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**Effect of digestate application on microbial respiration and
bacterial communities’ diversity during bioremediation of
weathered petroleum hydrocarbons contaminated soils**

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Highlights

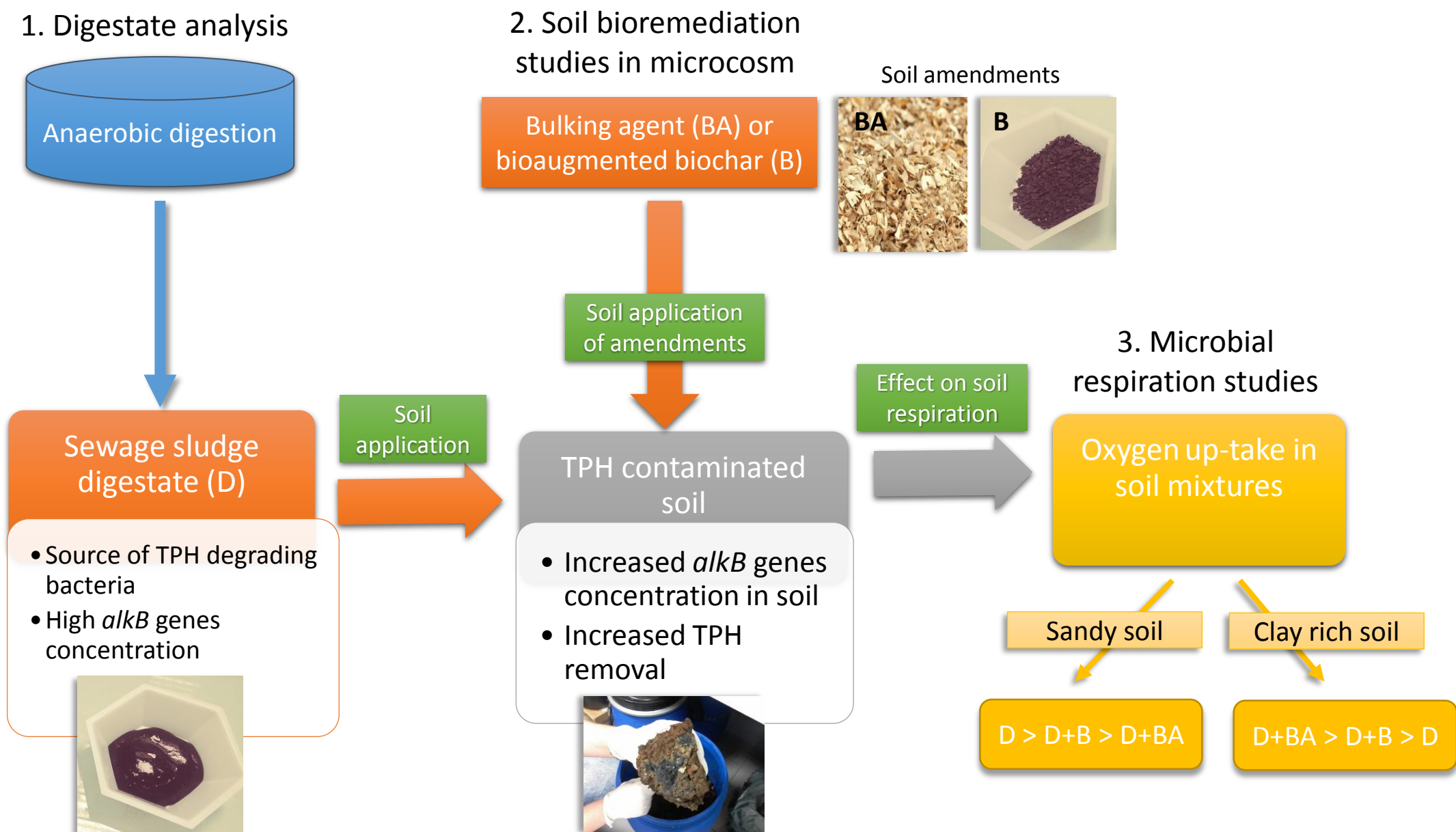
- Digestate application with bulking agent or immobilized bacteria improve TPH removal
- Digestate effect on soil respiration depends on soil texture
- Sewage sludge digestate contains high concentrations of *alkB* genes

- 31 • Application of digestate increases and maintains *alkB* genes content in the soil
- 32 • Distinct microbial groups developed in amended and non-amended soils

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Abstract

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

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

1. Introduction

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

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

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

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

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

2. Materials and methods

2.1. Characterization of soil and digestate samples

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

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.

