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Sydow, Mateusz; Szczepaniak, Zuzanna; Framski, Grzegorz; Staninska, Justyna; Owsianiak, Mikolaj; Szulc, Alicja; Piotrowska-Cyplik, Agnieszka; Zgoła-Grześkowiak, Agnieszka; Wyrwas, Bogdan; Chrzanowski, Lukasz

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6 Persistence of selected ammonium- and phosphonium-based ionic liquids in urban park soil 7 microcosms

- 8
- 9 Mateusz Sydow¹*, Zuzanna Szczepaniak², Grzegorz Framski³, Justyna Staninska⁴, Mikołaj
- 10 Owsianiak⁵, Alicja Szulc¹, Agnieszka Piotrowska-Cyplik², Agnieszka Zgoła-Grześkowiak¹,
- 11 Bogdan Wyrwas¹, Łukasz Chrzanowski¹
- 12
- ¹Faculty of Chemical Technology, Poznan University of Technology, Berdychowo 4, 60-965
- 14 Poznań, Poland
- ¹⁵ ²Institute of Food Technology of Plant Origin, Poznan University of Life Sciences, Wojska
- 16 Polskiego 31, 60-624 Poznań, Poland
- ¹⁷ ³Institute of Bioorganic Chemistry, Polish Academy of Sciences, Noskowskiego 12/14, 61-704
- 18 Poznań, Poland
- ⁴Department of Biotechnology and Food Microbiology, Poznan University of Life Sciences,
- 20 Wojska Polskiego 48, 60-627 Poznań, Poland
- ⁵Division for Quantitative Sustainability Assessment, Department of Management Engineering,
- 22 Technical University of Denmark, Produktionstorvet, Building 424, DK-2800 Kgs. Lyngby,
- 23 Denmark
- 24
- 25 *Corresponding author. Tel.: +48 61 665 37 16; fax: +48 61 665 36 49.
- 26 *E-mail address:* mateusz.sydow@gmail.com (M. Sydow)
- 27

28 Abstract

- 29 Knowledge about biodegradability of ionic liquids (ILs) in terrestrial systems is limited. Here,
- 30 using urban park soil microcosms spiked with either ammonium- or phosphonium-based ILs
- 31 [didecyldimethylammonium 3-amino-1,2,4-triazolate, benzalkonium 3-amino-1,2,4-triazolate,
- trihexyl(tetradecyl)phosphonium chloride, or trihexyl(tetradecyl)phosphonium 1,2,4-triazolate],
- 33 we studied their (i) 300-day primary biodegradation, and (ii) influence on CO_2 evolution from the
- 34 microcosms. The primary biodegradation ranged from 21 to 33% of total compound in the
- dissolved phase. The evolution of CO_2 from spiked microcosms was either lower or within the
- 36 range of background soil respiration, indicating no or small mineralization of the parent

37 compounds and/or their metabolites, and their negligible or small toxicity to soil microorganisms.

- 38 Our results suggest the potential for persistence of the four studied ILs in urban park soils.
- 39

40 **Keywords:** impact assessment; ionic liquids; risk assessment; terrestrial toxicity;

41 biotransformation;

42

43 **1. Introduction**

Ionic liquids (ILs) are a group of chemicals composed of an organic cation and an organic or 44 inorganic anion. Due to a large number of cation - anion combinations, the number of ILs which 45 may be obtained is tremendous (up to 10^{18}) (Rogers and Seddon 2003). Various ILs are currently 46 47 applied in catalysis and biocatalysis, and in organic synthesis (e.g., multi-ton scale use of imidazolium-based IL in the BASILTM process developed by BASF) (Plechkova and Seddon 48 49 2008; Quijano et al. 2010). Although there are no reports providing information about the presence of ILs in water or soil ecosystems yet, the increasing use of ILs in the industry is 50 51 expected to lead to environmental pollution. Consequently, there has been an increasing number of studies dealing with ILs behaviour and impact on the environment (Ranke et al. 2007; 52 53 Petkovic et al. 2010; Pham et al. 2010; Cvjetko Bubalo et al. 2014).

