DTU Library

Biodiversity of soil bacteria exposed to sub-lethal concentrations of phosphonium-based ionic liquids: Effects of toxicity and biodegradation

Sydow, Mateusz; Owsianiak, Mikołaj; Framski, Grzegorz; Woźniak-Karczewska, Marta; Piotrowska-Cyplik, Agnieszka; Ławniczak, Łukasz; Szulc, Alicja; Zgoła-Grześkowiak, Agnieszka; Heipieper, Hermann J; Chrzanowski, Łukasz

[Link back to DTU Orbit](#)

1 Sydow, M., Owsianiak, M., Framski, G., Woźniak-Karczewska, M., Piotrowska-Cyplik, A.,

2 Ławniczak, Ł., Szulc, A., Zgoła-Grześkowiak, A., Heipieper, H.J., Chrzanowski, Ł., 2018.

3 Biodiversity of soil bacteria exposed to sub-lethal concentrations of phosphonium-based ionic

4 liquids: Effects of toxicity and biodegradation. Ecotoxicol. Environ. Saf. 147, 157–164.

5 doi:10.1016/j.ecoenv.2017.08.026

Biodiversity of soil bacteria exposed to sub-lethal concentrations of phosphonium-based ionic liquids: effects of toxicity and biodegradation

Mateusz Sydow[1], Mikołaj Owsianiak[2], Grzegorz Framski[3], Marta Woźniak-Karczewska[1], Agnieszka Piotrowska-Cyplik[4], Łukasz Ławniczak[1], Alicja Szulc[1], Agnieszka Zgoła-Grześkowiak[1], Hermann J. Heipieper[5], Łukasz Chrzanowski[1,*]

[1]Faculty of Chemical Technology, Poznan University of Technology, Berdychowo 4, 60-965 Poznań, Poland

[2]Division for Quantitative Sustainability Assessment, Department of Management Engineering, Technical University of Denmark, Produktionstorvet, Building 424, DK-2800 Kgs. Lyngby, Denmark

[3]Institute of Bioorganic Chemistry, Polish Academy of Sciences, Noskowskiego 12/14, 61-704 Poznań, Poland

[4]Institute of Food Technology of Plant Origin, Poznan University of Life Sciences, Wojska Polskiego 31, 60-624 Poznań, Poland

[5]Department of Environmental Biotechnology, Helmholtz Centre for Environmental Research – UFZ Permoserstrasse 15, 04318 Leipzig, Germany

*Corresponding author - tel.: +48 61 665 37 16; fax: +48 61 665 36 49.

E-mail address: lucaschrz@gmx.de (Ł. Chrzanowski)

27 **Abstract**

28 Little is known on the effect of ionic liquids (ILs) on the structure of soil microbial

29 communities and resulting biodiversity. Therefore, we studied the influence of six

30 trihexyl(tetradecyl)phosphonium ILs (with either bromide or various organic anions) at

31 sublethal concentrations on the structure of microbial community present in an urban park soil

32 in 100-day microcosm experiments. The biodiversity decreased in all samples (Shannon's

33 index decreased from 1.75 down to 0.74 and OTU's number decreased from 1399 down to

34 965) with the largest decrease observed in the microcosms spiked with ILs where

35 biodegradation extent was higher than 80%. (i.e. $[P_{66614}][Br]$ and $[P_{66614}][2,4,4]$). Despite this

36 general decrease in biodiversity, which can be explained by ecotoxic effect of the ILs, the

37 microbial community in the microcosms was enriched with Gram-negative hydrocarbon-

38 degrading genera e.g. *Sphingomonas*. It is hypothesized that, in addition to toxicity, the

39 observed decrease in biodiversity and change in the microbial community structure may be

40 explained by the primary biodegradation of the ILs or their metabolites by the mentioned

41 genera, which outcompeted other microorganisms unable to degrade ILs or their metabolites.

42 Thus, the introduction of phosphonium-based ILs into soils at sub-lethal concentrations may

43 result not only in a decrease in biodiversity due to toxic effects, but also in enrichment with

44 ILs-degrading bacteria.

45

46 **Keywords:** biodegradation; ionic liquids; microbial community; biodiversity; toxicity;

47 Illumina NGS**.**

1. Introduction

Ionic liquids (ILs) are a group of chemical compounds composed of an organic cation and an organic or inorganic anion, which have melting point below $100^{o}C$. The salts based on imidazolium or ammonium cations are among the two most popular and well-studied groups of ILs (Coleman and Gathergood, 2010; Cvjetko Bubalo et al. 2014). In the recent years, however, the phosphonium-based ILs became popular due to relatively low costs of their synthesis and relatively good thermal stability. Tetraalkylphosphonium ionic liquids are used as solvents, catalysts, electrolytes and corrosion inhibitors (Fraser and MacFarlane 2009). This group of ILs has been used in industrial processes, such as the isomerisation of 3,4-epoxybut-1-ene to 2,5-dihydrofuran carried out by the Eastman Chemical Company (IL used as catalyst) or the production of pharmaceutical intermediates by utilizing Sonogashira coupling conducted by the Central Glass Co., Ltd., Japan (IL used as solvent) (Plechkova and Seddon, 2007). In general, ILs can be ecotoxic when they enter aquatic or terrestrial ecosystems (Pham et al. 2010). Several papers focused on the evaluation of the environmental impacts of ILs (Ferlin et al. 2013a, 2013b, Liwarska-Bizukojc and Gendaszewska 2013, Peric et al. 2013, Pernak et al. 2011, Ventura et al. 2013, Borkowski et al. 2016). However, the number of scientific reports studying the impact of ILs on the structure of indigenous microbial communities inhabiting soil is still insufficient (Ławniczak et al. 2016), as the majority of the studies is focused on the effects of ILs on single microbial species (Piotrowska et al. 2017).

The influence of ILs on complex microbial communities inhabiting soil can be evaluated using Illumina Next-Generation Sequencing Technology (Illumina NGS), which produces useful high-throughput 16S amplicon data. Thereby, Illumina NGS enables an insight into the diversity of microbial taxa at the great scale and coverage (Caporaso et al. 2012; You et al. 2016). While most studies focused on the assessment of ecotoxicity reports regarding their fate and exposure, including biodegradability and persistence, are limited. Biodegradation tests are mainly conducted with the use of imidazolium-, ammonium-, and pyridinium-based ionic liquids, whereas the number of studies dedicated to phosphonium-based ILs is still limited. Moreover, most of the biodegradation assays are predominantly based on the use of short-term OECD tests (with a 28-day test time window) and there is little information regarding the long-term (>28 days) biodegradability of phosphonium-based ILs. Furthermore, the data from biodegradation studies carried out in the terrestrial environment with respect to based ILs are scarce, as the number of reports dedicated to this topic is limited

4

81 (Modelli et al. 2008; Pham et al. 2010). The results obtained in our previous study showed
82 that primary biodegradation of selected phosphonium-based ILs in urban park microcosms
83 was low and reached 25 and 29% for [P$_{66614}$][Cl] and [P$_{66614}$][Tr], respectively (Sydow et al.
84 2015).

85   The aim of this study was to determine the effect of six selected
86 trihexyl(tetradecyl)phosphonium ILs with either inorganic or different organic anions
87 supplied at sub-lethal concentrations on the structure of soil bacterial and resulting changes in
88 biodiversity. The experiments were carried out in soil microcosms and lasted for 100 days.
89 The soil has document biodegradation potential toward other phosphonium-based ionic
90 liquids (Sydow et al. 2015). Yet, apart from [P$_{66614}$][Br], the studied ILs are antifungal agents
91 and are expected to influence biodiversity mainly through ecotoxic effects of the attached
92 anions (Walkiewicz et al. 2010). The determination of structural changes within the
93 community was assessed using Illumina NGS genetic assay, supported by determination of
94 ILs' biodegradation in the soil combined with determination of 100-day CO_2 evolution from
95 the soils spiked with the ILs. The soil used in the experiments was an urban park soil with
96 some potential for biodegradation of ionic liquids (Sydow et al. 2015).

