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Assessment of Bioaugmentation and Biostimulation Efficiencies for Petroleum Contaminated Sediments

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Abstract
The effectiveness of hydrocarbon bioremediation strategies approaches is depending on various issues such as type and volume of pollution, nutrient accessibility in the target ecosystem, time, biodiversity of microorganisms, pollutant bioavailability and many others. In the present research, laboratory studies were carried out on the bioremediation of coastal sediment samples artificially contaminated with weathered crude oil. The efficiency of natural attenuation, bioaugmentation and biostimulation for removal of Total Petroleum hydrocarbons (TPH) were compared in 90 days experiments. Three oil concentrations of 3, 30, 60 g oil/kg soil were studied to investigate the effect of oil concentration on the bioremediation process. The average degradation, in biostimulation tests were 52.11, 58.36 and 43.02% whereas in bioaugmentation tests were 73.89, 73.76 and 58.31% for 3, 30 and 60 g oil respectively. The results indicated that excessive crude oil (more than 30 g/kg in this case) is not suitable for bioremediation presumably duo to inhibitory or toxicity to the microorganisms. By supplementation of acclimatized microorganism as well as nitrogen and phosphorus, a satisfactory amount of biodegradation is reachable in two months.

Key words Biodegradation, Degradation, Petroleum hydrocarbons, Crude oil, Oil spill, Marine environment

Introduction

The spill of crude oil has created a wide range of environmental problems; particularly marine ecosystems are sensitive to contaminant impacts. When oil spills into the sea, change its properties and behaviors such as volatility, solubility, tendency to emulsion and degradation capacity (Laffon et al., 2006). To recover spilled oil from the environment, several techniques including physical, chemical, and biological methods, are used (Si-Zhong et al., 2009). When the oil layer is thick enough mechanical devices, such as skimmers, are used in oil spill cleanup process (Ghannam and Chaalal, 2003). However physical/mechanical methods can recover 10-15 % of spilled oil (OTA, 1991).

Bioremediation, recommended as an alternative technology to degrade toxic waste, in treatment of environmental contamination. This technology successfully used for petroleum

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hydrocarbon contamination with less environmental damages (Milic et al., 2009; Mohajeri et al., 2011).

Economical strategies for bioremediation are including natural attenuation, biostimulation, bioventing, bioaugmentation, composting, and phytoremediation (Bento et al., 2005). Natural attenuation is a variety of processes that naturally act to reduce the mass, toxicity, mobility, volume, or concentration of contaminants in the environment, it is include biodegradation, dispersion, sorption, dilution, volatilization, and chemical or biological stabilization, transformation, or destruction of contaminants (US-EPA, 1999a). Crude oil bioremediation can be supported by increasing the indigenous microorganisms, through introduce nutrients into the ecosystem (biostimulation) or addition of microbial consortium (bioaugmentation) as highlighted in several bioremediation reports (Qiao et al., 2014; Mohajeri et al., 2013; Tsai et al., 2009; Zhou et al., 2008; Wang et al., 2008; Bento et al., 2005).

The effectiveness of bioremediation strategies approaches and applications is depending on various issues such as type of hydrocarbon pollution (Eg: amount, Density, API gravity, etc.), nutrient accessibility in the target ecosystem, time, biodiversity and richness of microorganism, pollutant bioavailability and many others.

Biodegradation is not efficient in very low concentrations of hydrocarbons; therefore natural attenuation may be a more possible option. On the other hand, microbial degradation in high concentrations of hydrocarbons limited due to toxic effects (Lin et al., 2009; Venosa and Zhu, 2003; Zekri and Chaalal, 2000).

Deferent results have been achieved about selection of appropriate bioremediation approach in case of light and heavy oil spill in the marine environment, and still it is lack of knowledge on application of biostimulation and/or bioaugmentation in coastal sediments clean-up. Ruberto et al. (2003) showed that bioaugmentation is a valuable alternative tool to improve hydrocarbon bioremediation and Mancera-López et al., (2008) confirmed that bioaugmentation was more effective than biostimulation.

The objective of this research was determination of sufficient range of initial wreathed crude oil contamination in coastal sediment samples. Additionally success of natural attenuation, biostimulation and bioaugmentation were compared in different crude oil concentrations.

