Applications and Limitations of Bioassay Methods for Contaminants of Emerging Concern in Environmental Monitoring

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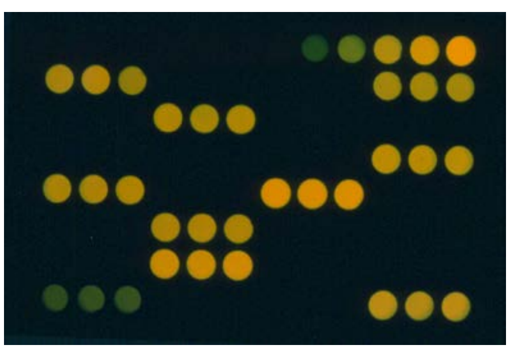
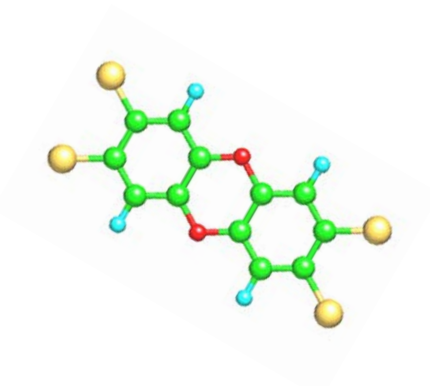
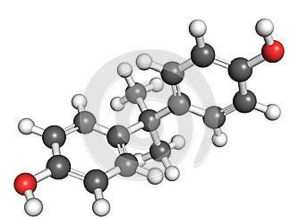
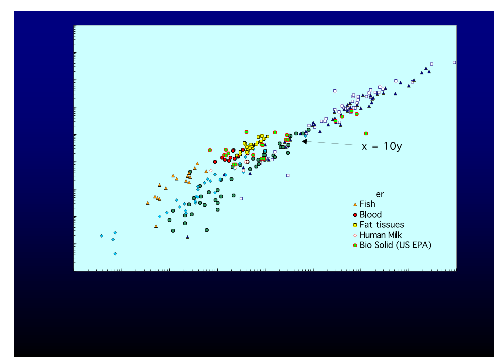
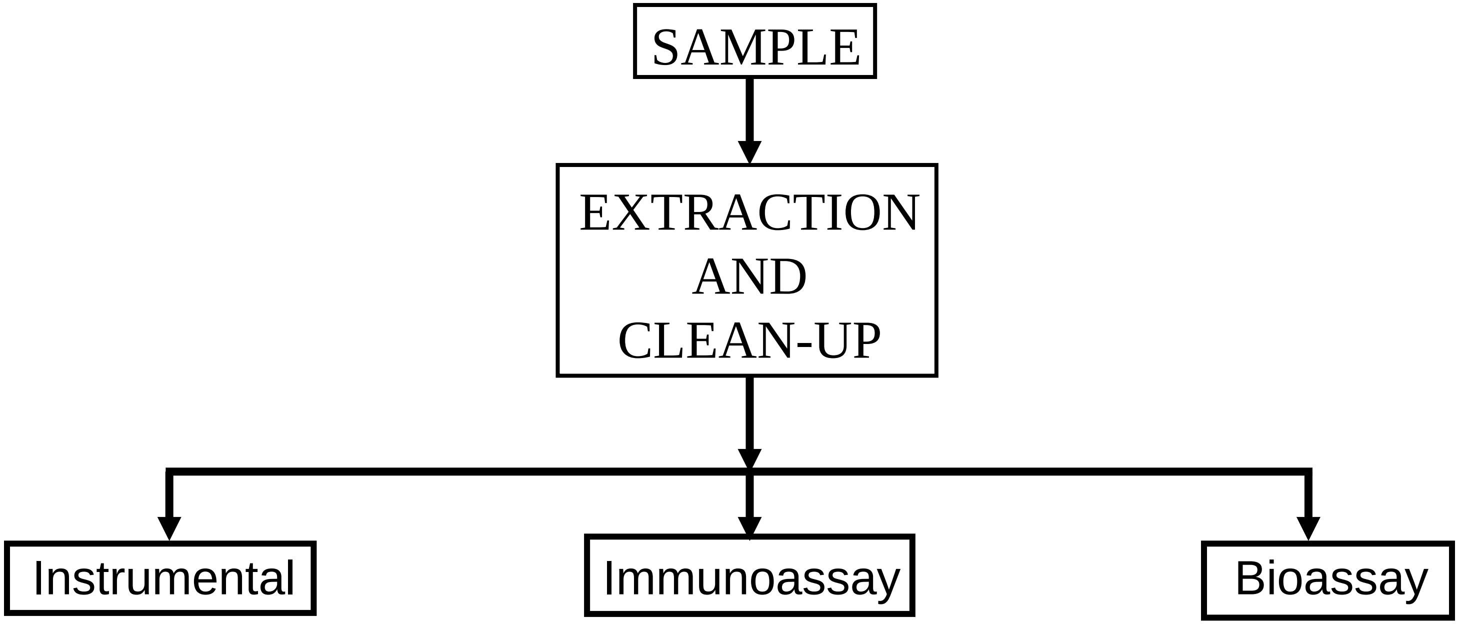








Screening and Monitoring Approaches for Contaminants of Concern (Known and Unknown)

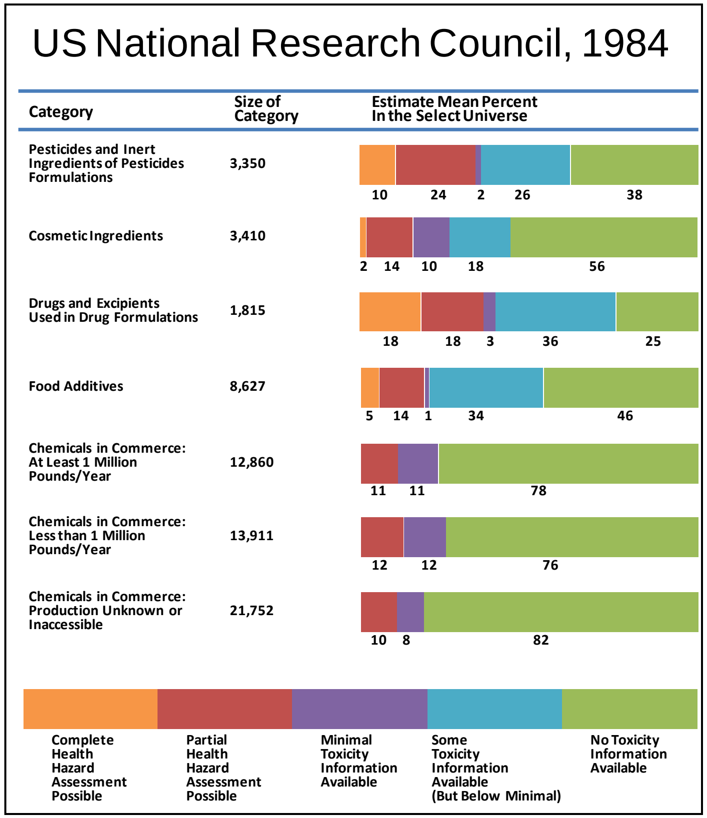


Issues to consider:

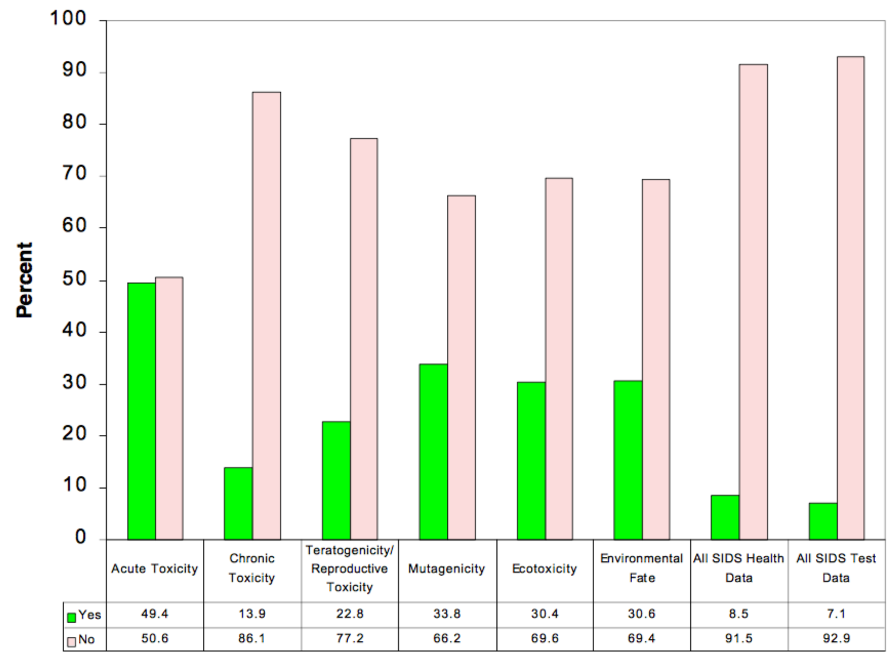
1. Chemicals to be Measured (known and unknown)
2. Measurement and Screening (speed, cost, accuracy, precision)
3. Biological/toxic Potency Estimates (TEQs, EEQs, BEQs, etc)
4. Mixture Interactive Effects (inhibition, additivity, synergism)

Toxicology and Chemicals
We know a lot about a little and little about a lot!

Ability to Conduct Health Hazard Assessment On a Select Universe of Chemicals



US High Production Volume (HPV) Chemicals (2,863 produced at > 1 million lbs per year)



Chemical Hazard Data Availability Study (USEPA,1998)

Need biological/toxicological effects information on many chemicals
Since it's open-ended on effects endpoints, many bioassays are needed

Environmental Monitoring: HTS Bioassays and Endpoints

(detection of chemicals affecting mechanisms related to adverse health effects)

- Acute Toxicity
- Genotoxicity (Mutagenicity)
- Endocrine Disruption
- Neurotoxicity
- Reproductive & Developmental Toxicity

- Enzyme (stimulation/inhibition)
- Cell-based reporter gene (stimulation/inhibition)
  (nuclear receptors, transcription factors)
- Cell signaling pathways (NFkB, RTKs, PKs, p53)
- Cell growth, cell viability, cytotoxicity
- Stress response (DNA damage, oxidative stress, inflammation)

Bioassays can ‘t be comprehensive – some mechanisms and assays not amenable to HTS, multifactorial mechanisms are problematic. What are characteristics of useful bioassays?