Low volatility of ILs excludes emission into air as an exposure route, but there can be 54 direct release routes to freshwater or soil. It is generally thought that relatively high thermal and 55 chemical stability of ILs contributes to their low biodegradability (Quijano et al. 2011; Neumann 56 et al. 2012), primarily on aquatic environments (Coleman and Gathergood 2010; Ford et al. 2010; 57 Stolte et al. 2011). In soils, Modelli et al. (2008), demonstrated that at the end of a 180-day 58 respirometric experiment, none of the four imidazolium-based ILs was mineralized completely, 59 and the amount of CO₂ evolved depended on both the type of cation and the type of anion. 60 Primary biodegradation, defined as an alteration in the chemical structure of a substance, brought 61 about by biological action, resulting in the loss of a specific property of that substance (OECD 62 301, 1992), has been studied and formation of biotransformation products has also been 63 64 documented (Stolte et al. 2008; Pham et al. 2009; Docherty et al. 2010). Sorption of ILs onto the soil matrix can occur, depending on soil parameters (such as organic carbon content or cation 65 exchange capacity), which can lead to a decrease of their bioavailability and biodegradability and 66 contribute to the potential persistence (Stepnowski et al. 2005; Studzińska et al. 2008; Mrozik et 67

al. 2012). Most published studies focused on toxicity of ILs to single, isolated microbial strains

69 (Pham et al. 2010; Cvjetko Bubalo et al. 2014), but little is known about the influence of ILs on

- the activity of soil microbiota. Recently, Peric et al. (2014), showed that aprotic ILs inhibited soil
- respiration in the concentration range from 100 mg kg⁻¹ to 10,000 mg kg⁻¹ depending on the ILs
- 72 structure.

The aim of our study was to investigate primary biodegradation of ammonium- and 73 74 phoshonium-based ILs in urban park soil microcosms, simulating an accidental spill, and their influence on CO_2 evolution from the microcosms. The four ILs studied were: (i) 75 didecyldimethylammonium 3-amino-1,2,4-triazolate ([DDA][3AT]), (ii) benzalkonium 3-amino-76 77 1,2,4-triazolate ([BDA][3AT]), (iii) trihexyl(tetradecyl)phosphonium chloride ([P₆₆₆₁₄][Cl]), and (iv) trihexyl(tetradecyl)phosphonium 1,2,4-triazolate ([P₆₆₆₁₄][Tr]). Apart from [P₆₆₆₁₄][Cl], the 78 studied ILs have antifungal properties. The triazole-based ILs were previously described as novel 79 80 anti-microbial and anti-fungal plant protection agents with a broad spectrum of activity (Walkiewicz et al. 2010), and are thus expected to be toxic to the microorganisms present in the 81 82 soil. An urban park soil of known biodegrading potential toward petroleum hydrocarbons (Sydow et al. unpublished results) was employed. The primary biodegradation (300-day) was studied by 83 measuring ILs' residues in the soil, while CO₂ evolution from spiked microcosms was measured 84 using a respirometric test. 85

86

87 2. Materials and methods

88

89 **2.1. Synthesis of ionic liquids**

The ammonium-based ILs were synthesised with the use of quarternary ammonium bromides, 90 which were obtained in reaction of equimolar amount of amine (decyldimethylamine or 91 benzyldimethylamine) with appropriate bromoalkane (1-bromodecane or 1-bromododecane 92 respectively). The reaction was carried out using anhydrous acetonitrile as solvent at 70°C for 24 93 h. After cooling the reaction mixture to room temperature, quaternary ammonium bromides were 94 precipitated by adding cold ethyl acetate. The bromides were filtered and dried under vacuum at 95 50°C. Product yield was over 95%. Next, 3-amino-1,2,4-triazolate ILs were prepared by in situ 96 deprotonation of corresponding azole with sodium or potassium hydroxide in methanol and 97 mixed with an equimolar amount of appropriate quaternary ammonium bromides, according to 98

- the method described in Walkiewicz et al. (2010). The phosphonium-based ILs were prepared
 according to method described by Cieniecka-Rosłonkiewicz et al. (2005). Briefly,
- 101 trihexyl(tetradecyl)phosphonium chloride was prepared in reaction of trihexylphosphine and 1-
- 102 bromotetradecane. The 1,2,4-triazolate was obtained by *in situ* deprotonation of corresponding
- azole with sodium hydroxide in methanol, followed by reaction with chloride precursor. Water
- 104 content of the synthesized ILs was determined by Karl Fisher method for all prepared
- 105 compounds, and was less than 0.1%. The list of the studied ILs is given in Table 1.
- 106

