Biomarkers & Biochemical Endpoints of Toxicological Studies

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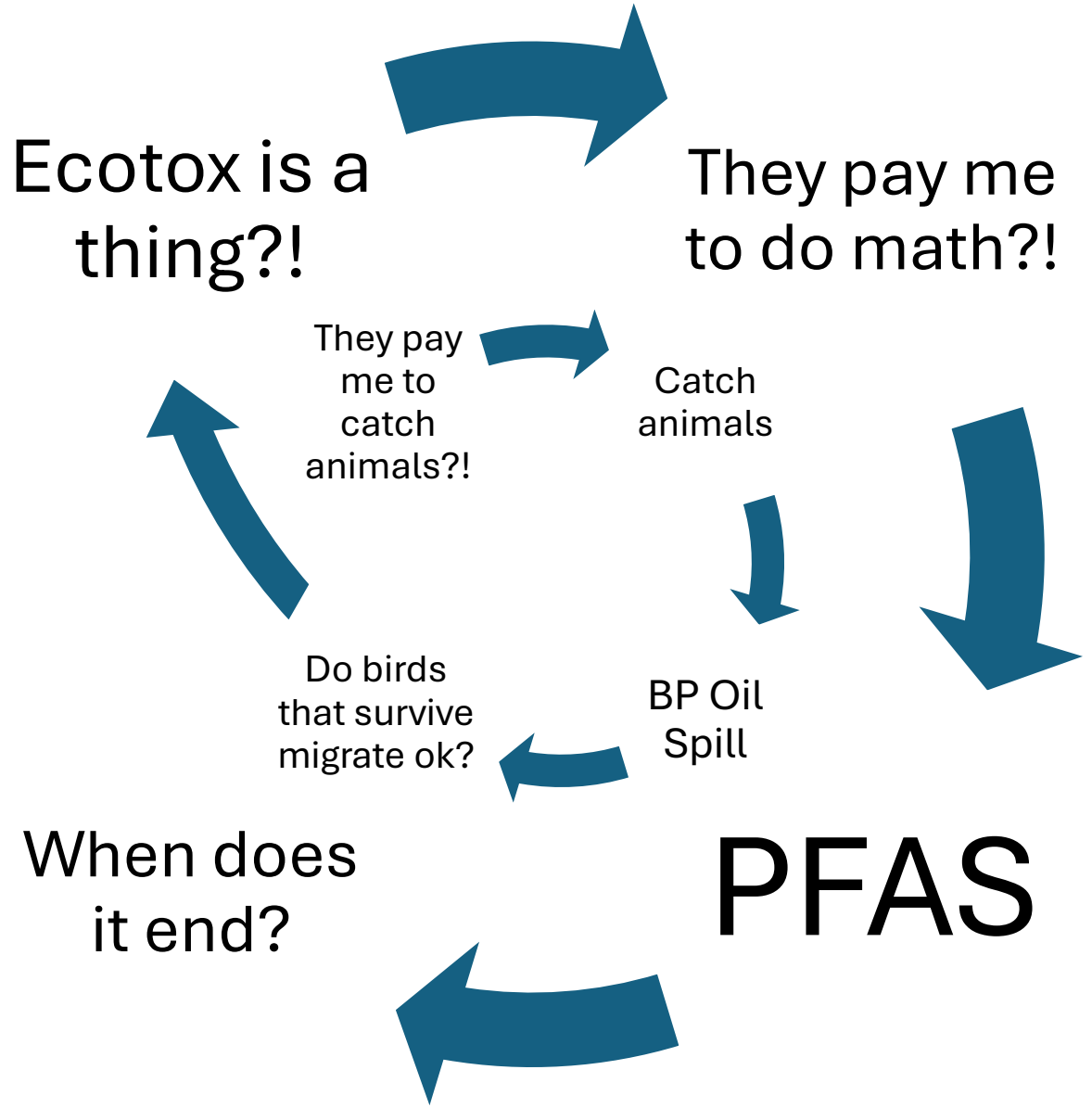
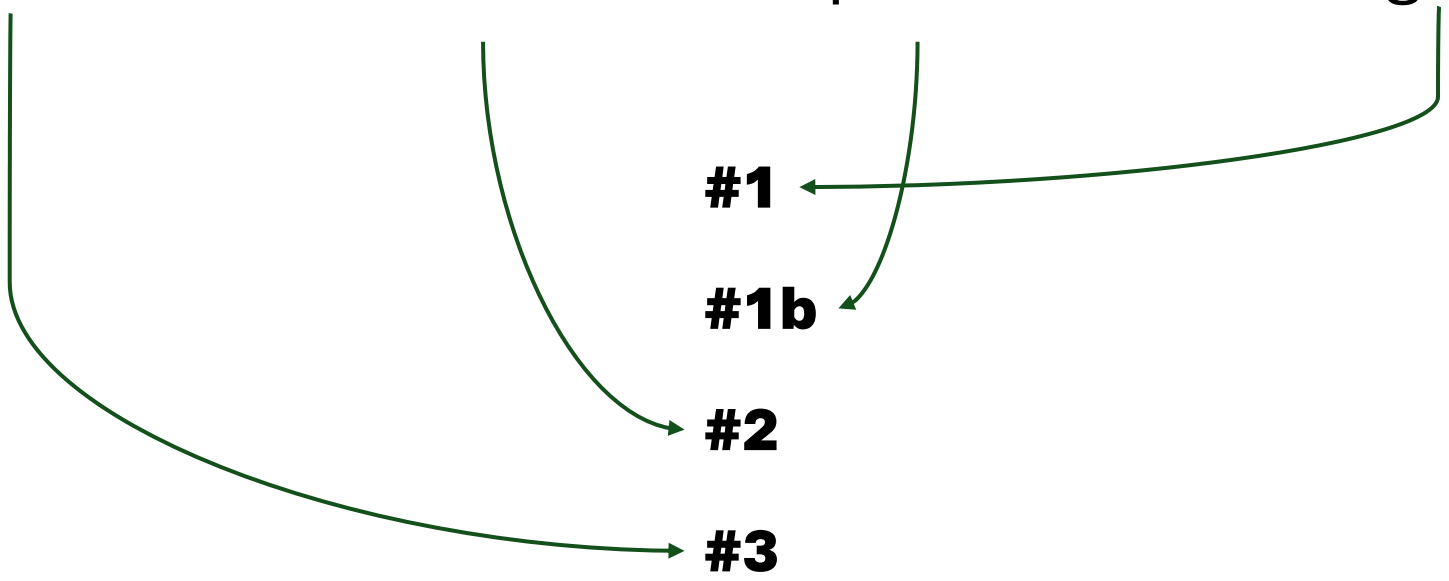
Who am I?

- Quantitative EcoToxicologist
- Work with Dr. Yonkos
  - Terrestrial organism exposure to PFAS
- DoD Biologist in Public Health Toxicology Directorate.
  - Support risk+hazard assessments



