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Physicochemical properties, heavy metal content and fungal characterization of an old gasoline-contaminated soil site in Anand, Gujarat, India

Shamiyan R. Khan[1], J.I. Nirmal Kumar[1]*, Rita N. Kumar[2], Jignasha G. Patel[1]

[1]Department of Environmental Science andTechnology, Institute of Science and Technology for Advanced Studies and Research (ISTAR), Vallabh Vidya Nagar -388 120, Gujarat, India
[2]Department of Biological & Environmental Sciences, N.V. Patel College of Pure & Applied Sciences, Vallabh Vidya Nagar, Gujarat, India

*Corresponding author, E-mail: arunajoshi@gmail.com

Abstract

The current investigation was aimed to study physicochemical properties, heavy metal concentration and fungal communities of top soil samples taken from an old gasoline-contaminated site and from uncontaminated garden site in Anand, Gujarat, India. The total petroleum hydrocarbon concentrations were 11 500 and 142.65 mg kg^{-1} for gasoline contaminated and uncontaminated soils, respectively. The contamination was associated with increased soil organic carbon, nitrogen concentration and clay content (2.77, 0.58 and 35.70%, compared to 1.50, 0.15 and 32.4% in the garden soil, respectively), i.e., increased organic and inorganic nutrient contents. Increased concentration of heavy metals like cadmium, copper, iron, zinc and lead in contaminated soil was evident. Nine native fungal species belonging to a total of six genera, included *Aspergillus terreus, Aspergillus versicolor, Aspergillus niger, Fusarium oxysporum, Penicillium janthinellum* from garden soil, and in addition *Aspergillus niger, Fusarium oxysporum, Cladosporium bruhnei* and *Galactomyce geotrichum* from the aged gasoline-contaminated soil were identified based on 18S rRNA. The changes created by gasoline contamination resulted in change of intenisty of some physicochemical properties, soil conductivity, pH, fungal growth and soil fertility indices.

Key words: 18S rRNA, fungal growth, gasoline contamination, heavy metals, soil physicochemical analysis, total petroleum hydrocarbons.
Abbreviations: CFU, colony forming units; TOC, total organic carbon; TOM, total organic matter; TPH, total petroleum hydrocarbons.

Introduction

Gasoline,like all fossil fuels, primarily consists of a complex mixture of molecules called hydrocarbons, including straight chain, branched, cyclic hydrocarbons, polycyclic aromatic hydrocarbons and inorganic substances. In large concentrations, petroleum products are highly toxic to many organisms, including humans (Alexander 1994). The dominance of petroleum products in the world economy creates conditions for distributing large amounts of these toxins into populated areas and ecosystems around the globe (Ojumu 2004). With continued utilization, greater quantities of gasoline are being transported over long distances. Therefore, gasoline can enter into the environment through spillage, may occur as a result of oil pipe corrosion, pipeline/flow leakage, rupture of tanks, effluents from sabotage and human errors (Chikere, Chijioke–Osuji 2006). Since petroleum contains some gaseous components, these fractions will volatilize from oil polluted soil leaving the non-volatile components as residues (Odu 1977). It has been demonstrated that oil spillage affects the physical and chemical nature of soils (Minai-Tehran, Herfatmanesh 2007). The economic and environmental impacts of oil pollution on soil are enormous, causing serious damage to vegetation, soil fertility (Nwachukwu, Ugorji 1995) and soil-borne microorganisms; the toxicity varies depending on the type of oil and additives used during refining and also on the biota of spillage (Reddy 2001).

Bioremediation of such soils involves intentional release of microorganisms to the contaminated site for clearance of the pollutants. Fungi plays an important role in removing hazardous compounds from water and soil. Sediment particles contaminated with petroleum products from spills is one of the ecological niches for fungi, which use carbon from hydrocarbons in polluted sediment particles, leading to their biodegradation. Fungi have been found to be better degraders of petroleum than those used in traditional bioremediation techniques, such as bacteria (Al-Nasrawi 2012). The size of the microbal biomass is generally considered to be important in bioremediation. The microbial biomass itself represents a considerable pool of nutrients, which is continuously diverted into growth cycles of micro- and macrophytes.

