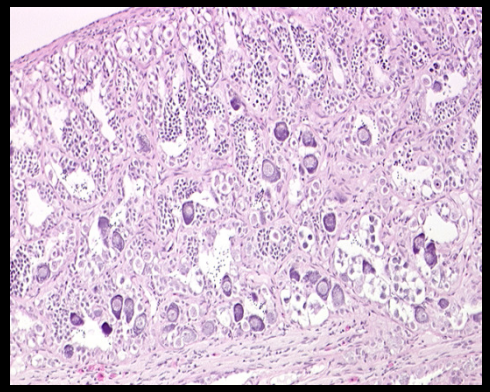
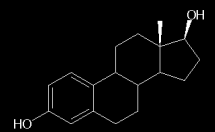
Application of *in vitro* and *in vivo* bioassays to facilitate the identification of EDC sources in environmental waters: a brief history of intersex research in the Potomac watershed





Luke R Iwanowicz

USGS, Leetown Science Center, National Fish Health Research Laboratory, Kearneysville, WV

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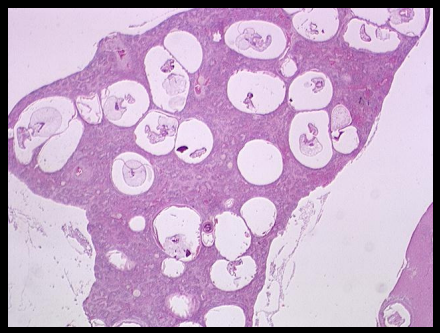
South Branch of the Potomac River 2002-2003

- Fish kill reported in the South Branch of the Potomac River during 2002
- Variety of species affected, but primarily Centrarchids
- The diversity of opportunistic pathogens suggested environmental stressors that may lead to immunosuppression
- Complete histological examination led to an unexpected finding.



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Blazer et al (2007) Intersex (Testicular Oocytes) in Smallmouth Bass *Micropterus dolomieu* From the Potomac River and Selected Nearby Drainages. Journal of Aquatic Animal Health. 19.242-253.

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Pollution Blamed for Intersex Fish

By The Associated Press
posted: 21 December 2004 08:59 am ET

Male fish bear eggs in Potomac

Sewage or factory effluent may be cause of 'intersex' abnormality

Tuesday, December 21, 2004 Posted: 10:14 AM EST

SHARPSBURG, Maryland (AP) -- Male fish that are growing eggs have been found in the Potomac River near Sharpsburg, a sign that a little-understood type of pollution is spreading downstream from West Virginia, a federal scientist says.

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Intersex Fish Linked To Population And Agriculture In Potomac River

ScienceDaily (Feb. 11, 2008) — For several years, scientists have been working to determine why so many male smallmouth bass in the Potomac River basin have immature female egg cells in their testes - a form of intersex. They are closer to finding an answer.

Smallmouth bass. Scientists have found new clues as to why so many male smallmouth bass in the Potomac River basin have immature female egg cells in their testes - a form of intersex. (Credit: iStockphoto)

See also:

Plants & Animals
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- Agriculture and Food

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- Ecology
- Recycling and Waste
- Sustainability

Research by the U.S. Geological Survey (USGS) shows that a high incidence of intersex occurs in the Potomac watershed at sites where farming is most intense and where human population density is highest. The study also shows the greatest prevalence of this form of intersex, known as testicular oocytes (TO), occurs in the spring, just before and during the spawning season.

"We collected smallmouth bass

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Intersex Fish Are Found at High Rate in a Region

By THE ASSOCIATED PRESS
Published: September 7, 2006

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'Intersex' Fish in Potomac River Raises Questions of Impact on Humans

Wednesday, September 06, 2006
Associated Press

FOX NEWS.com

Study Links Population to Intersex Fish

February 8, 2008 - 6:13pm

BALTIMORE (AP) - A form of intersex fish, which have both male and female traits, were found more often by researchers in areas with more farming and population density, according to a new study by the U.S. Geological Survey.

So-called intersex fish have been found in U.S. waters over the past decade, including the southern Great Lakes, the Potomac River watershed, which includes the Eastern Panhandle in West Virginia, and the Southern California coast. The cause isn't fully understood, but researchers suspect wastewater and farm runoff polluted with chemicals that stimulate estrogen production are at fault.

U.S. Geological Survey researchers found the frequency of male smallmouth bass with immature female eggs in their testes was highest where farming is most intense and where human population density is highest. The study also found the prevalence of the form of intersex, known as testicular oocytes, was greatest just before and during the spring spawning season.

The results were published in the current edition of the Journal of Aquatic Animal Health.

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In W.Va., Some Male Fish Lay Eggs

Posted 2/10/2005 06:00 AM

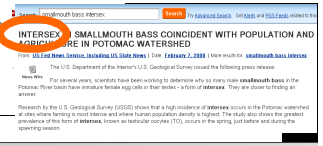
Researchers are trying to figure out why this is happening in the South Branch of the Potomac River.

Potomac's 'Intersex' Fish Raise Pollution Fears

September 6, 2006

Release from: David A. Fahrenthold
The Washington Post

WASHINGTON - Abnormally developed fish, possessing both male and female characteristics, have been discovered in the Potomac River in the District and in tributaries across the region, federal scientists say -- raising alarms that the river is tainted by pollution that drives hormone systems haywire.



INTERSEX ... SMALLMOUTH BASS COINCIDENT WITH POPULATION AND AGRICULTURE IN POTOMAC WATERSHED

Intersex Fish Study Finds Endocrine Disruptors In Potomac Basin

January 18, 2007

Release from: Associated Press

CHARLESTON, W.Va. - Scientists investigating intersex fish in the Potomac River basin have found endocrine-disrupting contaminants in several tributaries, including pesticides, flame retardants and personal-care products, the U.S. Geological Survey said.

One substance, the fungicide hexachloro-benzene, has been banned from use in the United States, the USGS said in a report released Wednesday.

The daily journal of life in and around water
UnderwaterTimes.com

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Report Intersex Fish Linked to Human Pollution; 'All Samples' Contained Endocrine-disrupting Compounds

Underwatertimes.com News Service

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Reston, Virginia (Jan 17, 2007 18:20 EST) The discovery of a high incidence of intersex (male fish exhibiting female characteristics) in smallmouth bass of the Potomac River Basin has prompted an investigation of water quality and wastewater discharge into the upper Potomac, and blood-plasma studies on this fish species. Recently, U.S. Geological Survey (USGS) scientists found pesticides, flame retardants, and personal-care products containing known or suspected endocrine-disrupting chemicals in several tributaries to the Potomac River and in the smallmouth bass that inhabit them.

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ENVIRONMENT

What Do 'Intersex' Fish Mean for Water Quality?

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Talk of the Nation, September 15, 2006 · Fish with both male and female sex traits are found in the Potomac River. Fish pathologist Vicky Blazer talks about whether hormones in the water might be to blame.

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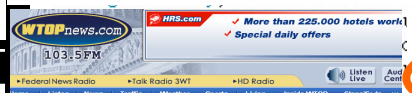
Male Fish Producing Eggs in Potomac River

William Cocke
for National Geographic News
November 3, 2004

Something fishy is happening in the headwaters of the Potomac River. Scientists have discovered that some male bass are producing eggs—a decidedly female reproductive function.

WTOPnews.com 103.5FM



Fairfax Wants Help Solving Intersex Fish Issue

abcNEWS start here

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U.S.

Intersex Fish Found in Potomac River

Pollution Fears to Cause Mutation

By LAURA VARQUEZ
Sept. 6, 2006

ABCNEWS

PRINT | EMAIL | LINK

Intersex bass a problem in Potomac.(SPORTS) (OUTDOORS)

Washington Times, The, February, 2007

PREMIUM

Content provided in partnership with HighBeam RESEARCH

Byline: Gene Mueller, THE WASHINGTON TIMES ANNAPOLIS - During a Black Bass Roundtable a yearly meeting comprising local anglers, fishing guides, bass federation members and a host of Maryland Department of Natural Resources biologists the top item easily was a subject on every freshwater angler's mind these days: the intersex of smallmouth bass in the Potomac River watershed.

