Design Of High-Throughput Screens And Their Applications In The Biomedical And Environmental Sciences

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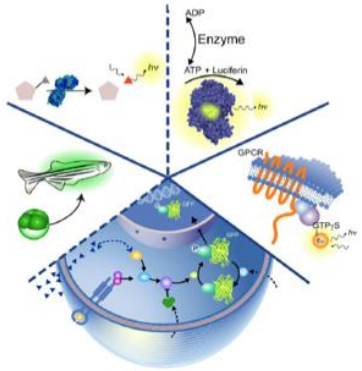
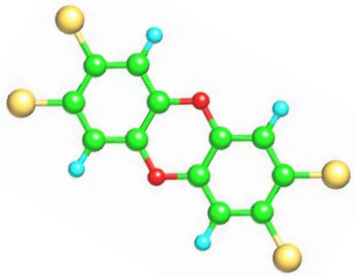
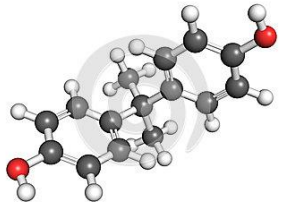
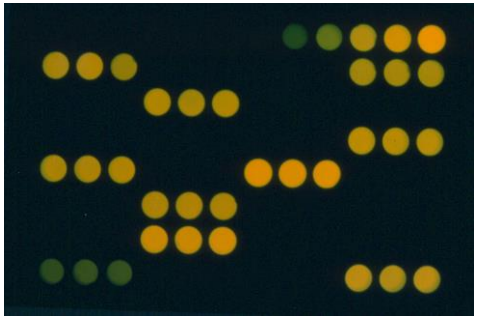








High Throughput Screening

High Throughput Screening (HTS) is most often thought of as the drug-discovery process widely used in the pharmaceutical and biotech industry.

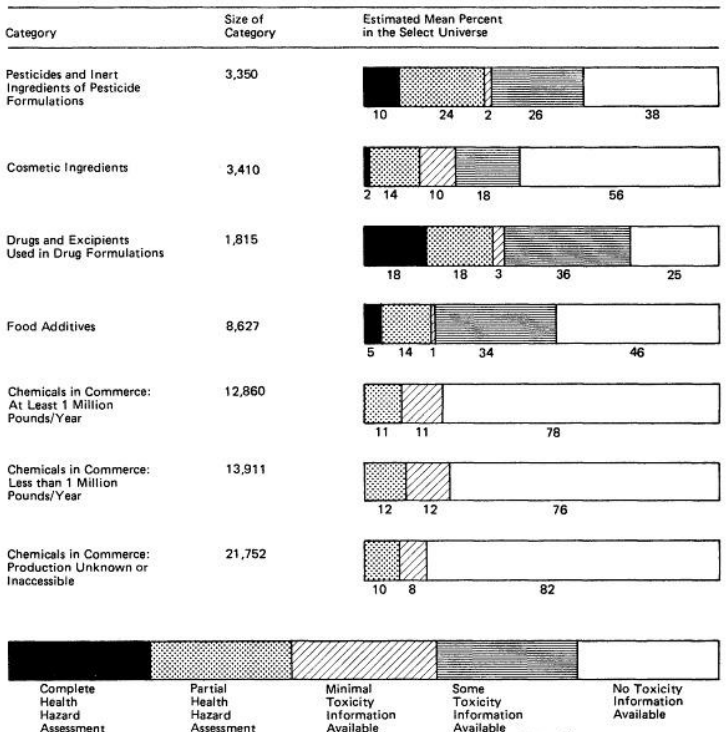
It leverages robotics and automation to quickly assay the biological or biochemical activity of a large number of chemical compounds **against a desired therapeutic target**. Commonly 10,000 to >1,000,000 compounds are screened per day.

It is a useful for discovering specific chemicals (lead compounds) that can interact with receptors, enzymes or other pharmacological targets, or to profiling a cellular or biochemical pathway of interest. Compounds further optimized for optimal drug design.

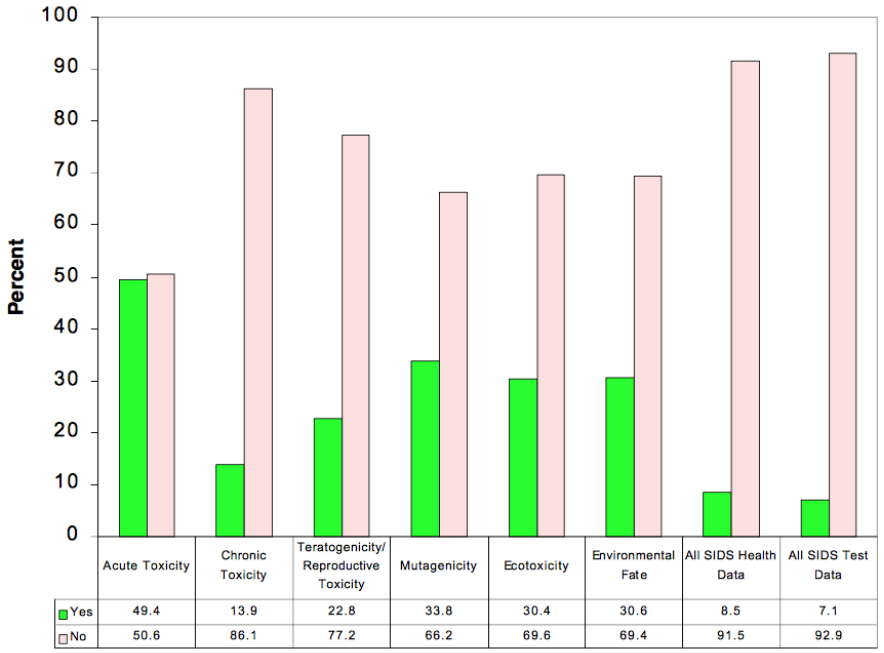
This type of HTS is not suitable for toxicity testing or toxicology screening since the specific biological or toxicological activity and/or mechanism of action of a compound or class of compounds is typically not well understood or known or the chemicals are unknown.

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



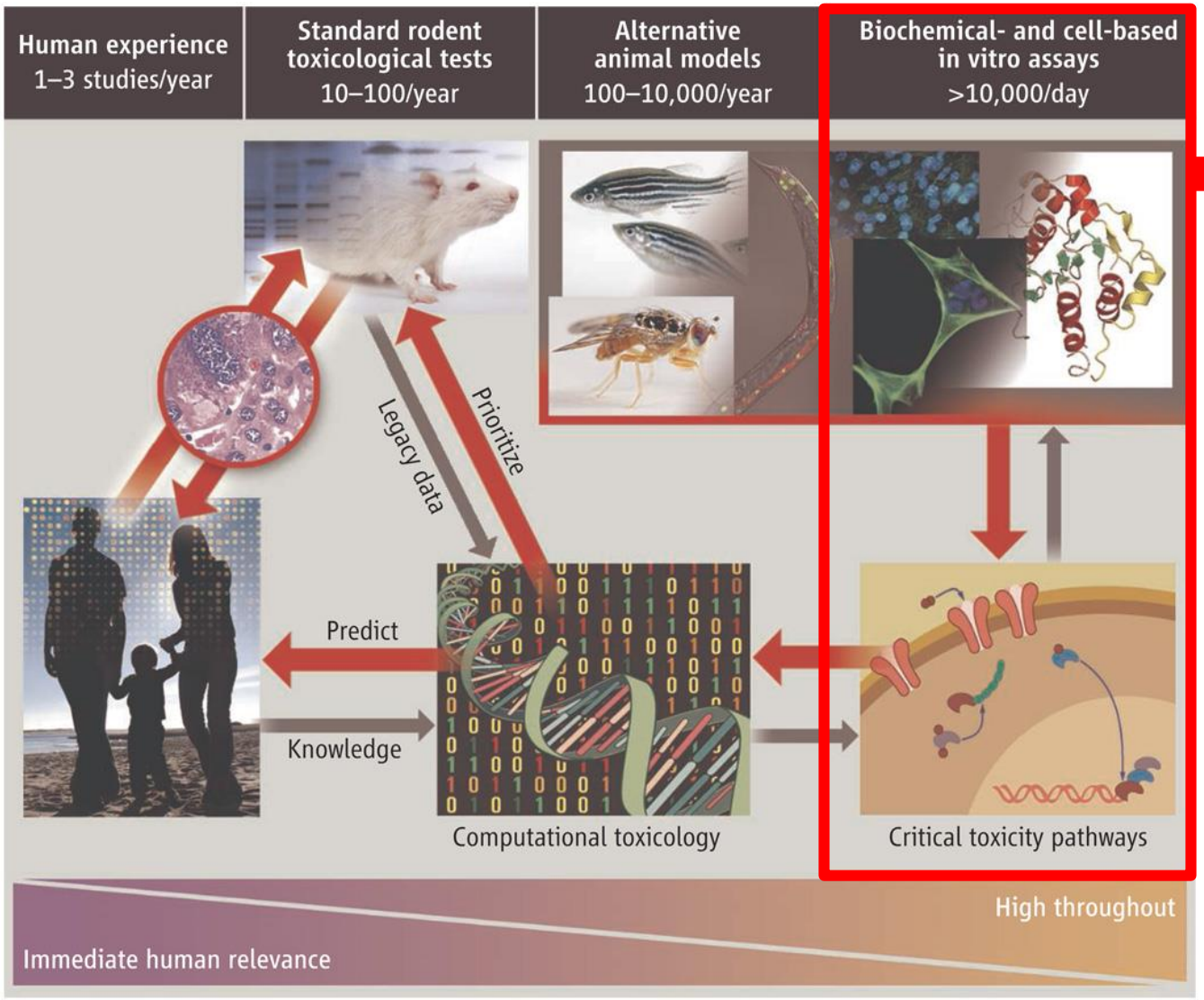
US High Production Volume (HPV) Chemicals
(2,863 produced at > 1 million lbs per year)



Chemical Hazard Data Availability Study (USEPA,1998)

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

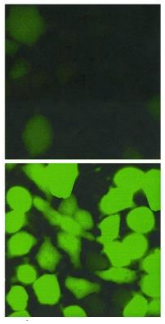
Can High Throughput and Computational Toxicology Approaches Provide the Toxicological Data Needed to Help Understand or Predict the Adverse Effects of Chemicals In Vivo?



Depends on Number of Bioassays & In Vitro ↓ to ↓ In Vivo Extrapolation of Bioassays

Collins et al. Science 319,906 (2008)

Development of a HTS Screening Assay



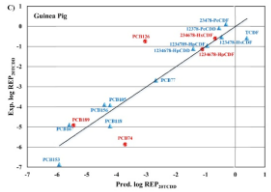
Assay developed in a research lab setting

Identify potential HTS-compatible assay formats

Develop the assay protocol and reagents

Adapt the screening assay to automation and scale up

Optimize and validate assay performance



Develop secondary assays to validate and confirm HTS positives

(environmental screening assays with instrumental analysis confirms)

Modified from Dexheimer, 2014

Variety of Modes For HTS Bioassay Output Detection

Detection methods simplified

- Fluorescence
- Luminescence
- Absorbance
- Fluorescence Polarization
- FRET; TR-FRET
- AlphaScreen (Perkin Elmer)
- FLIPR (calcium channel sensing)
- High content microscopy (fluorescence)

Example assay formats

Purified molecular targets

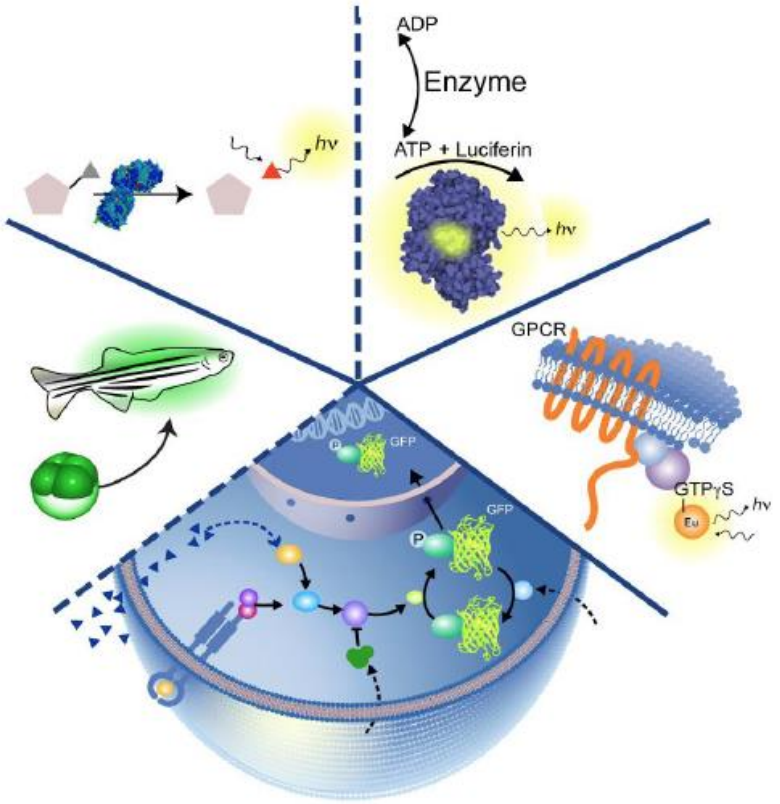
- pro-florescent substrates
- coupled-enzyme reporters

