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Evaluating the role of free living nematodes in hydrocarbons biodegradation by microbiological techniques

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Abstract: Microbial motility in soil is considered as one of the main obstacles reducing the efficiency of hydrocarbon bioremediation technology. Free living soil nematodes have a strong effect on the transport of seed applied bacterial inoculate mediating colonization of the rhizosphere. Therefore, the aim of current investigation is to evaluate the role of free-living soil nematodes in hydrocarbon bioremediation using a suite of microbiological and chemical methods. A biopile method has been adopted to investigate the ability of free-living nematodes to improve the microbial motility in a hydrocarbon contaminated soil. Enumeration of hydrocarbon-degrading microorganisms was carried out using the Most Probable Number (MPN) technique followed by measurements of soil microbial activity and toxicity. Hydrocarbon concentration was detected by GC-FID analysis. Assessment of hydrocarbon bioremediation showed that the nematode treated biopile was associated with a greater hydrocarbon reduction, comparing with control. This confirms that the addition of soil nematodes to the hydrocarbon contaminated soil had a strong influence on the general soil microbial activity. The overall results of this study concluded that the soil nematodes could play an important role in hydrocarbon bioremediation

Keywords: Bioremediation, Microbial Motility, Soil Toxicity, Bioavailability

1. INTRODUCTION

Motility is not alleged to be a mechanism of long distance movement of bacteria through soil [1]. Though, the action of transporting agents, in particular percolating water and soil animals is deliberated to be liable for long distance bacterial transport in soil. Bacteria can be transported through the soil profile to long distance through percolation [2], but the ability of this mechanism to distribute bacteria throughout soils is restricted as the mass flow of water tends to move through certain flow pathways. Thus, horizontal spreading of soil microbes is more likely to be carried out by soil animals or plant roots than water flux [1].

Contaminant degradation is carried out largely by microbes thus one may argue that in bioremediation processes soil animals are not imperative. However, soil animals can play indirect significant role in bioremediation processes. Soil animals can be utilised in bioremediation processes to improve soil structure through their comminution and burrowing activity [3]. Furthermore, nutrient cycling at the site can be improved though animal grazing resulting in an increase of soil fertility and thus increase nutrient availability to soil microorganisms [4].

In addition to their role in soil health restoration, soil animals, such as nematodes and earthworms, are important agents of bacterial transport in soil, particularly under drier conditions. A number of studies have been performed on microbial transport by earthworms [5] and optimistic effects of earthworms in bioremediation programmes were documented [6]. However, one of the main restrictions of using earthworms in bioremediation technology is that they are known to be intolerant to constraints such as soil concentrations above 1% [7] and to low pH [1], conditions often found in contaminated sites. The use of earthworms in bioremediation has some other limitations, i.e., difficult to mass production and require higher O2 levels than are often found in contaminated soils.

Another main group of soil organisms is nematodes which are most abundant and are present at very high population densities (> 100 g-1 soil) throughout the soil profile [8]. A lot of attention has been focused on nematodes responsible for plant diseases [9], but far less is known about the majority of the nematode community that plays numerous beneficial roles in soil. Moreover, some studies showed their use in bioremediation technology, although there is an abundance of evidence suggesting that nematodes

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could play a significant role in soil mixing, aeration and restoration of soil health [8, 10, 11].

In last decade, it was found that nematodes have a strong effect on the transport of seed applied bacterial inocula mediating colonization of the rhizosphere [8]. Therefore, the present study aimed for investigating and evaluating the role of free living soil nematodes in hydrocarbon bioremediation using a suite of microbiological and chemical methods.

2. MATERIALS AND METHODS

Soil preparation and experimental plan

Approximately 3.6 kg of a hydrocarbon contaminated soil was collected from a petrol station site, Abha city, Saudi Arabia, in June 2016. To procure homogenous material the soil was sieved using a 3.5 mm sieve. The moisture content of the soil was maintained at 60 % water holding capacity. The experiment was conducted over a period of 40 d. The two soils used in the experimental design were, the control soil and the nematode treated soil were amended with inorganic nutrient in order to simulate soil microbial activity (see below). Approximately 100 g wet weight of soil, in triplicate, was placed into a 500 ml glass jar and the jars sealed with stretched parafilm. The total number of jars used over the period of bioremediation was 36. Jars were kept at 25°C in the dark at 70% relative humidity. Sampling was carried out on days 1, 5, 10, 20, 30 and 40. The jars were arranged in a random order and rearranged at each sampling time to compensate for any local temperature fluctuations in the incubator.

