Development of a Tiered Approach to Assess Bioaccumulation of Chemicals
Project Committee

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Committee History

- Formed in April 2005 as an Emerging Issues Committee to examine the state of bioaccumulation science, and to determine top research needs to improve the accuracy of assessments.
  - Collaboration with SETAC (Society of Environmental Toxicology & Chemistry).
  - Information sharing with industry (ECETOC, CEFIC-LRI, APAG), government (TC-NES, OECD, JRC), and societies (ACS, INVITOX, ISSX, SOT).

- Established as a HESI Project Committee at 2007 Annual Meeting
- Took over leadership from Annie Weisbrod in 2007
- HESI Program Strategy and Stewardship Committee review to seek extension of charter for 2 more years. Sunset in 2011.
Drivers for this Work

- National and International regulatory programs focused on identifying and controlling (including banning) chemicals that are Persistent, Bioaccumulative and Toxic (PBTs).

- Although data are missing for P and T, B data are extremely scarce (< 3% of all chemicals have any type of data)
Why the interest in PBTs?

- 1962 Rachel Carson’s book “Silent Spring”
- As early as the 1960’s scientists had already found growing numbers of dead birds
- Raptor bird populations had been decimated in a short period of time
- Culprit – DDT/DDE found in dead birds and raptor eggs that had not hatched.
Environmental analysis found DDT/DDE but also other substances, e.g., PCBs, in remote regions and animal tissues.

Concentrations higher in raptor birds than in the fish that they ate.

For example, PCBs were primarily used in electrical equipment, not released deliberately to the environment, yet found distributed worldwide.

Why?
Questions

- How did these chemicals get to remote areas?
- Were they the cause of observed population declines in wildlife?
- Reversible?
- “Canary” for human exposure and effects?
- Were there a set of properties for chemicals that could be used to proactively identify?
Answer

- Yes
- They were persistent (on order of years), can undergo long-range transport, **biomagnified (higher concentrations in raptors than in the fish that they ate)** AND toxic (caused adverse effects not necessarily acutely).
- Toxic effects included changes in hormone systems leading to failure to reproduce
Timeline of “PBT” Related Activities

- **1962**: Rachel Carson’s Book *Silent Spring*
- **1972**: DDT banned in US
- **1982**: Convention on Long-Range Transboundary Air Pollution
- **1992**: Arctic Monitoring and Assessment Programme
- **2008**: REACh

Key Events:
- **Earth Summit**
- **Canadian DSL Categorization**
- **Great Lakes Water Quality Agreement**
- **Great Lakes Bi-national Agreement**
- **Unece Aarhus POPs Protocol**
- **NAFTA Sound Management of Chemicals**
- **Canada/US Bi-national Toxic Strategy**
- **Stockholm Convention on POPs**
The United Nations Stockholm Convention (i.e. POPs Protocol) has led to significant increase in activity in the assessment of Persistent, Bioaccumulative, Toxic substances (PBT) worldwide because the Signatories agree to implement PBT assessments into their chemical management programs.

Thousands of chemicals will need to be evaluated under these programs.

- **Sept 2006, Canada:** > 97% of the initial Categorization Decisions on Bioaccumulation potential of organic compounds (~10,000) on the Domestic Substance List were solely based on model predictions.
  - Now must address the categorization decisions or substances will be banned

- **2006-2012, Europe:** ~ 3025 chemicals anticipated requiring B testing
Bioaccumulation Assessments

Because *in vivo* bioaccumulation data are relatively scarce, assessments must rely on:

- Computer models which are not appropriate for all chemical classes
  
  **OR**

- OECD TG 305 fish bioconcentration factor (BCF) test costs ~$125,000 per chemical, uses >100 fish, takes 40+ days and significant chemical analyses.
### Estimated Cost and Fish Use for BCF tests: EU REACH

<table>
<thead>
<tr>
<th>OECD 305e (est. 3025 tests)</th>
<th>Fish used for REACh (est. 3025 tests)</th>
<th>Total # Fish</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>108</td>
</tr>
<tr>
<td>Fish used for a test</td>
<td></td>
<td>326,700</td>
</tr>
<tr>
<td>Fish used for REACh</td>
<td></td>
<td>326,700</td>
</tr>
</tbody>
</table>

