



HAL
open science

Bacterial seeding potential of digestate in bioremediation of diesel contaminated soil

Anna Gielnik, Yoan Pechaud, David Huguenot, Aurélie Cebron, Giovanni Esposito, Eric D. van Hullebusch

► **To cite this version:**

Anna Gielnik, Yoan Pechaud, David Huguenot, Aurélie Cebron, Giovanni Esposito, et al.. Bacterial seeding potential of digestate in bioremediation of diesel contaminated soil. *International Biodeterioration and Biodegradation*, 2019, 143, pp.104715. 10.1016/j.ibiod.2019.06.003 . hal-02341826

HAL Id: hal-02341826

<https://hal.science/hal-02341826>

Submitted on 9 Dec 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

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

3
4 Anna Gielnik ^{1,2*}, Yoan Pechaud ¹, David Huguenot ¹, Aurélie Cébron ³, Giovanni Esposito ², Eric D.
5 van Hullebusch ^{4,5}

6 ¹Université Paris-Est, Laboratoire Géomatériaux et Environnement (LGE), EA 4508, UPEM, 77454
7 Marne-la-Vallée, France

8 ²University of Napoli “Federico II”, Department of Civil, Architectural and Environmental
9 Engineering, 80125 Napoli, Italy

10 ³Université de Lorraine, CNRS, LIEC, F-54000 Nancy, France

11 ⁴IHE Delft Institute for Water Education, Department of Environmental Engineering and Water
12 Technology, P.O. Box 3015, 2601 DA, Delft, The Netherlands

13 ⁵Université de Paris, Institut de Physique du Globe de Paris, CNRS, UMR 7154, F-75238 Paris,
14 France

15 *Corresponding author: Anna Gielnik

16 E-mail address: aniagielnik@gmail.com

17
18 **International Biodeterioration & Biodegradation**

19 **Special Issue: Biotechnologies and Nanotechnologies for Sustainable Environment and**
20 **Sponge Cities (BNSuE-Sponge)**

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; Katakaki 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: MgSO₄ (0.2),
102 CaCl₂ (0.02), KH₂PO₄ (1.0), (NH₄)₂HPO₄ (1.0), NH₄Cl (3.5) and FeCl₃ (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 NaN₃ 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[®]
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[®] gas tight flasks equipped with a CO₂ 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[®] 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 O₂ L⁻¹); α: Bunsen absorption coefficient; M(O₂): molecular
135 mass of O₂ (mg mol⁻¹); V_b: bottle volume (L); V_s: sample volume (L); Δρ(O₂): 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 μL of freeze samples using Fast DNA Spin Kit for Soils (MP Biomedicals). Extracted DNA
142 was eluted in 100 μL 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°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 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 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 β -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 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.

389 Acknowledgements

390 The authors acknowledge the financial support from the European Union's Horizon 2020
391 research and innovation programme under the Marie Skłodowska-Curie grant agreement No.
392 643071 ('Advanced Biological Waste-to-Energy Technologies – ABWET').

