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Effects of Environmentally Relevant Concentrations of 4-methyl-1-cyclohexanemethanol (MCHM) on General Esterase and Glutathione-S-Transferase Activity in Fathead Minnows (Pimephales promela)

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Abstract

In 2014, several thousand gallons of coal-processing chemicals, which included 4-methyl-1- cyclohexanemethanol (MCHM), spilled into the Elk River in West Virginia. The location of this spill in West Virginia's chemical valley is historically significant in defining the exploitations of people residing in this area. The concentration of crude MCHM in the river was approximated at 0.15 µg/L. Although some initial studies did consider the effects of MCHM exposure on humans, little attention has been given to aquatic wildlife. In this project, I will expose fathead minnows, *Pimephales promelas*, an environmentally relevant species, to concentrations of MCHM between zero and fifty ppm for 72 hours. General Esterase and Glutathione-*S*-Transferase enzyme activities will be measured using whole body protein extracts, and compared to the control groups. I expect to see a significant increase in the activity of both systems. Increased activity could indicate that the fish have exhibited a detoxification response, which has potential implications for the endogenous functions of these enzyme systems.

Historical Context

West Virginia has the lowest median annual household income of any U.S. state (Suneson, 2018). Currently, the coal industry in West Virginia is responsible for creating more in-state jobs than any other field, with the exception of transportation corporations (U.S. Bureau of Labor Statistics, 2021). Because its geographical location renders the state inherently rich in resources, both West Virginia's economy and cultural identity are centered around, if not largely dependent on, the production of coal (Blaacker, 2012; York, 2010). Although productive for the economy, this interdependency often results in the labor force itself being taken advantage of, as prominent coal mining companies manipulate the political grounds surrounding chemical

regulation (York, 2010). Resultingly, the threshold for acceptance of chemical contamination is dramatically increased. State required permits for modern coal mining practice do not limit toxic metal usage, and the subsequent water quality standards are significantly weaker than those stipulated by federal guidelines. Modern primary concerns include acid mine drainage and sedimentary run offs containing persistent toxic pollutants, which routinely enter rivers and streams (Coalition, 2003). Overall, the elasticity of modern chemical regulation in West Virginia can be explained by a shift in the ownership and purpose of residential land, which occurred in the late 19th and early 20th centuries.

Historically an overwhelmingly rural state, farming in early West Virginia played a central role in its cultural and economic development. In the late 19th century, the majority of West Virginian land usage was agricultural (Lewis, 1993). Although originally meant to feed their families and neighbors (proximal kin), farmers began raising more crops and livestock to support the population influx at the turn of the century. The number of cattle, sheep, swine, and poultry increased in an attempt to supply exportation efforts as well as native pantries. However, the resource limitations of the rocky Appalachian terrain numbered the days of West Virginia's agricultural progression. While farm sizes increased in the late 19th century, by the mid 1900s expansion shifted to match the presence of more sustainable resources; namely coal and timber. As the value of their land increased, so did the number of small farmers willing to sell (Lewis, 1998).

Although the land has always been rich in natural resources, the transfer of control of these resources from the agricultural to the industrial sector drastically influenced blue-collar residents (Lewis, 1993). Industrialization led to an increase in absentee landowning, as out-of-state investors began targeting coal resources for financial gain in the mid-1800s.

Consequentially, landowners began to care less about the ramifications of resource exploitation, as it often did not directly impact their daily lives. These investments proved their value rapidly, and the average farm size dramatically decreased around 1910 (Rasmussen, 1994). As farmers began to sell their land to the highest bidder, the demand for alternative sources of employment increased. Fortunately for the common man, the burgeoning coal industry was ready to receive him (York, 2010).

