

Use of the Zebrafish Developmental Screen and Estimation of Internal Concentration to Assess Toxicity

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Introduction

- Chemical lipophilicity contributes to bioconcentration in aquatic species. Lipophilicity correlates with developmental toxicity in various aquatic models.
- Zebrafish is being used as a model organism to screen thousands of chemicals in the ToxCast and Tox21 research programs for potential to induce developmental defects or overt toxicity.
- The partition coefficient (log P) is an indicator of lipophilicity.
- We examined the relationship between log P, estimated body burden, and developmental toxicity in zebrafish for 309 environmental chemicals from the ToxCast Phase I library.
- We then used hepatic clearance, protein binding data, and reverse toxicokinetic models to compare zebrafish toxicity and ToxCast high-throughput screening (HTS) activity to *in vivo* rat data.

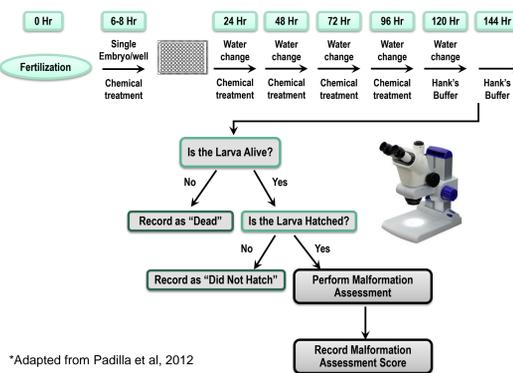
Conclusions

- Lipophilicity (log P) contributes substantially to bioavailability and bioaccumulation in the developing zebrafish embryo/larva and influences toxicity accordingly.
- Certain classes of chemicals, such as pyrethroids, are predicted to bioconcentrate significantly in zebrafish based on their log P values.
- For most chemicals tested, zebrafish assays provide a conservative estimate of developmental toxicity lowest effect levels. However, the developmental toxicity of certain chemical classes, such as conazoles, may be underpredicted by zebrafish studies.
- For all chemicals tested, the ToxCast HTS assays were more sensitive than zebrafish or rat prenatal studies.
- ToxCast *in vitro* assay targets may provide insight into the biological relevance of zebrafish assays for predicting mammalian developmental toxicity.

Experimental Methods

- Chemicals were screened by immersing zebrafish embryos in media containing chemical concentrations from 0.001 to 80 µM and determining the half-maximal activity concentration (AC₅₀) for toxicity (lethality, non-hatching, or dysmorphology) (Figure 1; Padilla et al. 2012).

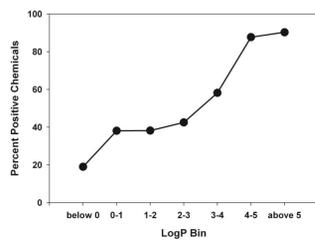
Figure 1. Zebrafish Developmental Assay Exposure and Evaluation Schema



*Adapted from Padilla et al, 2012

- There was a clear relationship between log P and incidence of developmental toxicity among the ToxCast Phase I chemicals (Figure 2).

Figure 2. Relationship Between Partition Coefficient (Log P) and Developmental Toxicity

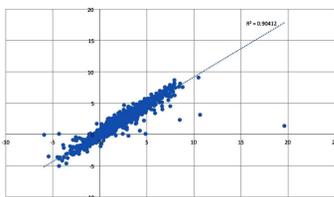


Chemicals were placed in log P bins (log P less than 0, 0 to 1, etc.). Points on the plot represent the percent of chemicals that were developmentally toxic to zebrafish in each bin. (From Padilla 2013)

Estimating Log P

- We compared experimental log P values for 2335 Tox21 chemicals to predicted values based on chemical structures from EpiSuite (<http://www.epa.gov/oppt/exposure/pubs/episuite.html>).
- The correlation between experimental and predicted log P values (R² > 0.9, Figure 3), suggests that predicted values can be used when experimental values are not available.

