Nigerian Journal of Environmental Sciences and Technology (NIJEST)
www.nijest.com
ISSN (Print): 2616-051X | ISSN (electronic): 2616-0501
Vol 1, No. 2 July 2017, pp 256- 264

Biodegradation Potentials of Bacterial Isolates from Auto-Mechanic Workshops in Oluku, Edo State, Nigeria

Oshoma C.E.[1], Eze E.E.[1], Omonigho S. E.[1,*]
[1]Department of Microbiology, University of Benin, Benin City, Nigeria
*esharegoma.omonigho@uniben.edu

ABSTRACT

The frequent discharge of used petroleum products from automobiles has become a major source of concern due to unguided discharge into the soil environment, hence the need for biodegradation of the products. Bacterial species were isolated from contaminated soil in mechanic workshops and screened for their for hydrocarbon degradation potentials using standard microbiological procedures. Physicochemical properties of the contaminated soil were also analysed using standard techniques. The highest and lowest heterotrophic bacterial counts of 2.82 ± 0.16 ×10^8 and 2.09 ± 0.32 ×10^8 cfu/g were from the control soil and site 1 respectively. For hydrocarbon utilizing bacterial, Site 2 had the highest load of 8.33 ± 2.55 ×10^5 while the control had the least 1.35±0.33× 10^4 cfu/g. The bacterial isolates from the contaminated soil were found to be Corynebacterium kutsceri, Escherichia coli, Bacillus licheniformis, Bacillus subtilis, Bacillus megaterium, Klebsiella oxytoca, Staphylococcus aureus, Pseudomonas aeruginosa and Micrococcus luteus. The highest and lowest in the frequency of occurrence among the isolates were B. subtilis (27.5 %) and E. coli (1.4%) respectively. The screened hydrocarbon utilizing bacterial isolates were C. kutseri, B. subtilis and P. aeruginosa. The ability to degrade crude oil revealed that bacterial consortium had the highest growth profile of 12.90 ×10^5 while the least was C. kutsceri with values of 8.20 ×10^5 cfu/g. The consortium bacteria had the highest percentage of hydrocarbon products degradation. The ability of the consortium bacteria to remove a high percentage of crude oil components makes it potentially useful for bioremediation of site highly contaminated with petroleum hydrocarbon.

Keywords: Bacteria, crude oil, mechanic workshops and contaminated soil

1.0. Introduction

With an over increasing world population, petroleum products are extensively used all over the globe that apparently have strong connection to environmental pollution due to hydrocarbon discharge (Bidoia *et al*., 2010). Petroleum spillage occurs from several causes such as, leakages from pipelines and storage tanks, waste disposal, blowout, accidental spills through transportation and uses (Obayori *et al*., 2014). Environmental pollution by petroleum products remain an unavoidable consequence of oil exploitation, transportation and distribution activities. Petroleum and its products toxicity on contaminated soil, depends on their concentration, composition, environmental factors and biological state of the organisms at the time of contamination (Eze *et al*., 2014).

The Nigerian environment is known for indiscriminate, nonchalant and highly unregulated petroleum products disposal (Odjegba and Sadiq, 2000), causing a decline in soil quality. Mechanic workshop often dispose-off used oil into open grounds, from where it finds its way into canals, drainages and underground water (Obayori *et al*., 2014). It may be of interest to note that small amount of petroleum hydrocarbon released into aquifers could results to concentrations of dissolved hydrocarbons which exceed regulatory limits (Spence *et al*., 2005). Also, exposure of petroleum hydrocarbons for a long time can cause liver or kidney disease, damage to bone marrow and high risk of cancer (Lloyd and Cackette, 2001). To overcome the environmental risk associated with petroleum and its products, research is now geared toward remediating the contaminated soil.

Indiscriminate discharge of used petroleum products from vehicles is a source of pollution in mechanic workshops and its environs. Hence, the need for a concerted effort in studying the feasibility of using oil degrading bacteria for remediation. Okon (2006) advocated for biological remediation of petroleum contaminated soil due to the negative consequences of physicochemical approaches. Bioremediation of petroleum by natural population of microorganisms is one of the effective mechanisms of reclaiming soil and aquifers. The clean-up of hydrocarbon by this approach is very attractive because it is easy to maintain, applicable over a large area, cost effective and destruction of the contaminant (Bento *et al*., 2005). A wide range of bacteria and fungi have been implicated to exhibit the ability to degrade or utilize hydrocarbon as substrates (Challian *et al*., 2004; Ekhaise and Nkwelle, 2011).

