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RESEARCH ARTICLE

Bioremediation assessment, hematological, and biochemical responses of the earthworm (*Allolobophora caliginosa*) in soil contaminated with crude oil

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Abstract

Soil contamination with crude oil is a major environmental problem. The aim of this study was to assess whether the earthworm *Allolobophora caliginosa* could improve the degradation of petroleum hydrocarbons while enriching soils that were contaminated with crude oil. In addition, the toxic effects of crude oil on earthworms during bioremediation will be assessed. The soil samples were experimentally contaminated with two different quantities of light crude oil 5 or 7.5 ml for 60 days. Activities of *A. caliginosa* resulted in 33.56% and 54.98% total petroleum hydrocarbon (TPH) losses from the soil contaminated with 5 ml of crude oil after 22 and 60 days; respectively. While in 7.5 ml crude oil-contaminated soil, there was a loss of 32.24% and 71.05% of TPH over the same period of time. During the experiment, however, there were no signs of improvement in soil physicochemical properties. Earthworm tissue analyses at the end of the experiment showed significant bioaccumulation of petroleum hydrocarbons in their tissues and changes in their metabolic and hematological parameters. Earthworms exposed to crude oil showed a significant increase in protein, malondialdehyde, and glutathione but decreased in catalase levels and total antioxidant capacity compared to control earthworm after 60 days of exposure. There was a significant decrease in the Hgb, RBC_S, Hct, MCV, MCH, platelet count, and WBCs. As a result, the earthworm *Allolobophora caliginosa* has been shown to be good bioremediator for oil-contaminated soils and also has potential as a bioindicator for contamination.

Keywords Bioremediation · Earthworm · *Allolobophora caliginosa* · Crude oil · Antioxidant parameters · Hematological parameters

Introduction

Oil pollution in the earth's biosphere can be caused either by natural phenomena or by human activity. Petroleum pollution has the potential to damage the ecosystem. It poses risks to the characteristics of ground water, air and soil and could also harm human health (Schaefer and Juliane 2007; Lopez et al. 2008). Even at low levels of contamination, carcinogenic and mutagenic crude oil compounds could cause severe chromosomal damage due to their persistence and biological toxicity (Aguilera et al. 2010; Robertson and Hansen 2015).

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The production, transportation, storage, and exploration of oil have led to contamination of soil by Petroleum hydrocarbons (Bento et al. 2005). Crude oil is a variable mixture of thousands of compounds; alkanes, aromatics, resins, and asphaltenes being the four main fractions. Due to crude oil structural complexity, the measurements of petroleum from environmental samples are recorded as total petroleum hydrocarbons (TPHs) (Faustorilla et al. 2017).

.The presence of oil in soil may reduce aeration and water permeability by filling the soil pore spaces (Jia et al. 2017). It results in changes in soil physicochemical properties such as moisture content, pH value, total organic carbon, electric conductivity, and soil mineral nutrients, thereby indirectly affecting the development of microorganisms and plants (Wang et al. 2013; Devatha et al. 2019).

Following oil leakage to the soil, several physical and chemical techniques can be applied to reduce the level of pollution. However, these procedures are expensive, require high energy, and cause secondary contamination (Mrozik and

Piotrowska-Seget 2010). On the other hand, bioremediation has been reported as an economic and environmentally friendly method by which microorganisms with enzymatic activity are involved in the degradation of petroleum hydrocarbons into less toxic products (Wang et al. 2019; Nneji et al. 2016).

The application of earthworm in a method known as vermiremediation in order to clean polluted soil is one of the most effective bioremediation technologies. Earthworms may reduce, or contribute to the reduction of various inorganic and organic contaminants such as heavy metals, polycyclic aromatic hydrocarbons (PAHs), crude oil, and pesticides from the soil (Schaefer and Juliane 2007; Hickman and Reid 2008). Earthworms could also improve the quality of the soil they inhibit by adding vermicast to the soil after reprocessing the polluted soil in the gut of the earthworm (Drake and Horn 2007).

Several studies have shown the possible use of earthworm to enhance soil depletion of hydrocarbons. By improving microbial soil activity, earthworms have increased the elimination of PAHs: phenanthrene, benzo(a)pyrene, and anthracene from polluted soil (Contreras-Ramos et al. 2008 and 2009). Earthworms also have improved microbial biomass in oil-contaminated soil, while TPH decreased by 28 day compared to controls (Schaefer et al. 2005; Schaefer and Filser 2007). Chachina et al. (2015) reported that the use of earthworms decreased by approximately 99% of hydrocarbons content from petroleum- and diesel-contaminated soil of 20–60 g/kg, after 22 weeks.

Although it appears clear that earthworms could accelerate and increase the rate of TPH degradation, a number of variables also need to be assessed in order to establish vermiremediation as a possible technique. Further studies at higher scales are required to demonstrate the efficacy of earthworm in the degradation of crude oil fractions. Hydrocarbon exposure to earthworms is also a major challenge to the continuation of the remediation process, as light crude oil has been reported to be more harmful to earthworms than heavy crude oil (Martinkosky et al. 2016).

The need to identify and assess the effect of contamination on the quality of the environment has led to the development of biological markers. Theoretically, biomarkers have significant advantages in ecotoxicological studies. They are used as measures of adverse effects in organisms below the individual level (molecular or cellular) (Van Gestel and Van Brummelen 1996). In earthworm ecotoxicology, there are a variety of biomarkers of toxic compounds such as detoxification cation enzymes, metallothioneins, cholinesterases, oxidative stress parameters, and others (Novais et al. 2011). Examination of novel and efficient biomarkers in earthworms is advancing in soil pollutant impact assessment phase.

Allopophora caliginosais earthworm (Savigny, 1826) is the dominant local type in Egypt (El-Duweini and Ghabbour 1965). *Allopophora caliginosa* is an endogeic species, living in topsoil mineral layers in horizontal burrows. Endogeic worms consume large amounts of soil during burrowing; this feeding pattern could increase the contact of soil particles with the worm gut and result in more TPH degradation. Several studies on bioremediation using other species of earthworms have been reported. However, research on the bioremediation of crude oil using *A. caliginosa* was limited. The purposes of this study were to (1) investigate the potential of *A. caliginosa* to remove TPH from crude oil-contaminated soils, in parallel with the measurement of variations in soil physicochemical properties. (2) study the impacts of crude oil on earthworms during remediation and to critically evaluate their utility as a bioindicator for crude oil toxicity in the soil; this has been carried out based on two directions; first, determine the concentration of hydrocarbons in earthworm tissues. Second, investigate biochemical and hematological changes in the tissues of the earthworm.

