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US EPA CSS-HERA Board of Scientific Counselors Chemical Safety Subcommittee Meeting

US EPA CSS-HERA BOSC Meeting – February 2-5, 2021

CSS Session 1C Slides

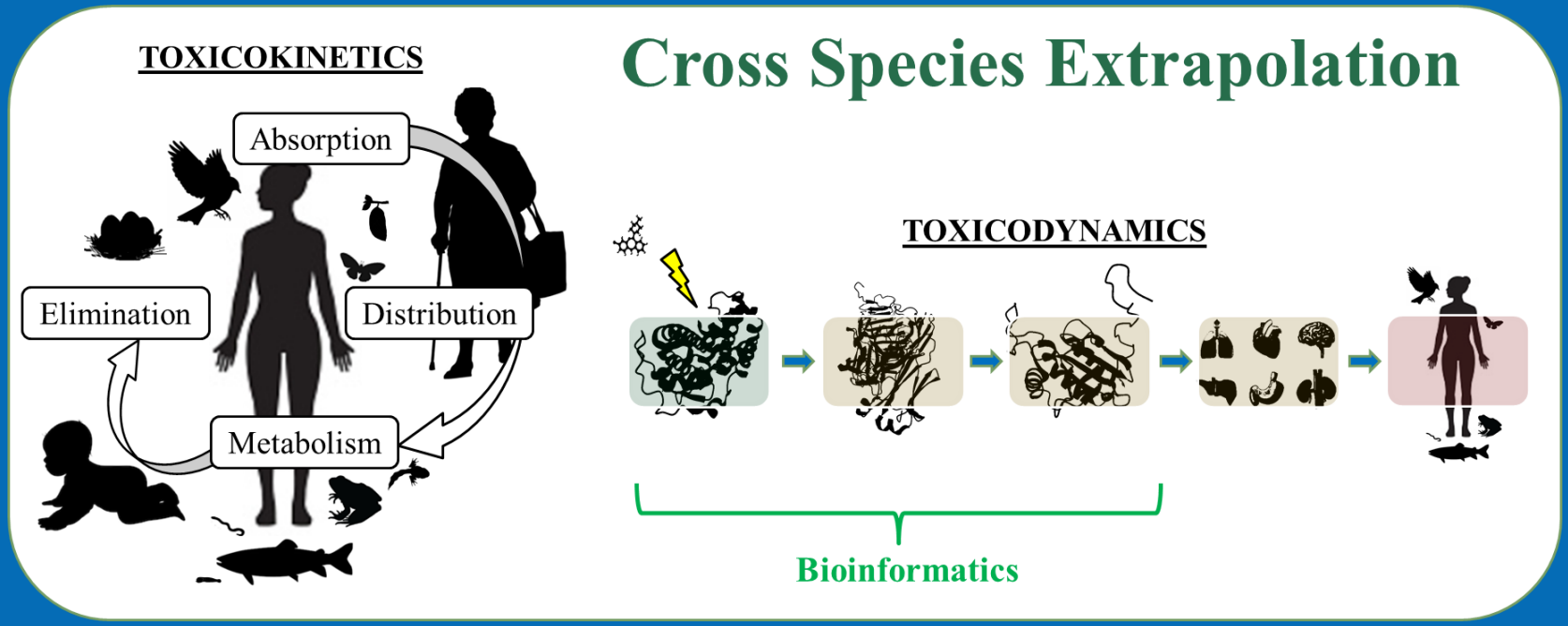
The work presented within represents US EPA Office of Research and Development research activities. Material includes both peer reviewed, published results and work-in-progress research. Please do not cite or quote slides.

Table of Contents

EPA United States Environmental Protection Agency

Approaches and Models for Species Extrapolation

Carlie A. LaLone, Ph.D.
Research Bioinformaticist



Office of Research and Development
Center for Computational Toxicology and Exposure, Great Lakes Toxicology and Ecology Division

February 2nd, 2021

Partners in Regulatory Decision-Making

- Capitalize on available data

Clean Air Act
Clean Water Act
Resource Recovery Act
Endangered Species Act
Food Quality Protection Act
Endocrine Disruptor Screening Program
Federal Insecticide, Fungicide, and Rodenticide Act
Frank R. Lautenberg Chemical Safety for the 21st Century Act
Comprehensive Environmental Response, Compensation, and Liability Act
Guidelines for Deriving Numerical National Water Quality Criteria for the Protection of Aquatic Organisms and Their Uses

Confidence in decisions, with limited data available, limited resources for testing, strong backing to reduce animal use

Stakeholder Identified Challenges

- Limited or no toxicological data for the animal or plant species of interest – reliance on surrogate (model organisms)
- Impractical to generate new data for all species
  - Testing resources are limited
  - EPA directive aligns with international interest to reduce animal use
  - Ever-increasing demand to evaluate more chemicals in a timely and sometimes expedited manner
- Sensitivity of species must be estimated based on scientifically-sound methods of cross-species extrapolation
  - Immense diversity of species in the wild
  - Important challenge for species listed under the Endangered Species Act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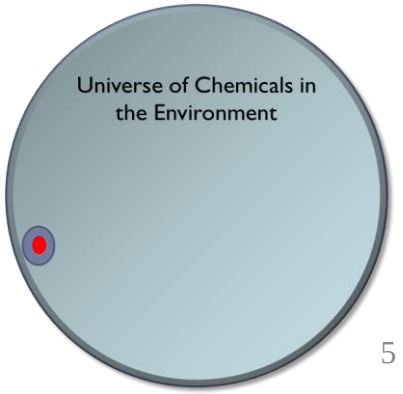
Chemical Safety Evaluation

- Protect human health and the environment
  - Ensure that chemicals in the marketplace are reviewed for safety

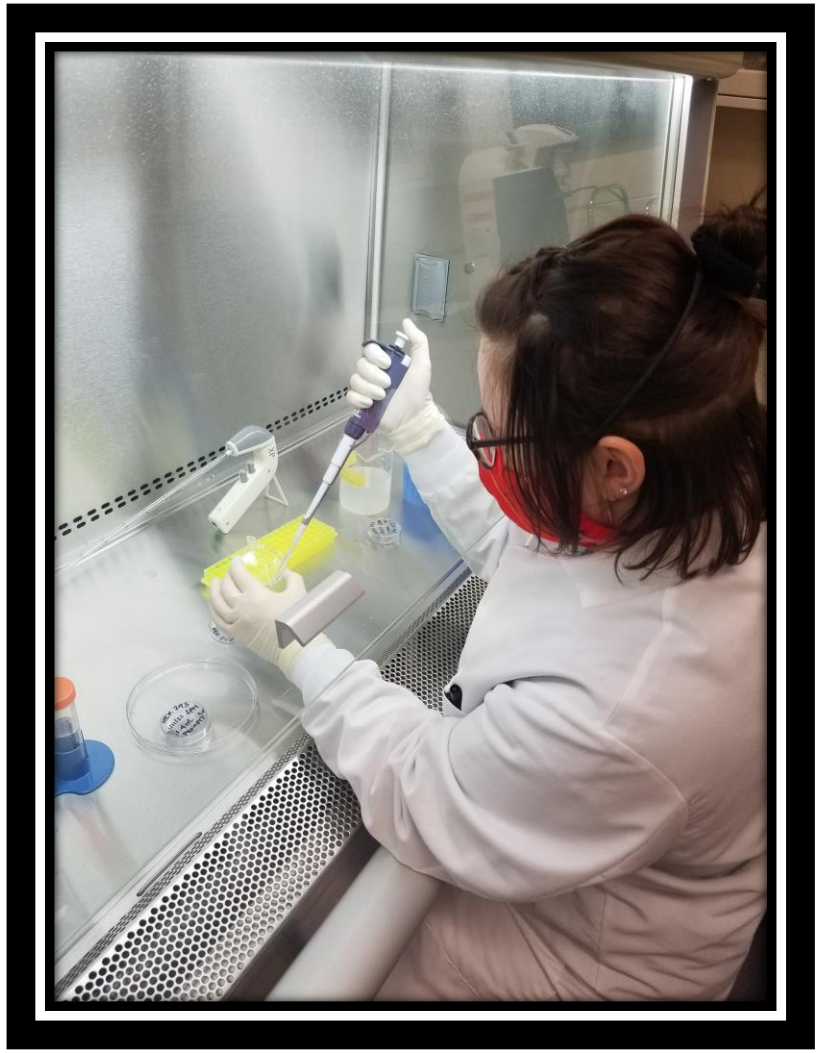
- Challenging mission:
  - Tens of thousand of chemicals are currently in use and hundreds are introduced annually
  - Many have not been thoroughly evaluated for potential risk to human health and the environment
    - *Chemicals tested across species: Even more sparse*



Surrogate species (model organism)

Strategic Approach to Species Extrapolation

Computational:
Bioinformatics (Session 2 Demo)
Systematic review

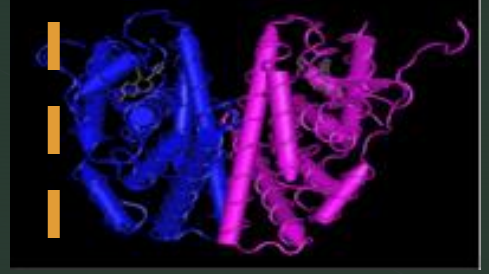


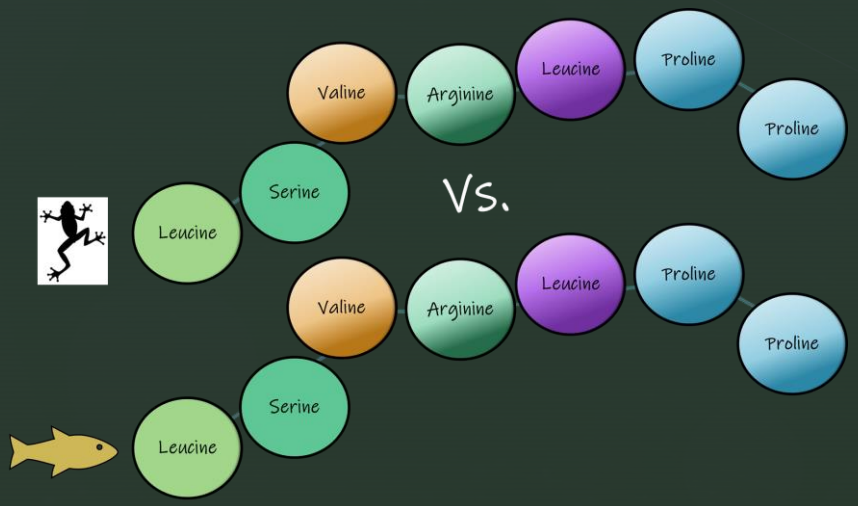
Experimental:
Site-directed mutagenesis
Attagene XS-2 Factorial assay (Dr. Blackwell)

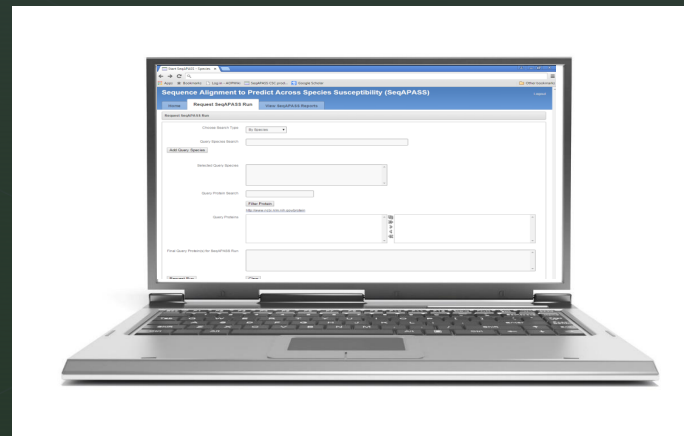
Case Examples:
PFAS targets
Endocrine pathways
Pollinators

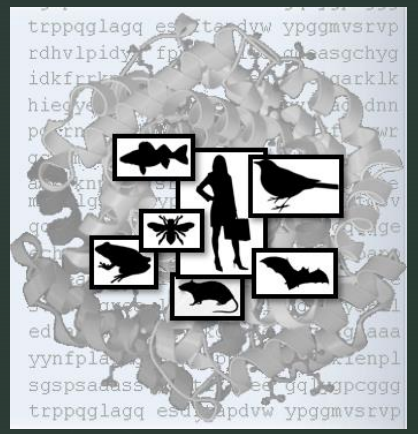
Sequence

MTMTLHTKASGMALLHQIQGNELEPLNRPQLKIPLERPLGE
VYLDSSKPAVYNYPEGAAYEFNAAAAANAQVYGQTGLPYG
PGSEAAAFGSNGLGGFPPLNSVSPSPLMLLHPPPQLSPFLQ
PHGQQVPYYLENEPSGYTVREAGPPAFYRPNSDNRRQGGR
ERLASTNDKGSMAMESAKETRYCAVCNDYASGYHYGVWSC
EGCKAFFKRSIQGHNDYMCPATNQCTIDKNRRKSCQACRLR
KCYEVGMMKGGIRKDRRGGRMLKHKRQRDDGEGRGEVG
SAGDMRAANLWPSPLMIKRSKKNSLALSLTADQMVSALLA
EPPILYSEYDPTRPFSEASMMGLLTNLADRELVHMINWAKV
PGFVDLTLHDQVHLLECAWLEILMIGLVWRSMEHPGKLLFA
PNLLLDRNQGKCVEGMVEIFDMLLATSSRFRMMNLQGEEF
VCLKSIILLNSGVYTFLSSTLKSLEEKDHIHRVLDKITDTLIHLM









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www.toxsci.oxfordjournals.org

TOXICOLOGICAL SCIENCES, 153(2), 2016, 228–245

doi: 10.1093/toxsci/kfw119
Advance Access Publication Date: June 30, 2016
Research article

Sequence Alignment to Predict Across Species Susceptibility (SeqAPASS): A Web-Based Tool for Addressing the Challenges of Cross-Species Extrapolation of Chemical Toxicity

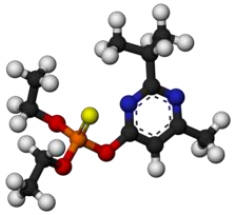
Carlie A. LaLone,*,[1] Daniel L. Villeneuve,* David Lyons,† Henry W. Helgen,‡ Serina L. Robinson,§,[2] Joseph A. Swintek,¶ Travis W. Saari,* and Gerald T. Ankley*

https://seqapass.epa.gov/seqapass/

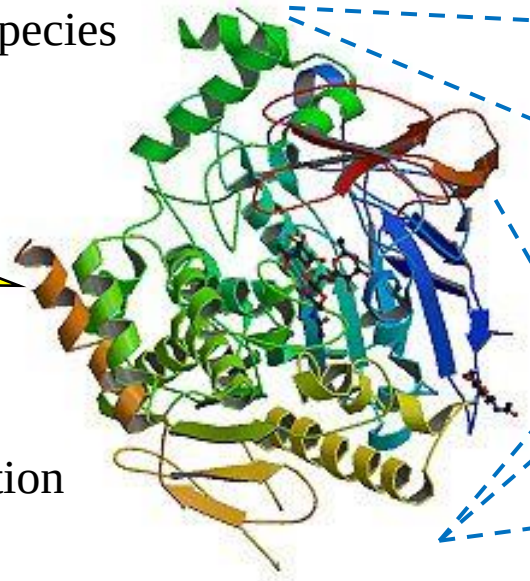
Sequence Alignment to Predict Across Species Susceptibility (SeqAPASS)

What information is required for a SeqAPASS query?

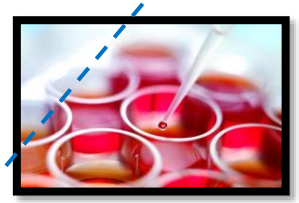
1. Protein
2. Species



Chemical-Protein Interaction



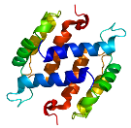
Knowledge of a sensitive or targeted species



Knowledge of the model organism used in an *in vitro* assay

Knowledge of the species for which the Key Event was developed

Chemical Molecular Target in Target Species



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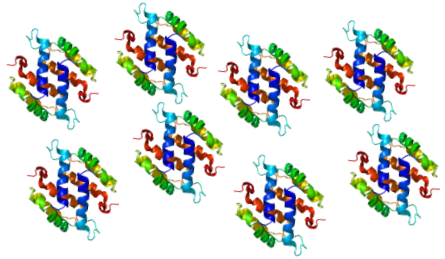


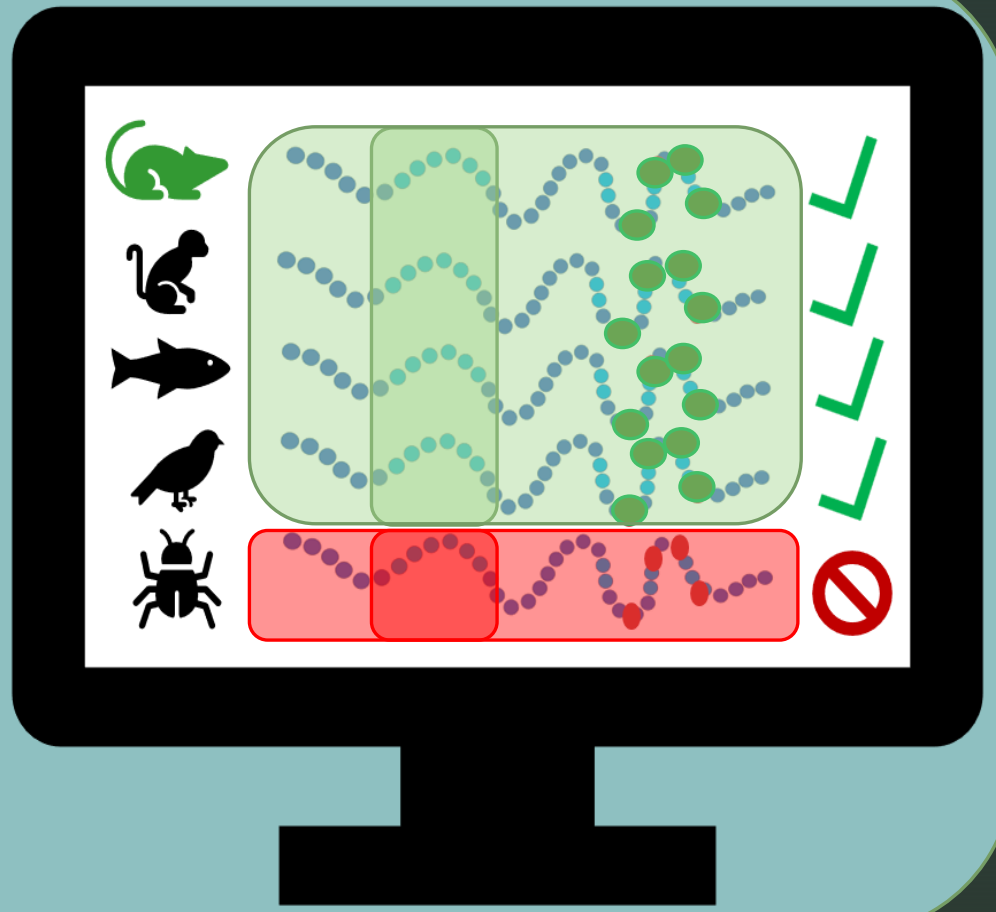
Compare to Millions of Proteins
From Thousands of Species

Greater similarity = Greater likelihood that chemical can act on the protein
Line of Evidence: Predict Potential Chemical Susceptibility Across Species

