**Report:** Zebrafish developmental toxicity profiles for UML solvents and solvent mixtures

**Conducted by:** Oregon State University, Sinnhuber Aquatic Research Laboratory (OSU/SARL)

**For:** Greg Morose, University of Massachusetts Lowell (UML)

**Date Assessed:** February, 2018 and May, 2018

**Assessment Types:** Embryo and larval mortality, morphology, embryo and larval photomotor response behavior, and larval startle response behavior

**Report Date:** February 27, 2018

**Addended Report Date:** June 1, 2018
**Purpose:** To assess the vertebrate developmental hazard potential of various common organic solvents and defined mixtures thereof.

**Experimental background:** These *in vivo* assays used the zebrafish embryo (*D. rerio*) as a biological sensor to evaluate a comprehensive battery of developmental endpoints for chemical hazard via multiple mechanisms of action [1, 2]. The developmental zebrafish assays 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 (roughly 19-29 hours post-fertilization, hpf), spontaneous tail flexions occur as the muscles in this region are innervated [5]. This spontaneous behavior at 22 hpf is sensitive to light perturbation via photoreceptors in the developing hindbrain and has been designated the embryo photomotor response (EPR) [6]. The EPR is an early, fast and sensitive assay to detect chemical perturbation of development. While the EPR readout is behavioral, later stage developmental defects predicted by an abnormal EPR are not restricted to behavioral outcomes but often include morphological deficits as well [7]. Neuronal effects of developmental chemical exposure on larval photomotor response (LPR) activity and behavior following an acoustic startle (LSR) can be easily measured in zebrafish, widening the potential field of bioactivity that we can detect [8-10]. We have found that developmental mortality and morphology endpoints, combined with the embryo and larval photomotor responses and startle response, serves as a robust biological sensor for chemical hazard potential.

**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 [5]. 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 [11].

*Chemical Preparation.* All February sample preparations were provided by UML as neat preparations of single compounds or as defined mixtures. The singular solvent samples were diluted in ultrapure water to a maximum test concentration of 80 µM. The solvent mixtures were diluted for testing such that the most abundant component was anchored to the 80 µM maximum concentration. The maximum test concentration of 80 µM is consistent with previous developing zebrafish assays conducted by the U.S. EPA to assess chemical hazard.[2] The May samples included re-use and testing of the Methyl acetate and DMSO preparations reported in February, and addition of 1,3-dioxolane (Sigma-Aldrich cat# 271020) supplied by
Oregon State University. The May testing included two samples: 1) 1,3-dioxolane as a single solvent and 2) a Methyl acetate/DMSO/1,3 Dioxolane mixture. We note that the 1,3-dioxolane preparation came from the manufacturer amended with 75 ppm butylated hydroxytoluene (BHT) as a stabilizer. To control for any potentially confounding effects of BHT bioactivity in the ensuing zebrafish assays, BHT was also tested alone for developmental effects at the highest concentration resulting from testing of the Methyl acetate/DMSO/Dioxolane mixture. We also included a 10 fold higher BHT test concentration in the unlikely event that the targeted concentration was at the threshold for bioactivity, rendering detection more prone to stochastic effects. All component test concentrations are shown in Table 1.

Chemical exposures. The 7-concentration curve was run in triplicate to obtain N = 36 animals per concentration; 1 embryo was exposed per well, 12 embryos exposed per concentration per plate. Zebrafish embryos without the chorion were loaded 1 per well at 6 hpf into 100 µl of embryo medium/test solvent solution in 96-well plates by two technicians using flame-polished Pasteur pipettes. The embryos were allocated first to the control row wells, then to the successively higher concentration rows to avoid carryover. The pipette was changed between each sample. Each 96 well plate was covered with a sheet of parafilm sandwiched between the plate and the plate lid. Thus, the parafilm sealed the plate en masse, but did not seal individual wells.

Table 1. Individual and mixture component tested concentrations.

