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Sydow, Mateusz; Owsianiak, Mikoaj; Framski, Grzegorz; Woniak-Karczewska, Marta; Piotrowska-Cyplik, Agnieszka; awniczak, ukasz; Szulc, Alicja; Zgoa-Grzekowiak, Agnieszka; Heipieper, Hermann J; Chrzanowski, ukasz

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

8

9 Mateusz Sydow¹, Mikołaj Owsianiak², Grzegorz Framski³, Marta Woźniak-Karczewska¹,
10 Agnieszka Piotrowska-Cyplik⁴, Łukasz Ławniczak¹, Alicja Szulc¹, Agnieszka Zgoła-
11 Grześkowiak¹, Hermann J. Heipieper⁵, Łukasz Chrzanowski^{1,*}

12

13 ¹Faculty of Chemical Technology, Poznan University of Technology, Berdychowo 4, 60-965
14 Poznań, Poland

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

18 ³Institute of Bioorganic Chemistry, Polish Academy of Sciences, Noskowskiego 12/14, 61-
19 704 Poznań, Poland

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

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

24

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

26 *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. [P₆₆₆₁₄][Br] and [P₆₆₆₁₄][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.

48 **1. Introduction**

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

68 The influence of ILs on complex microbial communities inhabiting soil can be
69 evaluated using Illumina Next-Generation Sequencing Technology (Illumina NGS), which
70 produces useful high-throughput 16S amplicon data. Thereby, Illumina NGS enables an
71 insight into the diversity of microbial taxa at the great scale and coverage (Caporaso et al.
72 2012; You et al. 2016). While most studies focused on the assessment of ecotoxicity reports
73 regarding their fate and exposure, including biodegradability and persistence, are limited.
74 Biodegradation tests are mainly conducted with the use of imidazolium-, ammonium-, and
75 pyridinium-based ionic liquids, whereas the number of studies dedicated to phosphonium-
76 based ILs is still limited. Moreover, most of the biodegradation assays are predominantly
77 based on the use of short-term OECD tests (with a 28-day test time window) and there is little
78 information regarding the long-term (>28 days) biodegradability of phosphonium-based ILs.
79 Furthermore, the data from biodegradation studies carried out in the terrestrial environment
80 with respect to based ILs are scarce, as the number of reports dedicated to this topic is limited

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 [P₆₆₆₁₄][Cl] and [P₆₆₆₁₄][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 [P₆₆₆₁₄][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 CO₂ 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 ¹H and ¹³C 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

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

120

121 **2.3. Determination of sub-lethal concentrations**

122 In order to assess the potential toxicity of the used ILs and estimate sub-lethal concentration
123 of each ILs which could be used in biodegradation tests (ion residues, CO₂ evolution) and
124 genetic assay (Illumina NGS), the preliminary test – seed germination assay – with the use of
125 grass species was conducted. The preliminary test was chosen to be carried out using plants,
126 as the most convenient method of toxicity assessment in soil. The EC₅₀ values (the
127 concentration of a chemical at which 50% of its effect is observed) of ILs were determined by
128 assessing seeds germination with increasing (total) concentrations (125; 250; 500; 1000;
129 2000; 4000; 8000 mg kg⁻¹) of a particular IL in soil. A mix of seeds (*Festuca rubra* 40%;
130 *Festuca arundinacea* 20%; *Agrostis capillaris* 4%; *Poa pratensis* 6%; *Festuca trachyphylla*
131 30%) was used in the test. After 14 days of growth, above-ground parts of germinated seeds
132 were collected and weighed. Triplicate sets were performed for each treatment. The EC₅₀
133 values were determined using the Trimmed Spearman–Karber method (An 2004). The
134 SPEARMAN program (EPA’s Center for Exposure Assessment Modeling, USA), was used to
135 calculate the EC₅₀ values.

