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Zebrafish developmental screening of the ToxCast™ Phase I chemical library

S. Padilla[a,*], D. Corum[b,1], B. Padnos[a], D.L. Hunter[a], A. Beam[b,2], K.A. Houck[b], N. Sipes[b], N. Kleinstreuer[b], T. Knudsen[b], D.J. Dix[b], D.M. Reif[b]

[a] National Health and Environmental Effects Research Laboratory, Office of Research and Development, U.S. Environmental Protection Agency, Research Triangle Park, NC, USA
[b] National Center for Computational Toxicology, Office of Research and Development, U.S. Environmental Protection Agency, Research Triangle Park, NC, USA

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ABSTRACT

Zebrafish (*Danio rerio*) is an emerging toxicity screening model for both human health and ecology. As part of the Computational Toxicology Research Program of the U.S. EPA, the toxicity of the 309 ToxCast™ Phase I chemicals was assessed using a zebrafish screen for developmental toxicity. All exposures were by immersion from 6–8 h post fertilization (hpf) to 5 days post fertilization (dpf); nominal concentration range of 1 nM–80 μM. On 6 dpf larvae were assessed for death and overt structural defects. Results revealed that the majority (62%) of chemicals were toxic to the developing zebrafish; both toxicity incidence and potency was correlated with chemical class and hydrophobicity (logP); and inter-and intra-plate replicates showed good agreement. The zebrafish embryo screen, by providing an integrated model of the developing vertebrate, compliments the ToxCast assay portfolio and has the potential to provide information relative to overt and organismal toxicity.

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1. Introduction

Traditional toxicity testing requires collecting data on one chemical at a time using common laboratory species (*e.g.*, rats, rabbits, mice). With tens of thousands of chemicals now in commerce, it is apparent that the toxicological community cannot continue testing in this manner. Instead, higher throughput testing needs to be employed to collect data on hundreds to thousands of chemicals [1]. These higher throughput designs include *in vitro* assays, *in silico* modeling and the use of small model organisms as alternative species for toxicity testing.

The zebrafish (*Danio rerio*), a small, freshwater fish native to the Ganges River, is one alternative vertebrate species that has become popular in embryology, pharmacology and biomedical research and is particularly amenable to large-scale screening of chemical libraries [2–4]. Not only are these animals easy to rear and maintain, producing hundreds of offspring per week, but they mature rapidly (6 days) and are small enough for sustaining in 96-well microtiter plates. Beyond the technical advantages of working with a small fish model, there are scientific advantages to assessing zebrafish as a prototype for delineating the functional activity of specific biological pathways and their regulatory controls. Because many key developmental signaling pathways and their regulation are conserved between fish and mammals, the zebrafish provides a model for studying mammalian disease as well as for molecular dissection of developmental pathways (*e.g.*, [5–12]). This also extends to the potential to assess *in vivo* toxicity because zebrafish develop relevant structures such as liver for metabolic conversions [13,14], a thyroid gland that controls development [15–17], and blood-brain barrier [18,19]. In addition, the transparency of the zebrafish embryo is convenient for imaging and dysmorphology assessment.

The technical and scientific advantages of zebrafish have led to the notion that zebrafish would be an appropriate model for developmental toxicity screening [20–32]. Several laboratories have correlated zebrafish general developmental toxicity with mammalian developmental toxicity of select chemicals [33–37]. To our knowledge these comparisons have not been made for a large number of chemicals, especially environmental chemicals.

In the present study, we used zebrafish embryos to screen 309 environmental chemicals, largely pesticides and antimicrobials from the ToxCast™ Phase I chemical library (http://epa.gov/ncct/toxcast/). Adding to the >500 assay portfolio of ToxCast [38], the zebrafish development assay uniquely provides an integrative model of development that can be merged into the ToxCastDB database to support predictive modeling of developmental

* Corresponding author at: Integrated Systems Toxicology Division (B105-03), U.S. Environmental Protection Agency, Research Triangle Park, NC 27712, USA. Tel.: +1 919 541 3956.
E-mail address: Padilla.Stephanie@epa.gov (S. Padilla).
[1] Present Address: Medical University of South Carolina, Department of Regenerative Medicine and Cell Biology, 173 Ashley Ave, Charleston, SC 29425.
[2] Present Address: Department of Statistics, North Carolina State University, Raleigh, North Carolina.

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toxicity. As such, the goals of the present study were to test the ToxCast™ Phase I 309 chemicals for toxicity toward the zebrafish embryo using a developmental assay with general, phenotypic endpoints (lethality, non-hatching, and malformations). We first tested each of the chemicals in a Single Concentration Study at a relatively high nominal dose (80 μM) and followed this by a definitive Concentration-Response Study of each chemical that spanned 5 orders of magnitude. As part of a quality assurance plan we assessed the reproducibility of the assay by (1) comparing inter- and intra-plate replicate chemicals, (2) comparing our data with previously published data, and (3) assessing how well the Single Concentration Study results predicted the Concentration-Response Study. We then focused on identifying classes of chemicals which showed particular toxicity toward developing zebrafish, chemical characteristics that influenced the toxicity profiles, and potential metabolic capabilities with respect to protoxicants and active metabolites. Longer term goals not included in the present report are to compare the zebrafish embryo assay results with the rich database of *in vitro* and mammalian *in vivo* testing that has been used to profile the development of toxicity pathways of these same chemicals [38–45]. In fact an initial analysis comparing these zebrafish toxicity data to the mammalian *in vivo* testing data has just been accepted for publication [46]; also the potency data presented herein are publically available (http://www.epa.gov/ncct/toxcast/data.html) for additional analyses or comparisons.

2. Materials and methods

2.1. Chemicals

The chemical library consisted of the EPA's Phase I ToxCast™ library of 320 substances. This set contains 309 unique chemicals, five duplicates that were obtained from various sources and three triplicates as plating replicates for internal quality control [47]. A table and Structure Data Format (SDF) file of the Phase-I chemical library is available for download at: http://www.epa.gov/NCCT/toxcast/chemicals.html. Most of the chemicals are food-use pesticides for which extensive animals testing results are available and that met physicochemical requirements including solubility in dimethyl sulfoxide (DMSO), molecular weight range of 250–1000 kilodaltons and commercially availability with purity >90% (90% of compounds met these criteria with exceptions allowed for a number of higher or lower molecular weight compounds and several defined mixtures). The library was structurally diverse with over 40 functional classes and more than 24 pesticidal modes of action represented [38,40]. Stock solutions of all chemicals were prepared in 100% DMSO at a concentration of 20 mM. Quality control (QC) of the chemical information, structures and testing substances was performed as described [40]. Results of the QC analysis are available on the ToxCast website: http://www.epa.gov/ncct/toxcast/chemicals.html. Analysis of the chemical solutions showed degradation of 8% of the chemicals over time, in particular for some members of the sulfuron class of pesticides.

2.2. Zebrafish husbandry

Wild type adult zebrafish (*Danio rerio*; undefined outbred stock obtained from Aquatic Research Organisms, Hampton, NH, 03842) were housed in an AAALAC-approved animal facility at 28 °C with a 14:10 h light:dark cycle (lights on at 08:30 h). Adult fish (2–3 females per male; density = 15–20 adults per tank) were kept in one of several 9-liter (L) flow-through colony tanks (Aquaneering Inc., San Diego, CA). All adults in a colony tank were placed in a 2 L (static) breeding tank (Aquatic Habitats, Apopka, FL) one hour prior to light onset. Typically, adults from two to three colony tanks were mated on the same day. Two hours after light onset the adults were returned to the colony tank. All embryos were gathered from each breeder tank, pooled, and placed in a 28 °C water bath for 2 h, followed by two washes [48] with 0.06% bleach (v/v) in 10% Hanks' Balanced Salt Solution [48] (13.7 mM NaCl 0.54 mM KCl, 25 μM Na_2HPO_4, 130 μM $CaCl_2$ 100 μm $MgSO_4$ and 420 μm $NaHCO_3$,), hereafter referred to as Hanks' solution, for 5 min in order to remove any residual bacteria or fungi.

All studies were carried out in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals [49], and were approved by the Institutional Care and Use Committee at the U.S. EPA National Health and Environmental Effects Research Laboratory.

2.3. Chemical exposures

Zebrafish embryos were exposed in 96-well plates as shown in Fig. 1. Briefly, on day 0, approximately 6–8 h after fertilization, zebrafish embryos were placed 1 embryo per well in Millipore Multiscreen Nylon mesh plates (catalog number MANMN4050, Millipore Corp, Bedford, MA) and exposed to nominal concentrations of the chemicals. In each well, 1 μl of the chemical in DMSO from the stock plate was diluted with 250 μl of 10% Hanks' solution; the final DMSO concentration was 0.4% (v/v) in all wells; vehicle controls receives DMSO only. Each plate was sealed with a non-adhesive material (Type A, BioRad, Hercules, CA), covered with a lid, and wrapped in Parafilm® to minimize evaporation. All embryos and larvae were kept in a 26 ± 0.1 °C incubator with a 14:10 h light-dark cycle (with lights on at 08:30 h and off at 22:30 h).

