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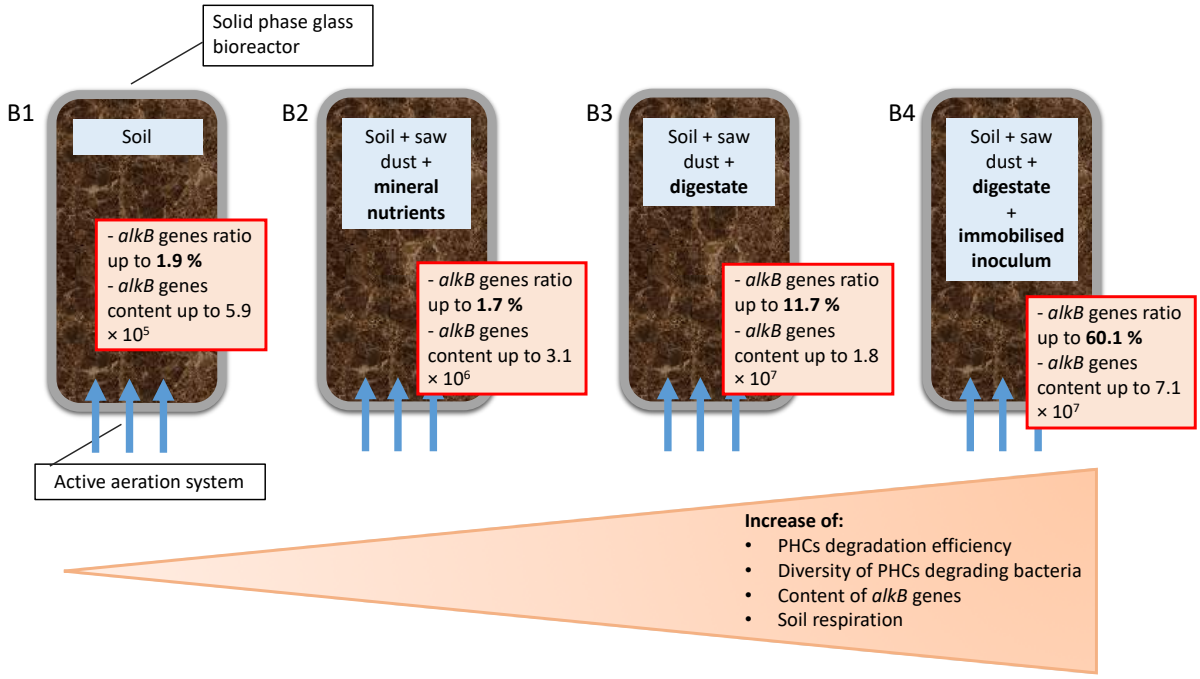

Solid phase glass bioreactor
B1
Soil
- *alkB* genes ratio up to **1.9 %**
- *alkB* genes content up to 5.9 × 10^5
B2
Soil + saw dust + **mineral nutrients**
- *alkB* genes ratio up to **1.7 %**
- *alkB* genes content up to 3.1 × 10^6
B3
Soil + saw dust + **digestate**
- *alkB* genes ratio up to **11.7 %**
- *alkB* genes content up to 1.8 × 10^7
B4
Soil + saw dust + **digestate** + **immobilised inoculum**
- *alkB* genes ratio up to **60.1 %**
- *alkB* genes content up to 7.1 × 10^7
Active aeration system
Increase of:
• PHCs degradation efficiency
• Diversity of PHCs degrading bacteria
• Content of *alkB* genes
• Soil respiration

23

24

Highlights

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

Abstract

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

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

1. Introduction

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

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

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

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

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

Materials and methods

1.1 Experimental set-up

The experiments (Figure 1) were performed in 12 L glass solid phase bioreactors with active aeration designed according to Namkoong et al. (2002). To provide appropriate oxygen amount, bioreactors were aerated from the bottom, with air flow rate ranging between 190 and 200 L h^{-1} m^{-3} soil. Before entering into the reactors, CO_2 was removed from the inlet air by a NaOH trap. Next, the air passed through a humidifier to remove aspirated alkali solution and to increase humidity content in order to prevent reactors from drying. Exhaust air from the reactors was filtered by an Amberlite® column in order to adsorb volatile organic compounds, which could be released due to hydrocarbons volatilization. Further, the exhaust air passed through 5M NaOH trap to accumulate CO_2 generated during bacterial respiration. The amount of trapped CO_2 was quantified by titration with HCl (Namkoong et al., 2002).

