The Effect of Acetaminophen (Analgesic) and Diphenhydramine (Antihistamine) on Nociception Response of *Caenorhabditis elegans,* Heart Rate of *Eisenia fetida,* and Mortality of Both *E. fetida* and *C. elegans*

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Pharmaceutical pollution continues to increase each year but its adverse effects on the environment are still largely unknown. Pharmaceuticals can enter the terrestrial ecosystem when animal manure and sewage sludge are applied to land as a fertilizer or when irrigated with contaminated water. To understand the extent to which common drugs impact the ecosystem, two commonly polluting pharmaceuticals, acetaminophen and diphenhydramine, which have been found in many cities such as Chicago, Dallas, and most notably The Great Lakes, were studied. Various concentrations were tested on *E. fetida* and *C. elegans*. Indicators such as heart rate were observed for *E. fetida*, nociception response for *C. elegans,* and mortality for both. It was hypothesized that when acetaminophen and diphenhydramine were introduced, all indicators would be negatively affected. Thirty trials were conducted for each test. The results indicated the pharmaceuticals had a significant impact on heart rate, nociception response, and mortality of both *E. fetida* and *C. elegans* respectively: ($F(6,48)=262.33$, $p<0.001$), ($F(6,27)=169.67$, $p<0.001$), ($F(8,48)=2.98$, $p=0.0086$), ($F(6,27)=169.67$, $p<0.001$). With heart rates increasing by 171% for *E. fetida* in some cases and nociception response decreasing by over 46% for *C. elegans*, it was concluded that both populations were negatively affected by acetaminophen and diphenhydramine. Based on experimentation, even small concentrations of pharmaceuticals in the environment can have detrimental effects to certain organisms, severely impacting the ecosystem.

Introduction

While pharmaceutical drugs are critical for curing and preventing harmful diseases both in humans and animals, they can be detrimental to the surrounding environment if not disposed properly. The most common pharmaceuticals that are found in the environment are oral contraceptives, antibiotics, antifungals, statins or cytotoxins, nonsteroidal, metabolites, anti-depressants, anti-inflammatory drugs (Boxall, 2004). Many of these drugs reach the environment unintentionally as human drugs are metabolized and then excreted into sewer systems. Up to 80% of these pharmaceuticals are still present in sewer systems, as facilities are not built to extensively filter them out. These active pharmaceutical ingredients may be released into the soil environment when contaminated sewage sludge or animal manure is applied to land. (Carter, et al., 2014). Increased concentration of various drugs on land or in sewage systems can have devastating effects on certain organisms leading to lasting impacts on the food chain.

Concentrations of acetaminophen, also known as paracetamol, are on the rise. Used as both an analgesic, to treat minor pains/headaches, and antipyretic, to reduce fever, one in four people in the U.S. take some form of an acetaminophen containing product. With over 27 billion doses sold in 2009, it has become one of the most widely used over the counter drugs (Smith 2015). As a result, acetaminophen has become a growing pollutant over the years, with levels of up to 10,000 ng l^{-1} have found in surface water (Boxall 2004). Since analgesic drugs are rapidly excreted in urine, up to 2-5% of acetaminophen is excreted as an unchanged metabolite through urine, which can potentially harm the surface water of aquatic or terrestrial organisms (Ziylan, et al., 2011).

While acetaminophen itself is not toxic, the medicine can be converted into a toxic metabolite in the liver causing a potential overdose in the organism by xenobiotic metabolism (Mazaleuskaya, et al., 2015). Through this process, the harmful byproduct, N-acetyl-p-benzoquinone imine (NAPQI), is formed by the oxidative action of cytochrome P450 enzymes or prostaglandin H synthase. Which allows NAPQI to bind to hepatic proteins causing hepatotoxicity. Usually, in small doses, our body will negate this harmful byproduct by producing glutathione which NAPQI will attach to forming a nontoxic glutathione conjugate. However, when too much acetaminophen is consumed, our body is not able to detoxify or negate all the NAPQI which leads to an overdose (Mazaleuskaya, et al., 2015). When acetaminophen levels rise, the concentration may increase to the level where animals cannot produce enough glutathione to negate NAPQI, therefore leading to death. While some wastewater treatment plants have high removal rates for acetaminophen, regardless of the efficiency, acetaminophen and other drugs still continue to be found in the environment from other means. This growing concern has been present for other pharmaceuticals as well, such as diphenhydramine.

Another common over the counter drug, diphenhydramine is an antihistamine, mainly used for allergy relief and as a sedative to alleviate coughing; it blocks the effects of histamines at the H1 receptor (Topp, et al., 2012). When consumed 2-15% of diphenhydramine can be excreted completely unchanged even after hepatic metabolism. Moreover, sewage treatment plants are unable to completely break down the pharmaceutical as 25% of nearby surface water contained some amount of diphenhydramine (Topp, et al., 2012). Previous research has shown a mean concentration of diphenhydramine in nature that ranges from 1.2-10 ng g^{-1} which was detected from tissue analysis of fish. Consequently, when soil is irrigated by this surface water, research has shown that diphenhydramine begins to form irreversible bonds with the soil properties. According to Topp (2012), following biosolid application, diphenhydramine remained in the soil even after three years. This can potentially have unintended effects on organisms that live in the terrestrial ecosystem.

One such common organism is *E. fetida* or earthworms. *E. fetida,* like humans, can perform xenobiotic metabolism, as they also possess cytochrome P450 enzymes, allowing them to break down acetaminophen into its harmful byproduct NAPQI (Cao et al., 2017). Few studies have been done to understand the effects of drugs on worms but many have been done to see how they respond to heavy metals. Cao et al., (2017) research shows *E. fetida's* CYP450 enzymes try to metabolize heavy metals such as Cadmium (Cd), Lead (Pb), or Zinc (Zn) rather than excrete them right after consumption, negatively impacting the worms. A combination of these heavy metals and large doses of drugs can be very detrimental to the earthworm which plays a large role in the environment.

