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# 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$^{o}$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

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

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

## 2. Materials and methods

### 2.1. Chemical reagents

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

### 2.2. Characterization of soil

Mollic gley soil was collected from a city park in the center of Poznan city (N 52.4011445, E 16.9222993) in September 2013 from the depth of 10-20 cm according to the procedure 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$^{o}$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 ± 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

subsamples were collected from each set. These subsamples were stored at –80 ºC 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

selected IL was added to 10 g of sterilized soil. Next, methanol was evaporated and untreated sterilized soil in the amount of 90 grams was added. Finally, samples were mixed vigorously. The set-up for the sorption tests consisted of 18 samples contaminated with ILs (i.e. 3 samples for each IL) and 1 additional sample for monitoring of the soil moisture. All of the samples contained the base traps to provide constant moisture content of the soil (equal to 18 ± 2%). After 100 days of incubation at 20$^{o}$C under sterile conditions, 0.5 g portion of soil from each replicate was subjected to a three-step ultrasound assisted extraction with methanol (3 x 1 mL) and analyzed by HPLC-MS to determine the fraction of ILs that was not permanently sorbed onto soil matrix. During the calculation of ions residual masses, it was assumed that the value of sorbed fraction is constant and does not change over time. Moreover, we assumed linear relationship between sorption and concentration of the used ILs (although in reality the relationship is not linear), which is a simplified, but still relevant assumption for organic compounds (Moyo et al. 2014). The recovery efficiency of the extraction of the ILs from the soil matrix dependent on the used ILs and reached (with respect to both cation and anion) 99% for [$P_{6661}$4][2,4,4], 89% for [$P_{66614}$][Br], 89% for [$P_{66614}$][$Ntf_2$], 86% for [$P_{66614}$][$N(CN)_2$], 70% for [$P_{66614}$][3AT] and 73% for [$P_{66614}$][Bt].

### 2.7. HPLC-MS analysis

For HPLC-MS analysis of residual ions can we followed the procedure of Sydow et al. (2015). Briefly, three 1 mL soil extracts (all three obtained via three-step extraction of each soil sample) were combined, filtered through a 0.2 μm PTFE syringe filter and diluted with methanol : water solution (80:20 v/v). The HPLC-MS analyses were performed with the UltiMate 3000 RSLC chromatograph from Dionex (Sunnyvale, CA, USA). Five μL samples were injected into a Hypersil GOLD column (100 mm 2.1 mm I.D.; 1.9 μm) with a 2.1 mm I.D. pre-filter cartridge (0.2 μm) from Thermo Scientific (Waltham, MA, USA). The mobile phase consisted of $5x10^{-3}$ mol $L^{-1}$ ammonium acetate in water (phase A) and methanol (phase B) at a flow rate of 0.2 mL $min^{-1}$.

### 2.8. Evolution of $CO_2$ from the microcosms

The $CO_2$ evolution tests were carried out in the same sealed 1-L glass bottles as described in section 2.4. Overall, 18 soil samples contaminated with ILs (i.e. 3 samples for each IL), 3 soil samples for moisture monitoring and 3 control samples (containing 100 g of park soil spiked 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

studied ILs in urban soil. Following the approach presented in Horel and Schiewer (2011) and Sydow et al. (2015), a carbon mass balance was performed in order to estimate the mineralization of primarily degraded [$P_{66614}$][Br]. Assuming a yield of 0.4 g of microbial carbon per 1 g of IL carbon, it was calculated that approximately 42% of the primarily degraded [$P_{66614}$][Br] was mineralized. This corresponds to 31% mineralization of the total compound. For the majority of the used ILs, the measured $CO_2$ evolution was not statistically different as compared to control, which is in agreement with our previous study dedicated to biodegradability of other phosphonium- and ammonium-based ILs (Sydow et al. 2015). In that study, it was observed that the only mineralized compound was [$P_{66614}$][Cl] – the only IL with inorganic anion and a similar chemical composition to [$P_{66614}$][Br] (both are composed of halide anion). Although it is possible that the presence of complex organic anions attached to [$P_{66614}$] cation may have inhibited mineralization of the cation, and simultaneously, the whole compound, the opposite mechanism suggesting the negative influence of cation on biodegradation of organic anions cannot be excluded. Although the presence of long alkyl chains (as in the case of [$P_{66614}$] cation) may facilitate biodegradation of the whole compound, it was previously observed that such ions may simultaneously be more toxic than homologues with shorter chains (Stolte et al. 2011).

