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Growth Behaviour of Heterotrophic Bacterial in Crude Oil Contaminated Soil under *Pseudomonas sp*. Amendment

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ABSTRACT

Bioaugmentation involves the introduction of exogenous microorganisms to help accelerate natural attenuation processes. It is crucial to understand the growth behaviour of total heterotrophic bacteria (THB) while carrying out bioremediation of soils polluted with petroleum hydrocarbons. Hence, the growth of THB in petroleum hydrocarbon contaminated wet and dry soils under remediation by in Pseudomonas sp. was investigated. The wet and dry soils artificially polluted with crude oil were treated with 20-50 ml volumes of Pseudomonas sp. Total heterotrophic bacteria (THB) populations were monitored over 84 days. Growth patterns, rates and a predictive model were analyzed. Results were compared to untreated controls. From the results, THB counts increased significantly in treated soils compared to controls. The THB growth exhibited shorter lag phase and longer exponential and stationary phases. THB growth rates rose substantially in treated soils over time, while the predictive model reasonably estimated the growth of THB, but deviation increased after 70 days. Generally, bioaugmentation with Pseudomonas sp. effectively enhanced THB proliferation in hydrocarbon-polluted soil, which exhibited a rapid exponential growth within 14 days and remained stationary from 28 days and throughout. Therefore, Pseudomonas sp. is a potential bioaugmentation agent with capacity to sustain THB growth for a prolonged time required to improve biodegradation of TPH in contaminated sites.

KEYWORDS: Bioaugmentation, *Pseudomonas sp*., THB Growth, Predictive model

1. INTRODUCTION

The use of microorganisms offer great advantages in removing pollutants from soil or water as they are non toxicity to the environment. The microorganismsfeedon the pollutants thereby remediating the contaminated environment. In some bioremediation studies, microorganisms can be cultured and introduced directly to the pollutedenvironment or stimulated using agricultural and biological wastes, or fertilizer to supply nutrient necessary to produce and grow thenative microorganisms in the environment like soil (Borah and Yadav, 2016; Ere & Amagbo, 2019). As the microorganisms populates, they used the substrate (pollutant) as food, thereby reducing the of pollutant concentration level (Obiakalaije *et al*., 2015; Varjani, 2017a).

When a nutrient supplying agent like fertilizer, surfactant and oxygen are used to promote the growth of microorganisminbioremediation process, it is called bio-stimulation, butwhen microorganism isdirectlyintroduced to the pollutedenvironment, it is called bio-augmentation (Varjani & Upasani, 2017). In bio-augmentation, genetically modified bacteria with specific activities are tailored to enhance the biodegradation rate (Varjani, 2017b).

In this study, *Pseudomonas Sp.*is used as bio-augmentation agent to biodegrade TPH in soils. However, the growth of microorganism differs in the environmentin which it is applied. For instance, soil properties like texture, water retention capacity, salinity and chemical constituents among othersinfluence thegrowth rate and survival of native organism (Tharmer *et al*., 2013; Ebadi *et al*., 2017; Al-Hawash *et al*., 2018).

Various studies have evaluated the mechanisms and growth of microorganisms through mathematical modeling. Manzoni et al. (2012) developed a mathematical model coupling partial differential equations to simulate microbial growth and transport in unsaturated soil. It considered various physical, chemical and biological mechanisms. Both partial analytic solutions and full numerical simulations were presented and validated against infiltration experiments. Another study by Resat et al. (2012) developed a hybrid kinetic model for substrates/enzymes interaction in continuous system and individualcells to study how microbial dynamics are affected by heterogeneous environments and different carbon hydrolysis strategies. A more recent work by Gerke et al. (2021) discussed current approaches to represent biogeochemical processes like substrate transport, microbial activity and interactions with the pore space in digital soil models across micro to field scales. Specifically, this study will evaluate the growth rate and phases associated with total heterotrophic bacteria (THB) in crude oil polluted wet and dry soils as characteristic nature of soils in the Niger Delta area of Nigeria.

2. MATERIALS AND METHODS

2.1 Soil contamination

340 ml of crude oil was dispersed in wet and dry soil samples weighing 5kg with initial TPH concentrations recorded as 35981.09 mg/kg and 32563.39 mg/kg, respectively. The soils with crude oil were then treated with 20 to 50 ml of *Pseudomonas sp*. and incubated at room temperature for a period of 84 days. Another wet and dry soil samples were prepared without the addition of *Pseudomonas sp*., serving as control samples.

