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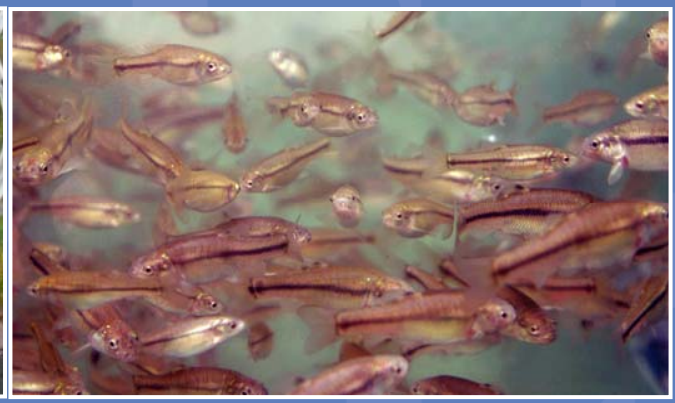
Emerging Contaminants and Protection of Aquatic Life: Prioritization and Identification

Gary Ankley

USEPA, Duluth, MN



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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

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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

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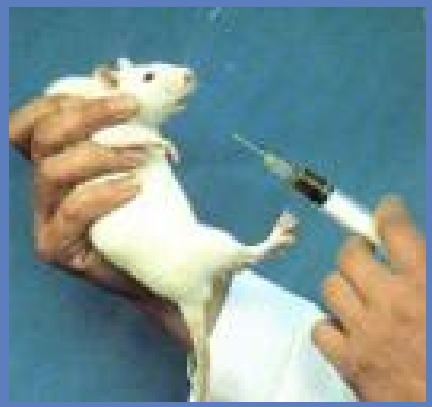
Contaminants of Emerging Concern

- 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 → 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

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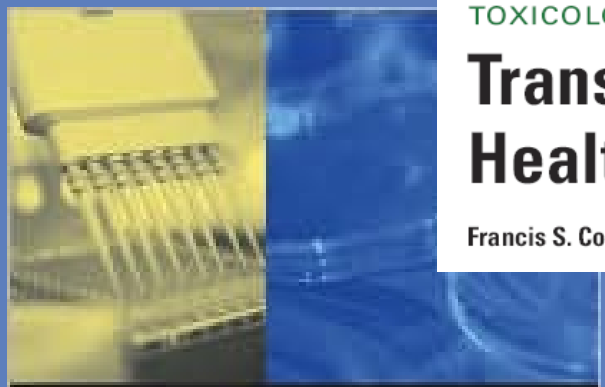

TOXICITY TESTING IN THE 21ST CENTURY
A VISION AND A STRATEGY

POLICYFORUM

TOXICOLOGY

Transforming Environmental Health Protection

Francis S. Collins,[1*†] George M. Gray,[2*] John H. Bucher

15 FEBRUARY 2008 VOL 319 SCIENCE www.sciencemag.org

Meeting *the* Scientific Needs *of* Ecological RISK Assessment *in* a Regulatory Context

STEVEN P. BRADBURY
U.S. EPA

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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

Increasing efficiency, cost-effectiveness, and focus

Risk assessment is a tiered process distinguished by levels of increasing complexity, beginning with the preliminary

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

Technical Report No. 102

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

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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)

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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

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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

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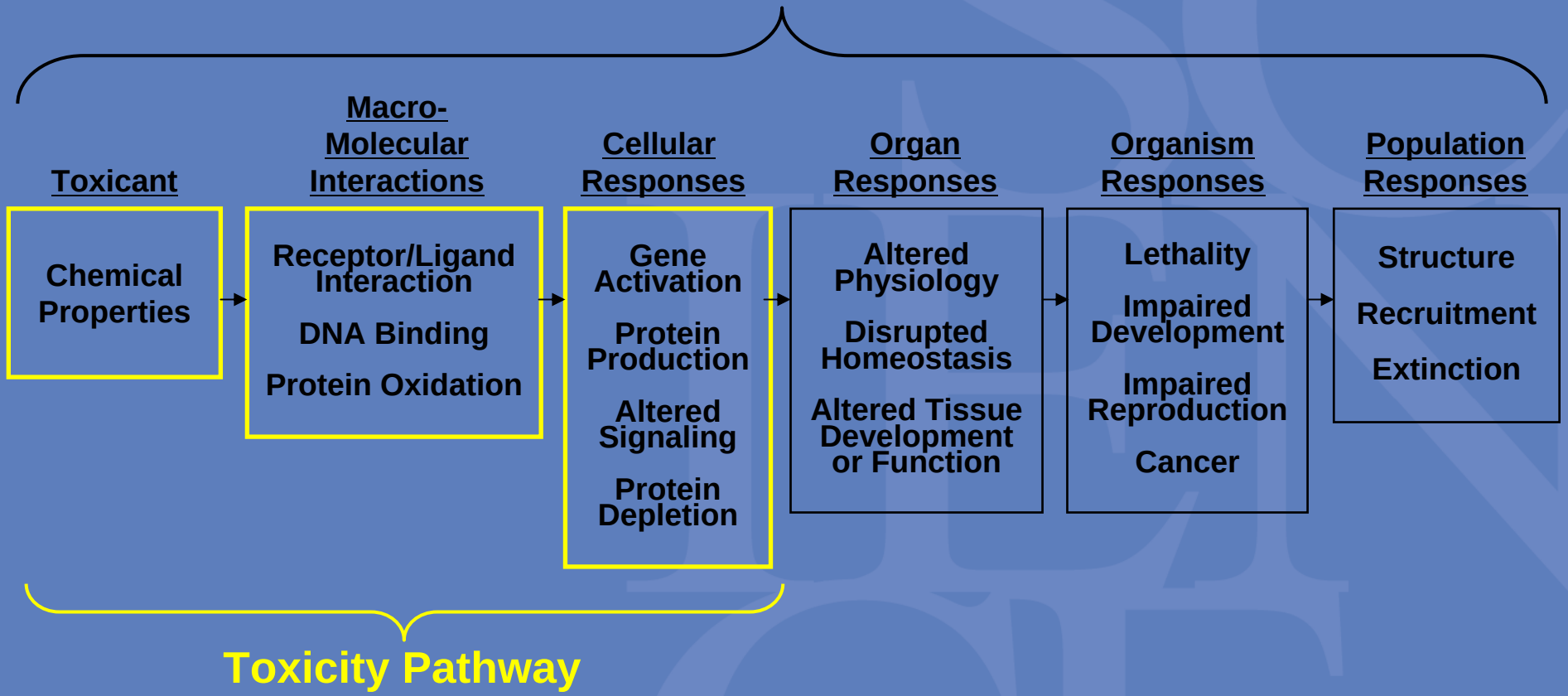
Adverse Outcome Pathway



"Cellular response pathways that when sufficiently perturbed are expected to result in adverse health effects"

Toxicity Testing in 21st Century, NRC 2007.

