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Effect of bioaugmentation of crude oil polluted tropical soils on the growth of *Panicum maximum*

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Abstract

The effect of bioaugmentation of crude oil polluted soils on the growth of *Panicum maximum* was examined in a green house study. Weathered polluted soil samples (2% w/w) in experimental pots were planted and inoculated with hydrocarbon utilizing bacterial species (*Micrococcus* sp. RZ1, *Pseudomonas* sp. RZIII, *Bacillus* sp. RZIII, and *Bacillus* sp. GSIII). Samples of unpolluted soil and polluted soil (2% w/w) were also maintained as controls. At intervals from the second to the tenth week after planting (WAP), three replicates each of the plants in soil samples with different treatments were randomly chosen, and then analyzed for plants' shoot height, plants' biomass and plants' leaf area. The results of the study showed that the inoculation of polluted soils with competent hydrocarbon utilizing species offered some advantages to growth stimulation. The shoot height, shoot weight and root weight of *P. maximum* in polluted soil bioaugmented with *Pseudomonas* sp. RZIII and *Bacillus* sp. RZIII increased significantly more than those in polluted soil samples 10 WAP. The result highlights the importance of bioaugmentation with indigenous bacterial isolates that have adapted to the environment of application.

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Introduction

As long as industrialization relies on petroleum and its allied products, the problem of petroleum pollution with its attendant ecological consequences would remain a major environmental issue.

In order to restore contaminated sites, forms of remediation available include physical, chemical and bioremediation. Bioremediation techniques harness the natural activities of microorganisms and higher organisms to degrade, transform and/or accumulate a wide range of compounds including hydrocarbons, polychlorinated biphenyls, polyaromatic hydrocarbons (PAHs), radionuclides and metals (Diaz, 2008), leading ultimately to removal of the pollutants from the environment. They have proved successful in enhancing the cleanup of pollutants in contaminated environments (Adieze *et al*., 2003; Odokuma and Dickson, 2003; Adieze, 2012). Bioremediation can be spurred on via biostimulation or bioaugmentation (Das and Chandran, 2011). Biostimulation involves the addition of fertilizers to increase the population of microbes able to utilize the contaminants within the medium. Bioaugmentation involves the addition of competent contaminant degrading microbial strains to contaminated media to enhance the resident population's ability to break down contaminants.

Microbial ability to degrade hydrocarbons appears to be a promising tool to cope with petroleum pollution. It is therefore not surprising that much research effort is channeled towards studying the dynamics of the interactions between the microbes and the pollutants that leads to environmental restoration.

It is well known that plants stimulate hydrocarbon degrading organisms in contaminated soils (Tesar *et al*., 2002). Rhizodegradation combines the physical and chemical modifications in the rhizosphere, which affect pollutants' bioavailability and stimulate microbial processes (Corgie *et al*., 2004).

The inoculation of hydrocarbon degrading bacteria into the rhizosphere of plants present in contaminated systems, ensures that the hydrocarbon contaminants are utilized by the inoculants, decreasing the plant stressors in soil, thus protecting the plants from the toxic effects of the contaminants (Siciliano *et al*., 2001). These thus increase the success of plant and inoculants introduced in contaminated sites.

Although the introduction of bacteria to contaminated media enhance the degradation of the contaminants in the media, certain species of oil-degrading bacterial inoculants will fail to enhance hydrocarbon degradation if they are unable to compete with indigenous soil populations and survive (Atlas, 1995). Thus, the survival of the plants and inoculants is a deciding factor in the rate of degradation of hydrocarbons in soil (Mishra *et al.,* 2001).

In this study, the suitability of four different bacterial species as inoculants respectively, of *P. maximum*-microbial system to overcome possible restrictions on the growth of *P. maximum* (a phytoremediator plant) in crude oil-polluted soil were investigated in a greenhouse study. The experiment was part of a larger investigation aimed at evaluating the efficiency of bioaugumentation of crude oil polluted non-rhizosphere soil, and rhizosphere soil as a bioremediation strategy for crude oil-polluted soil. The aim of this study was to assess the efficacy of four bacterial inoculatants to improve the growth of *P. maximum* in crude oil polluted soil. The results of the first part of the study have been reported in details in a previous paper (Adieze, 2012) which studied the effect of bioaugumentation on soil microbial populations and residual crude oil concentration of a polluted tropical soil. There is also the need to assess the effect of bioaugmentation on residual concentrations of PAHs in planted crude oil polluted tropical soils.