Mechanic workshops used various petroleum products such as engine oil, diesel and kerosene on daily bases. The disposal of these products results in hardening or change in the texture of the soil, which has an effect on the microbiological and physicochemical quality of the contaminated soil. Hence, in view of the high number of mechanic workshops and their unguided disposal of used petroleum products into the environment, there lies the need to ameliorate the environmental risk associated with these products. Therefore, this investigation aims at assessing the potential of hydrocarbon degrading bacterial isolates from contaminated soil in mechanic workshops.

2.0. Materials and Methods

2.1. Study Area and Soil Sample Collection

The study areas were two auto mechanic workshops located at Site 1 and Site 2 within Ovia North East Local Government area, Edo State. Soil samples within these areas were collected into sterile polyethene bags using a soil auger at a depth of 0 – 15 cm. The samples were immediately transported to the laboratory for microbiological and physicochemical analysis.

2.2. Isolation and Enumeration of Bacteria

A 10 grams of contaminated soil sample was suspended in 90 ml of sterile distilled water in a conical flask. The soil suspension was thoroughly mixed and 10 fold serial dilution was carried out. Using pour plate technique, 0.1 ml from the dilutions were plated in duplicates on sterile Nutrient agar (NA), amended with Nystatin to discourage fungi growth, for total heterotrophic bacterial counts. The NA plates were incubated aerobically at 37 ^{o}C for 24 – 48 h. The number of viable cells in the samples were calculated from the colonies formed, inoculum size and dilution factor, then expressed as colony forming unit per gram (Cain *et al*., 2013).

2.3. Enumeration, Characterization and Identification of Hydrocarbon Utilizing Bacteria (HUB)

This involved cultivation of hydrocarbon degraders in minimal salt medium (with crude oil as carbon source). Minimal salt medium was prepared according to the composition formulated by Mills *et al*. (1978) as modified by Okpokwasili and Okorie (1988). One gram (1.0 g) of the oil contaminated soil sample was dissolved in test tubes containing 9 ml of sterile MSM and serially diluted (10-fold) using MSM as diluent. An aliquot (0.1 ml) of the dilutions was plated out in duplicate under aseptic condition using pour plate technique in the modified minimal salt agar (MSA) with 1 % crude oil as carbon source, incorporated with 0.5 ml Nystatin solution. The plates were incubated at 37 ^{o}C for 5 days.

After incubation, HUBs were enumerated, colonies of bacteria grown on the agar plates were counted, isolated, purified by streaking on NA plates and stored on NA slants for cultural characterization and identification. The bacterial isolates were characterised and identified on the basis of taxonomic schemes published in Bergey's manual of Determinative Bacteriology (Holt *et al*., 1994). Bacterial isolates were characterised on the basis of their cultural, morphological and biochemical characteristics. The identified isolates were maintained on NA slants at 4 ^{o}C for further studies.

2.4. Screening of Isolated Bacterial for Utilization of Hydrocarbon

Degradation potential of HUB was screened according to the modified method of Hanson *et al*. (1993); Bidoia *et al*. (2010) using 2,6- dichlorophenol indophenol (DCPIP) as redox indicator. Minimal salt medium (MSM) (100 ml) was transferred into 250 mL Erlenmeyer flasks and sterilized at 121 ^{o}C for 15 min. After cooling, 1 % of blended crude oil, filter sterilized, was added and 0.55 w/v DCPIP as redox indicator. The bacterial isolates of 1 ml was inoculated, incubated at 28±2 ^{o}C for 144 h on a Gyrator Shaker at a speed of 180 rpm. One of the flask was uninoculated and served as the control. The flasks were monitored daily for colour change (from deep blue to colourless). Absorbance of all the assays was measured using UV visible Spectrophotometer (model 752), at an optical density of 600nm, to test for degradation ability of the isolates.

2.5. Biodegradation Potentials of Screened Isolation

The bacterial isolates which had scored the most reduced optical density during the screening test were used for degradation of crude oil. Time-course degradation of the crude oil was carried out in MSM according to the method of Mills *et al*. (1978) as modified by Okpokwasili and Okorie (1988). The medium was supplemented with 1 % crude oil and inoculated with overnight culture of isolates according to Ekhaise and Nkwelle (2011) method. All the flasks were incubated at 28±2 ^{o}C in a Gyratory shaker at 150 rpm and monitored for a period of 15 days. The bacterial growth was monitored using pour plate technique on a nutrient agar plates. After incubation period, residual oil components concentrations were determined by Gas chromatography.

