e-ISSN No.: 2582-4228

Journal of Indian Association for Environmental Management

Journal homepage: www.http://op.niscair.res.in/index/php/JIAEM/index

Bioremediation of Petroleum Hydrocarbon from Soil using Earthworm species *Eisenia fetida* in a Vermiwash System

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Submitted: October 04, 2021 Revised: March 06, 2022 Accepted: March 08, 2022

Abstract: **Soil contamination by petroleum hydrocarbon-based compounds continues to present an environmental hazard in many countries. More so, in many developing countries there is a lack of proper disposal mechanisms for products such as used vehicular engine oil. The investigation and application of appropriate biological methods to mitigate and possibly irradicate the adverse effects of hydrocarbon contaminants on soil quality and productivity is critical for environmental sustainability. The study reports on the application of earthworm species *Eisenia fetida* in the remediation of petroleum hydrocarbons from clay loam soil and their effects on native microbial populations under vermiwash conditions. The earthworm species were obtained from the soil in the community of Nismes, Guyana. The results revealed that *Eisenia fetida* strived well in 5 % v/w used engine oil, 92 % survival was recorded in engine oil-contaminated soil. *Eisenia fetida* exhibited an ability to biodegrade petroleum hydrocarbon from the soil and bioaccumulate these compounds within their tissue. The results attained showed a reduction of 68.95 % of TPH in contaminated soil that consisted of *Eisenia fetida* and a 4.50 % reduction in contaminated soil without the *Eisenia fetida*. Further, *Eisenia fetida* in used engine oil contaminated soils possessed 66.9 % more TPH in their tissues compared to *Eisenia fetida* in uncontaminated soil. Investigations revealed exemplary support for the use of *Eisenia fetida* in the remediation of petroleum hydrocarbon, promotion of microbial population, and improvement of soil quality.**

***Keywords*: Bioremediation, *Eisenia fetida,* Total Petroleum Hydrocarbon, Used Engine Oil**

I. Introduction

The natural equilibrium of our ecosystem is incessantly disturbed by environmental pollution. A major source of environmental pollution is linked to petroleum and petroleum products. Many developing countries continue to encounter pollution caused by the improper disposal of used motor vehicular engine oil in soil (Zitte *et al.,* 2015), a common source of highly toxic petroleum hydrocabons. Petroleum hydrocarbons in used engine oil has been linked to reproductive damage in animals and potentially humans (Akintunde *et al.,* 2015). These compounds have also been linked to cancer in humans (Gay *et al.,* 2010). The adverse environmental and human effects of petroleum hydrocarbons have led to increasing research towards its remediation.

Bioremediation strategies have gained a lot of attention towards the remediation of petroleum hydrocarbon from soil (Das and Das, 2015), with vermi-remediation being an environmentally friendly and economically viable application (Sinha *et al.,* 2010). The use of earthworms in soil remediation was linked to their ecological, biological, and behavioral characteristics in their natural environs (Ma *et al.,* 2003). The body of the earthworm acts as biofilters for the intake of chemical contaminants from the soil along with the consumption of contaminated soil through their buccal cavity to facilitate the bioconversion and biodegradation of contaminants (Sinha, Herat, Bharambe, & Brahambhatt, 2009). The desorption and degradation of contaminants in earthworms was linked to microbial and enzyme actions (Azadeh and Zarabi, 2015). Sinha *et al* (2009), postulated that earthworms enhance soil quality through their burrowing actions and the excretion of nutrient-rich materials constituting nitrogen, phosphorous, potassium (NPK), and micronutrients in the form of vermicasts.

The use of earthworms in soil remediation has gained a lot of recognition over the years, but not without its limitations. Hamby (1997), postulated that earthworms are diverse and very cooperative organisms given that they function both independently and also aid the function of bacteria (bioaugmentation) and plants (phytoremediation) in the removal of harmful chemicals from the environment. However, some parameters may influence the bioaccumulation rate and effect of earthworms in the environs. Soil type and soil compounds were found to affect the bioavailability of contaminants such as Polycyclic Aromatic Hydrocarbons (PAHs) for microbial degradation and degrading capabilities of microbial communities. However, studies indicate that indigenous soil microbial activity is critical for changes in hydrocarbon availability to invertebrates. It was observed that in non-sterile soils, microorganisms degraded readily available contaminants very quickly and after several days only the non-available fraction was left for uptake by earthworms or other invertebrates (Šmídová, 2013). Hence, natural, chemically unaltered soils would provide better bioaccumulation of hydrocarbon compounds by earthworms. Further, the effect of soil type on bioaccumulation suggests an understanding that the soil textural class is essential for the bioremediation of petroleum hydrocarbons.

The earthworm species *Eisenia fetida* is recognized as an important species in soil remediation (Coutino-Gonzalez *et al*. 2010; Asgharnia *et al.*, 2014; Njoku *et al.*, 2016). However, few studies have examined their use in clay loam soil with a focus on their interaction with native soil bacteria during the petroleum hydrocarbon bioremediation process. More so, under vermiwash conditions which allow for the natural soil macro-nutrient (NPK) and bacteria analysis to be investigated simultaneously. In essence, the study investigated the potential of *Eisenia fetida* to aid the bioremediation of petroleum hydrocarbon in native soil (clay-loam) and determined whether there are significant differences between the natural breakdown of petroleum hydrocarbon in soil and bioremediation of petroleum hydrocarbon aided by earthworms. Additional investigation was conducted on the effect *Eisenia fetida* has on the physico-chemical characteristics and native bacteria population of soil during bioremediation.

