Zebrafish as a Model System for Research and Teaching
2019 *Eastern Biologist* Special Issue 1:101–116

4-Methylcyclohexanemethanol (4-MCHM) Influences *Danio rerio* (zebrafish) Larval Behavior and Plankton Community Composition

Ashley Williams[1], Danielle Turner[1], Rebecca Li[1], Danielle Lott[1], Steven F. Riera[1], Anna Wagner[1], Abid Shaikh[2], Risa A. Cohen[1*], and Vinoth Sittaramane[1*]

Abstract - 4-Methylcyclohexanemethanol (4-MCHM) spilled into the Elk River in Charleston, WV, USA, in early 2014. While the local human population experienced adverse dermatological and gastrointestinal effects and toxicity assays were performed on a variety of organisms, the full public health and ecological consequences of 4-MCHM exposure remains unknown. The goals of this study were to examine 1) the effects of 4-MCHM on development and behavioral of zebrafish larvae and 2) the toxicity of 4-MCHM to the plankton community which provides food resources to fish. Five-day zebrafish developmental toxicology assays tracked morphological, physiological, and behavioral changes induced by 4-MCHM and identified the concentration that induced lethality to 50% of the exposed population (LC_{50}). We observed significant increase in *hsp70* and decrease in *cyp1a* and *p53* expression in 4-MCHM treated larvae indicating potential oxidative stress. Severe behavioral changes such as reduced swimming occurred at environmentally relevant concentrations of 1 and 5 ppm, while 10 ppm 4-MCHM induced sedation in zebrafish larvae due to potential Na^+ channel interference. Plankton communities were exposed to environmentally relevant concentrations of 4-MCHM for one week to examine changes in abundance and community composition. Concentrations of 0.5 and 1 ppm decreased phytoplankton abundance and shifted zooplankton species composition from larger to smaller-bodied taxonomic groups. This potential for behavioral changes in fish that affect foraging coupled with altered trophic structure in aquatic systems indicates ecological consequences of 4-MCHM that can inform the development of risk assessments and improve safety measures.

Introduction

On January 9, 2014, more than 10,000 gallons of 4-Methylcyclohexanemethanol (4-MCHM), an industrial coal processing and cleaning chemical, spilled into the Elk River upstream of the Kanawha County municipal water intake in Charleston, WV, resulting in reports of rashes, nausea, diarrhea, vomiting, and other symptoms from human residents in the area (CSB 2017, Phetxumphou et al. 2016, Schade et al. 2015, Thomasson et al. 2017). Though the spilled mixture contained propylene glycol phenyl ether (PPH), dipropylene glycol phenyl ether (DiPPH), and 4-MCHM, 4-MCHM comprised 69–89% of the mixture (Eastman 2016a, b; Horzmann et al. 2017; Whelton et al. 2017). Furthermore, some 4-MCHM remained in pipes and carbon filters allowing continued release into the water supply. Thus, there is considerable interest in studies focused on potential health and ecological impacts of 4-MCHM and its metabolites (CDC 2014a, b; NTP 2015a, b, c; NTP 2016a, b, c; Pastenbauch et al. 2015).

Existing data suggests crude MCHM (mixture of *cis* and *trans* 4-MCHM) is a dermal and eye irritant and depresses central nervous system (CNS) activity in mice, rats, and is moderately harmful to fathead minnows (Eastman 2016a, b; Johnson et al. 2017; Terhaar 1977). Because of the potential deleterious effects of 4-MCHM, toxicity, behavioral assays, and

[1]Department of Biology, [2]Department of Chemistry and Biochemistry, Georgia Southern University, Statesboro, GA 30460. [*]Corresponding authors: vsittaramane@georgiasouthern.edu and rcohen@georgiasouthern.edu

mechanistic studies are needed to understand the transient and chronic effects of 4-MCHM on the CNS. Recent studies conducted by the National Toxicology Program (NTP) using up to 128 ppm 4-MCHM did not identify significant changes in developmental, morphological, or touch response endpoints in zebrafish (NTP 2015a, b, c; NTP 2016a, b, c). Photomotor response (PMR) assays conducted at 24 hours post fertilization revealed 11 ppm 4-MCHM induced defects in embryo movements inside the chorion in response to light pulses (NTP 2015a, b, c). While the PMR assay is a robust indicator of embryo movement, it may not truly reflect larval or adult swimming behavior. Horzmann et al. (2017) observed that 4-MCHM, crude MCHM, and the tank mixture from the spill site were all lethal to zebrafish larvae at 100 ppm, and doses of 1 ppm and above were able to induce morphological alterations and decreased activity in zebrafish larvae at 120 hpf. These findings are interesting, however, further investigations are required to identify potential mechanisms underlying the behavioral alterations and their implications in the toxicological and ecological context.

The 4-MCHM release contaminated the Elk River, therefore assessing effects of 4-MCHM on aquatic ecosystems is important. Surface water concentrations 3 days after the spill ranged from 6–36 ppm and concentrations of 3.4 ppm persisted for ~20 days. MCHM was detected in the sediments 10 months past the date of the spill (Cozzarelli et al. 2017). Thus, it is imperative that both acute and chronic effects of 4-MCHM on aquatic microorganisms be investigated (Lan 2015, Paustenbach et al. 2015). Previous MCHM concentrations reported to impair *Daphnia magna* were high (48-hr EC_{50} of 98 ppm and NOEC of 50 ppm) (Dunstan et al. 1975, Terhaar 1977). However, more recent studies after the 2014 spill revealed 48-hr EC_{50} of 57 ppm and NOEC of 6.25 ppm, within the range of observed environmental concentrations (Foreman et al. 2015, Whelton et al. 2015). Despite this renewed interest in MCHM, all testing on freshwater plankton to date appears to have been conducted using a single species (*D. magna*) over acute time frames.

MCHM exposure has the potential to influence a planktonic species at environmentally relevant concentrations (CDC 2014b, Ott et al. 1978). This indicates a need to examine community-level effects. Removal of even one zooplankton taxonomic group, like copepods, can increase the abundance of their prey (e.g., rotifer and cladoceran grazers), ultimately decreasing phytoplankton availability (Byron et al. 1984, Lay et al. 1985, Lynch 1977). Furthermore, not all species within a group likely have the same sensitivity to MCHM; for example, some copepod species suffered increased mortality rates following 30 d of exposure to hydrocarbons while others increased in abundance (Millward et al. 2004). Replacement of larger zooplankton groups (e.g., copepods) with smaller groups (e.g., rotifers) can reduce abundance and quality of food for visual predators such as planktivorous fish that rely on larger bodied plankton (Brooks and Dodson 1965).

The focus of this study was to investigate 4-MCHM behavioral toxicity using zebrafish (*Danio rerio*) as the model organism and potential effects to aquatic food webs by examining changes in plankton abundance and species composition. This multifaceted approach provides valuable information about the effects of 4-MCHM on cellular, organismal, and population levels and the potential for decreases in overall plankton abundance and loss of particular planktonic groups to alter resource availability to higher trophic levels.

Methods

Zebrafish husbandry and chemical treatments

Embryos were obtained from a breeding colony of wild-type AB strain laboratory zebrafish (*Danio rerio*). Adult zebrafish were bred according to previously established protocols (Westerfield 2007). Embryos were collected immediately after spawning and reared in E3

(Embryo medium) at 28°C for 120 hours post fertilization (hpf). Treatments were created using purified 4-MCHM (100 parts per million (ppm) stock solutions of >98% purity, CAS 34885-03-5, TCI-America). Different concentrations of 4-MCHM were prepared as required with filtered E3. Embryos were treated in uncoated 6-well or 24-well plates (Falcon #353046 or #353226) with appropriate E3 (untreated control) or Dimethylsulfoxide (DMSO) treated controls. This work was approved by the institutional animal care and use committee of Georgia Southern University.