Cell extracts

- membrane preparations
- reconstituted signaling cascades

Cellular/organism phenotypes

- reporter-gene cellular sensors
- model organisms
- high-content imaging



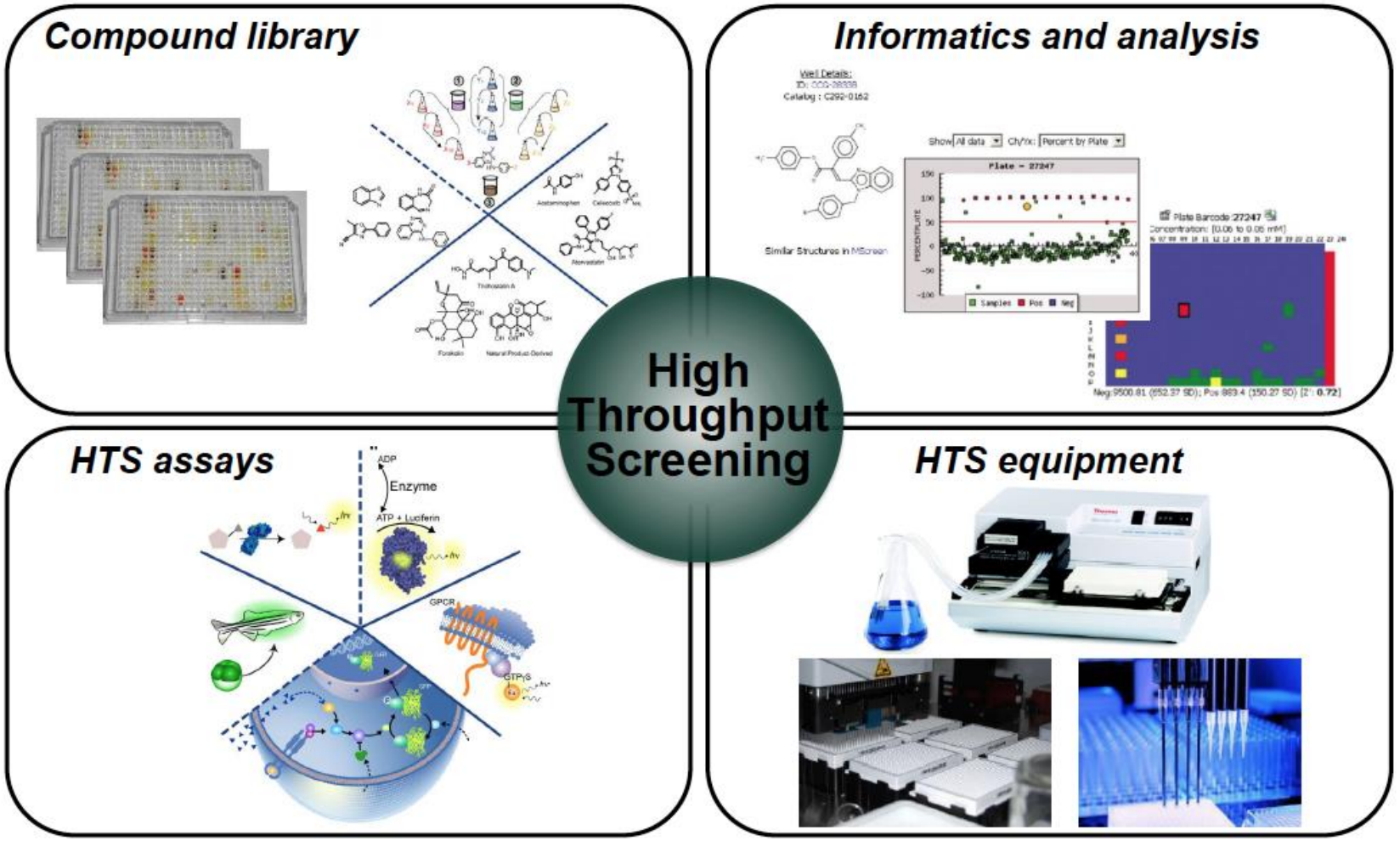
Inglese et al. *Nature Chemical Biology* (2007).

HTS Screening Assay Examples

- Receptor-binding
- Enzyme (stimulation/inhibition)
  - proteases, kinases, phosphatases, lipases, esterases, others
- Cytochrome P450 inhibition
- Protein binding
- Bacterial growth
- Cell-based reporter gene (stimulation/inhibition)
  - nuclear receptors, transcription factors
- Cell signaling pathways
  - NFkB, RTKs, PKs, p53
- Cell growth, cell viability, apoptosis, cytotoxicity
- Stress response
  - DNA damage, heat shock, hypoxia, oxidative, inflammation

Bioassays can't be comprehensive (some mechanisms and assays not amenable to HTS), multifactorial mechanisms can be problematic, in vitro bioassay is not a tissue or animal

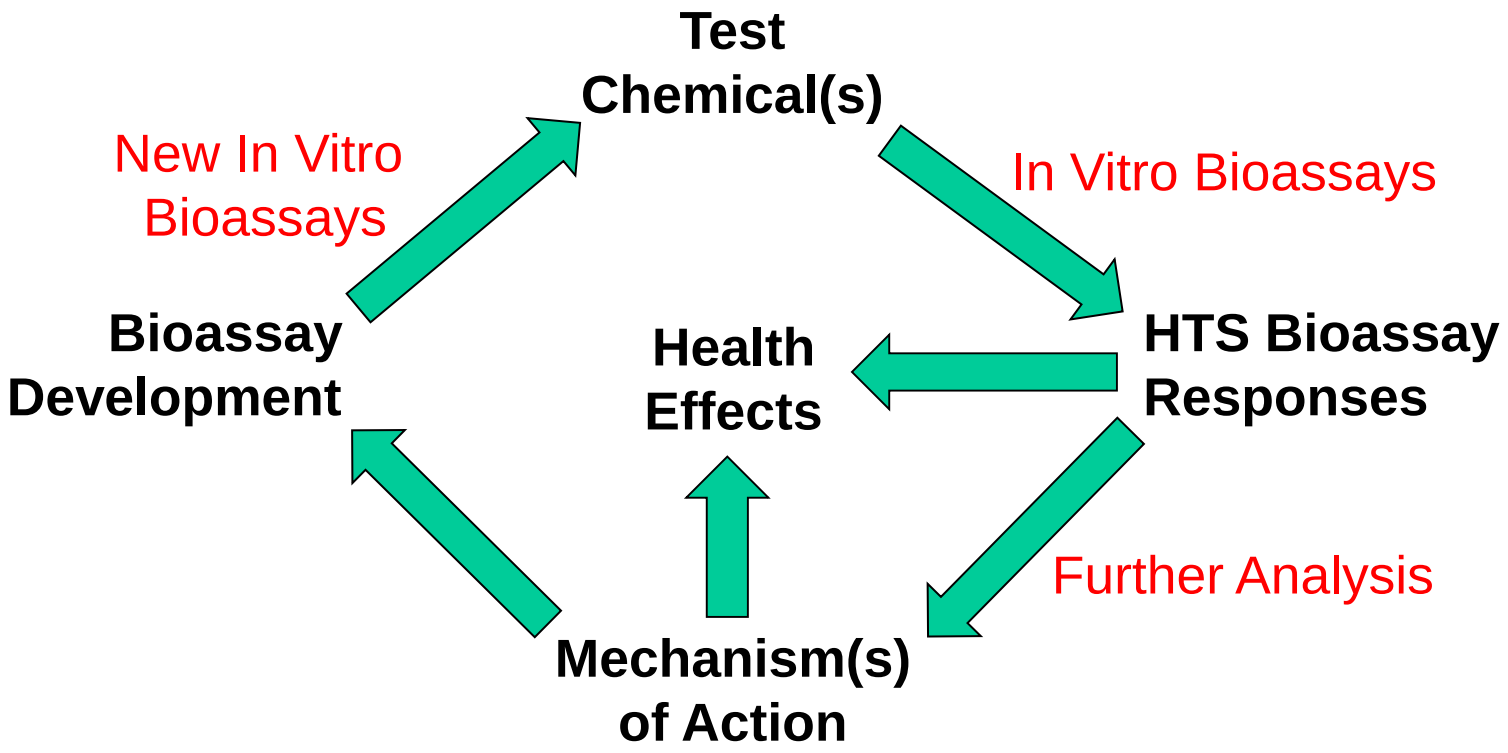
HTS Allows Targeted Biological/Toxicological Effects of Chemicals to be Determined Using Diverse In Vitro Bioassays



The Tox21 and ToxCast Screening Programs are currently doing just that.

HTS of Chemicals With Multiple In Vitro Bioassays

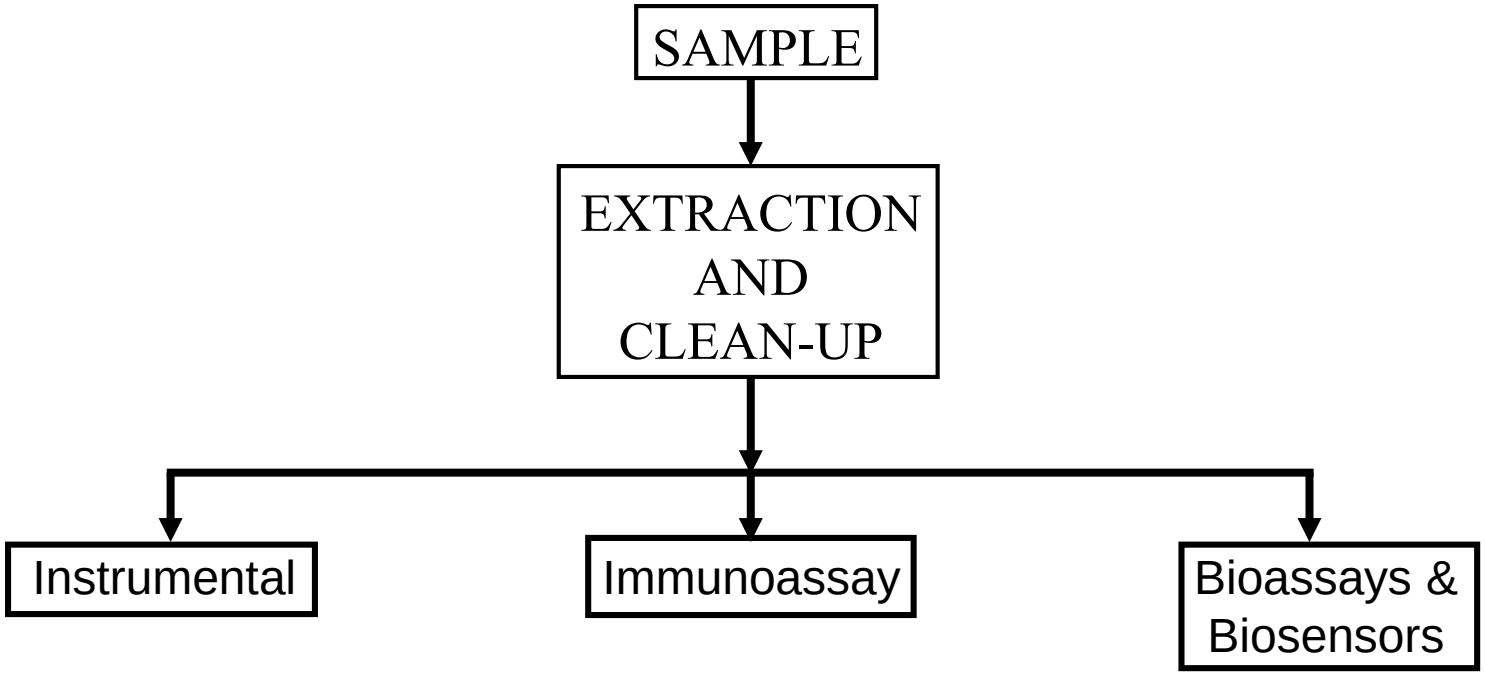
- **Identifies Some Biological Toxicological Effects and Mechanisms of Action Based on Available Bioassays**
- **Allows Development of Other Chemical Selective Bioassays**



Application of in vitro bioassays for detection and relative quantitation of bioactive chemicals in environmental and biological samples and consumer products.

