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Bacterial seeding potential of digestate in bioremediation of diesel contaminated soil

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Abstract

Petroleum contaminated soils constitute an environmental problem which may be solved with the help of bioremediation. Soil bioaugmentation with petroleum degrading bacteria is an efficient clean-up strategy. Currently scientific interest focuses on finding sources of microbial agents able to degrade hydrocarbons which may serve as species pools for enrichments during inoculum preparation. Seven microbial enrichments were obtained from various digestates, from a petroleum contaminated soil and from mix of both: soil and digestate. Diesel degrading capacities of microorganisms from digestates, which may serve as inoculum and nutrient source for bioremediation, were assessed. Additionally, the presence and abundance of alkane monooxygenase encoding genes (*alkB*) was estimated in enrichments. The highest proportion of *alkB* genes was found for enrichments originating from composted digestate of organic fraction of municipal solid waste and petroleum contaminated soil having the most efficient diesel removal rates (78 and 77 % diesel removal, respectively). Enrichments obtained from digestate mixed with soil have a lower performance than single source enrichments. Strains belonging to *Rhodococcus* and *Achromobacter* genus were found in all enrichments, and *Rhodococcus* dominated in enrichment with the higher diesel degradation potential. All those results indicate a great potential of digestate as a source of diesel degraders for soil bioremediation.

Key words: alkanes; *alkB* genes; bioaugmentation; hydrocarbons; organic fertilizers

1. Introduction

Soil contaminated with petroleum products is a problem all over the world (Lu et al., 2014). Due to toxicological risks, the use and management of polluted lands are limited. Among soil treatment strategies, bioremediation gains a lot of interest (Megharaj et al., 2011). Due to combination of natural ability of microorganisms to metabolize the contaminants with environmental engineering approaches, bioremediation represents a cost-efficient strategy (Aburto-Medina et al., 2012).

The most efficient bioremediation strategies are based on soil bioaugmentation with contaminant degrading microorganisms and nutrient supplementation (Mrozik and Piotrowska-Seget, 2010; Agnello et al., 2016). Oil reservoir production water (Song et al., 2018) or total petroleum hydrocarbons (TPH) contaminated soils can be sources of petroleum degrading microorganisms used as inoculum for bioremediation. However, in case of high concentration of petroleum products, and thus limited growth of soil microflora or previous implementation of chemical oxidation treatments, other sources of inoculum need to be implemented.

According to organic waste recycling strategies, organic fertilizers constitute a resource saving alternative over mineral products and have lower environmental impact. In last years, the use of digestate as organic fertilizer gains interest in soil science due to high concentration of nutrients in bioavailable form and low level of easily degradable carbon (Tambone et al., 2010; Walsh et al., 2012; Kataki et al., 2017). Although bioremediation has been studied for last 25 years, still little is known about contribution of bacteria from organic fertilizers to the overall efficiency of contaminant removal. On the one hand, bacteria from aged contaminated soils have already developed necessary metabolic apparatus to degrade hydrocarbons (*e.g.* alkane monooxygenases encoded by *alkB* genes) but on the other hand bacteria from digestate are adapted to degrade various types of organic compounds and have wide metabolic capacities. It is thus interesting to compare microbial diversity and petroleum degrading efficiency of both matrices. Here, for the first time, we compared diesel degradation efficiency of microbial enrichments originating from different types of digestates with enrichments from industrial hydrocarbon contaminated soil as well as we studied the structure of enriched microbial communities.

The goals of the study were to: (i) assess if digestate can be a potential source of diesel degrading microorganisms for soil bioremediation as well as (ii) study the microbial diversity

and verify which bacteria were dominant in mixed enrichments (bacteria originating from soil or from digestate) or if new species were promoted. In this study we have performed liquid batch experiments to study diesel degradation efficiency of bacterial enrichments originating from sewage sludge digestate, composted digestate of organic fraction of municipal solid waste (OFMSW), a TPH contaminated soil and mixed sources.

2. Materials and Methods

2.1. Soil and digestate

The hydrocarbon contaminated soil was obtained from a petroleum refinery located in north-east of France. Before use, the soil was air dried under fume hood and sieved through <2 mm. Fresh and powder sewage sludge digestate were obtained from a biogas plant (Limoges, France). Powder digestate was prepared from fresh digestate by solid liquid separation and dehydration in the biogas plant. Composted digestate of OFMSW was produced by a biogas plant (southern Italy). Detailed characteristics of soil and digestates are specified in Table 1. Initial physicochemical characterization of soil and digestates was performed by Synlab (France) which is a certified laboratory.

Table 1 Characteristics of soils and digestates used in the study. Mean (n = 3) and standard deviation. TOC: total organic carbon; TN: total nitrogen; TPH: total petroleum hydrocarbons; P: total phosphorus; n.d. not determined.

2.2. Experimental design

To evaluate diesel degradation potential of different enrichments, digestates and soil were incubated in variants presented in Table 2. In brief, fresh sewage sludge digestate (2 g) and/or powder sewage sludge digestate (0.4 g) and/or dry composted OFMSW digestate (0.4 g) and/or soil (5 g) were added to sterile glass flasks containing 50 mL of modified Bushnell-Haas (BH) medium broth. The composition of the BH broth was as follows: MgSO₄ (0.2), CaCl₂ (0.02), KH₂PO₄ (1.0), (NH₄)₂HPO₄ (1.0), NH₄Cl (3.5) and FeCl₃ (0.05) grams per liter. The broth was adjusted to a final pH of 7.2 ± 0.1 and autoclaved. Diesel obtained from a local gas station was used as a supplemented carbon source in 1% (v/v) concentration. Prior to addition, diesel was sterilised by filtration using 0.2 µm syringe filter. The flasks were placed on a rotary incubator shaker at 160 rpm and 21°C and incubated for one week. After this time, 1 mL of enrichment was transferred into the fresh BH medium with diesel as sole carbon

source. The procedure was repeated 3 times. Before use, bacterial enrichments were harvested by centrifugation for 15 min at 5000 rpm and washed 4 times with phosphate buffer saline (PBS) pH 7.2. Then, bacteria were resuspended in 10 mL of BH broth and adjusted to 1.5 optical density (600 nm) to ensure a similar amount of biomass initially added in the degradation study. Inoculum (1 mL) was then added to 50 mL of BH broth with 1% (v/v) concentration of diesel and incubated for 21 days to monitor TPH degradation efficiency (Song et al., 2018). Abiotic controls were run in parallel using 0.05 g of NaN₃ as microbial growth inhibitor (Table 2). All the experiments were performed in triplicate.