While a great number of West Virginian residents became and still are dependent on the coal industry for their jobs, they were no longer involved in the regulation of the process. Once able to control how their own farming practices affected their land, many in the working class sequentially found themselves employed by absentee landowners with private agendas (Lobao et al., 2016). As the industrial influx proved beneficial for the state's economy, governmental development progressed in support of big coal. Unfortunately, when it came to environmental regulation policy this meant that an extremely generous amount of power was given to private interest holders. As an increasing amount of decision-making power was allocated towards industry administrators, the influence of coal interests in the government became endemic (Perdue & Pavela, 2012). The predominantly rural state population no longer corresponded to an agriculturally based economy, leaving many who worked the land unaware of their ecological footprint. By the time modern science caught up to the significance of chemical contamination, the West Virginian government was already ingrained with competing interests (Carley, Evans, & Konisky, 2018).

The historical context surrounding West Virginia as a state surely plays a role in the continued lapse of strong regulatory influence in coal mining. In the modern era, the lackadaisical atmosphere surrounding chemical regulations has been fostered by the

disproportionate ratio of residential and absentee landownership, as political power favors the remaining primary employers (Rasmussen, 1994). Because there is less land available for residential interests, the primary and most secure employment opportunities for local West Virginians have shifted to industry, where they remain today. This in turn creates an effective cap on the earning capabilities for state residents, limiting their influence in policy decisions and keeping them confined to this local cycle of exploitation (Lewin, 2019; Perdue & Pavela, 2012).

The socioeconomic dichotomy created by the coal industry spreads beyond making millionaires of some and paupers of many. For generations, coal has provided food, shelter, education, and the hope of something more. However, the simultaneous contamination, destruction, and death have gone largely unnoticed (Burns, 2011). When compared to other U.S. state populations, West Virginians have one of the highest incidence rates for cancer and resulting mortalities (Hendryx, Fedorko, & Anesetti-Rothermel, 2010). Moreover, children of rural West Virginian residents in these coal mining communities have been reported to have significantly lower birth weights than the national average (Ahern, 2011). Another tangible representation of the modern consequences of this dichotomy can be seen in the Elk River spill of 2014 (McDonald, 2014). Although the deficit in regulatory legislation for coal mining practices indirectly promotes negligence like that seen in 2014, these incidences rarely effect the government officials or company administrators. As most government officials live in more urbanized areas removed from the direct effects of coal practice and many landowners live out of state entirely, it becomes easy to ignore even glaringly obvious implications (Perdue & Pavela, 2012). Instead, the rural working class often becomes the victim of chemical malpractice without even knowing it, further masking the significant need for reform (Coalition, 2003).

Introduction

Contamination of fresh water sources has many origins. Common sources of contamination for freshwater ecosystems include run offs from cattle lots (Sischo, Atwill, Lanyon, & George 2000), fertilizers (Bouchard, Williams, & Surampalli, 1992) and pesticides (Anderson, Opaluch, & Sullivan, 1985) from agricultural fields, pulp waste contamination from papermills (Chandra, Singh, & Raj, 2006), storm water run offs (Van Donsel, Geldreich, & Clarke, 1967), and industrial chemical spills (McGowan and Song, 2012).

On January 9, 2014, a storage tank containing 10,000 gallons of coal extraction chemicals ruptured and leaked into the Elk River outside of Charleston, West Virginia (Lan, Hu, Gao, Alshawabkeh, & Gu, 2015; Whelton, Mcmillan, L-R Novy, 2016; White, & Huang, 2016). The central chemical in this spill was 4-methyl-1-cyclohexanemethanol, abbreviated MCHM (Lan et al., 2015). The concentration of crude MCHM in the river was approximated at 0.15 µg/L (U.S. National Toxicology program, 2016). The spill was initially assessed by the CDC and U.S. National Toxicology Program, both of which focused primarily on the possible toxicological effects on humans (Rosen et al., 2014; U.S. National Toxicology Program, 2016). In humans, MCHM exposure caused oxidative stress and DNA degradation in lung tissues (Lan et al., 2015). Additional studies have been conducted assessing MCHM effects on mammalian species besides humans.

