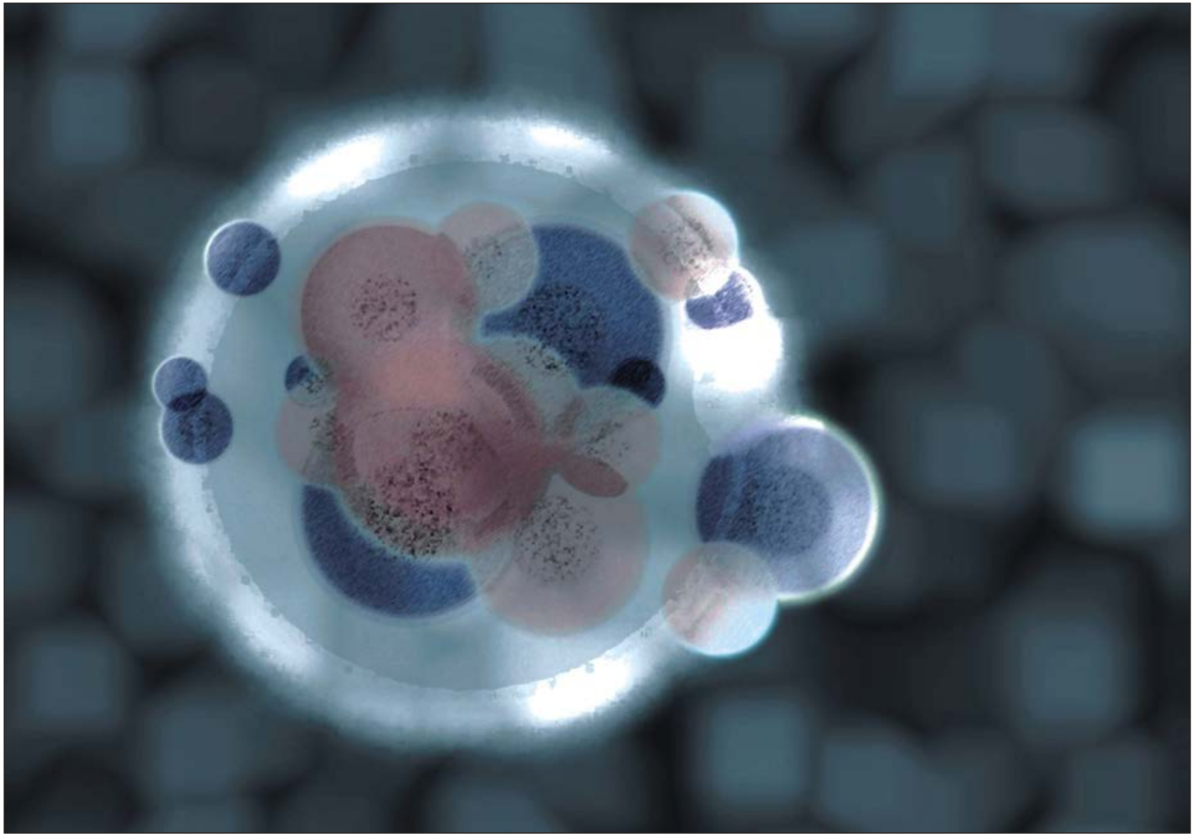

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Use of Microbiological and Chemical Methods for Assessment of Enhanced Hydrocarbon Bioremediation

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Abstract: A suite of microbiological tools, complementing the standard chemical analysis, was used for evaluating the effect of steam and surfactant on the rate of hydrocarbon biodegradation. The microbiological monitoring tools were soil microbial counts, soil microbial respiration, and microbial toxicity biosensor. The correlations between these parameters and with the levels of hydrocarbon residues were investigated. The overall assessment showed that bioremediation was an effective method for reducing hydrocarbon concentration. However, the monitoring tools used showed that the steam and surfactant had no significant effect in increasing the rate of hydrocarbon bioremediation or the toxicity reduction comparing with the control. Consequently, alternative techniques for enhancing hydrocarbon bioavailability must be investigated in order to establish a successful bioremediation of heavy hydrocarbons in soil. This study also demonstrated that the combination of different classes of biological and chemical tools would be more effective in monitoring hydrocarbon bioremediation that any single approach.

