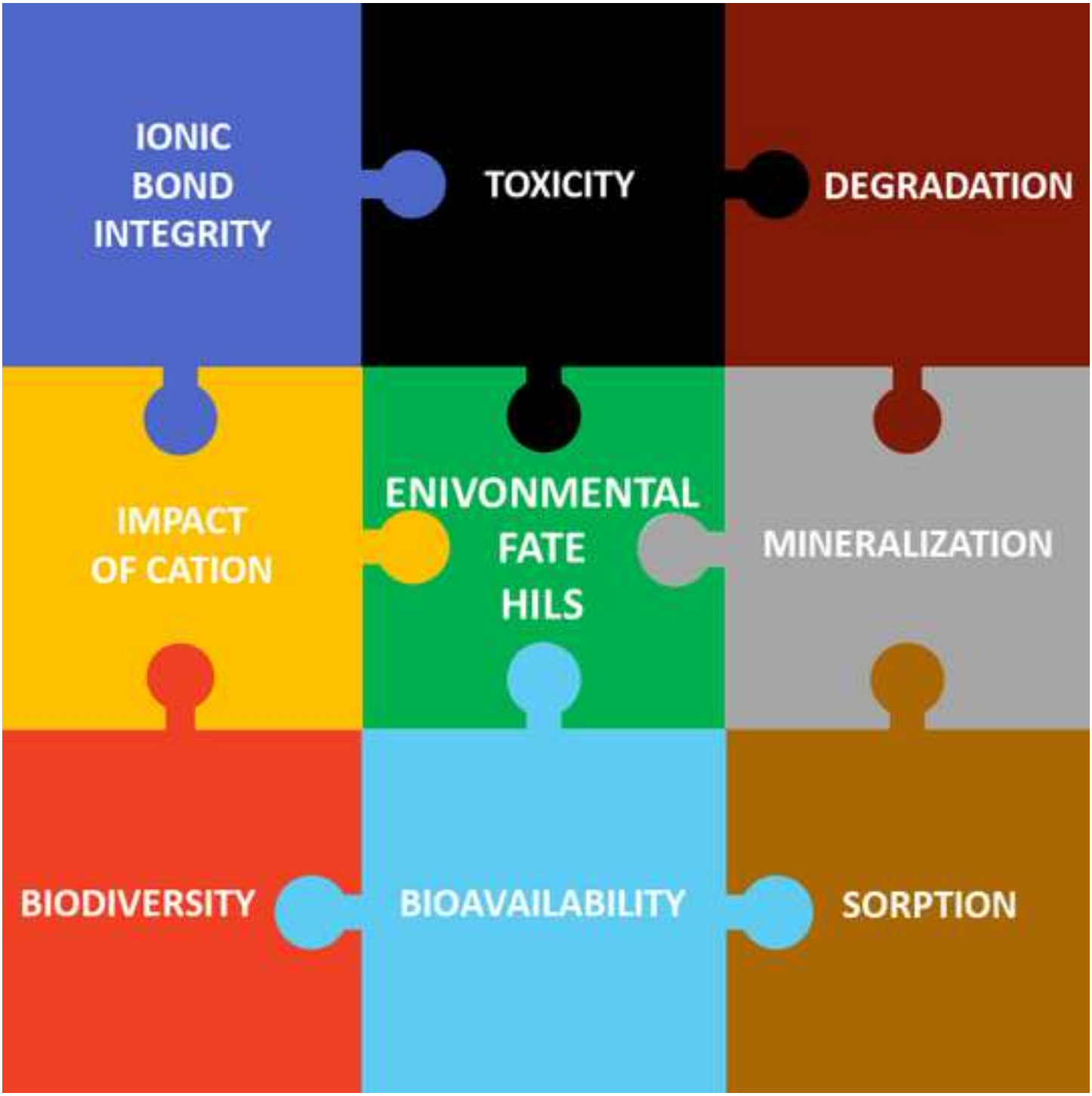
- Cations and anions in HILs act as separate moieties in soils.
- Quaternary ammonium cations in HILs are toxic and tend to sorb in soils.
- Bioaugmentation approach was insufficient to minimise cation’s impact.
- Toxicity and biodegradation of HILs are reflecting cation’s properties.

Graphical Abstract



Glyphosate *versus* Glyphosate based ionic liquids: Effect of cation on glyphosate biodegradation, *soxA* and *phnJ* genes abundance and microbial populations changes during soil bioaugmentation

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Abstract

The applicability of herbicidal ionic liquids (HILs) as an alternative form of herbicides is currently evaluated. Yet, the available research is lacking information on the behaviour of herbicidal ionic liquids upon addition to the environment, *i.e.*, if cations and anions act as separate moieties or remain an ionic salt. Hence, we tested degradation of five HILs with the glyphosate anion, their bioavailability in soil, toxicity towards microorganisms, impact on the biodiversity and the abundance of *phnJ* and *soxA* genes. The cations were proven to be slightly or moderately toxic. The properties of cations determined the properties of the whole formulation, which it might suggest that cations and anion act as the independent mixture of ions. The mineralisation efficiencies were in the range of 15–53%; however, in the case of cations (except non-toxic choline), only 13–20% were bioavailable for degradation. The hydrophobic cations were proven to be highly sorbed, while the anion was readily available for microbial degradation regardless of its counterion. The approach to enrich test samples with isolated microorganisms specialised in glyphosate degradation resulted in higher degradation efficiencies, yet not high enough to mitigate the negative impact of cations. In addition, increased activity of enzymes participating in glyphosate degradation was observed. In the view of obtained results, the use of cationic surfactants in HILs structure is not recommended, as sorption was shown to be determining factor in HILs degradation efficiency. Moreover, obtained results indicate that corresponding ions in HILs might act as separate moieties in the environment.

1. Introduction

Over the last few years, novel application form of herbicides has been proposed, namely the herbicidal ionic liquids (HILs) (Pernak et al., 2011). The majority of them is composed of commercial herbicides in anionic form paired with cations of desired secondary properties, such as good surface activity (Wilms et al., 2020b). While this synthetic approach allows to eliminate the use of toxic additives in the herbicidal mixtures, at the same time HILs contain cationic surfactants, which might also have negative environmental influence, as they are known to disrupt cellular membranes of microorganisms (Cierniak et al., 2020). This in turn might have a vast impact on microbial biodiversity, as well as degradation of surface-active cations and herbicidal anions in the environment. Since the use of large cations might facilitate their sorption to soil particles, it might translate into their lower bioavailability for microorganisms and, consequently, lower biodegradability potential (Niemczak et al., 2017a; Stachowiak et al., 2021).

The properties of anions and cations in HILs are expected to be modified due to the interactions of these moieties in synthesised new formulations (Hough et al., 2007), which means that cation selection might affect the resulting properties of an anion. However, not only these reports have not been proven to date, but also first evidences stating that cation and anion act separately and differently upon introduction to the environment has been published (Parus et al., 2022a; Wilms et al., 2020a; Woźniak-Karczewska et al., 2022). Namely, it has been shown that in soils herbicidal anions were degraded well, while cations were subjected only to partial biotransformation (Wilms et al., 2020a). Moreover, the hydrophobicity and sorption potential of cations in HILs has virtually no effect on the anion's hydrophobicity and resulting mobility in soil (Parus et al., 2022a, 2022b; Woźniak-Karczewska et al., 2022). This, in turn, calls for further examination of the behaviour of HILs in the environment and their impact on native soil microbiota, due to the incorporation into the structures of these compounds cations

68 that are suspected of high antimicrobial activity (Parus et al., 2020). Up to date, the study using
69 ^{13}C-labelling has proven that in soils, only herbicidal anion was incorporated into bacterial
70 phospholipid-derived fatty acids (Wilms et al., 2020a). Yet, the assessment on how native
71 microbiota capable of herbicides' degradation will cope with additional stress caused by above-
72 mentioned cationic surfactants in HILs is still needed in order to fully understand their influence
73 on microbial community structures. It is also a unique opportunity to analyse practical
74 performance of commercial formulations on the agricultural fields, as the synthesis of HIL
75 allows to observe environmental impact of both herbicide and the surfactant (Wilms et al.,
76 2020a).

