CHANGES IN BEHAVIOR AS A RESULT OF EXPOSURE TO NAPROXEN:
MIMICKING NATURAL SYSTEMS

Alexandra E. Neal

A Thesis
Submitted to the Graduate College of Bowling Green
State University in partial fulfillment of
the requirements for the degree of

MASTER OF SCIENCE

August 2016

Committee:

Paul Moore,  Advisor
Rachelle Belanger
Jeff Miner
ABSTRACT

Paul Moore, Advisor

Animals living within aquatic habitats regularly encounter chemical pollution as a result of anthropogenic activities. Typically, the toxicity of chemical pollutant or toxicants is determined by the median lethal concentration (LC50). However, LC50’s do not provide an accurate representation of exposure to a pollutant within natural systems. In their native habitats, animals experience exposure as a fluctuating concentration as a result of turbulent mixing. Edwards and Moore (2014) showed that more turbulent environments produce exposures with a high degree of fluctuation in frequency, duration, and intensity. In order to more effectively evaluate the effects of pollutants, we created a more ecologically relevant exposure paradigm, utilizing both flow and substrate within a small mesocosm. A commonly used pharmaceutical, naproxen, was used as the toxicant and female crayfish (Orconectes virilis) as the target organism to investigate changes in fighting behavior as a result of dynamic exposure. Crayfish underwent either a static or a dynamic exposure to naproxen in 23 hour long trials. Following exposure, the target crayfish and an unexposed size matched opponent underwent a 15 minute fight trial. These fight trials were recorded and later analyzed using a standard ethogram. Results indicate that exposure to sublethal concentrations of naproxen, in both static and flowing conditions, negatively impact aggressive behavior. Results also indicate that a dynamic exposure paradigm has a greater negative impact on behavior than a static exposure. Turbulence and habitat structure play important roles in shaping chemical exposure. Research in the future should incorporate features of chemical signals, such as intermittency and number of peaks above the mean concentration in order to form a more comprehensive image of chemical exposure and predict the resulting sublethal effects from exposure.
ACKNOWLEDGMENTS

To everyone involved in making this project happen, thank you. None of this would be possible without you.

First, I want to thank (repeatedly) my advisor and mentor, Dr. Paul Moore. His scientific knowledge and experience, his desire to challenge me, and wisdom about life have helped to transform me into a better scientist and thinker. I am also forever grateful for his patience and continual support through what has been the most difficult two years of my life.

I would like to thank my committee members Dr. Rachelle Belanger and Dr. Jeff Miner. Dr. Belanger’s knowledge about ecotoxicology has been a great help in my research and she is a positive role model of what women in science can achieve. I would never have come to visit Bowling Green State University without Dr. Miner and am especially thankful for his willingness to be on my committee.

I would like to thank all of my lab mates in the Laboratory for Sensory Ecology. This group of people has been one of the most welcoming and supportive groups I have ever worked with. Thank for you all for pushing me to be the best scientist I can be, while keeping me laughing through the worst days of research.

None of my research during the summer would have been possible without the University of Michigan Biological Station community. I am sincerely thankful for the opportunity to work at the Stream Lab Research Facility and the funding provided by the student research fellowship.

I would like to thank my friends who helped me grow personally throughout this graduate school journey, especially Natalie Kopan, Jessica Imhoff, and Michael Conrad. Natalie and Jess, who have been working right alongside me from home to pursue their academic goals and remind me to keep going. I am beyond thankful to have Mike as my best friend who has picked me up every time I think that science has me beat.
Finally, to my family; thank you for the love and support from far away. My father, Lee Neal, has taught me a strong work ethic and has always encouraged me. My grandparents, Marshall and Lora Feldpusch, helped to make my undergraduate career a reality. My siblings, Samantha and Michael Neal, for laughs, love, and everything else.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>METHODS</td>
<td>6</td>
</tr>
<tr>
<td>Animals (Collection and Holding)</td>
<td>6</td>
</tr>
<tr>
<td>Experimental Design</td>
<td>6</td>
</tr>
<tr>
<td>Exposure Arenas</td>
<td>7</td>
</tr>
<tr>
<td>Dynamic Exposure Arenas</td>
<td>8</td>
</tr>
<tr>
<td>Static Exposure Arenas</td>
<td>8</td>
</tr>
<tr>
<td>Exposure Paradigm</td>
<td>9</td>
</tr>
<tr>
<td>Hydrodynamic and Electrochemical Measurements</td>
<td>10</td>
</tr>
<tr>
<td>Behavioral Assay</td>
<td>12</td>
</tr>
<tr>
<td>Data Analysis</td>
<td>12</td>
</tr>
<tr>
<td>Fight Trials</td>
<td>12</td>
</tr>
<tr>
<td>Flow Data</td>
<td>13</td>
</tr>
<tr>
<td>Chemical Plume Data</td>
<td>13</td>
</tr>
<tr>
<td>RESULTS</td>
<td>14</td>
</tr>
<tr>
<td>Chemical Data</td>
<td>14</td>
</tr>
<tr>
<td>Overall Effects</td>
<td>14</td>
</tr>
<tr>
<td>Univariate Results</td>
<td>14</td>
</tr>
<tr>
<td>Exposure Type Results</td>
<td>14</td>
</tr>
<tr>
<td>Concentration Effects on Aggressive Behavior</td>
<td>15</td>
</tr>
<tr>
<td>Interaction Effects</td>
<td>15</td>
</tr>
</tbody>
</table>
INTRODUCTION