Environmental Monitoring With Bioassays

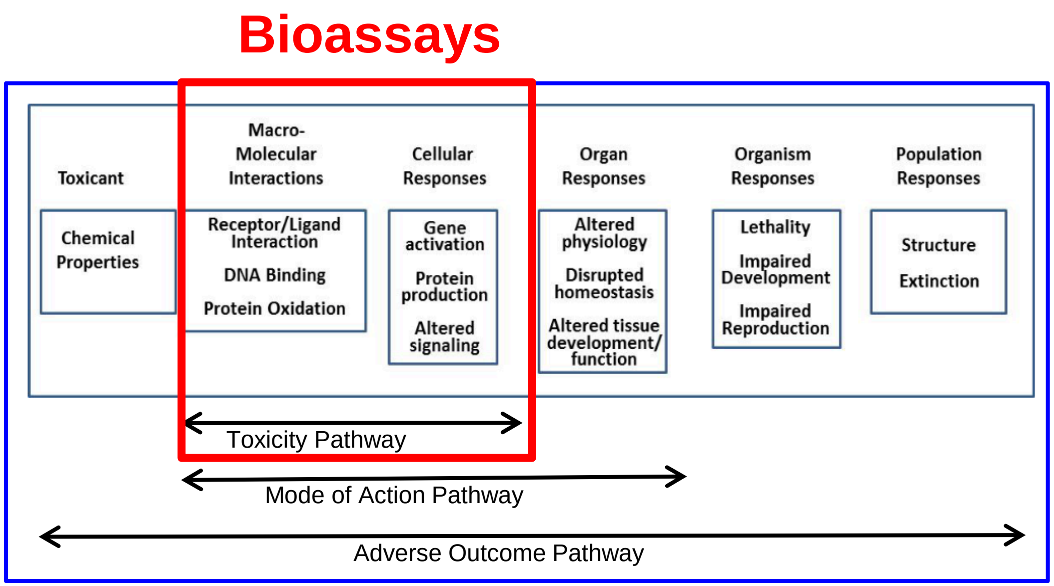
* **Chemical/Chemical Class Detection Method (selective screening)**

  Few chemical selective bioassays available (dioxin-like chemicals)

* **"Hazardous" Chemical Detection Method (open-ended Screening)**

  Requires some qualitative/quantitative relationship with risk

  - First step requires relating the bioassay and bioassay result to an adverse outcome pathway (AOP).



Environmental Monitoring With Bioassays

* **Chemical/Chemical Class Detection Method (selective screening)**

  Few chemical selective bioassays available (dioxin-like chemicals)

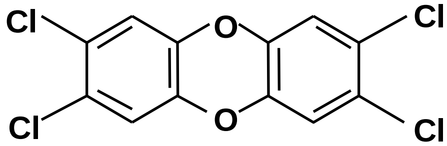
* **“Hazardous” Chemical Detection Method (open-ended Screening)**

  Requires some qualitative/quantitative relationship with risk

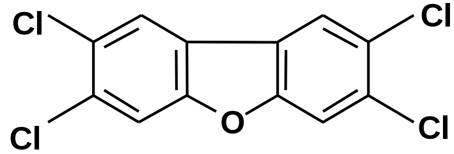
  - First step requires relating the bioassay and bioassay result to an adverse outcome pathway (AOP).
  - Second, confirm relationship the concentration-response of the bioassay with the dose-response for adverse health outcomes produced *in vivo* (animals or humans).
  - Third, toxicokinetic analyses will be necessary to normalize doses (usually blood plasma vs. media concentrations).
  - These bioassays respond to chemicals that act through a common mechanism and/or AOP.

Example of bioassays for environmental monitoring/screening?

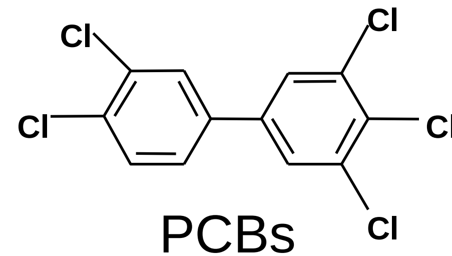
Health Effects of Dioxin-Like HAHs



PCDDs



PCDFs



PCBs

Toxicity

- Cancer
- Immunotoxicity
- Heart disease
- Liver toxicity
- Skin toxicity
- Birth defects
- Wasting syndrome
- Lethality

Biochemical

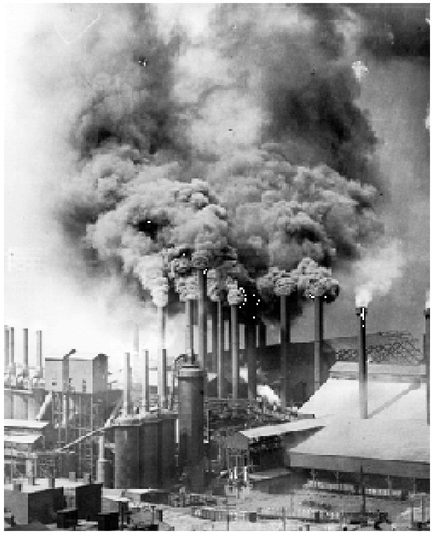
- Endocrine disruption (estrogen/testosterone)
- Inhibit cell division
- Alter gene expression (induction/repression)
- Alter chemical and drug degradation
- Oxidative stress

HAHs – Halogenated Aromatic Hydrocarbons

Exposure to Dioxin-Like HAHs From Diverse Sources

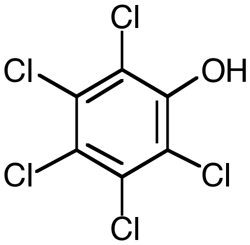
Herbicide Spraying (i.e. Agent Orange)

Meat & Dairy



Combustion

Environmental Contamination

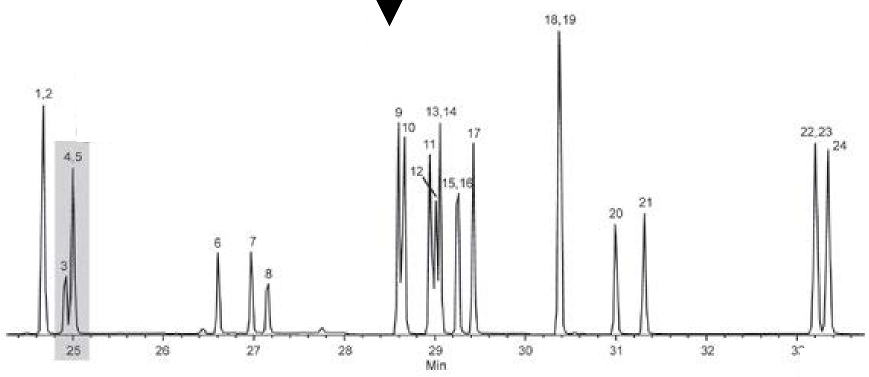


Chlorophenols

Transformers (PCBs)

DLCs: "Gold Standard" Analysis by High Resolution GC:MS

Environmental and Biological Samples → **Extraction and Clean-up Procedures** → **Halogenated Aromatic Hydrocarbons (HAHs)** → **Gas Chromatography High Resolution Mass Spectrometry**

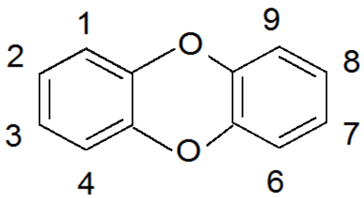


Allows Determination of Exact PCDD, PCDF and PCB Concentrations

But What About the Overall Biological/Toxicological Potency of the sample?

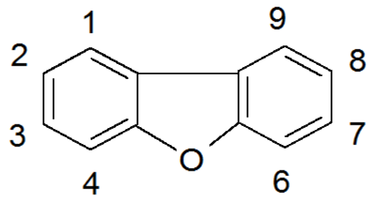
Calculation of the Relative Toxic Potency of a Complex Mixture of Dioxin-Like Halogenated Aromatic Hydrocarbons (TEFs are derived from in vivo toxicity results)

PCDDs



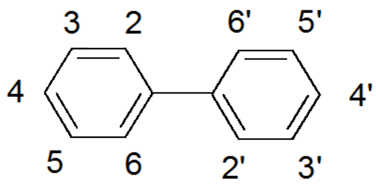
Congeners	TEF
2,3,7,8-TCDD	1.0
1,2,3,7,8-PeCDD	1.0
1,2,3,4,7,8-HxCDD	0.1
1,2,3,6,7,8-HxCDD	0.1
1,2,3,7,8,9-HxCDD	0.1
1,2,3,4,6,7,8-HpCDD	0.01
OCDD	0.0003

PCDFs



Congeners	TEF
2,3,7,8-TCDF	0.1
1,2,3,7,8-PeCDF	0.03
2,3,4,7,8-PeCDF	0.3
1,2,3,4,7,8-HxCDF	0.1
1,2,3,6,7,8-HxCDF	0.1
1,2,3,7,8,9-HxCDF	0.1
2,3,4,6,7,8-HxCDF	0.1
1,2,3,4,6,7,8-HpCDF	0.01
1,2,3,4,7,8,9-HpCDF	0.01
OCDF	0.0003

