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1 **Effect of digestate application on microbial respiration and**
2 **bacterial communities' diversity during bioremediation of**
3 **weathered petroleum hydrocarbons contaminated soils**

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5 Anna Gielnik^{1,2,3}, Yoan Pechaud¹, David Huguenot¹, Aurélie Cébron⁴, Jean-Michel Riom¹, Gilles
6 Guibaud³, Giovanni Esposito², Eric D. van Hullebusch^{1,6,7}

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

9 ²University of Napoli "Federico II", Department of Civil, Architectural and Environmental
10 Engineering, 80125 Napoli, Italy

11 ³University of Limoges, PEIRENE, Équipe Développement d'indicateurs ou prévision de la qualité
12 des eaux, URA IRSTEA, 123 Avenue Albert Thomas, 87060 Limoges Cedex, France

13 ⁴Université de Lorraine, CNRS, LIEC, F-54000 Nancy, France

14 ⁶IHE Delft Institute for Water Education, Department of Environmental Engineering and Water
15 Technology, P.O. Box 3015, 2601 DA, Delft, The Netherlands

16 ⁷Institut de Physique du Globe de Paris, Sorbonne Paris Cité, Université Paris Diderot, UMR 7154,
17 CNRS, F-75005 Paris, France

18
19 Corresponding author: Anna Gielnik

20 E-mail: (aniagielnik@gmail.com)

21 Postal address: UPEM - Université Paris-Est Marne-la-Vallée; 5 Boulevard Descartes - Champs-Sur-
22 Marne; 77454 Marne-la-Vallée, France

23

24 **Revised version resubmitted to Science of the Total Environment**

25

26 **Highlights**

- 27 • Digestate application with bulking agent or immobilized bacteria improve TPH
28 removal
- 29 • Digestate effect on soil respiration depends on soil texture
- 30 • Sewage sludge digestate contains high concentrations of *alkB* genes

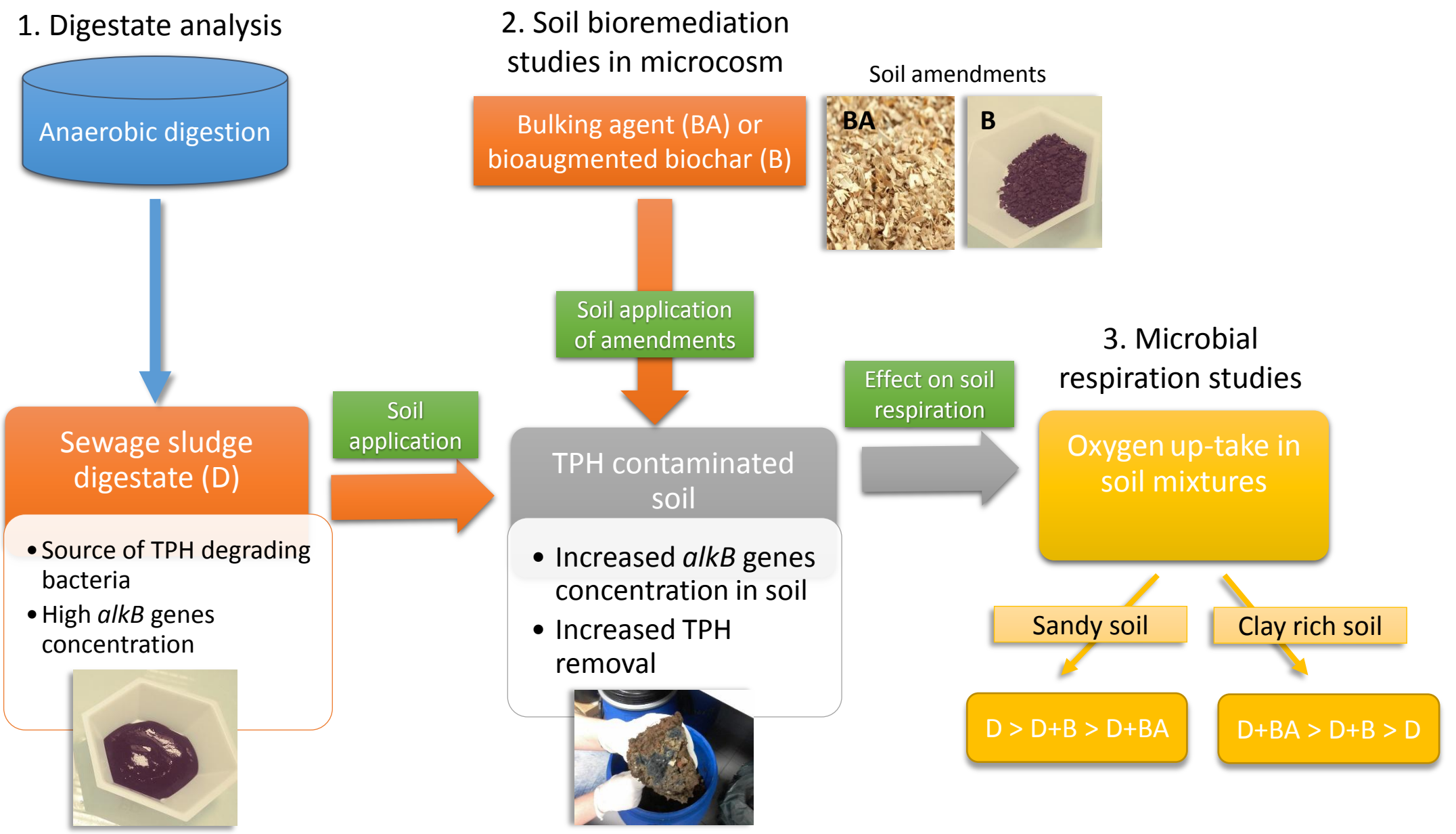
31 • Application of digestate increases and maintains *alkB* genes content in the soil

32 • Distinct microbial groups developed in amended and non-amended soils

33

34

35



Highlights

- Digestate application with bulking agent or immobilized bacteria improve TPH removal
- Digestate effect on soil respiration depends on soil texture
- Sewage sludge digestate contains high concentrations of *alkB* genes
- Application of digestate increases and maintains *alkB* genes content in the soil
- Distinct microbial groups developed in amended and non-amended soils

36 **Abstract**

37 Digestate is an organic by-product of biogas production via anaerobic digestion processes and
38 has a great potential as soil fertilizer due to concentrated nutrients. In this study, we examined
39 digestate as a potential nutrient and microbial seeding for bioremediation of weathered (aged)
40 petroleum hydrocarbon contaminated soils. We analyzed 6 different treatments in microcosm
41 using two industrial soils having different textures: a clay rich soil and a sandy soil. After 30
42 days of incubation, the highest total petroleum hydrocarbons (TPH) removal was observed in
43 microcosms containing digestate together with bulking agent (17.8 % and 12.7 % higher than
44 control in clay rich soil and sandy soil, respectively) or digestate together with immobilized
45 bacteria (13.4 % and 9 % higher than control in clay rich soil and sandy soil, respectively).
46 After digestate application microbial respiration was enhanced in sandy soil and inhibited in
47 clay rich soil due to aggregates formation. After bulking agent addition to clay rich soil
48 aggregates size was reduced and oxygen uptake was improved. Application of digestate to
49 soil resulted in the development of distinct microbial groups in amended and non-amended
50 soils. Genera containing species able to degrade TPH like *Acinetobacter* and *Mycobacterium*
51 were abundant in digestate and in soil amended with digestate. Quantification of *alkB* genes,
52 encoding alkane monooxygenase, revealed high concentration of these genes in digestate
53 bacterial community. After application of digestate, the level of *alkB* genes significantly
54 increased in soils and remained high until the end of the treatment. The study revealed great
55 potential of digestate as a nutrient and bacteria source for soil bioremediation.

56

57

58 Key words: *alkB* genes, soil remediation, organic fertilizers, TPH removal, qPCR

59 **1. Introduction**

60 Soils contaminated with petroleum products is a well-recognized worldwide problem
61 (Lu et al., 2014). Among soil treatment methods, bioremediation constitutes a promising and
62 economical approach (Beškoski et al., 2011; Coulon et al., 2010). A body of literature exists
63 on the topic of total petroleum hydrocarbons (TPH) bioremediation analysing various
64 biostimulation (addition of nutrients) and bioaugmentation (application of cultivable
65 degrading agents) strategies (Masy et al., 2016; Safdari et al., 2018; Sayara et al., 2010a).
66 Application of organic matter to soil is known to improve soil quality by *e.g.* stabilization of
67 pH and enrichment in soil organic matter, which supports microbial growth (Nardi et al.,
68 2004; Tambone et al., 2010). However, not much attention is paid on the use of organic
69 amendments (*e.g.* organic wastes) for soil treatment.

