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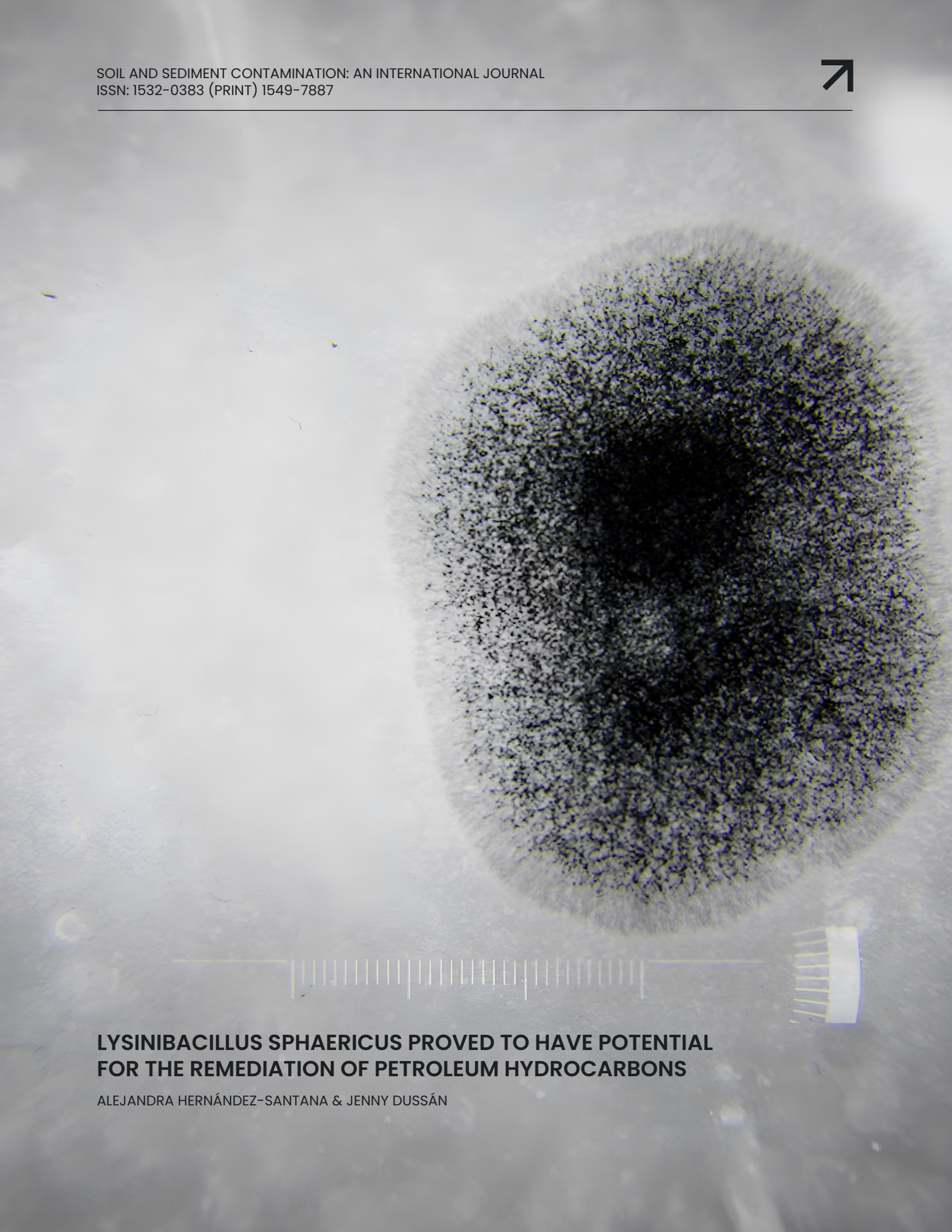

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ALEJANDRA HERNÁNDEZ-SANTANA & JENNY DUSSÁN

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ALEJANDRA HERNANDEZ-SANTANA AND JENNY DUSSAN
CENTRO DE INVESTIGACIONES MICROBIOLOGICAS (CIMIC), UNIVERSIDAD DE LOS ANDES, CRA 1 N. 18 A-12, BOGOTÁ, COLOMBIA