2.2 Measurement of THB counts

The total heterotrophic bacteria (THB) were determined after pollution and during the treatment. Thus, prepared nutrient agar culture plates were made according to the manufacturer's specification (HIMEDIA) M001-500G, HIMEDIA Laboratories Pvt. LTD

Number-400086, India). The culture plates were dried and 0.1ml of the 10^1 diluted soil samples was placed on it and it was spread using a glass rod spreader to dryness on the plate. This was incubated in an incubator at 37ºC for 24 hours and the counting of the bacteria was made on the plate after the bacteria have shown growth. The bacteria that did not grow after 24 hours was further allowed in the incubator for another 24 hours and readings were made on them.

2.3 THB growth rate model

The rate of THB growth is expressed by Fogler (2009) as:

$$r_{THB} = \mu C_{THB} \tag{1}$$

However, the Monod equation can be used to study the specific rate of bacteria growth. This is expressed as:

$$\mu = \frac{\mu_{\max} S}{K_s + S} \tag{2}$$

Combining equations (1) and (2), a new expression for THB growth rate can berestated as:

$$r_{THB} = \mu_{\max} \frac{C_{THB} S}{K_s + S} \tag{3}$$

Where, r_{THB} is the rate (cfu/g.day) $\mu_{\max}$ is the specific growth rate, $\mu_{\max}$ is the maximum specific growth rate (mg/kg.day), C_{THB} is THB concentration (cfu/g), S is the concentration of TPH (mg/kg), *t* is time (day) and K_s is the degradation rate constant (mg/kg).

2.4 THB growth prediction model

The predictive model for THB growth is formulated using regression analysis. This multiple polynomial model is expressed as:

$$C_{THB} = \beta t^n N^m \tag{4}$$

Where, C_{THB} is THB concentration (cfu/g), *t* is remediation time (day), N is the amount oftreatment (*Pseudomonas sp*.) (ml) and β is the model constant, while *n* and *m* are power indices relating to remediation time and amount of treatment, respectively.

The constants were evaluated by linearizing equation (4) as follows:

$$\ln C_{THB} = \ln \beta + n \ln t + m \ln N \tag{5}$$

The model coefficients were determined using Microsoft Analysis ToolPak and then substituted in equation (4) to predict the THB counts at any given time and amount of *Pseudomonas sp*.

3. RESULTS AND DISCUSSION

Thepopulationof total heterotrophic bacteria (THB) recorded during thetreatment of soil samples polluted by total petroleum hydrocarbon (TPH) at different quantities of *Pseudomonas sp*. (20 – 50 ml) over a period of 84 days as compared to control soil samples is presented inTable 1 forwet soil and Table 2 for dry soil.

Table 1: Total heterotrophic bacteria growth (cfu/g) in wet soil

	THB (cfu/g)				
Time (Day)	Control	20ml	30ml	40ml	50ml
0	384.368	384.368	384.368	384.368	384.368
14	3418.2	174348	288576	394788	833664
28	155710	1613220	2695380	4098180	4659300
42	652302	2454900	3857700	4729440	5521020
56	769536	2917820	6152280	8366700	9609180
70	833664	3616220	5641260	7924420	9126220
84	883764	3745480	5338660	7282540	8745480

Table 2: Total heterotrophic bacteria growth (cfu/g) in dry soil

	THB (cfu/g)				
Time (Day)	Control	20ml	30ml	40ml	50ml
0	196.392	196.392	196.392	196.392	196.392
14	2665.24	157314	271542	406812	732462
28	97734.9	1482960	2434860	3296580	3987960
42	806610	1825500	3266520	4367720	5649080
56	1683360	3086760	5771520	7354660	8228420
70	1733460	3461520	5569300	7262020	7844860
84	1943880	3573060	5618220	6937020	7642660

3.1 THB phase growth analysis

The various phases associated with bacteria growth under metabolic process was studies as illustrated in the Figures 1 and 2 for wet and dry soils under treatment using bioaugmentation technique.