77 In our study, we synthesized HILs composed of selected cations paired with one of the
78 most commonly applied broad-spectrum herbicide, glyphosate (*N*-(phosphonomethyl)glycine)
79 (Benbrook, 2016). Despite the fact that glyphosate is considered as a compound that is rapidly
80 mineralised in soils, however, literature data indicate varying half-life times for glyphosate and
81 its main metabolite, aminomethylphosphonic acid (AMPA), from less than 24 hours to 280
82 days, and 10 – 98 days, respectively, depending on soil sorption properties (Accinelli et al.,
83 2004; Al-Rajab and Schiavon, 2010; Bai and Ogbourne, 2016; Bento et al., 2016; Bergström et
84 al., 2011; Grunewald et al., 2001; Mamy et al., 2005; Shushkova et al., 2009; Syan et al., 2014;
85 Yang et al., 2013; Zhang et al., 2015a). Such long half-lives may promote increased
86 environmental risks, *e.g.*, presence of permanent contamination due to the accumulation of this
87 compound or its toxicity towards non-targeted organisms after desorption (Al-Rajab and
88 Schiavon, 2010). Additionally, it has been reported that the prolonged presence of glyphosate
89 may severely disrupt soil biodiversity, posing threat not only to overall health of soil
90 microbiome but also to crops themselves (Arango et al., 2014; Bai and Ogbourne, 2016; Druille
91 et al., 2013; Duke et al., 2012; Lorch et al., 2021).

The aim of this study was to evaluate the impact of cations of different hydrophobicity in HILs on the degradation of herbicidal anion, exemplified by glyphosate. In order to do that, we employed mineralisation experiment of HILs composed of selected cations paired with glyphosate anion in terrestrial systems. In addition, despite monitoring the activity of indigenous microorganisms, we also tested the efficiency of bioaugmentation with previously isolated glyphosate degraders. Moreover, changes in soil microbiome structure were investigated, along with identification of genes responsible for glyphosate degradation, in order to evaluate the impact of cations in HILs on functioning of soil microbiome.

2. Materials and Methods

2.1. Materials

2-hydroxyethyltrimethylammonium chloride (purity 98%), 1-chlorododecane (purity 97%), 2-(dimethylamino)ethanol (purity >99%), benzalkonium chloride (purity 95%), didecyldimethylammonium chloride (50% solution in isopropanol:water, 2:3 (v:v)), 1-bromooctadecane (purity 97%), N-(phosphonomethyl)glycine (glyphosate, purity 96%) and deuterium oxide (99.9 atom % D) for NMR analyses were purchased from Sigma-Aldrich (Saint Louis, Missouri, USA). Hexadecyltrimethylammonium chloride (50% solution in water) was obtained from Brenntag (Essen, Germany). All following solvents: methanol (purity >99%), acetonitrile (purity 99%), isopropanol (purity 98%), ethyl acetate (purity 99%) and potassium hydroxide (purity 85%) were obtained from Avantor (Gliwice, Poland). Deionized water with a conductivity lower than 0.1 $\mu S \cdot cm^{-1}$, from HLP Smart 1000 demineralizer (Poznań, Poland) was used.

2.2. Syntheses

2.2.1. Synthesis of compounds with glyphosate anion

The potassium salt of glyphosate and herbicidal ionic liquids [Chol][Glyph] (2-hydroxyethyltrimethylammonium N-(phosphonomethyl)glycinate), [C_{12}Chol][Glyph]

(dodecyl(2-hydroxyethyl)dimethylammonium N-(phosphonomethyl)glycinate), [DDA][Glyph] (didecyldimethylammonium N-(phosphonomethyl)glycinate), [C_{16}TMA][Glyph] (hexadecyltrimethylammonium N-(phosphonomethyl)glycinate) and [BA][Glyph] (benzalkonium N-(phosphonomethyl)glycinate) were synthesized and characterized within this study based on the procedure developed in a framework of our previous works (**Table 1**), according to synthesis procedure illustrated in **Fig. 1**.

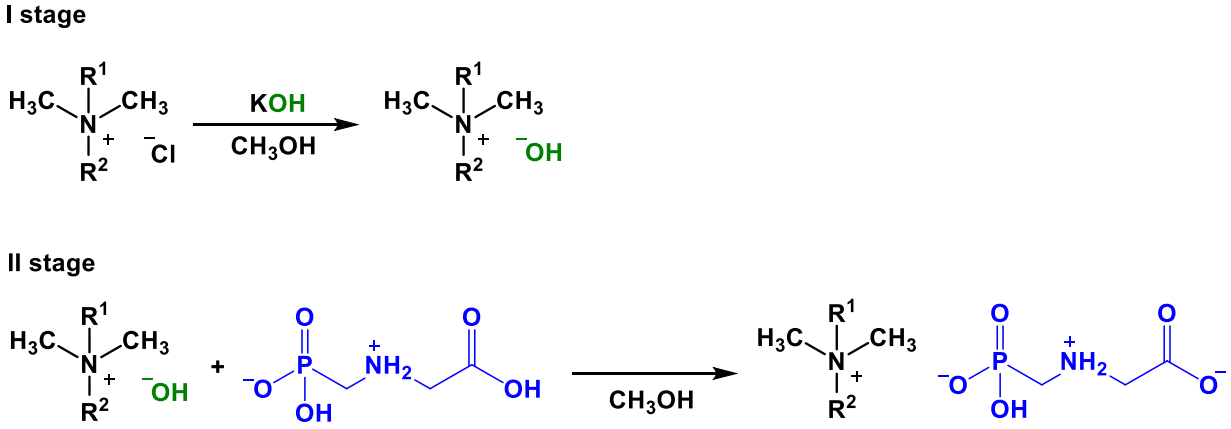


Fig. 1. Synthesis of HILs containing glyphosate as the anion

Table 1 Synthesized HILs containing glyphosate as the anion

Acronyms	R^1	R^2	Yield	Appearance at 25 °C	CAS	Ref.
[Chol][Glyph]	CH_3	CH_2CH_2OH	91	Liquid	1253049-57-8	a
[C_{12}Chol][Glyph]	$C_{12}H_{25}$	CH_2CH_2OH	95	Wax	---	---
[DDA][Glyph]	$C_{10}H_{21}$	$C_{10}H_{21}$	93	Wax	1354726-32-1	b, d
[C_{16}TMA][Glyph]	$C_{16}H_{33}$	CH_3	89	Wax	95014-89-4	c
[BA][Glyph]	$C_{12}H_{25}$	CH_2Ph	92	Liquid	---	b, d

[a] WO2010123871 A1 (Li et al., 2010); [b] WO2012006313 A2 (Pernak et al., 2012); [c] EP124351 A1 (Prisbylla, 1984); [d] Pernak et al., 2014

2.2.2. Spectral analysis

^{1}H NMR spectra were obtained *via* use of a Varian VNMR-S 400 MHz spectrometer (operating at a frequency of 400 MHz) and a Mercury Gemini 300 spectrometer (operating at a frequency

of 300 MHz), with the tetramethylsilane (TMS) applied as internal standard. ^{13}C NMR and ^{31}P NMR spectra were recorded with the same instruments, at 75 and 100 MHz, and 121 MHz, respectively. Spectra for all synthetized compounds are presented in Supplementary Material (Section *1. Spectra of herbicidal ionic liquids*).

2.3. Isolation and identification of glyphosate-degrading microbial community

Samples of agricultural soils, which had contact with herbicides in the past, were collected from a field located in Kamionki, Poland (N 52.16467, E 16.59464) to sterile packages from the depth of 10 – 20 cm (Alef and Nannipleri, 1995). After transport to laboratory, they were sieved through 1.6. mm sieve and stored at 4 °C. The isolation procedure started within 24 hours from the moment of samples collection. Approx. 5 g of soil (wet weight) served as an inoculum. The microorganisms were cultivated in 150 mL sterile Erlenmeyer flasks filled with 25 mL of sterile mineral medium (MM) amended with 1 g/L of glyphosate (H, herbicide) and supplemented with 100 µL of microelements solution (MM+H). The herbicide acted as a sole source of carbon and energy for isolated microorganisms, and its concentration was chosen based on literature data (Benslama and Boulahrouf, 2013; Fan et al., 2012; McAuliffe et al., 1990; Nourouzi et al., 2011). Mineral medium consisted of 7.0 g/L $Na_2HPO_4 \times 2H_2O$, 2.8 g/L KH_2PO_4, 0.5 g/L NaCl, 1.0 g/L NH_4Cl. After sterilisation, it was supplemented with microelements solution, sterilised *via* membrane filter 0.22 µm (MCE, Mixed Cellulose Ester), of following composition: 200 mg/L $MgSO_4 \times 7\ H_2O$, 20 mg/L $FeSO_4 \times 7\ H_2O$, 10 mg/L $MnSO_4 \times 4\ H_2O$, 12.8 mg/L ZnCl, 2 mg/L $CaCl_2 \times 6\ H_2O$, 1.2 mg/L $BaCl_2$, 0.72 mg/L $CoSO_4 \times 7\ H_2O$, 0.072 mg/L $CuSO_4 \times 5\ H_2O$, 13 mg/L H_3BO_3, 20 mg/L EDTA, and 0.292 mL/L HCl 37% (Woźniak-Karczewska et al., 2018). Thus prepared culture was incubated at 28 °C on a rotary shaker (120 rpm). After 3rd transfer to a fresh medium (MM+H), enrichment culture was stored at −80 °C in sterile 20% (v/v) glycerol stocks until used.

