Emerging Contaminants and Protection of Aquatic Life: Prioritization and Identification

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There are known knowns. These are things we know that we know. There are known unknowns. That is to say, there are things that we know we don't know. But there are also unknown unknowns. There are things we don't know we don't know.

Donald Rumsfeld
Assessing Risks of Contaminants of Concern

- **Known knowns**: “Conventional” pollutants, e.g., pesticides, PCBs, PAHs, metals, etc.
  - Know how to measure them and have the data to assess risk
  - Require effective exposure monitoring

- **Known unknowns**: PBDEs, PPCPs (including some EDCs), nanomaterials
  - Suspect or know (increasingly) they are present, but don’t have data to assess risk
  - Require prospective assessments

- **Unknown unknowns**: ???
  - Chemicals we either can’t or don’t know to measure—could include mixtures—but may be causing effects
  - Require retrospective (or diagnostic) assessments
Contaminants of Emerging Concern

• Known unknowns (prospective)
  – Daunting “laundry lists” of chemicals for which little/no data exist
  – Need to identify those substances of most concern and acquire data required to assess risk
  – Reliance only on fate/exposure (production volume, persistence, residues) to identify these chemicals problematic
  – Requires ability to estimate possible effects without extensive testing

• Unknown unknowns (retrospective)
  – Adverse effects inferred either from field observations or controlled testing of field samples (including in situ studies)
  – Effects generally associated with complex mixture of stressors
  – Requires ability to associate (chemical) stressors with observed response(s)

Conventional toxicology approaches alone not well suited to meeting these challenges
Conventional Approach to Toxicology

- Empirical emphasis focused on whole animal testing
- “Apical” endpoints
  - Survival, development/growth, reproduction, cancer
- Dose $\rightarrow$ Observe
  - Adverse effects assessed without necessarily understanding how or why they occur
- Test all possible outcomes to determine which are relevant
Traditional Approach to Toxicology

Problems:

1. Costs of testing (money, time, animals)
   - Example: pesticide registration – total costs around $50 M
   - Example: EDSP – Tier 1 $200-400K, Tier 2 $1.25 M, 600-1200 animals

2. Tens of thousands of chemicals to evaluate

3. Species extrapolation challenges (25,000-30,000 species of fish alone)

4. Difficult to address environmental mixtures
TOXICOLOGY

Transforming Environmental Health Protection

Francis S. Collins, George M. Gray, John R. Deeley

Meeting the Scientific Needs of Ecological Risk Assessment in a Regulatory Context

Steven J. Hladonov
U.S. EPA

Tom C. J. Peijs
Procter & Gamble
Services Company NV-SA
(Belgium)

Cornelis J. van Leeuwen
European Commission

Three strategies could move both science and regulation forward.

During the past decade, the field of ecological risk assessment has progressed considerably. Advances have come from such international bodies as the OECD, IUPAC, U.S. EPA, and the European Commission. Critical to these advances is a different scientific approach that changes the present focus on the laboratory to one that assesses risk in a more ecological context. Three strategies could move both science and regulation forward.

Increasing efficiency, cost-effectiveness, and focus
Risk assessment is a laborious process distinguished by levels of increasing complexity, beginning with the laboratory and moving to field observations of ecosystems.

Intelligent Testing Strategies in Ecotoxicology: Mode of Action Approach for Specifically Acting Chemicals

Technical Report No. 102

www.sciencemag.org
Predictive Toxicology

- The science of making predictions of toxicity outcomes based on previously untested relationships  
  (Ramos et al. 2007)

- Identify organizing principles that underlie biological response to chemicals

- Use that knowledge in a systematic fashion to predict, based on physical/chemical properties, a priori knowledge, and/or simplified bioassays, the likelihood that a given chemical will elicit an adverse effect or that an observed response might be associated with a given chemical

  - Grounded in established and verifiable theory
  - Transparent
  - Reasonable and quantifiable uncertainty
  - Optimal use of available resources and data
Predictive/Mechanistic Toxicology in Ecological Risk Assessments

• Prioritization (P)
  – Depending on degree of allowable uncertainty, could be used to eliminate chemicals from testing

• Focus testing (P)
  – Species, endpoints, experimental design

• Cross-species/chemical extrapolations (P,R)

• Exposure analysis/reconstruction (R)
  – Critical for non-persistent chemicals

• Support diagnostic approaches to ascertain chemicals (or chemical classes) responsible for observed effects (R)
Challenges in the Application of Mechanistic Toxicology to Ecological Risk Assessment

• Many of the “tools” require specialized training/facilities
  – Histological analyses
  – In vitro (tissue, cell) assays
  – Alterations in gene/protein/metabolite expression or abundance

• Complex data analysis
  – Bioinformatic challenges can be substantial (e.g., “omics”)
  – Confusing or contradictory information (e.g., due to lack of baseline knowledge)

