be absorbed directly across the respiratory tract epithelium. There is some evidence to suggest that substances with higher log P values may have a longer half-life within the lungs but this has not been extensively studied (Cuddihy and Yeh, 1988). Very hydrophilic substances might be absorbed through aqueous pores (for substances with molecular weights below around 200) or be retained in the mucus and transported out of the respiratory tract. For poorly water-soluble dusts, the rate at which the particles dissolve into the mucus will limit the amount that can be absorbed directly. Poorly water-soluble dusts depositing in the nasopharyngeal region could be coughed or sneezed out of the body or swallowed (Schlesinger, 1995). Such dusts depositing in the tracheo-bronchial region would mainly be cleared from the lungs by the mucocilliary mechanism and swallowed. However a small amount may be taken up by phagocytosis and transported to the blood via the lymphatic system. Poorly water-soluble dusts depositing in the alveolar region would mainly be engulfed by alveolar macrophages. The macrophages will then either translocate particles to the ciliated airways or carry particles into the pulmonary interstitium and lymphoid tissues.

Table R.7.12—2 Interpretation of data regarding respiratory absorption

Data source	What it tells us
Vapour Pressure	Indicates whether a substance may be available for inhalation as a vapour. As a general guide, highly volatile substances are those with a vapour pressure greater than 25 KPa (or a boiling point below 50°C). Substances with low volatility have a vapour pressure of less than 0.5 KPa (or a boiling point above 150°C)
Particle size	Indicates the presence of inhalable/respirable particles. In humans, particles with aerodynamic diameters below 100 μm have the potential to be inhaled. Particles with aerodynamic diameters below 50 μm may reach the thoracic region and those below 15 μm the alveolar region of the respiratory tract. These values are lower for experimental animals with smaller dimensions of the structures of the respiratory tract. Thus the quantitative deposition pattern of particles in the respiratory tract varies with the particle size distribution of the inspired aerosol and may further depend on physical and physico-chemical properties of the particles (e.g. shape, electrostatic charge). Nonetheless general deposition patterns may be derived (Snipes, 1989; US EPA, 1994)
Log P	Moderate log P values (between -1 and 4) are favourable for absorption directly across the respiratory tract epithelium by passive diffusion. Any lipophilic compound may be taken up by micellular solubilisation but this mechanism may be of particular importance for highly lipophilic compounds (log P >4), particularly those that are poorly soluble in water (1 mg/l or less) that would otherwise be poorly absorbed.
Water Solubility	Deposition: Vapours of very hydrophilic substances may be retained within the mucus. Low water solubility, like small particle size enhances penetration to the lower respiratory tract. For absorption of deposited material similar criteria as for GI absorption apply
Inhalation toxicity data	If signs of systemic toxicity are present then absorption has occurred. This is not a quantitative measure of absorption.
Oral toxicity data	If signs of systemic toxicity are present in an oral toxicity study or there are other data to indicate the potential for absorption following ingestion it is likely the substance will also be absorbed if it is inhaled.
Hydrolysis Test	Hydrolysis data are not always available. The hydrolysis test (EU C.7[30], OECD TG 111) conducted for >10 tons substances notified under REACH (Annex VIII) provides information on the half-life of the substance in water at 50°C and pH values of 4.0, 7.0 and 9.0. The test is conducted using a low concentration, 0.01 M or half the concentration of a saturated aqueous solution (whichever is lower). Since the temperature at which this test is conducted is much higher than that in the respiratory tract, this test will not provide an estimate of the actual hydrolysis half-life of the substance in the respiratory tract. However, it may give an indication that the parent compound may only be present in the respiratory tract for a limited period of time. Hence, toxicokinetic predictions based on the characteristics of the parent compound may be of limited relevance.

Dermal absorption

The skin is a dynamic, living multilayered biomembrane and as such its permeability may vary as a result of changes in hydration, temperature, and occlusion. In order to cross the skin, a compound must first penetrate into the *stratum corneum* (non-viable layer of corneocytes forming a complex lipid membrane) and may subsequently reach the viable *epidermis*, the *dermis* and the *vascular network*. The stratum corneum provides its greatest barrier function against hydrophilic compounds, whereas the viable epidermis is most resistant to penetration by highly lipophilic compounds (Flynn, 1985).

Dermal absorption represents the amount of topically applied test substance that is found in the epidermis (stratum corneum excluded) and in the dermis, and this quantity is therefore taken as systemically available. Dermal absorption is influenced by many factors, e.g. physico-chemical properties of the substance, its vehicle and concentration, and the exposure pattern (e.g. occlusion of the application site) as well as the skin site of the body (for review see ECETOC, 1993; Howes *et al.*, 1996; Schaefer and Redelmaier, 1996) (Table R.7.12—3). The term *percutaneous penetration* refers to *in vitro* experiments and represents the amount of topically applied test substance that is found in the receptor fluid – this quantity is taken as systemically available.

Substances that can potentially be taken up across the skin include gases and vapours, liquids and particulates. A tiered approach for the estimation of skin absorption has been proposed within a risk assessment framework (EC, 2007): Initially, basic physico-chemical information should be taken into account, i.e. molecular mass and lipophilicity (log P). Following, a default value of 100% skin absorption is generally used unless molecular mass is above 500 and log P is outside the range [-1, 4], in which case a value of 10%[31] skin absorption is chosen (de Heer *et al.*, 1999). A flow diagram outlining this tiered approach is presented in Appendix R.7.12-4.

Table R.7.12—3 Interpretation of data regarding dermal absorption

Data source	What it tells us
Physical State	Liquids and substances in solution are taken up more readily than dry particulates. Dry particulates will have to dissolve into the surface moisture of the skin before uptake can begin. Absorption of volatile liquids across the skin may be limited by the rate at which the liquid evaporates off the skin surface (Pryde and Payne, 1999).
Molecular Weight	Less than 100 favours dermal uptake. Above 500 the molecule may be too large.

[30] See Test Methods Regulation (Council Regulation (EC) No 440/2008).

[31] The lower limit of 10% was chosen, because there is evidence in the literature that substances with molecular weight and/or log P values at these extremes can to a limited extent cross the skin. If data are available (e.g. data on water solubility, ionogenic state, 'molecular volume', oral absorption and dermal area dose in exposure situations in practice) which indicate the use of an alternative dermal absorption percentage value is appropriate, then this alternative value can be used. Scientific justification for the use of alternative values should be provided.

Data source	What it tells us
Structure	As a result of binding to skin components the uptake of substances with the following groups can be slowed: certain metal ions, particularly Ag^{+}, Cd^{2+}, Be^{2+} and Hg^{2+} acrylates, quaternary ammonium ions, heterocyclic ammonium ions, sulphonium salts. A slight reduction in the dermal uptake of substances belonging to the following chemical classes could also be anticipated for the same reason: Quinines, dialkyl sulphides, acid chlorides, halotriazines, dinitro or trinitro benzenes.
Water Solubility	The substance must be sufficiently soluble in water to partition from the stratum corneum into the epidermis. Therefore if the water solubility is below 1 mg/l, dermal uptake is likely to be low. Between 1-100 mg/l absorption is anticipated to be low to moderate and between 100-10,000 mg/l moderate to high. However, if water solubility is above 10,000 mg/l and the log P value below 0 the substance may be too hydrophilic to cross the lipid rich environment of the stratum corneum. Dermal uptake for these substances will be low.
Log P	For substances with log P values <0, poor lipophilicity will limit penetration into the stratum corneum and hence dermal absorption. Values <–1 suggest that a substance is not likely to be sufficiently lipophilic to cross the stratum corneum, therefore dermal absorption is likely to be low. Log P values between 1 and 4 favour dermal absorption (values between 2 and 3 are optimal) particularly if water solubility is high. Above 4, the rate of penetration may be limited by the rate of transfer between the stratum corneum and the epidermis, but uptake into the stratum corneum will be high. Above 6, the rate of transfer between the stratum corneum and the epidermis will be slow and will limit absorption across the skin. Uptake into the stratum corneum itself may be slow.
Vapour Pressure	The rate at which gases and vapours partition from the air into the stratum corneum will be offset by the rate at which evaporation occurs therefore although a substance may readily partition into the stratum corneum, it may be too volatile to penetrate further. This can be the case for substances with vapour pressures above 100-10,000 Pa (ca. 0.76-76 mm Hg) at 25°C, though the extent of uptake would also depend on the degree of occlusion, ambient air currents and the rate at which it is able to transfer across the skin. Vapours of substances with vapour pressures below 100 Pa are likely to be well absorbed and the amount absorbed dermally may be more than 10% of the amount that would be absorbed by inhalation.

Data source	What it tells us
Surface Tension	If the surface tension of an aqueous solution is less than 10 mN/m, the substance is a surfactant and this will enhance the potential dermal uptake. Surfactants can also substantially enhance the absorption of other compounds, even in the absence of skin irritant effects.
Skin irritation / Corrosivity	If the substance is a skin irritant or corrosive, damage to the skin surface may enhance penetration.
Dermal toxicity data	Signs of systemic toxicity indicate that absorption has occurred. However, if steps have not been taken to prevent grooming, the substance may have been ingested and therefore signs of systemic toxicity could be due to oral rather than dermal absorption.
Skin sensitisation data	If the substance has been identified as a skin sensitiser then, provided the challenge application was to intact skin, some uptake must have occurred although it may only have been a small fraction of the applied dose.
Trace elements	If the substance is a cationic trace element, absorption is likely to be very low (<1%). Stable or radio-isotopes should be used and background levels determined to prevent analytical problems and inaccurate recoveries.

Even though many factors (Table R.7.12—3) are linked to the substance itself, one should bear in mind that the final preparation or the conditions of its production or use can influence both rate and extent of dermal absorption. These factors should also be taken into account in the risk assessment process, including at the stage of estimating dermal absorption[32]. Also, the methods described are focused on the extent of absorption, and not on its rate (with the exception of *in vitro* studies), which can play a major role in determining acute toxicity.

Distribution

The concentration of a substance in blood or plasma (blood level) is dependent on the dose, the rates of absorption, distribution and elimination, and on the affinity of the tissues for the compound. Tissue affinity is usually described using a parameter known as volume of distribution, which is a proportionality factor between the amount of compound present in the body and the measured plasma or blood concentration. The larger the volume of distribution is, the lower the blood level will be for a given amount of compound in the body. A particularly useful volume term is the volume of distribution at steady-state (Vd_{ss}). At steady-state, all distribution phenomena are completed, the various compartments of the body are in equilibrium, and the rate of elimination is exactly compensated by the rate of absorption. In non steady-state situations, the distribution volume varies with time except in the simplest case of a single-compartment model. In theory, steady-state can be physically reached only in the case of a constant zero-order input rate and stable first-order distribution and elimination rates. However, many real situations are reasonably close to steady-state, and reasoning at steady-state is a useful method in kinetics.

[32] In determining the dermal penetration the dosing vehicle seems to be of great importance!

The rate at which highly water-soluble molecules distribute may be limited by the rate at which they cross cell membranes and access of such substances to the central nervous system (CNS) or testes is likely to be restricted by the blood-brain and blood-testes barriers (Rozman and Klaassen, 1996). It is not clear what barrier properties the placenta may have. However, species differences in transplacental transfer may occur due to differing placental structure and also differing metabolic capacity of the placenta and placental transporters in different species.

Although protein binding can limit the amount of a substance available for distribution, it will generally not be possible to determine from the available data which substances will bind to proteins and how avidly they will bind. Furthermore, if a substance undergoes extensive first-pass metabolism, predictions made on the basis of the physico-chemical characteristics of the parent substance may not be applicable.

Table R.7.12—4 Interpretation of data regarding distribution

Data source	What it tells us
Molecular Weight	In general, the smaller the molecule, the wider the distribution.
Water Solubility	Small water-soluble molecules and ions will diffuse through aqueous channels and pores. The rate at which very hydrophilic molecules diffuse across membranes could limit their distribution.
Log P	If the molecule is lipophilic (log P >0), it is likely to distribute into cells and the intracellular concentration may be higher than extracellular concentration particularly in fatty tissues.
Target Organs	If the parent compound is the toxicologically active species, it may be possible to draw some conclusions about the distribution of that substance from its target tissues. If the substance is a dye, coloration of internal organs can give evidence of distribution. This will not provide any information on the amount of substance that has distributed to any particular site. Note that anything present in the blood will be accessible to the bone marrow.
Signs of toxicity	Clear signs of CNS effects indicate that the substance (and/or its metabolites) has distributed to the CNS. However, not all behavioural changes indicate that the substance has reached the CNS. The behavioural change may be due to discomfort caused by some other effect of the substance.

Accumulative potential

It is important to consider the potential for a substance to accumulate or to be retained within the body, because as they will then gradually build up with successive exposures the body burden can be maintained for long periods of time.

Lipophilic substances have the potential to accumulate within the body if the dosing interval is shorter than 4 times the whole body half-life. Although there is no direct correlation between the lipophilicity of a substance and its biological half-life, substances with high log P values tend to have longer half-lives unless their large volume of distribution is counter-balanced by a high clearance. On this basis, there is the potential for highly lipophilic substances (log P >4) to accumulate in individuals that are frequently exposed (e.g. daily at work) to that substance. Once exposure stops, the concentration within the body will decline at a rate determined by the half-life of the substance. Other substances that can accumulate within the body include poorly soluble particulates that deposited in the alveolar region of the lungs, substances that bind irreversibly to endogenous proteins and certain metals and ions that interact with the matrix of the bone (Rozman and Klaassen, 1996).

Table R.7.12—5 Interpretation of data regarding accumulation

Site	Characteristics of substances of concern
Lung	Poorly water and lipid soluble particles (i.e. log P values around 0 and water solubility around 1 mg/l or less) with aerodynamic diameters of 1 μm or below have the potential to deposit in the alveolar region of the lung. Here particles are likely to undergo phagocytosis by alveolar macrophages. The macrophages will then either translocate particles to the ciliated airways or carry particles into the pulmonary interstitium and lymphoid tissues. Particles can also migrate directly to the pulmonary interstitium and this is likely to occur to the greatest extent where the particle is toxic to alveolar macrophages or inhaled in sufficient quantities to overwhelm the phagocytic capabilities of alveolar macrophages. Within the pulmonary interstitium clearance depends on solubilisation alone, which leads to the possibility of long-term retention (Snipes, 1995).
Adipose tissue	Lipophilic substances will tend to concentrate in adipose tissue and depending on the conditions of exposure may accumulate. If the interval between exposures is less than 4 times the whole body half-life of the substance then there is the potential for the substance to accumulate. It is generally the case that substances with high log P values have long biological half-lives. On this basis, daily exposure to a substance with a log P value of around 4 or higher could result in a build up of that substance within the body. Substances with log P values of 3 or less would be unlikely to accumulate with the repeated intermittent exposure patterns normally encountered in the workplace but may accumulate if exposures are continuous. Once exposure to the substance stops, the substance will be gradually eliminated at a rate dependent on the half-life of the substance. If fat reserves are mobilised more rapidly than normal, e.g. if an individual or animal is under stress or during lactation there is the potential for large quantities of the parent compound to be released into the blood.
Bone	Certain metals e.g. lead and small ions such as fluoride can interact with ions in the matrix of bone. In doing so they can displace the normal constituents of the bone, leading to retention of the metal or ion.
Stratum corneum	Highly lipophilic substances (log P between 4 and 6) that come into contact with the skin can readily penetrate the lipid rich stratum corneum but are not well absorbed systemically. Although they may persist in the stratum corneum, they will eventually be cleared as the stratum corneum is sloughed off.

Metabolism

Differences in the way substances are metabolised by different species and within different tissues is the main reason for species and route specific toxicity. The liver has the greatest capacity for metabolism and is commonly causing route specific presystemic effects (first pass) especially following oral intake. However, route specific toxicity may result from several phenomena, such as hydrolysis within the GI or respiratory tracts, also metabolism by GI flora or within the GI tract epithelia (mainly in the small intestine) (for review see Noonan and Wester, 1989), respiratory tract epithelia (sites include the nasal cavity, tracheo-bronchial mucosa [Clara cells] and alveoli [type 2 cells]) and skin.

It is very difficult to predict the metabolic changes a substance may undergo on the basis of physico-chemical information alone. Although it is possible to look at the structure of a molecule and identify potential metabolites, it is by no means certain that these reactions will occur *in vivo* (e.g. the molecule may not reach the necessary site for a particular reaction to take place). It is even more difficult to predict the extent to which it will be metabolised along different pathways and what species differences may exist. Consequently, experimental data shall help in the assessment of potential metabolic pathways (see Section R.7.12.2.2).

Excretion

The major routes of excretion for substances from the systemic circulation are the urine and/or the faeces (via bile and directly from the GI mucosa; see Rozman, 1986).

The excretion processes involved in the *kidney* are passive glomerular filtration through membrane pores and active tubular secretion via carrier processes. Substances that are excreted in the urine tend to be water-soluble and of low molecular weight (below 300 in the rat, mostly anionic and cationic compounds) and generally, they are conjugated metabolites (e.g., glucuronides, sulphates, glycine conjugates) from Phase II biotransformation. Most of them will have been filtered out of the blood by the kidneys though a small amount may enter the urine directly by passive diffusion and there is the potential for re-absorption into the systemic circulation across the tubular epithelium.

Biliary excretion (Smith, 1973) involves active secretion rather than passive diffusion. Substances that are excreted in the bile tend to have higher molecular weights or may be conjugated as glucuronides or glutathione derivatives. In the rat it has been found that substances with molecular weights below around 300 do not tend to be excreted into the bile (Renwick, 1994). There are species differences and the exact nature of the substance also plays a role (Hirom *et al.*, 1972; Hirom *et al.*, 1976; Hughes *et al.*, 1973). The excretion of compounds via bile is highly influenced by hepatic function as metabolites formed in the liver may be excreted directly into the bile without entering the bloodstream. Additionally, blood flow as such is a determining factor.

Substances in the bile pass through the intestines before they are excreted in the faeces and as a result may undergo enterohepatic recycling (circulation of bile from the liver, where it is produced, to the small intestine, where it aids in digestion of fats and other substances, back to the liver) which will prolong their biological half-life. This is a particularly problem for conjugated molecules that are hydrolysed by GI bacteria to form smaller more lipid soluble molecules that can then be reabsorbed from the GI tract. Those substances less likely to re-circulate are substances having strong polarity and high molecular weight. Other substances excreted in the faeces are those that have diffused out of the systemic circulation into the gastrointestinal tract directly, substances which have been removed from the gastrointestinal mucosa by efflux mechanisms and non-absorbed substances that have been ingested or inhaled and subsequently swallowed. However, depending on the metabolic changes that may have occurred, the compound that is finally excreted may have few or none of the physico-chemical characteristics of the parent compound.

Table R.7.12—6 Interpretation of data regarding excretion

Route	Favourable physico-chemical characteristics
Urine	Characteristics favourable for urinary excretion are low molecular weight (below 300 in the rat), good water solubility, and ionisation of the molecule at the pH of urine.
Exhaled Air	Vapours and gases are likely to be excreted in exhaled air. Also volatile liquids and volatile metabolites may be excreted as vapours in exhaled air.
Bile	In the rat, molecules that are excreted in the bile are amphipathic (containing both polar and nonpolar regions), hydrophobic/strongly polar and have a high molecular weight. In general, in rats for organic cations with a molecular weight below 300 it is unlikely that more than 5-10% will be excreted in the bile, for organic anions e.g. quaternary ammonium ions this cut off may be lower (Smith, 1973). Substances excreted in bile may potentially undergo enterohepatic circulation. This is particularly a problem for conjugated molecules that are hydrolysed by gastrointestinal bacteria to form smaller more lipid soluble molecules that can then be reabsorbed from the GI tract. Those substances less likely to re-circulate are substances having strong polarity and high molecular weight. Little is known about the determinants of biliary excretion in humans.
Breast milk	Substances present in plasma generally also may be found in breast milk. Lipid soluble substances may be present at higher concentrations in milk than in blood/plasma. Although lactation is minor route of excretion, exposure of neonates via nursing to mother’s milk may have toxicological significance for some substances.
Saliva/sweat	Non-ionised and lipid soluble molecules may be excreted in the saliva, where they may be swallowed again, or in the sweat.
Hair/nails	Metal ions may be incorporated into the hair and nails.
Exfoliation	Highly lipophilic substances that have penetrated the stratum corneum but not penetrated the viable epidermis may be sloughed off with skin cells.

R.7.12.2.2 Generating and Integrating Toxicokinetic information

In vivo studies provide an integrated perspective on the relative importance of different processes in the intact biological system for comparison with the results of the toxicity studies. To ensure a valid set of TK data, a TK *in vivo* study has to consist of several experiments that include blood/plasma-kinetics, mass balances and excretion experiments as well as tissue distribution experiments. Depending on the problem to be solved, selected experiments (e.g. plasma-kinetics) may be sufficient to provide needed data for further assessments (e.g. bioavailability).