160

161 *2.4. Toxicity determination of HILs towards enrichment culture used in the mineralisation*
162 *experiment*

163 The half maximal effective concentration (EC_{50}) values of tested HILs and their cations'
164 chlorides were determined for the enrichment culture used in the mineralisation experiment.
165 Tested culture was hence transferred from glycerol stocks 20% (v/v) to 50% Tryptic Soy Broth
166 medium (TSB, Sigma Aldrich, Poland) and then incubated for 24 h at 28 °C. The biomass was
167 transferred threefold, and the cell suspension was adjusted to optical density OD_{600} = 0.100 ±
168 0.010. Then, 200 µL of biomass was placed in a sterile 96-well plate and incubated with shaking
169 (120 rpm) for 3 h to reach exponential growth stage. After that, 50 µL of tested formulations in
170 concentrations of active substance ranging from 1–1000 mg/L (1, 5, 10, 50, 100, 250, 500, 1000
171 mg/L) was added to specific wells in triplicates and incubation was continued for additional 5.5
172 h. In case of cation's chlorides ([Chol][Cl], [C_{12}Chol][Cl], [DDA][Cl], [C_{16}TMA][Cl] and
173 [BA][Cl]), the concentrations were recalculated to reach the same cation mass as in
174 corresponding HILs. Microorganisms lacking analysed compounds (biotic control) and
175 compounds' solutions without microorganisms (abiotic controls) were used as controls. After
176 incubation, EC_{50} values were determined based on the equation presented in Syguda et al., 2020.
177

178 *2.5. Characterisation of soil*

179 The soil utilised in experiments was collected from an agricultural field in Rzgów, Poland (N
180 52.151102, E 18.050041) from the depth of 10–20 cm. Prior to experiments, the soil was stored
181 in secured container (no longer than 3 days), then sieved through 1.6 mm sieve and
182 characterised according to USCS (Unified Soil Classification System) as sandy loam (*Standard*
183 *Practice for Classification of Soils for Engineering Purposes (Unified Soil Classification*
184 *System)*, 2006). The specification of soil was as follow: organic carbon: 1.3%; porosity: 0.4

185 m^3/m^3; bulk density: 1.3 Mg/m^3; field water capacity: 0.21 m^3/m^3; relative field capacity: 0.565
186 m^3/m^3; soil moisture during sampling 19%; total sulphur: 276 mg/kg; $N\text{-}NO_3$: 7.9 mg/kg d.w.s.;
187 $N\text{-}NH_4$: 1.4; P: 81 ± 1.1 mg/kg; K: 88 ± 2.3 mg/kg; Mg: 69 ± 1.3 mg/kg. Grain size distribution:
188 2.0–0.05 = 70.7%, 0.05–0.002 = 27.4%, <0.002 = 1.9%.
189
190 *2.6. Bioavailability of herbicidal ionic liquids in soils*
191 The herbicides at concentration of 1000 mg/kg of soil dry weight (set by active substance,
192 glyphosate) were added the soil (100 g), thoroughly mixed and dried at 60 °C to constant
193 weight. Then, the first two steps of the Community Bureau of Reference (BCR) sequential
194 extraction were applied (Jakubowska et al., 2008; Wali et al., 2014) to determine the amount of
195 xenobiotics that are readily available/leachable with mild agents. In the first step, herbicides
196 dissolved in water were extracted. Briefly, 1 g of soil samples treated with corresponding
197 herbicides were placed in 50 mL polyethylene centrifuge tubes, filled with 40 mL of distilled
198 water, and then shaken (280 rpm, 16 h, 20 °C) and centrifuged (10,000 rpm, 10 min). The
199 supernatants were then collected into fresh containers. In the second step, 40 mL aliquots of
200 0.11 mol/L acetic acid solution were added to the residual soil and treated as in the first step.
201 Then, supernatants were filtered through 0.22 μm syringe filter (PTFE) and analyzed for the
202 content of herbicidal anions and cations by LC-MS/MS.
203
204 *2.7. Mineralisation experiment*
205 Previously isolated enrichment culture was incubated at 28 °C on a rotary shaker (120 rpm).
206 After 3rd transfer to a fresh medium (MM+H), preculture was transferred to 1 L of sterile Tryptic
207 Soy Broth (Sigma-Aldrich, Germany) with the addition of glyphosate (1 g/L) (TSB+H). After
208 72 h of incubation (28 °C, 120 rpm), the biomass was washed three times with sterile 0.85%

(v/v) NaCl solution, centrifuged (4500 rpm, 15 min, 4 °C) and then resuspended in 0.85% (v/v) NaCl solution.

The experiment was carried out in sealed 1 L glass bottles, containing 100 g of non-sterile soil. The traps containing 0.75 M NaOH solution (10 mL in a 20 mL vial) were placed inside each bottle for CO_2 evolution tests. The soil was prepared by sieving it through 1.6 mm sieve and vigorous mixing with 20 mL of aqueous solution, to ensure optimal moisture (field water capacity 0.22 m^3/m^3). The concentration of HILs in soil were set as 1 g of active compound/1 kg of soil, and the composition of liquid added to soil was either 18 mL of HIL and 2 mL of inoculum suspended in sterile 0.85% (v/v) NaCl (approach with bioaugmentation – B), 18 mL of HIL and 2 mL of sterile 0.85% (v/v) NaCl (approach without bioaugmentation – NB), 18 mL of distilled water + 2 mL of inoculum suspended in sterile 0.85% (v/v) NaCl (biotic control) or 18 mL of distilled water + 2 mL of sterile 0.85% (v/v) NaCl (abiotic control). The HILs' concentration of 1 g of active compound/1 kg of soil was significantly higher than field concentration of glyphosate (up to 1080 g/ha) as it was experimentally established to be suitable to observe differences in degradation of these compounds. All samples were prepared in triplicates. Finally, the microcosms were incubated for 12 weeks at 20 ± 2 °C. Mineralisation extent was determined according to Warder titration with 0.1 M HCl of diluted NaOH and Na_2CO_3 solutions from traps placed inside bottles, with the use of automatic titrator (Metrohm titroprocessor 686, Herisau, Switzerland). After each measurement, the vials were rinsed with distilled water, dried and filled with fresh NaOH solution. The controls were prepared to investigate background respiration of soil without compounds' addition.

2.8. LC-MS/MS analysis

After 28 and 90 days of the experiment, soil samples were subjected to two-step extraction to determine contents of cations and anions. To prepare the extracts, soil samples in bottles were

thoroughly mixed prior to weighing approx. 5 g of soil into 50 mL centrifuge tubes, then 10 mL aliquots of distilled water were added and vortexed for 10 s. The centrifuge tubes were then shaken for 30 min (320 rpm) and centrifuged (10,000 rpm, 15 min). The extracts were then filtered into fresh tubes (50 mL) through quantitative strainers. To the soil sediments, 10 mL aliquots of distilled water were added and the samples were shaken for 15 min (320 rpm), followed by centrifugation (5,000 rpm, 15 min) and decanting the solution through a quantitative filter. The two extracts were combined. The samples were then filtered through 0.22 μm PTFE syringe filters (Advantec, Tokyo, Japan) and stored in a refrigerator. The LC-MS/MS analyses were performed with the UltiMate 3000 RSLC chromatographic system (Dionex, Thermo, Waltham, MA, USA) coupled with an API 4000 QTRAP triple quadrupole mass spectrometer with electrospray ionization (ESI) (AB Sciex, Foster City, CA, USA) in positive mode (LC-MS/MS). For the analysis the Luna C18 column ((150 mm×2.0 mm, 3 μm particle size); Phenomenex, USA) was used. The composition of phase A (CH_3COONH_4, 5 mM in water) and phase B (methanol) eluents was different depending on the type of analyte. Detailed parameters of analysis are presented in Supplementary Material (Section *3. Parameters of LC-MS/MS analysis*). The extraction method was previously validated by performing extraction from the whole sample to check whether the 5 g samples are representative.