136

137 **2.4. Preparation of soil samples**

138 The experiments in soil were carried out in sealed 1-L glass bottles (one bottle corresponds to
139 one sample), which contained 100 g of urban park soil and were not inoculated. The samples
140 were prepared as follows: 10 g of non-sterilized soil were added into bottles and then spiked
141 with a methanol solution (5 mL) of each IL to reach a final concentration equal to previously
142 determined EC₅₀ (i.e. 3010 – 3960 mg kg⁻¹, which corresponds to 0.0237 – 0.0401 [M]). Next,
143 methanol was evaporated with nitrogen. Afterwards, untreated soil in the amount of 90 grams
144 was added. The soil was later vigorously mixed. Finally, the microcosms were incubated at
145 20°C for 100 days. The set-up for the tests consisted of 18 samples contaminated with ILs
146 (i.e. 3 replicate samples for each IL), 3 additional samples for monitoring of the soil moisture

147 and 3 control samples (spiked only with methanol, which was then evaporated with nitrogen).
148 The base traps containing NaOH solution were placed inside each bottle (mostly to be used
149 for CO₂ evolution tests) to maintain full saturation in the microcosms, as it provided
150 equilibrium between the headspace phase and the soil. Therefore the moisture content of the
151 soil was constant during the experiments and was equal to 18 ± 2%. Each of the bottle
152 replicates was used for three different tests i.e. one bottle with soil was used for genetic assay
153 (20g of the soil was used for Illumina NGS assay) biodegradation test (0.5 g of the soil was
154 used for HPLC-MS analysis) and CO₂ evolution tests (base traps were placed inside the
155 bottles).

156

157 **2.5. Assessment of bacterial community structure in soil using Illumina sequencing**

158 Illumina Next-Generation Sequencing (NGS) enables to study qualitative and quantitative
159 composition of microbial samples at all taxonomic ranks – from kingdom to species level.
160 Here, Illumina genetic assay was employed in order to assess the effects of the used ILs on
161 the structure of the microbial community inhabiting urban park soil. Although it can be
162 expected that some filamentous fungi are resistant to ionic liquids (Petkovic et al. 2009), this
163 study was limited only to Bacteria and Archaea kingdom. It is mostly caused by the fact that
164 the studied phosphonium-based ILs were designed as antifungal agents (mostly due to
165 antifungal properties of the attached anions) and are toxic toward fungi (Walkiewicz et al.
166 2010). In this study, the contribution of the particular microbial taxon was presented as % of
167 total taxa (regarding the same taxonomic rank). Class, family and genus taxonomic ranks
168 were chosen to be presented in results section, as changes on these levels enable the
169 comparison of the microbial community structure between samples. The detailed NGS data
170 containing the information about the contribution of taxa in all taxonomic ranks can be found
171 in the NCBI Nucleotide Archive database under the project number PRJNA389990
172 (<https://www.ncbi.nlm.nih.gov/sra/SRP109755>). BioSample accessions: SAMN07257075
173 (Control), SAMN07257076 ([P₆₆₆₁₄][Ntf₂]), SAMN07257077 (P₆₆₆₁₄][Br]), SAMN07257078
174 ([P₆₆₆₁₄][3AT]), SAMN07257079 ([P₆₆₆₁₄][2,4,4]), SAMN07257080 ([P₆₆₆₁₄][N(CN)₂]),
175 SAMN07257081 ([P₆₆₆₁₄][Bt]).

176

177 **2.5.1. DNA extraction**

178 After termination of the studies, one soil sample (20 g of soil) from each replicate was
179 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 $-80\text{ }^{\circ}\text{C}$ 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 Ławniczak 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°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 (3×1
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 $[\text{P}_{66614}][2,4,4]$, 89% for $[\text{P}_{66614}][\text{Br}]$, 89% for $[\text{P}_{66614}][\text{Ntf}_2]$, 86% for
228 $[\text{P}_{66614}][\text{N}(\text{CN})_2]$, 70% for $[\text{P}_{66614}][3\text{AT}]$ and 73% for $[\text{P}_{66614}][\text{Bt}]$.