Consequently, soils that maintain a high level of

microbial biomass are capable of storing more nutrients, as well as cycling more nutrients through the ecosystem (Torstensson et al. 1998). For optimization of effective gasoline bioremediation processes, it is essential to consider environmental and biological factors affecting the process. The environmental factors include availability of nutrients, pH, soil texture and extent of gasoline contamination in the polluted soil, whereas the biological factors encompass the bacterial, fungal or algal species that are responsible for bioremediation of gasoline in the contaminated soils (Bahuguna et al. 2011). The present study was carried out to study the effect of refined petroleum hydrocarbons on physicochemical properties, heavy metal enrichment and fungal load of aged gasoline-contaminated soil as compared with garden soil. The obtained results can be further useful for standardization of in situ bioremediation as well as establishment of biodegradation protocols.

Materials and methods

Collection of soil samples

Aged gasoline-contaminated soil samples were collected from a subsurface automobile garage located in Vallabh Vidyanagar, Anand, Gujarat, India. The soil in this sample area was chronically polluted with gasoline for at least fifteen years. Uncontaminated garden soil was used as control. At both sites, 10 top and sub-soil samples were collected randomly within a 0.5-m plot and mixed into a composite representative sample with a sterile spatula into a sterile polyethylene bag and taken to the laboratory immediately. Further, stones were removed from the samples and soils were homogenized through a 2 mm sieve. The soil samples were designated as gasoline and garden, and were stored in dark bottles until further analysis. All parameters were measured in triplicate and the mean value of three measurements was used for data analysis.

Determination of soil physicochemical properties

Soil samples were analysed for particle size by the international pipette method (Gee, Bauder 1986), bulk density by metal core sampler method (Blake, Harte 1986), and porosity and moisture content according to Maiti (2003). The pH and electric conductivity of the soil samples were determined in soil/water (1:1, v/v) suspension by a pH meter and a conductivity meter (Sension 5, HACH, USA). Total organic carbon (TOC) concentration was determined with the titration method of Walkey and Black (1934). Total organic matter (TOM) concentration was quantified by multiplying TOC with 1.724 (Osuji, Nwoye 2007). Total nitrogen was measured by Kjeldahl digestion (Gerhardt, Turbotherm, Germany) and steam distillation method (Black 1965), sodium and potassium were determined using a flame photometer. Total salinity, sulphate, phosphate, magnesium and calcium were estimated using procedures of Maiti (2003). Soil fertility indices were calculated considering the N, P, K, TOC and TOM (Osuji, Nwoye 2007).

Analysis of heavy metals

For estimation of heavy metal (Cd, Pb, Cu, Zn, Co, and Fe) concentrations, 0.5 g of dried soil was digestied with concentrated HNO_3, H_2SO_4 and H_2O_2 (2:6:6) as prescribed by Nirmal Kumar et al. (2008). The blanks were run in a set, and samples were analysed with an Inductive Coupled Plasma Analyzer (Optima 3300 RL, Perkin-Elmer, USA) at Sophisticated Instrumentation Centre for Applied Research and Testing, Vallabh Vidya Nagar, Gujarat, India. The concentrations of heavy metals were expressed as mg kg^{-1}. The enrichment factor was calculated to derive the degree of soil contamination and heavy metal accumulations in soil from the contaminated site with respect to uncontaminated soil (Kisku et al. 2000).

Determination of total petroleum hydrocarbons

The soil samples were cleaned of roots, thoroughly mixed, and 2 g of each soil sample was weighed into a clean extraction container. Then 10 mL of extraction solvent (chloroform/dihloromethane, 1:1, v/v) was added into each sample, mixed thoroughly and allowed to settle. The mixtures were carefully filtered into a clean solvent extraction vessel using filter paper fitted into buchner funnels. The extracts were concentrated to 2 mL and then transferred for cleanup/separation. The concentrated aliphatic fractions were transferred into labelled vials with teflon caps for gas chromatograph analysis. The amount of TPH was measured following the USEPA Method 8015B for GC analysis with a FID detector and a HP-5 column, at the Sophisticated Instrumentation Centre for Applied Research and Testing. External calibration was conducted with original gasoline fuel.