<table>
<thead>
<tr>
<th>Sample Components</th>
<th>Single component µM</th>
<th>Mix component 1 µM</th>
<th>Mix component 2 µM</th>
<th>Mix component 3 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone</td>
<td>80, 10, 1, 0.1, 0.01, 0.001, 0</td>
<td>63.7, 8, 0.8, 0.08, 0.008, 0.0008, 0</td>
<td>27.8, 3.5, 0.35, 0.035, 0.0035, 0.0003, 0</td>
<td></td>
</tr>
<tr>
<td>Methyl acetate</td>
<td>80, 10, 1, 0.1, 0.01, 0.001, 0</td>
<td>59.7, 7.5, 0.75, 0.075, 0.0075, 0.0007, 0</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Thiophene</td>
<td>80, 10, 1, 0.1, 0.01, 0.001, 0</td>
<td>50.6, 6.3, 0.63, 0.063, 0.006, 0.0006, 0</td>
<td>NA</td>
<td></td>
</tr>
</tbody>
</table>

Butylated hydroxytoluene (BHT) 0.02µM, 0.002 µM

Mix components in order of decreasing abundance

Mix component 1 µM | Mix component 2 µM | Mix component 3 µM

Methyl acetate/DMSO/Thiophene (Sample 4) 80, 10, 1, 0.1, 0.01, 0.001, 0

Methyl acetate/DMSO (Sample F) 80, 10, 1, 0.1, 0.01, 0.001, 0

Acetone/Cyclohexane (Sample 18) 80, 10, 1, 0.1, 0.01, 0.001, 0
Embryo Photomotor Response behavior (EPR). At 22–24 hpf, embryos were assessed, in plate, for photomotor response using a custom photomotor response analysis tool (PRAT)[12]. For every exposure plate, 850 frames of digital video were recorded at 17 frames s⁻¹ from beneath a custom 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, and 9 seconds later, a 2nd pulse of light, and then 10 more seconds of dark. A representative wildtype EPR is shown in Figure 1. Animals dead or malformed at the 24 hpf timepoint were excluded from the behavior data sets.

Larval Photomotor Response behavior (LPR). At 120 hpf (5 days post fertilization) zebrafish are free swimming larvae and the photomotor response assayed total movement (swim distance) in response to multiple light -> dark transitions. Briefly, a Zebrabox behavior chamber (ViewPoint Life Sciences, Montreal, CA) with an infrared backlit stage was used to track total movement in 96 wells during a 24 minute assay. HD video was captured at 15 frames s⁻¹ and processed in real time by the manufacturer’s software. A representative wildtype
LPR is shown in Figure 2. Additional animals dead or malformed at the 120 hpf timepoint were excluded from the larval behavior data analysis.

*Larval Startle Response behavior (LSR).* The LSR assay was an audible tone (400 Hz) occurring 10 seconds after conclusion of the LPR assay (same instrument platform) with motion tracking commencing at the tone and for the following 10 seconds. The assay is performed in visible light. The speaker delivering the tone was physically coupled to the assay plate stage so that the acoustic energy was transmitted as directly as possible into particle motion of water rather than via air to water. A typical wildtype LSR behavior profile is shown in Figure 3. Analysis was similar to the LPR data.

*Mortality and morphology responses.* Embryos were statically exposed until 120 hpf. At 24 hpf, embryos were assessed for 4 developmental toxicity endpoints (MO24: mortality at 24 hpf, DP: developmental progression, SM: spontaneous movement, and NC: notochord distortion) [13]. At 120 hpf, 18 developmental endpoints were assessed [13]. The zebrafish acquisition and analysis program (ZAAP), a custom program designed to inventory, acquire, and manage zebrafish data, was used to collect developmental endpoints as either present or absent (i.e., binary responses were recorded). Evaluation of the morphology endpoints was conducted by solely 3 senior Tanguay lab staff persons (G. Gonnerman, M. Simonich and L. Truong) who are the most experienced evaluators in the lab. The Tanguay lab does not conduct formal, on-going measurement of inter-evaluator variability but indirectly checks this parameter by randomly assigning replicate plates to different evaluators and verifying inter-plate consistency at the time of data analysis. An internal QAQC plate consisting of 48 control animals and 48 animals exposed to 0.2 uM Ziram was run as an internal check for response consistency in the animals.
For quality assurance, negative controls exhibited less than 20% cumulative mortality and morbidity, and for the positive control, at least 80% of the Ziram-exposed animals displayed adverse effects.

