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Effect of Aerating Duration on Hydrocarbon Biodegradation in a Simulated Crude-Oil Polluted Aquatic Environment Undergoing Bioremediation

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

The aim of this research work was to determine the aerating duration that would be effective in enhancing hydrocarbon biodegradation rate during bioremediation of crude-oil polluted river. Sediment and river-water were placed in four glass troughs labeled CT (control), A, B, and C. The setups were polluted with crude-oil, and allowed undisturbed for 2 weeks. Subsequently, accessible crude-oil on the surface was removed; bacteria and nutrients were then added. Air was bubbled for 3 hours into setups A, B, and C, at daily, 3 days, and 7 days interval respectively. Aeration was not applied to setup CT. On day 1, 7, 14, and 21, hydrocarbon concentration was determined; populations of total heterotrophic bacteria (THB) and hydrocarbon-utilizing bacteria (HUB) were also determined. The time it will take for hydrocarbons in the setups to biodegraded "completely" was calculated using first-order reaction equation. The results obtained showed that 71.43, 86.39, 83.17, and 15.42 % hydrocarbon degradation were obtained in setup A, B, C, and CT respectively. The time it will take for hydrocarbons in the setups to biodegrade "completely" were 129, 89, 101, and 1079 days for A, B, C, and CT respectively. There was slight reduction in population of HUB in setup CT, fairly stable population in setup A, and increase in population of HUB in setups B and C. It is concluded that aerating crude-oil polluted aquatic environment for 3 hours at 3 days interval will be more effective in enhancing hydrocarbon biodegradation rate during bioremediation.

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Keywords: Aerating duration; aquatic environment; bioremediation; crude-oil pollution; hydrocarbon biodegradation rate.

1. INTRODUCTION

Much have been documented on the dangers associated with aquatic environments polluted with crude-oil [1-3] from negative impacts on aquatic life to negative impacts on humans due to dependency on aquatic life and water. It is thus necessary to remediate crude-oil polluted aquatic environments within the least possible time. The first set of remedial action usually taken following a large oil spill on water is containment and recovery. Containment is achieved with booms, while recovery is achieved with booms, skimmers, and sorbents [4]. These do not however remove all the oil. Thus, chemical or biological treatments are employed to achieve complete removal of the remaining/inaccessible oil. Biological treatment will usually involve the use of bio-agents such as nutrients, enzymes, or microorganisms that increase the rate of biodegradation of hydrocarbons. There are certain factors however that influence the rate of biodegradation of hydrocarbons; oxygen been a notable factor. Anaerobic hydrocarbon utilizing microorganisms exhibit slower growth than their aerobic counterpart [5]. Thus, biodegradation of hydrocarbons is often slow in the absence of oxygen. Hydrocarbons generally float on water forming thin films and sometimes thick films. These films limit oxygen diffusion into water, and will thus limit the rate of biodegradation of the hydrocarbons.

Aeration, a process by which air is forcefully circulated through a liquid, tends to enhance oxygen diffusion into liquids. Aeration has been used in the United States of America to remediate polluted urban waterways [6]. It increases oxygen levels in the water column thereby leading to increased pollutant removal. For enhanced bioremediation of crude-oil polluted aquatic environment, a form of aeration is therefore necessary.

In a previous research work, the application of aeration in the bioremediation of petroleum hydrocarbon polluted water was investigated [7]. The duration of aeration was however not considered in the research. Aeration involves the use of equipment that relies on electricity or fuel. Therefore, use of continued aeration during bioremediation of crude-oil polluted water will increase the cost associated with bioremediation. The aim of the research was thus to determine the duration of aeration that would be effective in enhancing the rate of hydrocarbon biodegradation during bioremediation of crude-oil polluted water bodies.

2. MATERIALS AND METHODS

2.1 Collection of Water and Sediment Samples

Water and sediment samples were collected from a river located near the Rivers State University (RSU), Port Harcourt, Nigeria. The water samples were collected at about 2 m from the shoreline at a depth of 0 – 30 cm. The water and sediment samples were collected using disinfected 10 L Jerry-can and polyethylene-bags respectively. The collected samples were transported to the Microbiology laboratory, RSU for bioremediation experimentation.