107 **2.2. Soil**

108 The soil used in the experiments was collected from a city park in Poznań, Poland (N

- 109 52.4011445, E 16.9222993) and according to Unified Soil Classification System, was
- 110 characterized as fine grained silt loam type OL belonging to organic silts and organic silty clays
- of low plasticity. The composition of experimental soil was as follows: clay 4 ± 1 [%]; silt 83 ± 3
- 112 [%]; sand 13 ± 2 [%]. Detailed characteristics of the soil: organic carbon 5.44 ± 0.31 [g kg⁻¹];
- 113 nitrogen 0.57 ± 0.07 [g kg⁻¹]; phosphorous 0.080 ± 0.005 [g kg⁻¹]; pH 6.95 ± 0.7; bulk density
- 114 1.41 ± 0.06 [Mg m⁻³]; porosity 0.455 ± 0.03 [m³ m⁻³]; moisture 18 ± 1 [%]; cation exchange
- 115 capacity 22.1 ± 0.8 [cmol_c kg⁻¹]. A sign \pm represents standard deviation (n = 3).
- 116

117 2.3.1. Primary biodegradation

Each primary biodegradation experiment was set up with four replicates. Three replicates were 118 119 used to analyse ions residues with the use of HPLC-MS, while one replicate was used for moisture monitoring over the course of the experiment. The samples were prepared as follows: 10 120 g of non-sterilized soil was added into bottles and then spiked with a methanol solution of each 121 IL to reach an approximate concentration of 4000 mg kg⁻¹ of IL per sample. The concentration in 122 this range is typically used in hydrocarbon biodegradation tests (Lisiecki et al. 2014). Next, 123 methanol was evaporated with nitrogen. Afterwards, untreated soil in the amount of 90 grams 124 125 was added. The soil was later vigorously mixed. Finally, the microcosms were incubated at 20°C for 300 days. The soil moisture was determined once a week by using moisture analyser 126 (RADWAG MA 110.R, Poland), and adjusted to 18% if necessary. After 300 days three 0.5 g 127 samples of each bottle replicate (giving nine sampling points for each studied IL) were subjected 128 to three-step ultrasound assisted extraction with methanol (3 x 1 mL) and analysed by HPLC-MS 129

to determine the residual masses of ILs ions. Controls were prepared as above, except that the 130 131 urban soil was previously sterilized in order to investigate the influence of sorption on primary biodegradation of the compounds. The soil was divided into aliquots of 30 g, frozen, placed in 132 sealed polyethylene bags and irradiated at 40,000 grey using a ¹⁹²Ir source (Alef and Nannipleri 133 1995). Afterwards, a solution of selected IL was added to 10 g of sterilized soil. Next methanol 134 was evaporated and untreated sterilized soil in the amount of 90 grams was added and samples 135 were mixed vigorously. After 300 days of incubation under sterile conditions, soil from each 136 replicate was used in order to determine the fraction of ILs that was not sorbed onto soil matrix. 137 For this purpose three 0.5 g samples of each bottle replicate (giving nine samples in total) were 138 subjected to three-step ultrasound assisted extraction with methanol (3 x 1 mL) and analysed by 139 HPLC-MS. 140

The primary biodegradation of selected ILs was calculated with regard to initial masses of 141 142 ILs at the beginning of the experiment, residual masses of cations and anions at the end of the experiment and percentage values of ILs fraction sorbed onto studied soil. It was not possible to 143 144 determine the residual anion amount for [P₆₆₆₁₄][Tr], since the employed analytical method did not allow it. The residues of [Cl] anion were not analysed, since halide ILs undergo hydrolysis 145 146 reaction in the environment. Therefore, in the case of both phosphonium-based ILs primary biodegradation was calculated ignoring the final mass of the anion. Thus, primary biodegradation 147 148 of ammonium- and phosphonium-based ILs cannot be compared directly. The primary 149 biodegradation was calculated as presented in Eq. 1.