Material and method

Collection of soil and acclimatization of earthworm

Soil samples were obtained from an agricultural farm in Zagazig, Sharkia Governorate, Egypt, at a depth of 10–12 cm. The soil was dark brown in color; soil texture was clay loam with 34.6% sand, 30.5% silt, and 34.9% clay (according to Gee and Bauder 1986) and with water holding capacity (WHC) 79%. The background TPH concentration was 1963 ± 14 mg/kg. The collected soil was sundried by spreading it for 48 h on a flat, clean board surface. The dried soil was sieved using a 5-mm mesh plastic filter to remove debris and large stones.

Adult *Allolobophora caliginosa* earthworms (About 90 individuals) weighing 1.5–2.8 g and with length 10–12 were also collected from the same farm by digging and hand sorting with hand trowel. The collected specimens were transported to the laboratory within an hour accompanied by the moist soil and acclimatized for 7 days in holding rectangle containers covered with netting wire and fed under controlled laboratory conditions. The experimental animals were maintained at 20 °C ± 2 °C and relative humidity 70–80%. Only adult earthworms with well-developed clitella were used in this study.

Crude oil

The crude oil used in this experiment was a light crude oil with specific gravity 0.84 and API gravity 37. The oil obtained from Misr petroleum company, Cairo, Egypt, and its characterization was performed using the standard test method of analysis ASTM (2003). The concentrations used were 5 and 7.5 ml. The concentrations used in this study were analogous to other ecotoxicological studies, such as Ekperusi and Aigbodion (2015).

Experimental design

One kilogram of sun-dried soil was weighed and placed into nine rectangular plastic boxes (1 kg in each box) (18 × 9 × 10 cm). In addition to the control, three replicates of each concentration (5 and 7.5 ml) were prepared. Using a 10-ml glass beaker, 5 and 7.5 ml of crude oil were added and extensively mixed into each of the six boxes. Treatments with crude oil-contaminated soil were extensively mixed and remained exposed to the elements in the laboratory for 7 days. After 7 days, cow dung was freshly collected by the traditional ways from the campus, and about 25 g cow dung weighed was thoroughly mixed with both controlled and contaminated soils in the boxes. Immediately after the addition of cow dung, earthworms were removed from the holding containers, washed with clean water, and then measured and weighed. Earthworms were inoculated into the crude oil polluted soils and controlled soils (10 earthworms in each container). Distilled water was added to the boxes twice a week to keep the soil moistened, with water content between 69 and 82%.

Preparation of earthworm tissues

According to the experimental plan, on day 60, earthworms from control and contaminated breeding boxes have been removed from the soil and washed. The blood samples were collected directly into K3 EDTA tubes prior to other procedures. The earthworms were placed on wet filter paper in a petri dish to get rid of their gut contents (Dalby et al. 1996). After which, they were killed rapidly by putting them on ice. A set of procedures involving the weighing and dissecting of earthworms have been executed. One gram of tissue was homogenized in phosphate-buffered saline. The homogenates were centrifuged at 8000 rpm at 5 °C and the supernatants were stored at −80 °C for biochemical analysis. In parallel, 5 g of tissue was used for determination of TPH by GC-MS system.

Determination of concentration of total petroleum hydrocarbon in soil and earthworm tissues

The TPH content was extracted twice from the treated and untreated soil samples and earthworm tissues using the method outlined by Njoku et al. (2016). Five grams of each sample were weighed, and 10 ml of the mixed solution of hexane and dichloromethane was added as a solvent with a ratio of 3:7. The mixture was shaken for 30 min using a mechanical shaker. The filtrate was purified by a pasture pipette packed with anhydrous sodium sulfate. Then, it was filtered, and the filtrate was pooled together and allowed to concentrate to 1 ml. The concentration of total petroleum hydrocarbons in soil and earthworm tissues was determined by the GC-MS system (Agilent Technologies) and was equipped with gas chromatograph (7890B) and mass spectrometer detector (5977A) at Central Laboratories Network, National Research Centre, Cairo, Egypt. The GC was equipped with HP-5MS column (30 m × 0.25 mm internal diameter and 0.25-μm film thickness). Analyses were performed using helium as a carrier gas at a flow rate of 1.0 ml/min at a splitless, injection volume of 2 μl, and the following temperature program: 50 °C for 10 min; rising at 8 °C/min to 300 °C and held for 10 min. The injector and detector were held at 280 °C and 220 °C, respectively. The data were estimated by integration of the area under the resolved chromatographic profiles using the TotalChrom software (Villas-Bôas et al. 2006).

Determination of physicochemical parameter of soil

Soil samples were taken at the three randomly replicated treatments at different exposure periods (t_0, t_{22}, t_{60}) to determine physicochemical parameters such as pH, electric conductivity (EC), nitrate (N), phosphate (P), sodium (Na) potassium (K), calcium (Ca), magnesium (Mg), and organic matter (OM). These parameters have been determined using procedures by Sparks (1996).

Antioxidant biomarkers

Malondialdehyde (MDA) was determined according to methods of Uchiyama and Mihara (1978), catalase (CAT) was measured according to Aebi (1974), total antioxidant capacity (TAC) was estimated according to Nemec et al. (2000), and glutathione (GSH) was carried out according to the method Beutler et al. (1963) according to the corresponding assay kit protocol (Sat 450, AMS, Italy). Total proteins were measured using total proteins kit according to Biuret method.

Hematological parameter

The hematological parameters; hemoglobin concentration (Hgb), red blood cell count (RBCs), hematocrit (Hct), mean cell volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC), platelet (Plt), and white blood cell count (WBCs) were measured according to the methods of Brown (1993). These parameters were obtained at once for each blood sample using an automated hematology analyzer (Celltac α/MEK-6420K).