2.2 Bioremediation Setups

Four glass troughs were used for the bioremediation setups. The troughs had the following dimension: length – 30 cm, width – 20 cm, height – 20 cm. The four troughs were labeled CT (control), A, B, and C. Sediment of 100 g were placed in each trough, then 2 L of the river water was added. The setup was allowed undisturbed for a day. Subsequently, the setups were artificially polluted with 100 ml crude-oil. The artificially polluted simulated aquatic environment was allowed undisturbed for 2 weeks. On the expiration of the two weeks, accessible crude-oil floating on the water surface was removed with the aid of sterile 250 ml capacity glass beakers. Then, 1 ml bacterial culture and 5 ml nutrient solution in the form of mineral salts broth (MSB) were added to the setups. The composition of the MSB is as outlined in Odokuma & Dickson [8] $MgSO_4.7H_2O$ - 0.42 g/L, KH_2PO_4 - 0.83 g/L, NaCl - 10.0 g/L, KCl - 0.29 g/L, Na_2HPO_4 - 1.25 g/L, $NaNO_3$ - 0.42 g/L. After addition of bacteria and nutrients, each setup was then treated as follows: Setup A – air was bubbled into trough A for 3 hours daily, Setup B – air was bubbled into trough B for 3 hours at 3 days interval, Setup C – air was bubbled into trough C for 3 hours at 7 days interval. Air bubbling was achieved with the aid of an aquarium air pump (Sea Star, HX-106A), drip-

set tubing, and Y-tubes (Plate 1). Aeration was not applied to the control setup (CT).

2.2.1 Preparation of the bacterial culture

The bacterial culture added to the setups was prepared by placing 1 g garden soil into 10 ml nutrient broth. The inoculated broth was incubated at ambient temperature (29 – 31 °C) for 24 hours. After incubation, the ensuing turbid broth (now containing increased bacterial population) was decanted from the soil sediment into a sterile test tube, and used for the bioremediation setup.

2.3 Monitoring of the Setups

The setups were maintained for 21 days. The total hydrocarbon concentration (THC) in the setups was determined on day 1, 7, 14, and 21. The population of total heterotrophic bacteria (THB) and hydrocarbon utilizing bacteria (HUB) were determined on day 1 and day 21.

2.3.1 Determination of THC

The water and sediment in the setups were agitated by mixing using a sterile long L-shaped glass rod. About 10 ml sample was then collected with the aid of a sterile 10 ml pipette, then 5 ml of the collected sample placed in a 250 ml separating funnel and the remaining 5 ml placed in another 250 ml separating funnel. About 10 ml N-hexane was added to the content of the first separating funnel; for the second separating funnel, 10 ml n-hexane was used to rinse the oil in and around the pipette used in sample collection into the separating funnel. The mixture in the separating funnels were agitated for about 1 minute, and then allowed to stand for separation of the aqueous phase from the solvent phase. The aqueous phase in the funnels were removed, while the solvent phase were collected and subjected to absorbance measurement using a 721 VIS Spectrophotometer (Huanghua Faithful Instrument Co. Ltd, China) set at 420 nm. The absorbance readings were used to determine the THC through extrapolation from the plot of crude-oil concentration versus crude-oil absorbance at 420 nm which was previously obtained. The THCs from the divided samples were used to calculate the average THC.

2.3.2 Determination of population of THB and HUB

The water and sediment in the setups were agitated by mixing using a sterile long L-shaped glass rod. About 1 ml sample was then collected with the aid of a sterile 1 ml pipette and placed into 9 ml sterile normal saline to obtain 10^{-1} dilution. This dilution was agitated and then subjected to 10-fold serial dilution till a dilution of 10^{-4} was obtained. Aliquots of 0.1 ml of the different dilutions were spread inoculated on plates of nutrient agar (NA) and mineral salts agar (MSA) supplemented with 100 $mg.ml^{-1}$ ketoconazole, in duplicates. The composition of the MSA is as outlined in Odokuma & Dickson [8]. Inoculated MSA plates were supplied with petroleum hydrocarbons using the vapour phase transfer technique [9]. Inoculated NA plates were incubated at ambient temperatures (29 - 31° C) for 24 hours; inoculated MSA plates were also incubated at ambient temperatures but for 7 days. After incubation, counts of ensuing colonies on NA and MSA plates were used to calculate the THB and HUB population respectively.

2.4 Assessment of Hydrocarbon Degradation

The extent of hydrocarbon degradation (EHD) in the setups was determined with the aid of Eq. 1.

EHD (%) = (initial THC – final THC) × 100 ÷ initial THC   Eq. 1.