Toxic effects of MCHM in mammals have been documented. Consumption of various MCHM concentrations through drinking water in pregnant rats resulted in low birth weights, while direct injection of 1ppm MCHM resulted in lymph node swelling among mice (Johnson et al., 2017; U.S. National Toxicology Program, 2016). Although there is little information regarding MCHM effects in non-mammals, there is a report of alternations in swim patterns for

zebrafish larvae exposed to MCHM concentrations between 1ppm-100ppm (Horzmann, Freeman, de Perre, Lee, & Whelton, 2017). Overall, little is known regarding the toxicological effects of MCHM on aquatic biota, and Zebra Fish are not environmentally relevant to the MCHM spill of interest. Fish are model organisms for studying the toxicological outcomes of water contaminates because of their anatomy, but only specific fish species were present in the Elk River at the time of the spill (Adeyemi et al., 2013; Kroon, Streten, & Harries, 2017; Rodrigues, Antunes, Correia, & Nunes, 2015).

The Elk River in West Virginia is home to several well documented fish species, including the *Pimephales promelas,* commonly known as the Fathead Minnow (Hocutt, Denoncourt, & Stauffer, 1978; Blazer et al., 2007). Because of their environmental relevance, I chose to use the Fathead Minnow ("Fishes of West Virginia," 2018) to analyze the effects of MCHM on two enzyme systems; Glutathione-S-Transferase (GST) and General Esterase (GE). These respective enzyme families have many significant endogenous functions in organisms, and are also involved in detoxification. General Esterase and GST are known biomarkers of exposure for pesticide contamination, and changes in enzymatic activity have the potential to effect numerous biological activities which are potentially problematic to organisms (Domingues, Agra, Monaghan, Soares, & Nogueira, 2010).

Glutathione-S-Transferase encompasses a group of detoxification enzymes, which have shown enzymatic activity in all fish species tested thus far (Aksoy et al., 2016). The enzyme family is useful clinically for resisting environmental pollutants in the human body (Adeyemi et al., 2013; Aksoy et al., 2016; Kroon et al., 2017; Rodrigues et al., 2015). Moreover, GST possesses a number of endogenous functions involved in hormone synthesis and is able to degrade the amino acid tyrosine (Aksoy et al., 2016). Additionally, GST is genetically relevant

due to its involvement in transcription (Wiencke, Pemble, Ketterer, & Kelsey, 1995) and plays a role in the cellular metabolism of plants (Edwards, Dixon, & Walbot, 2000). The essential endogenous roles GST possesses are likened to General Esterases, which are similar detoxification enzymes that have critical endogenous functions (Goncharov et al., 2017).

General Esterase, in addition to its detoxification functions, aids in the process of gluconeogenesis (York, Ponder, & Majerus, 1995) as well as protects against organic toxins in insects (Alemayehu et al., 2017; Goncharov et al., 2017; Wang et al., 2015). Endogenously, GE plays a role in neurotransmitter processes and is able to detoxify lipid, phosphate, and carbamate containing compounds (Goncharov et al., 2017; Hamers et al., 2000; Zhou et al., 2004). Furthermore, GE is involved in essential metabolic functions in termites (Davis, Kamble, & Prabhakaran, 1995). Because of these differing endogenous functions, disruption of GE or GST could have significant negative effects on a variety of organisms.

Based on this information, I see relevancy in exposing fathead minnows to MCHM concentrations of 5, 10, 20, or 50 ppm for 72 hours in order to assess possible changes in activity levels of GST and/or GE enzymes. The potential implications of this study in relating to the endogenous effects of MCHM contamination could be widespread. Implications of changes in enzyme activity will be discussed in analyzing the results of this investigation.