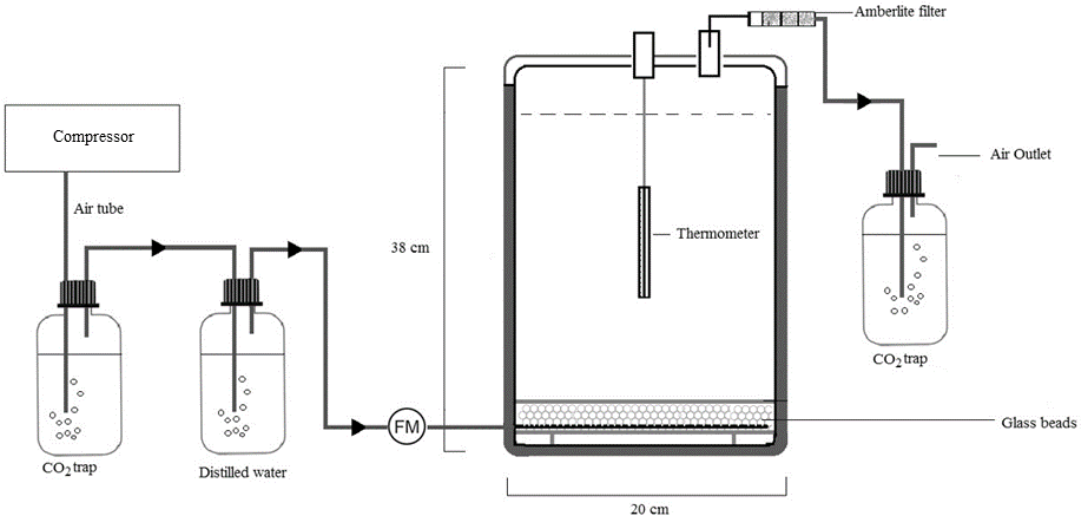


Figure 1. Scheme of bioreactor set up used in the study. FM: flow meter. Arrows indicates the direction of the airflow.

To ensure high porosity of the soil mixture, treated soil was mixed with bulking agent (sawdust). The ratio of added sawdust was adjusted to favor a homogeneous airflow throughout reactor (Rhykerd et al., 1999). Apart from control condition (B1), three biostimulation and bioaugmentation treatment conditions were tested in duplicate (Table 1). Biostimulation treatments covered addition of nitrogen in the form of NH_4Cl (B2) or sewage sludge digestate (B3). The digestate to soil ratio was optimized based on previous studies (Gielnik et al., 2018; Namkoong et al., 2002). In bioaugmented treatments, bacteria immobilized on biochar were added together with digestate (B4). The ratio of added biochar was suggested by other studies (Brown et al., 2017). Bioreactors were incubated during 63 days at 20 °C (±2 °C). To ensure the homogeneity of the mixture, the content of bioreactors was mixed once a week and humidity level was checked by drying the samples overnight at 110 °C and further adjusting reactors content to fit 60% of the water holding capacity (WHC). Temperature inside the reactors was measured during the treatment by thermocouple systems. To study the volatile fraction of PHCs, exhaust column with 25 g of Amberlite® was extracted every week and the sorbent was replaced.

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

Table 1. Treatments analyzed during the experiment.

Set-up	Description
B1	Control: Soil (5.00 kg) + sawdust (0.49 kg = 500 cm^3) + water (60% WHC)
B2	Soil (5.00 kg) + sawdust (0.49 kg = 500 cm^3) + NH_4Cl (C:N:P 100:10:2) + water (60% WHC)
B3	Soil (3.75 kg) + sawdust (0.49 kg = 500 cm^3) + digestate (1.25 kg) + water (60% WHC)
B4	Soil (3.50 kg) + sawdust (0.49 kg = 500 cm^3) + digestate (1.25 kg) + bacteria immobilized onto biochar* (0.25 kg) + water (60% WHC)