What is Intersex?

An **abnormal** condition characterized by the presence of male and female reproductive components in a gonad that should be exclusively male OR female.

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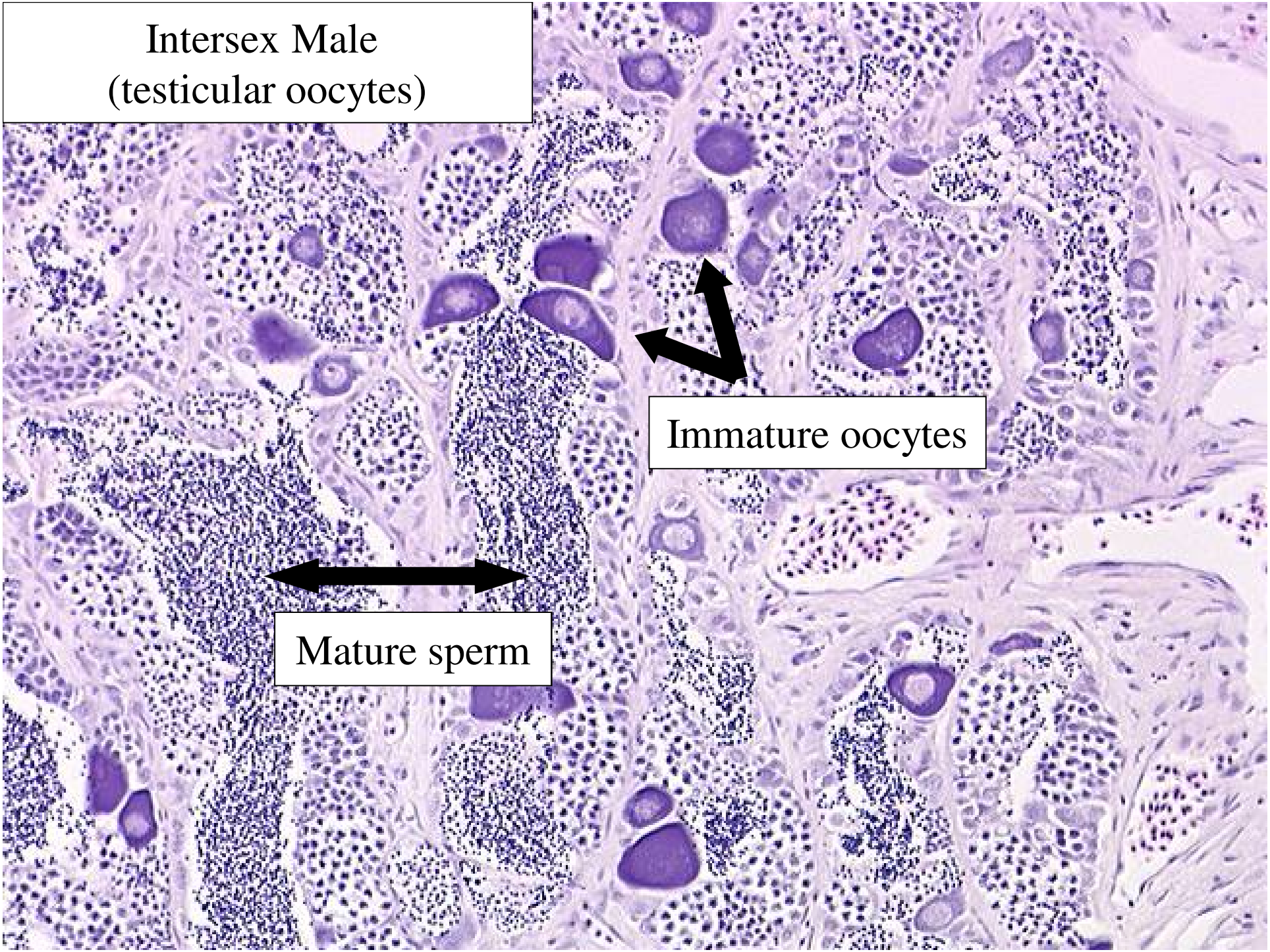
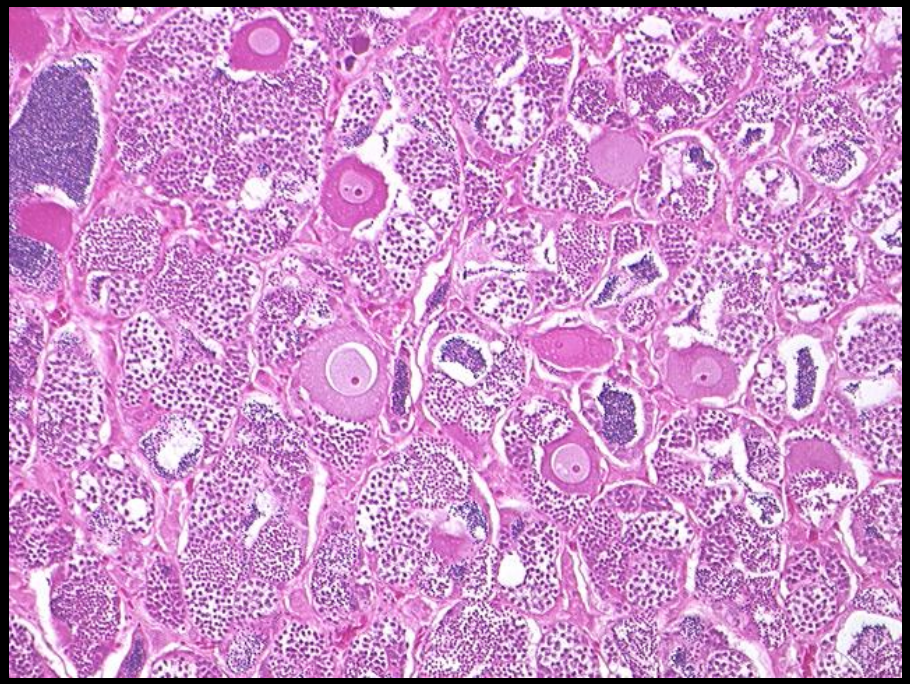

Intersex Male
(testicular oocytes)
Immature oocytes
Mature sperm

Intersex in Gonochorist Fishes



- Immature oocytes within testes
- Used as an indicator/ biomarker of exposure to estrogenic compounds
- Probably induced early in development, but can possibly occur later

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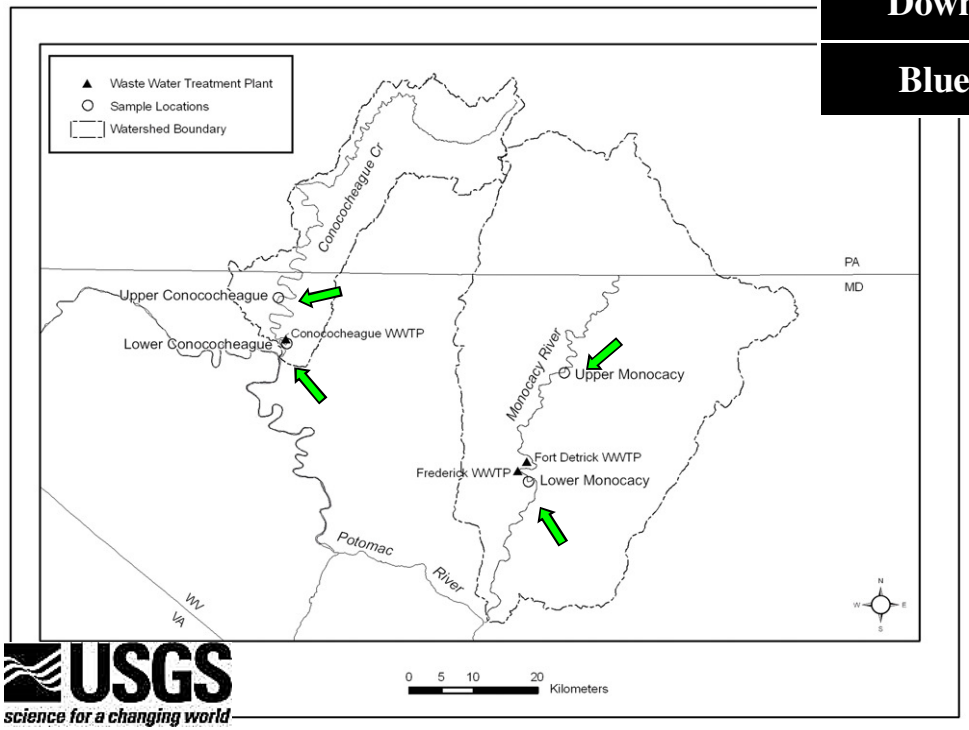
USFWS collaborative project 2005-2006