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 $0.2 \mu\text{m}$ 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. ; $1.9 \mu\text{m}$) with a 2.1 mm
237 I.D. pre-filter cartridge ($0.2 \mu\text{m}$) from Thermo Scientific (Waltham, MA, USA). The mobile
238 phase consisted of $5 \times 10^{-3} \text{ mol L}^{-1}$ 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

246 prepared to investigate the background respiration of the used park soil. The amount of
247 emitted CO₂ from the microcosms was determined by measuring CO₂ content in a base trap
248 (10 mL of 0.75 M NaOH in a 20-mL vial) placed in the microcosms (as described by Szulc et
249 al. (2014) and Sydow et al. (2015)). Briefly, titration of the diluted NaOH and Na₂CO₃
250 solution from the trap was done with 0.1 M HCl using an automatic titrator (Metrohm
251 titroprocessor 686). The content of the base traps was replaced with fresh NaOH solution after
252 each measurement.

253

254 **2.9. Statistical analysis**

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

258

259 **3. Results and Discussion**

260 **3.1. Biodegradation of ionic liquids and evolution of CO₂ from soil microcosms**

261 The EC₅₀ values (corrected for sorbed fraction in soil) reached 3010 mg kg⁻¹ (0.0323 [M]) for
262 [P₆₆₆₁₄][Br], 3290 mg kg⁻¹ (0.0237 [M]) for [P₆₆₆₁₄][2,4,4], 3540 mg kg⁻¹ (0.0258 [M]) for
263 [P₆₆₆₁₄][Ntf₂], 3590 mg kg⁻¹ (0.0332 [M]) for [P₆₆₆₁₄][Bt], 3840 mg kg⁻¹ (0.0377 [M]) for
264 [P₆₆₆₁₄][3AT] and 3960 mg kg⁻¹ (0.0401 [M]) for [P₆₆₆₁₄][N(CN)₂].

265 As can be seen in Figure 1, after 100 days of incubation, residual ions (both cation and
266 anion) were detected in all contaminated soil samples (bromide anion was not investigated).
267 The presence of different anions had significant influence on the residual amount of the
268 [P₆₆₆₁₄] cation. The lowest amount of cation residue was observed when the anion was [Br]
269 (9%) and [2,4,4] (11%). On the other hand, the highest amount of cation residue was observed
270 when the anion was [N(CN)₂] (60%). For other soil samples spiked with ILs, the residual
271 amounts of cations were as follows: 31% ([P₆₆₆₁₄][3AT]), 42% ([P₆₆₆₁₄][Bt]) and 47%
272 ([P₆₆₆₁₄][Ntf₂]). Moreover, the lowest amount of anion residue was observed in the case of
273 [2,4,4] (12%) and the highest amount of anion residue was detected in samples contaminated
274 with [P₆₆₆₁₄][N(CN)₂] (46% of anion residue). The amount of other residual anions were as
275 follow: 38% ([3AT]), 41% ([Bt]) and 26% ([Ntf₂]).

276 In order to elucidate mechanism determining the shape of community structure and
277 biodiversity changes we carried out biodegradation and CO₂ evolution experiments. The CO₂
278 evolution curves for all the used ILs are presented in Figure 2. At the end of the experiment,

