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# Dynamics and Hydrocarbon Degrading Potential of Microorganisms Associated With Long-Term Polluted Site in South-South Nigeria

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## ABSTRACT

The dynamics and hydrocarbon degrading potential of microorganisms associated with long-term polluted soils near automobile workshops in Uyo metropolis was evaluated using standard microbiological techniques. The result indicates total heterotrophic and hydrocarbon utilizing bacterial counts of 5.74 and 4.92 $Log_{10}$CFU/g, whereas heterotrophic mould and yeast counts were 4.06 and 5.19 $log_{10}$CFU/g respectively. Heterotrophic microbial abundance was higher than the hydrocarbon degraders and the difference was significant at p = 0.05. Hydrocarbon degrading bacteria identified were members of genera *Pseudomonas, Bacillus, Micrococcus, Proteus,* and fungal species *Yarrowia lipolytica, Candida tropicalis, and Mucor hiemalis. Micrococcus, Pseudomonas, Candida tropicalis* and *Candida pseudotropicalis* exhibited high degradative potential with growth diameter of 15,10, 10 and 9 mm respectively. The respiration rate in the control soil was 0.02 mg $CO_2$ g $d^{-1}$ compared to 0.24 mg$CO_2$ g $d^{-1}$ in the polluted soil. The total petroleum hydrocarbon concentration of 1.3 mg/kg in the polluted soil was 6.5 times higher than 0.2 mg/kg in the control. The heavy metal concentration which ranged from 0.1 to 3.26 mg/kg in the order Zn>V > Pb > Fe > Ni > Co were within the WHO permissible limits and caution should be taken to avoid accumulation and biomagnification in the environment. Overall, hydrocarbon exposed microorganisms, *Pseudomonas* sp. and *Candida tropicalis* exhibited competitive advantage in their degrading potentials and are good candidates to be harnessed in remediation of contaminated site.

**Keywords:** Hydrocarbon, Hydrocarbonoclastic microorganisms, Heavy metals, Degradative potential.

## Introduction

An existential concern relative to petroleum hydrocarbon is the pollution of the environment on a large scale from intentional and accidental spills (Nkanang *et al*., 2021). The high number of malfunctioning automobiles with subsequent increase in the release of harmful substances into the environment poses a great environmental concern in Nigeria. The soil in many automobile workshops in Nigeria serves as a sink for the disposal of used petroleum products such as oil, and a source of pollution to other environmental compartments which raises public health concerns (Olowu and Adebayo, 2023). The petrogenic hydrocarbon products are mobile, toxic with mutagenic and carcinogenic properties, and the consequence of their presence in the soil is profound (Zhang *et al*., 2022). As a carcinogen, petroleum hydrocarbons have been implicated in skin, lung, bladder, liver and stomach cancers, poor growth and nerve functions and infertility in exposed individuals (Vermeulen, *et al*., 2023).

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Autochthonous microorganisms in the soil are involved in the breakdown of petroleum hydrocarbon for growth, reproduction, and as a requirement to relieve physiological stress (Varjani, 2017). The microorganisms that are able to degrade hydrocarbon in the soil are diverse with varying catalytic ability. Many genera of bacteria with the potential to breakdown petroleum hydrocarbon include *Arthrobacter, Rhodococcus, Pseudomonas, Achromobacter, Acinetobacter, Pandoraea, Burkholderia, Enterobacter, Bacillus Dietzia, Staphylococcus, Mycobacterium,* and *Streptococcus (*Varjani, 2017; Xu *et al*., 2017). However, bacteria such as *Cycloclasticus, Alcanivorax, Thallassolituus, Oleispira,* and *Marinobacter* are regarded as keystone species in relation to their ability to degrade and remove of hydrocarbon in the soil (Zhang *et al.,* 2022).

Also, yeasts and mould are able to degrade and use hydrocarbons as a source of energy for growth, and the species include *Candida tropicalis*, *C. albicans*, *Debaryomyces hansenii* and *Yarrowia lipolytica* (Hassanshahian *et al.,* 2012). The extracellular enzymes released by the moulds enhance degradation of recalcitrant hydrocarbons and the upregulation of remediation process (Ramdass *et al.,* 2021), especially if the microbial consortium used were previously exposed to petroleum hydrocarbon (Briffa *et al,* 2020).

