*Title Page

Effect of bioaugmentation on long-term biodegradation of diesel/biodiesel blends in soil microcosms

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STOTEN-D-18-14407 Response to the reviewer comments

Dear Dr. Zhang,

We are very grateful for the evaluation of our revised manuscript. In accordance with the comments of Reviewer #2, several additional comments were added to the manuscript. All the corresponding changes in green.

Reviewer #2:

COMMENT: Thanks for authors' responses, which addressed most of my concerns. However, it seem like that there were no biological replicates although the authors mentioned that two additional samples were collected for 16S rRNA amplicon sequencing. One of the disadvantages of lack of replicate sequencing data is that multivariate statistical analysis can't be used to evaluate the changing pattern/trend of microbial communities at the statistical level. So, I suggest the authors may add several sentences in the manuscript to address this limitation. Or please make a more clear description for this part, and show the replicate sequencing data if I misunderstood this point.

Response: We wish to thank the Reviewer for his insightful suggestion and an overall positive reception of our revised manuscript. In accordance with the Reviewers' suggestion, an additional paragraph has been added into the manuscript in order to address the statistical limitations associated with the lack of replicates for sequencing data (p. 11, l. 245-252):

It should be noted that the results of the Illumina MiSeq sequencing may be limited by the lack of replicates of sequencing data. This prevented the possibility to employ a multivariate statistical analysis and evaluate the statistical significance of the observed differences. In consequence, it was not possible to assess the trends of microbial community shifts at a statistical level. The highlighted issue may be of particular importance in case of complex terrestrial matrices, in case of which the isolation of DNA is challenging. In the framework of this study the data obtained based on Illumina MiSeq sequencing was primarily used to evaluate the efficiency of the bioaugmentation process. Additionally, an attempt to elucidate the "key players" which participate in the biodegradation of various diesel/biodiesel blends.

Thank You once again for Your valuable contribution to our manuscript.

Abstract

We studied long-term (64.5 weeks) biodegradation of diesel fuel, diesel/biodiesel blends (B10-B90) and biodiesel fuels in urban soil microcosms containing indigenous microorganisms, or indigenous microorganisms augmented with a hydrocarbon-degrading bacterial community. Mineralization extent (mmol of CO_2 per day) of B10-B30 blends was smaller compared with diesel fuel at both short- (28 days) and long-term (109 days), and increased with biodiesel content. Priming with hydrocarbon degraders accelerated mineralization in the short-term (by up to 140%), with highest influence using blends with lower biodiesel content, but did not significantly influence kinetics and mineralization extent in the long-term. Although the biodiesel fraction was degraded completely within 64.5 weeks, 3-12% of the total aromatic and aliphatic hydrocarbons remained in the microcosms. Barcoded 16S rRNA gene MiSeq sequencing analysis revealed a significant effect of blend type on the community structure, with a marked enrichment of *Sphingobacteriia* and *Actinobacteria* classes. However, no significant influence was determined in the long-term, suggesting that the inoculated bacterial community may not have survived. Our findings show that biodiesel is preferentially degraded in urban soil and suggest that the value of bioaugmentation for bioremediating biodiesel fuels with hydrocarbon-degrading bacteria is limited to short-term exposures to lower (B10-B30) blends.

Keywords

Bacterial community, fuel blends, hydrocarbons, mineralization, MiSeq sequencing

1. Introduction

Petroleum diesel fuel is often blended with biodiesel [fatty acid methyl esters (FAMEs)] before being introduced to the market (Luque et al., 2010). Biodiesel mixed with petroleum diesel fuel can be used in unmodified diesel engines in different proportions ranging from 2% to 20% depending on government policy (DeMello et al., 2007; Luque et al., 2010). In Germany, the pure biodiesel is available and used in transportation without being taxed (Demırbas, 2017). However, in the rest of the European Union, the addition of biodiesel to conventional fuel is approximately 5% (Bücker et al., 2011; Schleicher et al., 2009). This blending generally has a positive influence on biodegradation rates of fuel (Horel and Schiewer, 2011; Silva et al., 2012). Several studies have focused on the effect of biodiesel in accelerating the biodegradation in sediments and soils (Miller and Mudge, 1997; Taylor and Jones, 2001). Miller and Mudge (1997) reported the addition of biodiesel to enhance biodegradation of petroleum hydrocarbons in sediments contaminated with crude oil. This phenomenon is generally explained by the fact that the FAMEs are preferentially utilized by microorganisms over the petroleum hydrocarbons. For example, Horel and Schiewer (2011) measured that biodiesel stimulated microbial populations in sandy soil, thereby increasing biodegradation rates of the blends. This effect is usually explained by the structural similarities between FAMEs and *n*-alkanes, as well as similarities in their metabolic mechanisms (Yassine et al., 2013). DeMello et al. (2007) reported the degradation rate constants for FAMEs and *n*-alkanes in seawater were comparable. This corroborates with the study by Yassine et al. (2013) which described higher *n*-alkane degradation rates in biodiesel blends with acclimated microbial cultures as attributed to the ability of FAMEs to be co-solubilized with *n*-alkanes. Moreover, these studies emphasized that biodegradation of aromatic compounds was also affected by biodiesel blending. A key factor when considering the influence of biodiesel on biodegradation of diesel in soil is the ability of the former to act

as solubilizing agent (Fernández-Álvarez et al., 2007; Miller and Mudge, 1997). According to Fernández-Álvarez et al. (2007), among the different bioremediation agents (microorganisms, nutrients and biodiesel) that can be used, only biodiesel has been shown to accelerate the biodegradation of both aliphatic and aromatic fractions of heavy fuel oil. On the other hand, Mariano et al. (2008) observed no effect of biodiesel on diesel biodegradation in soil and water in an experiment lasting over 120 days. Leme et al. (2012) showed the mutagenic and genotoxic effects of biodiesel and its diesel blends in soil matrix, emphasizing the potential harmful effects of biodiesel. However, there remains a paucity of knowledge regarding the long-term influence of biodiesel on the biodegradation of different hydrocarbon fractions in diesel/biodiesel blends in complex soil matrix.

The use of isolated microbial communities, consortia or specific populations of microorganisms (El Fantroussi and Agathos, 2005) for the *in situ* treatment of polluted sites – also called bioaugmentation – has been considered a useful approach to increase bioremediation efficiency (Atashgahi et al., 2018; Di Gregorio et al., 2016; Lladó et al., 2012; Meyer et al., 2014). Positive results were described by Teng et al. (2010), who showed that addition of hydrocarbon-degrading strains enhanced the bioremediation of soil contaminated with polycyclic aromatic hydrocarbons (PAHs), while Szczepaniak et al. (2016) showed the effectiveness of using PAH-degrading consortia during the early stage of bioaugmentation treatment. Both studies highlighted the stimulatory effect of autochthonous microorganisms with the addition of exogenous hydrocarbon-degrading microorganisms over the short-term. However, there are also contradictory studies that reported either a negative or no effect by bioaugmentation (Bouchez et al., 2000; Saponaro et al., 2001; Silva et al., 2009). No significant effect on biodegradation of PAHs after fungal and bacterial consortia introduction into soil were observed by Silva et al. (2009). The study by Bouchez et al. (2000) indicated the difficulties in adaptation of augmented microorganisms to a well-adapted initial bacterial

population. According to El Fantroussi and Agathos (2005), bioaugmentation is still in the experimental phase with no general guidelines for how to efficiently introduce external microorganisms to treat a contaminated site. Recently, however, Horemans et al. (2016) presented a three-step approach emphasizing the importance of compatibility of microorganisms and soil selection to the success of bioaugmentation treatments. This was also mentioned by Bento et al. (2005), who showed that an effective bioaugmentation approach for treatment of diesel oil contaminated sites can depend on soil properties as well as indigenous soil microorganisms. Bioaugmentation treatments with bacteria (Meyer et al., 2014, 2012) and fungi (Junior et al., 2009) have been successfully applied for diesel/biodiesel blends, where the biodegradation of different blends were higher compared with non-bioaugmented set-ups. However, many studies concern the biodegradation of only a limited range of blends, such as B2, B5, B20 or B50 (Bücker et al., 2011; Meyer et al., 2014; Schleicher et al., 2009) or the experiments were conducted over short periods of 28, 60 or 84 days (Horel and Schiewer, 2011; Schleicher et al., 2009; Silva et al., 2012). Therefore, it is difficult to generalize about the effectiveness of bioaugmentation on degradation of wide range of diesel/biodiesel blends during long-term exposure, as well as due to the variability in soil types, their autochthonous microbial communities, and the experimental approaches performed across different laboratories.

Here, we examined the effects of biodiesel on the biodegradation of aliphatic and aromatic fractions in a wide range of diesel/biodiesel blends. Long-term biodegradation experiments were conducted in urban soil microcosms in two parallel variants: autochthonic microcosms *versus* autochthonic microcosms bioaugmented with a hydrocarbon-degrading community that was previously isolated from contaminated soil. The response of the autochthonic microbial community towards increasing biodiesel concentration, and that of the

exogenously-added hydrocarbon-degrading community, was analyzed by 16S rRNA gene sequencing using Illumina MiSeq technology.

2. Materials and Methods

2.1. Fuels

Diesel fuel (EN 590:2004), assigned as D was purchased from a petrol station (PKN Orlen, Poland). Biodiesel (assigned as B100) was produced from rapeseed oil (DIN V 51606) and obtained from PetroTec AG in Germany. In addition to these two types of fuels, nine diesel/biodiesel blends with increasing by 10% biodiesel content that is from 10 to 90% (v/v) (assigned B10, B20, B30, B40, B50, B50, B60 B70, B80, and B90) were prepared by batching in laboratory and mixing volumetric portions of diesel and biodiesel fuels. Two methyl ester of oleic acid (C18:1) and linoleic acid (C18:2) constituted a majority of 68% and 21% of the biodiesel respectively, while the remaining 11% consisted of methyl esters of C16:0, C18:0, C20:0 and C20:1 (Lisiecki et al., 2014).

2.2. Microorganisms

The bacterial community that was used in this study – designated BC125 – was isolated from crude oil-contaminated soil (Gorlice, Małopolska, Poland). The selectively enriched community was maintained using only mineral medium with diesel fuel as a sole carbon and energy source. Metagenomic analysis of V4 hypervariable region of the 16S rRNA gene identified 22 classes. The most dominant microbial classes detected in BC125 were *Alphaproteobacteria* (47.85%), followed by *Bacilli* (22.71%), *Gammaproteobacteria* (13.31%), *Actinobacteria* (8.58%), *Clostridia* (3.37%), *Betaproteobacteria* (2.08%) and *Flavobacteriia* (1.36%). The community was tested with respect to structural and functional robustness when exposed to different hydrocarbons according to the report provided by

Sydow et al. (2016). It was proved to maintain both structural and functional integrity when exposed to various aliphatic, cyclic and aromatic hydrocarbons. The bacterial community was able to efficiently degrade hydrocarbons in a pH range of 6.5-7.5.

The BC125 was stored as glycerol stocks (20% v/v) at -80°C until used. A 1 ml of stock suspension was transferred to Erlenmeyer flask (300 mL, SIMAX, Sazava, Czech Republic) with 50 mL of mineral medium supplemented with 0.5% (v/v) diesel fuel as described in Sydow et al. (2016). The culture was incubated with shaking (120 rpm; 25 °C, Multitron; Infors HT, Bottmingen, Switzerland) for 24 h. Subsequently, the cell suspension (1 mL) was transferred into fresh mineral medium (50 mL) and cultivated for 72 h in conditions described above. The final enrichment culture was obtained after three transfers. The fresh pre-culture (50 mL) for mineralization experiments were washed three times in sterile NaCl (0.85% v/v) and subsequently incubated on mineral medium (500 mL) with 0.5% (v/v) diesel fuel as described in Sydow et al. (2016). The BC125 was incubated (120 rpm; 25°C) for to 48 h. When optical density (OD_{600}) of the pre-culture reached approximately 3.0 ± 0.1, the cell suspension was centrifuged (10,000 *g*; 4°C; 15 min, Heraeus Multifuge 3S-R, Hanau, Germany) and washed three times with mineral medium. The resuspended cells in medium served as inoculum for subsequent experiments.

2.3. Characterization of soil

Mollic gley soil used in this study was collected from a city park in Poznan, Poland (N 52.4011445, E 16.9222993) and previously characterized in Sydow et al. (2015). Briefly, the soil samples were taken from the depth of 10-20 cm and sieved (2.0 mm). The soil was characterized as fine-grained silt loam type OL (United Soil Classification System). The detailed composition of soil was as follows: clay, 4 ± 1 [%]; silt, 83 ± 3 [%]; sand, 13 ± 2 [%]. The characteristics of the soil were as follows: organic carbon 5.44 ± 0.31 [g kg^{-1}]; nitrogen

147 0.57 ± 0.07 [g kg^{-1}]; phosphorous 0.080 ± 0.005 [g kg^{-1}]; pH 6.95 ± 0.7; bulk density 1.41 ±
148 0.06 [Mg/m^3]; porosity 0.455 ± 0.03 [m^3/m^3]; moisture during sampling 18 ± 1 [%]; cation
149 exchange capacity 22.1 ± 0.8 [cmolc kg^{-1}]. A symbol ± represents standard deviation from
150 three independent replicates.
151
152 **2.4. Microcosms and mineralization measurements**
153 To evaluate the mineralization extent of diesel (D) and biodiesel blends (B10-B100), 50 g of
154 soil was placed in sterile pre-weighed 1000 mL SIMAX bottles (SIMAX, Sazava, Czech
155 Republic). Subsequently, fuels (0.75 mL of D or B10-B100) were spiked on the soil surface.
156 The bottles were weighed again to determine the exact amount of fuels added to each bottle,
157 which was essential for further analytical protocols (0.1 mg accuracy). Average concentration
158 of D and B10-B100 fuel was 12 g/kg soil (approx. 1% v/w, a level at which biological
159 treatment is typically feasible). Each experimental setup was performed in triplicates, thus
160 overall 33 samples with diesel/biodiesel blends were prepared. Another 33 samples with
161 microcosms (50 g of soil) were first spiked with diesel/biodiesel blends as described above
162 and then augmented with BC125 suspension (1 mL; with final concentration 2×10^8 CFU g^{-1})
163 – further assigned as D+, B10+, B20+ etc. The non-augmented samples were amended with 1
164 mL of sterile mineral medium to maintain the soil field capacity at 85% v/v in all microcosms
165 (augmented and non-augmented samples). Additionally, three biotic, non-spiked soil controls,
166 three non-spiked, augmented with active BC125 soil controls and three non-spiked,
167 augmented with killed inoculum (autoclaved immediately before inoculation) controls were
168 also prepared. All samples were gently mixed and finally, all microcosms were incubated at
169 20°C for 64.5 weeks.
170 The mineralization extent of fuels was assessed by measurements of CO_2 trapped in the base
171 trap (10 mL of 0.75 M NaOH in a 20-mL vial), and placed in each microcosm as described in

Szulc et al. (2014). Titration with 0.1 M HCl of diluted NaOH and Na_2CO_3 solution from the trap, according to Warder method, was carried out with the use of automatic titrator (Metrohm titroprocessor 686, Herisau, Switzerland). After each measurement the content of the base trap was replaced with fresh NaOH solution. The samples were measured in different time intervals: every 1-3 days (I month), once to twice a week (II-III month), every two weeks (IV-V month), once a month (VI-XII month), and the last measurements were performed 102 days after the penultimate measurement was taken (day 452).

