DEVELOPMENT OF BIOANALYTICAL TECHNIQUES FOR MONITORING OF CHEMICALS OF EMERGING CONCERN IN RECYCLED WATER

JOINT MEETING BETWEEN CALIFORNIA AND WATEREUSE RESEARCH FOUNDATION PROJECT TEAMS

JANUARY 23 - 24, 2014
MEETING AGENDA

To be held at:
Southern California Coastal Water Research Project
3535 Harbor Blvd. Suite 110, Costa Mesa, CA 92626

For Audio Access: Dial +1 (213) 493-0007
Access Code: 682-658-022
Audio PIN: Shown after joining the meeting

Thursday, January 23

8:30	Coffee & pastries	
9:00	Welcome & Introductions	Stephen Weisberg (SCCWRP) Julie Minton (WRF)
9:15	Opening Remarks	Jonathan Bishop (CA Water Board) David Smith (WateReuse CA) Vickie Wilson (EPA ORD) John Printen (Life Technologies)
9:30	WRF10-07 Project Summary (Goals, Key Results)	Beate Escher, Fred Leusch
10:15	BREAK	
10:30	CA Project Summary (Goals, Key Results)	Nancy Denslow, Sandy Westerheide
11:15	Discussion "*Which bioassays are ready for pilot implementation*?"	Moderators: N. Denslow, B. Escher
12:00	LUNCH	

1:00	Analysis & Interpretation of Bioassay Results WRF Intercalibration Exercise & Effect-Based Monitoring Trigger Development	B. Escher
2:00	Comparing Bioassay & Analytical Chemistry Results – CA Intercalibration Exercises	Shane Snyder
2:30	Standardization of Bioassay Protocols	Alvina Mehinto
3:00	BREAK	
3:15	Discussion - "*How do we implement bioassays for monitoring of recycled water*?"	Moderators: K. Maruya, B. Escher
4:00	Bioassays/MOA Wish List for CA	Dan Schlenk
4:30	Promising Endpoints in the Development Phase	F. Leusch
5:00	Discussion – "*What tools/data are needed to make monitoring more comprehensive and robust?"*	Moderators: V. Wilson, F. Leusch
5:45	Adjourn	

DINNER WITH THE GROUP

Friday, January 24

7:30	Coffee & pastries	
8:00	Summary of Day 1; Breakout Assignments	K. Maruya, B. Escher
8:30	Breakout Session (by Project)	
10:00 – 10:15	BREAK	
10:15	Meeting Summary and Consensus Building Bioassays to move forward Implementation Strategy Next Steps	Moderators: K. Maruya, B. Escher
11:30	Project Deliverables, Action Items & Wrap Up	
11:45	Adjourn	

WateReuse Research Foundation – SCCWRP Collaboration Meeting #2

Costa Mesa, CA

January 23-24, 2014

Julie Minton

Director of Research Programs

The Foundation's Mission

To conduct and promote applied research on the reclamation, recycling, reuse and desalination of water.

The Foundation's Vision

2011- 2020

- Annual Budget of $5-10 Million
- Funding Partners
  - DPR Initiative Donors
  - Utilities/Manufacturers
  - Bureau of Reclamation
  - CA SWRCB/DWR/CEC
  - Pentair Foundation
  - Subscribers
  - Partners: AWRCE/Singapore PUB
  - Multinational Corporations
  - Charitable Foundations

- A Global Presence and Reach
- The Respected Voice for Research on Water Reuse and Desalination

WATEREUSE RESEARCH

WateReuse Research Foundation : History

- Incorporated on **September 13, 1993** to:
  - Develop the Science & Technology Necessary to Support the Water Recycling Needs of the 21st Century
- Foundation Specializes in Conducting “Leading Edge” Applied Research
- Address Following: Chemical & Microbiological Agents, Treatment Technology, Economics, Marketing, Public Perception
- Push Back the Frontiers in Technology

WATEREUSE RESEARCH

Significant Events

- Hired FT Executive Director on August 1, 2000
- Secured $180,000 in Funding from USBR in September, 2000
- Secured “Earmark” of $1MM in FY 2001
- Received Matching Funding of $1MM from CA-SWRCB in 2002
- Expanded Mission to Include Desalination in 2003
- Reconstituted, Strengthened RAC in 2004
- Developed Equitable Sustainable Funding Model in 2007
- Changed Name in 2010
- Launched the CA DPR Initiative in June 2012
- New Executive Director to start March 1, 2014

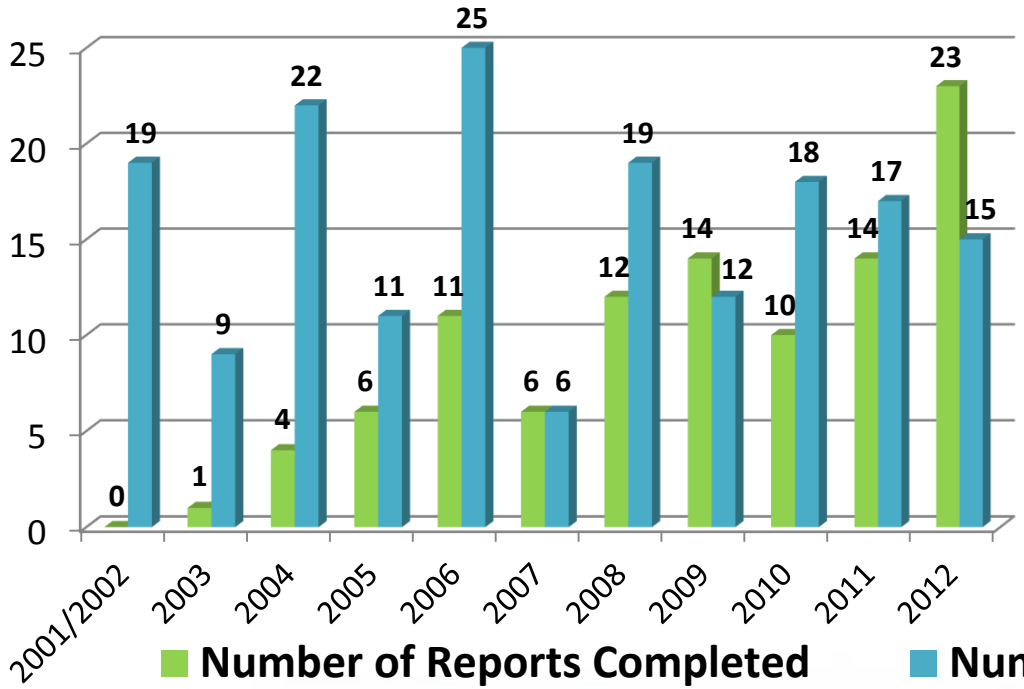
WATEREUSE RESEARCH

Outreach is an Important Element of Foundation Work

- Number of Outreach Pieces to Date: 500+
  (reports, presentations, proceedings, peer-reviewed publications)

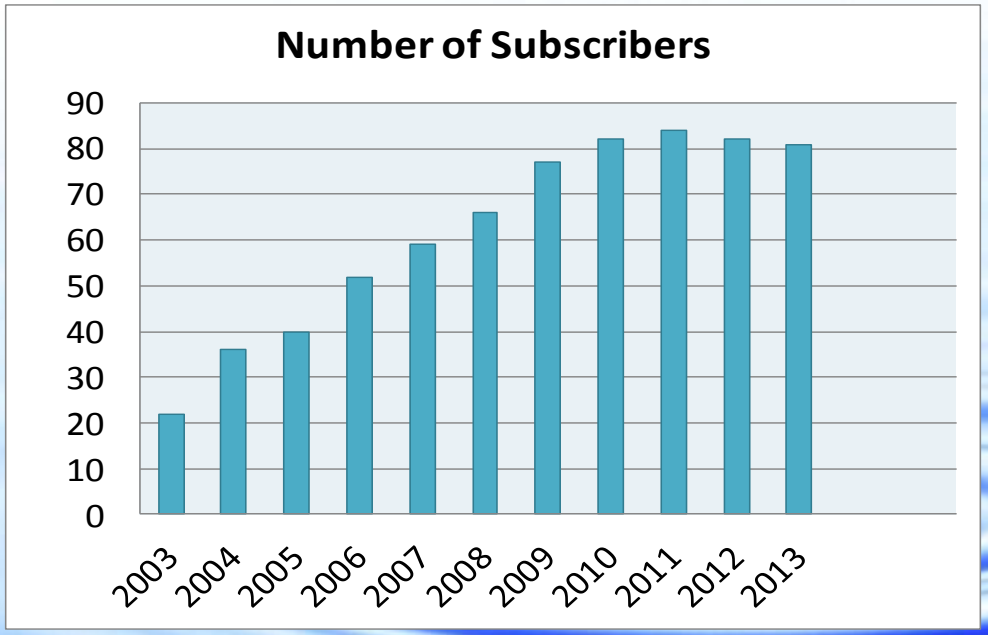
- New Journal Initiated in 2012– *WorldWater: Water Reuse and Desalination*

- Webcast Program initiated in 2011: 60-90 min program on hot topic held on the second Thursday of each month (free for Subscribers)



Since 2000…

172 projects commissioned
Over $50M in funding leveraged
120 published works
50 projects still active



In 2013…

12 projects launched
$1.9M in funding awarded
36 published reports

WATEREUSE RESEARCH

Annual WateReuse Research Foundation Conference

- First Conference held on June 5-6, 1997
- Theme was ”Merging Our Resources”
- Will Convene 18th Annual Conference
  - May 19-20, 2014 in Las Vegas, NV
- Conference provides opportunity to:
  - Showcase results of WRRF research
  - Hear presentations from federal agencies, researchers from partner organizations
  - Identify future research needs
- EPA’s OR&D and Water Research Foundation have been Conference Sponsors for 13 Consecutive Years

Research Categories

- Direct Potable Reuse
- Business Economics & Industrial Reuse
- Public Acceptance & Policy
- Desalination

Thank you!

Julie Minton

jminton@watereuse.org

703-548-0880 x 108

WATEREUSE'S FORWARD-LOOKING DIRECTION

- **RAC re-focused its framework for regular research to place more emphasis on socio-economic research angles, and public policy implications, to generate more ROI for subscribers – a sharpened focus**

- **The Foundation has made a major commitment to philanthropy, to replace previous government/agency funding lost – trying to attract more non-dues donors from within our community, but also from humanitarians/philanthropists**

- **Have raised $5.3 million in philanthropy since June 2012 towards DPR Adoption – tremendous opportunities**

- **Future research priorities will center around:**
  - **-- Potable reuse as a supply solution to water scarcity/availability across the US, not just in CA, TX, AZ and CO**
  - **-- Industrial reuse, especially the water-food-energy nexus**
  - **-- International water reuse, as it impacts the human condition**
  - **-- Championing innovation and new technology in reuse – for all water portfolios**

Goals and Key Results of the Project

Sandy Westerheide and Nancy Denslow
University of South Florida &
University of Florida

Goals of the project

- Characterize the response of selected *in vitro* bioassays for samples representing a range of recycled water quality
- Quantify the relationship, if any, between bioassay response and higher order impacts that are relevant to human health
- Identify the appropriate use and role for bioassays that exhibit acceptable performance in a recycled water monitoring program

Approach

Tasks

1. Literature review → identify most promising assays
2. Evaluate bioassays and optimize them. Validate with water samples of known chemistry
3. Compare bioassay response to reference doses – Predict BEQ's
4. Provide data interpretation and implementation guidance

Selecting Relevant Endpoints

Assay	Acronym	Mechanism	Potential Health Implications
Estrogen receptor activity	ER	Estrogen signaling	Reproduction, cancer
Androgen receptor activity	AR	Maintenance of male sexual phenotype	Androgen insensitivity syndrome
Progesterone receptor activity	PR	Embryonic development, cell differentiation, homeostasis	Cancer, diabetes, hormone resistance syndromes
Peroxisome proliferator-activated receptor gamma	PPARg	Fatty acid storage and glucose metabolism	Obesity, diabetes, atherosclerosis, and cancer
Glucocorticoid receptor	GR	cortisol, glucocorticoids	Development, metabolism, immune response, neuroendocrine integration
Genotoxicity		DNA mutations	Cancer
Cytotoxicity		General toxicity	Tissue integrity

Bioassay Comparison

- Relevance
  - specificity (MOA, CEC)
  - link to tox pathways, apical endpoints
- Robustness
  - specificity, sensitivity, precision
  - historical usage
- Simplicity
  - protocol complexity
- Time & Cost
  - set-up, incubation, data interpretation, reporting
  - capital & recurring costs
- Vendor support
  - co-investment, leveraging
  - ready resources & expertise

Commercial Assays Table

Vendor	Assay Name	Cell Type	Assay Description
Invitrogen	GeneBLAZER	293T cells (kidney)	FRET-based reporter assay
BioDetection Systems	CALUX	U2-OS (bone)	Luciferase reporter assay
SwitchGear Genomics	LightSwitch	HT1080 (fibrosarcoma)	Multiplexed luciferase reporter assay
Attagene	Factorial TM	Transfect into cells of choice	Multiplexed reporter assay using capillary electrophoresis
DiscoverX	PathHunter	MD453 (breast) U2OS (bone)	Split beta-Gal reporter assay
Indigo BioSciences-Axxora	Nuclear Receptor Assays	Unspecified	Luciferase reporter assay

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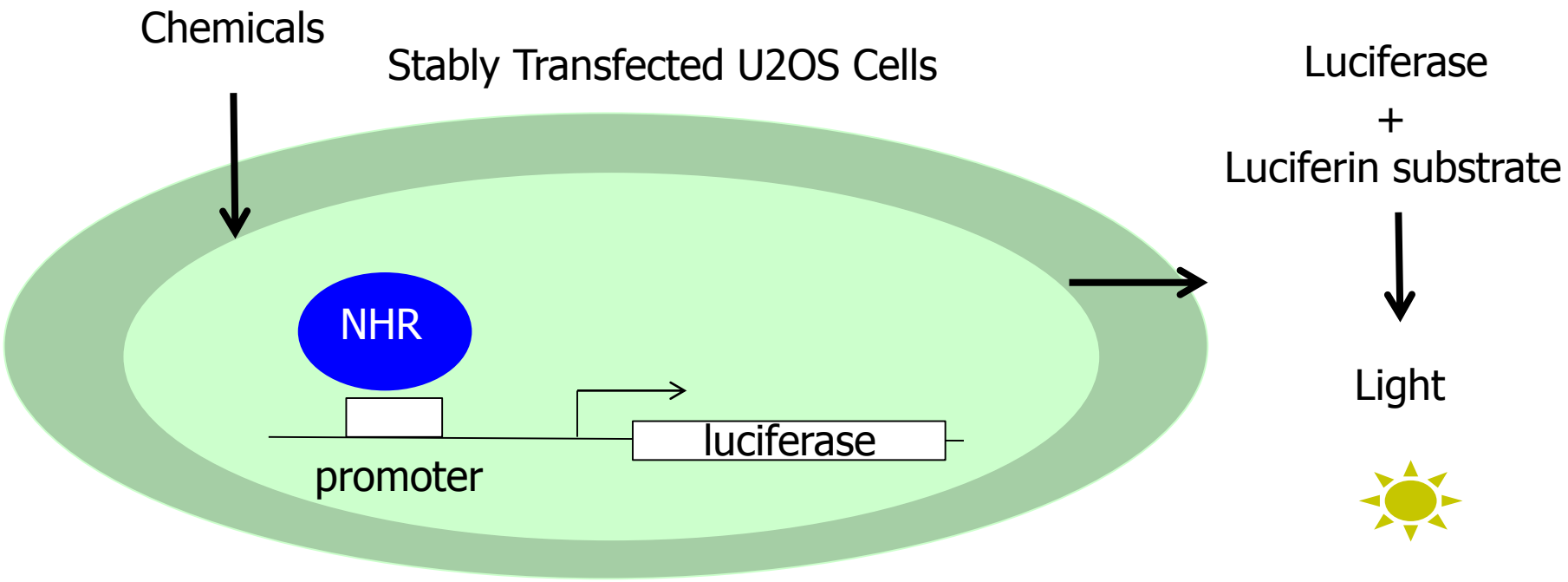
Vendor Assay Availability

Bioassay	Invitrogen	BDS-CALUX	SwitchGear
Estrogenicity- ER	Yes	Yes	Yes
Androgenicity- AR	Yes	Yes	Yes
Progesterone activity- PR	Yes	Yes	Yes
Genotoxicity- p53	Yes	Yes	Yes
Peroxisome proliferator activated receptor- PPARg	Yes	Yes	Yes
Glucocorticoid receptor activity- GR	Yes	Yes	Yes
Cytotoxicity	Yes-separate assay	No	Yes- integrated assay

BDS CALUX assays

- Stable U2OS (bone) cells
  - Express nuclear hormone receptor
  - Contain luciferase reporter with optimized DNA binding site for nuclear hormone receptor
- Cells are plated, treated with compounds, and then assayed for luciferase activity

BDS CALUX Assays



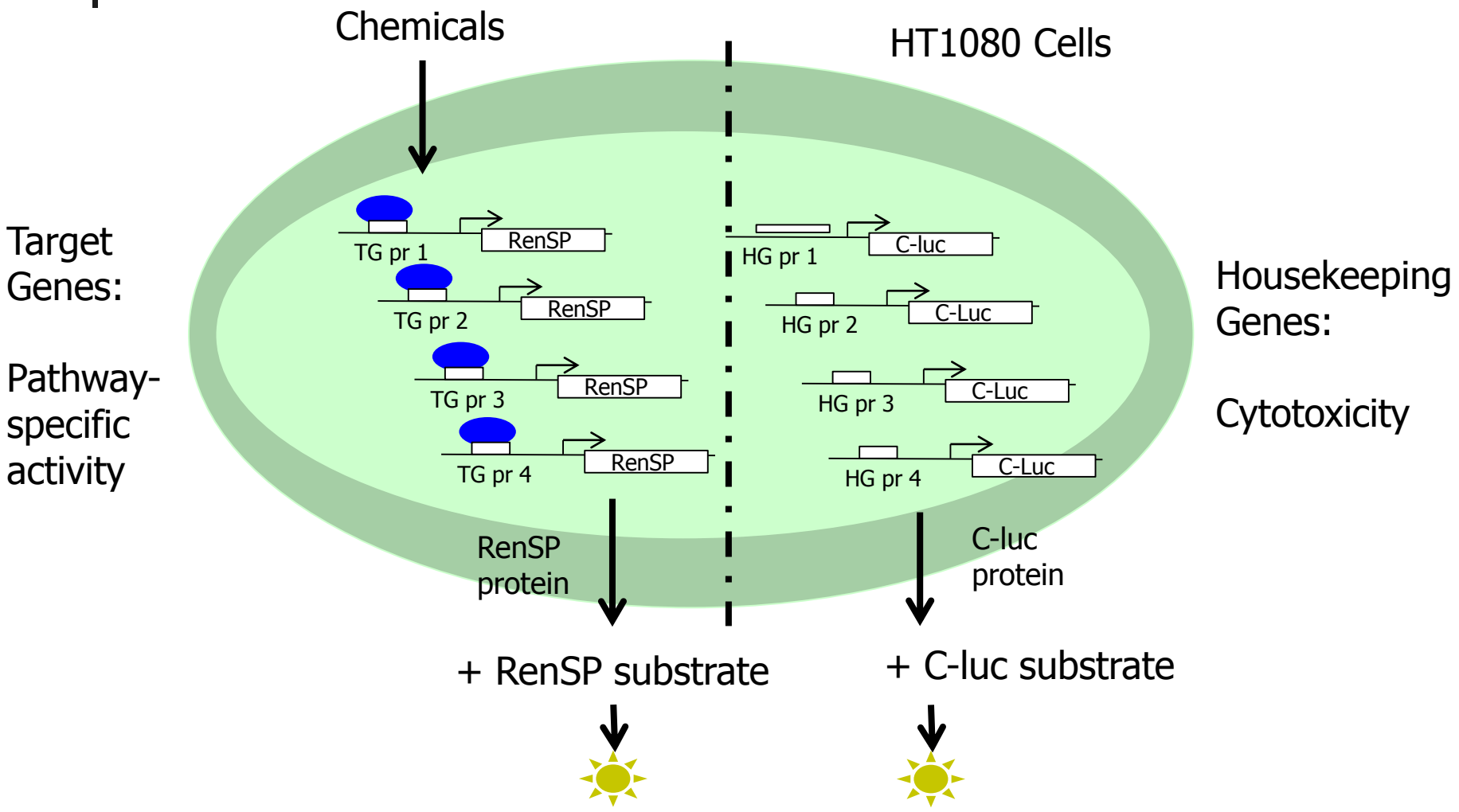
- Individual stable cell lines: ER, AR, PR, p53, PPARγ, GR
- No cytotoxicity assay

SwitchGear LightSwitch Assays

- HT1080 cells
  - Highly transfectable fibrosarcoma cells
  - Contain normal number of chromosomes
  - Can also use any other cell type of choice

- Cells are plated, transfected with pooled reporters, treated with compounds, and then assayed for dual luciferase activity

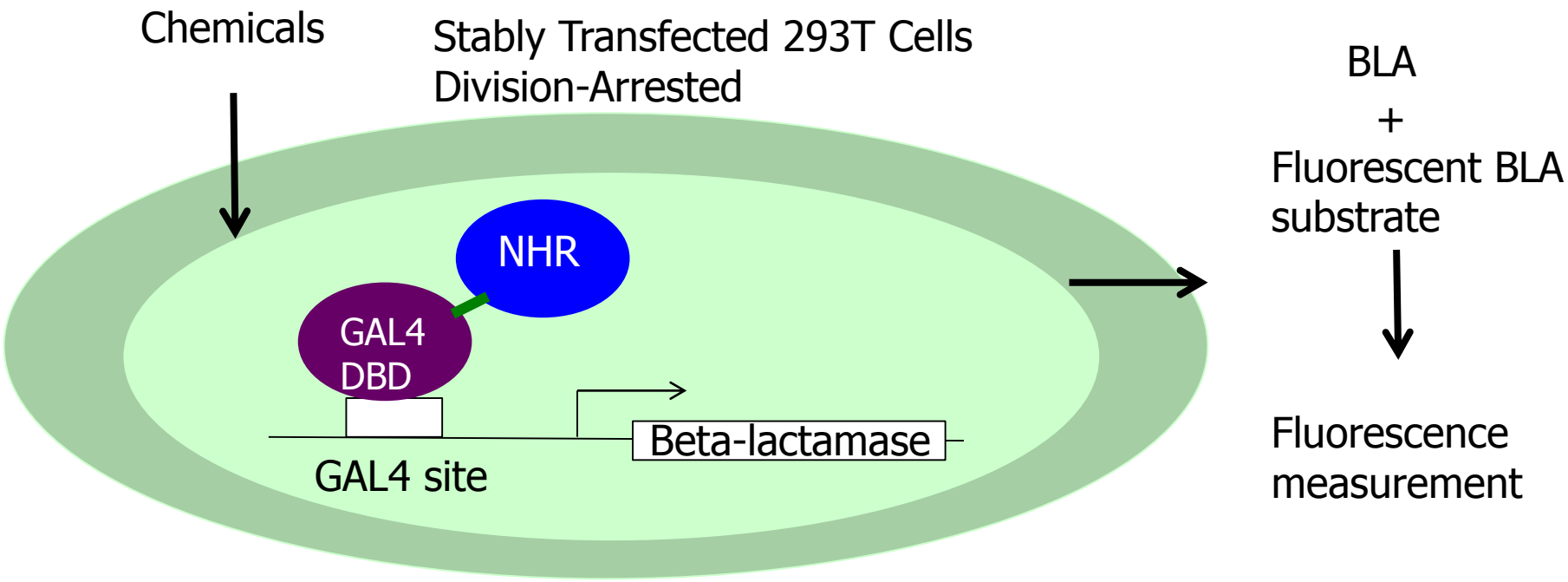
SwitchGear LightSwitch Assays



Invitrogen GeneBLAzer Assays

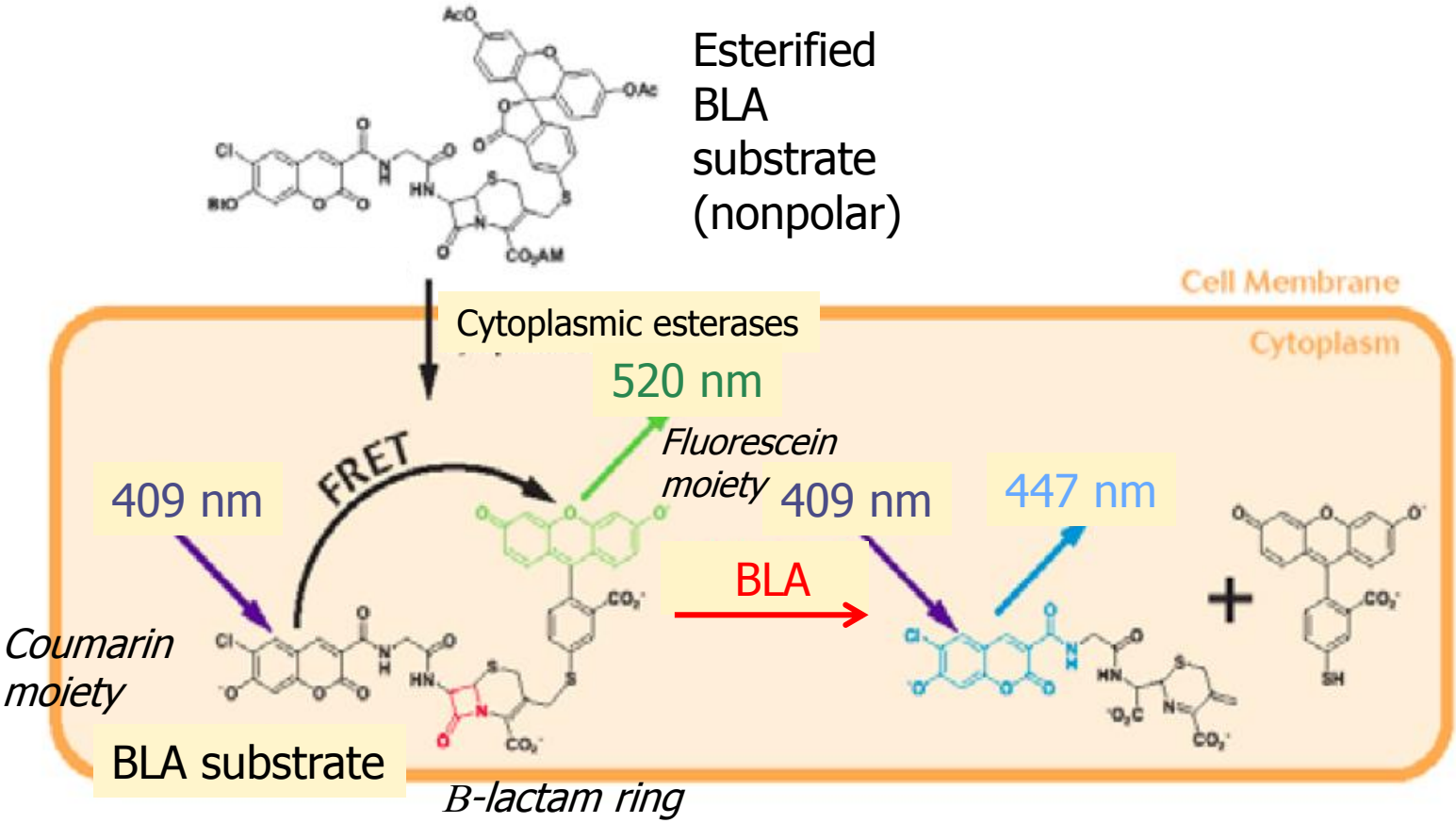
- Stable 293T cells
  - Transfected with GAL4-NHR and beta lactamase reporter containing GAL4 DNA binding site
- Cells are plated, treated with compounds, treated with fluorescent substrate, and then assayed for fluorescence activity