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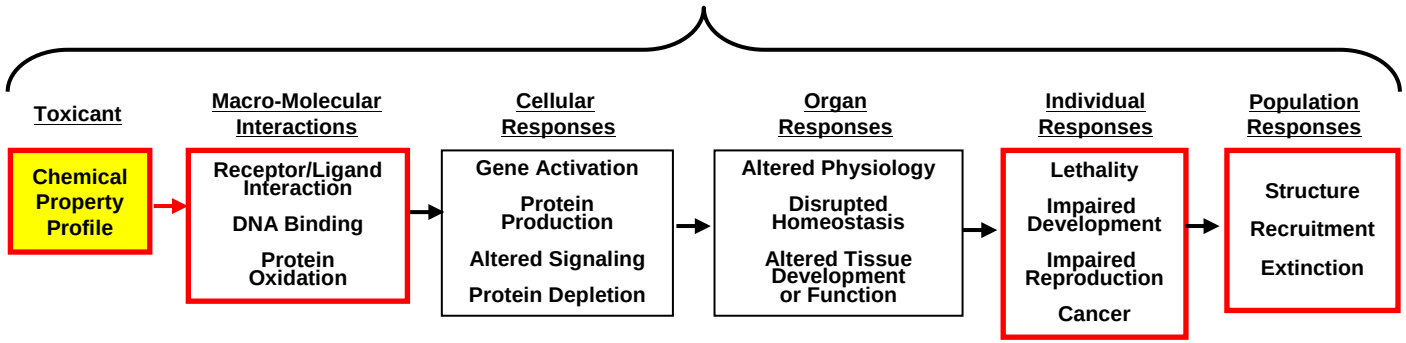
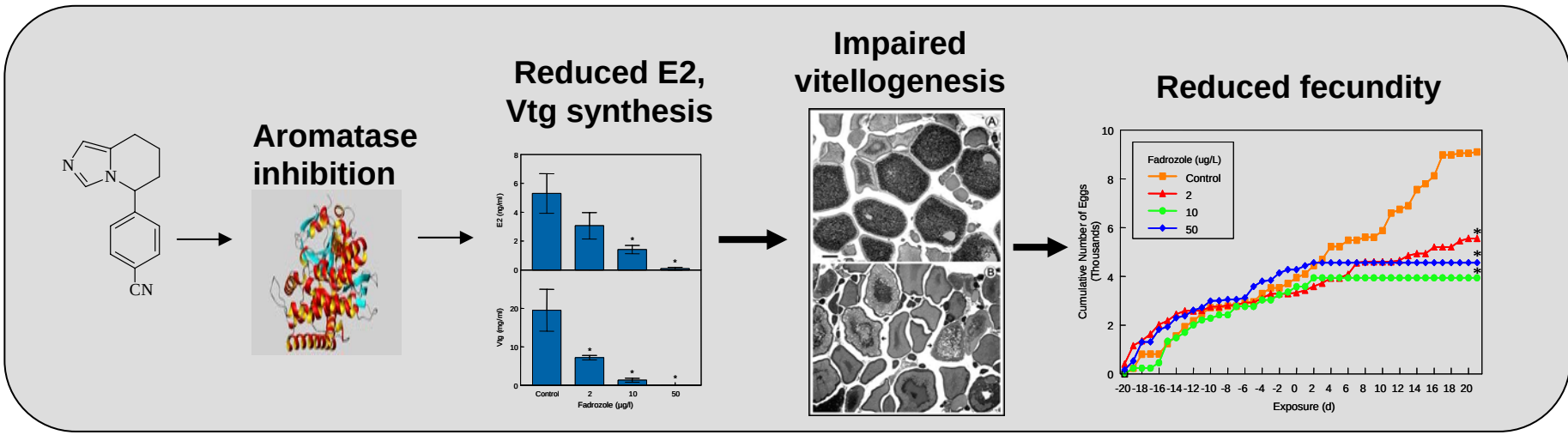
AOP Examples (Ankley et al. in press)

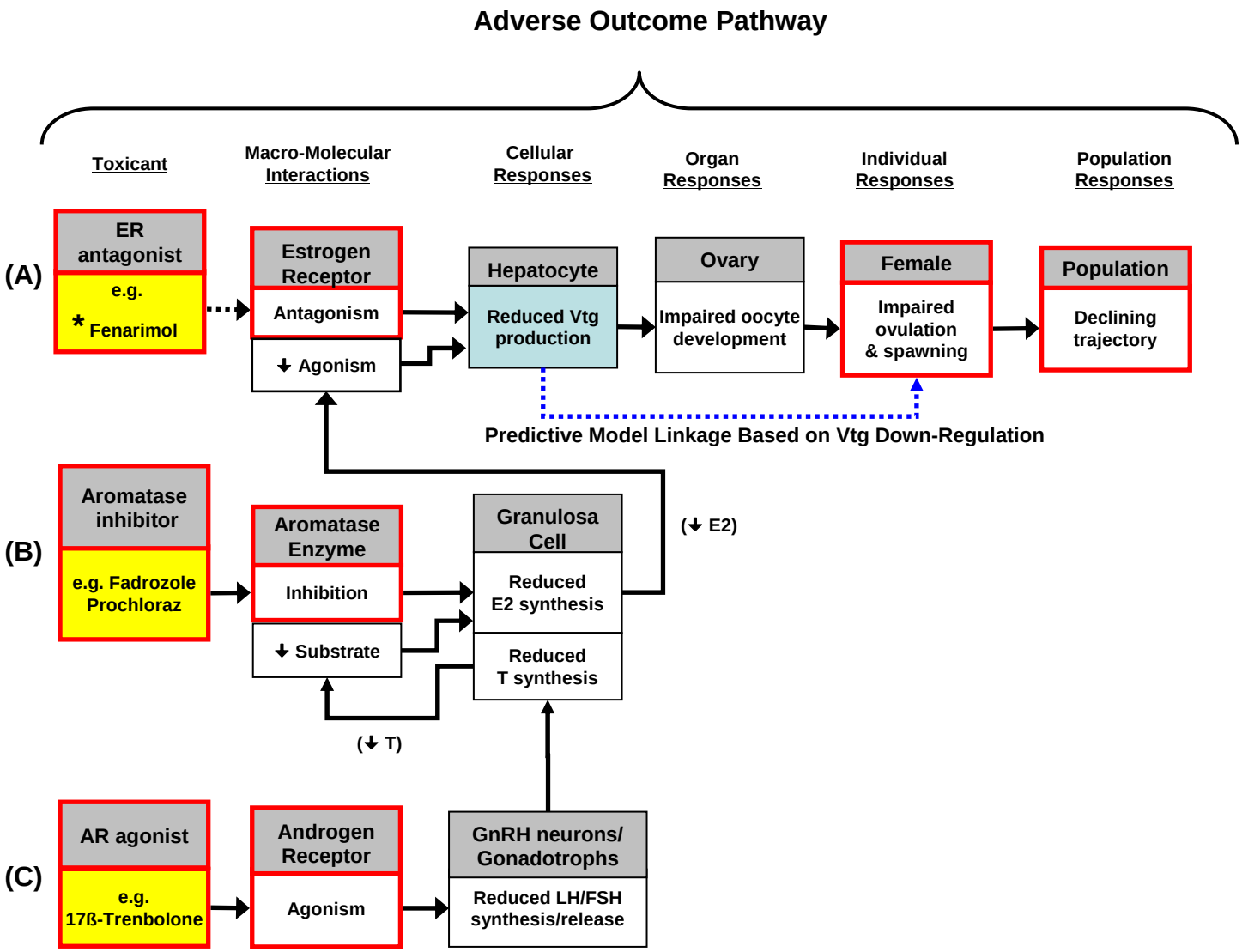
- Narcosis
- Photo-Activated Toxicity
- AhR Mediated Toxicity
- Estrogen Receptor Activation
- **Impaired Vitellogenesis**

Adverse Outcome Pathway





Example of an AOP in fathead minnows exposed to an aromatase inhibitor



Multiple AOPs converging at common insult of impaired vitellogenesis

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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

