

Southern Adventist University
KnowledgeExchange@Southern

Research in Biology

Biology Department

2017

4-Methylcyclohexanemethanol Acute Toxicity to Daphnia and Its Effects on General Esterase and Glutathione S-Transferase Activity in Fathead Minnows

Rachel Clark

Southern Adventist University, rachelnclark@southern.edu

Jared Lewis

Southern Adventist University, jaredl@southern.edu

Jeehoon Jung

Southern Adventist University

Benjamin Thornton

Southern Adventist University

Follow this and additional works at: https://knowledge.e.southern.edu/research_bio



Part of the [Toxicology Commons](#)

Recommended Citation

Clark, Rachel; Lewis, Jared; Jung, Jeehoon; and Thornton, Benjamin, "4-Methylcyclohexanemethanol Acute Toxicity to Daphnia and Its Effects on General Esterase and Glutathione S-Transferase Activity in Fathead Minnows" (2017). *Research in Biology*. 4.
https://knowledge.e.southern.edu/research_bio/4

This Article is brought to you for free and open access by the Biology Department at KnowledgeExchange@Southern. It has been accepted for inclusion in Research in Biology by an authorized administrator of KnowledgeExchange@Southern. For more information, please contact jspears@southern.edu.

**4-Methylcyclohexanemethanol Acute Toxicity to Daphnia and Its Effects on General Esterase and
Glutathione S-Transferase Activity in Fathead Minnows**

Jared Lewis, Rachel Clark*, Jeehoon Jung, Benjamin Thornton, Ph.D.

Department of Biology

Southern Adventist University, Collegedale, TN 37315.

*Corresponding author – rachelnclark@southern.edu, (813)-418-2111.

Acknowledgements

We thank the Department of Biology, Southern Adventist University (SAU), for the use of its facilities, and G. Suarez for assisting with lab work. We thank Drs. H. Odens, T. Trott, and A. Corbit for providing assistance in methods and procedures. Funding for this project was provided by an internal academic research grant from Southern Adventist University.

Abstract – In 2014, 10,000 gallons of crude MCHM spilled into the Elk River in West Virginia. Due to a paucity of information about the toxicity of this chemical prior to the spill, our research aimed to verify the published 48-h EC_{50} for *Daphnia magna* (Daphnia) examine the effects of MCHM on two enzyme detoxification systems, general esterase and glutathione S-transferase, in *Pimephales promelas* (Fathead Minnow). We used pure MCHM instead of the crude form used in Eastman’s toxicity tests because the composition of crude MCHM is variable. The EC_{50} we obtained for pure MCHM did not differ significantly from the EC_{50} provided by Eastman for the crude form. We exposed Fathead Minnows to sublethal environmentally-relevant concentrations of MCHM for 96 h prior to performing the enzyme assays. The experimental groups in these assays did not show significant difference from control groups. Although our results add to the toxicity information, there is still a concerning deficit in chronic toxicity testing because of possible prolonged human exposure following the spill.

Keywords

MCHM: 4-methylcyclohexanemethanol

NOEC: No observable effect concentration

GE: general esterase

GST: glutathione s-transferase

Introduction

Background

On 9 January 2014, roughly 10, 000 gallons of an organic chemical mixture spilled into the Elk River in West Virginia. The contaminants, originating from a leak at a facility owned by Freedom Industries, contained primarily MCHM and other components listed by Bailey (2014). This chemical mixture is largely hydrophobic, and its primary use is as a flotation agent in coal processing. Following the spill, the contaminated water was drawn up at the Kanawha Valley Water Treatment Plant at Charleston, West Virginia, and distributed to nine counties, affecting 300,000 residents (Rosen et al. 2014). The Centers for Disease Control and Prevention (CDC) initially released a

safety threshold of 1000 ppb (CDC 2014), which was corroborated by multiple sources (CDC 2014, Paustenbach et al. 2015, U.S. National Toxicology Program (NTP) 2016a). The West Virginia Test Assessment Project (WVTAP) independently advised that the threshold be lowered to 120 ppb (Rosen et al. 2014, Whelton et al. 2015), and the Executive Department of West Virginia lowered the threshold to 10 ppb because of the uncertainty (Stolze et al. 2015). Residents in affected areas were advised to avoid consumption of all local water until 18 January (Rosen et al. 2014). Data from Whelton et al. (2015) revealed that, between 18 January and 29 January, water concentrations of MCHM measured at some water distribution facilities remained higher than the threshold set by WVTAP.