279 the highest amount of emitted CO₂ (29.8 mmol) was observed in the sample containing
280 [P₆₆₆₁₄][Br]. The emission of CO₂ 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 [P₆₆₆₁₄][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 CO₂. 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 [P₆₆₆₁₄] 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 CO₂ evolution results did not confirm full
297 mineralization of the [P₆₆₆₁₄][2,4,4], as the amount of emitted CO₂ was not statistically
298 different from the control. Therefore, similarly to other studied ILs, [P₆₆₆₁₄][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 [P₆₆₆₁₄][Br] were characterized by significantly higher
311 CO₂ 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 [P₆₆₆₁₄][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 [P₆₆₆₁₄][Br] was mineralized. This corresponds to 31% mineralization of the total
317 compound. For the majority of the used ILs, the measured CO₂ 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 [P₆₆₆₁₄][Cl] – the only IL
321 with inorganic anion and a similar chemical composition to [P₆₆₆₁₄][Br] (both are composed of
322 halide anion). Although it is possible that the presence of complex organic anions attached to
323 [P₆₆₆₁₄] 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 [P₆₆₆₁₄] 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 [P₆₆₆₁₄][N(CN)₂]. The contribution of Gammaproteobacteria increased only in case of
338 [P₆₆₆₁₄][N(CN)₂] (by 5%), while the *Bacilli* class increased in case of [P₆₆₆₁₄][2,4,4] and
339 [P₆₆₆₁₄][Ntf₂] (by up to 10%). The increase of contribution of *Clostridia* class was observed
340 for [P₆₆₆₁₄][Bt] (by 7%), [P₆₆₆₁₄][3AT (by 2%)] and [P₆₆₆₁₄][Br] (by 1%), while the
341 contribution of Deltaproteobacteria increased only in case of [P₆₆₆₁₄][Bt] (by 5%),
342 Betaproteobacteria class slightly increased its contribution in case of all samples with
343 exception of [P₆₆₆₁₄][Ntf₂]. The contribution of *Sphingobacteria* class increased only in
344 samples spiked with [P₆₆₆₁₄][3AT] (by 12%) and [P₆₆₆₁₄][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 *Xanthomonadaceae* (most abundant), followed by
352 *Planctomycetaceae*, *Bacillaceae*, *Caulobacteraceae* and *Hyphomicrobioaceae*. In the case of
353 soil samples spiked with [P₆₆₆₁₄][N(CN)₂], [P₆₆₆₁₄][Ntf₂] and [P₆₆₆₁₄][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 *Xanthomonadaceae* 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 [P₆₆₆₁₄][Br] and [P₆₆₆₁₄][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 [P₆₆₆₁₄][Bt] this family was not detected as top five abundant). Moreover, with
362 respect to soil samples contaminated with [P₆₆₆₁₄][N(CN)₂] and [P₆₆₆₁₄][Ntf₂], 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 [P₆₆₆₁₄][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 [P₆₆₆₁₄][Bt], 2.78% for [P₆₆₆₁₄][3AT], 3.09% for [P₆₆₆₁₄][Br], 6.41% for
370 [P₆₆₆₁₄][2,4,4], 17.57% for [P₆₆₆₁₄][Ntf₂] and 13.70% for [P₆₆₆₁₄][N(CN)₂]; *Pseudomonas*:
371 0.05% for control; 0.79% for [P₆₆₆₁₄][Bt], 3.62% for [P₆₆₆₁₄][3AT], 1.12% for [P₆₆₆₁₄][Br],
372 6.72% for [P₆₆₆₁₄][2,4,4], 2.98% for [P₆₆₆₁₄][Ntf₂] and 0.49% for [P₆₆₆₁₄][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 [P₆₆₆₁₄][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 [P₆₆₆₁₄][3AT] and
384 [P₆₆₆₁₄][Bt] (p = 0.71), and between [P₆₆₆₁₄][Ntf₂] and [P₆₆₆₁₄][N(CN)₂] (p = 0.14).

385 In contrast to the results obtained in this study, Lawniczak 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 CO₂ 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 phosphonium-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 [P₆₆₆₁₄][2,4,4] (contribution equal to
422 15.61%) and [P₆₆₆₁₄][Ntf₂] (contribution equal to 15.08%). Yet, only one IL, [P₆₆₆₁₄][N(CN)₂],
423 exhibited significantly lower CO₂ 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 [P₆₆₆₁₄] 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 [P₆₆₆₁₄][Br] and [P₆₆₆₁₄][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**