E. fetida provide numerous benefits through their burrowing and feeding behavior. An increase in overall soil quality is shown, resulting in greater crop production. The following soil properties can be improved by *E. fetida*: available nutrients, better drainage, soil aeration, and soil organic

matter. *E. fetida* feed on plant debris, where their digestive system allows them to concentrate these available nutrients and organic matter into their stool (Duiker, 2017). As *E. fetida* burrow in the soil, they often lay their nutrient rich stool in these tunnels, thus providing a favorable environment for root growth (Duiker, 2017). The importance of *E. fetida* to mix surface nutrients with soil becomes very evident, for when they are introduced to the environment, an increase in soil organic matter begins to show, resulting in substantial crop growth. Moreover, these tunnels allow plant roots to pervade deeper into the soil, where they can gain access to extra moisture and nutrients. Through their extensive channelling, the soil is loosened and aerated, thus improving soil drainage. Since worms are essential and beneficial to the environment, it is important to understand any factors that threaten the population. While in reality both heavy metals and drugs in combination have an effect on earthworms, it is important to understand how drugs individually impact the worms. To understand some of the subtle effects heart rate was measured. This is an excellent indicator, for a high or low heart rate can provide a deeper understanding on some of the subtle effects these pharmaceuticals have. Mortality was also measured to understand how these pharmaceuticals affect the population. Much like earthworms, nematodes help contribute to the overall structure and function of the terrestrial ecosystem.

Nematodes or *C. elegans* are commonly found in anthropogenic habitats located in compost or the soil itself. *C. elegans* are also essential to environment since they enhance soil quality in three major ways: mineralizing nutrients for plants, providing a food source for other soil organisms, and lastly regulating population of other organisms as well as disease causing organisms. *C. elegans* feed on smaller organisms and distribute any bacteria or fungi in their digestive system when consumed. When consuming bacteria or fungi, these nematodes release excess ammonium (NH_4^+) which is released in a plant available form (Ferris, 2010).

Like earthworms, *C. elegans* can be threatened by the presence of these pharmaceutical pollutants. Moreover, *C. elegans* are also able to perform xenobiotic metabolism, as they posses CYP450 enzymes, thus allowing them to metabolize acetaminophen into NAPQI but also overdose when there is too much (Gotoh, 1998). The subdued effect of acetaminophen would also affect *C. elegans* nociception response which is a rapid withdrawal behavior designed to protect the organism from potential danger. Since *C. elegans* are preyed upon by other nematodes, insects, microarthropods, bacteria and fungi, as well as earthworms such as *E. fetida,* a slower escape response could decrease its chance of survival. Due to the importance of nociception response, it was used to test the effect of acetaminophen (analgesic) and diphenhydramine (antihistamine) on the escape behavior of *C. elegans*. This dependent variable can act as an indicator to clearly show any significant effects of these pharmaceuticals. Likewise, Leung et al. (2016) studied the effects of various analgesics such as ibuprofen on stereotypical escape behavior for *C. Elegans*. This was done by quantifying the escape behavior from the center of velocity (pixels/s), showing it negatively affected nociception response with a decrease in escape responses to 13 ± 9 pixels per second (Leung et al., 2016). This same methodology can be used to understand the effects of acetaminophen and diphenhydramine on *C. Elegans*. Moreover, equivalent to *E. fetida,* mortality was also be measured, to indicate how varying concentrations affect life span of the nematode.

Pharmaceutical pollution continues to increase year to year but its adverse effects on the environment are still largely unknown. Human medicines are now widely detected at low concentrations in surface freshwater, groundwater, and coastal saltwater. Pharmaceuticals can enter the soil environment when animal manure and sewage sludge are applied to land as a fertilizer or during irrigation with contaminated water. Crowe (2014) states that researchers have already detected medicines in the Great Lakes at concentrations high enough to be a concern such as acetaminophen, codeine, antibiotics, hormones, steroids, and antiepileptic compounds, and dozens of other chemicals. Moreover, as stated by the Environmental Health Division (2014) acetaminophen have been found in trace amounts of the drinking water in Minnesota. However, acetaminophen isn't the only pharmaceutical polluted, as the U.S. Environmental Protection Agency has found diphenhydramine near Chicago, Dallas, Philadelphia, Phoenix and Orlando, Florida. Rivers in these areas receive large amounts of wastewater discharge from nearby sewage treatment plants (Fish in U.S. Rivers Tainted With Common Medications, 2009). Studies have shown that acetaminophen interferes with normal embryonic development, reproduction, growth, behavior, survival, and endocrine system functions of fish (Minnesota Department of Health, 2014). Aquatic ecosystems are mainly tested, for wastewater primarily ends up polluting rivers. However, through biosolid application and irrigation, soil and the terrestrial ecosystem are impacted as well. Little is known of pharmaceutical effects on soil dwelling organisms. The purpose of this study was to test the pharmaceuticals acetaminophen and diphenhydramine on the subtle effects they may impose on *E. fetida* and *C. elegans.* Mortality, heart rate, and nociception response are measured to gain a deeper understanding on what these medications may impose.

It was hypothesized that if acetaminophen was used on *E. fetida* and *C. Elegans,* then the mortality would increase, because both possess CYP450 enzymes which breaks down the drug into its harmful byproduct NAPQI. However, if diphenhydramine was used on *E. fetida* and *C. Elegans,* then mortality would remain the same for *E. fetida*, but an increase in mortality would be shown for *C. Elegans* due to its relative size. Moreover, if both pharmaceuticals were used on *E. fetida,* then the heart rate would increase, because one of the side effects for both drugs in humans is elevated heart rate and hypotension. If both pharmaceuticals were used on *C. elegans* than nociception response would decrease as both medicines induce relief.

