

# Zebrafish: A Predictive Model For Assessing Developmental Neurotoxicity

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## 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 transparency, rapid development, and simplicity of chemical delivery. Previous studies at Phylonix demonstrated a high correlation between developmental neurotoxicity observed in zebrafish and findings in mammalian models (Ton et al. 2006. Birth Defects Research (Pt. A) 76: 553-567; Parg et al. 2005. Meths. Cell Biol. 76: 75-85). In these studies, we characterized brain apoptosis and tail motor neuron defects and correlated results with behavioral defects. To further establish the validity of a zebrafish model for assessing developmental toxicity, we recently surveyed 120 environmental toxicants selected from the Voluntary Children's Chemical Evaluation Program (VOCCEP) list and the EPA Emergency Planning and Community Right-to-Know Act (EPCRA). We assessed several quantitative parameters of neurotoxicity, including: 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. We identified several compounds that showed effects on one or more of the neurotoxicity parameters examined and some compounds showed broad toxicity across all neuroanatomical endpoints. We also identified compounds that increased or decreased motor activity in day 6 zebrafish. These results combined with our previous research show that the zebrafish model has predictive value as an alternative model for developmental neurotoxicity screening (Parg et al. 2007. J. Pharm. Tox. Meth. 55: 103-112). Adoption of zebrafish developmental neurotoxicity assays can speed characterization of environmental toxicants and streamline pre-clinical drug development. This work was supported by National Science Foundation SBIR award # 0548057.

## 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 (Parg 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 120 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

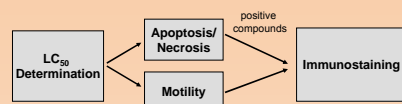
**LC<sub>50</sub> Determination:** Zebrafish (n=20) were exposed by static immersion from 6-96 hours post fertilization (hpf) at compound concentrations of 1, 10, 100, and 500 µM. Zebrafish treated with 0.1% DMSO were used as controls. Determination of LC<sub>50</sub> values was accomplished through logistic regression using JMP 7.0 (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 6-96hpf 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).

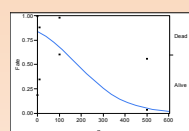
**Whole-Mount Neuron Immunostaining:** Albino zebrafish (n=10) were exposed to selected compounds by static immersion from 6-96hpf at ½ LC<sub>50</sub>. 0.1% DMSO treated zebrafish were used as controls. Axon tracts were visualized with fluorescently labeled anti-acetylated tubulin (Sigma). Motor neurons 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



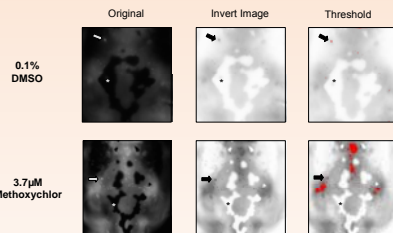
**Figure 1: Screening paradigm for environmental toxicants.** Compounds with an identifiable LC<sub>50</sub> between 1 and 500µM were both subjected to apoptosis/necrosis and motility assays. Compounds that tested positive to both assays were subject to immunostaining of motor neurons, axon tracts, and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

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



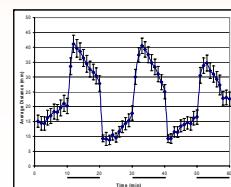
**Figure 2: LC<sub>50</sub> identification.** The LC<sub>50</sub> of a compound was identified through logistic regression, where the probability of a given fate (alive/dead) is equal to the vertical distance underneath the blue line. In this example for p-cresol, the whole-model regression was significant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

### II. Apoptosis / Necrosis Assay

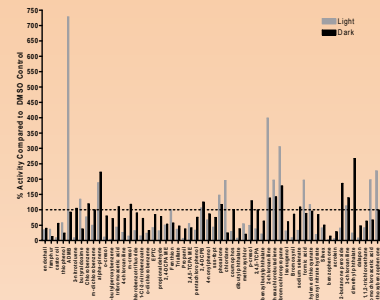


**Figure 3: Morphometric analysis of apoptosis/necrosis.** Zebrafish (n=5) were stained with acridine orange, and dorsal images of the brain (anterior at the top of the image) were acquired using a fluorescent scope with the same exposure time and gain. Images were inverted and thresholded using ImageJ 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