Zebrafish behavior and motor response assays

To test the effects of 4-MCHM on zebrafish larval behavior, we performed assays using a high throughput behavior analyses system (DanioVision with Ethovision 11 software, Noldus Information Technologies, Inc. Netherlands). Five biological replicates (n = 5) were performed with three treatments (1, 5, and 10 ppm of 4-MCHM) and three controls (1, 5 and, 10 ppm DMSO). Briefly, 5 days post fertilization (dpf) free-swimming larvae with swim bladders were selected and exposed to 1, 5, or 10 ppm concentrations of 4-MCHM or a solvent control Dimethylsulfoxide (1, 5, and 10 ppm DMSO) for 3 hours. All treatments were performed in 24-well plates at 28°C and placed inside the DanioVision Observation Chamber for recording of infrared activity. Behavior recording occurred over 30-minute acclimatization, 60-minute dark, 30 minutes light, 60 minutes dark, and 90 minutes light periods. Alternating periods of light and dark were chosen to simulate day and night, thereby influencing locomotion. Video recordings were used to quantify behavioral differences between DMSO and 4-MCHM treated larvae. The software was used to measure distance moved, velocity, and to create heat maps of larval locomotion within each well.

To determine the effects of 4-MCHM on larval motor response, we tested the escape or touch response of 5 dpf larvae after exposure to DMSO or 4-MCHM at 10 ppm for 3 hours (Budik and O'Malley 2000). Larvae in glass depression slides containing test solution were placed under a microscope and touched using a pin while recording responses for one minute, using a Zeiss monochrome camera attached to the microscope. Finally, larvae washed with embryo medium three times for 5 minutes each were observed to determine whether 4-MCHM effects could be reversed.

Quantitative PCR analysis

For quantitative PCR assays, total RNA from pooled zebrafish larvae samples were extracted using TRIzol reagent (#1559626, Thermo Fisher Scientific Inc, USA), according to standard procedures. The concentration of RNA was determined using a NanoDrop™ (Thermo Fisher Scientific, USA). Total 1 µg RNA was reverse-transcribed to cDNA using Superscript III First-Strand Synthesis SuperMix (# 18080400, Thermo Fisher Scientific, USA) according to kit instructions. Real-time PCR with SYBR green detection was performed using an Applied Biosystems™ QuantStudio™ 6 and 7 Real-time PCR system (Thermo Fisher Scientific, USA) using standard protocols. Reactions were run in triplicates in three independent experiments. Expression of selected biomarkers (*hsp70*, *cox17*, *catalase*, *cyp1a*, and *p53*) in 4-MCHM treated zebrafish larvae samples were determined as log2 ratio (fold change) compared to untreated E3 controls. Gene-specific primers from published zebrafish primer sequences (Table 1) were obtained from Integrated DNA Technologies, Coraville, IA. PCR efficiencies for each primer were determined from a standard curve using dilutions of embryo cDNA ($R^2 > 0.98$ for all primers). To calculate expression levels, normalization was performed by subtracting the mean threshold cycle (Ct) value for the housekeeping gene β-actin gene, from the Ct value of the target biomarker (ΔCt value). The ΔCt values of

treated samples were calibrated against the untreated ΔCt values for all biomarker genes. The relative expression of biomarker molecules relative to the untreated control was calculated by 2−ΔΔCt. The mRNA expression level of the different biomarker genes are expressed as fold change (log2) according to the formula: $2^{-(\Delta Ct(\text{treated sample})-\Delta Ct(\text{untreated sample})}$ and presented in Figure 1. Experiments were performed at least three times and standard errors and means were calculated (*P < 0.01).

CoroNa Green assay

To visualize *in vivo* Na^+ ion distributions in zebrafish larvae, CoroNa Green, AM, cell permeant dye (C36676, ThermoFisher Scientific, USA) resuspended in DMSO was used to stain zebrafish ionocytes at 3 dpf. Ionocytes are specialized cells distributed over the yolk during early larval stages that accumulate ions for homeostasis. Dye solution was prepared by adding 100 µl of resuspended CoroNa Green dye to 3 mL of E3 embryo medium with or without 4-MCHM. Zebrafish larvae were incubated in the dye solution at room temperature for 30 minutes and then observed for green fluorescence indicating Na^+ binding at 488nm using the Zeiss Axio fluorescent microscope. Embryos were then mounted onto depression slides in 3% methylcellulose. Mounted embryos were analyzed and imaged using confocal microscopy Zeiss LSM 710 with ZEN Black software.

Plankton community

To test whether 4-MCHM affects aquatic plankton communities at concentrations measured in the environment, assemblages of freshwater plankton were exposed to concentrations of 0, 0.5 or 1 ppm 4-MCHM. Water containing plankton was pumped from a pond located on the Georgia Southern University campus, Statesboro, GA, USA (32.41° N, 81.78° W) and transported to the Biological Sciences greenhouse where 10 L aliquots were dispensed into each of 24, 20 L cylindrical opaque plastic microcosms. Treatments were created using purified 4-MCHM added to individual microcosms. Each microcosm was assigned to one of the three 4-MCHM treatments, one of two sampling time points (initial or one week) with fourfold replication and randomized by location in the greenhouse.

Table 1. Primers for Biomarkers

Target	Primer Sequences	Gene bank number
β-actin	F:5'-CGA GCA GGA GAT GGG AAC C-3' R:5'-CAA CGG AAA CGC TCA TTG C-3'	BC063950 (Hill et al. 2005)
catalase	F:5'-AGG GCA ACT GGG ATC TTA CA-3' R:5'-TTT ATG GGA CCA GAC CTT GG-3'	AF170069 (Cruz et al. 2013)
cox17	F:5'-GCA GCG CAG AAA AAG CCA CT-3' R:5'-ACG CAA GCA GTC ACA CAC AT-3'	NM001004652 (Alsop and Wood 2011)
cyp1a	F:5'-CGT AAT CTG CGG GAT CTG TT-3' R:5'-TTC TCA TCG GAC ACT TGC AG-3'	BC094977.1 (Handley-Goldstone et al. 2005)
hsp70	R:5'-CAA GAA TCC CAA AGA AGC T-3' F:5'-AGG GTT AAT GCT CTT GTT CAG TTC T-3'	AF210640.1 (Hill et al. 2005)
p53	F:5'-CTG AAG TCC GCA GAT G-3' R:5'-CGT TTG GTC CCA GTG GTG G-3'	XM_005165101.2 (Shi et al. 2008)

Initially and after one week of exposure, on October 31 and November 7, 2014, the microcosms were stirred gently to homogenize plankton that might have settled, and a 100 mL water sample was collected to determine phytoplankton abundance using pigment concentration (chlorophyll *a*) as a proxy. Water samples were vacuum filtered to concentrate algal cells on Whatman GF/F glass fiber filters (nominal pore size 0.7 µm). Pigments were extracted from the collected cells in 90% acetone, followed by analysis using a TD Trilogy fluorometer (Turner Designs, Sunnyvale, CA) according to EPA method 445.0 (Arar and Collins 1997). The remaining volume in each microcosm was filtered through a plankton net (80 µm mesh) to concentrate all of the zooplankton into a 40 mL sample and preserved in 70% ethanol for subsequent identification using microscopy. The zooplankton samples were then stained with Rose Bengal, followed by counting and identification in a Bogorov chamber under a dissecting microscope at 60x (Goswami 2004, Parsons et al. 1984). Copepods were identified to order, cladocerans to genus, and rotifers to family (Postel et al. 2000).

Analytical verification of 4-MCHM

We determined the concentration of 4-MCHM stock solutions used for treatment using NMR spectrometry and GC/MS (Foreman et al. 2015).

NMR Spectrometer method. A 1L stock aqueous solution of 25 ppm 4-MCHM was extracted in dichloromethane. A known amount of toluene (10 µL, 8.65 mg) was then added as internal standard to the above extracted sample. The mixture was then dissolved in deuterated chloroform and 1 H NMR spectra was recorded on a 400 MHz NMR spectrometer. For quantification analysis, methyl signal (δ 2.34) from toluene was compared against methyl signal (δ 0.90) of 4-MCHM and the ratio was found to be (1:2.26). The actual concentration of 4-MCHM was determined as 19.54 milligrams per liter.

