OPTIMISATION OF BIOSTIMULATION BY USING DIFFERENT NUTRIENT RATIOS TO IMPROVE BIOREMEDIATION OF PETROLEUM CONTAMINATED SOIL

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Abstract: Petroleum contamination of sandy soils is a severe environmental problem in Libya, but relatively little work has been carried out to optimize the bioremediation of such heavily contaminated soil, particularly at a pilot scale.

This study assessed the effect of varying nutrient (NPK) levels and types (urea) on bioremediation of diesel contaminated soil by a combination of chemical and toxicological analyses. Varying nutrient C:N:P ratios were used 100:10:10, 100:10:1, 100:1:1 and 100:1:0.1that contained both NPK fertilizer and urea as an extra source of nitrogen,the optimum C:N:P ratio found was 100:10:1 (and urea was found to be a good nutrient source). In this study (achieved approximately 69% TPH removal). The amounts of petroleum removed as revealed by chemical analysis appeared to correspond well to bacterial counts and the residual toxicity of soil as estimated by the Microtox assay. The highest amounts of nutrients used (C:N:P ratio of 100:10:10) did slightly reduce bioremediation effectiveness. GC analysis revealed that hydrocarbons of chain length C9-C20 were most effectively removed and that the higher chain length petroleum compounds (>C20) remained in the soil. Overall the work demonstrated the clear potential of nutrient stimulation to reduce levels of hydrocarbons present and to reduce the soil toxicity.

Keywords: Nutrient, NPK, toxicity, bioremediation, biostimulation

INTRODUCTION

The comparison of PH transformation levels between bioaugmentation and biostimulation as carried out in the previous studies (data not shown) revealed that biostimulation was the best potential treatment method for the bioremediation of the Zawia Oil Refinery soils. However, only one nutrient level was used and bioremediation could be enhanced further if nutrient levels were optimized. In petroleum-contaminated soils, the availability of nitrogen and phosphorus are often limited due to the excessive carbon input from the hydrocarbons (Jin and Fallgren, 2007). The importance of obtaining the correct nutrient level for bioremediation is emphasized in several previous studies. For example Sarkar *et al* (2005) showed that the microbial population was decreased in the fertilizer-amended soils, suggesting NH3 overdosing and/or the toxic effect of the used fertilizer. Chaillan *et al* (2006) reported that the addition of urea appeared to be toxic. In addition, several authors have reported the negative effect of high nitrogen, phosphorus and potassium concentrations on the biodegradation of hydrocarbons. For instance, Eke and Scholz (2008) concluded that biodegradation activity can be inhibited by the addition of excessive nutrient concentrations. It has been demonstrated that microbial activity and petroleum degradation in contaminated soils can be depressed as result of excess nitrogen due to osmotic soil water potential depression (Walworth *et al*, 2007). Thus, in order to avoid any inhibition excessively high nutrient levels were not used in this study. Overall, it appears that both the level and type of nutrients added can either improve or decrease the level of bioremediation achieved.

There are no specific methods for determining the exact nutrient sources and ratios to utilize at a site and indeed nutrient optimization for bioremediation appears to have received relatively little attention despite its' clear importance. Therefore, the optimal C:N:P ratio frequently recommended for

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bioremediation is 100:10:1 as used in this work (Liebeg and Cutright, 1999). Other work has reported that the optimal C:N:P ratio is 100:15:3 (33:5:1) for hydrocarbon biodegradation (Sarkar *et al*, 2005).

The principle aim of the work in this study was to determine the best nutrient level to reduce PH in the soils to a safe level. The determination of the success of bioremediation (i.e. reduction of risk from the contamination) is an interesting area and most studies only chemically analyse soil for compounds present. However, there is a need to also determine soil toxicity as toxic transformation intermediates may be produced during bioremediation. This is indicated by several previous studies e.g. pentachlorophenol transformation in soil and microbial transformation of diesel oil (Mariano *et al* (2007). The presence of toxic intermediates produced during biodegradation of crude oil caused luminescence inhibition (Plaza *et al*, 2008). There are a number of methods that can be used for determining soil toxicity e.g. bacterial bioluminescence, plant growth, earthworm etc. Dorn and Salanitro (2000) assessed bioremediation using different ecotoxicity methods, the earthworm (*Eiseniafetida*) 14-d lethality assay, the modified Microbics Microtox Solid-Phase assay, and the 14-d plant seed germination and growth assays.

In this research, a microbiological luminescence-based bacterial kit was applied to assess the toxicity of petroleum hydrocarbon contaminated soil during and after nutrient assisted bioremediation. Specifically, this involved the use of Vibrio fischeri inhibition assay (Microtox TM). This toxicity test has been used previously in several soil toxicity studies and has the advantages of being rapid and economical to carry out. Light- emitting bacteria can be a suitable tool for environmental studies. In vivo luminescence is a sensitive indicator of xenobiotic toxicity to microorganisms and reflects the metabolic status of the cell as result of being directly coupled to respiration via the electron transport chain (Girotti *et al*, 2008).

The use of a combination of both chemical and toxicological analysis was considered to be the best method to determine the optimum nutrient level for bioremediation of the PH contaminated the Zawia Refinery soils.

Aims and Objectives

The aim of this study was to optimise bioremediation of PH contaminated the Western Refinery soils by altering nutrient levels. A secondary aim was to assess the success of bioremediation by a combination of chemical and toxicological methods.

Objectives

To optimize nutrient levels for bioremediation, by using different types and ratios of nutrient amendments (Nitrogen based fertilizer and urea).

To assess the use of a bacterial biosensor-based tool kit to monitor hydrocarbon bioremediation.