Materials and methods

Collection of samples

Soil samples are uncontaminated surface soil (0 – 20 cm) from a fallow patch of land within the National Root Crops Research Institute Complex, Umudike. The samples were collected and taken to the laboratory following procedures described in Adieze (2012).

Seedlings of *P. maximum*, were obtained from those growing in uncontaminated soil in the wild within the National Root Crops Research Institute Complex, Umudike. They were transplanted into uncontaminated soil (150 g) in thin membrane polyethylene bags, watered and grown under about 90% shading for 24 hours before transplanting into experimental pots containing 2% (w/w) crude oil polluted soil samples.

Microbial inocula used (*Micrococcus* sp. RZ1, *Pseudomonas* sp. RZIII, *Pseudomonas* sp. GSIII, *Bacillus* sp. RZIII, and *Bacillus* sp. GSIII) were hydrocarbon utilizing bacterial species isolated and stored on agar slants as described in Adieze (2012).

Standardisation of microbial inocula and adaptation in crude oil

A loopful of each of the bacterial isolate was inoculated into 50 ml nutrient broth contained in 250 ml conical flasks and incubated in a shaker (150 rpm) at 30 °C for 24h. The cells were harvested by centrifugation at 10,000 g for 20 mins. The cell pellets collected were washed twice in 20 ml of sterile tap water. Washed cells were re-suspended in 10 ml sterile normal saline (0.85% NaCl) in 20 ml sterile test tubes (Adieze, 2012).

To standardize the bacterial inocula in normal saline, the optical density (OD) readings of different dilutions of bacteria species suspension were determined using a colorimeter set at 660 nm. The numbers of viable organisms per ml of the different dilutions of the bacterial species suspensions were determined by plating out 0.1 ml aliquots on nutrient agar (duplicate plates for each dilution). A standard curve was then drawn matching specific OD readings to specific numbers of viable organisms (Adieze, 2012). Inocula used were standardised by adjusting their cell suspensions to an optical density of 0.40 at 660 nm. This corresponded to between 10^7 and 10^8 cfu/ml based on a standard curve of the numbers of viable organisms per millilitre of cell suspension earlier plotted.

Bacterial isolates were then assayed for adaptation to crude oil by sub culturing a standardised suspension of each of the isolates into sterile 190 ml mineral salt broth supplemented with 1% Bonny light crude oil contained in 500 ml conical flask. The mineral salt broth was composed of g/l: NaCl, 10.0; $MgSO_4.7H_2O$, 0.42; KCl, 0.29; KH_2PO_4, 0.8; K_2HPO_4, 1.25 and $NaNO_3$, 0.42 per litre of deionized water (Okpokwasili and Amanchukwu, 1988). This culture was incubated on a rotary shaker (150 rpm) at 28±2°C for five days (Odokuma and Dickson, 2003). The pH of the cultures were monitored and maintained at between 7 – 7.2 by adjusting the culture with standard phosphate buffer (APHA, 1985). The five hydrocarbon utilizing bacterial species (*Micrococcus* sp. RZ1, *Pseudomonas* sp. RZIII, *Pseudomonas* sp. GSIII, *Bacillus* sp. RZIII, and *Bacillus* sp. GSIII) adapted and grew in mineral salt broth supplemented with 1% Bonny light crude oil. These five isolates and their consortium were used in this study.

Soil preparation

Artificial pollution of the soil was done by mixing crude oil and acetone (3:1), prior to mixing with 10% of the total soil (Adieze *et al.*, 2012). The crude oil laddered soil was then added to the bulk of the soil and the mixture homogenised to obtain the final concentration of 2% w/w crude oil in soil. The crude oil-polluted soil was stirred several times for 2 days to remove the acetone (Banks *et al.,* 2000).

Planting of P. maximum

Eight hundred grams of 2% (w/w) crude oil polluted soil were weighed into seven black cylindrical plastic planting pots (8cm x 20cm). Thereafter, soil samples were moistened with tap water (80% water holding

capacity) and allowed to undergo weathering for six weeks in a greenhouse.