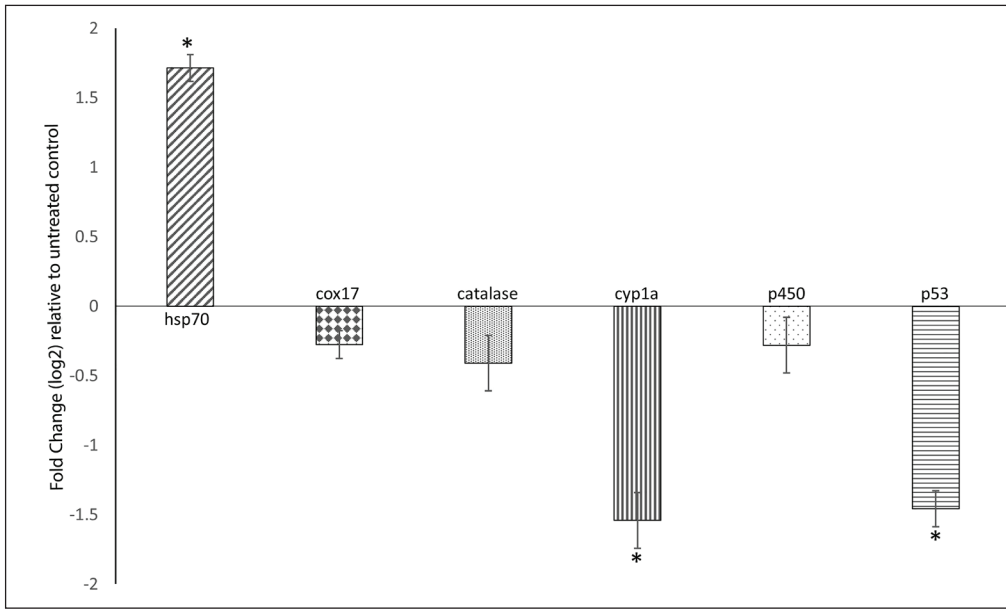

Figure 1. 4-MCHM Affects Toxicity Biomarker Expression in Zebrafish Larvae. Relative fold changes of toxicity biomarker genes in 10 ppm 4-MCHM treated zebrafish larvae compared to untreated controls. Results are presented as fold changes (log2 ratio) of selected biomarker genes, *hsp70*, *cox17*, *catalase*, *cyp1a* and *p53* normalized to housekeeping gene *β-actin* levels. Values represent means ±SD (n = 3, biological replicates). Asterisks indicate significantly increased or decreased expression levels ($p < 0.01$).

GC/MS method. A 10 ppm 4-MCHM solution was made using 10.9 microliters of 4-MCHM for every liter of distilled water. This stock was then diluted to create the remaining concentrations; 200 milliliters of 10 ppm stock was mixed with 1800 mL of distilled water to create 2 liters of 1 ppm solution. This mixture was then bottled in 1-liter glass amber bottles (Fisher Scientific, USA) and sent overnight on ice to TestAmerica, Inc in Canton, OH. TestAmerica Inc analyzed the sample by GC/MS and the concentration of 4-MCHM in the solution was determined as 0.88 ppm.

Statistical analysis

The dependent measures corresponding to each assessment are described above. All statistical analyses were performed with JMP Pro 10 (SAS Institute Inc., Cary, NC, USA). Data were tested for normality and homogeneity of variances, and log transformed if assumptions of parametric tests were not met. Lethality, heart rate per minute, hatching rate, and morphometric assessments were analyzed using one-way analysis of variance (ANOVA). The mean value of distance travelled (mm) velocity (mm/sec) were calculated from the individual values of each fish for each treatment group and compared with the DMSO control group and embryo water group using ANOVA followed by Dunnett's test for post hoc comparisons of controls to exposed groups. All data are expressed as mean ± standard error of the mean (SEM) for a group of size "n". $P < 0.001$ was considered as a criterion for statistical significance. Plankton data were tested for normality and homogeneity of variances, and log transformed if assumptions of parametric tests were not met. 4-MCHM effects on total zooplankton abundance and log-transformed chlorophyll *a* data were analyzed using ANOVA. To assess whether 4-MCHM altered zooplankton community composition by changing either the abundance or the presence/absence of multiple zooplankton species, zooplankton data were analyzed using permutational multivariate analysis of variance (PERMANOVA), followed by pairwise comparisons using PRIMER v7 with PERMANOVA+ (PRIMER-E LTD., Plymouth, UK) (Clarke and Gorley 2006).

Results and Discussion

Zebrafish embryo toxicity assay

To determine the toxicity of 4-MCHM, we exposed 6 hpf embryos to various concentrations of 4-MCHM up to 120 hpf. While concentrations of less than 100 ppm produced minimal lethality (data not shown), concentrations above 100 ppm produced consistent lethality (see Supplemental Figure S1 A, available online at https://eaglehill.us/ebioonline/suppl-files/ebio-028-Sittaramane-s1.pdf). Our results are consistent with Horzmann et al. (2017), indicating that concentrations above 100 ppm are lethal. However, Horzmann et al. (2017) did not identify an LC_{50} (Lethal concentration for 50% of the exposed population) for 4-MCHM. We therefore treated zebrafish embryos with 4-MCHM concentrations of 100, 200, 400, and 1000 ppm. These experiments identified 200 ppm as the LC_{50} for 4-MCHM, inducing consistent mortality in ~50% of the zebrafish larvae. Using 200 ppm as LC_{50}, we investigated the physiological parameters such as heart rate per minute and hatching success rate in zebrafish embryos. We determined that at 200 ppm, both heart rate per minute and hatching rate were significantly reduced compared to untreated E3 controls (Fig. S1 B and C). We further investigated the morphological changes in the developing zebrafish embryos as a result of 4-MCHM treatment (see Supplemental Figure S2, available online at https://eaglehill.us/ebioonline/suppl-files/ebio-028-Sittaramane-s2.pdf).

4-MCHM treated larvae develop pericardial edema (arrow head in Fig. S2 B). Consistent with the edema, 4-MCHM treated larvae showed significantly increased pericardial area (37.2 ± 3.2 μm^2) compared to untreated E3 controls (19.3 ± 2.2 μm^2) (Fig. S2 C). Not surprisingly, trunk length and eye diameter of the 4-MCHM treated zebrafish larvae at 120 hpf were significantly reduced compared to untreated E3 controls (Fig. S2 C). Taken together, our results were consistent with Horzmann et al. (2017), that prolonged developmental exposure of embryos to 4-MCHM at LC_{50} concentration leads to profound toxicity. However, thus far, the molecular mechanisms underlying 4-MCHM toxicity in zebrafish have not been studied. We analyzed the expression of several toxicity biomarker genes in 120 hpf zebrafish larvae treated with 4-MCHM at LC_{50} (Fig. 1). Relative fold change of *hsp70*, *cox17*, *catalase*, *cyp1a*, and *p53* expression levels compared to untreated E3 controls were identified by qRT-PCR assay. The expression of heat shock protein 70 (*hsp70*) was significantly elevated (1.75-fold, Fig. 1) in 4-MCHM treated larvae compared to E3 controls. *Hsp70* is an important marker of oxidative stress response during development (Hahn et al. 2014, Hallare et al. 2004, Hill et al. 2005, Ton et al. 2003, Yan Hu et al. 2010) and increased levels of *hsp70* in 4-MCHM treated larvae indicate a potential oxidative stress response. Expression levels of cytochrome c oxidase subunit 17 (*cox17*) and catalase (*cat*) were decreased (Fig. 1) in 4-MCHM treated larvae compared to the E3 controls, again indicating a potential oxidative stress in 4-MCHM treated larvae (Alsop and Wood 2011, Craig et al. 2007, Cruz et al. 2013, Xiong et al. 2011). Toxic agents such as dioxins and aromatic hydrocarbons are known to induce the expression of cytochrome p450, family 1 subfamily A (*cyp1a*) in zebrafish embryos (Bugel et al. 2013, Handley-Goldstone et al. 2005, Liu et al. 2016, Sloman and McNeil 2012, Voelker et al. 2008) to mediate the activation of Aryl hydrocarbon receptor (AhR). However, 4-MCHM treated larvae showed a downregulation (1.5-fold, Fig. 1) of *cyp1a* expression compared to E3 controls, indicating that 4-MCHM induced toxicity is not mediated through AhR activation. Another important mechanism of toxicity in zebrafish embryos is by induction of *p53* dependent apoptosis (Felix et al. 2018, Ho et al. 2013) of cells. However, 4-MCHM treated larvae showed a downregulation (1.6-fold, Fig. 1) of *p53* expression compared to E3 controls, indicating that 4-MCHM induced toxicity is not mediated through *p53* induced apoptosis. Taken together, our investigations confirmed the toxicity of 4-MCHM, identified the LC_{50} for 4-MCHM in zebrafish larvae, and provided the first set of mechanistic evidence underlying 4-MCHM toxicity in zebrafish larvae.