To compare the bacterial based toxicity of treated soil with more traditional chemical analysis of PH e.g. does a decrease in soil toxicity (as measured by Microtox) correlate with a lower level of petroleum compounds as determined by chemical analysis?

To choose the optimum nutrient type and ratio for use in subsequent pilot scale bioremediation studies.

MATERIALS AND METHODS

Site Description and soil collection

Target soil samples were obtained from the Zawia Oil Refinery. A total of twenty four fresh soil samples were collected at 20-30 cm depth from a contaminated diesel-refuelling area, from the spill around pipelines which carry the refined products to the shipping points situated at the studied site the Zawia Refinery, Libya. The soil contained a total petroleum hydrocarbon (TPH) level of 35000 mg/kg (see results section below).Samples were placed in plastic bags and cooled for transport(see details analyses below).

Overview of experiments on nutrient optimization for bioremediation

The initial nutrient levels (available C, N and P) in the soil samples were measured in order to evaluate the amount of nutrient addition needed to achieve the optimal C:N:P ratio.The amount of nutrient required for microbes are approximately the same as their cell composition. Though, carbon is needed at higher quantities by cells, this can be supplied by the contaminant present (Liebeg and Cutright, 1999). Degradation tests were set up in small-scale microcosms simulating soil conditions, to examine the response of the indigenous hydrocarbon degraders to different nutrient supplements in soil by manipulating the C/N/P ratio. The initial nutrient levels in the soil samples were measured (see nutrient analysis's section) and results indicated that nitrogen and phosphorus levels were very low.

Gas chromatography analysis showed that the contaminants consist of an average carbon chain length of approximately between C_{23}- C_{27}. This indicates that the contaminant was mainly diesel fuel and/or crude oil. Based on this result the required amount of nitrogen and phosphorus were calculated accordingly. The required amount of nitrogen and phosphorus was based on the typical C:N:Pratio of 100:10:1.

Soil Analysis

Soil texture: Particle size was determined using a Master Sizer 2000 (Malner International) at a chemical analysis laboratory of the Libyan Petroleum Institute (LPI). Libyan soils are essentially of the texture of "sandy loam". They are typically poor in essential nutrients (N, P) and have low microbial counts. Loamy sand soils tend to be dry and need to be humidified continuously during biological treatment. They do, however, have good permeation properties and thus facilitate aeration and gas exchange during bioremediation. The petroleum products that contaminated the soil samples were extracted using a Soxhlet Extraction System (SES) (see petroleum analysis of contaminated soil samples below). Dichloromethane was used for the extraction.

Soil pH

Soil pH was determined using a pH meter (Jenway 3020) on soil suspensions in water in triplicates as described by ISO 10390 (1994). Twenty five grams of soil was stirred continuously with 50ml of deionised water d.d.H_2O for 15 minutes and allowed to equilibrate for another 15 minutes. The pH meter was immersed in the supernatant and was rotated gently. Then a pH reading was recorded, with the pH value being expressed in terms of pH= $\log_{10}\{H^+\}$, where $\{H^+\}$ = hydrogen ion concentration.

Moisture Content and Water Holding Capacity (WHC)

Moisture content was determined by drying 10g of the soil sample in an oven at 105°C.In triplicate, 10g of soil was added on filter papers (Whatman No. 42) and fitted into Buchner funnels. Deionised water was added slowly (at a rate of 1cm hr-1) until the water level was just above the soil surface and the soil was saturated and dripping into the flask below. The funnel was then removed and left to drain overnight until no further drainage occurred. The soil was left for 24 hours, rewetted to saturation and the whole apparatus was reweighed. The percentage of moisture content of the soil in triplicate was then determined. The 100% water holding capacity could then be calculated as follows:

$$100\%\ \text{WHC} = \frac{\text{mean total water (g) taken up by the soil samples}}{\text{Average amount of oven dried soil (ODS) in the funnel}}$$

Nutrient Analyses

The soils were dried, sieved (2mm) and analysed for available K, N, and P. Available potassium was measured by exchangeable cation extraction with NH_4NO_3 at 1:5 w/v (Anon, 1986). The extractable potassium in the soil was measured using flame photometric determination. Available nitrogen was determined after extraction with 50ml 2M KCL (potassium chloride), (Keeney and Bemner, 1966). Available phosphorus was measured as Olsen-P (Olsen *et al*, 1954), extracted by sodium bicarbonate at a pH of 8.5 for 30min.

Soil Microcosms

Nine different nutrient treatments were carried out with varied C:N:P ratios and the use of urea as an additional nutrient source.

Microcosm experiments were prepared. All experiments were run in triplicate. For each set of experiments, 100g of soil (dry wt) were placed in a 250ml dark jars, the control soil was left un-amended (no nutrients added) whilst the amended soils were mixed with different types and levels of nutrients to give varying C:N:P ratios 100:10:10, 100:10:1, 100:1:1, and 100:1: 0.1, respectively.

Another set of nutrient amended microcosms containing varying nutrient ratios was also set up that contained both NPK fertilizer and urea as an extra source of nitrogen. 0.76g urea was added to 100g soil. This gives 100gC:10g N, and the rest of N and P were made to the required C:N:P ratio by adding the commercial NPK fertilizer. The following C:N:P ratios were used 100:10:10, 100:10:1, 100:1:1 and 100:1:0.1.

For all treatments, moisture was adjusted (added water to 60% WHC) and kept constant throughout the duration of the experiment. Soils were incubated for 130 days at 30°C.

Chemical and microbiological assessment of contaminated soil samples were carried out as described below.

Microbial Enumeration

The estimation of total bacterial viable counts in the bioremediation soil samples was carried out using a dilution plate count technique.