ABSTRACT

In this study, the ability of Lysinibacillus sphaericus to degrade aromatic hydrocarbons as well as complex hydrocarbon mixtures, such as diesel oil and oily sludge, was evaluated. L. sphaericus was able to grow when toluene, naphthalene, or phenanthrene were used as a sole carbon source in minimal salt medium. Removal efficiencies of up to 95% were found for C10-C28 hydrocarbons in the biodegradation assays of diesel oil. The biodegradation of oily sludge was evaluated in landfarming-like experiments in the open air and in completely covered containers in the field. After 50 days of treatment, the removal efficiency of total petroleum hydrocarbons in open-air and closed assays was of 84.1% and 60.1%, respectively. Furthermore, L. sphaericus was able to degrade volatile hydrocarbons (benzene, toluene, ethylbenzene, and phenol) in the headspace of closed containers, preventing the emission of these compounds to the atmosphere. L. sphaericus was herein proposed as a promising candidate to be used in bioremediation strategies of petroleum hydrocarbons.

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1. Introduction

Crude oils are composed of a diversity of aliphatic and aromatic hydrocarbons, which nowadays constitute the primary energy supply around the world (International Energy Agency, 2016). Most of these hydrocarbons are toxic, widely distributed, and highly persistent (Fuentes et al., 2016). In fact, some aromatic hydrocarbons, like benzene, tol- uene, ethylbenzene, naphthalene, and phenanthrene, have been listed as priority pollu- tants by the U.S. Environmental Protection Agency (U.S. Environmental Protection Agency, 2014) because of their known toxicity, carcinogenicity, mutagenicity, and many other adverse health effects (Bolden et al., 2015). The increasing demand for petro- leum-based products makes the exposure of all living forms to these organic com- pounds more frequent and might bring devastating consequences for the maintenance of the ecosystem.

CONTACT JennyDussan jdussan@uniandes.edu.co CentrodeInvestigacionesMicrobiologicas(CIMIC),Universidad de los Andes, Bogotá, Colombia Cra 1 N. 18 A-12.
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Taking into account the harmful effects of many hydrocarbons, there is a need for novel technologies that are able to recycle these compounds and convert them in less- or non-toxic substances. Besides, these novel technologies should overcome the operational drawbacks of existing techniques, such as high capital, operating and maintenance costs, high energy input, and production of toxic byproducts (Hu et al., 2013). In this context, bioremediation, which exploits the ability of microorganisms to degrade pollutants, is a remarkable alterna- tive. It has proven to be an efficient, cost-effective, and environmentally safe technology that offers valuable advantages, such as direct degradation, transformation of contaminants in less toxic forms, and in some cases, complete mineralization of contaminants (El-Naas et al., 2014).

The significant role of indigenous microbiota in reducing the environmental impact of hydrocarbon spills has been demonstrated (Atlas and Hazen, 2011). Since the first isolation of hydrocarbon degrading bacteria in 1913 (See S€ohngen, 1913), many other microorganisms with this ability have been reported, especially those belonging to the phyla Actinobacteria, Proteobacteria, and Firmicute (Fuentes et al., 2016). Hydrocarbon can be selectively degraded either by pure strains or consortiums of microorganisms (Bacosa and Inoue, 2015; Santisi et al., 2015; Tao et al., 2017). Although it has been demonstrated that microbial consortiums might degrade hydrocarbon more effectively than single strains (Rahman et al., 2002; Sathishkumar et al., 2008), few consortiums have been tested in the field.