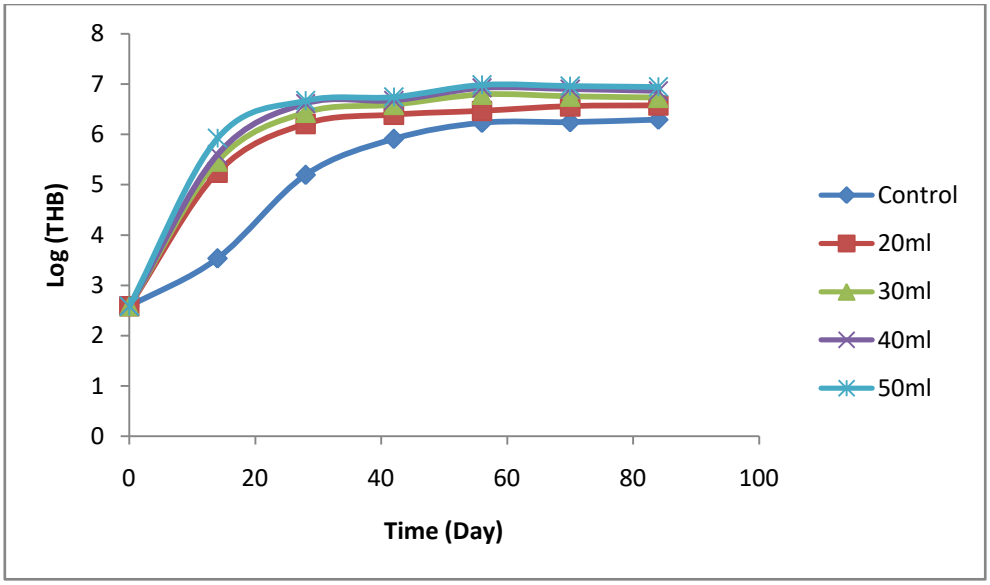


Figure 1: Bacteria growth incrude oil polluted wet soil

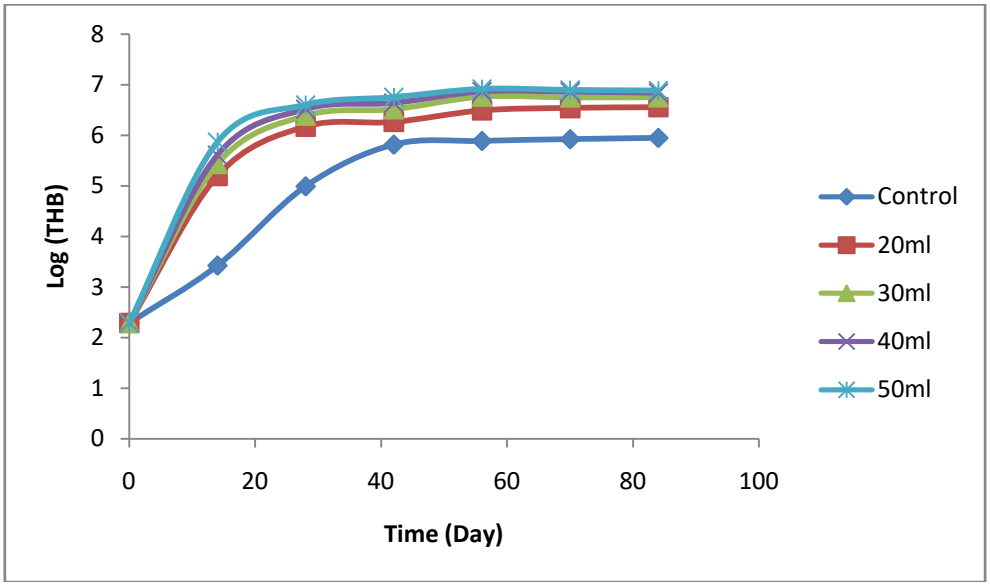


Figure 2: Bacteria growth in crude oil polluted dry soil

Figures 1 and 2 show the profiles of the phases of bacteria growth in crude oil polluted wet and dry soil samples, respectively, over a period of 84 daysunder bioremediation for control and inoculated soil samples with 20 – 50ml of *Pseudomonas sp*. The profiles of the natural logarithm of total heterotrophic bacteria (THB) versus remediation time indicated that the THB growth in the polluted wet and dry soils under treatment can be analyzed in different phases. Thus, for both wet and dry soils, the control sample showed a slower total bacteriagrowth over the entire periods of the remediation compared to the samples with treatment. The analysis of the growth phases indicates that all the samples has very short lag phase, indicating that bacteria in soil adapted quickly to the crude oil polluted soil environment.

