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Crayfish as a biomonitor for the algal toxin microcystin-LR

Kayla McRobb

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Crayfish as a Biomonitor for the Algal Toxin Microcystin-LR.

by

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Submitted to the Department of Biology

Eastern Michigan University

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

in

Ecology, Evolution, and Organismal Biology

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Yspilanti, Michigan
Dedication

For Seth, Bones, and Foxy, none of whom saw enough of me as I made my way through graduate school. Thank you for loving me even in my absence and tolerating me throughout this process.
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Dr. Robert Huber of Bowling Green State University, for teaching us to set up, allowing us to use, and helping me troubleshoot his custom crayfish monitoring Java software.

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Abstract

The bloom-forming alga *Microcystis* produces the hepatotoxin microcystin-LR. Removing this toxin from drinking water requires expensive treatments; current analytical methods are incapable of real-time monitoring. Crayfish are resistant to microcystin-LR toxicity and respond well to operant conditioning. I hypothesized that crayfish could sense and be trained to respond to microcystin-LR via electroshocks for use as biomonitor. In the microcystin detection experiment, *Procambarus clarkii* moved away from microcystin-LR (p < 0.001) while *Orconectes rusticus* did not respond (p = 0.28). Neither species could be reliably trained to move to the tank’s center when microcystin-LR was present. To understand why, I tested *Procambarus clarkii*’s ability to respond to a neutral scent using positive and negative reinforcement. They associated the scent with positive reinforcement (p < 0.001) but not negative reinforcement (p = 0.21), suggesting crayfish may be incapable of associating scents with negative tactile stimuli.
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Chapter One: Introduction

Microcystin-LR: Origin and Structure

Blooms of the blue-green alga (or cyanobacterium) *Microcystis* are becoming larger and more common in many regions of the world. As nutrient loads increase due to farming practices there is more nitrogen and phosphorus available to support cyanobacterial growth (Michalak et al. 2013). Increased water temperature due to climate change favors growth of cyanobacteria, which grow well at 25 degrees Celsius or more, and also causes lakes to stratify earlier in the growing season and stay stratified for longer into the autumn, which allows the top layer to remain warmer (Paerl and Huisman 2008; Michalak et al. 2013). This stratification also allows cyanobacteria to use their gas vesicles to float at the surface undisturbed, shading out other species of phytoplankton and creating dense surface blooms (Paerl and Huisman 2008). Climate change models predict increases in spring runoff followed by extended periods of summer drought which increases the residence time of nutrients brought into the system during the wet spring period, further encouraging bloom growth (Michalak et al. 2013). Microcystis and other cyanobacteria also produce toxins which can be harmful to people and animals. Although we know the factors that encourage bloom growth in *Microcystis* we are still far from understanding what encourages its toxin production. Toxic and non-toxic strains of *Microcystis* have been shown to grow together and toxic strains do not always produce toxins (US EPA 2014 Apr 3).
Microcystins are a family of toxins produced by cyanobacteria primarily of the genus *Microcystis*, but also of the genera *Anabaena*, *Nostoc*, and *Oscillatoria* (Dawson 1998; US EPA 2014 Apr 3). There are eighty known microcystins, and structurally they are cyclic heptapeptides, meaning they are a cyclic molecule formed by seven amino acids (Hayama et al. 2012). Five of these amino acids remain essentially the same in each microcystin variant, aside from some changes in methylation and stereochemistry that can have effects on toxicity. These amino acids are D-alanine, D-methylaspartic acid, 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (also known as Adda), D-glutamic acid, and N-methyldehydroalanine (Carmichael et al. 1988). The remaining two amino acids differ in each microcystin variant (See Fig. 1). In the most toxic and well-studied variant, microcystin-LR, L-leucine is the R₁ group and L-arginine is the R₂ group (Carmichael 1997; Pyo and Kim 2013). Microcystin-LR ingestion can cause gastrointestinal distress, liver failure, neurological problems, and death in humans and other mammals, and skin contact can cause

![Microcystins](image)

**Figure 1.** Basic structure of microcystins. In all three of the most common microcystins, the R₂ group is arginine. In microcystin-LR, the R₁ group is leucine, while in microcystin-YR it is tyrosine, and in microcystin-RR it is another arginine (Pyo and Kim 2013).
contact dermatitis (US EPA 2014 Apr 3). Because of these toxic effects and the growing presence of *Microcystis* in water resources around the globe, the World Health Organization has created a drinking water safety limit for microcystin-LR of 1 µg/L (Thompson et al. 2007). This limit was developed by using mice to determine the Tolerable Daily Intake (TDI) of 0.04 µg/kg/d and then calculating the daily intake of a 60 kg (132 lb) person assuming that 80% of the two liters of water consumed is from a contaminated source (Guidelines for Drinking Water Quality 2004).

\[
0.04 \text{ µg microcystin/kg body weight/day} \times 60 \text{ kg person} / (2L \text{ water per day} \times 0.80) = 1.5 \text{ µg/L}
\]

Equation 1. Formula to calculate the drinking water safety limit of microcystin-LR from the tolerable daily intake (TDI) derived from mice. This number is rounded down to one significant digit to leave a safe margin of error (Guidelines for Drinking Water Quality 2004).

Although no human fatalities have resulted from ingestion of this toxin thus far, it has caused the deaths of numerous domesticated animals in at least seven states in the U.S. as well as in Canada (California EPA 2009).

**Microcystin LR: Monitoring for Drinking Water Safety**

The toxin produced by algal blooms puts drinking water at risk and is expensive to remove from the water supply. For example, in August of 2014, the City of Toledo, Ohio, closed down its municipal water system for three full days due to elevated levels of microcystin-LR (Cyanobacterial Harmful Algal Blooms 2014). This was partly due to the location of Toledo’s water intake in Lake Erie’s Western Basin, which sees very large and highly variable blooms (Fig. 2.) (Michigan Tech 2013; Lee 2014). To complicate the issue,
normal water treatment methods do not remove microcystins or other algal toxins from drinking water, so they must be treated with alternative chemicals (Hitzfeld et al. 2000; US EPA 2013 Sep 10). The City of Toledo spends $150,000 per month on activated carbon treatment for algal toxins during bloom season, which can last from June until late October depending on conditions in the lake (Michalak et al. 2013; Troy and Henry 2013 Sep 24). This treatment is administered directly into the water intake pipe to facilitate maximum removal of microcystins by the time water reaches the treatment facility, but managers have no real-time way to test toxin levels (US EPA 2013 Sep 10; Aranda-Rodriguez et al. 2015).

Many government agencies and universities are researching the causes of algal blooms in Lake Erie and have come together to make recommendations on how to decrease their likelihood, such as meeting phosphorus loading targets in the Maumee River by decreasing fertilizer use (US EPA et al. 2013). Until recently, progress toward these phosphorus initiatives was impeded by farm politics in the largely rural Maumee River basin (Wines 2014 Aug 4). However, Ohio Senate Bill 150 now requires education and certification of farmers who apply fertilizer to more than 50 acres of land, and Ohio Senate Bill 1 restricts the timing of fertilizer application and the disposal of dredge material in Lake Erie to help reduce phosphorus loads (US EPA and Environment Canada 2015). Additionally, the 2015-2019 Western Lake Erie Basin Phosphorus Reduction Initiative is a voluntary program in Michigan, Ohio, and Indiana that helps to fund best soil conservation
practices in critical watersheds to reduce nitrogen, phosphorus, and sediment runoff into the lake (US EPA and Environment Canada 2015).

As farmers work to reduce nutrient loads, scientists continue to work toward faster and more accurate monitoring options for microcystins. The City of Toledo and most water treatment facilities in developed countries currently use an ELISA (enzyme-linked immunosorbent assay) test to monitor toxin levels on a day to day basis (US EPA 2013 Sep 10). In this type of test, microcystin and an enzyme compete for binding sites on an antibody that sticks to a coating on the wells of a microplate. A substrate that turns blue in the presence of the enzyme is added to the plate, and then a stop solution which changes the color to yellow is added. The test is then read by a microplate reader at 450 nm, with darker yellow color indicating less microcystin-LR. The readings are compared to a standard curve to give the total amount of microcystin-LR present in each sample (Beacon Analytical Systems, Inc). This test must be run with standards and replicates, and samples must be pre-treated to ensure all algal cells have been broken open before testing, which can delay results for up to 24 hours (WHO 2003). Worse, this test cannot differentiate between the very toxic

Figure 2. Algal bloom photo series. This series of photos spans a single week and illustrates the dramatic shifts in algal blooms that are possible over short periods of time. Yellow-green color indicates blooms that are a water quality concern with brighter color indicating areas of more pressing concern. The areas in red are areas of public health concern that may have concentrations of microcystin-LR up to 20 µg/L (Michigan Tech 2013).
microcystin-LR and less toxic variants, and thus reports up to ten times the amount of toxin that is actually present due to interactions with other variants (Rivasseau et al. 1999).

There are other microcystin-LR monitoring options, but none that fit the needs of water treatment facilities. Liquid chromatography offers several more accurate monitoring options, but all have their pitfalls. Normal liquid chromatography has limited microcystin standards available (Hayama et al. 2012). Ultraviolet liquid chromatography lacks the sensitivity to detect microcystin-LR at the drinking water safety level (WHO 2003; Pyo and Kim 2013). High pressure liquid chromatography with pre-treatment of samples and mass spectrometer analysis can differentiate between microcystin variants (Fig. 3) and is very sensitive (thresholds as low as 0.1 µg/L) but is also prohibitively expensive, requires laborious pre-treatment, and is too complex to be run in-house by water treatment facility employees (Hayama et al. 2012; Pyo and Kim 2013). Chemiluminescence immunochromatographic analysis of algal toxins uses antibodies and a chemiluminescent substrate to bind microcystin-LR and find its concentration based on the amount of light given off (Pyo and Kim 2013). While this method is very fast (only ten minutes) and can

![Figure 3. Microcystin variant chromatograph. The results from this chromatograph show differentiation between the three most common microcystin variants, microcystin-LR, microcystin-YR, and microcystin-RR (Pyo and Kim 2013).](image-url)
differentiate between variants, the luminometer needed to read the samples is too expensive for use in a water treatment facility (Pyo and Kim 2013). Electronic nose technology uses odors released by growing cyanobacteria to identify toxic from non-toxic strains, but struggles to predict algal growth patterns accurately and cannot differentiate between toxin variants (Shin et al. 2000). Further, electronic noses have not been tested outside of controlled laboratory environment, which may introduce further error (Shin et al. 2000). Overall, analytical experts agree that multiple methods must be used to accurately identify and quantify microcystins in a sample (WHO 2003).