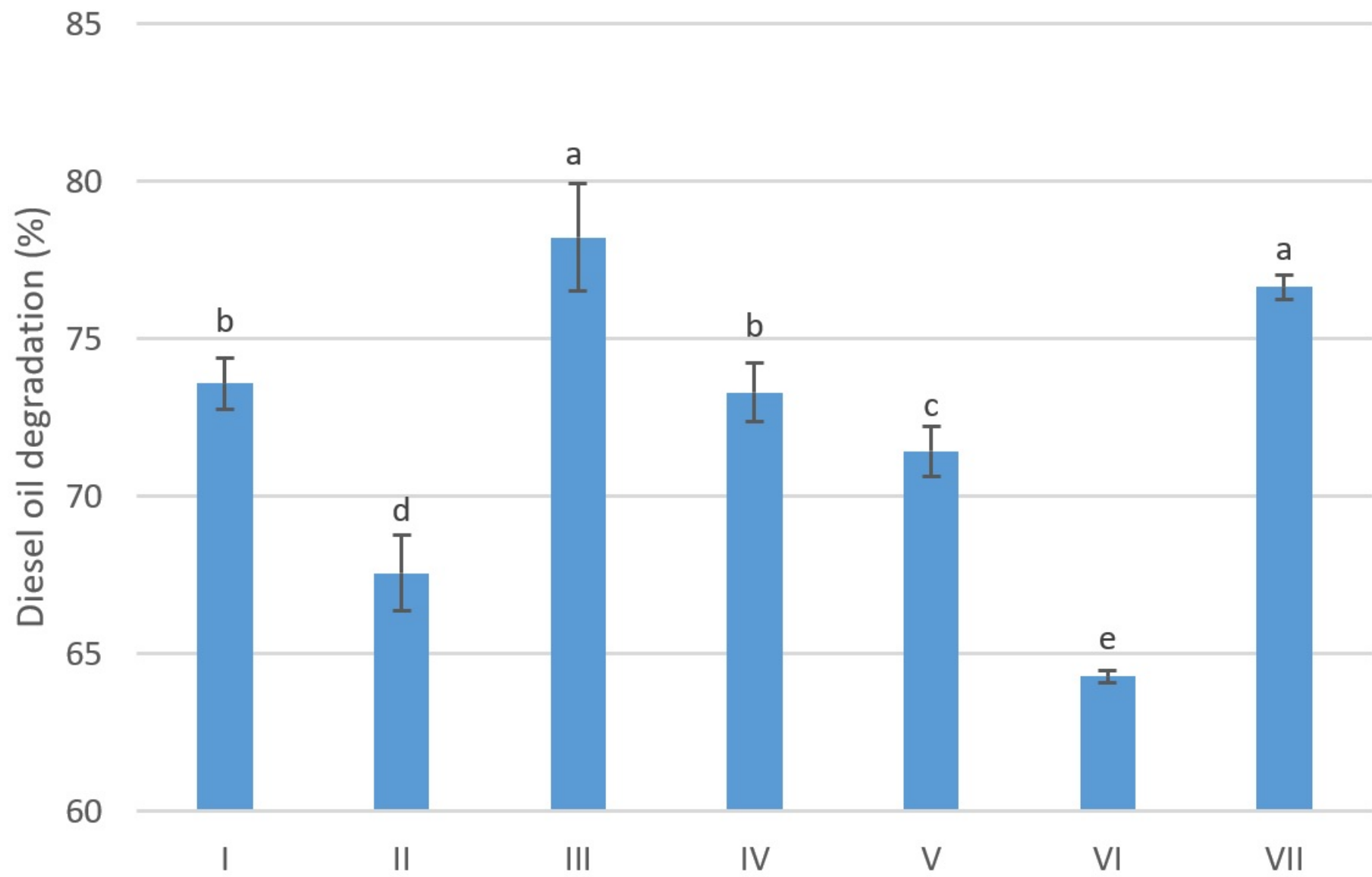
393 References

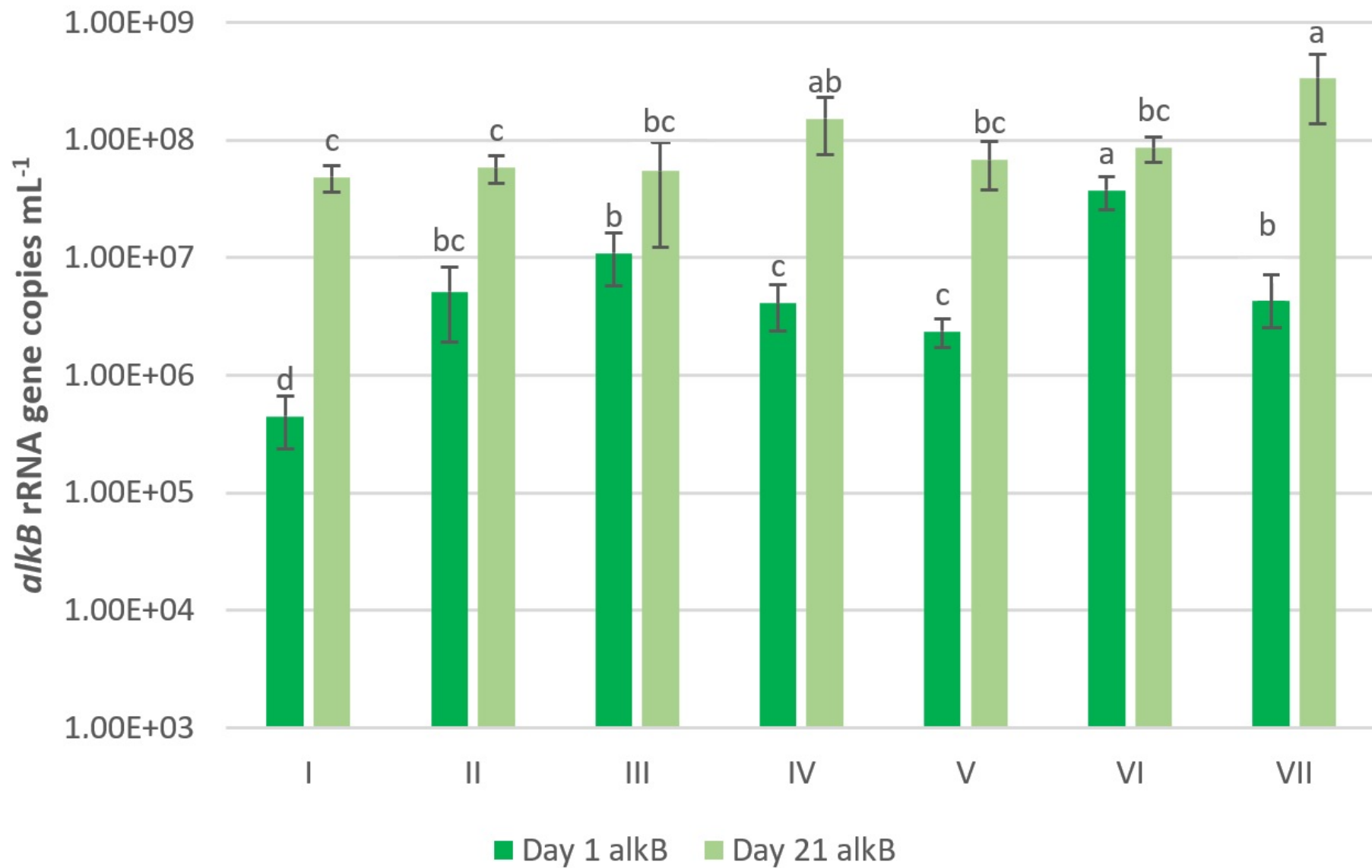
- 394 Abass O.K., Fang F., Zhuo M., Zhang K., 2018. Integrated interrogation of causes of
395 membrane fouling in a pilot-scale anoxic-oxic membrane bioreactor treating oil
396 refinery wastewater, *Sci. Total Environ.* 642, 77–89.
- 397 Abed R.M.M., Al-kind S., 2015. Diversity of Bacterial Communities Along a Petroleum
398 Contamination Gradient in Desert Soils, *Microb. Ecol.* 69, 95–105.
- 399 Aburto-Medina A., Adetutu E.M., Aleer S., Weber J., Patil S.S., Sheppard P.J., Ball A.S.,
400 Juhasz A.L., 2012. Comparison of indigenous and exogenous microbial populations
401 during slurry phase biodegradation of long-term hydrocarbon-contaminated soil,
402 *Biodegradation.* 23, 813–822.
- 403 Agnello A.C., Bagard M., van Hullebusch E.D., Esposito G., Huguenot D., 2016.
404 Comparative bioremediation of heavy metals and petroleum hydrocarbons co-
405 contaminated soil by natural attenuation, phytoremediation, bioaugmentation and
406 bioaugmentation-assisted phytoremediation, *Sci. Total Environ.* 563–564, 693–703.
- 407 Biache C., Oualia S., Cébron A., Lorgeoux C., Colombano S., Faure P., 2017.
408 Bioremediation of PAH-contaminated soils: Consequences on formation and
409 degradation of polar-polycyclic aromatic compounds and microbial community
410 abundance, *J. Hazard. Mater.* 329, 1–10.
- 411 Cappelletti M., Frascari D., Pinelli D., Mezzetti F., Fedi S., Zannoni D., 2017. Consortium
412 (C2): Diversity of alkane monooxygenase genes and design of an on-site
413 bioremediation process, *Int. Biodeterior. Biodegradation.* 119, 649–660.
- 414 Cébron A., Beguiristain T., Bongoua-Devisme J., Denonfoux J., Faure P., Lorgeoux C.,
415 Ouvrard S., Parisot N., Peyret P., Leyval C., 2015. Impact of clay mineral, wood
416 sawdust or root organic matter on the bacterial and fungal community structures in two
417 aged PAH-contaminated soils, *Environ. Sci. Pollut. Res.* 22, 13724–13738.