Prior MCHM toxicity research – *Daphnia magna* Straus (*Daphnia*)

Very little was known about the toxicity of MCHM prior to the spill. The limited available information was from the safety data sheet (SDS) and a few basic toxicological tests, mainly provided by the manufacturer, Eastman Chemical Company. Several errors and conflicts regarding Eastman's SDSs provided reason for concern over the general deficit of knowledge on the toxicity of MCHM. For example, the NOEC for *Daphnia* found in the SDS, 1998 edition (Rosen et al. 2014), was 50 mg/L while in the 2011 edition, the NOEC was reported as 40 mg/L (Eastman Chemical Company 2011). In the 2016 SDS edition, the value was removed altogether (Eastman Chemical Company 2016). In an independent assessment, Dr. Andrew Whelton, who was also a project leader for the WVTAP (Rosen et al. 2014), exposed *Daphnia* to crude MCHM and found an NOEC of 6.25 mg/L (compared to Eastman's value of 40 or 50 mg/L) and an EC₅₀ of 50 mg/L (compared to Eastman's value of 98.1 mg/L) (Whelton 2014). As a second example of concern, the percent composition of 1,4-cyclohexanedimethanol (CHDM) in crude MCHM reported by Eastman (1-2%, Eastman Chemical Company 2011) has been disputed, finding 5.3% CHDM in samples taken from the holding tank at Freedom Industries (Bailey 2014). Since the Freedom Industries holding tank contained more than just crude MCHM (Rosen et al. 2014), we expect the actual percent composition of CHDM in crude MCHM to be greater than 5.3%. Due to these apparent discrepancies, we chose to repeat the toxicity tests performed by Eastman. Both Eastman and Whelton used crude MCHM for their toxicity tests, but because of the variations in crude MCHM composition, we chose to perform the tests using pure MCHM.

General esterase and glutathione s-transferase introduction

A comprehensive review of the available literature found that crude MCHM and its constituents were generally low to moderately toxic over acute and subchronic exposure periods (Paustenbach et. al. 2015). The review included references to toxicity studies performed by the NTP that found several physiological side effects of exposure to the chemicals. At the highest dose of crude MCHM (500 mg/kg/day), rats experienced an increase in liver weight; an increase in creatinine, albumin, and red blood cell volume, and a decrease in blood glucose and eosinophils. An increase in triglycerides in the blood was observed at a lower dose of crude MCHM (300 mg/kg/day). The triglyceride increase was also seen at the highest dose of pure MCHM (500 mg/kg/day), although the other effects associated with crude MCHM were not observed. The magnitude of all the changes seen was considered minor (NTP 2016b). However, the observations do raise questions concerning sub-lethal biological effects of the chemicals.

When the toxicology data of a chemical compound is lacking, often a broad exploration into the effects of the chemical on enzyme activity is initiated. From there, mechanisms of toxification can be demonstrated and the knowledge base increased for the compound. This can be demonstrated by several experiments with PCBs (polychlorinated biphenyls) where multiple activities of enzymes were observed after exposure. Tang (et al) found varying PCB concentrations in samples of soil around an e-waste facility and determined how these affected the activity of urease, phosphatase, and catalase found in soil bacteria. These activities were used as “indicators of the general activities of soil microorganisms” and possible “indicator(s) of the soil quality” (2014). In another paper, enzymes from two populations of seals from regions with varying levels of PCBs were compared to discover mechanisms of toxic effects. Activities of enzymes from Phase I and Phase II classes (including glutathione-s-transferase) were used to determine the route of metabolism of the PCB compounds (Routti et. al., 2008). Each of these studies demonstrates the common procedure of testing differing concentrations of a lesser-known compound (in this case, PCBs) against commonly found enzymes of detoxification to increase our knowledge of the toxicity of the compound. These concepts were applied to our study of MCHM with two different enzymes.

Examination of enzyme activity can lead to advances in the knowledge of the biological properties of MCHM. Any significant changes that may occur in the activity of enzymes can serve as biomarkers of exposure (Abass, 2014; Hodge et al, 2000). The enzymes used in this study are the first in a series of enzymes to be examined with MCHM. We examined the effects of pure MCHM on two enzyme detoxification systems, GE and GST, in an

environmentally relevant species, *Pimephales Promelas* Rafinesque (Fathead Minnow; West Virginia Department of Environmental Protection, 2017).

General esterases make up a broad spectrum of enzymes that use water to hydrolyze esters into carboxylic acid and alcohol. Some have high specificity and clear endogenous functions, (Kua et al. 2002), while others have very little molecular specificity and can catalyze hydrolysis for a wide range of molecules (Aldridge 1993). This is indicative of detoxification systems because it allows the enzymes to identify and attach to various xenobiotics. Glutathione S-transferases, likewise, make up a very broad spectrum of detoxifying enzymes. This enzyme system requires glutathione (GSH) and catalyzes the GSH sulfhydryl group's nucleophilic attack on an electrophilic substrate. Like GE, the enzymes have endogenous functions (Armstrong 1997, Eaton and Bammler 1999). As a detoxification system, GST has been shown to target a wide variety of substrates (Eaton and Bammler 1999).

