Innovative Use of Earthworms for the Remediation of Soil Contaminated with Crude Oil

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Abstract

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Crude oil soil contamination is widespread, and due to the complex chemical nature of crude oil, remediation can be expensive and challenging. Crude oil that remains in soil for long periods of time can become sequestered in the soil matrix and become difficult to biodegrade. Heavier, more complex hydrocarbons are persistent in the environment due to resistance to biodegradation, which makes remediation problematic. Earthworms ingest soil, exposing it to intense physical and chemical processes and can increase soil microbial activity and therefore are promising candidates for enhancement of crude oil bioremediation. I examined if different earthworm species could tolerate and degrade crude oil. Toxicity tests established that *Eisenia fetida and Apporectodea caliginosa* were crude oil tolerant and suitable for use in crude oil degradation experiments. Two primary degradation experiments were performed. In the first, laboratory made soil was mixed with crude oil at concentrations of approximately 30,000 mg/kg, with and without the addition of a microbial inoculum consisting of contaminated, native soil from an active remediation site. Petroleum degradation was examined in the presence and absence of *E fetida*. This experiment measured the importance of a hydrocarbon adapted microbial community to crude oil degradation rates. In the second, petroleum degradation in the contaminated, native soil from the remediation site was examined in the presence and absence of the *E. fetida* and *A. caliginosa*. The second experiment measured the effectiveness of earthworms on real-world soils that had weathered in the field for several decades. In the first degradation experiment, petroleum concentrations declined significantly ($p<0.01$) in the

presence of *E. fetida* compared to controls. After 342 days, concentrations declined by 56% without the microbial inoculum and 63% with the microbial inoculum. Heavier and more complex hydrocarbons were more resistant to degradation. In the second experiment, petroleum concentrations declined by approximately 93% in the native soil in the presence of each of the worm species, significantly more than the declines observed in the controls. The results of these experiments show that earthworms accelerate degradation of crude oil and are a promising candidate for the enhancement of crude oil bioremediation (vermiremediation).

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INTRODUCTION

Petroleum is the centerpiece of global transportation and chemical synthesis, requiring constant production and processing. It has a wide range of uses after refinement: transportation fuel, electricity generation, asphalt, and as a chemical feedstock, especially for plastics. Because of petroleum's importance in global commerce, petroleum is extracted worldwide, with 21 different countries each producing more than 1 million barrels of petroleum per day (EIA 2014). Crude oil is the primary source of petroleum and reflecting its importance, worldwide crude oil production has increased every year for the past five years (EIA 2014). While crude oil production has been part of human civilization for almost 6,000 years, the first modern extraction wells were not drilled until the mid-1800s and by the late 1800s crude oil had established itself as an important part of modern society (Totten 2004a; Totten 2004b). Given its long history and ubiquitous global presence, it is no surprise that crude oil contamination has become a major concern.

Crude oil contains a variety of different compounds, including many that are toxic to living organisms. There are currently 85 different petroleum hydrocarbon Superfund remediation sites in the United States (USEPA 2014). Because crude oil production and refinery facilities are rarely in the same location, spills during transport with tankers, pipelines, and trains regularly occur. Oil storage facilities can leak into soil; extraction wells can leak over the course of many years or dramatically erupt and release millions of gallons of crude oil in a few days. Many of these sources of crude oil and mechanisms of release are terrestrial and crude oil can persist in soil for many years (Atlas 1981; Alexander 1995; Schaefer & Filser 2007). For this reason many methods to remediate crude oil contaminated soils have been developed, however the variety of compounds found in crude oil make remediation a challenge.

Chemical Composition of Crude Oil

Crude oil is composed of numerous hydrocarbons and related compounds. The composition of crude oil also varies depending on its extraction location; oil from Kuwait has a different chemical make-up from crude oil pumped in California. Crude oil is classified based on its percent composition of paraffins, aromatics, naphthenes, and asphaltenes (Westlake et al. 1974; Atlas 1981; Sugiura et al. 1997). Paraffins, also called *n*-alkanes or saturates, are straight or branched saturated hydrocarbon chains (Figure 1A). This fraction is considered to be the most easily degraded component of crude oil (Westlake et al. 1974; Atlas 1981; Sugiura et al. 1997). Aromatic compounds are classified on the presence of the benzene ring; this class includes polyaromatic hydrocarbons (Figure 1B). Lighter aromatic hydrocarbons can be biodegraded (degradation via microbes, see below) with resistance to degradation increasing with the number of aromatic rings and greater molecular weights (Atlas 1981; Blackburn et al. 1993; Sugiura et al. 1997). Naphthenes are non-aromatic rings, including the cycloalkanes, and occur in a wide range of structures of varying complexity (Figure 1C). Simple naphthenes are relatively biodegradable, with resistance to biodegradation increasing with greater complexity (more functional group substitutions) and molecular weight (Atlas 1981; Blackburn et al. 1993). Finally, the asphaltenes are a catch-all group of heavier, viscous compounds in oil which are not well described but often contain metals, sulfur and other elements (Figure 1D)(Atlas 1981; Podgorski et al. 2013). For all four classes of hydrocarbons, as the carbon number increases the vapor pressure and solubility decrease. Each of these classes of compounds is present in oil to a greater and lesser degree, depending on the origins of the crude oil, and all contribute to soil contamination.

A. Paraffins/Saturates

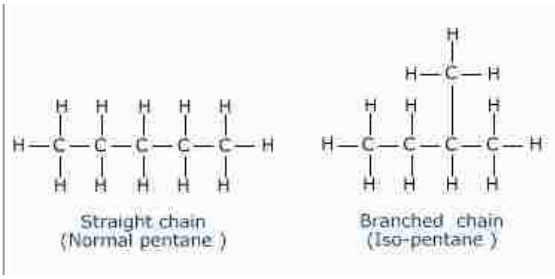


Figure from Tutorvista.com

B. Aromatics

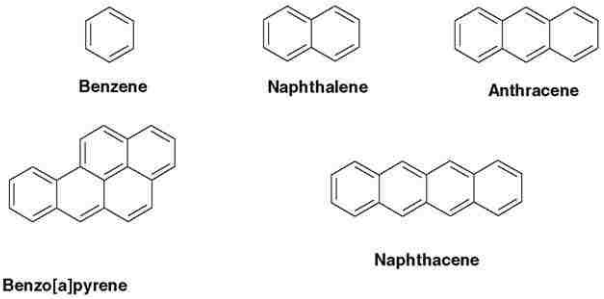
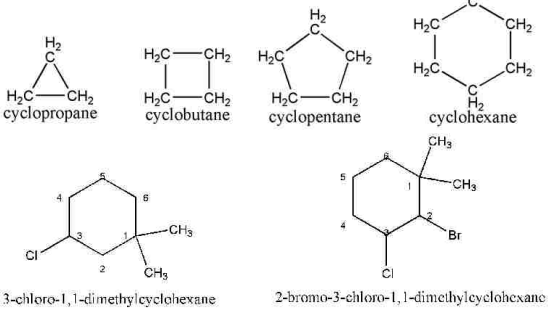


Figure from What-when-how.com

C. Napthenes



D. Asphaltenes

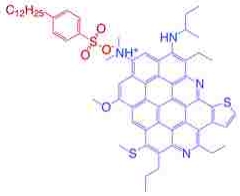


Figure from http://en.wikibooks.org/wiki/Organic_Chemistry/Cycloalkanes

Figure 1: Different chemical structures of hydrocarbons

Soil Remediation Strategies

Existing soil remediation strategies can be grouped into three categories: physical, chemical, and biological. However, many remediation technologies utilize more than one of these categories in concert. Physical remediation technologies remove the contaminant mass from the contaminated site prior to treatment or disposal. These technologies often relocate the contamination rather than actually eliminating it, such as moving benzene vapors from the soil onto activated carbon filters and then landfilling the filters. Chemical remediation technologies introduce a reactive chemical, often an oxidizer or chelating agent, which alters the chemical structure or state such that it is no longer toxic. Biological remediation technologies utilize living things, such as bacteria or plants, to metabolize or accumulate the contaminant in its tissue.

Each of these strategies can be effective for a range of contaminants and contaminated media, both separately and when used together. Here, I discuss some of the existing technologies for crude oil contaminated soils.

All of the ex-situ treatment technologies require excavation of the crude oil contaminated soils, which can then be transported to a treatment facility or be remediated on-site. A classic way to remediate petroleum contaminated soils is simply to place them in a landfill. This has the advantage of being straight-forward and relatively quick, as the soil remediation project can proceed as quickly as soils can be removed, transported, and landfilled. The disadvantages are just as obvious, as the contamination is only being moved to a specialized location and the soil cannot be re-used.

Thermal desorption is a technique wherein the contaminated soil is placed in a "thermal desorber", which heats the soil, allowing contaminants to volatilize (USEPA 2012). However, thermal desorption can have high labor and energy costs and does not function as well in soils with high amounts of fine particles or organic matter (Khan et al. 2004; Van Deuren et al. 2002). Incineration is a similar technology, but operates at temperatures high enough to oxidize the contaminant(s) (Khan et al. 2004). Incineration has high energy needs, requires a great deal of regulatory involvement, and produces an ash that is typically not suitable for re-use (Van Deuren et al. 2002).

Soil vapor extraction is a proven technology utilized at many petroleum contaminated sites. Wells are installed in the vadose zone and negative pressure is applied, pulling contaminated soil vapor from the subsurface and through a filter, allowing additional hydrocarbons to volatilize from soil particles and be extracted (Van Deuren et al. 2002; USEPA 2004). This process is highly effective in permeable soils with volatile contaminants (such as low molecular weight

aromatics), but is less effective for compounds with low vapor pressures or in finer, less permeable soils (Van Deuren et al. 2002)

There are numerous variations of bioremediation, but all focus on adding and/or stimulating organism growth in contaminated soil, often through the addition of nutrients and oxygen. Biodegradation of most hydrocarbons occurs aerobically and the insertion of oxygen into the hydrocarbon molecule can be the limiting step in the degradation of many compounds (Atlas 1981; Blackburn et al. 1993). Thus, many bioremediation systems require excavation and/or mixing to maximize soil exposure to the air or the air is injected into the contaminated soil.

Bioslurry reactors are ex-situ systems that combine excavated soil, air, water, and nutrients in a regularly mixed container (Kuyukina et al. 2003; Khan et al. 2004). These systems can produce rapid degradation (Kuyukina et al. 2003). This technology works for many petroleum types, but has difficulty treating clay particles, requires dewatering after treatment, and can be very expensive due to equipment and labor (Kuyukina et al. 2003; Khan et al. 2004).

Landfarming is also an ex-situ treatment and involves spreading petroleum contaminated soil in thin layers and stimulating microbial growth through plowing and the addition of nutrients and moisture (Kuyukina et al. 2003; USEPA 2004). This technique can effectively treat a range of petroleum products and can lead to TPH decreases of up to 90% in a short time (Kuyukina et al. 2003). Landfarming takes large amounts of land, extensive labor for tilling and nutrient applications, and is less effective for higher molecular weight hydrocarbons, such as PAHs (USEPA 2004; Van Deuren et al. 2002).

Composting is another ex-situ remediation treatment where contaminated soils are mixed with organic amendments such as leaves, vegetable waste or wood chips to stimulate microbial growth (Van Deuren et al. 2002; Hickman & Brian Reid 2008). The compost and soil mixture must be aerated using blowers, mixing in a vessel or through tilling and has been used to

degrade several types of hydrocarbons, including oil refinery sludge and PAHs (Van Deuren et al. 2002; Hickman & Brian Reid 2008; Gandolfi et al. 2010; Ceccanti et al. 2006). Composting bioremediation has several disadvantages: it increases the contaminant mass due to the compost addition, requires labor to mix the soil and compost, incompletely degrades more complex hydrocarbons, and typically is not able to reduce contaminant concentrations by more than 80-90% (Ceccanti et al. 2006; Hickman & Brian Reid 2008; Gandolfi et al. 2010).

There are drawbacks common to most bioremediation systems. Often measurable hydrocarbons remain after the remediation is completed and the treatment of contaminated fine soils is especially challenging, often resulting in longer remediation timelines (Atlas 1981; Sugiura et al. 1997; Kuyukina et al. 2003; Khan et al. 2004; Ceccanti et al. 2006; Hickman & B. J. Reid 2008; Hickman & Brian Reid 2008; USEPA 2004). The problems of residual hydrocarbons and difficulty in treating fine soils may be closely related. As organic contaminants such as hydrocarbons remain in soil they become sequestered within the soil matrix itself (Alexander 1995; Hatzinger & Alexander 1995; Hatzinger & Alexander 1997; Ehlers & Luthy 2003; Semple et al. 2003). Organic contaminants such as hydrocarbons adsorb and diffuse into soil organic matter and are difficult to then desorb for degradation (Brusseau et al. 1991; Hatzinger & Alexander 1995; Semple et al. 2003; Ehlers & Luthy 2003). These sequestration processes make pollutants less available to microbes and thus less biodegradable (Hatzinger & Alexander 1997; Reid et al. 2000; Alexander 2000; Semple et al. 2003).

Contaminants also remain after bioremediation due to preferential microbial degradation of the more energetically favorable compounds, such as straight chain alkanes, resulting in a remaining fraction of crude oil with an increased proportion of resistant PAHs, naphthenes, and asphaltenes (Atlas 1981; Sugiura et al. 1997; Semple et al. 2003; Hickman & B. J. Reid 2008; Blackburn et al. 1993; Westlake et al. 1974). This combination of contaminant sequestration

and increasing degradation resistance leads to a biphasic degradation curve that has been observed in many bioremediation studies (Song et al. 1990; Hatzinger & Alexander 1995; Hatzinger & Alexander 1997; Alexander 2000; Loehr et al. 2001; Kuyukina et al. 2003). Biphasic degradation is characterized by a relatively rapid initial decline in contaminant concentrations, followed by a period of little to no reduction in concentrations. This is pattern is often called a "hockey stick" due to the resemblance of the degradation curve to a hockey stick.

In summary, the complex and mixed nature of crude oil make it difficult to remediate by chemical, physical, or biological means. Expensive and/or wasteful physical remediation systems are currently the only way to consistently remove more than 95% of crude oil contaminant mass. For biological remediation systems to be as effective at many crude oil sites, a method to remove hydrocarbons from soil sequestration sites and expose microbially resistant compounds to attack in favorable conditions is needed. As I explore below, the humble earthworm has the required qualities to improve bioremediation of crude oil.

Earthworm Ecology

Earthworms are commonly divided into three categories: epigeic, anecic, and endogeic. Epigeic earthworms (such as the composting earthworm, *E. fetida*) live on or near the soil surface, typically do not form burrows and feed on organic litter such as leaves or animal dung. Anecic earthworms (such as the nightcrawler, *L. terrestris*) live in vertical soil burrows and move to the surface to feed on litter, often bringing their food into the burrow with them (Tiunov et al. 1997; Bohlen et al. 2004). Endogeic earthworms (such as the gray garden worm, *A. caliginosa*) spend their lives in deeper mineral soil horizons, moving in horizontal burrows and feeding on soils containing small amounts of organic matter and dead root material.

Earthworms interact with the soil environment through the drilosphere, which is the earthworm, its casts and burrows, and the microbes and other invertebrates present in these structures

(Lavelle et al. 2004). Earthworms move throughout the soil column, consuming and shifting soil particles and creating burrows that can reach the surface and aerate the subsurface soil (Li et al. 2002; Lavelle et al. 2004). These burrows are lined with a mucus that has a high water holding capacity, making the burrows very humid (Tiunov et al. 1997). Earthworm mucus is very high in carbon, which can act as a food source for microbes and *L. terrestris* burrows, which are lined with mucus, have been found to have as much as ten times more bacterial cells than nearby soils (Tiunov et al. 1997; Bohlen et al. 2004). The increased bacterial population effect of the *L. terrestris* burrows was found to extend up to 1 cm into the soil surrounding the burrow (Tiunov et al. 1997). Earthworms move organic matter down through the soil column in their burrows and via feeding activities and their casts tend to have more organic matter and nutrients in them than the surrounding mineral soils (Li et al. 2002; Lavelle et al. 2004; Curry & Schmidt 2007).