Lysinibacillus sphaericus, formerly known as Bacillus sphaericus, is a gram-positive, aero- bic, sporulating, and functionally diverse bacterial species, widely known for its toxic activity against mosquito larvae (Berry, 2012; Pen~a-Montenegro et al., 2015). Biotechnological potential in the areas of toxic metals bioremediation (Bafana et al., 2015; Rahman et al., 2015) and xenobiotics degradation (Bahuguna et al., 2011; Hu et al., 2014; Misal et al., 2014) has been discovered in the Lysinibacillus group. In a recent metabolic reconstruction at genome scale, genes involved in the hydrocarbon degradation are identified in L. sphaericus, suggesting novel traits for the biodegradation of petroleum compounds (G⊠omez-Garz⊠on et al., 2017). Previous works have proved that some of the strains belonging to this species are able to degrade petroleum hydrocarbons and to produce biosurfactants (Chaudhary et al., 2015; Manchola and Duss⊠an, 2014; Mnif et al., 2015, 2011). However, there is a lack of a characterization of the full range of hydrocarbons that L. sphaericus is able to use as carbon source for growing. In this study, the ability of L. sphaericus to degrade aromatics and com- plex mixtures of hydrocarbons was evaluated.

2. Materials and methods

2.1. Lysinibacillus sphaericus strains

Two L. sphaericus strains (2362 and OT4b.31) were selected from the culture collection at the Centro de Investigaciones Microbiol□ogicas (CIMIC) at Universidad de los Andes (Bogot□a, Colombia). The selection of these two strains was made on the basis of a recent comparative genomic analysis that revealed that the L. sphaericus group comprises a novel species (G□omez-Garz□on et al., 2016). Strain 2362 is the most commonly studied representa- tive for the proposed novel species, whereas OT4b.31 is the most related strain to the type strain of L. sphaericus, KCTC 3346.

2.2. Biodegradation assays

The inoculum was prepared in nutrient broth as follows: a single colony was placed in 5 mL of nutrient broth [2.0 g yeast extract, 2.0 g peptone, and 5.0 g NaCl per liter] and incubated at 30 C and 150 rpm for 17 h. After that, a subculture was made (1% v/v) and incubated at 30 C and 150 rpm until reaching an optical density of 0.5 at 600 nm. The culture was then centrifuged for 10 min at 5,000 rpm and the cell pellet was washed twice in minimal salt medium (MSM) [0.5 g KH2PO4, 2.0 g Na2SO4, 2.0 g KNO3, 0.001 g CaCl2¢2H2O, 1.0 g MgSO4¢7H2O and 0.0004 g FeSO4 per liter] before inoculation. A bacterial inoculum con- taining 10% of the total assay volume was added to all non-control experiments to reach a final cell density of 107 cell/mL as reported by Manchola and Dussan (2014).

Hydrocarbon biodegradation assays were carried out in 30 mL of liquid MSM in capped flasks at 30□C without agitation. Assays were carried out with toluene, naphthalene, phenan-threne, and diesel oil. Toluene was supplemented to a final concentration of 50 mg L¡1 and diesel oil in a concentration of 5% (v/v). These concentrations were selected because they are normally used in biodegradation studies (de Souza Pereira Silva et al., 2015; Woods et al., 2011), and L. sphaericus was able to successfully grow under those conditions. For solid hydrocarbons, such as naphthalene and phenanthrene, a solution in acetone that contained the proper amount of hydrocarbon to reach a final concentration of 50 mg L¡1 was previously made up. This solution was aseptically added to autoclaved glass flasks, allowing the acetone to evaporate. After complete evaporation of acetone, a solution of sterile culture media and inoculum was added under a laminar flow hood so as to reach the desired final concentration of organic compound (50 mg L¡1) in the final assay volume. Abiotic controls were prepared in the same way with sterile MSM without bacterial inoculation. Control experiments without a carbon source were also set up. All experiments were run in triplicate and incubated at 30 C without agitation.

Bacterial growth was monitored by serial dilution plating on Standard Plate Count (SPC) agar every 7 days. Samples were incubated at 30 C for 24 h and the colony-forming units (CFU) enumerated.