Figure 3. Experimental vs. Predicted Partition Coefficient (Log P) Values for 2335 Tox21 Chemicals

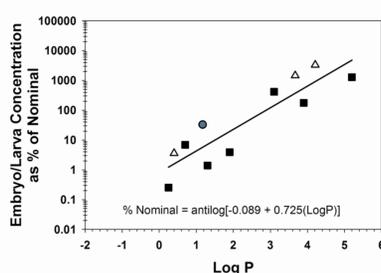


The log P values predicted by EpiSuite (x-axis) were plotted against experimentally derived log P values (y-axis). There was a high degree of correlation between predicted and experimental values (R² = 0.904).

Applying Bioconcentration Factor

- The linear relationship between log P and bioconcentration was derived from multiple studies (Figure 4; adapted from Padilla 2013).
- The regression equation from these data was applied to the AC₅₀ values from the ToxCast screen and used to estimate a body burden associated with developmental toxicity (EC₅₀).

Figure 4. Relationship Between Partition Coefficient (Log P) and Bioconcentration in the Fish Embryo



Literature values for embryo/larval chemical concentrations were plotted against log P to define the relationship between log P and bioconcentration. Values represented by solid squares are from Berghmans et al. (2008), open triangles from Gustafson et al. (2012), and gray circle from Thomas et al. (2009). The solid line represents the relationship between log P of the chemical and concentration in the embryo.

Chemical Potency Shifts

- Table 1 shows the ten most toxic ToxCast Phase I chemicals to developing zebrafish, based on either nominal half-maximal activity concentration (AC₅₀), or estimated internal half-maximal activity concentration (EC₅₀).
- Thiram, butafenicil, fluthiacet-methyl, rotenone, and fentin are toxic at submicromolar concentrations, regardless of whether external exposure or estimated internal body burden is considered.
- Tefluthrin was toxic at low concentrations and was predicted to have high bioconcentration in zebrafish. This observation was consistent among all pyrethroids tested (Table 2: 13 pyrethroids in ToxCast Phase I, log P range 3.31–8.15).
- For the five chemicals in Table 2 with lowest adverse effect levels (LOAELs) in rat prenatal studies, there appears to be a relationship between potency in the zebrafish embryo and developmental toxicity LOAEL in the rat.

Table 1. Most Toxic Chemicals to Developing Zebrafish Embryos

Chemical ^a	AC ₅₀ (µM)	AC ₅₀ Rank	Log P	EC ₅₀ (µM)	EC ₅₀ Rank	Chemical Structural Category
Thiram	≤0.0014	1	1.73	0.0002	1	thiocarbamate
Rotenone	≤0.0014	2	4.1	0.0107	4	isoflavone
Tefluthrin	0.0046	3	6.5	1.9331	38	pyrethroid ester
Butafenicil	0.0069	4	3.05 ^b	0.0091	3	uracil phenyl halide carboxylate
Pyridaben	0.0114	5	6.37	3.8562	48	diazine phenyl sulfide halide ketone
Flumetralin	0.0123	6	5.45	0.8957	25	aniline alkylate dinitro fluoro
Fluthiacet-methyl	0.0148	7	3.77	0.0652	7	conazole (imidazoles)
Abamectin	0.0173	8	NA	NA	NA	mectin
Fentin	0.0763	9	3.53	0.2253	14	organometallic
Propargite	0.1279	10	5	4.3941	51	phenyl ether sulfate
Dazomet	0.2814	19	0.63	0.0066	2	thiocarbamate
Fluoxastrobin	0.1873	16	2 ^b	0.0430	5	strobin
Daminozide	66.5075	183	-1.5	0.0443	6	carbamate carboxylic acid amine
Methylene bis(thiocyanate)	3.9125	76	0.62 ^b	0.0897	8	thiocyanate
Imazamox	3.5	71	0.73	0.0965	9	imidazolone pyridine carboxylic acid
Thiophanate-methyl	1.2252	47	1.4	0.1033	10	benzimidazole carbamate

Abbreviations: AC₅₀ = nominal half-maximal activity concentration; EC₅₀ = estimated internal half-maximal activity concentration; log P = partition coefficient; NA = no experimental or predicted log P value available in EpiSuite.

^a Chemicals shown are the top ten most toxic, ranked first by AC₅₀ and then by EC₅₀. There was an overlap of four chemicals in the top ten by each measure.

^b Log P values were predicted with EpiSuite.