The test included ranges of doses of acetaminophen and diphenhydramine from 1, 5, and 10 ppm, and a controlled group kept at 0 ppm. The experiment lasted over a 3 week period. Morality was studied in both *E. fetida* and *C. elegans*. A random grid analysis was used for *C. elegans* to measure a sample as calculating the true sample size was not feasible. Heart rates of each of the thirty *E. fetida* were measured by locating the clittelium and counting the beats for thirty seconds and multiplying it by two to get bpm. Lastly, nociception response was quantified by using an infrared laser and measuring the speed of *C. elegans* over fifteen seconds. Data were then statistically analyzed using a repeated measures ANOVA test at alpha equal to .05.

Methods

Both *E. fetida* (earthworms) and *C. elegans* (nematodes) are soil dwelling organisms that are essential to the terrestrial ecosystem. They are used as model organisms to show the impact pharmaceuticals can have on organisms in the soil. Varying concentrations of common over the counter drugs were tested on these organisms to understand the contextual impact the drugs may have on the organisms when polluted in the environment.

Both *E. fetida* and *C. elegans* remain active in depths of greater than or equal to 7 cm. While *E. fetida* can be kept in soil, *C. elegans,* on the other hand, can be cultured in nematode agar plates using aseptic techniques. The terrestrial environment for *E. fetida* was created using plastic containers with ventilated lids allowing oxygen through. In each container, soil was sifted into 2 mm particles until 1 kg was obtained. Since soil has varying water sorption capacities, making a homogenous mixture for each test will remove any variability. When administering the pharmaceuticals into the ecosystem, water was used as the solvent to dissolve both acetaminophen and diphenhydramine. It was important for the earthworm habitats to have moistures of 60-80%, as this is most suitable for these organisms (Carter, et al., 2014). Through preliminary tests it was discovered that 200 mL of water was needed per kg. This being said, 1 mL of water was necessary for the nematode cultures where a proportional ppm concentration was created. In addition, to sustain constant moisture as well as enough food in the *E. fetida* habitats, necessary amounts of distilled water was given to each container proportionally, and 5 grams of potato starch was given daily. *C. elegans* are cultured on nematode agar plates with *E. Coli*

as the food source. *C. elegans* are cultured for 3 days prior to experimentation to ensure nematodes of varying stages up to L4 were established. Lastly, *E. fetida* were randomly assigned to a container till a sample size of 30 was reached. Each organism had 48 hours to accustom to the new environment. The experiment was conducted over a 21 day period.

When culturing *C. elegans* the following procedures were followed. First, Nematode growth agar from Carolina Biological was used to fill 10 Petri dishes two thirds the way full. Next, using *Escherichia coli*, OP50, Living, Plate Culture from Carolina Biological, a inoculating loop was then used to isolate single colonies on a streak plate of agar. Only two streak plates were initially seeded and left to grow overnight at room temperature. The next step was to culture the *C. elegans* on the petri plates. Using aseptic techniques an inoculating loop was used to streak the initial two seeded plates with *C. elegans* in the opposite direction. It takes 3 days for *C. elegans* to mature and complete its life cycle. On day 2 of culturing seven more streak plates were seeded with *E. Coli* and left to grow overnight. Now on day 3, the initial active *C. elegans* cultures were ready to be chunked and added to the seven seeded streak plates. Using a sterile spatula, a small block of agar from an active plate of *C. elegans* was cut out and placed face down on the surface of a new agar plate. This was done for all 7 of the plates being tested. After, another 3 days of culturing, the seven *C. elegans* culture plates were now ready to be used for testing.

To understand the effects of the medicines at varying concentrations, mortality of both *E. fetida* and *C. elegans* was one of the dependent variables. Varying doses of acetaminophen and diphenhydramine of 1 ppm, 5 ppm, and 10 ppm are applied to the soil (see figure 2). Data were recorded every 2 days by counting the number dead. When measuring the mortality of *E. fetida*, after analyzing each organism they were placed in an extra container housed with 1 kg of soil, this eliminated counting the same worm twice. After each worm was analyzed and data were recorded, the worms were placed back into their experimental habitat. However, to study the mortality of *C. elegans* the population was rapidly reproducing, this makes finding the true population size in the sample not feasible. Moreover, a sample of each test was chosen using a random grid analysis. Through each sample a total of 30 *C. elegans* was first to counted to ensure sample sizes were equal, if not a new random sample was selected. Data were then recorded by counting how many were dead.

In addition, measuring pulse rates produce deeper understanding to some of the subtle effects these medications have. *E. fetida* have five hearts which pump blood throughout the body. They are generally located right above the Clitellum. Each time the worm contracts was equal to one beat. By locating the Clittellum the rate was determined by counting the worm's pulse for 30 seconds and multiplying it by 2 to get beats per minute.

Lastly, nociception response of *C. elegans* was tested using a Infrared Laser Diode, of 780 nm and working current of 100mA. The laser diode was attached to the a dissecting microscope and then focused at the focal plane of the *C. elegans* (Wittenburg, et. al., 1999). The laser was aimed at the head of the nematode for 1 second and then the speed (meters/second) was analyzed over a 15 second time frame (see Appendix A, Figure 7).

Figure 1: Experimental Design Diagram

Hypothesis:
H_o: There is no difference between Heart Rate, Mortality, and Nociception Response with the control *E. fetida/C. elegans* and the treatments.
H_a: There is a significant difference between Heart Rate, Mortality, and Nociception Response with the control *E. fetida/C. elegans* and the treatments.