2.2. Biochar and bacteria immobilization

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

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

Bacteria for immobilization were enriched separately from both contaminated soils using a modified Bushnell Haas medium (pH 7.2) containing 0.2 g of MgSO_4 , 0.02 g of CaCl_2 , 1 g of KH_2PO_4 , 1 g of Na_2HPO_4 , 1 g of NH_4NO_3 and 1 mg of $\text{FeSO}_4 \times 7 \text{H}_2\text{O}$ per 1 L of UPW. Diesel oil obtained from commercial gas station was used in final concentration of 1% v/v in the medium as a sole carbon and energy source.

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

2.3. Experimental design and treatments

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

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

Table 2. Experimental setups and treatment strategies.

2.4. Analytical methods

2.4.1. TPH analysis

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

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

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

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

2.4.2. Microbial respiration

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

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

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,

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

2.4.6. Statistical analysis

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

3. Results and discussion

3.1. TPH removal

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

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

Fig. 1. Percentage of TPH in clay (panel A) and sandy (panel B) soil after 30 days of treatments.

C: soil + water; CF: soil + mineral nutrients; CD: soil + digestate; CDA: soil + digestate + bulking agent; CDBI: soil + digestate + bacteria immobilized on biochar; S: day 1; E: day 30. Mean (n=3) and standard deviation. The same letters represent no significant differences among treatments for each soil, respectively (one-way ANOVA; $p > 0.05$).

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

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

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

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

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

3.2. Impact of digestate addition on microbial respiration

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Table 3. Microbial diversity indicators from sequencing in digestate (D) and treatments of soil S1 and soil S2. C: soil + water; CDA: soil + digestate + sawdust; 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 C from soil S2 having low read number were not included in analysis.

3.4.2. Bacterial diversity on the phylum level

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

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

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

Fig. 5. Relative abundance of main bacterial phyla in treatments of soil S1 and soil S2 and in digestate (D). C: soil + water; CDA: soil + digestate + sawdust; 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 (n=3) and standard deviation. Treatment CDBI contains immobilized bacteria enriched respectively from both soils. Samples C from soil S2 were not included in analysis due to low read number.

3.4.3. Bacterial diversity on the genus level

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

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

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

Fig. 6. Heatmap profile showing dominant ($\geq 0.1\%$) bacterial genera, based on relative abundance in logarithmic values, in digestate (D) and treatments of soil S1 and soil S2. C: soil + water; CDA: soil + digestate + sawdust; 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). Numbers 1, 2 and 3 indicate the three replicates. Treatment CDBI contained immobilized bacteria enriched respectively from both soils. Samples C from soil S2 were not included in analysis due to low quantity of extracted DNA.

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

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

Bacteria communities changes

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

Fig. 7. Principal coordinates analysis (PCoA) showing bacterial beta-diversity among digestate (D) and treatments of soil S1 and soil S2. C: soil + water; CDA: soil + digestate + sawdust; 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). Numbers 1, 2 and 3 indicate the three replicates. Calculations were based on weighted UniFrac distances. Treatment CDBI contain immobilized bacteria enriched

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

4. Conclusions

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online.