Invitrogen GeneBLAzer Assays



- Individual stable cell lines: ER, AR, PR, p53, PPARγ and GR
- Cytotoxicity measured separately (i.e. Presto Blue assay)

Fluorescence measurement



In the presence of beta lactamase expression (BLA), BLUE fluorescence is produced due to elimination of FRET

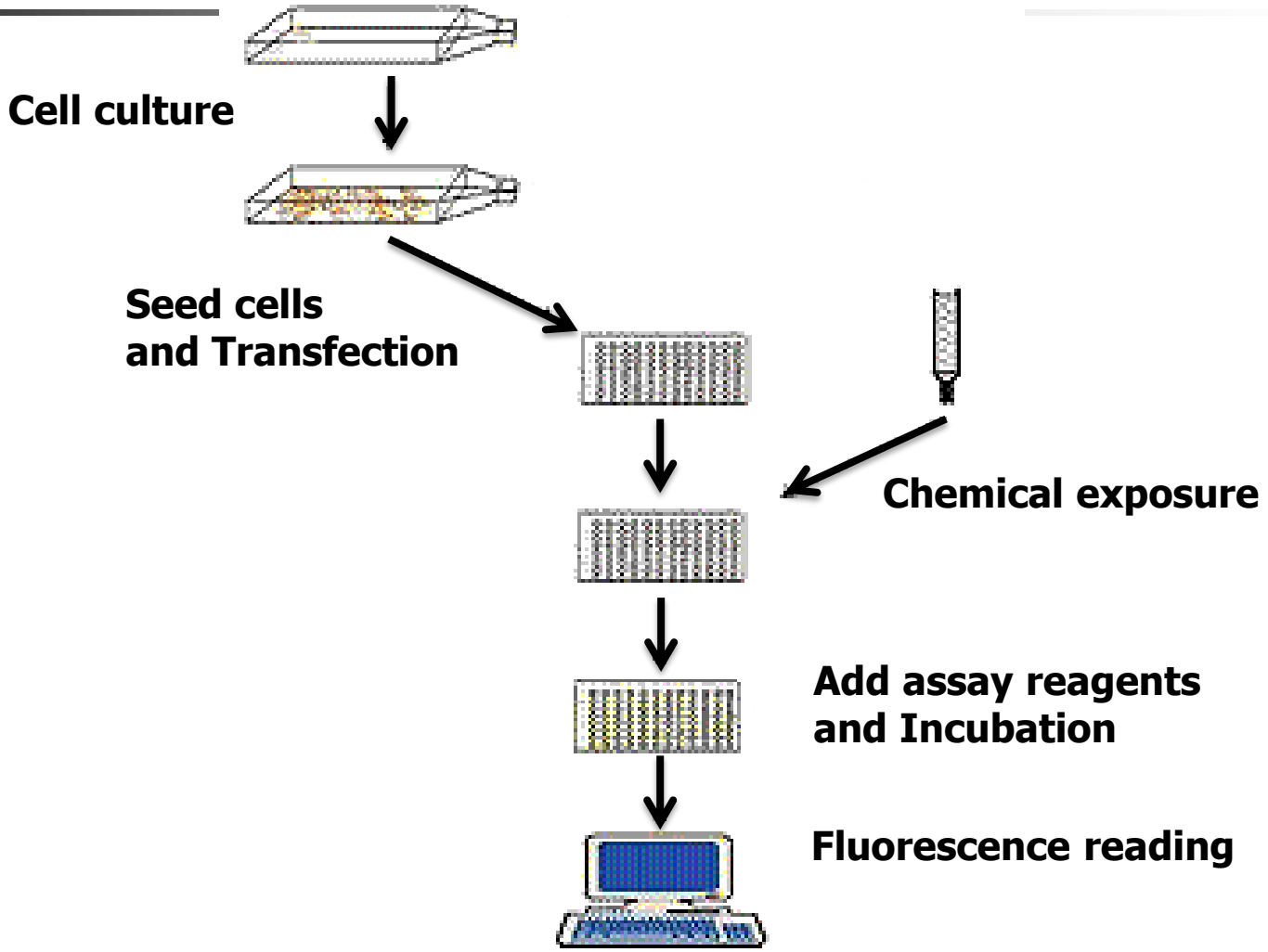
Characteristics of systems

- LightSwitch
  - Endogenous genes
  - Built-in cytotoxicity readout
  - Requires transfection
- CALUX
  - Artificial but sensitive
  - Widely used in Europe
  - Robust
  - Requires yearly license and MTA
- GeneBLAzer
  - Artificial but sensitive
  - Robust
  - Simplest and fastest
  - Best “kit” format

Optimization of GeneBLAzer Assays

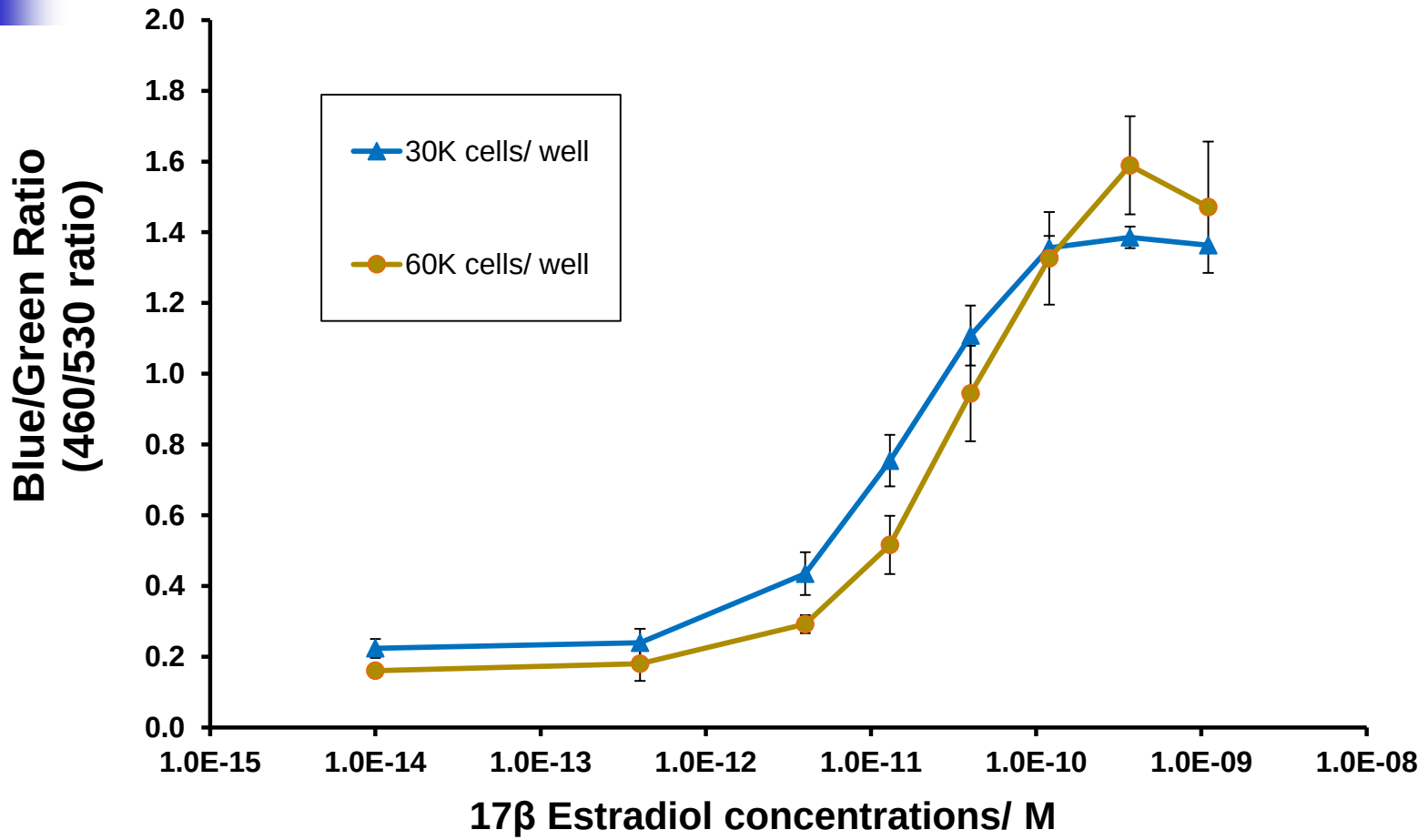
- Estrogen receptor -- ER
- Androgen receptor -- AR
- Progesterone receptor -- PR
- Glucocorticoid receptor -- GR
- Peroxisome proliferator activated receptor-- PPARα
- Peroxisome proliferator activated receptor --PPARγ
- Aryl hydrocarbon receptor -- AhR
- Cytotoxicity – Presto blue
- Genotoxicity – p53

In vitro assay protocol



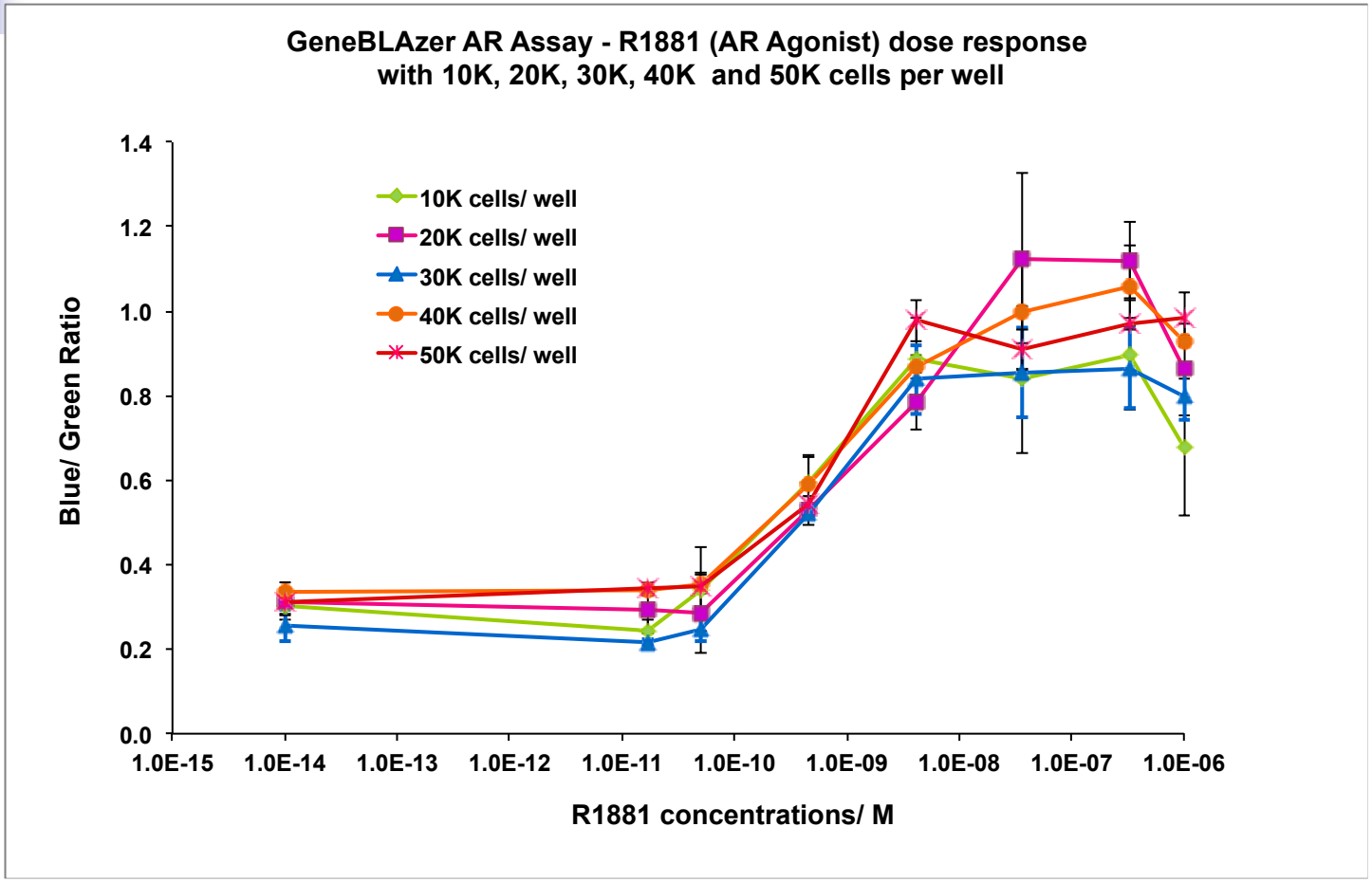
GeneBLAzer ERα Assay

E2 dose response with 30K and 60K cells per well

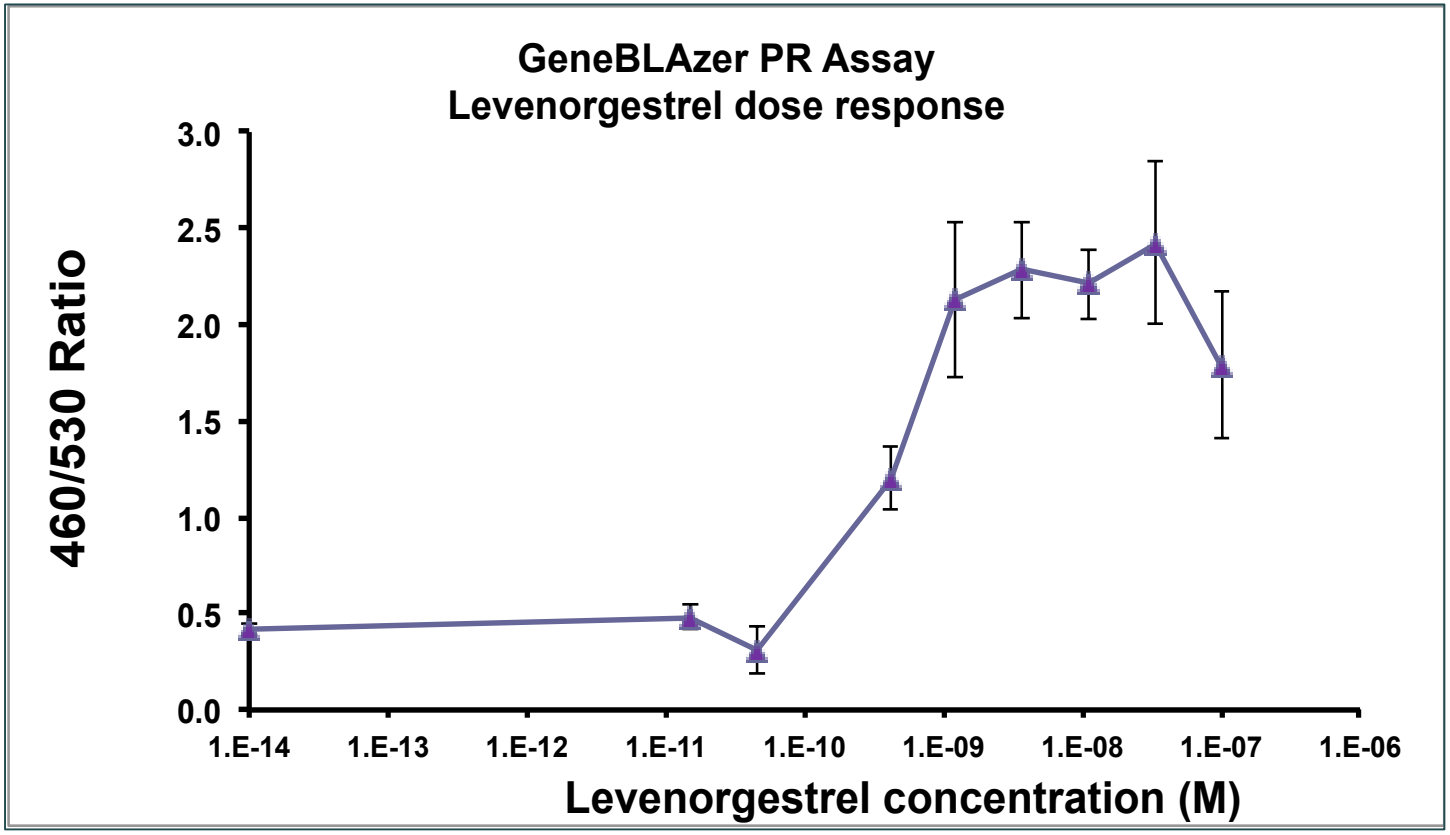


(Sumith Jayasinghe)

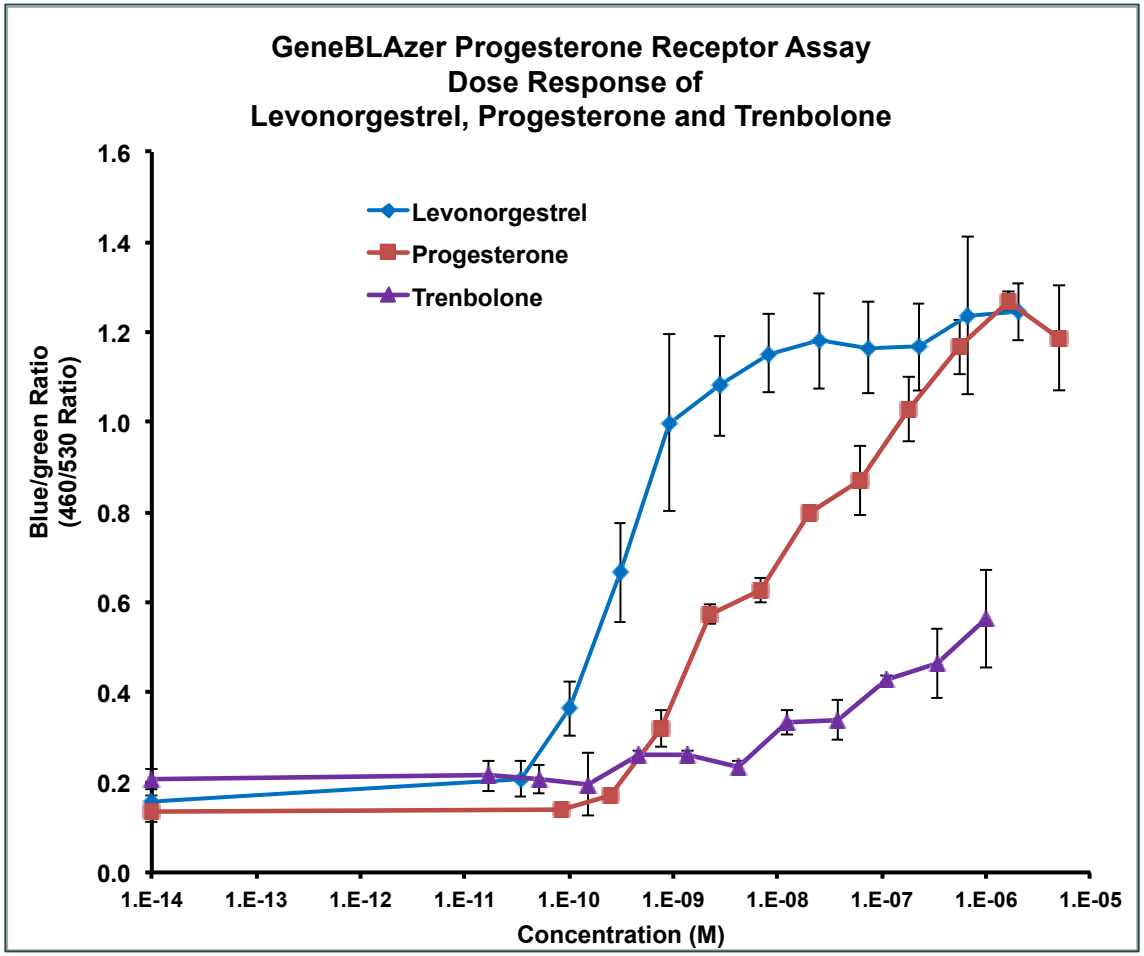
Invitrogen AR assay



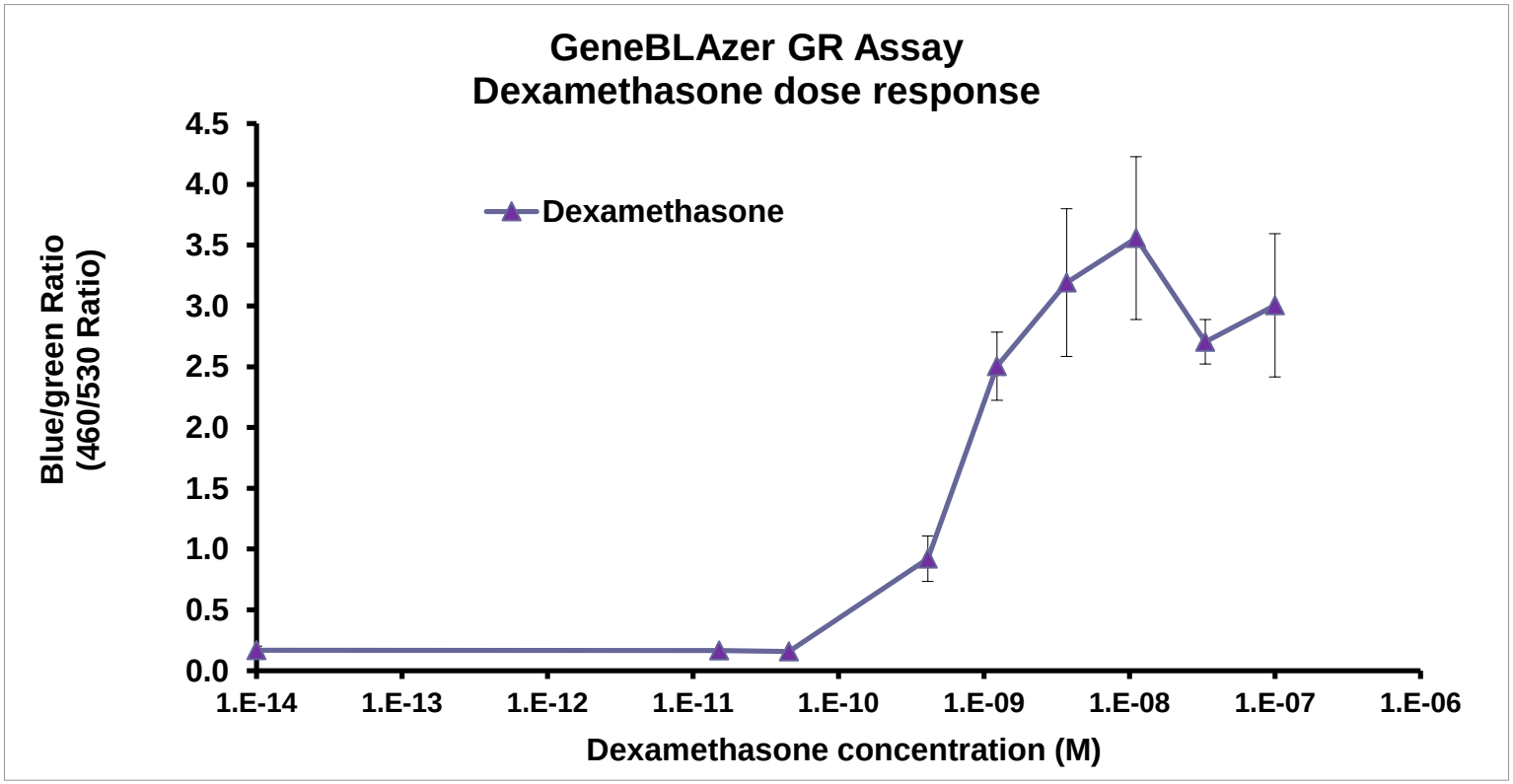
Invitrogen PR assay



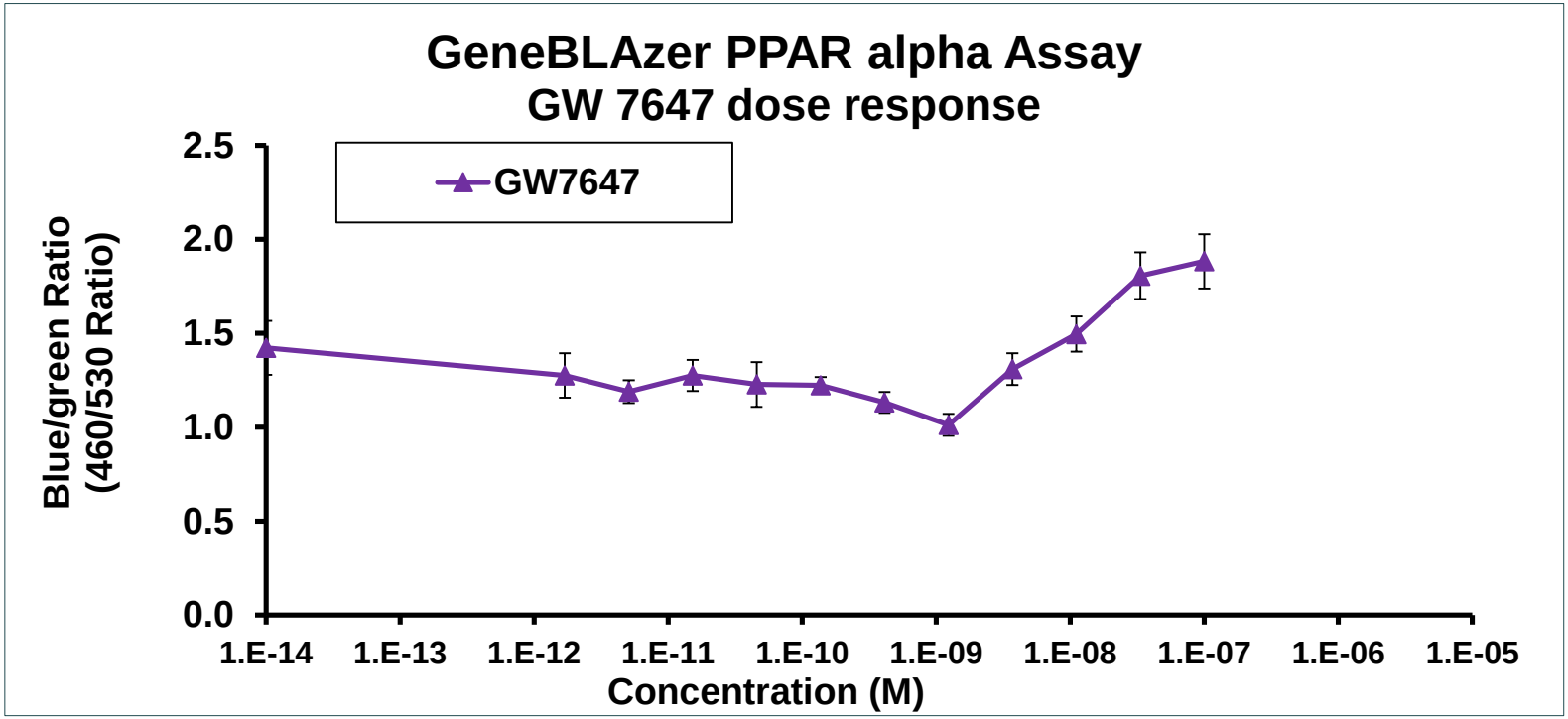
Progesterone receptor Levonorgestrel, progesterone, and trenbolone



