Molecular Analysis of MCHM Toxicity in Zebrafish

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Molecular Analysis of MCHM Toxicity in Zebrafish

By

Cassandra Pelton

Under the mentorship of Dr. Vinoth Sittaramane

ABSTRACT

In early 2014 Freedom Industries, located in Charleston, WV, leaked approximately 10,000 gallons of 4-methylcyclohexane methanol into the Elk River. This river serviced approximately 300,000 people as a source of municipal water. Its effects on the people and surrounding wildlife is largely unknown.

Chemicals in surface runoff have the potential to induce aquatic animal trait changes, such as altered movement and feeding, that can ultimately alter predator-prey dynamics in aquatic systems. However, chemicals with high potential to enter aquatic systems, like 4-methylcyclohexane methanol (MCHM) used to clean coal, were tested using only short-term, single-species studies prior to use. Data from short-term MCHM exposures to zebrafish (*Danio rerio*) suggests that three-hour exposures to 1 ppm MCHM yield 50% reduction in zebrafish activity. However, cellular level changes in molecular toxicity is unknown.

The goal of this experiment was to determine how 4-Methylcyclohexanemethanol (MCHM) impacted the expression of hsp70, p450, and ahr2 genes in *Danio rerio* species. This was done by extracting RNA using Invitrogen TRIzol protocol. From there cDNA was synthesized and analyzed with qPCR to determine level of gene expression.

The results from this experiment show there was an increase in all 3 biomarkers at 0 ppm, 1 ppm, and 5 ppm compared to the housekeeping gene beta-actin. There was also an increase in all 3 biomarkers between week 1 and 3 of exposure compared to the housekeeping gene beta-actin.

Keywords: MCHM, toxicity, hsp70, ahr2, cytochrome p450, Zebrafish
1. Acknowledgements

I would like to thank the Georgia Southern University Honor’s Program to allow me to complete a capstone project. I would like to thank the Department of Biology for allowing me to use the qPCR machine. Most importantly, I would like to thank my mentor Dr. Vinoth Sittaramane for guiding me and allowing me to use his lab to complete this project.
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2. Introduction

2.1: Elk River Chemical Spill

On January 9th, 2014 approximately 10,000 gallons of chemicals were leaked into the Elk River in Charleston, West Virginia. Calls reporting a licorice smell immiting from Freedom Industries tank farm began at approximately 8:15 in the morning. Inspectors reported to the site and were informed by a Freedom Industries employee that there was no problem. However, after further investigation, a leak was discovered at tank 396. The employee that initially stated there was no problem did not call in the spill to the West Virginia Department of Environmental Protection until forced to do so just before noon. The inspectors were only then informed that the chemical spilling from tank 396 was crude 4-methylcyclohexane methanol (MCHM).

Around this same time a West Virginia American Water official was brought on site and began testing the water. Around 4 PM the tests came back stating the chemical in the water was crude MCHM. A do-not-use order was issued at 5:50 in the evening and the public was addressed around 6. The community had been using contaminated water all day and were very unhappy that they were informed so late in the day. People were posting comments like this one to social media stating their unhappiness as to how the situation was handled: “The leak happened when & they’re just now deciding this? Everyone in my house has already bathed in it today, including my 3 month old daughter. I’ve washed her bottles in it. My animals drank it. Ughhh. My babies better not get sick!”

The information that Freedom Industries was providing was not credible from the start. When the spill was originally detected officials were informed that the spill had not yet reached the Elk River, which was false. It was also revealed, almost two weeks later,
that MCHM was not the only chemical in that tank. Another chemical, containing two propylene glycol phenyl ethers (PPM), was not reported to have been in the tank even though employees were aware of its presence.

This chemical spill left 300,000 residents without clean water to drink or bathe in. In the weeks following 600 people went to the ER reporting symptoms they claimed were related to the spill. 13 people ended up being hospitalized. The spill had a financial effect on the city as well. Businesses of Charleston, WV lost approximately $61 million in the first month according to a study done by the Marshall University Center for Business Research.