Embryos were exposed to the chemicals for 5 days post fertilization (dpf) (*i.e.*, 120 h post fertilization) with daily dosing (*i.e.*, complete solution change with

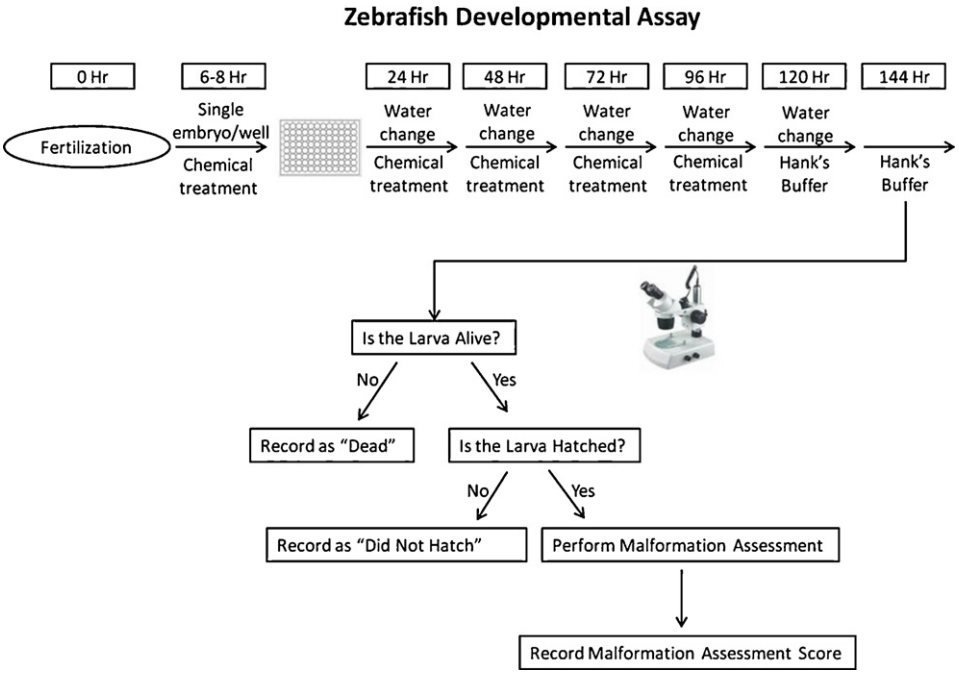

Fig. 1. Experimental design of zebrafish developmental assay. Fertilized eggs were harvested and treated as shown. Microscopic assessments of larvae were performed on day 6 using a decision tree.

chemical renewal every 24 h), followed by a wash-out in Hanks' buffer for 1 day prior to the lethality, hatching, and malformation assessments performed on 6 dpf (see Fig. 1).

Note about the use of DMSO: there appear to be two main issues surrounding the use of DMSO: (1) the possible toxicity of the DMSO and (2) the concern that the DMSO may be "facilitating" the passage of the chemical into the embryo. With regard to the toxicity, our own assays on the toxicity of the DMSO vehicle have consistently shown that 0.4% DMSO elicited no change in mortality, malformations or hatching, or, in other assessments, behavior. This agrees well with the published literature [50–54]. With regard to the DMSO acting as penetrating agent, what data that we were able to locate indicate that much higher concentrations would be required for that to occur [55–57], although none of these studies were done in zebrafish embryos.

2.4. Embryo/larval assessments

On 6 dpf (144 h post fertilization), each larva was assessed by visual inspection under a dissection microscope (Olympus SZH10 Research Stereo). Fig. 1 shows the decision tree for collecting endpoints from each larva. If a larva was dead, no more assessments were made. If a larva was viable, it was then determined if it had hatched or not. If the larva had not hatched, then that information was recorded as an endpoint. If a larva was alive and hatched, an assessment of the degree of malformation was made.

Embryos/larvae were considered dead at 6 dpf if there were signs of coagulation, decay, or no visible heartbeat. Embryos/larvae were considered not hatched if they remained encased in the chorion. If a larva was alive and hatched it was assessed by an observer, blinded to the treatment. Larva was assessed for malformations of general categories as previously described [58]. In brief this involved the following assessments: (1) spine (*e.g.*, stunted skeletal growth, curved spine, kink in tail), (2) fins (*e.g.*, malformed or stunted fins), (3) cranial/facial (*e.g.*, abnormal head, eyes, or otoliths), (4) thorax (*e.g.*, distension, heart malformations), (5) abdomen (*e.g.*, edema, emaciation), and (6) position in the water column (*e.g.*, floating, lying on side). These features were scored for each of the categories, which thus may contain a number of possible malformations that could occur. Some malformations were scored in binary fashion (1,0 for present or not) while others were scored by relative degree, from not present (0) through severe (4). The aggregated scores across all categories of malformations were then summed for each condition and defined as the "Malformation Index". Higher Malformation Indices denote more severely malformed fish, and the indices for the present study went as high as 34, with the historical control values normally between 0 and 3. The Malformation Index mean (±SEM; standard error of the mean) for the controls in the Single Concentration Study was 0.51 ± 0.10 ($n = 217$ embryos that were alive and hatched), and for the Concentration-Response Study the mean was 0.66 ± 0.09 ($n = 706$ embryos that were alive and hatched).

2.5. Single concentration study

The chemicals were arrayed on a 96-well stock plate, 80 chemicals per plate, 20 mM concentration of each, with 16 DMSO (vehicle) controls. As described in detail above, the embryos were exposed to the chemicals (80 μM final concentration, renewed daily) by immersion from 0 dpf until 5 dpf. Eighty micromolar was chosen as the highest concentration for two reasons (1) because the stock solutions were prepared at 20 mM in 100% DMSO. Therefore to limit the amount of DMSO in the rearing solution, we used the smallest accurate amount possible: 1 μl diluted in 250 μl of 10% Hanks' which gave a final, highest concentration of 80 μM: and (2) we felt increasing the concentration above 80 μM would be beyond environmentally or pharmacologically relevant concentrations.

On 6 dpf, each embryo/larva was assessed for viability, hatching status and malformations. There were 4 embryos per concentration per chemical (each embryo on a separate microtiter plate). If more than two controls on a plate (*i.e.*, ≥2/16 = 12.5%) showed lethality or significant malformations, the data from that entire plate were rejected, and the experiment was repeated. Less than 4% of the plates were rejected.

2.6. Concentration-response study

Each stock plate consisted of 7–8 chemicals, arrayed in an 11-point (semi-log) concentration-response with the highest stock concentration (20 mM), the same as in the Single Concentration Study. Adding 1 μl of the chemical solution from the stock plate to the treatment plate and diluting with 250 μl of Hanks' buffer resulted in a descending concentration-response curve spanning 5 orders of magnitude (80.000, 26.600, 8.800, 2.960, 1.000, 0.320, 0.110, 0.030, 0.012, 0.004, and 0.001 μM). There were also eight vehicle controls (*i.e.*, DMSO only; final concentration 0.4%) on each plate. Approximately on every 5th plate a positive reference chemical (chlorpyrifos ethyl) was included [59]. As described in detail above, the embryos were exposed to the chemicals (renewed daily) by immersion from 0 dpf until 5 dpf, and on 6 dpf, each embryo/larva was assessed for viability, hatching status and malformations. Usually, there were 2 embryos per concentration per chemical (each embryo on a separate microtiter plate). If more than one control on a plate (≥12.5%, *i.e.*, ≥1/8) showed lethality or significant malformations, the data from that entire plate were rejected, and the experiment was repeated. Less than 4% of the plates were rejected.

2.7. Toxicity score

In order to formalize the descriptive data (lethality and hatching status) with the numerical data (Malformation Index), we assigned the descriptive data a numerical score: 40 for lethality and 20 for non-hatching, and if the larva was alive and hatched, then the Toxicity Score was equal to the Malformation Index. A chemical was considered active in the Single Concentration Study if the mean Toxicity Score of the four technical repeats for each chemical was greater than the overall mean Toxicity Score of the control fish in the study. For example, in the Single Concentration Study there were 228 controls; the overall mean Toxicity Score for the controls was 2.24 ± 9.53 (SD; standard deviation). Therefore any chemical with a mean Toxicity Score above 2.24 was considered active in the Single Concentration Study.