Materials and methods

Sampling

After a primary study, sediment samples were collected from the top 20 cm surface of Butterworth Beach, Northwest Malaysia and transported to the laboratory in a cool box. The Physical properties of sediment sample are shown in Table 1. The moisture content was in the range of 60 ± 3%. Sediment cores were sectioned into 2 cm intervals to remove large roots, macro fauna and stones. Sediment samples were oven dried for 24 hours at 105 ºC and passed through a 2 mm sieve (Roy and McGill, 1998). Seawater was periodically collected from same place. Properties of seawater sample are displayed in Table 2.

Table 1. Physical properties of sediment sample

Sieve size (μm)	Component (%)
>2000	0.0 (Gravel)
50- 2000	19.4 (Sand)
2-50	57.7 (Silt)
<2	22.9 (Clay)
Total	100.0

Table 2. Properties of seawater sample

Parameter	Amount ±StD[1]
DO (mg/L)	4.1±0.6
COD (mg/L)	760±120
TPHs (mg/L)	3.4±1.2
Total Nitrogen (mg/L)	2.0±0.4
Total phosphorus (mg/L)	0.04±0.02

[1]StD= Standard deviation

Bacterial concertina acclimatization

One liter seawater, 10 g soil sample and 1 mL crude oil were inoculated into a 2 L conical flask containing growth medium under static condition. The acclimatized sample was stirred, aerated and maintained at room temperature under natural light conditions and pH 7.0. The modified medium containing 1 g/L NH_4NO_3, 1 g/L KH_2PO_4, 1 g/L K_2HPO_4, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 0.05 g/L $FeCl_3$, and 0.02 g/L $CaCl_2$ was used to culture bacteria (Ghazali et al., 2004). The bacterial strains were identified as describe by (Barrow and Feltham, 1993; Holt et al., 1994). The total number of microorganisms was determined by plating serially diluted samples on nutrient agar plates in room temperature and neutral pH (Chen et al., 2005; Ruberto et al., 2003).

Reactors and supplements

Three type of bioremediation technologies; (a) biostimulation (b) bioaugmentation and (c) natural attenuation were performed in cylindrical plexiglas reactors (30 cm length, 15 cm ID) in three different oil concentrations (3, 30 and 60 g per kg sediment) as represented in Table 1. In addition a control was carried out using sterilized soils to ensure the quality assurance of bioremediation study. One kilogram of dried sediment was placed in each reactor. Light crude oil was obtained from the Shell Refining Company, Port Dickson-Malaysia; characteristics of the crude oil are shown in table 3. Crude oil artificially weathered according to (Mills et al., 2004; Page et al., 2002). NH_4Cl and K_2HPO_4 were used as N and P source respectively and were added to reactors 24 h after oil addition.

Bioremediation experiments were carried out in room temperature (28±2 ° C). Reactors were manually mixed daily to enhance oxygenation and kept moist during the experimentation time by addition of oxygenated seawater.

TPHs were measured in days 0, 7, 14, 28, 50, 70 and 90 after introduction of nutrients and bacteria to monitor the bioremediation process. Table 4 shows reactors and conditions for bioremediation process.

Table 3. Characteristics of the crude oil

Characteristic (Unit)	Tapis	Bintulu	Miri Light	Sutu den
API gravity	45.8	36.5	32.3	35.1
Barrel factor(BBL/T)	7.898	7.483	7.296	7.423
Pour point (°C)	12	-6	-9	36
Viscosity at 20 °C (Pa.s)	3.51	4.08	4.69	73.6
Percentage	54	17	5	24

Table 4. Reactors and conditions for bioremediation process

Reactor			Addition			
No	Process	Code	Oil (g)	N (g)	P (g)	Bacteria
1	Biostimulation	Stm 3	3	0.3	0.03	+
2	Bioaugmentation	Aug 3	3	0.3	0.03	+
3	Natural attenuation	Att 3	3	-	-	-
4	Biostimulation	Stm 30	30	3	0.3	+
5	Bioaugmentation	Aug 30	30	3	0.3	+
6	Natural attenuation	Att 30	30	-	-	-
7	Biostimulation	Stm 60	60	6	0.6	+
8	Bioaugmentation	Aug 60	60	6	0.6	+
9	Natural attenuation	Att 60	60	-	-	-
10	Control	Con	-	-	-	-