Determination of physicochemical properties

Physicochemical properties of the contaminated soil are shown in Table 1. Soil pH was determined by placing 25 ml of soil into a 100 ml beaker and adding 50 ml of dH_2O [12]. The mixture was stirred for about 15 min and then allowed to equilibrate for an extra 15 min. The pH meter (HI 8424 microcomputer pH mater, HANNA Instruments) was submerged in the mixture and the pH value was then recorded. Available NH_4^+ and NO_3^- were extracted from 5 g soil with 50 ml 2M KCl [13]. The samples were shaken end-over-end for 30 min at 60 rpm. The samples were then centrifuged at a speed of 3000 *g*, for 30 min in a cool-spin centrifuge. Ten ml of the suspension was transferred into new vials and the samples were then analysed using a Flow Injection Analyser (Tecator FIA, Star, UK). Soil organic matter (SOM) was determined by a gravimetric method based on soil weight loss which follows the loss of organic matter on ignition. Fifty gram of each soil sample was oven dried for 24 h at 105˚C, and then was placed into a furnace for an extra 4 h at 400˚C.

Preparation of treatments

Nutrient supplement

An initial analysis of C:N:P concentrations was executed to determine the experimental supplement of nutrients to ensure a C:N:P ratio of 100:10:1 (5). NH_4NO_3 and K_2HPO_4 (Fischer Scientific) were used as a nitrogen source (11 g N kg^{-1} dry weight of soil) and phosphorus source (2 g P kg^{-1} dry weight of soil), respectively. Using a fine sprayer, nutrient solution was sprayed evenly over the soil surface. The soil was then continuously 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 [14].

Growth and preparation of Nematodes

Nematode growth medium was modified from the method described by Lewis and Fleming [15]. 1.2 g of Na Cl, 20 g of peptone and 25 g of agar were added to 1 l dH_2O and the mixture was then autoclaved. The mixture was left to cool (55˚C) and then 1 ml cholesterol (5 mg ml^{-1} in EtOH), 1 ml of 1 M MgSO4, and 25 ml of 1 M potassium phosphate were sterilely and added to the mixture. Petri dishes of nematode growth medium were inoculated with free living soil nematodes. The nematodes were grown at 15 ˚C for about 2 w and then they were transferred to a Baermann funnel apparatus for extraction into water. A dissecting microscope was used to count nematodes and then they were added to microcosms at a rate of 10 g^{-1}soil.

Soil microbial analysis

Enumeration of hydrocarbon-degrading microorganisms

Hydrocarbon-degrading microorganisms enumera-tion was achieved using the most probable number (MPN) technique as described by Braddock and Catterall [16]. The MPN plates were wrapped with foil and incubated at 22˚C for 14 d. The positive wells (pink colour) were used to ascertain the MPN of microorganisms per ml after incubation. MPN calculator (Version 4.0) computer program was adopted for calculations.

Assessment of soil microbial activity

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. The produced Co_2 was determined by GC as described by Paton *et al.* [17]. Respiration values were determined as mg CO_2 g $soil^{-1}$ d^{-1} following subtraction of a blank vacuette containing atmospheric CO_2.

Assessment of soil toxicity

Soil ecotoxicity assessment was performed as described by Paton *et al.* [17]. 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 0.1M sterile KCl and placed into an orbital incubator shaker set at 25˚C and 200 rpm for 1 h. Following incubation, 0.9 ml of each sample in triplicate was mixed with 0.1 ml cell suspension in luminometer cuvettes at 15 s intervals between each sample. After 15 min exposure to the sample, light output was measured using a JADE luminometer (Labtech Instrumental, UK).

