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HISTOPATHOLOGICAL IMPACTS OF TOTAL PETROLEUM HYDROCARBON CONTAMINATED SOIL ON EARTHWORM EUDRILUS EUGENIAE

Environmental Science

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ABSTRACT

The Eudrilus eugeniae (Kinberg) species of soil-dwelling native African night crawler earthworms are a crucial variation for vermicomposting in tropical and subtropical areas. Biological functions of earthworms include eating, breaking down, and absorbing organic wastes. They then excrete castings through their metabolic system, which boosts the quantity of nutrients for plants. Earthworms are great biological indicators for toxicology studies and environmental surveillance, especially for research on the effects of pollutants on the soil ecosystem. Earthworms were treated to various concentrations of total petroleum hydrocarbon (0, 15, 30, 45, 60 and 75%) with vegetable wastes with varying percentages of effective microorganisms. According to the experiment, greater concentrations of total petroleum hydrocarbon (75%) are highly affected the histopathological alterations of earthworm and had a hazardous impact meanwhile when earthworms were exposed to 45 and 60%, interestingly low health risk of TPH to Eudrilus eugeniae and below 45% concentrations of TPH showed no menace to earthworm. Therefore, the modest hazards connected to TPH treatments on soil get a lot of intriguing attention. The results of this investing action can provide important findings about how to use locally available, potent microorganisms to upgrade TPH-contaminated soil using earthworms.

KEYWORDS

Total petroleum hydrocarbon, effective microorganisms, vermicomposting, histopathology, Eudrilus eugeniae.

INTRODUCTION

Earthworms have a significant impact on soil fertility and permeability, but little is understood about the variables that affect earthworm population, species diversity, and impact on soil processes in agricultural soil. The macro- and micronutrient-rich casting of earthworms contributes to the fertility and health of the soil. Vermicasting has significantly reduced the usage of pesticides and virtually eliminated the input of artificial fertilizers. Earthworm death, development, and reproduction have been linked to petroleum hydrocarbon pollution of agricultural soil in recent years as shown by a number of studies. Vermicomposting is becoming increasingly important in addressing the serious issues related to the disposal of vast amounts of organic waste. It serves as the best bioconversion technique for recycling organic waste and disposing of solid waste materials (Moorthi et al. 2016; Moorthi et al. 2018).

Total petroleum hydrocarbons (TPH), according to the Environmental Protection Agency, are mixtures of chemicals like gasoline, kerosene, mineral oil, etc. that are thick, dark liquids or semisolid hydrocarbon compounds that are obtained from petroleum sources (Eom et al. 2007). TPH that has been discharged into the soil might travel through the soil to the subsurface water and become trapped there as vapour. Although the addition of TPH onto the soil ecosystem to modify the ability of macro and micro fauna. Consequently, it's critical to understand how contaminated soils impact and threaten the ecological receptors (Adetutu et al. 2013; Shen et al. 2016).

According to Siddiqui et al. (2001), diesel pollution affects soil respiration, microbial population shifts, and plant germination. Hydrocarbons also impede plant development. It is becoming increasingly difficult to analyze pollution at certain sites in order to remediate, monitor these cleaning processes, and assess the ultimate quality of the soil due to the harmful consequences that soil contamination with petroleum-based chemicals has on human health. TPH is an important environmental toxin that can harm ecosystem.

Earthworms play an essential role in controlling dynamics of the soil materials and its structure. They make up a sizable fraction of the biomass of the micro fauna and are a significant part of the soil fauna ecosystems in the majority of habitats. They are segmented, tube-shaped animals that consume both living and dead organic substances. It uses the skin to carry out respiration. Earthworms have an essential effect on soil fertility and permeability, but little is understood about the variables that affect earthworm population, species diversity, and impact on soil processes in agricultural soil. The macro and micronutrient-rich casting of earthworms contributes to the fertility and health of the soil. Vermicasts have significantly decreased the usage of pesticides and virtually eliminated the input of artificial fertilizers.

