Resistance of the Freshwater Microbiome to a Combination of Pesticides

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Acceptance of Senior Honors Thesis

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Abstract

Pesticides are used prevalently throughout the United States and can negatively affect non-target organisms. Rain run-off can move multiple pesticides into freshwater ecosystems and bring them in contact with non-target organisms that have variable tolerances to pollution. This study seeks to improve our understanding of how an acute exposure to a combination of pesticides may restructure microbial communities by examining the microbiome before and after a 48-hour exposure to 2000 ppb of herbicides (glyphosate, atrazine, and 2,4-D). Pesticide exposure did not significantly alter the alpha or beta diversity of the sample types, which demonstrates the ability of freshwater microbiomes to resist acute pesticide disturbance. This finding is important when considering questions of freshwater conservation.

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Pesticides

For thousands of years, humans have utilized pesticides in various forms, from using sulfur compounds to kill insects to applying salt to control weed growth (Graham, 2019; Meyer & Scribner, 2009). Over the centuries the use of pesticides has become more complex and prevalent. Pesticide use in the Unites States has increased dramatically following the production of organic synthetic pesticides after World War II (de Castro Marcato et al., 2017; Graham, 2019). By the 1980s and 1990s, farmers switched from mainly utilizing insecticides to relying heavily on various types of herbicides (Graham, 2019). Currently, nearly 6 billion pounds of pesticides are used each year worldwide with over a billion pounds being used in the United States (Atwood & Paisley-Jones, 2017; Hayes & Hansen, 2017). Herbicides now make up 57% of the pesticides used in the United States with a large proportion being used for agricultural purposes (Atwood & Paisley-Jones, 2017; Figure 1). Three of the most prevalently used herbicides in the United States are glyphosate, atrazine, and 2,4-D (Figures 2-3; Hayes & Hansen, 2017).

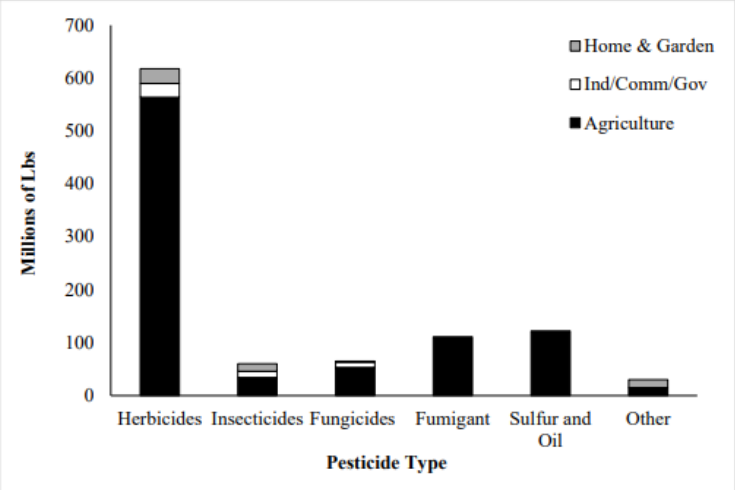

Figure 1. Herbicides are the most utilized pesticides in the United States, the majority of which are used for agricultural purposes. Image from Atwood & Paisley-Jones, 2017. (https://www.epa.gov/sites/production/files/2017-01/documents/pesticides-industry-sales-usage-2016_0.pdf). In the public domain.

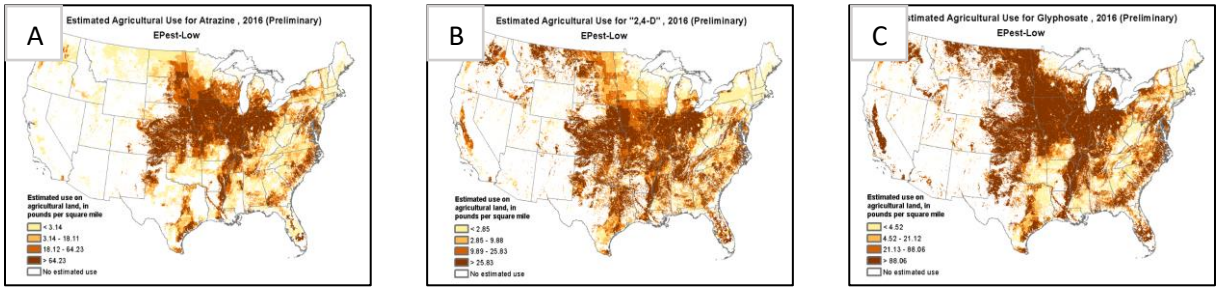

Figure 2. Yearly use of the herbicides atrazine (A), 2,4-D (B), and glyphosate (C) in 2016. Images obtained from United States Geological Survey (2017). (https://water.usgs.gov/nawqa/pnsp/usage/maps/compound_listing.php). In the public domain.

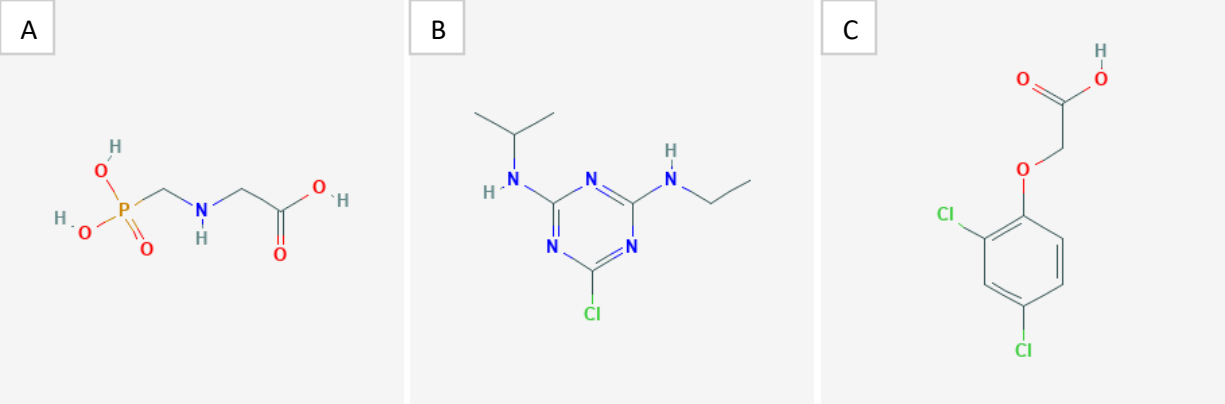

Figure 3. 2-D structures of glyphosate (A), atrazine (B), and 2,4-D (C). Structures obtained from PubChem and the National Center for Biotechnology Information. (https://pubchem.ncbi.nlm.nih.gov/#query= CIDs 3496, 2256, and 1486 respectively)

Glyphosate

Glyphosate, the active ingredient in Roundup, is a broad-spectrum, nonselective organophosphorus herbicide that is commonly used on crops such as soybeans, maize, and cotton and can be used as a harvest aid for other crops such as wheat (Banaee et al., 2019; Benbrook, 2016; Meyer & Scribner, 2009; Song, 2013). This herbicide interrupts aromatic amino acid production in plants by inhibiting the 46 kDa enzyme 5-enolpyruvylshikimate-3-phosphate synthase, which is also present in bacteria and fungi (Fernanda Moreira et al., 2019; Hove-Jensen et al., 2014; Lozano et al., 2019; Thiour-Mauprivez et al., 2019). Glyphosate has been commonly used since it was first commercially produced in 1974, but its utilization has dramatically increased since the introduction of genetically modified glyphosate tolerant crops in 1996 (Benbrook, 2016; Meyer & Scribner, 2009). Glyphosate was originally only applied to areas where all vegetation was to be killed, but this innovation allowed it to be applied over various types of crops (Benbrook, 2016). This technology of glyphosate tolerant crops is now prevalent, with 80-90% of corn and soy in the US being “Roundup-ready” (Hayes & Hansen, 2017). Other

factors, such as the proliferation of glyphosate resistant weeds and the reduction of glyphosate price, have further increased the use of glyphosate, making it the most widely applied pesticide in the United States at over 270 million pounds used each year (Atwood & Paisley-Jones, 2017; Benbrook, 2016).