97

98 **2. Materials and methods**

99 **2.1. Chemical reagents**

100 The phosphonium-based ILs were prepared according to method described by Cieniecka-
101 Rosłonkiewicz et al. (2005). Briefly, trihexyl(tetradecyl)phosphonium bromide was prepared
102 in the reaction of trihexylphosphine and 1-bromotetradecane. The azolate ILs were
103 synthesized according to the method described by Walkiewicz et al. (2010). The water content
104 of synthesized ILs was determined by Aquastar volumetric Karl-Fischer titration with
105 Composite 5 solution as the titrant and anhydrous methanol as solvent. The water content of
106 each of the ILs reached values lower than 500 ppm. The compounds were also characterized
107 by ^{1}H and ^{13}C NMR spectroscopy and elemental analysis as described in Walkiewicz et al.
108 (2010). The list of the studied ILs as well as their chemical structures is presented in Table 1.

109

110 **2.2. Characterization of soil**

111 Mollic gley soil was collected from a city park in the center of Poznan city (N 52.4011445, E
112 16.9222993) in September 2013 from the depth of 10-20 cm according to the procedure
113 described by Alef and Nannipleri, (1995). According to United Soil Classification System, the

soil used in the experiments is characterized as fine grained silt loam type OL belonging to organic silts and organic silty clays of low plasticity. The soil was stored in closed 5-L polypropylene containers for one week at constant temperature equal to 20°C. Prior to the experiments, the soil was sieved and analyzed according to the procedures described by Adeboye et al. (2011). The composition and full characteristics of the soil can be found in Sydow et al. (2015).

2.3. Determination of sub-lethal concentrations

In order to assess the potential toxicity of the used ILs and estimate sub-lethal concentration of each ILs which could be used in biodegradation tests (ion residues, CO_2 evolution) and genetic assay (Illumina NGS), the preliminary test – seed germination assay – with the use of grass species was conducted. The preliminary test was chosen to be carried out using plants, as the most convenient method of toxicity assessment in soil. The EC_{50} values (the concentration of a chemical at which 50% of its effect is observed) of ILs were determined by assessing seeds germination with increasing (total) concentrations (125; 250; 500; 1000; 2000; 4000; 8000 mg kg^{-1}) of a particular IL in soil. A mix of seeds (*Festuca rubra* 40%; *Festuca arundinacea* 20%; *Agrostis capillaris* 4%; *Poa pratensis* 6%; *Festuca trachyphylla* 30%) was used in the test. After 14 days of growth, above-ground parts of germinated seeds were collected and weighed. Triplicate sets were performed for each treatment. The EC_{50} values were determined using the Trimmed Spearman–Karber method (An 2004). The SPEARMAN program (EPA's Center for Exposure Assessment Modeling, USA), was used to calculate the EC_{50} values.

2.4. Preparation of soil samples

The experiments in soil were carried out in sealed 1-L glass bottles (one bottle corresponds to one sample), which contained 100 g of urban park soil and were not inoculated. The samples were prepared as follows: 10 g of non-sterilized soil were added into bottles and then spiked with a methanol solution (5 mL) of each IL to reach a final concentration equal to previously determined EC_{50} (i.e. 3010 – 3960 mg kg^{-1}, which corresponds to 0.0237 – 0.0401 [M]). Next, methanol was evaporated with nitrogen. Afterwards, untreated soil in the amount of 90 grams was added. The soil was later vigorously mixed. Finally, the microcosms were incubated at 20°C for 100 days. The set-up for the tests consisted of 18 samples contaminated with ILs (i.e. 3 replicate samples for each IL), 3 additional samples for monitoring of the soil moisture

and 3 control samples (spiked only with methanol, which was then evaporated with nitrogen). The base traps containing NaOH solution were placed inside each bottle (mostly to be used for CO_2 evolution tests) to maintain full saturation in the microcosms, as it provided equilibrium between the headspace phase and the soil. Therefore the moisture content of the soil was constant during the experiments and was equal to $18 \pm 2\%$. Each of the bottle replicates was used for three different tests i.e. one bottle with soil was used for genetic assay (20g of the soil was used for Illumina NGS assay) biodegradation test (0.5 g of the soil was used for HPLC-MS analysis) and CO_2 evolution tests (base traps were placed inside the bottles).

2.5. Assessment of bacterial community structure in soil using Illumina sequencing

Illumina Next-Generation Sequencing (NGS) enables to study qualitative and quantitative composition of microbial samples at all taxonomic ranks – from kingdom to species level. Here, Illumina genetic assay was employed in order to assess the effects of the used ILs on the structure of the microbial community inhabiting urban park soil. Although it can be expected that some filamentous fungi are resistant to ionic liquids (Petkovic et al. 2009), this study was limited only to Bacteria and Archaea kingdom. It is mostly caused by the fact that the studied phosphonium-based ILs were designed as antifungal agents (mostly due to antifungal properties of the attached anions) and are toxic toward fungi (Walkiewicz et al. 2010). In this study, the contribution of the particular microbial taxon was presented as % of total taxa (regarding the same taxonomic rank). Class, family and genus taxonomic ranks were chosen to be presented in results section, as changes on these levels enable the comparison of the microbial community structure between samples. The detailed NGS data containing the information about the contribution of taxa in all taxonomic ranks can be found in the NCBI Nucleotide Archive database under the project number PRJNA389990 (https://www.ncbi.nlm.nih.gov/sra/SRP109755). BioSample accessions: SAMN07257075 (Control), SAMN07257076 ([P_{66614}][Ntf_2]), SAMN07257077 (P_{66614}][Br]), SAMN07257078 ([P_{66614}][3AT]), SAMN07257079 ([P_{66614}][2,4,4]), SAMN07257080 ([P_{66614}][$N(CN)_2$]), SAMN07257081 ([P_{66614}][Bt]).

2.5.1. DNA extraction

After termination of the studies, one soil sample (20 g of soil) from each replicate was collected. Afterwards, all samples belonging to the same set were homogenized and three 10 g

180 subsamples were collected from each set. These subsamples were stored at −80 ºC until

181 further processing (less than two weeks). Each subsample was subjected to extraction of total

182 DNA and further analyzes separately and the data obtained for each set of subsamples was

183 combined. Total DNA was extracted from 500 mg of each soil using Genomic Mini AX Soil

184 kit (A&A Biotechnology) according to manufacturer's instruction. Extracted DNA were

185 quantified using Quant-iT HS ds.-DNA assai kit (Invitrogen) on Qubit2 fluorometer; 2 µl of

186 extracts were examined on a 0.8% agarose gel.

187

188 **2.5.2. Biodiversity assessment**

189 For PCR amplification and calculation of OTU abundance and Shannon's index we followed

190 the procedure presented in Ławniczak et al. (2016). Briefly, we targeted Region IV of

191 bacterial 16S RNA gene amplified using a set of primers with Illumina adapters, followed by

192 sequencing using primers as described in Caporaso et al. (2012). The sequencing was done

193 using paired-end (2x250) MiSeq Reagent Kits v2 (Illumina, USA). For processing of the

194 sequencing data, as in Lawniczak et al. (2016), we used CLC Genomic Workbench 8.5 and

195 CLC Microbial Genomics Module 1.2. (Qiagen, USA), followed by clustering of the

196 sequencing reads against the SILVA v119 99% 16S rRNA gene database (July 24, 2014,

197 Quast et al. 2013). OTU abundance and Shannon's index were calculated for rarefaction

198 analysis with a depth of 100 000 sequences per sample.

199

200 **2.6. Biodegradation in soil**

201 The biodegradation experiments in soil were carried out in sealed 1-L glass bottles as

202 described in section.2.4. Therefore, the set-up for the tests consisted of 18 samples

203 contaminated with ILs (i.e. 3 samples for each IL), 3 additional samples for monitoring of the

204 soil moisture and 3 control samples (spiked only with methanol, which was then evaporated

205 with nitrogen). The microcosms were incubated at 20°C for 100 days. After 100 days, one soil

206 portion (0.5 g) from each bottle contaminated with IL was subjected to three-step ultrasound

207 assisted extraction with methanol (3 x 1 mL) and analyzed by HPLC-MS.

208 In order to include sorbed fraction of the used ILs onto soil matrix during calculation

209 of the ions residual masses, additional control tests with sterilized soil (to inhibit

210 biodegradation) contaminated with ILs were performed. First, the urban park soil was divided

211 into aliquots of 30 g, frozen, placed in sealed polyethylene bags and irradiated at 40,000 grey

212 using a ^{192}Ir source (Alef and Nannipleri 1995). Afterwards, a methanol solution (5 mL) of

213   selected IL was added to 10 g of sterilized soil. Next, methanol was evaporated and untreated

214   sterilized soil in the amount of 90 grams was added. Finally, samples were mixed vigorously.

