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Functional potential of sewage sludge digestate microbes to degrade aliphatic hydrocarbons during bioremediation of a petroleum hydrocarbons contaminated soil

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

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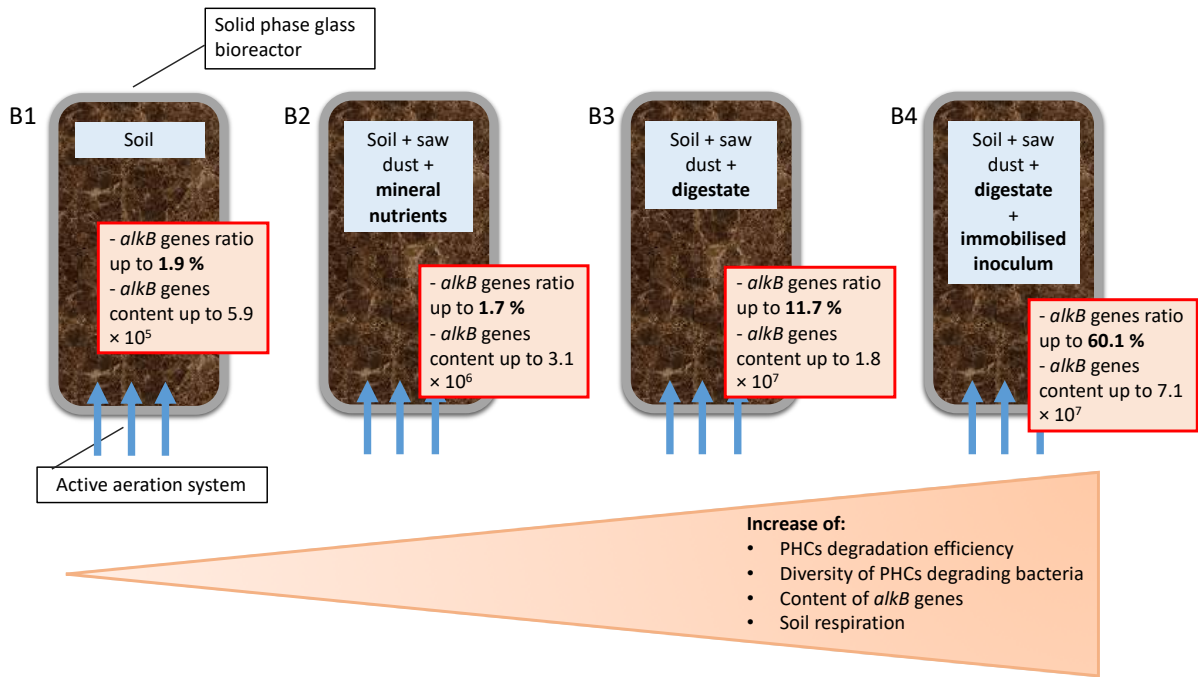
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25 **Highlights**

- 26 • PHCs contaminated soil was treated with digestate in actively aerated bioreactors
- 27 • Digestate application increased alkB genes content in soil
- 28 • Digestate was more efficient in bioremediation compared to mineral nutrients
- 29 • Application of immobilised bacteria together with digestate increased PHCs removal
- 30 • Digestate increased density and diversity of PHCs degraders in soils

31

32 **Abstract**

33 Sewage sludge digestate is a valuable organic waste which can be used as fertilizer in
34 soil bioremediation. Sewage sludge digestate is not only a good source of nutrients but is also
35 rich in bacteria carrying *alkB* genes, which are involved in aliphatic hydrocarbons metabolism.
36 Increase of *alkB* genes ratio in polluted soils has been observed to improve bioremediation
37 efficiency. In this study, for the first time, the genetic potential of indigenous microorganisms
38 of digestate to degrade petroleum products was assessed. The objectives were to study
39 petroleum hydrocarbons (PHCs) removal together with shifts in soil taxa and changes in the
40 concentration of *alkB* genes after digestate application. Initial *alkB* genes concentration in
41 contaminated soils and digestate was 1.5% and 4.5%, respectively. During soil incubation with
42 digestate, *alkB* genes percentage increased up to 11.5% and after the addition of bacteria
43 immobilized onto biochar this value increased up to 60%. Application of digestate positively
44 affected soil respiration and bacterial density, which was concomitant with enhanced PHCs
45 degradation. Incubation of soil amended with digestate resulted in 74% PHCs decrease in 2
46 months, while extra addition of bacteria immobilized onto biochar increased this value up to
47 95%. The use of digestate affected the microbial community profiles by increasing initial
48 bacterial density and diversity, including taxa containing recognized PHCs degraders. This
49 study reveals the great potential of digestate as a soil amendment which additionally improves
50 the abundance of *alkB* genes in petroleum contaminated soils.

51 Key words: *alkB* genes, bioreactors, organic wastes, soil clean-up, PHCs

52

53 1. Introduction

54 Intensive exploration and refinery of crude oil have resulted in a worldwide legacy of
55 soils polluted with crude oil composed mostly of alkanes and its derivatives, which constitutes a
56 threat for human health, disables soil services and affects land management (Shahi et al., 2016).
57 Bioremediation is an efficient strategy to treat petroleum hydrocarbons (PHCs) contaminated
58 soils (Lu et al., 2014). A successful bioremediation treatment requires regulation of soil nutrient
59 ratio (C:N:P), oxygen supply and most of all, dense, specialized microbial biomass able to
60 metabolize the contaminants (Safdari et al., 2018). One of the most important enzymes group
61 involved in PHCs oxidation are alkane monooxygenases encoded by *alkB* genes, responsible
62 for the first step of alkanes oxidation (Fuentes et al., 2014). Actual research and development
63 efforts are focusing on the increase of the 'soil bioremediation potential' which is connected
64 with the abundance of petroleum degraders, and thus functional genes, responsible for
65 contaminant degradation (Abtahi et al., 2020; Powell et al., 2006).

66 An efficient way to increase the density of PHCs metabolizing bacteria in soil, and thus,
67 to increase soil *alkB* genes content, is bioaugmentation. This method includes addition of liquid
68 biomass suspensions or immobilized bacteria (e.g. onto biochar) to soil (Agnello et al., 2016;
69 Mrozik and Piotrowska-Seget, 2010). Immobilization of inoculum on porous organic carriers
70 becomes popular practice due to significant improvement of microbial cells preservation
71 (Mrozik and Piotrowska-Seget, 2010). Dense and diverse microbial populations with a wide
72 metabolic capacity, which may serve for bioremediation purposes are also found in organic
73 fertilizers (Barra Caracciolo et al., 2015; Megharaj et al., 2011). However, those fertilizers are
74 poorly investigated in terms of functional genes content allowing alkanes degradation.

75 Digestate is a by-product of anaerobic digestion of organic residues (e.g. sewage sludge,
76 animal manures, organic fraction of municipal solid wastes) and constitutes a valuable soil
77 amendment with undiscovered potential in bioremediation (Kataki et al., 2017). Compared to
78 raw feedstock, nutrients in digestate are concentrated and present in bioavailable form (e.g.
79 ammonia) (Gómez-brandón et al., 2016), additionally the amount of easily biodegradable
80 compounds is reduced which is beneficial for bioremediation as they constitute a preferable
81 carbon source for microorganisms (Sayara et al., 2010).