*indigenous hydrocarbon degrading bacteria were enriched from bioreactor B3

1.2 Soil, digestate and biochar

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

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

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

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

	Soil	Sewage sludge digestate	Method
pH (H_2O)	6.2 (0.2)	7.1 (0.1)	NF ISO 10693
Water content (%)	0.4 (0.1)	95.6 (0.2)	NEN-ISO 11465
WHC (mL cm^{-3})	0.2 (0.1)	n.a.	OECD test No. 222
OM (%)	2.2 (0.1)	87.3 (0.7)	NF ISO 14235
TOC (g kg^{-1} DW)	15 (1)	120 (7)	NEN-EN 13137
TN (g kg^{-1} DW)	0.3 (0.1)	20.8 (0.8)	NEN-EN-ISO 11732
P (g kg^{-1} DW)	0.3 (0.1)	10.0 (0.2)	NEN 6961, CEN/TS 16171, NF-EN 16179
C:N:P	100:2.3:2.0	100:17.3:8.3	-
PHCs (mg kg^{-1} DW)	13200 (124)	1.8 (0.1)	Section 2.4
US-EPA 16 PAHs (mg kg^{-1} DW)	25 (1)	n.a.	Internal method (SynLab)
Total elements content (mg kg^{-1} DW)			NEN 6961, NEN-EN-ISO17294-2
Fe	7600 (124)	54000 (210)	
Al	2700 (35)	7600 (14)	
Cd	0.3 (0.0)	0.7 (0.0)	
Cr	8.7 (0.1)	34.0 (0.3)	
Cu	57 (1.0)	110 (1.2)	
Hg	6.5 (0.2)	0.7 (0.1)	
Pb	120 (4.4)	27 (2.3)	
Ni	7.0 (0.1)	8.9 (0.4)	
Zn	88 (1.5)	270 (6.2)	

n.a.: not analyzed

1.3 PHCs quantification

For PHCs analysis 10 g of air-dried and grinded sample was extracted with 10 mL of hexane. To study PHCs volatilization, 25 g of Amberlite® from the exhaust gas column was extracted with a mix of 15 mL methanol and 15 mL hexane. The extraction procedure was based on USEPA 8015B and 3550s methods (USEPA, 2007, 1996), with mechanical shaking combined with ultrasonic extraction. PHCs were 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. Maximal oven temperature was 325 °C. 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 $mL.min^{-1}$ with pressure 113.5 kPa. Diesel oil was used as quantification standard.

1.4 DNA extraction

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

1.5 Real-time PCR

The extracted genomic DNA was used to quantify total bacterial and fungal populations. Primers selected for total bacteria were 968F/1401R (Felske et al., 1998) targeting 16S rDNA and Fung5F/FF390R (Smit et al., 1999; Vainio and Hantula, 2000) targeting 18S rDNA for fungi. Functional genes, i.e. alkanes hydroxylating monooxygenases genes (*alk*B), were amplified using primers AlkBF: 5'-AACTACATCGAGCACTACGG-3' and AlkBR: 5'-TGAAGATGTGGTTGCTGTTCC-3' (Powell et al., 2006) using 50°C for annealing temperature. Real-time polymerase chain reaction (qPCR) quantifications were performed using CFX96 Real Time PCR detection system (Bio-Rad) and SybrGreen detection (iQ sybr green Supermix, Bio-Rad), according to the procedure described elsewhere (Cébron et al., 2015, 2008) and using dilution series (10^1 to 10^8 $copies.\mu L^{-1}$) of standard plasmids.

1.6 Sequencing analysis

Ilumina MiSeq v3 Sequencing (2 × 300 bp) of the V3-V4 region of the 16S rDNA was performed by MicroSynth AG (Switzerland) on previously isolated DNA. The company is ISO certified according to 9001:2008 and ISO / IEC 17025. Library preparation included sample quality control and Nextera two-step PCR amplification using primer set 341f_ill/802r_ill, PCR product purification, quantification and equimolar pooling. Bioinformatic analysis included demultiplexing, merging of forward and reverse reads, quality filtering, trimming, chimera removal, operational taxonomic unit (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 of the most abundant genera and clustering with Pearson method were performed using free Heatmapper software (heatmapper.ca).