2.5. Hydrocarbon and FAME analyses

After 64.5 weeks, the microcosms (three replicates for each setup) were sacrificed and the residual hydrocarbons and FAME were determined. Briefly, after removal of base traps, 12.5 mL of acetone was added into each bottle and the samples were vortexed for 1 min (Vortex-Genie 2 Shake, Scientific Industries, New York, US). Subsequently, 5 g of anhydrous $MgSO_4$ was added and the samples were vortexed again. Next, 7.5 mL portion of *n*-hexane was added and vortexed for another 1 min. The bottles were sonicated for 20 min in order to promote desorption of the analytes from solid matrix. The samples were shaken vigorously (Multitron; Infors HT, Bottmingen, Switzerland) after the first 10 min to homogenize soil sticking on the bottom of the flask. The samples were then shaken on a horizontal shaker (250 rpm; 15 min). Subsequently, the obtained extract (1 mL) was washed with 0.1 M NaOH (3 mL) to remove acetone and co-extracted acidic interferences and the upper phase further processed. One fraction of the extract was taken and cleaned on a Florisil column (Sigma Aldrich, St. Louis, US) for total hydrocarbon and FAME analysis; another fraction was also taken, but this time cleaned and fractionated on a Ag-impregnated silca gel column (Merck, Darmstadt, Germany) into saturated (aliphatic) and non-saturated (aromatic and FAME) fraction as described by Lisiecki et al. (2014). The resultant hydrocarbon fractions (aliphatic and aromatic) were

finally determined with gas chromatography (GC-FID and GC×GC-TOF-MS, Agilent, Palo Alto, US) according to the procedures described elsewhere (Lisiecki et al., 2014). The results were presented as a ratio of remaining to initial masses of each fraction (total diesel/biodiesel blends, total hydrocarbons, aliphatic hydrocarbons, aromatic hydrocarbons and FAME). The presented error bars for the GC analysis results represent confidence intervals for $p = 0.05$.

2.6. Evaluation of bacterial community structure in the soil

The influence on qualitative and quantitative composition of microbial community samples was assessed using Illumina MiSeq sequencing (Illumina, San Diego, US). Here, Illumina genetic analysis was applied in order to investigate the potential changes in the bacterial community structure due to biodiesel content as well as bioaugmentation treatment. The contribution of most abundant microbial phyla and classes were presented as % of total taxonomic rank.

Two additional samples of each treatment were setup for Illumina MiSeq sequencing, as described in section 2.4 above. After termination of the soil experiments, approximately 20 g of soil from central area of each experimental microcosm (ten random samples from depth of approx. 10 cm) were collected and homogenized. The subsamples were divided into three equal portions and then stored at -80 °C until used (no more than two weeks). Extraction of DNA and PCR amplification using universal primers were performed according to the procedure provided by Ławniczak et al. (2016) and Szczepaniak et al. (2016). Briefly, the isolation of the genetic material from analyzed samples was performed using appropriate Genomic Mini AX kits (A&A Biotechnology, Gdynia, Poland), as recommended by the manufacturer. The validation of isolation efficiency was conducted with a fluorometric method by means of a Qubit™ dsDNA HS Assay Kit and Qbit 2.0 apparatus (ThermoFisher Scientific, Waltham, US). For PCR amplification and sequencing the universal prokaryote

primers 515F-806R were applied to amplify the V4 region of the 16S rRNA gene (Caporaso et al., 2012). The PCR reaction (25 µl) contained the following: 5 µl microbial template genomic DNA, 5 µl of each primer, 2.5 µl of PCR-grade water (ThermoFisher Scientific, Waltham, US) and 12.5 µl of PCR Master Mix with the Taq polymerase (ThermoFisher Scientific, Waltham, US). The thermocycler (ThermoFisher Scientific, Waltham, US) program was employed with initial denaturation at 95°C for 3 min, followed by 35 cycles of 95°C for 1 min, 52°C for 30s, 72°C for 1 min and final extension at 72°C for 10 min. The amplicons were purified on Clean-Up columns (A&A Biotechnology) and used for library construction. Sequencing was carried out with a MiSeq Reagent Kit v2 (2x250 bp) using a MiSeq (Illumina) platform. Details concerning the preparation of libraries were presented in our previous study (Szczepaniak et al., 2016). After sequencing, the raw data in FASTQ format were imported to the CLC Genomics Workbench 8.5 software with the CLC Microbial Genomics Module 1.2 (CLCbio, Qiagen Bioinformatics, Aarhus, Denmark). The reads were demultiplexed, and paired ends were merged (mismatch cost = 2, min score = 8, Gap cost = 3, max unaligned end mismatches = 5). Primer sequences were trimmed (quality limit = 0.05, ambiguous limit = 'N'), and the identification and elimination of chimeric reads was performed. The output data were clustered independently based on two reference databases, namely SILVA v119 (Quast et al., 2013) and GreenGenes 13.5 (DeSantis et al., 2006) at a 97% probability level of OTUs (operational taxonomic units). The alpha-biodiversity (number of OTUs) factor was determined based on the merged abundance table (clustered against SILVA v119). The final sequencing datasets generated and analyzed within the framework of this study are available in the SRA repository, with the identifier SRP156685 (https://www.ncbi.nlm.nih.gov/sra/SRP156685).

Overall, we selected three microcosms supplemented with D, B20 and B100 non-augmented and augmented treatments (D+, B20+, B100+). B20 has received significant attention and is

247 one of the most commonly investigated biodiesel blend (Cyplik et al., 2011; Demirbas, 2007;
248 Junior et al., 2009; Meyer et al., 2012; Silva et al., 2012). According to our study,
249 mineralization extent in B20 blend microcosms presented the most unexpected pattern and
250 therefore this microcosm was selected for further genetic analysis.
251 It should be noted that the results of the Illumina MiSeq sequencing may be limited by the
252 lack of replicates of sequencing data. This prevented the possibility to employ a multivariate
253 statistical analysis and evaluate the statistical significance of the observed differences. In
254 consequence, it was not possible to assess the trends of microbial community shifts at a
255 statistical level. The highlighted issue may be of particular importance in case of complex
256 terrestrial matrices, in case of which the isolation of DNA is challenging. In the framework of
257 this study the data obtained based on Illumina MiSeq sequencing was primarily used to
258 evaluate the efficiency of the bioaugmentation process. Additionally, an attempt to elucidate
259 the "key players" which participate in the biodegradation of various diesel/biodiesel blends.
260

261 **2.7. Mineralization kinetics and statistical analysis**

262 As the experiment proceeded, it was observed that the curves expressing the increase of
263 cumulative CO_2 evolution were neither linear nor logarithmic. Hence, for a matter of
264 simplicity, two sections (namely from day 0 until day 28, as a beginning of the experiment,
265 and from day 33 to day 109, as the most intensive period), where mineralization curves were
266 approximately linear ($R^2 \geq 0.95$), were selected for further analysis. Subsequently, zero-order
267 kinetics model was applied to describe and compare the kinetics of organic matter
268 mineralization (associated mainly with the fuels additions), between the investigated
269 experimental setups. Similar approaches to characterizing mineralization kinetics in porous
270 media were presented previously (Dechesne et al., 2010; Owsianiak et al., 2010). The one-
271 way ANOVA with $p < 0.05$ were used for statistical comparisons. This approach was also

employed for statistical analysis of metagenomic data in order to establish the significance of differences for untreated vs non-bioaugmented and non-bioaugmented vs bioaugmented systems.

3. Results

3.1. Evolution of CO_2 and mineralization kinetics

Mineralization extent of the different fuel blends was measured as amount of CO_2 released in the microcosms (corrected for the background, substrate-unamended control), as summarized in Table 1 and Fig. 1. In non-augmented microcosms, mineralization extent increased with increasing biodiesel content, and ranged from 44.1 ± 2.3 for B10 to 48.8 ± 2.4 mmol CO_2 for B100 (Table 1). For diesel, mineralization extent was the highest and equal to 49.9 ± 3.8 mmol. The evolution of CO_2 in all samples differed significantly from that in the controls (9.7 ± 1.1 mmol) without any fuel addition (Fig. 1). In bioaugmented microcosms, the mineralization extent did not increase with increasing biodiesel content as in non-augmented samples. The highest CO_2 evolution were observed for B20 (48.5 ± 3.1 mmol), while the lowest for B50 (42.9 ± 2.1 mmol). However, there were no statistically significant differences between the mineralization extent of non-augmented and augmented diesel/biodiesel blends, apart from pure diesel microcosms ($p = 0.047$).

Regression performed on non-augmented and augmented mineralization curves presented the influence of biodiesel content on mineralization extent during short- (days 0-28) and long-term (days 33-109) mineralization phases (Table 1). Linear regressions applied on the mineralization curves for non-augmented samples revealed that mineralization rate constants were higher for higher biodiesel blends. This was generally true for both mineralization phases. However, it is worth noticing that the mineralization rate constants of non-augmented B10-B30 microcosms were lower than of microcosms spiked with pure diesel (D) in both

297 phases. On the other hand, regressions for augmented samples showed that mineralization rate
298 constants were higher in the short-term mineralization phase compared with non-augmented
299 samples (apart from D+, B80+ and B100+). In the long-term phase, however, the opposite
300 was observed. There were statistically significant differences in rate constants during short-
301 term mineralization phase of non-augmented and augmented samples for lower biodiesel
302 blends from B10 to B60 ($p < 0.05$), while in long-term phases the significant differences were
303 observed only for B40 ($p = 0.046$) and B50 ($p = 0.041$).
304
305 **3.2. Fate of hydrocarbons and FAME**
306 Based on GC-FID and GC×GC-TOF-MS studies after 64.5 weeks, biodiesel was completely
307 degraded in all diesel/biodiesel blends (Fig. S1. Supporting Information). Depending on the
308 blends, the total petroleum hydrocarbon residues ranged from 3 to 12% of the introduced
309 hydrocarbon fractions in samples without bioaugmentation, and from 4 to 8% in samples with
310 bacterial augmentation. After 64.5 weeks, there were no statistical differences between blends
311 in case of total hydrocarbon residues ($p > 0.05$) in non-augmented and augmented treatments.
312 No clear effect of the type of blend on ratio of remaining to initial masses of hydrocarbon
313 fractions (aliphatic and aromatic factions) was observed, apart from B80-B90 blends where
314 the increase in this ratio were determined. Moreover, the ratio of residual aromatic to aliphatic
315 fraction at the end of the experiment remained unchanged for all treatments (Fig. S2.
316 Supporting Information).
317
318 **3.3. Bacterial community structure in non-augmented and augmented soil**
319 Figure 2A shows the contribution of ten most abundant bacterial phyla in bacterial community
320 (BC125), untreated soil sample (control) and microcosms supplemented with different fuels
321 without (B100, B20, D) and with (B100+, B20+, D+) bioaugmentation treatment.

The most dominant microbial phyla detected in untreated urban soil (Fig. 2A control) were *Proteobacteria* (45.64%), followed by *Planctomycetes* (15.41%), *Clostridia* (10.11%), *Chloroflexi* (12.63%), *Acidobacteria* (8.78%) and *Actinobacteria* (5.54%). The rest of the identified microbial taxa were estimated below 5% of total detected taxonomic ranks ($p = 0.011$). The microbial community structure changed between the treatments (i.e. controls vs treatments with B100, B20 and D soil samples) after 64.5 weeks exposure. The relative abundance of *Bacteroidetes* increased in case of samples spiked with B100, B20 and D by 5, 12 and 6% respectively. The increase in abundance of *Actinobacteria* was also observed for soils supplemented with fuels (B100 by 8%, B20 by 2% and D by 3%). On the other hand the contribution of *Planctomycetes* deceased in each B100, B20 and D spiked soils by 7, 5 and 3%, respectively, while the contribution of both *Chloroflexi* and *Acidobacteria* deceased by 1-3% depending on the fuel. No changes were determined for *Proteobacteria*, the most abundant phylum ($p = 0.123$). The supplementation of urban soil with different fuel and oil-degrading bacteria (B100+, B20+, D+) did not affect significantly the composition of their bacterial community structure compared with non-augmented samples (B100, B20, D) ($p = 0.094$). However, the relative abundance of *Proteobacteria* increased by 7 and 8% for B20+ and D+ with reference to samples without bioaugmentation treatments. The highest increase (by 15%) was observed for *Bacteroidetes* in soil supplemented with pure diesel (D+), even though the abundance of *Bacteroidetes* decreased by 5% in B20+ samples. The contribution of *Planctomycetes* increased by 2% for B100+, while for B20+ and D+ the contribution decreased by 2 and 6%, respectively. The abundance of *Actinobacteria* and *Chloroflexi* deceased with the increased amount of diesel fuel (even by 7% depending on phylum).

