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

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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 Organization for Economic Cooperation and Development (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 use read-across, chemical grouping, and quantitative structure–activity relationships with an assessment of the key processes in bioconcentration: Adsorption, distribution, metabolism, and excretion. 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 bioaccumulative (B) categorization schemes are provided. Assessment of biotransformation with in vitro assays and in silico approaches holds significant promise. Further research is needed to identify their variability and confidence limits and the ways to use this as a basis to estimate bioconcentration factors. 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

The use of 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 nonanimal alternative approaches of testing should be used in the place of animal procedures wherever possible. Council Directive 86/609/EEC (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 Replace, Reduce, and Refine principles (3Rs). “Replacement” means the substitution of insentient material for conscious living higher animals. “Reduction” means reduction in the number 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 that 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 Relevance (meaningfulness and usefulness of a test for a particular purpose), Reliability (reproducibility of results within and between laboratories), and Regulatory acceptability (suitability of a test for risk assessment purposes [human health/environment]).

Fish are typically secondary consumers or predators and therefore considered to represent a high trophic level and to be organisms of choice for assessing the bioconcentration potential of chemicals in aquatic organisms. Because 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 Organization for Economic Cooperation and Development Test Guideline 305 (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 with the organic chemical’s physicochemical properties (log of the octanol–water partitioning coefficient \(\log K_{ow}\)). This screening procedure assumes no substantial bioconcentration for compounds with log \(K_{ow}<3\). Above log \(K_{ow}=6\), nonlinear relationships can be applied, and in most of these cases, a chemical-by-chemical evaluation is more appropriate (Nendza 1991). The quantitative structure–activity relationship (QSAR) approach on the basis of log \(K_{ow}\) is not reliable for all chemical classes: surface active agents, organic colorants (ECETOC 1998), or lipophilic chemicals that are biotransformed (de Wolf et al. 1992), for example.

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

The European Commission has recently adopted a draft legislative text describing the registration, evaluation, authorization, 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). Nonanimal testing is promoted in REACH, although strategies for the use of alternative information methodologies have not been spelled out. However, such strategies are needed not only to achieve the 3Rs, but to keep the REACH testing costs at manageable levels.

The main objective of this paper is to present a bioconcentration testing strategy on the basis of the work of a European Centre for Ecotoxicology and Toxicology of Chemicals (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). This act defines a “protected animal” as any living vertebrate, other than man (Section I[1] of the Act). This was extended to the invertebrate species *Octopus vulgaris* via an amendment (Animals [Scientific Procedures] Act 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 *O. vulgaris*, from the time at which they become capable of independent feeding. Several other definitions for an animal do exist (e.g., Animal Welfare Act 1966; EEC 1986) but will not be used here.

It is more important that the spirit of the 3Rs is applied than 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 (Guidance on the Operation of the Animals [Scientific Procedures] Act 1986). As a consequence, use of the organs/cells would constitute 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 according to Hodgeson and Levi (1994). Absorption is transport across a biological membrane into systemic circulation (e.g., across fish gills, intestine, skin) after the introduction of a chemical through food, water, air, sediment, or soil. After absorption, a chemical can 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 can be distributed to a tissue and elicit a toxic response; other tissues might serve as permanent sinks (e.g., fat) or as temporary depots, allowing for slow release into circulation. After reaching a tissue, enzymes can biotransform the chemical. During phase I biotransformation reactions, a polar group is introduced into the molecule that 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 is eliminated from the body through endogenous waste. Chemicals can be exhaled directly through the gills or broken down (biotransformed) and ultimately exhaled as CO₂. Polar molecules that are freely soluble in plasma can be removed through renal filtration and passed into urine. Lipophilic (fat-soluble) chemicals can be conjugated and excreted in bile (feces). In addition to excretion, growth of the organism might also be relevant, affecting the chemical concentration in the organism in the case in which the rate of other excretion processes is on 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 BCFs 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 sub lethal concentrations of the test substance. During exposure, both fish and water are sampled at regular time intervals, and the concentration of the (parent) test substance is measured. During the 1st 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 can also be expressed as a function of total lipid weight. Specific chemical analysis and radiotracer techniques can 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 on at least 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 1-compartment model, bioconcentration can be described with a simple 1st-order kinetic model

\[ C_t = C_w \times k_u/k_d (1 - e^{-k_d t}) \]

where \( C_t \) is the substance concentration in fish (mg/g wet fish), \( C_w \) is the substance concentration in water (mg/L), \( k_u \) is the uptake clearance (mL/[g wet fish]^{-1}•d^{-1}), \( k_d \) is the elimination rate constant (L/d), and \( t \) is the exposure time (d). 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, 1st-order 1-compartment kinetics have been found to adequately describe bioconcentration (Kristensen and Tyle 1991; Sijm 1991).