Recently, a great deal of study has been done to find out the toxicity from petroleum contamination on earthworm growth, reproduction and death. On the histopathological impact of various points of view on earthworms, relatively few researches have been documented. The most effective method for identifying the effects of commercial insecticides, industrial contaminants and other wastes at the cellular level of an earthworm is histology since it offers important details on the development, destruction, and disarray of tissues. The goal of the current investigation is to ascertain how treated the soil polluted with total petroleum hydrocarbons with vegetable wastes and effective microorganisms and subsequently how affected the histopathological alterations of the earthworm E. eugeniae.

MATERIALS AND METHODS

Collection and Evaluation of Soil Contaminated with TPH

TPH leakage from moving cars and heating fuels near an oil refinery in the Erode District of Tamil Nadu, India, polluted the soil that was used for this investigation. The recovered contaminated soil was thoroughly mixed, dried for two hours at 150°C, then ground. According to accepted procedures, the samples were described in the lab (APHA 2005).

Collection of Vegetable Wastes

The vegetable wastes were collected from Chithode Market, Erode District, Tamil Nadu, India. Vegetable wastes originating from potato cabbage, cauliflower, bitter gourd, tomato, carrot, radish, ladies finger, brinjal etc. were shadow dried and assessed. After mixing completely the random wastes were collected and dried at 110°C in order to determine the dry weight of the wastes (Shah et al. 2013).

Collection and Preparation of Effective Microorganisms

The effective microorganisms (EM) were procured from Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India. Collected EM were cultured using mineral medium and molasses. The EM consists of lactic acid bacteria, yeast, actinomycetes bacteria and fermenting fungi. The various microorganisms are Streptococcus lactis, Candida utilis, Streptomyces albus, Penicillium sp. Aspergillus oryzae, and Mucor hiemalis. Effective microorganisms include high populations of lactic acid bacteria at 1×10^8 CFU mL^{-1}, yeast at 2×10^6 CFU mL^{-1}, actinomycetes at 2×10^6 CFU mL^{-1}, and fermenting bacteria at 1×10^3 CFU mL^{-1}. About 100 ml of the EM solution were introduced to 1 lt of sterile distilled water, together with 50 ml of molasses and 100 ml of mineral medium, in order to revive the EM. The pH of EM solution was tested every day for 5 to 10 days and maintained at 26±2°C temperature. The solution attained a pH from 6.5 to 4.0 and pleasant odour (Zakaria et al. 2010). In the present study, 1:100 ratio (1 part EM and 100 parts water) was used.

Preparation of Mineral Media

The media contained 2.12g of K_2HPO_4, 2.12g of KH_2PO_4, 2g of NaCl, 1g of $MgSO_4.7H_2O$, 0.1g of $CaCl_2$ and 4g of KNO_3 in 1 L of double distilled water. They were sterilized using autoclave at 120°C for 15 minutes. The media were kept at room temperature for further use. HCl or NaOH were used to keep the pH of media at 7±0.2.

Collection of Molasses

The Sakthi Sugars Factory in Erode, Tamil Nadu, India provided the molasses that was gathered. It was added to the polluted soil as source of carbon to encourage microbiological activity. 20g of molasses contains carbohydrate (14.95g), sugar (11.10g), glucose (2.38g), sucrose (5.88g), and Fructose (2.56g).

Collection and Identification of Earthworm E. Eugeniae

The vermicomposting unit at Nasiyanur, Erode, Tamil Nadu, India, is where the earthworm E. eugeniae was procured and further identified using Manual of earthworm identification (Julka, 2009). The earthworms were housed in clay pots with a capacity of 5 liters in the laboratory conditions. Cow dung was moistened at a rate of 60 to 70% and provided as an extra carbon source. Similar-sized E. eugeniae were carefully chosen from the clay pots for further investigation.

Earthworm Inoculation

Approximately 25 numbers of juvenile non-clitellated E. eugeniae earthworms with a weight at least 200-250 mg were introduced into the containers which were filled with various mixtures of soil (0, 15, 30, 45, 60 and 75%) that had been polluted with total petroleum hydrocarbons and vegetable wastes (25%). The containers added with 25% concentrations of effective microorganisms. Throughout the experiment, the humidity of the containers was kept from 35 to 40 per cent by sprinkling them with water. They were incubated at room temperature in a damp and dark environment to mature the vermicompost. To keep the worms inside and shield them from vermin, netting was placed over them.