The toxicity of a chemical is typically determined by its median lethal concentration (LC_{50}) which is the concentration at which approximately half of a test population dies from exposure after a specified amount of time (Stephan 1977). Traditionally, these exposure trials are conducted using static concentrations of pollutants (Anderson et al. 1980; Williams & Dusenbury 1990; Diamond 2006; Lovern & Klaper 2006). A recent addition to this style of ecotoxicology has begun to recognize that toxicant concentrations in natural systems are dynamic in time (Handy 1994; Ashauer et al. 2006; Hoang et al. 2007; Wang 2011). The exact dynamic nature of toxicant concentrations in natural systems are due to a number of factors including pulsed addition of a toxicant to an environment (Stoeckel et al. 2012), precipitation events that deliver toxicants to aquatic systems (Hangen et al. 2001), and the movement of toxicants through a system due to flow mechanics (Giller & Malmqvist 1998). These newer studies have incorporated a pulsatile testing paradigm where model organisms are exposed for a shorter length of time to a single concentration multiple times (Cold & Forbes 2004; Diamond 2005; Zhao & Newman 2006; Earl & Whiteman 2009). Although an improvement over static models, toxicity values derived from static or pulsatile trials do not take into account environmental factors that alter the intensity, frequency, and duration of exposure.

The concentration of a toxicant in the environment will fluctuate with respect to intensity and duration as a result of the three major factors listed above that create temporal variation in toxicant concentrations. Intensity of a toxicant can change in response to an influx of a chemical into the system or chemical mixing (Miller 1997). Frequency and duration can both alter toxicant concentrations as a result of habitat structure preventing continuous transport of toxicants downstream (Finelli 2000). Given this level of understanding on the dynamic nature of toxicant
concentration in natural systems (Murlis et al. 1992; Murlis & Jones 1981), LC50 values derived from static or even slow pulsatile tests do not accurately represent potential threats experienced by organisms in under ecologically relevant conditions (Chapman 2002). Given the disparity between previous LC50 work with static concentrations over extended periods of time and the types of exposure (temporally and spatially dynamic) experienced by organisms in natural systems, a significant gap in understanding arises (Gordon et al. 2012). The current definitions and assessments of exposure are ill-formed when applied to a field setting. Exposure (as a set concentration of ppm or ppm over a time period) is not relevant to the spatial and temporal scales at which organisms experience toxicants primarily as a result of fluid mechanics of dispersal of chemicals or toxicants (Murlis et al. 1992). Thus, to understand exposure from an ecological point of view, understanding the role of flow in dispersing chemicals in any singular habitat is crucial (Sanford 1997; Hart & Finelli 1999). Fortunately, the fluid dynamics (air or water) are fairly well understood and a theoretical concept can be developed.

Both terrestrial and aquatic systems are composed of fluid mediums that serve to move toxicants through their environment (Denny 1993). Fluid movement determines both the amount of time that toxic chemicals reside within a habitat and the fine-scale fluctuations in concentration that occur as a result of dispersal (Vogel 1994; Sanford 1997). Two processes determine the temporal dynamics of chemical dispersion in habitats: molecular diffusion and bulk advection (Denny 1993). Given the time (< days) and size scales (> 0.1 mm) under consideration for macroscopic organisms, diffusion can be ignored and the distribution of toxicants in an environment are due to bulk advection. Bulk advection can be subdivided into laminar and turbulent flow (Vogel 1994). Based on the physical conditions of most habitats (wind or water speed and roughness elements), toxic exposure paradigms are almost universally
turbulent and thus, the distribution of toxicants within an environment are due to the mechanics associated with turbulence.

Because nearly all dispersion and transport of fluids in natural freshwater systems happens due to turbulence, flow can be considered of equal importance to other abiotic factors, such as light or temperature (Sanford 1997). Additionally, the physical characteristics of aquatic habitats, including the topography of the stream bed and substrate composition impact the turbulent structures contained within the flow (Fischer et al. 1979; Wolf et al. 2009; Johnson & Rice 2014). Given that each habitat is unique in these two fundamental characteristics, the residence time and concentration of toxicants will vary significantly across habitats (Edwards & Moore 2014). Consequently, animals present within highly turbulent habitats will be exposed to chemical plumes that vary significantly (Moore & Atema 1991; Finelli 2000; Weissburg 2011).

Thus, exposure in natural systems is more complex than static trials can model, since chemical plume structure has been linked with hydrodynamics (Moore & Grills 1999; Finelli et al. 1999, 2000; Weissburg et al. 2002; Webster & Weissburg 2009; Wolf et al. 2009; Edwards & Moore 2014). Chemical distributions will not be homogeneous as static tests suggest; they are patchy and change as they move downstream and interact with objects. As a result of the physical dispersion of chemicals by turbulence, two animals within any flowing habitat located at different locations within a habitat will be subjected to different and heterogeneous exposures from the same chemical plume (Weissburg 2010). However, current toxicity assessment does not account for variations in exposure due to turbulence or habitat structure, despite ongoing discussion about the need for more realistic exposure paradigms (Erickson et al. 1989; Handy 1994; Delos 1999; Diamond et al. 2005). Rethinking chemical exposure becomes especially important when assessing toxicants that are both prevalent in flowing freshwater systems and
potent in low doses. Therefore, construction of an exposure paradigm that mimics flowing habitats is necessary in assessing the sublethal effects of chemical exposure.