Soil pollution by indiscriminate disposal of spent engine oil (SEO) in Low- and Middle-Income Countries (LMIC) has been traced to Automobile Mechanic Workshops (AMWs). The polluted soils serve as a sink and source of microorganisms with varying potentials which are able to adapt and tolerate heavy metal and hydrocarbon stress including antibiotic resistance. (Akpoduado *et al*., 2022). Although it has been estimated that about 0.08 to 0.46 % of the total oil produced is released into the environment, which eventually pollute the waters and shorelines (Saravana and Amruta, 2013), the long-term contamination of a specific site with hydrocarbon presents a major environmental concern. Therefore, knowledge of the abundance and diversity of hydrocarbon degrading microorganisms associated with long-term polluted site is critical for effective and efficient bioremediation strategies and environmental management.

## Materials and methods

### Sample Collection

The soil samples were obtained at three different hydrocarbon polluted automobile repair workshop in Uyo mechanic village located at Obio Offot, Uyo, Akwa Ibom State (5.0199º N, 7.8867º E). Composite samples at 15 cm soil depth were collected from predetermined sites using the auger into sample bags and labelled B, C and D respectively. The control soil sample (sample A) was obtained from garden soil from University of Uyo, Nwaniba road. The samples were transported to microbiology laboratory and analysed within 2 hours.

### Isolation and Enumeration of Culturable Aerobic Bacterial and Fungal Isolates

The total heterotrophic bacteria in the samples were enumerated using the pour plate technique (Antai *et al*., 2014) on nutrient agar (NA) supplemented with 50 µg/mL of Nystatin. Sabouraud Dextrose agar (SDA) and yeast agar fortified with Streptomycin (0.5 mg/L) to inhibit bacterial contaminants was used to enumerate mould and yeast respectively. The seeded NA and SDA plates were incubated at 28 ± 2 °C for 24 hours and 5 to 7 days for bacteria and fungi respectively. Microbial enumeration was aided by the use of Quebec colony counting (560 Suntex).

### Isolation and Enumeration of Hydrocarbon Utilizing Microorganism

We used a method previously described else where (Nkanang *et al*.,2021). Briefly, four sets of 45 ml of mineral salt medium were prepared in conical flasks, sterilized by autoclaving at 121 °C for 15 minutes and allowed to cool. Thereafter, 5 g of each of the soil samples and 0.5 ml of petroleum hydrocarbon was added to the mineral salt medium in the conical

flask. The flasks were incubated in a rotary shaker (SGM-300 Gallemkamp, England) operated at 120 rpm (28 ± 2 ºC) for seven days. Total heterotrophic counts were determined by pour-plating the serially diluted sample on nutrient agar, Sabouraud Dextrose agar (SDA) and yeast agar supplemented with the respective antimicrobial agent, incubated at room temperature (28 ± 2 °C) for 24 hours for bacteria and 5-7 days for fungal isolates respectively. Visible colonies were counted and expressed as colony forming unit per gram (CFU/g) of the soil sample.

### Purification and Maintenance of Pure Culture

The discrete colonies of isolates were purified by repeated subculturing into a freshly prepared medium. The pure cultures were inoculated on agar slants and incubated appropriately and preserved in the refrigerator at 4°C for further analysis.

### Determination of Hydrocarbon Degradative Potentials

The ability of the isolates to metabolize hydrocarbon was determined using oil agar method. The degradative potential of the microbial consortium in the soil sample was determined using drop culture method and respiration rate.

### Oil agar method

Method earlier reported by Nkanang *et al.*, 2021 were employed. Mineral salt medium was prepared and sterilized by autoclaving. On cooling, 1% of sterilized crude oil was added and aseptically poured into the sterile Petri dishes and allowed to solidify at room temperature. The microbial isolates were streaked on the medium and incubated at 28 ± 2°C for 5-7 days. The ability of the isolates to breakdown the petroleum hydrocarbon was graded as +++ (strong), ++ (moderate), + (weak), while (-) indicated no growth.

### Drop Culture Method

The drop collapse method is a rapid approach to detect biosurfactants production and was determined as described by Bodour and Miller-Maier (1998) with a slight modification. Briefly, the enriched sample was subjected to centrifugation at 2000 rpm for 10 minutes and the supernatants were used. A drop of sterile petroleum hydrocarbon was placed on a clean white tile and a drop of each supernatant laid on the oil and allowed to react. The oil displacement spread diameter was measured after 40 minutes.