Physico-chemical Characterization

Vermicompost was regularly collected, shade dried, ground and examined the following physico-chemical parameters like pH, electrical conductivity (EC), organic carbon (TOC), carbon/nitrogen ratio (C/N ratio), total nitrogen, phosphorus, potassium (T NPK), available nitrogen, phosphorus, potassium (A NPK), calcium (Ca) and magnesium (Mg) at regular intervals (0, 30 and 60 days). The vermicompost samples were analyzed using the following standard techniques. With a 1:10 ratio, the pH and EC in the sample water suspension were measured by a pH meter and a conductivity meter (Falcon et al. 1987). The chromic acid wet digestion method for TOC analysis (Walkley and Black 1934) and Macrokjeldahl method for the analysis of TN (APHA, 2005). TP and TK were analysed by Spectrophotometry - Vanado molybdate yellow colour method and flame photometric method (Jackson 1973) respectively. ANPK were analyzed by the methods followed by Subbiah and Asija (1956), Olsen et al (1954) and Black (1965) respectively. Triple acid digestion and extraction–versenate methods adopted for analysis of Ca and Mg respectively (Piper 1966).

Histopathological Examinations

Histopathological examinations on earthworms from soil polluted with various concentrations of TPH were undertaken in order to examine reactions to exposure in more detail. Following autopsy, the clitellar area of the earthworms from the control and experimental categories was carefully washed in physiological saline solution; cut into sections the necessary size, subsequently fixated using Bouins fixative. The tissue was placed in a 70% alcohol solution after being fixed at the ambient temperature for 24 hours. The tissues were subjected to 70% alcohol on several times until the yellow colour disappeared. After that, the tissues were washed in xylene, dipped in molten paraffin, penetrated with it, and lastly submerged in paraffin wax (58°C melting point). This process dehydrated the tissue. Dehydroxy phthalate xylol (DPX) and hematoxylin and eosin (HPE) were used to stain tissue slices that were 5 micrometers thick. The common paraffin technique was used to study the histopathology of the clitellar region of E. eugeniae (Humason 1979).

Statistical Analysis

To determine if there were significant variations between the treatments, one-way analysis of variance (ANOVA) and a low significant difference test were used at the $p<0.05$ level. The statistical analysis was done using the software SPSS V21.0.

RESULTS AND DISCUSSION

The physico-chemical values of the vermicomposted TPH contaminated soil with vegetable wastes displayed in Table 1 to 5. Different time intervals were used for the characterization (0, 30 and 60 days). In the beginning, the pH was acidic, but by the 30th day, it was almost neutral. The pH eventually approached neutral in all the treatments on 60th day (Table 1). From day 0 to day 60, EC was progressively decreasing. On day 60, over half of the EC was decreased (Table 1). When vermicompost is used as manure, its neutral pH and lower EC promote plant development. The TOC level decreased as the vermicomposting process developed. On the first day, the TOC varied in the all ratios of TPH contaminated soil. In comparison to the initial, the level of TOC was decreased on 60th day vermicomposts. The explanation for the decreased unpleasant odour of TPH could be attributed to the much lower level of TOC (Table 2). As a result, following vermicomposting, C/N ratios changed significantly from day 0 to day 60. This indicates that the continuous degradation was stronger and a suitable C/N ratio was obtained in vermicomposted TPH. C/N ratio is much lower than their initial condition and the wastes are reused as the fertilizer (Table 2).

The most important nutrients, total and available NPK contents analyzed in TPH contaminated soil with vegetable wastes are presented in Table 3 and Table 4 respectively. Higher nutrient contents were showed at the completion of vermicomposting process in all the concentrations of TPH. The increment was progressive as the days increased. In this way, the increment in nitrogen levels was found to be steady in all the concentrations. In common, the basic plant supplements, NPK, were found to extend in vermicomposted TPH. Over time, the levels of Ca and Mg dropped. The quantity on the last day was discovered to be lower than the other because the 45 and 60% of TPH already included a reduced level of Ca and Mg (Table 5). As a result, TPH vermicompost is also rich in micronutrients, proving that soil polluted by solid TPH waste may yield high-quality vermicompost.