Figure 2B shows the ten most abundant bacterial classes in non-augmented (B100, B20, D) and augmented (B100+, B20+, D+) soil spiked with appropriate fuels. The most dominant microbial classes detected in the untreated soil (control) were *Alphaproteobacteria* (19.41%),

Gammaproteobacteria (15.45%), *Planctomycetacia* (14.91%), *Acidobacteria* (7.69%) and *Betaproteobacteria* (6.84%). All other classes that were identified represented <5% of total identified taxonomic ranks ($p = 0.018$). These results revealed that both *Sphingobacteriia* and *Actinobacteria* increased their relative abundance in all samples supplemented with B100, B20 and D by 5, 12, 6% and 11, 2, 4%, respectively. Notably, the contribution of both classes did not exceed 1% in untreated soil sample ($p = 0.016$). The increase of the abundance of *Sphingobacteria* was caused by the increased ratio of bacteria belonging to the *Chitinophagaceae* genus in this class. This genus was predominant and its ratio exceeded 95% in this class. In turn, the increased ratio of bacteria belonging to the *Actinobacteria* class was caused by the increased abundance of the following genera: *Arthrobacter*, the increase of which was particularly high in case of addition of biodiesel, and *Corynebacteriales*. A decrease of bacteria belonging to the *Gaiellales* genus was also observed in this class, for which the contaminants introduced into soil were toxic. The ratio of this genus in the *Actinobacteria* class decreased from 52% (control soil) to 2-7% in contaminated soil samples. The relative abundance of *Gammaproteobacteria* increased by 2% for B100, while that in the B20 and D treatments decreased by 2 and 9%, respectively. The following bacterial genera were predominant in the *Gammaproteobacteria* class: *Aquicella* (46%), *Arenimonas* (15%), *Lysobacter* (15%) and *Thermomonas* (7.4%). The ratio of *Aquicella* and *Thermomonas* did not change in case of soils supplemented with diesel, however the abundance of *Arenimonas* and *Lysobacter* decreased significantly to 2.7 and 2.4%, respectively. In case of samples supplemented with biodiesel (B20 and B100) a notable decrease of all the above-mentioned genera was observed. Changes were also noted in case of the *Pseudomonas* genus, the ratio of which in control soil amounted to 0.35%. The addition of B100 caused a significant increase to 40%, which decreased in case of B20 (27%) and diesel (2.7%). A significant increase (by 7%) of the *Betaproteobacteria* for samples spiked with pure diesel was detected. In case of

the *Betaproteobacteria* class, the following genera were predominant in control soil: *Acidovorax* (47%), *Noviherbaspirillum* (21%) and *Ralstonia* (2.8%). In the sample supplemented with diesel (D), the abundance of *Acidovorax* did not change, whereas the ratio of *Noviherbaspirillum* and *Ralstonia* increased to 30 and 7.6%, respectively. On the other hand, the decrease in abundance of *Planctomycetacia* (by 7% for B100, 5% for B20, and 3% for D) and *Acidobacteria* (by 3% for B100, 3% for B20, and 2% for D) was also observed. No significant changes were estimated in the most abundant class, *Alphaproteobacteria* ($p = 0.131$).

Within bacterial classes, the differences between non-augmented and augmented samples were more visible, however still bioaugmentation treatment did not affect significantly the community structures ($p = 0.097$). Similar to non-augmented soil, the increase in abundance of *Sphingobacteriia* (by 9% for B100+, 9% B20+ and 14% D+) and *Actinobacteria* (by 6% for B100+, B20+, D+) were determined with reference to untreated soil (control). The increased ratio of bacteria belonging to the *Sphingobacteriia* class resulted from the increased abundance of uncultured bacteria belonging to the *Chitinophagaceae* family. These bacteria were part of the autochthonous population and were not present in BC125. In control soil, this genus comprised 50% of bacteria belonging to *Sphingobacteriia*, whereas in case of samples supplemented with diesel (D), B20 and B100 their abundance was equal to 55, 83 and 96%, respectively. The increased ratio of the *Actinobacteria* bacterial class was caused by the increase of the following genera: *Arthrobacter*, which was particularly predominant in case of biodiesel (58%), and *Cellulosimicrobium* (18%). In the framework of this class the decrease of bacteria belonging to the *Gaiellales* genus was observed, for which the contaminants were toxic. Its ratio in the *Actinobacteria* class decreased 52% (control soil) to 5.3% in samples supplemented with diesel oil. However, compared to soil without bioaugmentation, the highest increase (by 4, 5 and 16 % for B100+, B20+ and D+, respectively) were determined

for *Gammaproteobacteria*. It is worth noting that the contribution of *Gammaproteobacteria* in BC125 reached 13.31% (see Materials & Methods section, 2.2. Microorganisms). In contrast to samples without bioaugmentation, the *Pseudomonas* genus was predominant in the *Gammaproteobacteria* class. Its ratio in the soil microbiome was equal to 82% (D+), 62% (B20) or 29% (B100). Interestingly, its ratio in BC125 was low (equal to 0.6%). The ratio of genera *Aquicella*, *Arenimonas*, *Lysobacter* and *Thermomonas*, which were predominant in control soil, was notably decreased in samples supplemented with diesel (D+) or biodiesel (B20+ and B100+). The abundance of *Alphaproteobacteria* decreased by 4% for B100+, while for B20+ and D+ members of this class increased by 3 and 4%, respectively. *Sphingomonas* genus was predominant in the *Alphaproteobacteria* class. In BC125 it comprised 46% of all bacteria, and up to 92% of bacteria belonging to the *Alphaproteobacteria* class. In comparison with control soil (34% among *Alphaproteobacteria*) its ratio decreased to 24% (B100+), 11% (B20) or 7.4% (D), respectively. It should be highlighted that these changes were not significant ($p = 0.134$), considering that *Alphaproteobacteria* was the most abundant bacterial class in BC125 (46.85%). The increased abundance of *Acidobacteria* for B20+ (by 6%) was also identified. In case of *Acidobacteria*, all the changes of resulted from the increased abundance of uncultured bacteria belonging to Subgroup 4 and 6. However, the most visible changes were observed for soil (D+) spiked with pure diesel and BC125, where an increase in *Flavobacteriia* (by 8%) and a simultaneous decrease in *Planctomycetacia* (by 6%) and *Betaproteobacteria* (by 9%) compared with soil (D) without addition of bacterial community were determined. Changes in the *Flavobacteriia* class were caused by shifts of the abundance of bacteria belonging to the *Flavobacterium* genus. It can be assumed that this genus was introduced into the soil with the biopreparation, since its ratio in the control soil was below 0.01%. Furthermore, it did not occur in any sample of soil contaminated with hydrocarbons. It

is difficult to explain its high ratio. The decrease of *Planctomycetacia* in D+ soil relative to D soil was caused by the decreased ratio of the *Planctomycetaceae* family, particularly of uncultured genera belonging to this family. In case of *Betaproteobacteria*, The decreased ratio in D+ soil relative to D soil was associated with the decrease abundance of *Acidovorax* and *Noviherbaspirillum* families. No significant changes ($p = 0.119$) were observed for *Bacilli*, which was second most abundant class (22.71%) in BC125.

After 64.5 weeks, the alpha diversity estimates were also determined for untreated soil, BC125, autochthonic microcosms (B100, B20, D) and bioaugmented autochthonic microcosms (B100+, B20+, D+). The mean value of the observed OTU's for the untreated soil samples was equal to 2,268. The microcosms supplemented with B100 and B20 caused significant increase ($p < 0.05$) in the values of OTUs and reached 2,592 and 2,314; respectively. The enhancement was also established for the same microcosms supplemented with bacterial community, however no considerable differences between augmented and non-augmented samples were observed (B100+ = 2,516; B20+ = 2,363). For diesel treated soil with and without bacterial inoculation the mean values of observed OTUs were the lowest and did not differ significantly ($p > 0.05$) in comparison to untreated soil (D = 2,214; D+ = 2,219).

4. Discussion

4.1. Long-term mineralization of diesel/biodiesel blends in urban soil

Lisiecki et al. (2014) demonstrated that in porous matrices (sterile sand) the increase of biodiesel content in blends was positively correlated with an increase in their mineralization extent after 82.5 weeks. Here, the results showed that after long-term exposure the mineralization extents in urban soil with autochthonous microorganisms were similar and clearly not dependent on the amount of biodiesel in fuels. Many authors emphasized the tremendous adaptation capacity of autochthonous microorganism to harsh conditions

(Bouchez et al., 2000; Vogel, 1996), especially when the time is sufficient enough to fully adapt and consequently degrade exogenously added xenobiotics. According to Thompson et al. (2005), indigenous microorganisms are the most suitable candidates for slow and continuous degradation of pollutants during long-term exposure. Prior studies have also noted that the former oil contaminated soils are often the most promising source for isolation of efficient hydrocarbon-degrading bacteria (Owsianiak et al., 2009b; Rahman et al., 2002; Szczepaniak et al., 2016). Hence, in the soil from city park placed next to the main road, the presence of hydrocarbon-degrading community among autochthonous microorganisms was expected. Based on Illumina MiSeq sequencing more than one third of microbial classes abundance detected in the untreated soil belonged to *Alphaproteobacteria* and *Gammaproteobacteria*. Plethora of studies indicated that both *Alphaproteobacteria, Gammaproteobacteria* as well as *Bacilli* and *Actionbacteria* which were also the most dominant classes in bacterial community (BC125), are in fact well-known hydrocarbon degraders in soil and have been often enriched during biodegration of hydrocarbons (Fuentes et al., 2015; Marchand et al., 2017; Tiralerdpanich et al., 2018).

Although, the mineralization extent after long-term exposure was almost equal for each fuel, we revealed that the increase of biodiesel content in blends caused the enhancement of mineralization extent, especially at short- and long-term mineralization phases. The presence of FAMEs has been already reported to accelerate the biodegradation of diesel in experiments (up to 28 and 60 days) in different types of porous matrixes, such as sand soil (Horel and Schiewer, 2011), oxisol (Meyer et al., 2014) or soil from rain forest (Silva et al., 2012). Several studies emphasized that biodegradation of both FAMEs and *n*-alkanes undergo similar metabolism via β-oxidation mechanism (Lisiecki et al., 2014; Sydow et al., 2016; Yassine et al., 2013), thus the acceleration in mineralization in the presence of biodiesel might be expected. Our findings are consistent with Yassine et al. (2013), who suggested that this

was a result of co-solubilization mechanisms rather than cometabolism, for which the latter occurs mainly when one of the substrates is not readily biodegradable. The authors clearly determined that the ability of FAMEs to co-solubilize the *n*-alkanes is associated with reduction of interfacial surface tension and enhancement of their bioavailability for microorganisms. However, DeMello et al. (2007) presented that the acceleration of *n*-alkanes degradation in the presence of FAMEs in seawater microcosms took place only in early stage of the experiment. After longer time (53 days), the authors determined no effect of biodiesel on composition of the residual mixtures. They emphasized that the long period of time caused this lack of differences in terms of hydrocarbon composition between diesel and its biodiesel blends, which might be also explain our results. Mariano et al. (2008) also showed that in experiments lasting up to 120 days, no stimulation effect of FAMEs (B2, B5, B20) on diesel degradation in both soil from a petrol station and water samples were found. Taken collectively, it can be concluded that in short-term exposure, FAMEs is expected to increase the mineralization extents of different kinds of diesel/biodiesel blends, whereas in the long-term FAMEs had no visible influence on their mineralization extent.

Our study also revealed that the mineralization rate constants of B10-B30 blends in urban soil were lower than of diesel fuel (D) during short- and long-term exposure, while generally for higher diesel/biodiesel blends (above B30) the higher mineralization rates were determined. This is in accordance with Owsianiak et al. (2009a), who noticed that only the introduction into petroleum diesel above 30% of biodiesel contribute to the enhancement of biodegradation efficiency in aqueous media. No positive effect of low content of biodiesel (even up to B20) on diesel degradation were also observed in other study (Mariano et al., 2008). Thus, it might be concluded that the positive effect on the biodegration efficiency of diesel/biodiesel blends in soil microcosms can be expected only after exceeding a certain concentrations of biodiesel added to conventional fuel.

No correlation between introduced and residual amount of hydrocarbons were determined after long-term exposure, which might suggest that biodiesel addition had neither stimulating nor inhibiting effect on hydrocarbon biodegradation. However, it is highly probable that in short-term period this observation would be different. According to Yassine et al. (2013), FAMEs enhanced the mineralization rates of both aliphatic (C_{10}-C_{21}) and aromatic (toluene, *o*-xylene, tetraline) hydrocarbons in acclimated activated sludge within 7 days. Such observation was explained by better solubilization of hydrocarbons in the presence of FAMEs. But it was also shown that biodiesel was a better growth substrate than diesel (Bücker et al., 2011; Owsianiak et al., 2009a), and thus FAMEs were able to increase the degradation rates of *n*-alkanes by enhancing beforehand the biomass growth (Yassine et al., 2013).

The microbial community analysis revealed that after 64.5 weeks exposure to different diesel/biodiesel blends, the bacterial profiles changed in comparison to untreated soil. The observation provided by Szczepaniak et al. (2016) indicated no significant differences in soil microbiome after 3 months of PAHs degradation in relation to uncontaminated soil. Although in our study the bacterial community structure returned partially to their initial composition, the significant increase in contribution of *Actinobacteria* and *Sphingobacteriia* were determined. Both classes are well-known hydrocarbon degraders (Isaac et al., 2015; Janbandhu and Fulekar, 2011; Lisiecki et al., 2014). *Actinobacteria* is widely described to be able to degrade aliphatic and aromatic hydrocarbons in both aquatic and soil environments (De Pasquale et al., 2012; Isaac et al., 2015), while Sydow et al. (2016) clearly showed that *Sphingobacterium* spp. can be *n*-alkane-degrading specialists. Previous studies have reported that fatty acids from FAMEs revealed structural and metabolic similarities with *n*-alkanes and their metabolites of biological oxidation (alcohols, aldehydes and acids) (Fulco, 1983; Lisiecki et al., 2014; Wentzel et al., 2007; Yassine et al., 2013). Thus, it was expected that *n*-

alkane-degraders able also to successfully degrade FAMEs will appear. Moreover, Lisiecki et al. (2014) determined that there was neither inhibiting nor stimulating effect of different FAMEs content on *Sphingobacterium* during degradation of broad range of diesel/biodiesel blends in sand microcosms. On the other hand, several studies demonstrated that the increased growth of *Gammaproteobacteria* was stimulated by the presence of biodiesel (Cyplik et al., 2011; Lisiecki et al., 2014; Sydow et al., 2016). Although we did not observe an increased abundance in the *Gammaproteobacteria* in the presence of pure biodiesel, the significant decrease for members of this class was observed with a decreased FAMEs content in urban soil. Furthermore, our results are also in agreement with those reported by Cyplik et al. (2011), who presented the suppression effect of biodiesel on the abundance of *Betaproteobacteria*. Here *Betaproteobacteria* increased two-fold to its contribution when urban soil was spiked with pure diesel. Lors et al. (2012) found that in soil polluted by coal tar, *Betaproteobacteria* appeared in bacterial community after three months when concentrations of PAHs were non-toxic and low enough to maintain such conditions. They suggested that *Betaproteobacteria* taxa could act as a bio-indicator for the endpoint of the bioremediation processes. Therefore, more work is needed to determine the influence of diesel/biodiesel blends on bacterial community in field conditions as limitation in carbon source and nutrients availability may play a critical role in community structure changes.