• Translation of information into endpoints meaningful to risk assessment not always apparent
  – Both a science and communication issue
In Press:
*Environmental Toxicology and Chemistry*

**Adverse Outcome Pathways:**
A Conceptual Framework to Support Ecotoxicology Research and Risk Assessment.

Gerald T. Ankley, Richard S. Bennett, Russell J. Erickson, Dale J. Hoff, Michael W. Hornung, Rodney D. Johnson, David R. Mount, John W. Nichols, Christine L. Russom, Patricia K. Schmieder, Jose A. Serrano, Joseph E. Tietge, Daniel L. Villeneuve

http://www3.interscience.wiley.com/journal/122596462/issue
Adverse Outcome Pathway

Definition:

Adverse Outcome Pathway (AOP):

*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*

Builds on the “toxicity pathway” concept described by NRC (2007)

Designed for the translation of mechanistic information into endpoints meaningful to ecological risk
“Cellular response pathways that when sufficiently perturbed are expected to result in adverse health effects”

AOP Examples (Ankley et al. in press)

- Narcosis
- Photo-Activated Toxicity
- AhR Mediated Toxicity
- Estrogen Receptor Activation
- Impaired Vitellogenesis
Example of an AOP in fathead minnows exposed to an aromatase inhibitor
Multiple AOPs converging at common insult of impaired vitellogenesis
Insights from the Impaired Vitellogenesis AOP

• Prospective Assessments (prioritizing)
  – Support endpoint selection for short-term in vivo screens (VTG in females)
  – Focus in vitro assay and QSAR model development (inhibition of aromatase, ER antagonism, AR activation)

• Retrospective Assessments (diagnosing)
  – Identification of classes of causative chemical stressors (e.g., inhibitors of steroidogenesis)
  – Interpretation of biomarker data (sex steroids, VTG) relative to possible population responses
Mechanistic Toxicology Approaches in Prospective Ecological Assessments

• Prioritization/extrapolation based on existing knowledge
  – Use of high-quality, searchable sources of ecotoxicology data
  – Consideration of data from human health-oriented studies
    • Evaluation of biological targets, pathway conservation and potency (e.g., pharmaceuticals)

• Pathway-specific computational models to predict effects

• Prioritization/extrapolation based on pathway identification for untested chemicals
  – Short-term in vivo and in vitro assays
  – Responses reflective of specific pathways
Welcome to ECOTOX Release 4.0. The ECOTOX (ECOTOXicology) database provides single chemical toxicity information for aquatic and terrestrial life.

For information on the latest data releases please see the Recent Additions.

View the Quick User Guide (PDF, 2 p. 244 KB) to help get you started.

You will need to turn off pop-up blockers for this site.

You should consult the original scientific paper to ensure an understanding of the context of the data retrieved from the ECOTOX database.

http://cfpub.epa.gov/ecotox/
In ECOTOX’s Advanced Database Query one can access independently compiled data sets.

ECOTOX has been adding data fields quarterly (12/09)
Pharmaceuticals in the Environment (PiE)

• PiEs considered CECs for several reasons
  – Increasingly detected in drinking and surface waters
  – Potentially 1000s of parent compounds and metabolites
  – High public visibility (human health)
  – Potential risk to fish/wildlife populations (EE2, diclofenac)

• May not be highly persistent in conventional sense
  – Pseudo-persistence significant issue

• Often target conserved pathways

• Tend not to be acutely toxic but can be extremely potent
  – Fish ACRs (acute-to-chronic ratio) >1000

• Little useful exposure/effects data for directly assessing ecological risk
Prioritizing PiEs for Assessment and Monitoring

• Valuable data exist for many drugs collected as part of development/human health safety testing (e.g., www.drugbank.ca)
  – Basic physico-chemical properties
  – Major degradates and metabolites
  – Biological targets/pathways (primary & side effects)
  – Potency

• Considered in a systematic manner, this information can provide an basis for a screening-level assessment of risk

SETAC Pellston reports on human and veterinary drugs (2005; 2008)

Prioritizing PiEs for Potential Ecological Risk

- Which occur (or likely could occur) in the environment?
  - Empirical data (e.g., Benotti et al.; Wu et al.; Ramirez et al.) or estimates from stability, Kow, etc.

- Is there knowledge of stable and/or biologically-active degradates/metabolites?

- Are the known biological targets/pathways directly relevant to processes controlling populations?
  - Survival, growth/development, reproduction

- What is the degree of conservation of pathways across species?

- How potent are the chemicals?
Conservation of Drug Targets Across Species
Narcosis is “non-specific toxicity resulting from weak and reversible hydrophobic interactions” (Overton, 1901)

Baseline toxicity: if a chemical does not produce toxicity by some more specific mechanism it will act by narcosis, providing it is sufficiently soluble in water at high enough concentrations to achieve required chemical activity

Narcosis is theorized to result from hydrophobic interactions between chemicals and cellular membranes.