Effects of WWTP

Site	Prevalence TO	TO Severity Index	Mean Vitellogenin Conc. mg/ml*
Conococheague Upstream	100% [A]	2.1 ± 0.3[A]	0.117 ± 0.051[A]
Conococheague Downstream	90% [A]	1.8 ± 0.4[A]	0.050 ± 0.036[B]
Monocacy Upstream	82%[a]	1.2 ± 0.3[a]	0.059 ± 0.010[a]
Monocacy Downstream	100%[a]	1.9 ± 0.3[a]	0.143 ± 0.085[a]
Blue Plains	23%	0.2 ± 0.2	0.577 ± 0.165



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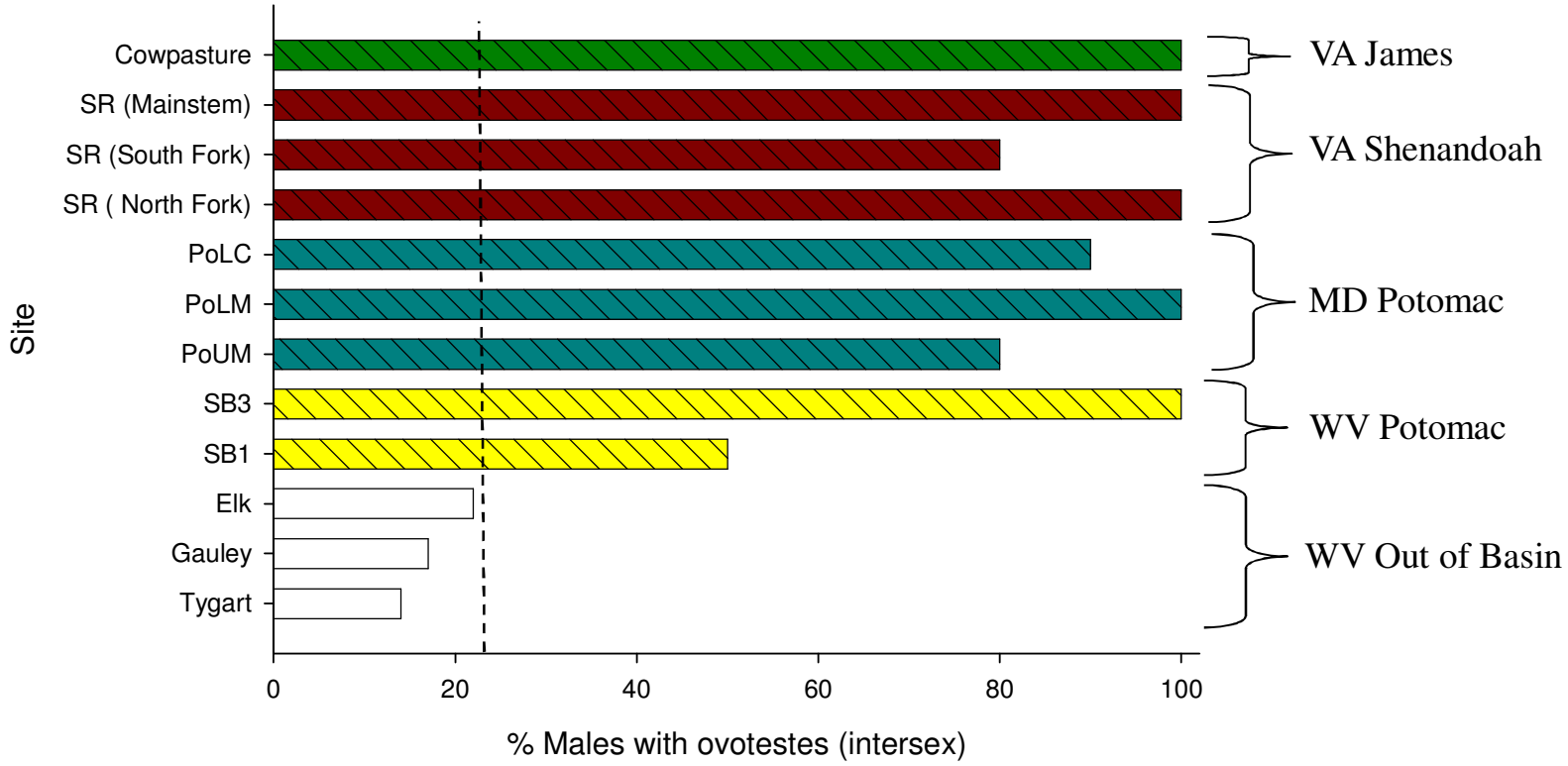
- Sample above and below WWTP
- Elevated Vitellogenin
- Species differences
- Considerable agriculture influence
- EDCs identified, Estrogenic activity

Iwanowicz et al (2009) *Environ Toxicol Chem* 28: 1072-1083
Alvarez et al (2009) *Environ Toxicol Chem* 28: 184-1095

Regional Prevalence of Intersex



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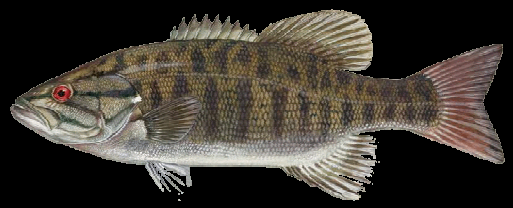
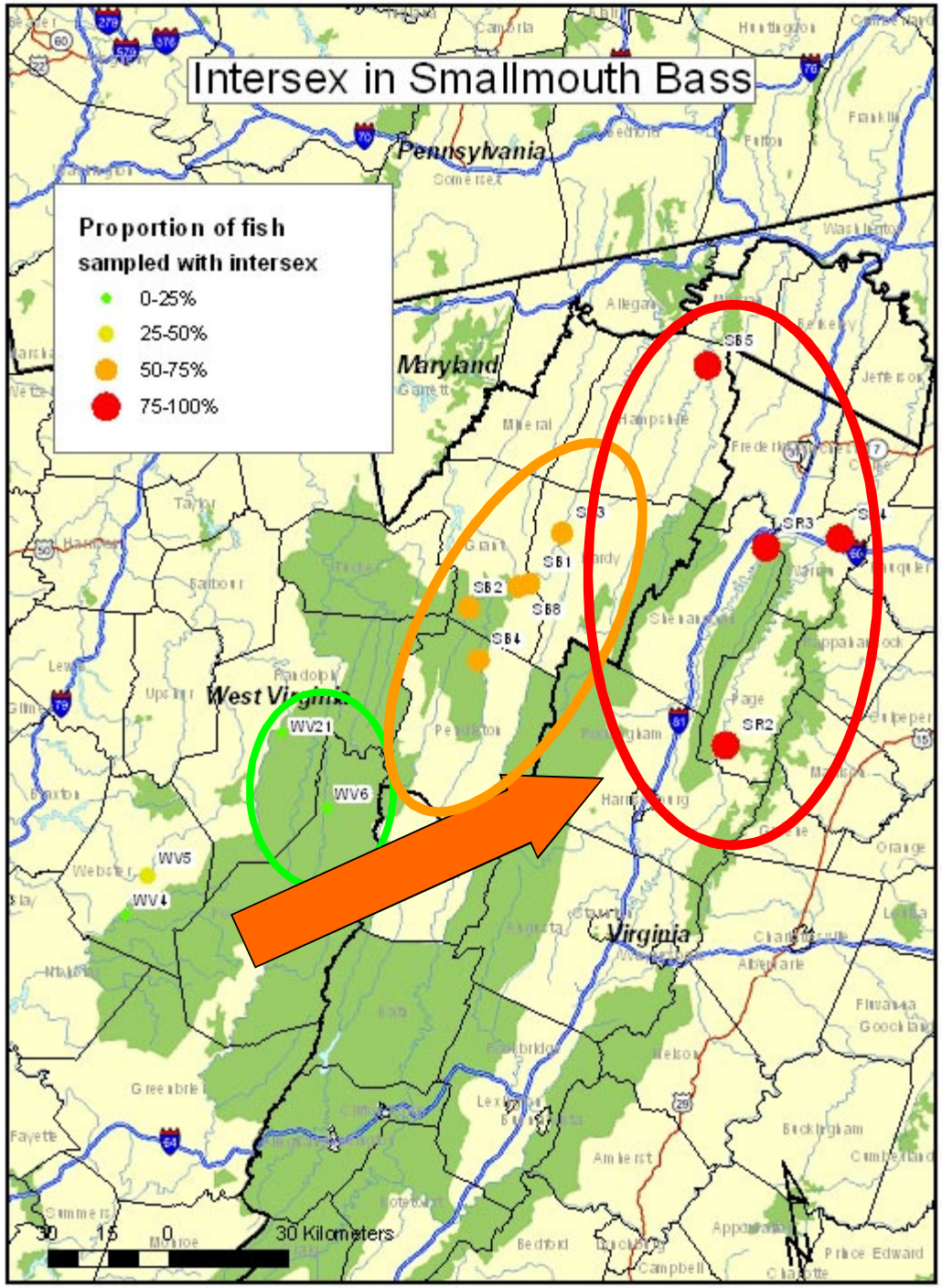
Positive correlation between prevalence/ severity of intersex and human population density/ agriculture land-use