70 Reasonable practice of waste management encourages recycling of organic waste by
71 soil application (Tampio et al., 2016). Digestate is a by-product of anaerobic digestion
72 processes which constitutes a valuable organic amendment with several advantages over
73 mineral fertilizers (Gómez-brandón et al., 2016; Walsh et al., 2012). First of all, digestate
74 contains high density and diversity of microorganisms with wide catabolic capacities (Wang
75 et al., 2018). Secondly, during anaerobic digestion nutrients become concentrated in
76 bioavailable form (Gómez-brandón et al., 2016; Kataki et al., 2017) and easily biodegradable
77 organic carbon quantity is reduced (Risberg et al., 2017). Moreover, it was observed that
78 humic acids which are present in digestate can support desorption of organic contaminants
79 from soil matrix increasing their bioavailability (Liang et al., 2007; Sayara et al., 2010b).
80 However, despite the promising physico-chemical and microbial properties of digestate, to the
81 best of our knowledge it has never been tested before as an amendment in soil bioremediation.
82 Thus, the influence of the application of digestate on soil microbial activity is not known.
83 Particularly, to better understand the role of digestate in the process, its influence on microbial

84 diversity and on the concentration of functional genes need to be addressed. Organic matter
85 present in digestate may be degraded by monooxygenases encoded by *alkB* genes (Sutton et
86 al., 2013). Interestingly, the same enzymes are crucial for metabolism of alkanes and other
87 TPH constituents and could thus play a key role in the process (Sutton et al., 2013).

88 The final effect of digestate on soil microbial activity may be affected by factors like
89 soil texture, physico-chemical and hydraulic properties as well as contaminants concentration,
90 composition and weathering stage. For example in clay rich soils low permeability limits fluid
91 flow and can affect oxygen transfer due to the formation of soil aggregates (Yeh and Young,
92 2003) which may be enhanced after digestate application. Thus, the influence of soil
93 composition and texture on the treatment efficiency and on the microbial activity needs to be
94 addressed.

95 The main objectives of this study were i) to assess the value of digestate as microbial
96 inoculum for the remediation of TPH contaminated weathered soils, ii) to study the effect of
97 digestate on activity and diversity of soil microflora, and iii) to evaluate the impact of soil
98 composition and texture on the efficiency of microbial respiration. In order to study how soil
99 particle size affects microbial activity after digestate application we have examined two
100 common types of industrial TPH contaminated soils, a clay rich soil and a sandy soil.
101 Together with digestate application, bioaugmentation was performed by addition of soil
102 indigenous TPH degrading bacteria immobilized on biochar. The bacterial diversity was
103 monitored through high throughput sequencing of 16S rDNA (Illumina MiSeq). The
104 functional property of the bacterial communities in these conditions was assessed through
105 quantification of *alkB* genes (qPCR).

106

107 **2. Materials and methods**

108 **2.1. Characterization of soil and digestate samples**

109 Soil S1 used in the experiment was obtained from an oil refinery site located in the
110 north-east of France (Dunkerque) and soil S2 originated from an industrial site contaminated
111 with motor oil (Lyon, France). Both soils were air dried and sieved through < 2 mm before
112 use. Soil S1 was classified as loam with 26.3 % clay content and soil S2 had a texture of fine
113 sand (94.6 %). Soil S1 was characterized by a slightly alkaline pH while soil S2 was acid,
114 which is in agreement with the soil types. TPH level was 6.1 and 32.6 g kg⁻¹ DW in soil S1
115 and S2, respectively. In soil S1 TPH was present in the form of black and viscous nuggets
116 located in soil aggregates while for soil S2 contamination was yellow and less viscous.
117 Sewage sludge digestate was obtained from biogas plant in Limoges, France. Digestate was
118 concentrated by centrifugation to decrease water content from 96 to 81 %. Digestate was
119 stored at 4°C before being applied to soil. Detailed characteristics of soils and digestate are
120 specified in Table 1. Initial physicochemical characterization of soil and digestate was
121 performed by Synlab (France) which is a certified laboratory (ISO/IEC 17025:2005). Aliquots
122 of soil and digestate were stored at -20°C for further DNA extraction.

123 **Table 1. Soils and digestate characteristics.** WHC: water holding capacity; OM: organic matter;
124 TOC: total organic carbon; TN: total nitrogen; TPH: total petroleum hydrocarbons, PAH: polycyclic
125 aromatic hydrocarbons.

126

127 **2.2. Biochar and bacteria immobilization**

128 Biochar used in the study was produced from sewage sludge digestate by pyrolysis at
129 350°C using the Biogreen[®] technology (Wongrod et al., 2018). The detailed characteristics of
130 the biochar are given elsewhere (Wongrod et al., 2018). Before use, biochar was washed 3

131 times with ultra-pure water (UPW) to remove impurities and stabilize pH, sterilized and dried
132 according to recommendations (Xu and Lu, 2010).

133 Bacteria for immobilization were enriched separately from both contaminated soils
134 using a modified Bushnell Haas medium (pH 7.2) containing 0.2 g of MgSO₄, 0.02 g of
135 CaCl₂, 1 g of KH₂PO₄, 1 g of Na₂HPO₄, 1 g of NH₄NO₃ and 1 mg of FeSO₄ × 7 H₂O per 1 L
136 of UPW. Diesel oil obtained from commercial gas station was used in final concentration of
137 1% v/v in the medium as a sole carbon and energy source.

138 In brief, 10 g of each soil was added to 100 mL of medium and cultivated for 3 weeks
139 on rotary shaker at 21°C and 180 rpm in Erlenmeyer flasks. Each week, 1 mL of culture was
140 transferred on fresh medium. At the end, 10 mL of bacterial enrichment culture was added to
141 1 L of medium and cultivated in 1.5 L bottles for 1 week. Before use, bacteria were harvested
142 by centrifugation for 15 min at 5,000 rpm and washed 4 times with saline phosphate buffer
143 pH 7.0. After all, bacteria were resuspended in fresh medium, adjusted to fit the optical
144 density of 1 measured at 600 nm (UV-1800, Shimadzu) and incubated with 0.5 kg of biochar
145 on rotary shaker at 150 rpm during 4 days (Zhang et al., 2016). When incubation was
146 finished, biochar was drained and dried at room temperature under a sterile hood. Bacterial
147 counts were performed on Bushnell Haas agar plates to check immobilization efficacy
148 (Labana et al., 2005). Colony-forming unit of immobilized bacteria was 2.1×10^8 and $4.9 \times$
149 10^8 g^{-1} biochar for soil S1 and S2, respectively. Before use immobilized biochar was stored at
150 4°C no longer than 3 days (Xu and Lu, 2010).

151 **2.3. Experimental design and treatments**

152 Glass bottles (1 L) were filled with contaminated soil and mixed with different
153 additives (mineral fertilizer, digestate, bulking agent, biochar and immobilized bacteria) to the
154 total weight of 1 kg. The seven tested conditions done in triplicates, are listed in Table 2.

155 Fresh sewage sludge digestate was amended to soil in 25 % w/w ratio, bulking agent (pine
156 and poplar tree saw dust, sieved at > 5 mm) was added in 25 % v/w ratio while biochar was
157 applied in 5 % w/w ratio. For treatment with mineral nutrients, $(\text{NH}_4)_2\text{SO}_4$ and K_2HPO_4 at a
158 C:N:P ratio of 100:10:2 were applied (Xu and Lu, 2010). Ammonium sulphate was selected
159 as a nitrogen source as it is the most common mineral fertilizer applied to soils worldwide due
160 to its low price. Humidity of samples was adjusted to fit 70 % of the water holding capacity
161 (WHC) of each soil mixture. Soils were incubated for 30 days. Every 6 days the content of the
162 bottles was manually mixed to maintain oxygen conditions and 15 g of soil mixtures were
163 sampled for respiration studies. At the beginning and at the end of the experiment 15 g of
164 samples were taken for TPH quantification and DNA extraction. Clay soil exhibited high
165 aggregation tendency, at the end of the experiment soil aggregates were dried and the size was
166 measured.

167 **Table 2. Experimental setups and treatment strategies.**

168

169 **2.4. Analytical methods**

170 **2.4.1. TPH analysis**

171 Before TPH extraction, soil samples were air dried and homogenised by grinding in a
172 mortar. The percentage of the decrease of contaminant was based on the initial TPH
173 concentration of each treatment. Extraction procedure was based on USEPA 8015B and 3550s
174 methods (USEPA, 2007, 1996), with the use of hexane as a solvent and mechanical shaking
175 combined with ultrasonic treatment. The extraction was performed three times and the
176 extracts were pooled.

177 The differences between contaminant desorption characteristics (soil washing test) in
178 both soils was tested. In Erlenmeyer flask 100 g of soil was mixed with 100 mL of UPW with

179 0.01 % of NaN₃. The flasks were incubated on rotary shaker for 5 h at 100 rpm and 21°C.
180 After that time the liquid phase was recovered and extracted three times with hexane. To
181 study the extent of TPH sorption on sawdust, 500 g of each soil was mixed with sawdust (size
182 > 5 mm) in 25% v/w ratio and 60 % of WHC and incubated for 10 days in 21°C. Next, soil
183 was air dried, sawdust was separated from soil by sieving and extracted with hexane as
184 described earlier.