Novy (2015) tested the effects of gene expression on *Daphnia* using the tank liquid from the chemical spill. They found that a 48-hour exposure at 3.7 ppm and 50 ppm, there was a significant difference in GST expression for the control and exposed groups, with the exposed groups expression upregulated. This suggests that there are some sublethal effects that can affect the activity of detoxification enzymes. While we recognize that we tested against different species and with pure MCHM instead of the tank liquid, this brings into question the effects on Fathead minnows. It is expected that an increase in gene expression will affect enzyme activity, and we expected to observe a difference in the GST activity of Fathead Minnows.

Objectives of Study

The first objective of this investigation was to determine the 48-hour EC_{50} of *Daphnia* to pure MCHM for comparison with the results from Eastman (2016) and Whelton (2014). The second objective was to determine if an exposure for 96 hours at maximum estimated concentrations (20 ppm) found in the Elk River following the spill (Ziemkiewicz 2014) would alter the enzyme activity for GE and GST for Fathead Minnows. Our purpose was to determine whether MCHM in the spill's concentrations would alter these enzyme's activity in Fathead Minnows.

Methods

Chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

MCHM toxicity to Daphnia

Culture. Daphnia (Sachs Systems Aquaculture, St. Augustine, FL) were kept at approximate concentrations of 150 Daphnia/L in Elendt M4 medium (Elendt 1990, Samel et. al. 1999) made from reverse osmosis (RO) water. The culture was maintained at a temperature of 20°C, pH of 7.0 to 8.5, and photoperiod of 16:8 h light:dark, according to EPA specifications (United States Environmental Protection Agency (US EPA) 2002). One fourth of the medium was siphoned off and replaced three times per week. Dissolved oxygen (DO) levels were measured before water changes and maintained at levels greater than 3 mg/L. Cultures were fed four to five times per week as needed (spirulina, chlorella, active baker's yeast; 1:1:1 by volume).

Exposure. In each experiment, Daphnia were exposed to pure MCHM (Tokyo Chemical Industry Co., Tokyo, Japan) for 48 h using 11 treatments (162, 142, 128, 116, 104, 92, 80, 66, 46, 23, 0 ppm). Each treatment contained 10 Daphnia in 200 mL of Elendt M4 medium and was performed in triplicate. Treatments were not aerated nor were Daphnia fed during exposure. After 48 h, effective response was measured by counting Daphnia that remained immobile after gentle disturbance. The experiment was repeated three times. Effective concentrations were calculated using probit analysis with the software POLO Plus (LeOra Software, Petaluma, CA). All glassware used in exposures was washed with 10% HCl, rinsed in RO water, and allowed to air dry before use.

Enzymatic effect of MCHM on Fathead Minnows

Culture. Fathead Minnows (Anderson Minnow Farm, Lonoke, AR) were held in a 100 L aquarium in RO water with excess of 100 ppm calcium carbonate at the following conditions: $21 \pm 2^\circ\text{C}$, photoperiod of 16:8 light:dark, DO level ≥ 5 ppm. Ammonia levels were mitigated with API Ammo-Lock and Ammo-Carb (Mars Fishcare North America, Inc., McLean, VA). Fathead Minnows were fed frozen brine shrimp twice per day. Water changes were performed as needed (Denny 1988, US EPA 2002).

Exposure. The exposures were performed in four 10 L aquaria with circulating water, using the same conditions as in the culture. Aquaria were rinsed with 10% HCl, acetone, and RO water before each exposure. Adult non-sexed Fathead Minnows (eight to ten individuals per treatment) were exposed to four concentrations of pure MCHM (20, 13, 6, 0 ppm) for 96 h, and each aquarium received 0.5 – 1.0 g frozen brine shrimp twice per day. After exposure, Fathead Minnows were sacrificed in a 250 mg/L solution of tricaine methane sulfonate, massed, measured, and

sexed. The viscera were extracted, flash frozen in liquid nitrogen, and stored at -80°C . Exposures were repeated in triplicate.

Protein quantification. Each protein sample was homogenized on ice in glass homogenization tubes (10 strokes for 45 seconds using an electric stirrer at 750 rpm). Five hundred microliters of homogenization buffer (pH 7.2, 100 mM sodium phosphate, 10% glycerol, 1 mM EDTA, 0.1 mM DTT in sodium phosphate, 1.0 mM PMSF in 2-methoxyethanol, 1.0 mM PTU in 2-methoxyethanol) was used for every 0.11 grams of tissue sample. The homogenate was transferred to a 1.5-mL microcentrifuge tube and centrifuged at 10 000 g for 10 min at 4°C . The supernatant was homogenized by hand for 30 strokes, centrifuged a second time under the same conditions, and quantified using Coomassie Plus Protein Assay Reagent Kit (Pierce Biotechnology, Rockford, IL) in Nunc MicroWell F-bottom 96-well microplates and with a Multiskan Go spectrophotometer (Thermo Fisher Scientific, Waltham, MA). After quantification, each protein sample was divided into two equal aliquots and stored at -80°C .