### 3.2. Structure of soil bacteria after exposure to ionic liquids

Figure 3a shows the contribution of dominant classes identified within soil microbial community after exposure to either of the studied ILs at sub-lethal concentrations. The obtained results indicate that the studied phosphonium-based ILs had significant influence on the structure of soil microbial community as the contribution of dominant classes changed after 100-day exposure. In four out of six soil samples spiked with ILs, the contribution of Alphaproteobacteria increased with the highest increase (by 24%) was observed for [$P_{66614}$][$N(CN)_2$]. The contribution of Gammaproteobacteria increased only in case of [$P_{66614}$][$N(CN)_2$] (by 5%), while the *Bacilli* class increased in case of [$P_{66614}$][2,4,4] and [$P_{66614}$][$Ntf_2$] (by up to 10%). The increase of contribution of *Clostridia* class was observed for [$P_{66614}$][Bt] (by 7%), [$P_{66614}$][3AT (by 2%)] and [$P_{66614}$][Br] (by 1%), while the contribution of Deltaproteobacteria increased only in case of [$P_{66614}$][Bt] (by 5%), Betaproteobacteria class slightly increased its contribution in case of all samples with exception of [$P_{66614}$][$Ntf_2$]. The contribution of *Sphingobacteria* class increased only in 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% ($p = 0.015$) of the contribution was represented by five most abundant bacterial families. By
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).

The lowest value of OTU's and Shannon's index was determined for $[P_{66614}][2,4,4]$ (OTU's = 965, Shannon's index = 0.73). In general, the introduction of the studied ILs contributed to significant reduction of the microbial biodiversity in soil. The PCA plot of weighted Unifrac distances indicate that the bacterial community structure changed significantly ($p = 0.017$) upon treatment with the studied ILs compared to control soil (Fig. 4). There were no significant differences between microbial structures of soils treated with $[P_{66614}][3AT]$ and $[P_{66614}][Bt]$ ($p = 0.71$), and between $[P_{66614}][Ntf_2]$ and $[P_{66614}][N(CN)_2]$ ($p = 0.14$).

In contrast to the results obtained in this study, Lawniczak et al. (2016) did not observe an effect of the herbicidal ionic liquids on biodiversity (both OTU's and Shannon's index were not significantly different from the control). However, similarly to our study, they observed that the structure of the community (assessed using Illumina NGS) was significantly affected (on the phylum level) by the exposure to herbicidal ILs. This difference may be explained by either shorter exposure time (100 days in this study, 28 days in Ławniczak et al. (2016)) not allowing occurrence of significant changes in biodiversity, or differences in applied ILs concentration and differences in initial biodiversity of the studied soils (control Shannon's index in this study was 1.75, while control Shannon's index in Ławniczak et al. (2016) was 4.95). A higher biodiversity is usually associated with higher resistance to different perturbations (Isbell et al. 2015). Deive et al. (2011) also observed a decrease in biodiversity of microbial communities isolated from the salt marsh and industrial soils exposed to various imidazolium- and cholinium-based ILs. The authors observed higher survival of microbial strains isolated from industrial soils, which were contaminated in the past by petroleum hydrocarbons. Also Sun et al. (2017) observed a significant decrease in biodiversity and alternation of the structure of soil microbial community exposed to 1-octyl-3-methylimidazolium tetrafluoroborate for 40 days. On the other hand, Guo et al. (2015) observed a significant decrease in biodiversity of soil microbial community only in higher concentrations of the alkyl-imidazolium-based ionic liquid with chloride anion.

### 3.3. Explaining changes in community structure and decrease in biodiversity

Basing on the genetic assay and biodegradation and $CO_2$ evolution experiments, it is hypothesized that decrease in biodiversity is explained by a combination of two factors (i) a toxic effect of the phosphonium-based ILs or their metabolites towards non-resistant microbial taxa within the community, and/or (ii) an emergence of few ILs-degrading taxa, 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**

