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The Toxicity of Acetaminophen, Caffeine and Carbendazim in Earthworms (Eisenia fetida)

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The Toxicity of Acetaminophen, Caffeine and Carbendazim in Earthworms (*Eisenia fetida*)

BY

Matthew M. Bulman

THESIS

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Abstract

There has been a need for the assessment of ground water contamination risk on terrestrial organisms. One terrestrial organism, *Eisenia fetida*, was chosen as a test species and exposed to three common pharmaceutical and/or pesticide related contaminants: acetaminophen (CAS#103-90-2), caffeine (CAS#58-08-2) and carbendazim (CAS#10605-21-7), respectively. Levels of contamination varied from grams per liter to micrograms per liter (ppb) using distilled water as the solvent. *Eisenia fetida* was examined through a 28 day range/index toxicity test. A Benchmark Dose (BMD) dichotomous probit analysis, using a 95% confidence interval, was calculated for all three contaminants. All three contaminants were found to have Benchmark Dose Lower 95 % Confidence Intervals (BMDL's) well below levels of full saturation (BMD/BMDL of Acetaminophen: 160ppm/100pm; Caffeine: 120ppm/50ppm; Carbendazim: 71ppb/41ppb), with hazard quotients of 3.8×10^{-6}, 2.6×10^{-6} and 2.9×10^{-3}, respectively. These three contaminants showed that despite low levels of ground water contamination found currently in the United States several terrestrial organisms and ecosystems could be severely impacted.

Acknowledgements

First and foremost I want to thank my advisor Dr. Doug Klarup for his interest in the contamination of the United States ground water. Without his foresight and encouragement in the summer of 2008 this project would have never come into existence. To Dr. Karen Gaines, thank you for the encouragement and excitement in talking about worms, their ecological role and statistical analysis. I have learned much in my short time here from the both of you. To Dr. Andrew Methven, thank you for the understanding and flexibility that has become part of this wonderful MSNS program here at Eastern Illinois University. Your dedication to the program and its students is simply remarkable and dually appreciated. Thank you to the graduate school and Eastern Illinois University for its graduate assistantship program and the use of its facilities, both were pivotal to the completion of this project.

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To the MSNS crew thank you for keeping me sane through this process and all the laughs throughout the program. A special thanks to Laurie Nannini, Kyle Farley and Steve Zownorega. You three made this program a joy to attend and always provided constant insight and humility during this process.

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Introduction

Recently developed technology has allowed for incredibly precise measurements of chemical contaminants not thought possible in past experimentation. High performance liquid chromatography with mass spectroscopy has allowed scientists to view contaminants in the parts per billion ranges (Cahill et al., 2004). In using such technology, scientists have begun to examine the world around them with the utmost precision. The results of such investigations have been alarming.

Of recent note was the investigation of the United States ground water supply. Early investigations found over one hundred pharmaceutical, personal care product (PCP) and pesticide related contaminants within the nationwide ground water supply (Barnes et al., 2008). Due to these highly developed experimental techniques, and a widening view of environmental assessment, it has become evident that much, if not all, of the United States ground water supply has become contaminated with chemicals primarily used for medicinal, personal and veterinary purposes (Lienert et al., 2007).

Nearly every contaminant found in ground water has been detected in the parts per billion range, which has led many experts to debate the true danger of such small levels of contamination (Barnes et al., 2008) and whether this level of contamination could impact local ecosystems and/or human exposure to these ground water supplies.

One terrestrial organism found to be highly susceptible to ground water contaminations is *Eisenia fetida*, commonly known as the red wiggler earthworm

(Ruz-Jerez et al., 2002). Due to the prevalence of *E. fetida* in soil ecosystems, and the high dependence on nutrient cycling of said ecosystems, *E. fetida* remains a highly investigated organism for soil contamination (USEPA, 1994).

Earthworms, like *E. fetida*, have remained key species within terrestrial food webs (Devillers, 2009) and indicators of soil quality (Frund et al., 2011). Devillers (2009) described the role of earthworms as "[performing] a number of essential functionalities like decomposition of organic litter, tillage and aeration of the soil, and enhancement of microbial activity." Terrestrial ecosystems have relied heavily on the soil on which they exist and by association the earthworms that live within that soil. Devillers (2009) described the soil food web as a "set of organisms that work underground to help sustain the essential functions of soil." While billions of organisms have helped to facilitate this process, earthworms were considered unique, in that, they consumed every particle of the soil, but more importantly have left behind castings which have been a valuable fertilizer for plants (Devillers, 2009).

Since earthworms have had such a huge role in terrestrial ecosystems, they have become a species that has helped to identify soil quality (Frund et al., 2011) and environmental risk assessment (USEPA, 1994). *Eisenia fetida* should give insight to the level of concern necessary for current ground water contaminations. Through testing of various ranges of concentrations of specific ground water contaminants, it should be possible to infer at what level pharmaceutical contaminations become problematic for terrestrial ecosystems and human beings by association.

Literature Review

Recent technological breakthroughs in chemical analysis has allowed for contaminant detection in the parts per billion (ppb) scale. This incredible precision in analysis has allowed for acute, sensitive testing of drinking water in the United States. In a 1999-2000 nationwide sampling of various ground and surface water areas within the United States, Kolpin et al. (2002) found nearly 100 organic waste contaminants of varying concentrations, occurrences and types. The contaminants consisted of four main classes: antibiotics, prescription/non-prescription drugs, wastewater-related compounds (OWC's) and synthetic hormones (Kolpin et al., 2002). This study utilized 139 United States stream locations, thereby analyzing United States surface waters in a survey method. Additional studies by Barnes et al. (2008) and Focazio et al. (2008) used similar methodologies, collaboratively, to analyze 47 different ground water locations and 74 untreated drinking water sources, respectively, across the United States. All three studies found similar levels of contamination of the four primary groups of contaminants (Barnes et al., 2008; Focazio et al., 2008; Kolpin et al., 2002). In all three studies the non-prescription drugs, caffeine and acetaminophen were highly prevalent (Barnes et al., 2008; Focazio et al., 2008; Kolpin et al., 2002).

The analytical methodology involved in these studies consisted of the identification of non-prescription drugs through the use of solid-phase extraction (SPE). Extracted compounds were then analyzed by using high-performance liquid chromatography (HP/LC) (Cahill et al., 2004). This highly selective and

precise methodology can also be used to monitor specific drug metabolites. Drawbacks to this method of analysis involved the selection of specific drug components to be identified and separated. For example, similar structural metabolites were difficult to distinguish from parent drug forms. Corrections were made if known metabolites were identified and specifically searched for via these analytical methods. However, these means of analytical methods required the investigator to search for given contaminants rather than just providing an entire view of what contaminants existed in a given sample.

Through further analysis and selection, these studies have also detected relatively high concentrations of two main non-prescription drug metabolites; 1,7-dimethylxanthine (caffeine metabolite, shown in ***Figure 1***) and cotinine (nicotine metabolite, shown in ***Figure 2***) (Barnes et al., 2008; Focazio et al., 2008; Kolpin et al., 2002).

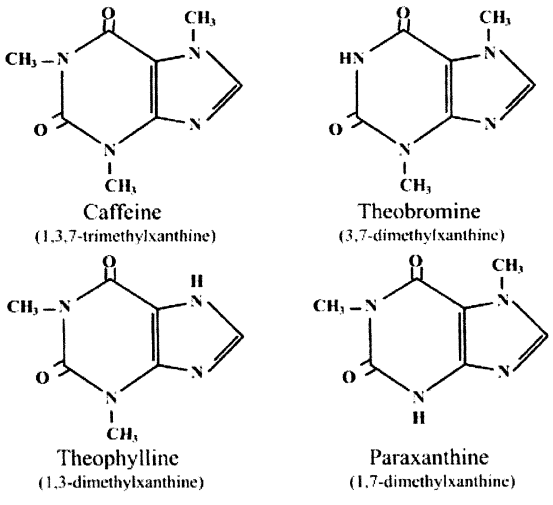

Figure 1: Caffeine and associated metabolites (Hawke et al., 2000)

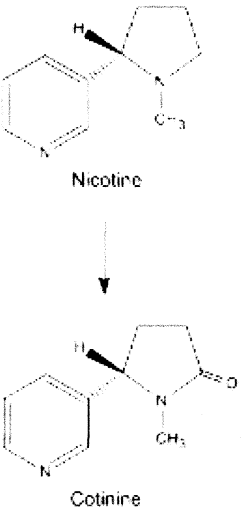

Figure 2: Nicotine and associated metabolite (Tricker, 2002)

The detection of these metabolites suggested some form of organism interaction with parent drug contaminants. Further research suggested that the metabolism of caffeine to 1,7-dimethylxanthine would be characteristic of human metabolic pathways (Sved et al., 1976). However, research suggested that the metabolism of nicotine remained highly specific to individual drug use and genetic polymorphisms (Sved et al., 1976). Even so, the most prevalent detected human metabolite was C-oxidation to cotinine (Tricker 2002), the metabolite detected in water resources. Research and prevalence correlation would suggest that much of the metabolite detection could be due to human excretion. However, the research also suggested similar metabolism of non-pharmaceutical drugs via soil microorganisms. Microbial ecological studies have shown that soil microorganisms can metabolize pharmaceuticals like caffeine into varying metabolic forms, similar to the most prevalent human metabolic forms (Mazzafera et al., 1995). While individual genetic variations could affect the minor metabolites of drugs, research consistently showed that the most prevalent metabolized form of non-prescription pharmaceuticals was the same form regardless of metabolic pathway studied.

