

# Developmental neurotoxicity assessment in zebrafish: a survey of 200 environmental toxicants

Louis J. D'Amico, Chunqi Li, Wen Lin Seng, and Patricia McGrath

Phylonix Pharmaceuticals, Inc., Cambridge, Massachusetts, U.S.A.

## Abstract

Conventional assessment of neurotoxicity in mammalian models relies on histological, neurophysiologic, and behavioral studies that are laborious and time consuming. The zebrafish (*Danio rerio*), is an increasingly attractive alternative model for developmental neurotoxicity, in part due to its embryonic transparency, rapid development, and simplicity of chemical delivery. Previous studies at Phylonix demonstrated a high correlation between developmental neurotoxicity observed in zebrafish with findings in mammalian models. In particular we characterized brain apoptosis and tail motor neuron defects that correlated with behavioral defects. To further establish the feasibility of a zebrafish model for developmental toxicity, we surveyed developmental neurotoxicity of 200 environmental toxicants chosen from the Voluntary Children's Chemical Evaluation Program (VCCPEP) list and/or EPA Emergency Planning and Community Right-to-Know Act (EPCRA). We looked at the following parameters of neurotoxicity: 1) lethality, 2) brain apoptosis, 3) axon tract disruption in the brain and tail, 4) motor neuron formation in the tail, 5) catecholaminergic neurotoxicity, and 6) motility. From the 200 toxicants surveyed, we identified several compounds that showed effects on one or more of the parameters examined. Some compounds showed broad toxicity across all neuroanatomical endpoints. We also identified compounds that increased and decreased motor activity in day 6 zebrafish. Our results show that the zebrafish model has predictive value as an alternative model for developmental neurotoxicity screening. This work was supported by National Science Foundation SBIR award # 054957.

## Introduction

**The scope of agricultural and industrial pollutants:** Every year, billions of pounds of toxic chemicals are released by industrial facilities and agricultural practices, much of which ends up in air or groundwater; ¼ of these chemicals are known or suspected neurotoxins (Schettler *et al.* 2000). Currently, more than 85,000 industrial chemicals are produced in the US every year, with an additional 2000-3000 new chemicals registered each year. More than 70% of these chemicals have little or no toxicity data (Claudio *et al.* 2000).

**Zebrafish as a new model for developmental neurotoxicity:** Conventional neurotoxicity assessment in mammalian models using histological, neurophysiologic, and behavioral studies are expensive, laborious, and time consuming. Due to the large number of compounds that require testing, a more rapid yet informative model would facilitate screening of potential toxicants. The zebrafish (*Danio rerio*) has previously been shown to be a useful model for assessing drug and developmental toxicity (Parrig *et al.* 2002; Zhang *et al.* 2003). Since the embryo is transparent and develops rapidly, visualizing development of the central and peripheral nervous system is possible. Behavioral assays are also being developed which look at motor activity and startle response. Zebrafish assays can be performed relatively quickly on a large number of animals.

**Developing a zebrafish developmental neurotoxicity model:** To demonstrate the utility of zebrafish screens for developmental neurotoxicity, we initiated a pilot screen of 200 environmental toxicants. We focused on measuring lethality, apoptosis/necrosis in the brain, as well as surveying axon tracts, motor neurons, and the catecholaminergic system. We also developed a high-throughput screen for motor activity.

## Materials and methods

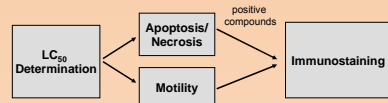
**LC<sub>50</sub> Determination:** Zebrafish (n=20) were exposed by static immersion from 6-96 hours post fertilization (hpf) at compound concentrations of 500, 100, 10, and 1 µM. Zebrafish treated with 0.1% DMSO were used as controls. Determination of LC<sub>50</sub> values was accomplished through logistic regression using JMP 5.1 (The SAS Institute, Cary, NC) based on total mortality throughout the entire 96hr treatment period.