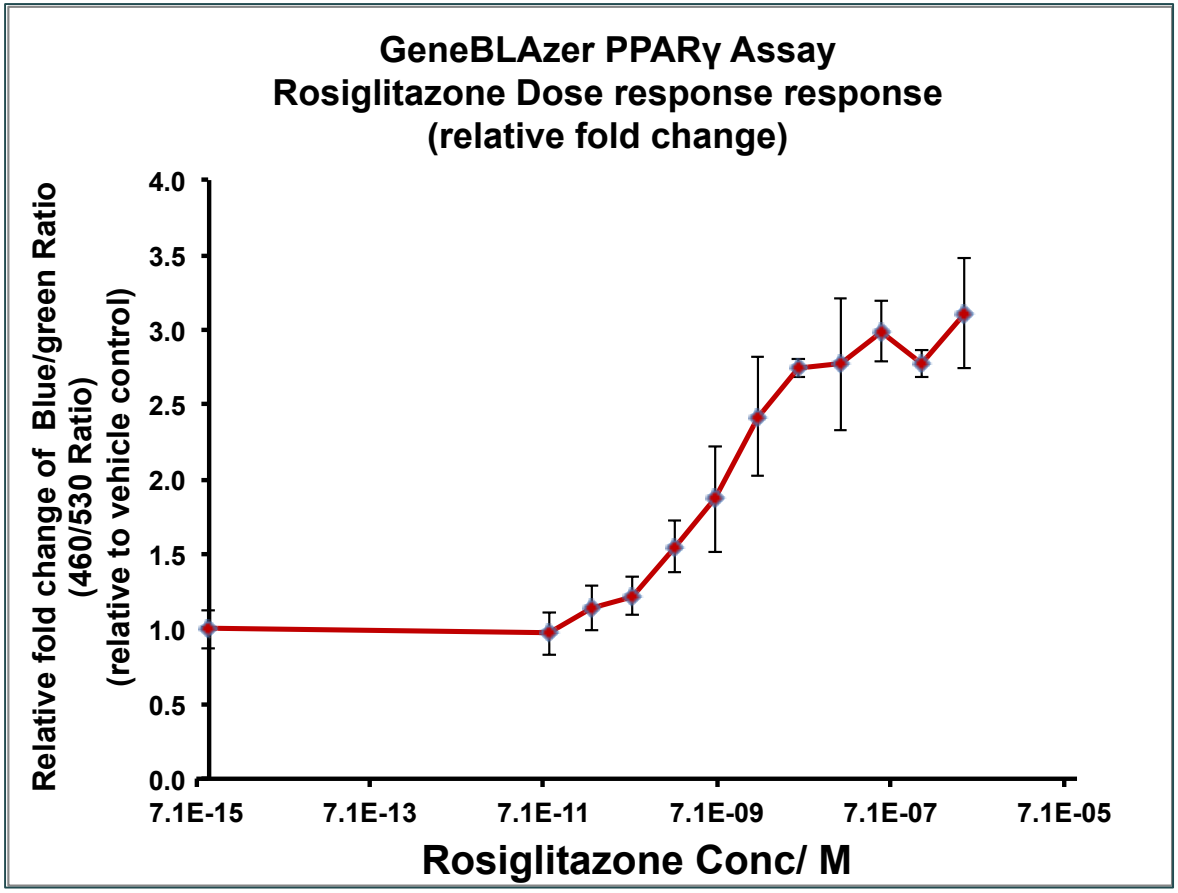
Invitrogen GR assay



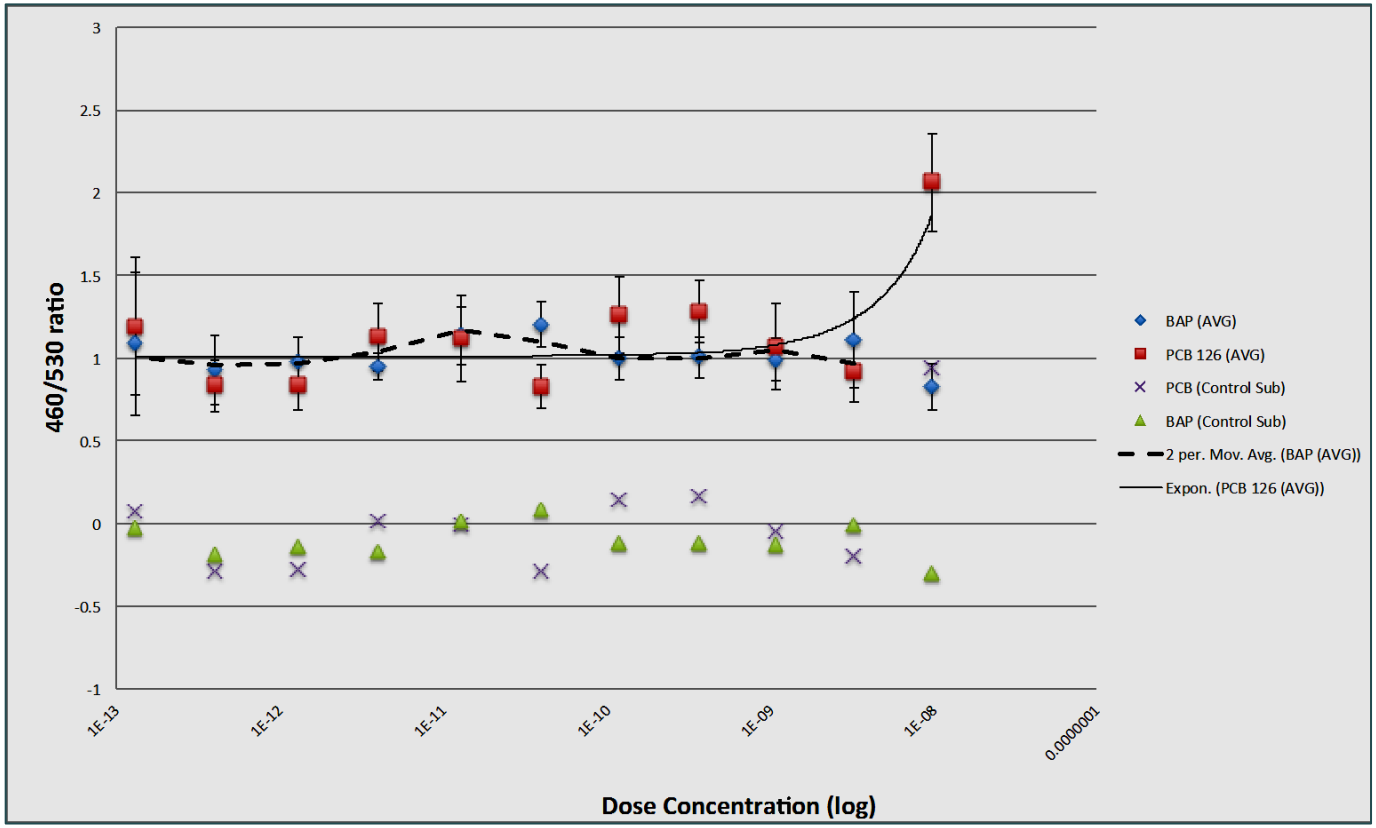
Invitrogen PPARα Assay



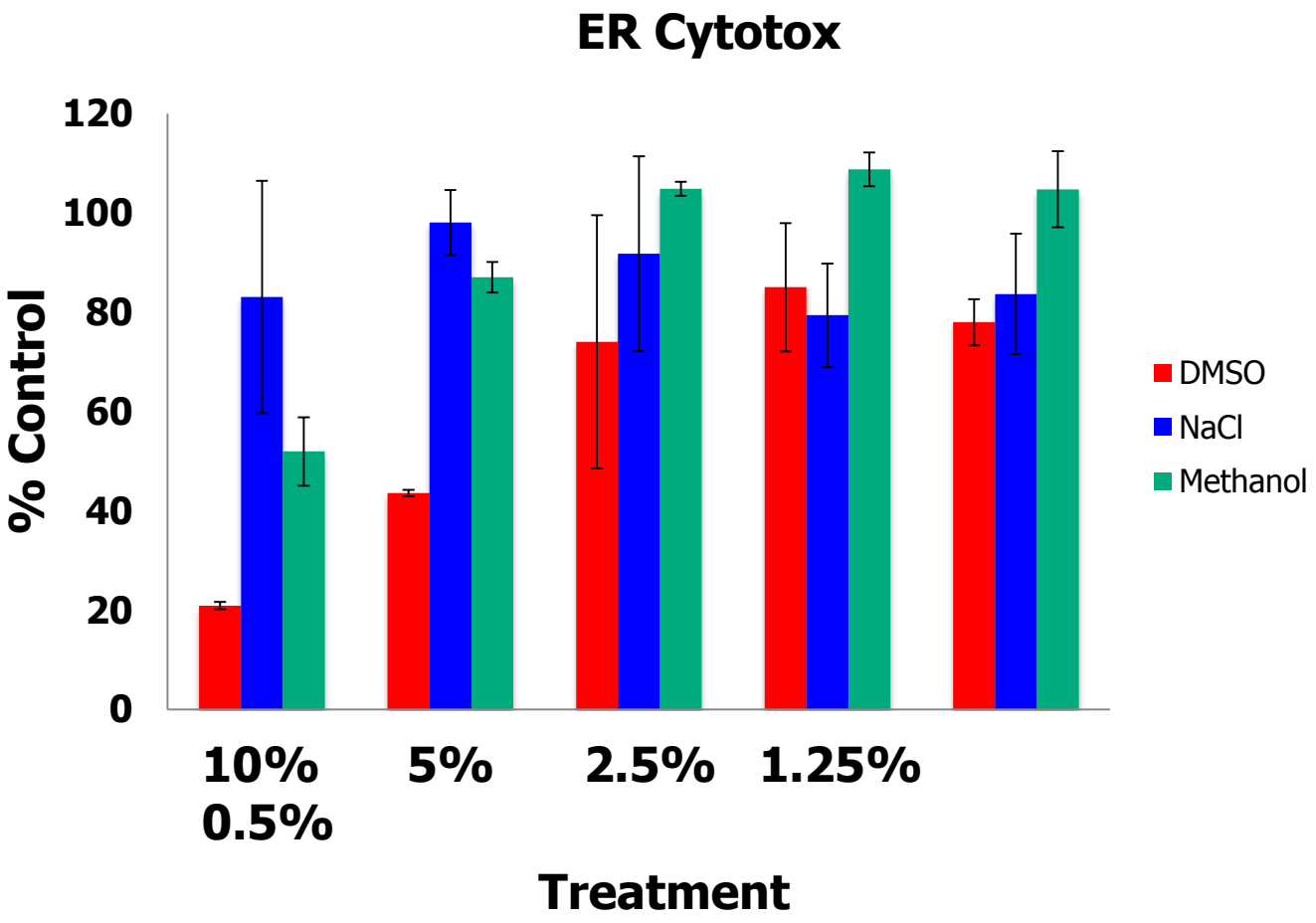
Invitrogen PPARγ Assay

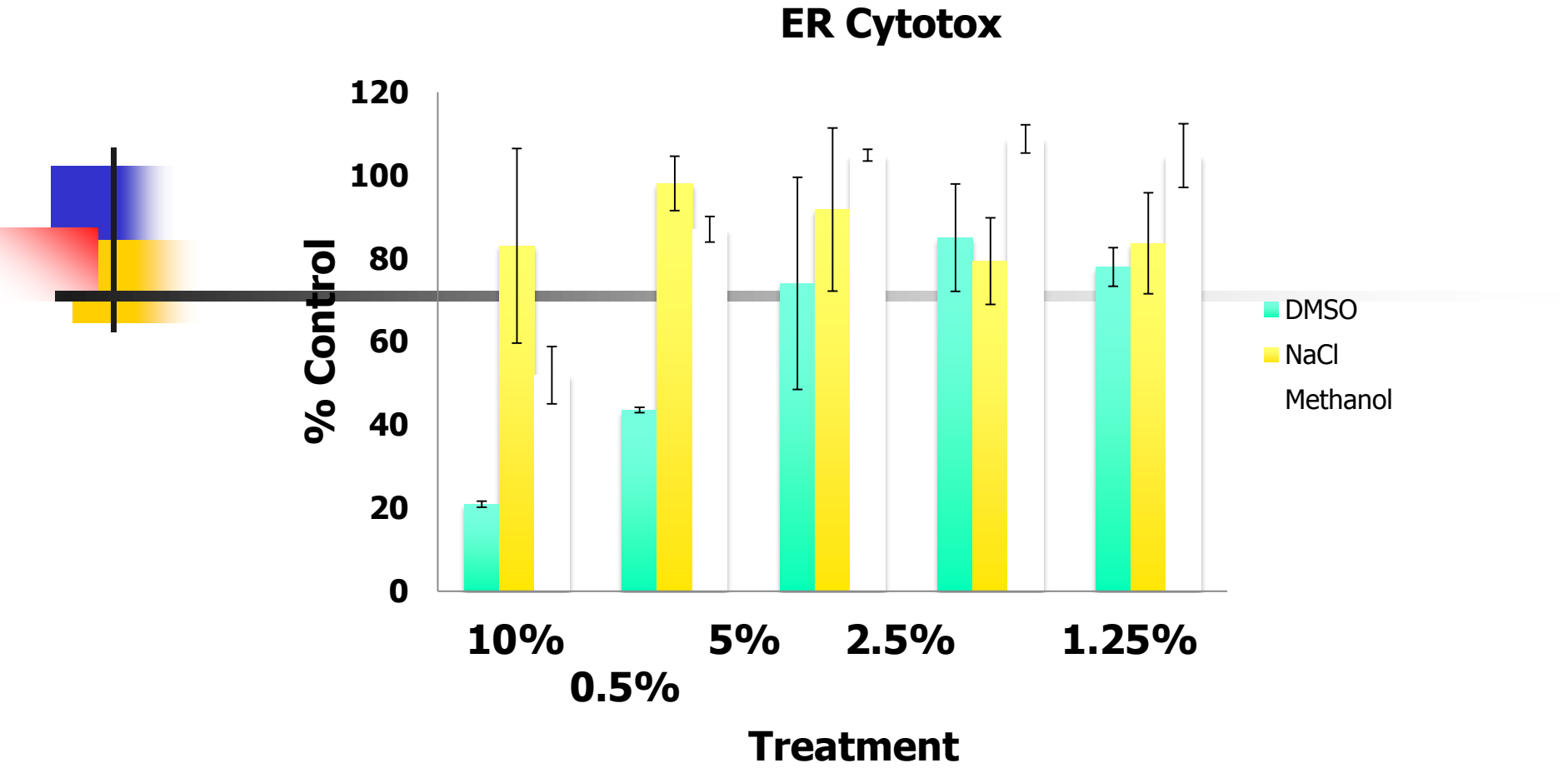


Invitrogen AhR assay

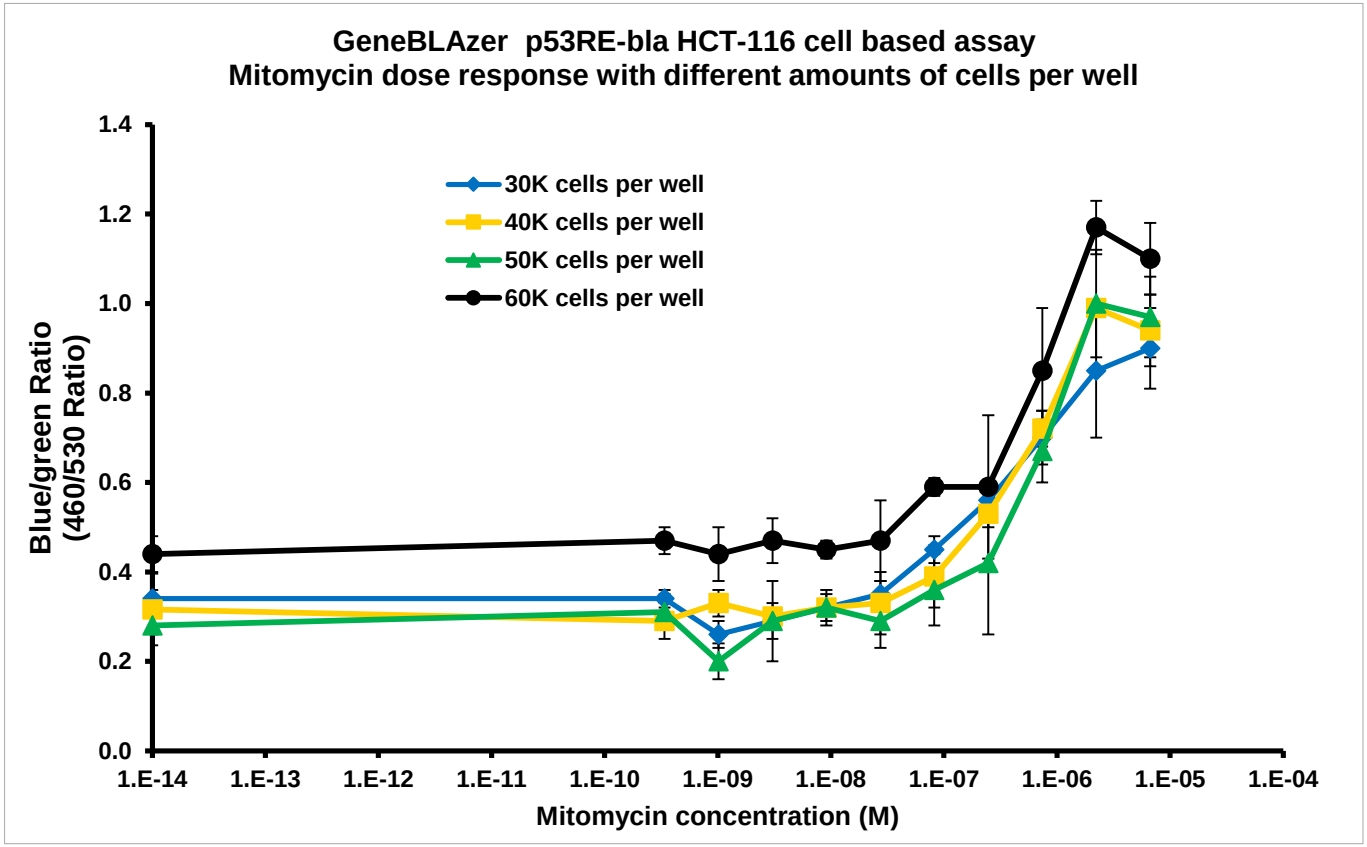


Invitrogen cytotoxicity

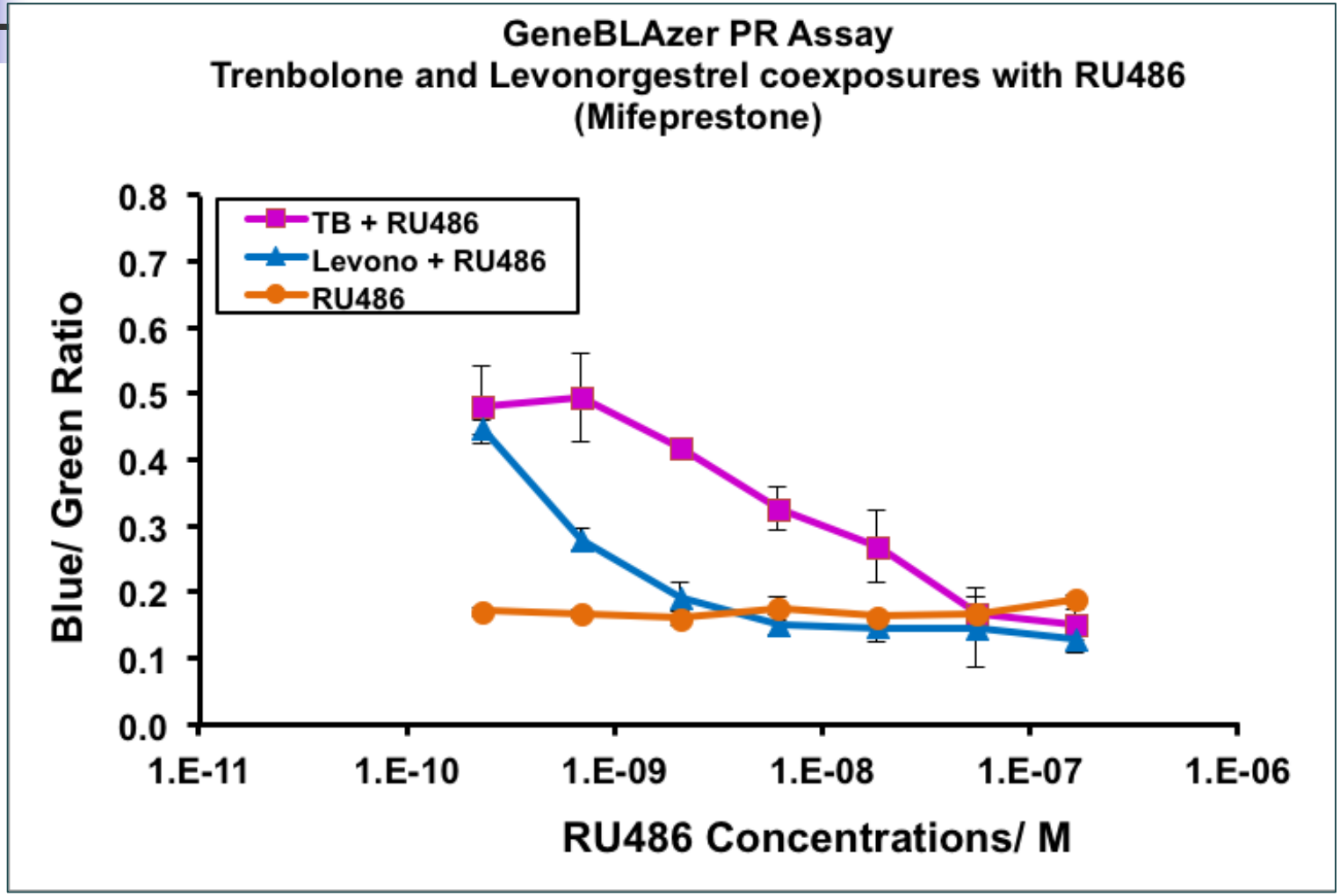



ER Cytotox
120
100
80
60
40
20
0
% Control
10%
5%
2.5%
1.25%
0.5%
Treatment
DMSO
NaCl
Methanol