2.3. In-field assessment of oily sludge biodegradation

2.3.1. Soil sample and oily sludge

Soil samples were collected from Palmarito exploration site, Casanare Department, Colombia (E 1.185.964, N 1.055.444); This is the same site where the oily sludge bioremediation pro- cesses were carried out. The soil type was composed of 38% clay (< 0.002 mm), 42% silt (0.02-0.002 mm), and 20% sand (2-0.02 mm). Soil characteristics were as follows: pH D 4.56; organic matter content D <25%; total nitrogen D 0.00465 ppm; total phosphorous D <4 ppm; cation exchange capacity D 8.78 meq/100 g. Soil characteristics were determined by the National Laboratory of Soils (Instituto Geografico Agustin Codazzi, 2013). Oily sludge had an API gravity of 39.3 and was collected from the Palmarito petroleum exploration site as well.

2.3.2. Experimental setup

Field experiments were carried out in the Palmarito petroleum exploration site (E 1.185.964, N 1.055.444), which has a mean temperature of 26.4C and an annual precipitation of 200–300 mm (Instituto de Hidrolog□ıa, 2014). Twelve 20 m2 plots were prepared for the landfarm- ing process. Five hundred kg of oily sludge and 1,000 kg of soil were homogeneously mixed and laid on the surface of each plot. Four different treatments were evaluated:

I. Container open to the atmosphere and inoculated with the bacterial consortium.
II. Container open to the atmosphere and not inoculated with the bacterial consortium.
III. Container totally coated with impermeable membrane and inoculated with bacterial consortium.
IV. Container totally coated with impermeable membrane and not inoculated with bacte- rial consortium.

The bacterial consortium was made up by the two strains of L. sphaericus herein studied (2362 and OT4b.31). Closed containers were made with a GSE ProFlex Geomembrane (pro- pylene/ethylene copolymer) with a thickness of 2.00 mm, density of 0.87 g cm¡3, and capac- ity of 2,000 kg. Each experiment was run in triplicate. The mixture of soil and oily sludge was daily mixed for 7 days before microbial consortium inoculation. After the addition of the microbial consortium, soil in plots with treatments open to the atmosphere were aerated weekly by tilling. Soil samples from all experiments were taken on days 8, 15, and 50 to eval- uate hydrocarbon degradation and bacterial number. Serial dilutions from 1.0 g of soil were made and bacterial growth was assessed by the plate count method on SPC agar. Samples were incubated at 30□C for 24 h and the CFU enumerated. Ten-mL samples of headspace from close containers (Treatment III and IV) were also taken on days 8, 15, and 50 to evalu- ate volatile organic compounds (VOC) concentration.

2.4. Analytical methods

2.4.1. Diesel oil

Diesel oil removal was evaluated using gas chromatography on day 28, given that the oily appearance in the degradation flasks severely diminished by the fourth week of experimenta- tion. The percentage of hydrocarbon degradation was determined by comparing the amount of hydrocarbon in inoculated samples with the amount in abiotic controls. Hydrocarbon removal was calculated as percent

recovery compared to the initial sample.
Residual hydrocarbons were extracted three times using 20 mL of dichloromethane, and the solvent extract was subjected to gas chromatography analysis in the Hewlett Packard 5890 series II plus GC system (Agilent Technologies). The system was equipped with an FID detector and Agilent HP-5MS column. The carrier gas was helium, and the rate was 2.5 mL min¡1. The oven temperature was initially set at 40 C and then raised to 100 C at the rate of 2 C min¡1. The temperature was later raised to 290 C at a rate of 10 C min¡1. Quantifica- tion was made by the addition of the external standard 47518-U (Supelco).

2.4.2. Total petroleum hydrocarbons in oily sludge

Residual hydrocarbons in 25.0 g of soil were extracted three times using dichloromethane, and the solvent extract was subjected to a gas chromatography analysis in the Shimadzu Gas Chromatograph GC-2014. The system was equipped with a FID detector and a DB-1 column. The carrier gas was helium and the rate was 1 mL min¡1. The oven temperature was initially set at 35 C for 14 min and then raised to 210 C at a rate of 10 C min¡1. Finally, the temperature was raised to 320 C at a rate of 40 C min¡1. The detector's temperature was set at 300 C and the injector temperature at 250 C. The percentage of hydrocarbon degradation was determined by comparing the amount of hydrocarbon in inoculated samples with the amount in abiotic controls. Hydrocarbon removal was calculated as percent recovery compared to the initial sample. Quantification was made by the addition of the external standard n-Hydrocarbon mix (46855-U Supelco).