Statistical analysis

Data were analyzed by using the SPSS version 20 (SPSS, Richmond, VA, USA). Two-way ANOVA was used to determine the impact of crude oil concentration and time exposure on TPH concentrations in soil. One-way ANOVA was used to compare the effects of crude oil concentrations on TPH accumulation, hematological parameters, and biochemical biomarkers in earthworms. Significant differences at $P < 0.05$ among treatments were conducted using Duncan test as a post hoc test.

Results

Earthworm mortality

After exposure to 7.5 ml of crude oil, the percentage of dead earthworm was 40% after 60 days (at the end of the experiment). No mortality was recorded after 60 days of exposure to 0.0 (control) or 5 ml of crude oil.

Effect of earthworm *A. caliginosa* on concentration of TPH in crude oil-contaminated soil

As shown in Table 1, the concentration of TPH was significantly reduced in soil samples with earthworm at t_{22} and t_{60} compared to the treatments without worms at t_0 ($P < 0.05$). The concentration TPH was significantly affected by crude oil concentrations and duration of exposure. In addition, the TPH concentration showed time and dose-dependent patterns. After treatment with 5 ml of crude oil, TPH decreased by 33.56 and 54.98% after 22 and 60 days of exposure. On the other hand, TPH decreased by 32.24 and 71.04 % after treatment with 7.5 ml.

Effect of earthworm *A. caliginosa* on physicochemical parameters of crude oil-contaminated soil

Results in Table 2 showed that physicochemical soil parameters decreased after crude oil treatment after 22 and 60 days of the experiment. Statistical analysis showed that there was no significant difference ($P > 0.05$) between pH, EC, Na, K, Ca, Mg, and organic matter at t_0 (soil without earthworm) and the treatments (soils with earthworms) at different concentrations. On the other side, there was a significant difference ($P < 0.05$) between nitrate, phosphate, and humidity at t_0 (soil without earthworm) and the treatments (soils with earthworms) with 5 and 7.5 ml crude oil.

Bioaccumulation of TPH in the tissues of earthworm *A. caliginosa*

The concentrations of TPH in tissues of crude oil exposed earthworms for 60 days have been shown in Fig. 1. Total petroleum hydrocarbon accumulation was significantly affected by crude oil concentrations. The highest concentrations of TPH were detected in earthworms after 60 days of exposure to 7.5 ml crude oil (216800 mg/kg) compared to control. The lowest oil residues were retained in earthworm after 60 days of exposure to 5 ml crude oil were (175,800 mg/kg) compared to control (16,400 mg/kg).

Evaluation of the effect of different concentrations of crude oil on earthworm *A. caliginosa*

Biochemical biomarkers in earthworm *A. caliginosa* exposed to different concentrations of crude oil

Table 3 shows that the levels of MDA, GSH, and TP in earthworm treated with 5 and 7.5 ml of crude oil were significantly higher than that of the control group. On the other hand, levels of CAT and TAC were significantly decreased ($P < 0.05$) in earthworm after 60 days of exposure. After exposure to 5 and 7.5 ml of crude oil, MDA, GSH, and TP were increased by 29.4–131.3%, 53.9–129.5%, and 8.9–12.7%, respectively,

Table 1 Concentrations of total petroleum hydrocarbon (mg/kg) in oil-contaminated soil samples with the earthworm *A. caliginosa* at different exposure periods

The tested concentrations (ml)	Concentrations of TPH (mg/kg soil dry w.) in oil-contaminated soil with the earthworm *A. caliginosa* at different exposure periods (days)		
	t_0	t_{22}	t_{60}
5	45,200 ± 3863[a]	30,030 ± 2566[b]	20,350 ± 1967[c]
7.5	62,410 ± 4299[a]	42,290 ± 3715[b]	18,070 ± 1359[c]
Two-way ANOVA		***F*-value**	***P*-value**
Time of exposure		49.58	0.000
Oil concentration		11.97	0.001
Concentration × time of exposure		4.367	0.02

Data are represented as means of 3 samples ± SE. Mean values with different letters are significantly different at $P < 0.05$ (two-way ANOVA and subsequent post hoc multiple comparison with Duncan's multiple range test). Mean values with the same alphabetical superscripts are not statistically significant at $P > 0.05$. t_0 oil-contaminated soil without worms at test start, t_{22} oil-contaminated soil with worms at 22 days of exposure, t_{60} oil-contaminated soil with worms at 60 days of exposure

Table 2 Physicochemical parameters of oil-contaminated soil with earthworm *A. caliginosa* through 60 days

Parameters	Conc.						
	Control	Soil after exposure to 5 ml of crude oil at different exposure periods			Soil after exposure to 7.5 ml of crude oil at different exposure periods (days)		
		t_0	t_{22}	t_{60}	t_0	t_{22}	t_{60}
PH	7.9 ± 0.00	7.7 ± 0.00	7.1 ± 0.00	6.8 ± 0.00	7.1 ± 0.00	6.9 ± 0.00	6.1 ± 0.00
EC (s/m)	1517 ± 0.00	960 ± 0.00	852 ± 0.00	688 ± 0.00	808 ± 0.00	715 ± 0.00	584 ± 0.00
N (mg/g)	2.67 ± 0.11	2.46 ± 0.10	1.94 ± 0.13	1.45 ± 0.10*	2.49 ± 0.11	1.74 ± 0.12	1.31 ± 0.10*
P (mg/kg)	30.56 ± 0.81	21.52 ± 0.57	21.45 ± 0.57	19.87 ± 0.53*	20.37 ± 0.54	19.79 ± 0.53	17.52 ± 0.57*
Na (mq/l)	2.78 ± 0.00	2.36 ± 0.00	1.95 ± 0.00	1.85 ± 0.00	2.57 ± 0.00	1.68 ± 0.00	1.74 ± 0.00
K (mq/l)	1.05 ± 0.00	0.90 ± 0.00	0.88 ± 0.00	0.74 ± 0.00	0.92 ± 0.00	0.63 ± 0.00	0.55 ± 0.00
Ca (mq/l)	5.20 ± 0.00	4 ± 0.00	3.90 ± 0.00	3 ± 0.00	3.80 ± 0.00	2.80 ± 0.00	1.20 ± 0.00
Mg (mq/l)	2.80 ± 0.00	2.10 ± 0.00	2 ± 0.00	1 ± 0.00	2.20 ± 0.00	1.89 ± 0.00	0.80 ± 0.00
OM (%)	2.24 ± 0.00	2.09 ± 0.00	2.12 ± 0.00	2.10 ± 0.00	2.09 ± 0.00	2.08 ± 0.00	1.41 ± 0.00

Data are represented as means of 3 samples ± SE

EC electric conductivity, *N* nitrate, *P* phosphate, *Na* sodium, *K* potassium, *Ca* calcium, *OM* organic matter, t_0 oil-contaminated soil without worms at test start, t_{22} oil-contaminated soil with worms at 22 days of exposure, t_{60} oil-contaminated soil with worms at 60 days of exposure

*Significant difference (Duncan test, $P < 0.05$)

while CAT levels were reduced to −16.3% and –34.9% after treatment with 5 and 7.5 ml, respectively.