Key words: Steam, surfactant, biodegradation, toxicity biosensor, microbial respiration, bioavailability, water holding capacity

INTRODUCTION

Petroleum hydrocarbons are widely used in our daily life as fuel and chemical compounds. As a result of this massive use, petroleum has become one of the most common contaminants of large soil surfaces, and eventually is considered as a major environmental problem (Sanscartier *et al.*, 2009). The increase in public awareness towards the conservation of the environment has led to the development of various physiochemical techniques for clearing up contaminated sites. Although most of the physicochemical methods can be efficient for treating a wide range of pollutants, they are extremely expensive (Joo *et al.*, 2008). Consequently, bioremediation has become a valuable alternative technology to many physiochemical methods as it is a cost-effective, cheap and environmentally friendly treatment.

Bioremediation can be defined as the use of soil microbes to degrade pollutants to harmless substances (Collin, 2001). However, there are a wide range of factors known to reduce the ability of soil microbes to breakdown contaminants. These factors include nutrients, pH, temperature, moisture, oxygen, soil characteristics and contaminant bioavailability (Bundy *et al.*, 2002). So, optimizing these environmental conditions could enhance contaminants biodegradation in soil (Margesin *et al.*, 2000).

Contaminant bioavailability is a key factor responsible for the persistence of contaminants which otherwise should be readily biodegraded (Qin *et al.*, 2009). The key factors reducing the hydrocarbon bioavailability involve their absorption into the soil organic matter, their presence in non-aqueous phase liquids, and their entrapment within the physical matrix of soils (Semple *et al.*, 2003). To improve hydrocarbon bioavailability, steam flushing technique which elevates temperatures sufficiently to distill the organic contaminants from the non-aqueous phase can be used (Schmidt *et al.*, 2002). However, the high temperature may kill some of the soil microbes which are able to degrade hydrocarbons. To avoid this, surfactants, which are chemical compounds, can be used to increase the contaminant bioavailability to soil microbial degraders through reducing contaminant viscosity and thus increasing hydrocarbon solubility (Gao *et al.*, 2008; Qin *et al.*, 2009). The disadvantages of using surfactant involve factors such as surfactant could be used as a preferred substrate or its toxicity effect when present at high concentrations (Gao *et al.*, 2008).

Chemical methods are usually used to evaluate the efficiency of hydrocarbon bioremediation (Bhattacharyya *et al.*, 2005). However, this approach does not provide enough information about the toxicity status during the period of bioremediation. To overcome the

limitations of the standard chemical techniques a suite of microbiological methods was used in this study for monitoring and evaluating the progress of hydrocarbon bioremediation. The monitoring methods employed were soil microbial counts, bacterial biosensor (for assessing toxicity changes) and gas chromatography (for CO_2 and hydrocarbon analysis). Although it is not unusual for these methods to be used as a single monitoring tool, there are few case studies where they have been used in combination. Hence, the aim of the present study was to evaluate several soil microbiological methods, complementing the standard chemical analysis, for monitoring and evaluating the effect of steam and surfactant on the rate of hydrocarbon biodegradation and toxicity reduction.

MATERIALS AND METHODS

Soil preparation: Approximately 10 kg of a hydrocarbon contaminated soil was collected in April 2008, from an old petrol station site, Abha city, Saudi Arabia. The soils were sieved, using a 3.5 mm sieve, to obtain a homogenous material as possible. Initial physiochemical characteristics of the soil were determined as shown in Table 1. The moisture content of the soil was maintained at 60% Water Holding Capacity (WHC).