215   The set-up for the sorption tests consisted of 18 samples contaminated with ILs (i.e. 3 samples

216   for each IL) and 1 additional sample for monitoring of the soil moisture. All of the samples

217   contained the base traps to provide constant moisture content of the soil (equal to $18 \pm 2\%$).

218   After 100 days of incubation at $20^{\circ}C$ under sterile conditions, 0.5 g portion of soil from each

219   replicate was subjected to a three-step ultrasound assisted extraction with methanol (3 x 1

220   mL) and analyzed by HPLC-MS to determine the fraction of ILs that was not permanently

221   sorbed onto soil matrix. During the calculation of ions residual masses, it was assumed that

222   the value of sorbed fraction is constant and does not change over time. Moreover, we assumed

223   linear relationship between sorption and concentration of the used ILs (although in reality the

224   relationship is not linear), which is a simplified, but still relevant assumption for organic

225   compounds (Moyo et al. 2014). The recovery efficiency of the extraction of the ILs from the

226   soil matrix dependent on the used ILs and reached (with respect to both cation and anion)

227   99% for $[P_{66614}][2,4,4]$, 89% for $[P_{66614}][Br]$, 89% for $[P_{66614}][Ntf_2]$, 86% for

228    $[P_{66614}][N(CN)_2]$, 70% for $[P_{66614}][3AT]$ and 73% for $[P_{66614}][Bt]$.

229

230   **2.7. HPLC-MS analysis**

231   For HPLC-MS analysis of residual ions can we followed the procedure of Sydow et al.

232   (2015). Briefly, three 1 mL soil extracts (all three obtained via three-step extraction of each

233   soil sample) were combined, filtered through a 0.2 µm PTFE syringe filter and diluted with

234   methanol : water solution (80:20 v/v). The HPLC-MS analyses were performed with the

235   UltiMate 3000 RSLC chromatograph from Dionex (Sunnyvale, CA, USA). Five µL samples

236   were injected into a Hypersil GOLD column (100 mm 2.1 mm I.D.; 1.9 µm) with a 2.1 mm

237   I.D. pre-filter cartridge (0.2 µm) from Thermo Scientific (Waltham, MA, USA). The mobile

238   phase consisted of 5×10^{-3} mol L^{-1} ammonium acetate in water (phase A) and methanol (phase

239   B) at a flow rate of 0.2 mL min^{-1}.

240

241   **2.8. Evolution of CO_2 from the microcosms**

242   The CO_2 evolution tests were carried out in the same sealed 1-L glass bottles as described in

243   section 2.4. Overall, 18 soil samples contaminated with ILs (i.e. 3 samples for each IL), 3 soil

244   samples for moisture monitoring and 3 control samples (containing 100 g of park soil spiked

245   with methanol, which was then evaporated with nitrogen) were prepared. The controls were

prepared to investigate the background respiration of the used park soil. The amount of emitted CO_2 from the microcosms was determined by measuring CO_2 content in a base trap (10 mL of 0.75 M NaOH in a 20-mL vial) placed in the microcosms (as described by Szulc et al. (2014) and Sydow et al. (2015)). Briefly, titration of the diluted NaOH and Na_2CO_3 solution from the trap was done with 0.1 M HCl using an automatic titrator (Metrohm titroprocessor 686). The content of the base traps was replaced with fresh NaOH solution after each measurement.

2.9. Statistical analysis

All experiments were carried out in triplicates. Each error margin range represents standard errors of the mean (SEM). Analysis of variance (at $\alpha=0.05$) in Statistica 6.0 was employed for statistical comparisons.

3. Results and Discussion

3.1. Biodegradation of ionic liquids and evolution of CO_2 from soil microcosms

The EC_{50} values (corrected for sorbed fraction in soil) reached 3010 mg kg^{-1} (0.0323 [M]) for $[P_{66614}][Br]$, 3290 mg kg^{-1} (0.0237 [M]) for $[P_{66614}][2,4,4]$, 3540 mg kg^{-1} (0.0258 [M]) for $[P_{66614}][Ntf_2]$, 3590 mg kg^{-1} (0.0332 [M]) for $[P_{66614}][Bt]$, 3840 mg kg^{-1} (0.0377 [M]) for $[P_{66614}][3AT]$ and 3960 mg kg^{-1} (0.0401 [M]) for $[P_{66614}][N(CN)_2]$.

As can be seen in Figure 1, after 100 days of incubation, residual ions (both cation and anion) were detected in all contaminated soil samples (bromide anion was not investigated). The presence of different anions had significant influence on the residual amount of the $[P_{66614}]$ cation. The lowest amount of cation residue was observed when the anion was [Br] (9%) and [2,4,4] (11%). On the other hand, the highest amount of cation residue was observed when the anion was $[N(CN)_2]$ (60%). For other soil samples spiked with ILs, the residual amounts of cations were as follows: 31% ($[P_{66614}][3AT]$), 42% ($[P_{66614}][Bt]$) and 47% ($[P_{66614}][Ntf_2]$). Moreover, the lowest amount of anion residue was observed in the case of [2,4,4] (12%) and the highest amount of anion residue was detected in samples contaminated with $[P_{66614}][N(CN)_2]$ (46% of anion residue). The amount of other residual anions were as follow: 38% ([3AT]), 41% ([Bt]) and 26% ([Ntf_2]).

In order to elucidate mechanism determining the shape of community structure and biodiversity changes we carried out biodegradation and CO_2 evolution experiments. The CO_2 evolution curves for all the used ILs are presented in Figure 2. At the end of the experiment,

10

279   the highest amount of emitted CO_2 (29.8 mmol) was observed in the sample containing

280     $[P_{66614}][Br]$. The emission of CO_2 in other samples did not differ significantly from the

281   emission observed in the case of control sample (25.7 mmol) without any ILs addition.

282   Furthermore, in the case of $[P_{66614}][N(CN)_2]$ (i.e. IL that was primarily degraded to the lowest

283   extent) respiration of the soil was significantly lower than in control sample, reaching 22.6

284   mmol of emitted CO_2. The obtained results showed that the studied ILs generally did not

285   significantly inhibit the respiration activity of soil microbiota.

286   The biodegradation results showed that biodegradation of the studied phosphonium-

287   based ILs was different depending on the attached anion. The lowest amount of $[P_{66614}]$ cation

288   residue was observed in the case of ILs with inorganic [Br] anion, which surely did not

289   influence the biodegradation of the cation. The lowest amount of anion residue was observed

290   in the case of [2,4,4] anion, which consists of two 2,4,4-trimethylpentyl chains. The [2,4,4]

291   anion is structurally similar to branched alkanes and may potentially be utilized by iso-alkanes

292   degraders, which were likely present in the studied soil (Sydow et al. 2016). Previous studies

293   confirmed that branched alkanes utilizers are present in soils permanently polluted by

294   petroleum hydrocarbons and may induce growth of some bacterial taxa (Sydow et al. 2016).

295   The observed rapid biodegradation of [2,4,4] anion may also explain its low inhibitory effect

296   toward biodegradation of cation. Nevertheless, the CO_2 evolution results did not confirm full

297   mineralization of the $[P_{66614}][2,4,4]$, as the amount of emitted CO_2 was not statistically

298   different from the control. Therefore, similarly to other studied ILs, $[P_{66614}][2,4,4]$ was most

299   probably transformed by the microbial community to metabolic intermediates. The metabolic

300   pathway associated with transformation of the molecules similar to branched alkanes are less

301   known than those for linear alkanes, but may involve an ω-oxidation of the compound with

302   formation of dicarboxylic acids in the first step, leading to shorter-chained products (Stolte et

303   al. 2008; Rojo, 2010). In general, the phosphonium-based ILs with antifungal properties are

304   not expected to be readily degraded by indigenous soil microbial communities inhabiting soils

305   even in longer periods of time (i.e. 300 days) (Sydow et al. 2015). Also Deive et al. (2011)

306   observed low biodegradability (degradation rate lower than 25%, in many cases equal to 0%)

307   of several 1-ethyl-3-methylimidazolium- and cholinium-based ILs in 2-months test conducted

308   with the use of microbial communities isolated from the industrial and salt marsh soil and

309   cultivated on peptone solution.