### Respiratory Rate

We determined the CO produced by respiration of the microbial biomass in a moist soil using a simple laboratory respirometer (Rowell,1995). Briefly, 50 g of each sample were measured and transferred into separate Erlenmeyer flasks. Ten milliliters of 0.3 M NaOH in a test tube was tied with thread and suspended in each of the conical flask. The conical flask was corked and stored in a dark room for seven days, thereafter 10 ml NaOH was transferred into a beaker using a 10 ml pipette. Ten ml of 1M $BaCl_2$ solution was added to give a precipitation. Thereafter 3 drops of phenolphthalein indicator were introduced to the solution to give a pink colour indicating an alkaline solution. This was titrated with 0.1M of HCI until the colour changes from pink to colourless. The end point of the acid was recorded and used to determine the amount of $CO_2$ evolved. Respiration rate of the treatments was calculated using the equation:

Respiration rate =

$$\frac{\text{milligram of } CO_2}{\text{gram of soil x Number of days}} \quad (1)$$

### Heavy Metal Analysis

One gram of the composite soil sample was placed in Teflon beaker and digested with perchloric acid and nitric acid ratio (1: 1) $HNO_3$-$HCLO_4$, followed by sulphuric acid. The mixture was heated at 200 ºC for 30 minutes. The digested sample was cooled to 28 ± 0.2ºC and made up to 50 ml with distilled water and analysed for Fe, V, Zn, Pb, Co, and Ni using Atomic Absorption Spectrophotometer (AAS model AgilentAA55) after selecting the wavelengths at which the heavy metals were determined. The

Instrument was calibrated with standard solutions prepared from analytical grade chemicals (Merck, Germany). The results are expressed as mg/kg of the net weight.

Statistical analysis: The data was subjected to the two-factor unpaired t-Test at 5% significant level to determine the effects on the soil microbial flora.

## Result and Discussion

The result of the microbial abundance (Figure 1a) revealed that the total heterotrophic bacterial count (THBC) of samples ranged from 5.60 to 5.70 $Log_{10}$CFU/g and a mean of 5.65 ± 0.18 $Log_{10}$ CFU/g compared to the control with 6.0 $Log_{10}$CFU/g. Hydrocarbon utilizing bacterial count ranged from 4.53 to 5.32 $Log_{10}$CFU/g and a mean of 4.99 ± 0.37 $Log_{10}$CFU/g compared to the control with 4.70 $Log_{10}$CFU/g. The difference was 1.05 to 1.07 higher in the control than polluted soils and significant at p = 0.05.

The heterotrophic mould count (Figure 1b) ranged from 3.69 to 4.78 $Log_{10}$CFU/g and a mean of 4.42 ± 0.80 $Log_{10}$CFU/g compared to the control with 3.0 $Log_{10}$CFU/g. The total heterotrophic yeast count ranged from 4.95 to 5.66 $Log_{10}$CFU/g and a mean of 5.23 ± 0.31 $Log_{10}$CFU/g compared to the control with 5.09 $Log_{10}$CFU/g. The hydrocarbon utilizing mould count ranged from 3.0 to 3.30 $Log_{10}$CFU/g and a mean of 3.10 ± 0.15 $Log_{10}$ CFU/g compared to the control with 2.95 $Log_{10}$CFU/g. The hydrocarbon utilizing yeast count ranged from 4.0 to 4.93 $Log_{10}$CFU/g and a mean of 4.61 ± 0.44 $Log_{10}$CFU/g compared to the control with 4.78 $Log_{10}$CFU/g. There were no