Table 2 Composition of enrichments originating from different samples.

2.3. Diesel quantification

To quantify remaining diesel at the end of the experiment, full flasks were extracted with 15 mL of hexane by mixing (1 min) on a vortex. Extraction procedure was repeated 3 times and the extracts were pooled. Diesel was quantified on gas chromatography with flame ionization detector (Shimadzu) with capillary column 30 m × 0.25 mm × 0.25 μm (ZB5HT Inferno, Phenomenex) according to the procedure described elsewhere (Gielnik et al., 2019).

2.4. Microbial respiration

Microbial respiration was monitored during the incubation with the use of Oxitop[®] system. Briefly, a volume of 20 mL BH broth supplemented with diesel (1 % v/v) and 0.4 mL of appropriate inoculum was placed in Oxitop[®] gas tight flasks equipped with a CO₂ trap (solid NaOH) and incubated on rotary shaker at 160 rpm and 21 °C for 21 days in parallel with experimental batch for diesel degradation. Oxygen up-take in the flasks was measured every 4 hours and registered in the measuring Oxitop[®] heads as a pressure drop in hPa. Calculations were based on to the equation 1.

$$BR = \frac{M(O_2)}{R \cdot T_m} \cdot \left(\frac{V_b - V_s}{V_s} + \alpha \frac{T_m}{T_0} \right) \cdot \Delta p(O_2) \quad (1)$$

where BR: basal respiration (mg O₂ L⁻¹); α: Bunsen absorption coefficient; M(O₂): molecular mass of O₂ (mg mol⁻¹); V_b: bottle volume (L); V_s: sample volume (L); Δp(O₂): pressure

difference (mbar); R: perfect gas constant ($\text{L mbar mol}^{-1} \text{K}^{-1}$); T_0 : reference temperature (273.15 K); T_m : measured temperature (K).

2.5. DNA extraction

Genomic DNA was extracted at the beginning and at the end of experiment from 200 μL of freeze samples using Fast DNA Spin Kit for Soils (MP Biomedicals). Extracted DNA was eluted in 100 μL of DNA free ultra pure water. DNA concentration and purity were determined using spectrophotometer UV-1800 (Shimadzu) equipped with a TrayCell adaptor for micro-volumes (Hellma) (Biache et al., 2017). DNA was stored at -20°C for further analyses.

2.6. Real-time PCR analysis

Real-time PCR analysis were performed according to previous studies (Cébron et al., 2008; Cébron et al., 2015). The extracted genomic DNA was used to quantify total bacterial and fungal populations using 968F/1401R (Felske et al., 1998) and Fung5f/FF390r (Smit et al., 1999; Vainio et al., 2000) primer sets targeting bacterial 16S rRNA and fungal 18S rRNA genes. Functional genes, *i.e.* alkanes monooxygenases genes, were quantified using primers alkBFd/alkBRd (Powell et al., 2006). Real-time PCR quantifications were performed using CFX96 C1000TMRReal Time system (Bio-Rad) and dilution series of standard plasmids.

2.7. Sequencing analysis

Next Generation Sequencing Illumina MiSeq v3 run ($2 \times 300 \text{ bp}$) of the V3-V4 region of the 16S rDNA was performed by MicroSynth (Switzerland) on previously isolated DNA. The company is ISO certified according to 9001:2008 and ISO / IEC 17025. Library preparation included sample quality control and Nextera two step PCR amplification using primer set 341f_ill/802r_ill, PCR product purification, quantification and equimolar pooling. Bioinformatic analysis included demultiplexing, merging of forward and reverse reads, quality filtering, trimming, chimera removal, operational taxonomic unit (OUT) clustering (97 % identity threshold) and subsampling for even sample size (rarefaction to the lower number of reads per sample). Taxonomy assignment was based on the SILVA 16S database v.123 (> 60

% confidence). Alpha diversity calculation and comparative statistics were done using Phyloseq and DeSeq2 (R packages). Heat map was constructed using Heatmapper software. Sequencing reads are available in the GenBank database with submission number SUB5445613 and BioProject ID PRJNA532594.

3. Results and discussion

3.1. Diesel degradation and oxygen uptake

Diesel degradation by the seven tested enrichments is presented in Figure 1 as a value relative to control. The obtained results have shown significant diesel removal for all enrichments compared to control (ANOVA; $P \leq 0.05$). Diesel removal efficiency was as follows: III=VII>I=IV>V>II>VI (ANOVA; $P \leq 0.05$). Diesel removal by bacteria originating from soil (VII) and from OFMSW composted digestate (III) have reached the highest values (77 and 78 %, respectively), which were not significantly different (ANOVA; $P \leq 0.05$). The lowest contaminants removal was observed for variants representing fresh sewage sludge digestate with soil (VI) and powder sewage sludge digestate with soil (II), reaching 64 and 68 %, respectively.

Figure 1 Percentage of diesel decrease after 21 days for all tested variants relative to the control. Symbols explained in Table 2. Mean ($n = 3$) and standard deviation. Values followed by the same letter are not statistically different (ANOVA; $p > 0.05$).