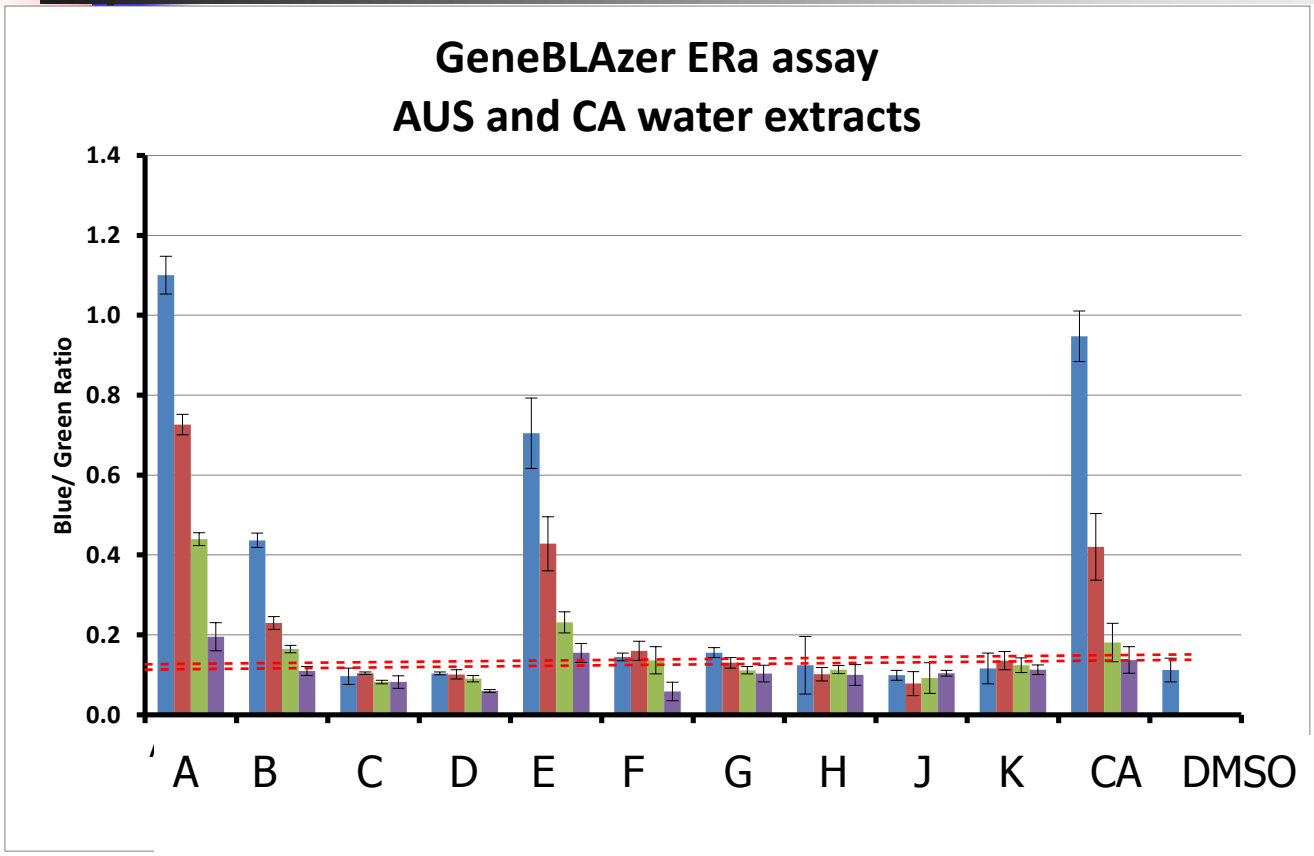
p53 assay using agonist mitomycin



Antagonism of PR Assay

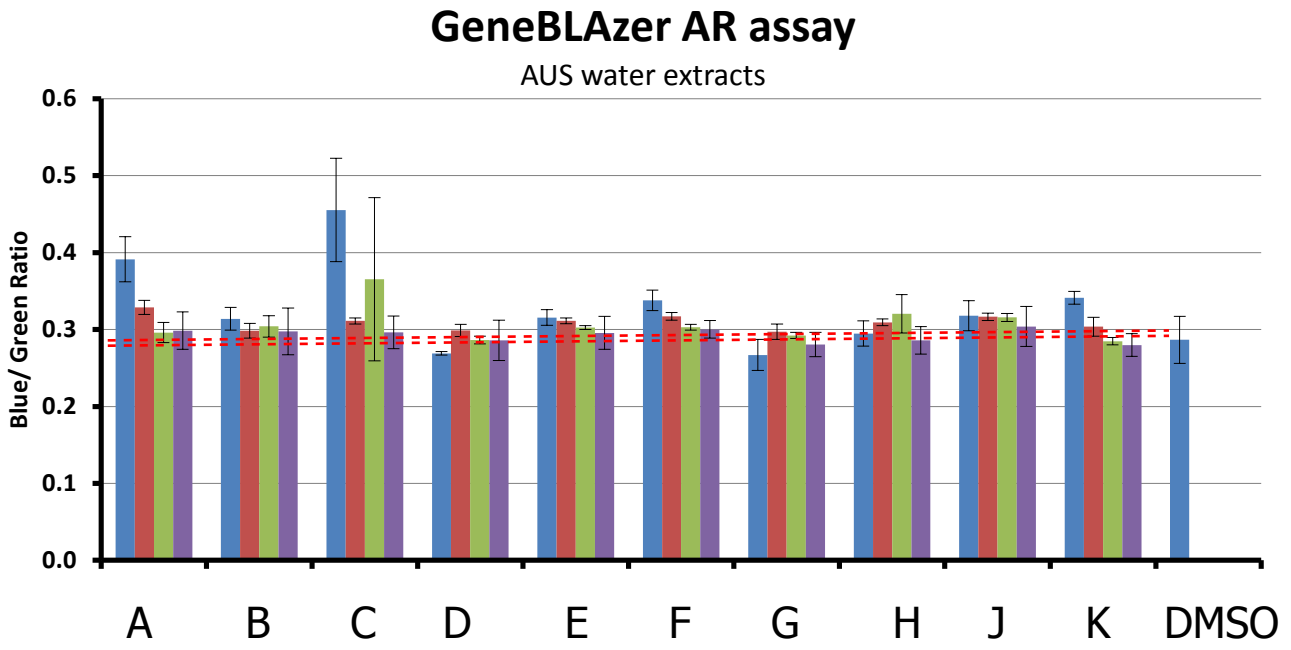


Round Robin Results -- ERα



Legend for samples
A= Effluent 2
B= Effluent 1
C= Ozonation
D= Storm water
E= Membrane
F= RO
G= River Water
H = AO
J= Blank
K= Drinking water
CA= SCCWRP proj

AR assay



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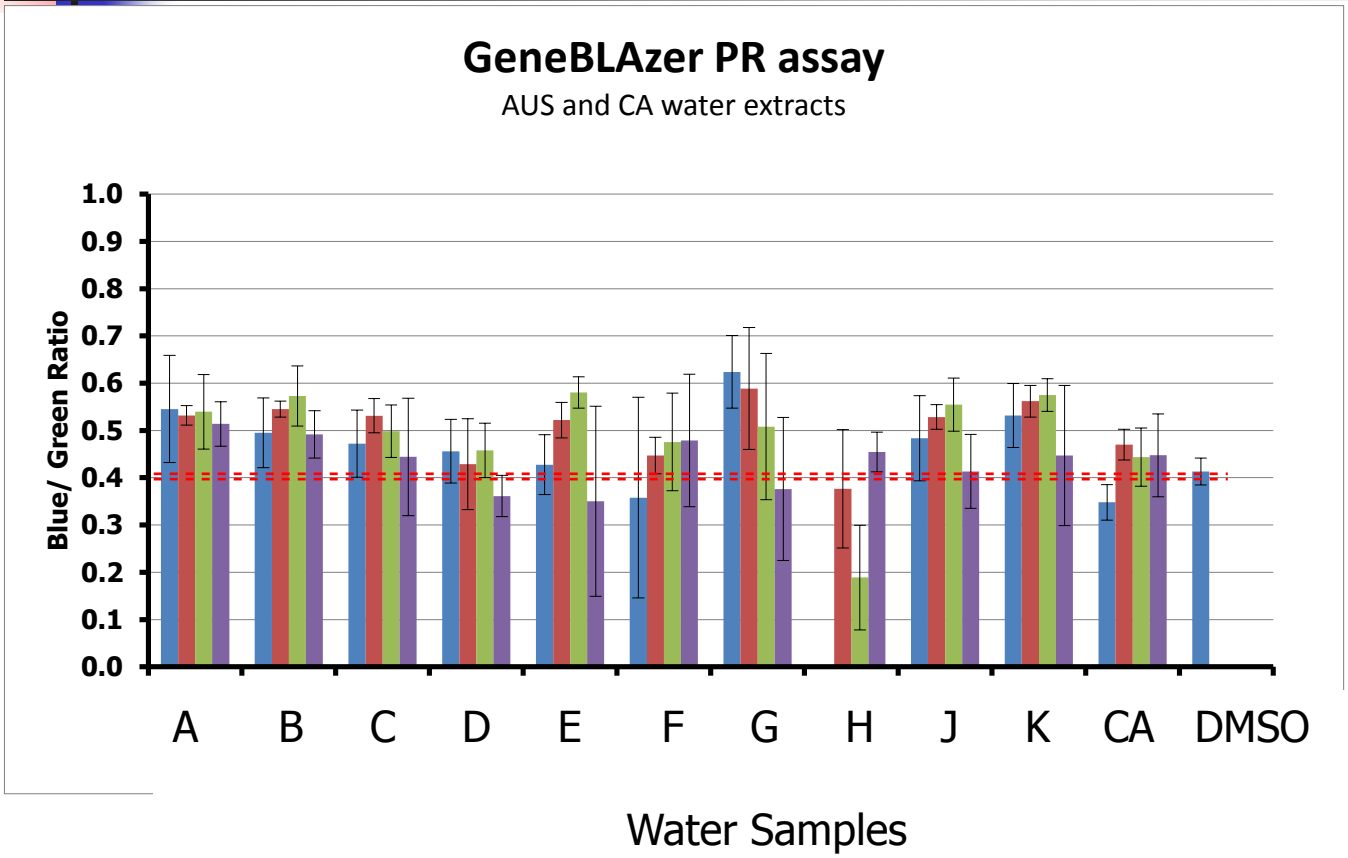
PR Assay



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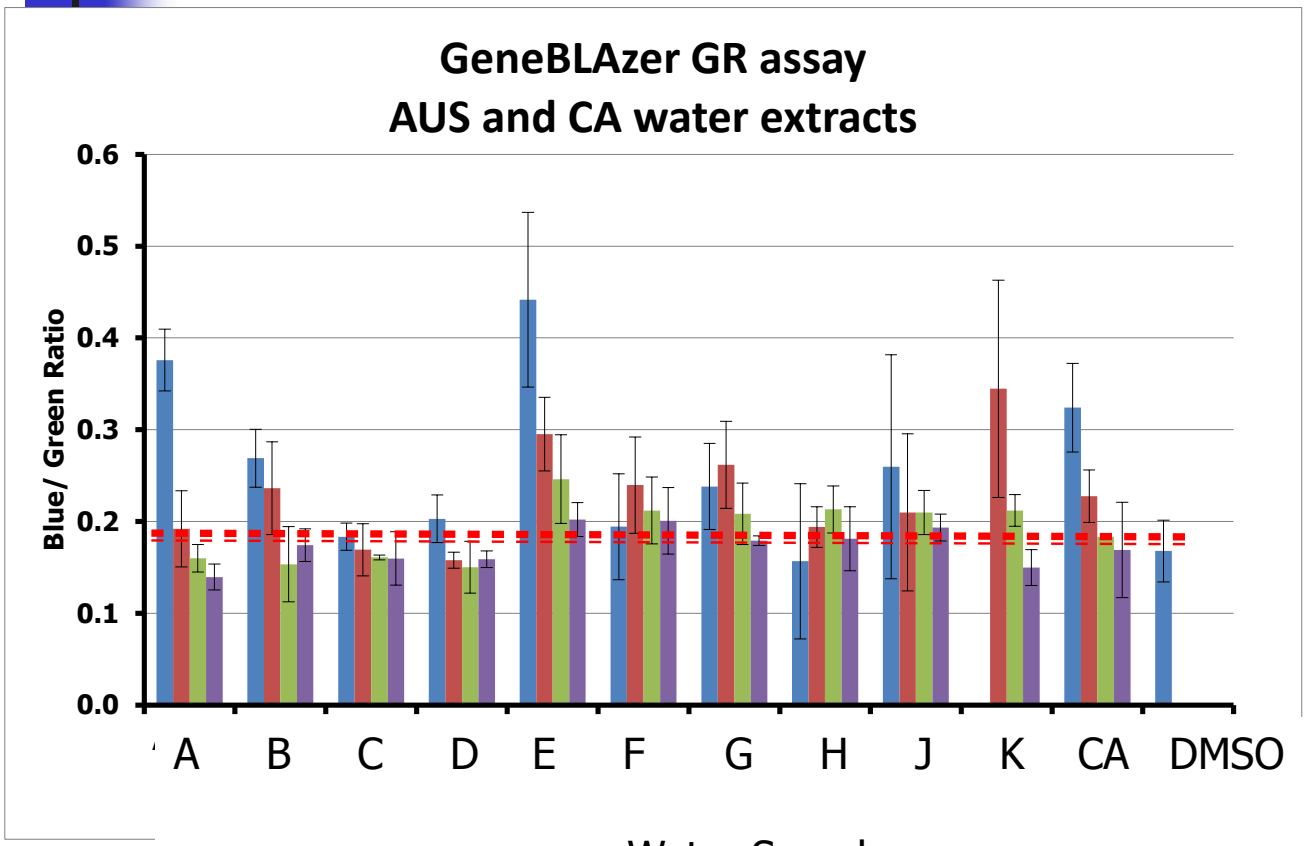
GR assay



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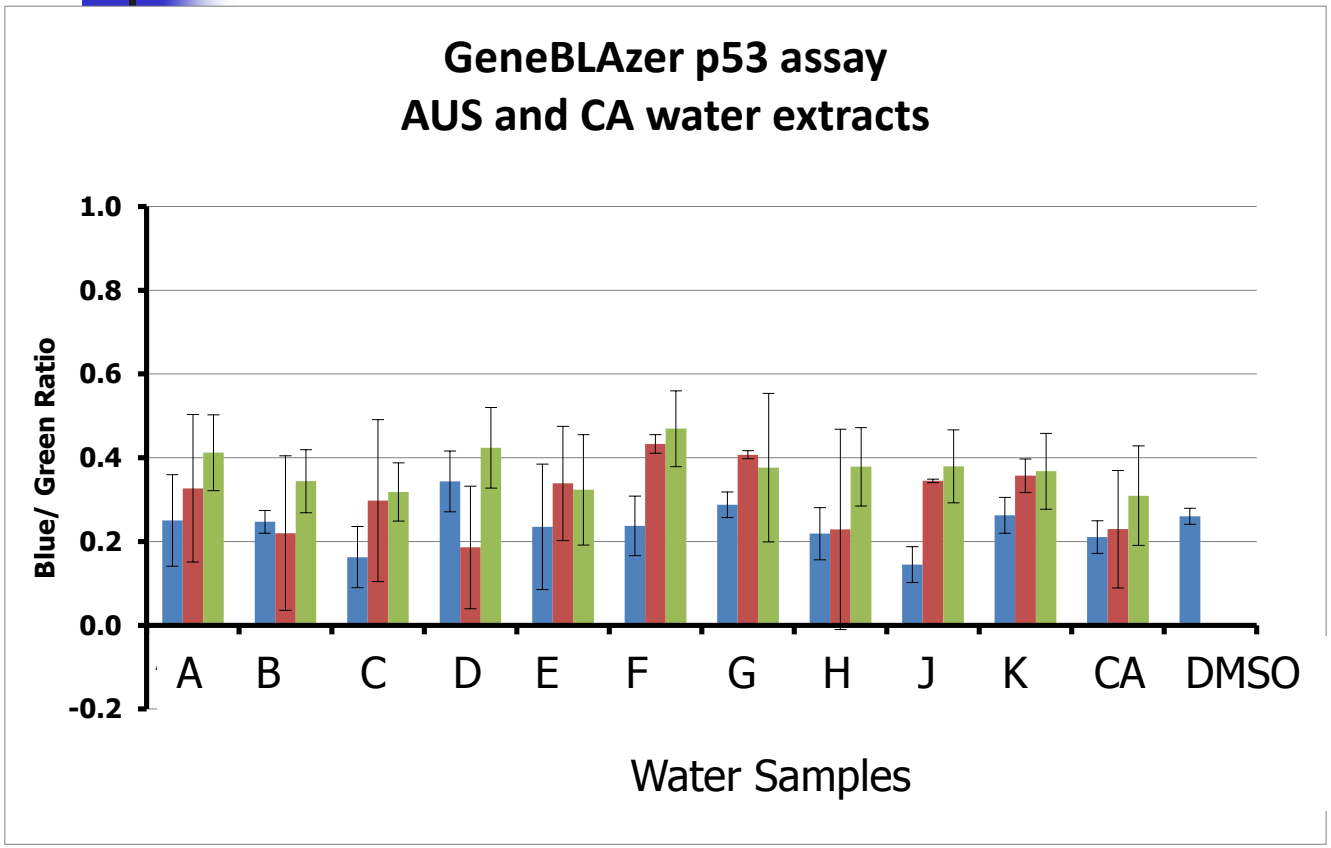
P53 Genotoxicity Assay



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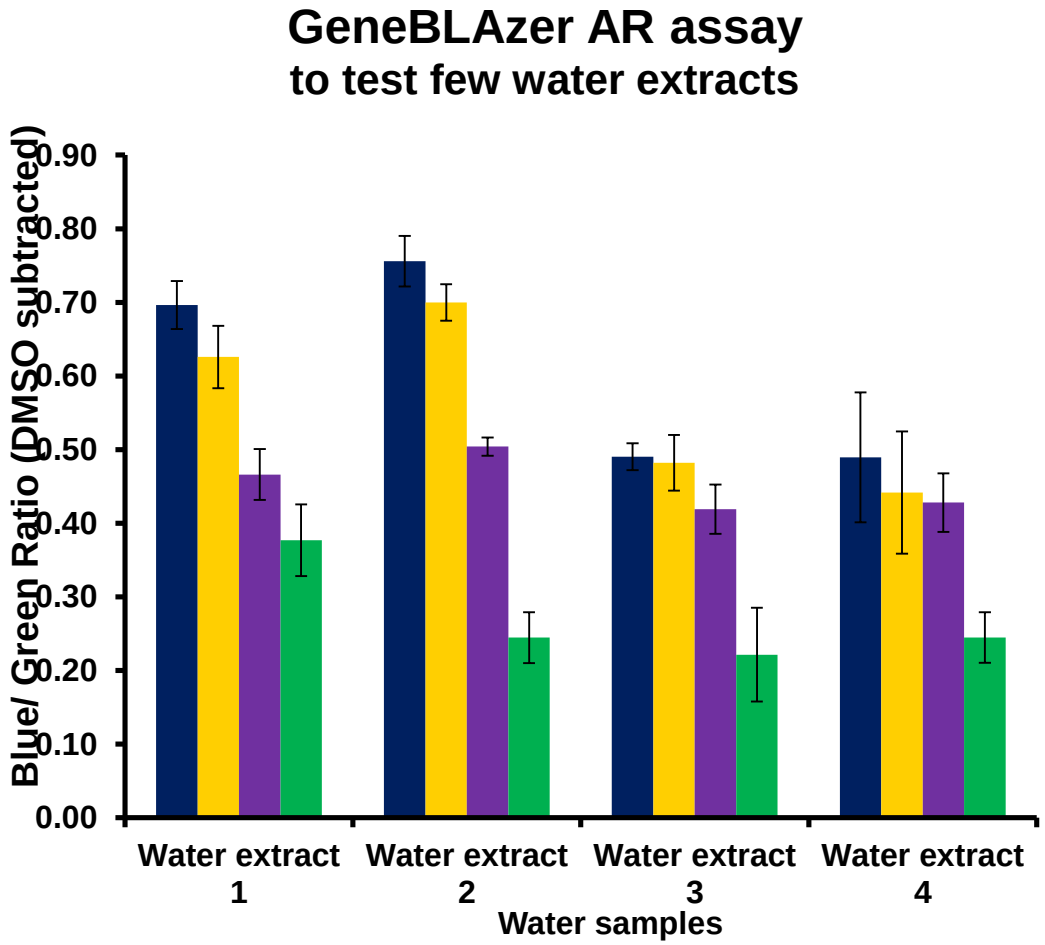
Fenholloway river- Florida Androgens and progesterone

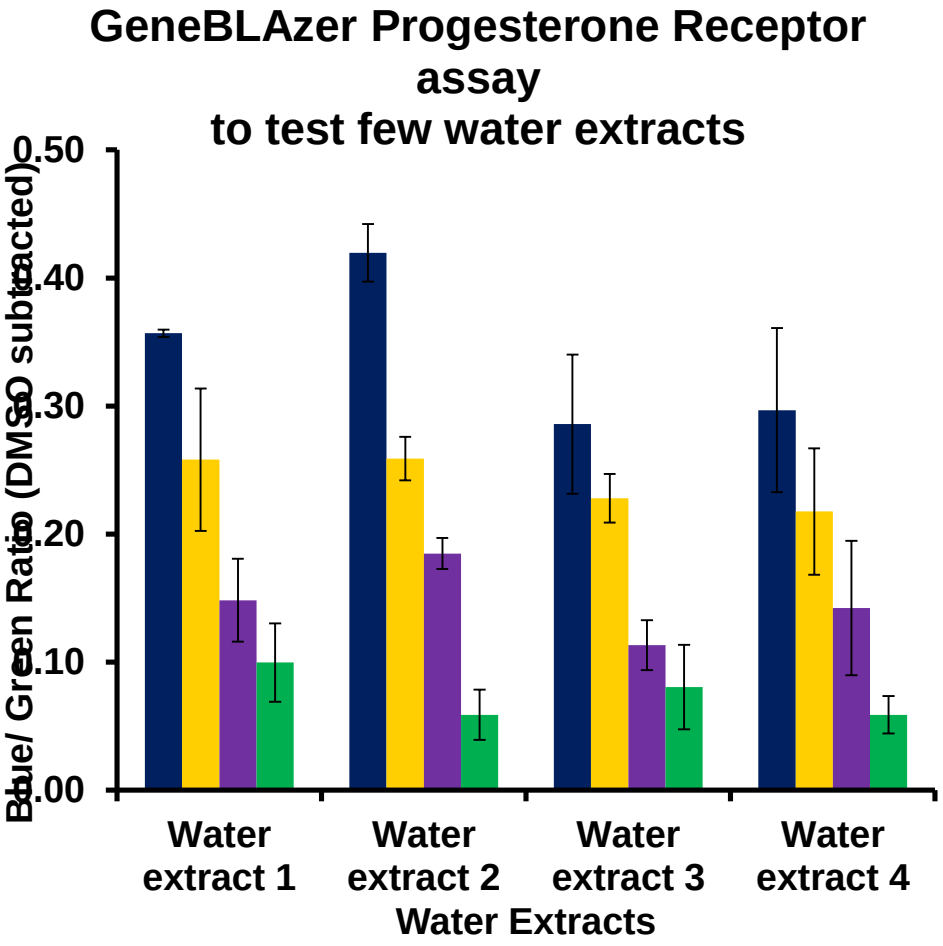
	Androstenedione	Progesterone
Water column	0.04 ±0.02 ug/L	2.06 ±0.38 ug/L
Sediments	0.7 ± 0.02 ug/L	48.8 ±7 ug/L

Jenkins, 2001

Fenholloway River and Econfina River in Florida



Fenholloway River and Econfina River in Florida



Conclusions

- Bioanalytical assays work well with standard chemicals and also work with water extracts
- Can be used to help inform the chemist about the analytes that should be investigated
- Multiple commercial assays are available
- Assays are relatively easy to perform – training required – mostly careful pipetting
- Still need to find a functional AhR assay

Acknowledgements

- UF team: Sumith Jayasinghe
- USF team: Jamie Mendez, Chris Menzie
- UCR team: Dan Schlenk, Jordan Crago
- UA team: Shane Snyder, Ai Jia
- SCCWRP team: Keith Maruya, Alvina Mehinto

Analytical Methods & Results

Ai Jia, Shimin Wu, Tarun Anumol, Bingfeng Dong,
Darcy VanDervort, & Shane Snyder

The University of Arizona

23rd January 2014

http://snyderlab.arizona.edu/

Challenges

- Extraction Method has limitations
  - Inappropriate for inorganics and highly-polar organics
  - Loss of highly-volatiles
  - Assumed recovery/stability for unknowns
  - Recovery not corrected for bioassays
- Analytical data from extracts less robust
  - No surrogates for recovery & suppression correction
  - Modern instrumental methods use <2 mL sample vol.
    - ➢ If mass balance good, instruments are faster/easier

Sample Collection-SCCWRP

Sampling Date:	**2012.6.18**	**Roger Road Effluent (1st round)**
	2012.8.28	**Green Valley AOP Pilot (2nd round)**
	2013.7.01	**West Basin recycle water (2nd round)**

+

Washed with MeOH and Milli-Q water

Ice inside

Sample Collection-1st round

Roger Road Wastewater Reclamation Facility (1st Round)

Treatment process consists of:

1) Headworks
2) Clarifiers
3) Biotowers
4) Chlorination

RR effluent is used for the irrigation of golf courses and also infiltrated.