Methods

Rearing

Fathead minnows (Southeastern Pond Management, Birmingham, AL) were kept in 38 L aquaria, with 25-30 fish per aquarium. Each tank was filled with tap water, which was dechlorinated using Aqueon water conditioner (Central Aquatics, Franklin, WI). Water temperature was maintained at 21 ± 2°C and all tanks were adequately aerated. Photoperiod was

established by ambient lighting and tank water was changed weekly. Fathead minnows were fed Deworming Green Flake (Angels Plus, Olean, NY); approximately no more than they could consume in five minutes. Fish remained in good health for a minimum of two months prior to exposures.

Exposures

Exposures were conducted in 12 L aquaria with identical conditions to the rearing tanks. Prior to and following each exposure, aquaria were disinfected using 70% ethanol and rinsed using tap water, deionized water, and distilled water, respectively. Pure MCHM concentrations for exposure were 50, 20, 10, 5, or 0 ppm. MCHM concentrations for exposures were mixed in volumetric flasks and stirred for 12 hours using magnetic stir bars. Each tank contained seven unsexed fathead minnows, which were exposed to one of the above concentrations for 72 hours, food withheld. Following exposure, fathead minnows were sacrificed using 0.01 M ethyl-3-aminobenzoate methanesulfonate. Each exposure was repeated four times. Whole fish samples were flash frozen using liquid nitrogen and stored at -80ºC until protein extraction approximately 2-7 days later.

Protein Preparation and Quantification

Frozen minnows were thawed on ice and their caudal fins and heads (starting directly behind the operculum) were removed before wet-massing. Each fish was homogenized on ice in a glass homogenization tube (30 strokes using an electric stirrer at 750 rpm) with 500 µL 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) added for every 0.11 grams of tissue sample. The homogenate was then relocated to a 1.5 mL microcentrifuge tube and centrifuged at 10,000 g for 10 min at 4°C. The

supernatant was then removed and placed in a new glass homogenization tube, and the homogenization process was repeated on ice for 30 strokes by hand. The homogenate was then centrifuged again under the same conditions and the supernatant was stored at -80ºC in a 1.5 mL microcentrifuge tube. Approximately 24-72 hours later, the protein samples were thawed on ice and quantified in Nunc MicroWell F-bottom 96-well microplates and with a Multiskan Go spectrophotometer (Thermo Fisher Scientific, Waltham, MA) using Coomassie Plus Protein Assay Reagent Kit (Pierce Biotechnology, Rockford, IL).

General Esterase Activity Assay

Protein samples were diluted to 5 μg/μL with 113 mM sodium phosphate buffer, pH 7.2, and incubated at 30°C for 5 min. General esterase activity levels were measured by adding 20 μL of the diluted sample to each of three wells on a Nunc MicroWell F-bottom 96-well microplate. Two hundred microliters of 1 mM para-nitrophenyl acetate (PNPA) were added to each well. Control wells contained 20 μL of the sodium phosphate buffer with 200 μL PNPA. The rate of hydrolysis of PNPA was recorded by taking 60 readings at two second intervals (λ = 405 nm), following shaking at medium speed (15 s). The slope (absorbance/time) was calculated from the readings and converted to specific activity (μmol of product/min/mg) using a molar extinction coefficient of 6.53/mM/cm and path length of 0.6135 cm.

Glutathione S Transferase Activity Assay

Using 850 μL of 113 mM sodium phosphate buffer, pH 7.2, 100 μL of 50 mM reduced glutathione (GSH), and 50 μL of the protein sample, a master mix was prepared and incubated at 30°C for 5 min. After incubation, 190 μL of the master mix was added to each of four wells of a Costar 3635 UV compatible microplate (Corning, Inc., Corning, NY). The microplate was then shaken for 25 seconds at medium speed in the Multiskan Go and incubated for 2 minutes at

30°C. Ten microliters of fresh substrate (40 mM 1-chloro-2,4-dinitrobenzene, CDNB; incubated at 30°C) were added to three wells and 10 μL of sodium phosphate buffer was added to the control. The plate was shaken a second time for 25 s at medium speed, and absorbance read (λ = 405 nm) for 30 readings. The slope (absorbance/time) was determined from readings 4 – 20 and converted to specific activity (μmol of product/min/mg) using a molar extinction coefficient of 6.53/mM/cm and path length of 0.5756 cm.