Chemical analysis and QA/QC

The inorganic nutrient contents were determined with a HACH DR2000 direct reading spectrophotometer using HACH proprietary reagents (Hach Methods -10071 and 8190, 1995 a, b). All Quality assurance and quality control (QA/QC) were performed following the standard methods (APHA, 2005). US-EPA method 1660 (US-EPA, 1999b) were used to measure TPH concentration. For each reactor 5 g replicate samples were collected and extracted by analytical grade n-hexane and dichloromethane (Merck, Darmstadt, Germany). Spiked sample were analyzed for checking the percent recovery. The average recovery for total hydrocarbons was 87.19 ± 12.31%. Method Detection Limit (MDL) was 5 mg/kg soil.

To confirm the results and by using US-EPA method 8000B (US-EPA, 1991), a GC/FID analysis were carried out with a GC 2000 Series (Fisons Instruments, Milan, Italy) Chrom-Card data system version 2.1 (Thermo Electron, Rodano, Italy). Capillary column was a DB-5 (J&W Scientific, Folsom, CA, USA) (60 m length, 0.25 mm I.D., film thickness 0.25 µm) injector and detector temperature were set to 300 °C. Oven temperature program was 1 min at 60 °C, then increasing by 10 °C/min up to 160 °C then 10 min in this temperature followed by 4 °C/min up to 300 °C, and finally 10 min at 300 °C. Carrier and make-up gas were helium and nitrogen respectively. Average RSD in this study was 6.58%. Detection limit (signal/noise = 3) was 1 µg/g.

Results and discussion

Natural attenuation

All oiled sediments showed a trend of decreasing TPH concentrations over time which could be attributed to both physical and biological processes.

Weathered crude oil (WCO) degradation began during the first week of experiment in all reactors and continued up to 90 days. The natural attenuation demonstrated a WCO removal of 15.33, 15.48 and 13.01 % for initial oil concentrations of 3, 30 and 60 g per kg soil, respectively. This amount of oil removal is mainly due to evaporation, photo-oxidation, volatilization and biodegradation. The rate of degradation in natural attenuation after two weeks stayed approximately constant and removal by natural attenuation decreases with increasing oil concentration. No hydrocarbon was observed in control reactor.

Biostimulation and bioaugmentation in different oil concentration

Indigenous acclimatized microorganisms used in bioaugmentation examination. The bacterial inoculums were identified as species of *Acinetobacter*, *Alcaligenes*, *Bacillus*, *Pseudomonas* and *Vibrio* and counted in the final culture of 1.2×10^6 cells/mL. The biodegradation capability of the indigenous microorganisms seems to be adequate for the process with supplemented nutrients. The addition of indigenous bacteria increased biodegradation in comparison to the other bioremediation methods.

Comparison natural attenuation, biostimulation and bioaugmentation in 3 g oil concentration are presented in Fig. 1. The figure demonstrated that oil removal by natural attenuation without nutrients supplementation or bacteria addition was much slower compared to the biostimulation and bioaugmentation. After 90 days, only about 15.33 % of 3 g petroleum hydrocarbons were removed by natural attenuation; whereas in the same period and oil concentration, 52.11 % and 73.89 % removal was obtained thorough biostimulation and bioaugmentation respectively.

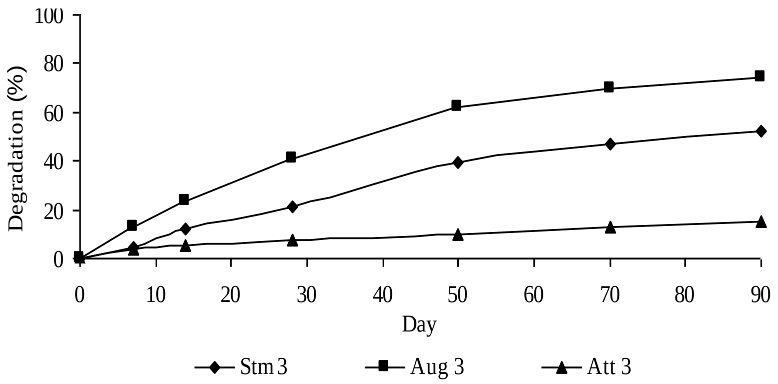

Figure 1. Comparison natural attenuation, biostimulation and bioaugmentation in 3 g oil concentration

Almost half of the oil in bioaugmentation reactors was utilized in the first month in 3 g oil concentration. Degradation was fast in first month and after day 50 it was significantly slower, it clearly means that in present of acclimatized microorganism as well as nitrogen and phosphorus, a satisfactory amount of biodegradation is reachable in about two months and more time does not efficient compared to first 50 days.