Examples of Validated (Regulatory Accepted) High-Throughput Environmental Screening/Monitoring Bioassays

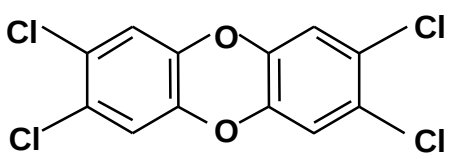
Chemical and Biological Techniques to Detect and Quantitate Dioxin-Like Chemicals (DLCs) and Endocrine Disruptor Chemicals (EDCs)



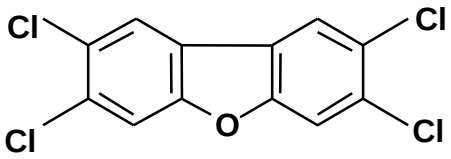
Issues to consider:

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)

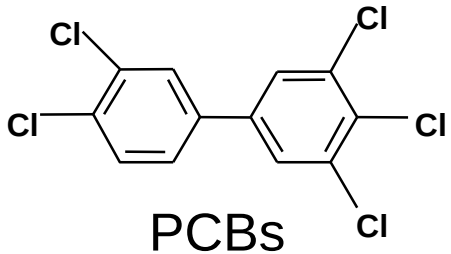
Health Effects of Dioxin-Like HAHs



PCDDs



PCDFs



PCBs

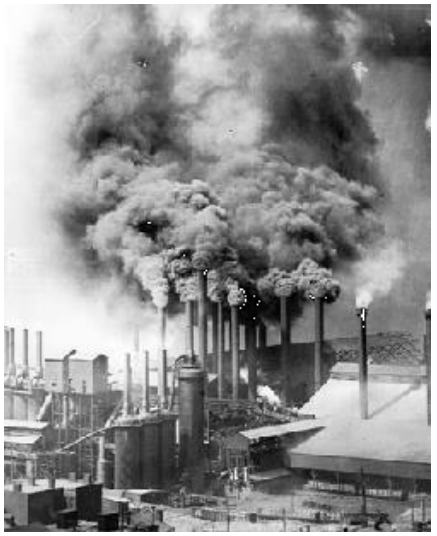
Toxicity	Biochemical
Cancer	Endocrine disruption (estrogen/testosterone)
Immunotoxicity	Inhibit cell division
Heart disease	Alter gene expression (induction/repression)
Liver toxicity	Alter chemical and drug degradation
Skin toxicity	Oxidative stress
Birth defects	
Wasting syndrome	
Lethality	

HAHs – Halogenated Aromatic Hydrocarbons

Exposure to Dioxin-Like HAHs From Diverse Sources

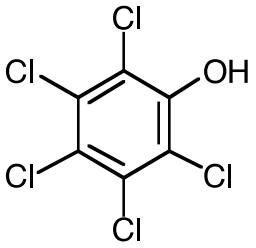
Herbicide Spraying
(i.e. Agent Orange)

Meat & Dairy



Combustion

Environmental Contamination



Chlorophenols

Transformers (PCBs)

Dioxin-Like HAHs: "Gold Standard" Analysis by Instrumental Analysis

Environmental and Biological Samples

→

Extraction and Clean-up Procedures

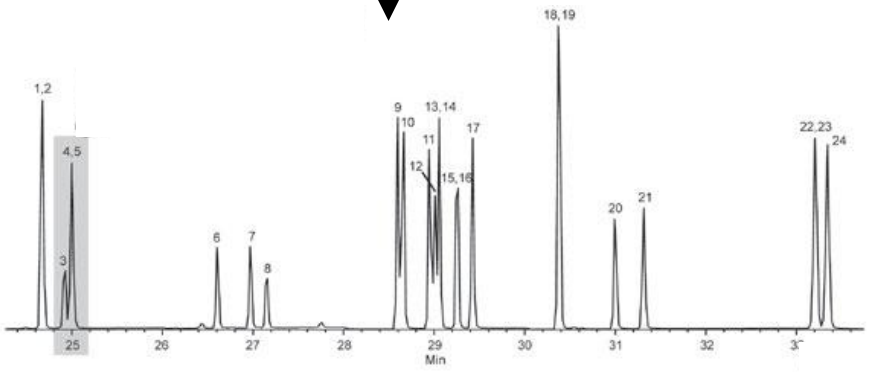
↓

Halogenated Aromatic Hydrocarbons (HAHs)

←

Gas Chromatography High Resolution Mass Spectrometry

↓

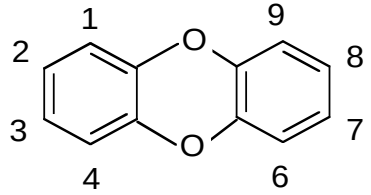


Allows Determination of Exact PCDD, PCDF and PCB Concentrations

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

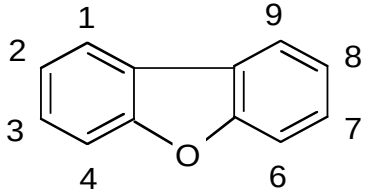
Calculation of the Relative Toxic Potency of a Complex Mixture of Dioxin-Like Halogenated Aromatic Hydrocarbons (TEFs are derived from in vivo toxicity results)

PCDDs



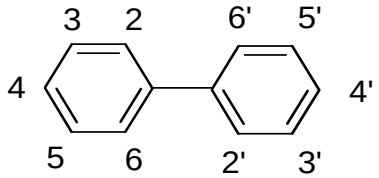
Congeners	TEF
2,3,7,8-TCDD	1.0
1,2,3,7,8-PeCDD	1.0
1,2,3,4,7,8-HxCDD	0.1
1,2,3,6,7,8-HxCDD	0.1
1,2,3,7,8,9-HxCDD	0.1
1,2,3,4,6,7,8-HpCDD	0.01
OCDD	0.0003

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



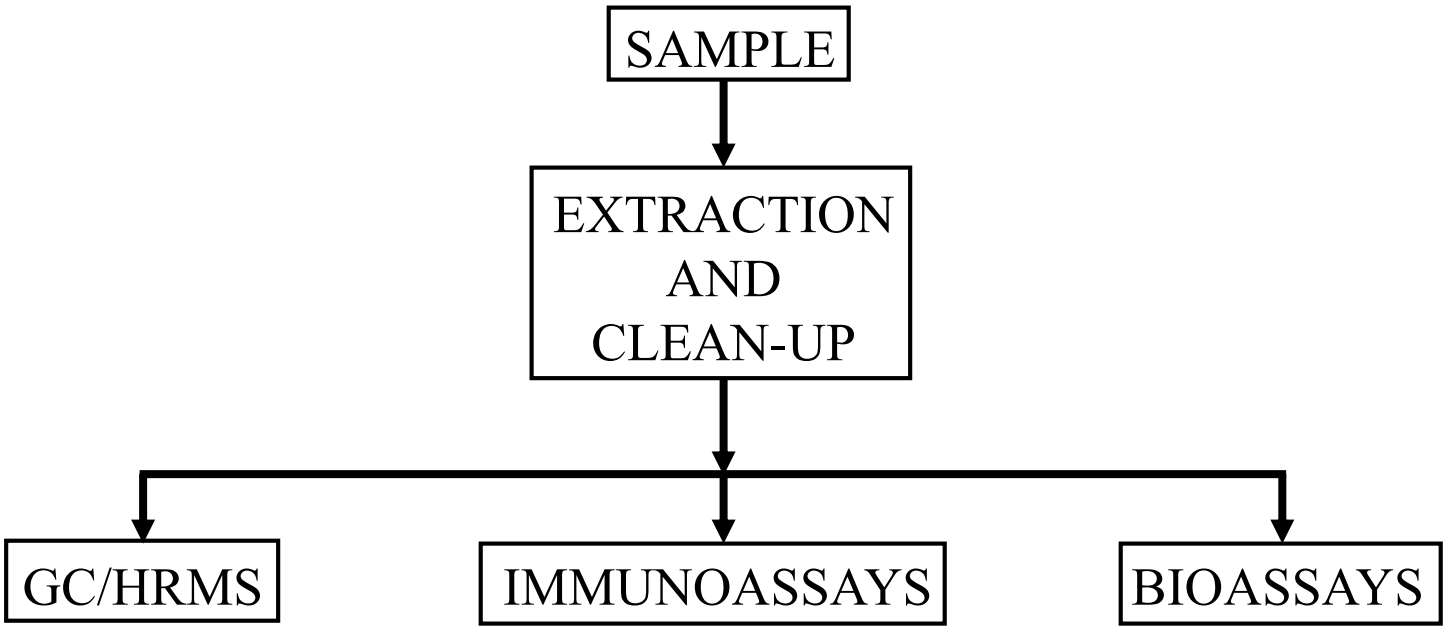
Congeners	TEF
3,3',4,4'-TCB	0.0001
3,4,4',5-TCB	0.0003
3,3',4,4',5-PeCB	0.1
3,3',4,4',5,5'-HxCB	0.03

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

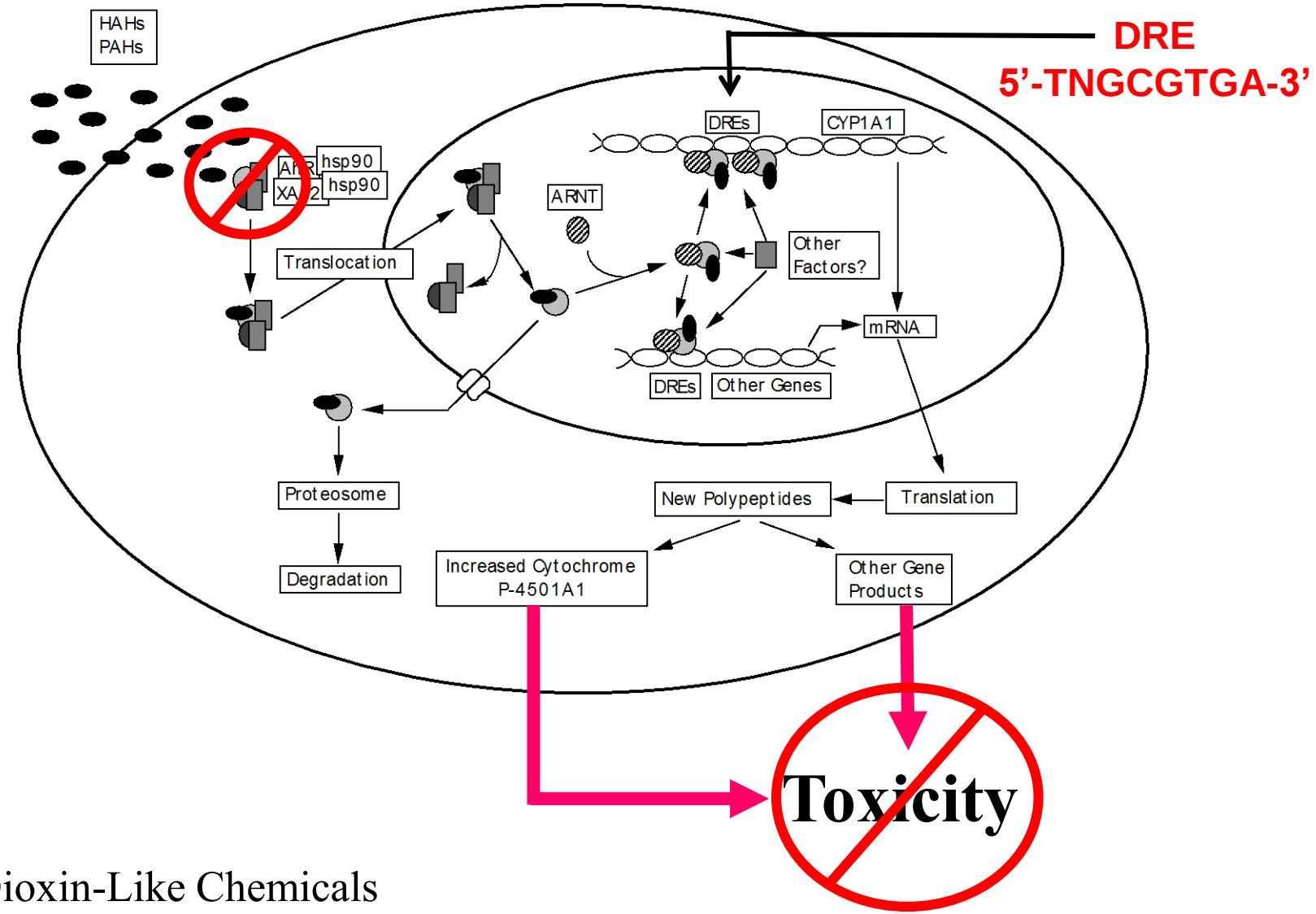
Chemical and Biological Techniques to Detect and Quantitate Halogenated Dioxins and Related Chemicals



While GC/HRMS produces an exact measure of the concentration of target chemicals in a sample and an estimate of its toxic potency, it has limitations for large scale screening applications.

