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(RESEARCH ARTICLE)

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Studies on the ecological succession of microorganisms during bioremediation of hyper-toxic crude oil polluted soil using five amendments

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Abstract

Dangerous impact of crude oil in the environment is a common knowledge and its spillage has been controlled using both chemical and biological methods. Bioremediation however, is most advocated because of its environmental friendliness and sustainability. The present study sought to identify and monitor successions, and diversity of microorganisms elicited by five amendments in the bioremediation of hypertoxic crude oil pollution. Five amendment patterns which incorporated 200 g each of saw dust, wood chip, compost, N.P.K. fertilizer and poultry droppings; were used to stimulate biodegradation in 50% crude oil contaminated soil, and their respective microbial counts and microbial successions were monitored over a period of eight months. Statistical analyses were conducted using SPSS version 21 software. Poultry droppings amendment had the highest significant ($p<0.05$) bacterial and fungal counts. *Bacillus subtilis*, *Stenotrophomas maltophilia* and *Pseudomonas aeruginosa* were the predominant bacteria observed during the microbial succession studies across the amendment microcosms, while *Aspergillus flavus*, *Penicillium citrinnum* and *Fusarium solani* were the predominant fungal isolates. It was observed that the hyper-toxic concentration of the crude oil impacted the diversity and succession of the microbes across all the amendments used when compared to the control sample.

Keywords: Amendments; Bioremediation; Crude oil; Hydrocarbon; Microbial succession.

1. Introduction

Crude oil bio-remediation is a technique used for the cleaning up of deleterious impact of crude spill in the environment. Bio-remediation is seen to be environmental friendly and sustainable when compared to the use of chemical compounds for crude oil spill clean-up. Studies have shown several researchers carrying out bio-remediation of crude oil spillage using individual microorganisms or organic amendments like compost, poultry droppings, cow dung for bio-augmentation which in turn drives the bioremediation process (Nwankwegu et al., 2017; Marie et al., 2020; Yu et al., 2022). These studies have shown the individual capacities of various microorganisms in bioremediation of crude oil polluted soils, their mechanisms for crude oil degradation and kinetics behind their degradation capacities. However, a knowledge gap exists in figuring out what best combinations of microorganisms give the most efficient bio-remediation in shortest time possible. This is important because hydrocarbon spills lead not just to arable soil pollution, but can contribute to green house gas emission thus creating complex environmental toxicities (Opetubo et al., 2022a).

This led to the need to study the ecological succession of these microorganisms to help identify the predominant organisms, transient organisms and also the most persistent indigenous organisms that drive the bio-remediation process. Yu et al. (2022) monitored the bacterial succession of crude oil enriched soil using next generation sequencing so as to identify even unculturable organisms that may have participated in the bioremediation. This present study carried out bio-remediation of hypertoxic crude oil concentration using the following amendments: wood chip, poultry

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droppings, saw dust, saw dust and N.P.K. fertilizer; and monitored the succession of microorganisms that participated in the bio-remediation process over a period of eight months. Nwankwegu et al. (2017) opined that crude oil concentration plays a role in selecting the type of microorganisms that participate in its degradation and also in impacting the organisms that persist longer in the habitat than the other. They also opined that different amendments whether organic or inorganic impact the microbial succession observed in a particular bioremediation regime. Thus, this present study sought to confirm the possibility of these claims by employing a hypertoxic crude oil concentration of 50% and the usual amendments applied (wood chip, poultry droppings, saw dust, saw dust and N.P.K. fertilizer) more commonly by researchers to see if the organisms present will correspond or differ from reports from other studies.

2. Material and methods

2.1. Bioremediation Amendment Design

Six amendments to evaluate the efficiency of crude oil biodegradation in the contaminated soils were carried out using randomized block design as shown in Table 1. The treatments were grouped into microcosms as follows:

- M1: Compost amendment microcosm.
- M2: Poultry droppings amendment microcosm.
- M3: N.P.K. fertilizer amendment microcosm.
- M4: Saw dust amendment microcosm.
- M5: Wood chip amendment microcosm.
- M6:Control experiment which used remediation by enhanced natural attenuation (RENA), which involves soil's natural ability to remove the crude oil.