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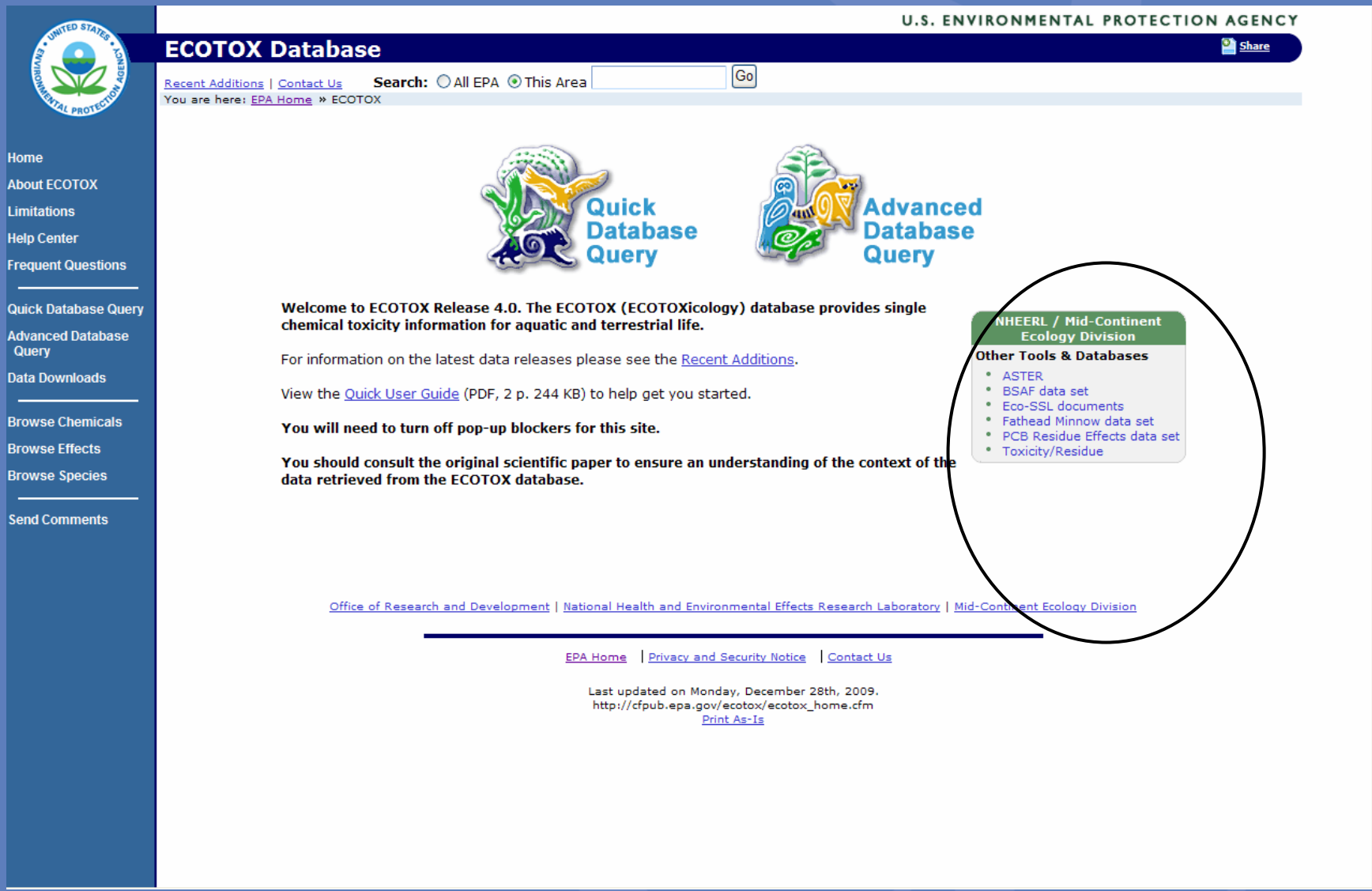
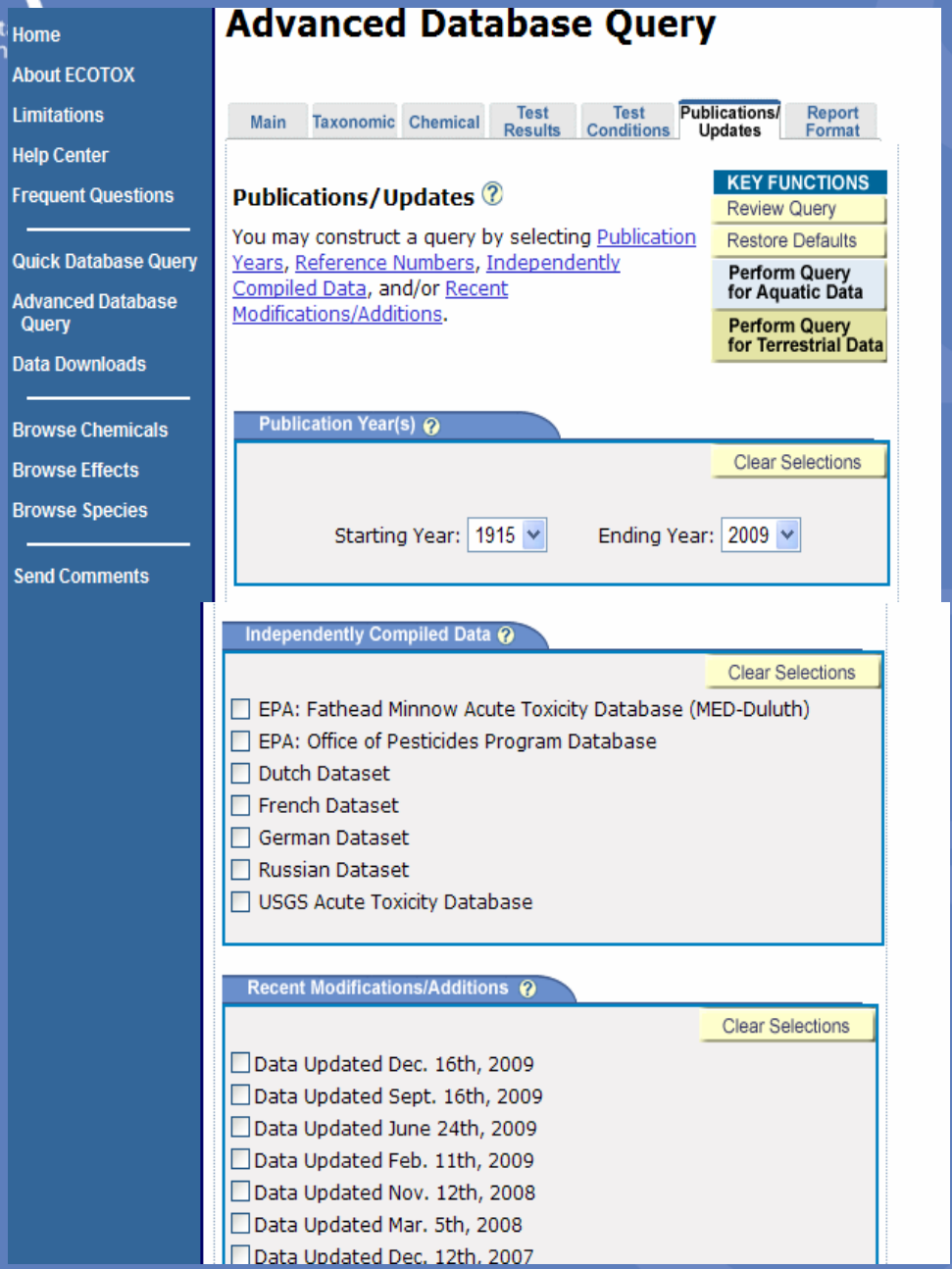
Mechanistic Toxicology Approaches in Prospective Ecological Assessments

- 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

ECOTOX: A Freely Available Data Resource

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)

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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

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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)

Draft CENR report “Pharmaceuticals in the Environment: An Interagency Research Strategy” (2009)

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?

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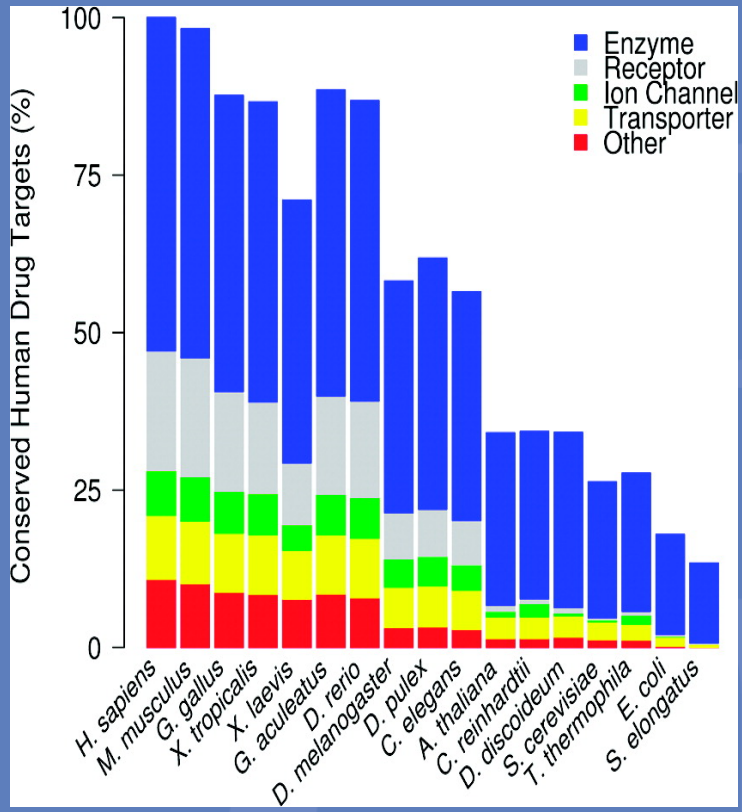
Conservation of Drug Targets Across Species



Published in: Lina Gunnarsson; Alexandra Jauhiainen; Erik Kristiansson; Olle Nerman; D. G. Joakim Larsson; *Environ. Sci. Technol.* **2008,** 42, 5807-5813.
DOI: 10.1021/es8005173

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Computational Models for Predicting Effects: Narcosis Example

Narcosis is “non-specific toxicity resulting from weak and reversible hydrophobic interactions” (Overton, 1901)