It is through their interactions with organic matter that earthworms most strongly affect soil microbes and can change the size and distribution of microbial biomass (Bohlen et al. 2004). There is some question about how passage through the earthworm gut affects soil microbial populations. Bohlen et al (2004) indicate that gut processes may reduce microbial biomass and Lavelle et al (2004) state that earthworm casts are compact, resulting in low microbial activity. However, Brown and Doube (2004) argue that passage through the gut increases both microbial density and diversity. Scheu (1987) found that *A. caliginosa* casts had a greater microbial biomass and higher respiration than the surrounding soil, while Singleton et al (2003) found that *L. rubellus* casts had greater numbers of bacterial cells and higher bacterial diversity than the intestine. The microbes associated with earthworm gut varies by ecological category, due to food choices and differences in gut morphology (Thakuria et al. 2010). Near surface forest soils in the Northeastern United States exhibited a doubling of microbial biomass and

increases in microbial respiration rates of three to seven times due to an earthworm invasion (Li et al. 2002).

Earthworms can also affect soil structure. Their feeding activities create organic matter and mineral aggregates, while their movements create burrows, both of which can continue to exist long after the earthworm has died (Lavelle et al. 2004; Bohlen et al. 2004). A study of *E. fetida* found that passage of feldspar particles through the earthworm gut reduced the size of the particles and that gut passage created more rounded quartz particles (Suzuki et al. 2003). Another study found ingestion and digestion of fine slate particles (used as a generic clay particle) by *L. terrestris* resulted in mineral degradation and production of new clay particles (Needham et al. 2004).

Thus, earthworms are able to produce moist, aerated environments with large and diverse microbial populations that are ideal conditions for bioremediation while also degrading organic matter and soil particles to expose sequestered contaminants.

Previous Vermiremediation Studies

Due to the qualities highlighted above, there have been a number of studies using earthworms in organic pollutant remediation systems (vermiremediation) and the field has been the subject of several review papers to date (Hickman & B. J. Reid 2008; Sinha et al. 2008; Dendooven et al. 2011; Rodriguez-Campos et al. 2014). These studies have examined the effect of earthworms on contaminant sequestration and biodegradation. The research examining non-hydrocarbon organics has frequently reached conflicting conclusions, while the hydrocarbon research has generally shown that the presence of earthworms results in greater contaminant degradation over short time periods.

Thirteen different studies using earthworms to treat herbicide contamination have been

performed, with mixed results, some studies showing increased contaminant availability and removal and other studies showing the opposite (Hickman & B. J. Reid 2008; Rodriguez-Campos et al. 2014). Earthworm casts were found to increase sorption of chlorophenols and decreased bioavailability (Shan et al. 2011), while another study found that earthworms made sorbed residues of DDT and HCH (two common pesticides) mobile and bioavailable (Verma & Pillai 1991). The reasons behind these contrary findings are not clear. Studies utilizing earthworms in the remediation of polychlorinated biphenyls (PCBs) have also been performed, with two studies showing increased PCB degradation in soil in the presence of earthworms and a third study showing no additional change in PCB degradation , and bioaccumulation of PCBs in earthworm tissues (Singer et al. 2001; Luepromchai et al. 2002; Tharakan et al. 2006). However, the vast majority of organic pollutant vermiremediation studies have focused on petroleum hydrocarbons, with specific attention paid to PAHs (Hickman & B. J. Reid 2008; Sinha et al. 2008; Dendooven et al. 2011; Rodriguez-Campos et al. 2014).

Numerous studies have been performed that focus on the effect of earthworms on the degradation of specific PAHs or PAH mixtures, with a notable increase in studies over the past decade (Hickman & B. J. Reid 2008; Sinha et al. 2008; Dendooven et al. 2011; Rodriguez-Campos et al. 2014). Several studies have shown accelerated removal of PAHs in the presence of earthworms, especially at lower initial concentrations, and in some cases 100% of the PAH was removed (Ma et al. 1995; Contreras-Ramos et al. 2006; Hickman & Brian Reid 2008; Sun et al. 2011; Tejada & Masciandaro 2011; Jing et al. 2013). Two follow-up studies showed that microbes in the earthworm gut were important in PAH degradation (Contreras-Ramos et al. 2008; Contreras-Ramos et al. 2009).

Several vermiremediation studies have focused on petroleum mixtures, including crude oil, with mixed results (Hickman & B. J. Reid 2008; Rodriguez-Campos et al. 2014). A study from 2002 using “landfarm soils” found that the addition of *E. fetida* increased soil respiration, but did not

reduce TPH concentrations (Callaham et al. 2002). A 28 day study of crude oil contaminated soil found that the presence of earthworms increased soil respiration, increased microbial biomass and resulted in TPH declines ranging from 17-42%, while a follow-up study showed that heavier hydrocarbons were strongly degraded (Schaefer et al. 2005; Schaefer & Filser 2007). A study combining earthworms and compost mixed with hydrocarbon contaminated soil found that earthworms reduced hydrocarbon concentrations by up to 65% without compost (Hickman & Brian Reid 2008).

Vermiremediation shows promise for treatment of petroleum hydrocarbons, however the experiments in the literature have not addressed issues with mortality and most tend to be of short duration. Vermiremediation is unlikely to become a viable bioremediation technology if the earthworms must constantly be replaced due to mortality. Methods to reduce mortality are needed before vermiremediation can be applied in the field. Real-world remediation sites often have long time lines, from several months to several decades. The short duration of many of the vermiremediation studies means the rate of degradation after one or several months is unknown, resulting in a major data gap. Determining the length of time that petroleum continues to degrade in the presence of earthworms is key knowledge for assessing the applicability of vermiremediation. Very few of the reviewed studies looked directly at crude oil; however vermiremediation has shown promise in this application. Insufficient follow-up studies have prevented this remediation technology from moving to commercial application. In this study I address the following questions:

Will earthworms stimulate microbial soil populations?

Is it possible to reduce TPH concentrations in soil containing crude oil using vermiremediation and if so, what time scales are required?

Are certain earthworm species more appropriate for vermiremediation?

What factors might cause the biphasic degradation curve during vermiremediation?

MATERIALS & METHODS

Many of the methods explained below were developed in 2011-2012 during preliminary tests and in Degradation Trial 1 (see below). *E. fetida* was chosen initially as the test earthworm of documented success in several other studies (Schaefer et al. 2005; Schaefer & Filser 2007; Ceccanti et al. 2006; Contreras-Ramos et al. 2006; Contreras-Ramos et al. 2008). *E. fetida* are tolerant of crude oil, survives in a wide range of environmental conditions, is widely available for purchase, and is relatively easy to raise in laboratory conditions. *E. fetida* are epigeic worms, tending to spend the majority of their time in surface and near-surface soils.

Two other earthworm species (*Apporectodea caliginosa* and *Lumbricus terrestris*), representing two additional ecotypes, were also tested for TPH degradation effectiveness. *A. caliginosa* is an endogeic worm, primarily living in the deeper, mineral soil layers in horizontal burrows. Endogeic worms feed on soil particles, a feeding pattern that could increase contact between the worm gut and soil particles and cause greater TPH degradation. *L. terrestris* is an anecic worm, making permanent, vertical burrows in the soil column. *L. terrestris* has been used with mixed success in several other studies (Schaefer et al. 2005; Ceccanti et al. 2006; Schaefer & Filser 2007). It was thought that the greater vertical movement of *L. terrestris* would more effectively stimulate microbial growth deeper in the soil and increase TPH degradation.

Earthworm Procurement and Cultivation

The earthworms were obtained from several sources over the course of the experiments. Most of the *E. fetida* earthworms used in the experiments were purchased from Sky Nursery in Shoreline, Washington. Additional *E. fetida* were obtained from outdoor stock maintained by our collaborators at the University of California-Berkeley (UCB) and from Dr. Davidson's outdoor compost bin in Seattle, Washington. Both of these *E. fetida* stocks originated with earthworms from the Yelm Earthworm and Castings Farm (in Yelm, Washington), which also supplies Sky

Nursery. *A. caliginosa* earthworms were collected from organic backyard gardens in Seattle, Washington, and *L. terrestris* were ordered from a commercial supplier (Berkshire Biological) and delivered via UPS.

In the laboratory, all earthworms were maintained in the defined artificial soil (see below) mixed with their native soil or bedding and additional hydrated coconut coir. The earthworms were fed a 3:1 mixture of carrot pulp and coffee grounds, in addition *A. caliginosa* and *L. terrestris* were provided with aged leaves and grass. All earthworms were acclimated to laboratory conditions for at least two weeks prior to addition to experimental treatments.

Preparation of the Defined Artificial Soil

The defined artificial soil (referred to as "artificial soil" throughout this document) was prepared by hand mixing 70% sand by dry mass (from Salmon Bay Sand & Gravel), 20% clay (from Seattle Pottery Supply), and 10% dried coconut coir (Beats Peat™) until the soil was visually homogenous (OECD 1984) For experimental treatments requiring hydrocarbon contamination, San Joaquin Valley (SJV) crude oil provided by the Chevron Corporation (Chevron) was used. Stock contaminated soil was made by weighing SJV crude oil and dissolving it in dichloromethane (DCM) and adding it to the artificial soil at approximately 15% (w/w). The contaminated soil was mixed with a stainless steel spoon until visually homogenous and no soil particles without oil were observed. This contaminated soil was then ventilated in a fume hood for 14 days and the soil was mixed every 2-3 days. The venting eliminated most of the toxic, more volatile SJV components, remaining DCM, and mimicked the weathering of crude oil in a native soil.

Analysis of TPH Degradation Trial 1 Data

The data set from the first vermiremediation trial (Degradation Trial 1) was reviewed. Degradation Trial 1 was conducted from 2012-2013. TPH concentrations had been determined at time points beginning with initial sampling at the beginning of the experiment and concluding after 396 days, following the methods detailed below.

The effect of the SJV crude oil on *E. fetida* egg hatch rates was also determined during this previous experiment. Egg capsules were collected from *E. fetida* that had not previously been exposed to crude oil. Each egg capsule treatment took place in 20 g of artificial soil with 32-33% moisture. Two groups of 15 egg capsules each were placed in separate petri dishes with soil containing 1.7% crude oil (w/w). A second treatment using 15 egg capsules in a single petri dish with soil at 3% crude oil (w/w) was also conducted. The control was conducted using two sets of 15 egg capsules each that were placed in separate petri dishes with uncontaminated soil. All the egg capsules used were between 0 and 6 days old. The petri dishes were sealed with parafilm to maintain soil moisture throughout the experiment. The petri dishes were observed for 33 days to determine hatch rate.

Selection and Preparation of Native, Contaminated Soils

Chevron provided native soils from a remediation site in Michigan (undisclosed location) contaminated with crude oil from historic oil drilling and extraction. Three different soil types from different areas of the site were provided: WYANTTB 1-2', ACB5 4-6', and IBBI1 3-5'. The TPH concentrations in these soil types varied from ~7,000 – 46,000 mg/kg (Table 1). To obtain a native soil representative of the site and to prevent earthworm toxicity from high TPH concentrations, the three soil types were mixed together using a cleaned and decontaminated steel shovel. This native soil mixture ("native soil") was then ventilated for approximately 2

weeks in a fume hood to remove the light and volatile fraction of hydrocarbons prior to experimental use. During ventilation the soils were stirred approximately every 3 days.

Table 1: Concentrations of Native Soils

Soil	TPH Concentration (mg/kg)
WYANTTB 1-2'	46,114 (±3,938)
ACB5 4-6'	22,470 (±6,487)
IBBI 3-5'	7,656 (±844)
Native Soil	30,772 (±900)

A particle size analysis of the native soil was performed using the American Society for Testing and Materials (ASTM) D421-85 and ASTM D422-63 standards. The native soil consisted of 85% sand, 6.2% silt and 7.7% clay and contained approximately 1% organic matter, determined by combustion of oven dry soil at 550°C.

Characterization of Hydrocarbons in Residual and Native Soils

Approximately 100g soil samples from the Degradation Trial 1 experimental treatments and the native soils were collected in glass jars and submitted to Eurofins – Lancaster Laboratories in Lancaster, Pennsylvania. The soil samples from the Degradation Trial 1 treatments were collected 239 days after the completion of the experiment and 635 days after the experiment began. The samples were analyzed by Method MA DEP EPH 5/04 (Massachusetts Department of Environmental Protection Extractable Petroleum Hydrocarbons) to determine the concentrations of specific compounds and concentrations within specific hydrocarbon ranges and classes. EPH, extractable petroleum hydrocarbons, consists of the aliphatic and aromatic

hydrocarbons from C10-C35. In contrast, TPH (Total Petroleum Hydrocarbons) includes all hydrocarbons in a sample including those lighter and heavier than the EPH fraction, as well as naphthenes and asphaltenes. The EPH analysis was undertaken to determine if particular groups of hydrocarbons were degraded preferentially in the presence or absence of earthworms.

Toxicity Tests

Toxicity tests were conducted on the artificial soils with crude oil and the native soils prior to experimental use, following established protocols (OECD 1984). 1 kg of the test soil was placed in a 1 liter glass Mason jar and ten individual earthworms were added per jar. The toxicity tests were performed in duplicate. The jars were incubated for 14 days without feeding, and the earthworm survivors were counted at 7 and 14 days. Each of the three earthworm species (*E. fetida*, *A. caliginosa,* and *L. terrestris*) was tested. The native soils (with approximately 30,000 mg/kg TPH) were hand-mixed with uncontaminated, artificial soil to determine a range of toxicities for each species at the following ratios: 100% native soil (30,000 mg/kg TPH), 1:1 native soil to artificial soil (15,000 mg/kg TPH), and 100% uncontaminated, artificial soil.

A second set of toxicity tests was conducted following high mortality in the first round of native soil experiments to determine the reason for the toxicity (detailed below in Results); only *E. fetida* and *A. caliginosa* were used in this toxicity test (Table 2). Following the first round of native soil experiments, the TPH concentrations had declined. In addition to earthworms incubated in the laboratory, *E. fetida* from UCB were also obtained and incubated for two weeks to acclimate to laboratory conditions. Three experiments were conducted using the native soil (now containing approximately 19,000 mg/kg TPH): 1. a series of soil dilutions (2:1, 1:1, and 1:2 native soil to artificial soil by dry weight) were tested to determine if the native soil was toxic due to chemical concentrations. 2. The native soils were mixed with 5% and 10% coconut coir by

dry weight to determine if low organic matter content contributed to mortality. 3. The UCB earthworms were tested separately from those that had been present in the laboratory for over a month to determine if insufficient laboratory acclimation was the reason for mortality. A 100% artificial soil control was also performed for both species and the UCB earthworms.