P. maximum's seedlings obtained and propagated in uncontaminated soil were then transplanted into the planting pots as previously described (Adieze, 2012) by embedding in the top 8 cm of the seven planting pots. Thereafter, the seedlings were grown in a greenhouse under about 90% shading for one week, followed by 55% shading for another two weeks before transferring to the green house with zero percent shading. The seedlings were moistened intermittently to avoid drying.

Survival of bacterial species in the rhizosphere of P. maximum

Inoculation of planted pots

Six holes (each 6 cm deep) were made in each planted pot. Standardised suspension of each of the five hydrocarbon utilizing bacterial species and their consortium (20 ml each) was then inoculated each into a respective planted pot by spray irrigation. Six planted pots were thus inoculated. The seventh planted pot was left as a control.

Assay for survival of inoculated bacterial species

Inoculated bacterial species were assayed for survival in the rhizosphere of *P. maximum* by comparing populations of hydrocarbon utilizers in the rhizosphere of inoculated planted pots with that in the un-inoculated planted control pot. This was estimated on the day of inoculation and two weeks after by plate count. Inoculants' that maintained in rhizosphere soils population greater than or equal to 40% of their initial population were selected (Adieze, 2012) and used for bioaugmentation studies of vegetated polluted soil samples.

Experimental Set up and inoculation of planted soil microcosm

Eight hundred grams (800 g) each of weathered crude oil polluted soil (2% w/w) were placed in plastic planting pots. These constituted the polluted pots. A total of 60 polluted pots were set up. The pots were then planted with *P. maximum* following the procedure described in Adieze *et al.* (2012). Of the 60 planted polluted pots, 15 pots each were inoculated with each of the four (4) different selected bacterial inoculants following procedures described in Adieze (2012). Briefly, standardized inoculum in sterile distilled water was introduced into holes made on the surface of the planted crude oil polluted soils.

Pots of planted unpolluted and planted polluted soil samples (15 each) were also set up and maintained as controls. Control treatments were treated as the experimented units except that inoculation was by sterile distilled water alone.

Six sets of 15 plastic planting pots each were thus set up. These are:

Set 1: Pots of vegetated unpolluted soil.

Set 2: Pots of vegetated polluted soil.

Set 3: Pots of vegetated polluted soil + *Micrococcus sp*. RZ1

Set 4: Pots of vegetated polluted soil + *Pseudomonas* sp. RZIII

Set 5: Pots of vegetated polluted soil + *Bacillus* sp. RZIII

Set 6: Pots of vegetated polluted soil + *Bacillus* sp. GSIII.

After bioaugmentation, the pots were transferred to the green house with approximately 12h day light and incubated for ten weeks following procedures described in Adieze, *et al.* (2012).

Sample analysis

At intervals from the second week to the tenth week of incubation, three replicate pots of each of the various treatments chosen randomly were destructively sampled. The plant samples obtained were analysed for plants' growth indices. Plants' shoot height and biomass were measured as described in Adieze, *et al.* (2012). The plants' leaf area was obtained by determining the product of the length and width of the leaf measured at its broadest portion (Amadi and Bari, 1992). The plants' shoot height and

leaf area were determined at 4, 6, 8 and 10 weeks after planting (WAP), while the plant's biomass was determined on the 4th and 10th WAP.

Results

Survival of Inoculated bacterial species in the rhizosphere of P. maximum.

The result of the population of bacterial species introduced into and recovered from the rhizosphere of *P. maximum* in 2% w/w oil polluted soil samples two weeks after inoculation is presented in Table I.

Of the six bacteria species inoculated into the rhizosphere of *P. maximum* in polluted soil, four (*Micrococcus* sp. RZ1, *Pseudomonas* sp. RZIII, *Bacillus* sp. RZIII, and *Bacillus* sp. GSIII) maintained a population 40% and above (of the initial hydrocarbon utilizing bacterial population) 2 weeks after inoculation (Table I). The consortium of the five bacteria species showed the poorest population recovered (19.2% of their initial population). It was followed by the *Pseudomonas* sp. GSIII (31.7%). However, *Bacillus* sp. RZIII had the best percentage recovery (53.8%).

The growth response of P. maximum in bioaugmented crude oil polluted soil samples The results of the *P. maximum* shoots' height response to crude oil polluted soil and its bioaugmentation are shown in Figure 1.

