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Animal Use Replacement, Reduction and Refinement: Development of an Integrated Testing Strategy for Bioconcentration of Chemicals in Fish[@]

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@ This paper is dedicated to Tom C.J. Feijtel (1959 – 2005), who inspired many of us.

ABSTRACT

When addressing the use of fish for the environmental safety of chemicals and effluents there are many opportunities for applying the principles of the 3Rs: Reduce, Refine and Replace. The current environmental regulatory testing strategy for bioconcentration and secondary poisoning has been reviewed and alternative approaches that provide useful information are described.

Several approaches can be used to reduce the number of fish used in the OECD Test Guideline 305, including alternative *in vivo* test methods such as the dietary accumulation test and the static exposure approach. The best replacement approach would seem to utilise read-across, chemical grouping, and (Q)SARs, with an assessment of the key processes in bioconcentration: adsorption, distribution, metabolism and excretion (ADME). Biomimetic extraction has particular usefulness in addressing bioavailable chemicals and is in some circumstances capable of predicting uptake. Use of alternative organisms such as invertebrates should also be considered. A single cut-off value for molecular weight and size beyond which no absorption will take place cannot be identified. Recommendations for their use in B-categorisation schemes are provided. Assessment of biotransformation with *in vitro* assays and *in silico* approaches hold significant promise. Further research is needed to identify their variability, confidence limits, and ways to use this as a basis to estimate BCFs.

A tiered bioconcentration testing strategy has been developed taking account of the alternatives discussed.

Keywords: Integrated testing strategy, Bioconcentration, Animal testing, 3Rs, OECD 305

INTRODUCTION

Using animals for safety testing represents a dilemma about balancing the need to ensure chemicals can be handled and used safely, against legitimate and widely felt societal concerns about animal testing. A range of testing is required to provide data for product hazard assessments by the chemicals industry. Tests are based on regulations and voluntary industrial initiatives designed to protect human and wildlife health as well as the surrounding environment. Testing for environmental effects includes assessment of bioconcentration, notably with fish.

European legislation requires that non-animal, alternative approaches of testing should be used in the place of animal procedures wherever possible (EEC 1986) states that ‘an experiment shall not be performed if another scientifically satisfactory method of obtaining the result sought, not entailing the use of an animal, is reasonably practically available’.

Russell and Burch (1959) originally defined the **R**eplace, **R**educe and **R**efine principles (3Rs). ‘Replacement’ means the substitution for conscious living higher animals of insentient material. ‘Reduction’ means reduction in the numbers of animals used to obtain information of given amount and precision. ‘Refinement’ means any decrease in the incidence or severity of inhumane procedures applied to those animals which still have to be used.

An additional '3Rs' known as the 'Solna principles' (OECD 1996a) have been identified. These 3Rs state that tests for regulatory purposes need to reflect the following: biological **R**elevance (meaningfulness and usefulness of a test for a particular purpose), **R**eliability (reproducibility of results within and between laboratories), and **R**egulatory acceptability (suitability of a test for risk assessment purposes (human health / environment).

Fish are typically secondary consumers or predators, therefore considered to represent a high trophic level, and organisms of choice for assessing the bioconcentration potential of chemicals in aquatic organisms. Since fish are an important part of the diet of humans, they also represent a potential route of exposure of chemicals to humans.

The usual procedure in a regulatory context for determining a bioconcentration factor (BCF) is to apply the OECD 305 (Bioaccumulation: flow-through fish test) (OECD 1996b). However, many of the existing chemical legislative frameworks around the world, except in Japan, do not require experimental determination of bioconcentration at basic tiers of the risk assessment; they rely on extrapolation using the organic chemical's physico-chemical properties (log K_{ow}). This screening procedure assumes no substantial bioconcentration for compounds with a log $K_{ow} < 3$. Above a log K_{ow} of 6, non-linear relationships can be applied and in most of these cases a chemical by chemical evaluation is more appropriate (Nendza 1991). The log K_{ow} based QSAR approach is not reliable for all chemical classes, e.g. surface active agents, organic colorants (ECETOC 1998) or lipophilic chemicals that are biotransformed (de Wolf *et al*. 1992).

Bioconcentration factors are used in classification of substances dangerous for the aquatic environment (UN 2003) and in regulatory B-assessments and prioritization schemes (EC 2003; DGEE 2003; CEPA 1999). For example in Europe, if a substance has a BCF > 2,000, it fulfils the criterion for being bioaccumulative (B). If it has a BCF > 5,000, it fulfils the criterion for being very bioaccumulative (vB).

The European Commission has recently adopted a draft legislative text, describing the Registration, Evaluation, Authorisation and Restrictions of Chemicals (REACH) (DGEE 2003). Chemicals produced at above 1 tonne per year will be subjected to a registration procedure, and information relevant to health and environmental safety should be provided. This could mean approximately 12-13 million animals being used for the assessment of approximately 30,000 chemicals by 2012 (IEH 2001). Non animal testing is promoted in REACH, although strategies for using alternative information methodologies have not been spelt out. However, such strategies are needed not only to achieve the 3Rs, but also to keep the REACH testing costs at manageable levels.

The main objective of this paper is to present a bioconcentration testing strategy based on the work of an ECETOC Task Force (ECETOC 2005) that can be applied in a regulatory context taking account of alternative information methodologies.

BACKGROUND AND CURRENT APPROACHES

In the context of animal testing in Europe, the definition used for an animal is that contained in the UK Guidance on the Operation of the Animals (Scientific Procedures) Act, 1986 (UK 1986). This act defines a ‘protected animal’ as any living vertebrate, other

than man [Section 1(1) of the Act]. This was extended to the invertebrate species *Octopus vulgaris* via an amendment (UK 1993). The protection also extends to certain immature forms of development of mammals, birds and reptiles - from halfway through the gestation or incubation period; and for fish, amphibians and *Octopus vulgaris* - from the time at which they become capable of independent feeding. Several other definitions for an animal do exist (e.g. EEC 1986; USA 1966) but will not be used here.

It is more important that the spirit of the 3Rs is applied, as opposed to which specific category an alternative approach fits. For instance, the use of fish for harvesting of organs or cells is an area for possible confusion as to whether the test is a replacement or a refinement. Fish held in an aquarium and humanely sacrificed are not counted as animals used in an experimental procedure (UK 1986). As a consequence, the use of the organs/cells would constitute a replacement.

Accumulation of a chemical is the result of a mix of physiological and physical processes - absorption, distribution, metabolism, and excretion (ADME). The processes are described, based on Hodgeson et al. (1994). Absorption occurs after the introduction of a chemical through food, water, air, sediment, or soil, and it is the transport across a biological membrane into systemic circulation e.g. across fish gills, intestine, skin. After absorption, a chemical may bind to plasma proteins for circulation throughout the body, as well as to tissue components like fat or bone. This is called distribution. The chemical may be distributed to a tissue and elicit a toxic response; other tissues may serve as permanent sinks (e.g. fat), or as temporary depots allowing for slow release into circulation. After reaching a tissue, enzymes may biotransform the chemical. During phase I biotransformation reactions a polar group is introduced into the molecule, which

increases its water solubility and renders it a suitable substrate for phase II reactions. In phase II biotransformation reactions the (parent or altered) molecule combines with an endogenous substrate and can be readily excreted. Biotransformation is generally a detoxification mechanism. Excretion refers to the process by which a chemical gets eliminated from the body through endogenous waste. Chemicals may be exhaled directly through the gills, or may be broken down (biotransformed) and ultimately exhaled as CO_2. Polar molecules that are freely soluble in plasma can be removed through renal filtration and passed into urine. Lipophilic (fat soluble) chemicals may be conjugated and excreted in bile (faeces). In addition to excretion, growth of the organism may also be relevant affecting the chemical concentration in the organism, in the case when the rate of other excretion processes is in the same order of magnitude as the growth (dilution) rate. Furthermore, other “excretion” processes could be the transfer of lipophilic chemicals to the offspring via the eggs.

For the experimental determination of bioconcentration factors (BCF) in fish, a number of test guidelines have been documented; the most generally applied being OECD 305 (OECD 1996b). OECD 305 is conducted in 2 phases: an exposure phase followed by a depuration phase. In the exposure phase, a sufficient number of fish is exposed to 2 sublethal concentrations of the test substance. During exposure both fish and water are sampled at regular time-intervals and the concentration of (parent) test substance measured. During the first phase the concentration of test substance in the water should be kept constant within narrow limits (± 20%). Hence, the guideline recommends the use of a flow-through system. After having reached an apparent steady-state (or after 28 d), the remaining fish are transferred to clean water and the depuration is

followed. The BCF is expressed as a function of total wet weight of the fish and may also be expressed as a function of total lipid weight. Specific chemical analysis and radiotracer techniques may be used as analytical methods. If the latter technique is applied, a specific chemical analysis (or a selective cleaning-up procedure) of the parent compound should be used at the end of the exposure period.

OECD 305 requires 3 groups of fish, 2 exposure groups and a control group held under identical conditions. A minimum of 4 fish are sampled on at least 5 occasions during the uptake phase, and at least on 4 occasions during the elimination phase. (Table 1)

The guideline does not specify whether it is acceptable to reduce fish sampling in the control group, hence it has to be assumed that the sampling protocol for the control group is similar to that of the 2 exposure groups.

Assuming that aquatic organisms can be mathematically represented as a homogenously mixed one-compartment then bioconcentration can be described with a simple first-order kinetic model:

$$C_f = C_w * k_u/k_d * (1-e^{-t*kd})$$

where C_f is the substance concentration in fish (mg/g wet fish), C_w the substance concentration in water (mg/l), k_u the uptake clearance (ml/g wet fish/day), k_d the elimination rate constant (1/day), and t the exposure time (day). In this model, k_u and k_d are independent of C_w and t, but dependent on the properties of the chemical being bioconcentrated. Usually, first order one compartment kinetics have been found to adequately describe bioconcentration (Sijm 1991; Kristensen et al. 1991).

