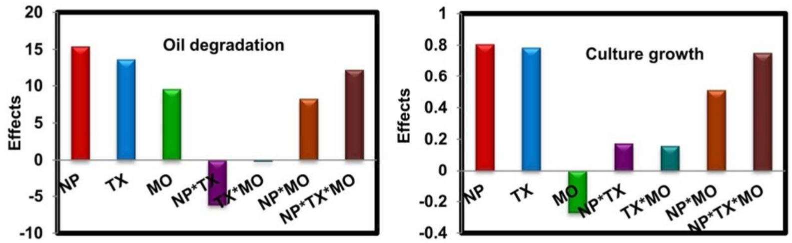
Graphical Abstract:

While each of the strategies nutrient addition (NP), surfactant addition (TX) and bioaugmentation with microorganisms (MO) can enhance oily sludge biodegradation, employing these strategies simultaneously leads to enhanced biodegradation through synergistic effects.



Environmental Impact:

Proper management of oily sludge is essential for preventing soil and groundwater contamination. In this study, a 2^3 full factorial design is used to evaluate the impact of various bioremediation strategies for oily sludge decontamination, i.e., bioaugmentation with indigenous microorganisms (MO), biostimulation with nutrients (NP) and biostimulation with surfactants (TX). This design reveals effective strategies while minimizing the experimental runs. Batch biodegradation studies were conducted over a period of 30 days. The effects were computed based on the 2^3 design and statistically significant effects were identified based on ANOVA. The main effects of nutrients, surfactant and bioaugmentation were all positive and significant for oil degradation. Significant synergistic effects among the various strategies were also observed.

Evaluation of Bioaugmentation and Biostimulation Effects on the Treatment of Refinery Oily Sludge using 2^n Full Factorial Design

by

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Abstract

Bioremediation approaches for treatment of oily sludge from a refinery were evaluated using the 2^3 factorial design. The three strategies tested were bioaugmentation with indigenous microbial consortia (MO) isolated from oily sludge, biostimulation with nutrients (NP) and biostimulation with the surfactant Triton X-100 (TX). Eight experimental runs were conducted in triplicate with factor settings +/- (high/low) as per the 2^3 design. The main effects and various interaction effects of the factors on oil degradation and microbial growth in suspension were evaluated during a 30 day study. Multifactor ANOVA could reveal the significant effects while the normal order score approach failed in this scenario. The main effect of biostimulation with nutrients in the form of nitrate and phosphate as well as biostimulation with Triton X-100 was positive and significant when both oil degradation and microbial growth in suspension were chosen as the response variable. However, the main effect of bioaugmentation was only significant for oil degradation but was insignificant for microbial growth at 90% confidence level. The MO-NP binary interaction and the MO-NP-TX ternary interaction were positive and significant indicating the synergistic effect of these strategies on oil degradation and microbial growth. All other binary interactions were found to be insignificant.

Keywords: ANOVA; Bioaugmentation; Biostimulation; Indigenous microbial consortium; Refinery oily sludge

1. Introduction

Oily sludge is generated from petroleum refineries in huge quantities as a byproduct of various processes and operations. Massive amount of oily sludge is also disposed off from the crude oil storage tank bottoms during cleaning and maintenance. Handling and disposal of such huge volume of waste becomes a major challenge. Oily sludge is a complex mixture of petroleum hydrocarbons and other solids including heavy metals that are carcinogenic and potent immunotoxicants. Apart from physicochemical treatment technologies, bioremediation is a clean, environmentally friendly treatment technology that can be applied for degradation of oily sludge generated in oil refineries [1-3]. Although successfully applied, landfarming approaches has certain limitations in terms of large space and time requirements and air pollution due to emission of volatile organic compounds. In contrast, slurry phase degradation can provide rapid and extensive degradation of oil by enhancing mass transfer rates and promoting interaction among microorganisms, pollutants and nutrients [4-6].

Two approaches can be used to enhance the bioremediation process i.e., bioaugmentation with native or tailored microbial consortium and biostimulation with nutrients and surfactants in controlled batch slurry systems [4,7-11]. However, effect of such approaches is not beneficial in all scenarios. The possible reasons could be site specific features such as soil or sludge type, distribution of contaminated hydrocarbons and presence/distribution of indigenous microorganisms capable of degrading the contaminants [8,12,13].

