Welcome to the CLU-IN Internet Seminar

Assessing Ecological Risk of Endocrine Disrupting Chemicals: State-of-the-Science Approaches

Sponsored by: National Institute of Environmental Health Sciences, Superfund Research Program

Delivered: April 22, 2010, 1:30 PM - 3:30 PM, EDT (17:30-19:30 GMT)

Instructors:
Nancy Denslow, University of Florida (ndenslow@ufl.edu)
Gerald Ankley, U.S. EPA, Office of Research and Development, Mid-Continent Ecology Division (Ankley.Gerald@epa.gov)

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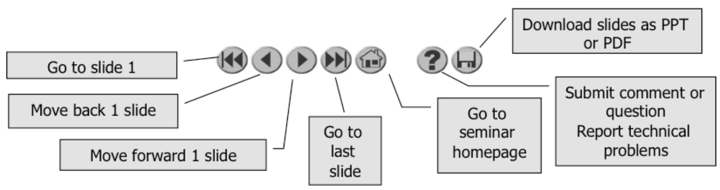
Housekeeping

- Please mute your phone lines, Do NOT put this call on hold
- Q&A
- Turn off any pop-up blockers
- Move through slides using # links on left or buttons



- This event is being recorded
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Although I'm sure that some of you have these rules memorized from previous CLU-IN events, let's run through them quickly for our new participants.

Please mute your phone lines during the seminar to minimize disruption and background noise. If you do not have a mute button, press *6 to mute #6 to unmute your lines at anytime. Also, please do NOT put this call on hold as this may bring delightful, but unwanted background music over the lines and interupt the seminar.

You should note that throughout the seminar, we will ask for your feedback. You do not need to wait for Q&A breaks to ask questions or provide comments. To submit comments/questions and report technical problems, please use the ? Icon at the top of your screen. You can move forward/backward in the slides by using the single arrow buttons (left moves back 1 slide, right moves advances 1 slide). The double arrowed buttons will take you to 1st and last slides respectively. You may also advance to any slide using the numbered links that appear on the left side of your screen. The button with a house icon will take you back to main seminar page which displays our agenda, speaker information, links to the slides and additional resources. Lastly, the button with a computer disc can be used to download and save today's presentation materials.

With that, please move to slide 3.

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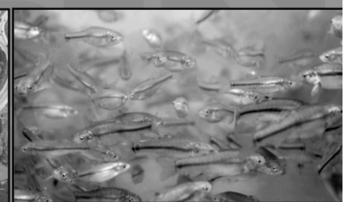
Assessing EDCs in the Field: New Approaches to Old Challenges

G. Ankley



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NHEERL, Mid-Continent Ecology Division, Duluth, MN

April 22, 2010

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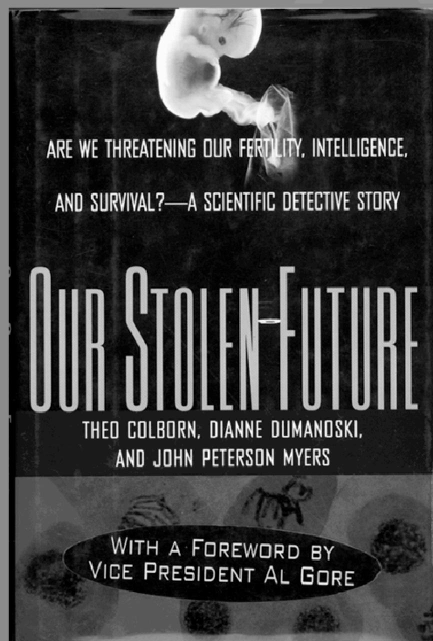

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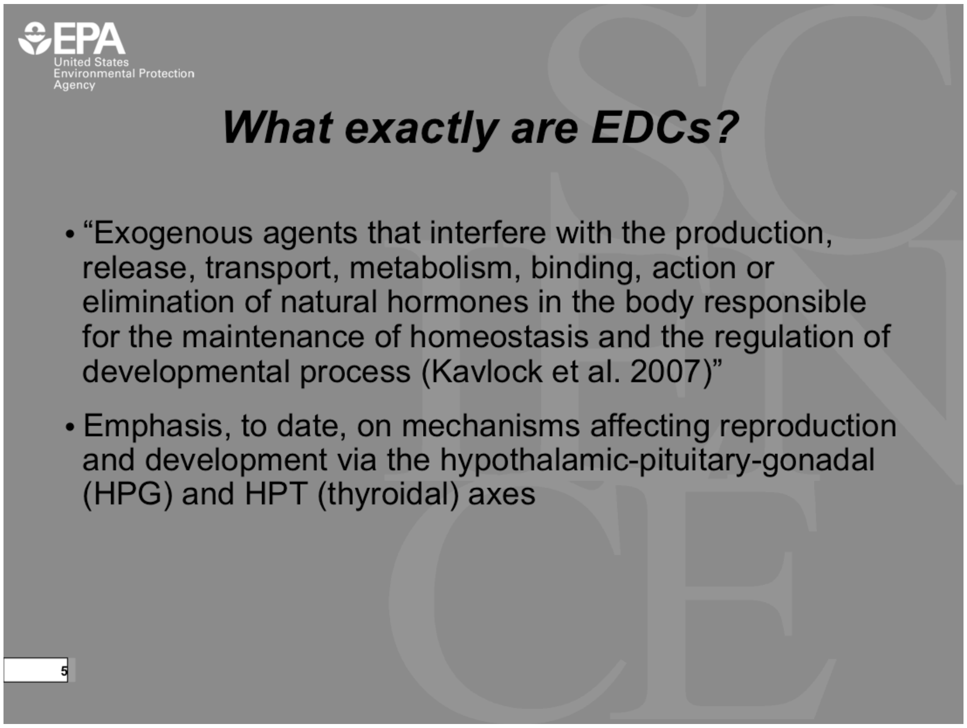
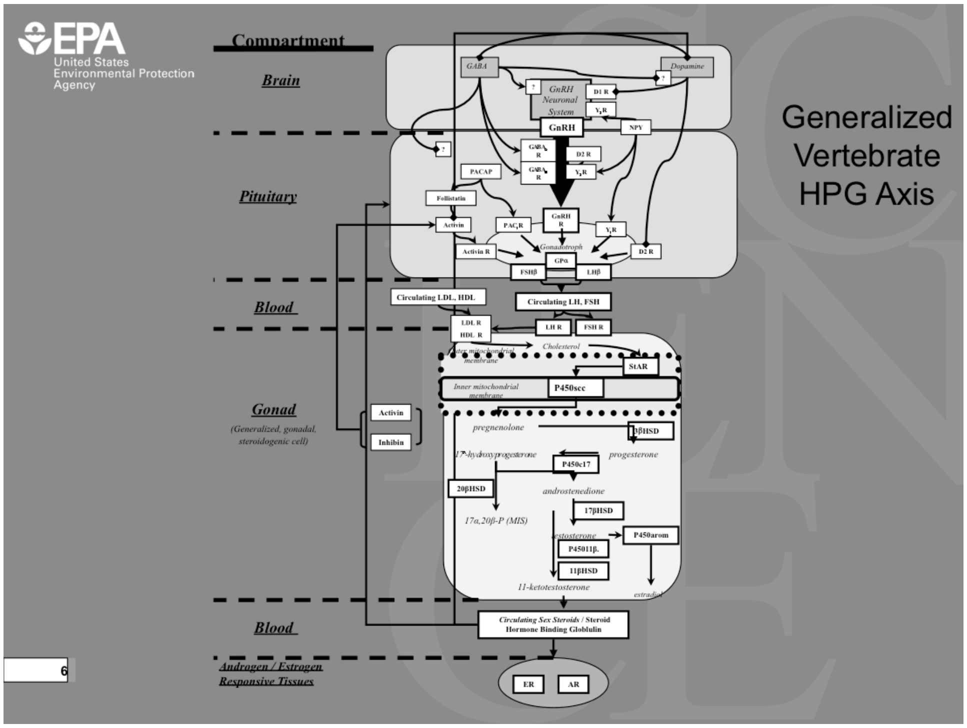


Refer to Mikes talk


EPA
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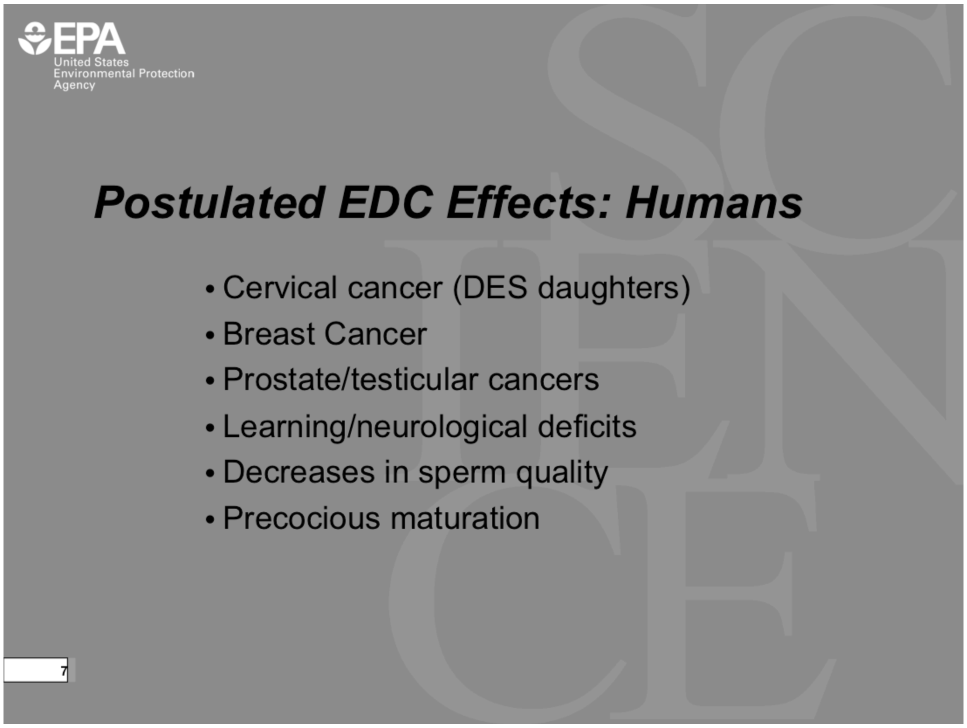
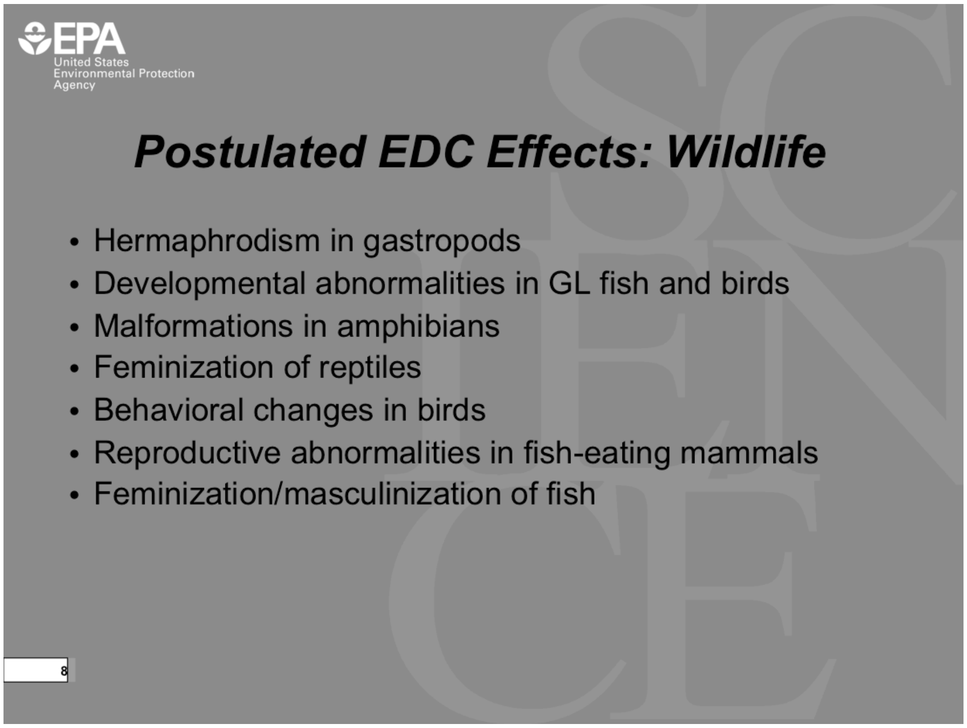
Generalized
Vertebrate
HPG Axis
Compartment
Brain
Pituitary
Blood
Gonad
(Generalized, gonadal, steroidogenic cell)
Blood
Androgen / Estrogen
Responsive Tissues
GABA
Dopamine
GnRH
Neuronal
System
D1 R
NPY
GnRH
PACAP
D2 R
Follistatin
Activin
GnRH
R
Gonadotroph
Activin R
D2 R
GPα
FSHβ
LHβ
Circulating LDL, HDL
Circulating LH, FSH
LDL R
HDL R
LH R
FSH R
Cholesterol
membrane
StAR
Inner mitochondrial
membrane
P450scc
Activin
Inhibin
pregnenolone
3βHSD
progesterone
P450c17
20βHSD
androstenedione
17α,20β-P (MIS)
17βHSD
P450arom
P45011β.
11βHSD
11-ketotestosterone
Circulating Sex Steroids / Steroid
Hormone Binding Globlulin
ER
AR
6