Hence, there are 2 different methods to evaluate BCF. The first is to calculate it from the concentration of a chemical in fish divided by the concentration in water (under steady-state conditions). The second method uses kinetic data, i.e. uptake clearance and elimination rate.

$$BCF = k_u/k_d = C_f/C_w$$

Experience from a ring test of the former OECD 305E between European laboratories showed that the variations in BCF estimates between the 2 methods was less than the inter-laboratory variation (Kristensen et al. 1991). This is further improved when a correction for the bioavailable fraction in water is made (Schrap et al. 1990), (e.g. for sorption to suspended or dissolved organic materials).

Most of the earlier studies to determine the BCF of highly hydrophobic substances did not always follow the OECD 305 test protocol possibly introducing artefacts in the testing and in the interpretation of the BCFs from these studies. These artefacts may include difficulties in measuring the 'true' aqueous concentration due to sorption of the substances to particulate and dissolved (organic) matter; adsorption processes to glass walls or other materials; volatilisation; etc. (Anonymous 2004).

For less hydrophobic compounds (log $K_{ow} < 3$) passive diffusion of freely dissolved, bioavailable material through the cell membrane (i.e. the hydrophobic phase) is considered to be the rate limiting step for uptake. For more hydrophobic compounds diffusion is limited by the aqueous boundary layers between the fish membrane and the bulk water (Gobas et al. 1987).

The uptake clearance (k_u; ml/g wet fish/day) is relatively constant between log K_{ow} of 3 and 6 but varies as a function of fish weight (Sijm et al. 1995). For nonionic organic chemicals with log K_{ow} > 6, there is some evidence to suggest that the uptake clearance may decline with increasing hydrophobicity.

An estimate of the rate of depuration k_d may be obtained from empirical relationships between k_d and log K_{ow}. These relationships apply only to chemicals with log K_{ow} values between 2 and 6.5 (Hawker et al. 1988). An important elimination factor in bioconcentration is the possible biotransformation of substances (Sijm et al. 1997) which is ignored when estimating k_d via empirical relationships with K_{ow} (de Wolf et al. 1992). In such cases $k_d = k_e + k_m$ where, k_e represents excretion of the parent molecule and k_m elimination by biotransformation.

Since many substances that bioconcentrate distribute themselves within the organism's body into the fat or lipids, the first estimation that may be carried out for a BCF is of a chemical's potential to partition between octanol and water. QSARs and experimental techniques for measuring this parameter are available (ECETOC 1998, 2003; EC 2003). The recommended model for log K_{ow} up to 6 is Veith et al. (1979), while for chemicals with log K_{ow} > 6, a parabolic equation, re-calculated from that described by Connell et al. (1988), is recommended. In general (Q)SAR-models should only be used for those chemicals which fall within the domain of the model and for which the descriptors are suitable (EC 2003; ECETOC 2003). Surfactants are clear examples of organic materials outside the scope of (Q)SAR models which use log K_{ow} as this is not an appropriate physico-chemical descriptor for such materials. Metals also fall outside most QSAR-models as active uptake and sequestration can occur in biological systems. In

cases where uptake is hindered or elimination via biotransformation is increased EU-accepted QSAR-models will overestimate bioconcentration.

REVIEW OF ALTERNATIVE APPROACHES

As part of REACH, it is possible that many chemicals in the tonnage level of more than 100 tonne per annum will need to be tested for their potential to bioconcentrate. Pedersen et al. (2003) estimated that 5,500 chemicals in Europe are manufactured or imported at this tonnage level. Taking into account the 55% percent of HPV-chemicals with a log K_{ow} > 2.7 (Beek 1991), the number of chemicals for BCF-testing can be calculated as 3,025. With a minimum number of fish for an OECD 305 study estimated at 108 (Table 1), the minimum required number of fish for REACH bioconcentration testing is 326,700. The following sections will review reduction and replacement approaches to assess the bioconcentration potential of chemicals in fish. So far, no refinement strategy has been identified.

Reduction of animal use

This can be achieved by exposing less fish per replicate/concentration to the minimum that can be statistically justified, or by limiting the numbers of concentration exposures to 1. If the latter is applied, the number of fish used for testing can immediately be reduced by 33% (Table 1).

Alternatively, the number of sampling points can be reduced to a number sufficient for estimating the kinetic parameters from the slopes of the uptake and depuration curves (Hinderleiter 2004). Unlike the standard OECD test, steady state does

not need to be achieved. This design can lower the animal usage by approximately 55% (Table 1). Benefits would further include lower cost, faster execution, less waste, and less chemical usage.

Another approach to reducing the number of animals used in OECD 305 depends upon the purpose for which the test is being conducted. In some regulatory schemes all that is necessary is to know whether the BCF is greater than a particular trigger value. In such circumstances conducting a depuration phase may not be necessary, reducing the animal usage by approximately 45%.

Static exposure procedures allow for determination of uptake clearance and depuration rate constants during bioconcentration of stable substances (Banerjee et al.1984; de Wolf et al. 1998). It requires the exposure of fish to an aqueous solution of the substance under static conditions, and measurement of loss of substance from the exposure system as a function of time. The rate constants are obtained from fitting the time-concentration profile to a simple mathematical model describing the exchange of substance between fish and water. The original approach by Banerjee et al. (1984) measured the substance in water and assumed removal processes such as biotransformation, sorption and volatilisation are not likely to occur. De Wolf et al. (1998) adapted this approach to study volatile materials by exposing fish to an aqueous solution in a fully closed system while measuring loss of substance from the air as a function of time. These approaches use less than 20% of the number of animals as compared to the OECD 305 study (Table 1).

A mathematical analysis of the robustness of static exposure systems (de Wolf et al. 1995) showed reasonably accurate estimates of uptake clearance and elimination rate constants are obtained when the substance concentration in fish is determined at the end of the exposure period, even in cases where (limited) loss occurs due to sorption and biotransformation. Further research comparing empirical data for metabolised substances from both static and flow-through experiments is required to assess the full applicability of the static exposure method.

The elimination rate constants measured in a dietary bioaccumulation study takes account of possible biotransformation reactions and may provide information helpful for estimation of the bioconcentration factor. In combination with a conservative estimate of the uptake clearance a reasonable estimate of the BCF can be obtained.

In a dietary bioaccumulation study fish are fed chemical-spiked food at a fixed concentration over a specified period of time depending on the expected half-life (T½) (Parkerton et al. 2001). At the end of this dietary exposure period some fish are analysed for parent substance (time = 0 of the depuration phase). The remaining fish are transferred to clean diet, and sequentially sampled and analysed over time so that a depuration curve can be established. From these data the half-life, dietary assimilation efficiency and BAF, defined as the steady-state ratio of the concentration in fish to that in the diet, can be readily derived.

Dietary bioaccumulation tests are, in practice, much easier to conduct for poorly water-soluble substances than the OECD 305 test, because a higher and more constant exposure to the substance can be administered via the diet than via water. A pitfall could

be the possibility for overestimation of the BCF in cases where gill uptake clearance rate is reduced. These experiments require approximately 40% of the number of animals used in the OECD 305 (Table 1).

Replacement of animals

A replacement strategy can be achieved by considering information from other species, or from related chemicals, (Q)SAR modelling, biomimetic or surrogate approaches, *in vitro* and embryos assays. These approaches are acceptable when validated and fit for the regulatory purpose.

Read-across/analogue and chemical grouping/category approaches typically involve using information on one chemical or a group of chemicals, respectively, and making some assessment about the relevance of that information for the unknown value of the non-tested chemical. QSARs for predicting BCF have been extensively evaluated and are mainly based on correlations with K_{ow} (ECETOC 1995, 1998, 2003). Eighty percent of chemicals to be registered under REACH can be covered using a combination of these techniques for estimating bioconcentration (Pedersen et al. 2003).

Based on a review of all available BCF data in the literature a computer program that allows for the estimation of BCF-values for a wide range of organic chemicals has been developed (BCFWIN by Meylan et al. 1999). This program estimates the BCF using the substance's log K_{ow} and correction factors to take into account certain structural and molecular factors that influence bioaccumulation by hindering uptake, and other factors that consider biotransformation (www.epa.gov/oppt/p2framework/docs/envfate.htm#Sub4). The approach adopted was to group chemicals and derive relationships for each group. It

was reported that some of these factors could be rationalised on the basis that they were related to some degree of reactivity or known biotransformation behaviour.

Arnot and Gobas (2003) have developed a bioaccumulation QSAR based on a mass-balance approach for assessing the bioaccumulation potential of organic chemicals in aquatic food webs. Processes of chemical absorption, distribution, biotransformation and egestion can be accounted for using values representative of a so called “generic fish”. As a result, the QSAR can be adapted to include the effect of metabolic transformation and trophic dilution on the calculated BCF and BAF. The model has been used by Environment Canada to categorize discrete organic substances on the Canadian Domestic Substances List (DSL) for bioaccumulation potential (Environment Canada 2003).

Another approach aims to address biotransformation starting from first principles (Dimitrov, Dimitrova et al. 2002; Dimitrov, Mekenyan et al. 2002; Dimitrov et al. 2006). Here, BCF is first modelled as a maximum value, ignoring any mitigating factors and based only on log Kow as an indicator of partitioning behaviour. Then the other factors are included, thus size, maximum diameter of 1.5 nm (Dimitrov, Dimitrova et al. 2002) and potential metabolism by fish (Dimitrov et al. 2006) are used to reduce the predicted BCF (www.oasis-lmc.org/software.php).

Södergren (1987) described a system based on a semi-permeable membrane device (SPMD) composed of a dialysis bag filled with hexane which has been further developed based on low density polyethylene bags which contain natural lipids or the model lipid triolein (1,2,3-tri[cis-9-octadecenoyl]glycerol) (see Huckins et al. 1997) to mimic the way organisms extract chemicals from water (i.e. biomimetic extraction).

SPMDs are relatively easy to use and will extract only bioavailable chemicals from the water in proportion to their partitioning coefficients simulating the potential for aquatic organisms to bioconcentrate chemicals. However, the equilibration time can be very long thus it has been suggested that results from SPMDs exposed for less than 2 months should be treated with caution (Booij et al. 1998).

Arthur et al. (1990) described another biometic extraction approach in which they constructed a Solid Phase Micro Extractor (SPME), composed of a thin polymer coating on a fused silica fibre. This fibre accumulation (and kinetics) is analogous to the bioconcentration of chemicals observed in aquatic organisms (Leslie et al. 2002). The process is very fast, due to the high surface area to volume ratio and generally easy to set up and use (Arthur et al. 1990; Vaes et al. 1996, 1997; Mayer et al. 2003).