The high dose level administered in an ADME study should be linked to those that cause adverse effects in toxicity studies. Ideally there should also be a dose without toxic effect, which should be in the range of expected human exposure. A comparison between toxic dose levels and those that are likely to represent human exposure values may provide valuable information for the interpretation of adverse effects and is essential for extrapolation and risk assessment.

In an *in vivo* study the systemic bioavailability is usually estimated by the comparison of either dose-corrected amounts excreted, or of dose-corrected areas under the curve (AUC) of plasma (blood, serum) kinetic profiles, after extra- and intravascular administration. The systemic bioavailability is the dose-corrected amount excreted or AUC determined after an extravascular substance administration divided by the dose-corrected amount excreted or AUC determined after an intravascular substance application, which corresponds by definition to a bioavailability of 100%. This is only valid if the kinetics of the compound is linear, i.e. dose-proportional, and relies upon the assumption that the clearance is constant between experiments. If the kinetics is not linear, the experimental strategy has to be revised on a case-by-case basis, depending of the type of non-linearity involved (e.g. saturable protein binding, saturable metabolism etc.).

Generally *in vitro* studies provide data on specific aspects of pharmacokinetics such as metabolism. A major advantage of *in vitro* studies is that it is possible to carry out parallel tests on samples from the species used in toxicity tests and samples from humans, thus facilitating interspecies comparisons (e.g., metabolite profile, metabolic rate constants). In recent years methods to integrate a number of *in vitro* results into a prediction of ADME *in vivo* by the use of appropriate PBK models have been developed. Such methods allow both the prediction of *in vivo* kinetics at early stages of development, and the progressive integration of all available data into a predictive model of ADME. The resulting information on ADME can be used both to inform development decisions and as part of the risk assessment process. The uncertainty associated with the prediction depends largely on the amount of available data.

Test substances and analytical methodology

TK and metabolism studies can be carried out using non-labelled compounds, stable isotope-labelled compounds, radioactively labelled compounds or using dual (stable and radio-) labelling. The labels should be placed in metabolically stable positions, the placing of labels such as ^{14}C in positions from which they can enter the carbon pool of the test animal should be avoided. If a metabolic degradation of the test substance may occur, different labelling positions have to be taken into account to be able to determine all relevant degradation pathways. The radiolabelled compound must be of high radiochemical purity and of adequate specific activity to ensure sufficient sensitivity in radio-assay methods.

Separation techniques are used in metabolism studies to purify and separate several radioactive fractions in biota such as urine, plasma, bile and others. These techniques range from relatively simple approaches such as liquid-liquid extraction and column chromatography to more sophisticated techniques such as HPLC (high pressure liquid chromatography). These methods also allow for the establishment of a metabolite profile. Quantitative analytical methods are required to follow concentrations of parent compound and metabolites in the body as a function of time. The most common techniques used are LC/MS (liquid chromatography/ mass spectroscopy) and high performance LC with UV-detection, or if ^{14}C-labelled material is used, radioactivity-detection-HPLC. It is worth mentioning that kinetic parameters generally cannot be calculated from measurement of total radioactivity to receive an overall kinetic estimate. Nevertheless, to generate exact values one has to address parent compound and metabolites separately. An analytical step is required to define the radioactivity as chemical species. This is usually faster than cold analytical methods. Dual labelling (e.g. ^{13}C and $^{14}C/^{12}C$) is the method of choice for structural elucidation of metabolites (by MS and NMR [nuclear magnetic resonance] spectroscopy). A cold analytical technique, which incorporates stable isotope labelling (for GC/MS [gas chromatography/ mass spectroscopy] or LC/MS), is a useful combination. Unless this latter method has already been developed for the test compound in various matrices (urine, faeces, blood, fat, liver, kidney, etc.), the use of radiolabelled compound may be less costly than other methods.

In any TK study, the identity and purity of the substance used in the test must be assured. Analytical methods capable of detecting undesirable impurities will be required, as well as methods to assure that the substance of interest is of uniform potency from batch to batch. Additional methods will be required to monitor the stability and uniformity of the form in which the test substance is administered to the organisms used in the TK studies. Finally, methods suitable to identify and quantify the test substance in TK studies must be employed.

In the context of analytical methods, *accuracy* refers to how closely the average value reported for the assay of a sample agrees with the actual amount of substance being assayed in the sample, whereas *precision* refers to the amount of scatter in the measured values around the average result. If the average assay result does not agree with the actual amount in the sample, the assay is said to be *biased*, i.e., lacks specificity; bias can also be due to low recovery.

Assay *specificity* is perhaps the most serious problem encountered. Although *blanks* provide some assurance that no instrument response will be obtained in the absence of the test substance, a better approach is to select an instrument or bioassay that responds to some biological, chemical, or physical property of the test substance that is not shared with many other substances.

Besides, it is also necessary that the assay method is usable over a sufficiently wide range of concentrations for the toxic substance and its metabolites. The lower limit of reliability for an analytical method has been perceived in different ways; frequently, the term *sensitivity* has been used to indicate the ability of an analytical method to measure small amounts of a substance accurately and with requisite precision. It is unlikely that a single analytical method will be of use for all of these purposes. Indeed, it is highly desirable to use more than one method, at times. If two or more methods yield essentially the same results, confidence in each method is increased.

Important Methods for Generation of ADME data

Evaluation of absorption

Absorption is normally investigated by the determination of the test substance and/or its metabolites in excreta, exhaled air and carcass (i.e. radioactivity balance). The biological response between test and reference groups (e.g. oral versus intravenous .) is compared and the plasma level of the test substance and/or its metabolites is determined.

Dermal Absorption

Technical guidelines on the conduct of skin absorption studies have been published by OECD in 2004 (EU B.44[33], OECD TG 427; EU B.45, OECD TG 428; OECD GD 28). Advantages of the *in vivo* method (EU B.44, OECD TG 427) are that it uses a physiologically and metabolically intact system, uses a species common to many toxicity studies and can be modified for use with other species. The disadvantages are the use of animals, the need for radiolabelled material to facilitate reliable results, difficulties in determining the early absorption phase and the differences in permeability of the preferred species (rat) and human skin. Animal skin is generally more permeable and therefore may overestimate human percutaneous absorption (US EPA, 1992). Also, the experimental conditions should be taken into account in interpreting the results. For instance, dermal absorption studies in fur-bearing animals may not accurately reflect dermal absorption in human beings.

In vitro systems allow us to apply to a fixed surface area of the skin an accurate dose of a test substance in the form, volume and concentration that are likely to be present during human exposure. One of the key parameters in the regulatory guidelines in this field is that sink conditions must always be maintained, which may bias the assay by build-up of the substance in the reservoir below the skin[34]. A major issue of concern in the *in vitro* procedure turned out to be the presence of test substance in the various skin layers, i.e., absorbed into the skin but not passed into the receptor fluid. It was noted that it is especially difficult to examine very lipophilic substances *in vitro*, because of their low solubility in most receptor fluids. By including the amount retained in the skin *in vitro*, a more acceptable estimation of skin absorption can be obtained. Water-soluble substances can be tested more accurately *in vitro* because they more readily diffuse into the receptor fluid (OECD GD 28). At present, provided that skin levels are included as absorbed, results from *in vitro* methods seem to adequately reflect those from *in vivo* experiments supporting their use as a replacement test to measure percutaneous absorption.

If appropriate dermal penetration data are available for rats *in vivo* and for rat and human skin *in vitro*, the *in vivo* dermal absorption in rats may be adjusted in light of the

[33] See Test Methods Regulation (Council Regulation (EC) No 440/2008).

[34] A build up of substance in the reservoir below the skin is not such a problem if a flow through cell is used for *in vitro* testing.

relative absorption through rat and human skin *in vitro*. The latter adjustment may be done because the permeability of human skin is often lower than that of animal skin (e.g. Howes *et al.*, 1996). A generally applicable correction factor for extrapolation to man can, however, not be derived, because the extent of overestimation appears to be dose, substance, and animal specific (ECETOC, 1993; Bronaugh and Maibach, 1987).

In silico models might also improve the overall knowledge of crucial properties significantly. Mathematical skin permeation models are usually based on uptake from aqueous solution which may not be relevant to the exposure scenario being assessed. In addition, the use of such models for quantitative risk assessment purposes is often limited because these models have generally been validated by *in vitro* data ignoring the fate of the skin residue levels. However, these models may prove useful as a screening tool or for qualitative comparison of skin permeation potential. On a case-by-case basis, and if scientifically justified, the use of (quantitative) structure activity relationships may prove useful, especially within a group of closely related substances.

It is notable that a project on the Evaluation and Prediction of Dermal Absorption of Toxic Chemicals (EDETOX) was conducted (Williams, 2004). A large critically evaluated database with *in vivo* and *in vitro* data on dermal absorption/penetration of chemicals has been established. It is available at http://edetox.ncl.ac.uk. Based on this data, existing QSARs were evaluated (Fitzpatrick *et al.*, 2004). Furthermore new models were developed: a mechanistically based model, which was used to interpret some of the newly generated data, a simple membrane model and a diffusion model of percutaneous absorption kinetics. All these models have mostly been based on and applied to rather large organic molecules and have thus limited relevance for assessment of inorganic substances. Furthermore, a guidance document was developed for conduct of *in vitro* studies of dermal absorption/penetration and can be obtained via http://www.ncl.ac.uk/edetox/. Although mainly based on the experiences gathered with organic substances, parts of this practical guidance on conduct of such studies are also applicable to inorganic substances.

Evaluation of Distribution

For determination of the distribution of a substance in the body there are two approaches available at present for analysis of distribution patterns. Quantitative information can be obtained firstly, using whole-body autoradiographic techniques and secondly, by sacrificing animals at different times after exposure and determination of the concentration and amount of the test substance and/or metabolites in tissues and organs (EU B.36[35], OECD TG 417).

Evaluation of the Accumulative Potential

Bioconcentration refers to the accumulation of a substance dissolved in water by an aquatic organism. The static *bioconcentration factor* (BCF) is the ratio of the concentration of a substance in an organism to the concentration in water once a steady state has been achieved. Traditionally, bioconcentration potential has been assessed using laboratory experiments that expose fish to the substance dissolved in water (EU

[35] See Test Methods Regulation (Council Regulation (EC) No 440/2008).

C.13[35], OECD TG 305). The resulting fish BCF is widely used as a surrogate measure for bioaccumulation potential.

Another possibility to assess the accumulative potential of a substance is to expose rats repeatedly to a substance (e.g. 4 week daily administration) and determine the body burden or the amount in a relevant compartment in a time course.

Accumulating substances can also be measured in milk and therefore additionally allow an estimation of transfer to the breast-fed pup.

Evaluation of Metabolism

In vivo TK studies generally only determine the rates of total metabolic clearance (by measurement of radiolabelled products in blood/plasma, bile, and excrements) rather than the contributions of individual tissues. It has to be taken into account that the total metabolic clearance is the sum of the hepatic and potential extrahepatic metabolism.

In vitro tests can be performed using isolated enzymes, microsomes and microsomal fractions, immortalised cell lines, primary cells and organ slices. Most frequently these materials originate from the liver as this is the most relevant organ for metabolism, however, in some cases preparation from other organs are used for investigation of potential organ-specific metabolic pathways.

When using metabolically incompetent cells an exogenous metabolic activation system is usually added in to the cultures. For this purpose the post-mitochondrial 9000x g supernatant (S9 fraction) of whole liver tissue homogenate containing a high concentration of metabolising enzymes is most commonly employed - the donor species needs to be considered in the context of the study. In all cases metabolism may either be directly assessed by specific identification of the metabolites or by subtractive calculation of the amount of parent substance lost in the process.

Evaluation of Excretion

The major routes of excretion are in the urine and/or the faeces (via bile and directly from the GI mucosa; see Rozman, 1986). For this purpose urine, faeces and expired air and, in certain circumstances, bile are collected and the amount of test substance and/or metabolites in these excreta is measured (EU B.36[35], OECD TG 417).

The excretion of substances (metabolites) in other biological fluids such as *saliva*, *milk*, *tears*, and *sweat* is usually negligible compared with renal or biliary excretion. However, in special cases these fluids may be important to study either for monitoring purposes, or in the case of milk allowing an assessment of the exposure of infants.

For volatile substances and metabolites exhaled air may be an important route of elimination. Therefore, exhaled air shall be examined in respective cases.

In silico methods - Kinetic modelling

In silico methods for toxicokinetics, can be defined as mathematical models, which can be used to understand physiological phenomena of absorption, distribution, metabolism and elimination of substances in the body. These methods gather, for example, QSAR models, compartmental models, or allometric equations (Ings, 1990; Bachmann, 1996). Their main advantages compared to *classical* (*in vitro*, *in vivo*) methods is that they estimate the toxicokinetics of a given agent quicker, cheaper and reduced the number of experimental animals. A detailed discussion of the approaches that integrate information generated *in silico* and *in vitro* is presented in Appendix R.7.12-2 of this document.

When using kinetic models, two opposite situations can be schematically described:

- either the values of some or all parameters are unknown, and the model is adjusted (fitted) to data in order to extract from the dataset these parameter values: this is the fitting situation.
- or the parameter values are considered as known, and the model is used to generate simulated datasets: this is the simulation situation.

Appropriate algorithms, implemented in validated suitable software, are available to perform fitting and simulation operations. Both model fitting and simulation operations have specific technical problems and pitfalls, and must be performed by adequately trained scientists or scientific teams. Simulation is an extremely useful tool, because it is the only way to predict situations for which it is not, and often will never be possible to generate or collect real data. The results of carefully designed simulations, with attached uncertainty estimations, are then the only available tools for quantitative risk assessment. The better the model-building steps will have been performed, the better defined will be the predictions, leading ultimately to better-informed regulatory decisions.

In a risk assessment context, to identify TK relationship as best as possible, TK information collected from *in vitro* and *in vivo* experiments could be analysed on the basis of *in silico* models. The purpose of TK *in silico* models is to describe or predict the concentrations and to define the internal dose of the parent substance or of its active metabolite. This is important because internal doses provide a better basis than external exposure for predicting toxic effects. The prediction of pharmaco- or toxicological effects from external exposure or from internal dose rests upon *in silico* pharmaco- or toxicodynamic modelling. The combined used of pharmacokinetic models (describing the relationships between dose / exposure and concentrations within the body), with pharmacodynamic models (describing the relationship between concentrations or concentration-derived internal dose descriptors and effects), is called pharmacokinetic / pharmacodynamic modelling, or PKPD modelling. The term toxicokinetic / toxicodynamic modelling, or TKTD, covers the same concept.

TK models typically describe the body as a set of compartments through which substances travel or are transformed. They fall into two main classes: *empirical* models and physiologically-based kinetic models (PBK) (Andersen, 1995; Balant and Gex-Fabry, 1990; Clewell and Andersen, 1996; Gerlowski and Jain, 1983). All these models simplify the complex physiology by subdividing the body into compartments within which the toxic agent is assumed to be homogeneously distributed (Gibaldi and Perrier, 1982). Empirical TK models represent the body by one or two (rarely more than three) compartments not reflecting the anatomy of the species. These models are simple (with a low number of parameters), allow describing many kinds of kinetics and can be easily fitted to experimental data.

The structure and parameter values of *empirical kinetic models* are essentially determined by the datasets themselves, whether experimental or observational.

Datasets consist generally in concentration versus time curves in various fluids or tissues, after dosing or exposure by various routes, at various dose or exposure levels, in various individuals of various species. Classic kinetic models represent the body by a small number of compartments (usually 1 or 2 per compound or metabolite, rarely 3, exceptionally more than 3) where ADME phenomena occur. Phenomena are described using *virtual* volume terms and transfer rates, which are the parameters of the models. The function of the volume parameters is to relate the concentrations measured, e.g. in plasma, to the amounts of xenobiotic present in the body. The volumes described in the model usually have no physiological counterpart.

The structure of the model itself is largely determined by the datasets which they are intended to describe. This is why these models are often said to be *data-driven*, or *top to bottom*. Compared to physiologically based models, classic kinetic models are usually better adapted to fitting model to data in order to extract parameter values.

A *physiologically based (PBK) model* is an independent structural mathematical model, comprising the tissues and organs of the body with each perfused by, and connected via, the blood/lymphatic circulatory system. PBK models comprise four main types of parameter:

- Physiological
- Anatomical
- Biochemical
- Physico-chemical

Physiological and anatomical parameters include tissue masses and blood perfusion rates, estimates of cardiac output and alveolar ventilation rates. Biochemical parameters include enzyme metabolic rates and polymorphisms, enzyme synthesis and inactivation rates, receptor and protein binding constants etc. Physico-chemical parameters refer to partition coefficients. A partition coefficient is a ratio of the solubility of a substance in a biological medium, usually blood-air and tissue-blood. Anatomical and physiological parameters are readily available and many have been obtained by measurement. Biochemical and physico-chemical parameters are compound specific. When such parameters (see e.g. Brown *et al.*, 1997; Clewell and Andersen, 1996; Dedrick and Bischoff, 1980) are measured and used to construct an *a priori* model that qualitatively describes a dataset, then confidence in such a model should be high. In the absence of measured data, such as partition coefficients, these may be estimated using tissue-composition based algorithms (Theil *et al.*, 2003). Metabolic rate constants may be fitted using a PBK model, although this practice should only be undertaken if there are no other alternatives. A sensitivity analysis (see below) of these models (Gueorguieva *et al.*, 2006; Nestorov, 1999) may be performed for identifying which parameters are important within a model. It helps prioritizing and focusing on only those parameters which have a significant impact on the risk assessment process and to identify sensitive population. A discussion on the applicability of PBK Modelling for the development of assessment factors in risk assessment is presented in Appendix R.7.12-3 of this document and in the IPCS project document Characterisation and Application of Physiologically Based Pharmacokinetic Models in Risk Assessment (2010).

The potential of PBK models to generate predictions from *in vitro* or *in vivo* information is one of their attractive features in the risk assessment of substances. The degree of later refinement of the predictions will depend on the particular purpose for which kinetic information is generated, as well as on the feasibility of generating additional data. When new information becomes available, the PBK model should be calibrated; Bayesian techniques, for example, can be easily used for that purpose.

PBK models are very useful when the kinetic process of interest cannot be directly observed and then when extrapolations are needed. Indeed, inter-species, inter-individual, inter-dose or inter-route extrapolations are more robust when they are based on PBK rather than on empirical models. The intrinsic capacity for extrapolation makes PBK models particularly attractive for assessing the risk of substances, because it will be usually impossible to gather kinetic data in all species of interest, and particularly in man, or by all relevant exposure schemes. More specifically, PBK models also allow to evaluate TK in reprotoxicity, developmental and multi-generational toxicological studies. PBK model can be developed to depict internal disposition of substance during pregnancy in the mother and the embryo/foetus (Corley *et al.*, 2003; Gargas *et al.*, 2000; Lee *et al.*, 2002; Luecke *et al.*, 1994; Young *et al.*, 2001). Lactation transfer of toxicant from mother to newborn can also be quantified using PBK models (Byczkowski and Lipscomb, 2001; Faqi *et al.*, 1998; You *et al.*, 1999). The main interests of PBK are also the ability to check complex hypothesis (such as, for example, the existence of an unknown metabolism pathway or site) and to give predictions on the internal doses (which is not always observable in human). Finally, they also allow estimation of kinetic parameter (e.g. metabolism constant) and dose reconstruction from biomarkers.

The rationale for using PBK models in risk assessment is that they provide a documentable, scientifically defensible means of bridging the gap between animal bioassays and human risk estimates. In particular, they shift the risk assessment from the administered dose to a dose more closely associated with the toxic effect by explicitly describing their relationships as a function of dose, species, route and exposure scenario. The increased complexity and data demands of PBK models must be counter-balanced by the increased accuracy, biological plausibility and scientific justifiability of any risk assessment using them. It follows from this that PBK models are more likely to be used for substances of high concern.

<u>Sensitivity analysis</u>

As biological insight increases, more complex mathematical models of physiological systems that exhibit more complex non-linear behaviour will appear. Although the governing equations of these models can usually be solved with relative ease using a generic numerical technique, often the real strength of the model is not the predictions it produces but how those predictions were produced. That is, how do the hypotheses, that fit together to make the model, interact with each other? Which of the assumptions or mechanisms are most important in determining the output? How sensitive is the model output to changes in input parameters or model structure? Sensitivity analysis techniques exist that can address these questions by giving a measure of the effects on model output caused by variation in its inputs. SA can be used to determine:

- Whether a model emulates the organism being studied,
- Which parameters require additional research to strengthen knowledge,

- The influence of structures such as *in vitro* scalings,
- Physiological characteristics/compound specific parameters that have an insignificant effect on output and may be eliminated from the model,
- Feasible combinations of parameters where model variation is greatest,
- Most appropriate regions within the space of input parameters for use in parameter optimisation,
- Whether interaction between parameters occurs, and which of them interact (Saltelli *et al.*, 2000).

Predictions from a complex mathematical model require a detailed sensitivity analysis in order that the limitations of the predictions provided by model can be assessed. A thorough understanding the model itself can greatly reduce the efforts in collating physiological and compound specific data, and lead to more refined and focused simulations that more accurately predict human variability across a population and identify groups susceptible to toxic effects of a given compound.