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

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453

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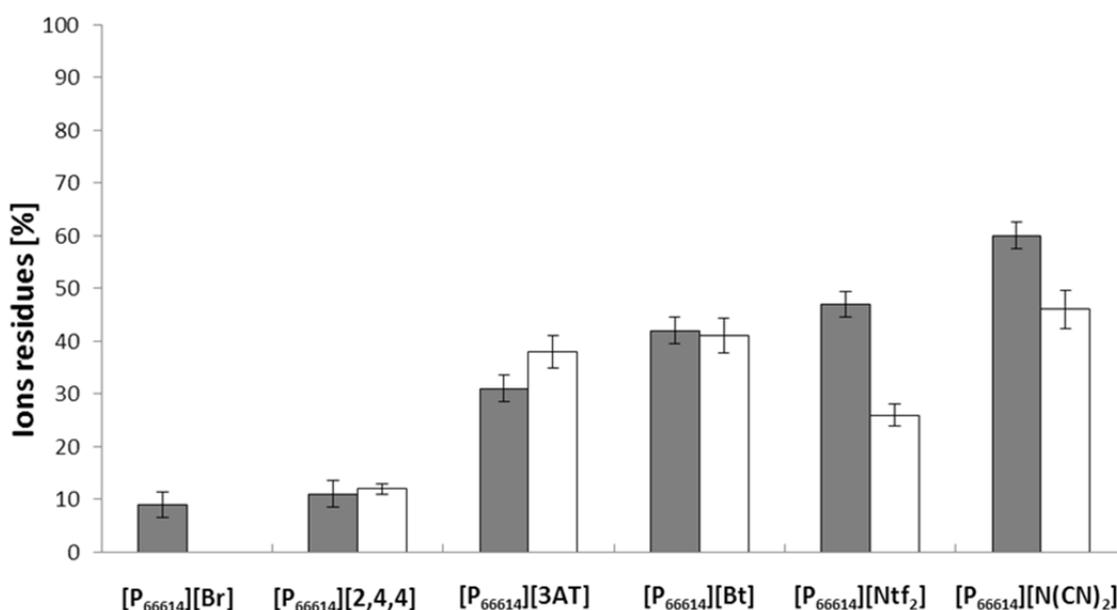