However, in wet soil, as indicated by Figure 1, the logarithmic value of 2.5847 was recorded for control sample in day 1 andthen increased to a value of 5.814449 in day 42.This growth phase can be described as exponential growth. After day 42, the growth transitioned to the stationary phase till day 84 of the analysis. This growth trend was also observed in all the treated samples, except that thetreated soil samples depicted a shorter lag and longer exponential growth phases compared to the control sample. Thus, the *Pseudomonas sp*. treated wet soil experienced an accelerated exponential increase in THB count from the initial population to day 14, but the population remained relatively constant with time, indicating that the growth has entered the stationary phase.

Similar trends and growth phases were recorded in the dry soil (Figure 2) for both control and treated soils. However, the bacteria growth in the wet soil slightly edged the growth in dry soil, as shown in Tables 1 and 2, while the highest THB count was recorded in soil samples inoculated with 50 ml *Pseudomonas sp*. Generally, there was no indication of death phase within the period of the remediation, which implied the availability of nutrients sustaining the bacteria to metabolize on the substrate hydrocarbons. These phased growth patterns align with Ye et al. (2019) who inoculated PAH-contaminated soil with a PAH-degrading consortium. A work by Udari et al. (2019) also reported similar growth trends of PAH-degrading bacterium B2 inoculated to aged PAH-contaminated soil over 90 days. Overall, the log growth curves delineate the typical microbial growth dynamics in polluted soils, and

Pseudomonas sp. supplementation improved the soilrestoration which affirms its bioaugmentation potential for TPH-bioremediation.

3.2 Growth Rate Evaluation

The THB growth rate was evaluated by using the model in equation (1). The model constants used were determined as shown in Table 3.

Table 3: Evaluated constants for THB growth rate

	U_{max} (mg/kg. day)		K_s (mg/kg)	
Treatment (ml)	Wet	Dry	Wet	Dry
Control	-250	-416.67	-52480	-55629
20	1111.11	1000	7721.89	9461.2
30	1000	1111.11	4257.3	7840.22
40	1428.57	1250	4655	6888.25
50	1428.57	1000	2651	2960.3

