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

8
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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,
32 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 CO₂ evolution from the
34 microcosms. The primary biodegradation ranged from 21 to 33% of total compound in the
35 dissolved phase. The evolution of CO₂ 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**

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

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

68 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
70 the activity of soil microbiota. Recently, Peric et al. (2014), showed that aprotic ILs inhibited soil
71 respiration in the concentration range from 100 mg kg⁻¹ to 10,000 mg kg⁻¹ depending on the ILs
72 structure.

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

86

87 **2. Materials and methods**

88

89 **2.1. Synthesis of ionic liquids**

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

99 the method described in Walkiewicz et al. (2010). The phosphonium-based ILs were prepared
100 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
103 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
111 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**

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

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

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

150

$$151 \quad PB = 100\% - \frac{M_C \cdot X_C + M_A \cdot X_A}{M_{IL}} - FS_{IL} \quad (\text{Eq. 1})$$

152 where:

153 PB – primary biodegradation of IL [%];

154 M_C – residual mass of cation at the end of the experiment [g];

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 [%].

160

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

163

$$164 \quad FS_{IL} = \left(1 - \frac{M_E}{M_I}\right) \cdot 100\% \quad (\text{Eq. 2})$$

165 where:

166 M_E – mass of extracted IL [g];

167 M_I – mass of introduced IL [g].

168

169 **2.3.2. HPLC-MS analysis**

170 Three 1 mL soil extracts of each sampling point (obtained via three-step extraction of each 0.5 g
171 soil sample) were combined, filtered through a 0.2 μm PTFE syringe filter and diluted with
172 methanol : water solution (80:20 v/v). The HPLC-MS analyses were performed with the UltiMate
173 3000 RSLC chromatograph from Dionex (Sunnyvale, CA, USA). Five μL samples were injected
174 into a Hypersil GOLD column (100 mm \times 2.1 mm I.D.; 1.9 μm) with a 2.1 mm I.D. pre-filter
175 cartridge (0.2 μm) from Thermo Scientific (Waltham, MA, USA). The mobile phase consisted of
176 5×10^{-3} mol L⁻¹ ammonium acetate in water (phase A) and methanol (phase B) at a flow rate of 0.2
177 mL min⁻¹. Gradient elution was performed by linearly increasing the percentage of phase B from
178 85 to 100% in 4 min and maintained at 100% for 3 min. The LC column effluent was directed to
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
182 each mass transition detected in the MS/MS multiple reaction monitoring mode was set to 200
183 ms. Nitrogen was used as curtain gas (10 psi), nebulizer gas (40 psi), auxiliary gas (45 psi) and
184 collision gas (medium). The source temperature was 400°C and the ion spray voltage was 4500 V
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**

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

199 The evolution of CO₂ in each soil sample was determined periodically by measuring CO₂
200 content in base traps (10 mL of 0.75 M NaOH in a 20-mL vial) placed in the microcosms (bottles
201 with the used urban park soil) as described elsewhere (Szulc et al. 2014; Lisiecki et al. 2014).
202 Each bottle contained one base trap. NaOH and Na₂CO₃ from each trap were titrated with 0.1 M
203 HCl using an automatic titrator (Metrohm titroprocessor 686). The content of the base traps was
204 replaced with fresh NaOH solution after each measurement.

205

206 **2.5. Statistical analyses**

207 One-way analyses of variance (ANOVA), $\alpha=0.05$ 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**

213 Below, we present results for primary biodegradation, sorption, CO₂ evolution, and carbon
214 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**

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

235

236 **3.2. Evolution of CO₂ and carbon balance**

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

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

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

265

266 **3.3. Potential biotransformation mechanisms**

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

279 explain primary biodegradation of up to ca. 30% in our systems with no or small mineralization.
280 Although some potential biotransformation products, such as aldehydes or ethanol are toxic
281 toward microorganisms, (Stolte et al. 2008; Pham et al. 2009; Zhang et al. 2011),
282 biotransformation products with shorter side chains and ring hydroxylation products are typically
283 less toxic as compared to the parent compound (Docherty et al. 2010, Ranke et al. 2004; Stolte et
284 al. 2011). This may explain why no or very small inhibition of CO₂ evolution from spiked
285 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
289 persistent in urban park soil microcosms. Primary biodegradation ranging from 21 to 33% of total
290 compound in the dissolved phase, combined with CO₂ evolution from the microcosms being in
291 range of soil background respiration rate, suggest no or very small mineralization of the primary
292 compounds and/or their potential metabolites and no or negligible toxicity to soil
293 microorganisms.

