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# ENVIRONMENT CONTAMINATION WITH PETROLEUM HYDROCARBONS

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***Abstract***

*Important quantities of petroleum hydrocarbons are frequently spilled into the environment harming the environment, as well as human health. Furthermore, the interaction of petroleum hydrocarbons with the environment significantly disturbs the activity of the microorganisms, including bacteria that exist in petroleum hydrocarbons polluted environments. Using two different microbiological methods four groups of bacteria, such as heterotrophic, hydrocarbon-tolerant, hydrocarbon-degrading, as well as enterobacteria were detected in the analyzed samples collected from an old petroleum products storage. The detection of these bacteria, especially hydrocarbon-degrading, as well as hydrocarbon-tolerant bacteria was not unexpected since the concentration of the petroleum hydrocarbons in the analyzed samples was above the limit allowed by international environmental standards. Up to now, different treatment technologies have been developed to remove toxic hydrocarbons from environments contaminated with petroleum products. Therefore, because of the use of different remediation strategies, like bioremediations, the affected areas can be recovered and returned to their natural circuit.*

***Key words****: bacteria, bioremediation, contamination, environment, petroleum hydrocarbon.*

## INTRODUCTION

Petroleum and petroleum products (e.g., diesel, gasoline, lubricants) are all over the world the principal source of energy for many industries and our daily lives. Consequently, accidents, leaks, and spills occur during petroleum exploration, production, transport, and storage, and petroleum hydrocarbons are frequently released into the environment (Onutu & Tita, 2018). As a result of the contamination of soil and groundwater with petroleum hydrocarbons diversity and activity of indigenous microorganisms are frequently perturbed. Furthermore, petroleum hydrocarbon contamination also has a negative impact on human health, animals, and plants (Das & Chandran, 2010; Chikere et al., 2011; Xu et al., 2018; Logeshwaran et al., 2018; Petruta & Drăcea, 2023). Nowadays, different physical, chemical, and biological methods are used for the remediation of environments contaminated with petroleum and petroleum products. Biological methods are considered eco-friendly, cost-effective, and more efficient in the complete degradation of petroleum hydrocarbons, as compared with other methods. Furthermore, strategies that ensure adequate concentration of oxygen and nutrients can create suitable environmental conditions for the growth of bacteria capable of degrading petroleum and petroleum products. Analyzing the effects of petroleum hydrocarbons on bacterial community diversity and dynamics in contaminated sites has been reported to be valuable for remediation practices and to help in evaluating the recovery potential of petroleum and petroleum products contaminated sites (Logeshwaran et al., 2018).
As bacteria play a significant role in the degradation of petroleum hydrocarbons investigations into the bacterial communities within contaminated samples are essential to find adequate strategies for bioremediation of the petroleum hydrocarbon contaminated sites (Truskewycz et al., 2019). The purpose of this research was to investigate the presence of several bacterial groups, such as heterotrophic, hydrocarbon-tolerant, hydrocarbon-degrading, and enterobacteria in two environmental

samples collected from an old site contaminated with petroleum products.

## MATERIALS AND METHODS

### Samples collection

The groundwater and soil samples analyzed in the present study were collected from an old site contaminated with petroleum products (Figure 1) located in Constanta County. The water table level at the time of taking the aquifer sample was located at a depth of -4.73 m, and the soil sample was taken from the vicinity of the monitoring well at a depth of about -0.8 m. We observed that in the soil sample, an advanced degree of degradation of petroleum products was found, and in the groundwater sample, a film of petroleum hydrocarbons was observed, which indicates an old pollution. Petroleum and petroleum products that are accidentally released into the environment due to different human activities can often contaminate the soil, and by vertical migration, they reach the groundwater (Ossai et al., 2020). When petroleum hydrocarbons contaminate the environment, they undergo a variety of weathering that involves physicochemical processes (e.g., dispersion, evaporation, dissolution, adsorption, photolysis) and biological processes (biodegradation and biotransformation). According to the literature data, environmental factors, for instance, temperature, humidity, and precipitation affect the biodegradation process of petroleum hydrocarbons (Abena et al., 2019; Truskewycz et al., 2019; Ossai et al., 2020).