Hematological parameters

Table 4 illustrates hematological indices in earthworm *A. caliginosa* after exposure for 60 days to 5 and 7.5 ml of crude oil. Hgb, RBC_S, Hct, MCV, MCH, platelet count, and WBCs were significantly decreased ($P < 0.05$) in earthworm exposed to 5 and 7.5 ml crude oil. On the other hand, MCHC value was significantly increased after exposure to 7.5 ml crude oil. The result showed that earthworm treated with 7.5 ml crude oil was more effective on hematological indices than those treated with 5 ml oil.

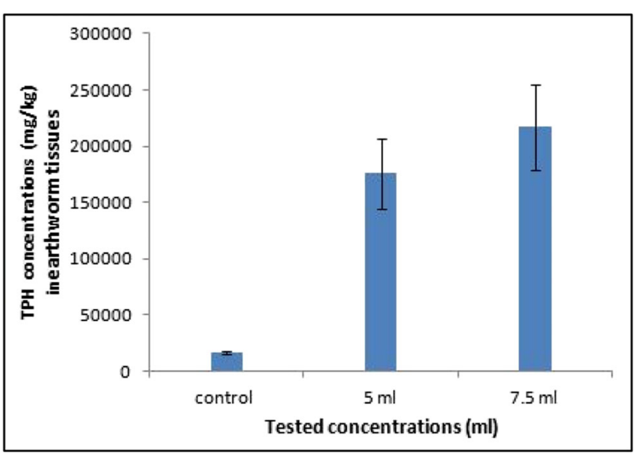

Fig. 1 Accumulation of TPH (mg/kg) inside body of the earthworm *A. caliginosa* exposed to different concentrations of crude oil for 60 days

Discussion

Earthworms are reported to either biotransform or biodegrade the inorganic and organic pollutants in the soil, rendering them nontoxic to their tissues and to the ecosystem (Sinha et al. 2010). At the end of the present study, it was found that TPH concentrations were significantly decreased by 54.98 and 71.05%, respectively, in soil samples contaminated with 5 and 7.5 ml of crude oil. These findings revealed that *Allolobophora caliginosa* inoculation has an effect on the rate of degradation of petroleum hydrocarbons in contaminated soils. This was consistent with previous research using different species of earthworm. After 28 days, the analysis of crude oil polluted soils that inhibited by three different species of earthworms: *Allolobophora chlorotica*, *Lumbricus terrestris*, and *Eisenia fetida* showed that TPH decreased between 17 and 42% (Schaefer et al. 2005). However, the activities of *Eudrilus eugeniae and L. terrestris* led to 88.50 and 76.42% TPH losses, respectively, from the soil polluted with 3 ml crude oil after treatment for 30 days (Njoku et al. 2016). In another study by Almutairi (2019) upon inoculation of *E. eugeniae* earthworm into crude oil-contaminated soil of 30,000 mg/kg, TPH dropped by 42% after 150 days. Chachina et al. (2015) reported that, in crude oil-contaminated soil with petroleum concentration of 20–60 g/kg and in the presence of *Eisenia fetida*, 99% of the hydrocarbon content had been removed after 22 weeks. However, in diesel polluted soils with a concentration of 1014 $\mu L\ kg^{-1}$, the hydrocarbons decreased by 43–52% after 90 days (Fernández et al. 2011). The reduction of hydrocarbons from

Table 3 Biochemical biomarkers in gut of earthworm *A. caliginosa* treated with different concentrations of crude oil for 60 days

Parameter	Concentrations of crude oil (ml)			*F*-value	*P*-value
	0.0 control	5	7.5		
TP (mg/gm)	0.79 ± 0.03^{a}	0.86 ± 0.01^{b}	0.89 ± 0.01^{b}	10.64	0.04
MDA (nmol/g)	1.6 ± 0.1^{a}	2.07 ± 0.16^{ab}	3.70 ± 0.70^{b}	13.73	0.03
CAT (U/g)	0.86 ± 0.02^{a}	0.72 ± 0.02^{b}	0.56 ± 0.03^{c}	39.77	0.007
TAC (μmol/l)	0.17 ± 0.01^{a}	0.09 ± 0.02^{b}	0.06 ± 0.01^{b}	16.17	0.02
GSH (μg/mg)	18.60 ± 1.40^{a}	28.63 ± 1.97^{b}	42.68 ± 1.33^{c}	57.76	0.004

Each value is mean of 3 samples ± SE. Mean values with different alphabetical superscripts at each row differed significantly at $P < 0.05$ (one-way ANOVA and subsequent post hoc multiple comparison with Duncan's multiple range test). Mean values with the same alphabetical superscripts are not statistically significant at $P > 0.05$

TP total proteins, *MDA* malondialdehyde, *CAT* catalase, *TAC* total antioxidant capacity, *GSH* glutathione

contaminated soil may be attributed to the mineralization of petroleum products with the aid of earthworms (Contreras-Ramos et al. 2008). However, Dabke (2013) informed that increasing the activity of microorganisms in the digestive tract of earthworms may lead to clean up of hydrocarbons. Another possible mechanism for hydrocarbon degradation was the stimulation of soil microbe activity as a result of earthworm activity (Schaefer et al. 2005). In this study, the loss of TPH in soil treatments could be due to any or a combination of such mechanisms.