Within the last twenty years, pharmaceutical compounds, (i.e., prescription drugs, hormones, and over the counter medications) have been reported to contribute to freshwater pollution, primarily as effluent from sewage treatment plants (Santos et al. 2010; Arnold et al. 2014). Over 600 different pharmaceuticals have been detected in several aquatic systems including surface water, effluents from sewage treatment plants, groundwater, and drinking water (Segura et al. 2009; Meredith-Williams et al. 2012; Hughes et al. 2013). Pharmaceuticals pose a significant threat to wildlife, both due to loose regulations for discharge and a lack of knowledge about their uptake and effects in aquatic organisms (Brodin et al. 2014; Carlsson et al. 2006; Shore et al. 2014). Pharmaceuticals are biologically active compounds designed to modify the physiology, and possibly the behavior, of their intended target without killing those targets (Monteiro & Boxall 2010; Meredith-Williams et al. 2012; Boxall et al. 2012). As a result of this sublethal effect, impacts on ecological systems are difficult to quantify.

The purpose of this study was to investigate how sublethal concentrations of chemicals dispersed by turbulence impact an organism’s behavior. Naproxen, a non-steroidal anti-inflammatory drug (NSAID), commonly used for pain relief was selected for this study. Naproxen has been detected in varying concentrations in freshwater bodies across the globe and the intended therapeutic use, side effects, and detection in stream effluents make naproxen a relevant pharmaceutical to investigate (Ternes 1998; Isidori et al. 2005; Carlsson et al. 2006; Nikolaou et al. 2007; Carballa et al. 2008; Straub & Stewart 2007; Corcoran et al. 2010).

Crayfish were used due to their ecological importance in stream environments (Momot 1995; Usio & Townsend 2004). Crayfish are sensitive to pollution, making them important
bioindicators of overall ecosystem health. Additionally, crayfish are found in a broad range of aquatic habitats, indicating a high probability of exposure to anthropogenic chemicals. Within stream communities, they play a significant role at multiple trophic levels, acting both as predators and detritivores (Parkyn et al. 2001; Schofield et al. 2001; Dorn & Wojdak, 2003). Consequently, the absence of crayfish from a stream has the potential to negatively affect the other organisms present. Crayfish behaviors, including aggression, have been extensively studied (Blake & Hart 1993; Bergman & Moore 2003; Davis & Huber 2007; Lahman et al. 2015). Agonistic interactions among crayfish are ecologically important, as fighting is key in determining social hierarchies (Goessmann et al. 2000). This social behavior is an important component to acquisition of resources (food and shelter), as well as sexual selection. Because a well-established fight ethogram exists (Bergman & Moore 2003) and crayfish frequently engage in agonistic behaviors (Moore & Grills 1999; Wolf & Moore 2002; Bergman et al. 2003), they are a model organism to assess behavioral changes due to chemical exposure.

In order to assess behavioral changes due to chemical exposure, crayfish were exposed to various concentrations of naproxen under one of two exposure paradigms: static and dynamic. Our hypothesis was that crayfish in the dynamic exposure would show greater impairments to all concentrations of naproxen due to the large-scale fluctuations in exposure as compared to the stable concentrations in static conditions.
METHODS

Animals (Collection and Holding)

Female crayfish, Orconectes virilis, were collected from the Maple Bay in Burt Lake in Cheboygan County, MI, USA (45.48°N, 84.70°W), during June, July, and August of 2015 using wire hand-nets. Prior to use, animals were isolated in plastic containers with aeration holes for a minimum of seven days to minimize the effects of any previous social experiences (Karavanich & Atema, 1998; Bergman et al. 2003). All containers were labeled to identify individuals and placed in either a flow-through metal horse trough or a flow-through stream. Water from the Maple River was pumped into the troughs and stream to provide fresh water as well as detritus for crayfish consumption. Adult non-reproductive female crayfish with intact appendages were used in this study. The intraorbital carapace length was measured in order to size-match (less than 10% size difference in combatants) crayfish for fight trials.

Experimental Design

To investigate the effects of sublethal exposure of naproxen on crayfish behavior, a fully 2 x 4 factorial experimental design was performed. The first factor was the type of exposure (dynamic or static) and the second factor was the exposure concentration (control: 0.0 µg/L; low: 0.027 µg/L; medium: 2.30 µg/L and high: 14.0 µg/L). The toxicant used in this study was naproxen, a non-steroidal anti-inflammatory drug (NSAID). The concentrations selected for trials are representative of the range at which naproxen has been detected in surface water and sewage treatment plant effluents in several freshwater bodies (Ternes 1998; Metcalfe et al. 2003; Carballa et al. 2004; Fent et al. 2006; Quinn et al. 2008; Carocci et al. 2014). The medium concentration treatment of 2.30 µg/L was determined by averaging several reported concentrations together, while the low and high concentrations were taken directly from detected concentrations (Nikolaou et al. 2007; Brun et al. 2006). Naproxen has a half-life in surface
waters of 24-27 days depending on temperature and light conditions (Grenni et al. 2013; Durán-Álvarez et al. 2015). Thus, the concentrations of naproxen within our static treatments remain relatively constant throughout the 23 hour trial.

Experimental conditions consisted of the following:

**Dynamic Exposure:**
- Control concentration (0.0 µg/L) \( N = 15 \)
- Low concentration (0.027 µg/L) \( N = 15 \)
- Medium concentration (2.30 µg/L) \( N = 15 \)
- High concentration (14.0 µg/L) \( N = 15 \)

**Static Exposure:**
- Control concentration (0.0 µg/L) \( N = 15 \)
- Low concentration (0.027 µg/L) \( N = 15 \)
- Medium concentration (2.30 µg/L) \( N = 15 \)
- High concentration (14.0 µg/L) \( N = 15 \)

**Exposure Arenas**
All trials were conducted at the University of Michigan Biological Station Stream Research facility in Pellston, MI. Two different exposure types were tested, static and dynamic, in order to investigate the influence of turbulent mixing on chemical exposure. The static exposures were conducted by exposing animals in aquaria filled with a naproxen laced solution, similar to a traditional LC\(_{50}\) toxicity test. The dynamic exposure trials included flow and substrate components by placing animals in an artificial flow-through stream in order to mimic natural instream conditions for exposure. A naproxen solution was fed continuously into each stream for the duration of the trial.
Dynamic Exposure Arenas
Flow-through exposure streams measured 165 × 20 × 40 cm (L:W:H) and were constructed using cinder blocks measuring 40.6 × 20.3 × 20.3 cm (L:W:H) and lined with 6 mm polyethylene plastic sheeting. Sand and gravel were collected from the Maple River and spread across the bottom of the streams to a consistent depth of 3 cm.