Atrazine

Atrazine is a triazine herbicide that has been widely used for over half a century to control broadleaf plants in crops such as maize, sugar cane, and pineapple (de Albuquerque et al., 2020; Parada et al., 2019; Stara et al., 2018). This herbicide inhibits weed growth by blocking electron transport in photosystem II of photosynthesis (de Albuquerque et al., 2020; Thiour-Mauprivez et al., 2019). Atrazine's mode of action can also target bacteria such as cyanobacteria that undergo photosynthesis (Thiour-Mauprivez et al., 2019). Atrazine is highly persistent and soluble in water and is resistant to microbial decomposition (Britt et al., 2020; de Albuquerque et al., 2020; Stara et al., 2018). This pesticide was banned from the European Union in 2005 but is still the second most heavily used pesticide in the United States (Atwood & Paisley-Jones, 2017; Meyer & Scribner, 2009; Stara et al., 2018).

2,4-D

2,4-D, which was first commercially produced in 1946, is an herbicide in the chlorophenoxy family that is applied on crops such as wheat, corn, and rice and on lawns and golf courses to limit the growth of broad leaf weeds and grass (Boivin et al., 2005; de Castro Marcato et al., 2017; Meyer & Scribner, 2009; Song, 2013). 2,4-D is a selective herbicide that imitates the plant hormone auxin (de Castro Marcato et al., 2017; Lozano et al., 2019; Song, 2013). This herbicide is not degraded rapidly like natural auxin is in plants, which leads to

uncontrolled plant growth and eventual death through the overproduction of reactive oxygen species and the plant hormone ethylene (de Castro Marcato et al., 2017; Song, 2013). With glyphosate being used so heavily, use of herbicides such as 2,4-D have increased to counter the growth of glyphosate resistant weeds in countries such as Argentina (Lozano et al., 2019). 2,4-D is a highly water-soluble pesticide that is biodegraded quickly (Lushchak et al., 2018), although its degradation rate is influenced by the availability of other carbon sources for microbes (Lozano et al., 2019).

Impact of Pesticides

Pesticides, including these three previously mentioned, have been used in very beneficial ways to reduce weed growth, kill harmful insects, and destroy pathogenic fungi. Their use has increased crop yield and growth (Parada et al., 2019) and protected human health (Graham, 2019). For example, DDT, which was banned in 1972 for its negative environmental impact (Davis, 2019), was used in World War II to limit malaria and insect-borne diseases (Graham, 2019). Pesticides have brought consequential benefits to the human population, and this should not be overlooked when considering pesticides' impact in the environment and what regulatory policies are needed.

However, with the dramatic increase in pesticide utilization, it is critical to examine what effects and unintended negative consequences may be occurring in public health and various environments. Pesticides do not only contact weeds and pathogens, increasing crop yield and limiting disease. They can also encounter many non-target environments and organisms and act as environmental disturbances (Lushchak et al., 2018). Much research has been conducted on how pesticides affect various ecosystems and organisms. In 1962, Rachel Carson's influential book *Silent Spring* laid out the negative impacts DDT and other insecticides had on humans and

non-target organisms such as birds, mammals, and fish (Davis, 2019; Graham, 2019). This and subsequent research led to DDT's eventual ban by the EPA in 1972 (Davis, 2019; Graham, 2019). Other pesticides have been found to negatively impact non-target organisms such as birds (Davis, 2019) and pollinator populations (Sponsler et al., 2019). Herbicide use has also been linked with altered microbial susceptibility to antibiotics, as exposure to toxins can activate the production of efflux pumps that are sometimes used by bacteria in antibiotic resistance (Kurenbach et al., 2017). This poses a risk for pesticides to accentuate the public health problem of multi-drug resistant bacteria.

The specific herbicides used in this experiment have been found to have negative unintended effects. Glyphosate can negatively impact non-target organisms by inhibiting cholinesterase (Banaee et al., 2019). This inhibition impacts the nervous system function, which could lead to lethal inhibition of vital functions. Atrazine has been found to act as a feminizing endocrine disruptor in many organisms by artificially increasing estrogen production and limiting androgen production (Britt et al., 2020; Hayes & Hansen, 2017; Loughlin et al., 2016). This occurs despite the fact atrazine's regular mechanism targets photosynthesis in plants. This illustrates that pesticides can have negative consequences through unexpected alternate mechanisms (Hayes & Hansen, 2017). 2,4-D can have genotoxic effects and can negatively impact reproductive function depending on its dose (de Castro Marcato et al., 2017; Ikechukwu et al., 2012).

Quantifying the impact of pesticides is complex as it is influenced by many factors including environment temperature, pH, mineral composition, microbial makeup, and dissolved oxygen levels (de Castro Marcato et al., 2017; Lushchak et al., 2018; Parada et al., 2019; Thiour-Mauprivez et al., 2019; Tsui & Chu, 2008).

In addition, organisms do not live in isolation, but instead interact in complex interrelated ecosystems. Therefore, determining the effect of pesticides within these complex and dynamic ecological communities can be difficult to measure. For pesticides to be approved for use, they are tested in various in vitro and in vivo settings on certain important species (Mancini et al., 2019; Relyea, 2009). While this is very helpful in determining toxicologic safety, the impact in interdependent ecological systems may be difficult to predict (Mancini et al., 2019; Relyea, 2009), which leads some to call for further testing to determine pesticide impact (Thiour-Mauprivez et al., 2019; Timmis et al., 2019). Pollutant disturbance at one level of a community often indirectly impacts other aspects of that community (Fleeger et al., 2003). If contamination alters the abundance of predators or grazers, the abundance of lower trophic levels may increase (Fleeger et al., 2003). Alternatively, if the contamination directly impacts primary producers and lower trophic levels, this can indirectly alter higher trophic levels (Fleeger et al., 2003). For example, some farmers utilize pesticides to kill rodent populations that negatively impact their crop yield (Baudrot et al., 2020). These rodents, although damaging to farmers, are important in the overall ecosystem health, and their destruction can lead to a reduction in the predator population through a decrease in the predator's food source and secondary poisoning (Baudrot et al., 2020). This can be counterproductive because this negative impact on non-target predators, which help naturally limit the pest population, can lead to reliance on pesticides over the long term to control the rodent population (Baudrot et al., 2020). These events, called trophic cascades (Fleeger et al., 2003), are just one-way pesticide exposure can cause a disturbance in an entire population that was not expected when the pesticide was first applied.

Another factor that further complicates the ability to quantify the impact of pesticides is that often multiple pesticides are used in a single area (Figure 2; Relyea, 2009), which can cause

non-target organisms to be exposed to a combination of herbicides. The effect of pesticides in isolation may be different than when they are present in a mélange with other chemicals and pesticides (Cedergreen, 2014). These interactions can result in synergistic, additive, or antagonistic effects through alterations to bioavailability, metabolism, or uptake and transport (Cedergreen, 2014). Cedergreen (2014) stated synergy occurs relatively rarely at environmentally relevant concentrations of pesticides, and 95% of those synergistic cases contain either a cholinesterase inhibitor or an azole fungicide. Cedegreen (2014) argued there may be a greater threat posed by pesticides having an additive effect than two chemicals having a synergistic effect. As Figure 2 illustrates, application of multiple pesticides in the same area is common. Therefore, additive effects should not be viewed as an unusual event but instead as a likely scenario that should be further evaluated (Relyea, 2009).

