2,4-D versus 2,4-D based ionic liquids: Effect of cation on herbicide biodegradation, *tfdA* genes abundance and microbiome changes during soil bioaugmentation.

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Abstract

The commercial formulations of herbicides rely on surfactants which increase the efficiency of active substance. Herbicidal ionic liquids (ILs), in which cationic surfactants are combined with herbicidal anions, allow for additives' reduction and ensure very good herbicide performance with lower doses. We aimed to test the impact of synthetic and natural cations on biological degradation of 2,4-dichlorophenoxyacetic acid (2,4-D). Although primary biodegradation was high, the mineralization in agricultural soil indicated incomplete conversion of ILs to CO_2. Even the introduction of naturally-derived cations resulted in an increase in the herbicide's half-lives – from 32 days for [Na][2,4-D] to 120 days for [Chol][2,4-D] and 300 days for the synthetic tetramethylammonium derivative [TMA][2,4-D]. Bioaugmentation with 2,4-D-degrading strains improves the herbicides' degradation, which was reflected by higher abundance of *tfdA* genes. Microbial community analysis confirmed that hydrophobic cationic surfactants, even those based on natural compounds, played a negative role on microbial biodiversity. Our study provides a valuable indication for further research related to the production of a new generation of environmentally friendly compounds. Moreover, the results shed a new light on the ionic liquids as independent mixtures of ions in the environment, as opposed to treating them as new type of environmental pollutants.

Environmental implication:

Increasing weed resistance to crop protection products favours the use of ionic liquids with herbicidal activity (HILs) since they reduce the number of additives and provide great performance at lower doses. However, HILs are still a combination of well-known field contaminants present in commercial formulations. Our study gives a multidimensional environmental fate assessment of 2,4-D anion and surfactant cations of natural or synthetic origin. Determining the degradation extent, mineralization kinetics, microbial toxicity and soil microbiome structure with gene abundance provides a valuable indication for further research on use of naturally-derived substrates to produce a new generation of environmentally friendly compounds.

1. Introduction

In recent years, the topic of environmentally friendly solutions in agrochemistry gains an increasing attention of scientific community. One of the wider discussed issues is addition of adjuvants to commercial herbicidal mixtures. Historically, the discovery of adjuvants dates back to the end of the XIX century, where a solution of soap was found to enhance the performance of arsenical formulations on weeds. Nowadays adjuvants are still being extensively used with postemergence herbicides, to help overcome the barriers that impede absorption of the herbicide from the leaf surface to the interior of the tissues [1]. Theoretically, adjuvants are intended to be deprived from biological activity; however, various reports revealed their detrimental impact on existing ecosystems as well as the human health. The most infamous case refers to glyphosate-based formulations that contained ethoxylated tallow amines as adjuvants. They were recognized not only as active principles of human cell toxicity, but also contributors to microbiome disruption more than glyphosate alone [2,3].

Consequently, the synthesis of ionic liquids with herbicidal activity (HILs) was proposed as a unique possibility to combine herbicidal anions with quaternary surface-active cations, thus eliminating the necessity of toxic adjuvants' addition to the mixtures [4]. However, since cationic surfactants themselves might also pose a threat to microbiome due to disruption of cellular membranes [5], non-toxic cations of natural origin are often used (*e.g.*, carnitine, betaine, choline) [6,7]. Nevertheless, even the presence of harmless cations might affect soil biodiversity, which in turn might also have a vast impact on xenobiotics' degradation due to changing ratio of microbial degraders. As a result, the need to analyse their behaviour in the environment before they can be used on a mass scale, arose.