To provide a continuous flow of river water to dynamic arenas, unfiltered water was pumped from the east branch of the Maple River into each of the exposure arenas. Water was delivered to each stream via a polyvinyl chloride (PVC) pipe manifold which split into four separate 3.175 cm diameter pipes and water flow was controlled by ball valves. A collimator, constructed of two egg crate styrene lighting panels (1.69 cm² holes) wrapped in a plastic mesh screen (0.40 cm² holes) was placed at the upstream end of the exposure arenas. A 50 x 35 cm (L:W) plywood board was placed at the downstream end of the stream to control water depth and outflow. Each board was drilled with 10 holes with each hole fitted with 2.54 cm long pieces of 1.27 cm diameter PVC pipes. Water level was maintained at a depth of 13.0 ± 2.0 cm throughout trials. Additionally, the flow velocity of water moving through the exposure streams was 2.7 ± 0.20 cm/s.

Crayfish were tethered in a single spatial location 65 cm downstream from the point source of naproxen. This location was chosen based off of the chemical measurement of dilution (see below).

Static Exposure Arenas
Static exposure arenas consisted of 37.8 L glass aquarium tanks placed within a flow through stream (identical to the dynamic exposure streams). The stream served to ensure that light and temperatures in the static conditions matched those in the dynamic conditions. The aquaria were filled with 19 L of unfiltered Maple River water along with the appropriate naproxen dose
Crayfish were placed within the arena after the aquaria were filled with naproxen laced water.

**Exposure Paradigm**

Crayfish were exposed to naproxen in either dynamic or static arenas for a period of 23 hours. Animals were secured using tethers tied to 3 brick tile stack, each brick measuring 3.5 × 5.0 cm (W:L). Each tether was constructed of a 1.5 × 1.5 cm (L:W) Velcro square tied to a 10 cm long piece of monofilament fishing line (0.36 mm). Each crayfish had the opposite piece of Velcro square superglued in the center of the carapace in order to attach the tether.

For the dynamic exposure, the naproxen solution was delivered to the streams from reservoirs at the upstream end. Reservoirs consisted of 22.7 L plastic buckets, fitted with a plastic tube connector at the bottom. The flow rate of 1.2 × 10⁻⁴ L/s was regulated by fitting each tube with a metal Hoffman closed compressor screw clamp. A source concentration of naproxen solution was gravity fed into the upstream end of the exposure arena via a plastic Tygon tube (5.0 mm OD, 4.5 mm ID). One end of the tube was buried 65 cm in front of the tether and 0.5 cm beneath the substrate to mimic a groundwater fed entry into a stream. Source concentrations for each treatment were calculated from the flow rate, desired average concentration of naproxen at the point of the animal, length of exposure period, and dilution factor. Naproxen solutions were prepared by crushing 220 mg naproxen sodium caplets into a fine powder, dissolving the powder into 1.0 L beaker of water, and sonication of the solution until well mixed (~ 5 mins). Following sonication, the 1.0 L stock solutions were added to an additional 9.0 L of water in reservoirs at least 12 hours prior to exposure trials.

For the static exposure, naproxen solutions were added to the exposure tanks immediately before placing the crayfish in the tanks. Stock solutions of naproxen were made by crushing 220
mg naproxen sodium caplets using a mortar and pestle. The crushed naproxen was then weighed and divided into separate glass scintillation vials which was subsequently added to 1.0 L of filtered water from the Maple River to produce 0.001 M solutions. To produce the desired concentration in the static exposure tanks, the appropriate volume of the stock solution was added to aquaria using a 10 mL, 25 mL graduated cylinders, or a 25 µL micropipette. After addition of the solution, each tank was stirred for 1 minute before beginning the exposure trial.