Vermicomposting significantly changed the physicochemical properties of the different additives. Because humic compounds act as a buffer, the pH of the entire vermicompost was nearly neutral and sustained throughout the period of the 60 day maturation phase (Allison 1973). Nagavallemma et al. (2005) reported similar outcomes. Similarly the experiment on the vermicomposting of TPH revealed that the earthworm population expanded significantly and the offensive odour was reduced throughout the decomposition phase (Karmegam and Daniel 2009).

From the initial day, there was a noticeable drop in the EC value in all ratios of vermicompost. The utilization of soluble salts by efficient microorganisms for the production of microbial biomass, ingesting of soluble salts by earthworms and elevated microbial activity might all be contributing factors to the degradation of EC (Kumar and Singh 2001; Madan and Yadav 2013). Because of the enhanced microbial activity, the vermicomposted TPH showed a considerably reduced TOC, indicating higher oxidation of organic carbon. The conversion of CO2 during microbial respiration and the resulting mineralization of organic matter, which raises the total nitrogen concentration, may be the cause of TOC loss. Effective microorganisms use carbon as an energy source for the enzyme activities by breaking down organic matter (Tripathi and Bhardwaj 2004).

One of the most popular measures of vermicompost maturation is the C/N ratio, which drastically dropped as vermicomposting progressed. Vermicomposting typically has a C/N ratio of 14–22. The C/N ratio is within this range in all of the investigations that are incorporated in the present findings. Senesi (1989) said that a declining C/N ratio denotes a good degree of organic matter stabilization and an adequate quantity of organic wastes. E. eugeniae was superior at composting, as evidenced by a notably higher TN content. In comparison to other large organic manures, vermicompost has relatively high nitrogen content. The increased rate of mineralization that happens throughout the vermicomposting process could be the cause of the rise in both total and readily available NPK. With the aid of solubilizing gut-dwelling bacteria, earthworms transform insoluble and particulate organic materials into soluble forms, increasing its availability (Cabrera et al. 2005; Suthar 2009). The humification processes used in these treatments may be the reason for the noticeably larger level of nutrients found in vermicomposts (Sathisha 2000). Lower Ca and Mg values were found in the final vermicomposts made through E. eugeniae when micronutrient level was examined. Previous researchers have also noted that vermicompost has a higher concentration of micronutrients (Selladurai et al. 2010).

The fundamental anatomy of E. eugeniae was similar to that of other earthworms. From outer to inner layers, the body wall was made up of a cuticle layer, the epidermis a spherical muscle, longitudinal muscles and the peritoneum. A cuticle is a noncellular, colourless layer. One layer of columnar epithelium supported the epidermis. Movement was made possible by the circular and longitudinal muscle layer. Only adult earthworms, like E. eugeniae, which reach maturity after 10 weeks, exhibit the clitellum. Its transverse slice revealed glands that secreted mucus, cocoons, and albumin. The cocoon-secreting gland secretes a proteinaceous sleeve that encases the ovum while the mucous gland produces mucus to preserve earthworm posture during copulation. The albumin-secreting gland releases albumin to provide the embryo with nutrients.

The function of the pharynx, which is to transfer food to the gizzard, is supported by its ciliated columnar epithelium. The gizzard's thick cuticle is a result of its role in food crushing. The typhlosole involves folding the gut to improve the area available for absorption. The form of the typhlosole can vary according on the species of earthworm. The dorsal blood channel flow blood vice versa, whereas the ventral blood vessel flows the blood, antero-posterior direction of the body as well as to the majority of organs.

The present study examined the histopathological alterations of earthworms after exposure of TPH (0% 15%, 30%, 45%, 60% and 75%) with vegetable wastes (25%) (Fig 1). It has been determined that histopathological alterations in earthworms are significant indicators of toxicity (Kilic 2011; Doherty et al. 2019). Earthworms may be exposed to total petroleum hydrocarbons by dermal contact or consumption of polluted soil litter. These substances can permeate the epidermis to reach the coelomic fluid and then disperse throughout the organism's body. The present results were examined and compared the clitellum tissue samples with those of the control earthworms. After the experiment was finished, an examination of the EM concentrations of 0, 15, and 30% revealed that the clitellum was intact and positioned properly. Furthermore, all of their interior viscera were clearly defined; there were no inclusion bodies, lesions, necrosis, pigments, cancer, or inflammation (Fig 1). In the meantime, interestingly the degrees of damage at 45% and 60% of TPH concentrations were extremely low.