As illustrated in Fig.2, After 90 days, in reactors with 30 g/kg hydrocarbon concentration the natural attenuation processes reached to 15.48 % of the total hydrocarbon removed. Biodegradation was notably greater in the reactors supplemented with nutrient compared to the non nutrient oiled sediments resulting. In compare of biostimulation and bioaugmentation, the oil degradation was approximately doubled in 50 days by bacteria supplements. The biological treatment rate during 70 days reached the high value and was very low during the 70 to 90 days.

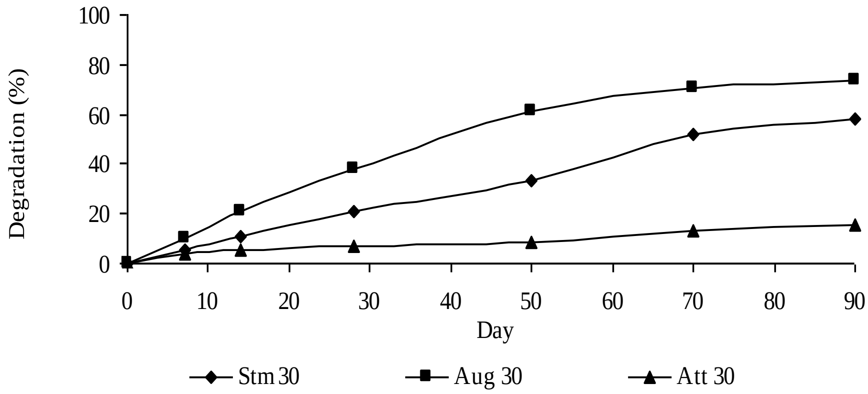

Figure 2. Comparison natural attenuation, biostimulation and bioaugmentation in 30 g oil concentration

The effectiveness of biostimulation depends on delayed contact between the added nutrients and the oil within the bioremediation. The C: N: P ratio could affect the rates of degradation of weathered oil as well.

Fig. 3 demonstrated degradation percentage in 90 days for oil concentration of 60 g. Among all, the lowest WCO biodegradation were observed in these concentrations; presumably duo to deficient of oxygen and available nutrients. Degradation rate was not reasonable in first week in both biostimulation and bioaugmentation experiments since high concentrations of hydrocarbons may delay adaptation phase and cause inhibition of biodegradation due to toxic effects. The same results also reported by Nikolopoulou and Kalogerakis (2008); Zhu et al. (2001). The biodegradation does not occur in very high oil concentrations, or occurs in very low rate as reported by Trindade et al. (2002).

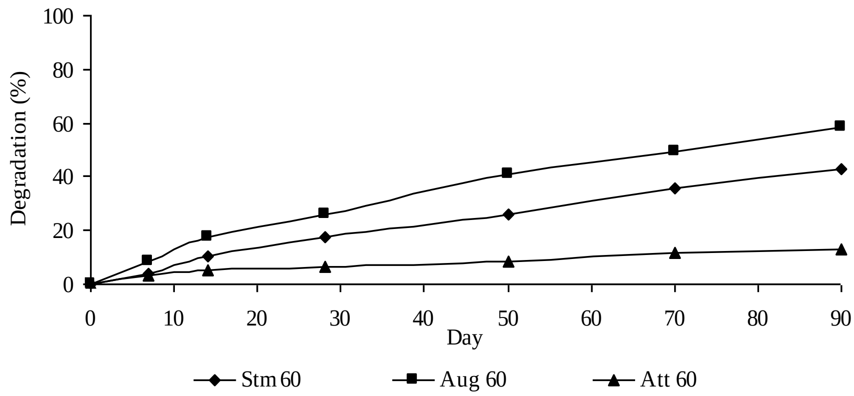

Figure 3. Comparison natural attenuation, biostimulation and bioaugmentation in 60 g oil concentration