This spill had such a huge impact on the people of Charleston because there was not much information known about the compound being leaked into the Elk River. At the time of the spill MCHM was not handled as a transportation hazard but the Safety Data Sheet said that it was a skin and eye irritant and harmful if ingested (Manuel). The current Safety Data Sheet states the same thing but adding that it is suspected to harm unborn children (Horzmann). Various studies on animals have been done to fill in the unknowns on the toxicity of crude MCHM. One such study, done by Purdue University, was performed on Danio rerio (zebrafish) species, the same model that was used in this study. This study analyzed the toxicity of crude MCHM, 4-MCHM, and the chemical solution found in tank 396 of Freedom Industries. Overall, crude MCHM and the tank mixture were more toxic in reference to hatch rates and mortality. 4-MCHM demonstrated changes in body morphology at as little as 1 ppm. Visual motor response assays demonstrated both hypoactivity and hyperactivity compared to controls (National Toxicology Program). The National Toxicology Program (NTP) also did some
toxicology studies on zebrafish, focusing mainly on photomotor responses. Of the many chemicals this study analyzed, 4-MCHM was one of the 3 chemicals that induced change in the zebrafish photomotor response. This is not necessarily indicative of any kind of neurological impairment (Light), but it is supported by the Purdue University study as well as unpublished studies done in this lab.

Another study, sponsored by the Eastman Chemical Company, analyzed the effect of crude MCHM on *Pimephales promaels* (fathead minnow) and found the lethal concentration 50 was approximately 57.4 mg/L and would be a “moderate concern level” according to the U.S. EPA criteria.

Though the Elk River chemical spill sparked a lot of interest in this compound not a lot is understood as to how it impacts ecosystems and human populations. The purpose of this study is to further analyze the toxicity of MCHM on the *Danio rerio* model using three biomarkers: hsp70, p450, and ahr2.

### 2.2 Hsp70

70-dKa heat shock proteins, or hsp70, are part of a group of protein folding catalysts and molecular chaperones that assist in the folding of new proteins, misfolded proteins, moving proteins across membranes, and help control regulatory proteins (Mayer). Due to their function hsp70 proteins have the ability to proofread other proteins to make sure they are folded properly. It is used as a biomarker because it has been shown to elevate during times of stress (Ireland *et al*).

### 2.3 Cytochrome p450

Cytochrome p450, or CYPs, function as metabolites for endogenous compounds and foreign substances (Hong). When organisms are exposed to toxic substances this
family of hemoproteins work to detoxify them (Hong). This protein is used as a biomarker because it is shown to elevate when the organism is exposed to a toxic substance.

2.4 Aryl Hydrocarbon Receptor

Aryl hydrocarbon receptor, or Ahr2, is a ligand-gated transcription factor. It plays a role in development, homeostasis in environment, and circadian rhythms (Hahn). Traditionally ahr2 is used as a toxicity biomarker for 2,3,7,8- tetrachlorodibenzo-p-dioxin (TCDD) (Goodale et al) and similar compounds, but may be used as an indicator for other endogenous chemicals.

2.5 Hypothesis

We hypothesize that there will be an increase in expression of all three biomarkers at 1 parts per million (ppm) of MCHM and 5 ppm compared to the control sample of 0 ppm. We also hypothesize there will be an increase in expression of all three biomarkers from week 1 to week 3 in 1 ppm and 5 ppm.

3. Materials and Methods

3.1 RNA Separation

Three fish from each of the following categories were removed from the -80 degree Celsius freezer to thaw: 0 parts per million (ppm) of MHCM at week 1 of exposure, 0 ppm at week 3, 1 ppm at week 1, 1 ppm at week 3, 5 ppm at week 1, and 5 ppm at week 3. Each sample was transferred to a 2 mL tube and 1 mL of Trizol was added. All samples were vortexed for 15 minutes. Another mL of Trizol was attempted to the tube containing fish 1 from the 0 ppm at 1 week (0.1.1) but was unsuccessful. The contents of that tube was split in half and a total of 2 mL of Trizol was used between the two tubes.
0.5 mL of Trizol was added to the remaining 17 tubes and all samples were vortexed until mostly dissolved. Samples were stored in the -20 degree Celsius freezer.