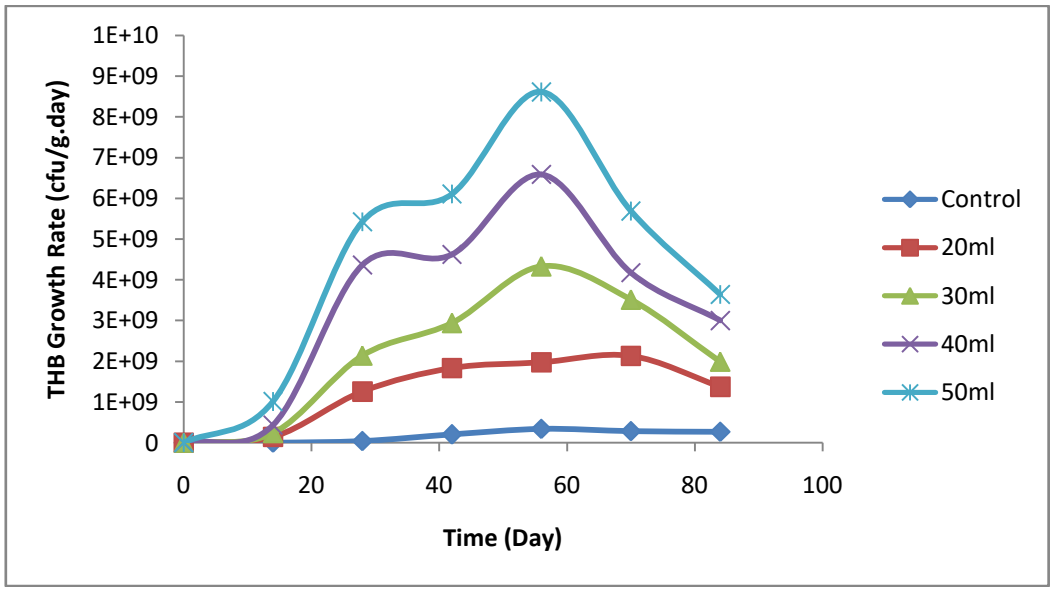


Figure 3: The growth rate oftotal heterotrophic bacteria (THB) in wet soil

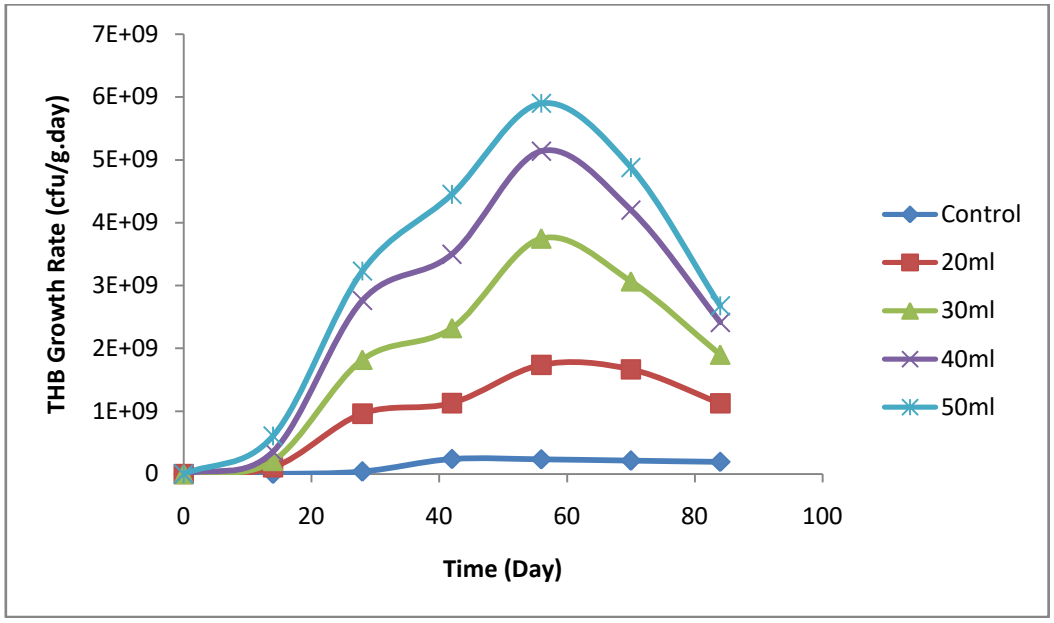


Figure 4: The growth rate oftotal heterotrophic bacteria (THB) in dry soil

The growth rates of total heterotrophic bacteria (THB) in crude oil polluted wet and dry soils treated with *Pseudomonas sp*. over 84 days at 20 – 50 ml of quantity is presented in Figures 3 and 4. Asdemonstratedby the profiles in the Figures 3 and 4, all treated samples exhibited significantly higher growth rates compared to the control wet or dry soil samples. Thus, in wet soil, THB growth rate increased from 2.096 x 10^5 to 2.718x 10^8cfu/g.day, while for dry soil it increased from 1.155 x 10^5 to 1.924x 10^8cfu/g.day. However, with 20 ml addition of *Pseudomonas sp*., the rate increased exponentially from 3.516 x 10^5 to 1.405x 10^9cfu/g.day for wet soil and from 1.522 x 10^5 to 1.137x 10^9cfu/g.day for dry soil over the remediation period of 84 days.With further addition of 50 ml *Pseudomonas sp*.,thegrowth rate wasamplified with similar trend, recording the maximum growth rate of 8.623x 10^9cfu/g.day and 5.903x 10^9cfu/g.day after 56 daysfor wet and dry soils, respectively.Importantly, from 70 to 84 days, there was a decline of THB growth rate, indicating a period of nutrient reduction necessary for microbial activities.

These findings correlate with AlKharusi et al. (2019), who reported escalated rates of hydrocarbon degradation in contaminated sandstone treated with a microbial consortium of *Bacillus subtilis* J2, *Micrococcus yunnanensis* NTU-12 and *Pseudomonas aeruginosa* NTU-5 as compared to the control soil sample. A similar study also reported a high growth rate of THB in diesel-polluted mangrove sediment amended with PAH-degrading bacterial consortium (Shukor et al., 2019). Sabra et al. (2020) also observed that PAH-polluted soil receiving fermentate of *Bacillus sp*. displayed higher degradation rates compared to the un-amended control, attributing it to indigenous microbial proliferation stimulated by bacterial metabolites. Another study by Prapagdee et al. (2020), who inoculated PAH-contaminated soil with a PA20 isolate, observed a maximum growth rate of 3.6 x 10^5cfu/g.day for the treated soil compared to negligible change in the control sample. The elevated positive growth rates over time with increasing *Pseudomonas sp*. imply its viability as a bioaugmentation agent for accelerating bioremediation of TPH-contaminated sites by enhancing native THB activity.

