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EVALUATION OF MICROFLORA IN PETROL CONTAMINATED SOIL

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ABSTRACT
The present paper intends to know the infertility of soil caused by hydrocarbon contamination. To understand this, soil sample was taken in three different pots and was polluted with 0 mL, 50 mL, and 100 mL of petrol. The pot with 0 mL petrol served as control. Quantitative analysis like checking the bacterial counts and qualitative analysis, like biochemical tests was done on day 10 and day 20 after incubation with the fuel. The bacterial count decreased initially during the incubation period but gradually increased as the incubation period progressed to day 20. The bacterial population in control sample was positive for nitrogen fixation test and did not show any positive result for phosphate solubilization. The bacterial population in petrol treated soil samples was negative for both nitrogen fixation test and phosphate solubilization test which showed that those microorganisms which were present in fertile soil were absent here. The samples which were incubated for longer period of time, though gave negative results for biochemical analysis, showed majority of gram-positive cocci population, which are known to be one of the potential degraders of hydrocarbon (Toledo *et al.,* 2006).

INTRODUCTION

Soil is essentially a natural body of mineral and organic constituents produced by solid material recycling during a myriad of complex process of solid crust modifications, which are closely related to the hydrologic cycle (Ibrahim, 2004). It is the interface at which all the forces, acting on the earth's crust meet to produce a medium unconsolidated material that acts as an environment for further changes and developments keeping pace with the evolution of global earth system as a whole. It offers shelter and habitat for countless number of organisms and provides incubation and a living medium for plants, while perfectly playing its role in the universal cycle of material flow between four main geospheres (atmosphere, lithosphere, hydrosphere, and biosphere). The soil microbial community (Tuhá ková *et al.,* 2001) is impacted by proximity to busy highways, with the number of hydrocarbon degraders showing clear response within a few meters of the road side.

Soil pollution is caused by means other than the direct addition of xenobiotic compounds such as agricultural runoff waters, industrial waste materials, acidic precipitates, and radioactive fallout. Both organic and inorganic contaminants are important in soil. The most prominent chemical groups of organic contaminants are fuel hydrocarbons, Polynuclear Aromatic Hydrocarbons (PAHs), Polychlorinated Biphenyls (PCBs), chlorinated aromatic compounds, detergents, and pesticides. Inorganic species include nitrates, phosphates and heavy metals such as cadmium, chromium and lead; inorganic acids; and radio nuclides (radioactive substances) (Yaron *et al.,* 1996). PAHs constitute a class of organic compounds containing two or more fused benzene rings in linear, angular, and cluster arrangements. The environmental fate of these ubiquitous contaminants is of concern because of the mutagenicity, ecotoxicity, and carcinogenic potential of high molar mass PAHs (Papa, *et al.,* 2008). Most of the research on the degradative biochemical pathways, the genes involved in PAH metabolism and genetic regulation has been on *Pseudomonas, Sphingomonas, Burkholderia* and *Comamonas* strains (Ramos, 2004). PAHs enter the air mostly as releases from volcanoes, forest fires, burning coal, and automobile exhaust. Altered number of bacteria growing in petroleum hydrocarbon contaminated (PHC) soil has also been documented (Hans-Holger and Felgentreu, 2006). The principal aim of this study was to evaluate the microflora in petrol contaminated soil.

MATERIALS AND METHODS

The soil sample was collected from an area where the plants were well grown. The sample was taken from the area N. R. Colony, Bangalore. Around 15cm soil was dug and was transferred to polythene covers. 3Kg of soil was transferred to three different pots each and they were named as Pot A, Pot B, and Pot C respectively. Pot A was maintained as control sample. Pot B and Pot C were treated with 50 mL and 100 mL of petrol respectively. The pots were incubated under normal sunlight and temperature conditions. Treated samples from Pot B and C were taken for analysis on day 10 and day 20.

Microbiological analysis was done at 10 -day and 20-day interval after addition of petrol. Accordingly, A10, B10 and C10 samples referred to control sample, sample from pot B on day 10, and sample from pot C on day 10 respectively. A similar designation followed to samples drawn on day 20. The analysis was done in two phases, quantitatively and

qualitatively.