Herbicides' Effects in Freshwater Ecosystems

One specific non-target ecosystem, lotic systems, are exposed to many types of pesticides. Activities such as rain runoff, soil erosion, or spray drift can cause herbicides, that were originally applied in fields, to flow into freshwater streams (Banaee et al., 2019; de Albuquerque et al., 2020; Lozano et al., 2019; Lushchak et al., 2018; Stara et al., 2018; Widenfalk et al., 2008). In the United States, pesticide levels commonly exceed the no-effect level for aquatic health set by the EPA in agricultural and urban streams (57% and 83% respectively) (Staley et al., 2015). Pesticide exposure can negatively impact organisms in these lotic systems. For example, a 14-day chronic exposure to atrazine significantly negatively altered biochemical markers in the hemolymph and induced tissue damage such as cell hypertrophy and tissue disintegration in crayfish (Stara et al., 2018). Banaee et al. (2019) also found chronic

glyphosate exposure in crayfish significantly altered biochemical markers that are indicative of cellular stress.

Just as it is difficult to measure how pesticides impact non-target organisms generally, there are multiple factors that make it difficult to quantify pesticide impact in freshwater ecosystems. First, current flow may cause the concentration of a pesticide to vary within different areas of a lotic system. Even where there is less flow of water, such as in lentic systems, there can still be variance in pesticide concentration due to the unpredictability inherent in spray application and wind speed and direction (Tsui & Chu, 2008). A seemingly small aspect such as water turbidity can impact the effects of herbicide treatment (Lozano et al., 2019). Therefore, the impacts of pesticides across freshwater ecosystems are complex and variable.

Microbial Communities in Lotic Systems

One critical component of an ecosystem is its microbiome. Microorganisms make up most of the biodiversity on the planet (Zimmerman et al., 2014) and are important in determining the overall health of host organisms and the greater surrounding environment (Britt et al., 2020; Girvan et al., 2004; Rossi et al., 2018). Microbial composition plays a critical role in soil fecundity (Fierer et al., 2012) and in the growth and health of plant life due to impacting the availability of important nutrients such as carbon, nitrogen, and phosphorous (Fierer et al., 2012; Heijden et al., 2007; Thiour-Mauprivez et al., 2019; Timmis et al., 2019; Zimmerman et al., 2014). Almost all macroorganisms have a microbial community on and/or in them that plays a large role in the health and identity of the organism (Timmis et al., 2019). Normal resident microbes play an active or indirect protective role for many macroorganisms, so a disturbance of the microbiome can allow pathogenic microbes to colonize and jeopardize the health of the organism (Harris et al., 2009; Skelton et al., 2017). In lotic systems microorganisms play an

important role in cycling nutrients and are a foundational component of lower trophic levels (Tornwall et al., 2015). Microbes also have a critical role in breaking down chemical pollutants (Thiour-Mauprivez et al., 2019; Timmis et al., 2019). Disturbance of this microbial community can lead to harm in the organism and greater environment (Timmis et al., 2019). Despite the critical role it plays, microbial communities in freshwater systems have been understudied (Tornwall et al., 2015). Therefore, research in this area is critical to truly understand the impact pesticides are having on microbial communities and on the lotic ecosystem.

There is conflicting research on how herbicides affect microbial communities based on the environment they are applied in, the combination of pesticides that are applied, and the amount of time the pesticides are in the environment (Muturi, Orindi, & Kim, 2013; Muturi, Donthu, Fields, Moise, & Kim, 2017; Rossi et al., 2018; Stachowski-Haberkorn et al., 2008). Pesticides can act as a disturbance that causes some bacterial populations to diminish, while tolerant bacteria may grow and fill the space left by the diminished populations (Thiour-Mauprivez et al., 2019; Widenfalk, Bertilsson, Sundh, & Goedkoop, 2008). For example, researchers found atrazine levels as low as 3-30 ppb altered the microbial community of oysters that allowed an increase of a pathogenic bacterial species in some of the treatment groups (Britt et al., 2020).

Microbial response to pesticide exposure is dynamic, because it depends on the specific types of bacteria in each sample and the mechanism of action of each pesticide used (Thiour-Mauprivez et al., 2019). The presence of a tolerant species, through pesticide degradation or actions of enzymes such as efflux pumps (Thiour-Mauprivez et al., 2019), may impact the entire community response to a pesticide (Lozano et al., 2019). Researchers discovered repeated exposure to atrazine and 2,4-D can lead to the selection of genes in bacteria for enzymes that

degrade the herbicides (Thiour-Mauprivez et al., 2019). This pesticide degradation can be a source of carbon for the microbes and lead to bacterial growth (Thiour-Mauprivez et al., 2019). Glyphosate can also act as a carbon and phosphate source for certain types of bacteria (Hove-Jensen et al., 2014). Microbial community response also depends on the diversity present in a community. More diverse communities are better able to functionally withstand environmental disturbances because different species have redundancy in roles (Lozano et al., 2019; Shade et al., 2012).

Pesticides can impact microbial communities, but microbiomes can also be seemingly unaffected by pesticide pollution. When a microbiome is unchanged by an environmental disturbance it is termed resistant, and when it is affected, but then returns to pre-disturbance levels it is called resilient (Shade et al., 2012).

Previous Research

This current study has been motivated by previous research conducted by our lab. The first study investigated the growth and development of juvenile crayfish under environmentally relevant chronic Atrazine exposure (0.5-5.0 ug/L). The findings suggested Atrazine suppressed growth and higher exposure levels led to tissue degradation in the hepatopancreas tissue. This study was expanded to examine the impact of acute exposure to environmentally relevant doses (50 and 500 ug/L) of Atrazine and Glyphosate on the ectosymbionts of crayfish called branchiobdellidan worms. The findings revealed sub-lethal tissue degradation when branchiobdellidan worms were exposed to isolated pesticides and 100% mortality in 48 hours when branchiobdellidan worms were exposed to both pesticides, suggesting synergistic interactions between atrazine and glyphosate (Table 1). Based on these previous findings, we

chose a combined treatment of herbicides and used an acute exposure to determine their impacts on microbial communities.

Table 1. The combined atrazine and glyphosate treatments resulted in a greater ectosymbiont mortality than individual pesticide treatments.

Treatment Group	Time (hours) Percent Dead Over Time					Total number out of 10 dead after 48 hours
	0	8	24	32	48	
Control	0	0	0	0	0	0
ATZ 50 µg/L	0	0	0	0	0	0
ATZ 500 µg/L	0	0	0	0	0	0
GLY 50 µg/L	0	0	0	0	0	0
GLY 500 µg/L	0	0	0	0	0	0
ATZ 50 & GLY 50 µg/L	0	10	10	10	100	10
ATZ 500 & GLY 500 µg/L	0	10	90	100	100	10

In addition to the associated ecotoxicology studies, our lab also researched the microbiomes of crayfish and the surrounding environment. We determined there were distinct communities found on crayfish, sediment, and water (Figure 4). The water's microbiome was very unique, while the crayfish and sediment environments appeared to be associated with each other. Therefore, we did not merely want to examine the microbial communities on crayfish, but also in the water and on the sediment because their microbiomes appear to be distinct and could respond differently to pesticide exposure.