2,4-dichlorophenoxyacetic acid (2,4-D) is one of the most commonly used selective herbicides [8–10]. This chemical is considered as moderately persistent in the environment and prone to volatilisation and mobility [8,11,12]. Recently, Schortgen et al. stated also that weed control by 2,4-D dimethylamine salts strongly depends on mixture's water hardness and adjuvant inclusion [13]. Hence, 2,4-D is often paired with cations to form ionic liquids, which are by definition less volatile than herbicidal anion itself and due to their structure might affect the mobility of the whole formulation [4,6,14–29]. However, environmental tests in previous works have already proven that degradation of anions varies depending on their form, *i.e.* active substance *vs* HIL, questioning their ionic integrity upon introduction to environment [30]. In the case of 2,4-D paired with 4,4-dialkylmorpholinium, degradation of anion was lower (9–61 %) than cations (52–94 %) [21]. Analogous trend might be observed also in HILs with different anions (*e.g.*, MCPA, MCPP) [30]. In addition, degradation studies of 2,4-D anion paired with

cationic fungicides propiconazole and tebuconazole have proven that anion was not mineralised at all, while cations were degraded at approx. 65 and 94 %, respectively [24].

The results of previous studies are deemed inconclusive as to how the cation affects anion's degradation. Namely, despite the fact that the studies on HILs' biodegradation exist, it is less common to test cations and anions biodegradation separately [31]. Moreover, the interactions between ions in HILs upon introduction to the environment were not thoroughly examined. Hence, the first studies on whether they act as separate moieties or a whole formulation have appeared [31,32]. Taking into account previous findings, we decided to test the impact of cations on anion's degradation. In order to do that, we chose cations of supposedly minor toxicity, and combined them with herbicidal anion, 2,4-D. The mineralisation efficiencies of synthesised salts were then evaluated, along with their primary degradation and toxicity. Furthermore, we examined the differences in degradation between systems bioaugmented with previously isolated microorganisms specialised in 2,4-D degradation and these without bioaugmentation. Finally, we determined shifts in microbial community structures and abundance of *tfdA* genes that encodes the α-ketoglutarate-dependent dioxygenase, which catalyzes the first step of the 2,4-D degradation pathway, in bioaugmented and non-bioaugmented soil treated with herbicidal ionic liquids with synthetic and naturally-derived cationic surfactants. The results of this study will aid in understanding whether ionic liquids when introduced into the environment are just a mixture of independent ions. Since many authors indicate that they are a new type of emerging pollutants, the behaviour of both cations and anions separately is rarely analysed, so this type of work is required in order to understand the fate of ionic liquids introduced into the environment.

2. Materials and Methods

2.1. Materials

Betaine hydrochloride (99 %), *N*-dodecylbetaine (35 % aqueous solution, EMPIGEN® BB detergent), D,L-carnitine hydrochloride (98 %), 2-hydroxyethyltrimethylammonium chloride (choline chloride, 99 %), 2-dimethylaminoethanol (99 %), 1-chlorododecane (97 %), tetramethylammonium chloride (98 %), benzyltrimethylammonium chloride (97 %), tetrabutylammonium chloride (97 %), 2,4-dichlorophenoxyacetic acid (97 %), methanol (LC-MS grade) and ammonium acetate (LC-MS grade) were purchased from Sigma-Aldrich (St. Louis, MO, USA). *N*-(3-cocoamidopropyl)betaine (30 % aqueous solution, Dehyton® PK 45) was purchased from BASF (Ludwigshafen, Germany). Potassium hydroxide (>85 %), hydrochloric acid (35 %) and all solvents were purchased from Avantor Performance Materials

Poland S.A (Gliwice, Poland). All solvents were used without further purification, whereas quaternary ammonium chlorides as well as *N*-dodecylbetaine and *N*-(3-cocoamidopropyl)betaine were thoroughly dehydrated/dried and stored over P_4O_{10} before the synthesis.

2.2. Synthesis

Five 2,4-D-based salts: [Bet][2,4-D] (betainium 2,4-dichlorophenoxyacetate), [C_{12}Bet][2,4-D] (dodecylbetainium 2,4-dichlorophenoxyacetate), [CAPBet][2,4-D] (cocamidopropylbetainium 2,4-dichlorophenoxyacetate), [Car][2,4-D] (carnitinium 2,4-dichlorophenoxyacetate), [Chol][2,4-D] (cholinium 2,4-dichlorophenoxyacetate) were synthesized and identified according to the previously described methods [6,26,27]. Dodecyl(2-hydroxyethyl)dimethylammonium chloride was obtained according to the protocol described recently [33].