2.5 Assessment of the Time Required for Complete Biodegradation

The time it took for the hydrocarbons in the setups to degrade completely through biodegradation as a result of the treatments was determined using the first order reaction equation (Eq. 2; [10].

$$\text{Log } [A]_t = (-K\,t / 2.303) + \text{Log } [A]_0 \qquad \text{Eq. 2.}$$

In the equation, $[A]_t$ is the concentration at time t, $[A]_0$ is the initial concentration, t is the time required for $[A]_t$ to reduce to $[A]_0$, and K is the degradation constant.

For compound [A] to be biodegraded completely, i.e. for $\text{Log } [A]_t = 0$, Eq. 2 becomes,

$$t = 2.303 \times \text{Log } [A]_0 \div K \ \ldots \qquad \text{Eq. 3.}$$

Note, $\text{Log } [A]_t = 0$ implies that A = 1 (Log [1] = 0). Eq. 3 was thus used in calculating the time it will take for hydrocarbon concentrations in the setups to reduce to 1 mg/L.

Plate 1. Air bubbling into the simulated crude-oil polluted water body with the aid of aquarium air pump, drip-set tubing, and Y-tubes. [The control setup is the glass trough at the left-back, setup A is at the right-back, setup B at the front-right, and setup C at the front-left]

2.6 Isolation and Identification of Hydrocarbon Utilizing Bacteria

Bacterial colonies on enumerated MSA plates were isolated and sub-cultured onto fresh sterile NA plates and coded. Sub-cultured isolates were identified based on colonial morphology, fluorescence under ultra-violet (UV) light, Gram stain reaction, microscopic morphology, and reaction to the following biochemical/ physicochemical tests: catalase, oxidase, starch hydrolysis, motility, salt (7% NaCl) tolerance, Methyl Red, Vogues-Proskauer, glucose fermentation, and lactose fermentation. These tests were carried out as described in Prescott *et al*. [11] and Madigan *et al*. [12].

3. RESULTS AND DISCUSSION

3.1 Reduction in Hydrocarbon Concentration in the Bioremediation Setups

The total hydrocarbon concentration (THC) in the bioremediation setups in the course of the experimental period is presented in Fig. 1. In the Figure, it can be seen that the different setups had different THC on day 1. However, the Analysis of variance (ANOVA) of the THC showed that there was no significant difference (P > 0.05) between the THCs. Also, it can be seen in Fig. 1 that there was reduction in THC in all the setups including the control, and the reduction was highest in setup B and lowest in setup CT (the control setup). The ANOVA of the different THC in the different setups on day 21 showed that there was no significant difference (P > 0.05) between the THC in the different setups. However, ANOVA of the THCs in each experimental setup and the control setup on day 21 showed that there was significant difference (P < 0.05) between the THCs.

The extents of hydrocarbon degradation in setups A, B, C, and CT at the end of the experiment were 71.43, 86.39, 83.17, and 15.42 % respectively (Table 1). In a related study, a reduction in THC of about 53.3 % was obtained after 4 weeks of treatment of crude oil polluted water with aeration and culture of a hydrocarbon degrading bacterium [7]. In another related study where near complete reduction in THC was obtained after 8 weeks of treatment of crude oil polluted waters with mixed microbial culture, nutrient, aeration, and agitation [13], about 52 % THC reduction was achieved on the 3rd week. In the present study, the highest THC reduction obtained after 3 weeks of treatment was 86.39 %. The much difference in the extent of THC

reduction observed in this study and those observed in the other studies cited here can be attributed to the intervals between aeration; in this study, aeration was applied for 3 hours at 3 days interval in the setup having the highest THC reduction, whereas in the other studies aeration was applied on a daily basis. Biodegradation of hydrocarbons in water has been shown to occur under aerobic and anaerobic conditions, with the highest hydrocarbon reduction observed in aerobic conditions [14]. Also, microorganisms such as *Bacillus*, *Citrobacter*, and *Pseudomonas*, which partake in aerobic and anaerobic degradation of hydrocarbons are facultative anaerobes [15-18]. Products of anaerobic biodegradation of hydrocarbons include volatile and long-chain fatty acids, e.g. acetate, formate, and stearate [19]. Fatty acids are metabolized by microorganisms to yield acetyl-CoA which is further metabolized to yield energy and precursors for cellular biosynthesis [20]. In the presence of oxygen, acetyl-CoA enters the tricarboxylic acid cycle and undergoes a series of enzymatic reactions leading to the release of CO_2 [11]. A cycle of anaerobic and aerobic phase may thus be required by facultative anaerobes which are hydrocarbon degraders to achieve complete degradation of hydrocarbons within a relatively short time. The time in-between which aeration was applied may have thus instigated the aerobic biodegradation of products from anaerobic biodegradation.