Control (No Drug)		acetaminophen		diphenhydramine		acetaminophen		diphenhydramine		acetaminophen		diphenhydramine	
0 ppm		1 ppm				5 ppm				10 ppm			
E. fetida	*C. Elegans*	*E. fetida*	*C. Elegans*	*E. fetida*	*C. Elegans*	*E. fetida*	*C. Elegans*	*E. fetida*	*C. Elegans*	*E. fetida*	*C. Elegans*	*E. fetida*	*C. Elegans*
30	30	30	30	30	30	30	30	30	30	30	30	30	30

Independent variable: Varying concentrations of acetaminophen and diphenhydramine
Dependent Variable: Mortality, Pulse Rate (*E. fetida)*, Nociception Response (*C. elegans)*
Control Variable: No pharmaceutical applied
Constants: Container size, amount of soil, soil depths, amount of water, soil moisture, species of organisms, testing period, number of organisms in each container

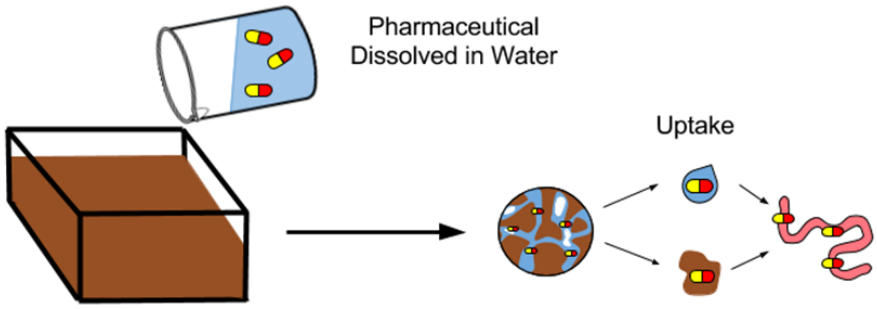

Figure 2: Experimental Setup for *E. fetida*. The figure above shows the experimental design for introducing the pharmaceuticals to *E. fetida*. Each drug is water soluble and was administered when dissolved. The uptake process is also represented above.

Results

The research consisted of *C. elegans* and *E. fetida* with varying Treatments: Control (Treatment 1), Acetaminophen 1 ppm (Treatment 2), Acetaminophen 5 ppm (Treatment 3), Acetaminophen 10 ppm (Treatment 4), Diphenhydramine 1 ppm (Treatment 5), Diphenhydramine 5 ppm (Treatment 6), and lastly Diphenhydramine 10 ppm (Treatment 7). 30 trials was conducted for each treatment. Data were collected every two days for twenty one days for each treatment (see Figure 1). A repeated measures ANOVA was then conducted at alpha equal to 0.05.

The descriptive statistics clearly show that the mean heart rate of *E. fetida* for treatment: 2 (M=7.59, SD=0.480), 3 (M=5.733, SD=0.502), 5 (M=9.252, SD=0.349), 6 (M=8.325, SD=0.529), and 7 (M=7.978, 0.307) were lower than the control (M= 10, SD=0.672). The shows an overall decrease in heart rate when compared to the control except for treatment 4, acetaminophen 10 ppm, where it drastically increased. However, in treatment 4 (M=17.104, SD=1.270) the mean heart rate was significantly higher than the control. When looking at mortality, it seems an increase was shown respectively with dosage. Furthermore, as concentrations rose from 5 ppm to 10 ppm with acetaminophen, increase in mortality was shown as treatment 3 (M=0.222, SD=0.441) was less than treatment 4 (M=1.11, SD=1.453). The same trend was shown with diphenhydramine, for treatment 6 (M=0.222, SD=0.441) was less than treatment 7 (0.556, 0.294). When looking at nociception response of *C. elegans*, the average speed (meters/second) tended to be lower when varying concentrations of the drugs were introduced. This was shown as treatment: 2 (M=0.050, SD=.0007), 3 (M=0.035, SD=0.0015), 4 (M=0.021, SD=0.0011), 5 (M=0.058, SD=0.0019), 6 (M=0.049, SD=0.0016), and 7 (M=0.044, SD=0.0018) were lower than the control (M=0.067, SD=0.0040). Lastly, with mortality the same trend was shown with *C. elegans*. For acetaminophen the average mean mortality increased on average from least to greatest respectively: treatment 2 (M=3.750, SD= 1.982), 3 (M=5.000, SD=1.673), and 4 (M=7.50, SD=2.65). The same trend was shown for diphenhydramine from least to greatest respectively: treatment 5 (M=3.750, SD=1.581), 6 (M=4.751, SD=1.718), and lastly treatment 7 (M=5.000, SD=2.191).

Table 1: Descriptive statistics of means on heart rate of *E. fetida*

Variable	Treatment	Mean	SE Mean	StDev	Min	Q1	Median	Q3	Max
Measurement	1	10.000	0.224	0.672	8.867	9.498	9.900	10.666	10.900
	2	7.590	0.160	0.480	7.110	7.132	7.467	7.966	8.467
	3	5.733	0.167	0.502	5.000	5.300	5.733	6.233	6.467
	4	17.104	0.423	1.270	15.667	16.033	16.867	17.700	19.800
	5	9.252	0.116	0.349	8.667	9.000	9.333	9.467	9.867
	6	8.325	0.180	0.539	7.600	7.963	8.333	8.467	9.533
	7	7.978	0.102	0.307	7.333	7.833	8.000	8.200	8.400

The descriptive statistics for heart rate of *E. fetida* are given above. Treatments 2-3 lowered the heart rate and induce relief, while treatment 4 increased exponentially by 171%. Treatments 5-7 lowered heart rate.