This paper found that *Allolobophora caliginosa* earthworms have a potential for decomposition of total petroleum hydrocarbons (TPHs) in crude oil-contaminated soils ranging from 45,200 to 62,410 mg/kg. It was noted that the TPH of 45,200 mg/kg was not harmful to earthworm survival, but TPH of 62,410 mg/kg reduced their survival to 40% after 60 days. This means that they can tolerate high concentrations of hydrocarbons in contaminated soils compared to other species mentioned in previous studies. According to Hentati et al. (2013), earthworm *Eisenia andrei* reported a 67% mortality rate after 14 days when exposed to a concentration of 1000 mg/kg of oil-contaminated soil. Another research also concluded that 0.5% of the oil content had no toxic impact on the survival of *Eisenia fetida* earthworm, but when the concentration was increased to 1.5%, the survival rate decreased by 40% after 7 days (Shakir Hanna and Weaver 2002). Thirty percent of *Eisenia fetida* died after exposure to 40,000 mg kg^{-1} concentration of diesel after 14 days (Chachina et al. 2016). Another study with the same earthworm, contaminant, and exposure time showed that there was no earthworm survived at 30,000 mg kg^{-1} (Moon et al. (2013). The lower mortality rate in this study could be due to the physiological adaptation of earthworms to the pollutants and conditions.

In this study, all measured soil physicochemical parameters: PH, OM, EC, and soil mineral nutrients decreased after crude oil treatment, and this decrease continued overtime for

Table 4 Hematological parameters of earthworm *A. caliginosa* treated with different concentrations of crude oil for 60 days

Parameters	Concentrations of crude oil (ml)			*F*-value	*P*-value
	Control	5	7.5		
Hgb (g/dl)	2.7 ± 0.1^{a}	2 ± 0.2^{b}	1.3 ± 0.1^{c}	24.50	0.01
RBC_S (million/ul)	1 ± 0.02^{a}	0.96 ± 0.03^{ab}	0.90 ± 0.02^{b}	7.94	0.06
Hct (%)	9 ± 0.5^{a}	6 ± 0.2^{b}	4 ± 0.71^{c}	32.36	0.009
MCV (fl)	90.5 ± 1.5^{a}	85 ± 2.5^{a}	44.4 ± 2.68^{b}	123.04	0.001
MCH (pg)	27 ± 2.5^{a}	21.78 ± 1.22^{b}	14.25 ± 0.75^{b}	15.96	0.03
MCHC (g/dl)	30 ± 0.54^{a}	30.61 ± 0.40^{a}	32.5 ± 0.14^{b}	16.74	0.02
Plt ($\times 10^3$/ul)	150 ± 10^{a}	124.5 ± 5.5^{a}	91 ± 4^{b}	17.96	0.02
WBC_S ($\times 10^3$/ul)	54.8 ± 1.18^{a}	47.9 ± 2.1^{a}	11.5 ± 1.5^{b}	202.1	0.001

Each value is mean of 3 samples ± SE. Mean values with different alphabetical superscripts at each row differed significantly at $P < 0.05$ (one-way ANOVA and subsequent post hoc multiple comparison with Duncan's multiple range test). Mean values with the same alphabetical superscripts are not statistically significant at $P > 0.05$

Hgb hemoglobin, RBC_S red blood cells, *Hct* hematocrit, *MCV* mean cell volume, *MCH* mean cell hemoglobin, *MCHC* mean cell hemoglobin concentration, *Plt* platelet, WBC_S white blood cells all treatments. This could mean that, due to the increase in soil worm activity in the contaminated soil to improve remediation process, the soil properties tend to decrease. This was supported by the results of Ceccanti et al. (2006) which detected a decrease in EC, total carbon, and phosphorous of soil polluted by hydrocarbon, indicating a progressive decomposition of the contaminants. However, the use of organic carbon as a source of energy by earthworms and microorganisms has led to a reduction in organic carbon in polluted soils (Azizi et al. 2013).

Earthworms perform different functions in soil. They recover soil structure and contribute to the decomposition of organic matter and nutrient cycling. They also enhance the biological, physical, and chemical characteristics of the soil in order to improve its fertility (Richardson et al. 2009). According to Ekperusi and Aigbodion (2015), it was found that earthworms *Hyperiodrilus africanus* significantly changed the physicochemical characteristics of the crude oil-contaminated soil after 12 weeks. This was not in accordance with our observations, as by the end of 60 days, there were no signs of improvement in the soil, and the results are still so far from the standard levels of control soil. Also, there was a significant decrease in nitrate and phosphate concentrations which was proportional to the crude oil concentration. This finding proved the presence of a correlation between the nutrient level in the soils and their petroleum hydrocarbon content. The TPH in the soil could increase the carbon concentration which in turn could influence the nutrients equilibrium in the soil (Devatha et al. 2019). However, Njoku et al. (2016) reported that the more take-up of nutrients by earthworms, the more influence they will have on soil pollutant content and consequently more remediation. Phosphorus is an essential micronutrient for soil fauna; the reduction in soil levels may be attributed to the soil microbes that could utilize large amounts of AP during the degradation of hydrocarbons (Wang et al. 2009). However, reduction in soil nitrate in soils contaminated with petroleum hydrocarbons could be due to earthworms, because they absorb considerable quantities of nitrogen for digestion in order to maintain their biological function (Iordache and Borza 2012; Sandor and Schrader 2012).

Earthworms are known as good bioindicators of soil quality. Endogenic species can tolerate a wide range of soil contaminants than epigeic ones (Paoletti 1999). Epigeic earthworms are known to be able to degrade or accumulate hydrocarbons (Contreras-Ramos et al. 2009; Parrish et al. 2006). The results of this study further confirmed the suggestion that endogenic species would be useful to the same extent or to a greater extent than epigeal species (OECD 2010). Significant amounts of TPH were detected in tissues of *A. caliginosa* at the end of the experiment when compared to the control. This point suggested that the accumulation of petroleum hydrocarbons in earthworm tissues could be another possible mechanism for accelerating TPH degradation in the soil.

Hydrocarbon accumulation was found to be higher in soil polluted with 7.5 ml of crude oil than in soil polluted with 5 ml; this may be due to body wall or intestinal absorption of soil hydrocarbons. According to Belfroid et al. (1996), the earthworms accumulate several lipophilic organic compounds from their surrounding soil by passive absorption via the body wall and even by intestinal absorption during the passage of the soil through the intestine. This deposition increased as the surrounding soil content of the pollutant increased. The results of this study were in accordance with Ekperusi and Aigbodion (2015), who detected elevated amounts of TPH and BTEX in earthworm tissues after 12 weeks from exposure to crude oil.