Isolation of the indigenous fungal population

For isolation of the indigenous fungal population, 1 g of each sieved soil sample was homogenously mixed with 1 drop (approximately 0.1 mL) of Tween 80. About 1.0 g of homogenized, soil sample was aseptically transferred using a flame-sterilized steel spatula into a sterile test tube containing 9.0 mL of sterile distilled water. This gave a 10^{-1} dilution, and subsequently, three-fold (10^{-3}) serial solutions were prepared from the 10^{-1} dilution. Diluted samples (1 mL) was poured on Potato Dextrose Agar plates and Sabouraud Dextrose Agar plates. Streptomycin (500 mg L^{-1}) as an antibiotic to inhibit bacterial growth was added to media after sterilization (Harrigan, McCance 1990). Then, the plates were incubated at temperature 28 to 31 °C for 48 h or more depending on the rate of fungal growth. To obtain pure cultures of the fungal isolates, fungal cultures were aseptically subcultured into fresh plates and incubated until the fungus began to sporulate, followed by subsequent subculturing to obtain pure cultures consisting of only one

type of fungal isolate. A part of the pure culture was then aseptically transferred into sterile agar slants which had previously been prepared in sterile sugar tubes. The sugar tubes were then incubated till full growth of the fungus and they then served as stock cultures.

Estimation of total fungal population

Both the contaminated and uncontaminated soils were assessed for total fungal population using the colony forming unit (CFU) method (Lily et al. 2009). For that, 1 g of each soil sample was suspended into 10 mL of sterile distilled water and was aseptically serially diluted further up to 10-7 dilution. An aliquot of 0.1 mL from each diluted soil suspension was poured onto Potato Dextrose agar plates using the spread plate technique. Plates were incubated for 3 to 5 days at 30 °C. Results were recorded as CFU per gram of soil.

Identification of fungal isolates

Fungal genera were identified according to morphological characters and classified according to taxonomical keys published in the literature (Nelson-Smith 1973; Malloch 1997). The inoculated plates were identified on the basis of cultural (colour and colonial appearance of the fungal colony) and morphological characteristics.

Species were identified by DNA sequination method. A suitable mass of inoculum of fungal isolate was prepared carefully by removing the upper surface of the isolate, without agar medium. DNA was extracted following the technique of Al-Nasrawi (2012). Genomic DNA was isolated from fungal samples using the Chromous fungal genomic DNA isolation kit following manufacturer's protocol (Chromous Biotech, Banglore, India). Mechanical lysis was enhanced using a Talboys High Throughput Homogenizer (Troemner, Thorofare, NJ, USA) at 1600 rpm for 3 min.

DNA extracts were assessed using a Nano-drop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Fungal 18S rRNA genes were PCR-amplified using 5'-GTAGTCATATGCTTGTCTC-3' and 5'-GAAACCTTGTTACGACTT-3' primers. Reactions were performed in 100 µL volume containing 4 µL dNTPS, 400 ng of each primer, 10X Taq DNA Polymerase Assay Buffer 10 µL, 1 µL of Taq DNA Polymerase enzyme, and 1 µL template DNA. Thermo cycling conditions consisted of an initial denaturation stage of 94 °C for 5 min followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, and a final stage of 72 °C for 5 min. PCR products obtained were Gel eluted using Chromous Gel extraction kit and sent for sequencing on an ABI 3500 XL Genetic Analyzer (Applied Biosystems Inc., Foster City CA, USA). The basic local alignment search tool-BLAST was used to classify and identify closely related fungal sequences (Al-Nasrawi 2012).

Nucleotide sequence accession numbers

The nucleotide sequences reported in this paper have been submitted to the NCBI, GenBank database under the accession numbers KC545846-KC545855, and KC545868-KC545875.

