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MCHM (4-methylcyclohexane Methanol) Influences the Predator-Prey Interaction Between Danio Rerio and Daphnia Magna

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MCHM (4-METHYLCYLOCHEXANE METHANOL) INFLUENCES THE PREDATOR-PREY INTERACTION BETWEEN *DANIO RERIO* AND *DAPHNIA MAGNA*

by

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ABSTRACT

Chemical contamination alters organism-level traits, such as activity and feeding, that can ultimately affect aquatic trophic interactions. Despite the importance of predator-prey relationships in aquatic communities, chemical toxicity is often tested on single species prior to use. For example, 4-methylcyclohexane methanol (MCHM) used in industrial coal-cleaning enters the environment regularly from low-level contamination during disposal and occasionally in high concentrations following accidental spills, but its effects on fish-zooplankton interactions remain unknown. It was hypothesized MCHM exposure affects zebrafish and *D. magna* swimming behavior differently when exposed individually or together, and ultimately affects their relationship. Zebrafish and *D. magna* were exposed individually and together to various environmentally relevant concentrations of MCHM. In the single-species experiments, zebrafish and *D. magna* swimming distance, velocity, and activity, as well as *D. magna* mortality, were quantified 1, 3, 5, and 7 days post-exposure to 0, 0.5, 1, 3, or 5 ppm MCHM. In single species tests, zebrafish in all MCHM treatments experienced an immediate and consistent decrease in all measured parameters. *Daphnia magna* swimming distance, velocity and activity also decreased by approximately 30-50%; however, unlike the zebrafish, mortality occurred after 3 days, reaching 100% by the end of the experiment. When exposed together, *D. magna* exhibited similar results to the individual test. In contrast, only zebrafish in the 1 and 5 ppm concentrations experienced decreased swimming distance and velocity when prey was present. To examine how decreased mobility in both organisms affected their predator-prey interaction, a feeding study

was conducted. Zebrafish were exposed to MCHM concentrations of 0, 0.5, 1, 3, or 5 ppm. After 1, 3, 5, and 7 days, 15 live *D. magna* were released into a tank containing one fish. The number of *D. magna* remaining after 30 minutes and one hour was used to calculate feeding rate. The number of strikes performed by each zebrafish was also quantified. Zebrafish feeding rate decreased by 40% in the 1, 3 and 5 ppm MCHM treatments compared to the control throughout the exposure period. Zebrafish in those MCHM treatments also performed more strikes per *D. magna* consumed. The individual species tests suggest MCHM exposures longer than three days could lead to loss of zebrafish food resources. Short-term exposures led to decreased mobility in both organisms, and the feeding study results indicated these changes altered the zebrafish-*D. magna* predator-prey relationship.

INDEX WORDS: Predator-prey, 4-methylcylcohexane methanol, Zebrafish, *Daphnia magna*, Feeding, Toxicity

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CHAPTER 1

INTRODUCTION

Chemical pollution is one of the most common disturbances to aquatic ecosystems (Miller et al. 1989, NRC 2017). Industrial effluent, such as waste from manufacturing, frequently enter aquatic environments via spills or waste disposal, where they can negatively affect aquatic organisms (Samuelson, 2009). For example, populations of primary producers or consumers may experience direct lethal effects of chemical exposure, causing an immediate decrease in population size (Kuyvenhoven, 2016). A mixture of industrial detergents and corrosion inhibitors increased mortality in larval fish (*Tilapia* sp) due to respiratory failure (Ezemonye et al., 2007). Decreased abundance of one type of organism can then induce density-mediated indirect effects on other populations (Relyea & Hoverman, 2006). The herbicide terbutryn decreased periphyton growth, thereby reducing food availability to grazers (Rybicki and Jungmann, 2018). Chemicals can also cause trait-mediated indirect effects by altering behavior that ultimately affects an organism's ability to survive. Organophosphates caused erratic swimming in crayfish, making them unable to feed and creating an increase in the abundance of their white shrimp prey source (Pandey et al., 2011). Altered population densities and traits, including movement and feeding, can ultimately affect the composition of an aquatic community by changing species interactions such as those between predators and prey (Newman, 2009).

Altered predator-prey relationships influence energy movement between trophic levels within food webs (Carpenter et al., 2001). Salt marsh fish (*Fundulus heteroclitus*) exposed to mercury consumed less shrimp due to decreased mobility, resulting in a population decrease, followed by decreased abundance of piscivorous predators (Smith & Weis, 1997). Additionally, Rasmussen et al. (2013) observed similar effects on the predator-prey interaction between the

crustacean *G. pulex* and brown trout *S. trutta*; *G. pulex* exhibited erratic swimming and consumed fewer prey after exposure to a pyrethroid pesticide, ultimately causing a decrease in the brown trout population. Decreased prey activity after exposure to chemicals can also alter predator-prey interactions (Abdel-Moneim et al., 2015). Cadmium decreased the mobility of brine shrimp, increasing ease of capture by larger grass shrimp (Wallace et al., 2000). In contrast, decreased *Daphnia magna* mobility after exposure to copper led to decreased feeding on the *D. magna* by larval freshwater zebrafish (*Danio rerio*) and ultimately increased mortality in the zebrafish population (Abdel-Moneim et al., 2015). Thus, testing chemical effects on interacting organisms is key to understanding how aquatic ecosystems may be affected (McPartland et al., 2015).

The alicyclic primary alcohol 4-methylcyclohexane methanol (MCHM) used to clean coal commonly enters freshwater rivers (He et al., 2015). From here forward, MCHM will refer specifically to 4-methylcylohexane methanol rather than any of its isomers or other forms. This chemical MCHM is used as a foaming agent to separate usable coal from clay and rock (Foreman et al., 2015), resulting in a MCHM-debris waste mixture that is released into small tributaries (Wills 2006, He et al. 2015, Toxnet 2017). Routine disposal results in MCHM concentrations of 0.5-1 ppm in freshwater systems, but spill concentrations can be much higher. Approximately 10,000 gallons of MCHM spilled into the Elk River in West Virginia in January 2014, contaminating the drinking water supply with a maximum concentration of 13.7 ppm (Rosen et al. 2014, Scaggs et al. 2015, US Chemical Safety and Hazard Investigation Board 2016,). Although MCHM has been used in the coal industry since the mid-20th century (Osnos 2014), relatively little is known about the potential consequences of MCHM contamination on aquatic organisms and their interactions.

Despite the potential for regular MCHM entry into aquatic ecosystems, existing toxicity data are derived from short-term, high-concentration testing (Eastman 1998, Whelton et al. 2014). After exposing *D. magna* to MCHM for 48 hours, a commonly used acute time frame, concentrations greater than 50 ppm decreased mobility (Eastman, 1998). Subsequent experiments revealed that concentrations greater than 40 ppm decreased mobility, 20% lower than the previous study (Eastman, 2014). However, Whelton et al. (2014) found that concentrations greater than 6.25 ppm increased *D. magna* immobility after 48 hours. The cause of the discrepancies between the Eastman (2014) and Whelton et al. (2014) studies is unclear. Environmental test conditions may have differed between the two experiments; specifically, the amount of ventilation allowed by the containers holding the *D. magna* may influence the effects of MCHM. The high concentrations used in both studies also make it difficult to apply the results to real-world aquatic ecosystems. To investigate effects of MCHM at environmentally relevant concentrations and exposure duration, I performed a pilot study examining *D. magna* responses to 6-day exposures of 0.5, 1, or 3 ppm of MCHM in ventilated containers. Decreased mobility (by $\geq 42\%$) occurred in all concentrations after 96 hours, in addition to $\geq$ 6-fold higher mortality relative to the control. These results suggest that MCHM may be more harmful than previously thought, particularly over chronic exposure times, affecting *D. magna* populations and the abundance of their predators.

Fish predators of *D. magna* may be affected by MCHM exposure at commonly found environmental concentrations, as well as similar concentrations as the *D. magna.* Photomotor responses of larval zebrafish (*Danio rerio*) were reduced when exposed to 4.5 ppm MCHM for 24 hours (National Toxicology Program 2016). Larval zebrafish may be affected by lower concentrations; exposing 5-day old zebrafish to MCHM for 3 hours reduced activity by

approximately 50% during light periods at 1 ppm (V. Sittaramane, unpublished data). Zebrafish and *D. magna* are both affected by low concentrations of MCHM measured in the environment but the mortality observed in preliminary studies suggest that *D. magna* may be more sensitive than zebrafish. This difference may alter the predator-prey relationship between the two organisms, and therefore investigating their interaction in the presence of MCHM is warranted.

Single-species toxicity tests are often easier to reproduce and interpret than microcosm tests, but the results are more difficult to extrapolate to aquatic environments (Pascoe et al., 2000). For example, while mobility in catfish and snails exposed separately to the pesticide endosulfan is unaffected, catfish feeding rate on the snails decreased to zero (Monde et al., 2016). Similarly, prawns (crustacean *M. borellii*) and zooplankton exposed to the pesticide chlorpyrifos separately experienced mortality at concentrations ≥ 0.01 µg l^{-1} while prawn predation on the zooplankton was decreased between 0.002 - 0.01 µg l^{-1} (Gutierrez & Negro, 2014). Clearly testing predator-prey relationships may reveal toxic effects in addition to those observed in single species studies. In both single species and mesocosm experiments containing primary producers, invertebrates and vertebrates exposed to the herbicide diquat, mortality occurred at a concentration 2-fold lower for organisms in the mesocosm experiments than the single species tests due to change in food availability (Van den Brink et al., 2006). In these studies, testing organisms together revealed effects that were not observed in single species tests, and were necessary to understand the toxicity of the chemicals on species interactions.

To make predictions about MCHM effects on aquatic communities, testing responses of both single species and species interactions is essential. The effects of MCHM exposure on zebrafish, *D. magna,* and their predator-prey interactions were examined. I hypothesized that MCHM exposure affects zebrafish and *D. magna* swimming behavior differently when exposed

individually or together. *Daphnia magna* and zebrafish were separately exposed to MCHM for seven days, and it was predicted that as MCHM concentration increases, zebrafish and *D. magna* swimming activity should decrease. To examine swimming behavior in a multi-species setting, zebrafish exposed to MCHM were fed MCHM treated *D. magna.* Zebrafish feeding rate was expected to decrease, and swimming mobility in both organisms should differ when they are tested together and separately. The results from this study show the importance of utilizing multiple interacting organisms to understand a chemical's potential effects on predator-prey relationships within a food web.