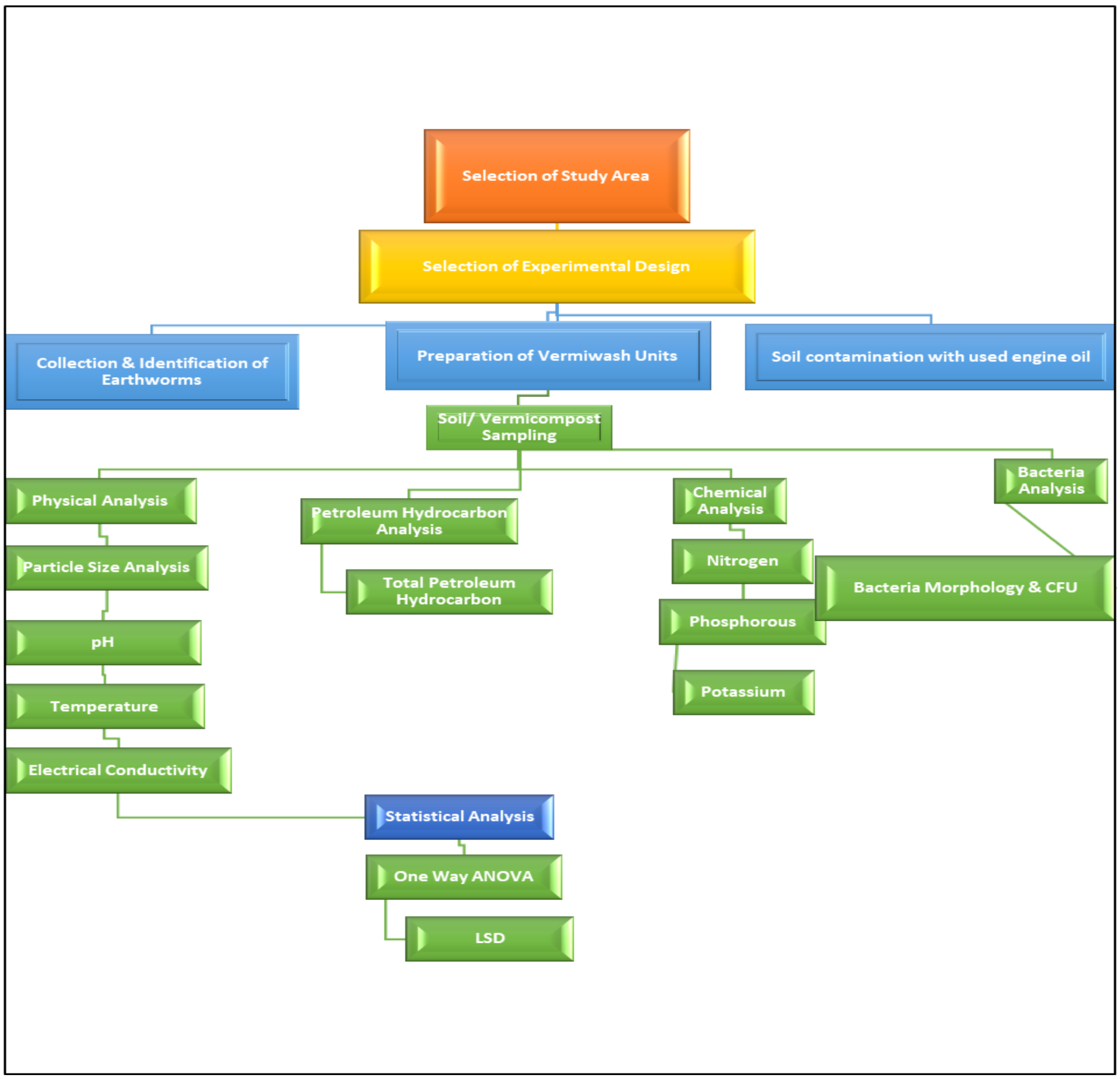

Figure 1. Outline of Methodology

II. Material and Methods

Design of experiment

The research conducted employed the complete randomized design (CRD). The design constituted four treatments and four replicates. Each treatment title was labeled on plain paper and placed in a black plastic bag. The treatments were then randomly assigned by handpicking from the list of papers placed within the bag by four colleagues. The treatments of experimental design and diagrammatic representation were as indicated in Table 1 and Figure 1-2.

TABLE 1
Treatments

State of condition/Treatment	T1	T2	T3	T4
Natural mode (Cow manure)	+			
Earthworm Only (Cow manure)		+		
Earthworm + Used Engine Oil (Cow Manure)			+	
Used Engine oil Only (Cow manure)				+

Treatments	T1	T4	T1	T3
	T3	T1	T2	T1
	T2	T3	T4	T2
	T4	T3	T2	T4

Figure 2. Simple diagrammatic representation of complete randomized experimental design

Phase 1: Field experiment

Collection and identification of Eisenia fetida

The digging and hand sorting method was used for the collection of the earthworms. The litter component of soil (2-5cm) was thoroughly searched, using a fork to remove topsoil within a demarcated area. The soil was placed on a plastic sheet within a wheelbarrow and sorted for earthworms. The earthworms found were placed within plastic containers with soil and transported to the study site for identification and application to vermiwash units (Nxele *et al.*, 2015). The earthworm species (*Eisenia fetida*) was identified based on its morphology and anatomy as well as its ecological feature with the aid of an established annotated key for identification (Ansari and Saywack, 2011).

Preparation of experimental set-up process (Figure 3)

Collection and preparation of the soil

Soil samples were collected from areas surrounding earthworm sampling plots from a depth of 10 - 30 cm using a fork. The samples were weighed and stored in plastic buckets at room temperature.

Figure 3: Preparation of Experimental Units

Collection and preparation of used mineral-based engine oil

Two gallons of used mineral-based engine oil was collected from a local mechanic shop on the West Bank of Demerara. The oil was collected after being drained from the engine of a motor vehicle that was serviced. The oil was then placed in a plastic bottle and transported to the University of Guyana where it was stored and used during the study.

Contamination of soil with Used Engine Oil

Sixteen (16) containers were set up with (2000 g) of soil being placed in each. 10 grams of used oil was weighed and added to eight (8) of the containers with soil. The oil was then mixed with a sterilized spatula until visually homogeneous with soil to give approximately 0.5% (w/w). 50 grams of cattle dung was ground and incorporated into contaminated soil. The containers were left to stand for seven days in a fume hood to allow for binding and removal of more volatile components of the engine oil. After seven days, a composite sample was made by collecting 10 g of soil from each container and taken for an initial analysis of the TPH and physicochemical parameters. A sample was also taken for soil bacteria analysis. The constituents of each container were transferred to individual vermiwash units after construction which were randomly assorted in the study area.

Preparation of vermiwash units

Sixteen (16) square five gallon bottles were selected for the units. The top of the bottles was then cut off at the height of 8 cm using a hand saw. An electric drill was used to insert a hole of diameter 2 cm, approximately 6 cm from the base. A tap with mesh vents was affixed within the hole on each container in such a way that allowed effortless drainage of vermiwash. Each unit was placed on a stand in such a way to facilitate the collection of vermiwash. 7 cm of pebbles were placed at the bottom of each container followed by 7 cm of coarse sand. Water was allowed to flow through these layers enabling the settling of the basic filter unit. Subsequently, a 10 cm layer of heat sterilized loamy soil was placed on top of the filter bed. Seventy-five adult earthworms were introduced into the soil of each container. The prepared contaminated soils (5 cm layer) was added to each of the vermiwash units. These units were sprinkled with water at regular intervals (alternate days) to keep moist and continuously monitored. Soil samples were also analyzed every 20 days for TPH and physico-chemical parameters.

Soil (vermicompost) sampling

The composite sampling method was used to retrieve samples from the units. Soil samples were retrieved from units using a soil auger. The soil auger was inserted to a depth of 10 inches to sample each column of the unit (excluding the pebbles). Five sub-samples were taken from random points in the units. These samples were placed in a container and mixed to form a single sample. 100 g of the sample was placed in a glass gar and transported for nutrient and TPH analysis (via a cooler with gel ice-pack), another 100 g sample was used for physicochemical analysis (Woods End Laboratories Incorporated, 2000).

Phase 2: Laboratory experiment

Total petroleum hydrocarbon analysis of soil

The Gravimetric Method (specifically the Soxhlet Extraction Method) was used for the determination of the total petroleum hydrocarbon content in the soil. This method was divided into two parts which included the extraction of oil and grease from soil followed by the determination of total petroleum hydrocarbons present (U.S. Environmental Protection Agency, 1995).

Total petroleum hydrocarbon analysis of earthworm tissue

The earthworms were dried and ground into powder, and the Gravimetric Method (specifically Soxhlet Extraction Method) was used for the determination of the total petroleum hydrocarbon content in earthworms (U.S. Environmental Protection Agency, 1995).

Nutrient and physico-chemical analysis of soil

The physico-chemical characteristics of the soil assessed were as follows: Particle size distribution, pH, EC, temperature, and nutrient analysis [nitrogen, phosphorous, and potassium (NPK)].

Physico-chemical analysis

The Hydrometer method was used for the analysis of particle size distribution. The hydrometer method involved measuring the specific gravity of the soil suspension at its bulb center (ASTM, 2017). pH and Electrical conductivity (EC in mS/cm) was measured using a multimeter branded "Estik 11". Soil temperature was measured using a soil thermometer.

Nutrient analysis

The total amount of nitrogen (N) was analyzed using the total kjeldahl nitrogen and total nitrogen in acid digests by spectrophotometry technique (Bremner & Mulvaney, 1982). Phosphorous (P) was analyzed using the Bray exchangeable phosphorous method (SERA-IEG 17, 2000). Potassium (K), was extracted using one molar ammonium acetate and measured spectrophotometrically (Knudsen, Peterson, & Pratt, 1982).

Microbial analysis of soil

The bacteria population of the soil was analyzed via Culturing and Identification of morphological features. Bacterial count was conducted on the initial and final soil samples. The methods used for determining colony count and morphology were adapted from (Frankland, Latter, & Poskitt, 1995).

Techniques of data analysis

The data attained from the research were presented using descriptive analysis in the form of graphs. These were created utilizing microsoft excel. Additionally, the data were analyzed using analysis of variance (ANOVA) at a 95% confidence level (α = 0.05), with a post hoc test executed using least significant difference (LSD. The statistical package used for analysis was stastistix ten.

III. RESULTS AND DISCUSSION

Earthworm remediation in this research was aided by the use of cow manure, a carbon-rich nutrient source for the soil and earthworms in the vermiwash system. The theory around the remediation process was viewed from a biphasic viewpoint, which involved internal and external processes for petroleum hydrocarbon breakdown from the soil.