Table 2. Chemical Class Bioconcentration Example: Pyrethroids

Chemical ^a	Log P	AC ₅₀	EC ₅₀	Rat Prenatal LOAEL (mg/kg/day)
Cyfluthrin	5.95	0.33	55.32	0.14
Tefluthrin	6.5	0.01	1.93	5
S-Bioallethrin	4.78	1.05	25.08	50
Resmethrin	6.14	2.80	645.42	80
Permethrin	6.5	3.00	1261.86	150
Esfenvalerate	6.21	0.29	76.11	Null
Fenprothrin	5.85	0.32	46.05	Null
Cypermethrin	6.24	0.33	88.08	Null
Bifenthrin	8.15	0.57	3730.77	Null
Prallethrin	4.49	1.57	23.00	Null
Cyhalofop-butyl	3.31	2.94	6.02	Null
d-cis,trans-Allethrin	4.78	6.57	156.43	Null
Tetramethrin	4.73	10.33	226.18	Null

Abbreviations: AC₅₀ = nominal half-maximal activity concentration; EC₅₀ = estimated internal half-maximal activity concentration; LOAEL = lowest adverse effect level from the EPA's Toxicological Reference Database; log P = partition coefficient; Null = rat prenatal studies were performed but no developmental toxicity effects were seen.

^a Chemicals are ranked first by rat prenatal LOAEL, where applicable, then by AC₅₀ in the zebrafish embryo.

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A summary of NICEATM and ICCVAM activities at the Ninth World Congress is available on the National Toxicology Program website at <http://ntp.niehs.nih.gov/go/41583>.

Comparison to *In Vivo* and HTS Data

- ToxCast Phase I compounds were also screened in >600 HTS assays (Kavlock et al. 2012), including:
 - Human primary cell assays measuring protein signaling
 - Cell-free biochemical assays measuring enzymatic activation and receptor binding
 - Assays for nuclear receptor target activity
 - Transcription factor activation assays
 - Assays measuring cytochrome P450 induction
- Most of these chemicals have *in vivo* rodent toxicity data (prenatal, multigenerational, chronic/cancer, and/or subchronic studies) available in ToxRefDB (<http://actor.epa.gov/toxrefdb/>).
- A subset of 27 compounds active in the zebrafish and having *in vivo* prenatal ToxRefDB data also had hepatic clearance and protein binding data (Wetmore et al. 2013).
- We computed the rat oral equivalent values from the zebrafish data and the most sensitive ToxCast HTS assay target. We compared these values to the LOAELs from ToxRefDB (Table 3).
- Seven chemicals (highlighted in pink in Table 3) were developmentally toxic to zebrafish but not rats (i.e., these chemicals had rat prenatal studies in ToxRefDB but no recorded LOAEL).
- From the remaining 20 with rat prenatal LOAELs:
 - Thirteen chemicals had rat oral equivalent values from the zebrafish data (AC₅₀ or EC₅₀) that were lower than the prenatal LOAEL in the rat.
 - Three chemicals (fenbuconazole, permethrin, and resmethrin, highlighted in green in Table 3) had rat prenatal LOAELs that fell between the oral equivalents estimated from the zebrafish AC₅₀ and EC₅₀.
 - Four conazoles (cyproconazole, flufenacet, flusilazole, and hexaconazole, highlighted in orange in Table 3) had rat oral equivalent values from the zebrafish data (AC₅₀ or EC₅₀) that were higher than the prenatal LOAEL in the rat.