**REACH**: Number of chemicals > 100 tons/year = 5,500
Percentage with log KOW > 2.7 = 55%
Number of chemicals for BCF-testing (X1 * X2) = 3,025


€302 ($378) Million just for ‘B’ testing
Reduce or Restrict Animal Testing

EU Commission for the Environment

“The Commission considers that the most pragmatic approach to reduce experiments on animals is by the introduction of alternative methods that eventually replace animal testing (Replacement alternatives). Whenever replacement is not possible, all efforts should be made to apply those methods which use fewer animals (Reduction alternatives) and which cause least harm to the animals (Refinement alternatives). Newly developed alternative methods have to be validated in order to assess their relevance and reliability. Subsequently they can be made available for regulatory purposes.”

As REACH concepts expand globally, restrictions in animal testing will follow.

From: http://ec.europa.eu/environment/chemicals/lab_animals/alternative_en.htm
Bioaccumulation testing

- Adult fish are vertebrates (thus are not alternatives to mammals)
  - OECD 305 test for one chemical uses over 100 fish!!

- Alternative methods are not “a nice to have” option they are a necessity
  - In some cases, chemicals tested using vertebrates will be penalized, if alternative methods are available
  - Resources required (time and money) will be much less
Alternative Methods

- Since alternative methods that meet 3Rs will soon be the only option or the preferred option globally

- HESI B project committee has been focused on how to meet the needs of the chemical industry to respond to the pressure for more B assessments while at the same time reducing animal testing.
Initial Goals

- To examine the state of bioaccumulation science, and to determine top research needs to develop and improve the accuracy of assessments.
- Assemble an international, cross-sector team to address this goal and ensure that the necessary tools are developed to conduct scientifically valid bioaccumulation assessments.
- Step 1: Coordinate and fund international workshops.
  - Identify data gaps and research needs.
  - Align on a tiered approach (models, *in vitro* and *in vivo* tests and extrapolations).
  - Recommend which models and methods need to be refined and developed.
  - Communicate and publish recommendations for incorporating the tiers approach and models/methods into PBT evaluations, globally.
HESI Workshops

- April 2005 - Cincinnati, OH (P&G, HESI)
  - Developed strategy and initiated planning for four additional workshops

- November 2005 - Baltimore, MD (HESI, SETAC-NA)
  - PURPOSE: Identify *in vivo* fish B data sources, and discuss how to improve B data use and modelling globally.

- March 2006 - San Diego, CA (HESI, SOT)
  - PURPOSE: Identify *in vitro* ADME tests that can be used to improve B assessment.

- May 2006 - Netherlands (HESI, ECB, RIVM, SETAC)
  - PURPOSE: Involve REACH policy makers into global effort to develop tiered approaches for B assessment.
Tiered approach for ‘B’ assessment

**Low Tier**: Kow based models (e.g., BCFWIN)

**Low Tier**: Improved models and evaluation of bioaccumulation potential using physical/chemical parameters

**Mid Tier**: *In vitro* methods to evaluate ADME properties

**High Tier**: reduced *in vivo* methods to measure BCF

**High Tier**: Standard OECD 305E fish BCF test

**Reality**: Field monitoring of trophic transfer & biodilution
## 2005-6 Workshop Participating Organizations

### ACADEMIA
- Bourgas Univ, Bulgaria
- EAWAG Switzerland
- Louisiana State University
- Ohio State University
- Simon Fraser University
- Trent University
- Univ of Bern
- Univ of Guelph
- University of Florida
- University of Montreal
- University of N. Texas
- University of Texas

### CONSULTING / CONTRACT LAB
- ADMET Technologies
- CanTest
- CellzDirect
- EURAS, Arcadis
- Leadscope
- Syracuse Research Corp.
- Wildlife International
- Research Institute for Fragrance Materials