DES used to prevent miscarriages-illustrates a couple hallmarks: (1) sensitive windows, (2) latent effects

Others examples more speculative



Effects in wildlife more certain in many cases-TBT, PCBs and (germane to this talk), feminization of fish exposed to EEs

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Assessing Ecological Risks of EDCs

- Emphasis to date at EPA has been more on predicting possible effects of new chemicals (EDSP) rather than diagnosing impacts in field, where several very challenging questions exist:
  - –-Analytical (What to measure at what detection?)
  - –-Evaluation of mixtures (Endocrine MOA of concern? Interactive effects?)
  - –-Biological endpoints (Biomarker vs. apical?)
  - –-Population-level responses (How to discern?)

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Tools Needed for Assessing Risk of EDCs in the Field

- Short-term *in vitro* and *in vivo* assays suitable for complex mixtures
- Analytical/fractionation approaches to identify specific chemicals causing biological effects
- Diagnostic endpoints (biomarkers) indicative of chemical MOA
- Approaches to link diagnostic responses to alterations in individuals and populations

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Assessing EDC Mixtures: 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)

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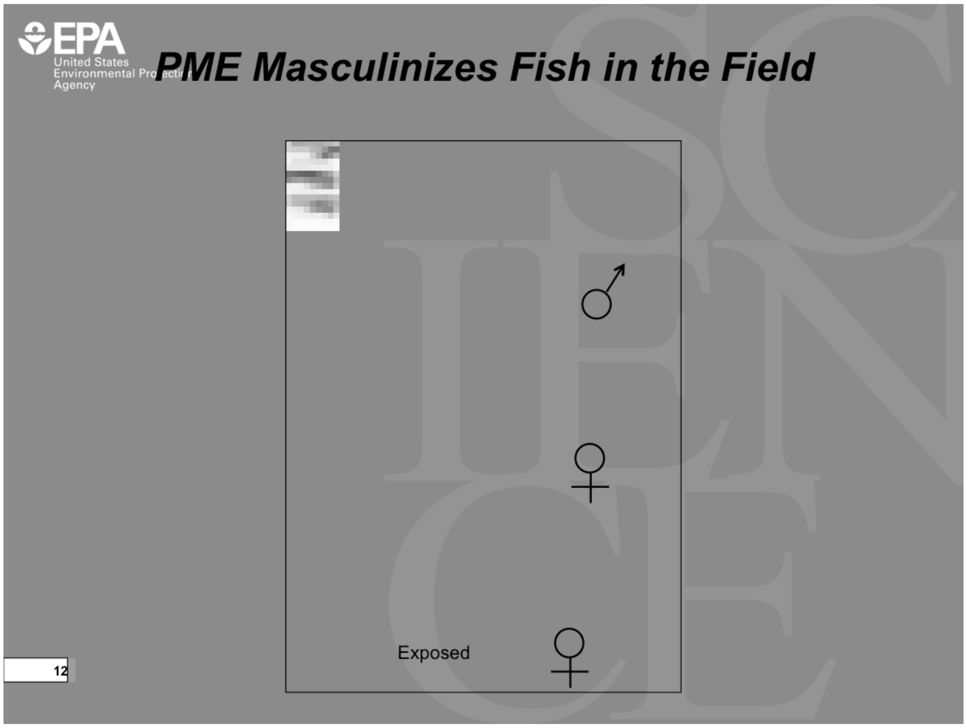
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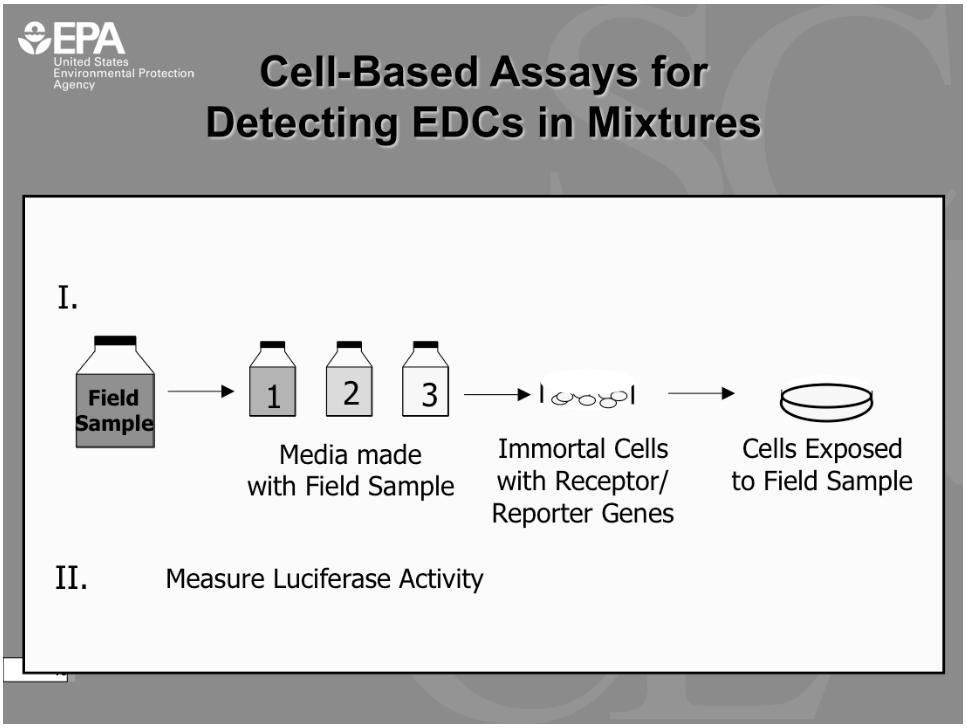
PME Masculinizes Fish in the Field
♂
♀
Exposed
♀
12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Cell-Based Assays for Detecting EDCs in Mixtures
I.
Field Sample
1
2
3
Media made with Field Sample
Immortal Cells with Receptor/ Reporter Genes
Cells Exposed to Field Sample
II.
Measure Luciferase Activity

The MDA-kb2 assay is a cell line which endogenously expresses the androgen receptor. It was stably transfected with the luciferase reporter gene. Luciferase is produced and when the cell is lysed and luciferin and ATP is added, light is produced and can be quantified. It is produced in a dose dependent manor.

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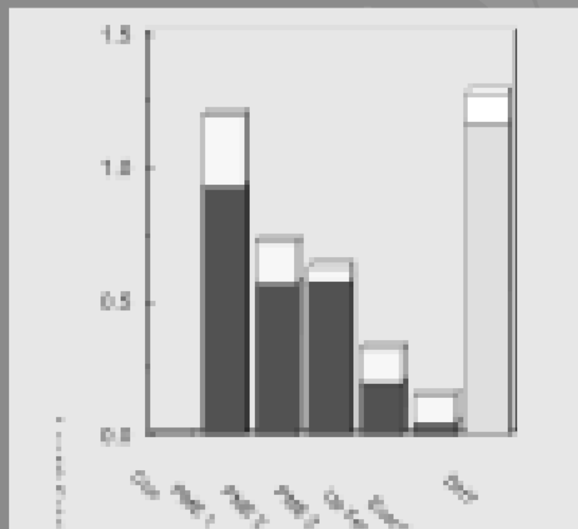
PME Androgenic Activity



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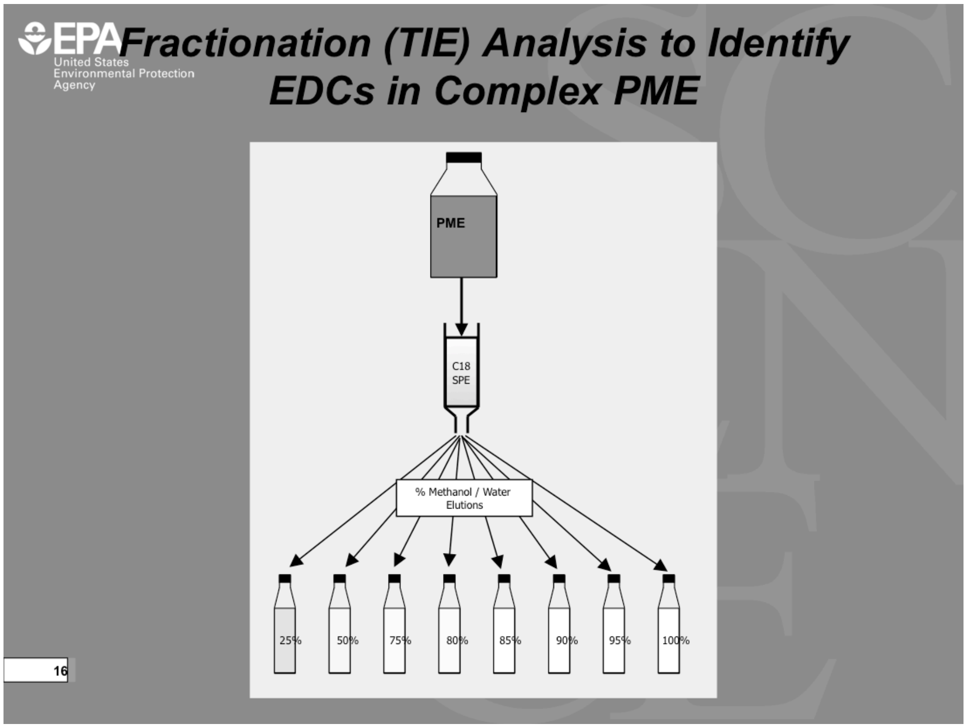
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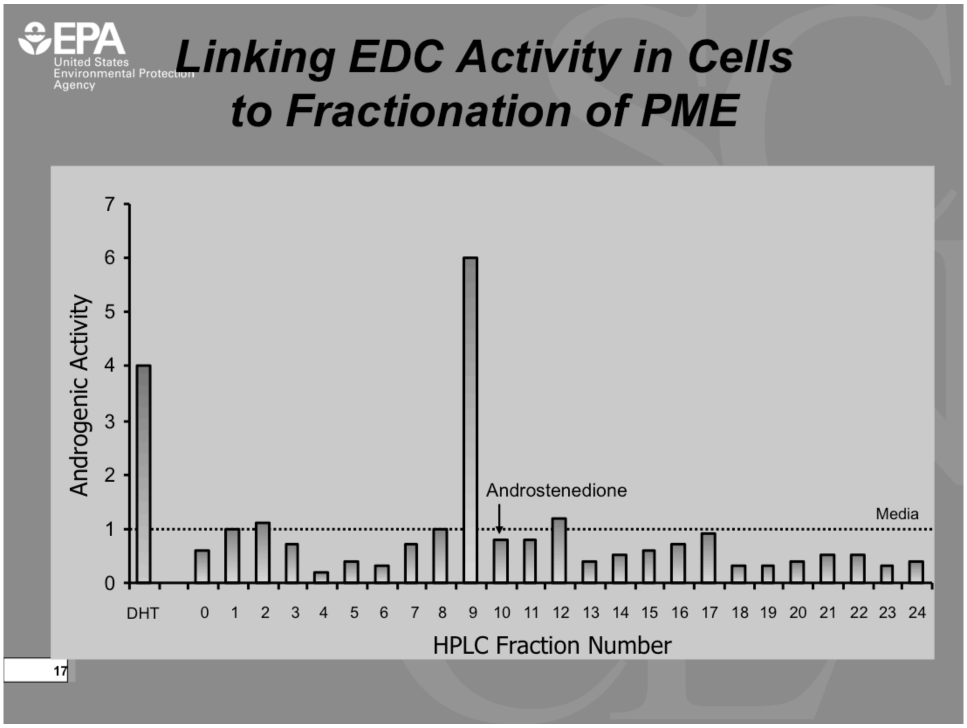
Fractionation (TIE) Analysis to Identify
EDCs in Complex PME
PME
C18
SPE
% Methanol / Water
Elutions
25%
50%
75%
80%
85%
90%
95%
100%
16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Linking EDC Activity in Cells to Fractionation of PME
Androgenic Activity
7
6
5
4
3
2
1
0
Androstenedione
Media
DHT 0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
HPLC Fraction Number
17

