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DETERMINATION OF MICROBIAL CONTAMINANTS RECOVERED FROM BRAZILIAN PETROL STATIONS

DETERMINACIÓN DE CONTAMINANTES MICROBIANOS EN ESTACIONES DE SERVICIOS BRASILERAS

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Abstract

Soils from sites surrounding petrol stations are prone to hydrocarbon contamination and the selective pressure exerted on existing organisms may facilitate the establishment of a tolerant microbiota. Thus, the detection of some key microbial groups may serve as one way to detect soil conditions around petrol stations. The aim of this study was to quantify 5 microbial groups in soil and fuel samples from 7 facilities in João Pessoa, Brazil: total heterotrophic bacteria, hydrocarbonoclastic bacteria, acid-producing bacteria, iron bacteria and total filamentous fungi. In soil samples, the microbial density was low (10^0-10^7 CFU/g) revealing a pronounced impact on the microbiota (TPH in soil ranged from 10,000 to 12,000 mg/Kg). In diesel samples, a density ranging 10^{-1}-10^3 MPN/mL was detected, especially hydrocarbonoclastics and acid-producing bacteria, which suggests involvement of microbes in possible corrosion processes inside the tank. The results indicated that the microbial groups analyzed are potential indicators of soil quality in petrol stations. Phytotoxicity tests indicated a high degree of contamination among 42% of the facilities and *Zea mays* was the best ecotoxicity indicator.

Keywords: biodeterioration, hydrocarbonoclastic microbes, soil, Paraíba.

Resumen

Los suelos de las áreas alrededor de estaciones de servicio son propensos a la contaminación por hidrocarburos y la presión selectiva ejercida puede permitir el establecimiento de una microbiota tolerante. En este contexto, la detección de algunos grupos microbianos claves puede servir como una forma de diagnóstico medioambiental del estado del suelo de estaciones de servicio. El objetivo de este estudio fue cuantificar cinco grupos microbianos en muestras de suelo y diesel provenientes de 7 estaciones de servicio en João Pessoa, Brasil: bacterias heterotróficas totales, bacterias hidrocarbonoclásticas, bacterias productoras de ácido, bacterias oxidadoras del hierro y hongos filamentosos. En las muestras de suelo, la concentración microbiana fue baja (10^0-10^7 CFU/g) siendo indicador de contaminación. (TPH en el suelo osciló entre 10.000 y 12.000 mg/Kg). En las muestras de diesel fue detectada una concentración entre 10^{-1}-10^3 NMP/ml, principalmente de bacterias hidrocarbonoclásticas y bacterias productoras de ácido, indicando la posible participación de microorganismos en los procesos de corrosión del interior de tanques. Los resultados indicaron que los microrganismos evaluados son promisorios como indicadores de calidad del suelo de estaciones de servicio. Pruebas de fitotoxicidad indicaron un alto grado de contaminación en 42% de los puestos y *Zea mays* fue el mejor indicador de ecotoxicidad.

Palabras clave: biodeterioración, microorganismos hidrocarbonoclásticos, estación de servicio, suelo, Paraíba.

1 Introduction

Oil hydrocarbons are introduced into the environment via a variety of contaminant sources. Contamination can occur during any processing stage, including crude oil extraction, storage, fuel transportation or at the point of sale. This means that petrol stations constitute a significant risk of leaking because the tanks are underground and are subject to corrosion. In addition, many of these tanks are old and not properly maintained, which can contribute to the contamination of soil and underground water bodies (Rosales *et al*., 2014; Liu *et al*., 2006).

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Petrol stations are the main sources of hydrocarbon contamination in urban areas and these compounds can migrate horizontally and vertically when they contact the soil (Bachu, 2008). Additionally, petrol stations and their surroundings exert significant selective pressures on existing biota due to the recalcitrant and/or persistent nature of most of the organic compounds involved, as well as a variable degree of mutagenicity, which can alter existing microbiota (Jiang *et al*., 2012).