- 418 Cébron A., Norini M.P., Beguiristain T., Leyval C., 2008. Real-Time PCR quantification of
419 PAH-ring hydroxylating dioxygenase (PAH-RHD α) genes from Gram positive and
420 Gram negative bacteria in soil and sediment samples, *J. Microbiol. Methods.* 73, 148–
421 159.
- 422 de Souza Pereira Silva, D., de Lima Cavalcanti, D., de Melo, E.J.V., dos Santos, P.N.F., da
423 Luz, E.L.P., de Gusmão, N.B., de Fátima de Queiroz Sousa, M.V., 2015. Bio-removal of
424 diesel oil through a microbial consortium isolated from a polluted environment. *Int.*
425 *Biodeterior. Biodegrad.* 97, 85–89.
- 426 Felföldi T., Székely A.J., Gorál R., Barkács K., Scheirich G., András J., Rácz A., Márialigeti
427 K., 2010. Polyphasic bacterial community analysis of an aerobic activated sludge
428 removing phenols and thiocyanate from coke plant effluent, *Bioresour. Technol.* 101,
429 3406–3414.
- 430 Felske W.M., Akkermans A., de Vos A.D.L., 1998. Quantification of 16S rRNAs in complex
431 bacterial communities by multiple competitive reverse transcription-PCR in
432 temperature gradient gel electrophoresis fingerprints, *Appl. Environ. Microbiol.* 64,
433 4581–4587.
- 434 Gielnik A., Pechaud Y., Huguenot D., Riou J.-M., Guibaud G., Esposito G., van Hullebusch
435 E.D., 2019. Effect of digestate application on microbial respiration and bacterial
436 communities' diversity during bioremediation of weathered petroleum hydrocarbons
437 contaminated weathered soils, *Sci. Total Environ.* 670, 271-281.
- 438 Hu J., Zhang L., Chen J., Luo Y., Sun B., Chu G., 2016. Performance and microbial analysis
439 of a biotrickling filter inoculated by a specific bacteria consortium for removal of a
440 simulated mixture of pharmaceutical volatile organic compounds, *Chem. Eng. J.* 304,
441 757–765.
- 442 Huang L., Ma T., Li D., Liang F., Liu R., Li G., 2008. Optimization of nutrient component
443 for diesel oil degradation by *Rhodococcus erythropolis*, *Mar. Pollut. Bull. J.* 56, 1714–
444 1718.
- 445 Jin C.E., Kim M.N., 2017. Change of bacterial community in oil-polluted soil after
446 enrichment cultivation with low-molecular-weight polyethylene, *Int. Biodeterior.*
447 *Biodegradation.* 118, 27–33.
- 448 Joy S., Rahman P. K.S.M., Sharma S., 2017. Biosurfactant production and concomitant
449 hydrocarbon degradation potentials of bacteria isolated from extreme and hydrocarbon
450 contaminated environments, *Chem. Eng. J.* 317, 232-241.
- 451 Jung E., Fathoni A., Jeong G., Do H., Nam T., Kong I., Kyun J., 2013. *Microbacterium*
452 *oxydans*, a novel alginate- and laminarin-degrading bacterium for the reutilization of
453 brown-seaweed waste, *J. Environ. Manage.* 130, 153–159.
- 454 Kaczorek E., Smu W., Zdzarta A., Sawczuk A., Zgo A., 2016. Influence of saponins on the
455 biodegradation of halogenated phenols, *Ecotoxicol. Environ. Saf.* 131, 127–134.
- 456 Kataki S., Hazarika S., Baruah D.C., 2017. Investigation on by-products of bioenergy
457 systems (anaerobic digestion and gasification) as potential crop nutrient using FTIR,
458 XRD, SEM analysis and phyto-toxicity test, *J. Environ. Manage.* 196, 201–216.
- 459 Li H., Wang X., Mu B., Gu J., Liu Y., Lin K., Lu S., Lu Q., Li B., Li Y., Du X., 2013.
460 Molecular detection, quantification and distribution of alkane-degrading bacteria in