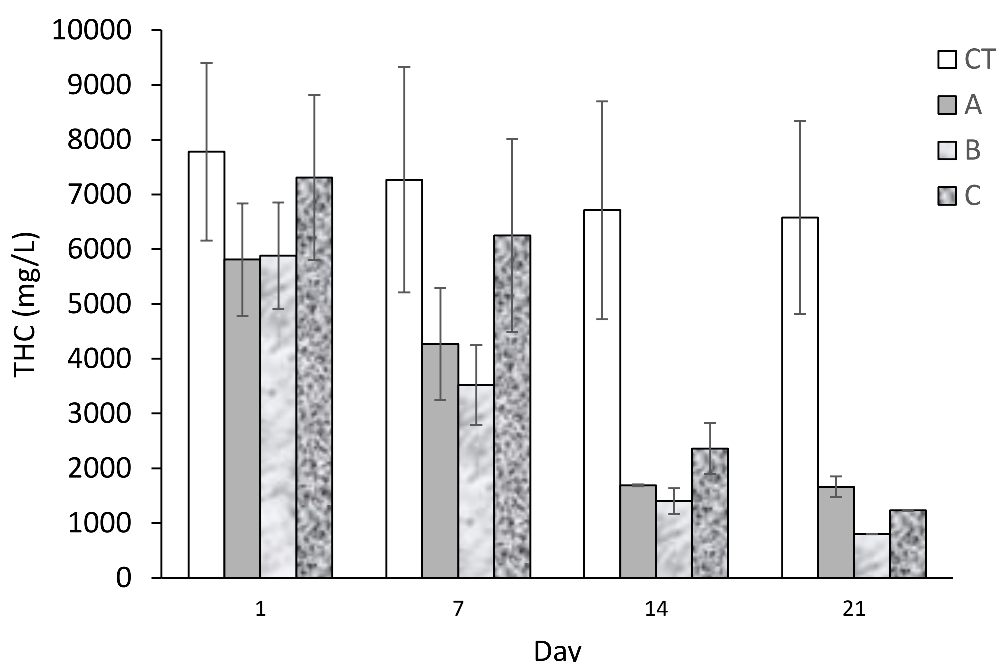

Fig. 1. Total hydrocarbon concentration (THC) in the setups at the different days of analysis
CT: Control setup, A: setup in which air was bubbled for 3 hours daily, B: setup in which air was bubbled for 3 hours at 3 days interval, C: setup in which air was bubbled for 3 hours at 7 days interval

Table 1. Extent of hydrocarbon degradation in the setups

Setup	Total hydrocarbon concentration on…		
	day 0 (mg/L)	day 21 (mg/L)	EHD (%)
A	5810	1660	71.43
B	5880	800	86.39
C	7310	1230	83.17
CT	7780	6580	15.42

EHD: Extent of hydrocarbon degradation, CT: Control setup, A: setup in which air was bubbled for 3 hours daily, B: setup in which air was bubbled for 3 hours at 3 days interval, C: setup in which air was bubbled for 3 hours at 7 days interval

3.2 Predicted time for Complete Biodegradation

Using the linear function equations of the plots of Log THC versus time (Fig. 2a and 2b) in conjunction with Equation 2, the degradation constants, *k*, of the total hydrocarbon concentration (THC) in setups CT, A, B, and C, were determined to be 0.0083, 0.0670, 0.0986, and 0.0903 per day respectively. Substituting for *K* in Equation 3, the time it will take for THC in setups CT, A, B, and C, to reduce to 1 mg/L were calculated to be 1079 days, 129 days, 89 days, and 101 days respectively. From the values obtained, it can be seen that complete biodegradation of hydrocarbons will first be achieved in bioremediation setup B.

Attenuation of petroleum hydrocarbons in terms of biodegradation has been shown to follow a first order reaction [21-22]. The plots in Fig. 2a & b are graphical depictions of first order reaction equation (Equation 2).