A mixed consortium of microorganisms may exhibit various modes of hydrocarbon uptake, such as, uptake of soluble hydrocarbons, uptake of emulsified forms or direct interfacial uptake facilitated by development of hydrophobic cell surfaces. Bioaugmentation with mixed microbial consortia is preferred due to their wide metabolic networks, through which they can easily assimilate the complex hydrocarbons in oily sludge or oil contaminated soils [9,14-18]. Moreover, enrichment of indigenous microorganisms and bioaugmentation with these enriched microorganisms well adapted to the contaminated environment has been recommended by various researchers [10,15]. Biostimulation with surfactants may increase the bioavailability of hydrocarbons by emulsification and also by altering the microbial cell surface properties so as to increase the interaction between the microbes and hydrocarbon contaminants [2,11,12,17-20]. Often oil contaminated soil or oily sludge is found to have much higher carbon content in comparison to nitrogen (N) and phosphorous (P). Adequate supply of N and P is essential for microbial growth and contaminant degradation. Moreover, during the course of natural attenuation nutrient depletion may lead to reduction in the indigenous microbial population. Thus, biostimulation with nutrients in the form of N and P is often found to induce contaminant degradation by the native microbial population while the activity of bioaugmented cultures is also enhanced [2,11,12,21].

Laboratory feasibility tests for bioremediation are essential to determine the potential of indigenous microbes to degrade the pollutants and to evaluate strategies for optimizing the rate and extent of degradation before pilot/full scale design of in-situ or ex-situ treatment schemes. Various studies focusing on comparative treatment studies of different bioremediation strategies make use of statistical tools to evaluate the significant differences between treatments [3,11,12]. In this study a 2^n full factorial design was used to investigate the effect of various factors so as to identify an appropriate treatment scheme for bioremediation of oily sludge from a refinery. This design can be used for screening a number of independent factors while minimizing the number of experimental runs. Statistical analysis of the results can provide useful information on bioremediation strategies. Three factors likely to influence biodegradation of oily sludge in laboratory batch systems were identified as bioaugmentation with indigenous microorganisms, biostimulation with nutrients and biostimulation with surfactants. Two different response variables were chosen, i.e., oil degradation in the system and microbial growth in suspension after 30 days. A key objective was to determine the main effect of each of these factors and the interaction effect between various factors so as to reveal possible synergism/antagonism among the three strategies. Although these strategies are commonly employed for oily sludge bioremediation, synergistic/antagonistic interactions among these strategies have not been explored by other researchers. The effects were quantified based on the 2^3 design and the significance of these effects was determined based on Analysis of variance (ANOVA).

2. Materials and Methods

2.1 Source of chemicals

The chemical surfactant TritonX-100 was procured from SD Fine Chemicals Pvt. Ltd. (Mumbai, India). The various chemicals used for preparation of mineral media, nutrient broth and bacteriological agar were procured from SD Fine Chemicals Pvt. Ltd., SRL industries Ltd., Merck and Hi Media Pvt. Ltd. High purity dichloromethane (DCM) used for extraction was obtained from Merck (India).

2.2 Source of oily sludge

The oily sludge was obtained from the weathering pits of a petroleum refinery in Mumbai (India) during August, 2010. The sludge was dewatered and centrifuged under high pressure to recover almost 90% of the crude oil prior to its disposal in the weathering pits. The sludge was collected from the refinery site, dried, sieved, homogenized and stored at 4 $^{\circ}$C. The batch biodegradation studies reported here were conducted almost two years after the sludge was collected (October-November, 2012).

2.3 Isolation and Enrichment of microorganisms from refinery oily sludge

Laboratory experiments were conducted to isolate and enrich microorganisms from the oily sludge. Enrichment was conducted in 500 mL flasks containing 100 mL mineral medium [17] with 0.5% (w/v) oil extracted from the sludge as the sole substrate. Isolation of pure cultures was carried out both by spread plating and streak plating in nutrient agar plates. The isolates were identified based on 16S rDNA sequencing (Macrogen, Inc., Korea) followed by BLAST analysis (NCBI) and were identified based on closest match with available sequences in the database. Five pure cultures were combined and used for bioaugmentation in the oily sludge biodegradation studies.

2.4 2^n Full Factorial Design

A 2^3 full factorial design was utilized in which eight runs were conducted at appropriate setting of each factor. The three factors were chosen as bioaugmentation with indigenous cultures isolated from sludge (MO), biostimulation with nutrients (NP) and biostimulation with Triton X-100 (TX), a chemical surfactant. The effect of the three factors were studied on two response variables i.e., % degradation of total petroleum hydrocarbons (TPH) in sludge over 30 days and increase in viable count in the aqueous phase (Ln(N/No), where No and N are viable count in the aqueous phase at 0 and at 30 days). Eight runs were conducted as per the design matrix for 2^3 full factorial design to determine the main effects, and binary and ternary interaction effects [22]. The level of the three factors (+/-) in each run was controlled as per the design matrix. The normal plot approach and analysis of variance (ANOVA) approach were both used to evaluate the significant main effect and interaction effects. The ANOVA was performed using STATISTICA ver.8.