- 150
- 151

$$PB = 100\% - \frac{M_{C} \cdot X_{C} + M_{A} \cdot X_{A}}{M_{II}} - FS_{IL}$$
(Eq. 1)

eriment [g];

152 where:

153	PB – prii	nary	biodegr	adat	tion o	of IL [[%];		
154	M_C – res	idual	mass of	f cat	tion a	t the	end	of th	e exp
				<u>c</u>				0.1	

- 155 M_A residual mass of anion at the end of the experiment [g];
- 156 M_{IL} initial mass of IL at the beginning of the experiment [g];
- 157 X_C percentage of molar mass of cation in overall molar mass of IL [%];
- 158 X_A percentage of molar mass of anion in overall molar mass of IL [%];
- 159 FS_{IL} the sorbed fraction of IL onto soil matrix [%].

161 The sorbed fraction of each IL was calculated with regard to mass of extracted IL and mass of162 introduced IL as presented in Eq. 2.

163

$$FS_{IL} = \left(1 - \frac{M_E}{M_I}\right) \cdot 100\% \tag{Eq. 2}$$

165 where:

166 M_E – mass of extracted IL [g];

 M_I – mass of introduced IL [g].

168

169 **2.3.2. HPLC-MS analysis**

Three 1 mL soil extracts of each sampling point (obtained via three-step extraction of each 0.5 g 170 soil sample) were combined, filtered through a $0.2 \,\mu m$ PTFE syringe filter and diluted with 171 methanol : water solution (80:20 v/v). The HPLC-MS analyses were performed with the UltiMate 172 3000 RSLC chromatograph from Dionex (Sunnyvale, CA, USA). Five µL samples were injected 173 174 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 175 5x10⁻³ mol L⁻¹ ammonium acetate in water (phase A) and methanol (phase B) at a flow rate of 0.2 176 mL min⁻¹. Gradient elution was performed by linearly increasing the percentage of phase B from 177 85 to 100% in 4 min and maintained at 100% for 3 min. The LC column effluent was directed to 178 179 the API 4000 QTRAP triple quadrupole mass spectrometer from AB Sciex (Foster City, CA, 180 USA) through the electrospray ionization source (Turbo Ion Spray) that operated in positive ion 181 mode for analyses of cations and in negative ion mode for analyses of anions. The dwell time for each mass transition detected in the MS/MS multiple reaction monitoring mode was set to 200 182 183 ms. Nitrogen was used as curtain gas (10 psi), nebulizer gas (40 psi), auxiliary gas (45 psi) and collision gas (medium). The source temperature was 400°C and the ion spray voltage was 4500 V 184 185 for cations and -4500 V for anions. The declustering potential was 50 V for cations and -50 V 186 for anions.

187

188 2.4. Evolution of CO₂ from the microcosms

Each experiment was performed in triplicate. Microcosms were prepared as follows: 10 g of non-189 190 sterilized soil was added to 1L glass SIMAX bottles closed with PP screw caps and later spiked with a methanol solution of each IL to reach an approximate concentration of 4000 mg kg⁻¹ of IL 191 per sample. Next, methanol was evaporated with nitrogen. Afterwards, 90 grams of untreated soil 192 were added and the soil was vigorously mixed. Finally, the microcosms were incubated at 20° C 193 for 300 days. The amount of emitted CO₂ was determined according to respirometric tests 194 procedure. Additionally, a set of three control replicates - bottles containing 100 g of non-195 196 sterilized urban park soil without any addition of IL – was prepared. The controls were prepared to investigate the background respiration of the used soil. Natural soil respiration was determined 197 according to respirometric tests procedure. 198

The evolution of CO_2 in each soil sample was determined periodically by measuring CO_2 content in base traps (10 mL of 0.75 M NaOH in a 20-mL vial) placed in the microcosms (bottles with the used urban park soil) as described elsewhere (Szulc et al. 2014; Lisiecki et al. 2014). Each bottle contained one base trap. NaOH and Na₂CO₃ from each trap were titrated 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.

205

206 **2.5. Statistical analyses**

207 One-way analyses of variance (ANOVA), α =0.05 were performed to detect significant 208 differences between the treated groups and the respective controls. All of the presented error bars 209 represent standard errors of the mean (for primary biodegradation tests n = 9, for CO₂ evolution 210 tests n = 3).

211

212 **3. Results and discussion**

Below, we present results for primary biodegradation, sorption, CO₂ evolution, and carbon

balance of the four studied ILs in soil microcosms. Formation of biotransformation products and

215 potential biotransformation mechanisms, are then discussed.