Quantitative analysis: One g of soil from each of the pots was added to 10mL sterile distilled water and serially diluted. Dilutions 10^{-3}, 10^{-4} and 10^{-5} were selected to obtain countable number of colonies. 1mL of each of above dilutions was plated on nutrient agar plates by pour plate method in triplicates. Incubation was done at 38ºC for 48 hrs. The readings of these plates are depicted in Table 1, 2, 3, 4, and 5.

The dilution 10^{-4} was chosen as representative plate since the colonies were distinct and most of the colony types seen in the other two dilutions were also seen in the chosen plate.

Table 1: Readings of sample A

Dilution	Number of bacterial colonies in the sample			Average
10^{-3}	115	99	121	111.6
10^{-4}	42	31	26	33
10^{-5}	1	1	0	0.66

Table 2: Readings of sample B10

Dilution	Number of bacterial colonies in the sample			Average
10^{-3}	112	160	170	147.3
10^{-4}	15	23	17	18.33
10^{-5}	6	2	7	5

Table 3: Readings of sample C10

Dilution	Number of bacterial colonies in the sample			Average
10^{-3}	50	23	35	36
10^{-4}	10	20	19	21.66
10^{-5}	2	3	5	3.33

Table 4: Readings of sample B20

Dilution	Number of bacterial colonies in the sample			Average
10^{-3}	23	49	31	34.33
10^{-4}	18	20	13	17
10^{-5}	0	0	0	0

Table 5: Readings of sample C20

Dilution	Number of bacterial colonies in the sample			Average
10^{-3}	90	60	88	79.33
10^{-4}	28	26	36	30
10^{-5}	0	0	0	0

The population of bacteria per gram of the sample was tabulated as shown in the Table 6.

Table 6: Colony forming units of representative plates

Samples	Bacterial population represented as: 10^3 x cfu/g of soil sample
A	33.00
B10	18.33
B20	21.66
C10	17.00
C20	30.00

In qualitative analysis, colony morphology, pigmentation of the colonies, gram reaction and biochemical activities were studied. The colony characters were tabulated for each of the plates as shown in the Table 7.

Similar tabulation was done for the samples B10, C10, B20, and C20. Evaluation of each of the isolates from all the representative plates was done for nitrogen fixing ability and phosphate solubilising capacity.

Nitrogen fixing ability: The stock culture (16hr old culture stored at 4ºC) was diluted 10 times by taking 1mL of stock culture in 10mL of nutrient broth. 100 mL of each of the strain was inoculated in Norris Glucose Nitrogen free agar plates by spread plate method. The plates were incubated at 37ºC for 24 hrs, after which readings were taken. Plates showing growth on the media indicate positive result and those showing no growth indicate negative result.

Phosphate solubility test: Inoculation of the strains on Pikovskaya's Agar was done similar to nitrogen fixing test. The plates were incubated at 37ºC 24 – 48 hrs. The readings were taken after 48 hrs. Plates showing growth and dissolution of media indicated positive result while plates showing only growth and no dissolution of media indicated negative result.

RESULTS AND DISCUSSION

The results of the above treatments of the soil sample can be studied under various categories as follows:

Bacterial Enumeration - The population of bacteria in the representative dilution (10^{-3}) was studied as number of colony forming units (cfu) per gram of the soil.

Bacterial Characterization: Characterization was done at two levels. First level involved studying the colony morphology and Gram's reaction. The second level involved testing nitrogen fixing ability and phosphate solubilizing capacity. First level

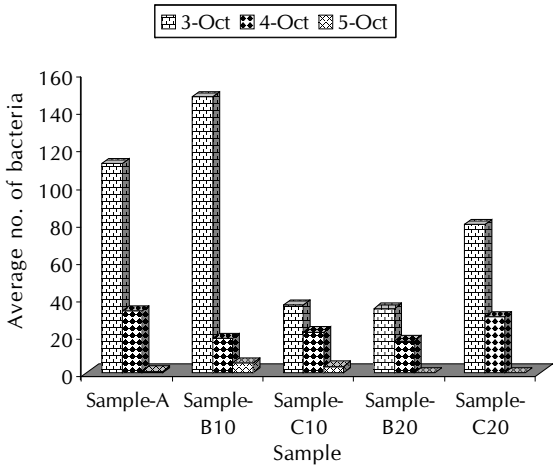

Figure 1: Depiction of average no. of bacteria from different samples

Table 7: Sample A – representative plate

Isolate No.	Edge	Form	Elevation	Surface	Transparency	Colour	Gram reaction
A1	Irregular	Curled	Flat	Smooth	Transparent	Green	Negative Rods
A2	Circular	Entire	Convex	Smooth	Opaque	Orange	Negative Rods
A3	Irregular	Curled	Convex	Smooth	Opaque	Whitish	Positive cocci
A4	Irregular	Curled	Flat	Mucoid	Opaque	Brownish	Positive cocci