2.5 Biodegradation Study

A thirty day long biodegradation study was set-up as per the full factorial design matrix. For each run as specified in this matrix, triplicate batches were set-up. Each batch flask

(500 mL) contained 10% (w/v) oily sludge in 100 mL mineral media (MM). Biostimulation consisted of addition of nutrients in the form of nitrate-nitrogen (NO_3-N) and phosphate-phosphorous (PO_4-P). The MM already contained some N and P at the baseline level (-) of 222.4 mg/L N as NH_4-N and 198.66 mg/L P as PO_4-P such that the N:P ratio was 1.21:1 (mass basis). For nutrient addition (NP +) additional N and P i.e., 70 mg/L NO_3-N and 31.2 mg/L PO_4-P was supplemented in the medium in the form of KNO_3 and KH_2PO_4, respectively. A nonionic surfactant, TritonX-100 was also used to study its effect on the extent of oily sludge degradation. Flasks biostimulated with Triton X-100 (TX +) contained the surfactant at twice the critical micelle concentration (CMC). For bioaugmentation (MO +), 5 mL of the 5-membered reconstituted consortium adjusted to unit absorbance at 600 nm was added as inoculum. These cultures enriched and isolated from the oily sludge were maintained in the laboratory using 0.5% (w/v) oil extracted from the sludge. The reconstituted consortium was prepared by addition of each of the strain in equal proportion after growing them up to end of log phase.

The flasks were sacrificed initially (i.e., 0 day) and after 30 days of incubation at 35ºC and 150 rpm in a rotary shaker. The sludge slurry contained in the flasks was filtered. The residual sludge collected in the filter paper was further analyzed for the residual oil content at initial i.e., 0 day and after 30 days. The aqueous suspension obtained was analyzed for pH, viable cell count and total organic carbon (TOC) content. Moreover, liquid-liquid extraction using dichloromethane as solvent (in the ratio of 1:1)[19] was also performed to estimate oil/total petroleum hydrocarbon (TPH) in the aqueous suspension. Residual TPH in sludge was estimated by soxhlet extraction using dichloromethane as solvent followed by gravimetric analysis and %degradation of oil was computed based on the zero day values. Standard error (SE) was based on propagation of error for studies conducted in triplicate setup. Viable counts in the aqueous phase of the sludge slurry set-up were determined through standard plate count procedure at 0 and 30 days, respectively and Ln(N/No) was determined as a measure of culture growth in suspension.

3. Results and Discussion

Oily sludge is a natural material that is often reported to have microbial cultures associated with it. In batch biodegradation studies with this oily refinery sludge Jasmine and Mukherji [23] have earlier demonstrated that in batch systems where no microbial cultures were added (un-spiked controls) a large variability in oil biodegradation was observed (44±10% over 30 days). In contrast, systems containing 0.1% sodium azide added as a biocide showed negligible oil degradation (6±4.5% over 30 days). Loss of oil in the un-spiked controls was found to increase progressively with time and this was coupled with increase in culture count in the aqueous phase. In some cases, biodegradation in the un-spiked controls were almost comparable to systems where specific aliphatic and aromatic hydrocarbon degrading *Burkholderia* cultures were added extraneously. The predominant cultures found in the aqueous phase of the un-spiked controls were isolated and identified. The large variability observed in the unspiked controls indicated heterogeneity in distribution of these microorganisms in the sludge. The strains isolated from oily sludge were identified through 16 S rRNA analysis. The predominant cultures were identified as *Microbacter* sp., *Bacillus* sp., *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Stenotrophomonas* sp. Each of these strains exhibited good growth when 0.5 %(w/v) of the oil extracted from oily sludge was provided as sole source of carbon and energy (Fig. 1) and end of log phase was reached within 7 days. Ongoing studies have revealed remarkable ability of these cultures to utilize hydrocarbons across various groups as sole substrate, including n-alkanes, cycloalkanes, and 2-, 3- and 4-ring PAHs (unpublished results). Although degradation of hydrocarbons, crude oil and oily sludge by *Pseudomonas* sp. *Acinetobacter* sp. and *Bacillus* sp. is widely reported [7, 14, 24-27], no previous studies have reported the role of *Microbacter sp.* and *Stenotrophomonas sp.* in oily sludge degradation.