Flexible Analysis Based On Available Data

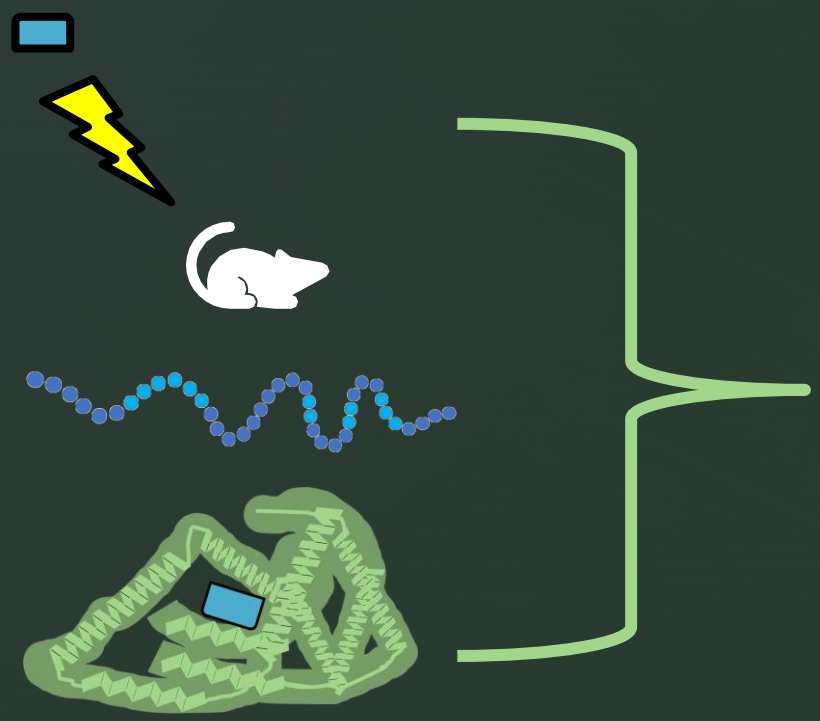
Level 1	Primary Amino Acid Sequence Alignments
Level 2	Conserved Functional Domain Alignments
Level 3	Critical (Close Contact) Amino Acid Conservation

seqapass.epa.gov/seqapass/

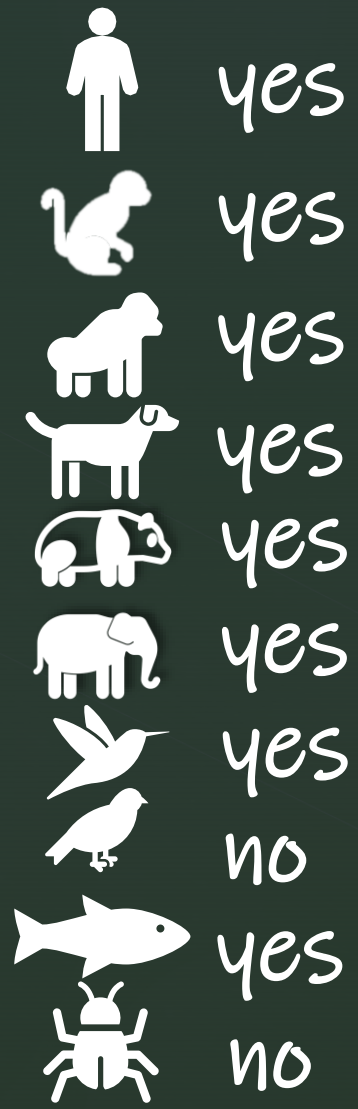


Gather Lines of Evidence Toward Protein Conservation

SeqAPASS Predicts Likelihood of Similar Susceptibility based on Sequence Conservation:



yes



Line(s) of evidence indicate

- The protein is conserved
- The protein is NOT conserved

Evolution of the SeqAPASS tool

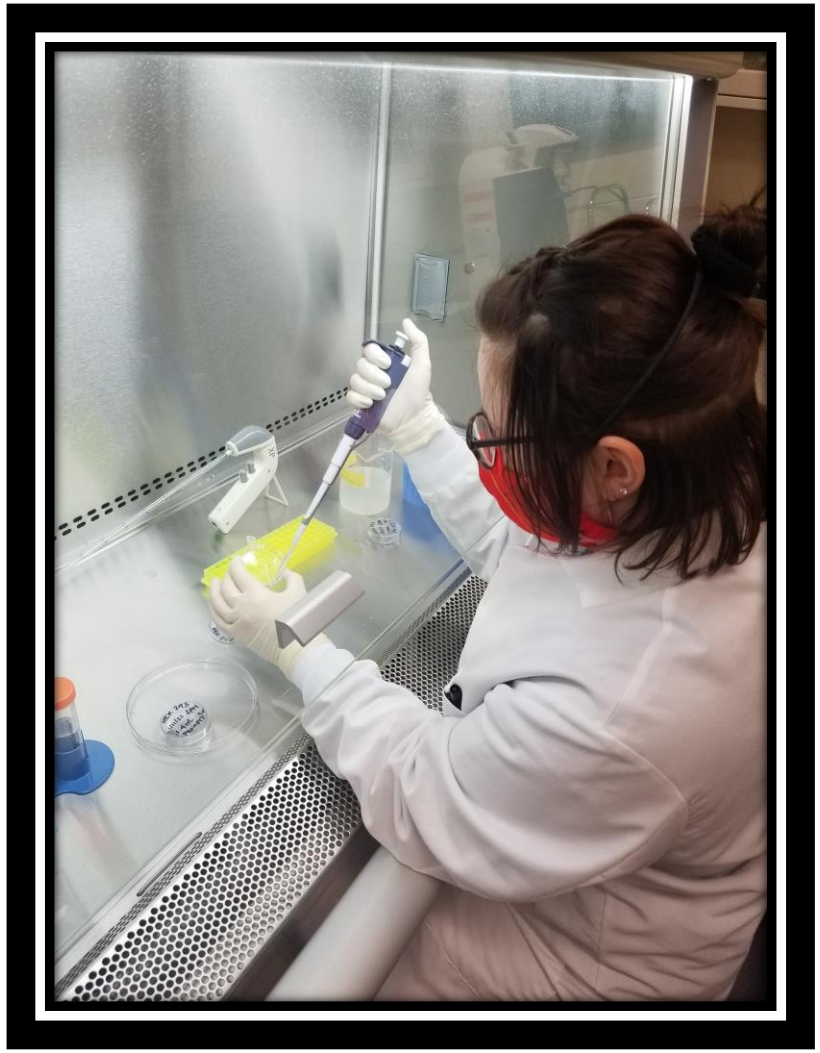
- v5.0 (Nov. 2020): Develop visualization (Level 3), Develop Decision Summary Report
- v4.0 (2019): Improve visualization, user guidance, summary tables, interoperability
- v3.0 (2018): Develop visualization (Level 1 & 2), automate Level 3 Susceptibility Predictions
- v2.0 (2017): develop Level 3 Susceptibility Predictions
- v1.0 (2016): Develop interface Level 1 & 2 and integrate essential functionality

Strategic Approach to Species Extrapolation

Computational:

Bioinformatics (Session 2 Demo)

Systematic review



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Site-directed mutagenesis

Attagene XS-2 Factorial assay (Dr. Blackwell)

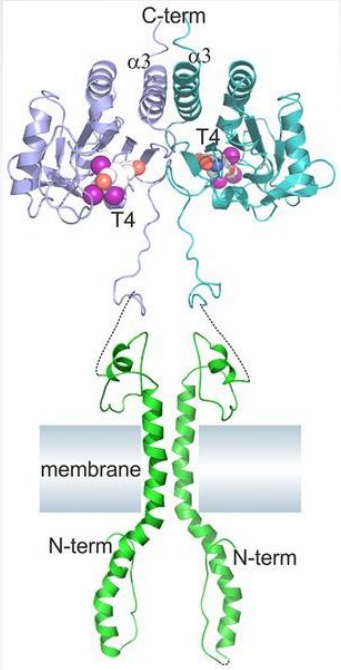
Case Examples:

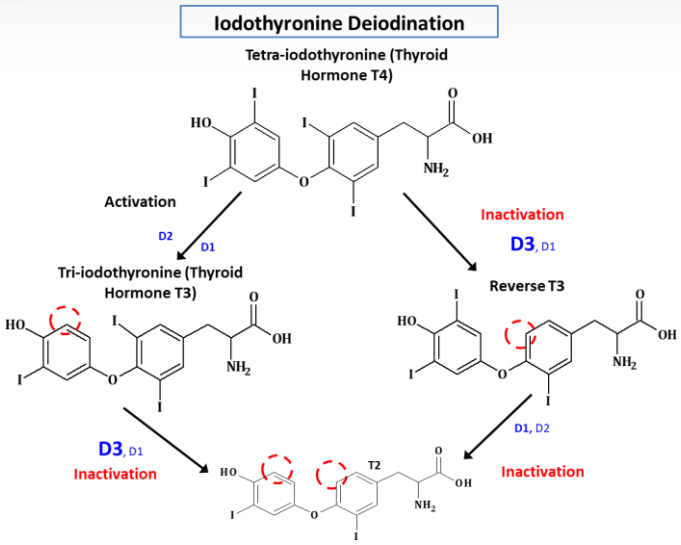
PFAS targets

Endocrine pathways

Pollinators

Deiodinase 3: Important enzyme in thyroid function



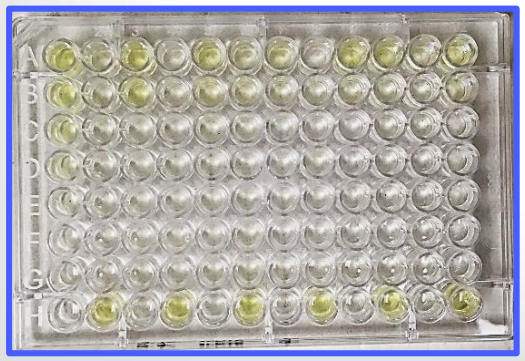


SeqAPASS Critical Amino Acid Comparison

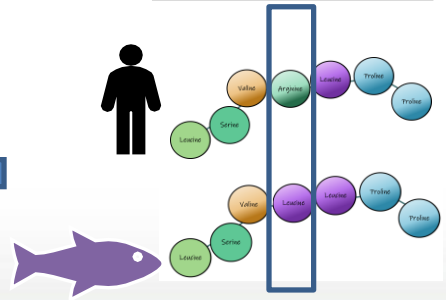
Total Match
Partial Match
Not a Match
Susceptible Yes
Susceptible No

Common Name	Similar Susceptibility	Amino Acid 1	Amino Acid 2	Amino Acid 3	Amino Acid 4	Amino Acid 5
Human	Y	168C	169T	239C	240A	257Y
Red drum	N	130C	131S	201S	202N	219Y
Blue tilapia	Y	123C	124T	194C	195L	212Y
Tongue sole	N	130C	131S	201G	202N	219Y
Zebra mbuna	N	130C	131S	201T	202N	219Y
Nile tilapia	N	130C	131S	201T	202N	219Y
Senegalese sole	N	130C	131S	201G	202N	219Y
Ballan wrasse	N	130C	131S	201S	202N	219Y
Gilthead seabream	N	130C	131S	201S	202N	219Y
Monterrey platyfish	N	124C	125T	195C	196R	213Y
Amazon molly	N	124C	125T	195C	196R	213Y
Shortfin molly	N	124C	125T	195C	196R	213Y
Sapphire devil	N	129C	130S	200G	201N	218Y
Southern platyfish	N	124C	125T	195C	196R	213Y
Goldlined spinefoot	N	130C	131S	201C	202E	219Y
Torafugu	N	130C	131S	201S	202N	219Y
Sailfin molly	N	130C	131S	201S	202N	219Y
Threespot wrasse	N	110C	111S	181S	182N	199Y
Princess parrotfish	N	97C	98S	168S	169N	186Y
Striped parrotfish	N	85C	86S	156S	157N	--
Frogs and toads	N	132C	133T	203C	204R	221Y
Two-lined caecilian	Y	129C	130T	200C	201P	218Y
Puerto Rican coqui	N	130C	131T	201C	202R	219Y
Caecilians	Y	129C	130T	200C	201P	218Y
Tropical clawed frog	N	130C	131T	201C	202R	219Y
African clawed frog	N	128C	129T	199C	200R	217Y
Gabon caecilian	Y	130C	131T	201C	202P	219Y
American bullfrog	N	131C	132T	202C	203R	220Y
Coelacanth	N	131C	132T	202C	203F	220Y
Sea lamprey	N	143G	144S	215C	216P	233A

Site Directed Mutagenesis to Probe SeqAPASS Level 3



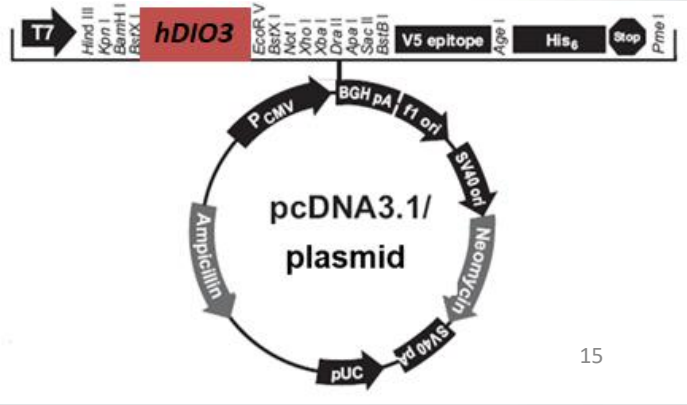
Mutate hDIO3



hDIO3 mutants:

Sea lamprey	C168G
Fish	T169S
Fish	C239S
Frog	A240R
Lungfish	Y257A
Sea lamprey	Y257F

Human Deiodinase 3 Enzyme

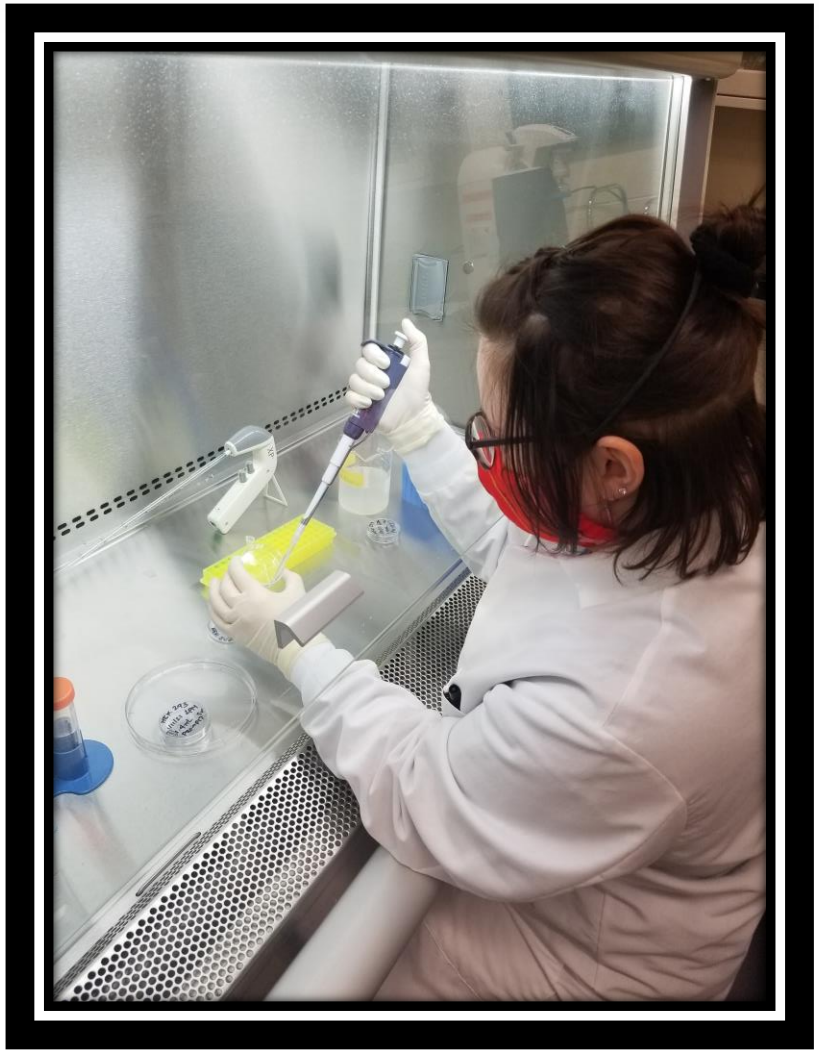


Strategic Approach to Species Extrapolation

Computational:

Bioinformatics (Session 2 Demo)

Systematic review



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Site-directed mutagenesis

Attagene XS-2 Factorial assay (Dr. Blackwell)

Case Examples:

PFAS targets

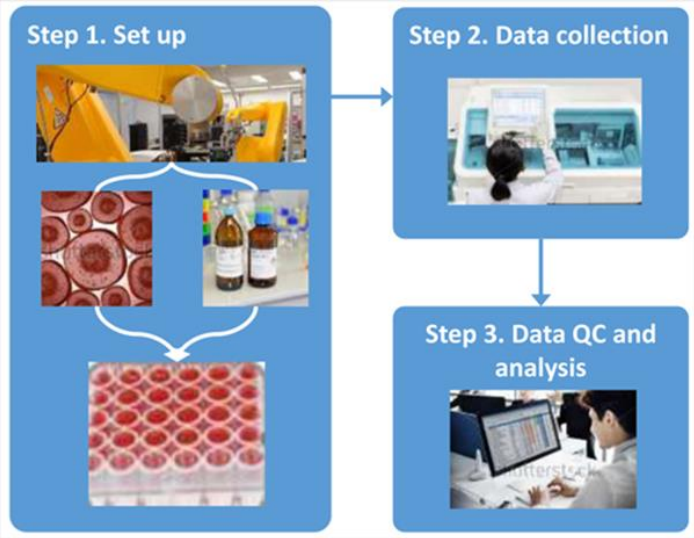
Endocrine pathways

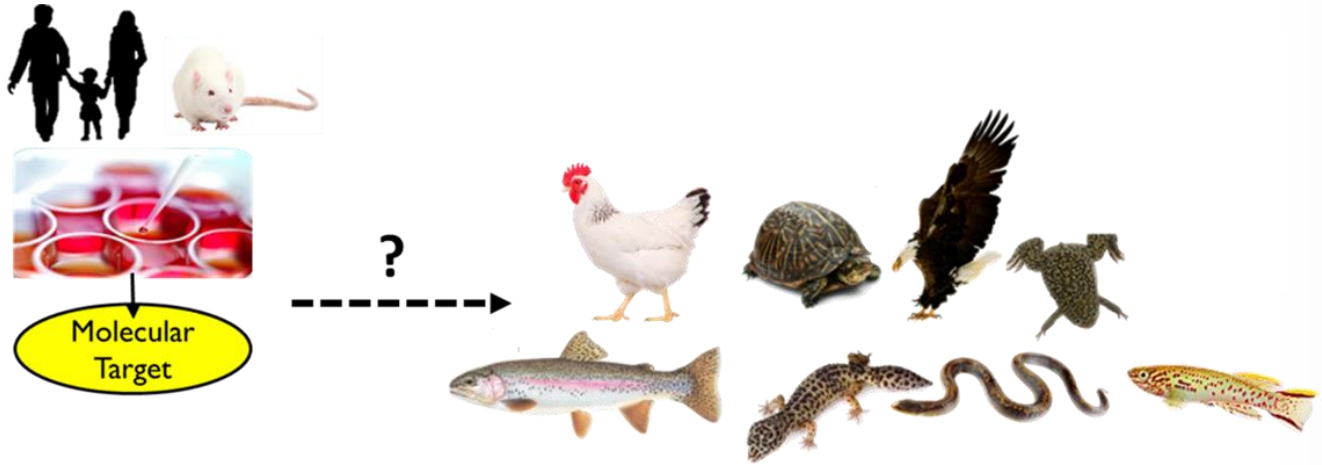
Pollinators

U.S. EPA Toxicity Forecaster (ToxCast)