185 TPH was quantified on gas chromatography with flame ionization detector
186 (Shimadzu) with capillary column 30 m × 0.25 mm × 0.25 μm (ZB5HT Inferno,
187 Phenomenex) and hydrogen as a carrier gas. Initial oven temperature was hold for 3 min at
188 70°C and increased by 20°C/minute until reaching 325°C. Run time for analysis was 22 min.
189 Injection was done with 1 μL of sample in the split mode with split ratio 20 and temperature
190 285°C. Column flow was set at 3.0 mL/min with pressure 113.5 kPa. Diesel oil obtained from
191 a local gas station was used as quantification standard.

192 **2.4.2. Microbial respiration**

193 Microbial respiration was monitored during the incubation with the use of the Oxitop[®]
194 system. Fifteen grams of fresh sample was placed in Oxitop[®] gas tight flasks equipped with a
195 CO₂ trap (solid NaOH and 50 mL of 1 M NaOH solution) and incubated at 21°C for 6 days.
196 After this time sample was replaced with a fresh one and procedure was repeated until the end
197 of the experiment. Oxygen up-take in the flasks was measured every 4 hours and registered in
198 the measuring Oxitop[®] heads as a pressure drop in hPa. Calculations were performed
199 according to the equation:

$$200 \quad SR = \frac{M(O_2)}{R \cdot T} \cdot \frac{V_{fr}}{M_s} \cdot \Delta\rho \quad (1)$$

201 where SR: soil respiration ($\text{mg O}_2 \text{ g}^{-1} \text{ DW}$); $M(\text{O}_2)$: molar mass of oxygen (mg mol^{-1}); V_{fr} :
202 free gas volume (L); Δp : pressure difference (mbar); R: general gas constant ($\text{L mbar mol}^{-1} \text{ K}^{-1}$);
203 T : measuring temperature (K); M_{S} : soil dry mass (g).

204 **2.4.3. DNA extraction**

205 Genomic DNA was extracted from 500 mg of freeze samples using Fast DNA Spin
206 Kit for Soils (MP Biomedicals). Extracted DNA was eluted in 100 μL of DNA-free UPW.
207 DNA concentration and purity was determined using spectrophotometer UV-1800 (Shimadzu)
208 equipped with a TrayCell adaptor for micro-volumes (Hellma) (Biache et al., 2017). DNA
209 was stored at -20°C before further analyses.

210 **2.4.4. Real-time PCR analysis**

211 The extracted genomic DNA was used to quantify total bacteria (16S rDNA) and fungi
212 (18S rDNA) using 968F/1401R (Felske et al., 1998) and Fung5f/FF390r (Smit et al., 1999;
213 Vainio and Hantula, 2000) primers, respectively. Functional genes, *i.e.* alkanes hydroxylating
214 monooxygenases genes, were quantified using primers described elsewhere (Powell et al.,
215 2006). Real-time PCR quantifications were performed using CFX96 Real Time PCR system
216 (Bio-Rad) according to previously described procedure (Cébron et al., 2015, 2008).

217 **2.4.5. Sequencing analysis**

218 Illumina Sequencing MiSeq v3 run ($2 \times 300 \text{ bp}$) of the V3-4 region of the 16S rDNA
219 was performed by MicroSynth (Switzerland) on previously isolated DNA. The company is
220 ISO certified according to 9001:2008 and ISO / IEC 17025. Library preparation included
221 sample quality control and Nextera two step PCR amplification using primer set
222 341f_ill/802r_ill, PCR product purification, quantification and equimolar pooling.
223 Bioinformatic analysis included demultiplexing, merging of forward and reverse reads,

224 quality filtering, trimming, chimera removal, OTU clustering (97 % identity threshold) and
225 subsampling for even sample size (rarefaction to the lower number of reads per sample).
226 Taxonomy assignment was based on the SILVA 16S database v.123 (> 60 % confidence).
227 Alpha diversity calculation and comparative statistics were done with the use of Phyloseq and
228 DeSeq2 (R packages). Heat map was constructed using Heatmapper software.

229 **2.4.6. Statistical analysis**

230 Statistical analyses were performed using XLStat statistical software for Excel.
231 Significant differences of parameters among the treatments were detected with one-way
232 ANOVA ($p < 0.05$) followed by Tukey test. All experiments were performed in triplicates.

233

234 **3. Results and discussion**

235 **3.1. TPH removal**

236 In soil S1, TPH removal in control (C) and treatment CF and CD did not differ
237 significantly (ANOVA; $p > 0.05$) and reached about 10 % of removal compared to C_S
238 (initial soil) (Fig. 1). In soil S2, control (C) and treatments CF and CD also displayed a similar
239 TPH removal (about 24 % compared to C_S). This decrease can be caused by natural
240 attenuation which includes spontaneous biodegradation, volatilization and formation of non-
241 extractable residues (Megharaj et al., 2011). Significant TPH removal in comparison to
242 control was not observed in treatments with mineral nutrients (CF). This may be caused by
243 inhibition of hydrocarbon degrading bacteria adapted to oligotrophic conditions (Cerqueira et
244 al., 2014). Application of bulking agent and bioaugmentation together with digestate has
245 significantly enhanced TPH removal in both soils. In soil S1, addition of bacteria immobilized
246 on biochar to the soil amended with digestate (CDBI) has resulted in 23.4 % of TPH decrease.

247 For the treatment with digestate and bulking agent (CDA), 27.8 % of contaminant decrease
248 was observed. In sandy soil (S2), TPH removal in treatments CDBI and CDA reached 35.0 %
249 and 36.7 %, respectively. Greater TPH removal in soil S2 is connected with higher initial
250 TPH concentration in comparison with soil S1 (Table 1).

251 **Fig. 1. Percentage of TPH in clay (panel A) and sandy (panel B) soil after 30 days of treatments.**
252 C: soil + water; CF: soil + mineral nutrients; CD: soil + digestate; CDA: soil + digestate + bulking
253 agent; CDBI: soil + digestate + bacteria immobilized on biochar; S: day 1; E: day 30. Mean (n=3) and
254 standard deviation. The same letters represent no significant differences among treatments for each
255 soil, respectively (one-way ANOVA; $p > 0.05$).

256 In all treatments in comparison to the initial state in soil S1 a significant decrease for
257 four studied TPH fractions was observed while in soil S2 only for fractions C10-C12, C12-
258 C16 and C16-C21. For treatments CDA and CDBI in comparison with control in soil S1 a
259 significant loss was observed for the tree first fractions while in soil S2 a significant loss was
260 only observed for fraction C21-C40 (Table S1).

261 For both soils, different losses of each fraction were observed. These losses were not
262 proportional to the total contaminant or initial concentration of each fraction. However, for
263 both soils the degradation level of the different fractions was as follows: C10-C12 > C12-C16
264 > C16-C21 > C21-C40, corresponding to the dependency between increase of TPH mass and
265 decrease in biodegradability and microbial preference (Mao et al., 2009; Sutton et al., 2013).
266 In soil S1, the decrease of heavy fractions (C16-C21 and C21-C40) was significantly higher
267 than in soil S2, whereas the decrease of light fractions (C10-C12 and C12-C16) was
268 significantly higher in soil S2 than S1.

269 In order to verify if TPH decrease in treatments containing biochar was not caused by
270 sorption phenomena and creation of non-extractable residues, additional treatments containing

271 soil with sterile biochar (CB) were performed. Results obtained for both soils in treatment CB
272 did not differ significantly from the control (C) (supplementary materials, Table S1), which
273 excludes non-reversible sorption of TPH on used biochar. For both soils, efficiency of
274 treatment with immobilized bacteria (CDBI) was higher than for a treatment without
275 bioaugmentation (CD), which is in accordance with other authors (Xu and Lu, 2010).

276 Different properties of contaminant in both soils may also affect degradation patterns.
277 In soil S2 due to high TPH level, the sorption on sawdust (CDA) represented 35.4 ± 3.1 % of
278 the total TPH while for soil S1 the sorption was about 2.8 ± 0.9 %. These values correspond
279 to the extent of TPH released to the aqueous phase. Soil washing tests with UPW showed 1.2
280 ± 0.8 % of TPH desorption to the aqueous phase for soil S1 and 31.8 ± 5.3 % for soil S2.
281 These fractions can be considered as bioavailable and this explains why TPH removal was
282 greater in soil S2.

283 **3.2. Impact of digestate addition on microbial respiration**

284 For clay rich soil (S1) three different respiration patterns may be distinguished (Fig.
285 2A). First pattern with the highest oxygen up-take was observed for treatment with digestate
286 and bulking agent (CDA). Next, moderate oxygen uptake was noted for treatments with
287 digestate (CD) and digestate with immobilized bacteria (CDBI). Control (C) and treatment
288 with mineral fertilizer (CF), biochar (CB) and immobilized biochar with mineral nutrients
289 (CFBI) followed third respiration pattern with the lowest respiration rates (for CF, CB, CFBI
290 data not shown).