3.3 Predictive model for THB growthin crude oil environment

The predictive model for THB growth was formulated using regression model in equation (2), with quantity of *Pseudomonas sp*. and degradation time used as the independent variables. The estimatedvariable coefficients were substituted andthen used to predict the THB counts at any given time. The measured and predicted values are shown in Figures 5 to 8 for both wet and dry soils.

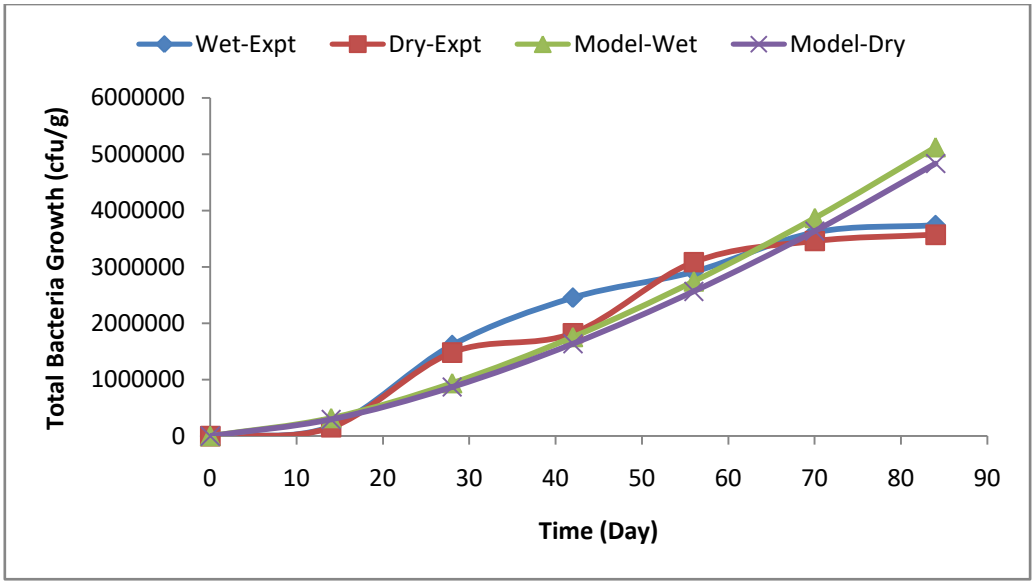


Figure 5: Predicted THB growth at 20 ml *Pseudomonas sp*.treated soil

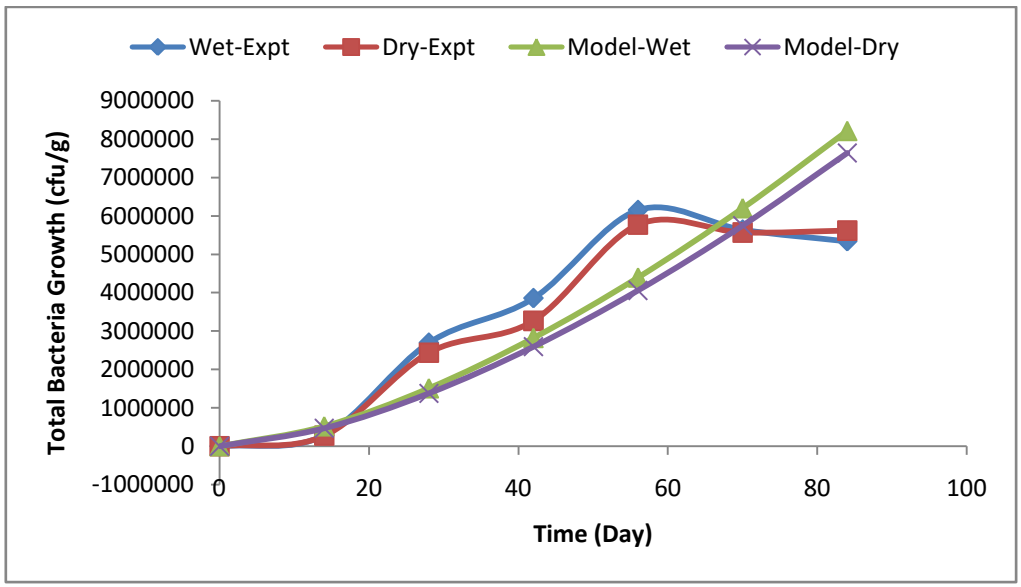


Figure 6: Predicted THB growth at 30 ml *Pseudomonas sp*.treated soil

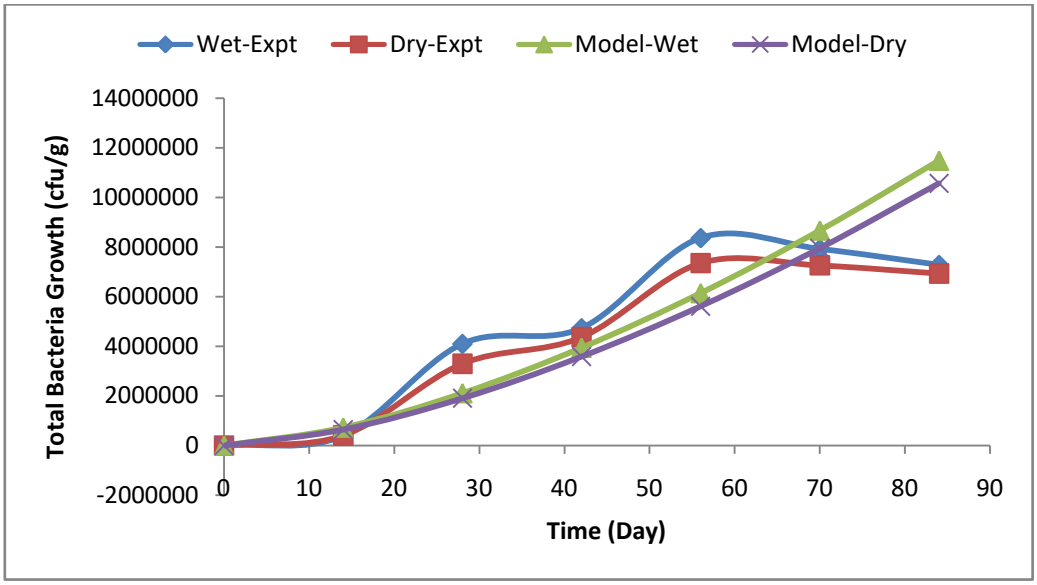


Figure 7: Predicted THB growth at 40 ml *Pseudomonas sp*.treated soil

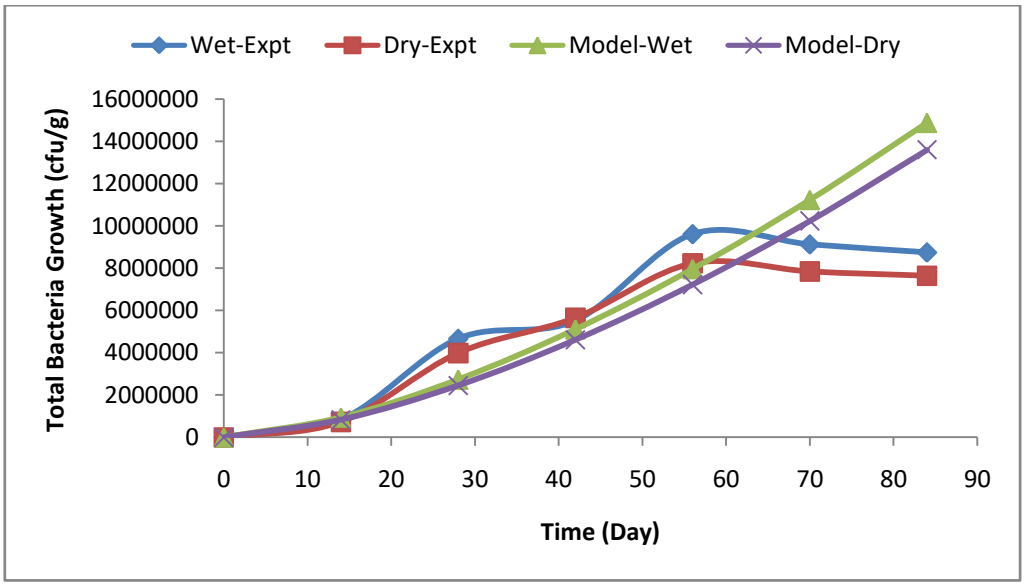


Figure 8: Predicted THB growth at 50 ml *Pseudomonas sp*.treated soil

Figures 5 to 8 compare the experimentally measured (Wet-Expt, Dry-Expt) and predicted (Wet-Model, Dry-Model) THB counts in wet and dry soils treated with 20 – 50 ml *Pseudomonas sp*. with time. In Figure 5, the experimental and predicted THB values at 20 ml *Pseudomonas sp*. addition for both wet and dry soils showed increasing trends over time with correlation coefficients obtained as 0.8816 and 0.8929 respectively, though between day 70 and day 84, there was increasing deviation between the model valueand the actual value from the experiment. For instance, at 84 days for wet soil, the measured THB count was 3.7455 x 10^5cfu/g as against the predicted 5.1283x 10^6cfu/g.