Earthworms through their body walls as well as by intestinal surfaces are always exposed to pollutants in the soil. Thus, they have a consistent detoxification system for their survival (Kilic 2011). Biochemical examinations were used to evaluate the possible mode of action of stressors and the ability of the organism to adapt to the external environment (Abdel–Tawwab, M. 2016). Many biochemical parameters are estimated to be used as biomarkers in the present study. These include total protein, antioxidant enzyme, and hematological parameters.

Proteins play an essential role in metabolic pathways and are used as a diagnostic tool to assess the physiological status of the organism (Prasath and Arivoli 2008). The current study clearly showed that total proteins increased significantly in earthworm *A. caliginosa* treated with crude oil. This increase may be due to cellular defense mechanisms that are stimulated to resist these toxicants (El-Sayed et al. 2020).

Antioxidant enzyme activities are found broadly distributed in the tissues of invertebrate organisms (Livingstone 2001). Assessing antioxidant enzymes can demonstrate the antioxidant status of organisms by using them as biomarkers for contaminant-mediated oxidative stress. Furthermore, they are suitable biomarkers reflecting not only exposure to contaminants but also their toxicity (Valavanidis et al. 2006). Malondildhyde (MDA) is one of the final products of cell peroxidation of polyunsaturated fatty acids and is used as a biomarker for oxidative stress (Davey et al. 2005). This study showed a significant increase in MDA levels in *A. caliginosa* earthworm after exposure to crude oil. Similarly, Doherty et al. (2019) found that MDA levels increased in earthworm, *Eudrilus eugeniae*, after exposure to benzene, toluene, ethylbenzene, and xylene. This increase may be due to an increase in free radicals that cause MDA overproduction.

Catalase (CAT) provides the first line of protection against reactive oxygen species and is used as an oxidative stress biomarker (Van der Oost et al. 2003). In the present study, CAT activity was decreased in earthworm *A. caliginosa* after exposure to crude oil for 60 days. Such decreases in CAT activities may result from the accumulation of H_2O_2 levels. In the same study, Doherty et al. (2019) found that CAT activity decreased in the earthworm, *Eudrilus eugeniae*, after exposure to benzene, toluene, ethylbenzene, and xylene for 28 days. Total antioxidant capacity (TAC) is used to assess the antioxidant status of the organism and to evaluate the antioxidant response against the free radicals produced in the tissue sample (Richetti et al. 2011). The present study showed a decline in TAC levels for crude oil-exposed earthworms. This decrease may be due to its protective role against damages induced by free radicals. GSH maintains the redox status of the cell and protects cells from oxidative stress (Dickinson and Forman 2002). The GSH levels in this study have been significantly increased. These results were contrary to the findings of Doherty et al. (2019) who stated a decrease in GSH levels in the earthworm, *Eudrilus eugeniae*, after exposure to benzene, toluene, ethylbenzene, and xylene for 28 days. This increase in GSH levels can be attributed to their protective functions against oxidative stress.

Hematological changes are reported to be early warning indicators of the toxic effects of pollutants in vertebrates (Dauwe et al. 2006); however, they are not well studied in invertebrates and in earthworms for comparison. Earthworms have a circulatory system which is closed. Blood consists of hemoglobin, a large extracellular hemoprotein that flows through a closed circulatory system. Despite the fundamental function of the respiratory pigment in the physiology of the earthworm, little is known about its susceptibility to environmental contaminants. In this study, a significant decrease in the Hgb, RBC_S, Hct, MCV, MCH, platelet count, and WBCs was detected in earthworms exposed to crude oil (5 and 7.5 ml) for 60 days when compared with the control. This was in agreement with the findings of Calisi et al. (2011) which showed that heavy metals including cadmium, mercury, and copper cause changes in hematological parameters in the earthworm *Lumbricus terrestris*. Finally, it can be said that the blood analysis can be suitable biomarker of exposure/effect to be involved in a multibiomarker strategy in earthworm in soil monitoring assessment.

The present study has shown that *Allolobophora caliginosa* earthworms along with their success in being a good bioremediator have been able to tolerate high concentrations of hydrocarbons in crude oil-contaminated soils compared to other species mentioned in previous studies. Additionally, the severe effects detected in antioxidant and hematological parameters in earthworms during the remediation process have not often been discussed in this type of subject in previous studies.

Conclusion

The results of this study have shown that the endogenic earthworms *Allolobophora caliginosa* have the potential to clean up petroleum hydrocarbons from crude oil polluted soils. Higher removal efficiency was found in soils containing 7.5 ml crude oil compared to 5 ml. In order to achieve a higher level of remediation and therefore restore the physical-chemical properties of contaminated soils, longer experiments would be needed as 60 days were not sufficient time for complete degradation. Also, it was found that the significant accumulation of petroleum hydrocarbons in earthworm tissues could lead to severe effects in their hematological and biochemical parameters.

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Author contribution S.E.Nassar conceived and designed the experiment. R. M.Said conceived and performed the experiment. All authors contributed in writing, reading, and approving the final manuscript.

Data availability Data included in the article.

Declarations

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References

Abdel–Tawwab, M. (2016) Effect of feed availability on susceptibility of Nile tilapia, *Oreochromis niloticus* (L.) to environmental zinc toxicity: growth performance, biochemical response, and zinc bioaccumulation. Aquac. 464:309–315

Aebi, H., 1974. Catalase. Methods enzymatic analysis .In: Bergmeyer HU (Ed.),Academic Press. New York, 673-678.

Aguilera F, Méndez J, Pásaro E, Laon B (2010) Review on the effects of exposure to spilled oils on human health. J Appl Toxicol 30:291–301

Almutairi M (2019) Vermiremediation strategy for remediation of Kuwaiti oil contaminated soil. Appl Sci 1:1312. https://doi.org/10.1007/s42452-019-1297-3

ASTM (2003). "Annual book of standard petroleum products and lubricants:. American Society For Testing And Material,Philadphia, USA.