4.2. Influence of bioaugmentation approach on diesel/biodiesel blends

The concept of inoculating the hydrocarbon-polluted areas with fast-degrading microorganisms in order to increase the biodegradation rate and reduce the time to enhance the bioremediation efficiency has been developed for many years (Gentry et al., 2004; Mukherjee and Bordoloi, 2011; Szulc et al., 2014). In previous studies, single strains, mixed cultures or consortia were used as inocula (Cerqueira et al., 2011; Junior et al., 2009; Rahman

et al., 2002). Tyagi et al. (2011) suggested that strategies involving the use of microbial consortia, rather than a single culture, is more beneficial for bioremediation as it provides biodiversity and robustness, as is depictive for the real environment. Following this assumption we used a hydrocarbon-degrading bacterial community isolated from oil-contaminated soil, as we determined a high biodegradation potential.

The biodegradation kinetics presented the intensive activity only within first 28 days (short-term phase), while during long-term phase (33-109 days) no enhancement in mineralization rates compared with non-augmented microcosms were determined. This finding suggested that the microbial community had a positive effect on biodegradation of diesel/biodiesel blends only after inoculation, while over time the efficiency of bioaugmentation had decreased. Our results are in accordance with Szczepaniak et al. (2016), who determined that the bioaugmentation of soil contaminated with PAHs was successful only during the early stage of treatment, while after a few months the bacterial community composition returned to the previous conditions. In the present study, after 64.5 weeks the bacterial profile of diesel/biodiesel-contaminated soil, when augmented with bacterial community, was found to be comparable to non-augmented samples. One possible explanation is that the microbial community did not adapt sufficiently to survive this long-term exposure. Goldstein et al. (1985) described that possible failure of bioaugmentation might be justified by low growth rates of supplemented microorganisms in relation to indigenous microorganisms, when in soil microcosms various easy available carbon sources were presented. Prior studies emphasized also the significant importance of interaction between inoculated and autochthonous microorganisms in terms of their viability, activity and proliferation (El Fantroussi and Agathos, 2005; Goldstein et al., 1985; Thompson et al., 2005), indicating that supplementation of contaminated site with autochthonous microorganisms is more beneficial in long-term degradation of pollutants. Within this work, the applied bacterial community was

non-indigenous microorganisms, isolated from different environmental conditions. Hence, this might be the reason why the bioaugmentation was diminished after some time. However, the procedure using non-autochthons fast degraders has been already successfully applied in previous studies (Junior et al., 2009; Stella et al., 2017; Teng et al., 2010).

On the other hand, Johnsen et al. (2007) determined that priming the PAH-polluted soil by adding as inoculum bioremediated soil with a high hydrocarbon degradation potential resulted in the increase even up to 1,000 times the number of cultivable PAH-degraders. This means that the soil-adapted community has demonstrated the high survival rate, persistence and proliferation in PAH-contaminated soil during the experiment lasting 16 weeks. Although, the introduction to hydrocarbon polluted microcosms soil-adapted degraders seems to be beneficial, such treatment had no significant effect on hydrocarbon degradation, which accords with our observations. The higher degradation rates of phenanthrene, fluoranthen and pyrene were determined only within few weeks after inoculation, in the end the degradation rates of primed and not primed microcosms were comparable. Recent studies have described the significant impact of soil matrices on biodegradation success (Bento et al., 2005; Horemans et al., 2016). This issue was described by Horemans et al. (2016), who determined the biodegradation potential of phenanthrene-degrading bacterial on twenty uncontaminated, sterile soils with various physico-chemical characteristics. The authors revealed that there were differences in the extent of phenanthrene degradation, and that this was dependent on the soil properties. Although, to simplify the models, they did not consider the influence of biotic factors, which might strongly affect activity and survival of supplemented microorganisms; they hence developed a three-step tool for predicting the bioaugmentation success. Based on models described in their study, the soil used within the framework of this research was classified as soil with potential to survival with medium degrading activity of bioaugmented strain. However, in terms of our soil, the authors recommended the bioaugmentation together

with biostimulation as a good and effective biodegradation strategy. Therefore, the effectiveness of bioaugmentation approach of diesel/biodiesel contaminated site depend on both selection of appropriate microorganisms treatment and compatible soil to successfully enhance the chances of bioaugmentation in urban microcosms.

5. Conclusions and practical implications

The present study demonstrated that after long-term exposure (64.5 weeks), the mineralization extent of different diesel/biodiesel blends in urban soil does not depend on biodiesel concentration in fuel. This finding suggests that giving sufficient time for biodegradation of such blends from soil might be an effective bioremediation strategy. However, the addition of biodiesel to conventional diesel fuel increases the biodegradation kinetics. Thus, during short periods of time diesel/biodiesel blending higher than 30% seems to be beneficial for bioremediation of petroleum mixtures spills. This study has shown that bioaugmentation can potentially be effective only during the early stages of treatment, whereas after long-term exposure no differences in mineralization extent and bacterial community structure between augmented and non-augmented microcosms occur. It would therefore seem that a beneficial approach in our long-term treatment would be to use successive bioaugmentation. Corroborating this, Colla et al. (2014) suggested that successive bioaugmentation was an effective strategy in bioremediation of soil polluted with diesel/biodiesel blends. Several studies (Łebkowska et al., 2011; Tahhan et al., 2011) demonstrated that multiple inoculation of hydrocarbon-contaminated soil with autochthonous and non-autochthonous microorganisms revealed satisfactory results, and such approaches could be applied as a powerful tool in bioremediation. Moreover, according to Tahhan et al. (2011), additional supplementation of bacterial consortium into soil during petroleum hydrocarbons degradation significantly improved the removal of aromatic and asphaltic fractions, whose biodegradation

is usually much slower. Collectively, our findings suggest that single bioaugmentation treatment might not be enough to significantly accelerate the removal of hydrocarbon contaminations from urban soil matrix. Therefore, in order to enhance biodegradation, when time is not a limiting factor, the use of bioaugmentation approach may not be an adequate and justifiable solution.

Acknowledgment

The research work was funded by the National Science Centre in Poland in the years 2014-2018 with the research project Opus no 2013/11/B/NZ9/01908.

Supporting Information. Fig. S1 – Effect of the amount of biodiesel in blends on the residual of total diesel/biodiesel blends and hydrocarbons fractions; Fig. S2 - Ratio of saturated to unsaturated fraction of diesel residues.

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Figure and table captions:

Fig. 1. Mineralization extent of diesel (D) and diesel/biodiesel blends (B10-B100) in urban soil microcosms without bioaugmentation (1A, 1B - mineralization within first 28 days) and with bioaugmentation (2A, 2B - mineralization within first 28 days). Error bars represents confidence intervals for $p = 0.05$.

Fig. 2. Relative abundance of the most dominant microbial phyla (A) and classes (B) inhabiting soil (control) and soil spike with diesel/biodiesel blends with autochthonic microcosms (B100, B20, D) versus autochthonic microcosms bioaugmented with specialized bacterial community BC125 (B100+, B20+, D+).

Table 1. Mineralization extent and rate constants for different fuels and biodegradation conditions (augmented vs, non-augmented).

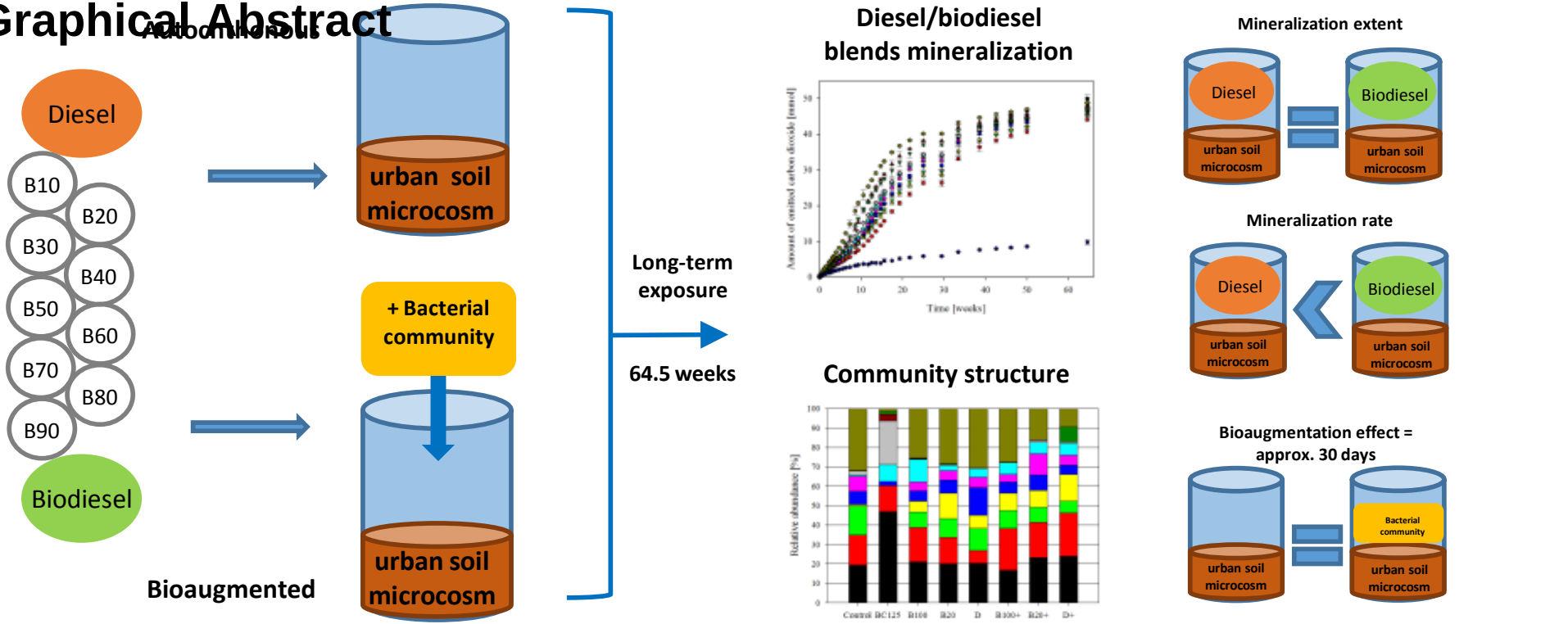
Supplementary materials:

Fig. S1. Effect of the amount of biodiesel in blends on the residual of total diesel/biodiesel blends (●), total hydrocarbons (○), aliphatic hydrocarbons (Δ), aromatic hydrocarbons (■) and FAME (▼) after 64.5 weeks without (A) and with (B) bioaugmentation. m/m_0 express the residual content of different fractions to their initial masses. The error bars are omitted, in order to make figure more clear and legible.

Fig. S2. Ratio of saturated to unsaturated fraction of diesel residues in soil matrix after 64.5 weeks without (A) and with (B) bioaugmentation. The error bars represent standard error of

889 the mean (n=3). As a reference the ratio of saturated to unsaturated fraction of fresh diesel
890 fuel was determined.

*Graphical Abstract



***Highlights (for review : 3 to 5 bullet points (maximum 85 characters including spaces per bullet point)**

- Long-term (64.5 weeks) biodegradation of diesel/biodiesel in urban soil was studied
- 3-12% of the total aromatic and aliphatic hydrocarbons remained in the microcosms
- Effect of bioaugmentation was evaluated
- MiSeq sequencing analysis revealed a significant effect of blend type
- No significant influence of bioaugmentation was determined in the long-term

Abstract

We studied long-term (64.5 weeks) biodegradation of diesel fuel, diesel/biodiesel blends (B10-B90) and biodiesel fuels in urban soil microcosms containing indigenous microorganisms, or indigenous microorganisms augmented with a hydrocarbon-degrading bacterial community. Mineralization extent (mmol of CO_2 per day) of B10-B30 blends was smaller compared with diesel fuel at both short- (28 days) and long-term (109 days), and increased with biodiesel content. Priming with hydrocarbon degraders accelerated mineralization in the short-term (by up to 140%), with highest influence using blends with lower biodiesel content, but did not significantly influence kinetics and mineralization extent in the long-term. Although the biodiesel fraction was degraded completely within 64.5 weeks, 3-12% of the total aromatic and aliphatic hydrocarbons remained in the microcosms. Barcoded 16S rRNA gene MiSeq sequencing analysis revealed a significant effect of blend type on the community structure, with a marked enrichment of *Sphingobacteriia* and *Actinobacteria* classes. However, no significant influence was determined in the long-term, suggesting that the inoculated bacterial community may not have survived. Our findings show that biodiesel is preferentially degraded in urban soil and suggest that the value of bioaugmentation for bioremediating biodiesel fuels with hydrocarbon-degrading bacteria is limited to short-term exposures to lower (B10-B30) blends.