CHAPTER 2

METHODS

Experimental Setup

To evaluate the effects of MCHM on the swimming behavior of zebrafish and *D. magna* individually, a behavioral experiment was conducted. Organisms were exposed to one of five treatments: 0 (control), 0.5, 1, 3, or 5 ppm MCHM for 7 days. MCHM commonly enters the environment as a single pulse during routine disposal and spills (Rosen et al., 2014), and the 7-day exposure period is similar to the length of time high MCHM concentrations were observed in the 2014 spill; MCHM was still found in drinking water 10 days after the spill occurred (Rosen et al., 2014). Treatment solutions for zebrafish were made with filtered water treated with 100 g L^{-1} of calcium chloride, sodium bicarbonate, and Instant Ocean solution (Instant Ocean, Blacksburg, VA) to maintain appropriate osmotic pressure for zebrafish, followed by mixing with calculated amounts of 98% crude 4 methyl-cyclohexane methanol (CAS: 34885-03-5, TCI America, Portland OR). Treatment solutions for *D. magna* were made using the same amounts of MCHM mixed with spring water. Initial and final MCHM concentrations were verified according to methods 8270C and 8270D (EPA 1998); MCHM was extracted from aqueous solutions with methylene chloride using a separatory funnel and analyzed using GC/MS (TestAmerica Laboratories, Inc., Canton, OH) (Table 1).

Study Organisms and Care

Male, adult wildtype zebrafish reared at Georgia Southern University (V. Sittaramane, pers. comm.) were used for the experiments. The fish were approximately 6 months old, and averaged 2.5 ± 0.2 cm standard length and 0.32 ± 0.1 g wet weight. Male fish were used to avoid possible differences in mobility due to sex (Engeszer et al., 2007). Water changes were

performed daily to maintain adequate oxygen and tank cleanliness for the zebrafish. A large volume of each MCHM solution was mixed at the beginning of the exposure period and stored in ventilated containers; this allowed MCHM to volatilize out of solution over time, and tank cleanliness could be maintained without sacrificing the single pulse design. All experiments took place in the department of Biology aquarium facility at Georgia Southern University (Statesboro, GA, USA). Zebrafish were maintained on a 12:12 hour light-dark cycle with an average irradiance intensity of 49.6 ± 5.1 µmol m^{-2} s^{-1}, and a mean temperature of 19 ± 0.4 °C.

Individual *Daphnia magna* (10 days old, 2-5 mm; Carolina Biological, Burlington, NC) were exposed to 5 ml of MCHM solution in well plates. Each daphnid received a nonlimiting concentration (approximately 3.2 x 10^5 cells ml^{-1}) of green algae (*Chlorella* sp., Carolina Biological, Burlington, NC) as a food source at the beginning of the experiment (Barata et al. 2008, Pablos et al. 2015). Cell density was verified using a BD Accuri C6 flow cytometer (Becton-Dickinson, CA, U.S.A.), which passes individual algal cells by lasers, which counts the cells based on their chlorophyll autofluoresence (Veldhuis and Kraay, 2000). The plates were kept on a 12 hr light-dark cycle under daylight with an average intensity of 52.6 ± 0.6 µmol m^{-2} s^{-1} (Natural Daylight 5000K, Sylvania, Wilmington, MA).

Experiment 1: Zebrafish behavior during MCHM exposure

To determine whether MCHM exposure affected zebrafish mobility over time, individual zebrafish (n = 9) were immersed in 0.8 L of treatment solution in clear, 1 liter plastic tanks with ventilated lids from 6-27 July 2017 (Figure 1). Zebrafish activity is stimulated by light and decreases in the dark (Dai et al., 2014). Light stimulus was applied after exposure to MCHM treatments on day 0 and then after 1, 3, 5, and 7 days. At each sampling time, the tanks were

illuminated and zebrafish swimming was video recorded (ICD-49: 1/2″ CCD Super Cube DSP Monochrome Camera, Ikegami) for one hour to determine average zebrafish swimming distance and velocity (Cachat et al., 2010). The experiment was repeated three times (n = 3) for a total of 9 replicates of each treatment. The fish were provided 2 grams of egg yolk flake fish food 7 hours prior to filming, allowed to feed for two hours, and then the tank water was replaced with clean MCHM treatment solution.

Experiment 2: Effects of MCHM on Daphnia magna Behavior

The goal of this experiment was to determine whether MCHM exposure decreases *D. magna* swimming distance, velocity, and activity. *Daphnia magna* initiate escape behaviors in response to predatory cues such as vibrations in the water caused by fish swimming and striking at prey (Sarma & Nandini 2006). Therefore, a standardized vibration was administered by dropping a 1.3 kg weight from 25 centimeters above the countertop after exposure to MCHM treatments on day 0 and after 1, 3, 5, and 7 days. *Daphnia magna* (n = 6) swimming distance, velocity, and activity in response to the vibration were video recorded for 10 minutes (von Elert & Pohnert, 2000).

Experiment 3: MCHM effects on zebrafish predation of D. magna

The effect of MCHM exposure on the predator-prey interaction between zebrafish and *D. magna* was tested by observing the organisms together. The experiment consisted of two trials (n=2) performed over two weeks. After zebrafish (n=6) were exposed to MCHM treatments for 24 hours, 15 *D. magna* that were exposed to the same treatments were placed into each tank. Feeding occurred again on days 3, 5, and 7 post exposure. Fish were not fed the day between

trials to ensure hunger (Abdel-Moneim et al., 2015). The number of *D. magna* remaining were visually confirmed after 30 and 60 minutes.

$$Feeding\ rate = \frac{Starting\ number\ of\ live\ D.\ magna - number\ remaining}{time}$$

Each bout of feeding was recorded with a video camera (ICD-49: 1/2″ CCD Super Cube DSP Monochrome Camera, Ikegami), and the number of strikes performed by each fish in that hour was scored visually. A strike was defined as an approach and attempt to capture prey (New & Kang, 2000). The number of *D. magna* consumed by each fish and the number of strikes performed were used to calculate the number of strikes *D. magna* consumed^{-1}.

Ethovision Analysis

The video recordings from each experiment were used to analyze swimming distance, velocity, and activity for each zebrafish and *D. magna* with Ethovision XT behavioral software (Ethovision XT, Noldus, Leesburg, VA). The EthoVision program was used to distinguish tracked objects from their background based on their brightness.

To obtain swimming distance and velocity, the center of each object was determined and its position tracked in each frame of the video. Ethovision determined the position of the fish 25 times in one second, and detection thresholds were set with the dynamic subtraction center point detection (range 160-255).

Swimming activity conveys the amount of movement in a given time frame, as a percentage, and can be a more accurate measurement when analyzing shorter time frames (< 15 minutes) (Prober et al., 2008). To obtain swimming activity, fifteen minutes of the hour-long zebrafish recording and the entire 10 minute *D. magna* post-stimulus recording was analyzed. To

calculate swimming activity, every pixel in the video was compared between the current frame and the previous one for the duration of the recording.

$$Activity = \frac{the\ number\ of\ changed\ pixels\ for\ the\ current\ frame}{Total\ number\ of\ pixels\ in\ the\ frame}$$

Swimming activity was only obtained for *D. magna* swimming behavior without a predator present; Ethovision behavioral software was unable to detect swimming activity with multiple *D. magna* present in one tank.

Statistical Analysis

The data were tested for assumptions of normality and homogeneity of variances using the Shapiro-Wilk *W* Test and Levene's Test, respectively. Sphericity of the data was determined using Mauchly's test. Only swimming activity for zebrafish and *D. magna* required a square root transformation to meet assumptions of parametric tests. Zebrafish swimming, feeding rate, and striking were analyzed for the entire duration of the experiment both with and without *D. magna* present. Due to *D. magna* mortality, the sample size became too small for statistical analysis after day 3; therefore, *D. magna* swimming velocity, distance, and activity were analyzed from day 0 to day 3 when tested alone. The *D. magna* used in the feeding study were only analyzed at one time point (24 hours post exposure) to avoid complications due to mortality. Therefore, change over time was not a factor when analyzing *D. magna* mobility with a predator present.

With zebrafish present, *D. magna* were only observed 24 hours post exposure. Effects of MCHM treatment on zebrafish or *D. magna* swimming distance, velocity, and square root of activity over time were analyzed using two-way repeated measures ANOVA, followed by Tukey-Kramer post-hoc multiple comparisons (JMP Pro 10.0.0, SAS Institute Inc., Cary, NC). Significance level was set at $\alpha = 0.05$ for all tests. Only significant interactions are reported.

CHAPTER 3

RESULTS

Zebrafish movement during MCHM exposure

Zebrafish exposed to MCHM exhibited reduced swimming distance (Table 2, Figure 2). The severity of the decrease, as well as the magnitude of the distance travelled, was dependent on the presence or absence of prey; furthermore, MCHM concentration was affected swimming distance when both organisms were tested together. All fish travelled farther with prey present (Table 3). Control zebrafish traveled an average of 152.8 ± 7.5 m without prey, 18% less than the distance travelled with *D. magna* present (Figure 2). All MCHM treated fish exhibited a 35% decrease in swimming distance when exposed alone (All Pairs, Tukey-Kramer post-hoc multiple comparisons, $p < 0.01$). In contrast, when *D. magna* were present, only swimming distance for fish in the 1 and 5 ppm treatments was reduced by 37% compared to the control (All Pairs, Tukey-Kramer post-hoc multiple comparisons, $p < 0.02$). Both individually and together, the reduction in swimming distance was seen at least 24 hours after exposure and remained consistent over time (Table 2)

Swimming velocity decreased due to MCHM exposure, both in the presence and absence of prey (Table 2, Figure 3). Swimming velocity increased in all fish with *D. magna* present (Table 3). Control zebrafish swam an average 5.85 ± 0.18 cm s^{-1} without prey, and an average of 7.46 ± 0.1 cm s^{-1} after *D. magna* were introduced. When tested alone, all treated fish had an average decrease of 34% compared to the control and there was no difference in swimming velocity among treatments containing MCHM (All Pairs, Tukey-Kramer post-hoc multiple comparisons, $p < 0.03$, Figure 3). After *D. magna* were fed to the zebrafish, all treated fish except those in the 3 ppm concentration had an average 4.86 ± 0.7 cm s^{-1}, a 35% decrease

compared to the control (All Pairs, Tukey-Kramer post-hoc multiple comparisons, $p > 0.05$). The decrease in velocity occurred at least 24 hours after exposure with and without prey present, and remained the same throughout the experiment.

Swimming activity decreased in MCHM exposed fish (Table 2, Figure 4). While swimming activity was higher in all fish when prey was introduced (Table 3), zebrafish swimming activity declined in all MCHM treatments whether prey was present or absent. Without *D. magna*, control fish averaged 12.7 ± 2% swimming activity on day 0; with prey present, control zebrafish exhibited an average 18.9 ± 1.2% swimming activity. In both tests, zebrafish exposed to 0.5, 1, and 5 ppm had an average 8.7 ± 1.6% (All Pairs, Tukey-Kramer post-hoc multiple comparisons, $p < 0.02$). However, zebrafish in the 3 ppm treatment had a higher swimming activity than the other concentrations when prey was present, with an average 13.2 ± 0.6% swimming activity. The decrease in swimming activity was observed at least 24 hours after exposure both with and without prey. Swimming activity decreased by approximately 50% between day 5 and day 7 in the zebrafish exposed to 0.5, 1, and 5 ppm treatments when *D. magna* was not present (All Pairs, Tukey-Kramer post-hoc multiple comparisons, $p > 0.05$, Figure 4, Table 2). However, with prey present there was no change in activity over time (Table 5, Figure 4).