A general disadvantage of biomimetic extractions is that the ability of fish to metabolise chemicals is not simulated, thus the bioconcentration of chemicals will be over-estimated. Furthermore, the potential for chemicals to be actively taken up via the gut is not addressed.

The physiological processes that govern bioconcentration in invertebrates may differ greatly from those in fish (e.g. the biotransformation systems are less developed in most invertebrates). Therefore, the use of invertebrates to assess bioconcentration potential of chemicals in fish cannot be routinely recommended. However, if there is only a need to demonstrate that the BCF in fish is below a certain value, then it may be possible to utilise BCFs from invertebrates as conservative estimations of the BCF in fish. Analogues for risk assessment, the BCF derived from an invertebrate could also be

used as a maximal value, and if the risk assessment indicated no concern then the use of fish to derive a BCF for fish would be difficult to justify.

Reduced absorption. Lipinski et al. (1997) first identified 5 physical chemical characteristics that influence solubility and absorption across the intestinal lumen using more than 2,200 drug development tests. These characteristics have been rigorously reviewed (Wenlock et al. 2003; Proudfoot 2005), used to develop commercial models to estimate absorption in mammals, and are commonly used by the human and veterinary pharmaceutical industry. Although less research has been conducted in fish, data indicate significant similarity among all vertebrates, as described below.

"Lipinksi's Rule of 5" allowed the prediction of poor solubility, and poor absorption from chemical structure. A chemical is not likely to cross a biological membrane in quantities sufficient to exert a pharmacological or toxic response when it has more than 5 Hydrogen (H)-bond donors, 10 H-bond acceptors, molecular weight greater than 500, and has a Log K_{ow} value greater than 5 (Lipinksi et al. 1997). Wenlock et al. (2003) studied about 600 additional chemicals and found that 90% of the absorbed compounds had fewer than 4 Hydrogen (H)-bond donors, < 7 H-bond acceptors, molecular weight less than 473, and had a Log D value less than 4.3. More recent work by Vieth et al. (2004) and Proudfoot (2005) supports the lower numbers. Molecular charge and the number of rotational bonds will also affect absorption by passive diffusion across a membrane or diffusion between cells.

The "leakiness" of a tissue, or its ability to allow a chemical to passively diffuse through it, is measured using trans-epithelial electrical resistance (TEER) and can be used

382 to compare tissue capabilities. A low TEER value indicates the tissue has greater
383 absorption potential. Although the studies by Lipinski et al. (1997), Wenlock et al.
384 (2003), Vieth et al. (2004) and Produdfoot (2005) focussed on absorption across the
385 intestinal lumen, the more restrictive TEER for fish gills (Table 2) implies that the
386 equations and concepts can be reapplied to conservatively estimate absorption in fish.

387 Molecular weight. Several values have been suggested for the molecular weight
388 (MW) cut-off for absorption across fish tissues. The EU TGD (EC 2003) indicates that
389 molecules with a MW greater than 700 g/mol are less likely to be absorbed and
390 bioconcentrate, whereas the US EPA, exempts chemicals with a molecular weight of
391 above 1,100 g/mol in the PBT assessment conducted under the Toxic Substances Control
392 Act (US EPA 1999). Anliker et al. (1988) suggested that a pigment could be excluded
393 from a fish bioaccumulation test if it has both a molecular weight of greater than 450 and
394 a cross section of over 1.05 nm (as the second smallest van der Waals diameter or C_{eff}).
395 Rekker et al. (1992) suggested that a calculated log K_{ow} of > 8 can be used on its own, or
396 in combination with a molecular weight of > 700-1,000 to conclude (with confidence)
397 that the compound is unlikely to bioaccumulate. While there has been limited
398 experimental evidence for a molecular weight cut-off, Burreau et al. (2004) did
399 demonstrate reduced bioconcentration and no biomagnification for high molecular weight
400 polybrominated diphenyl ethers, with 6 or more bromines, molecular weight 644-959.
401 Considering that molecular size and shape vary versus MW, molecular weight alone is
402 insufficient to allow absorption predictions. However, it does suggest that once the
403 molecular weight is in the region of 700 - 1,100, depending on other factors, a reduced
404 BCF may be expected. Hence, while recognising the uncertainties in the interpretation of

experimental results, we recommend that to demonstrate a reduced BCF a substance should have either:

- a molecular weight in excess of 1,100 g/mol, or
- a molecular weight of 700 – 1,100 g/mol with other indicators (see later discussion).

Molecular size. Molecular size may be considered as a more refined approach, taking into account molecular shape and flexibility explicitly rather than molecular weight alone. Opperhuizen et al. (1985) suggested a limiting cross sectional diameter for gill membrane permeation of 0.95 nm. in their study on polychlorinated naphthalenes (PCNs) bioconcentration Loonen et al. (1994) studied the bioconcentration of polychlorinated dibenzo-p-dioxins and polychlorinated dibenzofurans and found that the laterally substituted (2,3,7,8 substituted) were bioconcentrated while the non-laterally substituted were not. The main reason for this was attributed to metabolism (previously reported by Opperhuizen et al. 1990; Sijm et al. 1993), however, lower lipid solubility and lower membrane permeability were also considered to have played a role in the reduced BCFs observed. The non-accumulating structures would all have exceeded the effective cross-sectional diameter of 0.95 nm.

Anliker et al. (1987) studied the limits of bioconcentration of azo pigments in fish and their relation to the partition coefficient and the solubility in water and octanol. Despite a high calculated log K_{ow} for 2 pigments, the experimentally determined log BCFs were low. The explanation for this apparent inconsistency is the very limited fat (lipid) storage potential of these pigments, indicated by their low solubility in n-octanol

(< 1 and < 0.1 mg/L; see below) and their large molecular size (i.e. cross sectional diameters of 0.97 and 1.68 nm).

Anliker et al. (1988) assessed 23 disperse dyestuffs, 2 organic pigments and a fluorescent whitening agent, for which the experimental BCFs in fish were known. Sixteen halogenated aromatic hydrocarbons were included for comparison. None of the disperse dyestuffs, even the highly lipophilic ones with log K_{ow} > 3, accumulated significantly in fish. Their large molecular size was suggested to prevent their effective permeation through biological membranes and thus limit their uptake during the time of exposure. Anliker proposed that a second largest cross section of over 1.05 nm with molecular weight of greater than 450 would suggest a lack of bioconcentration for organic colourants.

Although lack of bioconcentration of some chemicals with a cross section of > 0.95 nm has been explained by limited membrane permeability, other studies have demonstrated uptake by fish and other species of substances with large cross sections (e.g. some dioxin and PBDE congeners) (Opperhuizen et al. 1987; Morris et al. 2004). Therefore a simple parameter may not be sufficient to explain when reduced BCF/BAF occurs. Dimitrov, Dimitrova et al. (2002**)** have tried to develop a more mechanistic approach to address this concept, using of molecular weight, size, and flexibility in their BCF estimates.

Dimitrov, Mekenyan et al. (2002) found that for compounds with a log K_{ow} > 5.0, a threshold value of 1.5 nm for the maximum cross-sectional diameter (i.e. molecular length) could discriminate between chemicals with BCF > 2,000 from those with BCF

449 < 2,000. This critical value was found to be comparable with the architecture of the cell
450 membrane, i.e. half the thickness of the lipid bilayer of a cell membrane. This is
451 consistent with a possible switch in uptake mechanism from passive diffusion through the
452 bilayer to facilitated diffusion or active transport. In a later paper, Dimitrov et al. (2003)
453 used this parameter to assess experimental data on a wide range of chemicals. The
454 conclusion was that a chemical with maximum cross-sectional diameter larger than 1.5
455 nm would not have a BCF > 5,000, i.e. would not meet the EU PBT criteria for vB
456 chemicals (EC 2003). In unpublished work, following further assessment of their data set,
457 they have changed this value to 1.74 nm (Dimitrov et al. 2004).

458 Earlier Opperhuizen et al. (1987) proposed that a substance with an effective
459 molecular length > 4.3 nm would not pass membranes, either in the gills or in the gut, at
460 all, based on a series of bioaccumulation and bioconcentration studies with linear and
461 cyclic polydimethylsiloxanes (silicones) varying in chain length. Membrane crossing is
462 very unlikely since such large molecules would disturb the entire interior strucuture of
463 the lipid membrane. Molecular weight did not explain reduced uptake, since 1 of the
464 substances with a molecular weight of 1,050 was detected in fish. The cross sectional
465 diameter of silicones could in itself not explain the reduced uptake since these diameters
466 were smaller or equal to those of PCBs that did bioaccumulate strongly.

467 Opperhuizen et al. (1987) also referred to a study by Hardy et al. (1974) where
468 uptake in codlings of long chain alkanes was disturbed for alkanes with corresponding
469 molecular lengths of > 4.3 nm. Tolls et al. (2000) observed uptake in fish of some
470 nonionic surfactants with an apparent equal length to long chain alkanes which seems
471 contradictory to the earlier proposed cut-off molecular length by Opperhuizen et al.

(1987). However, the uptake of the long nonioinc surfactants may be explained by internal molecular flexibility reducing the effective molecular length below 4.3 nm.

In conclusion there would appear to be no clear cut-off value for molecular size beyond which no absorption will take place. While recognising the uncertainties in the interpretation of experimental results, it is recommended:

- a maximum effective molecular length of 4.3 nm indicates no uptake and indicates a chemical is not bioconcentrating;
- a maximum cross-sectional diameter of 1.74 nm indicates a chemical would not have a BCF > 5,000;
- a maximum cross-sectional diameter of 1.74 nm plus a molecular weight of 700 – 1,100 would suggest a chemical would not have a BCF > 2,000.

Other indicators. There are other indicators for low uptake that could also be used to suggest that a chemical, despite having a log K_{ow} in excess of 4.5, has a low bioconcentration potential such as lack of experimentally observed gill or skin permeability, and low or reduced uptake in mammalian studies e.g. OECD 420, 423, 425 and 435 (OECD 2001a, 2001b, 2001c, 2004). Cell culture models offer many advantageous features for the analysis of chemical transport across membranes and can be used to expedite identification of compounds with less favourable uptake properties, and to evaluate structure-absorption relationships.