2.9. Assessment of bacterial community structure in soil via barcoded 16S rRNA gene MiSeq sequencing analysis

2.9.1. DNA extraction, library construction and NGS sequencing

Bacteria were harvested from 100 μL of glycerol stock by centrifugation for 10 min at 14,100 x g. The pellet was dissolved in 50 μL of 1 mM Tris-EDTA buffer. Lysozyme (A&A Biotechnology, Gdańsk, Poland) was added to the final concentration of 0.1 mg/mL and

samples were incubated for 30 min at 37 °C. Then 360 µL of ATL lysis buffer (Qiagen, Hilden Germany) and 40 µL of 2 mg/mL Proteinase K (Bio Basic, Markham, ON, Canada) were added and samples were incubated for 30 min at 56 °C. Subsequently, 200 µL of the lysate from each sample was used to isolate total genomic DNA using the DNeasy Blood & Tissue Kit (Qiagen, Hilden Germany) according to the manufacturer's protocol for animal tissues. Before PCR amplification, DNA extracts were normalized with sterile water to a concentration of 10 ng/µL. Blank DNA extraction was prepared and sequenced as a negative control.

Bacterial 16S rRNA gene fragments (16S) were amplified using V4F (CGATCAGCAGCCGCGGTAATA) and V4R (ATGGACTACCAGGGTATCTAA) primers targeting the V4 region (Makowska et al., 2020). Primers were tailed at 5'-ends with dual-indexed Ion Torrent adapters for sequencing using the Ion Torrent system (Life Technologies, Carlsbad, CA, USA). PCRs were done in two technical replications, each in a total volume of 10 µL containing Hot FIREPol DNA Polymerase, 0.25 µM of each primer and 1 µL of template DNA. The PCR program was as follows: 95 °C for 12 min, followed by 30 cycles at 95 °C for 15 s, 50 °C for 1 min and 72 °C for 45 s, with a final extension step at 72 °C for 5 min. Blank PCRs were prepared and sequenced as negative controls. After PCR, technical replications were pooled and, for each sample, 3 µL was electrophoresed on a 2% agarose gel to check amplification efficiency. Then, all samples were pooled in equal quantities and purified using the 2% E-Gel SizeSelect II Agarose Gels system (Invitrogen, Waltham, MA, USA), according to the manufacturer's instructions.

DNA concentration and fragment length distribution of the library were established using the High Sensitivity D1000 Screen Tape assay on the 2200 Tape Station system (Agilent, Santa Clara, CA USA). Clonal template amplification was performed using the Ion Torrent One Touch System II and the Ion Torrent OT2 Kit according to the manufacturer's instructions.

Sequencing was carried out using the Ion 540 Kit-OT2 and Ion Torrent S5 system according to the manufacturer's instructions.

2.9.2. Read processing and data analysis

Raw sequence data were pre-filtered by Ion Torrent Suite software version 5.12.2 (Life Technologies, Carlsbad, CA, USA) to remove polyclonal and low-quality sequences. Further bioinformatic analyses were conducted using fastq data and custom workflow. Sequence reads shorter than 200-bp were removed from the dataset using Geneious R11.1.5 (Biomatters Ltd. Auckland, New Zealand). Leading and trailing low-quality bases were removed using Trimmomatic version 0.39 (Bolger et al., 2014). FASTX-Toolkit (Hannon, 2010) was used to extract sequences with a minimum of 50% bases with a quality score of ≥ 25. Quality-filtered sequences were separated by barcodes and trimmed at 5'- and 3'-ends to exclude PCR primers in Geneious R11.1.5. The singletons (<10 reads) were removed using the FASTX_UNIQUES and SORTBYSIZE algorithms (Edgar, 2013). Chimeras were removed using the default settings in UCHIME2 version 4.2.40 (Edgar, 2016a).

Operational taxonomic unit (OTU) clustering at 97% similarity was done in USEARCH version 11.0.667 (Edgar, 2013). Sequences were denoised into zero-radius operational taxonomic units (ZOTUs) and, subsequently, a ZOTU table was constructed according to the DENOISING STEPS (Edgar, 2016a). The ZOTU table was then corrected for the 16S copy number based on the UNIBAS algorithm. Phylogenetic affiliations were analysed by the USEARCH SINTAX algorithm using a confidence threshold of 0.8 (Edgar, 2018a, 2018b, 2016b; Edgar and Flyvbjerg, 2018). ZOTUs were compared against the SILVA database for ARB for small subunit ribosomal RNAs version 138 (Glöckner et al., 2017; Quast et al., 2013; Yilmaz et al., 2014). The UNCROSS2 algorithm was used to remove ZOTUs detected in control samples from

the dataset (Edgar, 2018c). Then, the reads were normalized by OTUTAB_RARE algorithm (Edgar and Flyvbjerg, 2018) to compare sample diversities.

The potential orthologs of prokaryotic communities in all samples was predicted using the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) version 2.4.1 software package (Douglas et al., 2020). The ZOTU table normalised by 16S rRNA gene copy number was used for orthologs prediction, generating a table of Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthologs (KOs) (Kanehisa, 2019; Kanehisa et al., 2021; Yi et al., 2020). The predictions were categorized at KEGG Orthology. As an indicator for the PICRUSt2 prediction accuracy, the Nearest Sequenced Taxon Index (NSTI) for each sample was estimated and calculated (Langille et al., 2013). A ratio comparison of *soxA* and *phnJ* orthologs were prepared in statistical analysis of metagenomic profiles (STAMP) version 2.1.3 (Parks et al., 2014). Visualization of the family-level bacterial microbiome composition profiles and cluster tree based on unweighted pair-group method with arithmetic median (UPGMA) were conducted in STAMP version 2.1.3. MetacodeR package version 0.3.5 was used to visualize the bacterial diversity in heat tree format, while Wilcoxon Rank Sum test was used to compare differences between the taxa abundances among samples (Foster et al., 2017). The charts were edited in Corel Draw Graphics Suite 2021.

2.10. Statistical analysis

All tests were performed in triplicates. Each of the presented error bars represent standard errors of the mean (n = 3). Additionally, one-way ANOVA, $\alpha = 0.05$, was employed to detect statistical differences significance in all systems.

3. Results and discussion

3.1. Bioavailability and degradation of herbicidal ionic liquids in soils

The main advantage of HILs' synthesis reported by some authors is the tunability of these compounds, and hence, the possibility to eliminate addition of toxic adjuvants (Pernak et al., 2015, 2011). It is due to the fact that quaternary cationic surfactants, which are most commonly present in these formulations, in theory should form ionic pairs with herbicidal anions and, consequently, influence the properties of the whole compound, resulting in numerous benefits in field applications (Niemczak et al., 2017b; Paul and Moulik, 2015; Pernak et al., 2015, 2013, 2011; Zajac et al., 2018). However, the indications are starting to arise that in reality cation and anion in these formulations exhibit different environmental fates and thus degradation potential, *i.e.*, herbicides in anionic form were degraded preferentially in soils, while cations were subjected to quantitative sorption and were only partially biotransformed (Wilms et al., 2020a). These differences in degradation of cations and anions in HILs might be attributed to a few factors. Firstly, high sorption of quaternary cationic surfactants to various materials (*e.g.*, activated sludge, natural sediments, clay materials, minerals, proteins, cell walls of microorganisms) makes them less susceptible to microbial degradation (Boethling and Lynch, 1992; Cierniak et al., 2020; Khan et al., 2015). Secondly, it has been well established that quaternary ammonium surfactants are toxic to microorganisms, since they might inhibit respiratory enzymes and disrupt cellular membranes, which in turn could have a vast impact on cation's half-lives in the environment (Bergero and Lucchesi, 2018; Conidi et al., 2019; Zhang et al., 2015b; Zhang et al., 2011). Moreover, microorganisms in agricultural soils, which are accustomed to the presence of herbicides, will cope with degradation of these compounds better than with the breakdown of cationic surfactants. It is because they are far more rarely present on fields, especially when compared to anionic and non-ionic surface-active additives utilised in almost all commercial formulations (Wilms et al., 2020a).

