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1 **Bacterial seeding potential of digestate in bioremediation of diesel**  
2 **contaminated soil**

3  
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17  
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21

22

23 **Abstract**

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

41

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

43

44

## 45 1. Introduction

46 Soil contaminated with petroleum products is a problem all over the world (Lu et al.,  
47 2014). Due to toxicological risks, the use and management of polluted lands are limited.  
48 Among soil treatment strategies, bioremediation gains a lot of interest (Megharaj et al., 2011).  
49 Due to combination of natural ability of microorganisms to metabolize the contaminants with  
50 environmental engineering approaches, bioremediation represents a cost-efficient strategy  
51 (Aburto-Medina et al., 2012).

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

59 According to organic waste recycling strategies, organic fertilizers constitute a resource  
60 saving alternative over mineral products and have lower environmental impact. In last years,  
61 the use of digestate as organic fertilizer gains interest in soil science due to high concentration  
62 of nutrients in bioavailable form and low level of easily degradable carbon (Tambone et al.,  
63 2010; Walsh et al., 2012; Katakai et al., 2017). Although bioremediation has been studied for  
64 last 25 years, still little is known about contribution of bacteria from organic fertilizers to the  
65 overall efficiency of contaminant removal. On the one hand, bacteria from aged contaminated  
66 soils have already developed necessary metabolic apparatus to degrade hydrocarbons (*e.g.*  
67 alkane monooxygenases encoded by *alkB* genes) but on the other hand bacteria from digestate  
68 are adapted to degrade various types of organic compounds and have wide metabolic  
69 capacities. It is thus interesting to compare microbial diversity and petroleum degrading  
70 efficiency of both matrices. Here, for the first time, we compared diesel degradation efficiency  
71 of microbial enrichments originating from different types of digestates with enrichments from  
72 industrial hydrocarbon contaminated soil as well as we studied the structure of enriched  
73 microbial communities.

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

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

81

## 82 **2. Materials and Methods**

### 83 *2.1. Soil and digestate*

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

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

95

### 96 *2.2. Experimental design*

97 **To evaluate diesel degradation potential of different enrichments, digestates and soil**  
98 **were incubated in variants presented in Table 2.** In brief, fresh sewage sludge digestate (2 g)  
99 and/or powder sewage sludge digestate (0.4 g) and/or dry composted OFMSW digestate (0.4  
100 g) and/or soil (5 g) were added to sterile glass flasks containing 50 mL of modified Bushnell-  
101 Haas (BH) medium broth. The composition of the BH broth was as follows: MgSO<sub>4</sub> (0.2),  
102 CaCl<sub>2</sub> (0.02), KH<sub>2</sub>PO<sub>4</sub> (1.0), (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub> (1.0), NH<sub>4</sub>Cl (3.5) and FeCl<sub>3</sub> (0.05) grams per liter.  
103 The broth was adjusted to a final pH of 7.2 ± 0.1 and autoclaved. Diesel obtained from a local  
104 gas station was used as a supplemented carbon source in 1% (v/v) concentration. Prior to  
105 addition, diesel was sterilised by filtration using 0.2 µm syringe filter. The flasks were placed  
106 on a rotary incubator shaker at 160 rpm and 21°C and incubated for one week. After this time,  
107 1 mL of enrichment was transferred into the fresh BH medium with diesel as sole carbon

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

116 **Table 2 Composition of enrichments originating from different samples.**

117

### 118 2.3. Diesel quantification

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

124

### 125 2.4. Microbial respiration

126 Microbial respiration was monitored during the incubation with the use of Oxitop<sup>®</sup>  
127 system. Briefly, a volume of 20 mL BH broth supplemented with diesel (1 % v/v) and 0.4 mL  
128 of appropriate inoculum was placed in Oxitop<sup>®</sup> gas tight flasks equipped with a CO<sub>2</sub> trap (solid  
129 NaOH) and incubated on rotary shaker at 160 rpm and 21 °C for 21 days in parallel with  
130 experimental batch for diesel degradation. Oxygen up-take in the flasks was measured every 4  
131 hours and registered in the measuring Oxitop<sup>®</sup> heads as a pressure drop in hPa. Calculations  
132 were based on to the equation 1.