**Biomonitoring as an Alternative Monitoring Option**

In situations where chemical testing is not sufficiently specific or is cost prohibitive, biomonitoring may be a functional alternative. Biomonitoring uses a model organism to determine environmental toxicity and may involve measuring bioaccumulation in tissues, biochemical changes within the organism, or morphological or behavioral changes (Zhou et al. 2008) Aquatic organisms are often used as biomonitors because they can be very sensitive to changes in the aquatic environment (Ferrao-Filho and Kozlowsky-Suzuki 2011; Pavagadhi and Balasubramanian 2013). Bioaccumulation, consistent biochemical changes within organisms, and morphological changes have not been found in relation to microcystin-LR, but behavioral changes have been noted in many studies (Malbrouck and Kestemont 2006; Ferrao-Filho and Kozlowsky-Suzuki 2011; Pavagadhi and Balasubramanian 2013). Assays like the LD$_{50}$ (the concentration at which half of the organisms present die) are often used as standards for biomonitoring, but avoidance assays, in which live organisms move away from the substance in question, have been shown to be more sensitive and may be a cost-effective solution for effluent monitoring (Zhou et al. 2008).
Basic behavioral changes like avoidance can be encouraged or even developed into more complex indicator behaviors through operant conditioning. Operant conditioning is defined as the study of behaviors that are controlled or maintained by reinforcement schedules (Staddon and Cerutti 2003). In simpler terms, in operant conditioning, the subject is rewarded for performing a specific behavior using either positive or negative reinforcement (Schultz 2015). A positive reinforcement is a reward, such as food, given after the completion of the desired behavior, while negative reinforcement is the removal of a negative stimulus, such as a shock, after the completion of the desired behavior (Schultz 2015). In operant conditioning, these rewards are linked to a neutral stimulus so that the subject learns to associate the neutral stimulus with the reinforcement (Staddon and Cerutti 2003). For example, if a rat receives a shock after a light turns on until it presses a lever, it will associate the light with the threat of a shock and push the lever to avoid it. Experiments like these are often used in both vertebrates and invertebrates to investigate learning abilities (Wight et al. 1990; Blake and Hart 1993; Staddon and Cerutti 2003; Kawai et al. 2004; Hazlett 2007; Tomina and Takahata 2010).

**Focal Animals for Biomonitoring**

Both fish and crustaceans have wide behavioral repertoires and are sensitive to natural chemical stimuli (Derby and Sorensen 2008), making them good candidates for biomonitoring of microcystin-LR. Fish react negatively to exposure to cyanobacteria and associated metabolites, often showing stress responses like upward orientation, gaping of the mouth, gill explosion, hectic swimming, and increased mortality (Baganz et al. 1998; Baganz et al. 2004; Ernst et al. 2006; Malbrouck and Kestemont 2006; Ernst et al. 2007). Unfortunately, these responses are non-specific and are seen in response to other stressors,
such as heavy metals, making them less useful as monitoring tools (Yilmaz et al. 2003; Eissa et al. 2009; Svecevicius 2009; Wang et al. 2013). In contrast to fish, microcystin-LR concentrations under 1 mg/L in water sources have not caused toxicity effects in any crayfish species (Ferrao-Filho and Kozlowsky-Suzuki 2011). Aside from their ability to tolerate high concentrations of microcystins, crayfish display olfactory abilities that make them potentially useful for biomonitoring of microcystin-LR. Crayfish and other crustaceans show olfactory sensitivity to many amino acids, which are the building blocks of heptapeptides like microcystins (Mellon 1996; Steullet et al. 2000; Corotto and O’Brien 2002). Crayfish are also capable of distinguishing between different substrates and show clear substrate preferences, which can be useful for predicting baseline behavior (Viau and Rodriguez 2010). Crayfish are even capable of learning to avoid specific substrates via negative operant conditioning, indicating the ability to learn from conditioning paradigms (Bhimani 2014).

The two crayfish species of particular interest to us are *Procambarus clarkii* and *Orconectes rusticus*. *Procambarus clarkii*, or the red swamp crayfish, is native to North America from Mexico in the south to Ohio in the north and has reported invasive populations on almost every continent (Global Invasive Species Database 2016a). *Procambarus clarkii* has a low detection threshold for leucine, one of the R groups of microcystin-LR (Corotto and O’Brien 2002). Because other common microcystin variants lack this amino acid, it is possible that *P. clarkii* is capable of distinguishing between microcystin variants (Hayama et al. 2012; Pyo and Kim 2013). Further, the crayfish species *Procambarus clarkii* preferred eating toxic strains of *Microcystis* over non-toxic ones and showed improved growth when eating toxic strains (Vasconcelos et al. 2001). The crayfish used in this experiment accumulated 2.9 µg of microcystin-LR per milligram of dry crayfish weight and still showed
improved growth, lipid stores, and protein levels compared to control groups and crayfish fed non-toxic *Microcystis* strains. This positive response to the toxin of interest makes this species an especially promising candidate for further experimentation with microcystin-LR.

*Orconectes rusticus*, commonly called the rusty crayfish, is native to the Midwest, specifically Ohio, Illinois, Indiana and Kentucky (Global Invasive Species Database 2016b). It is also highly invasive when introduced. *Orconectes rusticus* showed increased responsiveness to food odors (which often include amino acids) than other species of crayfish in the same genus, which could indicate that this species would respond well to olfactory training regimes (Willman et al. 1994). *Orconectes* species have successfully been taught to respond to neutral olfactory stimuli through operant conditioning paradigms that use negative reinforcement (Hazlett 2003; Hazlett 2007). Further, when conditioned to a stimulus, *O. rusticus* showed retention of the conditioned response for five days after removal of the stimulus (Nathaniel et al. 2010). These characteristics make this crayfish species an excellent choice for behavioral biomonitoring via operant conditioning.

Both *Orconectes rusticus* and *Procambarus clarkii* show predictable responses to stimuli commonly used in operant conditioning and consistent baseline behaviors that make them attractive candidates for behavioral biomonitoring. *Orconectes rusticus* has been shown to preferentially spend time in proximity to arena walls, indicating a baseline spatial behavioral preference (Daws et al. 2011). Both *P. clarkii* and *Orconectes* species have been shown to respond spatially to stimuli, moving away from light, olfactory alarm cues, and electrical shocks respectively, and moving toward food odors (Bouwma and Hazlett 2001; Yamane and Takahata 2002), indicating the ability to associate stimuli with specific locations. Each of these species are easy to acquire due to their invasive status, are sensitive
to amino acids, and have been used successfully in previous training experiments, suggesting they will also be useful for biomonitoring training.

**Hypotheses**

Taking advantage of these qualities in crayfish, I hypothesized that crayfish could be trained via operant conditioning to respond to microcystin-LR at concentrations close to the WHO safety limit with specific behaviors. To test this hypothesis, I first investigated whether crayfish could detect microcystin-LR at 5μg/L, and then tested whether crayfish could be trained via operant conditioning using negative reinforcement to respond at these levels. Based on the lack of response to the second test, I then examined whether crayfish are capable of associating a neutral scent with any type of operant conditioning, positive or negative.

*Hypothesis I:*

I first determined whether individuals of the species *Procambarus clarkii* and *Orconectes rusticus* could discern between microcystin-LR at 5 μg/L and a control using a place preference experiment. In this experiment I hypothesized that *P. clarkii* is able to differentiate between microcystin-LR and the control due to its sensitivity for the amino acid leucine, which is present in the algal toxin. I hypothesized that *O. rusticus* is also sensitive to the amino acids in microcystin-LR at such a low concentration because these odors represent food to omnivorous species and *O. rusticus* has been shown to be more sensitive to food odors than other species in the same genus. Further I predicted that both species would move toward the toxin because amino acids could be interpreted as food by omnivorous crayfish.
species, and *O. rusticus* especially has been shown to move aggressively toward food odors (Willman et al. 1994).

**Hypothesis II:**

Then, I hypothesized that crayfish could be trained by operant conditioning to avoid a location when microcystin-LR is present. I used electroshock programming to teach *Procambarus clarkii* and *Orceonectes rusticus* to move from their preferred location at the edges of the tank into the center third of the tank in the presence of microcystin-LR. I evaluated later responses to the toxin in the absence of shocks against a behavioral control taken before the application of any negative reinforcement. I predicted that both species would spend more time in the center of the tank in the presence of microcystin-LR when trained to do so. I predicted that the time spent in the center in the presence of microcystin-LR would increase from the earliest training trial to the last training trial.