In Brazil, little is known about the quality of petrol stations. A study carried out in the state of São Paulo found that 78% of these facilities suffered leaks. Gasoline was the most abundant compound released into the environment in 90% of those cases. In other states, these data are not described, but the former information should correspond to reality in most of them (Souza *et al*., 2012).

Microbes are the key of the process of oil-contaminated soil recovery (Colin *et al*., 2012). Bacteria comprise the largest portion of biomass and are largely responsible for the elimination of oil derivatives from natural environments. However, other classes of microorganisms also act as hydrocarbon degraders, including fungi (George-Okafor *et al*., 2009). The hydrocarbon mineralization process occurs more effectively under aerobic conditions and isolation and identification of some microbial populations able to grow on petroleum contaminated sites may serve as a basis for managing interventions in oil impacted environments (Koshlaf and Ball, 2017; Dashiti *et al*., 2015, Cisneros-de La Cueva *et al*., 2014).

Interventions on oil-contaminated soils start from a characterization based on reference values regulated by environmental agencies of each country, however these analyzes have a high cost (Almeida Jr *et al*., 2016). By assigning to certain microbial groups the title of hydrocarbon-contamination indicators can act as an alternative to minimize expenses as well as to monitor putatively contaminated areas. This study aimed to propose some aerobic groups as indicators of hydrocarbon contamination by detecting them in soil and/or from samples from fuel supply tanks in the municipality of João Pessoa. A secondary goal of this study was to evaluate the ability of two plants to reveal the ecotoxicity index of the soil samples.

2 Materials and methods

2.1 Sites and sampling

Petrol stations were selected based on two criteria. First, the sites had to be visually deteriorated and easily identified based on rusty pumps and/or metallic support structures, broken or cracked floors, oil stains and/or fuel and water leaks. Second, the facility location had to be characterized by superficial water bodies, surrounding vegetation and significant customer flow. A total of 7 stations matched the outlined criteria.

Approximately 1 Kg of sandy soil were aseptically extracted from a depth of 15 cm. Samples were collected near the pumps and/or the area surrounding the petrol station. 10 g of soil were used for microbial quantifications (Genhardt *et al*., 1994). Liquid fuel samples represented by 1 L of regular gasoline and diesel oil, were directly obtained from the pumps. 100 mL were used for the microbiological analysis (Passman, 2003).

Table 1. Aerobic groups and conditions of microbial isolation from soil samples

Microbial groups	Conditions	
	Culture media	Incubation time at 29±1ºC
Total heterothrophic bacteria	Tryptose Soy Agar	48h
Acid-producing bacteria	Phenol red broth	48h
Hydrocarbonoclastic bacteria	Mineral Medium[1]	30 days
Ironbacteria	Ammonium ferric citrate	14 days
Total filamentous fungi	Sabouroud-Dextrose agar	72-96h

[1] Composition: K_2HPO_4 (0.5g/L); $(NH_4)_2SO_4$ (0.5 g/L); $MgSO_4$ (0.5 g/L), $FeCl_2$ (10.0 mg/L); $CaCl_2$ (10.0 mg/L); $MnCl_2$ (0.1 mg/L), $ZnSO_4$ (0.01 mg/L) and 2 drops of a B vitamin complex solution, pH 7.2±0.2