$$133 \quad BR = \frac{M(O_2)}{R \cdot T_m} \cdot \left( \frac{V_b - V_s}{V_s} + \alpha \frac{T_m}{T_0} \right) \cdot \Delta\rho(O_2) \quad (1)$$

134 where BR: basal respiration (mg O<sub>2</sub> L<sup>-1</sup>); α: Bunsen absorption coefficient; M(O<sub>2</sub>): molecular  
135 mass of O<sub>2</sub> (mg mol<sup>-1</sup>); V<sub>b</sub>: bottle volume (L); V<sub>s</sub>: sample volume (L); Δρ(O<sub>2</sub>): pressure

136 difference (mbar); R: perfect gas constant ( $L \text{ mbar mol}^{-1} \text{ K}^{-1}$ );  $T_0$ : reference temperature  
137 (273.15 K);  $T_m$ : measured temperature (K).

138

#### 139 2.5. *DNA extraction*

140 Genomic DNA was extracted at the beginning and at the end of experiment from 200  
141  $\mu\text{L}$  of freeze samples using Fast DNA Spin Kit for Soils (MP Biomedicals). Extracted DNA  
142 was eluted in 100  $\mu\text{L}$  of DNA free ultra pure water. DNA concentration and purity were  
143 determined using spectrophotometer UV-1800 (Shimadzu) equipped with a TrayCell adaptor  
144 for micro-volumes (Hellma) (Biache et al., 2017). DNA was stored at  $-20^\circ\text{C}$  for further  
145 analyses.

146

#### 147 2.6. *Real-time PCR analysis*

148 Real-time PCR analysis were performed according to previous studies (Cébron et al.,  
149 2008; Cébron et al., 2015). The extracted genomic DNA was used to quantify total bacterial  
150 and fungal populations using 968F/1401R (Felske et al., 1998) and Fung5f/FF390r (Smit et al.,  
151 1999; Vainio et al., 2000) primer sets targeting bacterial 16S rRNA and fungal 18S rRNA  
152 genes. Functional genes, *i.e.* alkanes monooxygenases genes, were quantified using primers  
153 alkBFd/alkBRd (Powell et al., 2006). Real-time PCR quantifications were performed using  
154 CFX96 C1000TMRReal Time system (Bio-Rad) and dilution series of standard plasmids.

155

#### 156 2.7. *Sequencing analysis*

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

166 % confidence). Alpha diversity calculation and comparative statistics were done using  
167 Phyloseq and DeSeq2 (R packages). Heat map was constructed using Heatmapper software.  
168 Sequencing reads are available in the GenBank database with submission number  
169 SUB5445613 and BioProject ID PRJNA532594.

170

### 171 3. Results and discussion

#### 172 3.1. Diesel degradation and oxygen uptake

173 Diesel degradation by the seven tested enrichments is presented in Figure 1 as a value  
174 relative to control. The obtained results have shown significant diesel removal for all  
175 enrichments compared to control (ANOVA;  $P \leq 0.05$ ). Diesel removal efficiency was as  
176 follows: III=VII>I=IV>V>II>VI (ANOVA;  $P \leq 0.05$ ). Diesel removal by bacteria originating  
177 from soil (VII) and from OFMSW composted digestate (III) have reached the highest values  
178 (77 and 78 %, respectively), which were not significantly different (ANOVA;  $P \leq 0.05$ ). The  
179 lowest contaminants removal was observed for variants representing fresh sewage sludge  
180 digestate with soil (VI) and powder sewage sludge digestate with soil (II), reaching 64 and 68  
181 %, respectively.

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

185

186 During incubation time, microbial metabolic activity was studied through respiration  
187 measurements (i.e. the amount of oxygen uptake is displayed in Fig. S3). The observed diesel  
188 removal values for the different enrichments were correlated with the respiration intensity  
189 (Pearson correlation;  $r = 0.898$ ;  $p = 0.006$ ). For all the enrichments, except IV, intense  
190 respiration was observed within the first 10 days of the experiment representing more than 75  
191 % of total oxygen up-take of the 21-days incubation (Fig. S3). This increase reflects the phase  
192 of exponential growth occurring in microbial cultures at the beginning of incubation. After this  
193 time, activity started to stabilize, and this trend continued until the end of the experiment. This  
194 may be related to the depletion of preferable carbon source among diesel components and  
195 possible accumulation of toxic metabolites. Observed respiration intensity suggests that most  
196 of diesel was degraded in the first 10 days of the experiment.



