ILSI HESI / JRC / SETAC-EU Workshop on Bioaccumulation Assessments

May 5-6, 2006

Dutch Congress Centre
The Hague, The Netherlands

Organized by:

ILSI Health and Environmental Sciences Institute (HESI)
The Joint Research Centre (JRC)
SETAC-Europe

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International Life Sciences Institute (ILSI)

ANTITRUST STATEMENT

The Branches and Institutes of ILSI, including their respective Boards of Trustees, Scientific Advisors, Scientific Directors, Members, Committees, Subcommittees, Task Forces, and Working Groups, meet to promote understanding and resolution of significant health, nutrition, and safety issues that confront the public, industry, and government. With this goal in mind, ILSI meetings should be occasions where members' representatives and other invited participants:

1. Discuss scientific solutions to problems affecting the health, nutrition, and safety of the public.
2. Develop means to contribute to proper analysis of public health, nutrition, and safety issues by regulatory bodies.
3. Review industrial activities and problems with implications for public health, nutrition, and safety, and review new scientific developments.
4. Support and promote research and educational programs to enhance public health, nutrition, and safety.
5. Develop objective and voluntary industry standards to promote health and safety and compliance with regulatory requirements.

ILSI meetings shall not be occasions where members' representatives and other invited participants:

1. Discuss prices or pricing policies, or any marketing policy with a direct or indirect effect on pricing or any other terms of sale.
2. Confer about division or allocation of sales territories or customers.
3. Establish blacklists or boycotts of suppliers, purchasers, or competitors.
4. Coerce members to implement particular programs or policies.
5. Resolve problems unique to a single member or a small, select group of members.
6. Exchange or disseminate information relating to costs of production, distribution, or marketing.

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International Life Sciences Institute Code of Ethics and Organizational Standards of Conduct

Statement of Purpose

The goal of the International Life Sciences Institute's (ILSI) Code of Ethics and Organizational Standards of Conduct is to assure that ILSI members, scientific advisors, consultants, other key stakeholders in ILSI scientific activities, and users of ILSI's scientific work products are aware of the ethical principles guiding the organization's structure and the tenets behind the organization's adherence to rigorous, peer-reviewed scientific investigation and scientifically balanced, evidence-based work products.

Introduction

The International Life Sciences Institute is an international organization that seeks to promote the public health through the advancement of peer-reviewed scientific investigation and application of evidence-based decision-making in the areas of nutrition, food safety, toxicology, risk assessment, and the environment. ILSI accomplishes its mission through support of scientific research, publications, and workshops and conferences. The principles listed below provide a framework to guide ethical decision-making. (Note: Reference below to policies applicable to "ILSI" includes ILSI, ILSI branches, the ILSI Research Foundation, and the ILSI Center for Health Promotion.)

Principle 1. Scientific Integrity

All ILSI projects must have a primary public purpose and benefit, and must address issues of broad public health interest.

The ILSI and ILSI branch Boards of Trustees must be composed of at least 50% public sector members (primarily academic); the remaining trustees represent ILSI's member companies. ILSI's trustees serve in a voluntary capacity; they are not paid for their time.

ILSI shall only support animal and human subject research that has been approved by the appropriate bodies responsible for ensuring humane and ethical treatment of the animals or human subjects (e.g., Institutional Review Boards, Ethical Clearance Committees, Animal Care and Use Assurance Committees, etc.). All ILSI-supported research shall be conducted to meet the highest standards as well as all applicable legal standards.

ILSI encourages publication of all research results, regardless of outcome. ILSI entities shall not control the content of publications of research grantees or commissioned authors, but shall encourage academic freedom.

All ILSI research grantees must include language in their grant-related publications identifying the sponsor and providing appropriate sponsor contact information.

All ILSI committees and task forces must have scientific advisors from academia or government to ensure multi-sector input and balance, and ILSI will only undertake activities for which there is broad interest and support.

Members of ILSI committees or task forces who are in attendance at meetings, symposia, or workshops must identify themselves on registration forms and materials by their primary affiliation (i.e., employer).

ILSI will be transparent in the disclosure of its funding sources.

Principle 2. Conflict of Interest/Bias

ILSI believes that ensuring balance of perspectives is the most appropriate way to ensure that the impact of any potential conflict of interest or bias is minimized and does not exert an undue influence on the scientific process.

With respect to publications, grant reviews, and expert panels, ILSI asks the scientists with whom it works to disclose any potential conflicts of interest. ILSI may ask scientists to excuse themselves from an activity.

Scientists who work with ILSI are expected to act in accordance with their own institution's conflict of interest policies and with applicable laws, as well as to comply with the conflict of interest policies of any journal or organization with which they may work, including ILSI.

Principle 3. Advocacy

ILSI does not conduct lobbying activities. Advocacy of any kind is strictly limited to promotion of the use of evidence-based science as an aid in decision-making.

Principle 4. Publications

ILSI-sponsored manuscripts must undergo stringent peer-review by qualified reviewers. Editors and reviewers will treat manuscripts under review as confidential. Scientists will not serve as editors or reviewers of a manuscript if past or present connections with the author(s) preclude an objective evaluation of the work.

All ILSI publications, including proceedings from workshops or symposia sponsored by ILSI branches or entities will utilize appropriate attribution language to denote funding sources and sponsors, and ILSI entities shall provide contact information in all publications they produce for anyone interested in obtaining additional information about the organization or the specific sponsors of a particular project.

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Workshop Program

HESI

ILSI Health and Environmental Sciences Institute

HESI/JRC/SETAC-EUROPE WORKSHOP ON BIOACCUMULATION ASSESSMENTS MAY 5-6, 2006

THE HAGUE, THE NETHERLANDS

WORKSHOP PROGRAM

May 5, 2006

8:30	Welcome and Introductions	Theo Traas RIVM Tanya Netzeva European Chemicals Bureau/JRC
9:15	HESI Overview	Karluss Thomas HESI
9:30	EU Experience and REACH Requirements	Peter Lepper European Chemicals Bureau/JRC
10:30	**Break**	
10:45	POPs Protocol Requirements	Jose Tarazona INIA
11:15	Experience from Canada	Mark Bonnell Environment Canada
11:45	Insight from PBT Group 3.3	Steve Dungey EA Science Group
12:15	Grouping – top themes/needs – what is needed for REACH? POPs?	All
12:30	**Lunch**	
1:30	Summary of HESI In Vivo B Database Workshop and In Vivo Test Types	Annie Weisbrod The Procter & Gamble Company
2:15	Summary of HESI In Vitro/ADME Workshop and In Vitro Test Types	Sue Erhardt The Dow Chemical Company Birgit Hoeger ECVAM / IHCP

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3:00	**Break**	
3:15	ECETOC Report on PBT: Focus on ITS for B	Sylvia Gimeno The Procter & Gamble Company-Belgium
4:00	Modeling of Bioaccumulation	Sabcho Dimitrov University of Bourgas Tanya Netzeva European Chemicals Bureau/JRC Jose Tarazona INIA
5:00	Poster Social (current and developing tests)	Beate Escher Eawag Birgit Hoeger ECVAM/IHCP
7:30	Dinner	All
May 6, 2006		
9:00	Review of Day One: Talks, Selected Themes	Annie Weisbrod The Procter & Gamble Company Theo Traas RIVM Tanya Netzeva European Chemicals Bureau/JRC Mark Bonnell Environment Canada
9:30	Grouping (select ideas to resolve top themes/needs identified on Day one)	All
10:30	Working Groups (develop action plans)	All
12:30	**Lunch**	
1:30	Present Proposed Action Plans	Working Group Coordinators
3:00	**Break**	
3:15	Present Proposed Action Plans (Continued)	Working Group Coordinators
4:30	Integrate Feedback and Revise Plans	All
6:00	Adjourn	

Participants List

HESI

ILSI Health and Environmental Sciences Institute

ILSI HESI / JRC / SETAC-EU WORKSHOP ON BIOACCUMULATION ASSESSMENTS
MAY 5-6, 2006

THE HAGUE, THE NETHERLANDS

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Summary of HESI Bioaccumulation In Vivo Database Workshop

HESI
ILSI Health and Environmental Sciences Institute

DRAFT 4: April 11, 2006

Models Section from submission to *Environmental Health Perspectives*

Title

Workgroup Report: Review of Fish Bioaccumulation Databases used for Identifying Persistent, Bioaccumulative, Toxic Substances

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Models for Predicting Bioconcentration and Bioaccumulation

Bioconcentration models

In 1974, the first relationship based upon the K_{ow} of the chemical was established for predicting BCFs of nonionic organic chemicals(Neely WB, Branson DR, Blau GE, 1974). This relationship was of the general form:

$$\log \mathrm{BCF} = a \log K_{ow} + b$$

where a and b are empirical constants derived by regression analysis of BCF-K_{ow} data sets. Numerous regression equations have been developed since then with varying amounts of bioconcentration data (Bintein S, Devillers J, Karsher W, 1993;Schüürmann G and Klein W, 1988;Veith GD, Defoe DL, Bergstedt BV, 1979). Based upon the analyses of BCF data and underlying partitioning theory (de Wolf W, de Bruijn JHM, Seinen W, Hermens J, 1992), the slope of the regression equation should be close to one and the intercept should be approximately zero for BCF-K_{ow} data sets of organic chemicals with these specific characteristics: nonionic, small molecular weight (< 1000 g/mol), very slowly or non-metabolized, and when BCF values are expressed by the chemical concentration in fish normalized to its lipid content and the bioavailable (or freely dissolved) concentration of the chemical in water.

BCFWIN: This QSAR model is contained within the Estimation Programs Interface (EPI) Suite®, developed by the U.S.-Environmental Protection Agency's Office of Pollution Prevention and Toxics and the Syracuse Research Corporation (SRC). The suite of models is publicly available on the Internet (http://www.epa.gov/oppt/p2framework/docs/epiwin.htm). The EPI Suite contains eleven programs for estimating physical-chemical properties, rate constants, and partition coefficients for organic chemicals. One of these programs is BCFWIN, which estimates the chemical's bioconcentration factor based upon its K_{ow} and structural features (e.g., functional groups and elemental composition) (Meylan WM, Howard PH, Boethling RS, Aronson D, Printup H, Gouchie S, 1999). The BCFWIN predictive algorithm is built upon a database of 694 chemicals; i.e., 610 nonionic organic compounds (which include 18 organometallics) and 84 ionic organic compounds (which include carboxylic acids, sulfonic acids and their salts, and quaternary nitrogen compounds).

The BCFWIN predictive model is, in essence, a refinement of the regression equation approach presented by Neely et al. with a much larger database of BCFs that permitted the development of correction factors for specific chemical class and structure molecular arrangements (Neely WB, Branson DR, Blau GE, 1974). The model reasonably predicts BCF values for chemicals within the model’s domain of applicability; based upon comparison of estimated and measured BCFs in the BCFWIN training set (i.e., 694 chemicals), 50 percent, 82 percent, and 90 percent the estimated log BCFs are one half, three quarters, and one log unit of their measured values, respectively.

As discussed previously, the BCF database assembly process did not evaluate the quality of individual studies incorporated into the database. Rules were developed for assigning a chemical's BCF value from the list of reported values assembled, and these assignments were made for the 694 chemicals. Any uncertainties incorporated into the list of 694 selected BCF values are directly translated into the predictive model. Uncertainties also arise from the quality of the K_{ow} data for individual chemicals used in the BCF-K_{ow} training set.

CONCAWE: The algorithms used by the BCFWIN program were extended to hydrocarbons by developing a correction factor for the hydrocarbon chemical class

(Stewart S, Aronson D, Meylan W, Howard P, 2005). The hydrocarbon correction factor was developed using the new set of recommended BCF values for 84 hydrocarbons. For the hydrocarbons, the mean absolute error and standard deviation for the log BCF was 0.43 ± 0.54.

Base-line Model, a.k.a. "POPs": The base-line concept for modeling the bioconcentration of chemicals is based on a reference curve delineating the empirically observed maximum bioconcentration driven by hydrophobicity of chemicals (Dimitrov S, Dimitrova N, Parkerton T, Comber M, Bonnell M, Mekenyan O, 2005). In fact, this is the highest log *BCF* (log BCF_{Max}) which can be reached for a given log K_{OW} value assuming that small sized, nonionized molecules exhibit maximal bioavailability and are not metabolized (Dimitrov S, Dimitrova N, Walker J, Veith G, Mekenyan OG, 2002;Dimitrov S, Dimitrova N, Walker J, Veith G, Mekenyan OG, 2003) . The base-line model was theoretically justified by the multi-compartment diffusion model:

$$\log BCF_{MAX} = \log\left(\frac{{K_{OW}}^{n}}{(aK_{OW}+1)^{2n}} + F_W\right) \qquad \text{(Equation 1)}$$

where *a* and *n* are fitted model parameters, and F_w is the water content of the organism.