Importance of Uncertainty and Variability

Uncertainty and variability are inherent to a TK study and affect potentially the conclusion of the study. It is necessary to minimise uncertainty in order to assess the variability that may exist between individuals so that there is confidence in the TK results such that they can be useful for risk analysts and decision-makers.

Variability typically refers to differences in the physiological characteristics among individuals (inter-individual variability) or across time within a given individual (intra-individual variability). It may stem from genetic differences, activity level, lifestyles, physiological status, age, sex *etc*. Variability is inherent in animal and human populations. It can be observed and registered as information about the population, but it cannot be reduced. An important feature of variability is that it does not tend to decrease when larger samples of a population are examined.

Variability in the population should then be taken into account in TK studies. Regarding PBK models, it may be introduced by the use of probability distributions for parameters representing the distribution of physiological characteristics in the population. The propagation of these variability to model predictions may be evaluated using Monte Carlo simulations methods.[36]

Uncertainty can be defined as the inability to make precise and unbiased statements. It is essentially due to a lack of knowledge. Uncertainty in the information may decrease with the size of the sample studied. It can be theoretically, eliminated and at least

[36] These methods consist of specifying a probability distribution for each model parameter; sampling randomly each model parameter from its specified distribution; running the model using the sampled parameter values, and computing various model predictions of interest. Instead of specifying independent distributions for parameters, a joint probability distribution may be assigned to a group of parameters to describe their correlation.

reduced by further optimised experiments or by a better understanding of the process under study.

Uncertainty may be related to:

The experimental nature of the data. Indeed, uncertainty comes from errors in experimental data. Experimental data are typically known with finite precision dependent of the apparatus used. However such uncertainties may be easily assessed with quality measurement data. They can be modelled with probability distributions (*e.g.*, the measured quantity is distributed normally with mean the actual quantity and a given standard deviation). Uncertainty may also be generated by the data gathering process and errors made at this stage (reading errors, systematic measurement errors, *etc*).

The modelling procedure. Uncertainty is most of the time inescapable due to the complexity and unknown nature of the phenomena involved (model specification). The source of uncertainty in the model structure (and more particularly in PBK models) is primarily a lack of theoretical knowledge to correctly describe the phenomenon of interest on all scales. In this case, the world is not fully understood and therefore not modelled exactly. Summing up, in a model, a massive amount of information can in itself be a technical challenge. An organism may be viewed as an integrated system, whose components correlations are both strong and multiple (e.g., a large liver volume might be expected to be associated with a large blood flow). Given the complexity of an organism, it is not feasible to integrate all the interactions between its components (most of them are not even fully known and quantified) in the development of a model. Therefore modellers have to simplify reality. Such assumptions will however introduce uncertainty. A general statistical approach to quantify model uncertainty is first to evaluate the accuracy of the model when predicting some datasets. Models based on different assumptions may be tested and statistical criteria (such as the Akaike criterion[37]) may be used to discriminate between models

The high inherent variability of biological systems. The variability itself is a source of uncertainty. In some cases, it is possible to fully know variability, for example by exhaustive enumeration, with no uncertainty attached. However, variability may be a source of uncertainty in predictions if it is not fully understood and ascribed to randomness.

[37] Measure of the logarithm of the likelihood.

R.7.12.2.3 Include human data when available to refine the assessment

Human biological monitoring and biological marker measurement studies provide dosimetric means for establishing aggregate and/or cumulative absorbed doses of substances following specific situations or exposure scenarios or for establishing baseline, population-based background levels (Woollen, 1993). The results from these studies, e.g., temporal situational biological monitoring, provide a realistic description of human exposure.

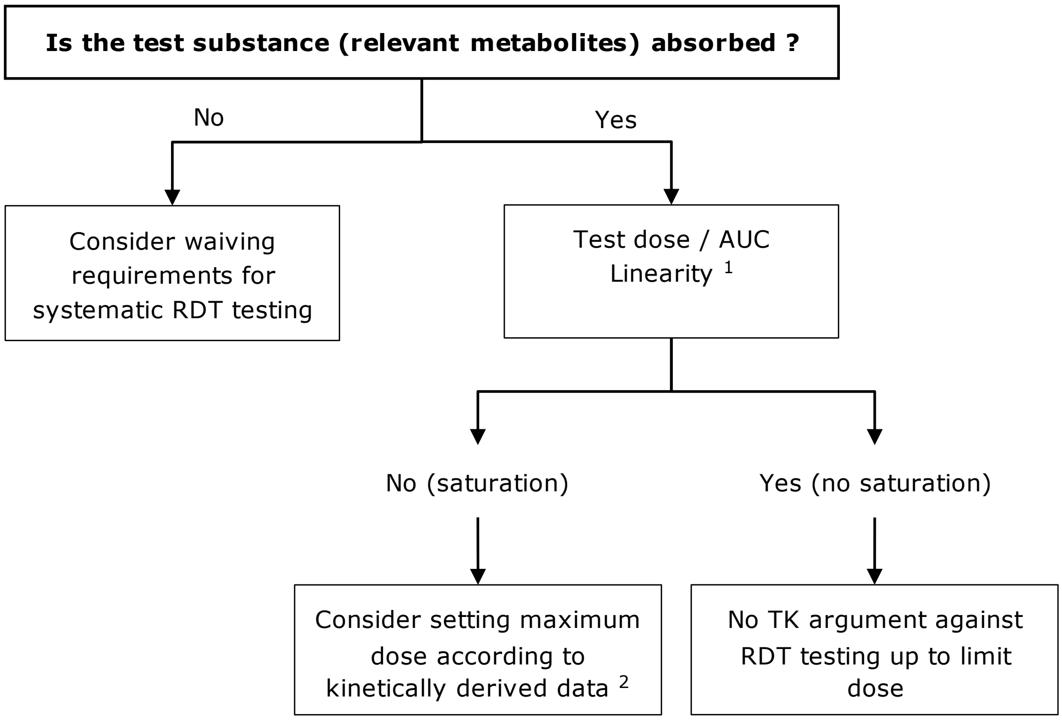
Biomonitoring, the routine analysis of human tissues or excreta for direct or indirect evidence of human exposures to substances, can provide unique insights into the relationship between dose and putative toxicity thresholds established in experimental animals, usually rats. Pioneering research by Elkins *et al.* (1954) on the relationship between concentrations of substances in the workplace and their concentrations in body fluids helped to establish the Biological Exposure Index (ACGIH, 2002). Urine is the most frequently used biological specimen, due to its non-invasive nature and ease of collection and its importance as a route of excretion for most analytes. The analyte to be monitored should be selected depending on the metabolism of the compound, the biological relevance, and feasibility considerations, in order to maximise the relevance of the information obtained.

R.7.12.2.4 Illustration of the benefit of using Toxicokinetic information

The understanding of the mode of action of a substance or at least the estimation through a category of substances with a similar structure and action supports argumentation on specific modulation of testing schemes (even waiving) and the overall interpretation of the biological activity of a substance. The following diagrams shall illustrate the way of thinking that can be applied regarding making use of TK information when this is available. It should be acknowledged that just in very rare cases a *yes-no* answer could be applied. Often a complex pattern of different information creates specific situations that deviate from the simplified standard procedures given below. The answer *no* can be understood in regard to *no significant* effect based on substance dependent expert judgment and detection limits of sensitive test methods (compare REACH Annex VIII, Section 8.7). Therefore, experts need to be consulted for use of TK data for designing tests individually, interpretation of results for elucidating the mode of action or in a grouping or read-across approach and also regarding the use of computational PBK model systems.

Use of TK information to support Dose Setting Decisions for Repeated Dose Studies

TK data, especially information on absorption, metabolism and elimination, are highly useful in the process of the design of repeated dose toxicity (RDT) studies. Repeated dose toxicity studies should be performed according to the respective OECD or EU guidelines. The highest dose level in such studies should be chosen with the aim to induce toxicity but not death or severe suffering in the test animals. For doing so, the OECD or EU guidelines suggest to test up to a standardised limit dose level called maximum tolerated dose (MTD). It is convenient to remember that such doses may, in certain cases, cause saturation of metabolism and, therefore, the obtained results need to be carefully evaluated when eventually assessing the risk posed by exposure at levels where a substance can be readily metabolised and cleared from the body. Consequently, when designing repeated dose toxicity studies, it is convenient to consider selecting appropriate dose levels on the basis of results from metabolic and toxicokinetic investigation. Figure R.7.12—1 illustrates how TK data could assist in dose setting decisions for repeated dose toxicity studies.



[1] In the dose-range under consideration for RDT testing

[2] Meaning that the highest dose-level should not exceed into the range of non-linear kinetics.

Figure R.7.12—1 Use of TK data in the design of RDT studies

The question which needs to be addressed initially is whether the substance is absorbed. If it can be demonstrated that a substance is not absorbed, it cannot induce direct systemic effects. In such a case, from the kinetic point of view, there is no need for further repeated dose testing[38]. If the substance is absorbed the question arises whether there is a linear relationship between the administered dose and the AUC in the blood. If this is the case and the substance is not metabolised, then there is no kinetic argument against testing at the standardised MTD suggested by OECD or EU guidelines.

Often the dose/AUC relationship deviates from linearity above a certain dose. This is illustrated in Figure R.7.12—2. In both cases described the dose level corresponding to the inflexion point can be regarded as the kinetically derived maximally tolerated dose (MTD) If information in this regard is available, it might be considered setting the highest dose level for repeated doses studies according to the kinetically derived MTD.

[38] Secondary effects misinterpreted, as primary toxic effects need to be excluded.

In example 1 the AUC does not increase beyond a certain dose level. This is the case when absorption becomes saturated above a certain dose level. The dose/AUC relationship presented in example 2 can be obtained when elimination or metabolism becomes saturated above a certain dose level, resulting in an over proportional increase in the AUC beyond this dose.

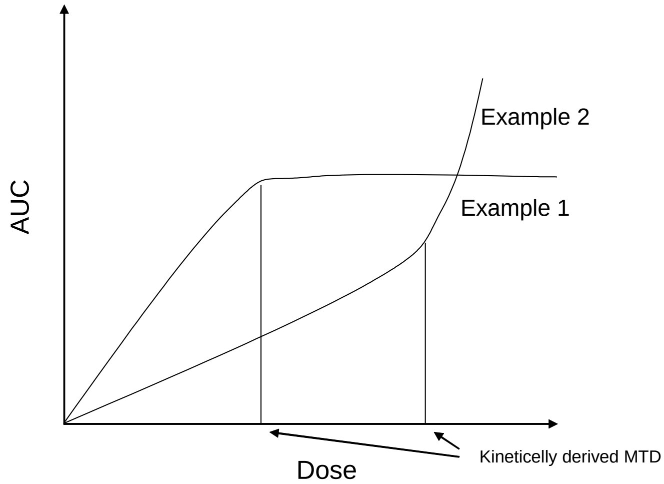


Figure R.7.12—2 Departure from linearity at certain doses

Use of kinetic information in the design and validation of categories

Information on kinetics *in vivo* will assist the design of categories. Candidate category substances can be identified, with which to perform *in vitro or in vivo* tests, thus making extrapolation of toxicological findings between substances more relevant.

Where there is uncertainty or contradictory information within a category, the category or membership of a certain substance to a category can be verified using kinetics information.

Metabolism Studies as basis for Internal Dose considerations

Biotransformation of a substance produces metabolites that may have different toxicological properties than the substrate from which they are formed. Although metabolism is generally referred to a detoxification purpose, there are also many examples for which metabolites have a higher intrinsic toxicity than the parent compound itself (metabolic activation). Therefore, the knowledge if the test substance is metabolised and to which metabolites is necessary to enable the assessment of the results from toxicity studies in respect to waiving and grouping approaches as well as to define an internal dose (see Table R.7.12—3).

If the test substance is not metabolised, the parent compound is the relevant marker for the measurement and the definition of the internal dose. If the test substance is metabolised, the knowledge which metabolites are formed is essential for any further step in an assessment. When this information is not available, it can be investigated by appropriate *in vitro* and/or *in vivo* metabolism studies (see Section R.7.12.2.1). In special cases metabolites may show a high degree of isomeric specificity and this should be born in mind in the design and interpretation of mixtures of isomers, including racemates. If the metabolites are known and if toxicity studies are available for these metabolites, risk assessment may be carried out based on these data and an assessment on the basis of the definition of the internal dose can be made. If the toxicity profile for the metabolites is unknown, studies that address the toxicity of these metabolites may be performed under special considerations of potential group approaches (especially if a chemical substance is the metabolite of different compounds, e.g. like a carboxylic acid as a metabolite of different esters).

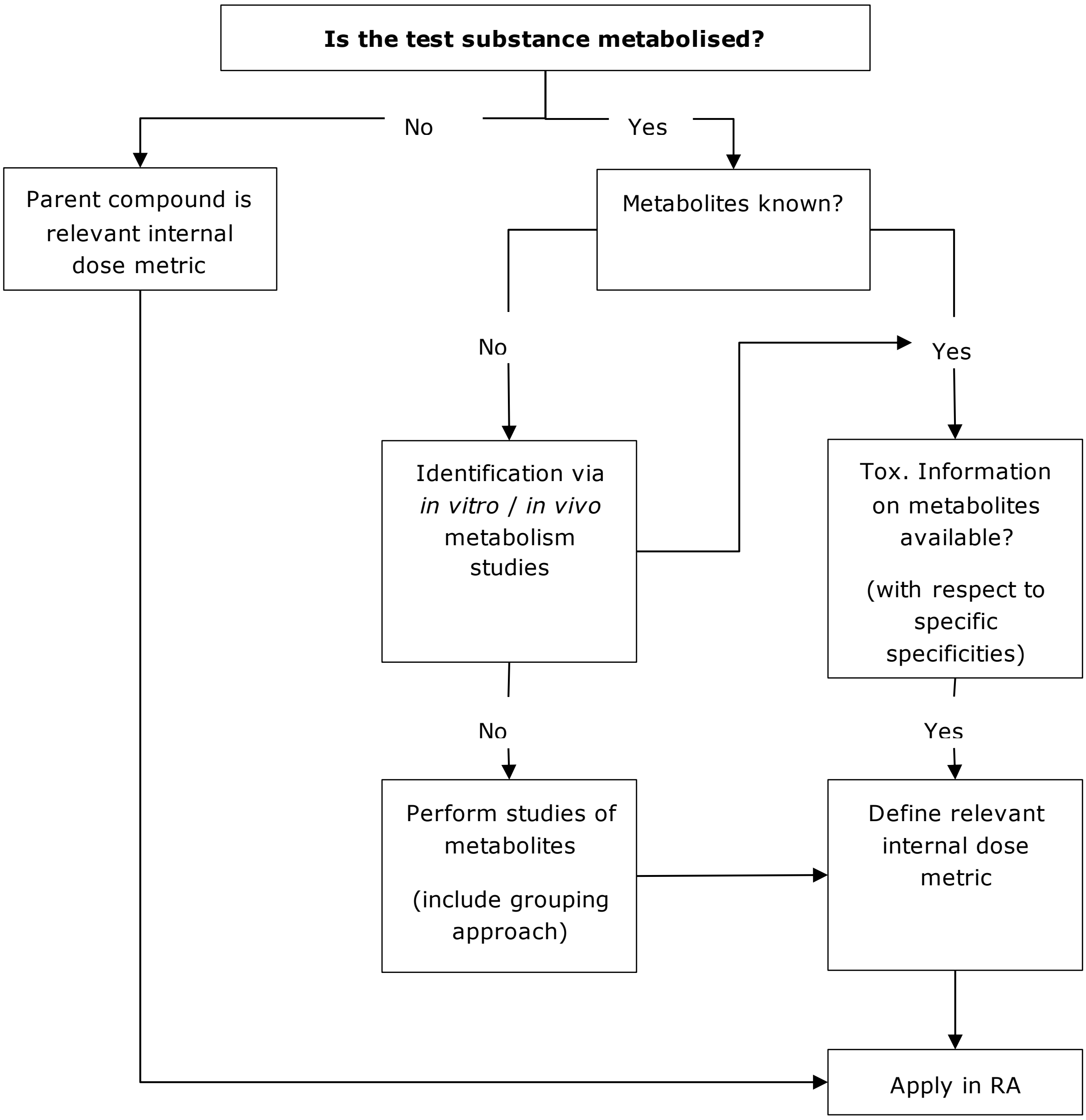


Figure R.7.12—3 Use of increasing knowledge on substance metabolism

TK information can be very helpful in bridging various gaps as encountered in the whole risk assessment, from toxicity study design and biomonitoring[39] setup to the derivation

[39] Biological monitoring information should be seen as equivalent (i.e. as having neither greater nor lesser importance) to other forms of exposure data. It should also be remembered that biological monitoring results

of the DNEL (Derived No-Effect Level) and various extrapolations as usually needed (cross-dose, cross-species including man, cross-exposure regimens, cross-routes, and cross-substances). The internal dose is the central output parameter of TK studies and therefore the *external exposure – internal dose – concept* is broadly applicable in the various extrapolations mentioned (see also Section R.7.12.2.4). In addition, under REACH, derivation of DNELs is obligatory. If, for that purpose, route-to-route extrapolation is necessary and in case assessment of combined exposure (via different routes) is needed, for systemic effects, internal exposure may have to be estimated.

Exposure should normally be understood as external exposure which can be defined as the amount of substance ingested, the total amount in contact with the skin or either the amount inhaled or the concentration of the substance in the atmosphere in combination with the exposure duration, as appropriate. In cases where a comparison needs to be made with systemic effects data (e.g. when inhalation or dermal toxicity values are lacking or when exposures due to more than one route need to be combined) the total body burden has to be estimated and expressed as an internal dose.

Determination of the level of systemic exposure is considered synonymous to determination of bioavailability of a substance to the general circulation. Depending on the problem considered and other concomitant information such as exposure scenarios, this could be expressed as a fraction bioavailable (F), a mass bioavailable, a concentration profile, an average concentration, or an AUC. It should be emphasised that it is usually not possible to show that the amount of a substance bioavailable is zero, apart from favourable cases by dermal route, considering only intact skin. This should be assessed in terms of thresholds, the objective being to establish whether or not the bioavailability of a substance is predicted to be below a certain threshold. The degree of certainty of the prediction will depend on each case, important factors being the accuracy and reliability of the *in vivo, in vitro* or *in silico* model used, the performance of the methods used to assay the substance or its metabolites, the estimated variability in the target population etc.

Tissue distribution characteristics of a compound can be an important determinant of its potential to cause toxicity in specific tissues. In addition, tissue distribution may be an important determinant of the ability of a compound to accumulate upon repeated exposure, although this is substantially modified by the rate at which the compound is cleared. Correlation of tissue distribution with target tissues in toxicity studies should be accomplished while substantial amounts of the substance remain present in the body, for example, at one or more times around the peak blood concentration following oral absorption. Such data should quantify parent compound and metabolites, to the extent feasible. If the metabolites are unknown or difficult to quantify, subtracting parent compound from total radioactivity will provide an estimate of the behaviour of the total metabolites formed.

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reflect an individual's total exposure to a substance from any relevant route, i.e. from consumer products, and/or from the environment and not just occupational exposure. Data from controlled human exposure studies are even more unlikely available. This is due to the practical and ethical considerations involved in deliberate exposure of individuals.

Extrapolation

For ethical reasons, data allowing estimating model parameters are poor, sparse, and do not often concern human populations; recourse to extrapolation is then needed. TK data are mostly gathered for few concentrations (usually less than 5 different concentrations) and limited number of different exposure times. However, risk evaluation should also status on different doses (exposure concentrations and times). Inter-dose/inter-exposure time extrapolation is a common way to satisfy this request - mathematical methods (e.g. linear regression) are used for this purpose. The non-linear kinetic behaviour of substances in a biological organism is the result of a number of mechanisms e.g., saturable metabolism, enzyme induction, enzyme inactivation and depletion of glutathione and other cofactor reserves. High-dose-low-dose extrapolation of tissue dose is accomplished with PBK modelling by accounting for such mechanisms (Clewell and Andersen, 1996).

In the rare case where data on human volunteers are available, they only concern a very limited number of subjects. Extrapolation to other body and to the global population should be done (inter-individual extrapolation). The problem of sensitive populations also raises and TK study should status on other gender, age or ethnic groups, for example. As it is practically nearly impossible to control internal dose in humans, alternative animal study is often proposed. Since risk assessment aims at protecting human population, inter-species extrapolation (Davidson *et al.*, 1986; Watanabe and Bois, 1996) should be done. For practical reasons, the administration route in experimental study can be different from the most likely exposure route. Risk assessment implies then to conclude on another route than the one experimentally studied. Inter-route extrapolation should then be performed.