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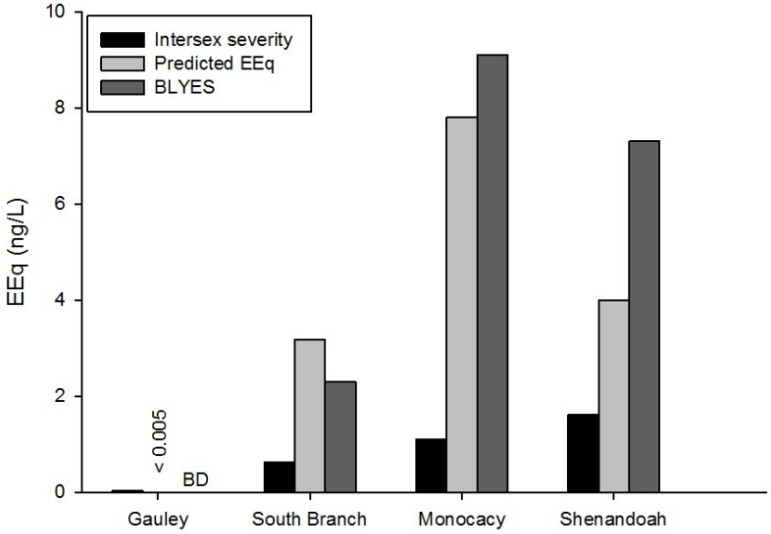
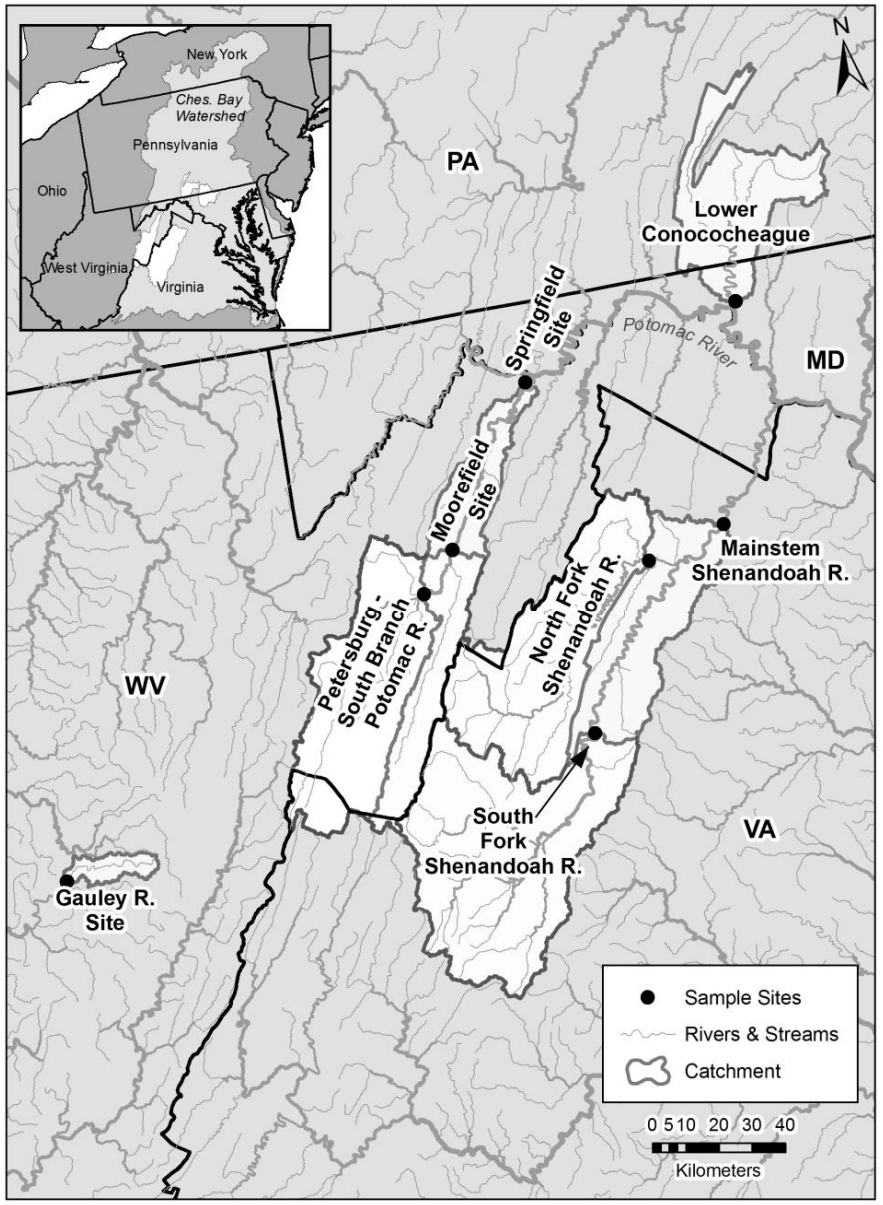
Blazer et al (2007) *J Aquat Anim Health* 19:242-253.

Spawning Study 2007



Compound	Gauley	SB	Mon	Shen
17a-estradiol	--		Y	Y
17b-estradiol	--	Y	Y	
estrone	--	Y	Y	Y
androstenedione	Y	Y	Y	Y



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Blazer et al. (*2012)* Environmental Monitoring and Assessment.

Are there EDCs and emerging chemicals present?