2.4.3. VOC emissions

Four volatile organic compounds were evaluated in the headspace of closed containers: ben- zene, ethylbenzene, toluene, and phenol. These compounds were considered because they are readily volatilized, persistent in nature, and have high potential to exert carcinogenic, toxic, and mutagenic effects (Masih et al., 2016; Miri et al., 2016). Samples from the head- space of closed containers were taken by injecting a needle and extracting 10 mL of the gas- eous phase of the containers in a syringe on days 8, 15, and 50. The concentration of the four volatile compounds was determined by using the ppbRAE MiniRAE plus (10.6 eV lamp, RAE systems) calibrated with 10 ppm isobutylene (RAE Systems, 2016). The correc- tion factor for each gas was called up from the internal memory of the instrument, and the concentration of each organic compound was calculated. This instrument was chosen given that it is easily portable and is designed for field use.

2.5. Statistical analysis

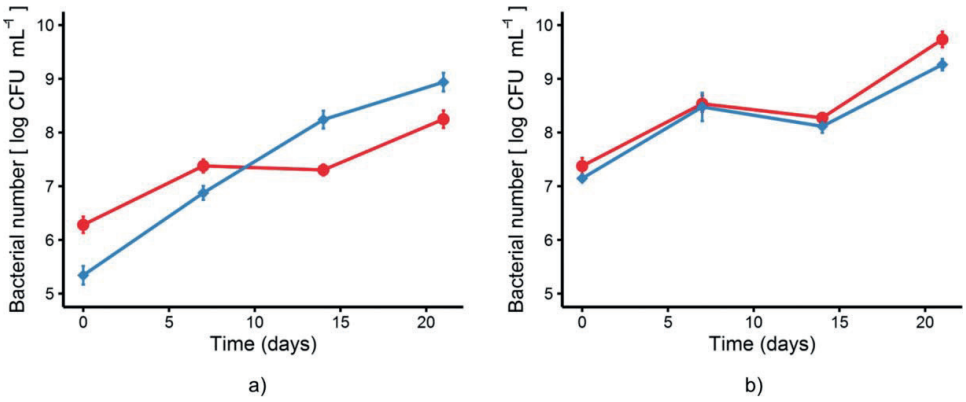
Statistical analysis and graphs were made in R statistical package (R Development Core Team, 2016). A significance level of 0.05 was chosen in all cases. Significance was evaluated by means of ANOVA. Assumptions for normality and homoscedasticity were evaluated prior to use of ANOVA.

3. Results and discussion

3.1. Biodegradation assays of aromatic hydrocarbons and diesel oil

The utilization of hydrocarbons by L. sphaericus was evidenced by significant increases in cell numbers in the experiments in which different aromatic hydrocarbons and diesel motor fuel were individually provided as sole carbon source (Figure 1). Increases of up to four orders of magnitude in the cell number were observed in 21 days of experimentation. Both strains 2362 and OT4b.31 exhibited similar growth patterns in the tested hydrocarbons. By contrast, in the control experiment with no carbon source, the bacterial number remained nearly constant (data not shown), which dismissed nutrient reserves as being responsible for growth in biodegradation assays. Thus, even though the concentration of aromatic hydrocar- bons was not monitored throughout time, L. sphaericus was able to use the provided hydro- carbons for growth. This was assumed as a metabolic activity towards the tested aromatic and aliphatic hydrocarbons.