U.S. EPA ToxCast Program:

US EPA ToxCast Program: Uses **mammalian cell-based assays** to rapidly screen chemicals, identify putative molecular targets, and identify those most likely to be endocrine disruptors





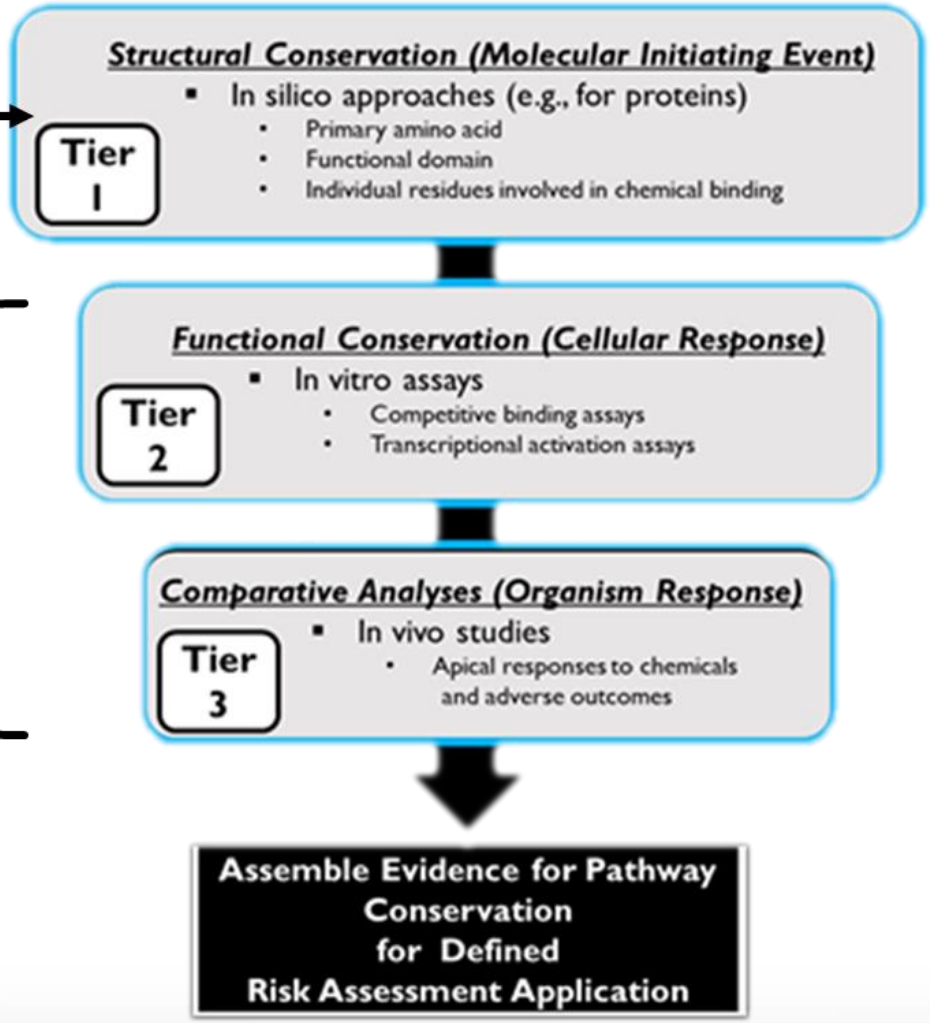
Key Questions for Consideration:

- How well does this mammalian-based prioritization approach reasonably reflect potential impacts on other vertebrates?
- Can we expect chemicals that interact with mammalian receptors to also interact with receptors of other species?

Hierarchal Framework for Evaluating Pathway Conservation Using Existing Evidence

Analysis of structure across species using in silico computational tools

Literature review and comparison of available in vitro and in vivo estrogen receptor data



Environ Toxicol Chem. 2016 Nov;35(11):2806-2816. doi: 10.1002/etc.3456. Epub 2016 Jun 28.

Evaluation of the scientific underpinnings for identifying estrogenic chemicals in nonmammalian taxa using mammalian test systems

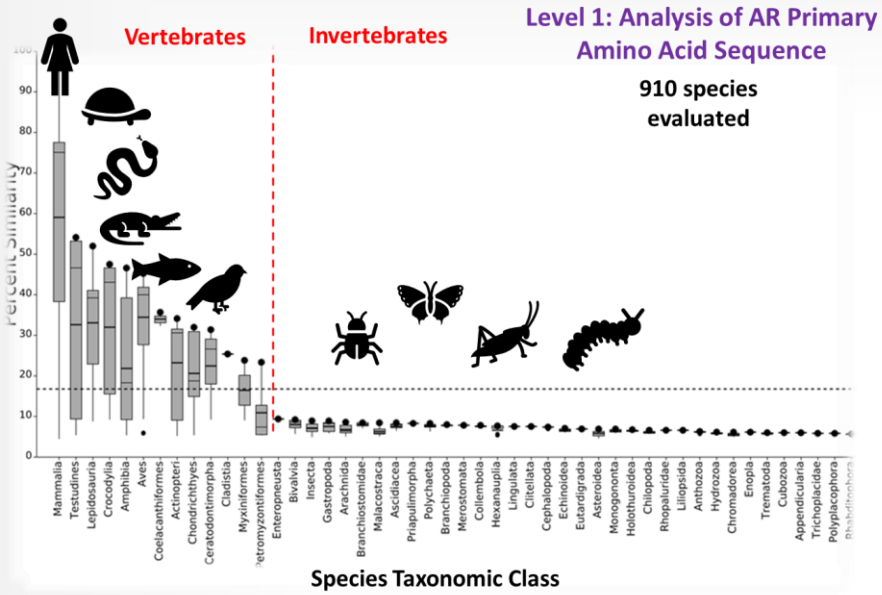
Gerald T Ankley [1], Carlie A LaLone [2], L Earl Gray [3], Daniel L Villeneuve [2], Michael W Hornung [2]

What other important endocrine targets have a large base of pre-existing structural, molecular target, and toxicity data?

→ **Androgen Receptor (AR)**

Assessing AR Conservation Across Species Using the SeqAPASS Tool

1.



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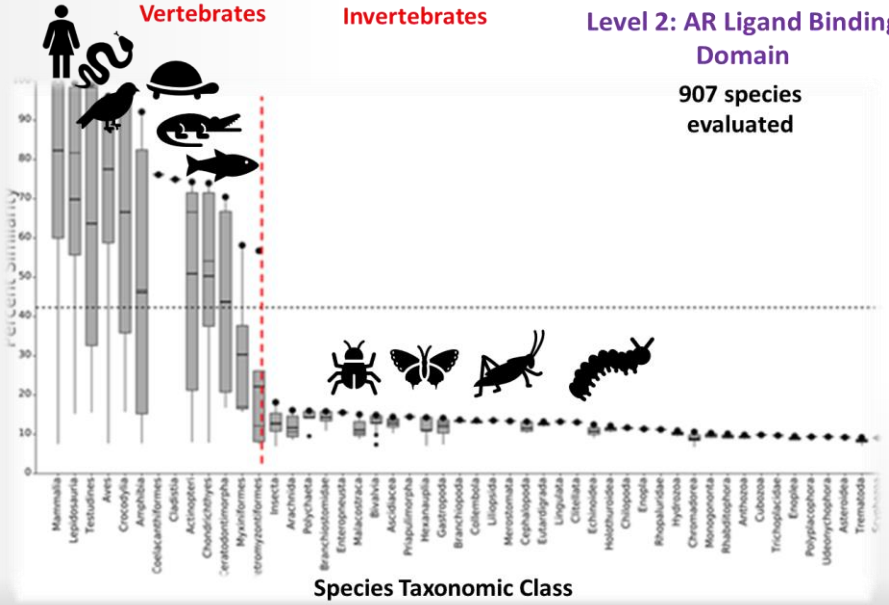


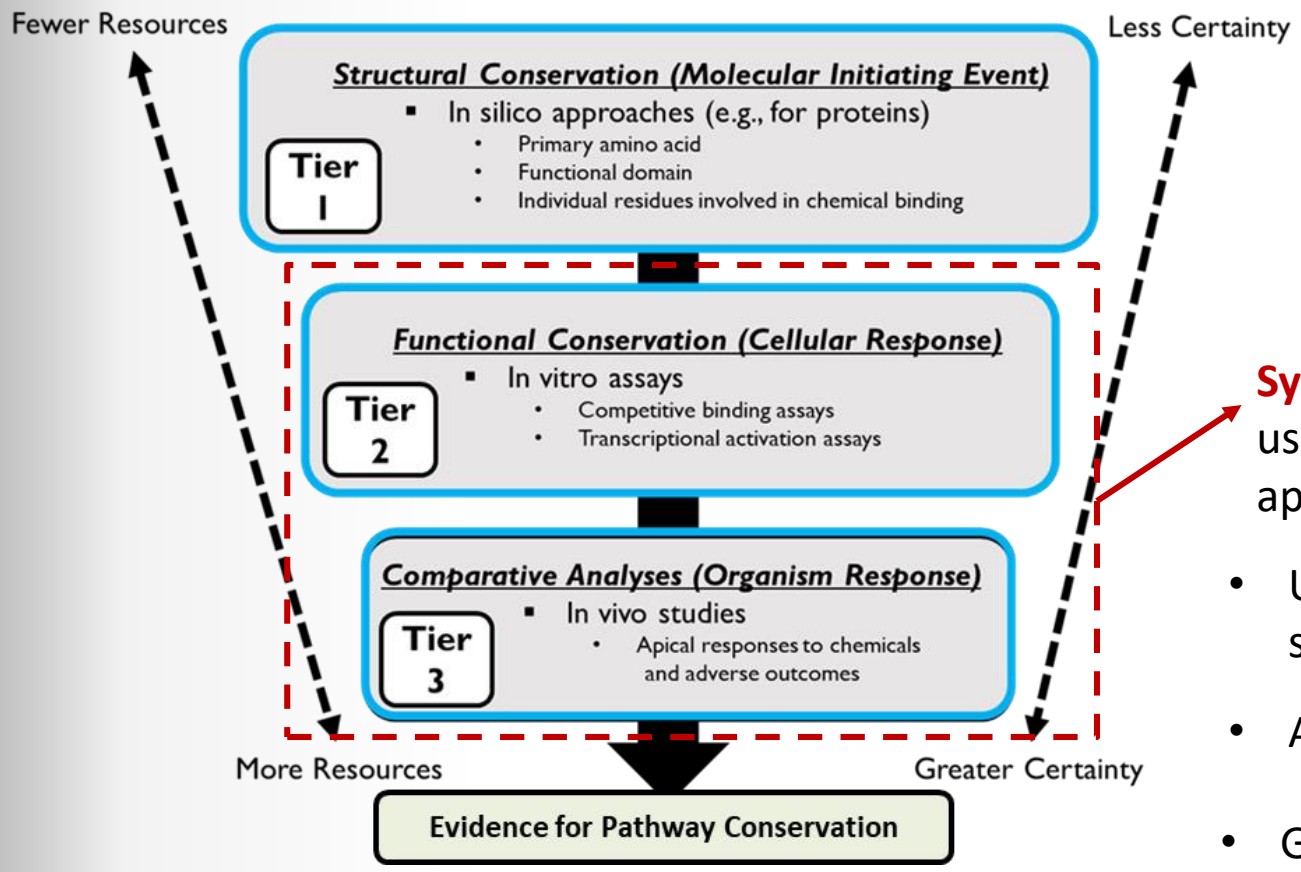
3. **Level 3: Analysis of Conservation of Individual Amino Acid Residues**

250 species evaluated

Taxonomic Group	# of Spp.	Shared Susceptibility
Mammals	117/1	Yes/No
Lizards, Snakes	11	Yes
Turtles	3	Yes
Birds	58	Yes
Crocodiles, Alligators	4	Yes
Amphibians	13	Yes
Coelacanths	2	Yes
Eel-shaped	1	Yes
Bony Fish	87/1	Yes/No
Sharks, Rays	4	Yes
Lungfish	2	Yes

- Across all three levels, SeqAPASS results suggest conservation of AR across vertebrate species
- Overall, these predictions suggest that chemicals that bind and activate AR in mammalian-based assays, are likely to interfere with AR in other vertebrate species
- Line of evidence for pathway conservation

Evaluating Existing Data to Extrapolate High-Throughput Androgen Receptor Screening Data Across Species



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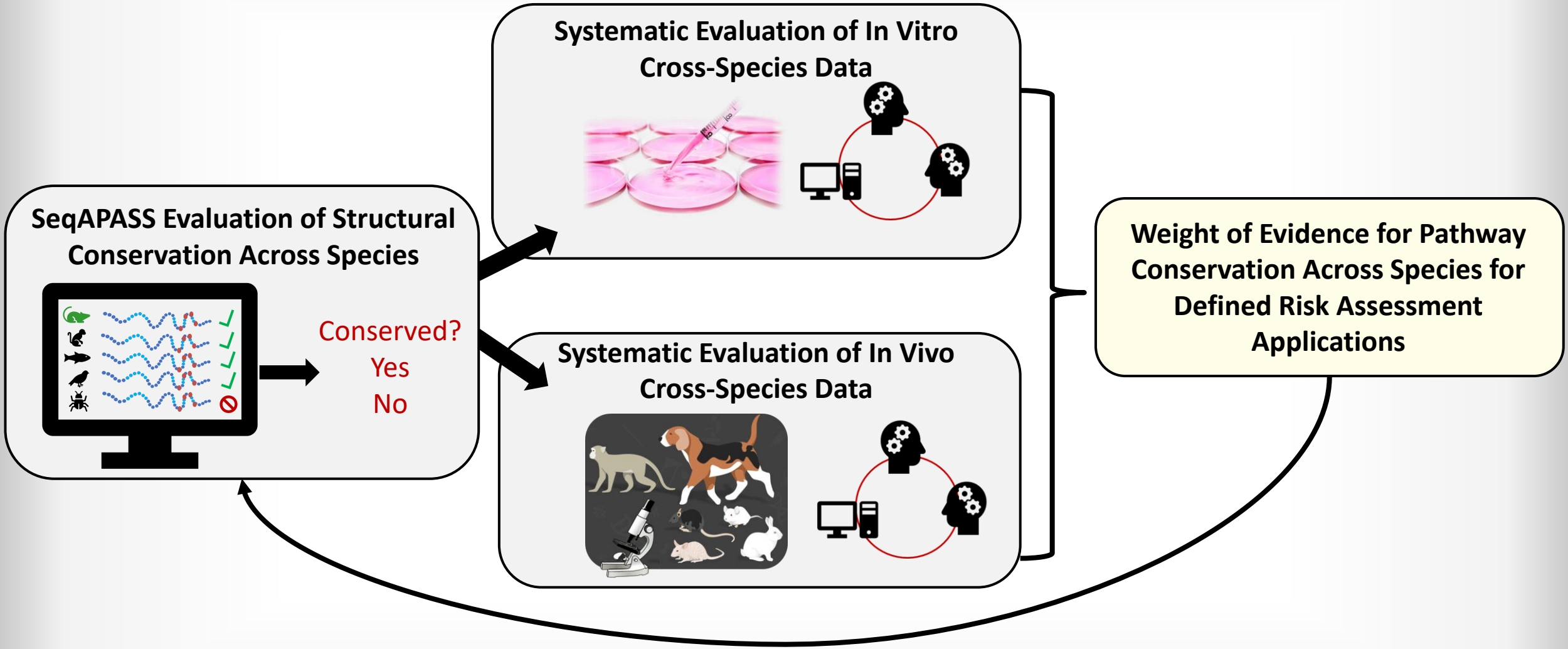
Systematic Literature Review: A type of literature review that uses systematic methods to collect secondary data, critically appraise research studies, and synthesize findings

- Using existing evidence (literature), we can evaluate the scientific basis of our cross-species predictions
- Advances in data science can improve this workflow
- Gathering in vivo and in vitro data from vertebrate species exposed to known androgenic compounds provides **additional lines of evidence** for the conservation of the biological pathway across species

Evaluating Existing Data to Extrapolate High-Throughput Androgen Receptor Screening Data Across Species



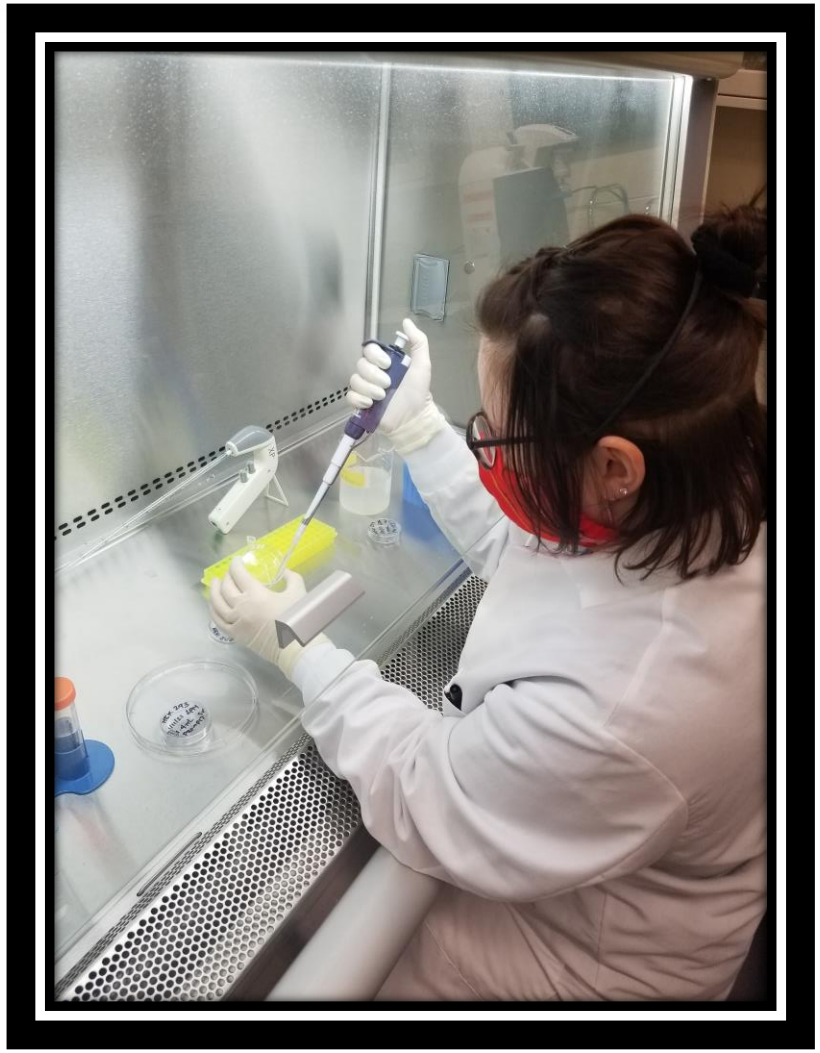
- Apply pathway to other targets of interest
- Repeat process to account for the emergence of new information

Strategic Approach to Species Extrapolation

Computational:

Bioinformatics (Session 2 Demo)