104 of the 158 chemicals measured had a least one detection.
31 pesticide compounds (25 insecticides, 5 herbicides, 1 fungicide)
22 PAHs
10 prescription pharmaceuticals
7 antibiotics
7 flavors and fragrances
7 industrial wastewater compounds
6 nonprescription pharmaceuticals
4 hormones
3 household wastewater compounds
3 detergent metabolites
2 plant/animal sterols
2 flame retardants

Chemical group	Gauley	South Branch	Monocacy	Shenandoah
herbicides	2	3	5	4
insecticides	14	14	22	20
antibiotics	0	5	7	8
Flavors/fragances	3	3	7	5
Industrial (waste water)	5	5	6	6
Nonprescription	1	1	3	3
Prescription	0	2	9	5
PAH	5	6	18	15
Household (waste water)	3	2	3	2
Detergent metabolites	3	0	0	3
Flame retardants	1	1	2	2
hormones	1	3	4	3
sterols	1	0	1	1

Kolpin et al. (*In press)* Sci. Tot. Environ

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Associations of Land-use with Intersex

(Spawning Study 2007)

Site	Human Density[1]	WWTP[2]	WWTP Flow[3]	% Ag[4]	AFO[5]	Animal Numbers[6]	Intersex[7]
Gauley River	0.06	0	0	0.5	0	464	11.3% 0.02 (0.07)
South Branch Petersburg	0.07	3	0.95	16.4	296 (296)	1,450,120	74.3% 0.97 (0.95)
South Branch Moorefield	0.07	4	1.43	15.2	497 (496)	7,384,685	54.5% 0.50 (0.50)
South Branch Springfield	0.08	5	1.93	15.2	565 (562)	8,719,093	82.2% 1.02 (0.76)
Shenandoah North Fork	0.28	50	1.59	32.7	1,174 (960)	11,757,596	90.0% 1.16 (0.78)
Shenandoah Mainstem	0.43	101	25.66	32.6	3,655 (2,539)	33,928,442	93.0% 1.64 (0.93)
Shenandoah South Fork	0.56	19	20.84	35.9	2,029 (1,176)	14,788,173	100.0% 1.83 (0.65)
Conococheague Creek (lower)	0.69	13	8.31	50.3	10 (1)	1,819,225	87.5% 1.03 (0.78)

Land-use Characteristics	Intersex Prevalence		Intersex Severity	
	r^2	*p*	r^2	*p*
Human population density	0.39	0.10	0.42	0.08
Number of WWTP	0.22	0.24	0.34	0.13
WWTP Flow	0.32	0.15	0.63	0.02
Percent agriculture	0.63	0.02	0.50	0.05
Tot. Animal Feeding Operations	0.28	0.17	0.56	0.03
Poultry houses	0.27	0.18	0.50	0.05
Total animals	0.27	0.18	0.48	0.06
Animal density	0.49	0.05	0.58	0.03

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Blazer et al. (*In press)* Environ Mon Assess.

Bioactivity of chemical extracts from water samples

Polar Organic Chemical Integrative Sampler (POCIS)
Semipermeable Membrane Device (SPMD)

Grab water sample
OASIS HLB

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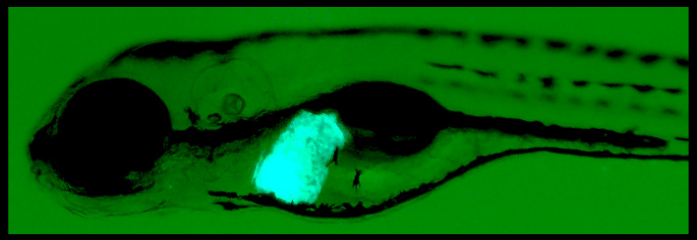
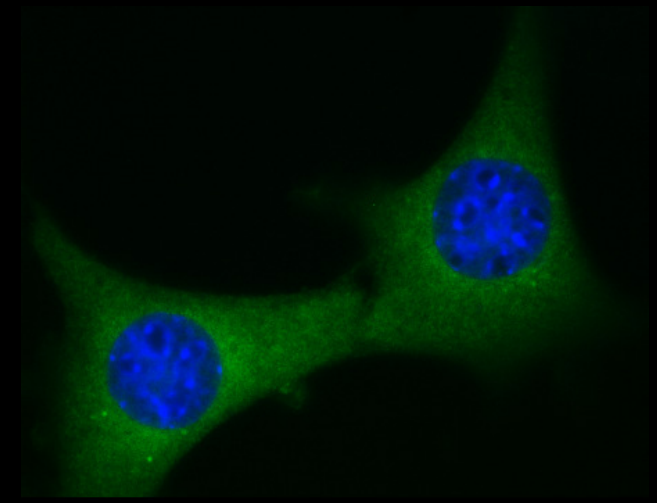
Development and Application of *in vitro/ vivo* based screening

(Collaborative Research)





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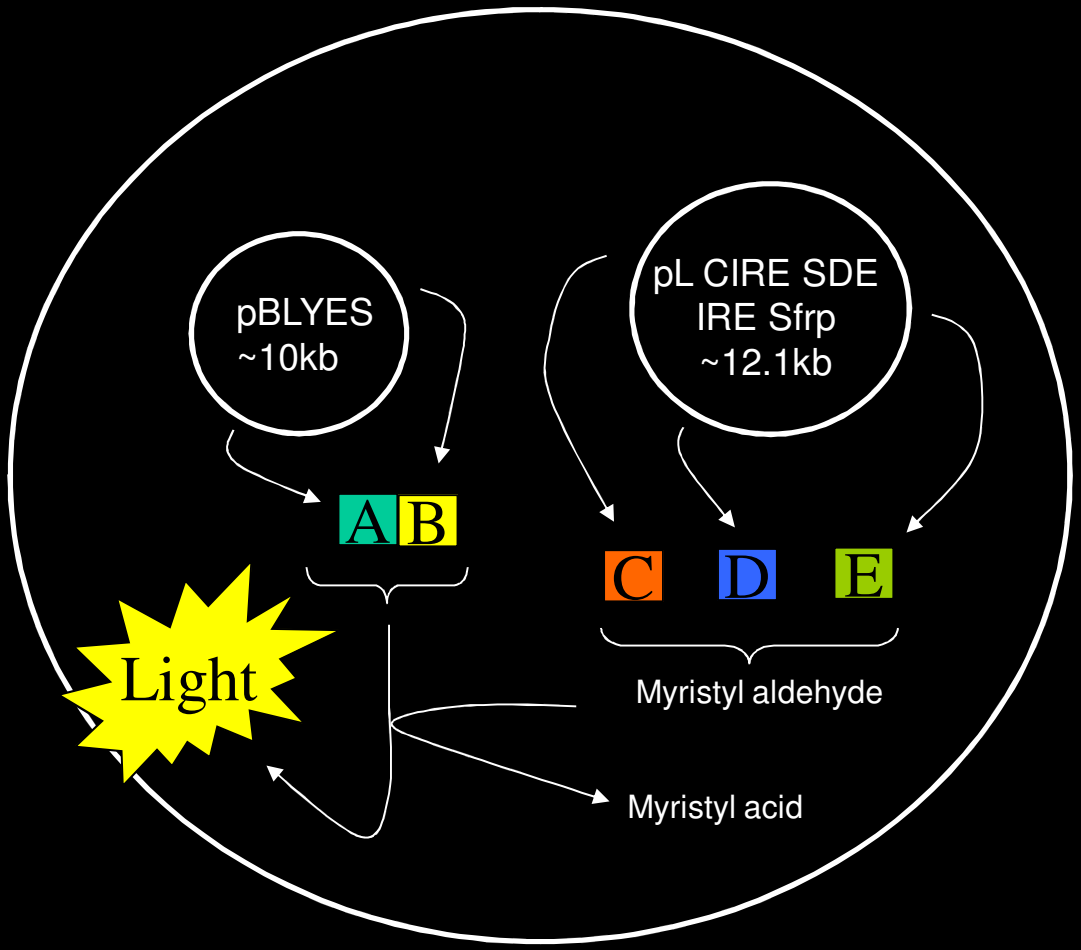
BLYES Reporter System

'Estrogenicity'