Default values have been derived to match the extrapolation idea in a general way. The incorporation of quantitative data on interspecies differences or human variability in TK and TD into dose/concentration-response dose assessment through the development of chemical specific adjustment factors (CSAFs) might improve risk assessment of single substances. Currently, relevant data for consideration are often restricted to the component of uncertainty related to interspecies differences in TK. While there are commonly fewer data at the present time to address interspecies differences in TD, inter-individual variability in TK and TD, it is anticipated that the availability of such information will increase with a better common understanding of its appropriate nature (IPCS, 2001). The type of TK information that could be used includes the rate and extent of absorption, the extent of systemic availability, the rate and extent of presystemic (first-pass) and systemic metabolism, the extent of enterohepatic recirculation, information on the formation of reactive metabolites and possible species differences and knowledge of the half-life and potential for accumulation under repeated exposure.

The need for these extrapolations can lead one to prefer physiological TK models to empirical models (Davidson *et al.*, 1986; Watanabe and Bois, 1996; Young *et al.*, 2001). Indeed, PBK models facilitate the required extrapolations (inter-species, inter-subject etc). By changing anatomical parameters (such as organ volumes or blood flows), a PBK model can be transposed from rat to human, for example.

Interspecies extrapolation

The use of animal data for toxicological risk assessment arises the question of how to extrapolate experimentally observed kinetics to human subjects or populations - the ability to compare data from animals with those from humans will enable defining chemical-specific interspecies extrapolation factors to replace the default values. One possibility to do so is the calculation of allometric factors by extrapolation based on different body sizes. The most complex procedure for inter-species extrapolation is the collection of different data and use these in a PBK modelling.

Allometric scaling is a commonly employed extrapolation approach. It is based on the principle that biological diversity is largely explained by body size (Schneider *et al.*, 2004). Allometric scaling captures the correlations of physiological parameters or TK with body size. More precisely, allometric equations relate the quantity of interest (e.g., a tissue dose) to a power function of body mass, fitted across species:

$$Y = a\ BM^b$$

where Y is the quantity of interest, a is a species-independent scaling coefficient[40], BM is body mass and b is the allometric exponent. Values of b depend upon whether the quantity of interest scales approximately with body mass (b=1), metabolic rate[41] (b=0.75), or body surface area (b=0.67[42]) (Davidson *et al.*, 1986; Fiserova-Bergerova and Hugues, 1983; West *et al.*, 1997). As it is easy to apply, the allometric scaling is probably the most convenient approach to interspecies extrapolation. However, it is very approximate and may not hold for the substance of interest. As such it can be conceived only as default approach to be used only in the absence of specific data in the species of interest.

For a substance that demonstrates significant interspecies variation in toxicity in animal experiments, the most susceptible species is generally used as the reference for this extrapolation. Uncertainty factors up to 1000 or more have been applied in recognition of the uncertainty involved. Whereas a metabolic rate constant estimated in this way may be used in a PBK model, it is preferable, where possible, to determine such parameters *in vitro* using tissue subcellular fractions or estimate them by fitting a PBK model to an appropriate dataset.

Consequently, to better estimate tissue exposure across species, PBK models may be used for the considered toxicant (Watanabe and Bois, 1996). These models account for transport mechanisms and metabolism within the body. These processes are then modelled by the same equation set for all species considered. Differences between species are assumed to be due to different (physiological, chemical, and metabolic) parameter values. Extrapolation of PBK models then relies on replacing the model parameter values of one species with the parameter values of the species of interest. For physiological parameters, numerous references (Arms and Travis, 1988; Brown *et al.*,

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[40] Fits single data points together to form an appropriate curve.

[41] In this context not metabolism of compounds! The factor adapts different levels of oxygen consumption.

[42] This scaling factor is generally justified on the basis of the studies by Freireich *et al* (1966), who examined the interspecies differences in toxicity of a variety of antineoplastic drugs.

1997; ICRP, 2002) give standard parameter values for many species. Chemical (partitioning coefficient) and metabolic parameter values are usually less easily found. When parameter values of PBK model are not known for the considered species, recourse to *in vitro* data, Quantitative Structure-Property Relationships (QSPR) predictions or allometric scaling of those parameters is still possible. To take into account population variability in the extrapolation process, probability distributions of parameters may be used rather than single parameter values. PBK models can be particularly useful where data are being extrapolated to population subgroups for which the little information is available e.g. on pregnant women or infants (Luecke *et al.*, 1994; Young *et al.*, 2001).

Inter-route Extrapolation

Route-to-route extrapolation is defined as the prediction of the total amount of a substance administered by one route that would produce the same systemic toxic response as that obtained for a given amount of a substance administered by another route.

In general, route-to-route extrapolation is considered to be a poor substitute for toxicity data obtained using the appropriate route of exposure. Uncertainties in extrapolation increase when it becomes necessary to perform a risk assessment with toxicity data obtained by an administration route which does not correspond to the human route of exposure. Insight into the reliability of the current methodologies for route-to-route extrapolation has not been obtained yet (Wilschut *et al.*, 1998).

When route-to-route extrapolation is to be used, the following aspects should be carefully considered:

- *nature of effect:* route-to-route extrapolation is only applicable for the evaluation of systemic effects. For the evaluation of local effects after repeated exposure, only results from toxicity studies performed with the route under consideration can be used;
- *toxicokinetic data (ADME):* The major factors responsible for differences in toxicity due to route of exposure include:
- differences in bioavailability or absorption,
- differences in metabolism (first pass effects),
- differences in internal exposure pattern (i.e. internal dose).

In the absence of relevant kinetic data, route-to-route extrapolation is only possible if the following assumptions are reasonable:

- Absorption can be quantified

- Toxicity is a systemic effect not a local one (compound is relatively soluble in body fluids, therefore systemically bioavailable) and internal dose can be estimated[43]
- First-pass effects are minimal

Provided the listed criteria are met, the only possibility for route-to-route extrapolation is to use default values. If route-to-route extrapolation is required or if an internal N(O)AEL/starting point needs to be derived in order to assess combined exposure from different routes, information on the extent of absorption for the different routes of exposure should be used to modify the starting point. On a case-by-case basis a judgement will have to be made as to whether the extent of absorption for the different routes of exposure determined from the experimental absorption data is applicable to the starting point of interest. Special attention should be given to the dose ranges employed in the absorption studies (e.g. very high dose levels) compared to those (e.g. much lower dose levels, especially in the case of human data) used to determine the starting point. Consideration should also be given to the age of the animals employed in the absorption studies (e.g. adult animals) compared to the age of the animals (e.g. pups during lactation) used to determine the starting point. For substances that undergo first-pass metabolism by one or more routes of administration, information on the extent of the presystemic metabolism and systemic availability should also be considered. This could lead to an additional modification of the starting point.

In practice, in the absence of dermal toxicity factors, the US EPA (2004) has devised a simplified paradigm for making route-to-route (oral-to-dermal) extrapolations for systemic effects. This approach is subject to a number of factors that might compromise the applicability of an oral toxicity factor for dermal exposure assessment. The estimation of oral absorption efficiency, to adjust the toxicity factor from administered to absorbed dose, introduces uncertainty. Part of this uncertainty relates to distinctions between the terms absorption and bioavailability. Typically, the term absorption refers to the disappearance of substance from the gastrointestinal lumen, while oral bioavailability is defined as the rate and amount of substance that reaches the systemic circulation unchanged. That is, bioavailability accounts for both absorption and pre-systemic metabolism. Although pre-systemic metabolism includes both gut wall and liver metabolism, for the most part it is liver first pass effect that plays the major role.

In the absence of metabolic activation or detoxification, toxicity adjustment should be based on bioavailability rather than absorption because the dermal pathway purports to estimate the amount of parent compound entering the systemic circulation. Simple adjustment of the oral toxicity factor, based on oral absorption efficiency, does not account for metabolic by-products that might occur in the gut wall but not the skin, or conversely in the skin, but not the gut wall.

The efficiency of first pass metabolism determines the impact on route-to-route extrapolation. The adjusted dermal toxicity factor may overestimate the true dose-

[43] It needs to be ensured that systemic effects are not secondary to local ones. E.g. dermal contact with a substance may also result in direct dermal toxicity, such as allergic contact dermatitis, chemical irritation or skin cancer – effects that might in an early stage lead to systemic responses that consequently are misinterpreted as such.

response relationship because it would be based upon the amount of parent compound in the systemic circulation rather than on the toxic metabolite. Additionally, percutaneous absorption may not generate the toxic metabolite to the same rate and extent as the GI route.

In practice, an adjustment in oral toxicity factor (to account for absorbed dose in the dermal exposure pathway) is recommended when the following conditions are met: (1) the toxicity value derived from the critical study is based on an administered dose (e.g., delivery in diet or by gavage) in its study design; (2) a scientifically defensible database demonstrates that the GI absorption of the substance in question, from a medium (e.g., water, feed) similar to the one employed in the critical study, is significantly less than 100% (e.g., <50%). A cut-off of 50% GI absorption is recommended to reflect the intrinsic variability in the analysis of absorption studies. Thus, this cut-off level obviates the need to make comparatively small adjustments in the toxicity value that would otherwise impart on the process a level of accuracy that is not supported by the scientific literature.

If these conditions are not met, a default value of complete (i.e., 100%) oral absorption may be assumed, thereby eliminating the need for oral toxicity-value adjustment. The Uncertainty Analysis could note that employing the oral absorption default value may result in underestimating risk, the magnitude of which being inversely proportional to the true oral absorption of the substance in question.

The extrapolation of the kinetic behaviour of a substance from one exposure route to another can also be performed by using PBK models. This extrapolation procedure is based on the inclusion of appropriate model equations to represent the exposure pathways of interest. Once the substance has reached the systemic circulation, its biodistribution is assumed to be independent of the exposure route. To represent each exposure pathway different equations (or models) are typically used. The oral exposure of a substance may be modelled by introducing a first order or a zero order uptake rate constant. To simulate the dermal absorption, a diffusion-limited compartment model may represent skin as a portal of entry. Inhalation route is often represented with a simple pulmonary compartment and the uptake is controlled by the blood over air partition coefficient. After the equations describing the route-specific entry of substances into systemic circulation are included in the model, it is possible to conduct extrapolations of toxicokinetics and dose metrics.

In conclusion, route-to-route extrapolation can follow the application of assessment factors as long as the mentioned pre-conditions are met. Any specific TK information may refine the assessment factor in order to meet the precautionary function of the application of the factors as such.

Appendices to Section R.7.12

Appendix R.7.12-1 Toxicokinetics– Physiological Factors

This inventory has been compiled to provide a source of information on physiological parameters for various species that may be useful for interpreting toxicokinetic data. The list is not exhaustive and data from other peer-reviewed sources may be used. If study-specific data are available then this should be used in preference to default data.

Zwart *et al.* (1999) have reviewed anatomical and physiological differences between various species used in studies on pharmacokinetics and toxicology of xenobiotics. A selection of the data presented by these authors that may be relevant in the context of the EU risk assessment is quoted below. The tables are adapted from Zwart *et al.* (1999).

The authors however, focus on the oral route of administration and data relevant for other routes may have to be added. Some of those are already quoted in the section on repeated dose toxicity and are therefore not repeated here.

Data on stomach pH-values

<u>Qualitative Aspects to be considered in the stomach</u>

Rodents have a non-glandular forestomach that has no equivalent in humans. It is thin-walled and transparent. In the non-glandular stomach the pH is typically higher than in the glandular part and it contains more microorganisms. The glandular stomach has gastric glands similar to the human stomach but is a relatively small part of the total rodent stomach. Data on stomach pH for different species are rare and most stem from relatively old sources.

Table R.7.12—7 Data on stomach pH for different species

	Human	Rhesus monkey	Rat	Mouse	Rabbit	Dog	Pig
Median							2.7 (3.75-4)
Median anterior portion	2.7 (1.8-4.5)	4.8	5.0	4.5	1.9	5.5	4.3
Median posterior portion	1.9 (1.6-2.6)	2.8	3.0	3.1	1.9	3.4	2.2
Fasted	1.7 (1.4-2.1)					1.5	1.6-1.8 (0.8-3.0)
Fed	5.0 (4.3-5.4)					2.1± 0.1 [1)]	<2 [2)]

1) Standard deviation

2) Data from one animal only

Data on intestine pH and transit times

Table R.7.12—8 Data on intestine pH

pH (fasted)	Human	Rat (Wistar)	Rabbit	Dog	Pig	Monkey
Intestine		6.5-7.1	6.5-7.1	6.2-7.5	6.0-7.5	5.6-9
Duodenum	5-7	6.9[1]		4.5-7.5	7.2	
Jejunum	6-7					
Ileum	7-8					
Jejunum/ileum		7.8[1]				
Caecum	5.9	6.8	6.6	6.4	6.3	5.0
Colon	5.5-7	6.6, 7.1 [1)]	7.2	6.5	6.8	5.1
Rectum	7					

1) Fed state

Table R.7.12—9 Calculated transit times in the intestine

Transit time (hours)	Human	Rat	Rabbit	Dog
small intestine	2.7 to 5 [1)] Children (8 to 14 years): 5.1-9.2	1.5		0.5-2
Colon	Children (8 to 14 years): 6.2-54.7	6.0-7.2	3.8	

1) From various authors, after fasting or a light meal

Physiological parameters for inhalation

Table R.7.12—10 Comparison of physiological parameters relating to the upper airways of rat, humans, monkeys

Species	body weight (kg)	Body surface area (m^2)	Nasal cavity volume (cm^3)	Nasal cavity surface area (cm^2)	Relative nasal surface area	Pharynx surface area (cm^2)	Larynx surface area (cm^2)	Trachea surface area (cm^2)	Tidal volume (cm^3)	Breaths per min	Minute volume (l/min)
Human	70	1.85	25	160	6.4	46.6	29.5	82.5	750-800	12-15	9-12
Rhesus monkey	7	0.35	8	62	7.75	-	-	-	70	34	2.4
Rat	0.25	0.045	0.26	13.44	51.7	1.2	0.17	3	2	120	0.24

(from De Sesso, 1993)

The US EPA in the Exposure factors handbook (1997) has reviewed a number of studies on inhalation rates for different age groups and activities. The activity levels were categorised as resting, sedentary, light, moderate and heavy. Based on the studies that are critically reviewed in detail in the US EPA document, a number of recommended inhalation rates can be derived. One bias in the data is mentioned explicitly, namely that most of the studies reviewed were limited to the Los Angeles area and may thus not represent the general US population. This should also be born in mind when using those data in the European context. The recommended values were calculated by averaging the inhalation rates (arithmetic mean) for each population and activity level from the various studies. Due to limitations in the data sets an upper percentile is not recommended. The recommended values are given below:

Table R.7.12—11 Summary of recommended values from US EPA (1997)

Population	Mean ventilation rates [m^3/24 h]
Long-term exposures	
Infants <1 year [1)]	4.5
Children 1-2 years [1)]	6.8
3-5 years [1)]	8.3
6-8 years [1)]	10
9-11 years males females	 14 13
12-14 years males females	 15 12

Population	Mean ventilation rates [m^3/24 h]
15-18 years males females	 17 12
Adults 19 – 65+ years males females	 15.2 11.3
Short-term exposures	**m^3/h**
Children	
Rest	0.3
Sedentary activities	0.4
Light activities	1.0
Moderate activities	1.2
Heavy activities	1.9
Adults	
Rest	0.4
Sedentary activities	0.5
Light activities	1.0
Moderate activities	1.6
Heavy activities	3.2
Outdoor workers	
Hourly average	1.3 (3.3 m^3/h) 2)
Slow activities	1.1
Moderate activities	1.5
Heavy activities	2.5

1) No sex difference found

2) Upper percentile

The document also mentions that for a calculation of an endogenous dose using the alveolar ventilation rate it has to be considered that only the amount of air available for exchange via the alveoli per unit time has to be taken into account, accounting for approximately 70% of the total ventilation. This should also be considered in the risk assessment.

Using a respiratory tract dosimetry model (ICRP66 model; Snipes *et al.*, 1997) calculated respiration rates for male adults. Based on these breathing rates estimated daily volumes of respiration were derived for different populations:

- General population: 8 h sleep, 8 h sitting, 8 h light activity: 19.9 m^3
- Light work: 8 h sleep, 6.5 h sitting, 8.5 h light activity, 1 h heavy activity: 22.85 m^3
- Heavy work: 8 h sleep, 4 h sitting, 10 h light activity, 2 h heavy activity: 26.76 m^3

The same authors also mention that in humans breathing pattern changes from nose breathing to nose/mouth breathing at a ventilation rate of about 2.1 m^3/h (60% through nose, 40% through the mouth). At a ventilation rate of 5 m^3/h about 60% of air is inhaled through the mouth and 40% through the nose. However these model calculations seem to overestimate the ventilation rates compared to the experimental data reviewed by US EPA (1992).

Physiological parameters used in PBK modeling

Literature on PBK modelling also contains a number of physiological parameters that are used to calculate tissue doses and distributions. Brown *et al.* (1997) have published a review of relevant physiological parameters used in PBK models. This paper provides representative and biologically plausible values for a number of physiological parameters for common laboratory species and humans. It constitutes an update of a document prepared by Arms and Travis (1988) for US EPA and also critically analyses a compilation of representative physiological parameter values by Davies and Morris (1993). Those references are therefore not reviewed here, but given in the reference list for consultation. In contrast to the other authors Brown *et al.* (1997) also try to evaluate the variability of the parameters wherever possible, by giving mean values plus standard deviation and/or the range of values identified for the different parameters in different studies. The standard deviations provided are standard deviations of the reported means in different studies, in other words they are a measure of the variation among different studies, not the interindividual variation of the parameters themselves. This variation may therefore include sampling error, interlaboratory variation, differences in techniques to obtain the data. The authors also provide some data on tissues within certain organs, which will not be quoted here.

Table R.7.12—12 Organ weights as percent of body weight

(adapted from Brown *et al.* (1997)) (Typically the values reflect weights of organs drained of blood)

Organ	Mouse mean ± standard deviation	Mouse range	Rat mean ± standard deviation	Rat range	Dog mean ± standard deviation	Dog range	Human reference value mean ± standard deviation	Human range
Adipose tissue[1]		5-14 [1a)]		5.5-7 [1b)]			13.6 ± 5.3 [1c)] 21.3 [1d)], 32.7 [1e)]	5.2-21.6 [1c)]
Adrenals	0.048 [2)]		0.019 ± 0.007	0.01 - 0.031	0.009 ± 0.004	0.004 - 0.014	0.02 [3)]	
Bone	10.73 ± 0.53	10.16 - 11.2		5-7 [4)]	8.10 [2,5)]		14.3 [3)]	
Brain	1.65 ± 0.26	1.35-2.03	0.57 ± 0.14	0.38 - 0.83	0.78 ± 0.16	0.43 - 0.86	2.00 [3)]	
Stomach	0.60 [2)]		0.46 ± 0.06	0.40 - 0.60	0.79 ± 0.15	0.65 - 0.94	0.21 [3)]	
Small intestine	2.53 [2)]		1.40 ± 0.39	0.99 - 1.93	2.22 ± 0.68	1.61 - 2.84	0.91 [3)]	
Large intestine	1.09 [2)]		0.84 ± 0.04	0.80-0.89	0.67 ± 0.03	0.65 - 0.69	0.53 [3)]	
Heart	0.50 ± 0.07	0.40-0.60	0.33 ± 0.04	0.27 - 0.40	0.78 ± 0.06	0.68 - 0.85	0.47 [3)]	
Kidneys	1.67 ± 0.17	1.35-1.88	0.73 ± 0.11	0.49 - 0.91	0.55 ± 0.07	0.47 - 0.70	0.44 [3)]	
Liver	5.49 ± 1.32	4.19-7.98	3.66 ± 0.65	2.14 - 5.16	3.29 ± 0.24	2.94 - 3.66	2.57 [3)]	
Lungs	0.73 ± 0.08	0.66-0.86	0.50 ± 0.09	0.37 - 0.61	0.82 ± 0.13	0.62 - 1.07	0.76 [3)]	
Muscle	38.4 ± 1.81	35.77-39.90	40.43 ± 7.17	35.36 - 45.50	45.65 ± 5.54	35.20 - 53.50	40.00 [3)]	
Pancreas	No reliable data		0.32 ± 0.07	0.24 - 0.39	0.23 ± 0.06	0.19 - 0.30	0.14 [3)]	

Organ	Mouse mean ± standard deviation	Mouse range	Rat mean ± standard deviation	Rat range	Dog mean ± standard deviation	Dog range	Human reference value mean ± standard deviation	Human range
Skin	16.53 ± 3.39	12.86-20.80	19.03 ± 2.62	15.80 - 23.60	no representative value		3.71 3) (3.1 female, 3.7 male) 3)	
Spleen	0.35 ± 0.16	0.16 - 0.70	0.20 ± 0.05	0.13 - 0.34	0.27 ± 0.06	0.21 - 0.39	0.26 3)	
Thyroid	no data		0.005 ± 0.002	0.002 - 0.009	0.008 ± 0.0005	0.0074 - 0.0081	0.03 3)	

1) Defined mostly as dissectible fat tissue,

1a) Strongly dependent on strain and age in mice,

1b) Male Sprague Dawley rats equation: Fat content = 0.0199·body weight + 1.664, for male F344 rats: Fat content = 0.035·body weight + 0.205

1c) Males, 30-60 years of age

1d) ICRP, 1975 reference value for 70 kg man,

1e) ICRP, 1975 reference value for 58 kg women

2) One study only

3) ICRP, 1975 reference value

4) In most of the studies reviewed by the authors

5) Mongrel dogs

To derive the organ volume from the mass for most organs a density of 1 can reasonably be assumed. The density of marrow free bone is 1.92 g/cm^3 (Brown *et al.*, 1997).