Neither study (Kolpin et al., 2002 or Focazio et al., 2008) reported the detection of acetaminophen metabolites in ground water samples as a result of detection methods and selectivity of contaminants used in these experiments.

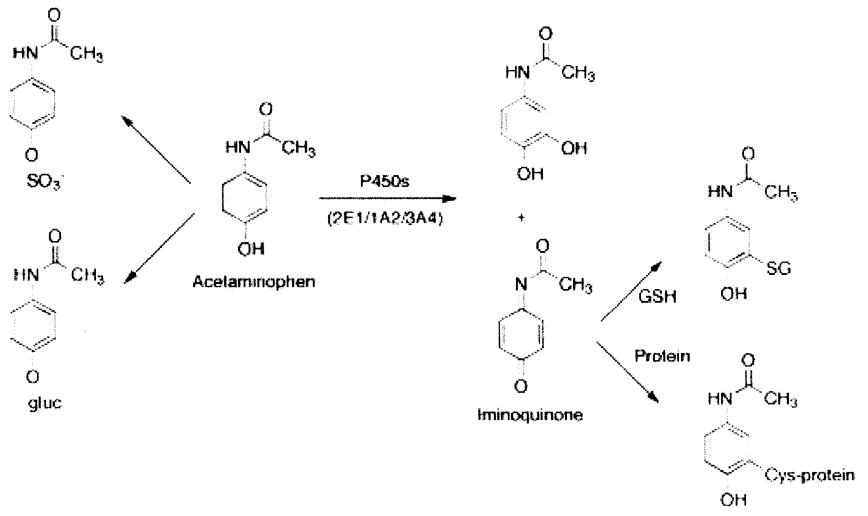

Figure 3: Acetaminophen and associated metabolites (Guengerich, 2006)

Efforts were not made to detect acetaminophen metabolites (shown in ***Figure 3***), but rather the parent forms of the drugs. However, it would be reasonable to suggest that this drug would also exist in the metabolized form. All non-prescription drugs list a half-life (in the human body) timeframe indicating 50 percent of remaining parent drug existing in the non-metabolized form. Most non-prescription drugs list half-lives on an hour scale, meaning rapid metabolism of the parent drugs via various metabolic pathways.

Non-prescription ibuprofen metabolically interacts in a manner similar to most non-prescription pharmaceuticals and is primarily metabolized within the liver by a combination of enzymes belonging to the cytochrome P450 family (Chang et al., 2008), as shown in ***Figure 4***.

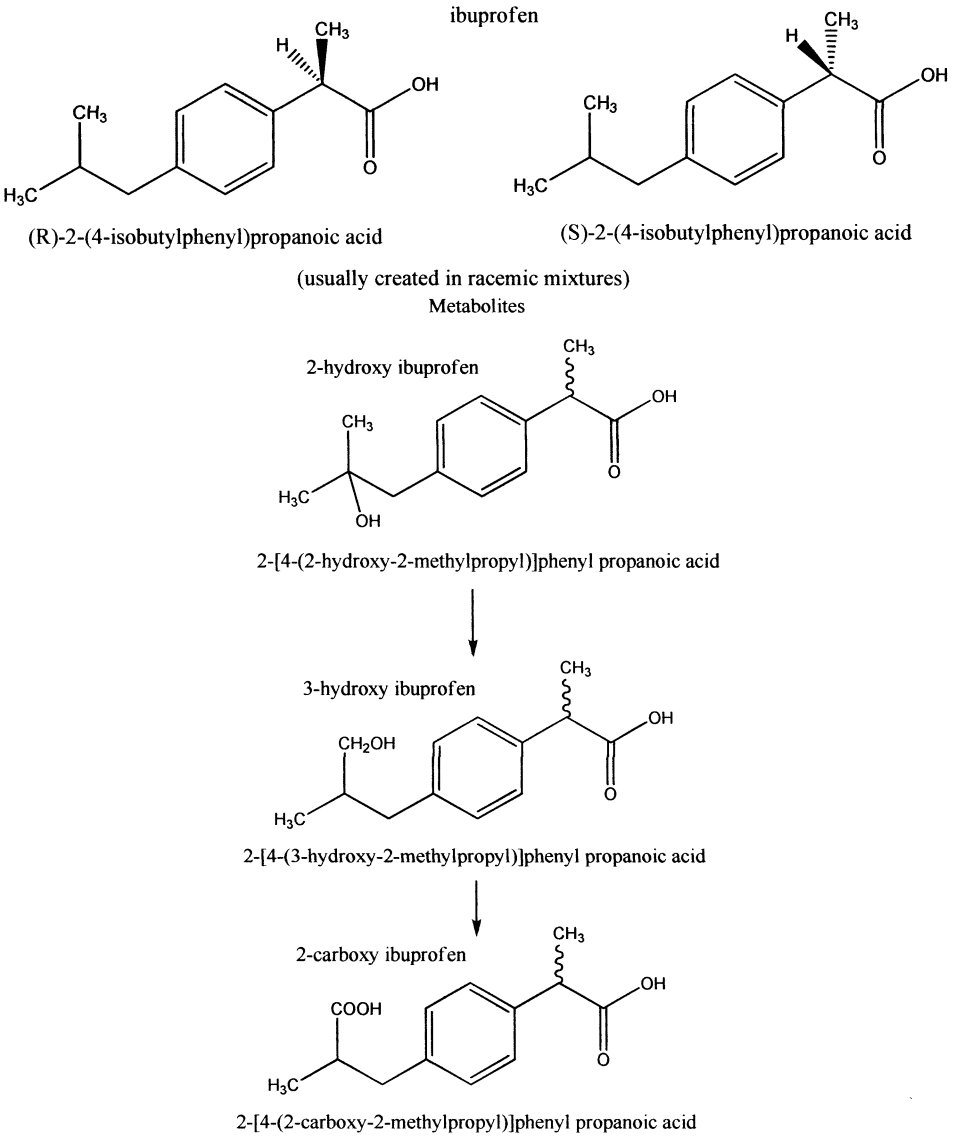

Figure 4: Ibuprofen and associated metabolites (Chang et al., 2008)

Major metabolites of ibuprofen, and other non-prescription pharmaceuticals, were determined via analysis of human urine using high performance liquid chromatography (Chai et al., 1988). Models and experimental determination usually correlate to explain and suggest prevalent parent drug metabolites. In the case of ibuprofen, both models and experimental determination suggested that the primary human metabolites of ibuprofen are (OH)-ibuprofen (2-[4-(2-

hydroxy-2-methylpropyl)phenyl] propionic acid) and (COOH)-ibuprofen (2-[4-(2-carboxypropyl)phenyl] propionic acid) (Chang et al., 2008; Chai et al., 1988). While microorganisms metabolize ibuprofen using a different pathway, research has suggested that the primary metabolites would be very similar. In an investigation using 24 different microbial cultures it was found that several soil microorganisms metabolized ibuprofen to the common human metabolic form, OH-ibuprofen (2-[4-(2-hydroxy-2-methylpropyl)phenyl] propionic acid) (Hutt et al., 1993). This was consistent with the notion that regardless of metabolic pathway the most prevalent metabolites would likely be identical for non-prescription pharmaceuticals.