Hence, 2 different methods are used to evaluate BCF. The 1st is to calculate it from the concentration of a chemical in fish divided by the concentration in water (under steady-state conditions). The 2nd method uses kinetic data (i.e., uptake clearance and elimination rate).

\[ \text{BCF} = k_u/k_d = C_t/C_w \]

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 interlaboratory variation (Kristensen and Tyle 1991). This is further improved when a correction for the bioavailable fraction in water is made (Schrap and Oppenhuizen 1990), that is, 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 artifacts in the testing and in the interpretation of the BCFs from these studies. These artifacts might include difficulties in measuring the true aqueous concentration because of sorption of the substances to particulate and dissolved (organic) matter, adsorption processes to glass walls or other materials, and volatilization, for example (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 and Mackay 1987).

The uptake clearance (\( k_u \) mL/[g wet fish]^{-1}•d^{-1}) is relatively constant between a 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 \( K_{ow} > 6 \), some evidence suggests that the uptake clearance might decline with increasing hydrophobicity.

An estimate of the rate of depuration \( k_d \) can 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 and Connell 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 \( k_m \) is elimination by biotransformation.

Because many substances that bioconcentrate distribute themselves within the organism’s body into the fat or lipids, the 1st estimation that can 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), whereas for chemicals with log \( K_{ow} > 6 \), a parabolic equation, recalculated from that described by Connell and Hawker (1988), is recommended. In general (Q)SAR models should only be used for those chemicals that 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 that use log \( K_{ow} \) because it is not an appropriate physicochemical descriptor for such materials. Metals also fall outside most QSAR models because active uptake and sequestration can occur in biological systems. In cases in which 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 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% of high production volume chemicals with log \( K_{ow} > 2.7 \) (Beek 1991), the number of chemicals qualifying for BCF testing is calculated to be 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**

Reduction of animal use can be achieved by exposing fewer 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 (P. Hinderleiter, personal communication). Unlike the standard OECD test, steady state does not need to be achieved. This design can lower animal usage by approx-
approximately 55% (Table 1). Benefits would further include lower cost, faster execution, less waste, and less chemical used.

Another approach to reducing the number of animals used in OECD 305 depends on 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 might not be necessary, reducing 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 and Lieder 1995). It requires the exposure of fish to an aqueous solution of the substance under static conditions and measurement of the loss of substance from the exposure system as a function of time. The rate constants are obtained by fitting the time–concentration profile to a simple mathematical model describing the exchange of substance between fish and water. The original approach of Banerjee et al. (1984) measured the substance in water and assumed that removal processes such as biotransformation, sorption, and volatilization are not likely to occur. De Wolf and Lieder (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 compared with the OECD 305 study (Table 1).

A mathematical analysis of the robustness of static exposure systems (de Wolf and Lieder 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.

### Table 1. Minimum number of fish sampled in a bioconcentration test

<table>
<thead>
<tr>
<th></th>
<th>Uptake phase</th>
<th>Depuration phase</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>OECD 305 bioconcentration: flow-through test (OECD 1996b)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nr of fish per sampling occasion</td>
<td>4</td>
<td>4</td>
<td></td>
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<tr>
<td>Nr of sampling occasions</td>
<td>5</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Subtotal</td>
<td>20</td>
<td>16</td>
<td>108</td>
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<tr>
<td>Nr of exposure and control groups</td>
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<td>3</td>
<td></td>
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<tr>
<td>Subtotal</td>
<td>60</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td><strong>Abbreviated OECD 305 study (Hinderleiter 2004)</strong></td>
<td></td>
<td></td>
<td>48</td>
</tr>
<tr>
<td>Nr of fish per sampling occasion</td>
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<td>4</td>
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<td>Nr of sampling occasions</td>
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<td></td>
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<tr>
<td>Subtotal</td>
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<td>16</td>
<td></td>
</tr>
<tr>
<td>Nr of exposure and control groups</td>
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<td>2</td>
<td></td>
</tr>
<tr>
<td>Subtotal</td>
<td>16</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td><strong>Static bioaccumulation study (Banerjee et al. 1984; de Wolf and Lieder 1995)</strong></td>
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<td>Nr of fish per sampling occasion</td>
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<tr>
<td>Nr of exposure and control groups</td>
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<td></td>
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<tr>
<td>Subtotal</td>
<td>20</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><strong>Dietary bioaccumulation study (Parkerton et al. 2001)</strong></td>
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<td>40</td>
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<tr>
<td>Nr of sampling occasions</td>
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<tr>
<td>Nr of exposure and control groups</td>
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<td>Subtotal</td>
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period, even in cases in which (limited) loss occurs because of sorption and biotransformation. Further research comparing empirical data for metabolized 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 might provide information helpful for estimation of the BCF. 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 period over a specified period of time depending on the expected half-life ($T_{1/2}$; Parkerton et al. 2001). At the end of this dietary exposure period, some fish are analyzed for parent substance (time = 0 of the depuration phase). The remaining fish are transferred to a clean diet and sequentially sampled and analyzed over time so that a depuration curve could be established. From these data, the half-life, dietary assimilation efficiency, and bioaccumulation factor (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 is 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 in which 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 modeling, biomimetic or surrogate approaches, and 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 the use of information on 1 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 evaluated extensively 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 with a combination of these techniques for estimating bioconcentration (Pedersen et al. 2003).