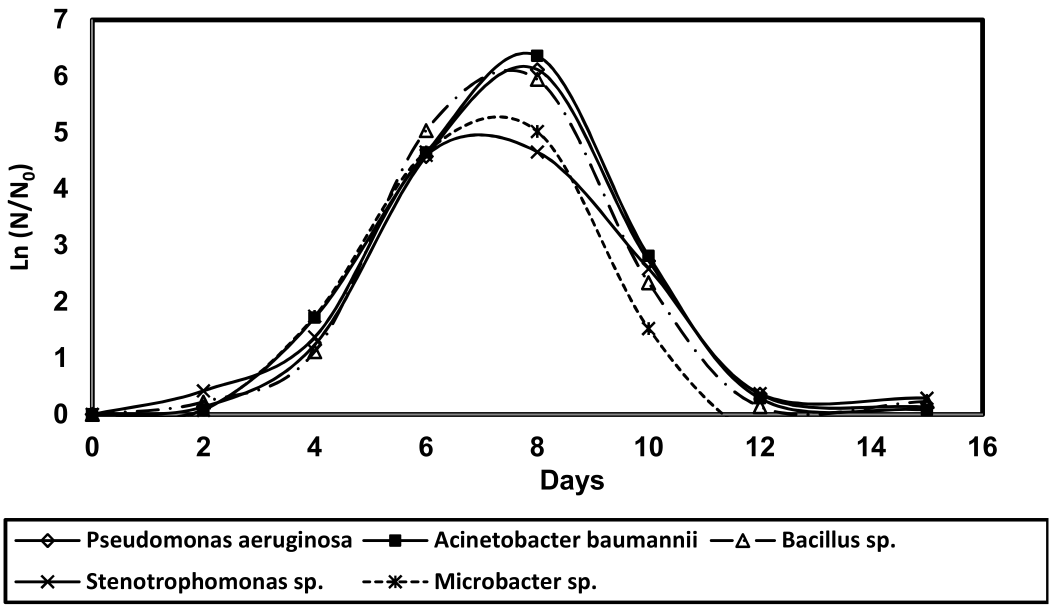

Fig. 1 Growth of pure cultures isolated from oily sludge on 0.5%(w/v) of extracted oil provided as sole substrate in batch cultures

In this study, a reconstituted consortium comprised of these strains was used for bioaugmentation. The use of mixed indigenous microbial consortia may be advantageous due to broader metabolic capacity, synergistic effects and co-metabolic effects [7,21]. These indigenous microorganisms are expected to be better acclimatized to the oily sludge and may exhibit better tolerance to co-contaminants [11] compared to other extraneous oil degrading cultures. Nitrate was supplemented as nitrate-nitrogen in the batches with nutrient supplementation since an excess of ammonium nitrogen is known to be toxic to some microorganisms. An excess of phosphates is also sometimes reported to be toxic to microorganisms, such that microbial degradation may be adversely affected in nutrient supplemented systems. The nonionic surfactant Triton X-100 was used in this study since nonionic surfactants are comparatively less toxic and Triton X-100 has been reported to enhance microbial degradation of oil [17,19,20].

The study was designed as per 2^3 factorial design. The conditions prevailing in each run/treatment is illustrated in Table 1. The oily sludge contained 9-10.5% oil on dry weight basis. Significant variation was observed in the experiments due to heterogeneous distribution of oil and microorganisms in the sludge as illustrated by the SE values. TPH associated with the aqueous phase was consistently found to be very low (0.35 ± 0.17%), hence, %degradation was estimated solely based on TPH determined by soxhlet extraction of the sludge. This study revealed that bioaugmentation with microorganisms and biostimulation with nutrients and surfactants increased the extent of degradation of oil in oily sludge to 57 (±9.3) % over 30 days. In contrast, the individual treatments (i.e., only addition of microorganisms or only addition of nutrients or only addition of surfactants) yielded much lower degradation of oil. Oil degradation over 30 days was only 6.7±3.1% in the controls where neither bioaugmentation nor biostimulation was performed. Interestingly, for studies conducted with the sludge within a year of sludge collection, TPH degradation in the un-inoculated controls was much higher, i.e., 44±10% [23]. Thus, prolonged storage at 4°C decreased the activity of the indigenous cultures. With addition of nutrients only, oil degradation with respect to initial was 32.4 ±9.7% whereas only addition of Triton X 100 increased oil degradation to 39.1±4.6% over 30 days. Only bioaugmentation with microbial consortium caused 20.5±3.0% oil degradation over 30 days. Further interpretation regarding the impact of using multiple strategies, i.e., simultaneous addition of nutrients and surfactants and simultaneous bioaugmentation and biostimulation with nutrients were interpreted using factorial design and ANOVA concepts.