All three studies (Barnes et al., 2008; Focazio et al., 2008; Kolpin et al., 2002) indicated contamination on a wide scale across several United States water resources. While all three reports have raised questions and give cause for concern, ground water contamination has been shown to be one of the highest threats for terrestrial ecology. Ground water has been described as an important source of drinking water, irrigation, and flow in many streams and rivers (Alley et al.,1999). The use of ground water in irrigation has represented a direct link to terrestrial (non-wetland) organisms. Ground water studies, specifically, have shown ibuprofen to have a maximum concentration of 3.11 μg/L, higher than all but one contaminant, *N-N*-diethyltoluamide (insect repellent) (Barnes et al., 2008). While both of these concentration ranges were in the parts per billion ranges (3.11ppb and 13.5ppb, respectively), early detection may help to identify which drugs, if any, would be cause for future concern. It would be

logical to conclude that pharmaceuticals found in the highest range of contamination and prevalence would likely remain in the highest level of contamination in future studies. If ecological risk assessment for highly prevalent drugs could be assessed, future problems could be minimized.

Since surface water was the first water system to be tested for pharmaceutical contaminants, freshwater aquatic environments were the first ecological systems to be subjected to toxicity testing. Escher et al. (2005) found that ibuprofen slightly impaired the function of some aquatic life forms. While the mortality rates remained low, non-lethal impairments were noted in the experiments. This discovery should spur additional testing involving several species over prolonged periods of time to help identify the accumulation of pharmaceutical contaminants and the harm these contaminants may have caused. Terrestrial life forms were not tested in the Escher et al. (2005) study and neither were human ibuprofen metabolites.

Contamination via excretion models suggests that not only non-metabolized ibuprofen could enter the ground water, but also the metabolites (OH)-ibuprofen and (COOH)-ibuprofen. Surface water toxicity research has suggested that most metabolized forms of drugs were reduced in toxic potential when compared to the parent drug. However, ibuprofen and its metabolites could pose some ecological risk (Lienert et al., 2007). In a study of 42 different pharmaceuticals by Lienert et al. (2007), it was found that ibuprofen and acetaminophen were two of eight pharmaceuticals that actually increased in toxicity in *Daphnia*, algae and fish in the metabolized form. This could pose a

serious ecological threat because the levels of metabolized ibuprofen and acetaminophen have not been heavily monitored. Due to the high consumption of ibuprofen and acetaminophen in the United States, and the lack of monitoring the main metabolite contaminants, both drugs may pose one the greatest ecological threats of all the pharmaceutical contaminants (Lienert et al., 2007).

While the effects of caffeine metabolites were not studied by Lienert et al. (2007), similar cellular oxidative problems in the liver of rainbow trout were detected by Gagne et al., 2006. Even at low environmental levels of contamination, this study suggested that the oxidative effects of non-prescription drugs could be detrimental to cellular function over a prolonged period of time (Gagne et al., 2006). Additional studies involving *Daphnia magna* and *Vibrio fischeri* with pharmaceuticals like acetaminophen have shown similar outcomes and were consistent with other research at environmentally low levels of concentration (Kim et al., 2007). In aquatic life forms, lethal levels of contamination have not been a primary concern, although further investigation have been suggested in an effort to help determine prolonged effects (Kim et al., 2007).

Despite the evidence of contamination found in aquatic organisms, little has been done in the realm of terrestrial organisms. There have been many reasons for the lack of terrestrial testing ranging from disbelief in the impact of pharmaceuticals in trace amounts to the belief that the primary contamination factor has been improper disposal, which would only affect aquatic life forms in surface water. However, if contamination existed through means of human

excretion and wastewater sewage sludge, terrestrial life forms may be impacted more than previously believed. Toxicity testing of highly consumed (and excreted) drugs could provide insight to the ecological effects of pharmaceutical contamination of terrestrial organisms.

The origin of ground water contaminants has remained highly debated. Disposal of unused medication via household toilets does not account for the variety and complexity of the contaminants detected (Herberer, 2002) because medicinal products were not only disposed of in unused form, but also in metabolized form in human excrement. Unused medicine disposed of in the trash would likely make its way to waste disposal sites and thereby leach into the ground water. Research has also shown that several drug metabolites, thought inactive, were actually further metabolized by microorganisms, in some cases, back to the parent form of the drug (Clark et. al, 2006). Excreted human medicine has been found to make its way to sewage treatment plants and either into sewage sludge or possibly into surface waters if untreated. Sewage sludge, often mixed with manure, which has been found to contain excreted animal medications, has often been applied to farm land as fertilizer. Deposition of sewage sludge on large areas of land in terrestrial environments could eventually release contaminants into the ground water (Herberer, 2002). This could explain the high prevalence of not only human antibiotic contaminants but also veterinary contaminants detected in ground water samples. The wastewater sewage sludge, mixed with terrestrial manure, would expose microorganisms to both

parent and metabolic forms of pharmaceuticals. Through irrigation over farm lands these contaminants could then reach ground water supplies.

Despite the recent discovery of pharmaceutical contaminants within ground water, there have been few, if any, regulations on acceptable levels, or concentrations, of given contaminants. The lack in regulation has been due primarily to the lack of data suggesting any reason for immediate concern since contaminant concentrations in the ground water range in the parts per billion. For example, the USEPA lists relative lethal dosages of half populations (LC/LD50's) for rats for such contaminants. The oral LD50's for rats for acetaminophen, caffeine and carbendazim are 1944mg/kg, 192mg/kg and 6400mg/kg, respectively (Sigma-Aldrich, 2012). These levels of contamination are several thousand times more concentrated than current ground water contamination. Thus, there would be little correlation to human mortality and current ground water contamination. However, if the level of contamination were effecting small, large quantity species like *E. fetida*, that occur in large numbers in the soil, the effect on human may not be direct, but could be just as detrimental.

The success and diversity of terrestrial organisms has been known to rely heavily on the condition of the soil within a given ecosystem. While, several organisms have been found to be responsible for the health and maturity of soil, only one such organism can be highly linked to the soil health: *Eisenia fetida*. *Eisenia fetida* have been described as "engineers" of the soil environment in which they live (Devillers, 2009). Earthworms have been responsible for both the

buildup of new terrestrial material and the breakdown of old materials. The worm, in turn, could be considered a meal for small, consumers. However, the true link of the worm to terrestrial food webs has been described by the way in which they develop in the environment in which they live.

Earthworms have been shown to till the ground in which they live and in doing so have created tunnels or holes underground. These tunnels have allowed for higher water retention, less water runoff and higher rates of nutrients being able to enter the soil (Devillers, 2009). Devillers (2009) has described earthworms as "[keeping] the soil healthy by moving organic matter from the surface into the soil. By speeding up the breakdown of plant material, earthworms also [have sped] up the rate at which nutrients [were] recycled back to plants." Due to their high prevalence and important ecological role, earthworms have remained an important part of a healthy, functioning terrestrial ecosystem.

A large amount of ground water contaminants have been shown to originate from wastewater sewage that is often combined with various forms of manure and spread across terrestrial land for fertilizer (Barnes et al., 2008). This application would put ground burrowers, like *E. fetida*, in direct contact with a large source of ground water contamination. *Eisenia fetida* would then consume quantities of these ground water contaminants through the exposure to wastewater sludge on the surface of terrestrial environments. Norr and Riepert (2007) described earthworms as "appropriate test system(s) to assess the bioaccumulation potential of substances in the terrestrial environment." In a

study involving ^{14}C-sulfadiazine, a possible contaminant, the uptake of the ^{14}C-sulfadiazine was clearly detected in the tissue of the earthworm, *Eisenia fetida* (Norr & Riepert, 2007). If these contaminants were found to be harmful to *E. fetida* there could be substantial effect to local terrestrial ecosystems. Initially, there would be noticeable detrimental effects to lower level consumers that have ingested affected earthworms. However, these effects would likely remain small and isolated due to the relatively short life-span of *E. fetida* and its primary residence below ground. Of major concern would be the effects on the surrounding soil environment. *Eisenia fetida* has been shown to play a crucial role to the physical development of soil and the recycling of organic material. Without this low level recycler/builder the soil health could be drastically affected. A change in soil health would logically bioaccumulate up the food chain first to plant species and microorganisms and then to higher level consumers.

Since earthworms have been shown to be such good recyclers of organic material, the accumulation of large amounts of contaminants within their tissues could result in those contaminants being left as worm castings for plants to uptake. Kipper et al. (2010) has shown that contaminants found in sewage sludge compost taken up and bioaccumulated in plants treated with contaminated soil. If worms were carriers of these contaminants, they could also easily pass these contaminants on to plant materials they come in to contact with. This could mean that over long periods of time earthworms could be ingesting terrestrial contaminants from ground water resources and passing them

on at higher levels of concentration to species higher in the food chain either through direct consumption or plant uptake.