Interestingly, however, the presence of cationic surfactants in HILs' structure might influence the degradation of anion as well, since quaternary ammonium cations are known to

form complexes with anions (Brycki et al., 2014; Sütterlin et al., 2008; Zhang et al., 2015b). When considering formation of such complexes of non-ionic character, cationic surfactants, due to their positive charge, after sorption in soil might act similarly to anion-exchanging resins, so it is possible for them to bind herbicides (in anionic form) in soils and, consequently, decrease their degradation efficiency. It is also expected outcome for HILs with glyphosate anion, as this herbicide is known for its strong sorption in soils, explained by binding its phosphonate groups to the cations or formation of hydrogen bonds between glyphosate and humic substances (Dollinger et al., 2015).

It has been already reported that hydrophobic cationic surfactants exhibit the highest sorption potential among other surfactants, namely of even 90% of higher, while at the same time anionic and non-ionic surface-active chemicals are subjected to negligible sorption (Cierniak et al., 2020). Due to the this fact, distinguishing between cation sorption, primary degradation and complexation might pose a scientific challenge for herbicidal ionic liquids (Boethling and Lynch, 1992; Cierniak et al., 2020; Zhang et al., 2015b). In the case of biodegradation studies, obtained results most commonly show only the disappearance of the analytical signal of the main compound, without considering its degradation by-products (Sydow et al., 2015; Zembrzuska et al., 2016). Moreover, such analyses do not indicate whether the amount of removed compound corresponds to its degradation or rather sorption. In most cases, only the bioavailable part is recovered from matrix, and especially in the case of larger and more hydrophobic cations, only application of different, more aggressive solvents allows to obtain fraction bound to soil (Boethling and Lynch, 1992; Cierniak et al., 2020). Additionally, it is well-established that the more hydrophobic cation, the higher its sorption and the more difficult it is to recover it from soil matrix or from activated sludge (Bergero and Lucchesi, 2018). Hence, quaternary ammonium cations, often utilised in HILs, are prone to exhibit significant sorption in soils, which is directly connected to their lower bioavailability

and in consequence – lower degradation potential (Bergero and Lucchesi, 2018; Conidi et al., 2019; Ying, 2006).

Table 2 Primary degradation vs bioavailability – after 28 days

Compound	Cation		Anion	
	bioavailable part [%][a]	primary degradation of bioavailable part [%]	bioavailable part [%][a]	primary degradation of bioavailable part [%]
[K][Glyph]	[-]	[-]	104.3 ± 0.1	98.1 ± 0.9
[Chol][Glyph]	102.5 ± 0.2	98.9 ± 0.2	100.3 ± 0.1	96.1 ± 0.8
[C_{12}Chol][Glyph]	21.6 ± 0.1	99.0 ± 0.1	97.2 ± 0.2	99.1 ± 0.3
[C_{16}TMA][Glyph]	13.8 ± 0.2	99.7 ± 0.3	99.1 ± 0.3	94.4 ± 0.9
[DDA][Glyph]	12.9 ± 0.1	99.2 ± 0.8	101.8 ± 0.1	99.4 ± 0.4
[BA][Glyph]	13.1 ± 0.2	99.3 ± 0.6	98.3 ± 0.2	99.6 ± 0.6

[a] Details presented in Supplementary Material (Section *4. Bioavailability of HILs*).

As presented in **Table 2**, the primary degradation values for all tested compounds indicated almost complete degradation of cations and anions during standard 28-day test. However, taking into account abovementioned problems with performing such analyses, we decided to compare obtained values with bioavailability of these substances in used soil. As it can be clearly seen, glyphosate anion was highly bioavailable, but at the same time over 80% of each cation (except choline, [Chol]) was sorbed to soil matrix, which means that only 13–20% was bioavailable for microorganisms in degradation process. This phenomenon might be attributed to the presence of hydrophobic chains present in all HILs except choline, and it stands in accordance with literature data on high sorption of hydrophobic cationic surfactants, as discussed above. This in turn means that obtained primary degradation values did not actually translate into biodegradation of cations, but rather showed the recovery of only small amount of bioavailable part, which was susceptible to degradation. Hence, the expected mineralisation

(ultimate biodegradation) results were expected to be significantly lower. Indeed, as presented in **Table 3**, mineralisation efficiencies were within range of 15–53%. These results stands also in agreement with previous research performed by Sydow et al., 2015, which has proven that herbicidal ionic liquids have reached very small ultimate biodegradation values, of 4–7%.

Table 3 *Mineralisation efficiencies*

Compound	non-bioaugmented [%]	bioaugmented [%]
[K][Glyph]	15.42 ± 0.8	28.04 ± 1.8
[Chol][Glyph]	36.12 ± 0.9	47.96 ± 1.0
[C_{12}Chol][Glyph]	53.01 ± 0.1	53.34 ± 0.7
[C_{16}TMA][Glyph]*	[-][a]	47.96 ± 0.1
[BA][Glyph]	32.79 ± 0.1	36.74 ± 0.6
[DDA][Glyph]	39.13 ± 0.1	44.15 ± 0.3

[a] Data vary from other due to the unexpected microbial activity in all three replicates.

It has been proven that addition of specialised enrichment culture to a system with quaternary ammonium cations improves their degradation (Conidi et al., 2019). In our study, bioaugmentation with microorganisms with confirmed ability to degrade glyphosate resulted in higher CO_2 evolution; however, these changes were not substantially high, as presented in **Fig. 1** below. The sole exception was [C_{16}TMA][Glyph], illustrated on separate plot, where unexpected presence of fungi was observed in all replicates in non-bioaugmented approach. This explains the exceedingly high CO_2 emissions, obtained due to high respiratory activity of fungi. Interestingly enough, its presence was not observed in the approach with bioaugmentation, possibly due to the competitive interactions between microorganisms.

In general, the only thing that had an influence on the amount of released CO_2 was the initial carbon content in the sample – as expected, samples containing [K][Glyph], which only has 3 carbon atoms in its structure, exhibited the lowest CO_2 emissions. The introduction of

specialised microorganisms to soil containing compounds characterised by high bioavailability ([K][Glyph] and [Chol][Glyph], **Table 2**), resulted in an increase in their degradation efficiencies (**Table 3**). Specifically, in case of sorbed chemicals (HILs with hydrophobic cations), characterised by low bioavailability (**Table 2**), even addition of specialised microorganisms did not result in substantially enhanced mineralisation efficiencies, since the contaminant was simply inaccessible for microorganisms. Sorption of chemicals to soil was discussed by many authors in terms of contaminants' removal from the environment by surfactant utilisation (Brown et al., 1999; Ghosh and Mukherji, 2016; Singh et al., 2018; Singleton et al., 2016; Wolf et al., 2019). Namely, addition of surface-active chemicals can stimulate desorption of xenobiotic bound to matrix (Brown et al., 1999; Ghosh and Mukherji, 2016; Singh et al., 2018; Singleton et al., 2016; Wolf et al., 2019). Only that way, contaminant is becoming bioavailable and susceptible to microbial degradation. Interestingly enough, the structure of HILs incorporate cationic surfactants, known for their high sorption potential. This, in turn, should result not only in virtually no effect in stimulating herbicide desorption from soil, but also in increased sorption of cations in soil and, consequently, accumulation of contamination, especially compared to commercially used herbicidal formulations with adjuvants of anionic or non-ionic character.