Mitigating chemical properties (molecular size and flexibility, ionization, volatilization, and adsorption) and organism specific properties (biotransformation and permeability) are used as reducing factors of the maximum bioconcentration determined via the base-line model:

$$\log BCF = \log\left(BCF_{MAX} \prod_i F_i\right) \qquad \text{(Equation 2)}$$

where F_i are the mitigating factors. Specific submodels have been developed for estimating $F_{\text{Metabolism}}$, $F_{\text{Ionization}}$ and $F_{\text{Molecular Size}}$. An example of the effect of the different mitigating factors on the predicted BCF value is provided for octadecenylsuccinic acid in Figure 1.

The model parameters were optimised by making use of the training set of experimental BCF values from 542 chemicals. The model performance for the training set showed a correlation coefficient of $R^2 = 0.84$; residual sum of squares SSR = 139.8 and variance $s^2 = 0.294$. For 88 percent of the training set chemicals, the difference between observed and calculated BCF values was found to be within 0.75 log unit. In an external validation exercise with 176 chemicals, the model demonstrated similar predictability of 80 percent, for chemicals belonging to model applicability domain (Dimitrov SD, Dimitrova GD, Pavlov T, Dimitrova N, Patlewics GY, Niemela G, Mekenyan OG, 2005;Mekenyan OG, Pavlov TS, Grancharov V, Todorov M, Schmieder P, Veith GD, 2005) .

The analysis of the relative importance of the three mitigating factors showed that passive diffusion has a 69 percent contribution, metabolism 27 percent, whereas the rest of all mitigating factors was 4 percent. Unequivocally, these contributions show the primary importance of metabolism as compared to other mitigating factors. A screening exercise recently performed on the ~10,000 organic substances for Environment Canada DSL revealed that by including all mitigating factors, the number of chemicals identified as potentially B was reduced significantly, compared with the model using molecular size as

only mitigating factor (Dimitrov S, Dimitrova N, Walker J, Veith G, Mekenyan OG, 2002;Dimitrov S, Dimitrova N, Walker J, Veith G, Mekenyan OG, 2003) . About 12.5 percent of the chemicals were identified as potentially B with only molecular size as a mitigating factor, versus 1.5 percent of the chemicals identified as potentially B with all the mitigating factors accounted for.

Bioaccumulation models

Food web models can predict BCFs, BAFs, and BSAFs for aquatic organisms, and are being used increasingly in regulatory-driven assessments because they incorporate dietary sources and other environmentally relevant processes that contribute to exposure. Since the 1970s, food web models have been created using data from persistent organic pollutants. Many of these chemicals are very slowly metabolized by aquatic species, which has enabled greater understandings of key bioavailability, uptake, and elimination mechanisms in the environment. For substances that are subject to metabolic biotransformation, BAF values may be over-predicted if this loss rate is not included in the model's parameters(Burkhard LP, Endicott DD, Cook PM, 2003). Food web models have not been evaluated for all chemical classes, i.e., ionizing substances, as these field data are not available.

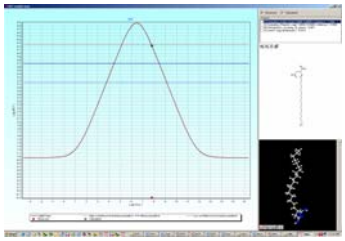
Application of food web models requires the specification of the food web, ecosystem conditions (e.g. sediment-water column disequilibria of the chemical, organic carbon content of the sediment, dissolved and particular organic carbon concentrations in water, average temperature), the biotransformation rates and other related factors for all organisms of the food web (e.g., weights, lipid and water contents, prey species). When properly constructed with high quality input data, predicted BAFs from food web models can be highly accurate. Based upon comparison of estimated and measured BAFs for three ecosystems, 60 percent and 96 percent of the estimated log BAFs were within 0.3 and one log unit of their measured values, respectively(Arnot JA and Gobas FAPC, 2003). Improving the accuracy of food web models beyond that obtained with current models will be difficult, because contaminant concentration vary widely among individual organisms in the environment. This variability is a key factor controlling the model accuracy when comparing estimated and measured BAFs (Arnot JA and Gobas FAPC, 2003).

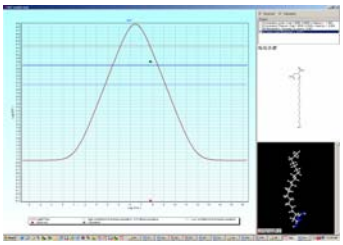
The application of typical food web models for screening large numbers of chemicals, such as for chemical management programs, is an arduous task because of the variability in site-specific ecosystem conditions and the input data required to simulate specific food webs. A semi-empirical mass balance bioaccumulation model was developed to address these limitations, providing a generic site assessment method (Arnot JA and Gobas FAPC, 2003) . The model circumvents many of the required site-specific input parameters by calibrating BAF predictions to measured BAF data. The model delivers a BAF prediction for a selected generic trophic level (e.g., lower, middle, upper) in a generic aquatic food web, requiring only a K_{OW} value for the chemical. Calibrating the model to BAF data for poorly metabolized chemicals allows for estimates of food web bioaccumulation potential. If reliable metabolic biotransformation data and scaling

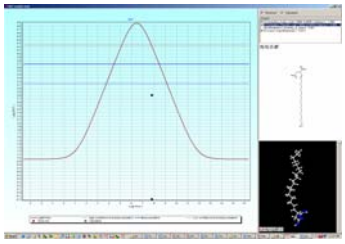
factors are available, these can be included in the mass balance calculations. The model can also provide BCF estimates by excluding dietary uptake. Environment Canada uses this model in their evaluations of bioaccumulation potential for new and existing substances.

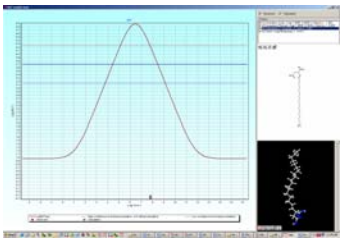
The growing field of determining the chemical absorption, distribution, metabolism, and excretion (ADME) processes in fish is the subject of the ILSI-HESI SETAC *In-vitro*/ADME Workshop conducted in March 2006. Those workshop participants explored the development and validation of techniques for extrapolating subcellular or *in vitro* measurements to whole body biotransformation rates or enzymatic activity rates across species, which could then be used as "stand-alone" assessments or incorporated into BCF and BAF model predictions.

Fig 1. Predicted BCF values for octadecenylsuccinic acid (CAS 028299-29-8) using the baseline model with no mitigating factors (a), molecular size as an mitigating factor (b), molecular size and ionization as mitigating factors (c), and molecular size, ionization, and metabolism as mitigating factors (d).

a 

b 

c 

d 

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Summary of HESI In Vitro ADME Bioaccumulation Workshop

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Decision Tree for Evaluating Bioaccumulation Potential in Fish

Introduction

Due to the limitations of computer models and *in vivo* fish bioconcentration data, a cross-sector HESI SETAC Working Group (March 4, 2006) was requested to design a decision tree that could be used to improve how the potential for bioaccumulation ("B") of chemicals is assessed. The aim of the decision tree is to indicate how additional information about various properties of a chemical could be incorporated into current B assessment methods, and by doing so, improve both the understanding of a chemical's environmental risk and categorization decisions for PBT programs. Further, this effort could help to focus and prioritize research and funding on what will most greatly influence the decision-making process concerning materials that have a potential for "B" in the environment.

The "Decision Tree" provides a step-by-step guide to decisions on the next steps to take when determining the potential for "B" for any material.

The specific goals are to:

1) Maximize our understanding of bioaccumulation potential while minimizing the use of animal testing.
2) Minimize testing requirements through the use physicochemical data and structure-activity relationships (SAR).
3) Reduce the time needed for xenobiotics to be evaluated.
4) Improve prioritization and identification of chemicals for further study.
5) Increase understanding of SAR.

To accomplish these goals, "B" assessment is done in a tiered fashion. As described here:

Tier 1 – Initial Screen for "B"

Within the regulatory arenas where "B" categorization is mandated, they are deciding on a process that could include basic empirical bioconcentration data as well as computer models for estimating "B" potential. The results of these models or data are then compared to the pre-determined criteria for "B". If the chemical is determined to be "B" based on this approach, the chemical should pass to further evaluation in the subsequent tiers of the "Overall "B" Decision Tree (Figure 1).

Tier 2 – Paper Screening Exercise

The first step in the subsequent evaluation process is to gather all the data on the chemical and conduct two types of paper screenings. The first screening is to determine if the substance is present in the aquatic environment and then if the substance is likely to remain in the aquatic environment; both indicate whether aquatic organisms can be exposed. Multi-compartment fate models, such as a Level III fugacity model, can provide a useful screen of the potential of a compound to occur in various compartments in the environment, including the aquatic environment. If this first screening identifies that organisms are unlikely to be exposed, further tiers of assessment are unnecessary. However, if exposure is possible, a second

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screening is conducted to determine if absorption across biological membranes is impeded (e.g., chemical is not bioavailable, too large, etc.). If absorption is possible, do Tier 3.

Tier 3- Absorption potential

This part of the decision tree aims to provide estimates on two processes that govern the absorption potential of chemicals:

- Environmental availability of the compound for absorption by the organism.
- Ability of the compound to cross the biological membranes/epithelia to enter the organism.

Considering the currently available tools, a three-pronged approach appears feasible:

- Physicochemical parameters to provide baseline information (i.e., "Lipinski's Rule of Five" adapted for use with fish).
- Biomimetics or passive samplers such as SPMD, SPME, EVA.
- Biological models.

If a chemical appears to be bioavailable and absorbed, its metabolic lability is then considered.

Tier 4 – Metabolism Assessment
(Figure 2)

The first step would be to use a simple *in vitro* screen for metabolic potential (e.g., in S9 liver fractions or hepatocytes). If no significant biotransformation of the compound is observed or greater certainty is needed, then some type of *in vivo* study (e.g., cannulated fish) or *ex vivo* study (e.g., liver perfusion) could be initiated to estimate the impact of low metabolism on bioaccumulation potential. If the revised "B" estimate is greater than the regional criteria of interest, then a risk assessment may be performed. If the revised "B" value is less than the criteria of interest, consideration should be given to extrapolating the metabolic data across species (e.g., using PBPK modeling) and using these data as part of a "Weight of Evidence" approach to explain why the compound of interest is not bioaccumulative. If necessary, additional studies may be performed to examine the potential effects of major metabolites.

Tier 5- Risk Assessment

If a compound still meets criteria for "B" it will then be important to move on to a more formal risk assessment. The overall objectives will follow a risk-based approach to understand the true potential for "B" in the environment, and conducting an *in vivo* test (e.g., OECD 305) or field studies may be required. In this effort, the database of species "B" values may need to be expanded to include several levels in the food chain. It will be important to combine exposure, potential for absorption and metabolic clearance with relevant organism or population level data to evaluate the risk to organisms in the environment, especially predatory animals, based on the "B" evaluation.

Figure 1 - Overall "B" Decision Tree

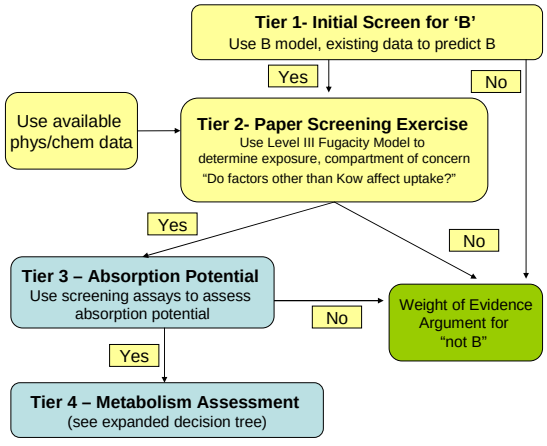
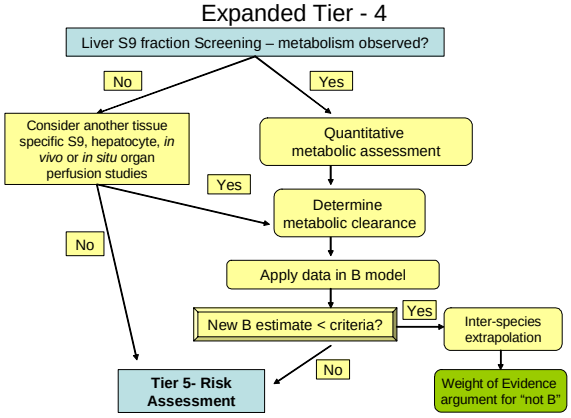


Figure 2- Metabolism Assessment Decision Tree
Expanded Tier - 4



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Development of a Strategy to Assess the Potential of *In Vitro* Methods to Predict Bioaccumulation

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Session Charge for HESI SETAC In vitro ADME Workshop (4 March 2006)

Design a research strategy that will lead to the determination of how and when diverse *in vitro* methods may be used to predict bioaccumulation in fish. Results of this session were needed to provide background and status information for the upcoming HESI sponsored workshop in Den Haag, The Netherlands (conducted prior to SETAC-Europe, May, 2006) and as an outline for a future publication.