Systematic review



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VYLDSSKPAVYNYPEGAAYEFNAAAAANAQVYGQTGLPYG
PGSEAAAFGSNGLGGFPPLNSVSPSPLMLLHPPPQLSPFLQ
PHGQQVPYYLENEPSGYTVREAGPPAFYRPNSDNRRQGGR
ERLASTNDKGSMAMESAKETRYCAVCNDYASGYHYGVWSC
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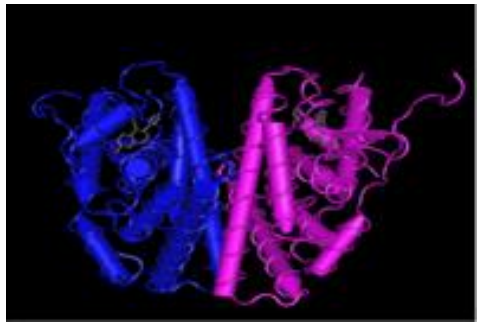
EPPILYSEYDPTRPFSEASMMGLLTNLADRELVHMINWAKV
PGFVDLTLHDQVHLLECAWLEILMIGLVWRSMEHPGKLLFA
PNLLLDRNQGKCVEGMVEIFDMLLATSSRFRMMNLQGEEF
VCLKSIILLNSGVYTFLSSTLKSLEEKDHIHRVLDKITDTLIHLM

Yes or No
Susceptible or Not Susceptible

Structure

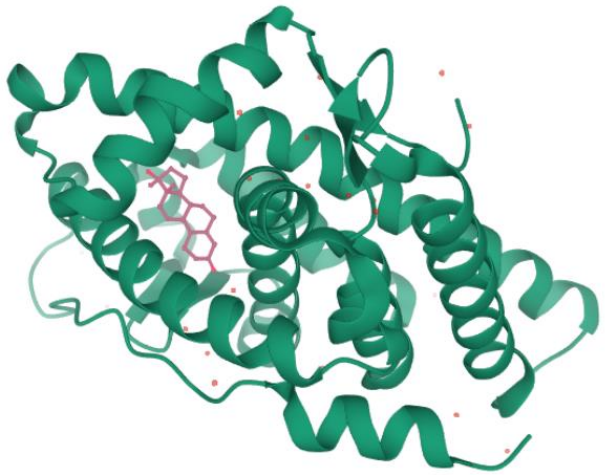


Structural-based
comparisons of similarity
Predicted binding affinity

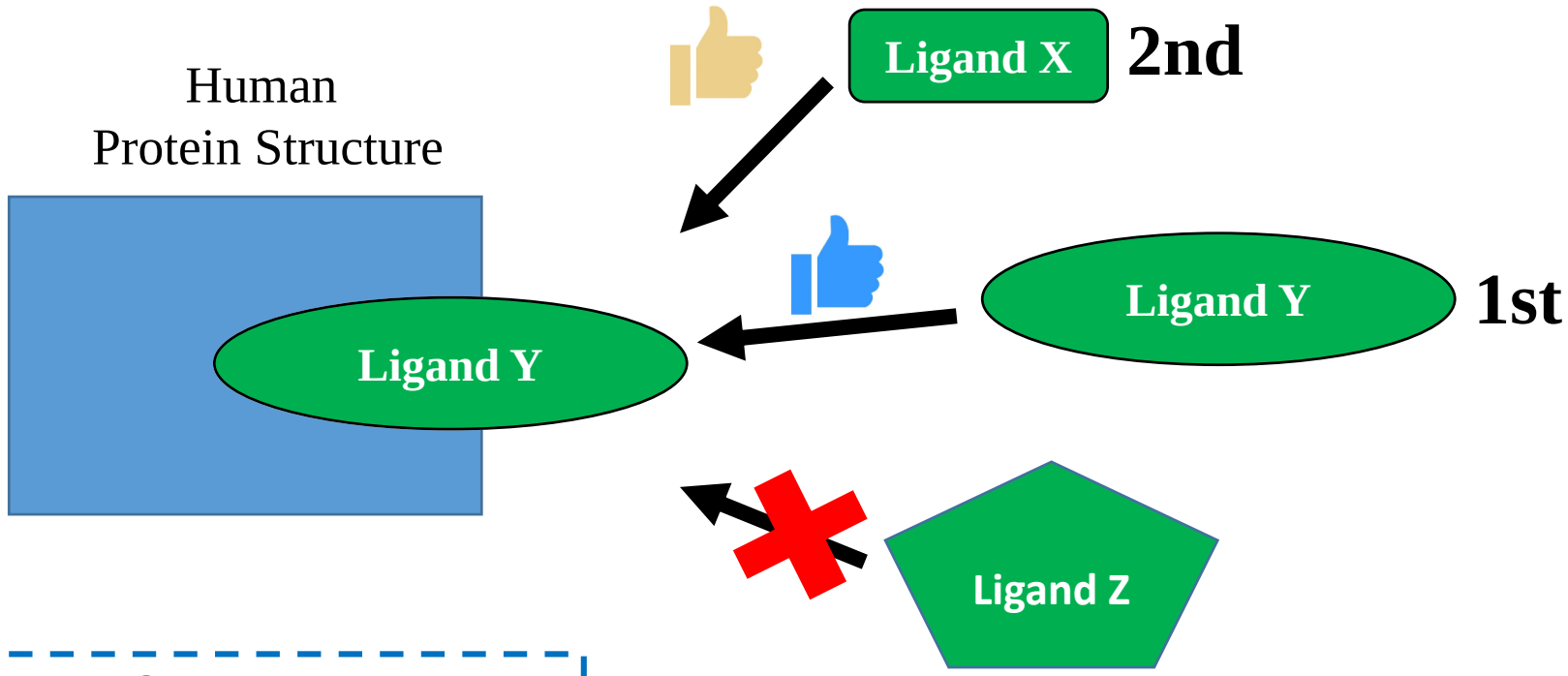
Function

Improvements in bioinformatics

Advances in Drug Discovery/Development



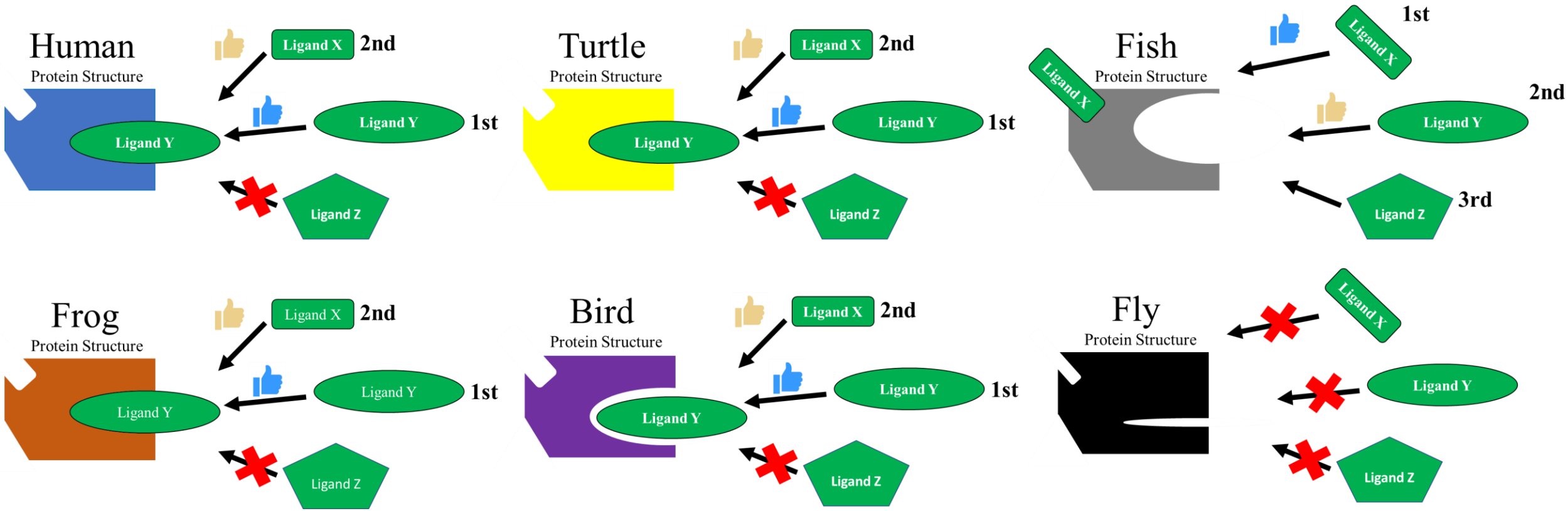
Structure derived from X-ray crystallography



Bioinformatics Toolbox:

Molecular modeling
Molecular docking
Virtual screening
Molecular dynamic simulations

Application to Species Extrapolation



Bioinformatics Toolbox:

Molecular modeling

Molecular docking

Virtual screening

Molecular dynamic simulations

Sequence

MTMTLHTKASGMALLHQIQGNELEPLNRPQLKIPLERPLGE
VYLDSSKPAVYNYPEGAAYEFNAAAAANAQVYGQTGLPYG
PGSEAAAFGSNGLGGFPPLNSVSPSPLMLLHPPPQLSPFLQ
PHGQQVPYYLENEPSGYTVREAGPPAFYRPNSDNRRQGGR
ERLASTNDKGSMAMESAKETRYCAVCNDYASGYHYGVWSC
EGCKAFFKRSIQGHNDYMCPATNQCTIDKNRRKSCQACRLR
KCYEVGMMKGGIRKDRRGGRMLKHKRQRDDGEGRGEVG
SAGDMRAANLWPSPLMIKRSKKNSLALSLTADQMVSALLA
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PGFVDLTLHDQVHLLECAWLEILMIGLVWRSMEHPGKLLFA
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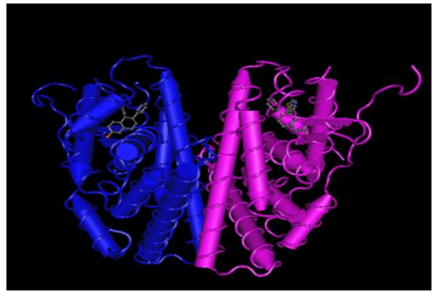
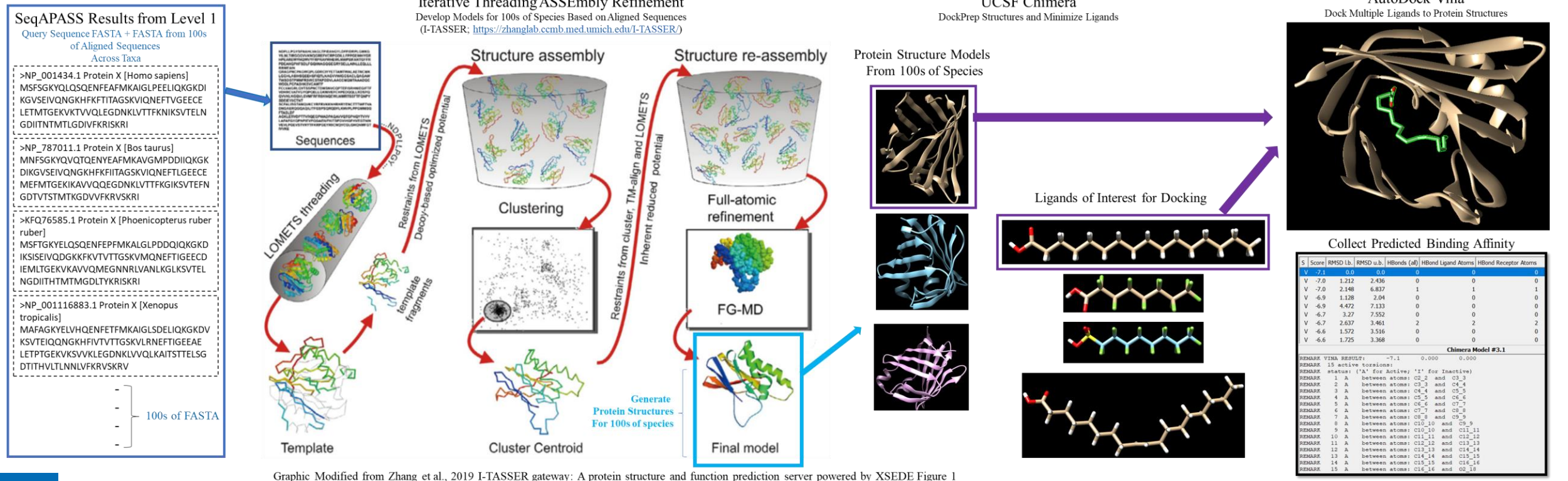
Structure





Graphic Modified from Zhang et al., 2019 I-TASSER gateway: A protein structure and function prediction server powered by XSEDE Figure 1

Acknowledgements

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Audrey Wilkinson

SeqAPASS v5.0

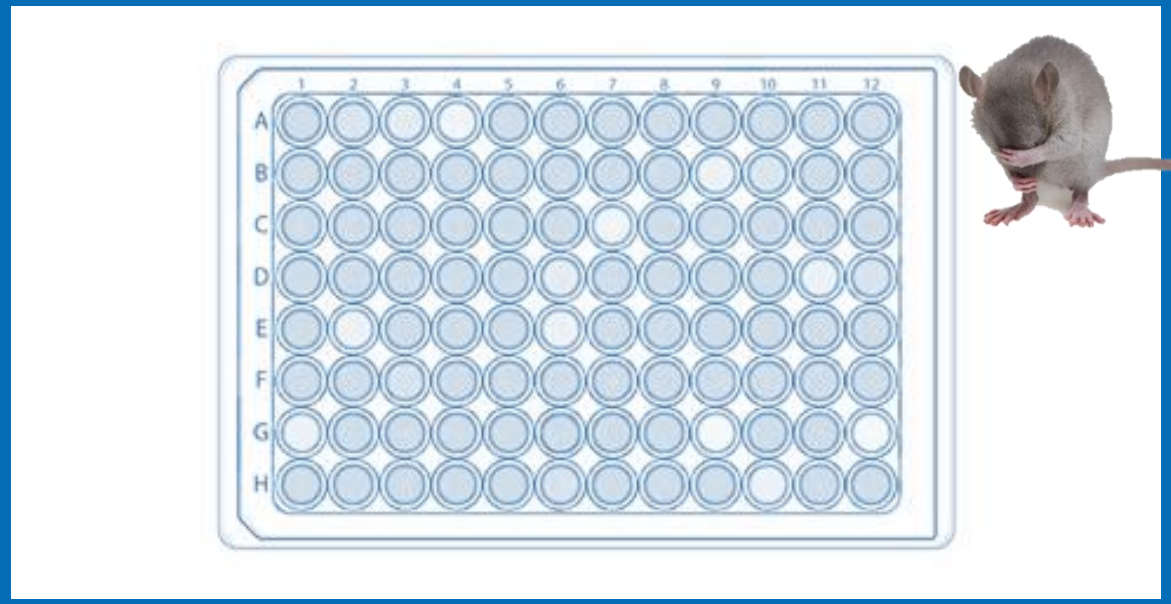
LaLone.Carlie@epa.gov

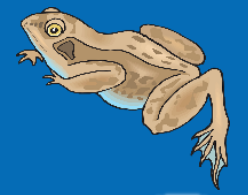
https://seqapass.epa.gov/seqapass/

EPA United States Environmental Protection Agency

Novel in vitro Methods for Ecological Species: Evaluating Cross-species Differences in Nuclear Receptor-Ligand Interactions

B.R. Blackwell, D.L. Villeneuve, G.T. Ankley, C.A. LaLone, J.A. Doering

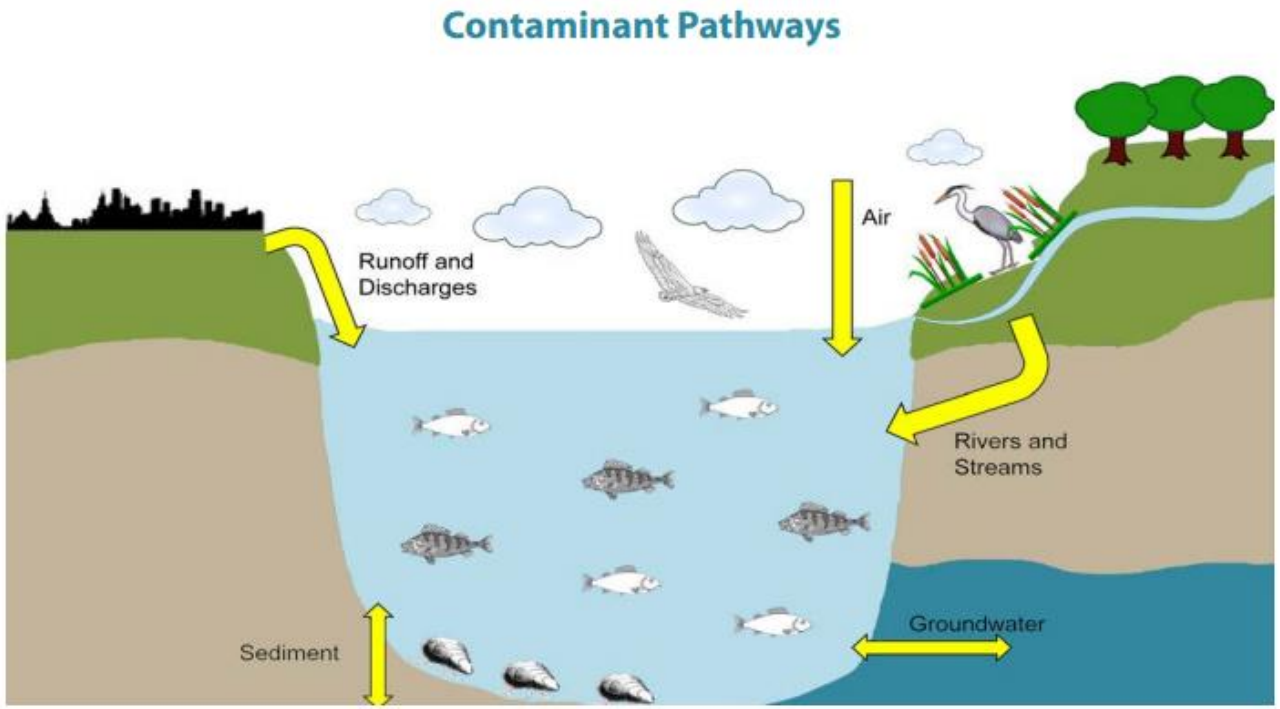




Office of Research and Development
Center for Computational Toxicology and Exposure, Great Lakes Toxicology and Ecology Division

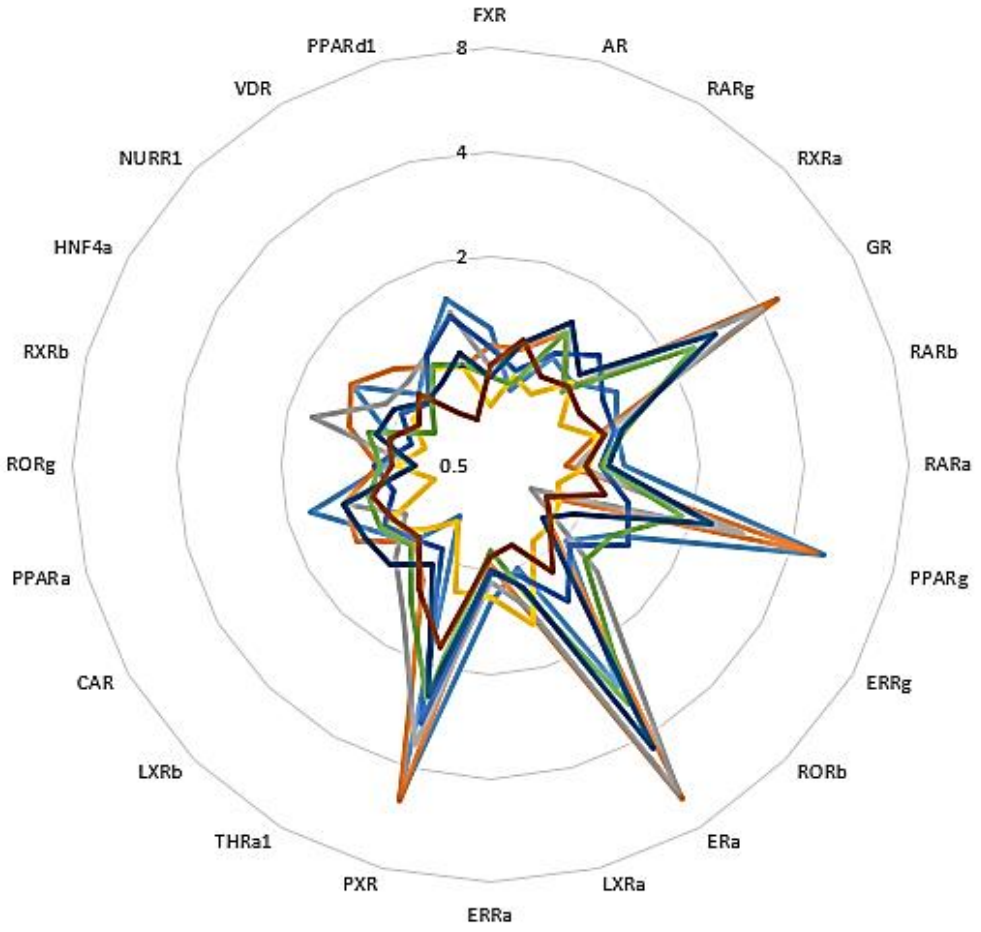
50 EPA at Progress for a Stronger Future

Chemicals in the Environment



- Hundreds of chemicals detected in the environment
- Contaminants of emerging concern (CECs) include those with limited toxicity data
- Recognized as critical data gap by stakeholders:
  - OCSPP; OW; Regions: Great Lakes National Program Office (GLNPO)
- Effects-based monitoring a tool for understanding impacts and potential risks of contaminants

Environmental Monitoring with Attagene TRANS-FACTORIAL Assay



- PXR
- ERα
- PPARγ
- GR
- PPARα
- RXRβ

Do the human receptors adequately represent sensitivity of aquatic vertebrate receptors?