Lecture Map

- Biomarkers & Biochemical Endpoints of Toxicological Studies

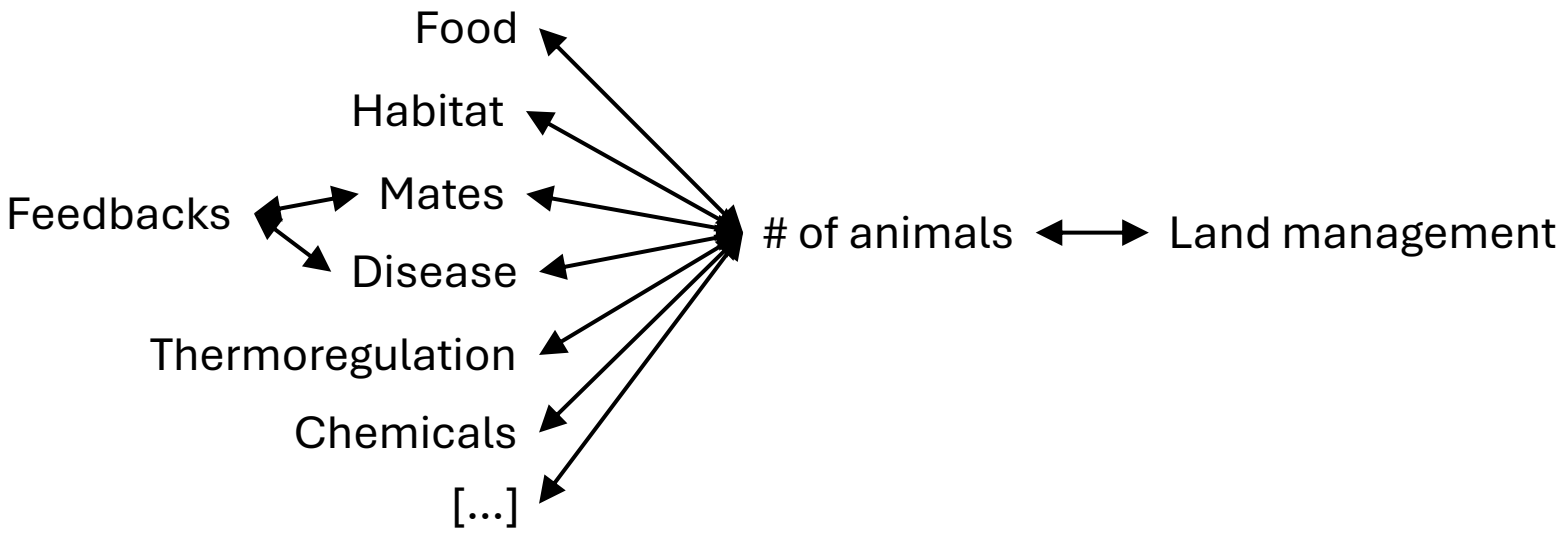


Toxicological paradigms to consider

- Interdisciplinary and translational science

Chemical Exposure → Effect

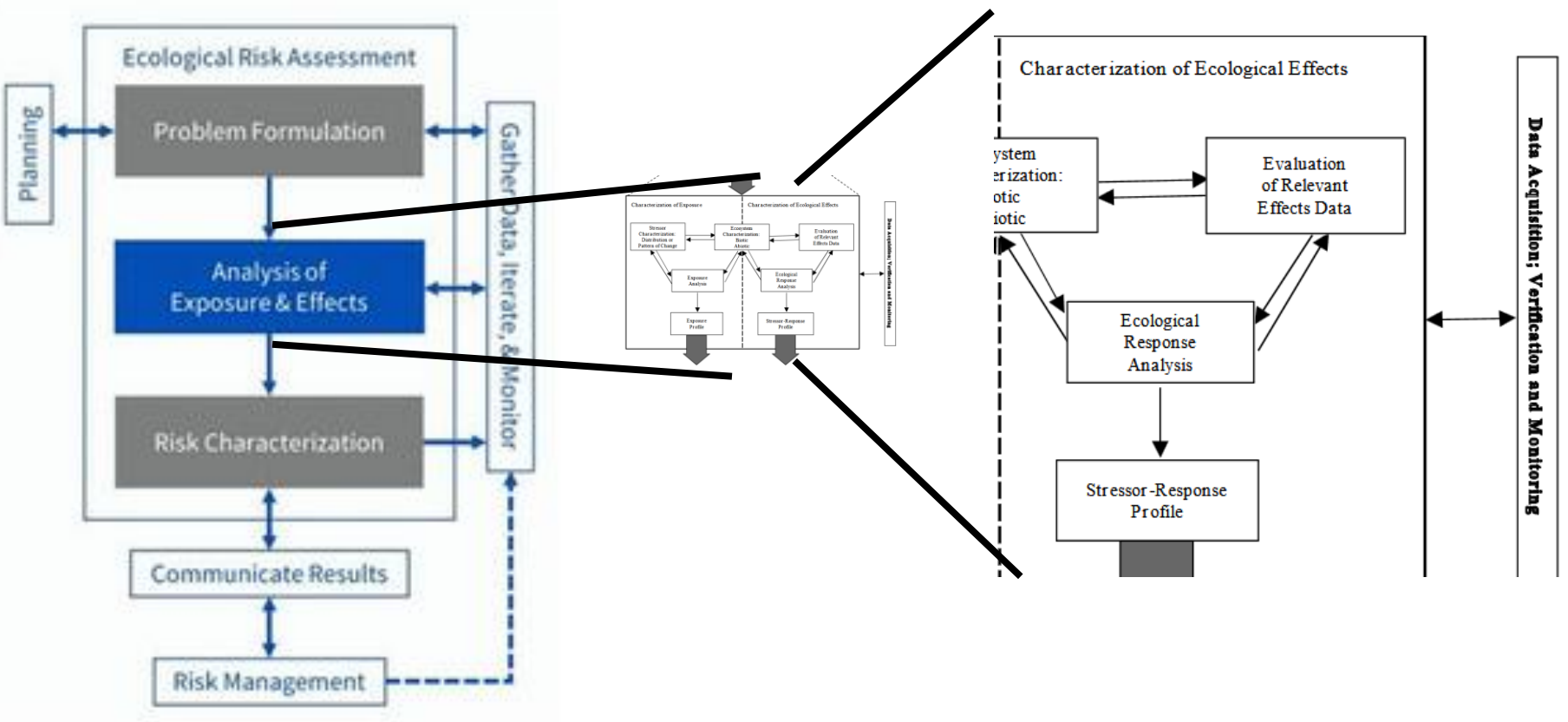
Data you have.



Your data in context.

Toxicological paradigms to consider

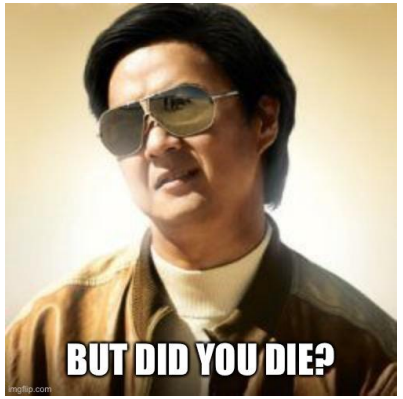
- Basic to applied translation has a purpose



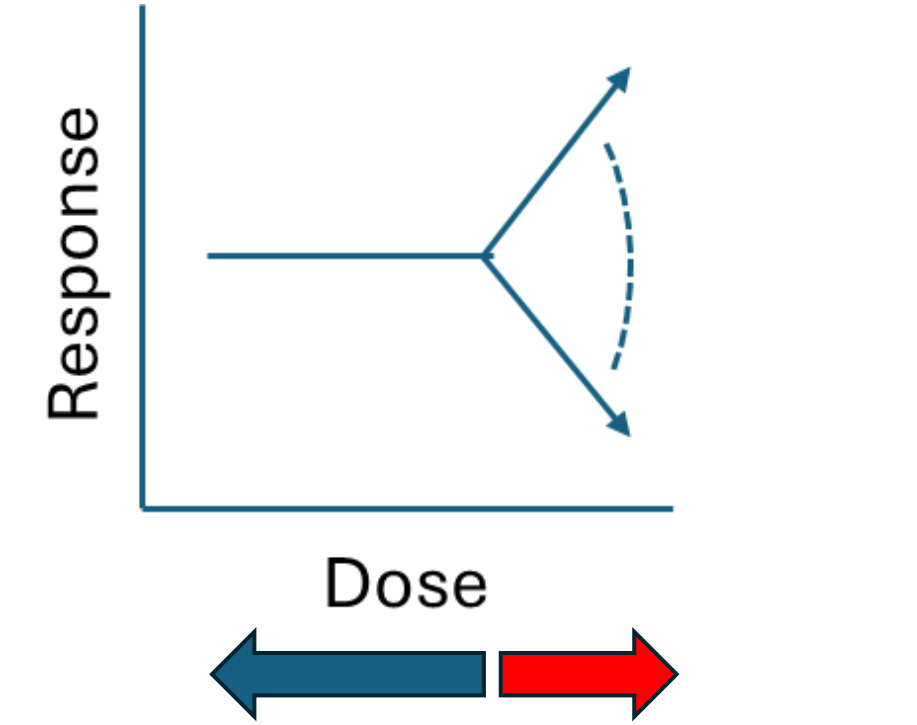
The goal is to predict effects given exposure

Toxicological paradigms to consider

- “Dose makes the poison”
- Regs are usually based on the identification of the ***highest safe dose***

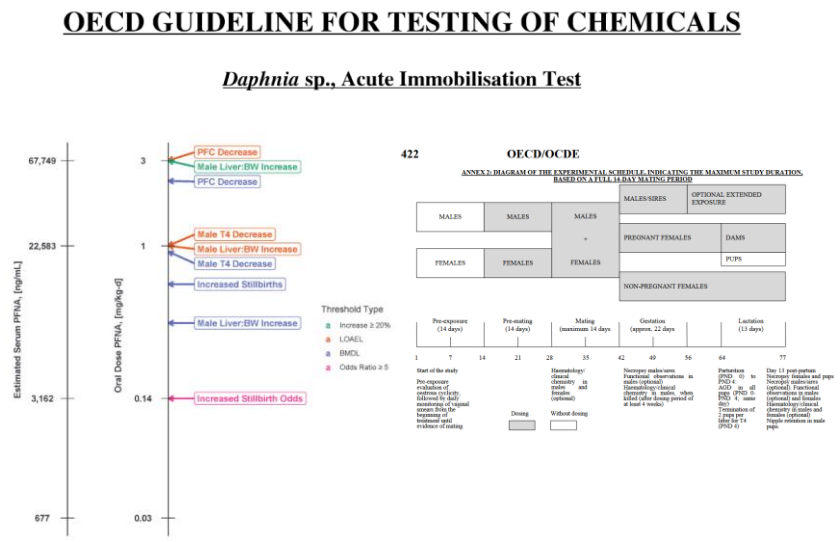


Supposed to be “adverse.”
What does adverse mean?



Toxicity tests

- Purposes
  - Regulatory requirements
    - Standardized protocols + endpoints +reporting
    - Comparative and threshold setting
  - Research
    - Mechanisms
    - Refined exposures + endpoints
    - Co-stressors + modifying factors
    - Comparative and threshold setting



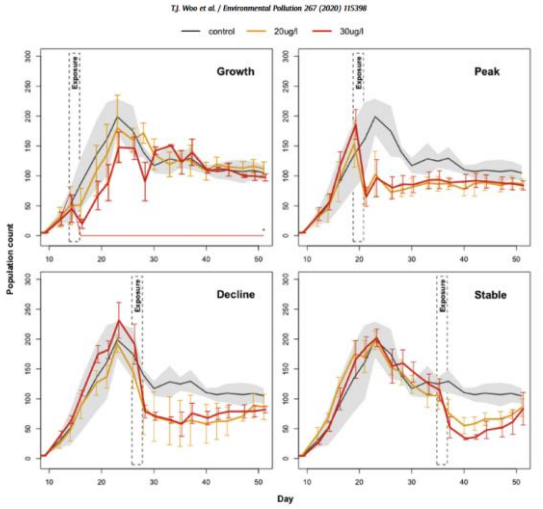


TABLE 3.—Absolute liver weight, relative liver weight (% body weight), hepatocyte hypertrophy, and labeling index (LI) of PFOA- and WY-treated PPAR-α knockout mice (mean ± SD).

