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

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Abstract

Little is known on the effect of ionic liquids (ILs) on the structure of soil microbial communities and resulting biodiversity. Therefore, we studied the influence of six trihexyl(tetradecyl)phosphonium ILs (with either bromide or various organic anions) at sublethal concentrations on the structure of microbial community present in an urban park soil in 100-day microcosm experiments. The biodiversity decreased in all samples (Shannon's index decreased from 1.75 down to 0.74 and OTU's number decreased from 1399 down to 965) with the largest decrease observed in the microcosms spiked with ILs where biodegradation extent was higher than 80%. (i.e. [P$_{66614}$][Br] and [P$_{66614}$][2,4,4]). Despite this general decrease in biodiversity, which can be explained by ecotoxic effect of the ILs, the microbial community in the microcosms was enriched with Gram-negative hydrocarbon-degrading genera e.g. *Sphingomonas*. It is hypothesized that, in addition to toxicity, the observed decrease in biodiversity and change in the microbial community structure may be explained by the primary biodegradation of the ILs or their metabolites by the mentioned genera, which outcompeted other microorganisms unable to degrade ILs or their metabolites. Thus, the introduction of phosphonium-based ILs into soils at sub-lethal concentrations may result not only in a decrease in biodiversity due to toxic effects, but also in enrichment with ILs-degrading bacteria.

Keywords: biodegradation; ionic liquids; microbial community; biodiversity; toxicity; 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°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

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 $20^{\circ}C$. 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 $20^{\circ}C$ 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

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 CO_2 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 $18 \pm 2\%$. 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 CO_2 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 ([P_{66614}][Ntf_2]), SAMN07257077 (P_{66614}][Br]), SAMN07257078

174 ([P_{66614}][3AT]), SAMN07257079 ([P_{66614}][2,4,4]), SAMN07257080 ([P_{66614}][N(CN)$_2$]),

175 SAMN07257081 ([P_{66614}][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

subsamples were collected from each set. These subsamples were stored at −80 ℃ until further processing (less than two weeks). Each subsample was subjected to extraction of total DNA and further analyzes separately and the data obtained for each set of subsamples was combined. Total DNA was extracted from 500 mg of each soil using Genomic Mini AX Soil kit (A&A Biotechnology) according to manufacturer's instruction. Extracted DNA were quantified using Quant-iT HS ds.-DNA assai kit (Invitrogen) on Qubit2 fluorometer; 2 µl of extracts were examined on a 0.8% agarose gel.

2.5.2. Biodiversity assessment

For PCR amplification and calculation of OTU abundance and Shannon's index we followed the procedure presented in Ławniczak et al. (2016). Briefly, we targeted Region IV of bacterial 16S RNA gene amplified using a set of primers with Illumina adapters, followed by sequencing using primers as described in Caporaso et al. (2012). The sequencing was done using paired-end (2x250) MiSeq Reagent Kits v2 (Illumina, USA). For processing of the sequencing data, as in Lawniczak et al. (2016), we used CLC Genomic Workbench 8.5 and CLC Microbial Genomics Module 1.2. (Qiagen, USA), followed by clustering of the sequencing reads against the SILVA v119 99% 16S rRNA gene database (July 24, 2014, Quast et al. 2013). OTU abundance and Shannon's index were calculated for rarefaction analysis with a depth of 100 000 sequences per sample.

2.6. Biodegradation in soil

The biodegradation experiments in soil were carried out in sealed 1-L glass bottles as described in section.2.4. Therefore, the set-up for the tests consisted of 18 samples contaminated with ILs (i.e. 3 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 microcosms were incubated at 20°C for 100 days. After 100 days, one soil portion (0.5 g) from each bottle contaminated with IL was subjected to three-step ultrasound assisted extraction with methanol (3 x 1 mL) and analyzed by HPLC-MS.

In order to include sorbed fraction of the used ILs onto soil matrix during calculation of the ions residual masses, additional control tests with sterilized soil (to inhibit biodegradation) contaminated with ILs were performed. First, the urban park soil was divided into aliquots of 30 g, frozen, placed in sealed polyethylene bags and irradiated at 40,000 grey 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,

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, 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 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