Experimental set-up: The experiment included four different treatments monitored over a period of 30 day. The treatments applied were as follow: control, steam, surfactant and a combination of steam and surfactant. Approximately 100 g wet weight of soil was placed without packing into a 500 mL glass jar and sealed with parafilm. The jars were kept at 25°C in the dark at 70% relative humidity. All microcosms including the control were treated with nutrient solution in order to simulate microbial degraders (see below). Triplicate microcosms were destructively sampled at each sampling time. Therefore, there were 84 microcosms in total used over the period of bioremediation. The microcosms were arranged in a random order, and rearranged every week throughout the experiment to compensate for any local temperature fluctuations in the incubator. The microcosms were sampled every five days starting from day 1 (seven sample points over 30 day).

Table 1: Soil characteristics

Soil characteristics	Values
Texture	Sandy loam
pH (d.d.H_2O)	6.30
WHC	0.4 g g^{-1}
Bulk density	1.12 g cm^{-3}
Available N	Not detectable mg g^{-1}
Available P	Not detectable mg g^{-1}
Hydrocarbon content	30 g kg^{-1}

Preparation of treatments: An initial determination of C:N:P concentrations was performed to estimate the experimental supplement of nutrients to ensure a C:N:P ratio of 100:10:1 (Bundy *et al*., 2004). NH_4NO_3 (Fischer Scientific) was used as a nitrogen source (8.5 g N kg^{-1} dry weight of soil), whereas K_2HPO_4 (Fischer Scientific) were used as a phosphorus source (1.6 g P kg^{-1} dry weight of soil). Using a fine sprayer, nutrient solution was sprayed evenly over the soil surface. The soil was then continuously hand-mixed for 5 min with a stainless-steel spatula. Additional water was added to bring the final moisture content of each soil to 60% of WHC. Steam treatment was carried out by evenly spreading the contaminated soils on a tray and the contaminated soil was then steamed for 3 min using a steam generator (Sleep and McClure, 2001). The surfactant treatment (1% of surfactant) was prepared by dissolving 1 mL of Tween 80 (BDH lab supplies) into 100 mL of d.d.H_2O and was then applied during the amendment of soil moisture content (Aronstein and Alexander, 1993).

Soil pH: Soil pH measurements were carried out on soil suspensions in d.d.H_2O (Dawson *et al*., 2007). The mixture was stirred regularly for 15 min and then allowed to equilibrate for an extra 15 min. The pH meter (HI 8424 microcomputer pH meter, HANNA Instruments) was then submerged in the mixture, spinning gently and the pH value was then recorded.

Determination of extractable NH_4^+ and NO_3^-: NH_4^+ and NO_3^- were extracted (1:10 ratio of matrix:solution) using 2M KCl (Allen, 1989). The samples were then over and under shaken for 30 min at 60 rpm and the samples were then centrifuged at a speed of 3000 x g, for 30 min in a Cool Spin Centrifuge. Ten milliliter of the suspension was transferred into new vials and the samples were then analysed using a Flow Injection Analyser (FIA).

Total hydrocarbon extractions: Chemical analysis of total hydrocarbon was preformed as described by Dawson *et al.* (2007). Five grams of soil were weighed into glass Wheaton vials and 22.5 mL dichloromethane (DCM) and 2.5 mL acetone added. Samples were sonicated for 5 min (applied energy on soil suspension = 1200 J mL^{-1}) and shaken end-over-end for 16 h. After settling, 10 mL of solution was added to a clean vial, evaporated under N_2 and re-suspended in 10 mL hexane. From each sample, 4.9 mL was transferred to a new vial and mixed with a 100 μL of squalane as an internal standard. Two milliliter of the extraction was then transferred into GC-vials, which were then analysed on the Gas Chromatograph (CE instruments GC 8000) coupled with a Flame Ionisation

Detector, Phenomenex ZB1 capillary column (30 m length, 0.32 mm inside diameter, and 0.5 μm ft film thickness) and an Autosampler AS 8000 1 μL injection. The detector temperature was set as 310°C, injector temperature at 250°C, oven temperature at 60°C for 2 min, increased at 5°C per min, stable at 110°C for 1 min, increased at 10°C per min to 300°C maintained for 15 min and with a total running time of 47 min.