Results and discussion

Physicochemical properties of gasoline-contaminated and uncontaminated soil

The total volume of petroleum products used today surpasses all other chemicals of environmental and health concern. Due to the number of facilities, individuals and processes and the various ways the products are stored and handled, environmental contamination by petroleum products is potentially widespread. In this study the total petroleum hydrocarbon (TPH) concentration found in gasoline-contaminated soil was 11 500 mg kg^{-1}, in comparison to 143 mg kg^{-1} in soil of uncontaminated garden site (Table 1). The high TPH is sa result of soil contamination with gasoline through the operation of the automobile garage on the site. These concentrations of TPH create soil conditions unsatisfactory for plants and microbial growth (Dejong 1980). The negative effect of contamination can be increased by the presence of other toxic materials, such as cresol, phenols, and chlorine which might inhibit growth of hydrocarbon-oxidizing microorganisms (Ujowundu et al.2011).

Soil texture as an important physical parameter and plays

Table 1. Physicochemical properties of uncontaminated garden soil and gasoline–contaminated soil

Parameter	Uncontaminated garden soil	Gasoline-contaminated soil
Sand (%)	42.4	37.9
Silt (%)	25.2	26.4
Clay (%)	32.4	35.7
Texture class	Clay loam	Clay loam
Moisture content (%)	18.09	13.49
Bulk density (g mL^{-1})	0.68	0.90
Porosity (%)	69.95	66.30
pH	7.2	5.9
Electrical conductivity (µS cm^{-1})	428	212
Salinity (%)	1.4	0.9
Na (mg kg^{-1})	90.3	58.0
Ca (mg kg^{-1})	33.7	13.6
Mg (mg kg^{-1})	141.3	74.7
Sulphate (mg kg^{-1})	879	1781
Total petroleum hydrocarbons (mg kg^{-1})	143	11 500

a very important role in establishment and development of microorganisms and plants. Gasoline-contaminated soil had a slightly higher clay and silt content, in compariosn to uncontaminated garden soil (Table 1). Both soils were classified as clay-loam. The higher percentage of clay and silt in the gasoline-contaminated soil caused lower soil aeration and porosity, as compared to uncontaminated garden soil, which could affect crop productivity, yield and growth (Sztompka 1999). Also, moisture content was lower for contaminated soil. The low moisture content of contaminated soils could be due to the presence of hydrocarbons and polycyclic aromatic hydrocarbons, which can cause an increase in soil hydrophobicity, leading to a decrease in the moisture holding capacity of soil (Balks et al. 2002).

A review of existing data on the Niger Delta by Osuji (2001) showed that extremely high hydrocarbon levels in soil affect both above- and belowground flora and fauna, which are essential factors in the biogeochemical cycle, as they affect availability of plant nutrients. Among soil fertility indices, the concentrations of macronutrients N, P and K in both contaminated and uncontaminated soils were low (Fig. 1),as compared to acceptable ranges of 15 000, 2 000 and 10 000 mg kg^{-1} for N, P, and K respectively, as recommended for agricultural soils (HSEENV 2004). The concentrations of extractable macronutrients P and K, as well as Ca and Mg in the gasoline-impacted soil were significantly lower than in uncontaminated garden soil. This could be due to utilization of the nutrients by resident microflora. Osuji and Nwoye (2007) suggested that it is unlikely that the oil release is directly responsible for the loss of macronutrients from soil. However, higher concentration of sulphate and nitrogen (5.8 mg kg^{-1}) in gasoline-contaminated soil, in comparison to uncontaminated soil (1.5 mg kg^{-1}), supports the findings of Ujowundu et al. (2011), who studied the biochemical and physical properties of diesel-contaminated soil in southeastern Nigeria. The increase of soil extractable nitrogen could be due to the nitrogen content of refined gasoline fuel (Slavica et al. 2003). In addition, the high amount of organic carbon and organic matter in the contaminated soil samples (Fig. 1) could be due to gasoline fuel, which is composed of hydrocarbon and polycyclic aromatic hydrocarbons (Atlas 1981).