Chemical potencies were estimated for each compound in the Concentration-Response Study as half-maximal activity concentrations (AC_{50}). The "response" was the combined Toxicity Score (described above), which ranged from 0 to a maximum imputed value of 40. Standard sigmoidal curves were fit using a 4-parameter Hill model, where the response was defined in terms of the four parameters {T, B, AC_{50}, W} given in Eq. (1):

$$f(X) = T - \frac{T - B}{1 + (X/AC_{50})} \quad (1)$$

The parameters T and B are the upper ("top") and lower ("bottom") asymptotes of each assay response, respectively. The W parameter, or "Hillslope", dictates the curve slope (change in response relative to concentration) between B and T, where higher numbers indicate steeper curves. The parameters were fit using a custom R implementation (R Development Core Team, Vienna, 2011) of the Evolutionary Algorithm Dose Response Modeling (EADRM) algorithm [60]. The EADRM algorithm has been shown to handle complex concentration-response spaces and can overcome the challenge of highly variable initial parameter values. Using such a flexible approach to computing the AC_{50} value for each response provided a systematic way to compare compound potencies, even in the face of heterogeneous response patterns. Positive AC_{50} "hit" acceptance criteria were applied as a combination of efficacy (*i.e.*, response at the top asymptote of the sigmoidal fit), and goodness-of-fit (R^2). The minimum R^2 cutoff was 0.4, and the minimum efficacy cutoff was 6.5 (calculated as one standard deviation above the mean of the vehicle control response values). For chemicals where Toxicity Score responses were significant at the lowest concentrations (*e.g.*, rotenone and thiram), thus precluding curve fits, AC_{50} values were heuristically set to the minimum concentration tested. AC_{10} values were estimated using the same curve-fitting procedure, except that the equation was solved to find the concentration at which the response was 10% of the "top" activity.

3. Results

A Single Concentration Study was conducted as a preliminary to identify any shortcomings in the design of the experimental protocol, and to determine how well the single concentration results predicted the concentration-response results. Table 1 contains a list of all the chemicals tested, their CAS numbers, and the results from the Single Concentration and Concentration-Response Studies. Supplemental information, Fig. 1, shows all concentration-response curves from the Concentration-Response Study as well as the individual data points for each chemical, and Supplemental Table 1 provides detailed curve parameters for all chemicals, including AC_{10} estimates.

3.1. Chemical AC_{50} determination

In the Concentration-Response Study, 191 of the 309 chemicals (62%) were judged to be active for toxicity in developing zebrafish embryos based on a calculated AC_{50} value (Table 1). A graphical presentation of all data is shown in Supplemental Fig. 1, and a histogram showing the frequency distribution of the AC_{50} values is presented in Fig. 2. The two most potent chemicals, rotenone and thiram, each had AC_{50} values below 1 nM, but most of the chemicals (80%) had an AC_{50} above 1 μM, and a significant portion (49%) had AC_{50} values above 10 μM.

Examples of the shapes of the concentration-response curves are shown in Fig. 3. Many chemicals elicited a zebrafish embryo toxicity curve exemplified by Butafenacil, where the lower concentrations produced no effect on embryonic development, and the next higher concentration was lethal to the embryos/larvae. Other chemicals showed more gradual transitions between no effect and lethality, where the intermediate concentrations between the

Table 1
Results for all chemicals tested. For each row, the columns (from left to right) contain the following information on a given chemical: name, CAS number, the Toxicity Score from the Single Concentration Study, summary results from the Single Concentration Study ("+" = positive; "–" = negative; "N/A" = not tested), and the estimated AC_{50} from the Concentration-Response Study.

Chemical	CAS number	Single concentration study Toxicity Score Mean of $n = 4$	Result of single concentration study	Concentration-Response Study AC_{50} (μM)
(Z,E)-Fenpyroximate	111812-58-9	40.00	+	0.1381
2,2-Bis(4-hydroxyphenyl)-1,1,1-trichloroethane (HPTE)	2971-36-0	40.00	+	24.6796
2,4-D	94-75-7	2.50	+	–
2,4-DB	94-82-6	40.00	+	19.2327
2,5-Pyridinedicarboxylic acid, dipropyl ester	136-45-8	40.00	+	1.1894
2-Phenylphenol	90-43-7	35.00	+	35.4398
3-Iodo-2-propynylbutylcarbamate	55406-53-6	40.00	+	1.0726
6-Deisopropylatrazine	1007-28-9	11.25	+	–
Abamectin	71751-41-2	40.00	+	0.0173
Acephate	30560-19-1	10.00	+	–
Acetamiprid	135410-20-7	0.50	–	–
Acetochlor	34256-82-1	40.00	+	23.6834
Acibenzolar-S-Methyl	135158-54-2	40.00	+	10.8380
Acifluorfen	50594-66-6	0.25	–	–
Alachlor	15972-60-8	40.00	+	25.9260
Aldicarb	116-06-3	9.75	+	54.4689
Ametryn	834-12-8	21.75	+	–
Amitraz	33089-61-1	40.00	+	14.4975
Anilazine	101-05-3	40.00	+	26.3814
Asulam	3337-71-1	0.00	–	–
Atrazine	1912-24-9	0.00	–	–
Azamethiphos	35575-96-3	40.00	+	23.9139
Azinphos-methyl	86-50-0	40.00	+	9.2867
Azoxystrobin	131860-33-8	40.00	+	3.6014
Bendiocarb	22781-23-3	34.50	+	34.0551
Benfluralin	1861-40-1	7.50	+	4.6768
Benomyl	17804-35-2	40.00	+	1.0802
Bensulfuron-methyl	83055-99-6	0.00	–	71.5274
Bensulide	741-58-2	40.00	+	10.2381
Bentazone	25057-89-0	11.75	+	–
Bifenazate	149877-41-8	40.00	+	10.1389
Bifenthrin	82657-04-3	10.75	+	0.5650
Bisphenol A	80-05-7	40.00	+	46.2207
Boric acid	10043-35-3	0.00	–	60.6850
Boscalid	188425-85-6	40.00	+	1.1337
Bromacil	314-40-9	0.00	–	–
Bromoxynil	1689-84-5	40.00	+	11.5812
Buprofezin	69327-76-0	40.00	+	3.0563
Butachlor	23184-66-9	40.00	+	3.8532
Butafenacil	134605-64-4	40.00	+	0.0069
Butralin	33629-47-9	40.00	+	10.0961
Butylate	2008-41-5	1.75	–	–
Cacodylic acid	75-60-5	1.00	–	–
Captafol	2939-80-2	40.00	+	1.7329
Captan	133-06-2	40.00	+	1.1926
Carbaryl	63-25-2	7.75	+	58.4918
Carboxin	5234-68-4	40.00	+	5.1264
Carfentrazone-ethyl	128639-02-1	40.00	+	0.5697
Chlorethoxyfos	54593-83-8	27.75	+	–
Chloridazon	1698-60-8	0.00	–	–
Chloroneb	2675-77-6	3.75	+	70.4583
Chlorothalonil	1897-45-6	40.00	+	0.3141
Chlorpropham	101-21-3	33.25	+	70.8950
Chlorpyrifos (ethyl) oxon	5598-15-2	40.00	+	0.4046
Chlorpyrifos (ethyl)	2921-88-2	N/A	N/A	8.4936
Chlorpyrifos-methyl	5598-13-0	40.00	+	30.6466
Chlorsulfuron	64902-72-3	6.25	+	–
Cinmethylin	87818-31-3	40.00	+	42.1315
Clodinafop-propargyl	105512-06-9	40.00	+	0.3187
Clofentezine	74115-24-5	0.75	–	–
Clomazone	81777-89-1	0.50	–	–
Cloprop	101-10-0	0.00	–	–
Clopyralid	1702-17-6	0.50	–	–
Clopyralid-olamine	57754-85-5	1.00	–	–
Clorophene	120-32-1	40.00	+	8.2515
Clothianidin	210880-92-5	0.25	–	–
Coumaphos	56-72-4	40.00	+	–
Cyanamide	420-04-2	1.25	–	–

Table 1 (*Continued*)

