West Virginia Chemical Spill:
Zebrafish Developmental Toxicity and Photomotor Response Studies

Materials and Methods

The zebrafish developmental toxicity and photomotor response studies use zebrafish embryos (*Danio rerio*) as a biological sensor to evaluate developmental endpoints for chemical hazard via multiple mechanisms of action.[1,2] These studies are conducted in physiologically intact organisms, and the embryos develop in a short window in which there is a high probability of detecting adverse outcomes such as developmental delays, morphological abnormalities, and behavioral alterations. Zebrafish is a highly prolific, small, complex organism that shares a highly conserved anatomy and physiology with all vertebrates.[3] Importantly, the critical processes of zebrafish neurodevelopment are homologous to those in humans.[4] Early in zebrafish embryogenesis, which is 19-29 hours post-fertilization (hpf), spontaneous tail contractions occur as the muscles in this region are innervated.[5] This spontaneous behavior at 24 hpf is sensitive to light perturbation via photoreceptors in the developing hindbrain and has been designated as the photomotor response (PMR).[6] The normal PMR at 24 hpf is sensitive to chemical perturbation and amenable to rapid screening for behavior-modifying compounds.[7] It has been shown that developmental mortality or morphology endpoints, combined with the PMR, serves as a robust biological sensor for chemical hazard potential.[1]

Methods

Zebrafish. Tropical 5D wild-type adult zebrafish were housed at an approximate density of 1000 per 100 gallons. Spawning funnels were placed into the tanks the night prior, and embryos were collected and staged. To increase bioavailability, the chorion was enzymatically removed using pronase (63.6 mg/ml, ≥ 3.5 U/mg) at 4 hpf using a custom automated dechorionator.

Chemical Preparation. All chemicals (Table 1) were obtained, prepared, and coded. The chemical code was only broken once all data were evaluated including statistical analyses. Chemicals were provided at 40 mM in 150 uL of DMSO and in 96-well plates. Upon arrival, chemicals were diluted to 20 mM and stored at -20°C in amber HPLC vials. Samples were thawed 30 minutes prior to exposure.

Chemical Exposures. The 8-concentration curve and 96-well plate layout for the NTP compounds is shown in Table 1. The tests were completed twice: Round 1 and Round 2. In each round, three identical plates were run to obtain N = 36 animals (1 embryo exposed per well, 12 embryos exposed per concentration per plate, see Table 2). Because the dosimetry is unknown for these compounds, a broad

[1] Truong, L., et al., Multidimensional in vivo hazard assessment using zebrafish. Toxicol Sci, 2014. 137(1): 212-33.
[2] Knecht, A.L., et al., Comparative developmental toxicity of environmentally relevant oxygenated PAHs. Toxicol Appl Pharmacol, 2013. 271(2): 266-75.
[3] Howe, K., et al., The zebrafish reference genome sequence and its relationship to the human genome. Nature, 2013. 496(7446): 498-503.
[4] Tropepe, V. and H.L. Sive, Can zebrafish be used as a model to study the neurodevelopmental causes of autism? Genes Brain Behav, 2003. 2(5): 268-81.
[5] Kimmel, C.B., et al., Stages of Embryonic Development of the Zebrafish. Developmental Dynamics, 1995. 203: 253-310.
[6] Kokel, D., et al., Identification of nonvisual photomotor response cells in the vertebrate hindbrain. J Neurosci, 2013. **33**(9): 3834-43.
[7] Raftery, T.D., et al., High-content screening assay for identification of chemicals impacting spontaneous activity in zebrafish embryos. Environ Sci Technol, 2014. 48(1): 804-10.

dose range was selected. The top dose was set with the constraints of using no more than 1 percent dimethyl sulfoxide (DMSO), which is the maximum tolerable concentration of DMSO in developing zebrafish. Zebrafish embryos without the chorion were loaded 1 per well at 6 hpf into 100 µl of embryo medium in 96-well plates by an automated embryo placement system which ensured allocation to study groups was random. A digital dispenser was used to dispense the NTP compounds immediately after embryo placement. Picoliter-sized droplets of compounds carried in 100% DMSO vehicle were dispensed, using inkjet technology. The DMSO concentration was normalized to 0.64% in all wells. All testing conditions were identical across plates, chemicals, and testing days.