Zebrafish larval locomotor behavior and touch response assay

Doses of 4-MCHM at 1 ppm dramatically reduced the activity of zebrafish larvae during light periods (Fig. 2A). Heat map analysis of larval locomotion revealed 4-MCHM treated larvae exhibited multiple extended non-locomotion periods as indicated by areas of dark blue (Fig. 2B), in comparison to control larvae. Further, DMSO treated control larvae moved on average nearly twice the distance (19500 vs. 10000 mm) and velocity (1.8 vs. 0.8 mm sec^{-1}) of the 4-MCHM treated larvae (Fig. 2 C and D). Effects of 4-MCHM on larval locomotion also appeared to be dose dependent; increasing concentrations to 5 ppm, 10 ppm and 25 ppm resulted in more defects in movement pattern (See Supplemental Figure S3 A, B, and C, available online at https://eaglehill.us/ebioonline/suppl-files/ebio-028-Sittaramane-s3.pdf). Increasing concentrations of 4-MCHM resulted in decreased mean distance moved and mean velocity of larvae. Taken together, these data strongly suggest a dose-dependent and dynamic decrease in zebrafish larval locomotion due to 4-MCHM exposure.

The 4-MCHM-induced lack of movement in larvae at concentrations of 5 and 10 ppm suggests 4-MCHM could be triggering sedative/anesthetic effects. Typically, free-swimming

zebrafish larvae swim away from the point of external contact such as pin touch. When larvae are sedated or anesthetized, they lose this ability. DMSO treated or untreated control larvae responded to touch by moving away from the pin (Fig. 3A), while 10 ppm 4-MCHM treated larvae either failed to move away or showed complete lack of touch response (Fig. 3B). Surprisingly, even in an airtight plastic box, zebrafish larvae in clean medium next to a dish with 10 ppm 4-MCHM solution exhibited complete lack of touch response after overnight incubation compared to control larvae next to 10 ppm DMSO, likely due to volatility of 4-MCHM (Fig. 3E and F). Regardless of 4-MCHM exposure method, washing larvae with clean embryo medium resulted in recovery of touch response (Fig. 3C and D). These data indicate that both airborne and aqueous 4-MCHM exposure can induce a reversible state of sedation or anesthesia in zebrafish larvae. While the 4-MCHM induced decreased movement is consistent with the previous findings (Horzmann et al. 2017), our experiments have identified a dose dependent effect of 4-MCHM on the movement of zebrafish larvae. Horzmann et al. (2017) identified the 4-MCHM induced decrease in locomotion behavior using a shorter assay involving alternating light and dark periods of 10 minutes each. However, our experiments were performed over a period of 4 hours with at least 30–60 minutes of light and dark cycles. Our experiments reveal the decreased movement and velocity specifically in the light period could be the major cause of overall decreased locomotion. Our experiments also provide the first evidence that 4-MCHM induces a strong sedative/anesthetic effect on zebrafish larvae rather than a mere decrease in locomotion. Further, vapor induced lack of touch response and movement defects have not been reported.

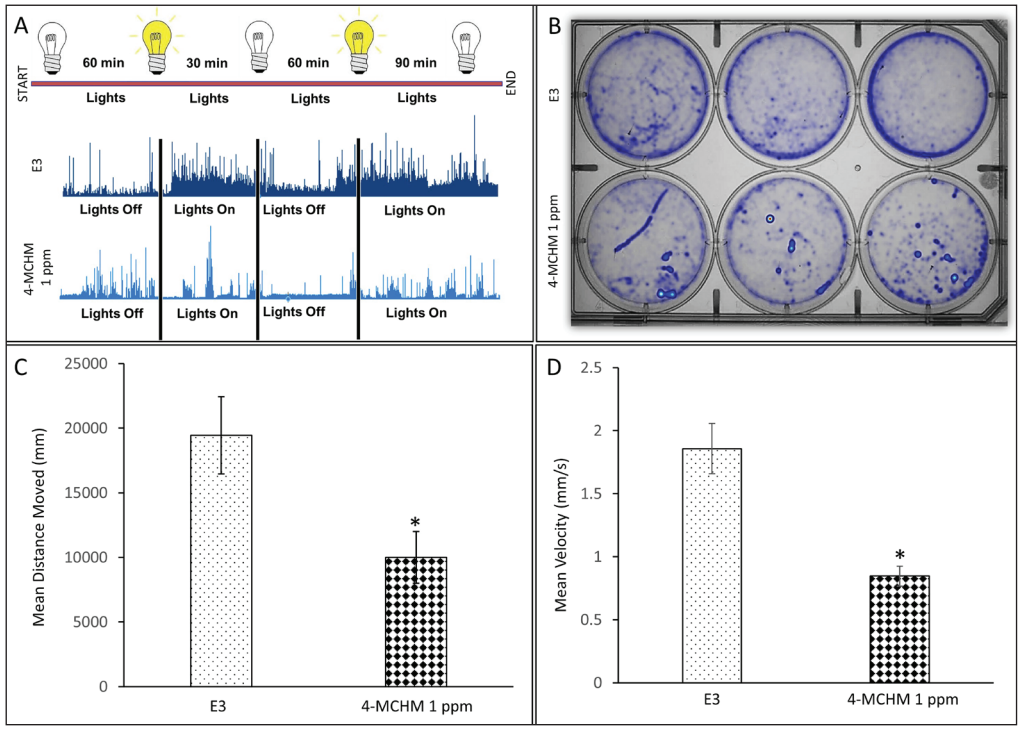

Figure 2. 4-MCHM Reduces Zebrafish Larval Locomotor Behavior. 4-MCHM (1 ppm) exposure reduced zebrafish larval locomotor behavior compared to controls (1 ppm DMSO). (A) Larval activity pattern under light and dark phases (n = 27), (B) Heat map visualization of larval activity (n = 24), and (C, D) Total distance (mm) and mean velocity of larval movement (mm/sec), respectively (n = 24). Asterisks indicate significantly reduced movement and velocity ($p < 0.001$).