A Short-Term *in vivo* Test for Detecting Reproductive Toxicity of EDCs

- Conducted with fathead minnow, a model species used for research and regulatory work in labs throughout the world
- Three-week test includes endpoints reflective of specific classes of EDCs *and* apical responses useful to risk assessment
  - -Vitellogenin, steroids, SSC, histology
  - -Egg production, fertility, hatch
- Developed for EPA's EDSP (Endocrine Disruptor Screening and Testing Program), but applicable to assessment of complex mixtures as well

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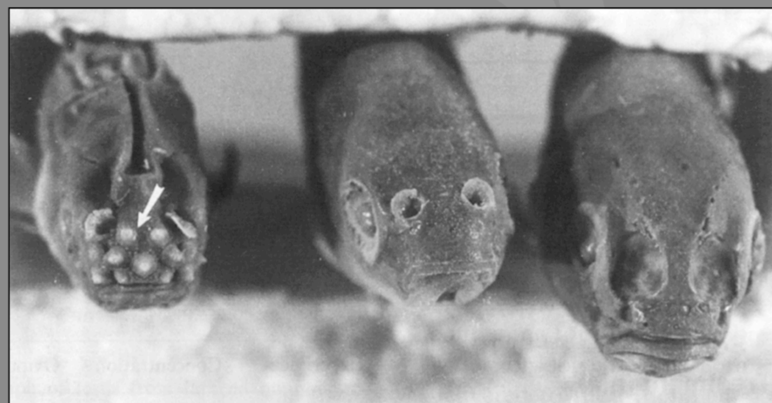
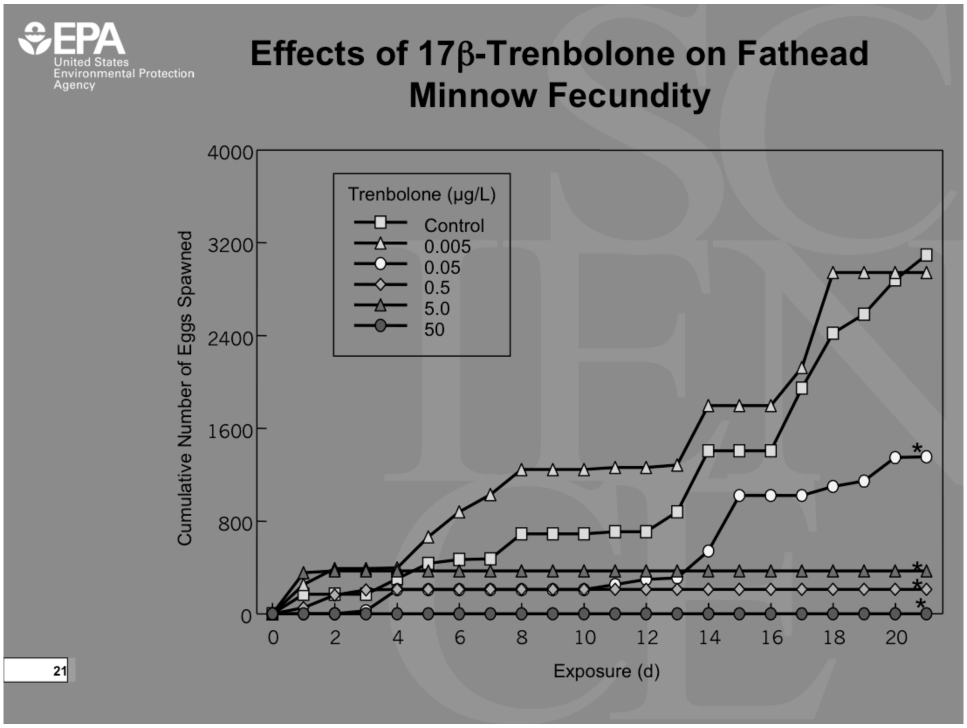
Effects of an Estrogen on Male Secondary Sex Characteristics



Miles-Richardson et al. (1999) Aquat. Toxicol.

20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Effects of 17β-Trenbolone on Fathead Minnow Fecundity
Trenbolone (µg/L)
Control
0.005
0.05
0.5
5.0
50
Cumulative Number of Eggs Spawned
0
800
1600
2400
3200
4000
0
2
4
6
8
10
12
14
16
18
20
Exposure (d)
*
21

Example Applications to Field Assessments of EDCs

- Monitoring Nebraska CAFO (Concentrated Animal Feeding Operation) samples for effects on reproduction and endocrine function (Kolok, Sellin et al.)
- Evaluating UK municipal effluents for estrogenicity (Thorpe, Tyler et al.)
- Monitoring Canadian pulp/paper mill effluents for reproductive and endocrine impacts (Kovacs, Parrott et al.)

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Linkage of Exposure and Effects Using Genomics, Proteomics, and Metabolomics in Small Fish Models

- USEPA – Cincinnati, OH
  - D. Bencic, M. Kostich, D. Lattier, J. Lazorchak, G. Toth, R. Wang,
- USEPA – Duluth, MN, and Grosse Isle, MI
  - G. Ankley, E Durhan, M Kahl, K Jensen, E Makynen, D. Martinovic, D. Miller, D. Villeneuve
- USEPA – Athens, GA
  - T. Collette, D. Ekman, M. Henderson, Q. Teng
- USEPA-RTP, NC
  - M. Breen, R. Conolly
- USEPA STAR Program
  - N. Denslow (Univ. of Florida), E. Orlando, (Florida Atlantic Univ K. Watanabe (Oregon Health Sciences Univ.), M. Sepulveda (F Univ.)
- USACE – Vicksburg, MS
  - E. Perkins, N. Garcia-Reyero
- Other partners
  - Joint Genome Institute, DOE (Walnut Creek, CA)
  - Sandia, DOE (Albuquerque, NM)
  - Pacific Northwest National Laboratory (Richland, WA)
  - O. Mekenyan (University of Bourgas)

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Project Objectives

Investigate effects of EDCs with different MOA using the fathead minnow 21-d test to establish adverse outcome pathways

Identify the genomic responses to the same chemicals using shorter-term exposures to support development of specific biomarkers indicative both toxic MOA and adverse outcome

Concurrently integrate data in a systems and network modeling contexts, as well as relevant population modeling, as a basis for prediction

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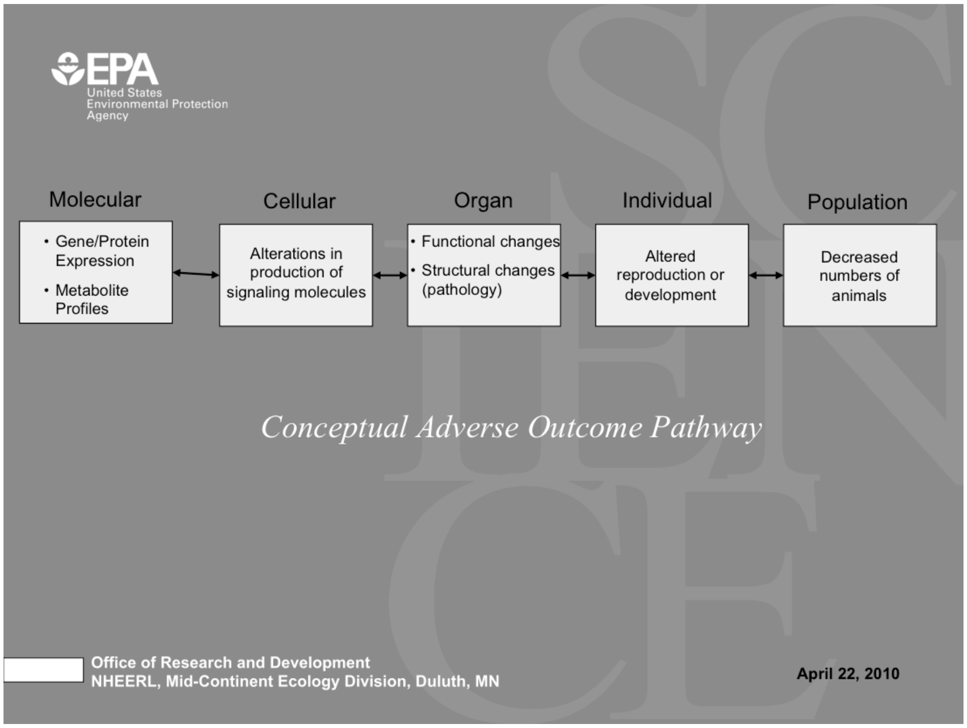