- 461 production water from low temperature oil fields, *Int. Biodeterior. Biodegradation*. 76,
462 49–57.
- 463 Lu L., Yazdi H., Jin S., Zuo Y., Fallgren P.H., Ren Z.J., 2014. Enhanced bioremediation of
464 hydrocarbon-contaminated soil using pilot-scale bioelectrochemical systems, *J.*
465 *Hazard. Mater.* 274, 8–15.
- 466 Maddela R. N., Burgos R., Kadiyala V., Riofrio A., Bangeppagari M., 2016. Removal of
467 petroleum hydrocarbons from crude oil in solid and slurry phase by mixed soil
468 microorganisms isolated from Ecuadorian oil fields, *Int. Biodeterior. Biodegradation*.
469 108, 85–90.
- 470 Marecik R., Chrzanowski Ł., Piotrowska-cyplik A., 2015. Rhizosphere as a tool to introduce
471 a soil-isolated hydrocarbon-degrading bacterial consortium into a wetland
472 environment, *Int. Biodeterior. Biodegradation*. 97, 135–142.
- 473 Martin-Sanchez, P.M. Gorbushina A.A., Toepel J., 2018. Quantification of microbial load in
474 diesel storage tanks using culture- and qPCR-based approaches, *Int. Biodeterior.*
475 *Biodegradation*. 126, 216–223.
- 476 Máthé I., Benedek T., Tánicsics A., Palatinszky M., Lányi S., 2012. Diversity, activity,
477 antibiotic and heavy metal resistance of bacteria from petroleum hydrocarbon
478 contaminated soils located in Harghita County (Romania), *Int. Biodeterior.*
479 *Biodegradation*. 73, 41–49.
- 480 Megharaj M., Ramakrishnan B., Venkateswarlu K., Sethunathan N., Naidu R., 2011.
481 Bioremediation approaches for organic pollutants: A critical perspective, *Environ. Int.*
482 37, 1362–1375.
- 483 Morales-Guzmán G., Ferrera-Cerrato R., Rivera-Cruz C., Torres-Bustillos L.G., Arteaga-
484 Garibay R.I., Mendoza-López M.R., 2017. Diesel degradation by emulsifying bacteria
485 isolated from soils polluted with weathered petroleum hydrocarbons, *Appl. Soil Ecol.*
486 121, 127–134.
- 487 Mroziak A., Piotrowska-Seget Z., 2010. Bioaugmentation as a strategy for cleaning up of soils
488 contaminated with aromatic compounds, *Microbiol. Res.* 165, 363–375.
- 489 Muthukamalam S., Sivagangavathi S., Dhrishya D., Rani S.S., 2017. Characterization of
490 dioxygenases and biosurfactants produced by crude oil degrading soil bacteria,
491 *Brazilian J. Microbiol.* 48, 637–647.
- 492 Nkem B.M., Halimoon N., Yusoff F.M., Johari W.L.W., Zakaria M.P., Medipally S.R.,
493 Kannan N., 2016. Isolation, identification and diesel-oil biodegradation capacities of
494 indigenous hydrocarbon-degrading strains of *Cellulosimicrobium cellulans* and
495 *Acinetobacter baumannii* from tarball at Terengganu beach, Malaysia, *Mar. Pollut.*
496 *Bull.* 107, 261–268.
- 497 Powell S.M., Ferguson S.H., Bowman J.P., Snape I., 2006. Using Real-Time PCR to Assess
498 Changes in the Hydrocarbon-Degrading Microbial Community in Antarctic Soil
499 During Bioremediation, *Microb. Ecol.* 52, 523–532.
- 500 Reyes-Sosa M.B., Apodaca-Hernández J.E., Arena-Ortiz M.L., 2018. Bioprospecting for
501 microbes with potential hydrocarbon remediation activity on the northwest coast of the
502 Yucatan Peninsula, Mexico, using DNA sequencing, *Sci. Total Environ.* 642, 1060–
503 1074.