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Table 1-3
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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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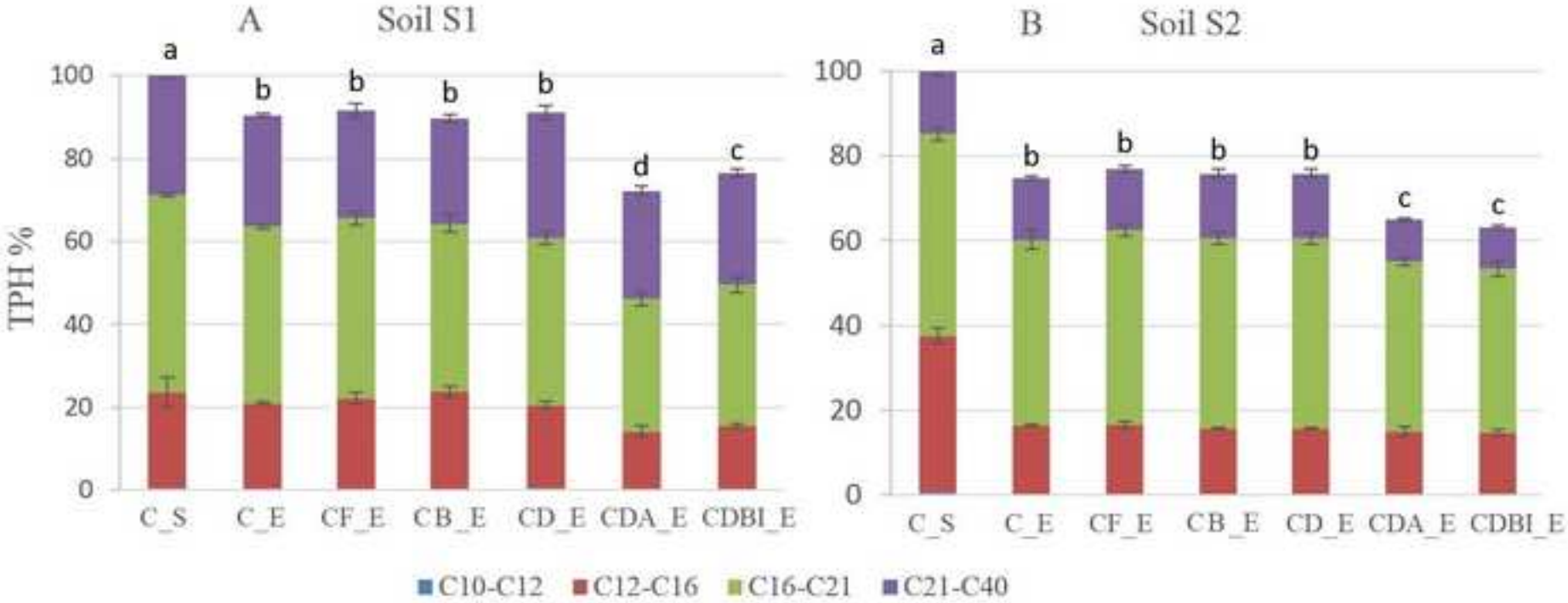


Figure 2
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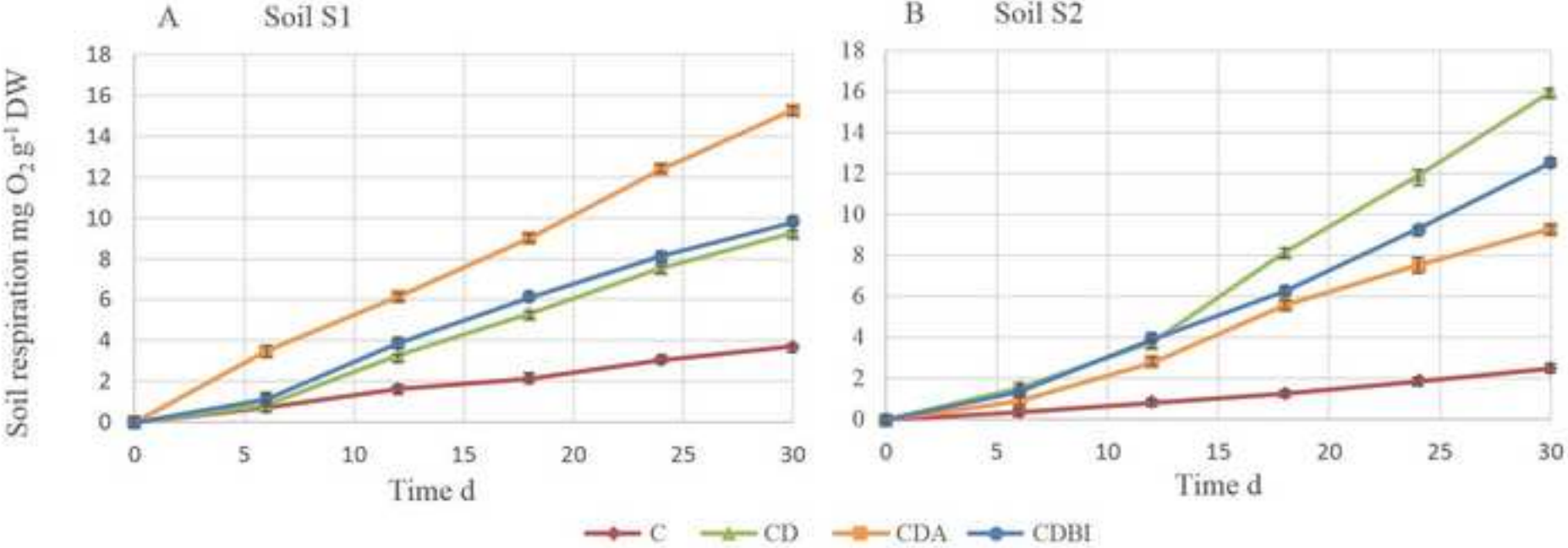


