West Virginia Chemical Spill:
Nematode (*Caenorhabditis elegans*) Toxicity Studies

Materials and Methods

The National Toxicology Program (NTP) evaluated the toxicity of 12 Elk River spill chemicals, structurally related chemicals, and related chemical mixtures (Table 1) to the nematode or roundworm *Caenorhabditis elegans* (*C. elegans*). These assays evaluated larval development, feeding, and reproduction.

Table 1. Elk River Spill Chemicals and Structurally Related Compounds Tested in *C. elegans* Assays

CASRN*	Compound Name	Notes
34885-03-5	4-Methylcyclohexanemethanol (MCHM)	a
51730-94-0	Dipropylene glycol phenyl ether (DiPPh)	a
770-35-4	Propylene glycol phenyl ether (PPH)	a
105-08-8	1,4-Cyclohexanedimethanol	a
98955-27-2	4-Methoxymethylcyclohexanemethanol	a
4331-54-8	4-Methylcyclohexanecarboxylic acid	b
114651-37-5	Cyclohexanemethanol, 4-[(ethenyloxy)methyl]-	b
498-81-7	Cyclohexanemethanol, alpha, alpha, 4-trimethyl-	b
94-60-0	Dimethyl 1,4-cyclohexanedicarboxylate	a
4169-04-4	Phenoxyisopropanol	b
NA	Crude 4-Methylcyclohexanemethanol (Crude MCHM)	c
NA	DOWANOL™ DiPPh	d

* CASRN = CAS Registry Number; [a]Major or minor constituent of the spilled liquid; [b]Not a component of the spilled liquid, but included because the compound is structurally related to MCHM or PPH; [c]A commercial mixture containing >70% MCHM along with lesser amounts of five other chemicals; [d]A proprietary commercial mixture of DiPPH isomers

Nematode Culture

Bristol N2 (wild-type) *C. elegans* (*Caenorhabditis* Genetic Center, Minneapolis, MN) were maintained at 20 °C on K-agar plates (2% bacto-agar, 0.25% bacto-peptone, 51 mM sodium chloride, 32 mM potassium chloride, 13 µM cholesterol) seeded with *E. coli* OP50 as the food source [Williams and Dusenbery 1988]. Age-synchronized cultures were prepared using a sodium hypochlorite solution (250 µM sodium hydroxide, 1% bleach), as previously described [Khanna et al. 1997]. After a final rinse with K-medium (51 mM sodium chloride, 32 mM potassium chloride), embryos were transferred to a complete K-medium solution (K-medium plus 3 mM calcium chloride, 3 mM magnesium sulfate, 13 µM cholesterol) [Boyd et al. 2009] to hatch and arrest as L1s for the growth assay, or were placed on K-agar plates with food and allowed to develop to the appropriate life stage for the reproduction and feeding assays.

Larval Development, Feeding, and Reproduction Assays

Larval development and growth, feeding, and reproduction assays were performed as previously described using the COPAS Biosort (Union Biometrica Inc., Somerville, MA, USA) [Rice et al. 2014]. Age-

synchronized nematodes were added to 96-well plates containing the test compound (Table 1), *E. coli* food source, and either K-medium (reproduction and feeding) or complete K-medium (larval development and growth). All chemicals were received as 20 mM stock solutions in 100% DMSO and stored at 4 °C. The chemicals were tested up to the maximum DMSO concentration (1%) that does not affect the *C. elegans* endpoints, which resulted in maximum test concentrations of 20 µM.

To assess larval development and growth, 50 synchronized L1 larvae were placed in each well of a 96-well plate and incubated in the presence of test chemical for 48 h at 20 ºC. Negative control animals developed to the final L4 larval stage during incubation. After exposures, nematodes were aspirated using the COPAS ReFlx option where two size characteristics of individual nematodes, the length or 'time of flight' (TOF) and optical density or 'extinction' (EXT) were measured [Smith et al. 2009].

To assay reproduction, 5 L4 larvae were placed into each well of a 96-well plate and allowed to grow and lay embryos in the presence of test chemical for 48 h at 20 ºC. The total numbers of adults and offspring were then quantified using the COPAS Biosort [Boyd et al. 2010b].

For the feeding assay, 25 young adult nematodes (3 day old adults) were loaded into each well of a 96-well plate and incubated for 24 h at 20 ºC in the presence of test chemical. To measure feeding, 5 mL fluoresbrite polychromatic red 0.5-mm microspheres (Polysciences) diluted 20-fold in deionized water were added to each well. Nematodes were allowed to feed for 15 min, after which 5 mL of 170mM sodium azide was added to arrest feeding. Nematodes were collected, and the levels of red fluorescence in the nematode pharynx and intestine were measured [Boyd et al. 2007].

Data Analysis
All three assays were run; dichlorvos was used as positive control. Three independent replicates were conducted on different days for each chemical-assay combination.

Larval development assay

- Response variable was log(EXT), the log transformation of the light absorbed by each worm as it passed through a laser.
- Data were fit to a mixture of a lognormal distribution (for detritus) and a population distribution. The estimated domain of the population distribution was used to identify nematode observations.
- Observed well means were subtracted from the mean of the negative controls on the same plate, eliminating plate effects and calculating effect sizes. The effect sizes were used as statistical unit weighted by the count of nematodes in the well.
- Least Effective Concentrations (LEC) were found by comparing the sample of effect sizes at each concentration against the negative control effect sizes on that plate, using a weighted t-test with the adjustment for unequal variances. The Bonferroni multiple correction adjustment was used. LEC was defined as the lowest concentration with a significant difference between the treated and negative control group such that no insignificant difference was found at a higher concentration.
- Summary plots were created using box plots for the effect sizes at each concentration together with negative and positive controls.

Feeding assay

- Response variable was log(red fluorescent signal intensity) from the microspheres used in the assay.

- Observed well means were subtracted from the mean of the negative controls on the same plate, eliminating plate effects and calculating effect sizes. The effect sizes were used as statistical unit weighted by the count of nematodes in the well.
- Least Effective Concentrations (LEC) were found by comparing the sample of effect sizes at each concentration against the negative controls on that plate, using a weighted t-test with the adjustment for unequal variances. The Bonferoni multiple correction adjustment was used. LEC was defined as the lowest concentration with a significant difference between the treated and negative control group such that no insignificant difference was found at a higher concentration.
- Summary plots were created using box plots for the effect sizes at each concentration together with negative and positive controls.

Reproduction assay

- Response variable was the count of observations in each concentration group.
- Least Effective Concentrations (LEC) were found using all 3 replicates with Dunnett's test testing against the common control group. LEC was defined as the lowest concentration with a significant difference between the treated and negative control group such that no insignificant difference was found at a higher concentration.
- Summary plots were created using scatter plots for the observed counts at each concentration together with negative and positive controls.

References

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