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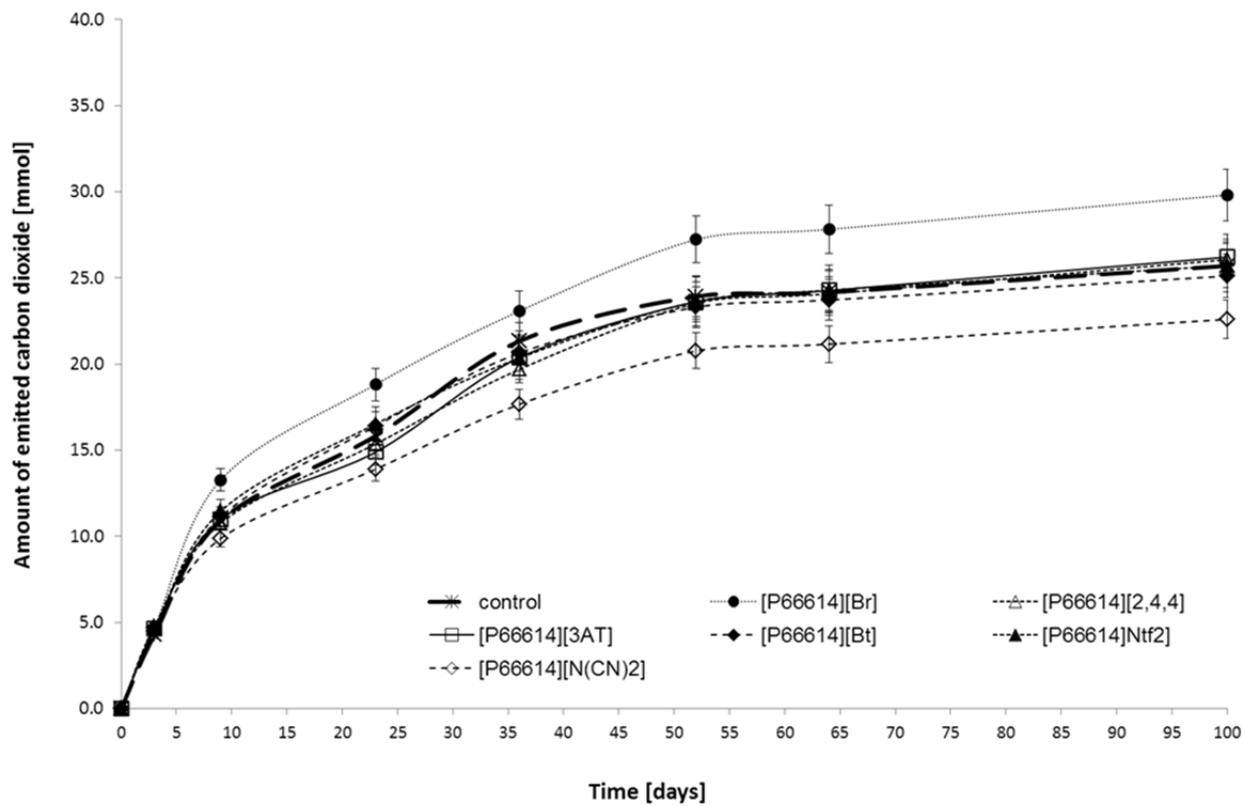
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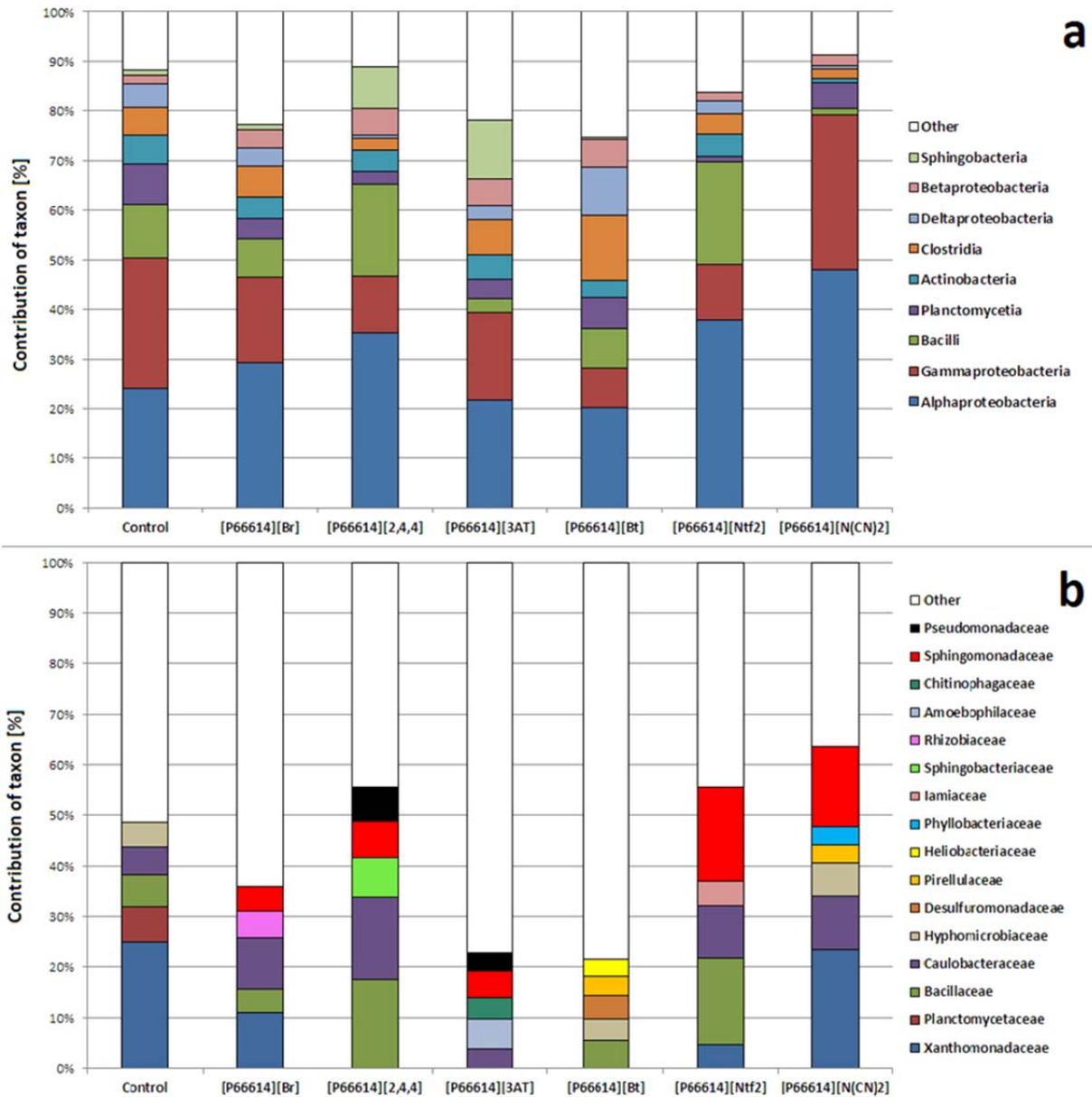