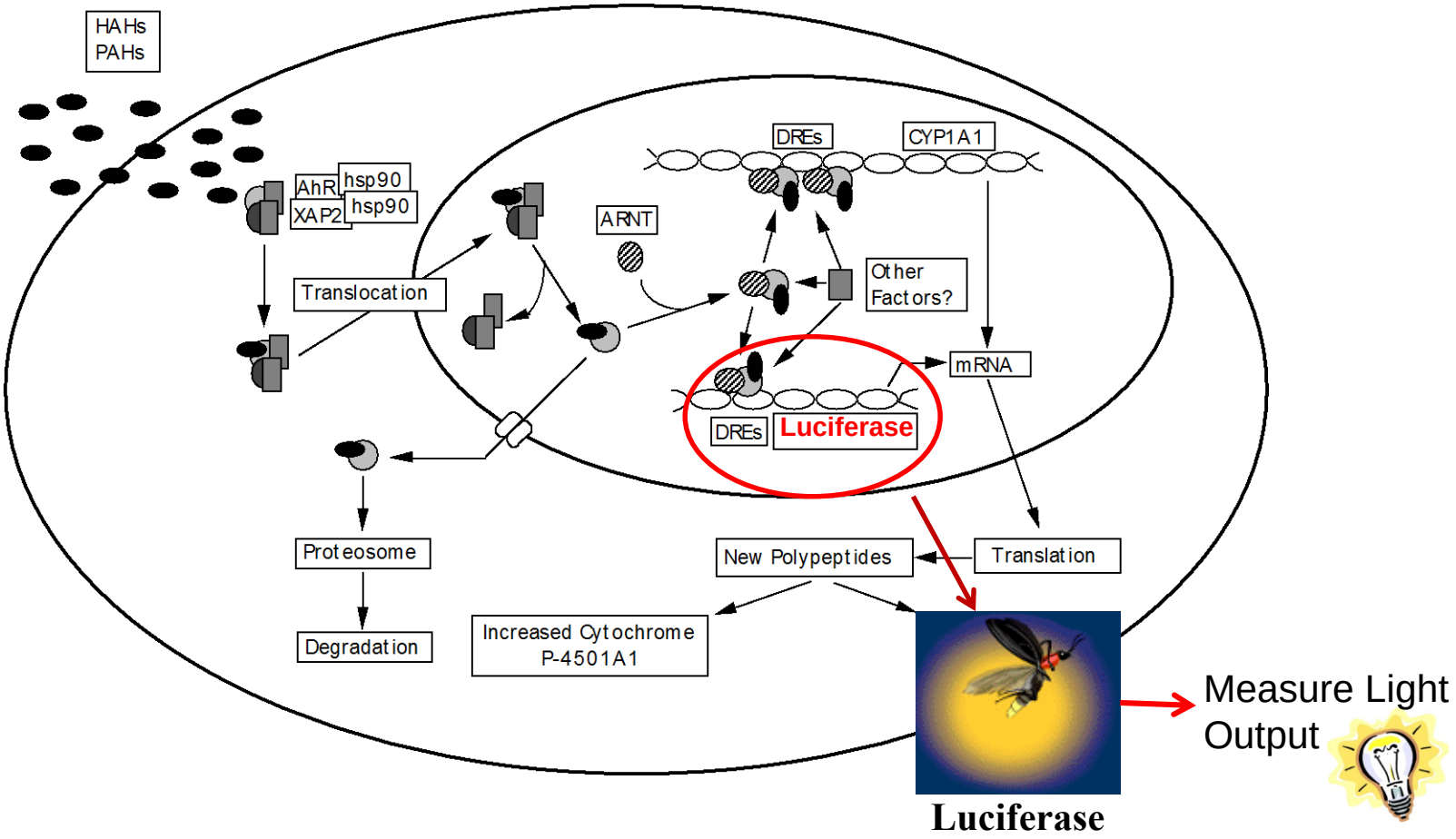
Develop and Utilize a Mechanism-Based Bioassay for Screening

The Ah (Dioxin) Receptor (AhR) Signaling Pathway is Responsible for the Toxic and Biological Effects of TCDD and DLCs



DLCs - Dioxin-Like Chemicals

The Ah Receptor (AhR) Signal Transduction Pathway: Development of AhR-Based CALUX Bioassays for Detection of Dioxin-Like Chemicals



CALUX: Chemically-**A**ctivated **LU**ciferase e**X**pression

USEPA (Method 4435)

CALUX Cell Bioassay Procedure

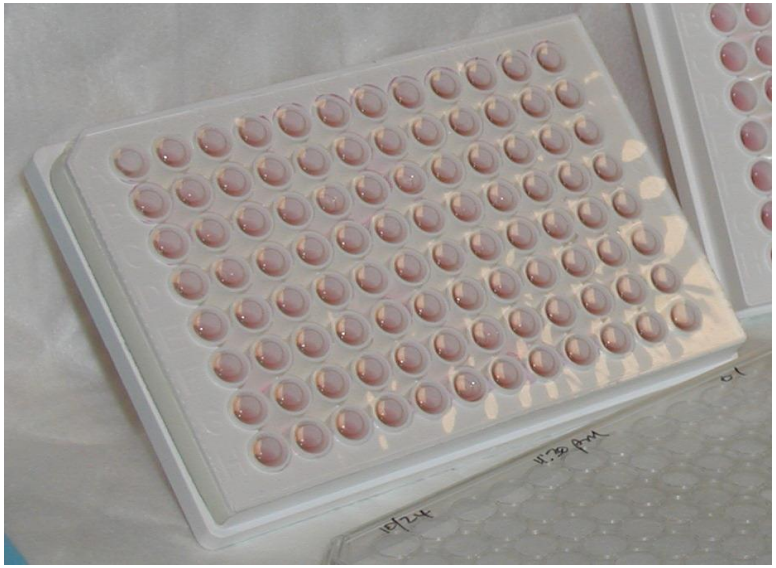
Recombinant Mouse Hepatoma (H1L6.1c3) Cells Plated into 96-Well Microplates

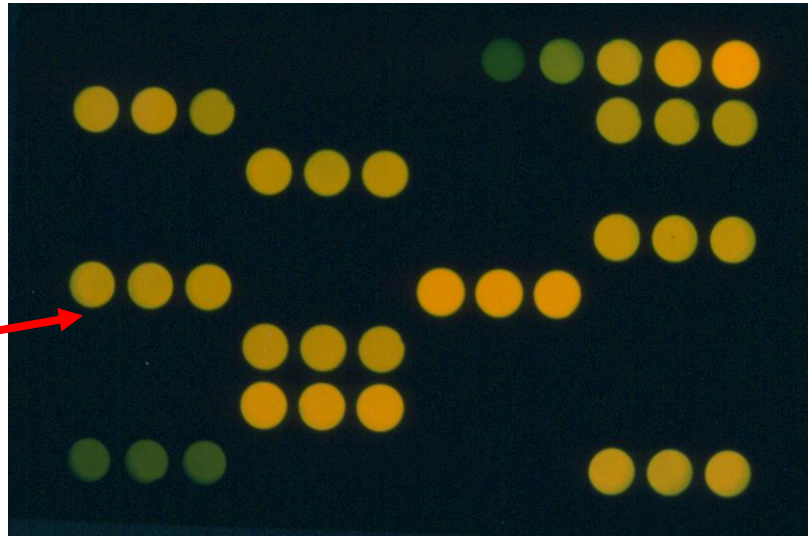
↓

Chemicals or Extracts Added to Each Well and Incubated for 24 Hours

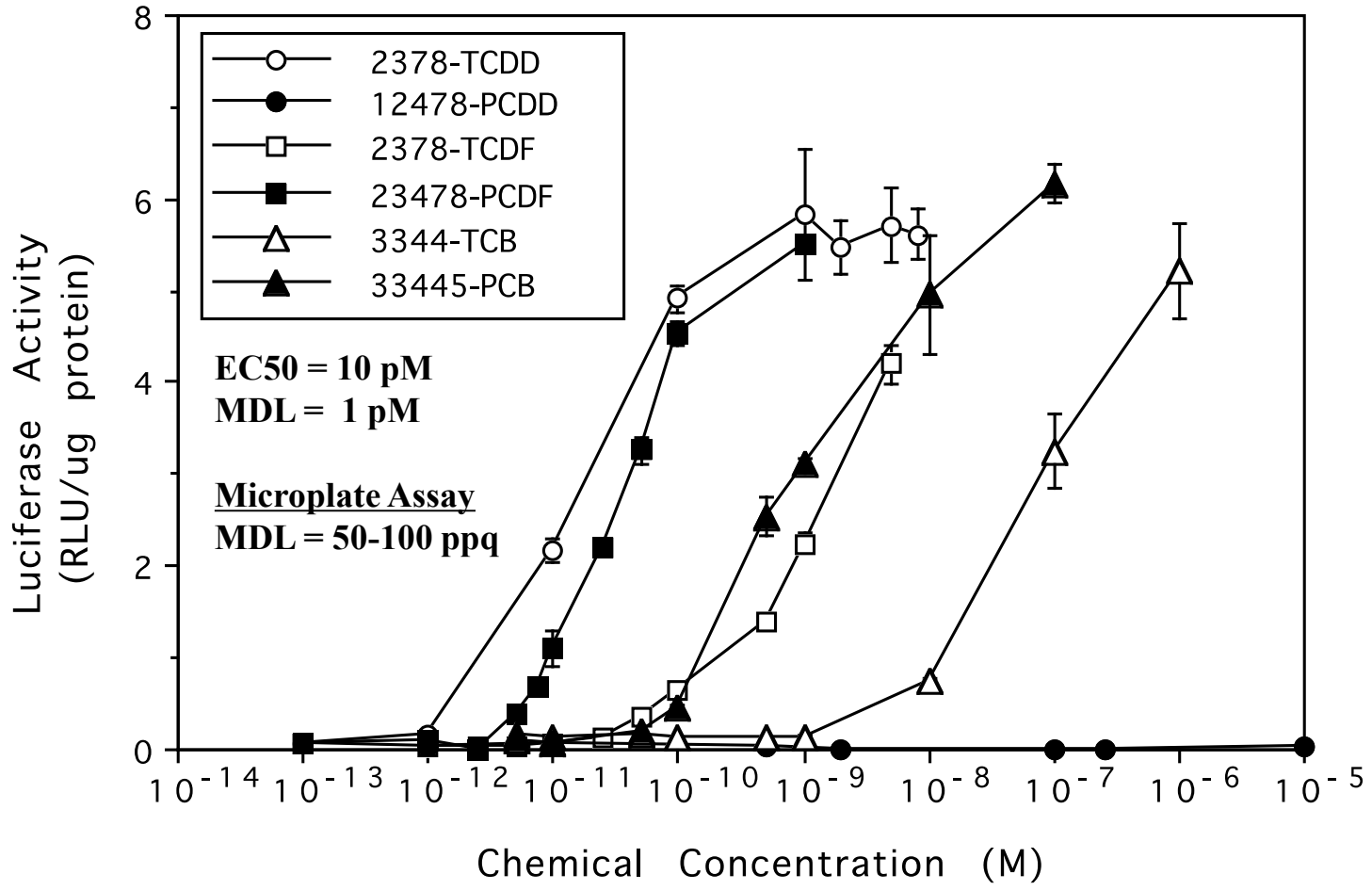
↓

Wells are Washed, Cells Lysed, and Luciferase Activity Measured in a Microplate Luminometer





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

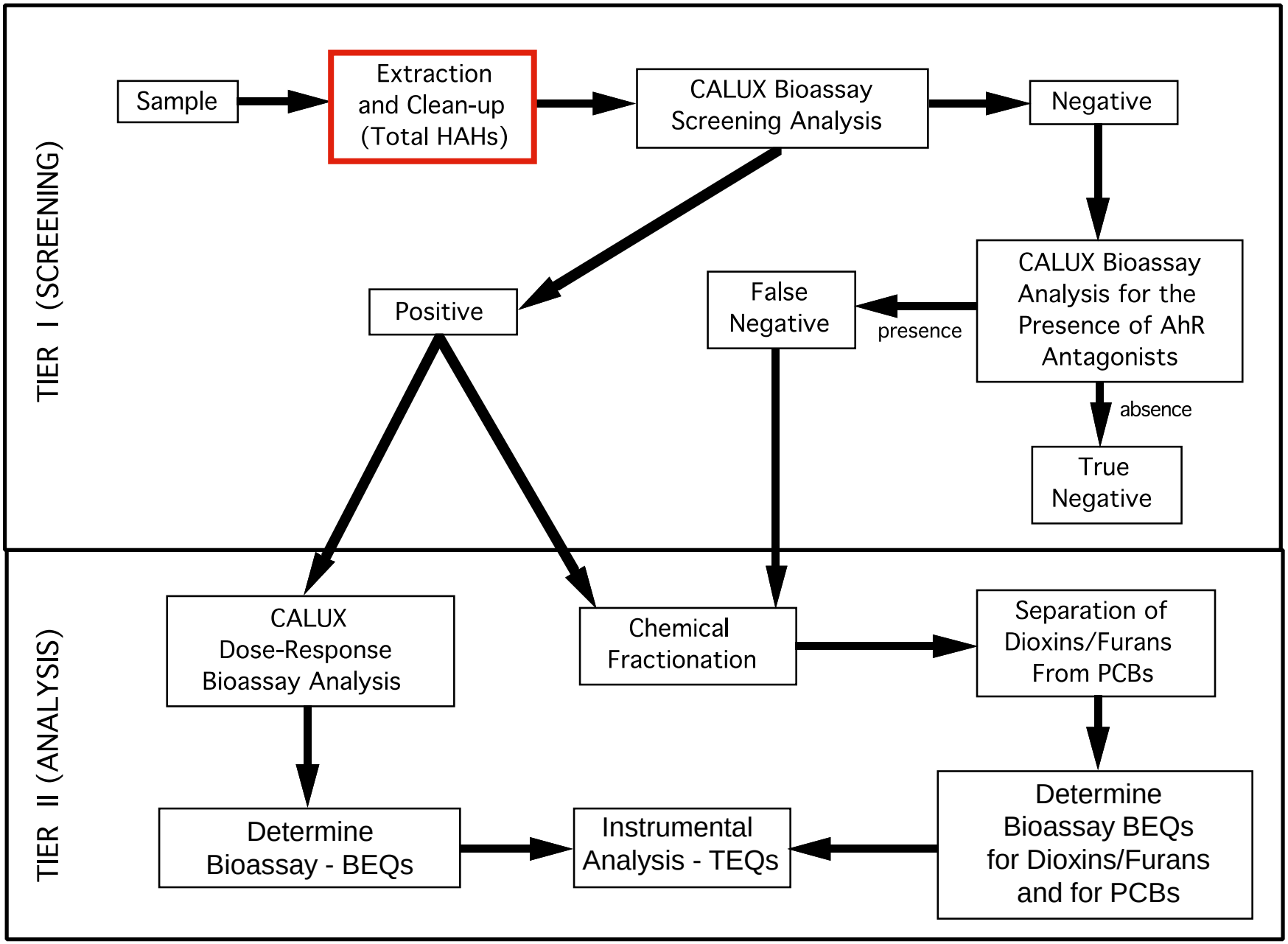


WHO Toxic Equivalency Factors (TEFs) and CALUX Relative Potency (REP) Factors for Chlorinated Dibenzo-p-Dioxins, Dibenzofurans and Biphenyls.