**Hypothesis III:**

Lastly, I trained crayfish to respond to a neutral stimulus (goldfish scent) using both positive and negative operant conditioning paradigms. I hypothesized that the crayfish would learn to associate the neutral stimulus with the positive reinforcer (food) faster than they would learn to associate the negative reinforcer (shocks) with the neutral stimulus because crayfish routinely use scent cues to locate food, while other sensory cues are more commonly used to avoid predation (Willman et al. 1994; Keller and Moore 1999; Bouwma and Hazlett 2001; Corotto and O’Brien 2002).
Chapter Two: Methods

Crayfish Acquisition and Surgery

*Procambarus clarkii* (6.25 cm in length on average) were bought (Pet Solutions Plus, Beavercreek, OH, USA), while *Orconectes rusticus* (~5.20 cm in length on average) were trapped from the Huron River in Ypsilanti, MI, just downstream of the Peninsular Paper Dam, using baited traps. Each species was kept in a separate tank of aerated dechlorinated tap water and fed rabbit food pellets every three days. Individuals used in Experiments 2 & 3 underwent surgical implantation of an electrode prior to the start of the training regimen. Individuals were kept on crushed ice for 20 minutes before surgery. A dissecting needle was flame sterilized with 95% ethanol, allowed to cool, and used to puncture the crayfish carapace at a fifteen degree angle to avoid puncturing the pericardium (Fig. 4). Fourteen

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Figure 4. Crayfish anatomy. This figure shows the position of crayfish internal organs where red includes the heart and blood vessels, pink indicates the gonads, orange indicates the digestive tract, and green indicates the brain and associated neurons. A) Angle at which the dissecting needle was inserted into the carapace in order to avoid the heart. B) Final position of the inserted electrode which is held in place with super glue (Squicie 2010).
gauge insulated solid copper wire was inserted 3 mm into the carapace, directly above and parallel to the pericardium (Fig. 4). This wire was sealed in place with cyanoacrylate glue. The wire was inserted above the pericardium to ensure electroshocks were effective, but the wire was not inserted into the pericardium to avoid possible damage or death during surgery or shock training (Squie 2010). Crayfish were then allowed to recover from surgery for at least 24 hours prior to any training.

**Experiment 1: Testing for Behavioral Response to Microcystin-LR Odor**

The purpose of this experiment was to determine whether *Procambarus clarkii* and *Orconectes rusticus* can detect 15 µg microcystin-LR introduced into one side of a tank by analyzing their spatial reaction to the toxin via a place preference experiment.

**Experimental Design**

A USB camera (Microsoft) was placed 0.75 meters above the water level of two 36 x 22 x 24.5 centimeter plastic tanks (Lee’s Kritter Keepers, San Marcos, CA, USA). The water depth was 5.5 cm, light level was less than 1 µmol/m²/s (Li-Cor 250A photometer, Lincoln, Nebraska, USA) and temperature was 23 ºC. The USB camera interfaced with an Apple computer. Real time tracking of crayfish movement used custom software developed by Dr. Robert Huber of Bowling Green University using the JavaGrinders library, a public-domain set of programming functions that can be used for the analysis of behavioral experiments. The code needed to run this experiment is included in Appendix A1.

Ice cubes were prepared with one of two treatments: 15 µg of microcystin-LR dissolved in 90 µg of 95% ethanol (Cayman Chemical) or 90 µg of 95% ethanol as a control. In both cases, the ethanol was evaporated using a concentrated flow of air and the resulting
residue was dissolved in water and frozen. The focal animal (*Procambarus clarkii* n = 13, *Orconectes rusticus* n = 16) was allowed to acclimate to the 3 liter tank for one hour prior to beginning the experiment. The computer program was started, and a tea diffuser containing an ice cube was immediately introduced to either end, one containing microcystin-LR and one containing the control treatment as described above. The computer program then tracked the two dimensional position of the crayfish for 15 minutes, the amount of time it took for the microcystin-LR to become uniformly distributed throughout the tank at 5 µg/L (ELISA assay, see Appendix B). The tanks were then emptied and cleaned before being used again.

*Data Analysis and Statistics*

The resulting x-y coordinate data were analyzed to determine the proportion of time spent on the side of the tank containing the experimental treatment (Microsoft Excel 2013). The data were analyzed using a t-test (Systat) in which the null hypothesis was that the mean is equal to fifty percent (H₀: x = 50), indicating an equal amount of time spent near and far from microcystin-LR, and the alternative hypothesis is that the mean is not equal to fifty percent (Hₐ: x ≠ 50), indicating that crayfish tended to spend more time either near to or far from the toxin. Data less than 50% indicates more time was spent on the side containing the control and data greater than 50% indicates more time was spent on the side containing microcystin-LR.

**Experiment 2: Operant Conditioning using Negative Reinforcement**

The purpose of this experiment was to determine whether *Procambarus clarkii* or *Orconectes rusticus* can learn to associate microcystin-LR with negative reinforcement.
provided by electroshocks. Their spatial response to the toxin before and after conditioning acts as a measure of learning.

**Experimental Design**

For this experiment, the same camera, computer, and tank arrangement described above were used. Individual tanks were outfitted with a small plastic grates filled with white aquarium gravel on the bottom of the two outer thirds of the tank (Fig. 5). The inner third of the tank was left as smooth plastic. This encourages the crayfish to spend more time in the two outer thirds and less time in the center due to substrate preferences (Viau and Rodriguez 2010). They are also expected to spend more time in the outer two thirds of the tank due to their preference for spending time on the edges of the tank (Daws et al. 2011).

![Figure 5](image)

Figure 5. Experiment 2 tank schematic. The outer two thirds which are shown here as textured contain white aquarium gravel, a preferred crayfish substrate, while the white center third is smooth plastic, an undesirable substrate. Black circles inside the tank indicate water input points. The black circle outside of the tank is the drain location.

The individual tanks received water at six points (each corner and in the center of each long side) to ensure even distribution and flushing of water and added chemicals for training. This water flowed via gravity from a large aerated holding tank into the individual tanks (3L holding capacity) at a rate of one liter per minute. At this rate, the time needed to flush microcystin-LR from the initial concentration (5 µg/L) to the WHO safety limit of 1 µg/L was six minutes (See Appendix B). All test chemicals (microcystin-LR or ethanol) were
added via syringe to the main water supply tube and were fully mixed into the experimental tank after 30 seconds.

The implanted electrode, which served as the anode, was connected to a relay (Model 1014 0/0/4, Phidgets Inc., Alberta, Canada) controlled by the computer program using 14 gauge stranded copper speaker wire. Electroshocks were applied using an SM6 Simulator DC power supply (Grass Medical Instruments, Quincy, Massachusetts, USA) set to 1 volt, which was attached to the relay’s outputs. Two cathode wires were inserted into each tank, one along each short side, and a multimeter was used to test whether the shock remained uniform throughout the tank. Real-time tracking and shock administration used custom software developed by Dr. Robert Huber of Bowling Green University using the JavaGrinders library. The code needed to run this experiment is included in Appendix A2.

Training for each crayfish (Procambarus clarkii n = 10, Orconectes rusticus n = 10) was carried out over three days as outlined below (Fig. 6). The crayfish were connected to the inactive computer relay and allowed to acclimate to the tank for 24 hours. A naïve baseline was then collected for six minutes using the computer code from Appendix A1. This information allowed us to quantify the normal behavior of each individual crayfish for later comparison. Two conditioning treatment types were then carried out such that each individual received eight microcystin-LR conditioning experiences and eight control conditioning experiences in random order spread over two days of training. A rest period of at least fifteen minutes was observed between each treatment.
The first treatment type was training with the chemical of interest, microcystin-LR. During this treatment, the desirable behavior is for the crayfish to move into the center third of the tank in spite of its natural tendency to remain in the outer areas. Therefore, the computer program was designed to shock the crayfish when present in either end of the tank and to cease delivering shocks when the crayfish moved into the center (Fig. 7). The code for this is included in Appendix A2. In this case, 15 µg of microcystin-LR dissolved in ethanol was injected into the delivery tube for the tank for an initial concentration of 5 µg/L when fully mixed and the computer program was started immediately afterward. For six minutes, until the concentration of microcystin-LR dropped below the level of interest (1 µg/L), the crayfish was shocked for a duration of 0.5 seconds every 3 seconds while present in either end of the tank. At the end of six minutes the program was halted and the coordinate data saved for analysis.

Figure 6. Experiment 2 schematic of the experimental procedure for each crayfish. Red areas indicate locations where crayfish received shocks. A naïve baseline behavior sample was taken with no shocks present. Eight microcystin-LR and eight control conditioning trials using shocks were performed in random order for a total of sixteen conditioning trials. The behavior of each crayfish was evaluated in the presence of microcystin-LR and the control with no shocks present to determine the efficacy of conditioning.

The first treatment type was training with the chemical of interest, microcystin-LR. During this treatment, the desirable behavior is for the crayfish to move into the center third of the tank in spite of its natural tendency to remain in the outer areas. Therefore, the computer program was designed to shock the crayfish when present in either end of the tank and to cease delivering shocks when the crayfish moved into the center (Fig. 7). The code for this is included in Appendix A2. In this case, 15 µg of microcystin-LR dissolved in ethanol was injected into the delivery tube for the tank for an initial concentration of 5 µg/L when fully mixed and the computer program was started immediately afterward. For six minutes, until the concentration of microcystin-LR dropped below the level of interest (1 µg/L), the crayfish was shocked for a duration of 0.5 seconds every 3 seconds while present in either end of the tank. At the end of six minutes the program was halted and the coordinate data saved for analysis.
The second treatment type was training with a control, 95% ethanol, in which microcystin-LR is normally dissolved when purchased. During this treatment, the desirable behavior is for the crayfish to stay out of the center third of the tank. The computer program was modified in this case to deliver shocks when the crayfish was present in the center third of the tank and to cease shocks when the crayfish moved to either end (Fig. 8). This treatment ensured the crayfish were not responding to the ethanol alone, but to the microcystin-LR, and that crayfish did not simply remain in the center third of the tank perpetually in the interest of avoiding shocks. In this case, 150 µL of 95% ethanol was

Figure 7. Microcystin conditioning schematic. Schematic of the experimental tank as used in microcystin-LR conditioning trials, where red indicates areas where the crayfish would be shocked and white indicates safe areas. When microcystin-LR was introduced into the tank, a crayfish residing in the outer two thirds of the tank was shocked, but a crayfish that moved into the center of the tank would avoid punishment.