Table 1. Elk River Spill Chemicals Tested in Zebrafish

CASRN*	Compound Name	Notes
34885-03-5	4-Methycyclohexanemethanol (MCHM)	a
NA	Crude 4-Methycyclohexanemethanol (Crude MCHM)	b
770-35-4	Propylene glycol phenyl ether (PPH)	a
94-60-0	Dimethyl 1,4-cyclohexanedicarboxylate	a
51181-40-9	Methyl 4−methylcyclohexanecarboxylate (MMCHC)	a
98955-27-2	4−(Methoxymethyl)cyclohexanemethanol (MMCHM)	a
4331-54-8	4−Methylcyclohexanecarboxylic acid	a
2105-40-0	2−Methylcyclohexanemethanol (2MCHM)	a
105-08-8	1,4-Cyclohexanedimethanol	a
4169-04-4	Phenoxyisopropanol	c
114651-37-5	Cyclohexanemethanol, 4−[(ethenyloxy)methyl]−	c
498-81-7	Cyclohexanemethanol, alpha, alpha, 4-trimethyl-	c
NA	DOWANOL™ DiPPh	d

* CASRN = Chemical Abstracts Service Registry Number. [a]Major or minor constituent of the spilled liquid (a minor constituent is considered to be approximately 10% or less of the spilled material); [b]A commercial mixture containing >70% MCHM along with lesser amounts of five other chemicals; [c] Not a component of the spilled liquid, but included because the compound is structurally related to MCHM or PPH; [d]A proprietary commercial mixture of dipropylene glycol phenyl ether isomers.

Table 2. NTP Zebrafish Dose Curve and Sample Layout in 96-Well Plates

Concentration (µM) in Each Well of a 96-Well Plate											
100	100	100	100	100	100	100	100	100	100	100	100
83.7	83.7	83.7	83.7	83.7	83.7	83.7	83.7	83.7	83.7	83.7	83.7
67.3	67.3	67.3	67.3	67.3	67.3	67.3	67.3	67.3	67.3	67.3	67.3
51.0	51.0	51.0	51.0	51.0	51.0	51.0	51.0	51.0	51.0	51.0	51.0
34.7	34.7	34.7	34.7	34.7	34.7	34.7	34.7	34.7	34.7	34.7	34.7
18.3	18.3	18.3	18.3	18.3	18.3	18.3	18.3	18.3	18.3	18.3	18.3
2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