216

217 **3.1.** Primary biodegradation and sorption

Figure 1a shows that 300-day primary biodegradation of the four studied ammonium- and 218 phosphonium-based ILs in urban park soil microcosms was small. It reached 33 and 21% of the 219 total compound in the dissolved phase for the two ammonium-based ILs ([DDA][3AT] and 220 [BDA][3AT], respectively), and 25 and 29% for the two phosphonium-based ILs ($[P_{66614}][Cl]$ 221 and [P₆₆₆₁₄][Tr], respectively). Figure 1b shows that the sorbed fraction of the studied ILs in soils 222 was generally higher for the two ammonium-based ILs, reaching 44 and 64% for [DDA][3AT] 223 and [BDA][3AT], respectively, as opposed to 13 and 29% for [P₆₆₆₁₄][Cl] and [P₆₆₆₁₄][Tr], 224 respectively. Thus, the IL with longer alkyl chain (i.e., [BDA][3AT]) exhibited higher sorption 225 226 onto soil as compared to the shorter-chain alternative (i.e., [DDA][3AT]), and furthermore the IL with inorganic anion (i.e., $[P_{66614}][C1]$) is the least sorbed among the four studied ILs. Both 227 228 findings are in agreement with Studzinska et al. (2008) who showed how sorption of ILs onto soil depends on the side chain length and on the cation-anion combination. In soils, biodegradation of 229 organic compounds can be influenced by their bioavailability for microbial uptake from the 230 dissolved phase (Alexander 2000). However, differences in sorption between the studied ILs do 231 232 not translate directly into apparent differences in primary biodegradation, suggesting that the persistence of the four ILs is rather due to their low inherent biodegradability than due to 233 234 bioavailability restrictions in the soil matrix.

235

3.2. Evolution of CO₂ and carbon balance

Figure 2a shows that CO₂ evolution from spiked microcosms was slightly below or within range 237 of background soil respiration, indicating no or small negative effect of the studied ILs on 238 respiratory activity of microbiota in the soil. Only in the case of [BDA][3AT], were statistically 239 240 significant differences between the microcosms spiked with ILs and the control microcosm observed. These findings from a long-term soil microcosm experiment are not in disagreement 241 with findings from biodegradation tests performed in aquatic systems for structurally similar ILs. 242 In a manometric respiratory test with activated sludge, Pisarova et al. (2012) showed that one 243 (i.e., (2-hydroxyethyl)-trimethylammonium methanesulphonate) out of three ammonium-based 244 245 ILs reached 74% of ultimate biodegradation within 28 days. The two other ILs (i.e., tributylmethylammonium methanesulphonate and butyl-trimethylammonium methanesulphonate) 246 reached 7 and 4%, respectively. In a CO_2 headspace test, also Atefi et al. (2009) showed that 247

ultimate biodegradation of various tri-*n*-hexylphosphonium-based ILs in an aqueous medium
after 28 days ranged from 0 to 30%, depending on the side chain functionality.

The largest CO_2 evolution was measured for the microcosms spiked with [P₆₆₆₁₄][Cl]. We 250 therefore performed carbon balance to see how much of the primarily biodegraded [P₆₆₆₁₄][Cl] 251 could be mineralized. Even if the CO₂ evolution from that microcosms had been statistically 252 different from that measured for the control, and assuming yield of 0.4 g of microbial carbon per 253 1 g of IL carbon, 63% of the primarily biodegraded [P₆₆₆₁₄][Cl] in the dissolved phase could be 254 mineralized. This corresponds to 16% mineralization of total compound. For the three other ILs, 255 256 measured CO₂ evolution was much lower compared to theoretical CO₂ evolution expected if all primarily biodegraded ILs had been mineralized (and assuming a yield of 0.4 g of microbial 257 258 carbon per 1 g of IL) (Figure 2b). This confirms no, or very small, ultimate biodegradation of the four studied ILs in our soil microcosms and suggests that biotransformation products must have 259 260 formed in the microcosms. Further, one IL (i.e., [BDA][3AT]), showed CO₂ evolution values lower as compared to the control, suggesting some, albeit small, inhibition of soil microbial 261 activity in the microcosm spiked with this IL at total concentration of 4000 mg kg⁻¹. Peric et al. 262 (2014) showed, that for various imidazolium-based ILs, inhibition of respiratory activity of the 263 soil can occur in concentration range of 100 to 10,000 mg kg⁻¹, depending on the IL structure. 264 265

