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USE OF WASTE SLUDGE IN THE IMPROVEMENT OF THE QUALITY OF SOILS CONTAMINATED WITH PETROLEUM PRODUCTS

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Abstract

The ecological reconstruction of the sites contaminated with petroleum products is necessary to restore the geological environments affected by the economic activities specific to the petroleum industry. The purpose of ecological reconstruction is for the site to be returned to the environment for the resumption of economic and landscape functions, without presenting risks to the environment and human health. Decontamination technologies are based on various bioremediation methods. In many cases, significant amounts of soil are required to fill the resulting excavations and to systematize the land to complete the ecological reconstruction work. In the present study, the possibility to use sludge collected from a municipal wastewater treatment plant in the ecological reconstruction is presented. Both, the presence of hydrocarbon-degrading bacteria and the geotechnical characteristics make it possible to use the waste dehydrated sludge for the ecological reconstruction of the sites contaminated with petroleum products.

Key words*: contaminated site, decontamination, ecological reconstruction, sewage sludge.*

INTRODUCTION

It is well known that the oil and gas industry play an important role in the world economy. Its products represent both an important source of energy and a source for raw materials needed in other fields of activity. Large areas of land are occupied by the activities of this industry. In the entire technological chain for the exploitation of oil there is a risk of contamination of the marine and terrestrial environment, both with oil and with the products obtained from its processing. Oil spills affect large areas of land and have a negative impact on ecosystems and human health. Lands affected by oil pollution can no longer be used (Poliak et al., 2018).

The decontamination, remediation of the soil, the subsoil and the ecological reconstruction of a site contaminated with petroleum products appeared as a necessity to reduce the negative effects on the environment, eliminate any risk to the health of the population and return the land to the natural circuit for the resumption of ecological, economic and landscape functions. Agricultural lands that have been polluted with oil products, forest areas, pastures must resume their productive functions, and the most difficult mission in ecological reconstruction is to restore the soil. Pollution with petroleum products affects soil quality by modifying microbial activity (Li et al., 2007; Silva-Castro et al., 2015), but certain microorganisms can metabolize petroleum hydrocarbons.

The use of microorganisms for the decontamination of soils polluted with hydrocarbons has started to be a method used on a large scale as an ecological and versatile solution (Bento et al., 2005; Silva-Castro et al., 2013). For the bioremediation of soils contaminated with hydrocarbons it is necessary creating a favorable environment that provides the nutrients and the carbon source necessary for the development of microorganisms.

It is known from specialized literature that sewage sludge contains nutrients, microorganisms, many beneficial substances and can be used in agriculture as a natural fertilizer (Iticescu et al., 2021). All these characteristics could make sewage sludge play an important role in the bioremediation of soils contaminated with petroleum hydrocarbons. Sewage sludge results from wastewater treatment and is continuously increasing due to

investments in new treatment plants (Iticescu et al., 2021).

Presently, the management of sewage sludge is an enormous challenge in the field of environmental engineering (Rorat et al., 2019). The increase of sewage sludge produced by wastewater treatment plants around the world needs their proper disposal (Duan et al., 2017). After sewage sludge discarding in the sea, land use of dehydrated sludge for soil bioremediation of the contaminated sites or as fertilizers in agriculture becomes a common disposal way, due to the abundance of valuable components like organic matter and nutrients (e.g., nitrogen, phosphorus, potassium) (Duan et al., 2017; Rorat et al., 2019). In several European Union countries, more than 50% of the sludge is used in several applications. It is well-known, that land application of sewage sludge can improve soil properties (e.g., pH, organic matter, nutrients, porosity, moisture, stability) and finally increase plant growth (Duan et al., 2017). However, sewage sludge can also contribute to soil pollution since they contain organic and inorganic contaminants, as well as pathogens that pose a potential danger to human and animal health and the quality of ecosystems, if used as a fertilizer (Iticescu et al., 2021).

The aim of this study was to investigate the presence of several bacterial groups, including hydrocarbon-tolerant and hydrocarbon-degrading bacteria, in two sewage sludge samples taken from a municipal wastewater treatment plant. The presence of heterotrophic bacteria and enterobacteria was also investigated in the present work.

MATERIALS AND METHODS

Sampling. The sewage sludge samples, named sludge 1, and dehydrated sludge 2 were taken from the Galati wastewater treatment plant (Galati County, Romania).