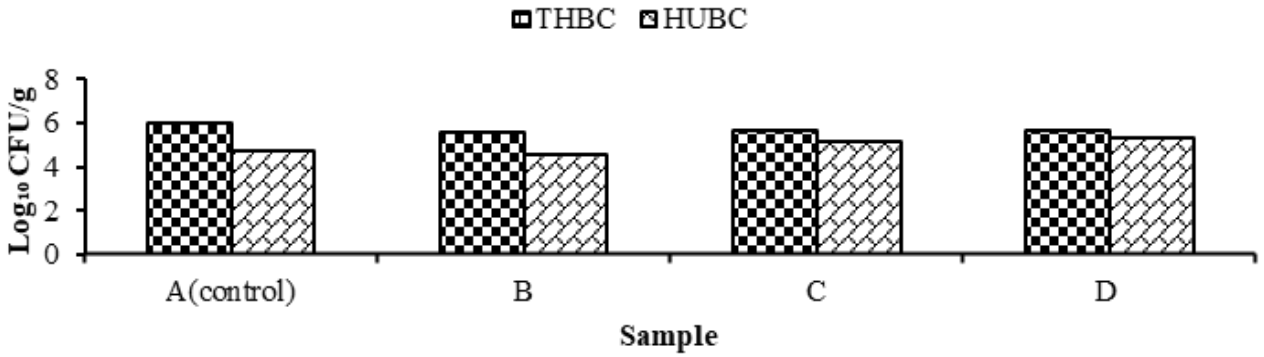


**Figure 1a.** Total heterotrophic and hydrocarbon utilizing bacterial count of the samples
**Key:** THBC = total heterotrophic bacterial count; HUBC = hydrocarbon utilizing bacterial

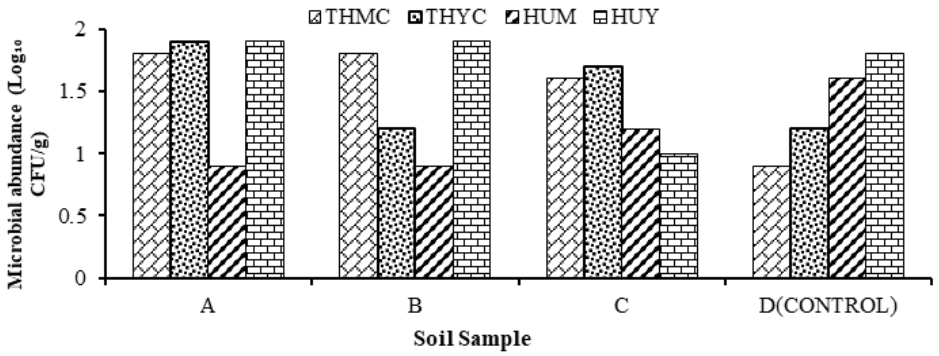


**Figure 1b:** Abundance of mould and yeast in the soil sample
**KEY: THMC**- Total heterotrophic mould count; **THYC**-Total heterotrophic mould count; **HUM**-Hydrocarbon Utilizing Mould; **HUY-** Hydrocarbon Utilizing Yeast.

significant differences ($p$ = 0.05) between the mould and yeast microbial communities.

The bacteria isolates identified were members of the genera *Flavobacterium, Proteus*, *Bacillus*, *Staphylococcus*, *Micrococcus, Lactobacillus, Corynebacterium, Pseudomonas, Arthrobacter,* and *Leuconostoc.* Mould isolates identified were *Aspergillus clavatus, Candida pseudotropicalis, Aspergillus nidulans, Candida albicans, Penicillium expansum, Sclerotorium sp., Torula sp., Penicillium sp., Aspergillus niger, Aspergillus ochraceus, Aspergillus candidus, Sacchromyces sp., Moniella sp., Mucor hiemalis* and *Candida lipolytica,* whereas the yeast were *Candida pseudotropicalis, Geotrichium sp., Yarrowia lipolytica, Candida tropicalis, Sacchromyces sp., Candida maltose, Pichia pastoris* and *Candida oleophila.*

The distribution of the bacteria in the different samples (Table 1) and the hydrocarbon degradative potential of bacterial and fungal isolates (Table 2) indicates that *Micrococcus sp* and *Pseudomonas* sp demonstrated a high degradative potential (+++) with growth diameter of 15 mm and 10 mm respectively. *Bacillus sp* and *Staphylococcus sp* had moderate degradative potential, whereas *Proteus sp, Flavobacterium sp, Arthrobacter sp* and *Corynebacterium sp* demonstrated weak degradative potential. There was no observed growth for *Lactobacillus sp* and *Leuconostoc sp.* Similarly, *Yarrowia lipolytica***,** *Candida tropicalis* and *Candida pseudotropicalis* had strong degradative potential (+++) with growth diameter of 8.6 ,9 and 10 mm respectively while *Candida albicans***,** *Candida maltose* and *Mucor hiemalis* showed moderate degradative potential (++).