Table 2: Treatment Design of Second Toxicity Tests

Toxicity Treatment	UW *E. fetida*	*UCB E. fetida*	*A. caliginosa*	Purpose
100% Artificial Soil	X	-	-	Determine background mortality in uncontaminated soil under test conditions
100% Artificial Soil	-	X	-	Determine background mortality in uncontaminated soil under test conditions
100% Artificial Soil	-	-	X	Determine background mortality in uncontaminated soil under test conditions
100% Native Soil	X	-	-	Determine earthworm tolerance to petroleum toxicity in native soil
100% Native Soil	-	X	-	Determine earthworm tolerance to petroleum toxicity in native soil
100% Native Soil	-	-	X	Determine earthworm tolerance to petroleum toxicity in native soil
2:1 (Native soil: artificial soil)	X	-	-	Determine earthworm tolerance to petroleum toxicity in native soil at varying concentrations
2:1 (Native soil: artificial soil)	-	X	-	Determine earthworm tolerance to petroleum toxicity in native soil at varying concentrations
2:1 (Native soil: artificial soil)	-	-	X	Determine earthworm tolerance to petroleum toxicity in native soil at varying concentrations
1:1 (Native soil: artificial soil)	X	-	-	Determine earthworm tolerance to petroleum toxicity in native soil at varying concentrations
1:1 (Native soil: artificial soil)	-	X	-	Determine earthworm tolerance to petroleum toxicity in native soil at varying concentrations
1:1 (Native soil: artificial soil)	-	-	X	Determine earthworm tolerance to petroleum toxicity in native soil at varying concentrations
1:2 (Native soil: artificial soil)	X	-	-	Determine earthworm tolerance to petroleum toxicity in native soil at varying concentrations
1:2 (Native soil: artificial soil)	-	X	-	Determine earthworm tolerance to petroleum toxicity in native soil at varying concentrations
1:2 (Native soil: artificial soil)	-	-	X	Determine earthworm tolerance to petroleum toxicity in native soil at varying concentrations

Toxicity Treatment	UW *E. fetida*	*UCB E. fetida*	*A. caliginosa*	Purpose
5% Organic Matter	X	-	-	Determine if lack of organic matter in native soil was cause of mortality
5% Organic Matter	-	X	-	Determine if lack of organic matter in native soil was cause of mortality
5% Organic Matter	-	-	X	Determine if lack of organic matter in native soil was cause of mortality
10% Organic Matter	X	-	-	Determine if lack of organic matter in native soil was cause of mortality
10% Organic Matter	-	X	-	Determine if lack of organic matter in native soil was cause of mortality
10% Organic Matter	-	-	X	Determine if lack of organic matter in native soil was cause of mortality

Degradation Experiments: Standard Methods

All degradation experiments were conducted in plastic bins with vented lids or one liter jars with paper towels held in place with screw tops. All experimental treatments were performed in duplicate except for the volatilization controls in the residual and inoculum degradation experiments which were used without replication. The experimental bins and jars were stored in a darkened portion of the laboratory at 20°C for the duration of the experiment. At the beginning of each experiment using 6 L plastic bins each food and earthworm bin received 100 g of 3:1 carrot:coffee feed mix. In experiments using the 12 L plastic bins each food and earthworm bin initially received 150 g of a 1:1 fruit/vegetable pulp:coffee feed mix. In experiments using 1 L glass jars initially received 14 g of 3:1 carrot:coffee feed mix. The following earthworm masses were added to the earthworm treatment bins in each experiment: 6 L bins received 30 g, 12 L bins received 120 g, and 1 L jars received 4.3 g.

Distilled water and a 3:1 carrot:coffee feed mix were added weekly to the food controls and earthworm treatment to maintain consistent soil moisture and to provide sufficient food for earthworm survival and reproduction. Distilled water was added to the volatilization controls on

a weekly basis. Moisture loss was determined by mass balance and bi-weekly moisture content measurements (see below in Methods). The experiments with 6 L bins received 50 g, the 12 L bins received 200 g and the 1 L jars received 8 g of feed weekly, respectively.

Contaminated, Artificial Soil with and without Inoculum Degradation Experiment

Hypothesis & Experimental Design

This experiment was conducted to determine the importance of an existing microbial population for TPH degradation. The artificial soil used in these experiments was created in a controlled environment from materials with a paucity of microbes, meaning there may be a smaller population and less microbial diversity from which to build a hydrocarbon degrading community. Soils from crude oil contaminated sites are likely to contain a microbial community adapted to hydrocarbon degradation (Westlake et al. 1974; Atlas 1981; Rahman et al. 2002). The experiment compared crude oil degradation in treatments with contaminated artificial soils and in treatments with contaminated artificial soils that also contained 10% (w/w) of soil from a crude oil contaminated site (native soil), which acted as a microbial inoculum. Table 3 details the experimental design of the inoculum degradation experiment. The experiment began in December 2013 and TPH concentrations were measured over time from all experimental bins for 342 days.

Table 3: Treatment Design of Inoculum Degradation Experiment

Bin Type[1]	~3% SJV	Feed	*E. fetida*	Native Soil Inoculum	Purpose
Volatilization control	X	-	-	-	Demonstrate volatilization rate of TPH in artificial soil
Feed alone treatment	X	X	-	-	Demonstrate contribution of organic amendment to TPH degradation
Earthworm treatment	X	X	X	-	Determine contribution of earthworms to TPH degradation
Volatilization control	X	-	-	X	Demonstrate volatilization rate of TPH during the experiment
Food alone treatment	X	X	-	X	Demonstrate contribution of organic amendment and native soil inoculum to TPH degradation
Earthworm treatment	X	X	X	X	Determines contribution of worms and native soil inoculum to TPH degradation

1. Duplicate bins were prepared for all treatments except volatilization controls.

The experiment was conducted in 6 L plastic bins. 3.6 kg of the stock contaminated and weathered artificial soil (15% crude oil w/w) described above was hand mixed with 20.4 kg of clean, artificial soil. The resulting artificial soil contained approximately 3% TPH by weight. Each of the treatments without inoculum received 2.5 kg dry weight of this contaminated, artificial soil. Each of the inoculum treatments received 2.5 kg dry weight of soil that consisted of 2.25 kg dry weight of the contaminated, artificial soil hand mixed with 0.25 kg dry weight (10% of total soil weight) of the native soil (microbial inoculum). Distilled water was added to each treatment bin such that the initial moisture content of each bin was between 36% and 37%. Due

to insufficient earthworm biomass at the initiation of the experiment, no earthworm mortality controls were included in this experiment.

Figure 2: Bins used for the inoculum degradation experiments. A, Earthworms are visible near the bottom (yellow arrow), and feed layer at top (double arrow). B, close up showing the dark feed layer at the top of the soil.

Contaminated, Native Soil Degradation Experiment

Hypothesis & Experimental Design

The contaminated native soils were used in a degradation experiment to test the effectiveness of earthworm mediated hydrocarbon degradation on real-world soils. Because *E. fetida* is an epigeic, compost earthworm, it was hypothesized that the endogeic, horizontally burrowing *A. caliginosa* would stimulate more hydrocarbon degradation due to its tendency to make greater use of the deeper soil layers. Table 4 details the experimental design of the native soil degradation experiment. Due to earthworm mortality, several phases of the experiment were required, see Figure 4.

Table 4: Treatment Design of Native Soil Degradation Experiment

Bin Type	Feed	*E. fetida*	*A. caliginosa*	Purpose
No Oil Control	+	+	-	Indicate *E. fetida* biomass (survival) in uncontaminated artificial soil
No Oil Control	+	-	+	Indicate *A. caliginosa* biomass (survival) in uncontaminated artificial soil
Volatilization control	-	-	-	Demonstrate volatilization rate of TPH in the native soil
Food treatment	X	-	-	Demonstrate contribution of organic amendment to TPH degradation in the native soil
Worm treatment	X	X	-	Determine contribution of *E. fetida* to TPH degradation in the native soil
Worm treatment	X	-	X	Determine contribution of *A. caliginosa* to TPH degradation in the native soil

Test 1a

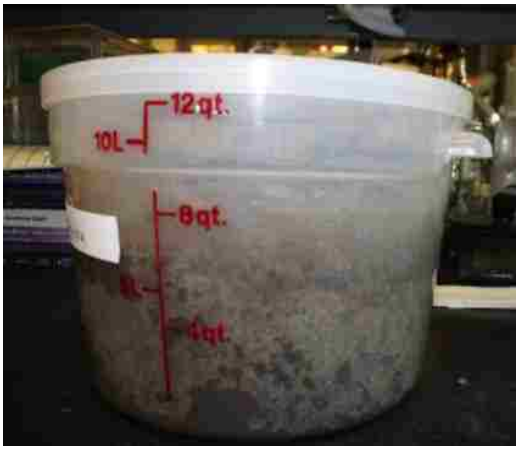


Figure 3: Twelve liter Bins initially used in native soil experiment.

This experiment was first conducted in 12 L plastic, food-grade bins. Each treatment bin received 10.0 kg dry weight of the native soil. Separate mortality controls using artificial soil controls were not conducted using 12 L bins. Due to the lower water holding capacity of the native soil, the initial moisture content of each bin was between 10% and 13%. Earthworms in this experiment experienced mortality exceeding 90% of biomass within 21 days of beginning the experiment.

Test 1b

The experimental bins were left undisturbed for approximately 24 days before the experiment was re-started. 2.5 kg dry weight of soil from each experimental bin was transferred into a 6 L container to continue the experiment and distilled water was added as need to maintain soil moisture at approximately 13%. Additionally, earthworm mortality control bins using 2.5 kg of uncontaminated, artificial soils mixed with distilled water were added to the experimental design. No uncontaminated, native soils were available for use as mortality controls.

After 17 days in the native soil *A. caliginosa* mortality was between 70-90% of biomass and after 19 days in the native soil *E. fetida* mortality was between 77-90% of biomass.

Bi-weekly soil sampling continued throughout the experiment (see below for soil sampling protocol), with the final soil sample collected on Day 70. The 70 experimental days include Test 1, Test 1b, and the time between Tests 1a and 1b. The *A. caliginosa* treatment bins contained earthworms for 47 days of the 70 day experiment and the *E. fetida* treatment bins contained earthworms for 40 days of the 70 day experiment.

Test 2

96 days after the completion of the initial experiment, a second native soil experiment was conducted with the native soils. The soils from Test 1b were placed in 6 L bins and earthworms and food were added as described above. All the controls and treatments in Test 2 used the same native soils as Tests 1 and 2, for example, the native soil that was used in the *E. fetida* treatment in Test 1 was also used in the *E. fetida* treatment in Tests 1b and 2. Mortality controls using uncontaminated, artificial soil were included in Test 2.

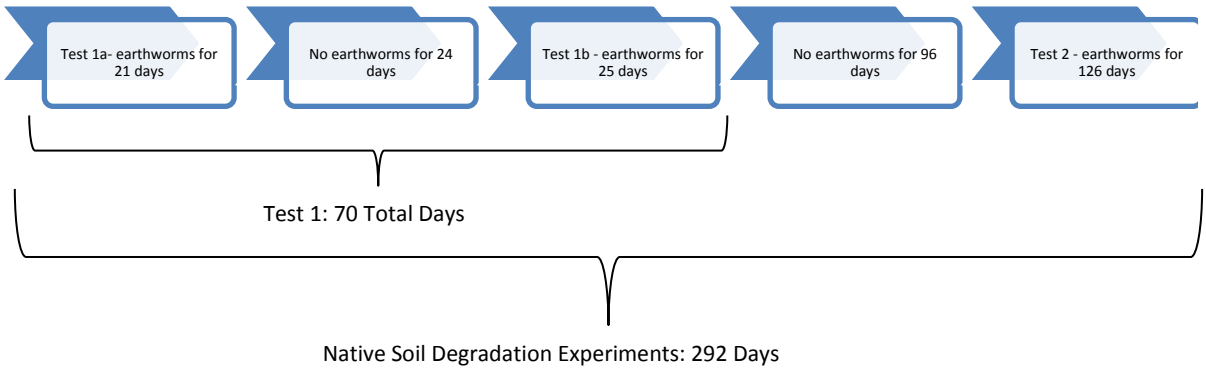


Figure 4: Native Soil Degradation Experiments Timeline.

Degradation Trial 1 Residual Soils

Degradation Trial 1 produced contaminated, artificial soil that had undergone vermiremediation for approximately 396 days reached a degradation plateau and still contained residual petroleum hydrocarbons. These remaining hydrocarbons were resistant to further degradation under current conditions. These residual soils were stored in separate treatment bins at 20°C from July to September 2013, at that time the soils remaining from the vermiremediation treatment bins were mixed together and homogenized.

Hypotheses

If lack of bioavailability due to contaminant sorption to soil particles was the reason for the degradation plateau then degradation would resume if the hydrocarbons were desorbed from the soil. To test the sorption and bioavailability hypothesis the residual soils were extracted with hexane to remove the hydrocarbons. The hexane extract was applied to previously uncontaminated, artificial soils to allow them to be bioavailable again.

If an unknown biodegradation byproduct had accumulated to toxic levels during Degradation Trial 1 and was inhibiting further biodegradation then degradation would resume if the inhibitor

was diluted. The residual soils were diluted with uncontaminated, artificial soils to dilute any byproduct that was present.

Crude Oil Extraction from Residual Soils

Half of the residual soil was extracted with hexane. Two-hundred grams of the residual soil was added to 400 ml of hexane, hand shaken for two minutes and then bath sonicated for 30 minutes. The soil solution was allowed to settle for 30 minutes and then the hexane extract was decanted through filter paper to another bottle. This procedure was repeated until all of the residual soils had been hexane extracted twice. The hexane extract was then allowed to volatilize in a fume hood until approximately 850 ml of the hexane extract remained and then was mixed with 1.84 kg of uncontaminated, artificial soil. GC-FID analysis showed that the TPH concentration of this soil contaminated with the residual crude oil (extract soils) was 21,258 ± 628 mg/kg.

Dilution of Residual Soils

The remaining half of the residual soil was hand-mixed with uncontaminated, artificial soils at dry weight ratios of 1:1 and 2:1 (diluted soils).

Residual Soils Degradation Experiments

Table 5 details the experimental design of the extract soils degradation experiment and Table 6 details the design of the diluted soils degradation experiment.

Table 5: Treatment Design of Extract Soils Degradation Experiment

Bin Type	Feed	Worms	Extracted SJV	Purpose
No Oil Control	+	+	-	Indicate earthworm survival in uncontaminated artificial soil
Volatilization Control	-	-	+	Demonstrate volatilization rate of extracted SJV crude in artificial soil
Feed alone Treatment	+	-	+	Demonstrate contribution of organic amendment to extracted SJV crude degradation
Worm Treatment	+	+	+	Determine contribution of worms to degradation of extracted SJV crude

Table 6: Treatment Design of Diluted Soils Degradation Experiment

Bin Type	Feed	Worms	Extracted SJV	Purpose
No Oil Control	+	+	-	Indicate earthworm biomass (survival) in uncontaminated artificial soil
Volatilization Control	-	-	+	Demonstrate volatilization rate of diluted residual SJV crude in artificial soil
Feed alone Treatment	+	-	+	Demonstrate contribution of organic amendment to diluted residual SJV crude degradation
Worm Treatment	+	+	+	Determine contribution of worms to degradation of diluted residual SJV crude

The experiments were conducted in 1 L glass jars with vented lids; insufficient residual soil was available for larger treatment volumes. The extract soils (1.84 kg) were mixed with 0.47 kg of uncontaminated artificial soil and then mixed with distilled water. Each glass jar received 0.36 kg dry weight of this soil mixture. The diluted soils were mixed at a 1:1 or 2:1 ratio of contaminated to non-contaminated soil as required for the experiment. The mortality control jars received 0.36 kg dry weight of uncontaminated, artificial soil mixed with distilled water. The soil moisture in all experimental treatments and controls was between 32%-34%. All treatments were performed in duplicate except the volatilization controls, which used a single jar.

Figure 5: Jars used for the residual degradation experiments (7 inches tall).

Protocols for Soil Sampling and Determination of Moisture Content and Volatile Solids

Soil samples for TPH analysis were collected from each treatment bin or jar in all the experiments at the beginning and end of each experiment and on an approximately bi-weekly schedule throughout the experiment. Four 5.00 g (±0.05 g) samples were collected into glass vials from four locations in the inoculum and native soil degradation experiment bins. The top 3-5 cm of soil and soil touching the sides of the bin were not sampled to avoid feed and edge effects, respectively. Three of the samples were analyzed by gas chromatography with a flame ionization detector (GC-FID) and the fourth sample was archived. Similar sampling methods were used in the residual soil experiments, but only three 5.00 g (±0.05 g) samples were collected. Two of the samples were analyzed and the third was archived.