The internal process would have involved naturally produced enzymes located within the intestinal walls of earthworms, along with adapted microbial endosymbionts within their gastrointestinal system. Chemical contaminants in the soil would be taken in by earthworms, by absorption through their moist body wall into their interstitial water or consumption of soil through their buccal cavity (Sinha *et al.*, 2009). The soil contaminants (petroleum hydrocarbons) will enter into direct contact with hydrocarbon-degrading enzymes. As the soil enters the mouth, it is passed to the gizzard which grounds it into very small particles (approx. 2 mm), thereby enhancing the surface area for microbial interaction (Karthikeyan *et al.*, 2004). The particles are then passed through the alimentary/ intestinal tract of the earthworm, where hydrocarbon-degrading microbes such as *Pseudomonas*, *Acaligenes,* and *Acidobacterium* may act on these compounds, and their enzymatic system would be activated resulting in enzyme secretion such as Cytochrome P450, Hydrolases and dehydrogenases (Azadeh and Zarabi, 2015) that would cause petroleum hydrocarbons in the soil to be biotransformed (altered into less toxic compounds) or biodegraded in the gut and possible bioaccumulation in their tissues. Moreover, contaminants such as fluoranthene and phenanthrene have been found to bioaccumulate within the tissues of earthworms (Ma *et al*., 1995).

The external process will involve the release of earthworm cast into the soil and within the burrows formed by earthworms (containing microorganisms and organic compounds) as they maneuvers throughout the soil. As earthworms' burrows throughout the soil, they alter the physical properties of the soil, via increasing soil aeration among other structural changes and therefore would allow for increased contact between micro-organisms and contaminants (Roriguez-Campos *et al.,* 2014). Moreover, the bioturbation of the soil allows for the degradation of petroleum hydrocarbons in the soil by oxidative processes. The vermicasts of earthworm consist of many microbes and are nutrient-rich. This provides suitable conditions for enhanced oxidation of contaminants by microbes in the soil while simultaneously increasing the available microbe population. Further, earthworms' ability to transport indigenous hydrocarbon-degrading microbes (Das and Chandran, 2010) throughout the soil will also aid the external biodegradation process.

In essence, the interaction between the earthworms and their surrounding physical, biological and chemical environment would provide a very suitable environment for the effective oxidation of the toxic petroleum hydrocarbons. Further, utilizing the digestive system of the earthworms to physically degrade the soil particles on consumption to a state accessible for initial microbial action coupled by enzymatic responses to biodegrade compounds into a less toxic form that would be bioaccumulated and excess released as beneficial mineralized forms to the environment for continued microbial action.

The common practice once employed for the remediation of sites contaminated with crude oil (petroleum hydrocarbons) was geared mostly towards physical and chemical methods which were often quite costly and not very environmentally friendly. However, as time progressed numerous techniques were adopted for remediation of petroleum hydrocarbon and recently attention towards different bioremediation strategies as an option to remove petroleum hydrocarbon from soil (Das and Das, 2015).

Vermiremdiation is a socially acceptable, environmentally friendly, and economically viable method for bioconversion of organic wastes into nutrient-rich compounds (Sinha *et al.,* 2010). Vermeremediation is based primarily on the unique ecological, biological, and behavioral characteristics of earthworms in soil (Ma *et al.,* 1995). Thus, earthworms play a significant role in the field of waste remediation, and they were also an essential component in this study since they would provide cost-effective and safer remediation techniques for contaminated soil.

The process of bioremediation for soil contamination has been in practice since the 1940s, but the field gained global recognition preceding the Exxon Valdez oil spill in 1989 (Margesin and Schinner, 1997). Exxon Valdez oil spill propelled widespread studies that proved bioremediation to be useful in the treatment of sites contaminated by hydrocarbons. Further, earthworm species, *Eisenia fetida* were found to double the reduction rate of some of the most abundant and persistent intermediate products of hydrocarbons present in soil within 28 days (Coutiño-Gonzále *et al.,* 2010).

Bioremediation functions primarily on biodegradation, defined as the total mineralization of organic contaminants into environmentally safe compounds or transformation of complex organic pollutants to simpler organic compounds by biological agents such as bacteria in the genus *Acinetobacter, Aeromicrobium, Brevibacterium, Burkholderia, Dietzia, Gordonia,* and *Mycobacterium* (Das and Chandran, 2010). Many indigenous bacteria and fungi in the aquatic and soil environs facilitate the degradation of hydrocarbon contaminants based on the type of hydrocarbon chain (Das and Chandran, 2010). Within the domain of bioremediation, bio-augmentation and bio-stimulation are the two important sub-categories. Bio-stimulation focuses on organisms present within an area of contamination; it refers to the introduction of organic or inorganic compounds to stimulate indigenous microorganisms to break down pollutants in the environment. Bio-augmentation involves the isolation and transferral of foreign organisms to the contaminated site to achieve restoration (Gentry *et al.,* 2004). Bio-stimulation and bio-augmentation may be used to enhance bioremediation based on the type of environment and the availability of hydrocarbon-degrading bacteria. Researchers purport that bioremediation strategies provide more benefits in comparison to physical and chemical approaches since their implementation can be in situ, straightforward, less intrusive, and often more cost-effective (Das and Das, 2015).

Earthworms hold significant benefits towards a sustainable society. They are thought to have the potential of offering cost-effective solutions to many social, economic, and environmental issues being imposed on the human environs. More specifically, they possess the ability to aid in the natural management of municipal and industrial organic waste away from landfill sites and reduce greenhouse gas emissions (Sinha *et al.,* 2010b).

The anatomy and physiology of earthworms aid in their capacity to bioremediate toxic chemicals from the soil and aquatic environment. Their bodies act as biofilters that can purify, disinfect and detoxify municipal and industrial wastewaters. Thus, facilitates significant reductions in the biological oxygen demand, chemical oxygen demand, and total dissolved solids of wastewater. They can eradicate endocrine-disrupting chemicals from sewage, bio-accumulate and bio-transform chemical contaminants from the soil, and rejuvenate contaminated lands for development. Their excreta and secretions are utilized for the restoration and improvement of soil fertility. Furthermore, earthworms are being explored even within the medical fields for the development of cures for major ailments affecting humans (Sinha *et al.,* 2010a).

Earthworms possess the ability to hasten the removal of contaminants from the soil through the regulation of soil properties via their mechanical actions increasing contact between contaminants and soil microorganisms (Roriguez-Campos *et al*., 2014); and internal biological processes inclusive of desorption of contaminants as it passes through their gut and enzyme action to facilitate breakdown (Azadeh and Zarabi, 2015). Earthworms' ability to exist in a highly toxic environment renders them beneficial in the rejuvenation of diverse ecosystems.

Sinha *et al.,* (2009) articulated that earthworms have the innate ability to absorb chemicals through their moist body wall into their interstitial water and also via their mouth during the passage of soil through their gut. The chemical contaminants passed through their stomach are either bio-transformed or biodegraded rendering them harmless in their bodies. Earthworms simultaneously enhance soil quality through their burrowing actions and the excretion of nutrient-rich materials constituting nitrogen, phosphorous, potassium (NPK), and micronutrients in the form of vermicasts.

It was promulgated that earthworms utilize their digestive system in the biodegradation of heavy metals by the detachment of complex aggregates between ions and humic substances in waste as it decays. Enzyme-driven processes facilitate the assimilation of metal ions by earthworms trapping them within their tissues preventing their release into the environment. Thus, preventing the entry of heavy metals into organic waste (Pattnaik and Reddy, 2012).

In the field of environmental sciences, earthworms were investigated for their use in the bioremediation of petroleum hydrocarbons. Earthworms are cosmopolitan and thus give scope for more global research. To date, research has labeled various earthworm species as beneficial organisms in bioremediation, notable species include *Lumbricus rubellus*, *Pheretima hawayana*, *Perionyx excavates*, *Hyperiodrilus africanus*, *Eudrilus eugeniae and Eisenis fetida*.

Soil texture

The soil is a critical component of terrestrial systems that consists primarily of weathered rocks and minerals (Alloway, 1990). The characteristics of soil play an essential role in its productive capacity and suitability for the existence of organisms and symbiotic associations. The productive capacity of soil tells of its ability to facilitate agricultural and other beneficial output to man and the environment. The soil texture and nutrient availability are of grave importance for the application of agricultural and environmental practices. Thus, given that soil texture is known to influence the presence of nutrients and organisms within the soil, an analysis was conducted on selective chemical characteristics (Table 2) and particle size distribution of the soil used during the study (Table 3). Soil texture gives a classification of the physical components of the soil particles that falls below two millimeters (2 mm) in size. These particles fall into three main categories, that is; sand, silt, and clay (USDA-NRCS, 2018). Additionally, soil type influences the response of micro-organisms to hydrocarbon compounds in the soil; as it relates to its breakdown (Hamamura, Olson, Ward, & Inskeep, 2006).