Microbiological analysis of the sludge samples. Samples were mixed (1:2 v/v or g/v) with phosphate buffer saline (PBS, Sambrook and Russel 2001) and incubated at room temperature on a rotary shaker (200 rpm) for one hour. Then, serial dilutions (10^{-1}-10^{-12}) were done in PBS. The pH of the samples was determined using a Hanna pH 213 (Woonsocket, Rhode Island, USA).

In the **plate count agar** (PCA) method, serial dilutions of each sample were inoculated onto different culture media: LB agar (Sambrook and Russel 2001) for heterotrophic bacteria account, LB agar added with diesel fuel (5% v/v) for hydrocarbon-tolerant bacteria, minimal agar (Stancu, 2022) added with diesel (5% v/v) for hydrocarbon-degrading bacteria, and EMB agar (Levine agar) for enterobacteria account. Petri plates were incubated at 30°C, for 1-5 days. Then, the number of bacteria present per ml or g (CFU ml^{-1} or CFU g^{-1}) of the sample was determined.

In the **most probable number** (MPN) method, serial dilutions of each sample were inoculated (as described previously by Stancu & Grifoll, 2011) onto 96-multiwall plates containing different culture media: LB broth (Sambrook and Russel 2001) for heterotrophic bacteria account, LB broth added with diesel (5% v/v) for hydrocarbon-tolerant bacteria, and minimal broth (Stancu, 2022) added with diesel (5% v/v) for hydrocarbon-degrading bacteria account. Multiwall plates were incubated at 30°C, for 1-14 days. The bacterial population growth was determined using triphenyl tetrazolium chloride (TTC 0.3% w/v) dye as an indicator of cellular respiration (Stancu & Grifoll, 2011). The multiwall plates were incubated at room temperature for one more day in the dark. When red color was developed (because of the TTC reduction) wells were considered positive for bacterial growth (cel. ml^{-1} or cel. g^{-1}).

Isolation of hydrocarbon-tolerant, hydrocarbon-degrading bacteria. The bacteria were isolated by the enrichment culture method (Stancu and Grifoll, 2011; Stancu, 2020). Each sample (5% v/v) was used to initiate enrichment cultures in LB broth added with diesel (5% v/v), as well as in minimal broth added with diesel (5% v/v) as the sole carbon source. Tubes were incubated at 30°C, on a rotary shaker (200 rpm) for 14 days. The enrichment cultures (5% v/v) were transferred into fresh LB broth added with diesel for hydrocarbon-tolerant bacteria isolation, and minimal broth added with diesel for hydrocarbon-degrading bacteria isolation. Then, the tubes were incubated in the same conditions for another 14 days. The isolated bacteria were stored frozen in 25% (v/v) glycerol at -80°C.

The growth of the isolated bacteria in the presence of diesel was confirmed by the

measurement of the optical density at 660 nm (OD_{660}), as well as by the spot method (as described by Stancu, 2020). In the spot method, the cultures (25 µl) were inoculated onto LB agar, and then the Petri plates were incubated at 30°C, for 1-3 days. The diesel biodegradation by the isolated bacteria was confirmed by fragmenting the oil film on the surface of the growth medium and by monitoring the carbon dioxide production (CO_2 mg l^{-1}) (as described by Stancu, 2022).

RESULTS AND DISCUSSIONS

In the sewage sludge samples, sludge 1 and dehydrated sludge 2 it was revealed, by the plate count agar method, the presence of the following groups of bacteria: heterotrophic, hydrocarbon-tolerant, hydrocarbon degrading, and enterobacteria. The number of these four bacterial groups varied from one sample to another (10^3-10^7 CFU ml^{-1} or g^{-1}) (Table 1).

The heterotrophic bacteria were present in higher numbers (10^5, 10^7 CFU ml^{-1} or g^{-1}) in the two samples, as compared to the number of hydrocarbon-tolerant bacteria (10^4, 10^5 CFU ml^{-1} or g^{-1}). The enterobacteria were present in higher numbers in the dehydrated sludge 2 sample (10^7 CFU g^{-1}), as compared to their numbers in the sludge 1 sample (10^3 CFU ml^{-1}). When the LB agar and MM agar were supplemented with diesel, we observed diffuse bacterial colonies or cloth bacterial growth on the plates (Table 1, Figure 1). For these two groups of bacteria, as well as for the heterotrophic bacteria we also used the most probable number (MPN) method for more precise quantification of the viable bacteria that can tolerate and/or degrade petroleum hydrocarbons that exist in the composition of diesel.