Lee and levy (1987) tested two concentrations 3% (v/v) and 0.3% (v/v) to sand beach sediments and concluded that low concentration of petroleum hydrocarbons is rapidly removed by natural attenuation while at the higher concentrations, crude oil biodegradation rates showed nutrient limitation and the bioremediation treatments enhanced the rate of oil removal and supported chemical changes in oil composition on the sandy beach.

Lepo et al. (2003) examined the biodegradation of different concentration of artificially weathered crude oil in laboratory and shows that lower doses of oil had more efficient degradation than high oil concentration. Same results also documented by (Kim et al., 2005) who determine the most effective bioremediation strategies for different levels of contamination using Arabian light crude oil of 3% and 6%. The influences of oil concentration on hydrocarbon biodegradation were also investigated by (Del'Arco and de Franca, 2001). They tested bioremediation of initial oil concentration of 14, 21 and 28 g/kg and were resulted 33.7, 32.9 and 28.9 removals.

In our study, the reduction of TPH in reactors supplemented with nutrients and bacteria was significantly higher than that in reactors with no supplements. Bioaugmentation shows fast action from starting trials and became slow after 50 days. the rate of hydrocarbon utilization was low in biostimulation and natural attenuation tests during first week because bacteria was not adapted with new environment, the rate is developed into fast till end of 90 day experimentation. In spite of this, biostimulation is demonstrating adequate degradation in long time.

Bioaugmentation efficiency

The effectiveness of bioaugmentation is dependent on several factors including effective attachment, retention, and metabolic adaptation of the added microbial population in treatment systems (Satoh et al., 2003). Additionally, type of oil and environmental factors play a critical role in bioaugmentation success.

The comparison bioaugmentation and biostimulation for hydrocarbons were documented by Mancera-López et al. (2008); Mathew et al. (2006) and Bento et al. (2005). Most of the studies have been shown that addition of both microorganism and nutrient significantly increases hydrocarbons removal but there is still a gap regarding comparison efficiency of different techniques for petroleum hydrocarbons treatment. Since natural attenuation, biostimulation and bioaugmentation are capable to remove petroleum hydrocarbons, the bioaugmentation efficiency (E_{Aug}) is imperative to select the most appropriate remediation strategy. The following formula was used to estimate the E_{Aug} percentage:

$$E_{Aug} = \frac{R_{Aug} - R_{Stm}}{R_{Aug} - R_{Att}} \times 100 \quad (1)$$

where R_{Aug} is the percent removal by bioaugmentation, R_{Stm} is the percent removal by biostimulation and R_{Att} is the percent removal by natural attenuation. Bioaugmentation efficiency was 37.19, 26.42 and 33.75 for 3, 30 and 60 g oil respectively. Bioaugmentation performed well in oil concentration of 3 g, therefore the highest E_{Aug} were demonstrated in these bioreactors. Both bioaugmentation and biostimulation exhibited superior removal in oil concentration of 30 g; consequently in medium range oil pollution biostimulation can be effective enough for clean-up of a shoreline. On the other hand a high E_{Aug} were observed for 60 g initial oil concentrations, indicating that bioaugmentation is more effective in high crude oil concentrations. It also representative that lack of microorganisms is a great limiting factor in case of heavily oil spill in the marine environment.

Conclusion

The results of this study concluded that bioremediation is not appropriate for high concentration of petroleum. The biostimulation process experiments show oil degradation of 52.11, 58.36 and 43.02%, while the bioaugmentation trials show oil degradation of 73.89, 73.76 and 58.31 % for initial oil concentrations of 3, 30 and 60 g per kg soil respectively. Therefore, bioaugmentation technique is recommended for bioremediation of crude oil contaminated soil. Although the all experiments were carried out in 90 days, the superior removals was happened in first 50 days thus two mounts may be appropriate time for reasonable degradation percentage for this type of crude oil. Future investigations should be concentrated on improvements of bioaugmentation technique using dispersants and biosurfactants.

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