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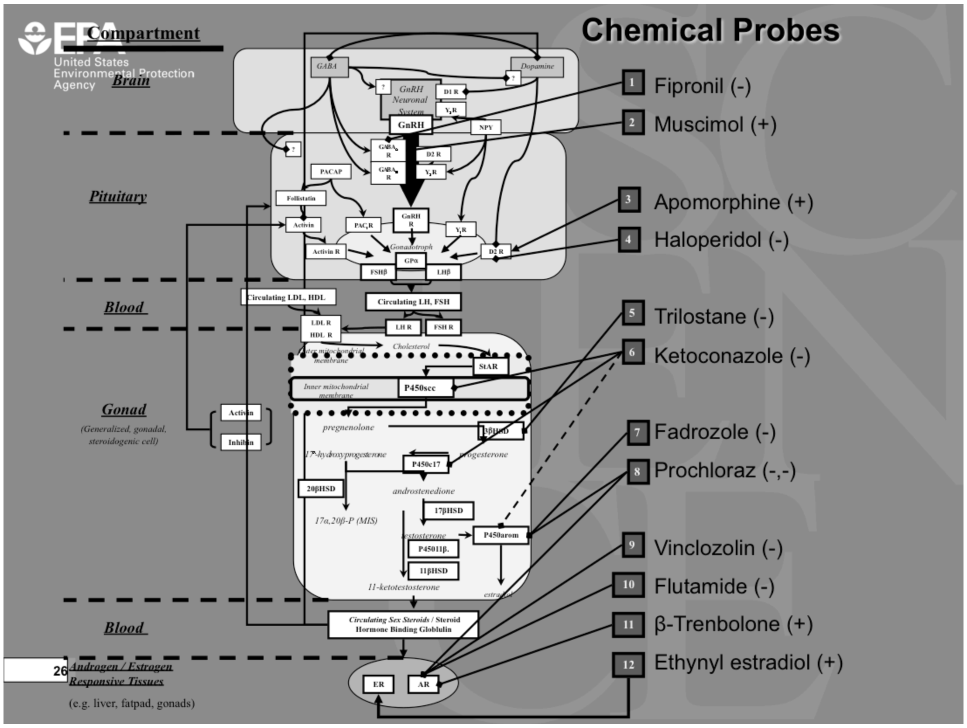
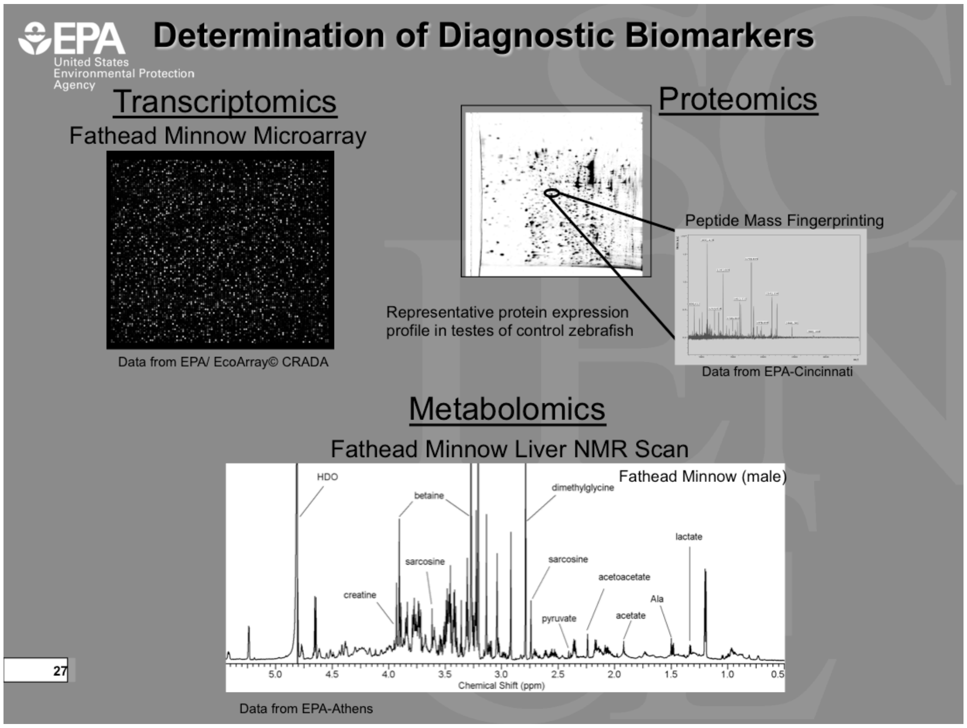
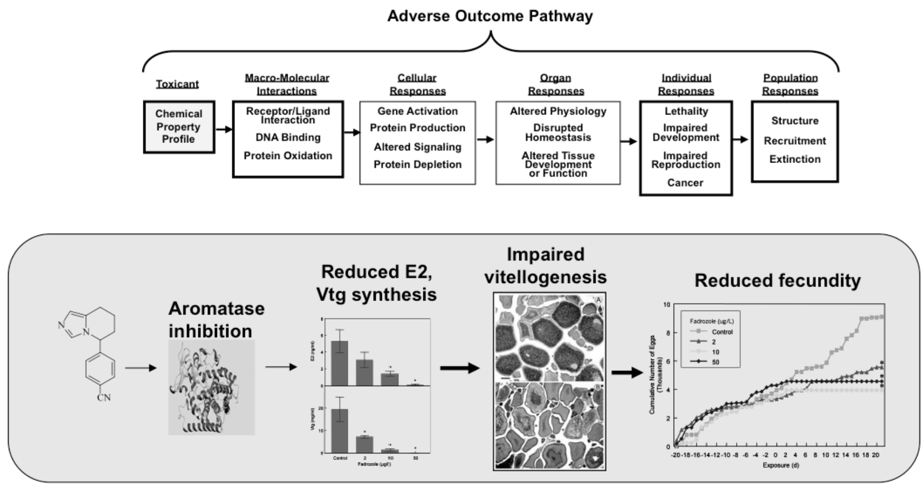
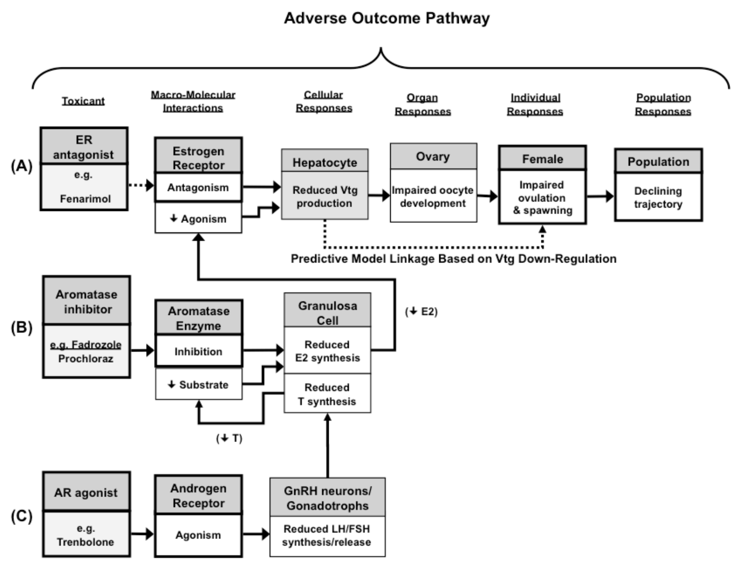
Molecular
Cellular
Organ
Individual
Population
• Gene/Protein Expression
• Metabolite Profiles
Alterations in production of signaling molecules
• Functional changes
• Structural changes (pathology)
Altered reproduction or development
Decreased numbers of animals
Conceptual Adverse Outcome Pathway
Office of Research and Development
NHEERL, Mid-Continent Ecology Division, Duluth, MN
April 22, 2010


Chemical Probes
Compartment
Brain
Pituitary
Blood
Gonad
(Generalized, gonadal, steroidogenic cell)
Blood
Androgen / Estrogen Responsive Tissues
(e.g. liver, fatpad, gonads)
GnRH
Circulating LDL, HDL
Circulating LH, FSH
StAR
P450scc
P450c17
20βHSD
17βHSD
P450arom
11βHSD
Activin
Inhibin
Circulating Sex Steroids / Steroid Hormone Binding Globlulin
ER
AR
1 Fipronil (-)
2 Muscimol (+)
3 Apomorphine (+)
4 Haloperidol (-)
5 Trilostane (-)
6 Ketoconazole (-)
7 Fadrozole (-)
8 Prochloraz (-,-)
9 Vinclozolin (-)
10 Flutamide (-)
11 β-Trenbolone (+)
12 Ethynyl estradiol (+)
26

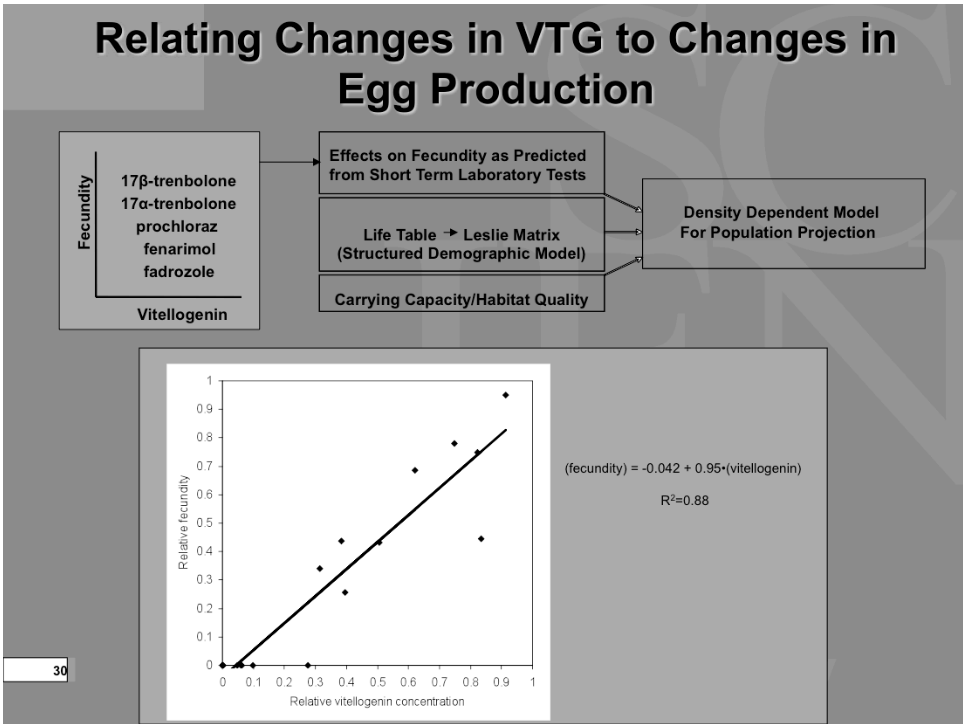

Determination of Diagnostic Biomarkers
United States Environmental Protection Agency
Transcriptomics
Fathead Minnow Microarray
Data from EPA/ EcoArray© CRADA
Proteomics
Peptide Mass Fingerprinting
Representative protein expression profile in testes of control zebrafish
Data from EPA-Cincinnati
Metabolomics
Fathead Minnow Liver NMR Scan
Fathead Minnow (male)
HDO
betaine
dimethylglycine
sarcosine
creatine
pyruvate
acetoacetate
acetate
Ala
lactate
Chemical Shift (ppm)
Data from EPA-Athens
27

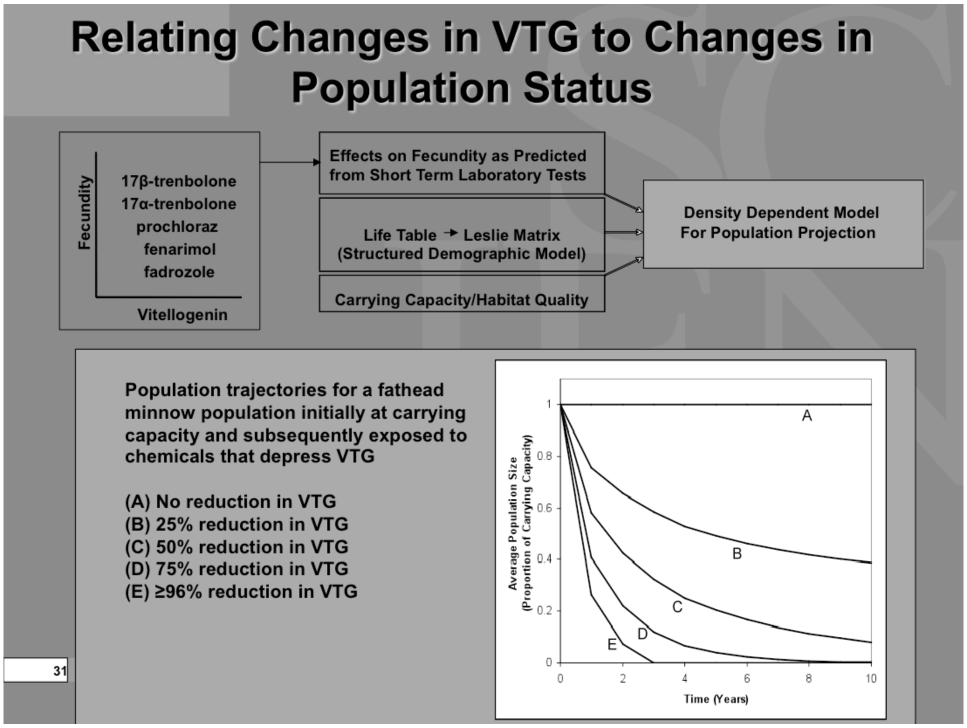


Example of an AOP in fathead minnows exposed to an aromatase inhibitor



Multiple AOPs converging at common insult/node of impaired vitellogenesis


Relating Changes in VTG to Changes in Egg Production
Fecundity
17β-trenbolone
17α-trenbolone
prochloraz
fenarimol
fadrozole
Vitellogenin
Effects on Fecundity as Predicted from Short Term Laboratory Tests
Life Table → Leslie Matrix (Structured Demographic Model)
Carrying Capacity/Habitat Quality
Density Dependent Model For Population Projection
Relative fecundity
Relative vitellogenin concentration
(fecundity) = -0.042 + 0.95•(vitellogenin)
R²=0.88
30