Figure 8. Ethanol control conditioning schematic. Schematic of the experimental tank as used in control conditioning trials, where red indicates areas where the crayfish would be shocked and white indicates safe areas. When the ethanol control was introduced into the tank, a crayfish residing in the outer two thirds of the tank would avoid punishment, but a crayfish that moved into the center of the tank would be shocked.
injected into the main delivery tube of the tank and the program was started immediately. This treatment was also run for six minutes, with crayfish remaining in the center receiving shocks of 0.5 second duration every 3 seconds. After six minutes the program was halted and the data saved for analysis. After each training treatment, the tanks were flushed for 15 minutes to ensure all introduced chemicals were removed.

After the full sixteen training experiences, the crayfish were allowed to rest for one hour. After the rest period, 15 µg of microcystin-LR in ethanol (MC) or 150 µL of 95% ethanol (ET) were introduced into the tank one at a time in random order, with at least fifteen minutes between treatments. These treatments were recorded with no shocks using the code present in Appendix A1. The x-y coordinate data from the microcystin-LR treatment (MC) and from the ethanol treatment (ET) were compared to the naïve data for each individual crayfish to determine whether they spend more time in the center when in the presence of microcystin-LR or spend more time in the two outer areas in the presence of the ethanol control treatment. Using these comparisons, I can determine whether the crayfish have made the appropriate connections between their location in the tank and the chemical stimulus present based on their operant conditioning experiences with shocks as negative reinforcement.

Data Analysis and Statistics

Statistical analysis of data from this experiment was two-fold. The microcystin-LR (MC), ethanol (ET), and the naïve (N) data were analyzed to determine the proportion of time spent in the center of the tank. These were then compared statistically using repeated measures ANOVA (Systat) to determine whether the amount of time spent in the center of the tank differed significantly among the three treatments. Randomized block ANOVA
separates the error associated with the differences in behavior of each individual crayfish from the error associated with the treatments themselves (the MC, ET, and N data) to allow for more sensitive analysis of the differences between treatments. The null hypothesis was that there was no difference between any of the treatments ($H_0$: $MC = N = ET$) and the alternative hypothesis was that there was a difference between at least some of the treatments ($H_a$: $MC \neq N \neq ET$ or $MC = N \neq ET$ or $MC \neq N = ET$).

The data from the first and last microcystin-LR conditioning treatments for each individual were analyzed to determine what proportion of time was spent in the center of the tank during each period. These data were statistically analyzed using a paired t-test (Systat). In the paired t-test, the proportion of time spent in the center during the first treatment was subtracted from the proportion of time spent in the center of the tank during the last treatment. Since I expected individuals to spend more time in the center when conditioning was complete, I expected that these differences would be positive. Because of this, I analyzed the data using the null hypothesis that there was no difference between them or that the mean difference ($x$) was less than zero ($H_0$: $x \leq 0$) and the alternative hypothesis that the mean difference was greater than zero ($H_a$: $x > 0$). The data from the first and last control (ethanol) conditioning treatments for each individual were analyzed to determine the proportion of time spent in the outer two areas of the tank. These data were also statistically analyzed using a paired t-test in which the null hypothesis was that the mean difference was zero or less ($H_0$: $x \leq 0$) and the alternative hypothesis was that the mean difference was greater than zero ($H_a$: $x > 0$).
Experiment 3: Comparing Positive and Negative Reinforcement Paradigms

The purpose of this experiment was to compare the ability of *Procambarus clarkii* to learn to associate a neutral scent with a change in spatial location using both positive and negative operant conditioning reinforcement paradigms.

Experimental Design

For this experiment, the same camera and computer arrangements from the previous experiments were utilized. Clay pots (7.5 cm in height) were secured on their sides in the center rear of each tank (Fig. 9). Duct tape was used to block areas behind the pots to ensure crayfish hid inside the pots rather than beside them. Long vinyl tubes were secured above the opening to each clay pot and were used to deliver the desired scent treatment into the tank. Water for flushing the tank was delivered behind the clay pot via gravity from a large aerated holding tank. Crayfish used in this experiment were not fed for 48 hours prior to initiating trials.

![Figure 9. Experiment 3 tank schematic. Crayfish hid in the orange area which indicates the clay pot, but could not access the black areas next to it. The white area was freely accessible. The black circle indicates the tube used for scent introduction. The white circle indicates the tube used for tank flushing between treatments. The red “X” indicates where chicken pieces were introduced in the positive reinforcement paradigm. The black circle outside of the tank indicates the drain.](image-url)
In this experiment, I used two different operant conditioning paradigms to train crayfish to respond the same neutral smell: goldfish scent. This scent was obtained by placing four medium sized (x = 32 mm in length) goldfish \textit{(Carassius auratus)} into two liters of aerated water for 24 hours (Acquistapace et al. 2003).

To determine whether crayfish can associate a neutral smell with a positive stimulus, 2 ml of goldfish scent was added to each 3 L tank via the vinyl tube attached above the clay pot. Immediately afterward, a piece of chicken (~1 cm$^3$) attached to a pole via fishing line was then introduced into the far end (Fig. 5) of the tank and a timer was started. The time it took the crayfish to fully leave the shelter of the clay pot in order to move toward the chicken was recorded for each trial (n = 10). The crayfish were allowed to feed on the chicken for thirty seconds. The tank was then flushed with water for five minutes at a rate of 2 L per minute. This process was repeated four times per day at random intervals for four days for a total of sixteen training units. These same methods were followed using water instead of goldfish scent on an additional ten crayfish (n =10) to act as a control and ensure the scent became the conditioned stimulus rather than some other variable, such as the presence of the experimenter.

To determine whether crayfish can associate a neutral scent with a negative stimulus, 2 ml of goldfish scent was added to each 3 L tank via the vinyl tube attached above the clay pot. The computer program was then used to shock each crayfish (n = 10) for 0.5 seconds every three seconds until it fully left the shelter, at which point the shocks ceased. The tank was then flushed with water for five minutes at a rate of 2 L per minute, and the process was repeated four times per day at random intervals for four days. These same methods were followed using water instead of goldfish scent (n = 10) to act as a control and ensure the
scent became the conditioned stimulus instead of any other variable. The availability of control data also allows me to compare the positive and negative reinforcement experiments by allowing me to remove the effects of any differences in response caused by the differences in the two experiment types.

Data Analysis and Statistics

The response times were analyzed using repeated measures ANOVA in three combinations: positive reinforcement (PR) vs. positive reinforcement control (PRC), negative reinforcement (NR) vs. negative reinforcement control (NRC), and positive reinforcement (PR) vs. negative reinforcement (NR) after subtracting the baseline control values (PR-PRC and NR-NRC). In a repeated measures design, I can determine the effect of two different independent variables as well as whether there is a significant interaction between those two variables. I can determine the effect of the treatment type, in this case positive reinforcement, positive reinforcement control, negative reinforcement, or negative reinforcement control (PR, PRC, NR, NRC). I can determine the effect of treatment sequence, or in other words, whether there is a difference between the first training unit and the last training unit regardless of treatment type. In this case, a graph of the treatment sequence would show a negative slope to indicate faster response time from training unit one to training unit sixteen. Lastly, I can determine whether there is an interaction between treatment type and treatment sequence, or whether the change between the first and last training units for one treatment type are different than that same change for another treatment type. In this case, the negative slope for one treatment type would be steeper or less steep than the negative slope for a second treatment type. The null hypothesis for each test was that there was no difference between the two treatments (H₀: PR=PRC, NR=NRC, and PR=NR).
The alternative hypothesis for the first comparison (PR vs. PRC) was that naïve crayfish would learn to respond to the positive reinforcement treatment faster than they would learn to respond to the positive reinforcement control treatment. In a repeated measures design, this would appear as a significant interaction between the treatment type and the treatment sequence. The alternative hypothesis for the second comparison (NR vs. NRC) was that the naïve crayfish would learn to respond to the negative reinforcement treatment faster than they would learn to respond to the negative reinforcement control treatment. In a repeated measures design, this would also appear as a significant interaction between the treatment type and the treatment sequence. For the third comparison, the baseline slope for each control treatment was subtracted from each data point of the experimental treatment to facilitate comparison of the two different methods (PR data – PRC slope and NR data – NRC slope). This process is referred to as detrending. The alternative hypothesis in this case (PR vs NR) was that the naïve crayfish would learn to respond to one treatment paradigm faster than they would learn to respond to the other treatment paradigm. In a repeated measures design, this would also appear as a significant interaction between the treatment type and the treatment sequence.
Chapter Three: Results

Experiment 1: Behavioral Response to Microcystin-LR Odor

The purpose of this experiment was to determine whether *Procambarus clarkii* and *Orconectes rusticus* can detect microcystin-LR at 5 µg/L by analyzing their spatial reaction to the toxin via a place preference experiment. Once the two ice cubes were introduced into the tank, the crayfish immediately moved back and forth between the two ends of the tank, typically taking between one and five minutes to choose a side of the tank that they preferred and then remaining on that side for the remainder of the 15 minute trial period.

In all cases, *Procambarus clarkii* spent significantly less time on the side of the tank that contained microcystin-LR, spending only 19.08 ± 14.4% S.D. of their time near the toxin (Fig. 10). A t-test (two-tailed) was performed on the x-y coordinate data recorded when

![Figure 10. *Procambarus clarkii* Experiment 1 results. Proportion of time spent near microcystin-LR (dark blue) and near the control (light blue) in a rectangular tank by 13 individuals of *Procambarus clarkii* over 15 minutes of exposure, indicating a clear preference for moving away from the toxin (t-test, mean = 19.08%, df = 12, t = -7.73, p_{0.05/2} < 0.001).](image-url)
testing the olfactory response of *Procambarus clarkii* to microcystin-LR. The null hypothesis is that the mean proportion of time spent near the microcystin-LR (positive) is equal to fifty percent (H₀: x = 50), indicating an equal amount of time spent near and far from the toxin. The alternative hypothesis is that the mean proportion of time spent near the toxin is not equal to fifty percent (Hₐ: x ≠ 50). The results of the t-test indicate that *P. clarkii* spent a significantly larger proportion of time away from the microcystin-LR (t-test, mean = 19.08%, df = 12, t = -7.73, p₀.05(2) < 0.001).