Basal respiration: One gram dry weight of each soil sample was placed in a 10 mL vacuette sealed with a screw cap embodying a PTFE septum. The samples were then incubated for 24 h at 25°C. Carbon dioxide produced was determined by GC as described by Paton *et al.* (2006). Respiration values were determined as mg CO_2 g^{-1} soil day^{-1} following subtraction of a blank vacuette containing atmospheric CO_2.

Ecotoxicity assessment: Soil ecotoxicity assessment was carried out as described by Sousa *et al.* (1998). The toxicity of the samples was determined using the luminescence-based microbial biosensor *Pseudomonas fluorescens* 10586r pUCD607 containing *V. fischeri lux* CDABE genes as a multi-copy plasmid. Freeze dried cells were resuscitated by adding 10 mL 0.1 M sterile KCl and placed into an orbital incubator shaker set at 25°C and 200 rpm for 1 h. From each sample, 10 μL was transferred to each well of a black 96-well microtiter plate containing 170 μL of dH_2O. Twenty microlitre of the resuscitated cells were then added to each microtiter well and were mixed thoroughly for 5 sec. The control wells contained 180 μL of dH_2O and 20 μL of the biosensor cells. The luminescence reading was measured with a Lucy Anthos microtitre luminometer (Labtech, Uckfield, UK). The luminescence was recorded after 15 min of exposure.

Enumeration of hydrocarbon-degrading microbes: Enumeration of soil hydrocarbon-degrading microbes was carried out using Minimal Salt Medium (MSM) as described by Jørgensen *et al.* (2000) with some modification. One milliliter (0.1%) of sterilised (using a 0.2 μm cellulose acetate membrane) diesel was used as a carbon source. Twenty milliliter of a stock solution of 1% w/v sodium pyrophosphate buffer in 25% Ringers solution (Townsend *et al.*, 2000) was added to 3 g dry weight samples in Wheaton vials and the samples were vortexed for 30 sec. The samples were sonicated for 1 min, and then allowed to settle for 2 min. Culturable cell counts were carried out by serially diluting 100 μL of the cell suspension in sterile micro-centrifuge tubes containing 900 μL of 25% Ringer's solution and the suspension was then vortexed. Ten microlitre aliquots of each dilution, in triplicate, was placed onto plates and allowed to dry thoroughly, then incubated for 48 h. The number of colony forming units (cfu's) per gram oven dry weight of the initial cell suspension was then calculated.

Statistical analysis: The parametric testing and analysis of variance were used for data analysis. If the data and residuals were not normally distributed or did not have equal variance, even after transformation, then the Kruskal-Wallis test was used. All analysis were performed at $p \leq 0.05$ using MINITAB, version 13.1.

RESULTS

Assessment of soil microbial respiration: On day 1, the surfactant treated biopiles had the highest microbial respiration whereas the steam and steam/surfactant biopiles had the lowest microbial respiration (Fig. 1). Between day 1 and 10, all the treated biopiles including the control showed a sharp increase in the soil microbial respiration. This increase, however, was significantly greater in the surfactant and steam/surfactant treated biopiles than the control and steam treated biopiles alone. Following day 10, the respiration rate for all the biopiles declined rapidly until day 15 which was significantly less than the previous sample point. The respiration rate then increased slightly until day 20 which was significantly higher than day 15. Following day 20, the respiration remained constant for the following sample points.

Assessment of hydrocarbon microbial degraders: There were significant changes in the microbial numbers during the experiment, suggesting a response of the soil microbial population to the progress of hydrocarbon biodegradation. On day 1, the microbial numbers were

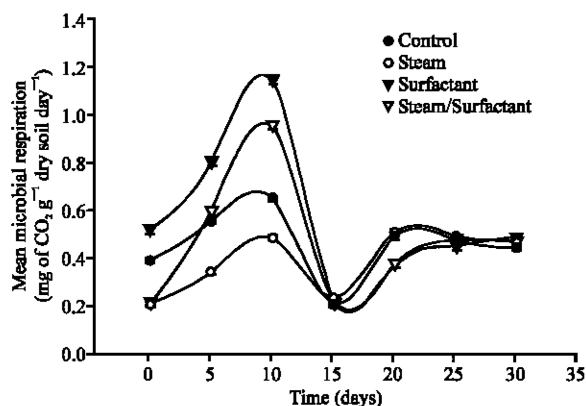

Fig. 1: Mean soil respiration (mg CO_2 g^{-1} of dry soil day^{-1}) over 30 days. Bars indicate standard errors of the mean (n = 3)