Four 2,4-D-based salts: [C_{12}Chol][2,4-D] (dodecyl(2-hydroxyethyl)dimethylammonium 2,4-dichlorophenoxyacetate), [TMA][2,4-D] (tetramethylammonium 2,4-dichlorophenoxyacetate), [BTMA][2,4-D] (benzyltrimethylammonium 2,4-dichlorophenoxyacetate) and [TBA][2,4-D] (tetrabutylammonium 2,4-dichlorophenoxyacetate) were synthesised according to procedure described previously [26,34].

Products isolation and purification

After synthesis, the solvents were evaporated from the post-reaction mixture and the obtained products were additionally purified through dissolution in a small portion (10–15 mL) of acetone. The precipitated impurities were filtered off and the solvent was evaporated from the filtrate. Finally, the obtained products were dried at 40 °C for 24 h under reduced pressure (1–2 mbar). After the synthesis, all 2,4-D-based salts were stored in a vacuum desiccator over a drying agent (P_4O_{10}).

2.3. Characterization of herbicidal ionic liquids

Spectral analysis

^{1}H NMR spectra were recorded on a VNMR-S spectrometer (Varian, USA) operating at 400 MHz with TMS as the internal standard (DMSO was used as a solvent for analyses). ^{13}C NMR spectra were obtained with the same instrument operating at 100 MHz. Resulting spectra are presented in **Fig. S1-S6**.

154

155 *Melting point*

156 MP 90 melting point system (Mettler Toledo, Switzerland) was used in order to determine the
157 melting points of the obtained salts. The precision of the measurements was ensured by
158 calibration of the apparatus using certified reference substances.

159

160 *Water content*

161 The water content in the synthesized salts was measured with a TitroLine 7500 KF trace
162 apparatus (SI Analytics, Germany) using the Karl Fischer titration method. The water content
163 was determined in pure methanol as well as in the obtained methanolic solutions containing
164 appropriate salt. Based on the collected results, the water content in pure products was
165 calculated.

166

167 *Toxicity evaluation*

168 Tested HILs were evaluated for antimicrobial activity towards environmental strains utilised in
169 bioaugmentation study with half maximal effective concentration (EC_{50}) assay [35]. The culture
170 was transferred from glycerol stocks 20 % (v/v) to sterile mineral medium (MM) (0.5 g/L NaCl,
171 1.0 g/L NH_4Cl, 2.8 g/L KH_2PO_4, 7.0 g/L $Na_2HPO_4 \times 2H_2O$) amended with 0.5 g/L of 2,4-D and
172 cultured at 28 ± 2 °C for 24 h. After three transfers, the cell suspension in fresh medium was
173 adjusted to reach optical density OD_{600} equal to 0.100 ± 0.010. Following, 200 µL of microbial
174 solution was placed in a sterile 96-well plate and incubated (30 °C, 120 rpm, 4 h) in order to
175 reach exponential growth stage. Then, each of tested formulations (50 µL) were added in
176 triplicates to a specific well in a plate, in active substance concentrations of 1 – 1000 mg/L (1,
177 5, 10, 50, 100, 250, 500, 1000 mg/L), and incubated in the same conditions for 12 h. Abiotic
178 control (compound solutions without microorganisms) and biotic control (microorganisms
179 without compounds) were prepared as well. Based on optical density OD_{600} measurements,
180 EC_{50} values were determined according to procedure described by Piotrowska et al. (2017) [35],
181 using the following formulas [36]:

182 $$G_R = \frac{\ln(ODx) - \ln(ODy)}{t} \quad (1)$$

183 $$G_I = \frac{G_R sample}{G_R control} \times 100\ \% \quad (2)$$

184 where G_R – microorganisms' growth rate, G_I – microorganisms' growth rate inhibition, t –
185 growth time, ODx – optical density at the time of substance addition, ODy – optical density at
186 time y after substance addition.