Azizi AB, Liew KY, Noor ZM, Abdullah N (2013) Vermiremediation and mycoremediation of polycyclic aromatic hydrocarbons in soil and sewage sludge mixture: a comparative study. J Environ Sci Devel 4(5):565–568. https://doi.org/10.7763/IJESD.2013.V4.414

Belfroid A, Sijm D, Van Gestel C (1996) Bioavailability and toxicokinetics of hydrophobic aromatic compounds in benthic and terrestrial invertebrates. Environ Rev 4:276–299

Bento FM, Camargo FAO, Okeke BC, Frankenberger WT (2005) Comparative bioremediation of soils contaminated with diesel oil by natural attenuation, biostimulation and bioaugmentation. Bioresour Technol 96:1049–1055

Beutler E, Duron O, Kelly BM (1963) Improved method for the determination of blood glutathione. J.Lab.Clin.Med 61:882–890

Brown BA (1993) Haematological principles and procedures, 6th edn. Lippincot, Williams & Wilking. Philadelphia

Calisi A, Lionetto MG, Sanchez-Hernandez JC, Schettino T (2011) Effect of heavy metal exposure on blood haemoglobin concentration and methemoglobin percentage in Lumbricus terrestris. Ecotoxicol. 20(4):847–854

Ceccanti B, Masciandaro G, Garcia C, Macci C, Doni S (2006) Soil bioremediation: combination of earthworms and compost for the ecological remediation of a hydrocarbon polluted soil. Water Air Soil Pollut 177(1–4):383–397

Chachina SB, Voronkova NA, Baklanova ON (2015) Biological remediation of the engine lubricant oil-contaminated soil with three kinds of earthworms, *Eisenia Fetida*, *Eisenia Andrei*, *Dendrobena Veneta*, and a mixture of microorganisms. Procedia Eng 113:113–123

Chachina SB, Voronkova NA, Baklanova ON (2016) Biological remediation of the petroleum and diesel contaminated soil with earthworms *Eisenia Fetida*. Procedia Eng 152:122–133

Contreras-Ramos SM, Alvarez-Bernal D, Dendooven L (2008) Removal of polycyclic aromatic hydrocarbons from soil amended with biosolid or vermicompost in the presence of earthworms (*Eisenia fetida*). Soil Biol. Biochem. 40:1954–1959

Contreras-Ramos SM, Alvarez-Bernal D, Dendooven L (2009) Characteristics of earthworms (*Eisenia fetida*) in PAHs contaminated soil amended with wastewater sludge or vermicompost. Appl Soil Ecol 41:269–276

Dabke SV (2013) Vermiremediation of heavy metal-contaminated soil. Blacksmith Institute. J Healt Pollut 3(4):4–10. https://doi.org/10.5696/2156-9614-3.4.4

Dalby P, Baker GH, Smith SE (1996) "Filter paper method" to remove soil from earthworm intestines and to standardise the water content of earthworm tissue. Soil Biol Biochem 28:685–687

Dauwe T, Janssens E, Eens M (2006) Effects of heavy metal exposure on the condition and health of adult great tits (*Parus major*). Environ Pollut 140(1):71–78

Davey MW, Stals E, Panis B, Keulemans J, Swennen RL (2005) High-through put determination of malondialdehyde in plant tissues. Anal Biochem 347(2):201–207

Devatha CP, Vishnu Vishal A, Purna Chandra Rao J (2019) Investigation of physical and chemical characteristics on soil due to crude oil contamination and its remediation. ApplWater Sci 9(4). https://doi.org/10.1007/s13201-019-0970-4

Dickinson DA, Forman HJ (2002) Cellular glutathione and thiols metabolism. Biochem Pharmacol 64:1019–1026

Doherty VF, Idowu A, Otitoloju AA (2019) Histopathological and biochemical alterations in Eudrilus eugeniae (Kinberg 1867) as biomarkers of exposure to monocyclic aromatic hydrocarbons in oil impacted site. J Basic Appl Zool 80:63

Drake H, Horn M (2007) As thewormturns: the earthworm gut as a transient habitat for soil microbial biomes. Annu Rev Microbiol 61:169–189

Ekperusi OA, Aigbodion FI (2015) Bioremediation of petroleum hydrocarbons from crude oil-contaminated soil with the earthworm. *Hyperiodrilus africanus Biotech* 5(6):957–965

El-Sayed, A.S., Fathalla, M., Yassin, M.A., Zein, N., Morsy, S., Sitohy, M., Sitohy, B., .(2020). Conjugation of *Aspergillus flavipes* taxol with porphrin increases the anticancer activity of taxol and ameliorates its cytotoxic effect. Molec. 25 (263).

Faustorilla MV, Chen Z, Dharmarajan R, Naidu R (2017) Determination of total petroleum hydrocarbons in Australian groundwater through the improvised gas chromatography–flame ionization detection technique. J Chromatogr Sci 55(8):775–783

Fernández MD, Pro J, Alonso C, Aragonese P, Tarazona JV (2011) Terrestrial microcosms in a feasibility study on the remediation of diesel-contaminated soils. Ecotoxol Environ Saf 74:2133–2140

Gee GW, Bauder JW (1986) Particle size analysis. In: Klute A (ed) Methods of soil analysis. Part 1. Physical and mineralogical methods. 2nd edition. Soil Science Society of America Inc. American Society of Agronomy, Inc., Madison, WI, pp 383–411

El-Duweini,A.K. and Ghabbour, S.I., 1965. Population density and biomass of earthworms in different types of Egyptian soils. J Appl Ecol 2(2), 271-287.

Hentati O, Lachhab R, Ayadi M, Ksibi M (2013) Toxicity assessment for petroleum-contaminated soil using terrestrial invertebrates and plant bioassays. Environ Monit Assess 185:2989–2998

Hickman ZA, Reid BJ (2008) Earthworm assisted bioremediation of organic contaminants. Environ Int 34(7):1072–1081

Iordache M, Borza I (2012) The bioremediation potential of earthworms (Oligochaeta: Lumbricidae) in a soil polluted with heavy metals. J Food Agric Environ 10(2):1183–1186

Jia J, Zong S, Hu L, Shi S, Zhai X, Wang B, Li G, Zhang D (2017) The dynamic change of microbial communities in crude oil-contaminated soils from oil fields in China. J Soil Contam 26: 171–183

Kilic AK (2011) Histopathological & biochemical alterations of earthworm (*Lumbricus terrestris*) as biomarker of soil pollution along Porsuk River Basin (Turkey). Chemo. 83(8):1175–1180