**Apoptosis/Necrosis Determination:** Zebrafish (n=20) were exposed to selected compounds by static immersion from 6-96hpf at ½ LC<sub>50</sub>. Embryos were stained with acridine orange, washed, and imaged within 1 hour of staining. Quantification of fluorescence was accomplished using an inverse thresholding function and particle counting with ImageJ (NIH, Bethesda, MD). Images were analyzed for total fluorescent area above threshold in an oval area from a line posterior to the developing nasal pits to posterior of the developing ears. The medial edges of the developing eyes defined the borders of the analyzed area. Fluorescence in compound-treated zebrafish (n=5) was compared with control zebrafish treated with carrier (DMSO) alone. The average fluorescent area was determined and compared between compound treated and controls with a two-sample T-test assuming unequal variances.

**Motor Activity Assay:** Zebrafish (n=15) were exposed to selected compounds by static immersion from 6-144hpf at ½ LC<sub>50</sub>. After treatment zebrafish were washed and placed into a 96-well plate. After a 1 hour period of equilibration to the testing room (under constant light), zebrafish activity was recorded over a 1 hour period consisting of alternating 10 min photoperiods. The total distance moved each minute was recorded.

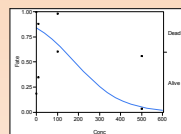
**Whole-Mount Neuron Immunostaining:** Albino zebrafish (n=10) were exposed to selected compounds by static immersion from 6-96hpf at ½ LC<sub>50</sub>. 0.1% DMSO treated zebrafish were used as controls. Axon tracts were visualized with fluorescently labeled anti-acetylated tubulin (Sigma). Motor neurons were visualized with fluorescently labeled anti-ZNF1 (Developmental Studies Hybridoma Bank, U. of Iowa). Catecholaminergic neurons were visualized using fluorescently labeled anti-tyrosine hydroxylase (Millipore/Chemicon).

## Results



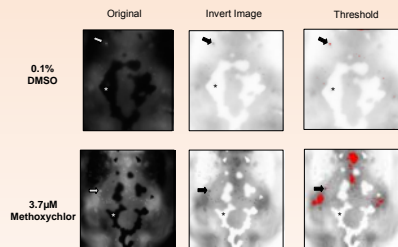
**Figure 1: Screening paradigm for environmental toxicants.** Compounds with an identifiable LC<sub>50</sub> between 1 and 500µM were both subjected to apoptosis/necrosis and motility assays. Compounds that tested positive to both assays were subject to immunostaining of motor neurons, axon tracts, and the catecholaminergic system.

### I. LC<sub>50</sub> Determination



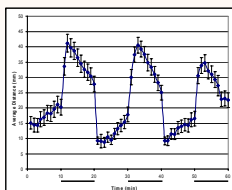
**Figure 2: LC<sub>50</sub> identification.** The LC<sub>50</sub> of a compound was identified through logistic regression, where the probability of a given fate (alleviated) is equal to the vertical distance underneath the blue line. In this example for p-cresol, the whole-model regression was significant (p<0.0001). Inverse prediction based on the regression equation for a fate of 0.5 gave a predicted LC<sub>50</sub> of 171.5µM.

### II. Apoptosis / Necrosis Assay

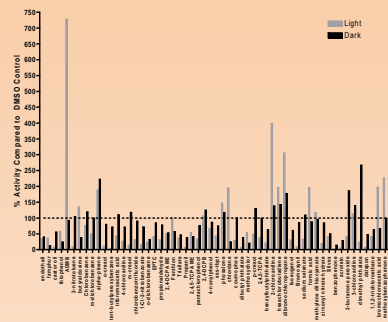


**Figure 3: Morphometric analysis of apoptosis/necrosis.** Zebrafish (n=5) were stained with acridine orange, and dorsal images of the brain (anterior at the top of the image) were acquired using a fluorescent scope with the same exposure time and gain. Images were inverted and thresholded using ImageJ software. Positive signals were defined by particle size (in pixels) and fluorescent intensity. Arrows indicate apoptotic/necrotic cells or tissue in the brain. An \*\* indicates the location of pigment bands that normally occur during development.