Figure 3
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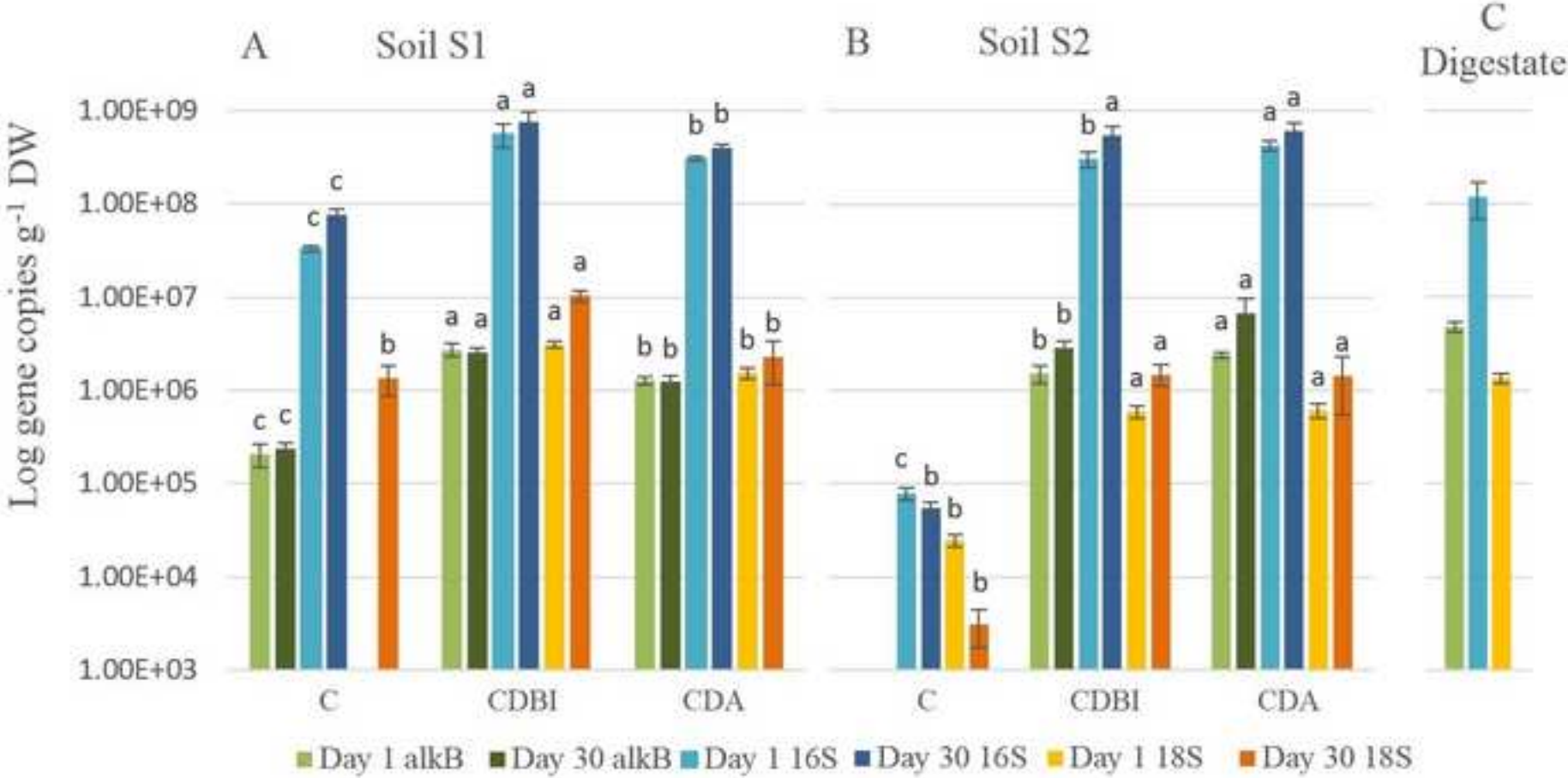