Grab water or POCIS extracts screened using a yeast reporter system

Light measured as an endpoint

Available reporters designed to measure estrogens/ androgens



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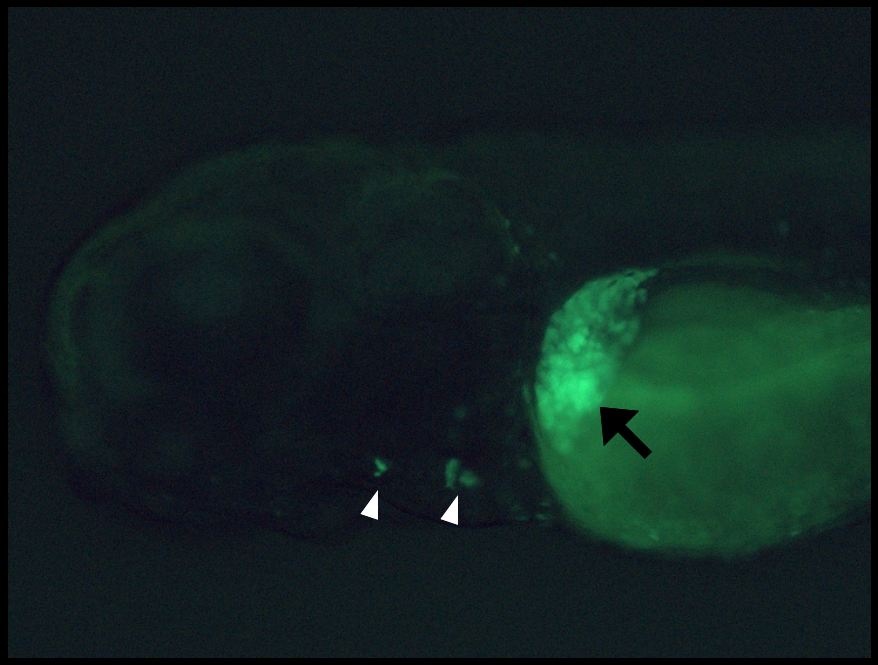
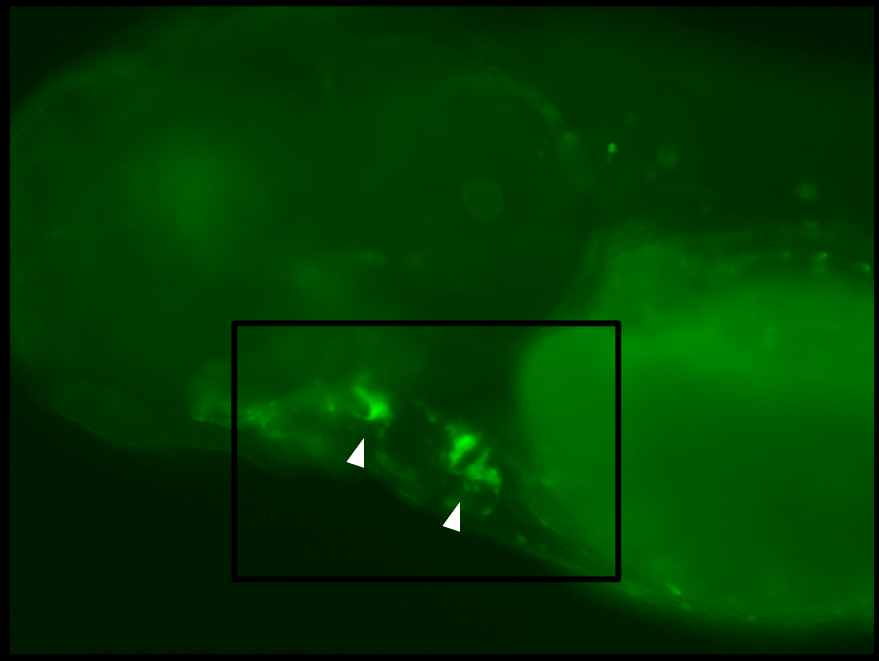
Sanseverino et al. (2005). Applied and Environmental Microbiology, p. 4455-4460, Vol. 71, No. 8