The United States Environmental Protection Agency (USEPA) has adopted a protocol involving *Eisenia fetida* as an indicator species for soil environments. Research has suggested that due to its relatively short life-span, the environmental uptake of a soil environment by *E. fetida* could readily be related to terrestrial environment soil contamination (Sample et al., 1998). Furthermore, the bioaccumulation of soil contaminants using *E. fetida* as a test indicator species in several test experiments has already been demonstrated (Kinney et al., 2007). The development of artificial soil and the introduction of various chemical contaminants over a short period of time could allow for possible risk assessment of terrestrial contaminants when compared to the effects on *E. fetida*. Previous research has also indicated that *E. fetida* facilitates the indication of sublethal side effects that can easily be observed in toxicity testing (Van Gestel & Weeks, 2003). Through the use of *E. fetida* accurate terrestrial ecological threats can be identified and related to ground water pharmaceutical contaminants. The response of *E. fetida* to acetaminophen and caffeine could help to determine what ecological threat, if any, exists in the recent discovery of pharmaceutical ground water contamination. While *E. fetida* mortality or threat does not necessarily mean direct threat or mortality to humans, a serious effect on *E. fetida* could mean a serious threat to terrestrial ecosystems.

Methodology

The purpose of this research was to determine at what levels acetaminophen, caffeine and carbendazim become environmentally hazardous to terrestrial life forms. The emphasis of this research was placed on terrestrial organisms and their exposure. In selecting specific terrestrial indicator organisms, the potential for environmental risk can be related to not only improper pharmaceutical disposal but also improper treatment of human wastewater. Through this analysis the ecological/environmental risk of pharmaceutical contaminated ground water can be better understood in relation to specific terrestrial organisms. The methodology provided was used to create a reference, or index, range for the toxicity of *E. fetida* when exposed to three different pharmaceutical contaminants.

In order to address the potential environmental implication of pharmaceutical contaminants a test organism was analyzed. The red worm (*Eisenia fetida*), a common composting worm, was used as a test (indicator) species for soil toxicity by the Environmental Protection Agency of the United States (USEPA, 1994). *Eisenia fetida* has been shown to move, eat and reproduce through the use of the soil environment. Due to this particular relationship, *E. fetida* has been shown to readily come into contact with and uptake soil contaminants.

It was important to consider the experimental environment necessary for *E. fetida*. To experimentally control the level of soil contamination artificial soil was used. Through collaboration with several environmental specialists, the EPA

has established artificial soil for experimental testing. The EPA artificial soil contained 68% No. 70 mesh silica sand, 20% kaolin clay, 10% sphagnum peat moss, and 2% calcium carbonate (dry weight). In order to add water to the dry mixture, the artificial soil was moistened to 35% (by weight) with deionized/distilled water (USEPA, 1996).

It was imperative that test organisms were not exposed to contaminants before the testing process began. In order to control the contamination of test organisms, all test organisms were cultured in artificial soil and fed non-contaminated food. The food source for the *E. fetida* was saturated alfalfa (*Medicago sativa)* pellets. Through this means of culture, *E. fetida* was not exposed to contaminants and became acclimated to the artificial soil before the testing process began. *Eisenia fetida* was cultured for six months to allow for multiple generations to propagate before testing began.

The temperature of the soil was maintained at approximately 25°C and was not altered more than ±3°C on any given day in an effort to minimize organism stress. The pH of the soil was maintained at about pH 7 through the use of the calcium carbonate in the dry mix. The pH did not vary by more than ±0.5 to ensure no additional stresses were placed on *E. fetida*. The cultures were kept in a ventilated, covered container to provide an environment with proper lighting and ensure adequate moisture for *E. fetida*.

In an effort to control the maturity level of individual organisms, only adult organisms were used. The EPA recommended using earthworms of approximately 300-600mg in weight with a clearly defined clitellum (USEPA,

1996). This control ensured that only adult worms were analyzed and that the maturity level of the test species was kept constant.

The lifespan of *E. fetida* was considered when selecting a time frame for the testing process. To ensure that the test organism was only affected by laboratory contamination, a life span range of 28 days was used. This ensured that, on average, the mortality rate of the test species was primarily due to exposure to contaminants. The contaminants were added with the 35% water (by mass) used to moisten the soil containers. The contamination was done in this manner to mimic environmental contamination of varying concentrations. Observations were taken on days 7, 14, 21 and 28 to ensure that all effects of the contaminants were measured in this experimental timeframe (USEPA, 1996). Test chambers consisted of glass Mason jars with ten adult organisms placed into a container with 200 grams (dry weight) artificial soil.

The highest concentration tested was the point of total saturation for the given contaminant in distilled water. Saturated solutions were made by dissolving the solid form of the contaminant in distilled water (at 25°C) until excess solid formed in the bottom of the solution. A magnetic stir bar and stir plate were used to ensure proper mixing of the solution until full saturation had been reached. The saturated solution was then filtered three times using grade 2 filter paper (medium porosity, medium flow rate, 8µm particle retention). Each saturated solution was then diluted by a factor of ten until each contaminant had reached the parts per billion range (µg/L). Only one trial of each test solution and one control group was used to verify accurate mortality rates of *E. fetida*, as

suggested by USEPA (1996). Since this experiment was designed as a reference/index experiment, final solution concentrations were not quantifiably verified after the dilutions.

The contaminants used were acetaminophen (CAS# 103-90-2), caffeine (CAS# 58-08-2) and carbendazim (CAS# 10605-21-7), shown in ***Figure 5***.

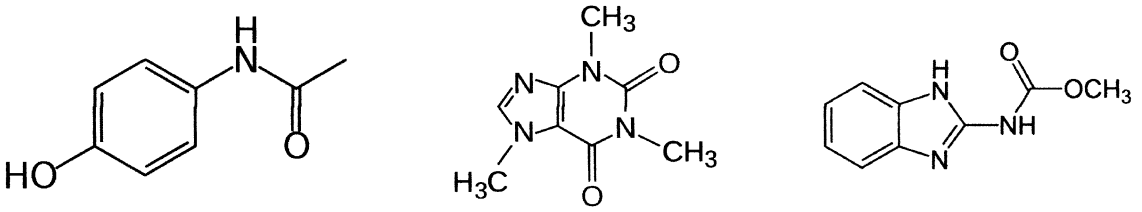

Figure 5: Acetaminophen, caffeine and carbendazim structures, respectively (O'Neil, 2006)

Acetaminophen and caffeine were selected due to their prevalence in ground water studies (Barnes et al. 2008). Carbendazim was used because of its high toxicity in soil ecosystems and lethal effects on organisms even in small dosages (Burrows and Edwards, 2004 and Muthuviveganandavel et al., 2008).

The test chambers were kept at a temperature near 22°C with constant lighting to encourage *E. fetida* to remain buried within the test soil during the testing period. The chambers were made of glass to minimize leachates to test soil chambers. The relative humidity of the test chambers was maintained at 85% for the entire 28 days.

The test species were observed on days 7, 14, 21 and 28. Observations specified organism location (below or above surface), behavioral symptoms (writhing, stiffened, shortened, elongated, pulsing on surface; or inactive below

surface in a ball), pathological symptoms (lesions, midsegment swelling, ulcerated areas) and mortality.

Benchmark Dose (BMD) methodology was used to analyze the mortality rates of *E. fetida* versus the exposure of the three contaminants. BMD was an alternative to the No Observed Adverse Effected Level (NOAEL) methodology. Traditional NOAEL analysis allows for the calculation of the highest experimental dose that does not produce a statistical increase in adverse effects in sensitive individuals (National Research Council, 2000). NOAEL's have been found to be sensitive to sample sizes and vary considerably across different experiments (National Research Council, 2000 and Filipsson et al., 2003). The BMD was defined by Crump (1995) as the specific dose that corresponded to a moderate increase over some defined background rate of response. Using the BMD statistical analysis the increase over the background rate could be easily altered to fit the small amount of data represented in this experiment and provide a risk assessment for the three contaminants on an index level with errors and propagations minimized.