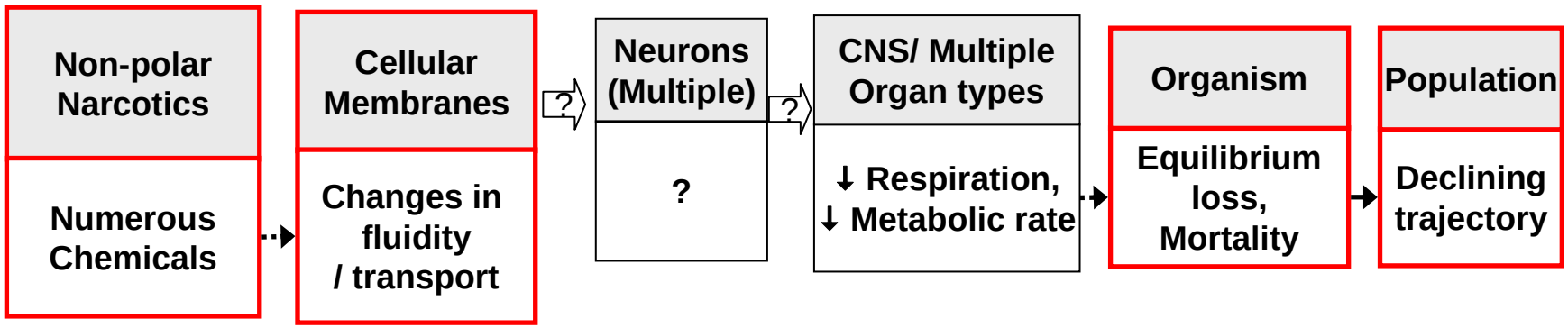
Baseline toxicity: if a chemical does not produce toxicity by some more specific mechanism it will act by narcosis, providing it is sufficiently soluble in water at high enough concentrations to achieve required chemical activity

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



… but the relationship between chemical property and adverse outcome is well established

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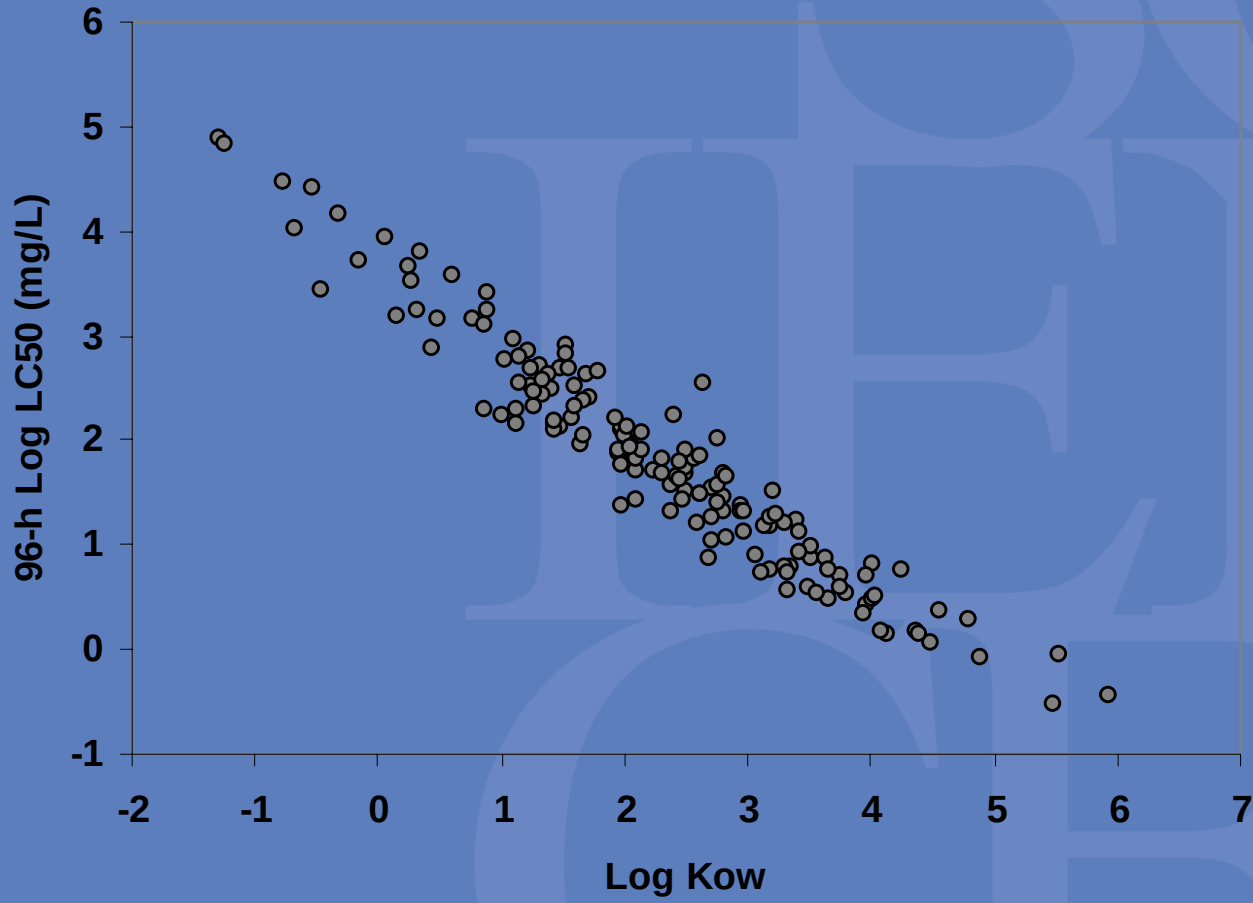
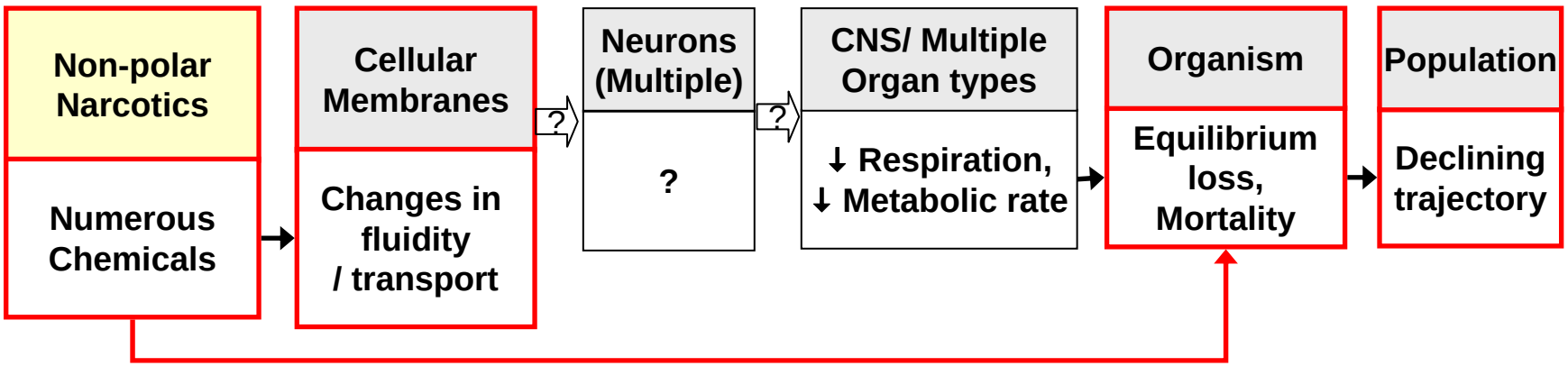
Fathead Minnow 96-h Toxicity



Data from Russom et al. 1997. *ET&C*, 16, 948-967

Robust Predictive QSAR



Log Kow

96 h LC50

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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)

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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