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

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

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

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

228

229 3.2. *Microbial density and presence of functional genes*

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

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

245 **Composition of diesel is diverse and differs according to the production procedures.**  
246 **Diesel contains many different chemical components including branched chain alkanes, alkyl**  
247 **benzenes and polycyclic aromatic hydrocarbons (PAHs), however its main components are**  
248 **linear alkanes. *AlkB* genes encoding alkane hydroxylases belonging to monooxygenases are**  
249 **proved to enable degradation of alkanes with different carbon chain length (Powell et al., 2006;**  
250 **Jin and Kim, 2017). The hydroxylation of alkanes may occur in terminal or sub-terminal**  
251 **position and is initiated by alkane hydroxylases which oxidise alkane to alcohol (Fuentes et al.**  
252 **2014). Alcohol is further oxidized to aldehyde by alcohol dehydrogenase. Aldehyde is**  
253 **subsequently oxidized to carboxylic acid by aldehyde dehydrogenase and serves as a substrate**  
254 **for acetyl coenzyme A (acyl-CoA) synthesis which is further funnelled into  $\beta$ -oxidation**  
255 **pathway (Fuentes et al., 2014).**

256 **Figure 2 Copy number of *alkB* genes at the beginning and at the end of the experiment.** Symbols  
257 correspond with Table 2. Mean ( $n = 3$ ) and standard deviation. Values that are annotated with the same  
258 letter among one sampling time are not significantly different (Tukey's multiple range test with  $p =$   
259 0.05).

260 In all tested enrichments, initial concentration of *alkB* genes has increased after 21 days  
261 and the final concentration has exceeded  $10^7$  gene copies  $\text{mL}^{-1}$ . Hence, there is no doubt that

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

268

### 269 3.3. Microbial community structure

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

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

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

293 (Cappelletti et al., 2017). Species within the genera possess wide spectrum of alkane  
294 monooxygenases and can utilize wide range of organic contaminants as primary substrates or  
295 as co-substrates. Bacteria within *Rhodococcus* genus are also capable of biosurfactant  
296 production which facilitate the contact with hydrophobic compounds and thus improves  
297 substrate availability (Cappelletti et al., 2017). *Achromobacter* species were already isolated  
298 from crude oil contaminated soils and have proved ability to metabolize petroleum products  
299 (Marecik et al., 2015), such as *Achromobacter xylosoxidans* growing on alkanes (Tanase et al.,  
300 2013).

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

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

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

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

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

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

325 species with crude oil degrading capacities (Muthukamalam et al., 2017). However,  
326 *Microbacterium oxydans* which was detected during the present study was observed to degrade  
327 complex polysaccharides like alginic acid, which is widely distributed in cell walls (Jung et al.,  
328 2013). This may suggest growth on dead biomass produced during incubation.

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

335 Species of *Raoultella* were found in samples II IV VI VII. This genus was previously  
336 isolated from crude oil contaminated sites and was associated with phenols biodegradation  
337 (Kaczorek et al., 2016). *Raoultella ornithinolytica* strains, found in samples II, IV, VI and VII,  
338 could degrade diesel and exhibits strong oil emulsification activity (Morales-guzmán et al.,  
339 2017).