- 504 Salminen J.M., Tuomi P.M., Jørgensen K.S., 2008. Functional gene abundances (*nahAc*,
505 *alkB*, *xylE*) in the assessment of the efficacy of bioremediation, Appl. Biochem.
506 Biotechnol. 151, 638–652.
- 507 Shahi A., Aydin S., Ince B., Ince O., 2016. Evaluation of microbial population and functional
508 genes during the bioremediation of petroleum-contaminated soil as an effective
509 monitoring approach, Ecotoxicol. Environ. Saf. 125, 153–160.
- 510 Smit K., Leeftang E., Glandorf P., van Elsas B., Wernars J.D., 1999. Analysis of fungal
511 diversity in the wheat rhizosphere by sequencing of cloned PCR-amplified genes
512 encoding 18S rRNA and temperature gradient gel electrophoresis, Appl. Environ.
513 Microbiol. 65, 2614–2621.
- 514 Song W., Wang J., Yan Y., An L., Zhang F., Wang L., Xu Y., Tian M., Nie Y., Wu X., 2018.
515 Shifts of the indigenous microbial communities from reservoir production water in
516 crude oil- and asphaltene-degrading microcosms, Int. Biodeterior. Biodegrad. 132, 18–
517 29.
- 518 Tambone F., Scaglia B., D'imporzano G., Schievano A., Orzi V., Salati S., Adani F., 2010.
519 Assessing amendment and fertilizing properties of digestates from anaerobic digestion
520 through a comparative study with digested sludge and compost, Chemosphere. 81,
521 577–583.
- 522 Tanase A., Ionescu R., Chiciudean I., Vassu T., Stoica I., 2013. Characterization of
523 hydrocarbon-degrading bacterial strains isolated from oil-polluted soil, Int.
524 Biodeterior. Biodegradation. 84, 150–154.
- 525 Vainio J., Hantula E.J., 2000. Direct analysis of wood-inhabiting fungi using denaturing
526 gradient gel electrophoresis of amplified ribosomal DNA, Mycol. Res. 104, 927–936.
- 527 Walsh J.J., Jones D.L., Edwards-Jones G., Williams A.P., 2012. Replacing inorganic
528 fertilizer with anaerobic digestate may maintain agricultural productivity at less
529 environmental cost, J. Plant Nutr. Soil Sci. 175, 840–845.
- 530 Wang S., Kuo Y., Hong A., Chang Y., Kao C., 2016. Bioremediation of diesel and lubricant
531 oil-contaminated soils using enhanced landfarming system, Chemosphere. 164, 558–
532 567.
- 533 Wang X.-B., Chi C.-Q., Nie Y., Tang Y.-Q., Tan Y., Wub G., Wua X.-L., 2011. Degradation
534 of petroleum hydrocarbons (C6–C40) and crude oil by a novel *Dietzia* strain,
535 Bioresour. Technol. 102, 7755–7761.
- 536