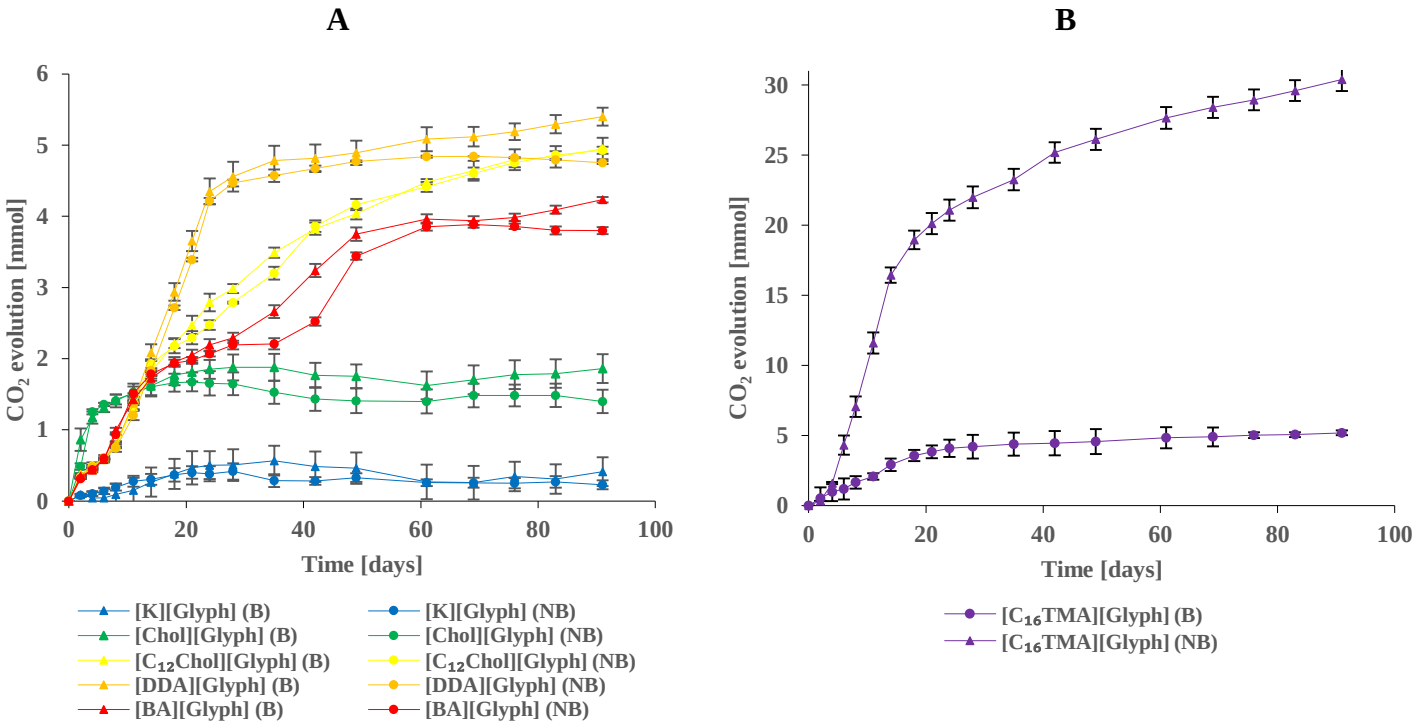


Fig. 1 CO_2 evolution curves for tested HILs (A). The $[C_{16}TMA][Glyph]$ mineralisation curves are presented as a unique example (B). NB – non-bioaugmented samples, B – bioaugmented samples. The CO_2 evolution values of respective bioaugmented and non-bioaugmented controls were subtracted from curves to illustrate signals resulting only from compounds' degradation.

3.2. Toxicity determination of HILs towards enrichment culture used in the mineralisation experiment

The results of antimicrobial activity test towards enrichment culture used in the experiment are presented in **Table 4**. As it can be clearly seen, pure glyphosate was harmless for microorganisms, which stands in agreement with other reports on its toxicity (Amorós et al., 2007; Busse et al., 2001). The addition of choline, hydrophilic cation of natural origin, to formulation did not result in increased toxicity (Zeisel and Canty, 1993). As it has been already established, choline is as a neurotransmitter acetylcholine precursor, and as a compound present

in metabolic pathways is considered as not toxic itself (Gadilohar and Shankarling, 2017; Zeisel and Da Costa, 2009). However, introduction of hydrophobic cationic surfactants to herbicidal formulations resulted in significantly higher toxicity of obtained compounds. Even simple addition of hydrophobic aliphatic chain to the choline (forming [C_{12}Chol]) caused a considerable decrease in EC_{50} values – from over 1000 mg/L to approx. 50 mg/L. Further modifications and formation of hydrophobic compounds translated into even higher toxicity. Moreover, the toxicity of HILs seemed to reflect only the toxicity of cation. Namely, the glyphosate alone ([K][Glyph]) was proven to be non-toxic, while toxicities of cations with chlorine anion and their corresponding HILs (with glyphosate anion) were very similar. This in turn might support the theory that cations and anions in HILs act as separate moieties. In any other case, the toxicity of the whole compound would differ from that of a cation and anion, and would not be simply a sum of their toxicities.

Table 4 Antimicrobial activity of HILs with glyphosate anion and their respective precursors towards enrichment culture used in the mineralisation experiment.

Precursor	**EC_{50} [mg/L][a]**	**Toxicity[b]**	**HIL**	**EC_{50} [mg/L][a]**	**Toxicity[b]**
[K][Glyph]	>1000	harmless	[-]	[-]	[-]
[Chol][Cl]	>1000	harmless	**[Chol]**[Glyph]	>1000	harmless
[C_{12}Chol][Cl]	47.5 ± 0.9	slightly toxic	**[C_{12}Chol]**[Glyph]	49.8 ± 1.3	slightly toxic
[C_{16}TMA][Cl]	23.8 ± 0.2	slightly toxic	**[C_{16}TMA]**[Glyph]	26.2 ± 0.3	slightly toxic
[BA][Cl]	6.1 ± 0.1	moderately toxic	**[BA]**[Glyph]	7.0 ± 0.1	moderately toxic
[DDA][Cl]	1.2 ± 0.1	moderately toxic	**[DDA]**[Glyph]	2.0 ± 0.1	moderately toxic

[a] The tested concentrations were set by active substance (Glyph) and the amounts of cations in precursors are equal to those in HILs. Classification of toxicity according to (Passino and Smith, 1987); >1000 mg/L – harmless, 100–1000 mg/L – practically harmless, 10–100 mg/L – slightly toxic, 1–10 mg/L – moderately toxic, <1 mg/L – toxic.

These results fit the observations made by some authors (Kaczmarek et al., 2019; Pernak et al., 2016, 2011; Pęziak-Kowalska et al., 2017), who as an advantage of HILs listed the possibility to apply lower doses of these herbicides on fields. It is perfectly reasonable statement, since high toxicity of cations combined with that of an herbicidal anion will result in creation of a formulation that is harmful to weeds. However, as it was discussed earlier, these substances upon entering the environment (*i.e.*, soils) will most likely strongly adsorb to the matrix and hence decrease their biodegradation potential. This in turn calls for a debate whether combining herbicidal anions with cationic surfactants is desired in terms of their environmental fate.

3.3. Assessment of bacterial community structure in soil via barcoded 16S rRNA gene MiSeq sequencing analysis

Metagenomic analysis of V4 hypervariable region of the 16S rRNA gene (details described in Section *2.9. Assessment of bacterial community structure in soil via barcoded 16S rRNA gene MiSeq sequencing analysis*) identified 16 classes. The most dominant microbial classes in enriched culture utilised in bioaugmentation approach were *Bacilli* (44.17%), followed by *Gammaproteobacteria* (28.00%), *Clostridia* (26.44%), *Actinobacteria* (0.72%) and *Alphaproteobacteria* (0.35%). The bacterial community was able to efficiently degrade glyphosate as a sole carbon source (details in Supplementary Material, Section *2. Biodegradation in aqueous environment*).

The changes in the structure of bacterial communities isolated from experimental samples were established in the same manner. At class level, mostly members of *Bacilli* and *Gammaproteobacteria* dominated the soil bacterial communities, followed by *Clostridia*. Besides, members of the class *Alphaproteobacteria* were present with a relative abundance mostly between 0–8%, with a few exceptions. After 28 days, in the bioaugmented