Summary

There was a consensus that while in vitro methods provide great potential to estimate physical/chemical properties important for 'B' estimations, they will require further evaluation to verify their ultimate usefulness. The group believed this was best done via demonstration projects. Demonstration projects involve testing selected chemicals in abiotic and biotic systems that produce partitioning, absorption, and metabolism data; these data are scaled up and put into a generic fish bioaccumulation model (Arnot and Gobas, 2003) to estimate BCF/BAF ('B'). The 'B' model predictions incorporating the abiotic and biotic system data are ultimately compared to measured 'B' data. The following provide a brief description of the key tasks and methods that will be employed in conducting the demonstrations.

Selection of Chemicals

The potential success of any *in vitro* method can only be assessed in the context of the method choices available and type of chemical properties. Solubility, sorption, volatility, potential for biotransformation, metabolic pathways, availability of analytical methods and existence of reliable 'B' data (with species noted) are all factors that can affect the choice of chemicals to be evaluated. Table 1 provides a 'read-across' of the diverse factors that may be used to select chemicals to demonstrate how in vitro methods might be used in B assessments.

Table 1. Factors that can be used to select chemicals for a demonstration project.

Chemical	'B' (Kow-only)*	Measured 'B'**	Predicted *Kmet***	Species	Predicted Metabolic Pathway(s)****	Analytical Method Available*****
W	High	< Kow-only	High	From measured 'B' data	Phase I and II	
X	High	~ Kow-only	Low		Phase I	
Y	Low	< Kow-only	High		Phase I and II	
Z	Low	~ Kow-only	Low		None	

* 'B' (Kow-only) = predicted 'B' from a computer model using Kow as the only input and assumes no metabolism.
** 'B' is a measured value from reliable tests, e.g., OECD 305E BCF test, or field monitoring study.
*** 'Kmet', rate of whole-fish metabolism that is either quantitatively measured in vivo or in vitro (with scale up), or qualitatively estimated by subtracting measured 'B' from predicted 'B'.
****Metabolic pathways for mammals are available in text books and can be estimated by computer models, e.g.,TIMES, METEOR. However for fish, metabolism models are not available, hence potential pathways could be proposed based on best professional judgment (M James, K Kleinow, J Nichols and I Schultz volunteered to provide more details on this selection factor for interested parties).
***** A chemical's priority for selection is reduced if significant analytical method development is needed.

Systems that Describe Exposure and Dose

Suspected 'B' chemicals are typically highly sorptive, hence a primary route of exposure for fish to 'B' materials is via ingestion in the real world. Systems that can provide measures of sorption or bioavailability are critical for the proper estimation of 'B' and for understanding the results of various in vitro methods. These systems can be Abiotic or Biotic. An abiotic system typically refers to a solid phase that test chemicals absorb or adsorb to, and can be used to measure sorbed and free (soluble, unabsorbed) fractions. Biotic systems provide measures of transference across biological barriers (e.g., lumen, blood brain barrier). While several abiotic systems were discussed, two were favored by the session participants: EVA (ethylene vinyl acetate film) and SPME (solid phase micro-extraction). Since free and sorbed concentrations in the diverse in vitro methods are necessary for proper interpretation, EVA and SPME should be used in: sub-cellular and cellular media, water (for fish tests) and fish blood. Caco-2 and fish intestinal preps were the two biotic systems discussed. It was noted that although expert techniques are required to extract the tissue and conduct the test, only the intestinal preps are derived from fish and therefore thought to produce permeability data directly related to whole fish.

Subcellular Systems

Three different subcellular systems were discussed for metabolism studies: liver S9, microsomes, and homogenates. Only S9 and microsomes were considered worthy of further consideration because of the assays' higher degree of sensitivity and thus, measurability, of biotransformation. These systems are viewed as screening tools to assess the potential for biotransformation in higher biological systems (cellular, tissue and whole fish). While biotransformation (as metabolite generation and/or loss of parent material) rates can be measured in these test tube fractions, the consensus was that results should best be communicated as positive/negative (yes/no) or binned (high/medium/low) potentials. It was recommended that the same species of fish be used to compare utility of an in vitro test with in vivo measurements. For example, if a reliable measured 'B' value from Rainbow trout is used, then microsomal and S9 fractions from Rainbow trout should be used to evaluate how metabolism affects 'B' potential. Alignment on protocols (how to create the subcellular fractions, incubation temperatures, etc) will be necessary, particularly as investigations per fish species are compared across the diverse chemicals tested in

different laboratories. Efforts should be made to measure free and sorbed fractions in media. Parent chemical loss rates should be based on total and free fractions.

Cellular

Since 'B' materials are likely to enter fish via the intestine, there is a long-term need to assess the importance of intestinal uptake and metabolism on 'B' in fish. However at this time, fish intestinal preps are less commonly used, requiring method development. On the other hand, the use of fish hepatocytes to assess biotransformation is growing rapidly. Primary hepatocytes have increased realism in estimating 'B' beyond subcellular liver based preps because they include membrane transport (active and passive mechanisms). Their use has been primarily limited to only a few labs and species (Common carp and Rainbow trout). A current limitation of using hepatocytes is the need to have fresh fish cultures and their small tissue yield. To facilitate method transfer, there is a need to investigate the development of cryopreserved fish hepatocytes. Presently, cryopreserved hepatocytes from lab mammals and humans are commercially available. Parent chemical loss rates (hepatic clearance) should be based on total and free fractions.

In Situ Isolated Liver Preparations

Isolated liver preps provide the greatest integrative measure of uptake, distribution and biotransformation of chemicals in the liver. These preps have only been developed for catfish and Rainbow trout, species that have encapsulated livers and clearly defined hepatic and portal blood vessels. To enable a 'read-across' of methods exposure should be based on free and total fractions as dosed via blood.

Ed. 26 April 2006, A Weisbrod

5

Reporting Formats

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ILSI-HESI, ECB, JRC-ECB and JRC-ECVAM workshop, 5-6 May 2006

Reporting formats from the QSAR Experience project

Theo Traas and Betty Hakkert, Chemical Substances Bureau RIVM

Introduction

The QSAR experience project is an initiative from regulators in the European Commission to gain experience with the use of QSARs in risk assessment of chemicals. The project is currently under the guidance of the European Chemicals Bureau (ECB) and a subcommittee of the EU technical committee for new and existing substances.

In chemicals risk assessment, large-scale regulatory programs are underway such as the OECD HPVC program, the Canadian DSL program and the new European Chemicals legislation (REACH). It is expected that in the near future, alternatives for in vivo-testing such as *in silico* and *in vitro* methods will be used much more frequently in risk assessment. Both industry (as responsible entities or registrants) and regulators will need to deal with the question how the results of these alternative methods need to be interpreted, how these are reported and how they can be evaluated (and weighted).

As part of the experience project, reporting formats were suggested to exchange experience between regulators on the use and interpretation of QSAR models in risk assessment. It became clear that reporting on the use and outcome of alternative methods but can be placed in a wider context. If the results of alternative methods are not reported consistently, it will be very difficult to evaluate if the methods used are valid for a specific risk assessment context, if they have been applied correctly and if they have been interpreted correctly. Therefore, we feel it is a joint interest for both industry and regulatory bodies to develop a system for reporting alternative methods, such that they can be easily interpreted and evaluated in the risk assessment. This should also be considered when designing a testing strategy.

Considerations on the goal of formats

The goal of the formats is to streamline how alternative methods are reported, and in no way tries to limit or fix which methods are used. The underlying (database of) methods that are described in some detail (see section on levels) can be easily expanded once new methods have been developed.

For industry, it is vital that they can report findings of alternative methods in an accepted format and that the underlying models or methods used are described and stored somewhere to avoid duplication of effort.

For regulators, it is vital that they can see how a certain result is achieved, that results of different methods are reported and that the underlying methods or models can be traced and scrutinized. This is needed to evaluate whether the alternative methods provided in the risk assessment are adequate for the test endpoint in question and provide sufficient certainty for regulatory decision making.

Levels of reporting formats

The current reporting formats have three levels.

- *Level one* – Reporting of end conclusion of using alternative methods, based on the summaries for each method or model, for a specific substance and endpoint (e.g., Substance Y for bioaccumulation).
- *Level two* – Reporting of the prediction and conclusion for a specific substance and endpoint, for a single method or model.
- *Level three* – Description of a specific method or model, based on the OECD criteria.

Level one is the top level reporting format that provides essential information and the conclusions for a specific substance and endpoint. The conclusions from each underlying method or model are repeated so the reasoning and weight of evidence is transparent. As part of this level, a summary of essential substance characteristics (as input to the models or methods) can be given.

Some of the information is dependent on the regulatory framework in question. It can also addresses cut-off criteria, screening criteria, thresholds, classification and labeling issues.

Level two is the reporting level for an individual model or method, for a specific substance and endpoint. The format states the basic

Examples

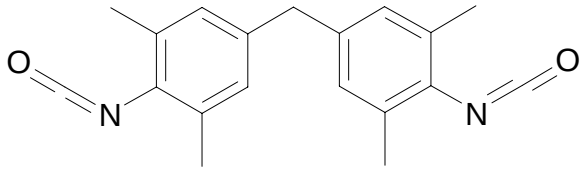
Unfortunately, examples of reporting formats for the endpoint bioaccumulation are not yet available. For illustration purposes, we have provided examples of reporting formats for the endpoint of Skin Irritation.

The example consists of separate parts that are electronically linked (but collated for this example)

- Substance identity (Cas nr. 101657-77-6)
- Level 1 report for skin irritation, purpose of classification and labeling
- Level 2 report for the Gerner model (specific)
- Level 2 report for the DerekfW model (specific)
- Level 3 report for the Gerner model (generic)
- Level 3 report for the DerekfW model (generic)

Hopefully, these examples will stimulate the discussion on how to use the results from alternative methods in risk assessment and allow others to evaluate the results.

Identity

Chemical Name (English)	4,4'-methylenebis(2,6-dimethylphenyl cyanate)
CAS RN	101657-77-6
EINECS/ELINCS-nr.	CAS RN not found in ESIS
SMILES	O=C=Nc1c(C)cc(cc1C)Cc2cc(C)c(c(C)c2)N=C=O
Structure (2D):	
Molecular Weight	306.36 g/mol
Bruto Formula	$C_{19}H_{18}N_2O_2$

Physico-Chemical parameters

Parameter	Value	Unit	Source
Melting point	135 107	°C	(estimate) confidential test
Water Solubility	5.3 6.5	mg/l	(estimate) confidential test
Log Kow	7.4 7.6		(estimate) confidential test
Surface tension	37.8	mN/m	est. Chemsketch 8
Lipid solubility	3.87	??	Confidential test
Hydrolysis	Unknown		
pH in water solubility test	Unknown		

LEVEL 1 EU Classification & Labelling – Skin Irritation

Substance

ITS for substance:	4,4'-methylenebis(2,6-dimethylphenyl cyanate), Identity – Example 2.doc

Endpoint

Regulatory endpoint:	EU Classification and Labelling for dangerous substances and preparations: http://ecb.jrc.it/Legislation/1967L0548EC.htm

Data – QSARs, category approach, in-vivo & in vitro test data

Does the intended use of the chemical give any indication for corrosive properties?	Result	Yes, reactive chemicals – skin corrosion or irritation is likely
	Reliability	2
	Reasoning	No data is available on the use of this substance but isocyanates are known to spontaneously react with water, forming a primary amine (known alert for skin irritancy) and carbondioxide.
Is the pH of the substance indicative of corrosive properties (2>pH>11.5)?	Result	No data available. Skin corrosion not likely
	Reliability	2
	Reasoning	No strongly acidic or basic functionality is present, also not after reaction with water.
Is the substance an organic hydroperoxide?	Result	No – Not corrosive to skin (not R34)
	Reliability	1
	Reasoning	Substance is not an organic hydroperoxide
Is the substance an organic peroxide?	Result	No – Not irritant to skin (not R38)
	Reliability	1
	Reasoning	Substance is not an organic peroxide
Does the substance contain impurities (> 0.1%) that are known skin irritants or corrosives?	Result	No – No classification needed for impurities
	Reliability	1
	Reasoning	
Results of the Gerner exclusion rules for skin irritation:	Level 2:	L2 - Gerner - Example 2.doc
	Result	Not a skin irritant (NOT R38), and not a skin corrosive (NOT R34/35)
	Reliability	1
	Reasoning	The combination of four applicable rules is thought to be give sufficient evidence of the absence of skin irritation potential.
Results of the DEREKfW 8.0 prediction for skin irritation:	Level 2:	L2 - DEREKfW - Example 2.doc
	Result	Skin irritant (mammalian)
	Reliability	1-2
	Reasoning	The isocyanide alert (2X) indicates potential skin irritation. The evaluation of the potential for skin penetration is invalidated by a suspect log K_{ow} estimation. When the experimental value is used, the evaluation would be that skin penetration of the substance is NOT favorable.
Available *in-vitro* data	Result	No data available

	Reliability	
	Reasoning	
Available *in-vivo* data	Result	No data available
	Reliability	
	Reasoning	

Conclusion

Weighted summary of the presented data	Result	Not a skin irritant, NOT R38 or R34/35
	Reliability	1
	Reasoning	pH, chemical class and purity of the substance do not require classification. Physico-chemical properties of the substance indicate absence of skin irritation potential (Gerner rules). The presence of a structural alert (isocyanide, DEREKfW) indicates potential for skin irritation, but this potential is diminished by the phys.chem. properties. DEREKfW also indicates the importance of physico-chemical properties favouring or hindering skin penetration in the interpretation of the validity of the alert. Overall the substance is evaluated as not requiring C&L for skin irritation or skin corrosion.
Need for further testing? > Physico-chemical or related to model input > *In vitro* testing > *In vivo* testing	>. >.	