The moisture content of the soil in each bin or jar was measured at the same time as soil samples were collected; volatile solids were measured bi-monthly during the experiments. Soil moisture was determined using an approximately 10g soil sample collected from the center of the bin (5g in the 1 L jars). The sample was weighed and then dried in a 105°C oven for at least 24 hours and then weighed again. The weight loss from the oven drying was the soil moisture. To determine the volatile solids content of the soils, the oven dried soils were ignited in a 550°C muffle furnace for one hour and the weight loss was the volatile solids content.

Crude Oil Extraction and Gas Chromatography Flame Ionization Detector Protocols for TPH Analysis

To determine TPH concentrations in the experiment soils, approximately 5 g of moisture absorbent Na_2SO_4 was added to the glass vial containing 5.00 g (±0.05 g) of soil (wet weight). The hydrocarbons were extracted from the soil samples by adding 10ml of hexane to the glass vial. 16µl of a 40 mg/L solution of the small aromatic compound decafluorobiphenyl (DFBP) was added to each vial as a surrogate to measure extraction efficiency and dilution accuracy (surrogate recovery between 85%-115% was considered acceptable). The vials were capped, hand shaken for 2 minutes and bath sonicated for 10 minutes. The hexane extract was then transferred to a 1.8 ml auto-sampler (AS) vial. Dilution of the extract in the AS vial with hexane was performed as needed to maintain sample concentrations within the instrument calibration range (500 to 5,000 mg/kg TPH, see below). Initial samples (25,000 - 30,000 mg/kg TPH) were diluted 5-fold, dilutions later in the experiments were based on the result of the previous analysis of the sample.

The extract was analyzed with a Perkin Elmer Autosystem GC-FID using a 15-meter RTX-1 column with a 0.32 mm inner diameter and a 0.25 µm film thickness. The injector and detector ports were maintained at 320°C during analysis and the oven initial temperature was 40°C. The

initial oven temperature was held for 2 minutes and then increased by 20°C per minute to 330°C and held for 8 minutes. Sample injections were 1 µl using an autosampler syringe. The column carrier gas was helium, with an initial pressure of 5 psi (pressure decreased above 40°C). A TPH calibration curve was constructed by analyzing solutions of known concentrations of SJV crude (6 samples between 500 and 5,000 mg/L TPH) with each sample set. A plot of the concentration vs. area under the curve for the calibration standards yielded an r^2 value of 0.995 or greater, if the r^2 value was less than 0.995 the sample set results were discarded as unreliable. DFBP surrogate recoveries were determined using the same method with a calibration curve constructed with 4 samples between 5 and 50 mg/L.

A hexane blank, blank spike (2,000 mg/L TPH spike), blank spike duplicate, artificial soil matrix control blank, matrix spike (artificial soil + 2,000 mg/L TPH spike) and matrix spike duplicate were also analyzed with each sample set to determine the hexane background and determine that accurate TPH was recovered during extraction. Continuing calibration (CC) standards (2,000 and 3,000 mg/L TPH) were analyzed approximately every 12 samples, TPH recoveries of 85%-115% were considered sufficient. If samples were not bracketed by passing CC standards the samples were re-analyzed. TPH concentrations were calculated from chromatograms by subtracting the hexane background and DFBP surrogate peak area from the area under the sample curve between the beginning of the surrogate peak and 22 minutes. All TPH soil concentrations were corrected for dilution and for soil moisture by dividing by the percent dry weight. Hydrocarbon size standards of C10 to C40 were also run periodically with samples to establish the size range of hydrocarbons in the soil.

Statistics

MS Excel 2010 was used for statistical analysis of the TPH degradation results. One-tailed Student's T-test was used for comparison of TPH concentrations between treatment and

controls in each experiment. A two-tailed Student's T-test was used for comparison of TPH concentrations between the earthworm treatments in the native soil experiments. Differences were considered significant at p-values ≤ 0.05. Any deviations from this protocol are described in the relevant experimental section above.

Bacterial Soil DNA Isolation Methods

Bacterial soil DNA isolation was performed using the Powermax® Soil DNA Isolation Kit. Approximately 5 grams of soil composited from 3 locations from one bin in each of the treatments and controls in the inoculum and native soil degradation experiments. Due to low DNA recovery, two 10 gram samples of soil were collected for isolation from the volatilization bins in both experiments and from uncontaminated soil bins without earthworms in the inoculum experiment as a control for the artificial soil. The method associated with this kit was followed, except rather than using a vortex adapter, an MP-Fast Prep shaker was used for 30 seconds, followed by a hand shaker for 5 minutes. In the case of low DNA recovery, Millipore centrifugal filter units were used to concentrate DNA. The DNA solution was then centrifuged for 10 minutes at 3220 rpm to reduce the solution volume to approximately 300 µl.

DNA quantification was performed using a Thermo Scientific NanoDrop™ Spectrophotometer. One µl of DNA solution was placed on the NanoDrop™ and analyzed. Multiple readings were taken when the DNA concentration was less than 30 nanograms per µl.

RESULTS

Analysis of TPH Degradation Trial 1 Data

In TPH Degradation Trial 1 TPH concentrations were measured at regular intervals in three experimental treatments for 396 days. The volatilization control TPH concentration began at 33,928 ±259 mg/kg and fluctuated between 23,310 ±1,608 mg/kg (Day 92) and 44,322 ±5,164 mg/kg (Day 85). The TPH concentration in the volatilization control did not decline appreciably over the course of the experiment. The fluctuations in TPH concentration appeared to be due to instrument variability (seen as concentration increases and decreases in all samples on a given day) and heterogeneity in the artificial, contaminated experimental soils. Due to the large variability in the TPH concentrations between time points, the concentrations in each of the experimental treatments (with food and with food and *E. fetida*) are presented as percentages of the TPH present in the volatilization control.

Figure 6 shows the percent degradation over time in the experimental treatments. The volatilization control did not degrade appreciably and therefore was held at 100% and compared to the feed and worm treatments, both of which exhibited substantial TPH degradation. The rate of TPH degradation was significantly greater in the earthworm treatment compared to the food alone and earthworm treatment beginning on Day 71 ($p<0.01$, student's t-test). Day 287 was the final day that the earthworm treatment contained significantly less TPH ($p<0.05$) than the feed alone treatment. The slope of the TPH concentration degradation in the earthworm treatment was consistently greater than the feed only treatment until day 287. By this time the TPH concentration in the earthworm treatment had plateaued at approximately 16,000 mg/kg (50% of the initial concentration). In the earthworm treatments, ~55 % of the TPH was removed in 244 days, whereas with feed alone about 25% TPH was removed (Figure 6, dotted line). The

feed only treatment continued to exhibit TPH degradation from day 244 until day 396, whereas the earthworm treatment TPH concentration did not continue to decline.

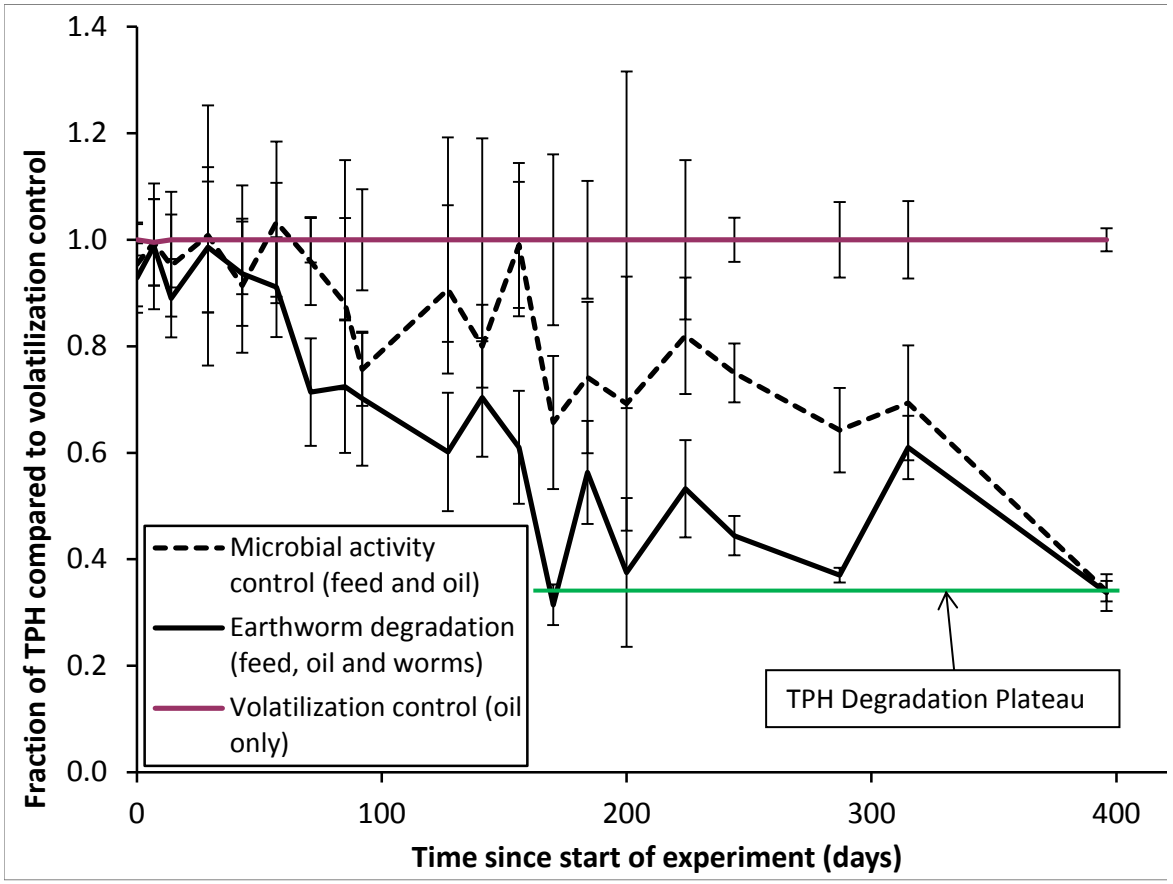


Figure 6: Percentage of TPH remaining in experimental treatments compared to the volatilization control over time (Initial TPH concentrations: Volatilization control = 33,517 mg/kg, microbial activity control = 31,323 mg/kg, earthworm degradation = 31,826 mg/kg).

Earthworm Hatch Test

Earthworm eggs and hatchlings were tolerant of the SJV crude oil at 1.7%. In the control treatment (no crude oil), 87% of the egg capsules hatched after 15-20 days while 90% of the egg capsules exposed to 1.7% (w/w) SJV crude oil hatched after 15-20 days (Figure 7). Only a single egg capsule (7%) exposed to 3% (w/w) SJV crude oil hatched during the 33 day hatch test.

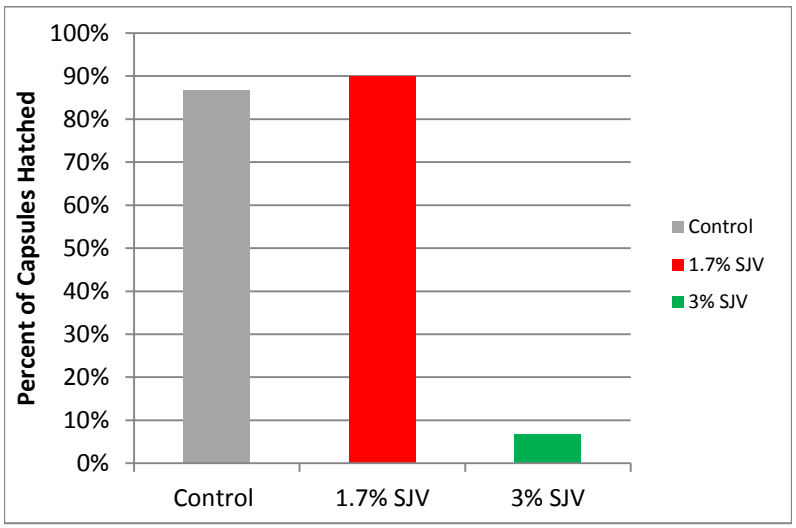


Figure 7: Results of the E. fetida egg capsule hatch toxicity test.

Characterization of Hydrocarbons in Residual Soils

Residual soils from the previous year's crude oil degradation experiment were analyzed for EPH by Eurofins – Lancaster Laboratories and grouped into three sizes or carbon number ranges for aliphatics (>C10 – C12, >C12 – C16, >C16 – C35) and four sizes or carbon number ranges for aromatics (>C10 – C12, >C12 – C16, >C16 – C21, >C21 – C35). The volatilization control contained aliphatics ranged from >C12 – C35 and aromatics >C12 – C35, no aliphatics and aromatics <C12 were detected. The two treatments (earthworms and feed only) contained similar concentrations of aromatic and aliphatic hydrocarbons to each other and substantially lower concentrations than those measured in the volatilization control (Figure 8A). The treatments showed complete degradation of the aromatics from >C12 – C16, and large declines in concentrations of aliphatics >C12-C35 and aromatics >C16-C35 (Figure 8A), indicating strong degradation of these fractions. The >C16-C35 aromatic range concentration declined by 35% and the >C16-C35 aliphatic range declined by almost 80%.

While degradation occurred in both aliphatics and aromatics from C12-C35 in feed and earthworms treatments, there was an increase of the relative abundance of the aromatics >C21-C35 in the earthworm and food only treatments relative to the Crude Oil and volatilization control (Figure 8B). This shift in is due to preferential degradation of the smaller hydrocarbons, instead of the heavier and more resistant >C21-C35 aromatics. Following the experimental treatments, the soils contained approximately 35% heavy aromatics and 55% heavier aliphatics (>C16-C35) as compared to approximately 23% heavy aromatics and 58% heavier aliphatics in the volatilization control.

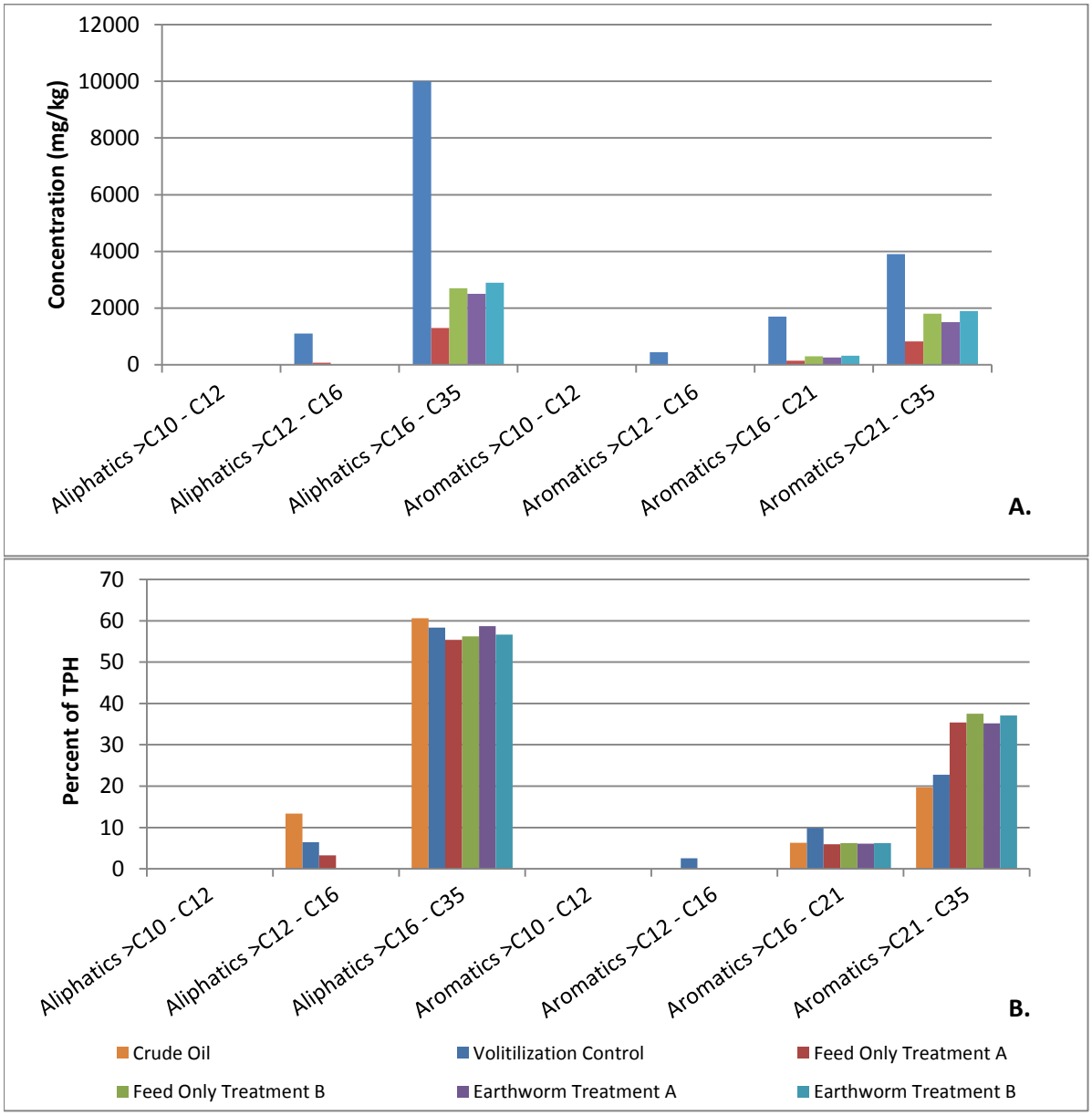


Figure 8: Hydrocarbon analysis of SJV crude oil and soils from degradation experiments. A. concentrations of hydrocarbons in contaminated soils (SJV crude oil not included). B. proportions of aliphatic and aromatic hydrocarbons present in SJV crude oil and experimental soils.