Wood et al. (1997) developed a method for the primary culture of gill epithelial cells from freshwater rainbow trout. Application in quantitative analysis of chemical transport

493 across membranes is currently limited since primary epithelial cells are used, which
494 increases the possibility of high inter-experimental variability (Wood et al. 2002).

495 One *in vitro* model system that has proven useful in chemical gastro-intestinal
496 absorption studies is the Caco-2 cell line (Hidalgo et al. 1996). Caco-2 cells are human in
497 origin, and can be manipulated in culture so that they exhibit many characteristics of the
498 human small intestinal epithelium. Caco-2 monolayers have been extensively used in the
499 prediction of intestinal absorption *in vivo* (Bailey et al. 1996), and have been found
500 specifically useful in identification of pharmaceuticals with potential absorption problems
501 (Artursson et al. 1996).

502 Use of Caco-2 monolayers for prediction of fish gill absorption *in vivo* may
503 overestimate potential absorption of a chemical though the gill (Table 2). Use of these
504 cellular models can decrease the number of animals needed for bioconcentration studies
505 by identifying those chemicals which have limited uptake. An additional advantage of
506 this cell culture model is that multiple studies can be performed with a relatively small
507 amount of radiolabelled test chemical.

508 Pärt (1990) developed a perfused gill preparation from rainbow trout
509 (*Oncorhynchus mykiss*) as an alternative for studies *in vivo*. The perfused gill allows
510 direct measurements of *in vivo* absorption rates of chemicals across the gill epithelium
511 (Pärt et al. 1992). Uptake rate constants of different classes of hydrophobic organic
512 chemicals determined in isolated perfused gills of rainbow trout (*O. mykiss*) are higher
513 than those determined in guppy (*Poecilia reticulata*) *in vivo* (Sijm et al. 1995). Both
514 systems show relatively high variation, however this can be significantly reduced and the

uptake rate constants determined once they are normalised with a reference chemical. Subsequent extrapolation to fish of different sizes can be through use of allometric relationships (Sijm et al. 1995; Sijm et al. 1999; Hendriks et al. 2001; Hendriks and Heikens 2001).

Reduced distribution. The concept of having a value relating a chemical's solubility in octanol to reduced BCF/BAF is derived from 2 considerations. Firstly, that octanol is a reasonable surrogate for fish lipids, and secondly, that if a substance has a reduced solubility in octanol this may result in a reduced BCF/BAF (and reduced or no effect to the animal). The former forms the basis of the majority of models for predicting BCF using log K_{ow}. When a substance has a low solubility in octanol (S_{oct}) as well as a low solubility in water (S_w), the resulting ratio S_{oct}/S_w could range from very low to very high, with no clear idea on how this would affect the magnitude of the BCF/BAF. Still, it could be argued that a very low solubility in octanol could be used as an indication that only low body burdens build up in an aquatic organism.

Chessells et al. (1992) demonstrated a decrease in lipid solubility with increasing K_{ow} values for highly hydrophobic compounds (log $K_{ow} > 6$). It was suggested that this led to reduced BCFs. Banerjee et al. (1991) demonstrated that by introducing a term for lowered octanol/lipid solubility into the calculated log K_{ow} BCF relationship, they could significantly improve the prediction of bioconcentration for highly hydrophobic chemicals. Experimental K_{ow} values already reflect the lower octanol solubility.

The meaningful implication of bioaccumulation is to identify the maximum concentration(s) in organisms that would give rise to concern. The concept of critical

body burdens (CBB) for acute effects is reasonably well-established (McCarty et al. 1993; McCarty 1986) especially for chemicals that act via a narcosis mode of action. Recent reviews of this concept (Barron et al. 1997, 2002; Sijm et al. 1999; Thompson et al. 2003) can be summarised as follows:

- There are, still, very little data available, especially for specifically acting chemicals and for chronic effects, upon which to make decisions relating to generic CBBs;

- It is likely that much of the variability in CBBs can be explained by species sensitivities, biotransformation, lipid content, the measurement of organ versus whole body measurements and whether the chemical is correctly assigned to a mode of action category;

- It is possible to identify ranges of CBB values for specific modes of action. This is easier for narcosis type mode of actions, and becomes increasingly prone to error moving towards more specifically acting chemicals.

Table 3 summarises 4 sources of information for CBBs, and when comparing the expert judgement of Sijm to the ranges indicated and to the figures in the respective publications, it is clear that the values chosen are in the median values of the ranges/data. However, there is a lot of variability and therefore uncertainty in deciding on the actual CBB value to use. Choosing the value of 0.001 mmol/kg ww (mid-point for respiratory inhibitors) allows for approximate protection for all the modes of action with the exception of the most toxic chemicals. The rationale for this would be that chemicals that act by the lowest and most specific mode of action are very likely to be toxic (T) and

559 hence sufficiently bioaccumulative to be of immediate concern. The choice is therefore
560 pragmatic but protective.

561 Lipid normalising the chosen CBB of 0.001 mmol/kg ww, and assuming a lipid
562 content of 5%, gives a lipid normalised CBB of 0.02 mmol/kg lipid or 0.02 * Molecular
563 weight mg/l lipid. However, given the uncertainty involved it is suggested to introduce an
564 application factor of 10to account for species differences and organ versus body
565 differences.

566 Based on the above it is proposed that where a chemical has a solubility of less
567 than (0.002 * Molecular weight) mg/l in octanol it should be assumed that the compound
568 has only a limited potential to establish high body burdens and to bioaccumulate. If it
569 does bioaccumulate, it would be unlikely to give rise to levels in biota that would cause
570 significant effects.

571 Increased elimination/depuration. De Wolf et al. (1992) demonstrated a
572 significant reduction in the bioconcentration of chlorinated anilines compared to log K_{ow}-
573 based predictions which was attributed to increased elimination via biotransformation. *In*
574 *vitro* assays can provide information on both the range of metabolites as well as their
575 relative importance, and provide data useful for input into fish-specific physiologically
576 based pharmacokinetic (PBPK) modelling efforts. Several types of studies are available
577 that assess the influence of biotransformation on the BCF in fish such as measuring the
578 decrease of parent compound (mass balance approach) (e.g. Opperhuizen 1986),
579 comparison of total elimination of biotransformable and non-biotransformable chemicals

with a similar K_{ow} (de Wolf et al. 1993a), and estimation of the *in vivo* biotransformation rates from *in vitro* assays.

Biotransformation activity has been measured in fish liver, intestine, gill, kidney and brain (Lindström-Seppä et al. 1981; Miller et al. 1989; Van Veld et al. 1990; Hegelund et al. 2003). Since metabolism processes take place primarily in the liver, this is the organ of choice to study the biotransformation of chemicals.

In vitro estimation of biotransformation potential. Biotransformation potential of fish has been investigated in liver slices (Schmieder et al. 2000), whole liver homogenates (de Wolf et al. 1993b), liver subfractions (Kolanczyk et al. 1999; Dyer et al. 2003; Perdu-Durand et al. 2004), isolated hepatocytes and cell lines (Cravedi et al. 2001; Segner 1998; Segner et al. 2001; Dyer et al. 2004). The xenobiotic metabolite pattern produced by fish hepatocytes *in vitro* is generally similar to that observed *in vivo* (Segner et al. 2001).

Biotransformation is strongly taxa- and species-specific which may be due to endogenous or exogenous factors (Sijm et al. 1997).. Negligible biotransformation higher up in the food chain implies a potential risk of biomagnification (Sijm et al. 1997). The following types of compounds can be distinguished: a) compounds which are poorly biotransformed as a general rule, b) compounds which are poorly biotransformed by specific organisms/groups e.g. PAH in mussels, c) compounds which are easily biotransformed across phyla.

The rate of biotransformation of chemicals through enzymatic reactions can be monitored either by an increase in the activity of enzymes involved, by the decrease in

602 the amount of substrate (parent compound), or by an increase in products. The faster the
603 rate of parent biotransformation the less likely it is that the chemical will bioaccumulate,
604 with the influence of biotransformation on the overall elimination and BCF value more
605 pronounced for hydrophobic chemicals (de Wolf et al. 1992). However, because of the
606 lack of data there are no generally accepted approaches to use the *in vitro* rates to
607 estimate potential BCFs. This is an area for further research.

608 Dyer et al. (2003) applied an approach to derive a BCF_{cell} for various surfactants
609 using carp primary hepatocytes, and cultured hepatocytes (PLHC-1 cells). The rates of
610 uptake and loss of the test chemical from the cellular systems were estimated assuming
611 first order kinetics and the BCF in the cells determined by a ratio of uptake rate to the rate
612 of loss. For LAS the calculated BCF_{cell} were approximately 4-fold less than the BCF_{fish}
613 measured by Tolls et al. (1997) *in vivo* in fathead minnow. For the linear alcohol
614 ethoxylate ($C_{13}EO_8$) the corresponding BCF_{cell} were 2 to 30-fold less compared to the
615 fathead minnow results generated by Tolls et al. (2000).

616 An advantage of *in vitro* methodologies for assessing biotransformation is that
617 they are rapid and less expensive than *in vivo* tests. A compromise between conducting *in*
618 *vivo* BCF testing and exposing liver systems would be to measure the same parameters in
619 livers extracted from exposed fish. This may allow for a reduction in the number of fish
620 used in assessing bioconcentration of a chemical, however this has not yet been
621 investigated.

622

INTEGRATED BIOCONCENTRATION TESTING STRATEGY

We reviewed the current environmental regulatory testing strategies for bioaccumulation and the alternative approaches that could provide (elements of) the required information on bioconcentration. Based on this an integrated bioconcentration testing strategy can be developed taking account of alternative approaches including existing data (e.g. read-across and extrapolation), QSARs, *in vitro* and other techniques for implementing the 3Rs (Figure 1). The testing strategy developed is a tiered process; Tier 1 uses estimation models and Tier 2 using non-animal experimental systems. Depending upon the quality of the prediction, these tiers can lead to a replacement of animals used for assessing bioconcentration within environmental assessment. Tier 3 makes use of experimental systems but with a reduced number of animals. The full BCF test performed according to OECD 305 is Tier 4 (the final step in the strategy). Validation of alternative approaches from Tiers 1, 2 and 3 should include a comparison of performance against results for the Tier 4 test.