310   Only soil samples spiked with $[P_{66614}][Br]$ were characterized by significantly higher

311   CO_2 evolution compared to the control. This indicates none or marginal mineralization of the

312   studied ILs in urban soil. Following the approach presented in Horel and Schiewer (2011) and

313   Sydow et al. (2015), a carbon mass balance was performed in order to estimate the

314   mineralization of primarily degraded $[P_{66614}][Br]$. Assuming a yield of 0.4 g of microbial

315   carbon per 1 g of IL carbon, it was calculated that approximately 42% of the primarily

316   degraded $[P_{66614}][Br]$ was mineralized. This corresponds to 31% mineralization of the total

317   compound. For the majority of the used ILs, the measured CO_2 evolution was not statistically

318   different as compared to control, which is in agreement with our previous study dedicated to

319   biodegradability of other phosphonium- and ammonium-based ILs (Sydow et al. 2015). In

320   that study, it was observed that the only mineralized compound was $[P_{66614}][Cl]$ – the only IL

321   with inorganic anion and a similar chemical composition to $[P_{66614}][Br]$ (both are composed of

322   halide anion). Although it is possible that the presence of complex organic anions attached to

323    $[P_{66614}]$ cation may have inhibited mineralization of the cation, and simultaneously, the whole

324   compound, the opposite mechanism suggesting the negative influence of cation on

325   biodegradation of organic anions cannot be excluded. Although the presence of long alkyl

326   chains (as in the case of $[P_{66614}]$ cation) may facilitate biodegradation of the whole compound,

327   it was previously observed that such ions may simultaneously be more toxic than homologues

328   with shorter chains (Stolte et al. 2011).

329

330   **3.2. Structure of soil bacteria after exposure to ionic liquids**

331   Figure 3a shows the contribution of dominant classes identified within soil microbial

332   community after exposure to either of the studied ILs at sub-lethal concentrations. The

333   obtained results indicate that the studied phosphonium-based ILs had significant influence on

334   the structure of soil microbial community as the contribution of dominant classes changed

335   after 100-day exposure. In four out of six soil samples spiked with ILs, the contribution of

336   Alphaproteobacteria increased with the highest increase (by 24%) was observed for

337    $[P_{66614}][N(CN)_2]$. The contribution of Gammaproteobacteria increased only in case of

338    $[P_{66614}][N(CN)_2]$ (by 5%), while the *Bacilli* class increased in case of $[P_{66614}][2,4,4]$ and

339    $[P_{66614}][Ntf_2]$ (by up to 10%). The increase of contribution of *Clostridia* class was observed

340   for $[P_{66614}][Bt]$ (by 7%), $[P_{66614}][3AT$ (by 2%)] and $[P_{66614}][Br]$ (by 1%), while the

341   contribution of Deltaproteobacteria increased only in case of $[P_{66614}][Bt]$ (by 5%),

342   Betaproteobacteria class slightly increased its contribution in case of all samples with

343   exception of $[P_{66614}][Ntf_2]$. The contribution of *Sphingobacteria* class increased only in

344   samples spiked with $[P_{66614}][3AT]$ (by 12%) and $[P_{66614}][2,4,4]$ (by 8%). On the other hand,

345 the contribution of *Actinobacteria* and *Planctomycetia* class decreased by 1 to 7% (depending

346 on the ILs) in all samples compared to control.

347   Figure 3b shows the contribution of the five most abundant bacterial families

348 identified in soils spiked with particular ILs and control. The structure of the microbial

349 community changed significantly in all of the studied samples spiked with ILs, as new

350 families became dominant within microbial community. The most dominant microbial

351 families detected in control sample were *Xanthomonodaceae* (most abundant), followed by

352 *Planctomycetaceae*, *Bacillaceae*, *Caulobacteraceae* and *Hyphomicrobioaceae.* In the case of

353 soil samples spiked with [P$_{66614}$][N(CN)$_2$], [P$_{66614}$][Ntf$_2$] and [P$_{66614}$][2,4,4] more than 50% (p

354 = 0.015) of the contribution was represented by five most abundant bacterial families. By

355 contrast, in the case of soil samples spiked with other ILs, the dominant families represented

356 no more than 35% of all identified families. The contribution of *Xanthomonoddaceae* family

357 (the most abundant in control sample) decreased in all soil samples spiked with ILs, but its

358 dominance was maintained in samples spiked with [P$_{66614}$][Br] and [P$_{66614}$][N(CN)$_2$].

359 Compared to control sample, the contribution of bacterial family belonging to

360 *Sphingomonadaceae* became significant in majority of the samples contaminated with ILs

361 (only in case of [P$_{66614}$][Bt] this family was not detected as top five abundant). Moreover, with

362 respect to soil samples contaminated with [P$_{66614}$][N(CN)$_2$] and [P$_{66614}$][Ntf$_2$], the most

363 abundant genus was *Sphingomonas*, which represented 13.70 (p = 0.012) and 17.57% (p =

364 0.008) of all identified genera, respectively (data not shown). Also, in the case of the soil

365 samples spiked with [P$_{66614}$][2,4,4], a significant percent of contribution was represented by

366 *Sphingomonas* (6.41%, p = 0.027) and *Pseudomonas* (6.72%, p = 0.014). In general, in the

367 case of all studied samples spiked with ILs the contribution of *Sphingomonas* and

368 *Pseudomonas* genera was higher compared to the control sample (*Sphingomonas*: 1.46% for

369 control; 1.83% for [P$_{66614}$][Bt], 2.78% for [P$_{66614}$][3AT], 3.09% for [P$_{66614}$][Br], 6.41% for

370 [P$_{66614}$][2,4,4], 17.57% for [P$_{66614}$][Ntf$_2$] and 13,70% for [P$_{66614}$][N(CN)$_2$]; *Pseudomonas*:

371 0.05% for control; 0.79% for [P$_{66614}$][Bt], 3.62% for [P$_{66614}$][3AT], 1.12% for [P$_{66614}$][Br],

372 6.72% for [P$_{66614}$][2,4,4], 2.98% for [P$_{66614}$][Ntf$_2$] and 0.49% for [P$_{66614}$][N(CN)$_2$]).

373   The Shannon's diversity estimates differed significantly (p < 0.05) among control and

374 the treatments, but also among some of the treatments, with a mean Shannon's index value of

375 1.33 (Table 2). Moreover, the highest value of Shannon's index was obtained for control soil.

376 Additionally, the mean value of the observed OTU's was also significantly different among

377 control and ILs treated soils and reached a maximum value for control soil (1399) (Table 2).

378   The lowest value of OTU's and Shannon's index was determined for $[P_{66614}][2,4,4]$ (OTU's =

379   965, Shannon's index = 0.73). In general, the introduction of the studied ILs contributed to

380   significant reduction of the microbial biodiversity in soil. The PCA plot of weighted Unifrac

381   distances indicate that the bacterial community structure changed significantly (p = 0.017)

382   upon treatment with the studied ILs compared to control soil (Fig. 4). There were no

383   significant differences between microbial structures of soils treated with $[P_{66614}][3AT]$ and

384   $[P_{66614}][Bt]$ (p = 0.71), and between $[P_{66614}][Ntf_2]$ and $[P_{66614}][N(CN)_2]$ (p = 0.14).

385   In contrast to the results obtained in this study, Ławniczak et al. (2016) did not

386   observe an effect of the herbicidal ionic liquids on biodiversity (both OTU's and Shannon's

387   index were not significantly different from the control). However, similarly to our study, they

388   observed that the structure of the community (assessed using Illumina NGS) was significantly

389   affected (on the phylum level) by the exposure to herbicidal ILs. This difference may be

390   explained by either shorter exposure time (100 days in this study, 28 days in Ławniczak et al.

391   (2016)) not allowing occurrence of significant changes in biodiversity, or differences in

392   applied ILs concentration and differences in initial biodiversity of the studied soils (control

393   Shannon's index in this study was 1.75, while control Shannon's index in Ławniczak et al.

394   (2016) was 4.95). A higher biodiversity is usually associated with higher resistance to

395   different perturbations (Isbell et al. 2015). Deive et al. (2011) also observed a decrease in

396   biodiversity of microbial communities isolated from the salt marsh and industrial soils

397   exposed to various imidazolium- and cholinium-based ILs. The authors observed higher

398   survival of microbial strains isolated from industrial soils, which were contaminated in the

399   past by petroleum hydrocarbons. Also Sun et al. (2017) observed a significant decrease in

400   biodiversity and alternation of the structure of soil microbial community exposed to 1-octyl-3-

401   methylimidazolium tetrafluoroborate for 40 days. On the other hand, Guo et al. (2015)

402   observed a significant decrease in biodiversity of soil microbial community only in higher

403   concentrations of the alkyl-imidazolium-based ionic liquid with chloride anion.