Toxicity Tests

Toxicity tests were performed using the native soils with three candidate species of earthworms, *L. terrestris, A. caliginosa,* and *E. fetida*. The results of the toxicity tests indicated that *E. fetida* and *A. caliginosa* were acceptable candidates for TPH degradation tests with the contaminated, native soil (Figure 9). *L. terrestris* did not survive well in the lab under any conditions, with mortality greater than 50% in non-contaminated soils and 100% mortality in the native soil tests.

A. caliginosa showed high tolerance to laboratory conditions, with 100% survival in the uncontaminated, artificial soil, and tolerance to the native soils (75% survival in the 50% native soil, and 35% survival in 100% native soil after two weeks). *E. fetida* showed the highest tolerance to the native soils, with 80% and 95% survival in 100% and 50% native soil after two weeks, respectively - comparable to the 90% survival observed in the uncontaminated control. With these results it was determined that both *A. caliginosa* and *E. fetida* would be used in the native soil degradation experiment, while *L. terrestris* would not, due to the high observed mortality.

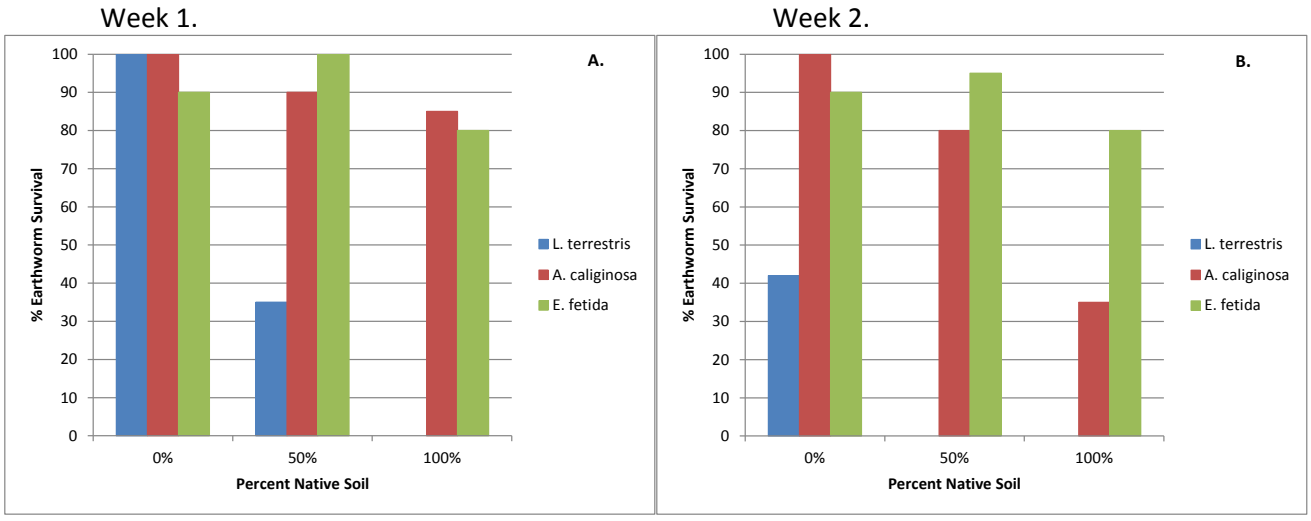


Figure 9: Percent survival rates of L. terrestris , A. caliginosa, and E. fetida in artificial, non-contaminated soil (0%), 1:1 mixture of natural and artificial soil (50%), and all natural soil (100%) after one (A) and two weeks (B).

After three weeks in the native soil experiment, both *E. fetida* and *A. caliginosa* experienced over 90% mortality. As noted above in Materials & Methods, the native soil experiment was reduced in scale to the 6 L bins from the 12 L bins and *A. caliginosa* and *E. fetida* were added. Mortality of approximately 80% was observed for both species, including in the clean artificial soil control bins where 14% and 60% mortality was observed for *A. caliginosa* and *E. fetida*, respectively.

To investigate the cause of the unexpected mortality, additional toxicity tests with the native soils were conducted using *A. caliginosa* and *E. fetida* earthworms that had been reared in the laboratory for at least one month. *E. fetida* from UCB were also tested after acclimating to laboratory conditions for two weeks. These toxicity tests were performed using the same methods described for the first toxicity set of toxicity tests. Five different dilutions of native soil, along with 100% and 0% controls were tested (see Table 2).

The results show that extended acclimation to laboratory conditions is an important determinant of earthworm mortality in the native soil (Figure 10). *E. fetida* that had been present in the laboratory for one month experienced very little mortality, while *E. fetida* that had only been in the laboratory for two weeks experienced much higher mortality, despite their larger size. *A. caliginosa* experienced higher mortality than the well acclimated *E. fetida* in almost all treatments, but generally had better survival than the *E. fetida* that had been in the laboratory for a shorter period of time.

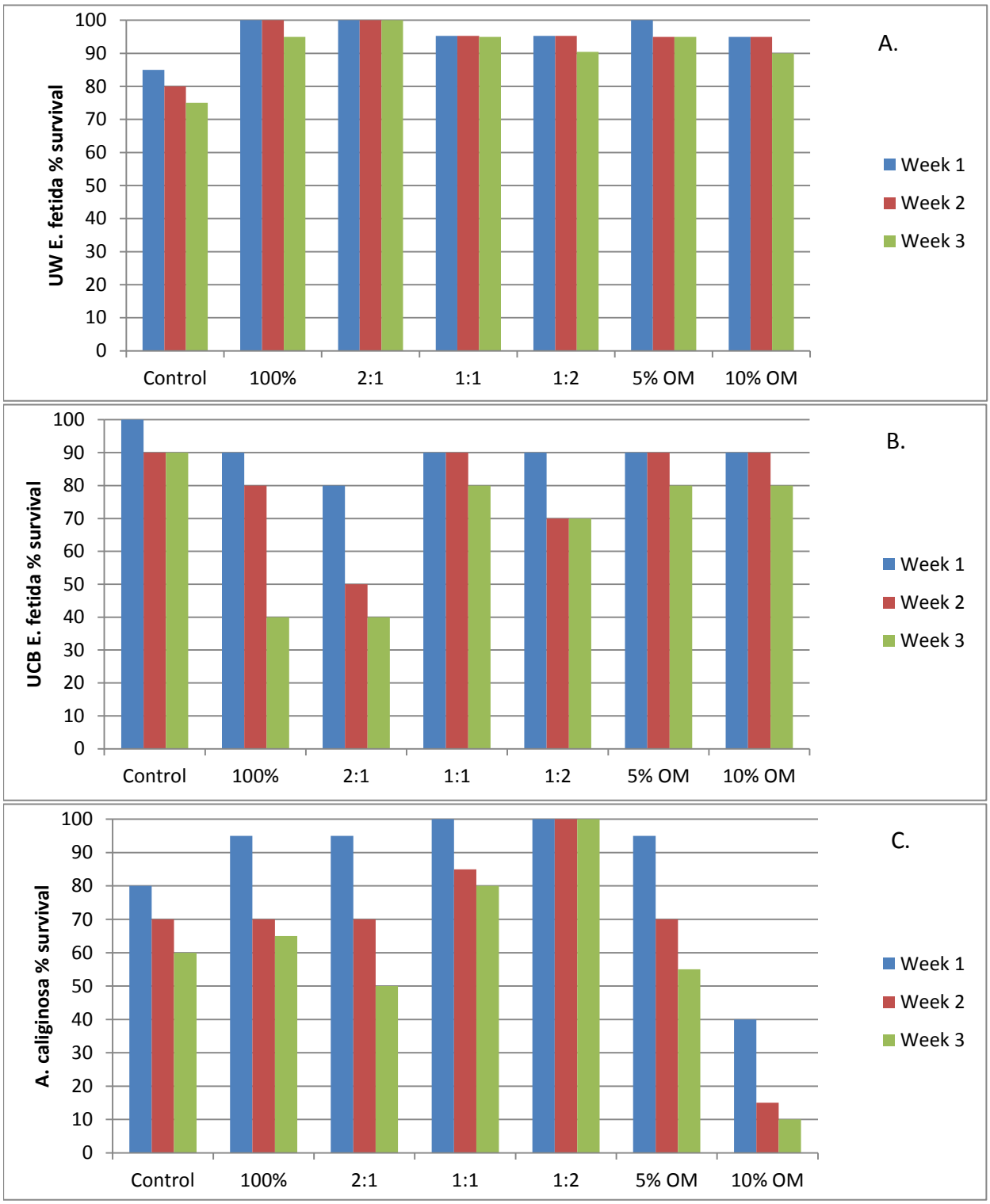


Figure 10: Percent survival rates of E. fetida reared at the UW (A.), E. fetida reared at UCB (B.), and A. caliginosa (C.) in artificial, non-contaminated soil (Control), all natural soil (100%), a 2:1 mixture of natural and artificial soil (2:1), a 1:1 mixture (1:1), a 1:2 mixture (1:2), natural soil mixed with 5% organic matter by weight (5% OM), and mixed with 10% organic matter by weight (10% OM) after one, two, and three weeks.

Inoculum Degradation Results

Microbes in the earthworm gut are derived from the surrounding soils (Thakuria et al. 2010). The laboratory made, artificial soil does not contain an existing microbial community as a native soil from the field. However, by providing a microbial inoculum (essentially "seeding" the artificial soil) in the form of the native soil from a crude oil remediation it was hypothesized that access to oil-adapted microbes would improve degradation in the presence of earthworms as compared to with artificial soil alone.

The results of the TPH analyses from the inoculum degradation experiments are presented below in Figure 11. During data analysis, outlier data points (defined as greater than 35% difference from the treatment mean) were removed from the results. There was considerable variability within each of the treatments, likely due to soil heterogeneity and GC-FID instrument variability between analysis runs. However, clear trends were visible in the data for all of the treatments. The TPH concentrations in the volatilization controls remained fairly constant (Table 7). The application of the organic carrot:coffee feed resulted in a decline in TPH concentrations, albeit at a significantly slower rate than in the presence of *E. fetida.* The earthworm treatment with the inoculum had a greater rate of TPH degradation compared to the controls than the earthworm treatment without the inoculum throughout the majority of the experiment (Figure 12 & Figure 13)

The initial concentration of the food with inoculum control was significantly less than the earthworm with inoculum treatment ($p=0.0026$). Because of the significantly lower initial TPH concentration, statistical tests comparing the two treatments were based on the rate of TPH decline.

The native soil inoculum treatments (dashed lines in Figure 11 and Figure 13) all had higher degradation rates compared to the artificial soil only treatments (solid lines in Figure 11 and

Figure 12). The TPH concentrations in the volatilization controls were generally the same throughout the experiment and no significant differences in TPH degradation between the food controls were present through 342 days. The earthworm treatments with inoculum had higher TPH degradation rates than the earthworm with no inoculum treatments at 23 days ($p=0.02$) and from 98 days to 252 days ($p<0.05$) (Figure 11). The TPH concentrations in the earthworm treatments began converging after Day 252

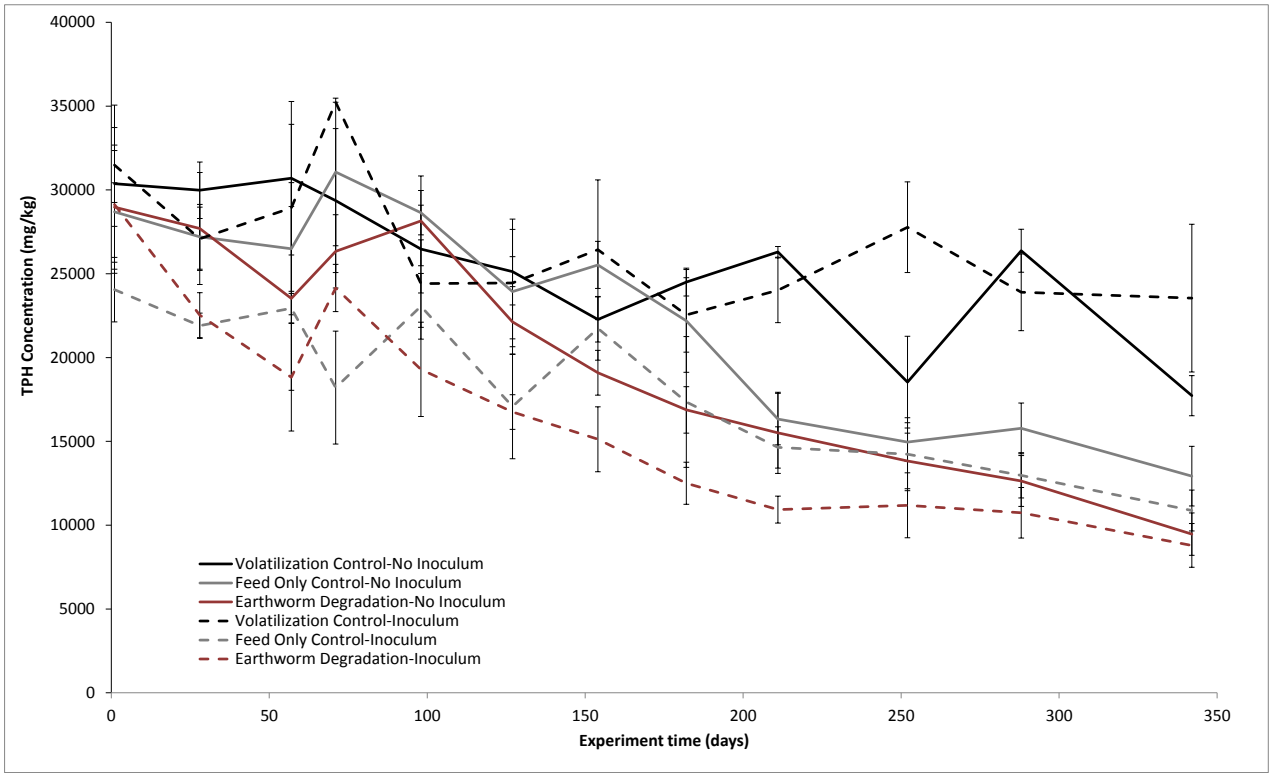


Figure 11: The influence of earthworms and inoculum on TPH concentrations in artificial soil contaminated with SJV crude oil. Beginning with approximately 30,000 mg/kg TPH (w/w) concentrations of TPH were measured over time. Solid lines show treatments without the "natural" soil inoculum and dashed lines show treatments with the inoculum.