Sample Collection-SCCWRP

Green Valley AOP Pilot Plant

1. GV-pilot influent (secondary eff)
2. GV-pilot UV (500mJ/cm2)
3. GV-pilot UV/H2O2 (500mJ/cm2, 10mg/L)
4. GV-pilot ozone (3mg/L)
5. GV-pilot ozone/UV (3mg/L, 500mJ/cm2)
6. GV-Chlorine (10mg/L HOCl, 2h contact)

Sample Collection-SCCWRP

West Basin Little Water Recycling Facility

1. Field Blank
2. WB-Influent
3. WB-Ozone
4. WB-MF
5. WB-RO
6. WB-UV AOP

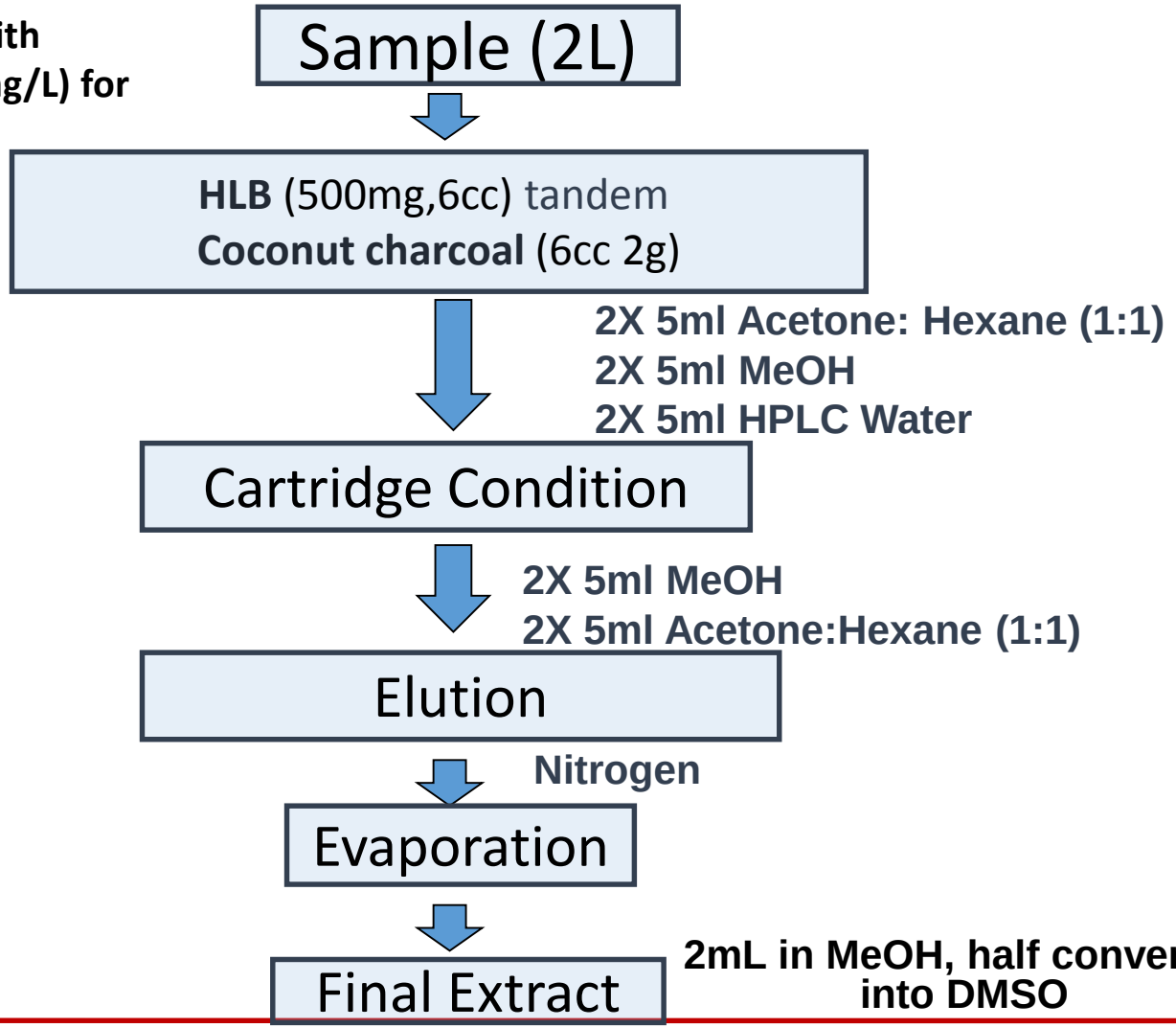
Sample Preparation

Samples as well as field blanks were moved into the lab and filtered immediately using the glass fiber filters (1.0um, Whatman)

Before SPE, all samples were stored at 4^{o}C.
Extraction was conducted within one week.

Sample Preparation

Dechlorinated with thiosulfate (50 mg/L) for specific samples.



Sample Preparation

1. Cartridge Conditioning

2. Loading Samples

3. Cartridge Elution

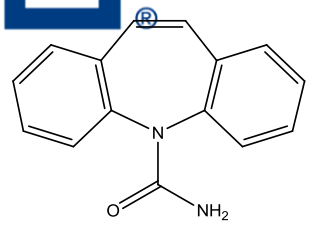
4. Evaporation

5. Transfer

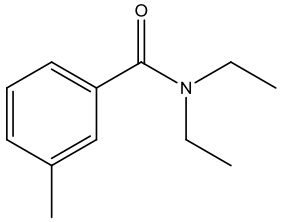
Target CECs

Acesulfame	Fluoxetine	PFBS	Sucralose
Atenolol	Gemfibrozil	PFDA	Sulfamethoxazole
Atrazine	Ibuprofen	PFDoA	TCEP
Benzophenone	Iohexol	PFHxA	TCPP
Benzotriazole	Iopamidol	PFHxDA	Testosterone
Caffeine	Iopromide	PFHxS	Triclocarban
Carbamazepine	Meprobamate	PFOA	Triclosan
DEET	Naproxene	PFOS	Trimethoprim
Diclofenac	Norethindrone	Primidone	
Diphenhydramine	Norgestrel	Propylparaben	
Ditiazem	PFBA	Simazine	

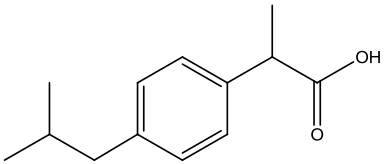
Target CECs



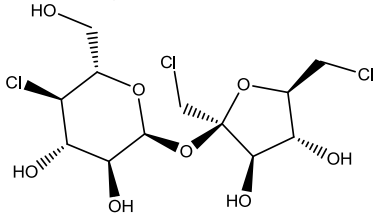
Carbamazepine (Anticonvulsant)



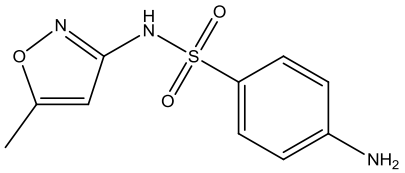
DEET (Insect Repellent)



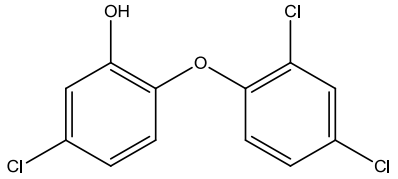
Ibuprofen (Anti-inflammatory Drug)



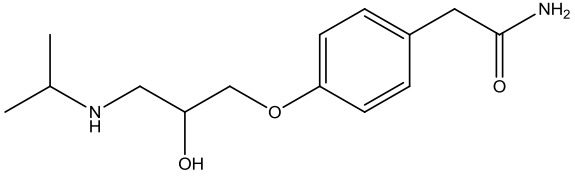
Sucralose (Artificial Sweetener)



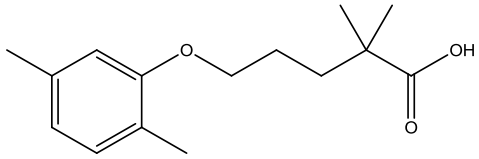
Sulfamethoxazole (Antibiotic)



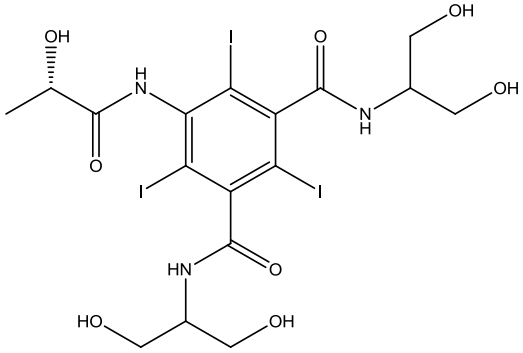
Triclosan (Antibacterial/Antifungal Agent)



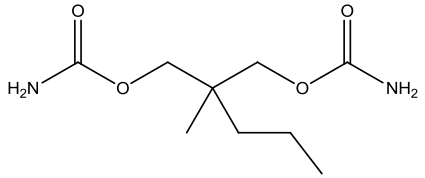
Atenolol (β-blocker)



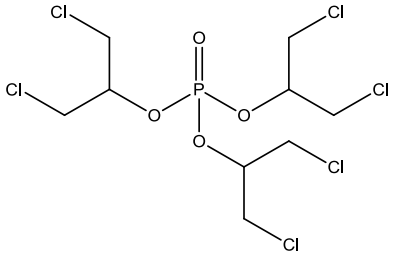
Gemfibrozil (Lipid-lowering Drug)



Iopamidol (Contrast Agent)



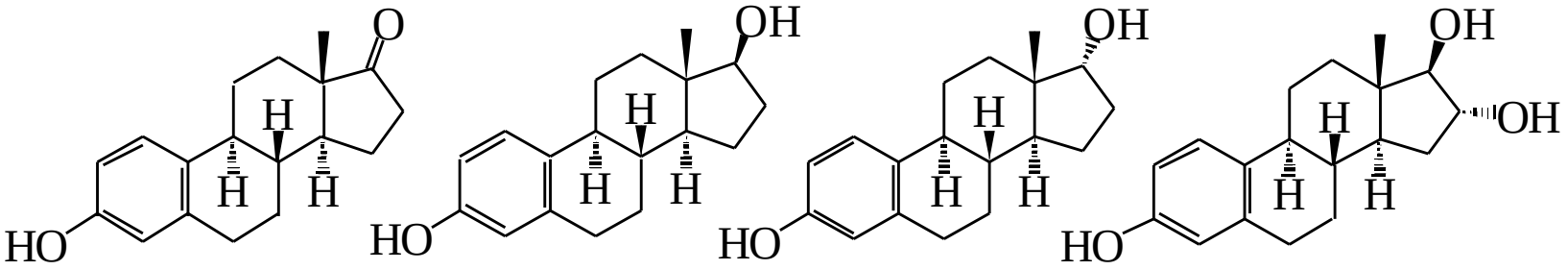
Meprobamate (Anxiolytic Drug)



TCPP (Flame Retardant)

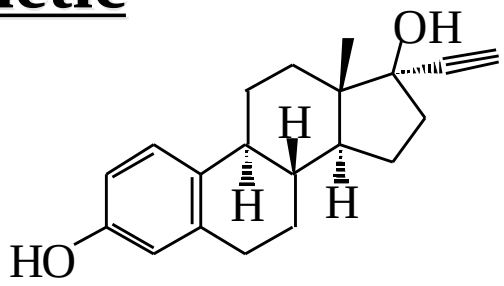
Target Hormones

Natural

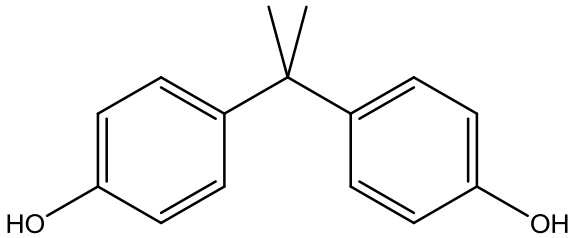


Estrone | 17β-estradiol (E2) | 17α-estradiol (E2) | Estriol (E3)

Synthetic



17α-Ethynylestradiol (EE2)



Bisphenol A (BPA)

Target Glucocortcoids

Target Analytes		
Aldosterone	Budesonide	Spironolactone
11-Deoxycorticosterone	Deflazacort	6-α-Methylprednisolone
Fludrocortisone	Flunisolide	Fluocinonide
Cortisone	Amcinonide	Betamethasone
Dexamethasone	Fluticasone Propionate	Fluorometholone
Triamcinolone	Mometasone Furoate	Triamcinolone Acetonide
Prednisone	Beclomethasone	Hydrocortisone
Prednisolone	Flumethasone	Fluocinolone Acetonide
Corticosterone	Clobetasol Propionate	Clobetasone Butyrate
Beclomethasone Dipropionate		
Surrogate		
Hydrocortisone-d_4	Dexamethasone-d4	Cortisone-d8
Prednisone-d4	Corticosterone-d8	Fludrocortisone-d5
Methylprednisolone-d2	Prednisolone-d6	

LC-MS/MS

Recovery: 88-122%

Method Performance for common CECs

Recoveries

(spike: 100 ng/L)

No Surrogates

Compounds	Recovery
Atrazine	63±4
TCPP	66±6
TCEP	66±2
Simazine	68±3
PFOS	71±2
Sulfamethoxazole	75±2
Sucralose	76±5
Caffeine	77±2
Primidone	78±4
PFBS	86±3
PFOA	88±2
Gemfibrozil	88±14
Carbamezapine	88±3
Trimethoprim	96±1
Sucralose	100±2
Triclosan	120±8
Sulfamethoxazole_13C6	97±2
Triclosan_d3	99±6
Sucralose_d6	79±17
Carbamezapine_d10	101±1
PFOA_C13	116±3

Compounds	Recovery
Fluoxetine	11±5
PFBA	28±2
DEET	38±9
Triclocarban	40±12
Fluoxetine d5	19±7

Method is good for common CECs

Detection Summary on 2nd round samples

- No compounds were detected in the field blank.
- Of the 12 samples analyzed, 29 of 41 (70%) target CECs were detected in the samples.
- 25 compounds were detected in more than 50% of Green Valley samples; while 24 were detected in more than 60% of West Basin samples (Raw, post ozone, post MF).
- Two compounds were detected in all of the samples except blank (Atenolol, Benzophenone).

CECs Concentration on 2nd round samples

<MRL

	Green Valley Pilot						West Basin					
ng/L	**Influent**	**UV**	**UV/H_2O_2**	**O_3**	**O_3/UV**	**Cl_2**	**Influent**	**O_3**	**MF**	**RO**	**UV**	**FB**
Acesulfame	13.9	<6.7	<7.0	<7.3	<6.2	<6.7	191	167	141	<7.0	<7.4	<6.9
Atenolol	1730	1670	1210	994	568	547	514	310	325	3.1	3.0	<0.2
Atrazine	<0.3	<0.4	<0.4	<0.4	<0.4	<0.4	14.4	12.1	12.1	<0.4	<0.4	<0.4
Benzophenone	184	63.4	11.1	54.9	8.7	10.4	880	334	280	150	130	<0.5
Benzotriazole	120	191	67.2	76.0	52.9	77.4	<16	<14	<15	<9.1	<9.1	<9.1
Caffeine	<3.1	<3.5	<3.5	<3.6	<3.2	<3.3	73.6	61.4	66.2	32.4	31.5	<3.0
Carbamezapine	290	224	265	10.4	28.6	23.8	118	16.4	30.2	<0.4	<0.3	<0.3
DEET	54.5	32.6	49.7	27.0	24.2	23.5	96.9	60.8	74.5	<0.5	<0.5	<0.5
Diclofenac	1360	378	240	<2.2	<1.9	273	120	10.9	70.0	<2.1	<2.0	<1.8
Diphenhydramine	512	485	456	<0.1	196	35.9	470	<0.2	265	<0.1	<0.1	<0.1
Ditiazem	266	184	174	<0.1	<0.1	165	262	47.7	56.1	<0.1	<0.1	<0.1
Fluoxetine	199	173	164	130	112	89.2	<0.2	<0.2	<0.2	<0.1	<0.1	<0.1
Gemfibrozil	148	135	130	14.1	45.7	87.9	633	221	319	<1.0	<0.9	<0.9
Ibuprofen	58.2	55.9	30.8	33.5	28.7	52.4	180	77.4	99.6	<7.6	<8.3	<7.8

CECs Concentration on 2nd round samples

<MRL

	Green Valley Pilot						West Basin					
ng/L	**Influent**	**UV**	**UV/H_2O_2**	**O_3**	**O_3/UV**	**Cl_2**	**Influent**	**O_3**	**MF**	**RO**	**UV**	**FB**
Iohexol	860	206	256	699	153	721	1830	1400	1320	<16	<15	<16
Iopamidol	294	79.8	52.8	168	40.3	147	387	277	324	<4.7	<4.5	<4.6
Iopromide	50.8	16.9	24.1	33.4	<15	37.4	44.3	54.1	39.9	<16	<15	<16
Meprobamate	540	402	417	324	313	404	370	300	336	<0.2	<0.1	<0.1
Naproxene	135	128	137	<3.5	19.2	40.3	854	163	267	<3.4	<3.2	<3.1
PFBA	6.8	6.0	6.4	4.9	4.7	4.7	<0.8	<0,8	<1.0	<0.8	<0.6	<0.4
PFOS	<0.7	<0.7	<0.7	<0.8	<0.7	<0.7	530	261	290	200	<0.6	<0.6
Primidone	709	812	711	449	471	595	49.0	33.9	42.1	<0.5	<0.5	<0.4
Sucralose	1810	1480	1610	282	346	216	12100	11100	19700	38.7	32.9	<8.5
Sulfamethoxazole	2270	537	129	41.4	27.3	<0.2	510	366	400	<0.2	<0.2	<0.2
TCEP	380	196	308	339	271	235	381	417	410	<0.3	<0.3	<0.3
TCPP	3960	1240	1930	1970	1230	693	731	718	859	<0.4	<0.4	<0.4
Triclocarban	185	99.7	93.9	60.5	42.5	37.0	30.8	15.1	18.3	<0.1	<0.1	<0.1
Triclosan	211	26.2	23.2	<2.5	<2.3	<2.3	346	<9.2	11.2	<2.4	<2.2	<2.2
Trimethoprim	288	269	269	<0.1	<0.1	<0.1	878	194	264	<0.1	<0.1	<0.1

CECs Concentration on 2nd round samples

Compounds not detected in any of the samples:

	Green Valley Pilot						West Basin					
ng/L	**Influent**	**UV**	**UV/H_2O_2**	**O_3**	**O_3/UV**	**Cl_2**	**Influent**	**O_3**	**MF**	**RO**	**UV**	**FB**
Norethindrone	<1.8	<2.1	<2.1	<2.2	<1.9	<1.9	<7.1	<6.7	<5.9	<1.9	<1.7	<1.7
Norgestrel	<0.7	<0.8	<0.7	<0.9	<0.7	<0.7	<1.7	<1.6	<1.6	<0.7	<0.7	<0.7
PFBS	<3.4	<4.0	<3.9	<4.1	<3.6	<3.7	<4.1	<3.9	<3.9	<3.9	<3.7	<3.7
PFDA	<0.6	<0.5	<0.7	<0.8	<0.6	<0.7	<0.4	<0.4	<0.4	<0.6	<0.5	<0.4
PFDoA	<2.8	<1.3	<2.8	<2.9	<2.5	<3.7	<1.2	<1.4	<1.2	<2.3	<1.1	<1.1
PFHxA	<46	<31	<37	<37	<23	<37	<45	<32	<25	<34	<31	<21
PFHxDA	<2.6	<3.6	<2.5	<2.8	<2.4	<4.3	<1.9	<2.1	<1.2	<2.1	<2.6	<1.5
PFOA	<0.8	<0.7	<0.9	<1.0	<0.7	<0.9	<0.7	<0.7	<0.8	<1.0	<0.9	<0.9
Propylparaben	<0.3	<0.4	<0.3	<0.4	<0.3	<0.3	<0.3	<0.3	<0.3	<0.4	<0.3	<0.3
Simazine	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.2	<0.2	<0.2	<0.1	<0.1	<0.1
Testosterone	<0.7	<0.8	<0.8	<0.8	<0.7	<0.7	<2.2	<1.9	<2.1	<0.7	<0.7	<0.7

<MRL

Steroid Hormone Concentration

- **Of the target estrogen compounds, only BPA was detected.**
- **Five glucocorticoid compounds were detected in some samples.**

<MRL

	Green Valley Pilot						West Basin					
ng/L	**Influent**	**UV**	**UV/H_2O_2**	**O_3**	**O_3/UV**	**Cl_2**	**Influent**	**O_3**	**MF**	**RO**	**UV**	**FB**
Bisphenol A	<2.7	3.2	<2.1	2.5	<2.2	<2.4	35.3	6.5	7.6	0.5	1.2	<0.4
Prednisolone/ Cortisone	0.06	<0.02	<0.02	<0.02	<0.02	<0.02	<0.05	<0.02	<0.02	<0.02	<0.02	<0.02
Amcinonide	0.4	0.68	0.49	0.5	0.62	0.47	<0.5	<0.2	<0.1	0.36	0.48	<0.1
Hydrocortisone	<0.2	<0.05	<0.05	<0.05	<0.05	<0.05	<0.2	<0.05	<0.05	<0.05	<0.05	<0.05
Fluticasone Propionate	<0.5	<0.1	<0.1	0.57	<0.1	<0.1	<0.5	<0.1	<0.1	<0.1	<0.1	<0.1
Fluocinonide	0.37	0.46	0.47	0.35	0.32	0.29	<0.4	<0.1	<0.1	<0.1	<0.1	<0.1
Betamethasone/ Dexamethasone	0.07	0.13	0.09	0.08	0.07	0.06	<0.05	<0.02	<0.02	0.03	<0.02	<0.02

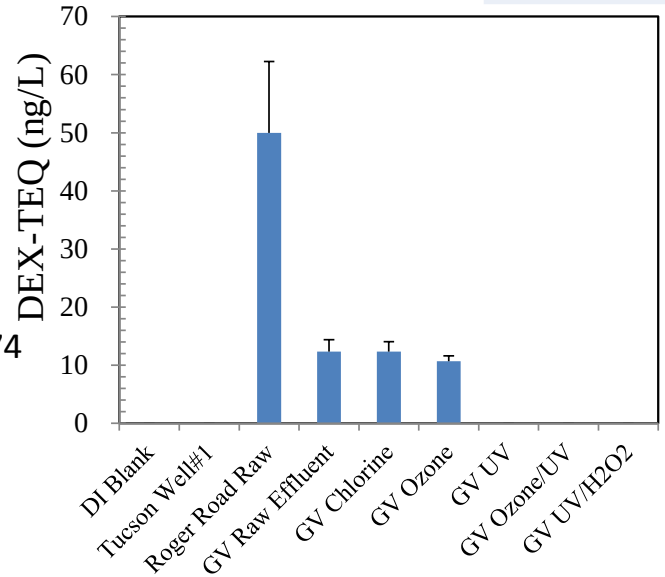
Chemistry & Bioassay

GR Analysis-TEQ value

compound	GR CALUX EC50 (nM)	REP[a]
aldosterone	112.2 ± 4.84	0.008 ± 0.06
amcinonide	0.49 ± 0.04	1.7 ± 0.09
betamethasone[b]	1.02 ± 0.05	0.8 ± 0.06
cortisol	11.4 ± 0.87	0.07 ± 0.08
cortisone	>1000[c]	<0.0008 ± 0.00006
desoximetasone	0.66 ± 0.03	1.3 ± 0.06
dexamethasone	0.84 ± 0.03	1 ± 0.05
flunisolide	0.49 ± 0.03	1.7 ± 0.07
fluorometholone	0.59 ± 0.03	1.4 ± 0.06
6α-methylprednisolone	2.25 ± 0.14	0.4 ± 0.07
paramethasone[b]	1.14 ± 0.04	0.7 ± 0.05
prednicarbate	4.75 ± 0.20	0.2 ± 0.06
prednisolone	3.68 ± 0.34	0.2 ± 0.1
prednisone	>500[c]	<0.002 ± 0.0004
rimexolone	0.83 ± 0.04	1 ± 0.06
triamcinolone	5.67 ± 0.23	0.2 ± 0.05
triamcinolone acetonide	0.37 ± 0.01	2.3 ± 0.04
triamcinolone hexacetonide	3.40 ± 0.17	0.3 ± 0.06

Environ. Sci. Technol. **2010,** *44,* 4766–4774

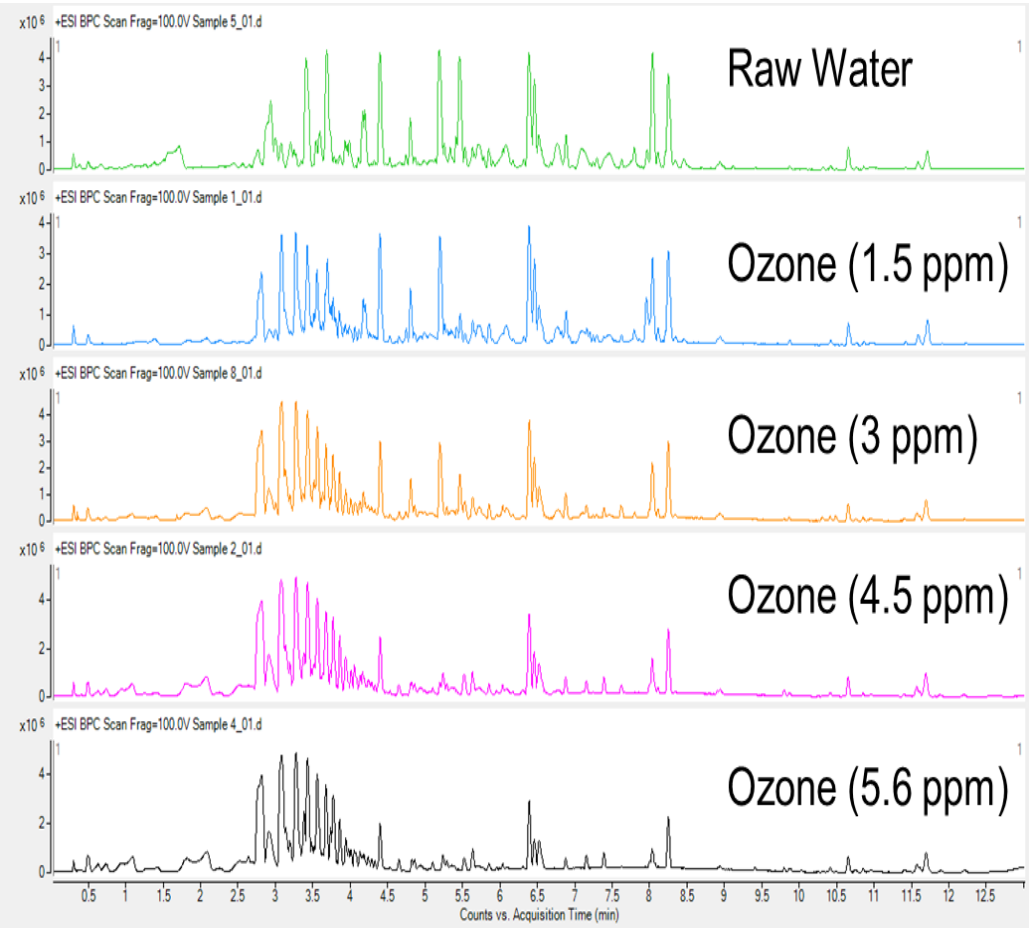
	DEX-EQ (ng/L)
GV Eff	1.1
UV	1.8
UV/H2O2	1.6
O3	2.3
O3/UV	2.0
Chlor	2.0



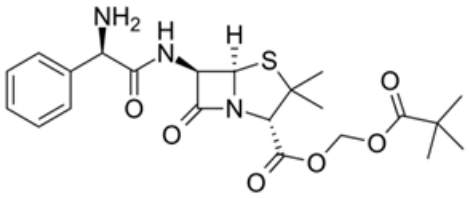
Chem-EQ << Bio-EQ

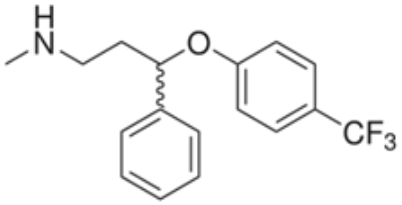
What if you don’t know the cause???