LEVEL 2 Gerner skin irritation model

MODEL

Model Name	Gerner physico-chemical exclusion rules for skin irritation
Level 3 Description	L3 - GERNER SKIN IRRITATION.doc
Endpoint description (dependent variable)	NOT Classifying for EU C&L as R38 (irritant to skin) and/or R34/R35 (corrosive to skin)
Model Descriptors (independent variables)	Physico-chemical parameters, see Identity – Example 2.doc

DOMAIN

Prediction for substance	4,4'-methylenebis(2,6-dimethylphenyl cyanate), Identity – Example 2.doc	
Model Domain	Chemical:	EU New Substances, no organometallic compounds Purity of the substance should be >95%
	Descriptor:	See Level 3 Description, L3 - GERNER SKIN IRRITATION.doc

PREDICTION

Applicable classes	Class All – organic substances, not salts or metal containing Class CN – compounds only containing C,H,O and N atoms			
Algorithm (rules that apply to this substance)	General algorithm of the exclusion rules: **IF (rule) THEN substance is NOT R38 and/or R34/45**			
	Class	**Rule**	**Result**	**Goodness of fit**
	CN	mol.weight > 290 g/mol	NOT R34/35	338/338
	CN	log K_{ow} > 4.5	NOT R34/35	119/119
	CN	aqueous solubility < 0.1 mg/l	NOT R38	104/104
	CN	log K_{ow} > 5.5	NOT R38	85/85
Remarks				
Structural analogues from training set	Not given – no means available to search the training set for structural analogues.			

CONCLUSION

Result	NOT R38 (irritant to skin) or R34/35 (corrosive to skin)
Reliability (Klimitsch)	1
Reasoning	The aqueous solubility rule for the CN class gave one false negative in the external validation set (borderline substance). However in combination with the three other applicable rules the quality of the prediction is thought to be sufficient. The rules based on molecular weight and log K_{ow} don't have exceptions in the training set, and did not give any false negatives in the external validation set.

LEVEL 2 DEREKfW skin irritation model

MODEL

Model Name	DEREKfW8.0
Level 3 Description	L3 - DEREKfW SKIN IRRITATION.doc
Endpoint description (dependent variable)	Skin Irritation (mammalian). Not necessarily strong enough to lead to classification (alert dependent)

DOMAIN

Prediction for substance	4,4'-methylenebis(2,6-dimethylphenyl cyanate), Identity – Example 2.doc	
Domain	Chemical:	Organic substances that contain at least one alert. The substance is a diisocyanate and thus contains the isocyanate structural alert for skin irritation. The examples show that the alert (isocyanate) can be a substituent of benzylic ring systems. Therefore the chemical is clearly within the domain of the structural alert.

PREDICTION

Algorithm	There is no algorithm, only a qualitative evaluation of structural alerts (leading to skin irritation) and parameters for skin penetration (favouring or hindering the potential skin irritation caused by the structural alert.	**Result**
	Alert identified: R1-N=C=O, R1= carbon atom (2X)	**Irritant to skin (mammals)**
	Parameters calculated for skin uptake evaluation ***Log Kp:*** *-2.036 Calc. by the Potts & Guy equation.* ***Log P:*** *3.596 Calc. by the Moriguchi estimation* ***MW****:. 306.37 g/mol* *Skin penetration is favoured by relatively lipophilic molecules (Log K_{ow} = 1-4) of low molecular weight (<500).*	**Skin penetration favorable for skin irritation**
Remarks	The presence of two isocyanate alerts in one structure strengthens the prediction of skin irritation potential. The estimation of log P (=log K_{ow}) differs strongly from the experimental value and other estimations (ClogP and KOWWIN QSARs).	
Structural analogues	The structural alert is illustrated with 5 analogues. These are however smaller than the submitted chemical. See Annex 1 (DEREKfW result): *Known irritants which fire the alert include:* *Methyl isocyanate* *Ethyl isocyanate* *Phenyl isocyanate* *Toluene diisocyanate*	

CONCLUSION

Result	Skin irritant
Reliability (Klimitsch)	1-2
Reasoning	The presence of an alert for skin irritation (2X) indicates potential skin irritation. The alert is thought to be valid, the substance is well within the structural domain of the alert. The evaluation of the potential for skin penetration is hampered by a suspect log K_{ow} estimation. When the experimental value is used, the evaluation would be that skin penetration of the substance is NOT favorable. The interpretation of the combination of the effect of the structural alert and the influence of skin penetration is left completely to the end user, no definite prediction is given by the algorithm. The quality of the overall prediction is therefore thought to be 1-2 (structural alert 1, skin penetration evaluation 2).

Annex I

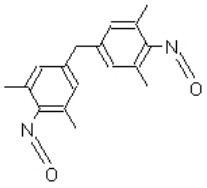
DEREK for Windows report

Version: *8.0.1*

Species: *human*
mammal
SuperEndpoints: *Irritation*

Compound Name:
Log Kp: *-2.036 Calculated by the Potts & Guy equation*
Log P: *3.596 Calculated by the Moriguchi estimation*
Molecular Weight: *306.365 Calculated by LPS*

Submitted Compound:



List of alerts found:

211 Isocyanate. Irritation (of the skin, eye and respiratory tract). Number of matches = 2

Alert overview: 211 Isocyanate

R1—N=C=O

R1 = C

Known irritants which fire the alert include:
Methyl isocyanate
Ethyl isocyanate
Phenyl isocyanate
Toluene diisocyanate

Isocyanates are highly reactive substances and generally irritating to the skin, eyes and respiratory tract. Hydrolysis and reaction with biologically important molecules, including proteins, occurs rapidly. Irritation to the respiratory tract may occur at low concentrations. E.g. exposure of humans to 2ppm methyl isocyanate for 1-5 minutes produced tears and irritation to the nose and throat. Diisocyanates are generally stronger irritants than monoisocyanates. A polymeric isocyanate, polymethylene polyphenyl isocyanate, has been classified as irritating to the skin, eyes and respiratory tract.

N.B. A structural alert for irritancy indicates some potential for this effect. Additionally, except for highly reactive corrosive substances, the skin and eye irritation potential of a chemical is very dependent on physicochemical properties which influences the concentrations at and exposure to component tissues. Skin penetration is favoured by relatively lipophilic molecules (Log P(octanol/water)= 1-4) of low molecular weight (<500). For many classes of chemicals (e.g. aliphatic amines) eye irritation is greatest for the more water soluble compounds which readily dissolve in the aqueous tear film on the cornea and conjunctiva. Liquid substances (cf.solids) have good tissue contact and are more likely to be irritating, particularly to the skin. Highly reactive corrosive chemicals may penetrate tissue as a result of corrosive damage with a lower dependence on solubility characteristics.

References:

Title: *The Dictionary of Substances and their Effects on CD-ROM.*
Author: *Anonymous.*
Source: *The Dictionary of Substances and their Effects on CD-ROM, SilverPlatter Information, Boston, 1996.*

Title: *Toxicology of the Eye.*
Author: *Grant WM.*
Source: *Toxicology of the Eye, Grant WM, Charles C Thomas, Springfield, 1962.*

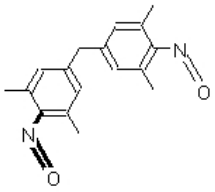
Title: *Cyanides and nitriles.*
Author: *Hartung R.*
Source: *Patty's Industrial Hygiene and Toxicology, 4th edition, volume 2D, Clayton GD and Clayton FE (editors), John Wiley, New York, 1994, 3119-3172.*

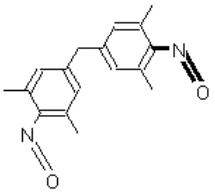
Title: *Respiratory effects of inhaled isocyanates.*
Author: *Karol MH.*
Source: *Critical Reviews in Toxicology, 1986, 16, 349-379.*

Title: *Mechanisms of activation of the sensory irritant receptor by airborne chemicals.*
Author: *Nielsen GD.*
Source: *Critical Reviews in Toxicology, 1991, 21, 183-208.*

Title: *Industrial hygiene.*
Author: *Schrenk HH.*
Source: *Industrial and Engineering Chemistry, 1955, 47, 107A-108A.*

Locations:





Examples: (211 Isocyanate)
(No examples)

Custom Examples: (211 Isocyanate)
(No examples)

LEVEL 3 QSAR model of Gerner et al.,

1. QSAR identifier

Literature model (2004/5) and software package DSS (2000), the latter is not evaluated

2. Source

The empirical rulebase model uses physical-chemical cut off values for specific empirical classes, that predicts the absence of skin corrosion or irritation. The model is developed by Gerner and co-workers at BfR in Berlin, Germany and was first reported in 2000 and updated in 2004 (Gerner et al. and Zinke et al.). More information and its potential use in testing strategies are described in (Walker et al, 2005).

2.1 Reference(s) to scientific papers and/or software packages

Gerner, I., Graetschel, G., Kahl, J., Schlede, E. Development of a Decision Support System for the Introduction of Alternative Methods into Local Irritation/Corrosion Testing Strategies: Development of a Relational Data Base. *ATLA* **2000**, 28, 11-28.

Gerner, I., Zinke, S., Graetschel, G., Schlede, E. Development of a Decision Support System for the Introduction of Alternative Methods into Local Irritancy/Corrosivity Testing Strategies. Creation of Fundamental Rules for a Decision Support System. *ATLA* **2000**, 28, 665-698.

Zinke, S., Gerner, I., Graetschel, G., Schlede, E. Local irritation/corrosion testing strategies: Development of a decision support system for the introduction of alternative methods. *ATLA* **1999**, 28, 29-40.

Zinke, S. and Gerner, I. Local irritation/corrosion testing strategies: Extending a decision support system by applying self-learning classifiers. *ATLA* **2000**, 28, 651-663.

Gerner, I., Walker J.D., Hulzebos, E., Schlegel, K., Use of physicochemical property limits to develop rules for identifying chemical substances with no skin irritation or corrosion potential, QSAR Comb. Sci., **23**, 726-733 (2004).

Walker, J.D., Gerner I., Hulzebos, E., Schlegel, K. (Q)SARs for predicting skin irritation and corrosion: Mechanisms, transparency and applicability of predictions, QSAR Comb. Sci., **23**, 721-725 (2004).

Walker, J.D., Gerner, I., Hulzebos, E.T., Schlegel, K. The skin irritation corrosion rules estimation tool (SICRET), QSAR Comb. Sci., **24**, 378-384 **(**2005).

2.2 Date of publication

A number of publications are given though key dates are notably 1999/2000.

2.3 Identification of the model developer(s)/authors

Dr. I. Gerner and co-workers at BfR. Matthias Herzler is the (Q)SAR contact point.

Dr. Matthias Herzler
Bundesinstitut für Risikobewertung (BfR)
Sicherheit von Stoffen und Zubereitungen
Toxikologie der Pestizide
Thielallee 88-92

14195 Berlin
Fon 01888 412 4402
Fax 01888 412 3260
Mail m.herzler@bfr.bund.de

2.4 Contact details of the model developer(s)/authors

The model can be derived from literature data.

2.5 Indication of whether the model is proprietary of non-proprietery

The model that predicts the chemicals is not proprietary, the details of the training set are.

3. Type of model

3.1 ■ 1-D (Q)SARs Empirical formulas

3.2 ☐ 2-D (Q)SARs

3.3 ☐ 3-D (Q)SARs

3.4 ☐ Battery of models

Overall prediction depends on applicability of multiple models/rules

3.5 ☐ Expert system

Overall prediction depends on application of multiple models/rules and use of data in knowledge base

3.6 ■ Empirical system

3.6 ☐ Neural network

3.7 ☐ Other

4. Definition of the model

The rabbit skin irritation test is the bases for the model (OECD404). The outcome of the test into a regulatory application is a two step process. The application of the chemical on the skin can result in erythema and oedema. The severeness and persistency of the effects is reflected in Draize irritation scores, that need to be reported in prescribed time intervals 1h, 24, 48 and 72h up to 21 days when effects are persisting. In the second step the scores are categorised using certain cut offs of the Draize scores, including persistency, for regulatory decision making in EU. The three categories are non-irritant, irritant, or corrosive. The classification and labelling of chemicals is used for risk reduction measures for workers and consumers that are exposed to these chemicals.