On the basis of 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 with the substance’s log $K_{ow}$ and correction factors that 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 rationalized on the basis that they were related to some degree of reactivity or known biotransformation behavior.

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 with the use of 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 (2003) to categorize discrete organic substances on the Canadian domestic substances list for bioaccumulation potential.

Another approach aims to address biotransformation starting from 1st principles (Dimitrov, Dimitrova, et al. 2002; Dimitrov, Mekenyan, et al. 2002; Dimitrov et al. 2006). Here, BCF is 1st modeled as a maximum value, ignoring any mitigating factors and based only on log $K_{ow}$ as an indicator of partitioning behavior. 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).

Söödergren (1987) described a system based on a semi-permeable membrane device (SPMD) composed of a dialysis bag filled with hexane that has been further developed with the use of low-density polyethylene bags that contain natural lipids or the lipid model trilienolein (1,2,3-tri[9-(octadecenyl)]glycerol; see Huckins et al. 1997) to mimic the way organisms extract chemicals from water (i.e., biomimetic extraction). Semi-permeable membrane devices 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 fewer than 2 months should be treated with caution (Booij et al. 1998).

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

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

The physiological processes that govern bioconcentration in invertebrates can 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 recommended routinely. However, if the need is only to demonstrate that the BCF in fish is below a certain value, then it might be possible to use BCFs from invertebrates as conservative estimations of the BCF in fish. As analogs 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 physicochemical characteristics that influence solubility and absorption across the intestinal lumen with the use of more than 2,200 drug development tests. These characteristics have been reviewed rigorously (Wenlock et al. 2003; Proudfoot 2005), have been 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.

Lipinski’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, more than 10 H bond acceptors, molecular weight > 500, and log $K_{ow}$ > 5 (Lipinski et al. 1997). Wenlock et al. (2003) studied about 600 additional chemicals and found that 90% of the absorbed compounds had fewer than 4 H bond donors, fewer than 7 H bond acceptors, molecular weight < 473, and log $K_{ow}$ < 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 by transepithelial electrical resistance (TEER) and can be used to compare tissue capabilities. A low TEER value indicates the tissue has greater absorption potential. Although the studies by Lipinski et al. (1997), Wenlock et al. (2003), Vieth et al. (2004), and Proudfoot (2005) focused on absorption across the intestinal lumen, the more restrictive TEER for fish gills (Table 2) implies that the equations and concepts can be reapplied to conservatively estimate absorption in fish.

**Molecular weight**—Several values have been suggested for the molecular weight (MW) cutoff for absorption across fish tissues. The EU Technical Guidance Document (EC 2003) indicates that molecules with MW > 700 g/mol are less likely to be absorbed and bioconcentrate, whereas the US Environmental Protection Agency (USEPA), exempts chemicals with MW > 1,100 g/mol in the persistence, bioaccumulation, and toxicity (PBT) assessment conducted under the Toxic Substances Control Act (USEPA 1999). Anliker et al. (1988) suggested that a pigment could be excluded from a fish bioaccumulation test if it has both MW > 450 and a cross section greater than 1.05 nm (as the 2nd smallest van der Waals diameter or $C_{vdw}$) 

Rekker and Mannhold (1992) suggested that a calculated log $K_{ow}$ > 8 can be used on its own or in combination with MW > 700–1,000 to conclude (with confidence) that the compound is unlikely to bio-accumulate. Although experimental evidence for a molecular weight cutoff is limited, Burreau et al. (2004) did demonstrate reduced bioconcentration and no biomagnification for high molecular weight polybrominated diphenyl ethers (PBDEs) with 6 or more bromines and MW = 644–959.