The soil treatments without inoculum showed limited TPH degradation prior to Day 127 and the TPH losses were generally similar to the volatilization control (Figure 11). By Day 127, the earthworm treatment began to diverge from the other no inoculum treatments and contained significantly less TPH than the volatilization control ($p=0.02$). The earthworm treatment

contained significantly less TPH than the food only treatment on Day 154 (p=0.0002). The earthworm treatment with no inoculum experienced a 67% decrease in TPH concentration through 342 days (Table 7).

Table 7: Percent Change in TPH in Inoculum Experiment Treatments by Day

Experiment Day	28	57	71	98	127	154	182	211	252	288	342
Treatment											
Volatilization-No inoculum	-1%	1%	-3%	-13%	-17%	-27%	-19%	-13%	-39%	-13%	-42%
Food -No inoculum	-5%	-8%	8%	0%	-17%	-11%ŧ	-23%	-43%ŧ	-48%ŧ	-45%ŧ	-55%ŧ
Worms-No inoculum	-4%	-19%	-9%	-3%	-24%ŧ	-34%ŧ*	-42%ŧ*	-46%ŧ	-52%ŧ	-56%ŧ*	-67%ŧ*
Volatilization + inoculum	-14%	-8%	12%	-22%	-22%	-16%	-28%	-24%	-12%	-24%	-25%
Food + Inoculum	-9%	-5%	-24%	-4%	-29%	-10%	-28%	-39%	-41%ŧ	-46%ŧ	-55%ŧ
Worms + Inoculum	**-23%ŧ***	-35%ŧ	-17%	**-34%***	**-42%ŧ**	**-48%ŧ***	**-57%ŧ**	**-62%ŧ***	**-62%ŧ***	-63%ŧ*	-70%ŧ*

ŧ – TPH concentration decline is significantly greater (p<0.05) than associated volatilization control

* – earthworm treatment TPH concentration decline is significantly greater (p<0.05) than associated food only control

Bolded entries indicate that TPH concentration decline is significantly greater (p<0.05) than earthworm without inoculum treatment

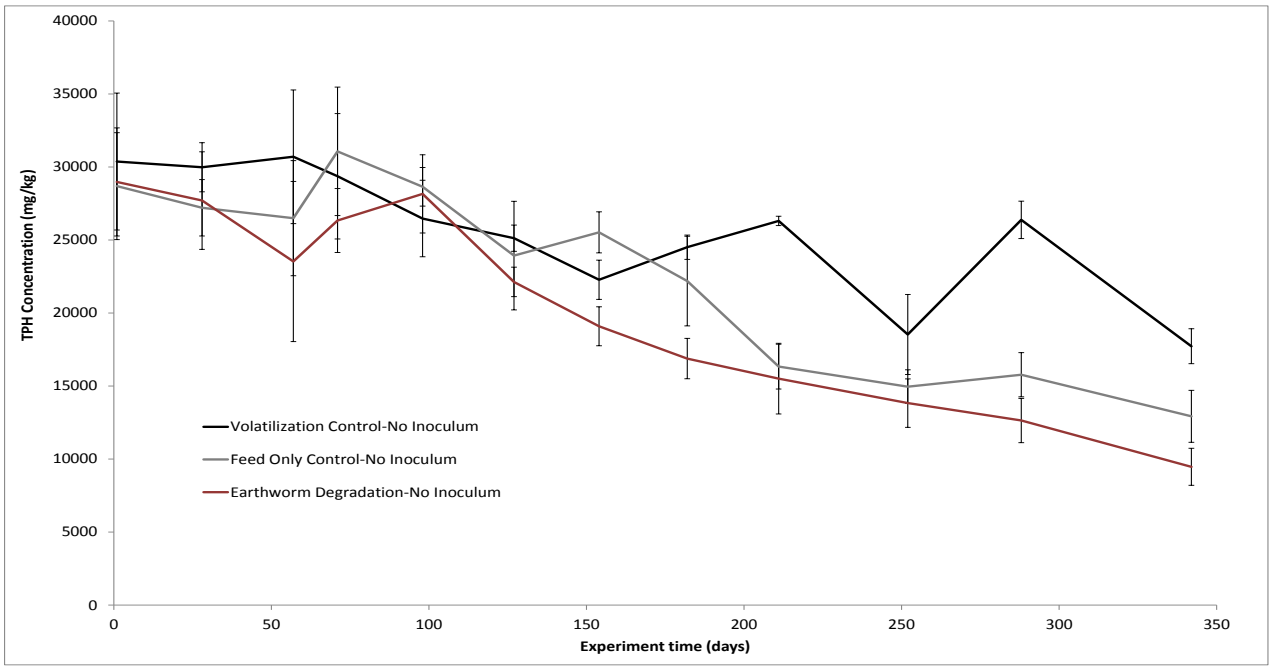


Figure 12: The influence of earthworms on TPH concentrations in artificial soil contaminated with SJV crude oil. Beginning with approximately 30,000 mg/kg TPH (w/w) concentrations of TPH were measured over time. Solid lines show treatments without the "natural" soil inoculum.

The increased TPH degradation in the presence of earthworms is clear in Figure 13 which shows only the experimental treatments with the native soil inoculum. The earthworm with inoculum treatment quickly diverged from the volatilization control and contained significantly less ($p<0.05$) TPH since Day 28. The earthworm treatment has generally contained less TPH (as a percent of the initial concentration) than the food alone treatment. This difference was been significant ($p<0.01$) at every time point from Day 154 through Day 342 (Table 7).

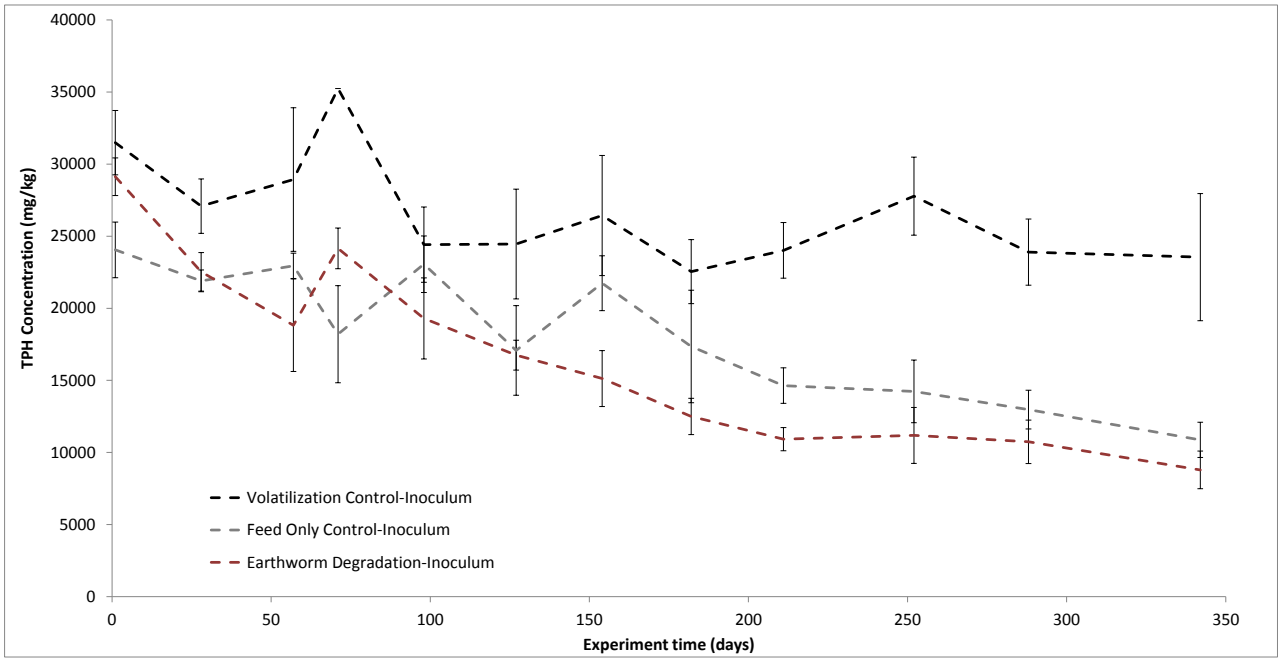


Figure 13: The influence of earthworms and inoculum on TPH concentrations in artificial soil contaminated with SJV crude oil. Beginning with approximately 30,000 mg/kg TPH (w/w) concentrations of TPH were measured over time. The dashed lines show treatments with the inoculum.

Figure 14 shows the chromatograms for SJV contaminated, artificial soils, before and after 288 days of vermitreatment. TPH declines in all portions of the crude oil curve are evident.

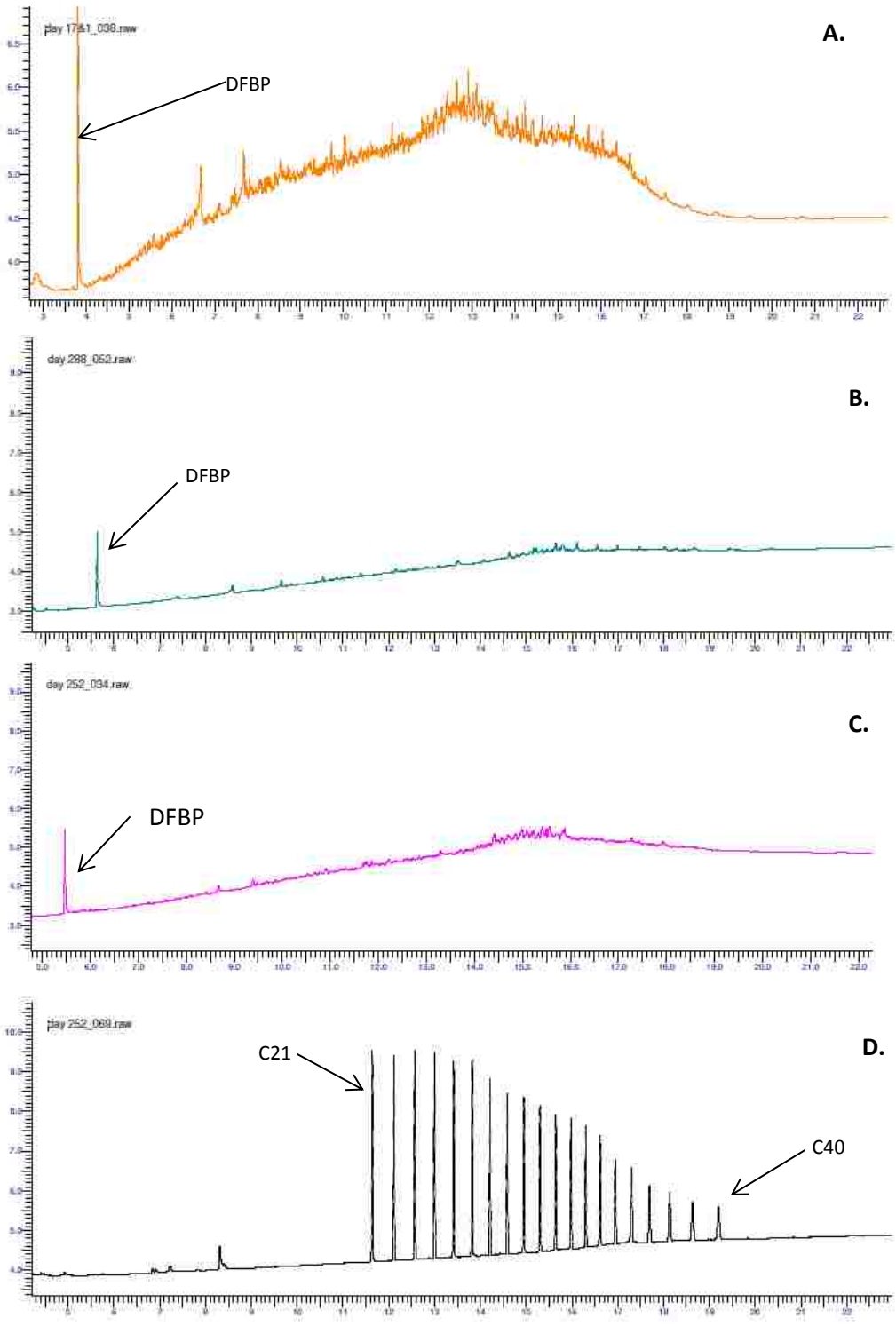


Figure 14: TPH chromatograms showing initial appearance of SJV crude oil (A), after 288 days in the presence of *E. fetida* and inoculum (B) and after 288 days in the presence of *E. fetida* without inoculum. An alkane standard from C21 to C40 is shown for comparison (D).

Native Soil Composition

The initial petroleum composition of the petroleum in the native soils is shown in Figure 15. The majority of the EPH (extractable petroleum hydrocarbons) is contained in the C16-C35 aliphatics, with a significant percent (16.4%) in C21-C35 aromatics (Figure 15A). EPH (aliphatics and aromatics from C10 – C35) accounts for 36% of the TPH in the native soil, with 64% being hydrocarbons and related compounds that are not included in the EPH analysis (Figure 15B). This 64% figure includes any hydrocarbons >C35, naphthenes and asphaltenes. C16-C35 aliphatics are approximately 22% of the TPH and C21-C35 aromatics are approximately 6% of the TPH. The initial concentration of C16-C35 aliphatics was 6,600 mg/kg and 1,800 mg/kg for C21-C35 aromatics.

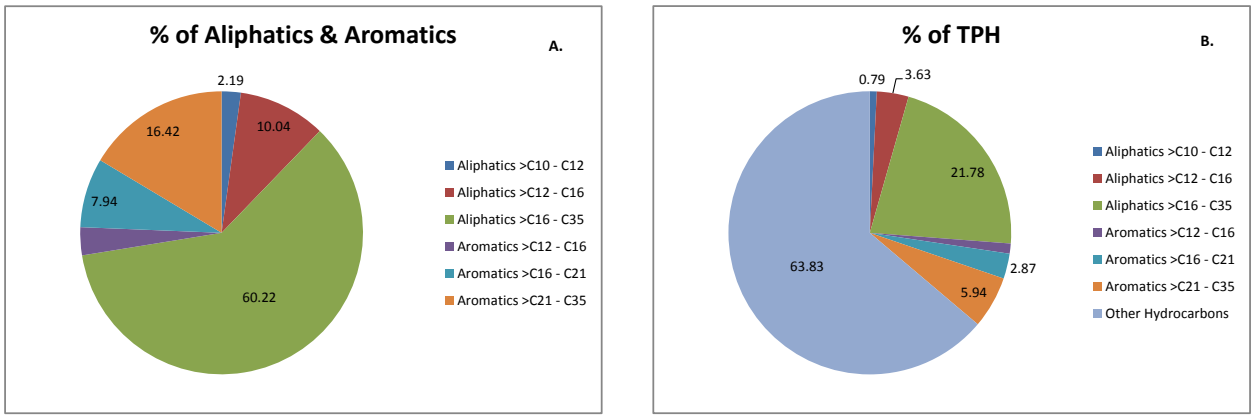


Figure 15: The initial composition of the petroleum hydrocarbons in the native soil treatments. (A) The percent of classes of aliphatics and aromatics by Method MA DE EPH (Massachusetts Department of Environmental Protection Extractable Petroleum Hydrocarbons) and (B) the percent components of overall TPH.