Effects of MCHM on Daphnia magna Swimming Behavior and Mortality

Determining MCHM effects on swimming distance, velocity, and activity, was complicated by mortality. During the single species test, no *D. magna* mortality occurred in the control during the 7 day exposure period, but 100% mortality was observed in MCHM treatments by the end of the experiment (Table 4, Figure 5). All MCHM treatments averaged

20.8 ± 8.3% mortality by day 3, which increased to 58.3 ± 21.5% on day 5 (Figure 5). The resulting decrease in sample size required that analysis be performed for the measured variables through day 3. The *D. magna* used in the feeding study were only analyzed at one time point (24 hours post exposure), and swimming activity was not analyzed.

Daphnia magna swimming distance decreased due to MCHM exposure (Table 4, Figure 6, Figure 7). *Daphnia magna* mobility was not affected by the presence or absence of predators (Table 5, Figure 8). In both experiments, control *D. magna* travelled approximately 87 centimeters in one minute. After 24 hours of MCHM exposure, treated *D. magna* moved an average of 55.3 ± 4.4 cm with a predator present (Figure 7), and this 38% decrease compared to the control was seen in all MCHM treatments (All Pairs, Tukey-Kramer post-hoc multiple comparisons, $p < 0.01$). Similarly, *D. magna* exposed to MCHM swam an average distance of 58.4 ± 19.6 cm in the absence of a predator, an approximately 30% decrease compared to the control (Figure 6). A change over time was seen when *D. magna* were tested alone; swimming distance decreased immediately on day 0, and was lowest on day 3 for MCHM treated *Daphnia magna* (Table 4, Figure 6).

Daphnia magna swimming velocity decreased due to MCHM exposure (Table 4, Figure 9, Figure 10). Both with and without a predator present, control *D. magna* swam approximately 3.5 mm s^{-1} throughout the exposure period (Table 5, Figure 11). With a predator, all MCHM treated *D. magna* moved with an average velocity of 2.4 ± 0.2 mm s^{-1} (Figure 9). Without zebrafish, MCHM-treated *D. magna* had a 23.8% reduced swimming velocity compared to the control immediately upon exposure, swimming an average of 2.6 ± 1.3 mm s^{-1} on days 0 and 1 (Figure 10). In both tests, there was no difference in swimming velocity across treatments containing MCHM (All Pairs, Tukey-Kramer post-hoc multiple comparisons, $p > 0.05$). Similar

to swimming distance, swimming velocity decreased immediately after exposure, but decreased over time. Without zebrafish, *D. magna* swimming velocity decreased by 40% between day 1 and day 3, resulting in an average swimming velocity of 1.5 ± 0.5 mm s^{-1} (Figure 10).

After 1 day of exposure, swimming activity decreased in all MCHM treated *D. magna* (Table 4, Figure 12). Control *D. magna* had an average 8.45 ± 3.27% swimming activity throughout the exposure period. While *D. magna* in the 3 ppm treatment exhibited a similar swimming activity to the control on day 0, by day 1 that similarity was gone and *D. magna* in all treatments had an average 5.5 ± 3.8% swimming activity. Swimming activity continued to decrease in *D. magna* exposed to MCHM as time passed, resulting in an average 4.7 ± 2.2% swimming activity by day 3 of exposure (Figure 12). This is an approximately 50% reduction in swimming activity compared to the control.

MCHM effects on zebrafish predation of D. magna

MCHM exposure decreased feeding rate in zebrafish (Table 6, Figure 13). Zebrafish in the 0.5, 1, and 3 ppm treatments consumed 35% fewer *D. magna* than the control. There was no difference between these treatments (All Pairs, Tukey-Kramer post-hoc multiple comparisons, $p < 0.05$). Zebrafish in the 5 ppm treatment consumed approximately 57% less *D. magna* than the control (Figure 13). Effects were observed immediately upon exposure to treatments, and there was no change in feeding rate over time.

There was no difference in the number of strikes exhibited by MCHM treated zebrafish and the control fish (Table 6). The control fish averaged 33.8 ± 6.3 strikes hr^{-1}, while treated fish used an average 27.4 ± 7.8 strikes hr^{-1} (Table 6, Figure 14). The high variation in number of strikes likely prevented any distinction between MCHM treated and control fish. However, fish

in the 1 and 3 ppm treatments performed 50% more strikes *D. magna* consumed^{-1} than the control (Table 6, Figure 15). Zebrafish in the 5 ppm treatments used an average of 5.6 ± 1.5 strikes *D. magna* consumed^{-1}, 78% more strikes than the control needed to consume almost double the amount of *D. magna* (Figure 15). There was no change in the number of strikes *D. magna* consumed^{-1} due to time, and the increase observed in the MCHM treatments was immediate.

CHAPTER 4

DISCUSSION

The hypothesis that MCHM affects the mobility of zebrafish and *Daphnia magna* was supported. Without prey, the decrease in zebrafish swimming distance, swimming velocity, and swimming activity in the presence of MCHM was not concentration dependent; there was no difference in magnitude of decrease across treatments. In previous photomotor response tests, larval zebrafish experienced an approximate 50% decrease in mobility after 3 hours of exposure and continued to decrease as concentrations increased (Sittaramane, pers. comm). In the present study, no dose-dependent response occurred. This difference may be attributed to the age of the zebrafish used (larval vs. adult); the smaller larval zebrafish take up chemicals through both the skin and the gills, and therefore could be more severely affected by MCHM exposure (Rubinstein, 2006). Additionally, Sittaramane (unpubl.) used a larger range of concentrations (1-25 ppm) than the current study, which may have led to the dose-dependent response. In contrast, Horzmann et al. (2017) found that larval zebrafish increased their swimming distance and velocity at 1 ppm after 5 days of exposure. These opposing results could be attributed to a wide range of possible disruptions in neural or muscular systems caused by MCHM exposure during larval development. The time frame used could also contribute to the differing results; Horzmann et al. (2017) utilized a 5 day exposure period, while Sittaramane (unpubl.) exposed larvae for 3 hours.

In addition to decreased swimming distance, velocity, and activity, *D. magna* exposed to MCHM experienced 100% mortality by day 7 at much lower concentrations than those observed in previous acute studies. The LC_{50} of *D. magna* exposed to MCHM was 98.1 ppm after 48 hours of exposure (Eastman, 2004), and a replication of that same study resulted in an LC_{50} of 50 ppm

(Whelton et al., 2014). While decreases in *D. magna* mobility would be evident during acute exposure to MCHM, mortality at lower concentrations would not have been observed. For example, *Daphnia magna* experienced no negative effects after 48 hours of exposure to antibiotics, but reproduction decreased and mortality increased over the course of 3 weeks at the lowest tested concentration (Wollenberger et al., 2000). Acute studies are often used to determine the amount of a toxin needed to kill or immediately alter an organism, while chronic studies can examine effects seen at lower concentrations over time (Barry & Meehan 2000). Amphipods experienced 50% mortality after 96 hours of exposure to 3, 045 µg l^{-1}; after 7 days of exposure, amphipods experienced increased mortality at concentrations 97% lower than detected in the acute study (Keithly et al., 2004). Studies with longer exposure periods are key to understanding how environmentally relevant concentrations may affect aquatic organisms.

When performing chronic tests, it is possible to investigate possible organism recovery from chemical effects. *Daphnia magna* exposed to 80 mg l^{-1} ibuprofen for 10 days experienced a 60% decrease in reproduction, but had a similar reproduction rate to the control after 10 days in 20 mg l^{-1} ibuprofen (Hayashi et al., 2008). In both experiments, MCHM concentration was decreasing throughout the exposure period. *Daphnia magna* swimming mobility worsened over time until all *D. magna* exposed to MCHM experienced 100% mortality. This effect did not occur in the zebrafish; the reduction in zebrafish mobility was consistent throughout the exposure period. Studies found that zebrafish embryos washed with clean medium appeared to recover from the effects of MCHM (Sittaramane, unpubl), suggesting that adult zebrafish may have regained normal mobility over time because the MCHM concentration would decrease to zero. The MCHM treated *D. magna* experienced mortality before recovery was possible, potentially causing a change in the interaction between the two species.

The hypothesis that response to MCHM would differ when zebrafish and *D. magna* were tested alone and together was supported. Without prey, all zebrafish exposed to MCHM exhibited reductions in both swimming distance and velocity; with prey, only fish in the 1 and 5 ppm treatments decreased in both parameters. However, all MCHM treated fish exhibited a lower swimming activity than the control regardless of whether prey was present. Low swimming activity, but similar swimming distance and velocity to the control, suggests that bursts of swimming were performed to catch prey; these bursts could have attributed to distance travelled and velocity, while remaining still for the duration of the analyzed recording and keeping swimming activity low. The change in zebrafish mobility in the presence of *D. magna* may be attributed to how fish respond to environmental cues that require a quick response, such as prey or predators, differently than environmental cues (Daggett et al., 2018). Zebrafish increased mobility when exposed to flake fish food, but exhibited even greater increases when exposed to a predator (Kim et al., 2015). Renick et al. (2016) found that killifish exposed to chlorpyrifos experienced no change in swimming performance when provoked with a change in lighting outside their tank, but did show concentration-dependent changes in mobility with a predator present.

Daphnia magna did not exhibit any difference in movement due to the presence or absence of a predator. The small size and physiological simplicity of *D. magna* compared to adult zebrafish, coupled with the increased mortality and decrease in swimming mobility over time due to MCHM exposure observed in the current study, implies that *D. magna* are more sensitive to MCHM than the zebrafish (Hanazato, 2001). It is possible that a predator was not sufficient to induce an increase in *D. magna* swimming. *Daphnia magna* exposed to silver nanoparticles experienced decreased mobility when tested individually, and did not increase mobility or

attempt to escape when a predatory dragonfly was introduced (Pokhrel & Dubey, 2012).In contrast, *D. magna* exposed to carbon nanomaterials experienced decreased mobility alone, but increased mobility after being exposed with a predator, brown trout (Brausch et al., 2011). Multi-species testing is needed to reveal real-world effects of a toxin due to an organism's behavioral response to predators or prey.

With both organisms present, zebrafish treated with MCHM consumed fewer *D. magna* than the control zebrafish. In a similar study utilizing juvenile fathead minnows, exposure to the antidepressant fluoxetine for 7 days resulted in a 50% decrease in feeding on brine shrimp and 25% decrease in growth rate (Stanley, 2007). This reduction in growth rate demonstrates that MCHM-exposed fish may get less nutrition to contribute to their growth or health maintenance. Despite consuming fewer *D.* magna, zebrafish exposed to MCHM performed a similar number of strikes at prey as the control. Perch strike rate on zooplankton was unaffected by exposure to toxic cyanobacteria for 30 days, but fewer zooplankton were consumed and fish exhibited lower overall fitness; reduced attack efficiency causes higher energy expenditure, possibly explaining the reduced fitness compared to the control (Persson et al., 2011). The observed decrease in zebrafish hunting efficiency in the current study may be linked to the way MCHM affects mobility. Previous experiments suggest that MCHM may interfere with the H^+-ATPase ionocytes, which are responsible for ion homeostasis and Na^+ accumulation (Sittaramane, unpubl.).