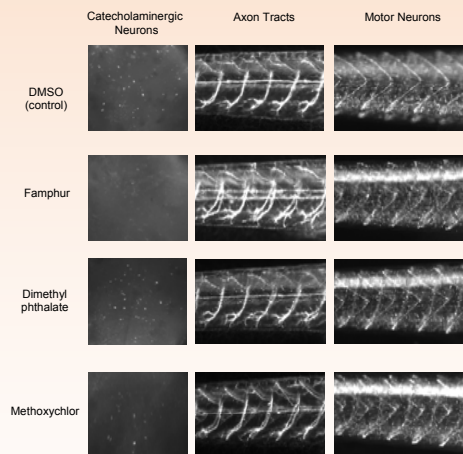


**Figure 4: Zebrafish motor activity under alternating photoperiods.** Day 6 zebrafish (n=200) were subjected to alternating periods of light and dark (indicated by black bars on the X-axis). The switch to no lighting resulted in increased motor activity, indicated by an increase in the mean distance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



**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 10 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%	-	-	-
Fampfur	27.45	+/-	-	-
Dimethyl phthalate	46.1	-	-	-
Methoxychlor	3.7	+	-	+/-
Cu Sulfate	5.2	+	+	+
Phthalaldehyde	54.5	+	-	+
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
α-Branine	147	Negative	Negative
Chlorobenzene	632	Negative	Negative
m-Dichlorobenzene	430	Negative	Negative
Benzylbutylphthalate	4.3	Negative	Negative
Chloroform	4.8	Negative	Positive*
n-Nonylphenol	2.6	Negative	Negative
2,4-Dichlorophenoxyacetic acid	2.8	Negative	Negative
Chloroform	17.8	Negative	Negative
Phthalone	2.7	Negative	Negative
2,4,5-Trichlorophenoxyacetic acid	3.7	Negative	Negative
S,S,S-Tributylphosphorotrithiate	5.3	Negative	Negative
Dimethylacetamide	7.4	Positive	Positive
Chloral hydrate	2.8	Negative	Negative
Coumaphos	12.2	Negative	Negative
Flutamide	4.9	Negative	Negative
Fenbuten	5	Negative	Negative
2,4-Dichlorophenoxyacetic acid, methyl ester	8.4	Negative	Negative
Paraldehyde	57	Negative	Negative
2,4,5-Trichlorophenoxyacetic acid, methyl ester	0.30	Negative	Negative
Propylal	15.1	Negative	Positive
Tribromoacetic acid	9.9	Negative	Negative
2-Ethoxy-α-methoxyacetophenone	14.9	Negative	Negative
Copper(II) sulfate pentahydrate	10.3	Negative	Positive*
4-Chlorobenzoic acid	34.1	Negative	Negative
4-Methylphenol (p-cresol)	171.8	Negative	Negative
3-Methylphenol (m-cresol)	14.7	Negative	Negative
4-Chloraniline	68.3	Negative	Negative
tert-Butyl mercaptopyruvate	22.8	Negative	Negative
2-methylphenol (o-cresol)	179.8	Negative	Negative
1-chloro-2-iodobenzene	17	Negative	Negative
Bromoacetic acid	238.9	Negative	Negative
Dimethyl phthalate	62.2	Positive	Positive
Epothilone	227.1	Negative	Negative
3-Chloroaniline	1.8	Negative	Negative
1,3,5-Trichlorobenzene	113.8	Positive	Negative
Silica	25.2	Negative	Positive
Benzophenone	48.3	Negative	Positive
Acetone	15.4	Negative	Positive
2-Chloroaniline	105.9	Negative	Positive
Hexachloro-1,3-sulfolane	151.3	Negative	Negative
2-Dimethylamino propanoic acid	14.4	Negative	Negative
Diazepam	298.3	Negative	Negative
1,2-dibromo-3-chloropropane	210	Negative	Negative
Zincory ribate hydrate	296.8	Negative	Negative
Hydroquinone	12	Negative	Negative
Formic acid	347.6	Negative	Negative
Methylene dichloride	53	Negative	Negative
Triphenylamine	15.9	Negative	Positive
Isobutyl methacrylate	206.2	Negative	Negative
2-nitrofluorene	144.1	Positive	Negative
4-Allyl-1,2-dimethylbenzamide	39.4	Positive	Negative
Hexachlorocyclopentadiene	29.3	Negative	Negative
Castor oil	17.2	Positive	Negative
Phthalaldehyde	52	Negative	Negative
Nicotinamide	242	Negative	Negative
Isomonyl	2	Negative	Negative
2-Dibromobenzene	178.1	Negative	Negative
Diethylmalonate	46.5	Negative	Negative
Dantrolene	54.5	Positive	Negative
Isic	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

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## For further information

Please contact 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 for more information.