**Hydrodynamic and Electrochemical Measurements**

To ensure that the average concentrations of the dynamic and static exposures were identical, electrochemical measurements were made in situ to determine the average dilution factor that would occur due to turbulent dispersion of the toxicant. Three-dimensional flow measurements were taken simultaneously with the electrochemical measurements. These measurements were used to determine the source concentration for the dynamic exposure paradigm to ensure that the average exposure concentration matched the correlated exposure in the static tanks. An acoustic Doppler velocimeter (ADV) (Nortek, AS, Rud, Norway), attached to a tripod, was used to measure velocity profiles in three dimensions where the exposed crayfish would be located in the dynamic exposure streams. The ADV sampled the flow velocity at a rate of 25 Hz and a 2 x 2 x 8 mm (W:D:L) sampling volume was used. Measurements were taken at three heights (2, 4, and 6 cm) above the substrate which correlate to heights related to locations of the chemosensory appendages of benthic organisms (Moore et al. 1989; Wolf et al. 2009; Edwards & Moore 2014). Because of the chaotic nature of turbulence, a 180 s sampling period was determined to be a long enough period to capture the large scale fluctuations in flow velocity in all three dimensions (Moore & Crimaldi 2004).
Simultaneous chemical measurements were done using a triple carbon fiber, graphite epoxy microelectrodes, as well as an Epsilon electrochemical detection system (Epsilon; Bioanalytical Systems, West Lafayette, Indiana, USA). Microelectrodes were constructed using three 30 µm diameter (approximately 1 mm in length) carbon fibers that were encased in the tip of a glass pipet. Details on the construction of the electrodes can be found elsewhere (Gerhardt et al. 1984; Brazell et al. 1987). To measure chemical concentrations at small spatial and temporal scales, DC potential amperometry was used. A step voltage (0 – 500 mV) was applied to the carbon fiber electrode at a rate of 20 Hz. While charged, any tracer molecules that came into contact with the electrode were oxidization and a current was formed at the electrode. At the 0 V, tracer molecules were reduced and produced a current flowing in the opposite direction. This method allowed for the quantification of currents at the sampling rate of 25 Hz. After all data points were collected, the electrode was calibrated in known concentrations of dopamine under laboratory conditions. These electrodes show a linear relationship between concentration and current at the concentration levels used in the experiment. More details on the sampling and calibration methods can be found elsewhere (Moore & Atema 1991; Moore et al. 1994). For stability purposes, the electrochemical microelectrode was attached to the ADV, with the tip of the ADV probe and microelectrode level to one another, in order to collect electrochemical and velocity data simultaneously at the same vertical position. Measurements were taken every 0.05 s during a 180 s sampling period for the electrochemical recordings. The ADV and microelectrode were placed at 2, 4, and 6 cm above the substrate as determined by a sub-function of the ADV. These positions were chosen to match the walking legs (2 cm), body (4 cm) and antennae (6 cm) of the crayfish. All calibration and post-processing of the electrochemical data were performed using in-house written software.
**Behavioral Assay**

Behavioral assays were conducted immediately after the 23 hour exposure period ended. Crayfish were size-matched for fight trials, based on no more than a 10% difference in carapace length (Pavey & Fielder 1996; Bergman & Moore 2005; Bywater et al. 2008). For each exposure treatment, an exposed crayfish fought a naïve opponent. Fight trials were performed in a Plexiglass fight arena measuring (40 x 40 x 14 cm) and divided into four quadrants, each having three opaque walls and one opaque removable wall (Berman & Moore 2005; Wofford et al. 2015). The fight arena was filled with clean water from the Maple River. One crayfish was placed in each quadrant and allowed to acclimate for 15 minutes. The removable wall was then taken out, allowing the crayfish to interact for a 15 minute period. Two fights were run simultaneously in the arena and videotaped. The two fighting pairs were visually and chemically unaffected by each other due to the central walls of the arena. Water was unable to cross between the central walls of the arena, preventing the mixing of water between simultaneous fights.

**Data Analysis**

*Fight Trials*

Video analysis was used to obtain the following measurements: contest start time, level of interaction, and contest stop time within the 15 minute trials. From these measurements, a total of eight dependent variables were calculated: the time to initiate a fight, amount of time to reach escalated vs. non-escalated behaviors, time spent at each intensity level, and total duration of interactions. Fight behaviors were analyzed using an ethogram from Bergman and Moore (2003). The differences in fight dynamics due to static and dynamic exposures as well as concentration effects of the toxicant were analyzed using a fully factorial 2 x 4 MANOVA. The first factor was exposure with two conditions (dynamic and static) and the second factor was concentration of toxicant with four conditions (control, low, medium, and high exposure). If any
differences were found in the overall and univariate tests, a Fisher-LSD post hoc test was run to determine where those statistical differences occurred.

**Flow Data**
Flow data collected using the ADV was analyzed with Explore V software (Nortek, AS, Rud, Norway). From this three-dimensional velocity data, we obtained the average flow velocities. Explore V was also used to obtain spectral density distributions, which describe the energy of a signal or times series is distributes across various frequencies (Wolf et al. 2009). Analysis of average velocity data was done by using a one-way analysis of variance (ANOVA) and Tukey HSD post hoc tests. Spectral density data was analyzed using a Fourier transformation covariance function in order to evaluate the time series data (Wolf et al. 2009).

**Chemical Plume Data**
Electrochemical measurements were analyzed using time-series approaches in order to understand the chaotic nature of the signal (Moore & Atema 1991). In order to calculate an average dilution factor based on the level of turbulence in the stream, a time averaged concentration using the entire 180 s recording. This average concentration was divided by the source concentration to produce a dilution factor which was 3000. Thus, to ensure that a crayfish in dynamic exposures received same average concentrations of naproxen as the crayfish in static trials, the inverse of the dilution factor was applied to each target concentrations for the static exposures to calculate a source concentration that was needed for the dynamic exposures. Additionally, temporal aspects of the chemical signal were characterized using an in house program (Moore & Atema 1991; Wolf et al. 2009; Edwards and Moore 2014).
RESULTS

Chemical Data
Chemicals being dispersed by turbulence fluctuate in space and time in a fairly chaotic manner (Fig. 1). Despite these fluctuations, an average concentration can be calculated and in our stream systems, the average tracer concentration was $29 \pm 7.5 \times 10^{-9}$ M. This concentration was used to calculate the dilution factor such that a reservoir concentration can be calculated to ensure that a particular static concentration can be replicated as an average concentration experienced by the crayfish over the experiment duration. This ensured that the exposure paradigm between the static and dynamic treatments differed only in the variability over time and not the mean exposure over time (Fig. 1). The dilution factor was calculated to be 3000. This number, along with the concentrations desired at the point of exposure in the streams (0.027, 2.3, and 14 µg/L), reservoir concentrations were determined.