PCBs



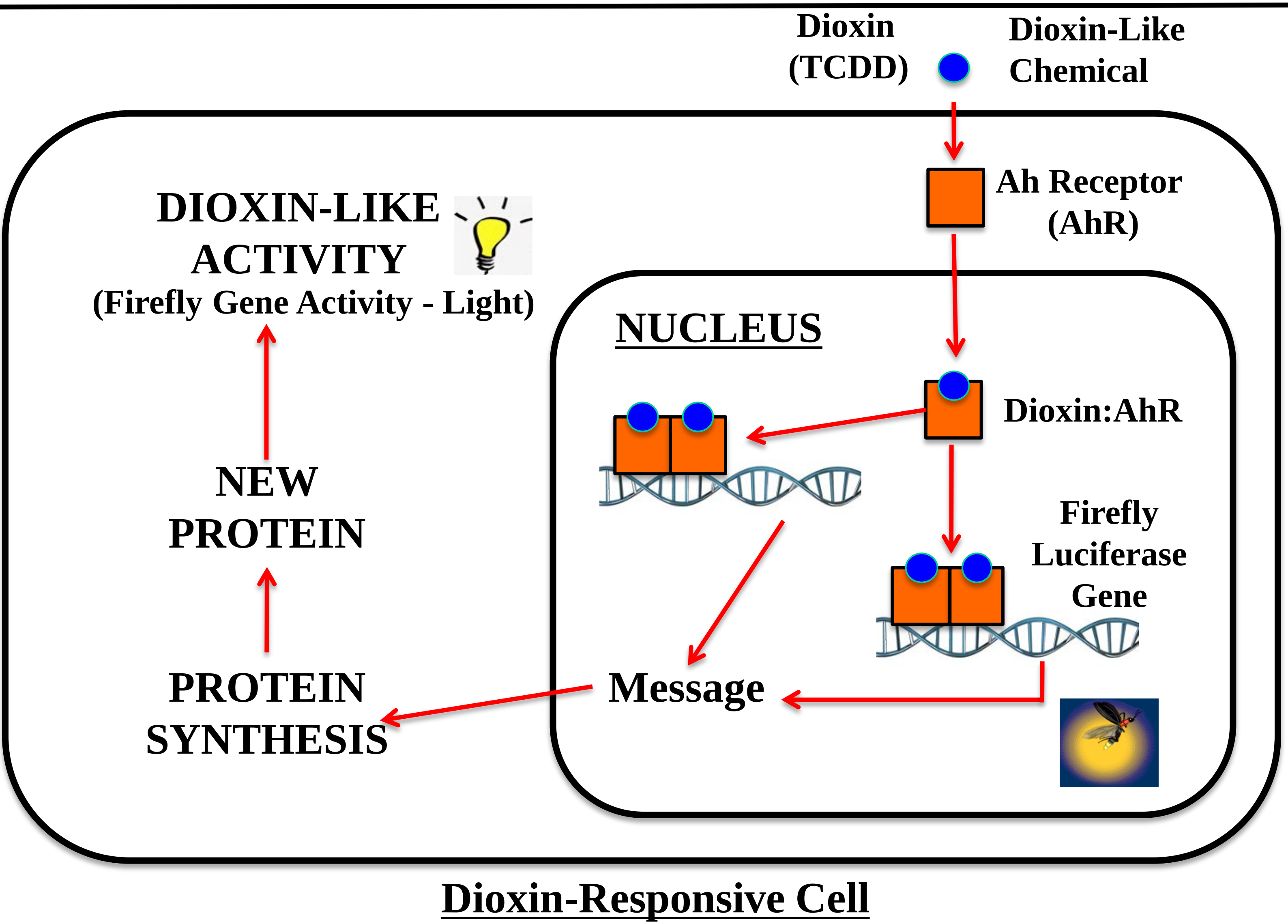
Congeners	TEF
3,3',4,4'-TCB	0.0001
3,4,4',5-TCB	0.0003
3,3',4,4',5-PeCB	0.1
3,3',4,4',5,5'-HxCB	0.03

TEF = Toxic Equivalent Factor
TEQ = Toxic Equivalent

$$TEQ = \sum([PCDD_i \times TEF_i]_n) + \sum([PCDF_i \times TEF_i]_n) + \sum([PCB_i \times TEF_i]_n)...$$

van den Berg et al. (2006) Toxicol. Sci. 93, 223-241

Mechanism of Dioxin Action – AhR CALUX Bioassay



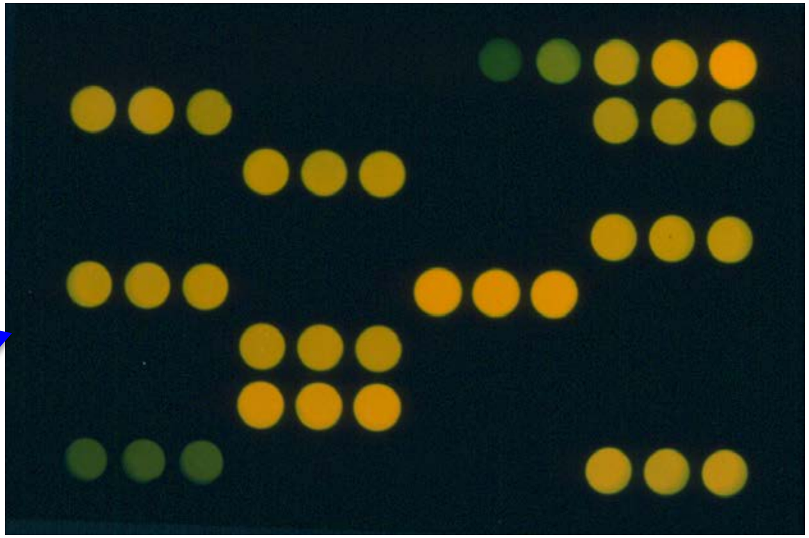
CALUX: Chemically-**A**ctivated **LU**ciferase e**X**pression [USEPA Method 4435]

CALUX Cell Bioassay Procedure

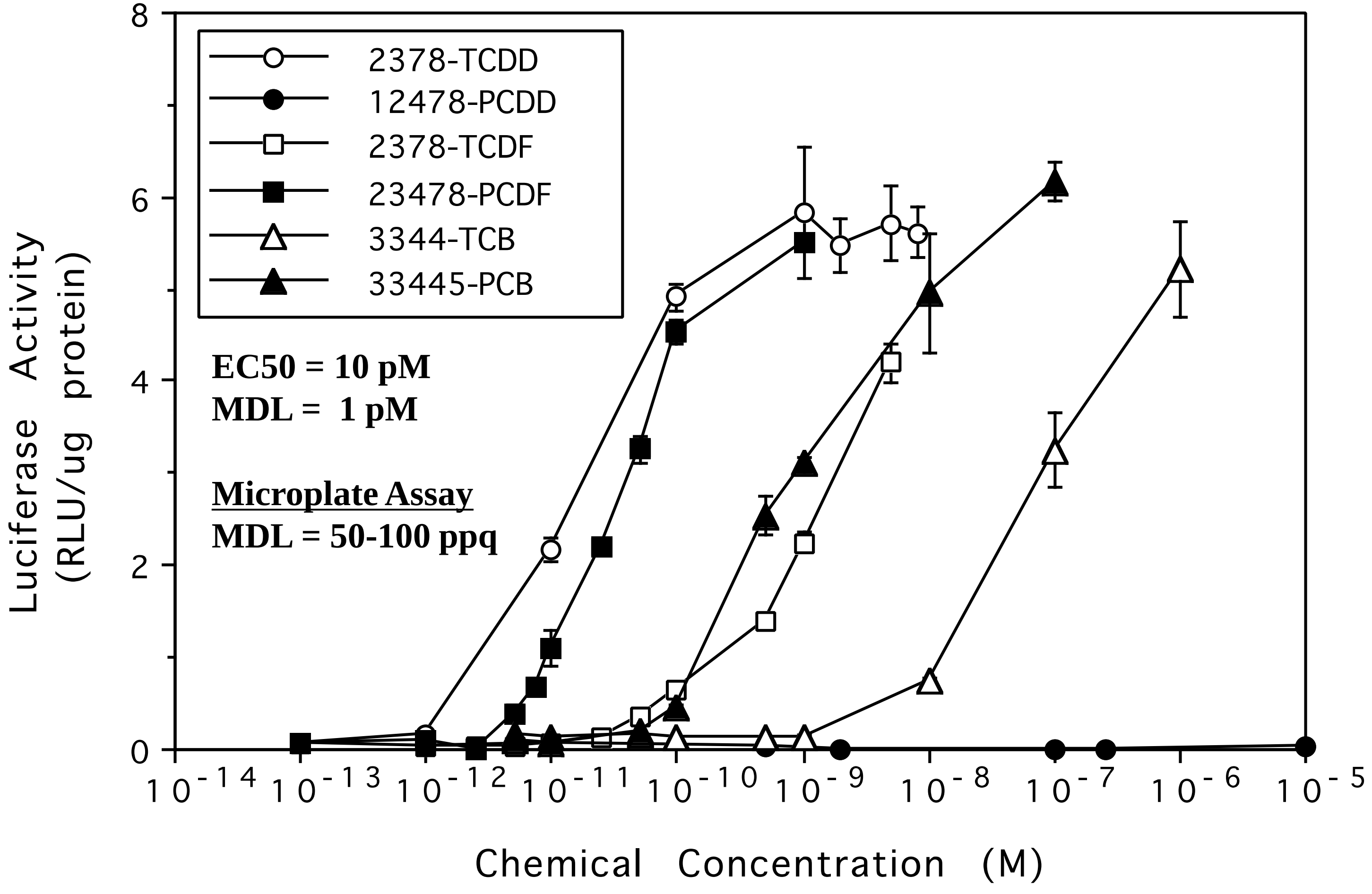
CALUX Cells Plated into 96-Well Microplates

Chemicals or extracts are added to each well and cells incubated for 24 hours

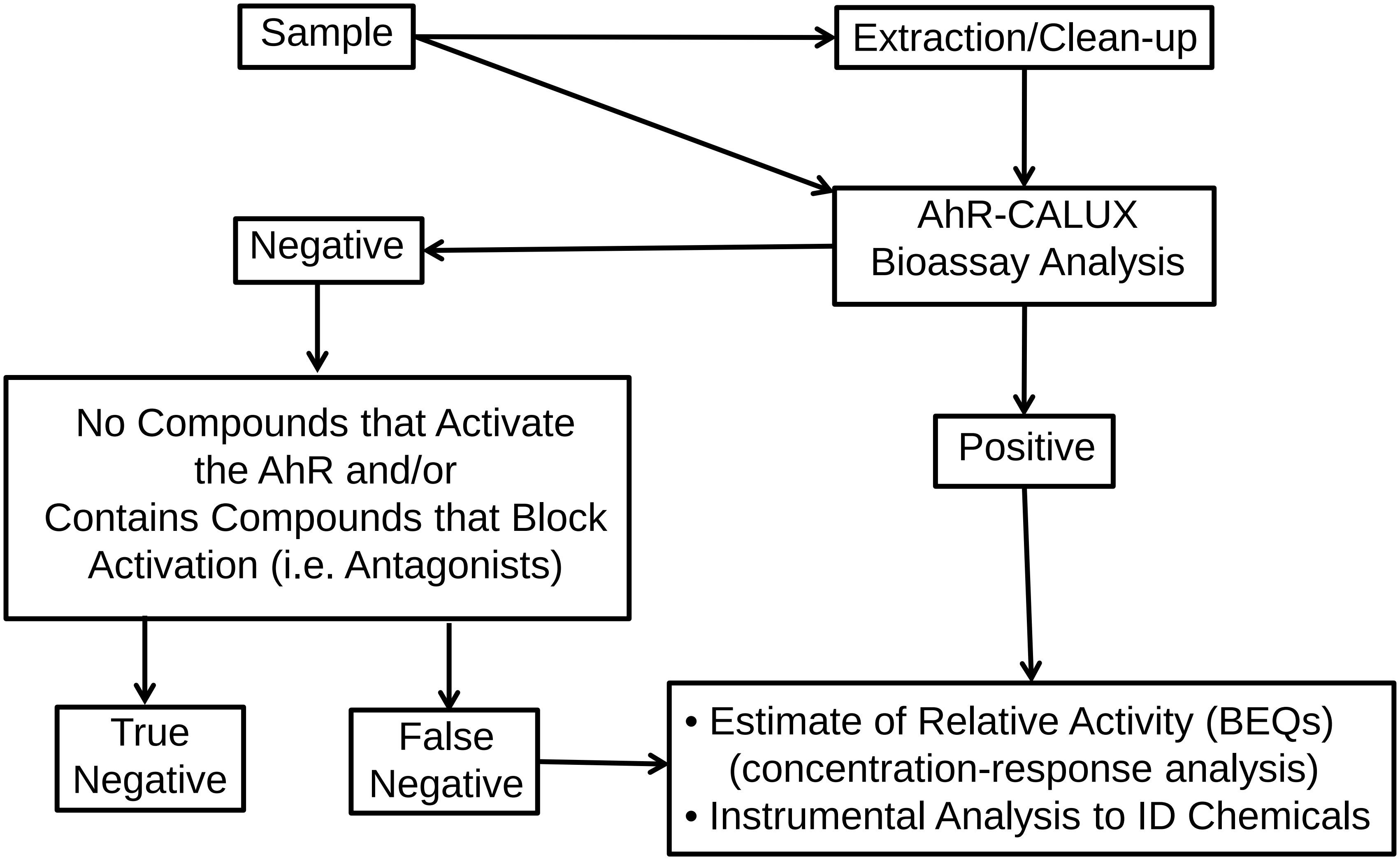
Amount of light produced is directly proportional to the concentration of active chemical added to the cells