**Statistical Analysis.**

*Embryo Photomotor Response (EPR).* The recorded periods at the beginning and end of the experiment (immediately surrounding the initiation/termination of camera recording) were truncated to assure equivalence in recorded experimental period for all chemicals. The statistical analysis 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 µg/ml] activity using a combination of percent change (50% peak difference from control in either direction) and a Kolmogorov-Smirnov test (Bonferroni-corrected p-value threshold = 0.05 /5 concentrations = 0.01).

*Larval Photomotor Response (LPR).* For the LPR assay, movement data from the 15 frames s⁻¹ capture was integrated into 6 sec bins and area under the curve for each treatment was statistically compared to the control movement by Kolmogorov-Smirnov test. The LPR at a given exposure concentrations was considered valid only when statistical significance (p < 0.05) was reached and the percent change in AUC was ≥ 50% above the control group AUC. Animals dead or malformed at the 120 hpf timepoint were excluded from the LPR data analysis.

*Larval Startle Response (LSR).* The LSR was statistically treated similar to the LPR data.

*Morphology.* All statistical analysis of the morphology endpoints was performed using code developed in R [14]. As described in Truong et al (2014), the data used were binary incidences recorded for each endpoint from ZAAP (as described above). 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 and is visualized on the plots where once the significant threshold is reach, the observations are indicated as red. 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. Slight differences in control incidence by endpoint and chemical were accounted for in our analysis method (described below). Outliers were defined as chemicals having an incidence rate greater than 3 SDs from the mean rate in controls across multiple endpoints.

To characterize responses for each chemical endpoint, we analyzed the morphology data using 2x2 contingency tables of the following form as an example:
Table 2. A 2 x 2 contingency for post-hoc comparison of treatment concentration vs. control for a given endpoint.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Endpoint</th>
<th>! Endpoint</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 µM</td>
<td>m1</td>
<td>n1 - m1</td>
</tr>
<tr>
<td>X µM</td>
<td>m2</td>
<td>n2 – m2</td>
</tr>
</tbody>
</table>

Here, X is the treatment concentration, m1/m2 are the number of observed fish at each concentration, and n1/n2 are the number of fish that presented the endpoint at each concentration. We chose to use Fisher's exact test because of its utility when there are low category counts. We believe this test 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 (FWER). We corrected for the testing of multiple concentrations but not for the testing of multiple endpoints or chemicals. The idea behind controlling the FWER was 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 of tests. We used Bonferroni to control the FWER by dividing alpha by x, so the error rate of the set of tests became 1-(1-alpha/x) ≤ alpha for all x ≥ 1. Statistical significance of endpoint incidence was reached where the stacked observations are shown as red in the plots.

Results

Embryo Photomotor Response behavior. The EPR is used as an early, fast and sensitive predictor of later, generalized, adverse outcomes, not restricted to behavioral endpoints. It is a valuable component in the capture of every practical metric of chemical bioactivity. The normal EPR pattern for wildtype zebrafish embryos at 22 – 24 hpf, based on thousands of observations in the Tanguay Laboratory, is a Baseline frequency of 0.1 – 0.2 flexions per sec.