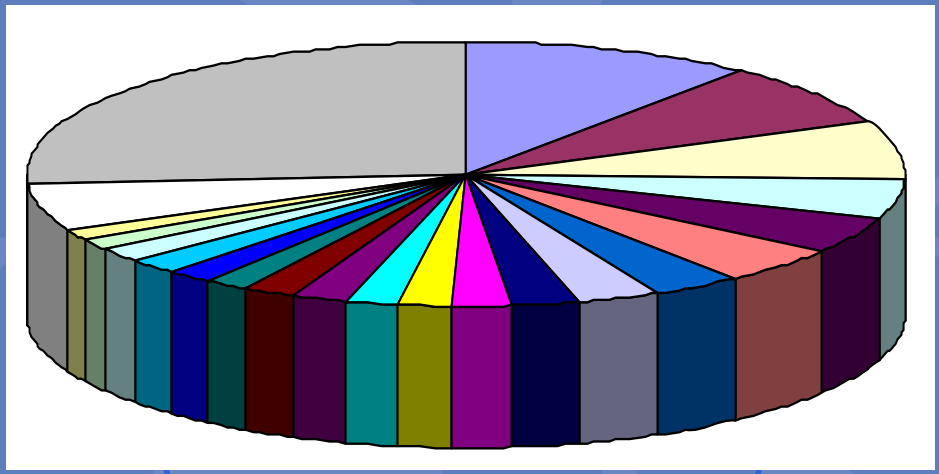
Cellular Assays

- **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



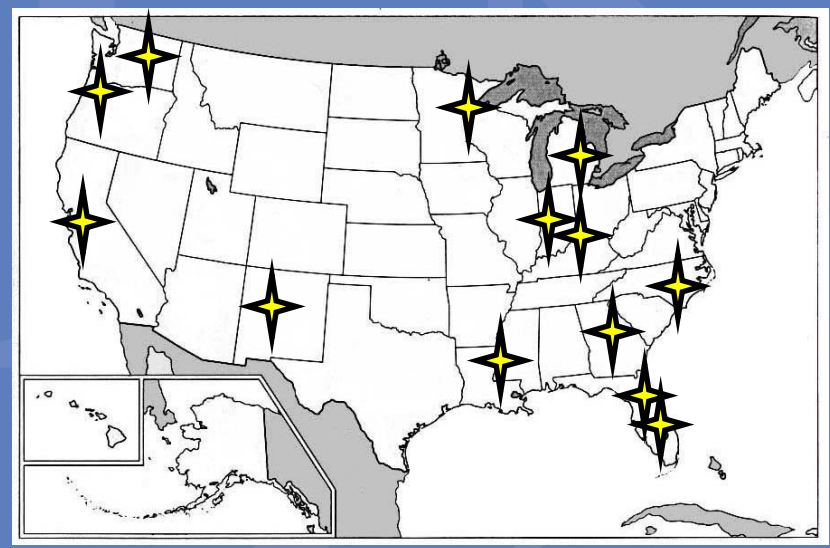
Chemical Class Distribution
(≥5/Class)

- Organophosphorus (39)
- Amide (26)
- Urea (26)
- Conazole (18)
- Carbamate (16)
- Phenoxy (15)
- Pyrethroid (12)
- Pyridine (11)
- Triazine (9)
- Dicarboximide (8)
- Phthalate (7)
- Dinitroaniline (7)
- Antibiotic (7)
- Thiocarbamate (7)
- Pyrazole (6)
- Nicotinoid (6)
- Dithiocarbamate (6)
- Aromatic Acid (6)
- Insect Growth Regulators (5)
- Imidazolinone (5)
- Unclassified (21)
- Other (93)

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
  - G. Ankley, E Durhan, M Kahl, K Jensen, E Makynen, D. Martinovic, D. Miller, D. Villeneuve
- 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)



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Zebrafish

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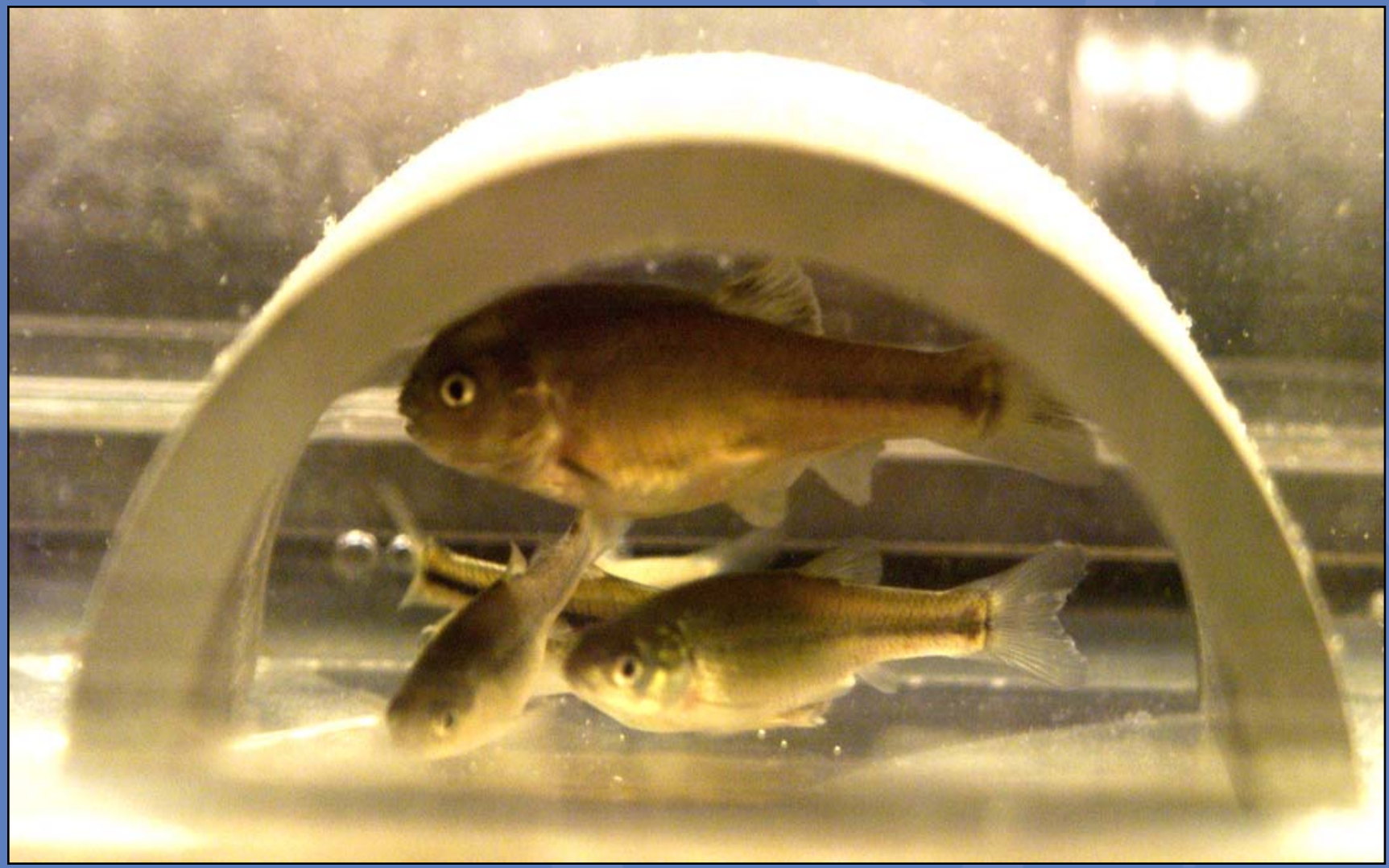
Fathead Minnows



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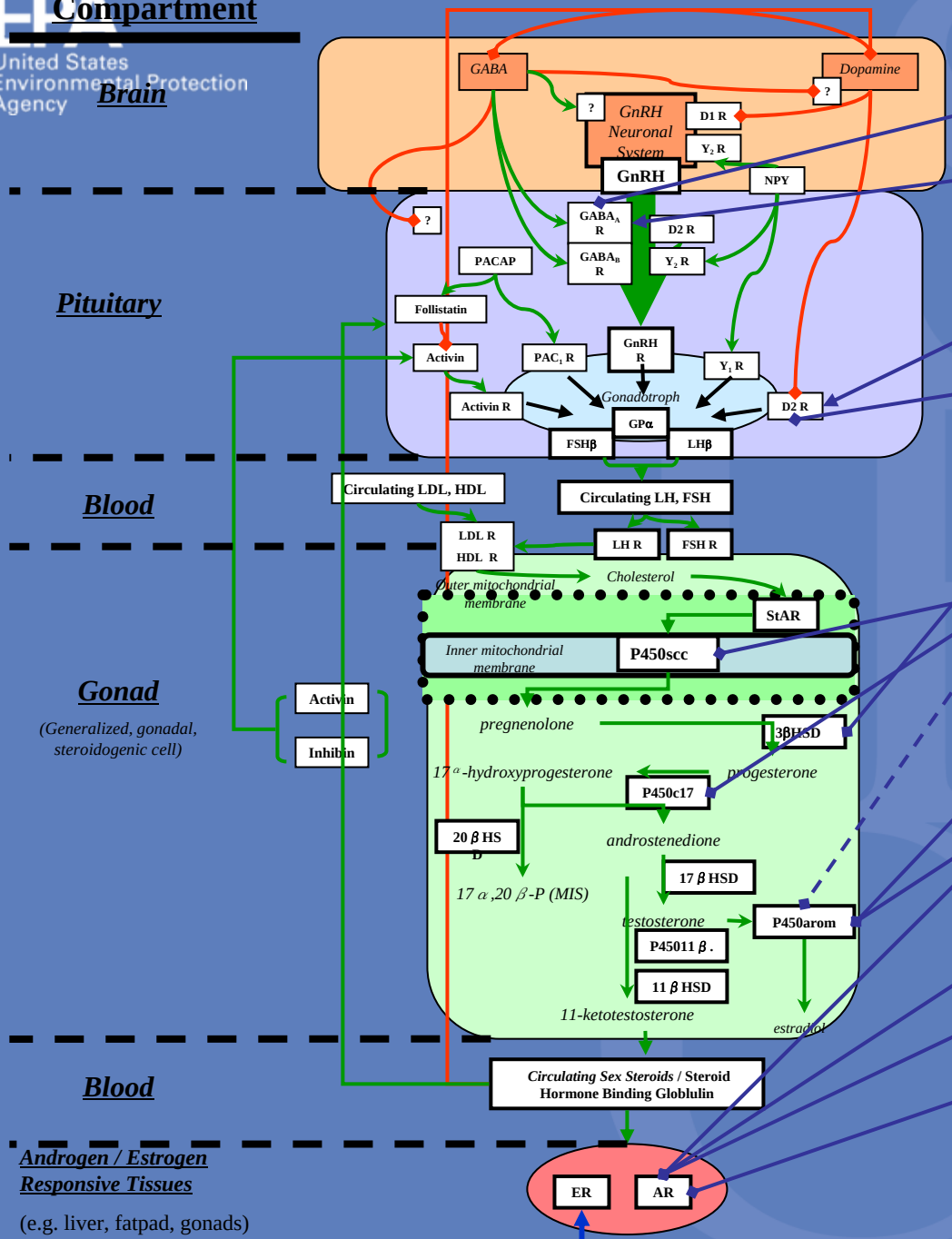
Compartment