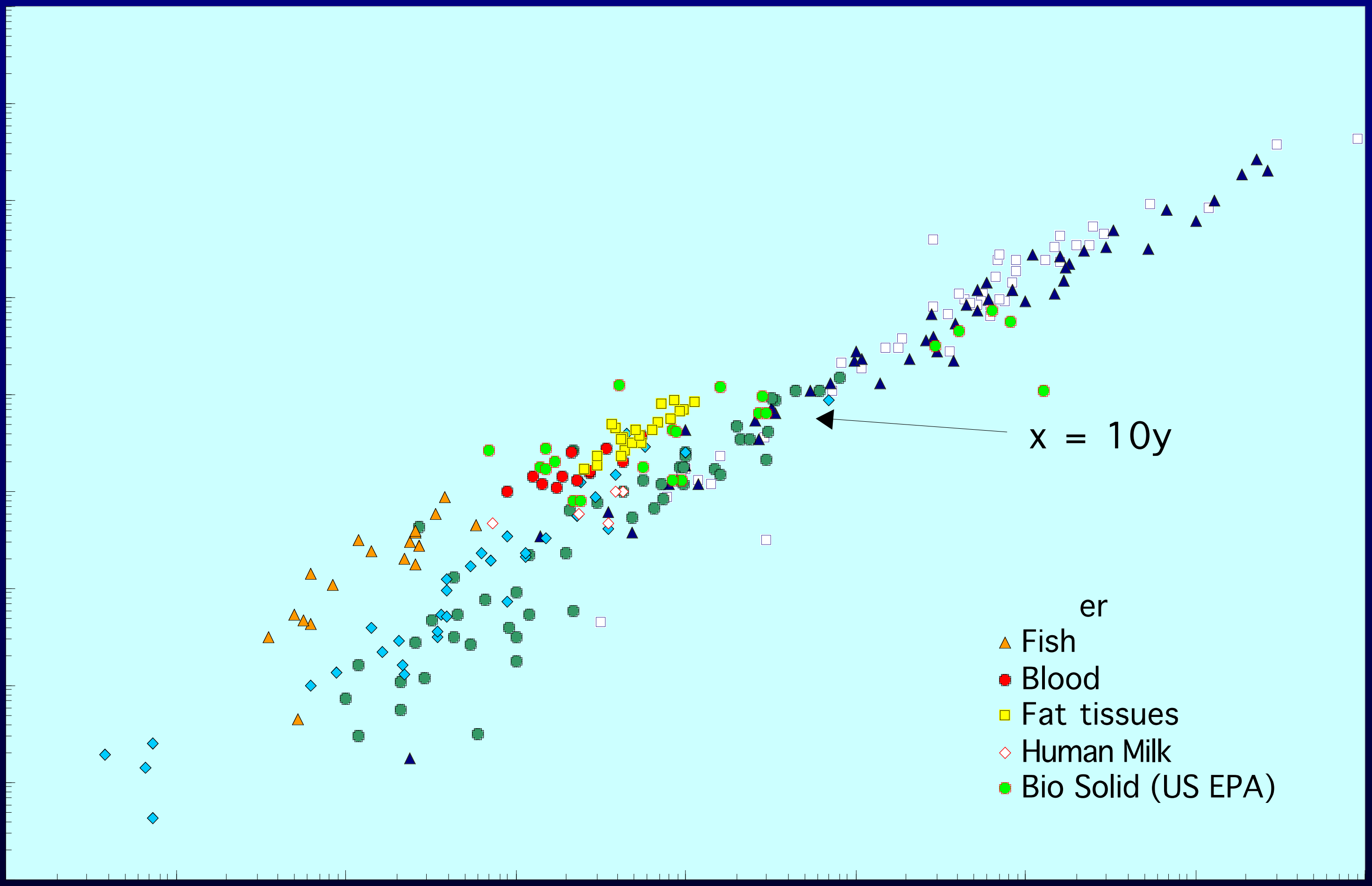
Dose-Dependent Activation of the CALUX Cell Bioassay by PCDDs, PCDFs and PCBs



Flow Diagram for CALUX Analysis of Unknown Chemicals & Extracts



BEQs – Bioanalytical equivalents

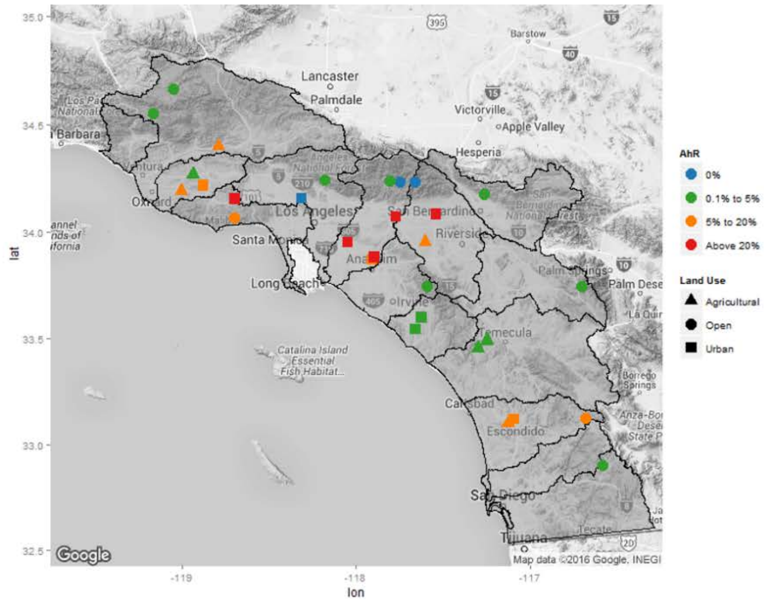


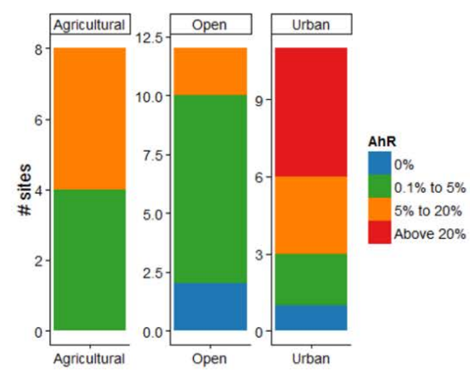
GC/HRMS TEQs (ppt)

Bioassay-BEQ (ppt)

Double-Blind CALUX Analysis of Biological and Environmental Matrices
CALUX BEQ Activity in Environmental Samples is Typically Greater than
TEQs Calculated from Instrumental Analysis (Additional AhR Active Chemicals)

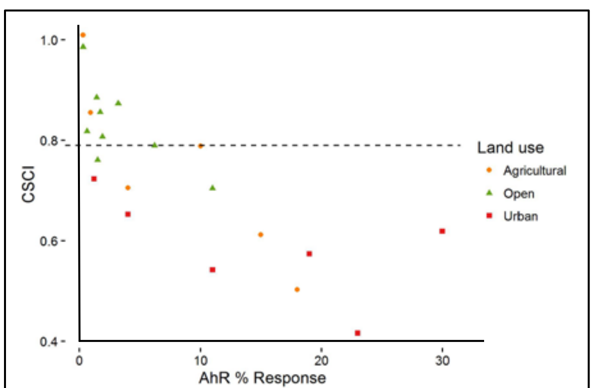
Application of the AhR-CALUX Bioassay for Water Quality Monitoring: Stream Samples From Southern California (2015)

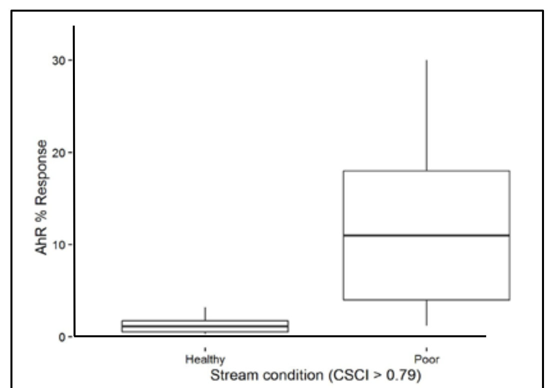




Negative correlation between AhR CALUX bioassay results and California Stream Condition Index (CSCI)

AhR active chemicals remain to be determined – GC:HRMS identified various flame retardants





Collaborative Study with Southern California Coastal Water Research Project (SCCWRP)

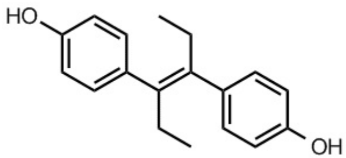
Biological, environmental, food and feed matrices/samples screened using DRE CALUX reporter gene cell bioassays for dioxins and related chemicals.