2.2. Microcosm Description

Soil sample (1000 g) was placed in six different set of pulverized pans each with a surface area of 441cm^2 and a volume of 1764cm^3. Crude oil (500 ml) was used to pollute each of the soil as prepared in six different pulverized pans. Equal quantity (200g) of each of the five selected amendments (compost, chicken droppings, N.P.K fertilizer, sawdust and wood chips) was added to each of the microcosms designated M1 – M5. Polluted soil without amendments was designated as M6 and served as control. These were thoroughly mixed with the aid of a hand trowel and process was continued weekly to provide sufficient air and oxygen in the microcosms. The soil was moistened by the addition of 200ml of water every week until the end of the experiment. Total petroleum hydrocarbon was analyzed monthly by gas chromatography equipped with flame ionization detector. Microbial enumeration was done using pourplate method on nutrient agar and spread plate method onSabouraud dextrose agar for bacteria and fungi respectively,using a 10-fold serial dilution. Vapour phase transfer method using mineral salt medium was used to enumerate the hydrocarbon degraders. Success of bioremediation was evaluated using enzyme assay dehydrogenase activity, reduction in total hydrocarbon content, biodegradation kinetic modelling and ecotoxicity test using maize and bean seed germination models.

Table 1 Biodegradation amendment design

Microcosm	Description
M1	1000 g soil + 500 ml crude oil + 200 g compost
M2	1000 g soil + 500 ml crude oil + 200 g chicken droppings
M3	1000 g soil + 500 ml crude oil + 200 g N.P.K fertilizer
M4	1000 g soil + 500 ml crude oil + 200 g saw dust
M5	1000 g soil + 500 ml crude oil + 200 g wood chips
M6	1000 g soil + 500 ml crude oil + no amendments (control

M- Microcosm

2.3. Isolation of Bacterial Hydrocarbon Degraders/Users

The enumeration of bacterial hydrocarbon degraders (BHD) was done by using the vapour phase method described by Bento *et al*. (2005)and Orji *et al*. (2012).A 10^5 dilution of each soil samples drawn from six different treatment options was inoculated into modified mineral salt medium. The medium components in gl^{-1} are: $MgS0_4 \cdot 7H_2O$,0.42; KCl, 0.297;

KH_2PO_4, 0.85; $NaNO_3$, 0.42; K_2HPO_4,1.27; NaCl, 0.1;agar powder (Oxoid, United Kingdom), 20 g. These were weighed out and were hydrated in 1000 ml distilled water in Erlenmeyer flask. The medium was sterilized by autoclaving at 121 °C, 15psi for 15mins before dispensing into sterile petri dishes. The solidified mineral salt agar (MSA) was inoculated separately with 10^{-1} and 10^{-5} dilutions of polluted soil sample. Whatman No. 1 filter papers were saturated with crude oil and the crude oil impregnated filter papers were aseptically placed onto the covers of the petri dishes while inverted. The hydrocarbon saturated filter papers supplied hydrocarbons by vapour phase transfer to the inoculum. The plates were incubated at room temperature (28 ± 2°C) for 5 – 7 days and colonies were counted from triplicates and mean values were recorded in colony forming unit per gram (cfu/g). Each colony that developed on plates inoculated with 10^{-1} dilution was sub-cultured and pure cultures were identified. This was repeated every month for the eight months duration of the experiment. The isolates appearing and disappearing within that period were used to deduce the succession of the microbes.