Treatment (n)	Liver Weight (g)	Relative Liver Weight (%)	Lesion Score	LI
Control (8)	0.92 ± 0.08	3.4 ± 0.4	1.1 ± 0.4	0.2 ± 0.2
1 mg/kg PFOA (8)	1.2 ± 0.14[a]	4.5 ± 0.2[a]	1.9 ± 0.6[a]	0.6 ± 0.4
3 mg/kg PFOA (7)	1.46 ± 0.21[a]	5.8 ± 0.3[a]	3.0 ± 0[a]	0.6 ± 0.3
10 mg/kg PFOA (7)	2.8 ± 0.18[a]	9.4 ± 0.6[a]	4.0 ± 0[a]	7.7 ± 3.0[a]
50 mg/kg Wyeth (7)	1.07 ± 0.24	3.9 ± 0.5	1.4 ± 0.5	0.6 ± 0.5

a. statistically different from control by Student's *t* test at $p < .05$

$$set\ LC50\ [(m * [algae] + b) + a * length\hat{}c]$$

$$resp = 1 - \exp(-\exp(b(\ln([pyra]) - \ln[LC50])))$$

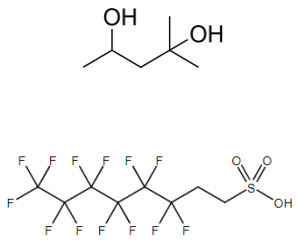
Toxicity test (firefighting foam example)

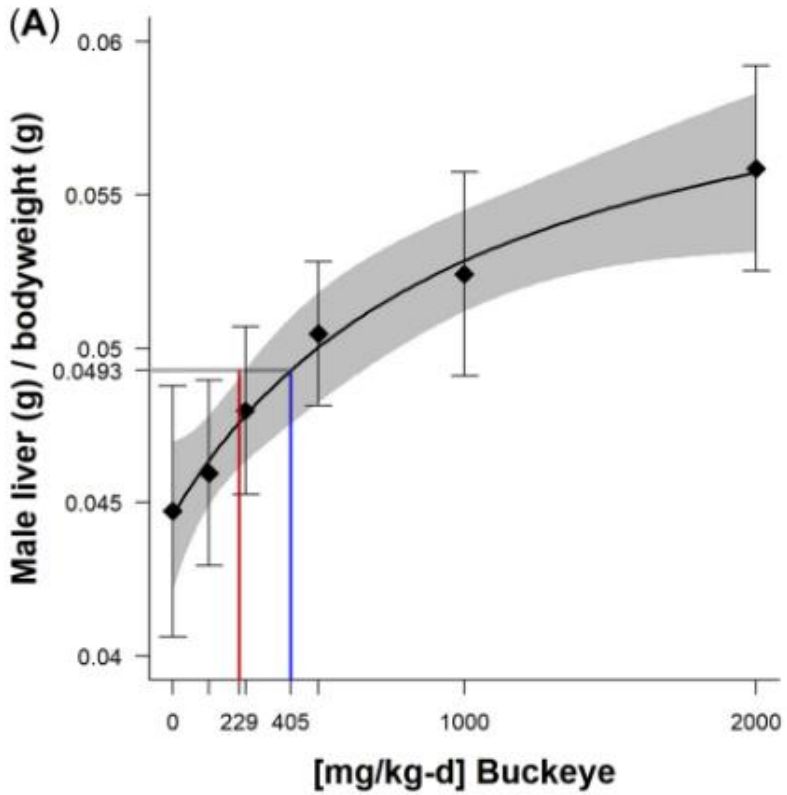
- Exposed mice to increasing concentration of fire fighting foams for 28 days.
- Is the toxicity profile (and threshold value) different between PFAS-free and PFAS-containing?
- Following basis of OECD#407 (Repeated dose 28-day oral toxicity study in rodents).

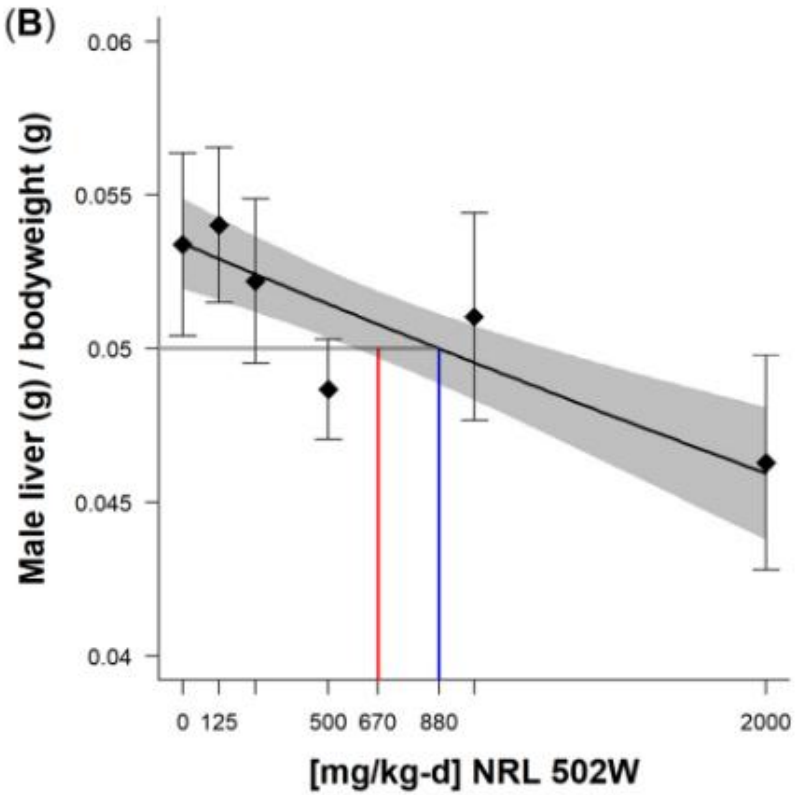
- Endpoints based on clinical observation, survival, body weight trends, organ weights, micronucleus assay, thyroid hormones, hematology, clinical chemistry, toxicological pathology.

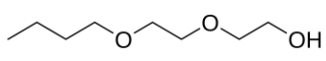
Two different results, can you explain it?

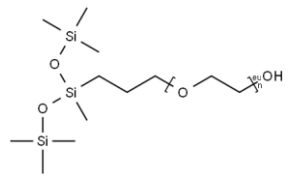
- The direction of the dose-response is opposite—why? And how do you make sense of it?





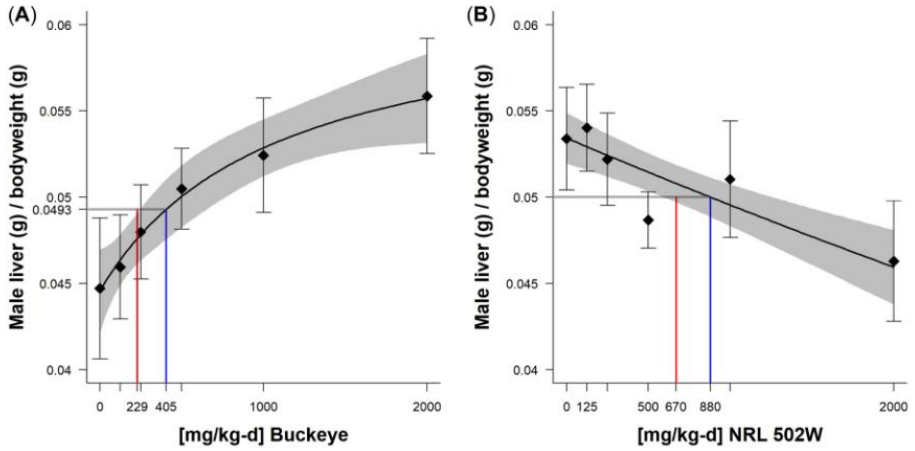






East AG, Narizzano AM, Holden LA, Bazar MA, Bohannon ME, Pervitsky D, Adams VH, Reinke EN, Quinn MJ. 2023. Comparative Toxicity of Seven Aqueous Film-Forming Foam to In Vitro Systems and *Mus*. Environmental Toxicology and Chemistry. 42(11):2364–2374. doi:10.1002/etc.5714.

The rest of the tox profile.



No influence on bodyweight over time, no increase in micronucleated reticulocytes or reticulocytes, no pathology

Increase in serum ALT, AST

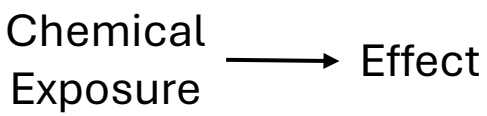
“Classic” hepatotoxicity

Reduction in serum total protein, increase in AST, reduction in RBC/HgB/MCV, increase in circulating thyroid hormones

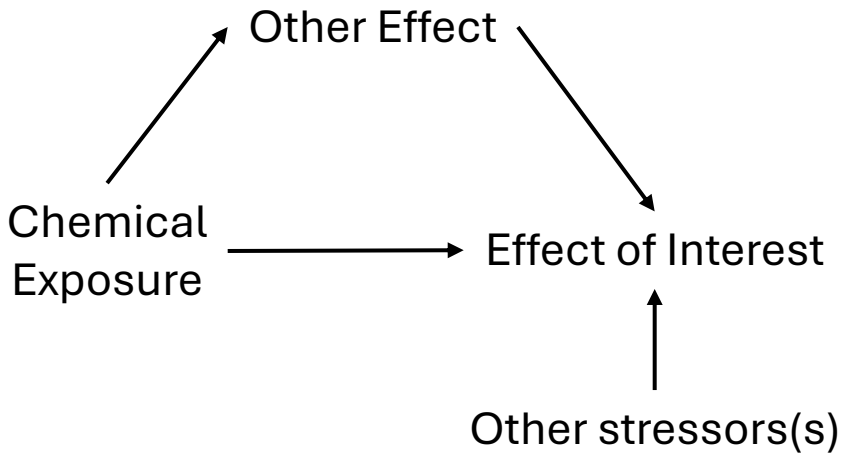
Poor body condition, solvent toxicity

Toxicity studies and causality

- Causality is usually the criteria we're striving for



Process expected.



Actual process.

Causality example

- There are multiple ‘routes’ by which a chemical could influence animal health if liver weight and bodyweight were the measured endpoints.