266 **3.3. Potential biotransformation mechanisms**

Metabolites of primary biodegradation of the studied ILs were expected to form in our 267 microcosms, because all four ammonium- and phosphonium-based ILs have long, unbranched 268 alkyl chains attached to the nitrogen or phosphorus atom in the organic cation. Stolte et al. (2008) 269 270 already showed that ω -oxidation on the terminal methyl group in alkyl chains of imidazoliumbased ILs occurs, leading to the formation of shorter-chained products with carboxylic group. 271 Also Pham et al. (2009) and Zhang et al. (2011) showed gradual degradation of ILs' alkyl chains 272 during biotransformation by activated sludge, or by Pseudomonas fluorescens, in aquatic media. 273 274 In addition, hydroxylation of the aromatic ring of pyridinium-based ILs has also been reported 275 (Docherty et al. 2010), just as in case of triazole pesticides (WHO 2006). Therefore, similar biotransformation mechanism cannot be ruled out for the three ILs containing triazolate-based 276 anion. Short-chained metabolites of ILs biotransformation typically have lower biodegradability 277 as compared to the parent compound (Jastorff et al. 2003; Neumann et al. 2014), which can 278

- explain primary biodegradation of up to ca. 30% in our systems with no or small mineralization.
- Although some potential biotransformation products, such as aldehydes or ethanol are toxic
- toward microorganisms, (Stolte et al. 2008; Pham et al. 2009; Zhang et al. 2011),
- biotransformation products with shorter side chains and ring hydroxylation products are typically
- less toxic as compared to the parent compound (Docherty et al. 2010, Ranke et al. 2004; Stolte et
- al. 2011). This may explain why no or very small inhibition of CO_2 evolution from spiked
- samples occurred during the course of the biodegradation experiment.
- 286

287 **4. Conclusions**

288 We showed that the four studied ammonium- and phosphonium-based ionic liquids were

- persistent in urban park soil microcosms. Primary biodegradation ranging from 21 to 33% of total
- 290 compound in the dissolved phase, combined with CO_2 evolution from the microcosms being in
- range of soil background respiration rate, suggest no or very small mineralization of the primary
- compounds and/or their potential metabolites and no or negligible toxicity to soil
- 293 microorganisms.
- 294

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422 Figure captions

- 423 Fig. 1. Primary biodegradation (a), and sorption (b) of the studied ILs in soil microcosms after
- 424 300 days. [DDA][3AT] is the didecyldimethylammonium 3-amino-1,2,4-triazolate, [BDA][3AT]
- 425 is the benzalkonium 3-amino-1,2,4-triazolate, [P66614][Cl] is the
- trihexyl(tetradecyl)phosphonium chloride, [P66614][Tr] is the trihexyl(tetradecyl)phosphonium
- 427 1,2,4-triazolate. Bars represent standard errors of the mean (n = 9). Note, that primary
- 428 biodegradation of ammonium- and phosphonium-based ILs cannot be compared directly because
- the phosphonium-based ILs anions could not be determined (see Methods section).

430

- 431 Fig. 2. Evolution of CO₂ from soil microcosms over 300 days (a), and theoretical and observed
- 432 CO_2 evolution at day 300 (b). The theoretical evolution is the expected CO_2 evolution if all
- 433 primarily biodegraded IL had been mineralized. [DDA][3AT] is the didecyldimethylammonium
- 434 3-amino-1,2,4-triazolate, [BDA][3AT] is the benzalkonium 3-amino-1,2,4-triazolate,
- 435 [P66614][Cl] is the trihexyl(tetradecyl)phosphonium chloride, [P66614][Tr] is the
- 436 trihexyl(tetradecyl)phosphonium 1,2,4-triazolate. Bars represent standard errors of the mean (n =

437 3).











Table 1. Ionic liquids used during the study.

445 Highlights 446 • The studied ILs show potential for long-term persistence in soils. 447 • Primary biodegradation of ILs ranged from 21 to 33%. 448 • CO₂ evolution from the microcosms was in range of soil background respiration rate. 449