Compound	WHO-TEF	CALUX REP
2378-TCDD	1	1.00 ±0.01
12378-PeCDD	1	0.73 ±0.1
123478-HxCDD	0.1	0.075 ±0.014
123678-HxCDD	0.1	0.098 ±0.017
123789-HxCDD	0.1	0.061 ±0.012
1234678-HpCDD	0.01	0.031 ±0.008
OCDD	0.0003	0.00034 ±0.00008
2378-TCDF	0.1	0.67 ± 0.01
12378-PeCDF	0.03	0.14 ±0.04
23478-PeCDF	0.3	0.58 ±0.08
123478-HxCDF	0.1	0.13 ±0.02
123678-HxCDF	0.1	0.14 ±0.03
123789-HxCDF	0.1	0.11 ±0.02
234678-HxCDF	0.1	0.31 ±0.06
1234678-HpCDF	0.01	0.024 ±0.007
1234789-HpCDF	0.01	0.044 ±0.010
OCDF	0.0003	0.0016 ±0.0005
PCB 77	0.0001	0.0014 ±0.0004
PCB 81	0.0003	0.0045 ±0.0012
PCB 126	0.1	0.038 ±0.007
PCB169	0.03	0.0011 ±0.0003

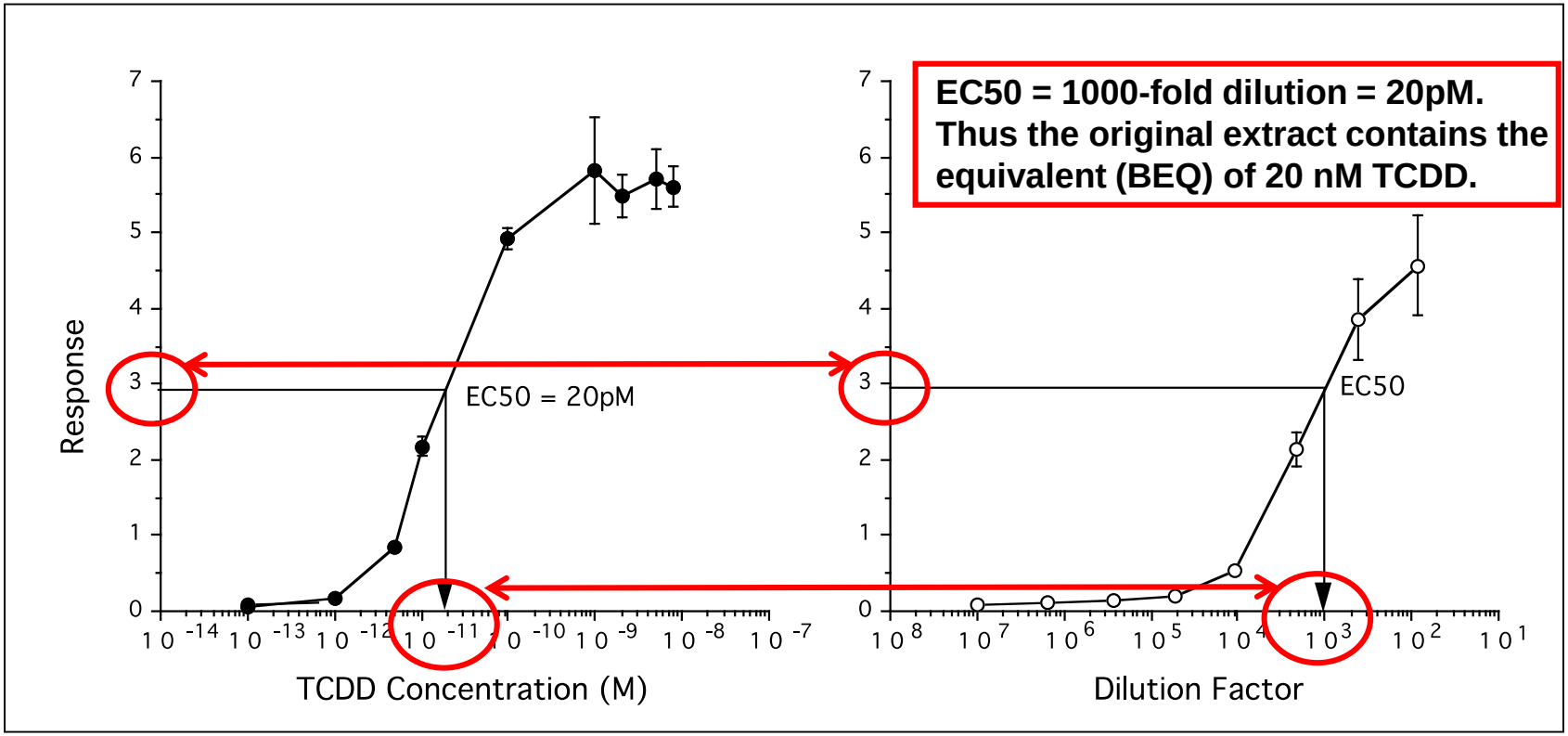
High correlation between CALUX Relative Potencies (REPs) and Toxic Equivalent Factors (TEFs) – Same mechanism (i.e. AhR)

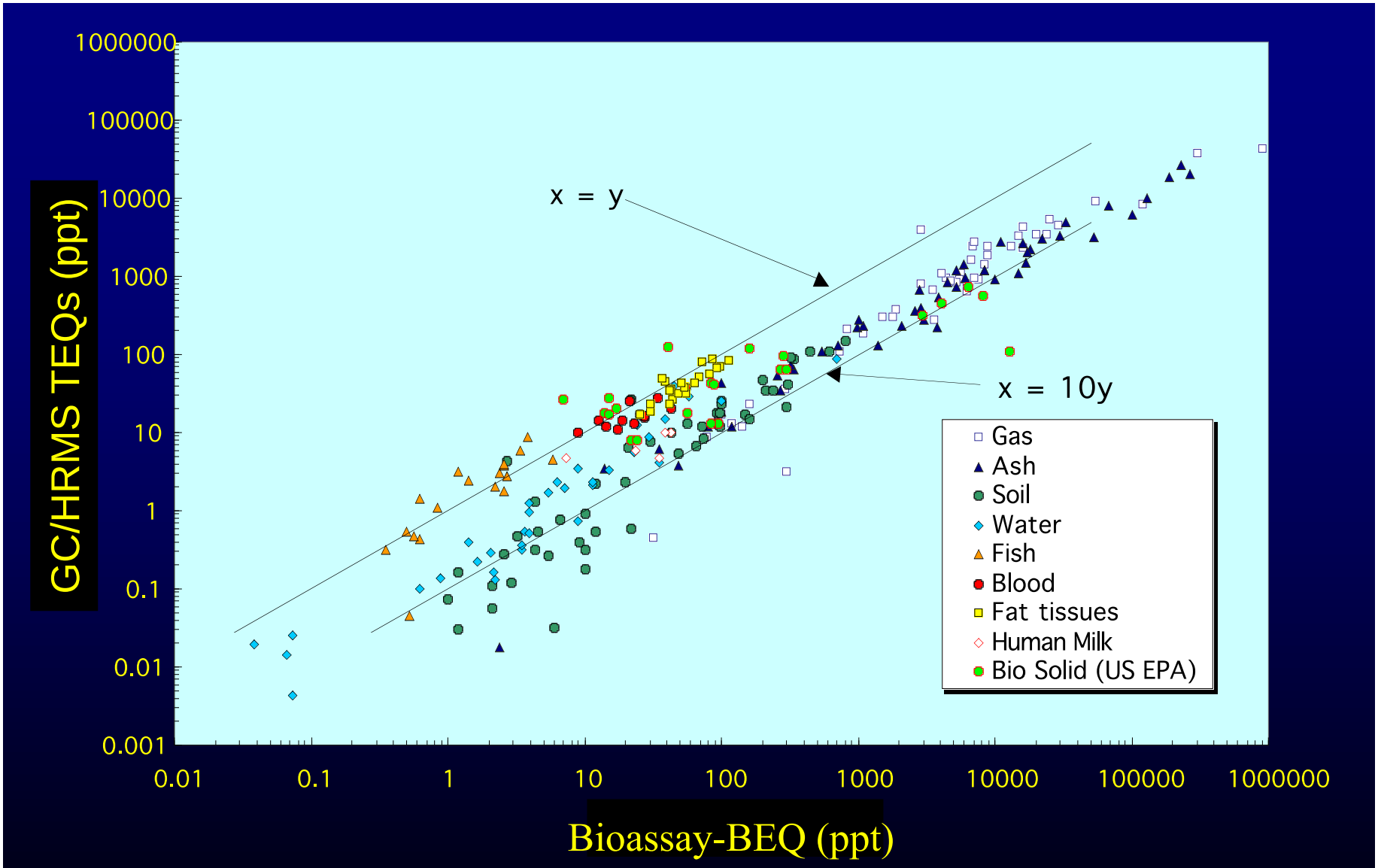
CALUX Bioassay Screening Procedure



BEQ – Bioanalytical Equivalents; TEQs – Toxic Equivalents

Calculation of the Relative Biological Potency (Bioanalytical Equivalent (BEQ)) of a Sample Extract Containing an Unknown Complex Mixture of Chemicals





Double-Blind CALUX Analysis of Biological and Environmental Matrices

From Hiyoshi Corporation and XDS

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

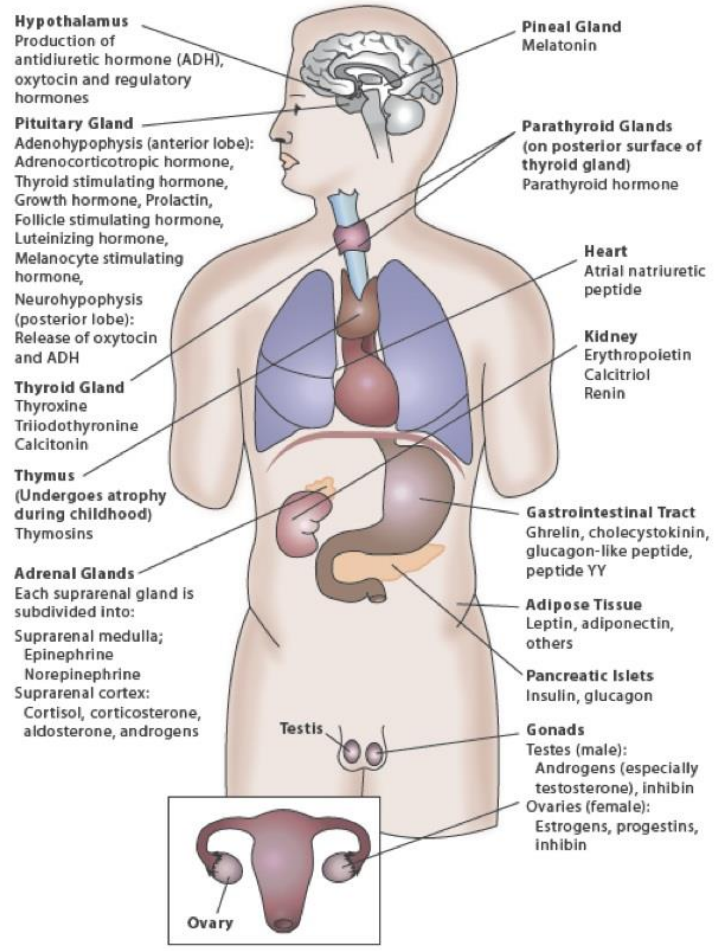
Biological Samples Screened	Reference	Environmental Matrices Screened	Reference
Human Tissues		**Sediment/Soils**	57,59,61,91,125-131
Blood Plasma/Serum	57-59,111-115		
		Water	15,57,111,132-133
Follicular Fluid	113		
Breast Milk	96,116,117	**Waste Management**	
		Effluent	134
Animal Tissues (various 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.

Not True For All Bioassays!