Soil (1g) was suspended in 9 ml of ¼ strength ringers solution, shaken for 10min (with a vortex mixer) to achieve a homogenized suspension. Following suspension, the supernatant containing bacterial cells was serially diluted with Ringers solution. A series of dilutions was then carried out up to 10^{-6}, which was used for the bacterial counts. A sample (0.1ml) of the appropriate dilution was then inoculated onto sterile, 20-ml petri dishes containing Nutrient Agar (DIFCO). Bacterial viable counts were estimated on days 0, 15, 31, 65, 130.

Petroleum Hydrocarbons Assays

Petroleum analysis of contaminated soil samples

Soil samples (triplicates) were analysed for petroleum hydrocarbons at the start and end of the experimental incubation.

10g of petroleum contaminated soil were weighed, dried, and extracted for 8 hours using soxhlet extraction apparatus (VELP Scientific, Model SER 148 Solvent Extractor) with 450 ml DCM (dichloromethane, 93%, 1:10). To absorb the moisture from the samples, extracts were decanted and concentrated to 40ml using a rotary evaporator. The dichloromethane was concentrated to 1.5-2mL, and the extracts were transferred into 2 ml gas chromatography (GC) vial for subsequent analysis. The organic phase of the extract was removed with a volumetric pipette and put in a sealed flask for further examination.

1) TPH fractions were analysed using a Varian CP-3800Gas Chromatograph, with flame ionization detection (FID). The determination of hydrocarbon concentrations is usually by capillary gas chromatography and a flame ionization detector (GC/FID). Theeffects of the bioremediation process have been followed by evaluating the changes in hydrocarbons content by gas chromatography and TPH analyser.
2 -An infra-red spectrophotometric method based on USA EPA method 418.1 (Spectrophotometric, Infrared): InfraCal TPH/TOG/ Model CVH.
Statistical analysis of the results was performed using SPSS version 14 STATISTICA for Windows release 5.1.

Gas Chromatograph Analysis

Analyses of n-alkanes and total petroleum hydrocarbons were performed at the beginning and end (130 days) of the chemostat incubation using a Chrompack Model 439 capillary gas chromatograph flame ionization detector (FID). The gas chromatographic analyses were conducted with a 300°C detector, 300°C injector, split ratio on 100:1 and samples of 0.1µl injections. A column temperature of 40°C was held for 2min and then ramped at a rate of 5°C/min to a final temperature of 300°C and held for 30min.

Degradation in the microcosms was estimated as the difference between the initial and final concentrations of total hydrocarbons (Rahman *et al*, 2002).

Bioluminescence bioassay for toxicity

Inhibition Assay

The assay is based on the analysis of light emission reduction of luminescent bacteria *Vibrio fischeri* (NRRL B-111777)when exposed to a contaminated environmental sample. In this work soil extract was exposed to *V.fischeri*: on days 0, 15, 31, 65, 130 of the bioremediation experiment.

Soil ethanol extract method: 2 g dry wt soil sample was placed in 10ml polypropylene centrifuge tubes and 2ml of 95% ethanol was added (in triplicate). They were mixed for 30 minutes in order to extract all the toxic components and then centrifuged at 6000rpm for 5 minutes. The supernatant was removed and the extract mixed with 3% sterile solution of NaCl (3% ethanol in 2% NaCl) following standard extraction procedure described by Girotti *et al*, (2008).

Freeze dried, bioluminescent cells of *V. fischeri* (as supplied by the manufacturer: SDIX Europe Ltd) were resuscitated by adding 1mL 0.1M sterile KCl and shaking at 25°C, 200rpm for 1 hour. In general assays were performed as described in Paton *et al*.(2006). After resuscitation, *V. fisheri* was used immediately. 100µl aliquot of this suspension was added to 900µl of extracted soil ethanol solution and mixed at 15-s intervals. The luminescence of the samples was measured after 15 min. exposure using the Microtox acute toxicity test Microtox Model 500 Analyzer (SDIX Europe Ltd.), as per the manufacturer's instructions. Microtox reagents and test solutions were also supplied by Strategic Diagnostics Inc. Three independent replicates of each assay were performed for each soil extract and the luminescence inhibition after 15 min exposure to each sample was taken as endpoint and recorded as relative light units (RLU).

Light emission was measured as RLU and calculated from the mean of the three replicates for each sample as % bioluminescence relative to control

samples (ethanol blank) for each assay (Girotti*et al*, 2008). Bioassays were carried out at room temperature which varied from 18 to 20°C.

The toxicity effect of different concentrations of nutrient amendments calculated by the following

$$\% \text{ INH} = \text{Be} - \text{Se/Be} \times 100 \quad \text{Or}$$
$$\text{INH } (\%) = (1-(\text{Se/Be})) \times 100$$

Where *Be* is the emission of the blank and Se that of the sample at the different times. The INH (Inhibition efficiency) values are the averages of at least three measurements, EC50 values are calculated corresponding to INH (%) = 50. If the inhibition caused by the extracted sample is below 20 %, the amount of toxicants is less than the detection limit. If the inhibition is between 20 - 50 %, the sample contains low amounts of toxicants and if over 50 % inhibition is observed, the sample contains high amount of toxicants (LaFarré *et al*, 2001; Girotti *et al*., 2008).