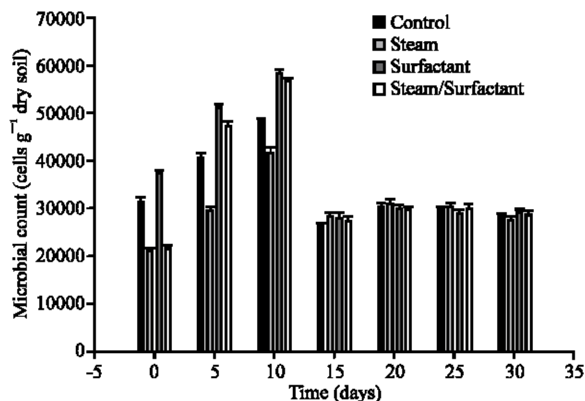

Fig. 2: The mean counts of hydrocarbon-degrading microorganisms (cfu's g^{-1} dry soil) over 30 days. Bars indicate standard errors of the mean (n = 3)

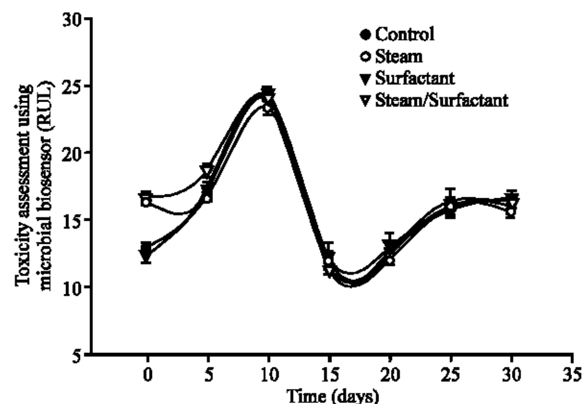

Fig. 3: Bioluminescence response for the toxicity biosensor *P. fluorescens* 10586r during hydrocarbon biodegradation. Bars indicate standard errors of the mean (n = 3)

significant different between the treated biopiles as the surfactant treated biopiles had the highest microbial numbers whereas the steam and steam/surfactant biopiles had the lowest microbial numbers. Between day 1 and 10, all the treated biopiles including the control showed an increase in the microbial counts, being significantly greater on day 10 than all the previous sample points. Although the treatments applied were associated with a significantly increase in the microbial counts during the initial stage of the bioremediation, the surfactant treated biopiles showed the greatest counts of microbial hydrocarbon degraders (Fig. 2). Following day 10, all the biopiles showed a sharp decline in the counts of microbial hydrocarbon degraders until day 15. This was then followed by no further significant changes in the microbial counts until day 30.

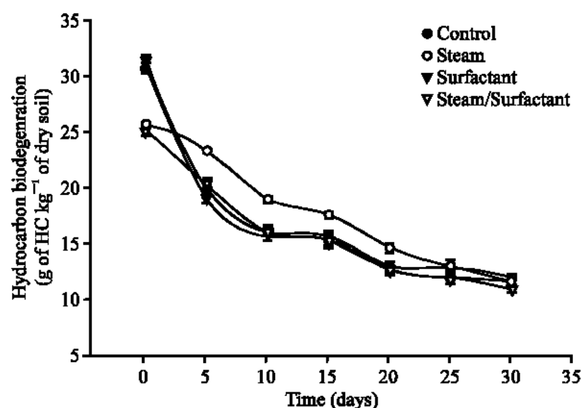

Fig. 4: The mean changes in hydrocarbon concentrations (g of HC kg^{-1} of dry soil) over 30 days. Bars indicate standard errors of the mean (n = 3)

Assessment of hydrocarbon toxicity: The initial toxicity assessment of hydrocarbon on day 1 showed that steam and steam/surfactant biopiles were significantly less toxic than the control and surfactant biopiles (Fig. 3). Between day 1 and 10, there was a significant decline in the soil toxicity for all the treated biopiles. Afterwards, the toxicity increased to its initial level by day 15. Following day 20, there was a slight reduction in the soil toxicity, being less than the initial toxicity level on day one.