Brown *et al.* (1997) also give values for cardiac output and regional blood flow as a percentage of cardiac output or blood flow/100 g tissue weight for the most common laboratory species and humans. The data used are derived from non-anaesthetised animals using radiolabelled microsphere technique. For humans data using various techniques to measure perfusion were compiled.

Table R.7.12—13 Cardiac output (ml/min) for different species

(adopted from Brown *et al.* (1997)).

Mouse mean ± standard deviation	Mouse range	Rat mean ± standard deviation	Rat range	Dog mean ± standard deviation	Dog range	Human reference value
13.98 ± 2.85	12 - 16	110.4 ± 15.60	84 - 134	2,936 [1)]	1,300 - 3,000 [1)]	5,200 [1)]

1) One study only

According to the authors giving blood flow in units normalised for tissue weight can result in significant errors if default reference weights are used instead of measured tissue weights in the same study.

Table R.7.12—14 Regional blood flow distribution in different species

(ml/min/100g of tissue) (adopted from Brown *et al.* (1997))

Organ	Mouse mean ± standard deviation	Mouse range	Rat mean ± standard deviation	Rat range	Dog mean ± standard deviation	Dog range
Adipose tissue[1]			33 ± 5	18 - 48	14 ± 1	13 - 14
Adrenals			429 ± 90	246 - 772	311 ± 143	171 - 543
Bone			24 ± 3	20 - 28	13 ± 1	12 - 13
Brain	85 ± 1	84 - 85	110 ± 13	45 - 134	65 ± 4	59 - 76
Heart	781 ± 18	768 - 793	530 ± 46	405 - 717	79 ± 6	57 - 105
Kidneys	439 ± 23	422 - 495	632 ± 44	422 - 826	406 ± 37	307 - 509
Liver	131					
Hepatic artery	20		23 ± 44	9 - 48	21 ± 3	12 - 30
Portal vein	111 ± 9	104 - 117	108 ± 17	67 - 162	52 ± 4	42 - 58
Lungs	35[1]		127 ± 46 [1)]	38 - 147 [1)]	79 ± 43 [1)]	36 - 122
Muscle	24 ± 6	20 - 28	29 ± 4	15 - 47	11 ± 2	6 - 18
Skin	18 ± 12	9 - 26	13 ± 4	6 - 22	9 ± 1	8 - 13

1) Bronchial flow

2) Based on animal studies

Table R.7.12—15 Regional blood flow distribution in different species

(% cardiac output) (adopted from Brown *et al.* (1997))

Organ	Mouse mean ± standard deviation	Mouse range	Rat mean ± standard deviation	Rat range	Dog mean ± standard deviation	Human reference value mean, male	Human reference value mean, female	Human range
Adipose tissue [1)]			7.0 [2)]			5.0	8.5	3.7-11.8
Adrenals			0.3±0.1	0.2-0.3	0.2[2]	0.3	0.3[2]	
Bone			12.2 [2)]			5.0	5.0	2.5-4.7
Brain	3.3±0.3	3.1-3.5	2.0±0.3	1.5-2.6	2.0 [2)]	12.0	12.0	8.6-20.4
Heart	6.6±.0.9	5.9-7.2	4.9±0.1	4.5-5.1	4.6 [2)]	4.0	5.0	3.0-8.0
Kidneys	9.1±2.9	7.0-11.1	14.1±1.9	9.5-19.0	17.3 [2)]	19.0	17.0	12.2-22.9
Liver	16.2		17.4	13.1-22.1	29.7 [2)]	25.0	27.0	11-34.2
Hepatic artery	2.0		2.4	0.8-5.8	4.6 [2)]			
Portal vein	14.1	13.9-14.2	15.1	11.1-17.8	25.1 [2)]	19.0	21.0	12.4-28.0
Lungs	0.5[1]		2.1±0.4 [1)]	1.1-3.0 [1)]	8.8 [1,2)]	2.5[1]		
Muscle	15.9±5.2	12.2-19.6	27.8 [2)]		21.7 [2)]	17.0	12.0	5.7-42.2
Skin	5.8±3.5	3.3-8-3	5.8 [2)]		6.0 [2)]	5.0	5.0	3.3-8.6

1) Bronchial flow

2) One study only

The blood flow to some organs such as the liver are highly variable and can be influenced by factors including anaesthesia, posture, food intake, exercise.

Gerlowski and Jain (1983) have published a compilation of different organ volumes and plasma flows for a number of species at a certain body weight from other literature sources.

Table R.7.12—16 Organ volumes, plasma flow used in PBK-models

Parameter	Mouse	Hamster	Rat	Rabbit	Monkey	Dog	Human
Body weight (g)	22	150	500	2,330	5,000	12,000	70,000
Volume (ml)							
Plasma	1	6.48	19.6	70	220	500	3,000
Muscle	10	-	245	1,350	2,500	5,530	35,000
Kidney	0.34	1.36	3.65	15	30	60	280
Liver	1.3	6.89	19.55	100	135	480	1,350
Gut	1.5	12.23	11.25	120	230	480	2,100
Gut lumen	1.5	-	8.8	-	230	-	2,100
Heart	0.095	0.63	1.15	6	17	120	300
Lungs	0.12	0.74	2.1	17	-	120	-
Spleen	0.1	0.54	1.3	1	-	36	160
Fat	-	-	34.9	-	-	-	10,000
Marrow	0.6	-	-	47	135	120	1,400
Bladder	-	-	1.05	-	-	-	-
Brain	-	-	-	-	-	-	1,500
Pancreas	-	-	2.15	-	-	24	-
Prostate	-	-	6.4	-	-	-	-
Thyroid	-	-	0.85	-	-	-	20
Plasma flow (ml/min)							
Plasma	4.38	40.34	84.6	520	379	512	3,670
Muscle	0.5	-	22.4	155	50	138	420
Kidney	0.8	5.27	12.8	80	74	90	700
Liver	1.1	6.5	4.7	177	92	60	800
Gut	0.9	5.3	14.6	111	75	81.5	700
Heart	0.28	0.14	1.6	16	65	60	150
Lungs	4.38	28.4	2.25	520	-	512	-

Parameter	Mouse	Hamster	Rat	Rabbit	Monkey	Dog	Human
Spleen	0.05	0.25	0.95	9	-	13.5	240
Fat	-	-	3.6	-	-	-	200
Marrow	0.17	-	-	11	23	20	120
Bladder	-	-	1.0	-	-	-	-
Brain	-	-	0.95	-	-	-	380
Pancreas	-	-	1.1	-	-	21.3	-
Prostate	-	-	0.5	-	-	-	-
Thyroid	-	-	0.8	-	-	-	20

Table R.7.12—17 A number of physiological parameters for different species

compiled by Nau and Scott (1987)

Parameter	Mouse	Rat	Guinea pig	Rabbit	Dog	Monkey	Human
Bile flow (ml/kg per day)	100	90	230	120	12	25	5
Urine flow (ml/kg per day)	50	200		60	30	75	20
Cardiac output (ml/min per kg)	300	200		150	100	80-300	60-100
Hepatic blood flow (l/min)	0.003	0.017	0.021	0.12	0.68	0.25	1.8
Hepatic blood flow (ml/min per kg)	120	100		50	25	25	25-30
Liver weight (% of body weight)	5.1	4.0	4.6	4.8	2.9	3.3	2.4
Renal blood flow (ml/min per kg)	30				22	25	17
Glomerular filtration (ml/min per kg)	5				3.2	3	1.3

Gad and Chengelis (1992) have summarised a number of physiological parameters for different species. The most important data of the most common laboratory test species are summarised below.

Table R.7.12—18 A number of physiological parameters for different species (Blaauboer *et al.*, 1996)

	Rat	Mouse	Guinea Pig	Rabbit	Dog (Beagle)
Blood volume whole blood (ml/kg)	57.5 - 69.9	78	75	45 - 70	-
Blood volume Plasma (ml/kg)	36.3 - 45.3	45	30.6 - 38.2	-	-
Respiratory frequency min^{-1}	66 - 114	84 - 230	69 - 160	35 - 65	10 - 30[1]
tidal volume (ml)	0.6 - 1.25	0.09 - 0.38	1.8	4 - 6	18 - 35[1]
Urine volume (ml/kg/24 h)	55			20 - 350	-
Urine pH	7.3 - 8.5	-	-	8.2	-

1) In Beagles of 6.8 to 11.5 kg bw

Appendix R.7.12-2 Prediction of toxicokinetics integrating information generated *in silico* and *in vitro*

The methods presented in this attachment are for the purpose to demonstrate the future use of *in silico* and/or *in vitro* methods in toxicokinetics. Although promising in the area of pharmaceutical research, most of the examples given have not been fully validated for the purpose of use outside this area. Further development and validation of these approaches are ongoing.

Techniques for the prediction of pharmacokinetics in animals or in man have been used for many years in the pharmaceutical industry, at various stages of research and development. A considerable amount of work has been dedicated to developing tools to predict absorption, distribution, metabolism, and excretion of drug candidates. The objective in drug development is to eliminate as early as possible candidate drugs predicted to have undesirable characteristics, such as being poorly absorbed by the intended route of administration, being metabolised via undesirable pathways, being eliminated too rapidly or too slowly. These predictions are done at various stages of drug development, using all available evidence and generating additional meaningful information from simple experiments. Although these techniques were developed in the particular context of drug development, there is no reason a priori not to use them for the safety assessment of substances. The toxicokinetic information generated can be used in particular to select substances to be further developed, to direct further testing and to assist experimental design, thus saving experimental efforts in terms of cost, time and animal use.

In practice, the prediction of the toxicokinetic behaviour of a substance rests upon the use of appropriate models, essentially physiologically-based compartmental pharmacokinetic models, coupled to the generation of estimates for the relevant model parameters. *In silico* models or *in vitro* techniques to estimate parameter values used to predict absorption, metabolic clearance, distribution and excretion have been developed. Blaauboer *et al.*(1996; 2002) reviewed the techniques involved in toxicokinetic prediction using physiologically-based kinetic models. The thorough discussion on the applicability of physiologically based pharmacokinetic models in risk assessment is provided by IPCS (2010). Also, a general discussion on the *in silico* methods used to predict ADME is provided by Boobis *et al.* (2002).

As for all predictions using models, these approaches must be considered together with the accompanying uncertainty of the predictions made, which have to be balanced against the objective of the prediction. Experimental validation *in vivo* of the predictions made and refinement of the models used is usually necessary (Parrott *et al.*, 2005; US EPA, 2007), and has to be carefully planned on a case by case basis. A strategy for integrating predicted and experimental kinetic information generated routinely during drug development is described by Theil *et al.* (2003), by Parrot *et al.* (2005), and by Jones *et al.* (2006). The principles presented by these authors are relevant to kinetics simulation and prediction in the field of chemical safety, since they allow the integration of the available kinetic or kinetically-relevant information from the very beginning of the risk assessment process. In the most initial stages of development, simulations can be generated using only physico-chemical characteristics, which themselves can be derived from *in silico* models (QSARs/ QSPRs).

The strategy proposed by Jones *et al.* (2006), in the compound set investigated, led to reasonably accurate prediction of pharmacokinetics in man for approximately 70% of the compounds. According to the authors, *these successful predictions were achieved mainly for compounds that were cleared by hepatic metabolism or renal excretion, and whose absorption and distribution were governed by passive processes. Significant mis-predictions were achieved when other elimination processes (e.g. biliary elimination) or active processes were involved or when the assumptions of flow limited distribution and well mixed compartments were not valid.*

In addition to the parent compound, in a number of cases metabolites contribute significantly or even predominantly, to the overall exposure-response relationship. In such cases, the quantitative *ex vivo* prediction of metabolite kinetics after exposure to the parent compound remains difficult. A separate study program of the relevant metabolites may then become necessary.

Models used to predict absorption / bioavailability

Gastro intestinal absorption models

In order to be absorbed from the GI tract, substances have to be present in solution in the GI fluids, and from there have to cross the GI wall to reach the lymph or the venous portal blood. Key determinants of gastrointestinal absorption are therefore:

- release into solution from solid forms or particles (dissolution),
- solubility in the GI fluids, and
- permeability across the GI wall into the circulatory system.

Dokoumetzidis *et al.* (2005) distinguish two major approaches in the modelling of the drug absorption processes involved in the complex milieu of the GI tract.

The first approach is the simplified description of the observed profiles, using simple differential or algebraic equations. On this basis, a simple classification for pharmaceutical substances, the Biopharmaceutics Classification System (BCS), resting on solubility and intestinal permeability considerations, has been developed by Amidon *et al.* (1995). The BCS divides pharmaceutical substances into 4 classes according to their high or low solubility and to their high or low intestinal permeability, and has been incorporated into FDA guidance (2000).

The second approach tries to build models incorporating in more detail the complexity of the processes taking place in the intestinal lumen, using either compartmental analysis, i.e. systems of several differential equations (Agoram *et al.*, 2001; Yu *et al.*, 1996; Yu and Amidon, 1999), dispersion systems with partial differential equations (Ni *et al.*, 1980; Willmann *et al.*, 2003 and 2004), or Monte Carlo simulations (Kalampokis *et al.*, 1999). Some of these approaches have been incorporated into commercial computer software (Coecke *et al.*, 2006; Parrott and Lave, 2002), or are used by contract research organisations to generate predictions for their customers. An attractive feature of these models is their ability to generate a prediction of extent and often rate of absorption in data-poor situations, i.e. at the initial stage of data generation, using a simple set of parameters describing ionisation, solubility and permeability.

Factors potentially complicating the prediction of absorption are:

- intra luminal phenomena such as degradation or metabolism, matrix effects, chemical speciation, which may reduce the amount available for absorption, or generate metabolites which have to be considered in terms of toxicological and toxicokinetic properties;
- intestinal wall metabolism, which may have similar consequences;
- intestinal transporters (efflux pumps), which may decrease the permeability of the GI wall to the substance.

These factors have to be considered and incorporated into absorption / bioavailability models on a case-by-case basis.

Parameter estimation for GI absorption models

A discussion on the *in vitro* approaches used to generate absorption parameters can be found in Pelkonen *et al.* (2001).

Where relevant, i.e. when dissolution from solid particles may be the limiting factor for GI absorption, estimates for the dissolution rate parameters can be obtained experimentally *in vitro* or using a QSAR/ QSPR approach (e.g. Zhao *et al.*, 2002). Potentially rate-limiting steps preceding dissolution (e.g. disaggregation of larger solid forms) are usually studied in to a greater extent in the pharmaceutical field than in chemical safety assessment, because they can be manipulated via formulation techniques. However, pre-dissolution events may also have a determining role in the absorption of substances, by influencing either its rate or its extent.

Solubility parameters can be estimated experimentally or using QSAR/ QSPR models. A discussion of *in silico* models can be found in Stenberg *et al.* (2002).

Permeability estimates can be obtained via:

- *in silico* models (QSAR/ QSPRs);
- *in vitro* permeation studies across lipid membranes (e.g. PAMPA) or across a monolayer of cultured epithelial cells (e.g. CaCO-2 cells, MDCK cells);
- *in vitro* permeation studies using excised human or animal intestinal tissues;
- *in vivo* intestinal perfusion experiments, in animals or in humans.

Discussion of the various *in silico* and *in vitro* methods to estimate intestinal permeability can be found in Stenberg *et al.* (2002), Artursson *et al.* (2001), Tavelin *et al.* (2002), Matsson *et al.* (2005).

<u>Dermal route</u>

Percutaneous absorption through intact skin is highly dependent on the physico-chemical properties of substances, and in particular of molecular weight and lipophilicity. Molecules above a certain molecular weight are unlikely to cross intact skin, and substances which are either too lipophilic or too hydrophilic have a low skin penetration. Cut off points at a molecular weight of 500 and log P values below -1 or above 4 have been used to set a conservative default absorption factor at 10 % cutaneous absorption

(EC, 2007). However, it should be emphasised that this is a default factor, and by no means a quantitative estimate of cutaneous absorption.

Predictive models have been developed to try and estimate the extent of dermal absorption from physico-chemical properties (Cleek and Bunge, 1993). An *in vitro* method has been developed and validated and is described in EU B.45[44] or OECD TG 428.

The EU founded project on the Evaluation and Prediction of Dermal Absorption of Toxic Chemicals (EDETOX) established a large critically evaluated database with *in vivo* and *in vitro* data on dermal absorption / penetration of substances. The data were used to evaluate existing QSARs and to develop new models including a mechanistically-based mathematical model, a simple membrane model and a diffusion model of percutaneous absorption kinetics. A guidance document was developed for conduct of *in vitro* studies of dermal absorption/penetration. More information on the database, model and guidance documents can be found at http://www.ncl.ac.uk/edetox/ .

Inhalation route

Together with physiological values (ventilation flow, blood flow), the key parameter needed to predict the passage into blood of inhaled volatile compounds is the blood/air partition coefficient (Blaauboer *et al.*, 1996; Reddy *et al.*, 2005). References to methods for estimating or measuring blood/air partition coefficients are indicated below together with the discussion of other partition coefficients. The parameters are included in physiologically-based models predicting the concentrations in the venous pulmonary blood, assimilated to the systemic arterial blood, and in the exhaled air.

Other factors may influence absorption by the inhalation route. For example, water solubility determines solubility in the mucus layer, which may be a limiting factor, and the dimensions of the particles are a key factor for the absorption of particulate matter.

Other routes

Other routes, e.g. via the oral, nasal or ocular mucosa, may have to be considered in specific cases.

Systemic bioavailability and first-pass considerations

After oral exposure, systemic bioavailability is the result of the cumulated effects of the absorption process and of the possible extraction by the liver from the portal blood of part of the absorbed dose, or first-pass effect. The first-pass effect can be incorporated into a suitably defined physiologically-based toxicokinetic model. Using estimates of both the absorption rate and of the intrinsic hepatic clearance, the systemic bioavailability of the substance can then be predicted. Metabolism at the port of entry can also occur within the gut wall, and this can be included in the kinetic models. At the model validation stage, however, it is often difficult to differentiate gut wall metabolism from liver metabolism *in vivo*.

[44] See Test Methods Regulation (Council Regulation (EC) No 440/2008).

Similarly, metabolism may occur in the epidermis or dermis. The current skin absorption test (EU B.45[45], OECD TG 428) does not take cutaneous metabolism into account. Specific studies may be necessary to quantify skin metabolism and bioavailability by dermal route.

Pulmonary metabolism of some substances exist (Borlak *et al.*, 2005), but few substances are reported to undergo a quantitatively important pulmonary first-pass effect.

Models to predict Distribution

Blood binding

Blood cell partitioning

Partitioning of compounds into blood cells, and in particular red blood cells (RBC), is an important parameter to consider in kinetic modelling (Hinderling, 1997).

Partitioning into leukocytes or even platelets may have to be considered in rare cases. A significant influence of such partitioning has been described for some drugs, e.g. chloroquine (Hinderling, 1997).

Partitioning into blood cells can be measured experimentally *in vitro* (Hinderling, 1997), or estimated using a QSAR/ QSPR approach based on physico-chemical properties.

Plasma protein binding

Plasma protein binding is an important parameter to be included in physiologically-based kinetic models, because plasma protein binding can influence dramatically distribution, metabolism and elimination. Plasma binding with high affinity will often restrict distribution, metabolism and elimination. However, this is by no means systematic, because the overall kinetics is a function of the interplay of all processes involved. Distribution will depend on the balance between affinity for plasma components and for tissues, and the elimination of compounds having a very high intrinsic clearance (i.e. very effective elimination mechanisms) will be hastened by high plasma protein binding, which causes more compound to be available for clearance in the blood compartment.

Plasma protein binding is measured using *in vitro* techniques, using either plasma or solutions of specific proteins of known concentrations. The most standard techniques are equilibrium dialysis and ultrafiltration, but numerous other techniques have been described. More detailed information and references are given by Zini (1991) and Roberts (2001). QSAR/ QSPR methods have also been used to predict of protein binding affinity (e.g. Colmenarejo, 2003).

Tissue distribution

Blood flow-limited distribution.