During incubation time, microbial metabolic activity was studied through respiration measurements (i.e. the amount of oxygen uptake is displayed in Fig. S3). The observed diesel removal values for the different enrichments were correlated with the respiration intensity (Pearson correlation; $r = 0.898$; $p = 0.006$). For all the enrichments, except IV, intense respiration was observed within the first 10 days of the experiment representing more than 75 % of total oxygen up-take of the 21-days incubation (Fig. S3). This increase reflects the phase of exponential growth occurring in microbial cultures at the beginning of incubation. After this time, activity started to stabilize, and this trend continued until the end of the experiment. This may be related to the depletion of preferable carbon source among diesel components and possible accumulation of toxic metabolites. Observed respiration intensity suggests that most of diesel was degraded in the first 10 days of the experiment.

In variants I, III, V and VII, the initial lag phase lasted a few hours, while in variants enriched from mixed sources (II, IV and VI), the initial lag phase was more prominent (Fig. S3). In variant IV, lag phase was observed in the first 4 days while in variants II and VI, the initial lag phase lasted for one day. It was reflected by significantly lower hydrocarbon degradation for these enrichments (II, IV, VI) in comparison with cultures developed from a single source (I, III, V, VII). The lag phase in mixed source enrichments suggests longer time needed for bacterial adaptation and development of new microecological balance as well as competition between the microbial populations of two different origins. In previous studies, addition of exogenous inoculum as activated sludge to TPH contaminated soil slurry solution has proved to increase treatment efficiency by 17 % in comparison with supplementation only with mineral nutrients (Aburto-Medina et al., 2012). However, higher efficiency of mixed culture over soil culture is not in accordance with our observations.

In the study of Souza et al. (2015), 69% of alkanes from diesel were removed in one week by a consortium composed of bacteria and yeasts isolated from petroleum contaminated environments. Other authors have observed that microbial consortia composed of *Bacillus* and *Geomyces* species isolated from contaminated soils were able to remove 87 % of TPH after 30 days of incubation (Maddela et al., 2016). Nkem et al. (2016) assessed diesel removal by *Cellulosimicrobium cellulans* and *Acinetobacter baumannii* species and noted respectively 64 and 58 % removal within 10 days. In the study of Joy et al. (2017), *Achromobacter* sp. and *Ochrobactrum* sp. removed respectively 46 and 39 % of crude oil within 7 days. Comparison of the above-mentioned experiments is presented in Table 3. Degradation values reported by other authors are comparable with those observed in the present study.

Table 3 Comparison of petroleum products biodegradation potential of different microbial consortia. TPH: total petroleum hydrocarbons; BH: Bushnell-Haas medium; MSM: minimal salt medium; MM: mineral nutrients

Our results show that all digestate enrichments contain bacteria able to degrade diesel. Interestingly, bacteria originated from weathered contaminated soil (i.e. biomass already adapted to high TPH concentrations) did not have a significant advantage over bacteria from composted OFMSW digestate, thus we can conclude that bacteria originating from digestate can contribute to the degradation of petroleum products in the same extent as microorganisms originating from hydrocarbons affected environments.

3.2. Microbial density and presence of functional genes

Initial fungal abundance in enrichments originating from powder sewage sludge digestate and composted OFMSW digestate (I, III) were low, while in enrichments from fresh sewage sludge digestate fungal counts were 3 orders of magnitude higher (Fig. S4). Fresh sewage sludge digestate with 81.1 % of water content is a suitable environment for fungi development. Interestingly, enrichments using a mixture of powder sewage sludge digestate or composted OFMSW digestate with soil (II, IV) revealed high fungal abundance, exceeding values observed in enrichment with soil alone (VII). For cultures with the highest removal rates (VII, III, I) the lowest fungal counts between 10^4 and 10^6 18S rRNA gene copies mL⁻¹ of enrichment were observed at the end of the experiment, while for the less efficient cultures (II, IV, V) final fungal abundance was significantly higher. In most of enrichments fungal abundance tended to decrease with time, confirming previous observations that fungal development is not favoured in batch cultures (Biache et al., 2017).

Bacterial counts increased during the experiment in all enrichments, and after 21 days bacterial concentration reached similar level above 10^8 16S rRNA gene copies mL⁻¹ in all samples (Fig. S4). Initial bacterial counts were not correlated to diesel removal efficiency.

Composition of diesel is diverse and differs according to the production procedures. Diesel contains many different chemical components including branched chain alkanes, alkyl benzenes and polycyclic aromatic hydrocarbons (PAHs), however its main components are linear alkanes. *AlkB* genes encoding alkane hydroxylases belonging to monooxygenases are proved to enable degradation of alkanes with different carbon chain length (Powell et al., 2006; Jin and Kim, 2017). The hydroxylation of alkanes may occur in terminal or sub-terminal position and is initiated by alkane hydroxylases which oxidise alkane to alcohol (Fuentes et al., 2014). Alcohol is further oxidized to aldehyde by alcohol dehydrogenase. Aldehyde is subsequently oxidized to carboxylic acid by aldehyde dehydrogenase and serves as a substrate for acetyl coenzyme A (acyl-CoA) synthesis which is further funnelled into β -oxidation pathway (Fuentes et al., 2014).

Figure 2 Copy number of *alkB* genes at the beginning and at the end of the experiment. Symbols correspond with Table 2. Mean ($n = 3$) and standard deviation. Values that are annotated with the same letter among one sampling time are not significantly different (Tukey's multiple range test with $p = 0.05$).

In all tested enrichments, initial concentration of *alkB* genes has increased after 21 days and the final concentration has exceeded 10^7 gene copies mL⁻¹. Hence, there is no doubt that

alkB genes were promoted during bacterial enrichment. The highest final concentration of *alkB* genes was observed for variant VII which was in accordance with the highest diesel removal rate. In other studies, it was observed that copy number of *alkB* genes was also positively correlated with TPH degradation (Salminen et al., 2008; Gielnik et al., 2019). In our study initial and final copy number of *alkB* genes for all variants were not directly correlated with diesel removal (Fig. 2).