Biological Samples Screened	Reference	Environmental Matrices Screened	Reference
Human Tissues		**Sediment/Soils**	57,59,61,91,125-131
Blood Plasma/Serum	57-59,111-115		
		Water	15,57,111,132-133
Follicular Fluid	113		
Breast Milk	96,116,117	**Waste Management**	
		Effluent	134
Animal Tissues (various species)		Fly Ash	91,92,97
Blood/Plasma Serum	118,119	Chemical	
Liver	119,120	Dechlorination	97
Blubber	119		
Wild Bird Eggs	121	**Atmospheric**	
Blue Mussel	122	Deposit Organic	
		Film	15,135
Food/Feed Samples		Particulate Matter	61,123,135,136
Feed	90,91,123,124		
Vegetables	120	**Miscellaneous**	
Meat	90,120	PCB Oil	91
Bovine Milk	68,90,120	Recycled paper	137
Fish	57		
Fat Samples	90,120		
Fish/Fisheries Products	120		

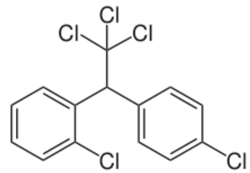
The AhR cell bioassay works for detection of dioxin-like HAHs in cleaned-up sample extracts because the target chemicals (HAHs) act by a common mechanism (AhR) that mediates the toxicity of these chemicals in vivo and we know the identity of the majority of dioxin-like HAHs.

Not True For All Bioassays!

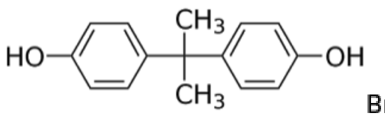
Health Effects of Endocrine Disruptor Chemicals (EDCs) (Estrogenic/Antiestrogenic EDCs)



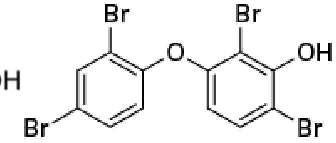
Diethylstilbestrol



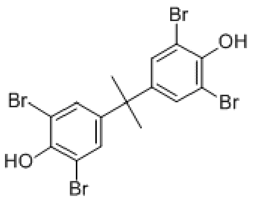
o,p-DDT



Bisphenol A



3-OH-BDE-47



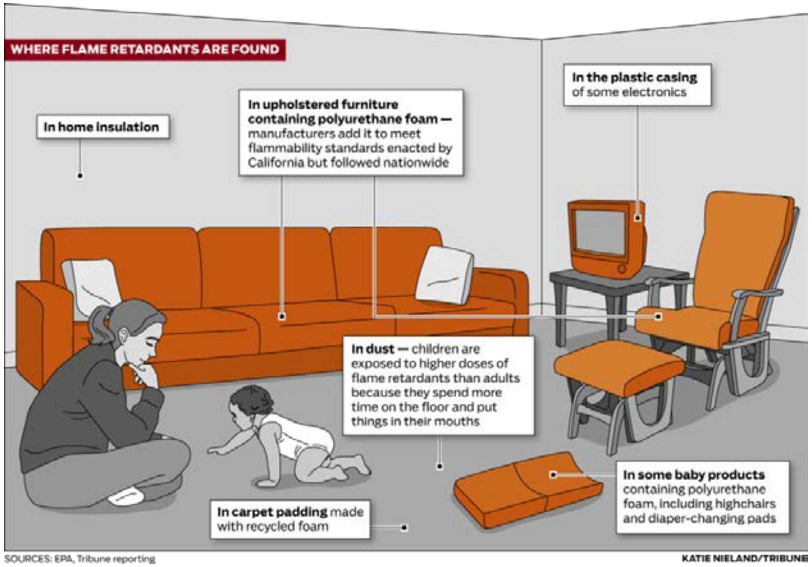
TBBPA

Wildlife and Humans (?)

- **Male reproductive issues: reductions in male fertility, sperm counts and number of males born.**
- **Female reproductive issues: fertility problems, early puberty, early reproductive senescence, endometriosis.**
- **Increased mammary, ovarian and prostate cancers.**
- **Altered sex-specific behaviors.**
- **Increased obesity, T2 diabetes and metabolic syndrome.**

Exposure to EDCs From Diverse Sources

Pharmaceuticals



Flame Retardants

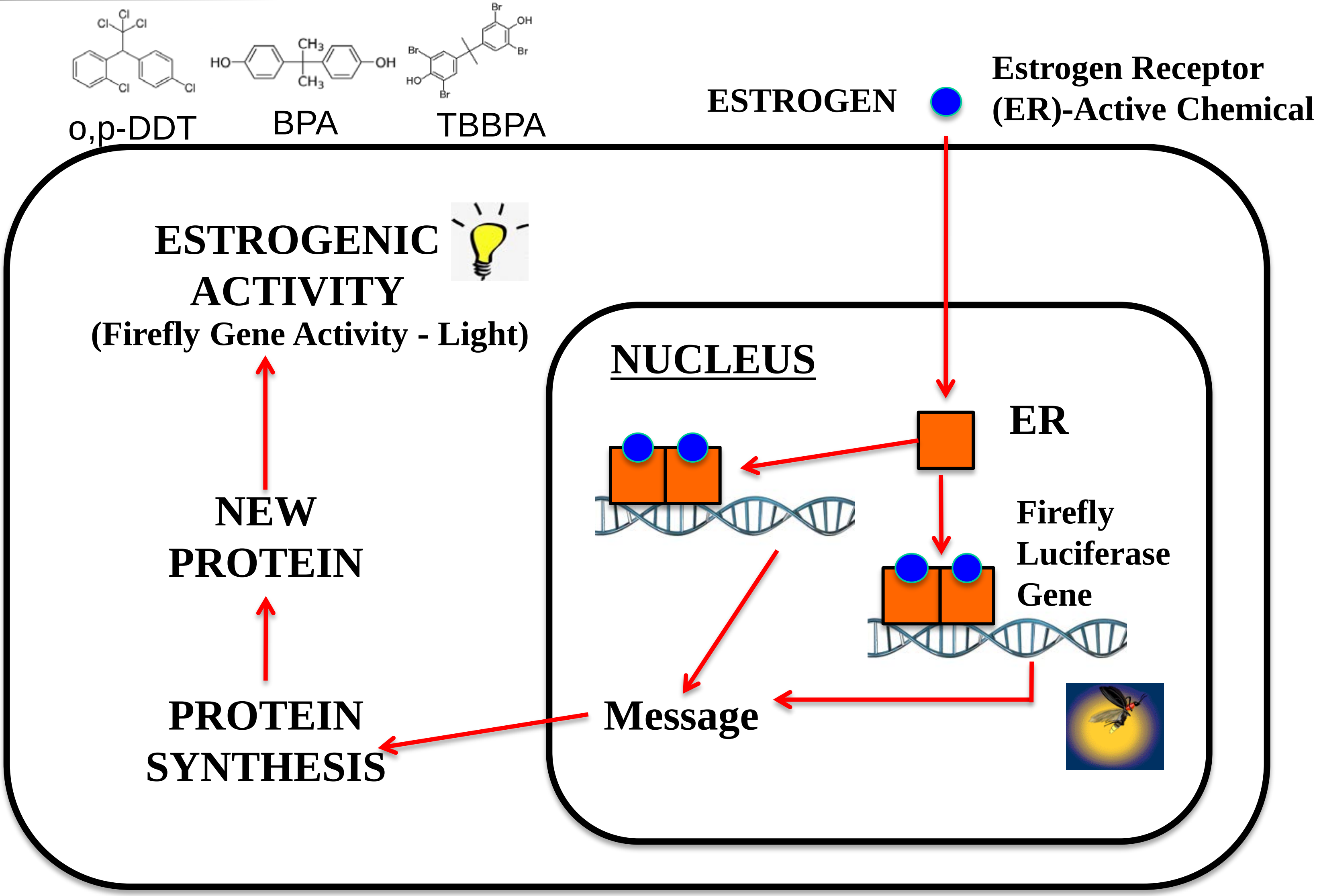
Pesticides

Dioxin-Like HAHs

Plastics and Plastic Products

Sunscreens

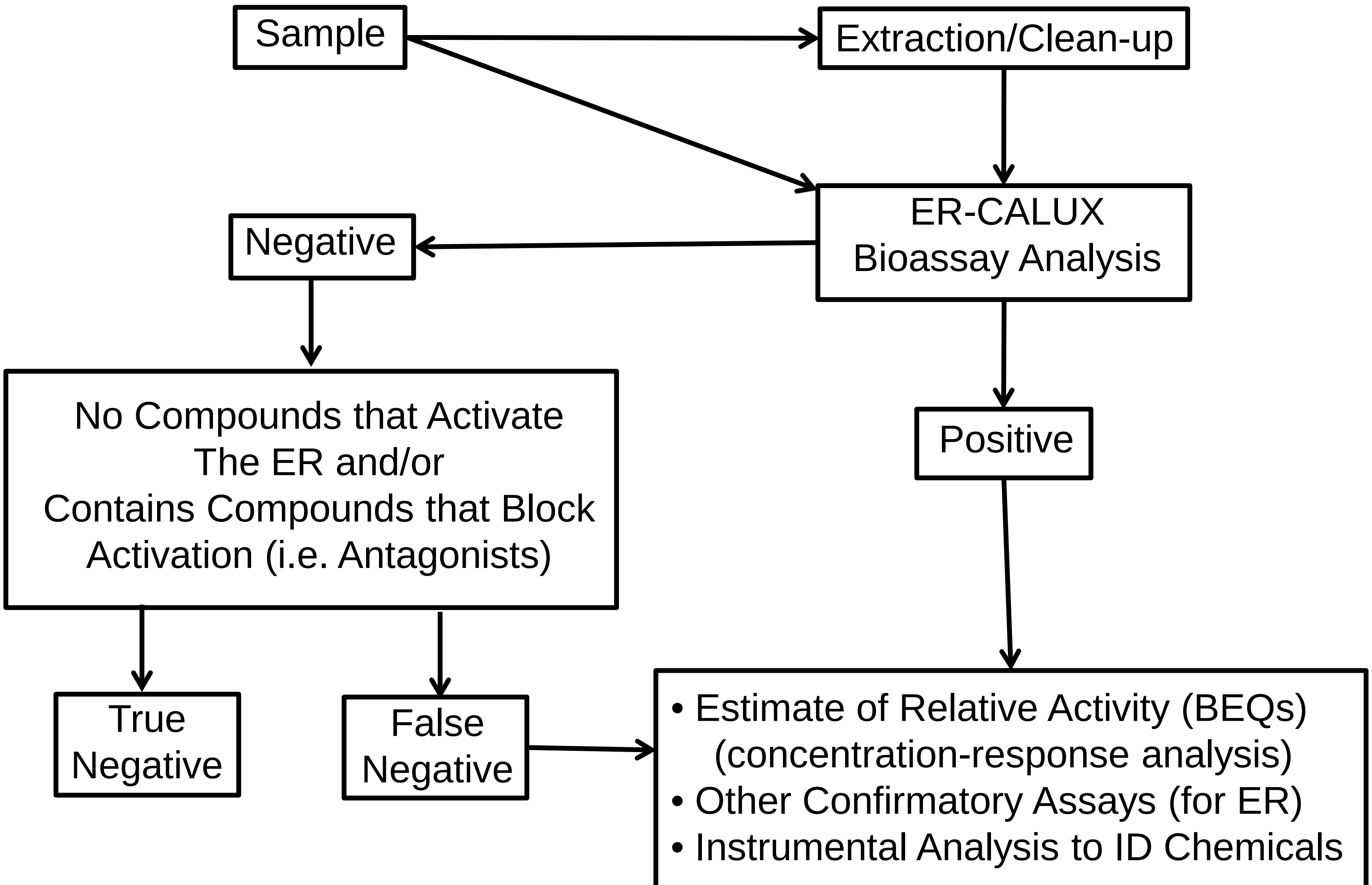
Estrogenic/Antiestrogenic Chemicals – ER CALUX Bioassay