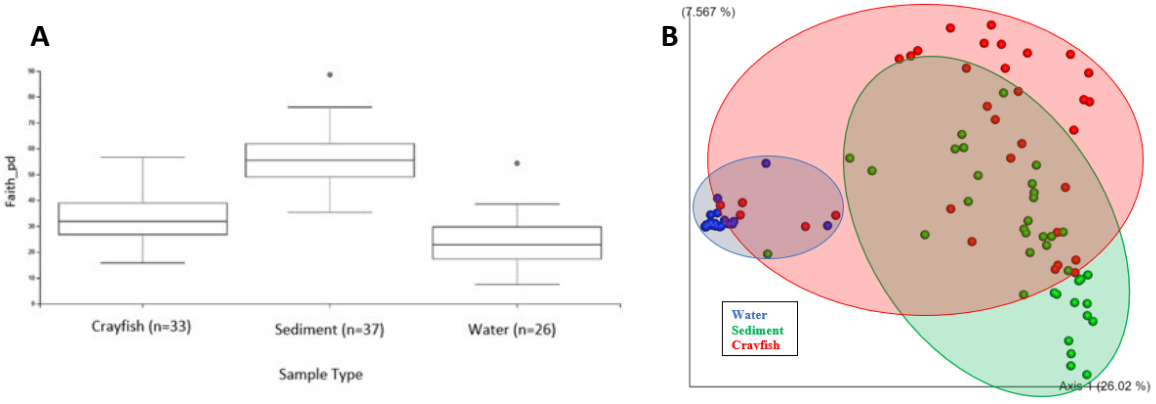

Figure 4. Microbes form distinct communities on different sample types (crayfish, sediment, and water). (A) Faith_pd plot illustrates that the alpha-diversity was significantly different ($p<0.001$) when comparing all sample types (crayfish, sediment, and water). (B) The water sample's microbiome appeared to vary from crayfish and sediment samples when comparing beta-diversity.

This study utilized the three herbicides glyphosate, atrazine, and 2,4-D because they are some of the most heavily used pesticides in the world (Atwood & Paisley-Jones, 2017; Meyer & Scribner, 2009) and often are applied in similar geographic areas (Figures 1-2), which opens the possibility of a combined exposure impacting non-target microbial communities. Crayfish were utilized in this experiment due to their roles as keystone species and ecosystem engineers and their frequent use as a model organism (Skelton et al., 2017). This experiment used the 48-hour time period and high pesticide amount to simulate a high intensity pulse disturbance (Shade et al., 2012), which reflects the conditions in a toxic spill. This experiment seeks to clarify how acute exposure to a combination of herbicides impacts microbial communities in a lotic system, specifically if a large acute pesticide exposure will reduce bacterial diversity and if it will shift which bacterial populations predominate in the resulting microbiome.

Materials and Methods

In August 2019, twelve adult crayfish (mean blotted wet mass of 3.40g) were collected from Opossum Creek: a forested second order stream near Liberty University (Figure 5) using a kick seine technique. The crayfish were collected and placed in Whirl-Pak bags that were filled with stream water and transported back to the lab.

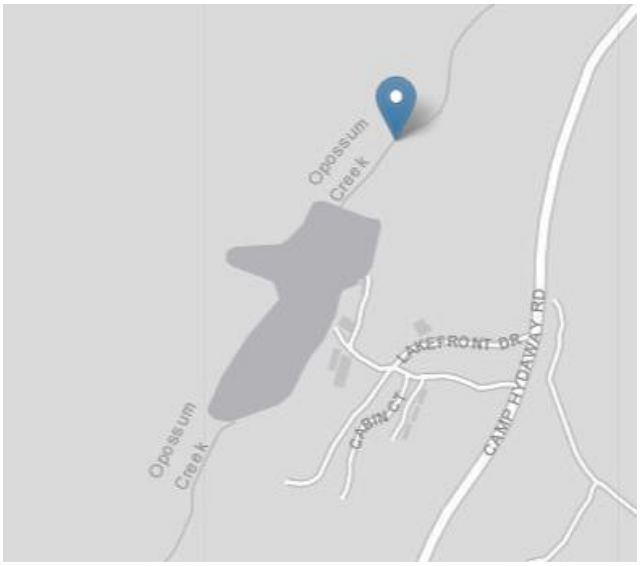

Figure 5. Collection site at Opossum Creek near Liberty University. Image obtained and adapted from Earth Explorer from U.S. Geological Survey, Department of the Interior/USGS (https://earthexplorer.usgs.gov/). In the public domain.

The crayfish were placed in individual tanks and allowed to acclimate for 9 days along with substrate from the collection site. Pre-exposure swabs were obtained in duplicate from the crayfish, sediment, and water. A total of 2000 ug/L (ppb) of atrazine (500 ppb), glyphosate (1000 ppb), and 2,4-D (500 ppb) were added to the 6 experimental tanks. 500 ppb of glyphosate were from a year-old mixture. This portion was significantly past the half-life of 45-60 days (Banaee et al., 2019) and was therefore not expected to impact the results of the experiment. The post-exposure swabs were then taken of the crayfish, sediment, and water at 48 hours and DNA was extracted from the swabs using a Qiagen DNeasy protocol. After extractions, unique forward primers were added to each sample, and PCR was conducted in duplicate and with a negative control using the 515F primer and unique 806R primers according to Illumina MiSeq protocol to amplify the 16s gene (Becker et al., 2014; Caporaso et al., 2010; Costello et al., 2009). The 16s gene is unique to each type of bacteria and allows researchers to determine species richness and

abundance within a sample (Paul et al., 2006). Gel electrophoresis was conducted on those samples in conjunction with PCR to check for contamination and to validate DNA presence. Samples were then quantified using the NanoDrop quantification protocol. The pooled sample was then sent off for Illumina sequencing at Molecular Biology Core Facilities using protocols similar to Caporaso et al. (2012). This specific sequencing technique was utilized due to its ability to measure a much greater number of microbial species than culturing techniques (Zimmerman et al., 2014). After the DNA was sequenced, it was analyzed using the bioinformatics software QIIME2, matching previously established protocol (Bolyen et al., 2019, see Appendix A) (Figure 6).

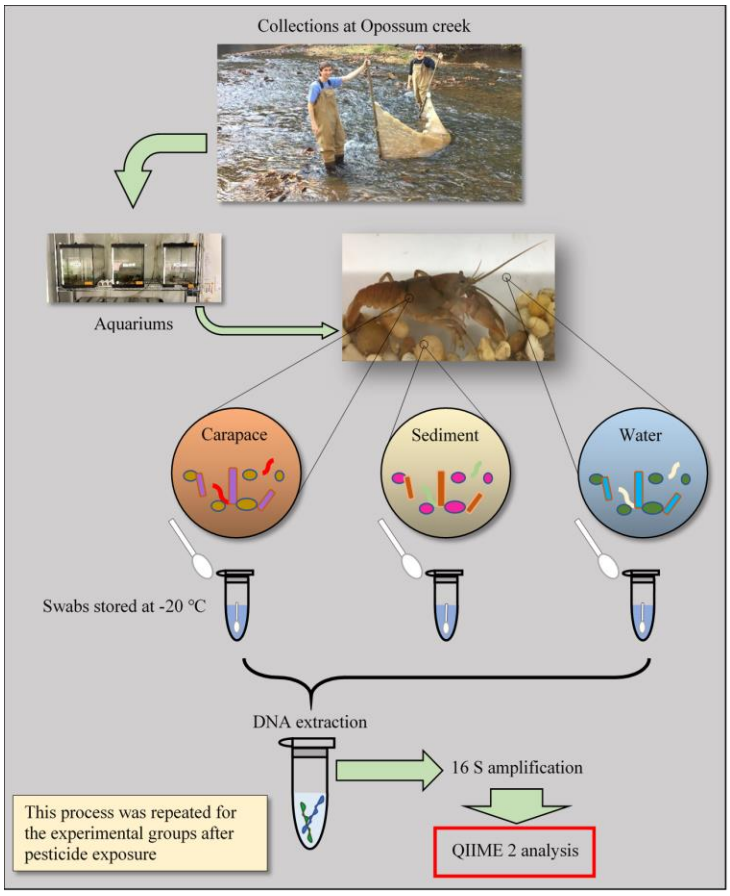

Figure 6. Experiment methodology. Crayfish were collected from Opossum creek, a forested stream, and placed in lab aquariums. Before and after acute pesticide exposure, samples were aseptically obtained from the crayfish, sediment, and water. Microbial DNA was extracted, the 16s gene was amplified using PCR, and the samples were sequenced and then analyzed using QIIME2.