### INDUSTRY
- 3M
- AkzoNobel
- AstraZeneca
- BASF
- Clariant
- Dow
- Dow Corning
- DuPont
- ExxonMobil
- Henkel
- L'Oreal
- Nova Chemicals
- P&G
- Pfizer

### GOVERNMENT
- Battelle Pacific Northwest Labs
- Env Canada - Existing Substances
- Env Canada - New Substances
- Env Canada - NWRI
- Environment Agency, UK
- ECB, European Commission
- ECVAM, European Commission
- INERIS, France
- INETA, Portugal
- INIA, Spain
- INRA, France
- Institute for Water Research, Norway
- IRAS, Netherlands
- METI-NITE, Japan
- RIVM, Netherlands
- US Army Corps of Engineers
- US EPA - OPPT
- US EPA - ORD
- US NOAA
Other Accomplishments in 2005 and 2006

- Improved the Env Canada process for evaluating potentially B substances.
- Built strong industry partnerships to ensure accurate B assignments.
- Expanded partnerships and trust across industry, government, and advisors.
- Improved cross-Atlantic communication and coordination / involvement.
- Established an extensive, collaborative network through SETAC and HESI.
Continued in 2007

- Established as a HESI Project Committee at 2007 Annual Meeting

- Started research projects based on results of workshops
  - Leverage HESI funding with outside partners
  - Focus on alternative methods – in vitro, improved models, etc.
HESI is contributing through advancing the science in RED areas
HESI Projects: Loss of Chemical via Metabolism

- Apparent $K_m$ database & QSAR development
- *In vitro* biotransformation test method development and standardization
  - Assessment of fish euthanasia techniques on the enzymatic activity of tissues used in *in vitro* tests
    - Trout S9 Assay
    - Trout/Carp Hepatocyte Assay
- Extrapolation model to predict BCF from *in vitro* test metabolism results
  - Fish Physiology measurements
Objective: Develop high quality BCF database with calculated apparent fish metabolism rate ($k_M$). Develop model to predict $k_M$ from chemical structure.

Database development: Jon Arnot Ph.D. graduate student at Trent University, Canada - Over 600 chemicals in database

QSAR developed by Syracuse Research Corporation (SRC)

Incorporated into US EPA EPIWIN model suite, Fall 2008

Three peer-reviewed publications

Conclusion: The model does well in separating out chemicals that are biotransformed slowly or quickly

Contributions: Env Canada ($10,000), U.S. EPA OPPT ($15,000), HESI (0$ in 2007, $10,000 in 2008)
**Objective:** Optimization and Standardization of *in vitro* metabolic rate assays using trout liver S9 fractions

- Secured funding: **ECVAM** (€ 100,000), **CEFIC** (€ 175,000), **HESI 2008 funding** $5,000
- Work to be completed 2010
- Up to 21 chemicals will be tested
- 7 labs in NA and EU: CellzDirect, CanTest, Dow, Dow Corning, Eawag, P&G, AstraZeneca

**Current status:** Optimized S9 incubation protocol; inter-laboratory testing completed on 3-6 chemicals
Fish Euthanasia Project

- **Objective:** Assessment of effects of fish euthanasia techniques on the enzymatic activity of tissues used in in vitro tests ([HESI 2008 funding - $20,000](#))

- Euthanasia methods examined:
  - MS-222 (200 mg/ml)
  - MS-222 (500 mg/ml)
  - CO2 asphyxiation (via sodium bicarbonate tablets, 31.7g/L water)
  - Spinal chord dislocation
  - MS-222 (200 mg/ml) + spinal chord dislocation + pithing [control]

- S9 metabolic activities tested (i.e., EROD, Testosterone hydroxylation, UGT, SULT)

- Results indicated some statistically significant (though small) differences between methods

- **Recommended method:** M-222 and spinal chord dislocation and pithing
Trout/Carp Hepatocyte Assay Subteam

- **Objective:** Development of *in vitro* metabolic rate assays using trout and carp hepatocytes.
  - Increased metabolic realism -- Phase I and II, including membrane-based processes
  - Research has shown that hepatocytes are more metabolically active than S9 fractions
  - Hepatocytes are not available for purchase and difficult to prepare