Determination of Hydrocarbon concentration

Chemical analysis of total hydrocarbon was adapted as designated by Dawson *et al.* [12]. 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 ml 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 (GC-FID), 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. Total hydrocarbons were determined as mg CO_2 g $soil^{-1}$ d^{-1}.

Statistical analysis

Firstly, parametric testing was performed and analysis of variance then used to compare data between different treatments. Kruskal-Wallis test was used in case the data and residuals were not normally distributed or did not have equal variance even after transformation. All analyses were performed at $P \leq 0.05$ using MINITAB, version 13.1.

3. FINDINGS AND DISCUSSION

Assessment of soil microbial activity

The control and nematode treated biopiles showed a rapid increase in the soil microbial respiration during the first 10 days. This increase, however, was significantly greater in the nematode treated biopile than the control biopile. This rapid increase in the microbial respiration during this initial stage was more likely due to the increase in the microbial numbers (Figure 2), responding to the addition of nutrients to the contaminated soil. The higher microbial activity in the biopile treated with nematodes observed during the first 10 days, comparing with the control, confirms the positive effect of soil nematodes in hydrocarbon bioremediation. Free living nematodes may have enhanced bacterial growth/activity by increasing the bioavailability of resources (i.e. carbon and nutrients) utilised by bacteria [4]. In addition, they may mediate distribution of soil microbes throughout the soil by carrying live and dormant bacteria on their surfaces a in their digestive systems [1]. Consequently, this will increase the direct contact between microbial degraders and organic contaminants in soil.

Following day 10, the microbial respiration declined rapidly until day 20 which was significantly less than all the previous sample points. This rapid decline in the soil microbial activity observed during this stage was more likely caused by the rapid depletion of readily biodegradable hydrocarbons (Figure 4). The increase in soil toxicity (Figure 3) during this period of bioremediation could also have had a negative effect on microbial activities and thus the production of CO_2.

Between day 20 and 30, the control biopile showed an increase in the rate of soil microbial respiration until day 30 which was significantly higher than day 20. This increase in the microbial respiration for the control during this period is might be because of the high concentration of hydrocarbons prompted in soil (Figure 4), which can be utilised by the soil microbial degraders. This would explain the observed increase in the soil microbial number (Figure 2), which will result in an increase in the rate of CO_2 production. On the other hand, the soil microbial respiration at the nematode treated biopile remained constant until day 30. Subsequently, the respiration rate of soil microbes dropped until day 40 which was considerably less than all the previous sample points. The reduction in the microbial activity recorded for the nematode treated biopile following day 30 until the end of the experiment may be attributed only to the slow rate of biodegradation of residual hydrocarbon (Figure 4).

Enumeration of hydrocarbon-degrading microorganisms

There were marked changes in the numbers of soil microbial population during the experiment (Figure 2). This suggests a response of the soil microbial degraders to the progress of hydrocarbon biodegradation. The control and nematode treated biopile showed a rapid increase in the microbial counts between day 1 and 10, being significantly greater on day 10 than all the pervious sample points. The rapid increase in the microbial count was probably due to the nutrients addition into the contaminated soil. Nutrients, in particular N and P, are essential for microbial growth and activity, as these allow microbes to synthesize the required enzymes degrading organic compounds [18]. This rapid increase in the soil microbial numbers was also linked with a rapid decline in hydrocarbon concentrations during the same period (Figure 4). This indicates that most of the contaminant reduction was due to biological processes rather than physical processes such as volatilisation or sequestration [19].

During the initial stage of bioremediation, nematode treated biopile showed higher growth in the microbial numbers than the control biopile (Figure 2). This confirms that the addition of nematodes strongly stimulated microbial growth by increasing substrate availability to soil microorganisms [3]. In addition, soil properties such as water-holding capacity and porosity can be improved by their movement though the soil. All these factors would result in an increase in the rate of hydrocarbon biodegradation by indigenous microbial degraders.

