Application of Computational Toxicology to Prospective and Diagnostic Ecological Risk Assessment

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* The contents of this presentation neither constitute nor, necessarily, reflect US EPA views or policies.
Toxicity Testing in the 20th Century

- Expensive
- Time-consuming
- Animal intensive

- Empirical
  - observation > understanding
Toxicity Testing in the 21st Century

Four competing objectives

• **Depth** – providing the most accurate, detailed, characterization possible.

• **Breadth** – providing data on the broadest universe of chemicals, endpoints, species, life-stages, etc.

• **Animal welfare** – using the fewest animals possible and minimizing suffering.

• **Conservation** – minimizing expenditure of money and time on testing and review.
“Transform toxicity testing from a system based on whole-animal testing to one founded primarily on in vitro methods that evaluate changes in biologic processes using cells, cell lines, or cellular components, preferably of human origin.”

“The vision emphasizes the development of suites of predictive, high-throughput assays ….” (p. 7)

“The mix of tests in the vision include tests that assess critical mechanistic endpoints involved in the induction of overt toxic effects rather than the effects themselves.” (p. 121)
Toxicity Testing in the 21st Century

How does the vision apply to Ecotoxicology?

- Ecotox is faced with the same competing objectives
- Need to generate useful hazard data cheaper, faster
- Need to prioritize what whole animal testing should be done

But….

- Human cell lines will not address species extrapolation
- Less willingness to apply precautionary approach – “show me the adversity”

- Units of concern are populations and ecosystem services/functions, not individuals.
Task 2.1.1: AOP Discovery and Definition

1. Developing AOP knowledge and populating an AOP knowledge-base.

2. Tools to evaluate conservation of molecular targets (i.e., molecular initiating events) as a basis for defining taxonomic susceptibility domains.

3. Supporting HTS assay development and application.

4. Supporting development of “virtual tissue” models.
Can we use in vitro data to predict eco hazards?
Aromatase (cyp19) inhibition

High throughput assay with human cell line

Sanderson et al. 2004, Toxicol. Sci. 82: 70-79
Mechanism-based categorization of aromatase inhibitors: a potential discovery and screening tool

P. I. Petkov a, S. Temelkov a, D. L. Villeneuve b, G. T. Ankley b, O. G. Mekenyan a

a Laboratory of Mathematical Chemistry, Bourgas As. Zlatarov University, Bourgas, Bulgaria
b US Environmental Protection Agency, Mid-Continent Ecology Division, Minnesota, USA

SAR and QSAR in Environmental Research, 20: 657–678.
IC50 = 7.0 ± 0.8 µM

So What?

Villeneuve et al. 2006. Aquat. Toxicol. 76:353-368

OPP-EFED
OPPT
Population Impacts

Forecast Population Trajectories

Average Population Size
(Proportion of Carrying Capacity)

Time (Years)

A 0%
B 25%
C 50%
D >75%
E >95%
Use in hazard/risk assessment has been limited by lack of well defined (predictive) linkages between these alternative types of data and adverse outcomes traditionally considered relevant to risk assessment.
An Adverse Outcome Pathway (AOP) is a conceptual framework that portrays existing knowledge concerning the linkage between a direct molecular initiating event and an adverse outcome, at a level of biological organization relevant to risk assessment.

A bit of fish reproductive biology

**ER Agonist**

- Estradiol Agonism
- Estrogen Receptor

**Hepatocyte**
- Vtg production

**Ovary**
- Oocyte development
- Ovulation & spawning

**Female**

**Population**
- Stable or increasing trajectory

**Cellular response pathway**

- **ERE-Vtg**

**Biologic inputs**

“Normal” Biological Function
Adverse Outcome Pathways – definition and example

Disturbed fish reproductive biology

Fadrozole

E2 (ng/ml) * * *

Vtg (mg/ml) * *

Control 2 10 50

Fadrozole (µg / L)