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

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

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

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

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

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

## 382 4. Conclusions

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

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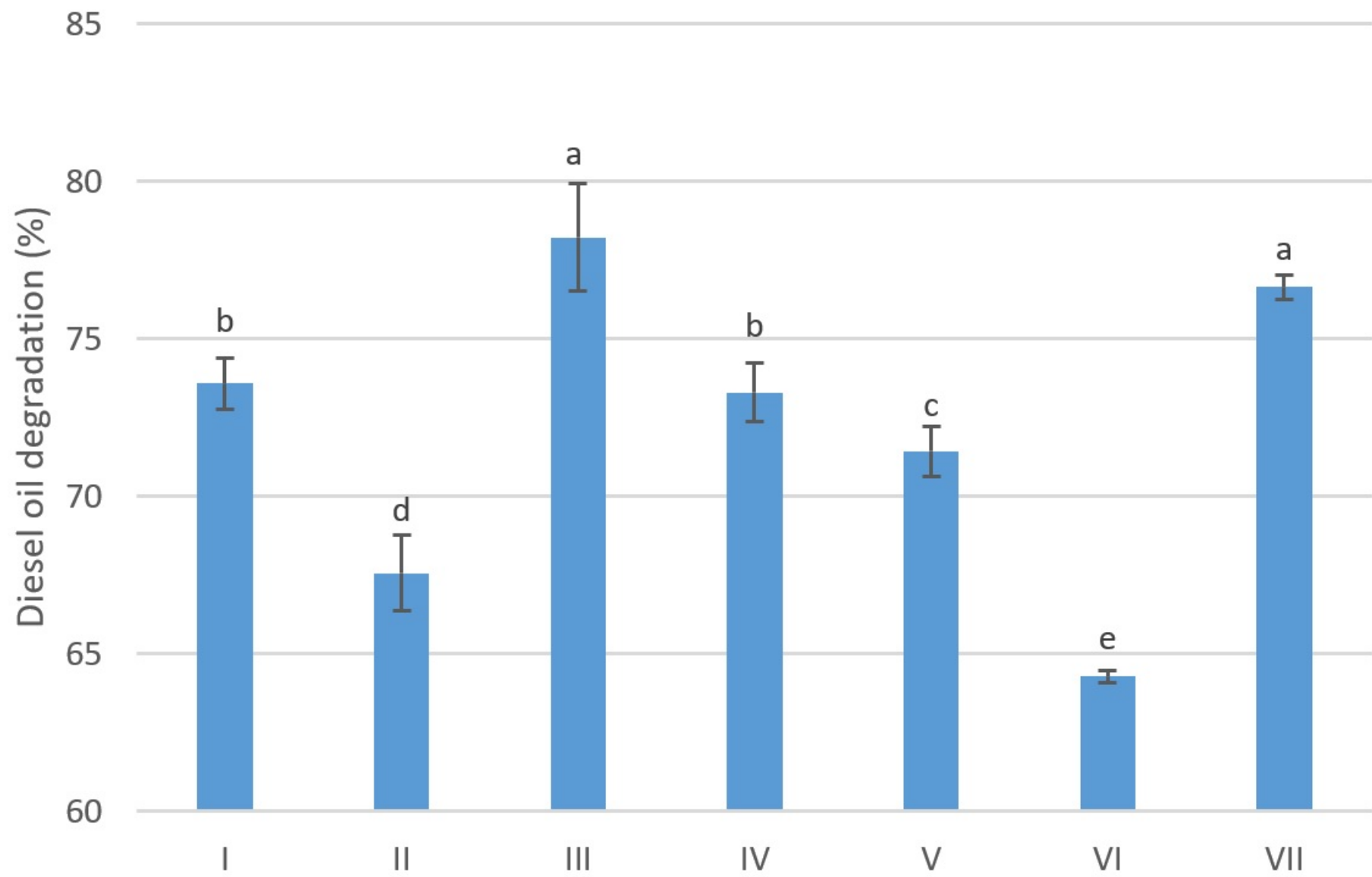