Table 2. Microbial density and soil characterization

Site	pH	TPH[1]	TBH[2]	IB[2]	APB[3]	HB[3]	TFF[2]
1	8.00±0.1	10,226	$5.0±1×10^4$	$2.3±0.1×10^7$	$1.4±0.1×10^5$	$1.4±0.1×10^3$	$2.0±1.0×10^4$
2	8.02±0.1	10,984	0	$4.0±0.2×10^5$	$1.5±0.1×10^3$	$1.4±0.1×10^3$	$2.0±1.0×10^4$
3	7.95±0.2	11,664	0	0	$2.0±0.1×10^3$	$3.0±1.0×10^1$	$2.0±1.0×10^4$
4	7.93±0.1	11,896	0	0	$3.0±0.1×10^3$	$7.0±1.1×10^0$	0
5	7.90±0.2	10,317	$6.0±2×10^4$	0	$1.1±0.1×10^5$	$2.0±0.1×10^1$	$1.0±0.1×10^4$
6	7.92±0.1	10,584	$1.0±1×10^5$	0	$4.0±1.0×10^3$	$1.5±0.2×10^1$	$4.0±1.2×10^4$
7	7.90±0.1	10,086	$2.2±1×10^7$	$2.3±0.1×10^7$	$6.0±1.0×10^2$	$6.0±1.0×10^0$	$2.1±1.1×10^7$

TBH - total heterothrophic bacteria; IB - ironbacteria; APB - acid producing bacteria; HB - hydrocarbonoclastic bacteria; TFF - total filamentous fungi. [1]TPH - total petroleum hydrocarbons (mg/kg); [2] - CFU/g; [3] - MPN/g

2.2 *Microbiological assays*

A total of 5 aerobic microbial groups were analyzed. Four bacterial and one fungal groups were quantified: total heterotrophic, hydrocarbonoclastic, acid-producing bacteria, iron bacteria and total filamentous fungi. The media properties and the incubation conditions are summarized in Table 1.

Two methods were used to investigate the microbial groups. The total heterotrophic bacteria, iron bacteria and total filamentous fungi were quantified using the pour plate technique (França *et al*., 2014) and the multiple tube technique was used to quantify the hydrocarbonoclastic bacteria and acid-producing bacteria (Silva *et al*., 2009). The results were expressed respectively in Colony Forming Units per soil gram (CFU/g) and Most-Probable-Number (MPN) per gram of soil or per milliliter of fuel. Analyses were performed in duplicate.

2.3 *Soil pH and ecotoxicity tests*

The pH was determined from the soil extracts, prepared mixing each sample with distilled water in proportions 1:25 (Vasconcelos *et al*., 2010). In order to correlate seed germination and root size in the presence of toxic components, compared to a distilled water control, seed germination index (I_G) values were determined according to Cavalcanti *et al*. (2016), using 10 seeds per plate (Toca do Verde, Canoas, Brazil) of *Zea mays* (corn) and *Cucumis anguria* (cackray). The assay was carried out in triplicate. I_G was measured as follows:

$$I_G = [(S_1 x R_1) \div (S_2 x R_2)] x 100$$

Where, S_1= number of germinated seeds on the soil extract, S_2= number of germinated seeds on the control, R_1= mean root length on the soil extract and R_2= mean root length on the control.

2.4 *Statistical analysis*

The results obtained were expressed as the mean plus or minus the standard deviation.

3 Results and discussion

3.1 *Microbial quantification*

The results of the microbial group quantifications in the soil and fuel samples are shown in Tables 2 and 3. The cultivable microbial densities identified in soil samples from all of the collection points were very diverse. Most analyses identified very low numbers of bacteria. This differs from natural soils ($> 10^{10}$ CFU/g) but is typical of oil-contaminated soils (Liu *et al*., 2006).

The Total Petroleum Hydrocarbons (TPH) concentration in soil samples, ranged from 10,000 to 12,000 mg/Kg (Table 2). The results indicated variations in contamination degree in different areas of the petrol stations, particularly when the samples collected from the surrounding area (petrol stations 1, 5, 6 and 7) were compared to those collected close to the pumps (petrol stations 2, 3 and 4).

A recent study investigated BTEX (benzene, toluene, ethylbenzene and xylene) concentration variations at different areas of a petrol stations in Thailand over three days. The study verified that the central zone of the facility exhibited the greatest contaminant concentrations. The contaminant content of the surrounding zone varied according to weather variables, such as temperature and relative humidity. According to the authors, these parameters often exhibit similar patterns during consecutive days, but differ during the same day in tropical regions (Rattanajongjitrakorna and Prueksasit, 2014).