In the end of the experiment, the body wall in the was partially destroyed due to the rupture of the layers that comprise the wall muscle, cellular deterioration, discoloration, moderate to major lesion areas, distortion of the body's muscle structure, and erosion of both internal and external tissues in 75%. Modest lesions and disruptions of the muscle layer walls were observed in the tissues at dosages of 45 and 60%. Comparable histological alterations have been reported in the intestines of E. fetida subjected to organophosphate pesticides in earlier research (Chakra Reddy and Venkateswara Rao 2008).

The observed alterations included the rupture of the layers that make up the wall muscle, cellular degeneration, pigmentation, moderate to severe lesion regions, distortion of the structure of the muscles in the body, and eroding of both internal and exterior tissues, ultimately resulting in the complete destruction of the body wall in the . In the tissues at doses of 5, 10, 15, 20% and 25% there were mild lesions and disruptions of the muscle layer walls. Previous studies have observed similar histological changes in the intestines of E. fetida exposed to organophosphate insecticides (Chakra Reddy and Venkateswara Rao 2008).

The control earthworms showed undamaged longitudinal and circular muscles as well as an unbroken epidermis, as seen in Fig 1. The earthworms' epidermis showed only minor alterations following treatment with 15, 30, 45, and 60% of TPH; nonetheless, extensive intercellular gaps of longitudinal and circular muscles were seen. After the studies were finished, E. eugeniae tissues subjected to 75% TPH showed the worst disturbances in their muscular layers and significant tissue disorganization, which led to epidermal damage, including expanded vacuoles and distorted epithelium cells. There was additional enlargement of the intercellular gap along with atrophied and deteriorated circular and longitudinal muscles. The body wall of earthworms treated to the maximum concentration (75% of TPH) was severely damaged.

A few cells were deteriorated, and the epicuticle was disorganized. There was visible rupture, a disorganized texture, and an expanded gap in the longitudinal and circular muscle, indicating additional atrophied tissue. High doses cause toxic symptoms in earthworms, including swelling, fragmentation, curling, and yellow mucus. A study conducted in compliance with Plytycz et al. (2009) examined a similar inference.

Jeyaprakasam et al. (2021) discovered similar outcomes in earthworms exposed to malathion. This results could potentially be explained by the concept of graphine oxide accumulates within the earthworm bodies, harming the intestines and impairing digestive system performance (Yang et al. 2018). Furthermore, earthworm health depends critically on stable and balanced gut microbes (Sommer et al. 2017). Yang et al. (2018) suggested that exposure to graphine oxide may reduce the diversity of gut microbes, change the composition of the gut microbes and ultimately cause intestinal damages.

CONCLUSION

The findings indicated that harmful effects of TPH are mediated by the morphological and physical stability of tissues. Its harmful impact may be largely attributed to its impact on other biological stress and pollution markers. It also demonstrates how the contaminants negatively impacted on the fertility of the soil. However, this investigation revealed that, even at 45 and 60% TPH concentrations, less harm was being done to the earthworm E. eugeniae. The findings showed that the rate of TPH degradation was increased when earthworms and beneficial bacteria were combined.