### III. Motility Assay



**Figure 4: Zebrafish motor activity under alternating photoperiods.** Day 6 zebrafish (n=200) were subjected to alternating periods of light and dark (indicated by black bars on the X-axis). The switch to no lighting resulted in increased motor activity, indicated by an increase in the mean distance traveled/minute. Error bars = ± 1 SEM.



**Figure 5: Normalized activity within each photoperiod.** The motor activity for each treatment is shown relative to 0.1% DMSO-treated controls. Compounds that increased activity will be greater than 100% (indicated by a dashed line), while those that decreased activity will be lower than 100%. For a compound to be classified as having a motor effect in our assay: 1) A two-sample T-test comparing the compound treated fish to controls in either photoperiod (light or dark) must determine the two populations are significantly different (p<0.05), and 2) the response of the compound treated fish must fall outside the value of the DMSO control ± ¼ the coefficient of variation of the DMSO control population.

### IV. Secondary Immunossays

	Catecholaminergic Neurons	Axon Tracts	Motor Neurons
DMSO (control)	Positive	Positive	Positive
Famphur	Negative	Negative	Negative
Dimethyl phthalate	Negative	Negative	Negative
Methoxychlor	Negative	Negative	Negative

**Figure 6: Neurotoxicity assessment by immunostaining.** 16x magnification images of catecholaminergic neurons (α-tyrosine hydroxylase), axon tracts (α-acetylated tubulin), and motor neurons (α-ZNF1), for some of the compounds studied. Dorsal images of catecholaminergic neurons (anterior at the top) and lateral images of axon tracts and motor neurons (anterior to the right) were acquired. Peripheral nervous system development appears less sensitive to environmental toxicants than central nervous system development (based on compound effects on catecholaminergic neurons).

**Table 1: Effects of selected compounds on secondary immunossay endpoints.**

Compound	Concentration (µM)	Catecholaminergic Neuron Toxicity	Axon Tract Toxicity	Motor Neuron Toxicity
DMSO (negative control)	0.1%	-	-	-
Famphur	27.45	+/-	-	-
Dimethyl phthalate	46.1	-	-	-
Methoxychlor	3.7	+	-	+/-
Cu Sulfate	5.2	+	+	+
Pentahydrate				
Tribromoacetic acid	9.5	+	+	+

**Table 2. Summary of Compound Effects on Zebrafish Developmental Neurotoxicity.** Of the 200 compounds assayed, 60 compounds had an identifiable LC<sub>50</sub> between 1 and 500µM. 9 of those compounds resulted in significant increases in brain apoptosis/necrosis, while 11 compounds resulted in motility defects. Three compounds were "hits" with an identifiable LC<sub>50</sub>, brain apoptosis/necrosis, and motility defects.