Analysis

An ANOVA test was performed to assess significant differences ($\alpha = 0.05$) in enzyme activity (R 3.4.1 with the package R Commander).

Results

I did not find a significant change in the specific activity levels of GE in fathead minnows exposed to MCHM, which is shown by an ANOVA, $F(4, 50) = 1.20$, $p = 0.323$. Because the ANOVA results were not significant, I did not run any post-hoc tests between concentrations.

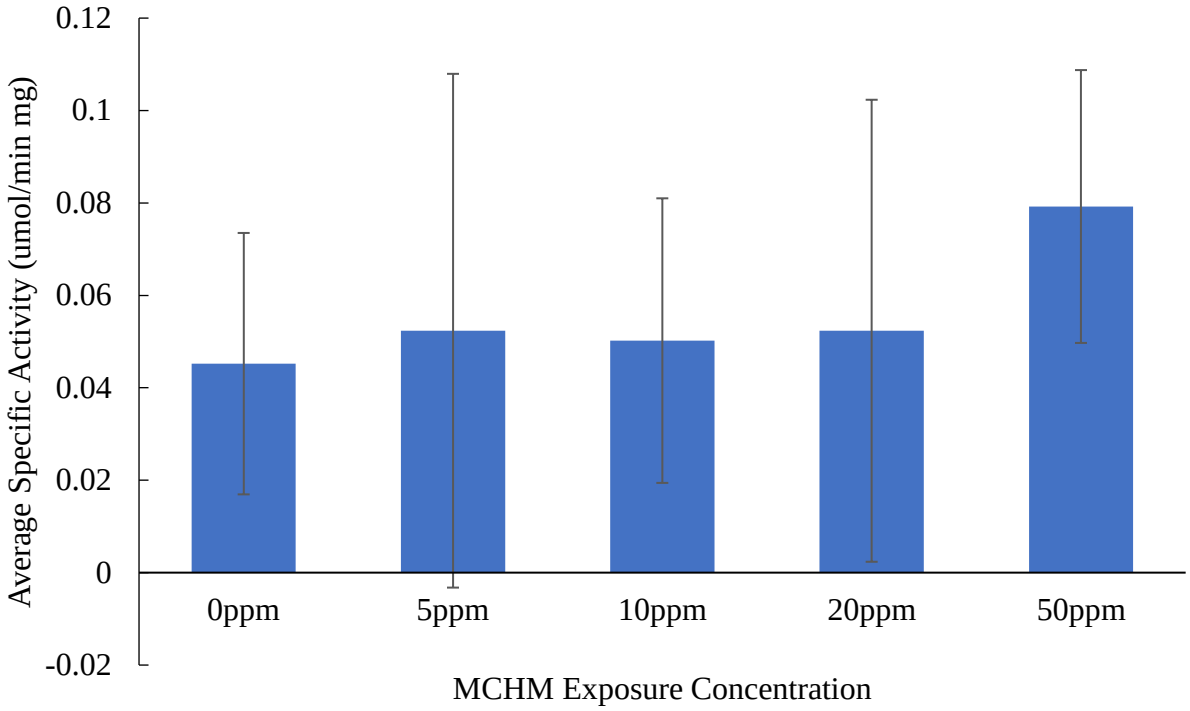

Figure 1. General esterase activity in tissue homogenate from fathead minnows, *Pimephales promelas*, exposed to various concentrations of MCHM for 72 hours; substrate – PNPA; absorbances read at 405 nm; $N = 56$; assay temperature 25° C. Bars represent standard error. The results of an ANOVA test did not show a significant difference between General Esterase activity levels and MCHM concentrations, $F(4, 50) = 1.20$, $p = 0.323$.