Figure 4
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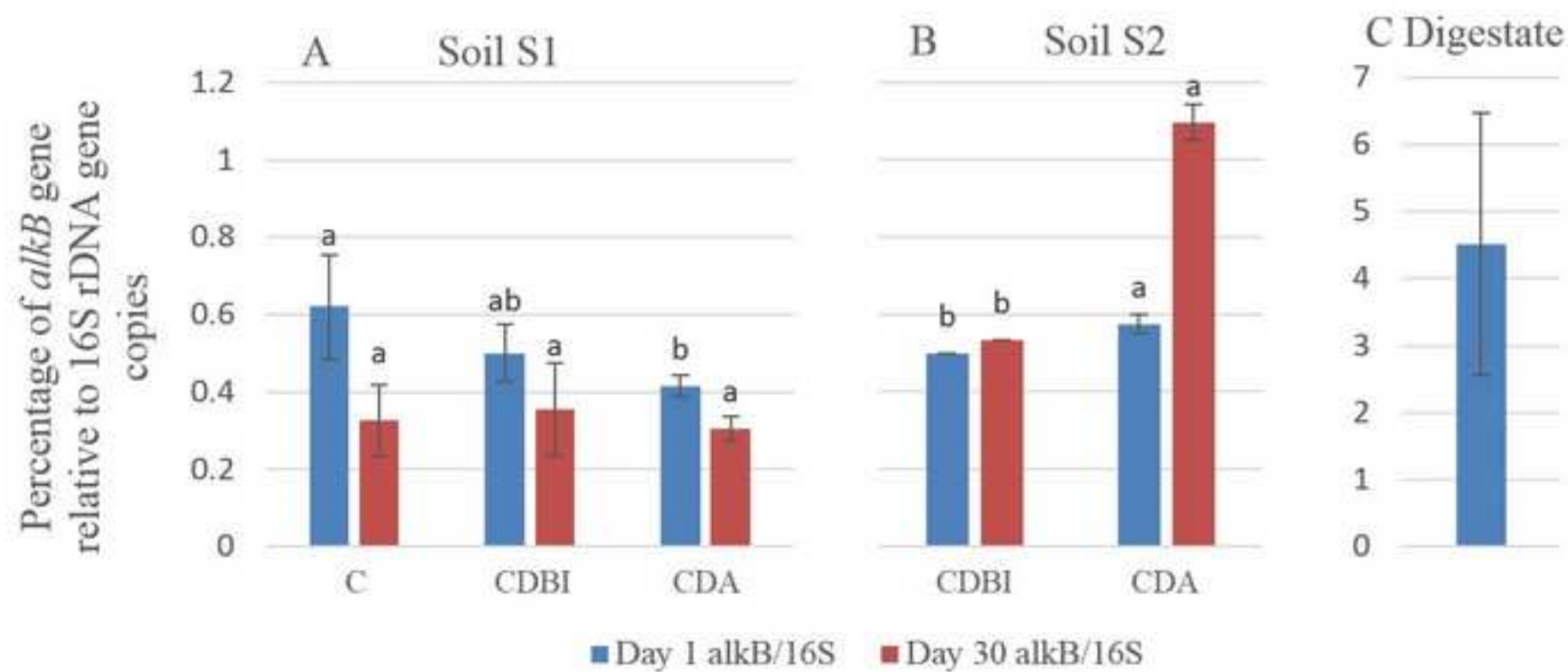