Table 3. Microbial density from fuel samples

Site	TBH[1]	IB[1]	APB[2]	HB[2]	TFF[1]
1	0	0	0	$2.3\pm0.2\times10^{3}$	0
2	0	$2.0\pm0.1\times10^{1}$	$1.5\pm0.2\times10^{1}$	$1.6\pm0.1\times10^{3}$	0
3	—	—	$1.1\pm0.1\times10^{0}$	$9.2\pm0.1\times10^{2}$	0
4	0	0	$1.4\pm0.2\times10^{2}$	$3.6\pm1.0\times10^{1}$	$1.5\pm0.2\times10^{1}$
5	0	0	0	$2.2\pm0.1\times10^{2}$	0
6	0	0	$7.0\pm1.1\times10^{-1}$	$>2.3\pm0.1\times10^{3}$	0
7	0	0	0	$>2.3\pm0.1\times10^{3}$	$1.5\pm0.1\times10^{1}$

TBH - total heterothrophic bacteria; IB - ironbacteria; APB - acid producing bacteria; HB - hydrocarbonoclastic bacteria; TFF - total filamentous fungi. [1] - CFU/mL ; [2] - MPN/mL

Intrinsic soil characteristics, such as texture, also influence the effects of hydrocarbons on microbiota. However, contaminant type and concentration as well as environmental factors are the most important variables. In bacteria and fungi, the transport of hydrocarbons from the environment into the cell can be carried out without energy expenditure, independent of temperature, by simple or facilitated diffusion. Preferably, the hydrocarbons are consumed in the following order: linear > branched > aromatic > polycyclic aromatic (Ruiz-Marín *et al*., 2013). The diffusion rate is slow and the passage of the compound through the membrane is not mediated by the concentration gradient formed and is therefore dependent on the presence of intermediate and/or final metabolites within the cell. The main mechanism of biodegradation involves the action of oxygenases leading to the formation of central metabolites that can be converted to citric acid cycle intermediates or used in the production of biomass (Fuentes *et al*., 2014).

Five microbial groups were tested in order to serve as potential candidates for indicators of soil contamination at petrol stations with a history of long-term pollution. These microorganisms represent key groups as they develop and replace the native microbiota by a new population composed of hydrocarbon-tolerant organisms (Yao *et al*., 2017). Under natural conditions, the microbial composition in soil, especially hydrocarboclastic, is much lower than that of total heterotrophs, but when the values begin to grow, a degree of contamination by hydrocarbons begins to appear (Alrumman *et al*., 2015; Márquez-Rocha *et al*., 2005).

Iron bacteria were not detected at petrol stations 3, 4, 5 and 6 but were quantified at petrol stations 1, 2 and 7. The density reached 10^{7} CFU/g at stations 1 and 7, differing by two orders of magnitude from the station 2. This difference may be related to the natural biological hydrocarbon removal processes, which require an oxidizing medium. Oxygen acts as an electron receptor, and the contaminant acts as a source of carbon and energy. As the available oxygen is consumed, microorganisms use other electron receptors in the soil, especially Fe^{3+} ions (Aelion *et al*., 1991).

Acid-producing bacteria were detected in all soils, varying in abundance from 10^{2} to 10^{5} CFU/g. This group is composed of microorganisms from different bacteria genera, which may not produce organic acids from natural substrates under natural conditions. However, this characteristic can be demonstrated in artificial media, and density values may be overestimated if compared to other studied groups (Dias *et al*., 2016).

Hydrocarbonoclastic bacteria were detected at all 7 petrol stations, varying from 10^{0} to 10^{3} MPN/g. This group of microorganisms possesses the metabolic tools to begin the oil derivative biodegradation process. These densities were smaller than those reported in the literature for soils subjected to long-term hydrocarbon contamination (10^{4}-10^{6} CFU/g). However, given the experimental conditions of this study, it was not possible to analyze the process of tolerance, where the hydrocarbonoclastic population gradually increases due to oil concentration variations over time. The low bacterial density may also be related to the bacteriostatic effect of hydrocarbons on the biota (Chen *et al*., 2007).