There was no significant increase in the specific activity of Glutathione-S-Transferase in fathead minnows exposed to MCHM as described by an ANOVA; $F(4, 49) = 1.36$, $p = 0.261$ (Figure 2). Post-hoc tests were not performed based on the lack of significance in the ANOVA.

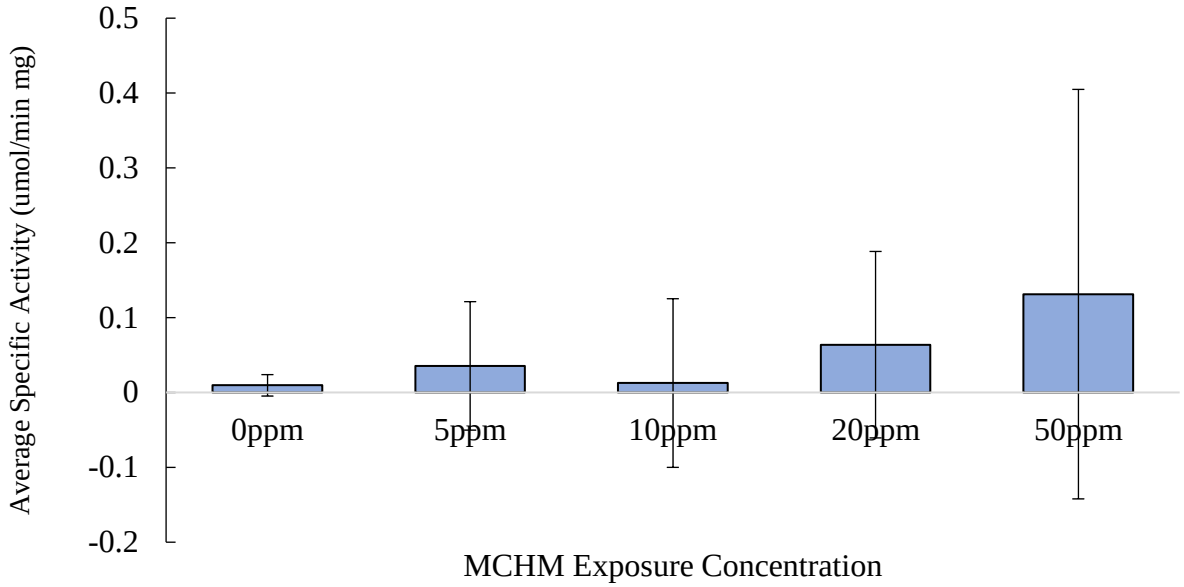

Figure 2. Glutathione-*s*-transferase activity in tissue homogenate from fathead minnows, *Pimephales promelas*, exposed to various concentrations of MCHM for 72 hours; substrate – CDNB; absorbances read at 405 nm; $N = 56$; assay temperature 25° C. Bars represent standard error. ANOVA test did not show a significant difference between Glutathione-S-Transferase activity levels and MCHM concentrations, $F(4, 49) = 1.36$, $p = 0.261$.

Discussion

Statistical analysis showed no significant differences between exposure groups for general esterase or glutathione-s-transferase. This is due to the high level of variance within each exposure group, even when obvious outliers are accounted for. There is some evidence of extreme esterase activity variation seen when testing fish species, such as salmon (Wheelock et al., 2005). Due to the global COVID-19 pandemic, I had to take a break in processing samples from March of 2020 until February of 2021, which contributed to a smaller total sample size than originally planned ($N = 56$). Repeating this study with larger sample sizes could be useful in ensuring the credibility of these results. Since the spill in 2014, subsequent studies analyzing the

toxicity of MCHM have been published that agree with this trend of low toxicity at relatively low concentrations (McMillan, 2015; Horzmann et al., 2017; U.S. Department of Health, 2020). As the primary focus of these studies has been risks associated with human exposure to MCHM, more examination of aquatic biota and other fish species could be useful.

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