4-MCHM impairs Na^+ influx

Interference with voltage gated sodium and other ion channels have been shown to induce rapid anesthetic effects (Scholz 2002). The rapid onset of sedative/anesthetic effects of 4-MCHM led us to test if 4-MCHM interferes with Na^+ movement across cell membranes using mitochondria rich cells (MRC) or ionocytes on the epidermis of zebrafish larvae. Ionocytes allow ion homeostasis in freshwater fishes by retaining salts within these cells and excreting excess water that enters the body (Hwang and Chou 2013). There are four different types of ionocytes in zebrafish larvae, but the H^+-ATPase rich (HR) ionocytes are primarily responsible for Na^+ accumulation using a Na^+/H^+ exchanger and H^+-ATPase (HA) channels (Hwang PP 2009). As expected, larvae reared in distilled water did not accumulate

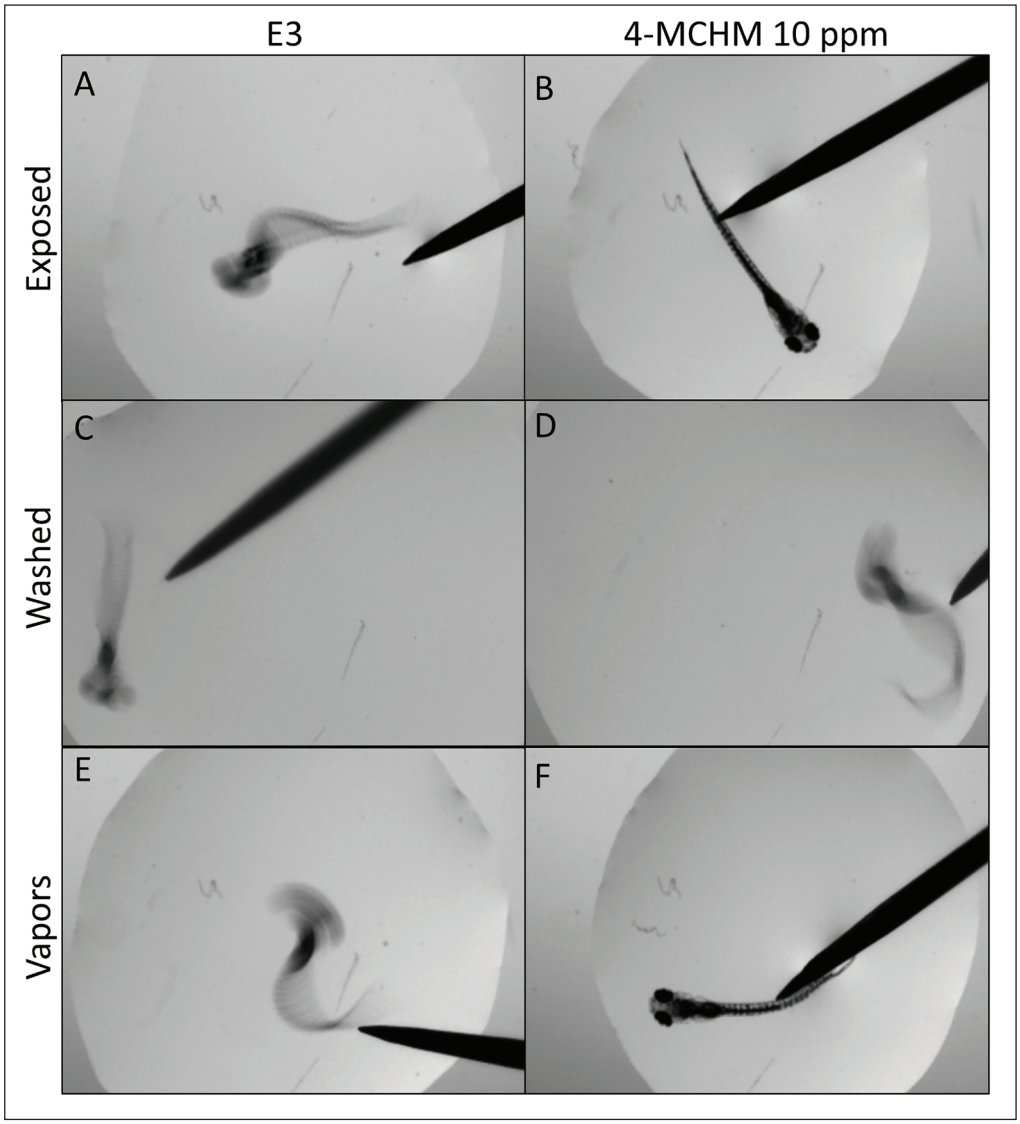

Figure 3. 4-MCHM Induces Anesthetic effects on Zebrafish Larvae. Zebrafish larval escape response to touch following exposure to 10 ppm DMSO (A, C, E) or 10 ppm 4-MCHM (B, D, F) (n = 10). Washed larvae recovered from anesthetic effect caused by 4-MCHM (D). DMSO vapors did not affect larval behavior (E), but 4-MCHM vapors induced an anesthetic effect (F).

Na^+ and therefore the HR cells were not stained with CoroNa Green (Fig. 4A) while the HR cells in DMSO treated control larvae were stained (Fig. 4B). In contrast, 4-MCHM treated larvae could not accumulate Na^+ (Fig. 4C), but recovered and accumulated Na^+ after being washed in embryo medium. Furthermore, when ionocytes were exposed to Tricaine, a commonly used anesthetic for zebrafish larvae that may function differently from 4-MCHM (Attili 2014), they were able to accumulate Na^+ (Fig. 4D). These results strongly suggest that 4-MCHM is a potent broad-spectrum Na^+ channel blocker capable of interfering with Na^+ transport across cell membranes and acts differently than Tricaine.

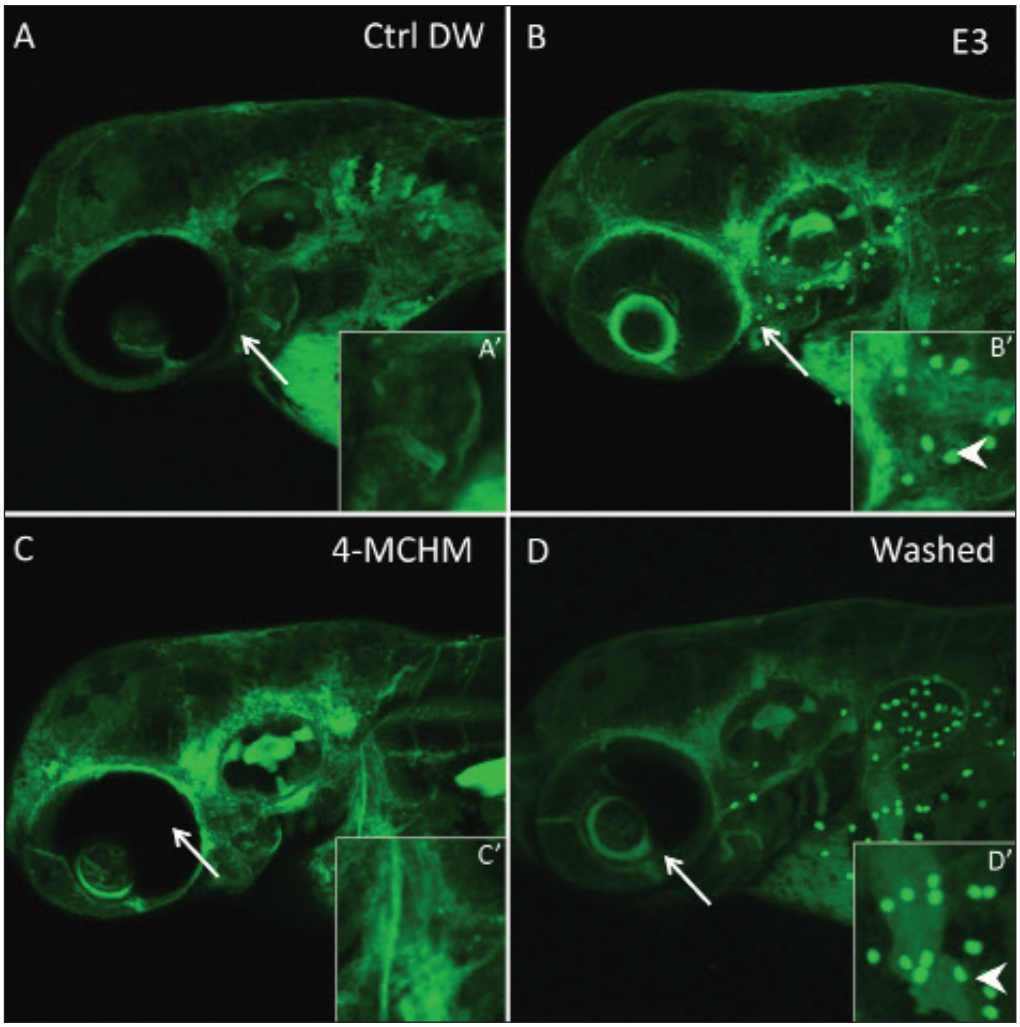

Figure 4. 4-MCHM Blocks Sodium Influx. All panels show lateral views of 3 dpf zebrafish larvae with CoroNa Green stained ionocytes (A-D). Insets show a higher magnification of stained ionocytes or lack thereof (A'-D'). (A) Zebrafish larvae reared in distilled water (n = 8) shows no stained ionocytes (A') due to lack of sodium ions, (B) larvae reared in embryo medium and exposed to DMSO (n = 8) shows stained ionocytes (white arrowhead, B') containing sodium ions, and (C) larvae raised in embryo medium treated with 4-MCHM (n = 10) shows no stained ionocytes (C') due to interference with sodium influx. Washed 4-MCHM treated embryos recovered and were able to allow sodium ion influx and stain ionocytes (white arrowhead, D', n = 10).