Due to the small sample sizes and high mortality rates, the Benchmark Response (BMR, also known as the specified effect) value was set to 10% (USEPA, 2012). This meant that the BMD was calculated using a 10% increase over the background rate of response. A Benchmark Dosage Lower Confidence Level (BMDL) was then calculated using the BMR, the calculated BMD and a 95% confidence interval calculation (USEPA, 2012). The BMDL for each contaminant represented the lowest level of contamination that caused a

statistically significant increased response, here 10%, when organisms were exposed to that dose of the contaminant (USEPA, 2012).

All calculations were run through the BMDS 2.2 program provided by the USEPA (2012). Since the data represented a percentage of effective organisms, a dichotomous, probit analysis was used. The USEPA (2012) labeled a dichotomous response as an analysis where the response varied on either the presence or absence of an effect. Probit analysis was used to take the mortality rate data and create a linear fit to non-linear data. A probit analysis, as described by Finney (1952), took the represented data and placed the data into a logistic form, assigned a probit value from statistical calculations and then took the data out of logistic form. USEPA (2012) provided all software necessary to run this analysis and provide a means of statistical fit. The USEPA (2012) suggested that a moderate to good fit required a P-value of greater than 0.1. An "Extra Risk" tool was used to represent the risk associated with no contamination exposure. The extra risk was defined as "additional risk divided by the predicted proportion of animals that will not respond in the absence of exposure" (USEPA, 2012). The "Extra Risk" tool was used because the control group did show response when not exposed to any contaminants.

The BMDL data was then compared to current levels of groundwater contamination for all three contaminants. A quotient was calculated between these two values and to produce a Hazard Quotient. USEPA OSW (2005) describes the Hazard Quotient as "the potential for developing...health effects as a result of exposure." Therefore, a Hazard Quotient above 1.0 would indicate

possible health related concerns for sensitive individuals (USEPA OSW, 2005). Since the Hazard Quotient utilized the BMDL values, the Hazard Quotient produces an index that considers the lower 95% confidence interval used to calculate the BMDL. Therefore a Hazard Quotient above 1.0 indicates possible risk to earthworm populations in lower 95% confidence interval of the BMDL.

Results

Acetaminophen Range Testing

A fully saturated solution of acetaminophen (CAS# 103-90-2), approximately 14 g/L at room temperature (~25°C) (O'Neil, 2006), was created and used as the base solution for the diluted versions of contaminated solution. The saturated solution of aqueous acetaminophen was diluted down by a factor of ten six times to reach a range concentration of 14µg/L or 14ppb.

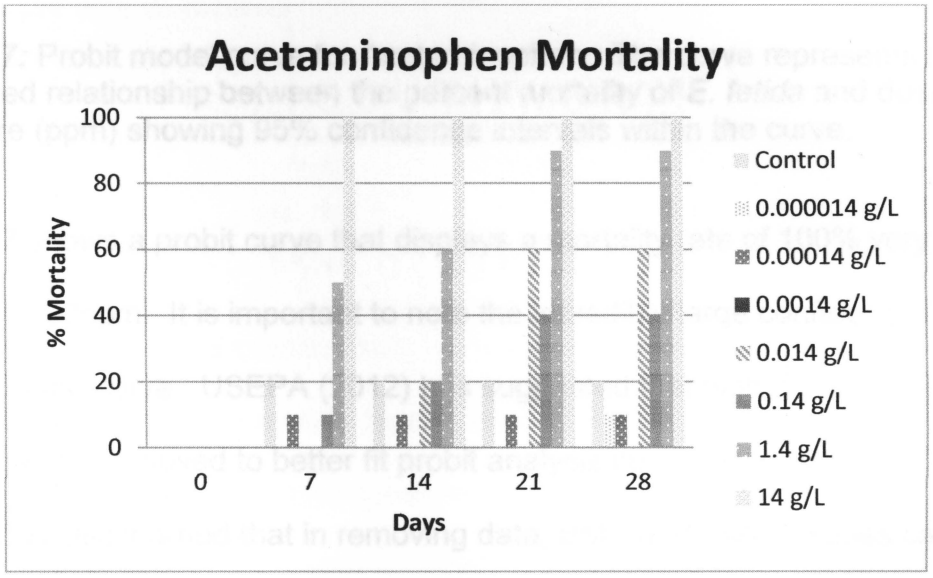

Figure 6: Acetaminophen Daily Percent Mortality. Temperature was maintained at 22.40 ± 0.67°C and pH was maintained at 7.02 ± 0.32.

The mortality percentage was viewed in daily increments and the most significant increases in percentage of mortality were found on days 7 and 21 (***Figure 6***).

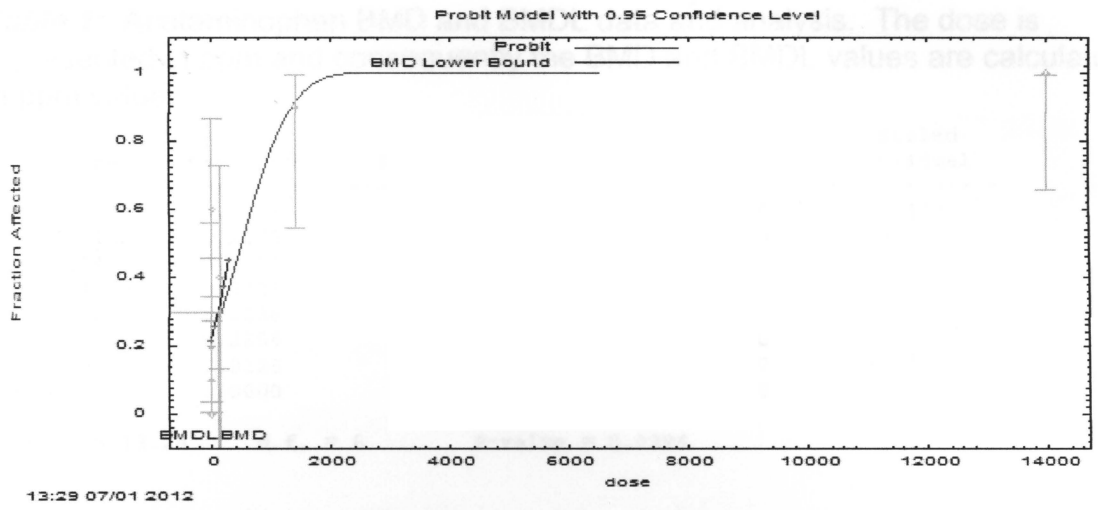

Figure 7: Probit model curve for Acetaminophen. The curve represents a calculated relationship between the percent mortality of *E. fetida* and dose exposure (ppm) showing 95% confidence intervals within the curve.

Figure 7 shows a probit curve that displays a mortality rate of 100% very near a dose of 2000ppm. It is important to note the incredibly large confidence interval values on the curve. USEPA (2012) has suggested that high concentration values can be removed to better fit probit analysis curve. However, USEPA (2012) has also warned that in removing data, BMD and BMDL values can be severely altered and less accurate. Due to the nature of this experiment, the small amount of data collected and low amount of significant figures noted, the high value concentration was not removed from the data.

Table 1: Acetaminophen BMD and BMDL data and analysis. The dose is represented in ppm and consequently the BMD and BMDL values are calculated in ppm values.

Dose	Est._Prob.	Expected	Observed	Size	Scaled Residual
0.0000	0.2175	2.175	2.000	10	-0.134
0.0140	0.2175	2.175	1.000	10	-0.901
0.1400	0.2175	2.175	1.000	10	-0.901
1.4000	0.2181	2.181	0.000	10	-1.670
14.0000	0.2238	2.238	6.000	10	2.854
140.0000	0.2854	2.854	4.000	10	0.803
1400.0000	0.9126	9.126	9.000	10	-0.141
14000.0000	1.0000	10.000	10.000	10	0.000

Chi^2 = 13.24 d.f. = 6 P-value = 0.0394

Benchmark Dose Computation

Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

BMD = 159.794

BMDL = 104.52

Since all solutions were created from full saturation and filtration only two significant figures can accurately be recorded. Therefore the BMD and BMDL values associated with acetaminophen were 160ppm and 100ppm (1.0 x 10^2ppm), respectively (see ***Table 1***). The p-value associated with this data was calculated to be 0.0394. USEPA (2012), suggests that good to moderate statistical fits for BMD analysis have critical p-values of 0.1. USEPA (2012) also suggests that dropping high dosage values can help to linearize probit analysis data. However, if the high concentration value was removed from the data, the p-value actually drops to 0.0212. However, with this change in statistical analysis the BMD and BMDL values did not change when significant figures were accounted for. Thus no data was excluded from this analysis.