294

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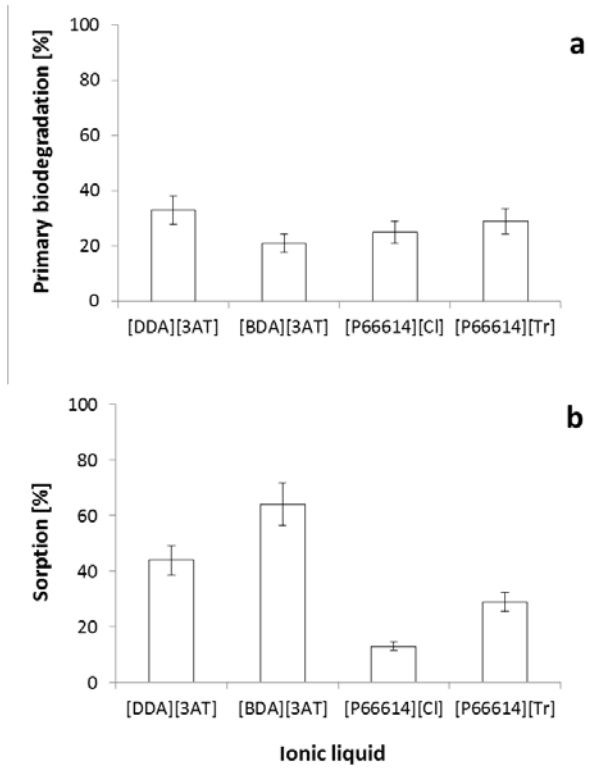
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422 **Figure captions**

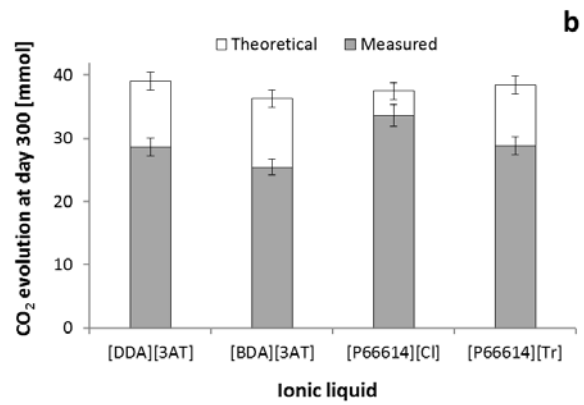
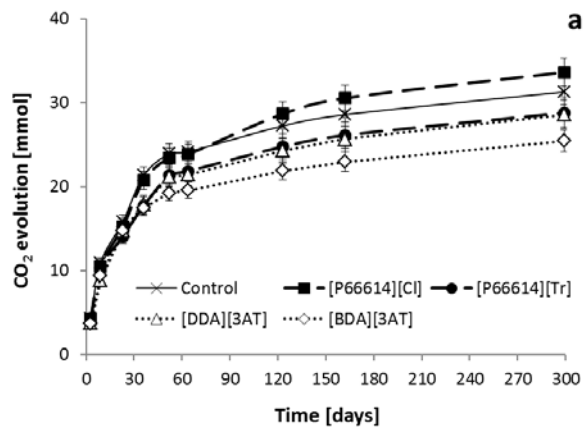
423 Fig. 1. Primary biodegradation (a), and sorption (b) of the studied ILs in soil microcosms after
424 300 days. [DDA][3AT] is the didecyldimethylammonium 3-amino-1,2,4-triazolate, [BDA][3AT]
425 is the benzalkonium 3-amino-1,2,4-triazolate, [P66614][Cl] is the
426 trihexyl(tetradecyl)phosphonium chloride, [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
429 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 CO₂ evolution at day 300 (b). The theoretical evolution is the expected CO₂ 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).



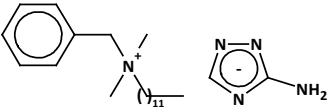
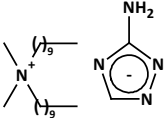
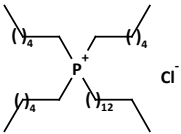
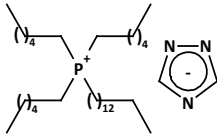
438

439 **Fig. 1.**



440

441 **Fig. 2.**

| Type | Name | Acronym | Structure |
|-------------------|-----------------------------------------------------|---------------------------|-------------------------------------------------------------------------------------|
| Ammonium-based | didecyldimethylammonium 3-amino-1,2,4-triazolate | [DDA][3AT] |  |
| | benzalkonium 3-amino-1,2,4-triazolate | [BDA][3AT] |  |
| Phosphonium-based | triethyl(tetradecyl)phosphonium chloride | [P ₆₆₆₁₄][Cl] |  |
| | triethyl(tetradecyl)phosphonium 1,2,4-triazolate | [P ₆₆₆₁₄][Tr] |  |

443

444 **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