TABLE 1
Chemical Characteristics of Contaminated Soil

Parameter	Value
pH	6.7
EC	0.99 ms/cm
Temperature	26^0C
Nitrogen	39.25 mg/kg
Phosphorous	42.7 mg/kg
Potassium	118 mg/kg

TABLE 2
Physical Characteristics of Contaminated Soil

Particle Size Distribution	Value (%)
Silt	45.56
Clay	36.35
Sand	14.95
Test Class	Clay Loam

Total petroleum hydrocarbon in soil

The chemical and physical characteristics of contaminated soil is listed in Table 2 and 3 respectively. The contamination of soil by used engine oil presents a threat to the ecosystem and potentially human health (OEHHA, 2006). Mineral-based engine oil contains hundreds of hydrocarbon compounds with chain lengths ranging from C_{15}- $C_{50.}$ These compounds are primarily mixtures of straight and branch chain hydrocarbons, cycloalkanes, and aromatic hydrocarbons which include a considerable fraction of nitrogen and sulfur-containing compounds. Poly-Aromatic Hydrocarbons (PAH), alkyl Poly-Aromatic Hydrocarbons, and metals are also central components of engine oils, with used oils having higher concentrations (ATSDR, 1997). The contaminated soil assessed over the study period showed a noticeable decrease in total petroleum hydrocarbon within the soil that contained earthworms as compared to that which did not. Some decrease was observed within soils without earthworm but not as comparable to the former. Moreover, Treatment 3 had an average reduction of 68.95 % overall TPH as compared to treatment 4 which had a 4.50% decrease (Figure 4).

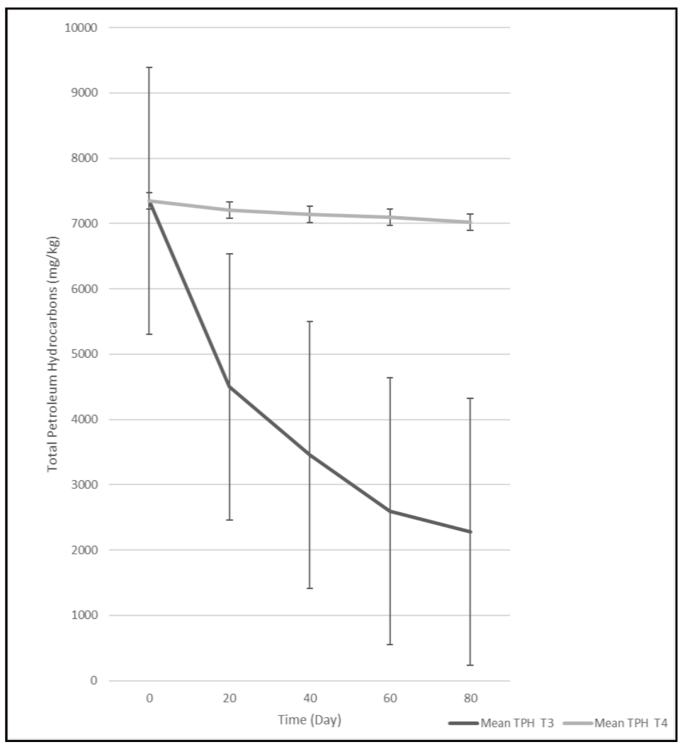

Figure 4. Mean Change in TPH (±Standard Deviation Bars) in soil with and without earthworms (T3 and T4)

Statistical analysis conducted using ANOVA showed that there was a significant difference $F(1, 38) = 36.11$ $(p < .0001)$ given that $p < .05$ in the means of the two treatments. Hence, suggests that earthworm species (*Eisenia fetida*) possess the potential to facilitate the bioremediation of petroleum hydrocarbon in soil. This result supports previous studies (Coutiño-González, Hernández-Carlos, Gutiérrez-Ortiz, & Dendooven, 2010; Njoku, Akinola, & Angibogu, 2016) which reported the same. The ability of earthworms to facilitate the bioremediation of petroleum hydrocarbons from the soil is postulated by Schaefer and Juliane (2007) to their mechanical (burrowing) characteristic which enhances the oxidative process in the soil thereby making available organic contaminants to microorganisms. Further, it was purported by Azadeh and Zarabi (2015) that earthworms' enzymatic mechanisms aid their ability to remediate petroleum hydrocarbons from the soil since earthworms such as *Eisenia fetida* contain the enzyme cytochrome P450, which degrades some hydrocarbon compounds. Hence, the consumption of soil by earthworms would reduce the level of petroleum hydrocarbons in the soil. Moreover, Munnoli *et al.* (2010) associated this phenomenon with microbial symbionts internally related to earthworms. Furthermore, Singleton (2003) conducted a study that found conclusive evidence of some bacteria genus associated with the intestine and cast of earthworms to be hydrocarbon degraders. However, Aira and Dominguez (2011) reported that the breakdown and removal of petroleum hydrocarbons in the soil are primarily due to soil microbes (bacteria and fungi) and earthworms acting as a supporting agents to enhance the microbial process. A previous study by Aira *et al.* (2007) suggests that earthworms work well in synergy with soil bacteria in hydrocarbon degradation and provide more efficiency than bacteria on their own. The results from this study support those as mentioned earlier based on the fact that both treatment soils constituted similar bacteria colonies at different population counts (**Error! Reference source not found.**5) as a result of the same origin, but results found treatment with earthworms significantly reduced TPH in the soil.

Total petroleum hydrocarbon in earthworm

Earthworms are good bioindicators of toxic contaminants in soil (Weber, 2007), based on their ability to successfully thrive within areas that pose death to most microorganisms (Satchell (1983); Haeba *et al*. (2013). Earthworms can bioaccumulate many highly toxic chemicals found in soil that pose a danger to the environment and human health, such as mercury (Zhang & Zheng, 2009) and cadmium (Sturzenbaum *et al.* (2004) among others. They possess the capacity to convert these chemicals into less harmful states via processes within their bodies (Sinha, Herat, Bharambe, & Brahambhatt, 2009; Zhang & Zheng, 2009). Additionally, they reduce contaminant levels by bioaccumulating them after bodily conversion through biological processes involving enzymes (Gao & Luo, 2005).

Earthworms' ability to bioaccumulate harmful chemicals is essential to their function as good biomonitors and bioindicators for pollutants in the soil. The research acquired data that supports earthworm (*Eisenia fetida*) capability to bioaccumulate petroleum hydrocarbons from local soil. The results showed that earthworms in contaminated soils, "Treatment 2" possessed an average of 66.9% ppm of TPH more than that of earthworms in uncontaminated soil, "Treatment 3" (Figure 5).

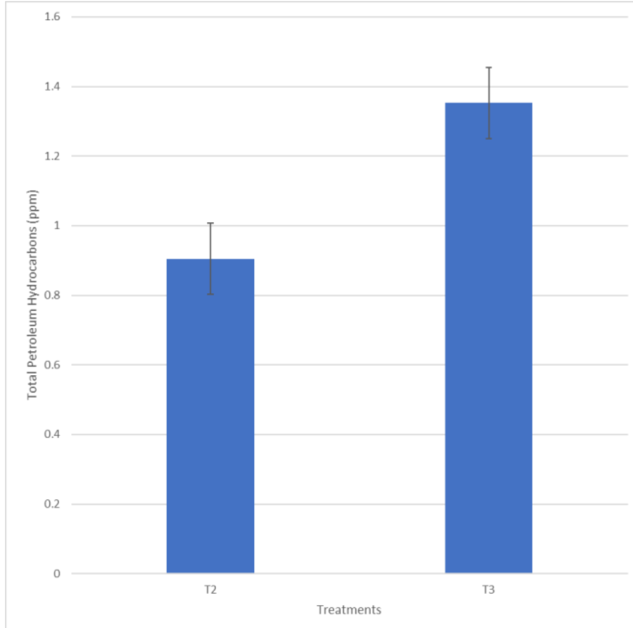

Figure 5. Total Petroleum Hydrocarbon (±Standard Deviation Bars) in earthworm tissue under different treatment

Soil pH

Soil pH is an accurate measurement of the hydrogen ion concentration within the soil; its value determines the degree of acidity or alkalinity of a given environment. The pH of soil influences the availability and absorption of nutrients by the plant and would have a direct effect on soil health and the macro and micro-ecosystems within it (Slessarev et al., 2016). The pH of the soil under the different treatment was observed to show variation based on treatment parameters; T1 which was the control remained constant over the study period, while T2 showed a small but consistent increase (1.01%), T3 indicated a downward variation with an overall decrease (0.60%) and T4 presented the most substantial reduction in pH (5.33%) (*Figure* 6).