Chemical assessment of hydrocarbon reduction: The initial hydrocarbon concentration (at day 0) in the steam and steam/surfactant biopiles was significantly less than the control and surfactant biopiles (Fig. 4). The overall results shown that all the treatments including the control showed a significant decline in the hydrocarbon concentration over time. The results also showed that the highest rate of hydrocarbon reduction was during the first 10 days. Furthermore, the results displayed that all the biopiles showed no significant reduction in the hydrocarbon concentrations between day 10 and 15. Between day 15 and 20, all the treated biopiles including the control showed a slow rate of hydrocarbon reduction. Following day 20, the hydrocarbon content remained level off until day 30.

DISCUSSION

Soil microbial respiration: The initial low soil microbial respiration observed on day 1 (Fig. 1) for the biopiles treated with steam comparing with the other treatments confirmed that the high temperature of the steam had probably a negative effect on the activity of soil

autochthonous microbes. The increase in the soil microbial respiration for all the biopiles including the control during the first 10 days was more likely due to the increase in the microbial numbers (Fig. 2), responding to the addition of nutrients to the contaminated soil. The positive effect of the nutrient addition on microbial activity and on the rate of hydrocarbon biodegradation has been reported by Atagana *et al.* (2003) and Molina-Barahona *et al.* (2004). Nutrients are important for microbial growth and activity, as they allow microbes to synthesize the required enzymes that degrade organic contaminants (Eweis *et al.*, 1998; Vidali, 2001). Therefore, initial analysis of nutrient concentrations in any hydrocarbon contaminated site is highly important, as bioremediation of hydrocarbons is often restricted by the imbalanced ratio between C:N:P (Atagana *et al.*, 2003).

The higher CO_2 production in the surfactant treated biopiles between day 1 and 10, comparing with the other treatments, could be a response to the addition of surfactant which could be utilised as a carbon source. The decline in CO_2 production observed for all the treated biopiles after day 10 was more likely caused by the rapid reduction in the hydrocarbon bioavailability. In addition, the increase in soil toxicity (Fig. 3) during this stage of bioremediation could also have had a negative effect on microbial activities and thus the production of CO_2.

Assessment of microbial numbers: The low microbial numbers observed on day 1 (Fig. 2) for the steam treated biopiles further confirmed the negative effect of high temperature of steam on the microbial population present in soil. The rapid increase in the soil microbial count during the first 10 days of the bioremediation for all the treatment applied was more likely to be a response to the addition of nutrients into the contaminated soil at day one. This suggests that nutrient concentration is a limiting factor restricting the hydrocarbon biodegradation at the original contaminated site. Therefore, it is highly recommended to amend the nutrients to the optimal concentration in order to support maximum growth and activity of microbial degraders at the contaminant site. The rapid increase in the microbial count of hydrocarbon degraders confirms that the indigenous microbial community is well adapted to the presence of hydrocarbon contaminants (Alexander, 1999). This rapid increase in the soil microbial numbers was also associated with a rapid decline in contaminant concentrations during the same time (Fig. 4), suggesting that most of the hydrocarbon reduction was due to biotic rather than abiotic processes such as volatilisation or ageing.