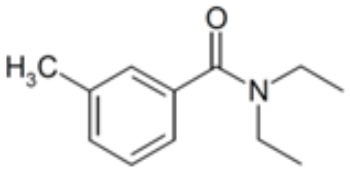
GC & LC QTOF for identification of unknowns



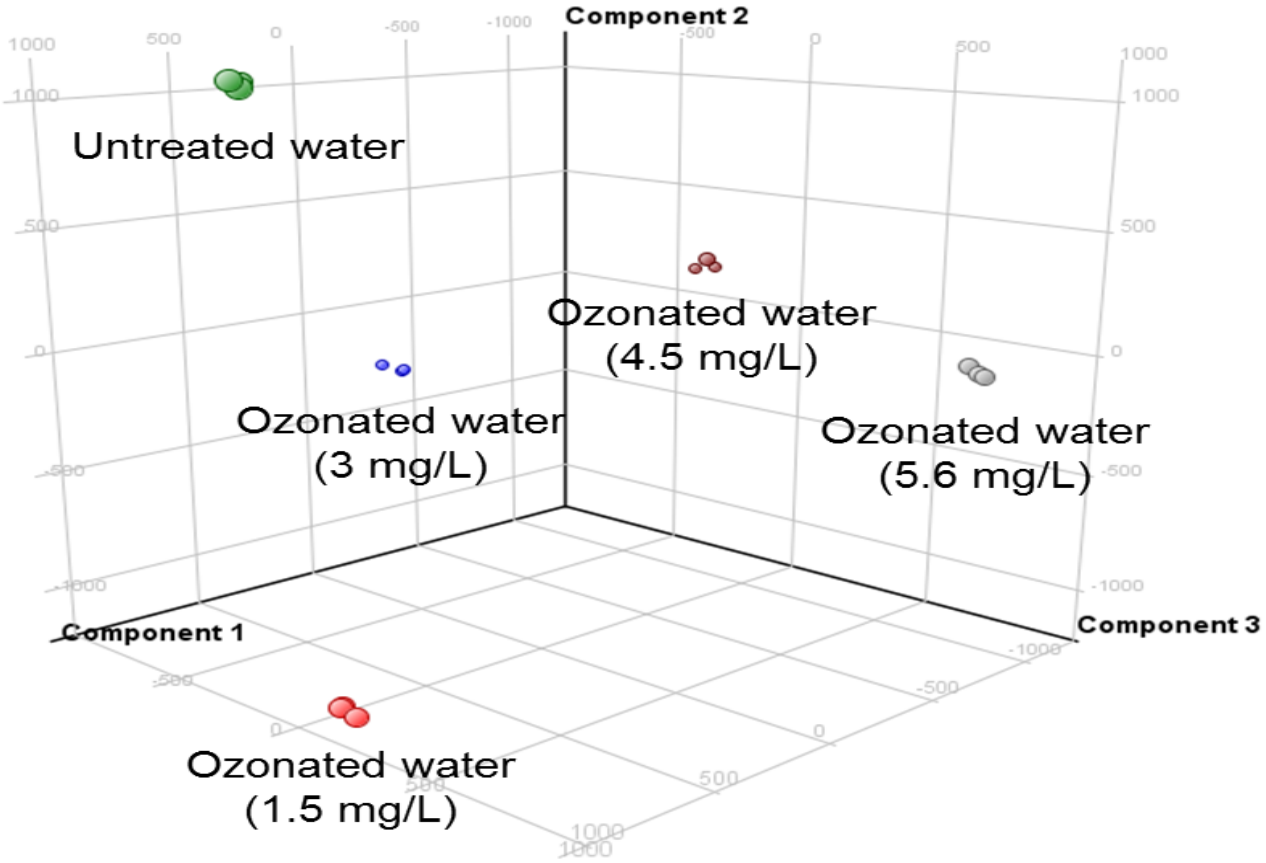
What if you don't know the cause???







PCA Plot for Different Ozone Doses

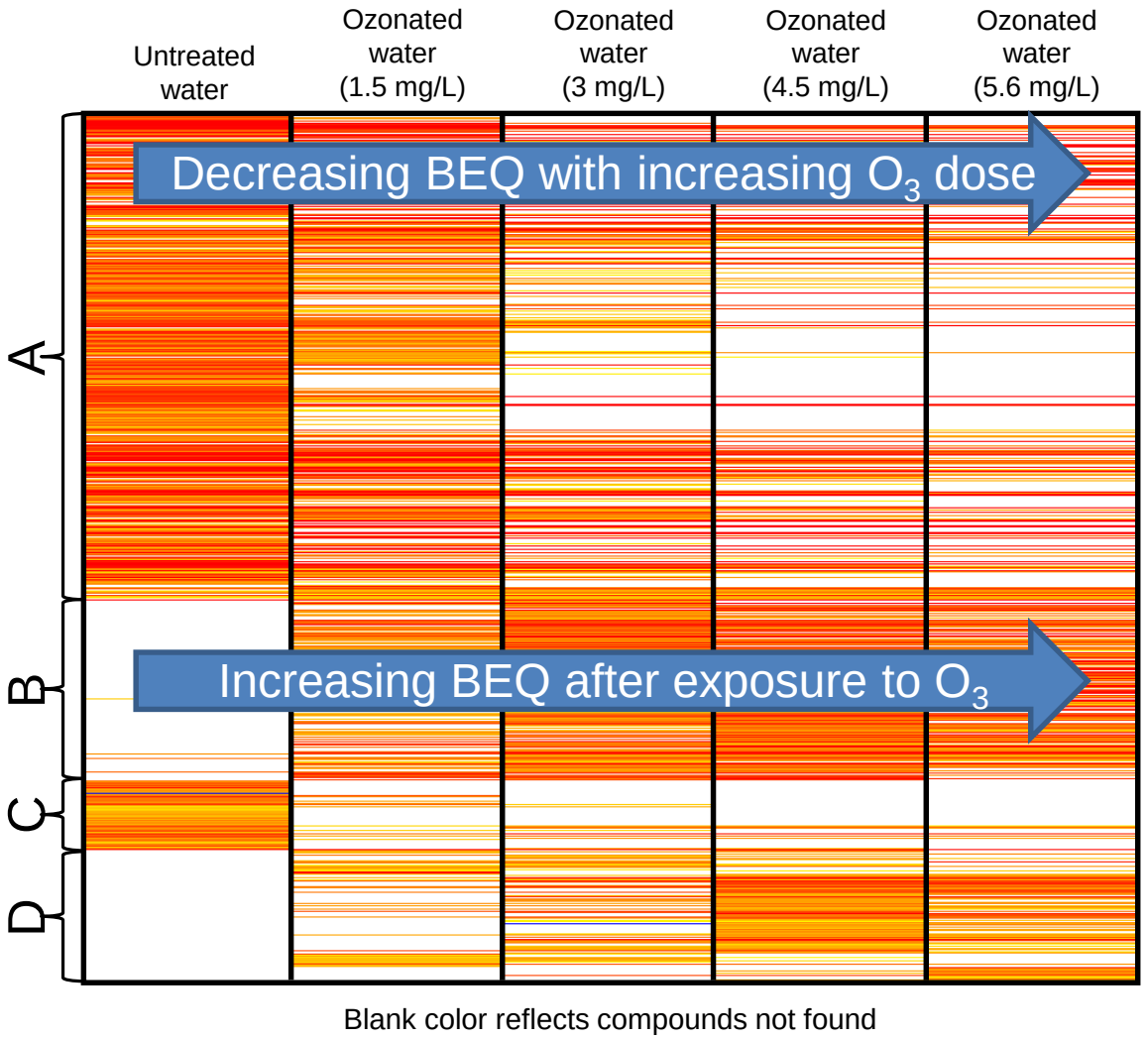


WATER TREATMENT & ANALYSIS OF UNKNOWNS

Although chromatograms were all similar for the analyst, clear differences appear on the heatmap

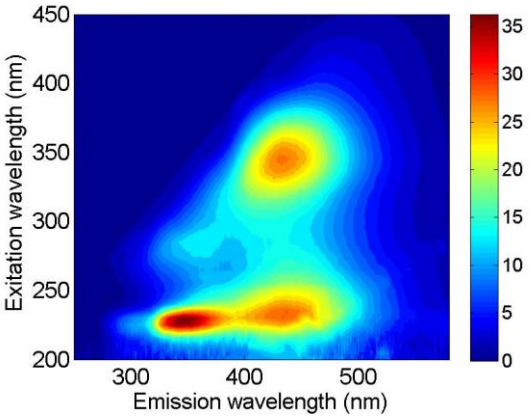
A & C are group of compounds in the raw water but at lower concentration or absent in ozonated water **(removed by ozone)**

B & D are compounds absent in raw water but present in treated water **(ozone by-products)**

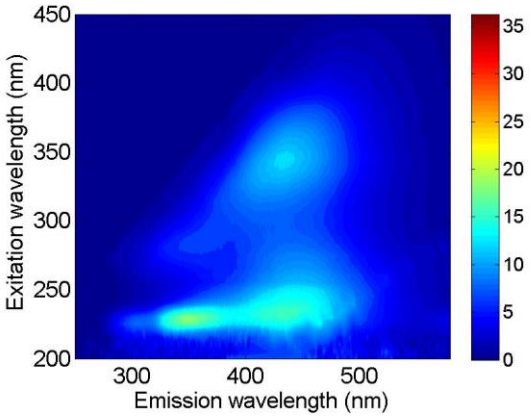


Application of Fluorescence indexes as surrogates for water quality

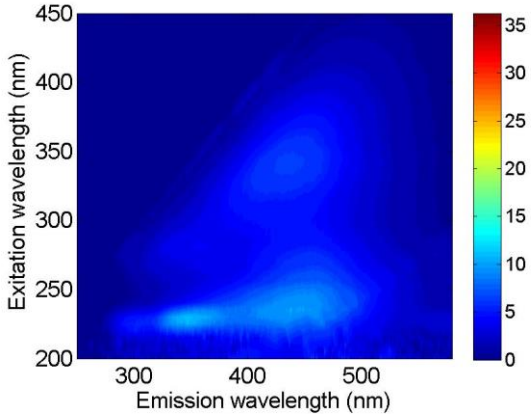
Wastewater Effluent on Ozone treatment



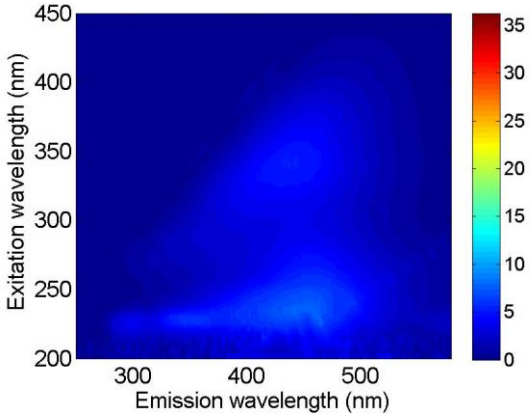
Control



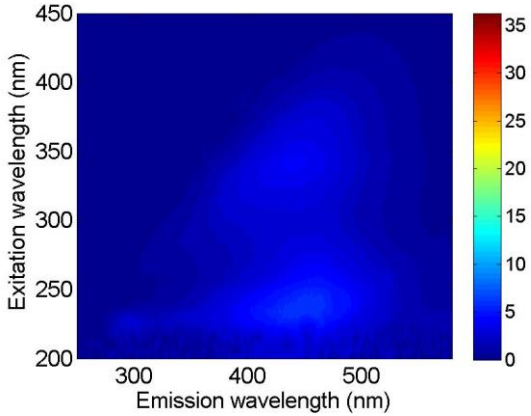
1.5 ppm



3 ppm

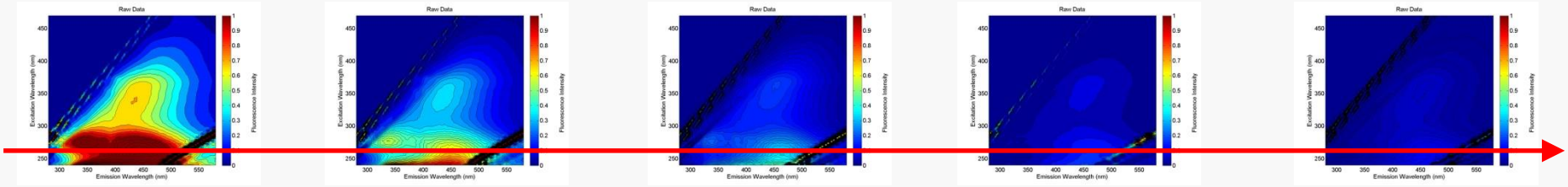


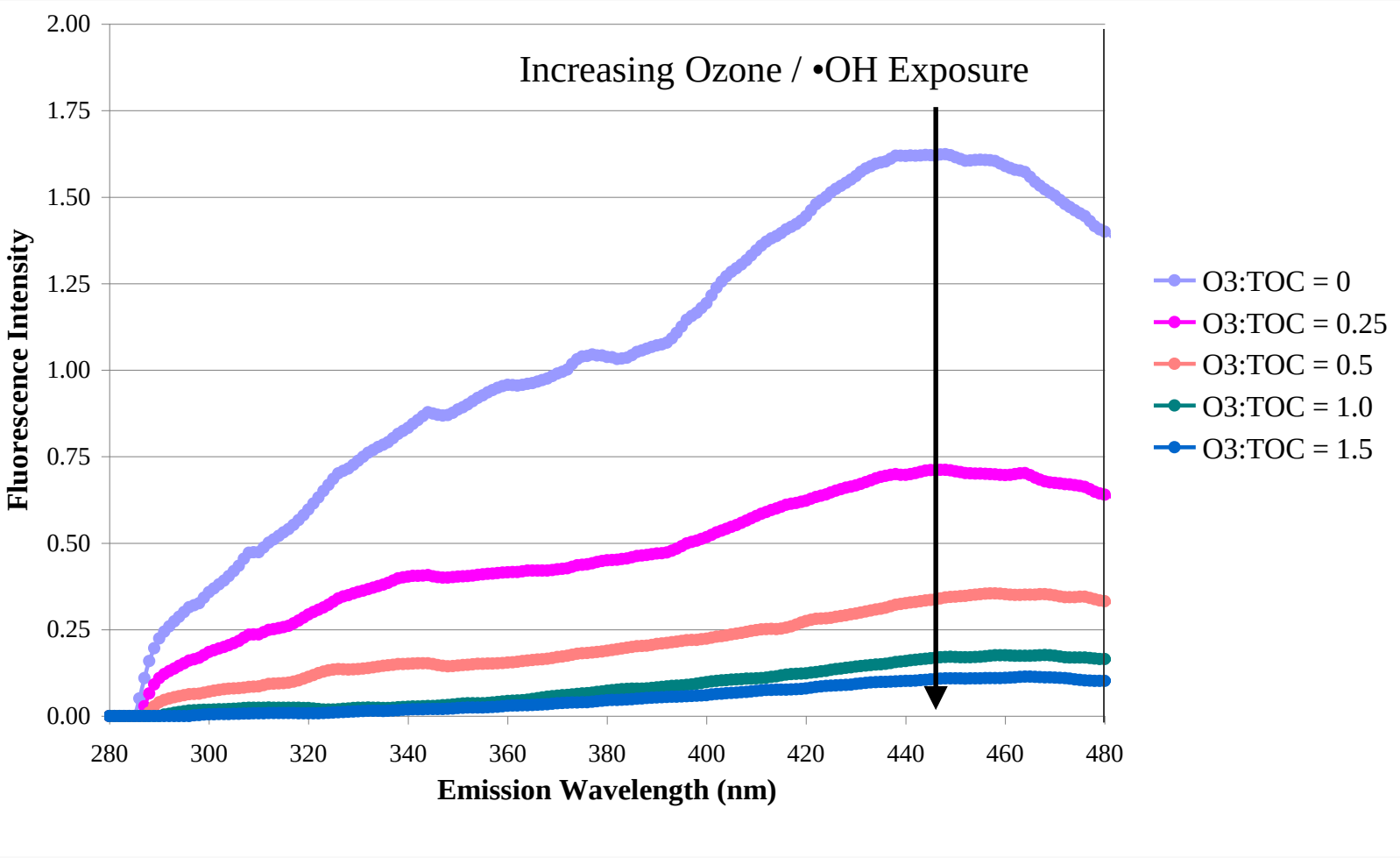
4.5 ppm



6 ppm

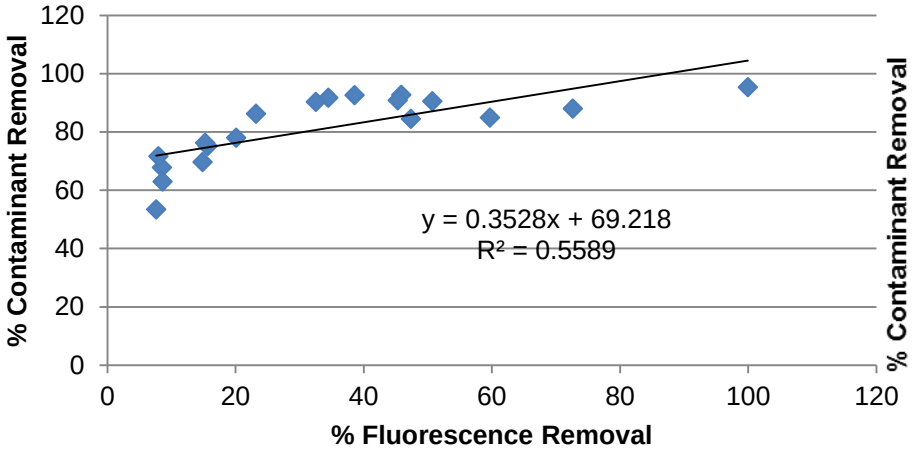
Fluorescence after O3 at 254 nm Excitation



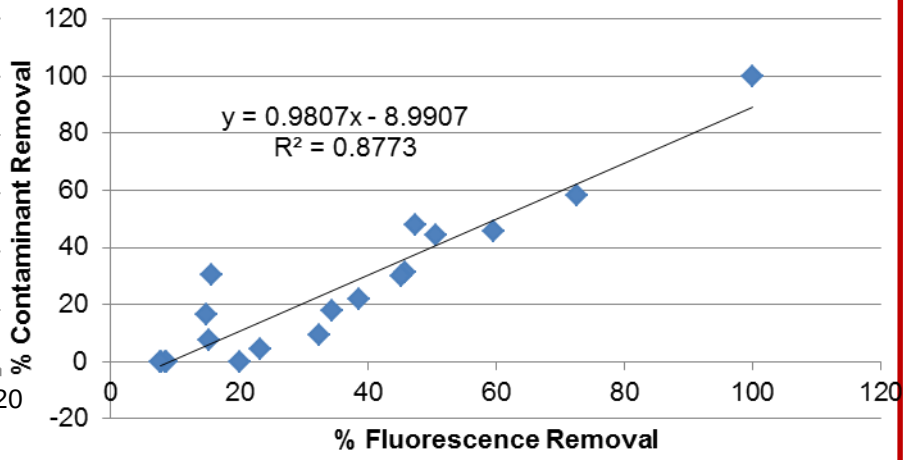


Fluorescence Excitation/Emission Pairs

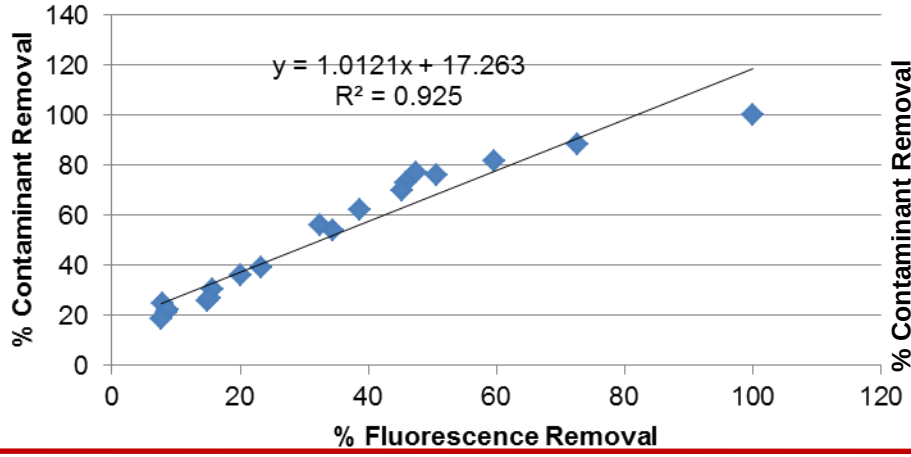
Group 1: Triclocarban



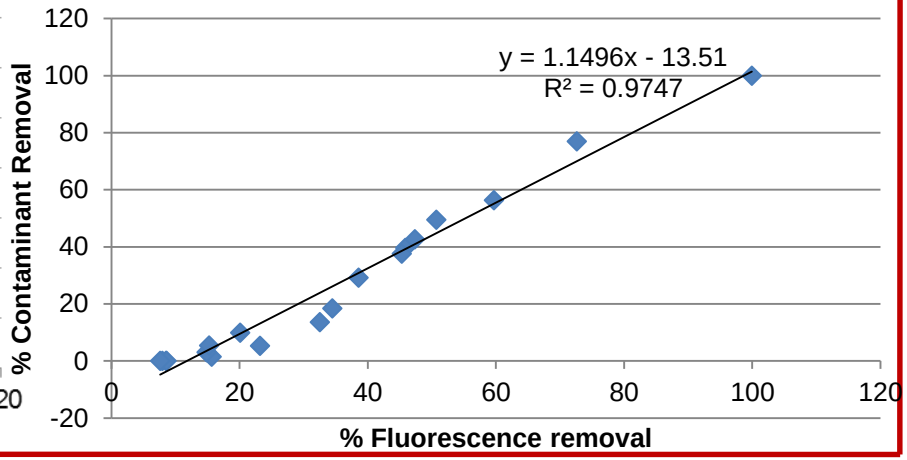
Group 2: PFOA



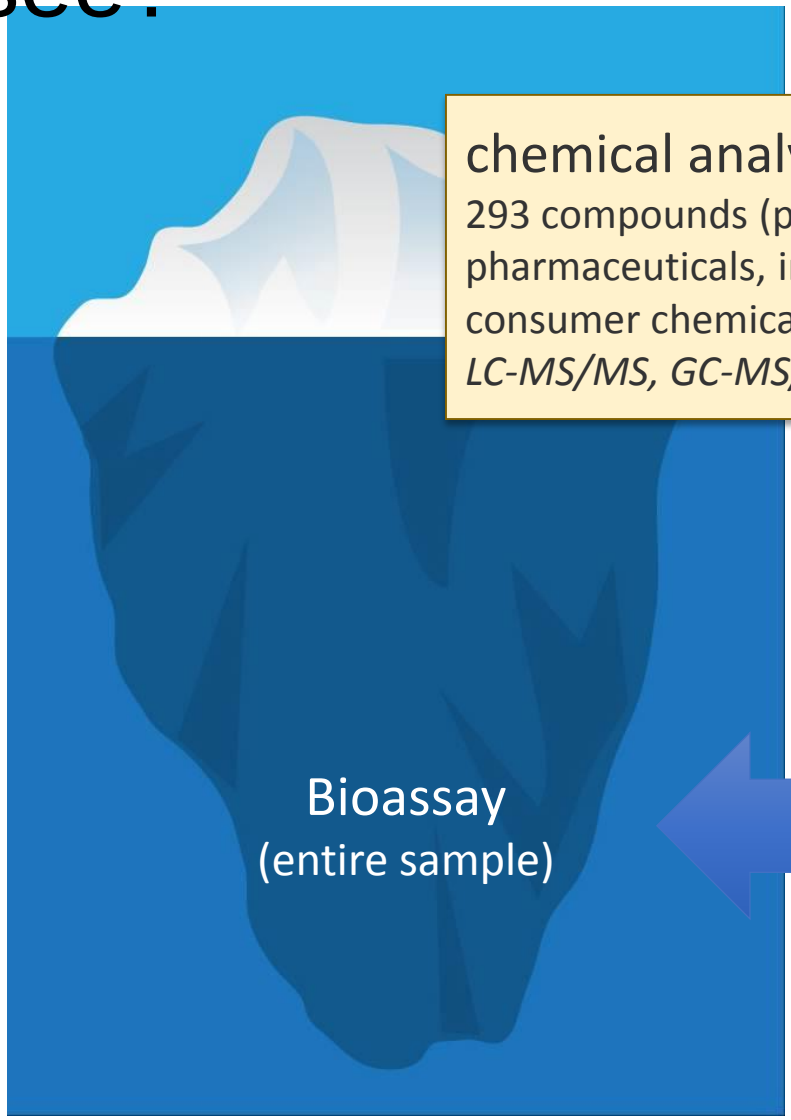
Group 3: Atenolol



Group 4: Primidone



Mixtures: how many micropollutants do we see?



chemical analysis
293 compounds (pesticides, pharmaceuticals, industrial and consumer chemicals)
LC-MS/MS, GC-MS/MS

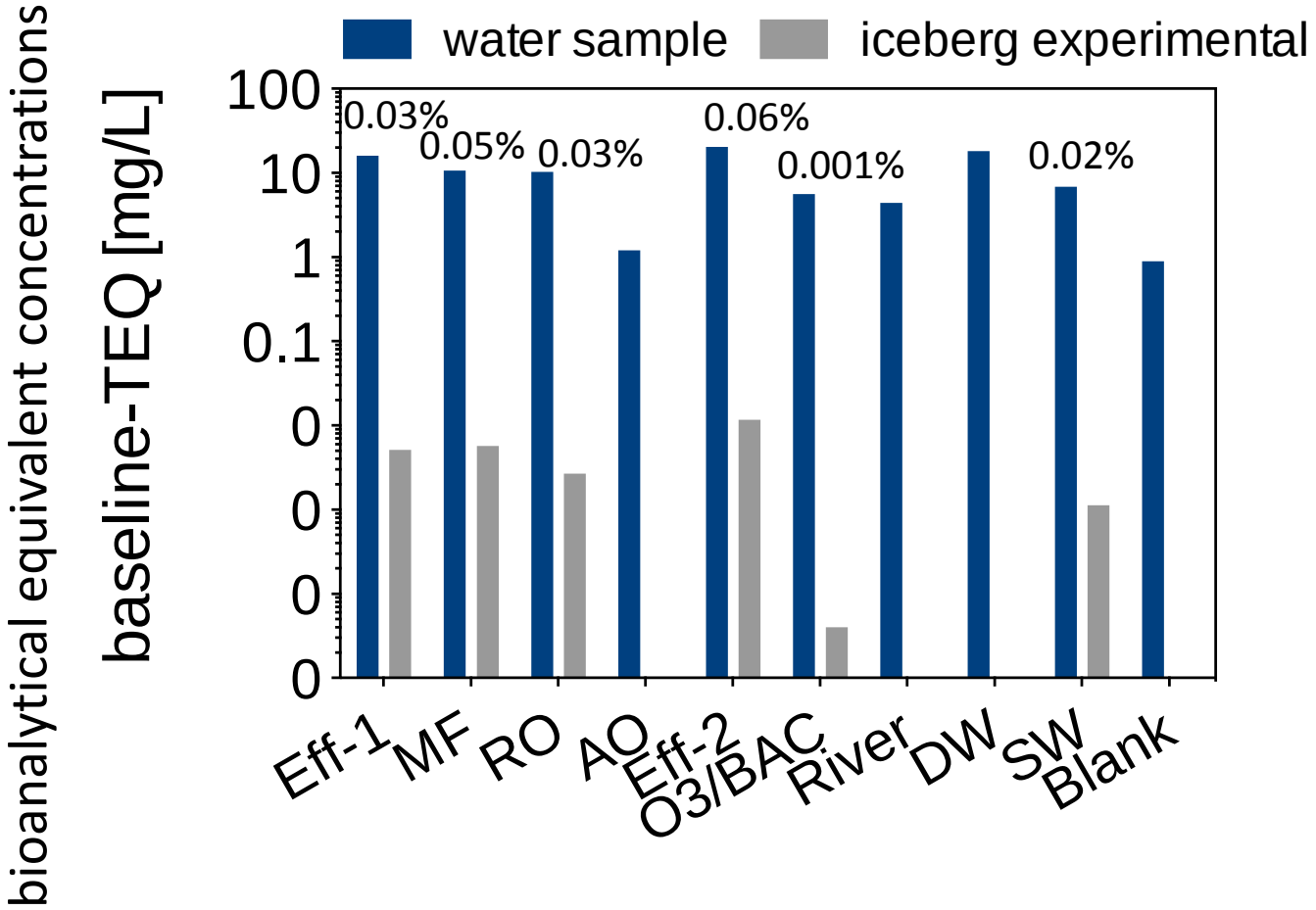
5 (SW) to 48 (Eff2) chemicals detected (<LOD in DW, blank)

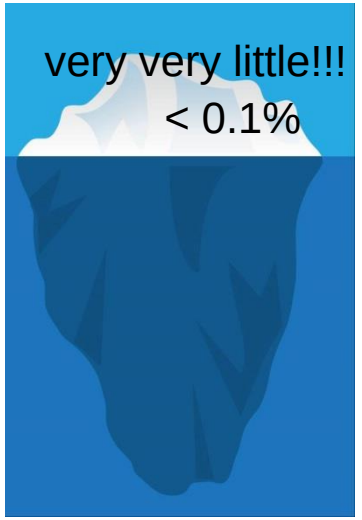
?