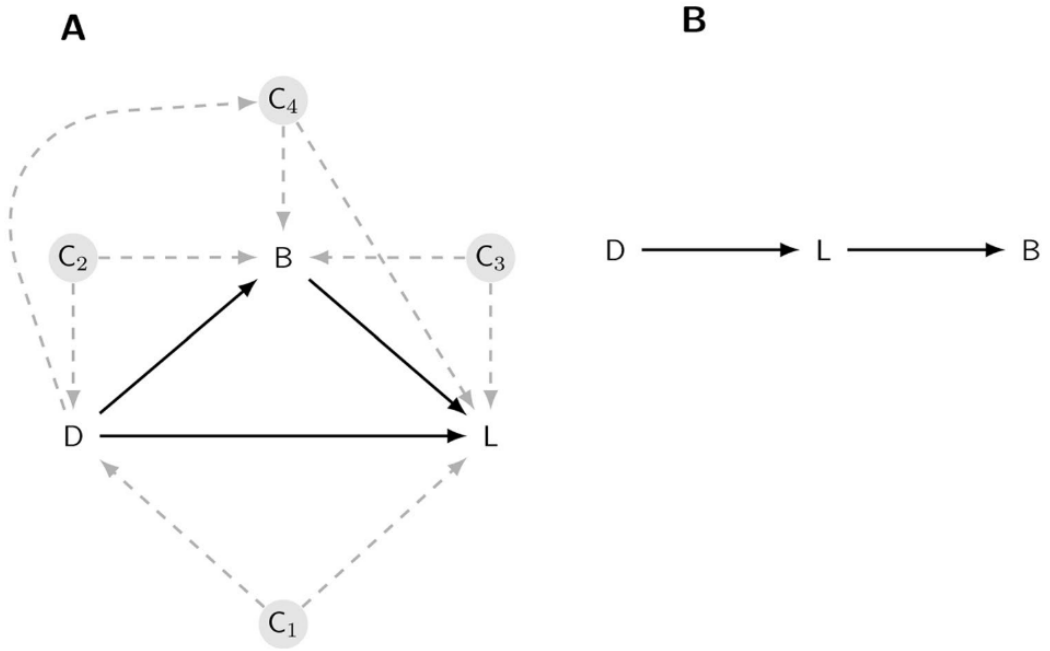
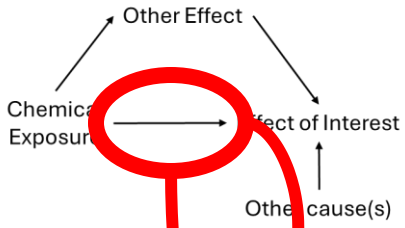


Figure 1. The causal model. A Drug (D) can affect liver weight (L) directly; by altering body weight (B), which in turn affects the liver weight; or through both mechanisms. Four potential confounding effects (C1–C4) must be ruled out before making causal mechanistic claims about how the drug affects liver weight (A). An alternative causal model is that the drug affects liver weight, making the animal ill, which then leads to a reduction in body weight.

Lazic SE, Semenova E, Williams DP. 2020. Determining organ weight toxicity with Bayesian causal models: Improving on the analysis of relative organ weights. Sci Rep. 10(1):6625. doi:10.1038/s41598-020-63465-y.

Endpoint(s) of test is a critical choice

- Zoom in and measure something better.
- This is the basis of “Adverse Outcome Pathways”



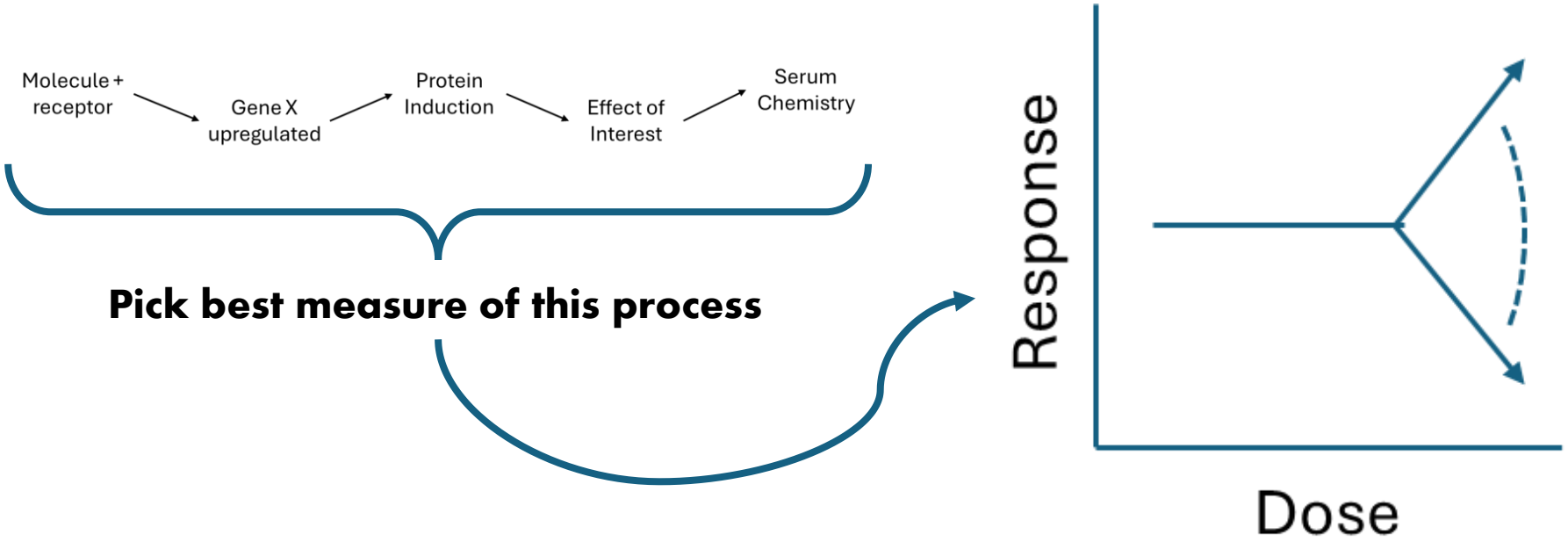
Very different goals can have the same starting point

Molecule + receptor → Gene X upregulated → Protein Induction → Organ weight → Serum Chemistry

Molecule + receptor → Oxidative stress → Reproduction rate → # of animals → Land mgmt. goals

High quality measures of effects

- Response is tied to process (specificity)
- Response is predictive of effects (sensitivity)



Beware!

- Be cognizant of your goals and your need for sensitivity/specificity.

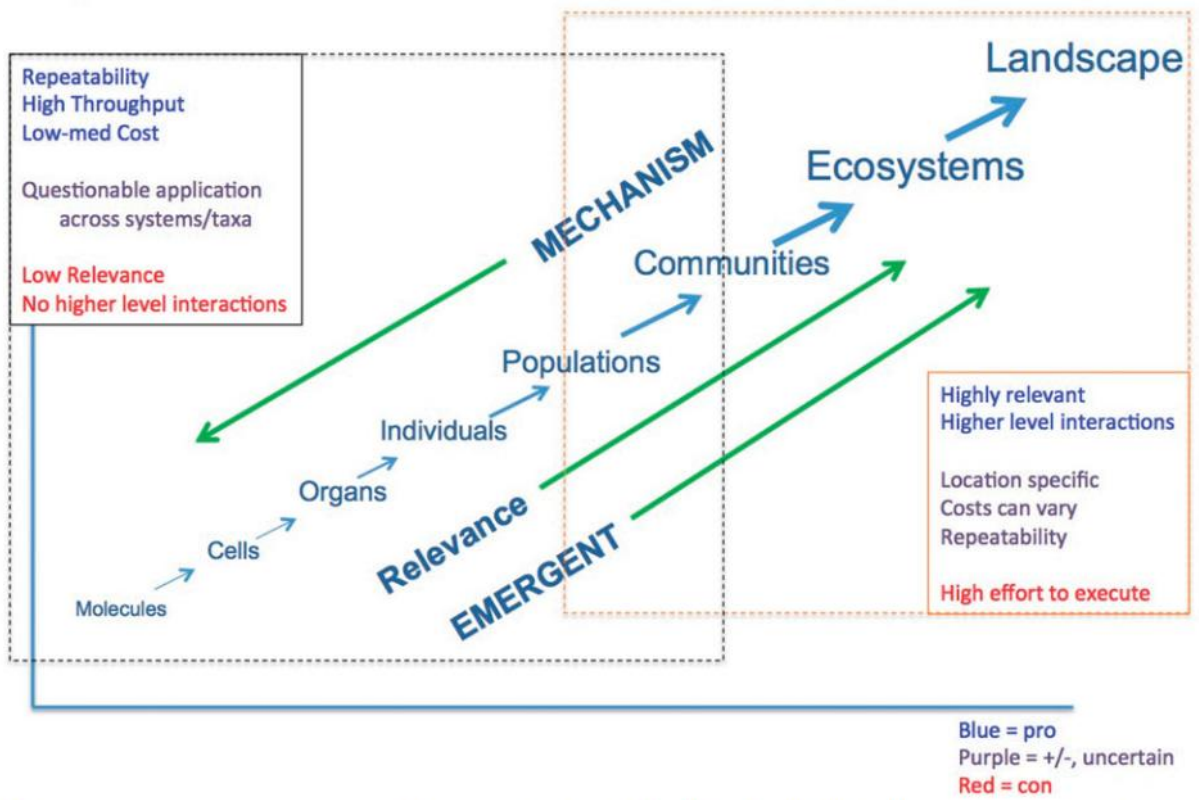


Figure 1. The pros, cons and uncertainties of ecological risk assessment based on data from different levels of biological organization. (This figure is presented in colour in the online version of the article.)