General esterase activity assay. One of the two aliquots for each protein sample was thawed on ice, diluted to 5 $\mu\text{g}/\text{ul}$ with 113 mM sodium phosphate buffer, pH 7.2, and incubated at 30°C for 5 min. The activity level for each sample was measured by adding 20 μl of the sample to each of three wells on a Nunc MicroWell F-bottom 96-well microplate. One additional well for each sample contained 20 μl sodium phosphate buffer as a control. Two hundred microliters of 1 mM para-nitrophenyl acetate (PNPA), in the same sodium phosphate buffer, was added to each well. After a 15-second medium shake, the rate of hydrolysis of PNPA was recorded by taking 60 readings at five second intervals ($\lambda = 405 \text{ nm}$). The slope (absorbance/time) was calculated from readings 20 – 36 and converted to $\mu\text{mol product}/\text{min}/\text{mg}$ using a molar extinction coefficient of 6.53/mM/cm and path length of 0.6135 cm.

Glutathione s-transferase activity assay. The second aliquot for each protein sample was thawed on ice and diluted to 1 $\mu\text{g}/\text{ul}$. A master mix, which included 850 μl of 113 mM sodium phosphate buffer, pH 7.2, 100 μl of 50 mM reduced glutathione (GSH), and 50 μl of the 1 $\mu\text{g}/\text{ul}$ protein solution, was prepared and incubated at 30°C for 5 min. The substrate, 40 mM 1-chloro-2,4-dinitrobenzene (CDNB), was kept at 30°C and remade daily. After incubation, 180 μl of the master mix was added to four wells of a Costar 3635 UV compatible microplate (Corning, Inc., Corning, NY). The microplate was shaken at medium speed in the spectrophotometer and incubated for 2 min at 30°C . Ten microliters of CDNB was added to three wells, and 10 μl of sodium phosphate buffer was added to the fourth well as a control. Four samples were run simultaneously on a plate. The plate was shaken at medium speed for 25 seconds, and the absorbance was recorded ($\lambda = 405 \text{ nm}$) for 30 readings. The slope (absorbance/time) was

calculated from readings 4 – 20 and was converted to $\mu\text{mol product}/\text{min}/\text{mg}$ using a molar extinction coefficient of $6.53/\text{mM}/\text{cm}$ and path length of 0.5756 cm .

Enzyme activity analysis. Significant differences in enzyme activity were assessed with multivariate factorial analysis and an ANOVA table using two different softwares Rcommander (R Foundation, Vienna, Austria) and SPSS (IBM, North Castle, NY).

Results

The observed percent immobilization for the exposure of *Daphnia* to MCHM for 48 h were used to create a dose response curve (Fig. 1). The EC_{10} , EC_{50} , and EC_{90} (with 95% Confidence Intervals) were calculated from that dose response curve and determined to be 56.0 ppm ($43.9 - 64.9$), 91.6 ppm ($83.1 - 99.3$), and 149.8 ppm ($134.2 - 177.9$), respectively.

General esterase activity (Fig. 2) and GST (Fig. 3) activity were not significantly different in exposure groups with respect to the control after 96-hour environmentally relevant exposure to MCHM for three trials. Activity between trials was significant for both GE, $F(2, 64) = 9.74$, $p < 0.001$, and GST, $F(2, 64) = 49.07$, $p < 0.001$.

Discussion

Daphnia toxicity tests

The EC_{50} published in Eastman Chemical Company's safety data sheet for crude MCHM (2016) falls within the range of the 95% confidence interval for the pure MCHM EC_{50} found by our study. Our results differ from those of Whelton (2014), who found crude MCHM to be more highly toxic than reported by Eastman. Novy (2015) experimented with pure MCHM, and found a EC_{50} of 84.3 ± 7.9 which is within our confidence interval. MCHM composition varies widely (e.g. 4-MCHM: 68-89%; 4-(methoxymethyl)cyclohexanemethanol: 4-22%; Eastman 2016). The variation in composition of crude MCHM may be the cause of the differing results.