The endpoint that the model predicts is not the outcome of the skin irritation test, the effects reported as Draize scores, but it predicts the categorisation of the chemical. The model can therefore be directly used for regulatory EU classification and labelling purposes.

4.1 Defined endpoint

4.1.1. Species:

The relevant test guideline determines the species being modelled though is typically a rabbit.

4.1.2 Endpoint:

The endpoint is EU classification and labelling for skin irritation.

4.1.3 Units of measurement:

The unit of measurement has to be interpreted as the chemical is corrosive, irritant and non-irritant.

4.1.4 Reference to a specific protocol:

The reference to the experimental protocol is OECD 404

4.2 Number of descriptors used as independent variables

Six, see below

4.3 Identification of descriptors (names, symbols)

Molecular weight (g/Mol)
Log Kow
Aqueous solution (a.s in g/l.)
Surface tension (s.t. in mN/m)
Lipid solubility (l.s. in g/kg)
Vapour pressure (v.p. in Pa)

4.4 Explicit algorithm for generating prediction from descriptors

4.5

The algorithm of the model is described as physical chemical cut-off values for specific empirical chemical classes above or below, which the absence of corrosive or irritation classification is predicted. Empirical classes are described as C or Chal, meaning that chemicals only contain C, H and O atoms, or only C, H, O and halogen atoms. For example, a physical chemical cut-off value is that C chemicals with a log Kow of < -3.1 will not be irritants or corrosives.

The model can be used to predict the absence of skin irritation classification of organic chemicals without any statistical methodology. All chemicals in the database that are classified for skin irritation are excluded from the rules.

Three prerequisites are stated. The pH of the aqueous solution of the chemical should be outside the corrosive boundaries meaning that the pH of the chemicals should not be above 11 or below 1.5, which already implies classification as a corrosive (OECD, 404). The chemical predicted should have at least a purity of 95%, as irritant or corrosive impurities might cause false negative predictions. When there are other reasons to assume high reactivity the rules (e.g. oxidisers) might give false negatives.

Though the physical limit values are empirically derived, the mechanism underlining these limit values is that most organic chemicals first have to penetrate the skin before being reactive is discussed in Walker et al. (2004).

Table 1. Chemical groups, physicochemical properties, number of chemicals in each group that were used to develop rules to identify chemicals with no skin irritation or skin corrosion potential (From Walker et al., 2005)

Chemical Group	Physicochemical property	# chem passed/ # chem tested	No Skin Irritation (I) or Corrosion (C)
All chemicals	melting point > 200°C	291/297*	No I or C
All chemicals	log P_{ow} or log K_{ow} < -3.1	56/56	No I or C
All chemicals	lipid solubility < 0.01 g/kg	60/60	No C
Group C ($C_xH_yO_z$)	melting point > 55°C	128/130*	No I or C
Group C ($C_xH_yO_z$)	molecul.weight > 350 g/Mol	93/93	No C
Group C ($C_xH_yO_z$)	surface tension > 62 mN/m	94/95**	No C
Group C ($C_xH_yO_z$)	vapour pressure < 0.0001 Pa	73/73	No I
Group CN ($C_xH_yO_zN_a$)	lipid solubility < 0.4 g/kg	56/56	No I or C
Group CN ($C_xH_yO_zN_a$)	molecul.weight > 290 g/Mol	338/338	No C
Group CN ($C_xH_yO_zN_a$)	aqueous solubility < 0.1 g/l	280/280	No C
Group CN ($C_xH_yO_zN_a$)	log P_{ow} or log K_{ow} > 4.5	119/119	No C
Group CN ($C_xH_yO_zN_a$)	vapour pressure < 0.001 Pa	273/273	No C
Group CN ($C_xH_yO_zN_a$)	molecul.weight > 540 g/Mol	86/86	No I
Group CN ($C_xH_yO_zN_a$)	melting point > 180°C	153/153	No I
Group CN ($C_xH_yO_zN_a$)	aqueous solubil. < 0.0001 g/l	104/104	No I
Group CN ($C_xH_yO_zN_a$)	log P_{ow} or log K_{ow} > 5.5	85/85	No I
Group CNHal ($C_xH_yO_zN_aF$,Cl,Br or I)	log P_{ow} or log K_{ow} > 3.8	70/70	No I or C
Group CNHal ($C_xH_yO_zN_aF$,Cl,Br or I)	aqueous solubility < 0.1 g/l	135/135	No C
Group CNHal ($C_xH_yO_zN_aF$,Cl,Br or I)	molecul.weight > 370 g/Mol	109/109	No C
Group CNHal ($C_xH_yO_zN_aF$,Cl,Br or I)	lipid solubil. < 400 g/kg	76/76	No C
Group CNHal ($C_xH_yO_zN_aF$,Cl,Br or I)	molecul.weight > 380 g/Mol	99/99	No I
Group CNHal ($C_xH_yO_zN_aF$,Cl,Br or I)	lipid solubil. < 4 g/kg	29/29	No I
Group CNHal ($C_xH_yO_zN_aF$,Cl,Br or I)	aqueous solubil. < 0.001 g/l	78/78	No I
Group CNS ($C_xH_yO_zN_aS_b$)	molecul.weight > 620 g/Mol	53/53	No C
Group CNS ($C_xH_yO_zN_aS_b$)	melting point > 50°C	179/180*	No C
Group CNS ($C_xH_yO_zN_aS_b$)	surface tension > 62 mN/m	92/92	No C
Group CNS ($C_xH_yO_zN_aS_b$)	melting point > 120°C	137/137	No I
Group CNS ($C_xH_yO_zN_aS_b$)	log P_{ow} or log K_{ow} < 0.5	96/96	No I
Group CHal ($C_xH_yO_zF$,Cl,Br or I)	molecul.weight > 370 g/Mol	24/24	No I or C
Group CHal ($C_xH_yO_zF$,Cl,Br or I)	molecul.weight > 280 g/Mol	59/59	No C

*chemicals that did not pass were organic salts which release strong inorganic acids or bases when in contact with aqueous substrates/organic media

**chemical that did not pass was a skin de-fatting ether with high vapour pressure at 20°C

Table 2: Additional rules for skin irritation/corrosion
(By Ingrid Gerner and Matthias Herzler not mentioned in the Gerner et al., 2004 and Walker et al., 2005 publications)

GROUP	IF				THEN NOT	REF.
	Parameter	Qualifier	Value	Unit		
All	log P_{OW} or log K_{OW}	>	9		R34, R35	[2]
C	a.s.	<	0.0001	g/mol	R34 or R35	[2]
CHal	log P_{OW} or log K_{OW}	>	4.5		R34 or R35	[2]
CHal	m.p.	>	65	°C	R34 or R35	[2]

[2] Gerner I, Herzler M. (2004) submitted to ECVAM on July 12, 2004

4.6 Goodness-of-fit statistics

The third column in table 1 shows the goodness-of-fit.

4.6 Information on the applicability domain of the model

4.6.1 Are full details of the training set given, including details of chemical names, structural formulae, CAS numbers (if available), and data for all descriptor and response variables.

Chemical names, structural formulae and CAS numbers are only available to the Competent Authorities of the EU member states. The German BfR has put these data in a database and data are confidential. However, the excel file containing the empirical formulas and outcome of the tests are not confidential and could be made available.

The information on descriptor and response values is available in an excel file and could be made publicly available as no confidential data are included. However this excel file is not yet made publicly available.

4.6.2 If the data used to develop the model were based on the processing of raw data (e.g., the averaging of replicate values)

For each chemical(one notification) one test was performed. No averaging of replicate values has been done.

4.6.3 Is there an adequate description of the data processing?

The data processing is adequately described.

4.6.4 Are the raw data provided?

The raw data are available and provided to the evaluator for the purpose of external validation by Rorije and Hulzebos (2005).

4.6.5 Does application of the appropriate statistical method(s) to the training set result in the same (Q)SAR model?

The results of the validation (Rorije and Hulzebos, 2005) show that application of the same method results in the same model.

The following remarks should be included:

Melting point and Vapour Pressure have their cut-off values set non-conservative. All rules based on **melting point** or vapour pressure have exceptions; sometimes a substantial part (44%) of the irritant/corrosive substances is not covered by the chosen cut-off value. It is suggested that the melting point rules are either removed, or that the cut-off values are set at more conservative values e.g., the values covering 100 percent or 100 percent. The rules using **vapour pressure** cannot be redefined using conservative cut-off values since these would in effect make the rules non-applicable to any substance (a cut-off value of 0 Pa would be needed). It is suggested that vapour pressure will be dismissed as a parameter to base exclusion rules for skin irritancy on.

Surface Tension The two exclusion rules based on Surface Tension have not been evaluated because of the limited applicability of the exclusion rules. These rules

apply only to 10 / 201 substances in the validation set, and only 2 of these 10 substances were not covered by any other rules.

4.6.6 Is there a specification of the statistical method(s)used to develop the QSAR (including details of any software packages used)?

There is a specification of the method used. This is however not a statistical method. It is a visual/graphical method that shows at which descriptor value no classification is noticed. No algorithm to determine the cut-off values for specific parameters has been used.

4.7 External validation/Predictivity

4.7.1 An indication whether the model has been validated by using a test set that is independent of the training set?

This has been done twice. First time the external validation is described in the publication of Zinke et al. [Zinke 1999] on the set of rules described in the same paper. The second external validation is presented in the present report by (Rorije and Hulzebos, 2005)

4.7.2.1 If an external validation has been performed, is the following information available Zinke et al:

In the first validation exercise the rule base, including the use of structural alerts was tested with 331 substances not used for the training the model, which contained 1000 chemicals (Zinke et al., 1999, tables VII and VIII). For skin corrosion a validation was carried out. For skin irritation no such validation was presented.

a) number of test structures;

   282 (already excluded the skin irritants (16) and the chemicals for which no experimental data was available (33)

b) the identity of the test structures;

c) the specific identity of the chemicals is not publicly available. More details are known to the CA's of the EU member states;

d) the approach for selecting the test structures;

   The next 331 chemicals submitted after deriving the rules were used;

e) the statistical analysis of the predictive performance of the model? (e.g., including sensitivity, specificity, and positive and negative predictivities for classification models);

   As the model only predicts the absence of effects, the prediction performance can only be given as specificity and false negatives. The specificity is expressed as the number chemicals that are correctly predicted as not classified divided by the number of chemicals that are negative based on the experimental test. False negative is the fraction of chemicals that showed to be irritating/corrosive, while the absence of skin irritant effects was predicted by the model.

f) the results of the prediction?
   The results were that the specificity was 63.2% (163/258), was correctly predicted not corrosive. The percentage false negatives was 4.2 % (1/24).

4.7.2.1 In the second validation exercise reported in the present report the following information was available:

a) **number** of test structures was available: 201.

b) **the identity**: this was only known as empirical formulas in the excel datasheet. More details are known to the CA's of the EU member states;

c) the approach for selecting the test structures;

the approach is known: the next 201 chemicals submitted after deriving the rules were used;

Definition of the applicability domain. The distribution of the test set among the empirical classes and descriptor values was compared with the training set. It was concluded that the test set was very similar to the training set and the test set can be considered a real external validation set.

e) **T**he statistical analysis of the predictive performance of the model? (e.g. including sensitivity, specificity, and positive and negative predictivities for classification models);

See above, only specificity and false negatives can be derived

f) a comparison of the predictive of the model against previously-defined quantitative performance criteria?
Rorije and Hulzebos, 2005 shows that
If a corrosive or irritant potential based on pH would be applied before applying the physico chemical exclusion (a prerquisite); if the recommended newly calculated cut-off values for melting point would be applied; and if the recommendation to remove the K_{ow} rule for CNS compounds is followed, the statistics for the performance of the exclusion rules on the external validation set improve as shown below:

Incorrect prediction of NOT R34/35	0	0%
Incorrect prediction of NOT R34/35/38	1	0.5%
Correct predictions of NOT R34/35	58	29.1%
Correct predictions of NOT R34/35/38	85	42.7%
No prediction – test result NOT R34/R35/R38	34	17.1%
No prediction – test result R34/R35 or R38	21	10.6%
total	199	100.0%

5 Mechanistic Interpretation, if possible

5.1 In the case of a SAR, is there a description of the molecular events that underlie the reactivity of the molecule (e.g. description of how substructural features could act as nucleophiles or electrophiles, or form part or all of a receptor-binding region)? See 5.2.

5.2 In the case of a QSAR, do the descriptors have a physicochemical interpretation that is consistent with a known mechanism (of biological action)?

The very reactive chemicals are excluded from the model (but included in the testing strategy according to OECD 404., because the model is empirically modelling skin absorption.