CRITICAL REVIEWS IN TOXICOLOGY, 2016
http://dx.doi.org/10.1080/10408444.2016.1190685

REVIEW ARTICLE

The pros and cons of ecological risk assessment based on data from different levels of biological organization

Jason R. Rohr[a], Christopher J. Salice[b] and Roger M. Nisbet[c]

[a]University of South Florida, Department of Integrative Biology, Tampa, FL, USA; [b]Towson University, Department of Biological Sciences, Towson, MD, USA; [c]University of California at Santa Barbara, Department of Ecology, Evolution, and Marine Biology, Santa Barbara, CA, USA

Biochemical endpoints

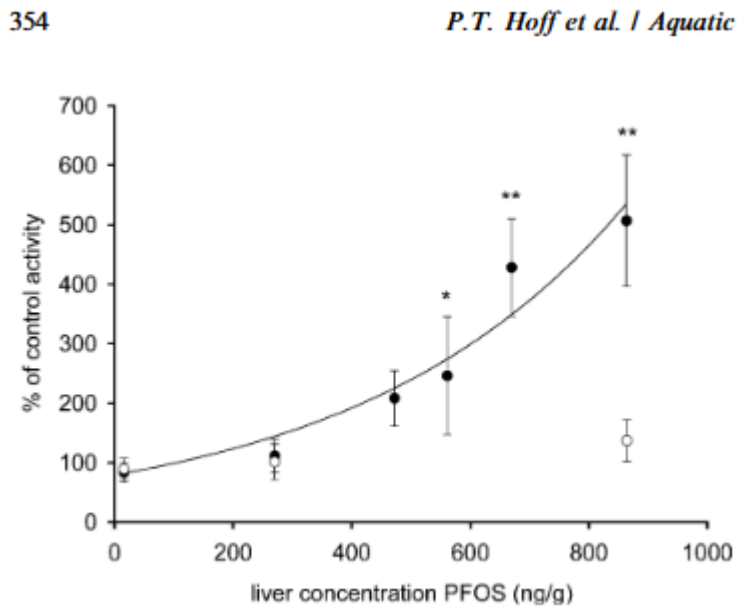
- Liver example
  - Liver is extremely important organ—first place where food contents meet bloodstream, metabolism (tox+food), lipid+protein regulation, handling wastes (e.g. bile excretion), etc.
- Specific markers of health have been developed.

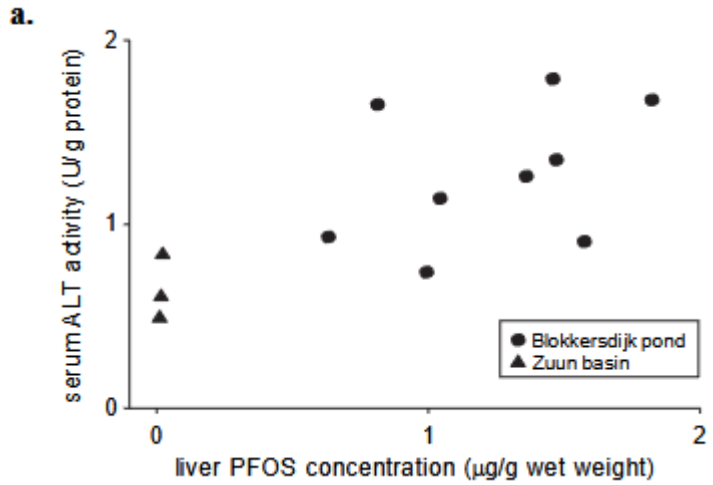
Table 2
Clinical biomarkers of liver toxicity.[59,126,117,161,162]

Biomarker	*Cellular localization	**Biological activity	***Tissue localization	$Injury	$$Specific damage markers	#Comments	##Disadvantage
ALT	Mitochondria in periportal and cytoplasm	Amino acid reductive transfer from amino acid	Primarily localized to liver	Increased in the presence of liver necrosis, cardiac dysfunction, and muscular damage.	Hepatocellular Necrosis	Standard method for evaluating liver cell damage	• Both enzymes activities can potentially exceed 100 times the upper reference limit. Maximum activity does not correlate with outcome
AST	Cytoplasm and mitochondria periportal	Amino acid reductive transfer from amino acid	Localized in heart, brain, skeletal muscle and liver	Elevated due to liver or extracellular tissue injury	Hepatocellular Necrosis	Less specific than ALT	• Peak enzymes activities do not affect prognosis.

Thakur S, Kumar V, Das R, Sharma V, Mehta DK. 2024. Biomarkers of Hepatic Toxicity: An Overview. Current Therapeutic Research. 100:100737. doi:10.1016/j.curtheres.2024.100737.

Turning an endpoint into a biomarker





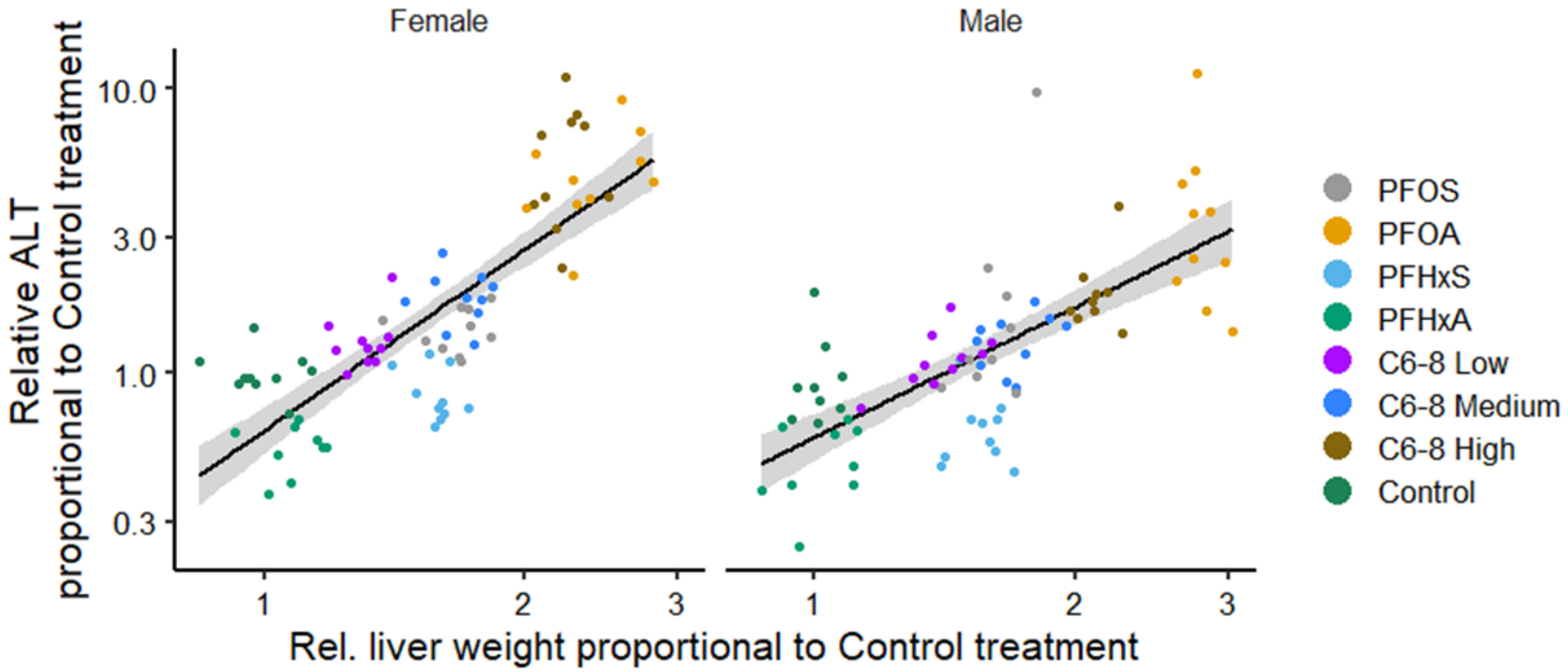
Laboratory relationship observed in the field!

Hoff PT, Van Dongen W, Esmans EL, Blust R, De Coen WM. 2003. Evaluation of the toxicological effects of perfluorooctane sulfonic acid in the common carp (Cyprinus carpio). Aquatic Toxicology. 62(4):349–359. doi:10.1016/S0166-445X(02)00145-5.

Hoff PT, Van Campenhout K, Van De Vijver K, Covaci A, Bervoets L, Moens L, Huyskens G, Goemans G, Belpaire C, Blust R, et al. 2005. Perfluorooctane sulfonic acid and organohalogen pollutants in liver of three freshwater fish species in Flanders (Belgium): relationships with biochemical and organismal effects. Environmental Pollution. 137(2):324–333. doi:10.1016/j.envpol.2005.01.008.

How about ‘adverse’ and ERA-relevant?

- Proportional increase in liver is predictive of proportional increase in ALT.



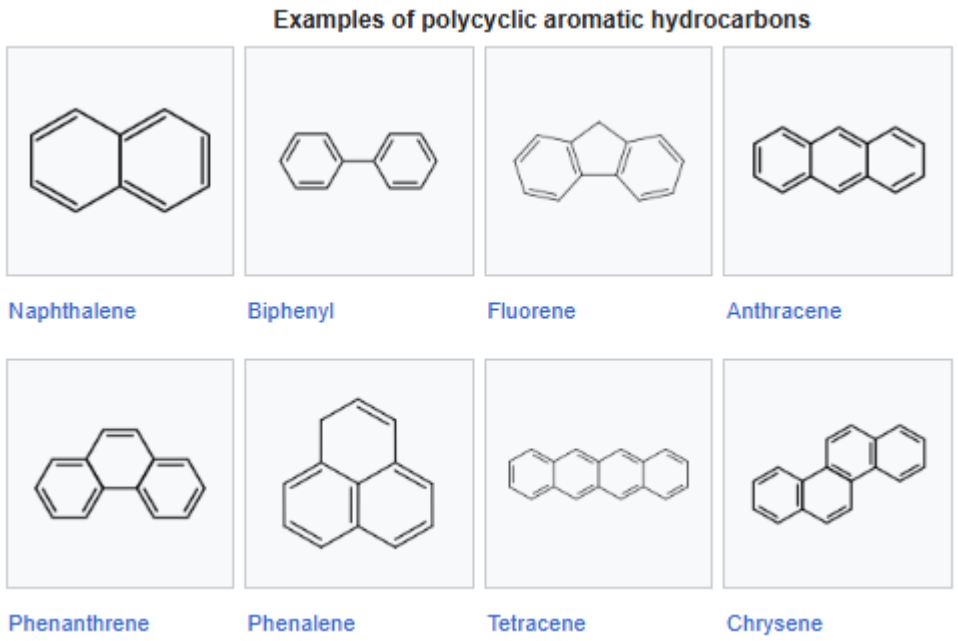
“Site A vs Site B show X-fold increase in ALT suggests Y-fold increase in liver weight”

Biomarker of damage vs biomarker of exposure?