We recognize that the effects of MCHM extended beyond the *Daphnia* immobilization endpoint used in our protocol. Abnormal behavior was often observed in individuals that were still motile. This may be an argument for a broader toxic effect than is established by the EC_{50} . Abnormal behavior in *daphnia* could lead to changes in

reproduction, changes in feeding habits, or changes in the interaction of their environment for the population. Further studies could observe differences in behavior related to MCHM to relate this toxicant to population effects.

Fathead Minnow enzyme activity analysis

Gene Expression Studies. Novy (2015) showed upregulation in GST gene expression after exposing *Daphnia* to the chemical spill liquid for 48 hours. However, we found that there was no difference between our controls and exposed fish for GST activity. One reason for this could be because we used pure MCHM instead of the multitude of concentrations found in the tank liquid. This may advocate that MCHM may not have been the toxic chemical in the chemical spill, and another component may have greater or an additive effect on detoxifying enzyme activity.

Confounding factors. In a study that focused on the effects of ammonia on aquatic macrophytes, researchers found that the activity of GST in those organisms was altered after a week-long exposure to varying concentrations of ammonia (Nimptsch and Pflugmacher 2007). We are uncertain whether those results can be applied to GST systems in other aquatic organisms. However, the possibility of finding a significant difference in GST activity of the Fathead Minnows may have been constrained by early complications with ammonia in the Fathead Minnow culture. Although the Fathead Minnows were maintained in conditions specified by the supplier, the large quantity of the fish kept together resulted in the accumulation of ammonia. In the study with aquatic macrophytes, the change in activity was unpredictable as well. Exposures having lower concentrations of ammonia showed decreased GST activity compared to controls while exposures having higher concentrations of ammonia showed increased GST activity compared to controls (Nimptsch and Pflugmacher 2007). As described in the methods, a detoxification agent was used in our cultures to address the problem of ammonia accumulation before the Fathead minnows were used in MCHM exposures. However, we recognize that ammonia may still have been a confounding factor, including in between trials.

An additional concern over whether the results accurately reflected MCHM's sub-lethal toxicity is the compound's poor water solubility. When added to water, the compound separates and forms a layer on top of the water. The method of MCHM exposure to Fathead minnows used in this experiment would be comparable to the aquatic exposure due to the spill in the Elk River. However, on an experimental basis, it is difficult to ensure every individual is exposed to identical quantities of the toxicant. A paper on aquatic toxicity testing recommends that poorly soluble substances be tested only up to the saturation limit because that is what limits aqueous exposure. Our

testing methodology was based on replicating environmental conditions and did not require us to establish the saturation limit for MCHM, but at least one source provided a water saturation limit for crude MCHM at 2000 mg/L (Ziemkowitz 2014). However, our observations suggested the saturation limit for pure MCHM was far below 2000 mg/L, indicated by our observation of distinct phases at MCHM concentrations approaching 200 mg/L. This makes us question the proposed saturation limit of 2000 mg/L for crude MCHM. Testing immiscible compounds above the saturation limit creates several other problems specific to our study, including *Daphnia* entrapment and blockage of fish gills (Weyman et al. 2012). These factors may have inhibited the determination of the intrinsic toxicity relating to the biochemical effects of the compound while still affecting the organisms externally. This concern has the most significance for the Fathead Minnow enzyme activity analysis where a low bioavailability may limit the enzymatic systems' access to the toxicant. However, no known sources used an alternative method of aquatic exposure or performed any research into the effect of MCHM on detoxification enzyme activity, in which bioavailability becomes more important than in acute toxicity tests.

Other concerns and possibilities for future research

A potential avenue of future research into the effects of MCHM on detoxification enzymes might be to examine the activity of the cytochrome P450 (CYP) enzyme family. These enzymes are involved in xenobiotic metabolism like general esterases and glutathione S-transferases (Ding and Kaminsky 2003). However, CYPs can hydroxylate alkanes in addition to having a hydrophobic substrate-binding site (Groves 2005). Because MCHM has a mostly hydrophobic alkane structure, it may be a suitable target for CYPs.

Even with a theoretically plausible enzyme that would target MCHM, the problem of low bioavailability should still be addressed. One alternative aquatic toxicity test is to use a water-sediment system that exposes organisms that live in the benthic zone. Insoluble or poorly soluble compounds present in aquatic environments above their saturation limit are likely to attach to solid particles in the water and precipitate out. This creates the possibility of accumulated toxicant concentration in the benthic zone and increased threat to bottom dwelling creatures (Weyman et. al. 2012).