Estrogen-Responsive Cell

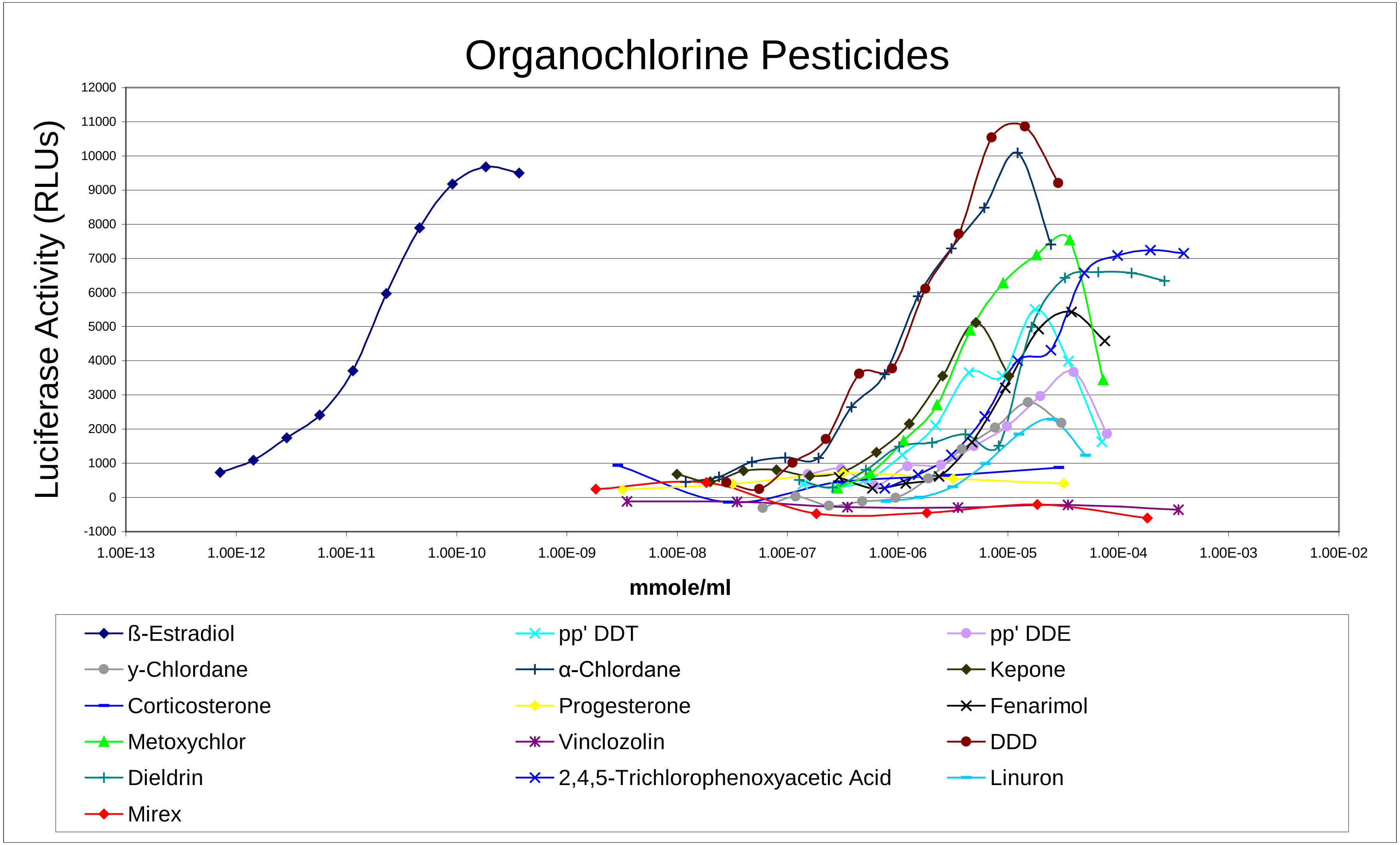
Method approved by OECD (TG455 and TG457) and USEPA (EDSP)

Flow Diagram for CALUX Analysis of Unknown Chemicals & Extracts

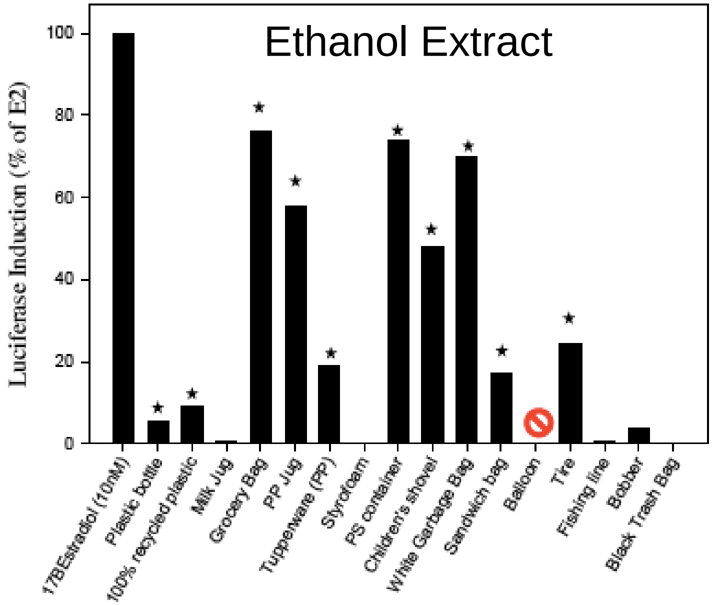


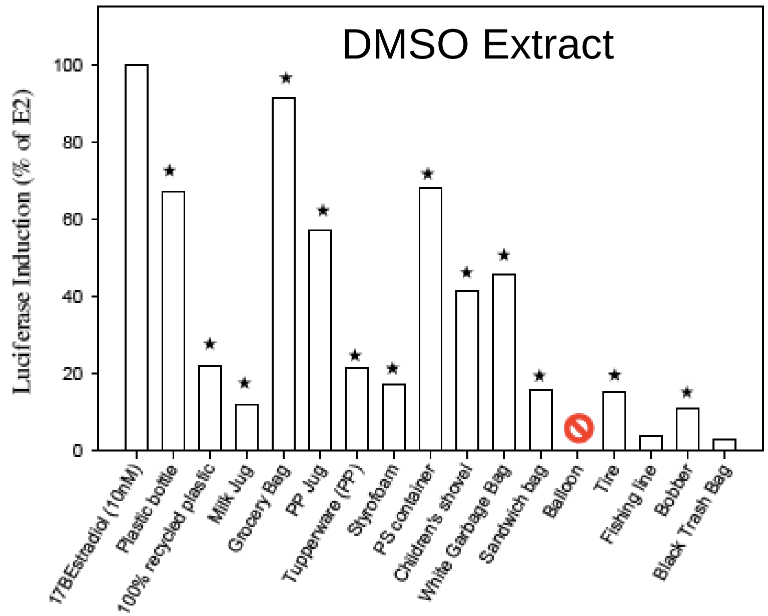
BEQs – Bioanalytical equivalents

Pure Chemical Screening



Extracts of Plastic and Rubber Products Contain Estrogenic Chemicals



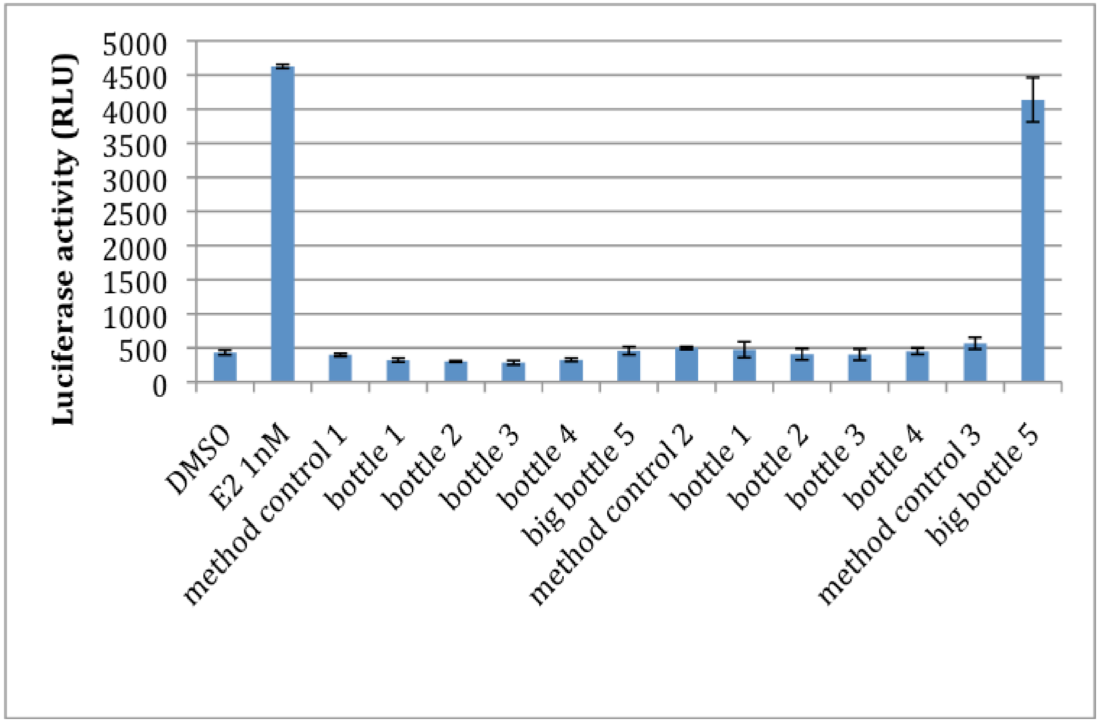


The level of estrogenic activity is dependent on the extraction solvent, suggesting different types of chemicals are being extracted

Bisphenol A (BPA)-Free Does Not Necessarily Mean
<u>Free of Estrogenic Activity (EA) Or That It Is An EDC!</u>