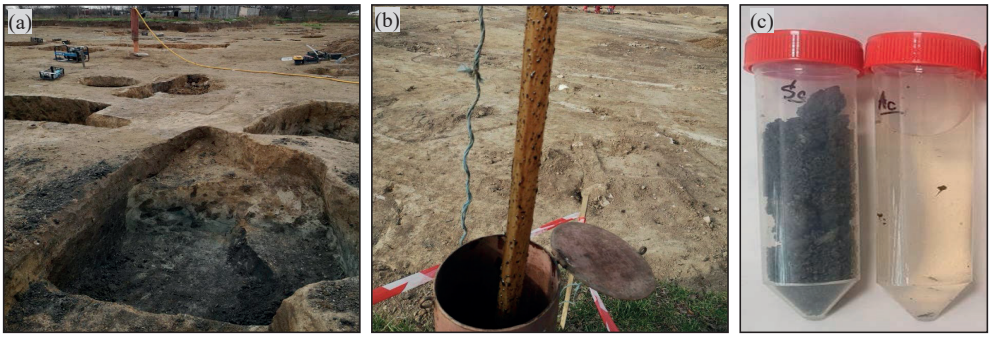


Figure 1. Samples collection from an old petroleum products storage:
(a) groundwater; (b) soil; (c) samples

### Microbiological analysis of samples

The microbiological analysis of the samples was made by the agar plate method and the most probable number method. The pH of the samples was measured by using a Hanna pH 213 (Woonsocket, Rhode Island, USA).

Plate count agar (PCA) method. Serial dilutions ($10^{-1}$-$10^{-6}$ in phosphate buffer saline or PBS) of each sample were inoculated on different culture media, such as LB agar (Sambrook et al., 1989) for heterotrophic bacteria, LB agar with 5% (v/v) diesel for hydrocarbon-tolerant bacteria, minimal agar (Stancu, 2023) with 5% diesel for hydrocarbon-degrading bacteria, and EMB agar for enterobacteria. Petri dishes were incubated at 30°C for 1-5 days. Then, it was determined the number of bacteria present per ml or g (CFU $ml^{-1}$, CFU $g^{-1}$).

The most probable number (MPN) method. Serial dilutions ($10^{-1}$-$10^{-12}$ in PBS) of each sample were inoculated into 96-multiwall plates (Stancu & Grifoll, 2011) containing different culture media, such as LB broth (Sambrook et al., 1989) for quantification of heterotrophic bacteria, LB broth with 5% diesel for hydrocarbon-tolerant bacteria, and minimal broth (Stancu, 2023) with 5% diesel for quantification of hydrocarbon-degrading bacteria. Multiwall plates were incubated at 30°C for 1-14 days. The growth of bacteria was determined (cell $ml^{-1}$, cell $g^{-1}$) using 0.3% triphenyl tetrazolium chloride (TTC) dye as a redox indicator of cellular respiration (Stancu & Grifoll, 2011).

**Isolation of hydrocarbon-tolerant, hydrocarbon-degrading bacteria**

The hydrocarbon-tolerant, hydrocarbon-degrading bacteria were isolated by the enrichment culture method (Stancu, 2020; Stancu, 2023). Each sample (5% v/v) was used to initiate enrichment cultures in LB broth as well as in minimal broth supplemented with 5% diesel as a sole carbon source. The tubes were incubated at 30°C on a rotary shaker (200 rpm) for 14 days. Then, the enrichment cultures (5% v/v) were transferred into fresh LB broth with 5% diesel for the isolation of hydrocarbon-tolerant bacteria and minimal broth with 5% diesel for the isolation of hydrocarbon-degrading bacteria. The tubes were incubated under the same conditions for another 14 days. Then, the growth of isolated bacteria in the presence of diesel was determined by measuring the optical density at 660 nm ($OD_{660}$), as well as by the spot method (Stancu, 2020; Stancu, 2023) using LB agar, EMB agar, and LB with diesel. The biodegradation of diesel by the isolated bacterial population was confirmed by diesel film fragmentation, as well as by monitoring the free carbon dioxide ($CO_2$ mg $l^{-1}$) (Stancu, 2023).

**Chemical analysis of samples**

The extraction of petroleum hydrocarbons from the aquifer sample was made using perchloroethylene, while the extraction of hydrocarbons from the soil was done by using S-316 solvent. Polar compounds were removed from extracts by adding activated aluminum oxide. To determine the hydrocarbon concentration in the aquifer (mg $l^{-1}$) and soil (mg $kg^{-1}$), the non-polar solvent extracts were analyzed by Fourier transform infrared spectroscopy (FTIR) (Falkova et al., 2016).

## RESULTS AND DISCUSSIONS

**Microbiological analysis of samples**

In the aquifer and soil samples which had an alkaline pH (7.8, 8.6), we revealed by plate count agar (PCA) method (Figure 2) the presence of the following groups of bacteria: heterotrophic, hydrocarbon-tolerant, hydrocarbon-degrading, and enterobacteria. The number of these bacteria varied from one sample to another ($10^5$-$10^7$ CFU $ml^{-1}$ or $g^{-1}$) (Table 1). Heterotrophic bacteria were present in high numbers in both the aquifer ($10^6$ CFU $ml^{-1}$) and soil ($10^7$ CFU $g^{-1}$) samples. Most of the bacteria present in the analyzed samples proved to be hydrocarbon-tolerant bacteria ($10^5$, $10^7$ CFU $ml^{-1}$ or $g^{-1}$). Also, enterobacteria were present in both the analyzed ($10^5$, $10^6$ CFU $ml^{-1}$ or $g^{-1}$) samples.