Considering that molecular size and shape vary with MW, molecular weight alone is insufficient to allow absorption predictions. However, it does suggest that once MW is in the region of 700–1,100, depending on other factors, a reduced BCF might be expected. Hence, although recognizing the uncertainties in the interpretation of experimental results, we recommend that to demonstrate a reduced BCF, a substance should have either

- MW > 1,100 g/mol or
- MW = 700–1,100 g/mol with other indicators (see later discussion).

**Molecular size**—Molecular size can be considered in 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 naphthalene 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, whereas the non–laterally substituted were not. The main reason for this was attributed to metabolism (previously reported by Opperhuizen and Sijm 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 and Moser (1987) studied the limits of bioconcentration of azo pigments in fish and their relation to the partition coefficient and 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, as 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

<table>
<thead>
<tr>
<th>Tissue</th>
<th>TEER $\Omega$ cm$^2$</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fish intestine</td>
<td>25–50</td>
<td>Trischitta et al. (1999)</td>
</tr>
<tr>
<td>Mammal intestine</td>
<td>20–100</td>
<td>Okada et al. (1977); Sinko et al. (1999)</td>
</tr>
<tr>
<td>Blood-brain barrier</td>
<td>400–2,000</td>
<td>Borchardt et al. (1996)</td>
</tr>
<tr>
<td>Fish gill</td>
<td>3,500</td>
<td>Wood and Pärt (1997)</td>
</tr>
<tr>
<td>Human skin</td>
<td>20,000</td>
<td>Potts and Guy (1997)</td>
</tr>
</tbody>
</table>

**Table 2. Tissue absorption potentials as measured by transepithelial electrical resistance (TEER)**
effective permeation through biological membranes and thus limit their uptake during the time of exposure. Aniker proposed that a 2nd largest cross section of more than 1.05 nm with MW > 450 would suggest a lack of bioconcentration for organic colorants.

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; Oppenhuizen et al. 1987; Morris et al. 2004). Therefore, a simple parameter might 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 molecular weight, size, and flexibility in their BCF estimates.

Dimitrov, Mekenyan, and Walker (2002) found that for compounds with \( \log K_{ow} > 5.0 \), 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 < 2,000. This critical value was found to be comparable to the architecture of the cell membrane (i.e., half the thickness of the lipid bilayer of a cell membrane). This is consistent with a possible switch in uptake mechanism from passive diffusion through the bilayer to facilitated diffusion or active transport. In a later paper, Dimitrov et al. (2003) used this parameter to assess experimental data on a wide range of chemicals. The conclusion was that a chemical with a maximum cross-sectional diameter larger than 4.3 nm would not have BCF > 5,000; that is, it would not meet the EU PBT criteria for vB chemicals (EC 2003). In unpublished work after further assessment of their data set, they have changed this value to 1.74 nm (S D Dimitrov, Bouras As, Zlatarov University, Bourgas, Bulgaria, personal communication).

Earlier, Oppenhuizen et al. (1987) proposed that a substance with an effective molecular length of more than 4.3 nm would not pass membranes, either in the gills or in the gut, at all, on the basis of a series of bioaccumulation and bioconcentration studies with linear and cyclic polydimethylsiloxanes (silicones) varying in chain length. Membrane crossing is very unlikely because such large molecules would disturb the entire interior structure of the lipid membrane. Molecular weight did not explain reduced uptake because 1 of the substances with MW = 1,050 was detected in fish. The cross-sectional diameter of siloxanes could in itself not explain the reduced uptake because these diameters were smaller or equal to those of PCBs that did bioaccumulate strongly.

Oppenhuizen et al. (1987) also referred to a study by Hardy et al. (1974) in which uptake in codlings of long-chain alkanes was disturbed for alkanes with corresponding molecular lengths of more than 4.3 nm. Tolls et al. (2000) observed uptake in fish of some nonionic surfactants with an apparent equal length to long-chain alkanes, which seems contradictory to the earlier proposed cutoff molecular length by Oppenhuizen et al. (1987). However, the uptake of the long nonionic surfactants could be explained by internal molecular flexibility reducing the effective molecular length below 4.3 nm.