Kossack and Denison, 2013

ER-CALUX Bioassay Analysis of Drinking Water From Plastic Containers for Estrogenic Chemicals



Water:

Method 1. Control (500ml MilliQ water)

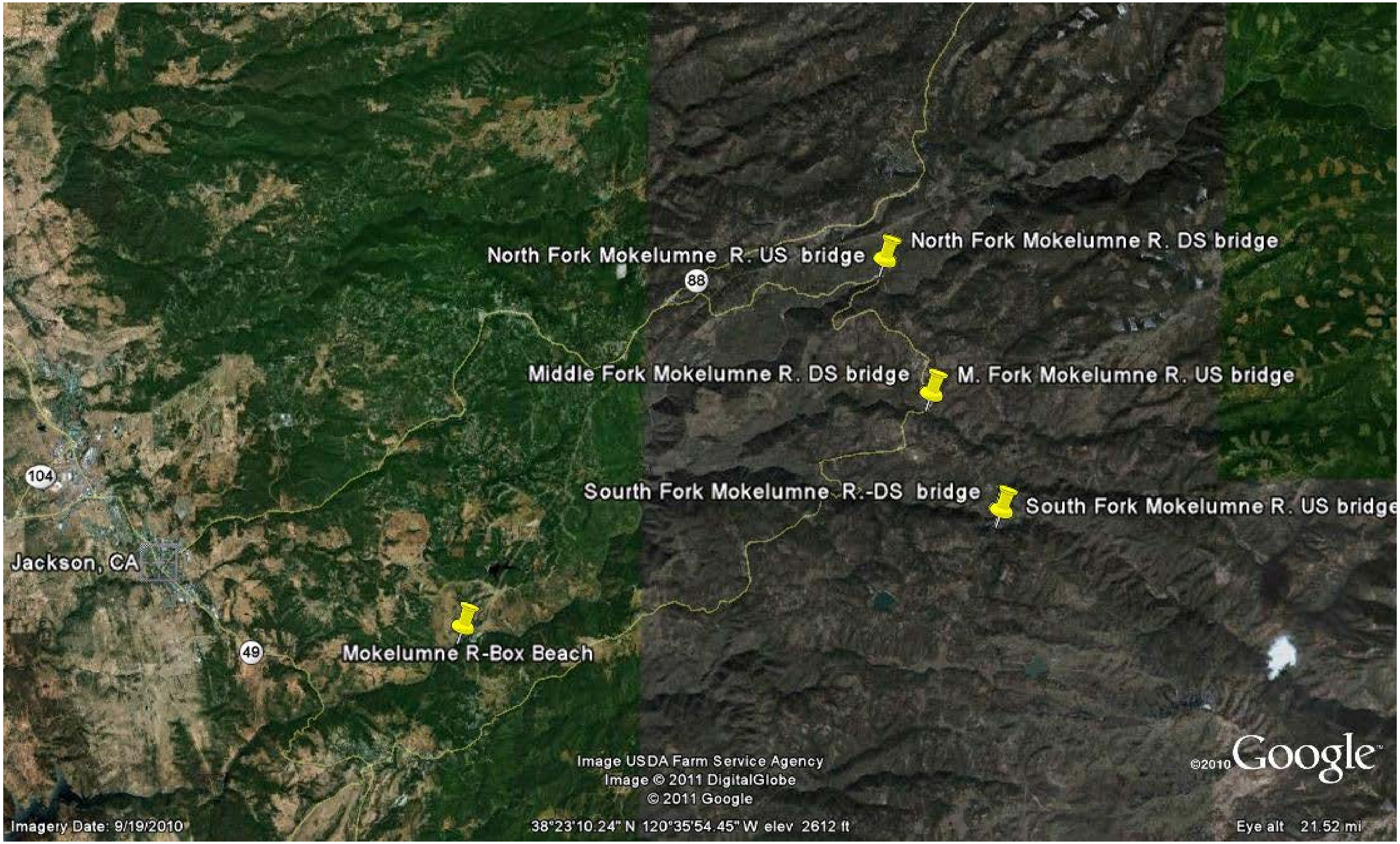
Test (500ml from water bottles (#1-4) or 5 gal. Carboy (#5))

Bottles:

Method 2. Control (20ml ethanol): Test (20ml ethanol in water bottles (#1-4))

Method 3. Control (40ml ethanol): Test (40ml ethanol in carboy (#5))

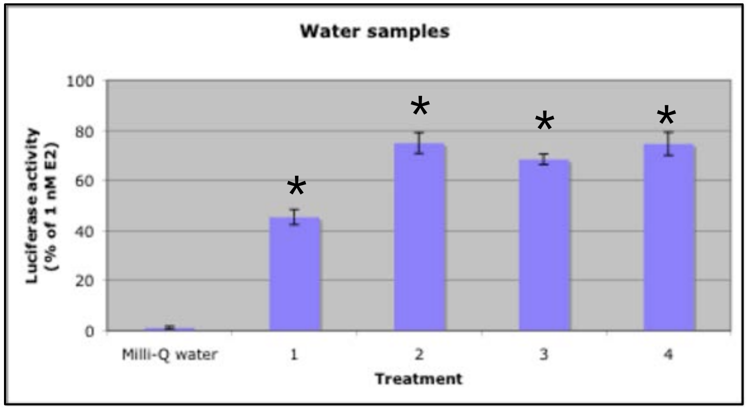
Environmental Monitoring For Estrogenic Activity Mokelumne River - Sampling Sites

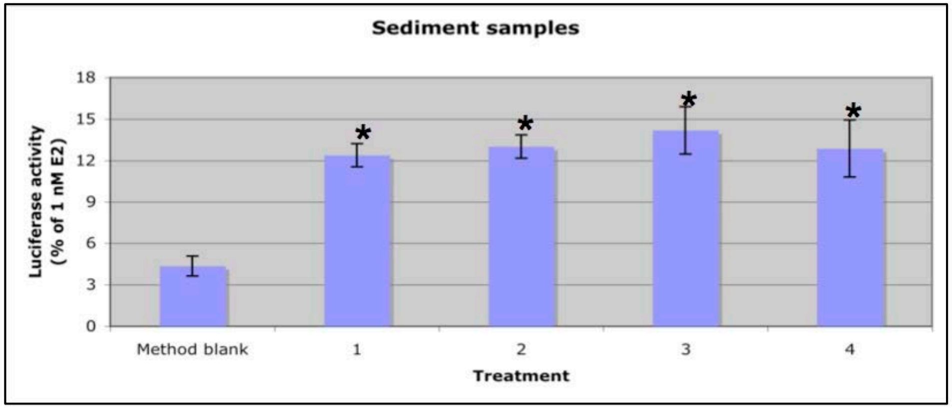


Estrogenic Activity of Water and Sediment Samples from Upper Mokelumne and Calaveras Rivers

Samples: Extracts of 1 liter of water or 10 g of sediment

1. Bridge, Sheep Ranch
2. South Fork, RRF Road
3. Middle Fork, Taylor Bridge
4. North Fork, Hwy 26 Bridge

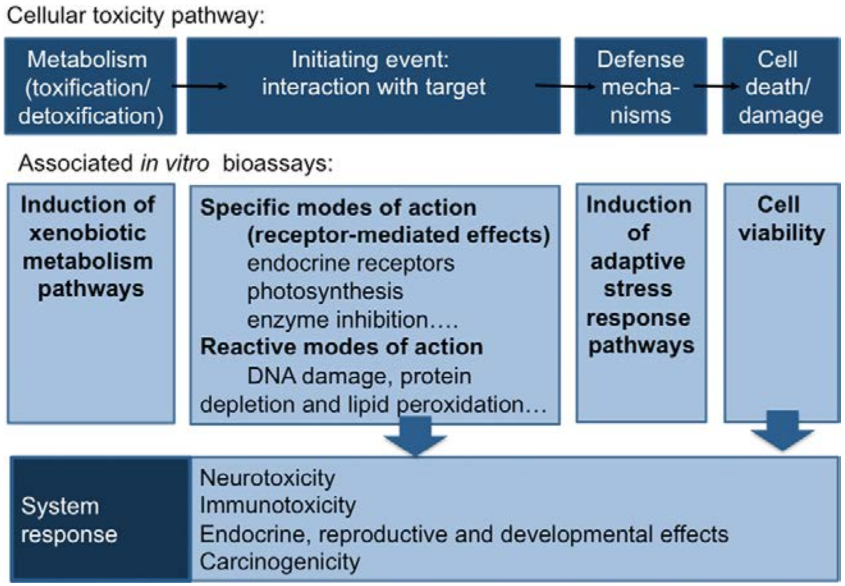


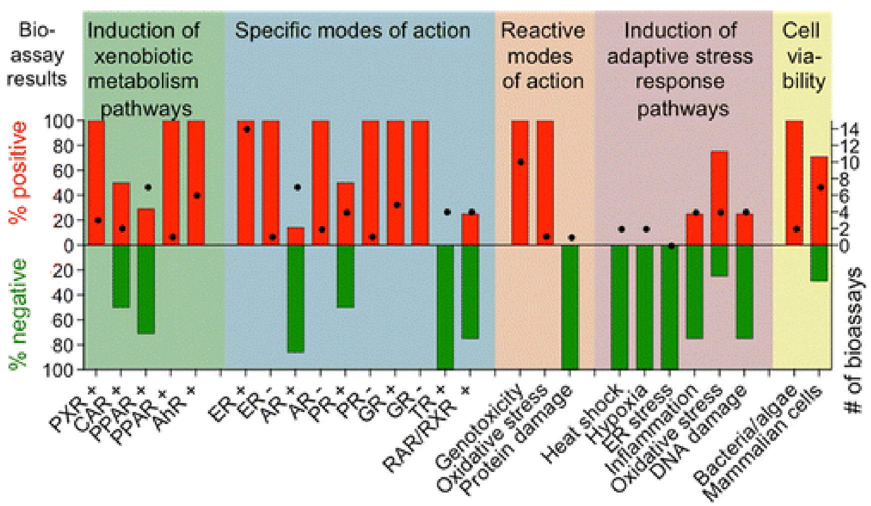


Significant levels of estrogenic activity in all Mokelumne River samples (equivalent activity in 10ml of water); sediment with relatively low activity. The responsible estrogenic chemical(s) remain to be identified.

Effects–directed analysis (EDA) - Combination of bioassays and chemical fractionation methods provides an avenue in which to identify the responsible bioactive chemical(s) in a complex mixture.