Chemical Probes

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

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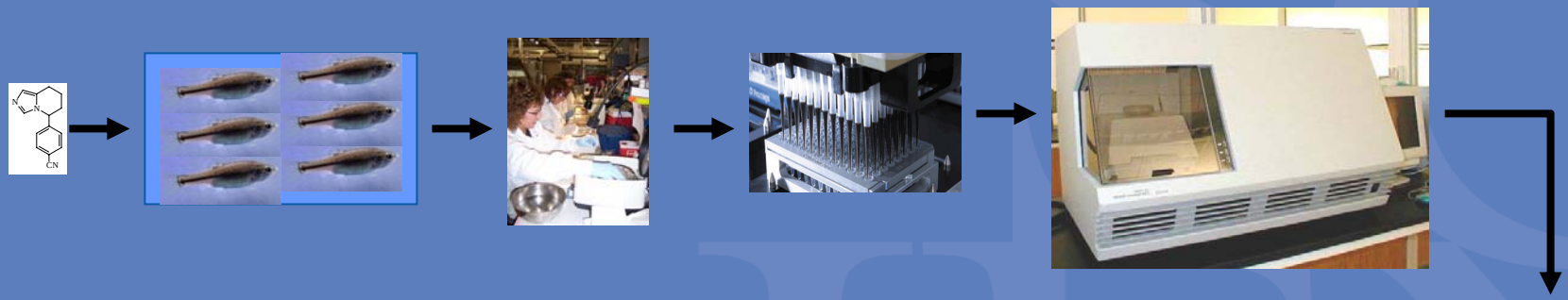
Endpoints and Analysis

- 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

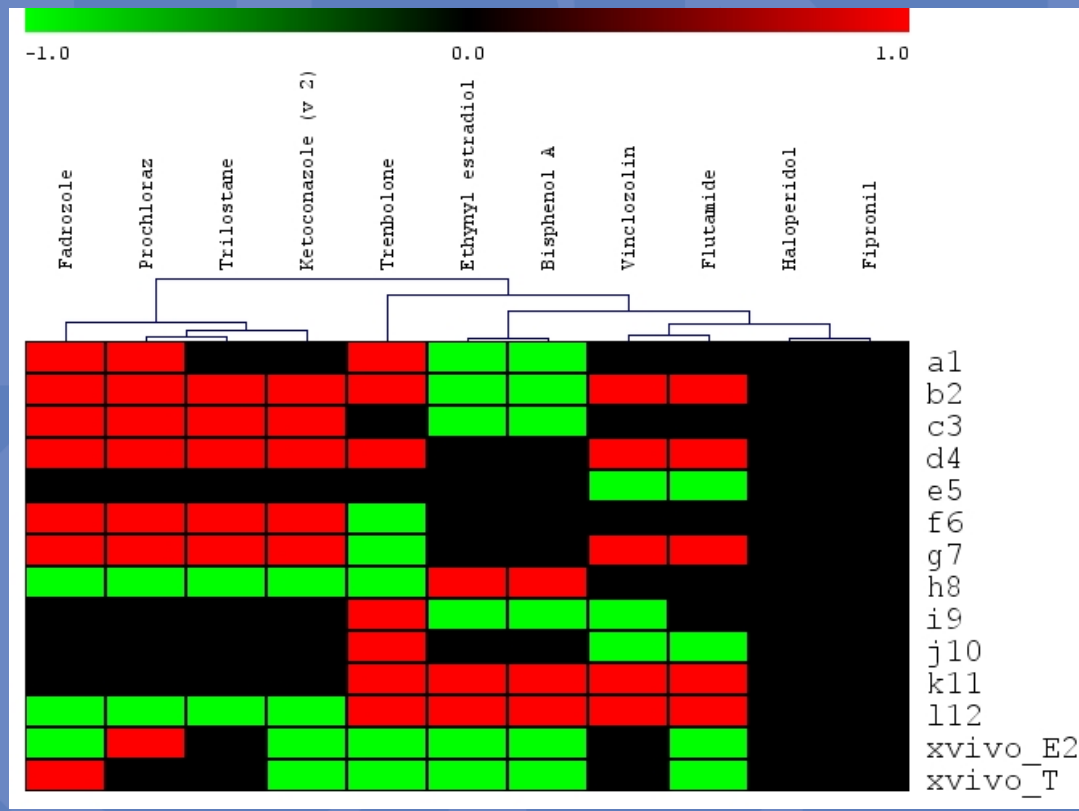
Prospective Assessment Application:

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**



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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

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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

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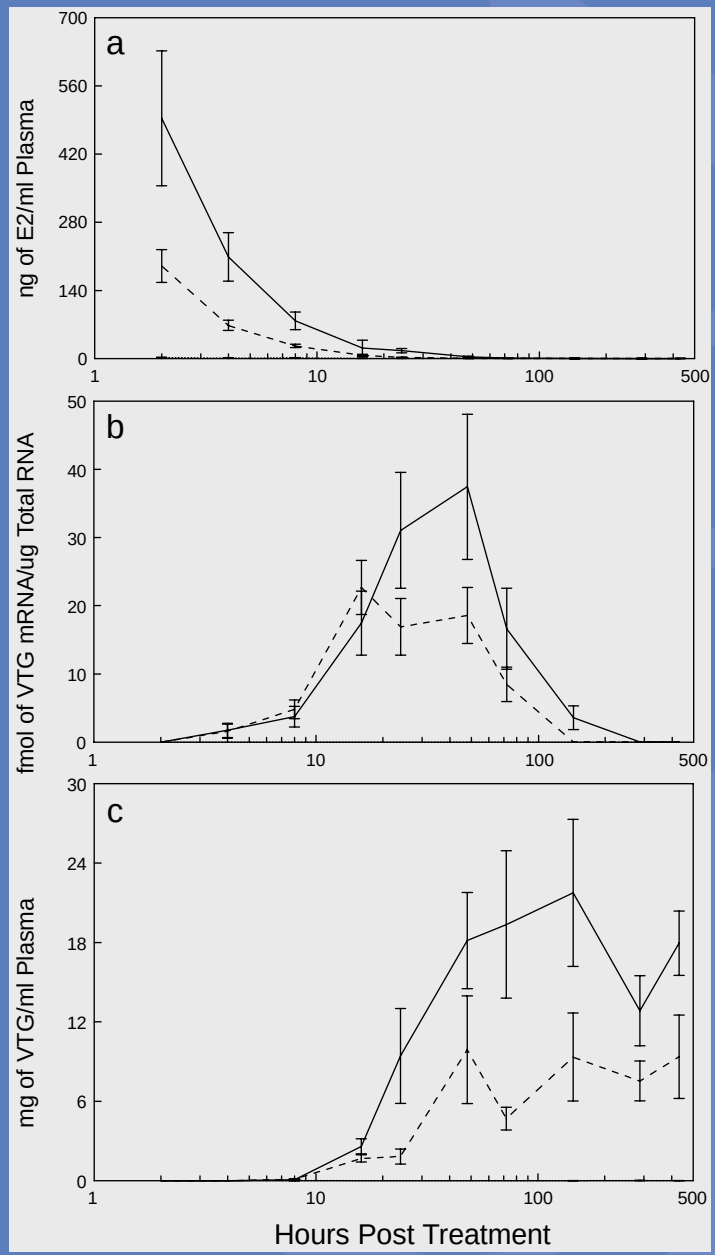
VTG Induction as an Indicator of Estrogen Exposure in Fish