Through the MPN method, the existence of the following groups of bacteria was detected in the sludge samples: heterotrophic, hydrocarbon-tolerant, and hydrocarbon-degrading bacteria (Table 1), and like in the previous assay, their number varied from one sample to another (10^4-10^{12} cells ml^{-1} or g^{-1}). One of the most important factors for the presence of bacteria in petroleum-polluted environments is frequently represented by their ability to adapt to new and changed environmental conditions (Stancu, 2019).

The heterotrophic bacteria were present in higher numbers (10^9, 10^{12} cells ml^{-1} or g^{-1}) in the two sludge samples.

Table 1. Microbiological analysis of the waste sludge samples

Sample	Number of bacteria by						
	PCA method (CFU ml^{-1} or g^{-1})				MPN method (cel. ml^{-1} or g^{-1})		
	Heterotrophic	Hydrocarbon-tolerant	Hydrocarbon-degrading	Enterob.	Heterotrophic	Hydrocarbon-tolerant	Hydrocarbon-degrading
Sludge 1	1.8×10^5	4.0×10^4, CBG	DBG	1.0×10^3	1.7×10^9	2.0×10^8	2.0×10^4
Sludge 2	2.4×10^7	1.8×10^5, CBG	DBG	1.6×10^7	2.5×10^{12}	2.5×10^{12}	2.0×10^6
The plate count agar (PCA) method; most probable number (MPN) method; enterobacteria (Enterob.); diffuse or cloth bacterial growth (DBG).							

Most of the bacteria present in the sludge 1 and dehydrated sludge 2 sample proved to be hydrocarbon-tolerant bacteria (10^8, 10^{12} cells ml^{-1} or g^{-1}). As observed (Table 1), the hydrocarbon-degrading bacteria were present in lower numbers in both sludge samples (10^4, 10^6 cells ml^{-1} or g^{-1}) proving that only some of them have the ability to degrade petroleum hydrocarbons which are commonly very toxic for most of the bacteria (Stancu, 2018; Stancu, 2019).

The enriched culture method was further used for the isolation of hydrocarbon-tolerant and hydrocarbon-degrading bacteria from the two sludge samples, sludge 1 and dehydrated sludge 2.

As previously reported, the enriched culture method is a very effective technique for the isolation of bacteria that exist in environments polluted with petroleum and petroleum products (Stancu & Grifoll, 2011; Stancu, 2020; Stancu, 2022). The growth of hydrocarbon-tolerant and hydrocarbon-degrading bacteria was revealed by the opalescence of the culture media and by the formation of black or brown sediment in the growth tubes (Figure 1). Most petroleum hydrocarbons faced in the environment are degraded by indigenous bacteria that use these

toxic organic compounds for their growth. There are over 70 bacterial genera capable to degrade petroleum hydrocarbons, and several of them, as are the bacteria from the genera *Achromobacter, Acinetobacter, Alcaligenes, Arthrobacter, Bacillus*, *Burkholderia*, *Corynebacterium*, *Enterobacter*, *Flavobacterium, Micrococcus*, *Mycobacterium*, *Pseudomonas*, *Rhodococcus, Vibrio*, etc. has a significant role in petroleum hydrocarbons degradation (Xu et al., 2018).

As expected, the growth of the hydrocarbon-tolerant and hydrocarbon-degrading bacteria in the presence of diesel (5%) varied from one sample to another (OD_{660} 0.46-1.87) (Table 2). In the case of hydrocarbon-tolerant bacteria, we observed higher growth (OD_{660} 1.61, 1.87), compared with the growth of hydrocarbon-degrading bacteria (OD_{660} 0.46, 0.77). The results were confirmed by the fact that the isolated bacteria showed good viability (25-100%) when they were grown in the presence of diesel (5%).

The fragmentation of the diesel film (Table 2), as a result of the hydrocarbon biodegradation, was observed both in the case of hydrocarbon-tolerant, as well as in the case of hydrocarbon-degrading bacteria. We observed that the amount of CO_2 released varied from one sample to another (704-770 mg l^{-1}), indicating petroleum hydrocarbon degradation. Since the sludge samples were contaminated with petroleum hydrocarbons (TPH 1370 mg l^{-1}), the isolation of hydrocarbon-tolerant and hydrocarbon-degrading bacteria from sludge 1 and dehydrated sludge 2, was not a surprise. From the hydrocarbon-tolerant and hydrocarbon-degrading bacterial cultures obtained, six isolates were purified through repeated passages on LB agar (Figure 1).

It was reported that certain physiological properties of the bacteria isolated from the petroleum-polluted environments can increase the availability and degradation of toxic hydrocarbons.