291 **Fig. 2. Microbial respiration in soil S1 and S2 analysed under 4 treatments.** C: soil + water; CD:
292 soil + digestate; CDA: soil + digestate + bulking agent; CDBI: soil + digestate + bacteria immobilized
293 on biochar. Mean (n=3) and standard deviation.

294 Clay rich soil has exhibited very high aggregation tendency, with soil aggregates
295 reaching up to 5.2 cm with average size of 3.2 ± 2.2 cm. Addition of digestate (CD) increased
296 the size of aggregates up to 10.6 cm length with average size of 5.7 ± 4.9 cm. The use of
297 bulking agent together with digestate decreased aggregates size to 3.1 cm with average size of
298 1.8 ± 1.4 cm. Smaller aggregates supported the oxygen uptake (Fig. 3A). In previous studies it
299 was observed that oxygen diffusion as well as nutrient transfer were limited in clay rich soil
300 which inhibited hydrocarbon degrading aerobes (Masy et al., 2016). These phenomena
301 probably occurred in treatments C, CF and CD where respiration was limited. Addition of
302 bulking agent can improve oxygen uptake in clay rich soils (Alvim and Pontes, 2018), as
303 observed in treatment BA.

304 For sandy soil (S2) control C and treatment CF had similar respiration patterns as for
305 soil S1. However, opposite order of respiration intensity was observed for the two soils for
306 treatments with digestate with the following order for soil S2: FD>DB>BA. In the first 6 days
307 of the experiment for soil S2, respiration rates were similar for all treatments. After 12 days of
308 treatment, evident increase in oxygen uptake was observed for treatments CD and DB. In both
309 soils, high respiration intensity in treatments CD was followed by low TPH degradation (Fig.
310 1) which suggests activity of indigenous digestate bacteria and utilization of digestate as a
311 carbon and energy source. Interestingly, addition of bulking agent together with digestate
312 (CDA) in soil S2 revealed lower respiration rate than in treatment with digestate alone (CD).
313 Respiration differences between two soils are connected with soil structure that governs
314 oxygen transfer (Yeh and Young, 2003). In the sandy soil S2 no aggregate formation was
315 observed which suggests that oxygen transfer rate was probably higher than in soil S1. Thus,
316 applications of sawdust could not evidently improve oxygen transfer. Increased TPH removal
317 after addition of sawdust may be in turn caused by contaminant dilution and possible local

318 decrease of soil toxicity. Sorption tests have confirmed that one third of contaminant in soil
319 S2 was sorbed on sawdust, which supports this finding.

320 **3.3. Amendment effect on microbial abundance and concentration of *alkB* genes**

321 Density and diversity of microbial populations as well as the presence of functional
322 genes in soil mixture are important features helping to understand bioremediation process.
323 Gene quantification analyses were performed only on control and samples from treatments
324 CDA and CDBI where a significant removal of TPH occurred. In all treatments, bacterial 16S
325 rDNA quantifications at the beginning and at the end of the treatment were prominently
326 higher than fungal 18S rDNA, which suggests a major role of bacteria in biodegradation (Fig.
327 3). At the beginning, microbial concentration in digestate was greater than in soils. For both
328 soils, addition of digestate has significantly increased bacterial and fungal populations (*i.e.*
329 ANOVA; $p \leq 0.05$) in comparison with control.

330 **Fig. 3. Quantification of *alkB*, 16S rDNA and 18S rDNA at the beginning and at the end of the**
331 **treatment.** C: soil + water; CDA: soil + digestate + bulking agent; CDBI: soil + digestate + bacteria
332 immobilized on biochar. Mean (n=3) and standard deviation. Separate ANOVAs were performed for
333 each gene class at one sampling time. Values that are annotated with the same letter among one gene
334 class and sampling time are not significantly different (Tukey's multiple range test with $p = 0.05$).

335

336 In spite of differences between both soils in structure, OM content, chemical
337 characteristics and TPH level, in treatments CDA and DB, both containing digestate, bacterial
338 and fungal densities are comparable. It suggests that soil amendment with digestate had major
339 effect on microbial populations. It is also interesting to notice, that in both soils, pH values
340 have changed after digestate application. The values of pH in S1 and S2 soil controls were 8.3
341 ± 0.2 and 5.9 ± 0.2 , respectively, while after digestate application in all treatments (CD, CDA,

342 CDBI) pH stabilized to 7.1 ± 0.3 and 6.8 ± 0.4 for soil S1 and S2, respectively, and did not
343 differ significantly (ANOVA; $p > 0.05$).

344 To study changes in TPH degrading potential in soils, *alkB* genes encoding alkane
345 monooxygenases were analysed (Powell et al., 2006). The presence of *alkB* genes was
346 detected in digestate, in soil amended with digestate and in S1 control soil. In S2 control soil
347 very low bacterial density was observed and *alkB* genes were below the detection limit. The
348 high initial TPH content could be toxic for bacteria and fungi (Khan et al., 2018) and the low
349 level of organic matter could explain the low density of microorganisms (Sutton et al., 2013).
350 Copy number of *alkB* genes was 10^5 copies/g in S1 control soil and 10^6 copies/g in digestate.
351 After digestate application, *alkB* content increased to more than 10^6 copies/g for all studied
352 treatments. Observed *alkB* genes contents are in accordance with other studies (Masy et al.,
353 2016; Sutton et al., 2013).

354 **Fig. 4. Percentage of *alkB* genes relative to the total bacteria represented by 16S rRNA genes in**
355 **clay soil (panel A), sandy soil (panel B), and digestate (panel C) at the beginning and end of**
356 **incubation.** C: soil + water; CDA soil + digestate + bulking agent; CDBI: soil + digestate + bacteria
357 immobilized on biochar Mean (n=3) and standard deviation. Separate ANOVAs were performed
358 according to the sampling time. Values that are annotated with the same letter among one sampling
359 time are not significantly different (Tukey's multiple range test with $p = 0.05$). In samples C from soil
360 S2, *alkB* genes were below the detection limit due to low quantity of extracted DNA.

361 Percentage of *alkB* genes relative to total 16S rRNA genes differed in both soils. In
362 soil S1, the percentage decreased with time for control and treatments (Fig. 4A). This was
363 expected to happen as the copy number of *alkB* genes is reported to decrease with the drop of
364 bioavailable fraction of TPH (Powell et al., 2006; Sutton et al., 2013). In soil S1, initial
365 concentration of TPH was low and due to high percentage of clay particles (Table 1), the
366 bioavailable fraction was supposed to be depleted quite fast. In soil S2, for treatment CDA the

391 The diversity of bacterial communities was examined through 16S rRNA gene
392 Illumina MiSeq sequencing of triplicates (Table 3). Due to very high concentration of TPH in
393 control S2 samples, DNA was extracted in low quantity (low bacterial counts), and its quality
394 avoided efficient quantification (low read number), thus these samples were excluded from
395 the analysis. The list of genera and species detected in the soil S2 (C) are presented in
396 supplementary materials (Tables S3 and S4) . For 33 samples, a total of 3,616,362 reads was
397 obtained. Data were normalized to 72,290 sequences per sample. As indicated by rarefaction
398 curves the 33 samples reached high diversity coverage (Fig. S2 and S3). Total number of
399 OTUs was 692, 21 OTUs were found in all samples (representing 3.0 % of the sequences), 80
400 OTUs were found in 90 % of samples (11.6 % of OTUs) and 579 OTUs were found in 50 %
401 of samples (83.7 % of OTUs). Shannon diversity index ranged from 3.45 to 4.53, while
402 Chao1 richness ranged from 275 to 611.

403 **Table 3. Microbial diversity indicators from sequencing in digestate (D) and treatments**
404 **of soil S1 and soil S2.** C: soil + water; CDA: soil + digestate + sawdust; CDBI: soil + digestate +
405 bacteria immobilized on biochar. Letters S and E indicate time of sampling with S: start (day 1),
406 and E: end (day 30). Mean values (n=3) and standard deviation (in bracket). Values of the
407 same column followed by the same letter are not statistically different (ANOVA; $p > 0.05$).
408 Samples C from soil S2 having low read number were not included in analysis.

409

410 **3.4.2. Bacterial diversity on the phylum level**

411 Sequences were assigned to 15 phyla, 20 classes, 29 orders, 34 families, 40 genera and
412 14 species. Relative abundance of main phyla is presented on Fig. 5. Initially, at the phylum
413 level, bacterial community exhibited qualitative and quantitative differences among control
414 soil and digestate. The diversity of digestate amended treatments (CDA, CDBI) was closer to

415 digestate diversity than to the control soil, then no major differences was detected between S1
416 and F soil treatments.