3.3. Microbial community structure

Bacterial diversity in the enrichments was analysed through 16S rRNA gene Illumina MiSeq sequencing. The diversity of the seven enrichments were analysed in triplicate at the end of the incubation. Total number of obtained reads was 1,204,354, the samples were normalized to 72,290 sequences per sample. All the samples reached good diversity coverage (see rarefaction curves as Supplementary materials, Figure S1). Total number of obtained OTUs was 35, 7 OTUs were found in all samples (representing 20.0 % of the sequences), 12 OTUs were found in 90 % of samples (34.3 % of OTUs) and 24 OTUs were found in 50 % of samples (68.6 % of OTUs). Shannon diversity index ranged from 1.2 to 2.2 and Chao1 richness ranged from 21.7 to 37.7 (Table S2).

Two bacterial phyla were detected in all samples: *Actinobacteria* and *Proteobacteria* (Fig. S5) in different ratios. The three most efficient cultures (III, VII and I) had significantly the highest level of *Actinobacteria* (50-60%) compare to *Proteobacteria*. In a previous study, analysis of crude oil degrading microbial consortia originating from reservoir production water showed a majority of bacteria assigned to *Proteobacteria* phyla (Song et al., 2018). Also, it was reported that *Proteobacteria* is the predominant phylum in highly petroleum contaminated soils when compared to uncontaminated soils (Abed and Al-kind, 2015). *Actinobacteria* is also a phylum common in soil (Song et al., 2018). Both detected phyla contain microbial taxa known to degrade petroleum products (Song et al., 2018).

The taxonomic specification of the cultures at the genus level is presented in Figure 3 and the list of identified species is given in the supplementary materials (Table S1). *Rhodococcus* and *Achromobacter* were the most common genera detected in all samples. Enrichments with the highest diesel degradations yields (III, IV, I) were dominated by *Rhodococcus* species. *Rhodococcus* genus is well known from TPH degrading capacities

(Cappelletti et al., 2017). Species within the genera possess wide spectrum of alkane monooxygenases and can utilize wide range of organic contaminants as primary substrates or as co-substrates. Bacteria within *Rhodococcus* genus are also capable of biosurfactant production which facilitate the contact with hydrophobic compounds and thus improves substrate availability (Cappelletti et al., 2017). *Achromobacter* species were already isolated from crude oil contaminated soils and have proved ability to metabolize petroleum products (Marecik et al., 2015), such as *Achromobacter xylosoxidans* growing on alkanes (Tanase et al., 2013).

Figure 3 Heatmap profile presenting samples diversity on genus level based on relative abundance. Clustering was based on Pearson correlation. Numbers 1, 2 and 3 indicate the three replicates.

Stenotrophomonas species were detected in samples I, II, III, VI and VII and were dominant in enrichments IV and V. This genus was found in diesel contaminated environments (Martin-Sanchez et al., 2018) and members of this genus were detected during crude oil degradation in batch treatments (Jin and Kim 2017).

Pseudomonas species were detected in samples II, IV, V, VI. This genus is also rich in well recognized TPH degraders, carrying various types of *alkB* genes and ability to produce biosurfactants (Wang et al., 2013). Detected during this study *Pseudomonas veronii* (cultures II, IV, V, VI) was observed to degrade wide spectrum of hydrocarbons including aliphatic hydrocarbons, BTX and various PAHs (Máthé et al., 2012). Interestingly the species was not detected in the most efficient diesel removing cultures (VII, III and I), suggesting that the process efficiency depends not only on the presence of single diesel degrading species but rather on the structural balance of the whole microbial community.

Members of *Gordonia* genus were detected only in samples enriched from sewage sludge fresh digestate (V) and constituted the most abundant bacterial group in this enrichment. This genus is ubiquitous in soils and has ability to degrade a wide range of organic compounds including petroleum products as well as can produce biosurfactants (Cappelletti et al., 2017).

Members of *Bordetella* genus were detected only in samples enriched from powder sewage sludge digestate (I). Species of *Bordetella* were previously isolated from TPH contaminated soils and are associated with TPH degradation (Wang et al., 2016).

Species belonging to *Microbacterium* genus were detected in all samples excluding enrichment from mixed soil and composted digestate (IV). This genus contains bacterial

species with crude oil degrading capacities (Muthukamalam et al., 2017). However, *Microbacterium oxydans* which was detected during the present study was observed to degrade complex polysaccharides like alginic acid, which is widely distributed in cell walls (Jung et al., 2013). This may suggest growth on dead biomass produced during incubation.

Members of *Rhodanobacter* genus were detected within samples V and VI, corresponding with enrichment originating from sewage sludge fresh digestate and sewage sludge fresh digestate with soil. This genus contains known hydrocarbon utilising bacterial strains (Abass et al., 2018). *Rhodanobacter lindaniclasticus*, identified in samples V, was previously observed to degrade phenol (Felföldi et al., 2010) and benzo(a)pyrene (Reyes-Sosa et al., 2018).

Species of *Raoultella* were found in samples II IV VI VII. This genus was previously isolated from crude oil contaminated sites and was associated with phenols biodegradation (Kaczorek et al., 2016). *Raoultella ornithinolytica* strains, found in samples II, IV, VI and VII, could degrade diesel and exhibits strong oil emulsification activity (Morales-guzmán et al., 2017).

Some species of *Pusillimonas* were detected in samples I, enriched from powder sewage sludge digestate. Various strains of *Pusillimonas* genera were able to grow using diesel as sole carbon source including *Pusillimonas noertemannii* (T3-5) and *Pusillimonas oleiphila* (T7-7) previously isolated from oil-polluted sea-bed mud (Huang et al., 2008). *Pusillimonas* sp. 5HP, detected here, was previously detected within specialised microbial community inside a biotrickling filter for the removal of pharmaceutical recalcitrant VOCs (Hu et al., 2016).