Table 2: Descriptive statistics of means on nociception response of *C. elegans*

Variable	Treatment	Mean	SE Mean	StDev	Min	Q1	Median	Q3	Max
Measurement	1	0.067	0.0014	0.0040	0.0620	0.0636	0.0677	0.0713	0.0730
	2	0.050	0.0002	0.0007	0.0497	0.0500	0.0503	0.0507	0.0517
	3	0.035	0.0007	0.0015	0.0337	0.0338	0.0340	0.0367	0.0370
	4	0.021	0.0006	0.0011	0.0207	0.0207	0.0210	0.0227	0.0227
	5	0.058	0.0007	0.0019	0.0543	0.0560	0.0583	0.0587	0.0600
	6	0.049	0.0007	0.0016	0.0473	0.0476	0.0495	0.0504	0.0517
	7	0.044	0.0008	0.0018	0.0417	0.0427	0.0444	0.0459	0.0462

The descriptive statistics for nociception response of *C. elegans* are given above. Response was measured by speed (meters per second). All treatments lowered nociception response.

Table 3: Descriptive statistics of means on mortality of *E. fetida*

Variable	Treatment	Mean	SE Mean	StDev	Min	Q1	Median	Q3	Max
Measurement	3	0.222	0.147	0.441	0.000	0.000	0.000	0.500	1.000
	4	1.111	0.484	1.453	0.000	0.000	1.000	2.000	4.000
	6	0.222	0.147	0.441	0.000	0.000	0.000	0.500	1.000
	7	0.556	0.294	0.882	0.000	0.000	0.000	1.500	2.000

When measuring mortality of *E. fetida*, treatments 1, 2, and 5 were taken out as no mortality was shown. Treatment 4 (acetaminophen 10 ppm) had the greatest impact with an average of 1.11, followed by treatment 7 with 0.556.

Table 4: Descriptive statistics of means on mortality of *C. elegans*

Variable	Treatment	Mean	SE Mean	StDev	Min	Q1	Median	Q3	Max
Measurement	1	3.333	0.333	1.000	2.00	2.50	3.00	4.00	5.00
	2	3.750	0.701	1.982	1.00	2.25	3.50	5.50	7.00
	3	5.000	0.683	1.673	3.00	3.75	5.00	5.75	8.00
	4	7.50	1.32	2.65	4.0	4.75	8.0	9.75	1000
	5	3.750	0.559	1.581	2.00	2.00	4.00	5.00	6.00
	6	4.571	0.649	1.718	2.00	3.00	5.00	6.00	7.00
	7	5.000	0.894	2.191	2.00	3.50	4.50	7.25	8.00

The descriptive statistics above show mortality of *C. elegans*. The average total death per day tended to be roughly the same for all treatment except treatment 4.

When looking at the inferential statistics on heart rate of *E. fetida* it was shown that a significant difference existed within the treatments ($F(6,48)=262.33$, $p<0.001$), however time ($F(8,48)=0.71$, $p=0.681$) was not (see table 5). Furthermore, when constructing a Tukey test, it was known that heart rate of treatments 1, 2, 3, 4, 6, and 7 were significantly different than the control as $p<0.05$ (see Appendix B, Table 9). As a result the hypothesis was supported. However, for treatment 5 $p=0.2174$, thus fail to rejecting the null hypothesis (see Appendix B, Table 9). When looking at mortality of *E. fetida* both time ($F(8,48)=2.98$, $p=0.0086$) and the treatments ($F(6,48)=4.13$, $p=0.020$) were significant (see table 6). However, once constructing a Tukey test only treatment 4 was significant when compared to the control as $p=0.018$ (see Appendix B, Table 10). When looking at the ANOVA test for nociception response of *C. elegans* it was shown that a significant difference existed within the treatments ($F(6,27)=169.67$, $p<0.001$), however time ($F(7,27)=0.56$, $p=0.779$) was not (see table 7). It was confirmed by the Tukey test that all treatments were significantly different than the control as $p=0.0002$ (see Appendix B, Table 11). Lastly when looking at the mortality of *C. elegans* it was shown that both time ($F(8,48)=5.02$, $p=0.0004$) and the treatments ($F(6,48)=3.16$, $p=0.00734$) were significant (see table 8). However, much like *E. fetida* using the Tukey Test it was concluded that only treatment 4 was significant with $p=0.006$ (see Appendix B, Table 12).

Table 5: General linear model ANOVA on heart rate of *E. fetida*

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Time	8	2.586	0.323	0.71	0.681
Treatment	6	716.616	119.436	262.33	<0.001
Error	48	21.854	0.455		
Total	62	741.056			

A repeated measures ANOVA was conducted between time and treatment on heart rate of *E. fetida* with alpha at 0.05. The high F-value and low p-value shows treatment was significant, while the opposite was shown for time, thus showing it was not significant.

Table 6: General linear model ANOVA on mortality of *E. fetida*

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Time	8	8.698	1.0873	2.98	0.0086
Treatment	6	9.048	1.5079	4.13	0.0020
Error	48	17.524	0.3651		
Total	62	35.270			

A repeated measures ANOVA was conducted between time and treatment on mortality of *E. fetida* with alpha at 0.05. The high F-value and low p-value shows both the treatments and time were significant.

Table 7: General linear model ANOVA on nociception response of *C. elegans*

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Time	7	0.000022	0.000003	0.56	0.779
Treatment	6	0.005724	0.000954	169.67	<0.001
Error	27	0.000152	0.000006		
Total	40	0.006731			

A repeated measures ANOVA was conducted between time and treatment on nociception response of *C. elegans* with alpha at 0.05. The high F-value and low p-value shows treatments were significant, while the opposite was shown for time, thus showing it was not significant

Table 8: General linear model ANOVA on mortality of *C. elegans*

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Time	8	71.17	8.897	5.02	0.00040
Treatment	6	38.40	6.400	3.61	0.00734
Error	33	58.54	1.774		
Total	47	189.67			

A repeated measures ANOVA was conducted between time and treatment on mortality of *C. elegans* with alpha at 0.05. The high F-value and low p-value shows both treatment and time were significant.