Plankton community

4-MCHM altered zooplankton community composition (PERMANOVA, Pseudo-$F_{2,11} = 4.70$, $p = 0.016$) rather than total zooplankton abundance (one-way ANOVA, $F_{2,11} = 1.13$, $p = 0.37$). Although the same taxonomic groups were present before and after 4-MCHM exposure, communities exposed to 0.5 and 1 ppm 4-MCHM differed from the control ($p < 0.05$) in terms of relative abundance of the species present. Copepods (adults and nauplii) comprised ~75% of the community in the control and decreased to <50% in 0.5 and 1 ppm treatments (Fig. 5). The 50–70% decrease in nauplii in 0.5 and 1 ppm treatments suggests early life stages are more sensitive to 4-MCHM, however altered adult copepod abundance also contributed to the decrease relative to the control.

One explanation for the loss of copepods could be higher sensitivity to 4-MCHM than other zooplankton groups in the community. Although copepods were generally equally sensitive to aromatic hydrocarbons and alcohols compared to cladocerans in single species toxicity tests, artificial pond communities exposed to hydrocarbons exhibited higher copepod mortality rates than cladocerans and rotifers (Kreutzweiser et al. 2002, Lay et al. 1985, Relyea 2005, Riera and Cohen 2016). In the present study, rotifers (Brachionidae) became the most abundant taxonomic group in both treatments that received 4-MCHM, increasing in proportion to 54% and 60% in the 0.5 and 1 ppm treatments respectively (compared to 18% in the control). This change may be only partially attributed to lower 4-MCHM sensitivity but enhanced by indirect effects associated with loss of copepod predators. For example, copepod removal via fish predation increases rotifer abundance, consequently altering community composition (Lynch 1977, McQueen et al. 1986). Finally, decreased food

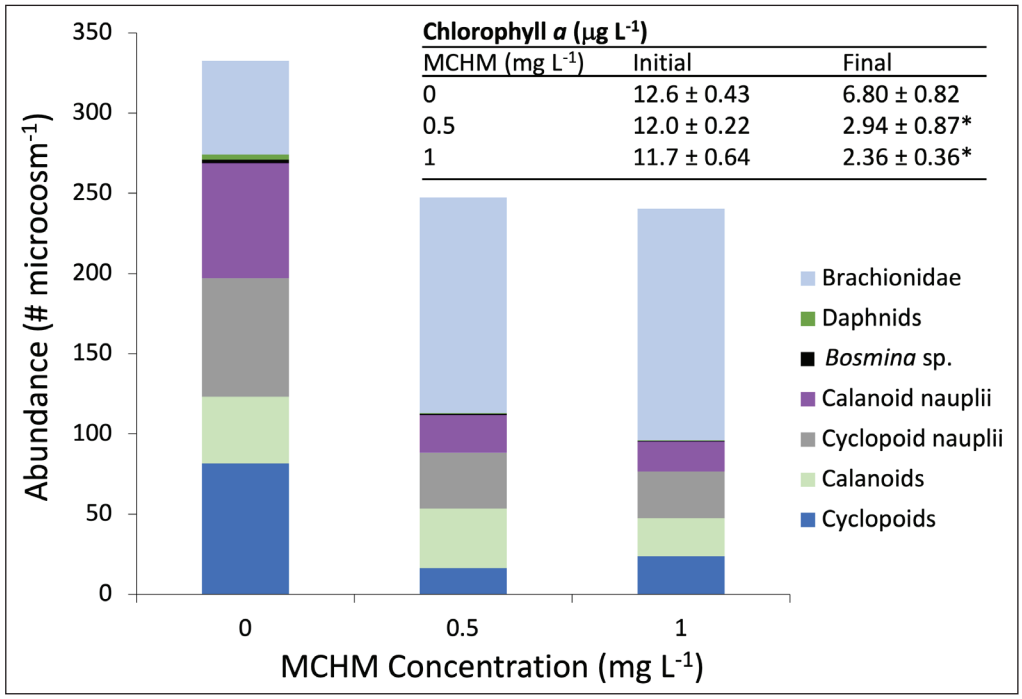

Figure 5. 4-MCHM alters Zooplankton Community Composition. Mean abundance of individuals belonging to each zooplankton group after one week of exposure to 4-MCHM treatments (n = 4 microcosms per treatment), and mean water column chlorophyll *a* concentration (µg L^{-1}) initially upon addition of 4-MCHM and after one week under greenhouse conditions ± one standard deviation (n = 4). Asterisks indicate significant differences from the control ($p < 0.05$).

availability may have contributed to copepod population decline. Phytoplankton abundance decreased across all treatments (one-way ANOVA, $F_{2,11} = 11.19$, $p = 0.004$), but chlorophyll *a* concentrations were 43 and 35% lower than the control in the 0.5 and 1 ppm treatments, respectively (Fig. 5). This decrease could have been due to direct toxicity of 4-MCHM; phytoplankton species display a range of sensitivities to aromatic hydrocarbons (Dunstan et al. 1975). However, the decrease in chlorophyll *a* concentration in the 0.5 and 1 ppm treatments coincided with increased rotifer (Brachionidae) abundance, which likely increased grazing pressure and competition for limited resources (Gilbert 1988, Lynch 1977, Sancher-Bayo 2006). 4-MCHM-induced reduction in copepod abundance and increase in rotifers could decrease food availability to higher trophic levels such as planktivorous fish that rely on vision to detect prey (Brooks and Dodson 1965).

Our findings provide important insights into the potential aquatic toxicity of 4-MCHM at the cellular (ionocytes), organism (zebrafish) and community levels in aquatic ecosystems. We have identified the LC_{50} for 4-MCHM in larval zebrafish. Expression analysis of toxicity biomarker genes strongly indicate oxidative stress in 4-MCHM treated zebrafish larvae. We identified swimming behavioral defects in zebrafish larvae due to 4-MCHM within a range of environmentally relevant concentrations that can affect their ability to evade predation and forage for food, and the likely Na^+ channel blocking neurotoxic mechanism of action for 4-MCHM. Finally, exposure to 4-MCHM concentrations at and below 1 ppm affected zooplankton community composition primarily by altering relative abundance of copepods, rotifers and phytoplankton, which has implications for resource availability to planktivorous fish like zebrafish.

Acknowledgements

This research was supported in part by the Georgia Environmental Protection Division Supplemental Environmental Project, Georgia Southern Interdisciplinary Pilot Grant, and Faculty Research Seed funds. We thank the Department of Biology at Georgia Southern University for continued support by providing space and facility to perform the experiments.

Literature Cited

Alsop D., and C.M. Wood. 2011. Metal uptake and acute toxicity in zebrafish: Common mechanisms across multiple metals. Aquatic Toxicology 105(3–4):385–93.

Arar E.J., and G.B. Collins. 1997. Method, 445: In vitro determination of chlorophyll a and pheophytin a in marine and freshwater algae by fluorescence. United States Environmental Protection Agency, Office of Research and Development, National Exposure Research Laboratory. 22 pp.

Attili S., and S.M. Hughes. 2014. Anaesthetic tricaine acts preferentially on neural voltage-gated sodium channels and fails to block directly evoked muscle contraction. PLoS One 9(8):e103751.

Brooks J.L., and S.I. Dodson. 1965. Predation, body size, and composition of plankton. Science 150:28–35.