1.7 Germination ecotoxicity tests

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

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

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

1.8 Microtox® ecotoxicity assay

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

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

1.9 Statistical analysis

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

2. Results and discussion

2.1 PHCs removal and mineralization extent

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

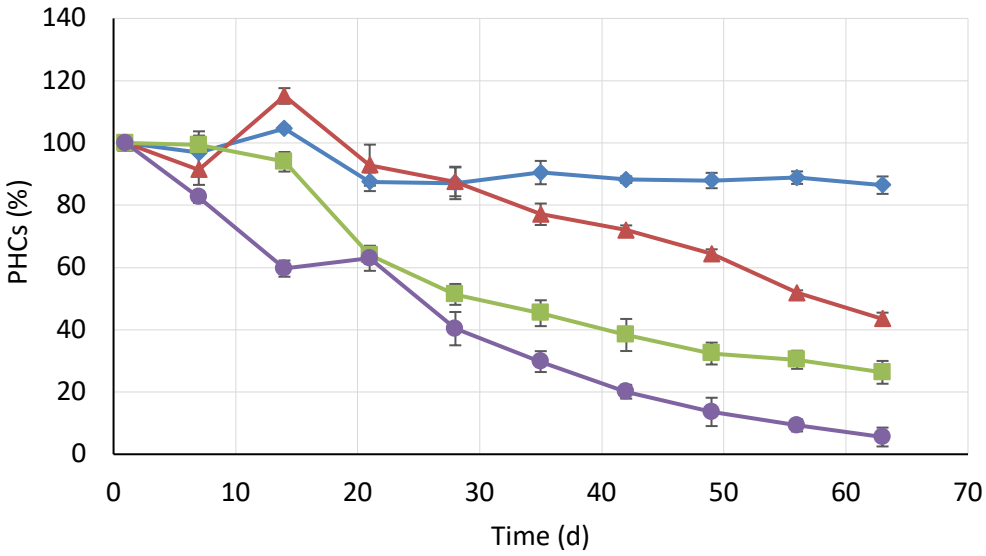


Figure 2. PHCs depletion during 63 days treatment for B1 condition; B2 condition; B3 condition; B4 condition. Abbreviations (B1, B2, B3, B4) refer to Table 1. Results are presented as a mean of samples collected from two reactors and analyzed in triplicate (n=3) with standard deviation.

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

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

PHCs removal was followed by the decrease of characteristic unresolved chromatographic peak (Fig. S1) representing weathered polar substances of highly recalcitrant nature (Bruckberger et al., 2018). For the treatments B1 and B2, PHCs increased above initial concentration in the first 15 days, which was not observed in treatments containing digestate. Similar observations were reported in another study (Dados et al., 2014), where the use of $(NH_4)_2SO_4$ as a nutrient source resulted in initial increase of PHCs concentration within the first 20 days, while for treatment with compost the increase did not occur. This suggests buffering capacities of organic amendments in terms of PHCs metabolites (*e.g.* aldehydes) (Xu and Lu, 2010b).

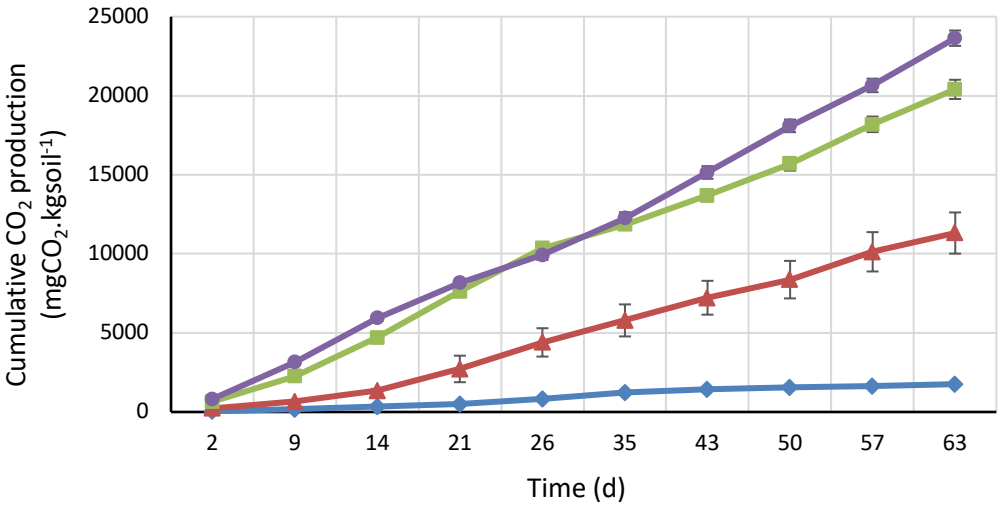


Figure 3. Carbon mineralization represented by cumulated CO_2 production for B1 condition; B2 condition; B3 condition; B4 condition. Abbreviations (B1, B2, B3, B4) refer to Table 1. Results are presented as a mean of samples collected from two reactors and analyzed in triplicate (n=6) with standard deviation.