82 Organic fertilizers were already studied in soil bioremediation, however mainly as
83 nutrient sources (Osei-Twumasi et al., 2020; Xi et al., 2020). If the microbial population
84 engaged in PHCs metabolism was studied, the data were restricted to simple analysis

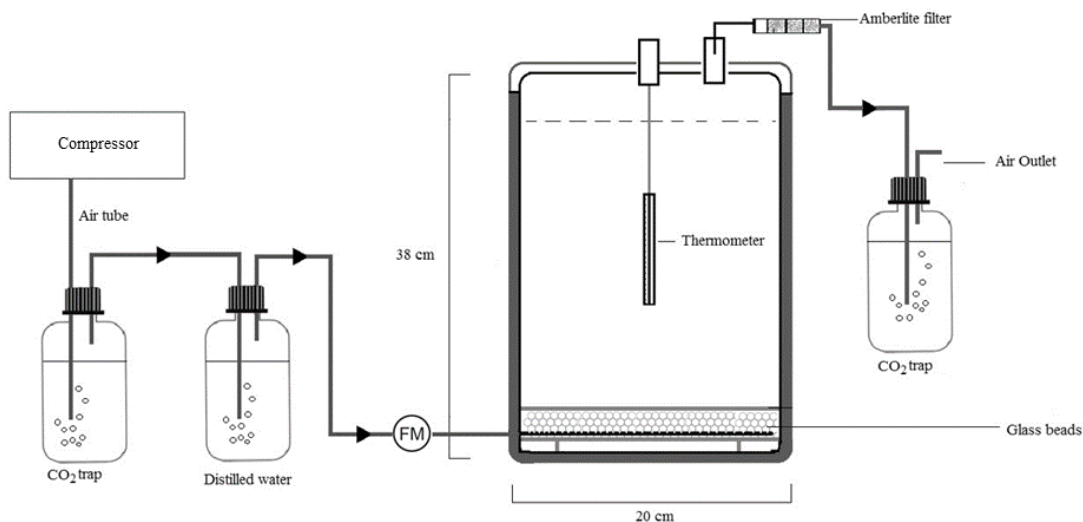
85 monitoring general changes of microbial community (DGGE, TTGE) or identification of
86 culturable bacterial strains (Abtahi et al., 2020). We hypothesized that bacteria contained in
87 digestate can enrich metabolic potential of soil providing new PHCs degrading taxa and thus
88 increase the content of *alkB* genes. We also hypothesized that addition of bacteria immobilized
89 onto biochar may further increase process efficiency if inoculated microorganisms have been
90 previously acclimated to treatment conditions. In this study, for the first time, we tested sewage
91 sludge digestate as soil inoculum in lab-scale aerated bioreactors. Our goal was to study how
92 addition of sewage sludge digestate to PHCs contaminated soil affects the concentration of *alkB*
93 genes and contaminants removal in time. We have also performed sequencing of total bacteria
94 in multiple time points to verify if addition of digestate affected the number of genera associated
95 with PHCs metabolism.

96

97 **Materials and methods**

98 **1.1 Experimental set-up**

99 The experiments (Figure 1) were performed in 12 L glass solid phase bioreactors with
100 active aeration designed according to Namkoong et al. (2002). To provide appropriate oxygen
101 amount, bioreactors were aerated from the bottom, with air flow rate ranging between 190 and
102 200 L h⁻¹ m⁻³ soil. Before entering into the reactors, CO₂ was removed from the inlet air by a
103 NaOH trap. Next, the air passed through a humidifier to remove aspirated alkali solution and to
104 increase humidity content in order to prevent reactors from drying. Exhaust air from the reactors
105 was filtered by an Amberlite[®] column in order to adsorb volatile organic compounds, which
106 could be released due to hydrocarbons volatilization. Further, the exhaust air passed through
107 5M NaOH trap to accumulate CO₂ generated during bacterial respiration. The amount of
108 trapped CO₂ was quantified by titration with HCl (Namkoong et al., 2002).



109
 110 **Figure 1. Scheme of bioreactor set up used in the study.** FM: flow meter. Arrows indicates the
 111 direction of the airflow.

112 To ensure high porosity of the soil mixture, treated soil was mixed with bulking agent
 113 (sawdust). The ratio of added sawdust was adjusted to favor a homogeneous airflow throughout
 114 reactor (Rhykerd et al., 1999). Apart from control condition (B1), three biostimulation and
 115 bioaugmentation treatment conditions were tested in duplicate (Table 1). Biostimulation
 116 treatments covered addition of nitrogen in the form of NH₄Cl (B2) or sewage sludge digestate
 117 (B3). The digestate to soil ratio was optimized based on previous studies (Gielnik et al., 2018;
 118 Namkoong et al., 2002). In bioaugmented treatments, bacteria immobilized on biochar were
 119 added together with digestate (B4). The ratio of added biochar was suggested by other studies
 120 (Brown et al., 2017). Bioreactors were incubated during 63 days at 20 °C (±2 °C). To ensure
 121 the homogeneity of the mixture, the content of bioreactors was mixed once a week and humidity
 122 level was checked by drying the samples overnight at 110 °C and further adjusting reactors
 123 content to fit 60% of the water holding capacity (WHC). Temperature inside the reactors was
 124 measured during the treatment by thermocouple systems. To study the volatile fraction of PHCs,
 125 exhaust column with 25 g of Amberlite[®] was extracted every week and the sorbent was
 126 replaced.

127 Once a week, 50 g of a representative sample (10 g of sample collected from 5 points
 128 and pooled together) originating from different reactor locations was collected from each
 129 reactor and homogenized for PHCs quantification and DNA extraction. Before PHCs
 130 extraction, soil mixture was air dried and grinded in a mortar. DNA analyses of every bioreactor
 131 were performed in triplicate. For DNA extraction, 5 g aliquots of the fresh samples were stored
 132 at -20 °C.

134

135

Table 1. Treatments analyzed during the experiment.

Set-up	Description
B1	Control: Soil (5.00 kg) + sawdust (0.49 kg = 500 cm ³) + water (60% WHC)
B2	Soil (5.00 kg) + sawdust (0.49 kg = 500 cm ³) + NH ₄ Cl (C:N:P 100:10:2) + water (60% WHC)
B3	Soil (3.75 kg) + sawdust (0.49 kg = 500 cm ³) + digestate (1.25 kg) + water (60% WHC)
B4	Soil (3.50 kg) + sawdust (0.49 kg = 500 cm ³) + digestate (1.25 kg) + bacteria immobilized onto biochar* (0.25 kg) + water (60% WHC)

136 *indigenous hydrocarbon degrading bacteria were enriched from bioreactor B3

137

138 **1.2 Soil, digestate and biochar**

139 Sewage sludge digestate was obtained from a biogas plant located in Limoges (France).
 140 Soil contaminated with petroleum products was obtained from an oil refinery site located in the
 141 north-east of France. Soil was air dried and sieved through < 2 mm according to the ISO 11464
 142 standard method. Fresh digestate was homogenized, sieved through < 2 mm and stored at 4 °C
 143 before analysis. Detailed parameters of both digestate and soil are presented in Table 2. Initial
 144 physico-chemical characterization of soil and digestate as well as concentration of polycyclic
 145 aromatic hydrocarbons (PAHs) in the soil was performed by Synlab (France, ISO/IEC
 146 17025:2005).

147 Biochar used for bacteria immobilization was produced from sewage sludge digestate
 148 by pyrolysis using the Biogreen[®] technology (Wongrod et al., 2018). Detailed characteristics
 149 of the biochar are given elsewhere (Wongrod et al., 2018). Before use, biochar was washed
 150 with ultrapure water to remove remaining impurities until the pH value of leaching water was
 151 stabilized, then sterilized by autoclaving and air dried under sterile hood (Xu and Lu, 2010a).
 152 Bacteria enriched for immobilization originated from the soil mixtures with the same
 153 composition as in bioreactor B3 (Table 1). Before enrichment, soil mixture was incubated for
 154 2 weeks to allow microbial adaptation and consequent community rearrangements. The
 155 immobilization procedure is given in details elsewhere (Gielnik et al., 2018). The final amount
 156 of immobilized bacterial cells represented by colony-forming unit was 3.4×10^8 cells per 1 g
 157 of biochar.

158 Addition of biochar to contaminated soil may result in irreversible sorption of PHCs
159 and formation of non-extractable residues. To verify if addition of biochar had significant effect
160 on PHCs concentration decrease, not due to biological processes, a simple test was performed.
161 Contaminated soil was mixed with non-inoculated biochar in a proportion corresponding with
162 bioreactor B4 (Table 1). Control constituted of a sole soil sample. Water content of both variants
163 was maintained at 60% of WHC and samples were incubated in triplicate for 2 months at 20
164 °C. After that time PHCs were quantified in all samples. Results have not revealed significant
165 differences in PHCs level between the control and sample with biochar (data not shown).
166

167 **Table 2 Characteristics of soil and digestate used in the study.** OM: organic matter; P: phosphorus;
 168 TN: total nitrogen; TOC: total organic carbon; PHCs: petroleum hydrocarbons, WHC: water holding
 169 capacity, DW: dry weight, US-EPA: United States Environmental Protection Agency.