Overall Effects
Crayfish aggressive behavior was significantly reduced as a result of static vs. dynamic exposure ($F(6,108,0.05) = 4.55, p < 0.001$), naproxen concentration ($F(18,306,0.05) = 1.92, p < 0.05$), and the interaction between these two factors ($F(18,306,0.05) = 2.1, p < 0.05$). In all cases, the rate of escalation to more aggressive behaviors was slowed and the amount of time the fight spent at escalated behaviors were reduced. There were no significant differences with fight initiation or wins and losses with any of the experimental factors listed above ($p > 0.05$).

Univariate Results
Exposure Type Effects
Crayfish exposed under a flowing (dynamic) condition were slower to escalate fights to more intense or aggressive behaviors than those crayfish exposed under stagnant (static) conditions ($F(6,108,0.05) = 4.55, p < 0.001$). For the rate of escalation to level two behaviors,
crayfish in dynamic exposure trials had significantly longer times (7.9 ± 1.1 s) than crayfish in static trials (5.2 ± 0.59 s; Fisher-LSD, p < 0.05, Fig. 2). In a similar fashion, escalation times to higher intensity levels (level 4) were longer for crayfish in dynamic exposure treatments (90.4 ± 15.7 s) compared to crayfish in static trials (55.8 ± 5.9 s; Fisher-LSD, p < 0.001, Fig. 3).

Similarly, crayfish in dynamic exposures spent significantly less time in non-escalated behaviors (Fisher-LSD, p < 0.05, Fig. 4). At lower intensity levels (level 2), crayfish spent an average of 37.8 ± 3.4s opposed to static animals, who spent 54.5 ± 5.3 s. No other behaviors were impacted by exposure type.

Concentration Effects on Aggressive Behavior
Exposure to different concentrations of naproxen decreased the aggressive behaviors of crayfish ($F_{(18, 306, 0.05)} = 1.92, p < 0.05$). Crayfish exposed to the medium concentration (2.3 µg/L) of naproxen, regardless of exposure type, had significantly longer escalation times to more aggressive behaviors than those in the highest concentration (14 µg/L) treatment (Fisher-LSD, p < 0.005, Fig. 5). In addition, the average time of animals spent in aggressive behaviors among the medium and high treatments was shorter (33.9 ± 8.9 s; 34.2 ± 13.3 s, respectively) than both the control and low treatments (48.6 ± 11.2 s; 52.1 ± 14.3 s, respectively; Fisher-LSD, p < 0.05, Fig. 6).

Interaction Effects
Exposure Type and Concentration
The interaction of exposure type and concentration significantly influenced aggressive behavior in crayfish ($F_{(18, 306, 0.05)} = 2.1, p < 0.05$). In a similar fashion to the results above, decreases in rate of escalation and fight intensity were observed as a result of the interaction between concentration and exposure type. Across all concentrations, the rate of escalation (initiation of a fight, escalation to non-aggressive and aggressive behaviors) demonstrated in
static trials was less than the similar times in dynamic trials. In level two behaviors (Fig. 7), the majority of these differences were not significant, with the exception of the dynamic control (8.5 ± 3.5 s) compared to the static medium treatment (4.1 ± 0.9 s; Fisher-LSD, \( p < 0.05 \)). A similar pattern between static and dynamic treatments was observed in the rate of escalation to more aggressive behaviors (level 4, Fig. 8), where all dynamic treatments were significantly longer than at least one of the static treatments (Fisher-LSD, \( p < 0.05 \)).

Results also showed that crayfish spent more time in non-escalated behaviors when in stagnant conditions under low toxic exposures (60.9 ± 9.6 s vs. 33.9 ± 6.7 s; Fisher-LSD, \( p < 0.05 \)). Additionally, crayfish under a flowing condition were more aggressive in low (69.4 ± 27.9 s vs. 38.7 ± 13.1 s) and medium concentrations (48.1 ± 15.8 vs. 15.8 ± 8.11 s) than static counterparts in higher intensity levels (level 4; Fisher-LSD, \( p < 0.05 \), Fig. 9).
DISCUSSION
The results of our study showed that dynamic exposure to a toxicant has a greater detrimental impact on crayfish agonistic behavior than static exposures of the same average concentration and duration. This effect can be seen by the decrease in rate of escalation to non-escalated behaviors (Fig. 2). Similarly, crayfish in dynamic exposures were slower to reach escalated behaviors than controls (Fig. 3). Thus, dynamic exposures appear to have greater behavioral and physiological impacts than the standard static exposure paradigms. In addition to differences due to dynamic exposure, increasing the exposure concentration of naproxen negatively impacted social behavior in crayfish. Crayfish exposed to higher concentrations of naproxen had spent less time in aggressive behaviors and escalated much slower to these aggressive behaviors (Fig. 5, 6). Similar findings are displayed in the interaction between exposure type and concentration (Fig. 7, 8). Finally, these results indicate that exposure to the pharmaceutical naproxen decreases aggression in crayfish at sublethal concentrations and that dynamic exposure to naproxen exacerbates that effect (Fig. 4).

A New Concept of Chemical Exposure
Given these differences in behavioral deficits between the two exposure paradigms, another factor beyond the average concentration of a toxicant must determine the impact of the toxicant on an organism. Direct in situ measures of exposure concentrations were used to calculate dilution factors and to ensure that both static and dynamic crayfish received the same average concentration of toxicants over the 23 hour period (Fig. 1). Given the turbulent dispersal of chemicals in flowing systems, in situ electrochemical measurements can provide a fine scale quantification of toxicant concentrations in flowing systems (Moore & Atema 1991; Moore et al. 1994; Wolf et al. 2009; Edwards & Moore 2014). The turbulent dispersal of chemicals in flowing systems is a fairly well studied area where the temporal dynamics of chemical fluxes can
be tied directly to several physical processes associated with flow which can easily quantified variables like eddy diffusivity (Denny 1993; Vogel 1994; Weissburg & Zimmer-Faust 1993; Weissburg et al. 2002; Roy et al. 2004; Webster & Weissburg 2009). Thus, we are confident that any differences in behaviors as a result of exposure is due to the temporally dynamic nature of the exposure experienced by crayfish and not potential differences in average concentrations during exposure.