-20 -18 -16 -14 -12 -10 -8 -6 -4 -2 0 2 4 6 8 10 12 14 16 18 20

Exposure (d)

Cumulative Number of Eggs

Forecast Population Trajectories

A0%

Average Population Size (Proportion of Carrying Capacity)

0.8 0.6 0.4 0.2 0.0

0 5 10 15 20

Time (Years)

E2 (ng/ml)

0 2 4 6 8

Time (Years)

Average Population Size (Proportion of Carrying Capacity)

0 25% 50% 75% >95%

0 5 10 15 20

Average Population Size (Proportion of Carrying Capacity)

0 25% 50% 75% >95%

0 5 10 15 20

Aromatase Activity

0 50

Fadrozole (µg / L)

0 75 150

Male Female

a b c

Ankley et al., 2002, Toxicol. Sci. 67:121-130
Adverse Outcome Pathway

**Toxicant**

**Macro-Molecular Interactions**
- Chemical Property Profile
  - Receptor/Ligand Interaction
  - DNA Binding
  - Protein Oxidation

**Cellular Responses**
- Gene Activation
- Protein Production
- Altered Signaling
- Protein Depletion

**Organ Responses**
- Disrupted Homeostasis
- Altered Tissue Development or Function

**Individual Responses**
- Lethality
- Impaired Development
- Impaired Reproduction
- Cancer

**Population Responses**
- Structure Recruitment
- Extinction

**Molecular initiating event**

**Key events or predictive relationships spanning levels of biological organization**

**Adverse outcome relevant to risk assessment**

![Aromatase inhibition](image)  
**Reduced E2, Vtg synthesis**

**Impaired vitellogenesis**

**Reduced fecundity**

<table>
<thead>
<tr>
<th>Exposure (d)</th>
<th>Control</th>
<th>2 ug/L</th>
<th>10 ug/L</th>
<th>50 ug/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>-20</td>
<td>0.8</td>
<td>0.9</td>
<td>1.0</td>
<td>1.1</td>
</tr>
<tr>
<td>-18</td>
<td>0.6</td>
<td>0.7</td>
<td>0.9</td>
<td>1.0</td>
</tr>
<tr>
<td>-16</td>
<td>0.4</td>
<td>0.5</td>
<td>0.6</td>
<td>0.7</td>
</tr>
<tr>
<td>-14</td>
<td>0.2</td>
<td>0.3</td>
<td>0.4</td>
<td>0.5</td>
</tr>
<tr>
<td>-12</td>
<td>0.0</td>
<td>0.1</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>-10</td>
<td>0.0</td>
<td>0.1</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>-8</td>
<td>0.0</td>
<td>0.1</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>-6</td>
<td>0.0</td>
<td>0.1</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>-4</td>
<td>0.0</td>
<td>0.1</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>-2</td>
<td>0.0</td>
<td>0.1</td>
<td>0.2</td>
<td>0.3</td>
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<tr>
<td>0</td>
<td>0.0</td>
<td>0.1</td>
<td>0.2</td>
<td>0.3</td>
</tr>
</tbody>
</table>
IC50 = 7.0 ± 0.8 μM

Population Impacts

Forecast Population Trajectories

Average Population Size (Proportion of Carrying Capacity)

Time (Years)

AOP

Hazard identification

Prioritization of testing

Aha!
Risk Assessment

• AOPs assume sufficient perturbation to cause the adverse outcome.

• Risk assessment – probability of an adverse outcome under defined circumstances.

• Requires an understanding of adaptive/homeostatic capacity of biological systems and their limits, relative to concentration and duration of exposure.
(i) Define the subset of genes, proteins, and other small molecules constituting the pathway of interest.

(ii) Perturb each pathway component. Detect and quantify the corresponding global cellular response to each perturbation.

(iii) Integrate the observed responses with the current, pathway specific model.

(iv) Formulate new hypotheses to explain observations not predicted by the model.