Results

Impact on Alpha-Diversity

There were several notable results from the acute pesticide exposure. First, the acute exposure did not significantly impact the alpha diversity of the control vs experimental samples with a p-value of 0.29429 for the overall comparison (Figure 7) and when comparing pre- and post-exposure samples (Figure 8; Table 2) and post-exposure samples specifically (Figure 9; Table 2).

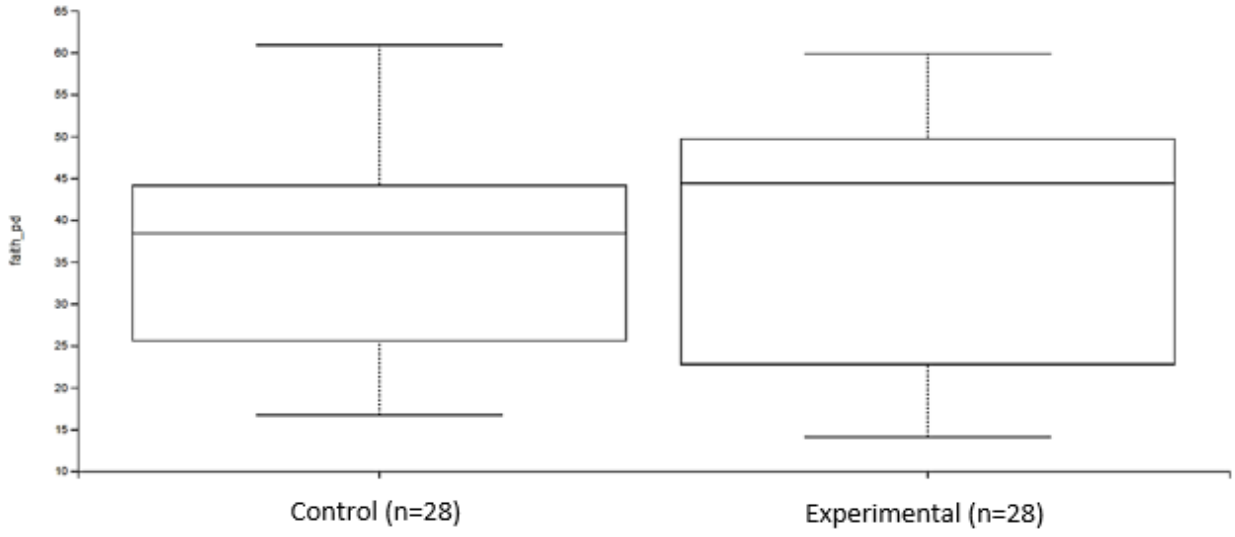

Figure 7. Faith_pd plot reveals there was not a significant difference in the alpha diversity of the control and experimental samples. P-value = 0.29429.

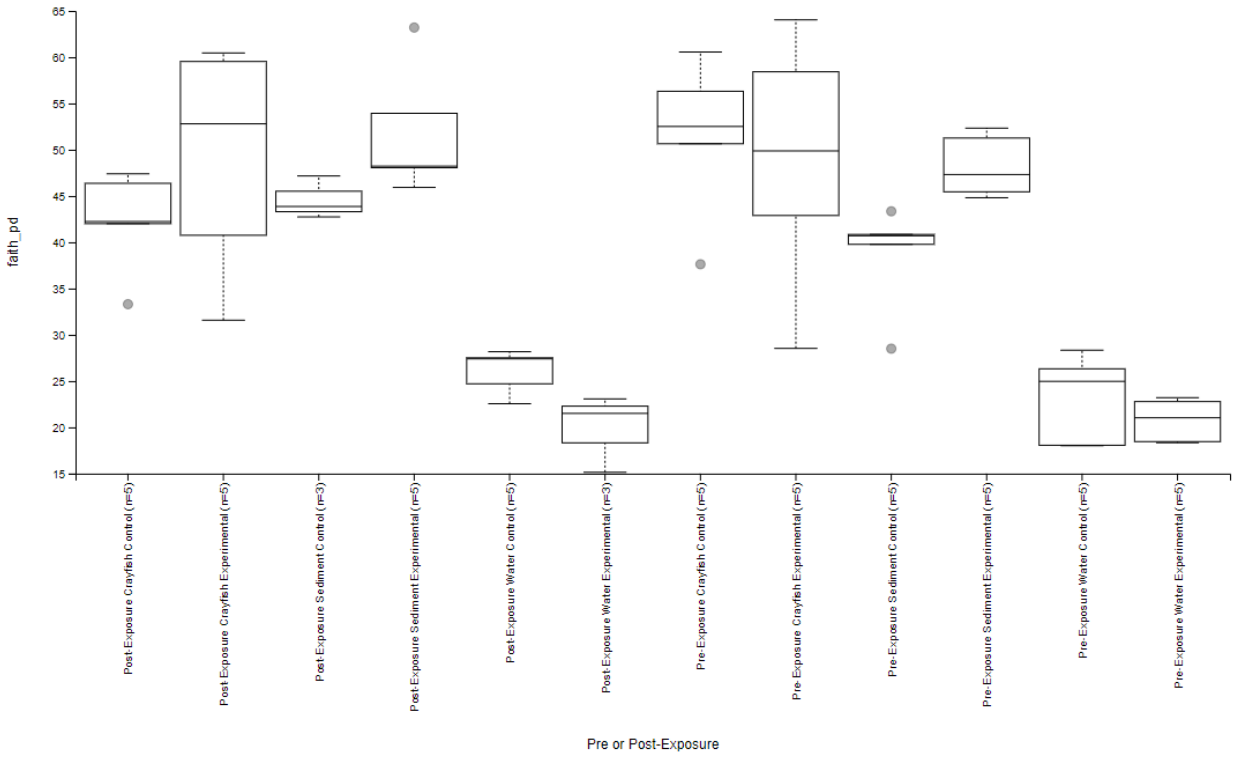

Figure 8. Comparison of control vs experimental and pre- and post-exposure samples shows there were no significant effects on the alpha diversity by acute pesticide exposure. The six box and whisker plots on the left side of the figure are post-exposure, and the six on the right side are pre-exposure. See Table 2 for p-values.