To determine chemical effects on aquatic ecosystems, it is necessary to consider how organisms interact with one another. Zooplankton may experience a 100% mortality rate after exposure to MCHM, depleting a food source that small freshwater fish are dependent on (McCann 2012). For example, when zooplankton were exposed to toxic phytoplankton,

decreased zooplankton abundance was followed by a decrease in planktivorous fishes (Turner and Tester, 1997). These results demonstrate that the effects of a toxin on one trophic level affects others within the food web. This can occur through direct population decrease or behavioral changes. Small freshwater fish exposed to low concentrations of MCHM may undergo changes in behavior that affect their ability to feed, and therefore affect their predators. When organisms on three trophic levels (brine shrimp, white-leg shrimp, and grunt fish) were exposed to lead, exposed brine shrimp had an increase in mortality, while white-leg shrimp began swimming erratically and decreased feeding; this ultimately reduced grunt fish feeding rate (Soto-Jiménez, 2011). Less available small fish may cause an increase in competition for food between piscivorous fish and birds, thereby causing a decreased abundance in other prey organisms that these predators consume. Kvitek and Bretz (2005) found that shorebirds changed their target prey type from aquatic snails to sand crabs when the snails became scarce, thereby lowering the sand crab population. If several prey populations are depleted, a density mediated indirect effect may be seen through a decrease in prey population abundance (Belgrano 2005).

The results from both single and multiple interacting species toxicity tests are important for making predictions about aquatic community responses to MCHM. While the single species tests showed how mobility and mortality was affected in both organisms, investigating their predator-prey relationship revealed information about zebrafish feeding rate and hunting behavior. Without looking at both zebrafish and *D. magna* together, the results from the single species test would have been insufficient to make accurate predictions about the effects of MCHM exposure on an aquatic community. This study demonstrates the importance of utilizing a combination of single and interacting species studies when investigating chemical toxicity, in order to understand potential changes in an ecosystem. Utilizing organisms that have a predator-prey

relationship allows toxicologists to gain insight into the effects of a toxin on not only single populations, but the food web as a whole.

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Table 1. Analysis of Initial and Final MCHM Concentration

Experiment	Nominal Concentration (ppm)	Initial Concentration (ppm)	Final Concentration (ppm)	% Loss
Zebrafish & *D. magna* Behavior	0.5	0.49	0.11	77.6
	1	1.10	0.26	76.4
	3	3.00	0.70	76.7
	5	4.95	1.20	75.8
Zebrafish Feeding	0.5	0.47	0.13	72.3
	3	2.80	0.65	76.8
	5	4.89	1.40	71.3

Table 2. Analysis of zebrafish swimming behavior (two-way repeated measures ANOVA) during MCHM exposure with and without prey present

	Factor	df (Effect, Total)	F Ratio	p Value
	Prey Absent			
Zebrafish Swimming Distance	MCHM	4, 20	11.13	<0.0001
	Time	4, 160	1.05	0.3821
	Time*MCHM	16,160	1.23	0.2505
Zebrafish Swimming Velocity	MCHM	4, 20	2.71	0.0317
	Time	4, 160	1.23	0.3132
	Time*MCHM	16, 160	0.61	0.8678
Zebrafish Swimming Activity	MCHM	4, 20	18.7	<0.0001
	Time	4, 160	3.0	0.0203
	Time*MCHM	16, 160	0.37	0.9857
	Prey Present			
Zebrafish Swimming Distance	MCHM	4, 20	9.58	<0.0001
	Time	4, 160	0.34	0.7978
	Time*MCHM	16,160	0.18	0.9988
Zebrafish Swimming Velocity	MCHM	4, 20	12.70	<0.0001
	Time	4, 160	0.46	0.7109
	Time*MCHM	16, 160	0.42	0.9531
Zebrafish Swimming Activity	MCHM	4, 20	72.97	<0.0001
	Time	4, 160	0.25	0.8644
	Time*MCHM	16, 160	0.63	0.8137

Table 3. Analysis of the difference in zebrafish swimming behavior during MCHM exposure with and without prey (two-way repeated measures ANOVA)

	Factor	df (Effect, Total)	F Ratio	p Value
Zebrafish swimming distance	Presence of Prey	1, 299	86.4	<0.0001
	Presence of Prey*MCHM	4, 299	1.87	0.1233
	Presence of Prey *Time	3, 299	0.17	0.9146
Zebrafish swimming velocity	Presence of Prey	1, 299	66.1	<0.0001
	Presence of Prey *MCHM	4, 299	1.62	0.1695
	Presence of Prey *Time	3, 299	0.27	0.8449
Zebrafish swimming activity	Presence of Prey	1, 299	106	<0.0001
	Presence of Prey *MCHM	4, 299	1.56	0.1014
	Presence of Prey *Time	3, 299	1.65	0.1769

Table 4. Analysis of *Daphnia magna* swimming behavior (two-way repeated measures and one-way ANOVA) during MCHM exposure with and without a predator present

	Factor	df (effect, total)	F ratio	p Value
	Without Predator			
D. magna swimming distance	MCHM	4, 20	5.96	0.0015
	Time	3, 60	4.49	0.0165
	Time*MCHM	12, 60	1.01	0.4387
D. magna swimming velocity	MCHM	4, 20	14.65	<0.0001
	Time	3, 60	18.29	<0.0001
	Time*MCHM	12, 60	1.03	0.4220
D. magna swimming activity	MCHM	4,20	3.31	0.0262
	Time	3,60	7.02	0.0021
	Time*MCHM	12,60	0.70	0.6889
D. magna mortality	MCHM	4,20	3.11	0.0271
	Time	3,60	5.83	0.0003
	Time*MCHM	12,60	1.25	0.4652
	With Predator			
D. magna swimming distance	MCHM	4, 20	19.2	<0.0001
D. magna swimming velocity	MCHM	4, 20	7.09	<0.0001

Table 5. Analysis of the difference in *D. magna* swimming behavior during MCHM exposure with and without prey (two-way repeated measures ANOVA)

	Factor	df (Effect, Total)	F Ratio	p Value
D. magna swimming distance	Presence of Predator	1,118	0.07	0.7862
	Presence of Predator*MCHM	4,118	0.27	0.8980
D. magna swimming velocity	Presence of Predator	1,118	0.19	0.6619
	Presence of Predator*MCHM	4,118	0.21	0.9315

Table 6. Analysis of zebrafish feeding rate and striking (two-way repeated measures ANOVA) during MCHM exposure

Experiment	Factor	df (Effect, Total)	F Ratio	P Value
Zebrafish Feeding Rate	MCHM	3, 20	22.25	<0.0001
	Time	3, 60	1.45	0.2364
	Time*MCHM	9, 60	0.72	0.7259
# of Strikes hour^{-1}	MCHM	3,20	1.69	0.1844
	Time	3,60	0.88	0.4514
	Time*MCHM	9,60	0.50	0.9084
# strikes *D. magna* consumed^{-1}	MCHM	3,20	7.86	0.0003
	Time	3,60	0.93	0.4290
	Time*MCHM	9,60	1.01	0.4570