- If ALT in serum shows up after the liver is damaged, we're not being protective.
  - Measure of adverse effect (i.e. evidence of harm or validation of risk)
- Are there biomarkers/bioindicators/biochemical endpoints that are informative before adverse effects?
- Is an adverse response the only useful response to differentiate toxicological profiles?

Polycyclic aromatic hydrocarbons

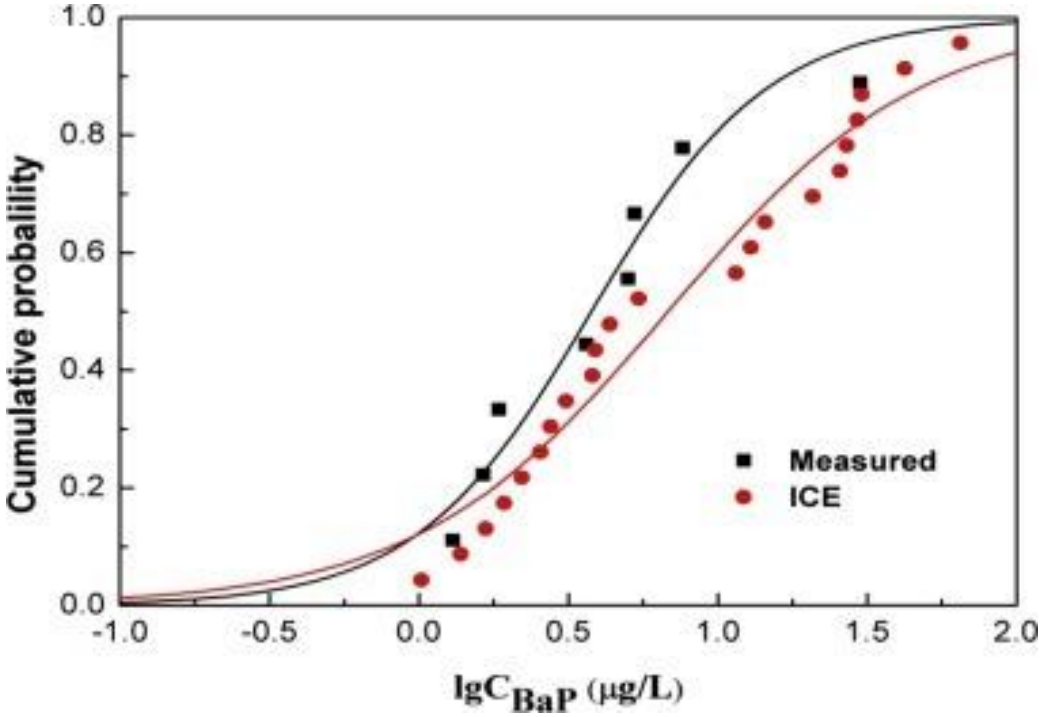
- Hydrocarbons (lots of CH bonds)
- Aromatic (“typified by benzene”) (old terminology)
- Cyclic (ring) (newer terminology that captures “ring” structure)
- Poly (many)
- Nonpolar, lipophilic, organic.
- Complex mixtures in natural hydrocarbons, combustion products.



https://en.wikipedia.org/wiki/Polycyclic_aromatic_hydrocarbon

Toxicity of PAHs

- Species sensitivity distribution of acute EC50s in various fish species exposed to benzo[a]pyrene.
- ~0.5 µg/L (ppb) HC_{50}



Wu, J., Yan, Z., Yi, X., Lin, Y., Ni, J., Gao, X., Liu, Z. and Shi, X., 2016. Comparison of species sensitivity distributions constructed with predicted acute toxicity data from interspecies correlation estimation models and measured acute data for Benzo [a] pyrene. *Chemosphere*, *144*, pp.2183-2188.

But what about the rest of the toxicology?

- PAHs are a classic example of nonpolar+lipophilic molecules that are easy in and hard to get out.
- Metabolism is key to excretion.
- What happens during metabolism?

PAH ‘bioactiviation’

- CYP450 proteins add polar components to aid the excretion of PAHs by increasing their water solubility (get into blood+urine).
- Reactive oxygen species bind to DNA…metabolism leads to increased toxicity…

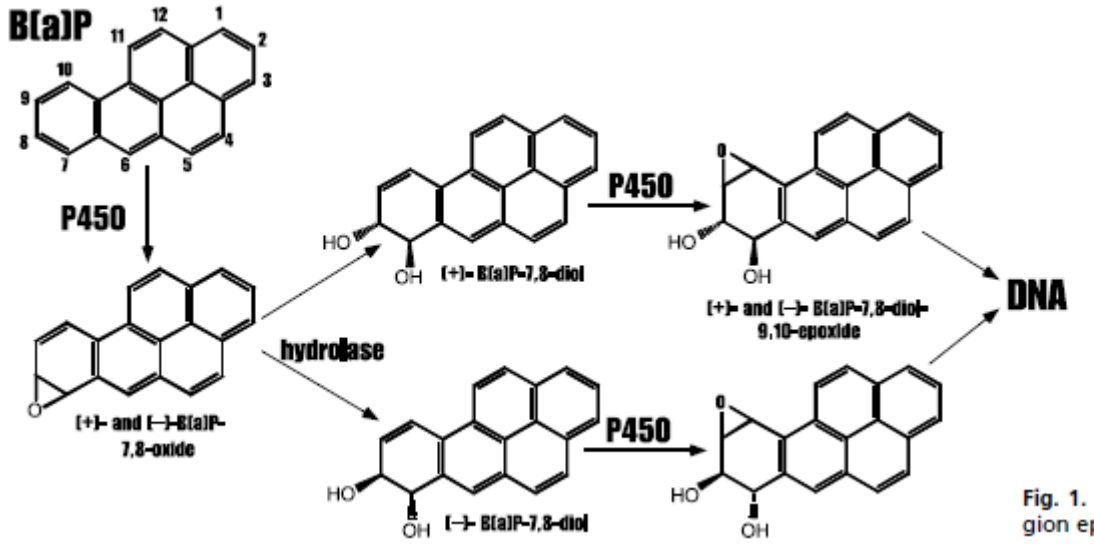


Fig. 1. Metabolic activation of B[a]P to bay region epoxides by P450 and epoxide hydrolase.

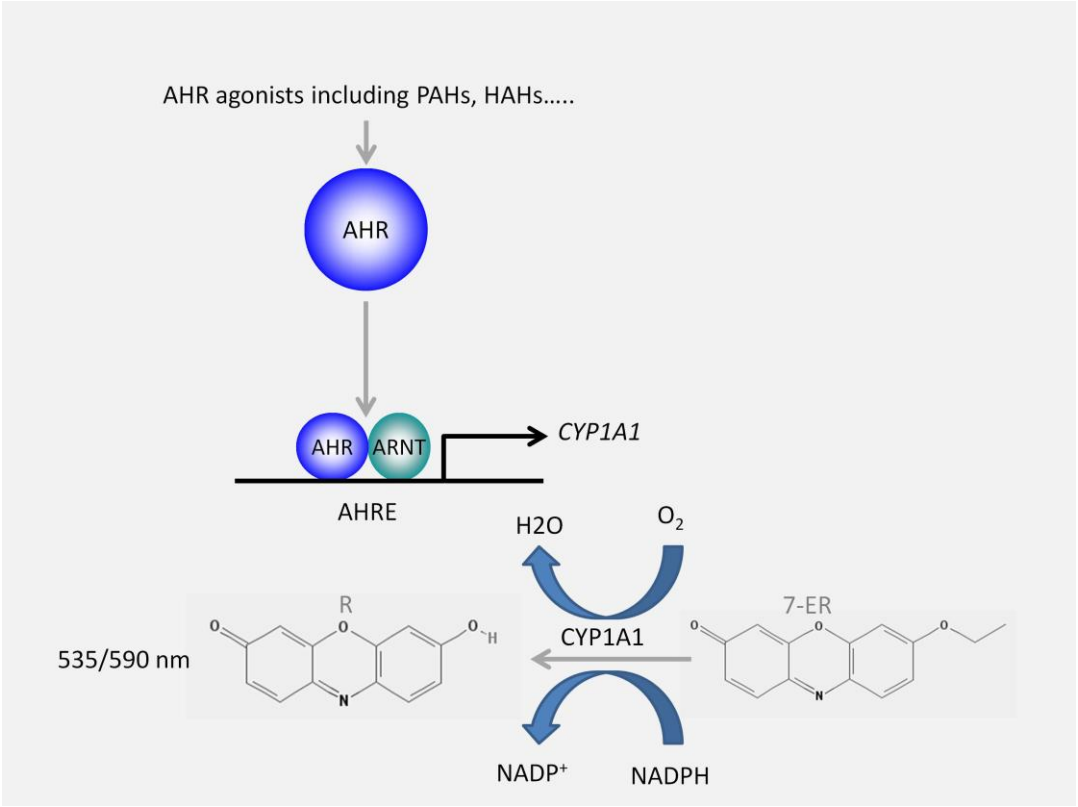
Metabolic activation of polycyclic aromatic hydrocarbons to carcinogens by cytochromes P450 1A1 and 1B1

Tsutomu Shimada[1,2] and Yoshiaki Fujii-Kuriyama[2,4,5]

[1]Osaka Prefectural Institute of Public Health, 1-3-69 Nakamichi, Higashinari-ku, Osaka 537-0025; [2]Department of Biochemistry and Center in Molecular Toxicology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232-0146, USA; [4]Center for Tsukuba Advanced Research Alliance, The University of Tsukuba, 1-1-1 Tennoudai, Tsukuba 305-8577; and [5]Core Research for Evolutional Science and Technology (CREST) Japan, Science and Technology Corporation, 4-1-8 Honcho, Kawaguchi 322-0012

How do we measure PAH metabolism?