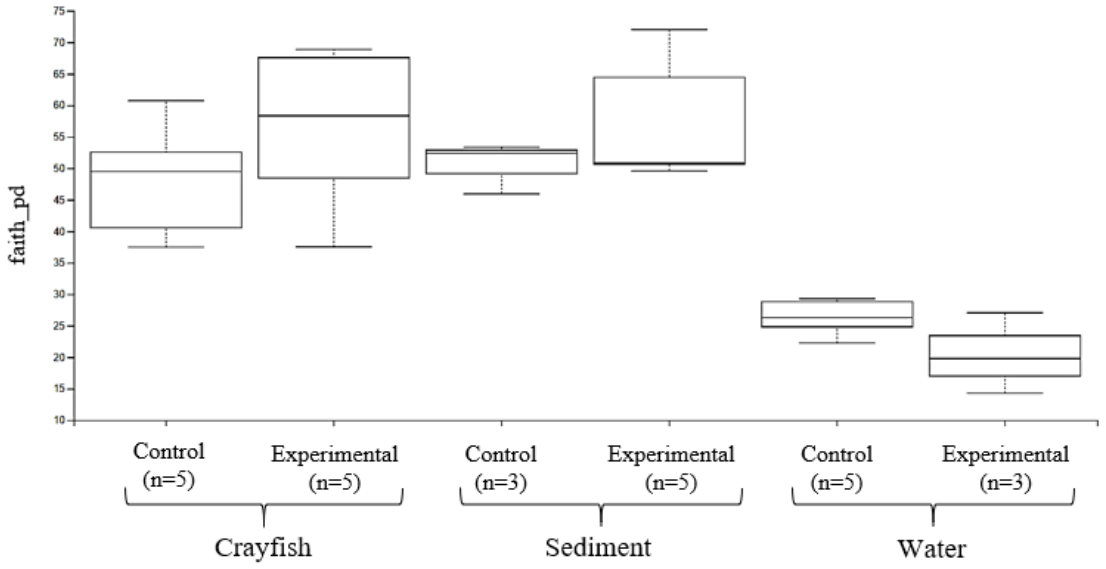

Figure 9. There was no significant difference in alpha diversity when comparing the control and experimental post-exposure sample types. Faith_pd plot measures species richness while taking into account phylogenetic distance between taxa. See Table 2 for p-values.

Table 2. p-values for comparison of sample types reveal acute pesticide exposure did not reach significance threshold of p-value = 0.05 for any sample type.

Sample Comparison	**p -value**
Pre- and Post-Exposure Crayfish Experimental	0.916815
Pre- and Post-Exposure Crayfish Control	0.117185
Pre- and Post-Exposure Sediment Experimental	0.075800
Pre- and Post-Exposure Sediment Control	0.296718
Pre- and Post-Exposure Water Experimental	0.654721
Pre- and Post-Exposure Water Control	0.250592
Post-Exposure Control vs Experimental Crayfish	0.347208
Post-Exposure Control vs Experimental Sediment	0.654721
Post-Exposure Control vs Experimental Water	0.179712

Impact on Beta-Diversity

Analyzing beta-diversity revealed a similar lack of significant impact. When comparing control versus experimental post-exposure sample types through Bray-Curtis and Jaccard ordination plots, the crayfish and water samples were not significantly different from each other (p-values > 0.05) (Figure 10). In contrast, post-exposure sediment samples were significantly different when comparing the control and experimental samples in the Jaccard similarity index

(Figure 10A). The comparison was not significant when viewed through the Bray-Curtis dissimilarity matrix (Figure 10B).

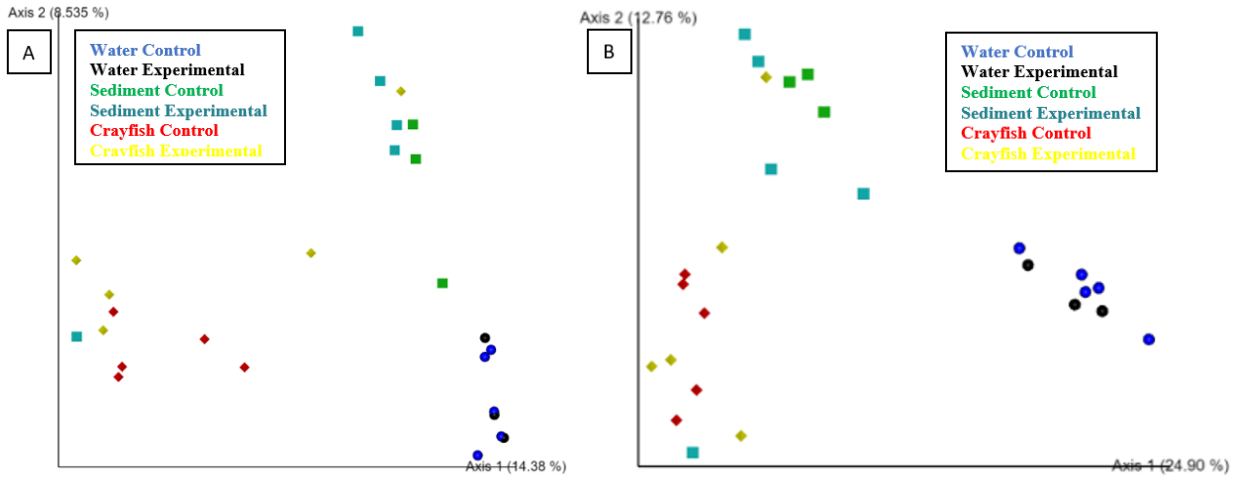

Figure 10. Bray Curtis and Jaccard ordination plots demonstrate differing effect on sediment samples. (A). The Jaccard similarity PCoA plot demonstrated a significant difference between control and experimental sediment samples (p-value = 0.041). The Jaccard plot is an unweighted test (does not consider taxa abundance), so it emphasizes unique microbial taxa. (B) The Bray-Curtis dissimilarity PCoA plot did not demonstrate a significant impact on the sediment post-exposure samples (p-value = 0.406). This plot is a weighted index, so it highlights taxa that make up a significant portion of the microbiome. The control and experimental post-exposure water and crayfish samples were not significantly different from each other (p-values > 0.05).

Difference Between Sample Types

Although acute pesticide exposure did not create a significant difference in the alpha diversity of pre- and post-exposure samples, sequencing data demonstrated that there was a significant difference between the water samples when compared to the crayfish and sediment samples when considering both alpha diversity (p-values: Crayfish to water = 1.422×10^{-7}.

Sediment to water = 6.580×10^{-7}) (Figure 11) and beta diversity (Figure 12). The sediment and crayfish samples were not significantly different with a p-value of 0.4649.

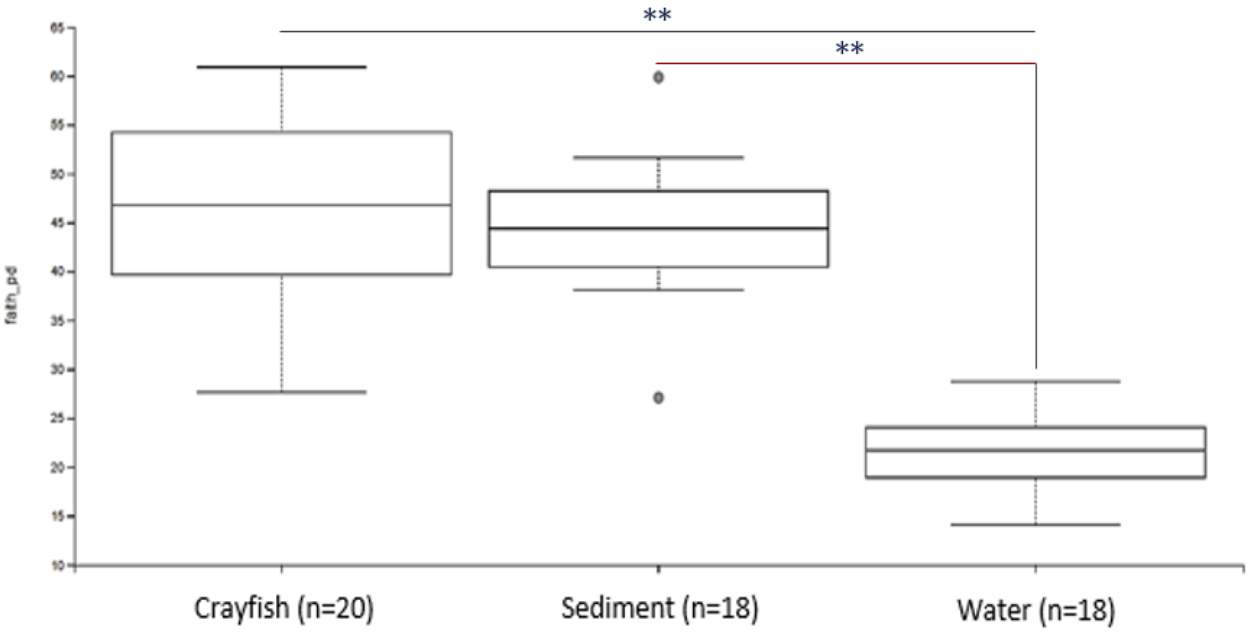

Figure 11. Crayfish and Sediment samples' alpha diversity were significantly different from the water samples, while crayfish and sediment samples were more similar in alpha diversity (** $p < 0.001$. p-values: Crayfish to water = 1.667×10^{-7}. Sediment to water = 3.509×10^{-7}. Crayfish to sediment = 0.4649).