RESULTS

Soil chemical and physical analysis

Although the soil samples used in the previous work and current experiments originated from the same site, namely the Western Refinery, Libya, the initial contamination level was not the same. The source of the soil used in this experiment was a diesel-refuelling area, and soil was contaminated by a mixture of crude oil and diesel oil. In order to obtain an indication of potential pollutants, samples were taken from oil-containing soil samples. A suite of chemical analyses and biological assessments carried out (Table 1) showed that soil texture was sandy and of a low nutrient level (N and P). Soil pH was neutral to slightly alkaline. The soil samples taken were mixed for homogenization to decrease the localized aggregates of contaminant levels. Total Petroleum Hydrocarbon (TPH) content was 35,000ppm, pH 7.6, 0.051ppm nitrogen, 19.76ppm phosphorus, and 1.29% moisture.

Table 1: Site Information and general properties of diesel contaminated soil studies

Contaminated soil property	
Sampling location	Western Refinery
Texture	Sand
pH	7. 6
Water holding capacity (%)	1. 29
Moisture (%) @105°C	3.16
Available C (ppm)	8.69 ppm
Available N (ppm)	0.051 ppm
NH_4-N	
Available P (ppm)	19.76 ppm
Total Petroleum Hydrocarbon (ppm)	35000 ppm
CFU	2.4×10^7 CFU g^{-1}

Change in microbial population during microcosm incubation

Generally there was a significant increase ($p<0.05$) in viable counts over the first 31 days of the bioremediation in all soil microcosms including the non-amended control (Figs.1 & 2). Counts decreased after this time in all microcosms to levels that were determined initially.

In soil microcosms treated with NPK the nutrient ratios of 100:10:10; 100:10:1 and 100:1:1gave significantly higher ($p<0.02$) bacterial counts than the control soils after 31 days incubation. For example, the cell count with C:N:P ratio of 100:10:1 was 7.33×10^{10} cfu/g soil after 31 days compared to the control which gave 4.2×10^{10} cfu/g soil.

In soil microcosms treated with urea and NPK fertiliser, bacterial counts achieved during the middle stages of incubation were significantly higher ($p<0.003$) than those observed with NPK fertiliser alone. Increases in bacterial counts to 4.1×10^{11} cfu/g soil and 3.6×10^{11} cfu/g soil during week 15 in amended microcosms by urea mixed C:N:P ratios (100:10:1 and 100:1:1 respectively) were observed interestingly. The highest ratio of urea mixed with NPK (100:10:10) appeared to restrict microbial counts to levels similar to those found in control soils after 2 weeks of incubation but the counts increased significantly after a further incubation period. The lowest level of urea plus NPK fertilizer did not increase bacterial counts relative to the control.

Effect of different nutrients ratios on bioremediation

The chemical analysis of the soil samples confirmed that the five soils had the same level of TPH contamination at the start of the experiment. The total content, before and after 4 months treatment, is reported in (Figs. 3 and 4).

During the bioremediation process it was possible to observe that the impact of NPK treated microcosms and urea mixed with NPK treated microcosms was clearly different to that of the control in relation to petroleum transformation. All treated microcosms with NPK alone, and urea mixed with NPK ratios after 4 months treatment gave a

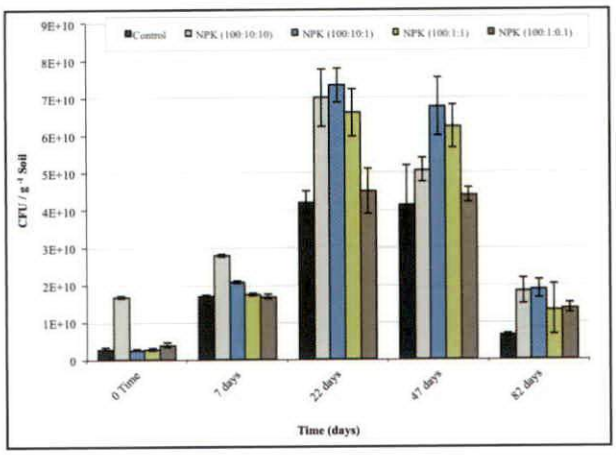

Fig. 1 Changes in heterotrophic bacteria counts (CFU g^{-1} soil) in treated soil microcosms with different ratios of NPK treatment.

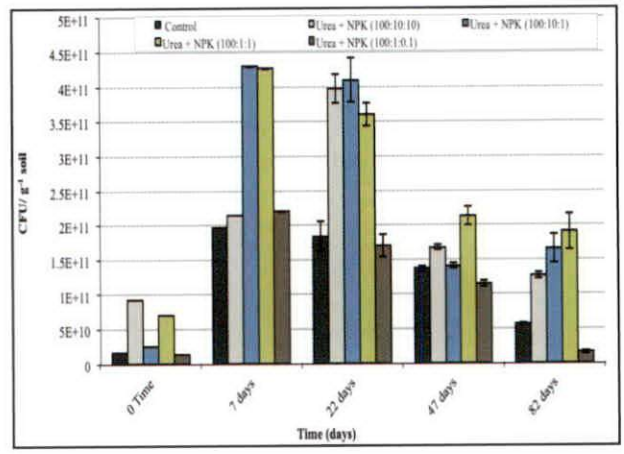

Fig. 2 Changes in heterotrophic bacteria counts (CFU g^{-1} soil) in treated soil microcosms with different ratios of Urea/NPK treatments.

significant decrease in total petroleum hydrocarbon content, with the only exception of lowest ratios 100:1:0.1.

NPK fertilizer microcosms: Soil microcosms treated with NPK only in the ratio 100:10:10 resulted in the highest TPH removal which was 46% by day 65 and 66% after four months treatment. 100:10:1 ratio gave significantly higher ($p<0.017$) transformation rate than the control and the other NPK ratio (100:10:10 and 100:1:0.1) after 130 days incubation.