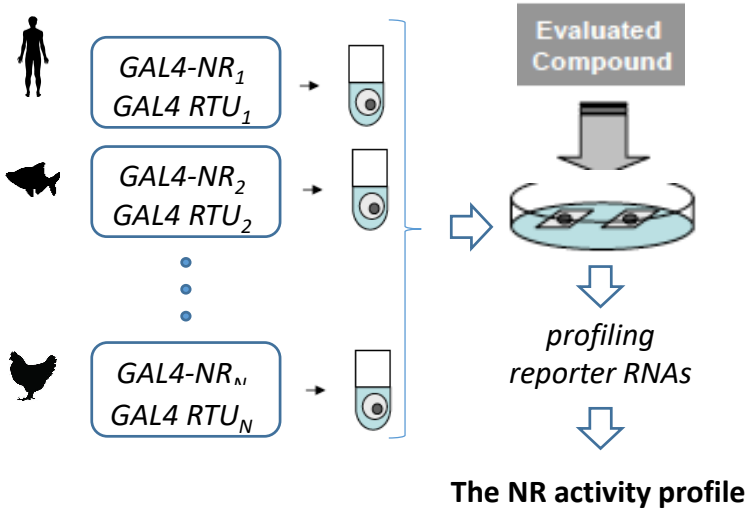
Among the most frequently detected nuclear receptor activities in surface water samples

Cross-species extrapolation

- To date, high throughput screening has been human centric
- Unclear how well mammalian HTS assays represent vertebrate diversity, let alone other phyla.
- Not feasible to include all taxa in a HTS screening program.

How can we strategically select the minimum number of representative species that cover the maximal range of variation in sensitivity and specificity?

Attagene EcoTox FACTORIAL Assay



NR	Class	Species	Sequence ID
ER1	Fish	*Danio rerio*	NM_152959.1
ER2α		*Danio rerio*	NM_180966.2
ER2β		*Danio rerio*	NM_174862.3
ER1	Amphibian	*Xenopus laevis*	NM_001089617
ER2		*Xenopus laevis*	NM_001130954
ER1	Reptilian	*Chrysemys picta*	NM_001282246
ER1	Avian	*Gallus gallus*	NM_205183
ERα	Mammalian	*Homo Sapiens*	NM_000125
ERβ		*Homo Sapiens*	NM_001437
AR	Fish	*Danio rerio*	NM_001083123
AR	Amphibian	*Xenopus laevis*	NM_001090884
AR	Reptilian	*Chrysemys picta*	XM_005279527
AR	Avian	*Gallus gallus*	NM_001040090
AR	Mammalian	*Homo Sapiens*	NM_000044
TRα	Fish	*Danio rerio*	NM_131396.1
TRβ		*Danio rerio*	NM_131340.1
TRα	Amphibian	*Xenopus laevis*	NM_001088126
TRα	Reptilian	*Chrysemys picta*	XM_005294120
TRα	Mammalian	*Homo Sapiens*	NM_199334
TRβ		*Homo Sapiens*	NM_000461
PPARγ	Fish	*Danio rerio*	NM_131467
PPARγ	Mammalian	*Mus musculus*	NM_001127330
PPARγ		*Homo Sapiens*	BC006811
PXR	Mammalian	*Mus musculus*	NM_010936

Considered 5 vertebrate classes
Focused on endocrine NRs

Differences in sensitivity among vertebrate classes were generally minor for ER, AR, TR.

Fish PPARγ was substantially less sensitive to classic PPARγ agonists than mammals.

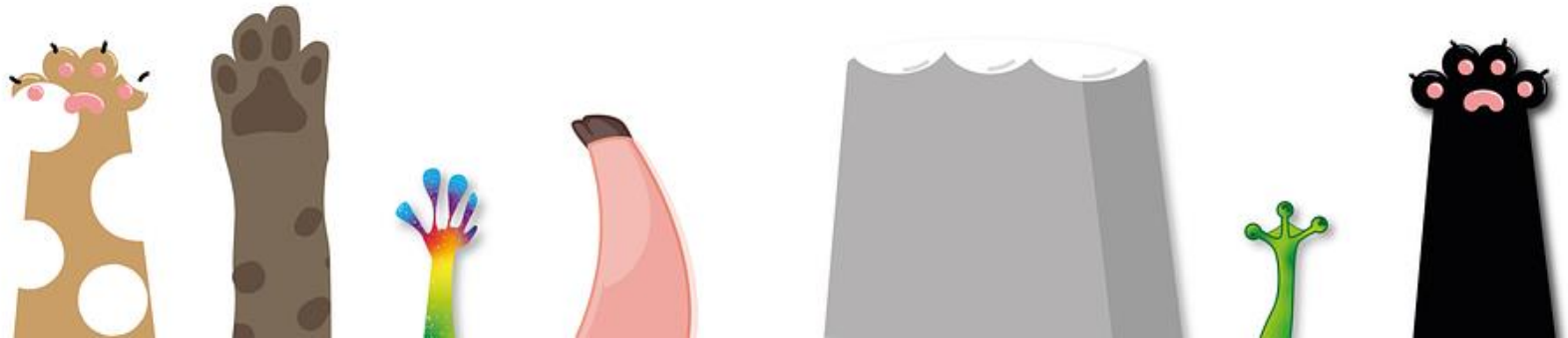
*Medvedev et al. Harmonized cross-species assessment of endocrine and metabolic disruptors by EcoTox FACTORIAL assay. Environmental Science & Technology **2020** 54 (19), 12142-12153. DOI: 10.1021/acs.est.0c03375.*

Species Selection

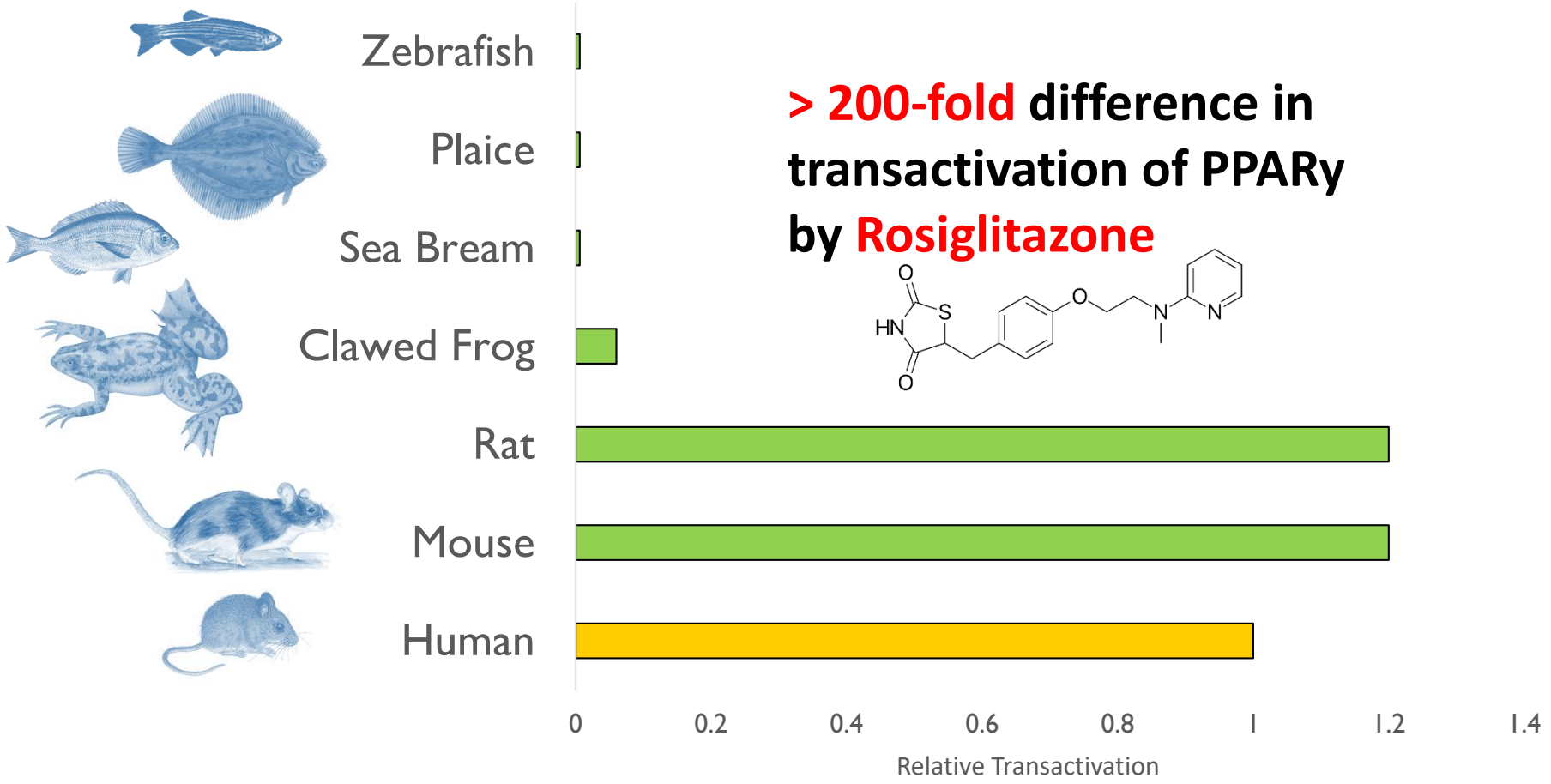
Is the selection of one representative vertebrate from each class the best way to cover the potential variability in sensitivity?

Could available information be used to guide a more strategic selection?

- Documented species differences in sensitivity to ligands
- Amino acid residues identified as critical to ligand binding in one or more species
- In silico analyses of conservation/variation in aa sequence using SeqAPASS



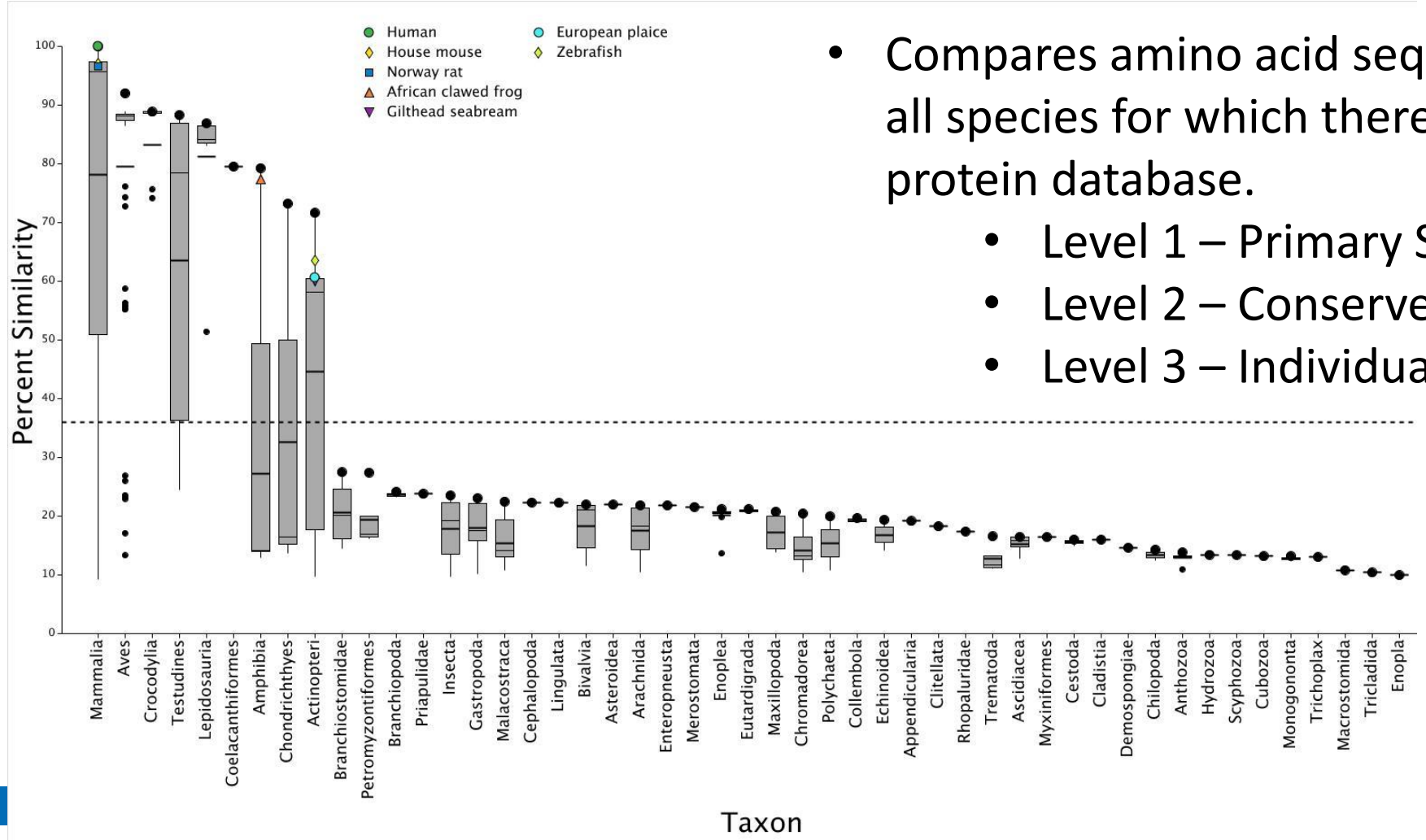
PPARγ – established cross-species differences



> 200-fold difference in transactivation of PPARγ by Rosiglitazone

SeqAPASS Analysis

https://seqapass.epa.gov/seqapass/



- Compares amino acid sequence information for all species for which there are data in the NCBI protein database.
  - Level 1 – Primary Sequence
  - Level 2 – Conserved domains
  - Level 3 – Individual amino acid residues

Example SeqAPASS Level 3 - PPARγ

- Only 4 positions showed important differences in amino acids among PPARγ
- 2 positions known to significantly alter interaction of ligand (rosiglitazone) with PPARγ

Taxa	# of Species	Position 1 (Ile309)	Position 2 (Gly312)	Position 3 (Cys313)	Position 4 (Tyr355)	Susceptibility Prediction
Human	1	I	G	C	Y	Yes
All Mammals	107	I	G	C	Y	Yes
Mallard-type	1	I	G	C	Y	Yes
Most Birds	70	I	R	C	Y	No
All Reptiles	19	I	R	C	Y	No
All Amphibians	2	I	R	C	F	No
Ancient Fishes	9	F	R	C	I	No
Most Fishes	61	F	S	C	I	No
Salmonid-type	3	V	R	I	T	No
Bonytongue-type	1	F	R	W	I	No
Zebrafish-type	2	F	S	Y	I	No

Strongly conserved among most birds, amphibians, reptiles

More variation among various orders of fishes than across other vertebrate classes

Example SeqAPASS Level 3 - PPARγ

- *In silico* mechanism for lack of Rosiglitazone binding to zebrafish PPARγ is severe steric hindrance from Gly312Ser and Cys313Tyr mutation
- Comparing positions 312 and 313 of human to other species

Taxa	Species	Position 312	Position 313	Susceptibility Prediction	Relative Transactivation
Mammal	Human	G	C	Yes	1.0
Mammal	Mouse	G	C	Yes	1.2
Mammal	Rat	G	C	Yes	1.2
Amphibian	Clawed Frog	R	C	No	0.06
Fish	Sea Bream	S	C	No	<0.006
Fish	Plaice	S	C	No	<0.006
Fish	Zebrafish	S	Y	No	<0.006

Strategic Approach

Similar types of analyses applied to

GR

PPARα

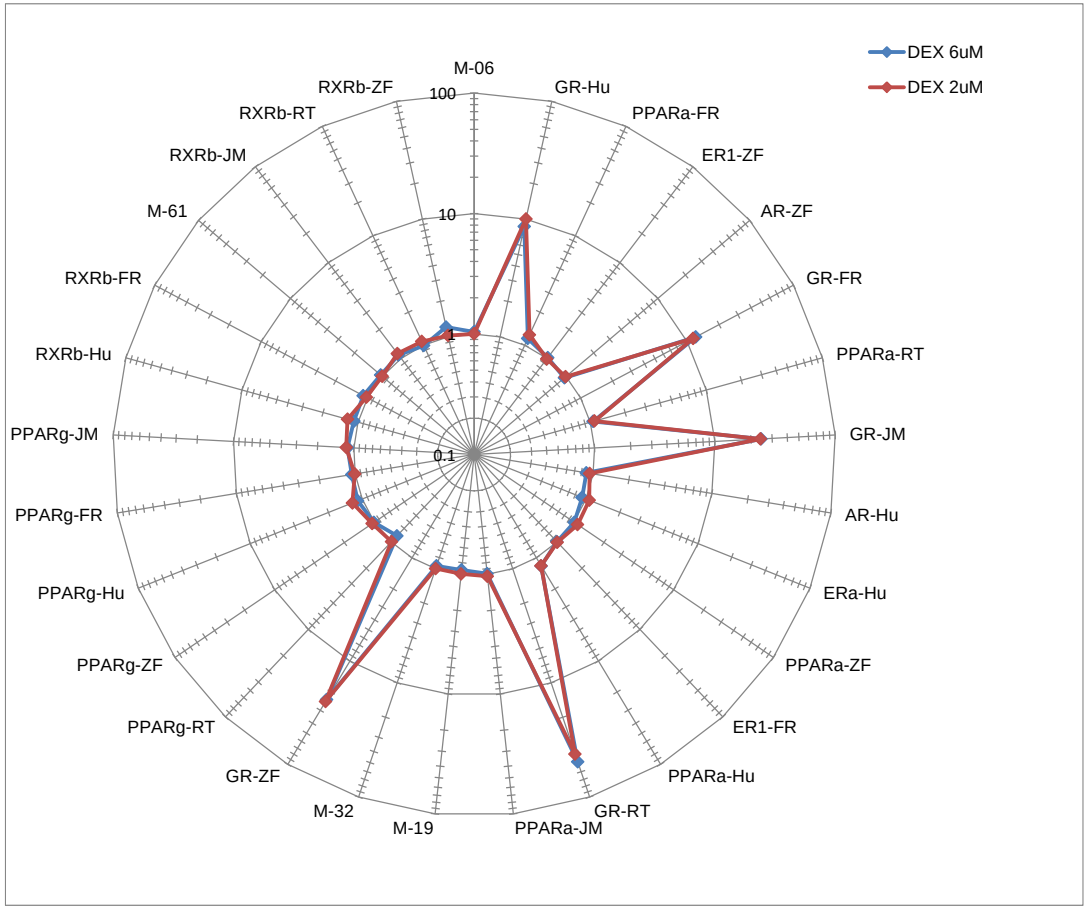
RXRb

Selected a group of species that should capture maximum diversity in response for these four NRs (& genomes available)