404

405   **3.3. Explaining changes in community structure and decrease in biodiversity**

406   Basing on the genetic assay and biodegradation and CO_2 evolution experiments, it is

407   hypothesized that decrease in biodiversity is explained by a combination of two factors (i) a

408   toxic effect of the phosphonium-based ILs or their metabolites towards non-resistant

409   microbial taxa within the community, and/or (ii) an emergence of few ILs-degrading taxa,

410   which outcompeted the other unable to utilize ILs or their metabolites. In the first case, the

411 contribution of more resistant species should increase after the perturbation. Gram-negative
412 bacteria are generally more resistant to toxic organic compounds, such as organic solvents or
413 antibiotics, which may be explained by their different structure resulting in the presence of the
414 efflux pump systems and outer cell membranes (Vermuë et al. 1993; Heipieper et al. 2007;
415 Heipieper and Martinez 2010; Stancu and Grifoll, 2011). As observed, the contribution of two
416 Gram-negative genera – *Pseudomonas* and *Sphingomonas* – increased in all samples spiked
417 with ILs, which may support the first hypothesis regarding the toxicity. Some of the studied
418 phospohonium-based ILs were found to be toxic to single bacterial species (especially these
419 containing halide anions) (Cieniecka-Rosłonkiewicz et al. 2005). However, it was observed
420 that the contribution of Gram-positive *Geobacillus* genus also increased in all soil samples
421 and became dominant in the samples spiked with [P_{66614}][2,4,4] (contribution equal to
422 15.61%) and [P_{66614}][Ntf$_2$] (contribution equal to 15.08%). Yet, only one IL, [P_{66614}][N(CN)$_2$],
423 exhibited significantly lower CO_2 evolution compared to control, indicating, in general, none
424 or low inhibition of the microbial activity in presence of the studied ILs. Thus, the observed
425 increase in abundance of bacteria belonging to the families *Sphingomonadaceae* and
426 *Pseudomonadaceae*, which consist of well-known hydrocarbon-degrading genera may
427 support the latter hypothesis, since all of the studied ILs consisted of [P_{66614}] cation with long
428 alkyl chains structurally similar to *n*-alkanes. Especially the genera *Sphingomonas* and
429 *Pseudomonas* are known for their ability to degrade various petroleum hydrocarbons, but also
430 other toxic compounds (White et al. 1996; Whyte et al. 1997). The highest reduction of
431 biodiversity was observed in soil samples spiked with [P_{66614}][Br] and [P_{66614}][2,4,4] -
432 compounds that were degraded to the highest extent. This may suggest that an efficient
433 biodegradation of ILs could induce the greater structural changes within microbial community
434 and the emergence of ILs-degraders. Guo et al. (2015) suggested that changes in biodiversity
435 of soil microbial community exposed to ILs may be caused by the intensified growth of some
436 microbial strains able to degrade new carbon source such as ILs. The presence of previously
437 unavailable carbon sources often induces the growth of specialists (also called r-strategists)
438 and decline of generalists (K-strategists) (Ciric et al. 2010; Sydow et al. 2016). Thus, the
439 observed reduction in biodiversity should be rather explained by the primary biodegradation
440 of ILs resulting from an emergence of ILs-degrading taxa within the microbial community.
441
442 **4. Conclusions**

15

443 We showed that when supplied at sub-lethal concentrations, the studied phosphonium-based
444 ILs could be a stress factor for soil microbial communities and impact their structure
445 diversity, especially by increasing the abundance of well-known hydrocarbon-degrading
446 genera such as *Sphingomonas* and *Pseudomonas*. Future studies should focus on
447 determination of possible ILs metabolites produced by environmental microbial consortia and
448 the effect of ILs on soil microbial communities at environmentally relevant concentrations.
449

453

454 **References**
455 Adeboye MKA, Bala A, Osunde AO, Uzoma AO, Odofin AJ, Lawal BA (2011) Assessment
456 of soil quality using soil organic carbon and total nitrogen and microbial properties in tropical
457 agroecosystems. Agr Sci 2:34–40.
458

459 An YJ, Kim YM, Kwon YI, Jeong SW (2004) Combined effect of copper, cadmium, and lead
460 upon *Cucumis sativus* growth and bioaccumulation. Sci Total Environ 326:857–863.
461

462 Alef K, Nannipleri P (1995) Methods in Applied Soil Microbiology and Biochemistry.
463 Academic Press, San Diego, USA.
464

465 Borkowski A, Ławniczak Ł, Cłapa T, Narożna D, Selwet M, Pęziak D, Markiewicz B,
466 Chrzanowski Ł (2016) Different antibacterial activity of novel theophylline-based ionic
467 liquids - Growth kinetic and cytotoxicity studies. Ecotoxicol Environ Saf 130:54–64.
468

469 Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, Fierer N, Owens SM,
470 Betley J, Fraser L, Bauer M, Gormley N, Gilbert JA, Smith G, Knight R (2012) Ultra-high-
471 throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. ISME
472 J 6:1621–1624.
473

474   Cieniecka-Rosłonkiewicz A, Pernak J, Kubis-Feder J, Ramani A, Robertson AJ, Seddon KR

475   (2005) Synthesis, anti-microbial activities and anti-electrostatic properties of phosphonium-

476   based ionic liquids. Green Chem 7:855-862.

477

478   Ciric L, Philip JC, Whiteley AS (2010) Hydrocarbon utilization within a diesel-degrading

479   bacterial consortium. FEMS Microbiol Lett 303:116–122.

480

481   Coleman D, Gathergood N (2010) Biodegradation studies of ionic liquids. Chem Soc Rev

482   39:600-637.

483

484   Cvjetko Bubalo M, Radošević K, Radojčić Redovniković I, Halambek J, Gaurina Srček V

485   (2014) A brief overview of the potential environmental hazards of ionic liquids. Ecotoxicol

486   Environ Saf 99:1–12.

487

488   Deive FJ, Rodriguez A, Varela A, Rodrigues C, Leitåo MC, Houbraken JAMP, Pereiro AB,

489   Longo MA, Sanromån MA, Samson RA, Rebelo LPN, Silva Pereira C (2011) Impact of ionic

490   liquids on extreme microbial biotypes from soil. Green Chem 13:687–696.

491

492   Ferlin N, Courty M, Gatard S, Spulak M, Quilty B, Beadham I, Ghavre M, Haiß A,

493   Kümmerer K, Gathergood N, Bouquillon S (2013a) Biomass derived ionic liquids: synthesis

494   from natural organic acids, characterization, toxicity, biodegradation and use as solvents for

495   catalytic hydrogenation processes. Tetrahedron 69:6150–6161.

496

497   Ferlin N, Courty M, Van Nhien AN, Gatard S, Pour M, Quilty B, Ghavre M, Haiß A,

498   Kümmerer K, Gathergood N, Bouquillon S (2013b) Tetrabutylammoniumprolinate-based

499   ionic liquids: a combined asymmetric catalysis, antimicrobial toxicity and biodegradation

500   assessment. RSC Adv 3:26241–26251.

501

502   Fraser KJ, MacFarlane DR (2009) Phosphonium-Based Ionic Liquids: An Overview. Aust. J.

503   Chem. 62:309–321.

504

505 Guo P, Zhu L, Wang J, Wang J, Liu T (2015) Effects of alkyl-imidazolium ionic liquid
506 [Omim]Cl on the functional diversity of soil microbial communities. Environ Sci Pollut Res
507 22:9059–9066.

508

509 Heipieper H.J, Martínez P. (2010) Toxicity of hydrocarbons to microorganisms. In: Timmis
510 K.N. (Ed.) Handbook of Hydrocarbon and Lipid Microbiology. Springer, Berlin, Heidelberg.
511 Vol. 2, Part 9, pp. 1565-1573.

512

513 Heipieper H.J, Neumann G, Cornelissen S, Meinhardt F. (2007) Solvent-tolerant bacteria for
514 biotransformations in two-phase fermentation systems. Appl Microbiol Biotechnol 74:961-
515 973.

516

517 Horel A, Schiewer S (2011) Influence of constant and fluctuating temperature on
518 biodegradation rates of fish biodiesel blends contaminating Alaskan sand. Chemosphere
519 83:652–60.

