Site-specific Profiles of Fish Feminization in Surface Waters of California Indicate Multiple Causes of Estrogenic Activities

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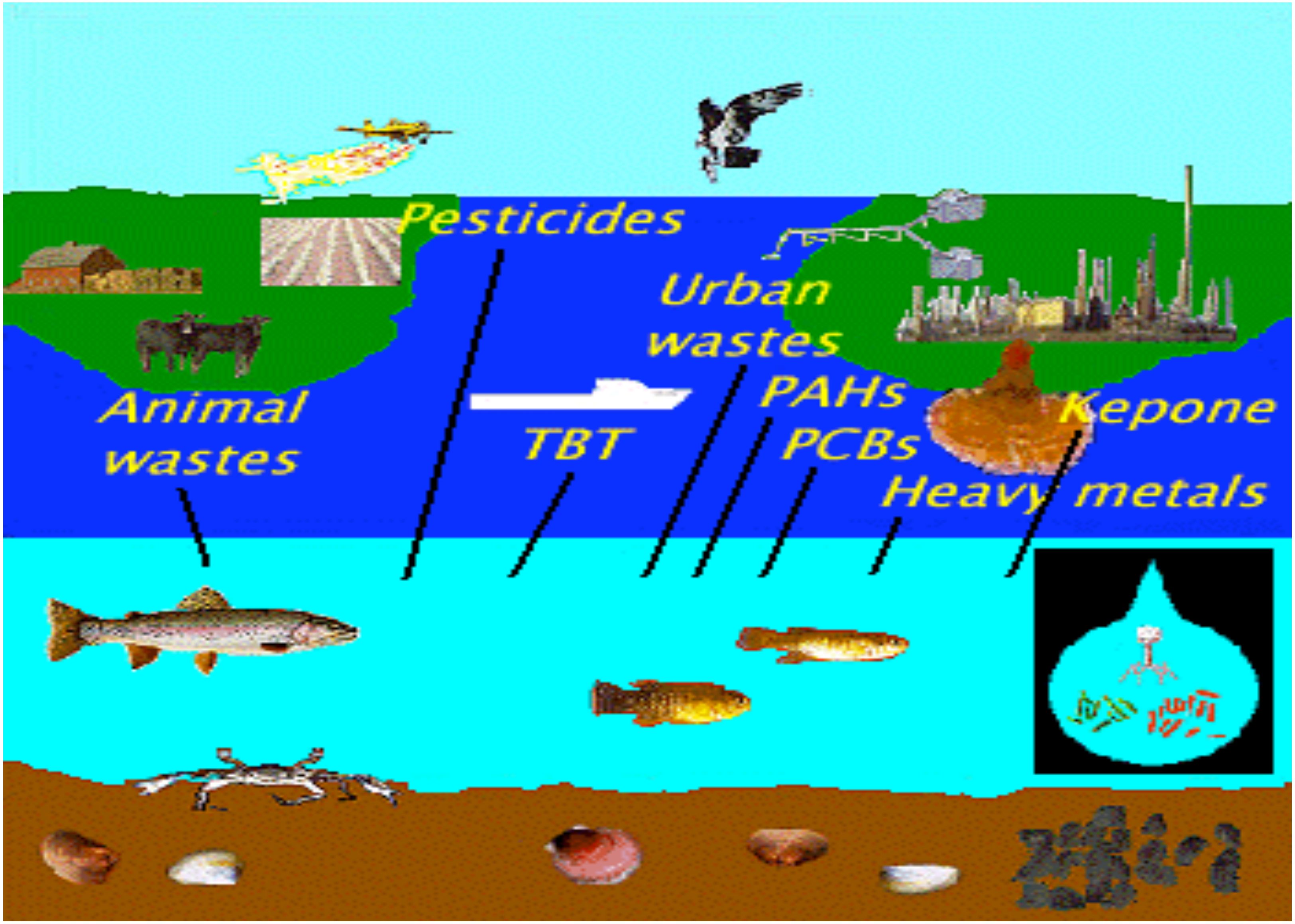
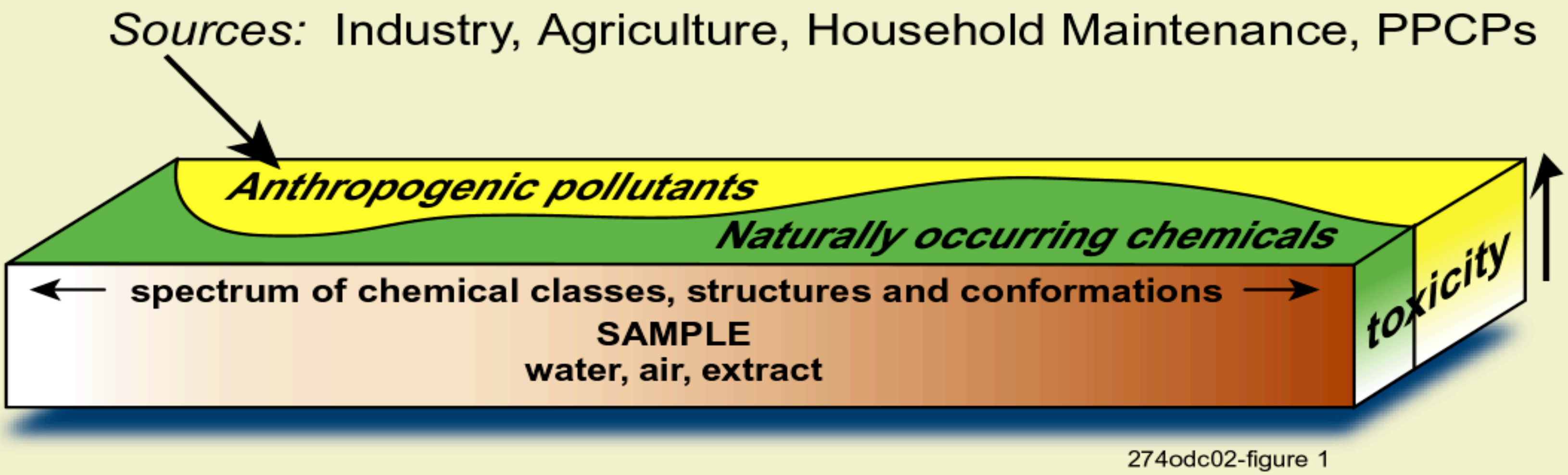
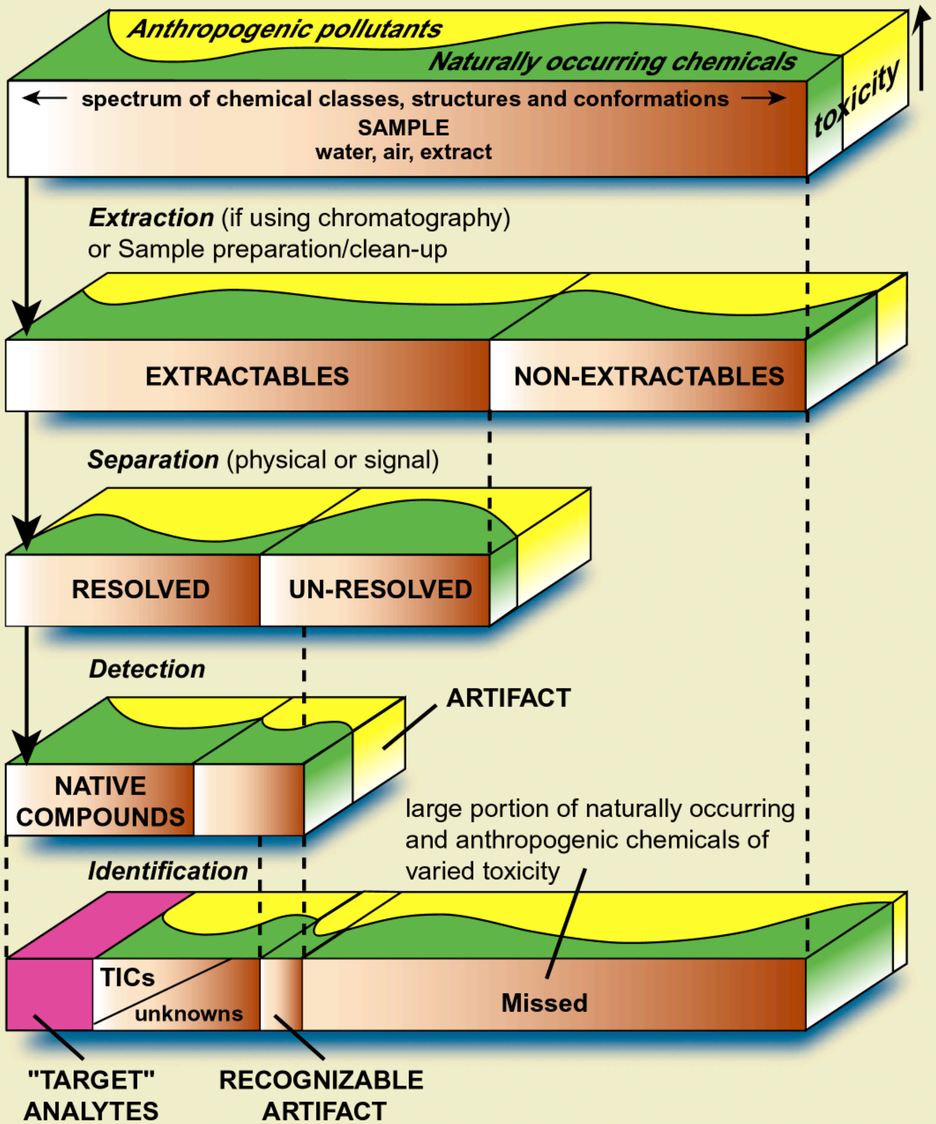