There was a noticeable distinction in the mean pH of the different treatments. Treatment 2 which consisted no used engine oil but only earthworms, saw an increase in pH towards neutrality, which can be supported by the school of thought that earthworms tend to reduce the salinity and enhance (neutralize) the pH of the soil (Bhawalkar & Bhawalkar, 1994). The change in pH is owed to the ability of earthworms to mechanically digest soil resulting in the secretion of calcium carbonates from their calciferous glands or by intestinal ammonia excretion or a combination of both activities within their internal structure (Edwards & Bohen, 1996). In T3 and T4 which both consisted of used engine oil and the former containing earthworms, it was observed that T3 resulted in a minute decline in pH(0.60%) as compared to

T4(5.33%). The decrease in initial soil pH which is supported by reports from previous studies (Leahy & Colwell, 1990; Kisic, Mesic, & Basic, 2009), may be resultant to the addition of used engine oil, which would cause the oil to inhibit the leaching of hydroxide compounds (salts) due to its hydrophobic nature, hence deterring any increase in H^+ ions. It is also possible that the production of organic acids by microbial metabolism has contributed significantly to the lowered pH (Osuji & Nwoye, 2007) since oil would cause the accumulation of this acid in the soil. However, the observed difference between the two treatments as it relates to the higher negligible change in pH of T3 as compared to T4 is likely the result of the earthworms buffering ability on the soil through its ingestion and secretion (Edwards & Bohen, 1996) and also the possible burrowing effect that provides aeration to allow for microbial metabolism to occur.

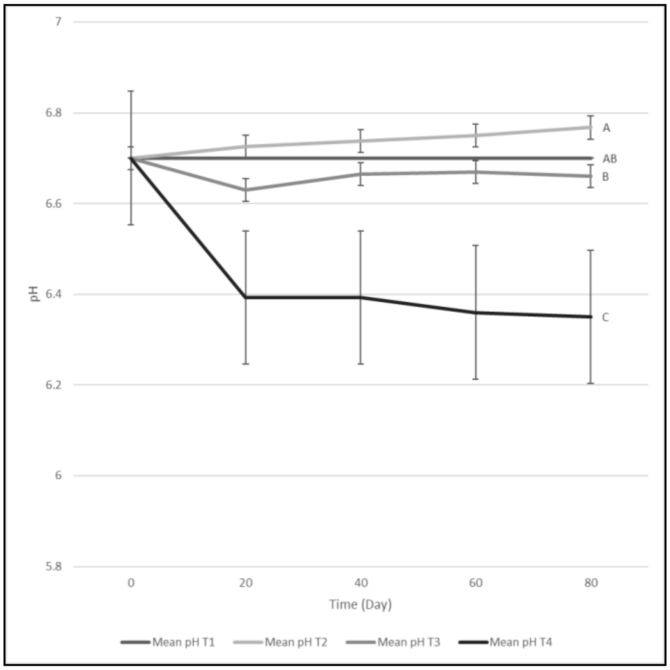

Figure 6. Mean Soil pH (±Standard Deviation Bars) under different Treatment at Day 0, 20, 40, 60 and 80

The numerical difference in pH across the different treatment were found to depict high statistical significance *F (3, 76) = 61.24 (p < .0001).* Further post hoc test (LSD) was conducted and found that there was a statistical difference between T3 and T4. Hence, earthworms significantly reduced the adverse effect that petroleum hydrocarbon would have on soil pH and thus improved this important soil parameter. Basker, Kirman, and Macgregor (1994) found results that were similar in their study which examined the effect of earthworm ingestion on potassium and soil properties.

Soil electrical conductivity (EC)

EC refers to the quantity of available salt in the soil. EC provides information on the salinity of the soil and acts as an indicator for soil health through inference on nutrient availability and loss (USDA- NRSC, 2014). The EC observed among the different treatments showed a consistent trend throughout the study (Figure 7).

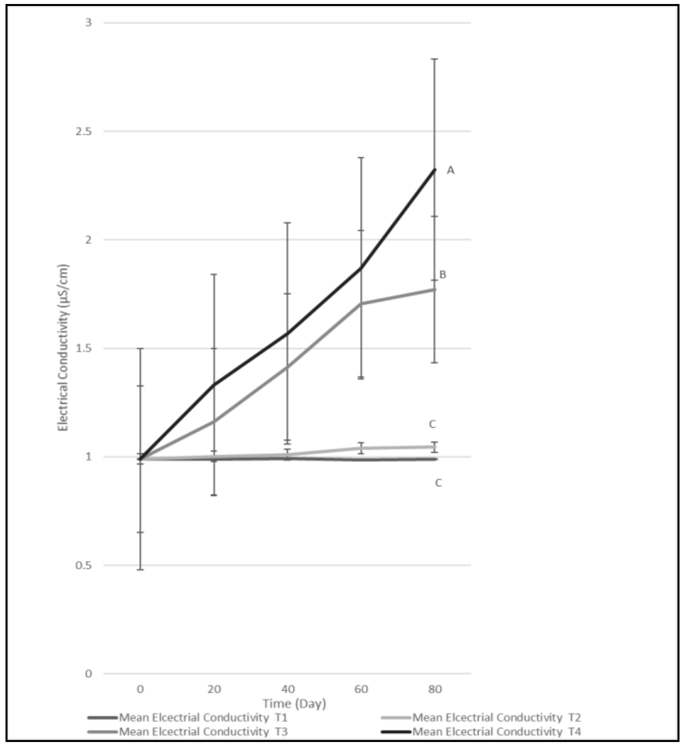

Figure 7. Mean EC (±Standard Deviation Bars) of soil under different treatment at Day 0, 20, 40, 60 and 80

Treatments 2, 3, and 4 showed an overall increase in EC over the research period having 5.56%, 78.79%, and 134.60% respectively. On examination of results between T1 and T2, it was found that there is a notable difference in the EC, though not as large as that of T3 and T4, it can be inferred that earthworms did account for an increase in the EC of soils, as previously reported by Chaduhuri *et al.* (2009). Thus, adds to the credibility of earthworms' beneficial effect on nutrient availability to plants. The high increase in EC for T3 and T4 may have been attributed to the addition of used engine oil since such an increase was not observed in the T1 and T2 which had no used engine oil added. This increase in electrical conductivity in T3 and T4 is possibly resultant to the organic constituents within the oil that acts more like insulators rather than conductors. However, the difference observed between T3 and T4 can be credited to the presence of earthworms aiding the reduction of the anoxic conditions through mechanical actions which allowed for aerobic metabolism to occur thus reducing the soil EC over time.

Statistical analysis conducted using ANOVA found that there was high statistical significance *F (3, 76) = 22.67 (p < .0001)* in the changes in EC across the various treatments. Further post hoc (LSD) test was conducted and found that there was a significant difference between T3 and T4. Thus, the presence of earthworms in soils contaminated with used engine oil had a significant positive effect on its EC.

Soil temperature

Temperature is an essential factor for the activity of most microbial organisms in soil (Nedwell, 1999). It is recognized as a necessary parameter in the biodegradation of hydrocarbons, which is influenced by the direct effect on the chemistry of the pollutant and the physiology and diversity of the microbial flora (Gibb, Chu, Wong, & Goodman, 2001; Venosa & Zhu, 2003). Moreover, temperature alters the viscosity of the oil which when reduced adversely affects bioremediation (Atlas R. M., 1995). Fogt *et al.* (1996) purported that the most suitable range for bioremediation in soil environment is 30-40^{o}C. The temperature of the soil was recorded over the study period within an average range of 24.8- 25.6^{o}C with the highest average in T3 (Figure). Though the temperature fell below the range outlined by Fogt *et al.* (1996), it was within the tolerable temperature range of 5^{o}C - 29 ^{o}C and the optimum range of 20 ^{o}C- 25 ^{o}C for good earthworm functionalities stated by Sinha *et al.* (2010). Hence, the results support the report by Sinha *et al.* (2010) since earthworms were successful in the remediation of petroleum hydrocarbons from the soil at the observed temperature.