Relating Changes in VTG to Changes in Population Status
Fecundity
17β-trenbolone
17α-trenbolone
prochloraz
fenarimol
fadrozole
Vitellogenin
Effects on Fecundity as Predicted from Short Term Laboratory Tests
Life Table → Leslie Matrix (Structured Demographic Model)
Carrying Capacity/Habitat Quality
Density Dependent Model For Population Projection
Population trajectories for a fathead minnow population initially at carrying capacity and subsequently exposed to chemicals that depress VTG
(A) No reduction in VTG
(B) 25% reduction in VTG
(C) 50% reduction in VTG
(D) 75% reduction in VTG
(E) ≥96% reduction in VTG
Average Population Size (Proportion of Carrying Capacity)
Time (Years)
A
B
C
D
E
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Tools Needed for Assessing Risk of EDCs in the Field

- Short-term *in vitro* and *in vivo* assays suitable for complex mixtures
- Analytical/fractionation approaches to identify specific chemicals causing biological effects
- Diagnostic endpoints (biomarkers) indicative of chemical MOA
- Approaches to link diagnostic responses to alterations in individuals and populations

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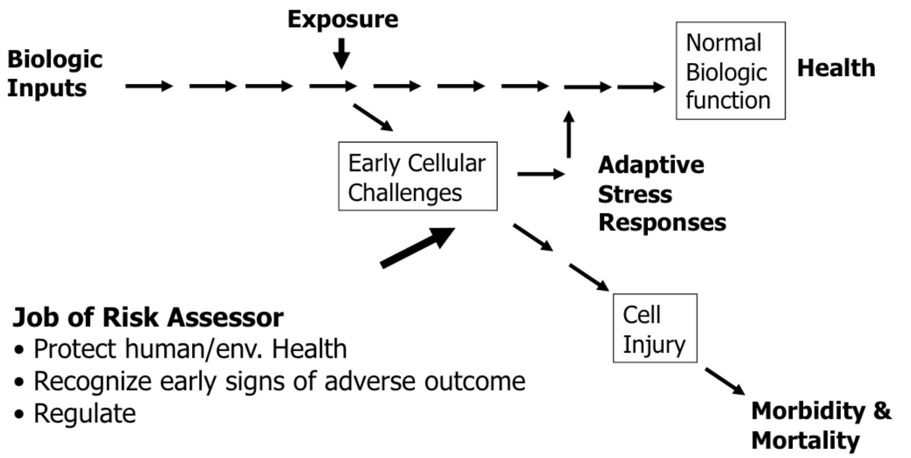
Molecular Biomarkers and Omics Technologies in Risk Assessment

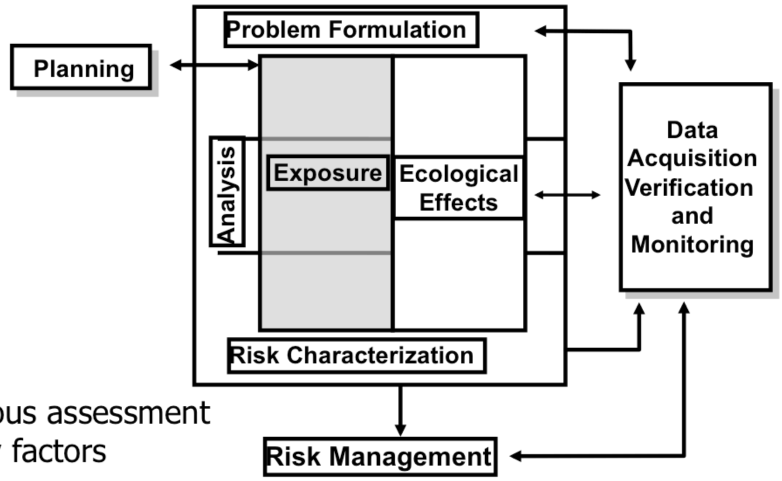
Nancy Denslow, Ph.D.
Center for Environmental and Human Toxicology
University of Florida

Intersection of exposure and biologic function



Adapted from Toxicity Testing in the 21st Century, NRC

Framework for Ecological Risk Assessment



- Rigorous assessment
- Safety factors

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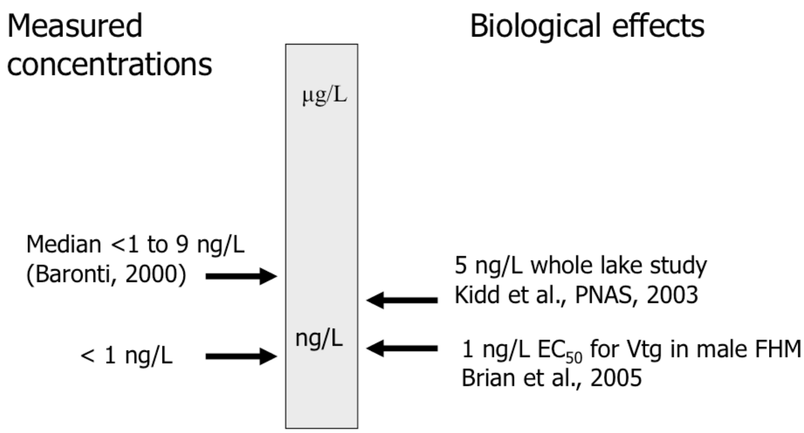
Unexplained issues

- Fish in superfund sites may be larger than in control sites
- Female fish may have smaller gonads but eggs appear mature
- Ovo-testis appears in some fish
- Some fish have increased susceptibility to disease
- Some fish show neurological/behavior disorders

Have we measured the right toxicity endpoints?
Do we recognize early signs of adverse effects?

Ethinylestradiol in surface waters



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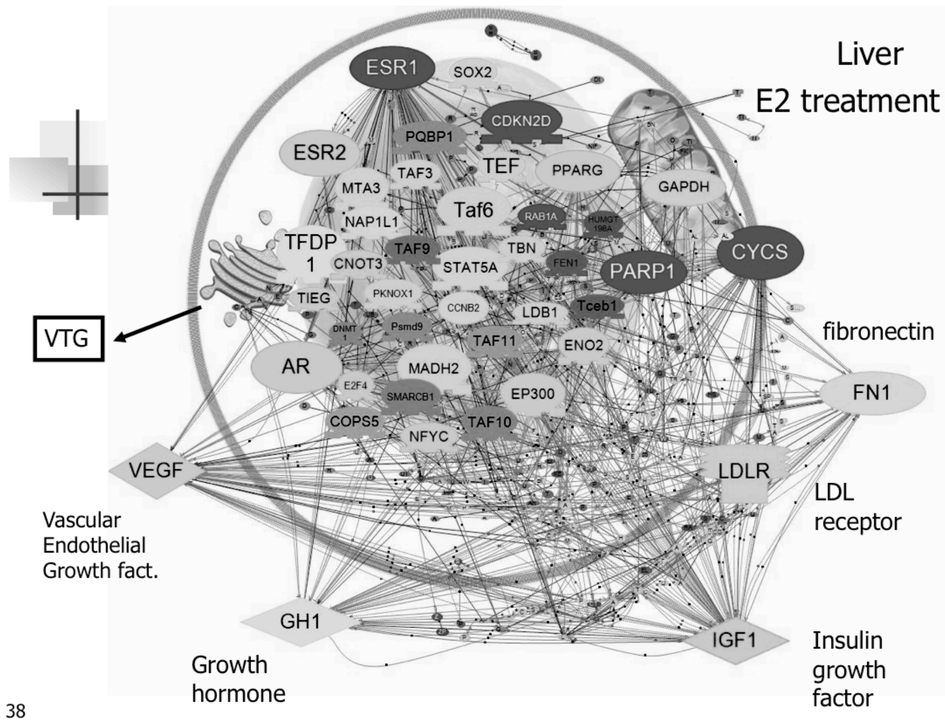

Liver
E2 treatment
ESR1
SOX2
CDKN2D
PQBP1
ESR2
TAF3
TEF
PPARG
GAPDH
MTA3
Taf6
RAB1A
NAP1L1
TFDP
1
TAF9
TBN
CYCS
CNOT3
STAT5A
FEN1
PARP1
TIEG
PKNOX1
CCNB2
LDB1
Tceb1
VTG
Psmd9
TAF11
ENO2
fibronectin
AR
MADH2
E2F4
SMARCB1
EP300
FN1
COPS5
TAF10
NFYC
VEGF
LDLR
LDL
receptor
Vascular
Endothelial
Growth fact.
GH1
IGF1
Insulin
growth
factor
Growth
hormone

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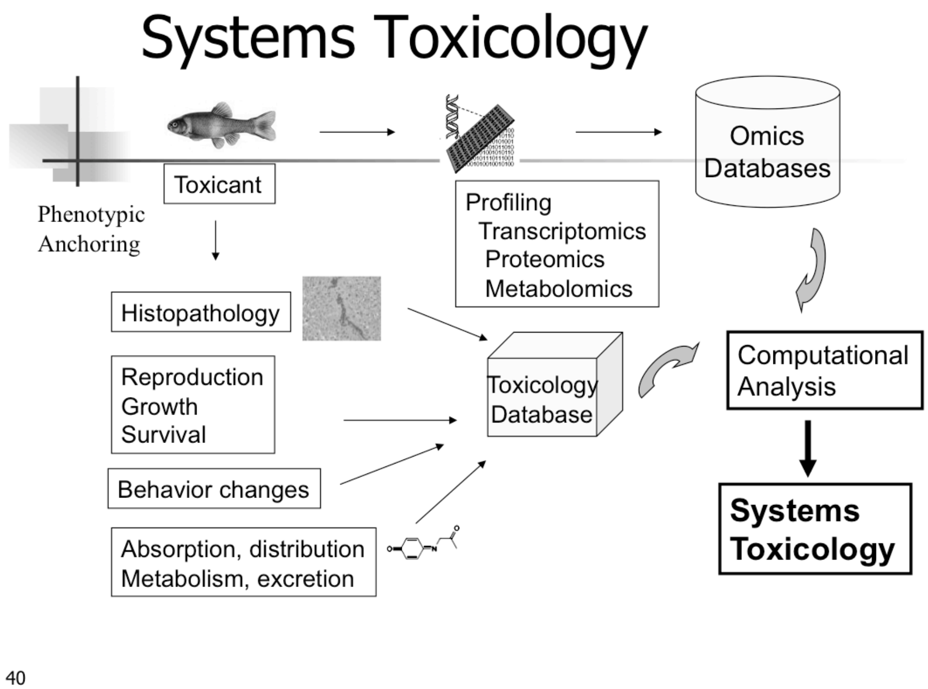
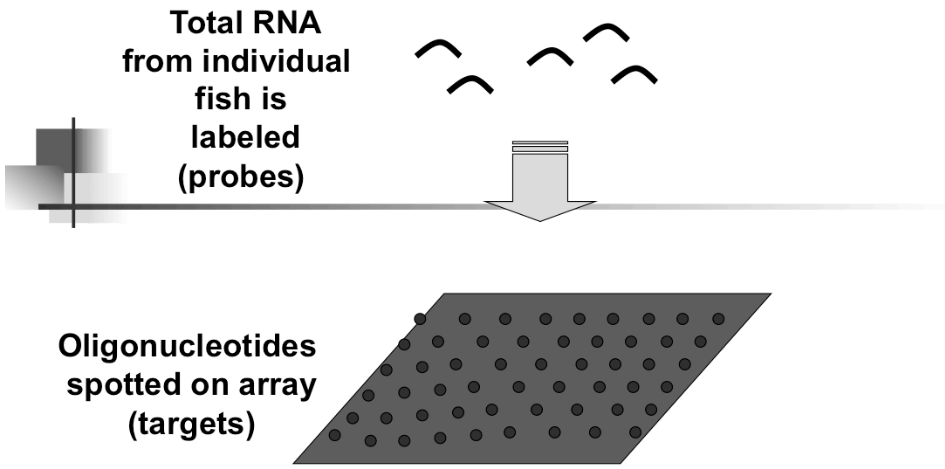
Endocrine Disruption