Presence of alkane hydroxylases was previously reported in the species belonging to all of the detected genera excluding *Rhodanobacter*, *Raoultella* and *Pusillimonas*. It may mean that these genera utilise other metabolic pathway to degrade alkanes e.g. cytochrome P450 or were degrading other components of diesel e.g. phenols or PAHs (Wang et al., 2011). It is also possible for bacterial cells to obtain functional genes from the environment due to horizontal gene transfer, thus bacterial strains can get ability to degrade diesel even if previously it was not observed (Shahi et al., 2016).

Figure 4 PCoA analysis based on UniFrac distances representing beta diversity. Green circles gather samples enriched from digestates and brown circles indicate samples enriched with the presence of soil.

Similarities among samples were measured by PCoA analysis (Fig. 4). The detailed characteristic showing correlation among samples can be found in supplementary materials (Figure S2). Beta diversity analysis demonstrated similarities among samples enriched from soil or soil with digestate (II, VI and VII), which suggest that presence of soil was determinative for microbial diversity. Samples originating from different digestates did not exhibited similarity to each other or to samples containing soil during enrichment step, which may indicate that all tested digestates had a specific pool of diesel degrading bacteria. Interestingly, samples IV enriched from composted digestate and soil were not similar to other samples enriched with the presence of soil or to samples originating from composted digestate. In this case, combination of both bacterial sources resulted in new community profile.

Similarities of samples II and VI (enriched from soil and digestate) to samples VII (enriched from soil) did not have curtail effect on diesel removal and final concentration of *alkB* genes, which differed significantly among those variants, while enrichments with the highest diesel removal (VII, III, I and IV) showed structural differences. This suggest that bacterial community structure needs more time for adaptation to utilise selective substrate, like diesel if the indigenous community (of soil or digestate) was previously disturbed by the presence of exogenous bacteria.

Our data confirm that digestate contains bacterial species beneficial for hydrocarbons remediation. This observation brings new possibilities regarding digestate application in soil treatment. TPH contaminated soils may be supplemented with digestate as a source of nutrient necessary for bacterial development as well as seeding of diesel degrading microbial species. In this way, soil digestate application may be considered as biostimulation and simultaneous bioaugmentation treatment which constitutes 'ready to use' solution. Furthermore, enrichments of digestate could be applied as inoculum for contaminated soils increasing the number of hydrocarbons degrading microorganisms. Hydrocarbon degrading enrichments of digestate may be also used as inoculum during slurry phase soil treatment in bioreactors.

4. Conclusions

In the present study, we have characterised diesel-degrading microorganisms in enrichments obtained from a TPH contaminated soil and different digestates. In all enrichments, diesel removal was significant and specific TPH degrading bacterial taxa were present. Bacteria from all enrichments had *alkB* genes and the content of the genes were promoted during the incubation. Enrichments with the highest diesel degradation were dominated by *Rhodococcus* species.