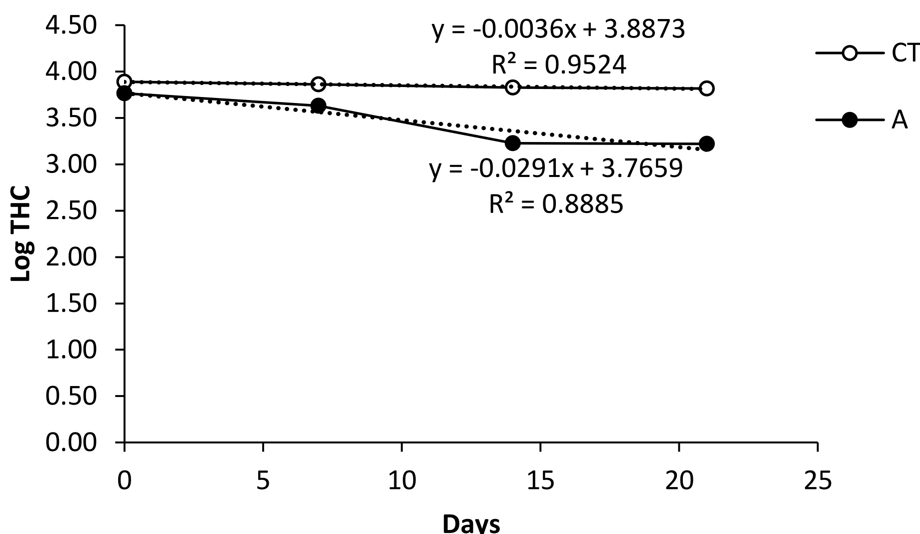

Fig. 2a. Plot of Log total hydrocarbon concentration (THC) in setup CT and A versus time

C: Control setup, A: setup in which air was bubbled for 3 hours daily

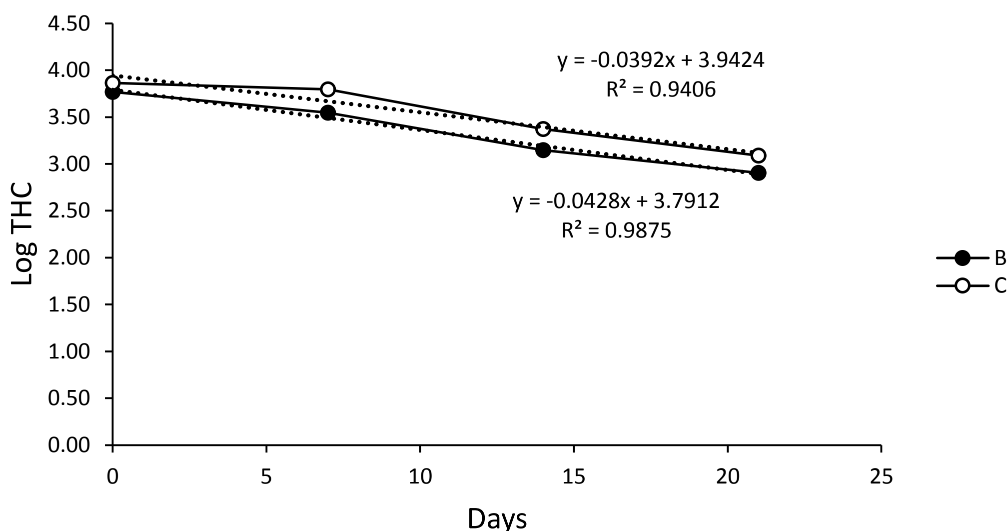

Fig. 2b. Plot of Log THC in setup B and C versus time

B: setup in which air was bubbled for 3 hours at 3 days interval, C: setup in which air was bubbled for 3 hours at 7 days interval

3.3 Change in Bacterial Populations in the Bioremediation Experimental Setup

The population of total heterotrophic bacteria (THB) and hydrocarbon utilizing bacteria (HUB) at the beginning and end of the experiment is presented in Fig. 3a and 3b. In Fig. 3a, it can be seen that there was reduction in the population of THB in the control setup, whereas in setup A, B, and C, there was an increase in the population of THB. In Fig. 3b, it can be seen that there was a slight reduction in the population of HUB in the control setup; the population of HUB in setup A was fairly stable, while in setups B and C there was an increase in the population of HUB. Studies have shown that there is increase in HUB population in hydrocarbon polluted habitats undergoing bioremediation [23-25]. Increase in population of HUB in setups B and C thus indicate that biodegradation of hydrocarbons was more in them, and thus the relatively high hydrocarbon degradation in them compared to setups A and CT (Table 1). This also supports the suggestion that a cycle of anaerobic and aerobic phase is required by facultative anaerobes which are hydrocarbon degraders to achieve complete degradation of hydrocarbons within a relatively short time.