Estimated that 60% of industrial chemicals act via this pathway
Narcosis – Baseline Toxicity

Not all linkages are known with absolute certainty in this AOP

- Non-polar Narcotics
- Cellular Membranes
  - Changes in fluidity / transport
- Neurons (Multiple)
- CNS/ Multiple Organ types
  - ↓ Respiration, ↓ Metabolic rate
- Organism
  - Equilibrium loss, Mortality
- Population
  - Declining trajectory

... but the relationship between chemical property and adverse outcome is well established
Fathead Minnow 96-h Toxicity

Data from Russom et al. 1997. ET&C, 16, 948-967
Robust Predictive QSAR

Non-polar Narcotics
Numerous Chemicals → Cellular Membranes → Changes in fluidity / transport → Neurons (Multiple) → CNS/ Multiple Organ types → Organism → Equilibrium loss, Mortality → Population Declining trajectory

Log Kow

96 h LC50
Identifying Pathways of Concern for Untested Chemicals

- No empirical data for majority of chemicals in commerce

- Computational models helpful for predicting baseline toxicity, but not available for many “reactive” pathways

- Collection of focused biological data for previously untested chemicals and comparison to responses for tested chemicals one viable option
  - Suites of in vitro assays for well-defined pathways
  - Short-term in vivo assays for pathway “discovery” and/or simultaneously monitoring multiple pathways (toxicogenomics)
ToxCast Background

• Coordinated through EPA/ORD National Center for Computational Toxicology
  • Phase 1 data publically available; Phase 2 ongoing

• Addresses chemical screening and prioritization needs for pesticidal inerts, anti-microbials, drinking water contaminants, HPVs, etc.

• Comprehensive use of HTS technologies to generate fingerprints and predictive signatures reflective of biological pathways of concern

• Oriented toward human health, but covers many conserved pathways
ToxCast *in vitro* HTS Assays

**Biochemical Assays**

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

- **Assay formats**
  - Radioligand binding
  - Enzyme activity
  - Co-activator recruitment

**Cellular Assays**

- **Cell lines**
  - HepG2 human hepatoblastoma
  - A549 human lung carcinoma
  - HEK 293 human embryonic kidney

- **Primary cells**
  - Human endothelial cells
  - Human monocytes
  - Human keratinocytes
  - Human fibroblasts
  - Human proximal tubule kidney cells
  - Human small airway epithelial cells

- **Biotransformation competent cells**
  - Primary rat hepatocytes
  - Primary human hepatocytes

- **Assay formats**
  - Cytotoxicity
  - Reporter gene
  - Gene expression
  - Biomarker production
  - High-content imaging for cellular phenotype

467 Endpoints
Phase I ToxCast
309 Unique Chemicals

- 276 Conventional Actives
- 16 Antimicrobials
- 9 Industrial Chemicals
- 8 Metabolites

www.epa.gov/ncct/toxcast/
Linkage of Exposure and Effects Using Genomics, Proteomics, and Metabolomics in Small Fish Models

- USEPA (NERL) – Cincinnati, OH
  - D. Bencic, M. Kostich, D. Lattier, J. Lazorchak, G. Toth, R.-L. Wang
- USEPA (NHEERL) – Duluth, MN, and Grosse Isle, MI
- USEPA (NERL) – Athens, GA
  - T. Collette, D. Ekman, M. Henderson, Q. Teng
- USEPA-RTP, NC
  - M.&M. Breen, R. Conolly (NCCT)
  - S. Edwards (NHEERL)
- USEPA (NCER) STAR Program
  - N. Denslow (Univ. of Florida), E. Orlando, (Florida Atlantic University), K. Watanabe (Oregon Health Sciences Univ.), M. Sepulveda (Purdue Univ.)
- USACE – Vicksburg, MS
  - E. Perkins, N. Garcia-Reyero
- Other partners
  - UC-SB, J. Shoemaker, K. Gayen (Santa Barbara, CA)
  - Joint Genome Institute, DOE (Walnut Creek, CA)
  - Sandia, DOE (Albuquerque, NM)
  - Pacific Northwest National Laboratory (Richland, WA)
Fathead Minnows
**Chemical Probes**

1. Fipronil (-)
2. Muscimol (+)
3. Apomorphine (+)
4. Haloperidol (-)
5. Trilostane (-)
6. Ketoconazole (-)
7. Fadrozole (-)
8. Prochloraz (-,-)
9. Vinclozolin (-)
10. Flutamide (-)
11. 17β-Trenbolone (+)
12. 17α-Ethynylestradiol (+)
Endpoints and Analysis