504 [C_{12}Chol][Glyph] treated soil, the relative abundance of *Alphaproteobacteria* reached an
505 extremely high value of 81.9%. The elevated abundance of these bacteria was also observable
506 in both non-bioaugmented and bioaugmented [C_{16}TMA][Glyph] treated soils (28 days) (25.3%
507 and 37.9%, respectively). In case of these treatments, the high abundance of
508 *Alphaproteobacteria* was due to the high abundance of *Rhizobiaceae*-related bacteria.
509 Nevertheless, after 90 days, the high abundance of *Rhizobiaceae* completely disappeared.
510 Interestingly, the abundance of class *Actinobacteria* was typically below 1% in the soil samples,
511 with the exception of two treatments. These were the bioaugmented [BA][Glyph] treated soil
512 samples (both 28 days and 90 days), in which members of the class *Actinobacteria* were
513 overwhelmingly dominated the bacterial communities with a relative abundance of 42.5% and
514 45.3%, respectively. These two microbial communities were considerably different from the
515 others, since members of the families *Propionibacteriaceae* and *Microbacteriaceae* were the
516 most dominant, while being completely missing from all the other samples. However, aside
517 from the few samples mentioned above, microbial community structures of the differently
518 treated soil samples were highly similar (**Fig. 2**, **Fig. 3**, additional heatmaps and heat trees for
519 the whole samples after 28 and 90 days in Supplementary Material, Section *5. Assessment of*
520 *bacterial community structure in soil via barcoded 16S rRNA gene MiSeq sequencing analysis*).
521 In vast majority of the soil samples, *Planococcaceae* (recently *Caryophanaceae*) of class *Bacilli*
522 were highly abundant. These bacteria showed a maximum abundance of 60.8% in
523 bioaugmented soil treated with [Chol][Glyph] (90 days), and a minimum of 0% in
524 bioaugmented soils treated with [BA][Glyph] (both 28 days and 90 days) and [C_{12}Chol][Glyph]
525 (28 days), respectively. Since *Planococcaceae* are known for their role in degradation of
526 organic chemicals, these were expected to be highly abundant in the case of samples with
527 organic cations (Sun et al., 2021). Yet, [BA] and [C_{12}Chol] cations of higher toxicity than

choline, might have been toxic to the *Planococcaceae* degraders, which in turn might have affected their degradation efficiency.

We have estimated the abundance of key genes of glyphosate biodegradation (*soxA* and *phnJ*) in the investigated bacterial communities by using PICRUSt2. The median relative abundance of *phnJ* gene for bioaugmented and non-bioaugmented samples after 28 and 90 days varied mostly between 0.001–0.005%. However, the maximum abundance (0.029%) was observable after 28 days in soil treated with [C_{12}Chol][Glyph] and having a bacterial community overwhelmingly dominated by *Rhizobiaceae*. In general, it was observable that the relative abundance of *phnJ* gene was >0.005% in those bacterial communities, where the abundance of *Rhizobiaceae* was relatively high (20–30%). Owing to the fact that *phnJ* genes are encoding alphaproteobacterial C-P bond lyases, this observation is not surprising (Kulikova et al., 2020). Regarding *soxA*, its relative abundance was around 0.05% in most of the samples. The highest abundance was predicted in case of non-bioaugmented soil treated with [C_{12}Chol][Glyph] (28 days), while the lowest values (<0.01%) were predicted for bioaugmented soils treated with [DDA][Glyph], [Chol][Glyph] or [BA][Glyph] (both 28 days and 90 days). The detrimental effect of these treatments on the *soxA* abundance is obviously observable in **Fig. 4**. Nevertheless, the reason for this remarkable decrease in the *soxA* abundance remained unclear since no treatment specific effect could be ascertained.

Overall, it can be concluded that no obvious change in the bacterial community structures was observable at class or family level, which undermine the unique integrity of herbicide ionic liquids in the soil at genetic level.

Samples non bioaugmented

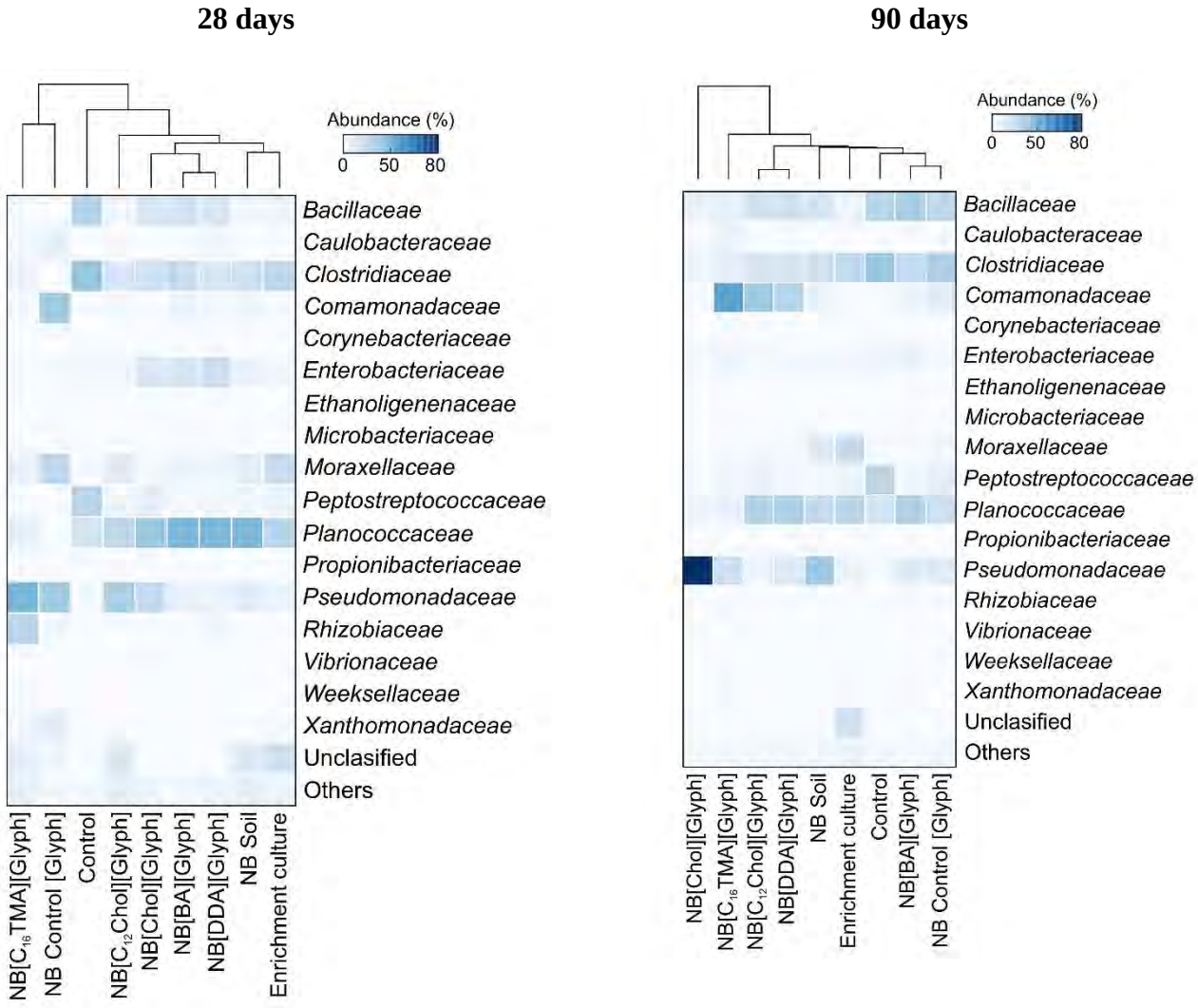
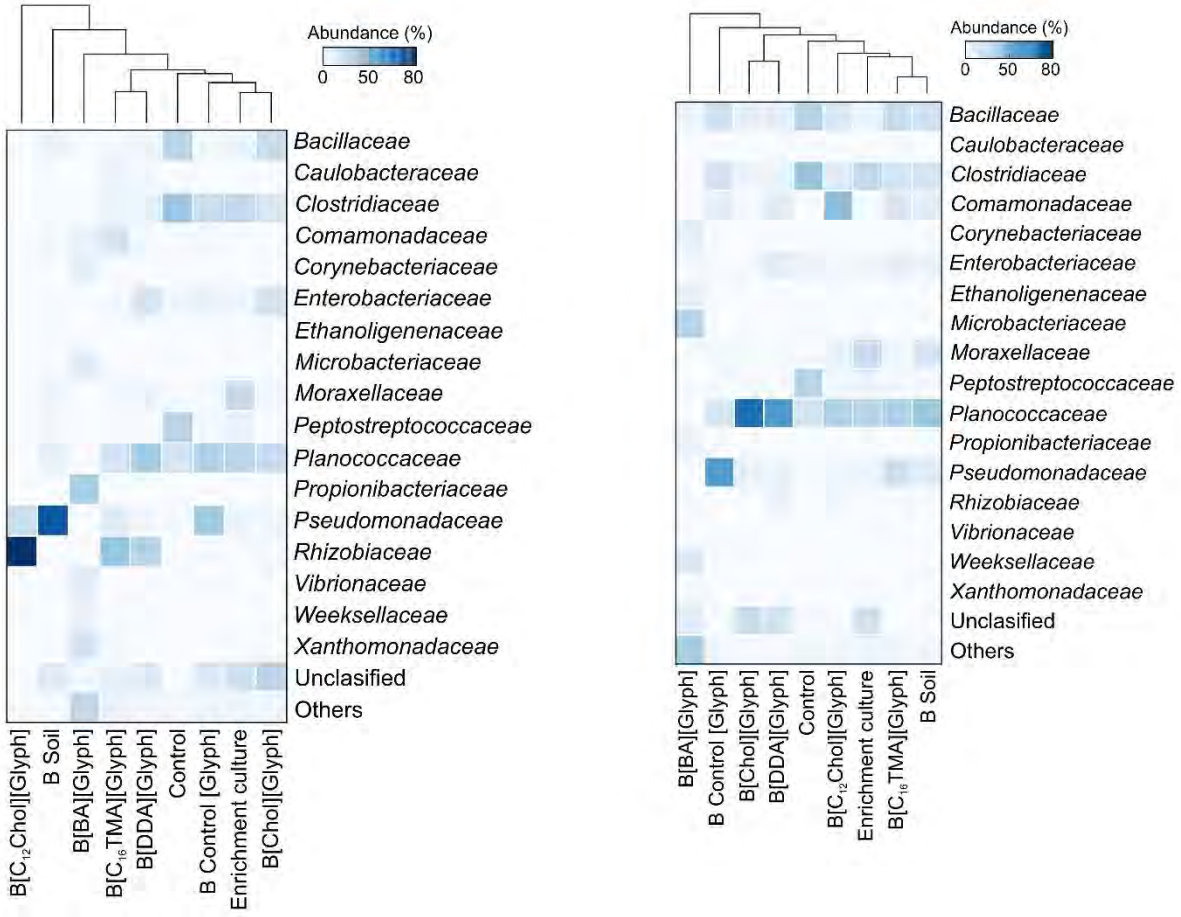