Table 3. Rat Oral Equivalent Values Across 27 Chemicals

Chemical ^a	ZF AC ₅₀ Rat Oral Equiv. (mg/kg/day)	ZF EC ₅₀ Rat Oral Equiv. (mg/kg/day)	Rat Prenatal LOAEL (mg/kg/day)	Chemical Category	ToxCast HTS AC ₅₀ Rat Oral Equivalent (mg/kg/day)	Most Sensitive ToxCast HTS AC ₅₀ Assay Target ^d
Flusilazole	7.69	30.16	0.4	conazole (triazoles)	0.018	NVS_ADME_hCYP2C19
Hexaconazole	77.79	426.01	2.5	conazole (triazoles)	0.057	NVS_ADME_hCYP2A2
Cyproconazole	53.73	55.43	12	conazole (triazoles)	0.026	NVS_ADME_hCYP2A2
Lindane	2.26	9.89	20	alkane cyclo chloro	0.503	ATG_VDRE_OIS
Fenarimol	1.02	3.38	35	phenyl-phenyl [C] sulfide alcohol diazine	0.004	NVS_ADME_hCYP2A2
Trifluralin	0.76	0.06	35	conazole (imidazoles)	0.010	NVS_ADME_hCYP2A2
Oxadiazon	0.47	11.54	40	oxadiazolone	0.267	ATG_PXRE_OIS
S-Bioallethrin	1.37	32.53	50	pyrethroid ester	0.488	NVS_ADME_hCYP3A5
Fenbuconazole	72.83	130.34	75	conazole (triazoles)	0.038	NVS_ADME_hCYP2A2
Resmethrin	2.97	685.22	80	pyrethroid ester	4.969	BSK_4H_VCAM1_down
Triadimefon	3.05	2.53	90	conazole (triazoles)	0.002	NVS_ADME_hCYP2A2
Tetraconazole	5.01	15.55	100	conazole (triazoles)	0.001	NVS_ADME_hCYP2C19
Flufenacet	148.08	248.66	125	conazole (imidazoles)	0.025	NVS_NR_hPXR
Permethrin	2.14	901.26	150	pyrethroid ester	2.571	BSK_LPS_PGE2_down
Cyprodinil	0.77	4.98	200	phenyl-diazine [N]	0.219	APR_CellCycleArrest_1hr_up
Acetochlor	240.09	307.70	600	phenyl acetanilide chloro	6.724	ATG_PXRE_OIS
Halosulfuron-methyl	1.08	0.01	750	sulfonyleurea	12.200	ATG_PPARG_TRANS
Fludioxonil	0.59	4.64	1000	phenyl-pyrrole ether nitrile fluoride	0.001	NVS_NR_hPXR
Triticoazole	2.63	5.21	1000	conazole (triazoles)	0.002	NVS_ADME_hCYP3A1
Chlorpropham	40.76	116.41	1000	phenyl carbamate chloro	2.974	NVS_MP_rPBR
Cyclanilide	0.17	0.03	Null	phenyl amide chloro carboxylic acid	0.003	APR_CellLoss_72hr_dn
Bensulfide	4.77	43.08	Null	phenyl sulfonamide thiophosphate	0.031	NVS_ADME_hCYP3A5
Dithiopyr	1.74	39.34	Null	pyridine thio ketone fluoride	0.046	NVS_NR_hPXR
Tricosan	0.91	20.91	Null	phenol-phenyl [C] halide	0.051	BSK_hDFCGF_CollagenIII_up
Bisphenol A	160.98	334.79	Null	phenol-phenol [C]	0.263	NVS_NR_hCAR_Antagonist
Diphenylamine	3.19	8.95	Null	phenyl-phenyl [N]	0.814	NVS_TR_hNET
Alachlor	390.23	1133.24	Null	phenyl acetanilide chloro	24.310	NVS_ADME_hCYP2B6

Abbreviations: AC₅₀ = nominal half-maximal activity concentration; EC₅₀ = estimated internal half-maximal activity concentration; HTS = high-throughput screen; LOAEL = lowest adverse effect level from the EPA's Toxicological Reference Database; log P = partition coefficient; ZF = zebrafish.

^a Chemicals are sorted by rat prenatal LOAEL in ascending order, with chemicals with no rat prenatal LOAEL in ToxRefDB listed last. Chemicals highlighted in green had rat prenatal LOAELs that fell between the oral equivalents estimated from the zebrafish AC₅₀ and EC₅₀. Chemicals highlighted in orange had rat prenatal LOAELs that were lower than the oral equivalents estimated from the zebrafish data. Chemicals highlighted in pink were not toxic in rat prenatal studies.

^b Calculated using the method of Wetmore et al. (2013).

^c Values are from ToxRefDB (<http://actor.epa.gov/toxrefdb/>).

^d Assay target definitions can be found in ToxCastDB (<http://actor.epa.gov/actor/faces/ToxCastDB/GenesAssocAssays.jsp>).

References

A reference list for this poster is available at <http://ntp.niehs.nih.gov/iccvam/meetings/9wcc/hamm-zebrafishlogp-refs.pdf>