Pesticides
Urban
wastes
PAHs
PCBs
Kepone
Heavy metals
Animal
wastes
TBT

Universe of Chemicals in the Environment



Limitations and Complexity of Environmental Chemical Analysis



TICs = tentatively identified compounds

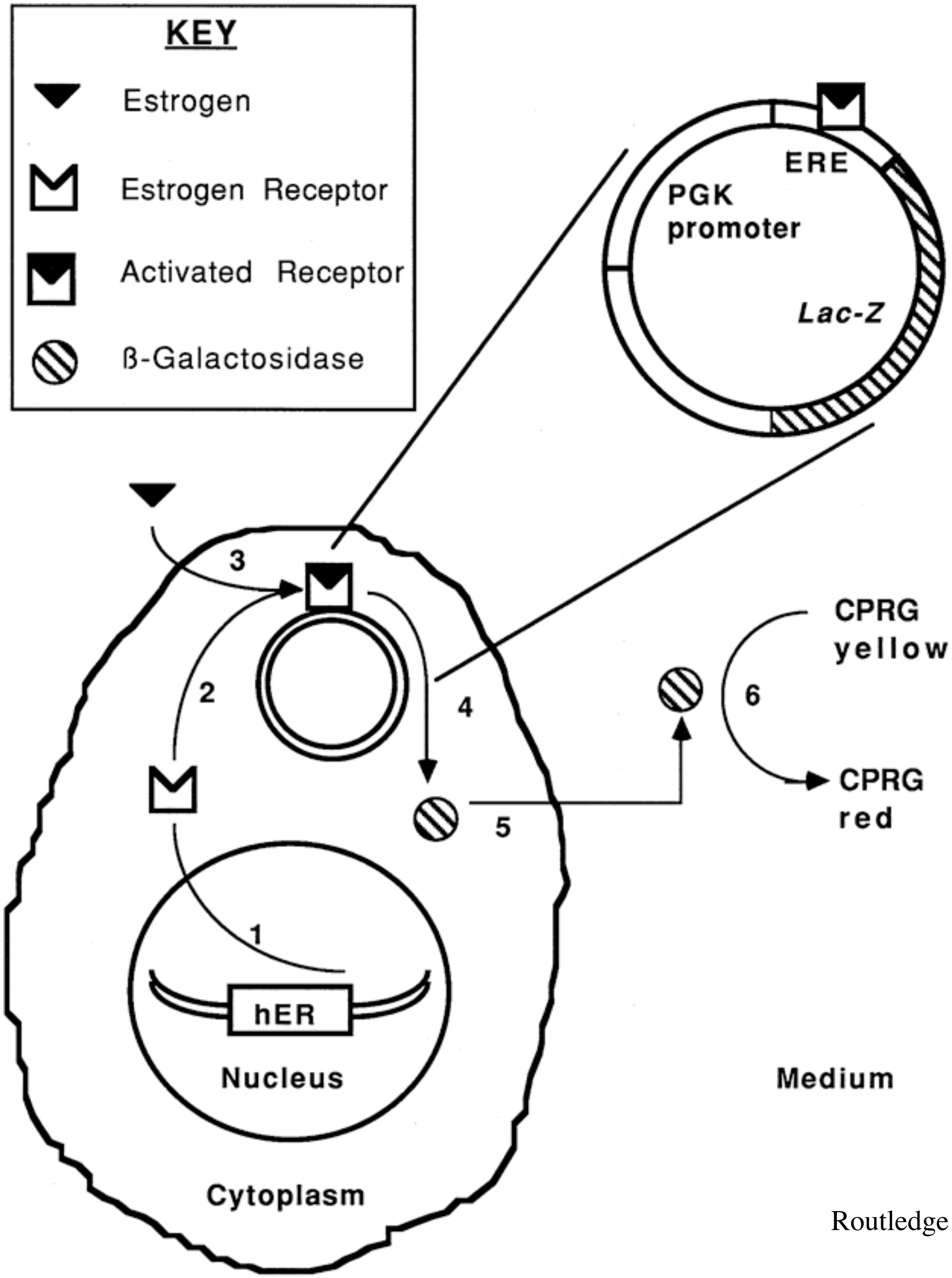
C.G. Daughton
U.S. EPA July 2002

274odc02-figure 2

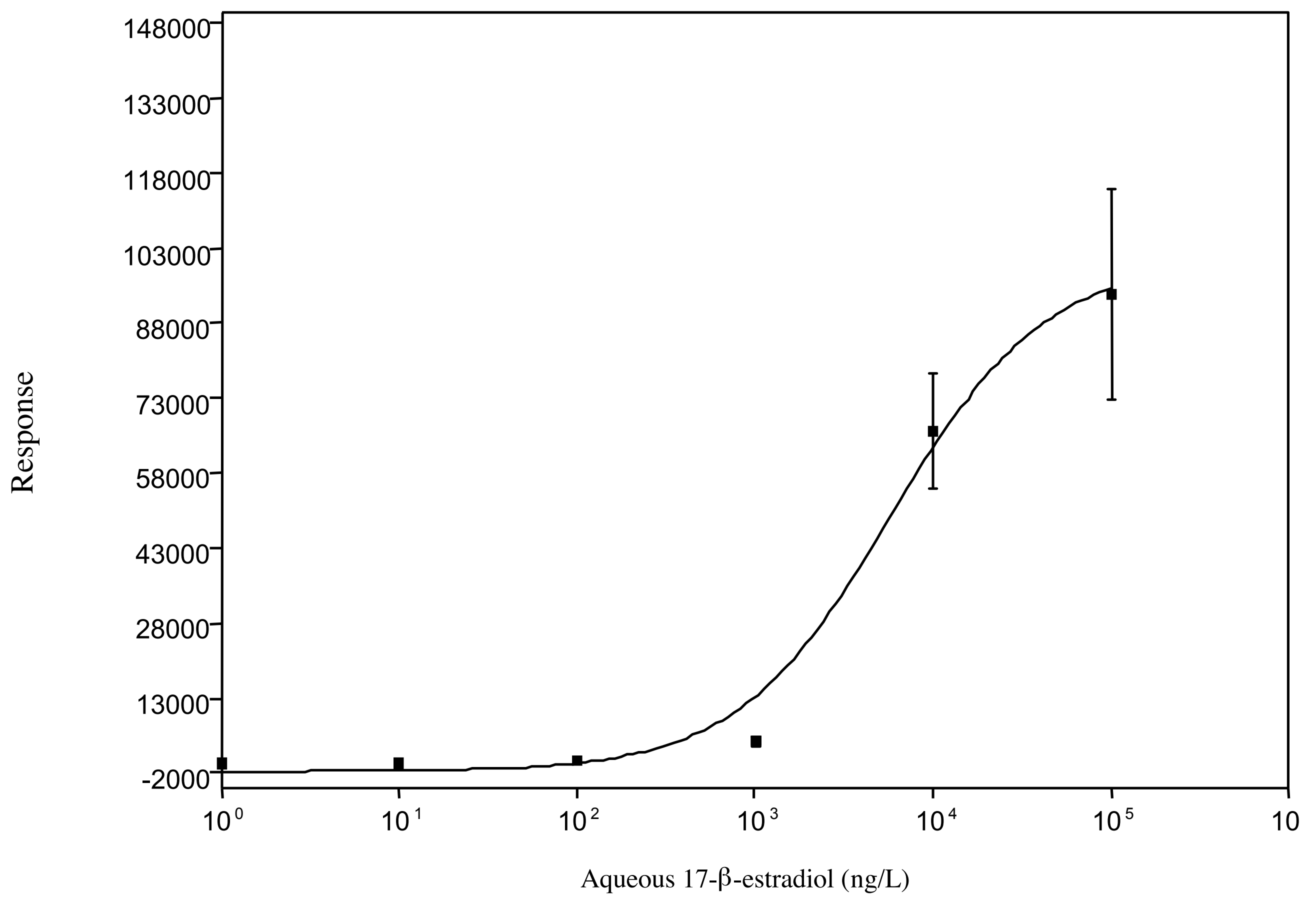
How to Measure Pharmaceutically Active Compounds

- Analytical Chemistry
  - Bioavailability?
  - Unknowns?
- Bioassay
  - In vitro (Receptor driven--Cell lines)
  - In vivo (Receptor driven--whole animal)

Yeast Estrogen Screen

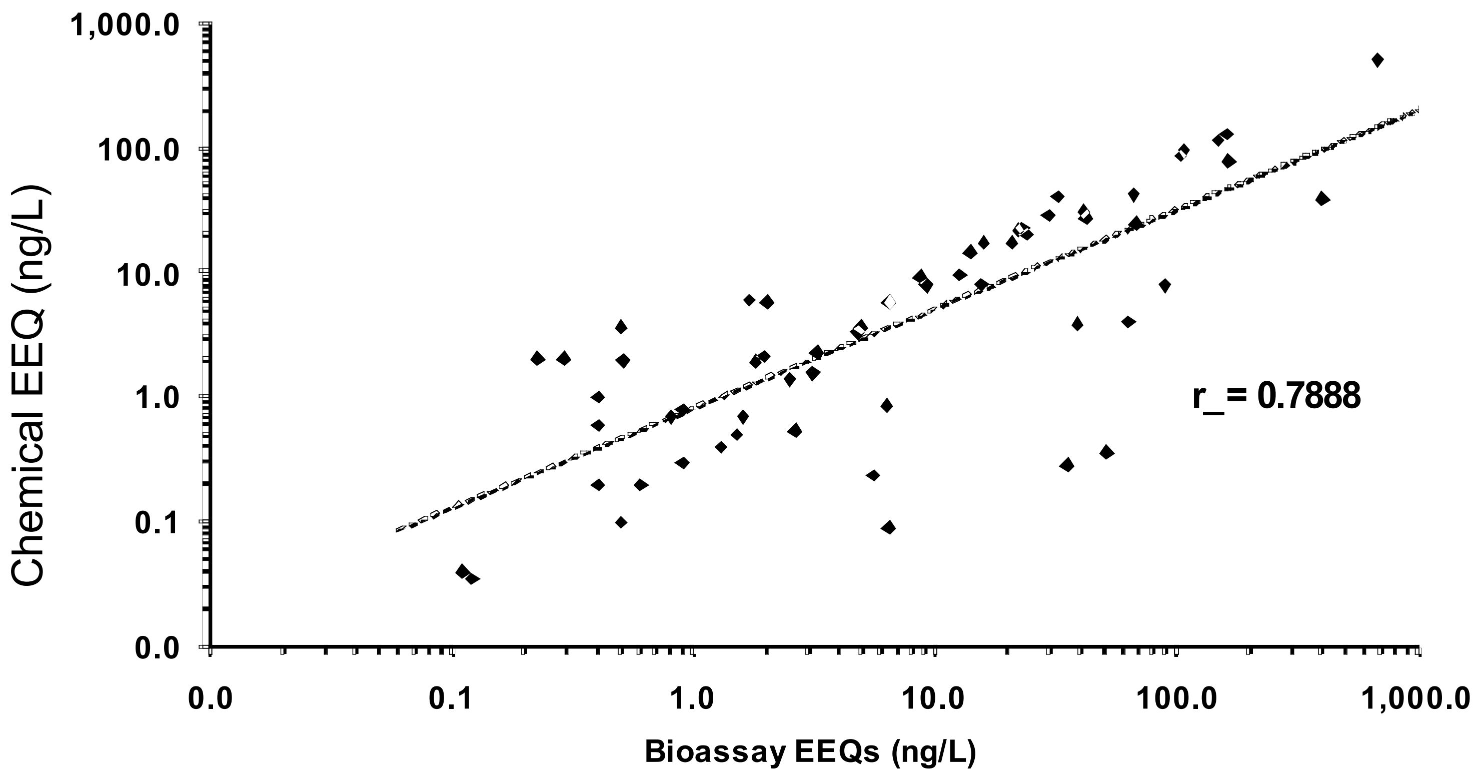


Routledge and Sumpter 1996

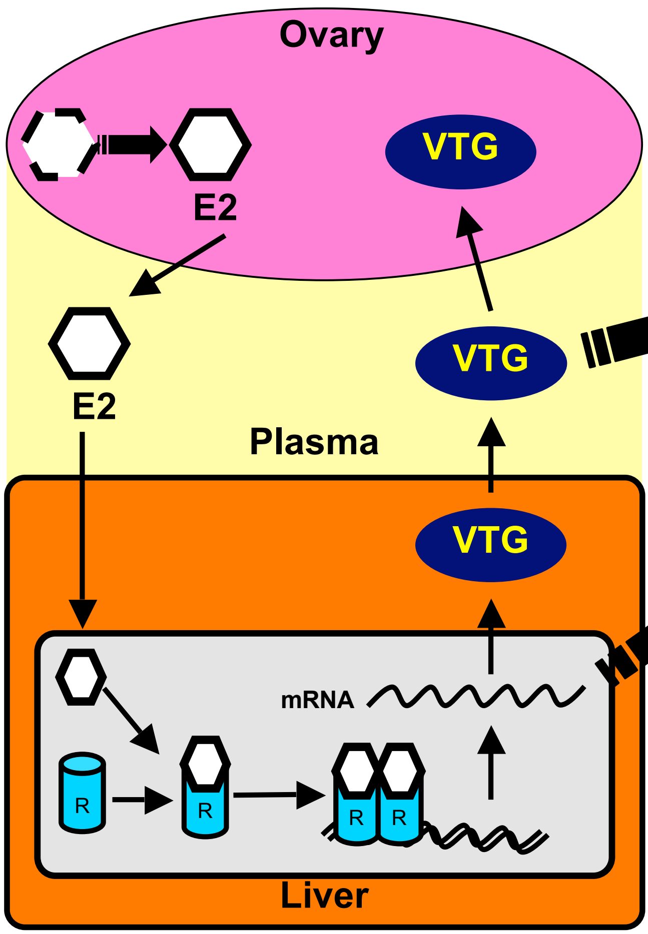


148000
133000
118000
103000
88000
73000
58000
43000
28000
13000
-2000
Response
10^0
10^1
10^2
10^3
10^4
10^5
10^6
Aqueous 17-β-estradiol (ng/L)

Figure II. Relationship between *in vitro* bioassay EEQs and chemically estimated EEQs in various studies (30, 33, 36, 38, 39, 64-67).

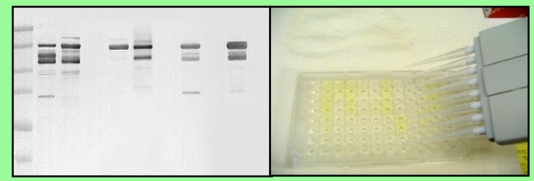


VTG induction as a tool to evaluate estrogenic exposure in fish



Determination of VTG

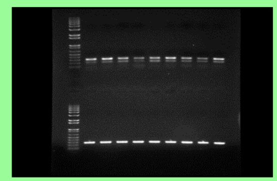
VTG protein detection



Western Blot

ELISA

VTG mRNA detection

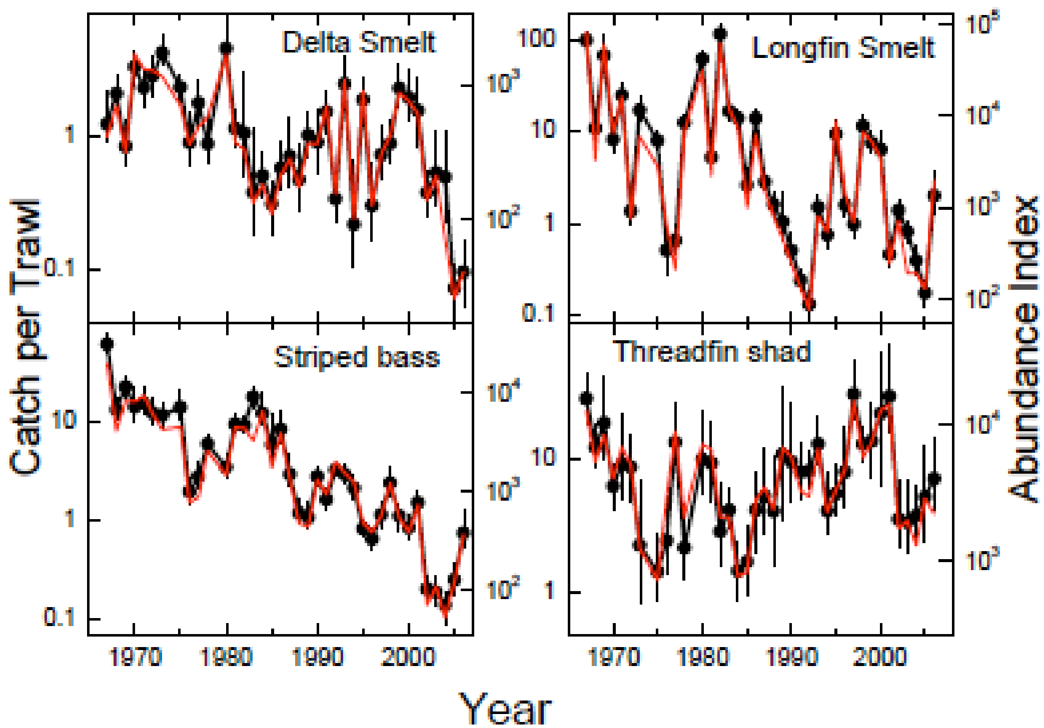


cDNA PCR

VTG induction in males:

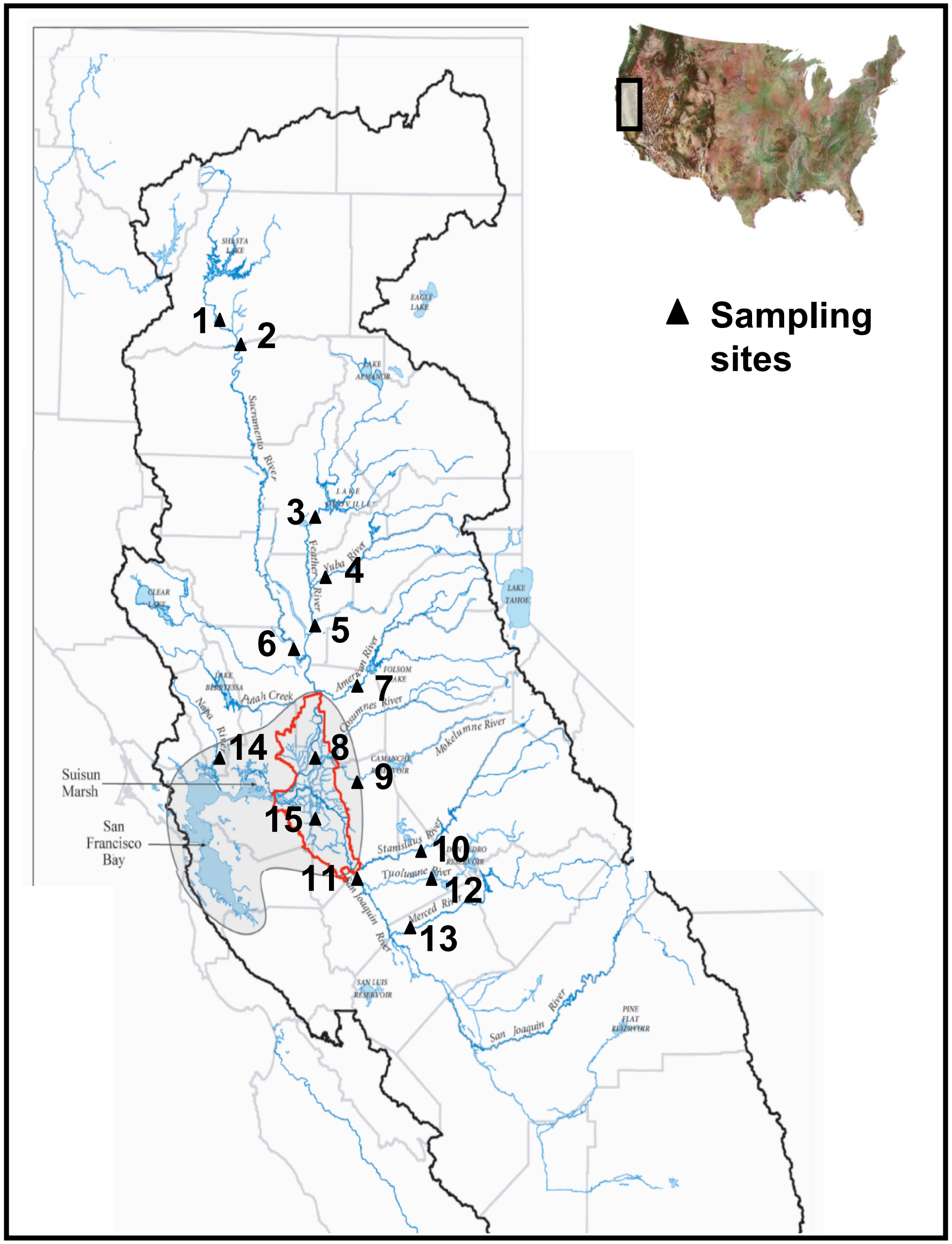
Biomarker of exposure to ESTROGENIC COMPOUNDS

Pelagic Organism Decline



Sommers et al. 2009

Material & Methods: Sampling sites



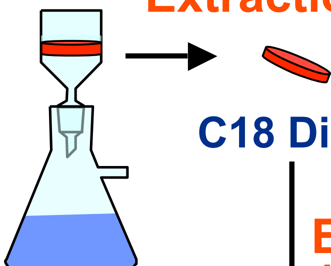
Site	Site name
1	Upper Sacramento River
2	Battle Creek
3	Upper Feather River
4	Yuba River
5	Lower Feather River
6	Lower Sacramento River
7	Lower American River
8	Sacramento River in Delta
9	Mokelumne River
10	Stanislaus River
11	San Joaquin River
12	Tuolumne River
13	Merced River
14	Napa River
15	Clifton Court Forebay

Material & Methods: Extracts & Exposures

Water extracts

Water (1 L)

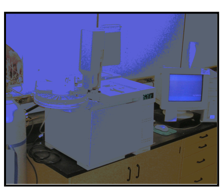
Solid-phase Extraction



C18 Disks

Elution
10 mL MetOH

Hormone determination (GC-MS)



Exposures *in vitro/in vivo*

Extract exposures

In vitro hepatocytes exposure

Primary Hepatocytes isolation and culture

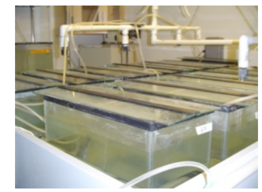
Extract added in the media (0.6% v/v)

Incubation
24 h

Total mRNA extract

VTG mRNA determination

In vivo whole fish exposure

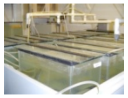


Intraperitoneal extract injection (x2)

Incubation
7 days

Plasma collection

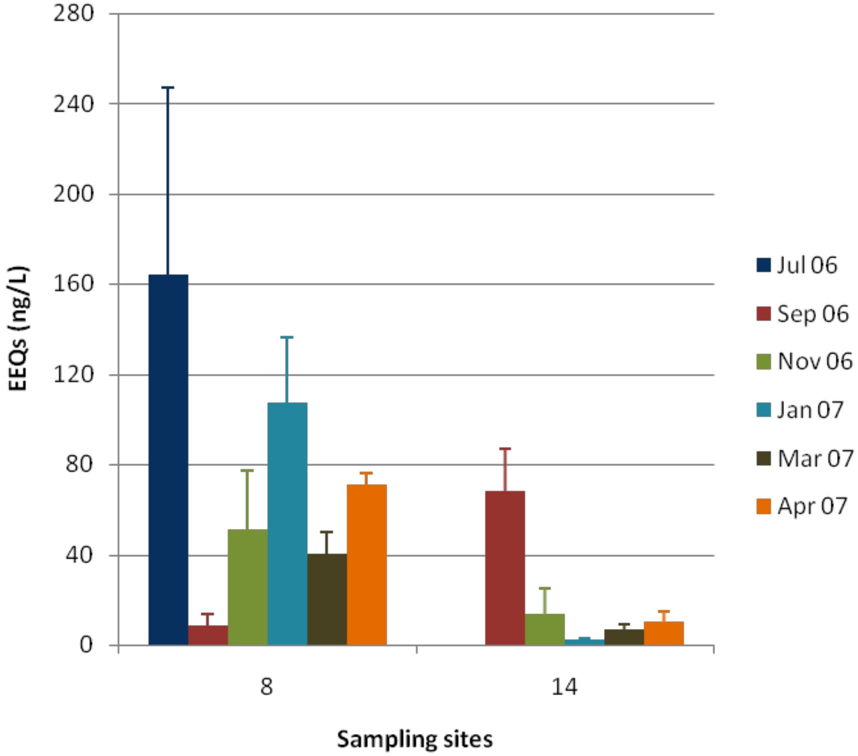
VTG protein levels determination

Results: Water extracts

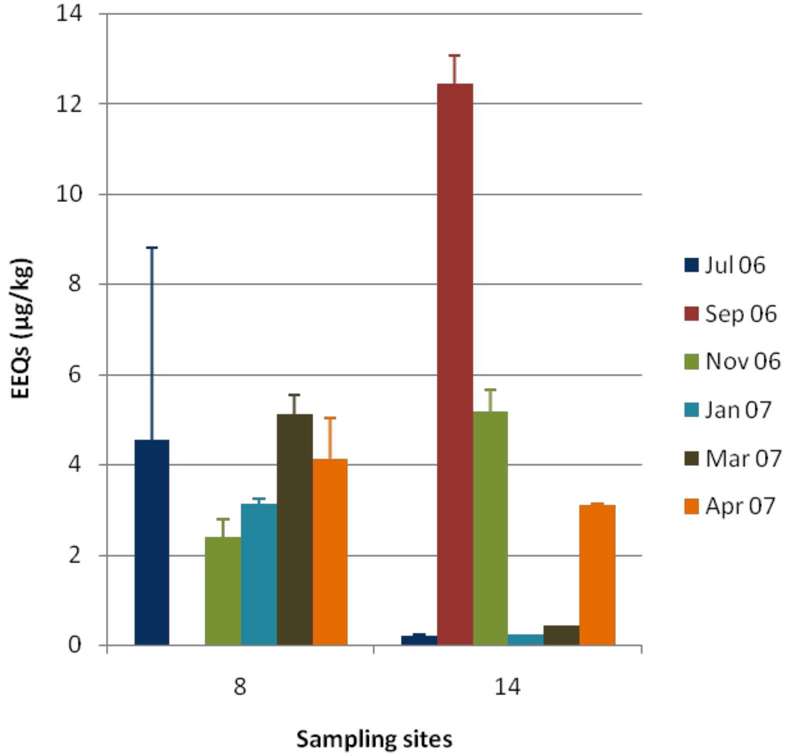
High Estrogenicity Areas (both methods)