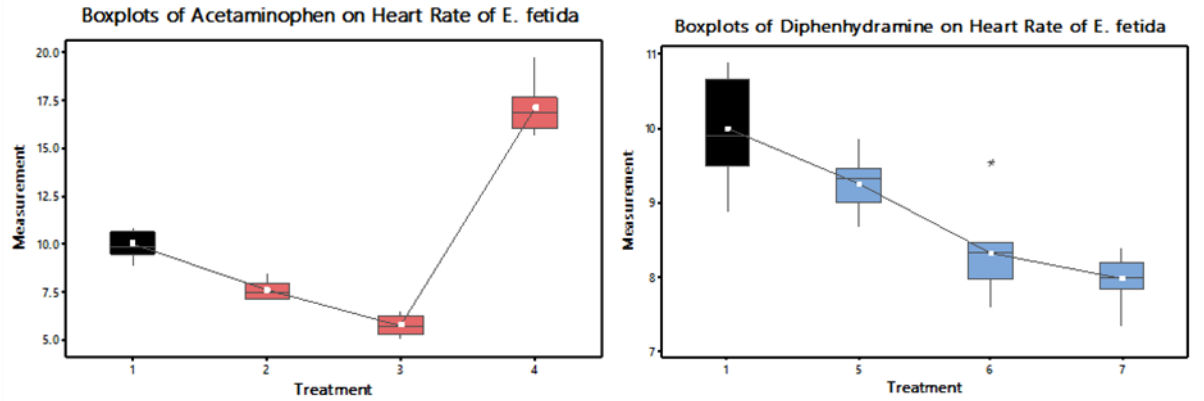

Figure 3: Boxplots on heart rate of *E. fetida.* This figure shows the control (treatment 1) compared to acetaminophen on heart rate on the left, while the boxplot on the right shows the same control compared to diphenhydramine on heart rate of *E. fetida*. Both graphs are at different scales to accurately compare each pharmaceutical to the control. Treatment 4 (acetaminophen 10 ppm) had a significantly higher heart rate than the control, while it was also shown that treatment 2, 3, 5, 6, and 7 have a mean lower than the control (treatment 1).

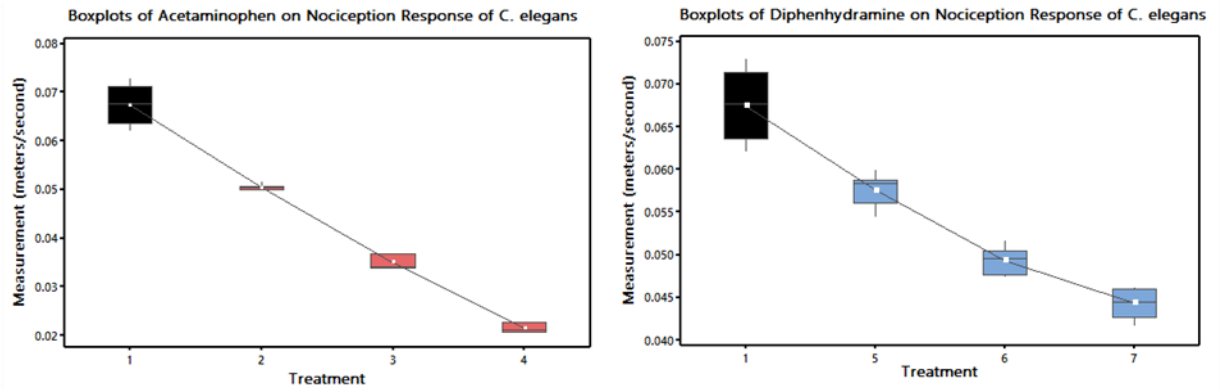

Figure 4: Boxplots on nociception response of *C. elegans.* This figure shows the control (treatment 1) compared to acetaminophen on nociception response on the left, while the boxplots on the right shows the same control compared to diphenhydramine on nociception response of *C. elegans*. Both graphs are at different scales to accurately compare each pharmaceutical to the control. All treatments with varying concentrations of acetaminophen and diphenhydramine negatively impacted nociception response, for speed (meters/second) decreased. Treatment 4 seems to affect the response speed the most for its mean was the lowest.

Discussion

The purpose of this study was to characterize the effects of two pharmaceutical drugs, acetaminophen and diphenhydramine, on two widely abundant organisms in the environment: *E. fetida* and *C. elegans.* Acetaminophen and diphenhydramine are found in popular over the counter drugs like Tylenol and Benadryl. Such drugs continue to be improperly disposed into the environment, and as pharmaceutical sales continue to rise, this will only continue. To understand how these drugs will impact the environment and organisms in it, varying concentrations of acetaminophen and diphenhydramine were tested on these organisms to understand the impact the drugs may have on the organisms when polluted in the environment. *E. fetida* and *C. elegans* were chosen as the model organisms due to their widespread abundance and critical role in their environment and food chain. For, *E. fetida* increases crop yield by 30% (Duiker, 2017), and *C. elegans* are prey for over 25 different species of animals (Ferris, 2010).

Previous research have shown an earthworm's uptake of pharmaceuticals is related to bioconcentration factor and pore water properties and how the overall soil pH is affected (Carter, et al., 2014). Many studies have looked at overall uptake of chemicals, however nobody has characterized general effects that could be occurring with these organisms. Crowe (2014) states that researchers have already found pharmaceutical pollution to be a growing problem; medicines detected in the Great Lakes, acetaminophen, codeine, antibiotics, hormones, steroids, and antiepileptic compounds, are at concentrations high enough to be a concern. However, acetaminophen isn't the only pharmaceutical polluted, as the U.S. Environmental Protection Agency has found diphenhydramine near Chicago, Dallas, Philadelphia, Phoenix and Orlando, Florida. In this study, *E. fetida* and *C. elegans* were used for their vital role in the environment, and to see whether these pharmaceuticals have any negative impact. To understand how the pharmaceutical pollution truly affects the organism, indicators such as mortality, heart rate, and nociception response were observed.