Chemical	CAS number	Single concentration study Toxicity Score Mean of $n=4$	Result of single concentration study	Concentration-Response Study AC_{50} (μM)
Cyanazine	21725-46-2	0.00	–	–
Cyazofamid	120116-88-3	40.00	+	3.5136
Cyclanilide	113136-77-9	40.00	+	16.3516
Cycloate	1134-23-2	0.50	–	–
Cyfluthrin	68359-37-5	19.75	+	0.3297
Cyhalofop-butyl	122008-85-9	40.00	+	2.9423
Cymoxanil	57966-95-7	40.00	+	42.3939
Cypermethrin	52315-07-8	18.25	+	0.3253
Cyproconazole	94361-06-5	29.75	+	67.8423
Cyprodinil	121552-61-2	40.00	+	10.3065
Cyromazine	66215-27-8	1.25	–	–
Daminozide	1596-84-5	0.00	–	66.5075
Dazomet	533-74-4	20.75	+	0.2814
d-cis,trans-Allethrin	584-79-2	40.00	+	6.5739
Diazinon	333-41-5	40.00	+	–
Diazoxon	962-58-3	40.00	+	28.9912
Dibutyl phthalate	84-74-2	40.00	+	1.4596
Dicamba	1918-00-9	0.00	–	–
Dichlobenil	1194-65-6	0.00	–	–
Dichloran	99-30-9	35.00	+	59.7135
Dichlorprop	120-36-5	22.50	+	0.9570
Dichlorvos	62-73-7	18.00	+	50.0260
Diclofop-methyl	51338-27-3	40.00	+	3.4244
Diclosulam	145701-21-9	0.00	–	–
Dicofol	115-32-2	40.00	+	10.3321
Dicrotophos	141-66-2	10.00	+	–
Diethylhexyl phthalate (DEHP)	117-81-7	0.00	–	–
Diethyltoluamide	134-62-3	0.00	–	–
Difenoconazole	119446-68-3	40.00	+	3.5568
Difenzoquat metilsulfate	43222-48-6	0.50	–	–
Dimethenamid	87674-68-8	40.00	+	30.4923
Dimethoate	60-51-5	0.00	–	–
Dimethomorph	110488-70-5	40.00	+	28.9286
Dimethyl phthalate	131-11-3	10.25	+	–
Diniconazole	83657-24-3	40.00	+	8.0916
Diphenylamine	122-39-4	30.00	+	33.9176
Diquat dibromide	85-00-7	13.25	+	–
Disulfoton	298-04-4	35.00	+	72.7532
Dithiopyr	97886-45-8	40.00	+	7.7709
Diuron	330-54-1	4.50	+	–
Emamectin benzoate	155569-91-8	40.00	+	2.7814
Endosulfan	115-29-7	40.00	+	0.9731
EPTC	759-94-4	1.00	–	–
Esfenvalerate	66230-04-4	26.00	+	0.2939
Ethalfluralin	55283-68-6	13.75	+	10.0532
Ethametsulfuron methyl	97780-06-8	0.00	–	–
Ethephon	16672-87-0	0.50	–	–
Ethofumesate	26225-79-6	22.25	+	–
Ethoprop	13194-48-4	1.25	–	–
Ethylenethiourea	96-45-7	2.50	+	–
Etoxazole	153233-91-1	40.00	+	1.7296
Etridiazole	2593-15-9	23.50	+	4.3080
Famoxadone	131807-57-3	40.00	+	0.1809
Fenamidone	161326-34-7	40.00	+	2.2795
Fenamiphos	22224-92-6	26.25	+	80.0000
Fenarimol	60168-88-9	40.00	+	14.6273
Fenbuconazole	114369-43-6	40.00	+	23.5665
Fenhexamid	126833-17-8	40.00	+	8.3498
Fenitrothion	122-14-5	40.00	+	33.5297
Fenoxaprop-ethyl	66441-23-4	40.00	+	2.5920
Fenoxycarb	72490-01-8	40.00	+	11.7816
Fenpropathrin	39515-41-8	16.75	+	0.3243
Fenthion	55-38-9	40.00	+	15.2726
Fentin	76-87-9	40.00	+	0.0763
Fipronil	120068-37-3	40.00	+	15.5011
Fluazifop-butyl	69806-50-4	40.00	+	8.8182
Fluazifop-P-butyl	79241-46-6	40.00	+	5.1253
Fluazinam	79622-59-6	40.00	+	0.8456
Fludioxonil	131341-86-1	40.00	+	1.8607
Flufenacet	142459-58-3	40.00	+	31.0648
Flufenpyr-ethyl	188489-07-8	40.00	+	0.4279
Flumetralin	62924-70-3	11.00	+	0.0123
Flumetsulam	98967-40-9	0.00	–	–
Flumiclorac-pentyl	87546-18-7	40.00	+	2.9252
Flumioxazin	103361-09-7	10.50	+	8.4308

Table 1 (*Continued*)

Chemical	CAS number	Single concentration study Toxicity Score Mean of $n = 4$	Result of single concentration study	Concentration-Response Study AC_{50} (μM)
Fluometuron	2164-17-2	20.00	+	–
Fluoxastrobin	361377-29-9	40.00	+	0.1873
Fluroxypyr	69377-81-7	1.25	–	–
Fluroxypyr-meptyl	81406-37-3	24.00	+	–
Flusilazole	85509-19-9	40.00	+	12.8890
Fluthiacet-methyl	117337-19-6	40.00	+	0.0148
Flutolanil	66332-96-5	40.00	+	11.6774
Folpet	133-07-3	40.00	+	8.8800
Foramsulfuron	173159-57-4	10.75	+	–
Forchlorfenuron	68157-60-8	40.00	+	46.1649
Formetanate hydrochloride	23422-53-9	3.50	+	–
Fosthiazate	98886-44-3	0.00	–	–
Halosulfuron-methyl	100784-20-1	4.00	+	34.0733
Hexaconazole	79983-71-4	40.00	+	18.7839
Hexazinone	51235-04-2	2.00	–	–
Hexythiazox	78587-05-0	19.25	+	–
Icaridin	119515-38-7	0.00	–	–
Imazalil	35554-44-0	40.00	+	2.4428
Imazamox	114311-32-9	0.25	–	3.5000
Imazapic	104098-48-8	0.00	–	–
Imazapyr	81334-34-1	1.25	–	–
Imazaquin	81335-37-7	0.00	–	–
Imazethapyr	81335-77-5	0.00	–	–
Imidacloprid	138261-41-3	0.00	–	–
Indoxacarb	173584-44-6	12.50	+	0.3385
Iodosulfuron-methyl-sodium	144550-36-7	10.25	+	–
Iprodione	36734-19-7	35.00	+	58.2788
Isazofos	42509-80-8	20.75	+	60.5566
Isoxaben	82558-50-7	27.25	+	34.0802
Isoxaflutole	141112-29-0	1.00	–	–
Lactofen	77501-63-4	40.00	+	0.3145
Lindane	58-89-9	20.75	+	33.7175
Linuron	330-55-2	35.75	+	35.7168
Malaoxon	1634-78-2	0.75	–	–
Malathion	121-75-5	40.00	+	23.5142
Maleic hydrazide	123-33-1	0.50	–	–
Mancozeb	8018-01-7	25.00	+	–
Maneb	12427-38-2	30.00	+	–
MCPA	94-74-6	8.00	+	–
Mepiquat chloride	24307-26-4	0.00	–	–
Mesosulfuron-methyl	208465-21-8	0.00	–	–
Mesotrione	104206-82-8	10.25	+	47.1237
Metalaxyl	57837-19-1	1.50	–	–
Metam-sodium hydrate	6734-80-1	29.50	+	21.6341
Methamidophos	10265-92-6	0.75	–	–
Methidathion	950-37-8	16.50	+	45.8907
Methomyl	16752-77-5	1.25	–	41.3661
Methoxychlor	72-43-5	40.00	+	2.6334
Methoxyfenozide	161050-58-4	4.75	+	–
Methyl cellosolve	109-86-4	1.50	–	–
Methyl hydrogen phthalate	4376-18-5	1.25	–	–
Methyl isothiocyanate	556-61-6	40.00	+	2.9635
Methylene bis(thiocyanate)	6317-18-6	40.00	+	3.9125
Metiram-zinc	9006-42-2	35.00	+	1.4400
Metolachlor	51218-45-2	34.50	+	33.7048
Metribuzin	21087-64-9	0.75	–	–
Metsulfuron-methyl	74223-64-6	10.00	+	–
Mevinphos	7786-34-7	11.50	+	55.8067
MGK	113-48-4	40.00	+	8.7261
Milbemectin (mix of >70% Milbemycin A4 CAS 51596-11-3; <30% Milbemycin A3 CAS 51596-10-2)	51596-10-2	40.00	+	0.1319
Molinate	2212-67-1	5.50	+	–
Monobutyl phthalate	131-70-4	10.50	+	–
Monocrotophos	6923-22-4	10.00	+	–
Myclobutanil	88671-89-0	30.00	+	39.8162
Naled	300-76-5	40.00	+	11.5512
Napropamide	15299-99-7	21.50	+	–
Niclosamide	50-65-7	40.00	+	0.9884
Nitrapyrin	1929-82-4	0.00	–	–
Norflurazon	27314-13-2	1.50	–	–
Novaluron	116714-46-6	0.50	–	79.7072
Oryzalin	19044-88-3	40.00	+	11.6812
Oxadiazon	19666-30-9	40.00	+	5.1219

Table 1 (*Continued*)