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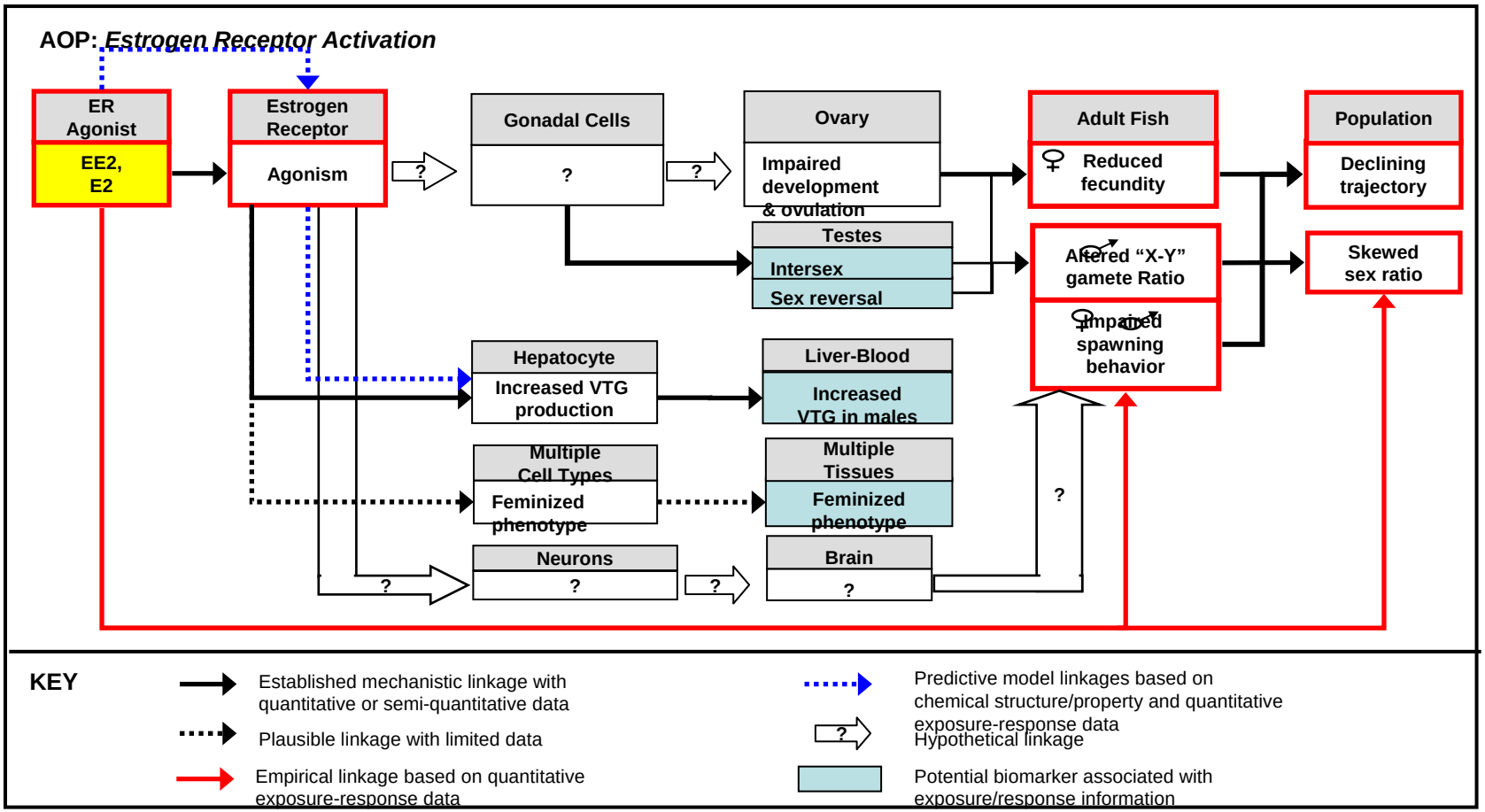
Considerations in Assay/Endpoint Selection: VTG Induction as a Case Example

- 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

AOPs for putting Biomarkers in Context: VTG Induction and Reproduction



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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

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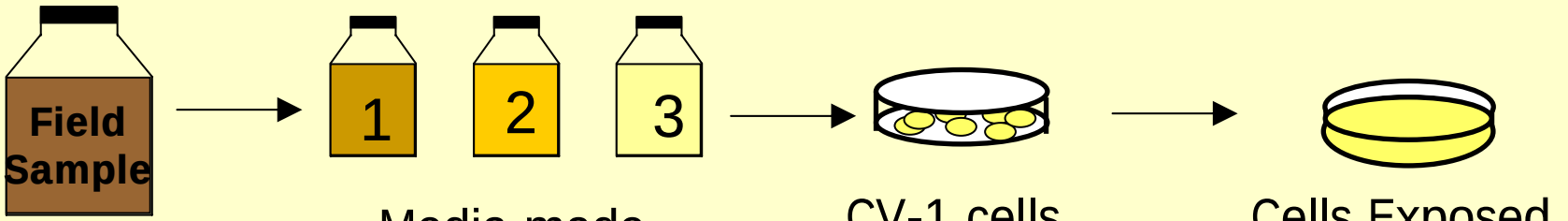
Mechanism-Based TIEs: A Pulp Mill Case Study

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.