Central to the strategy is the question “Is (refined) BCF suitable for purpose” (Figure 2). The purpose of this question is to ensure that the BCF being generated is either sufficiently accurate that an assessment of indirect exposure can be conducted or that regulatory decisions can be made with sufficient confidence. Clearly the closer a BCF estimate or measured value is to a boundary, either a regulatory defined criterion (e.g. BCF > 5,000) or an indicator of risk (e.g. the predicted environmental concentration to the no effect concentration (PEC/PNEC) = 1), the more confidence is needed that the BCF is reasonably accurate. In making this judgement the variability that occurs even with OECD 305 should be considered.

Tier 1

A. The first part of the assessment addresses whether the substance has a potential for restricted absorption. If unlikely to bioconcentrate, a surrogate or null BCF is estimated. The assessor then moves to the central question regards suitability of the estimate for its intended purpose.

If absorption does not seem to be restricted and biotransformation appears unlikely then the second question asked is whether log K_{ow} is an appropriate model or surrogate for describing the water-fish distribution process. In the case of metals and surfactants log K_{ow} is not an appropriate model and one should immediately move to C. If log K_{ow} is suitable, a measure of the octanol-water partition coefficient needs to be obtained. This can be done by model estimation (ECETOC 2003) or measurement methods (ECETOC 1998). Next is to evaluate whether there is an applicable (Q)SAR that includes the chemical in its domain. If yes, the log K_{ow} value can be used as input into the (Q)SAR to estimate the fish bioconcentration factor.

C. If log K_{ow} is not a suitable surrogate, but other approaches are (e.g. SPME), then they should be used at this stage. Other options include SPMD, dialysis bags, and biotic measurement systems (i.e. invertebrates). From this measurement an estimation of a fish bioconcentration factor is obtained. The confidence in the information is again addressed in the central question regards suitability of the estimate for its intended purpose. If there are no good alternatives it is suggested that a screening BCF study be conducted (move to Tier 3).

Tier 2

When a BCF has been estimated but there is significant uncertainty or not sufficient precision for the assessment, then go to point D below. However, in case there are no arguments for restricted uptake and no viable surrogates for partitioning behaviour then go to Tier 3.

D. The assessment at this point addresses to what extent biotransformation would impact the elimination of the substance from fish and thus reduce an estimated (maximum) BCF value. This can be approached by asking whether biotransformation occurs in other species with potential similarity in biotransformation pattern, or whether other, structurally related substances are known to be biotransformed. If so, a measure of biotransformation could be obtained either through the use of model estimations or *in vitro* measurements. In this way a refined BCF is obtained and the suitability of the new value assessed.

Tier 3

When there are no arguments for restricted uptake and no viable surrogates for partitioning behaviour available then testing is required. It is suggested that a fish BCF is estimated using *in vivo* screening tests before moving to a BCF measurement using the OECD 305 test guideline (Tier 4). If the estimate from the *in vivo* screening assays is suitable for purpose then one can exit the bioconcentration testing strategy. If not, the OECD 305 test will need to be performed before the testing strategy can be exited.

Tier 4

Conduct the OECD 305 study.

CONCLUSIONS AND RECOMMENDATIONS

The European Union Technical Committee for New and Existing Substances (TC NES) working group addressing persistent, bioaccumulative and toxic (PBT) substances considered the recommendations on molecular properties leading to reduced absorption and the influence of octanol solubility on distribution. They agreed to use them as part of their strategy of determining whether a chemical should be placed on a screening list and/or should be tested to determine whether it is B/vB. The criteria should be considered in a weight of evidence approach with other information, e.g. data derived from mammalian studies.

Several research needs can be identified upon further examination of the decision-tree proposed as a possible bioconcentration testing strategy (Figure 2). The use of relevant existing information on biotransformation can be considered a viable alternative replacing animals. Reduction measures, while still making use of a limited number of fish, can already be applied or may need rapid development for short-term application. In the mid to longer term, research programmes will be needed to enable the replacement tests to be fully accepted and implemented.

The domain of application of the standard *in vivo* bioconcentration test (OECD 305) should be more clearly defined. The uncertainties in the measurements obtained after conducting a standard *in vivo* bioconcentration test should be better assessed, without which the successful validation of alternatives methods to the fish bioconcentration test would be compromised. A database holding peer reviewed high quality BCF data, a “BCF Gold Standard Database” is under development and will

become a valuable resource for future development of alternative tests. The use of only 1 concentration or limited uptake/depuration phases should be evaluated and implemented for relevant chemical classes.

Other *in vivo* experimental approaches, e.g. the dietary accumulation protocol and abbreviated OECD 305 need to be investigated further to define their limits of applicability and eventually extend their domain. In addition, the assumptions regarding rates of uptake need to be confirmed and their limitations understood.

To better address the value of *in vitro* assays and their suitability for amending BCFs, additional research is needed to identify their variability and confidence limits. Research into the use of decision theory methods may also help by allowing for a better assessment of the uncertainty inherent in these techniques. Also some technical issues need to be addressed to better understand the use of *in vitro* methods. For the purpose of standardising protocols, recommended procedures for the isolation of fish cells, culture and exposure should be agreed and should be in compliance with the Good Cell Culture Practices. The development of *in vitro* assays, expert systems and models capable of incorporating ADME concepts should receive priority.

Absorption. The parameters governing physical restriction of cellular absorption of chemicals should be better described and the assumed constant rate of uptake, up to log K_{ow} 6, needs to be further investigated. Furthermore the applicability of using *in vitro* systems to assess absorption should be studied. The first step could be to evaluate whether the mammalian intestinal cells (Caco-2 cells) are representative of fish for understanding gill absorption, uptake from food and deriving assimilation factors. Future

734 research is needed to further assess the impact of gill biotransformation in the absorption
735 process. In addition, generation of information that provides more insight into the validity
736 of extrapolation from existing approaches to fish and/or the development of fish specific
737 absorption models is required.

738 Distribution and partitioning. The applicability domain of (Q)SARs for log
739 K_{ow}/BCF predictions should be better defined. Research into the conditions of use of
740 SPMD/SPME, within the context of the strategy outlined above, should be performed.
741 Their limitations and potential, for assessing poorly metabolisable chemicals and in
742 whole effluent assesment/environmental monitoring, should be explored.

743 Biotransformation. The use of available biodegradation data and
744 metabolism/biotransformation data from mammalian studies should be considered before
745 conducting any fish bioconcentration test. In order to ensure that extrapolation can be
746 done, a literature research study should capture differences and similarities between the
747 various classes. Bacteria, invertebrates and vertebrates are capable of chemical
748 biotransformation, but to various extents, and may be using various metabolic pathways.
749 The knowledge of biotransformation patterns and extent in diverse phyla may help
750 understand bioconcentration processes in fish (Sijm et al. 1997).

751 The existing (Q)SARs which address biotransformation in fish need to be
752 improved or further developed. The available *in vitro* biotransformation assays with
753 sub/cellular fish liver systems to address metabolism should be further investigated. In
754 order to allow the use of relevant information, the level of biotransformation potential in
755 the different *in vitro* systems, using different fish species or classes of organisms, should

be compared. The level of biotransformation potential *in vitro* should be compared to the level of biotransformation *in vivo*.

There are a number of issues in relation to the extrapolation from *in vitro* to *in vivo* for deriving a BCF. Ultimately it should be possible to relate, for example, the level of parent disappearance in microsomes with a factor that would refine the estimated BCF_{fish}, or a BCF_{cell} to BCF_{fish}. It is not yet obvious how absorption and metabolism in mammals relate to absorption and metabolism in fish. Another inherent difficulty of *in vitro* studies is the relation between responses in single cells to responses/effects in whole organisms. This is true for toxicological responses as well as for biotransformation processes. The acceptability of *in vitro* data could be enhanced provided that parallel studies are conducted *in vivo*, for example by comparing the level of enzymatic activity in the livers of exposed fish to that in exposed liver cells. This could also be used as a refinement and reduction of the number of fish used to assess fish bioconcentration.

In summary, it is clear when addressing the use of fish for the environmental safety of chemical products there are many opportunities for applying the principles of the 3Rs: Reduce, Refine and Replace. The current environmental regulatory testing strategy for bioconcentration and secondary poisoning can be significantly improved by use of alternative approaches that provide (elements of) the required information. We developed an Integrated Testing Strategy for bioconcentration assessment that can be applied in a regulatory context and takes into account these alternative information methodologies.

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REFERENCES

Anliker R, Moser P. 1987. The limits of bioaccumulation of organic pigments in fish: Their relation to the partition coefficient and the solubility in water and octanol. *Ecotox Environ Saf* 13:43-52.

Anliker R, Moser P, Poppinger D. 1988. Bioaccumulation of dyestuffs and organic pigments in fish. Relationships to hydrophobicity and steric factors. *Chemosphere* 17:1631-1644.

Anonymous. 2004. Fish, dietary bioaccumulation study protocol, based on a version adapted by the TC NES subgroup on PBTs of the original protocol developed for and used by ExxonMobil Biomedical Sciences, Inc (EMBSI).

Arnot J, Gobas FAPC. 2003. A generic QSAR for assessing the bioaccumulation potential of organic chemicals in aquatic food webs. *Quant. Struct.-Act. Realt*. 22: 1-9.

Arthur CL, Pawliszyn J. 1990, Solid phase microextraction with thermal desorption using fused silica optical fibers. *Anal Chem* 62:2145-2148.

Artursson P, Palm K, Luthman K. 1996. Caco-2 monolayers in experimental and theoretical predictions of drug transport. *Advanced Drug Delivery Reviews* 22:67-84.

796 Bailey CA, Bryla P, Malick AW. 1996. The use of the intestinal epithelial cell
797 culture model, Caco-2, in pharmaceutical development. *Advanced Drug Delivery Reviews*
798 22:85-103.

799 Banerjee S, Baughman GL. 1991. Bioconcentration factors and lipid solubility.
800 *Environ Sci Technol* 25:536-539.

801 Banerjee S, Sugatt RH, O'Grady DP. 1984. A simple method for determining
802 bioconcentration parameters of hydrophobic compounds. *Environ Sci Technol* 18:79-81.