	Soil	Sewage sludge digestate	Method
pH (H ₂ O)	6.2 (0.2)	7.1 (0.1)	NF ISO 10693
Water content (%)	0.4 (0.1)	95.6 (0.2)	NEN-ISO 11465
WHC (mL cm ⁻³)	0.2 (0.1)	n.a.	OECD test No. 222
OM (%)	2.2 (0.1)	87.3 (0.7)	NF ISO 14235
TOC (g kg ⁻¹ DW)	15 (1)	120 (7)	NEN-EN 13137
TN (g kg ⁻¹ DW)	0.3 (0.1)	20.8 (0.8)	NEN-EN-ISO 11732
P (g kg ⁻¹ DW)	0.3 (0.1)	10.0 (0.2)	NEN 6961, CEN/TS 16171, NF-EN 16179
C:N:P	100:2.3:2.0	100:17.3:8.3	-
PHCs (mg kg ⁻¹ DW)	13200 (124)	1.8 (0.1)	Section 2.4
US-EPA 16 PAHs (mg kg ⁻¹ DW)	25 (1)	n.a.	Internal method (SynLab)
Total elements content (mg kg ⁻¹ DW)			NEN 6961, NEN-EN-ISO17294-2
Fe	7600 (124)	54000 (210)	
Al	2700 (35)	7600 (14)	
Cd	0.3 (0.0)	0.7 (0.0)	
Cr	8.7 (0.1)	34.0 (0.3)	
Cu	57 (1.0)	110 (1.2)	
Hg	6.5 (0.2)	0.7 (0.1)	
Pb	120 (4.4)	27 (2.3)	
Ni	7.0 (0.1)	8.9 (0.4)	
Zn	88 (1.5)	270 (6.2)	

170 n.a.: not analyzed

171 **1.3 PHCs quantification**

172 For PHCs analysis 10 g of air-dried and grinded sample was extracted with 10 mL of
 173 hexane. To study PHCs volatilization, 25 g of Amberlite® from the exhaust gas column was
 174 extracted with a mix of 15 mL methanol and 15 mL hexane. The extraction procedure was based
 175 on USEPA 8015B and 3550s methods (USEPA, 2007, 1996), with mechanical shaking
 176 combined with ultrasonic extraction. PHCs were quantified on gas chromatography with flame

177 ionization detector (Shimadzu) with capillary column 30 m × 0.25 mm × 0.25 μm (ZB5HT
178 Inferno, Phenomenex) and hydrogen as a carrier gas. Maximal oven temperature was 325 °C.
179 Injection was done with 1 μL of sample in the split mode with split ratio 20 and temperature
180 285 °C. Column flow was set at 3.0 mL.min⁻¹ with pressure 113.5 kPa. Diesel oil was used as
181 quantification standard.

182 **1.4 DNA extraction**

183 Genomic DNA was extracted from 500 mg of freeze samples using Fast DNA Spin Kit
184 for Soils (MP Biomedicals). Extracted DNA was eluted in 100 μL of DNA free ultra-pure water.
185 DNA concentration and purity was determined using spectrophotometer UV-1800 (Shimadzu)
186 equipped with a TrayCell adaptor for micro-volumes (Hellma) (Biache et al., 2017). DNA was
187 stored at – 20 °C for further analyses.

188 **1.5 Real-time PCR**

189 The extracted genomic DNA was used to quantify total bacterial and fungal populations.
190 Primers selected for total bacteria were 968F/1401R (Felske et al., 1998) targeting 16S rDNA
191 and Fung5F/FF390R (Smit et al., 1999; Vainio and Hantula, 2000) targeting 18S rDNA for
192 fungi. Functional genes, i.e. alkanes hydroxylating monooxygenases genes (*alkB*), were
193 amplified using primers AlkBF: 5'-AACTACATCGAGCACTACGG-3' and AlkBR: 5'-
194 TGAAGATGTGGTTGCTGTTCC-3' (Powell et al., 2006) using 50°C for annealing
195 temperature. Real-time polymerase chain reaction (qPCR) quantifications were performed
196 using CFX96 Real Time PCR detection system (Bio-Rad) and SybrGreen detection (iQ sybr
197 green Supermix, Bio-Rad), according to the procedure described elsewhere (Cébron et al.,
198 2015, 2008) and using dilution series (10¹ to 10⁸ copies.μL⁻¹) of standard plasmids.

199 **1.6 Sequencing analysis**

200 Illumina MiSeq v3 Sequencing (2 × 300 bp) of the V3-V4 region of the 16S rDNA was
201 performed by MicroSynth AG (Switzerland) on previously isolated DNA. The company is ISO
202 certified according to 9001:2008 and ISO / IEC 17025. Library preparation included sample
203 quality control and Nextera two-step PCR amplification using primer set 341f_ill/802r_ill, PCR
204 product purification, quantification and equimolar pooling. Bioinformatic analysis included
205 demultiplexing, merging of forward and reverse reads, quality filtering, trimming, chimera
206 removal, operational taxonomic unit (OTU) clustering (97% identity threshold) and

207 subsampling for even sample size (rarefaction to the lower number of reads per sample).
208 Taxonomy assignment was based on the SILVA 16S database v.123 (> 60% confidence). Alpha
209 diversity calculation and comparative statistics were done with the use of Phyloseq and DeSeq2
210 (R packages). Heat map of the most abundant genera and clustering with Pearson method were
211 performed using free Heatmapper software (heatmapper.ca).

212 **1.7 Germination ecotoxicity tests**

213 Germination tests were performed at the beginning and at the end of the experiment
214 with the use of germination plates and seeds of garden cress (*Lepidium sativum*) and mustard
215 (*Sinapis alba*) as recommended by previous studies (Gargouri et al., 2014; Maila and Cloete,
216 2005; Mao et al., 2009). Germination plates were filled with 80 g of fresh homogenized sample
217 from each reactor. Control consisted of uncontaminated soil (obtained from the same industrial
218 area as contaminated soil) mixed with sawdust and digestate in the same ratio as soil from
219 bioreactors. Ten seeds were placed equally on each plate. Germination plates were incubated in
220 a growth chamber for 7 days, at 21 °C, with a photoperiod of 12/12 and photosynthetic photon
221 flux density at 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$. After 7 days, germinated plants were counted, and radicle
222 length was measured. Based on obtained data the germination index (GI) was calculated (Graj
223 et al., 2013) according to the equation:

$$224 \quad GI = \frac{Gs}{Gc} \times \frac{Ls}{Lc} \times 100[\%] \quad (1)$$

225 where: Gs and Gc are numbers of seeds germinated in the sample and control, respectively,
226 whereas Ls and Lc are the radicle lengths in the sample and control [mm], respectively.

227 **1.8 Microtox[®] ecotoxicity assay**

228 The toxicity of soil elutriates was determined using the Microtox[®] bioassay according
229 to previous studies (Khudur et al., 2015). Briefly, 1 g of air-dried soil was mixed with 9 mL of
230 ultra-pure water and incubated overnight in the dark at 150 revolutions per minute. After
231 incubation each sample was centrifuged at 4 500 revolutions per minute for 10 min. The
232 luminescence was measured on the dilutions of soil leaches at 15 °C after 5 and 15 min of
233 exposure, using a Microtox M500 Analyzer and with *Aliivibrio fischeri* (Hach, France) as a
234 biological reagent. Phenol (20 mg L⁻¹) and zinc sulfate (10 mg L⁻¹) standards, as well as reagent

235 control were run with each bath. Delta EC50 represents toxicity loss between samples at day 1
236 and day 63.