Table 3. Significant EPR behavior responses to solvent exposures.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>conc. µM</th>
<th>Interval</th>
<th>P</th>
<th>Delta</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone</td>
<td>0.1</td>
<td>Excitatory</td>
<td>&lt;0.0001</td>
<td>1.125</td>
<td>HYPER</td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>0.01</td>
<td>Excitatory</td>
<td>&lt;0.0001</td>
<td>0.866</td>
<td>HYPER</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>Excitatory</td>
<td>&lt;0.0001</td>
<td>0.759</td>
<td>HYPER</td>
</tr>
<tr>
<td>DMSO</td>
<td>0.01</td>
<td>Excitatory</td>
<td>&lt;0.0001</td>
<td>0.759</td>
<td>HYPER</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>Background</td>
<td>&lt;0.0001</td>
<td>0.792</td>
<td>HYPER</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>Refractory</td>
<td>&lt;0.0001</td>
<td>1.193</td>
<td>HYPER</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>Refractory</td>
<td>&lt;0.0001</td>
<td>0.794</td>
<td>HYPER</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>Background</td>
<td>&lt;0.0001</td>
<td>0.758</td>
<td>HYPER</td>
</tr>
<tr>
<td>Methyl Acetate</td>
<td>1</td>
<td>Background</td>
<td>&lt;0.0001</td>
<td>0.821</td>
<td>HYPER</td>
</tr>
<tr>
<td>Methyl Acetate Cyclohexane</td>
<td>0.1</td>
<td>Refractory</td>
<td>&lt;0.0001</td>
<td>1.017</td>
<td>HYPER</td>
</tr>
<tr>
<td>(sample 33)</td>
<td>0.01</td>
<td>Refractory</td>
<td>&lt;0.0001</td>
<td>1.624</td>
<td>HYPER</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>Excitatory</td>
<td>&lt;0.0001</td>
<td>0.873</td>
<td>HYPER</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Excitatory</td>
<td>&lt;0.0001</td>
<td>0.865</td>
<td>HYPER</td>
</tr>
<tr>
<td>N-Methyl-2-pyrrolidone</td>
<td>0.1</td>
<td>Refractory</td>
<td>&lt;0.0001</td>
<td>1.180</td>
<td>HYPER</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>Excitatory</td>
<td>&lt;0.0001</td>
<td>0.794</td>
<td>HYPER</td>
</tr>
</tbody>
</table>
The Excitatory phase, commencing with the first light flash, normally has a frequency of 3 – 6 flexions per sec., generally not sustained for more than 4 sec. of the 10 sec period. The Refractory phase, commencing at the second light flash, and 10 seconds after the first, normally has a flexion frequency of 0/sec.

There were significant effects on the EPR behavior associated with 8 of the 12 solvent samples (Table 3). These effects were almost exclusively modest hyperactivity with one instance of strong hyperactivity (NMP, 80 uM, Refractory phase). With the exception of Cyclohexane, all of the single solvents were associated with EPR hyperactivity. Only one mixture, Methyl Acetate Cyclohexane PCBTF (sample 33), was associated with EPR hyperactivity. Acetone, Methyl acetate and sample 33 exposures were only associated with hyperactive EPR at the lower end of the concentration range. Exposures to Dichloromethane, N-Methyl-2-pyrrolidone and Thiophene were each associated with hyperactivity along non-monotonic concentrations (low and high, but not mid-range concentrations). DMSO and PCBTF exposures were associated with EPR effects over all or most of the concentration range. No interval of the EPR assay (Background, Excitatory or Refractory) was preferentially affected by noted concentration responses associated with these 8 samples.

No significant EPR effects were associated with exposure to 1,3-Dioxolane, the Methyl acetate/DMSO/1,3 Dioxolane mixture, or BHT.

Larval photomotor response behavior. The significant effects on LPR behavior associated with exposure to the solvents samples are shown in Table 4.

### Table 4. Significant larval photomotor response behaviors at 120 hpf.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>conc. µM</th>
<th>Interval</th>
<th>N¹</th>
<th>P</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dichloromethane</td>
<td>0.01</td>
<td>Dark</td>
<td>32</td>
<td>&lt;0.001</td>
<td>HYPER</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>Light</td>
<td>32</td>
<td>&lt;0.001</td>
<td>HYPER</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>Dark</td>
<td>34</td>
<td>&lt;0.001</td>
<td>HYPER</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>Light</td>
<td>34</td>
<td>&lt;0.001</td>
<td>HYPER</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Dark</td>
<td>34</td>
<td>&lt;0.001</td>
<td>HYPER</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>Dark</td>
<td>35</td>
<td>&lt;0.001</td>
<td>HYPER</td>
</tr>
</tbody>
</table>

¹ Number of initial animals was 36 per concentration. Animals dead or malformed at 5 dpf were excluded from the LPR assay.
As a brief background, the normal LPR pattern for wildtype zebrafish larvae, based on thousands of observations in the Tanguay Laboratory, is one of slow, short distance swimming activity in the lighted phases and 2.5 - 4 fold more swimming activity in the dark phases. Samples and concentrations omitted from the table were not associated with an LPR significantly different from the control animals. Graphic representation of the solvent-associated LPR behavior in the dark and light phases is shown in Figure 5.

The concentration-LPR response profile associated with exposure to Dichloromethane was somewhat non-monotonic with no abnormal LPR activity at the lowest (0.001 µM) concentration or 10 uM, but hyperactivity in the dark (active) phase at 0.01, 0.1, 1 and 80 uM. Light (quiescent) phase hyperactivity was associated with 0.01 and 0.1 µM dichloromethane, but not the higher concentrations.