Between day 10 and 20, both biopiles exhibited a sharp decline in the counts of hydrocarbon microbial degraders. This rapid decline in the hydrocarbon microbial count could be related to the rapid decline in hydrocarbon bioavailability. In addition, it might have been as a response to the increase in soil toxicity (Figure 3). This observation is well agreed with some previous investigations [20, 21] reporting a similar pattern of microbial changes during the initial stage of hydrocarbon biodegradation.

Following day 20, the control biopile showed a slight increase in the microbial count which was significantly higher than day 20 (Figure 2). This increase in the microbial counts is might be due to the reduction in soil toxicity (Figure3). In addition, it may be correlated with the high available carbon substrates (Figure 4) that could support a high microbial population density. In contrast, the soil microbial counts at the nematode treated biopile remained constant until the end of the experiment. This was more likely due to the rapid reduction of the readily biodegradable hydrocarbons, as confirmed by the chemical analysis (Figure 4).

Assessment of soil toxicity

The *lux*-modified bacterial biosensor (*P. fluorescens* 10586r) showed that the toxicity level in the nematode treated biopile was significantly less than the control biopile during this experiment (Figure 3).This suggests that the addition of nematodes to the contaminated soil was able to achieve a higher reduction in the hydrocarbon toxicity by increasing the biodegradation rate of contaminant through improving the motility of microbial degraders in the contaminated soil. During the first 10 days, there was a rapid decline in the toxicity level for the control as well as the nematode treated biopiles, which was significantly less on day 10 than all the previous sample points. The significant reduction in the soil toxicity during this stage of bioremediation for both biopiles was more likely due to the high decline in hydrocarbon levels during the same period (Figure 4).

Between day 10 and 20, there was a significant reduction in the bioluminescence response for the control and the nematode treated biopiles revealing increased soil toxicity. This increase in the soil toxicity is might be due to the presence of metabolites which are more polar and thus more bioavailable [13]. Furthermore, such by products (e.g. carboxylic acids, catecholes etc) might be more toxic than the parent compounds [22]. The increase in soil toxicity was also associated with a reduction in the rate of hydrocarbon biodegradation (Figure 4). Following day 20, there was an increase in the bioluminescence response, being significantly higher on day 40 than the previous sample point (Figure3). This indicates to a progress in hydrocarbon biodegradation [23].

Chemical assessment of hydrocarbon reduction

The overall results of the chemical analysis showed that the control and nematode treated biopiles were associated with a significant decline in the total hydrocarbon concentration over the period of bioremediation. The decline in hydrocarbon concentrations in the nematode treated biopile was significantly greater than the control biopile. This confirms that the addition of nematodes to the contaminated soil had a positive effect on the bioremediation rate and thus reduced the time required to achieve acceptable concentrations of hydrocarbons.

The results showed that most of the hydrocarbon contaminants were biodegraded between days 1 and 10 which is in good agreement with previous study [20, 24] of hydrocarbon bioremediation. This might be due to the rapid decline in the low molecular weight hydrocarbons during the early stage of the bioremediation, as the low molecular weight hydrocarbons tend to be more biodegradable than the higher molecular weight hydrocarbons [25, 26].

No significant reduction in hydrocarbon concentrations between day 10 and 20 was observed which is due to the increase in soil toxicity, as confirmed by the biosensor-based toxicity test (Figure3). Whilst low solubility and diffusion of residual hydrocarbon remaining in the soil lead to slow rate of hydrocarbon reduction which was observed between day 20 and 40 (Figure 4).

4. CONCLUSION

The overall assessment of hydrocarbon bioremediation, using a suite of chemical and biological methods showed that the nematode treated biopile samples was associated with a greater hydrocarbon reduction, in comparison with control samples. This confirms that the addition of soil nematodes to the hydrocarbon contaminated soil had a strong influence on the general soil microbial activity. This should be carried out through the regulation of several important factors such as substrate bioavailability and microbial motility. So, under these conditions the degradation of hydrocarbons could be enhanced.