C:N:P ratios 100:10:1 & 100:1:1 showed similar results of 40% and 34% removal by day 65 of incubation and the transformation level increased to reach 52.5% and 49.5% by four months treatment respectively. The lowest petroleum hydrocarbon transformation was obtained with C:N:P ratio (100:1:0.1) treatment and control untreated soils. The former exhibited 27.6% removal by 65 days, then the removal increased to 31.7% by the end of experiment, whereas 17% TPH removal was obtained with control untreated soilsby middle of the treatment time and 29% removal by the end of the experiment.

Urea plus NPK fertiliser microcosms: Urea mixed with NPK treatments appeared to be more effective in TPH transformation than NPK treatment alone. However, the degree of TPH removal using urea mixed with C 100: N 10: P 1 ratio was significantly higher (69%) than other treatments urea mixed with C:N:P ratios (100:1:1) resulted in 67% TPH removal after 17 weeks treatment. Treatment with urea mixed with C:N: Pratios (100:10:10) and (100:1:0.1) reached 45.7%, and 49% by the end of incubation time respectively. The lowest TPH transformation was obtained again with control untreated soils which was 25.7%. The standard deviation for each analysis is presented in (Figs.3 and 4).

Gas Chromatograph analysis of the effective nutrient regime (Urea mixed with NPK)

A comparison study was made between the hydrocarbon composition of contaminant in the soil at the beginning of the study and after 130 days incubation in all soil microcosms. The gas chromatographic mass spectrophotometry analysis detected aliphatic compounds with carbon number between C14 and C27. Fig.5 shows the typical change (the urea plus NPK fertilizer treatments used as an example) in the distribution profile of n-alkanes during bioremediation in which a decrease in the middle chain carbon lengths (C14-C19) was observed. Fig. 5 was developed using GC/ MS chromatograms showing individual peaks of each petroleum compound. The higher molecular weight carbon compounds (C20 and upwards) were generally not reduced in level during bioremediation.

The addition of nutrients and increasing the nitrogen sources in the form of urea enhanced the removal of the short - and middle chain aliphatic compounds. The highest petroleum reduction was with middle chain C14-C19 when soil was amended with urea mixed with NPK ratios (100:10:1 & 100:1:1).

The C17/Pr and C18/ Ph ratios were calculated using the chromatograms of extracted soil samples from the treated soil microcosms to determine if

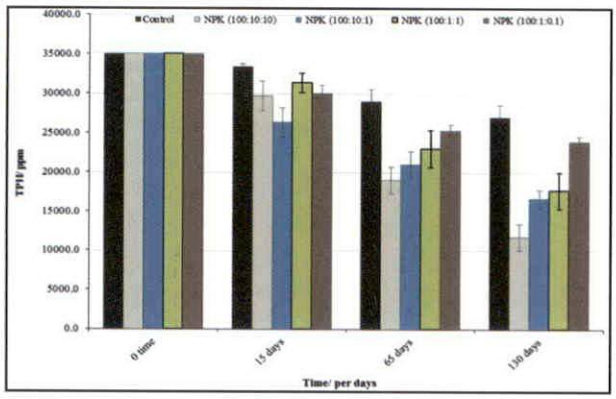

Fig. 3 Changes in TPH content in treated soil microcosmswith different ratio of NPK treatments determined by TPH analyzer before and after treatment. Error bars represent standard deviation of the mean from triplicate soil microcosms.

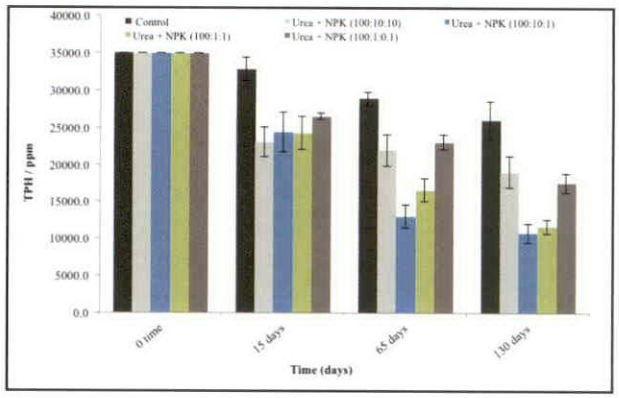

Fig. 4 Changes in TPH content in treated soil microcosmswith different ratio of Urea/NPK treatments determined by TPH analyzer before and after treatment. Error bars represent standard deviation of the mean from triplicate soil microcosms.

significant bioremediation was occurring. At the end of the 130 days study, results showed that urea mixed with C:N:P ratios (100:10:1 & 100:1:1) had removed pristane and phytane of their original values. The changes of C17/pristane from 13.4 to 10.5, and C18/phytane from 7.8 to 4.8, and changes of C17/pristane from 13.4 to 8.1, and C18/phytane ratios from 7.8 to 5.0, with both nutrient ratios respectively. Overall the results demonstrate that TPH removal was more extensive with short and middle chain aliphatic compounds compared to the longer chain hydrocarbons (C20-C27 chain length).

The effect of the different bioremediation treatments on PH contamination was estimated by gas chromatography and a TPH analyser (infra red spectrophotometry). Both methods gave good agreement in PH levels. The total PH content at the start of the experiment and after 130 days treatment for both nutrient treated soils, is reported in Figs. 3 and 4 (TPH analyser results) and (Fig. 5, GC results).