- **Coordinated by HESI with Sweat equity from:** DuPont, P&G, Univ. of Bern, Env Canada, ExxonMobil, CellzDirect

- Proposed project for 2009 – 2010 funding cycle
Extrapolation Model – Cowan et al 2008

Hepatocyte-based or S9 based Intrinsic clearance rate of parent chemical, CLm (ml/hr-cell)

- Liver Weight, LW (gm/Kg)
- Hepatocellularity, Hp (cells/gm of liver)
- Protein content, P (protein/gm of liver)

Intrinsic Clearance in Liver (L/d/Kg)
CLi = LW * Hp or P * CLm

- Cardiac Output, CO (L/d/Kg)
- Fraction of blood flow through liver, LF
- fu free fraction correction term

Hepatic Clearance (L/d/Kg)
CLh = (LF * CO * fu * CLi)/(LF * CO) + CLi * fu

- Volume of Distribution, Vd (L/Kg)

Elimination Rate Constant (1/d)
kM = CLh/Vd

Bioconcentration Factor (BCF)
Mass Balance Fish BCF model
(Arnot & Gobas, 2004)
Initial Verification of Model

- Start with high-quality measured BCF and $K_{ow}$ for representative chemicals. Measure intrinsic clearance rate, $CLm$, using an *in vitro* test
  - Carp hepatocytes
  - Trout S9
- Estimate BCF using $\log K_{ow}$ as only model input
- Estimate $k_M$ from in vitro data and resulting BCF
## Carp Hepatocyte and Trout S9

<table>
<thead>
<tr>
<th>Chemical (Log K\text{ow})</th>
<th>Measured BCF</th>
<th>Predicted BCF: log K\text{ow} only</th>
<th>Predicted BCF: k_M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haloxyfop-ME (3.5)</td>
<td>13</td>
<td>313</td>
<td>186</td>
</tr>
<tr>
<td>Zoxamide (3.8)</td>
<td>400</td>
<td>618</td>
<td>286</td>
</tr>
<tr>
<td>Chlorpyrifos (4.7)</td>
<td>1400</td>
<td>4288</td>
<td>1071</td>
</tr>
<tr>
<td>Fluroxypyr-MHE (4.7)</td>
<td>6</td>
<td>4288</td>
<td>355</td>
</tr>
<tr>
<td>C12 LAS (3.0)</td>
<td>90, 105, 359</td>
<td>99</td>
<td>85</td>
</tr>
<tr>
<td>C16EO8 (6.69)</td>
<td>388</td>
<td>11,923</td>
<td>622</td>
</tr>
</tbody>
</table>
Value of $k_M$ Extrapolation Model for Refining BCF

Red lines are BCF criteria = 5000 and 1000
Conclusion of Initial Validation

- Can extrapolate from *in vitro* test results to improved BCF estimate from that based on Kow only
  - S9
  - Hepatocytes
- Fish physiology data is limited
  - critical gap to gain more acceptance of model
Objective: Collect fish physiology data needed to extrapolate data from *in vitro* tests for use in refining *in vivo* extrapolation and BCF models.

Examined six fish species commonly used in BCF tests (i.e., trout, carp, goldfish, medaka, fathead minnow, zebrafish)

- Priority 1: Cardiac output and hepatic blood flow
- Priority 2: Blood and body constituents
- Priority 3: Liver parameters and blood binding characteristics

HESI 2007-8 funding ($50,000) plus some additional in 2009
Example of Initial Results

- Blood flow for trout is similar to previous measurements 17 ml/min/kg
- Hepatic blood flow is difficult to measure
- Measured blood flow is 32% of total blood flow
- These values resulted in improved estimates of BCF using the extrapolation model
HESI Projects: Bioavailability = Uptake of Chemical via Gills and Gut

**Objective:** Determine which physical/chemical factors limit bioavailability and uptake in fish and how this information can be incorporated into B assessments.