Chemical	CAS number	Single concentration study Toxicity Score Mean of $n=4$	Result of single concentration study	Concentration-Response Study AC_{50} (μM)
Oxamyl	23135-22-0	10.25	+	–
Oxasulfuron	144651-06-9	0.25	–	–
Oxyfluorfen	42874-03-3	15.00	+	12.0071
Oxytetracycline dihydrate	6153-64-6	1.00	–	–
Paclobutrazol	76738-62-0	40.00	+	26.8276
Parathion	56-38-2	30.50	+	8.6019
Parathion-methyl	298-00-0	40.00	+	26.1505
Pendimethalin	40487-42-1	17.25	+	42.5697
Penoxsulam	219714-96-2	0.50	–	–
Perfluorooctane sulfonic acid	1763-23-1	28.00	+	32.8835
Perfluorooctanoic acid	335-67-1	0.25	–	–
Permethrin	52645-53-1	12.00	+	3.0027
Phenoxyethanol	122-99-6	0.00	–	–
Phosalone	2310-17-0	40.00	+	9.3641
Phthalic acid, mono-2-ethylhexyl ester	4376-20-9	40.00	+	0.5665
Picloram	1918-02-1	0.00	–	–
Piperonyl butoxide	51-03-6	40.00	+	11.3428
Pirimicarb	23103-98-2	0.50	–	–
Pirimiphos-methyl	29232-93-7	40.00	+	11.2913
Prallethrin	23031-36-9	40.00	+	1.5687
Primisulfuron-methyl	86209-51-0	40.00	+	3.2547
Prochloraz	67747-09-5	40.00	+	4.4664
Prodiamine	29091-21-2	1.25	–	–
Profenofos	41198-08-7	40.00	+	8.6923
Prohexadione-calcium	127277-53-6	0.00	–	–
Prometon	1610-18-0	0.00	–	–
Prometryn	7287-19-6	23.00	+	–
Propamocarb hydrochloride	25606-41-1	24.25	+	–
Propanil	709-98-8	36.75	+	32.7022
Propargite	2312-35-8	40.00	+	0.1279
Propazine	139-40-2	13.50	+	–
Propetamphos	31218-83-4	35.00	+	–
Propiconazole	60207-90-1	40.00	+	26.6400
Propoxur	114-26-1	2.50	+	–
Propoxycarbazone-sodium	181274-15-7	1.00	–	15.4929
Propyzamide	23950-58-5	0.50	–	–
Prosulfuron	94125-34-5	0.33	–	21.0890
Pymetrozine	123312-89-0	1.25	–	17.4414
Pyraclostrobin	175013-18-0	40.00	+	0.1380
Pyraflufen-ethyl	129630-19-9	40.00	+	0.1873
Pyridaben	96489-71-3	40.00	+	0.0114
Pyrimethanil	53112-28-0	30.00	+	–
Pyriproxyfen	95737-68-1	40.00	+	26.1309
Pyrithiobac-sodium	123343-16-8	0.00	–	–
Quinclorac	84087-01-4	0.50	–	–
Quinoxyfen	124495-18-7	11.25	+	3.3917
Quintozene	82-68-8	25.00	+	18.8039
Quizalofop-ethyl	76578-14-8	40.00	+	1.2242
Resmethrin	10453-86-8	13.25	+	2.8012
Rimsulfuron	122931-48-0	1.25	–	–
Rotenone	83-79-4	40.00	+	<0.0014
S-Bioallethrin	28434-00-6	40.00	+	1.0541
Sethoxydim	74051-80-2	1.50	–	–
Simazine	122-34-9	0.00	–	–
Spirodiclofen	148477-71-8	40.00	+	1.7200
Spiroxamine	118134-30-8	22.25	+	–
Sulfentrazone	122836-35-5	31.50	+	33.8881
Symclosene	87-90-1	0.00	–	–
TCMTB	21564-17-0	40.00	+	0.4320
Tebufenozide	112410-23-8	20.00	+	–
Tebufenpyrad	119168-77-3	40.00	+	0.3200
Tebupirimfos	96182-53-5	40.00	+	9.6085
Tebuthiuron	34014-18-1	13.25	+	–
Tefluthrin	79538-32-2	18.00	+	0.0046
Tepraloxydim	149979-41-9	3.25	+	–
Terbacil	5902-51-2	1.50	–	–
Tetraconazole	112281-77-3	40.00	+	31.4710
Tetramethrin	7696-12-0	40.00	+	10.3323
Thiabendazole	148-79-8	40.00	+	30.2515
Thiacloprid	111988-49-9	0.50	–	–
Thiamethoxam	153719-23-4	1.25	–	74.7322
Thiazopyr	117718-60-2	40.00	+	32.6888
Thidiazuron	51707-55-2	0.50	–	–
Thiobencarb	28249-77-6	40.00	+	13.1923

Table 1 (*Continued*)

Chemical	CAS number	Single concentration study Toxicity Score Mean of $n = 4$	Result of single concentration study	Concentration-Response Study AC_{50} (μM)
Thiodicarb	59669-26-0	14.25	+	29.2314
Thiophanate-methyl	23564-05-8	8.00	+	1.2252
Thiram	137-26-8	40.00	+	<0.0014
Tralkoxydim	87820-88-0	23.25	+	46.1338
Triadimefon	43121-43-3	32.00	+	24.7762
Triadimenol	55219-65-3	40.00	+	29.9123
Tri-allate	2303-17-5	40.00	+	27.1595
Triasulfuron	82097-50-5	1.75	–	–
Tribenuron-methyl	101200-48-0	0.00	–	–
Tribufos	78-48-8	40.00	+	0.1439
Trichlorfon	52-68-6	16.25	+	–
Triclopyr	55335-06-3	10.00	+	–
Triclosan	3380-34-5	40.00	+	2.6589
Trifloxystrobin	141517-21-7	40.00	+	0.2664
Trifloxysulfuron-sodium	199119-58-9	0.00	–	–
Triflumizole	68694-11-1	40.00	+	1.6166
Trifluralin	1582-09-8	15.00	+	0.6292
Triflusulfuron-methyl	126535-15-7	0.25	–	–
Triticonazole	131983-72-7	7.00	+	13.0456
Vinclozolin	50471-44-8	20.50	+	37.5422
Zoxamide	156052-68-5	40.00	+	5.1268

lethal and no effect concentrations produced significant malformations or caused failure to hatch (*e.g.*, Mibemectin and Methyl Isothiocyanate in Fig. 3). A few chemicals did not cause lethality but produced extensive malformations across a wide concentration range (*e.g.*, Cypermethrin, Fig. 3).

3.2. Reproducibility and consistency

The data were reproducible among our own internal positive reference (chlorpyrifos ethyl) run on multiple plates, as well as among the duplicates and triplicates that were randomly included. Fig. 4 shows the mean set results for the eight inter- and intra-plate controls as well as for chlorpyrifos ethyl. Chlorsulfuron and EPTC were consistently negative, while bensulide, chlorpyrifos ethyl, 3-iodo-2-propynylbutylcarbamate, dibutyl phthalate, dichlfop-methyl, and fenoxaprop-ethyl were consistently positive. Only one chemical, prosulfuron, showed inconsistent results, with two of the replicates showing positive results, but one showing completely negative results. This may be due to the instability of the chemical, as analytical analysis of the stock solution showed evidence of decomposition, similar to other members of the sulfuron class (QC data available for all chemicals at http://www.epa.gov/ncct/toxcast/chemicals.html). Therefore, with the exception of the unstable prosulfuron, the binary activity (active or not) calls would have been consistent using any replicate of a given chemical. With respect to the AC_{50} values estimated for the stable active compounds, 3-iodo-2-propynylbutylcarbamate, bensulide, chlorpyrifos ethyl, dibutyl phthalate, and fenoxaprop-ethyl were nearly identical across replicate sets.

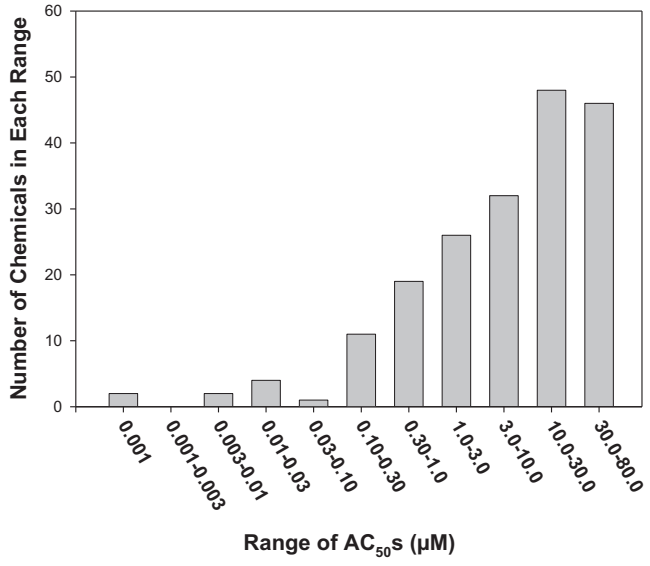

Fig. 2. Frequency distribution of the AC_{50}s that were positive in the zebrafish embryo toxicity Concentration-Response Study.

Comparison of the present data with the ECOTOX database (http://cfpub.epa.gov/ecotox/) and published literature revealed good agreement for the small number of comparisons available. From the ECOTOX database, the comparison was limited to zebrafish embryo studies where exposure to the chemical began by 8 h post fertilization and lasted for at least 48 h, but not more than 4 days, and the toxicity endpoints included were lethality and malformations. Using those criteria, there were 9 chemicals that could be compared with ToxCast Phase-I: atrazine, boric acid, diazinon, malathion, cypermethrin, thiram, permethrin, bifenthrin, and methylisothiocyanate. Additionally, six triazole derivatives tested in our system (flusilazole, hexaconazole, cyproconazole, triadimefon, myclobutanil, and triticonazole) were compared to developmental toxicity indices from the published literature [61,62]. Fig. 5 shows the comparison between the AC_{10} calculated in the present study with the LOEC (lowest effective concentration) in the 9 chemicals which matched the ECOTOX database and the BMC_T (benchmark concentration for teratogenicity at a 5% benchmark response) for the six triazole derivatives. Discordance was exhibited for two of the chemicals (atrazine and diazinon) that had no effect in our studies but were toxic in the ECOTOX database. Fitting a regression line to the remaining positive chemicals showed an excellent linear relationship ($R^2 = 0.79$) between our data and the previously published data. Furthermore, the slope of the line was close to 1 (1.07), an indication that the values obtained did not differ systematically from one another, even though the experimental designs were not identical.