Native Soil Degradation Results

Test 1a and 1b

The native soil TPH degradation experiment was initially begun in 12L bins (Test 1a) and switched to 6 L bins (Test 1b) following high severe earthworm mortality (described in above in

Materials and Methods – Contaminated, Native Soil Degradation Experiment). The experiment was stopped after 70 days due to high earthworm mortality. The *A. caliginosa* treatment bins contained earthworms for 47 days and the *E. fetida* treatment bins contained earthworms for 40 days of the 70 day experiment. Despite the limited amount of time the earthworms were present in the treatments, TPH concentrations declines in the presence of *E. fetida* were significantly greater than those observed in the volatilization control ($p<0.001$) and in the feed treatment ($p=0.006$). At the conclusion of Test 1b, TPH declined in the presence of *A. caliginosa* were significantly greater than the volatilization control ($p<0.001$) and feed treatment ($p<0.001$). The greater decrease in TPH in the presence of *A. caliginosa* was also significant compared to the decline with *E. fetida* ($p=0.02$).

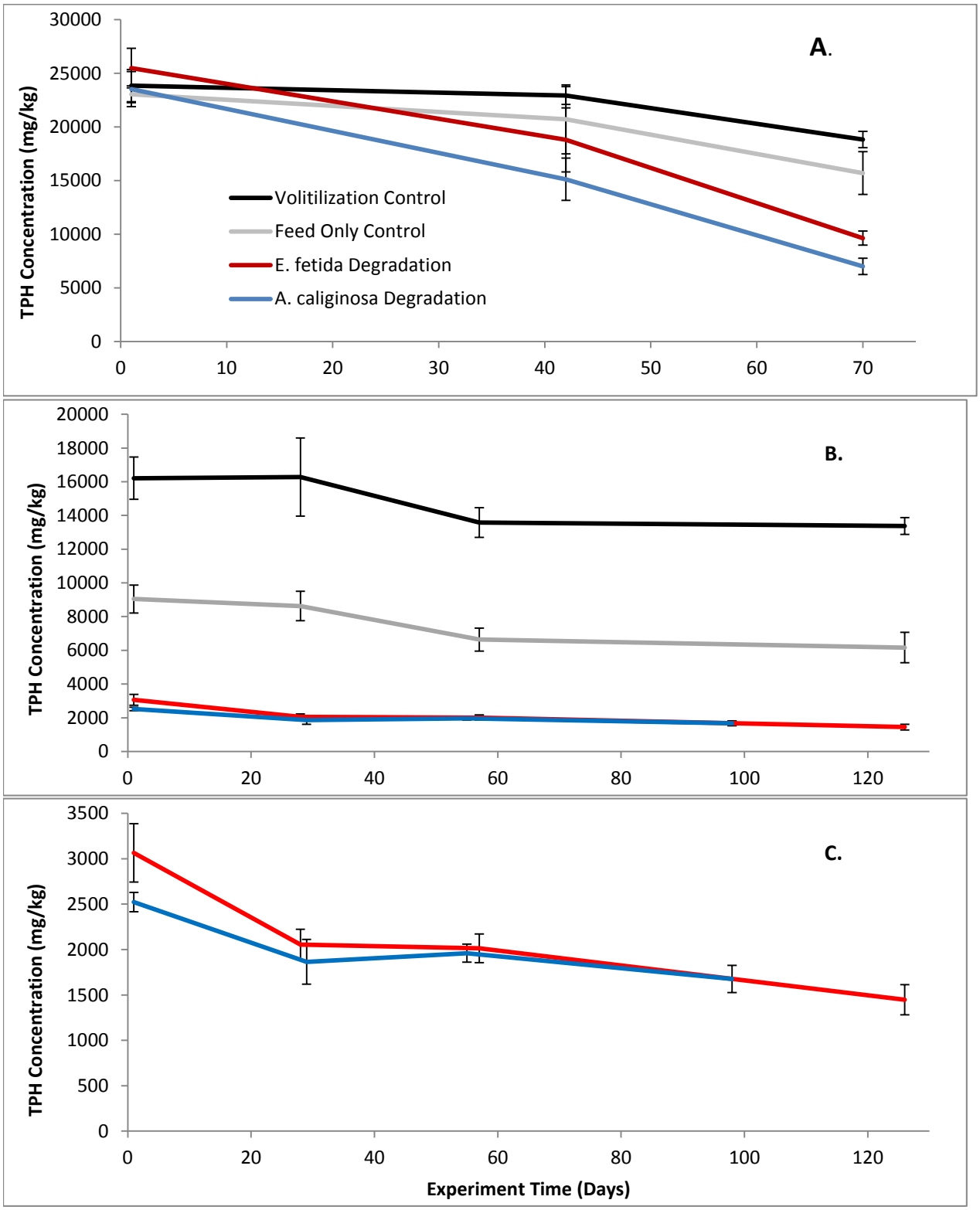


Figure 16: The influence of earthworms and feed on the native, contaminated soils. (A) Test 1a &1b results: The initial TPH concentration was similar in all treatments (25,000 mg/kg) and concentrations of TPH were measured over time. (B) Test 2 results: The same treatments, with differing initial concentrations after the first experiment. (C) Detail of the Test 2 earthworm treatments. Concentrations of TPH were measured over time.

Test 2

Soils from the bins with dead worms were reused in Test 2. The starting concentration of each treatment and control was significantly lower than the Test 1b ending concentrations (Figure 16 & Table 8). The TPH declined at a different rate in each treatment and control between Test 1b and Test 2 and the initial concentrations differed significantly ($p<0.01$). The initial TPH concentration in the *A. caliginosa* treatment was significantly less than the *E. fetida* and treatment ($p=0.01$).

The differences in TPH concentrations between the earthworm treatments and the volatilization and feed controls remained significantly different over the course of the 126 day experiment, however the TPH concentrations in the earthworm treatments converged and were not significantly different during Test 2 (Figure 16B & Table 8). All of the treatment and controls exhibited declines in TPH during Test 2 (Table 8).

All treatments and controls showed substantial reductions in TPH concentrations from the beginning of Test 1 through the end of Test 2 (Table 8). However, the presence of the both species of earthworms resulted in the greatest changes in TPH over the course of 292 days.

Table 8: Percent Change in TPH in Native Experiment Treatments

Treatment	Change in TPH During Test 1 (70 Days)	Change in TPH Between Experiments (96 Days)	Change in TPH During Test 2 (126 Days)	Total Change in TPH (292 Days)
Volatilization	-21%	14%	-18%	-44%
Food Only	-32%Ŧ	-42%Ŧ	-32%Ŧ	-73%Ŧ
E. fetida	-62%Ŧ*	-66%Ŧ*	**-53%Ŧ***	-94%Ŧ*
A. caliginosa	**-70%Ŧ***	**-64%Ŧ***	34%Ŧ* (98 days of treatment)	-93%Ŧ*

ŧ – TPH concentration decline is significantly greater ($p<0.05$) than volatilization control

* – earthworm treatment TPH concentration decline is significantly greater ($p<0.05$) than food only control

Bolded entries indicate that TPH concentration decline is significantly greater ($p<0.05$) than the other earthworm treatment

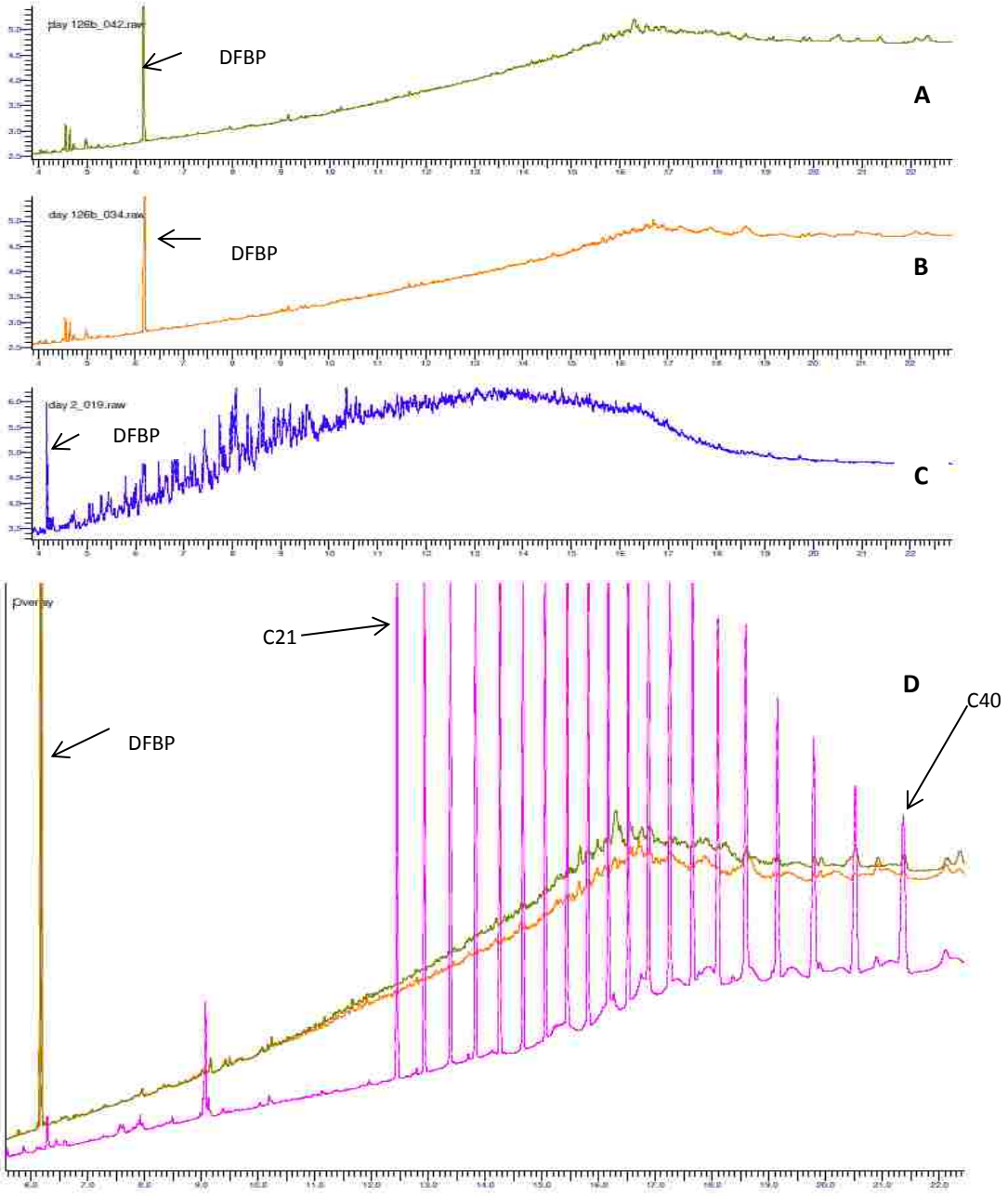


Figure 17: TPH chromatograms showing appearance of crude oil in native soil after 292 days in *E. fetida* treatment (A), 292 days in *A. caliginosa* treatment (B) and the initial appearance of the crude oil prior to treatment (C). The bottom panel (D) includes an alkane standard from C21 to C40 for comparison with the treatment samples from panels A & B (colors remain the same).

Residual Soils Degradation Results

The residual soil experiments were conducted in three separate ways, using extract soils, a 1:1 dilution, and a 2:1 dilution. After diluting the extract soils from 21,000 mg/kg using artificial, uncontaminated soil the concentration was expected to be ~15,000 mg/kg. However, TPH in the soil at the start of experiments only measured between 3,000-4,500 mg/kg, 75% lower than anticipated (Figure 18). TPH concentrations rose steadily from the initial concentration until Day 42 of the experiment and between Day 42 and Day 83 the three extract soil treatments all declined in concentration. On Day 42, when the peak concentration occurred, the soil samples were dried at 105°C for 24 hours prior to extraction and analysis to improve the extraction over the standard method. This drying process appears to have made more TPH available for extraction, however the measured TPH concentrations were between 7,300 and 7,800 mg/kg, approximately 50% of the expected concentration.

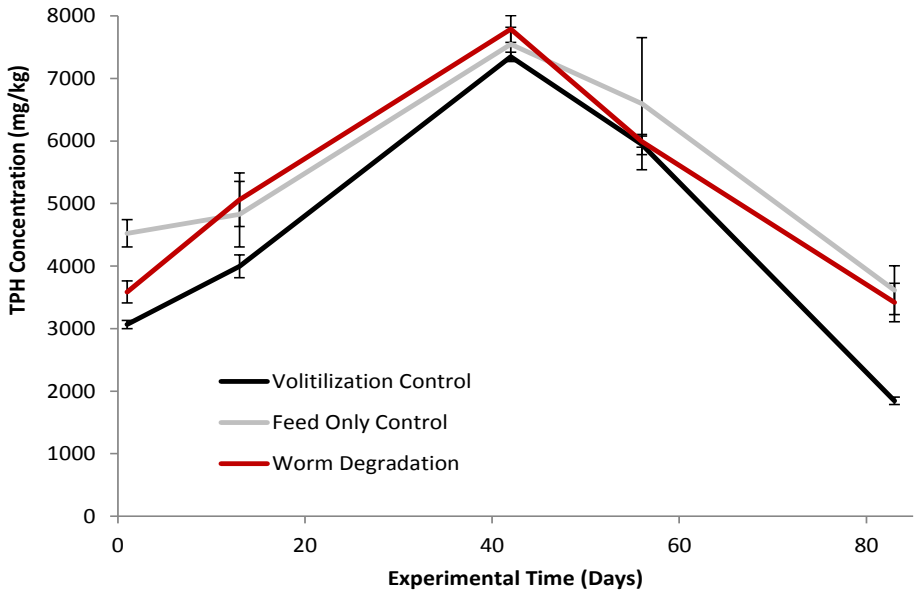
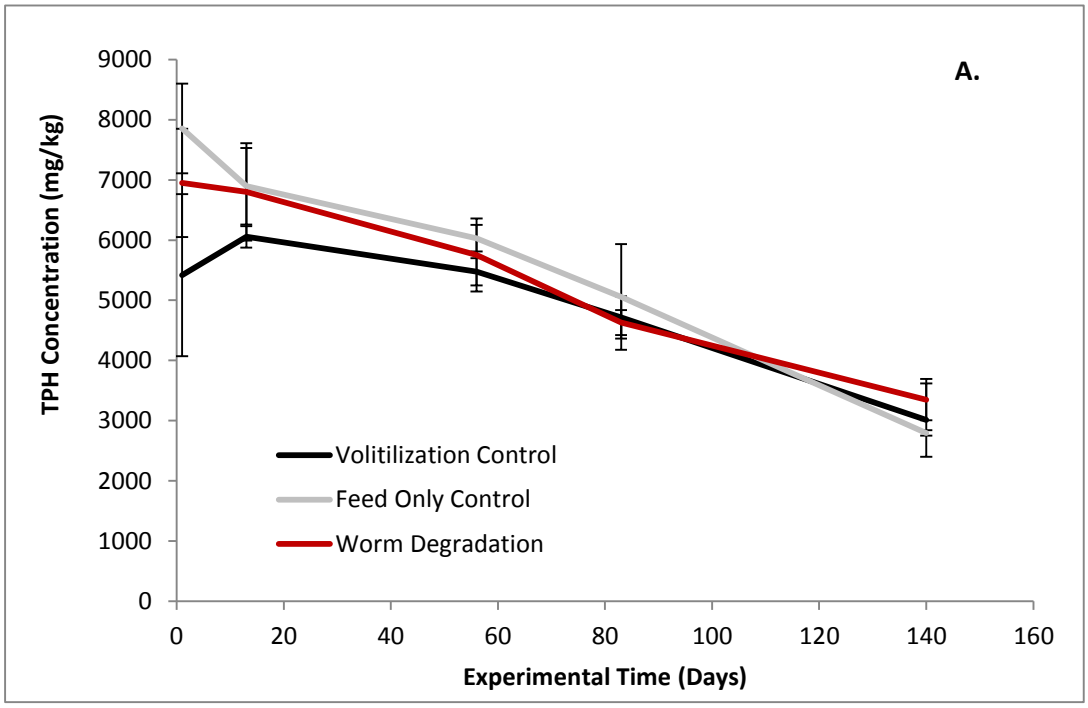


Figure 18: The influence of earthworms and feed on extracted TPH added to artificial soils (extract soils). Beginning with an initial TPH concentration of approximately 4,000 mg/kg (w/w), TPH concentrations were measured over time.