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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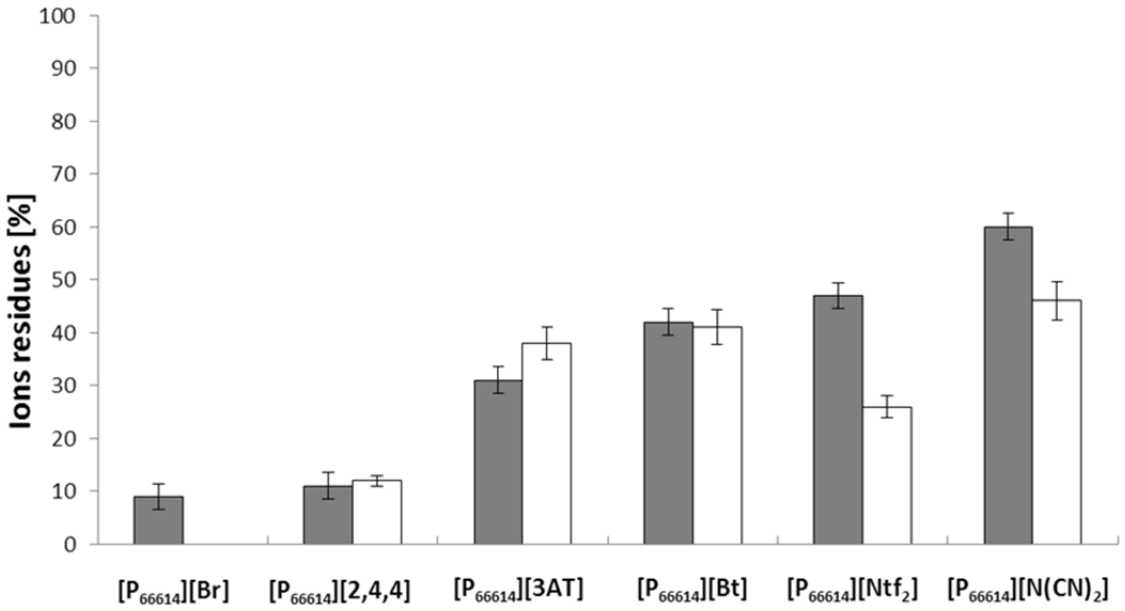
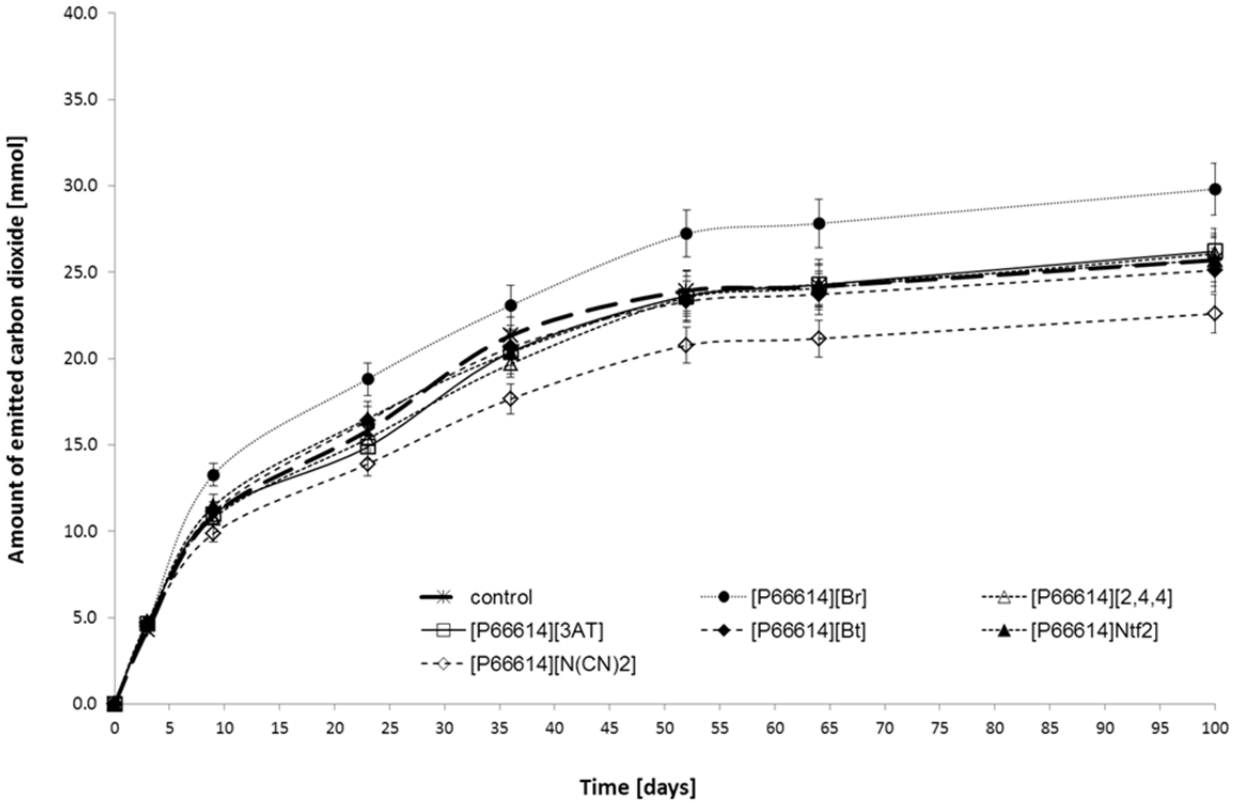