520

521 Isbell F, Craven D, Connolly J, Loreau M, Schmid B, Beierkuhnlein C, Bezemer T.M, Bonin
522 C, Bruelheide H, de Luca E, Ebeling A, Griffin J.N, Guo Q, Hautier Y, Hector A, Jentsch A,
523 Kreyling J, Lanta V, Manning P, Meyer S.T, Mori A.S, Naeem S, Niklaus P.A, Polley H.W,
524 Reich P.B, Roscher C, Seabloom E.W, Smith M.D, Thakur M.P, Tilman D, Tracy B.F, van
525 der Putten W.H, van Ruijven J, Weigelt A, Weisser W.W, Wilsey B.J, Eisenhauer N (2015)
526 Biodiversity increases the resistance of ecosystem productivity to climate extremes. Nature
527 526:574–577.

528

529 Jordan A, Gathergood N (2015) Biodegradation of ionic liquids – a critical review. Chem Soc
530 Rev 44:8200–8237.

531

532 Ławniczak Ł, Syguda A, Borkowski A, Cyplik P, Marcinkowska K, Wolko Ł, Praczyk T,
533 Chrzanowski Ł, Pernak J (2016) Influence of oligomeric herbicidal ionic liquids with MCPA
534 and Dicamba anions on the community structure of autochthonic bacteria present in
535 agricultural soil. Sci Total Environ 563–564:247–255.

536

537 Liwarska-Bizukojc E, Gendaszewska D (2013) Removal of imidazolium ionic liquids by
538 microbial associations: Study of the biodegradability and kinetics. J Biosci Bioeng 115:71–
539 75.
540
541 Modelli A, Sali A, Galletti P, Samorì C (2008) Biodegradation of oxygenated and non-
542 oxygenated imidazolium-based ionic liquids in soil. Chemosphere 73:1322-1327.
543
544 Moyo F, Tandlich R, Wilhelmi BS, Balaz S (2014) Sorption of hydrophobic organic
545 compounds on natural sorbents and organoclays from aqueous and non-aqueous solutions: A
546 mini-review. Int J Environ Res Public Health 11:5020–5048.
547
548 Mrozik W, Kotłowska A, Kamysz W, Stepnowski P (2012) Sorption of ionic liquids onto
549 soils: Experimental and chemometric studies. Chemosphere 88:1202-1207.
550
551 Paterson E, Sim A (1999) Rhizodeposition and C-partitioning of *Lolium perenne* in axenic
552 culture affected by nitrogen supply and defoliation. Plant Soil 216:155–164.
553
554 Peric B, Sierra J, Marti E, Cruãnas R, Garau MA, Arning J, Bottin-Weber U, Stolte S (2013)
555 (Eco)toxicity and biodegradability of selected protic and aprotic ionic liquids. J Hazard Mater
556 261:99–105.
557
558 Pernak J, Borucka N, Walkiewicz F, Markiewicz B, Fochtman P, Stolte S, Steudte S,
559 Stepnowski P (2011) Synthesis, toxicity, biodegradability and physicochemical properties of
560 4-benzyl-4-methylmorpholinium-based ionic liquids. Green Chem 13:2901-2910.
561
562 Petkovic M, Ferguson J, Bohn A, Trindade J, Martins I, Carvalho MB, Leitão MC, Rodrigues
563 C, Garcia H, Ferreira R, Seddon KR, Rebelo LPN, Silva Pereira C (2009) Exploring fungal
564 activity in the presence of ionic liquids. Green Chem 11:889–894.
565
566 Pham TPT, Cho CW, Yun YS (2010) Environmental fate and toxicity of ionic liquids: A
567 review. Water Res 44:352–372.
568

569 Piotrowska A, Syguda A, Wyrwas B, Chrzanowski Ł, Heipieper HJ (2017) Toxicity
570 evaluation of selected ammonium-based ionic liquid forms with MCPP and dicamba moieties
571 on *Pseudomonas putida*. Chemosphere 167:114–119.

573 Plechkova NV, Seddon, KR (2007) Applications of ionic liquids in the chemical industry.
574 Chem Soc Rev 37:123–150.

576 Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO (2013)
577 The SILVA ribosomal RNA gene database project: improved data processing and web-based
578 tools. Nucl Acids Res 41(D1):D590–D596.

580 Quijano G, Couvert A, Amrane A (2010) Ionic liquids: Applications and future trends in
581 bioreactor technology. Biores Technol 101:8923–8930.

583 Rojo F (2010) Enzymes for aerobic degradation of alkanes. In: Timmis K.N. (Ed.) Handbook
584 of Hydrocarbon and Lipid Microbiology. Springer, Berlin, Heidelberg. Vol. 1, p. 781-797.

586 Stancu M.M, Grifoll M (2011) Multidrug resistance in hydrocarbon-tolerant Gram-positive
587 and Gram-negative bacteria. J Gen Appl Microbiol 57:1–18.

589 Stepnowski P (2005) Preliminary assessment of the sorption of some alkyl imidazolium
590 cations as used in ionic liquids to soils and sediments. Aust J Chem 58:170–173.

592 Stolte S, Abdulkarim S, Arning J, Blomeyer-Nienstedt A.-K, Bottin-Weber U, Matzke M,
593 Ranke J, Jastorff B, Thöming J (2008) Primary biodegradation of ionic liquid cations,
594 identification of degradation products of 1-methyl-3- octylimidazolium chloride and
595 electrochemical wastewater treatment of poorly biodegradable compounds. Green Chem.
596 10:214–224.

598 Stolte S, Steudte S, Igartua A, Stepnowski P (2011) The biodegradation of ionic liquids e the
599 view from a chemical structure perspective. Curr. Org. Chem. 15:1946–1973.

600

Sun X, Zhu L, Wang J, Su B, Liu T, Zhang C, Gao C, Saho Y (2017) Toxic effects of ionic liquid 1-octyl-3-methylimidazolium tetrafluoroborate on soil enzyme activity and soil microbial community diversity. Ecotox Environ Safe 135:201–208.

Sydow M, Szczepaniak Z, Framski G, Staninska J, Owsianiak M, Szulc A, Piotrowska-Cyplik A, Zgoła-Grześkowiak A, Wyrwas B, Chrznowski Ł (2015) Persistence of selected ammonium- and phosphonium-based ionic liquids in urban park soil microcosms. Int Biodeterior Biodegradation 103:91–96.

Sydow M, Owsianiak M, Szczepaniak Z, Framski G, Smets B.F, Ławniczak Ł, Lisiecki P, Szulc A, Cyplik P, Chrzanowski Ł (2016) Evaluating robustness of a diesel-degrading bacterial consortium isolated from contaminated soil. N Biotechnol 33:852–859.

Szulc A, Ambrożewicz D, Sydow M, Ławniczak Ł, Piotrowska-Cyplik A, Marecik R, Chrzanowski Ł (2014) The influence of bioaugmentation and biosurfactant addition on bioremediation efficiency of diesel-oil contaminated soil: feasibility during field studies. J Environ Manage 132:121-128.

Ventura SPM, Marques CS, Rosatella AA,, Afonso CAM, Gonçalves F, Coutinho JAP (2012) Toxicity assessment of various ionic liquid families towards *Vibrio fischeri* marine bacteria. Ecotox Environ Safe 76:162−168.

Ventura SPM, Gurbisz M, Ghavre M, Ferreira FMM, Gonçalves F, Beadham I, Quilty B, Coutinho JAP, Gathergood N (2013) Imidazolium and pyridinium ionic liquids from mandelic acid derivatives: synthesis and bacteria and algae toxicity evaluation. ACS Sustainable Chem Eng 1:393−402.

Vermuë M, Sikkema J, Verheul A, Bakker R, Tramper J (1993) Toxicity of homologous series of organic solvents for the Gram-positive bacteria *Arthrobacter* and *Nocardia* sp. and the Gram-negative bacteria *Acinetobacter* and *Pseudomonas* sp. Biotechnol Bioeng 42:737−757.

633 Walkiewicz F, Materna K, Kropacz A, Michalczyk A, Gwiazdowski R, Praczyk T, Pernak J

634 (2010) Multifunctional long-alkyl-chain quaternary ammonium azolate based ionic liquids.

635 New J Chem 34:2281-2289.

636

637 White DC, Sutton ST, Ringelberg DB (1996) The genus *Sphingomonas*: physiology and

638 ecology. Curr Opin Biotech 7:301-306.