Endocrine (Hormone) Disrupting Chemicals (EDCs)

Endocrine Glands/Organs - Hormones



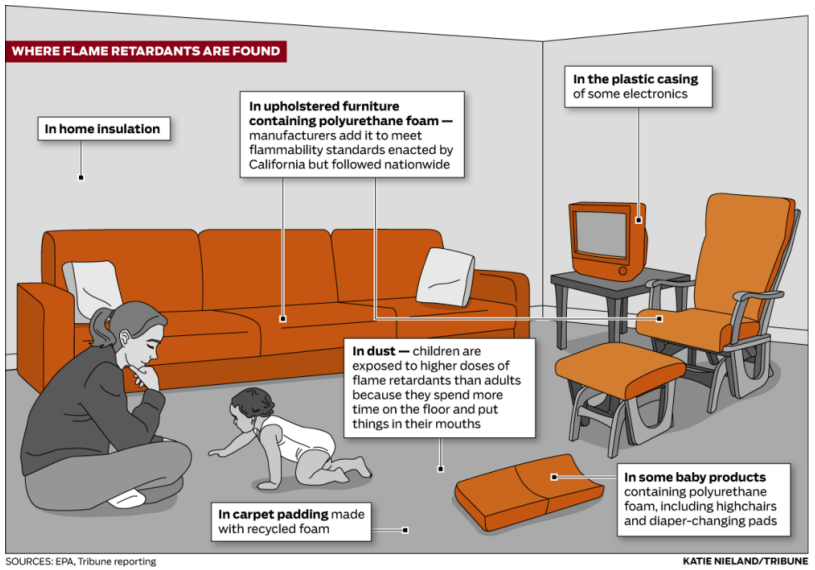
Definition of EDCs (IPCS, 2002)

*"An **endocrine disruptor** is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations."*

*"A **potential endocrine disruptor** is an exogenous substance or mixture that possesses properties that might be expected to lead to endocrine disruption in an intact organism, or its progeny, or (sub) populations."*

Exposure to EDCs From Diverse Sources

Pharmaceuticals



Flame Retardants

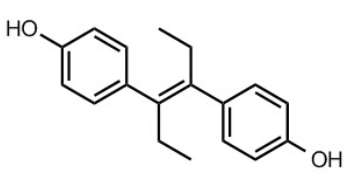
Pesticides

Dioxin-Like HAHs

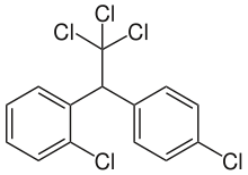
Plastics and Plastic Products

Sunscreens

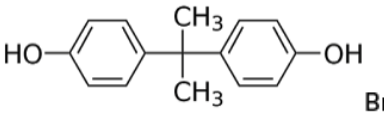
Health Effects of Endocrine Disruptor Chemicals (EDCs)
(Estrogenic/Antiestrogenic EDCs)



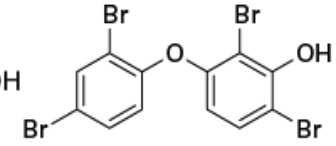
Diethylstilbestrol



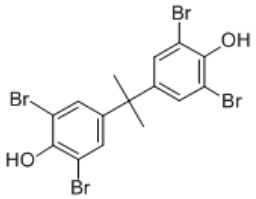
o,p-DDT



Bisphenol A



3-OH-BDE-47



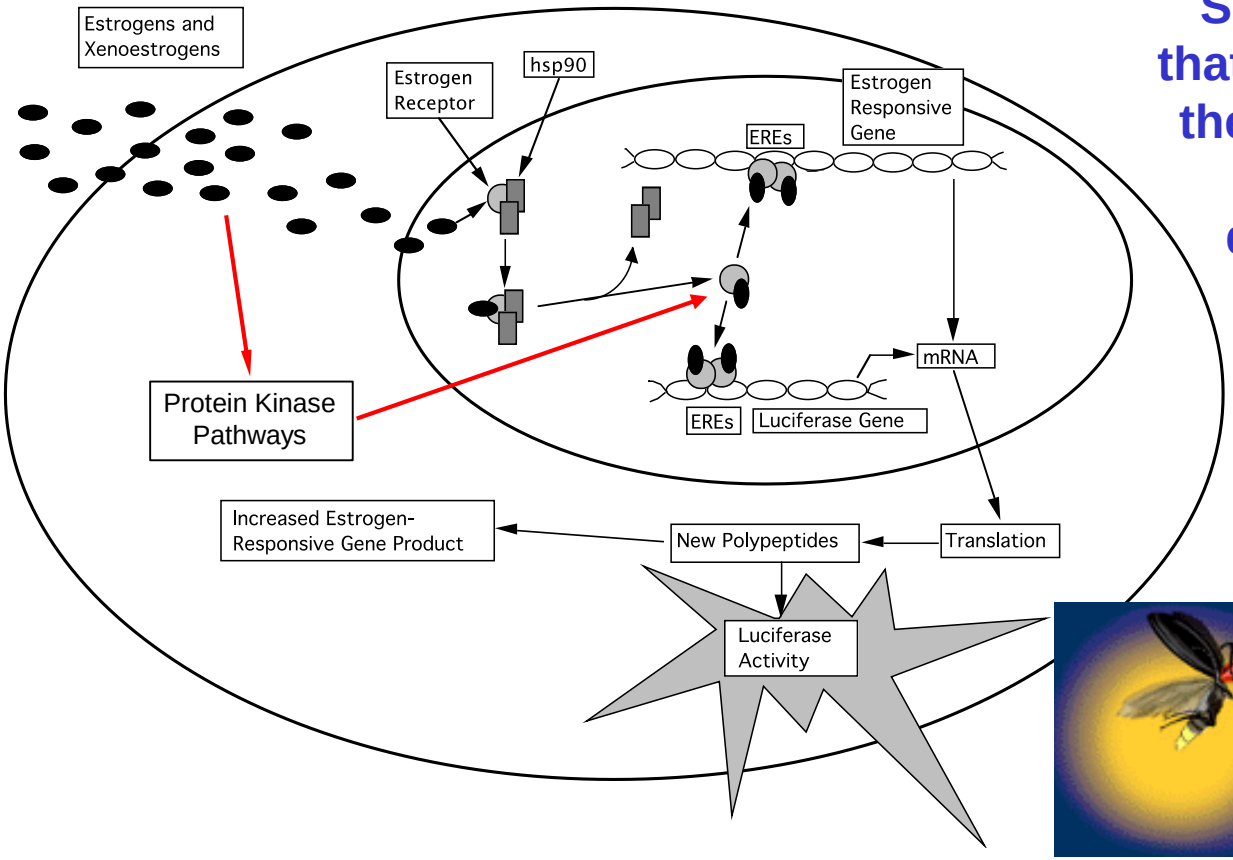
TBBPA

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.**

CALUX Cell Bioassay System for Detection of Estrogenic Chemicals

BG1Luc-ER-TA (BG1 Luciferase Estrogen Receptor Transactivation)



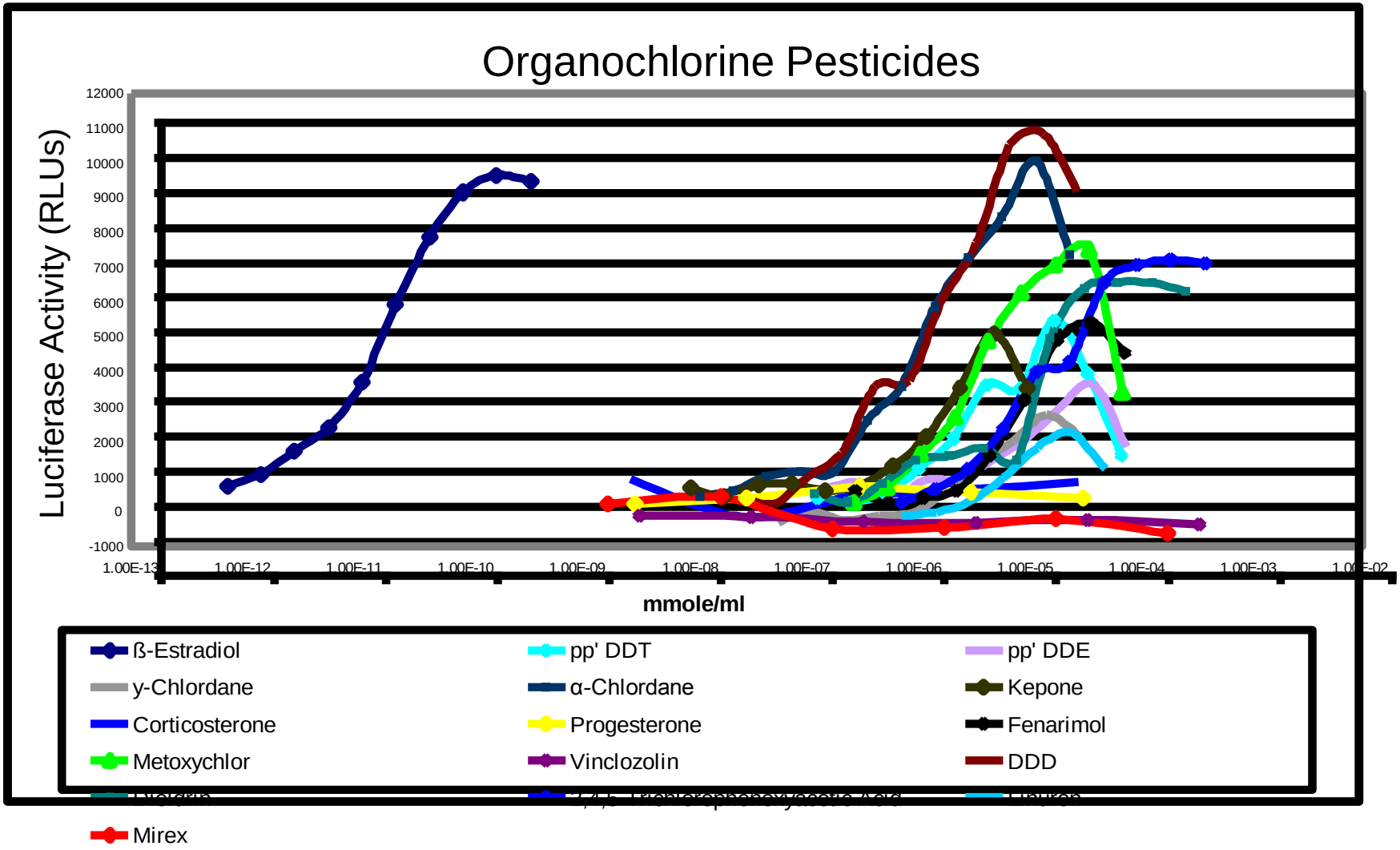
Screens for chemicals that can activate or inhibit the estrogen receptor(s) signaling pathway directly or indirectly

Firefly Luciferase

OECD Methods TG455/TG457 – USEPA EDSP

Human Ovarian Carcinoma (BG-1) Cells Containing a Stably Transfected Estrogen Receptor Responsive Luciferase Reporter Gene.

Pure Chemical Screening

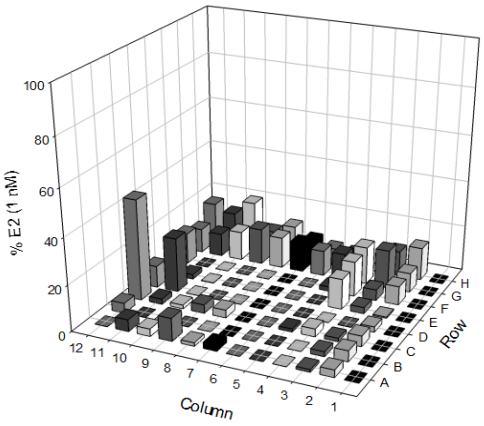


John Gordon (XDS)

Application of BG1Luc-ER-TA CALUX Cell Lines: High Throughput Screening for ER-Active Chemicals

BG1Luc4E2 (ERα) BG1LucERβc9 (ERα/ERβ)

Plate 1



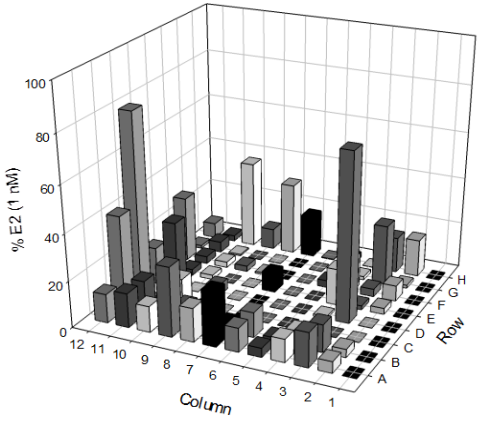
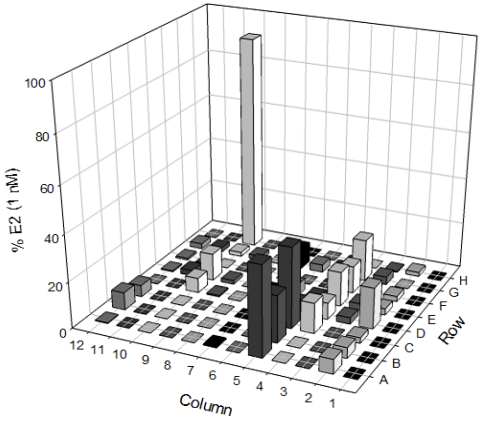
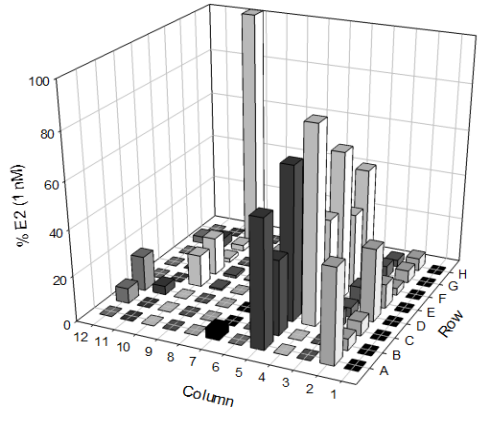