Table 1. Oil degradation and culture growth in suspension over 30 days for various treatments for the oily sludge biodegradation study as per 2^3 design

Run	Factors			Response variables			
	NP	TX	MO	% Degradation		Ln(N/N$_o$)	
				Mean	SE	Mean	SE
1	-	-	-	6.7	3.1	0.40	0.28
2	+	-	-	32.4	9.7	1.28	0.32
3	-	+	-	39.1	4.6	1.61	0.37
4	+	+	-	27.8	8.8	1.33	0.39
5	-	-	+	20.5	3	0.22	0.32
6	+	-	+	38.2	8.9	0.61	0.31
7	-	+	+	28.0	4.4	0.23	0.06
8	+	+	+	57.8	9.3	2.48	0.49

NP: nutrients, TX: Triton X 100, MO: microorganisms, SE: standard error, N: Viable count of microorganisms at 30 day, N_0: Viable count of microorganisms at 0 day

The influence of the various factors i.e., bioaugmentation with indigenous cultures isolated from sludge (MO), biostimulation with nutrients (NP) and biostimulation with Triton X-100 (TX) could be determined for both set of response variables, i.e., oil degradation and culture growth in suspension over 30 days based on the 2^3 factorial design as illustrated in Fig. 2a-b. The average oil degradation over 30 days across all the experimental runs was 31.3% and average culture growth in suspension (Ln(N/No) was 1.02. All the main effects and interaction effects elevated oil degradation over 30 days except for the binary interactions between nutrient and surfactant addition and bioaugmentation and surfactant addition. Surprisingly, the main effect of bioaugmentation was to reduce culture growth in suspension. All the other main effects and binary and ternary interaction effects were positive when culture growth in suspension was used as the response variable.

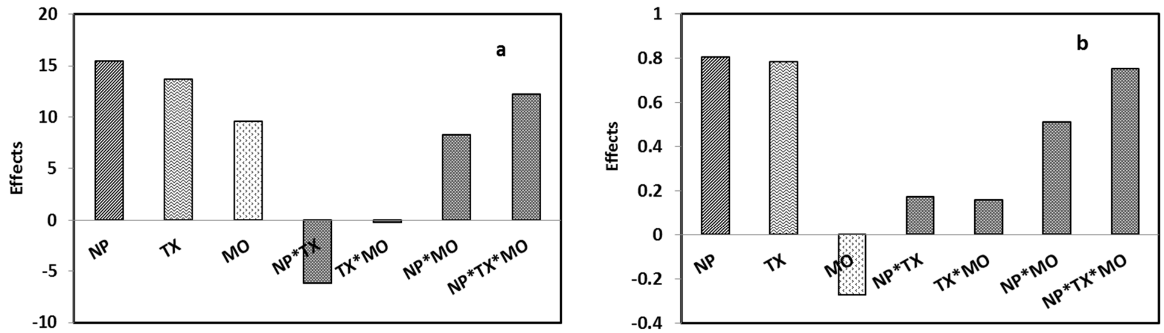

Fig. 2 Magnitude of main effects and binary and ternary interaction effects for the oily sludge biodegradation study as per 2^3 factorial design for (a) oil degradation and (b) culture growth in suspension as the response variable

After the average values of main effect, the two way interaction effects and the three way interaction effect was determined, an attempt was first made to determine if the normal order score approach could highlight the significant effects. However, this approach failed to provide much insight on the significant main and interaction effects as illustrated in Fig. 3a-b. In the normal order score approach, the effects that are random and normally distributed fall on a straight line when effects are plotted against the normal order score. In contrast, significant effects that are not randomly distributed fall off the straight line joining the random effects [22]. A straight line passing through the origin could not be fitted for the data illustrated in Fig. 3a-b. However, since each run was conducted in triplicate the significant and insignificant effects could be determined based on ANOVA. The ANOVA approach yields more insight since it reveals information contained in the replicates which is missing in the normal order score approach.

Multifactor ANOVA was performed using STATISTICA and the results are summarized in Table 2. The significance of main and interaction effects may be determined based on F-statistics and p-value. Statistically significant main effects of NP and Triton X-100 addition were found for both the measured response variables at 90% confidence level.

However, the main effect of bioaugmentation was significant for TPH degradation but insignificant in case of microbial growth in suspension.