417 At the beginning (samples S), the most abundant bacteria detected in S1 soil belong to
418 phyla *Proteobacteria* (34.6 %), *Actinobacteria* (26.3 %), *Firmicutes* (18.7 %) and *Chloroflexi*
419 (14.3 %) which are common for soils (Pezzolla et al., 2015). In contrast, in digestate and
420 amended soils the most frequent were *Microgenomates*, known previously as candidate
421 phylum OP11 (33.5 % in digestate). This taxa was previously found in anaerobic bioreactor
422 with artificial sewage and constituted around 30 % of microbial population (Gao et al., 2010)
423 and in corn stove digestate (around 1 %) (Li et al., 2018). Other phyla identified in digestate
424 belong to *Proteobacteria* (17.5 %), *Aminicenantes* (14 %) and *Actinobacteria* (10.8 %).
425 Comparatively at the beginning, in treatments CDA and CDBI of soil S1 *Microgenomates*
426 was dominant (brought by digestate) with initial abundance respectively 51.8 and 52.7 %,
427 *Proteobacteria* 9.3 and 15.8 %, *Aminicenantes* 7.3 and 7.9 % and *Actinobacteria* 9.4 % and
428 5.7 %. During time (difference between start and end of the incubation), changes within a
429 treatment were only quantitative. For example, in soil S1 CDA treatment we have observed
430 significant decrease of phyla characteristic for digestate e.g. *Microgenomates* and increase of
431 main soil taxa such as *Proteobacteria* (ANOVA; $P \leq 0.05$). In soil S2 only a decrease of
432 *Microgenomates* was observed with time, especially in bioaugmented treatments (CDBI).

433 **Fig. 5. Relative abundance of main bacterial phyla in treatments of soil S1 and soil S2 and in**
434 **digestate (D).** C: soil + water; CDA: soil + digestate + sawdust; CDBI: soil + digestate + bacteria
435 immobilized on biochar. Letters S and E indicate time of sampling with S: start (day 1), and E: end
436 (day 30). Mean (n=3) and standard deviation. Treatment CDBI contains immobilized bacteria enriched
437 respectively from both soils. Samples C from soil S2 were not included in analysis due to low read
438 number.

439

440 3.4.3. Bacterial diversity on the genus level

441 Clustering of genera has shown that digestate and amended soils had high level of
442 similarity, especially at the beginning of the treatment (Fig. 6). For example, *Psychrobacter*,
443 *Mycobacterium* and *Acinetobacter* were the most abundant genera (> 2 %) in digestate and
444 amended soils at the beginning of the treatment while in S1 control soil the most common
445 were *Bacillus*, *Agromyces*, *Patulibacter*, *Leptolinea* and *Longilinea*. At the end of the
446 treatment, in S1 control soil the main genera were *Pseudoxanthomonas*, *Agromyces*,
447 *Thiobacillus*, *Pseudomonas* and *Acinetobacter*. With time, differences in bacterial community
448 diversity became also visible between the two soils. In soil S1 for CDA treatment the most
449 abundant genera were *Arenimonas*, *Arthrobacter*, *Thermomonas* and *Mycobacterium* while
450 for treatment CDBI *Arenimonas*, *Thermomonas* and *Mycobacterium*. In soil S2 the dominant
451 genera in CDA treatment were *Dietzia*, *Mycobacterium*, *Halomonas* and *Stenotrophomonas*
452 while in CDBI treatment *Dietzia*, *Stenotrophomonas*, *Mycobacterium*, *Halomonas* and
453 *Microbacterium*.

454 In S1 control soil, genera not associated with TPH degradation were more often
455 present at the beginning of the study and replaced with time by taxa containing some known
456 species capable of alkane degradation. For instance at the beginning, among the top genera
457 only *Bacillus* has known ability to degrade hydrocarbons (Barra Caracciolo et al., 2015;
458 Reyes-Sosa et al., 2018), while after 30 days new genera appeared among which TPH
459 degraders are recognized e.g. *Pseudoxanthomonas*, *Pseudomonas* (*alkB* genes expression) and
460 *Acinetobacter* (*alkB* genes expression) (Barra Caracciolo et al., 2015; Liu et al., 2011; Nie et
461 al., 2014; Pepi et al., 2011; Reyes-Sosa et al., 2018). Species belonging to *Thiobacillus* were
462 also found in control soil, presence of this bacteria was already detected in petroleum
463 reservoirs (Reyes-Sosa et al., 2018). Within genera present in digestate and consequently in
464 amended soils at the beginning, *Psychrobacter* was the most abundant. This genus was not

465 previously assigned to TPH degradation activity and surprisingly it was linked with mercury
466 resistance (Pepi et al., 2011). Among other top genera, *Acinetobacter* species were shown to
467 be able to use alkanes as a carbon and energy source and possess *alkB* genes (Liu et al., 2011;
468 Nie et al., 2014; Wang et al., 2011), while *Mycobacterium* was previously observed to
469 catalyse different reaction and express wide range of catabolic genes including *alkB* genes
470 (Nie et al., 2014; Wang et al., 2016).

471 **Fig. 6. Heatmap profile showing dominant ($\geq 0.1\%$) bacterial genera, based on relative**
472 **abundance in logarithmic values, in digestate (D) and treatments of soil S1 and soil S2. C: soil +**
473 **water; CDA: soil + digestate + sawdust; CDBI: soil + digestate + bacteria immobilized on biochar.**
474 Letters S and E indicate time of sampling with S: start (day 1), and E: end (day 30). Numbers 1, 2 and
475 3 indicate the three replicates. Treatment CDBI contained immobilized bacteria enriched respectively
476 from both soils. Samples C from soil S2 were not included in analysis due to low quantity of extracted
477 DNA.

478

479 In treatments amended with digestate also an increase of taxa containing recognized
480 TPH degraders was observed. In S1 soil for treatments CDA and CDBI *Mycobacterium*
481 species were still present, however new dominant genera appeared including *Arenimonas*
482 which was previously observed in hydrocarbon degrading cultures and associated with oil
483 degradation (Reyes-Sosa et al., 2018; Wang et al., 2016), *Arthrobacter* (for CDA only) able to
484 degrade crude oil components (Reyes-Sosa et al., 2018) and *Thermomonas* which was
485 observed in microbial communities under intensive oil degradation (Al-Kharusi et al., 2016).
486 In S2 soil, microbial diversity evolved differently and the most abundant genera detected after
487 30 days were: *Dietzia*, known to express *alkB* like genes and having ability to degrade a wide
488 range of hydrocarbons (Wang et al., 2011), *Halomonas* with ability to degrade aliphatic
489 hydrocarbons (Reyes-Sosa et al., 2018), *Stenotrophomonas* which was previously observed in

490 soils amended with digestate (Wolters et al., 2018), *Microbacterium* previously described as
491 phenanthrene degrader (Reyes-Sosa et al., 2018) and *Mycobacterium*. At the end of the
492 treatment, we have also observed increase of other bacterial genera connected with
493 hydrocarbon degradation. For instance, in S2 soil treatment DB, a 2.2 % increase of *Gordonia*
494 known to possess *alkB* genes was observed (Liu et al., 2011). The list of identified species is
495 provided in the supplementary materials (Table S2).

496 **Bacteria communities changes**

497 Principal coordinates analysis (PCoA) based on weighted UniFrac distances reflecting
498 bacterial beta-diversity are in accordance with previous analysis. Fig. 7 shows distinct
499 changes in the microbial community diversity in S1 control soil and S1 treatments with
500 addition of digestate. As shown above, all amended soils were highly similar to digestate at
501 the beginning of the treatments. After 30 days, the similarity to digestate was still higher than
502 to soil, however, due to the creation of a new environment (mix of soil and digestate) the
503 bacterial community developed differently. This is in accordance with previous results
504 (Pezzolla et al., 2015) confirming that application of digestate had changed soil environment
505 and thus further bacterial development took different direction in control and amended
506 treatments. In both soils under CDA treatment the bacterial community composition was
507 similar and evolved similarly. For CDBI treatment, different taxa were promoted in S1 and S2
508 soils. Differences in CDBI treatment are likely a consequence of soil bioaugmentation with
509 diverse strains, native to each soil.

510 **Fig. 7. Principal coordinates analysis (PCoA) showing bacterial beta-diversity among digestate**
511 **(D) and treatments of soil S1 and soil S2.** C: soil + water; CDA: soil + digestate + sawdust; CDBI:
512 soil + digestate + bacteria immobilized on biochar. Letters S and E indicate time of sampling with S:
513 start (day 1), and E: end (day 30). Numbers 1, 2 and 3 indicate the three replicates. Calculations were
514 based on weighted UniFrac distances. Treatment CDBI contain immobilized bacteria enriched

515 respectively from both soils. Samples C from soil S2 were not included in analysis due to low quantity
516 of extracted DNA.