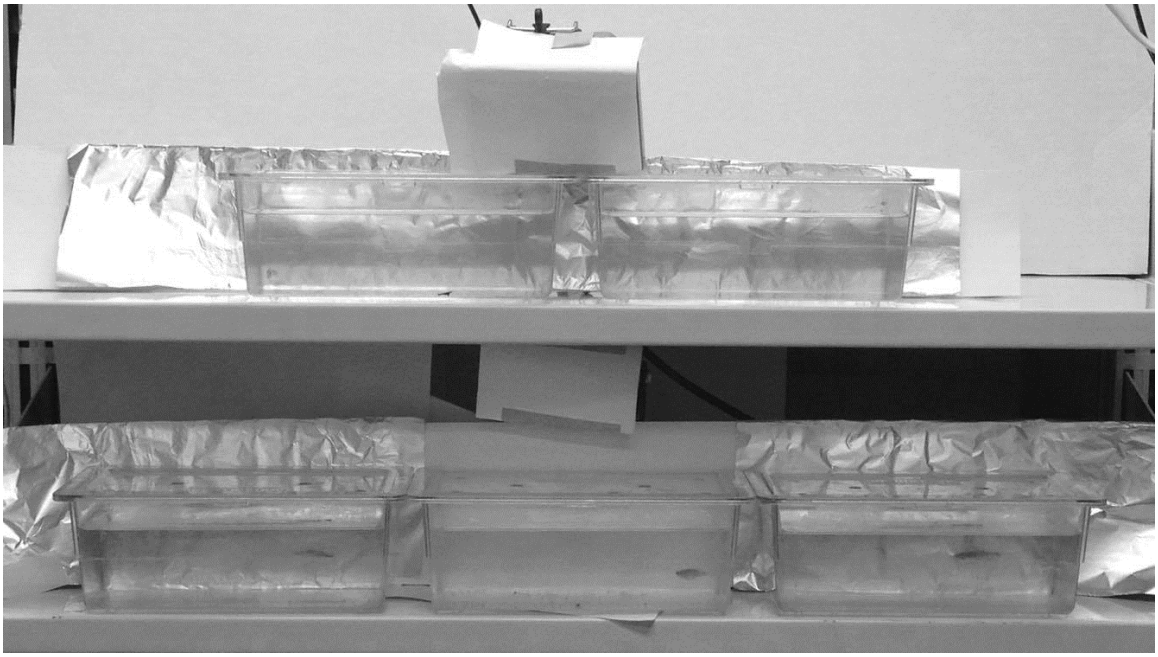

Figure 1. Example of experimental tank set-up for analysis of zebrafish mobility

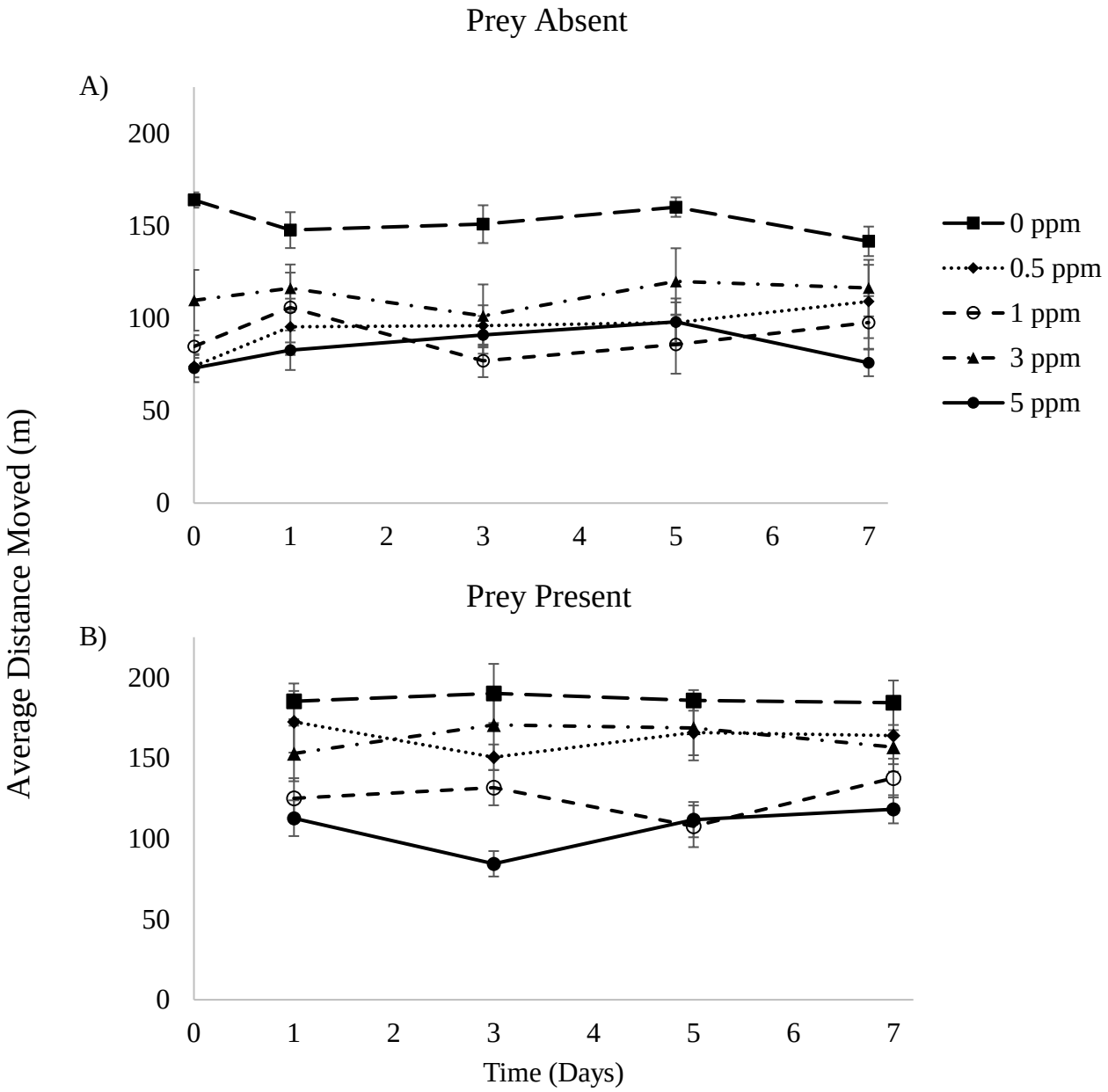

Figure 2. Average zebrafish swimming distance (m) over 7 days of exposure to one of 5 MCHM concentrations without (A) or with (B) prey. Error bars are ± one standard error of the mean (SEM) and n = 9.

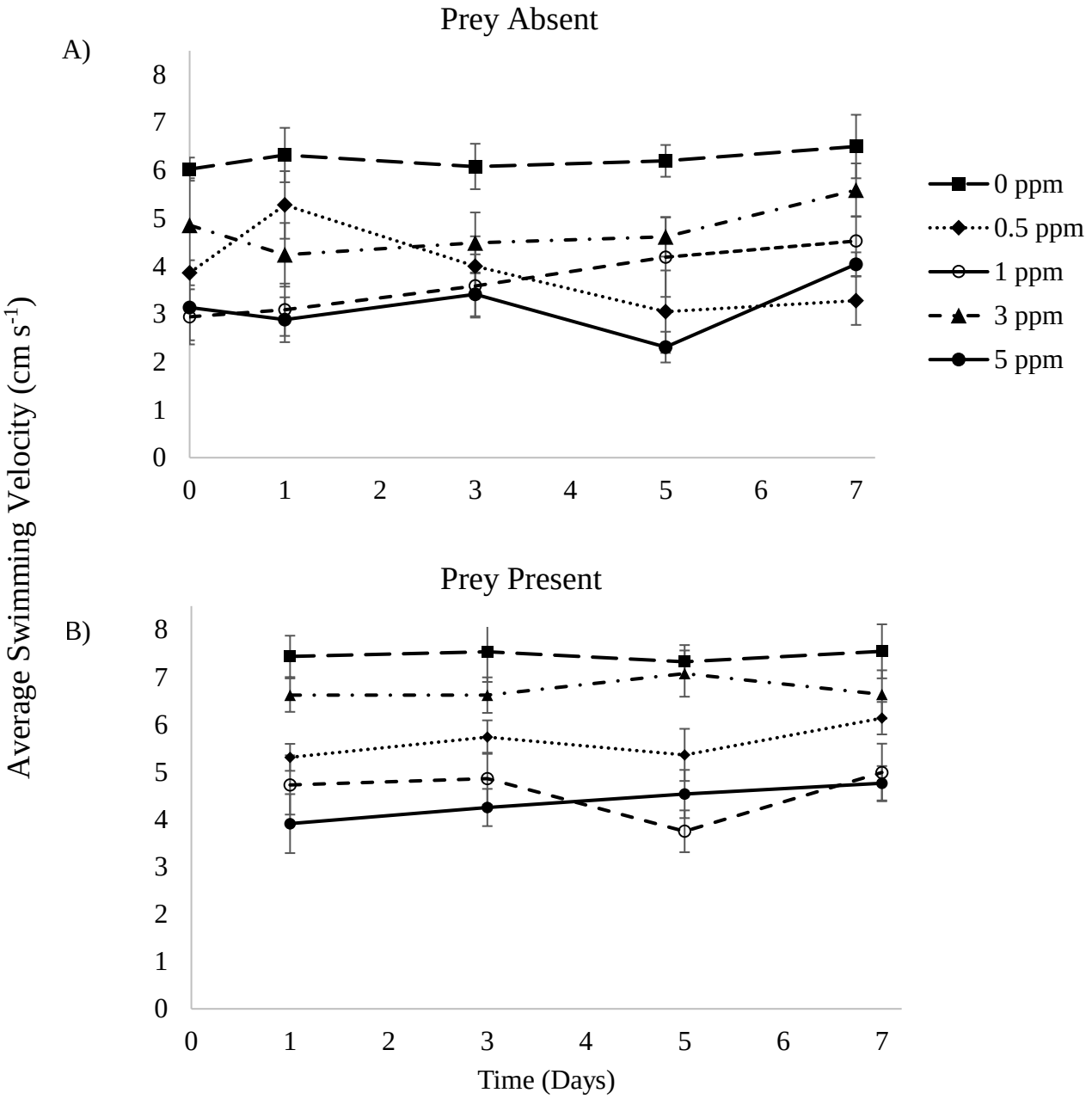

Figure 3. Average zebrafish swimming velocity (cm s^{-1}) over 7 days of exposure to one of 5 MCHM concentrations without (A) or with (B) prey. Error bars are ± one standard error of the mean (SEM) and n = 9.

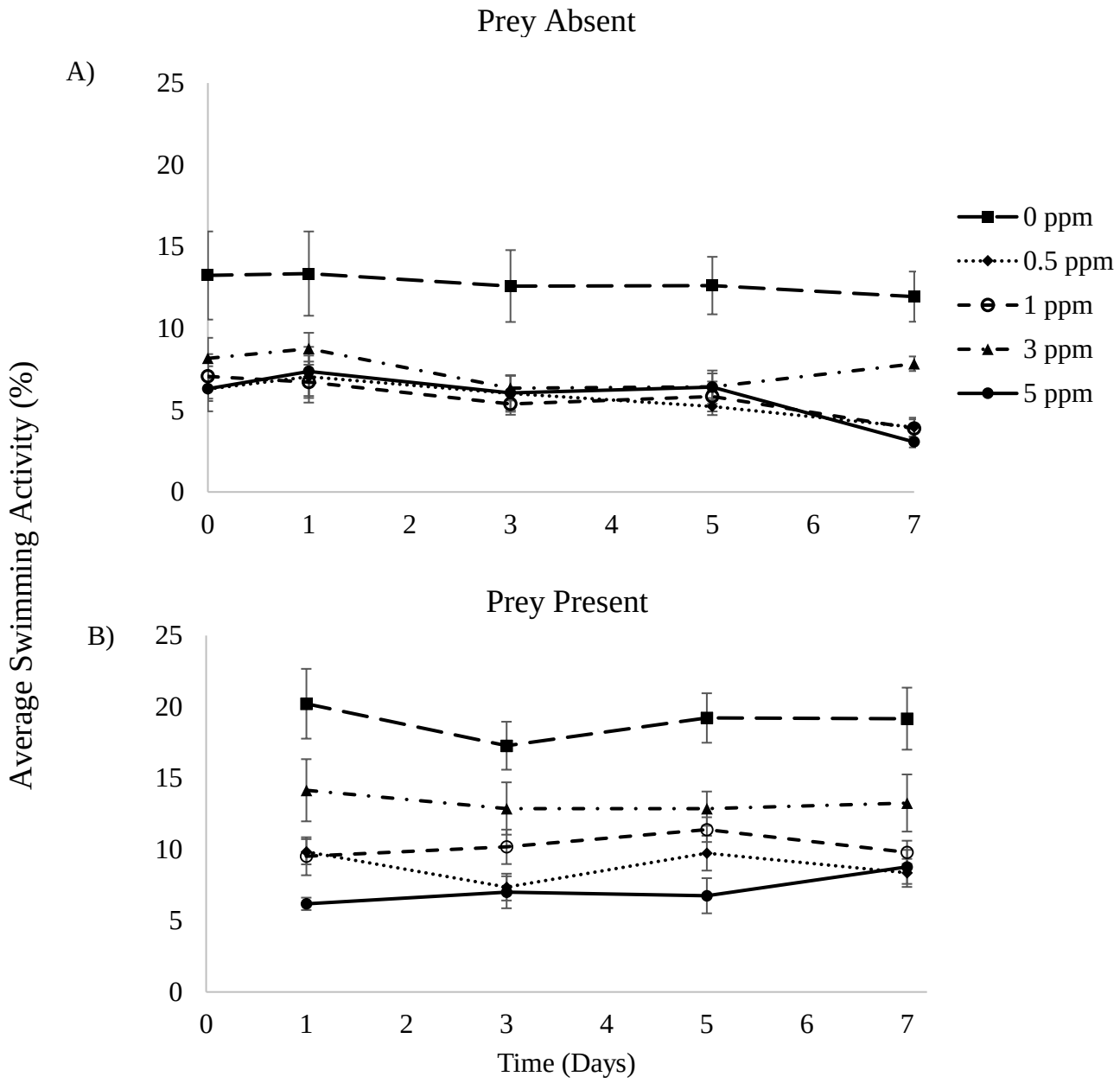

Figure 4. Average zebrafish swimming activity (%) over 7 days of exposure to one of 5 MCHM concentrations without (A) or with (B) prey. Error bars are ± one standard error of the mean (SEM) and n = 9.