803 Barron MG, Anderson MJ, Lipton J, Dixon DG. 1997. Evaluation of critical body
804 residue QSARs for predicting organic chemical toxicity to aquatic organisms. *SAR QSAR*
805 *Environ Res* 6:47-62.

806 Barron MG, Hansen JA, Lipton J. 2002. Association between contaminant tissue
807 residues and effects in aquatic organisms. *Reviews of Environmental Contamination and*
808 *Toxicology* 173:1-37.

809 Beek B. 1991. Welcome address and introduction. In: Nagel R, Loskill R, editors,
810 Bioaccumulation in aquatic systems. Weinheim, Germany: VCH Verlagsgesellschaft
811 mbH, p 1-6.

812 Booij K, Sleiderink HM, Smedes F. 1998. Calibrating the uptake kinetics of
813 semipermeable membrane devices using the exposure standards. *Environ Toxicol Chem*
814 17:1236-1245.

815 Borchardt RT, Smith PL, Wilson G, editors. 1996. Models for assessing drug
816 absorption and metabolism. New York (NY), USA: Plenum Press.

817 Burreau S, Zebuhr Y, Broman D, Ishaq R. 2004. Biomagnification of
818 polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs) studies
819 in pike (*Esox lucius*), perch (*Perca fluviatilis*) and roach (*Rutilus rutilus*) from the Baltic
820 Sea. *Chemosphere* 55:1043-1052.

821 CEPA. 1999. Persistence and Bioaccumulation Regulations (SOR/2000-107).
822 *Canada Gazette*134:607-612.

823 Chessells M, Hawker DW, Connell DW. 1992. Influence of solubility in lipid on
824 bioconcentration of hydrophobic compounds. *Ecotox Environ Saf* 23:260-273.

825 Connell DW, Hawker DW. 1988. Use of polynomial expressions to describe the
826 bioconcentration of hydrophobic chemicals by fish. *Ecotox Environ Saf* 16:242-257.

827 Cravedi JP, Boudry G, Baradat M, Rao D, Debrauwer L. 2001. Metabolic fate of
828 2,4-dichloroaniline, prochloraz and nonylphenol diethoxylate in rainbow trout: a
829 comparative *in vivo / in vitro* approach. *Aquat Toxicol* 53:159-172.

830 de Wolf W, Lieder PH. 1995. Analysis of the static exposure system to study
831 biotransformation and bioconcentration kinetics in aquatic organisms. *Toxicology*
832 *Modeling* 1:99-110.

833 de Wolf W, Lieder PH. 1998. A novel method to determine uptake and
834 elimination kinetics in fish of volatile chemicals. *Chemosphere* 36:1713-1724.

835 de Wolf W, de Bruijn JHM, Seinen W, Hermens JLM. 1992. Influence of
836 biotransformation on the relationship between bioconcentration factors and octanol-water
837 partition coefficients. *Environ Sci Technol* 26:1197-1201.

de Wolf W, Seinen W, Hermens JLM. 1993a. Biotransformation and toxicokinetics of trichloroanilines in fish in relation to their hydrophobicity. *Arch Environ Contam Toxicol* 25:110-117.

de Wolf W, Seinen W, Hermens JLM. 1993b. N-acetyltransferase activity in rainbow trout liver and the *in vitro* biotransformation of chlorinated anilines and benzenes in fish. *Xenobiotica* 23:1045-1056.

DGEE. 2003. Directorates General Enterprise and Environment. The new EU chemicals legislation REACH. DG Enterprise, Brussels, Belgium. (www.europa.eu.int/comm/enterprise/reach/index_en.htm).

Dimitrov SD, Dimitrova NC, Walker JD, Veith GD, Mekenyan OG. 2002**.** Predicting bioconcentration factors of highly hydrophobic chemicals. Effects of molecular size. *Pure Appl Chem* 74:1823-1830.

Dimitrov SD, Mekenyan OG, Walker JD. 2002. Non-linear modeling of bioconcentration using partition coefficients for narcotic chemicals. *SAR QSAR Environ Res* 13:177-184.

Dimitrov SD, Dimitrova NC, Walker JD, Veith GD, Mekenyan OG. 2003. Bioconcentration potential predictions based on molecular attributes - an early warning approach for chemicals found in humans, birds, fish and wildlife. *QSAR Comb Sci* 22:58-68.

Dimitrov SD, Dimitrova NC, Parkerton T, Comber MHI, Bonnell M, Mekenyan OG. 2006. Baseline model for identifying the bioaccumulation potential of chemicals. *SAR QSAR Environ Res* Accepted for publication.

Dyer SD, Bernhard M. 2004. Follow up to feasibility study on *in vitro* biotransformations systems: Determination of uptake, loss and bioconcentration of two surfactants. ERASM report, 35 p. www.erasm.org/study.htm

Dyer SD, Bernhard MJ, Versteeg DJ. 2003. Identification of an *in vitro* method for estimating the bioconcentration of surfactants in fish. In *ERASM final report.* Brussels, Belgium, p 1-66. www.erasm.org/study.htm

EC. 2003. 2nd edition of the Technical Guidance Document in support of Commission Directive 93/67/EEC on Risk Assessment for new notified substances, Commission Regulation (EC) No 1488/94 on Risk Assessment for existing substances and Directive 98/8/EC of the European Parliament and of the Council concerning the placing of biocidal products on the market. Office for Official Publications of the European Communities, Luxembourg.

ECETOC. 1995. The role of bioaccumulation in environmental risk assessment: the aquatic environment and related foodwebs. Technical Report No 67. European Centre for Ecotoxicology and Toxicology of Chemicals, Brussels, Belgium.

ECETOC. 1998. QSARs in the assessment of the environmental fate and effects of chemicals. Technical Report No 74. European Centre for Ecotoxicology and Toxicology of Chemicals, Brussels, Belgium.

ECETOC. 2003. QSARs: evaluation of the commercially available software for human health and environmental endpoints with respect to chemical management applications. Technical Report No 89. European Centre for Ecotoxicology and Toxicology of Chemicals, Brussels, Belgium.

ECETOC. 2005. Alternative testing approaches in environmental safety assessment. Technical Report No. 97. European Centre for Ecotoxicology and Toxicology of Chemicals, Brussels, Belgium.

EEC. 1986. Council Directive 86/609/EEC of 24 November 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes. *Official Journal of the European Communities* L 358.

Environment Canada. 2003. Guidance manual for the categorization of organic and inorganic substances on Canada's Domestic Substances List: Determining persistence, bioaccumulation potential, and inherent toxicity to non-human organisms. Existing Substances Branch. June. (http://www.ec.gc.ca/substances/ese/eng/dsl/cat_index.cfm)

Gobas FAPC, Mackay D. 1987. Dynamics of hydrophobic organic chemical bioconcentration in fish. *Environ Toxicol Chem* 6:495-504.

Hardy R, Mackie PR, Whittle KJ, McIntyre AD. 1974. Discrimination in the assimilation of *n*-alkanes in fish. *Nature* 252:577-578.

Hawker DW, Connell DW. 1988. Influence of partition coefficient of lipophilic compounds on bioconcentration kinetics with fish. *Wat Res* 22:701-707.

Hegelund T, Celander MC. 2003. Hepatic versus extrahepatic expression of *CYP*3A30 and *CYP*3A56 in adult killifish (*Fundulus heteroclitus*). *Aquat Toxicol* 64:277-291.

Hendriks JA, Heikens A. 2001. The power of size. 2. Rate constants and equilibrium ratios for accumulation of inorganic substances related to species weight. *Environ Toxicol Chem* 20:1421-1437.

Hendriks JA, van der Linde A, Cornelissen G, Sijm DTHM. 2001. The power of size. 1. Rate constants and equilibrium ratios for accumulation of organic substances related to octanol-water partition ratio and species weight. *Environ Toxicol Chem* 20:1399-1420.

Hidalgo IJ, Li J. 1996. Carrier-mediated transport and efflux mechanisms in Caco-2 cells. *Advanced Drug Delivery Reviews* 22:53-66.

Hinderleiter P. 2004. Haskell Laboratories (DE), USA. Personal communication.

Hodgeson E, Levi PE. 1994. Introduction to biochemical toxicology. Norwalk, (CT), USA: Appleton and Lange, p 11-48; 75-131; 177-192.

Huckins JN, Petty JD, Thomas J. 1997. Bioaccumulation: How chemicals move from the water into fish and other aquatic organisms. Health and Sciences Dept., API Publication Number 4656. API, Washington (DC), USA.

IEH. 2001. Testing requirements for proposals under the EC white paper Strategy for a future chemicals policy (Web report W6). Institute for Environment and Health, Leicester, UK. www.le.ac.uk/ieh/webpub/webpub.html (posted July 2001).

Kolanczyk R, Schmieder P, Bradbury S, Spizzo T. 1999. Biotransformation of 4-methoxyphenol in rainbow trout (*Oncorhynchus mykiss*) hepatic microsomes. *Aquat Toxicol* 45:47-61.

Kristensen P, Tyle H. 1991. The assessment of bioaccumulation. In: Nagel R, Loskill R, editors, Bioaccumulation in aquatic systems. Weinheim, Germany: VCH Verlagsgesellschaft mbH, p 189-227.

Leslie HA, Ter Laak TL, Busser FJM, Kraak MHS, Hermens JLM. 2002. Bioconcentration of organic chemicals: Is a solid-phase microextraction fiber a good surrogate for biota? *Environ Sci Technol* 36:5399-5404.

Lindström-Seppä P, Koivusaari U, Hanninen O. 1981. Extrahepatic xenobiotic metabolism in North-European freshwater fish. *Comp Biochem Physiol* 69C:259-263.

Lipinski CA, Lombardo F, Dominy BW, Feeney PJ. 1997. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Advanced Drug Delivery Reviews* 23:3-25.

Loonen H, Tonkes M, Parsons JR, Govers HAJ. 1994. Bioconcentration of polychlorinated dibenzo-p-dioxins and polychlorinated dibenzofurans in guppies after aqueous exposure to a complex PCDD/PCDF mixture: Relationship with molecular structure. *Aquat Toxicol* 30:153-169.