Cells Exposure



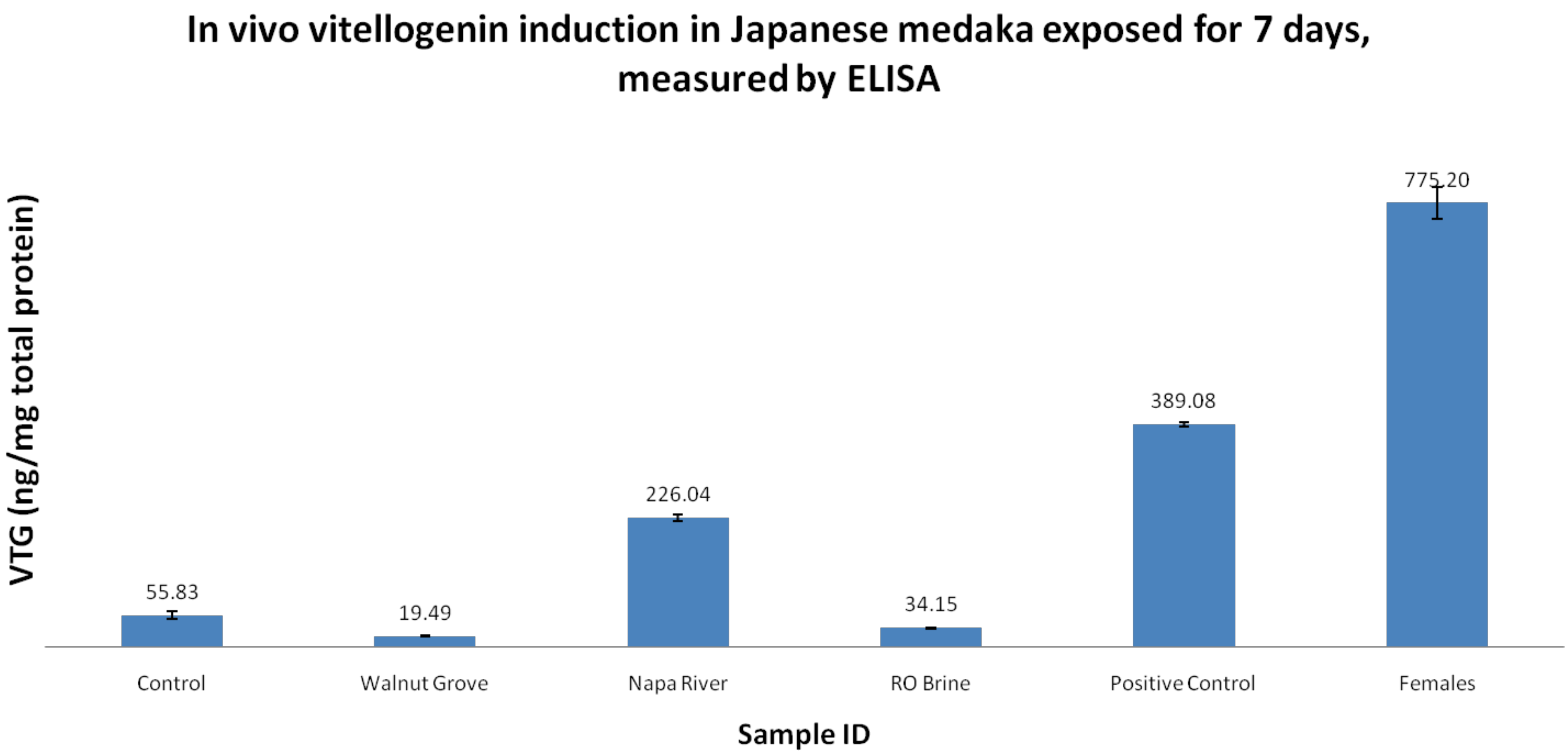
Data presented as Mean ± SEM

Whole Animal Exposure

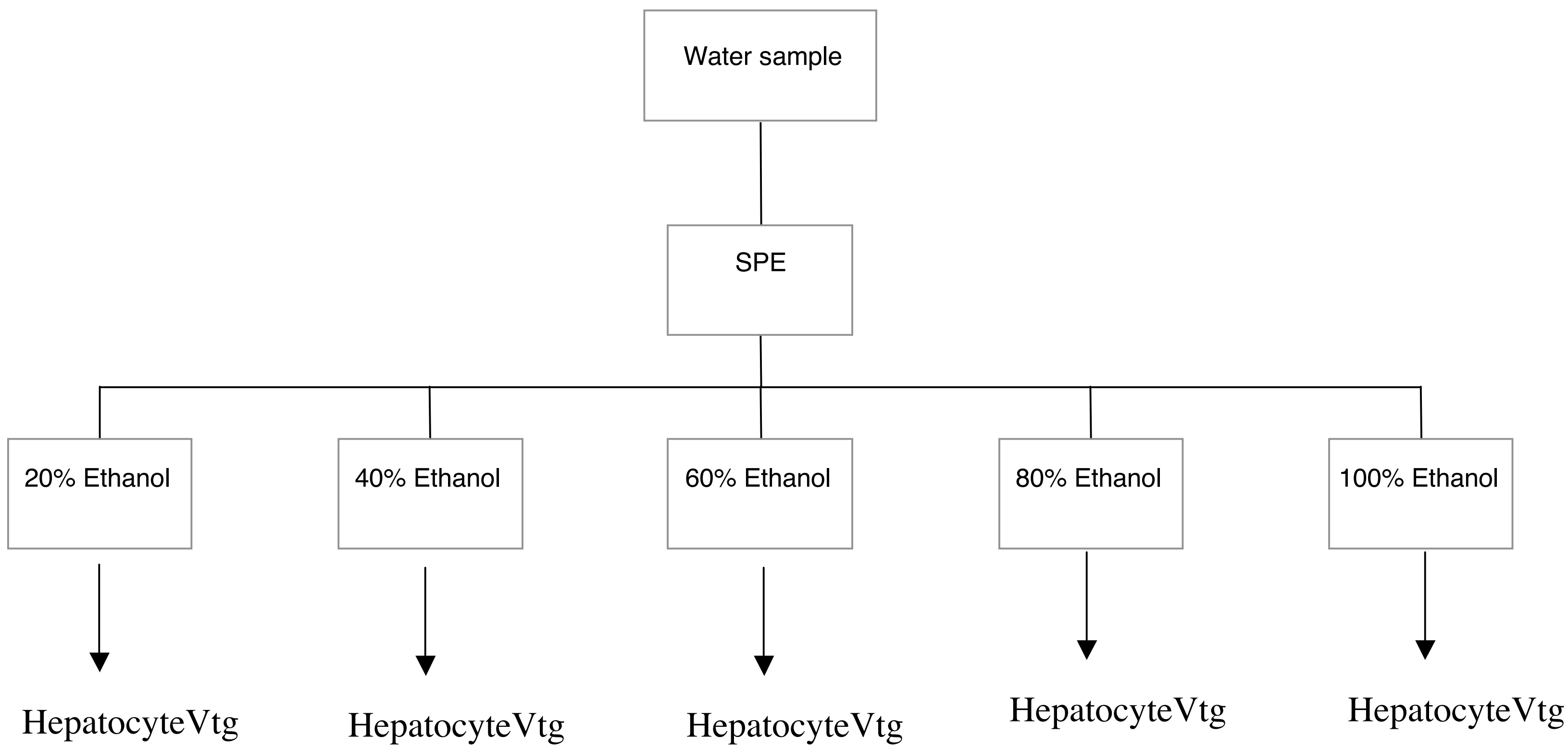


Data presented as Mean ± SEM

In Vivo Estrogenicity

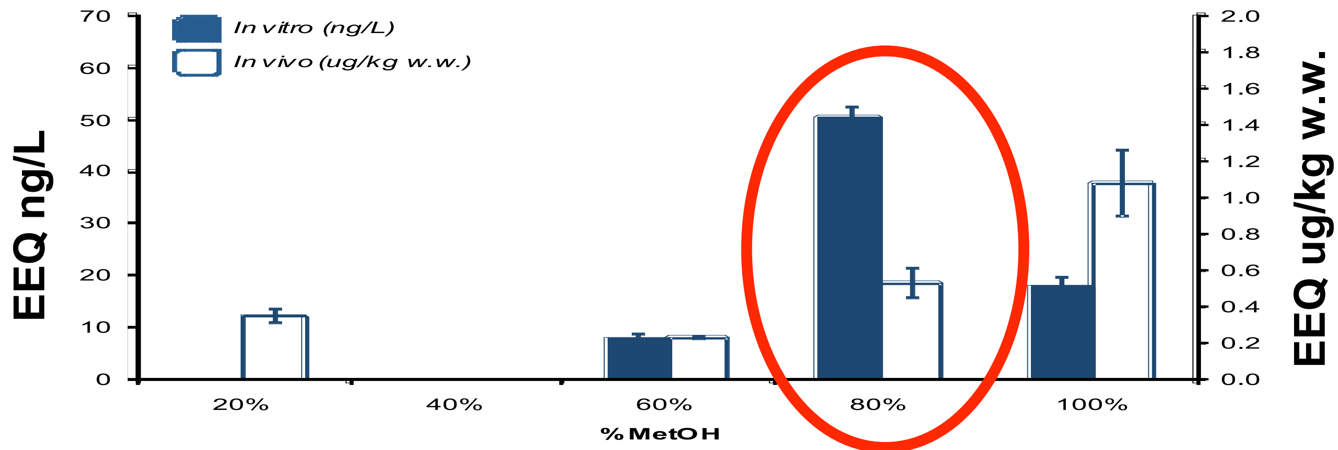


Fractionation of Water Extracts from OCSD plume and Reference site for the Determination of unknown estrogens.

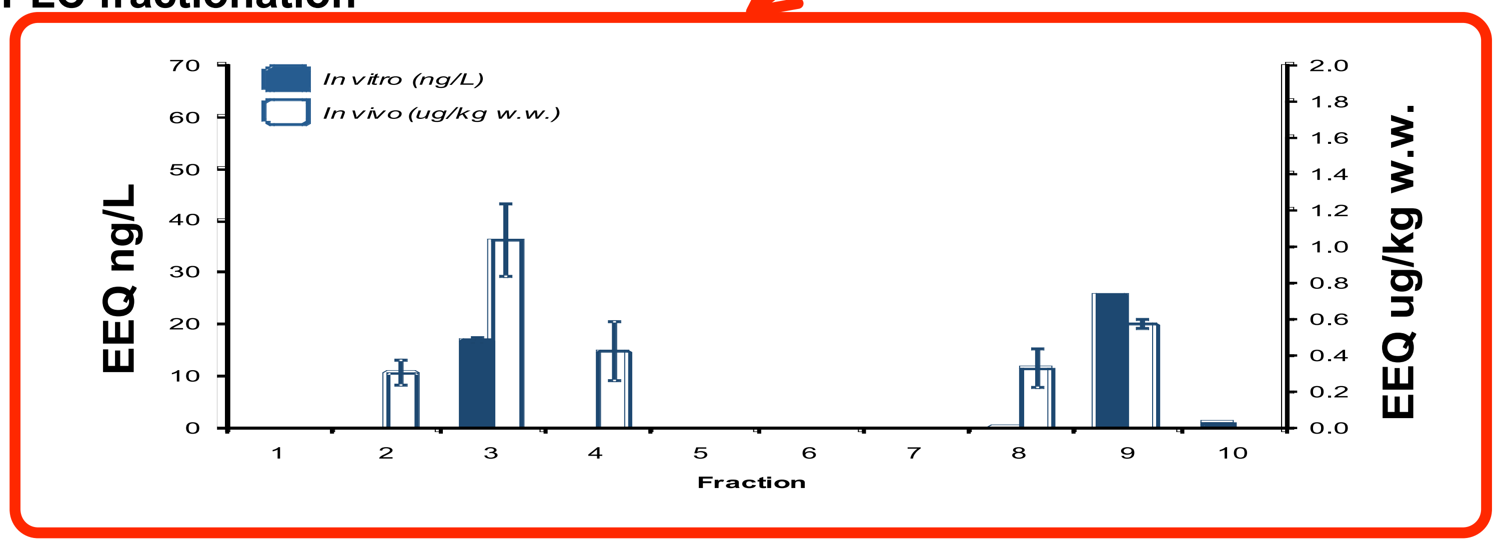


NAPA River

SPE fractionation

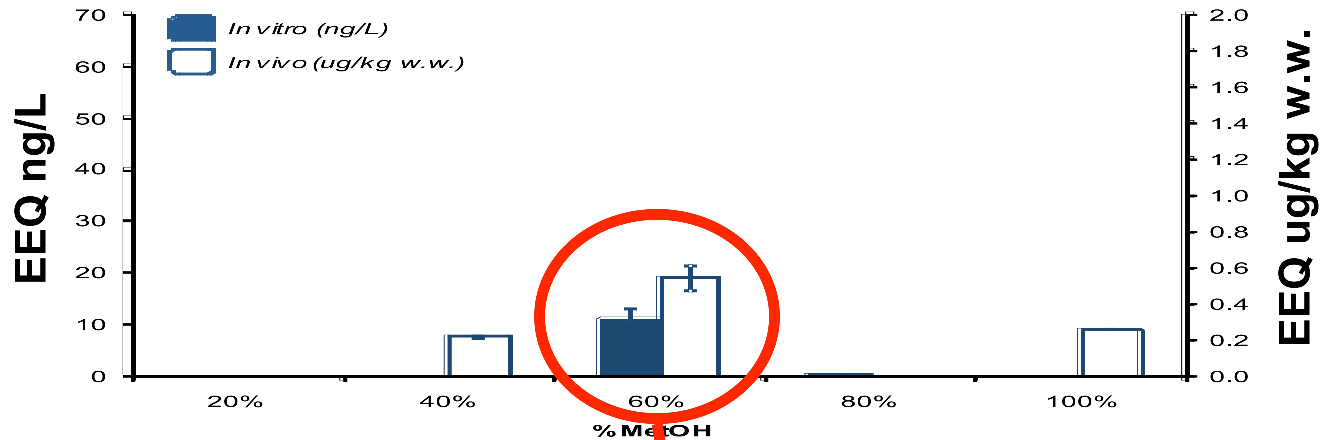


HPLC fractionation

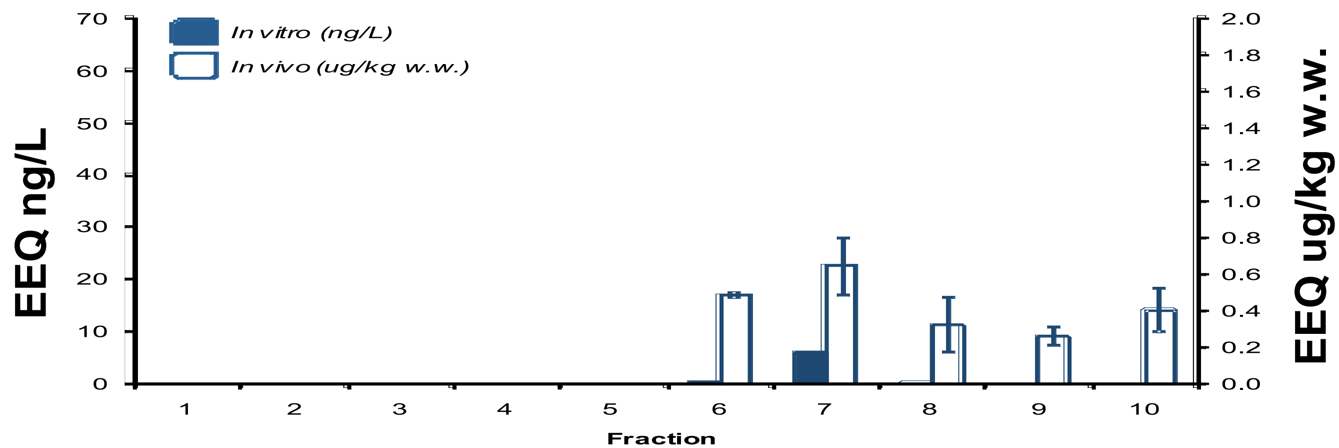


SACRAMENTO RIVER DELTA

SPE fractionation



HPLC fractionation



Measured Analytes for Bioactive Fractions

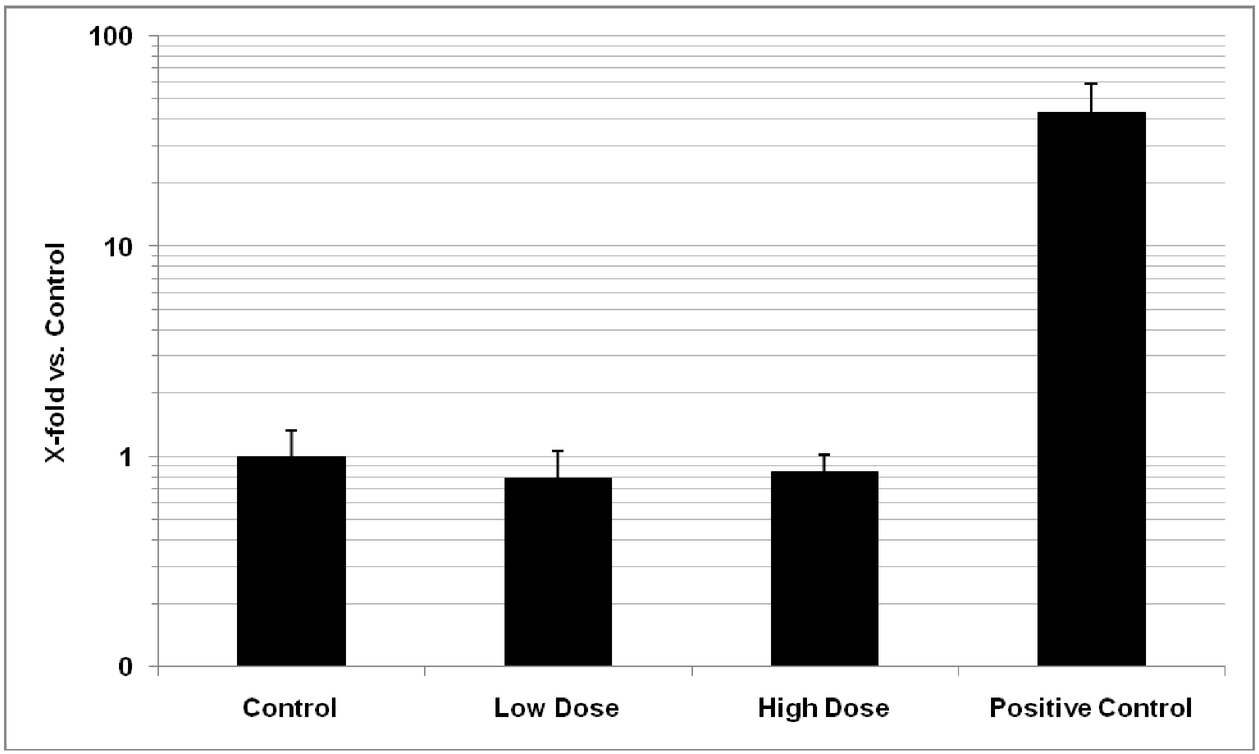
Phytoestrogens	Pharmaceuticals & Personal Care Products	Pesticides			Steroid Hormones	APEO/AP
Genistein	Sulfamethoxazole	Aldicarb	Promecarb	Secbumeton	17β-Estradiol	4-Octylphenol
Daidzein	Atenolol	Aldicarb Sulfone	Propham	Simazine	Estrone	OP1EO
Formononetin	Trimethoprim	Aldicarb sulfoxide	Siduron	Simetryn	Estriol	OP2EO
Biochanin A	Iopromide	Aminocarb	Swep	Terbuthylazine	Progesterone	4-Nonylphenol
Apigenin	Caffeine	Barbamate	Chlorpyrifos	Terbutryn	Testosterone	NP1EO
Naringenin	Fluoxetine	Baygon	Diazinon	Thiobencarb	Androstendione	NP2EO
Coumestrol	Meprobamate	Captan	Imidacloprid	DEET	Ethynylestradiol	
Chrysin	Dilantin	Carbaryl	Myclobutanil	Permethrin		
Matairesinol	Carbamazepine	Carbofuran	Oryzalin	Cypermethrin		
Equol	Diazepam	Carbofuran phenol-3-ketone	Oxyfluorfen	Deltamethrin		
Glycitein	Atorvastatin	Chlorpropham	Tebuconazole	Cyfluthrin		
	Benzophenone	Dioxacarb	Ametryn	Bifenthrin		
	Primidone	Diuron	Atraton	Triclopyr		
	TCPP	Fenuron	Atrazine	2,4-D		
	TCEP	Fluometuron	Cyanazine			
	Gemfibrozil	3-Hydroxycarbofuran	Deisopropyl-atrazine			
	Bisphenol A	Linuron	Desethyl-atrazine			
	Diclofenac	Methiocarb	Desmetryn			
	Naproxen	Methomyl	Dipropetryn			
	Triclosan	Monuron	2-Hydroxyatrazine			
	BHA	Neburon	Molinate			
	Musk ketone	Oxamyl	Prometon			
	Ibuprofen	Propazine	Prometryn			