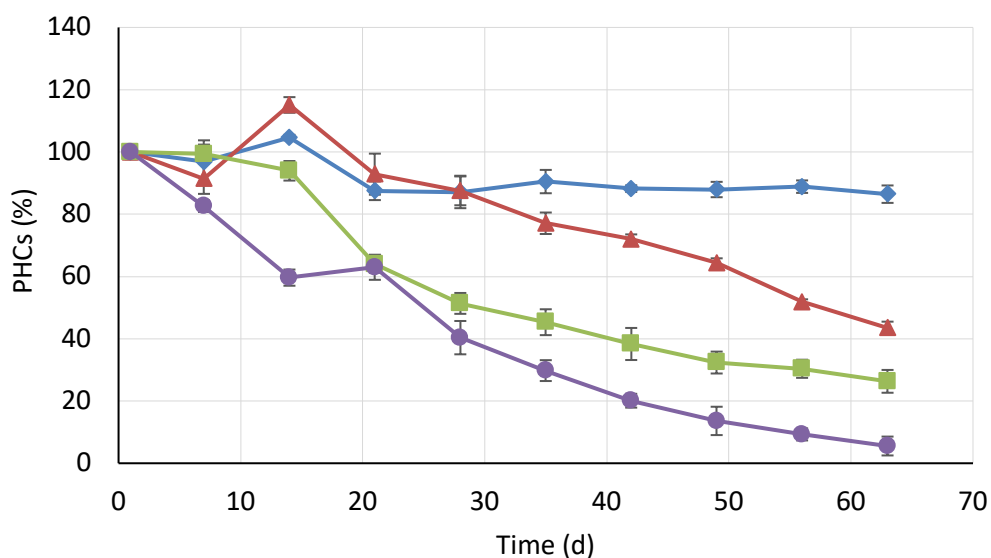
237 1.9 Statistical analysis

238 Statistical analyses were performed using XL Stat (Addinsoft 2015) statistical software for
239 Excel. Significant differences of parameters among the treatments were detected with one-way
240 ANOVA ($p < 0.05$) followed by Tukey *Post-Hoc* test.

241 2. Results and discussion

242 2.1 PHCs removal and mineralization extent

243 PHCs removal over time is presented in Fig. 2. The highest removal after 63 days was
244 observed for treatment B4 containing digestate and immobilized bacteria (94.5%), then for B3
245 with digestate (73.7 %), B2 with mineral nutrients (56.5 %) and the control B1 (13.6 %).
246 Differences observed after 63 days are significant (ANOVA; $P \leq 0.05$). During the first 30 days
247 more than 50% of PHCs were degraded in treatments containing digestate (B3 and B4), while
248 in treatment with mineral nutrients 50% of PHCs were degraded after 55 days.



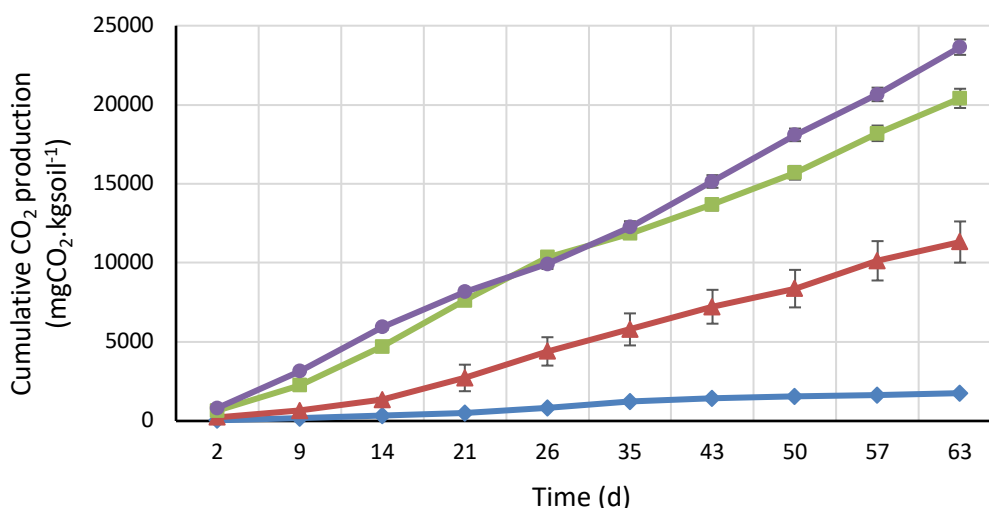
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250 **Figure 2. PHCs depletion during 63 days treatment** for ◆ B1 condition; ▲ B2 condition; ■
251 B3 condition; ● B4 condition. Abbreviations (B1, B2, B3, B4) refer to Table 1. Results are presented
252 as a mean of samples collected from two reactors and analyzed in triplicate (n=3) with standard
253 deviation.





254 PHCs degradation efficiencies reported in the literature vary from few weeks to few
255 months depending on soil and contamination characteristics as well as experimental scale

256 (Agnello et al., 2016; Dados et al., 2014; Megharaj et al., 2011). In the present study, 2 months
257 of treatment were sufficient for almost complete soil clean-up in the condition employing
258 digestate and immobilized bacteria (B4).

259 PHCs removal was followed by the decrease of characteristic unresolved
260 chromatographic peak (Fig. S1) representing weathered polar substances of highly recalcitrant
261 nature (Bruckberger et al., 2018). For the treatments B1 and B2, PHCs increased above initial
262 concentration in the first 15 days, which was not observed in treatments containing digestate.
263 Similar observations were reported in another study (Dados et al., 2014), where the use of
264 $(\text{NH}_4)_2\text{SO}_4$ as a nutrient source resulted in initial increase of PHCs concentration within the first
265 20 days, while for treatment with compost the increase did not occur. This suggests buffering
266 capacities of organic amendments in terms of PHCs metabolites (*e.g.* aldehydes) (Xu and Lu,
267 2010b).



268

269 **Figure 3. Carbon mineralization represented by cumulated CO₂ production for**  B1 condition;
270  B2 condition;  B3 condition;  B4 condition. Abbreviations (B1, B2, B3, B4) refer to
271 Table 1. Results are presented as a mean of samples collected from two reactors and analyzed in
272 triplicate (n=6) with standard deviation.

273 Petroleum compounds in contaminated soils can be removed by biodegradation,
274 biotransformation, volatilization or can be adsorbed on organic matter in a form of non-
275 extractable residues (Kästner et al., 2014). In the present study, volatilization values were below
276 0.5 % of PHCs loss and thus were not further considered. Mineralization of organic carbon
277 represented by CO₂ evolution was monitored to support information about microbial metabolic
278 activity and PHCs degradation (Fig. 3). We observed that CO₂ evolution was correlated with

279 PHCs removal, suggesting that biodegradation was the major force driving contaminant
 280 concentration decrease (Table 3). Mineralization values observed in the study are
 281 corresponding with the data of other authors reporting that about 10 g CO₂ kg⁻¹ soil was
 282 produced during first 30 days of soil treatment with sewage sludge in similar amendment ratio
 283 (Namkoong et al., 2002). Application of mineral nutrients often results in lower mineralization
 284 rates than organic fertilizers (Cerqueira et al., 2014; Tahhan and Abu-Ateih, 2009). In the
 285 present study, the use of digestate doubled respiration rates in comparison with mineral
 286 nutrients and was further increased by the application of immobilized bacteria which correlated
 287 with PHCs removal degree (Table 3).

288 **Table 3. Pearson correlation coefficients and p values between soil respiration and PHCs levels.**
 289 Abbreviations are reported in Table 1.

Reactors	r	p value
B1	0.737	0.015
B2	0.947	< 0.001
B3	0.968	< 0.001
B4	0.963	< 0.001

301 Intensive mineralization of organic matter during soil incubation is often followed by
 302 temperature rise (Antizar-Ladislao et al., 2006). However, in the present study, temperature
 303 inside reactors was equal to the temperature of the inlet air (20 ± 2 °C). As during anaerobic
 304 digestion easily biodegradable carbon is depleted and organic matter becomes stabilized,
 305 incubation of digestate may not result in rapid organic matter degradation and heat production.
 306 However, a significant organic fraction of organic matter can still be slowly degraded, which
 307 eventually contributes to observed CO₂ production. This is in accordance with the observations
 308 of other authors (Tambone et al., 2015), where composting of different pig slurry digestates did
 309 not always resulted in temperature rise but induced changes in Potential Dynamic Respiration
 310 Index (PDRI).