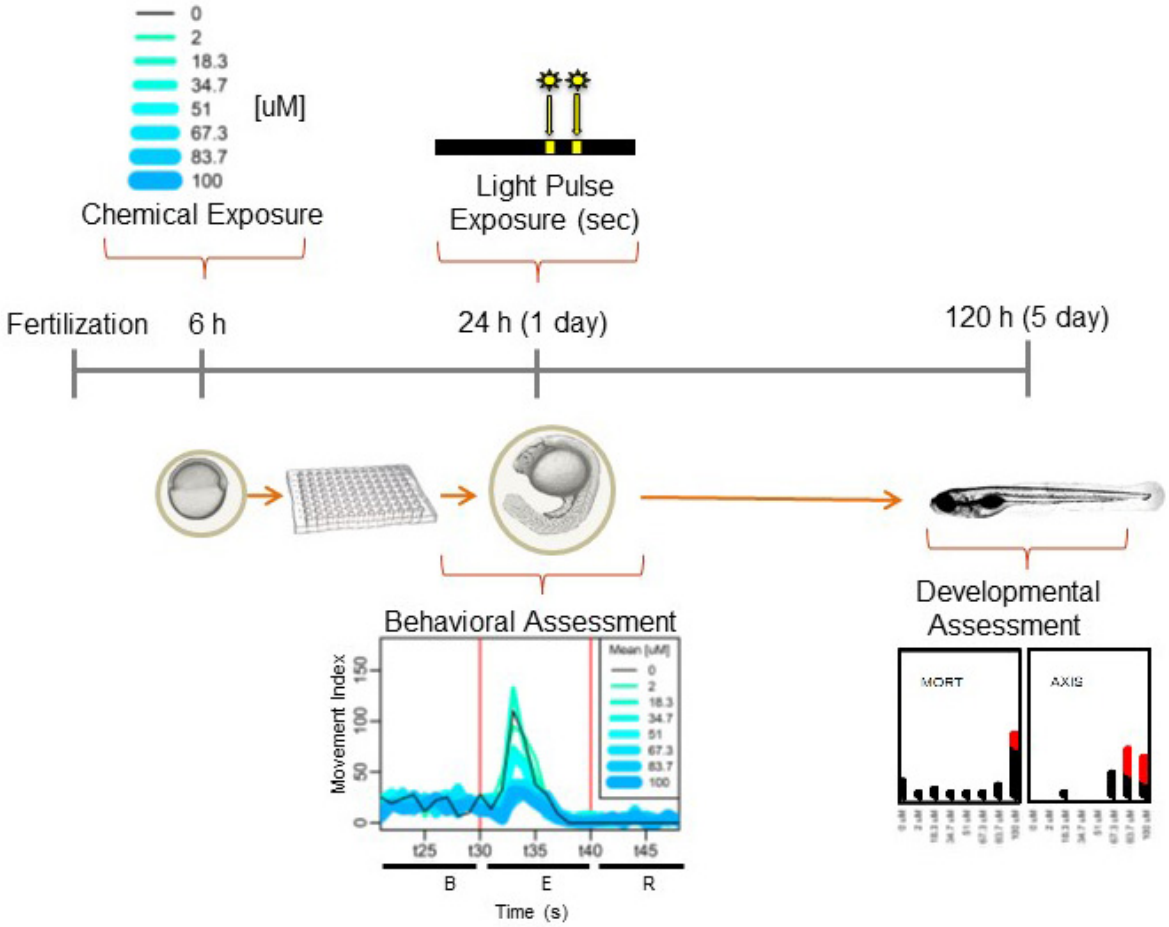

Figure 1[8]: At 6 hpf, concentrations of a given chemical were added to individual embryo wells in 96-well plates. At 24 hpf, plates were subjected to light pulse (at 30 s and 40 s) for the PMR assay. Movement was recorded during the 30 seconds of dark background, at and between the light pulses, and 10 seconds after the light pulse. The movement recordings were converted into the concentration–response profile shown under behavioral assessment. At 5 dpf, plates were assessed for specific developmental malformations, an example of which is shown under developmental assessment.

Zebrafish Photomotor Response Behavior. For experimental design, see Figure 1. At 24 hpf, embryos were assessed, in plate, for photomotor response. For every exposure plate, 850 frames of digital video were recorded at 17 frames per second from beneath a 96-well plate mount, and lighted from above with white LED and infrared lights. The light cycle consisted of 30 seconds of dark background (prior to the first light pulse), a short pulse of light, a second pulse of light at 9 seconds later, and then 10 more seconds of dark. Animals dead or malformed at the 24 hpf time point were excluded from the behavior data sets.

[8] Reif, D.M., et al. High-throughput characterization of chemical-associated embryonic behavioral changes predicts teratogenic outcomes. Arch Toxicol, 2015. Epub ahead of print.