Statistical Analysis conducted found that there was no statistical significance $F(3, 76) = 1.34$ $(p = 0.2684)$ in temperature across the various treatments. The absence of statistical significance indicates that the soil temperature was not significantly affected or altered by the presence of petroleum hydrocarbons (used engine oil) in the presence or absence of earthworm in a vermiwash system.

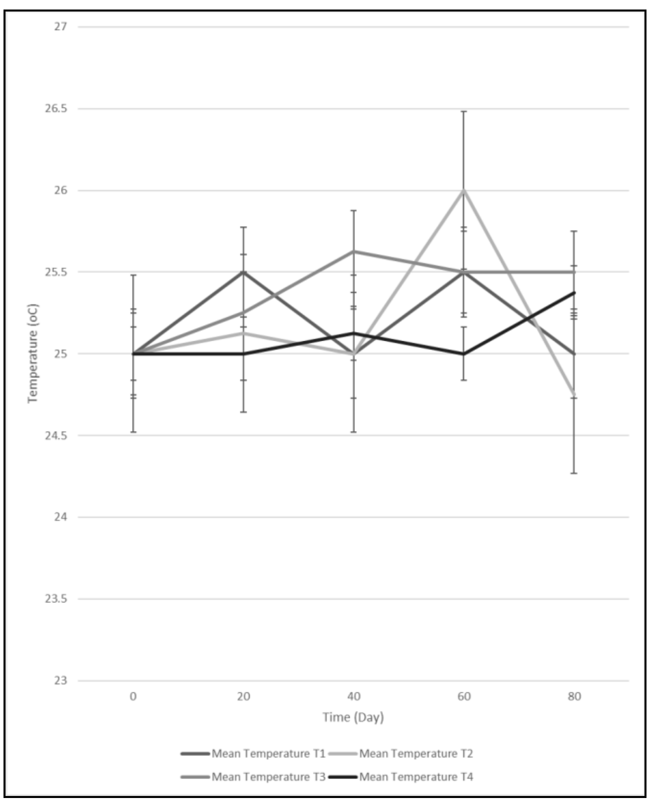

Figure 8. Mean Soil Temperature (^{o}C) (±Standard Deviation Bar) under different treatment on Day 0, 20, 40, 60 and 80

Soil nitrogen

Nitrogen is an essential nutrient required to support the productive ability of the soil. Moreover, nitrogen plays a critical role in the biochemical and physiological functions of plant development which directly affects the yield and quality of produce (Leghari, et al., 2016).

Nitrogen in soil is primarily available in organic forms. However, plants' uptake of nitrogen is facilitated by the mineralization of organic nitrogen by microorganisms in the soil (Carson & Phillips, 2018). Soil nitrogen is used in assessing the changes in the physical condition of the land by nutrient availability. The nitrogen levels in soil showed an average increase over the study period in all treatments except for T4 which had an average decrease of 21.20%; T1, T2, and T3 had corresponding increases of 7.17 %, 99.9% 13.91%. The changes in nitrogen levels across the different treatments are depicted in Figure . Earthworms enhance the nitrogen content of soil through ingestion of soil and excretion of nutrient-rich vermicast consisting of nitrogen-fixing microbes (Lee, 1985; Chaoui, Zibilske, & Ohno, 2003). Hence, the increase in soil nitrogen was observed in T2, as compared to the others.

Further, Singer *et al.* (2001) found similar results and posited that earthworms' efficiency improved under suitable conditions, which is due to their actions. The inhibitory factor that would have caused a decrease in soil nitrogen for T4 is probably the used engine oil, which contributed to the possible inhibition of nitrification. Odu *et al.* (1985) purported that the contamination of soil by petroleum hydrocarbon compounds causes an increase in hydrocarbon-degrading bacteria and a reduction in nitrifying bacteria, which may account for the reduced level of nitrogen in T4, accompanied by the presence of toxic constituents (ATSDR, 1997) that would suppress oxidation and microbial activities in the soil.

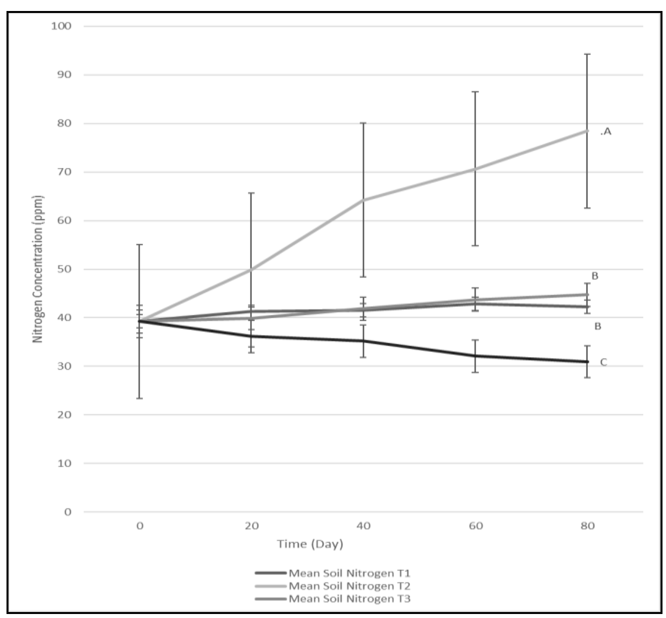

Figure 9. Mean Soil Nitrogen (±Standard Deviation Bars) under different treatment on Day 0, 20, 40, 60 and 80

Statistical analysis conducted found that there was high statistical significance *F (3, 76) = 44.59* (*P < 0.0001*) in the levels of nitrogen in soil among the different treatments. Further, a post hoc (LSD) test was conducted, which showed significant differences between T3 and T4. This result infers that earthworms made a substantial difference in mitigating the adverse effect of used engine oil on the soil nitrogen.

Soil potassium

Soil potassium is vital for plant growth and development since potassium is a macronutrient required by plants for photosynthetic function, stomal regulation, and enzyme activation (Prajapati & Modi, 2012). The presence of Potassium in natural soil is associated with their parent rocks. In agronomic practices, potassium is added through organic and inorganic fertilizers. This macronutrient is available in three forms in the soil but is primarily available to plants in solution form (Kaiser & Rosen, 2018). Soil tests for potassium were conducted to assess the nutrient status of the soil based on its classification to ascertain its health status. The concentration of potassium in the soil showed a decrease in all the treatments except T2 (Figure). Treatments T1, T3, and T4 showed an overall average reduction of 0.05 %, 1.39 %, and 12.08 % respectively whereas T2 saw an average increase of 14.72 %. Edward and Bowen (1996) asserted that earthworm casts constitute elevated levels of available potassium due to the selective feeding of earthworms on materials enriched in this cation.

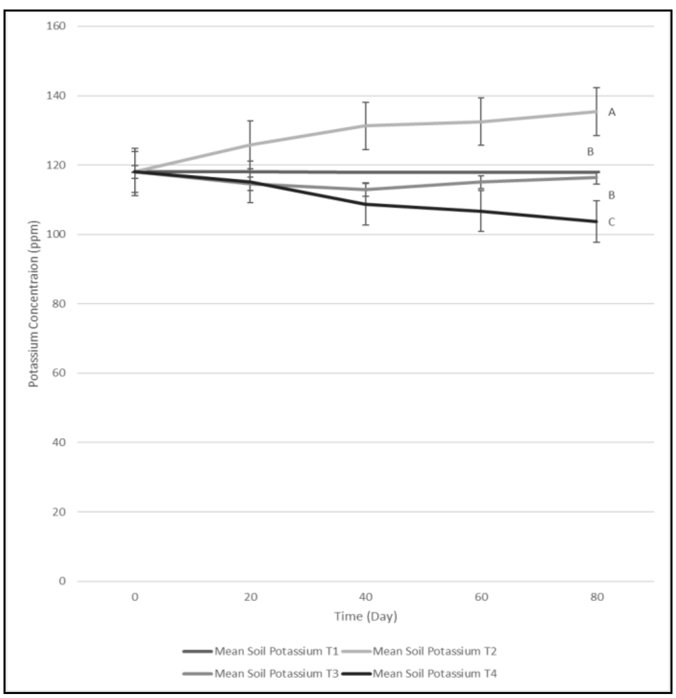

Figure 10. Mean Soil Potassium (±Standard Deviation Bars) under different treatment on Day 0, 20, 40, 60 and 80

Moreover, Edwards (2004) purported that gut transit of soil via earthworms enhances mineralization resulting in the conversion of cations, such as phosphorous and potassium to forms available for plant uptake. Manyuchi *et al.* (2013) reported similar results which found an elevated level of potassium in soil enriched with earthworms. Amadi *et al.* (1993) reported reduced levels of potassium and other essential soil nutrients in petroleum hydrocarbon contaminated soil, as seen in T4.