14

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**

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

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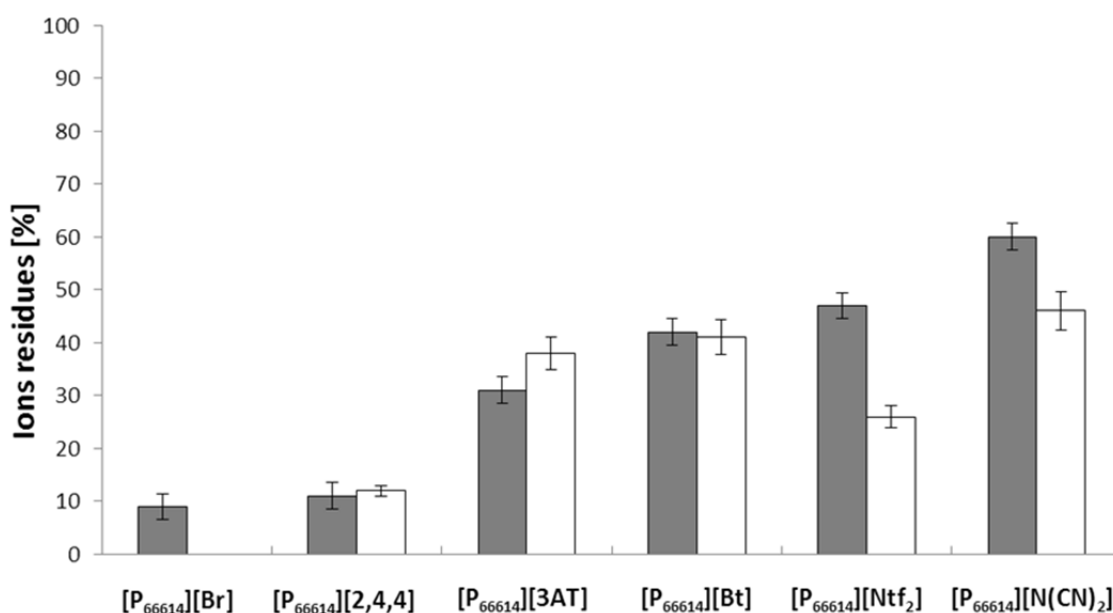
647
648 **Fig. 1.** The levels of ions residues of the selected phosphonium-based ILs after 100-day