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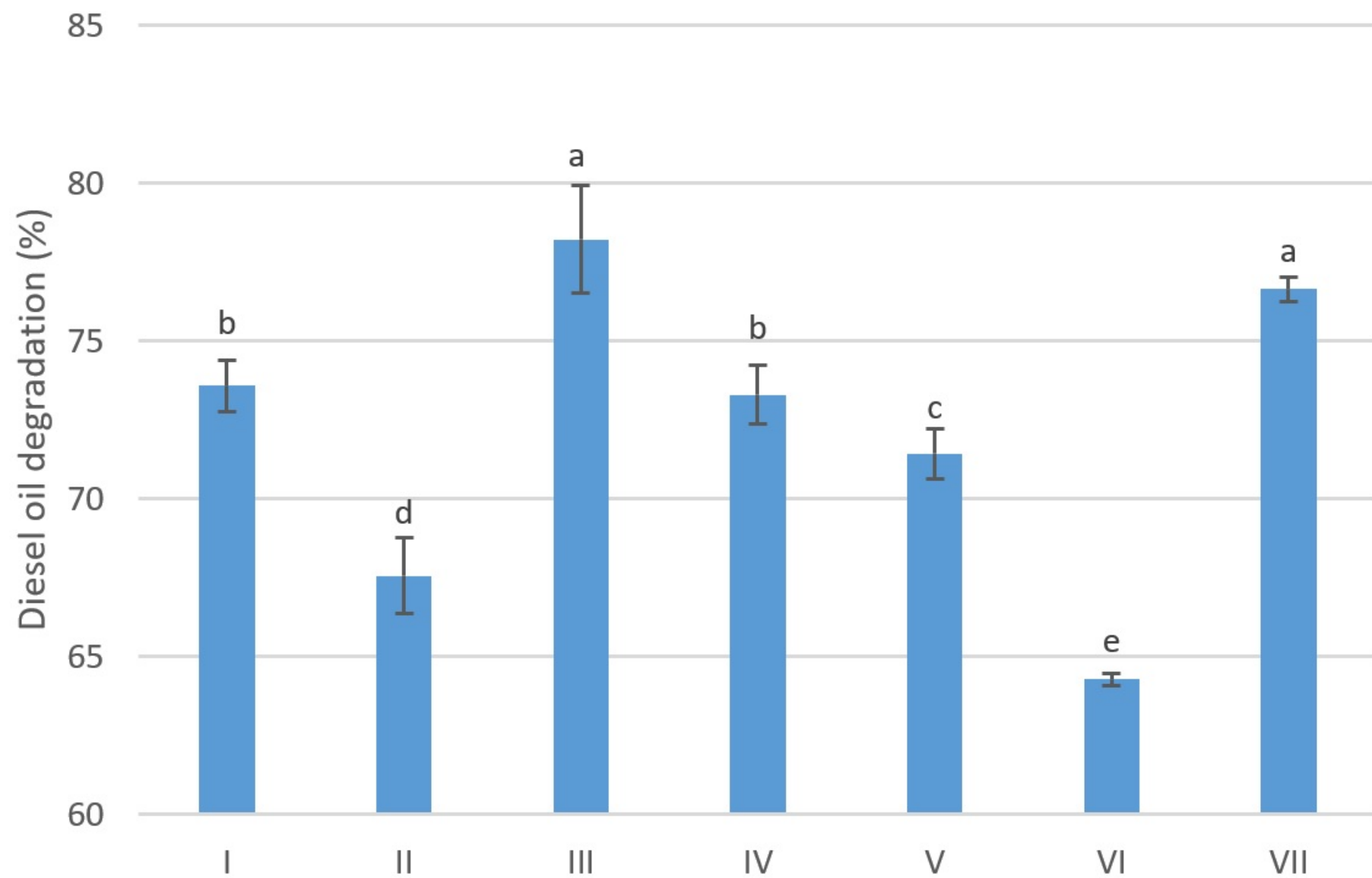
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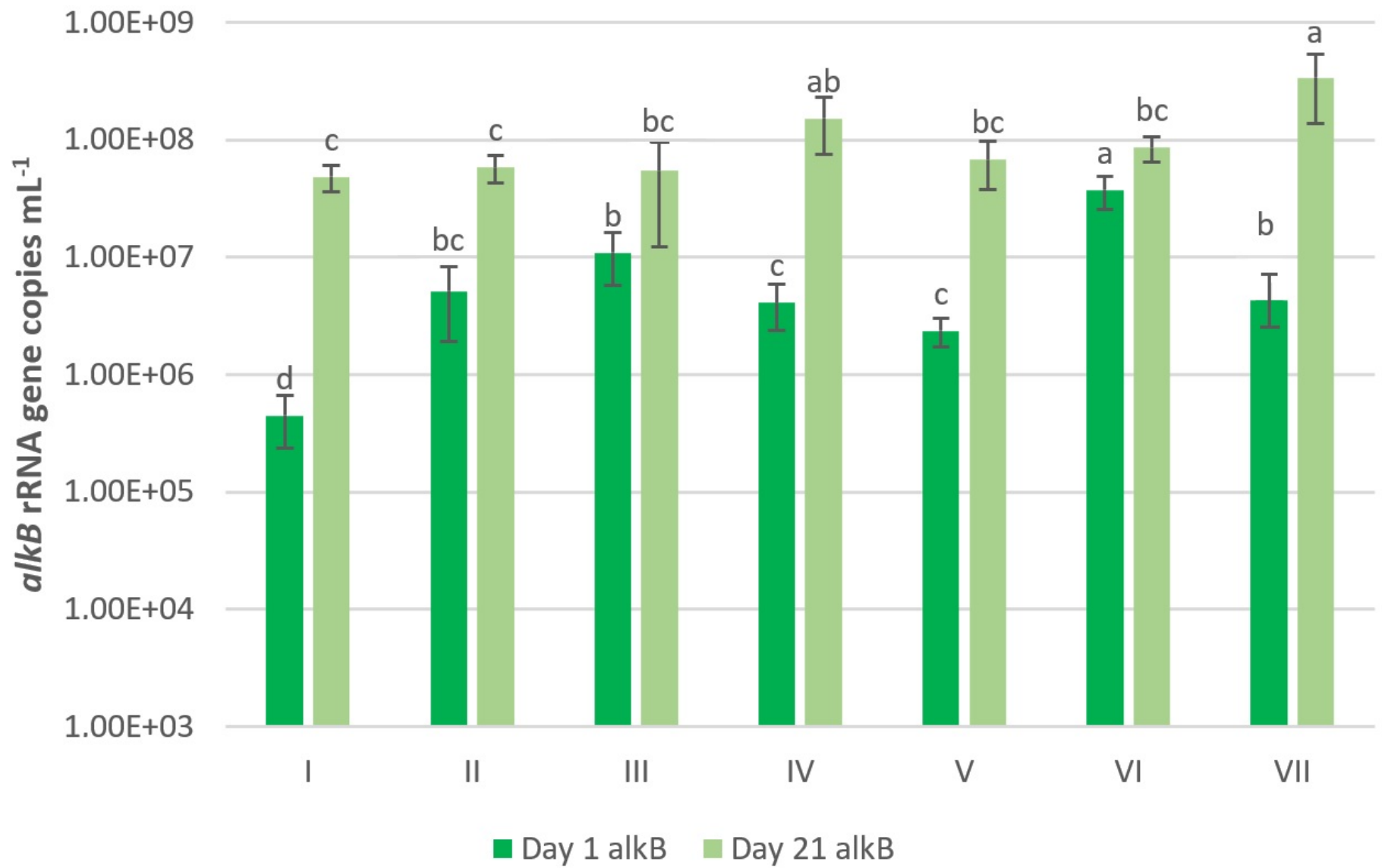