Budik S.A., and D.M. O'Malley. 2000. Locomotor repertoire of the larval zebrafish: Swimming, turning and prey-capture. Journal of Experimental Biology 210:2526–2539.

Bugel S.M., L.A. White, and K.R. Cooper. 2013. Inhibition of vitellogenin gene induction by 2,3,7,8-tetrachlorodibenzo-p-dioxin is mediated by aryl hydrocarbon receptor 2 (AHR2) in zebrafish (Danio rerio). Aquatic Toxicology 126:1–8.

Byron E.R., C.L. Folt, and C.R. Goldman. 1984. Copepod and cladoceran success in an oligotrophic lake. Journal of Plankton Research 6(1):45–65.

CDC. 2014a. Disaster Response and Recovery Needs of Communities Affected by the Elk River Chemical Spill, West Virginia. National Center for Environmental Health, Division of Environmental Hazards and Health Effects. Health Studies Branch, Atlanta, GA USA. 66 pp.

CDC. 2014b. Information about MCHM: 2014 West Virginia Chemical Release. Emergency Preparedness and Response. Centers for Disease Control and Prevention. 27 pp.

Clarke K., R. Gorley. 2006. User manual/tutorial PRIMER-E Ltd, Plymouth 93.

Cozzarelli I.M., D.M. Akob, M.J. Baedecker, T. Spencer, J. Jaeschke, D.S. Dunlap, A.C. Mumford, A.T. Poret-Peterson, and D.B. Chambers. 2017. Degradation of crude 4-MCHM (4-Methylcyclohexanemethanol) in Sediments from Elk River, West Virginia. Environmental Science and Technology 51(21):12139–12145.

CSB. 2017. Chemical spill contaminates public water supply in Charleston, West Virginia. US Chemical Safety and Hazard Investigation Board Investigation report: http://www.csb.gov/csb-releases-revised-freedom-industries-report-/. Accessed June 15, 2018.

Craig P.M., C.M. Wood, and G.B. McClelland. 2007. Oxidative stress response and gene expression with acute copper exposure in zebrafish **(***Danio rerio***)**. American Journal of Physiology- Regulatory, Integrative, Comparative Physiology 293(5):1882–1892.

Cruz F.F., C.E. Leite, T.C. Pereira, M.R. Bogo, C.D. Bonan, A.M. Battastini, M.M. Campos, and F.B. Morrone. 2013. Assessment of mercury chloride-induced toxicity and the relevance of P2X7 receptor activation in zebrafish larvae. Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology 158(3):159–64.

Dunstan W.M., L.P. Atkinson, and J. Natoli. 1975. Stimulation and inhibition of phytoplankton growth by low molecular weight hydrocarbons. Journal of Marine Biology 31(4):305–310.

Eastman. 2016a. Crude MCHM. SDS, No. 150000014291 [online]. Eastern Chemical Company, Kingsport, TN. http://ws.eastman.com/ProductCatalogApps/PageControllers/MSDS_PC.aspx?Product=71014291. Accessed July 8, 2018.

Eastman. 2016b. Eastman Crude MCHM Studies. Kingsport, TN. http://www.eastman.com/pages/Eastman-Crude-MCHM-Studies.aspx. Accessed July 8, 2018.

https://www.eastman.com/Pages/Eastman-Crude-MCHM-Studies.aspx. Accessed July 8, 2018.

Foreman W.T., D.L. Rose, D.B. Chambers, A.S. Crain, L.K. Murtagh, H. Thakellapalli, and K.K. Wang. 2015. Determination of (4-methylcyclohexyl)methanol isomers by heated purge-and-trap GC/MS in water samples from the 2014 Elk River, West Virginia, chemical spill. Chemosphere 131:217–224.

Felix L.M., A.M. Vidal, C. Serafim, A.M. Valentim, L.M. Antunes, S.M. Monteiro, M. Matos, and A.M. Coimbra. 2018. Ketamine induction of p53-dependent apoptosis and oxidative stress in zebrafish (Danio rerio) embryos. Chemosphere 201:730–739.

Gilbert J.J. 1988. Suppression of rotifer populations by Daphnia: A review of the evidence, the mechanisms, and the effects on zooplankton community structure. Limnology and Oceanography 33(6):1286–1303.

Goswami S.C. 2004. Zooplankton Methodology, Collection and identification: A field manual. National Institute of Oceanography, Dona Paula, Goa. 26 pp.

Hahn M.E., A.G. McArthur, S.I. Karchner, D.G. Franks, M.J. Jenny, A.R. Timme-Laragy, J.J. Stegeman, B.R. Woodin, M.J. Cipriano, and E. Linney. 2014. The Transcriptional Response to Oxidative Stress during Vertebrate Development: Effects of *tert*-Butylhydroquinone and 2,3,7,8-Tetrachlorodibenzo-*p*-Dioxin. PLoS ONE 9(11):e113158.

Hallare A.V., H.R. Kohler, and R Triebskorn. 2004. Developmental toxicity and stress protein responses in zebrafish embryos after exposure to diclofenac and its solvent, DMSO. Chemosphere 56(7):659–666.

Handley-Goldstone H.M., M.W. Grow, and J.J. Stegeman. 2005. Cardiovascular gene expression profiles of dioxin exposure in zebrafish embryos. Toxicological Sciences. 85(1):683–693.

Hill A.J., H. Teraoka, W. Heideman, and R.E. Peterson. 2005. Zebrafish as a model vertebrate for investigating chemical toxicity. Toxicological Sciences 86(1):6–19.

Ho N.Y., L. Yang, J. Legardi, O. Armant, M. Takamiya, S. Rastegar, and U. Strähle. 2013. Gene responses in the central nervous system of zebrafish embryos exposed to the neurotoxicant methyl mercury. Environmental Science and Technology 47(7):3316–25.

Horzmann K.A., C. de Perre, L.S. Lee, A.J. Whelton, and J.L. Freeman. 2017. Comparative analytical and toxicological assessment of methylcyclohexanemethanol (MCHM) mixtures associated with the Elk River chemical spill. Chemosphere 188:599–607.

Hwang P.P. 2009. Ion uptake and acid secretion in zebrafish (*Danio rerio*). Journal of Experimental Biology 212:1745–1752.

Hwang P.P., and M.Y. Chou. 2013. Zebrafish as an animal model to study ion homeostasis. Pflugers Archives. 465(9):1233–1247.

Johnson V.J., S.S. Auerbach, M.I. Luster, S. Waidyanatha, S.A. Masten, M.S. Wolfe, F.G. Burleson, G.R. Burleson, and D.R. Germolec. 2017. Evaluation of 4-methylcyclohexanemethanol (MCHM) in a combined irritancy and Local Lymph Node Assay (LLNA) in mice. Food and Chemical Toxicology 105:95–105.

Kreutzweiser D.P., R.C. Back, T.M. Sutton, D.G. Thompson, and T.A. Scarr. 2002. Community-level disruptions among zooplankton of pond mesocosms treated with a neem (azadirachtin) insecticide. Aquatic Toxicology 56(4):257–273.

Lan J., M. Hu, C. Gao, A Alshawabkeh, and A.Z Gu. 2015. Toxicity assessment of 4-Methyl-1-cyclohexanemethanol and its metabolites in response to a recent chemical spill in West Virginia, USA. Environmental Science and Technology 49(10):6284–6293.

Lay J.P., W. Schauerte, L. Peichl, W. Klein, and F. Korte. 1985. Influence of benzene on the phytoplankton and on Daphnia pulex in compartments of an experimental pond. Ecotoxicology and Environmental Safety 10(2):218–227.

Liu H., F.H. Nie, H.Y. Lin, Y. Ma, X.H. Ju, J.J. Chen, and R. Gooneratne. 2016. Developmental toxicity, EROD, and CYP1A mRNA expression in zebrafish embryos exposed to dioxin-like PCB216. Environmental Toxicology 31(2):201–210.

Lynch M. 1977. Zooplankton competition and plankton community structure. Limnology and Oceanography 22:775–777.