647
648 **Fig. 1.** The levels of ions residues of the selected phosphonium-based ILs after 100-day
649 experiment. Cation: grey bars, anions: white bars.
650




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

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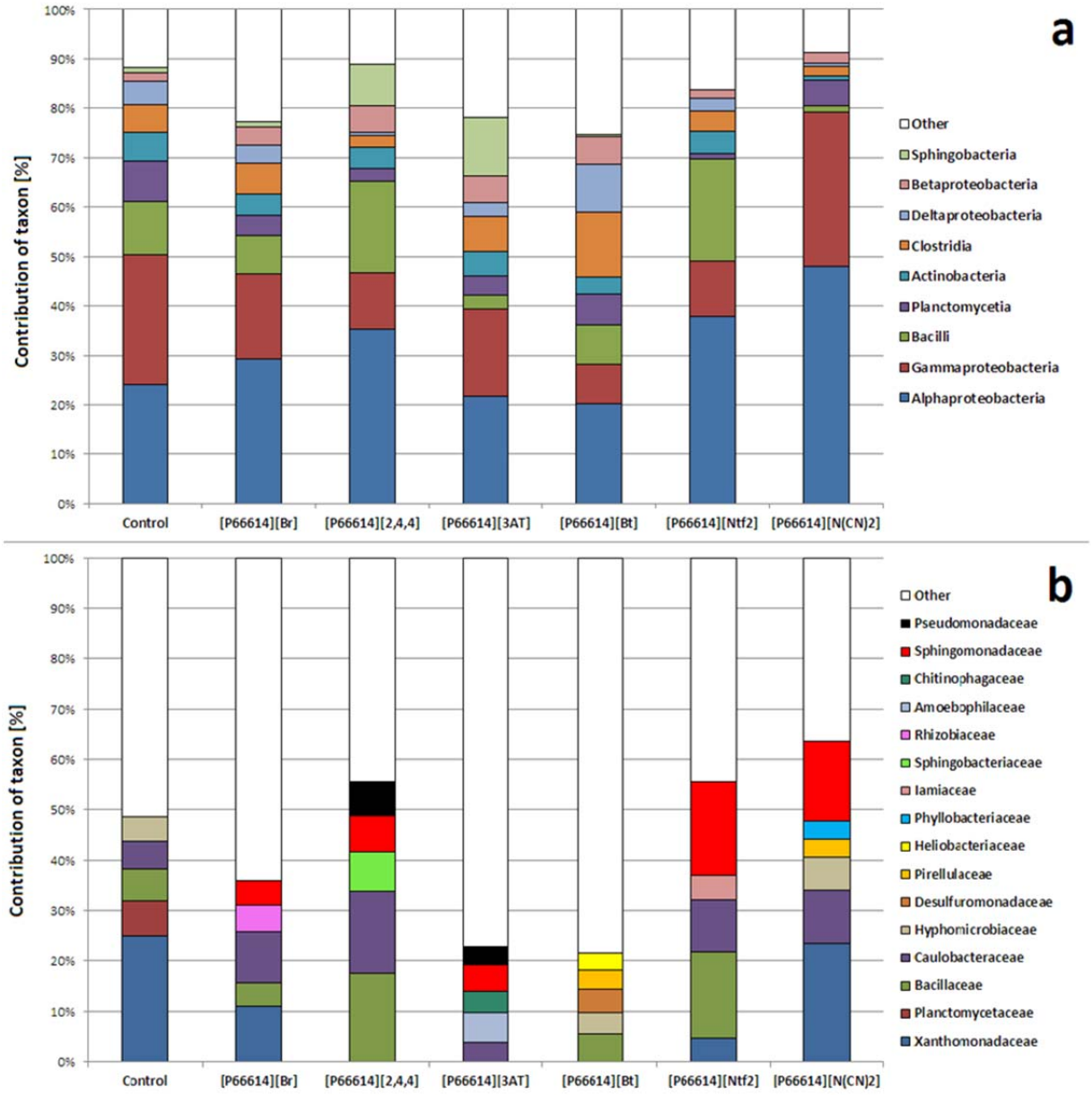



654

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).