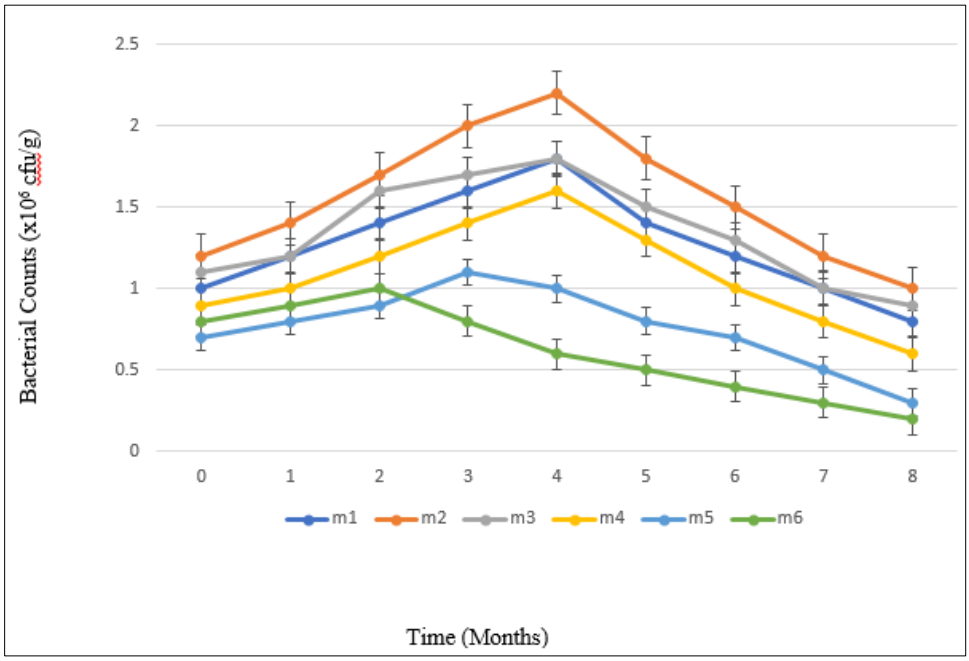
Bacterial isolates were identified using Gram stain, biochemical tests and 16S rDNA region sequencing.

2.4. Isolation and Identification of Fungal Hydrocarbon Degraders/Users

This was carried out using method described by Chukwura*et al*. (2005). The enumeration of fungal hydrocarbon degraders (FHD) was carried out using mineral salt agar (MSA) containing in gl^{-1}: NaCl, 0.1; $MgSO_4 \cdot 7H_2O$, 0.45; KCl, 0.42; KH_2PO_4, 0.29; Na_2HPO_4, H_2O, 0.86; $NaNO_3$ 0.43; agar, 25.0. The medium was sterilized by autoclaving at 121°C, 15psi for 15mins.Crude oil again, served as both sole carbon and energy sources. Three replicate plates inoculated by spread plating were inverted over sterile filter paper moistened with sterile crude oil placed on the lid of Petri dish covers containing the crude oil impregnated filter paper.The compounded medium for the hydrocarbon utilizing fungal count was amended with 250 mg of chloramphenicol. Incubation lasted for 7 days at room temperature (28±2°C). Isolates were identified using fungal atlas and ITS region DNA sequencing.

3. Results

3.1. Microbial Succession during Biodegradation Studies

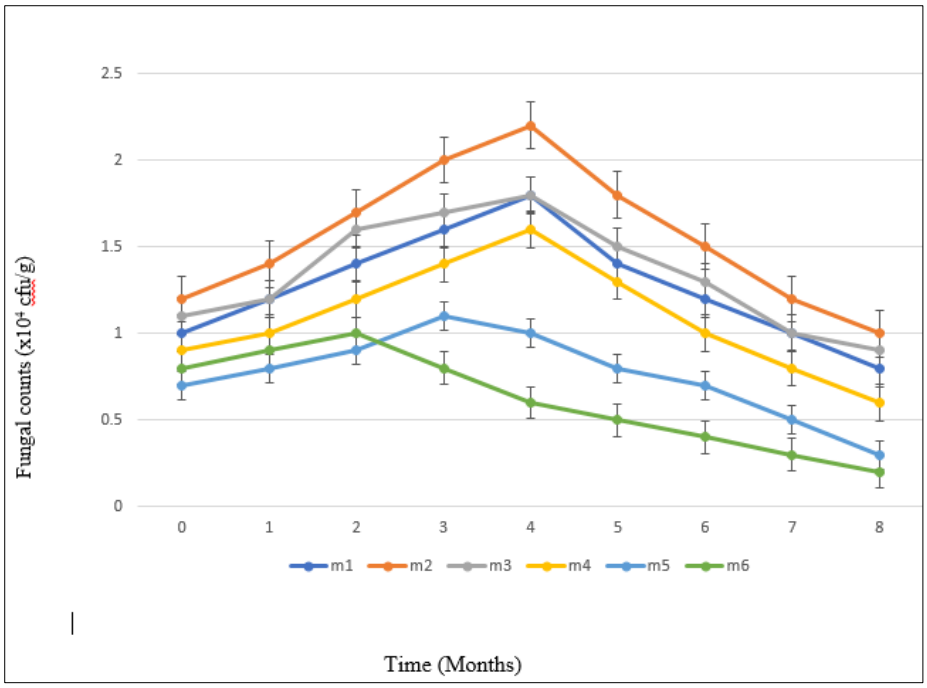


M1: Compost amended soil; M2: Poultry droppings amended soil; M3: N.P.K. fertilizer amended soil; M4: Saw dust amended soil; M5: Wood chip amended soil.;M6: Control experiment (RENA)