The result of the hydrocarbon degradative potential of the microorganisms by oil displacement spread of the consortium (Figure 2) indicates a diameter 0.45 mm and 3.5 mm for sample A (control) and C respectively. However, sample A(control) evolved the least amount of CO2 while sample D evolved the highest amount of CO2. The heavy metal detected in the samples were Zn, V, Pb, Fe, Ni, and Co in the polluted sample relative to the control soil (Table 3).

There were marked differences in bacterial population from polluted and unpolluted soils possibly as a direct consequence of the effects of the petroleum hydrocarbon discharged into the soils by the automobile mechanics. There was a higher microbial count of

**Table 1.** Distribution of Bacteria Isolates in the Soil Samples

| S/N | Bacterial isolates | Sample A | Sample B | Sample C | Sample D | Occurrence (%) |
|---|---|---|---|---|---|---|
| 1 | *Lactobacillus* sp | + | + | + | + | 100 |
| 2 | *Micrococcus* sp | - | + | + | - | 50 |
| 3 | *Pseudomonas* sp | + | + | + | + | 100 |
| 4 | *Flavobacterium* | - | - | - | + | 25 |
| 5 | *Bacillus* sp | + | + | + | + | 100 |
| 6 | *Proteus* sp | + | + | + | - | 75 |
| 7 | *Arthrobacter* sp | + | - | - | - | 25 |
| 8 | *Staphylococcus* sp. | + | + | - | + | 75 |
| 9 | *Corynebacterium* sp | - | - | + | - | 25 |
| 10 | *Leuconostoc* sp | + | - | - | - | 25 |

Key: - = Absent; + = Present

**Table 2.** Hydrocarbon Degradative Potential of Bacterial and Fungal Isolates

| Isolates | Growth Diameter (mm) | Hydrocarbon Degradative Potential |
|---|---|---|
| **Bacteria** | | |
| *Lactobacillus sp.* | Nil | - |
| *Micrococcus sp.* | 15 | +++ |
| *Pseudomonas sp.* | 10 | +++ |
| *Flavobacterium sp.* | 3 | + |
| *Bacillus sp.* | 5 | ++ |
| *Proteus sp.* | 3 | + |
| *Arthrobacter sp.* | 2 | + |
| *Staphylococcus sp.* | 5 | ++ |
| *Corynebacterium sp.* | 2 | + |
| *Leuconostoc sp.* | 0 | - |
| **Fungi** | | |
| *Yarrowia lipolytica* | 8.6 | +++ |
| *Candida tropicalis* | 10 | +++ |
| *Candida albicans* | 7 | ++ |
| *Candida pseudotropicalis* | 9 | +++ |
| *Candida maltose* | 5.4 | ++ |
| *Candida oleophilia* | 2.6 | + |
| *Pichia pastoris* | 2.5 | + |
| *Mucor hiemalis* | 6.9 | ++ |

Key; +++ = strong degrader (8 – 17 mm); ++ = moderate degrader (3.5 -7.4)
+ = weak degrader (1 -3 mm); -- = no growth

hydrocarbon utilizing bacteria from the mechanic workshop soil in relation to the control. The result corroborates with another study (Ndubuisi-Nnaji *et al*., 2015) in which hydrocarbon degraders are usually present in large numbers in many oils polluted soil and water compared to pristine environments. The result suggests that these organisms were able to effectively use hydrocarbon to generate energy (Nkanang *et al.,*2017 and Omonigho *et al*., 2017), adapt and tolerate the inhibitory effect of the oil component. This was consistent with another report that soils polluted with petrol usually have increased population of hydrocarbonoclastic mould and yeast (Agatha 2021). The *high oil displacement spread of 3.5 mm (sample C) and the high respiration rate of 0.24 mg/$CO_2$/g (sample D) reveals a high hydrocarbon degradative potential of the microbial consortium in the samples. Samples C and D harbored species with high potential for biosurfactant production which increased the soluble content and bioavailability of*