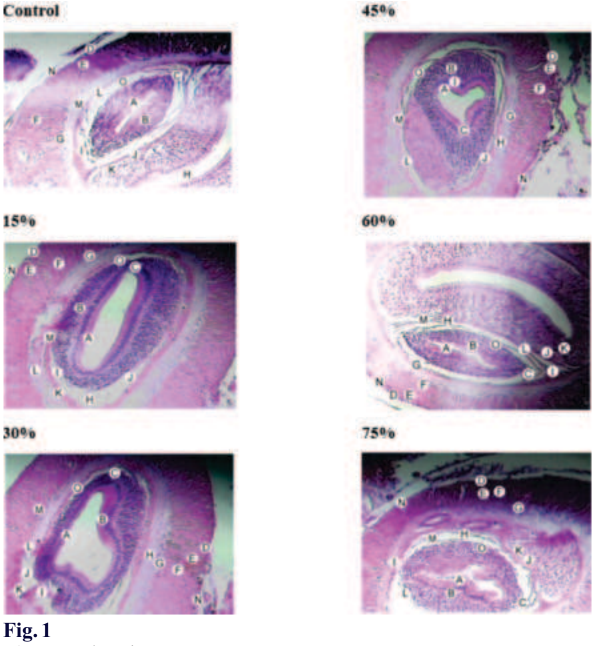

Fig. 1
A) Gastrodermis
B) Typhlosol

C) Dorsal blood vessel
D) Cuticle
E) Epidermis
F) Circular muscle
G) Longitudinal muscle
H) Coelom
I) Ventral blood vessel
J) Ventral nerve cord
K) Subneural blood vessel
L) Nephrostome
M) Nephridium
N) Setae
O) Chlorogogen cells

Table 1. pH and EC of different concentrations of total petroleum hydrocarbon contaminated soil (0, 15, 30, 45, 60 and 75%) treated with vegetable wastes (25%) and effective microorganisms using in E. eugeniae

TPH	Ph			EC (dSm-1)		
	0 day	30 days	60 days	0 day	30 days	60 days
0%	6.48±0.02	6.52±0.03	7.02±0.05	1.38±0.05	1.23±0.03	1.05±0.07
15%	6.59±0.03	6.66±0.02	7.05±0.03	1.42±0.05	1.27±0.04	0.97±0.07
30%	6.85±0.04	7.01±0.06	7.05±0.04	1.54±0.06	1.38±0.06	0.88±0.06
45%	6.96±0.03	7.08±0.04	7.18±0.02	1.60±0.06	1.48±0.04	0.95±0.06
60%	6.82±0.05	6.90±0.03	7.02±0.05	1.74±0.07	1.67±0.05	0.89±0.06
75%	6.78±0.03	6.85±0.02	6.92±0.03	1.69±0.06	1.53±0.07	0.80±0.06
P Value	0.001987*			0.00000*		

Two-way ANOVA of pH and EC
* Significance (P< 0.05)
** Insignificance (P>0.05)

Table 2. TOC and C/N ratio of different concentrations of total petroleum hydrocarbon contaminated soil (0, 15, 30, 45, 60 and 75%) treated with vegetable wastes (25%) and effective microorganisms using in E. eugeniae

TPH	TOC (mg/kg)			C/N ratio (%)		
	0 day	30 days	60 days	0 day	30 days	60 days
0%	40.36±4.05	39.51±3.03	36.39±2.05	24.02±2.01	22.84±2.04	20.44±2.03
15%	39.32±3.03	38.42±3.05	35.46±2.03	26.21±2.03	23.43±2.06	18.86±2.04
30%	38.56±3.04	37.64±2.04	34.56±3.04	24.72±2.07	22.40±2.05	18.58±3.06
45%	37.58±2.03	34.62±2.03	32.54±3.03	24.40±2.09	20.61±2.03	17.59±4.05
60%	37.48±2.05	36.56±3.05	34.48±2.05	23.72±2.04	20.77±2.08	18.15±3.04
75%	34.62±3.04	32.58±2.04	29.62±2.04	22.34±2.03	18.30±2.04	15.43±2.05
P Value	0.00000*			0.00000*		

Two-way ANOVA of TOC and C/N ratio
* Significance (P< 0.05)
** Insignificance (P>0.05)

Table 3. Total nitrogen, phosphorous and potassium of different concentrations of total petroleum hydrocarbon contaminated soil (0, 15, 30, 45, 60 and 75%) treated with vegetable wastes (25%) and effective microorganisms using in E. eugeniae