Livingstone DR (2001) Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. Mar Pollut Bull 42(8):656–666

Lopez E, Schuhmacher M, Domingo J (2008) Human health risks of petroleum-contaminated groundwater. Environ Sci Pollut Res Int 15:278–288

Martinkosky L, Barkley J, Sabadell G (2016) Earthworms (*Eisenia fetida*) demonstrate potential for use in soil bioremediation by increasing the degradation rates of heavy crude oil hydrocarbons. Sci Total Environ 580:734–743

Moon Y, Yim U-H, Kim H-S, Kim Y-J, Shin WS, Hwang I (2013) Toxicity and bioaccumulation of petroleum mixtures with alkyl PAHs in earthworms. Hum Ecol Risk Assess 19:819–835

Mrozik A, Piotrowska-Seget Z (2010) Bioaugmentation as a strategy for cleaning up of soils contaminated with aromatic compounds. Microbiol Res 165:363–375

Nemec A, Drobnič-Košorok M, Skitek M, Pavlica Z, Galac S, Butinar J (2000) Total antioxidant capacity (TAC) values and their correlation with individual antioxidants in healthy beagles. Acta Vet Brno 69: 297–303

Njoku KL, Akinola MO, Anigbogu CC (2016) Vermiremediation of soils contaminated with petroleum products using *Eisenia fetida*. J Appl Sci Environ Manag 20(3):771–779

Nneji, L.M., Somade, O.T., Adeyi, A.O., 2016. Earthworm-assisted bioremediation of petroleum hydrocarbon–contaminated soils from motorcar mechanic workshops in Ibadan, Oyo State, southwestern Nigeria. Bioremediat .J. 20: 4, 263-285, DOI: https://doi.org/10.1080/10889868.2016.1250723.

Novais SC, Gomes SIL, Gravato C, Guilhermino L, De Coen W, Soares AMVM, Amorim MJB (2011) Reproduction and biochemical responses in *Enchytraeus albidus* (Oligochaeta) to zinc or cadmium exposures. Environ Pollut 159(7):1836–1843

OECD, 2010.Guidline for the testing of chemicals: bioaccumulation in terrestrial Oligochaetes. OECD.Paris.France.

Paoletti MG (1999) The role of earthworms for assessment of sustainability and bioindicators. Agric Ecosyst Environ 74:137–155

Parrish, Z.D., White, J.C., Isleyen,M., Gent, M., Iannucci-Berger,W., Eitzer, B.D., Kelsey, J.W., Mattina, M.I., 2006. Accumulation of weathered polycyclic aromatic hydrocarbons (PAHs) by plant and earthworm species. Chemosphere .64: 609–618.

Prasath PMD, Arivoli S (2008) Biochemical study of freshwater fish *Catla catla* with reference to mercury chloride. Iran J Environ Health Sci Eng 3:109–116

Richardson DR, Snyder BA, Hendrix PF (2009) Soil moisture and temperature: tolerance and optima for a non-native earthworm species *Amyntas agrestis* (oligochaeta:Opisthopora: Megascolecidae). Southeast Nat 8:325–334

Richetti SK, Rosemberg B, VenturaLima J, Monserrat JM, Bogo MR, Bonan CD (2011) Acetylcholinesterase activity and antioxidant capacity of zebrafish brain is altered by heavy metal exposure. Neurotoxicol 32(1):116–122

Robertson LW, Hansen LG (2015) PCBs: Recent advances in environmental toxicology and health effects. The University Press of Kentucky, Lexington, KY, USA, pp 511–512

Sandor, M., Schrader, S., 2012. Interaction of earthworms and Enchytraeids in organically amended soil. North-Western. J .Zoo. 8(1), 46–56.

Schaefer M, Filser J (2007) The influence of earthworms and organic additives on the biodegradation of oil contaminated soil. Appl Soil Ecol 36(1):53–62

Schaefer M, Juliane F (2007) The influence of earthworms and organic additives on the biodegradation of contaminated soil. Appl Soil Ecol 36:53–62

Schaefer M, Petersen SO, Filser J (2005) Effects of *Lumbricus terrestris*, *Allolobophora chlorotica* and *Eisenia fetida* on microbial community dynamics in oil-contaminated soil. Soil Biol Biochem 37(11): 2065–2076

Shakir Hanna SH, Weaver RW (2002) Earthworm survival in oil contaminated soil. Plant Soil 240:127–132

Sinha RK, Chauhan K, Valani D, Chandran V, Soni BK, Patel V (2010) Earthworms: Charles Darwin's 'unheralded soldiers of mankind': protective and productive for man and environment. J Environ Prot Sci 1:251–260

Sparks, D.L., 1996. "Methods of soil analysis", part 3-chemical methods, (2nd Ed.), Agron. 9: A.S.A., Ins., Madison, Wisc., USA.

Uchiyama M, Mihara M (1978) Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. Anal Biochem 86:271–278

Valavanidis A, Vlahogianni T, Dassenakis M, Scoullos M (2006) Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. Ecotoxicol Environ Saf 64:178–189

Van der Oost R, Beyer J, Vermeulen NPE (2003) Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environ Toxicol Pharmacol 13:57–149

Van Gestel CAM, Van Brummelen TC (1996) Incorporation of the biomarkers concept in ecotoxicology calls for a redefinition of terms. Ecotoxicol. 5:217–225

Villas-Bôas SG, Noel S, Lane GA, Attwood G, Cookson A (2006) Extracellular metabolomics: a metabolic footprinting approach to assess fiber degradation in complex media. Anal Biochem 349: 297–305

Wang X, Feng Jiang F, Jing W (2009) Petroleum hydrocarbon contamination and impact on soil characteristics from oilfield in Momoge wetland. Environ Sci 30(8):2394–2401

Wang Y, Feng J, Lin Q, Lyu X, Wang X, Wang G (2013) Effects of crude oil contamination on soil physical and chemical properties in Momoge wetland of China. Chin Geogr Sci 23(6):708–715. https://doi.org/10.1007/s11769-013-0641-6

Wang X, Zheng J, Hana Z, Chenb H (2019) Bioremediation of crude oil-contaminated soil by hydrocarbon-degrading microorganisms immobilized on humic acid-modified biofuel ash: Chem. Technol Biotechnol 94:1904–1912