Pesticides detected (ng/L) in Estrogenically active fractions

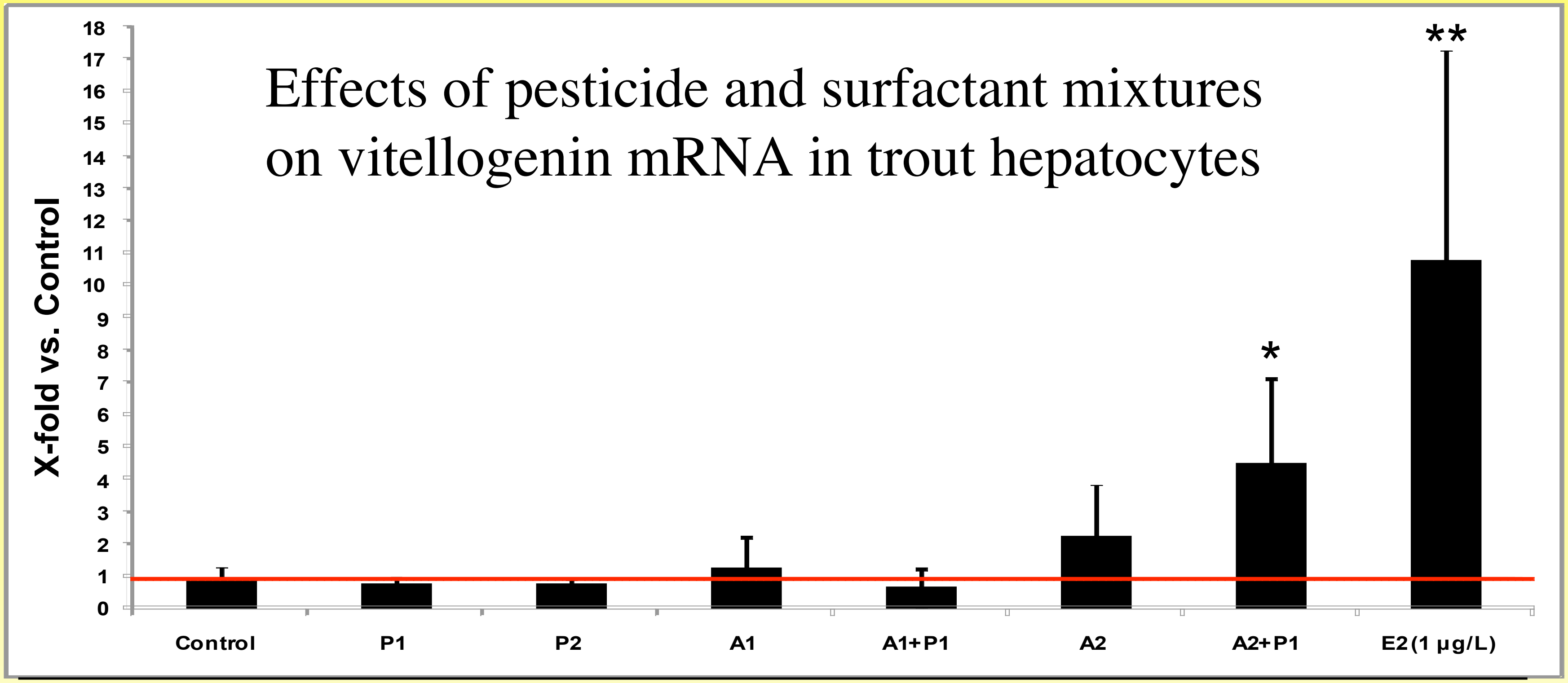
		diuron	simazine	2-hydroxyatrazine	*in vitro, EEQ, ng/L*	*in vivo, EEQ, ug/kg*
	Blk					
Delta	8/60	2.45		0.20	11.18	0.54
	8/80	0.31			0.49	BDL
Napa	14/80	6.16	4.13	2.77	50.4	0.53
	14/100	6.88	2.96	1.66	18	2.1

Evaluation of Reconstituted Pesticide Mixture by In vitro Hepatocyte Vtg mRNA assay

Pesticide	Concentration			
	Low Dose (1X)		High Dose (5X)	
	Water	In cells	Water	In cells
Simazine	7.2 µg/L	428 µg/L	36 µg/L	2.16 mg/L
Diuron	7 µg/L	420 µg/L	35 µg/L	2.1 mg/L
Atrazine	0.5 µg/L	30 µg/L	2.5 µg/L	150 µg/L
Deisopropylatrazine	4.3 µg/L	256 µg/L	21.5 µg/L	1.29 mg/L
Hydroxyatrazine	5.7 µg/L	342 µg/L	28.5 µg/L	1.71 mg/L

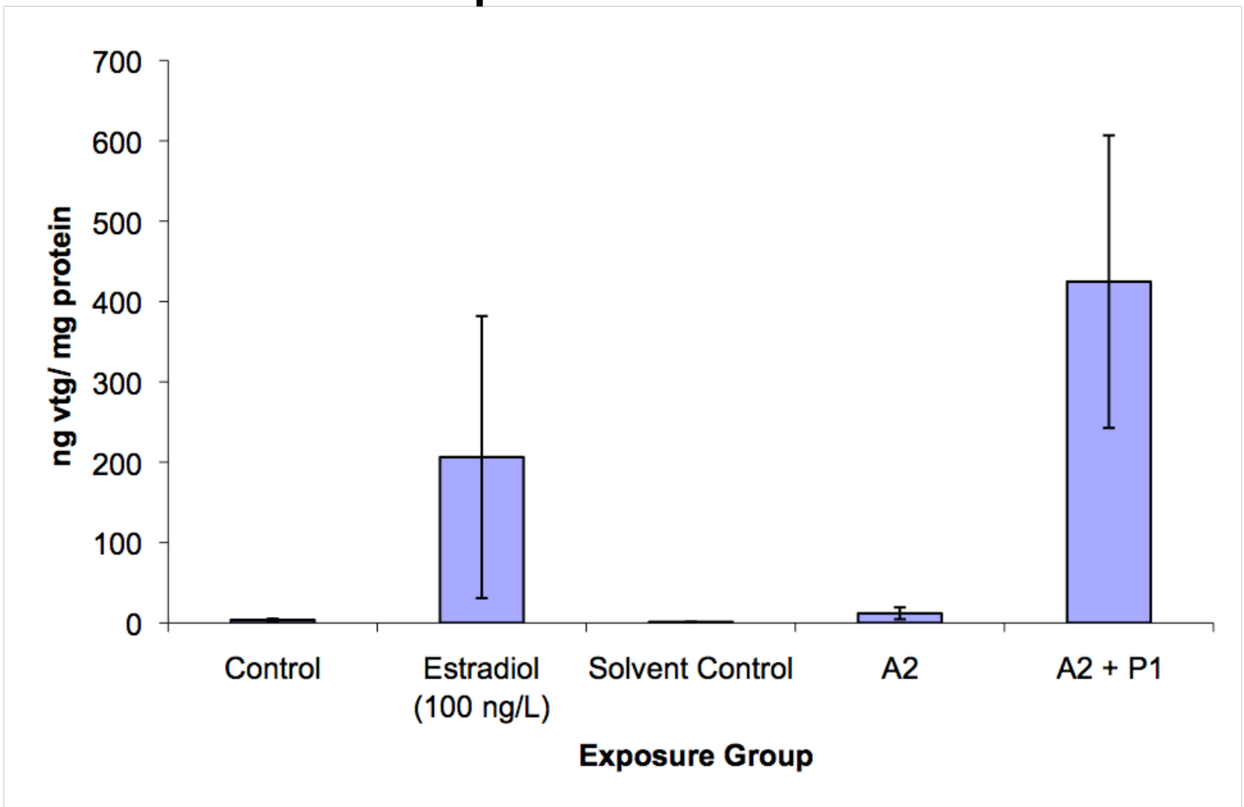


Effects of pesticide and surfactant mixtures on vitellogenin mRNA in trout hepatocytes



	Concentration						
	Control	P1	P2	A1	A1+P1	A2	A2+P1
Pesticides							
Simazine	-	7.2 µg/L	36.0 µg/L	-	7.2 µg/L	-	7.2 µg/L
Diuron	-	7.0 µg/L	35.0 µg/L	-	7.0 µg/L	-	7.0 µg/L
Atrazine	-	0.5 µg/L	2.5 µg/L	-	0.5 µg/L	-	0.5 µg/L
Deisopropylatrazine	-	4.3 µg/L	21.5 µg/L	-	4.3 µg/L	-	4.3 µg/L
Hydroxyatrazine	-	5.7 µg/L	28.5 µg/L	-	5.7 µg/L	-	5.7 µg/L
APEO							
Octylphenol (OP)	-	-	-	2.4 ng/L	2.4 ng/L	260 µg/L	260 µg/L
Octylphenol Polyethoxylates (OPEOs)	-	-	-	4.8 ng/L	4.8 ng/L	520 µg/L	520 µg/L
Nonylphenol (NP)	-	-	-	139.5 ng/L	139.5 ng/L	15.0 mg/L	15.0 mg/L
Nonylphenol Polyethoxylates (NPEOs)	-	-	-	155.5 ng/L	155.5 ng/L	16.6 mg/L	16.6 mg/L

Effects of Pesticide and Surfactant Mixtures on in vivo Vitellogenin Protein Expression in Japanese medaka



Effects of APE surfactants on the Estrogenic activity of 2,4 D

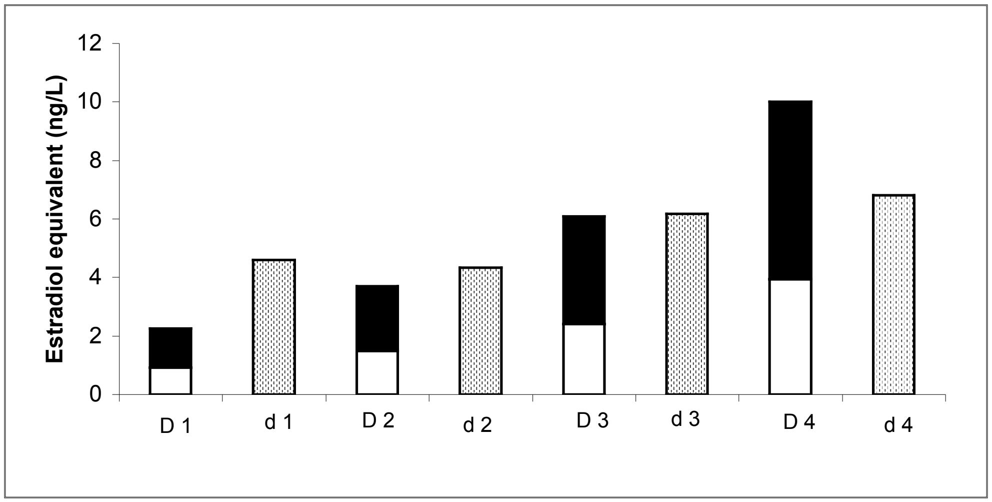
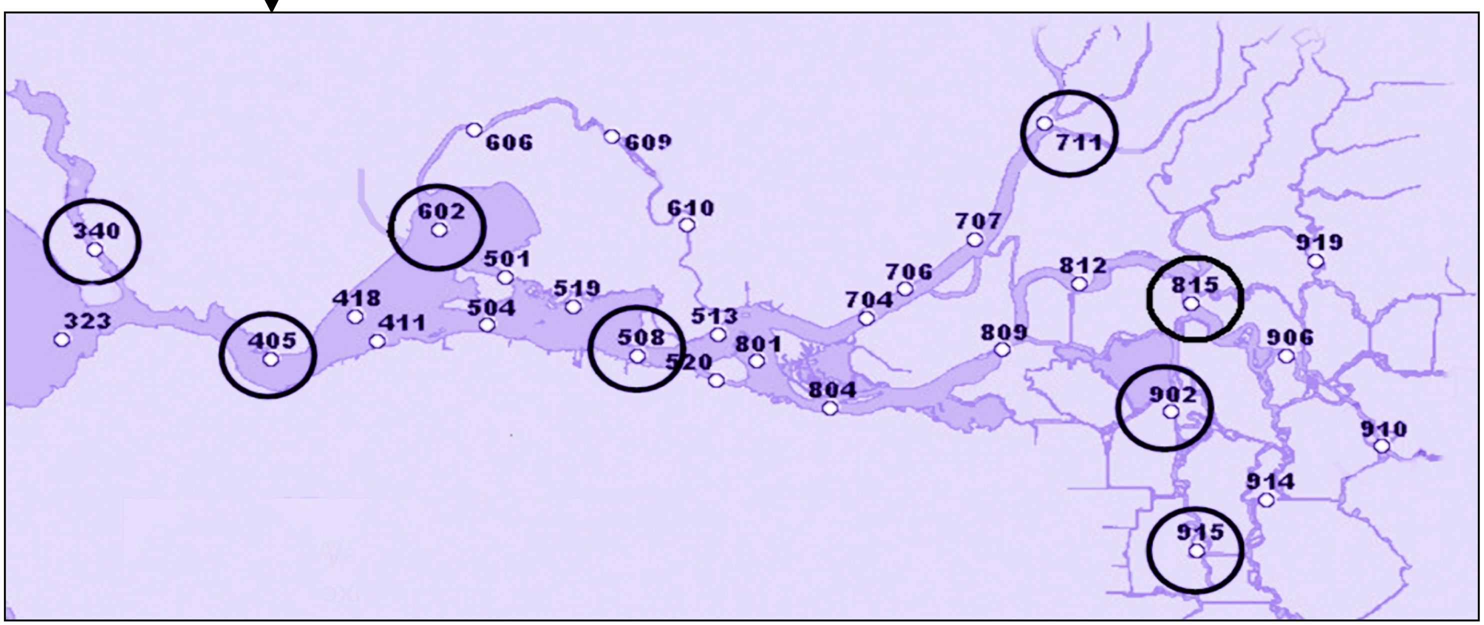


Figure 2. Estradiol equivalent concentrations (EEQs) of various concentrations of R-11 and 2,4-D. Solid bars are R-11, open bars are for 2,4-D, while dashlined bars are for the mixture of R-11 and 2,4-D.