In physiologically-based kinetic models, the most common model to describe distribution between blood and tissue is blood flow-limited distribution, i.e. the equilibrium between

[45] See Test Methods Regulation (Council Regulation (EC) No 440/2008).

tissue and blood is reached within the transit time of blood through the tissue. In this model, the key parameters are the partition coefficients. Partition coefficients express the relative affinity of the compound for the various tissues, relative to a reference fluid which may be the blood, the plasma or the plasma water. Tissue/ blood, tissue/ plasma, and tissue/ plasma water partition coefficients are inter-related via plasma protein binding and blood cell partitioning. Partition coefficients are integrated in the differential equations predicting blood and tissue concentrations, or in equations of models predicting globally the steady-state volume of distribution of the compound (Poulin and Theil, 2002).

Permeability-limited distribution

In some cases however, due to a low permeability of the surface of exchange between blood and a particular tissue (e.g. blood-brain barrier, placental barrier), the equilibrium between blood and tissue cannot be reached within the transit time of blood through the tissue, and a correction factor must be introduced in the differential equation describing distribution to that tissue. One common, simple way of doing this is to use the permeability area cross product. Thus, distribution is in this case determined by the arterial concentration and the three factors blood flow (physiological parameter), permeability per unit of surface (compound-specific parameter), and surface of exchange (physiological parameter; see Reddy *et al.*, 2005). Permeability-limited distribution makes prediction more difficult due to the lack of well-recognised, easy to use and robust models to quantify the necessary parameters.

<u>Determination of partition coefficients</u>

Experimental methods available to obtain blood/ air, tissue/ air and blood/ tissue partition coefficients are discussed by Krishnan and Andersen (2001). *In vitro* methods include vial equilibration (for volatile compounds), equilibrium dialysis and ultrafiltration. However, these methods require ex-vivo biological material, are time-consuming and often require the use of radiolabelled compound (Blaauboer, 2002).

Models to calculate predicted tissue/blood, tissue/plasma or tissue/plasma water partition coefficients from simple physico-chemical properties have been developed (Poulin and Theil, 2002; Rodgers *et al.*, 2005 and 2006). The necessary compound-specific input is limited to knowledge of the chemical structure and functionalities (e.g. neutral, acid, base, zwitterionic), the pKa or pKas where applicable, and the octanol-water partition coefficient at pH 7.4. Additional necessary parameters describe the tissue volumes and tissue lipid composition. Tissue volumes are usually available or can be estimated from the literature. There are less available direct data on tissue composition in terms of critical binding constituents, particularly in man, although some reasonable estimates can be made from the existing information.

QSAR/ QSPR models developed for the estimation of blood/air and tissue/blood partition coefficients have also been reported (Blaauboer, 2002).

Prediction of metabolism

<u>Numerous aspects of metabolism can and often should be explored using *in vitro* methods (Pelkonen *et al.*, 2005).</u>

Major objectives of the study of metabolism using *in vitro* methods are**:**

- determining the susceptibility of a substance to metabolism (its metabolic stability);
- identifying its kinetically and toxicologically relevant metabolites in the species of interest (including man);
- obtaining a quantitative global estimate of its metabolic clearance, to be included in toxicokinetic models.

Additional possible objectives are:

- characterising enzyme kinetics of the principal metabolic reactions, which can also be used for scaling up and predicting *in vivo* kinetics of a new substance;
- estimating the ability of the substance to act as a substrate for the different enzymes involved in biotransformation;
- exploring inter-species differences in metabolism;
- evaluating potential variability in metabolism in a given species, man in particular;
- identifying whether the substance and/or its metabolite(s) can act as an enzyme inducer;
- identifying whether the substance and/or its metabolite(s) can act as an enzyme inhibitor, and the type of inhibition involved.

Most methods have been developed in the pharmaceutical field, and focused on the cytochrome P isoforms (CYP), because these are the major enzymes involved in drug metabolism. The extension of existing methods to a wider chemical space, and to other enzymatic systems, such as other oxidation pathways, acetylation, hydrolysis, needs to be undertaken with caution, and methods are bound to evolve in this context. In any case, the study of metabolism *in vitro* is often an important step in the integrated risk assessment of substances. In many cases *in vitro* methods are the only option to study metabolism, due to the impracticality or sheer impossibility of *in vivo* studies.

<u>Relative role of different organs in metabolism</u>

Quantitatively, the most important organ for metabolism is by far the liver, although metabolism by other organs can be important quantitatively or qualitatively. The nature of the substance and the route of administration must be taken into account when assessing which organs are most relevant in terms of metabolism (Coecke *et al.*, 2006).

<u>*In vitro* methods to study metabolism</u>

In vitro methods to explore the metabolism, and particularly the hepatic metabolism of a substance are thoroughly discussed by Pelkonen *et al.* (2005) and Coecke *et al.* (2006). Depending on the objective, the different metabolising materials used are microsomes and microsomal fractions, recombinant DNA-expressed individual CYP enzymes, Immortalised cell lines, primary hepatocytes in culture or in suspension, liver slices.

Quantitative estimation of the intrinsic clearance of a substance.

One of the most important pieces of information in order to simulate the toxicokinetics of a substance is the intrinsic metabolic clearance *in vivo*, which has to be incorporated into the kinetic models. Intrinsic clearance can be estimated using quantitative *in vitro* systems (purified enzymes, microsomes, hepatocytes) and extrapolating the results to the *in vivo* situation.

If only a single or a few concentrations are tested, the intrinsic clearance can only be expressed as a single first-order elimination parameter, ignoring possible saturation phenomena. The latter can only be detected by testing a large enough concentration range in an appropriately chosen system. For instance, if a Michaelis and Menten model is applicable, both the V_{max} and the Km of the system may be thus determined.

Of particular importance are:

- the quality and characterisation of the metabolising system itself;
- the quality and characterisation of the experimental conditions, in particular as regards the system's capacity for binding the substances under study (Blanchard *et al.*, 2005) but obviously also as regards other parameters such as temperature, pH, etc.
- The use of appropriate scaling factors to extrapolate to predicted clearance values *in vivo*.

Scaling factors must be chosen taking into account the *in vitro* system utilised. They incorporate in particular information on the *in vitro* concentration of substance available to the metabolising system (unbound), the nature and amount of the enzymes present in the *in vitro* system, the corresponding amount of enzymes in hepatocytes *in vivo*, and the overall mass of active enzyme in the complete liver *in vivo*. Discussions on the appropriate scaling procedures and factors to be taken into account have been developed by Houston and Carlile (1997), Inoue *et al.* (2006), Shiran *et al.* (2006), Howgate *et al.* (2006), Johnson *et al.* (2005), Proctor *et al.* (2004).

In vitro screening for Metabolic interactions

In vitro screening procedures for the prediction of metabolic interactions have been developed for pharmaceuticals. They involve testing an *in vitro* metabolising system for a number of well characterised compounds, with and without the new substance (Blanchard *et al.*, 2004; Turpeinen *et al.*, 2005).

Prediction of excretion

The most common major routes of excretion are renal excretion, biliary excretion and, for volatile compounds, excretion via expired air.

There is at present no *in vitro* model to reliably predict biliary or renal excretion parameters. Determining factors include molecular weight, lipophilicity, ionisation, binding to blood components, and the role of active transporters. In the absence of specific a priori information, many kinetic models include non-metabolic clearance as a single first order rate excretion parameter.

Expired air (exhalation clearance)

Excretion into expired air is modelled using the blood/ air partition coefficient, as described in Appendix R.7.12-2 (Reddy *et al.*, 2005).

Biliary clearance

Current work on biliary excretion focuses largely on the role of transporters (e.g. Klaassen, 2002; Klaassen and Slitt, 2005). However, experimentally determined numerical values for parameters to include into modelling of active transport are largely missing, so that these mechanisms cannot yet be meaningfully included in kinetic models. Levine (1978), Rollins and Klaassen (1979) and Klaassen (1988) have reviewed classical information on the biliary excretion of xenobiotics. Information in man is still relatively scarce, given the anatomical and ethical difficulties of exploring biliary excretion directly in man. Compounds may be highly concentrated into the bile, up to a factor of 1000, and bile flow in man is relatively high, between 0.5 and 0.8 ml/min, so that considerable biliary clearance values of several hundred ml/min, can be achieved (Rowland and Tozer, 1989; Rowland *et al.*, 2004). It should be considered on a case-by-case basis whether biliary excretion and possible entero-hepatic recirculation should be included in the kinetic models used for prediction.

Renal clearance

In healthy individuals and in most pathological states, the renal clearance of xenobiotics is proportional to the global renal function, reflected in the glomerular filtration rate, which can be estimated *in vivo* by measuring or estimating the clearance of endogenous creatinine. Simple models for renal clearance consider only glomerular filtration of the unbound plasma fraction. However, this can lead to significant misprediction when active transport processes are involved. More sophisticated models have been described which include reabsorption and / or active secretion of xenobiotics (Brightman *et al.*, 2006; Katayama *et al.*, 1990; Komiya, 1986 and 1987), but there are insufficient input or reference data to both implement such models and evaluate satisfactorily their predictivity.

Kinetic modelling programs

A number of programs for toxicokinetics simulation or prediction are either available, or used by contract research companies to test their customer's compounds. A non-comprehensive list of such programs is given by Coecke *et al.*, (2006). Available physiologically-based modelling programs purpose-built for toxicokinetic prediction include (non-comprehensive list):

- SimCYP® (SimCYP Ltd, www.simcyp.com);
- PK-Sim® (Bayer Technology Services GmbH, www.bayertechnology.com);
- GastroPlus™ (Simulations Plus Inc, www.simulations-plus.com);
- Cloe PK® (Cyprotex Plc, www.cyprotex.com);
- Noraymet ADME™ (Noray Bioinformatics, SL, www.noraybio.com).

Numerous other simulation programs, either general-purpose or more specifically designed for biomathematical modelling, can be used to implement PBK models. A discussion on this subject and a non-comprehensive list can be found in Rowland *et al.* (2004).

Appendix R.7.12-3 PBK Modelling and the Development of Assessment Factors

A simple but fictional example of the development of an assessment factor for interspecies differences using PBK modelling is presented. A fictional substance, compound A, is a low molecular weight, volatile solvent, with potential central nervous system (CNS) depressant properties. Evidence for the latter comes from a number of controlled human volunteer studies where a battery of neurobehavioural tests were conducted during, and after, exposure by inhalation to compound A.

Compound A is metabolised *in vitro* by the phase I, mixed-function oxidase enzyme, cytochrome P450 2E1 (CYP2E1) by both rat and human hepatic microsomes. There are also some *in vivo* data in rats exposed by inhalation to compound A, with and without pre-treatment with diallyl sulphide, an inhibitor of CYP2E1, that are consistent with metabolism of compound A by this enzyme.

PBK models for the rat and standard human male or female for exposure by inhalation to compound A are built. The rat model was validated by simulating experimentally determined decreases in chamber concentrations of compound A following exposure of rats to a range of initial concentrations in a closed-recirculated atmosphere exposure chamber. The removal of chamber concentration of compound A over time is due to uptake by the rat and elimination, primarily by metabolism. The human PBK model was validated by simulating experimentally determined venous blood concentrations of compound A in male and female volunteers exposed by inhalation to a constant concentration of compound A in a controlled-atmosphere exposure chamber.

It is assumed that the following have been identified for the substance: 1) the active moiety of the substance, and 2) the relevant dose-metric (i.e., the appropriate form of the active moiety e.g., peak plasma concentration (Cmax), area-under-the-curve of parent substance in venous blood (AUCB), average amount metabolised in target tissue per 24 hours (AMmet), peak rate of hepatic metabolism (AMPeakMet), etc). In this case, it is hypothesised that the peak plasma concentration Cmax of compound A is the most likely surrogate dose metric for CNS concentrations of compound A thought to cause a reversible CNS depressant effect. However, Cmax, is dependent upon the peak rate of hepatic metabolism (AMPeakMet). Therefore, the validated rat and human PBK models were run to simulate the exposure time and concentrations of the human study where the neurobehavioural tests did not detect any CNS depressant effects. The dose metric, AMPeakMet for the rat would be divided by the AMPeakMet for the human. This ratio would represent the magnitude of the difference between a specified rat strain and average human male or female. This value may then replace the default interspecies kinetic value since it is based on substance-specific data. Therefore, the derivation of an appropriate assessment factor in setting a DNEL can be justified more readily using quantitative and mechanistic data.

Appendix R.7.12-4 Dermal absorption percentage†

† Based on *in vivo* rat studies in combination with *in vitro* data and a proposal for a tiered approach to risk assessment (Benford *et al.*, 1999).

Estimation of dermal absorption percentage. If appropriate dermal penetration data are available for rats *in vivo* and for rat and human skin *in vitro*, the *in vivo* dermal absorption in rats may be adjusted in light of the relative absorption through rat and human skin *in vitro* under comparable conditions (see equation below and Figure R.7.12—4). The latter adjustment may be done because the permeability of human skin is often lower than that of animal skin (e.g., Howes *et al.*, 1996). A generally applicable correction factor for extrapolation to man can however not be derived, because the extent of overestimation appears to be dose, substance, and animal specific (ECETOC, 2003; Howes *et al.*, 1996; Bronaugh and Maibach, 1987). For the correction factor based on *in vitro* data, preferably maximum flux values should be used. Alternatively, the dermal absorption percentage (receptor medium plus skin dose) may be used. Because, by definition, the permeation constant (Kp in cm/hr) is established at infinite dose levels, the usefulness of the Kp for dermal risk assessment is limited.

$$\textit{in vivo}\text{ human absorption} = \frac{\textit{in vivo}\text{ animal absorption} \times \textit{in vitro}\text{ human absorption}}{\textit{in vitro}\text{ animal absorption}}$$

Similar adjustments can be made for differences between formulants (e.g. *in vivo* active substance in rat and *in vitro* rat data on formulants and active substance)

Tiered Risk Assessment. The establishment of a value for dermal absorption may be performed by use of a tiered approach from a worst case to a more refined estimate (see Figure R.7.12—4). If an initial assessment ends up with a risk, more refinement could be obtained in the next tier if more information is provided on the dermal absorption. In a first tier of risk assessment, a worst case value for dermal absorption of 100% could be used for external dermal exposure in case no relevant information is available (Benford *et al.*, 1999). An estimate of dermal absorption could be made by considering other relevant data on the substance (e.g., molecular weight, log P_{ow} and oral absorption data) (second tier) or by considering experimental *in vitro* and *in vivo* dermal absorption data (third tier, see Section R.7.12.2.2). If at the end of the third tier still a risk is calculated, the risk assessment could be refined by means of actual exposure data (fourth tier) (Table R.7.12—5). This approach provides a tool for risk assessment, and in general it errs on the safe side.

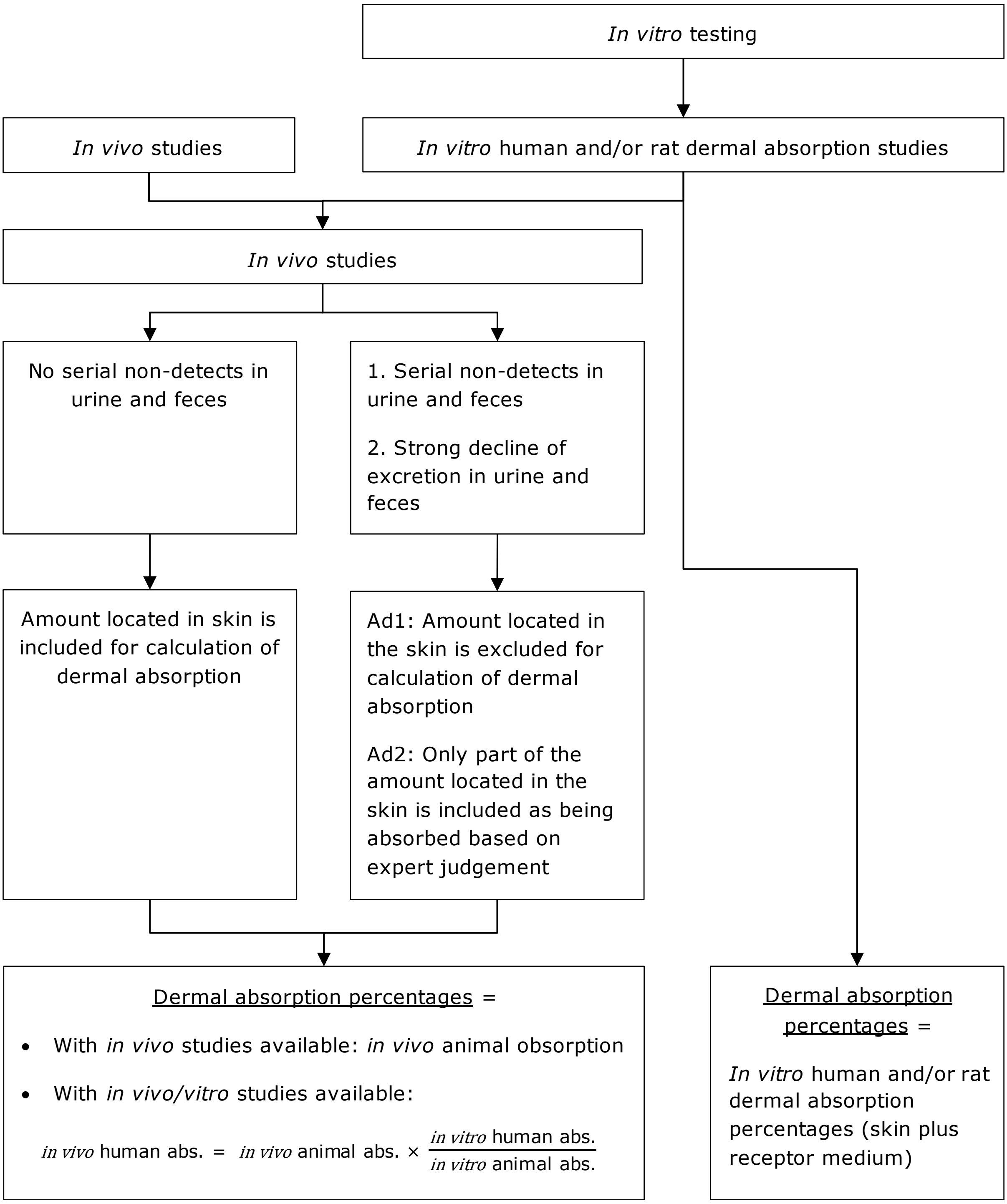

Figure R.7.12—4 Overview of the possible use of *in vitro* and *in vivo* data for setting the dermal absorption percentage.

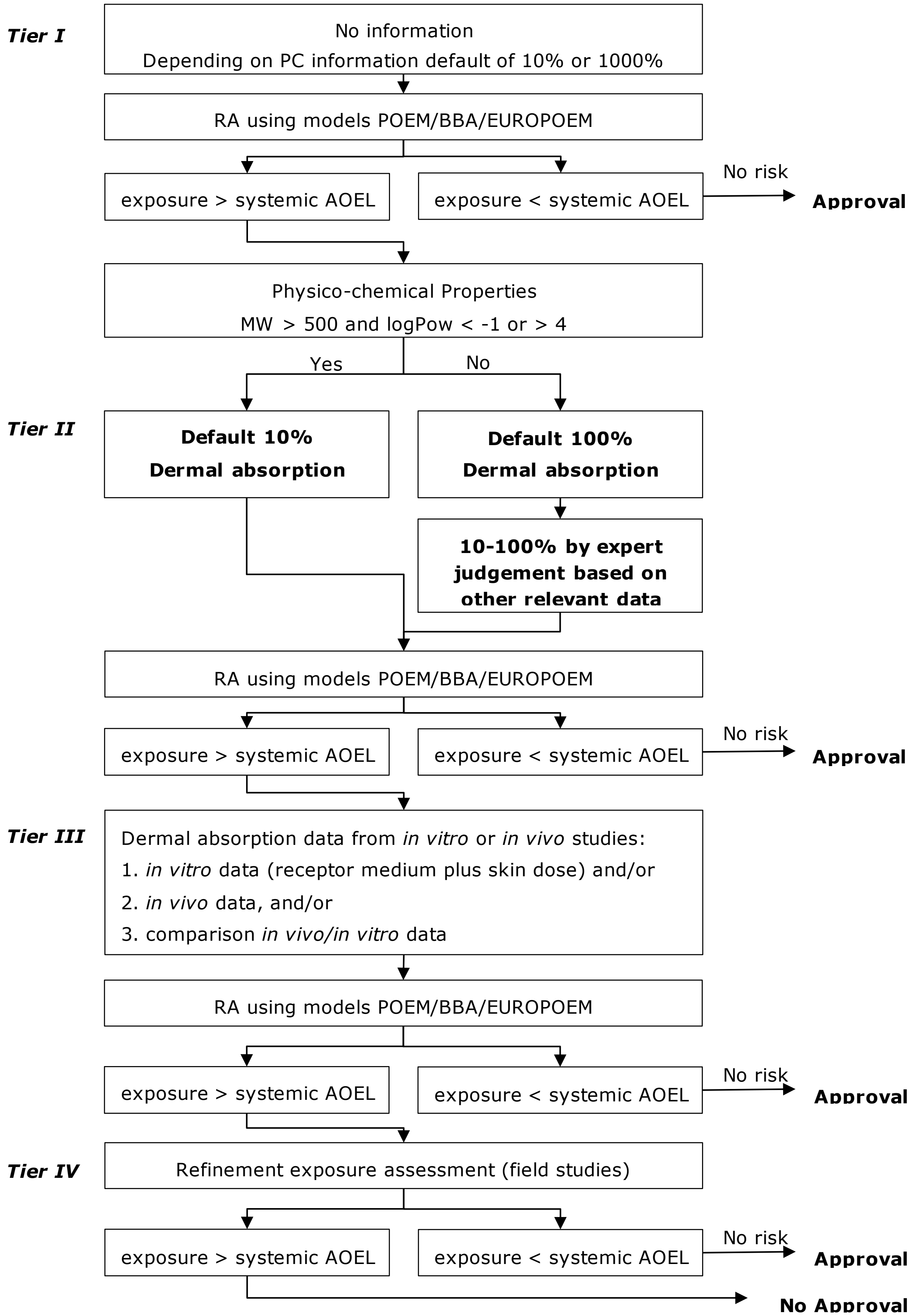

Figure R.7.12—5 Dermal absorption in risk assessment for operator exposure; a tiered approach

R.7.12.3 References for guidance on toxicokinetics

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R.7.13 Substances requiring special considerations regarding testing and exposure

Standard approaches for hazard and risk characterisation rely on the premise that human and/or environmental exposure to a certain substance is adequately represented by the exposure of the test substance used in standard test protocols. However, there may be situations where the composition of a substance to which human and/or environmental exposure occurs, could be different from that tested in the laboratory studies. For example substances with variability in composition may result in a similar variation in the exposure profile of the different components over time. Also the composition of a liquid that is a complex mixture might be very different from that of its associated vapour phase or the Water Accommodated Fraction (WAF) and it is therefore necessary to develop a specific testing strategy to ensure that the composition of the sample to be tested in the laboratory reflects fully the composition of the likely human or environmental exposure. Such substances are designated as *Non-standard substances*, *Complex Substances* or *Substance of Unknown or Variable composition, Complex reaction products or Biological material* (UVCB substances) and have generally the following characteristics:

- they contain numerous substances (typically closely related isomers and/or chemical classes with defined carbon number or distillation ranges), and cannot be represented by a simple chemical structure or defined by a specific molecular formula
- they are not intentional mixtures of substances.
- many are of natural origin (e.g., crude oil, coal, plant extracts) and cannot be separated into their constituent chemical species.
- the concept of *impurities* typically does not apply to complex substances.
- they are produced according to a performance specification related to their physico-chemical properties.