The results from this study support the concept that exposure cannot be simply reduced to the average concentration per unit time that an organism experiences during static bioassays. Static definitions of concentration ignore the temporal variations that occur during the turbulent dispersal of toxicant plumes in flowing systems. The rapid and sudden rises and decays in toxicant concentration that occur within flowing systems creates a more toxic exposure to animals. Temporal dynamics within toxic plumes can arise from two different sources: periodicity in input dynamics and turbulent dispersal.

More recent ecotoxicology work has attempted to incorporate the periodicity in input dynamics by performing intermittent exposure paradigms. Pulsed toxicant exposure on fathead minnows affected growth rates and the degree of the negative impacts were related to pulse frequency and duration of the toxicant (Diamond et al. 2005). Intermittent exposures to toxicants causes different impairments than static exposure paradigms, yet even these intermittent exposure paradigms do not reflect what occurs in natural systems (Kallander et al. 1996; Cold & Forbes 2004; Ashauer et al. 2006; Zhao & Newman 2006; Hoang et al. 2007; Welsh et al. 2008; Angel et al. 2015).

Thus, a new concept of exposure needs to be developed that incorporates realistic and temporally dynamic exposures that occur in natural systems. Some of the principal factors of
exposure include the dynamic features of turbulent dispersal of chemicals such as: magnitude of concentration (the intensity of a chemical), frequency of toxicant input (how often an animal is subject to exposure), and duration of exposure (the length of the exposure); all three of which were affected by turbulent mixing in the dynamic exposure trials (Gordon et al. 2012).

Furthermore, the several aspects of turbulent chemical plumes (e.g., intermittency, peak-to-mean ratios) should be considered fundamental elements of toxicity in temporally dynamic systems. Changes in each of these elements help elucidate the actual exposure that an organism experiences. Certainly, an exposure comprised of frequent pulses considerably higher than the average sampling concentration will impact organisms more severely, as pulses may exceed an individual’s threshold tolerance to a given toxicant (Pynnonen 1990; Earl & Whiteman 2009). Additionally, peak length and intermittency determines the length of intense exposure and recovery time between pulses (Handy 1994; Ashauer et al. 2006). Our electrochemical measurements of the chemical signal show peaks of significantly higher concentrations than the long term average concentration (Fig. 1). Over the 300s sampling period, we saw a minimum of 6 peaks with concentrations reaching 0.5 micromolar or greater. The presence and length of these peaks within the signal provide clear evidence of a unique and continually fluctuating exposure paradigm for crayfish within dynamic exposures. Subsequently, a more comprehensive definition of toxic exposure must address the abovementioned factors.

However, incorporating the temporal dynamics of exposure due to turbulent dispersal of toxicants in lotic systems has rarely been done (Edwards & Moore 2014). In order to understand exposure more comprehensively, environmental conditions, that alter exposure downstream, need to be considered and quantified. For instance, flow velocity heavily influences the intensity of turbulence in a system and consequently, the degree of mixing that occurs downstream from
the source of chemical release (Moore et al. 1994). Additionally, obstacles (such as rocks, downed trees, and riffle and pools) within the environment and type of substrate present (sand vs. gravel) additionally alter turbulent profiles which subsequently affect the profile of a chemical’s distribution downstream (Buffin-Bélanger et al. 2006; Wolf et al. 2009). Given that fluvial systems are heterogeneous with respect to flow patterns, exposure (as in concentration fluctuations over time) will also be heterogeneous as toxicants flow downstream (Moore et al. 2000; Edwards & Moore 2014). Ludington and Moore (in prep) found that crayfish downstream of an obstacle during exposure to pesticides exhibited different foraging behavior in comparison to crayfish in an upstream location. By changing physical characteristics, such as flow regime or substrate, of the experimental stream, a different degree of turbulence and exposure paradigm can be generated (Webster & Weissburg 2009).

The spatial and temporal fluctuations of a toxicant in our artificial streams explains the behavioral differences measured between the static and dynamic exposure conditions. Dynamic chemical plume structure is shaped by several quantifiable variables of flow as well as the mechanism by which toxicants enter freshwater habitats. Surface runoff or ground water flow are just one factor that determine how toxicants enter systems and under which turbulent regimes those toxicants will first be dispersed downstream (Hangen et al. 2001; Doležal & Kvítek 2004). In addition to mode of toxicant entry into freshwater bodies, roughness elements (substrate type), macrophyte cover, riffle/pool sequence also determine the type of fluid mechanism at play in dispersing toxicants. Any environmental condition that alters the amount of turbulent energy in habitat flow will dictate the degree of mixing as chemicals (Fischer et al. 1979; Hart & Finelli 1999; Wolf et al. 2009; Johnson & Rice 2014). Consequently, large fluctuations in exposure frequency, toxicant duration, and magnitude of a toxicant will be seen in more turbulent
environments (Edwards & Moore 2014). Thus, organisms located within the same stream, but in different hydrodynamic regimes, will have different exposure paradigms. Static toxicological trials do not capture this habitat heterogeneity and miss the dynamic nature of exposure that occurs in natural conditions. Our results show that removing the dynamic nature of toxic exposure and severely underestimate the impact of toxicants on behavior and presumably the underlying physiology that drives that behavior. In order to create more effective water impairment criteria, a shift away from static trials and LC50 values must happen. By incorporating the fine-scale measurements and utilizing existing knowledge of plume structure and the mechanisms of movement, the impacts of chemical exposure can be better predicted.
REFERENCES


and receiving waters, and potential for environmental effects as measured by acute and chronic aquatic toxicity. *Environmental Toxicology and Chemistry, 25*(8), 2163-2176.