Statistical analysis conducted using ANOVA found that there was high statistical significance *F (3, 76) = 46.15 (p < 0.0001)* in the levels of potassium in the soil**Error! Reference source not found.**. Further post hoc test (LSD) was conducted, which found significant differences between Treatments T3 and T4.

Soil phosphorous

Phosphorous is available in soil initially from the disintegration of rocks, with varying concentrations dependent on parent rock. Phosphorus serves significant functions in plant development, inclusive of but not limited to photosynthesis, genetic transfer, and nutrient transfer (Zaidi & Khan, 2009). Soil phosphorous is credited to decaying plant material on the soil surface and anthropogenic application of fertilizer for farming practices. Organic phosphorous in soil undergoes mineralization analogous to nitrogen. However, plants uptake phosphorous primarily in its soluble forms (Menziez, 2009). The concentrations of phosphorous gradually increased across treatments except for T3 which saw some fluctuation with an overall reduction (Figure) of 4.27 %. T1, T2, and T4 saw increases of 10.36 %, 82.08 %, and 246.60 % respectively. The increase in T2 as compared to T4 may be related to earthworm facilitated nutrient cycling and nutrient-enhancing process (Edwards & Bohen, 1996). However, the rise in T4 is likely owed to the addition of used engine oil, which was reported to increase following the input of crude oil to the soil, with a subsequent reduction after inoculation of earthworms (Ekperusi & Aigbodion, 2015). T4 consisted of no earthworms, and therefore no reduction occurred. The results acquired in T4 conflicted with a report by Atlas and Bartha (1993) which showed petroleum hydrocarbon (crude oil) contaminated soil depleting in the soil phosphorous and microbial inhibition. T3 results align with reports by Ekperusi and Aigbodion (2015) and Zavala-Cruz *et al.* (2013) that suggest that earthworms improved soil quality via increased nutrient availability.

Statistical Analysis conducted found that there was high statistical significance *F (3, 76) =45.09 (p < 0.0001)* for phosphorous in the soil. Further, a post hoc (LSD) test conducted found statistical differences between T3 and T4. This result exemplifies earthworms' beneficial effect on phosphorus in soils contaminated by the used engine oil.

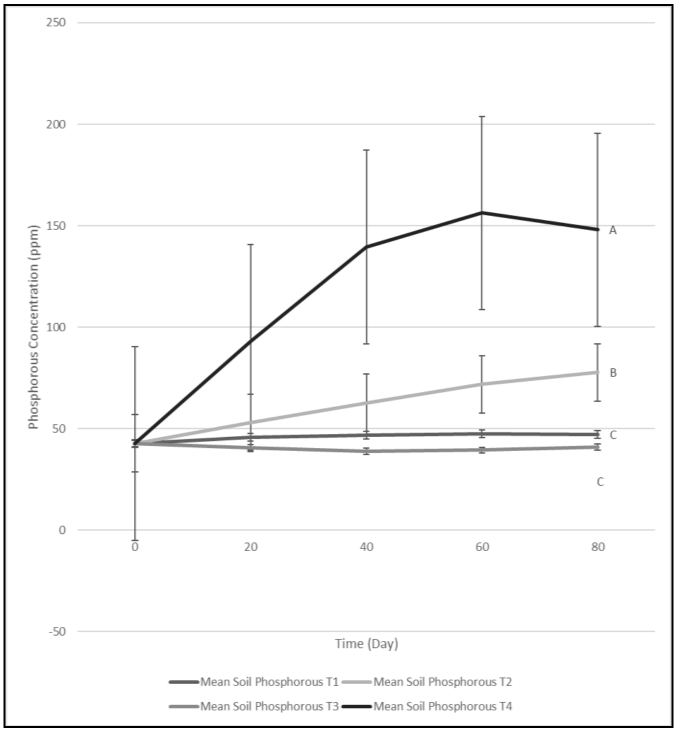

Figure 11. Mean Soil Phosphorous (±Standard Deviation Bars) under different treatment on Day 0, 20, 40, 60 and 80

Soil bacteria

Soil microbes execute a vital role in the biodegradation and bioremediation of contaminants in soils. These organisms are thought to work symbiotically with earthworms in the bioremediation of petroleum hydrocarbons (Aira, Monroy, & Domı´nguez, 2007). Some researchers theorize that soil microbes (bacteria and fungi) are primarily responsible for the biodegradation of contaminants with earthworms only acting as support catalysts (Munnoli, Teixeira da Silva, & Bhosle, 2010). The effect of petroleum hydrocarbons and earthworms on the bacteria community in soil was assessed, and three distinct colonies were preliminarily identified based on their morphological features (Table) with color (chromagenesis) being the key identifier. The CFU/g for each bacterium was tabulated at Day 0 and Day 80 for each treatment (Figure 12). At the initiation (Day 0), T4 was found to have the highest CFU /g ($4.96*10^6$) followed by T3 ($2.56*10^6$), T1 ($2.39*10^6$), and T2 ($2.05*10^6$) consecutively. At D80 T2 ($3.86*10^6$) was found to have the highest CFU /g followed by T3 ($2.89*10^6$), T1 ($2.44*10^6$), and T4 ($1.73*10^6$) respectively (Figure 12). The results showed a 65.2% reduction in CFU/g in T4, this reduction in bacteria CFU is likely consequential to the addition of used engine oil to the soil, which is a highly toxic source of petroleum hydrocarbon. The toxicity effect that petroleum hydrocarbon dispensed on soil microbes depends on the toxicity of the petroleum source (Labud, Garcia, & Hernandez, 2007). Bundy *et al.* (2002) contended that some hydrocarbons act as a source of carbon for microbial growth and activity, but this effect may not be exhibited in all soils or under different conditions. T1 had an increase of 2.30 % of CFU /g, which is indicative of the natural reproductive capacity of soil bacteria under the given circumstances, in absentia of added contaminants. T3 had an increase of 13.20% CFU /g when compared to T1 inference can be drawn on earthworm's contribution to microbial growth in the presence of petroleum hydrocarbon contamination. Hence, as reported by Wu *et al.* (2017) inference can be made that oil microbial activities and counts in soil were generally higher for bioremediation than for natural attenuation. T2 had an increase of 88.20 % of CFU /g, resultant of earthworms' ability to increase soil microorganisms by ingestion and excretion of vermicast as asserted by Edward and Fletcher (1988) and later supported by Edwards and Bohen (1996). Moreover, Munnoli *et al.* (2010) add the availability of food supplements and required conditions for their growth and reproduction as essential factors in the study.

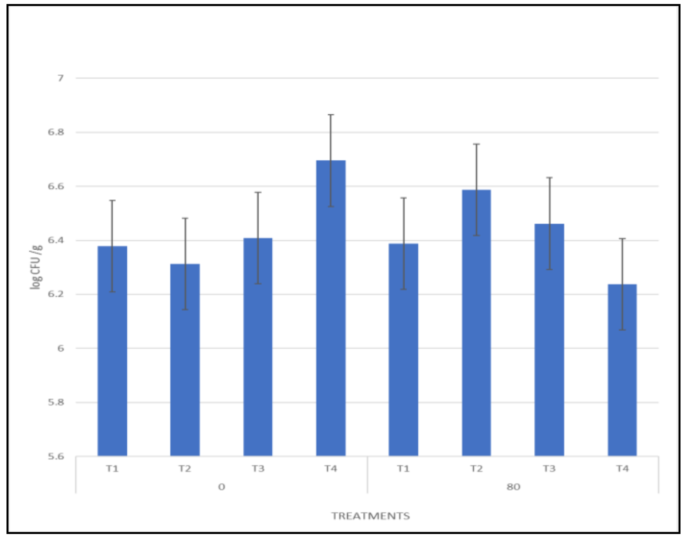

Figure 12. Bacteria Colony Forming Unit /g (±Standard Deviation Bars) under different treatment at Day 0 and 80

The Shannon Diversity (H) index was used to determine Species Richness, Diversity and Evenness at the initiation and completion of the study (**Error! Reference source not found.**5). The result showed consistency in species richness across all treatments, which implies that all three species of bacteria exhibited some tolerance for petroleum hydrocarbon contamination. Species Diversity, and Species Evenness increased in T1, T3, and T4 and decreased in T2.