2.6. Physicochemical Parameter Determination of Soil Samples

Several physical and chemical properties of soil samples were evaluated. Soil sample pH was determined in 1:1 soil/water suspension ratio using pH meter (Jenway 3051) as described by Cowan and Steel (2004). The conductivity and sulphate content were determined according to the method of Onojake and Osuji (2012). Available phosphorous, nitrogen, exchangeable cations (Na^{+}. Ka^{+}. Ca^{2+} and Mg^{2+}) and total hydrocarbon content (THC) were determined using the procedures stated by Akpoveta *et al*. (2011). The chloride content was determined using titrimetric procedure while total organic carbon (TOC) was by chronic acid titrimetric method of Eze *et al*. (2014). Heavy metal contents Fe^{2+}, Mn^{2+}, Pb^{2+}, Zn^{2+} and Ni^{2+}) were analysed by atomic absorption spectrophotometer (Alpha 4 AAS) after digestion of sample with nitric acid and distill water.

3.0. Results and Discussion

Degradation of petroleum components by microorganisms is possible due to the presence of enzymes secreted by them. The complex hydrocarbons can be degraded by those microorganisms which possess high enzymatic activities (Alexander, 1994). The heterotrophic bacterial (THB) counts showed that the soil (both contaminated and uncontaminated) contained bacteria. The highest and lowest bacterial load of $2.82 \pm 0.16 \times 10^{8}$ and $2.09 \pm 0.32 \times 10^{8}$ cfu/g was observed in the control and site 1 respectively. For the case of the hydrocarbon utilizing bacterial (HUB), site 2 had the highest load of $8.33 \pm 2.55 \times 10^{5}$ while the control site had the lowest load of $1.35 \pm 0.33 \times 10^{4}$ cfu/g (as seen in Table 1). Higher bacterial counts were observed in uncontaminated soil than the auto-mechanic workshop soil contaminated with petroleum products. Possible reason could be due to non-exposure of soil to petroleum products which did not distort the physicochemical and biological properties of this soil. The low bacterial counts in the mechanic workshops may be due to inability of some organisms to tolerate high concentration of the petroleum products in these environments. Same effect was observed in the report of Stephen *et al*. (2013).

The increase in HUB count from the mechanic workshop soil than the uncontaminated soil could be that these organisms have developed resistance effect against the hydrocarbon products found in the soil. It could also be a reflection of the soil microflora ability to strive and proliferate in these environments, despite the deliberate exposure of soil to varying dose of petroleum products (Selvakumar *et al*., 2014). The bacterial isolates from the contaminated soil were found to be

Corynebacterium kutsceri, *Escherichia coli*, *Bacillus licheniformis*, *Bacillus subtilis*, *Bacillus megaterium*, *Klebsiella oxytoca*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Micrococcus luteus* as shown in Table 2. The highest in the frequency of occurrence among the isolates was *B. subtilis* (27.5 %) while *E. coli* had the lowest percentage occurrence of 1.4 %. The identified bacterial isolates have also been reported by various authors as the most predorminant hydrocarbon degrading bacterial (Rahman *et al*., 2002; Selvakumar *et al*., 2014; Mbachu *et al* 2014). These bacterial identified had wide spread in the soil and can easily degrade hydrocarbon. The dominance of *B. subtilis* on the crude oil polluted soil may be due to the ability to produce spores which may shield them from the toxic effect of the hydrocarbon.

Table 1: Total Heterotrophic Bacterial (THB) and Hydrocarbon Utilizing Bacterial (HUB) Counts of Soil Samples Collected

Area	**THB ($\times 10^8$ cfu/g)**	**HUB (cfu/g)**
Site 1	2.09 ± 0.32	$4.07 \pm 1.10 \times 10^5$
Site 2	2.33 ± 0.48	$8.33 \pm 2.55 \times 10^5$
Control	2.82 ± 0.16	$1.35 \pm 0.33 \times 10^4$

Table 2: Frequency of Occurrence (%) of HUB Isolates from the Soil Samples Collected from Mechanic Workshop

Bacterial Isolates	**% frequency**
Corynebacterium kutsceri	7.2
Escherichia coli	1.4
Bacillus licheniformis	17.4
Bacillus subtilis	27.5
Bacillus megaterium	11.6
Klebsiella oxytoca	10.1
Staphylococcus aureus	4.3
Pseudomonas aeruginosa	13.0
Micrococcus luteus	7.2