639

640 Whyte LG, Bourbonnière L, Greer CW (1997) Biodegradation of petroleum hydrocarbons by

641 psychrotrophic *Pseudomonas* strains possessing both alkane (alk) and naphthalene (nah)

642 catabolic pathways. Appl Environ Microbiol 63:3719-3723.

643

644 You J, Wu G, Ren F, Chang Q, Yu B, Xue Y, Mu B (2016) Microbial community dynamics

645 in Baolige oilfield during MEOR treatment, revealed by Illumina MiSeq sequencing. Appl

646 Microbiol Biotechnol 100:1469–1478.
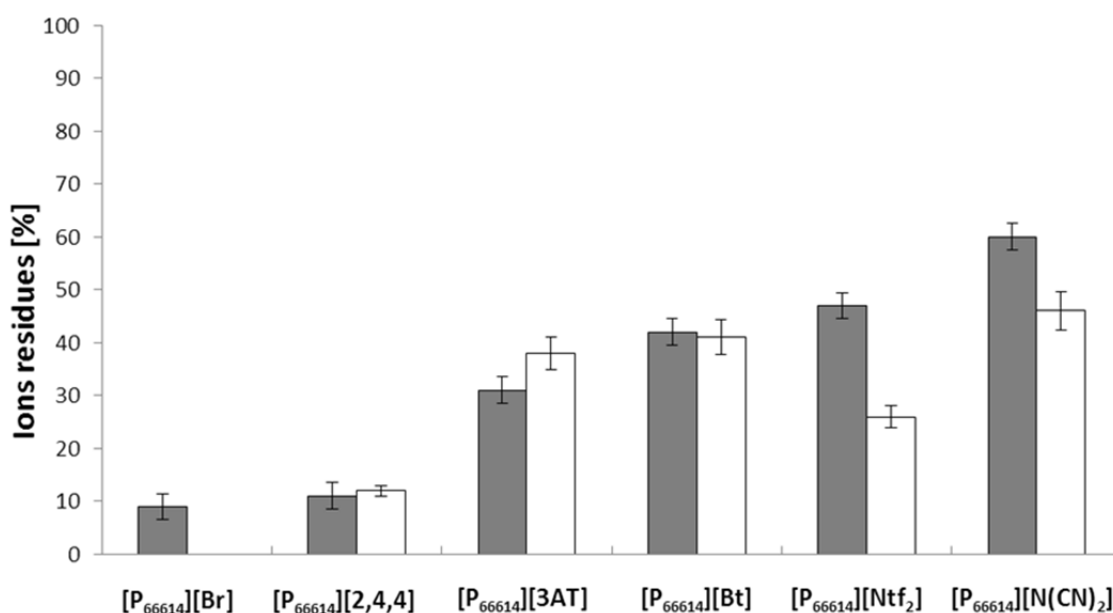
647
648 **Fig. 1.** The levels of ions residues of the selected phosphonium-based ILs after 100-day

649 experiment. Cation: grey bars, anions: white bars.

650

22
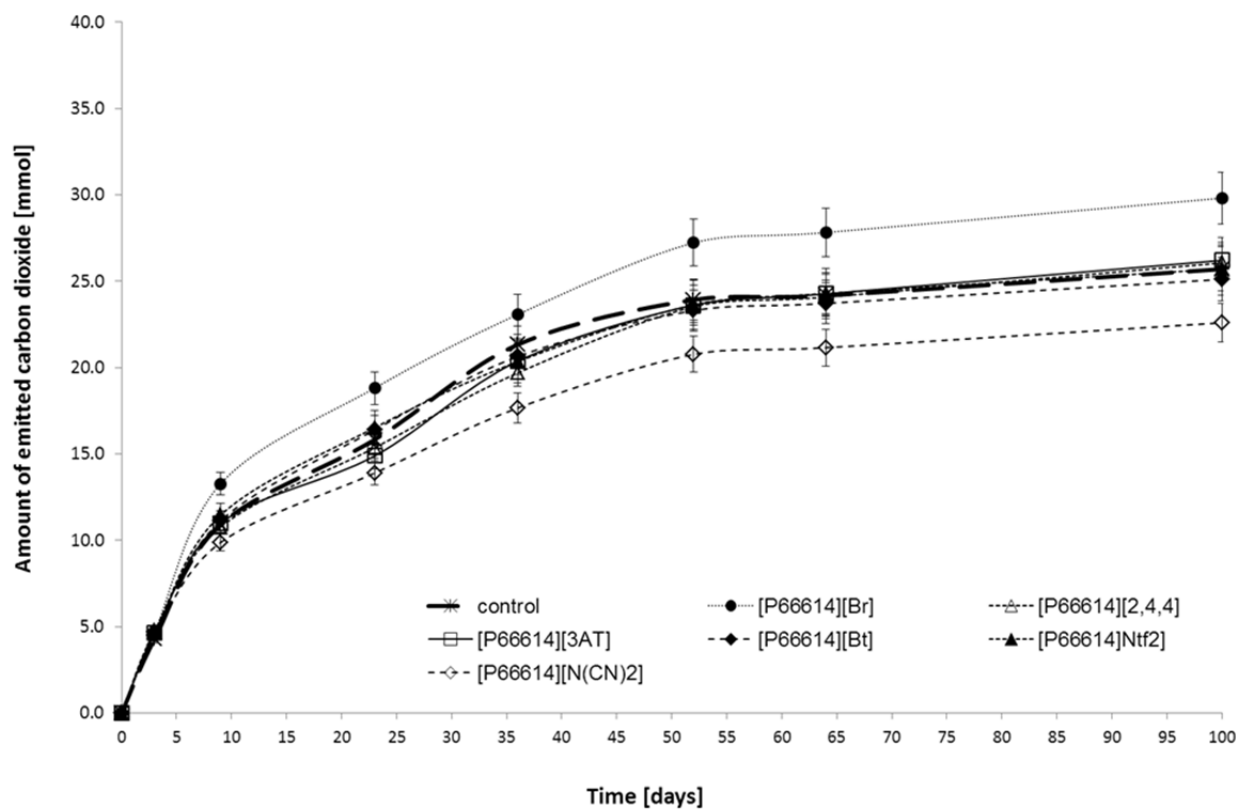
651
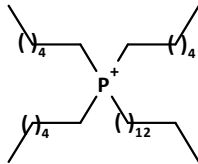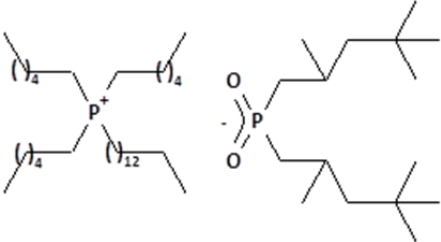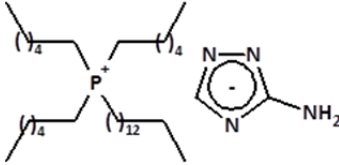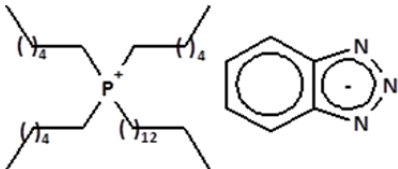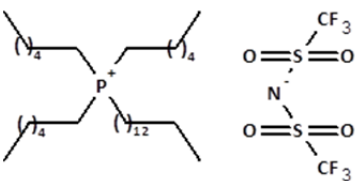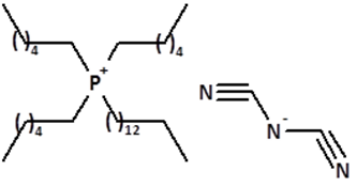652 **Fig. 2.** The evolution of CO_2 during the course of 100-day experiment.