Design additional perturbation experiments to test these, and iteratively repeat steps (ii), (iii), and (iv).
25°C, 12 h

Ekman et al. 2011, ET&C 30:319-329
Mechanistic Modeling

- To aid extrapolation from molecular perturbations to outcomes.
- How much perturbation is too much?

Measured Input: EE2 or TRB conc. in water

Predicted Output: Plasma VTG

Li et al. 2011. BMC Systems Biology 5:63
Mechanistic Modeling

- To aid extrapolation from molecular perturbations to outcomes.
- How much perturbation is too much?
- Predictive toxicology

Mechanistic Modeling

• To aid extrapolation from molecular perturbations to outcomes.
• How much perturbation is too much?
• Predictive toxicology

Predicted Input:
Average Fecundity

Predicted Output:
Population forecast


\[ M = \begin{pmatrix} 0.75 & 1.5 & 3 \\ 0.26 & 0 & 0 \\ 0 & 0.39 & 0 \end{pmatrix} \]

Life table for the labial midgut activity derived from populations (Weir, 1994; Zischke et al., 1993; Duda, 1989; Gleeson and Nacci, 2001)

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Survival (per year)</th>
<th>Fecundity (eggs/yr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>6.666</td>
<td>750</td>
</tr>
<tr>
<td>2</td>
<td>6.69039</td>
<td>1300</td>
</tr>
<tr>
<td>3</td>
<td>6.969321</td>
<td>3900</td>
</tr>
<tr>
<td>4</td>
<td>⬜</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Fig. 1. Leont matrix for the labial midgut activity derived from field studies (Wistrom, 1994; Zischke et al., 1993; Duda, 1989; Gleeson and Nacci, 2001) and developed using birth pulse survival and fertility rates and a potholing version (Gotelli, 1994; Currie, 2002).
Scaling it up:
Can we use human-oriented HTS data to predict eco hazards?
Assemble AOPs into AOP Knowledge-base

**Molecular Initiating Event**
- **ER** Antagonism
- **Aromatase Inhibition**
- **CYP17, CYP11A Inhibition**
- **AR Agonism**

**Key Events**
- Hepatocyte Reduced VTG production
- Ovary Impaired Oocyte Dev.
- Female Decreased ovulation/spawning
- Population Declining Trajectory

**Adverse Outcome**
- Population Declining Trajectory
ToxCast™ Assays

(Primarily Human / Rat)

- Protein families
  - GPCR
  - NR
  - Kinase
  - Phosphatase
  - Protease
  - Other enzyme
  - Ion channel
  - Transporter

- Assay formats
  - Receptor binding
  - Enzyme activity
  - Co-activator recruitment

~600 Total Endpoints

Biochemical Assays

Cellular Assays

- Cell lines
  - HepG2 human hepatoblastoma
  - A549 human lung carcinoma
  - HEK 293 human embryonic kidney
  - T47D human breast carcinoma
  - PC12 rat neuronal

- Primary cells
  - Human vascular endothelial cells
  - Human monocytes
  - Human keratinocytes
  - Human fibroblasts
  - Human proximal tubule kidney cells
  - Human small airway epithelial cells
  - Rat hepatocytes
  - Mouse embryonic stem cells

- Assay formats
  - Cytotoxicity
  - Reporter gene
  - Gene expression
  - Protein expression
  - High-content imaging

Model Organisms

- Zebrafish embryo development
- C. elegans growth
AOP Networks

- MIE (▲) and Key Events (●) aligned with molecular screening assays
Seven Test chemicals & Putative M.I.E.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Putative M.I.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prochloraz</td>
<td>Cyp19, cyp17 inhib.</td>
</tr>
<tr>
<td>Propiconazole</td>
<td>Cyp inhib.</td>
</tr>
<tr>
<td>Fenarimol</td>
<td>ER antagonist</td>
</tr>
<tr>
<td>Bisphenol A</td>
<td>ER agonist, AR ant.</td>
</tr>
<tr>
<td>Vinclozolin</td>
<td>AR antagonist</td>
</tr>
<tr>
<td>Prometon</td>
<td>Photosystem II inhib.</td>
</tr>
<tr>
<td>Fipronil</td>
<td>GABA-A receptor, chloride channel blocker</td>
</tr>
</tbody>
</table>

Post-hoc analysis

Did MS flag a MIE known to be relevant to fish reproduction?