This class of substances requires a case-by-case consideration of the approach to define the appropriate information and methods necessary for meeting the requirements of REACH. Pigments, surfactants, antioxidants, and complex chlorine substances are examples of classes of substances, which may require special considerations to take into account the testing requirements for complex substances. Recommendations for the assessment of natural complex substances like essential oils have been recently published (http://echa.europa.eu/support/substance-identification/sector-specific-support-for-substance-identification/essential-oils). Additional examples are presented in Section R.7.13.1 and R.7.13.2 for metal and inorganic substances and petroleum products, respectively.

R.7.13.1 Metals and Inorganics

Metals and inorganic metal compounds have properties which require specific considerations when assessing their hazards and risks. These considerations may include:

- The occurrence of metals as natural elements in food, drinking water and all environmental compartments
- The essentiality of some of the metals for humans and organisms living in the environment and their general relationship with the natural background
- The speciation of metals influencing bioavailability and for some even the hazard profile
- The short and long term bioavailability of metals and differing degrees of availability to humans and other organisms in the environment

The classical (eco-)toxicity tests do not necessarily consider the above properties and the results obtained may, therefore, be difficult to interpret. Taking specific considerations into account when testing metals and inorganic metal compounds could often prevent these. Extensive experience on hazard and risk assessment of metals was gathered under the Existing Substances Regulation programme and the technical and scientific knowledge with regard to metals has advanced significantly. These have been described in detail by Van Gheluwe *et al.* (2006) for the environment and Battersby *et al.* (2006) for human health. Specific guidance on testing and data interpretation for the hazard and risk assessment of metals and inorganic metal compounds is given in the chapters related to the individual endpoints.

R.7.13.2 Petroleum Substances

Petroleum substances belong to the group of UVCB substances: complex mixtures of hydrocarbons, often of variable composition, due to their derivation from natural crude oils and the refining processes used in their production. Many petroleum substances are produced in very high tonnages to a range of technical specifications, with the precise chemical composition of particular substances, rarely if ever fully characterised. Since complex petroleum substances are typically separated on the basis of distillation, the technical specifications usually include a boiling range. These ranges correlate with carbon number ranges, while the nature of the original crude oil and subsequent refinery processing influence the types and amount of hydrocarbon structures present. The CAS definitions established for the various petroleum substance streams generally reflect this, including details of final refinery process; boiling range; carbon number range and predominant hydrocarbon types present.

For most petroleum substances, the complexity of the chemical composition is such that it is beyond the capability of routine analytical methodology to obtain complete characterisation. Typical substances may consist of predominantly mixtures of straight and branched chain alkanes, single and multiple naphthenic ring structures (often with alkyl side chains), single and multiple aromatic ring structures (often with alkyl side chains). As the molecular weights of the constituent hydrocarbons increase, the number and complexity of possible structures (isomeric forms) increases exponentially.

Similar to the petroleum substances are the hydrocarbon solvents; they also consist of variable, complex mixtures of hydrocarbons and are described by EINECS numbers that are also used for petroleum refinery streams. Hydrocarbon solvents usually differ from petroleum refinery streams in the following ways:

- they are more highly refined;

- they cover a narrower range of carbon number;
- they contain virtually no substances of concern (e.g. benzene)
- they contain virtually no olefins.

Although compositionally somewhat better defined than the corresponding petroleum streams, hydrocarbon solvents require special consideration of the testing strategies similar to that of the petroleum substances.

Toxicity is defined via a concentration response and is dependant on the bioavailability of the individual constituents in a UVCB test substance. This may make interpretation for some substances very difficult. For example the physical form may prevent the dissolution of the individual constituents of such a substance to any significant extent where the whole substance is applied directly to the test medium. The consequence of this would be that toxicity may not be seen in such a test system. This would thus not allow for the toxicity assessment of these constituents to be addressed, were they to be released into the environment independent of the original matrix.

Testing strategies for environmental effects of petroleum substances necessarily reflect the complexity of their composition. Reflecting the properties of the constituent hydrocarbons, petroleum substances are typically hydrophobic and exhibit low solubility in water. However, reflecting the range of structures, constituent hydrocarbons will exhibit a wide range of water solubility. When adding incremental amounts of a complex petroleum substance to water, a point will be reached where the solubility limit of the least soluble component is exceeded and the remaining components will partition between the water and the undissolved hydrocarbon phases. Consequently, the composition of the total dissolved hydrocarbons will be different from the composition of the parent substance. This water solubility behaviour impacts on both the conduct and interpretation of aquatic toxicity tests for these complex substances, whilst the complex composition and generally low water solubility impacts on the choice and conduct of biodegradation studies.

For petroleum derived UVCBs, the lethal loading test procedure, also known as the WAF procedure provides the technical basis for assessing the short term aquatic toxicity of complex petroleum substances (Girling *et al.*, 1992). Test results are expressed as a lethal or effective loading that causes a given adverse effect after a specified exposure period. The principal advantage of this test procedure is that the observed aquatic toxicity reflects the multi-component dissolution behaviour of the constituent hydrocarbons comprising the petroleum substance at a given substance to water loading. In the case of petroleum substances, expressing aquatic toxicity in terms of lethal loading enables complex substances comprised primarily of constituents that are not toxic to aquatic organisms at their water solubility limits to be distinguished from petroleum substances that contain more soluble hydrocarbons and which may elicit aquatic toxicity. As a consequence, this test procedure provides a consistent basis for assessing the relative toxicity of poorly water soluble, complex substances and has been adopted for use in environmental hazard classification (UNECE, 2003). Complex substances that exhibit no observed chronic toxicity at a substance loading of 1 mg/l indicate that the respective constituents do not pose long term hazards to the aquatic environment and, accordingly, do not require hazard classification (CONCAWE, 2001; UNECE 2003).

There are two possible approaches for generating new information or interpreting existing information, bearing in mind the limitations on interpretation of the results mentioned above:

- First for petroleum substances, a model, PETROTOX, has been developed (Redman *et al.*, 2006), based on previous work assuming a non-polar narcosis mode of action (McGrath *et al.*, 2004; 2005). This model, which was developed to predict the ecotoxicity of petroleum substances and hydrocarbon blocks, could be used to address individual structures where no experimental data is available.
- The WAF loading concept may be used for environmental hazard classification (GHS 2005), but should not be used for PBT assessment.

The complex composition and generally low water solubility also impacts the choice and conduct of biodegradation studies.

A further complication impacting both the choice of test method and interpretation of results is the volatility of constituent hydrocarbons, which shows a wide variation across the range of carbon numbers and hydrocarbon structures present in petroleum substances. It has been the practise to assess the inherent hazards of petroleum substances by conducting testing in closed systems (going to great lengths to ensure that volatile losses are minimised), even though under almost all circumstances of release into the environment, there would be extensive volatilisation of many of the constituent hydrocarbons.

Health effects testing strategies for petroleum substances also reflect the complexity of their composition and their physico-chemical properties. Key factors impacting both the choice of test method and interpretation of results are:

- the vapour pressure of constituent hydrocarbons, which show a wide variation across the range of carbon numbers and hydrocarbon structures present in petroleum substances. This will influence the physical nature of the material to which exposure occurs
- the lipid solubility of constituent hydrocarbons, which show a wide variation across the range of carbon numbers and hydrocarbon structures present in petroleum substances. This will influence the potential for uptake into body tissues
- the viscosity of the complex petroleum substance which can significantly impact on potential for dermal absorption
- the presence of small amounts of individual *hazardous* constituents in complex petroleum substances eg Poly Aromatic Hydrocarbons (PAH’s), which may or may not be relevant to the toxicity of the complex petroleum substance
- the presence of other constituents in the complex mixture which may modify (inhibit or potentiate) the toxicity of hazardous constituents.

Toxicological evaluation of complex petroleum substances has normally been based on results of testing of the complete mixture, using OECD Guideline methods. Using this approach it has been possible to take account of the complex interactions that occur between individual constituents of the mixture and the various physico-chemical properties that influence potential for exposure and uptake. In some cases however it has been necessary to adopt modified or non-standard test methods to provide a more reliable indication of the toxicity of certain petroleum fractions. The use of non-standard methods to evaluate the health and environmental effects of petroleum substances is described in more detail in the endpoint specific chapters.

R.7.13.3 References for Substances requiring special considerations regarding testing and exposure

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Appendix to Section R.7.13

Appendix R.7.13-1 Technical Guidance for Environmental Risk Assessment of Petroleum Substances

1.0 Introduction

Petroleum substances typically consist of an unknown complex and variable composition of individual hydrocarbons. CAS numbers used to identify petroleum substances are based on various considerations including hydrocarbon type, carbon number, distillation range and the type and severity of processing used in substance manufacture.

To characterise hazards, CONCAWE (the oil companies' European organisation for environment, health and safety in refining and distribution) has grouped CAS numbers of petroleum substances derived from petroleum refining into generic categories of major marketed products (Boogard et. al, 2005). Further processing of these refinery streams can be performed to produce more refined hydrocarbon-based solvents. These products have also been further grouped to provide a consistent rationale for environmental hazard classification purposes (Hydrocarbon Solvents Producers Association, 2002).

Petroleum substances typically contain hydrocarbons that exhibit large differences in physio-chemical and fate properties. These properties alter the emissions and environmental distribution of the constituent hydrocarbons, and consequently it is not possible to define a unique predicted exposure concentration (PEC) for a petroleum substance. It is not, therefore, possible to directly apply current risk assessment guidance developed for individual substances to complex petroleum substances. To provide a sound technical basis to assess environmental exposure and risks of petroleum substances, CONCAWE devised the hydrocarbon block method (HBM) in which constituent hydrocarbons with similar properties are treated as pseudo-components or "blocks" for which PECs and predicted no effects concentrations (PNECs) can be determined (CONCAWE, 1996). Risks are then assessed by summing the PEC/PNEC ratios of the constituent blocks. While this conceptual approach has been adopted by the EU as regulatory guidance (EC, 2003) experience in applying this method was limited. Recent studies demonstrate the utility of the HBM to gasoline (MacLeod *et al.*, 2004; McGrath *et al.,* 2004; Foster *et al.*, 2005) and further work has been on-going to support the practical implementation of the HBM methodology to higher boiling petroleum substances. The following section provides a concise overview of the key steps which comprise the HBM and it's application to the risk assessment of petroleum substances.

2.0 Outline of Method

Risk assessment of petroleum substances using the HBM involves an eight step process:

<u>2.1. Analyse petroleum substance composition and variability</u>

The initial step involves analytical characterisation of representative samples with different CAS numbers included in the petroleum substance category (e.g. kerosines, gas oils, heavy fuel oils, etc.). Analytical approaches used for this purpose are generally based on chromatographic methodology and have been described previously (Comber *et al.*, 2006, Eadsforth *et al.*, 2006).

Options for analysis of petroleum substances that have been used include:

a. Full characterisation using GC can be performed on some simpler substances, e.g. gasoline. However, full characterisation of higher boiling point streams is not feasible due to the increased complexity of the substances and rapidly increasing number of hydrocarbon components present in such substances.

b. "Modified" Total Petroleum Hydrocarbon (TPH) in which the aromatic and aliphatic fractions of the sample are first separated via a HPLC column. The hydrocarbon distribution in both fractions is then quantified as a function of equivalent carbon number using flame ionisation detection. The equivalent carbon number (EC#) is defined by the elution time of the corresponding n-alkane standards. This approach has been adopted in risk-based assessment of petroleum contaminated sites (McMillen *et al.*, 2001). This method can be used to quantify hydrocarbons up to an EC# of ca. 120.

c. Two dimensional chromatography (2d-GC) uses the same initial fractionation step used in the above TPH method. Further resolution of the various aromatic (e.g. mono, di, tri, poly aromatic and partially hydrogenated aromatic ring classes) and aliphatic (e.g. n-paraffins, i-paraffins, monocyclics, dicyclics and polycyclic saturated ring structures) classes is achieved by the coupling of two columns, respectively based on volatility and polarity, in series. This high resolution method can be used to quantify hydrocarbons up to an EC# of ca. 35. However, this method is limited to petroleum substances that contain a significant fraction of hydrocarbons below EC# 35 (Eadsforth *et al.*, 2006).

<u>2.2 Select hydrocarbon blocks (HBs) to describe product composition</u>

Given the type of compositional data obtained using the methods above, HBs can be selected on the basis of EC# (i.e. boiling point range) and low (aromatic vs. aliphatic classes) or high (up to 16 hydrocarbon classes) resolution blocking schemes. Within aromatic and aliphatic classes or sub-classes, variation in physico-chemical properties depends on the range of EC# used to define the block. Analyses from multiple samples should be used to determine the mean and variance of HB mass fractions that are representative for the petroleum substance category under investigation.

<u>2.3. Define relevant physico-chemical and fate property data for HBs</u>

In order to perform environmental fate and effects modeling, physico-chemical and fate properties must be assigned to HBs. To estimate HB properties, CONCAWE has developed a library of ca. 1500 individual hydrocarbon structures that attempts to represent the structural diversity of the hydrocarbons present in petroleum substances. For each structure, publicly available quantitative structure property relationships (QSPR) have been used predict key properties (e.g. octanol-water partition coefficient, vapour pressure, atmospheric oxidation half-life, fish bioconcentration factor), (Howard *et al.*, 2006). To estimate primary biodegradation half-lives for various compartments, literature data on hydrocarbons tested in unacclimated conditions involving mixed cultures under environmentally realistic conditions have been used to develop a hydrocarbon-specific QSPR (Howard *et al.*, 2005). This new QSPR has been applied to estimate the half-life of representative library structures. Property data for individual library structures are then "mapped" to the corresponding HBs to assign HB property estimates. Due to the very low solubility of hydrocarbons with EC# > 35 in environmental media, these components are treated as inert constituents that are not considered further in exposure or effect assessment.

2.4. Estimate environmental emissions of HBs throughout product lifecycle stages

Once HBs have been selected and properties defined, an emission characterisation covering production, formulation, distribution, professional and personal use and waste life stages must be performed for the petroleum substance category. In addition to assessing the total magnitude of emissions into each environmental compartment (air, water and soil), it is also necessary to speciate these emissions in terms of the HB blocks selected that describe the petroleum product. As in the case of single substance risk assessments, emissions characterisation must be considered at different scales (local, regional and continental) and determined using either measured, modeled or, in the absence of other information, conservative default emission factors that are derived given HB properties and product use categories.

2.5. Characterise fate factors and intake fractions of HBs

To assess the environmental fate behavior of HBs, EUSES modeling has been performed for each library structure for different unit-emission scenarios (i.e. 100 kg/yr, 10 kg/yr or 1 kg/yr emission into air or water or soil at continental, regional and local scales, respectively). From these EUSES model runs, fate factors (fFs) and human intake fractions (iFs) for each emission scenario have been calculated. Fate factors for each compartment are defined as the calculated PEC in the compartment divided by the assumed emission for a given scenario. Intake fractions are defined as the predicted human exposure divided by the emission for a given scenario. This modeling exercise has provided a library of fFs and iFs for all representative hydrocarbon structures (van de Meent, 2007). This approach has the advantage that EUSES fate modeling only needs to be performed once so that results can then be consistently applied across different petroleum substance groups.

2.6. Determine environmental and human exposure to HBs

To calculate compartmental PECs and human exposures for different spatial scenarios, block emissions for the scenario are first equally divided among representative structures that "map" to that block. Emissions are then simply multiplied by the corresponding fFs or iFs that correspond to that structure to scale the model predicted exposure or human intake to the actual emission. PECs or human exposures for the block are then calculated by summing results for all of the representative structures that comprise the block.

For petroleum substances use of environmental monitoring data needs specific consideration. While data may be available for "total" hydrocarbons or specific hydrocarbon structures (e.g. naphthalene, chrysene), the source of these constituents may be multiple anthropogenic and natural sources. Therefore, such release or monitoring data may be only used to provide a worst-case, upper bound estimate of the concentration of a “block” for screening purposes. In contrast, model derived PECs are intended to provide a more realistic estimate for substance risk assessment since these values represent only the fraction of the observed total concentration of the “block" in the environment that is attributable to the specific petroleum substance under study.

2.7. Assess environmental effects of HBs

Since petroleum substances are comprised principally of only carbon and hydrogen, these substances will exert ecotoxicity via a narcotic mode of action (Verhaar *et al.*, 2000). Moreover, ecotoxicity endpoints for narcotic mixtures are generally observed and quantitatively modeled as simply additive (de Wolf *et al.*, 1988; McGrath *et al.*, 2005; Di Toro *et al.*, 2007). To assess the environmental effects of HBs comprising petroleum substances on aquatic and wastewater organisms, a modification of the target lipid model (McGrath *et al.*, 2004; Redman *et al.*, 2007) has been developed that builds on the work by Verbruggen (2003) in which toxicity relationships are related to membrane-water rather than octanol-water partition coefficients (Redman, 2007). This revision is needed to allow extension of the target lipid model to more hydrophobic constituents, beyond gasoline range hydrocarbons, that are present in many petroleum substances. The revised target lipid model has been used to derive PNECs for all CONCAWE library structures. If coupled with equilibrium partitioning theory, this model framework can also be used to support effects assessment in the soil/sediment compartment (Redman *et al.*, 2007b).

2.8. Evaluate *individual and aggregate risk of HBs*

To assess environmental risks, the PEC/PNEC ratio for each library structure within a block is calculated and then the ratios for different structures summed within each block. The additive risk contributed by all the blocks is then determined to estimate the risk of the petroleum substance group. This calculation is performed for each spatial scale.

Efforts are currently underway to automate the HBM method into a simple spreadsheet-based computational tool. This tool is intended to provide a generic methodology to support petroleum substance risk assessment that: (1) links analytical characterisation of petroleum substances to HB definition; (2) provides a consistent technical framework across different petroleum groups; (3) reflects the current state of science; and (4) is transparent and practical in scope. Availability of this tool will also allow the sensitivity of risk characterisation to be assessed in response to changes in compositional assumptions or alternative "blocking" schemes. Moreover, this tool will enable identification of HBs which are principal contributors to the PEC/PNEC ratio and where refinement in further data collection can be logically focused if the estimated PEC/PNEC > 1.

3.0 Limitations

At present the current HBM methodology does not quantitatively address effects on the air compartment due to lack of standardised laboratory hazard data. In addition, the method does not address heterocyclic compounds (e.g. carbazoles in cracked fuels) or metals (e.g. vanadium and nickel in fuel oils and asphalt) which may be present at low levels in certain petroleum substances. The potential for reduced exposure of certain polyaromatic hydrocarbons as a result of photodegradation or enhanced toxicity due to photoactivation is also not addressed due to the complexity and site-specific nature of these processes. Nevertheless, these issues may be considered on a case-by-case basis, at least in a qualitative manner.