A repeated measures ANOVA was then conducted at alpha equal to 0.05 with these observed indicators. The results indicated that the treatments were significantly different for heart rate ($F(6,48)=262.33$, $p<0.001$), however time ($F(8,48)=0.71$, $p=0.681$) was not (see table 5). When conducting a Tukey test for heart rate, all treatments were significant, except for diphenhydramine at 1 ppm (see Appendix B, Table 9). In up to 5 ppm of acetaminophen, heart rates for *E. fetida* decreased by approximately 43%, so the drugs effectively induced relief when given. However, as hypothesized the heart rates increased after 5 ppm which is indicative of an overdose in *E. fetida.* This is similar to a response acetaminophen has in humans as well where a human's heart rate decreases until he/she takes to high of dosage. When he/she overdoses, the heart rates spike. Likewise, when 10 ppm of acetaminophen was introduced, *E. fetida*'s heart rate increased by almost 171%. In comparison to the control, the higher doses of acetaminophen induced much higher heart rates which can be detrimental to the organism. Observing these changes in heart rate is important in understanding how *E. fetida* is reacting to the drugs. This extremely high heart rate is evidence that the worm needs to pump blood 71% faster in

order to complete the same activities. Since a higher heart rate means more energy is needed, death is more likely to occur because it is harder for an organism to fulfill its energy needs. However, unlike acetaminophen diphenhydramine had a decreasing trend for all concentrations, for heart rate fell by approximately 21%. This is probably because *E. fetida* needs a higher dosage of diphenhydramine for symptoms of an overdose to occur. Moreover, it is important to understand how the drugs can also impact an organism's nociception response or ability to react to noxious stimuli.

When measuring nociception response of *C. elegans*, the treatments were significant ($F(6,48)=169.67$, $p<0.001$), however time ($F(7,27)=0.56$, $p=0.779$) was not (see table 7). It was confirmed by the Tukey test that all varying concentrations of both drugs were significantly different than the control as $p=0.0002$ (see Appendix B, Table 11). With increase in concentration for both drugs, nociception substantially decreased. These same findings was also shown by Leung et al. (2016) as the analgesic, ibuprofen, showed that the velocity (pixels/s) decreased, thus showing nociception response was negatively affected as escape responses decreased by 13 ± 9 pixels per second. Likewise, nociception of *C. elegans* when introduced to acetaminophen and diphenhydramine decreased by over 46%. This indicates that when threatened by an external stimuli, *C. elegans* will react a lot slower, than when no pharmaceutical is introduced. This is harmful as this plays a key role in survival. If *C. elegans* were not able to respond faster and died as a result, than the food chain could be severely affected. Finally, when observing mortality it seems both populations, *E. fetida* and *C. elegans*, were only affected at the highest concentration tested for acetaminophen, 10 ppm, for it was the only treatment that had a *p*-value less than 0.05 (see Appendix B, Table 10, 12). This is likely because 10 ppm of acetaminophen showed the most effect on all variables measured. Higher concentrations of diphenhydramine need to be tested to likely see a more noticeable impact on mortality and heart rate.

One noticeable shortcoming in the experimentation was measuring mortality of *C. elegans*. With *E. fetida* mortality can be easily attained because there was a finite population size, however *C. elegans* had a rapidly increasing population, thus making day to day mortality data not as accurate. In the future, autofluorescence could be used, to accurately determine population size, and death total. There are several applications for future research. First, previous research has demonstrated that pharmaceuticals can be taken up from soil and accumulate in invertebrates such as earthworms (Carter, et al., 2014). Earthworms are at the base of many food chains and thus if chemicals are taken up into the earthworms they can facilitate the movement of chemicals into the food web via bioaccumulation and biomagnification processes. It would be interesting to test various organisms for bioaccumulation to see how much of the chemicals tend to travel from one organism to the next. Second, soil has many properties and variability that could potentially change the overall uptake of a pharmaceutical into an organism. A potential factor to study is the food *E. fetida* eat. Varying levels of soil organic matter (SOM) can be tested to test whether larger SOM amounts have an effect on chemical uptake. Third, *C. elegans* allow us to test many various indicators. Nociception response was conducted in this study, however in the future different approaches can be taken such as testing thermotaxis using a heat gradient. Lastly, acetaminophen and diphenhydramine are not the only pharmaceuticals being polluted; future studies could consist of varying drugs, doses, and over longer periods of time.

As pharmaceutical pollution continues to increase, awareness on proper disposal of drugs on any scale must increase. As medicines are becoming more available and widely cheaper, the potential risk of pollution begins to grow. U.S. hospitals and long-term care facilities annually flush millions of pounds of unused pharmaceuticals down the drain. The same goes for many households in America that flush down expired tablets. Even factories are allowed to do regulated dumping. By itself each may not be that harmful to the environment, but when put together, the situation can become very alarming. As seen by the worms and nematodes, even small dilutions of these pharmaceuticals will have adverse effects on the environment and organisms in it. This research characterizes the worst of what could happen if nothing is done to combat the current problem of pharmaceutical pollution, allowing us to identify the drastic effects that could occur. Currently, concentrations aren't high enough in the environment to see these effects, however, as pharmaceutical sales increase each year so does pollution, for two-thirds of pharmaceuticals are disposed of improperly (Boxall 2004). Through these characterized affects scientists can now identify symptoms and possible impacts that could occur in the environment as concentrations rise. In order to take future action, more proper filtration systems, as well as disposal opportunities for pharmaceuticals need to be established. By bringing awareness to pharmaceutical pollution and the negative impacts they have on the environment steps can be taken in the right direction to combat this problem.