Keywords

Bacterial community, fuel blends, hydrocarbons, mineralization, MiSeq sequencing

1. Introduction

Petroleum diesel fuel is often blended with biodiesel [fatty acid methyl esters (FAMEs)] before being introduced to the market (Luque et al., 2010). Biodiesel mixed with petroleum diesel fuel can be used in unmodified diesel engines in different proportions ranging from 2% to 20% depending on government policy (DeMello et al., 2007; Luque et al., 2010). In Germany, the pure biodiesel is available and used in transportation without being taxed (Demırbas, 2017). However, in the rest of the European Union, the addition of biodiesel to conventional fuel is approximately 5% (Bücker et al., 2011; Schleicher et al., 2009). This blending generally has a positive influence on biodegradation rates of fuel (Horel and Schiewer, 2011; Silva et al., 2012). Several studies have focused on the effect of biodiesel in accelerating the biodegradation in sediments and soils (Miller and Mudge, 1997; Taylor and Jones, 2001). Miller and Mudge (1997) reported the addition of biodiesel to enhance biodegradation of petroleum hydrocarbons in sediments contaminated with crude oil. This phenomenon is generally explained by the fact that the FAMEs are preferentially utilized by microorganisms over the petroleum hydrocarbons. For example, Horel and Schiewer (2011) measured that biodiesel stimulated microbial populations in sandy soil, thereby increasing biodegradation rates of the blends. This effect is usually explained by the structural similarities between FAMEs and *n*-alkanes, as well as similarities in their metabolic mechanisms (Yassine et al., 2013). DeMello et al. (2007) reported the degradation rate constants for FAMEs and *n*-alkanes in seawater were comparable. This corroborates with the study by Yassine et al. (2013) which described higher *n*-alkane degradation rates in biodiesel blends with acclimated microbial cultures as attributed to the ability of FAMEs to be co-solubilized with *n*-alkanes. Moreover, these studies emphasized that biodegradation of aromatic compounds was also affected by biodiesel blending. A key factor when considering the influence of biodiesel on biodegradation of diesel in soil is the ability of the former to act

as solubilizing agent (Fernández-Álvarez et al., 2007; Miller and Mudge, 1997). According to Fernández-Álvarez et al. (2007), among the different bioremediation agents (microorganisms, nutrients and biodiesel) that can be used, only biodiesel has been shown to accelerate the biodegradation of both aliphatic and aromatic fractions of heavy fuel oil. On the other hand, Mariano et al. (2008) observed no effect of biodiesel on diesel biodegradation in soil and water in an experiment lasting over 120 days. Leme et al. (2012) showed the mutagenic and genotoxic effects of biodiesel and its diesel blends in soil matrix, emphasizing the potential harmful effects of biodiesel. However, there remains a paucity of knowledge regarding the long-term influence of biodiesel on the biodegradation of different hydrocarbon fractions in diesel/biodiesel blends in complex soil matrix.

The use of isolated microbial communities, consortia or specific populations of microorganisms (El Fantroussi and Agathos, 2005) for the *in situ* treatment of polluted sites – also called bioaugmentation – has been considered a useful approach to increase bioremediation efficiency (Atashgahi et al., 2018; Di Gregorio et al., 2016; Lladó et al., 2012; Meyer et al., 2014). Positive results were described by Teng et al. (2010), who showed that addition of hydrocarbon-degrading strains enhanced the bioremediation of soil contaminated with polycyclic aromatic hydrocarbons (PAHs), while Szczepaniak et al. (2016) showed the effectiveness of using PAH-degrading consortia during the early stage of bioaugmentation treatment. Both studies highlighted the stimulatory effect of autochthonous microorganisms with the addition of exogenous hydrocarbon-degrading microorganisms over the short-term. However, there are also contradictory studies that reported either a negative or no effect by bioaugmentation (Bouchez et al., 2000; Saponaro et al., 2001; Silva et al., 2009). No significant effect on biodegradation of PAHs after fungal and bacterial consortia introduction into soil were observed by Silva et al. (2009). The study by Bouchez et al. (2000) indicated the difficulties in adaptation of augmented microorganisms to a well-adapted initial bacterial

population. According to El Fantroussi and Agathos (2005), bioaugmentation is still in the experimental phase with no general guidelines for how to efficiently introduce external microorganisms to treat a contaminated site. Recently, however, Horemans et al. (2016) presented a three-step approach emphasizing the importance of compatibility of microorganisms and soil selection to the success of bioaugmentation treatments. This was also mentioned by Bento et al. (2005), who showed that an effective bioaugmentation approach for treatment of diesel oil contaminated sites can depend on soil properties as well as indigenous soil microorganisms. Bioaugmentation treatments with bacteria (Meyer et al., 2014, 2012) and fungi (Junior et al., 2009) have been successfully applied for diesel/biodiesel blends, where the biodegradation of different blends were higher compared with non-bioaugmented set-ups. However, many studies concern the biodegradation of only a limited range of blends, such as B2, B5, B20 or B50 (Bücker et al., 2011; Meyer et al., 2014; Schleicher et al., 2009) or the experiments were conducted over short periods of 28, 60 or 84 days (Horel and Schiewer, 2011; Schleicher et al., 2009; Silva et al., 2012). Therefore, it is difficult to generalize about the effectiveness of bioaugmentation on degradation of wide range of diesel/biodiesel blends during long-term exposure, as well as due to the variability in soil types, their autochthonous microbial communities, and the experimental approaches performed across different laboratories.

Here, we examined the effects of biodiesel on the biodegradation of aliphatic and aromatic fractions in a wide range of diesel/biodiesel blends. Long-term biodegradation experiments were conducted in urban soil microcosms in two parallel variants: autochthonic microcosms *versus* autochthonic microcosms bioaugmented with a hydrocarbon-degrading community that was previously isolated from contaminated soil. The response of the autochthonic microbial community towards increasing biodiesel concentration, and that of the

exogenously-added hydrocarbon-degrading community, was analyzed by 16S rRNA gene sequencing using Illumina MiSeq technology.

2. Materials and Methods

2.1. Fuels

Diesel fuel (EN 590:2004), assigned as D was purchased from a petrol station (PKN Orlen, Poland). Biodiesel (assigned as B100) was produced from rapeseed oil (DIN V 51606) and obtained from PetroTec AG in Germany. In addition to these two types of fuels, nine diesel/biodiesel blends with increasing by 10% biodiesel content that is from 10 to 90% (v/v) (assigned B10, B20, B30, B40, B50, B50, B60 B70, B80, and B90) were prepared by batching in laboratory and mixing volumetric portions of diesel and biodiesel fuels. Two methyl ester of oleic acid (C18:1) and linoleic acid (C18:2) constituted a majority of 68% and 21% of the biodiesel respectively, while the remaining 11% consisted of methyl esters of C16:0, C18:0, C20:0 and C20:1 (Lisiecki et al., 2014).

2.2. Microorganisms

The bacterial community that was used in this study – designated BC125 – was isolated from crude oil-contaminated soil (Gorlice, Małopolska, Poland). The selectively enriched community was maintained using only mineral medium with diesel fuel as a sole carbon and energy source. Metagenomic analysis of V4 hypervariable region of the 16S rRNA gene identified 22 classes. The most dominant microbial classes detected in BC125 were *Alphaproteobacteria* (47.85%), followed by *Bacilli* (22.71%), *Gammaproteobacteria* (13.31%), *Actinobacteria* (8.58%), *Clostridia* (3.37%), *Betaproteobacteria* (2.08%) and *Flavobacteriia* (1.36%). The community was tested with respect to structural and functional robustness when exposed to different hydrocarbons according to the report provided by

Sydow et al. (2016). It was proved to maintain both structural and functional integrity when exposed to various aliphatic, cyclic and aromatic hydrocarbons. The bacterial community was able to efficiently degrade hydrocarbons in a pH range of 6.5-7.5.

The BC125 was stored as glycerol stocks (20% v/v) at -80°C until used. A 1 ml of stock suspension was transferred to Erlenmeyer flask (300 mL, SIMAX, Sazava, Czech Republic) with 50 mL of mineral medium supplemented with 0.5% (v/v) diesel fuel as described in Sydow et al. (2016). The culture was incubated with shaking (120 rpm; 25 °C, Multitron; Infors HT, Bottmingen, Switzerland) for 24 h. Subsequently, the cell suspension (1 mL) was transferred into fresh mineral medium (50 mL) and cultivated for 72 h in conditions described above. The final enrichment culture was obtained after three transfers. The fresh pre-culture (50 mL) for mineralization experiments were washed three times in sterile NaCl (0.85% v/v) and subsequently incubated on mineral medium (500 mL) with 0.5% (v/v) diesel fuel as described in Sydow et al. (2016). The BC125 was incubated (120 rpm; 25°C) for to 48 h. When optical density (OD_{600}) of the pre-culture reached approximately 3.0 ± 0.1, the cell suspension was centrifuged (10,000 *g*; 4°C; 15 min, Heraeus Multifuge 3S-R, Hanau, Germany) and washed three times with mineral medium. The resuspended cells in medium served as inoculum for subsequent experiments.

2.3. Characterization of soil

Mollic gley soil used in this study was collected from a city park in Poznan, Poland (N 52.4011445, E 16.9222993) and previously characterized in Sydow et al. (2015). Briefly, the soil samples were taken from the depth of 10-20 cm and sieved (2.0 mm). The soil was characterized as fine-grained silt loam type OL (United Soil Classification System). The detailed composition of soil was as follows: clay, 4 ± 1 [%]; silt, 83 ± 3 [%]; sand, 13 ± 2 [%]. The characteristics of the soil were as follows: organic carbon 5.44 ± 0.31 [g kg^{-1}]; nitrogen

147 0.57 ± 0.07 [g kg^{-1}]; phosphorous 0.080 ± 0.005 [g kg^{-1}]; pH 6.95 ± 0.7; bulk density 1.41 ±
148 0.06 [Mg/m^3]; porosity 0.455 ± 0.03 [m^3/m^3]; moisture during sampling 18 ± 1 [%]; cation
149 exchange capacity 22.1 ± 0.8 [cmolc kg^{-1}]. A symbol ± represents standard deviation from
150 three independent replicates.
151
152 **2.4. Microcosms and mineralization measurements**
153 To evaluate the mineralization extent of diesel (D) and biodiesel blends (B10-B100), 50 g of
154 soil was placed in sterile pre-weighed 1000 mL SIMAX bottles (SIMAX, Sazava, Czech
155 Republic). Subsequently, fuels (0.75 mL of D or B10-B100) were spiked on the soil surface.
156 The bottles were weighed again to determine the exact amount of fuels added to each bottle,
157 which was essential for further analytical protocols (0.1 mg accuracy). Average concentration
158 of D and B10-B100 fuel was 12 g/kg soil (approx. 1% v/w, a level at which biological
159 treatment is typically feasible). Each experimental setup was performed in triplicates, thus
160 overall 33 samples with diesel/biodiesel blends were prepared. Another 33 samples with
161 microcosms (50 g of soil) were first spiked with diesel/biodiesel blends as described above
162 and then augmented with BC125 suspension (1 mL; with final concentration 2×10^8 CFU g^{-1})
163 – further assigned as D+, B10+, B20+ etc. The non-augmented samples were amended with 1
164 mL of sterile mineral medium to maintain the soil field capacity at 85% v/v in all microcosms
165 (augmented and non-augmented samples). Additionally, three biotic, non-spiked soil controls,
166 three non-spiked, augmented with active BC125 soil controls and three non-spiked,
167 augmented with killed inoculum (autoclaved immediately before inoculation) controls were
168 also prepared. All samples were gently mixed and finally, all microcosms were incubated at
169 20°C for 64.5 weeks.
170 The mineralization extent of fuels was assessed by measurements of CO_2 trapped in the base
171 trap (10 mL of 0.75 M NaOH in a 20-mL vial), and placed in each microcosm as described in

Szulc et al. (2014). Titration with 0.1 M HCl of diluted NaOH and Na_2CO_3 solution from the trap, according to Warder method, was carried out with the use of automatic titrator (Metrohm titroprocessor 686, Herisau, Switzerland). After each measurement the content of the base trap was replaced with fresh NaOH solution. The samples were measured in different time intervals: every 1-3 days (I month), once to twice a week (II-III month), every two weeks (IV-V month), once a month (VI-XII month), and the last measurements were performed 102 days after the penultimate measurement was taken (day 452).

2.5. Hydrocarbon and FAME analyses

After 64.5 weeks, the microcosms (three replicates for each setup) were sacrificed and the residual hydrocarbons and FAME were determined. Briefly, after removal of base traps, 12.5 mL of acetone was added into each bottle and the samples were vortexed for 1 min (Vortex-Genie 2 Shake, Scientific Industries, New York, US). Subsequently, 5 g of anhydrous $MgSO_4$ was added and the samples were vortexed again. Next, 7.5 mL portion of *n*-hexane was added and vortexed for another 1 min. The bottles were sonicated for 20 min in order to promote desorption of the analytes from solid matrix. The samples were shaken vigorously (Multitron; Infors HT, Bottmingen, Switzerland) after the first 10 min to homogenize soil sticking on the bottom of the flask. The samples were then shaken on a horizontal shaker (250 rpm; 15 min). Subsequently, the obtained extract (1 mL) was washed with 0.1 M NaOH (3 mL) to remove acetone and co-extracted acidic interferences and the upper phase further processed. One fraction of the extract was taken and cleaned on a Florisil column (Sigma Aldrich, St. Louis, US) for total hydrocarbon and FAME analysis; another fraction was also taken, but this time cleaned and fractionated on a Ag-impregnated silca gel column (Merck, Darmstadt, Germany) into saturated (aliphatic) and non-saturated (aromatic and FAME) fraction as described by Lisiecki et al. (2014). The resultant hydrocarbon fractions (aliphatic and aromatic) were

finally determined with gas chromatography (GC-FID and GC×GC-TOF-MS, Agilent, Palo Alto, US) according to the procedures described elsewhere (Lisiecki et al., 2014). The results were presented as a ratio of remaining to initial masses of each fraction (total diesel/biodiesel blends, total hydrocarbons, aliphatic hydrocarbons, aromatic hydrocarbons and FAME). The presented error bars for the GC analysis results represent confidence intervals for $p = 0.05$.

2.6. Evaluation of bacterial community structure in the soil

The influence on qualitative and quantitative composition of microbial community samples was assessed using Illumina MiSeq sequencing (Illumina, San Diego, US). Here, Illumina genetic analysis was applied in order to investigate the potential changes in the bacterial community structure due to biodiesel content as well as bioaugmentation treatment. The contribution of most abundant microbial phyla and classes were presented as % of total taxonomic rank.