187 Finally, dependence between the concentration of compounds and microbial growth inhibition
188 was plotted, and EC_{50} values were determined.
189
190 *2.4. Isolation of 2,4-D-degrading enrichment culture*
191 The soil used for isolation purposes was collected into sterile containers from the depth of 10–
192 20 cm [37] from an agricultural field in Gorzów Wielkopolski, Poland (N 52.42337, E
193 15.17374). This soil had a proven history of herbicide treatments. Samples were sieved through
194 1.6 mm sieve and stored at 4 °C until isolation (no longer than 24 h). The cultivation was
195 performed in sterile Erlenmeyer flasks (150 mL) filled with 25 mL of MM [38] supplemented
196 with 0.5 g/L of 2,4-D as the sole source of carbon and energy for isolated microorganisms. The
197 concentration of herbicide in the cultivation medium was chosen based on literature data [38–
198 45]. Approximately 5 g (wet weight) of soil served as an inoculum. The cultures were then
199 incubated on a rotary shaker (120 rpm) at 28 °C for 7 days in darkness. Subsequently, they were
200 transferred three times to a fresh medium (25 mL, MM + 2,4-D).
201 In order to confirm the ability of isolated enrichment culture to degrade 2,4-D, the experiment
202 in aqueous environment was performed. Briefly, the cultivation was performed in sterile
203 Erlenmeyer flasks (250 mL), filled with sterile MM + 2,4-D (50 mL) and then inoculated with
204 enrichment culture prepared as described above, to reach optical density OD_{600} equal to 0.100
205 ± 0.010. In addition, biotic (MM + enrichment culture) and abiotic (MM + 2,4-D, without
206 microorganisms) controls were prepared. All microcosms were incubated in the dark, at 28 ± 2
207 °C with constant shaking (120 rpm) for 7 days. Samples were collected every 12 h until the end
208 of the experiment to determine the concentration of 2,4-D (LC-MS/MS analysis, *detailed*
209 *description in section 2.5.5. LC-MS analysis*). In the framework of this study, it has been
210 established that, with the addition of isolated microorganisms, [Na][2,4-D] samples (sodium
211 salt of 2,4-dichlorophenoxyacetic acid, concentration 0.5 g/L) half-lives were 14 days, without
212 simultaneous losses in abiotic controls.
213
214 *2.5. Biodegradation of HILs in soil environment*
215 *2.5.1. Preparation of inoculum*
216 The freshly isolated 2,4-D degrading microbial community was transferred to a 5,000 mL
217 SIMAX bottle filled with 1,000 mL of sterile TSB 50 % (Sigma Aldrich, Poland) with the
218 addition of 2,4-D (0.5 g/L). Thus prepared culture was then incubated (72 h, 28 ± 2 °C, 120
219 rpm), washed three times with sterile NaCl (0.85 %, v/v) solution, centrifuged (15 min, 4,500

rpm, 4 °C), and finally resuspended in sterile NaCl (0.85 %, v/v) in order to obtained final concentration of biomass in each sample equal to 2.01×10^8 CFU/g of soil.

2.5.2. Soil

Pristine soil, which was used throughout all the experiments, was collected from the depth of 10-20 cm from an agricultural field in Rzgów, Poland (N 52.151103, E 18.050041). Soil previously untreated with herbicides from an agroecological agriculture (other than that used to isolate the 2,4-D degrading enrichment culture) was selected in order to exclude the influence of previous contamination on obtained results. After sampling, the soil was stored in secured containers in order to prevent its contamination. The soil was then characterised according to USCS (Unified Soil Classification System) as sandy loam [46] and was described as follows: field water capacity: 0.23 m^3/m^3; relative field capacity: 0.562 m^3/m^3; porosity: 0.42 m^3/m^3; bulk density: 1.38 Mg/m^3; soil moisture during sampling 17 %; organic carbon: 1.5 %; N-NO_3: 7.8 mg/kg d.w.s.; N-NH_4: 1.5 mg/kg d.w.s.; Mg: 68.0 ± 1.3 mg/kg; K: 87.0 ± 2.3 mg/kg; P: 82.0 ± 1.1 mg/kg; grain size distribution: 2.0–0.05 = 71 %, 0.05–0.002 = 27 %, <0.002 = 2 %.

2.5.3. Mineralisation experimental setup

The mineralisation experiment was conducted in two variants: bioaugmented with microorganisms capable of 2,4-D degradation (B) and non-bioaugmented (NB). The tests were executed in sealed 1,000 mL glass bottles filled with 100 g of non-sterile soil.