Filamentous fungi values ranged from 10^{4}-10^{7} CFU/g, but were not detected at petrol station 4. Note that the soil pH values varied from 7.90 to 8.02, which would not favor the development of these organisms. In addition, eukaryotic organisms are more sensitive to oil compared to prokaryotic organisms. However, the presence of oil did not produce a fungistatic effect when compared to previous results in the presence of heavy oil, around 10^{1}-10^{3} (Gaylard *et al*., 1999).

Table 4. Seed germination index*

Plant	Petrol stations						
	1	2	3	4	5	6	7
C. anguria	103.3	112.5	130.3	94.3	110	124.3	121.7
Z. mays	164.5	129.4	12.4	23.5	78.8	141.6	126.9

* Standard deviation: *C. anguria* (±0.8) and *Z. mays* (±0.1)

Moreover, an intense inhibitory effect was found at petrol station 4, which was considered the most impacted facility from a microbiological point of view coinciding with the major TPH concentration. We highlight the importance of fungi at the beginning of the oil hydrocarbon degradation process, especially that of aromatic compounds attacked by non-specific enzymes (Torbor-Kaplon *et al*., 2005).

The detection of high densities of microorganisms involved in corrosive processes, especially iron bacteria and acid-producing bacteria, may indicate the degree of conservation of the storage tanks, resulting risks or potential spills. The microbial sampling was used to determine instances of biological contamination related to tank biocorrosion and product biodeterioration issues. No microorganisms were detected in the regular gasoline samples, which was possibly due to the presence of preservatives. However, the diesel oil samples exhibited contamination in all 7 petrol stations studied, confirming that the nature of the fuel directly influences the contamination susceptibility (Bacosa *et al*., 2010).

Products of microbial metabolism, such as organic acids, provide favorable conditions for the deterioration of coatings and metal used in storage tanks, resulting in potential leaks and reducing tank lifespan. Brazil regulates this activity via Resolution CONAMA # 273/00, but the actual scenarios at countless petrol stations across the country remain unknown (Souza *et al*., 2014).

This study identified an abundance of essentially hydrocarbonoclastic microbiota (10^1 to > 10^3 MPN/mL). No total heterotrophic bacteria were identified. In addition, a small number of iron bacteria were detected at station 2, and filamentous fungi were found in small quantities at petrol stations 4 and 7. Acid-producing bacteria (10^{-1}-10^2 MPN/mL) were detected at four of the seven facilities. Although present, the quantified hydrocarbonoclastic bacteria values were lower than the expected range, while the acid-producing bacteria values corresponded to literature reports, ranging from 10^1 to 10^4 MPN/mL (Bento *et al*., 2001; Passman *et al*., 2001; Gaylarde *et al*., 1999). The presence of acid-producing bacteria may suggest the relevance of tank conservation issues rather than an overestimation of microbial density.

The main biological contamination sources in stored fuels are soil microorganisms, which are carried by the air or released from biofilms on the tank walls. Microbial contamination in storage tanks at petrol stations is often due the lack of sterile conditions during transportation and fuel storage, as well as biomass accumulation in the water phase of the tank or at the water/fuel interface. Some authors stated that enough water typically exists within fuel storage tanks to maintain the appropriate conditions for microbial growth (Rodríguez-Rodríguez *et al*., 2010). This water can be part of the fuel mixture or be introduced via cleaning operations. However, precipitation is the major mechanism of microorganism transport. According to literature, a 25mm accumulation during one hour of rain increases the microorganism flow by a factor of 100, increasing the bacterial density from two to four orders of magnitude (Kaufman and Marsh, 1997).

Microbial contamination can compromise product quality and contribute to the degradation of storage systems. In this context, the definition of good practices of tank cleaning as well as the use of biocides in their coating can guarantee the reduction of impacts and the detection of these microbial groups can serve as a control of the management of these procedures.