Earthworm Survival

There was 100 % survival recorded in T2 and an average of 92 % survival of earthworms in T3 (Figure 13). Contreras-Ramos *et al.* (2009) found similar results using *Eisenia fetida* to remediate PAH-contaminated soil. *Eisenia fetida* sustained a highly toxic environment given an efficient food source (cow manure) under these study conditions. This high survival percentage (92 %) of earthworms observed in contaminated soil, supports the use of *Eisenia fetida* in petroleum hydrocarbon remediation of native lands and soil nutrient improvement. Their ability to sustain these conditions will aid the biodegradation and nutrient enhancement process.

TABLE 4
Morphological Characteristics of Bacteria Colonies

Colony ID	Form(Shape)	Elevation	Margin	Texture (Surface)	Opacity	Colour (Chromagenesis)	Gram Response
1W	Round	Convex	Entire	Mucoid	Translucent	White	Negative
2C	Irregular	Convex	Lobate	Smooth	Opaque	Crème	Positive
3Y	Irregular	Convex	Lobate	Mucoid	Opaque	Yellow	Positive

TABLE 5
Species Richness, Diversity and Evenness of Soil Bacteria at Initial and Final Stage of Study

Treatment	Species richness (S)		Species Diversity (H)		Species Evenness	
	Initial	Final	Initial	Final	Initial	Final
1	3	3	0.072	0.079	0.065	0.071
2	3	3	0.181	0.041	0.165	0.037
3	3	3	0.096	0.106	0.087	0.097
4	2	2	0.184	0.221	0.265	0.318

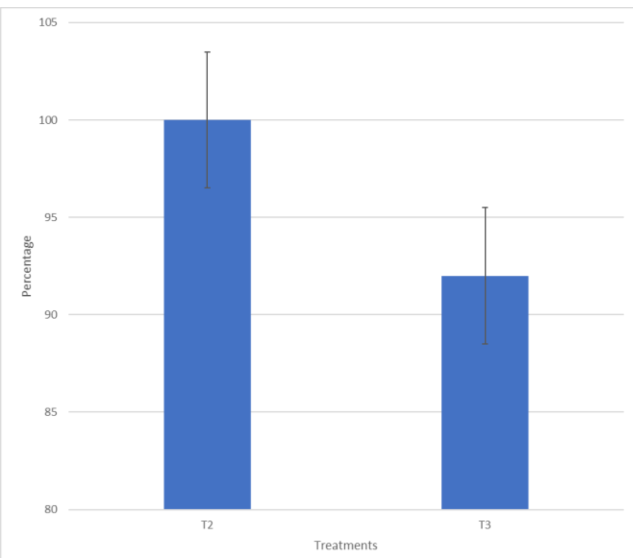

Figure 13. Percentage of earthworm survival (±Standard Deviation Bar) under different treatment

Hickman and Reid (2008), found earthworm-assisted bioremediation to be a viable approach for reclamation of soil contaminated by hazardous chemicals. They emphasized the significance of earthworm characteristics in the process, stating niche type (epigeic, endogeic, and anecic), food availability and soil type as significant environmental parameters that influence their response and behavior in soils.

Hongjian (2009) investigated the impacts of parameters; contact time on extractability, availability of petroleum hydrocarbon (hexachlorobenzene in different soils (paddy soil, red soil, and fluvo-aquic soil), and bioaccumulation in *Eisenia fetida*. The result showed that the aging rate of HCB varied depending on soil type. More so, most extractable HCB declined in the first 60 days after being spiked into soils. Paddy soil showed a higher aging rate than fluvoic-aquic soil or red soil. Hence, soil type was observed to affect earthworm bioremediation. Therefore, bioremediation studies requires specific emphasis on areas of varying soil type, geography, and climatic conditions.

Dendoovena *et al.*, (2011) found that earthworms' ability to enhance the removal of PAHs and the degradation products from soil may be limited by the number of earthworms, feed material, and moisture content of the soil. These factors are all essential for the successful bioremediation of polluted soil. They are all inextricably linked since the availability of feed material would influence the livelihood of earthworms and thus affect their survival which directly affects the quantity available for bioremediation. Further, the mechanical activity of earthworms and their casts affect soil moisture.

Krishna et al., (2011) assessed the potential of three earthworm species; *Eisenia fetida*, *Eudrillus eugeniae*, and *Anantapur species* for the bioremediation of phenol in the soil. *Eisenia fetida* and *Eudrillus eugeniae* were the most tolerant species, while the *Anantapur species* was found to be the least tolerant of the initial concentration of phenols from 20 ppm to 100 pm during the experiment. As the concentration of phenol increased from 20 ppm to 100 ppm across treatments, the bioremediation potential increased for *Eisenia fetida*, but the opposite occured in *Eudrillus eugenia and Anantapur species.* Further, *Eisenia fetida* was capable of uptaking 100 ppm of phenol from the soil within 72 hours. Hence, species type and contaminant level were limiting factors in the study. Species specificity was observed as a significant factor in bioremediation and pollutant type may also affect bioremediation based on species variation.

Al-Haleem and Khalid (2016) conducted a study that revealed the trend of degradation of total petroleum hydrocarbon in soil (when compounded with punch waste and nutrients) to increase significantly with an increase in earthworms number of five, ten, and twenty earthworms respectively per treatment and with an increase in tested days of 0, 7, 14, 28 days. The TPH concentration (20000 mg/kg) was reduced to 13200 mg/kg, 9800 mg/kg, and 6300 mg/kg in treatments with five, ten, and twenty earthworms respectively. Additionally, TPH concentration (40000 mg/kg) was reduced to 22000 mg/kg, 10100 mg/kg, and 4200 mg/kg in treatments with the above number of earthworms respectively. Hence, the factors of time and quantity of earthworms available were essential in the bioremediation process.

Guyana is now on the verge of exploiting its recently discovered petroleum hydrocarbon source. It would be amiss to overlook the potential impacts that could arise from this resource and its effect on the natural environment. Therefore, it is vital to explore preventative and remediation measures to preserve the environment. A consequence of this resource is a likely increase in the availability and use of petroleum-based products in Guyana. The improper disposal of petroleum-based products which occur daily by many small businesses and mechanic workshops is already of concern to environmental preservation. The production of petroleum products by Guyana is likely to accelerate this practice and also present the possibility of disasters faced by other countries. Thus, it is on the ideology of “failing to prepare is preparing to fail” that the researcher sought to investigate an environmentally friendly approach for the remediation of petroleum hydrocarbon in native soil. More so, the use of readily available species of earthworms to facilitate the rehabilitation of petroleum hydrocarbon from the soil will provide a cost-effective, ecologically safe, and socially acceptable method of protecting the environment.

IV. CONCLUSION

The potential for petroleum hydrocarbons bioremediation of local soils by indigenous earthworm species (*Eisenia fetida*) was evaluated. The study found that earthworms effectively reduced total petroleum hydrocarbon levels in the soil. Further, earthworms coexisted with soil bacteria from the beginning to the end of the experiment. They were thought to work synergistically to facilitate the bioremediation of petroleum hydrocarbons. Earthworms also reduced the adverse effect of used engine oil on soil parameters such as pH and EC. Moreoever they significantly improved three main macronutrients (nitrogen, phosphorus, and potassium) in soil essential for plant growth and development and increased microbial population over time. The increase in CFU/g for bacteria in soil that contained earthworms supports the cohesive relationship among these organisms in the bioremediation of petroleum hydrocarbons. The ability of earthworms to thrive under adverse conditions as indicated by their high survival percentage in soils contaminated by the used engine oil. This is a significant demonstration of the use of earthworms in environmentally safe technologies towards the clean-up of soils polluted with petroleum-based contaminants in Guyana.

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