The scope of the generic methodology is intended to address the risks posed by hydrocarbon components in petroleum substances. Therefore, additives that are intentionally introduced to modify the technical properties or performance of petroleum substances are outside the scope of this methodology, but in any event, these substances will be subject to independent risk assessments. Likewise, secondary constituents that are generated from reactions resulting from petroleum substance use (e.g. combustion by-products other than hydrocarbons components in the substance) are excluded and addressed by other EU and country-specific regulations.

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Appendix to Chapter R.7

Appendix R.7-1:	Threshold of Toxicological Concern (TTC) – a concept in toxicological and environmental risk assessment

Appendix R.7-1 Threshold of Toxicological Concern (TTC) – a concept in toxicological and environmental risk assessment

Human Health Aspects

Risk assessment for human health effects is based on the threshold of a critical toxicological effect of a substance, usually derived from animal experiments. Alternatively, a toxicological threshold may also be based on the statistical analysis of the toxicological data of a broad range of structurally-related or even structurally-different substances and extrapolation of the no effect doses obtained from the underlying animal experiments for these substances to levels considered to be of negligible risk to human health. This latter approach refers to the principle called Threshold of Toxicological Concern (TTC). Regarded in this way the TTC concept could be seen as an extension of such approaches read-across and chemical category. As such, the TTC concept has been incorporated in the risk assessment processes by some regulatory bodies, such as the U.S Food and Drug Administration (FDA) and the UN JMPR and EU EFSA in the assessment of flavourings and food contacts articles (SCF, 2001), as an approach to identify exposure levels of low regulatory concern, and as a tool to justify waiving of generation of animal data.

This section will briefly discuss different TTC approaches, their limitations, criteria for use, and finally their potential use under REACH.

<u>TTC approaches</u>

The TTC was implemented by the FDA as the *Threshold of Regulation* from food contact materials since 1995; a TTC value of 1.5 µg per person per day was derived for a chemical database that covered carcinogenicity (i.e. their calculated one per million risk levels; Gold *et al.*, 1995). This value is considered to be applicable for all endpoints except genotoxic carcinogens.

Munro *et al.* (1996) subsequently developed a structure-based TTC approach on principals originally established by Cramer *et al.* (1978). The structural classes of organic substances analysed showed significantly different distributions of NOEL's for subchronic, chronic and reproductive effects. Carcinogenic or mutagenic endpoints were not considered. Based on the chemical structure in combination with information on toxicity three different levels (90, 540 and 1800 µg per person per day, respectively) were derived. UN-JMPR and EU EFSA have implemented these values in the regulations for indirect food additives.

Another structure-based, tiered TTC concept developed by Cheeseman *et al.* (1999), extended the Munro *et al.* (1996) 3 classes approach by incorporated acute and short-term toxicity, mutagenic and carcinogenic potency (but exempting those of high potency).

More recently. Kroes *et al.* (2004) evaluated the applicability for different toxicological endpoints, including neurotoxicity and immunotoxicity, and proposed a decision tree with 6 classes of organic substances. Allergens or substances causing hypersensitivity could not be accommodated due to the lack of an appropriate database (enabling statistical analysis for this category of substances).

Apart from the two indicated cases, the other approaches have not been adopted by any regulatory body.

Recently, ECETOC has proposed a Targeted Risk Assessment approach for REACH including a series of threshold values for a wide variety of organic and non-organic substances (both volatile and non-volatile), i.e. so-called Generic Exposure Value (GEV), and Generic Lowest Exposure Value (GLEV) for acute and repeated dose toxicity (ECETOC, 2004). Category 1 and 1B carcinogens, mutagens and reprotoxins were excluded. The GEV is a generic threshold values for occupational exposure (and derived dermal values), derived from some most stringent Occupational Exposure Limits (OEL). The GLEV is based on classification criteria for repeated dose toxicity and extrapolation factors. It is noted that the derivation of GEV values was based upon an analysis of current published occupational exposure levels, and therefore also incorporated socio-economic and technical arguments in addition to the assessment factors applied to toxicological endpoints and other data on which the OELs were based. This approach has not been peer reviewed nor accepted by regulatory bodies.

<u>Basic requirements</u>

The TTC concept discussed above require a minimum set of information in order to be applied successfully. However it should be noted that the application of TTC excludes substances with certain structural elements and properties including:

- Non-essential, heavy metals and polyhalogenated dibenzodioxins, -dibenzofurans, or-biphenyls and similar substances:
  This class of substances cannot be addressed by the TTC concepts due to the bio-accumulating properties. Although the TTC approach is able to accommodate other categories of substances with bio-accumulating potential, within the regulatory context, substances with potential for bioaccumulation are 'of concern' and need to be assessed on a case-by-case basis. Potentially bioaccumulating or persistent substances are also excluded from default environmental risk assessments.

- Genotoxic carcinogens:
  A case-by-case risk assessment is required for genotoxic carcinogens, even though some carcinogens can be accommodated within the TTC concept if the estimated intake is sufficiently low (<0.15 □g/day).

- Organophosphates:
  This class of high potency neurotoxicants are excluded.

- Proteins:
  This class of substances is a surrogate to address specifically potential (oral) sensitisation, hypersensitivity and intolerances. There are no appropriate databases available which allow the derivation of a generic threshold for this type of endpoint.

Additionally, another very critical criterion concerns the knowledge on the handling and use of the substance. TTC is only applicable in case there is detailed information available on all anticipated uses and use scenarios for which the risk assessment is provided.

Limitations

The TTC has several limitations. First of all, they are derived on data bases covering primarily systemic effects from oral exposure. This is especially important concerning occupational situations where inhalation or dermal exposure is the main route of contact. Only some cover mutagenic, carcinogenic and acute effects, and in fact none (except for the proposed ECETOC approach) addresses local effects such as irritation and sensitisation.

As all TTC approaches (except for the proposed ECETOC approach) have oral exposure as the principle route, further substantial efforts are needed to explore its potential use for the exposures routes inhalation and skin contact, before any application may become realistic.

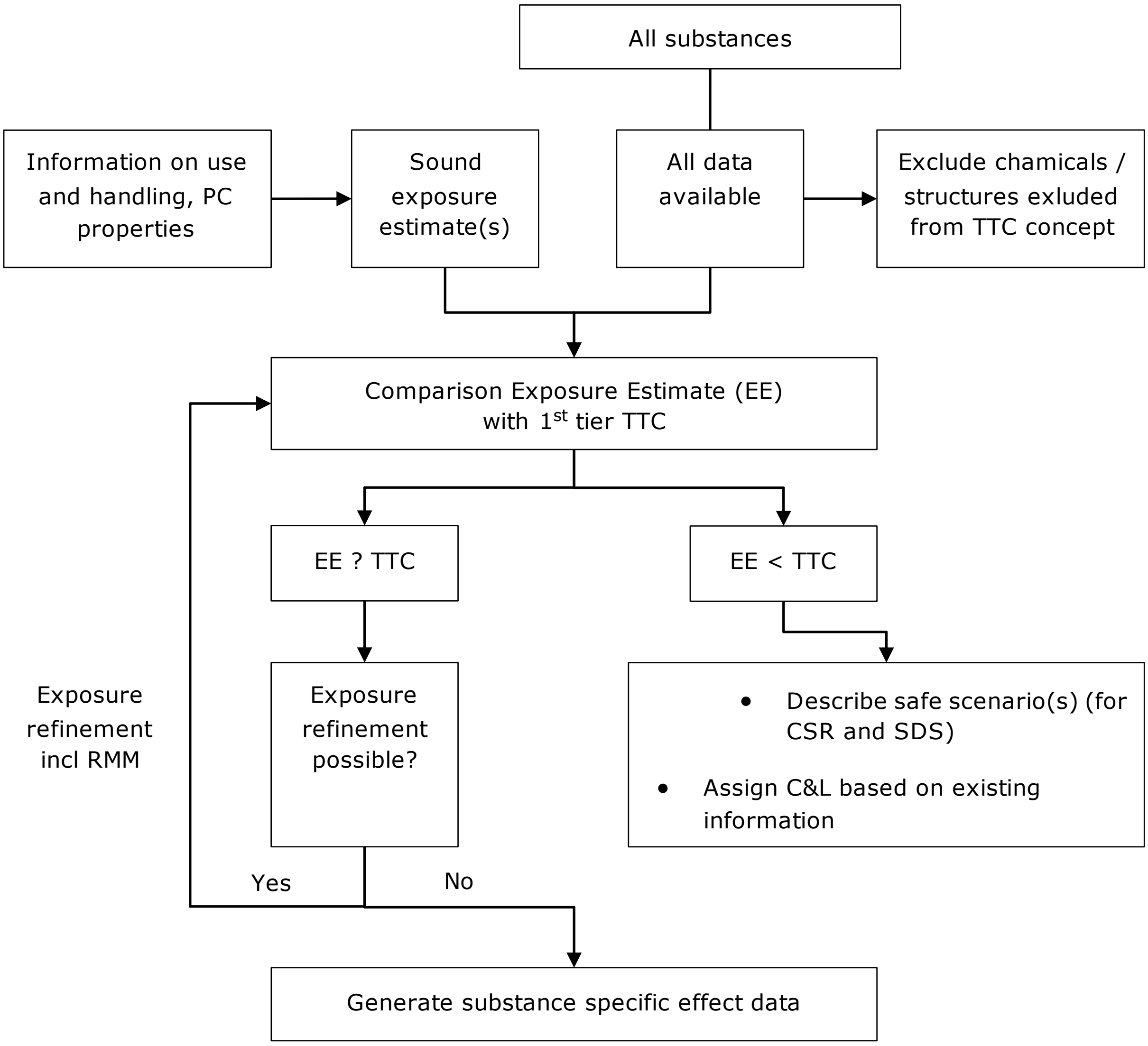
Several of the structurally-based approaches to TTC have limitations in applicability domain and cannot accommodate every chemical class. For instance, proteins, heavy metals, polyhalogenated-dibenzodioxins, aflatoxin-like substances, N-nitroso-compounds, alpha-nitro furyl compounds and hydrazins-, triazenes-, azides-, and azoxy-compounds have been excluded by the approach of Kroes *et al.* (2004). Also excluded are highly potent neurotoxicants, organophosphates and genotoxic carcinogens.

As indicated, the TTC approach is only applicable in case there is detailed information available on all anticipated uses and use scenarios for which the risk assessment is provided. Based on the experience of the EU Risk Assessment Programme for Existing Substances, robust exposure estimates will require a significant effort, even in cases where the uses were well characterised. In case of a multitude of (dispersive) uses and applications, it may not be feasible to generate overall exposure estimate with detail and precision necessary for use in a risk assessment relying on the thresholds based on the TTC concept. Therefore, a TTC will in practice only be applicable in those cases where there are only a few number of exposure scenario's that allow well characterisation.

Furthermore, the use of the TTC approach does not provide information on classification and labelling of a substance, or on its potency for a specific effect.

Use of the TTC concept

The TTC concept has been developed primarily for use within a risk assessment framework. As already indicated, the TTC concept is applied for regulatory purposes by the U.S FDA and the EU EFSA and UN JMPR in the assessment of food contact articles and flavourings, respectively. These specific TTC approaches underwent a critical review before being accepted on this regulatory platform. Clearly, in the same way, any other TTC approach should be agreed upon by the relevant regulatory body before use, and it should be clearly indicated for which endpoints, routes and population they apply.



The figure illustrates the way a TTC can be used: it precedes any substance-specific testing. One tier is shown, but one could apply additional tiering rounds (as clearly illustrated by the approach presented by Kroes *et al.*,2004) dependent upon the substance of interest.

Figure R.7.13—1 Generic TTC scheme/concept under REACH.

Potential use within REACH

It is feasible that within REACH the TTC concept may be of use for the chemical safety assessment at tonnage levels triggering limited information on repeated dose toxicity and/or reproduction: REACH clearly indicates the need for non-testing methods and provides the opportunity of waiving testing based on exposure considerations. When clearly documented and justified the following options could apply.

REACH Annex VII

The testing requirements specified in Annex VII would normally not trigger toxicity testing involving repeated exposures and the information at this tonnage level do provide insufficient information to determine a dose descriptor or any other starting point for the derivation of a DNEL for use in an assessment of the human health risks associated with repeated exposures. Although non-testing or *in vitro* methodologies may give insight in the toxicological properties of a substance, generally such methods are insufficiently specific to provide quantitative information on the potency and/or threshold of an adverse effect. In such a case the threshold derived from the TTC methodology might provide a reference value to assess the significance of the human exposure.

REACH Annex VIII-X

At these tonnage levels there may be circumstances triggering an adaptation of the REACH requirements that may lead to waiving of the repeated dose toxicity study and, consequently, the generation of a substance-specific dose descriptor or another starting point for the derivation of a DNEL:

- in Annex VIII, repeated dose toxicity (28 d test, 8.6) and reproductive toxicity testing (8.7) may be waived 'if relevant human exposure can be excluded in accordance with Annex XI section 3.
- in Annex IX and X testing could be waived in case there is no significant exposure, and there is low toxicity, and no systemic exposure.

In a case-by-case consideration, the appropriate threshold derived from the TTC methodologies agreed upon by the relevant regulatory body might be considered as a starting point to assess the significance of the human exposure. The level chosen will be critical to ensure a level of sufficient protection.

Final remark

Independent of the approach used in risk assessment of industrial chemicals it is important to maintain a sufficient level of protection. In the striving for alternatives to animal testing one suggested approach is the use of generic threshold values. However, application of TTC would imply that limited data may be generated and thus, that the level of protection might be influenced. From information on flavouring substances in the diet the TTC concept seems to be reasonable well based with respect to general toxicity and the particular endpoints examined. However, the possible application of TTC on industrial chemicals needs to be carefully considered. There may be some important differences between industrial chemicals and substances used for food contact articles or flavourings, such as differences in use pattern and composition (for a further discussion see Tema Nord, 2005; COC, 2004).

TTC concept for the environment[46]

Two approaches

Two different approaches have been used when deriving a TTC for the environment, i.e. the *action-limit* proposed by EMEA/CPMP (2001) and the environmental Exposure Threshold of No Concern (ETNC) proposed by ECETOC (2004) and de Wolf *et al.* (2005). Both these approaches are restricted to the pelagic freshwater compartment.

1. The first of these TTC-approaches, i.e. the *action-limit*, originates from a draft on environmental risk assessment of human pharmaceuticals (EMEA/CPMP, 2001), describing a tiered risk assessment process. The initial step is an environmental exposure assessment in which a coarsely predicted environmental freshwater concentration (PEC) for the pharmaceutical ingredient, or its major metabolites, is compared to an action limit (0.01 µg/L). In case the PEC is smaller than the action-limit and no environmental concerns are apparent, no further action is considered needed. On the other hand, when the PEC is larger than the action-limit, the assessment continues to a second phase, which involves an environmental fate and effect analysis. The action limit is based on an aquatic concentration below which it was concluded that no ecotoxicity data on drugs for relevant standard test organisms were reported (U.S. FDA, 1996). This concentration was further divided by an assessment factor of 100 to obtain the action limit. The action-limit has been questioned by the CSTEE (2001) since drugs with lower effect concentrations were found. In addition, the focus on acute toxicity in the draft was questioned, as chronic toxicity was considered more relevant for this kind of substances, i.e. pharmaceuticals.

2. A different TTC-approach was applied deriving an ETNC for the pelagic freshwater compartment, i.e. ETNCaquatic (ECETOC, 2004; de Wolf *et al.*, 2005). This approach was based on existing toxicological databases and substance hazard assessments for organisms in the freshwater environment, and a categorisation of substances into four different modes of action (MOA) according to the system by Verhaar *et al.* (1992). The stratified data was fitted to a lognormal distribution from which a fifth percentile, with a 50% confidence interval, was determined. This value was then divided by an assessment factor, ranging from 1 to 1000 depending on the data to obtain the ETNCaquatic. Metals, inorganics, and ionisable organic substances are not covered by this system, and thus not included when deriving the ETNCaquatic.

   The authors proposed an overall value of 0.1µg/L for MOA1-3. The authors considered that a broad application of the ETNCaquatic concept also needed to cover MOA4, and that the resulting ETNCaquatic likely would have to be much lower. This idea is substantiated by the fact that a substantially lower ETNCaquatic was observed when analysing the substances assigned a MOA4,

[46] Based on TemaNord 2005: 559.

as the resulting ETNCaquatic, MOA4 was 0.0004 µg/l. The lowest individual NOEC value in that particular database was 0.0006 µg/l (Fenthion).

Regulatory use

There is presently no use of the TTC concept as regards environmental assessments. However, in a draft by EMEA/CPMP (2001, 2005) a stepwise, tiered procedure for the environmental risk assessment of pharmaceuticals (for human use) is proposed. This approach would involve a TTC approach as it includes an action limit of 0.01 µg/l in pelagic freshwater environment.

The ETNC may be considered a risk assessment tool, and data might still be needed for classification or PBT assessment. In general, acute toxicity data will be available/predictable, and the resulting PNEC will often be above the ETNC. If it is lower, then the substance should be considered in more depth.

Discussion

The TTC-concept represents a new approach as regards environmental risk assessments since it results in a general PNEC (a non-effect threshold value) that is intended to be applied on an entire group of substances, as compared to the standard substance specific PNEC.

The TTC approach is developed only for direct effects on the pelagic freshwater ecosystem and not effects due to bioaccumulation, or accumulation in other compartments. In addition, the concept does not cover metals, other inorganic compounds, or ionisable organic compounds. The use of the threshold of no toxicological concern, as compared to experimental data, implies a higher risk of not considering the toxicity of degradation product(s)/metabolite(s), which may be unfortunate if they are more toxic than the parent compound.

It has been proposed by de Wolf *et al.*, 2005 to use the TTC concept as a tool for screening in order to select/prioritise substances for testing/further risk assessment, e.g. it may help to inform downstream users about the relative risk associated with their specific uses. The approach could also be valuable in putting environmental monitoring data into a risk-assessment perspective. For these applications the concept may work if the TTC is satisfactory determined. However, because only toxicity is considered, P and B criteria should also be consulted. The main reason using the TTC approach would be the saving of aquatic freshwater test organisms, including vertebrate species (mainly fish).

The method of deriving a PNEC, using the NOEC for the most sensitive species and an assessment factor, is the standard approach in TGD to derive a threshold value, i.e. Predicted No Effect Concentration (PNEC), for a substance. Instead of using NOECs for the most sensitive species, it has for some data rich substances (e.g. Zn in the Existing Substance Regulation) been accepted to instead use the 5th percentile and lognormal distribution, of all species from all phyla, to derive a NOEC. This since the traditional method of deriving PNEC, according to the TGD, for the data rich metals resulted in PNECs below background values. In these cases, ecotoxicity data for a number of species and phyla was used to derive a toxicity threshold (PNEC) for one substance. This differs from the ETNCaquatic (TTC)-approach, where instead an assessment factor is used on the fifth percentile of toxicity data for the many species for many substances (belonging to a defined group). In the first case, the concept accepts that 5% of the species NOECs will fall below the threshold. In the second case, the concept accepts that 5% of the substance PNECs will fall below the threshold. Is the safety level for the environment similar in these two cases? The consequences should be further evaluated.

What is the added value of using a generic PNEC as compared to (Q)SAR estimates, when no substance specific experimental toxicity data is available? As regards what Verhaar *et al.* (1992) defined as mode of action 1-2, available QSAR models exists, which are based on more specific data, which should be more relevant than a generic TTC. However, it should be stressed that QSARs are usually used as indicators of an effect, and not for confirmation of lack of effects (which is the opposite of how the TTC is proposed to be used!).

If the TTC-concept is to be used, should one or several threshold values be used? Using more than one threshold value implies a higher risk of using the wrong (not safe) threshold. The use of several thresholds put higher demands on the categorisation system. Substances may be categorised according to different systems. Considering the fact that the knowledge in this field has continued to grow over the years, is the approach suggested thirteen years ago by Verhaar *et al.* (1992), as proposed by ECETOC (2004) and de Wolf *et al.* (2005), presently the most appropriate way of grouping substances in order to derive a TTC? This method uses four modes of toxic action to differentiate between substances. Even though rules exists as to categorise that a substance exhibits one of the first of these three modes of action, it is however not possible, based on definite structural rules, to decide whether or not a substance exhibits the fourth of these modes. Inclusion in this fourth class must, and should, be based on specific knowledge on mode of toxic action of (groups of) substances. In addition, a substance may have more than one mode of action.

Hence, the use of only one threshold value appears to be the most transparent and conservative approach. As a consequence of the above, it seems reasonable to base this threshold value on chronic toxicity data for the most toxic substances, i.e. those categorised as having a specific mode of toxic action.

TTC can presently not be used as a stand-alone concept, but could perhaps in the future be included in a *Weight-of-Evidence* approach when deciding on potential derogations.

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