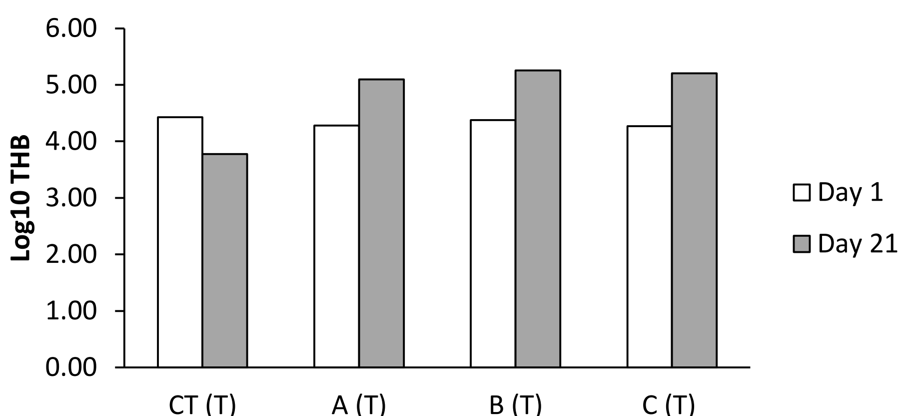

Fig. 3a. Change in population of total heterotrophic bacteria in the setups

CT(T): Total heterotrophic bacterial (THB) population in the control setup, A(T): THB population in setup A, B(T): THB population in setup B, C(T): THB population in setup C

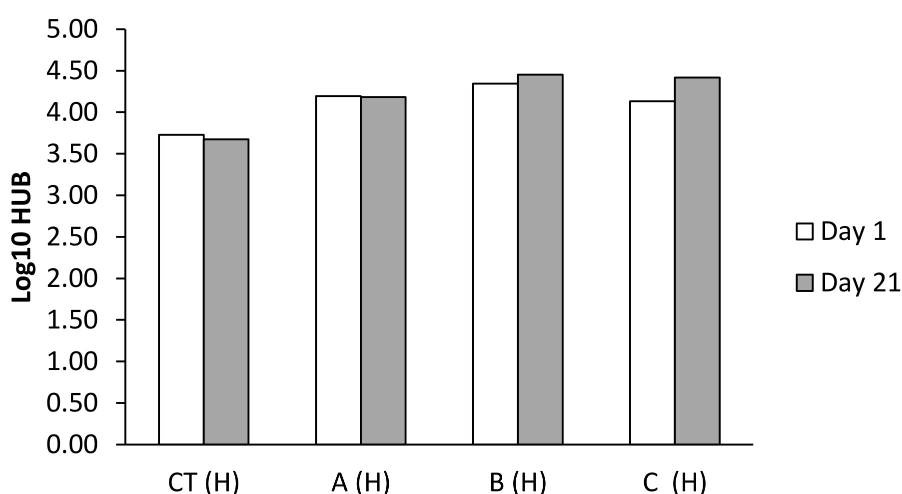

Fig. 3b. Change in population of hydrocarbon utilizing bacteria in the setups

CT(H): Hydrocarbon utilizing bacterial (HUB) population in the control setup, A(H): HUB population in setup A, B(H): HUB population in setup B, C(H): HUB population in setup C

3.4 Identity of the Hydrocarbon Utilizing Bacteria

The different isolated hydrocarbon utilizing bacteria (HUB) were 12 in number, and they were coded as Ct1, Ct2, HT7, HT1, C1, HT2, HT3, HT4, CtB1, CtB2, HT5, and HT6. They were coded according to the setup from which they were isolated from: Ct for isolates with unique colonial morphology from the control setup, HT for isolates from all the setups showing similar colonial morphology, C for isolate unique in colonial morphology from setup C, and CtB for isolates from the control setup showing similar colonial morphology with isolates from setup B. The colonial and microscopic morphology of the HUB isolates is presented in Table 2a, while the reaction pattern of the isolates to the biochemical and physicochemical tests used is presented in Table 2b. Comparing the colonial & microscopic morphology, and the reaction pattern of the isolates to information in selected Microbiology text books and Journal publications, the identity of the isolates is suspected as follows: Ct1 – *Arthrobacter* sp., Ct2 & HT1 – *Bacillus* spp., HT7 – *Pseudomonas fluorescens*, C1 & HT5 – *Staphylococcus* spp., HT2 & HT6 – *Corynebacterium* spp., HT3 – *Proteus* sp., HT4 – *Vibrio* sp., CtB1 – *Micrococcus* sp., CtB2 – *Serratia marcescens*. These bacteria have been shown to degrade petroleum hydrocarbons [26,17, 27-30].