- Human
- Xenopus laevis
- Rainbow trout
- Japanese medaka
- Zebrafish

Attagene EcoTox-2 Factorial assay

#	Name	Species	Latin names
1	GR	human	Homo Sapiens
2	GR	african clawed frog	Xenopus laevis
3	GR	rainbow trout	Oncorhynchus mykiss
4	GR	japanese medaka	Oryzias latipes
5	GR	Zebrafish	Danio rerio
6	PPARa	human	Homo Sapiens
7	PPARa	african clawed frog	Xenopus laevis
8	PPARa	rainbow trout	Oncorhynchus mykiss
9	PPARa	japanese medaka	Oryzias latipes
10	PPARa	Zebrafish	Danio rerio
11	PPARg	human	Homo Sapiens
12	PPARg	african clawed frog	Xenopus laevis
13	PPARg	rainbow trout	Oncorhynchus mykiss
14	PPARg	japanese medaka	Oryzias latipes
15	PPARg	Zebrafish	Danio rerio
16	RXRb	human	Homo Sapiens
17	RXRb	african clawed frog	Xenopus laevis
18	RXRb	rainbow trout	Oncorhynchus mykiss
19	RXRb	japanese medaka	Oryzias latipes
20	RXRb	Zebrafish	Danio rerio
21	ERa	human	Homo Sapiens
22	ER1	Zebrafish	Danio rerio
23	ER1	african clawed frog	Xenopus laevis
24	AR	human	Homo Sapiens
25	AR	Zebrafish	Danio rerio

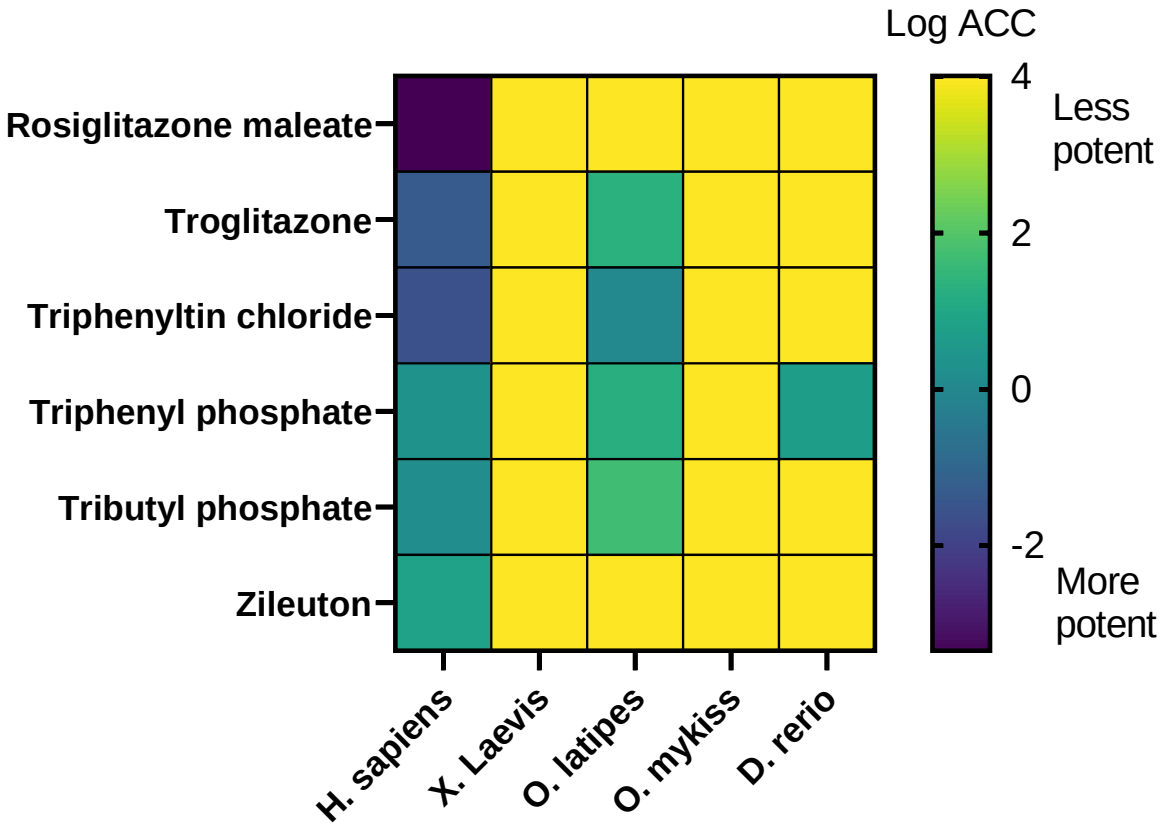


- 14 chemicals in concentration-response
- Surface water extracts

Test Chemicals

Test Chemical	Target
Rosiglitazone maleate	PPARg
Tributyl phosphate	PPARg, PXR
Prednisone	GR, AR
Troglitazone	PPARg, PPARa
Zileuton	PPARg; ALOX5
Bexarotene	RxRb
Gemfibrozil	PPARa
Butachlor	GR, AR (env)
Triphenyl phosphate	PPARg (env)
Fenofibrate	PPARa
Dexamethasone NaPO4	GR
Triphenyltin chloride	RxR, RAR
PFOA	PPARa (env)
Potassium PFHxS	PPARa (env)

Results - PPARγ

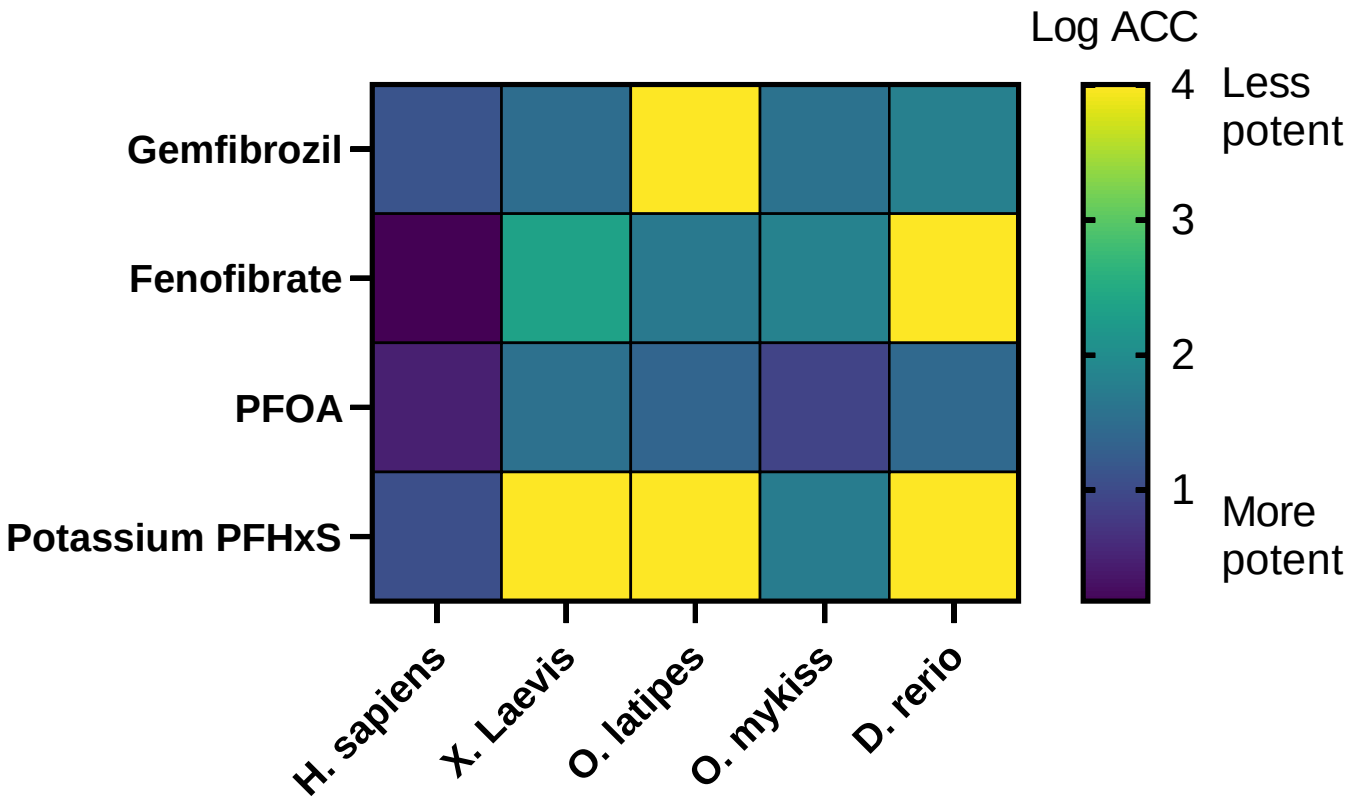


Predicted Susceptible

Taxa	Human (H. sapiens)	Frog (X. laevis)	Medaka (O. latipes)	Trout (O. mykiss)	Zebrafish (D. rerio)
Rosiglitazone	Y 1.0	N 0.06	N <0.006	N <0.006	N <0.006

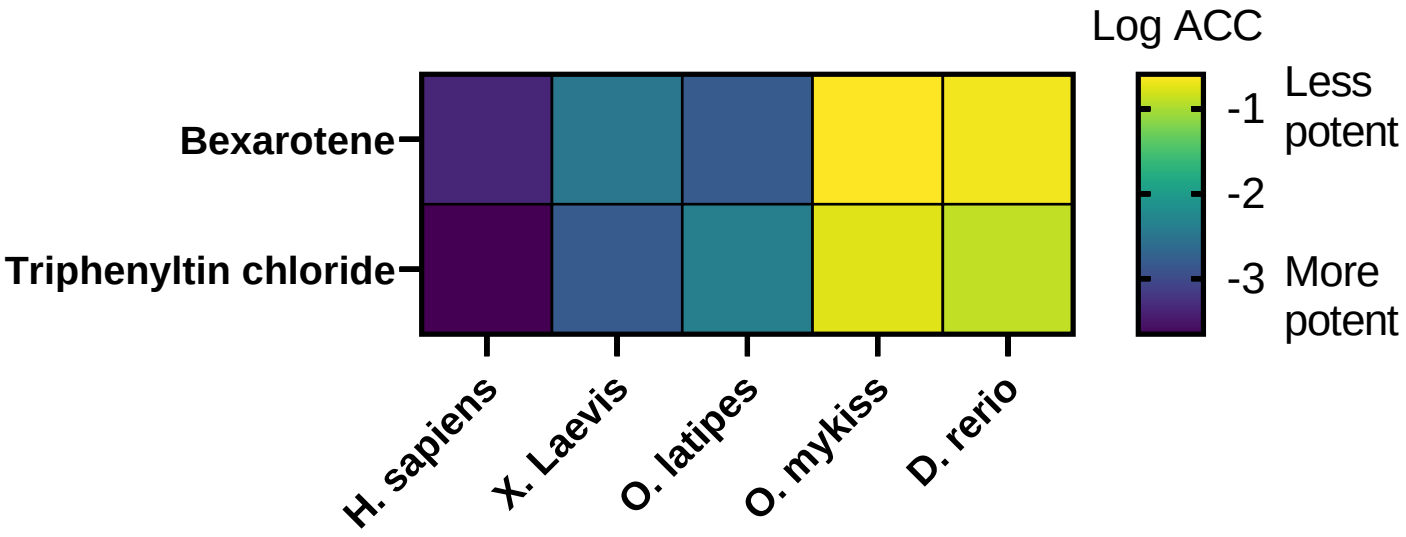
- As predicted, only human PPARγ was sensitive to rosiglitazone
- Among the other PPARγ agonists, Xenopus and rainbow trout were insensitive
- Japanese medaka, selected to represent “most fishes” showed partial sensitivity to some, but not all ligands.
- Zebrafish were sensitive to TPP, but not other ligands

Results - PPARα



- Medaka PPARα was insensitive to gemfibrozil
- Zebrafish PPARα was insensitive to fenofibrate
- Results suggest that aa residues critical to binding gemfibrozil and fenofibrate may differ
- All species sensitive to PFOA
- Human and rainbow trout demonstrated sensitivity to PFHxS

Results - RXRβ

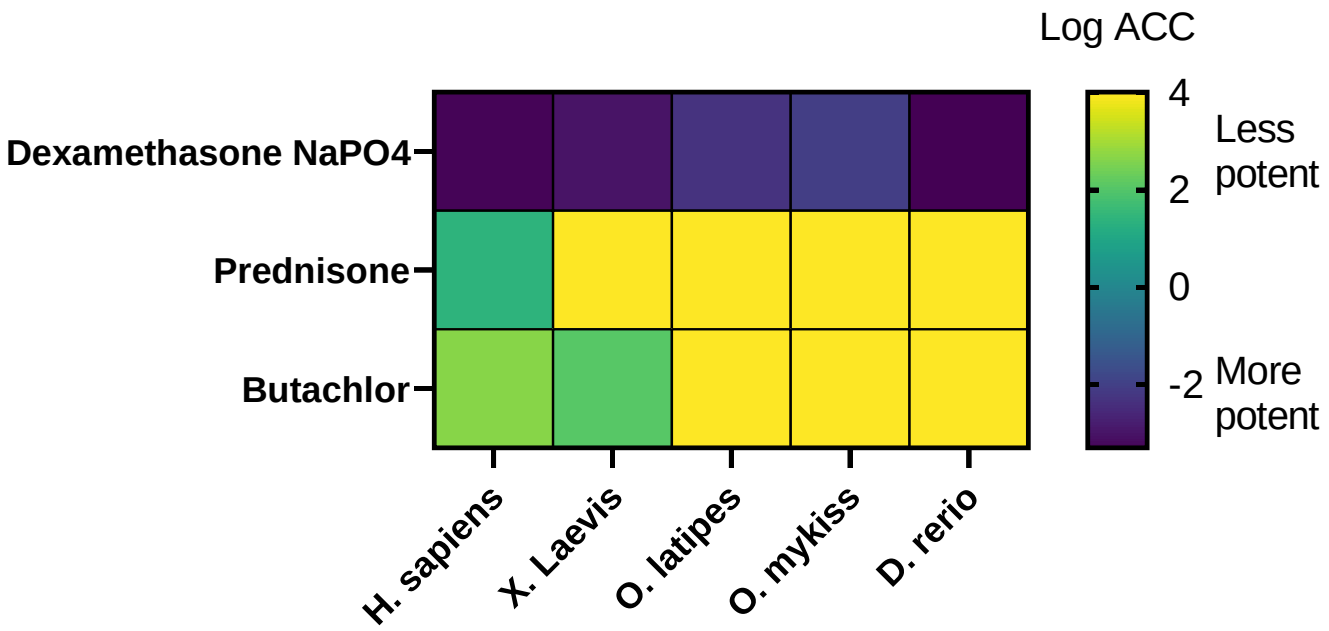


- Rainbow trout and zebrafish RXRb were less sensitive to RXRb ligands than the other species tested.

Results - GR

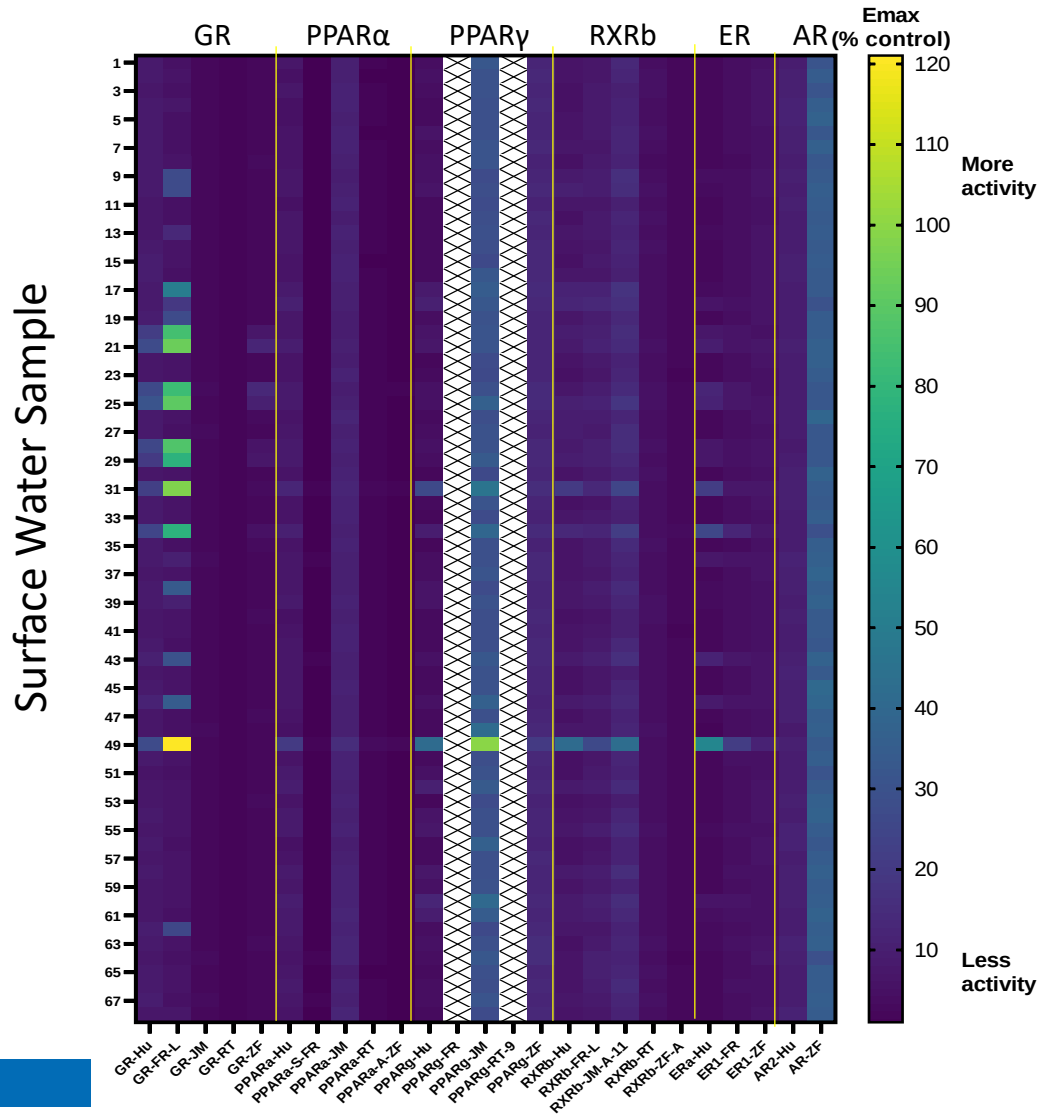
Predicted Susceptible

Taxa	Homo sapiens	Xenopus laevis	Oryzias latipes	Oncorhynchus mykiss	Danio rerio
Dexamethasone	Y	Y	N	N	Y



- Predictions were qualitatively accurate for dexamethasone but reflected different sensitivity, not overall susceptibility
- Need to metabolically activate prednisone to the GR-active prednisolone complicates interpretation

Application to Environmental Monitoring



- 68 surface water samples screened
- Among the GRs, Xenopus GR was the most responsive to GR-active compounds in environmental mixtures
- Among the PPARγ Japanese medaka PPARγ was the most responsive to the environmental mixtures
- Samples with the greatest activity were consistently elevated in all species, proportional to their intrinsic relative sensitivity.