311 **2.2 Soil toxicity changes**

312 The concentration of contaminants does not provide direct information about soil
 313 toxicity, due to possible presence of toxic substances other than PHCs (e.g. pesticides, trace
 314 elements) and toxic metabolites generated during hydrocarbons degradation, such as aldehydes

315 which are less hydrophobic and easily extracted into soil aqueous phase (Xu and Lu, 2010a).
316 The values of germination index (GI) for both tested plants followed the trend of PHCs removal
317 yield with high toxicity for condition B1 (GI < 20 %) and low toxicity values for conditions B3
318 and B4 (GI > 90%). In the same way, Microtox[®] assay, presented as the relative change of EC50
319 between the start and the end of the experiment, also revealed a toxicity decrease in accordance
320 with PHCs removal yields (B1 < B2 < B3 < B4).

321 High efficiency of PHCs biodegradation was followed by high soil toxicity decrease in
322 treatments containing digestate and digestate with immobilized bacteria. Observed soil toxicity
323 decrease is comparable with results of other authors analyzing bioremediation of industrially
324 contaminated soils treated with mineral fertilizers (Xu and Lu, 2010a). Moreover, in the study
325 of Nwankwegu et al. (2016) as well as in the present study, application of organic fertilizer
326 resulted in higher PHCs removal and toxicity decrease than the use of mineral nutrients pointing
327 that successful remediation may be performed with the use of sustainable nutrient sources like
328 digestate.

329

330 **Table 4 Toxicity changes after 63 days of incubation.** Abbreviations are reported in Table 1. GI:
 331 Germination Index. Results are presented as a mean of samples collected from two reactors and analyzed
 332 in triplicate with standard deviation in bracket. Values of the same line followed by different letter are
 333 significantly different (ANOVA $p < 0.05$).

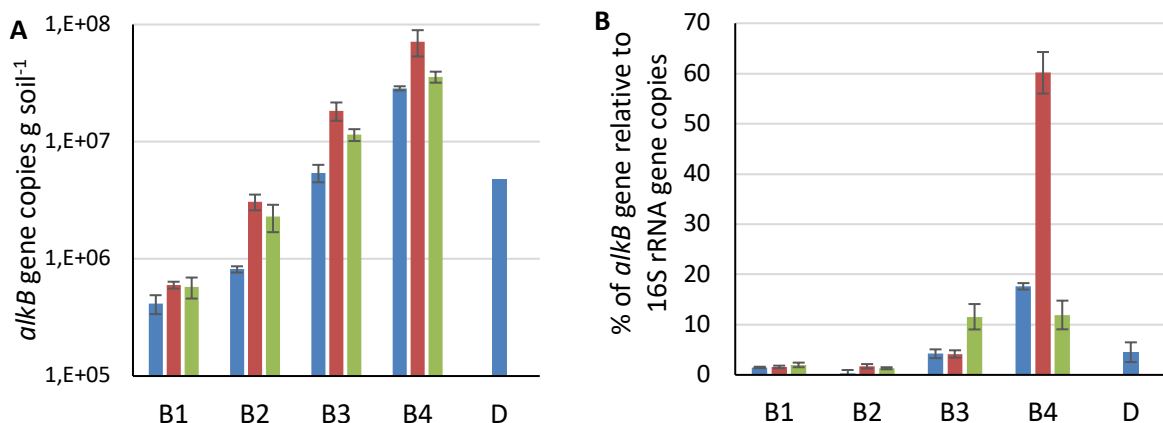
Toxicity assay	B1	B2	B3	B4
GI <i>Lepidium sativum</i> (%)	11.4 (2.6)c	58.2 (5.3)b	98.7 (1.7)a	96.2 (3.5)a
GI <i>Sinapis alba</i> (%)	16.5 (5.2)b	86.7 (6.3)a	93.0 (6.3)a	93.7 (1.9)a
Microtox[®] Δ EC50	16.7 (2.8)c	35.0 (5.0)b	63.3 (5.8)a	73.3 (5.8)a

334

335 2.3 Functional genes abundance

336 Genes copies corresponding to the total bacterial (16S rDNA) and fungal (18S rDNA)
 337 communities (Fig. S2), as well as *alkB* genes (Fig. S3) encoding monooxygenases crucial for
 338 alkanes oxidation (Powell et al., 2006) were measured at day 1, 30 and 63. The estimated
 339 abundance of fungi was around 2 orders of magnitude lower compared to bacteria (Fig. S2),
 340 which is a common observation in soil. Total bacterial population differed among treatments
 341 and slightly changed with time. Significantly more 16S rRNA gene copies were found in
 342 digestate and treatments B2, B3, B4 compared to the control (B1).

343 Figure 4A presents changes of the *alkB* genes concentrations with time. The content of
 344 *alkB* genes for all treatments was as follow B4>B3>B2>B1 (ANOVA; $p < 0.05$) at all
 345 monitoring time points, which corresponds with PHCs removal. For all treatments, excluding
 346 control, the lowest concentration of *alkB* genes was observed at the beginning of the
 347 experiment, then it greatly increased during the middle phase and further slightly decreased at
 348 the end. Addition of digestate (containing about 5.3×10^6 copies of *alkB* genes per g of dry
 349 weight), has significantly increased the copy number of *alkB* genes in soil. The initial number
 350 of *alkB* genes in control was 7.6×10^5 while in treatment B3 containing digestate the value
 351 exceeded 10^6 copies g^{-1} soil (Fig. 4A). Application of immobilized bacteria in treatment B4
 352 additionally raised the gene copies number above 10^7 copies g^{-1} soil. Such a high concentrations
 353 of *alkB* genes are outstanding in comparison to values obtained with mineral fertilizer as well
 354 as values reported in the literature (Masy et al., 2016; Shahi et al., 2016; Sutton et al., 2013).



355
 356 **Figure 4. A) *AlkB* genes copy number during the treatments; B) Percentage *alkB* genes relative to**
 357 **total bacteria represented by 16S rRNA gene copy number.** Treatment times: day 1 ■; day 30 ■;
 358 day 60 ■. Abbreviations are reported in Table 1. D: digestate. Results are presented as an average value
 359 of three samples collected from the same reactor with standard deviation.

360 At day 1, the percentage of *alkB* relative to 16S rRNA gene copies for control (B1) and
 361 treatment with mineral nutrients (B2) were 1.4% and 0.4%, respectively (Fig. 4B), which is in
 362 accordance with other studies where the observed values of *alkB*/16S rDNA for soil treatment
 363 with mineral nutrients ranged between 0.1 and 0.6% (Powell et al., 2006; Sutton et al., 2013).
 364 In treatment with digestate (B3) the initial *alkB*/16S rDNA ratio reached 4.2% while in
 365 treatment with digestate and inoculated biochar (B4) reached 17.6%, the differences between
 366 all treatments were significant (ANOVA; $p < 0.05$). The final values of the ratio were 2.0, 1.3,
 367 11.6, 11.9% for set-up B1, B2, B3 and B4, respectively, which indicates an important role of
 368 digestate in supplementation and maintenance of high proportion of bacteria carrying *alkB*
 369 genes in soil. These results are in accordance with a previous microcosm study (Gielnik et al.,
 370 2019).

371 The number of PHCs degrading bacteria, and thus the amount of *alkB* genes are
 372 connected to the amount of available substrate (e.g. PHCs) and bacterial activity. Thus, low
 373 contents of *alkB* genes were observed at the beginning of the experiment, before the intensive
 374 proliferation of biomass which is normally observed in the first half of the treatment. Intensive
 375 bioremediation in the first month was followed by lower activity in the second part of the
 376 experiment due to decrease of available substrate, which can be observed in Figure 2.
 377 Interestingly, very high ratio of *alkB*/16S rDNA (60.1%) was observed in the middle phase for
 378 treatment B4. It was probably due to introduction of inoculum already acclimated to treatment
 379 conditions which allowed fast microbial growth and dominance of bacteria carrying *alkB* genes
 380 over non-specialized soil microbial population. It also suggests that in other treatments, in the

381 phase of intensive proliferation, not only PHCs degraders, but also other bacterial species were
382 promoted.