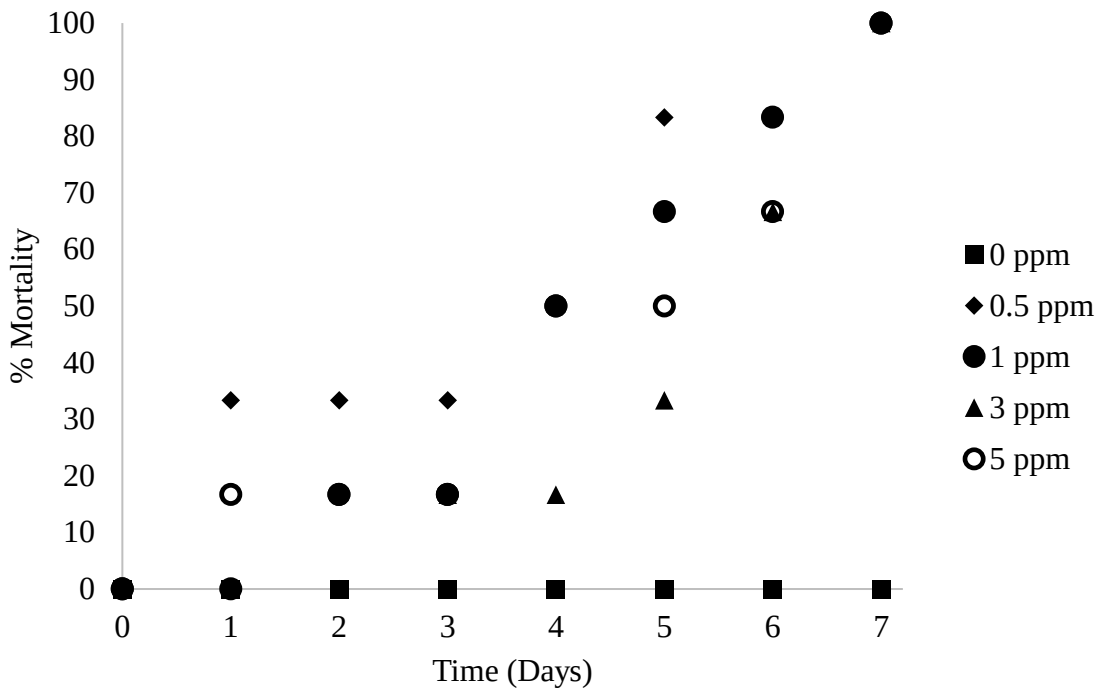

Figure 5. Average *Daphnia magna* percent mortality (%) over 3 days of exposure to one of 5 MCHM concentrations. Error bars are ± SEM and n = 6.

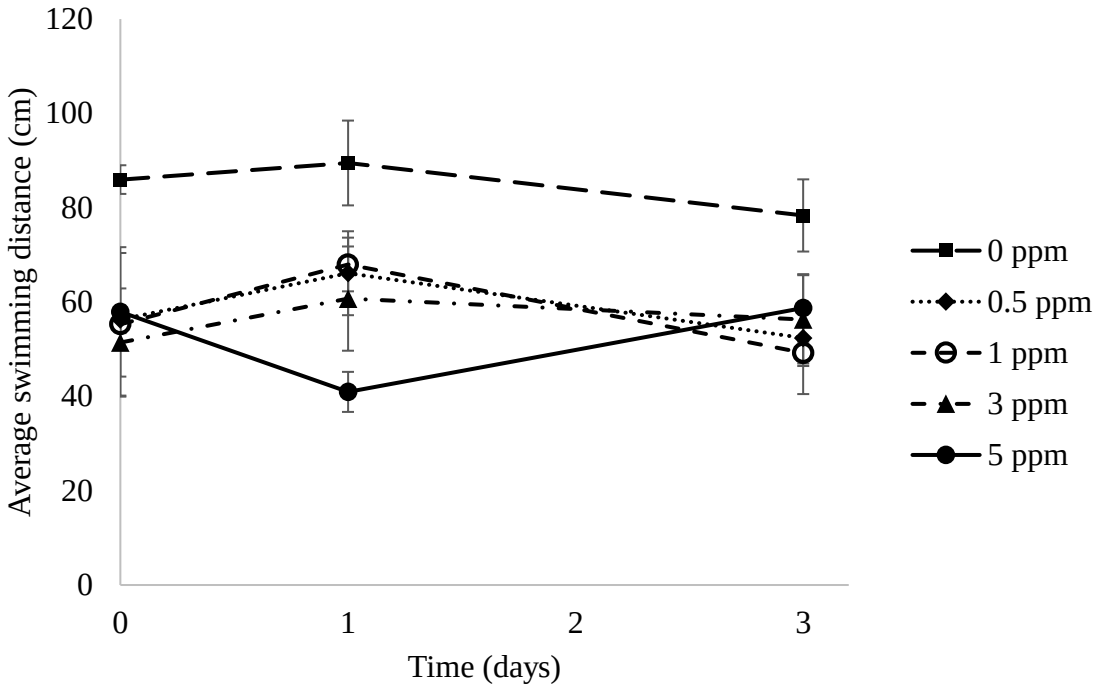

Figure 6. Average *Daphnia magna* swimming distance (cm) over 3 days of exposure to one of 5 MCHM concentrations. Error bars are ± SEM and n = 6.

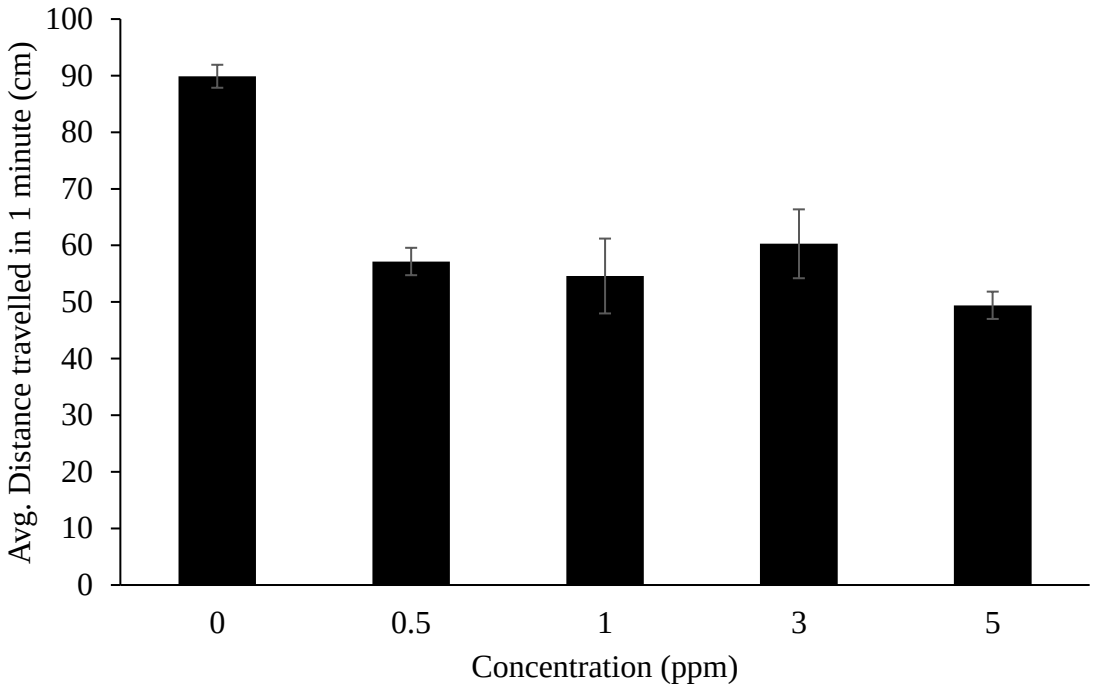

Figure 7. Average *Daphnia magna* distance travelled in one minute (cm) after 24 hours of exposure to one of 5 MCHM concentrations with a predator present. Error bars are ± SEM and n = 6.

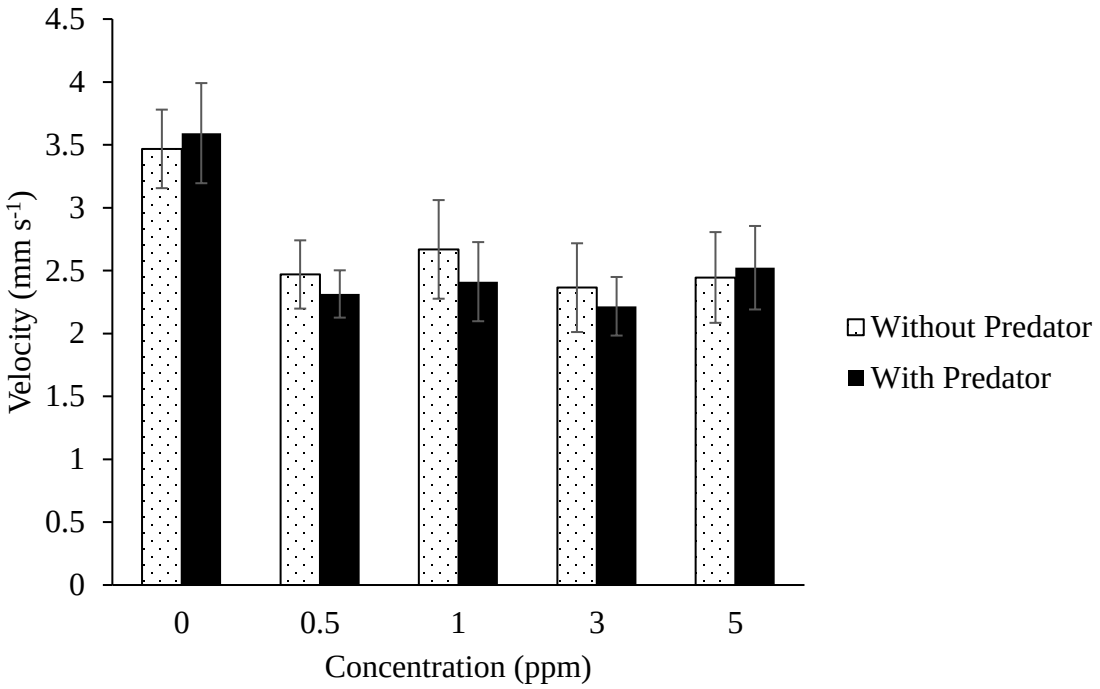

Figure 8. Average *Daphnia magna* distance travelled in one minute (cm) after 24 hours of exposure to one of 5 MCHM concentrations with and without a predator present. Error bars are ± SEM and n = 6.