Hoang, T. C., Gallagher, J. S., Tomasso, J. R., & Klaine, S. J. (2007). Toxicity of two pulsed metal exposures to *Daphnia magna*: relative effects of pulsed duration-concentration and


APPENDIX A - TABLES

**Table 1:** A fight ethogram adapted from Bergman & Moore (2003) used to analyze all videos by assigning intensity level to corresponding behaviors during fights. All behaviors at intensity level 3 and above are considered escalated behaviors, while behaviors below level 3 are non-escalated behaviors.

<table>
<thead>
<tr>
<th>Intensity Level</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>-2</td>
<td>Tailflip away from opponent or fast retreat</td>
</tr>
<tr>
<td>-1</td>
<td>Slowly back away from opponent</td>
</tr>
<tr>
<td>0</td>
<td>Ignore opponent with no response or threat display</td>
</tr>
<tr>
<td>1</td>
<td>Approach without a threat display</td>
</tr>
<tr>
<td>2</td>
<td>Approach with threat display using meral spread and/or antennal whip</td>
</tr>
<tr>
<td>3</td>
<td>Initial claw use by boxing, pushing, or touching with closed claws</td>
</tr>
<tr>
<td>4</td>
<td>Active claw use by grabbing opponent with open claws</td>
</tr>
<tr>
<td>5</td>
<td>Unrestrained fighting by grasping and pulling opponent’s claws or appendages</td>
</tr>
</tbody>
</table>
APPENDIX B - FIGURES

**Figure 1:** A 100 s (of 300 s record) representative display of concentration vs. time of a tracer being dispersed in the model exposure streams as measured by electrochemical electrode. Measurements are taken 20 times per second. The solid red line represents the mean concentration of tracer taken over the entire 300 s record.

![Figure 1](image1.png)

**Figure 2:** Mean (± SEM) time spent to reach fight intensity level 2 for crayfish exposed under dynamic (solid black) and static conditions (white). N = 55 for dynamic and N = 55 for static conditions and includes all treatments, including controls. Bars with different letters are significantly different from each other \(F_{(6,108,0.05)} = 4.55, p < 0.001\).
Figure 3: Mean (± SEM) time spent to reach fight intensity level 4 for crayfish exposed under dynamic (solid black) and static conditions (white). N = 30 for dynamic and N = 35 static conditions and includes all treatments, including controls. Bars with different letters are significantly different from each other ($F_{(6,108,0.05)} = 4.55, p < 0.001$).
**Figure 4:** Mean (± SEM) time spent at fight intensity level 2 for crayfish exposed under dynamic (solid black) and static conditions (white). N = 48 for dynamic and N = 58 for static conditions and includes all treatments, including controls. Bars with different letters are significantly different from each other ($F_{(6,108,0.05)} = 4.55, p < 0.001$).

**Figure 5:** Mean (± SEM) time spent by crayfish in increasing concentrations of naproxen (control N = 18; low = 0.027 µg/L, N = 12; medium = 2.3 µg/L, N = 17; high = 14 µg/L, N = 18) to reach fight intensity level 4. Data was pooled from both static and dynamic exposure types. Bars with different letters are significantly different from each other ($F_{(18,306,0.05)} = 1.92, p < 0.05$).
**Figure 6:** Mean (± SEM) time spent by crayfish in increasing concentrations of naproxen (control N = 19; low = 0.027 µg/L, N = 16; medium = 2.3 µg/L, N = 19; high = 14 µg/L, N = 21) at fight intensity level 4. Data was pooled from both static and dynamic exposure types. Bars with different letters are significantly different from each other ($F_{(18,306,0.05)} = 1.92$, $p < 0.05$).
Figure 7: Mean (± SEM) time spent to reach fight intensity level 2 based on exposure type and concentration of naproxen. Different shaded bars represent the mean time crayfish spent to level 2 by exposure type; dynamic (solid black) and static (white). For dynamic conditions, N = 15 (control), N = 13 (low), N = 13 (medium), N = 14 (high). For static conditions, N= 18 (control), N = 11 (low), N = 17 (medium), N = 14 (high). Bars with different letters are significantly different from each other ($F_{(18,306,0.05)} = 2.1, p < 0.05$).
Figure 8: Mean (± SEM) time spent to reach fight intensity level 4 based on exposure type and concentration of naproxen. Different shaded bars represent the mean time crayfish spent to level 4 by exposure type; dynamic (solid black) and static (white). For dynamic conditions, $N = 7$ (control), $N = 7$ (low), $N = 9$ (medium), $N = 7$ (high). For static conditions, $N = 11$ (control), $N = 5$ (low), $N = 8$ (medium), $N = 11$ (high). Bars with different letters are significantly different from each other ($F_{(18,306,0.05)} = 2.1, p < 0.05$).

Figure 9: Mean (± SEM) time spent at fight intensity level 4 based on exposure type and concentration of naproxen. Different shaded bars represent the mean time crayfish spent at level 4 by exposure type; dynamic (solid black) and static (white). For dynamic conditions, $N = 7$ (control), $N = 7$ (low), $N = 9$ (medium), $N = 7$ (high). For static conditions, $N = 12$ (control), $N = 7$ (low), $N = 8$ (medium), $N = 11$ (high).
9 (low), N = 10 (medium), N = 14 (high). Bars with different letters are significantly different from each other \(F_{(18,306,0.05)} = 2.1, p < 0.05\).