The samples were removed from the freezer and allowed to thaw. Once thawed all samples were centrifuged at 2,000 RPM for 15 minutes. The aqueous phase was removed and placed into a new 2 mL tube. The 0.1.1 sample that was split into two tubes remained in two tubes. The aqueous layer from each tube was put into a new one. 0.2 mL of chloroform per 1 mL of Trizol used was added to each tube. The 0.1.1 sample had 0.4 mL of chloroform added. All others had 0.3 mL added. All tubes were shaken vigorously for 15 seconds and stored in the -20 degree Celsius freezer.

The samples were allowed to thaw and then incubated for 3 minutes at room temperature. After incubation the samples were centrifuged at 12,000 RCF for 15 minutes at 4 degrees Celsius. The aqueous phase was removed, without disturbing the interphase, and placed in a new 2 mL tube. If the interphase was disturbed the tube was centrifuged again.

0.5 mL of 100% isopropanol was added per 1 mL of Trizol use to each tube. The 0.1.1 sample required 1 mL of 100% isopropanol while all the others required 0.75 mL of 100% isopropanol. All samples centrifuged at 12,000 RCF for 10 minutes at 4 degrees Celsius. Samples stored in -80 degree freezer.

Samples removed from freezer, allowed to thaw, and centrifuged again at 12,000 RCF for 10 minutes at 4 degrees Celsius. The solution was removed and discarded, leaving the RNA pellet at the bottom undisturbed. If it was disturbed the sample was centrifuged again and the remaining liquid was removed.

75% ethanol was made by mixing 100% ethanol with nuclease free water in a 3:1 ratio. 28 mL was needed for all the samples so 21 mL of ethanol was mixed with 7 mL of
nuclease free water. Each pellet was washed with 1 mL of 75% ethanol per 1 mL of Trizol used. The 0.1.1 sample required 2 mL of 75% ethanol while all others required 1.5 mL of 75% ethanol. All samples were vortexed briefly. The samples were then centrifuged at 7,500 RCF for 5 minutes at 4 degrees Celsius. Samples were then allowed to dry in the incubator at 37.1 degrees Celsius for 15 minutes.

400 microliters of nuclease-free water were added to each tube. The 0.1.1 sample tubes were combined. All 18 tubes were placed in the -20 degree Celsius freezer. Samples were removed from the freezer and allowed to thaw. They were incubated in a water bath set for 55 degrees Celsius for 5 minutes, vortexed, and placed in an ice bath for 5 minutes. This process was repeated 5 times. Once completed all of the samples were analyzed for RNA concentration and purity using Nanodrop (Table 1).

### 3.2 cDNA Synthesis

The two tubes with the highest purity from each parts per million and week number were used. These sample numbers were: 0.1.1, 0.1.3, 0.3.1, 0.3.3, 1.1.1, 1.1.2, 1.3.1, 1.3.3, 5.1.2, 5.1.3, 5.3.2, and 5.3.3. 2XRT Reaction Mix and RT Enzyme Mix were removed from the -80 degree freezer and were allowed to thaw on ice. 10 microliters of 2XRT Reaction Mix were added to each of 12 qPCR tubes. 2 microliters of RT Enzyme Mix were added to each of the 12 qPCR tubes. The RNA was then added. The NAC numbers for each of the 12 chosen samples were averaged and divided into 1000. This number was rounded to the tenths place and that many microliters was added into the qPCR tube with the 2XRT Reaction Mix and RT Enzyme Mix. This was repeated for each of the 12 samples. If the 1000/NAC result was over 8 only 8 microliters was added into the tube. Nuclease-free water was added to any of the tubes that were not up to 20
microliters (Table 2). After each of the sample tubes were made they were run thru a PCR machine for an hour and a half to make the cDNA. The cDNA was then analyzed for concentration and purity (Table 3).