5.3 Are any literature references cited in support of the proposed mechanistic basis of the (Q)SAR?

In other related publications literature references are supporting the empirical/mechanistic bases (e.g. Walker et al. 2004) and Hulzebos et al. 2005).

6. Applications of the model

Suggestions for possible applications for the model.

Skin irritation is predicted in terms of EU classification, chemicals are predicted as non-corrosives, non-irritants. The model can be applied to organic chemicals including the prerequisites on high reactivity, pH and purity for accepting negative predictions. Those chemicals that are predicted non-irritants are neither corrosives and need not be classified for skin irritation. The potential mechanism is often reactivity. Example chemicals are provided, which can possibly be used as analogues or categories, including EU classification if known.

7. Miscellaneous information

No additional information

9. References

OECD (Organisation for Economic Cooperation and Development). Guideline for Testing Chemicals No 404, Skin irritation, Paris, (2002). (http://www.oecd.org/dataoecd/45/25/2741642.doc).

Anon, Council Directive *67/548/EEC* of 27 June 1967 on the approximation of laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances, *Official Journal of the European Communities 196*, 16.8.1967, 1-98 (1967).

EC. Commission Directive 2001/59/EC of 6 August 2001 adapting to technical progress for the 28th time Council Directive 67/548/EEC on the approximation of the laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances *Official Journal L 225 , 21/08/2001 P. 0001 – 0333*,Office for Official Publications of the European Communities, Luxembourg, **2001**.

OECD, Harmonised integrated classification system for human health and environmental hazards of chemical substances and mixtures, *http://www.oecd.org/ehs/* **2001**.

Rorije E. and Hulzebos, E. (2005), Evaluation of (Q)SARs for the prediction of skin irritation/corrosion potential. SEC report, publicatie in prep. Available at the ECB website: http://ecb.jrc.it/QSAR/ Documents/Evaluation of skin irritation QSARs

LEVEL 3: DEREK FOR WINDOWS MODEL FOR SKIN IRRITATION

(copied from ECB proposal for sensitization , developed in consultation with LHASA Ltd)

Content

1. QSAR identifier

Derek for Windows skin irritation rulebase. Version No 8.

2. Source

2.1 Reference(s) to scientific papers and/or software package:

- Greene, N., Judson, N.P., Langowski, J.J., Marchant, C.A. (1999). Knowledge-based expert systems for toxicity and metabolism prediction: DEREKfW, StAR and METEOR. *SAR and QSAR in Environmental Research* 10, 299-314.
- Sanderson, D.M., Earnshaw, C.G. (1991). Computer prediction of possible toxic action from chemical structure; The DEREK system. *Human & Experimental Toxicology* 10, 261-273.
- Zinke, S., Gerner, I., Schlede, E. (2002). Evaluation of a rule base for identifying contact allergens by using a regulatory database: Comparison of data on chemicals notified in the European Union with ‘structural alerts’ used in the DEREKFW Expert System. *ATLA* 30, 285-298.
- Greene, N. (2002). Computer systems for the prediction of toxicity: an update. *Advanced Drug Delivery Reviews* 54, 417-431.

2.2 Date of publication:

A number of publications though key dates are notably 1986 when the first DEREK system was created at Schering Agrochemicals in the UK and 1989 when LHASA Ltd adopted the DEREK system and began coordinating the main development of the structure-toxicity knowledge base.

2.3 Identification of the model developer(s)/authors:

Lhasa Limited
LHASA is the acronym for Logic and Heuristics Applied to Synthetic Analysis)

2.4 Contact details of the model developer(s)/authors:

22-23 Blenheim Terrace,
Woodhouse Lane,
Leeds LS2 9HD, UK
Tel: +44 (0)113 394 6020
Fax: +44 (0)113 394 6099
Email: info@lhasalimited.org
Web: www.lhasalimited.org

2.5 Indication of whether the model is proprietary or non-proprietary: Proprietary

3. Type of model

3.1	☐	2D SAR
3.2	☐	3D SAR (e.g. pharmacophore)
3.3	☐	Regression-based QSAR
3.4	☐	3D QSAR
3.3	☐	Battery of (Q)SARs (overall prediction depends on application of multiple models/rules)
3.4	☑	Expert system (overall prediction depends on application of multiple models/rules and use of data in a knowledge base)
3.5	☐	Neural network
3.6		Other

4. Definition of the model

4.1 Dependent variable being modeled:

4.1.1 Species

The relevant test guideline (OECD404) determines the species being modeled though is typically rabbit

4.1.2 Endpoint (including exposure time)

The endpoint is defined as reactivity (acid, bases, oxidisers, reductors, surfactants) and for similar chemicals EU classification for irritation and corrosion is added. The model can be used as an indicator for reactivity and/or a supplier of analogues.

4.1.3 Units of measurement

Qualitative predictions are made which do not incorporate any specific unit of measurement.

4.1.4 Reference to specific experimental protocol(s):

The skin irritation knowledge encoded within Derek includes both public and proprietary data. Information about the experimental conditions is only given in the references associated with a given alert. Since only a subset of these are fully referenced, the quality of the data used in the derivation of an alert cannot be fully verified.

4.2 Number of descriptors used as independent variables:

Not applicable

4.3 Identification of descriptors (names, symbols):

Not applicable

4.4 Explicit algorithm for generating predictions from the descriptors:

DerekfW8.0 provides an explicit description of the substructure and substituents. When a query structure is processed, the alerts that match are displayed in a hierarchy called the prediction tree and are highlighted in bold in the query structure. The prediction tree includes the endpoint, and reasoning outcome, the number and name of the alert, and the example from the knowledge base if it exactly matches the query structure. The alert description provides a description depicting the structural requirement for the toxicophore detected and a reference to show the bibliographic references used. Some rules are extremely general with substructures only taking into account the immediate environment of a functional group. In other cases, the descriptions are much more specific. This means that remote fragments that may modulate skin irritation are not always taken into consideration in the assessment.

DEREKfW contains 25 structural alerts for skin irritation:

These alerts include some examples and the algorithms for the SAR are described including possible attachments.

4.5 Goodness-of-fit statistics

DEREKfW does not provide the full details of the training data used to develop an alert. Only a subset of the references and example chemicals used to develop the alert are provided for illustrative purposes.

4.6 Information on the applicability domain of the model

DEREKfW includes some inclusion/exclusion rules associated with an alert. These are documented in the alert description as particular substituents. For some skin irritation rules there are very clear descriptions of what is covered by a specific substructure. In other cases the rules are extremely general. Physical properties (Log P and MW) are used to limit the domain for skin irritation, by accounting for skin permeability (where dermal absorption is relevant). DEREKfW has limited means of flagging which chemistries are covered in the rulebase and which are not. The program is not suitable for polymers.

4.7 Information on the mechanistic basis/interpretation of the model

All the rules in Derek are based on either hypotheses relating to mechanisms of action of a chemical class or observed empirical relationships, the ideas for which come from a variety of sources, including published data or suggestions from the DEREK collaborative group.

This group consists of toxicologists who represent Lhasa Ltd and members who meet at regular intervals to give advice and guidance on the rule development work and predictions made by the program. The hypotheses underpinning each alert are documented in the alert descriptions as comments. These comments often include descriptions of features acting as electrophiles or nucleophiles. However, the detail depends on the specific alert. Some alerts contain no comments, aside from the modulating factors of skin penetration.

5. Development of the model

5.1 Explanation of the method (approach) used to generate each descriptor

Any information would be found in the comments section of the alert but this is not systemically provided.

5.2 Selection of descriptors

5.2.1 Indication of initial number of descriptors screened

Not applicable

5.2.2 Explanation of the method (approach) used to select the descriptors and develop the model from them

Not applicable

5.2.3 Indication of final number of descriptors included in the model:
Not applicable

5.3 Information on experimental design for data splitting into training and validation sets.

Not applicable

5.4 Availability of the training set

5.5.1 ☐ Chemical names (common names and/or IUPAC names)
5.5.2 ☐ CAS numbers
5.5.3 ☐ 1D representation of chemical structure (e.g. SMILES)
5.5.4 ☐ 2D representation of chemical structure (e.g. ISIS sketch file)
5.5.5 ☐ 3D representation of chemical structure (e.g. MOL file)
5.5.6 ☐ Data for each descriptor variable
5.5.7 ☐ Data for the dependent variable

DEREK rules describe generalised structure-activity relationships and do not record internally the specific chemical structures on which they are based. Derek is a knowledge base as opposed to a database. This does mean it is possible to use data from confidential sources as a basis for new rules without revealing exact chemicals to end-users. This provides a means by which proprietary data can be used without revealing potentially sensitive information.

This is a clear advantage for the purposes of securing business confidentially, but reduces the transparency of the system. The training set information available is limited to a few key example compounds to illustrate the scope of the alert.

6. Validation of the model

6.1 Statistics obtained by leave-one-out cross-validation
None

6.2 Statistics obtained by leave-many-out cross-validation
None

6.3 Statistics obtained by Y-scrambling
None

6.4 Statistics obtained by external validation
None

6.5 Definition of the applicability domain of the model

Evaluation exercise was performed by Hulzebos and Posthumus (2005) for DEREKfW 5.0, however the evaluation set of circa 50 chemicals were not detected, as the two alerts for skin irritation of that DEREKfW version were not present in the chemicals

6.6 Availability of the external validation set

6.6.1	☐	Chemical names (common names and/or IUPAC names)
6.6.2	☐	CAS numbers
6.6.3	☐	1D representation of chemical structure (e.g. SMILES)
6.6.4	☐	2D representation of chemical structure (e.g. ISIS sketch file)
6.6.5	☐	3D representation of chemical structure (e.g. MOL file)
6.6.6	☐	Data for each descriptor variable
6.6.7	☐	Data for the dependent variable

Not applicable for DEREKfW 8.0

7. Applications of the model

Suggestions for possible applications for the model.
Skin irritation is predicted as a potential hazard. The potential mechanism is often reactivity. Example chemicals are provided, which can possibly be used as analogues or categories, including EU classification if known.

8. Miscellaneous information

Needed?

- DerekfW is essentially a knowledge archive of structure-toxicity relationships.
- DerekfW is limited in that it identifies only ‘activating’ fragments, meaning the negative prediction is based solely on the lack of structural alerts. Only qualitative outcomes are provided, no measure of potency is provided. Training sets of chemicals containing these structural alerts are not provided. DerekfW does not provide a comprehensive list of references used in the development of each alert. Insufficient information is provided about the quality of the data used in the development of each alert.
- No clear explanation of the domain of applicability is provided that would alert the user as to when a query structure was within or outside the chemical domain of Derek.

- Some of the alerts within DerekfW are very general, explaining the high number of false positives in the external validation studies.
- DerekfW covers a small subset of chemical space, a huge number of rules would need to be developed in order to account for each chemical class. Development of DerekfW is incremental, focusing on each chemical class in turn. DerekfW would improve from adding more information about the modulating factors in the environment of an alert such as remote groups or by calculation of other physiochemical descriptors.

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6

Thought-Starter Document on Tools for Conducting Tiered Bioaccumulation Assessments

HESI

ILSI Health and Environmental Sciences Institute

Thought starter for Integrated Testing Strategies related to Bioaccumulation (B)
Based on ILSI-HESI and SETAC-NA / -Europe presentations (2004-2006)

These are the basic HESI-SETAC 'B' network messages:

1. We experts can help most by identifying a) what tools (e.g., cut-off values, models, tests) and qualifiers are helpful for assessors to use today to produce accurate B assessments and b) areas where flexibility in implementation is advisable to account for current concepts and new tools that should become available in 1-5 years as this field grows (e.g., in alternative testing methods).
2. Assessment processes need to be practical: conducting hundreds of OECD 305 BCF tests in 10 years is very unlikely due to the small number of contract labs doing these tests, the high cost of these tests, and pressure from animal welfare groups to reduce *in vivo* testing. Multiple computer models and test alternatives (e.g., *in vitro*) need to be allowed in some way so regulatory timings and valid information demands can be met. This is the case globally.
3. Bioaccumulation is not a simple hydrophobicity driven process; it results from ADME processes which should be considered in assessments of diverse chemical classes.