One final concern that is particularly relevant to humans is the deficit in chronic toxicity testing for MCHM. Most known toxicity tests focus on acute to subchronic toxicity, but there is good reasoning to pursue chronic testing. The WVTAP reported that low concentrations of MCHM were released from the Kanawha Valley Water

Treatment Plant for over two months after the spill. This finding was corroborated by the persistent odor at residences affected by the spill (Rosen et al. 2014). In another independent assessment of the health effects of MCHM, Whelton et al. (2015) found that all houses passed the CDC's MCHM safety screening level of 1 ppm, but residents still experienced adverse health effects at lower concentrations. Interestingly, Whelton et al. (2015) also reported finding possible sex-dependent effects of MCHM exposure with females having an increased likelihood of experiencing certain side effects. Sex-dependent effects can be particularly concerning because of the implications to reproductive systems. Even so, such speculation along with established long-term exposure and the health effects at levels below the official safety level, the need for chronic testing should be apparent.

Disclosure of Conflicts of Interest

The authors declare that they have no conflict of interest.

Ethical Approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

Literature Cited

Abass KS (2014) A method for fast assessment of OP/CB exposure in the Japanese Quail (*Coturnix coturnix japonica*) using combined esterases enzyme activity as biomarkers. *Enzyme Res* 2014:1-15.

Aldridge WN (1993) The esterases: Perspectives and problems. *Chemico-Biological Interactions* 87(1):5-13.

Armstrong RN (1997) Structure, catalytic mechanism, and evolution of the glutathione transferases. *Chemical Res in Toxicology* 10(1):2-18.

Bailey T (2014) Direct Testimony of Joseph A. Cotruvo, Ph.D., BCES. Bucci Bailey & Javins, L.C.

<http://www.psc.state.wv.us/scripts/WebDocket/ViewDocument.cfm?CaseActivityID=411178&NotType=%27WebDocket%27>. Accessed 12 February 2015.

Centers for Disease Control and Prevention (2014) Summary report of short-term screening level calculation and analysis of available animal studies for MCHM. CDC.

<https://emergency.cdc.gov/chemical/MCHM/westvirginia2014/pdf/MCHM-Summary-Report.pdf>. Accessed 1 December, 2016

- Denny JS (1988) Guidelines for the culture of Fathead Minnows, *Pimephales promelas*, for use in toxicity tests. US Environmental Protection Agency, Environmental Research Laboratory, Duluth, MN
- Ding X and Kaminsky LS (2003) Human extrahepatic cytochromes P450: Function in xenobiotic metabolism and tissue-selective chemical toxicity in the respiratory and gastrointestinal tracts. *Annual Rev of Pharmacology and Toxicology* 43:149-173
- Eastman Chemical Company (2011) Safety Data Sheet: Crude MCHM. Eastman Chemical Company, Kingsport, TN
- Eastman Chemical Company (2016) Safety Data Sheet: Crude MCHM. Eastman Chemical Company, Kingsport, TN
- Eaton DL and TK Bammler (1999) Concise review of the glutathione s-transferases and their significance to toxicology. *Toxicological Sciences* 49:156-1-64
- Elendt B (1990) Selenium deficiency in crustacea. *Protoplasma* 154:25-33
- Groves JT (2005) Models and mechanisms of cytochrome P450 action. *In* Cytochrome P450: Structure, Mechanism, and Biochemistry. Kluwer Academic/Plenum Publishers, New York
- Hodge S, Longley M, Booth L, Heppelthwaite V, and O'Halloran K (2000) An evaluation of glutathione s-transferase activity in the tasmanian lacewing (*Micromus tasmaniae*) as a biomarker of organophosphate contamination. *Bull of Environmental Contamination and Toxicology* 65(1): 8-15.
- Kua J, Zhang Y, and McCammon JA (2002) Studying enzyme binding specificity in acetylcholinesterase using a combined molecular dynamics and multiple docking approach. *J of the Amer Chemical Soc* 124:8260-8267.
- Nimptsch J and Pflugmacher S (2007) Ammonia triggers the promotion of oxidative stress in the aquatic macrophyte *Myriophyllum mattogrossense*. *Chemosphere* 66:708-714.
- Novy CL (2015) Acute aquatic toxicity of the chemicals spilled into West Virginia's Elk River. Dissertation, University of South Alabama.
- Paustenbach DJ, Winans B, Novick RM, and Green SM (2015) The toxicity of crude 4-methylcyclohexanemethanol (MCHM): Review of experimental data and results of predictive models for its constituents and a putative metabolite. *Critical Rev in Toxicology* 45:1-55.