517 **4. Conclusions**

518 Microcosm experiment have been performed to test the applicability of sewage sludge
519 digestate addition for enhancing the bioremediation of industrially TPH contaminated soils.
520 Digestate application to clay rich soil resulted in larger aggregates formation which limited
521 oxygen uptake. Addition of digestate together with saw dust decreased aggregates size and
522 improved soil respiration. In sandy soil no aggregates were formed and digestate efficiently
523 improved soil respiration rates. Addition of digestate has also significantly increased initial
524 level of *alkB* genes in the treated soils. Extra addition of sawdust as a bulking agent together
525 with digestate lead to a decrease of soil aggregates size and enhanced TPH dissipation in clay
526 rich soil. The use of bacteria immobilized onto biochar together with digestate also improved
527 bioremediation efficiency in both soils. For the 40 genera detected in the study, 8 have known
528 alkane degrading potential. Further studies are necessary to understand behaviour and
529 composition changes of microbial communities in contaminated soils amended with organic
530 fertilizers.

531

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535 **Appendix A. Supplementary data**

536 Supplementary data to this article can be found online.

537

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Table 1-3[Click here to download Table: Table 1-3.pdf](#)**Table 1. Soils and digestate characteristics.** WHC: water holding capacity; OM: organic matter; TOC: total organic carbon; TN: total nitrogen; TPH: total petroleum hydrocarbons, PAH: polycyclic aromatic hydrocarbons.

Parameter	Clay soil (S1)	Sandy soil (S2)	Digestate	Method
pH (H ₂ O)	8.3 (±0.1)	5.9 (±0.2)	n.a.	NF ISO 10693
Water content (%)	1.3 (±0.2)	0.8 (±0.2)	81.1 (±0.2)	NEN-ISO 11465
WHC (mL cm ⁻³)	0.44 (±0.01)	0.18 (±0.10)	n.a.	OECD test No. 222
OM (% DW)	3.6	2.8	87.3	NF ISO 14235
TOC (g kg ⁻¹ DW)	31	14	120	NEN-EN 13137
TN (g kg ⁻¹ DW)	0.9	0.1	20.8	NEN-EN-ISO 11732
P (g kg ⁻¹ DW)	0.6	0.1	10.0	NEN 6961, CEN/TS 16171, NF-EN 16179
C:N:P	100:2.8:1.8	100:0.7:0.6	100:17.3:8.3	-
TPH (g kg ⁻¹ DW)	6.1	32.6	1.8	Section 2.4.1.
Sand (%)	41.6	94.6	n.a.	NEN 5753
Silt (%)	32.1	5.4	n.a.	NEN 5753
Clay (%)	26.3	< 0.1	n.a.	NEN 5753
PAHs content (mg kg ⁻¹ DW)				Internal method; acetone-hexane extraction; GC-MS quantification
Phenanthrene	3.4	28	n.a.	
Pyrene	1.9	1.7	n.a.	
Fluoranthene	1.8	1.2	n.a.	
Acenaphthylene	2.5	0.9	n.a.	
Fluorene	1.8	7.4	n.a.	

Sum 16 US-EPA PAHs	19	45	n.a.
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Total elements content (mg kg⁻¹ DW)

NEN 6961,

NEN-EN-ISO17294-2

Fe	5400	7600	54000
Cu	150	<1	110
Cd	0.4	<0.2	0.7
Cr	15.1	1.2	34.2
Co	150	<1	110
Hg	2.4	<0.1	0.7
Pb	210	<10	27
Ni	13.0	1.6	8.9
Zn	180	<10	270

Table 2. Experimental setups and treatment strategies.

Setup	Setup composition	Treatment
C	Soil	Natural attenuation
CF	Soil + mineral fertilizer	Biostimulation
CD	Soil + digestate	Biostimulation
CDA	Soil + digestate + bulking agent	Biostimulation
CB	Soil + biochar	Bioaugmentation
CBIF	Soil + mineral fertilizer + bacteria* immobilized on biochar	Bioaugmentation + Biostimulation
CDBI	Soil + digestate + bacteria* immobilized on biochar	Bioaugmentation + Biostimulation

* *Bacteria enriched from soil S1 and S2 were inoculated in each soil, respectively.*

Table 3. Microbial diversity indicators from sequencing in digestate (D) and treatments of soil S1 and soil S2. C: soil + water; CDA: soil + digestate + bulking agent; CDBI: soil + digestate + bacteria immobilized on biochar. Letters S and E indicate time of sampling with S: start (day 1), and E: end (day 30). Mean values (n=3) and standard deviation (in bracket). Values of the same column followed by the same letter are not statistically different (ANOVA; $p > 0.05$). Samples S2_C having low read number were not included in analysis.

Sample	Number of reads	Observed OTU	Chao1 index	Shannon index
D	72430 (9344)a	429 (8)d	430 (8)d	3.67 (0.14)d
S1_C_S	142479 (12964)bc	285 (10)e	286 (10)e	4.40 (0.03)b
S1_C_E	146313 (13066)bc	274 (11)e	275 (11)e	3.77 (0.07)d
S1_CDA_S	133747 (21164)bc	606 (12)a	607 (12)a	3.58 (0.26)d
S1_CDA_E	136802 (8141)b	602 (3)a	599 (4)a	3.96 (0.13)c
S1_CDBI_S	128966 (12520)b	599 (10)a	599 (10)a	3.45 (0.41)d
S1_CDBI_E	176524 (29061)c	607 (12)a	611 (6)a	3.73 (0.30)d
S2_C_S	2724 (1781)d	n.a.	n.a.	n.a.
S2_C_E	1649 (174)d	n.a.	n.a.	n.a.
S2_CDA_S	139418 (4449)b	510 (3)c	510 (4)c	3.69 (0.13)d
S2_CDA_E	132827 (6381)b	533 (5)b	533 (5)b	3.93 (0.09)c
S2_CDBI_S	142724 (3447)b	505 (7)c	506 (7)c	3.73 (0.05)d
S2_CDBI_E	159279 (6393)c	511 (11)c	512 (11)c	4.53 (0.05)a

Figure 1
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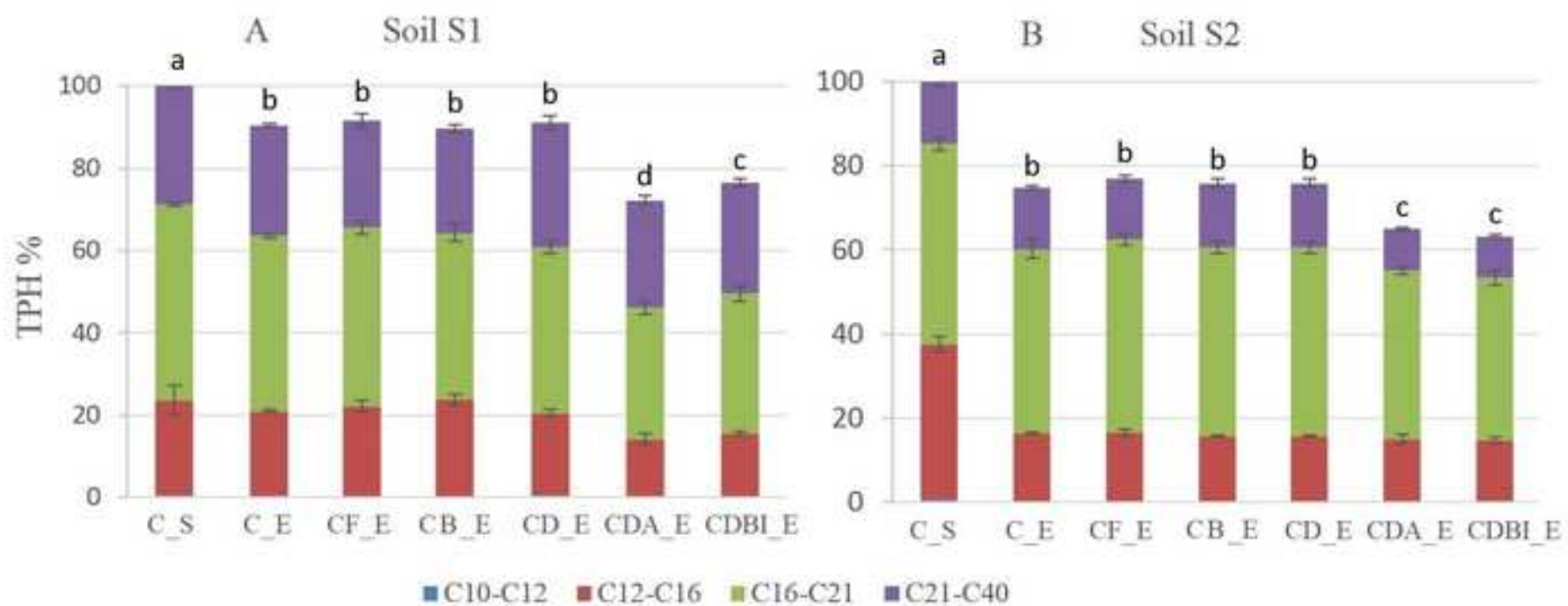