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

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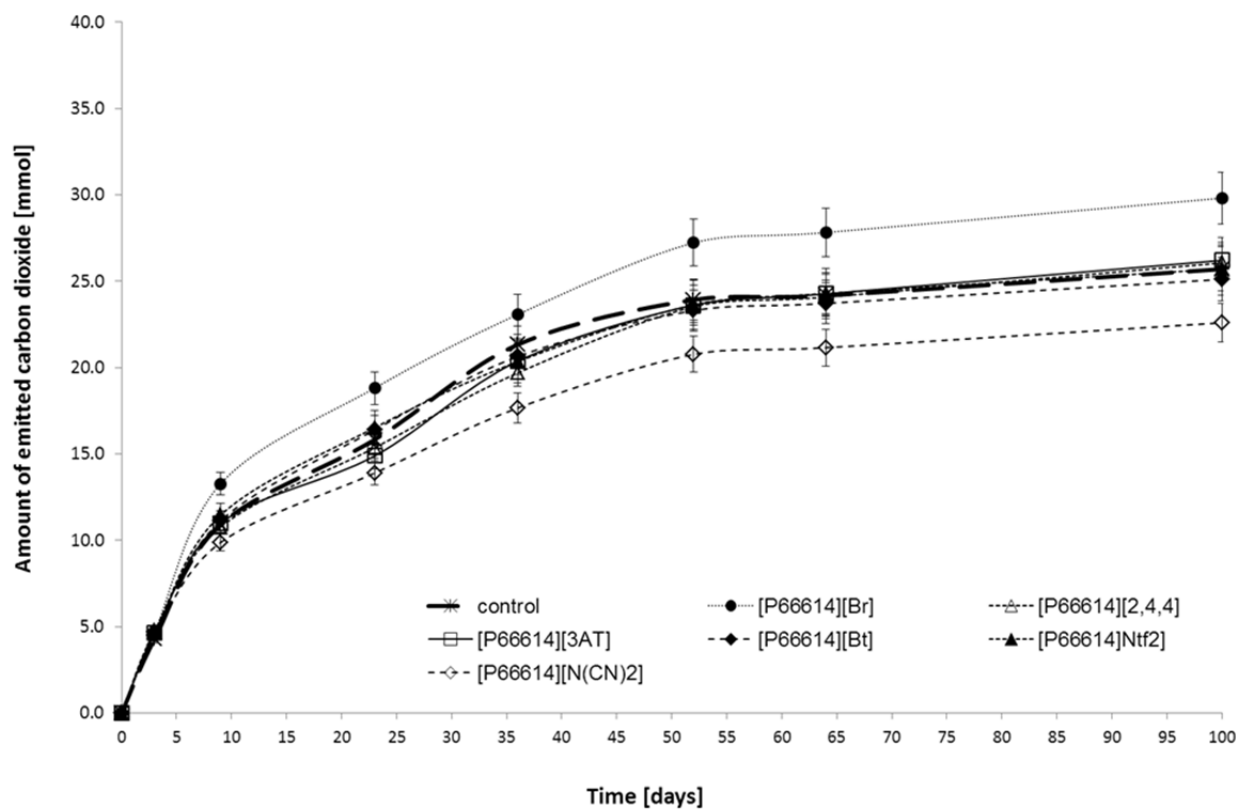
Fig. 2. The evolution of CO_2 during the course of 100-day experiment.
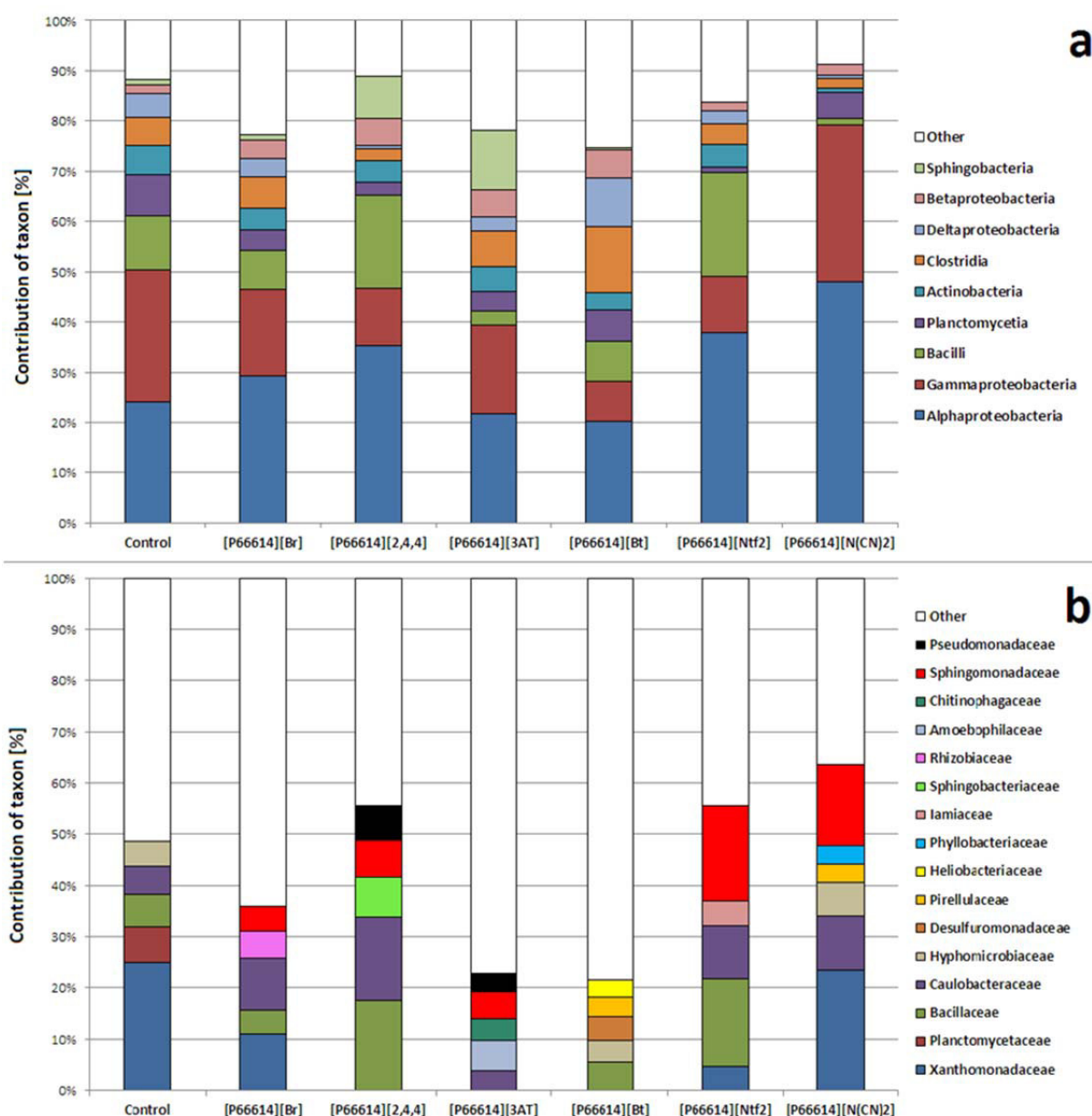
Fig. 3. The contribution of the most dominant microbial groups inhabiting urban park soil spiked with phosphonium-based ILs after 100-day exposure, presented with respect to (a) class, and (b) family taxonomic level (to facilitate the reading only five most dominant families among each soil treatment were presented).