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

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

Other indicators—There are other indicators for low uptake that could also be used to suggest that a chemical, despite having \( \log K_{ow} > 4.5 \), 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 favorable uptake properties and to evaluate structure–absorption relationships.

Wood and Pärt (1997) developed a method for the primary culture of gill epithelial cells from freshwater rainbow trout. Application in quantitative analysis of chemical transport across membranes is currently limited because primary epithelial cells are used, which increases the possibility of high interexperiment variability (Wood et al. 2002).

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

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

Pärt (1990) developed a perfused gill preparation from rainbow trout (Oncorhynchus mykiss) as an alternative for studies in vivo. The perfused gill allows direct measurements of in vivo absorption rates of chemicals across the gill epithelium (Pärt et al. 1992). Uptake rate constants of different classes of hydrophobic organic chemicals determined in isolated perfused gills of rainbow trout (O. mykiss) are higher than those determined in guppy (Poecilia reticulata) in vivo (Sijm et al. 1995). Both systems show relatively high variation; however, this can be reduced significantly and the uptake rate constants determined once they are normalized with a reference chemical. Subsequent extrapolation to fish of different sizes can be through use of allometric relationships (Sijm et al. 1995; 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—1st, that octanol is a reasonable surrogate for fish lipids, and 2nd, that a substance with reduced solubility in octanol could 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 with log $K_{ow}$. When a substance has a low solubility in octanol ($S_{oc}$) as well as a low solubility in water ($S_{w}$), the resulting ratio $S_{oc}/S_{w}$ could range from very low to very high, with no clear idea on how this would affect the magnitude of 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.

Chesells 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 and Baughman (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 in organisms that would give rise to concern. The concept of critical body burdens (CBBs) for acute effects is reasonably well established (McCarty 1986; McCarty and Mackay 1993), especially for chemicals that act by the lowest and most specific mode of action. 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 and hence sufficiently bioaccumulative to be of immediate concern. The choice is therefore pragmatic but protective.

Lipid normalizing the chosen CBB of 0.001 mmol/kg wet wt, and assuming a lipid content of 5%, gives a lipid normalized CBB of 0.02 mmol/kg lipid or 0.02 × MW (mg/L lipid). However, given the uncertainty involved, it is suggested to introduce an application factor of 10 to account for species differences and organ versus body differences.

On the basis of the above, it is proposed that for a chemical with a solubility of less than (0.002 × MW) mg/L in octanol it should be assumed that the compound has only a limited potential to establish high body burdens and to bioaccumulate. If it does bioaccumulate, it would be unlikely to give rise to levels in biota that would cause significant effects.

**Increased elimination/depuration**—De Wolf et al. (1992) demonstrated a significant reduction in the bioconcentration of chlorinated anilines compared with log $K_{ow}$–based predictions, which was attributed to increased elimination via biotransformation. In vitro assays can provide information on both the range of metabolites as well as their relative importance and provide data useful for input into fish-specific physiologically based pharmacokinetic modeling efforts. Several types of studies are available that assess the influence of biotransformation on the BCF in fish, such as measuring the decrease of parent compound (mass balance approach, e.g., Opperhuizen 1986), comparison of total elimination of bioaccumulative and non-bioaccumulative 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 and Celander 2003). Because 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 (Segner 1998; Cravedi et al. 2001; Segner and Cravedi 2001; Dyer and Bernhard 2004). The xenobiotic

<table>
<thead>
<tr>
<th>CBB lethality (mmol/kg wet wt) versus mode of action</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Narcosis</td>
<td>AChE inhibitors</td>
</tr>
<tr>
<td>2</td>
<td>0.01</td>
</tr>
<tr>
<td>2–8</td>
<td>0.000001–10</td>
</tr>
<tr>
<td>0.03–450</td>
<td>0.00004–29</td>
</tr>
<tr>
<td>1.7–8</td>
<td>0.05–2.7</td>
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Table 3. Summary of CBB lethality concentration ranges for different modes of action

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<table>
<thead>
<tr>
<th>CBB lethality (mmol/kg wet wt)</th>
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</tr>
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<tbody>
<tr>
<td>Narcosis</td>
<td>0.03–450</td>
</tr>
<tr>
<td>AChE inhibitors</td>
<td>0.00001–10</td>
</tr>
<tr>
<td>Respiratory inhibitors</td>
<td>0.00002–1.1</td>
</tr>
<tr>
<td>Toxicity</td>
<td>0.00005–0.02</td>
</tr>
</tbody>
</table>

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*References*

AChE inhibitors

- Respiratory inhibitors

- Narcosis

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metabolite pattern produced by fish hepatocytes in vitro is generally similar to that observed in vivo (Segner and Cravedi 2001).