Mayer P, Tolls J, Hermens JLM, Mackay D. 2003. Equilibrium sampling devices. *Environ Sci Technol* 37:184A-191A

McCarty LS. 1986. The relationship between aquatic toxicity QSARs and bioconcentration for some organic chemicals. *Environ Toxicol Chem* 5:1071-1080.

McCarty LS, Mackay D. 1993. Enhancing ecotoxicological modeling and assessment. *Environ Sci Technol* 27:1719-1728.

Meylan WM, Howard PH, Boethling RS, Aronson D, Printup H, Gouchie S. 1999. Improved method for estimating bioconcentration/bioaccumulation factor from octanol/water partition coefficient. *Environ Toxicol Chem* 18:664-672.

Miller MR, Hinton DE, Stegeman JJ. 1989. Cytochrome P-450 induction and localization in gill pillar (endothelial) cells of scup and rainbow trout. *Aquat Toxicol* 14:307-322.

Morris S, Allchin CR, Zegers BN, Haftka JJH, Boon JP, Belpaire C, Leonards PEG, Van Leeuwen SPJ, De Boer J. 2004. Distribution and fate of HBCD and TBBPA brominated flame retardants in North Sea estuaries and aquatic food webs. *Environ Sci Technol* 38:5497-5504.

Nendza M. 1991. QSARs of bioconcentration: validity assessment of log P_{ow}/log BCF correlations. In: Nagel R, Loskill R, editors, Bioaccumulation in aquatic systems. Weinheim, Germany: VCH-Verlagsgesellschaft.

OECD. 1996a. Final report of the OECD workshop on harmonization of validation and acceptance criteria for alternative toxicological test methods. Organisation for Economic Co-operation and Development, Paris, France.

960 OECD. 1996b. OECD guidelines for testing of chemicals. 305. Bioconcentration:
961 Flow-through fish test, last updated 14th June 1996. Organisation for Economic Co-
962 operation and Development, Paris, France.

963 OECD. 2001a. OECD guidelines for testing of chemicals. 420. Acute oral toxicity
964 - fixed dose procedure, last updated 17th December 2001. Organisation for Economic Co-
965 operation and Development, Paris, France.

966 OECD. 2001b. OECD guidelines for testing of chemicals. 423. Acute oral toxicity
967 - acute toxic class method, last updated 17th December 2001. Organisation for Economic
968 Co-operation and Development, Paris, France.

969 OECD. 2001c. OECD guidelines for testing of chemicals. 425. Acute oral
970 toxicity: Up and down procedure, last updated 17th December 2001. Organisation for
971 Economic Co-operation and Development, Paris, France.

972 OECD. 2004. OECD guideline for the testing of chemicals, draft proposal for a
973 new guideline (No. 435, 1st Version), *in vitro* membrane barrier test method for skin
974 corrosion. Organisation for Economic Co-operation and Development, Paris, France.

975 Okada Y, Irimajiri A, Inouye A. 1977. Electrical properties and active solute
976 transport in rat small intestine. II. Conductive properties of transepithelial routes. *J*
977 *Membr Biol* 31:221-232.

978 Opperhuizen A. 1986. Bioconcentration of hydrophobic chemicals in fish. In:
979 Poston TM, Purdy R, editors, Aquatic toxicology and environmental fate, Vol 9,

STP 921. Philadelphia, USA: American Society for Testing and Materials (ASTM), p 304-315.

Opperhuizen A, Sijm DTHM. 1990. Bioaccumulation and biotransformation of polychlorinated dibenzo-p-dioxins and dibenzofurans in fish. *Environ Toxicol Chem* 9:175-186.

Opperhuizen A, Van der Velde EW, Gobas FAPC, Liem DAK, Van der Steen JMD. 1985. Relationship between bioconcentration in fish and steric factors of hydrophobic chemicals. *Chemosphere* 14:1871-1896.

Opperhuizen A, Damen HWJ, Asyee GM, van der Steen JMD, Hutzinger O. 1987. Uptake and elimination by fish of polydimethylsiloxanes (silicones) after dietary and aqueous exposure. *Toxicol Environ Chem* 13:265-285.

Parkerton T, Letinski D, Febbo E, Davi R, Dzamba C, Connelly M, Christensen K, Peterson D. 2001. A practical testing approach for assessing bioaccumulation potential of poorly water soluble organic chemicals. Presentation at SETAC Europe, May 7-10, 2001, Madrid, Spain.

Pärt P. 1990. The perfused fish gill preparation in studies of the bioavailability of chemicals. *Ecotox Environ Saf* 19:106-115.

Pärt P, Saarikoski J, Tuurala H, Havaste K. 1992. The absorption of hydrophobic chemicals across perfused rainbow trout gills: methodological aspects. *Ecotox Environ Saf* 24:275-286.

Pedersen F, de Bruijn J, Munn S, van Leeuwen CJ. 2003. Assessment of additional testing needs under REACH: Effects of (Q)SARs, risk based testing and voluntary initiatives. IHCP, DG JRC, Ispra, Italy, 33 p.

Perdu-Durand E, Demmerle S, Cravedi JP. 2004. *In vitro* biotransformation rates of surfactants in carp and rainbow trout liver subcellular fractions. ERASM report, 28 p. www.erasm.org

Potts RO, Guy RH, eds. 1997. *Mechanisms of transdermal drug delivery*. Marcel Dekker, New York, NY, USA.

Proudfoot JR. 2005. The evolution of synthetic oral drug properties. *Bioorganic and Medicinal Chemistry Letters* 15:1087-1090.

Rekker RF, Mannhold R. 1992. Calculation of drug lipophilicity. Weinheim, Germany: VCH. (Cited at www.voeding.tno.nl/ProductSheet.cfm?PNR=037e)

Russell WMS, Burch RL. 1959. The principles of humane experimental technique. London, UK: Methuen.

Schmieder P, Tapper M, Linnum A, Denny J, Kolanczyk R, Johnson R. 2000. Optimization of a precision-cut trout liver tissue slice assay as a screen for vitellogenin induction: comparison of slice incubation techniques. *Aquat Toxicol* 49:251-268.

Schrap SM, Opperhuizen A. 1990. Relationship between bioavailability and hydrophobicity: Reduction of the uptake of organic chemicals by fish due to the sorption on particles. *Env Toxicol Chem* 9:715-724.

Segner H, Cravedi J. 2001. Metabolic activity in primary cultures of fish hepatocytes. *ATLA* 29:251-257.

Segner H. 1998. Isolation and primary culture of teleosts hepatocytes. *Comp Biochem Physiol* Part A 120:71-81.

Sijm DTHM. 2004. Personal communication to some members of the TCNES sub-group on PBTs.

Sijm DTHM, Hermens JLM. 1999. Internal effect concentrations: Link between bioaccumulation and ecotoxicity for organic chemicals. In: Beek B, editor, The handbook of environmental chemistry, Vol 2-J. Bioaccumulation: new aspects and developments. Berlin, Germany: Springer-Verlag, p 167-199.

Sijm DTHM, Wever H, Opperhuizen A. 1993. Congener-specific biotransformations and bioaccumulation of PCDDs and PCDFs from fly ash in fish. *Environ Toxicol Chem* 12:1895-1907.

Sijm DTHM, Verberne ME, de Jonge WJ, Pärt P, Opperhuizen A. 1995. Allometry in the uptake of hydrophobic chemicals determined *in vivo* and in isolated perfused gills. *Toxicol Appl Pharmacol* 131:130-135.

Sijm DTHM, de Bruijn J, de Voogt P, de Wolf W, eds. 1997. Biotransformation in environmental risk assessment. SETAC Europe, Brussels, Belgium.

Sijm DTHM. 1991. Extrapolating the laboratory results to environmental conditions. In: Nagel R, Loskill R, editors, Bioaccumulation in aquatic systems. Weinheim, Germany: VCH Verlagsgesellschaft, p 151-160.

Sinko PJ, Lee YH, Makhey V, Leesman GD, Sutyak JP, Yu H, Perry B, Smith CL, Hu P, Wagner EJ, Falzone LM, McWhorter LT, Gilligan JP, Stern W. 1999. Biopharmaceutical approaches for developing and assessing oral peptide delivery strategies and systems: *in vitro* permeability and *in vivo* oral absorption of salmon calcitonin (sCT). *Pharm Res* 16:527-533.

Södergren A. 1987. Solvent-filled dialysis membranes simulate uptake of pollutants by aquatic organisms. *Environ Sci Technol* 21:855-859.

Thompson RS, Stewart KM. 2003. Critical Body Burdens: A review of the literature and identification of experimental data requirements. BL7549/B, Brixham Environmental Laboratory, AstraZeneca Ltd, Brixham (Devon), UK.

Tolls J, de Graaf I, Thijssen MATC, Haller M, Sijm DTHM. 1997. Bioconcentration of LAS: Experimental determination and extrapolation to environmental mixtures. *Environ Sci Technol* 31:3426-3431.

Tolls J, Haller M, Labee E, Verweij M, Sijm DTHM. 2000. Experimental determination of bioconcentration of the nonionic surfactant alcohol ethoxylated. *Environ Toxicol Chem* 19:646-653.

Trischitta F, Denaro MG, Faggio C. 1999. Effects of acetylcholine, serotonin and noradrenalin on ion transport in the middle and posterior part of *Anguilla anguilla* intestine. *J Comp Physiol* B 169:370-376.

UK. 1986. Guidance on the Operation of the Animals (Scientific Procedures) Act 1986, Presented to Parliament by the Secretary of State for the Home Department

pursuant to Act Eliz. II 1986, C.14 Section 21, (Animals (Scientific Procedures) Act 1986). *Ordered to be printed by the House of Commons, 23 March 2000.*

UK. 1993. Statutory Instrument 1993 No. 2103, The Animals (Scientific Procedures) Act (Amendment) Order 1993. London, UK: The Stationery Office Limited.

UN. 2003. The globally harmonized system of classification and labelling of chemicals. UNECE (United Nations Economic Commission for Europe), Geneva, Switzerland. Available at www.unece.org/trans/danger/publi/ghs/ghs_rev00/00files_e.html

USA. 1966. Animal Welfare Act, Public Law 89-544 Act of August 24, 1966, 89th Congress, H. R. 13881, Act enabled 1966 and updated 1970, 1976, 1985, 1990. Annual updates available at www.aphis.usda.gov/ac/publications.html

US EPA. 1999. Category for persistent, bioaccumulative and toxic new chemical substances. Feb Reg 64:60194-60204. United States Environment Protection Agency, Office of Research and Development, Washington (DC), USA.