In contrast, *Orconectes rusticus* showed a much more variable response to microcystin-LR (mean time near toxin = 60.21 ± 36.9% S.D., Fig. 11). A t-test (two-tailed) was performed on the x-y coordinate data recorded when testing the olfactory response of *Orconectes rusticus* to microcystin-LR. The null hypothesis is that the mean proportion of time spent near the microcystin-LR is equal to fifty percent (H₀: x = 50) and the alternative

![Figure 11. *Orconectes rusticus* Experiment 1 results. Proportion of time spent near microcystin-LR (dark orange) and near the control (light orange) in a rectangular tank by 16 individuals of *Orconectes rusticus*, indicating no preference for spending time near or away from the toxin (t-test, mean = 60.21%, df = 15, t = 1.11, p₀.05(2) = 0.28).](image-url)
hypothesis is that the mean proportion of time spent near the toxin is not equal to fifty percent (Hₐ: x ≠ 50). The results of the t-test indicate that O. rusticus showed no preference for spending time near or far from microcystin-LR (mean time near toxin: x = 60.21%, df = 15, t = 1.11, p₀.₀₅(²) = 0.28).

**Experiment 2: Response to Operant Conditioning using Negative Reinforcement**

The purpose of this experiment was to determine whether *Procambarus clarkii* or *Orconectes rusticus* can learn to associate microcystin-LR with negative reinforcement provided by electroshocks. Their spatial response to the toxin before and after conditioning acts as a measure of learning. Both *Procambarus clarkii* and *Orconectes rusticus* performed the appropriate response of moving into the center when shocks were paired with microcystin-LR during conditioning trials, but neither continued to perform the behavior when shocks were absent. Further, there was no difference between the initial naïve screening (N) and post-conditioning treatments with microcystin-LR (MC) or ethanol (ET).

*Procambarus clarkii*

Individuals of *P. clarkii* spent most of their time in the outer two areas of the tank as expected during the first ethanol control trial, and continued to spend 9.79 ± 19.3% (S.D.) more of their time in the outer two areas during the last ethanol control trial (Fig. 12). A paired t-test (one-tailed) was performed on the x-y coordinate data recorded during the first and last control training trials administered to *Procambarus clarkii* to determine the proportion of time spent in the outer two areas of the tank at the start and end of the conditioning period. I expected the crayfish to spend more time in the outer two areas of the tank in the later trials, allowing us to use a one-tailed design. The null hypothesis for the one-
tailed test was that the difference between the mean of the first ethanol control conditioning treatment ($x_{\text{first}}$) and the last ethanol control conditioning treatment ($x_{\text{last}}$) for each individual is less than or equal to zero ($H_0: x_{\text{last}} - x_{\text{first}} \leq 0$). The alternative hypothesis was that the difference between the mean of the first microcystin-LR treatment and the mean of the last microcystin-LR treatment is greater than zero ($H_a: x_{\text{last}} - x_{\text{first}} > 0$). The results of the paired t-test indicated that there was no difference in the amount of time *P. clarkii* spent in the outer areas of the tank between the first and last control training trials (mean of difference = 9.79%, $df = 9$, $t = 1.6$, $p_{0.05(1)} = 0.072$).

Figure 12. *Procambarus clarkii* Experiment 2 results, ethanol control conditioning. The proportion of time spent in the outer two areas of the tank during the first ethanol control conditioning treatments for *Procambarus clarkii* is shown in dark blue. The proportion of time spent in these areas during the last ethanol control conditioning treatments is shown in light blue. There is no difference between the time spent in the outer areas between the first and last control conditioning treatments (paired t-test, mean = 9.79%, $df = 9$, $t = 1.6$, $p_{0.05(1)} = 0.07$).
Individuals of *P. clarkii* spent very little time in the center of the during the first microcystin-LR conditioning treatments, as expected. They spent 15.30 ± 25.6% (S.D.) more time in the center of the tank during the last microcystin-LR conditioning treatment (Fig. 13).

A paired t-test (one tailed) was performed on the x-y coordinate data recorded during the first and last microcystin-LR conditioning treatments administered to *Procambarus clarkii* to determine the difference between the proportion of time spent in the center of the tank at the start of the training period as compared to the end. I expected crayfish to spend more time in the center of the tank in later trials to avoid punishment, again allowing us to use a one-tailed design. The null hypothesis for the one-tailed test was that the difference between the mean of the first microcystin-LR treatment ($x_{\text{first}}$) and the last microcystin-LR trials ($x_{\text{last}}$) for each individual is less than or equal to zero ($H_0: x_{\text{last}} - x_{\text{first}} \leq 0$). The alternative hypothesis was that

![Figure 13. *Procambarus clarkii* Experiment 2 results, microcystin-LR conditioning. The proportion of time spent in the center of the tank during the first microcystin-LR conditioning treatments for *Procambarus clarkii* is shown in dark blue. The proportion of time spent in this area during the last microcystin-LR conditioning treatments is shown in light blue. There is a significant increase in the time spent in the center between the first and last microcystin-LR conditioning treatments (paired t-test, mean = 15.3%, df = 9, $t = 1.89$, $p_{0.05(1)} = 0.045$).](image-url)
the difference between the mean of the first microcystin-LR treatment and the mean of the last microcystin-LR treatment is greater than zero (Hₐ: \( x_{\text{last}} - x_{\text{first}} > 0 \)). The results of the paired t-test indicate that crayfish spent significantly more time in the center of the tank during the later conditioning treatments (mean = 15.3\%, df = 9, t = 1.89, p_{0.05(1)} = 0.045, Fig. 13).

Despite the correct responses to the conditioning treatments, individuals of *P. clarkii* did not form an association between the scent of microcystin-LR and moving to the center of the tank in the absence of shocks. Crayfish spent 40.6 \( \pm \) 28.7\% of their time in the center during naïve recordings. They spent 31.7 \( \pm \) 24.3\% of their time in the center in the presence of microcystin-LR and 46.5 \( \pm \) 35.4\% of their time in the center in the presence of the ethanol control (Fig. 14). A randomized block ANOVA was performed on the data collected after the completion of conditioning. The null hypothesis was that there was no difference between any of the treatments (H₀: ET = N = MC). I predicted that *Procambarus clarkii* would spend

![Figure 14. *Procambarus clarkii* naïve and conditioned behavior comparison. Time spent in the center of the tank during the naïve recordings (center), post-conditioning microcystin-LR exposure (right) and post-conditioning ethanol control exposure (left). There was no difference between any of the three treatments (Randomized Block ANOVA, means: ET = 46.5\%, N = 40.6\%, MC = 31.7\%, F_{(2, 18)} = 0.770, p_{0.05} = 0.478).](image-url)
more time in the center when microcystin-LR was introduced (MC) than during the naïve recordings (N) or ethanol control treatments (ET), which would be similar (Hₐ: MC = N ≠ ET). The results indicate that there was no difference between any of the treatments (means: ET = 46.5%, N = 40.6%, MC = 31.7%, F(2,18) = 0.770, p₀.05 = 0.478).

*Orconectes rusticus*

Individuals of *O. rusticus* spent most of their time in the outer two areas of the tank as expected during the first ethanol control trial, and continued to spend 12.95 ± 42.9% more of their time in the outer two areas during the last ethanol control trial (Fig. 15). A paired t-test (one-tailed) was performed on the x-y coordinate data recorded during the first and last control training trials administered to *Orconectes rusticus* to determine the proportion of time spent in the outer two areas of the tank at the start and end of the conditioning period. I

![Figure 15. Orconectes rusticus Experiment 2 results, ethanol control conditioning. The proportion of time spent in the outer two areas of the tank during the first ethanol control conditioning treatments for *Orconectes rusticus* is shown in dark orange. The proportion of time spent in these areas during the last ethanol control conditioning treatments is shown in light orange. There is no difference between the time spent in the outer areas between the first and last control conditioning treatments (paired t-test, mean = 12.9%, df = 9, t = 0.95, p₀.05(1) = 0.18).](image-url)
expected the crayfish to spend more time in the outer two areas of the tank in the later trials, allowing us to use a one-tailed design. The null hypothesis for the one-tailed test was that the difference between the mean of the first ethanol control conditioning treatment ($x_{\text{first}}$) and the last ethanol control conditioning treatment ($x_{\text{last}}$) for each individual is less than or equal to zero ($H_0: x_{\text{last}} - x_{\text{first}} \leq 0$). The alternative hypothesis was that the difference between the mean of the first microcystin-LR treatment and the mean of the last microcystin-LR treatment is greater than zero ($H_a: x_{\text{last}} - x_{\text{first}} > 0$). The results of the paired t-test indicated that there was no difference in the amount of time $O.\ rusticus$ spent in the outer areas of the tank between the first and last control training trials (mean of difference=12.9%, $df = 9$, $t = 0.95$, $p_{0.05(1)} = 0.183$).