Figure 5
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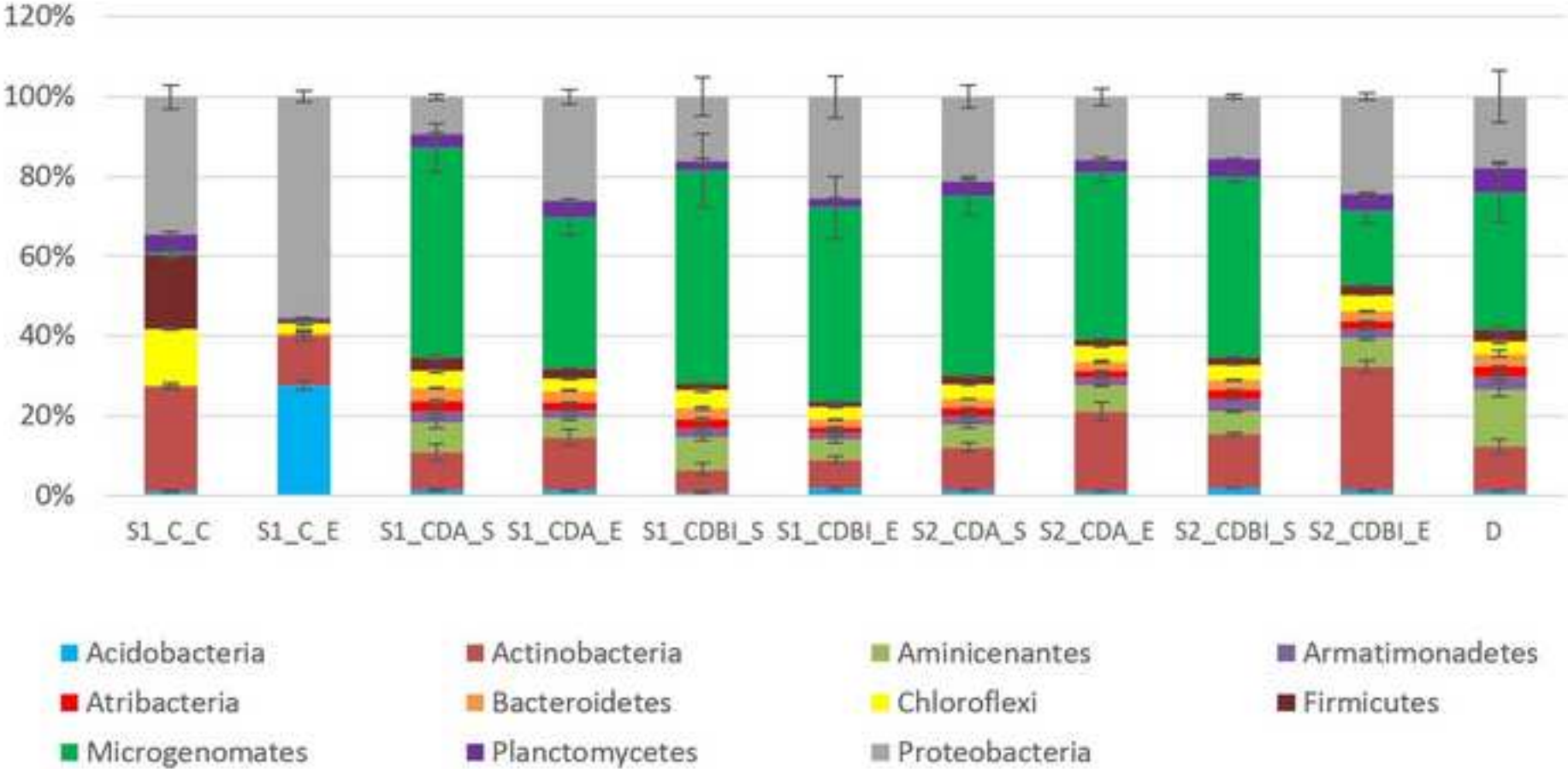