TABLE I: SOIL PHYSICOCHEMICAL PROPERTIES

Texture	**Sandy loam**
Soil pH	6.17
WHC	0.34 g g^{-1}
Bulk density	1.17 g cm^{-3}
Available N	< 0.003 mg g^{-1}
Available P	< 0.001 mg g^{-1}
Hydrocarbon content	39 g kg^{-1}

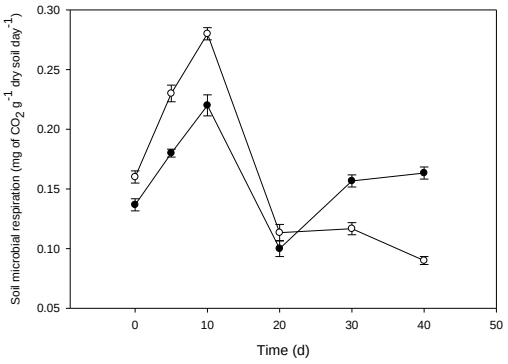

Fig. 1: Soil microbial respiration (mg CO_2 g^{-1} of dry soil day^{-1}) over 40 days, where (●) is the control biopile and (○) is the nematode treated biopile. Bars indicate standard errors of the mean (n=3).

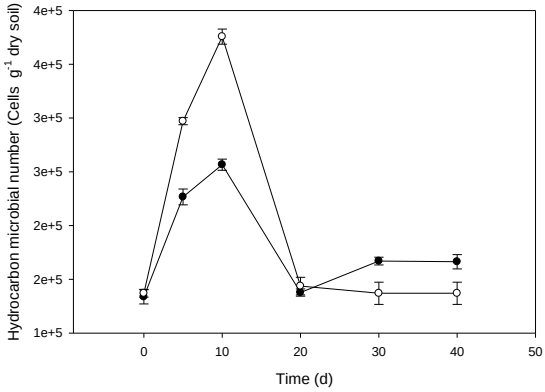

Fig. 2: The microbial counts of hydrocarbon-degrading microbes (CFU's g^{-1} dry soil) over 40 days,where (●) is the control biopile and (○) is the nematode treated biopile. Bars indicate standard errors of the mean (n=3).

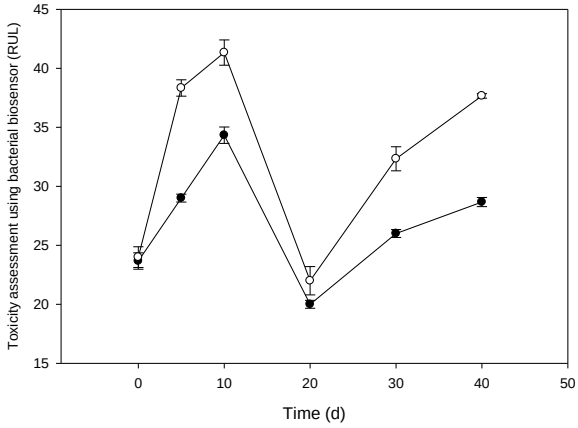

Fig. 3: Bioluminescence response for the toxicity biosensor *P. fluorescens* 10586r during hydrocarbon biodegradation,where (●) is the control biopile and (○) is the nematode treated biopile. Bars indicate standard errors of the mean (n=3).

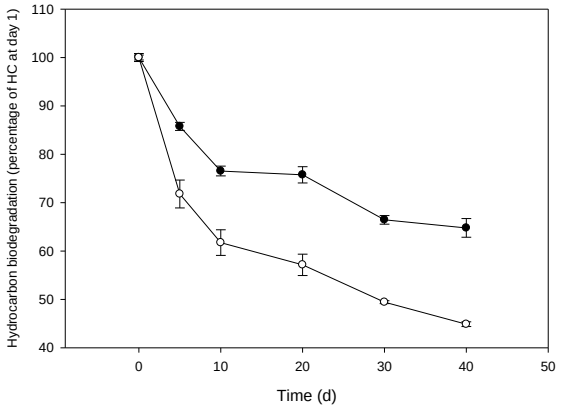

Fig. 4: Percentage changes (from an initial concentration of 39 mg HC g^{-1} of dry soil) in hydrocarbon concentrations over 40 days, where (●) is the control biopile and (○) is the nematode treated biopile. Bars indicate standard errors of the mean (n=3).

6. CONCLUSION

Both pollution.

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