Individuals of $O.\ rusticus$ spent very little time in the center of the tank during the first microcystin-LR conditioning treatments, as expected. They spent 22.96 $\pm$ 15.5% more time in the center of the tank during the last microcystin-LR conditioning treatment (Fig. 16). A paired t-test (one tailed) was performed on the x-y coordinate data recorded during the first and last microcystin-LR conditioning treatments administered to $Orconectes\ rusticus$ to determine the difference between the proportion of time spent in the center of the tank at the start of the training period as compared to the end. I expected crayfish to spend more time in the center of the tank in later trials to avoid punishment, again allowing us to use a one-tailed design. The null hypothesis for the one-tailed test was that the difference between the mean of the first microcystin-LR treatment ($x_{\text{first}}$) and the last microcystin-LR trials ($x_{\text{last}}$) for each individual is less than or equal to zero ($H_0: x_{\text{last}} - x_{\text{first}} \leq 0$). The alternative hypothesis was that the difference between the mean of the first microcystin-LR treatment and the mean of the last microcystin-LR treatment is greater than zero ($H_a: x_{\text{last}} - x_{\text{first}} > 0$). The results of the paired
t-test indicate that crayfish spent significantly more time in the center of the tank during the later conditioning treatments (mean = 22.96, df = 9, \( t = 4.68, p_{0.05(1)} = 0.0005 \)).

Figure 16. Orconectes rusticus Experiment 2 results, microcystin-LR conditioning. The proportion of time spent in the center of the tank during the first microcystin-LR conditioning treatments for Orconectes rusticus is shown in dark orange. The proportion of time spent in this area during the last microcystin-LR conditioning treatments is shown in light orange. There is a significant increase in the time spent in the center between the first and last microcystin-LR conditioning treatments (paired t-test, mean = 22.96, df = 9, \( t = 4.68, p_{0.05(1)} = 0.0005 \)).

Despite the correct responses to the conditioning treatments, individuals of *O. rusticus* did not form an association between the scent of microcystin-LR and moving to the center of the tank in the absence of shocks. Crayfish spent 68.6 ± 28.6% of their time in the center during naïve recordings. They spent 66.4 ± 16.1% of their time in the center in the presence of microcystin-LR and 73.8 ± 26.4% of their time in the center in the presence of the ethanol control (Fig. 17). A randomized block ANOVA was performed on the data collected after the completion of conditioning. The null hypothesis was that there was no difference between any of the treatments (\( H_0: ET = N = MC \)). I predicted that *Orconectes rusticus* would spend more time in the center when microcystin-LR was introduced (MC)
than during the naïve recordings (N) or ethanol control treatments (ET), which would be similar (H₀: MC = N ≠ ET). (means: ET = 73.8%, N = 68.6%, MC = 66.4%, F(2,18) = 0.369, p_{0.05} = 0.696).

**Experiment 3: Response to Positive and Negative Reinforcement in *Procambarus clarkii***

The purpose of this experiment was to compare the ability of *Procambarus clarkii* to associate a neutral scent with a change in spatial location using both positive and negative reinforcement. Response times in the positive reinforcement experiment decreased drastically from start to finish in tandem with exposure to goldfish scent, but the same decrease was not present in the positive reinforcement control treatment, in which the neutral scent was not present. Response times in the negative reinforcement experiment decreased from start to finish in the presence and absence of the neutral scent. Additionally, there was a significantly
decreased response time when paired with the positive reinforcement paradigm as compared to the negative reinforcement paradigm when the neutral scent was present in both cases.

**Positive Reinforcement**

Individuals of *Procambarus clarkii* who were conditioned using positive reinforcement with goldfish scent present displayed a decreased response time as the experiment progressed as shown by the steep negative slope (Fig. 18). In contrast, individuals that were conditioned using positive reinforcement with no goldfish scent displayed a less dramatic decrease in response time as show by the much shallower slope (Fig. 19). A repeated measures ANOVA was performed on the response time data from the positive reinforcement experiment (PR) and the positive reinforcement control (PRC) to determine whether crayfish response time differed between the two conditioning protocols. The null hypothesis was that there was no difference between the two conditioning protocols and no difference as treatment sequence progressed (H$_0$: PR = PRC). The alternate hypothesis was

![Figure 18. Experiment 3 positive reinforcement results. Change in response time to positive reinforcement conditioning protocol by individuals of *Procambarus clarkii* (n = 10) as the treatment sequence progressed.](image)

\[
y = -96.556x + 1749.4
\]

\[
R^2 = 0.8504
\]

0 2 4 6 8 10 12 14 16 18

Response Time (seconds)

Treatment Sequence
that there was a difference between the two conditioning protocols—and, specifically, that there would be a significant interaction between the conditioning protocol and the treatment sequence. The results indicate that there was a significant main effect of conditioning protocol $(F_{(1,18)} = 133.7, p < 0.001)$, or that there was a difference between the positive reinforcement experiment and the positive reinforcement control. The results also show that there was a significant main effect of treatment sequence $(F_{(14,252)} = 44.3, p < 0.001)$, or that response times decreased significantly as the experiment progressed from treatment 1 to treatment 16, regardless of conditioning protocol. Lastly, the results reveal that there was a significant interaction between conditioning protocol and treatment sequence $(F_{(14,252)} = 24.3, p < 0.001)$, or that the decrease in response time of the positive reinforcement experiment was significantly steeper than the decrease in response time of the positive reinforcement control.

Figure 19. Experiment 3 positive reinforcement control results. Change in response time to positive reinforcement control conditioning protocol by individuals of *Procambarus clarkii* (n = 10) as the treatment sequence progressed.

$$y = -17.986x + 1648.3$$
$$R^2 = 0.1353$$
Individuals of *Procambarus clarkii* who were conditioned using negative reinforcement with goldfish scent present displayed a moderate decrease in response time as the experiment progressed, as indicated by the negative slope (Fig. 20). Similarly, individuals that were conditioned using negative reinforcement with no goldfish scent displayed a strikingly similar moderate decrease in response time, and therefore slope (Fig. 21). A repeated measures ANOVA was performed on the response time data from the negative reinforcement experiment (NR) and the negative reinforcement control (NRC) to determine whether crayfish response time differed between the two conditioning protocols. The null hypothesis was that there was no difference between the two conditioning protocols and no difference as treatment sequence progressed (H₀: NR = NRC). The alternate hypothesis was that there was a difference between the two conditioning protocols, and specifically that there
would be a significant interaction between the conditioning protocol and the treatment sequence. The results indicate that there was not a significant main effect of conditioning protocol \( (F_{(1,18)} = 1.68, p = 0.21) \), or that there was no significant difference between the negative reinforcement experiment and the negative reinforcement control. The results also show that there was a significant main effect of treatment sequence \( (F_{(14,252)} = 80.98, p < 0.001) \), or that response times decreased significantly as the experiment progressed from treatment 1 to treatment 16, regardless of conditioning protocol. Lastly, the results reveal that there was no significant interaction between conditioning protocol and treatment sequence \( (F_{(14,252)} = 0.60, p = 0.86) \), or that the decrease in response time of the negative reinforcement experiment was not different from the decrease in response time of the negative reinforcement control.

Figure 21. Experiment 2 negative reinforcement results. Change in response time to negative reinforcement control conditioning protocol by individuals of *Procambarus clarkii* \((n = 10)\) as the treatment sequence progressed.
Comparison of Positive and Negative Reinforcement

To compare the positive and negative reinforcement paradigms statistically, I first had to adjust the data values to reflect the effects shown in the controls (PRC and NRC). I therefore found the line of best fit for each of the two control experiments and subtracted that from each data point of the related experimental data set (PR-PRC and NR-NRC). This allows us to compare the positive and negative reinforcement protocols by removing any baseline behavioral effects. All subsequent statistical tests were performed on the adjusted data sets. Individuals of *Procambarus clarkii* who were conditioned using positive reinforcement with goldfish scent present displayed a steep decrease in adjusted response time as the experiment progressed (Fig. 22). Conversely, individuals that were conditioned using negative reinforcement with goldfish scent displayed a small increase in adjusted response time as the experiment progressed (Fig. 23). A repeated measures ANOVA was performed on the adjusted response time data from the positive reinforcement experiment.
(PR) and the negative reinforcement experiment (NR) to determine whether crayfish response time differed between the two conditioning paradigms. The null hypothesis was that there is no difference between the two data sets ($H_0$: PR = NR). The alternate hypothesis was that there was a difference between the two conditioning paradigms, and specifically that there would be a significant interaction between the conditioning paradigm and the treatment sequence. The results indicate that there was a significant main effect of conditioning paradigm ($F(1,18) = 147.82, p < 0.001$), or that there was a difference between the positive reinforcement experiment and the negative reinforcement experiment. The results also show that there was a significant main effect of treatment sequence ($F(15,270) = 52.39, p < 0.001$), or that response times decreased significantly as the experiment progressed from treatment 1 to treatment 16, regardless of conditioning paradigm. Lastly, the results reveal that there was a significant interaction between conditioning paradigm and treatment sequence ($F(15,270) = 59.09, p < 0.001$), or that the decrease in response time of the positive reinforcement experiment

![Figure 23. Experiment 3 adjusted negative reinforcement results. Change in response time of individuals of Procambarus clarkii (n = 10) to negative reinforcement conditioning protocol adjusted to reflect the baseline change of the negative reinforcement control.](image-url)
experiment was steeper than the decrease in response time of the negative reinforcement experiment.
Chapter Four: Discussion

To my knowledge, this was the first study to explore the olfactory sensitivity of adult crayfish to microcystin-LR. It was also the first example of an attempt to use operant conditioning with negative reinforcement to condition crayfish to respond to a toxin for biomonitoring purposes. This was also the first study to compare the effects of positive and negative reinforcement operant conditioning paradigms on crayfish behavior and the first evidence for the lack of crayfish ability to link scent with tactile pain. Although the negative operant conditioning paradigm used was ineffective in eliciting the desired response from either crayfish species, the positive reinforcement paradigm employed showed some promise, and it is clear that Procambarus clarkii in particular is able to sense this toxin.