Petroleum compounds in contaminated soils can be removed by biodegradation, biotransformation, volatilization or can be adsorbed on organic matter in a form of non-extractable residues (Kästner et al., 2014). In the present study, volatilization values were below 0.5 % of PHCs loss and thus were not further considered. Mineralization of organic carbon represented by CO_2 evolution was monitored to support information about microbial metabolic activity and PHCs degradation (Fig. 3). We observed that CO_2 evolution was correlated with

PHCs removal, suggesting that biodegradation was the major force driving contaminant concentration decrease (Table 3). Mineralization values observed in the study are corresponding with the data of other authors reporting that about 10 g CO_2 kg^{-1} soil was produced during first 30 days of soil treatment with sewage sludge in similar amendment ratio (Namkoong et al., 2002). Application of mineral nutrients often results in lower mineralization rates than organic fertilizers (Cerqueira et al., 2014; Tahhan and Abu-Ateih, 2009). In the present study, the use of digestate doubled respiration rates in comparison with mineral nutrients and was further increased by the application of immobilized bacteria which correlated with PHCs removal degree (Table 3).

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

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

Intensive mineralization of organic matter during soil incubation is often followed by temperature rise (Antizar-Ladislao et al., 2006). However, in the present study, temperature inside reactors was equal to the temperature of the inlet air (20 ± 2 ºC). As during anaerobic digestion easily biodegradable carbon is depleted and organic matter becomes stabilized, incubation of digestate may not result in rapid organic matter degradation and heat production. However, a significant organic fraction of organic matter can still be slowly degraded, which eventually contributes to observed CO_2 production. This is in accordance with the observations of other authors (Tambone et al., 2015), where composting of different pig slurry digestates did not always resulted in temperature rise but induced changes in Potential Dynamic Respiration Index (PDRI).

2.2 Soil toxicity changes

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

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

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

329

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

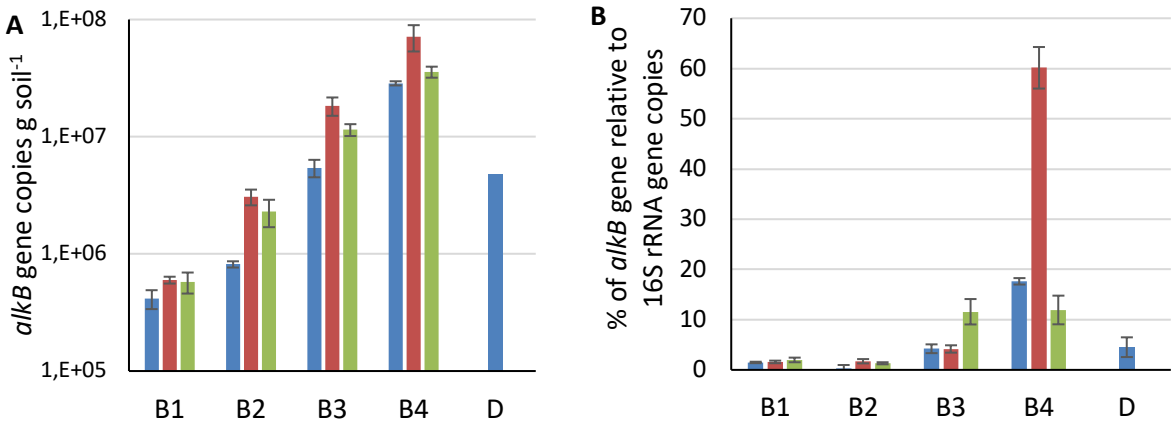
Toxicity assay	B1	B2	B3	B4
GI *Lepidium sativum* (%)	11.4 (2.6)c	58.2 (5.3)b	98.7 (1.7)a	96.2 (3.5)a
GI *Sinapis alba* (%)	16.5 (5.2)b	86.7 (6.3)a	93.0 (6.3)a	93.7 (1.9)a
Microtox® Δ EC50	16.7 (2.8)c	35.0 (5.0)b	63.3 (5.8)a	73.3 (5.8)a