Biotransformation is strongly taxa- and species-specific, perhaps because of 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: 1) Compounds that are poorly biotransformed as a general rule, 2) compounds that are poorly biotransformed by specific organisms/groups (e.g., polycyclic aromatic hydrocarbon in mussels), and 3) compounds that 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 the amount of substrate (parent compound), or by an increase in products. The faster the rate of parent biotransformation, the less likely it is that the chemical will bioaccumulate, with the influence of biotransformation on the overall elimination and BCF value more pronounced for hydrophobic chemicals (de Wolf et al. 1992). However, because of the lack of data, there are no generally accepted approaches to use the in vitro rates to estimate potential BCFs. This is an area for further research.

Dyer et al. (2003) applied an approach to derive a BCF_{cell} for various surfactants using carp primary hepatocytes and cultured hepatocytes (PLHC-1 cells). The rates of uptake and loss of the test chemical from the cellular systems were estimated assuming first-order kinetics and the BCF in the cells determined by a ratio of uptake rate to the rate of loss. For linear alkylbenzene sulfonate, the calculated BCF_{cell} were approximately 4-fold less than the BCF_{fish} measured by Tolls et al. (1997) in vivo in fathead minnow. For the linear alcohol ethoxylate (C_{13}EO_{8}), the corresponding BCF_{cell} were 2- to 30-fold less compared with the fathead minnow results generated by Tolls et al. (2000).

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

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 biocaccumulation. On this basis, an integrated bioconcentration testing strategy can be developed taking into account alternative approaches, including existing data (e.g., read-across and extrapolation), QSARs, and 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 uses nonanimal experimental systems. Depending on 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 the purpose?” (Figure 2). The purpose of this question is to ensure that the BCF being generated is either sufficiently accurate so that an assessment of indirect exposure can be conducted or regulatory decisions can be made with sufficient confidence. Clearly the closer a BCF estimate or measured value is to a boundary, either defined by a regulatory 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 judgment, the variability that occurs even with OECD 305 should be considered.

Tier 1

A—The 1st 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 with regard to suitability of the estimate for its intended purpose.

B—If absorption does not seem to be restricted and biotransformation appears unlikely then the 2nd 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 below. 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 BCF.

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 biot measurement systems (i.e., invertebrates). From this measurement, an estimation of a fish BCF is obtained. The confidence in the information is again addressed in the central question with regard to 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 with significant uncertainty or without 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 behavior, then go to tier 3.

D—The assessment at this point addresses to what extent biotransformation would affect 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 is assessed.
Tier 3
Without an argument for restricted uptake and with no viable surrogates for partitioning behavior available, testing is required. It is suggested that a fish BCF is estimated by in vivo screening tests before moving to a BCF measurement according to the OECD 305 test guideline (tier 4). If the estimate from the in vivo screening assays is suitable for the purpose, 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 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, should be tested to determine whether it is B/v, or both.

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 on 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 to replacing animals. Reduction measures, although still making use of a limited number of fish, can already be applied or might need rapid development for short-term application. In the mid to longer term, research programs 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 alternative 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.
Figure 2. Integrated testing strategy to assess fish bioconcentration.
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 might 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 standardizing protocols, recommended procedures for the isolation of fish cells, culture, and exposure should be agreed upon and should be in compliance with the Good Cell Culture Practices (Hartung et al. 2002; Coecke et al. 2005). 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 1st step could be to evaluate whether the mammalian intestinal cells (Caco-2 cells) are representative of fish for understanding gill absorption and uptake from food and for deriving assimilation factors. Future research is needed to further assess the effect of gill biotransformation in the absorption process. In addition, generation of information that provides more insight into the validity of extrapolation from existing approaches to fish, the development of fish-specific absorption models, or both is required.

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

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

The existing (Q)SARs that address biotransformation in fish need to be improved or further developed. The available in vitro biotransformation assays with sub-/cellular fish liver systems to address metabolism should be further investigated. To allow the use of relevant information, the level of biotransformation potential in the different in vitro systems with the use of different fish species or classes of organisms should be compared. The level of biotransformation potential in vitro should be compared with 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 and 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.

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.

Acknowledgment—The authors thank the members of the EU Technical Committee Working Group on PBT substances, specifically Steve Robertson.

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