Similar behaviour was observed for measured and predicted THB counts in wet and dry soils treated with 30, 40 and 50 ml *Pseudomonas sp*., as indicated in Figures 6, 7 and 8. However, at 30ml *Pseudomonas sp*., the measured and predicted THB values between day 14 and day 84 for wet soil ranged from 2.8858x 10^5– 5.3387x 10^6cfu/g and 5.1807x 10^5 – 8.2164x 10^6cfu/g, respectively, while for dry soil, measured and predicted THB values between day 14 and day 84 ranged from 2.7154x 10^5 – 5.6182x 10^6 and 4.6708x 10^5 – 7.6423x 10^6cfu/g,

respectively. Also, at 50 ml *Pseudomonas sp*., the measured and predicted THB values between day 14 and day 84 for wet soil ranged from 8.3366x 10^5 – 8.7455x 10^6cfu/g and 9.3817x 10^5 – 1.4879x 10^7cfu/g, respectively, while for dry soil, measured and predicted THB values between day 14 and day 84 ranged from 7.3246x 10^5 – 7.6427x $10^6$and 8.3131x 10^5 – 1.3602x 10^7cfu/g, respectively.

Generally, the models concurred with initial experimental values but over predicted the TPH beyond day 70. Nevertheless, the multiple linear regression model can be applied topredicted the growth of THB for crude oil polluted soil under pseudomonas sp. treatment over a given period of time.Corresponding previous studies also found reasonable agreement between predicted and actual values with tendencies for models to over predictthe actual results. For instance, Yin et al. (2020) observed that linear regression satisfactorily simulated PHAs degradation but overestimated mineralization at later stages. Chen et al. (2019) also observed a good prediction of bacteria growthwithinthe initial remediation period, but deviated subsequently. Hence, pattern of bacteria growth can lead to inaccurate prediction over time, except a model that can address the factors responsible for the deviations is used.