659

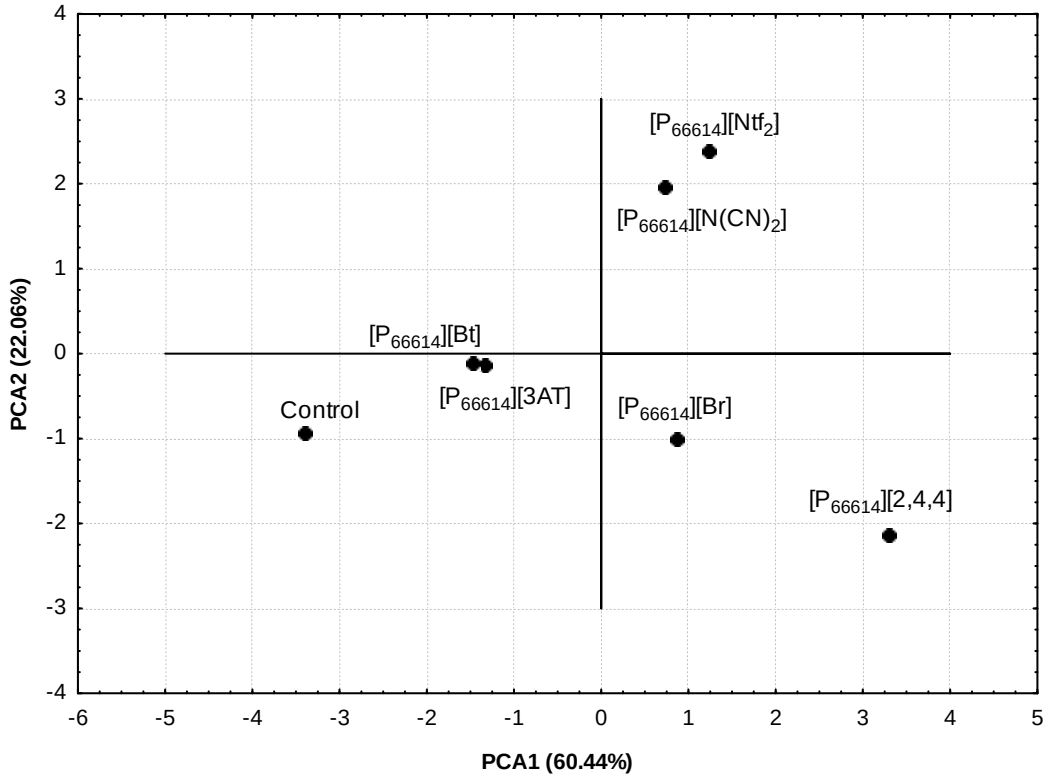
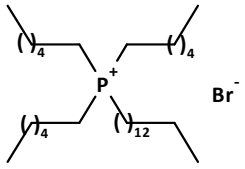
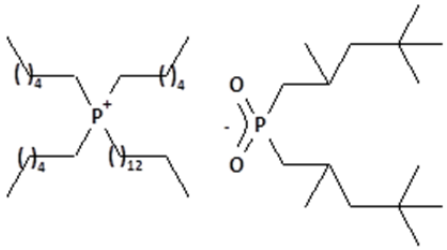
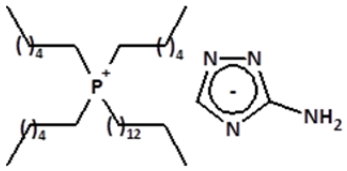
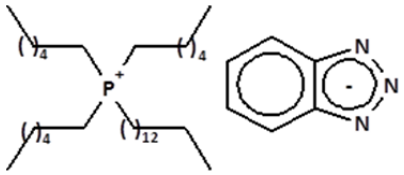
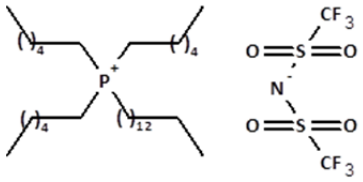
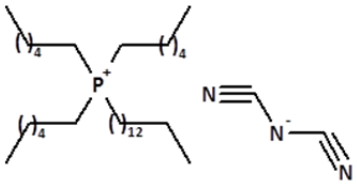



**Fig. 4.** PCA plot representing the weighted Unifrac distances for control and soils treated with the studied phosphonium-based ILs.

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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683

| 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 $p \leq 0.05$.