Were the in vivo effects consistent with our AOP(s)?
Prochloraz

1. CYP19, CYP17 Inhibition
2. Theca/Granulosa Reduced T & E2 Synthesis
3. Hepatocyte Reduced VTG production
4. Ovary Impaired Oocyte Dev.
5. Female Decreased ovulation/spawning
6. Population Declining Trajectory

Molecular Screening AC50s

In vivo effects related to AOP

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>Effects*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma E2</td>
<td>Sig ↓, 0.3 mg/L</td>
</tr>
<tr>
<td>Plasma Vtg</td>
<td>Sig ↓, 0.1 mg/L</td>
</tr>
<tr>
<td>Cumulative Fecundity</td>
<td>Sig ↓, 0.1 mg/L</td>
</tr>
</tbody>
</table>

*All effects are after 21 d of continuous exposure
See Ankley et al., 2005, Toxicol. Sci. 86:300-308

Derived from Knudsen et al. 2011, Toxicol. 282:1-15
**Propiconazole**

Propiconazole → CYP17, CYP11A Inhibition → Theca/Granulosa Reduced T & E2 Synthesis → Hepatocyte Reduced VTG production → Ovary Impaired Oocyte Dev. → Female Decreased ovulation/spawning → Population Declining Trajectory

**Molecular Screening AC50s**

- CYP2A2
- CYP2B1
- CYP2C19
- CYP3A5
- CYP2C11
- CYP3A1
- CYP2C6
- CYP2B6
- CYP2C18
- Opiate D1
- CYP2C9
- PBR
- CYP2D2
- PXR
- CYP2D1
- Opiate A

**In vivo effects related to AOP**

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma E2</td>
<td>Sig ↓, 0.5 mg/L</td>
</tr>
<tr>
<td>Plasma Vtg</td>
<td>Sig ↓, 0.5 mg/L*</td>
</tr>
<tr>
<td>Cumulative Fecundity</td>
<td>Sig ↓, 0.5 mg/L</td>
</tr>
</tbody>
</table>

* From 96 h range finding study, all other data are from a 21 d exposure. VTG data for 21 d exposure not yet available. From Skolness et al. (in press, Toxicol. Sci. 2012)

**Derived from** Knudsen et al. 2011, Toxicol. 282:1-15
**Prometon, Fipronil**

**Molecular Screening AC50s**

### Endpoint Effects

**Plasma E2**
- No effect (up to 1 mg/L)

**Plasma Vtg**
- No effect (up to 1 mg/L)

**Cumulative fecundity**
- No effect (up to 1 mg/L)

*Villeneuve et al. 2006, Environ. Toxicol. Chem. 25: 2143-2153*

### Endpoint Effects

**Plasma E2**
- No effect (up to 5 ug/L)

**Plasma Vtg**
- No effect (up to 5 ug/L)

**Cumulative fecundity**
- No effect (up to 5 ug/L)

*Bencic et al., in preparation*
Results

• Overall, when MS indicated perturbation of molecular target associated with AOP, adverse effects on reproduction and related key events were observed.

• Predicted pattern of effects observed, despite other targets being affected.

• Two chemicals with least impact on fish repro, also quite inactive in MS assays.

• Testing of *a priori* predictions based on MS and AOPs needed
Prospective Assessments

**Screening**
- Efficient/cost effective methods to predict hazard.
- Get more information from “alternative” data that are available.

**Prioritization**
- Conduct only the tests most likely to drive assessment.
- More effective use of testing resources increase efficiency.