647 **Fig. 1.** The levels of ions residues of the selected phosphonium-based ILs after 100-day
648 experiment. Cation: grey bars, anions: white bars.

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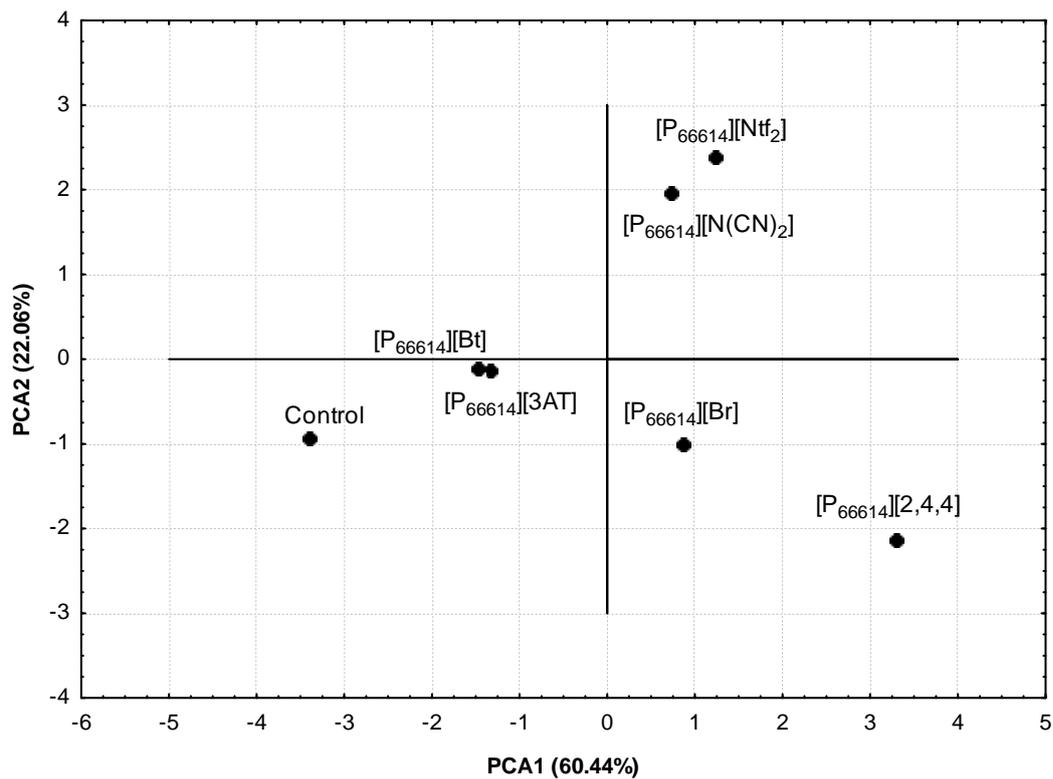
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 652 **Fig. 2.** The evolution of CO₂ during the course of 100-day experiment.
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654

655 **Fig. 3.** The contribution of the most dominant microbial groups inhabiting urban park soil
 656 spiked with phosphonium-based ILs after 100-day exposure, presented with respect to (a)
 657 class, and (b) family taxonomic level (to facilitate the reading only five most dominant
 658 families among each soil treatment were presented).