McQueen D.J., J.R. Post, and E.L. Mills. 1986. Trophic relationships in freshwater pelagic ecosystems. Canadian Journal of Fisheries and Aquatic Sciences 43(8):1571–1581.

Millward R.N., K.R. Carman, J.W. Fleeger, R.P. Gambrell, and R. Portier. 2004. Mixtures of metals and hydrocarbons elicit complex responses by a benthic invertebrate community. Journal of Experimental Marine Biology and Ecology 310(1):115–130.

NTP. 2015a. National Toxicology Program. West Virginia Chemical Spill: Zebrafish Developmental Toxicity Study August 2015 NTP Update. US Department of Health and Human Services, Research Triangle Park, NC.
https://ntp.niehs.nih.gov/ntp/research/areas/wvspill/zebrafish_update_aug2015_508_use.pdf. Accessed July 11, 2018.

NTP. 2015b. National Toxicology Program. West Virginia Chemical Spill: Zebrafish Developmental Toxicity Study July 2015 NTP Update. US Department of Health and Human Services, Research Triangle Park, NC.
https://ntp.niehs.nih.gov/ntp/research/areas/wvspill/zebrafish_update_july2015_508.pdf. Accessed July 11, 2018.

NTP. 2015c. National Toxicology Program. West Virginia Chemical Spill: Zebrafish Developmental Toxicity Study June 2015 NTP Update. US Department of Health and Human Services, Research Triangle Park, NC.
https://ntp.niehs.nih.gov/ntp/research/areas/wvspill/zebrafish_update_june2015_508.pdf. Accessed July 11, 2018.

NTP. 2016a. National Toxicology Program. West Virginia Chemical Spill: NTP Studies. US Department of Health and Human Services, Research Triangle Park, NC.
https://ntp.niehs.nih.gov/ntp/research/areas/wvspill/wv_finalupdate_july2016_508.pdf. Accessed July 11, 2018.

NTP. 2016b. National Toxicology Program. West Virginia Chemical Spill: Zebrafish Developmental Toxicity Study July 2016 NTP Update. US Department of Health and Human Services, Research Triangle Park, NC. https://ntp.niehs.nih.gov/ntp/research/areas/wvspill/zebrafish_update_july2016_508_use.pdf. Accessed July 11, 2018.

NTP. 2016c. US. National Toxicology Program. Chemical Methods - WV Chemical Spill NTP Studies: West Virginia Chemical Spill: NTP Chemical Procurement, Analysis, and Formulation. Research Triangle Park, NC USA. https://ntp.niehs.nih.gov/results/areas/wvspill/. Accessed July 15, 2018.

Ott F. S., R.P. Harris, and S.C. O'hara. 1978. Acute and sublethal toxicity of naphthalene and three methylated derivatives to the estuarine copepod, Eurytemora affinis. Marine Environmental Research 1(1):49–58.

Parsons F., P.R. Wood, and J. DeMarco. 1984. Transformations of tetrachloroethene and trichloroethene in microcosms and groundwater. Journal of American Water Works Association 76(2):56–59.

Paustenbach D.J., B. Winans, R.M. Novick, and S.M. Green. 2015. The toxicity of crude 4-methylcyclohexanemethanol (MCHM): Review of experimental data and results of predictive models for its constituents and a putative metabolite. Critical Reviews in Toxicology 45:1–55.

Phetxumphou K., A.M. Dietrich, N. Shanaiah, Smiley E., and D.L. Gallagher. 2016. Subtleties of human exposure and respnse to chemical mixtures from spills. Environmental Pollution 214:618–626.

Postel L., H. Fock, and W. Hagen. 2000. Biomass and abundance. Pp. 83–192, *In* R. Harris, P. Wiebe, J. Lenz, H.R. Skjoldal, and M. Huntley (Eds.). ICES Zooplankton Methodology Manual. Academic Press, London, UK. 684 pp.

Relyea R.A. 2005. The impact of insecticides and herbicides on the biodiversity and productivity of aquatic communities. Ecological Applications 15(2):618–627.

Riera S.F., and R.A. Cohen. 2016. Alkyl polyglucoside compound influences freshwater plankton community structure in floating field mesocosms. Ecotoxicology 25(8):1458–1467.

Sain A.E., A.M. Dietrich, E. Smiley, and D.L. Gallagher. 2015. Assessing human exposure and odor detection during showering with crude 4-(methylcyclohexyl)methanol (MCHM) contaminated drinking water. Science of the Total Environment 538:298–305.

Sanchez-Bayo F. 2006. Comparative acute toxicity of organic pollutants and reference values for crustaceans. I. Branchiopoda, Copepoda and Ostracoda. Environmental Pollution 139(3):385–420.

Schade C.P., N. Wright, R. Gupta, D.A. Latif, A. Jha, and J. Robinson. 2015. Self-reported household impacts of large-scale chemical contamination of the public water supply, Charleston, West Virginia, USA. PLoS One 10(5):e0126744.

Scholz A. 2002. Mechanisms of (local) anaesthetics on voltage-gated sodium and other ion channels. British Journal of Anaesthesia 89(1):52–61.

Shi X., Y. Du, K.S. Lam Paul, S.S. Wu Rudolf, and B. Zhou. 2008. Developmental toxicity and alteration of gene expression in zebrafish embryos exposed to PFOS. Toxicology and Applied Pharmacology 230:23–32.

Sloman K.A., and P.L. McNeil. 2012. Using physiology and behaviour to understand the responses of fish early life stages to toxicants. Journal of Fish Biology 81(7):2175–98.

Terhaar C. 1977. Basic Toxicity of Crude 4-Methylcyclohexanemethanol. Acc. No. 223270, HS&HFL No. 75e195. TL-77-12. Eastman Kodak Company, Rochester, NY. http://www.eastman.com/Literature_Center/Misc/ MCHM_acute_tox_1977.pdf. Accessed July 10, 2018.

Thomasson E.D., E. Scharman, E. Fechter-Leggett, D. Bixler, S. Ibrahim, M.A. Duncan, J. Hsu, M. Scott, S. Wilson, L. Haddy, A. Pizon, A.L. Burrer, A. Wolkin, and L. Lewis. 2017. Acute health effects after the Elk River chemical spill, West Virginia, January 2014. Public Health Reports 132(2):196–202.

Ton C., S. Stamatiou, and C.C. Liew. 2003. Gene expression profile of zebrafish exposed to hypoxia during development. Physiological Genomics 13(2):97–106.

Voelker D., N. Stetefeld, K. Schirmer, and S. Scholz. 2008. The role of cyp1a and heme oxygenase 1 gene expression for the toxicity of 3,4-dichloroaniline in zebrafish (*Danio rerio)* embryos. Aquatic Toxicology 86(1):112–120.

Whelton A.J., L. McMillan, M. Connell, K.M. Kelley, J.P. Gill, K.D. White, and C. Novy. 2015. Residential Tap Water Contamination Following the Freedom Industries Chemical Spill: Perceptions, Water Quality, and Health Impacts. Environmental Science and Technology 20(2):813–823.

Whelton A.J., L. McMillan, C.L.R. Novy, K.D. White, and X. Huang. 2017. Case study: The crude MCHM chemical spill investigation and recovery in West Virginia USA. Environmental Science: Water Research and Technology 3:312–332.

Xiong D., T. Fang, L. Yu, Z. Sima, and W. Zhu. 2011. Effects of nano-scale TiO2, ZnO and their bulk counterparts on zebrafish: Acute toxicity, oxidative stress and oxidative damage. Science of the Total Environment 409(8):1444–1452.

A. Williams, D. Turner, R. Li, D. Lott, S.F. Riera, A. Wagner, A. Shaikh, R.A. Cohen, and V. Sittaramane

Yan Hu, M. Plutz, and A.S. Belmont. 2010. Hsp70 gene association with nuclear speckles is Hsp70 promoter specific. Journal of Cell Biology 191(4):711.

Westerfield M. 2007. The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish (Danio rerio), 5th Edition. University of Oregon Press, Eugene.