Redox indicator is a common means of measuring hydrocarbon utilizing bacteria in a contaminated environment (Bidoia *et al*., 2010). Screening of bacteria capable of utilizing crude oil as a sole carbon source is by the use of DCPIP. This a rapid and low cost procedure (Selvakumar *et al*., 2014). The ability of bacteria to utilize crude oil was ascertain by mainly colour change observation of DCPIP, where we have the quantified decolouration time indicating the best strain for crude oil degradation (Bidoia *et al*., 2014). The ability of bacterial isolates to utilize crude oil was screened as shown in Table 3. The isolates that decolourized DCPIP in the shortest time were used for the growth profile test. The isolates were found to *C. kutseri*, *B. subtilis* and *P. aeruginosa* with an OD values of 0.207, 0.140 and 0.187 respectively. All the bacterial isolates had the ability to grow on crude oil as their sole carbon source and energy when screened for crude oil utilization. Amazingly, same genera of bacterial have been implicated in hydrocarbon utilization, mostly Bacillus and Pseudomonas spp, by many authors (Oboh *et al*., 2010; Selvakumar *et al*., 2014; Sebiomo *et al*., 2011; Ekhaise and Nkwelle, 2011).

Table 3: Screening of Bacterial Isolates for Crude Oil Utilization Potentials

Bacterial Isolates	Observed Colour Change	OD at 600 nm
Corynebacterium kutsceri	Colourless after 96 h	0.207
Escherichia coli	Blue after 144h	0.348
Bacillus licheniformis	Colourless after 120 h	0.212
Bacillus subtilis	Colourless after48 h	0.140
Bacillus megaterium	Colourless after 96 h	0.280
Klebsiella oxytoca	Colourless after 96 h	0.220
Staphylococcus aureus	Colourless after 120 h	0.290
Pseudomonas aeruginosa	Colourless after 48 h	0.187
Micrococcus luteus	Light blue after 144 h	0.513

Figure 1 shows growth profile of the bacterial isolates screened during the utilization of crude oil. Increase in growth over a period of 15 days showed the bacteria to utilize hydrocarbon as their sole carbon source. However, the highest growth profile on day 15 was found to be the consortium of the three bacteria, next was *B. subtilis* while the least was *C. kutsceri* with values of 12.90×10^5, 8.20×10^5 and 7.60×10^5cfu/g respectively. Eze *et al*. (2014) reported that soil environments contaminated with hydrocarbon are likely to contain microbial populations of diverse taxonomic characteristics capable of degrading the pollutant. Degradation of macromolecules in the contaminant to smaller molecules is influenced by range of useful enzymes expressed by the microorganisms that help in breaking down or decomposition of contaminant. The growth profile revealed no distinct lag phases exhibited by the bacterial isolates and their consortium. The increase in bacterial population of the crude oil degraders could be due to the fact that there is available nutrient supplied by the oil for their growth (Akpoveta *et al*., 2011). The crude oil served as sole carbon and energy sources to the bacteria.

The percentage removal of hydrocarbon components by the bacterial isolates is shown in Figure 2. The consortium of the three bacteria had the highest percentage hydrocarbon removal and the least was *C. kutsceri* with value 89.67 and 57.25% respectively. The utilization of hydrocarbon help the organism to increase in cell number with a concomitant decrease in hydrocarbon concentration during the incubation period (Sebiomo *et al*., 2011). This observation agreed with the work of Sebiomo *et al*. (2011) that the ability of microorganisms to grow in crude oil indicate effective hydrocarbon degradation potential. Worthy of note is that all the bacterial isolates were resident of the polluted environment hence, their biodegradation ability to the contaminants. For a complete removal of pollutant, biodegradation has been proven to be the best form. This potential should be improve in order to develop a faster means of clean-up of contaminated soil. The success of remediating polluted environment depends on microorganisms in close physical contact with the crude oil degrade. Therefore, to optimize biodegradation rate is to improve the growth rate of microflora that are indigenous to the soil. On a final analysis, the best way for successful removal/clean-up of pollutant is to increase the rate of biodegradation potentials of indigenous bacteria found in the contaminated soil. Based on the crude oil utilization capacity, the consortium is the most active degrader in the oil. This is suggestive of a synergistic relationship among individual members of the consortium that might have invariably boosted the degradative potential of the bacterial consortium (Ghazali *et al*., 2004).
Hydrocarbon degrading bacteria have been shown to attack more on the aliphatic and light aromatic fraction of the crude oil (Okerentugba and Ezeronya, 2003), hence the percentage increase in the removal of these products from the oil. The consortium had the highest percentage removal of various fractions of crude oil analysed followed by *B. subtilis* after 15 day of incubation. This agreed with the findings of Mbachu *et al*. (2014) who reported that *B. subtilis* exhibited the highest degradation potential (66.7%) on used engine oil. The result of this investigation is in line with the theory that individual member in a microbial community performs significant roles and also depend on the presence of different species and strains to effectively degrade crude oil in the environment (Ghazali *et al*., 2004). While in this study, high percentage crude oil components were removed by the microorganisms,