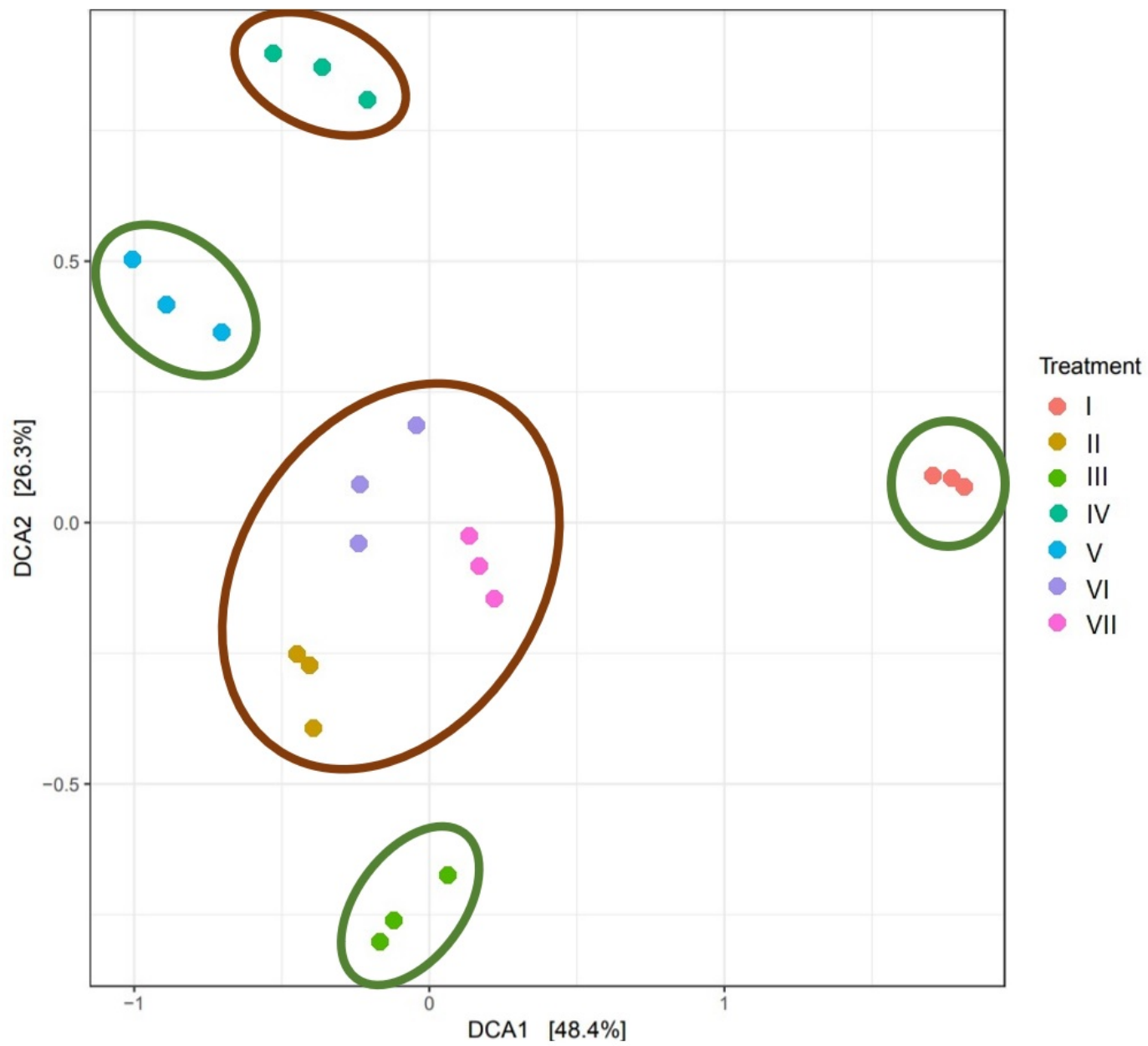


Table 1 Characteristics of soils and digestates used in the study. Mean (n = 3) and standard deviation. TOC: total organic carbon; TN: total nitrogen; TPH: total petroleum hydrocarbons; P: total phosphorus. n.d. not determined.

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