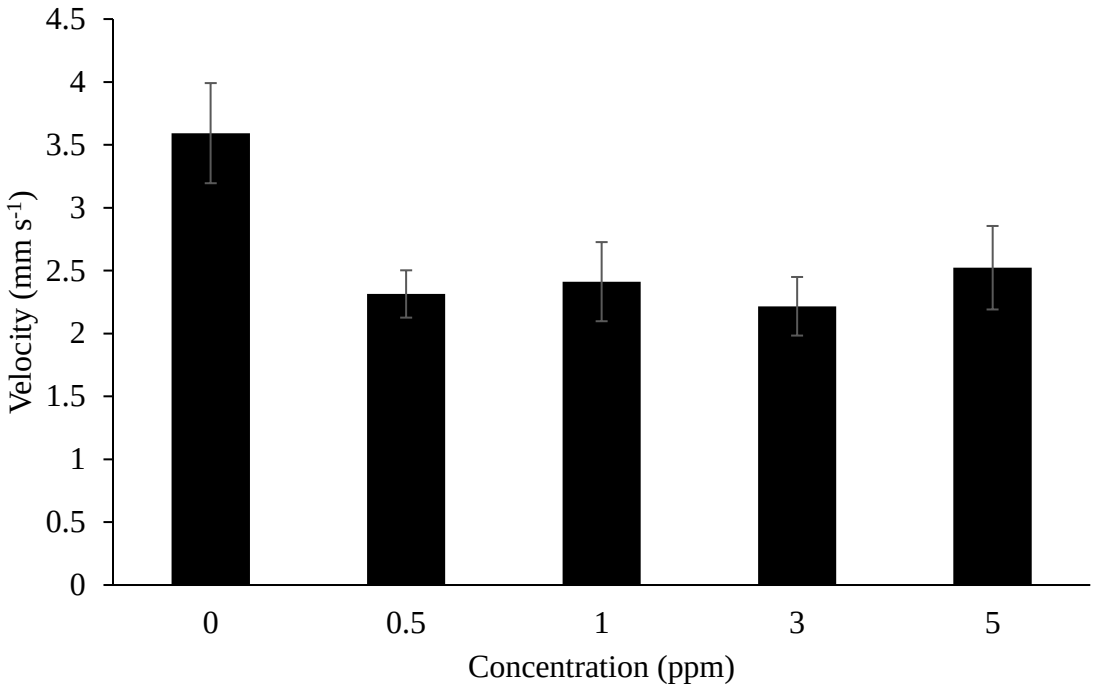

Figure 9. Average *Daphnia magna* swimming velocity (mm s^{-1}) after 24 hours of exposure to one of 5 MCHM concentrations with a predator present. Error bars are ± SEM and n = 6.

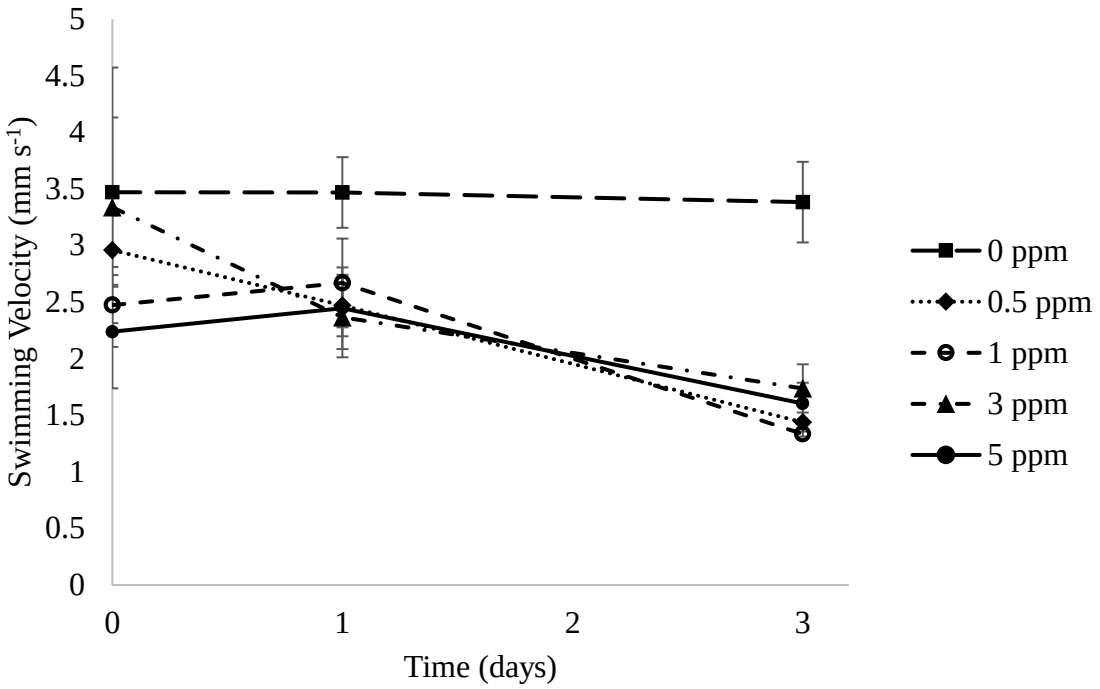

Figure 10. Average *Daphnia magna* swimming velocity (mm s^{-1}) over 3 days of exposure to one of 5 MCHM concentrations. Error bars are ± SEM and n = 6.

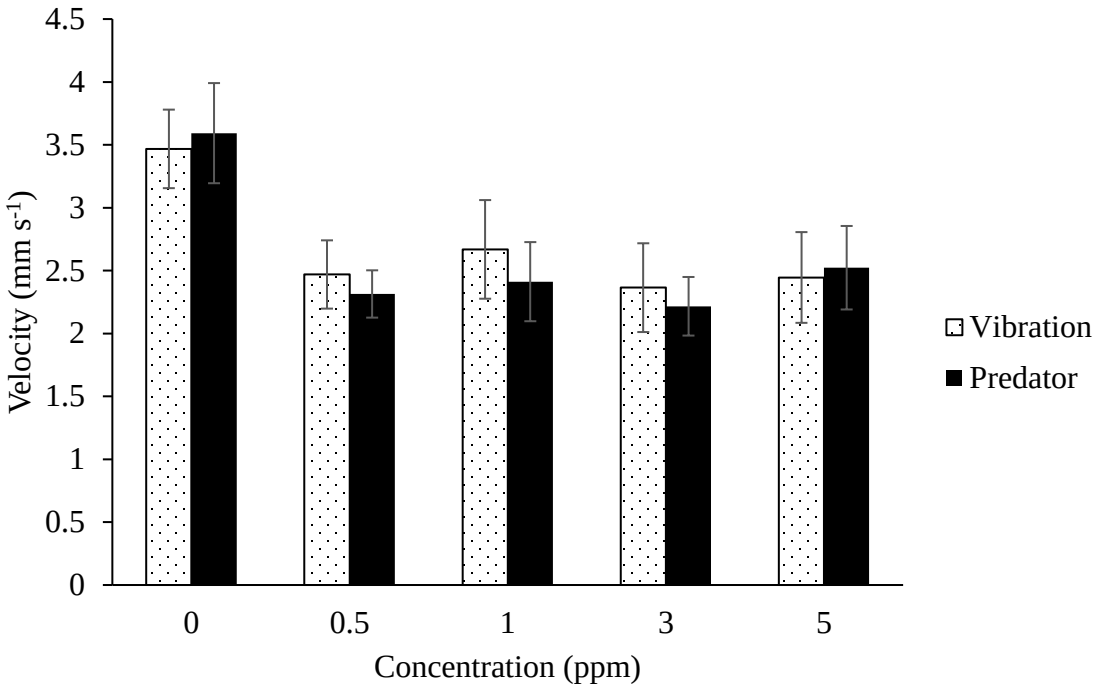

Figure 11. Average *Daphnia magna* swimming velocity (mm s^{-1}) after 24 hours of exposure to one of 5 MCHM concentrations with and without a predator present. Error bars are ± SEM and n = 6.

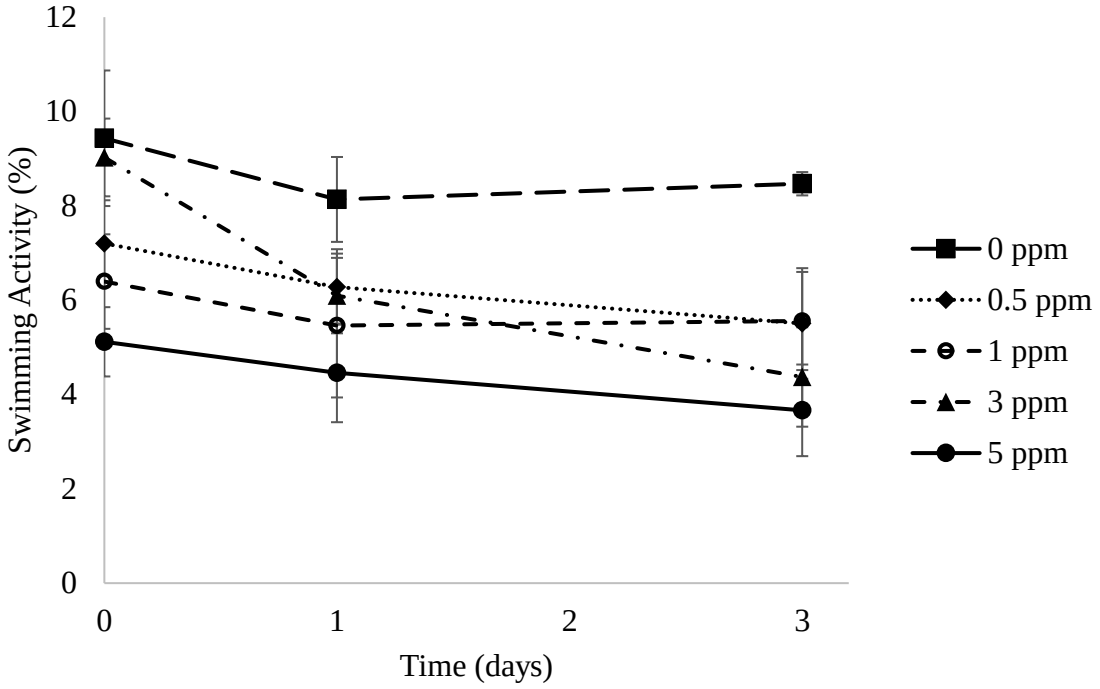

Figure 12. Average *Daphnia magna* swimming activity (%) over 3 days of exposure to one of 5 MCHM concentrations. Error bars are ± SEM and n = 6.