- Rosen JS, Whelton AJ, McGuire MJ, Clancy JL, Bartrand T, Eaton A, Patterson J, Dourson M, Nance P, and Adams C (2014) WV TAP Final Report. Corona Environmental Consulting, LLC.
<http://www.dhsem.wv.gov/wvtap/testresults/Documents/WV%20TAP%20Final%20Report.pdf>. Accessed 27 January, 2015.
- Routti H, Letcher RJ, Arukwe A, Van Bavel B, Yoccoz NG, Chu S, and Gabrielsen GW (2008) Biotransformation of PCBs in relation to phase I and phase II xenobiotic-metabolizing enzyme activities in ringed seals (*Phoca hispida*) from Svalbard and the Baltic Sea. *Environmental Science and Technology* 42:8952-8958.
- Samel A, Ziegenfuss M, Goulden CE, Banks S, and Baer KN (1999) Culturing and bioassay testing of *Daphnia magna* using Elendt M4, Elendt M7, and COMBO Media. *Ecotoxicol and Environmental Saf* 43:103-110.
- Stolze L, and Volpin F (2015) Modeling of the Elk River spill 2014. *Environmental Science and Pollut Res* 22:7980-7985.
- Tang X, Hashmi MZ, Long D, Chen L, Khan MI, and Shen C (2014) Influence of heavy metals and PCBs pollution on the enzyme activity and microbial community of paddy soils around an e-waste recycling workshop. *International J of Environmental Res and Public Health* 11:3118-3131.
- United States Environmental Protection Agency (US EPA) (2002) Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms. US EPA, Office of Water, Washington, DC
- U.S. National Toxicology Program (NTP) (2016) NTP research program on chemicals spilled into the Elk River in West Virginia final update. NTP.
http://ntp.niehs.nih.gov/ntp/research/areas/wvspill/wv_finalupdate_july2016_508. Accessed 29 November, 2016
- U.S. National Toxicology Program (NTP) (2016) West Virginia chemical spill: 5-day rat toxicogenomic studies July 2016 NTP update. NTP. http://ntp.niehs.nih.gov/ntp/research/areas/wvspill/tgmx_update_july2016_508.pdf. Accessed 29 November, 2016
- West Virginia Department of Environmental Protection (WVDEP). 2017. Fishes of West Virginia. WVDEP.
<http://www.dep.wv.gov/WWE/getinvolved/sos/Pages/Fishes.aspx>. Accessed September 3, 2017
- Weyman GS, Rufli H, Weltje L, Salinas ER, and Hamitou M (2012) Aquatic toxicity tests with substances that are poorly soluble in water and consequences for environmental risk assessment. *Environmental Toxicology and Chemistry* 31:1662-1669.

Whelton A (2014) New crude MCHM toxicity results presented at NACCHO 2014, Atlanta, Georgia. Whelton Group. <http://wheltongroup.org/?p=4503>. Accessed 25 January, 2015

Whelton AJ, McMillan L, Connell M, Kelley KM, Gill JP, White KD, Gupta R, Dey R, and Novy C (2015) Residential tap water contamination following the Freedom Industries chemical spill: Perceptions, water quality, and health impacts. *Environmental Science & Technology*, 49:813-823

Ziemkiewicz P (2014) Elk River chemical spill January 2014. West Virginia University. <http://wwri.org/wp-content/uploads/2014/03/Ziemkiewicz-Law-Forum-MCHM-24mar14.pdf>. Accessed 15 November, 2015

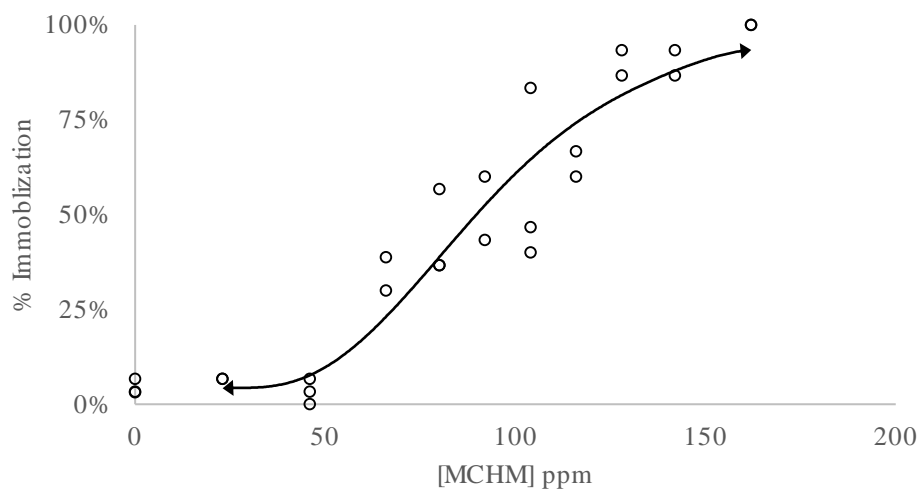


Fig. 1 Dose-response curve of adult *Daphnia magna* exposed to 4-methylcyclohexanemethanol for 48 h at 20°C in Elendt M4 medium; effective response - immobilization