- Hormone receptor activity
- Steroidogenesis
- Metabolism
- Immune system dysfunction
- Neuroendocrine control
- Apoptosis → oocyte atresia
- Cardiovascular function



Systems Toxicology
Omics
Databases
Toxicant
Phenotypic
Anchoring
Profiling
Transcriptomics
Proteomics
Metabolomics
Histopathology
Computational
Analysis
Reproduction
Growth
Survival
Toxicology
Database
Behavior changes
Systems
Toxicology
Absorption, distribution
Metabolism, excretion
40

How arrays work



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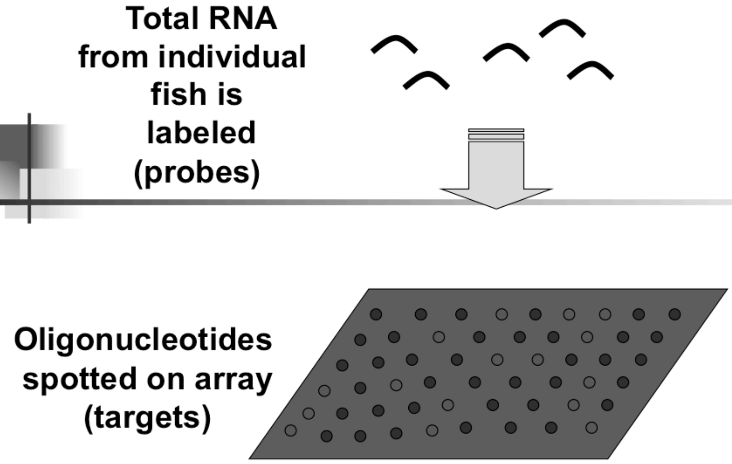
How arrays work



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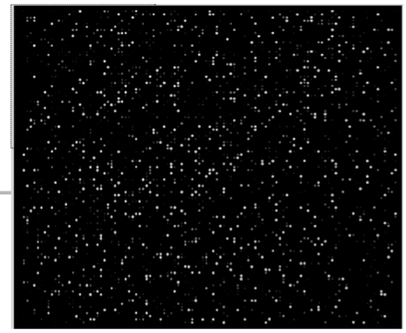
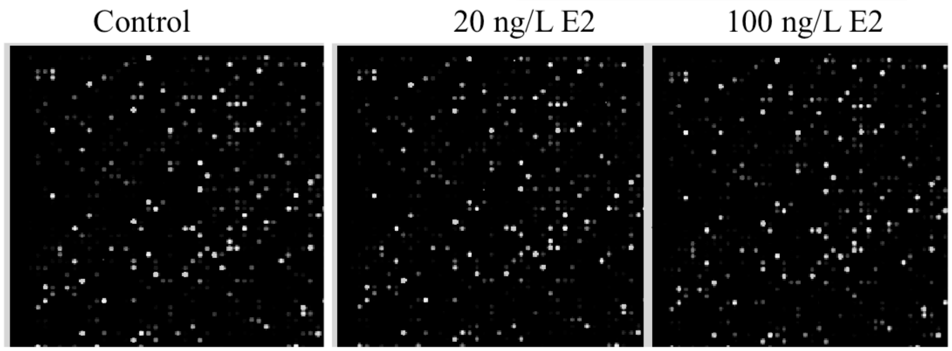
Analysis by microarray

Fish ± treatment → organ → total RNA → cDNA → array





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Largemouth bass studies

Superfund Research Program

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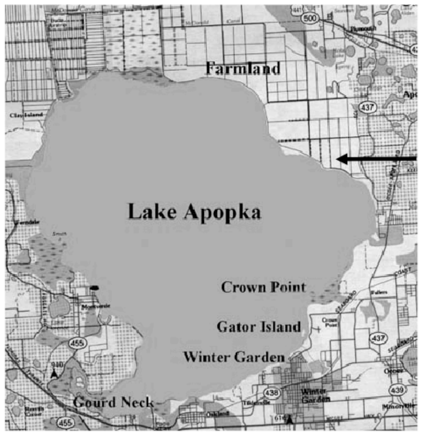
Lake Apopka

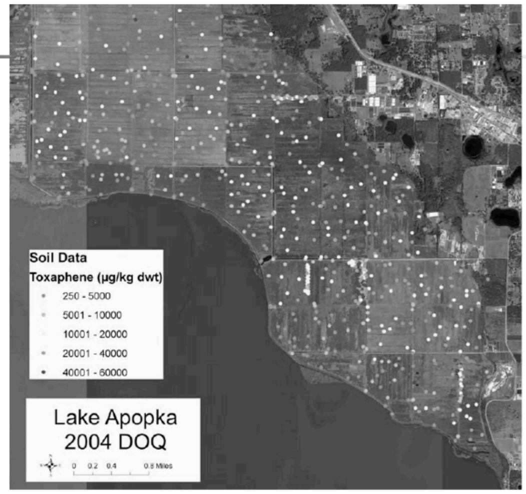


Amount per acre per yr
(13,000 acres)

- 68 lbs DDT
- 0.4 lbs Chlordane
- 13.3 lbs toxaphene

Dichlorobenzil → dichlorobenzophenone
chlorobenzilate

Toxaphene concentrations in soil



SJRWM Safe Levels
Report , 2006

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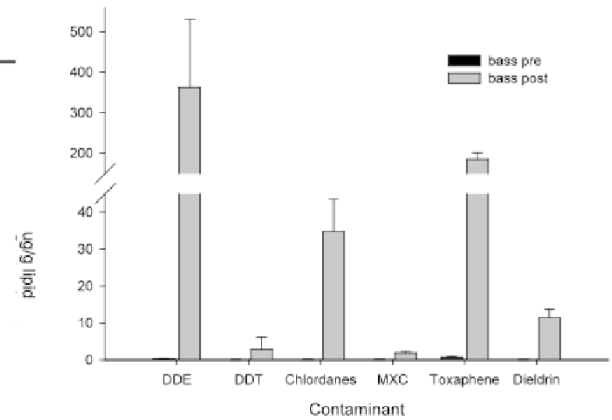

Contaminants in whole body analyses of Largemouth bass before and after living in mesocosms for 4 months.

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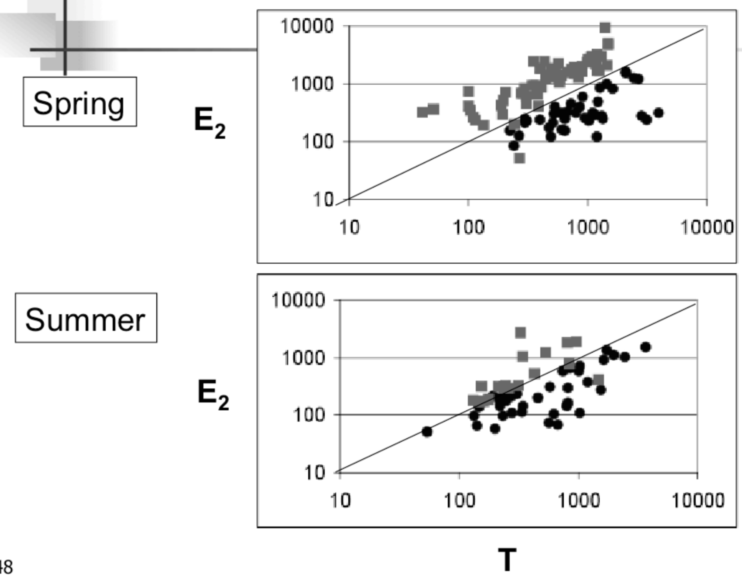
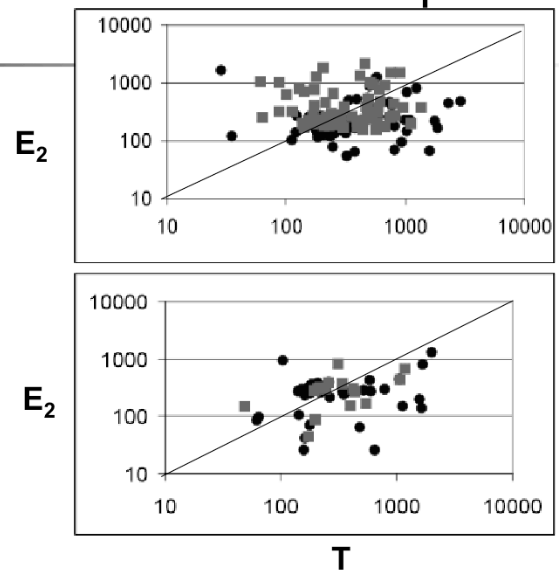
Steroid Hormones – Control Site



Steroid Hormones – Impacted Site

Spring

Summer



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Feeding study: p,p'-DDE, dieldrin and mixture

P,p'DDE	Dieldrin	P,p'-DDE +Dieldrin
Jan-Mar	Jan-Mar	Jan-Mar
15.7 ug/gm	0.32 ug/gm	15.7 & 0.32 ug/gm

Chemical analyses of contaminants (lipid normalized)

Contaminant levels	Mesocosm Study (ug/g wet weight)		Levels after 3 months (ug/g wet wt)		
	Levels before	Levels after	P,p′-DDE	Dieldrin	
P,p′-DDE	0.020 ± 0.006	**16.2 ± 8.6**	**15.7 ± 1.1**	ud	
Dieldrin	0.003 ± 0.0001	**0.50 ± 0.06**	ud	**0.32 ± 0.03**	
Methoxychlor	0.004 ± 0.000	0.08 ± 0.002	ud	ud	
Toxaphene	0.061 ± 0.008	8.2 ± 1.3	ud	ud	