Table 2a. Colonial and Microscopic morphology of isolated hydrocarbon utilizing bacteria

IC	CL	FM	SZ	EV	MG	SA	SX	FU	GS	MM
Ct1	Light orange	CC	MD	Convex	Entire	Smooth	Wet	-	+	R
Ct2	Gray white	IR	LG	Flat	Undulate	Rough	Dry	-	+	R
HT7	Cream*	CC	MD	Convex	Entire	Smooth	MC	+	-	R
HT1	Gray white	IR	LG	Flat	Undulate	Rough	Dry	-	+	R
C1	yellow	CC	MD	Convex	Entire	Smooth	Dry	-	+	C
HT2	Cream	CC	MD	Convex	Entire	Smooth	Wet	-	+	R
HT3	Cream	IR	LG	Flat	Entire	Rough	Dry	-	-	R
HT4	Cream	CC	MD	Convex	Entire	Smooth	Wet	-	-	R
CtB1	Yellow	CC	SM	Convex	Entire	Smooth	Dry	-	+	C
CtB2	Red	CC	MD	Convex	Entire	Smooth	MC	-	-	R
HT5	cream	CC	MD	Convex	Entire	Smooth	Dry	-	+	C
HT6	Cream	CC	MD	Convex	Entire	Smooth	Wet	-	+	R

IC: Isolate code, CL: colour, FM: form, SZ: size, EV: elevation, MG: margin, SA: surface appearance, SX: surface texture, FU: florescence under UV light, GS: Gram stain, MM: microscopic morphology, cream: with light-green pigment which diffused into the medium, CC: Circular, IR: Irregular, MD: medium, LG: Large, SM: small, MC: Mucoid, R: rods, C: cocci*

Table 2b. Result pattern of isolated hydrocarbon utilizing bacteria to the biochemical and physicochemical tests used

IC	CT	OX	SH	MT	ST	MR	VP	GF	LF	SO
Ct1	+	-	-	+	+	-	+	A	0	*Arthrobacter* sp.
Ct2	+	-	-	+	-	-	+	A	0	*Bacillus* sp.
HT7	+	+	-	+	+	-	-	A	0	*Pseudomonas fluorescens*
HT1	+	-	+	+	+	-	+	A	0	*Bacillus* sp.
C1	+	-	-	-	-	-	+	A	0	*Staphylococcus* sp.
HT2	+	-	-	+	+	-	+	A	A	*Corynebacterium* sp.
HT3	+	-	-	+	+	-	+	A	A	*Proteus* sp.
HT4	+	+	-	+	+	-	+	A	0	*Vibrio* sp.
CtB1	+	+	+	-	-	-	+	A	A	*Micrococcus* sp.
CtB2	+	-	-	+	+	+	+	AG	0	*Serratia marcescens*
HT5	+	-	+	-	+	-	+	A	A	*Staphylococcus* sp.
HT6	+	-	-	+	-	-	+	A	A	*Corynebacterium* sp.

IC: Isolate code, CT: catalase, OX: oxidase, SH: starch hydrolysis, MT: motility, ST: salt tolerance, MR: Methyl Red, VP: Vogues Proskauer, GF: glucose fermentation, LF: lactose fermentation, A: acid produced, AG: acid and gas produced, 0: no acid nor gas produced, SO: suspected organism

4. CONCLUSION

In this study, it has been shown that aeration enhances the biodegradation of petroleum hydrocarbons in crude-oil polluted water, and that the aerating duration influences this enhancement. Though there is no significant difference in the extent of hydrocarbon degradation between the different aerating duration, the time required to achieve complete biodegradation of hydrocarbons is between 2 to 6 weeks lesser for the setup aerated for 3 hours at 3 days interval compared to the other setups. It is thus concluded that for a crude-oil polluted aquatic environment undergoing bioremediation, aerating the water for 3 hours at 3 days interval will be more effective in enhancing biodegradation of the hydrocarbons.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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