Tissue-specific responses to environmental estrogens



Mill Creek
passive sampling



Hawksbill Creek
grab sampling

16 of 19 site samples activated reporter
3 displayed preference for heart valves

D. Gorelick
M. Halpern

Collaboration with NCI (Stavreva & Hagar)
In press; Scientific Reports

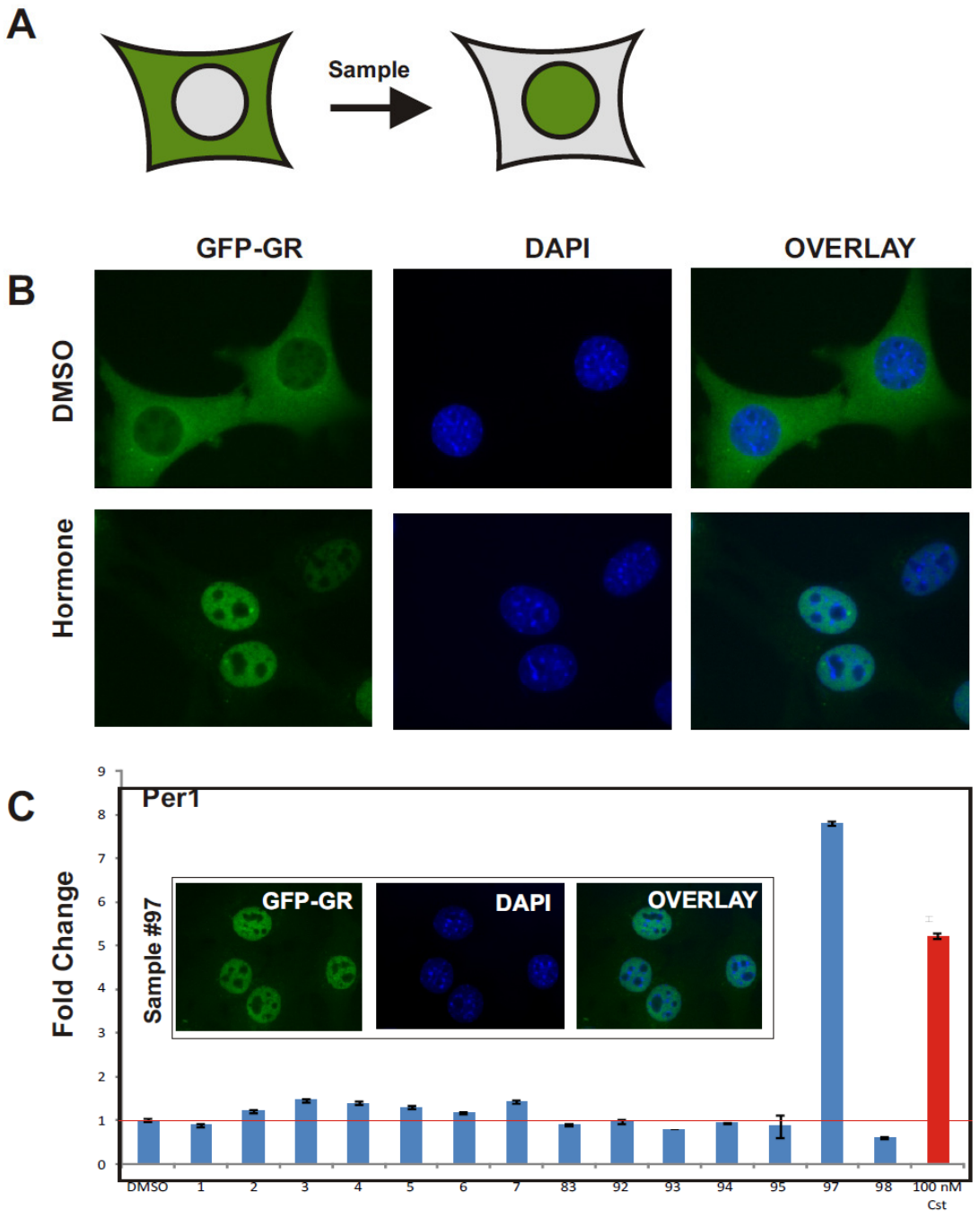
- Translocation assays

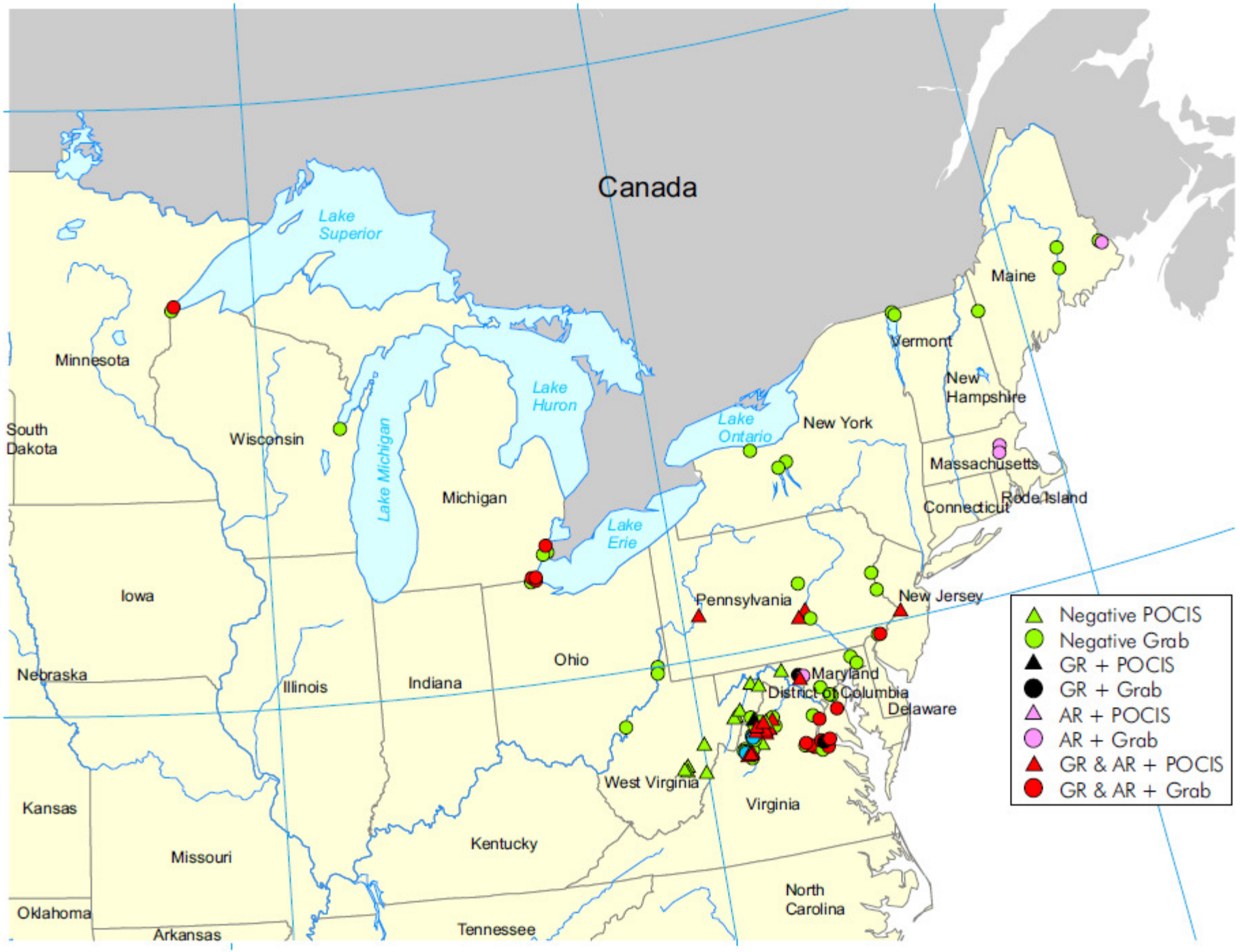
- Allow for high-throughput screening

- Identification of bioactive site prior to investment in chemical analysis

- Have already identified GR agonists at numerous sites

- Beyond estrogen-centric research

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Negative POCIS
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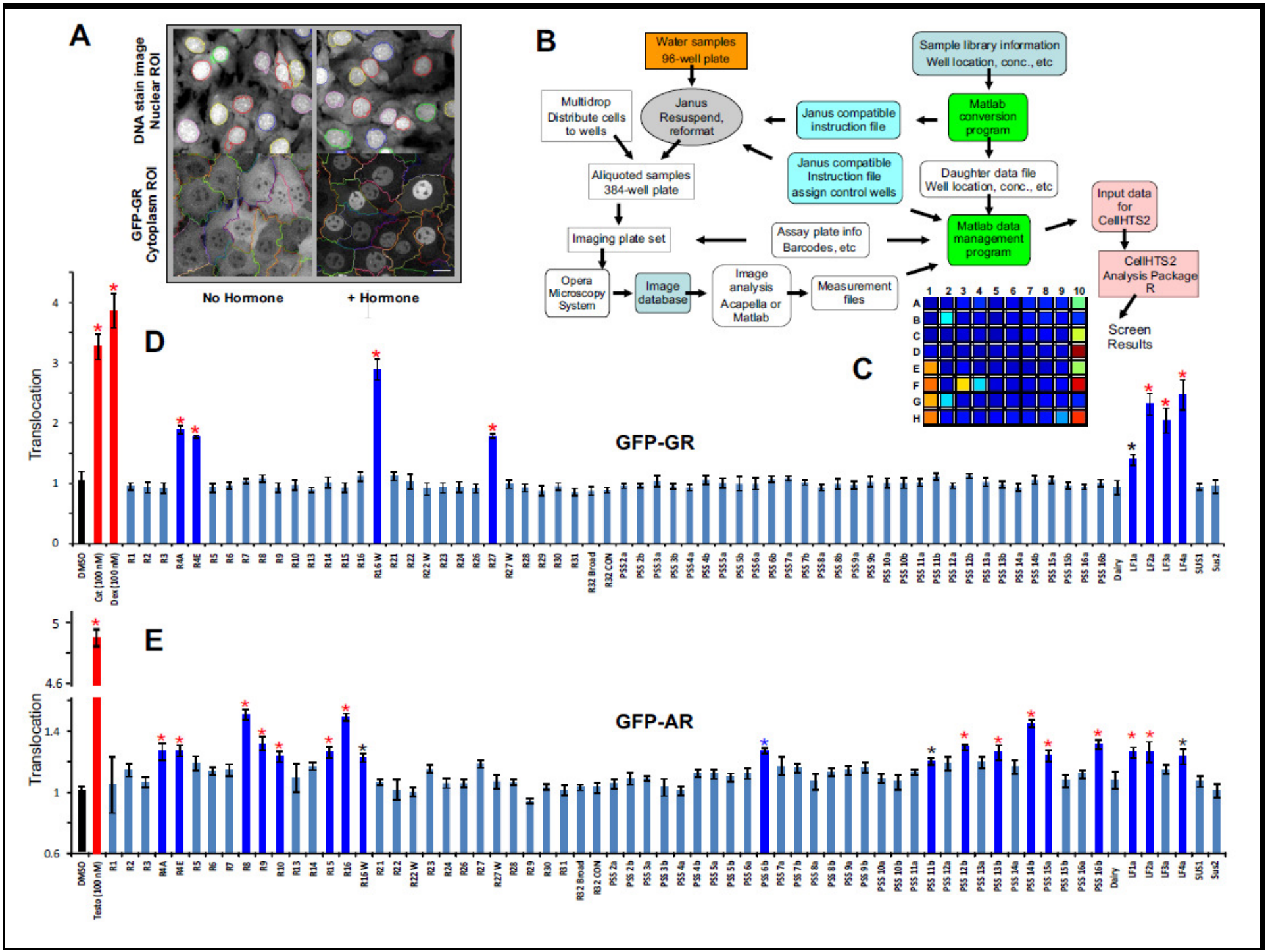
AR + Grab
GR & AR + POCIS
GR & AR + Grab