3.3. Chemical class evaluation

To determine which chemical classes tended to be toxic to developing zebrafish, we compared the AC_{50} values to the

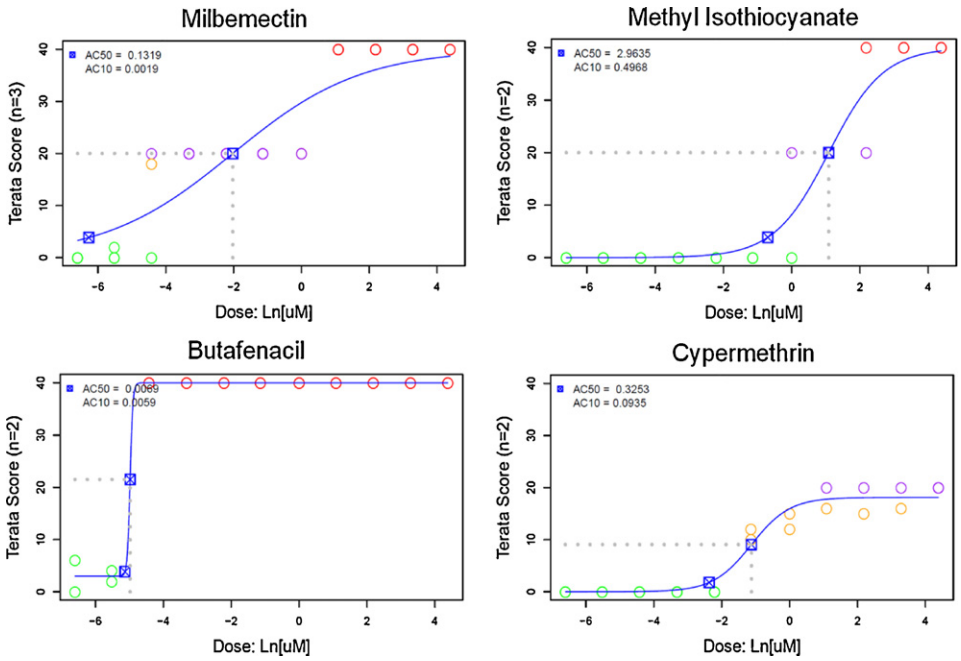

Fig. 3. Examples of different concentration-response curves. Green circles represent larvae that were within normal range, red circles represent nonviable larvae, purple circles represent larvae that did not hatch, and orange circles represent larvae that showed significant malformations. The blue line is the fitted concentration-response relationship as explained in Section 2. The calculated AC_{10} and AC_{50} (left and right boxes, respectively, on the blue line) are given in μM on each graph (upper left-hand corner) for each chemical. Each circle represents an individual embryo/larva, but in some cases if the circles are superimposed, one circle may represent more than one embryo/larva. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

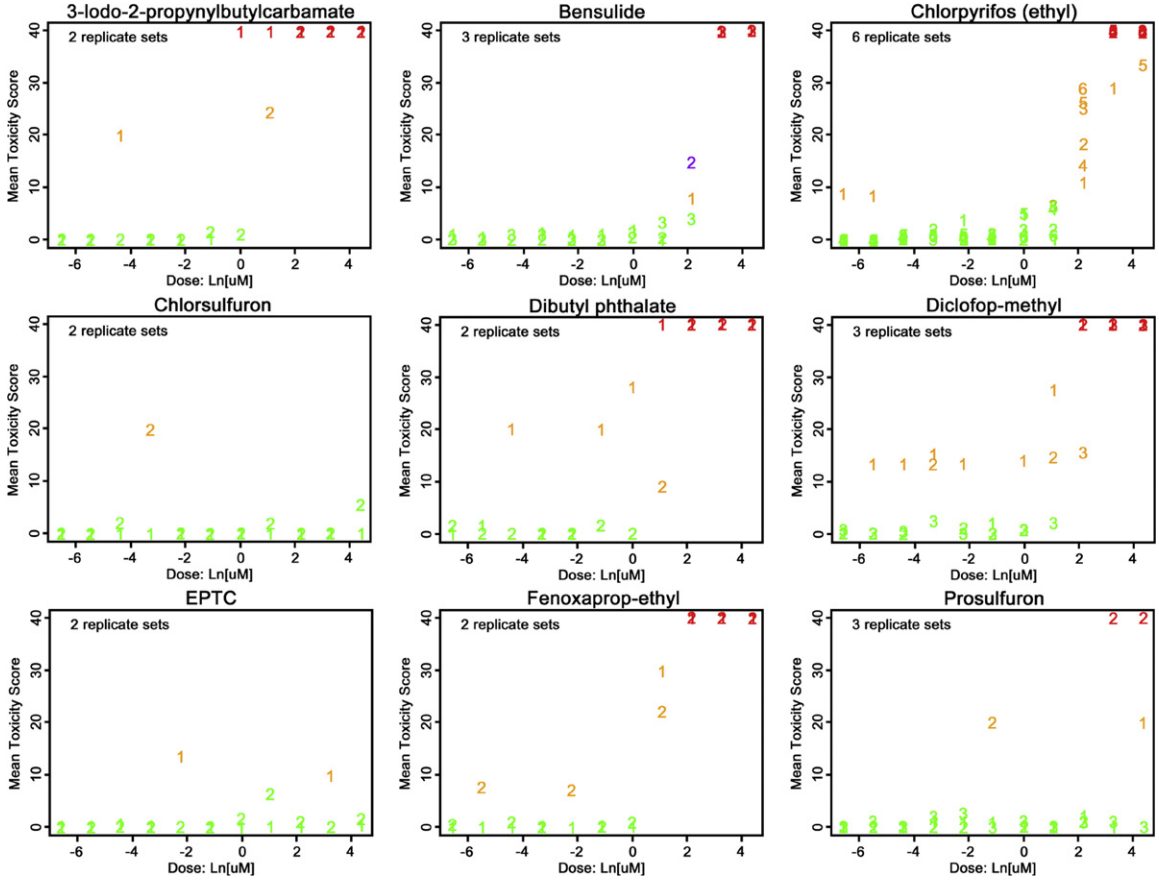

Fig. 4. Comparison of the reproducibility among plates. Each number represents a separate replicate set (mean Toxicity Score of 2–3 embryos per dose) for each chemical. Green numbers represent larvae that were within normal range, red numbers represent nonviable larvae, purple numbers represent larvae that did not hatch, and orange numbers represent larvae that showed significant malformations. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

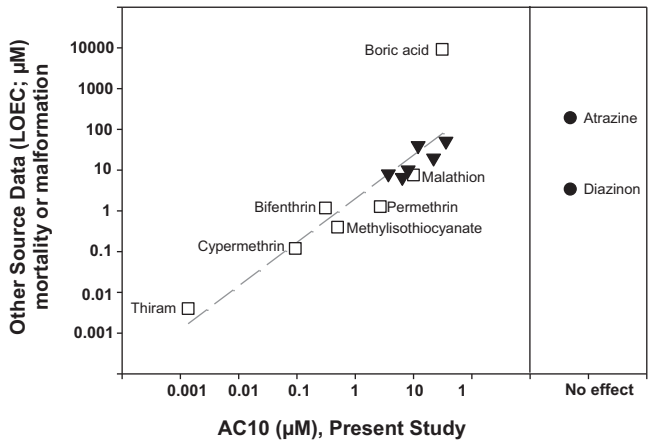

Fig. 5. Comparison of the present data with the zebrafish embryo toxicity data in the ECOTOX database as well as recently published papers on the toxicity of triazole derivatives [61,62]. □ = chemicals that were positive in the ECOTOX Database and in the present study; ● = chemicals that were positive in the ECOTOX Database, but negative in the present assay; ▼ = triazole derivatives tested in the above publications. The correlation line (dashed line) fit to the positive chemicals resulted in a slope 1.07 and $R^2 = 0.79$.

chemical class of each chemical compound (http://epa.gov/ncct/toxcast/data.html; for details on the chemical classes, see www.epa.gov/pesticides). For this comparison, the percentage of positive chemicals was determined for each class that had at least 3 members. Note that some chemicals fell into more than one chemical class. For example there were 18 total conazoles consisting of 12 triazoles and 6 imidazoles. Therefore, if a triazole was positive, it would be counted as positive in both the triazole class as well as in the conazole class. The percentage of positive chemicals for each class is represented by the gray bars in Fig. 6. There were 12 chemical classes in which 100% of the members were positive: triazolone (3 members), strobilurin (4), pyrethroid (12), phthalimide (3), phenyl organothiophosphate (5), organochlorine (4), macrocyclic-lactone (3), imidazole (6), dicarboximide (7), chloroacetanilide (4), aryloxyphenoxypropionic (7), and aromatics (4).

We next assessed the degree of toxicity for each class by averaging the AC_{50} values for the chemicals that were positive in each class. Only classes that had at least 2 positive chemicals were included in Fig. 6. There were four classes in which 100% of the chemicals were positive and the average AC_{50} was below 4 µM: strobilurin, pyrethroid, macrocyclic lactone, and aryloxyphenoxypropionic.