4. CONCLUSION

The study investigated the growth of total heterotrophic bacteria (THB) in crude oil contaminated soil under treatment using *Pseudomonas sp*. as a bioaugmentation agent. The total heterotrophic bacteria (THB) population was monitored over 84 days in crude oil polluted wet and dry soils treated with 20-50 ml of *Pseudomonas sp*., and compared to untreated control samples.The THB counts increased significantly in both wet and dry soils with treatment compared to the controls. Higher inoculation volumes of 30-50 ml *Pseudomonas sp*. led to the highest THB populations, indicating its effectiveness as a bioaugmentation agent in stimulating native soil microbes. Analysis of the THB growth patterns revealed characteristic lag, exponential, and stationary phases. The treated soils exhibited shorter lag phases and more prolonged exponential phases compared to controls, demonstrating the positive impact of *Pseudomonas sp*. supplementation. THB growth rates increased substantially in the treated soils over the 84 day period, with maximum rates reaching over 10^9cfu/g.day, while the predictive model reasonably estimated the THB counts in comparison to experimental measurements. However, the accuracy of the modeldecreased after 70 days. The study demonstrated that bioaugmentation with *Pseudomonas sp*. enhanced THB proliferation and the model can be applied to study the growth pattern of THB in crude oil polluted soil.

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