The diesel oil assays were also monitored for hydrocarbon degradation. Table 1 shows the reduction in the concentration of selected hydrocarbons by both L. sphaericus strains. Chro- matograms can be found in Fig S1 and Fig S2. The significant growth of L. sphaericus in diesel oil (Figure 1d) was accompanied by a reduction up to 95% in the concentration of light and medium hydrocarbons in 28 days of experimentation. This is comparable with the diesel degradation rate obtained by Morales-Guzm an et al. who reported 97.9% of diesel removal



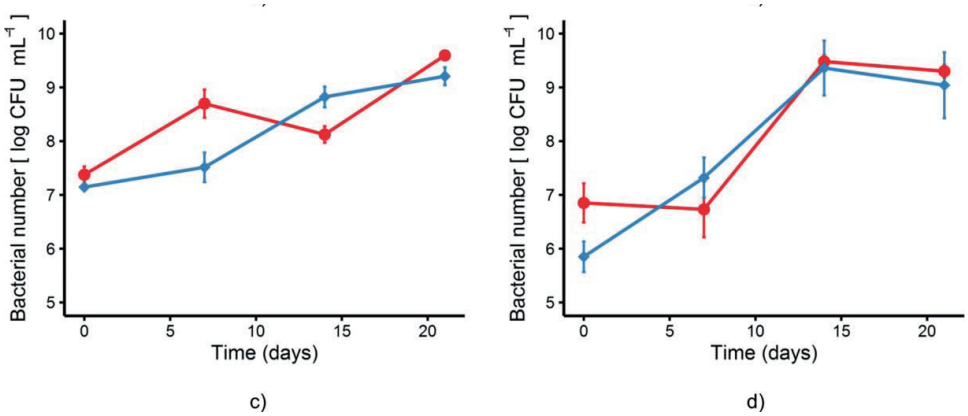


Figure 1.

Time course of L. sphaericus 2362 (circles) and OT4b.31 (diamonds) growth in MSM containing 50 mg L¡1 of a) toluene, b) naphthalene, c) phenanthrene, and d) diesel oil as a sole carbon source. Cells were grown at 30 C for 21 days. Values represent the average of triplicate determinations. Intervals at 95% of confidence are shown.

by Serratia marcescens C7S3A after 8 days of diesel biodegradation assays with 1% of diesel oil (v/v) (Morales-Guzm⊠an et al., 2017). Both L. sphaericus strains exhibited a similar behavior toward the evaluated hydrocar- bons, except for dodecane, in which strain 2362 showed a higher removal than strain OT4b.31. Although dodecane exhibits physical and chemical properties resembling other light-chain hydrocarbons, the degradation efficiency was not necessarily expected to be simi- lar to the one of closely related hydrocarbons. For example, Varjani and Upasani demon- strated that dodecane and tetradecane in crude oil are not degraded at comparable extents by Pseudomonas aeruginosa NCIM 5514, even when both compounds are similar in struc- ture and molecular weight (Varjani and Upasani, 2016).

3.2. In-field assessment of oily sludge degradation

The biodegradation of TPH in oily sludge by L. sphaericus was also determined. Bacterial growth was monitored throughout 50 days of the experiment and estimated by the plate count method. Figure 2a shows the time course of bacterial number in experiments carried out in the field. Bacterial numbers for Treatments II, III, and IV presented a small increase in the first 15 days, and a slight decrease by the end of the experiment. The bacterial number

Table 1.

Degradation of selected hydrocarbons of diesel motor fuel in L. sphaericus OT4b.31 and 2362. The assays were incubated at 30 C without agitation during 28 days. Data for abiotic control are also shown.