- **Publication:** Evaluation of 90 pesticide BCF data and initial development of QSAR – manuscript in press
- **Presentations and Publications in Preparation:** Evaluation of Lipinski Guidelines, and other physchem parameters, linked to absorption in fish using BCF data – 2 SETAC presentations (2006, 2007)

**Sweat equity:** BASF, P&G, Dow, Eawag, IRAS…

**Sub-Project:** Development of *in vitro* test methods to measure uptake across fish membranes -- Dosing and measurement methods
Outreach Examples

- February 2008 presentation to Environment Canada on *in vitro* S9 method and extrapolation model
  - Subsequently EC accepted S9 data to support non-B categorization for a chemical
- Presentations and Organized Sessions at
  - SETAC Europe, North America and Global meetings
Outreach Examples

- Chemicals Evaluation and Research Institute, Japan
  - Institute does all B testing for new and existing chemical registrations
  - Developed OECD 305 test
- Discussions on how they can participated in HESI and contribute to the research projects
  - Plan to initiate S9 testing in their laboratory in 2009
  - Conduct independent evaluation of Kmet QSAR
  - Shorter in vivo BCF test?
Example Publications to date


- Jackson et al. (in press). **Use of Structural Analysis To Predict Fish Bioaccumulation.** Pesticide Management Sciences.


Future Directions – 2009 and 2010

- Conduct final Workshop
  - Lab to field extrapolation
- Further work on intermediate tiers
  - Complete S9 method pre-validation (early 2010)
  - Initiate Hepatocyte research – establish cryopreservation method
  - Characterization of fish gut cell line – possible *in vitro* uptake model
  - Shortened *in vivo* test – reduce time, fish, cost
- Several papers
  - Criteria for judging quality of *in vitro* test results
Lab to Field Workshop

- Last of the workshop topics identified by the bioaccumulation committee in 2005.
- **Location:** November 2009 in conjunction with the SETAC North America Annual Meeting in New Orleans, LA.
- **Objectives:**
  - How do laboratory measured BCFs, BAFs, and BMFs compare to field measurements of bioaccumulation?
  - Why don’t laboratory measures of bioaccumulation data align with field data?
  - What are the main sources of variation of BCF/BAF/BSAF/BMF/TMF determined in the field?
- **Status:** A steering team has been formed which is working on identifying and inviting workshop participants and securing funding and sponsors.
- HESI will provide approximately $15K - $20K for the workshop.
Cryopreserved Hepatocytes

**Objective:** Development of fish primary hepatocyte cryo-preservation methods for two fish species (rainbow trout (*Oncorhynchus mykiss*) and common carp (*Cyprinus carpio*)

- Increased metabolic realism
  - Phase I and II, including membrane-based processes
  - Hepatic clearance rates from hepatocytes have been shown to exceed S9 rates
- Use of Hepatocytes
  - Requires fish stocks to be co-located with experimental labs
  - Many potential testing labs do not have fish culture facilities
  - Isolation is an art!

**Status:** Seeking research partners

**HESI funding:** **$50,000** in 2009-2010, anticipate some in-kind or partnership contributions
Characterization of Gut Cell Line

- Most important route of uptake for chemicals that could be bioaccumulative is through food and thus uptake through the gut
- Gut cell line for rainbow trout has been developed
- **Objective:** better understand the characteristics of the fish gut cell line and ability to plate
- **Status:** still in concept and discussion phase
**Shorter *in vivo* BCF test**

**Objective:** Develop a shorter, more cost effective in vivo BCF test that uses less fish

- Background work has been done to identify potential reductions in test using existing BCF test data
- Need for verification by conducting laboratory testing of the proposed protocol changes for several chemicals
- HESI is seeking partners to fund this work
  - $120,000
Time is right to make a difference

- Connections and groundwork laid for influencing future regulatory B assessments and acceptance of alternative methods
- International cross-sector partnerships are proving very important
  - Best B experts internationally are addressing how to improve assessments
  - Consulting on B assessment issues for specific chemicals
- Research is moving forward rapidly to meet the needs for alternative tests for B assessments
- Chemical companies who could be requiring B assessments need to continue funding and support of this work
Thanks

- Thanks to all the HESI member Companies who are and have supported this work
- Thanks to the multi-disciplinary, international team who has been active in conducting this research
Further Information and to Join the team

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