Table 1: Nucleic acid concentrations, in micrograms per microliter, and purity (260/280), repeated twice, of each sample. Highlighted value is an outlier.

<table>
<thead>
<tr>
<th>Sample</th>
<th>NAC (micrograms/microliter)</th>
<th>260/280</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1.1</td>
<td>327.2, 322</td>
<td>2.06, 2.07</td>
</tr>
<tr>
<td>0.1.2</td>
<td>146.7, 149.8</td>
<td>2.04, 2.04</td>
</tr>
<tr>
<td>0.1.3</td>
<td>687.9, 674.3</td>
<td>2.1, 2.14</td>
</tr>
<tr>
<td>0.3.1</td>
<td>84.9, 90.8</td>
<td>1.91, 1.91</td>
</tr>
<tr>
<td>0.3.2</td>
<td>63, 72.1</td>
<td>1.86, 1.86</td>
</tr>
<tr>
<td>0.3.3</td>
<td>414.4, 409.5</td>
<td>2.04, 2.05</td>
</tr>
<tr>
<td>1.1.1</td>
<td>140.5, 266.1, 142.2</td>
<td>1.96, 1.59, 1.99</td>
</tr>
<tr>
<td>1.1.2</td>
<td>89.1, 89.5</td>
<td>1.87, 1.88</td>
</tr>
<tr>
<td>1.1.3</td>
<td>82.5, 82.8</td>
<td>1.8, 1.82</td>
</tr>
<tr>
<td>1.3.1</td>
<td>175.1, 175.5</td>
<td>2.11, 2.11</td>
</tr>
<tr>
<td>1.3.2</td>
<td>68.3, 68.2</td>
<td>1.84, 1.85</td>
</tr>
<tr>
<td>1.3.3</td>
<td>807.4, 804.3</td>
<td>2.08, 2.08</td>
</tr>
<tr>
<td>5.1.1</td>
<td>81.6, 81.3</td>
<td>1.81, 1.81</td>
</tr>
<tr>
<td>5.1.2</td>
<td>108.6, 104.2</td>
<td>1.94, 1.94</td>
</tr>
<tr>
<td>5.1.3</td>
<td>134.3, 131.1</td>
<td>1.98, 1.98</td>
</tr>
<tr>
<td>5.3.1</td>
<td>74.1, 72.7</td>
<td>1.84, 1.83</td>
</tr>
<tr>
<td>5.3.2</td>
<td>161.7, 161</td>
<td>2.08, 2.07</td>
</tr>
<tr>
<td>5.3.3</td>
<td>429.3, 428.4</td>
<td>2.01, 2.00</td>
</tr>
</tbody>
</table>
Table 2: Components added to each qPCR tube

<table>
<thead>
<tr>
<th>Sample Number</th>
<th>2XRT Reaction Mix (microliters)</th>
<th>Nuclease-Free Water (microliters)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1.1</td>
<td>10</td>
<td>4.9</td>
</tr>
<tr>
<td>0.1.3</td>
<td>10</td>
<td>6.5</td>
</tr>
<tr>
<td>0.3.1</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>0.3.3</td>
<td>10</td>
<td>5.6</td>
</tr>
<tr>
<td>1.1.1</td>
<td>10</td>
<td>0.9</td>
</tr>
<tr>
<td>1.1.2</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>1.3.1</td>
<td>10</td>
<td>2.3</td>
</tr>
<tr>
<td>1.3.3</td>
<td>10</td>
<td>6.8</td>
</tr>
<tr>
<td>5.1.2</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>5.1.3</td>
<td>10</td>
<td>0.5</td>
</tr>
<tr>
<td>5.3.2</td>
<td>10</td>
<td>1.8</td>
</tr>
<tr>
<td>5.3.3</td>
<td>10</td>
<td>5.7</td>
</tr>
</tbody>
</table>

All components added to each qPCR tube
Table 3: Nucleic acid concentration and purity (260/280) of the cDNA samples.