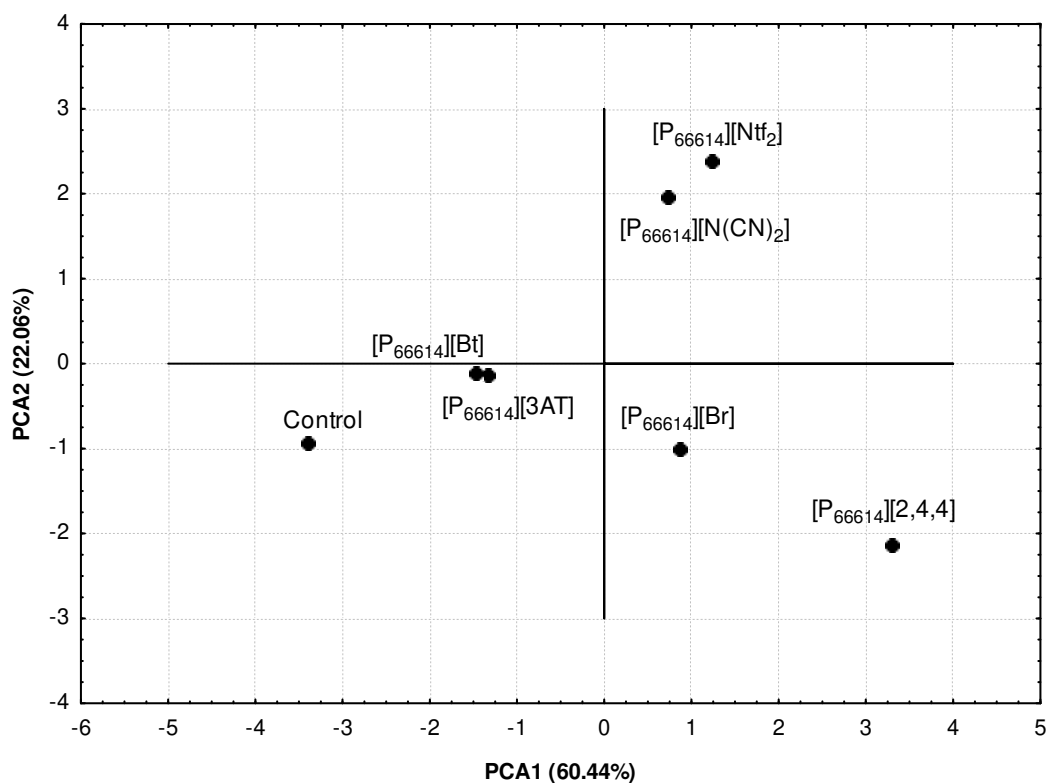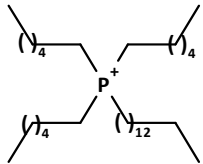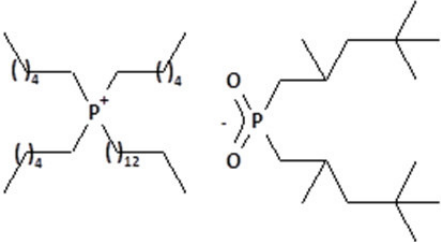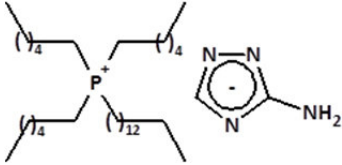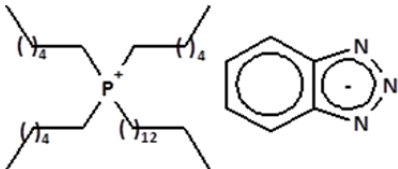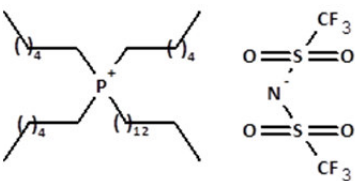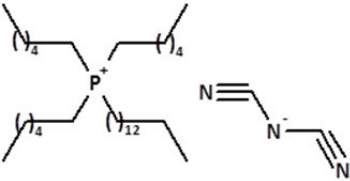
Fig. 4. PCA plot representing the weighted Unifrac distances for control and soils treated with the studied phosphonium-based ILs.

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

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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 $p \leq 0.05$.