Experiment 1: Behavioral Response to Microcystin-LR

When testing olfactory response, I predicted that both Procambarus clarkii and Orconectes rusticus would be able to detect microcystin-LR and that they would move toward the toxin because, as omnivores, both species would be attracted to the amino acid components of the toxin as they might be attracted to food. The results indicated that P. clarkii was definitely capable of sensing microcystin-LR, but contrary to my prediction, they moved away from the toxin in every case. These crayfish were raised in a lab, so this cannot be due to previous exposure to microcystin-LR. This species has been shown to tolerate up to 2.9 μg/g of microcystin-LR per crayfish dry weight as juveniles and even grow and develop better when fed toxic Microcystis strains instead of non-toxic strains (Vasconcelos et al. 2001), suggesting that toxicity effects would not play a role in this unexpected behavior. Crayfish are scavengers and the same fish that may represent food when dead may represent death when alive. Thus, sensing an unfamiliar grouping of amino acids may be cause for
caution and moving away rather than moving toward. In a group of three crayfish congeners, two species did not respond to bass odor at all, remaining inside their shelters (Willman et al. 1994; Derby and Sorensen 2008). This may explain the avoidant behavior displayed in my experiment. Regardless, the definitive ability to sense microcystin-LR at 400 µg/L or less makes *Procambarus clarkii* an attractive option for real time biomonitoring of this toxin if they can sense the toxin at slightly lower levels.

In contrast, *O. rusticus* did not show behavior that was significantly different from zero. The graph (Fig. 8.) shows that about two thirds of individuals of *O. rusticus* moved toward the toxin as expected, while the other one third moved away. *Orconectes rusticus* has been shown to spend more time outside of a shelter in the presence of the smell of predators (Willman et al. 1994). It has been suggested that because of this tendency toward boldness in the face of possible danger, this species also has more opportunities to feed on high-quality carrion that other crayfish shy away from, explaining its ability to crowd out native crayfish species in areas where it is invasive. This same tendency may explain so many of the individuals of this species moving toward the toxin rather than away. It is also possible that some of these crayfish have previous experience with *Microcystis* blooms since they were wild-caught from the Huron River (Ypsilanti, MI), which has natural lakes and is repeatedly dammed and thus provides the nutrient rich standing water necessary for the growth of *Microcystis*. If some of the crayfish have had negative experiences in the presence of toxin-producing strains, this may explain why some of the crayfish moved away from the toxin while others moved toward it. Alternatively, it is possible that *O. rusticus* simply is not sensitive to microcystin-LR at concentrations of 5 µg/L or less. However, this seems unlikely given the conserved sensitivity to amino acids that is seen in decapod crustaceans (Steullet et
Experiment 2: Response to Operant Conditioning Using Negative Reinforcement

When comparing the effects of training on crayfish behavior, I found that both *Procambarus clarkii* and *Orconectes rusticus* spent significantly more time in the center of the tank during the last microcystin-LR trial than they did during the first microcystin-LR trial. This agrees with my prediction that the crayfish would learn to avoid punishment by moving into the space that provided relief from negative reinforcement (electroshocks). However, there was no significant difference in the time spent in the outer areas of the tank between the first and last ethanol control trials. This was surprising because I expected the negative reinforcement to increase the amount of time spent in the outer areas during control trials as well. However, a difference may be difficult to detect because crayfish have been shown to prefer spending time near the edges of a tank (Daws et al. 2011) on substrates containing small stones (Viau and Rodriguez 2010), which is what the two outer areas provide. Because I expected crayfish to spend most of their time in these areas to begin with, significant differences in time spent in these areas are much more difficult to detect. It is possible that with a much larger sample size, these differences may become clear.

When comparing the naïve behavior of crayfish to post-training behavior in the presence of the conditioned stimuli (but the absence of the negative reinforcement), I found no difference between the naïve behavior, behavior of trained animals in the presence of microcystin-LR (MC), and behavior of trained animals in the presence of the ethanol control (ET). This indicates that neither species of crayfish was able to make an association between
the olfactory stimulus and the desired spatial response. There are four possible reasons for this failure to make the desired association.

The first possibility is that crayfish cannot make associations between their spatial location and the presence or absence of the negative reinforcement (electroshocks). This explanation is unlikely, as other studies have found that crayfish change their walking direction for up to six hours in response to a single electric shock and that crayfish learn to avoid areas with substrates that they have associated with shocks (Yamane and Takahata 2002; Bhimani 2014). This possibility is further diminished because I found that crayfish reacted with the desired spatial response more often as the training period continued during microcystin-LR training trials.

The second possibility is that crayfish cannot associate a smell with a location. This is unlikely as it has been shown that crayfish use olfaction to locate food, find mates, establish social hierarchies, and secondarily avoid predation via alarm cues (Willman et al. 1994; Bouwma and Hazlett 2001; Schneider et al. 2001; Derby and Sorensen 2008), all of which require moving toward or away from a location based on an olfactory cue. Further, *Procambarus clarkii* moved away from microcystin-LR during olfactory response trials I performed, indicating an unconditioned tendency to respond spatially to unfamiliar smells. However, in nature, there is a concentration gradient of scent which indicates which direction the crayfish should move. This gradient was lacking in my experiment since the scent was introduced everywhere at the same time.

The third possibility is that the operant conditioning paradigm used in this experiment was too complex for the crayfish, whose brains are very small and simple (Sandeman et al. 1992). This seems unlikely given the vast amount of literature that shows crustaceans are
capable of many types of learning, including food aversion (Wight et al. 1990), conditioned place preference (Panksepp and Huber 2004), classical conditioning (Orlosk et al. 2011), and operant conditioning (Tomina and Takahata 2010; Tomina and Takahata 2012). However, operant learning paradigms that employ punishments rather than rewards have seen mixed results in crayfish. One study found that crayfish could learn to avoid an electric shock by walking toward a light stimulus, but could not do the same when required to tail-flip backward toward the light stimulus instead (Kawai et al. 2004). Another study found that crayfish could learn to avoid specific substrates when paired with an electroshock punishment (Bhimani 2014). This evidence suggests it is possible, but unlikely, that operant conditioning with negative reinforcement is outside the abilities of the crayfish brain.

The fourth possibility is that crayfish are unable to associate scent with electroshock punishments. It has been found that rats may easily learn when a specific taste is followed by nausea or a specific sound is followed by an electric shock, but they have trouble learning that a shock follows a taste or illness follows a sound (Garcia et al. 1974). It is reasonable to assume that a rat needs to be able to learn to associate a taste with being ill to avoid being poisoned, but rats do not need to associate a sound with nausea in nature and thus have trouble doing so. I suspect that crayfish are similarly deficient in their need to associate scents with shocks or negative tactile sensations. It has been shown that crayfish respond strongly to visual predation cues and that this response is enhanced by alarm odors from conspecifics (Bouwma and Hazlett 2001). That study also showed that in the dark, alarm odors enhanced responses to tactile cues as well, but that during the day the combination of a tactile cue and an alarm odor was not enough to elicit a response. This suggests that crayfish respond to predation (which would naturally cause pain) primarily by visual cues in the light.
and by tactile cues in darkness, using odor only as a secondary cue. Since my experiment was conducted only during the day and purposefully excluded any visual cues through the use of a blind, it is reasonable to conclude that the crayfish were unable to associate the scent of microcystin-LR alone with the pain induced by the shock. To determine the most likely cause of the failure of the crayfish to associate the scent with the desired behavior, the third experiment, using both positive and negative reinforcement was devised.

**Experiment 3: Response to Positive and Negative Reinforcement in *Procambarus clarkii***

In this experiment, both positive and negative reinforcement were used to test the ability of *Procambarus clarkii* to respond to goldfish scent, a neutral stimulus that crayfish are known to sense (Hazlett 2007). Each protocol was performed with and without goldfish scent present to ensure no other variables were affecting the results. It was clear that the crayfish response times decreased in the presence of goldfish scent during the positive reinforcement treatment, indicating that the crayfish associated the scent with the desired behavior. This same decrease in response time was not seen in the positive reinforcement control experiment, indicating the association was made with the goldfish scent and not some other variable. This result indicates that crayfish are capable of learning from operant conditioning paradigms, which is also supported in the literature (Yamane and Takahata 2002; Hazlett 2007; Bhimani 2014).

In the negative reinforcement protocol, the crayfish also showed a decrease in response time as the experiment progressed. However, this was seen both in the presence and in the absence of the goldfish scent, indicating the crayfish learned to avoid the shocks in the control and experimental treatments—but did not appear to associate the goldfish scent with the negative reinforcement in a manner that showed enhanced reaction to the shocks. This
supports the idea that crayfish in experiment two failed to learn to associate shocks with microcystin-LR because crayfish are not capable of making an association between a tactile cue and a scent cue. Hazlett et al (2007) showed that crayfish would respond negatively to scents that they associated with conspecific alarm cues during operant conditioning, but their measure of negative response was an increase in hiding behavior accompanied by a decrease in feeding behavior. This indicates that the crayfish can make associations between scents and respond accordingly—but still shows no link between a tactile cue and a scent cue. Even correcting for baseline response times, the positive reinforcement experimental protocol showed faster learning by crayfish (as indicated by a steeper slope) than the negative reinforcement experimental protocol. In fact, the corrected negative reinforcement protocol indicated no learning by crayfish beyond the baseline effect of shocks. The success of the positive reinforcement protocol over the negative reinforcement protocol supports my hypothesis that crayfish would learn to associate a scent with a food reward more quickly than they would learn to associate a scent with a punishment.