A
DNA stain image
Nuclear ROI
GFP-GR
Cytoplasm ROI
No Hormone
+ Hormone
B
Water samples
96-well plate
Multidrop
Distribute cells
to wells
Janus
Resuspend,
reformat
Aliquoted samples
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Janus compatible
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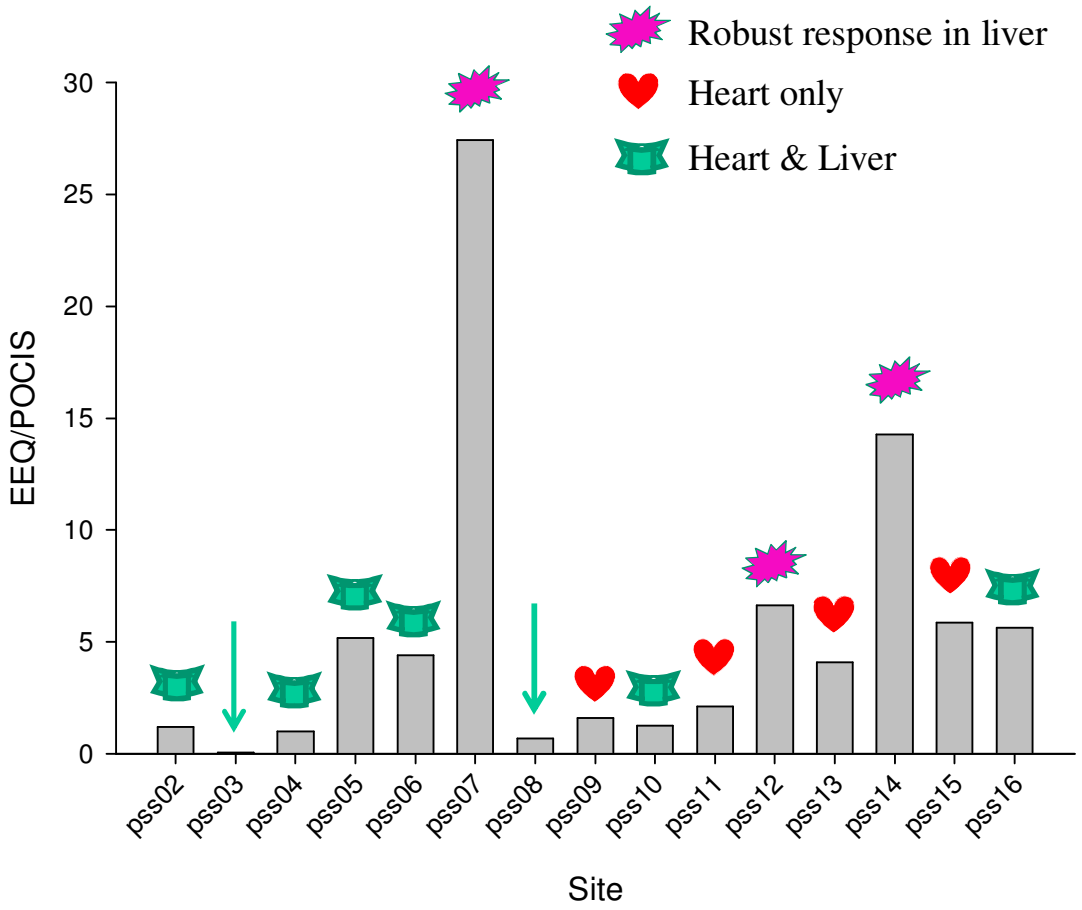
Matlab data
management
program
Input data
for
CellHTS2
CellHTS2
Analysis Package
R
Screen
Results
C
D
GFP-GR
Translocation
E
GFP-AR
Translocation

In vivo danio reporter (E2) bioassay

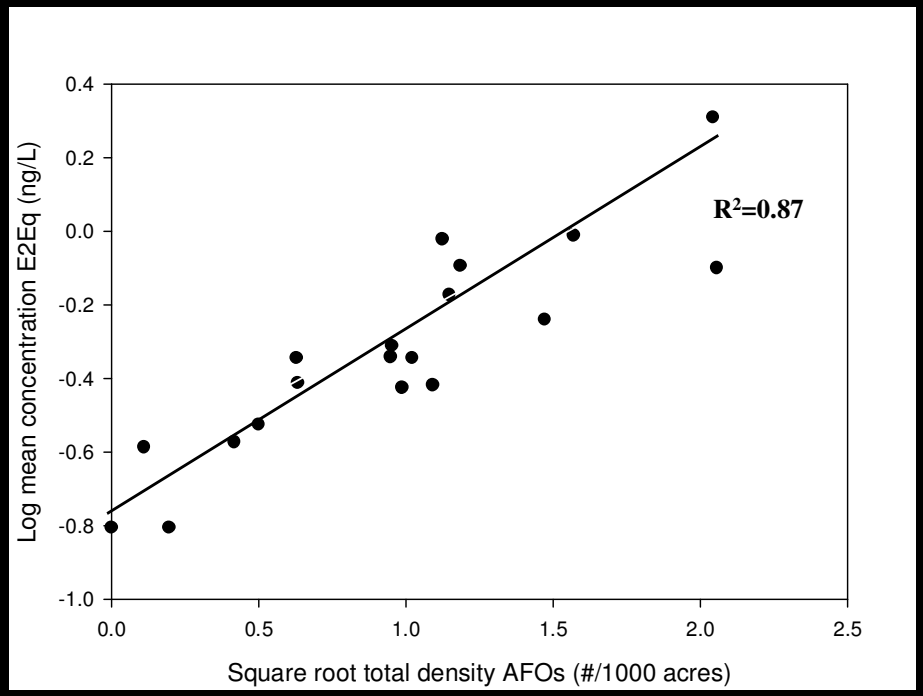
Biological relevance

ERα in heart
ERβ in liver

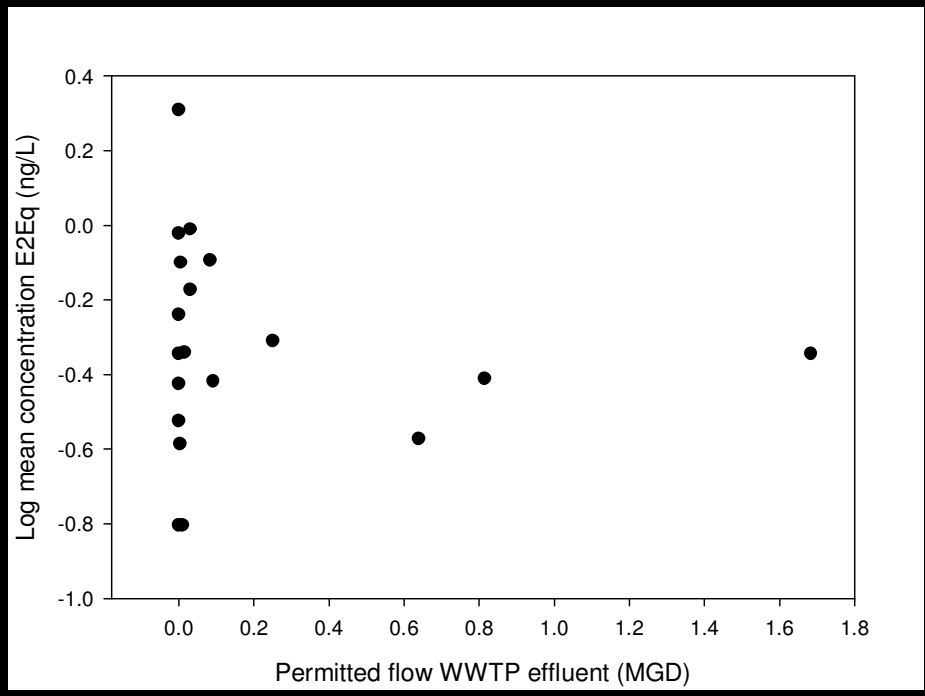


Estrogenicity (EEQ) of Water Extracts Smaller Tributaries of Shenandoah Drainage 2009

Agriculture



WWTP Effluent



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Ciparis et al. (*2012) Sci Tot Environ*

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Summary

- A number of EDCs and emerging contaminants have been identified in the Potomac River Watershed
- They are not exclusively associated with WWTPs; in fact, agricultural activities correlate better with estrogenicity
- Their effects on fish populations in the Potomac Watershed is unclear, but we suspect they may partially explain the increased observation of fish kills and intersex
- To date experimental sampling design has not been specifically tailored for source tracking, but this is a critical next step to make informed management decisions
- Intensive sampling and complimentary end-point methods required

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