By the most probable number method (MPN) (Figure 2) the existence of the following groups of bacteria was highlighted: heterotrophic bacteria, hydrocarbon-tolerant bacteria, and hydrocarbon-degrading bacteria, and their number varied from one sample to another ($10^7$-$10^{12}$ cells $ml^{-1}$ or $g^{-1}$) (Table 1). Heterotrophic and hydrocarbon-tolerant (HCT) bacteria were present in higher numbers ($10^{11}$-$10^{12}$ cells $ml^{-1}$ or $g^{-1}$) in the two analyzed samples. Unlike these two groups of bacteria, the hydrocarbon-degrading (HCD) bacteria were present in lower numbers ($10^7$ cells $ml^{-1}$ or $g^{-1}$) both in the soil and aquifer samples. These results can be explained because not all bacteria present in natural samples can degrade petroleum hydrocarbons which generally are very toxic for most microorganisms, including bacteria.

**Isolation of hydrocarbon-tolerant, hydrocarbon-degrading bacteria**

Previously it was observed (Stancu, 2020; Stancu, 2023), that the enriched culture method is a very effective technique for the isolation of bacteria that exist in environments polluted with petroleum and petroleum products. Consequently, we use the enriched culture method for the isolation of hydrocarbon-tolerant and hydrocarbon-degrading bacteria from soil and groundwater samples (Figure 2).

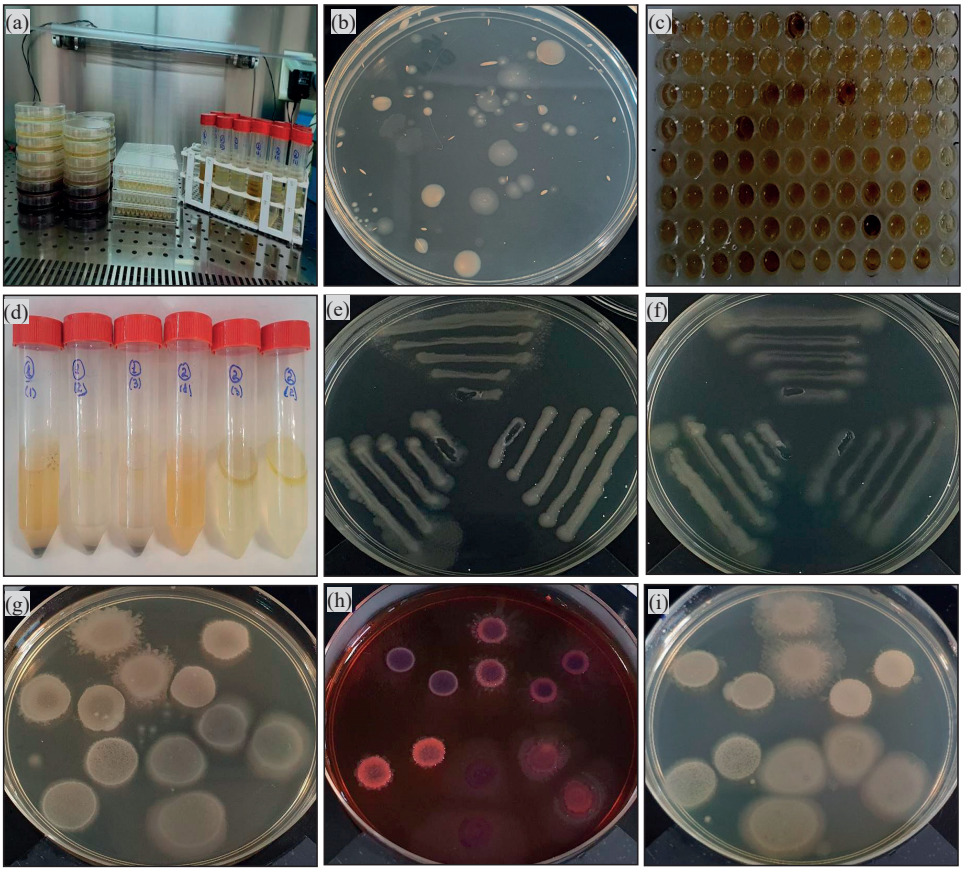


Figure 2. Enumeration and isolation of hydrocarbon-tolerant and hydrocarbon-degrading bacteria:
(a) microbiological analysis of samples; (b) plate count agar method; (c) most probable number method;
(d) bacteria isolation by enrichment culture method; (e, f) bacteria purification; (g) bacteria growth on LB agar;
(h) bacteria growth on EMB agar; (i) bacteria growth on LB agar with diesel