Dose	Conc.s of chemicals		dose	Conc.s of chemicals
	R-11 (mg/L)	2,4-D (mg/L)		R-11 (mg/L)+2,4-D (mg/L)
D 1	0.00089	0.00164	d 1	0.00089 R-11 + 0.00164 2,4-D
D 2	0.0089	0.0164	d 2	0.0089 R-11 + 0.0164 2,4-D
D 3	0.089	0.164	d 3	0.089 R-11 +0.164 2,4-D
D 4	0.89	1.64	d 4	0.89 R-11 + 1.64 2,4-D

Xie et al. 2005

Delta Sampling in 2008
(POD samples)



In vivo Estrogenic Activities of 2008 POD samples

Sample	*In vivo* **EEQs (ng/L)**
340	0.90 ± 0.03
405	**25.65 ± 4.01**
508	*bdl*
602	1.05 ± 0.10
711	**12.79 ± 1.65**
815	0.80 ± 0.12
902	*bdl*
915	2.02 ± 0.32

In vitro Vitellogenin induction of APEOs' plus selected pesticides (POD Samples)

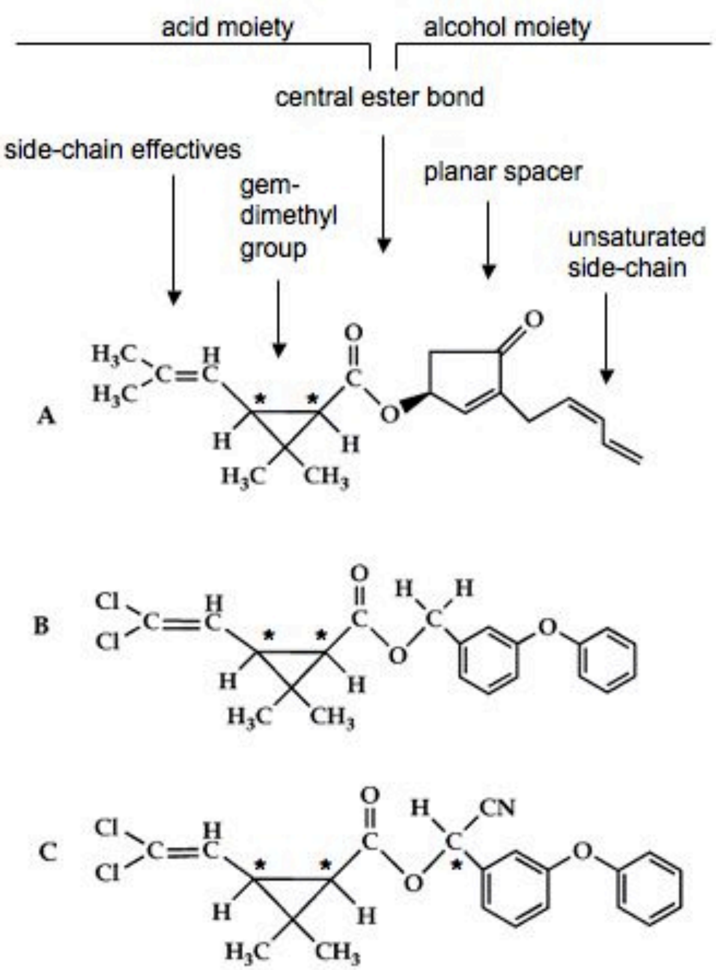
Pesticide	River water concentration	Code Cell Concentraion P1 (1x)	P5 (5x)
Bifenthrin	1 ng/L	24 ng/L	119 ng/L
Diuron	41 ng/L	972 ng/L	4862 ng/L

APEO	River water concentration	Code Cell Concentraion A1 (1x)	A5 (5x)
NP	90 ng/L	2.134 ug/L	10.672 ug/L
NPEOs	606 ng/L	14.372 ug/L	71.858 ug/L
OP	13 ng/L	308 ng/L	1.542 ug/L
OPEOs	84 ng/L	1.992 ug/L	9.960 ug/L

SYNTHETIC PYRETHROIDS

- Neurotoxicant
- OP substitutes
- Extensively used in agriculture and urban/household insect control
    - 40% of all registered insecticide products
    - Use in CA has nearly tripled in the last decade
        - 1.4M lbs total sales in 2004
        - 550k lbs (for PM in 2006; 30% agric.; 60% commercial; 10% household)
- Highly hydrophobic
- Broad-spectrum insecticides
- Low mammalian and avian toxicity
- Highly toxic to aquatic organisms
- EDC

CHIRALITY IN SYNTHETIC PYRETHROIDS



- Chiral
- Structural derivative of natural pyrethrins
- Conforms to a single SAR for insecticidal activity based on the shape of the 3-D configuration
- Activity results from the appropriate fit of the entire molecule at the site of action

THE SAME BUT NOT THE SAME

ENANTIOSELECTIVE NON-TARGET ACUTE TOXICITY

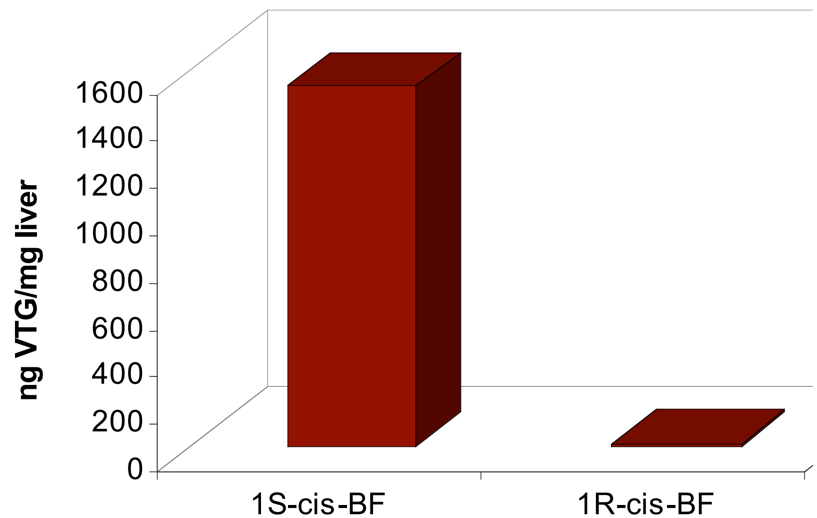
		D. pulex[2]	*D. magna*[3]		*C. dubia*[3]	
cis-Bifenthrin	*Selectivity ratio*[1]	14.0	22.0		18.0	
	Active enantiomer	(-)	*1R-cis*		*1R-cis*	
Permethrin	*Selectivity ratio*[1]	-	>15.5	>19.5	>38.5	>30.5
	Active enantiomer	N.D.[4]	*1R-cis*	*1R-trans*	*1R-cis*	*1R-trans*
Cypermethrin	*Selectivity ratio*[1]	-	-		>10	>8
	Active enantiomer	N.D.[4]	N.D.[4]		*1R-cis-αS*	*1R-trans-αS*
Cyfluthrin	*Selectivity ratio*[1]	-	-		>96	>47
	Active enantiomer	N.D.[4]	N.D.[4]		*1R-cis-αS*	*1R-trans-αS*

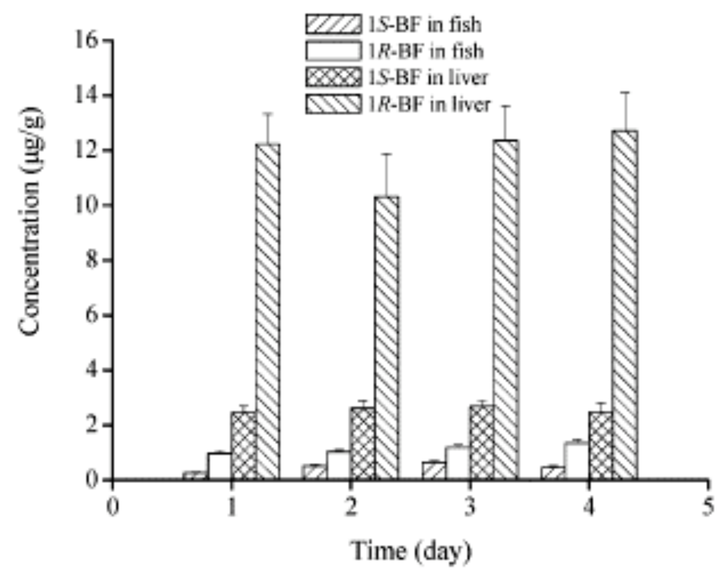
[1]Selectivity ratio is the ratio of the LC50 (or TLM) of less active enantiomer(s) to that of the active enantiomer(s); [2][44]; [3][11] ; [4]N.D. (no data)

Liu et al.(2005) PNAS 102:701-705; Liu et al. (2005) J. Food Drug Anal. 13:357-360

ESTROGENIC ACTIVITY AND PYRETHROIDS

1S-cis-BF is 123 times more active than 1R-cis-BF

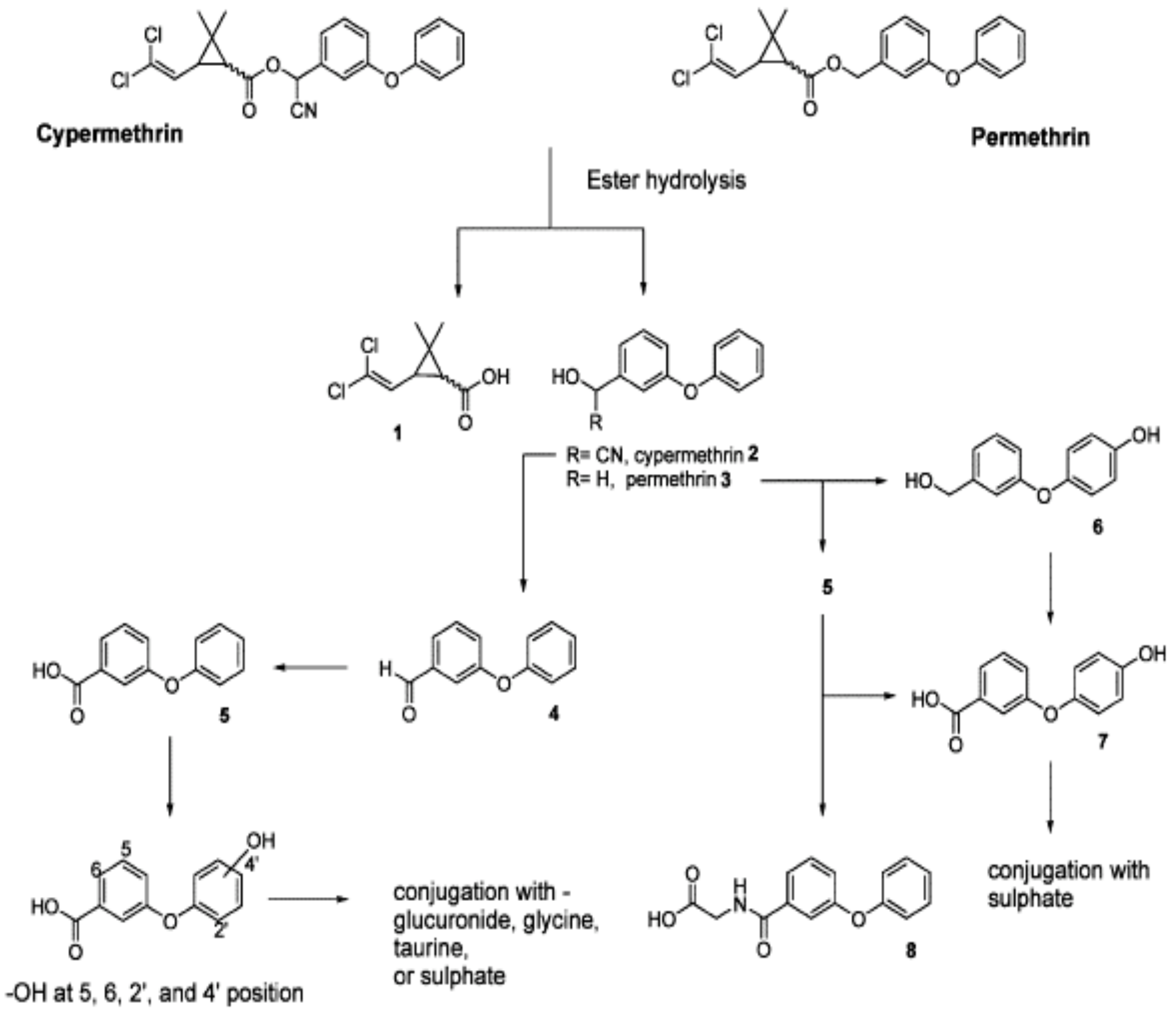




Enantioselectivity in in vivo vitellogenin induction in adult male Japanese medaka (ELISA Assay)

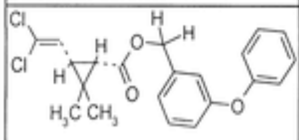
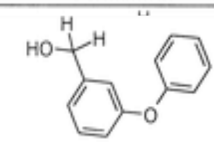
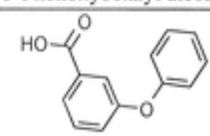
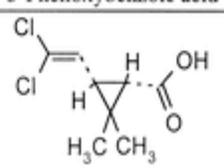
Wang et al., 2007, EST, 41: 6124-6128

ESTROGENIC ACTIVITY AND PYRETHROID BIOTRANSFORMATION



(McCarthy et al., 2006, J. Environ. Monit., 8: 197-202)