Fig. 2 Heatmap of hierarchical clustering of dominant (>5% in at least one sample) family-level bacterial microbiome composition profiles in the non-bioaugmented samples. ZOTUs not assigned to families were grouped as Unclassified, while ZOTUs with <5% abundance were grouped as Others. Darker colour represents higher abundance in the samples. NB: non-bioaugmented samples.

564 **Samples bioaugmented**

565

28 days **90 days**



566 **Fig. 3** Heatmap of hierarchical clustering of dominant (>5% in at least one sample) family-level bacterial
567 microbiome composition profiles in bioaugmented samples. ZOTUs not assigned to families were grouped as
568 Unclassified, while ZOTUs with <5% abundance were grouped as Others. Darker colour represents higher
569 abundance in the samples. B: bioaugmented samples.

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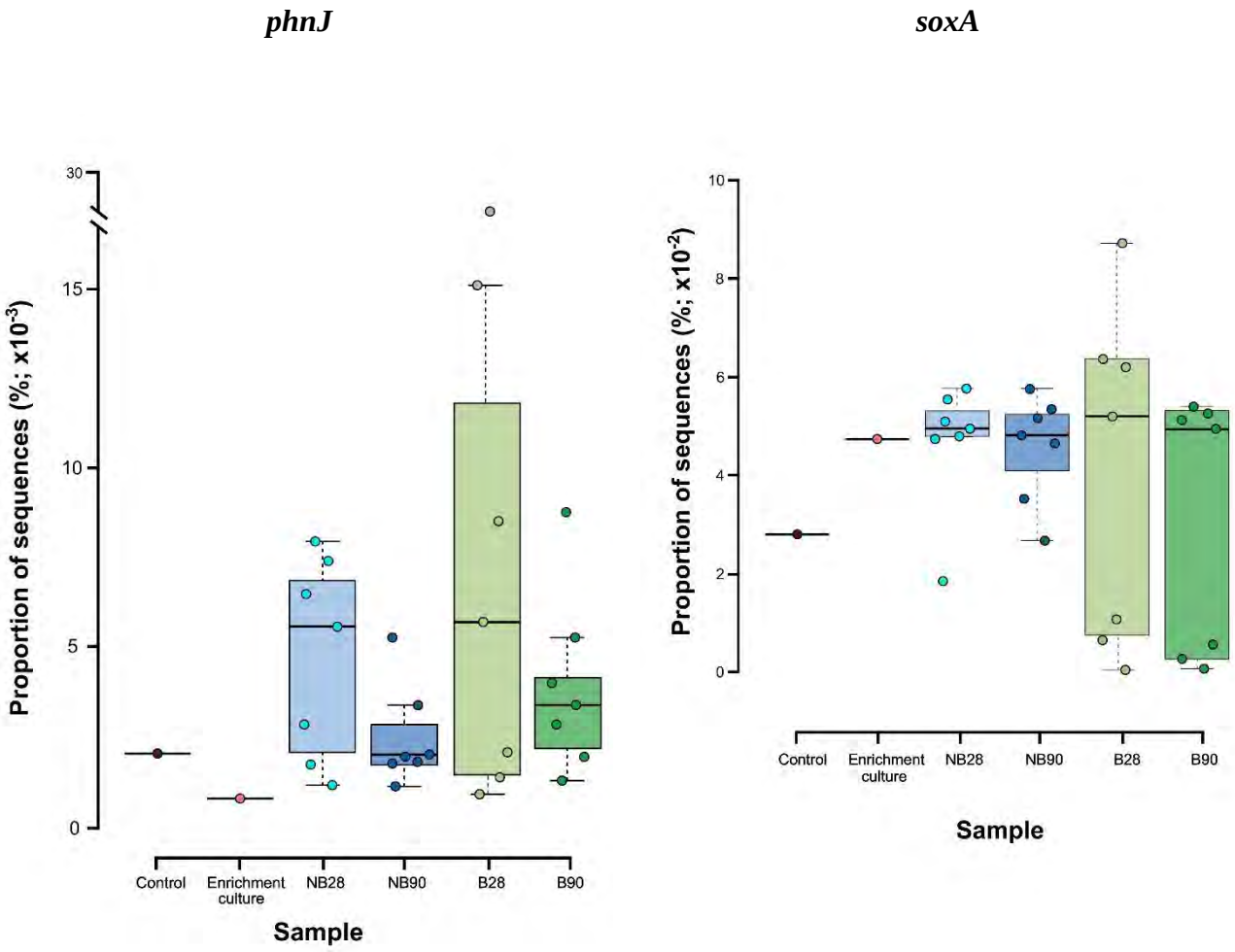

576 **Fig. 4** Boxplot representation of relative abundance of the *phnJ* and *soxA* orthologs related to KEGG. B28:
577 bioaugmented samples after 28 days, B90: bioaugmented samples after 90 days, NB28: non-bioaugmented
578 samples after 28 days, NB90: non-bioaugmented samples after 90 days.
579

580 **4. Conclusions**

581 In the course of performed experiments, the impact of cations on glyphosate degradation was
582 evaluated. The obtained results revealed that cations and anion might in fact act as separate
583 moieties in the process of glyphosate degradation. The toxicity studies has proven that the
584 toxicity of the whole formulation was strictly reflecting the toxicity of cation. Though, the
585 toxicity of resulting compound should differ from cation's toxicity if cations and anion in HILs
586 have ionic interactions in the environment. In addition, samples containing cationic surfactants,
587 unlike glyphosate anion, were proven to be highly sorbed to soil, which translated into

substantially lower bioavailability and poor mineralisation efficiencies. Furthermore, the lack of clear trends in the structure of bacterial community treated with herbicidal ionic liquids was another strong indication that in the terrestrial environment they behave more like mixture of independent ions than ionic pairs with unique properties.

The approach to enrich test samples with microorganisms specialised in glyphosate degradation allowed to observe higher degradation of HILs, visible both *via* mineralisation efficiencies and increased activity of selected enzymes taking part in glyphosate degradation. Although the abundance of *soxA* and *phnJ* genes increased in agricultural soil treated with certain HILs, these changes were not permanent and decreased over time. Thus, in view of efficiency of environmental pollutants removal, bioaugmentation did not result in significant degradation improvement, as the factor determining the rates of HILs' decomposition processes was sorption. This in turn led to the conclusion that utilising quaternary cationic surfactants in the structure of HILs is not recommended, as it may lead to sorption and accumulation of these toxic contaminants in the environment.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

The work was supported solely by funding from the National Science Centre, Poland conferred on the basis of the decision 2018/29/ B/NZ9/01136. This research was supported in part by PLGrid Infrastructure.

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