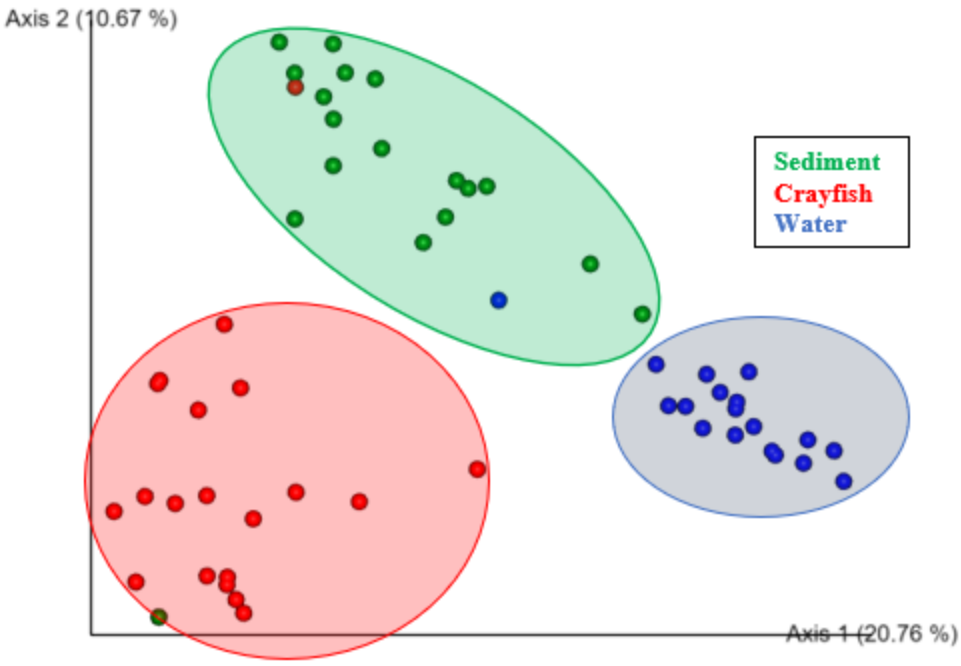

Figure 12. This Bray_Curtis principle coordinates analysis (PCoA) plot visualizes the distinct beta diversity for all sample types. PCoA plots measure similarity between samples, so samples that are closer together are more similar in beta diversity than those that are farther away from each other.

Specific taxa had unique distribution in the three sample types (Figure 13). There were several notable differences in the water samples. First, the bacterial family *Moraxellaceae* accounted for the most significant percentage of the water samples (from 6.3%-68.5%), while it accounted for a small percentage of the sediment and crayfish samples (largest percentage at 15.2%). Second, at the phyla level, the phylum Acidobacteriota, which was the 4th most frequent phyla among sample types, was virtually absent (<1%) in water samples. In addition, the bacterial family *Sphingomonadaceae* was most common in crayfish sample types.

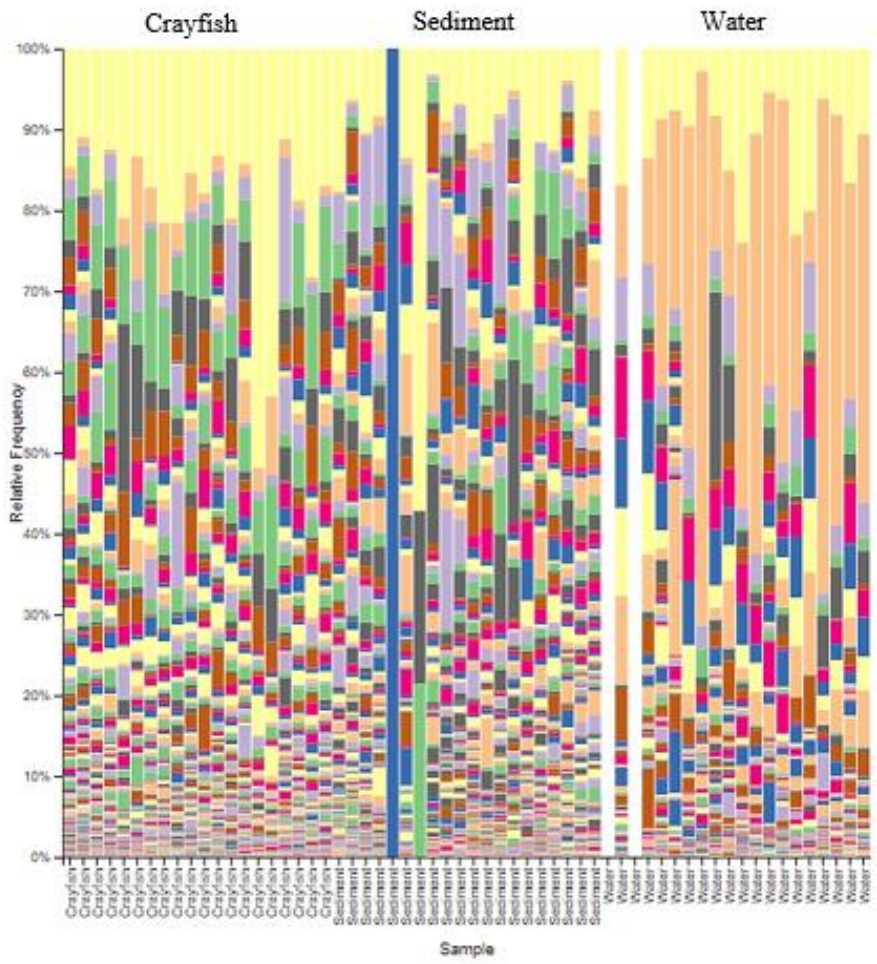

Figure 13. Taxa bar plot illustrating different taxa in sample types. This graph was measured at family taxonomic level.

Table 3. Top 10 most abundant microbial families.

Top 10 Most Abundant Microbial Families
1. *Comamonadaceae*
2. *Moraxellaceae*
3. *Pseudomonadaceae*
4. *Sphingomonadaceae*

5. *Flavobacteriaceae*
6. *Chitinophagaceae*
7. *Sanguibacteraceae*
8. *Devosiaceae*
9. *Rhizobiaceae*
10. *Enterobacteriaceae*

Discussion

Impact of Pesticides on Alpha Diversity

It was originally hypothesized that acute pesticide exposure would both significantly impact the diversity of the three sample types and alter the bacterial taxa that predominate. The results did not support the first hypothesis, as there were no significant differences in the alpha diversity when comparing pre- and post-exposure of the three sample types, with no experimental group having a p-value lower than 0.05 (Table 2). These findings also did not authenticate the second hypothesis that specific microbial taxa would be impacted. The taxa bar plot did not demonstrate any consistent alteration of specific phyla abundance for any sample type (Figure 13).