Below is a running list of models and assays that could be considered in tiered approach schemes for B assessment that the HESI-SETAC participants have used or are developing. References are generally available, but not included in the thought starter for simplicity:

Lowest Tier: We know nothing about the chemical

a) Cut-off values for chemicals with very high or very low Kow, large mol size, highly charged, highly metabolized by mammals & fish (Mekenyan et. al., De Wolf et. al.)

b) BCF, BAF, BSAF data for analogs within the chemical class (HESI B data sources manuscript, Weisbrod et. al.)

c) phys-chem analyses for Kow; use measured not predicted Kow in B models

d) BCF, BAF, BSAF models predictions for chemical

- use weight of evidence from several models to estimate B - not just one model - and models are appropriately used within chemical applicability domain (e.g., SRC BCFWIN, Mekenyan POPs, Gobas BCF, 2 TGD equations, Bonnell food web, Tarazona BAF, Nichols PBPK B model)

e) ADME information to evaluate the reliability of B model predictions, especially if they must be used for chemicals outside domain of applicability

- literature searches for existing fish & mammal data on membrane permeability and biotransformation within the chemical class
- predictions by ADME models developed for mammals and used for high through-put screening of these properties by pharmaceutical industry (e.g., ADMET for permeability potential, METEOR for metabolism potential)

Mid tier: Low tier indicates chemical could be absorbed and stored

Integrate HESI in vitro research tools, gaps & decision tree manuscript (Erhardt, Nichols, Plotzke, et. al.)

a) in vitro methods to evaluate absorption/bioavailability

- Caco2, PLHC-1, and other cell line assays to indicate permeability in specific tissues (e.g., ME Dowty et. al., M Moore & L Vasiluk)
- Semi-permeable membrane devices (SPMD) to indicate uptake potential (e.g., JH Kwon et. al., R Heltsley et. al.)
- bioaccumulation tests with aquatic invertebrates to indicate uptake potential for vertebrates (L Schuler et. al., Gunnarsson et. al.)

b) in vitro methods to evaluate metabolism

- fish or mammal liver S9, microsome, homogenate subcellular fractions to assess low or high metabolic capability (S Erhardt et. al., L Burkhard et. al.)
- fish perfused liver & GI (K Kleinow & M James)

c) in vitro methods to evaluate cell accumulation

- fish hepatocyte or other cellular cultures to examine accumulation and result in cells (S Dyer & JP Cravedi)

d) techniques to translate in vitro results into in vivo B predictions (J Nichols et. al., Cowan-Ellsberry et. al.)

High tier: Low and mid tier indicate chemical might be 'B'

a) Modified in vivo methods - Less slow, less resource intensive (<$70,000)

- cannulated fish to measure ADME rates (D Huggett et. al.)
- modified OECD 305 test, single concentration for exposure (not 2) (Woodburn et. al.)
- modified OECD 305 test, uptake OR depuration measurements only to calculate BCF (Woodburn & Springer),

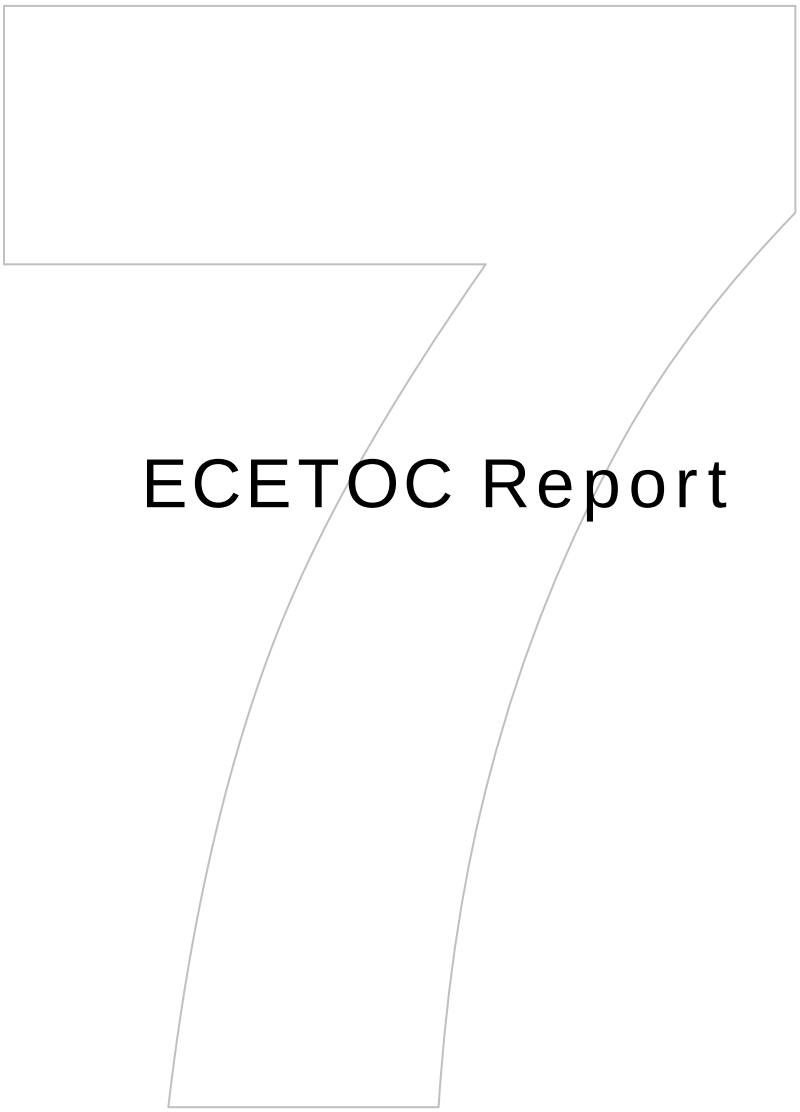
Annie Weisbrod (P&G) for ILSI-HESI; 11 April 2006

- modified OECD 305 test, with shorter duration of uptake & depuration for biotransformable organics - kinetic measurements to calculate BCF (W Bishop & A Maki)
- BAF via exposure through food, and ability to test mixtures (Parkerton et. al.)
- Uptake & depuration rates for invertebrates (Schuler et. al.)
- Critical body burden limit test (Clairant paper?)

b) Standard in vivo methods - Slow, resource intensive (~$125,000)

- OECD 305 Bioconcentration in Fish (1995)
    - HESI BCF study quality criteria manuscript (Parkerton et. al.)
- METI Bioconcentration Study with Medaka (1974, amended 1998)
- ASTM E1688-00 Bioaccumulation by Benthic Invertebrates (2000) - produces BSAF
- There are no standard tests for BAF to account for exposure through food (Parkerton et. al. could fill that gap)

Highest Tier: Field monitoring (little HESI-SETAC work in this area, so far)
D Muir & L Burkhard



ECETOC Report

Revised: 13 March 2006

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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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

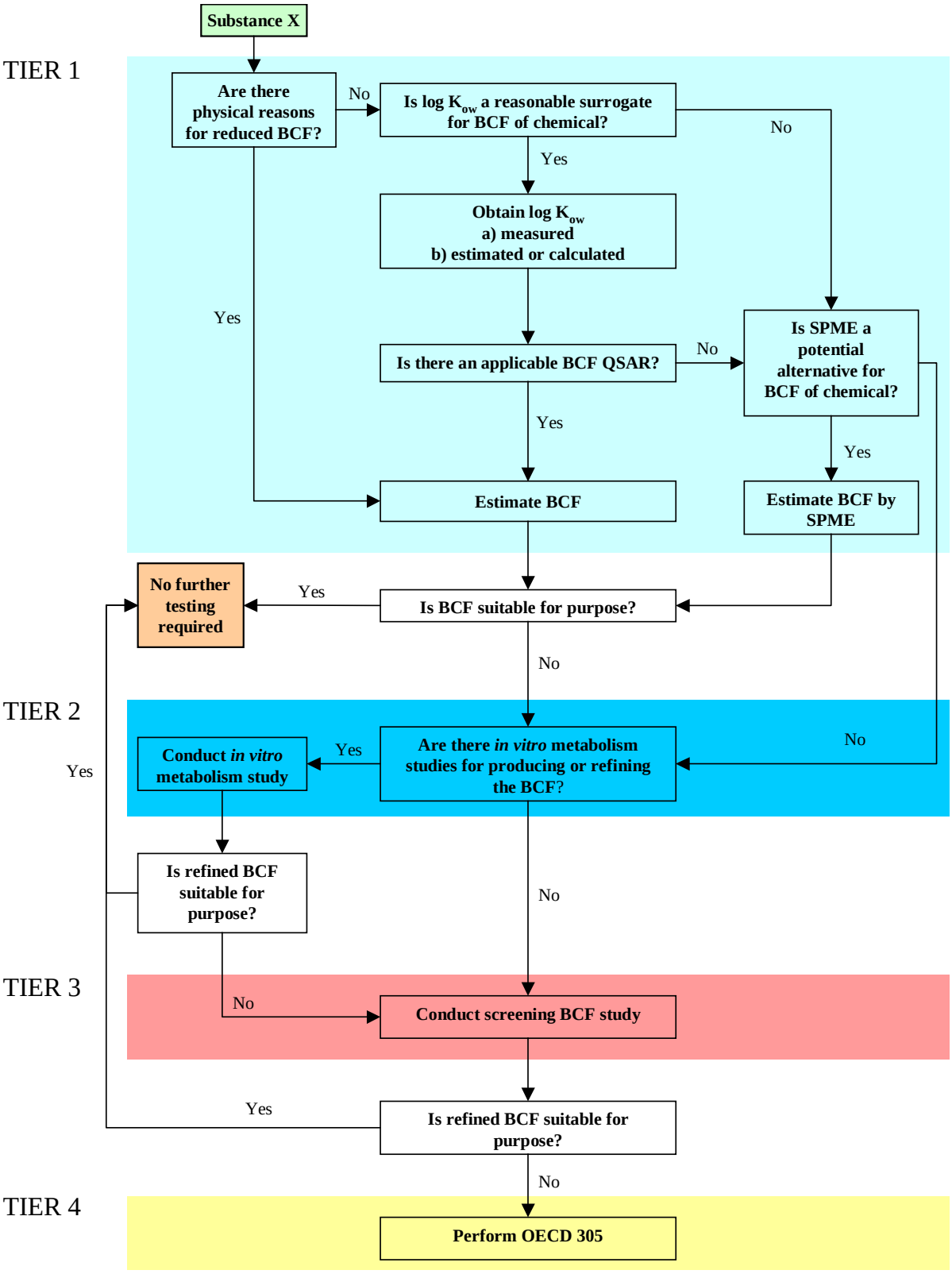
Tier III:

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

8

Poster Abstracts

HESI
ILSI Health and Environmental Sciences Institute

Overview of an International Workshop on In Vivo Fish Bioaccumulation Databases

AV Weisbrod[1], TF Parkerton[2], KB Woodburn[3]

[1]Procter & Gamble, Cincinnati, OH, USA, [2]ExxonMobil Biomedical Sciences, Annandale, NJ, USA, [3]The Dow Chemical Company, Midland, MI.

Assessing the bioaccumulation (B) potential of chemicals is an important current and future regulatory consideration in chemical management policies across the globe. A dataset of reliable in vivo B data is needed as a reference to support consistent regulatory assessments of specific chemicals, to serve as the basis for developing and/or validating models and alternative methods that are faster, cheaper and avoid animal use, and to give insights into inter-species and lab-field extrapolation of B data. To progress the development of a B in vivo database, a workshop sponsored by ILSI-HESI and involving B experts from industry, academia, and government, was conducted in November 2005. Workshop participants reviewed the availability and content of existing B databases worldwide, developed guidance for study quality criteria that should be considered when conducting B tests or judging their reliability, produced recommendations ! for the OECD on their BCF data template, and proposed steps to implement improved data sharing across government databases owners and modelers.

Overview of a Workshop Examining the Use and Development of *In vitro* Techniques for the Assessment of Bioaccumulation in Fish

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The bioaccumulation (B) of a chemical can be evaluated by exposing fish via water or food. Such an OECD 305 test can exceed $100,000, due to the need to provide consistent exposures, measure chemical concentrations, and observe >100 fish. The paucity of *in vivo* data on the large universe of chemicals that are lipophilic is driving development of reliable lower-cost approaches. Several approaches involve *in vitro* techniques to estimate the absorption, distribution, metabolism and excretion (ADME) of chemicals which are used to predict chemical behaviors that impact B. To progress the development of *in vitro* techniques for application to B assessments, a workshop sponsored by ILSI-HESI was conducted in March 2006. This presentation reviews the workshop presentations and guidance on the development and application of *in vitro* techniques for the assessment of bioaccumulation. Participants were from academia, industry, government research and

regulatory, representing a range of global perspectives. Over the course of two days, workshop participants reviewed existing *in vitro* techniques for mammals and fish, identified gaps in techniques and applications, and developed guidance to address gaps.

Topic: ER13
Keywords: PBT, bioaccumulation, fish

Lessons learned from the Evaluation of Bioconcentration and Bioaccumulation Factors of Commercial Chemicals

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The recently ratified UNEP protocol on Long Range Transport of Atmospheric Pollutants is causing various countries around the world to evaluate, categorize and prioritize thousands of commercial substances for their persistence, ability to bioaccumulate and toxicity. To assess the bioaccumulative properties, most jurisdictions make use of the octanol-water partition coefficient (Kow), the bioconcentration factor (BCF) and the bioaccumulation factor (BAF) and apply comparable criteria. This poster presentation summarizes a set of lessons learned from the evaluation of 5,317 bioconcentration factor (BCF) and 1,656 bioaccumulation factor (BAF) values in 219 aquatic species for 842 organic chemicals from 392 scientific literature and public database sources. The analysis indicates that there are empirical data available for less 3.7% of the chemicals in commerce in Canada, illustrating the importance of the selection of appropriate criteria and methods to evaluate the bioaccumulation behavior of the remaining chemicals. This poster summarizes several lessons we learned from the evaluation of bioaccumulation data and proposes a set of recommendations with regards to definitions, objectives, criteria values, data quality criteria, alternative experimental methods and the application of models and computational techniques that may be useful to regulators, industry and scientists interested in bioaccumulation.