Compound Name	LC <sub>50</sub> (µM)	Brain Apoptosis/Necrosis Result	Motility Result
αPiracetam	147	Negative	Negative
Chlorobenzene	432	Negative	Negative
DiChlorobenzene	439	Negative	Negative
Benzylpyridinium	4.3	Negative	Negative
Tribromobenzene	6.8	Negative	Positive
4-Nonylphenol	2.5	Negative	Negative
2,4-Dichlorophenoxyacetic acid	2.8	Negative	Negative
Chloroform	3.9	Negative	Negative
Phenacetone	6.1	Negative	Negative
2,4,5-Trichlorophenoxyacetic acid	3.7	Negative	Negative
2,4,5-Trichlorophenoxyethanol	6.3	Negative	Negative
Methoxychlor	3.7	Positive	Positive
Diethyl phthalate	2.8	Negative	Negative
Chloroform	3.9	Negative	Negative
Tribromobenzene	6.8	Positive	Negative
Famphur	3	Negative	Negative
2,4-Dichlorophenoxyacetic acid, methyl ester	6.4	Negative	Negative
Phenacetone	6.1	Negative	Negative
2,4,5-Trichlorophenoxyacetic acid methyl ester	0.99	Negative	Negative
Propylol	15.1	Negative	Positive
Tribromoacetic acid	9.5	Negative	Negative
2-Hydroxy-4-methylphenylacetone	14.4	Negative	Negative
Diethyl phthalate	16.3	Negative	Positive
4-Chlorobenzonitrile	24.1	Negative	Negative
4-methylphenol (p-cresol)	171.5	Negative	Negative
3-methylphenol (m-cresol)	14.7	Negative	Negative
4-toluenesulfonic acid	69.1	Negative	Negative
tert-butyl peroxide	22.8	Negative	Negative
2-methylphenol (o-cresol)	178.8	Negative	Negative
1-methyl-2-methylbenzene	17	Negative	Negative
Bromoacetic acid	238.9	Negative	Negative
Diethyl Phthalate	92.2	Positive	Positive
Fluorobenzene	321.1	Negative	Negative
Chlorobenzene	432	Negative	Negative
1,2-Dichlorobenzene	113.8	Positive	Negative
Styrene	25.2	Negative	Positive
Benzophenone	46.8	Negative	Positive
acetone	5.4	Negative	Positive
2-chlorobenzene	165.9	Negative	Negative
Phenacetone-3-sulfonate	151.3	Negative	Negative
2-sulfonamide peroxide	14.4	Negative	Negative
Diazepam	296.3	Negative	Negative
1,2-Dibromo-3-chloropropane	25	Negative	Negative
Zincopal nitrate hydrate	256.6	Negative	Negative
butylacetone	17.8	Negative	Negative
Benzoic acid	349.8	Negative	Negative
methylene dibisocyanate	5.3	Negative	Negative
Thiourea	18	Negative	Positive
iodoacetic anhydride	206.2	Negative	Negative
2-chlorobenzene	144.1	Positive	Negative
4-Acetyl-1,2-dimethylbenzene	36.4	Positive	Negative
benzophenone	25.2	Negative	Negative
iodol oil	112.2	Negative	Negative
diethylphthalate	32	Negative	Negative
endosulfan	242.8	Negative	Negative
tricyclohexyl	2	Negative	Negative
o-dichlorobenzene	178.1	Negative	Negative
propionamide	93.7	Positive	Negative
benzofur	54.9	Positive	Positive
urea	233.8	Negative	Negative

## Conclusions

- Zebrafish are amenable to developmental toxicity assessment, in particular neurotoxicity.
- While the peripheral nervous system may not show evidence of malformation, endpoints in the central nervous system may be more sensitive indicators of toxicant exposure.
- Future research will refine secondary immunossays and continue screening compounds to categorize the extent of neuronal malformation observed during toxicant exposure.

## Literature cited

Claudio, L., W. C. Kwa, A. L. Russell and D. Wallace (2000). Testing Methods for Developmental Neurotoxicity of Environmental Chemicals. *Toxicology and Applied Pharmacology* 164: 1-14.  
 Parrig, C., W. L. Sang, C. Semino and P. Madigan (2002). Zebrafish: a practical model for drug screening. *Assay and Drug Development Technologies* 1: 41-48.  
 Schettler, T., J. Sten, F. Reich, M. Valeri and D. Walling (2000). Chemicals, Regulations and the Environment. In *Health, Man, Toxic, Research* (Ed. by D. Walling). Cambridge, MA, Elsevier Boston.  
 Physicians for Social Responsibility Chapter 7 103-116.  
 Zhang, C., T. Fremgen and C. Wilent (2003). Zebrafish: an animal model for toxicological studies. *Current Protocols in Toxicology*. John W Wiley & Sons, Inc., NY: Unit 1.7, Supplement 17.

## For further information

Please contact [louis.damico@phylonix.com](mailto:louis.damico@phylonix.com). All studies were conducted in accordance with institutional animal care protocols consistent with the AVMA's panel on euthanasia. Phylonix offers a wide range of zebrafish assays for drug/compound screening. Please see [www.phylonix.com](http://www.phylonix.com) for more information.