<table>
<thead>
<tr>
<th>Sample Number</th>
<th>NAC (micrograms/microliter)</th>
<th>260/280</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1.1</td>
<td>1247.6</td>
<td>1.65</td>
</tr>
<tr>
<td>0.1.3</td>
<td>1143.7</td>
<td>1.66</td>
</tr>
<tr>
<td>0.3.1</td>
<td>1103.8</td>
<td>1.65</td>
</tr>
<tr>
<td>0.3.3</td>
<td>1021.5</td>
<td>1.64</td>
</tr>
<tr>
<td>1.1.1</td>
<td>1162.9</td>
<td>1.64</td>
</tr>
<tr>
<td>1.1.2</td>
<td>1055.9</td>
<td>1.61</td>
</tr>
<tr>
<td>1.3.1</td>
<td>1260.3</td>
<td>1.66</td>
</tr>
<tr>
<td>1.3.3</td>
<td>1062.7</td>
<td>1.69</td>
</tr>
<tr>
<td>5.1.2</td>
<td>1223.8</td>
<td>1.64</td>
</tr>
<tr>
<td>5.1.3</td>
<td>1021.1</td>
<td>1.67</td>
</tr>
<tr>
<td>5.3.2</td>
<td>1127.8</td>
<td>1.65</td>
</tr>
<tr>
<td>5.3.3</td>
<td>996.8 990.8</td>
<td>1.66</td>
</tr>
</tbody>
</table>

3.3 qPCR Setup

First the stock solution had to be created from the cDNA. The C1V1=C2V2 formula was used. C1 was replaced with the rounded concentration to the nearest hundred. C2V2 was replaced with 1000. V1 was determined for each sample, rounded to the nearest tenths place. The volume for each sample was subtracted from 10 to determine how much nuclease-free water to add to the dilution (Table 4). Once the stock solution was made for each sample the experimental solution was made for each sample. This was done in a 1 microliter of stock solution to 19 microliters of nuclease-free water. An experimental solution was made for each of the primers used as well. 1 microliter of stock primer was added to 19 microliters of nuclease-free water. The primers used were beta-actin, hsp70, cytochrome p450, and ahr2. Beta-actin was used as a housekeeping primer because it is always expressed. The other three primers were used as experimental
primers. Due to the number of samples that had to be prepared Beta-actin had to be
diluted in a 1:38 ratio for the last 2 of 3 qPCR plates.

Each sample used a total of 6 wells per primer being used. Three wells were
template wells, one well was a no-template well, and 2 wells were no primer wells. A
sample plate setup can be seen in Table 5. The template wells contained: 25 microliters of
Master mix, 9 microliters of forward primer dilution, 3 microliters of reverse primer
dilution, 2 microliters of template dilution (experimental solution), and 11 microliters of
nuclease-free water. The no template wells contained: 25 microliters of Master mix, 9
microliters of forward primer dilution, 3 microliters of reverse primer dilution, and 13
microliters of nuclease-free water. The no primer wells contained: 25 microliters of
Master mix, 2 microliters of template dilution, and 23 microliters of nuclease-free water.
All samples were placed on ice and added to the 96-well qPCR plate while it was also on
ice. After the plates were set up they were run thru the qPCR machine (Figure 9). The
first plate contained all of the 0 ppm samples, the second contained all the 1 ppm
samples, and the third contained all the 5 ppm samples. The first place was run for 40
minutes and the last two were run for an hour and a half. The amplifications were
analyzed and compared.
Table 4: volumes of cDNA and nuclease free water added together to make stock solutions.