The LPR profile for dichloromethane is shown in Figure 5 which also illustrates a somewhat erratic profile pattern relative to the canonical DMSO-LPR. DMSO, run herein as an experimental sample, is also a routine vehicle at 0.1% in most LPR data generated in the Tanguay Laboratory, thus we can attest to the canonical nature of that LPR profile. The erratic LPR profile was also associated with Methyl Acetate Cyclohexane PCBTF (sample 33) but without reaching a threshold for hyper- or hypoactivity relative to the control animals. None of the other solvent samples were associated with a erratic LPR profiles. We note that none of the solvent samples were associated with erratic EPR profiles at 24 hpf.

No significant LPR effects were associated with exposure to 1,3-Dioxolane, the Methyl acetate/DMSO/1,3 Dioxolane mixture, or BHT.

Larval startle response. Startle response is a relatively new assay in the developing zebrafish. An acoustic stimulus is transferred into particle motion of water detected by the larval zebrafish otoliths and mechanosensory cells of the lateral line. Thus, chemical disruption of these organs and related neuromotor systems, in otherwise normal appearing larvae, can manifest as an atypical startle response. A significantly altered startle response pattern was associated with exposure to 80 uM Cyclohexane (Figure 6). Solvent volatility associated effects on control animal LSR behavior were noted for Methyl Acetate Cyclohexane PCBTF (sample 33) and Dichloromethane, as was similarly noted in the LPR.

No significant LSR effects were associated with exposure to 1,3-Dioxolane, the Methyl acetate/DMSO/1,3 Dioxolane mixture, or BHT.

Mortality and morphology responses. Overt toxicity responses in a 22 endpoint battery that encompasses zebrafish development from 24 hpf – 120 hpf are summarized in Figure 7. The solvent exposures, including the May tests of 1,3-Dioxolane and the Methyl acetate/DMSO/1,3 Dioxolane mixture, were not associated with instances of overt toxicity. Note that had the incidence of any endpoint reached significance, those points would appear red in the Figure 7 graphs.
Conclusions
The solvents and mixtures thereof were largely developmentally benign at the concentrations and under test conditions used. We base this conclusion on the observation that modest but relatively abundant EPR effects were generally not associated with later stage adverse outcomes in the LPR, LSR and especially the overt toxicity endpoints. Abnormal mortality and morphology outcomes are not required for the detection of chemical hazard. However, given the frequency of abnormal (albeit modest) EPR outcomes, we would expect at least to see better correspondence between the EPR affecting solvents and those associated with abnormal LPR and LSR. The fact that we did not would suggest that the EPR results were largely transient. Whether this was due to actual non-toxicity or sample loss due to volatility, is not possible to conclude from this study design, except to note that obvious volatility effects on the control animals in each plate were only detected for 2 of the 12 samples. Our conclusions regarding volatility effects on the control wells are based on: 1) obvious solvent odor when the MA/CH/PCBTF plates and the DCM plates were opened for assay and 2) our previous experience with volatile compounds in our standard plate assays. When a sample-associated, irregular behavioral response, like the indicated erratic LPR and LSR here, is also apparent in the control animals, it is difficult to explain it any other way than volatility from the high concentration wells, likely owing to the elevated temp (28oC) during the entire exposure period. We note that we have not modeled well volatility, so can not offer robust support for sample volatility as the cause of the erratic responses.

The possible exception to a benign rating was Dichloromethane which, while associated with confounding volatility effects on the plate controls, was associated with consistent hyperactivity between the EPR and the LPR.

The additional May tests with 1,3-Dioxolane, the Methyl acetate/DMSO/1,3 Dioxolane mixture and controlling for potential confounding BHT effects, were not associated with bioactivity in the zebrafish model under the test conditions used.

References


Figure Legends

Figure 4. Larval photomotor response (LPR) behavior at 120 hpf associated with the test compounds. The free-swimming larval photomotor response was assayed over 3 cycles of 3 minutes visible light, 3 minutes dark (IR light) averaged together and displayed as a mean cycle response. Animals dead or malformed at the 120 hpf timepoint were excluded from the larval behavior data analysis.