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Appendix A

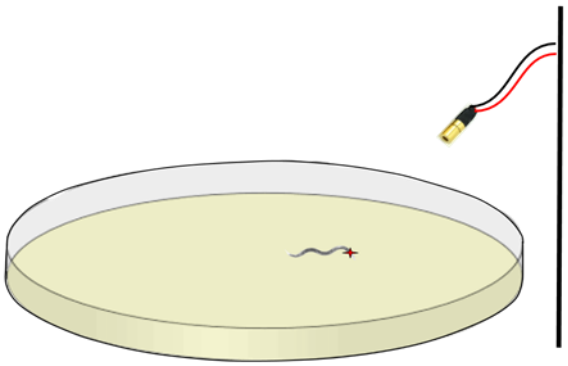

Figure 7: Experimental setup for *C. elegans*. Not drawn to scale.

Appendix B

Table 9: Tukey test on heart rate of *E. fetida*

Difference of Treatment Levels	Difference of Means	SE of Difference	Simultaneous 95% CI	T-Value	Adjusted P-Value
2 - 1	-2.410	0.318	(-3.388, -1.432)	-7.58	0.00002
3 - 1	-4.266	0.318	(-5.245, -3.288)	-13.41	0.00002
4 - 1	7.104	0.318	(6.126, 8.082)	22.33	0.00002
6 - 1	-1.674	0.318	(-2.653, -0.696)	-5.26	0.00005
7 - 1	-2.022	0.318	(-3.000, -1.044)	-6.36	0.00002
3 - 2	-1.856	0.318	(-2.835, -0.878)	-5.84	0.00002
4 - 2	9.514	0.318	(8.536, 10.492)	29.91	0.00002
5 - 2	1.662	0.318	(0.684, 2.641)	5.23	0.00005
4 - 3	11.370	0.318	(10.392, 12.349)	35.75	0.00002
5 - 3	3.519	0.318	(2.540, 4.497)	11.06	0.00002
6 - 3	2.592	0.318	(1.614, 3.570)	8.15	0.00002
7 - 3	2.244	0.318	(1.266, 3.223)	7.06	0.00002
5 - 4	-7.852	0.318	(-8.830, -6.873)	-24.69	0.00002
6 - 4	-8.778	0.318	(-9.757, -7.800)	-27.60	0.00002
7 - 4	-9.126	0.318	(-10.104, -8.148)	-28.69	0.00002
7 - 5	-1.274	0.318	(-2.252, -0.296)	-4.01	0.00253

Table 10: Tukey test on mortality of *E. fetida*

Difference of Treatment Levels	Difference of Means	SE of Difference	Simultaneous 95% CI	T-Value	Adjusted P-Value
4 - 1	1.111	0.323	(0.126, 2.096)	3.444	0.018
4 - 2	1.111	0.323	(0.126, 2.096)	3.444	0.018
5 - 4	-1.111	0.323	(-2.096, -0.126)	-3.444	0.018

Table 11: Tukey test on nociception response of *C. elegans*

Difference of Treatment Levels	Difference of Means	SE of Difference	Simultaneous 95% CI	T-Value	Adjusted P-Value
2 - 1	-0.01703	0.00117	(-0.02070, -0.01336)	-14.55	0.00002
3 - 1	-0.03241	0.00129	(-0.03645, -0.02837)	-25.13	0.00002
4 - 1	-0.04596	0.00153	(-0.05076, -0.04117)	-30.02	0.00002
5 - 1	-0.00989	0.00117	(-0.01356, -0.00622)	-8.45	0.00002
6 - 1	-0.01813	0.00122	(-0.02196, -0.01431)	-14.84	0.00002
7 - 1	-0.02308	0.00129	(-0.02712, -0.01904)	-17.90	0.00002
3 - 2	-0.01538	0.00132	(-0.01953, -0.01123)	-11.61	0.00002
4 - 2	-0.02893	0.00156	(-0.03382, -0.02405)	-18.54	0.00002
5 - 2	0.00714	0.00121	(0.00335, 0.01093)	5.91	0.00004
7 - 2	-0.00605	0.00132	(-0.01020, -0.00190)	-4.57	0.00112
4 - 3	-0.01355	0.00165	(-0.01873, -0.00838)	-8.20	0.00002
5 - 3	0.02252	0.00132	(0.01837, 0.02667)	17.00	0.00002
6 - 3	0.01428	0.00137	(0.00999, 0.01857)	10.42	0.00002
7 - 3	0.00933	0.00143	(0.00485, 0.01381)	6.52	0.00002
5 - 4	0.03608	0.00156	(0.03119, 0.04097)	23.11	0.00002
6 - 4	0.02783	0.00160	(0.02282, 0.03284)	17.40	0.00002
7 - 4	0.02288	0.00165	(0.01771, 0.02806)	13.85	0.00002
6 - 5	-0.00824	0.00126	(-0.01219, -0.00430)	-6.55	0.00002
7 - 5	-0.01319	0.00132	(-0.01734, -0.00905)	-9.96	0.00002
7 - 6	-0.00495	0.00137	(-0.00924, -0.00066)	-3.61	0.01518

Table 12: Tukey test on mortality of *C. elegans*

Difference of Treatment Levels	Difference of Means	SE of Difference	Simultaneous 95% CI	T-Value	Adjusted P-Value
4 - 1	4.17	1.07	(0.86, 7.48)	3.90	0.006
4 - 2	3.75	1.09	(0.38, 7.12)	3.44	0.021
5 - 4	-3.75	1.09	(-7.12, -0.38)	-3.44	0.021