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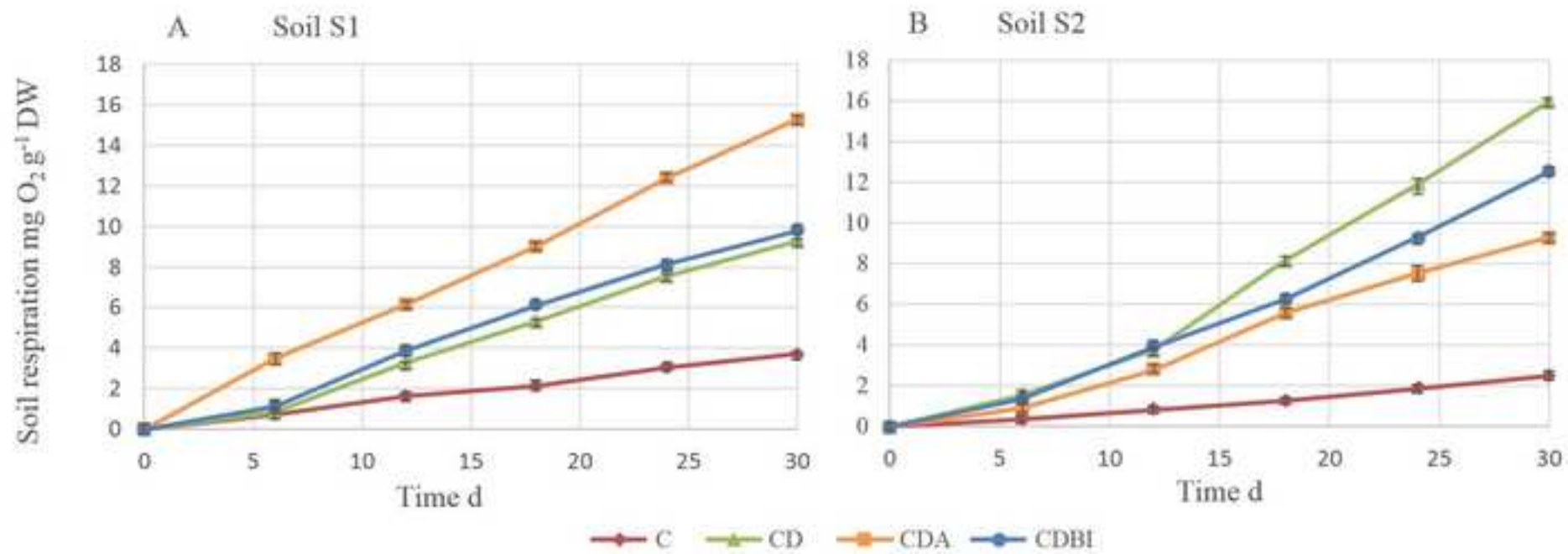


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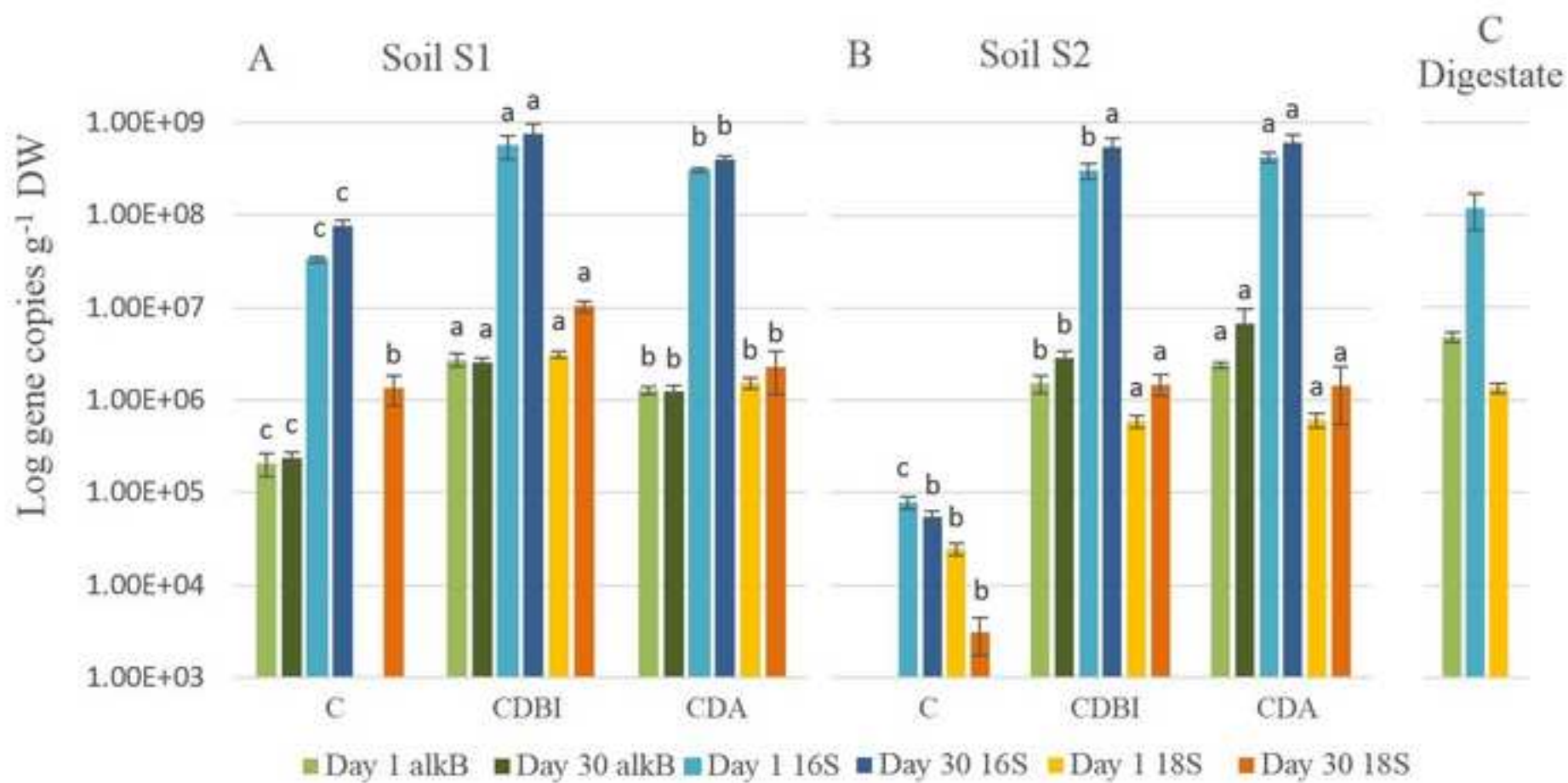


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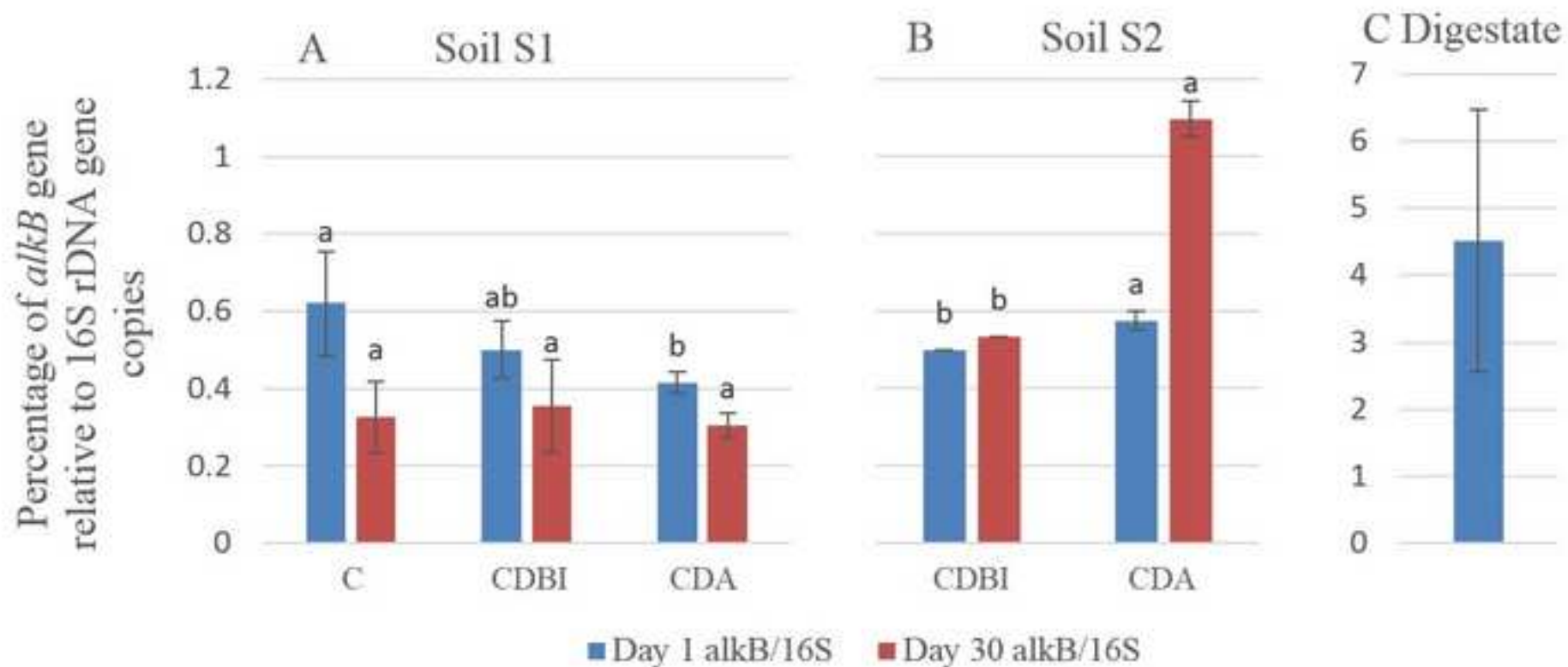