Figure 5. Larval startle response (LSR) of singulated larvae in a 96 well plate to an audible (400 Hz) tone where the speaker is coupled to the plate stage. The LSR assay occurred 10 seconds after conclusion of the LPR assay on the same instrument platform with motion tracking commencing at the tone and for the following 10 seconds. The assay is performed in visible light.

Figure 6. Mortality and morphology endpoint incidence (binomial presence/absence data) across all 32 replicates were plotted as stacked points, one point per observation. Any points at or above the threshold for binomial significance are red. The control incidence of all morphological and touch response endpoints was below the 20% (<6 of 32 control animals affected) cutoff for biological validity.

Key to endpoints: [24 hpf endpoints] MO24, mortality at 24 hpf; DP24, developmental progress delayed at 24 hpf; SM24, absent tail flexions by visual check; NC24, notochord abnormal at 24 hpf. [120 hpf endpoints] MORT, mortality at 120 hpf; YSE, excessive fluid accumulation around yolk sac, AXIS, body axis curvature; EYE, edema, size, location or number abnormal; SNOU and JAW, snout and jaw visibly malformed; PE, excessive fluid accumulation around heart; OTIC, ear malformed; BRAI, brain absent or malformed; SOMI, somite trunk muscle organization abnormal; PFIN, pectoral fins absent or abnormal; CFIN, caudal fin underdeveloped or malformed; PIG, pigmentation hyper- or hypo-development of melanocytes; CIRC circulation visibly slower/faster or less developed; TRUN, trunk body length shorter than normal; SWIM, swim bladder not present or not inflated; NC, notochord curvy or otherwise abnormal; TR, touch response, gentle touch of head or tail region fails to elicit an escape response; Any except mortality, summation of all non-mortality endpoint incidences; Any effect, summation of all endpoint incidences.
Figure 4.

Volatility effect on control animals
Hyperactivity apparent but insignificant

Canonical light/dark activity profile

Volatility effect on control animals
Hyperactivity significant
Figure 5A.

**DMSO**

Canonical startle response

**Cyclohexane**

Hyperactive startle 80 uM

**Methyl Acetate Cyclohexane PCBTF**

Volatility effect on control animals
Figure 5B.

Dichloromethane
conc  0   0.01 = 1   10
      0.001  0.1   10

Volatility effect on control animals
Figure 6A.
Figure 6B.
Figure 6C.
Figure 6D.

Methyl Acetate DMSO (sample F)

Counts

Conc

Methyl Acetate DMSO Thiophene (sample 4)

Counts

Conc
Figure 6E.
Figure 6F.

Thiophene

Counts

Dichloromethane

Counts

Figure 6F.
### 1 3-Dioxolane

<table>
<thead>
<tr>
<th>Conc</th>
<th>Counts</th>
<th>Mortality @ 24hpf</th>
<th>Dev Progression @ 24hpf</th>
<th>Spontaneous Movement @ 24hpf</th>
<th>Notochord @ 24hpf</th>
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<td></td>
<td></td>
<td>total Mort</td>
<td>Yolk Sac Edema</td>
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<td>Pigmentation</td>
<td>Circulation</td>
<td>Trunk</td>
<td>Swim Bladder</td>
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<td></td>
<td>Notochord @ 120 hpf</td>
<td>Touch Response</td>
<td>Any Effect Except Mortality</td>
<td>Any Effect</td>
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### Methyl acetate DMSO 1 3-dioxolane

<table>
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<th>Counts</th>
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<th>Dev Progression @ 24hpf</th>
<th>Spontaneous Movement @ 24hpf</th>
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<td></td>
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<td>total Mort</td>
<td>Yolk Sac Edema</td>
<td>Axis</td>
<td>Eye</td>
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<td></td>
<td></td>
<td>Snout</td>
<td>Jaw</td>
<td>Otic</td>
<td>Percardial Edema</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Brain</td>
<td>Somite</td>
<td>Pectoral Fin</td>
<td>Caudal Fin</td>
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<tr>
<td></td>
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<td>Pigmentation</td>
<td>Circulation</td>
<td>Trunk</td>
<td>Swim Bladder</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Notochord @ 120 hpf</td>
<td>Touch Response</td>
<td>Any Effect Except Mortality</td>
<td>Any Effect</td>
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