Two additional samples of each treatment were setup for Illumina MiSeq sequencing, as described in section 2.4 above. After termination of the soil experiments, approximately 20 g of soil from central area of each experimental microcosm (ten random samples from depth of approx. 10 cm) were collected and homogenized. The subsamples were divided into three equal portions and then stored at -80 °C until used (no more than two weeks). Extraction of DNA and PCR amplification using universal primers were performed according to the procedure provided by Ławniczak et al. (2016) and Szczepaniak et al. (2016). Briefly, the isolation of the genetic material from analyzed samples was performed using appropriate Genomic Mini AX kits (A&A Biotechnology, Gdynia, Poland), as recommended by the manufacturer. The validation of isolation efficiency was conducted with a fluorometric method by means of a Qubit™ dsDNA HS Assay Kit and Qbit 2.0 apparatus (ThermoFisher Scientific, Waltham, US). For PCR amplification and sequencing the universal prokaryote

primers 515F-806R were applied to amplify the V4 region of the 16S rRNA gene (Caporaso et al., 2012). The PCR reaction (25 µl) contained the following: 5 µl microbial template genomic DNA, 5 µl of each primer, 2.5 µl of PCR-grade water (ThermoFisher Scientific, Waltham, US) and 12.5 µl of PCR Master Mix with the Taq polymerase (ThermoFisher Scientific, Waltham, US). The thermocycler (ThermoFisher Scientific, Waltham, US) program was employed with initial denaturation at 95°C for 3 min, followed by 35 cycles of 95°C for 1 min, 52°C for 30s, 72°C for 1 min and final extension at 72°C for 10 min. The amplicons were purified on Clean-Up columns (A&A Biotechnology) and used for library construction. Sequencing was carried out with a MiSeq Reagent Kit v2 (2x250 bp) using a MiSeq (Illumina) platform. Details concerning the preparation of libraries were presented in our previous study (Szczepaniak et al., 2016). After sequencing, the raw data in FASTQ format were imported to the CLC Genomics Workbench 8.5 software with the CLC Microbial Genomics Module 1.2 (CLCbio, Qiagen Bioinformatics, Aarhus, Denmark). The reads were demultiplexed, and paired ends were merged (mismatch cost = 2, min score = 8, Gap cost = 3, max unaligned end mismatches = 5). Primer sequences were trimmed (quality limit = 0.05, ambiguous limit = 'N'), and the identification and elimination of chimeric reads was performed. The output data were clustered independently based on two reference databases, namely SILVA v119 (Quast et al., 2013) and GreenGenes 13.5 (DeSantis et al., 2006) at a 97% probability level of OTUs (operational taxonomic units). The alpha-biodiversity (number of OTUs) factor was determined based on the merged abundance table (clustered against SILVA v119). The final sequencing datasets generated and analyzed within the framework of this study are available in the SRA repository, with the identifier SRP156685 (https://www.ncbi.nlm.nih.gov/sra/SRP156685).

Overall, we selected three microcosms supplemented with D, B20 and B100 non-augmented and augmented treatments (D+, B20+, B100+). B20 has received significant attention and is

one of the most commonly investigated biodiesel blend (Cyplik et al., 2011; Demirbas, 2007; Junior et al., 2009; Meyer et al., 2012; Silva et al., 2012). According to our study, mineralization extent in B20 blend microcosms presented the most unexpected pattern and therefore this microcosm was selected for further genetic analysis.

It should be noted that the results of the Illumina MiSeq sequencing may be limited by the lack of replicates of sequencing data. This prevented the possibility to employ a multivariate statistical analysis and evaluate the statistical significance of the observed differences. In consequence, it was not possible to assess the trends of microbial community shifts at a statistical level. The highlighted issue may be of particular importance in case of complex terrestrial matrices, in case of which the isolation of DNA is challenging. In the framework of this study the data obtained based on Illumina MiSeq sequencing was primarily used to evaluate the efficiency of the bioaugmentation process. Additionally, an attempt to elucidate the "key players" which participate in the biodegradation of various diesel/biodiesel blends.

2.7. Mineralization kinetics and statistical analysis

As the experiment proceeded, it was observed that the curves expressing the increase of cumulative CO_2 evolution were neither linear nor logarithmic. Hence, for a matter of simplicity, two sections (namely from day 0 until day 28, as a beginning of the experiment, and from day 33 to day 109, as the most intensive period), where mineralization curves were approximately linear ($R^2 \geq 0.95$), were selected for further analysis. Subsequently, zero-order kinetics model was applied to describe and compare the kinetics of organic matter mineralization (associated mainly with the fuels additions), between the investigated experimental setups. Similar approaches to characterizing mineralization kinetics in porous media were presented previously (Dechesne et al., 2010; Owsianiak et al., 2010). The one-way ANOVA with $p < 0.05$ were used for statistical comparisons. This approach was also

employed for statistical analysis of metagenomic data in order to establish the significance of differences for untreated vs non-bioaugmented and non-bioaugmented vs bioaugmented systems.

3. Results

3.1. Evolution of CO_2 and mineralization kinetics

Mineralization extent of the different fuel blends was measured as amount of CO_2 released in the microcosms (corrected for the background, substrate-unamended control), as summarized in Table 1 and Fig. 1. In non-augmented microcosms, mineralization extent increased with increasing biodiesel content, and ranged from 44.1 ± 2.3 for B10 to 48.8 ± 2.4 mmol CO_2 for B100 (Table 1). For diesel, mineralization extent was the highest and equal to 49.9 ± 3.8 mmol. The evolution of CO_2 in all samples differed significantly from that in the controls (9.7 ± 1.1 mmol) without any fuel addition (Fig. 1). In bioaugmented microcosms, the mineralization extent did not increase with increasing biodiesel content as in non-augmented samples. The highest CO_2 evolution were observed for B20 (48.5 ± 3.1 mmol), while the lowest for B50 (42.9 ± 2.1 mmol). However, there were no statistically significant differences between the mineralization extent of non-augmented and augmented diesel/biodiesel blends, apart from pure diesel microcosms ($p = 0.047$).

Regression performed on non-augmented and augmented mineralization curves presented the influence of biodiesel content on mineralization extent during short- (days 0-28) and long-term (days 33-109) mineralization phases (Table 1). Linear regressions applied on the mineralization curves for non-augmented samples revealed that mineralization rate constants were higher for higher biodiesel blends. This was generally true for both mineralization phases. However, it is worth noticing that the mineralization rate constants of non-augmented B10-B30 microcosms were lower than of microcosms spiked with pure diesel (D) in both

297 phases. On the other hand, regressions for augmented samples showed that mineralization rate
298 constants were higher in the short-term mineralization phase compared with non-augmented
299 samples (apart from D+, B80+ and B100+). In the long-term phase, however, the opposite
300 was observed. There were statistically significant differences in rate constants during short-
301 term mineralization phase of non-augmented and augmented samples for lower biodiesel
302 blends from B10 to B60 ($p < 0.05$), while in long-term phases the significant differences were
303 observed only for B40 ($p = 0.046$) and B50 ($p = 0.041$).
304
305 **3.2. Fate of hydrocarbons and FAME**
306 Based on GC-FID and GC×GC-TOF-MS studies after 64.5 weeks, biodiesel was completely
307 degraded in all diesel/biodiesel blends (Fig. S1. Supporting Information). Depending on the
308 blends, the total petroleum hydrocarbon residues ranged from 3 to 12% of the introduced
309 hydrocarbon fractions in samples without bioaugmentation, and from 4 to 8% in samples with
310 bacterial augmentation. After 64.5 weeks, there were no statistical differences between blends
311 in case of total hydrocarbon residues ($p > 0.05$) in non-augmented and augmented treatments.
312 No clear effect of the type of blend on ratio of remaining to initial masses of hydrocarbon
313 fractions (aliphatic and aromatic factions) was observed, apart from B80-B90 blends where
314 the increase in this ratio were determined. Moreover, the ratio of residual aromatic to aliphatic
315 fraction at the end of the experiment remained unchanged for all treatments (Fig. S2.
316 Supporting Information).
317
318 **3.3. Bacterial community structure in non-augmented and augmented soil**
319 Figure 2A shows the contribution of ten most abundant bacterial phyla in bacterial community
320 (BC125), untreated soil sample (control) and microcosms supplemented with different fuels
321 without (B100, B20, D) and with (B100+, B20+, D+) bioaugmentation treatment.

The most dominant microbial phyla detected in untreated urban soil (Fig. 2A control) were *Proteobacteria* (45.64%), followed by *Planctomycetes* (15.41%), *Clostridia* (10.11%), *Chloroflexi* (12.63%), *Acidobacteria* (8.78%) and *Actinobacteria* (5.54%). The rest of the identified microbial taxa were estimated below 5% of total detected taxonomic ranks ($p = 0.011$). The microbial community structure changed between the treatments (i.e. controls vs treatments with B100, B20 and D soil samples) after 64.5 weeks exposure. The relative abundance of *Bacteroidetes* increased in case of samples spiked with B100, B20 and D by 5, 12 and 6% respectively. The increase in abundance of *Actinobacteria* was also observed for soils supplemented with fuels (B100 by 8%, B20 by 2% and D by 3%). On the other hand the contribution of *Planctomycetes* deceased in each B100, B20 and D spiked soils by 7, 5 and 3%, respectively, while the contribution of both *Chloroflexi* and *Acidobacteria* deceased by 1-3% depending on the fuel. No changes were determined for *Proteobacteria*, the most abundant phylum ($p = 0.123$). The supplementation of urban soil with different fuel and oil-degrading bacteria (B100+, B20+, D+) did not affect significantly the composition of their bacterial community structure compared with non-augmented samples (B100, B20, D) ($p = 0.094$). However, the relative abundance of *Proteobacteria* increased by 7 and 8% for B20+ and D+ with reference to samples without bioaugmentation treatments. The highest increase (by 15%) was observed for *Bacteroidetes* in soil supplemented with pure diesel (D+), even though the abundance of *Bacteroidetes* decreased by 5% in B20+ samples. The contribution of *Planctomycetes* increased by 2% for B100+, while for B20+ and D+ the contribution decreased by 2 and 6%, respectively. The abundance of *Actinobacteria* and *Chloroflexi* deceased with the increased amount of diesel fuel (even by 7% depending on phylum).

Figure 2B shows the ten most abundant bacterial classes in non-augmented (B100, B20, D) and augmented (B100+, B20+, D+) soil spiked with appropriate fuels. The most dominant microbial classes detected in the untreated soil (control) were *Alphaproteobacteria* (19.41%),

Gammaproteobacteria (15.45%), *Planctomycetacia* (14.91%), *Acidobacteria* (7.69%) and *Betaproteobacteria* (6.84%). All other classes that were identified represented <5% of total identified taxonomic ranks ($p = 0.018$). These results revealed that both *Sphingobacteriia* and *Actinobacteria* increased their relative abundance in all samples supplemented with B100, B20 and D by 5, 12, 6% and 11, 2, 4%, respectively. Notably, the contribution of both classes did not exceed 1% in untreated soil sample ($p = 0.016$). The increase of the abundance of *Sphingobacteria* was caused by the increased ratio of bacteria belonging to the *Chitinophagaceae* genus in this class. This genus was predominant and its ratio exceeded 95% in this class. In turn, the increased ratio of bacteria belonging to the *Actinobacteria* class was caused by the increased abundance of the following genera: *Arthrobacter*, the increase of which was particularly high in case of addition of biodiesel, and *Corynebacteriales*. A decrease of bacteria belonging to the *Gaiellales* genus was also observed in this class, for which the contaminants introduced into soil were toxic. The ratio of this genus in the *Actinobacteria* class decreased from 52% (control soil) to 2-7% in contaminated soil samples. The relative abundance of *Gammaproteobacteria* increased by 2% for B100, while that in the B20 and D treatments decreased by 2 and 9%, respectively. The following bacterial genera were predominant in the *Gammaproteobacteria* class: *Aquicella* (46%), *Arenimonas* (15%), *Lysobacter* (15%) and *Thermomonas* (7.4%). The ratio of *Aquicella* and *Thermomonas* did not change in case of soils supplemented with diesel, however the abundance of *Arenimonas* and *Lysobacter* decreased significantly to 2.7 and 2.4%, respectively. In case of samples supplemented with biodiesel (B20 and B100) a notable decrease of all the above-mentioned genera was observed. Changes were also noted in case of the *Pseudomonas* genus, the ratio of which in control soil amounted to 0.35%. The addition of B100 caused a significant increase to 40%, which decreased in case of B20 (27%) and diesel (2.7%). A significant increase (by 7%) of the *Betaproteobacteria* for samples spiked with pure diesel was detected. In case of

the *Betaproteobacteria* class, the following genera were predominant in control soil: *Acidovorax* (47%), *Noviherbaspirillum* (21%) and *Ralstonia* (2.8%). In the sample supplemented with diesel (D), the abundance of *Acidovorax* did not change, whereas the ratio of *Noviherbaspirillum* and *Ralstonia* increased to 30 and 7.6%, respectively. On the other hand, the decrease in abundance of *Planctomycetacia* (by 7% for B100, 5% for B20, and 3% for D) and *Acidobacteria* (by 3% for B100, 3% for B20, and 2% for D) was also observed. No significant changes were estimated in the most abundant class, *Alphaproteobacteria* ($p = 0.131$).