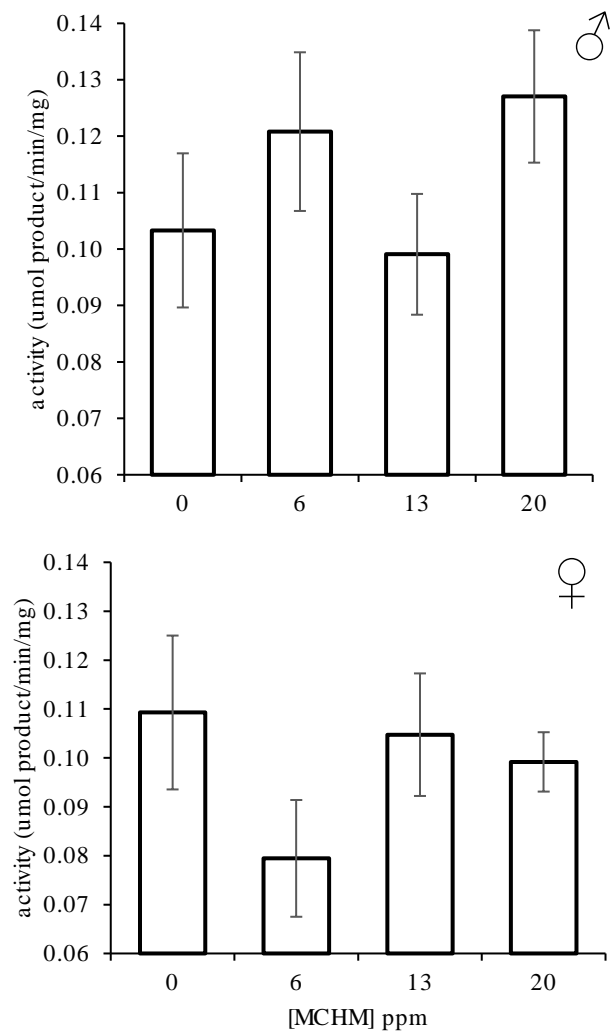


Fig. 2 General esterase activity in sexed adult *P. promelas* after 96-hr exposure to 4-methylcyclohexanemethanol (MCHM) in concentrations shown above; assay run at 30°C; activity was measured in 10 000 g supernatant from viscera homogenization with para-nitrophenyl acetate (PNPA) as a substrate; $\lambda = 405$ nm; standard error bars are shown; n value displayed within bar for each group

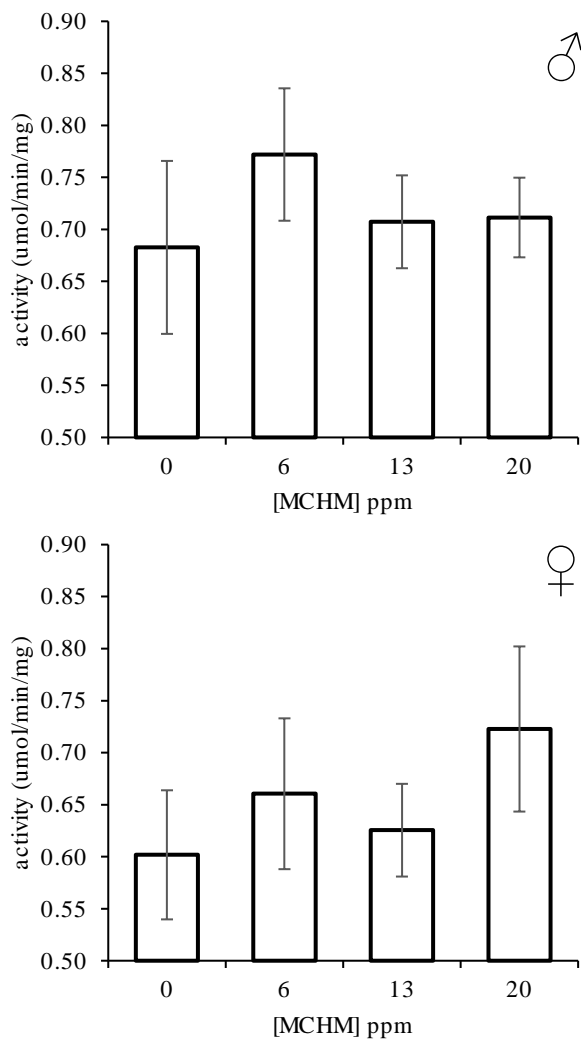


Fig. 3 Glutathione s-transferase activity in sexed adult *P. promelas* after 96-hr exposure to 4-methylcyclohexanemethanol (MCHM) in concentrations shown above; assay run at 30°C; activity was measured in 10 000 g supernatant from viscera homogenization with 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate; $\lambda = 340$ nm; standard error bars are shown; n value displayed within bar for each group.