<table>
<thead>
<tr>
<th>Sample Number</th>
<th>cDNA volume (microliters)</th>
<th>Nuclease-Free Water (microliters)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1.1</td>
<td>0.8</td>
<td>9.2</td>
</tr>
<tr>
<td>0.1.3</td>
<td>0.9</td>
<td>9.1</td>
</tr>
<tr>
<td>0.3.1</td>
<td>0.9</td>
<td>9.1</td>
</tr>
<tr>
<td>0.3.3</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>1.1.1</td>
<td>0.8</td>
<td>9.2</td>
</tr>
<tr>
<td>1.1.2</td>
<td>0.9</td>
<td>9.1</td>
</tr>
<tr>
<td>1.3.1</td>
<td>0.8</td>
<td>9.2</td>
</tr>
<tr>
<td>1.3.3</td>
<td>0.9</td>
<td>9.1</td>
</tr>
<tr>
<td>5.1.2</td>
<td>0.8</td>
<td>9.2</td>
</tr>
<tr>
<td>5.1.3</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>5.3.2</td>
<td>0.9</td>
<td>9.1</td>
</tr>
<tr>
<td>5.3.3</td>
<td>1</td>
<td>9</td>
</tr>
</tbody>
</table>

Table 5: Sample setup for 96 well qPCR for 1 fish sample.

<table>
<thead>
<tr>
<th>Beta-actin</th>
<th>Hsp70</th>
<th>P450</th>
<th>Ahr2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Template (T)</td>
<td>No template (NT)</td>
<td>T</td>
<td>NT</td>
</tr>
<tr>
<td>T</td>
<td>No primer (NP)</td>
<td>T</td>
<td>NP</td>
</tr>
<tr>
<td>T</td>
<td>NP</td>
<td>T</td>
<td>NP</td>
</tr>
</tbody>
</table>

4. Results and Discussion

Below are the expression plots for beta-actin, ahr2, hsp70, and p450 for 0 ppm, 1 ppm, and 5 ppm (Figures 1-4), and the expression plots for beta-actin, ahr2, hsp70, and p450 at week 1 and week 3 for 0 ppm, 1 ppm, and 5 ppm (Figures 5-8).
4.1 Expression plots comparing 0 ppm, 1 ppm, and 5 ppm for beta-actin and all three biomarkers

There was a small increase in expression for beta-actin between 0 and 1 ppm and again between 1 and 5 ppm.

There was a large increase in expression for ahr2 between 0 and 1 ppm. At 1 ppm there was one sample that showed constant expression and that sample is considered an outlier. More samples showed expression between 1 ppm and 5 ppm though the average amplitude appears to be about the same.

Figure 1: Expression plots for beta-actin at A) 0 ppm, B) 1 ppm, C) 5 ppm.

Figure 2: Expression plots for ahr2 at A) 0 ppm, B) 1 ppm, C) 5 ppm.

Figure 3: Expression plots for hsp70 at A) 0 ppm, B) 1 ppm, C) 5 ppm.
There was a large increase in expression for hsp70 between 0 ppm and 1 ppm. At 5 ppm it appears there were a greater number of samples that showed expression, though the average amplitude seemed remained about the same.

![Graph A](image1.png)
![Graph B](image2.png)
![Graph C](image3.png)

**Figure 4: Expression plots for cytochrome p450 at A) 0 ppm, B) 1 ppm, C) 5 ppm.**

It appears there was minimal increase in expression between 1 ppm and 5 ppm.

**4.2 Expression plots comparing week 1 to week 3 for 0 ppm, 1 ppm, and 5 ppm for beta-actin and all three biomarkers**

![Graph D](image4.png)
![Graph E](image5.png)
![Graph F](image6.png)

**Figure 5: Expression plots for beta-actin at: A) 0 ppm, week 1 B) 0 ppm, week 3 C) 1 ppm, week 1 D) 1 ppm, week 3 E) 5 ppm, week 1 F) 5 ppm, week 3.**

There was an increase in expression of beta-actin at 0 ppm between weeks 1 and 3. There appears to be very little change in expression between time point for 1 ppm and 5 ppm.
There was no change in expression in ahr2 at 0 ppm between week 1 and week 2 based on the expression plots alone. It appears there was a decrease in expression at 1 ppm between weeks 1 and 3 and a slight increase in expression at 5 ppm between weeks 1 and 3.