The remaining Year 2 earthworm treatment soils were diluted with artificial soil at a 1:1 and 2:1 dilution (contaminated:artificial), and results of each of treatment are shown in Figure 19. TPH concentrations in the 1:1 and 2:1 experiments have decreased only slightly over time in all treatments after 83 days.



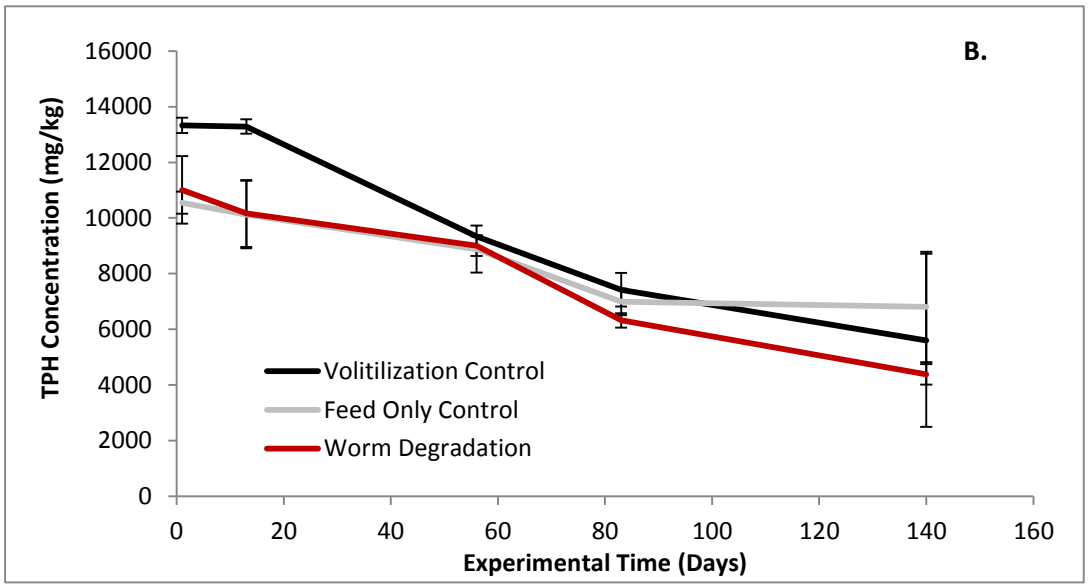


Figure 19: The influence of earthworms and feed on residual soils (A) diluted 1:1 with artificial soil. Initial TPH concentration was between 5,000 and 8,000 mg/kg (w/w), and (B) diluted 2:1 with artificial soil. Initial TPH concentration was between 10,500 and 13,500 mg/kg (w/w). TPH concentrations were measured over time.

DNA Concentrations in Experimental Soils

Microbial DNA was extracted from soils in the inoculum and native soil experiments and the DNA concentrations were quantified using a NanoDrop™ Spectrophotometer (Table 9). These are used as estimates of bacterial populations, however the NanoDrop™ results are not precise enough for detailed comparison.

Table 9: Soil Bacterial DNA Concentrations

Experimental Treatment	DNA Concentration (ng/g of soil)
Inoculum Degradation Experiment	
Volatilization Control –No Inoculum	127
Uncontaminated Artificial Soil, No Inoculum	79
Feed Treatment –No Inoculum	11,034
Worm Treatment –No Inoculum	27,242
Volatilization Control – With Inoculum	126
Uncontaminated Artificial Soil, With Inoculum	81
Feed Treatment – With Inoculum	14,296
Worm Treatment – With Inoculum	56,703
Native Soil Degradation Experiment	
Volatilization Control	1,592
Feed Treatment	23,036
E. fetida Treatment	41,348
A. caliginosa Treatment	17,982
E. fetida Mortality Control	14,404

Microbial DNA concentrations in the inoculum experimental treatments containing earthworms were two orders of magnitude greater than the volatilization controls, both with and without inoculum. The earthworm treatment containing inoculum had substantially more microbial DNA than the earthworm treatment without inoculum, while the feed treatments did not exhibit such a strong divergence in DNA concentrations between the inoculum and no inoculum treatments. The DNA concentrations were virtually identical in the volatilization controls. The native soil experimental treatments followed a similar pattern, with the *E. fetida* treatment exhibiting by far the greatest microbial DNA concentration and the volatilization control with the lowest DNA concentration. It should be noted that the DNA extraction and analysis occurred after more than 90% of the earthworms had died in the *A. caliginosa* treatment, which may be the reason that the bacterial DNA concentrations were lower than the native soil with feed control. The highest bacterial DNA concentrations found in these experiments were present in the *E. fetida* treatment containing contaminated artificial soil with the native soil inoculum and the second greatest DNA concentration occurred in the native soil with *E. fetida*.

DISCUSSION

The study showed that the presence of earthworms in these experiments enhanced crude oil degradation. Both the native soil and inoculum degradation experiments with earthworms produced significant declines in TPH compared to the respective controls. Earthworms also increased the degradation of hydrocarbons across the full range of carbon numbers (Figure 8, Figure 14 & Figure 17), an important finding because many bioremediation techniques do not perform well with heavier hydrocarbons (Van Deuren et al. 2002; Khan et al. 2004; USEPA 2004). The degradation of TPH in the native soil is particularly strong evidence for the potential efficacy of vermiremediation, since the native soil represents real site conditions with soil that had been contaminated for decades. This enhanced petroleum degradation appears to be due to increased microbial populations.

Microbial Stimulation

Earthworms can stimulate microbial soil populations by changing soil structure, digesting soil particles and leaving detritus and mucus that can be fed upon by microbes (Lavelle et al. 2004; Tiunov et al. 1997), and these experiments showed that earthworm mediated increases in bacterial populations (as measured by bacterial DNA concentrations) can enhance crude oil degradation (Figure 11 & Figure 16). Bacterial DNA concentrations increased in the presence of *E. fetida* compared to the associated feed and volatilization controls. The native soil inoculum in combination with earthworms produced even greater DNA concentrations, showing the importance of an existing hydrocarbon adapted microbial biomass to overall bacterial growth (Table 9). In all cases, higher bacterial DNA correlated with improved crude oil degradation. This increase in bacterial DNA is consistent with several other hydrocarbon bioremediation studies, which consistently found increased microbial activity in the presence of earthworms (Schaefer et al. 2005; Ceccanti et al. 2006; Schaefer & Filser 2007; Hickman & B Reid 2008).

Bacterial DNA concentrations also increased in the presence of *E. fetida* in the native soil experiment, likely contributing to the major TPH declines. It appeared that the effect of the microbial stimulation by the earthworms was strong enough that the TPH degradation continued during the 96 days between the two native soil treatments. The mucus and casts produced during the native soil degradation experiment, along with the decomposing earthworm bodies, likely remained after the experiment ended, continuing to support microbial activity even after the earthworms themselves died or were removed (Lavelle et al. 2004; Brown & Doube 2004).

The enhanced TPH degradation in the presence of increased bacterial populations in the native soil shows that vermiremediation can be used for effective bioremediation of crude oil. The increase in bacterial populations is consistent with the effect of other bioremediation techniques (Khan et al. 2004). However, the bacteria population increase occurs without tilling or the use of any other aeration, a major advantage of vermiremediation compared to other bioremediation technologies.

Differences in Degradation Rates: SJV Crude Oil vs. Native Soil

To be successful any approach to bioremediation must be applicable to a range of soil and contaminant conditions. These experiments showed that the presence of earthworms produced substantial declines in TPH concentrations in two different soil types with differing crude oil composition. The degradation rates differed however; TPH degradation in the native soil proceeded more rapidly and reduced TPH concentrations further than the artificially contaminated soils with earthworms, both with and without inoculum. The experiments differed in three ways that may explain the variation in crude oil degradation: the two soils have different particle size distributions and different percentages of organic matter; the native soils were more likely to have a full complement of adapted microbes at the beginning of the experiment; and the composition of the crude oil varied between the experiments.

The artificial soil contained 70% sand, 20% clay, and 10% organic matter, whereas the native soil contained 85% sand, 6.2% silt, 7.7% clay (13.9% total fines), and only 1% organic matter. Hydrophobic contaminants such as petroleum can sorb onto organic matter and soil particles, particularly fine particles, or diffuse into the organic matter matrix (Hatzinger & Alexander 1995; Alexander 1995; Hatzinger & Alexander 1997; Nam & Alexander 1998; Brusseau et al. 1991; Reid et al. 2000; Ehlers & Luthy 2003). Because the artificial soil contained an order of magnitude more organic matter and is composed of 6.1% more fine soil particles, it follows that there will be substantially more sequestration sites in this soil than in the native soil. Many of these sequestration sites can be pore spaces that are too small to be accessed by microbes, so biodegradation can only occur as the molecules diffuse out of these pore spaces (Hatzinger & Alexander 1995; Alexander 1995; Hatzinger & Alexander 1997; Nam & Alexander 1998; Reid et al. 2000; Ehlers & Luthy 2003). As shown by the characterization of the residual soils (Figure 8), the remaining TPH in the soil tends to consist of heavier hydrocarbons, which have a lower solubility than their lighter cousins (Sugiura et al. 1997). Based on this reasoning, it seems likely that more of the hydrocarbons were sequestered in the artificial soil and these low solubility hydrocarbons diffuse slowly, resulting in lengthier biodegradation timelines.

The native and inoculum soil experiments were also likely to have differing initial microbial biomasses, however no initial bacterial DNA concentrations were available. Using volatilization controls as a proxy for initial DNA concentrations, the native soil experiments began with an order of magnitude more bacteria than the inoculum experiments (Table 9). This higher initial bacterial biomass may have contributed to the more rapid TPH degradation in the native soil since the microbial population was already present and adapted to crude oil.

The crude oil composition in each experiment also differed. The proportions of C16-C21 and C21-C35 aromatics in the native soil crude oil (Figure 15) were substantially lower than those observed in the SJV contaminated soils (Figure 8). These two components of the crude oil

were shown to be the most difficult to degrade in Degradation Trial 1 (Figure 8B, earthworm and feed only treatments). The corresponding shift in relative abundance to the larger, more recalcitrant aromatic hydrocarbons over time may have contributed to a higher degradation plateau in the SJV contaminated inoculum experiment. Degradation of heavier hydrocarbons is difficult due to low solubility and high microbial resistance (Westlake et al. 1974; Atlas 1981; Sugiura et al. 1997; Reid et al. 2000), therefore the lower degradation plateau in the native soil may be partially due to crude oil composition.

The addition of the native soil as an inoculum of hydrocarbon adapted microbes was hypothesized to increase the total amount of TPH biodegradation and reduce the TPH concentration at which the degradation plateau appeared. However, the TPH degradation plateau appeared in the earthworm treatments. These results suggest that the soil inoculum did not affect the mechanism causing the TPH degradation plateau. The crude oil composition discussed above is one possible explanation for the degradation plateau. To explore additional possibilities, three experiments were executed with residual soils from Degradation Trial 1.

The soil dilution experiments did not support the hypothesis that a build-up of inhibitory compounds caused the degradation plateau. If an inhibitory compound was present at or above a threshold value, the 1:1 and 2:1 dilution of the residual soil could reasonably be expected to reduce the concentration of the inhibitor below the threshold and allow biodegradation to resume in the feed only control and earthworm treatment. The declining TPH concentrations in all treatments and controls with time (Figure 19) are most consistent with an increase in the non-extractable TPH fraction (Reid et al. 2000; Semple et al. 2003). I speculate that the addition of the uncontaminated, artificial soil for dilution created additional sequestration sites for the residual TPH. With time, the TPH has gradually diffused into these sites, with a corresponding decrease in extractable TPH.

The results from the extract soil experiment were insufficient to support or refute the hypothesis that the degradation plateau was due to contaminant sorption on soil and organic matter. The initial TPH concentrations were substantially lower than expected, indicating that sequestration of the TPH in organic matter and clays following soil mixing may have occurred, resulting in non-extractable TPH (Alexander 1995; Hatzinger & Alexander 1995; Hatzinger & Alexander 1997; Ehlers & Luthy 2003; Reid et al. 2000; Semple et al. 2003). The disappearance of the TPH confounds the test to determine if the residual TPH had become non-bioavailable due to sorption to soil and organic matter.

Toxicity & Reproduction

To be a competitive bioremediation technology vermiremediation cannot require constant rejuvenation of the earthworm population. Several other studies have noted high earthworm mortality (Schaefer et al. 2005; Schaefer & Filser 2007; Hickman & Reid 2008b; Fernández et al. 2011), which was also observed in the native soil experiments, particularly with *A. caliginosa*. The results of the toxicity tests and observations from the degradation experiments indicate that *A. caliginosa* was less tolerant to laboratory conditions and appeared to be more sensitive to petroleum toxicity than *E. fetida*. *E. fetida* maintained a steady population during the second native soil degradation experiment and showed high survivorship during all toxicity tests, indicating this species is a superior candidate for vermiremediation.

The ability of earthworms to reproduce in contaminated soils is also important for a sustainable vermiremediation system. The earthworm egg capsule toxicity test (Figure 7) showed that *E. fetida* can successfully reproduce in soil containing SJV crude oil. But in practice it will be challenging to maintain or expand earthworm populations in hydrocarbon degradation projects at crude oil concentrations greater than approximately 2% (w/w). Observations of the artificially

contaminated soils, native soils, and the residual soils support these findings. In all experiments, egg capsules and juvenile *E. fetida* were observed on and near the soil surface.

CONCLUSION

The results of these experiments show that earthworms, particularly *E. fetida,* can be used to enhance bioremediation and accelerate crude oil TPH degradation. Degradation of 90% or more of crude oil within 300 days is realistic even with TPH concentrations exceeding 25,000 mg/kg. This level of TPH degradation in a native soil from a remediation site is strong evidence that vermiremediation is a potentially viable treatment technology for crude oil contaminated soils. The current experimental results indicate that vermiremediation is the most promising in soils with low organic matter content and with a fines content of less than 15%. Due to the high variability between remediation sites, both in soil characteristics and in crude oil composition, pilot testing is needed to determine how applicable vermiremediation is to remediation sites currently present around the globe. Due to increased toxicity, soils with high initial TPH concentrations will likely need earthworm additions during remediation to maintain sufficient biomass until TPH is reduced below a toxicity threshold where reproduction is equal to or greater than mortality. However, at sites with lower initial soil TPH concentrations earthworm populations can be self-sustaining with reproductive rates sufficient to maintain a healthy earthworm population. Ongoing remediation may allow for establishment of a petroleum adapted population of earthworms with greater hydrocarbon tolerance, reducing biomass lost due to toxicity during early stage remediation.

Future Study

These experiments have shown that vermiremediation of crude oil is feasible in a laboratory setting. The next step is to test this technique on a larger scale in a field setting, where the environment is less controlled. The differences in TPH degradation efficiency between the artificial soil and native soil experiments also raise the question of how applicable vermiremediation can be at various contaminated sites. Testing the applicability of vermiremediation to fine soils is an important next step as well, these experiments did not determine if earthworms are able to decrease contaminant sorption or increase bioavailability. Experiments with soils containing higher percentages of clay will be important in showing how effective vermiremediation can be in highly challenging remediation conditions. Continued testing of vermiremediation with various crude oils is essential to establishment as a viable bioremediation technology. I showed that the presence of earthworms stimulates bacterial soil populations; the changes in microbial populations in contaminated soils due to earthworms should be explored. It may be possible to optimize vermiremediation with the addition of microbial inoculums to accelerate crude oil degradation in a variety of soils.

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