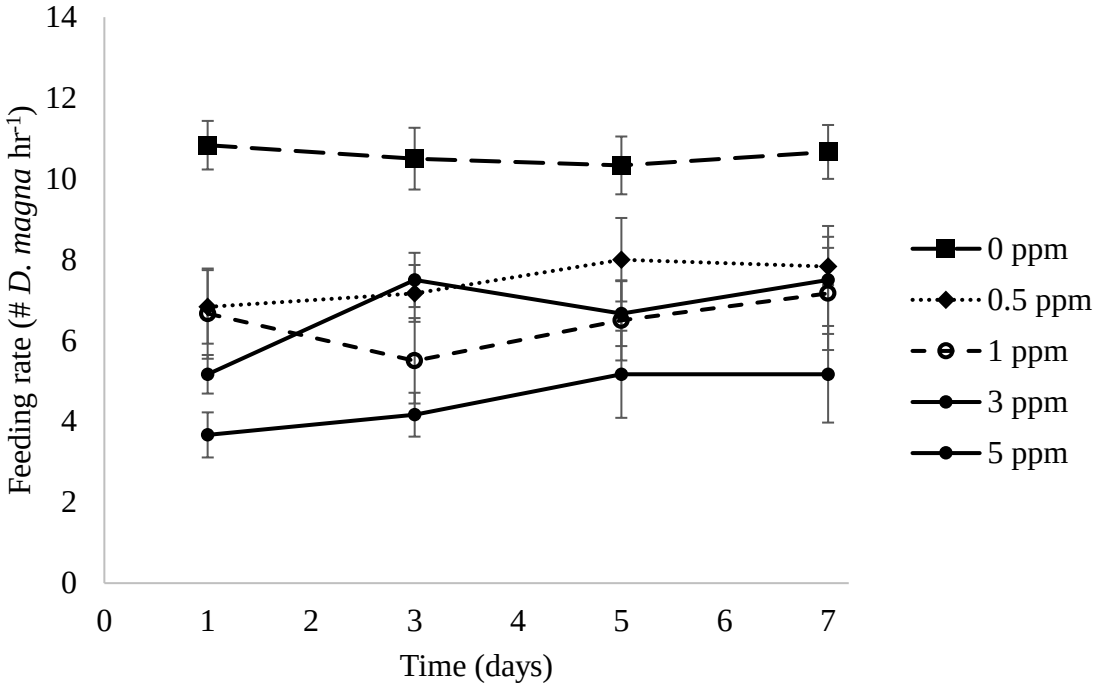

Figure 13. Average zebrafish feeding rate (# *D. magna* hr^{-1}) over 7 days of exposure to one of 5 MCHM treatments. Error bars are ± SEM and n = 6.

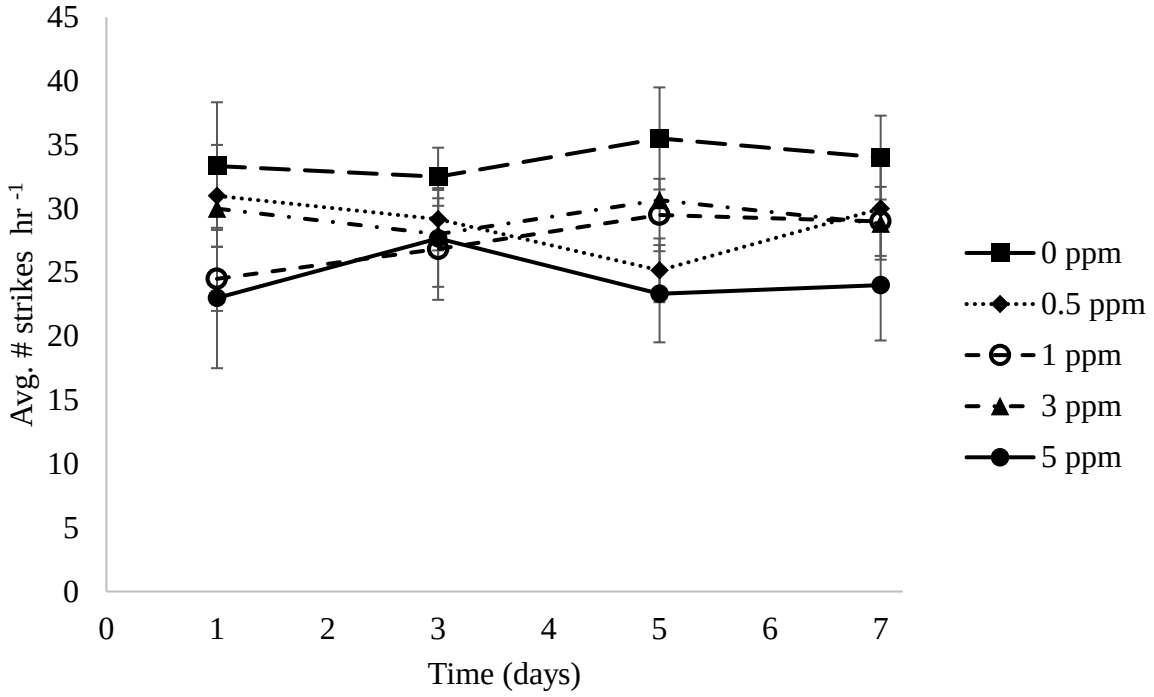

Figure 14. Average number of strikes hour^{-1} used by zebrafish over 7 days of exposure to one of 5 MCHM concentrations. Error bars are ± SEM and n = 6.

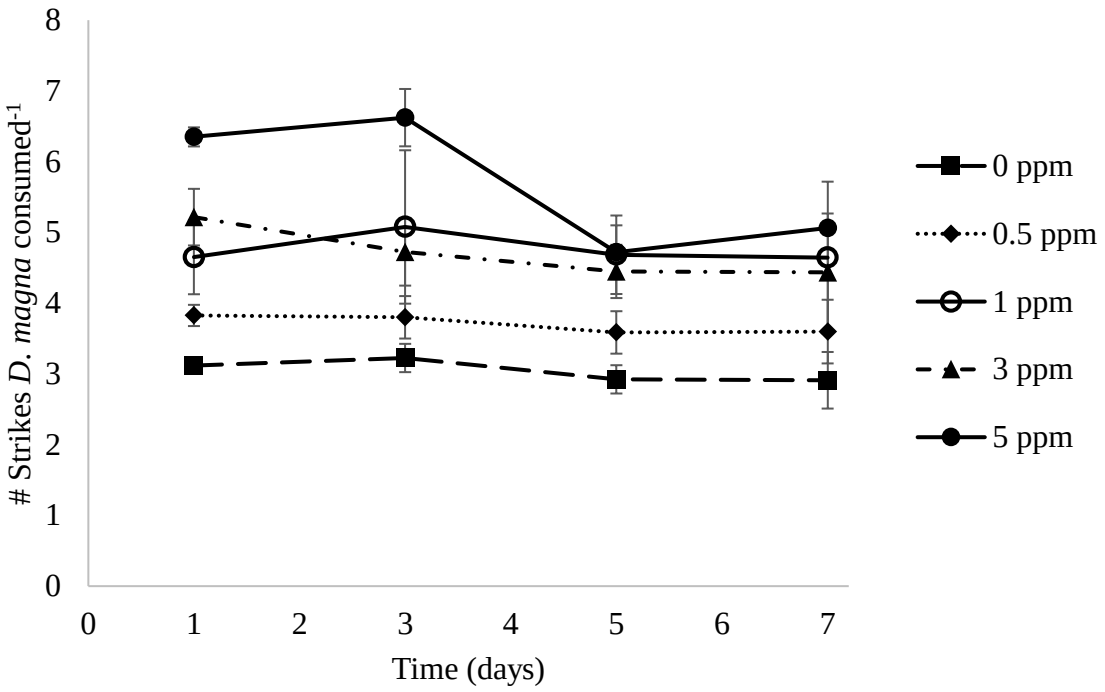

Figure 15. Average number of strikes *D. magna* consumed^{-1} used by zebrafish over 7 days of exposure to one of 5 MCHM concentrations. Error bars are ± one standard error of the mean (SEM) and n = 6.

Appendix 1

A preliminary feeding study was conducted in February 2018. The same methodology was used to conduct this experiment and the feeding study described in the methods section of this thesis, with minor alterations. The 1 ppm MCHM treatment was not used in the preliminary study, and I was unable to obtain striking or mobility data for the zebrafish and the *D. magna*.

The results from the preliminary study show that MCHM exposure decreased feeding rate in zebrafish (Table 1, Figure 1). Control zebrafish consumed an average of 10.5 ± 0.9 *D. magna* in one hour. Zebrafish in the 0.5 and 3 ppm treatments consumed 3% less than the control. There was no difference between these treatments (All Pairs, Tukey-Kramer post-hoc multiple comparisons, $p < 0.05$). Zebrafish in the 5 ppm treatment consumed an average of 3.16 ± 0.6 *D. magna* in one hour, eating approximately 60% less *D. magna* than the control. Effects were seen immediately when the zebrafish were exposed, and there was no change in feeding rate over time.

Table 1. Analysis of zebrafish feeding rate (two-way repeated measures ANOVA) during MCHM exposure

Experiment	Factor	df (Effect, Total)	F Ratio	P Value
Zebrafish Feeding Rate	MCHM	3, 20	24.39	<0.0001
	Time	3, 60	2.8	0.0657
	Time*MCHM	9, 60	0.5	0.87

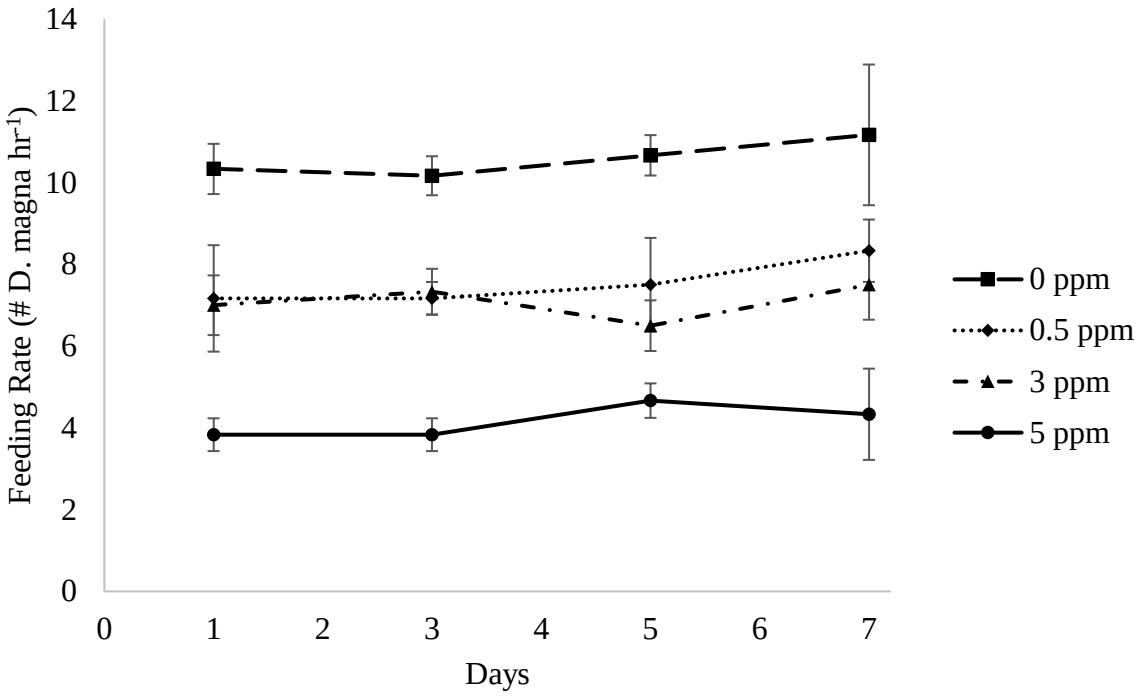

Figure 1. Average zebrafish feeding rate (# *D. magna* hr^{-1}) over 7 days of exposure to one of 4 MCHM treatments. Error bars are ± SEM and n = 6.