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

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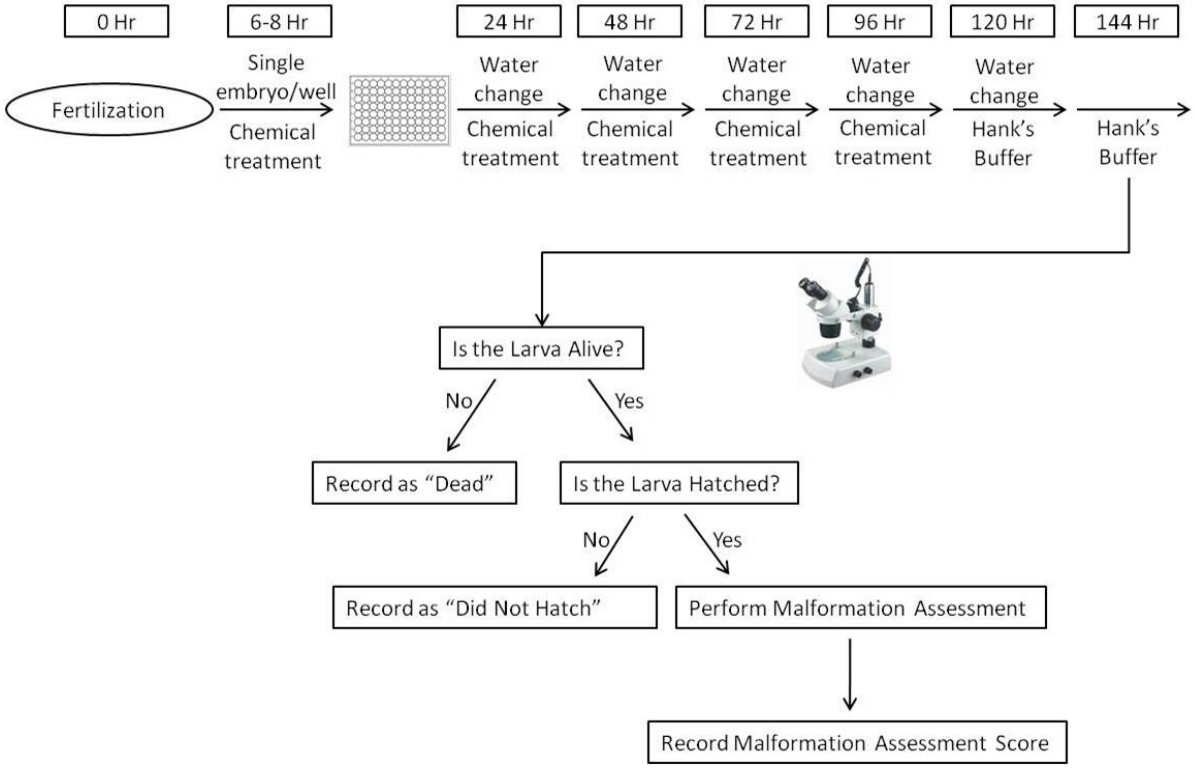
Introduction

- 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 embryos 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.

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 (AC_{50}) for toxicity (lethality, non-hatching, or dysmorphology) (**Figure 1**; Padilla et al. 2012).

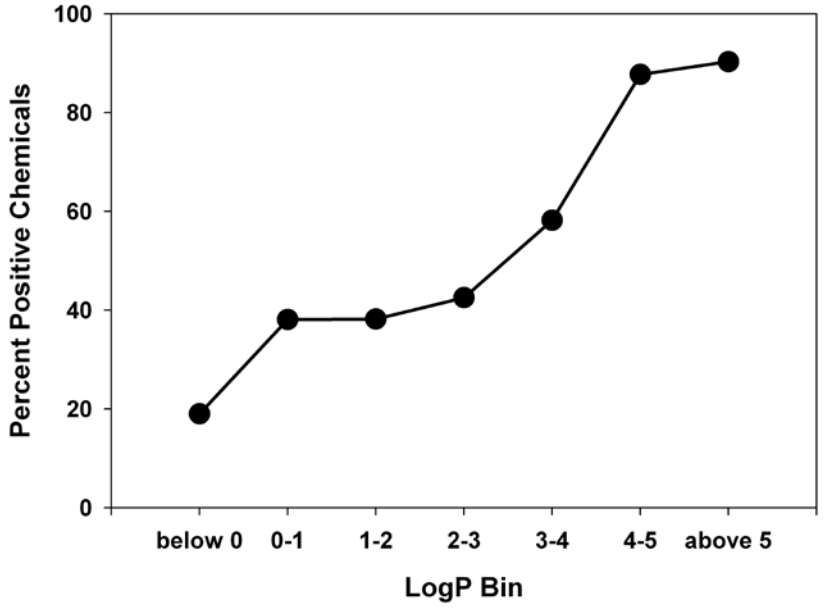
Figure 1 Zebrafish Developmental Assay Exposure and Evaluation Schema