Bioassay
"tip of the iceberg"

Tang, J.Y.M., McCarty, S., Glenn, E., Neale, P.A., Warne, M.S., Escher, B.I. 2013. Water Res., 47: 3300-3314.

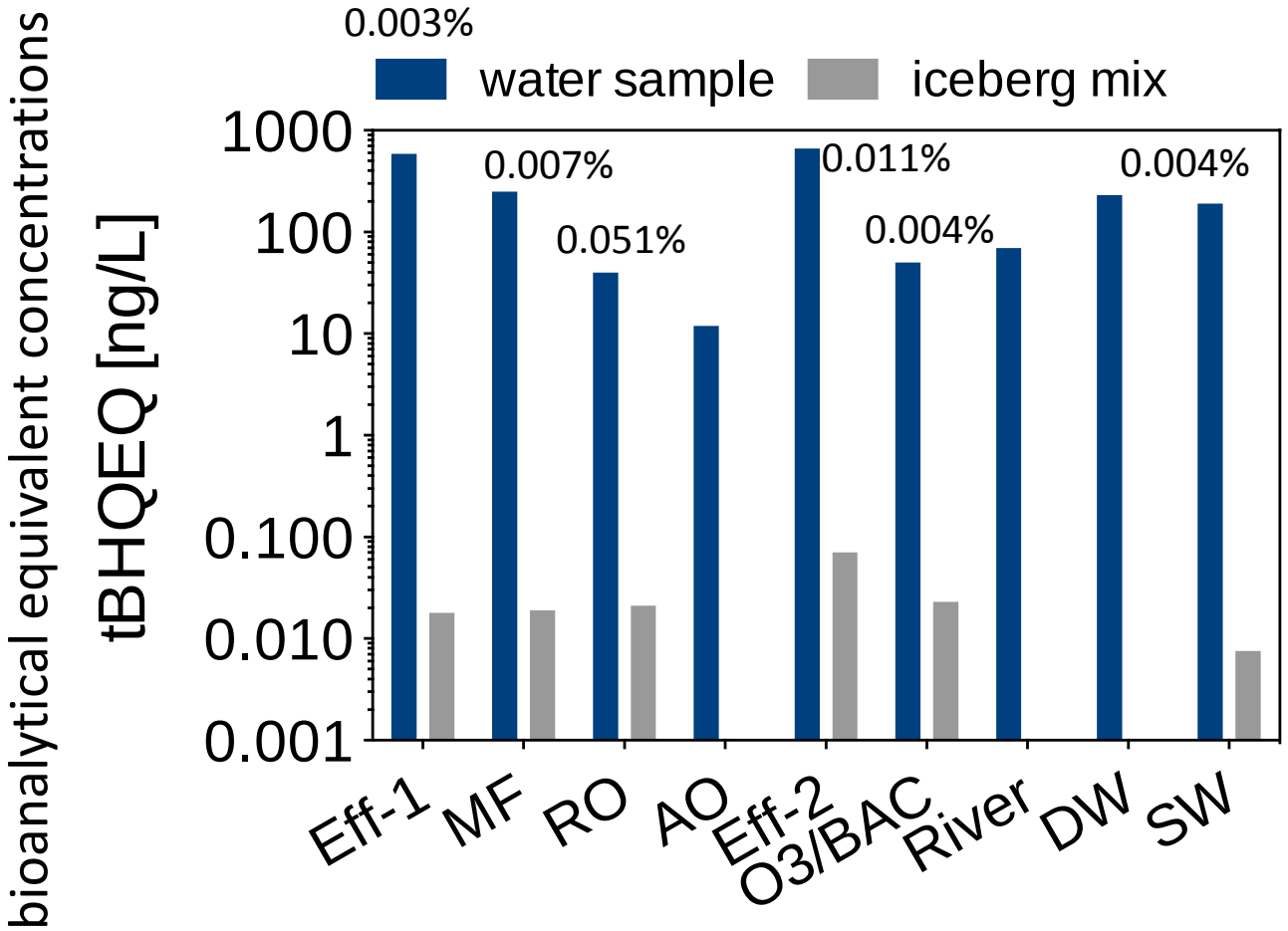
Which fraction of effect can be explained by known chemicals?
Example: Microtox





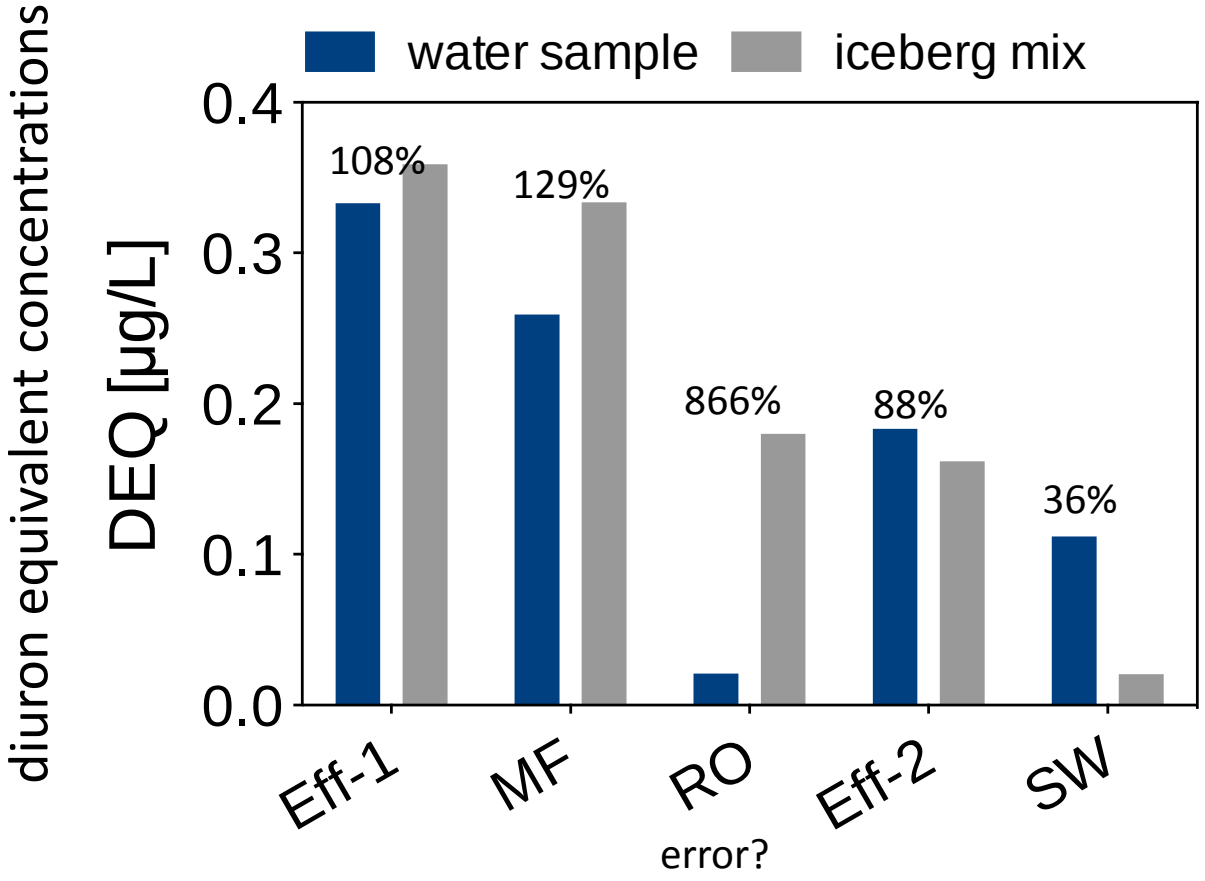
Tang, J.Y.M., McCarty, S., Glenn, E., Neale, P.A., Warne, M.S.,Escher, B.I. 2013. Water Res., 47: 3300-3314.

Which fraction of effect can be explained by known chemicals?
Example: oxidative stress response



Escher, B.I., van Daele, C., Dutt, M., Tang, J.Y.M. and Altenburger, R. (2013) Oxidative Stress Response Triggered By Pesticides, Pharmaceuticals And Their Mixtures Environmental Science & Technology, : 47(13): 7002-7011.

Which fraction of effect can be explained by known chemicals?
Example: photosynthesis inhibition



Tang, J.Y.M. and Escher, B.I. (2014). Realistic environmental mixtures of micropollutants in wastewater, recycled water and surface water: herbicides dominate the mixture toxicity towards algae. *Environmental Toxicology and Chemistry*: submitted 10 Oct 2013.

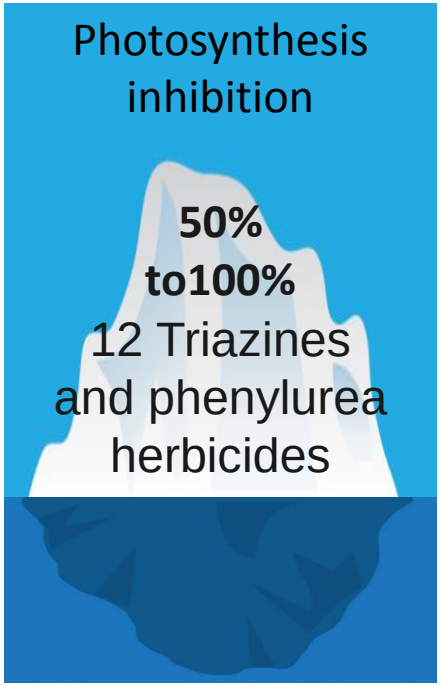
Which fraction of effect can be explained by known chemicals?

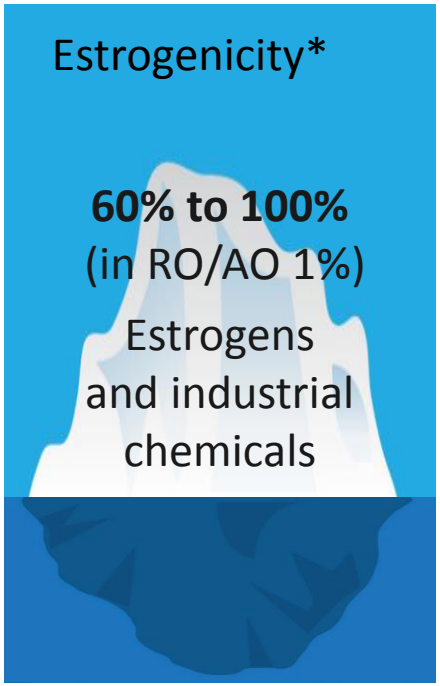
Cellular toxicity pathway

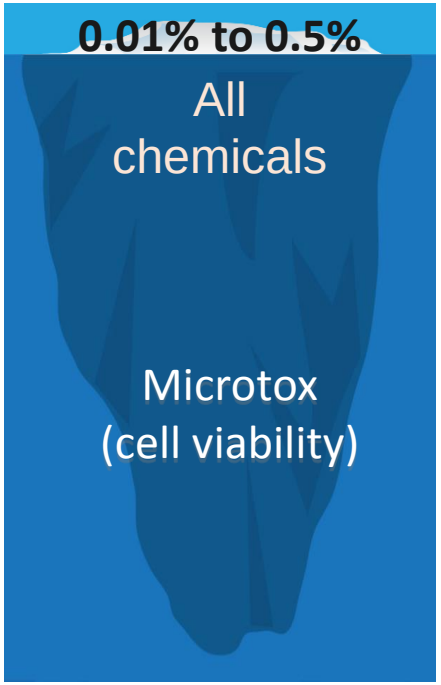
Metabolism → Interaction with target → Defense → Cell death

Receptor- mediated effects

Stress response and cytotoxicity







Escher, B.I., Lawrence, M., Macova, M., Mueller, J.F., Poussade, Y., Robillot, C., Roux, A.,Gernjak, W. 2011. Environ. Sci. Technol., 45: 5387-5394. Tang, J.Y.M., McCarty, S., Glenn, E., Neale, P.A., Warne, M.S.,Escher, B.I. 2013. Water Res., 47: 3300-3314.

Standardization of Bioassay Protocols

ALVINA MEHINTO

SCCWRP

Development of SOP

1. Background (cell lines, mode of action)
2. List of laboratory equipment, consumables, cell kit
3. Assay protocol
4. Data reporting (e.g. standard data entry spreadsheet)
5. Appendices (e.g. plate layout, preparation of dilutions)
6. Expected results
7. Troubleshooting

Laboratory Set-up

Molecular laboratory (centrifuges, microscopes, multichannel pipets...)

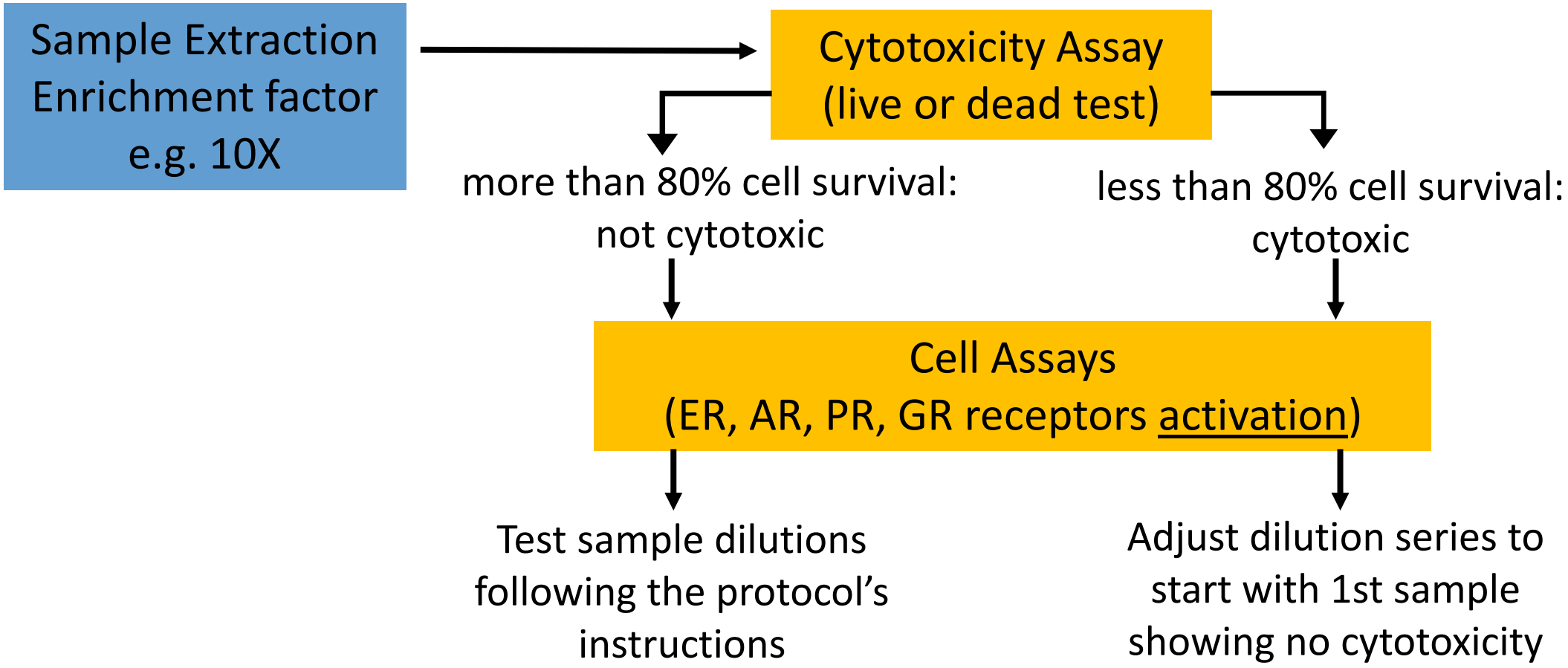
Specific equipment for bioassay:

- Biological safety cabinet class II
- Humidified cell culture incubator to maintain cells
- Cryogenic freezer
- Fluorescence plate reader, bottom read capabilities

Assay consumables

- Cell assay kit, assay media
- Cell culture plates
- Reference ompound

Bio-screening Workflow



Standardized Approach for CA Project

- Division arrested cells for ERa, AR, PR and GR
- Vehicle control: 0.5% DMSO
- Cell density: 50,000 cells/well (ERa, GR), 40,000 cells/well (AR, PR)
- Reference compounds: 9 concentrations for dose response curve
- Sample extracts: 4 dilutions in triplicate
- Set of QA/QC

Bioassay Preparation

Solutions:

- Assay media (different assay media may be required for different cell assays)
- Stock solutions for reference chemicals
- Working dilutions for reference chemicals and sample extracts

Cells:

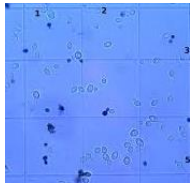
- Provided frozen, division-arrested
- Revived in assay media and plated the same day

Cell viability and count:

- Stain and count number of cells in known volume
- Dilute cell suspension to required cell density for the assay

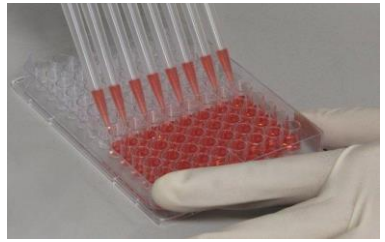
Cell Assay Protocol

Day 1



Cell count

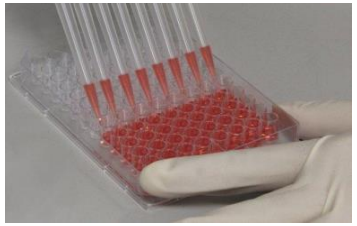
Plate cells @ specific density



Add diluted extracts

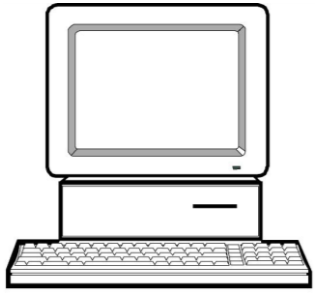
Overnight incubation (~16 hrs) at 37°C, 5% CO_2

Day 2



Add substrate

Incubation (~2 hrs) at room temperature



Measure fluorescence

QA/QC

Control for contribution of artifacts (blanks)

- Cell-free control – determine plate background
- Vehicle-free control – determine background of unstimulated cells
- Vehicle (e.g. DMSO) control – determine background caused by vehicle control
- Blank extract – chemical extraction blank sample
  - X3 replicates on EACH assay plate

➢ Control should not exceed e.g. 10% of EC_{10}

QA/QC – cont.

Calibrate assay response with reference compound

- Dose response curve with potent agonist (e.g. 17β-estradiol for ERα) to determine Bio-EQ
  - 9 dilutions X3 on first assay plate
  - 5 dilutions X2 on subsequent plates

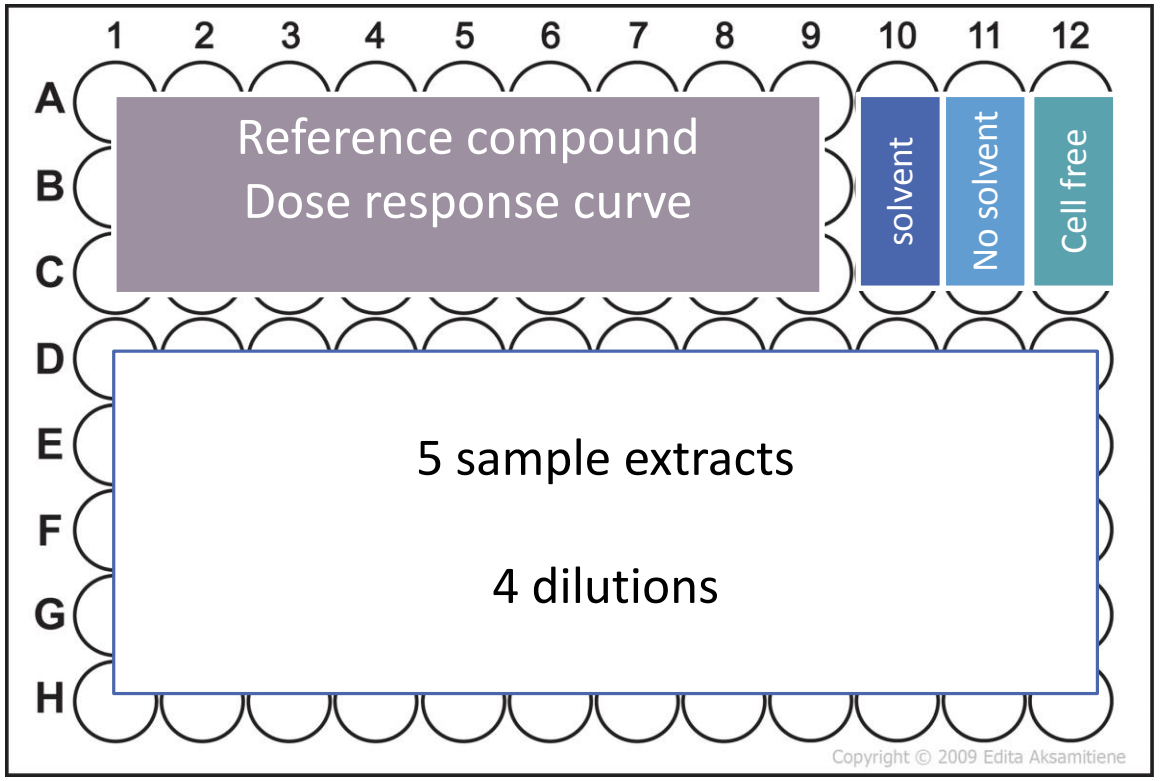
➢ EC50 should agree with historical/specified value, e.g. to within 30%