Caffeine Range Testing

A fully saturated solution of caffeine (CAS# 58-08-2), approximately 21 g/L at room temperature (~25°C) (O'Neil, 2006), was created and used as the base solution for the diluted versions of contaminated solution. The saturated solution of aqueous caffeine was diluted down by a factor of ten six times to reach a range concentration of 21µg/L or 21ppb.

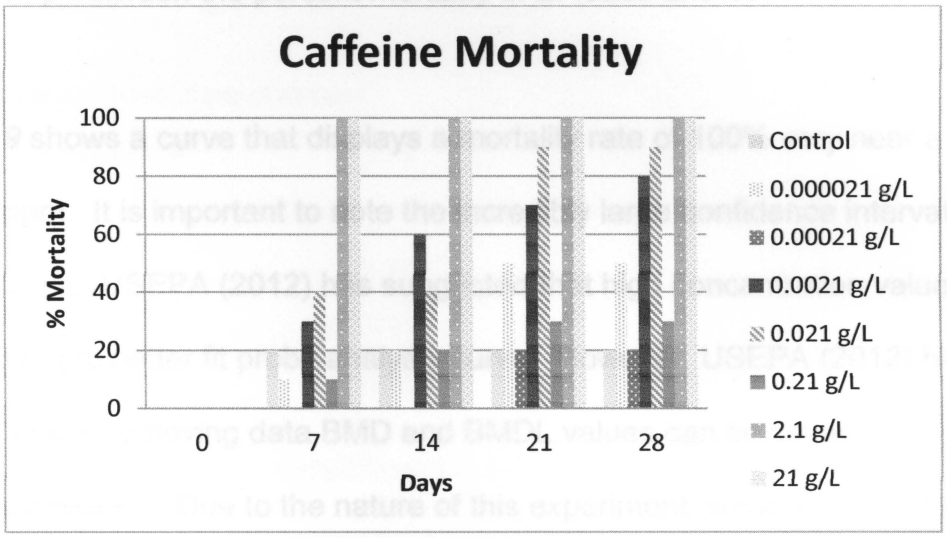

Figure 8: Caffeine Daily Percent Mortality. Temperature was maintained at 22.30 ± 0.72°C and pH was maintained at 7.13 ± 0.28.

The mortality percentage was viewed in daily increments and the most significant increases in percentage of mortality were found on day 7 and 21 (see ***Figure 8***). It is important to note that there was significant decrease in the percent of mortality in trial 3 (0.21 g/L) of caffeine contaminant.

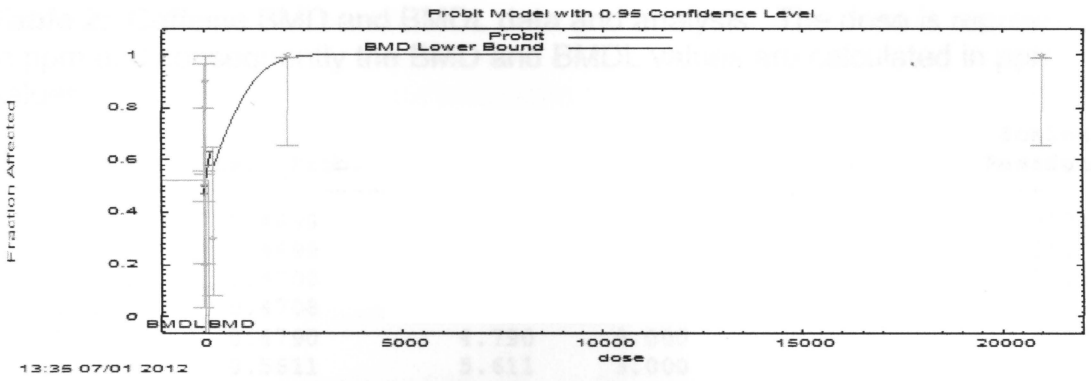

Figure 9: Probit model curve for Caffeine. The curve represents a calculated relationship between the percent mortality of *E. fetida* and dose exposure (ppm).

Figure 9 shows a curve that displays a mortality rate of 100% very near a dose of 2500ppm. It is important to note the incredibly large confidence interval values on the curve. USEPA (2012) has suggested that high concentration values can be removed to better fit probit analysis curve. However, USEPA (2012) has also warned that in removing data BMD and BMDL values can be severely altered and less accurate. Due to the nature of this experiment, small amount of data collected and low number of significant figures noted, the high value concentration was not removed from the data.

Table 2: Caffeine BMD and BMDL data and analysis. The dose is represented in ppm and consequently the BMD and BMDL values are calculated in ppm values.

Dose	Est._Prob.	Expected	Observed	Size	Scaled Residual
0.0000	0.4699	4.699	2.000	10	-1.710
0.0210	0.4699	4.699	5.000	10	0.191
0.2100	0.4700	4.700	2.000	10	-1.711
2.1000	0.4708	4.708	8.000	10	2.085
21.0000	0.4790	4.790	9.000	10	2.665
210.0000	0.5611	5.611	3.000	10	-1.664
2100.0000	0.9867	9.867	10.000	10	0.367
21000.0000	1.0000	10.000	10.000	10	0.000

Chi^2 = 20.24 d.f. = 6 P-value = 0.0025

Benchmark Dose Computation

Specified effect =	0.1
Risk Type =	Extra risk
Confidence level =	0.95
BMD =	121.791
BMDL =	50.1491

Since all solutions were created from full saturation and filtration only two significant figures can accurately be recorded. Therefore the BMD and BMDL values associated with caffeine were 120ppm and 50ppm (5.0×10^1ppm), respectively (see ***Table 2***). The p-value associated with this data was calculated to be 0.0025. USEPA (2012), suggests that good to moderate statistical fits for BMD analysis have critical p-values of 0.1. This was consistent was earlier observations noting a significant decrease in mortality rate for trial 3. If the high concentration value was removed from the data the p-value actually drops to 0.0011. However with this change in statistical analysis the BMD and BMDL values did not change when significant figures were accounted for. Thus no data was excluded from this analysis.

Carbendazim Range Testing

A fully saturated solution of carbendazim (CAS# 10605-21-7), approximately 8mg/L at room temperature (~25°C) (O'Neil, 2006), was created and used as the base solution for the diluted versions of contaminated solution. The saturated solution of aqueous carbendazim was diluted down by a factor of ten two times to reach a range concentration of 80µg/L, or 80ppb.

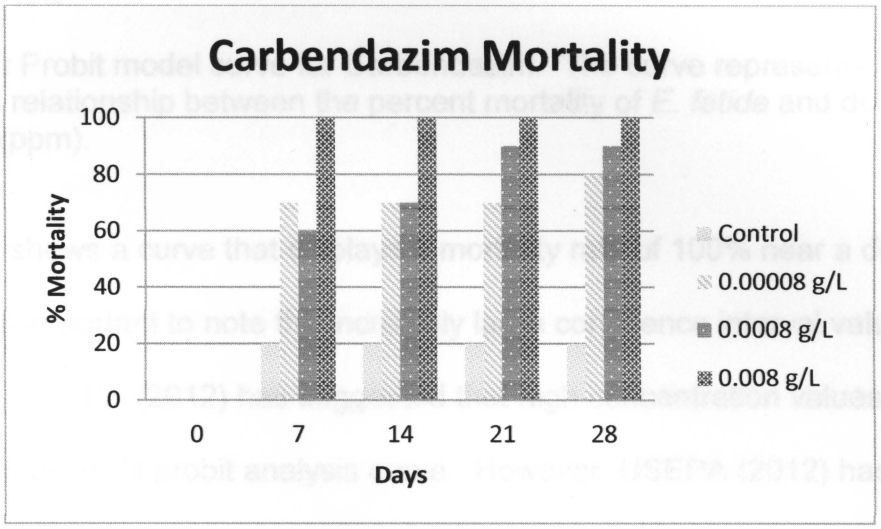

Figure 10: Carbendazim Daily Percent Mortality. Temperature was maintained at 22.10 ± 0.54°C and pH was maintained at 6.98 ± 0.27.

The percentage of mortality was viewed in daily increments and the most significant increases in percentage of mortality were found on day 7 with little change found in subsequent days following (see ***Figure 10***).