383 **2.4 Bacterial community composition**

384 The effect of microorganisms originating from organic fertilizers on bioremediation of
385 PHCs is poorly studied. It was previously observed that successful bioremediation of petroleum
386 products is connected with diverse bacterial community carrying functional genes (*e.g. alkB* or
387 cytochrome P450 monooxygenase genes) but also with ecological equilibrium among bacteria,
388 including species not directly engaged in bioremediation (Akbari and Ghoshal, 2014). Thus,
389 sequencing of 16S rRNA was performed to study in which extent soil indigenous microflora
390 may be affected by the presence of organic fertilizer and which bacterial groups (originating
391 from soil or from digestate) dominate.

392 The main bacterial phyla are presented in Figure S3. For all the treatments, dominant
393 phyla throughout the incubation were *Proteobacteria* or *Actinobacteria* which are common soil
394 phyla detected during bioremediation studies (Fuentes et al., 2014), while in digestate the
395 dominant phyla were *Proteobacteria* (35.9%) and *Aminicenantes* (30.0%). In treatments B1
396 and B2, an increase of *Verrucomicrobia* with time was observed. This phylum is common in
397 soil environments (Bergmann et al., 2011). *Verrucomicrobia* was already found in minority in
398 hydrocarbon affected soils and the phylum tended to diminish at the end of bioremediation
399 (Ramadass et al., 2018), which suggests its secondary role in PHCs degradation. *Aminicenantes*
400 and *Atribacteria* phyla were specific for fresh digestate, and were already detected during
401 anaerobic digestion of sewage sludge (Pan et al., 2019; Zhao et al., 2018). Probably due to
402 anaerobic preferences, representatives of the *Atribacteria* phylum were not detected after
403 mixing of digestate with soil and application of aeration, while *Aminicenantes* representatives
404 were detected in samples B4 but their relative proportion decreased with time.

405 In all treatments, except for B4, *Proteobacteria* dominated over *Actinobacteria* at the
406 beginning of the treatment, however with time *Actinobacteria* tended to increase.
407 *Actinobacteria* is the main phylum observed in oil bioremediation treatments and contains
408 many species recognized as hydrocarbons degraders (Ros et al., 2010). Interestingly, in
409 treatment B4, *Actinobacteria* was a dominant phylum at the beginning of the experiment which
410 can be explained by previous acclimation of microbial inoculum. This observation suggests that

411 introduced inoculum was well prepared to degrade hydrocarbons and thus its application
412 allowed to increase process performance.

413 Top genera differed among samples according to treatment and sampling time (Fig. 5). Detailed
414 information about genera and list of identified species are included in supplementary materials
415 (Tables S1 and S2).

416 Clustering analysis revealed a distinct bacterial population in digestate and treated soil.
417 Similarities between microbial profiles were initially observed for treatments B1 and B2 (B1_S
418 and B2_S), however in the middle phase of the treatment the two profiles became distinct which
419 suggests that addition of mineral nutrients shaped soil microbial profile. Samples from reactors
420 B4 displayed similar microbial profiles at the beginning and in the middle phase of the treatment
421 (B4_S and B4_M) which could be explain by the applied inoculum microbial populations that
422 were well adapted to degrade PHCs. A slight population shift in treatment B4 occurred at the
423 end of the incubation (B4_E) which was probably a consequence of PHCs depletion.

424 In the control (B1), the most abundant genera at the beginning of the experiment were
425 *Pseudomonas* (46.8%). *Pseudomonas* genus is a common soil bacterial taxa and contains many
426 well-known PHCs degrading species (Liu et al., 2011; Reyes-Sosa et al., 2018). After 30 days,
427 the population of *Pseudomonas* decreased to 1.6% and other species associated with PHCs
428 degradation were promoted including *Pseudoxanthomonas*, *Microbacterium* and *Nocardioides*
429 (Liu et al., 2011; Reyes-Sosa et al., 2018; Yu et al., 2018).

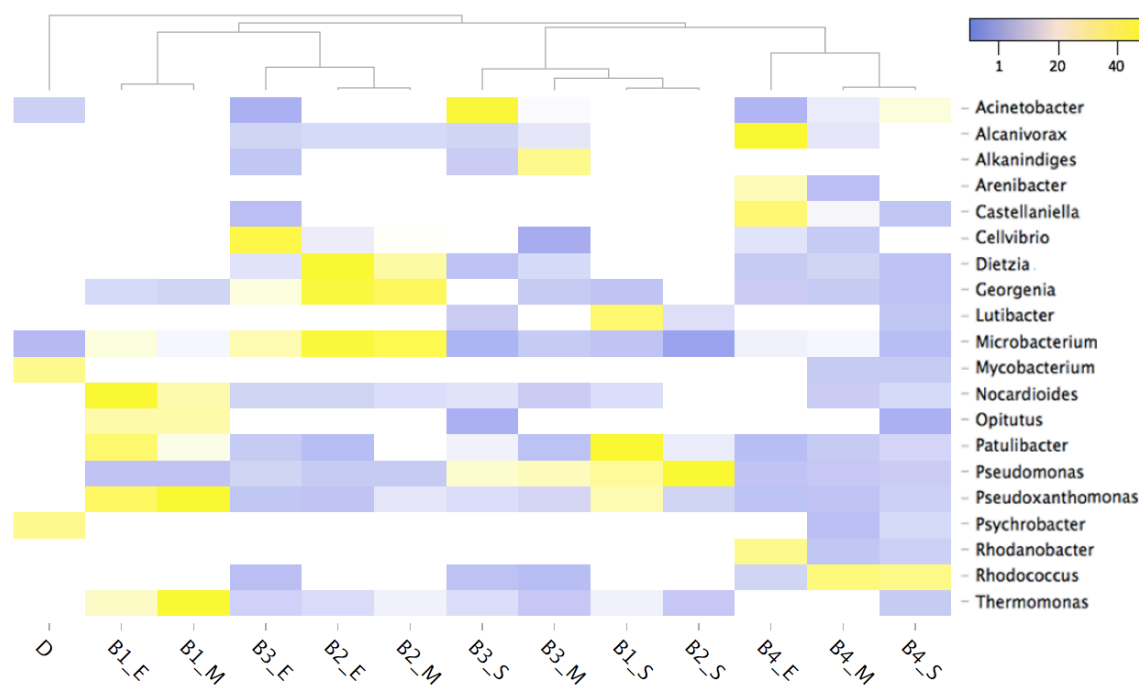
430 In the treatment B2, *Pseudomonas* were dominant in the first days of the study (90%)
431 and dropped to 4% after 30 days. At the end of the incubation the dominant genera included
432 *Microbacterium* (24.2%), *Cellvibrio* (13.6%), *Dietzia* (12.1%) and *Georgenia* (6%).
433 Interestingly, most *Dietzia* affiliated OTUs were assigned to *Dietzia maris* previously identified
434 as hydrocarbons degrading bacteria (Sutton et al., 2013).

435 In soil amended with digestate (B3), the most common genera at the beginning were
436 *Pseudomonas* (33%), *Acinetobacter* (32.3%) and *Rhodococcus* (12.4%), all of these genera
437 contain species with known ability to degrade PHCs (Liu et al., 2011; Reyes-Sosa et al., 2018;
438 Yu et al., 2018). Interestingly, in the middle part of the process, *Alkanindiges* reached a peak
439 abundance of 17.5% and almost totally disappeared after the next 30 days (0.2%). *Alkanindiges*
440 genera contains many PHCs degrading species (Bogan et al., 2003). The increase of
441 *Alkanindiges* population in the middle part of the treatment may be connected with the phase

442 of intensive PHCs degradation while decrease at the end of the treatment could be a result of
443 bioavailable hydrocarbons depletion.

444 In case of treatment B4, at the beginning, the dominant genera were *Rhodococcus*
445 (41.6%) and *Acinetobacter* (13.1%) associated with PHCs degradation (Liu et al., 2011; Reyes-
446 Sosa et al., 2018; Yu et al., 2018). After 63 days, the relative importance of *Rhodococcus*
447 decreased to 15.8% and other PHCs degrading genera became dominant including *Alcanivorax*
448 (21%), *Microbacterium* (7.6%) and *Arenibacter* (5%) (Reyes-Sosa et al., 2018). In all
449 treatments amended with digestate, initial richness of genera associated with PHCs degradation
450 and overall diversity measures were higher compared to reactors B1 and B2.