Conclusion

- Effects-based monitoring employing human cell lines using human nuclear receptors (hNR) are likely to yield different conclusions than if fish NRs were employed (at least for PPARγ, PPARα, RXRβ, and GR).

- Variations among different orders of fish may be as substantial as across other classes of vertebrates.

- Different chemical-specific profiles across species were consistent with a previous assumption that level 3 SeqAPASS analyses based on specific ligand-chemical interactions may not apply universally across relevant chemical space.
  - Complicates the ability to select a minimum number of species to capture maximum variability in sensitivity.

- Screening of additional chemicals using the XS-2 Factorial Assay may yield new insights that improve the ability to predict cross-species susceptibility based on aa sequence.

References

- *Cavallin et al. Effects-Based Monitoring of Bioactive Chemicals Discharged to the Colorado River before and after a Municipal Wastewater Treatment Plant Replacement. Environmental Science & Technology 2021, 55, 2, 974–984. https://doi.org/10.1021/acs.est.0c05269*
- *Lalone et al. Sequence Alignment to Predict Across Species Susceptibility (SeqAPASS): A Web-Based Tool for Addressing the Challenges of Cross-Species Extrapolation of Chemical Toxicity. Toxicological Sciences, Volume 153, Issue 2, October 2016, Pages 228–245, https://doi.org/10.1093/toxsci/kfw119*
- *Medvedev et al. Harmonized cross-species assessment of endocrine and metabolic disruptors by EcoTox FACTORIAL assay. Environmental Science & Technology 2020 54, 19, 12142-12153. https://doi.org/10.1021/acs.est.0c03375*

EPA United States Environmental Protection Agency

High Throughput Transcriptomics: A Multi-Species Approach

Presented by Kevin Flynn

to

US EPA BOSC

Chemical Safety Subcommittee Meeting




Office of Research and Development
Center for Computational Toxicology and Exposure

February 2, 2021

ORD Strategic Research Action Plan

CSS.1.7:

Develop, evaluate, and apply **non-mammalian** high-throughput toxicity tests for priority endpoints and pathways in ecological species for ecological risk assessment

CSS.4.4:

Develop rationale and case studies that apply AOPs and HTT data to inform test-order decisions and establish scientific support for waiving testing requirements for pesticides

Program Office Feedback & Support

Program Office support

- Office of Pesticide Programs (OPP)
- Office of Pollution Prevention and Toxics (OPPT)

EPA/ORD Partner Needs and Use

OCSPP; OW:

Ecological risk assessments address species across diverse taxonomic groups, many of which have limited or no available data. The clear majority of HTT methods are based on either human or mammalian in vitro systems, which results in an under-representation of pathways that are relevant and perhaps unique to non-mammalian taxa

OPP:

"The proposed research is responsive to EPA's commitment to reduce its reliance on resource intensive whole animal testing and to better allocate limited resources to where they are most needed through the use of more effective screening tools"

- evaluate taxonomic domain of applicability of HTT NAMs
- determine relevance to regulatory assessment endpoints
- provide evidence that risk estimates across taxa are suitable
- critical effort to turning theory into practice (risk estimates)

OPPT:

"HTT methods are required for OPPT to rapidly and efficiently screen and prioritize new and existing chemical substances under the Toxic Substances Control Act (TSCA)."

- meet scientific standards under section 26(h) of TSCA
- reduce testing on vertebrates under section 4(h) of TSCA
- determine unreasonable risk under sections 5 & 6 of TSCA

Demonstrating quantitative relationships between transcriptomic responses and phenotypic alterations in apical assessment endpoints across multiple taxa will ***provide a compelling means of promoting the adoption of such methods and incorporation of such data in ecological risk assessments*** – OPP comment to Eco-trans Output

The effort should provide an understanding of how the battery of assays relate an AOP where a molecular initiating event leads to a sequence of key events that culminate in an adverse outcome at the whole organism level and **how such methods can be used to effectively screen for effects across a wide taxonomic and chemical space**. OPP has a wide array of in vivo data on pesticides and these data could serve as a means of assessing the extent to which the battery of assays is predictive across chemicals/taxa - OPP comment to Eco-trans Output

OPP has a broad array of *in vivo* data across multiple taxa with which to compare transcriptomic responses once suitable linkages have been demonstrated. ***OPP is interested in working with ORD to identify "model" chemicals*** - OPP comment to Eco-trans Output

A Chemical Numbers Problem

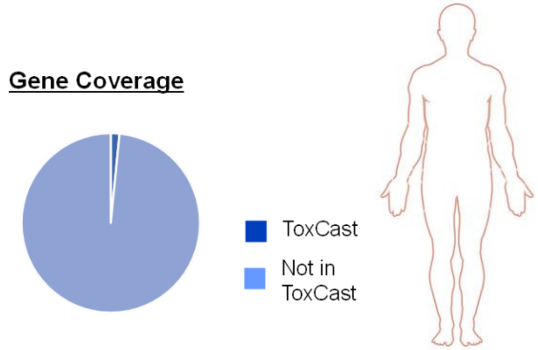
U.S. EPA Strategic Plan (2018-2022), Objective 1.4, Ensure Safety of Chemicals in the Marketplace

Problem Statement:

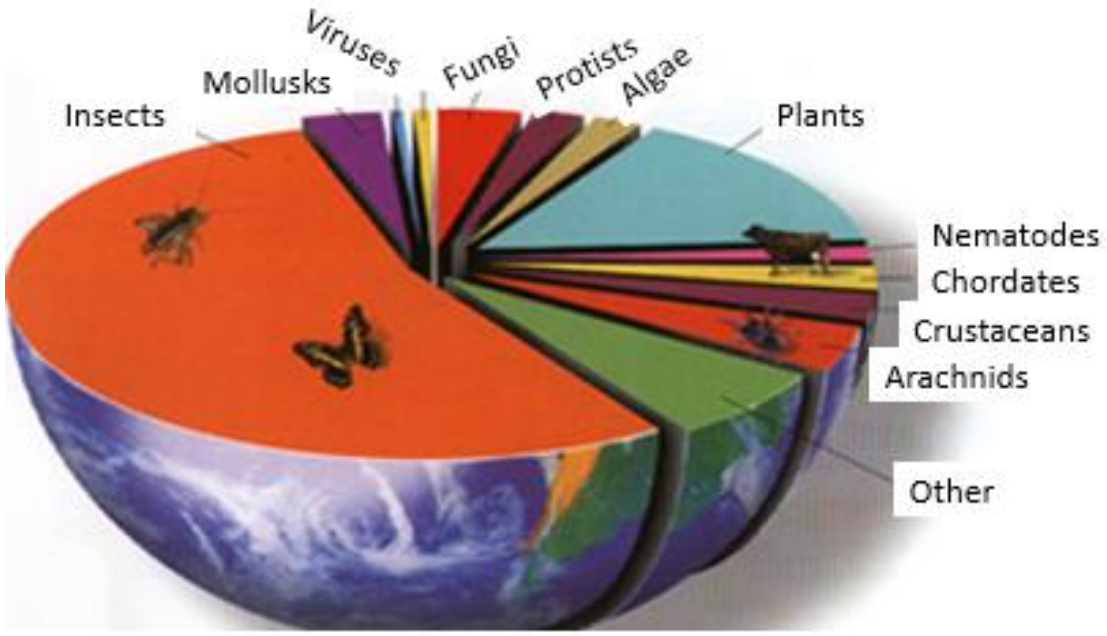
Tens of thousands of chemicals are currently in use and hundreds more are introduced to the market every year. Only a small fraction has been thoroughly evaluated for potential risks to human health and the environment.

“Too many chemicals, too little data”

A Biological Numbers Problem



"Throughout the development and execution of ToxCast and Tox21, key limitations of the current suite of HTS assays have been identified (Tice, et al., 2013). The limitations include ***inadequate coverage of biological targets and pathways***" Thomas et al. 2019



The Eco Data Gap:

- Humans are just a tiny fraction of the biological diversity we are charged to protect.
- Many genes/pathways are conserved
- Unique physiology in other kingdoms, phyla, classes…

HTP Eco Assay Development

Daphnia magna

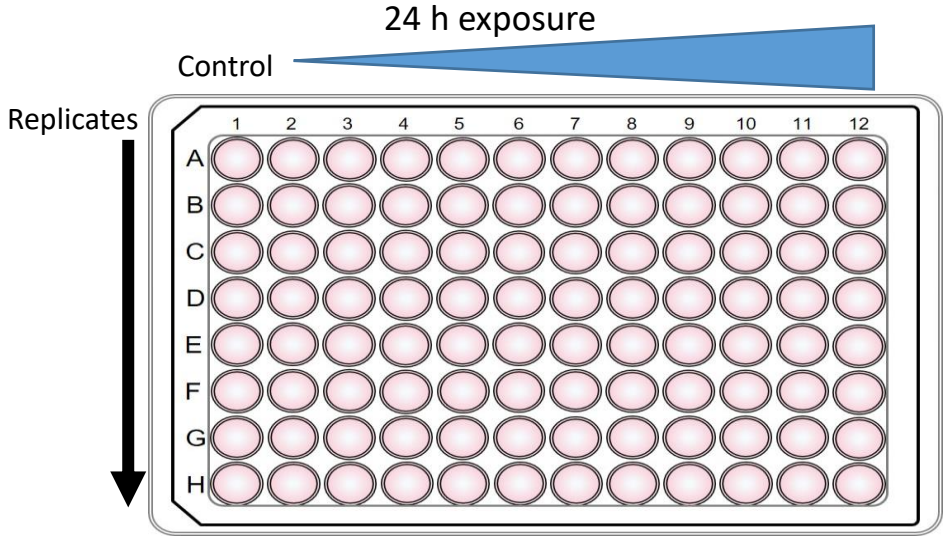
Pimephales promelas

Chironomus dilutus

Raphidocelis subcapitata

- Modify standard protocols and methods to allow rapid toxicity tests with small aquatic organisms in 96-well plates – 4 species
- Conduct exposures with diverse chemicals (ex. metals, neonics, pharmaceuticals, PFAS)
- Compare traditionally derived LC50 values to LC50 values calculated from 96-well plate-based exposures
- Use RNA-seq data to calculate transcriptomic-based point-of-departure (tPODs) that can be anchored to apical responses

HTP Eco Assay Development



Phenotypic anchoring

- survival
- behavior
- growth?

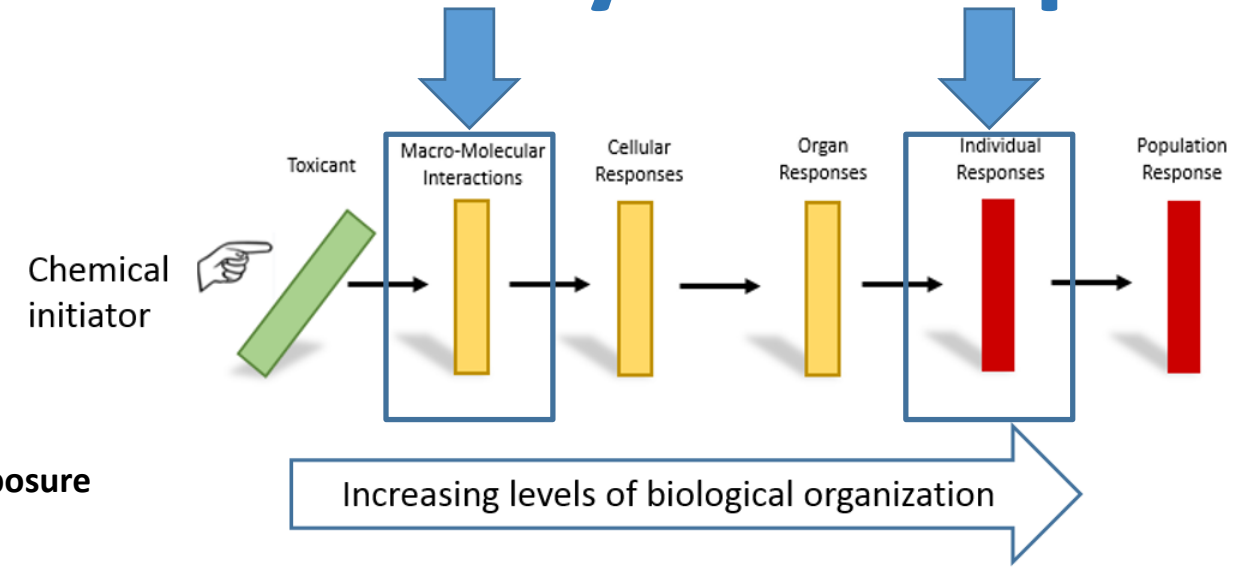
Species	Guideline Test Method	Age at Start	Temp
Daphnia magna	850.1010 Aquatic Invert Acute Toxicity	72-hour	20° C
Pimephales promelas	850.1075 Fish Acute Toxicity	24-hour	25° C
Chironomus dilutus	850.1790 Chironomid Sediment Toxicity	3rd instar	20° C
Raphidocelis subcapitata	850.4500 Algal Toxicity	Log-phase	24° C

Exposures Design

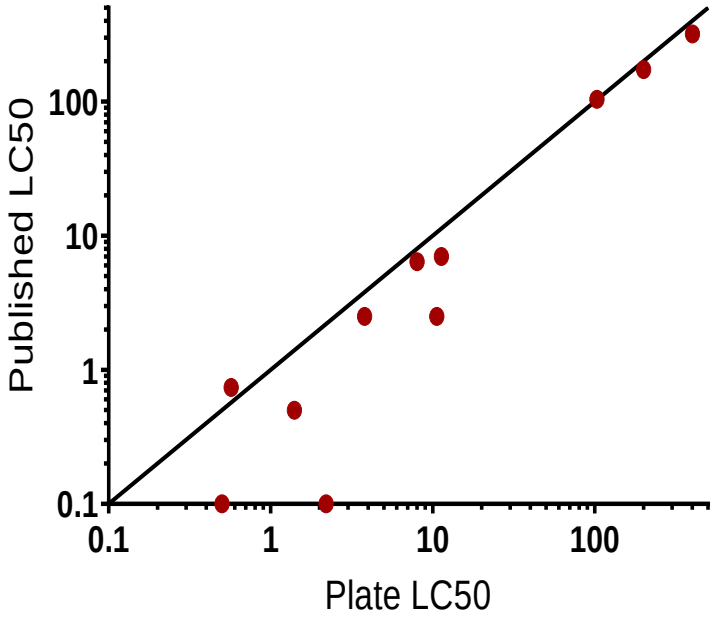
- 1 ml deep 96-well plates
- 12 concentration – 8 replicates per concentration
- 1 individual per well (algae ~5 x 10^4 cells/ml)
- 24-hour static exposures
- phenotypic endpoints assessed
  - animals: survival and behavior
  - algae: cell viability & division, photopigments
- then after homogenization, RNA extracted for transcriptomics

Species	Time to Load Plate	Control 24-hour Survival	RNA Qty per Well
Daphnia magna	~45 minutes	72-hour	~1000 ng
Pimephales promelas	~30 minutes	24-hour	~1500 ng
Chironomus dilutus	~60 minutes	3rd instar	~900 ng
Raphidocelis subcapitata	~10 minutes	Log-phase	~300 ng

HTP Eco Assay Development



LC50s:
Published vs 96-well Exposure



≈

- Linking to apical endpoints essential
- Apical Endpoints
  - Survival 👍
  - Reproduction 👎
  - Growth 👍 or 👎
- Behavior
- "Imageable" measurements

Eco Transcriptomics Applicability

NTP
National Toxicology Program
U.S. Department of Health and Human Services

NTP Research Report on
National Toxicology Program Approach to Genomic Dose-Response Modeling

NTP RR 5

APRIL 2018

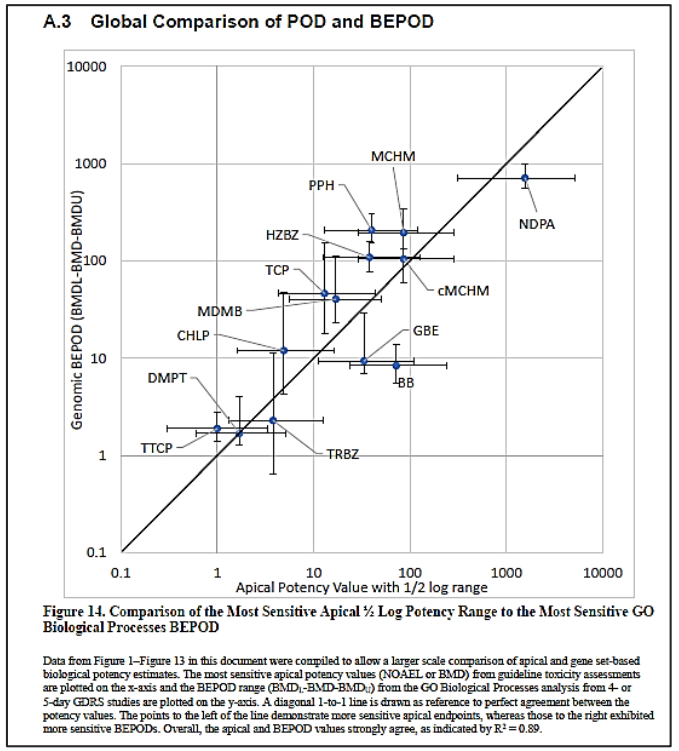


Figure 14. Comparison of the Most Sensitive Apical ½ Log Potency Range to the Most Sensitive GO Biological Processes BEPOD

Data from Figure 1–Figure 13 in this document were compiled to allow a larger scale comparison of apical and gene set-based biological potency estimates. The most sensitive apical potency values (NOAEL or BMD) from guideline toxicity assessments are plotted on the x-axis and the BEPOD range (BMD_L-BMD-BMD_U) from the GO Biological Processes analysis from 4- or 5-day GDRS studies are plotted on the y-axis. A diagonal 1-to-1 line is drawn as reference to perfect agreement between the potency values. The points to the left of the line demonstrate more sensitive apical endpoints, whereas those to the right exhibited more sensitive BEPODs. Overall, the apical and BEPOD values strongly agree, as indicated by $R^2 = 0.89$.

- Number of mammalian studies have shown short-term transcriptomics-based PODs are predictive of apical potency.
- Generally, within ½ log.