Vaes WHJ, Hamwijk C, Urrestarazu Ramos E, Verhaar HJM, Hermens JLM. 1996. Partitioning of organic chemicals to polyacrylate-coated solid phase microextraction fibers: Kinetic behavior and quantitative structure-property relationships. *Anal Chem* 68:4458-4462.

Vaes WHJ, Urrestarazu Ramos E, Hamwijk C, van Holsteijn I, Blaauboer BJ, Seinen W, Verhaar HJM, Hermens JLM. 1997. Solid phase microextraction as a tool to determine membrane/water partition coefficients and bioavailable concentrations in *in vitro* systems. *Chem Res Toxicol* 10:1067-1072.

Van Veld PA, Westbrook DJ, Woodin BR, Hale RC, Smith CL, Huggett RJ, Stegeman JJ. 1990. Induced cytochrome P-450 in intestine and liver of spot (*Leiostomus xanthurus*) from a polycyclic aromatic hydrocarbon contaminated environment. *Aquat Toxicol* 17:119-132.

Van Wezel AP, Opperhuizen A. 1995. Narcosis due to environmental pollutants in aquatic organisms: residue-based toxicity mechanisms and membrane burdens. *Critical Reviews in Toxicology* 25:255-279.

Veith GD, DeFoe DL, Bergstedt BV. 1979. Measuring and estimating the bioconcentration factor of chemicals in fish. *J Fish Res Board Can* 36:1040-1048.

Vieth M, Siegel MG, Higgs RE, Watson IA, Robertson DH, Savin KA, Durst GL, Hipskind PA. 2004. Characteristic physical properties and structural fragments of marketed oral drugs. *J Med Chem* 47:224-232.

Wenlock MC, Austin RP, Barton P, Davis AM, Leeson PD. 2003. A comparison of physiochemical property profiles of development and marketed oral drugs. *J Med Chem* 46:1250-1256.

Wood C, Pärt P. 1997. Cultured branchial epithelia from freshwater fish gills. *J Exp Biol* 200:1047-1059.

Wood CM, Kelly SP, Zhou B, Fletcher M, O'Donnell M, Eletti B, Pärt P. 2002. Cultured gill epithelia as models for the freshwater fish gill. *Biochim Biophys Acta - Biomembranes* 1566:72-83.

List of Tables and Figures:

Table 1: Minimum number of fish sampled in a bioconcentration test

OECD 305 bioconcentration: flow-through test (OECD 1996b)				
	Uptake phase		Depuration phase	Total
Number of fish per sampling occasion	4		4	
Number of sampling occasions	5		4	
Subtotal		*20*		*16*
Number of exposure and control groups	3		3	
Subtotal		*60*		*48*
				108

Abbreviated OECD 305 study (Hinderleiter 2004)				
	Uptake phase		Depuration phase	Total
Number of fish per sampling occasion	4		4	
Number of sampling occasions	2		4	
Subtotal		*8*		*16*
Number of exposure and control groups	2		2	
Subtotal		*16*		*32*
				48

Static bioaccumulation study (Banerjee et al. 1984; de Wolf et al. 1998)				
	Uptake phase		Depuration phase	Total
Number of fish per sampling occasion	10		0	
Number of sampling occasions	1		0	
Subtotal		*10*		*0*
Number of exposure and control groups	2		0	
Subtotal		*20*		*0*
				20

Dietary bioaccumulation study (Parkerton et al. 2001)				
	Uptake phase		Depuration phase	Total
Number of fish per sampling occasion	0		4	
Number of sampling occasions	0		5	
Subtotal		*0*		*20*
Number of exposure and control groups	0		2	
Subtotal		*0*		*40*
				40

Table 2: Tissue absorption potentials

Tissue	**TEER in Ω cm2**	**References**
Fish intestine	25-50	Trischitta et al. (1999)
Mammal intestine	20-100	Okada et al. (1977); Sinko et al. (1999)
Blood-brain barrier	400-2000	Borchardt et al. (1996)
Fish gill	3500	Wood et al. (1997)
Human skin	20,000	Potts et al. (1997)

Table 3: Summary of various ranges re CBB - lethality (mmol/kg ww)

Mode of action and source	Narcosis	AChE inhibitors	Respiratory inhibitors
Sijm (2004)	2	0.01	0.001
Thompson et al. (2003)	2 - 8	0.000001 - 10	0.000001 - 10
Barron et al. (2002)	0.03 - 450	0.00004 - 29	0.00002 - 1.1 (CNS seizure agents)
McCarty et al. (1993)	1.7 - 8	0.05 - 2.7	0.00005 - 0.02 (CNS seizure agents)

Sijm (2004) - an expert judgement view to arrive at an approximate single value based on 3 references(McCarty et al. 1993; Van Wezel et al. 1995; Sijm et al. 2000).

Thompson et al. (2003) - based on a literature review, the data range, beyond the narcosis mode of action, has been drawn from this report.

Barron et al. (2002) - based on Fig. 10 of Barron et al. (2002).

REPLACEMENT

No animals used

Tier I:

a) Physico-chemical analyses; literature search

b) Computer models to estimate ADME properties and BCF

Tier II:

In vitro assays to evaluate ADME properties

REDUCTION

Fewer animals used

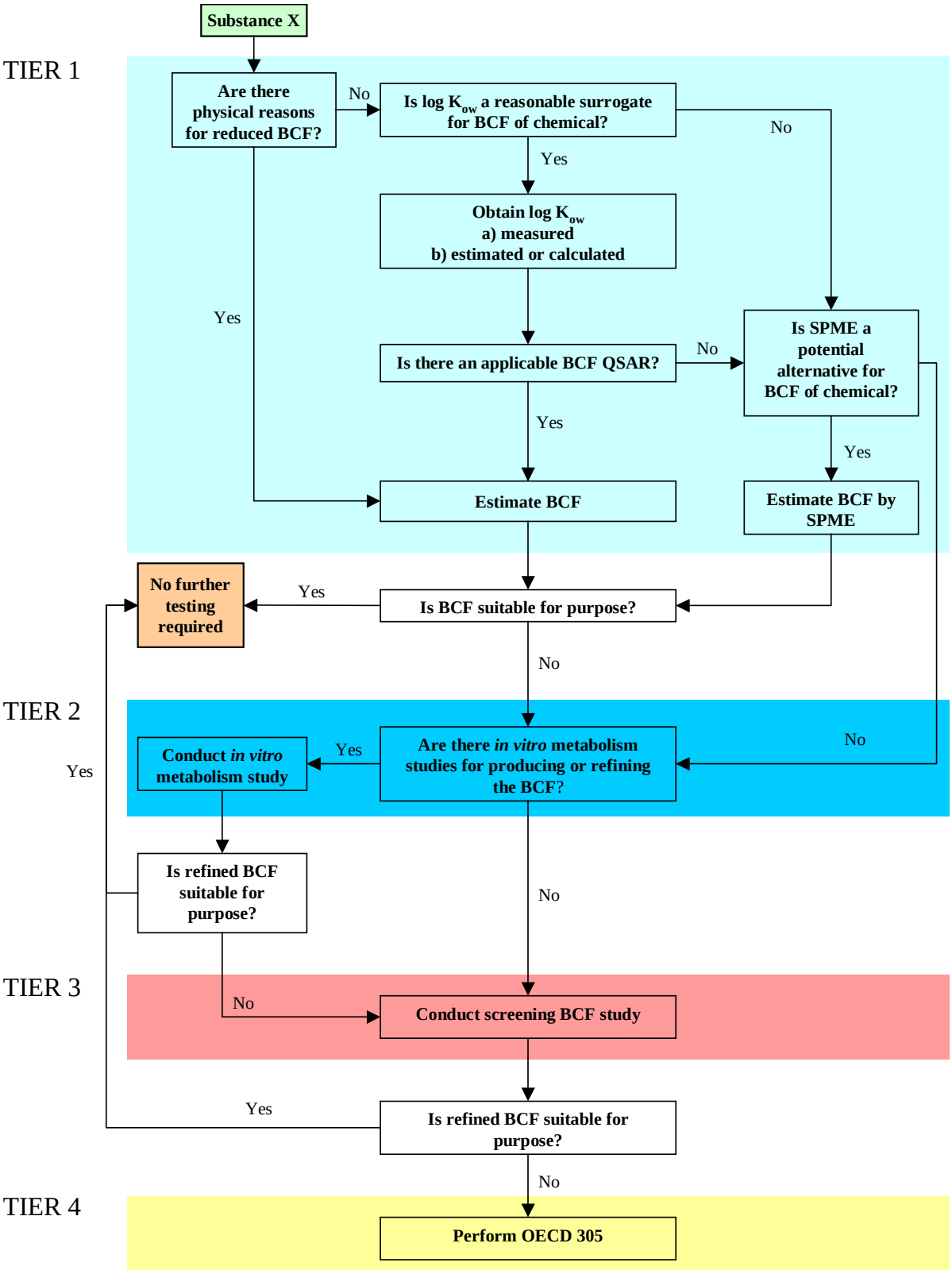
Tier III:

Modified *in vivo* methods to measure bioconcentration

108 animals used

Tier IV:

Gold standard OECD TG 305


Substance X
TIER 1
Are there physical reasons for reduced BCF?
No
Is log K_{ow} a reasonable surrogate for BCF of chemical?
No
Yes
Obtain log K_{ow}
a) measured
b) estimated or calculated
Yes
Is there an applicable BCF QSAR?
No
Is SPME a potential alternative for BCF of chemical?
Yes
Yes
Estimate BCF
Estimate BCF by SPME
No further testing required
Yes
Is BCF suitable for purpose?
No
TIER 2
Conduct *in vitro* metabolism study
Yes
Are there *in vitro* metabolism studies for producing or refining the BCF?
No
Yes
Is refined BCF suitable for purpose?
No
TIER 3
No
Conduct screening BCF study
Yes
Is refined BCF suitable for purpose?
No
TIER 4
Perform OECD 305