Measurements of Residual Toxicity during Petroleum Hydrocarbon transformation

The changes in toxicity as a function of petroleum transformation activity were also determined over 130 days. The results of the toxicity tests all given as averages for three replicates, are summarized in (Figs. 6 & 7). The biotoxicity analyses of the soil microcosms treated with NPK ratios of 100:10:10 & 100:10:1 and urea mixed with NPK ratios 100:10:1 & 100:1:1 performed during the treatment period initially showed little change. However, toxicity decreased markedly after three months treatment compared to control (non-treated soils). The greatest reduction in toxicity was observed with soil treated with urea mixed with NPK ratio (100:10:1), where toxicity was reduced to 75%, and 56.5% with urea mixed with NPK ratio (100:1:1).

DISCUSSION

The effect of nutrient treatment on bacterial counts

In this study, the effect of nutrient level (C:N:P ratio) over time on TPH transformation and microbial counts were investigated. Margesin *et al* (2007) found that hydrocarbon concentration and incubation time are important factors during bioremediation of diesel-contaminated soil. The higher the initial contamination, the more marked was the effect of fertilizer supplements.

Initial results from this study also indicated that simple nutrient addition was the most effective and economical potential bioremediation method for the Libyan soils studied. Given that the soils used in this work are low in nutrients (and the indigenous microbial population present potentially susceptible to nutrient shock). It was decided to investigate if different nutrient levels and different nitrogen sources (commercial NPK fertilizer and urea) had varied effects on microbial populations and bioremediation. Previous authors reported a range of different nutrient ratios to be optimum for different soils adding further reasons to optimize nutrient ratios in the soils studied here. Bacterial counts at eight different nutrient concentrations (C:N:P, ranging from 100:10:10, 100:10:1, 100:1:1, 100:1:0.1) were monitored over 130 days incubation at 30°C.

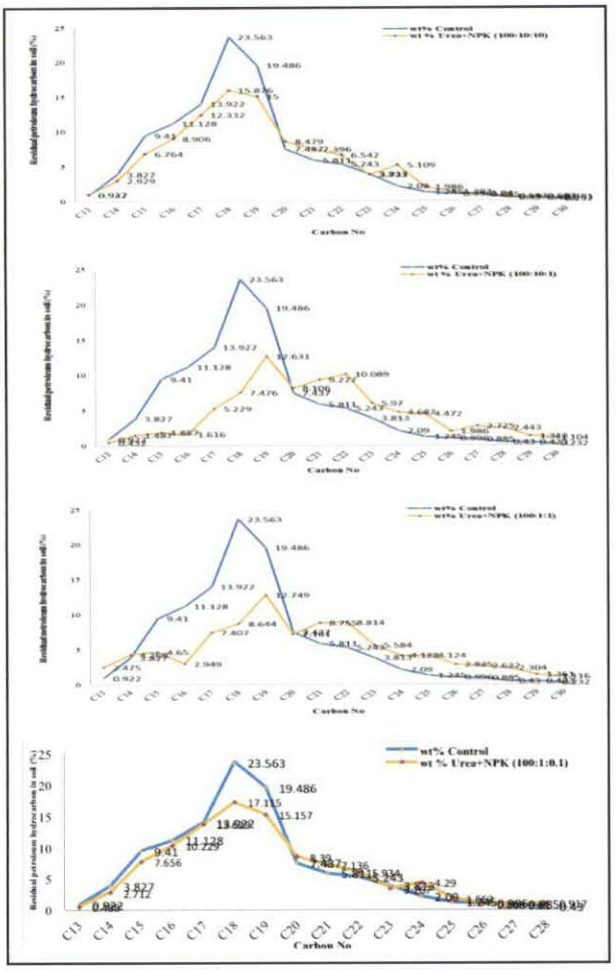

Fig. 5 Representative GC Chromatograms of TPH extracted from diesel-contaminated soil treated with urea mixed with NPK after 17 weeks incubation. The values in brackets represent the nutrient ratios.

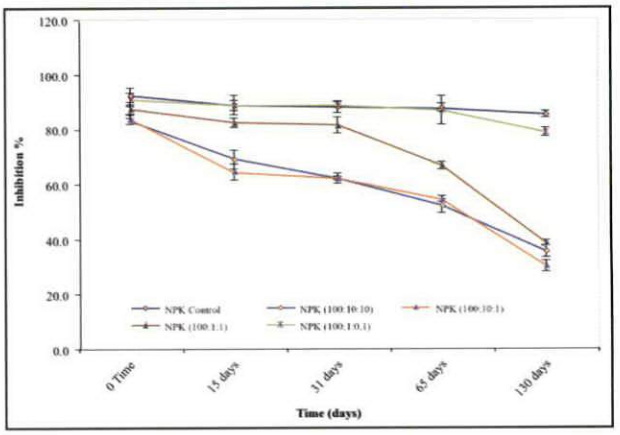

Fig. 6 Biotoxicity values during bioremediation treatment determined by V. fischeri bacteria. Error bars represent standard deviation of the mean from triplicate soil microcosms.

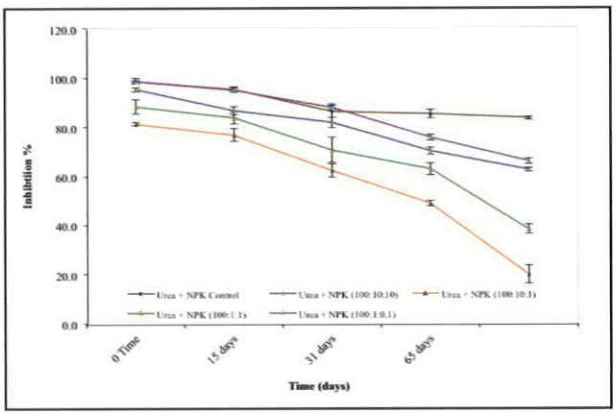

Fig. 7 Biotoxicity values during bioremediation treatment determined by V. fischeri bacteria. Error bars represent standard deviation of the mean from triplicate soil microcosms.