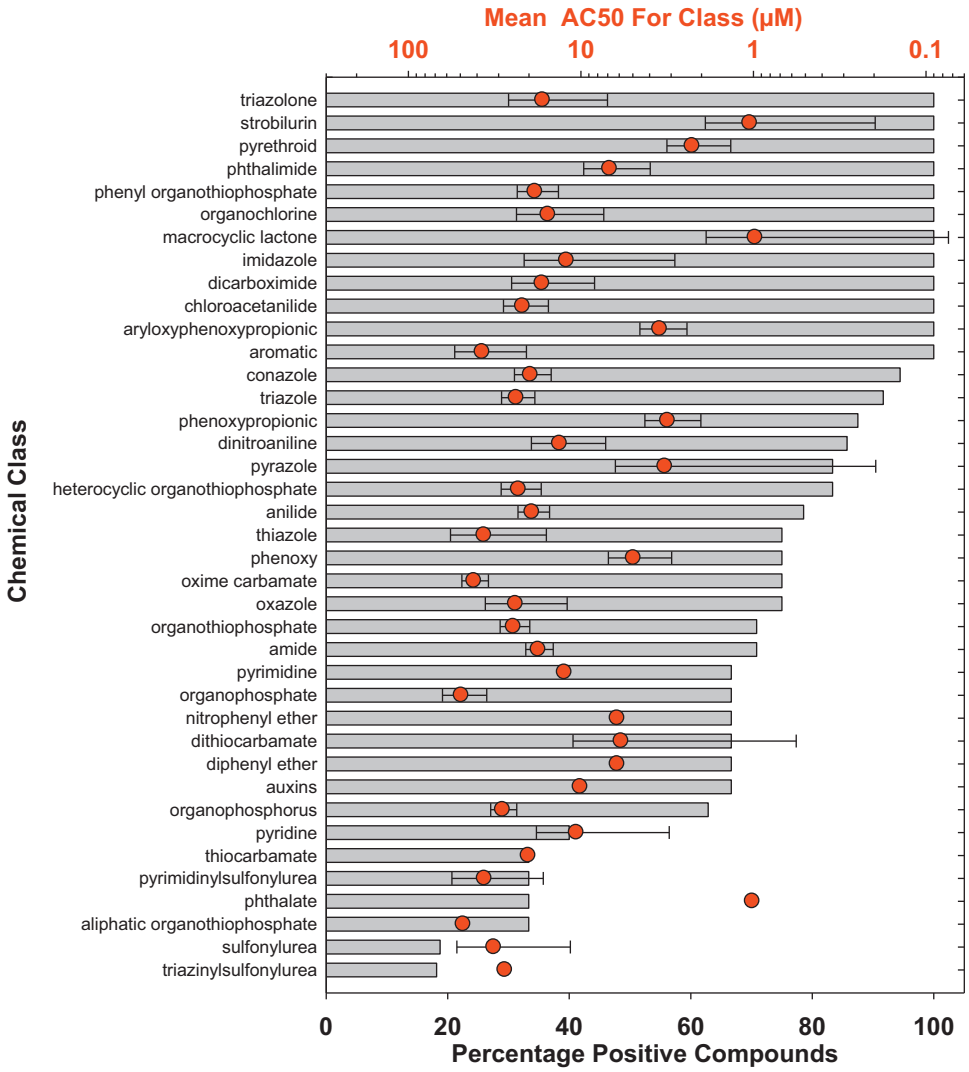

Fig. 6. Relationship between chemical class and toxicity to developing zebrafish. The percent positive chemicals in each class are represented by the gray bars (bottom axis), and the average AC_{50} for each group (±SEM) is indicated by the filled red circles (top axis). Only classes with three or more total members were analyzed, and only classes with at least 2 positive chemicals were included in the graph. If a class only had two positive chemicals, no error bars are shown, *i.e.*, triazinylsulfonylurea, aliphatic organothiophosphate, phthalate, thiocarbamate, auxins, diphenyl ether, nitrophenyl ether, and pyrimidine.

3.4. Toxicity profile versus logP

Another characteristic that we explored was the relationship between logP (octanol:water partition coefficient) and toxicity. The logPs for the chemicals [obtained from EPI (Estimation Programs Interface) Suite™; www.epa.gov/opptintr/exposure/pubs/episuite.htm] ranged between −2.82 and 8.15 (*i.e.*, 10 orders of magnitude). There was an overall pattern of increasing toxicity likelihood with increasing logP (Fig. 7, Panel A). As the hydrophobicity (*i.e.*, logP) of a chemical increased, the likelihood that the chemical would be toxic to the zebrafish embryo increased: for example, only 13% (2/15) of the chemicals with a logP less than or equal to 0 were positive in our assessments, but 89% (57/64) of the chemicals with a logP between 4 and 5 were positive. Panel B compares the average AC_{50} for all the positive chemicals within a logP bin to determine if the nominal concentration causing toxicity was also related to the chemical hydrophobicity. Not only did the chemicals with logPs at or below 0 tend to be nontoxic (Fig. 7, panel A), but if they were toxic, the mean AC_{50} was higher (*i.e.*, less potent) compared to the mean AC_{50} values of the other logP bins (Figure 7, Panel B). The chemicals with a logP above 0 but less than 4 varied little in AC_{50} (mean ≅20 μM), but chemicals with higher logPs tended to have lower (*i.e.*, more potent) AC_{50} values. In sum, both the likelihood that a chemical will be toxic to the developing zebrafish embryo and the degree of toxicity (*i.e.*, AC_{50}) were related to the hydrophobicity of the chemical.

3.5. Metabolic pairs

Several known mammalian protoxicants and their active metabolites were included in the group of 309 chemicals (Table 2). For some pairs, both the parent chemical and the metabolite were toxic to the developing zebrafish embryo (*e.g.*, methoxychlor, metam-sodium, chlorpyrifos ethyl and their active metabolites); in other cases, only the parent chemical was toxic (metriam-zinc, dibutyl phthalate, and malathion); and in others, only the active metabolite (but not the parent compound) was toxic (monoethylhexyl phthalate and diazoxon: metabolites of diethylhexyl phthalate and diazinon, respectively).

3.6. Single concentration screening versus AC_{50} determination

We compared results from the Single Concentration Study with the Concentration-Response Study from Table 1. The data in the Single Concentration Study consisted of an assessment of 4 larvae for each chemical at a concentration of 80 μM. We assigned a Toxicity Score to each animal in the Single Concentration Study in the same manner as the Concentration-Response Study, and then these individual values were averaged for each chemical (Table 1). The average Toxicity Score for the control larvae in the Single Concentration Study was 2.24; therefore, we considered any chemical in the Single Concentration Study with a Toxicity Score above 2.24 as active. There were only 10 chemicals classified as inactive in the Single Concentration Study, but later revealed to be active in the Concentration-Response Study (10 of 309 = 3% False Negative

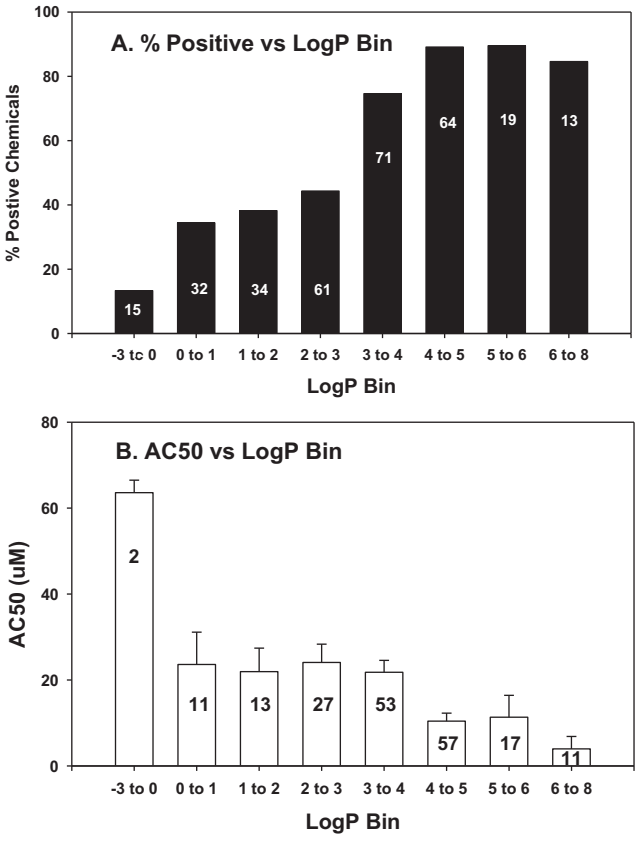

Fig. 7. The relationship between the hydrophobicity (octanol:water partition coefficient; logP) of a chemical and its toxicity to zebrafish embryonic development. Panel A: Relationship between logP of a chemical and the likelihood it will be toxic to the developing zebrafish embryo. Each bar has the number of chemicals with logP values in that range. There was a significant ($p < 0.0001$) relationship between logP bin and percentage of chemicals in that bin that were positive for toxicity (Kruskal–Wallis analysis on percentage positive *versus* logP bin). Panel B: Relationship of the mean AC_{50} for the positive chemicals in each logP bin. Each bar has the number of positive chemicals in each bin, and the height of each bar is mean (±SEM) of the mean AC_{50} for the positive chemicals in each bin. An ANOVA with AC_{50} as the dependent variable and logP as the independent variable showed a significant ($p = 0.044$) relationship between the two variables.