ENANTIOSELECTIVE ESTROGENIC ACTIVITY IN PYRETHROIDS

Chemical structure and name	Activity in Yeast Screens			
	Estrogenic	Anti-estrogenic	Androgenic	Anti-androgenic
 Permethrin	+ LOEC $3.1 \pm 0.4 \times 10^{-4}$ M EC50 2×10^{-3}M	-	-	+ LOIC $3 \pm 0.57 \times 10^{-5}$M IC50 $7.3 \pm 1.4 \times 10^{-4}$M
 3-Phenoxybenzyl alcohol	+ LOEC $3.2 \pm 0.58 \times 10^{-6}$M EC50 2×10^{-5}M	-	-	+ LOIC $3.5 \pm 0.5 \times 10^{-6}$M IC50 $3.67 \pm 0.67 \times 10^{-5}$M
 3-Phenoxybenzoic acid	-	+ LOIC $1.25 \pm 0.7 \times 10^{-5}$M IC50 $6.5 \pm 2.5 \times 10^{-5}$M	-	-
 Permethrin cyclopropane acid	-	+ LOIC $6 \pm 4 \times 10^{-5}$M IC50 $6.5 \pm 3.5 \times 10^{-4}$M	-	-

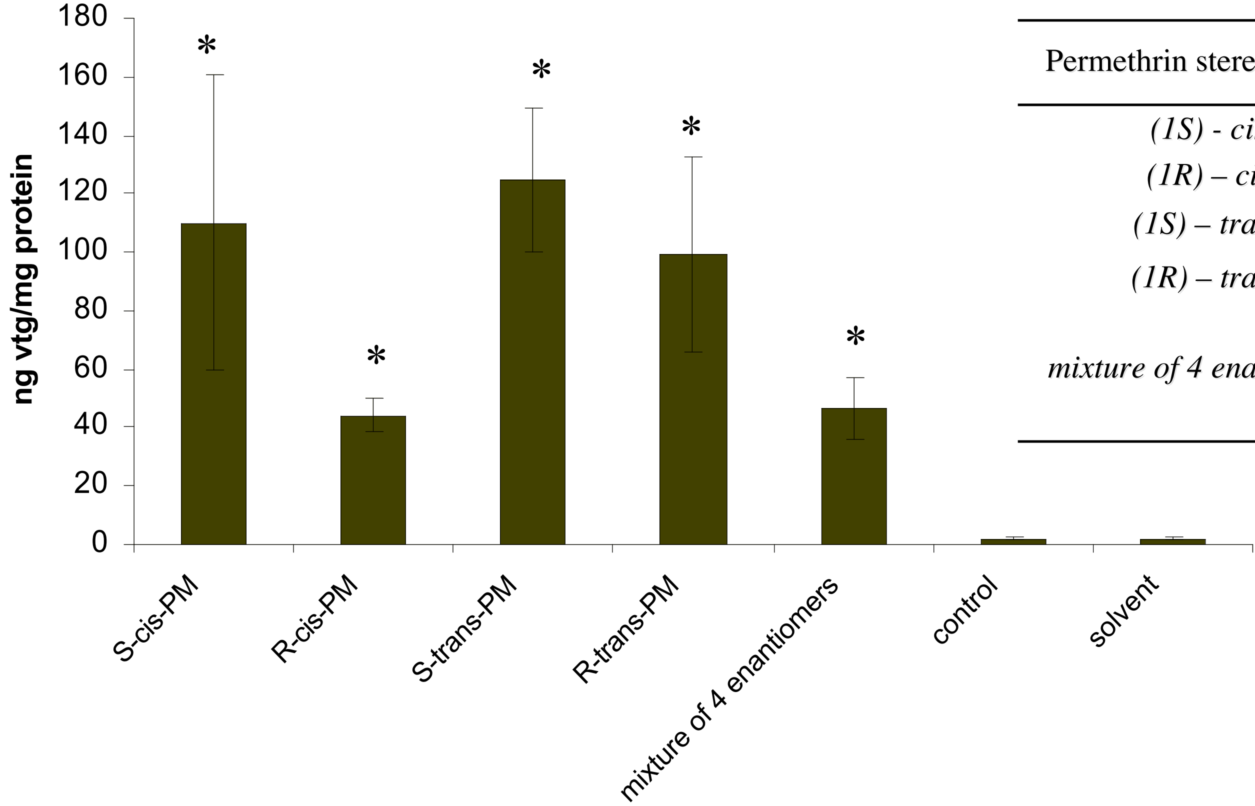
(Tyler et al., 2000, Environ Toxicol Chem, 19: 801-809)

Relative estrogenic potencies of pyrethroid parent compounds and degradation products:

Pyrethroid metabolite	Estrogenic activity (EC_{50}/ μM)	Relative potrency (estradiol = 1)
3-phenoxybenzyl alcohol	**6.67 ± 3.11**	**5×10^{-5}**
3-phenoxybenzaldehyde	**4.8 ± 3.42**	**7×10^{-5}**
3-phenoxybenzoic acid	**NA**	**-**
3-(4-hydroxy-3-phenoxy)-benzyl alcohol	**6.75 ± 2.28**	**5×10^{-5}**
3-(4-hydroxy-3-phenoxy)-benzoic acid	NA	-
N-3-(phenoxybenzoyl)glycine	NA	-

(McCarthy et al., 2006, J. Environ. Monit., 8: 197-202)

ENANTIOSELECTIVE ESTROGENIC ACTIVITY
- permethrin (*In vivo; Japanese medaka ELISA*)

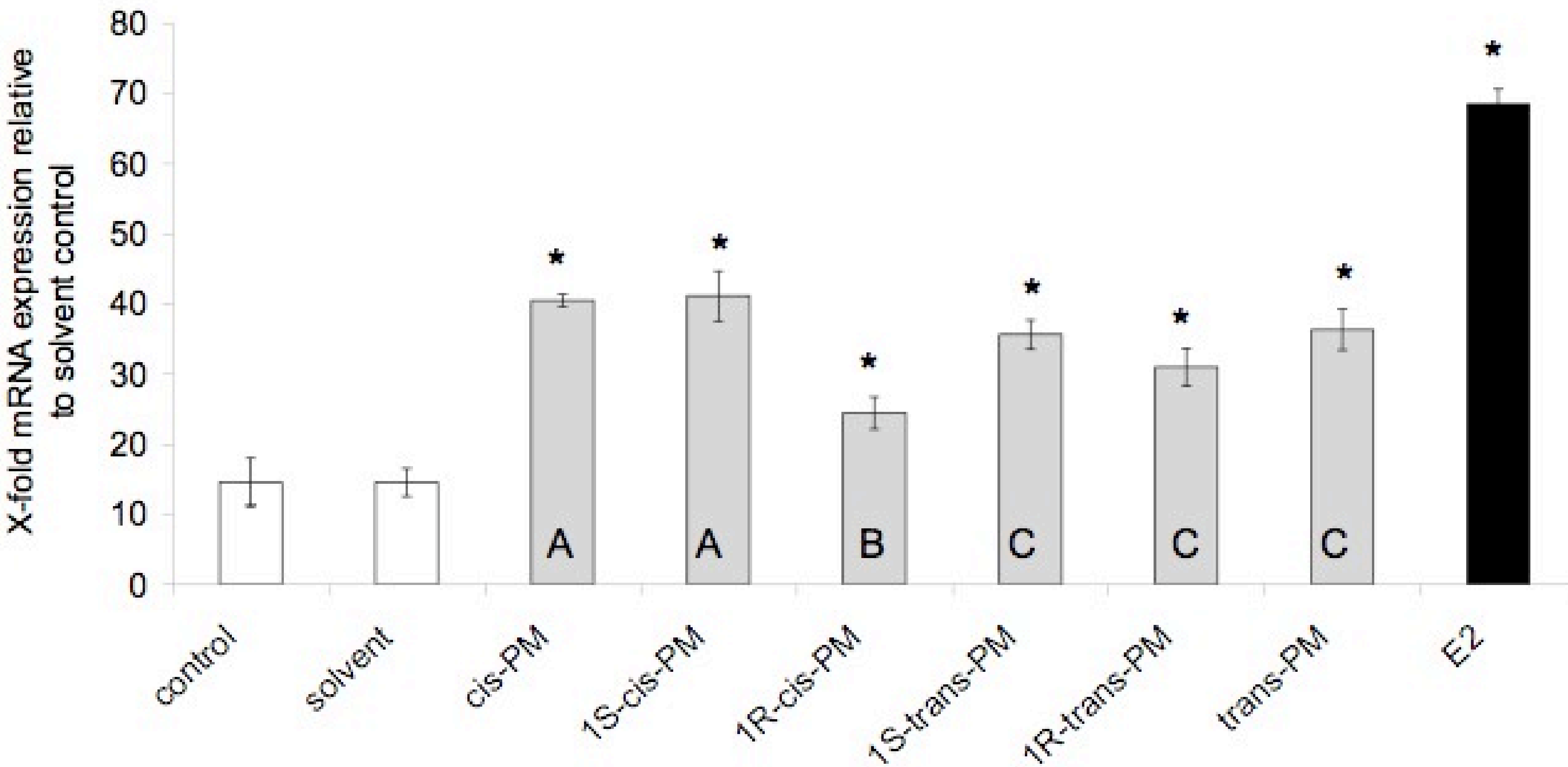


Permethrin stereoisomers	Induction vs. Control (fold ± SEM)
(1S) - cis	53.83 ± 24.73
(1R) – cis	21.71 ± 2.97
(1S) – trans	61.15 ± 12.06
(1R) – trans	48.49 ± 16.35
mixture of 4 enantiomers	22.78 ± 4.49

Values indicate mean ± SD; Treatment concentration = 10 ug/L; n = 3; positive control (E2) induction = $8.1 \times 10^3 \pm 3.3 \times 10^3$ ng Vtg/mg protein; E2 concentration = 0.10 μg/L); * Indicate significant difference from control (*P<0.05*); ** (*P<0.1*).

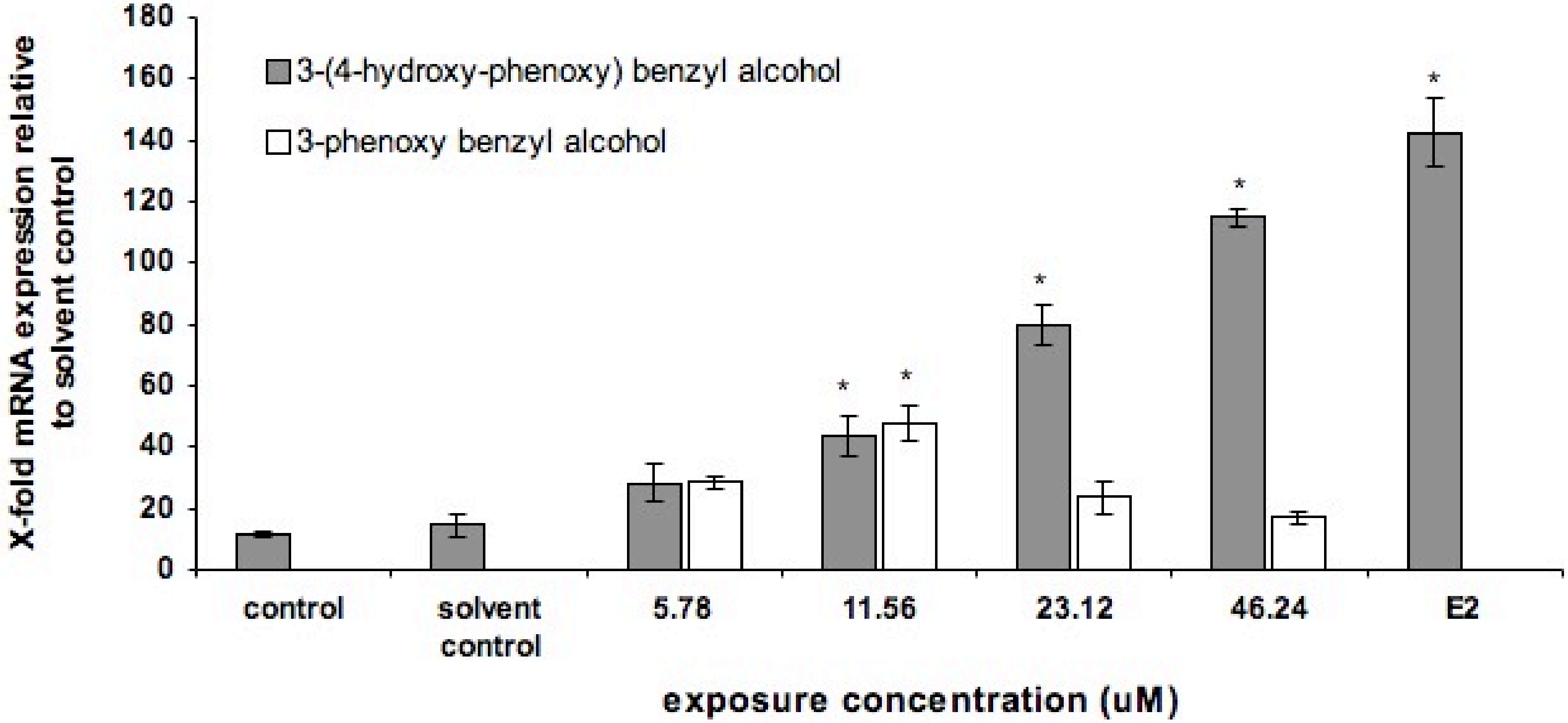
ENANTIOSELECTIVE ESTROGENIC ACTIVITY

- permethrin (*In vitro; primary hepatocytes*)