Mortality and Morphology Responses. For experimental design, see Figure 1. Embryos were statically exposed until 120 hpf. At 24 hpf, embryos were assessed for 4 developmental toxicity endpoints; at 120 hpf, 18 developmental endpoints were assessed.[9] An internal quality assurance plate consisting of 48 control animals and 48 animals exposed to 0.2 uM Ziram was run each day that NTP samples were run as an internal check for response consistency in the animals from different hatches. For quality assurance, negative controls must exhibit less than 20% cumulative mortality and morbidity, and for the positive control, at least 80% of the exposed animals must display adverse effects.

Statistical Analysis.
The tests were completed twice as Round 1 and Round 2 and compared; however, the tests were not combined, statistically.

Morphology. All statistical analysis of the morphology endpoints was performed using code developed in R. The data used were binary incidences recorded for each endpoint.[10] To visualize this data, stacking observations are plotted, for each concentration and endpoint incidence. A significance threshold is computed for each chemical-endpoint pair compared to the background (control) incidence rate. The data for each endpoint is binary (0, 1) and recorded for each well, which translates to a series (n=32) of Bernoulli trials. Therefore, the significance threshold is estimated using a binomial test. The binary information was used to test for confounding plate, well, and chemical effects across all controls and to identify outliers. Among the controls (concentration = 0), there were no statistically significant effects by plate or well location. There were slight differences in control incidence by endpoint and chemical, which were accounted for in the analysis method described below. Outliers were defined as chemicals having an incidence rate greater than 3 standard deviations from the mean rate in controls across multiple endpoints.

To characterize responses for each chemical endpoint, the morphology data was analyzed using 2x2 contingency tables (Table 3).

Table 3. A 2 x 2 Contingency for Post-Hoc Comparison of Treatment Concentration Versus Control for a Given Endpoint

Concentration	Endpoint	! Endpoint
0 µM	m1	n1 - m1
X µM	m2	n2 – m2

In Table 3, X is the treatment concentration, m1 and m2 are the number of observed fish at each concentration, and n1 and n2 are the number of fish that presented the endpoint at each concentration. The Fisher's exact test was used because of its utility when there are low category counts. This test is proposed to be more appropriate than using the chi-squared test, which makes distributional assumptions. Multiple comparison was used to control the family-wise error rate. The testing of multiple concentrations were corrected for; however, the testing of multiple endpoints or chemicals were not corrected for. The family-wise error rate was controlled to limit the chance of a false positive. When testing one variable, the objective was to limit the probability of a false positive below some level *alpha*. For multiple concentrations, the error rate of the set of tests was $1-(1-alpha)^x$, where x was the number

[9] See Table 2 in the NTP June 2015 update for a full list of endpoints: http://ntp.niehs.nih.gov/ntp/research/areas/wvspill/zebrafish_update_june2015_508.pdf.
[10] Truong, L., et al., Multidimensional in vivo hazard assessment using zebrafish. Toxicol Sci, 2014. 137(1): 212-33.

of tests. Bonferroni was used to control the family-wise error rate by dividing alpha by x, so the error rate of the set of tests became $1-(1-alpha/x)^x \leq alpha$ for all $x \geq 1$.

Photomotor Response Behavior. For the PMR at 24 hpf study, the recorded periods at the beginning and end of the experiment (immediately surrounding the initiation and termination of camera recording) were truncated to assure equivalence in recorded experimental period for all chemicals. The statistical analysis[11] of activity considered only the Background (B), Excitatory (E), and Refractory (R) intervals. The overall pattern of activity within each B, E, or R interval was compared to that interval's negative control (0 ppm) activity using a combination of percent change (50% peak difference from control in the negative direction, and 75% in the positive direction) and a Kolmogorov-Smirnov test (Bonferroni-corrected p-value threshold = 0.05/8 concentrations = 0.01).

[11] Reif, D.M., et al. High-throughput characterization of chemical-associated embryonic behavioral changes predicts teratogenic outcomes. Arch Toxicol, 2015. Epub ahead of print.