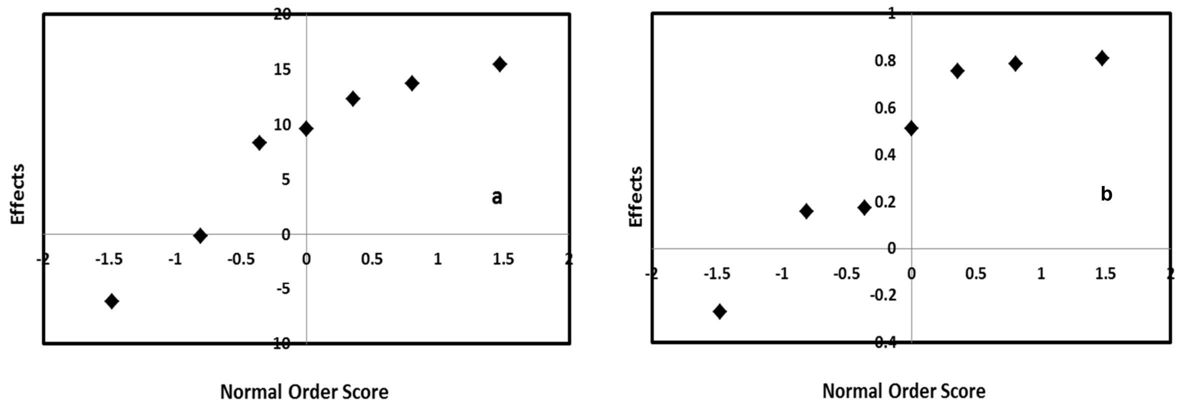

Fig. 3 Normal plot of effects for the 2^3 factorial design with (a) oil degradation and (b) culture growth in suspension over 30 days as response variables

Addition of nutrients had significant effects on the extent of degradation and also on microbial growth in the aqueous phase. Thus, the nutrients present in the baseline media poses a limitation in bioremediation of oily sludge over 30 day duration. Interestingly, most of the two way interactions other than that between bioaugmentation and nutrient addition were insignificant while the three way interaction term between all the three factors were significant. Thus, simultaneous addition of nutrients and surfactants along with bioaugmentation with indigenous cultures may offer significant benefit in bioremediation of this refinery sludge. Thus, complex interactions affect oil biodegradation and culture growth in the aqueous phase in this oily sludge bioremediation scenario. Various other researchers have utilized the analysis of variance approach for demonstrating the impact of various treatments on extent of biodegradation and microbial activity [3,11,12,21].

From the ANOVA results some variation is found between the significant and insignificant effects for the two response variables chosen, i.e., oil degradation and culture growth in suspension. The main effect of bioaugmentation with microorganisms was to increase oil degradation and this effect was found to be significant.

Table 2. Significant and insignificant effects at 90% confidence level based on ANOVA

Response	Factors	Significant		Factors	Insignificant	
		F-Statistics	P-value		F-Statistics	P-value
% Degradation	NP	10.869	0.005	NP*TX	1.677	0.214
	TX	9.896	0.006	TX*MO	0.012	0.914
	MO	4.227	0.056			
	NP*MO	3.225	0.091			
	NP*TX*MO	7.235	0.016			
Ln (N/N_0)	NP	10.192	0.006	MO	2.442	0.138
	TX	11.853	0.003	NP*TX	1.062	0.318
	NP*MO	4.156	0.058	TX*MO	0.369	0.552
	NP*TX*MO	11.133	0.004			

NP: nutrients, TX: Triton X 100, MO: microorganisms, N: Viable count of microorganisms at 30 day, N_0: Viable count of microorganisms at 0 day

In contrast, the main effect of bioaugmentation on culture growth in suspension was to reduce culture growth. However this reduction was found to be insignificant based on the ANOVA results. Increase in oil degradation corresponding with negligible increase in culture growth in suspension may be because of increase in N_o due to bioaugmentation causing nutrient deficiency. The enzymes produced by the microorganisms present at high concentration could have promoted higher oil degradation. It may also be due to different uptake mechanisms exhibited by the microbes responsible for oil degradation. In such sludges, oil mostly exists as sorbed oil rather than free phase oil. Microorganisms also associate with particulate matter in sludge and some sorbed hydrocarbons may have been utilized through direct interfacial uptake. Thus, viable count in the aqueous phase is not a true measure of microbial activity in the system. Degradation of oil is a better measure of the activity of oil degraders in this system although obtaining estimates on degradation is more laborious and time consuming. Biostimulation with nutrients and surfactants was found to cause increase in growth of microorganisms in the aqueous phase. Surfactants are known to facilitate micellar solubilization of oil and emulsification of oil and is thus likely to cause desorption of oil. Moreover, surfactants are known to alter microbial cell surface hydrophobicity [19,20] such that distribution of microorganisms in the system may be altered. Simultaneous addition of nutrients possibly promoted oil degradation by microorganisms suspended in the aqueous phase thereby leading to higher culture growth in the aqueous phase.