Figure 6
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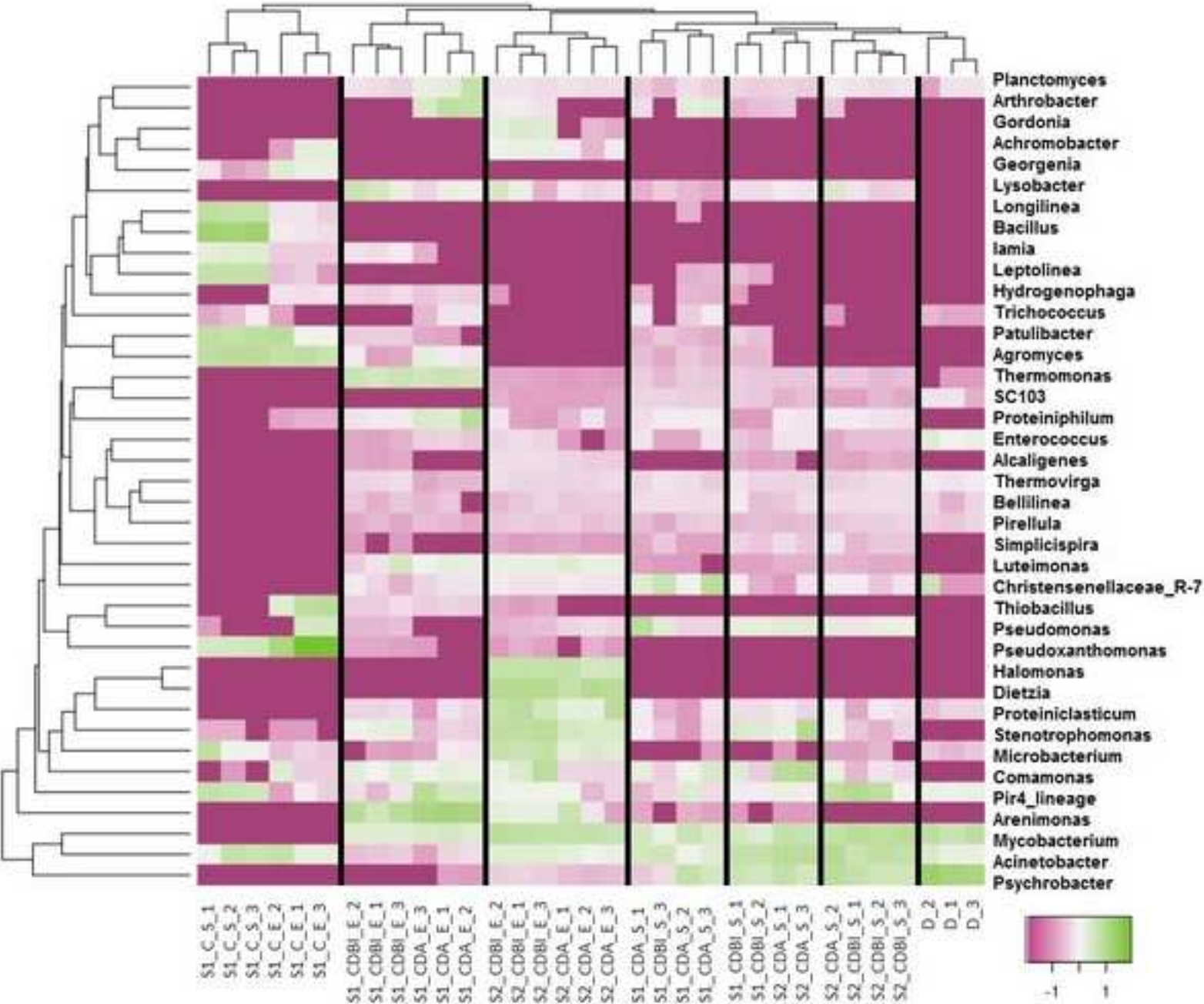


Figure 7

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