Significant increases over time in microbial populations in oil contaminated soil were recorded and results found in this work were generally similar to previous studies i.e. increases in counts were observed during the initial to middle stages of incubation followed by a decreases in counts. For example Margesin *et al* (2000) reported increased the number of hydrocarbon degraders in both nutrient stimulated samples and soil without nutrient amendments. Some authors have reported a decrease in counts with nutrient addition. Urea in particular has been known to cause a decreases in microbial counts, and the released of ammonia killed the fungal population and the hydrocarbon degraders (Chaillan *et al*, 2006; Peltola *et al*, 2006; Jin and Fallgren, 2007), but in this work, urea actually stimulated bacterial populations to the greatest extent.

Overall, the higher additions of nutrients used stimulated bacterial counts to the highest extent suggesting that the indigenous bacterial population was not susceptible to nutrient shock (apart from the highest level of urea plus NPK fertilizer used where counts were not as high). A viable count does not indicate any effects on bacterial diversity and it is possible that hydrocarbon transforming bacterial populations may have been affected detrimentally. If this was the case then petroleum analysis of the soil with nutrient amendment should indicate that nutrient addition decreased petroleum transformation; results actually showed that nutrient addition served to stimulate petroleum removal. It must be noted that as NPK fertilizer was used in this work then K levels in soil would also increases in nutrient amended soils. It is possible that added K

acted (in synergy with N and P) to boost microbial populations but it is assumed that K would have less effect on microbial growth than N and P as it is required in much less quantity by microbes.

The overall effect of nutrient treatment on TPH transformation

In this study a range of petroleum transformation levels were obtained as a result of using different nutrient levels emphasizing the importance of optimizing nutrient levels added for bioremediation. Liebeg and Cutright (1999) suggested that the optimal recommended C:N:P ratio for bioremediation is 100:10:1, and other authors have suggested similar and/or different nutrient ratios; e.g. Embar *et al*, (2006) obtained maximal reduction of TPH (91%) after 90 days in contaminated sandy soil supplemented with nitrogen and phosphorus at C:N:P ratio of 100:10:1, and the soil containing high concentration of crude oil 163,800ppm.

Atagana *et al* (2003) used different C:N ratios 5: 1, 10 : 1, 15 : 1, 20 : 1 and 25 : 1 and only 33% hydrocarbon removal was achieved using the highest nutrient supplementation (C : N 5 : 1), thus they concluded that the highest ratio was the least effective in supporting growth of microorganisms. This may be due to the high concentration of nutrient used being toxic to the microbial population.

Several authors have reported the negative effect of high nitrogen, phosphorus and potassium concentrations on the biodegradation. Walworth *et al* (2007) amended petroleum contaminated sandy soil with ammonium chloride (NH_4Cl) to levels 0, 125, 250, 375, 500, and 625 mg N / kg soil. They discovered that addition of greater levels of N (375, 500, or 675 mg N kg−1 soil) significantly depressed oxygen consumption to levels equivalent to that of the untreated control (no nitrogen). Also changes of residual petroleum levels in the nutrient amended soils were not significantly different from the untreated control soil. Other studies have showed that relatively high total nutrient addition had no inhibitory effects if the nutrients were added gradually and not in a single dose. Ferguson *et al* (2003) reported that the addition of nutrients in small dose kept nutrients concentration in the range required for microbial mineralisation of petroleum hydrocarbons. Nikolopoulou and Kalogerakis (2008) suggested using urea as a nitrogen source for bioremediation of petroleum compounds in open systems and our work would also support the use of urea as a nutrient source. It may have been possible that the indigenous microflora utilized the carbon in urea in preference to the pollutant carbon but in this case these levels of urea used appeared to have been stimulatory to petroleum removal.

Interestingly, the best petroleum removal rates observed in this study appeared to correlate with the highest increases in bacterial viable counts, which indicating that the less sophisticated but rapid and cheap bacterial count method is a good indicator of overall functionality of the population. in this case functionality refers to the ability of the bacterial population to reduce petroleum levels in soil. Clearly, the level and type of nutrient addition required for bioremediation is an area worthy of detailed investigation and it would appear that different soils have different nutrient requirements i.e. nutrient levels should be optimized for different soils. In this work nutrient levels of 100:10:1 (C:N:P) appeared to be the most suitable and urea was an effective nutrient source but this may not be the case for all soils.

Jin and Fallgren (2007) indicated that urea wasn't a good source of nutrient for bioremediation of petroleum contaminants and found to inhibit the enzyme responsible for petroleum degradation in contaminated sandy soil collected from an Egyptian site, and thus it was suggested that the application of urea in stimulating the petroleum degradation may be site-specific.

Effect of nutrient addition on transformation of individual petroleum hydrocarbons

As well as examining the effect of nutrients on the reduction of total petroleum hydrocarbon levels it was also considered important to examine the removal of individual petroleum compounds, to see if microbial transformation was restricted to any particular type. Or if the different nutrient levels caused a change in the types of petroleum compounds removed. The results showed that the aliphatic compounds were optimally reduced as result of addition of urea mixed with C:N:P ratios 100:10:, and 100:1:1 after 130 days treatment. This might due to the balance between nitrogen supply and the carbon supply during the oil degradation.

Significant reduction in hydrocarbon content (C9-C27) was observed in the soil samples amended ratios (100:10:1 and 100:1:1). It has been demonstrated that the intermediate length n-alkane chains (C10-C20) are the substrates most degradable by soil microorganisms (Balba *et al*, 1998). The long chains of n-alkanes (C20-C40) tend

to be recalcitrant due to their poor water solubility and lower bioavailability (Venosa and Zhu, 2003) and similar observations were made in this work.