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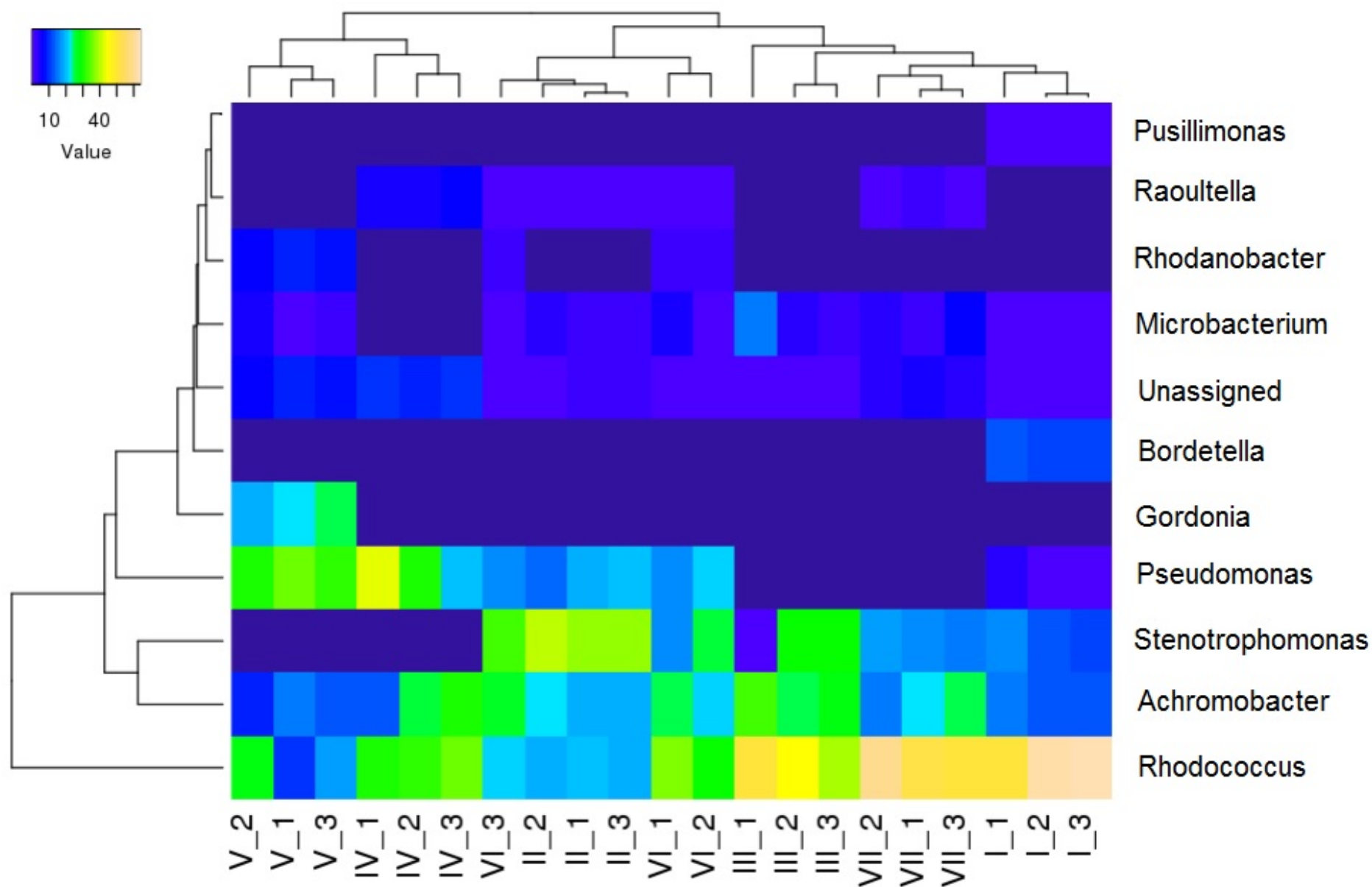
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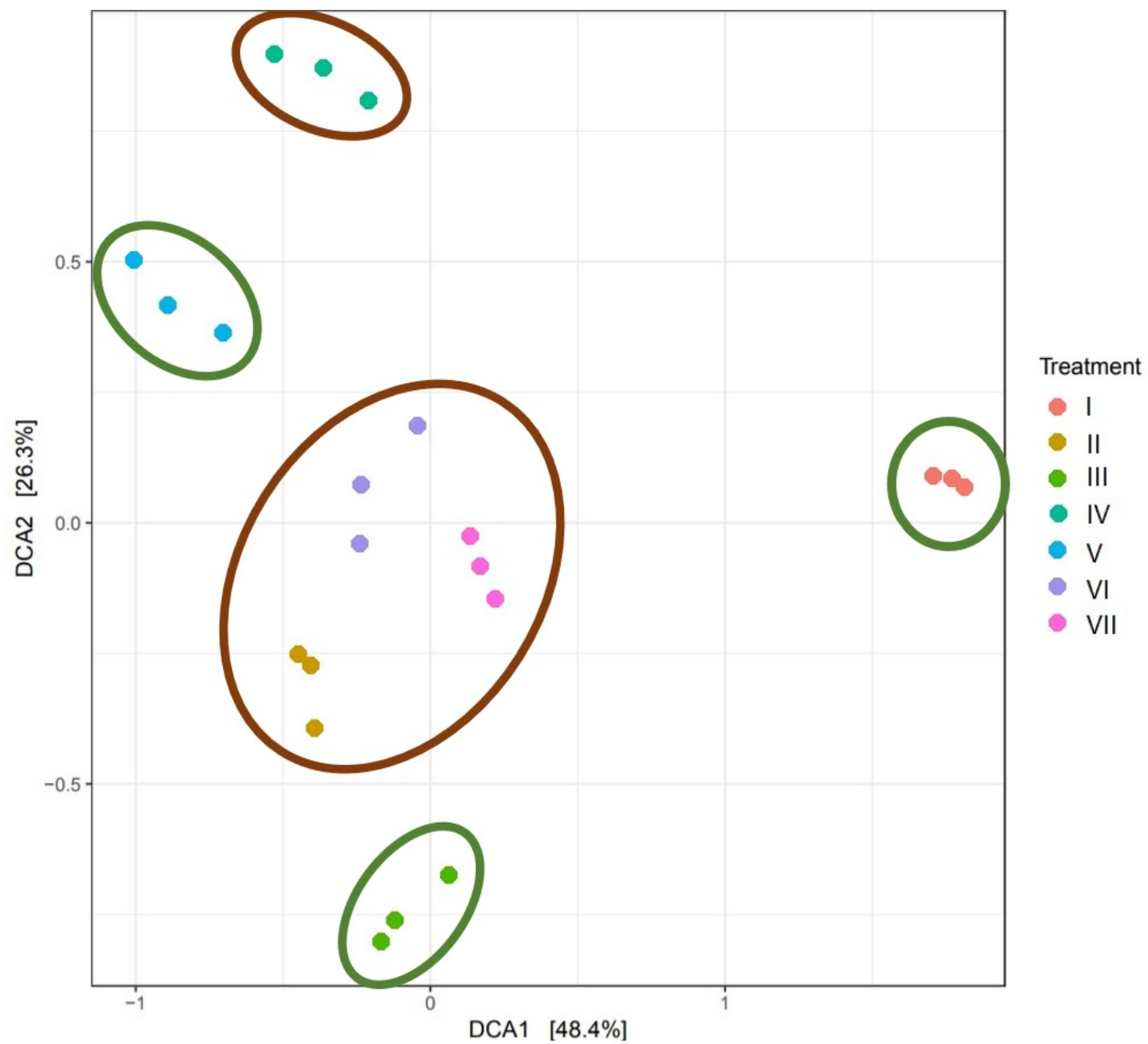