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661 **Fig. 4.** PCA plot representing the weighted Unifrac distances for control and soils treated with
 662 the studied phosphonium-based ILs.

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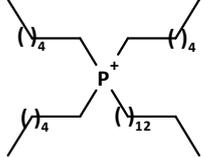
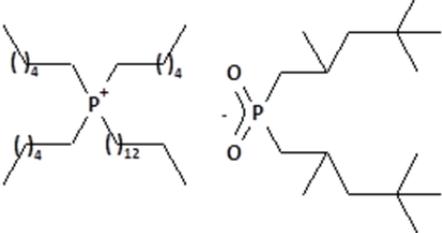
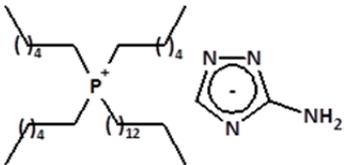
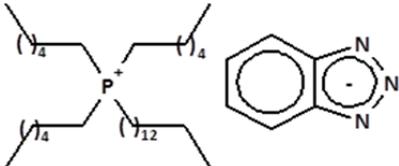
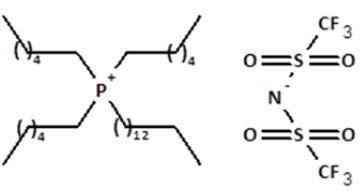
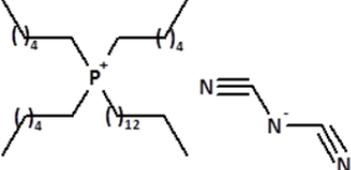
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679 **Table 1.** The acronyms, structures and names of the used phosphonium-based ILs.

Acronym	Structure	Full name
[P₆₆₆₁₄][Br]		trihexyl(tetradecyl)phosphonium bromide
[P₆₆₆₁₄][2,4,4]		tetradecyl(trihexyl)phosphonium bis(2,4,4-trimethylpentyl)phosphinate
[P₆₆₆₁₄][3AT]		trihexyl(tetradecyl)phosphonium 3-amino-1,2,4-triazolate
[P₆₆₆₁₄][Bt]		trihexyl(tetradecyl)phosphonium benzotriazolate
[P₆₆₆₁₄][Ntf₂]		tetradecyl(trihexyl)phosphonium bis(trifluoromethylsulfonyl)imide
[P₆₆₆₁₄][N(CN)₂]		tetradecyl(trihexyl)phosphonium (dicyano)imide

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Soil sample	OTU's observed	Shannon's index
Control	1399 ± 28 ^{b,c,d,e,f,g}	1.75 ± 0.04 ^{b,c,d,e,f,g}
P₆₆₆₁₄][Br]	1008 ± 62 ^{a,b,c,d,e}	1.34 ± 0,06 ^{a,b,c,d,g}
[P₆₆₆₁₄][2,4,4]	965 ± 55 ^{a,b,c,d,e}	0.73 ± 0,09 ^{a,b,c,d,e,f}
[P₆₆₆₁₄][3AT]	1205 ± 31 ^{a,e,f}	1.57 ± 0.08 ^{a,e,f,g}
[P₆₆₆₁₄][Bt]	1229 ± 43 ^{a,e,f}	1.59 ± 0.07 ^{a,e,f,g}
[P₆₆₆₁₄][Ntf₂]	1296 ± 58 ^{a,e,f}	1.48 ± 0,05 ^{a,e,f,g}
[P₆₆₆₁₄][N(CN)₂]	1258 ± 39 ^{a,e,f}	1.27 ± 0,04 ^{a,b,c,d,g}

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