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 Log P and Developmental Toxicity

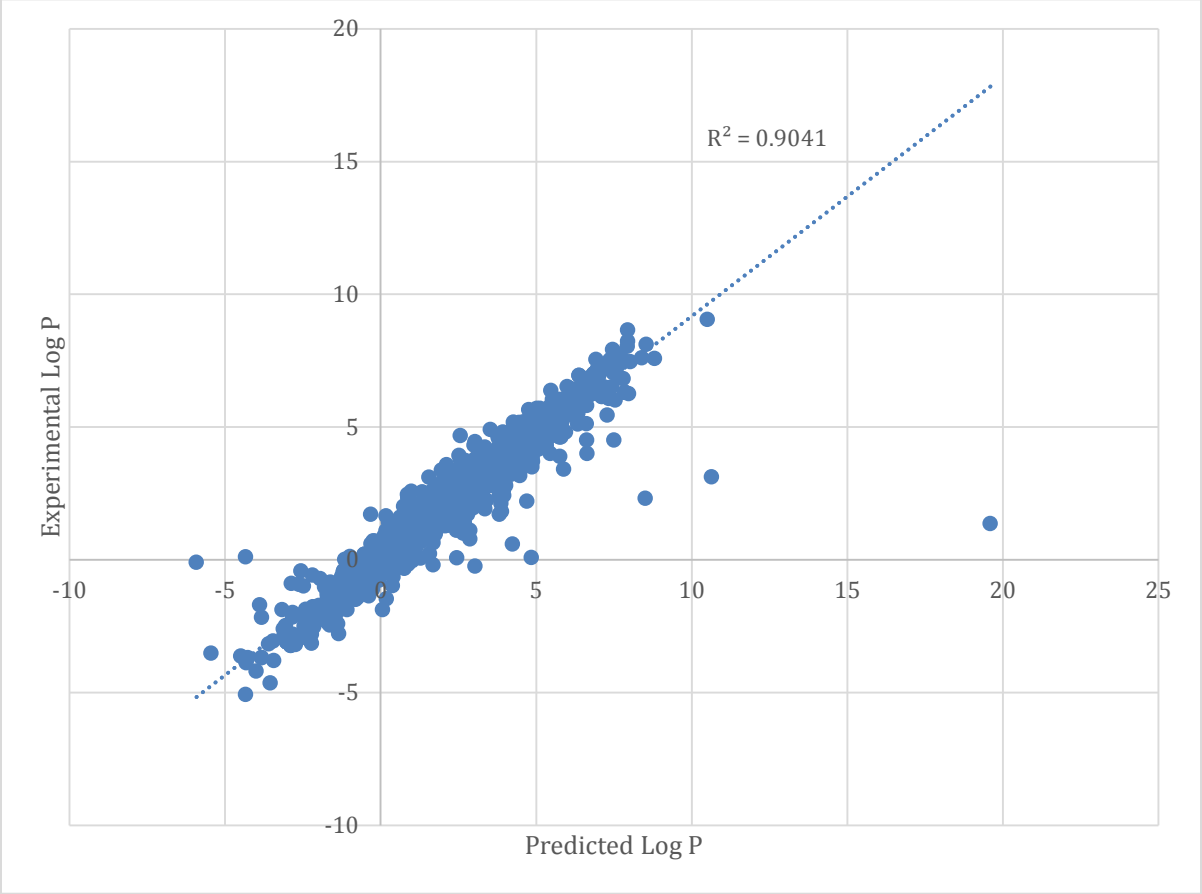


Chemicals were placed in log P bins (log P less than 0, 0 to 1, etc.). Points on the plot represent the percent of chemicals that were developmentally toxic to zebrafish in each bin. (From Padilla, 2013)

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/episuitedl.htm).
- The correlation between experimental and predicted log P values ($R^2 > 0.9$, **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