The higher microbial numbers observed in the surfactant treated biopiles between day 1 and 10 suggested that the surfactant had a positive effect on the microbial growth comparing with the steam treatment. However, the lack of a significant difference in the hydrocarbon reduction between the surfactant and control biopiles (Fig. 4) suggested that the addition of surfactant may favour the growth of specific groups of soil microbes that are able to utilise it as a carbon substrate (Wilson and Jones, 1992). This may also explain the rapid decline in microbial activity and numbers in the surfactant treated biopiles following day 10, as there was only a small concentration (1%) of surfactant added to the contaminated soil. In contrast, the lowest microbial numbers recorded in the steam treated biopiles during the same time may have been due to the negative effect of the high temperature of steam on the soil microbial population. This low numbers of microbes in the steam treated biopiles was also associated with the lowest hydrocarbon reduction (Fig. 4), confirming that the hydrocarbon reduction was due to biotic rather than abiotic pathways.

Toxicity assessment using microbial biosensor: The toxicity microbial biosensor (*P. fluorescens* 10586r) showed a significant change in hydrocarbon toxicity over time, indicating the progress of hydrocarbon biodegradation. The response of the toxicity microbial biosensor showed that the steam treated biopiles was less toxic than the other biopiles (the control and surfactant treatment alone) on day one. This suggests that the steam treatment reduced the soil toxicity (Fig. 3) by increasing the volatilisation rate of low molecular weight hydrocarbon which are more toxic than the higher molecular weight hydrocarbon (Alexander, 1999). The significant increase in the bioluminescence response for all the treatments applied including the control during the first 10 days of the bioremediation may be due to the high reduction in hydrocarbon concentrations during this period (Fig. 4).

On day 15, there was a significant decline in bioluminescence response, suggesting that hydrocarbon toxicity was greatest at this period for all the treated biopiles. This increase in soil toxicity may be due to the presence of by-products which are more water soluble and thus more bioavailable (Bundy *et al.*, 2004). Furthermore, such by-products might be more toxic than the parent compounds (Phillips *et al.*, 2000). This increase in soil toxicity was also correlated with a decline in the rate of hydrocarbon biodegradation (Fig. 4).

The slow reduction in soil toxicity shown by microbial biosensor between day 15 and 30 might be due to the slow rate of hydrocarbon biodegradation, which in sequence could be due to the low bioavailability of

residual hydrocarbons remained in the soil. However, the slow toxicity reduction seen between day 15 and the end of the experiment demonstrates that microbial remediation may not be possible unless the bioavailability of these heavy hydrocarbons is improved.

Chemical assessment of hydrocarbon reduction: The high rate of hydrocarbon biodegradation observed within the first 10 days of the bioremediation (Fig. 4) is more likely due to the presence of low molecular weights of hydrocarbons which tend to be more soluble and biodegradable than the high molecular weights of hydrocarbons (Balba *et al.*, 1998; Namkoong *et al.*, 2002). The overall assessment of hydrocarbon bioremediation over time displays that the steam and surfactant had no significant effect on the reduction of hydrocarbon comparing with the control. However, it was noticed that there was a slow hydrocarbon reduction between day 5 and 10 for the biopiles treated with steam. This result verifies that the steam treatment negatively affected the hydrocarbon reduction by affecting the growth and activity of soil microbial population (Fig. 1, 2). This additionally confirms that most of the hydrocarbon reduction was due to microbial biodegradation.

The lack of a significant hydrocarbon biodegradation, shown by all the treatments between days 10 and 15, was more likely to have been due to the increase in soil toxicity (Fig. 3). Following day 20, all the treatments applied were associated with a slow rate of hydrocarbon reduction which might be due to the low solubility and diffusion of residual hydrocarbons remained in the soil.

CONCLUSION

Bioremediation is a multi-disciplinary approach which includes many physical, chemical and biological factors determining the efficiency of hydrocarbon biodegradation. This study showed that the employment of chemical and biological methods in a holistic approach for evaluating the efficiency of hydrocarbon bioremediation is highly important, as any one approach alone does not provide an adequate assessment of the process and thus identifying a safe, environmental end-point. The overall assessment of hydrocarbon bioremediation showed no conclusive evidence to suggest that any of the treatments applied (steam or surfactant) had a significant effect on the bioremediation process. Consequently, alternative approaches, including physical, chemical or biological techniques for improving hydrocarbon bioavailability must be investigated in order to establish successful bioremediation of heavy hydrocarbons in soil.

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