West Virginia Chemical Spill: Zebrafish Developmental Toxicity Study
June 2015 NTP Update

Synopsis
The National Toxicology Program (NTP)[1] used zebrafish (*Danio rerio*) to evaluate the toxicity of four chemicals that were spilled into the West Virginia Elk River. This assessment evaluated effects on embryonic and larval development and behavior. With the exception of one minor spill constituent, all chemicals evaluated were inactive, including 4-methylcyclohexanemethanol (MCHM), the main component of the spilled liquid. NTP found in two independent studies that exposure of zebrafish embryos to dimethyl 1,4-cyclohexanedicarboxylate, a minor component of crude MCHM in the spilled liquid, resulted in developmental malformations, physical abnormalities, and mortality. This finding is in contrast to a previous study where there were no effects on survival or development of the offspring of pregnant rats administered dimethyl 1,4-cyclohexanedicarboxylate.

In addition to MCHM, the chemicals tested included propylene glycol phenyl ether (PPH), dimethyl 1,4-cyclohexanedicarboxylate, and 1,4-cyclohexanedimethanol, all minor components of the spilled liquid; two chemicals related in structure to MCHM or PPH; and a commercial mixture "crude MCHM" containing primarily MCHM and smaller amounts of other chemicals.

Zebrafish Developmental Toxicity Study

Background on Zebrafish
Zebrafish is a small, tropical, freshwater fish (*Danio rerio*) belonging to the minnow family that has been used extensively in biological research. It is useful for evaluating the effects of chemicals on developmental outcomes. It is a vertebrate, has a short life cycle, detailed information is available on its genetic code, and its embryonic development is similar to humans; i.e., zebrafish undergo many of the same morphological steps in embryonic development as humans and these steps are controlled by similar genes. In the studies described here, physical development, growth, and behavior were evaluated during the embryonic and larval period, which occurs over a period of five days.

Chemicals Tested in the Study
Zebrafish embryos were collected after fertilization and the chorion, or outer membrane surrounding the embryo, was removed. Six hours post fertilization (hpf), seven concentrations (2, 18, 35, 51, 67, 83, and 100 μM) of the pure chemicals or the mixture (see Table 1) were added to the liquid containing individual zebrafish embryos. The seven molar concentrations of 4-methylcyclohexanemethanol (MCHM) correspond approximately to 0.3, 2.3, 4.5, 6.5, 8.5, 10.6, and 12.8 ppm. At 24 and 120 hpf, the embryos were evaluated for specific endpoints related to embryonic and larval development and behavior (see Table 2).

[1] NTP is a federal, interagency program whose goal is to safeguard the public by identifying substances in the environment that may affect human health. NTP is headquartered at the National Institute of Environmental Health Sciences, which is part of the National Institutes of Health. For more information about NTP and its programs, visit http://ntp.niehs.nih.gov/.

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
105-08-8	1,4-Cyclohexanedimethanol	a
4169-04-4	Phenoxyisopropanol	c
498-81-7	Cyclohexanemethanol, alpha, alpha, 4-trimethyl-	c

* CASRN = Chemical Abstracts Service Registry Number; [a]Major or minor constituent of the spilled liquid (a minor constituent is considered to be approximately 20% 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.

Table 2. Endpoints Evaluated in Zebrafish

Endpoints Evaluated 24 Hours Post Fertilization (hpf)	
Endpoint	**Evidence of an adverse effect**
Mortality	Fish dies
Spontaneous movement	Fish exhibits no spontaneous movement
Progression of Development	Fish exhibits delayed development
Notochord	Notochord (embryonic anatomy structure that defines the center line of the body) is malformed (wavy notochord)
Endpoint Evaluated 120 Hours Post Fertilization (hpf)	
Endpoint	**Evidence of an adverse effect**
Mortality	Fish dies between 24 and 120 hpf
Axis	Axis (centerline of the body in larval fish) is curved or bent axis in either direction
Brain	Brain is malformed or tissue is dead
Caudal fin	Caudal fin is malformed or missing
Circulation	Fish exhibits reduced or no circulation or blood flow
Eye	Eyes are malformed, missing, or smaller/larger than normal
Heart	Fish has pericardial edema (fluid around the heart)
Jaw	Jaw is malformed
Otic	Auditory system is malformed or missing
Pectoral fin	Pectoral fin is malformed or missing
Pigmentation	Fish lacks pigmentation or is over pigmented
Snout	Snout is shortened or malformed
Somite	Somites (body sections) are malformed, disorganized, or missing
Swim bladder inflate	Swim bladder fails to inflate
Touch response	Fish is not responsive to touch
Trunk	Fish has short, malformed, or missing trunk
Yolk sac edema	Fish has fluid around the yolk sac

Study Findings

Chemicals from the spill were evaluated in two identical and independent studies. With the exception of one minor spill constituent, all chemicals evaluated were inactive in both studies, including MCHM, the main component of the spilled liquid.

In both studies, exposure of zebrafish to the highest three concentrations of dimethyl 1,4-cyclohexanedicarboxylate, a minor component (less than 1 percent) of the spilled liquid, caused increases in four types of malformations and physical abnormalities evaluated at 120 hpf, and also increased mortality at the highest concentration. The four developmental effects were a "curved or bent axis in either direction," "pericardial edema" (fluid around the heart), "pectoral fin is malformed or missing," and "yolk sac edema" (fluid around the yolk sac). In addition, an effect on "progression of development" was observed at 24 hpf in the second study, which was not observed in the first study. The lowest concentration that dimethyl 1,4-cyclohexanedicarboxylate produced effects was 67.3 µM (approximately 13 ppm). Thus, in these studies, dimethyl 1,4-cyclohexanedicarboxylate is considered to be toxic to developing zebrafish.

The finding that dimethyl 1,4-cyclohexanedicarboxylate is toxic to developing zebrafish does not by itself establish that it would cause similar effects in humans or in other established models used for assessing developmental toxicity (e.g., rats or mice). Thus, the significance to human health of the findings in developing zebrafish for this minor component of the spilled liquid is uncertain. Notably, a previous reproductive and developmental toxicity screening study of dimethyl 1,4-cyclohexanedicarboxylate found no effect on the offspring of pregnant rats administered doses greater than 1000 mg/kg/day (such doses are considered high in traditional, experimental animal toxicology studies).[2] While these results are not definitive, they do lessen the concern that exposure to dimethyl 1,4-cyclohexanedicarboxylate is a significant human developmental hazard. Many factors, including the amount and duration of exposure, differences in how the human body handles the chemical compared to other species, and whether the biological basis for the effect is similar between different species and humans, determine whether toxicity in animal studies would translate to similar effects in humans.

Next Steps

The first set of studies to evaluate the toxicity of the spilled chemicals in developing zebrafish is complete. Studies on additional spilled chemicals are being conducted, and their results will be reported separately. NTP will consider the findings reported here in any future, overall assessment of the spilled chemicals.

[2] http://apps.echa.europa.eu/registered/data/dossiers/DISS-d7f37488-f4c1-42a6-e044-00144f67d031/AGGR-3e1e46a8-2994-40f2-a42c-7de30b65c2ef_DISS-d7f37488-f4c1-42a6-e044-00144f67d031.html - AGGR-3e1e46a8-2994-40f2-a42c-7de30b65c2ef