The sediment post-exposure samples suggested a possibly significant impact by pesticides on beta-diversity (Figure 10A). This measurement was only significant in the Jaccard plot and not in the Bray-Curtis plot (Figure 10B). Jaccard is an unweighted index, so it highlights unique microbial taxa. In contrast the Bray-Curtis dissimilarity matrix is weighted, so minor, unique microbial taxa would not significantly alter the beta-diversity of a sample. When

comparing these plots together, it suggests that pesticide exposure may have significantly altered taxa that were minor contributors to the overall microbiome, while significant bacterial taxa, such as *Comamonadaceae* or *Moraxellaceae*, were unaffected by the acute exposure. It is not clear if the minor taxa that were affected play a significant physiological or ecological role in the freshwater ecosystem. This is important to understand when considering if this alteration is a significant factor to consider when looking to improve freshwater conservation.

These findings differ from previous research completed by our lab, which suggested that atrazine and glyphosate in combination appear to have a synergistic effect on crayfish ectosymbiont health (Table 1). Instead, this combination of pesticides did not appear to have an additive or synergistic effect on the microbiome alpha diversity despite the large pesticide dose. These current findings align with Cedergreen's (2014) conclusion that synergistic effects are rare. However, Cedergreen warned that additive effects of a combination of pesticides may pose a larger danger than possible synergistic effects. It is surprising then, that no additive effect was observed when combining the three pesticides.

In addition, this result is surprising when considering the ability of the microbiome to resist different types of disturbances. Shade et al. (2012) reviewed the topic of microbial resistance generally by examining microbiomes from 247 studies in multiple environments responding to different types of disturbance. They found that 82% of the studies demonstrated microbial sensitivity (i.e. was not resistant) to the disturbance (Shade et al., 2012). This percentage may demonstrate a tendency to publish positive findings, but it does suggest that microbial resistance is not the norm in the published literature. Our experiment suggest that microbial communities have remarkable ability to resist herbicide anthropogenic disturbance

despite pesticide exposure being at toxic spill levels. This is an important finding when considering the possible negative impacts of pesticide disturbances in freshwater ecosystems.

A possible explanation for the lack of significant findings was the shorter time period the effects were measured over. Murturi et al. (2017) found that significant impact of a combination of insecticides and pesticides was not observed on day 3 but was observed on day 7. This suggests there may be an effect that is time dependent, which was not captured in this experiment.

Difference in Alpha and Beta Diversity Between Sample Types

The finding that crayfish and sediment alpha and beta diversity differed significantly from water diversity (Figures 11-13) reinforces previous research conducted by our lab, which demonstrated the significant difference between the three microbial communities (Figure 4). In the past experiment the water microbiome was most distinct, and the sediment and crayfish communities were more associated with each other. In this experiment that similarity was continued. Because crayfish live and burrow in the sediment of freshwater streams it is understandable that their microbiomes are similar. Acidobacteria was an important taxon in the sediment and crayfish samples but was virtually absent from the water samples microbiome. Other researchers have found that this phylum is common in soil environments (Kielak et al., 2016). This phylum and others demonstrate the unique bacterial phyla that makes the water's microbiome distinct from the sediment and crayfish's microbiomes.

Limitations of Experiment

There are several limitations to this study that are important to note. First, the number of samples are limited (10 control and 10 treatment samples per sample type). Although these

results do provide helpful insight into this research question, the limited number of samples restricts the conclusions that can be drawn due to possible variation in samples. Second, a portion of the pesticides applied (500 ppb of glyphosate) was from 1 year old stock. It is not expected that this portion was toxicologically active, as it was significantly past its half-life, and therefore should not have impacted the microbiome. However, it is important to take this into consideration when examining the results. Third, there are limitations inherent in testing the impact of pesticides in a lab-based study. Although the aquarium experimental structure allows for more control of variables and there was a pump in each aquarium that circulated water in each tank, the aquarium set up does not replicate the aspect of flowing water that is inherent in lotic systems. Fourth, introducing the crayfish into a lab-based setting is a possible disturbance to their microbiome that is independent of pesticide exposure. The 9-day acclimation period was designed to minimize this possible variable by stabilizing the microbiome before pesticide exposure, but it is an aspect that should be considered. Fifth, the post-exposure measurement at 2 days serves as a snapshot of pesticide impact, but it does not measure later chronic effects on the microbiome from the acute exposure. Therefore, although there was no significant impact detected, pesticide exposure may have later chronically altered the microbiome. Sixth, measuring alpha diversity and abundance of individual taxa accounts for lethal effects on the microbiome, but sublethal effects and functional changes in the microbiome are not captured by this experiment (see Kurenbach et al., 2017 for example of functional change).

Future Research

Future research is imperative for two reasons. First, the limitations in this study prompt future research. Experiments with a greater number of samples and measurements at multiple points in time will help verify the findings of this study. Also, research should continue in this

area that seeks to mirror the in vivo nature of lotic systems, despite its inherent unpredictability (Tornwall et al., 2015).

Second, more analysis is needed due to the importance of this area of research and the scarcity of research examining microbial communities in lotic systems (Tornwall et al., 2015). Although lotic systems only account for a small portion of the water on earth (0.01%), they are home to a much larger portion of the earth's biodiversity (6%) and are often threatened due to human activity (Dudgeon et al., 2006; Tornwall et al., 2015). Therefore, research in this area is also an important consideration of future conservation efforts. Much of the research in lotic systems has focused on macroinvertebrates and fish with few focusing on microbial communities (Tornwall et al., 2015). As previously noted, microbial communities are very important for the health of an ecosystem. Therefore, policy makers are missing an important piece when considering questions of conservation and toxicology when they do not sufficiently understand how human activity is impacting the freshwater microbiome. Recent sequencing technology allows researchers much greater ability to research this question (Zimmerman et al., 2014) to discover how microbial communities are impacted by anthropogenic activities.

Conclusion

As a society, it is important to understand how our attempts to shape the environment impact various aspects of that environment. Pesticides have been used for good in the improvement of food production and in the protection of human health. However, the incredible amount and prevalent use of pesticides each year poses a threat to the health of non-target ecosystems, including microbes. Microbes are important in the health of organisms and the greater environment and are worthy of research when considering questions of freshwater conservation. In this experiment, acute exposure to a combination of pesticides did not

significantly impact the alpha diversity of the microbiome of the crayfish, sediment, and water. Although one plot suggested a significant impact to the sediment's microbiome, the overall beta-diversity was also not significantly altered. This demonstrates the ability of microbial communities to resist pesticide exposure and anthropogenic disturbances. This topic should continue to be investigated due to the limitations of the experiment and because of the vital importance of this topic for freshwater ecosystem health.

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Appendix A. Bioinformatics Methodology

Sequencing results were analyzed using Quantitative Insights Into Microbial Ecology 2 (QIIME2). A qza artifact was made using the Atacama soils protocol and the data was then demultiplexed and denoised. A sampling depth was set to maintain sample quality. The analysis considering both pre- and post-exposure samples had a sampling depth of 8542, while the post-exposure analysis had a larger sampling depth of 34090. Multiple output tables were then constructed using these samples and the metadata file: A faith_pd plot to measure alpha diversity and Bray-Curtis, Jaccard, and unifrac output tables to visualize beta diversity. Pairwise Kruskal-Wallis and PERMANOVA tests were utilized to quantify alpha and beta diversity significance, respectively. Sequences were then classified using the trained taxonomy database, and mitochondria and chloroplast sequences were removed using Silva.