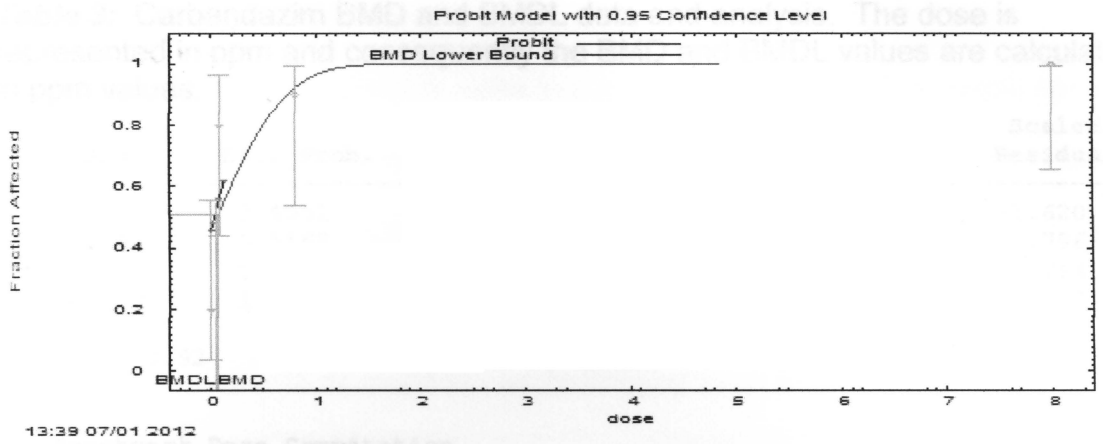

Figure 11: Probit model curve for Carbendazim. The curve represents a calculated relationship between the percent mortality of *E. fetida* and dose exposure (ppm).

Figure 11 shows a curve that displays a mortality rate of 100% near a dose of 1ppm. It is important to note the incredibly large confidence interval values on the curve. USEPA (2012) has suggested that high concentration values can be removed to better fit probit analysis curve. However, USEPA (2012) has also warned that in removing data BMD and BMDL values can be severely altered and less accurate. Due to the nature of this experiment, small amount of data collected and low number of significant figures noted, the high value concentration was not removed from the data.

Table 3: Carbendazim BMD and BMDL data and analysis. The dose is represented in ppm and consequently the BMD and BMDL values are calculated in ppm values.

Dose	Est._Prob.	Expected	Observed	Size	Scaled Residual
0.0000	0.4551	4.551	2.000	10	-1.620
0.0800	0.5162	5.162	8.000	10	1.796
0.8000	0.9223	9.223	9.000	10	-0.264
8.0000	1.0000	10.000	10.000	10	0.000

Chi^2 = 5.92 d.f. = 2 P-value = 0.0518

Benchmark Dose Computation

Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

BMD = 0.071372

BMDL = 0.0415903

Since all solutions were created from full saturation and filtration only two significant figures can accurately be recorded. Therefore the BMD and BMDL values associated with caffeine were 71ppb and 41ppb, respectively (note here the values are expressed in parts per billion, see ***Table 3***). The p-value associated with this data was calculated to be 0.0518. USEPA (2012), suggests that good to moderate statistical fits for BMD analysis have critical p-values of 0.1. If the high concentration value was removed from the data the p-value actually drops to 0.0150. However with this change in statistical analysis the BMD and BMDL values did not change when significant figures were accounted for. Thus no data was excluded from this analysis.

Sublethal Effects, EC50's and LOEC's

Due to the high mortality rates in a majority of trials and the small number of trials for each contaminant concentration, sublethal effects were noted during observational periods (see ***Figure 12***), but EC50's could not be calculated. In most trials *E. fetida* died before sublethal effects could be noted. As a result, a trend in sublethal effects could not be correlated to an EC50 value.

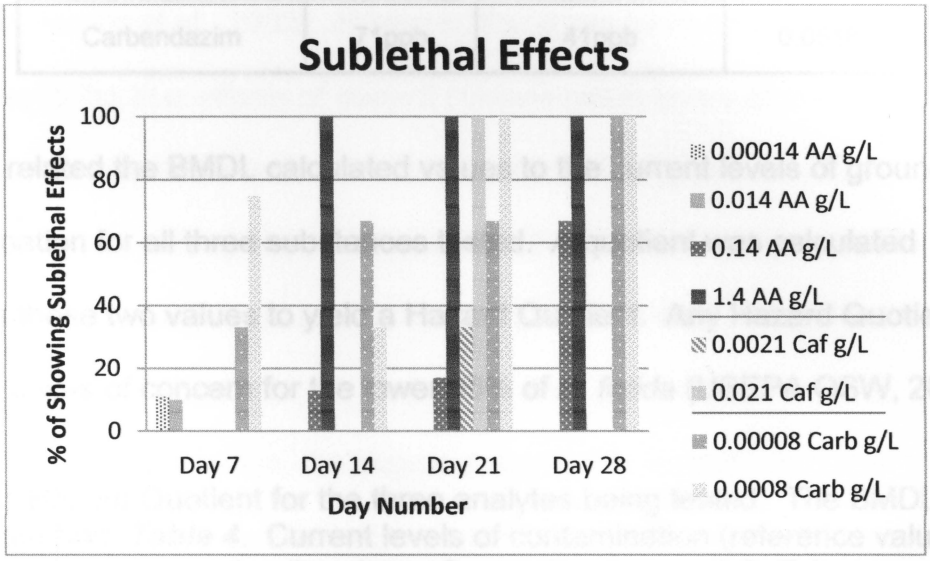

Figure 12: Sublethal Effects Given By Day. The only sublethal effects noted were inactivity (I) and shortened organism (SH) for the given concentrations above. Sublethal percentages were calculated based on the remaining living organisms for a given day. AA, Caf and Carb referred to Acetaminophen, Caffeine and Carbedazim, respectively.

Table 4 shows that all three contaminants were found to have BMDL's levels well below that of full saturation. The recommended critical p value is 0.1.

Table 4: Contaminant Comparison Data Table

Contaminant	BMD Value	BMDL Value	P-Value
Acetaminophen	160ppm	100ppm	0.0394
Caffeine	120ppm	50ppm	0.0025
Carbendazim	71ppb	41ppb	0.0518

Table 5 related the BMDL calculated values to the current levels of groundwater contamination for all three substances tested. A quotient was calculated between these two values to yield a Hazard Quotient. Any Hazard Quotient above 1.0 was of concern for the lower 95% of *E. fetida* (USEPA OSW, 2005).

Table 5: Hazard Quotient for the three analytes being tested. The BMDL values were taken from ***Table 4***. Current levels of contamination (reference values) were taken from supporting literature. Acetaminophen and Caffeine were taken from Barnes et al., 2008 and Carbendazim was taken from Dotson et al., 2012.

Contaminant	BMDL Value	Reference Value	Hazard Quotient
Acetaminophen	100ppm	3.8×10^{-4}ppm	3.8×10^{-6}
Caffeine	50ppm	1.3×10^{-4}ppm	2.6×10^{-6}
Carbendazim	41ppb	1.2×10^{-1}ppb	2.9×10^{-3}

Discussion

Evaluation and Explanation of Current Findings

Using a wide-range of contaminant concentrations allowed for a holistic approach to the determination of response dosages of ground water contaminants acetaminophen, caffeine and carbendazim, respectively. There has been little to no debate over the actual contamination of ground water supplies (Barnes et al., 2008). However, there has been much discussion and debate as to the true effects of current contamination levels of non-carcinogen based contaminants, such as over the counter (OTC) pharmaceuticals. The data presented (see ***Table 4***) showed that the relative levels of contamination for each of the three analytes has an impact the mortality of terrestrial organisms like *E. fetida*.

With acetaminophen, the largest increases in mortality occurred when observed at day 7 with another slightly smaller increase at day 21 (see ***Figure 6***). This would indicate that the first couple days of exposure were the most crucial to the mortality rate of *E. fetida* for exposure to acetaminophen. The second increase in mortality near day 21 would indicate that either residual contaminant was continuing to eliminate *E. fetida* or that *E. fetida* was consuming the contaminant and increasing its levels of contamination over time. Were the latter the case, it would be logical to assume that the organisms that died later in the trial would likely have shown some signs of sublethal affects earlier in the trial. The largest mortality increases occurred in trial 4 (0.014 g/L) and in trial 2 (1.4 g/L), both on day 21. This should have resulted from a drastic increase in

sublethal affects noted on day 14 for both trials. For trial 4 (0.014 g/L), no sublethal affects were noted at all during Day 14, however in trial 2 (1.4 g/L), 100 percent sublethal effects were noted on Day 14, which would be consistent with mortality rates on Day 21 as shown in ***Figure 6***.