334

335 ### 2.3 Functional genes abundance

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

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



355

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

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

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

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

2.4 Bacterial community composition

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

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

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

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

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

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

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

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

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

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

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

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

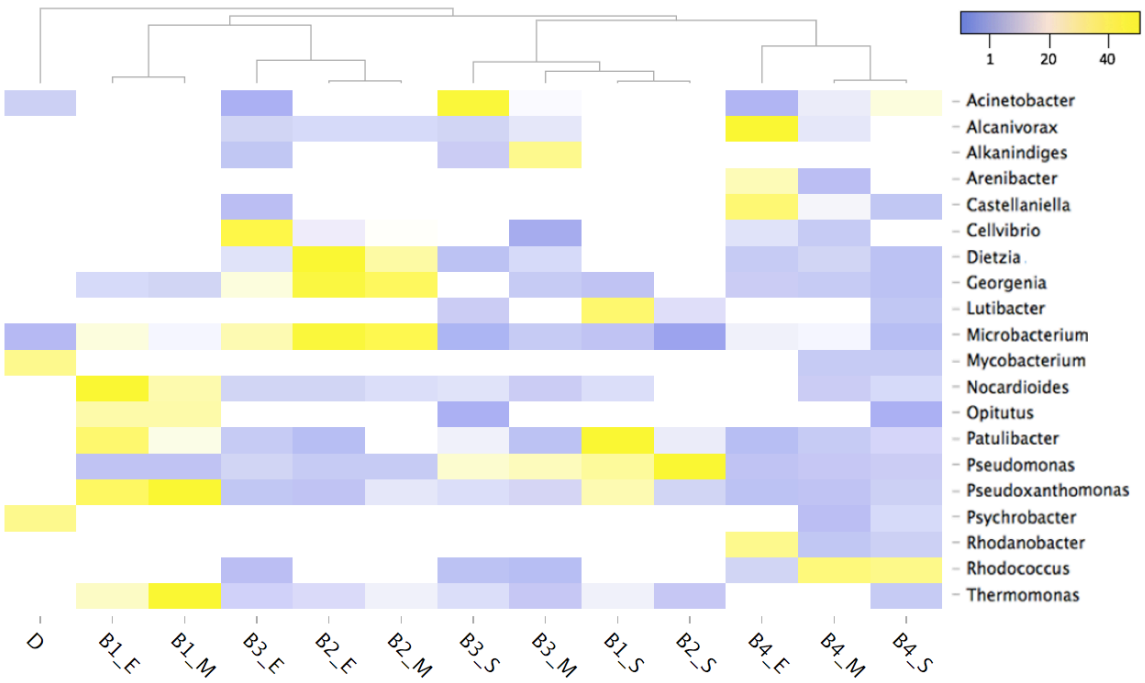


Figure 5. Heat map illustrating top genera (> 0.2%) based on relative abundance (%). Abbreviations are reported in Table 1. D: digestate; S: day 1; M: day 30; E: day 63. Results are presented as a mean of three samples collected from the same reactor. To show similarities in genera composition between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

Conclusions

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

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Supplementary materials

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

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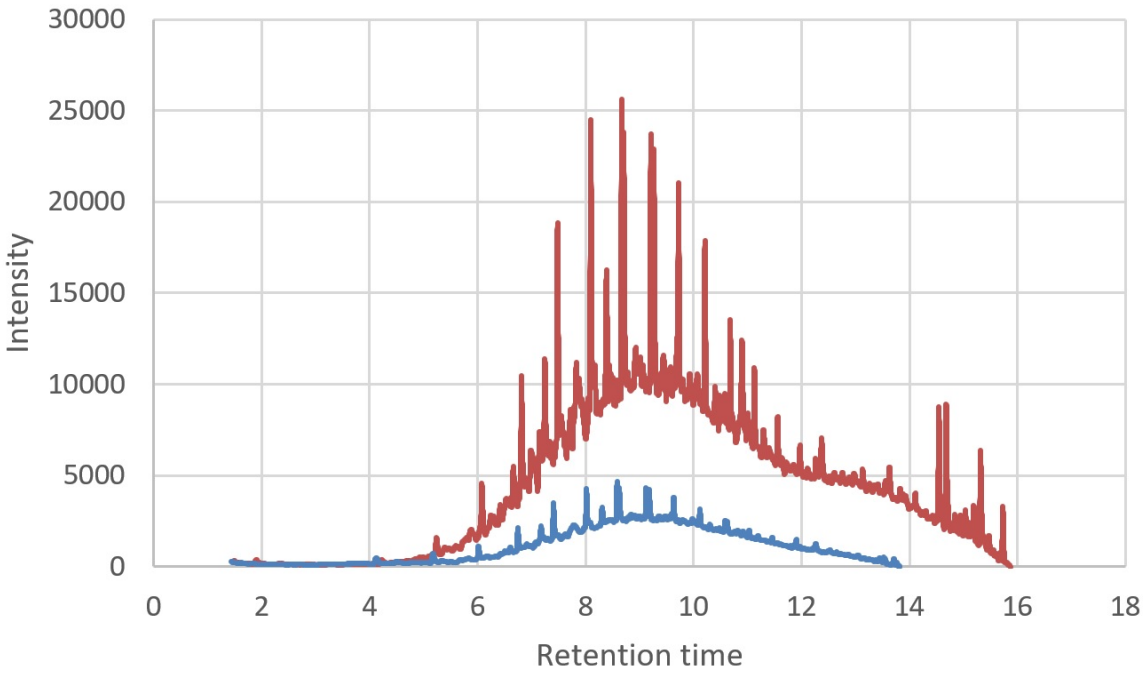