Based on my findings, it does not appear possible to condition these two crayfish species to respond to microcystin-LR using electroshocks. However, my findings illustrate that it may be more beneficial to train crayfish using positive reinforcement, as they are capable of associating a food reward with a scent. More importantly, crayfish and other crustaceans have been shown to be highly sensitive to amino acids (Willman et al. 1994; Mellon 1996; Steullet et al. 2000; Derby and Sorensen 2008), and *Procambarus clarkii* in particular has been shown to be sensitive to leucine at concentrations as low as 1.4 µg/L (Corotto and O’Brien 2002). This is significant because leucine is present in microcystin-LR, but not in other common microcystin variants (Hayama et al. 2012; Pyo and Kim 2013).
combination with my results, which indicate that *P. clarkii* can detect microcystin-LR at levels as low as 5 µg/L, this could indicate that *P. clarkii* is potentially capable of distinguishing between microcystin variants at useful levels. This is the most important next step in investigating the possibility of crayfish as a biomonitor for microcystin-LR. If *P. clarkii* is capable of differentiating between variants, it may be useful to study its olfactory systems in order to develop a sensitive and inexpensive way to monitor these toxins. Even though this species is capable of responding behaviorally to scent using positive reinforcement, studying its sensory tissues, specifically the pereopods known to be leucine sensitive, may be more useful for monitoring applications. From olfactory nerve recordings or other techniques it may be possible to uncover a more rapid and specific way to detect microcystin-LR than our current methods.

Though it is clear from my experiment that *Procambarus clarkii* are sensitive to microcystin-LR, more research on this species and its ability to differentiate between and respond to microcystin variants is needed to elucidate its possible uses as a biomonitor for microcystin-LR. I suggest using this species in conjunction with a positive reinforcement schedule to uncover its potential as a biomonitor. Choosing a response variable that is more sensitive than the time required for the crayfish to leave a shelter would also be helpful. It is actually extremely difficult to get crayfish to leave their shelter for any reason, which means my experiment may not have fully depicted the learning ability of *P. clarkii*. I would also suggest looking at nerve recordings as an alternative way to determine threshold sensitivity to microcystin-LR. These future experiments would create a much more accurate determination of whether *P. clarkii* could be useful for biomonitoring of microcystin-LR.
Chapter Five: Literature Cited


Beacon Analytical Systems, Inc. Microcystin Plate Kit Instructional Booklet.


Squcie. 2010. Procambarus clarkii. Lateral cross section of a crayfish or lobster showing the major internal organs of the reproductive, digestive, circulatory and excretory systems which have been identified with different colours.


Toledo residents warned against toxins in water. 2014. [accessed 2015 Mar 9]. https://www.youtube.com/watch?v=p2BrInmHWYQ&feature=youtube_gdata_player


Appendix A1: Monitor Programming

/*
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Online Documentation for this class can be found at
http://caspar.bgsu.edu/~software/Java/Docs/
*/
import java.awt.Rectangle;
import com.lobsterman.JavaGrinders.JavaGrinders;
import com.lobsterman.JavaGrinders.Tracker.*;

/**
 * demo class to control a frame grabber classes for tracking objects
 * @author <a href="mailto:lobsterman.bgsu@gmail.com">RH</a>
 @Version @(#)OpenCVTracker_simple.java 1/13/15
 */
public class OpenCVTracker_simple {
    static float fps = 5;
    static int camID = 0; // use usb webcam
    // static int camID = 1; // use camera built into monitor
    // static int camID = 2; // does not exist
    static int pixelThreshold = -1;
    static int objectSize = -1;
    static boolean darkObject = true;
    static int secsToRun = 1800;
    static boolean toFile = true;
//VideoCapture camera=new VideoCapture("test.mp4");

public static void main (String args[]) {
  try {
    JavaGrinders.listDetail=true;
    System.out.println("This demo application tracks a dark object in the frame for "+ secsToRun + " seconds at "+ fps + " fps.");
    System.out.println("Change tracker settings in file 'OpenCVTracker_simple.java'");
    JavaGrinders.listDebug = true;
    //JavaGrinders.listDetail = true;

    //specify what you are looking for
    Rectangle aRect = new Rectangle(0,1920,0,1080);
    TrackingJobSetting tj0 = new TrackingJobSetting(aRect,pixelThreshold,objectSize,darkObject);
    TrackingJobSettingsGroup trackJGroup = new TrackingJobSettingsGroup(tj0);

    //specify how you want that done or use the default RecordProc
    OpenCVRecordProc aProc = new OpenCVRecordProc();
    aProc.plotExtended = true; //plot simple dots for captured coordinates
    aProc.isGrayScale = true; //run the tracker in gray scale?
    aProc.drawObject = true;
    //
    aProc.fadeTrail();

    //make a tracker with these settings and run it
    OpenCVTracker cvTrackerDemo = new OpenCVTracker("OpenCVTracker",camID,fps,secsToRun,trackJGroup,toFile,false,true,null,
    aProc);
    cvTrackerDemo.setVisible(true);
  } catch (Exception e) { e.printStackTrace(); }
}

private static void VideoCapture(String string) {
  // TODO Auto-generated method stub
}
}
import com.lobsterman.JavaGrinders.JavaGrinders;
import com.lobsterman.JavaGrinders.Tracker.*; // include tracking functions
import com.lobsterman.JavaGrinders.Control.*; // include robotic functions
import com.lobsterman.JavaGrinders.spatial.*; // include spatial functions

/**
 * Minimal class to illustrate tracker control of shock relay
 * <a href="http://iEthology.com/install/">Required installations</a>
 * <a href="http://iEthology.com/hardware/">Supported hardware</a>
 */
public class OPC_Shock {
    public static void main(String args[]) {
        try {
            JavaGrinders.listDetail=true;
            // initialize robotic interface for PhidgetInterfaceKit 0/0/4 - 1014_2
            PhidgetRelayInterface intf = new PhidgetRelayInterface(1);

            // define control settings for first relay 0
            DeviceController theContr = new DeviceController(0);
            // define frame coordinates for punished area (min X, max X, min Y, max Y)
            // left top arena
            //theContr.setHotSpace(new GridSpace3D(1200,1500,500,800));
            // left bottom arena
            //theContr.addHotSpace(new GridSpace3D(393,765,606,849));
            // Safe Zone Shock
            //theContr.setHotSpace(new GridSpace3D(393,765,423,606));

            // define shock duration [ms]
            theContr.setOnDuration(1000);
            // define timeout period after shock [ms]
            theContr.setNotOnDuration(3000);
            // register controller with robotic interface
            intf.addDeviceController(theContr);

            DeviceController theContr1 = new DeviceController(1);
            // right top arena
            theContr1.setHotSpace(new GridSpace3D(500,800,500,800));
            // right bottom arena
            //theContr1.addHotSpace(new GridSpace3D(906,1305,588,849));
            // Safe Zone Shock
            //theContr1.setHotSpace(new GridSpace3D(906,1305,417,588));
        }
    }
}
// define shock duration [ms]
theContr1.setOnDuration(1000);
// define timeout period after shock [ms]
theContr1.setNotOnDuration(3000);
// register controller with robotic interface
intf.addDeviceController(theContr1);

// define settings for object tracking
int threshold = 60; // object contrast to background
int size = -1; // approximate object size in pixels
TrackingJobSetting theTJ = new TrackingJobSetting(null,threshold,size,true);
TrackingJobSettingsGroup tracks = new TrackingJobSettingsGroup(theTJ);

int camID = 1; // make OPC_shock tracker with input from default camera 0 is usb
float fps = 2; // requested # frames per second
OpenCVTracker theOPC = new OpenCVTracker(camID,fps,-1,tracks,true,false,true,intf,null);
theOPC.setVisible(true);
} catch (Exception e) { e.printStackTrace(); }
Appendix B: ELISA Testing

How to run the ELISA made by Beacon Analytical Systems, Inc Cat.#20-0068:

- Bring all reagents and samples to room temperature.
- Add 50 µL Mircocystin-HRP enzyme conjugate to each well in the microplate. Wells are coated with sheep anti-rabbit antibodies.
- Add 50 µL of calibrator, control, or sample to the appropriate wells.
- Add 50 µL of Rabbit anti-microcystin antibody solution to each well. Swirl vigorously and cover with Parafilm. Incubate for 30 minutes.
- Empty wells into the sink and rinse each well completely with wash solution five times. Invert the plate and tap out as much water as possible onto absorbent paper.
- Add 100 µL of substrate to each well. Cover and incubate for 30 minutes.
- Add 100 µL of stop solution (hydrochloric acid) to each well in the same order as the substrate was added.
- Read the microplate at 450nm and 650nm. Then subtract the 650 nm values from the 450 nm values.
- Average the replicates of each sample, calibrator, or control.
- Calculate %Bo (\%Bo = (Average of sample or calibrator x 100)/ Average of negative control))
- Graph the %Bo of each calibrator against its microcystin concentration on a semi-log graph. Draw a line of best fit.
- Use the line of best fit to determine the concentration of each sample using its individually calculated %Bo.
Determining trial length in Experiment 1:

To determine trial length for the first experiment, I needed to know how long it took for the microcystin-LR introduced into the tank via the ice cube to become uniformly incorporated throughout. To determine this I sampled each end of the tank (Microcystin-LR or MCLR end and ethanol end) and the very center of the tank just above the tank bottom using a micropipette. I sampled each location three times per minute for thirty minutes. I used plain tap water as a control. I then tested all samples and recommended calibrators as outlined in the protocol above. The graph below shows the resulting microcystin-LR concentrations from the samples collected. The maximum possible concentration the crayfish could encounter was 405 µg/L, which was the concentration of microcystin-LR in the ice cubes.
Based on the concentrations shown for each location in the tank in the graph above, I determined that any data taken after 15 minutes would not be useful since the concentration of microcystin-LR would be uniform in the tank at that point.

Determining flushing rate in Experiment 2:

To determine the flushing rate for the tanks in the second experiment, I took one sample per minute from the drain standpipe of three identical tanks for fifteen minutes. I then tested the samples using the ELISA protocol outlined above. I plotted the resulting composite means microcystin-LR concentrations over time and found a line of best fit.

![Graph showing concentration drop](image)

This graph shows that the concentration of the toxin drops below the level of interest (1 µg/L) at 6 minutes. For this reason, the length of each training period was set at 6 minutes.