653
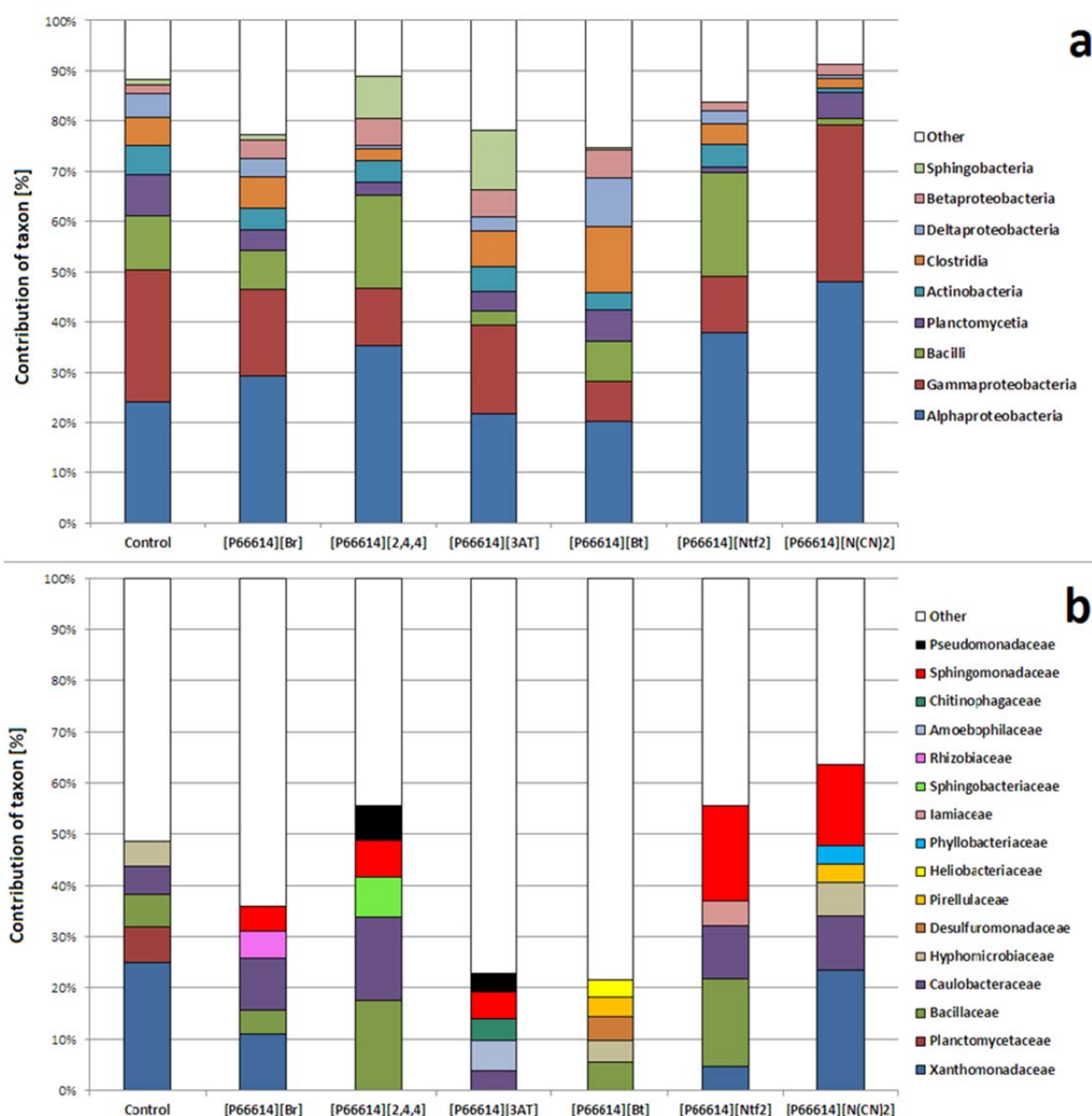
Fig. 3. The contribution of the most dominant microbial groups inhabiting urban park soil spiked with phosphonium-based ILs after 100-day exposure, presented with respect to (a) class, and (b) family taxonomic level (to facilitate the reading only five most dominant families among each soil treatment were presented).
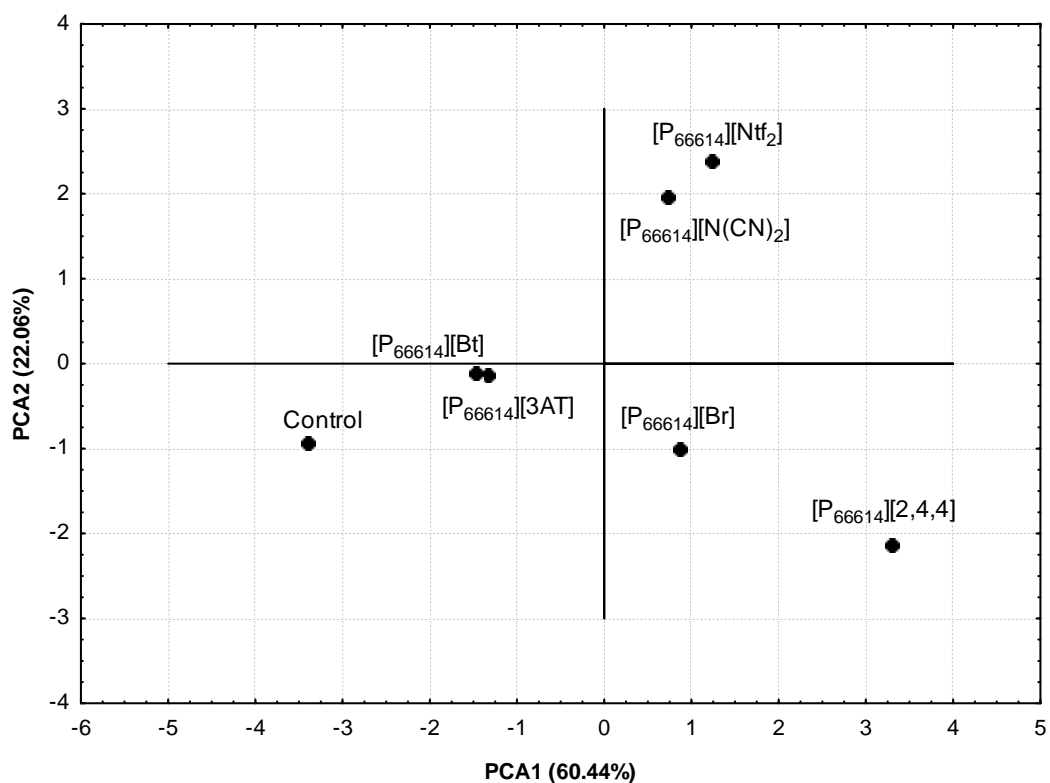
Fig. 4. PCA plot representing the weighted Unifrac distances for control and soils treated with the studied phosphonium-based ILs.

679   **Table 1.** The acronyms, structures and names of the used phosphonium-based ILs.

Acronym	Structure	Full name
[P$_{66614}$][Br]		trihexyl(tetradecyl)phosphonium bromide
[P$_{66614}$][2,4,4]		tetradecyl(trihexyl)phosphonium bis(2,4,4-trimethylpentyl)phosphinate
[P$_{66614}$][3AT]		trihexyl(tetradecyl)phosphonium 3-amino-1,2,4-triazolate
[P$_{66614}$][Bt]		trihexyl(tetradecyl)phosphonium benzotriazolate
[P$_{66614}$][Ntf$_2$]		tetradecyl(trihexyl)phosphonium bis(trifluoromethylsulfonyl)imide
[P$_{66614}$][N(CN)$_2$]		tetradecyl(trihexyl)phosphonium (dicyano)imide

680

681

682

683

26

Soil sample	OTU's observed	Shannon's index
Control	$1399 \pm 28^{b,c,d,e,f,g}$	$1.75 \pm 0.04^{b,c,d,e,f,g}$
P$_{66614}$][Br]	$1008 \pm 62^{a,b,c,d,e}$	$1.34 \pm 0,06^{a,b,c,d,g}$
[P$_{66614}$][2,4,4]	$965 \pm 55^{a,b,c,d,e}$	$0.73 \pm 0,09^{a,b,c,d,e,f}$
[P$_{66614}$][3AT]	$1205 \pm 31^{a,e,f}$	$1.57 \pm 0.08^{a,e,f,g}$
[P$_{66614}$][Bt]	$1229 \pm 43^{a,e,f}$	$1.59 \pm 0.07^{a,e,f,g}$
[P$_{66614}$][Ntf$_2$]	$1296 \pm 58^{a,e,f}$	$1.48 \pm 0,05^{a,e,f,g}$
[P$_{66614}$][N(CN)$_2$]	$1258 \pm 39^{a,e,f}$	$1.27 \pm 0,04^{a,b,c,d,g}$

Table 2. Alpha diversity estimates. Superscripts a,b,c,d,e,f,g correspond to the following table rows (1st row is *a*, 7th row is *g*) and describe which rows differ significantly at p $\leq$ 0.05.

687