Figure 1 Mean Hydrocarbon Degrading Bacteria Counts During Bioremediation

Hydrocarbon degrading bacterial and fungal counts from the ammended crude oil contaminated soil samples monitored over an 8-month period, showed significant ($p<0.05$) decrease in bacterial and fungal counts as shown in Figures 1 and 2.

Bacterial and fungal successions during bioremediation monitoring are shown in Tables 2 to 7. Predominant and persistent bacterial organisms identified were *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Bacillus thuringiensis*, while the predominant and persistent fungal organisms were *Penicillium citrinium*, *Aspergillus flavus*, *Rhizopus stolonifer* and *Fusarium solani*.



M1: Compost amended soil; M2: Poultry droppings amended soil; M3: N.P.K. fertilizer amended soil; M4: Saw dust amended soil; M5: Wood chip amended soil; M6: Control experiment (RENA).

Figure 2 Mean Hydrocarbon Degrading Fungal Counts During Bioremediation

Table 2 Microbial Succession Observed in Polluted Soil Remediated by Natural Attenuation

Period	Bacteria	Fungi
0	*Bacillus subtilis,* *Bacillus thuringiensis,* *Pseudomonas fluorescens,* *A. faecalis*	*A. flavus,* *F. solani,* *P. citrinium,* *Rhizopusstolonifer*
1	*B. subtilis,* *B. thuringiensis,* *P. fluorescens,* *A. faecalis*	*A. flavus,* *F. solani,* *P. citrinium,* *R. stolonifer*
2	*B. subtilis,* *B. thuringiensis,* *P. fluorescens,* *A. faecalis*	*A. flavus,* *F. solani,* *P. citrinium,* *R. stolonifer*
3	*B. subtilis,* *B. thuringiensis,* *A. faecalis*	*A. flavus,* *F. solani,* *P. citrinium*
4	*B. subtilis,*	*A. flavus*

	A. faecalis, *B. thuringiensis*	*F. solani* *P. citrinium*
5	*B. subtilis,* *B. thuringiensis*	*P. citrinium*
6	*B. subtilis,* *B. thuringiensis*	*P. citrinium,*
7	*Bi subtilis,* *B. thuringiensis*	*P. citrinium*
8	*B. subtilis,* *B. thuringiensis*	*P. citrinium*

Table 3 Microbial Succession Observed in Compost Amended Soil

Period	**Bacteria**	**fungi**
0	*Bacillus subtilis,* *Klebsiella pneumonia,* *P. aeruginosa*	*A. fumigatus,* *A. flavus,* *P. griseofulvin, C. utilis*
1	*B. subtilis,* *P. aeruginosa,* *C. amycolatium*	*A. fumigatus,* *A. flavus,* *P. griseofulvin, C. utilis*
2	*B. subtilis,* *P. aeruginosa*	*A. fumigatus,* *A. flavus,* *P. griseofulvin,* *C. utilis*
3	*B. subtilis*	*P. griseofulvin,* *A. flavus,* *C. utilis*
4	*B. subtilis*	*P. griseofulvin,* *A. flavus,* *F. solani*
5	*B. subtiis*	*P. griseofulvin,* *A. flavus,* *F. solani*
6	*B. subtilis*	*A. flavus,* *F. solani*
7	*B. subtilis*	*A. flavus,* *F. solani*
8	*B. subtilis*	*A. flavus,* *F. solani*