Strain	Hydrocarbon	Initial concentration (mgL-1)	Final concentration (mgL-1)	Removal (%)
OT4b.31	Decane	22.7	0.990	95.6
	Dodecane	51.0	47.8	6.29
	Tetracosane	14.0	2.50	82.2
	Hexacosane	9.33	1.47	84.2
	Octacosane	1.05	0.641	38.9
2362	Decane	30.9	1.38	95.5
	Dodecane	74.1	8.62	88.4
	Tetracosane	16.8	1.61	90.4
	Hexacosane	8.86	0.80	91.0
	Octacosane	1.48	0.42	71.6
Abiotic control	Decane	39.3	30.3	22.9
	Dodecane	74.7	72.9	2.41
	Tetracosane	16.1	15.8	1.86
	Hexacosane	4.73	2.97	37.2
	Octacosane	1.32	0.932	29.4

in open and inoculated assays (Treatment I) in the field decreased by around 3 orders of magnitude by the end of the experiment. Since most degradable fractions were depleted in the initial phase of the experiment – especially for Treatment I, in which degradation effi- ciency was the highest (84.1%) – the decrease in cell number might be caused by either low hydrocarbon availability or high recalcitrance of remaining compounds. This same trend has been observed when hydrocarbon-degrading consortia are used for the remediation of petroleum contaminated soils (Poi et al., 2017; Wang et al., 2016; Wu et al., 2017). In those cases, the drop in bacterial numbers has been attributed to the usage of the biodegradable organics.

Residual TPH in the soil decreased throughout time for all treatments (Figure 2b). Fig S3 and S4 present the final chromatograms for open and closed experiments, respectively. Treat- ment I, which consisted of open and inoculated containers, presented the highest removal rate (84.1%). However, Treatment II, which consisted of open but not-inoculated containers, had a removal rate of 52.6%, suggesting that either volatilization or biodegradation by indigenous microbiota contributed a great extent to the removal rates for Treatment I. Lower removal.

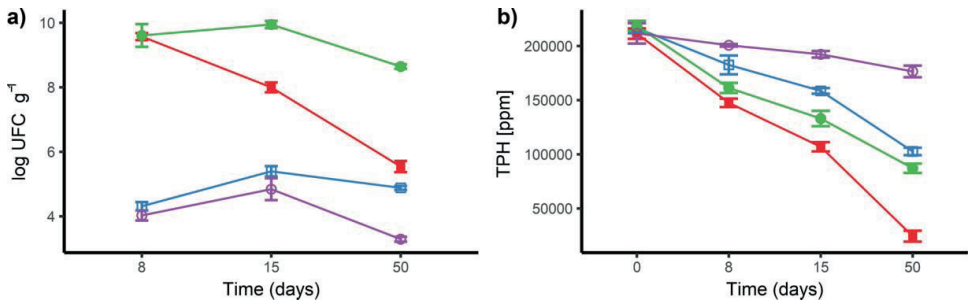


Figure 2.

Time course of a) bacterial number and b) TPH concentration throughout landfarming-like experiments in-field. Bars represent confidence interval at the 95% confidence level. Squares represent open assays and circles represent closed assays. Filled geometric shapes represent the assays where bacte- rial consortium was inoculated.

rates were obtained for Treatments III and IV, which consisted of inoculated and non-inocu- lated closed containers, respectively. Although the closed inoculated containers presented TPH removal to a lesser extent than the open inoculated containers, the percentage of TPH removal in closed inoculated containers (60.1%) was significantly ($p < 0.05$) higher than the percentage of TPH removal in closed containers without inoculation (16.6%). This supports the capability of the bacterial consortium under study to degrade TPH in soils contaminated with oily sludge.

When comparing the degradation rates for Treatments I and II with the ones for the Treatments III and IV, it seemed that volatilization –more than biodegradation by indige- nous microbiota – played a key role in TPH removal from the open containers. However, differences in oxygen availability between open and closed containers could also contribute to different degradation rates for both inoculated Treatments (I and III). Further analyses of organic compounds emissions were needed in order to determine the importance of volatili- zation in the TPH removal rates.

Benzene, ethylbenzene, toluene, and phenol were analyzed throughout the experiment in the headspace of closed containers (Figure 3). High concentrations of volatile hydrocarbons were found, suggesting a key role of volatilization in the removal of hydrocarbons during landfarming-like processes and raising concerns about the likeliness of air pollution during these processes. In fact, the concentrations of benzene and phenol were above the acceptable short-term exposure limit (STEL) for most part of the experiments. Acceptable STELs for benzene and phenol are 5 ppm and 15.6 ppm, respectively (The National Institute for Occu- pational Safety and Health (NIOSH), 2017). Similarly, Hejazi et al. stated that landfarming processes pose health risks through the inhalation exposure route to site workers due to the volatilization of light organic compounds (Hejazi et al., 2003).