Polluted bioaugmented soil samples stimulated more average shoot heights between 8 and 10 WAP than the polluted control samples and the unpolluted control samples. At 10 WAP, shoot heights of polluted bioaugmented samples ranged from 111.7±0.4 cm to 159±5.6 cm as against a range of 100.6±12.2 cm and 106.5±4.9 cm in the unpolluted control soil sample and polluted control sample respectively. Polluted soil bioaugmented with *Pseudomonas* sp. RZIII had the best growth stimulation 10 WAP with a shoot height yield of 159±5.6 cm.

Table 1. Population of bacterial species (cfu/g soil) introduced and recovered from the rhizosphere of *P. maximum* after two weeks.

Bacterial Species	Number introduced	Number recovered	% recovered
Micrococcus sp. RZ1	7.0×10^7	3.3×10^7	47.1
Pseudomonas sp. RZIII	1.3×10^8	6.7×10^7	51.5
Pseudomonas sp. GSIII	8.8×10^7	27.9×10^6	31.7
Bacillus sp. RZIII	8.0×10^7	4.3×10^7	53.8
Bacillus sp. GSIII	4.7×10^7	2.2×10^7	46.8
Consortium of the five isolates	5.3×10^7	10.2×10^6	19.2

Results of the *P. maximum* shoots' weight response to crude oil polluted soil and its bioaugmentation are shown in Figure 2. The results showed that at 10 WAP, polluted soil samples bioaugmented with *Pseudomonas* sp. RZIII had an average shoot weight of 10.46±0.9 g as against 5.87±0.6 g and 8.08±0.8 g in polluted control and unpolluted control soil samples respectively. Other polluted bioaugmented soil samples stimulated shoots' weight ranging from 8.52±0.8 g to 9.10±0.02 g.

The results of the *P. maximum* roots' weight response to crude oil pollution and bioaugmentation are shown in Figure 3. The results of the *P. maximum* roots' weight response to crude oil pollution and bioaugmentation 10 WAP shows that plants in polluted control soil samples had 108% weight increase from 0.99±0.02 g at 4 WAP to 2.06±0.21 g 10 WAP, as against 418.5% and 552.9% increase in weights recorded respectively in unpolluted soil and polluted soil samples bioaugmented with *Pseudomonas* sp.RZIII.

The results of the *P. maximum* leave areas' response to crude oil pollution and bioaugmentation are shown in Figure 4.

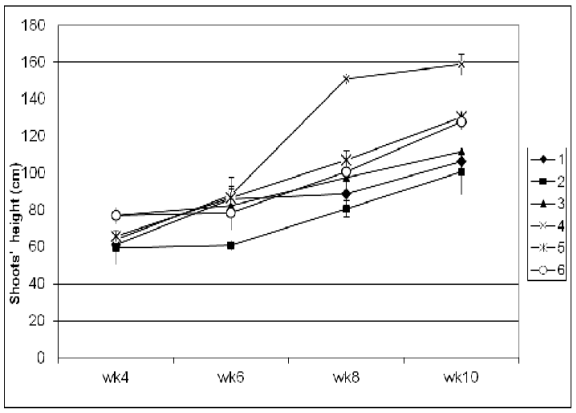

Fig. 1. *P. maximum* shoots' response to crude oil pollution and bioaugmentation.

Fig. 1. Legend: 1-Unpolluted soil, 2- Polluted control soil, 3- Polluted soil bioaugmented with *Micrococcus* sp. RZI, 4- Polluted soil bioaugmented with *Pseudomonas* sp. RZIII,5- Polluted soil bioaugmented with *Bacillus* sp. RZIII, 6- Polluted soil bioaugmented with *Bacillus* sp. GSIII.

The unpolluted soil samples had the best leaf areas throughout the study. It was followed by polluted bioaugmented soil samples. At 10 WAP, the soil samples had leaf areas' of 192.3±4.6 cm^2, 185.5±6.8 cm^2, 175±2.5 cm^2, 172.5±1.5 cm^2, 170.5±2.5 cm^2 and 147±5.0 cm^2, respectively for unpolluted soil, polluted soil bioaugmented with *Bacillus* sp. RZIII, polluted soil bioaugmented with *Pseudomonas* sp.RZIII, polluted soil bioaugmented with *Micrococcus* sp.RZI, polluted soil bioaugmented with *Bacillus* sp.GSIII, and polluted control soil.