Table 4 Microbial Succession Observed in Poultry Droppings Amended Soil

Period	Bacteria	Fungi
0	*Salmonella typhi,* *E. coli,* *Bacillus subtilis,* *K. pneumonia,* *S. faecalis*	*F. solani,* *A. fusidiodes,* *A. flavus,* *P. citrinium*
1	*S. typhi,* *E. coli,* *B. subtilis,* *Corynebacteriumamycolatium*	*F. solani,* *A. fusidiodes,* *A. flavus,* *P. citrinium*
2	*C. amycolatium,* *B. subtilis* *P. aeruginosa*	*F. solani,* *A.* *A. flavus,* *P. citrinium*
3	*C. amycolatium,* *Stenotrophonasmaltophila*	*F. solani,* *P. citrinium,* *A. flavus*
4	*S. maltophila,* *Flavobacteriumindologenes*	*F. solani,* *P. citrinium,* *A. flavus*
5	*S. maltophila,* *F. indologenes,* *S. maltophila,* *F. indologenes*	*F. solani,* *P. citrinium,* *A. flavus*
6	*S. maltophilia*	*F. solani,* *P. citrinium*
7	*S. maltophilia*	*F. solani,* *P. citrinium*
8	*S. maltophilia*	*F. solani,* *P. citrinium*

Table 5 Microbial Succession Observed in N.P.K. Amended Soil

Period	Bacteria	Fungi
0	*Corynebacteriumamycolatium,* *B. subtilis,* *P. aeruginosa*	*A. fusidiodes,* *P. citrinium,* *Geomycespannorium*
1	*C. amycolatium,* *B. subtilis,* *P. aeruginosa*	*A. fusidiodes,* *P. citrinium,* *G. pannorium*
2	*C. amycolatium,* *B. subtilis*	*A. fusidiodes,* *P. citrinium,*

	P. aeruginosa	*G. pannorium*
3	*Bacillus subtilis*	*P. citrinium,* *A. fusidiodes,* *G. pannorium*
4	*B. subtilis*	*P. citrinium,* *G. pannorium*
5	*B. subtilis*	*P. citrinium*
6	*B. subtilis*	*P. citrinium*
7	*B. subtilis*	*P. citrinium*
8	*B. subtilis*	*P. citrinium*

Table 6 Microbial Succession Observed in Saw Dust Amended Soil

Period	**Bacteria**	**Fungi**
0	*P. aeruginosa,* *B. subtilis,* *B. thuringiensis*	*C. sphaerosperinium,* *C. heteropogonus,* *C. lutanus,* *P. citrinium*
1	*B. subtilis,* *P. aeruginosa*	*C. sphaerosperinium* *C. heteropogonus* *C. lutanus* *P. citrinium*
2	*B. subtilis,* *P. aeruginosa*	*C. sphaerosperinium,* *C. heteropogonus,* *C. lutanus,* *P. citrinium*
3	*B. subtilis,* *P. aeruginosa*	*C. sphaerosperinum,* *C. heteropogonus*
4	*B. subtilis,* *Paeniclostridiumsordelli*	*C. lutanus,* *C. heteropogonus,* *R. mucilaginosa*
5	*P. sordelli* *B. subtilis*	*C. heteropogonus,* *C. lutanus*
6	*P. sordelli,* *B. subtilis*	*C. heteropogonus,* *C. lutanus*
7	*P. sordelli,* *B. subtilis*	*C. heteropogonus,* *C. lutanus*
8	*P. sordelli,* *B. subtilis*	*C. heteropogonus,* *C. lutanus*

Table 7 Microbial Succession Observed in Wood chip Amended Soil

Period	Bacteria	Fungi
0	*K. pneumonia,* *B. subtilis,* *P. aeruginosa*	*F. solani,* *P. citrinium* *C. utilis*
1	*B. subtilis,* *P. aeruginosa*	*F. solani,* *P. citrinium,* *C. utilis*
2	*B. subtilis,* *P. aeruginosa*	*F. solani,* *P. citrinium,* *C. utilis*
3	*Bacillus subtilis, P. aeruginosa*	*F. solani,* *P. citrinium,* *R. mucilaginosa*
4	*B. subtilis,* *FlavobacteriumIndologenes*	*F. solani,* *P. citrinium,* *R. mucilaginosa*
5	*B. subtilis*	*F. solani,* *P. citrinium*
6	*B. subtilis*	*F. solani,* *P. citrinium*
7	*B. subtilis*	*F. solani,* *P. citrinium*
8	*B. subtilis*	*F. solani,* *P. citrinium*