This data was consistent with the observation that with high mortality rates sublethal effects were extremely difficult to observe (see ***Figure 12***). Both trials should have shown significant increases in sublethal effects on Day 14 and only one trial did. This was consistent with statements made earlier that with the number of worms tested and the high rates of mortality made it nearly impossible to witness consistent levels of sublethal effects. Thus, it is not be possible to say whether the worms were consuming the contaminant in the soil or if residual contaminant remained within the soil. Further analysis of worm tissues and soil concentrations would be needed to verify if *E. fetida* was indeed consuming the contaminant.

With caffeine, the largest increases in percent mortality occurred at Day 7 and Day 21 (see ***Figure 8***), similar to that of acetaminophen. However, when specifically looking at sublethal effects of caffeine, only two trials showed any signs of sublethal effects and both those trials occurred on Day 21, which was consistent with previous statements that high mortality rates made it difficult to observe sublethal effects. Sublethal effects should have been noted in high quantities on Day 14 were not seen until Day 21, again indicating the lack of observation possible for given trials.

With carbendazim, which had the highest percentage of mortality in virtually all trials (see ***Figure 10***), it would be reasonable to conclude that the rise in sublethal effects would not be related to the increase in mortality rates. When looking at the mortality rate for *E. fetida* when exposed to carbendazim, the largest increase in mortality rates occurred at Day 7 with minimal increases the remaining days. Although sublethal effects were noted in the remaining living organisms (see ***Figure 12***), due to the high level of mortality of *E. fetida*, most trials had less than 10 percent of the population surviving, making it almost impossible to note sublethal effects accurately. In many cases only one organism remained alive and that organism showed some form of sublethal effect. This made the trial look as though the entire population were showing signs of sublethal effects when in fact only one organism had been.

Every contaminant tested showed that sublethal effects for *E. fetida* in small quantities are highly suspect. This would indicate that any calculations of EC50, LOEC and NOEC would be prone to error. The purpose of this investigation was to identify what ranges of contamination would be detrimental to *E. fetida*. With such small quantities of data taken, and such wide ranges of contamination exposure, it is reasonable to conclude that mortality data is the most accurate and least suspect data to associate when determining that range.

Benchmark Dose Methodology (BMD) and dichotomous probit analysis were used to take concentration values associated with various trials and generate Benchmark Dose Lower confidence levels (BMDL's) for *E. fetida* for all three contaminants (see ***Figures 7, 9*** and ***11*** and ***Tables 1, 2*** and ***3***). Since the

goal of this investigation was to identify a general area of concern for contaminants, and since there was only one trial of each contamination level tested, no data was excluded from the analysis regardless of trend fit.

All p-values in the three tested trials (0.0394, 0.0025, 0.0518, respectively) (see ***Table 4***) were below the critical p value of 0.1 level (USEPA, 2012). This was consistent with the nature of index/range experimentation. The goal of this experiment was to determine the need for true risk assessment for these contaminants in *E. fetida*. Since the diluted and saturated solutions were not verified after preparation, it was likely that there was error in their concentrations. This error would propagate through the preparation of the dilutions and thereby yield inconsistent data when related to worm mortality. Regardless of error, the dilution method would still give an index to the level of concern.

Despite slight fluctuations in experimental values/trends, all three contaminants were successfully analyzed and found to have response dosages well below the point of saturation. Carbendazim, specifically, was found to have a BMD and BMDL in the parts per billion ranges with little to no variance in trial data (see ***Table 3***). While acetaminophen and caffeine were detected at higher levels (ppm), both contaminants showed response in dosages well below full saturation (see ***Tables 1*** and ***2***). ***Table 5*** indicated that the hazard, or risk assessment, of the current levels of contamination on *E. fetida* remain relatively low. All three contaminants showed Hazard Quotients below a value of 1.0, however Carbendazim was significantly higher than Acetaminophen and Caffeine. These quotients were determined using the BMDL calculated data for

each contaminant. This meant that the hazard was compared to the highly susceptible, lower 95% confidence interval of *E. fetida*. All three quotients were well below 1.0 (see ***Table 5***). This indicates that the likelihood of detrimental health related effects on *E. fetida*, given current levels of contamination, would be rare. However, carbendazim showed a quotient almost 1000 times that of caffeine and acetaminophen. This meant that carbendazim was the contaminant of most risk to *E. fetida.*

While the use of carbendazim has not been approved in the United States, recent testing of internationally imported orange juice has found that other countries have continued to use the fungicide worldwide (Beru, 2012). This could mean that it would be highly possible for trace amounts of carbendazim to easily enter the local ecosystem in trace quantities. Since the BMDL for *E. fetida* was 41ppb, even small levels of contamination could be harmful to local ecosystems and ground water supplies.

While all three compounds did have varying BMDL's on *E. fetida*, it was alarming that all three contaminants did in fact show levels of response at concentrations well below full saturation (see ***Table 4***). The response dosage of all three compounds should not be taken lightly for a terrestrial organism as adaptable and durable as *E. fetida* (Edwards et al., 2011). *Eisenia fetida* is an organism that adapts well to changing environmental conditions and has lived, speaking on a phylogenetic level, for millions of years with only slight adaptions and moderations (Winnepenninckx et al., 1995 and Perez-Losada et al., 2005). This is one of the reasons that the effects noted on *E. fetida* are of such concern

Future Experiments and Research

All three contaminants showed BMDL's below full saturation and therefore should be tested using the EPA definitive range testing protocol (USEPA, 1996). Through the use of definitive range testing, all three contaminants could have more narrowly defined BMD's and BMDL's. Research would be needed to narrowly identify the exact levels of lethal and effect concentrations on *E. fetida*. Concentration would need to be quantified using instrumentation to verify accurate levels of contamination on associated trials. Through future investigations recommendations could be made for acceptable levels of contamination for both humans and terrestrial environments alike.

All organisms were purged and frozen after the range testing was completed. *Eisenia fetida* tissues and soil chambers should be tested for levels of contamination to determine if *E. fetida* was consuming the contaminant or if levels of contamination remain constant in soil samples. Through this analysis, methods of *E. fetida* metabolism could be hypothesized and possible means for pharmaceutical contamination clean up could be theorized. If *E. fetida* were, in fact, consuming the pharmaceutical contaminant this could allow for possible means of contamination removal from local water supplies and terrestrial ecosystems. However, if the levels of contamination remained constant in soil chambers it may be possible to conclude that the levels, while detrimental to invertebrate species, may not be bioaccumulating in higher level organisms.

Different terrestrial organisms and different contaminants should also be tested. Currently, there are over one hundred ground water pharmaceutical and

personal care product contaminants (Barnes et al., 2008). This experiment has only utilized one species and three specific contaminants. There must be widespread analysis and identification of all contaminants and associated effects on terrestrial ecosystems. There has been some focus on aquatic ecosystems and contaminant implications (Escher et al., 2005), however, there has been little to no research investigating the ramifications of ground water contamination on terrestrial organisms. As the United States continues to water farm fields, dispose of PCP's and pharmaceuticals, and humans excrete OTC drugs, terrestrial organisms will continue to be exposed to levels of contamination found by Barnes et al. (2008).

Conclusions

The data presented showed that levels of contamination in the parts per billion can be detrimental to the test species, *E. fetida*. Three different contaminants were tested against *E. fetida* and all three contaminants, currently found as ground water contaminants in the United States (Kolpin et al., 2002), were found to have BMDL's well below the point of saturation (see ***Table 4***). While the data shown has represented, specifically, a range testing approach to contamination, it has been clearly shown that detrimental levels of contamination were well below levels of full saturation. It should, however, be noted that these values are only suggestive as this experimentation was done as a method of index/range experimentation. The goal of this data was to show at what levels of contamination were there concerns for terrestrial organisms, specifically *E. fetida*.

Acetaminophen, caffeine and carbendazim were shown to have BMDL's values when tested against *E. fetida* of 100ppm, 50ppm and 41ppb, respectively (see ***Tables 1, 2*** and ***3***). Current levels of contamination of ground water are found to be lower than the data provided (Barnes et al., 2008), however, it would be reasonable to conclude that terrestrial ecosystems stand at risk for some contaminants like carbendazim (as shown by the Hazard Quotients in ***Table 5***.)

While the data presented here is brief and wide ranging, it can reasonably be concluded that current levels of pharmaceutical contamination are of concern to terrestrial ecosystems and, by association, human beings. Future research will show that terrestrial organisms, like *E. fetida*, are highly susceptible to such contaminants.

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