451 It was observed that treatments supplemented with digestate were characterized by more
452 dense and diverse microbial communities compared to treatments with mineral nutrients. It
453 could be a result of the dense digestate microbial population as well as improved soil conditions
454 including increase of soil organic matter, water holding capacity and contaminant dilution,
455 making soil more suitable environment for microbial development (Ren et al., 2018). Not only
456 bacterial taxa directly involved in the metabolism of PHCs were promoted during the study.
457 The relations between different bacterial taxa are not evident and may include symbiosis,
458 cooperation or mutualistic dependences additionally affected by abiotic conditions. Bacterial
459 community composition was initially influenced by digestate addition however it had not
460 crucial effect on the direction of microbial community changes. As mentioned in other studies,
461 the direction of the bacterial community evolution in amended soils does not only depend
462 strictly on the type of organic matter but also on soil type and environmental conditions (Pérez-
463 Piqueres et al., 2006).



464

465 **Figure 5. Heat map illustrating top genera (> 0.2%) based on relative abundance (%).**
 466 Abbreviations are reported in Table 1. D: digestate; S: day 1; M: day 30; E: day 63. Results are presented
 467 as a mean of three samples collected from the same reactor. To show similarities in genera composition
 468 between samples Pearson clustering method was employed.

469 **Conclusions**

470 Application of digestate to a PHCs contaminated soil in lab scale aerated bioreactors
 471 has enhanced soil bioremediation potential, as reflected by the higher concentration of *alkB*
 472 genes compared to mineral fertilizer. Digestate supplementation to soil improved activity,
 473 density and diversity of total microbial population. Moreover, use of digestate positively
 474 affected the density and diversity of bacterial taxa containing PHCs degrading species.
 475 Monitoring of the concentration of *alkB* genes during the treatments reflected the PHCs removal
 476 efficiency, which confirms role of *alkB* genes as biodegradation marker. Future studies could
 477 focus on *alkB* genes diversity in organic fertilizers, the kinetics of functional genes spreading
 478 in soil after fertilizer application and fluctuation of functional genes abundance according to
 479 the bioavailable contaminant concentration.

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Supplementary materials

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Functional potential of sewage sludge digestate microbes to degrade

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aliphatic hydrocarbons during bioremediation of a petroleum

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hydrocarbons contaminated soil

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

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van Hullebusch ^{4,5}

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¹Université Paris-Est, Laboratoire Géomatériaux et Environnement (LGE), EA 4508, UPEM, 77454

690

Marne-la-Vallée, France

691

²University of Napoli “Federico II”, Department of Civil, Architectural and Environmental

692

Engineering, 80125 Napoli, Italy

693

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

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⁴IHE Delft Institute for Water Education, Department of Environmental Engineering and Water

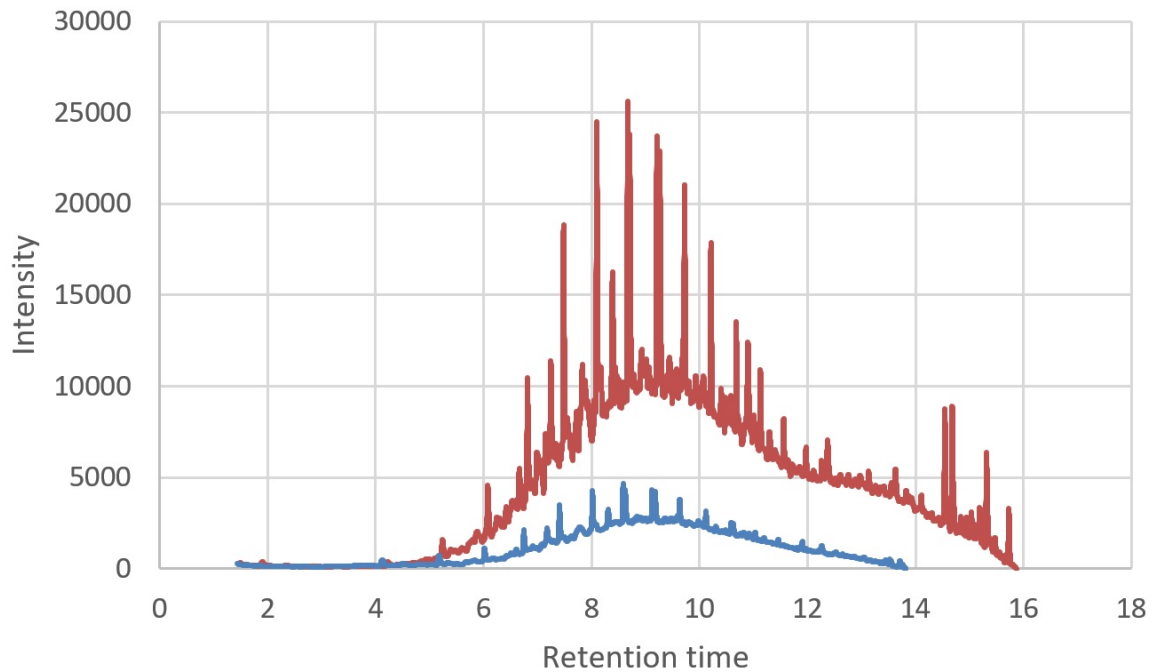
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Technology, P.O. Box 3015, 2601 DA, Delft, The Netherlands

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⁵Université de Paris, Institut de physique du globe de Paris, CNRS, F-75005 Paris, France

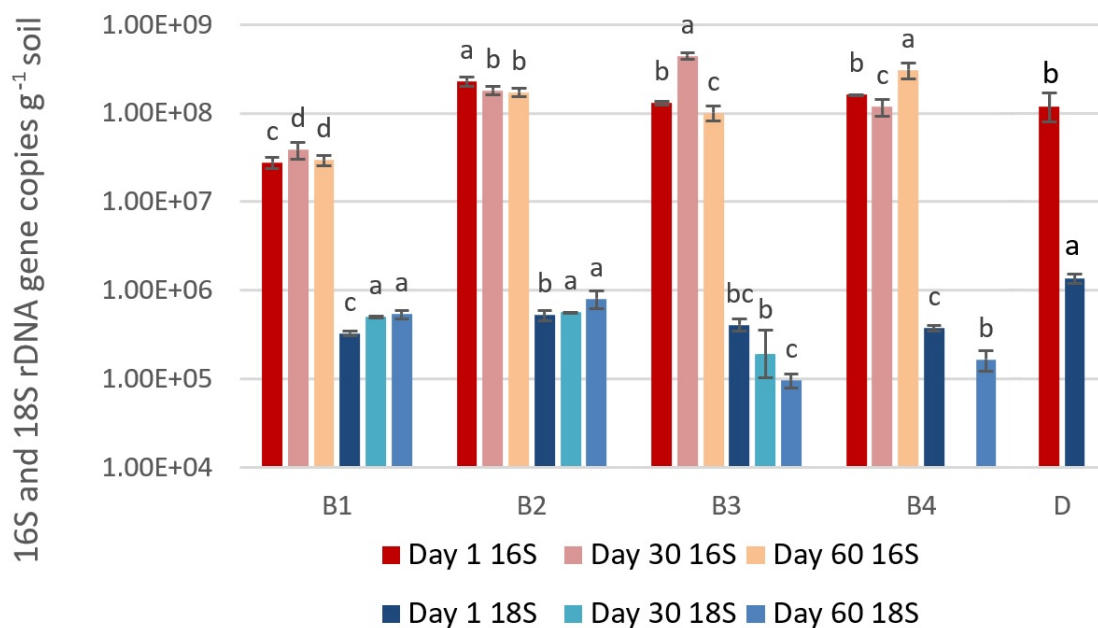
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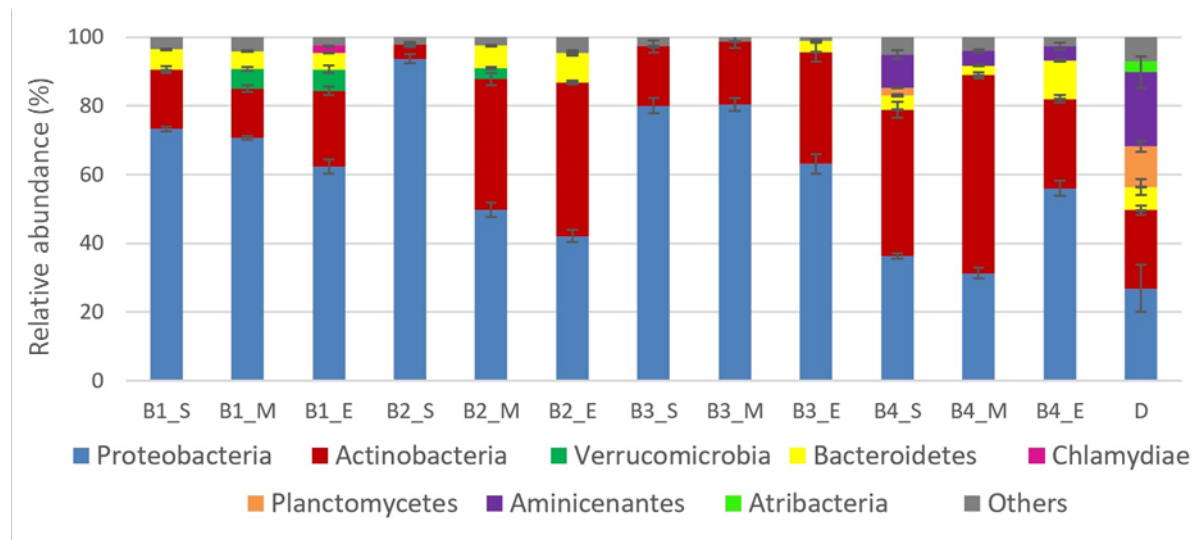
699 **Figure S1. GC-FID chromatogram presenting PHCs concentration before (red) and after (blue)**
 700 **63 days of treatment under conditions B3.**