Table 1. Microbiological analysis of aquifer and soil samples

| Samples | Number of bacteria by | | | | | |
|---|---|---|---|---|---|---|
| Ground water | PCA method (CFU $ml^{-1}$ or $g^{-1}$) | | | MPN method (cells $ml^{-1}$ or $g^{-1}$) | | |
| | Heterotrophic | HCT | Enterobacteria | Heterotrophic | HCT | HCD |
| Soil | $4.5 \cdot 10^{6}$ | $1.6 \cdot 10^{5}$ | $5.3 \cdot 10^{5}$ | $4.0 \cdot 10^{11}$ | $1.6 \cdot 10^{11}$ | $2.0 \cdot 10^{7}$ |
| | $5.9 \cdot 10^{7}$ | $6.5 \cdot 10^{7}$ | $5.6 \cdot 10^{6}$ | $1.6 \cdot 10^{12}$ | $1.2 \cdot 10^{12}$ | $9.5 \cdot 10^{7}$ |

As expected, in the soil and groundwater samples, the presence of both hydrocarbon-tolerant and hydrocarbon-degrading bacteria was observed. The growth of these bacteria varied from one sample to another depending on the culture conditions ($DO_{660}$ 0.99-2.21) (Table 2). In the case of hydrocarbon-tolerant bacteria, we observed better growth ($DO_{660}$ 1.45, 2.21), compared to that of hydrocarbon-degrading bacteria ($DO_{660}$ 0.99, 1.84). All these bacteria showed good viability on the LB agar, EMB agar, and LB agar with diesel (Figure 2).
The fragmentation of the diesel film, because of the biodegradation of the hydrocarbons, was observed for both the hydrocarbon-tolerant bacteria and hydrocarbon-degrading bacteria. The amount of free $CO_2$ released varied from one sample to another (1012, 1110 mg $l^{-1}$).

Table 2. Characterization of the isolated bacterial populations

| Samples | Hydrocarbon-tolerant | | | | Hydrocarbon-degrading | | | |
|---|---|---|---|---|---|---|---|---|
| | ($DO_{660}$) | Viability | Diesel degr. | $CO_2$ (mg $l^{-1}$) | ($DO_{660}$) | Viability | Diesel degr. | $CO_2$ (mg $l^{-1}$) |
| Groundwater | 1.45 | 100 | + | 1012 | 0.99 | 100 | + | 1100 |
| Soil | 2.21 | 100 | + | 1110 | 1.84 | 100 | + | 1210 |

**Chemical analysis of samples**

The isolation of hydrocarbon-tolerant and hydrocarbon-degrading bacteria from soil and aquifer was not surprising since both these samples were highly contaminated with petroleum hydrocarbons.

Based on the FTIR spectroscopy analysis, in the aquifer sample, the concentration of total petroleum hydrocarbons (TPH) was 273 mg $l^{-1}$, while in the soil their concentration was 17300 mg $kg^{-1}$ (Table 3).

Table 3. Petroleum hydrocarbon in aquifer and soil samples

| Samples | Petroleum hydrocarbons |
|---|---|
| | Concentration (mg $l^{-1}$ or mg $kg^{-1}$) |
| Groundwater | 273 |
| Soil | 17300 |

The TPH parameter is used as the first element in identifying sites contaminated with petroleum products, being an indicator of petroleum hydrocarbon contamination of the environment.

Based on the evaluation of this parameter, measures can be taken to remediate a contaminated site (Pinedo et al., 2013). As observed, the petroleum hydrocarbon concentrations were over the limit allowed by international environmental standards in both samples.

These results were not surprising, since in the past there was a deposit of petroleum products, such as gasoline, lighter liquid fuel, engine, and transmission oils in this area.

## CONCLUSIONS

In groundwater and soil samples, the presence of heterotrophic, hydrocarbon-tolerant, hydrocarbon-degrading, and enterobacteria was highlighted by using two classical microbiological methods.

All these bacteria were detected in lower numbers ($10^5$-$10^{11}$ CFU or cells $ml^{-1}$) in the aquifer sample, compared with their numbers in the soil sample ($10^6$-$10^{12}$ CFU or cells $g^{-1}$).

The isolation of hydrocarbon-degrading bacteria, as well as hydrocarbon-tolerant bacteria from the analyzed samples, was not unexpected, since both aquifer and soil samples were highly contaminated with petroleum hydrocarbons (273 mg $l^{-1}$, 17300 mg $kg^{-1}$) because of different human activities.

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