Within bacterial classes, the differences between non-augmented and augmented samples were more visible, however still bioaugmentation treatment did not affect significantly the community structures ($p = 0.097$). Similar to non-augmented soil, the increase in abundance of *Sphingobacteriia* (by 9% for B100+, 9% B20+ and 14% D+) and *Actinobacteria* (by 6% for B100+, B20+, D+) were determined with reference to untreated soil (control). The increased ratio of bacteria belonging to the *Sphingobacteriia* class resulted from the increased abundance of uncultured bacteria belonging to the *Chitinophagaceae* family. These bacteria were part of the autochthonous population and were not present in BC125. In control soil, this genus comprised 50% of bacteria belonging to *Sphingobacteriia*, whereas in case of samples supplemented with diesel (D), B20 and B100 their abundance was equal to 55, 83 and 96%, respectively. The increased ratio of the *Actinobacteria* bacterial class was caused by the increase of the following genera: *Arthrobacter*, which was particularly predominant in case of biodiesel (58%), and *Cellulosimicrobium* (18%). In the framework of this class the decrease of bacteria belonging to the *Gaiellales* genus was observed, for which the contaminants were toxic. Its ratio in the *Actinobacteria* class decreased 52% (control soil) to 5.3% in samples supplemented with diesel oil. However, compared to soil without bioaugmentation, the highest increase (by 4, 5 and 16 % for B100+, B20+ and D+, respectively) were determined

for *Gammaproteobacteria*. It is worth noting that the contribution of *Gammaproteobacteria* in BC125 reached 13.31% (see Materials & Methods section, 2.2. Microorganisms). In contrast to samples without bioaugmentation, the *Pseudomonas* genus was predominant in the *Gammaproteobacteria* class. Its ratio in the soil microbiome was equal to 82% (D+), 62% (B20) or 29% (B100). Interestingly, its ratio in BC125 was low (equal to 0.6%). The ratio of genera *Aquicella*, *Arenimonas*, *Lysobacter* and *Thermomonas*, which were predominant in control soil, was notably decreased in samples supplemented with diesel (D+) or biodiesel (B20+ and B100+). The abundance of *Alphaproteobacteria* decreased by 4% for B100+, while for B20+ and D+ members of this class increased by 3 and 4%, respectively. *Sphingomonas* genus was predominant in the *Alphaproteobacteria* class. In BC125 it comprised 46% of all bacteria, and up to 92% of bacteria belonging to the *Alphaproteobacteria* class. In comparison with control soil (34% among *Alphaproteobacteria*) its ratio decreased to 24% (B100+), 11% (B20) or 7.4% (D), respectively. It should be highlighted that these changes were not significant ($p = 0.134$), considering that *Alphaproteobacteria* was the most abundant bacterial class in BC125 (46.85%). The increased abundance of *Acidobacteria* for B20+ (by 6%) was also identified. In case of *Acidobacteria*, all the changes of resulted from the increased abundance of uncultured bacteria belonging to Subgroup 4 and 6. However, the most visible changes were observed for soil (D+) spiked with pure diesel and BC125, where an increase in *Flavobacteriia* (by 8%) and a simultaneous decrease in *Planctomycetacia* (by 6%) and *Betaproteobacteria* (by 9%) compared with soil (D) without addition of bacterial community were determined. Changes in the *Flavobacteriia* class were caused by shifts of the abundance of bacteria belonging to the *Flavobacterium* genus. It can be assumed that this genus was introduced into the soil with the biopreparation, since its ratio in the control soil was below 0.01%. Furthermore, it did not occur in any sample of soil contaminated with hydrocarbons. It

is difficult to explain its high ratio. The decrease of *Planctomycetacia* in D+ soil relative to D soil was caused by the decreased ratio of the *Planctomycetaceae* family, particularly of uncultured genera belonging to this family. In case of *Betaproteobacteria*, The decreased ratio in D+ soil relative to D soil was associated with the decrease abundance of *Acidovorax* and *Noviherbaspirillum* families. No significant changes ($p = 0.119$) were observed for *Bacilli*, which was second most abundant class (22.71%) in BC125.

After 64.5 weeks, the alpha diversity estimates were also determined for untreated soil, BC125, autochthonic microcosms (B100, B20, D) and bioaugmented autochthonic microcosms (B100+, B20+, D+). The mean value of the observed OTU's for the untreated soil samples was equal to 2,268. The microcosms supplemented with B100 and B20 caused significant increase ($p < 0.05$) in the values of OTUs and reached 2,592 and 2,314; respectively. The enhancement was also established for the same microcosms supplemented with bacterial community, however no considerable differences between augmented and non-augmented samples were observed (B100+ = 2,516; B20+ = 2,363). For diesel treated soil with and without bacterial inoculation the mean values of observed OTUs were the lowest and did not differ significantly ($p > 0.05$) in comparison to untreated soil (D = 2,214; D+ = 2,219).

4. Discussion

4.1. Long-term mineralization of diesel/biodiesel blends in urban soil

Lisiecki et al. (2014) demonstrated that in porous matrices (sterile sand) the increase of biodiesel content in blends was positively correlated with an increase in their mineralization extent after 82.5 weeks. Here, the results showed that after long-term exposure the mineralization extents in urban soil with autochthonous microorganisms were similar and clearly not dependent on the amount of biodiesel in fuels. Many authors emphasized the tremendous adaptation capacity of autochthonous microorganism to harsh conditions

(Bouchez et al., 2000; Vogel, 1996), especially when the time is sufficient enough to fully adapt and consequently degrade exogenously added xenobiotics. According to Thompson et al. (2005), indigenous microorganisms are the most suitable candidates for slow and continuous degradation of pollutants during long-term exposure. Prior studies have also noted that the former oil contaminated soils are often the most promising source for isolation of efficient hydrocarbon-degrading bacteria (Owsianiak et al., 2009b; Rahman et al., 2002; Szczepaniak et al., 2016). Hence, in the soil from city park placed next to the main road, the presence of hydrocarbon-degrading community among autochthonous microorganisms was expected. Based on Illumina MiSeq sequencing more than one third of microbial classes abundance detected in the untreated soil belonged to *Alphaproteobacteria* and *Gammaproteobacteria*. Plethora of studies indicated that both *Alphaproteobacteria, Gammaproteobacteria* as well as *Bacilli* and *Actionbacteria* which were also the most dominant classes in bacterial community (BC125), are in fact well-known hydrocarbon degraders in soil and have been often enriched during biodegration of hydrocarbons (Fuentes et al., 2015; Marchand et al., 2017; Tiralerdpanich et al., 2018).

Although, the mineralization extent after long-term exposure was almost equal for each fuel, we revealed that the increase of biodiesel content in blends caused the enhancement of mineralization extent, especially at short- and long-term mineralization phases. The presence of FAMEs has been already reported to accelerate the biodegradation of diesel in experiments (up to 28 and 60 days) in different types of porous matrixes, such as sand soil (Horel and Schiewer, 2011), oxisol (Meyer et al., 2014) or soil from rain forest (Silva et al., 2012). Several studies emphasized that biodegradation of both FAMEs and *n*-alkanes undergo similar metabolism via β-oxidation mechanism (Lisiecki et al., 2014; Sydow et al., 2016; Yassine et al., 2013), thus the acceleration in mineralization in the presence of biodiesel might be expected. Our findings are consistent with Yassine et al. (2013), who suggested that this

was a result of co-solubilization mechanisms rather than cometabolism, for which the latter occurs mainly when one of the substrates is not readily biodegradable. The authors clearly determined that the ability of FAMEs to co-solubilize the *n*-alkanes is associated with reduction of interfacial surface tension and enhancement of their bioavailability for microorganisms. However, DeMello et al. (2007) presented that the acceleration of *n*-alkanes degradation in the presence of FAMEs in seawater microcosms took place only in early stage of the experiment. After longer time (53 days), the authors determined no effect of biodiesel on composition of the residual mixtures. They emphasized that the long period of time caused this lack of differences in terms of hydrocarbon composition between diesel and its biodiesel blends, which might be also explain our results. Mariano et al. (2008) also showed that in experiments lasting up to 120 days, no stimulation effect of FAMEs (B2, B5, B20) on diesel degradation in both soil from a petrol station and water samples were found. Taken collectively, it can be concluded that in short-term exposure, FAMEs is expected to increase the mineralization extents of different kinds of diesel/biodiesel blends, whereas in the long-term FAMEs had no visible influence on their mineralization extent.

Our study also revealed that the mineralization rate constants of B10-B30 blends in urban soil were lower than of diesel fuel (D) during short- and long-term exposure, while generally for higher diesel/biodiesel blends (above B30) the higher mineralization rates were determined. This is in accordance with Owsianiak et al. (2009a), who noticed that only the introduction into petroleum diesel above 30% of biodiesel contribute to the enhancement of biodegradation efficiency in aqueous media. No positive effect of low content of biodiesel (even up to B20) on diesel degradation were also observed in other study (Mariano et al., 2008). Thus, it might be concluded that the positive effect on the biodegration efficiency of diesel/biodiesel blends in soil microcosms can be expected only after exceeding a certain concentrations of biodiesel added to conventional fuel.

No correlation between introduced and residual amount of hydrocarbons were determined after long-term exposure, which might suggest that biodiesel addition had neither stimulating nor inhibiting effect on hydrocarbon biodegradation. However, it is highly probable that in short-term period this observation would be different. According to Yassine et al. (2013), FAMEs enhanced the mineralization rates of both aliphatic (C_{10}-C_{21}) and aromatic (toluene, *o*-xylene, tetraline) hydrocarbons in acclimated activated sludge within 7 days. Such observation was explained by better solubilization of hydrocarbons in the presence of FAMEs. But it was also shown that biodiesel was a better growth substrate than diesel (Bücker et al., 2011; Owsianiak et al., 2009a), and thus FAMEs were able to increase the degradation rates of *n*-alkanes by enhancing beforehand the biomass growth (Yassine et al., 2013).

The microbial community analysis revealed that after 64.5 weeks exposure to different diesel/biodiesel blends, the bacterial profiles changed in comparison to untreated soil. The observation provided by Szczepaniak et al. (2016) indicated no significant differences in soil microbiome after 3 months of PAHs degradation in relation to uncontaminated soil. Although in our study the bacterial community structure returned partially to their initial composition, the significant increase in contribution of *Actinobacteria* and *Sphingobacteriia* were determined. Both classes are well-known hydrocarbon degraders (Isaac et al., 2015; Janbandhu and Fulekar, 2011; Lisiecki et al., 2014). *Actinobacteria* is widely described to be able to degrade aliphatic and aromatic hydrocarbons in both aquatic and soil environments (De Pasquale et al., 2012; Isaac et al., 2015), while Sydow et al. (2016) clearly showed that *Sphingobacterium* spp. can be *n*-alkane-degrading specialists. Previous studies have reported that fatty acids from FAMEs revealed structural and metabolic similarities with *n*-alkanes and their metabolites of biological oxidation (alcohols, aldehydes and acids) (Fulco, 1983; Lisiecki et al., 2014; Wentzel et al., 2007; Yassine et al., 2013). Thus, it was expected that *n*-

522 alkane-degraders able also to successfully degrade FAMEs will appear. Moreover, Lisiecki et
523 al. (2014) determined that there was neither inhibiting nor stimulating effect of different
524 FAMEs content on *Sphingobacterium* during degradation of broad range of diesel/biodiesel
525 blends in sand microcosms. On the other hand, several studies demonstrated that the increased
526 growth of *Gammaproteobacteria* was stimulated by the presence of biodiesel (Cyplik et al.,
527 2011; Lisiecki et al., 2014; Sydow et al., 2016). Although we did not observe an increased
528 abundance in the *Gammaproteobacteria* in the presence of pure biodiesel, the significant
529 decrease for members of this class was observed with a decreased FAMEs content in urban
530 soil. Furthermore, our results are also in agreement with those reported by Cyplik et al.
531 (2011), who presented the suppression effect of biodiesel on the abundance of
532 *Betaproteobacteria*. Here *Betaproteobacteria* increased two-fold to its contribution when
533 urban soil was spiked with pure diesel. Lors et al. (2012) found that in soil polluted by coal
534 tar, *Betaproteobacteria* appeared in bacterial community after three months when
535 concentrations of PAHs were non-toxic and low enough to maintain such conditions. They
536 suggested that *Betaproteobacteria* taxa could act as a bio-indicator for the endpoint of the
537 bioremediation processes. Therefore, more work is needed to determine the influence of
538 diesel/biodiesel blends on bacterial community in field conditions as limitation in carbon
539 source and nutrients availability may play a critical role in community structure changes.
540

541 **4.2. Influence of bioaugmentation approach on diesel/biodiesel blends**

542 The concept of inoculating the hydrocarbon-polluted areas with fast-degrading
543 microorganisms in order to increase the biodegradation rate and reduce the time to enhance
544 the bioremediation efficiency has been developed for many years (Gentry et al., 2004;
545 Mukherjee and Bordoloi, 2011; Szulc et al., 2014). In previous studies, single strains, mixed
546 cultures or consortia were used as inocula (Cerqueira et al., 2011; Junior et al., 2009; Rahman

et al., 2002). Tyagi et al. (2011) suggested that strategies involving the use of microbial consortia, rather than a single culture, is more beneficial for bioremediation as it provides biodiversity and robustness, as is depictive for the real environment. Following this assumption we used a hydrocarbon-degrading bacterial community isolated from oil-contaminated soil, as we determined a high biodegradation potential.

The biodegradation kinetics presented the intensive activity only within first 28 days (short-term phase), while during long-term phase (33-109 days) no enhancement in mineralization rates compared with non-augmented microcosms were determined. This finding suggested that the microbial community had a positive effect on biodegradation of diesel/biodiesel blends only after inoculation, while over time the efficiency of bioaugmentation had decreased. Our results are in accordance with Szczepaniak et al. (2016), who determined that the bioaugmentation of soil contaminated with PAHs was successful only during the early stage of treatment, while after a few months the bacterial community composition returned to the previous conditions. In the present study, after 64.5 weeks the bacterial profile of diesel/biodiesel-contaminated soil, when augmented with bacterial community, was found to be comparable to non-augmented samples. One possible explanation is that the microbial community did not adapt sufficiently to survive this long-term exposure. Goldstein et al. (1985) described that possible failure of bioaugmentation might be justified by low growth rates of supplemented microorganisms in relation to indigenous microorganisms, when in soil microcosms various easy available carbon sources were presented. Prior studies emphasized also the significant importance of interaction between inoculated and autochthonous microorganisms in terms of their viability, activity and proliferation (El Fantroussi and Agathos, 2005; Goldstein et al., 1985; Thompson et al., 2005), indicating that supplementation of contaminated site with autochthonous microorganisms is more beneficial in long-term degradation of pollutants. Within this work, the applied bacterial community was

non-indigenous microorganisms, isolated from different environmental conditions. Hence, this might be the reason why the bioaugmentation was diminished after some time. However, the procedure using non-autochthons fast degraders has been already successfully applied in previous studies (Junior et al., 2009; Stella et al., 2017; Teng et al., 2010).

On the other hand, Johnsen et al. (2007) determined that priming the PAH-polluted soil by adding as inoculum bioremediated soil with a high hydrocarbon degradation potential resulted in the increase even up to 1,000 times the number of cultivable PAH-degraders. This means that the soil-adapted community has demonstrated the high survival rate, persistence and proliferation in PAH-contaminated soil during the experiment lasting 16 weeks. Although, the introduction to hydrocarbon polluted microcosms soil-adapted degraders seems to be beneficial, such treatment had no significant effect on hydrocarbon degradation, which accords with our observations. The higher degradation rates of phenanthrene, fluoranthen and pyrene were determined only within few weeks after inoculation, in the end the degradation rates of primed and not primed microcosms were comparable. Recent studies have described the significant impact of soil matrices on biodegradation success (Bento et al., 2005; Horemans et al., 2016). This issue was described by Horemans et al. (2016), who determined the biodegradation potential of phenanthrene-degrading bacterial on twenty uncontaminated, sterile soils with various physico-chemical characteristics. The authors revealed that there were differences in the extent of phenanthrene degradation, and that this was dependent on the soil properties. Although, to simplify the models, they did not consider the influence of biotic factors, which might strongly affect activity and survival of supplemented microorganisms; they hence developed a three-step tool for predicting the bioaugmentation success. Based on models described in their study, the soil used within the framework of this research was classified as soil with potential to survival with medium degrading activity of bioaugmented strain. However, in terms of our soil, the authors recommended the bioaugmentation together

with biostimulation as a good and effective biodegradation strategy. Therefore, the effectiveness of bioaugmentation approach of diesel/biodiesel contaminated site depend on both selection of appropriate microorganisms treatment and compatible soil to successfully enhance the chances of bioaugmentation in urban microcosms.