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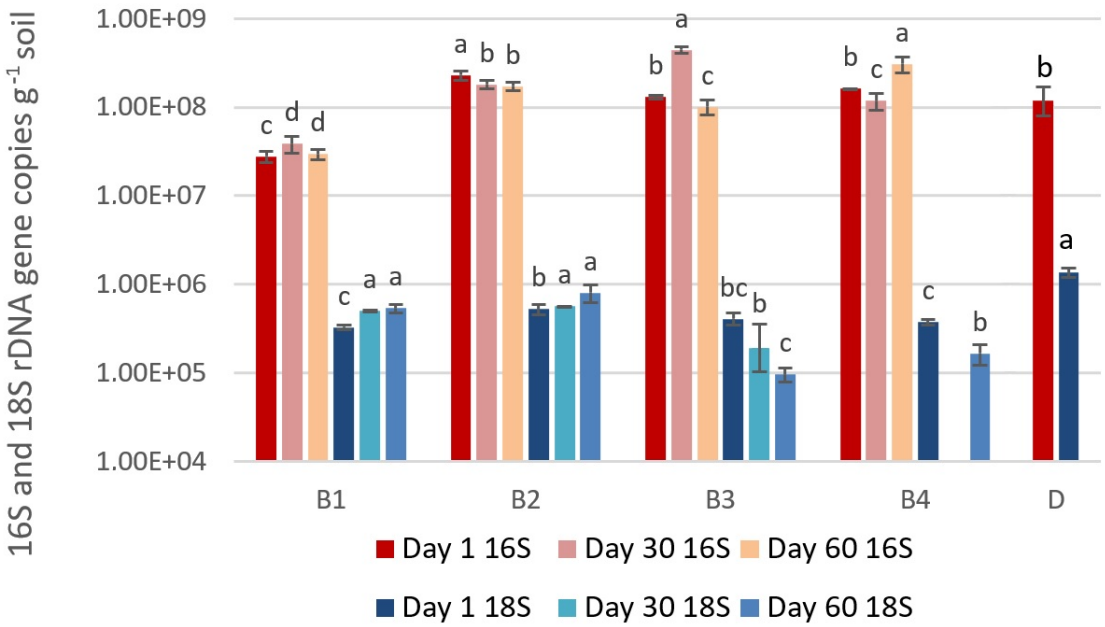
699 **Figure S1. GC-FID chromatogram presenting PHCs concentration before (red) and after (blue)**
700 **63 days of treatment under conditions B3.**