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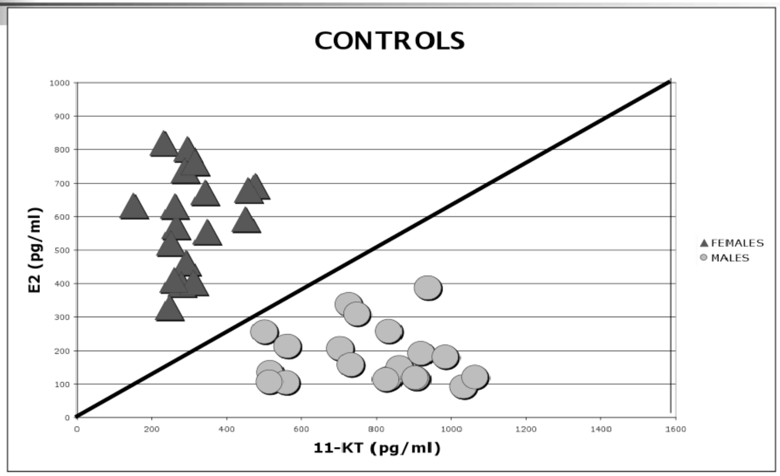
Feeding Study – Plasma hormones



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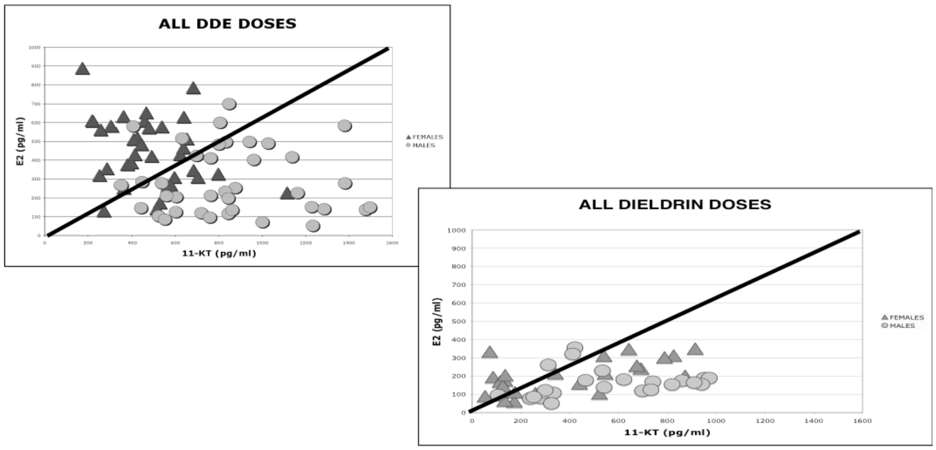
Plasma hormones: p,p'-DDE



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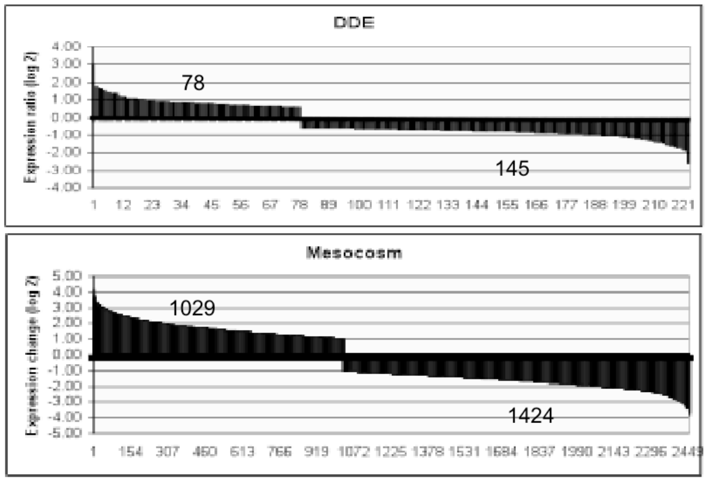
Gene expression profiles (P<0.01)

Gonad Tissue level: 15.7 ug DDE/g wet weight



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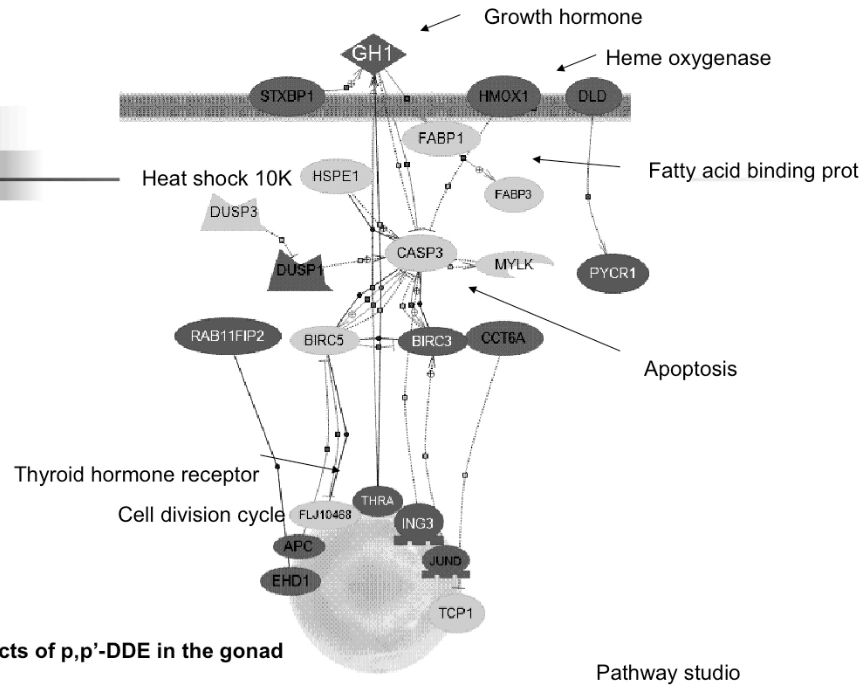
Biological processes regulated by exposure to the Mesocosm
Gonad

- Up regulated pathways
  - Glycolysis
  - Response to hypoxia
  - DNA repair
  - Chromatin assembly
  - Lipid biosynthesis

- Down regulated pathways
  - Cell cycle progression → growth of early life stages
  - Electron transport
  - Protein biosynthesis
  - Superoxide metabolism
  - Apoptosis
  - Immune response



Effects of p,p'-DDE in the gonad

Pathway studio

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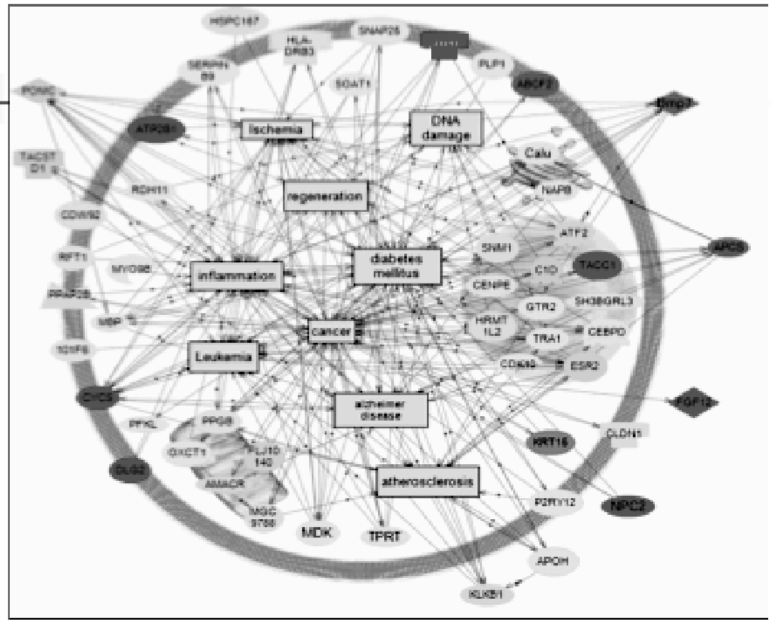
About 1/3 of proteins annotated to human homologs

Dieldrin effects in the brain



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Overall inferences from pesticide studies

1. Organochlorine pesticides are still found in the environment at very high levels and they are transferred to wildlife.
2. p,p'- DDE has pleiotropic functions –
   - Estrogen mimic → up-regulation of Vtg in males
   - Inhibits steroidogenesis
   - Positive effect on growth of organism
3. Dieldrin affects the brain – genes involved with neurological disease

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Fathead minnow studies

1. Obtain molecular biomarkers for
   Estrogen
   Androgen
   Anti-androgen
2. Field Studies

This research is funded by
U.S. EPA - Science To Achieve Results (STAR) Program
Grant # R 831848

National Sea Grant

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Fathead Minnow Study – EE2/ZM189,154 TB/FLU

EE2: 5, 10, 50 ng/L ZM: 100 ng/L	TB: 0.5, 5, 50 μg/L FLU: 50,100, 500 μg/L	Mixtures EE2/ZM = 5/100ng/L TB/FLU =0.5/500 μg/L

48 hr

Brain **Liver** **Gonad**

1. Evaluate gene expression changes
2. Evaluate chemical levels

(Garcia-Reyero et al., EST, 2009)
(Garcia-Reyero et al., BMC Genomics, 2009)

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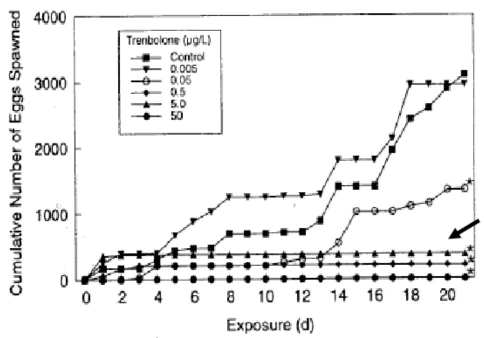
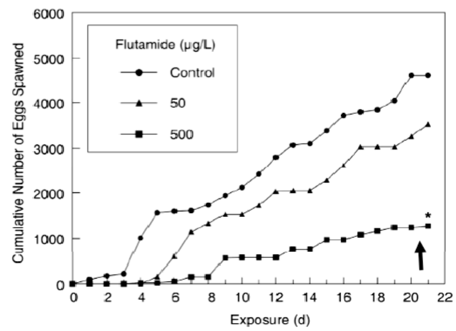
Measurement of fecundity in 21-d reproductive test

Trenbolone



Flutamide



(Ankley et al., 2003) (Jensen et al., 2004)

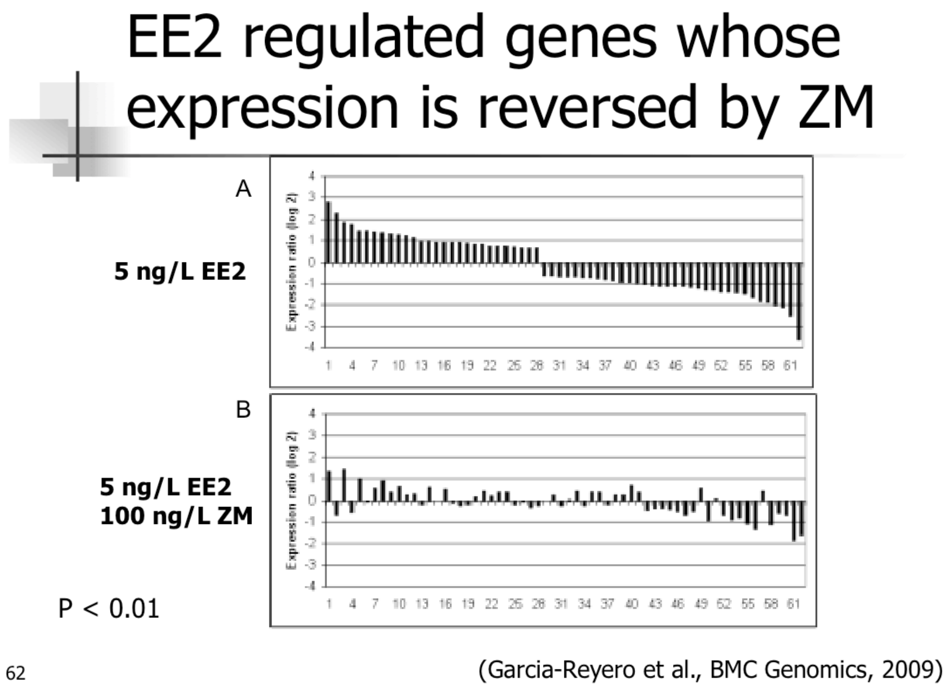


Figure 4. Plot of genes that were differentially regulated by 5 ng/L EE2 and rescued by the treatment with the mixture of 5 ng/L EE2 and 100 ng/L ZM 189,154 ($P < 0.01$) as determined on the 22K array. Genes are plotted in order of their change in expression with EE2.