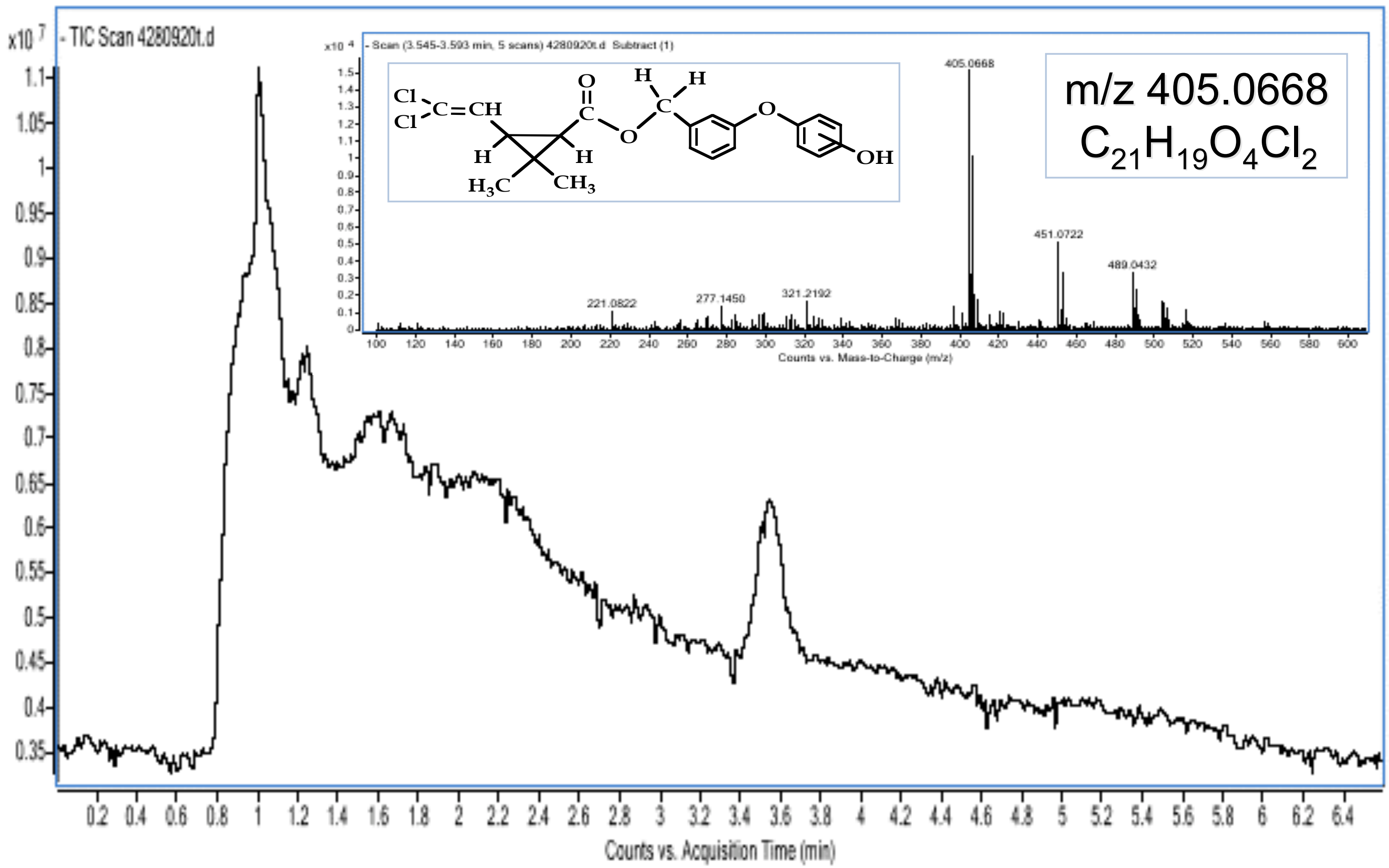
Values indicate mean ± SEM. Enantiomer concentration = 46 uM. Positive control (E2) concentration = 3.6 x 10^{-3} uM; solvent (acetone). Treatments between enantiomers that share the same letter are not significantly different (*P > 0.05*). * Indicate significant difference from solvent control (*P < 0.05*).

BIOACTIVATION TO ESTROGENIC METABOLITES



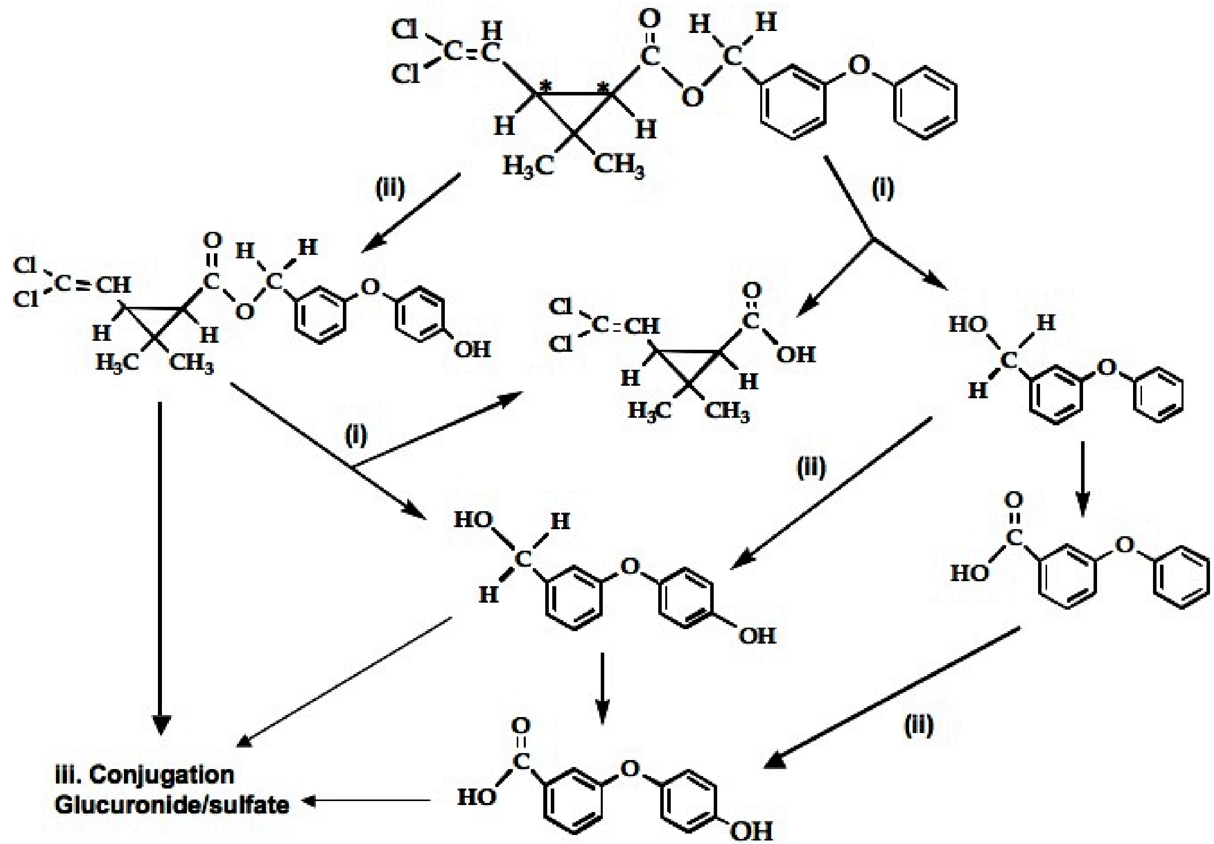
Values indicate mean ± SEM. Positive control (E2) concentration = 3.6 x 10^{-2} μM; solvent (acetone). * Indicate significant difference from solvent control ($p < 0.01$).

Permethrin - Enantioselective Biotransformation



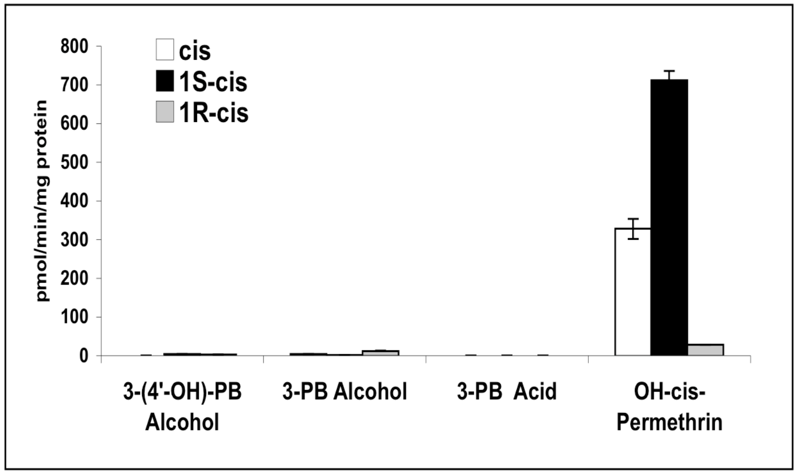
LC/MS TOF trace with accurate mass and molecular formula of major metabolite from *1S-cis*-permethrin metabolism in trout liver microsome. The most probable structure of metabolite is indicated. (Note: LC/MS trace and mass prectra of metabolite from *1S-trans*-permethrin metabolism is identical to the figure shown here).

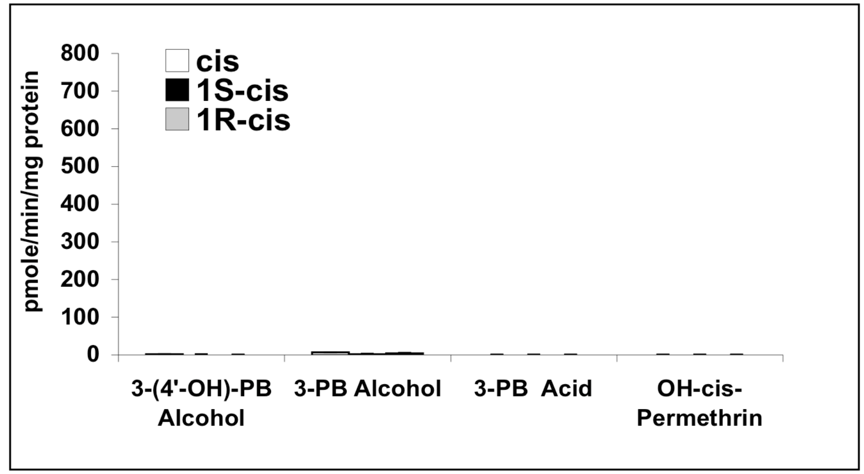
STEREOSELECTIVE BIOACTIVATION TO ESTROGENIC METABOLITES

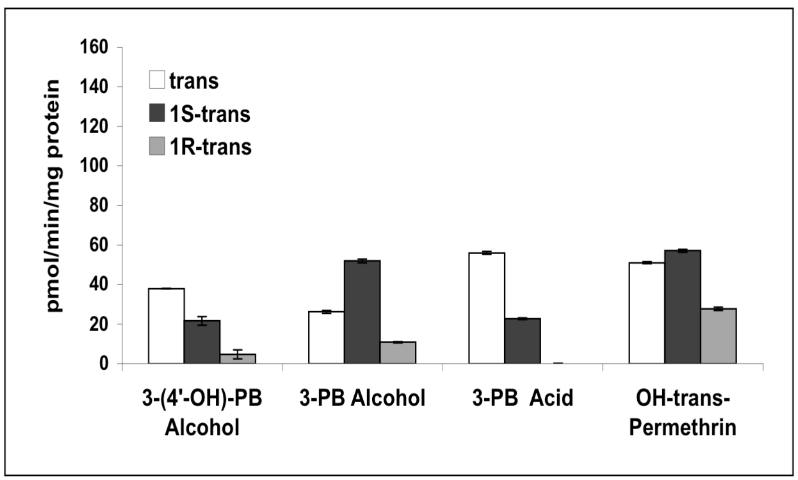


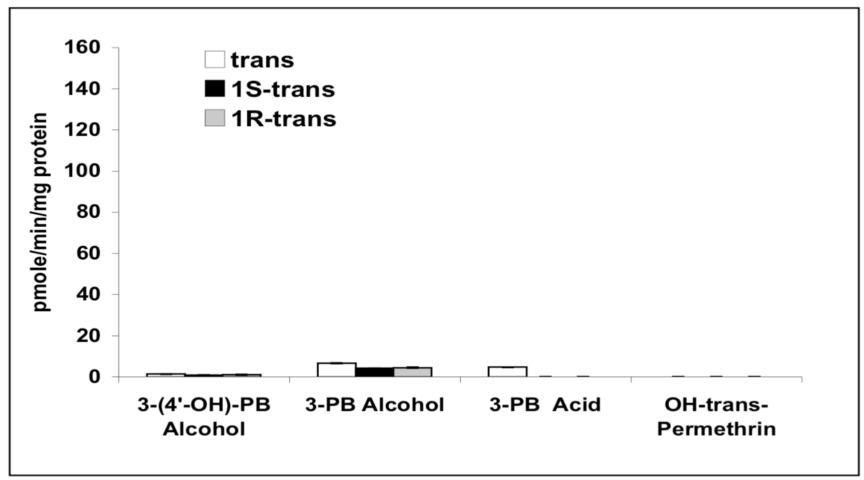
Biotransformation pathway of permethrin in fish

STEREOSELECTIVE BIOACTIVATION TO ESTROGENIC METABOLITES









NO INHIBITOR

KETOCONAZOLE INHIBITION

SUMMARY

• *In vivo* and *in vitro* evaluation in fish indicate stereoselective estrogenic activity of permethrin.

• The *1S*-*cis*-PM was more estrogenic than the *1R*-*cis* enantiomer. Similar trends have been observed in *trans*-PM enantiomers.

• PM biotransformation in trout liver microsome is more oxidative than hydrolytic.

• *trans*-PM was more susceptible to ester cleavage than *cis*-PM. *1S*-*trans*-PM was hydrolyzed more extensively than the *1R*-*trans* enantiomer.

SUMMARY

• *cis*-PM was more susceptible to hydroxylation than *trans*-PM. *1S*-*cis*-PM was hydroxylated more extensively than *1R*-*cis*-PM.

• Respective inhibition studies with ketoconazole confirmed that permethrin enantiomer hydroxylation is CYP-catalyzed.

• Results highlights the potential for target-inactive enantiomer(s) of chiral bioactive compounds to produce unintended effects in non-target species.

Delta Conclusions

- Individual sites carry unique TIE estrogenic signatures.
- Estrogenic activity was consistently observed in water extracts and whole water from surface waters.
- While several pesticides and surfactants were observed in fractions with estrogenic activity, reconstitution to environmental concentrations failed to demonstrate estrogenic activities observed in extracts or whole water exposures.

Delta Conclusions Cont

- Additional "unknown" estrogenic chemicals are likely sources of estrogenic activity particularly in systems receiving agricultural effluents/runoff.
- Greater than additive responses were observed in surfactant/herbicide, but in high surfactant concentrations.

Questions??