II. Measure Luciferase Activity

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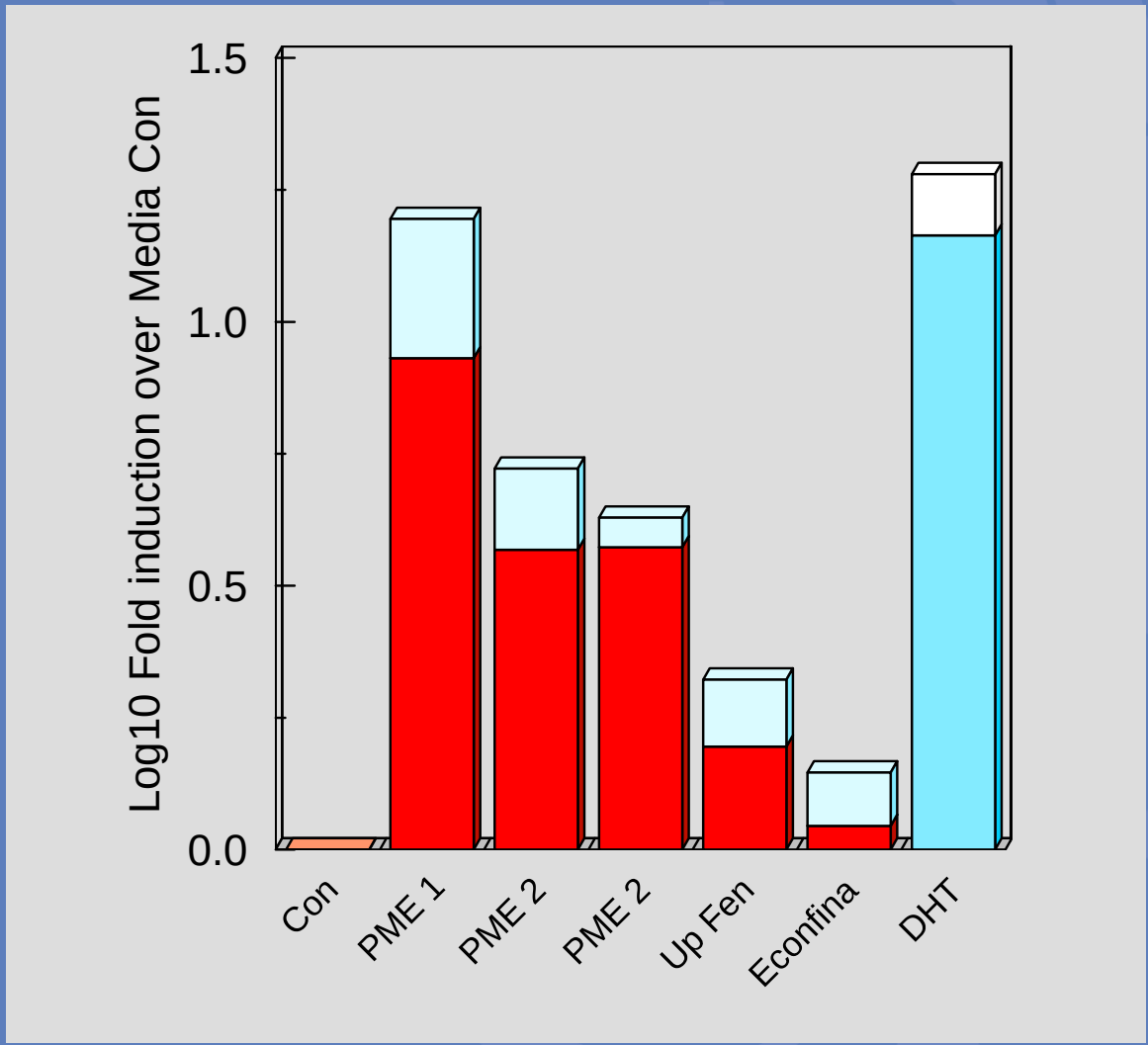
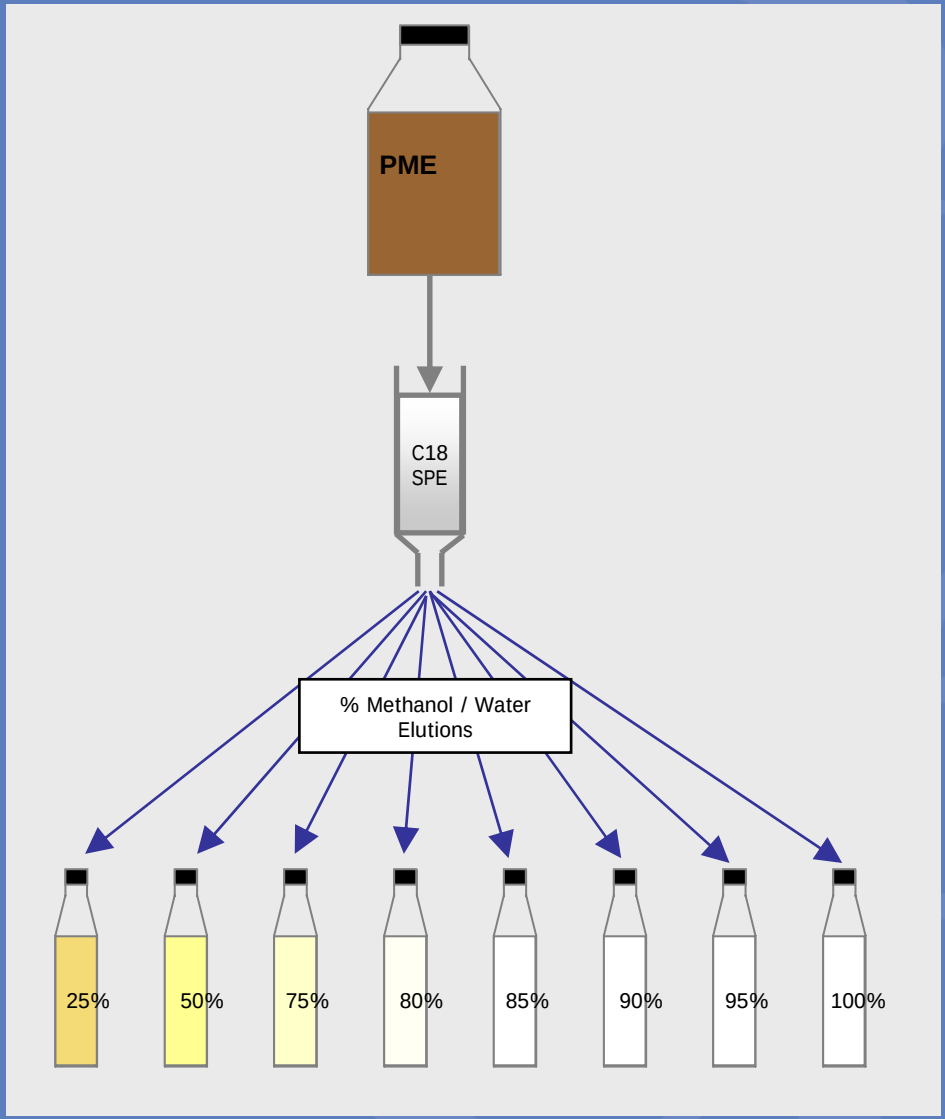
PME Androgenic Activity

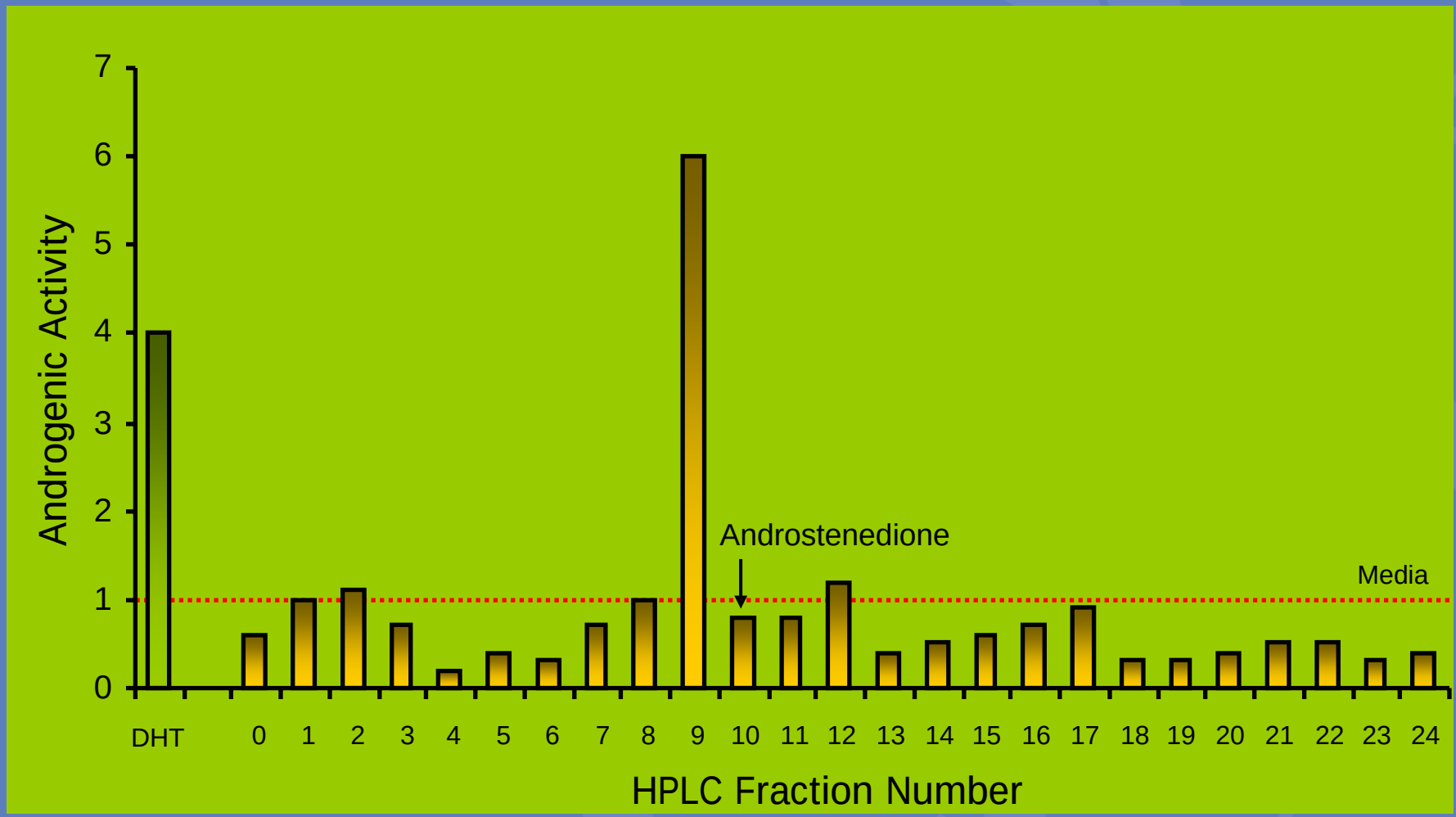


Application of TIE Analysis to Androgenic PME



EPA United States Environmental Protection Agency

Linking Androgenic Activity in Cells to Fractionation of PME



EPA United States Environmental Protection Agency

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