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Transcriptomic points-of-departure from short-term exposure studies are protective of chronic effects for fish exposed to estrogenic chemicals

Florence Pagé-Larivière, Doug Crump, Jason M. O'Brien*

National Wildlife Research Center, Environment and Climate Change Canada, Ontario, Canada

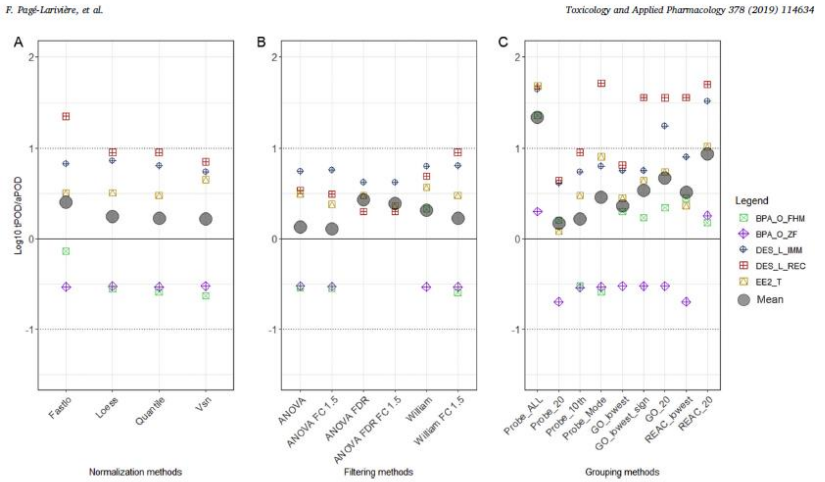


Fig. 6. Effect of the data processing methods on the log 10 ratio of transcriptomic PODs (tPOD) and referenced apical POD (aPOD, equivalent to the chronic LOEC). Representative examples for each data processing method are presented. A) The effect of the normalization method on the tPOD using only one filtering method (Williams Trend with FC ≥ 1.5 [$p < .05$]) and one grouping method ($Probe_{10th}$). B) The effect of the filtering method on the tPOD was determined using a single normalization (quantile) and grouping ($Probe_{10th}$) method. C) The effect of the grouping method on the tPOD using the quantile normalization and filtering with Williams Trend test with a fold-change filter ≥1.5-fold. A different color is assigned to each dataset to represent their specific ratio, and the grey dots represent the mean value of each column. The line at y = 0 represents a perfect situation where tPOD = aPOD. The dotted line at y = −1 and y = 1 represent a 10-fold difference between the transcriptomic and apical POD.

- 100% of tPODs were within 1 order of magnitude of chronic LOEC

HTP Eco Transcriptomics

Challenge.gov
Government Challenges, Your Solutions

EcoTox TARGET Challenge

Develop high quality, low-cost tools that assess global gene expression in common aquatic toxicity test organisms

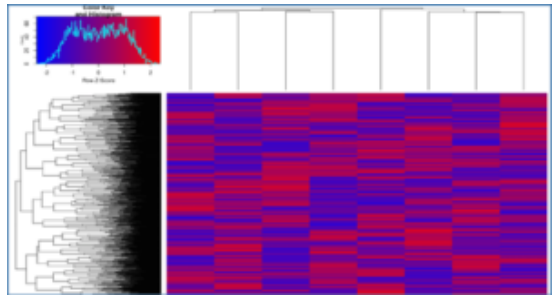
CHALLENGE DETAILS

- TOTAL CASH PRIZES OFFERED: $300,000
- TYPE OF CHALLENGE: Scientific
- PARTNER AGENCIES | FEDERAL: U.S. Army Engineer Research and Development Center
- PARTNER AGENCIES | NON-FEDERAL: DoW, Environment and Climate Change Canada, European Commission Joint Research Centre, Syngenta
- SUBMISSION START: 03/19/2020 12:00 PM ET
- SUBMISSION END: 06/15/2021 11:59 PM ET

Commercial Development

4 “Solvers”

Detection/analysis technology

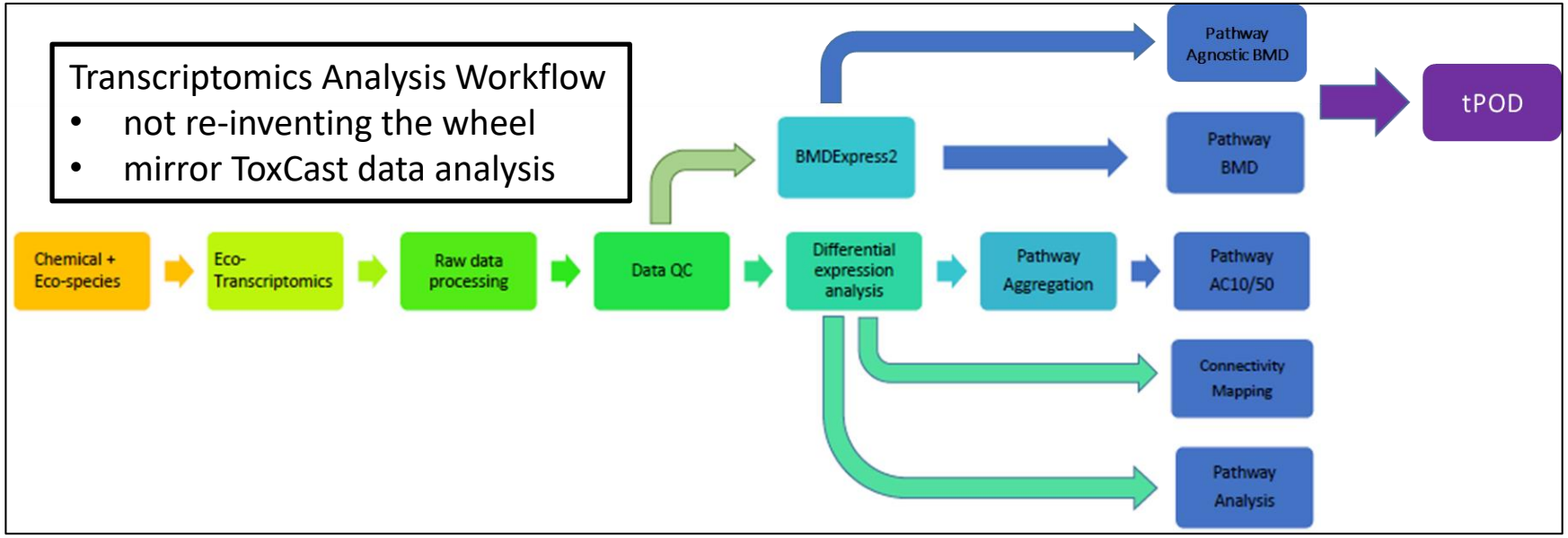


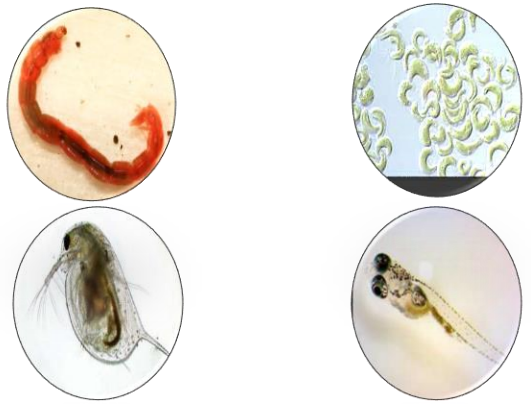
Commercially available
Low cost (<$50/sample)
High quality
Maximal coverage

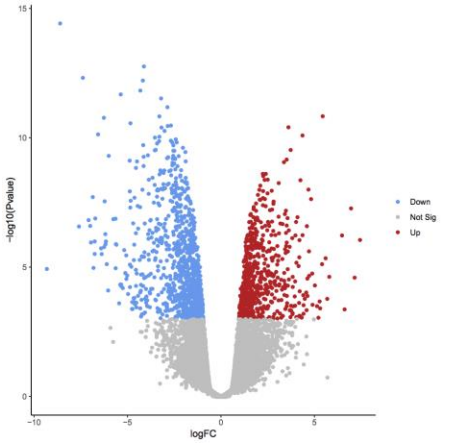
TARGET
EcoTox Challenge

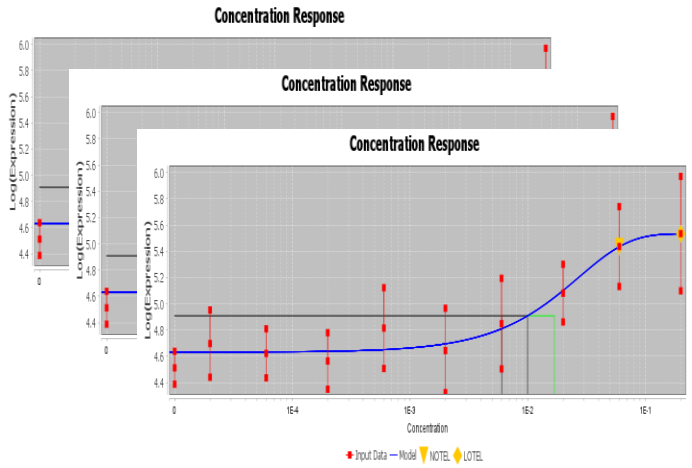
Eco Transcriptomics Data Analysis

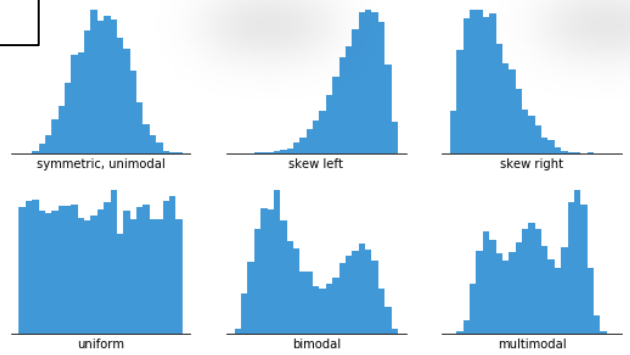
Transcriptomics Analysis Workflow
- not re-inventing the wheel
- mirror ToxCast data analysis











BMD Grouping

- All BMDs - median
- X lowest (ex. 20)
- Percentile (ex. 10th)
- 1st mode
- All BMDs from lowest GO terms or REACTOME pathways
- Median BMDs from 20 lowest GO terms or REACTOME pathways

Current Status

- Derive transcriptomics-based points of departure for 20 chemicals
- Testing with fathead minnow only
- Compare with traditional apical PODs
- Evaluate hypothesis that tPODs are protective relative to apical
- Includes chemicals of direct interest to Program Offices and partners

Workflow in Brief

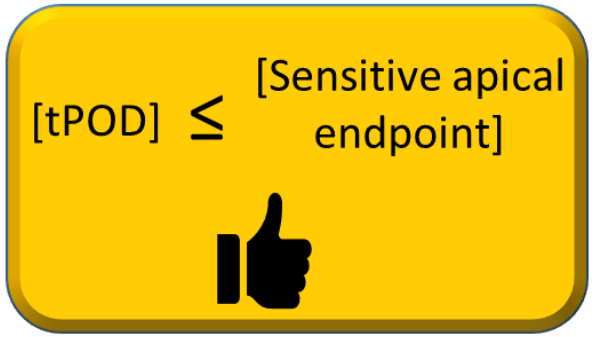
RNA-seq data was obtained from each well; all raw reads were assembled into transcript models, aligned with annotations, counted, normalized, and log2 transformed for each transcript

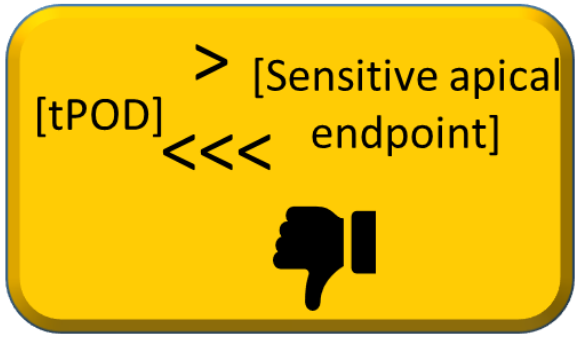
- Low count feature filtering: any given feature had to have a count of 10 or more in a minimum of 4 samples or that feature was filtered out
- Differentially expressed genes (DEGs) determined by NTP guidelines and transcriptomic POD for a chemical defined as median or 10th percentile POD of all DEGs (https://ntp.niehs.nih.gov/publications/reports/rr/rr05/index.html)

HTP Eco Assay Development

Chemicals	Chemical Class	Rationale	Data Product Support
CuSO4, NiSO4, ZnSO4	metal	OW; ease of exp.; mouse & RBT data	**APCRA case study**; 4 eco-species
Clothianidin, Thiacloprid, Imidacloprid	Neonicotinoid	OPP	**APCRA case study**; 4 eco-species; **Challenge**
Flupyradifurone	Butenolide	OPP	**APCRA case study**; 4 eco-species
Sertraline, Fluoxetine, Paroxetine	SSRI	Existing data at GLTED	**APCRA case study**; 4 eco-species
Atrazine, simazine, cyanazine	Herbicide	Herbicide	**Challenge**; 4 eco-species
Methoxyfenozide, tebufenozide, methoxyfenozide	Carbohydrazide	Insecticide	**Challenge**; 4 eco-species
Parathion, methidathion, fenthion	Organophosphate	mouse data	APCRA case study; 4 eco-species
dibutyl, diethylhexyl, butylbenzyl phthalates	Phthalates	TSCA high priority	APCRA case study; 4 eco-species
~20 specific PFAS	PFAS	PFAS plus up; small # *in vivo*	4 eco-species
50 – 100 additional	diverse	StRAP	4 eco-species

Draft Data





Chemical	Transcriptomic POD (10th and median)		96-hour LC50	Mortality-based POD
CuSO4	0.005 mg/L	0.03 mg/L	0.3 mg/L	0.2 mg/L
ZnSO4	0.063 ug/L	0.23 ug/L	2.2 mg/L	3.2 mg/L
NiSO4	0.146 mg/L	0.33 mg/L	6.2 mg/L	3.9 mg/L
Imidacloprid	0.03 µg/L	8.8 mg/L	173 mg/L	> 10 mg/L
Flupyradifurone	0.0226 µg/L	1.3 mg/L	Not in ECOTOX	> 10 mg/L
Clothianidin	0.2 µg/L	8.1 mg/L	0.5 (104) mg/L	> 10 mg/L
Thiacloprid	0.036 mg/L	57.2 mg/L	104 mg/L	85 mg/L
Sertraline	0.001 mg/L	0.6 mg/L	0.1 mg/L	0.9 mg/L
Fluoxetine	0.003 µg/L	0.02 mg/L	0.2 mg/L	0.8 mg/L
Paroxetine	0.002 mg/L	1.0 mg/L	3.5 mg/L	1.1 mg/L

Upcoming Work - Validation

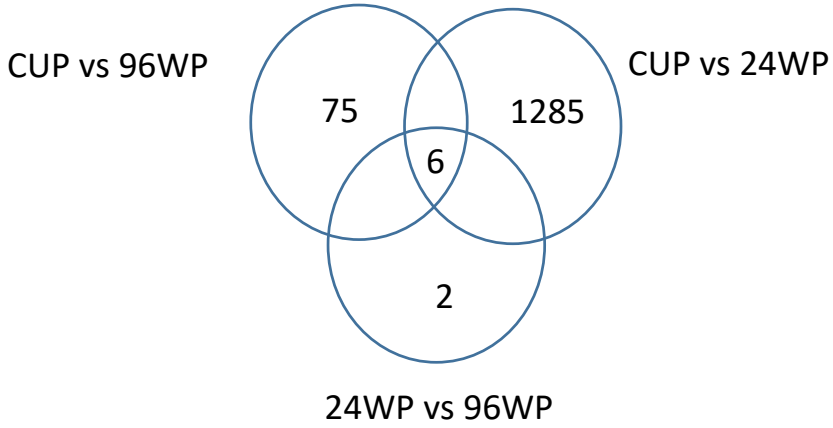
Assay Development

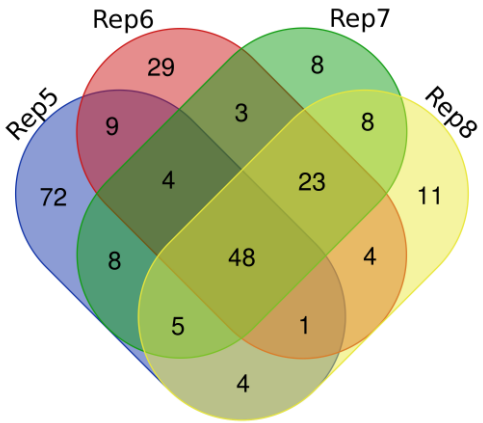
- Verify water quality parameters
  - dissolved oxygen
  - pH
  - ammonia
- Chemical bioavailability

Transcriptomics

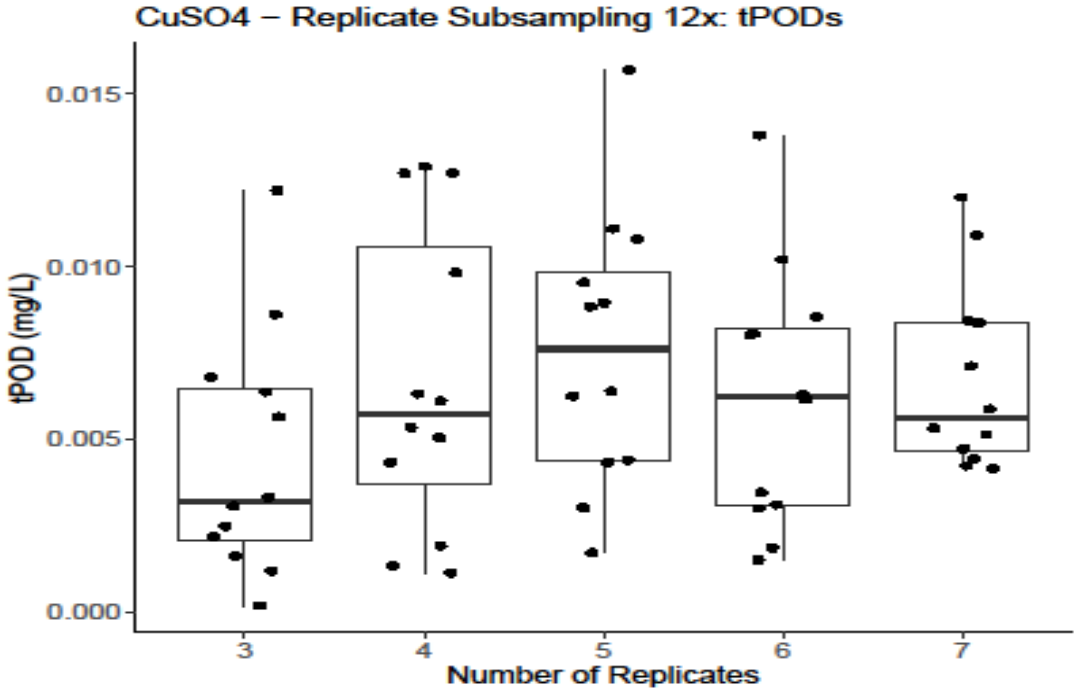
- Complete Challenge
  - platform development
  - genome annotation
- Definition/Implementation of analysis pipeline
- Assess variability focused on tPODs
  - intra/inter exposure plate
  - between exposure plates
  - appropriate replication

POD Calculation for CuSo4 in each Volume			
BMDExpress2 Results	Volume Format		
	CUP	24WP	96WP
#DEGs passing NTP filters[1]	128/369	52/159	108/208
Median POD (mg/L)	0.0445	0.045201	0.025





Overlap of DEGs



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Environment and Climate Change Canada

Environnement et Changement climatique Canada

References

- U.S. EPA Strategic Plan (2018-2022), Objective 1.4, Ensure Safety of Chemicals in the Marketplace

- Thomas, R. S., Bahadori, T., Buckley, T. J., Cowden, J., Deisenroth, C., Dionisio, K. L., ... & Williams, A. J. (2019). The next generation blueprint of computational toxicology at the US Environmental Protection Agency. Toxicological Sciences, 169(2), 317-332.

- Page-Lariviere, F., Crump, D., O'Brien, J. M. (2019). Transcriptomic points-of-departure from short-term exposure studies are protective of chronic effects for fish exposed to estrogenic chemicals. Toxicology and Applied Pharmacology, 378, 114634.

- EcoTox TARGET Challenge: https://www.challenge.gov/challenge/ecotox-challenge/

- National Toxicology Program Approach to Genomic Dose-Response Modeling: https://ntp.niehs.nih.gov/publications/reports/rr/rr05/index.html