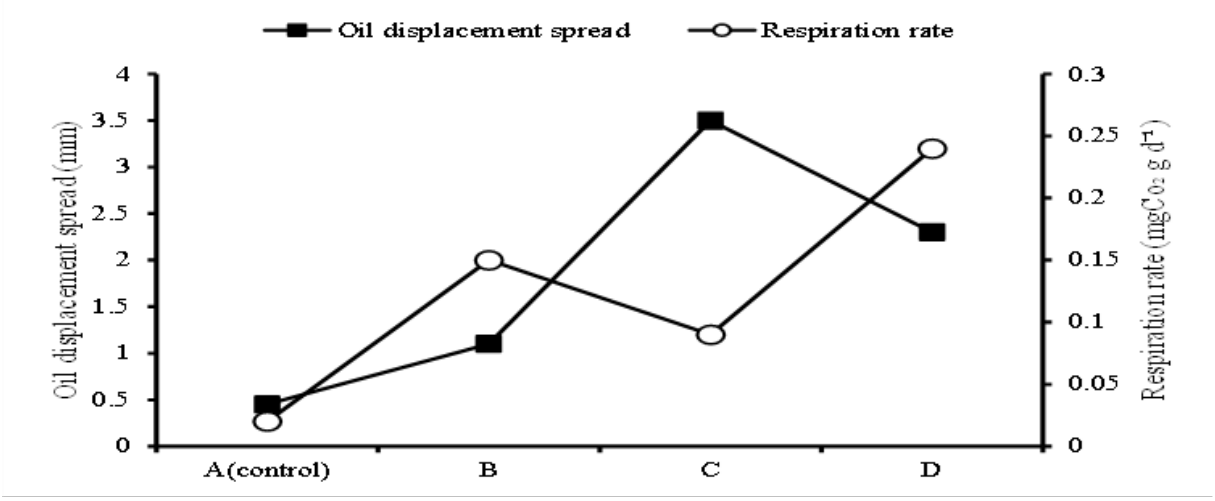


**Figure 2.** Hydrocarbon Degradative Potential of the Consortium

**Table 3:** Heavy Metal Analysis of the Samples

| | | | |
|---|---|---|---|
| Fe | 1.03 | 1.46 | 6 |
| V | 1.54 | 1.76 | 100 |
| Zn | 2.1 | 3.26 | 300 |
| Pb | 1.04 | 2.08 | 100 |
| Co | 0.08 | 0.1 | 10 |
| Ni | 0.22 | 0.32 | 35 |

**Key:** Fe is Iron, V is Vanadium, Zn is Zinc, Pb is Lead, Co is Cobalt, N is Nickel.

*the pollutants and improve degradation.*

*The microbial consortium produces crude enzymes, an efficient degradative factor accounting for the high degradation. Mixed microbial consortia are known to exhibit good performance in substrate tolerance and enhanced pollutant degradation (Kangaroo, 2021). T*he adaptation of yeast isolates to hydrocarbon pollutants is linked to the yeast's natural ability to thrive in diverse environment. The result agrees with another study (Gargouri, *et al*.,2019) in which *Candida tropicalis* and *Trichosporon asahii* efficiently degraded and removed petroleum hydrocarbon from the soil.

The concentrations of heavy metal in the polluted soil were higher than the unpolluted sample. The polluted soil samples contained the following heavy metals descending order: Zn > V > Pb > Fe > Ni > Co which were within permissible limits (WHO, 2021).

Zinc (Zn) showed exceptionally high concentration in the polluted sample. Presence of high concentration of metal ions contributes to greater electrical conductivity in the contaminated soil (Pathan 2011). High level of electrical conductivity indicates a high concentration of dissolved salts ions in the soil samples, an effective measure for assessing the contamination.

## Conclusion

In summary, there were empirical differences in bacterial and fungal populations between soils collected within operational mechanic workshops and unpolluted soil. These variations in microbial populations and the abundance of isolated genera are directly attributed to the continuous introduction of petroleum hydrocarbons into the workshop soils by

automobile mechanics. The study area with unique mechanic workshops, characterized by open spaces where various petroleum by-products including used engine lubricating oil, diesel, and petrol (PMS), are indiscriminately released during repair and maintenance services, have a profound impact on the composition of the soil microbiota. Soils within mechanic workshops, which are exposed to petroleum hydrocarbon pollutants, exhibit a higher prevalence of microorganisms that are able to metabolize them. The ability of bacterial and fungal populations to breakdown complex organic and harmful matter into simple and/or harmless compounds and assimilate them as food has made microbial application useful in the science of environmental restoration. Also, the selective advantage for hydrocarbon-utilizing microorganisms within oil-polluted environments has significant implications for bioremediation efforts in ecosystems affected by crude oil spills.

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