Discussion

Due to the toxicity of petroleum hydrocarbons in soils, and their potential to cause far reaching environmental and health impacts, there is a need for quick removal of these pollutants from the soil and for the restoration of the soil health. In this study, bacterial inoculation of the rhizospheres of *P. maximum* was assessed for improvement of the plant's growth in aged petroleum contaminated soils.

The assessment of the bacterial inoculants for survival in the plants' rhizosphere revealed poor percentage recovery in the inoculum composed of a consortium of five bacteria species. This could be as a result of poor survival of the consortium. Microorganisms used for soil inoculation however need to survive in the environment and be tolerant of the soil conditions at a contaminated site. Once the microorganisms are in a soil system, they need to persist over long periods of time to allow for sufficient levels of degradation. The survival of the plant and inoculants is a deciding factor in the rate of degradation of hydrocarbons (Mishra *et al.,* 2001).

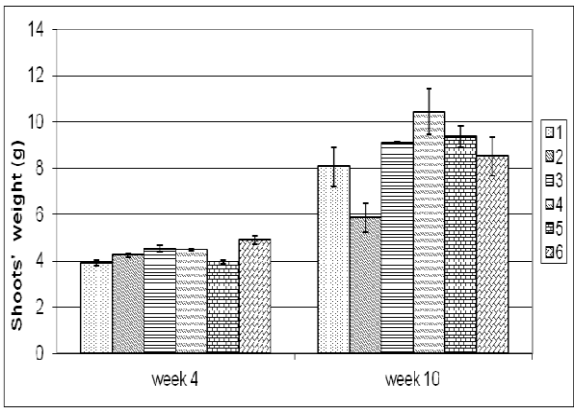

Fig. 2. *P. maximum* shoots' weight (g) response to crude oil pollution and bioaugmentation.

Fig. 2. Legend: 1-Unpolluted soil, 2- Polluted control soil, 3-Polluted soil bioaugmented with *Micrococcus* sp.RZI, 4-Polluted soil bioaugmented with *Pseudomonas* sp.RZIII, 5- Polluted soil bioaugmented with *Bacillus* sp. RZIII, 6- Polluted soil bioaugmented with *Bacillus* sp.GSIII.

The observed poor recovery above could be as a result of antagonism between the various species resulting from competition for nutrient and space. It may also be due to the production of antimicrobial agents by some isolates. Nweke *et al.* (2006) also observed that a pure culture of *Bacillus* sp. K9 utilized kerosene better than a consortium of *Bacillus* sp. C4, *Bacillus* sp. K9 and *Flavobacterium* sp.C11. It is noteworthy that although the individual microbes are from the same area and must have adapted to the temperature and soil type, the microenvironment from which they were isolated differs. This result highlights the need to assay the ability of a consortium to produce a required synergy before its use in field.

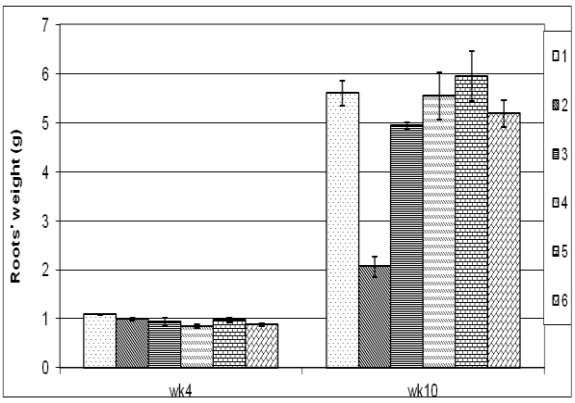

Fig. 3. *P. maximum* roots' weight (g) response to crude oil pollution and bioaugmentation.

Fig. 3. Legend: 1-Unpolluted soil, 2- Polluted control soil, 3-Polluted soil bioaugmented with *Micrococcus* sp.RZI, 4- Polluted soil bioaugmented with *Pseudomonas* sp.RZIII,5- Polluted soil bioaugmented with *Bacillus* sp. RZIII, 6- Polluted soil bioaugmented with *Bacillus* sp.GSIII.