It is important to highlight that the concentration of VOC in Treatment III assays decreased throughout the experiment (Figure 3a), whereas in Treatment IV, they remained almost constant except for the phenol which slightly decreased in concentration (Figure 3b). This was attributed to VOC degradation by the bacterial consortium, preventing volatile hydrocarbon emissions. Open assays showed higher removal rates (which were consistent with the high volatilization of VOC), but closed assays were a cleaner alternative to bioreme- diation processes. Moreover, the consortium under study degraded the volatile fraction in oily sludge, which led to the suggestion of the consortium herein studied as a powerful pro- spective tool for bioaugmentation in landfarming technologies.

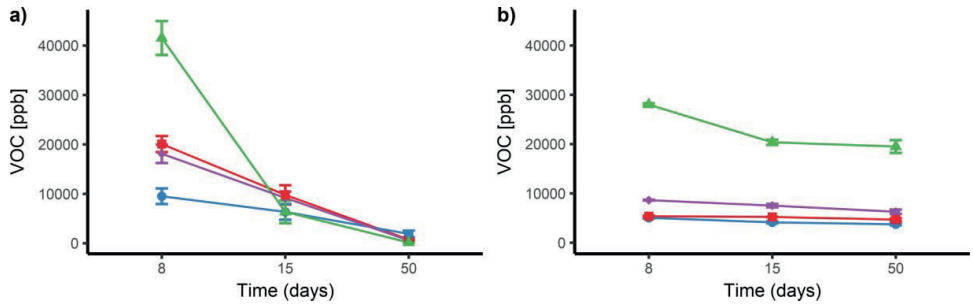


Figure 3.

Time course of VOC emissions in-field landfarming-like experiments. Treatments a) III and b) IV are shown. Bars represent confidence interval at the 95% confidence level. Triangles represent phenol, squares represent benzene, circles represent ethylbenzene, and diamonds represent toluene.

In comparison with other hydrocarbon-degrading organisms, L. sphaericus exhibits char- acteristics that make this species attractive for bioremediation of petroleum contaminated sites. Specifically, it has been demonstrated that L. sphaericus can grow in high concentra- tions of toxic metals such as arsenic, lead, and chromium (Lozano and Duss an, 2013), which are normal constituents of crude oil. Besides, L. sphaericus is able to produce biosurfactants that increase the bioavailability of hydrocarbons (Manchola and Duss an, 2014), which could make it easier for the cells to incorporate the hydrophobic hydrocarbons for degradation.

4. Conclusion

The functional potential of L. sphaericus for hydrocarbon biodegradation was demonstrated. It was found that L. sphaericus was able to grow using different mono- and polycyclic aro- matic hydrocarbons, as well as complex mixtures of hydrocarbons as a sole carbon source. Growth was accompanied by a reduction in the concentration of C10-C28 in the degradation flasks for diesel oil.
L. sphaericus also showed the ability to degrade TPH and volatile hydrocarbons in oily sludge in landfarming-like processes in-field. The highest percentage of TPH removal was obtained in the open containers where the consortium had been added. However, TPH removal in closed inoculated containers, though slower, prevented VOC emissions to the atmosphere. These results prompt us to propose the closed microbially-inoculated technique as a powerful prospective tool to overcome one of the current limitations in landfarming technologies: air pollution. Even though the molecular mechanisms underlying hydrocarbon degradation in L. sphaericus remain to be known, L. sphaericus proved to be able to degrade a wide array of petroleum hydrocarbons; therefore, this microorganism could be incorpo- rated in bioaugmentation strategies for hydrocarbons remediation.

Disclosure statement

The authors declare no conflict of interest.

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