Data poor Large Inventory  e.g., TSCA

Data-rich Programs  e.g., FIFRA
Environmental Monitoring

Regulatory context
- Complex and undefined exposures
- If apical responses are observed, it’s too late

Need
- Ability to cast a broad net
- Early warning signs
- Indication/elimination of cause(s) - diagnostic

Diagnostic Assessments

Early environmental risk assessors.
Chemical monitoring strategies are effective for chemicals whose hazards are well understood and for which sensitive analytical methods are available.

Biological effects monitoring can be a powerful complement to chemical monitoring.

- Can detect exposure to chemicals for which analytical methods are unavailable or impractical.
- Can provide insight into the potential biological consequences of those exposures.
Task 2.1.2: AOP-Based Effects Monitoring and Exposure Reconstruction

1. Apply AOP knowledge to development of effects-based monitoring approaches.

2. Methods for collecting and preparing environmental samples for HTS.

3. AOPs to support identification/elimination of causes of biological responses [exposure reconstruction]
One Common Approach to Effects-Based Monitoring

Environmental Sampling

Sample Extraction/Prep

In vitro bioassay
Supervised/Targeted Effects-based Monitoring

Looking under the biological lamp-post

- Will only detect the biological activities we look for
- Effective once a hazard of concern has been identified
- Not ideal for surveillance
- May miss activities that influence in vivo biological/ecological outcomes
Unsupervised Effects-based Monitoring

Ability to detect what we might not expect

- Take advantage of HTP to rapidly/cost effectively screen wide range of biological activities

- Ideal for surveillance

- Identification of targeted assays/endpoints for subsequent monitoring of status and trends

- More complete picture of how mixed biological activities influence in vivo biological/ecological outcomes
Fish exposed in situ

Surface water samples/extracts

Unsupervised

DNA-microarray Transcriptomics

Metabolomics

High throughput in vitro screening

Supervised

• Endpoints associated with established adverse outcome pathways:

  • E.g., Biochemical and molecular markers of endocrine disruption and adverse reproductive outcomes.

  • In vitro bioassays
    • MDA-kb2: (anti)androgenic activity
    • T47D: (anti)estrogenic activity
    • H4IIE: dioxin-like contaminants
    • H295R: steroidogenesis inhibitors
    • Others ……
Surveillance
Ambient water sample → Toxcast (subset of assays) → Response profile

Assay/Endpoint Selection
Response profile → AOP knowledge

Monitoring
Ambient water samples → Targeted assay/endpoint → status and trends
Predicting effects of mixtures

- Ambient water sample
- Extract
- Toxcast Phase 3 (subset of assays)
- Analytes detected in sample
- Chemicals analysed in Toxcast (I, II)
- Predicted profile of “positive” responses
- Empirical profile of “positive” responses
- Compare and discuss
Conclusions

• Computational toxicology has important role to play in 21st C ecotoxicology

• AOPs are a critical foundation
Alternative Data for Prospective Assessments

<table>
<thead>
<tr>
<th>Molecular</th>
<th>Cellular</th>
<th>Tissue</th>
<th>Organ</th>
<th>Organ System</th>
<th>Individual</th>
<th>Population</th>
<th>Ecosystem</th>
</tr>
</thead>
</table>

- QSAR, expert systems
- Biomarkers
- Screening & Prioritization
  - Toxcast, Tox21, in vitro

Demographic significance
- Survival
- growth-development
- reproduction
### Effects Data for Diagnostic Assessments

<table>
<thead>
<tr>
<th>Molecular</th>
<th>Cellular</th>
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<th>Organ</th>
<th>Organ System</th>
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<th>Population</th>
<th>Ecosystem</th>
</tr>
</thead>
</table>

#### Endpoint selection for monitoring
- Remediation/restoration
- Effectiveness of regulation

#### Diagnostic assessments
- High priority – chemical(s), class(es)

- **AOP Knowledge**

- **In situ**
  - Effects-based monitoring
- **In vitro and HT screening**
  - Environmental samples
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