Validate assay response

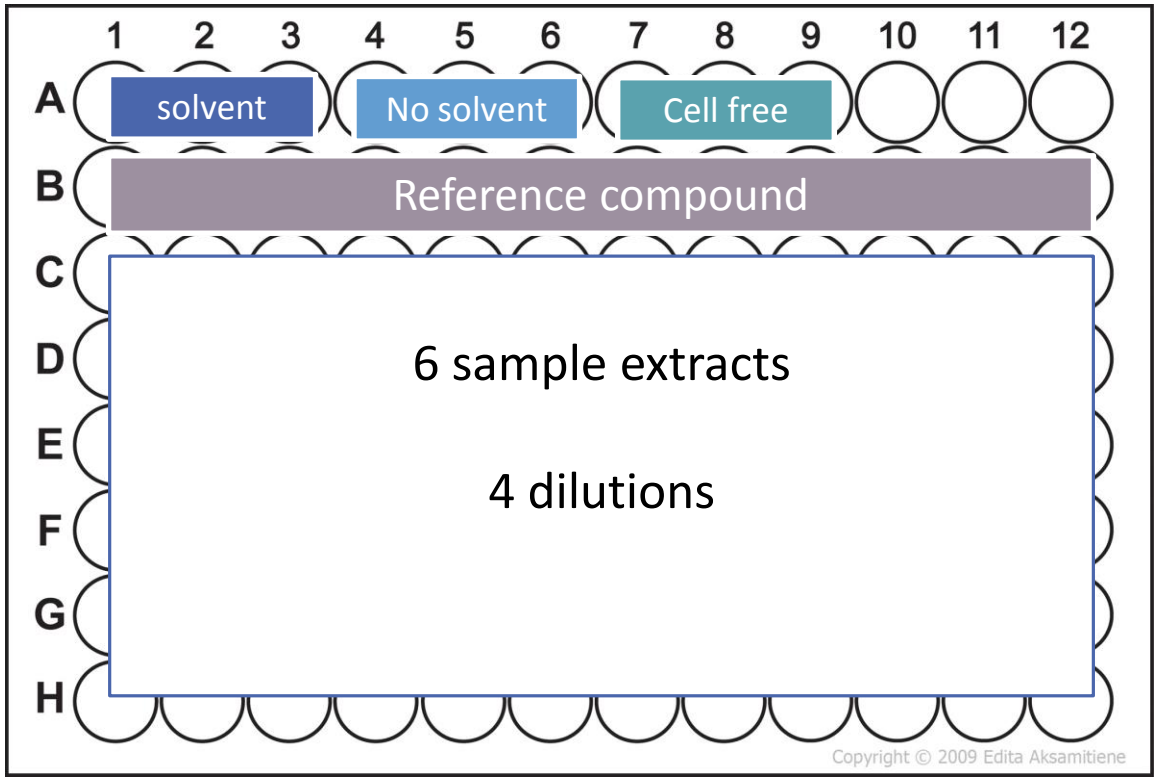
- Include spiked sample

➢ Response should be within the expected range of positive assay response

Cell Assay Protocol (96-well plate format)



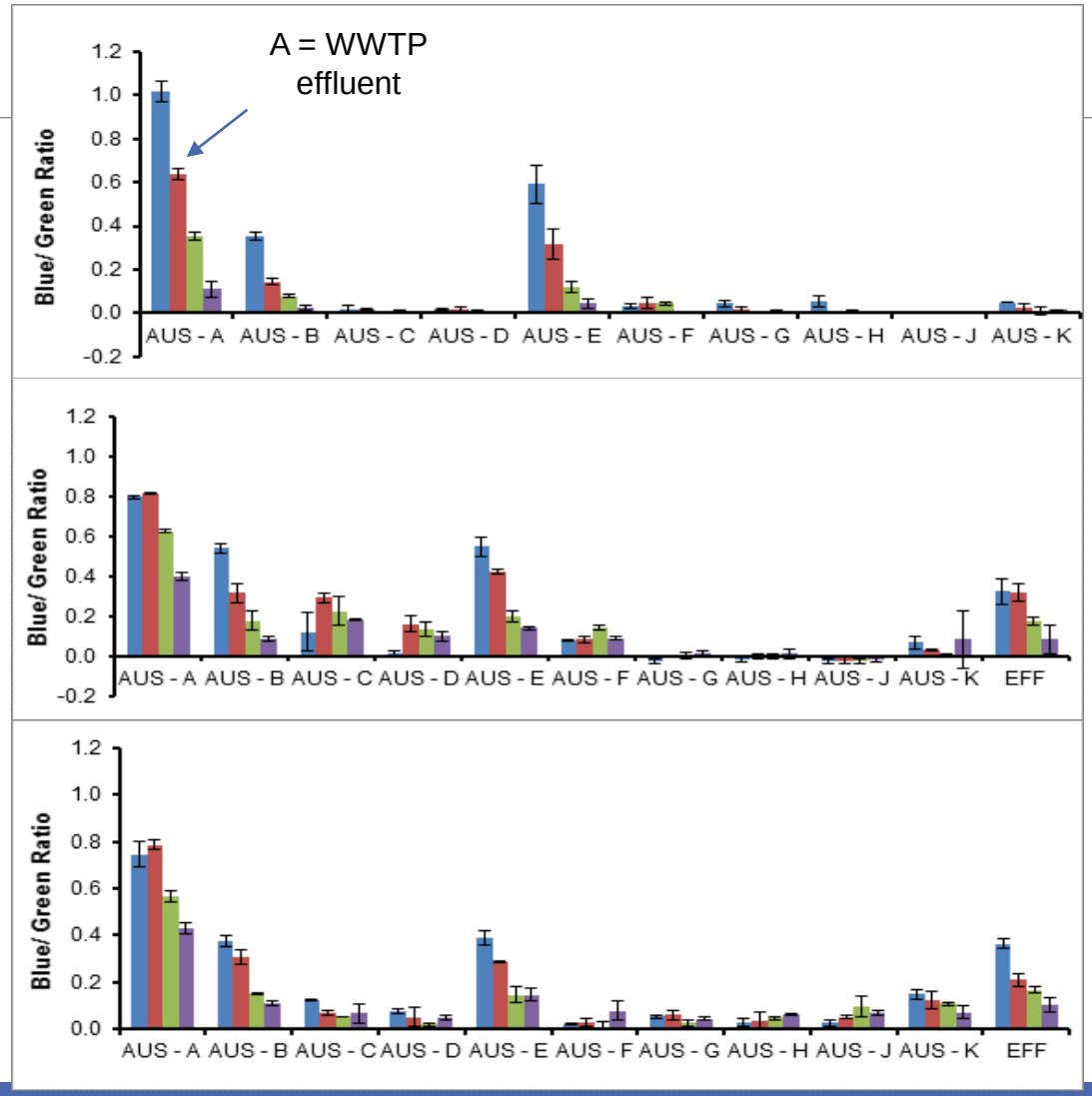
Standard plate



Additional plates

WRF Results

- Example with ERa cell assay
- Good agreement between CA team participating laboratories



Future Goals

Time/Cost Improvements:

- Customized kit with specific cell density, number of aliquots per plate, etc.
- Scale up to higher density plates to run samples more cost effectively
- Automation of protocol
- Multiplex endpoints for a given cell line

DATA INTERPRETATION & GUIDANCE

- **Translate bioassay results into quantifiable threshold**
  - total equivalent concentrations or quotients (TEQs)
- **Investigate relationship to priority CEC concentrations & health based trigger levels**
  - compile reference doses or “TTCs” for known/measured CECs
- **Develop tiered framework that best utilizes bioassay results**
  - first tier screening tool
  - bioassay threshold exceedances that trigger appropriate response
- **Conduct workshop for stakeholders**
  - appropriate role, implementation and use of bioassay results

DATA ANALYSIS

- **Step 1. Confirm bioassay results are valid (QA/QC checks)**

  Calculate EC_x (reference chemical) and compare to historical values

  If within specification, go to next step. If outside, take corrective action

  Assess blank contribution

  If within specification, go to next step. If outside, take corrective action

- **Step 2. Determine behavior of sample results**
  - Test for difference in fold response among sample dilution series
  - If dose-response exists, calculate EC_{10} and/or EC_{50}
  - If no dose-response, compare mean to blank
    - If no difference, report as "ND" (e.g. max REF * 2)

- **Step 3. Compute bioassay equivalents (BEQs)**
  - represent in units of ng/L based on reference chemical

    BEQ = EC_x (reference chemical) / EC_x (sample)

MONITORING THRESHOLDS

- **Step 1. Consult with regulators to identify current guidelines**
  - Fed, state MCLs for target analytes or analogs thereof
  - State, regional investigative benchmarks (e.g. notification levels)
  - International published thresholds based on human health effects

- **Step 2. Assess linkage of bioassay and higher order effects**
  - Compile relative potency factors (PFs) as $EC_{x,\text{in vitro}}$ / $EC_{x,\text{ in vivo}}$
  - Rank or weight PFs based on relevance/rigor of study
    - (epi > individual > organ > molecular)

- **Step 3. Apply margin of safety based on monitoring goals and uncertainty**
  - Action Level (AL) = PNEC or NOEC / (PF * SF)

DECISION MAKING

- **Step 1. Compare bioassay result to action level**

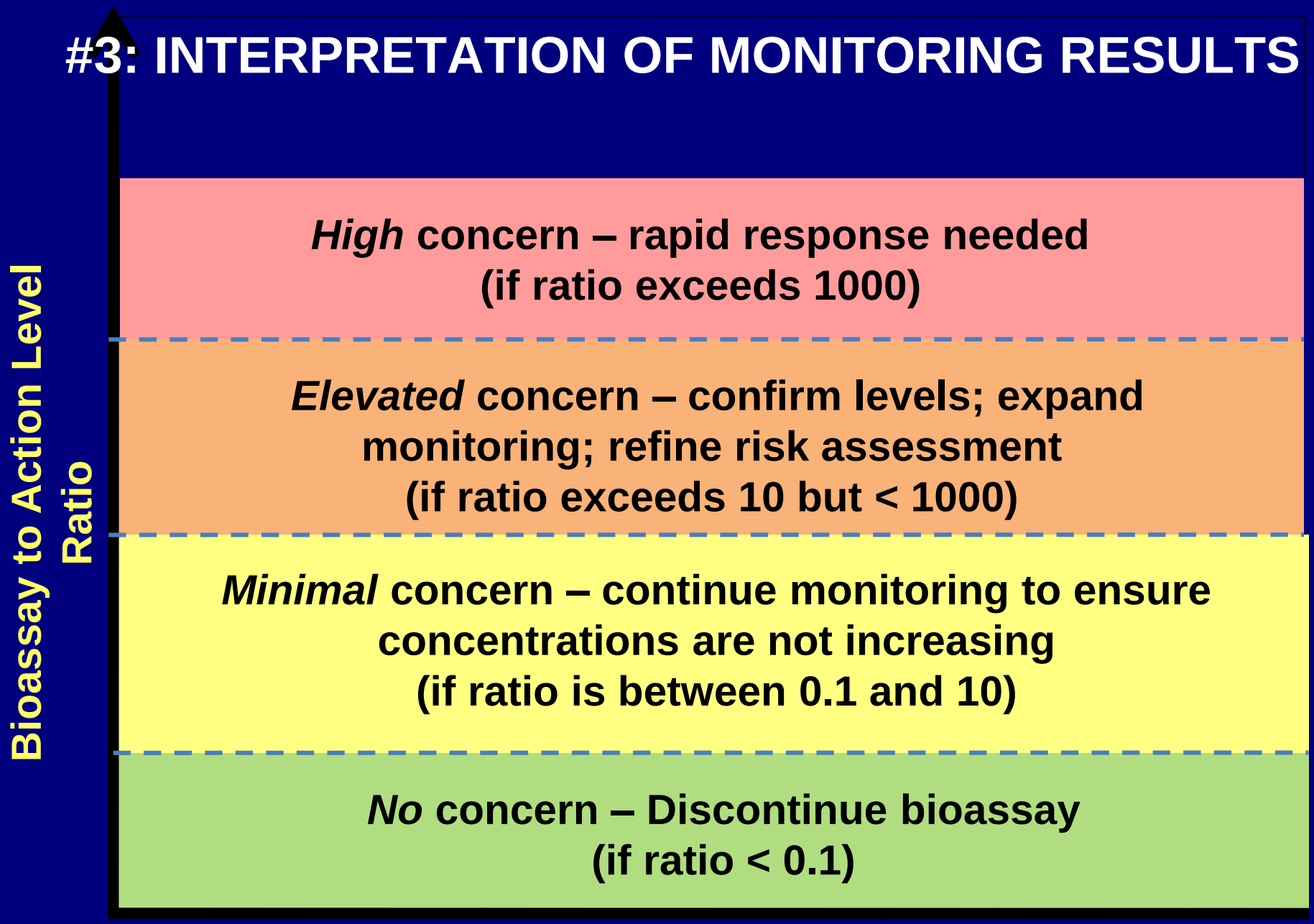
  If BEQ > AL, GO TO STEP 2

  If BEQ < AL, continue with baseline monitoring and GO TO STEP 3

- **Step 2. Define actions commensurate with exceedance**
  - Confirm a single exceedance within specified period of time (e.g. 72h)
  - If confirmed, initiate targeted chemical analysis “directed by bioassay”
  - Increase frequency of monitoring to see if exceedance persists
  - Notify regulatory agency and discuss/implement rigorous solutions

- **Step 3. Review monitoring data on a regular schedule**
  - Off ramp for bioassays that consistent exhibit “safe” response
  - Status quo monitoring for bioassays that show minimal/moderate response
  - Take action to reduce residuals causing consistent bioassay responses at higher levels of concern

#3: INTERPRETATION OF MONITORING RESULTS



Bioassay Wish List

Dan Schlenk
University of California, Riverside

Primary Uses of Bioassays

- Rapid and robust biological response that can be linked through MOA to a higher order adverse outcome
  - BEQ---TEQ----RfD
- Use a biological response that identifies exposure to mixtures of known and unknown stressors.
  - Focus chemical testing;
  - Screening/Tiered process

TOXICOLOGICAL SCIENCES 131(1), 40–55 (2013)
doi:10.1093/toxsci/kfs285
Advance Access publication September 28, 2012

In Vitro Perturbations of Targets in Cancer Hallmark Processes Predict Rodent Chemical Carcinogenesis

Nicole C. Kleinstreuer,* David J. Dix,* Keith A. Houck,* Robert J. Kavlock,* Thomas B. Knudsen,* Matthew T. Martin,* Katie B. Paul,† David M. Reif,* Kevin M. Crofton,† Kerry Hamilton,‡ Ronald Hunter,‡ Imran Shah,* and Richard S. Judson*,[1]

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[1]To whom correspondence should be addressed at National Center for Computational Toxicology, Office of Research and Development, U.S. EPA, 109 T.W. Alexander Drive (B205-01), Research Triangle Park, NC 27711. Fax: 919-541-1194. E-mail:judson.richard@epa.gov.

TOXICOLOGICAL SCIENCES 135(2), 277–291 2013
doi:10.1093/toxsci/kft164
Advance Access publication July 27, 2013

EADB: An Estrogenic Activity Database for Assessing Potential Endocrine Activity

Jie Shen,* Lei Xu,† Hong Fang,‡ Ann M. Richard,§ Jeffrey D. Bray,¶ Richard S. Judson,§ Guangxu Zhou,*
Thomas J. Colatsky,‖ Jason L. Aungst,‖‖ Christina Teng,‖‖‖ Steve C. Harris,* Weigong Ge,* Susie Y. Dai,# Zhenqiang Su,*
Abigail C. Jacobs,** Wafa Harrouk,†† Roger Perkins,* Weida Tong,* and Huixiao Hong*,[1]

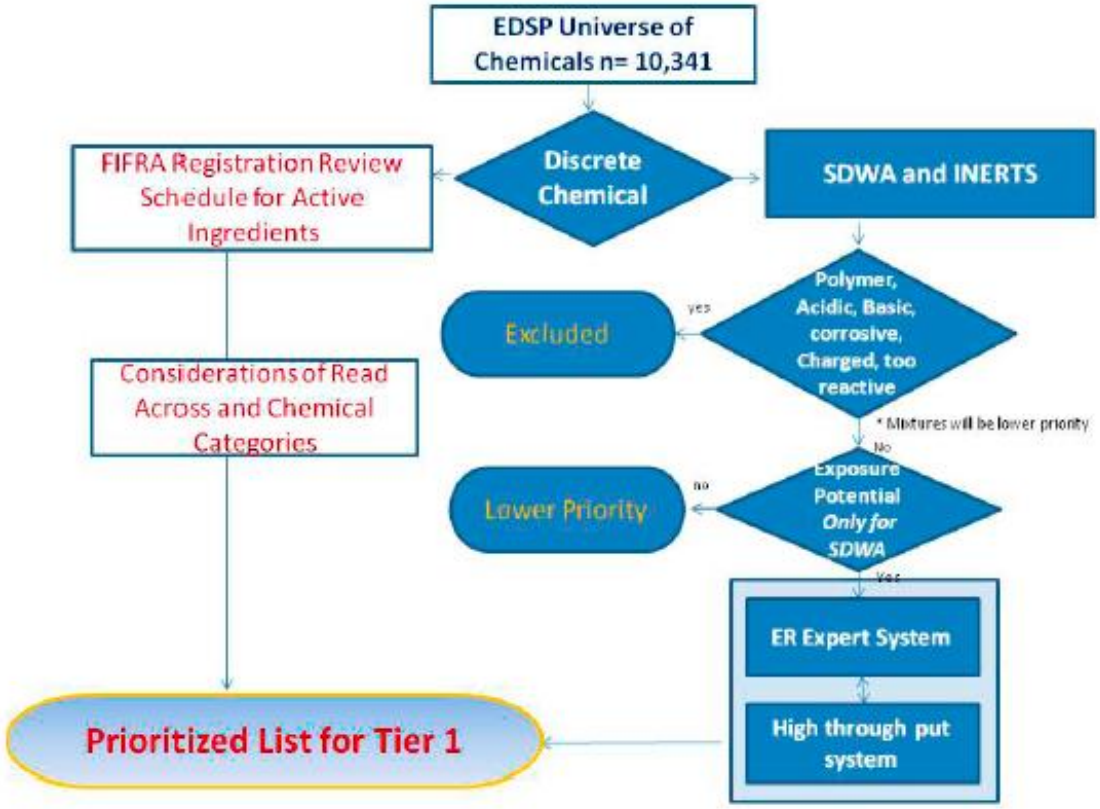


Figure 2.2.1: Proposed EDSP21 Prioritization Scheme

USEPA 2013

EATS Priority?

- Androgens
  - No Tier 1 transactivation assay for AR?
  - Anti-androgens > Androgens
    - Anti-Estrogens?
- Thyroid
  - Limited success with transactivation assays
  - Affinity/Sensitivity?
  - Thyroxine levels in vivo (mammals)
- Steroidogenesis
  - H295R
    - Translates well to steroid hormone concentrations/reproduction

Why EATS?

- EDSP targets
  - Large database and QSAR development Toxcast
- Vetted Protocols/Methods available
  - QA/QC
- Linkages to Adverse Outcomes better quantified

Glucocorticoid and Progesterone

- GR
  - Linkage to Immune/cardiovascular functions, developmental cellular proliferation
    - TEQ?
  - High sensitivity and robust assay that allows TIE analyses (WRF report)
- PR
  - Linkage to Reproductive and Neuroendocrine responses
    - TEQ
  - Environmental interest

Dioxin case study – Key Events Dose Response Framework

Application of National Research Council "Silverbook" Methodology for Dose Response Assessment of 2,3,7,8-Tetrachlorodibenzo(*p*)dioxin.

Authors: Simon T., Stephens M., Yang Y., Manning R.O., Budinsky R.A. and Rowlands J.C.

TEQ RfD for AhR Dysregulation = 30 pg/kg/d

Multiplex?

Nuclear Receptors/transcription factors		Level of activity
AhR	Aryl Hydrocarbon receptor	++++
AP1	Activator protein 1	+
AR	Androgen receptor	+
CAR	Constitutive androstane receptor	+++
ERa	Estrogen receptor alpha	++++
ERb	Estrogen receptor beta	+++
ERRg	Estrogen receptor related gamma	++
FXR	Farnesoid X Receptr	+
GR	Glucocorticoid receptor	++
HNF4a	Hepatocyte Nuclear factor 4 alpha	+
LXR	Liver X receptor	+
NRF2	Nuclear factor erythroid 2-related factor 2	+++

Nuclear Receptors/transcription factors		Level of activity
PPARa	Peroxisome proliferator-activated receptor	++++
PPARd1	Peroxisome proliferator-activated receptor	+
PPARg	Peroxisome proliferator-activated receptor	++
PXR	Pregnane-X-receptor	+
RARa	Retinoic Acid receptor, alpha	++++++
RARb	Retinoic Acid receptor, beta	+++++
RARg	Retinoic Acid receptor, gamma	++++++
RORb	Retinoid related orphan receptor beta	++++
RXRa	Retinoic-X receptor, alpha	+
RXRb	Retinoic-X receptor, beta	+++
VDR	Vitamin D receptor	+

Genotoxicity

- Chemicals of Concern
  - Cr^{VI}, 1,4 Dioxane, NDMA, DBPs (trihalos)
- Ames & uMu (SOS)
  - lack of sensitivity?
    - Exposure of known compounds (NDMA, BaP)
- P53 activities?
  - Adequate D/R
  - Chicken/egg?
  - TEQ?
- TIE?

Wish List Summary

- EDSP/Toxcast
  - ER redundancy
  - Anti-E; Anti-A
  - Thyroid?
  - Steroidogenesis
- Other NR
  - AhR
    - Life Tech Development
  - GR---TIE already performed
  - PR
- Genotoxicity Assays
  - P53

Promising endpoints in the development phase … and promising developments

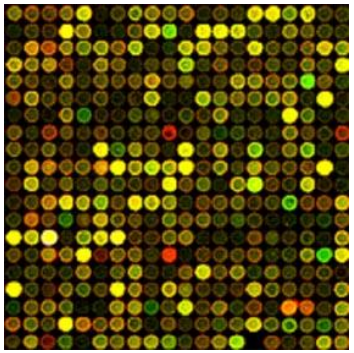
Frederic Leusch

Promising endpoints

- Based on interlab comparison:
  - Pregnane X receptor (PXR)
  - Oxidative stress (ARE-mediated)
- Based on known limitations of *in vitro* methods:
  - High throughput mammalian genotox assay
  - Non-genotoxic carcinogenicity
  - Neurotoxicity
  - Immunotoxicity
  - Developmental
  - Reproductive

Genomic methods
(*e.g.*, RT-PCR, gene arrays)

- Very versatile
- Can help discover new pathways relevant to contaminants in water
- But …
  - Limited throughput
  - Expensive



Metabolic activation

- Metabolic activation is important for:
  - Reactive toxicity
  - Thyroid active compounds (Murk et al)
  - Oxidative stress? Others?
- But often not incorporated in testing strategy
  - Cost: doubles number of analyses required
- Currently available:
  - Rat liver microsomes (S9 fraction)
  - Recombinant human CYP (Corning Supersome)

Moving towards true animal replacement

- Fetal Bovine Serum (FBS):
  - Contains hormones, growth factors, protease inhibitors, proteins, vitamins, amino acids, trace elements, lipids, attachment factors …
  - Significant source of variability, high ethical cost
- Development of serum free media
  - Would have big QAQC and ethical benefits
  - Any TK implications?

van der Valk et al 2010. Toxicol In Vitro 24: 1053-1063

A change in climate …

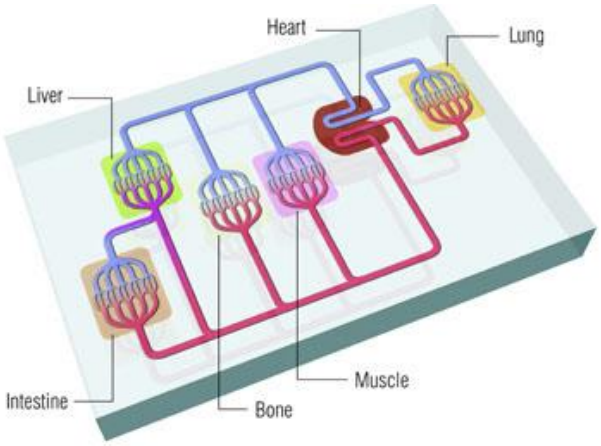
- Growing list of validated *in vitro* methods
  - Driven by ICCVAM and ECVAM (via OECD TG)
- Rapid increase in capacity
  - More than 100 commercial labs can conduct *in vitro* testing (most for drug discovery, but also for env samples)
  - http://www.alttox.org/ttrc/resources/in-vitro-testing.html
- Several projects (*e.g.*, DEMEAU) and publications devoted to development of bioassay guidelines

High throughput screening

- Many assays being adapted to 384-well format
- Electronic pipettes and pipetting robots are more widely available (and cheaper)

A little farther on the horizon …

- Implications of today's discoveries
  - Tox21: discovery of biological pathways induced by exposure to environmental pollutants
- Animal on a chip
  - Microfluidics to replicate organ systems
- 3D tissue and organ printing



Reif et al 2010 Environ Health Perspect 118(12): 1714-1720; Baker 2011 Nature 471: 661-665

MEETING OUTCOME

- **What endpoints are ready to move forward?**
  - Are there superior (commercially available) products that have not yet been tested
- **How should the bioassay results be used (e.g. screening vs. decision?)**
  - Propose a logical flow for use of screening data
  - Which applications?
- **How do we transfer this technology?**
  - Standardization, QA/QC guidelines
  - Lab certification
- **What more can these bioassays be used for?**
  - “hard” decision making
  - Receiving waters

Recommended Studies

- Ensure that water extraction efficiency is universal for all candidate endpoints
- Compare cost of bioanalytical assays vs chemistry
- Need to identify suitable AhR and genotoxicity assays

ER alpha

- Preferred MRL - 1 ng/L human relevance

  (0.1 ng/L ecological relevance

- Max REF - up to 50 depending on water quality
- Existing products: GeneBLAzer EC_{10}=5 ng/L

  BDS ERa-Calux EC_{10}= approx. 1 ng/L

  Possible non-commercial assays e.g. CAFLUX
- Reproducibility: Control charts over time ("Shewart log scale EC_{50}) should be within 2 standard deviations)
- Extraction: 1L using Oasis HLB 6cc recommended

ER data interpretation/ framework

1. Run in vitro assays
2. BEQ > action levels (1 ng/L)
    1. Confirm results
    2. Targeted analysis (e.g. hormomes, alkylphenols, etc.) to account for estrogenicity
    3. If BEQ > CEQ- do effect directed analysis (EDA)/TIE e.g. NTA
    4. If BEQ ~ CEQ: determine relevance to human health

1. CONSIDER BEQ/AL WHEN MOVING TO NEXT STEP

ERa application

- Testing of treatment efficacy
- **Screening**
- Decision making
- CONCLUSION : Do pilot evaluation of bioassay framework before taking next step (is it suitable for decision-making?)

Tech transfer

- General guidelines (performance-based)
  - Cell viability
  - Calibration
  - Required QA/QC
  - Cytotoxicity
  - Certified materials
- Standardized data evaluation (results reporting)
- Laboratory certification (inter-calibration exercises)
- Create & maintain information node
- Workshop

Future

- Additional applications
  - Receiving waters
  - Utility for human health assessment
  - Screening for EPA/TIE
  - Transition from screening to decision making tool
- Additional endpoints
  - GR assay is promising