Table 8: Results of biochemical tests

Isolate No.	Nitrogen Fixation test	Phosphate solubilization test
A1	Positive	Growth seen but no solubility of media
A2	Positive	Growth seen but no solubility of media
A3	Positive	Growth seen but no solubility of media
A4	Positive	Growth seen but no solubility of media
B10a	Positive	Growth seen but no solubility of media
B10b	Positive	Growth seen but no solubility of media
B20a	Positive	Growth seen but no solubility of media
B20b	Positive	Growth seen but no solubility of media
C10a	Negative	No growth
C10b	Positive	No growth
C10c	Negative	No growth
C20a	Negative	No growth
C20b	Positive	Growth seen but no solubility of media
C20c	Negative	No growth

of characterization of control sample (sample A) showed mixed population of gram negative and gram positive bacteria. Sample B showed comparatively less number of gram negative bacteria. Sample C showed predominantly gram positive population.

The second level of characterization was done on each of the isolates to check their ability to grow on a nitrogen free media and the capacity to solubilize phosphate in the media. The organisms or the isolates which grew on nitrogen free media implied that they possess the capacity to fix nitrogen. Similarly, the organism or the isolate that could grow and solubilize the phosphate component in the media implied the capacity of an organism to solubilize phosphate (presence of holo zone around the colonies indicated phosphate solubilization). The results are summarized in Table 8.

The soil activity and fertility are based on the soil microbial enzymes since the activities are mainly biochemical in nature. Most of the enzymes involved in soil functions and fertility are the oxidoreductase group involving dehydrogenases, phosphatase, and oxidase (Nwaugo *et al.,* 2007). Nitrogen fixation and phosphate solubilisation are carried out by the enzymes that belong to oxidoreductase group. A geographical region is tagged as pollution free region based on many factors of which one of them is population of flora. Plants grow well in fertile soil and fertility of soil can also be evaluated based on the presence of minerals. Nitrogen and phosphorus are two vital minerals which is essential for plant growth.

Quantitative analysis of sample A showed maximum number of colony forming units (Table 6). This could be attributed to the fertility of soil and fertile soil contains fantastic number of living microorganisms (Francis, 1951). Isolates from control sample showed positive result to nitrogen fixation test suggesting the capacity to fix nitrogen. Nitrogen fixers are one of the most essential group of bacteria which are ought to be present in fertile soil (Elmerich *et al.,* 2006). Quantitative analysis of treated samples B10 and C10 showed significant decrease in colony forming units. This could be because of the difficulty in survival and multiplication of the bacterial cells in the polluted environment with a new carbon source. Samples B and C showed more of gram positive cocci population compared to the control soil sample and research reports indicate gram positive cocci like *Micrococcus luteus* as active hydrocarbon degrader (Toledo *et al.,* 2006). Further the number of isolates, from the treated soil sample, showing nitrogen fixing ability is comparatively less than the isolates from control soil sample (Jamal Deni and Michel, 1999). Nevertheless, some bacterial isolates from samples B10 and C10 did show nitrogen fixing ability and this can be supported by literature review that some nitrogen fixers are potential hydrocarbon degraders (Ruth Eckford *et al.,* 2002). Further the samples which were incubated for longer duration (C20 and B20) showed significant increase in microbial population (Fig. 2). Research papers shows isolation of n-alkane degraders like *Nocardia, Rhodococcus* and *Gordonia* strains from hydrocarbon contaminated soil (Quatrini *et al.,* 2008). Most of the isolates from these samples gave negative results for biochemical tests (Table 8), indicating the absence or decline of the microbial population that were present in the control soil sample. This clearly indicates the decline in the fertility of the soil.

Soil being the best medium for plant growth, if contaminated with hydrocarbons becomes least supportive. One of the reasons for global warming is excessive usage of fossil fuels and one of the deleterious effects of usage of fossil fuel is infertile soil (Miller and Tayler, 2001).

The above research can be used for bioremediation programe by characterizing the isolates that survived the hydrocarbon pollution (treated soil samples).

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