Table 1 Characteristics of soils and digestates used in the study. Mean (n = 3) and standard deviation. TOC: total organic carbon; TN: total nitrogen; TPH: total petroleum hydrocarbons; P: total phosphorus. n.d. not determined.

Parameter	Soil	Fresh sewage sludge digestate	Powder sewage sludge digestate	Composted OFMSW digestate	Method
pH (H ₂ O)	7.4 (0.02)	n.d.	n.d.	n.d.	NF ISO 10693
Water content (%)	0.7 (0.1)	81.1 (1.1)	7.4 (0.1)	8.1 (0.7)	NEN-ISO 11465
TOC (g kg ⁻¹ DW)	16 (1)	273 (20)	316 (15)	256 (20)	NEN-EN 13137
TN (g kg ⁻¹ DW)	325 (5)	61 (2)	56 (1)	21 (7)	NEN-EN-ISO 11732
P (g kg ⁻¹ DW)	290 (15)	33 (1)	32 (2)	8 (1)	NEN 6961, CEN/TS 16171, NF-EN 16179
TPH (g kg ⁻¹ DW)	4.1 (0.2)	1.8 (0.3)	2.0 (0.1)	1.1 (0.2)	Gielnik et al., 2019
Trace elements content (mg kg ⁻¹ DW)					NEN 6961, NEN-EN- ISO17294-2
Fe	7500 (152)	54000 (2828)	57000 (1732)	10933 (1792)	
Cd	0.3 (0.0)	2.2 (0.2)	2.1 (0.1)	0.8 (0.1)	
Cr	8.1 (0.3)	135.1 (7.1)	42.6 (0.6)	24.7 (0.6)	
Hg	6.3 (0.4)	2.9 (0.2)	1.8 (0.0)	0.1 (0.0)	
Pb	120.1 (5.7)	115.2 (7.1)	81.1 (4.4)	67.3 (8.9)	
Ni	5.1 (1.5)	29.2 (2.8)	26.7 (0.6)	10.9 (1.2)	
Zn	84.2 (3.0)	895.1 (63.6)	803.3 (15.3)	1.1 (5.8)	

Table 2 Composition of enrichments originating from different samples.

N°	Enrichment conditions
I	BH broth + powder sewage sludge digestate + diesel oil
II	BH broth + soil + powder sewage sludge digestate + diesel oil
III	BH broth + composted OFMSW digestate + diesel oil
IV	BH broth + soil + composted OFMSW digestate + diesel oil
V	BH broth + fresh sewage sludge digestate + diesel oil
VI	BH broth + soil + fresh sewage sludge digestate + diesel oil
VII	BH broth + soil + diesel oil
C	Abiotic control = BH broth + diesel oil + NaN_3

Compound	Treatment method	Incubation time (d)	Degradation efficiency	Broth	Origin of consortium	Composition of consortium	Reference
Diesel 10 %	Aerated liquid phase bioreactors	7	69 % (of alkane components in diesel)	BH	TPH contaminated industrial side (Pernambuco, Brazil)	<i>S. saprophyticus</i> , <i>S. marcescens</i> , <i>R. aurantiaca</i> , <i>C. ernobii</i>	Souza et al., 2015
TPH in soil spiked in 10 % ratio with crude oil	Slurry phase bioreactors	30	88 %	MN	Crude oil contaminated soil (Lago Agrio , Ecuador)	<i>Bacillus cereus</i> , <i>Bacillus thuringiensis</i> , <i>Geomyces pannorum</i> , and <i>Geomyces</i> sp.	Maddela et al., 2016
TPH contaminated soil (145 g TPH kg ⁻¹ DW)	Slurry phase bioreactors	42	52 %	MN	Activated sludge	<i>Chitinophagaceae</i> , <i>Haliscomenobacter</i> sp., <i>Aquabacterium</i> sp., and others	Aburto-Medina et al., 2012
Diesel 2 %	Flasks incubated on rotary shaker	10	64 % (of alkane components in diesel)	MSM	tarball from contaminated beach (Terengganu, Malaysia)	<i>Cellulosimicrobium cellulans</i>	Nkem et al., 2016
Diesel 2 %	Flasks incubated on rotary shaker	10	58 % (of alkane components in diesel)	MSM	tarball from contaminated beach (Terengganu, Malaysia)	<i>Acinetobacter baumannii</i>	Nkem et al., 2016
Crude oil 2 %	Flasks incubated on rotary shaker	7	46 %	MSM	Hydrocarbon contaminated industrial samples	<i>Achromobacter</i> sp.	Joy et al.,2017
Crude oil 2 %	Flasks incubated on rotary shaker	7	39 %	MSM	Hydrocarbon contaminated industrial samples	<i>Ochrobactrum</i> sp.	Joy et al.,2017

Table 3 Comparison of petroleum products biodegradation potential of different microbial consortia. TPH: total petroleum hydrocarbons; BH: modified Bushnell-Haas medium; MSM: minimal salt medium; MM: mineral nutrients