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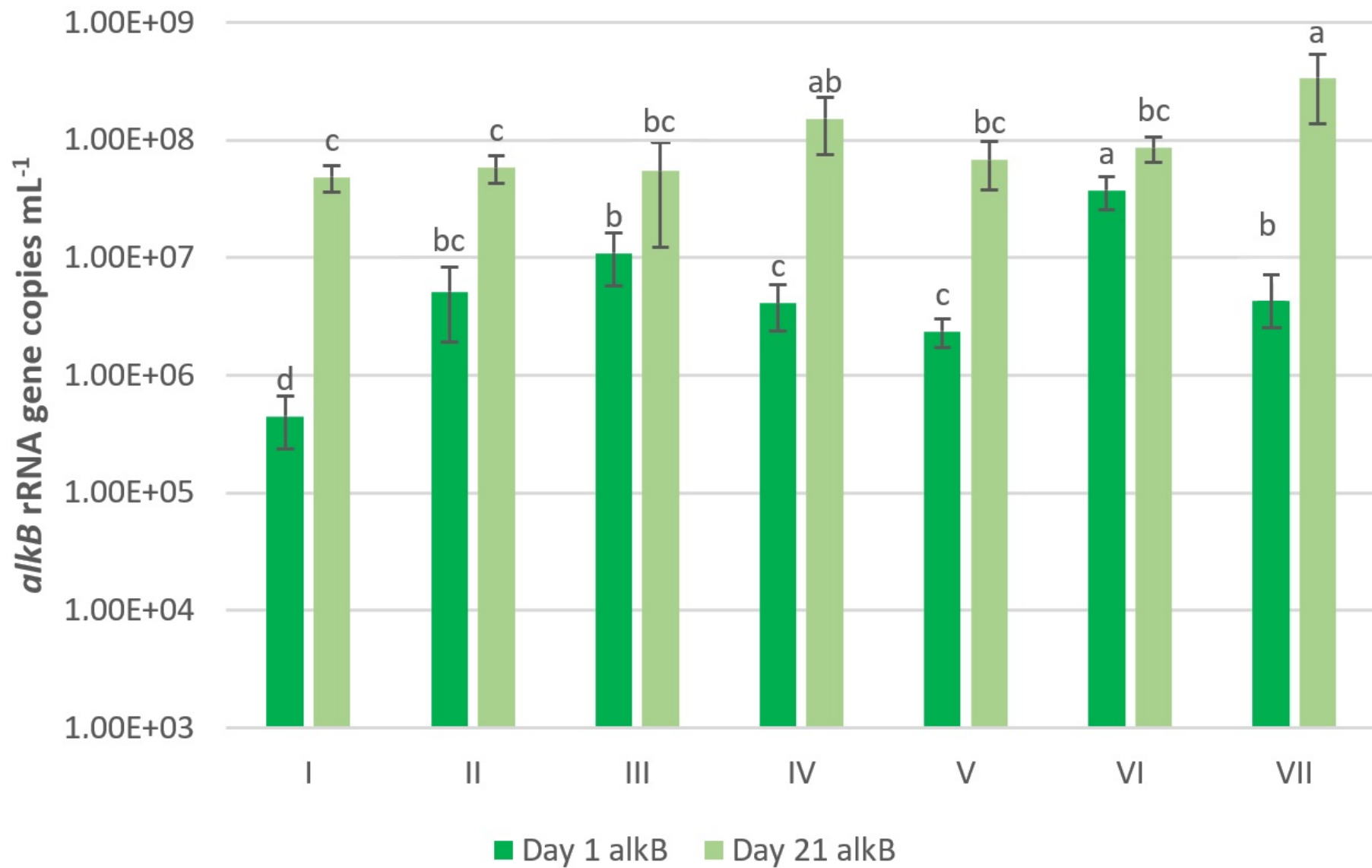
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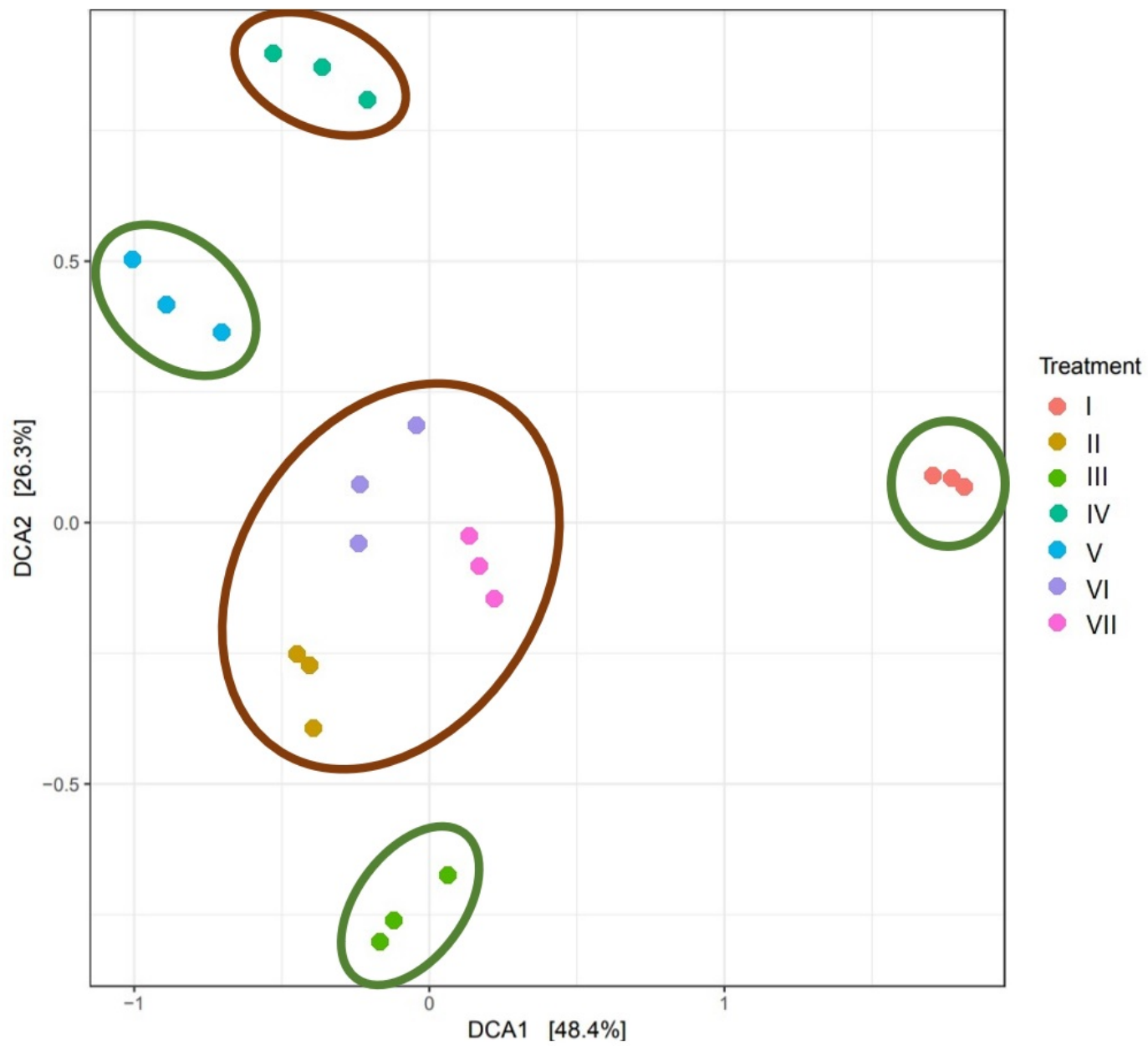
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**Table 1 Characteristics of soils and digestates used in the study.** Mean (n = 3) and standard deviation. TOC: total organic carbon; TN: total nitrogen; TPH: total petroleum hydrocarbons; P: total phosphorus. n.d. not determined.