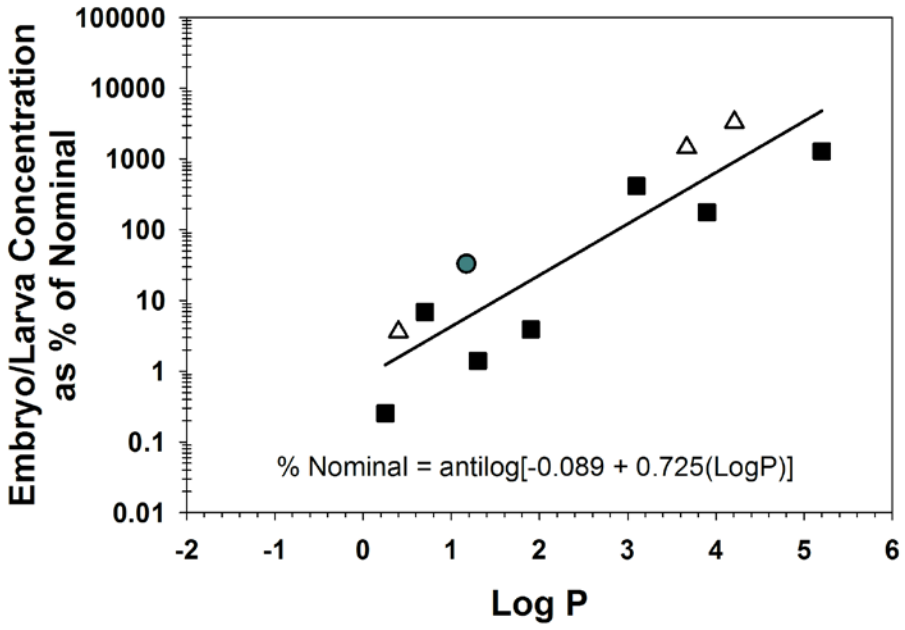


The log P values predicted by EpiSuite (x-axis) were plotted against experimentally derived log P values (y-axis). There was a high degree of correlation between predicted and experimental values (R^2 = 0.90412)

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 AC_{50} values from the ToxCast screen and used to estimate a body burden associated with developmental toxicity (EC_{50}).

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 (AC_{50}), or estimated internal half-maximal activity concentration (EC_{50}).
  - 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
Butafenacil	0.0069	**4**	3.05[b]	0.0091	**3**	uracil phanyl 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**	imidazolinone pyridine carboxylic acid
Thiophanate-methyl	1.2252	47	1.4	0.1033	**10**	benzimidazole carbamate

Abbreviations: AC_{50} = nominal half-maximal activity concentration; EC_{50} = 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 AC_{50} and then by EC_{50}. 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} (µM)**	**EC_{50} (µM)**	**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
Fenpropathrin	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: AC_{50} = nominal half-maximal activity concentration; EC_{50} = 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 AC_{50} in the zebrafish embryo

Comparison to *In Vivo* and HTS Data

- ToxCast Phase I chemicals 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 rat 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 chemicals with rat prenatal LOAELs:
  - Thirteen chemicals had rat oral equivalent values from the zebrafish data (AC_{50} or EC_{50}) that were lower than the prenatal LOAEL in the rat.
  - Three chemicals (fenuconazole, permethrin, and resmethrin highlighted in green in **Table 3**) had rat prenatal LOAELs that fell between the oral equivalents estimated from the zebrafish AC_{50} and EC_{50}.
  - Four conazoles (cyproconazole, flufenacet, flusilazole, and hexaconazole, highlighted in orange in **Table 3**) had rat oral equivalent values from the zebrafish data (AC_{50} or EC_{50}) 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 Equivalent (mg/kg/day)[b]	ZF EC_{50} Rat Oral Equivalent (mg/kg/day)[b]	Rat Prenatal LOAEL (mg/kg/day)[c]	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_rCYP2A2
Cyproconazole	53.73	55.43	12	conazole (triazoles)	0.026	NVS_ADME_rCYP2A2
Lindane	2.26	9.89	20	alkane cyclo chloro	0.503	ATG_VDRE_CIS
Fenarimol	1.02	3.38	35	phenyl-phenyl [C] halide alcohol diazine	0.004	NVS_ADME_rCYP2A2
Triflumizole	0.76	0.06	35	conazole (imidazoles)	0.01	NVS_ADME_rCYP2A2
Oxadiazon	0.47	11.54	40	oxadiazolone	0.267	ATG_PXRE_CIS
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_rCYP2A2
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_rCYP2A2

Tetraconazole	5.01	15.55	100	conazole (triazoles)	0.001	NVS_ADME_hCYP2C19
Flufenacet	146.08	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]	0.019	APR_CellCycleArrest_1hr_up
Acetochlor	240.09	307.7	600	phenyl acetanilide chloro	6.724	ATG_PXRE_CIS
Halosulfuron-methyl	1.08	0.01	750	sulfonylurea	12.2	ATG_PPARg_TRANS
Fludioxonil	0.59	4.64	1000	phenyl-pyrole ether nitrile fluoride	0.001	NVS_NR_hPXR
Triticonazole	2.63	5.21	1000	conazole (triazoles)	0.002	NVS_ADME_rCYP3A1
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
Bensulide	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
Triclosan	0.91	20.91	Null	phenol-phenyl [O] 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	24.31	NVS_ADME_hCYP2B6

Abbreviations: AC_{50} = nominal half-maximal activity concentration; EC_{50} = 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 AC_{50} and EC_{50}. 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 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)

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.

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