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703 **Figure S2. Bacterial and fungal abundance during the treatments represented by 16S and 18S**
 704 **rRNA genes copy number.** Abbreviations are reported in Table 1. D: digestate. Results are presented
 705 as an average value of three samples collected from the same reactor with standard deviation. Values
 706 that are annotated with different letter among one gene class and sampling time are significantly
 707 different (ANOVA $p < 0.05$ and Tukey's multiple range test).



709

710 **Figure S3. Relative abundance of main bacterial phyla in tested treatments during three**
 711 **sampling time points and in digestate.** Abbreviations are reported in Table 1. D: digestate; S: day 1;
 712 M: day 30; E: day 63. Results are presented as a mean of three samples collected from the same
 713 reactor with standard deviation.

Table S1. Relative abundance of main genera identified in the study at the 3 sampling times. D: digestate; B1: soil + sawdust; B2: soil + sawdust + mineral nutrients; B3: soil + sawdust + digestate; B4: soil + sawdust + digestate + immobilized bacteria. S – start (1 d); M –middle (30 d); E – end (63 d).

Genus	B1 E	B1 M	B1 S	B2 E	B2 M	B2 S	B5 E	B5 M	B5 S	B7 E	B7 M	B7 S	D
<i>Acinetobacter</i>	0	0	0.1	0	0	0.1	0.2	9.5	32.3	0.7	7.4	13.1	4
<i>Alcanivorax</i>	0	0	0	0.5	0.6	0	0.2	1.9	0.2	21	1.7	0	0
<i>Alkanindiges</i>	0	0	0	0	0	0	0.2	17.5	0.9	0	0	0	0
<i>Arenibacter</i>	0	0	0	0	0	0	0	0	0	5	0.5	0	0
<i>Castellaniella</i>	0	0	0	0	0	0	0.5	0	0	6.7	2.2	0.7	0
<i>Cellvibrio</i>	0	0	0	13.6	16.9	0	43.6	3	0	11.6	7.9	0	0
<i>Dietzia</i>	0.1	0	0	12.1	7	0	1.8	1.2	0.2	0.6	1.1	0.2	0
<i>Georgenia</i>	0.7	0.6	0.2	6	5.3	0.1	2.3	0.3	0.1	0.4	0.3	0.1	0
<i>Lutibacter</i>	0	0	6.8	0	0	1.1	0	0	0.4	0	0	0.2	0
<i>Microbacterium</i>	11.1	8	3.6	24.2	22.8	0.8	14.6	4.3	2.3	7.6	7.9	3.1	2.8
<i>Mycobacterium</i>	0	0	0	0	0	0	0	0	0	0.1	0.3	0.3	8.8
<i>Nocardioides</i>	5.1	2.3	0.5	0.3	0.5	0.1	0.3	0.2	0.6	0.1	0.2	0.4	0
<i>Opitutus</i>	5.6	5.5	0	0	0	0	0	0	0.4	0	0	0.3	0
<i>Patulibacter</i>	3.9	1.8	5.2	0.2	0.1	1.1	0.5	0.3	1.2	0.2	0.5	0.7	0
<i>Pseudomonas</i>	1.5	1.6	46.8	3.6	4	90	6.8	37.5	33	1.9	3.4	4.6	0
<i>Pseudoxanthomonas</i>	15.4	19.8	9.9	0.8	3.4	1.9	0.9	2.1	2.6	0.6	0.7	1.7	0
<i>Psychrobacter</i>	0	0	0	0	0	0	0	0	0	0.1	1.5	6.3	31.8
<i>Rhodanobacter</i>	0	0	0	0	0	0	0	0	0	7.2	0.2	0.6	0
<i>Rhodococcus</i>	0	0.1	0.1	0	0.1	0	12.1	11.5	12.4	15.8	44.1	41.6	0.1
<i>Thermomonas</i>	3.8	10.3	1.6	0.8	1.6	0.2	0.5	0.2	0.9	0	0.1	0.3	0

1 **Table S2. Relative abundance of species identified in the study at the 3 sampling times.** D: digestate; B1: soil + sawdust; B2: soil + sawdust + mineral
 2 nutrients; B3: soil + sawdust + digestate; B4: soil + sawdust + digestate + immobilized bacteria. S – start (1 d); M –middle (30 d); E – end (63 d).

Species	B1_S1	B1_M1	B1_E1	B2_S1	B2_M1	B2_E1	B5_S1	B5_M1	B5_E1	B7_S1	B7_M1	B7_E1	FD_1
(Unassigned)	60.1	98.0	97.9	13.0	80.5	76.2	77.9	71.9	90.8	96.1	90.2	91.3	100
<i>Arthrobacter_sulfureus</i>	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	3.3	1.4	0.0
<i>Cellvibrio_sp._E50</i>	0.0	0.0	0.0	0.0	1.4	1.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0
<i>Dietzia_maris</i>	0.0	0.0	0.1	0.0	7.0	12.1	0.2	1.2	1.8	0.2	1.1	0.6	0.0
<i>Georgenia_sp._2216.35.28</i>	0.2	0.6	0.7	0.1	5.3	6.0	0.1	0.3	2.3	0.1	0.3	0.4	0.0
<i>Lysobacter_concretionis_Ko07_=_DSM_16239</i>	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.7	1.3	1.6	0.0
<i>Pseudomonas_sp.</i>	0.0	0.0	0.0	0.0	0.0	0.0	13.0	18.5	0.1	0.0	0.0	0.0	0.0
<i>Pseudomonas_sp._G0919</i>	1.5	0.1	0.1	0.5	0.0	0.0	0.5	0.6	0.0	1.0	0.1	0.1	0.0
<i>Pseudomonas_stutzeri</i>	38.1	1.3	1.2	86.3	3.8	3.5	7.7	6.7	3.7	1.7	2.7	1.4	0.0
<i>Pusillimonas_noertemannii</i>	0.0	0.0	0.0	0.0	0.3	0.3	0.1	0.6	0.9	0.0	0.0	0.0	0.0
<i>Rhodanobacter_fulvus</i>	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.1	0.0
<i>Thermomonas_fusca_DSM_15424</i>	0.0	0.0	0.0	0.0	1.6	0.8	0.4	0.1	0.5	0.0	0.0	0.0	0.0

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