TPH	TN (mg/kg)			TP (mg/kg)			TK (mg/kg)		
	0 day	30 days	60 days	0 day	30 days	60 days	0 day	30 days	60 days
0%	1.68±0.05	1.73±0.02	1.78±0.01	0.19±0.01	0.23±0.03	0.24±0.04	0.13±0.01	0.14±0.01	0.12±0.04
15%	1.50±0.01	1.64±0.05	1.88±0.03	0.22±0.01	0.24±0.05	0.26±0.02	0.14±0.02	0.15±0.02	0.13±0.01
30%	1.56±0.01	1.65±0.01	1.86±0.01	0.23±0.02	0.25±0.01	0.26±0.01	0.13±0.02	0.14±0.04	0.15±0.01
45%	1.54±0.01	1.68±0.01	1.85±0.02	0.25±0.03	0.26±0.01	0.25±0.04	0.11±0.01	0.12±0.01	0.13±0.02
60%	1.58±0.01	1.76±0.01	1.90±0.01	0.24±0.01	0.23±0.04	0.25±0.01	0.12±0.03	0.13±0.04	0.14±0.02
75%	1.55±0.02	1.78±0.02	1.92±0.04	0.21±0.02	0.22±0.03	0.24±0.02	0.13±0.01	0.14±0.01	0.13±0.01
P Value	0.00033*			0.006867*			0.178315**		

Two-way ANOVA of Total NPK
* Significance (P< 0.05)
** Insignificance (P>0.05)

Table 4. Available nitrogen, phosphorous and potassium of different concentrations of total petroleum hydrocarbon contaminated soil (0, 15, 30, 45, 60 and 75%) treated with vegetable wastes (25%) and effective microorganisms using in E. eugeniae

TPH	AN (mg/kg)			AP (mg/kg)			AK (mg/kg)		
	0 day	30 days	60 days	0 day	30 days	60 days	0 day	30 days	60 days
0%	1.18±0.05	1.28±0.02	1.36±0.01	0.19±0.01	0.23±0.03	0.24±0.04	0.13±0.01	0.14±0.01	0.16±0.04
15%	1.26±0.01	1.38±0.05	1.42±0.03	0.22±0.01	0.24±0.05	0.26±0.02	0.14±0.02	0.15±0.02	0.18±0.01
30%	1.34±0.01	1.46±0.01	1.56±0.01	0.23±0.02	0.25±0.01	0.26±0.01	0.13±0.02	0.14±0.04	0.15±0.01
45%	1.47±0.01	1.54±0.01	1.65±0.02	0.25±0.03	0.26±0.01	0.25±0.04	0.11±0.01	0.12±0.01	0.15±0.02
60%	1.69±0.01	1.76±0.01	1.82±0.01	0.24±0.01	0.23±0.04	0.25±0.01	0.12±0.03	0.13±0.04	0.14±0.02
75%	1.59±0.02	1.65±0.02	1.78±0.04	0.21±0.02	0.22±0.03	0.24±0.02	0.13±0.01	0.14±0.01	0.16±0.01
P Value	0.00000*			0.006867*			0.00000*		

Two-way ANOVA of Available NPK
* Significance (P< 0.05)
** Insignificance (P>0.05)

Table 5. Calcium and magnesium of different concentrations of total petroleum hydrocarbon contaminated soil (0, 15, 30, 45, 60 and 75%) treated with vegetable wastes (25%) and effective microorganisms using in E. eugeniae

TPH	Ca (mg/kg)			Mg (mg/kg)		
	0 day	30 days	60 days	0 day	30 days	60 days
0%	620±8.9	548±10.1	440±10.2	452±7.8	350±9.6	178±9.6
15%	615±8.9	320±10.5	204±9.9	422±7.8	240±9.5	96±9.1
30%	608±8.9	360±10.2	190±10.0	430±7.8	230±9.5	92±9.5
45%	612±8.9	354±10.4	192±10.0	432±7.8	211±9.4	90±9.4
60%	610±8.9	380±9.9	185±10.5	428±7.8	195±9.5	82±9.6
75%	605±8.9	385±9.7	180±10.0	420±7.8	186±9.8	85±9.4
P Value	0.00000*			0.00000*		

Two-way ANOVA of Calcium and magnesium
* Significance (P< 0.05)
** Insignificance (P>0.05)

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