insignificant value of 3 % of the crude oil components was removed from the control medium. This could be influenced by physical factors such as run-off, flood and leaching, evaporation and photo-oxidation (Malik and Ahmed, 2011).

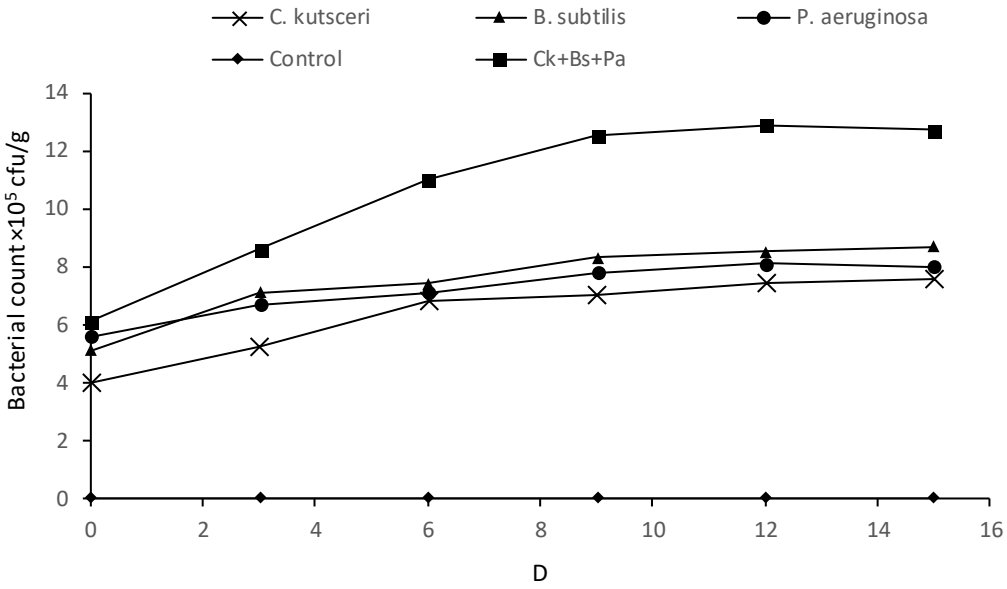

Figure 1: Total bacterial growth profile of the screened isolated and its consortium during the degradation of 1 % crude oil.

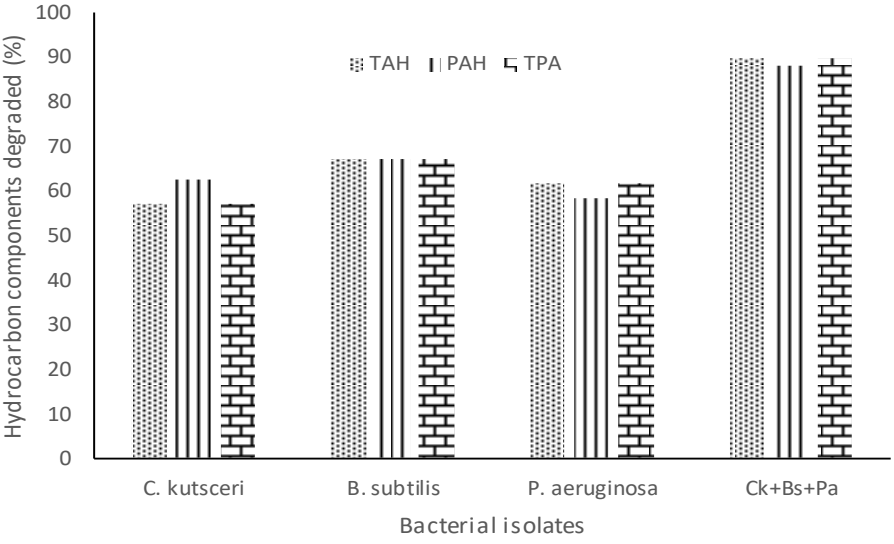

Figure 2: Percentage removal of 1 % crude oil components by bacterial isolates and its consortium after 15 days incubation. TAH, Total aliphatic hydrocarbon; PAH, Polycyclic aromatic hydrocarbon and TPH; Total petroleum hydrocarbon