3.2 *Ecotoxicity test*

Any plant may be used in ecotoxicity assays employing seeds. Particularly in the tests involving hydrocarbons, we must consider the use of plants whose seeds do not have their growth stimulated by oil after germination. There are eleven options reported in the literature (Cavalcanti *et al*., 2016).

The germination index values of *Z. mays* and *C. anguria* seeds in the presence of soil extract are shown in Table 4. Phytotoxicity is considered high when I_G < 50%, moderate when I_G = 50-80% and null when greater than 80% (Anastasi *et al*., 2009). Thus, *Z. mays* proved to be the most sensitive and indicated

a higher level of toxic compounds present in the soil at facilities 3, 4 and 5. Petrol station 5 was classified as of moderate toxicity, with I_G = 78.8±0.1%. The other two stations were classified as of high toxicity (I_G = 12.4±0.1 and 23.5±0.1%, respectively). No hydrocarbon influences on germination and plant development were detected at the other stations. A similar result was also observed for *C. anguria*, which was previously considered the best bioindicator (Vasconcelos *et al*., 2010). However, that study focused on aromatic polycyclic hydrocarbons, which are uncommon in the fossil fuels distributed at gas stations.

When comparing the results from the *Z. mays* ecotoxicity test with the results of soil microbiota quantification from the petrol stations (Table 2), the smallest I_G values coincide with the locations with the lowest microbial densities. This phenomenon reinforces the hypothesis that the soil is significantly impacted by contamination, causing stasis in the biota. Native edaphic microbiota imbalances are expected in areas with a history of oil derived contamination, which likely explains the increased number of hydrocarbonoclastic microbiota due to the exerted selective pressure of the contaminants (Hamamura *et al*., 2006). No heterotrophic bacteria were identified at petrol stations 3 and 4, coinciding with the I_G of *Z. mays* of less than 50%. This result is possibly related to the high contaminant levels compared to other facilities. The heterotrophic bacteria density was three orders of magnitude higher than the densities that have been commonly reported for soils with I_G values less than 50%. Therefore, the contaminant probably acted as a carbon source, decreasing the degree of toxicity.

In contrast, the hydrocarbonoclastic bacteria densities at stations 3 and 4 were one hundred times lower than those at petrol stations 1 and 2 (I_G >80%) and were identical to those at petrol stations 6 and 7. Despite the fact that selective pressures create inhospitable environments, other molecules can serve as preferred substrates, explaining the fact that hydrocarbonoclastic bacteria are still not dominant and other groups, such as acid-producing bacteria, are more distributed and common. Additionally, iron bacteria were absent at three petrol stations whose phytotoxicity was detected based on *Z. mays*. Iron bacteria populations varied between 10^5 – 10^7 at all other petrol stations, except station 6, suggesting that this group may be a potential bioindicator of soil hydrocarbon contamination at petrol stations.

Low fungal densities, even to the point of non-detection, suggest that filamentous fungi do not play an important ecological role in the contaminant degradation process, which relies on bacterial diversity. In addition, fungi are more sensitive to the presence of oil derivatives due to the smaller oxygen availability and the absence of cellulosic substrates (Ballaminut and Matheus, 2007; Merkl *et al*., 2004).

This study identified and quantified microbial groups that may be used to assess contamination levels, human health risks and environmental hazards near petrol stations. Additional protocols are needed to regulate effective indicators and microbiological quality standards at petrol stations, correlating biological indicators to environmental risk based on pollutant concentrations and the microbial density reductions.

Conclusion

The attempt to determine microbiological indicators in environments with a long-term oil contamination exhibited promising results in terms of soil quality at petrol stations. This study proposes five potential aerobic microbial groups as a tool to presume soil quality allowing basis to guide bioremediation strategies. Further investigation is needed to characterize contaminants and their correlations to the respective microbial groups.

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