4. Discussion

Microbial succession in response to crude oil pollution monitored over a period of eight months showed the bacterial and fungal species changes (Table 2- 7). *Corynebacterium*, *Bacillus* and *Pseudomonas* genera identified during the succession have also been reported by Jiao *et al*. (2016). They studied bacterial species succession in crude oil polluted soil and opined that ecological succession which occurs is as a result of environmental modification of factors such as soil pH, aliphatic alkane and aromatic compound concentration, and presence of other complex pollutants which fluctuate during bioremediation. They isolated and identified other bacterial genera which are not present in findings of this work such as *Gordonia* and *Dietzia* genera. This microbial succession observed suggested the need for microbial consortia to be adopted for bioremediation studies, instead of using monocultures as reported by Nwankwegu and Onwosi, (2017); Mohammadi-Sichani *et al*. (2017); Ojiabo, *et al*. (2019). Mukjang *et al*. (2022) evaluated microbial succession based on phylum for copost-amended crude oil polluted soil and natural attenuation set-up. They reported Actinobacteria, Proteobacteria and Firmicutes as the predominant phyla, with Proteobacteria and Firmicutes initially dominating the polluted soil in the first week, while from the second week Actinobacteria were reported to dominate and also persist throughout the period of bio-remediation monitoring; for the natural attenuation set-up. However, for the compost amendment set-up, Bacteriodes succeeded Actinobacteria and persisted all through the bio-remediation period. Specifically, *Acetobacter* was reported to have succeeded initial *Pseudomonas* community and persisted, which differed from the findings from the present study. The present study had *Bacillus subtilis* dominating in the compost amendment set-up.

This present study increased the crude oil concentration (50%) used in the pollution of the agricultural soil samples which differs from the average concentrations of 1-5% used in most studies, so as to see if crude oil concentration impacts the microbial diversity and also their ecological succession patterns. Marie *et al*. (2020) posited that crude oil

concentration as well as crude oil composition impacted on the microbial diversity observed from their study in China. They reported bacterial dominance of *Pseudomonas* and fungal dominance of *Penicillium* which partly corresponded with the findings of this study carried out in Nigeria. This is to say that whether heavy crude or light crude oil, the hydrocarbon contents possibly impact the microbial diversity of the polluted soil; secondly, certain microorganisms possess the capacity to hydrolyze the crude oil, whether light or heavy, low concentration or highly concentrated, regardless of geographical location. Huang *et al*. (2021) posited that crude concentrations impact metabolic capacities of hydrocarbon degrading organisms, and their alkane degrading enzyme production capacities as well, which directly affects microorganisms that persist or give way during hydrocarbon bioremediation process. They reported *Bacillus* as one of the dominant bacteria and *Stenotrophomas* as one of the less dominant bacteria, and this finding corresponded with that of this present study.

Galitskaya *et al*. (2021) carried out a comparative microbial succession study between 6% crude oil concentration and 25% crude oil concentration. They reported that increased concentration leads to higher concentration of heavier fractions of the crude oil, while not affecting the lighter fractions which are the alkanes and cycloalkanes. The consequence of this is that microorganisms concentrate on degrading the alkanes and cycloalkanes, and consequently their diversity and succession patterns arise from their efficiency in degrading these lighter fractions of alkanes and cycloalkanes in the crude oil. The microbes they identified from their bioremediation study partly corresponds with those we reported in this study. Hydrogen is basically liberated from hydrocarbon degradation, which consequently binds with atmospheric oxygen to give water formation (Opetubo et al., 2022b).

One of the hypotheses made in this study was that biostimulation of the crude oil polluted soil using different amendments would possibly impact the diversity and succession patterns of the hydrocarbon degrading microbes. It was however, observed in this study that only poultry droppings amendment impacted diversity while other amendments as well as the control experiment (RENA) had similar bacterial and fungal organisms present in the succession study.One would argue that this could have possibly been a result of constant crude oil concentration used in the study across the experimental microcosms, and it could also be possibly deduced that poultry droppings had more of enteric organisms that had the capacity for degrading 50% crude oil concentration; and thus, did not necessarily give way for the dominance of other microbes that re-occurred in other amendments and control experiment. Likewise, seeing that the control experiment had similar succession patterns with the other amendments aside poultry droppings, it could still be inferred that concentration possibly had impact on the microbial diversity and succession patterns for both bacteria and fungi.

5. Conclusion

Microbial succession and diversity during crude oil bio-remediation may not be significantly impacted by crude oil concentration used, however, biostimulation amendment used could partly impact the overall microbial diversity and succession patterns.

Compliance with ethical standards

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Disclosure of conflict of interest

Authors declare no conflict of interest.

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