| Parameter                                       | Soil        | Fresh sewage sludge digestate | Powder sewage sludge digestate | Composted OFMSW digestate | Method                              |
|---|-------------|-------------------------------|--------------------------------|---------------------------|-------------------------------------|
| pH (H <sub>2</sub> 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 <sup>-1</sup> DW)                     | 16 (1)      | 273 (20)                      | 316 (15)                       | 256 (20)                  | NEN-EN 13137                        |
| TN (g kg <sup>-1</sup> DW)                      | 325 (5)     | 61 (2)                        | 56 (1)                         | 21 (7)                    | NEN-EN-ISO 11732                    |
| P (g kg <sup>-1</sup> DW)                       | 290 (15)    | 33 (1)                        | 32 (2)                         | 8 (1)                     | NEN 6961, CEN/TS 16171, NF-EN 16179 |
| TPH (g kg <sup>-1</sup> 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 <sup>-1</sup> 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.**

| <b>N°</b>  | <b>Enrichment conditions</b>                                  |
|------------|---|
| <b>I</b>   | BH broth + powder sewage sludge digestate + diesel oil        |
| <b>II</b>  | BH broth + soil + powder sewage sludge digestate + diesel oil |
| <b>III</b> | BH broth + composted OFMSW digestate + diesel oil             |
| <b>IV</b>  | BH broth + soil + composted OFMSW digestate + diesel oil      |
| <b>V</b>   | BH broth + fresh sewage sludge digestate + diesel oil         |
| <b>VI</b>  | BH broth + soil + fresh sewage sludge digestate + diesel oil  |
| <b>VII</b> | BH broth + soil + diesel oil                                  |
| <b>C</b>   | Abiotic control = BH broth + diesel oil + NaN <sub>3</sub>    |



| 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 <sup>-1</sup> 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