The most readily degradable petroleum fractions are n-alkanes of intermediate length (C10-C20), whereas, shorter chain compounds are rather more toxic. Longer chain alkanes known as waxes (C20-C40) are hydrophobic and difficult to degrade. Results showed that urea mixed with NPK ratios (100:10:1 & 100:1:1) had further biodegraded C17/pristane from 13.4 to 8.3, and C18/phytane from 7.8 to 4.8, and C17/pristane from 13.4 to 8.1, and C18/phytane ratios from 7.8 to 5.0, of their original values respectively. Whereas, amended sludge contaminated soil with urea mixed with C:N:P ratios (100:10:10) only removed from C17/pristane from 13.4 to 10.5 and C18/phytane from 7.8 to 6.5. A similar change in pristane/ phytane ratios has been observed previously in sludge contaminated soil treated with nutrients, and evidence for biodegradation and changes in C17/Pr and C18/Ph ratios were determined using the GC-FID analysis. This confirms that nutrients are key parameters for promoting biodegradation (Hejazi and Husain, 2004).

Pristane has been reported to be a recalcitrant compound for attack by biodegrading microorganisms so any reduction in this compound (as observed in this work with urea plus NPK fertilizer) is an interesting result. It could be possible that more time is required for the microbial populations to degrade this branched-alkane to below detection levels.

The best petroleum (pristane and phytane) transformation rates in this work were obtained with urea and NPK fertilizer addition in a C:N:P ratio of 100:10:1. High levels of removal (approx 66%) were also seen with NPK fertilizer alone in a C:N:P ratio of 100:10:1. Addition of extra nutrients (C:N:P ratio of 100:10:10) reduced petroleum transformation slightly in comparison to 100:10:1 C:N:P ratios indicating a potential toxic effect of higher nutrient levels.

Ecotoxicity assessment

It has been demonstrated that ecotoxicity bioassays can be effectively used as supplementary tools for monitoring the effectiveness of remediating petroleum contaminated soils (Girotti *et al*, 2008). In this work a bacterial based luminescence bioassay was used to monitor the toxicity of soil samples taken during bioremediation. In all samples, toxicity levels were high at the beginning of bioremediation (100% inhibition was observed) indicating the high toxicity associated with the contaminated soil. The toxicity of the control soil samples remained high throughout the experiment and this was correlated with a small reduction in the petroleum hydrocarbons present. In contrast, nutrient amended microcosms showed a steady decrease in toxicity and the soils showing the highest reduction in hydrocarbons also gave the highest reduction in toxicity. The reduction in the smaller carbon compounds (C9-C20) clearly removed most of the associated soil toxicity and it appears the larger petroleum compounds not removed during bioremediation were less inhibitory. This may be due to the fact that the larger compounds are less soluble and therefore, are not removed during the ethanol extraction. Other workers have also shown that bioremediation using nutrient addition leads to a reduction in toxicity (Philp and Atlas, 2005). Generally, an increase in toxicity during the early stages of bioremediation is observed as pollutants are incompletely transformed into more toxic intermediates, but as bioremediation progresses the toxic intermediates are further transformed and toxicity reduces as a result. Girotti *et al* (2008) noticed that the toxicity of contaminant increased after short time of the treatment as result of existence of long chain hydrocarbons during the bioremediation of hydrocarbon contaminated soil, which decreased after long period of bioremediation. In this work as 100% toxicity of soil samples was observed initially it was impossible to see this initial toxicity increase. The percentage of light inhibition obtained by this assay was in good correlation with the reduction in total petroleum hydrocarbon determined, probably because both nutrient levels used representing the right level of amendment needed. However, it is important to realize the limitations of the toxicity methods used in this work ;the method only gives an indication of the short term toxicity of samples. For example, over a longer incubation period toxic residues may be released into soil due to changes in soil structure caused by rewetting/drying or freeze/ thaw cycles or microbial mineralization of that releases pollutants bound to organic materials in soil (e.g. humic matter). Also, it is important to test toxicity to other trophic levels such as plants and other eukaryotes (Salanitro *et al*, 1997). Millioli *et al*, (2009) used lettuce seeds of the *Lactucasativa species* to assess the soil toxicity of the addition

of rhamnolipid in a crude oil contaminated soil. However, the microbial toxicity test used here is quick and economical and does appear to reflect the level of hydrocarbons present as estimated by chemical analysis.

SUMMARY AND CONCLUSIONS

This study assessed the effect of varying nutrient (NPK) levels and types (urea) on bioremediation of diesel contaminated soil by a combination of chemical and toxicological analyses. In general, the optimum C:N:P ratio found was 100:10:1 (and urea was found to be a good nutrient source). Urea is a source of N and easily utilised carbon and appeared to stimulate bioremediation effectively. In this study (achieved approximately 69% TPH removal) despite previous studies finding it to cause inhibition of the microbial population present. The amounts of petroleum removed as revealed by chemical analysis appeared to correspond well to bacterial counts and the residual toxicity of soil as estimated by the Microtox assay. The highest amounts of nutrients used (C:N:P ratio of 100:10:10) did slightly reduce bioremediation effectiveness. GC analysis revealed that hydrocarbons of chain length C9-C20 were most effectively removed and that the higher chain length petroleum compounds (>C20) remained in the soil. Overall, the work demonstrated the clear potential of nutrient stimulation to reduce levels of hydrocarbons present and to reduce the soil toxicity. These promising results were used as the basis for subsequent scale-up studies: a pilot scale bioremediation experiment to treat a larger amount of contaminated soil.

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