Table 2
Toxicity outcomes for both the parent chemical and metabolite. A "+" indicates that the chemical was positive in the Concentration-Response Study; the number following the "+" sign is the AC_{50} (μM) for that chemical (see Table 1) A "−" sign indicates that the chemical was negative in the Concentration-Response Study.

Metabolic Pairs		Outcome	
Parent	Metabolite	Parent	Metabolite
Dimethyl phthalate	Methyl hydrogen phthalate	–	–
Atrazine	6-Deisopropylatrazine	–	–
Methoxychlor	2,2-Bis(4-Hydroxyphenyl)-1,1,1-Trichloroethane (HPTE)	+2.63	+24.68
Metam-sodium	Methyl isothiocyanate	+21.63	+2.96
Diethylhexyl phthalate	Monoethylhexyl phthalate	–	+0.5665
Metiram-zinc	Ethylenethiourea	+1.44	–
Maneb	Ethylenethiourea	-	–
Mancozeb	Ethylenethiourea	–	–
Dibutyl phthalate	Monobutyl phthalate	+1.46	–
Malathion	Malaoxon	+23.5	–
Diazinon	Diazoxon	–	+28.99
Chlorpyrifos (Ethyl)	Chlorpyrifos-oxon (Ethyl)	+8.5	+0.4

Table 3
Relationship between the results of the Single Concentration Study and the Concentration-Response Study. The Single Concentration Study was conducted with one concentration (80 μM, $n = 4$ embryos), while the Concentration-Response Study was conducted with an 11 point (semi-log) concentration-response curve for each chemical ($n = 2$ per chemical per dose). The Single Concentration Study predicted the results of the Concentration-Response Study in more than 80% of the cases for both positive and negatives. There was a significant relationship (Chi Square, $p < 0.001$) between the Single Concentration Study results and the results of the Concentration-Response Study.

Concentration-Response Study	Single Concentration Study	
	Positive	Negative
Positive	181/224 81%	10/85 12%
Negative	43/224 19%	75/85 85%

rate) (Table 3). The Single Concentration Study results were predictive of the concentration-response results with a sensitivity of 95%, specificity of 64% and a balanced accuracy of 83% [63].

4. Discussion

Assessing the toxicity of the ToxCast™ Phase I chemical library using a zebrafish embryonic developmental assay revealed that the majority (62%) of the chemicals were toxic to the developing embryo at concentrations at or below 80 μM. This toxicity, in terms of both incidence and potency, was correlated with chemical class as well as the hydrophobicity of the chemical. Furthermore, the conditions of the assay were such that the inter- and intra-plate consistency was good, and the data are in agreement with the few instances in the previously published data that we could locate where selected chemicals had been tested in zebrafish embryos using a similar protocol. We conclude that the zebrafish toxicity assay is a useful addition to the ToxCast portfolio, providing a unique integrative model of embryogenesis.

The fact that over 60% of the ToxCast™ Phase I chemicals affected embryonic development in the zebrafish is not surprising as these chemicals are mainly pesticides and pesiticide metabolites, and were designed to be biologically active, whether against fungi, worms, insects, fish, or plants. Most of the compounds were toxic in the assay within the micromolar range although a few were toxic to the developing zebrafish embryo in the submicromolar range. For several cases where data were available from an independent study, we obtained consistent results despite differences in the experimental details. The inter-plate positive reference (chlorpyrifos ethyl) and other concentration-response curves were highly consistent even when tested months apart. This consistency indicates that the experimental method was robust. On the other hand, two compounds, (atrazine and diazinon) gave discordant results with the literature. For atrazine the LOEC in the ECOTOX database was 185 μM and therefore well-above the highest concentration tested here (80 μM). For diazinon, the discrepancy could be due to our conservative analyses of the concentration-response curves.

The zebrafish embryo/larvae liver appears capable of cytochrome P450 activity very early in development [14,64–66]. It has also been recently shown that the embryonic zebrafish liver is able to metabolize protoxicants to their active toxic metabolites [67], although the extent of this activation is still unclear [34,68,69]. Our results comparing the toxicity of 12 pairs of protoxicants and their active metabolites shows that the zebrafish embryo/larva is able to convert protoxicants to their active metabolites, but that the conversion may be compound-specific. Taking the three organophosphate pesticides (malathion, diazinon, and chlorpyrifos ethyl) as an example: all three need to be activated to their oxon derivative for maximal anticholinesterase activity. There were, however, different patterns of toxicity for the organophosphate pesticides and their metabolites. Because both malathion and chlorpyrifos ethyl were toxic to the developing embryo, it can be assumed that in each case the embryo/larval zebrafish liver was able to convert the parent compound to the oxon metabolite. That is supported by the fact that chlorpyrifos oxon was also toxic to the development of the zebrafish embryo, but, curiously, the active metabolite of malathion, malaoxon, was negative. In any event, there is evidence from this small sampling that the zebrafish embryo/larva is capable of metabolic activation, an assay attribute which sets it apart from the typical *in vitro* screening assay.

Because so many chemicals were assessed here, it is possible to discern patterns of response that are difficult to see for smaller studies. Two of the patterns were the relationship between incidence and degree of toxicity relative to chemical class and logP. There were 18 chemical classes in which over 80% of the member chemicals were toxic to the developing zebrafish embryo, and in some cases, the potency of the group was also quite high. Four classes that showed considerable toxicity in the developing zebrafish, from the standpoint of both incidence and potency, were the strobilurins (plant fungicides; inhibitors of mitochondrial respiration), pyrethroids (primarily insecticides; sodium channel blockers), macrocyclic lactones (nematicides and insecticides; bind to glutamate-gated chloride ion channels), and aryloxyphenoxypropionics (herbicides; acetyl-CoA carboxylase inhibitors, decrease lipid biosynthesis).

LogP is just one of many chemical characteristics that could be assessed, but we concentrated on it because logP has often been linked to bioavailability of chemicals in fish [70–77]. The hydrophobicity of a chemical is well-known to have a powerful influence on the bioavailability of that chemical in an aquatic system [78]. Our results relating the two factors, logP and toxicity, support that observation. Chemicals that tended to be hydrophobic (*i.e.*, higher logP) tended to be more toxic to the developing zebrafish embryo and, conversely, chemicals that tended to be hydrophilic (*i.e.*, low logP) also tended not to be as toxic (considering either incidence or potency) to the developing zebrafish embryo. This is consistent with the notion that physiochemical characteristics of the chemical are a key determinant of the internal dosimetry. This implies that the nominal concentration of the chemical in the exposure medium does not necessarily reflect the tissue concentration in the embryo/larvae. In fact, this disconnect between nominal concentration and zebrafish embryo/larva body burden has been noted by other investigators for select chemicals [74,79–81]. This observation, if broadly applicable, has serious consequences for extrapolation of the results to mammalian data. If the zebrafish internal dose is not known, then it is extremely difficult to equate zebrafish dose with mammalian dose. If a systematic relationship exists between logP and internal concentration for the developing fish embryo as has been demonstrated in adult fish [70–73], then further studies are needed to uncover the relationship across a wide range of logP values that could be guided by the ToxCast 309 chemicals. It should be noted that this problem is not unique to small fish toxicity testing; there is some preliminary evidence that the same disconnect between nominal dose and actual dose may occur in *in vitro* testing [82]. We may provisionally conclude that zebrafish developmental toxicity is strongly related to both chemical class and logP, but it must be kept in mind that these two variables are likely interrelated.

Our results also suggest that a Single Concentration Study may be an efficient "pre-screen" for the Concentration-Response Study. The present data coupled with a very liberal definition of "hit" in the Single Concentration Study resulted in a sensitivity index of 95%, indicating excellent predictability of the Single Concentration Study for positive compounds in the Concentration-Response Study. We plan, therefore, to include this Single Concentration

Study as a pre-screen in future screening efforts against more diverse chemical collections.

The move toward greater use of *in vitro* assay systems for predicting toxicity greatly increases the number of chemicals that can be examined for potential adverse effects. While specific targets of toxicity can be rapidly assessed in this manner, the majority of toxicity targets has yet to be resolved [47]. This may be due to confounding factors such as simultaneous effects on multiple targets or to emergent properties of complex biological systems that remain challenging to replicate *in vitro*. Use of integrative model organisms such as the zebrafish as test systems provide the biological complexity of a vertebrate embryo but the simplicity of a moderate throughput platform. More detailed work is required to understand which types of toxicity are within the domain of applicability for this approach, particularly using a larger and more diverse chemical collection with many known toxicants and diverse mechanisms of action, as well as an analysis comparing general and specific effects across the concentration-response. This is a significant next step toward further validating this approach.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.reprotox.2011.10.018.

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