Relevance of free concentration measurements in bioaccumulation and toxicity studies

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Concentration measurements form the basis for the generation of numerous experimental parameters and many decisions are based on reported measured concentrations. Bioconcentration factors of highly hydrophobic compounds for example use aqueous

concentrations in the denominator, although it is known that concentration measurements in aqueous solutions of hydrophobic chemicals are extremely difficult to perform and are often subject to systematic errors. Total concentrations in soil and sediment are still often used by regulatory agencies in risk assessment decisions, while differences in bioavailability may strongly affect a site-specific risk. Also in vitro systems often report a dose or a concentration at a certain biological effect. However, as the precise exposure in these systems is often not investigated, such data and also the conclusions based on these data are often highly uncertain. This is one of the reasons why quantitative in vitro assays are poor in predicting quantitative in vivo effects. In this presentation we advocate the use of measured freely dissolved concentrations as a more intrinsic concentration parameter. Experimental data show that bioconcentration factors in soil and sediment organisms, calculated via freely dissolved concentration, do not show the often observed non-linearity with octanol-water partition coefficients. We will show that effect concentrations based on freely dissolved concentrations represent the more intrinsic potency of chemicals in in vitro assays. These data provide unbiased input for computational methods, and can shed an entirely different light on the activity of chemicals. Finally, examples will be presented on measured free concentrations in relation to bioavailability in soil. Besides the focus on examples, also techniques for measuring freely dissolved concentrations will be briefly discussed with an emphasis on solid phase micro-extraction

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Bioconcentration of primary amines in fish – too B or not too B?

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Key words: Cationic surfactant, adsorption, bioaccumulation, bioconcentration

Hexadecylamine (C16PA) is a component of a group of cationic surfactants currently under scrutiny in the EU existing substances (793/93) risk assessment process. Technical problems are associated with measuring the lipophilicity and bioaccumulation potential of these molecules. Laboratory measurements and off-the-shelf models do not provide accurate values of log Kow and no bioconcentration data exist. A modified OECD 305 bioconcentration test, reducing the number of fish, was performed to determine bioconcentration of C16PA in *Cyprinus carpio*. Plateau levels of C16PA were reached

within days of exposure but much C16PA was ionically or hydrophobically bound to the outer surfaces of the fish, notably to the mucous and scales while considerably less was within the fish tissues, including the gills. Remaining work was dedicated to the quantification of the internal and external fractions of C16PA to estimate the bioconcentration factor. Problems and techniques used to solve them are outlined.

Tiered approaches in silico assessment of bioaccumulation

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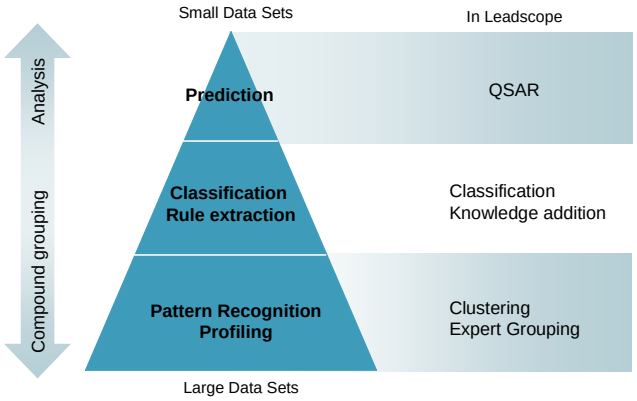
The assessment of the fate of a chemical as an environmental stressor requires a very complex process based on classifications of compound potentials in Persistence and Bioaccumulation. Due to the limited size and quality of the data from conventional testing, which often relies on animal testing, improved methods are being developed in relation with EU policies such as REACH and 7th Amendment to the Cosmetics Directives. A rough binary schematic diagram of a tiered approach can be devised for PB classifications. Further, the subsets of compounds within each tier can be quantitatively modeled. For quantitative modeling, metabolism is one of the most important factors in a chemical's ability to bio-accumulate. Any models relating $LogK_{OW}$ to LogBCF without reflecting the metabolic fate need to be improved. In this poster, a flexible data mining system that allows the user to add metabolic rules is demonstrated. By clustering the datasets for structures, two compound classes are compared for building models with and without the metabolic knowledge addition. For example, the biphenyl polychloride class modeled well without metabolic rules, while modeling the halophenols class was substantially improved by adding metabolic rules. Therefore, a predictive data mining system, where metabolic reactivity knowledge can be added on-demand, will greatly assess this tiered approach of bioaccumulation.



A Pragmatic Approach to Reduce the Workload in B Assessments

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Key words: B Cutoff value based on Kow, REACH PBT & vPvB Assessment

Article 13 of the REACH legislation (Council Draft December 2005) requires notifiers to carry out a PBT & vPvB Assessment. As REACH covers a huge amount of substances it is crucial for notifiers to have assessment guidance available preventing that substances have to be assessed on B which are in fact not bioaccumulative. In the EU the B Criteria is related to BCF (e.g. 2000 for PBT or 5000 for vPvB substances). As for very many substances BCFs are not available Kow is used as B criteria (potential B with Kow > 4.5 for PBT or > 5 for vPvB Substances). From available data is known that at higher Kow bioconcentration, bioaccumulation and biomagnification drops again. In the framework of the EU REACH Implementation Project RIP 3.2-2 'Commission Working Group on PBT & vPvB Guidance' a first draft proposal was delevoped for a Cutoff for B Assessment for substances with calculated Kow > 9.5. In addition if a substance fulfills this cutoff criteria it has to be checked if potential metabolites are not PBT or vPvB substances. This approach was tested with five substances from the EU PBT & vPvB List (originally 93 substances) and was found applicable. Another 10 substances from this list with calculated Kow > 9.5 are currently under investigation to check applicability.

Use of a Parallel Artificial Membrane System to Evaluate Passive Absorption and Elimination in Small Fish

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Evaluating bioconcentration has been of critical issue for decades. There are needs for developing alternative screening methods in a tiered approach due to the large number of synthetic chemicals that need to be evaluated and high cost of existing test methods using aquatic animals. For this reason, a parallel artificial lipid membrane permeability assay (PAMPA) that is a well established tool in pharmacokinetic research was explored and evaluated for its potential to mimic passive mass transfer of hydrophobic organic chemicals in fish. In this model system, a membrane filter-supported lipid bilayer separates two aqueous phases that represent the external and internal aqueous environments of fish. To predict bioconcentration kinetics in small fish using this system, literature absorption and elimination rates were analyzed using an allometric diffusion model to quantify the mass transfer resistances in the aqueous and lipid phases of fish. The impact of the aqueous phase mass transfer resistance was controlled by adjusting stirring intensity to mimic bioconcentration rates in small fish. Twenty three simple

aromatic hydrocarbons were chosen as model compounds the evaluation. For most of the selected chemicals, literature absorption/elimination rates fall into the range predicted from measured membrane permeabilities and elimination rates of the selected chemicals determined using the diffusion model system. After further refinement, this in-vitro system may be a valuable tool for bioconcentration assessment and prioritization of bioaccumulation testing.

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Bioaccumulation Poster

Advances in Bioaccumulation Assessment: Cross-sector Development of a Tiered Approach

Background

Challenging the paradigm**: "Bioaccumulation potential ('B') can be estimated by a chemical's Kow value."**

There are more than 100,000 chemicals in commerce globally. How many fit into that paradigm? What about lipophilic chemicals that are negatively charged, or large? Biotransformable substances? Molecules with weird groups on them that look nothing like the chemicals we in SETAC usually study? Recent efforts through the chemical management program in Canada surprised us all by revealing that many of the world's commercial chemicals do not fit into this paradigm, so it's harder to label the substance as a concern, or not. 'B' has become a huge and urgent challenge for the scientific and regulatory community to tackle, and new partnerships across disciplines, and entities like SETAC and HESI,,are focusing on advancing "B" science and keeping connected.

Although regulators and manufacturers use aquatic bioaccumulation potential to prioritize chemicals for risk evaluation and management, the resources involved with getting the data appear insurmountable, unless we can reasonably revise what we think we need. New national laws resulting from enactment of the United Nations Stockholm Convention (a.k.a. The POPs Protocol) in 2005 have led to significant new activity in the assessment of Persistent, Bioaccumulative, Toxic substances (PBTs). Canada is the first country to review its ~22,000 existing commercial substances for PBTs characteristics; they must publicly post their final list of substances that will undergo screening level risk assessment by September 2006. The REACh effort in Europe, although not fully dimensioned, is likely to expand this effort, as will the integration of PBT evaluation into reviews of new substances in the US, Japan, and Australia. Because bioaccumulation data are scarce relative to toxicity and biodegradation data, 99% of the preliminary bioaccumulation assessments in Canada have had to rely on QSAR and K_{OW}-based model estimates for fish. There is uncertainty in the assessments, as some chemical classes are outside the domain of some models used for evaluations, and others models do not have known domains. For example, initial results from the BCF models used in Canada found either 700 or 3000 discrete organics are potentially 'B', depending on the model used. Based on a pilot exercise, just collecting the data for 3000 categorized chemicals will require approximately 200 man years of effort. If we conduct the only internationally accepted B test (OECD 305) on the anticipated 3,025 PBTs in Europe, costs could exceed $378 million and 326,700 fish. The push for more data to understand PBT profiles is being met by animal welfare organizations, actively working to reduce or eliminate testing of vertebrates, including fish.

Therefore, there is a critical need to develop alternative approaches to investigate thousands of chemicals that require evaluation in the next 5 to 15 years. Methods using aquatic and mammalian species that focus on absorption, distribution, metabolism and excretion (ADME) are being explored, because bioaccumulation is the culmination of these multiple physiological processes and not solely based on a chemical's lipophilicity. New approaches under evaluation need to be verified and standardized. Development of, and international consensus on, a framework for using tiers of information will be critical to the advancement of a 'B' assessment. Determining what pieces of information are necessary and how they fit together to build a weight of evidence that guides further testing, and integration of data across tiers, is also important long term to meet regulatory deadlines, cost, and animal welfare concerns.

ILSI-HESI Emerging Issues Committee on Bioaccumulation Assessments

3 Workshops: Organizing Committee members
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*Kent Woodburn, Dow Chemicals
Andrew Worth, EU Chemicals Bureau - Joint Research Center

*Workshop co-chairs.

Tiered approach for 'B' assessment: *future* systems need work

Low Tier : BCF models (BCFWIN, POPs, Gobas)

Huge gap

future

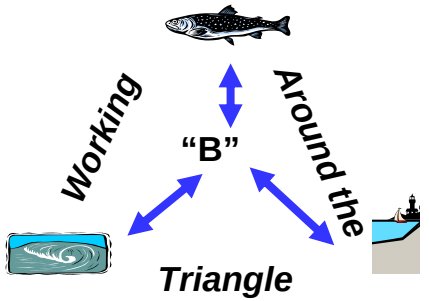
Low Tier : a) phys-chem analyses; literature searches: reapply fish & mammal data, b) ADME, BCF, BAF, BSAF models

Mid Tier : *In vitro* methods to evaluate ADME properties & cell accumulation

High Tier: Modified *in vivo* methods to measure BCF, BAF, BMF, BSAF

High Tier: Standard OECD 305 Bioconcentration factor (BCF) test

Reality: Field monitoring of "B", trophic transfer, biodilution



The "B" SAG: SETAC Advisory Group on Bioaccumulation Assessments

The purpose of the global 'B' SAG is to advance the state of bioaccumulation science, and increase the use of sound science in decision-making through the use of models, *in vitro*, and *in vivo* data for bench-scale, site-specific and regional bioaccumulation assessments.

Considered by SETAC World Council, 13 November 2005.
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Some Next Steps

- **Identify more collaborations for method development**
- **Publish posters & platforms from SETAC-NA Annual Meeting in "Advances in Bioaccumulation Assessment"**
- **Contribute to manuscript on alternative 'B' testing from ECETOC PBT task force.**
- **Publish Workshop Report from ILSI-HESI *In Vivo* Bioaccumulation Database Workshop (Nov 11-12, 2005, Baltimore, MD with SETAC)**
- **Hold ILSI-HESI ADME / *In Vitro* Tests for Bioaccumulation Assessments Workshop (Mar 3-4, 2006, San Diego, CA with SOT)**
- **Hold ILSI-HESI Tiered Approach for Bioaccumulation Assessments Workshop (May 2006, Netherlands with RIVM and ECB)**
- **Present at PBT Session (ER13) at SETAC-EU Annual Meeting (May 2006, The Hague)**

2005 SETAC-NA Annual Meeting, Baltimore MD USA