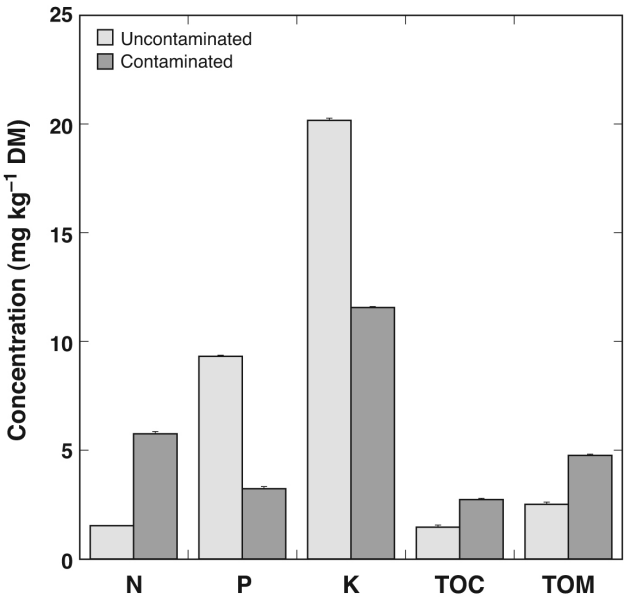

Fig. 1. Soil fertility indices in uncontaminated garden soil and gasoline-contaminated soil of Anand, Gujarat, India. TOC, total organic carbon; TOM, total organic matter. Bars indicate SE.

The contamination resulted in the soil pH (5.9) as compared to pH 7.2 in the uncontaminated soil (Table 1). The low pH may have affected fungal growth in the contaminated soil, which was observed to be low. It has been shown that optimal activity for microbial degradation occurs at pH 7.4 while considerable inhibition can be seen both at pH 4.5 and 8.5 (Verstrate et al. 1975). Similarly, the high content of TPH may have also caused the reduced fungal biomass observed in contaminated soil.

Soil electrical conductivity is a measure of soluble salt content in the soil and is used as an overall indicator of the level of macro- and micronutrients in the soil. Conductivity was estimated as 212 μS cm^{-1} in the gasoline-contaminated soil, compared to 428 μS cm^{-1} in the uncontaminated soil (Table 1). This indicates that the gasoline contaminantion affected soil structure and modified its physicochemical properties (Hawrot, Nowak 2006). The reduction in concentrations of sodium, calcium, potassium and magnesium (Table 1), which are suitable terminal electron acceptors affecting the indigenous microbial growth and

Table 2. Concentration of heavy metals (mg kg^{-1}) in gasoline-contaminated and uncontaminated garden soil in comparison to permissible limits. BDL, below detectable limit; N/A, not applicable

Heavy metal	Permissible Limits		Uncontaminated soil	Contaminated soil	Enrichment factor
	WHO/APHA	Indian standards			
Cadmium	0.3	3–6	BDL	1.3	0.00
Cobalt	N/A	N/A	12.92	24.14	1.86
Copper	150	135–270	79.08	935.22	11.82
Iron	N/A	N/A	1604.4	2545.6	1.58
Zinc	500	300–600	79.08	241.66	3.05
Lead	40	250–500	BDL	466	0.00

Table 3. Fungal taxa isolated from gasoline-contaminated (GC) and uncontaminated (UC) garden soils, identified by the length of 18S rRNA sequences by BLAST analysis

Soil	Sequence ID	Length	TOP BLAST	Similarity (%)	Class	Order	Family	Forward	Reverse
UC	SGX8	2713	*Penicillium janthinellum*	99%	Eurotiomycetes	Eurotiales	Trichocomaceae	KC545846	KC545847
	PGX9	1687	*Aspergillus niger* strain HKS11	99%	Eurotiomycetes	Eurotiales	Trichocomaceae	KC545848	KC545849
	SGX9	1714	*Fusarium oxysporum*	100%	Sordariomycetes	Hypocreales	Nectriaceae	KC545850	KC545851
	PGX10	1733	*Aspergillus terreus*	99%	Eurotiomycetes	Eurotiales	Trichocomaceae	KC545852	KC545853
	PGX11	1733	*Aspergillus versicolor*	99%	Eurotiomycetes	Eurotiales	Trichocomaceae	KC545854	KC545855
GC	PPX1	1687	*Aspergillus niger* strain HKS11	99%	Eurotiomycetes	Eurotiales	Trichocomaceae	KC545868	KC545869
	PPX2	1747	*Cladosporium bruhnei* strain USN 11	100%	Dothideomycetes	Capnodiales	Davidiellaceae	KC545870	KC545871
	SPX3	1767	*Fusarium oxysporum*	100%	Sordariomycetes	Hypocreales	Nectriaceae	KC545872	KC545873
	SPX4	1668	*Galactomyce geotrichum* strain SK15	100%	Saccharomycetes	Saccharomycetales	Dipodascaceae	KC545874	KC545875