- EROD activity assay
  - Expose liver tissue to 7-ethoxyresorufin, wait, measure fluorescence.
  - Activity scales with amount of 7-ethoxy-resorufin-O-deethylase present. EROD is present as a function of the amount of CYP1A1 induction through binding to aryl hydrocarbon receptor.



https://protocolexchange.researchsquare.com/article/nprot-3473/v1

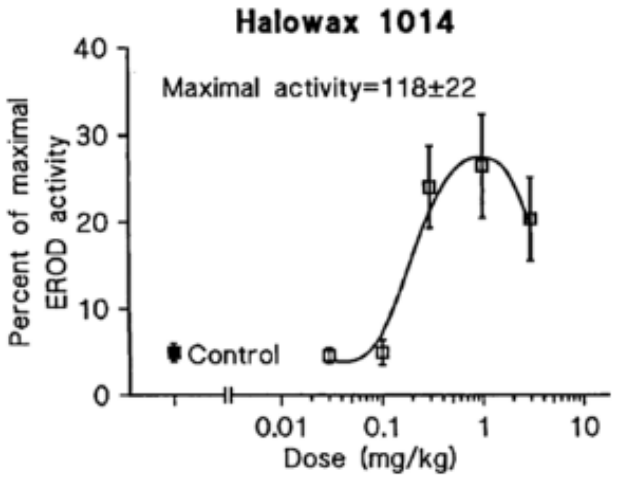
Activity increases before apical effects

- Chicken eggs were viable, but had large increases in EROD activity when exposed to mixtures of PAHs.

Table 1. Mortality on day 10 of development in chicken embryos treated with naphthalenes injected into the air sacs on day 7

Treatment	Dose (mg/kg)			
	Control	1.0	3.0	10.0
Hexachloronaphthalene-mix	0/20	0/7	6/12**	–
Halowax 1014	0/20	0/7	4/12*	6/8***
Heptachloronaphthalene	0/20	0/8	0/14	–

Fisher's exact probability test:
* $P < 0.05$
** $P < 0.01$
*** $P < 0.001$



Engwall, M., Brunström, B. and Jakobsson, E., 1994. Ethoxyresorufin O-deethylase (EROD) and aryl hydrocarbon hydroxylase (AHH)-inducing potency and lethality of chlorinated naphthalenes in chicken (Gallus domesticus) and eider duck (Somateria mollissima) embryos. Archives of toxicology, 68, pp.37-42.

In the field?

- Many, many, many uses of EROD for hydrocarbon exposure—think oil spills.
- Avian EROD activity predicts high concentration sites!

Table 2 SIMPER analysis of Low ethoxyresorufin-*O*-dealkylase (EROD) activity vs. High EROD activity following significant ANOSIM analysis of four categories of EROD activity compared to 33 organic and inorganic variables at 56 sites across the Great Lakes, 2010–2014

	SIMPER analysis Low EROD vs. High EROD		Geometric mean (95% Confidence Intervals) concentrations (ng/g wet weight)	
Variable	Percent contribution	Cumulative percent	Low EROD	High EROD
aPAHs	11.64	11.64	33 (23–45)	517 (265–1008)
pPAHs	11.13	22.78	14 (9.5–21.3)	221 (109–447)
Chlordane	9.2	31.98	12 (7.1–21.7)	102 (51–200)
Dieldrin	8.81	40.79	1.6 (1.0–2.7)	20 (10–39)
Heptachlor	8.73	49.52	2.8 (1.4–5.6)	28 (13–60)
PCBs	8.18	57.7	102 (45–228)	727 (521–112)
PAH TEQs	7.82	65.52	0.15 (0.06–0.38)	5.81 (2.21–15.30)
PFOS	6.21	71.74	65 (32–135)	172 (123–240)
PFCs	5.81	77.55	98 (50–193)	229 (162–325)
PBDEs	4.72	82.28	12.0 (6.8–21.4)	26.4 (18.5–37.7)
DDE	3.89	86.17	100 (75–135)	237 (180–312)
Benzenes	2.08	88.25	7.7 (3.2–18.3)	8.1 (6.1–10.8)
Mirex	1.98	90.23	7.5 (5.9–9.6)	8.0 (6.0–10.7)

Concentrations of contaminants in the Low and High EROD activity groups are also shown

SIMPER similarity percentage, *EROD* ethoxyresorufin-*O*-dealkylase, *aPAHs* alkylated polycyclic aromatic hydrocarbons, *pPAHs* parent PAHs, *PCBs* polychlorinated biphenyls, *TEQs* toxic equivalents, *PFOS* perfluorooctane sulfonate, *PFCs* perfluorinated chemicals, *PBDEs* polybrominated diphenyl ethers, *DDE p,p'*-dichlorodiphenyldichloroethylene

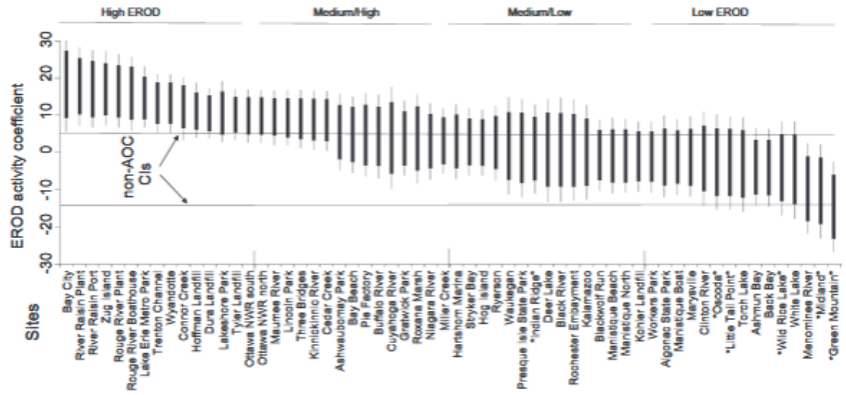
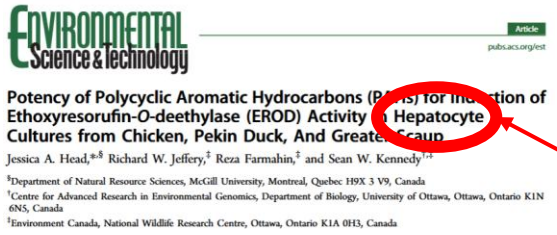


Fig. 2 Ninety-five percent confidence intervals (CIs, vertical lines), and adjusted CIs (black rectangles) for ethoxyresorufin-*O*-dealkylase (EROD) activity coefficients in tree swallow nestling livers at 54 sites within 27 Areas of Concern (AOCs) and six non-AOC locations, 2010–2014. Non-AOC sites are indicated by an asterisk. Horizontal lines indicate adjusted upper and lower confidence intervals (CIs) for six combined non-AOC sites. EROD activity was measured in pmol product/min/mg microsomal protein

Custer, T.W., Custer, C.M., Dummer, P.M., Bigorgne, E., Oziolor, E.M., Karouna-Renier, N., Schultz, S., Erickson, R.A., Aagaard, K. and Matson, C.W., 2017. EROD activity, chromosomal damage, and oxidative stress in response to contaminants exposure in tree swallow (Tachycineta bicolor) nestlings from Great Lakes Areas of Concern. Ecotoxicology, 26, pp.1392-1407.

Same idea but doesn't require lethal sampling?

- If EROD is downstream of gene induction and transcription, can't we just measure that (rtQPCR type methods)?
- Yep!
- Challenge is that liver tissue shows most activity, but as the appropriate sequences are known, the mRNA can be quantified.


ENVIRONMENTAL Science & technology

Article

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Potency of Polycyclic Aromatic Hydrocarbons (PAHs) for Induction of Ethoxyresorufin-O-deethylase (EROD) Activity in Hepatocyte Cultures from Chicken, Pekin Duck, And Greater Scaup

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Also in vitro techniques for screening!

Table 1. GenBank or Ensembl accession numbers of the studied transcripts.

Species	Gene	Number
Chicken	***CYP1C1***	**JN656933 (cloned)**
Turkey	*CYP1C1*-like	ENSMGAG00000015774
Mallad duck	*CYP1C1*-like	ENSAPLG00000001387
Anole lizard	*CYP1C1*-like	ENSACAG00000013750
Xenopus tropicalis	*CYP1C1*	HQ018042
Zebrafish	*CYP1C1*	NM001020610
Zebrafish	*CYP1C2*	NM001114849
Japanese quail	***CYP1B1***	**JN656934 (cloned)**
Chicken	*CYP1B1*	XP419515
Zebra finch	*CYP1B1*-like	XP002191325
Human	*CYP1B1*	NP000095
Anole lizard	*CYP1B1*-like	XP003216002
Xenopus tropicalis	*CYP1B1*	HQ018041
Japanese quail	***AHR1***	HM053555, **JN656935 (cloned)**
Chicken	*AHR1*	AAF70373
Turkey	*AHR1*	XP003207170
Albatross	*AHR1*	BAC87795
Zebra finch	*AHR1*	XP002188964
Common tern	*AHR1*	AF192503
Cormorant	*AHR1*	BAD01477
Mallard duck	*AHR1*	AF192501
Xenopus tropicalis	*AHR1*	CX900378
Mouse	*AHR*	NM013464
Human	*AHR*	AAH70080
Chicken	*AHR1B*-like	ENSGALG00000004322
Zebrafish	*AHR1A*	NP571103
Zebrafish	*AHR1B*	AAY42958
Chicken	*AHR2*	XP421887
Albatross	*AHR2*	BAC87796
Cormorant	*AHR2*	BAF64245
Zebrafish	*AHR2*	CAK11168
Japanese quail	***EF1A***	**JN656936 (cloned)**

doi:10.1371/journal.pone.0028257.t001

Jönsson, M.E., Woodin, B.R., Stegeman, J.J. and Brunström, B., 2011. Cytochrome P450 1 genes in birds: evolutionary relationships and transcription profiles in chicken and Japanese quail embryos. PLoS One, 6(12), p.e28257.

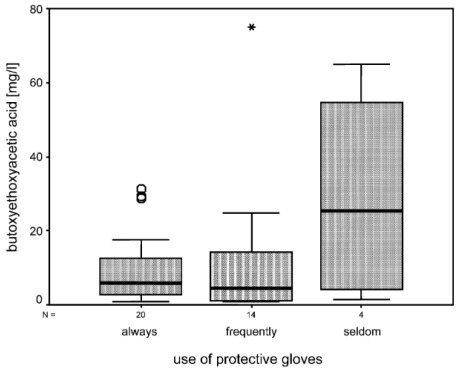
In standardized tox test?