5. Conclusions and practical implications

The present study demonstrated that after long-term exposure (64.5 weeks), the mineralization extent of different diesel/biodiesel blends in urban soil does not depend on biodiesel concentration in fuel. This finding suggests that giving sufficient time for biodegradation of such blends from soil might be an effective bioremediation strategy. However, the addition of biodiesel to conventional diesel fuel increases the biodegradation kinetics. Thus, during short periods of time diesel/biodiesel blending higher than 30% seems to be beneficial for bioremediation of petroleum mixtures spills. This study has shown that bioaugmentation can potentially be effective only during the early stages of treatment, whereas after long-term exposure no differences in mineralization extent and bacterial community structure between augmented and non-augmented microcosms occur. It would therefore seem that a beneficial approach in our long-term treatment would be to use successive bioaugmentation. Corroborating this, Colla et al. (2014) suggested that successive bioaugmentation was an effective strategy in bioremediation of soil polluted with diesel/biodiesel blends. Several studies (Łebkowska et al., 2011; Tahhan et al., 2011) demonstrated that multiple inoculation of hydrocarbon-contaminated soil with autochthonous and non-autochthonous microorganisms revealed satisfactory results, and such approaches could be applied as a powerful tool in bioremediation. Moreover, according to Tahhan et al. (2011), additional supplementation of bacterial consortium into soil during petroleum hydrocarbons degradation significantly improved the removal of aromatic and asphaltic fractions, whose biodegradation

is usually much slower. Collectively, our findings suggest that single bioaugmentation treatment might not be enough to significantly accelerate the removal of hydrocarbon contaminations from urban soil matrix. Therefore, in order to enhance biodegradation, when time is not a limiting factor, the use of bioaugmentation approach may not be an adequate and justifiable solution.

Acknowledgment

The research work was funded by the National Science Centre in Poland in the years 2014-2018 with the research project Opus no 2013/11/B/NZ9/01908.

Supporting Information. Fig. S1 – Effect of the amount of biodiesel in blends on the residual of total diesel/biodiesel blends and hydrocarbons fractions; Fig. S2 - Ratio of saturated to unsaturated fraction of diesel residues.

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Figure and table captions:

Fig. 1. Mineralization extent of diesel (D) and diesel/biodiesel blends (B10-B100) in urban soil microcosms without bioaugmentation (1A, 1B - mineralization within first 28 days) and with bioaugmentation (2A, 2B - mineralization within first 28 days). Error bars represents confidence intervals for $p = 0.05$.

Fig. 2. Relative abundance of the most dominant microbial phyla (A) and classes (B) inhabiting soil (control) and soil spike with diesel/biodiesel blends with autochthonic microcosms (B100, B20, D) versus autochthonic microcosms bioaugmented with specialized bacterial community BC125 (B100+, B20+, D+).

Table 1. Mineralization extent and rate constants for different fuels and biodegradation conditions (augmented vs, non-augmented).

Supplementary materials:

Fig. S1. Effect of the amount of biodiesel in blends on the residual of total diesel/biodiesel blends (●), total hydrocarbons (○), aliphatic hydrocarbons (Δ), aromatic hydrocarbons (■) and FAME (▼) after 64.5 weeks without (A) and with (B) bioaugmentation. m/m_0 express the residual content of different fractions to their initial masses. The error bars are omitted, in order to make figure more clear and legible.

Fig. S2. Ratio of saturated to unsaturated fraction of diesel residues in soil matrix after 64.5 weeks without (A) and with (B) bioaugmentation. The error bars represent standard error of

889 the mean (n=3). As a reference the ratio of saturated to unsaturated fraction of fresh diesel
890 fuel was determined.

Table 1
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Table 1. Mineralization extent and rate constants for different fuels and biodegradation conditions (augmented vs, non-augmented).

Fuel	Mineralization rates (period 0-28 days) [mmol CO_2 / day]		Mineralization rates (period 33-109 days) [mmol CO_2 / day]		Total mineralization extent [mmol CO_2]	
	non-augmented	augmented	non-augmented	augmented	non-augmented	augmented
Control	-	-	-	-	9.7 ± 1.1	10.1 ± 0.9
D	0.1480	0.1445	0.2091	0.1722	49.9 ± 3.8	43.2 ± 2.7
B10	0.1169	0.1711	0.1562	0.1875	44.1 ± 2.3	44.7 ± 2.3
B20	0.1338	0.1864	0.1951	0.1945	45.3 ± 3.3	48.5 ± 3.1
B30	0.1293	0.1589	0.1866	0.1644	45.9 ± 2.9	43.6 ± 3.2
B40	0.1534	0.1822	0.2102	0.1560	46.4 ± 2.6	43.2 ± 2.2
B50	0.1360	0.1844	0.2366	0.1742	48.0 ± 3.1	42.9 ± 2.1
B60	0.1574	0.1959	0.2492	0.2086	46.7 ± 2.8	46.3 ± 3.2
B70	0.1607	0.1707	0.2546	0.2479	47.0 ± 2.5	45.6 ± 2.3
B80	0.1756	0.1741	0.3154	0.3004	47.9 ± 2.5	44.0 ± 2.3
B90	0.1583	0.1702	0.2916	0.3372	46.8 ± 3.2	45.8 ± 2.4
B100	0.2452	0.2362	0.3242	0.3061	48.8 ± 2.4	45.9 ± 3.0

The rates are derived from the slope of the initial (0-28 days) and intermediate (33-109 days) phases of the mineralization curves; $R^2 > 0.95$ for all samples

Figure 1

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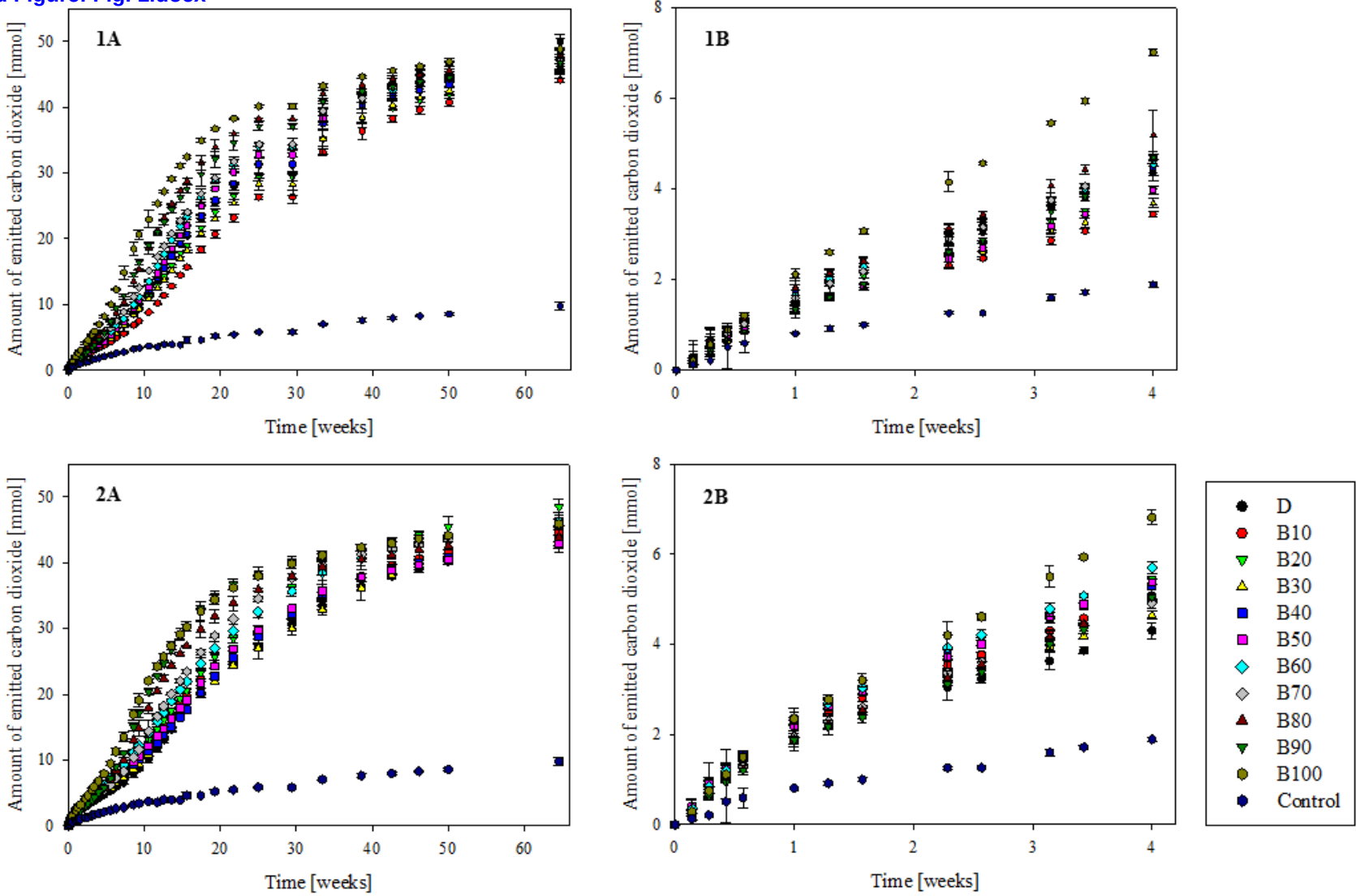

Figure 1. Mineralization of diesel (D) and diesel/biodiesel blends (B10-B100) in urban soil microcosms without bioaugmentation (1A, 1B - mineralization within first 28 days) and with bioaugmentation (2A, 2B - mineralization within first 28 days). Error bars represents confidence intervals for p = 0.05.

Figure 2
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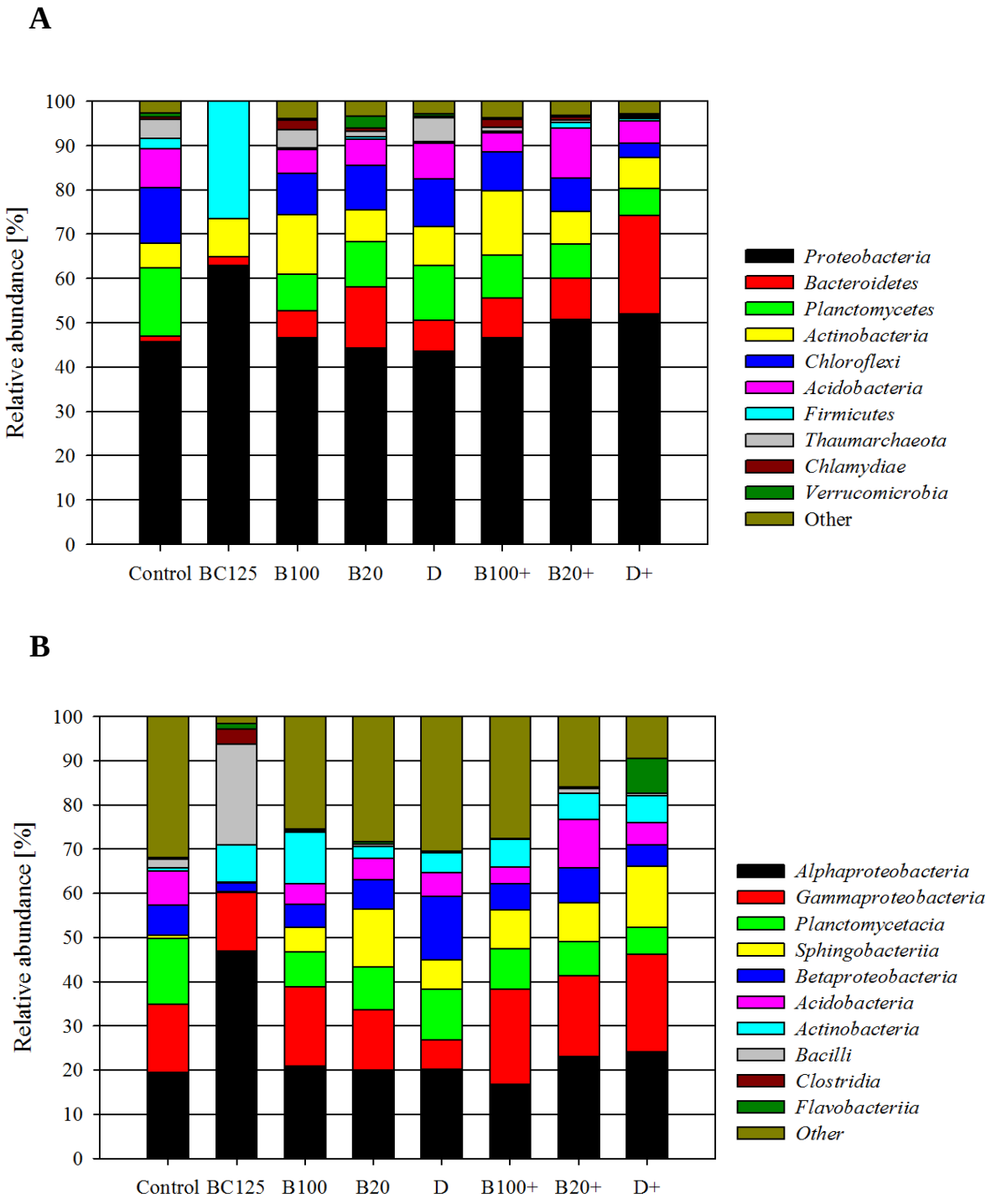

Figure 2. Relative abundance of the most dominant microbial phyla (A) and classes (B) inhabiting soil (control) and soil spike with diesel/biodiesel blends with autochthonic microcosms (B100, B20, D) versus autochthonic microcosms bioaugmented with specialized bacterial community BC125 (B100+, B20+, D+).

Supplementary material for on-line publication only

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