It is well-known that the bacteria which exist in petroleum-polluted environments have the ability to produce biosurfactants. These bacterial metabolites can emulsify petroleum hydrocarbons increasing the attack surface of bacteria and the accessibility of its enzymes (Stancu, 2015; Stancu, 2018; Stancu, 2020; Stancu, 2022).

CONCLUSIONS

In the sewage sludge samples, sludge 1 and dehydrated sludge 2 it was revealed by using the plate count agar method and MPN method, the presence of heterotrophic, hydrocarbon-tolerant, hydrocarbon-degrading, and enterobacteria.

All these four groups of bacteria were detected in higher numbers (10^5-10^{12} CFU or cells) in the dehydrated sludge 2, as compared to their numbers in the sludge 1 sample (10^3-10^9 CFU or cells).

The isolation of hydrocarbon-degrading, as well as hydrocarbon-tolerant bacteria from these two sludges, was not unexpected since the sewage samples were contaminated with petroleum hydrocarbons because of human activities.

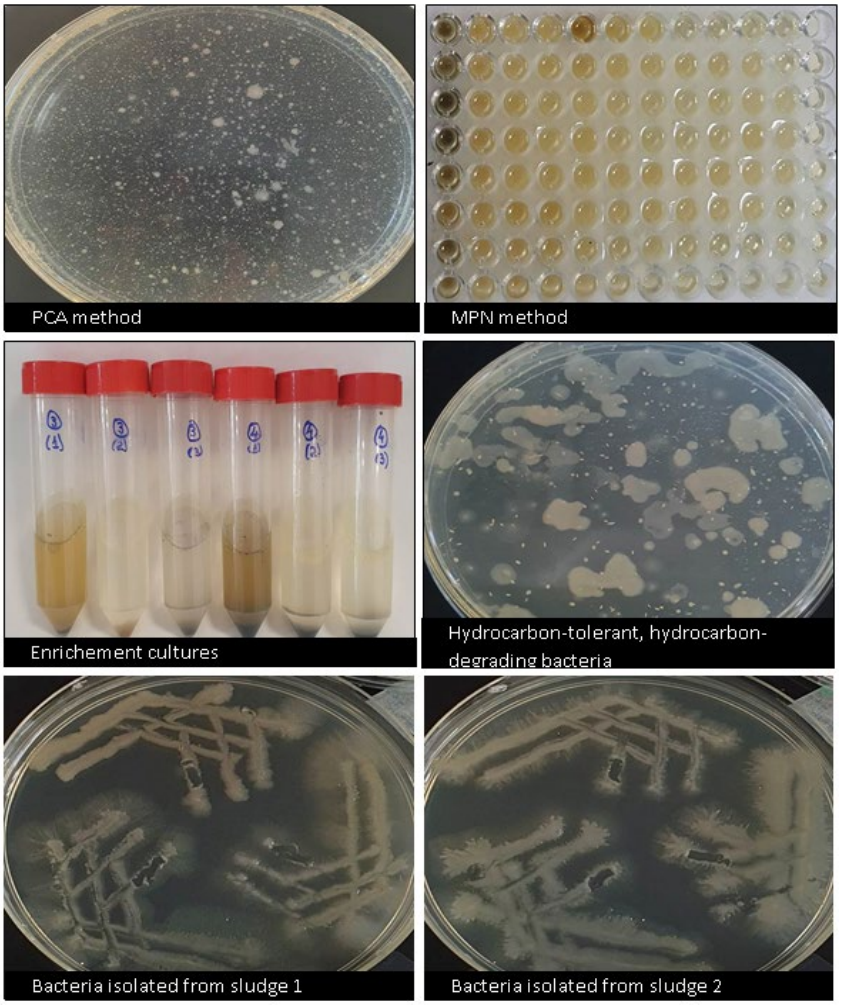

Figure 1. Enumeration and isolation of hydrocarbon-tolerant, hydrocarbon-degrading bacteria
Plate count agar (PCA) method; most probable number (MPN) method

Table 2. Characterization of the isolated bacterial populations

Sample	Bacteria							
	Hydrocarbon-tolerant				Hydrocarbon-degrading			
	Growth (OD_{660})	Viability	Diesel fragm.	CO_2 (mg l^{-1})	Growth (OD_{660})	Viability	Diesel fragm.	CO_2 (mg l^{-1})
Sludge 1	1.61	75	+	704	0.46	100	+	1100
Sludge 2	1.87	25	+	770	0.77	100	+	1230
Diesel film fragmentation (diesel fragm.), positive reaction (+); CO_2 production (CO_2 mg l^{-1}).								

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