701



702

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

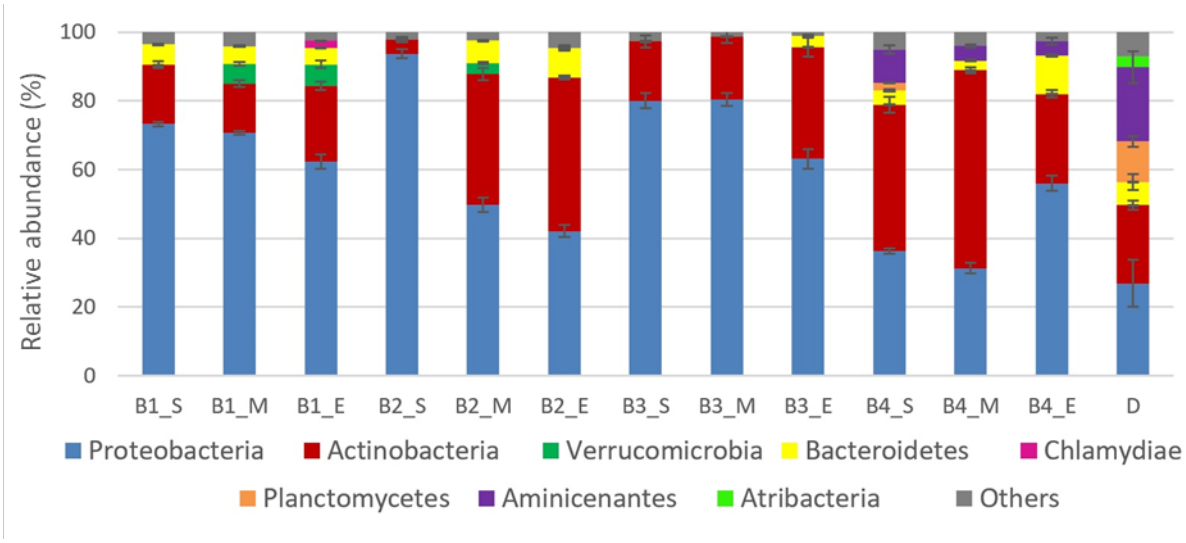


Figure S3. Relative abundance of main bacterial phyla in tested treatments during three sampling time points and in digestate. Abbreviations are reported in Table 1. D: digestate; S: day 1; M: day 30; E: day 63. Results are presented as a mean of three samples collected from the same reactor with standard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

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

Genus	B1_E	B1_M	B1_S	B2_E	B2_M	B2_S	B5_E	B5_M	B5_S	B7_E	B7_M	B7_S	D
Acinetobacter	0	0	0.1	0	0	0.1	0.2	9.5	32.3	0.7	7.4	13.1	4
Alcanivorax	0	0	0	0.5	0.6	0	0.2	1.9	0.2	21	1.7	0	0
Alkanindiges	0	0	0	0	0	0	0.2	17.5	0.9	0	0	0	0
Arenibacter	0	0	0	0	0	0	0	0	0	5	0.5	0	0
Castellaniella	0	0	0	0	0	0	0.5	0	0	6.7	2.2	0.7	0
Cellvibrio	0	0	0	13.6	16.9	0	43.6	3	0	11.6	7.9	0	0
Dietzia	0.1	0	0	12.1	7	0	1.8	1.2	0.2	0.6	1.1	0.2	0
Georgenia	0.7	0.6	0.2	6	5.3	0.1	2.3	0.3	0.1	0.4	0.3	0.1	0
Lutibacter	0	0	6.8	0	0	1.1	0	0	0.4	0	0	0.2	0
Microbacterium	11.1	8	3.6	24.2	22.8	0.8	14.6	4.3	2.3	7.6	7.9	3.1	2.8
Mycobacterium	0	0	0	0	0	0	0	0	0	0.1	0.3	0.3	8.8
Nocardioides	5.1	2.3	0.5	0.3	0.5	0.1	0.3	0.2	0.6	0.1	0.2	0.4	0
Opitutus	5.6	5.5	0	0	0	0	0	0	0.4	0	0	0.3	0
Patulibacter	3.9	1.8	5.2	0.2	0.1	1.1	0.5	0.3	1.2	0.2	0.5	0.7	0
Pseudomonas	1.5	1.6	46.8	3.6	4	90	6.8	37.5	33	1.9	3.4	4.6	0
Pseudoxanthomonas	15.4	19.8	9.9	0.8	3.4	1.9	0.9	2.1	2.6	0.6	0.7	1.7	0
Psychrobacter	0	0	0	0	0	0	0	0	0	0.1	1.5	6.3	31.8
Rhodanobacter	0	0	0	0	0	0	0	0	0	7.2	0.2	0.6	0
Rhodococcus	0	0.1	0.1	0	0.1	0	12.1	11.5	12.4	15.8	44.1	41.6	0.1
Thermomonas	3.8	10.3	1.6	0.8	1.6	0.2	0.5	0.2	0.9	0	0.1	0.3	0

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

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