The physicochemical properties of the soil samples showed varying values of the parameters analysed. The physicochemical parameters conducted on the oil polluted soil and oil free soil samples are shown in Table 4. The pH of the contaminated soil site 1was more acidic than site 2 and control with values 6.143, 6.233 and 7.160 respectively. However, there was no statistical difference in pH ($p > 0.05$). The polluted sites had the highest electric conductivity, heavy metals and total organic carbon than the

control soil. pH of the contaminated soil was found to be acidic. The low pH may be due to increased degradation of crude oil by the organisms in the soil, resulting in accumulation of acidic metabolites (Ijah and Abioye, 2003). Phosphorus and sulphate were higher in the contaminated than the control soil. Phosphorus availability for microorganisms in the soil may be restricted by its tendency to precipitate in the presence of certain metals such as Ca^{2+}, Mg^{2+} and Fe^{2+}. Increase in sulphate content can be attributed to the fact that there is continual replacement after being removed from soil (Eze *et al*., 2014). Nitrogen is an essential component of protein and nucleic acid. In most living being, nitrogen is taken up as assimilable nitrogen. Nitrogen can be lost from soil because some species of bacteria convert nitrate to gaseous nitrogen by using nitrate as a metabolic electron acceptor in place of oxygen (Nester *et al*., 2001). There was high level of electric conductivity in the polluted soils than the control soil, probably due to the presence of ions from the hydrocarbon introduced into the soil (Onojake and Osuyi, 2012). The high total organic carbon of the contaminated soil may be as a result of continuous released of the product into the soil. Same effect was found for THC, this could be a direct consequence of the unregulated discharged of petroleum products to the receiving soil.

Table 4: Physicochemical Properties of the Soil Samples

Parameter	Control	Site 1	Site 2
pH	7.160 ± 0.071	6.143 ± 0.203	6.233 ± 0.493
Electric conductivity (uS/cm)	28.667 ± 2.136	84.400 ± 9.322	81.800 ± 10.652
Total organic carbon (%)	1.277 ± 0.177	3.047 ± 0.307	2.733 ± 0.421
Phosphorus (mg/kg)	1.953 ± 0.189	3.483 ± 0.142	5.303 ± 0.273
Total nitrogen (mg/kg)	0.677 ± 0.015	0.057 ± 0.009	0.040 ± 0.006
Total hydrocarbon content (mg/kg)	55.127 ± 1.213	202.530 ± 3.514	286.170 ± 2.892
Sulphate (mg/kg)	0.180 ± 0.46	0.323 ± 0.035	0.817 ± 0.084
Cation exchange capacity (mg/kg)	2.686 ± 0.283	7.569 ± 1.179	5.103 ± 0.076
Fe^{2+}, (mg/kg)	0.275 ± 0.157	10.750 ±0.947	8.677 ± 0.667
Mn^{2+} (mg/kg)	0.078 ± 0.003	3.093 ± 0.321	1.464 ± 0.154
Pb^{2+}, (mg/kg)	0.150 ± 0.057	0.647 ± 0.0	0.620 ± 0.113
Zn^{2+} (mg/kg)	0.077 ± 0.019	0.763 ± 0.093	0.437 ± 0.047
Ni^{2+}, (mg/kg)	0	0.653 ± 0.101	0.350 ± 0.012
Cl^{-}, (mg/kg)	2.313 ± 0.193	7.787 ± 0.300	8.100 ± 1.489
Sand %	85.157 ± 2.079	73.763 ± 2.509	76.737 ± 2.039
Clay %	12.657 ± 0.315	23.070 ± 2.577	19.923 ± 1.390
Silt %	1.753 ± 0.122	3.167 ± 0.549	3.340 ± 0.655
Moisture content (%)	7.893 ± 1.036	14.267 ± 1.943	17.633 ± 2.898
Bulk density (g/cm^3)	0.730 ± 0.026	0.903 ± 0.003	0.973 ± 0.012

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