Microbial activity in the aqueous phase led to change in color of the aqueous suspension after 30 days. This phenomenon may be indicative of oil/hydrocarbons leaching out over time or possibly due to accumulation of intermediates formed during microbial degradation of oil. While the oil/hydrocarbon content in the aqueous suspension across the various treatments was negligible both initially and after 30 days, the TOC in the aqueous phase was significantly higher after 30 days (Fig. 4). Increase in TOC was also observed for the un-inoculated controls. Increase in TOC suggests accumulation of soluble intermediates formed during biodegradation of oil associated with the sludge. In most cases increase in TOC per unit decrease in TPH was found to vary from 4% to 12% while it was found to be 30% for the un-inoculated controls. Thus, the bioaugmentation and biostimulation strategies used led to more complete biodegradation and comparatively lower accumulation of intermediates. Increase in TOC per unit decrease in TPH was least (4%) where all the three strategies (bioaugmentation, addition of nutrients and addition of surfactant) were employed simultaneously.

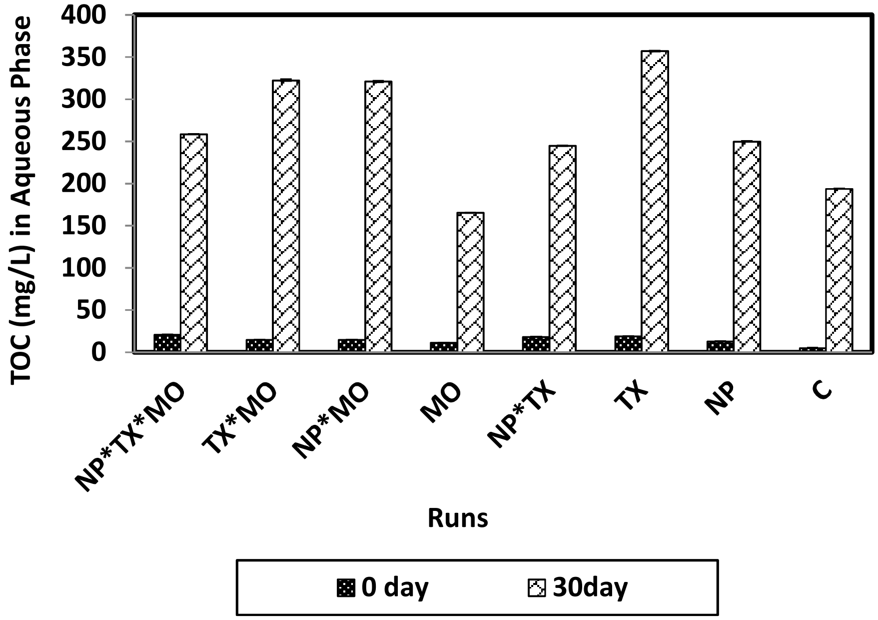

Fig. 4 Variation in TOC in the aqueous phase for the various treatments in the oily sludge biodegradation study as per 2^3 design

Various researchers have shown beneficial effects of bioaugmentation on degradation of oil associated with sludges and soil [1,15,28] as observed in this study. Bioaugmentation works best in scenarios where the indigenous population is very low [3]. In many scenarios, bioaugmented microorganisms have been found to be unable to adapt to the conditions prevailing in the contaminated environment. Bento *et al.*[12] demonstrated how presence of indigenous cultures in the soil limited the activity of bioaugmented microorganisms in a diesel contaminated soil system. They were unable to establish a correlation between TPH degradation, various treatments and activity of diesel degrading microorganisms since the indigenous microbial population degraded diesel more efficiently than the microbial consortium introduced during bioaugmentation. Tahhan *et al.*[28] also demonstrated that indigeneous microorganisms could degrade hydrocarbons when oily sludge was added to soil without bioaugmentation. However, successive bioaugmentation with the enriched indigenous consortium could improve the TPH removal rates. Alici *et al*.[15] reported that bioaugmentation with tailored microbial consortia could facilitate bioremediation of soil co-contaminated with diesel and heavy metals and up to 75% removal could be achieved in 42 days. Moriano *et al*. [3] reported that various amendments could enhance TPH removal to 45.5% over a period of 55 days; however, bioaugmentation with non-indigeneous cultures had no significant beneficial effect on TPH removal. Ayotamuno *et al*. [29] reported that bioaugmentation with extraneous microorganisms along with regular mixing and watering resulted in 63.7% - 84.5% reduction in TPH over a duration of six weeks. In general bioaugmentation is reported to be more successful in scenarios where the indigenous microorganisms native to the soil/sludge was enriched and added. Similar finding are revealed through our study. Low oil degradation (in run 1) due to depletion of the indigenous microorganisms originally present in the sludge during prolonged storage of the oily sludge could be partially overcome by bioaugmentation with indigenous microorganisms.