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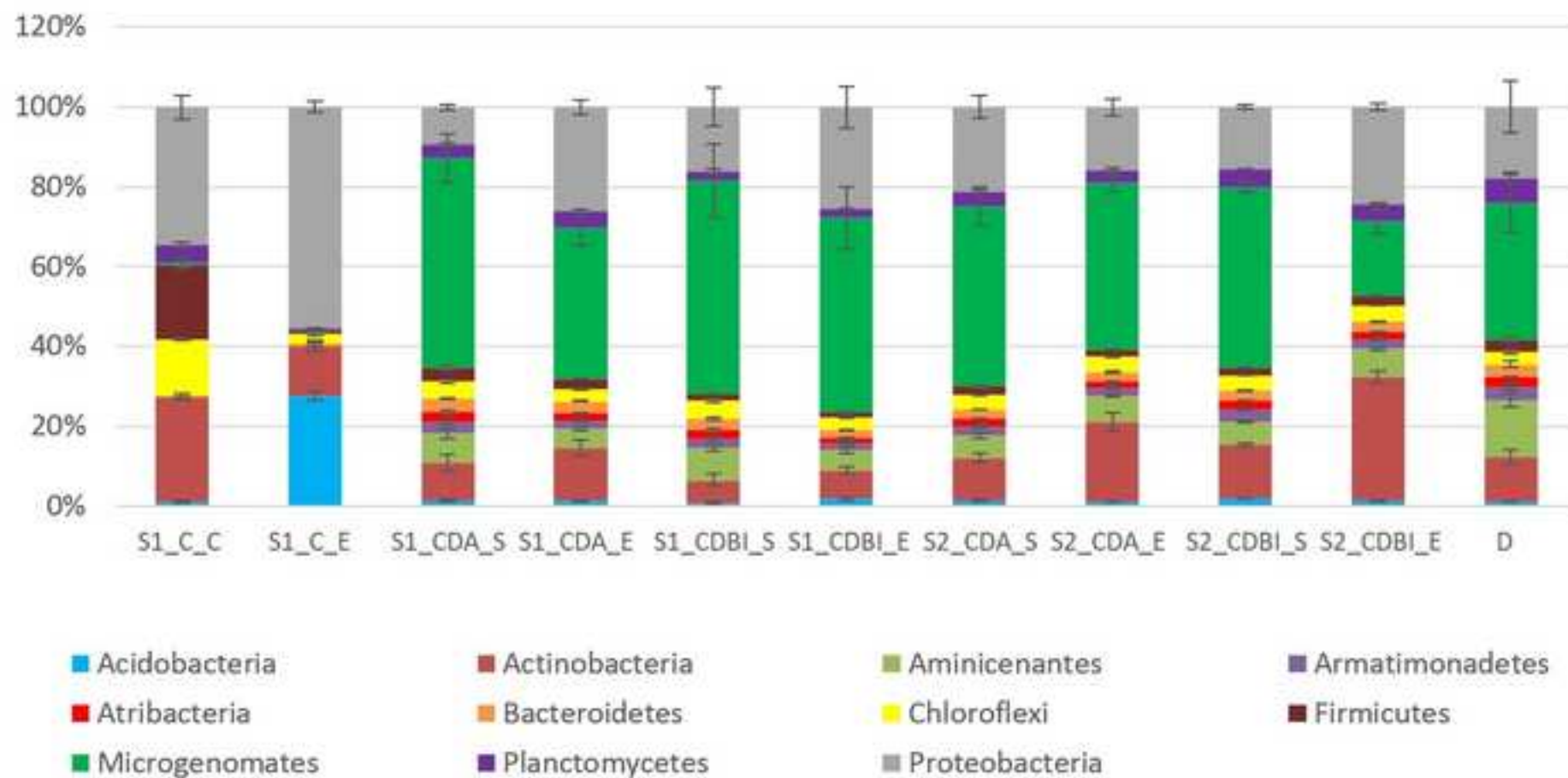


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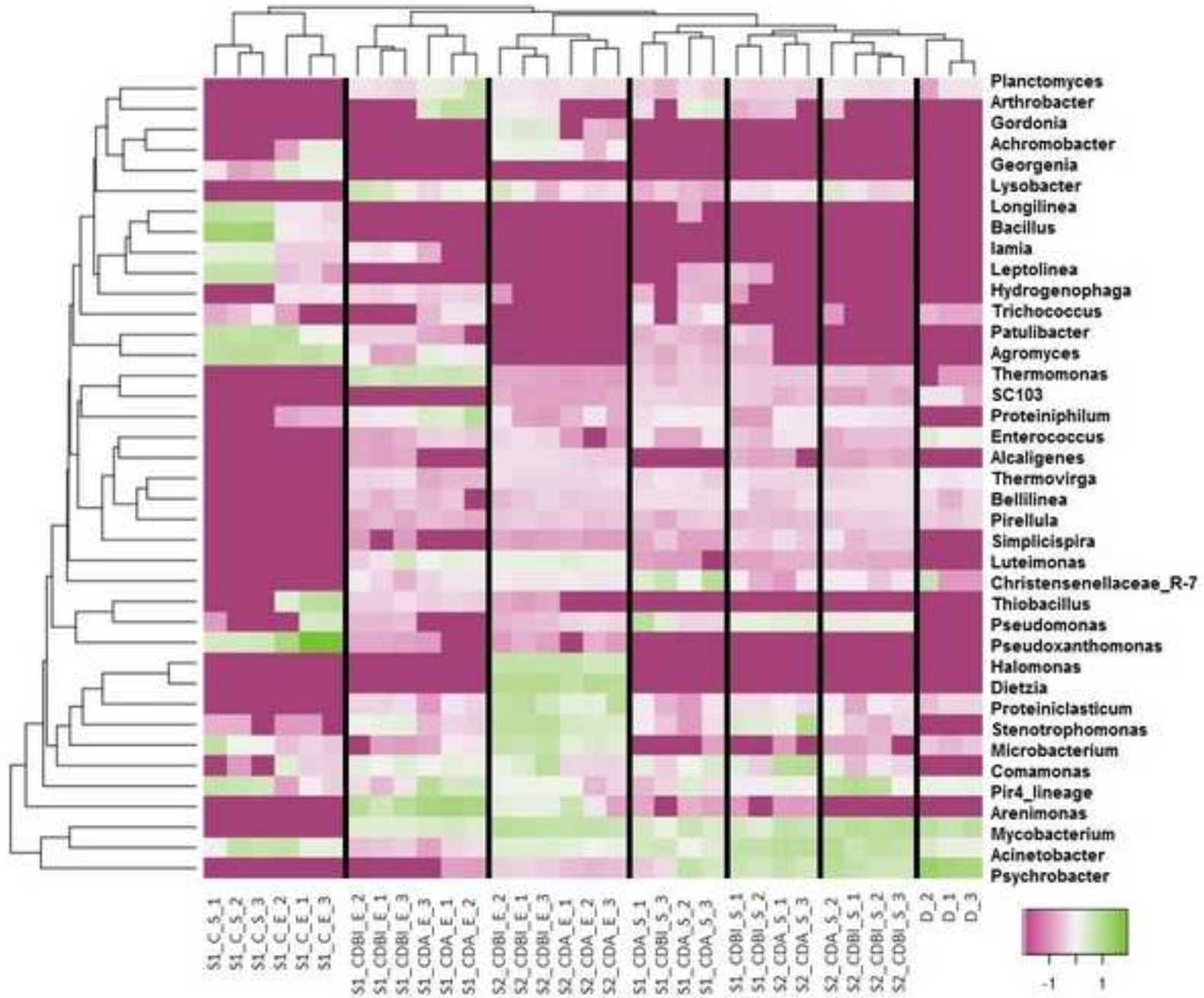


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