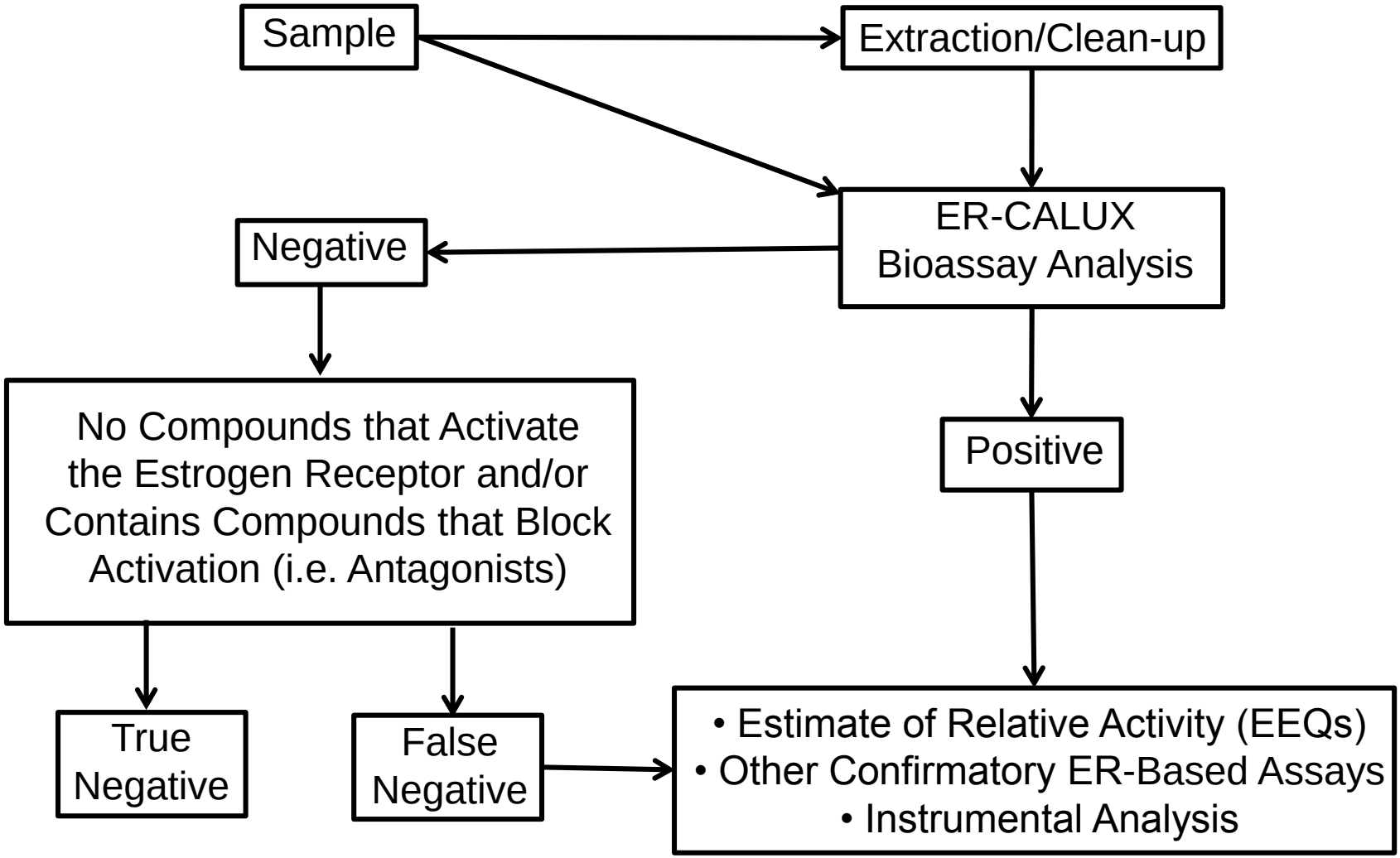
Plate 2

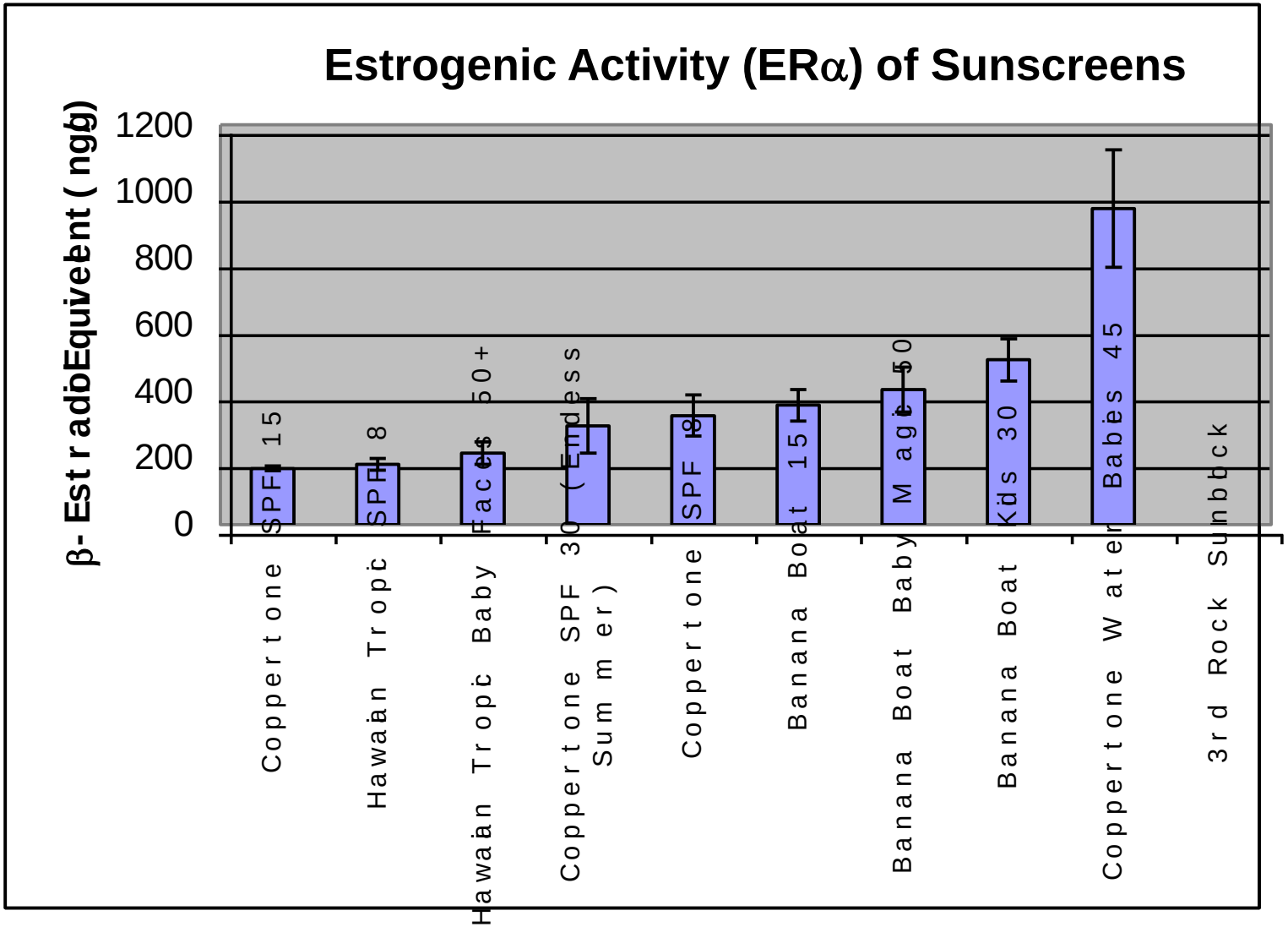




Chemical library: 176 chemicals ((2) 96-well plates, 10 μM test conc.)
(Pesticides, Herbicides, Fungicides, Industrial Chemicals, Drugs, Detergents, etc)

Flow Diagram for Analysis of Unknown EA Chemicals & Extracts

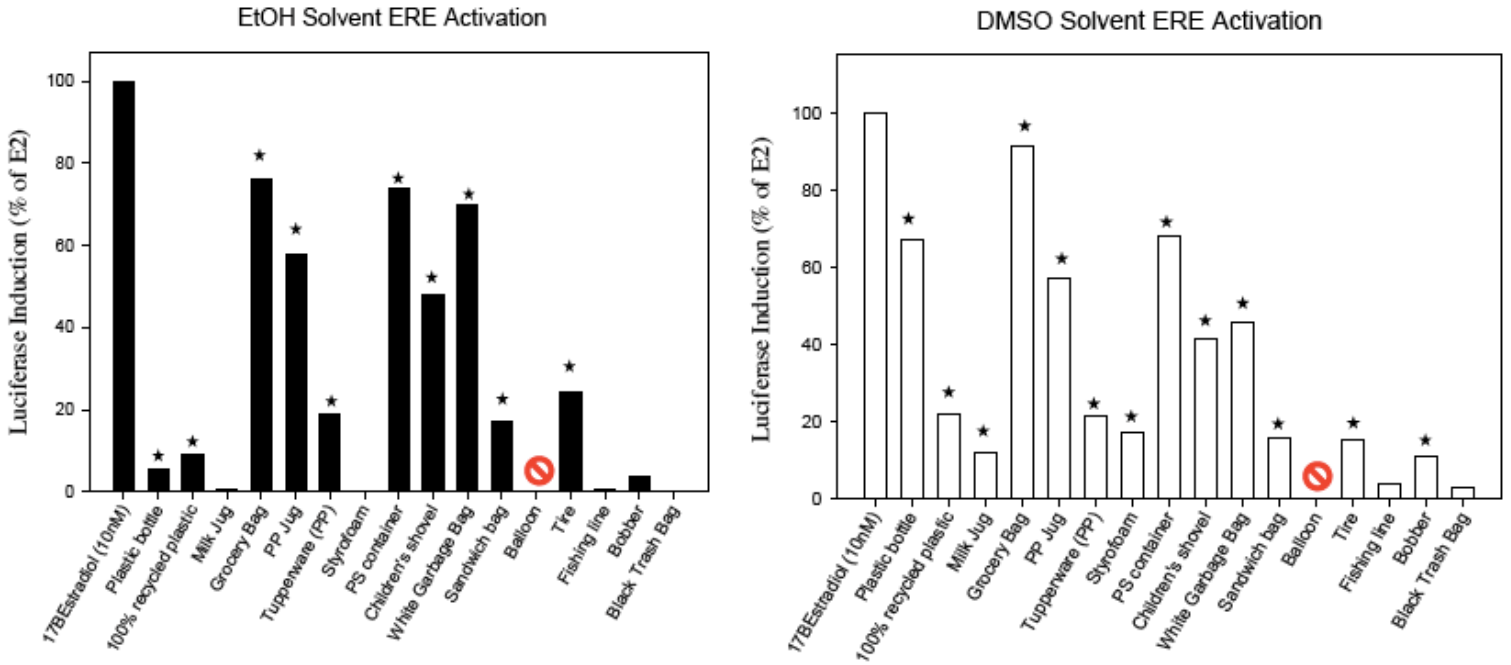




Benzophenone derivatives and parabens: 3-(4-methylbenzylidene)-camphor (4-MBC), octyl-methoxycinnamate (OMC), octyl-dimethyl-PABA (OD-PABA), bexophenome-3 (Bp-3) and homosalate (HMS)

John Gordon (XDS)

Simple Solvent Extracts of Diverse Plastic Products Contain Estrogenic Chemicals (ERα–Active)



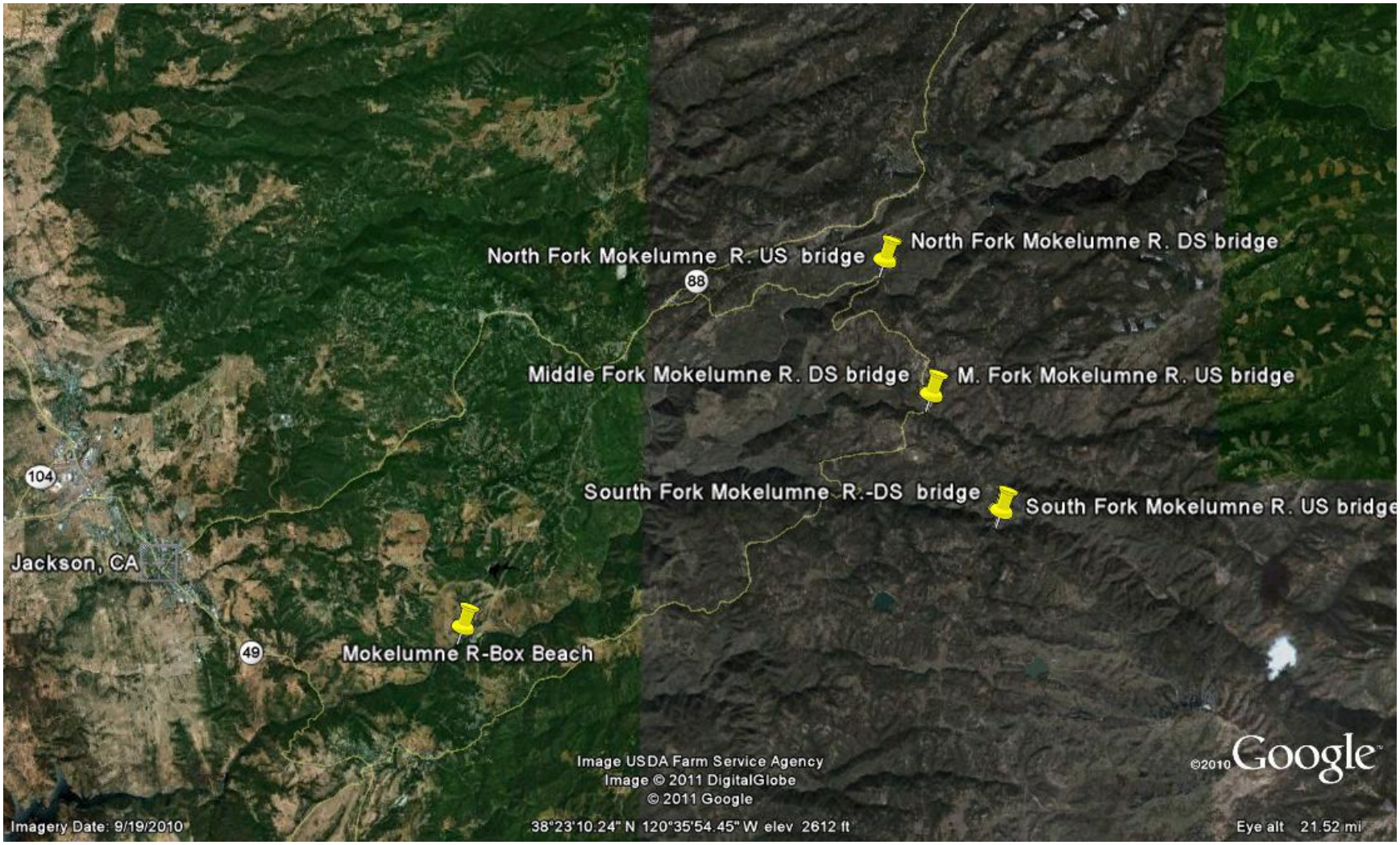
The level of estrogenic activity is dependent on the extraction solvent, suggesting different types of chemicals are being extracted