Combined Use of Many Bioassays for Water Quality Screening
(20 laboratories analyzed 10 water samples using 103 bioassays)

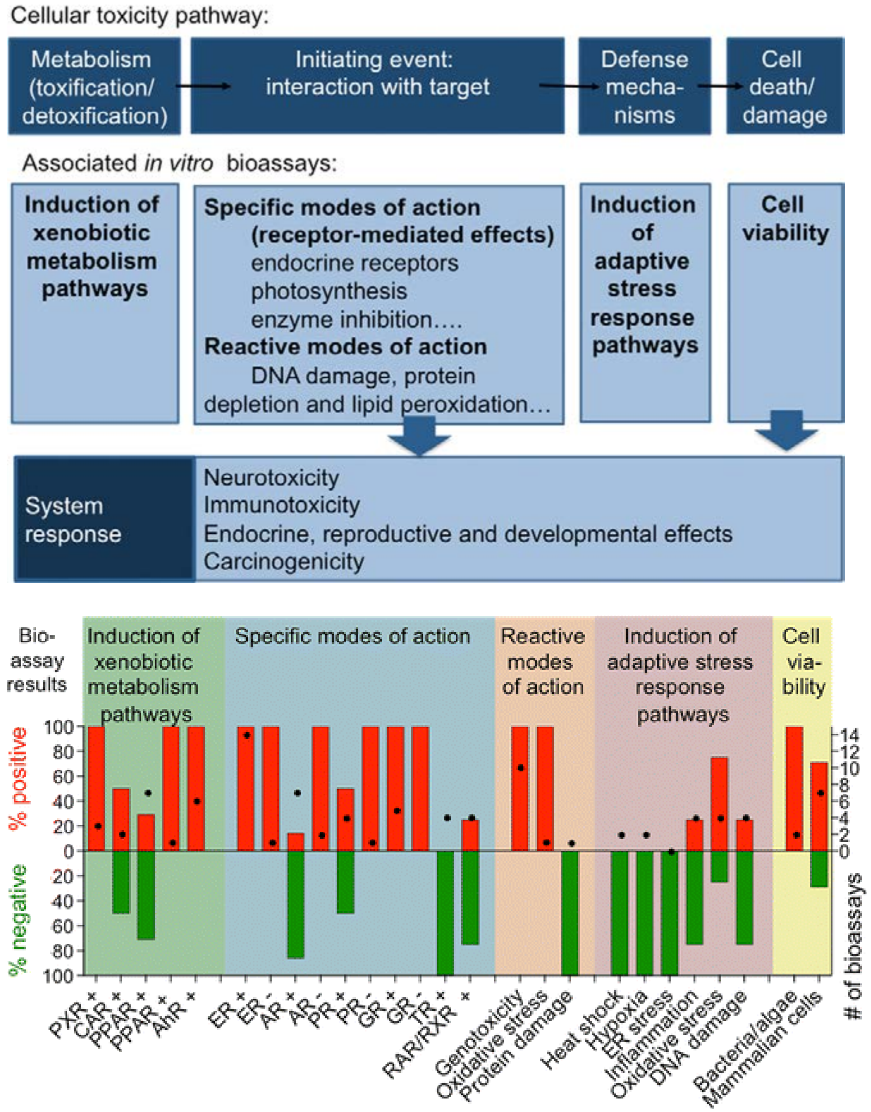




toxicity pathway	MOA	inducing chemicals/ *positive controls*	total	+	−
xenobiotic metabolism	pregnane X receptor (PXR)	steroids/	3	3	0
	constitutive androstane receptor (CAR)	phenobarbitol, various pharma-ceuticals	2 (1)	1	1
	peroxisome proliferator-activated receptor (PPAR)	phthalates, fibrate pharmaceuticals	7 (1)	2	5
	PPAR suppression		1	0	1
	aryl hydrocarbon receptor (AhR)	PAHs, PCDDs, coplanar PCBs	6 (1)	6	0
specific MOA	acetylcholinesterase (AChE)	insecticides	1	0	1
	photosystem II	herbicides	1 (1)	1	0
specific receptor-mediated MOA	estrogen receptor (ER)	human hormones and industrial chemicals (xenoestrogens), *17β-estradiol*	14 (9)	14	0
	ER suppression	*4-Hydroxy-tamoxifen*	1 (1)	1	0
	androgen receptor (AR)	*(Dihydro)-testosterone*	7 (6)	1	6
	AR suppresion	*Flutamide*	2 (1)	2	0
	progesterone receptor (PR)	*Levonorgestrel*	4 (5)	2	2
	PR suppression	*Mifepristone*	1	1	0
	glucocorticoid receptor (GR)	*Dexamethasone*	5 (6)	5	0
	GR suppression	*Mifepristone*	2	2	0
	thyroid receptor (TR)	*3,3′5-Triiodo-thyronine*	4 (1)	0	4
	RAR/RXR (Reproductive and developmental effects)	*Retinoic acid*	4	1	3
reactive modes of action	genotoxicity	*4-Nitroquinoline-N-oxide*	11 (4)	11	0
	oxidative stress	PAH, electrophilic chemicals, *t-butyl hydroquinone*	1	1	0
	protein damage	*Sea-Nine*	1	0	1
adaptive stress response pathway	heat shock response	oxygen depletion (can be caused by metals)	2	0	2
	hypoxia	tunicamycin, caplain	2	0	2
	endoplasmic reticulum stress	high salt, glycol	0	0	0
	inflammation	metals, PCBs, smoke, particles	4	1	3
	oxidative stress	reactive oxygen species, *t-butyl hydroquinone*	4	3	1

Escher et al. Env. Sci. Tech. 48, 1940 (2014)

Combined Use of Many Bioassays for Water Quality Screening
(20 laboratories analyzed 10 water samples using 103 bioassays)



Escher et al. Env. Sci. Tech. 48, 1940 (2014)

Tested: wastewater effluent, recycled water, stormwater, surface water and drinking water.

• Each water type had a characteristic bioanalytical profile with particular groups of toxicity pathways and were consistently positive or negative across test systems.

• The most responsive health-relevant endpoints were related to xenobiotic metabolism, hormone receptor pathways, genotoxicity, oxidative stress responses.

• The study demonstrated the utility of selected cell bioassays to benchmark water quality and the authors recommended a purpose-tailored panel of bioassays for routine monitoring.

Conclusions & Considerations

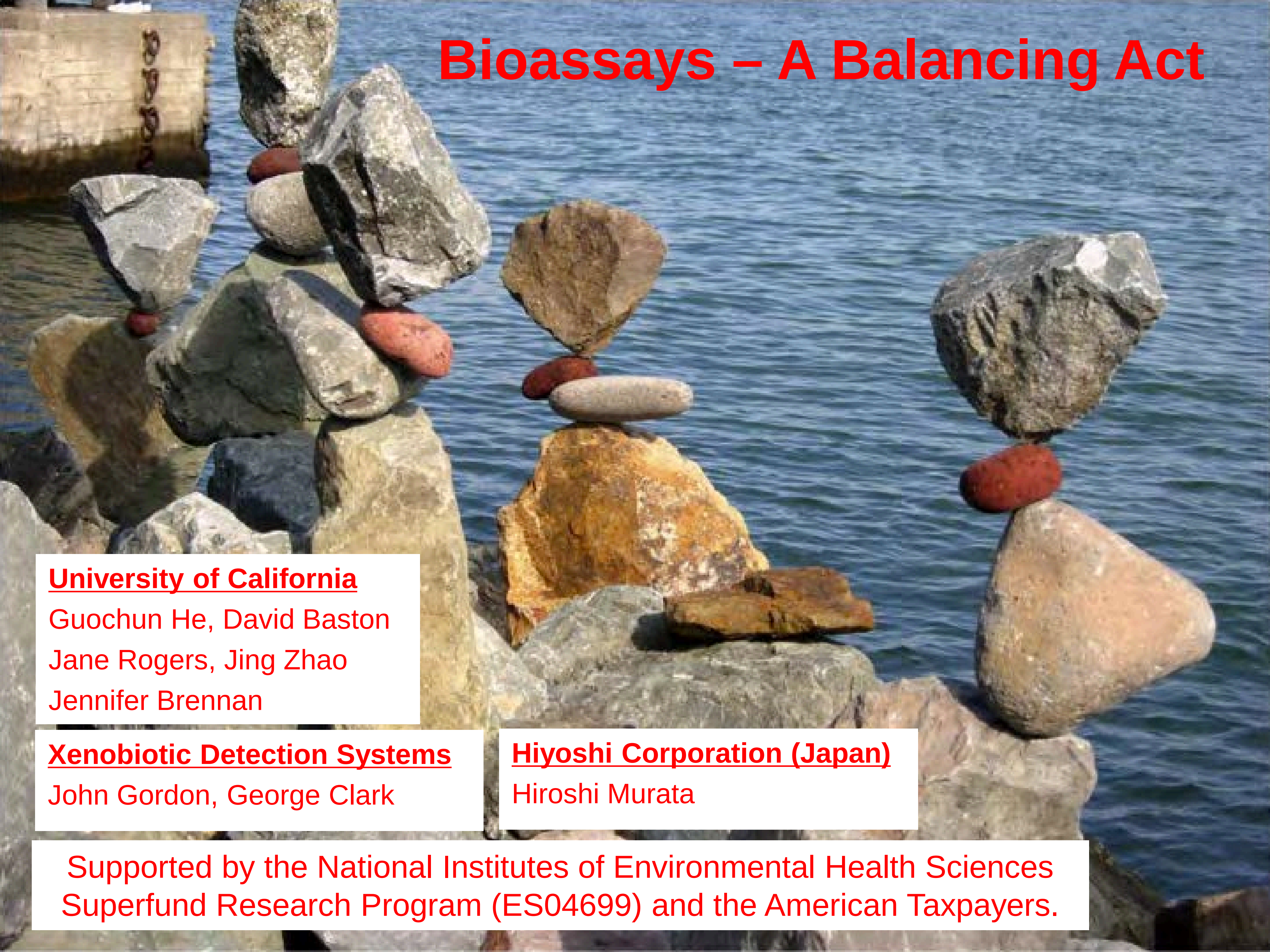
• Bioassays are not a replacement for instrumental analysis methods, but can complement those methods by allowing prioritization of sample analysis by the more costly and time-consuming instrumental analysis methods.

• Bioassays provide cost-effective and broad screening approaches for chemicals (known and unknown) affecting selected AOPs - some aspects must be considered:

- Extraction method used? [polar and nonpolar chemicals]
- Identity of chemical(s)? [unknown chemical mixtures – EDA]
- Toxic potential of chemical/extract in vivo? [AOP considerations of bioassay]
- Critical interpretation of results. What does a positive result tell you and what does a negative result tell you…. or not tell you?

• Given the current limitations of most bioassays for predicting adverse human health effects in vivo, they have been recommended for water monitoring applications and not for regulatory purposes.

Bioassays – A Balancing Act



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