- Global (microarray) and focused (QPCR) gene expression changes

- Protein and metabolite profiles

- Apical effects: steroids, gonad histology, reproduction

- Linked physiological and population models in a systems biology framework to facilitate prediction of effects
Prospective Assessment Application:
Screening to detect different classes of EDCs

Small battery of molecular responses measured by real-time PCR array

- Established linkage to impaired reproduction in fish & diagnostic utility
- Robust across species
- Short-term exposure duration (e.g., 4 d)
- Analyses amenable to HTS automation
- Design based on robust power analysis for all responses
Mechanistic Toxicology Approaches in Retrospective Assessments

- In vitro assays with complex samples (water, sediment) from the field
- Short-term in vivo assays conducted with field samples in the lab or in situ (caged animals)
- Collection of organisms from extant populations

Molecular/biochemical/histological endpoints reflective of defined biological pathways
Examples of Mechanistic Assays/Endpoints for Retrospective Assessment of EDCs

• Cell lines transfected with estrogen/androgen receptor-reporter (e.g., luciferase) gene constructs

• Measurement of changes in single gene/protein expression (e.g., vitellogenin [VTG] in fish)

• Evaluation of changes in multiple gene, protein, metabolite expression profiles (i.e., transcriptomics, proteomics, metabolomics)

• Determination of histological abnormalities (e.g., testis-ova) in fish collected from the field
VTG Induction as an Indicator of Estrogen Exposure in Fish

- **Chart a**: ng of E2/ml Plasma vs. Hours Post Treatment
- **Chart b**: fmol of VTG mRNA/ug Total RNA vs. Hours Post Treatment
- **Chart c**: ng of VTG/ml Plasma vs. Hours Post Treatment
Considerations in Assay/Endpoint Selection: VTG Induction as a Case Example

• Ease/cost of measurement
  – Commercial PCR (mRNA) or ELISA (protein) kits

• Sensitivity, rapidity and persistence of response
  – Occurs w/i hours at ng/L estrogen concentrations and can remain elevated long-term

• Specificity for pathway of concern
  – No chemicals other than estrogens induce VTG in males

• Linkage to biological impacts
  – Estrogens well established reproductive toxins in fish
  – Concentrations of EE2 (ca. 1 ng/L) that cause VTG induction comparable to those reducing production of fertile eggs
Impaired spawning behavior
Reduced fecundity
Altered "X-Y" gamete ratio
Increased VTG production
Hepatocyte
Increased VTG production
Multiple Cell Types
Feminized phenotype
Neurons
Impaired development & ovulation
Testes
Intersex
Sex reversal
Liver-Blood
Increased VTG in males
Multiple Tissues
Feminized phenotype
Brain
Established mechanistic linkage with quantitative or semi-quantitative data
Plausible linkage with limited data
Empirical linkage based on quantitative exposure-response data
Predictive model linkages based on chemical structure/property and quantitative exposure-response data
Hypothetical linkage
Potential biomarker associated with exposure/response information

AOPs for putting Biomarkers in Context: VTG Induction and Reproduction
Establishing Causation: The Role of TIE

- Retrospective assessments rely on observation of biological responses to indicate potential impacts
- Complex mixtures of chemical stressors are present
- Toxicity identification evaluation (TIE) techniques developed in late 80’s to identify toxicants causing lethality in WWTP effluents (NPDES)
- TIEs with mechanistic endpoints
  - Estrogenic WWTP effluent in UK (EE2)
  - Androgenic discharges from pulp/paper mills
  - Surface water associated with agriculture
Mechanism-Based TIEs: A Pulp Mill Case Study

Buckeye Plant, Fenhalloway River, FL

Collaborators: G. Ankley, L. Durhan (EPA, MED); E. Gray, P. Hartig, C. Lambright, L. Parks, V. Wilson (EPA, RTD); L. Guillette (Univ. Florida)
Cell-Based Assays for Detecting EDCs in Mixtures

I. Media made with Field Sample

II. Measure Luciferase Activity
PME Androgenic Activity

![Bar graph showing log10 fold induction over media control for different substances: Con, PME 1, PME 2, PME 2, Up Fen, Econfina, DHT. The y-axis represents log10 fold induction, and the x-axis represents different substances. The graph shows varying levels of induction for each substance.](image)
Application of TIE Analysis to Androgenic PME
Linking Androgenic Activity in Cells to Fractionation of PME

Androgenic Activity

HPLC Fraction Number

Androstenedione

Media
Summary and Conclusions

• Predictive/mechanistic toxicology tools have substantial potential for assessing the ecological risk of CECs

• Prospective assessments
  – Efficient use of existing knowledge/concepts
  – Computational (QSAR/SAR) models
  – Pathway-based in vitro/in vivo bioassays

• Retrospective assessments
  – Pathway-based bioassays with field samples
  – Mechanistic endpoints in organisms from the field
  – Fractionation/TIE to address mixtures