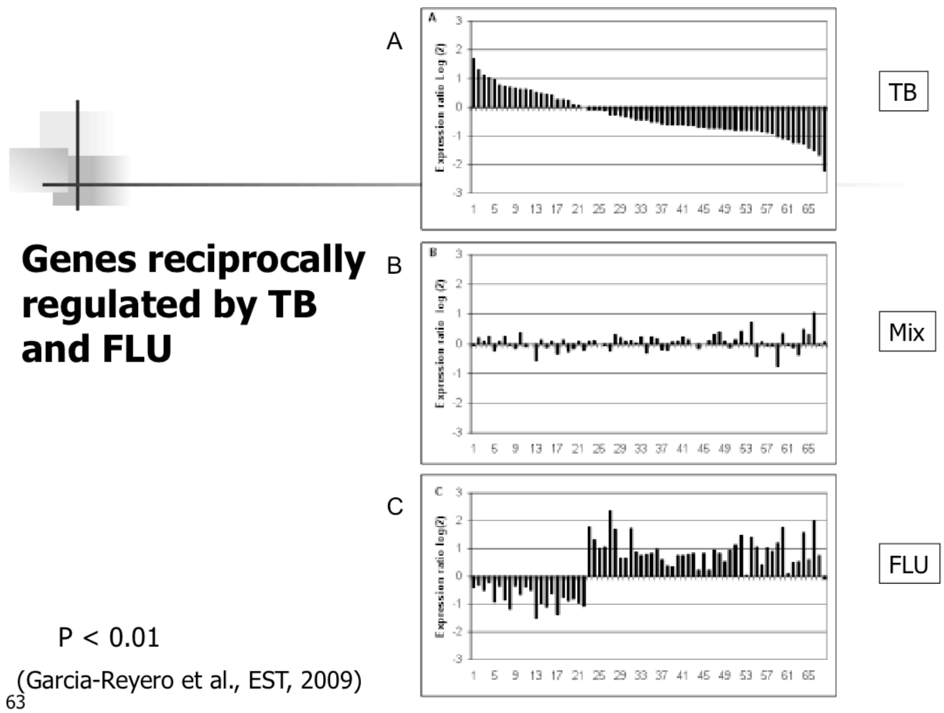
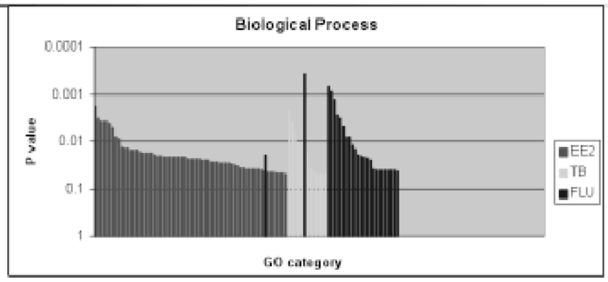


Figure 2. Genes that are reciprocally regulated by TB and FLU and the effect of mixing the two treatments. The genes were arranged in the same order in terms of the changes in expression levels for TB. (A) Expression of genes influenced by TB, (B) Expression of genes in a mixture of TB and FLU (C) Expression of genes influenced by FLU.

Biological Processes affected by EE2, TB, Flu



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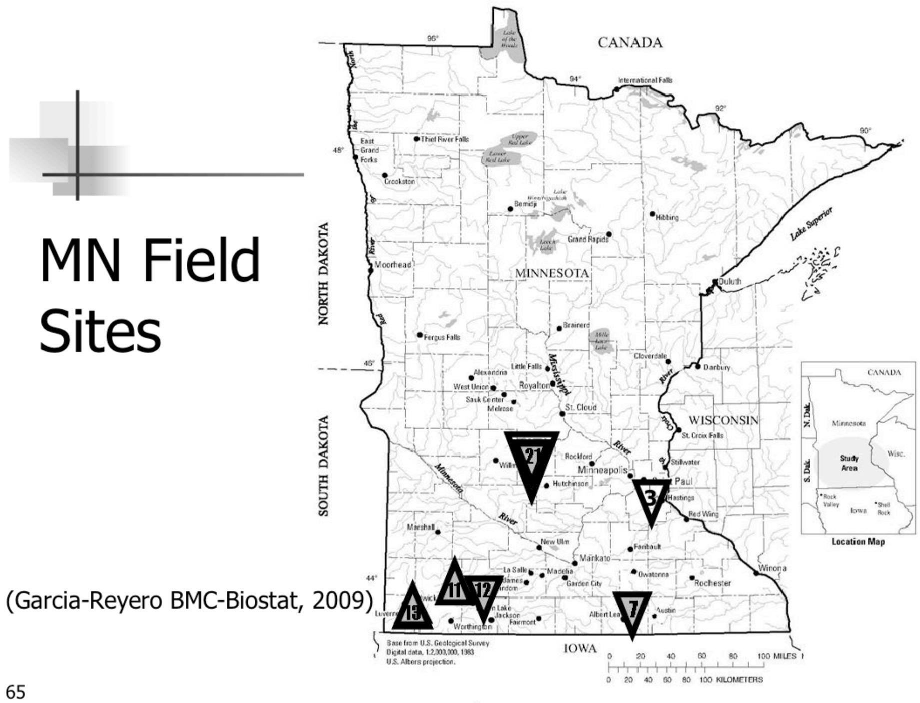


Fig 1: Location of the sites. Adapted from Lee et al. 1999.

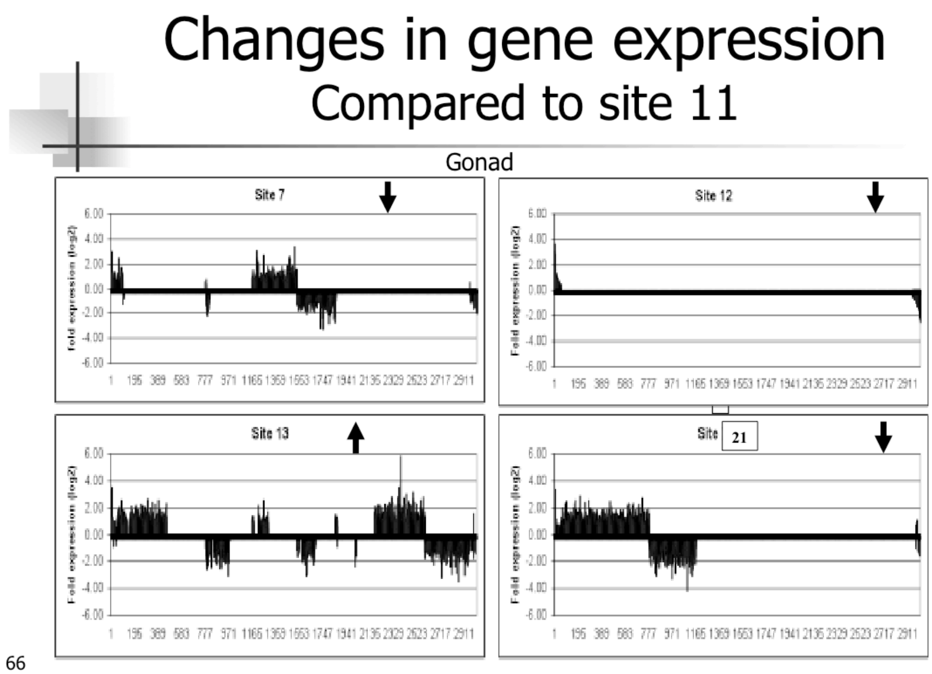
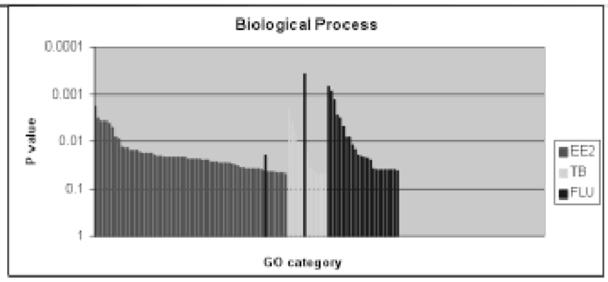


Figure 3. Expression fingerprints for gonad at each of the sites. Genes are expressed as fold change over expression at site #11 (similar to control). All genes were arranged from most highly expressed to most highly repressed for site#12 and the same order is kept for the other sites. For EE2 and TB profiles only those genes that are also present in the field sites as significant changes are included. Genes whose fold expression data was not significant at *p*-value < 0.05 were set to 0.

Biological Processes affected by EE2, TB, Flu



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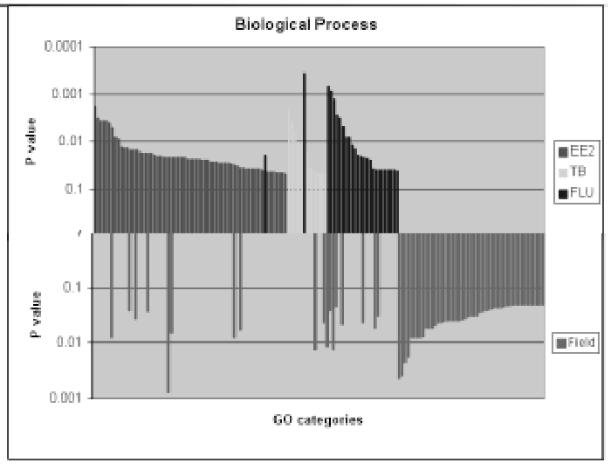
Biological Processes affected by EE2, TB, Flu



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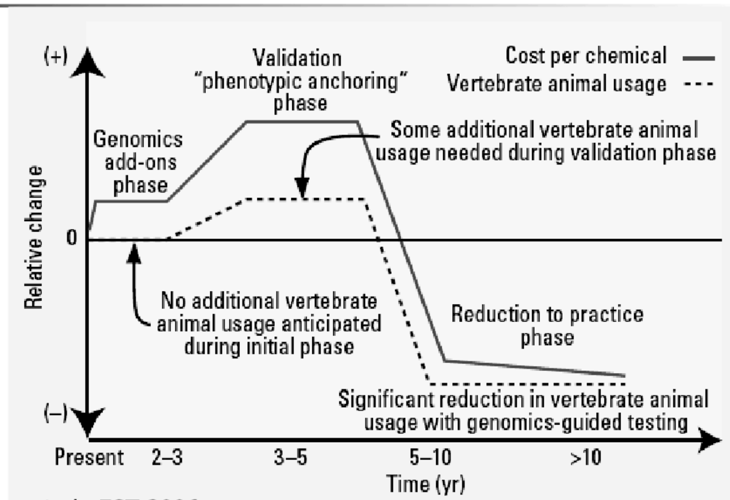
Resource requirements for integration of "Omics" technologies



Ankley et al., EST 2006

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Conclusions

- OMICS technologies provide a multilevel systems biology approach to safety assessment— from molecular to cellular, tissue, individual, and population-levels.
- Provides biomarkers with much improved predictive capacity
- Knowing the MOA can reduce uncertainties in chemical risk assessments
- Diagnostic studies to measure efficacy of site remediation

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- To view a complete list of resources for this seminar, please visit the **Additional Resources**
- Please complete the **Feedback Form** to help ensure events like this are offered in the future
- Link to "Improving Collaborations" Survey

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