- Useful exercise for studies of chemicals with unknown mechanisms or unknown types of effects.
- Remember back to solvent example?

Table 5
Liver metabolic enzymes—rats given DGBE for 13 weeks

Sex	Dose level (mg/kg/day)		Parameter (Units)				
			EROD (nmol/min/mg P)	MROD (pmol/min/mg P)	PROD (pmol/min/mg P)	UGT (pmol/min/mg P)	PNPH (nmol/min/mg P)
Males	0	Mean	24.731	13.018	1.514	1.096	0.296
		SD	2.476	1.256	0.149	0.057	0.038
	1000	Mean	30.592[a]	12.516	2.098[a]	1.287[a]	0.314
		SD	3.512	1.588	0.273	0.115	0.048
Females	0	Mean	38.481	26.032	2.328	0.779	0.244
		SD	4.881	6.966	0.419	0.055	0.039
	1000	Mean	47.636[a]	31.094	2.881[a]	0.906[a]	0.287
		SD	17.065	13.991	0.866	0.065	0.066

$N = 10$ for all parameters.
[a] Statistically identified, Dunnett's Test, two-way ANOVA, alpha = 0.05.

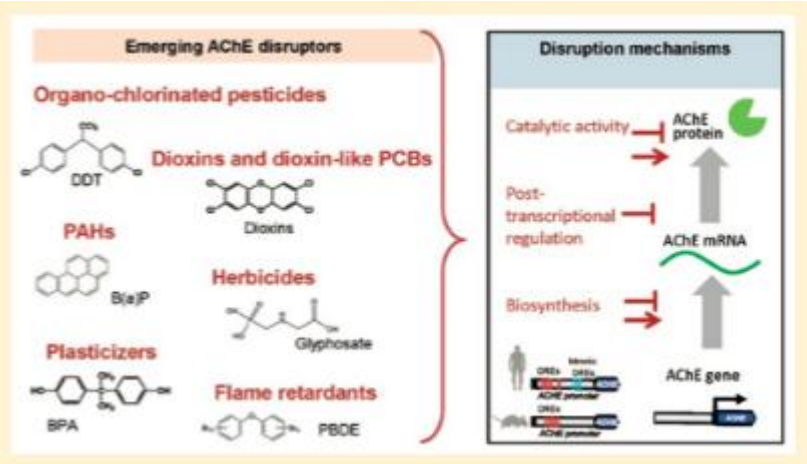


(In the DGBE example, the excreted metabolite is a better predictor of exposure—not necessarily effects though)

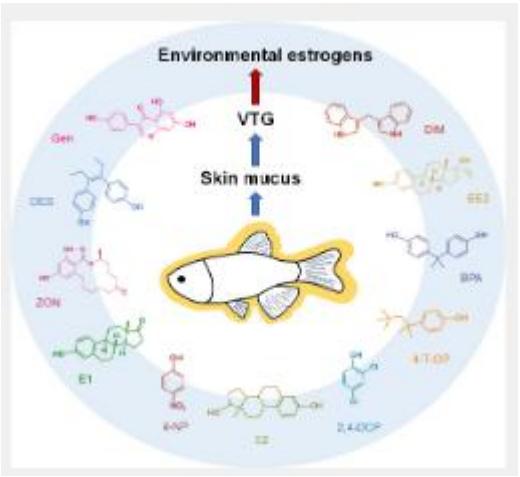
Johnson, K.A., Baker, P.C., Kan, H.L., Maurissen, J.P., Spencer, P.J. and Marty, M.S., 2005. Diethylene glycol monobutyl ether (DGBE): two-and thirteen-week oral toxicity studies in Fischer 344 rats. Food and chemical toxicology, 43(3), pp.467-481.
Göen, T., Korinth, G. and Drexler, H., 2002. Butoxyethoxyacetic acid, a biomarker of exposure to water-based cleaning agents. *Toxicology letters*, *134*(1-3), pp.295-300.

Other important examples

- Vitellogenin
  - Yolk protein precursor. Increase in males indicates exposure to estrogenic chemicals. Also, reduction in vitellogenin can reduce fecundity…
- Acetylcholinesterase
  - Terminates neurotransmission. Inhibition keeps nerve signaling “on.”
  - Is desired effect of some insecticides…



Fu, H., Xia, Y., Chen, Y., Xu, T., Xu, L., Guo, Z., Xu, H., Xie, H.Q. and Zhao, B., 2018. Acetylcholinesterase is a potential biomarker for a broad spectrum of organic environmental pollutants. Environmental science & technology, 52(15), pp.8065-8074.



Sun, B., Hu, C. and Chen, L., 2025. Fish Skin Mucus Vitellogenin as a Noninvasive, Sensitive Biomarker for Aquatic Xenoestrogens. Environment & Health.

Conclusions

- Be cognizant of causality, specificity, sensitivity, and desired inference.
- Biomarker is generally the word for quite validated methods
  - Bioindicator is something you're still studying
- Use in toxicity tests may be for a variety of purposes
  - Explore mechanisms, standard measures, useful in the field

Questions?

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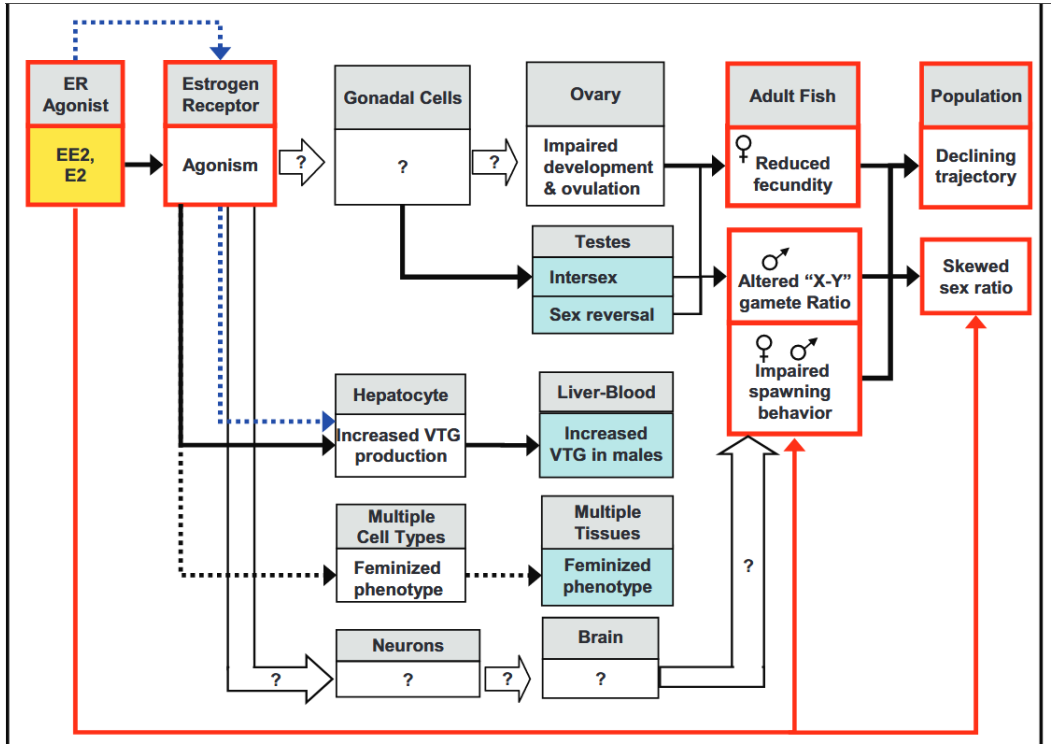
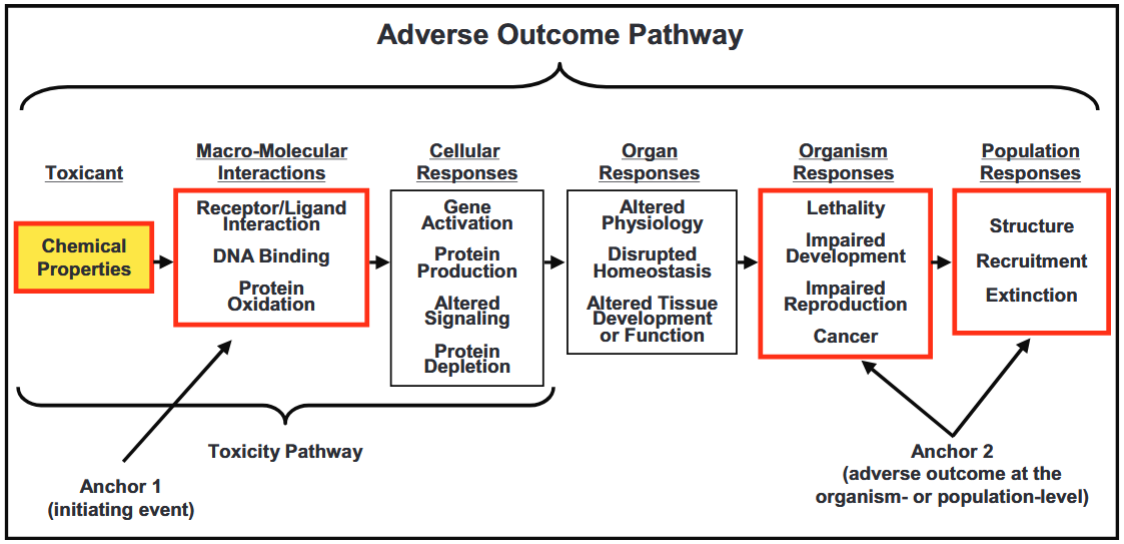
Adverse outcome pathways

- The recent(ish) development that gave a name to biological organization in toxicology.





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Hazard/Risk Assessment

ADVERSE OUTCOME PATHWAYS: A CONCEPTUAL FRAMEWORK TO SUPPORT ECOTOXICOLOGY RESEARCH AND RISK ASSESSMENT

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