Reduction in plant growth parameters (shoot height, biomass and leaf area) were observed in all the polluted control soils compared to the unpolluted control soils. This has also been reported by other studies; Adieze *et al.* (2012) reported reduced plants' height and biomass production with increase in percentage crude oil in planted soil. Merkl *et al.* (2004) observed a significant shoot length reduction in the presence of 3 and 5% crude oil. Liste and Felgentreu (2006) also reported 38.9% and 52.6% reduction in shoot and root biomass respectively of ryegrass in 1517 mg/kg TPHs contaminated soil over a 95 day period. The authors attributed inhibition of plant growth to such factors as toxic compounds in petroleum hydrocarbons especially low molecular weight hydrocarbons, physical barrier created by oil around plant roots, preventing oxygen, water and nutrients from getting to plant roots and immobilization of nitrogen. The inoculation of contaminated samples with competent hydrocarbon utilizing cells offered some advantages to growth stimulation. Shoot heights of *P. maximum* in polluted soil samples that were inoculated with hydrocarbon utilizing species had better growth than the un-inoculated plants and plants growing in unpolluted soil samples. Improved plant growth in this study could be as a result of increased degradation or removal of crude oil in polluted soils inoculated with indigenous hydrocarbon utilizing bacterial species. Crude oil-degrading bacteria such as *Pseudomonas, Micrococcus* and *Bacillus* sp. could metabolise the toxic components of crude oil leading to its degradation (Onwurah, 2003). Bentos *et al.* (2003) also observed 73 to 75% degradation of light and heavy fractions of TPH in soil with addition of pre-selected microbial consortium. They concluded that the best bioaugmentation performance can be approached by the selection and increasing of microbial species already present in the soil, this the observed reduced clean up time substantially.

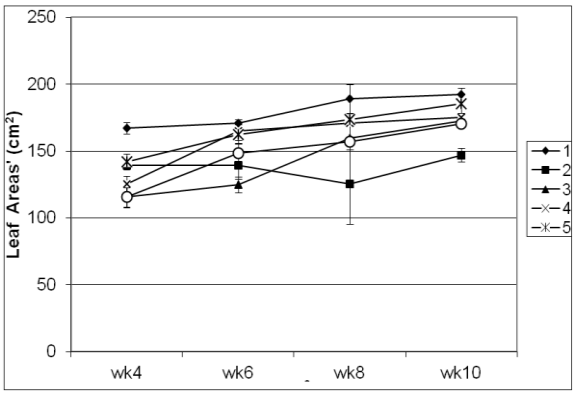

Fig. 4. *P. maximum* leaf areas' (cm^2) response to crude oil pollution and bioaugmentation.

Fig. 4. Legend: 1-Unpolluted soil, 2- Polluted control, 3-Polluted soil bioaugmented with *Micrococcus* sp.RZI, 4- Polluted soil bioaugmented with *Pseudomonas* sp.RZIII,5- Polluted soil bioaugmented with *Bacillus* sp. RZIII, 6- Polluted soil bioaugmented with *Bacillus* sp.GSIII.

The species used in this study may have some growth promoting attributes. Plant growth promoting species can influence plant growth directly through the production of phytohormones and indirectly through N_2 fixation and production of bio-control agents against soil-borne phytopathogens (Singh and Gaur, 1995; Glick and Pasternak, 2003). Glick (2003) also stated that plant growth promoting bacterial species increased plant's survival in heavily polluted soil, and

promoted plant root growth, thus that they can be used to facilitate the growth of plants used in phytoremediation. It could also be that the association of the plant and the microbes is mutual, thus enhancing plants growth.

Also the effect of the inoculants on the *P. maximum* shoots' and roots' biomass development in polluted soil showed that although bioaugmentation of the soil samples had a positive effect on both shoot and root biomass development, overtime the effects of the different inoculants species varied. The shoot had its best response from bioaugmentation with *Pseudomonas* sp.RZIII while the root had its best growth from bioaugmentation from *Bacillus* sp. RZIII. The response of *P. maximum* leaf areas' to bioaugmentation also showed that the inoculants stimulated increase in leaf area over that in polluted control samples. However, the best growth in leaf area was observed in the unpolluted control soil samples.

The results of this study, highlights the importance of bioaugmentation with indigenous microbial species that are well adapted to the microenvironment of application. It also shows the need to assay the ability of a consortium to produce a required synergy before its use in field.

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