In the present study, addition of nutrients alone or in combination with bioaugmentation could enhance oil/TPH degradation significantly. Impact of nutrient addition on oil biodegradation is reported to vary widely and is possibly dependent on system specific conditions. Gallego *et al*.[2] reported significant enhancement such that upon addition of inorganic nitrogen and phosphorous up to 90% degradation of diesel was observed under laboratory conditions. In 12 week long laboratory studies on degradation of petroleum sludge and contaminated soil, Cvijovic *et al*.[11] demonstrated that addition of nutrients (NPK) offered greater beneficial effect compared to surfactant addition when culture growth in the aqueous phase was used as the response variable. Admon et al.[34] found that degradation of oily sludge contaminated soil occurred only after application of nutrients in the ratio of C:N:P = 50:10:1. Liu *et al.*[35] found that the addition of manure (as nutrients) to oily sludge significantly increased the microbial activity and diversity; TPH in the treated sludge decreased by 58.2% over 365 days of bioremediation in comparison to only 15.6% in the control plot. Machin-Ramirez *et al.* [36] demonstrated that addition of commercial fertilizers enhanced the degradation of weathered oily sludge with removal of 24% TPH over a duration of 25 days. In contrast, Tahhan *et al.* [28] demonstrated inhibition of oily sludge biodegradation upon addition of nutrients possibly due to higher concentration of nitrogen and phosphorus already present in the sludge.

In addition to nutrients, TPH biodegradation is often limited due to hydrophobicity and low aqueous solubility of the constituent hydrocarbons. Surfactants may help in increasing the bioavailability of sorbed oil through micellar solubilization and emulsification. However, the use of surfactants in bioremediation experiments has been reported to both stimulate as well as inhibit hydrocarbon degradation influenced by various chemical properties of the surfactants and its interaction with microorganisms[17,19,20,37]. In the present study, the main effect of addition of surfactants was statistically significant both for microbial growth in suspension and oil degradation. The binary interactions of surfactant addition and nutrient addition and that of surfactant addition and bioaugmentation were insignificant for both the response variables chosen. The only significant binary interaction was that of nutrient addition and bioaugmentation with microorganisms, however it was only marginally significant ($0.1 >$ p-value > 0.05). In contrast, the 3-way interaction between surfactant addition, nutrient addition and bioaugmentation was significant even at 98% confidence level for both the measured response variables. Thus, the effect of simultaneous addition of nutrients, surfactants and indigenous microorganisms provided a synergistic effect on both oil degradation and culture growth.

Although synergism/antagonism has not been specifically explored by other researchers, several studies have reported enhanced TPH degradation when surfactant addition is combined with other strategies. Cameotra and Singh[38] reported that the effects of addition of nutrients with bacterial consortia and crude biosurfactant with bacterial consortia resulted in less TPH degradation (91-95%) in comparison to when nutrients and surfactants were added together (98% TPH degradation). Rahman *et al*.[21] reported that addition of rhamnolipid biosurfactant increased the degradation of light n-alkanes in a scenario where soil mixed with oily sludge was treated. However, simultaneous supplementation with inorganic nutrients and rhamnolipid biosurfactant resulted in more complete degradation.

5. Conclusion

Addition of enriched indigenous microbes, addition of nutrients and addition of surfactant significantly enhanced oil degradation in sludge over and above that observed in the controls. Sludge biodegradation studies using 2^3 factorial design with oil degradation and microbial growth over 30 days as response variables revealed that the main effect of nutrient addition and surfactant addition enhanced oil degradation and culture growth in suspension and these effects were significant at 90% confidence level. However, the main effect of bioaugmentation only enhanced oil degradation but did not increase microbial growth in suspension. This may indicate the possible role of microorganisms attached on to sludge solids in degrading sorbed oil. The binary interactions are found to be insignificant except for that between nutrient addition and bioaugmentation[21] which was found to have a synergistic effect. No other studies have revealed such a high ternary interaction effect between surfactant addition, nutrient addition and bioaugmentation indicating significant synergistic interaction among these strategies on oil degradation and microbial growth. Thus, the effect of the various factors is not solely additive. The characteristics of the sludge, the characteristics and composition of the oil in sludge and the nature of the indigenous microorganisms present possibly affected the results.

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