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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 about 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.

## 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., 2012, 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

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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 °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 g 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 (20 g 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