Bisphenol A (BPA)-Free Does Not Necessarily Mean Free of Estrogenic Activity (EA) or That It Is an EDC!

Kossack and Denison, 2013

ENVIRONMENTAL MONITORING

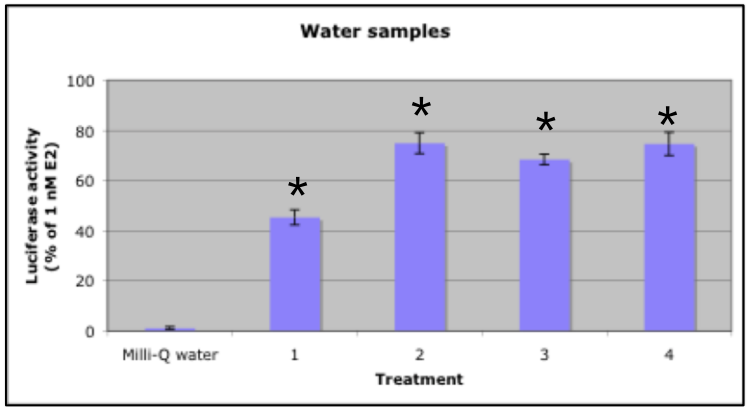
Mokelumne River Sampling Sites For Estrogenic (ERα) Activity

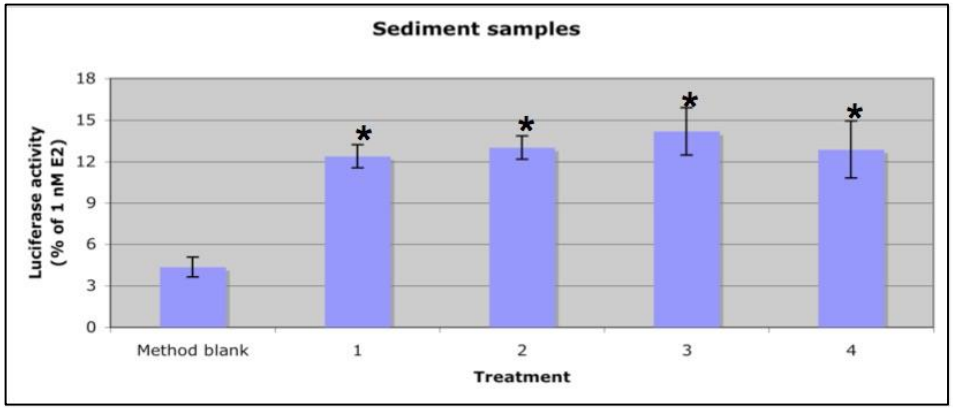


Measurement of Estrogenic Activity (ERα) of Water and Sediment Samples from Upper Mokelumne and Calaveras Rivers

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

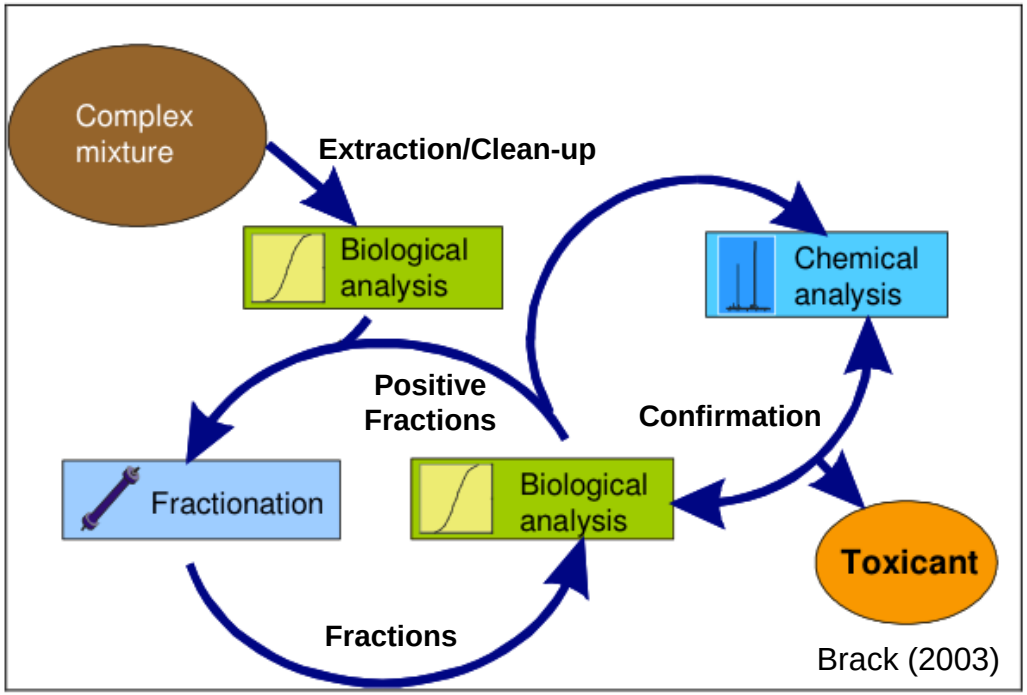




Significant levels of estrogenic activity in all Mokelumne River samples, but the sediment has significantly less activity.
The responsible chemical(s) remain to be identified.

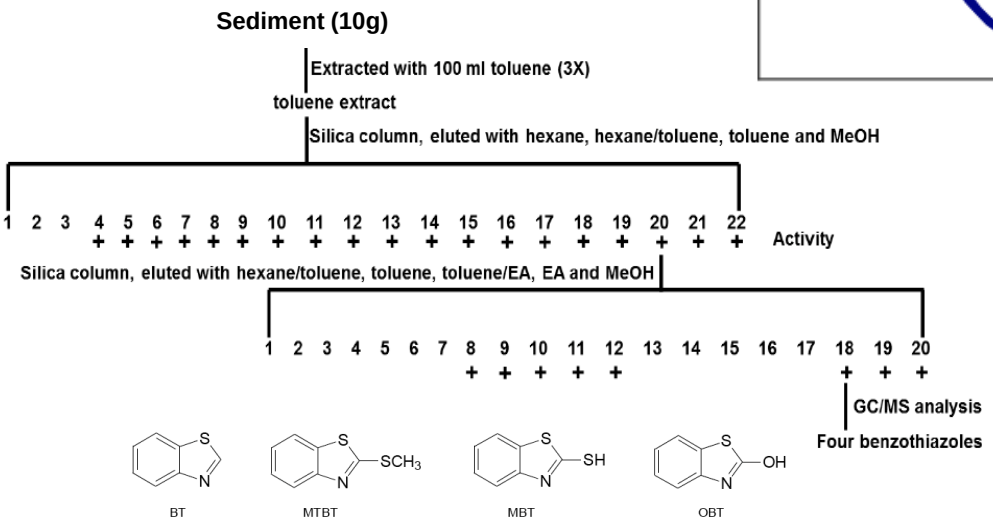
Effects–directed analysis (EDA) - Combination of bioassays and chemical fractionation methods provides an avenue in which to identify the responsible bioactive chemical(s) in a complex mixture.

Effects–Directed Analysis (EDA)



Brack (2003)

AhR CALUX - EDA



Selected Effects-Directed Analysis Studies on Various Matrices

Sample type	Biological endpoint	Chemical analysis	Toxicants identified	Reference
Surface waters/ sediments	Endocrine disruption, estrogenic activity, androgenic activity, dioxin and dioxin-like compound activity, cytochrome P-450 activity, mutagenicity, bioluminescence, algal growth, daphnid toxicity	GC-MS, LC-MS	1-dehydrotestosterone, 5β-androstane-3α-11β-diol-17-one, 4-androstenedione, 5α-androstanedione, androsterone, epi-androsterone, 17β-oestradiol, androsterone, nonylphenol, bis(2-ethylhexyl)phthalate, estrone, benzo[a]pyrene, perylene, benz[a]fluoranthrene, benzanthrone, galaxolide, tonalide, traseolide, tris-(2-chloroisopropyl) phosphate, nandrolone, 5α-Androst-16-en-3-one, methyl parathion, prometryn, n-tributyltin, n-phenyl-β-naphtalene amine, dinaphthofurans, naphthalenylbenzothiophene, benzaldehyde, tetradecanal, 2-nonenal, 1-hexyl-3-methylcyclopentane, 1,9-nonanediol, *o*-tolidine, nitroquinoline, nitroaniline, dichlorobenzidine, aromatic quinones	[14,15,26,29,36, 37,38,39,40]
Groundwater	Mutagenicity	GC-MS	2,4,6-trichlorophenol, 2,4-dichloro-6-methylphenol, 4-chlorobenzoic acid	[23]
Landfill leachate/soil	Bioluminescence, fish embryo toxicity	GC-MS	bisphenol A, 4-*t*-butylphenol, n-ethyl toluene sulfone amide, 9-methylacridine, 4-azapyrene, 2-phenylquinoline, 11-H-benzo[b]fluorine, retene	[17,41]
Fish bile	Endocrine disruption, anti-androgenic activity	GC-MS	2-naphthol, 2,2'-dihydroxybiphenyl, bisphenol A, chloroxylenol, dichlorophene, 1-hydroxypyrene, chlorophene, oxybenzone 9,10-di(chloromethyl) antracene, triclosan, 4-nonylphenol, abietic acid, pimaric acid, isopimaric acid	[32]

Burgess et al. ETC 32;1935 (2013)

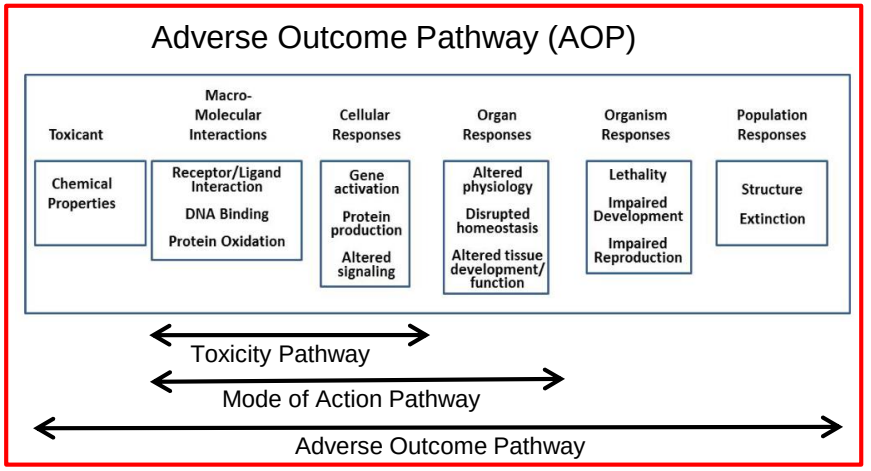
Conclusions

• HTP bioassays provide an avenue to identify the effects of known chemicals and mixtures on selected biological/toxicological pathways (i.e. available bioassays).

• Appropriate extraction and clean-up methods can be used with "toxic pathway" bioassays to identify samples with in vivo toxicity potential. Few available assays. AhR (chemicals and key target defined), ER (chemicals and target being defined)

• While HTP bioassays can be used to identify specific molecular and cellular responses affected by a chemical/mixture/extract, there are limtations that should be considered.

A. Toxic potential of chemical/extract in vivo?
[AOP considerations]
B. Extraction method used?
[polar and nonpolar chemicals]
C. Identity of chemical(s)?
[unknown chemical mixtures]
D. Mixture interactive effects?
[inhibition/additive/synergism]



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