There was a decrease in expression of hsp70 at 0 ppm between weeks 1 and 3. There was very little change in expression between time points for 1 ppm and 5 ppm based on the expression plots.

Figure 6: Expression plots for ahr2 at: A) 0 ppm, week 1 B) 0 ppm, week 3 C) 1 ppm, week 1 D) 1 ppm, week 3 E) 5 ppm, week 1 F) 5 ppm, week 3.

Figure 7: Expression plots for hsp70 at: A) 0 ppm, week 1 B) 0 ppm, week 3 C) 1 ppm, week 1 D) 1 ppm, week 3 E) 5 ppm, week 1 F) 5 ppm, week 3.
There was little change in expression in cytochrome p450 at 0 ppm between weeks 1 and 3. There appears to be a slight increase in expression at 1 ppm between weeks 1 and 3. There appears to be a slight decrease in expression at 5 ppm between weeks 1 and 3.

4.3 MCHM induced biomarker expression

There was a 4-5 fold increase in levels of expression for hsp70 compared to the housekeeping gene beta-actin. There was a 5-6 fold increase in levels of expression for
ahr2 compared to the housekeeping gene. There was a 6-7 fold increase in levels of expression for cytochrome p450 compared to the housekeeping gene.

There was a 5 fold increase in the expression of hsp70 compared to the housekeeping gene between the 1st and 3rd week. There was a 4 fold increase in expression of ahr2 compared to the housekeeping gene between the 1st and 3rd week. There was a 6 fold increase in expression of cytochrome p450 compared to the housekeeping gene between the 1st and 3rd week.

The hypothesis that there would be an increase in expression in the three experimental genes was correct. Cytochrome p450 had the greatest increase in expression compared to the housekeeping gene. The second-highest expression was seen in ahr2. The lowest increase in expression was seen in hsp70. The hypothesis that there would be an increase in expression between week 1 and week 3 for 1 ppm was also correct. The largest increase in expression between time points was seen in cytochrome p450. The
second-largest increase in expression between time points was seen in hsp70. The lowest increase in expression between time points was seen in ahr2.

Overall, cytochrome p450 showed the largest change in expression compared to the housekeeping gene. Since cytochrome p450 functions as a metabolite for endogenous compounds and foreign substances (Hong) this result would indicate the toxicity of MCHM. Ahr2 in involved in development as well as responding to chemicals in the environment (Hahn). It showed the second-largest increase in expression compared with the housekeeping gene (Figure 9) which indicates the fish was responding to chemicals to its environment. Hsp70 has been shown to elevate due to increased levels of stress (Ireland et al). Though it had the lowest increase in expression compared to the housekeeping gene it still indicates the organism was responding to a stressful environment.

The results seen here are in line with similar studies done on this same model organism. The study done by Purdue University showed morphological changes in zebrafish after exposure to MCHM, 4-MCHM, and the tank mixture from the Elk River spill (National Toxicology Program). The increase in gene expression for cytochrome p450 could explain this result. The NTP study, unpublished results from this lab, and the Purdue University study showed changes in photomotor and visual motor responses (Light), can be explained by both the increase in hsp70 and ahr2. The increase in stress can cause changes in visual responses. The increase in ahr2 could indicate the organism was responding to the lack of homeostasis in its environment. The ahr2 expression increase can also be explained by the organism metabolizing the endogenous chemical in its environment since ahr2 is similar in function to cytochrome p450.
This research can have implications for understanding the true toxicity of MCHM and how it will affect aquatic ecosystems. Future research can include similar experiments with MCHM exposure at higher ppm and longer exposure times.
5. Works Cited


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