metabolism (Ujowundu 2011). This could be the reason for the lower fungal growth in contaminated soil samples.

Heavy metals

The results of the heavy metal analysis are presented in Table 2. Gasoline-contaminated soil contained significantly higher concentrations for all analyzed heavy metals (Cd, Co, Cu, Fe, Zn, Pb) in comparison to uncontaminated garden soil. The concentration of Cd in the gasoline-contaminated soil was above the permisable limit. Fe had elevated concentration in both contaminated and uncontaminated soils. Although iron occurs naturally in groundwater, the higher concentration of iron has negative impact for human and animals. Pb concentration found in the contaminated soil also exceeded the limit. However, Cd, Cu, Zn and Pd concentrations in the uncontaminated garden soil were within the permissible limits of WHO and APHA. According to Indian standards (Awashti 2000; Sharma et al. 2006; Gupta et al. 2008) all the heavy metals, except Cu, in the gasoline-contaminated soil were within the permissible limits.

There was a significant difference in enrichment factor values between different heavy metals. Among the six metals estimated, the maximal enrichment was found in the case of Cu, and minimum in the case of Cd) and Pb (Table 2). An enrichment factor values above 1 indicates higher availability and distribution of metals in contaminated soil, thereby increasing the metal accumulation in plants species grown in contaminated soil (Kisku et al. 2000; Gupta et al. 2008).

Fungal characterization

In total, nine fungal strains belonging to six genera were isolated from the two different soil samples in the present investigation. Five strains, including *Aspergillus terreus, Aspergillus versicolor, Aspergillus niger, Fusarium oxysporum* and *Penicillium janthinellum* were found in the uncontaminated garden soil (Table 3). From the gasoline-contaminated soil, *Aspergillus niger, Fusarium oxysporum, Cladosporium bruhnei* and *Galactomyce geotrichum* were isolated.

The fungal growth rate expressed as the number of colony forming units was 5.4×10^6 CFU g^{-1} in the uncontaminated and 3.5×10^6 CFU g^{-1} in gasoline-contaminated soil, i.e. 35.18% lower in contaminated soil. The lower microbial population in the contaminated soil could be a direct or indirect effect of the gasoline. Considering direct effect, it was demonstrated that the presence of $C_5 - C_{10}$ homologues in the petroleum fraction is inhibitory to the majority of the hydrocarbon-degrading microorganisms (Okoh 2006). These solvents tend to disrupt membrane lipid structures of microorganisms. In addition, polycyclic aromatic hydrocarbons are highly toxic to microbial cell membranes, having both carcinogenic and mutagenic activity (Amellal 2001). Indirectly, an extremely high level of TPH in contaminated soil can result in impairment of gaseous

exchange and retention of soil carbon dioxide (Ujowundu 2011). These conditions in the present study might have resulted in increased acidity and decreased porosity of the contaminated soil.

In conclusion, it was shown that the aged gasoline-contaminated soil has extremely high concentration of petroleum hydrocarbons, which affected soil physicochemical properties, the fungal population and caused heavy metal enrichment. High organic carbon and total nitrogen concentration, a low soil fertility index, low pH and low moisture probably decreased fungal growth in the contaminated soil. These adverse changes can affect nutrient cycling, impede nutrient uptake by plant roots and subsequently lead to reduction in crop yield. The findings of the study could be utilized for the standardization of bioremediation protocols. Growth and activity of microorganisms in such sites could be enhanced by increasing moisture content and incorporating surfactants in the soil, which may further increase bioavailability of petroleum hydrocarbons in the soil for microbial degradation.

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