Each soil portion was sieved through a 1.6 mm sieve and mixed vigorously with 15 mL of aqueous solution to reach field water capacity. The composition of liquid added to soil varied depending on the sample and was as follows: **1)** 10 mL of HIL (at a concentration of 1 g of active substance/1 kg of soil), 2 mL of N/P solution (composition below), 3 mL of inoculum in sterile NaCl (0.85 %, v/v) (bioaugmented samples); **2)** 10 mL of HIL, 2 mL of N/P solution, 3 mL of sterile NaCl (0.85 %, v/v) (non-bioaugmented samples); **3)** 10 mL of deionised water, 2 mL of N/P solution, 3 mL of sterile NaCl (0.85 %, v/v) (abiotic control); **4)** 10 mL of deionised water, 2 mL of N/P solution, 3 mL of inoculum (biotic control). The N/P solution was added in each case in order to biostimulate microbial growth and its composition was established experimentally on the basis of the characteristics of soil utilized in experiments. The final amounts of salts added to each bottle with soil (100 g) were as follows: 191.5 mg NH_4NO_3, 238.3 mg KNO_3, 56.2 mg K_2HPO_4. In the last step of experiment preparation, the CO_2 traps containing 10 mL of 0.75 M NaOH solution were placed in each bottle. All samples were prepared in triplicates and incubated at 22 ± 2 °C for 90 days.

The extent of mineralisation in tested samples was determined in accordance with Warder titration with 0.1 M HCl of solutions from CO_2 traps (NaOH and Na_2CO_3) with the use of automatic titrator (Metrohm titroprocessor 686, Herisau, Switzerland). The vials were rinsed with distilled water after each measurement, and then dried and filled with 0.75 M NaOH solution (10 mL) prior placing them inside the bottles.

2.5.4. Primary biodegradation

In order to determine primary degradation efficiencies, soil samples were subjected to extraction after 28 and 90 days of the experiments according to the following procedure. Soil in experimental bottle was thoroughly mixed under sterile conditions, followed by weighing of 2.00 ± 0.05 g of it into 15 mL centrifuge tube. Then, 0.5 mL of HCl (0.1 M) and 5 mL of acetonitrile were added to the sample, vortexed for 10 s, homogenised with the use of ultrasound bath with cooling for 30 min, and finally centrifuged (10,000 rpm, 5 min). Thus prepared extracts were filtered into fresh centrifuge tubes (15 mL) through syringe filter (PTFE 0.22 μm, 0.22 in diameter, Advantec, Tokyo, Japan). Then, the soil precipitates were combined with fresh 0.5 mL HCl (0.1 M) and 5 mL of acetonitrile and the procedure was repeated. The two extracts were combined and stored at 4 °C prior to the LC-MS/MS analysis. The described method was validated by extraction from the whole mass of soil in the sample in order to confirm that the 2 g samples are representative, and the recovery efficacies of cations and anion were determined (**Table S1**).

Subsequently, in order to describe and compare the kinetics of soil treated with herbicidal salts bioaugmented and non-bioaugmented, the first-order kinetics model was applied according to the following formula [47]:

$$C(t) = C_0 \cdot \exp(-k \cdot t) \quad (3)$$

where C(t) (mg/kg) is the residual 2,4-D concentration, C_0 (mg/kg) is the initial 2,4-D concentration and t (days) is the time when the data was collected. Rearranging and solving for k (day^{-1}) gives:

$$k = \frac{\ln[C_0] - \ln[C(t)]}{t} \quad (4)$$

from which we finally obtain half-lives $\tau_{1/2}$ (days) of 2,4-D anion in analysed herbicidal salts:

$$\tau_{1/2} = \frac{ln2}{k} \quad (5)$$

2.5.5. *LC-MS/MS analysis*

For chromatographic separation, the LC-MS/MS system was used that contained the UltiMate 3000 RSLC chromatograph from Dionex (Sunnyvale, CA, USA) coupled with the API 4000 QTRAP triple quadrupole mass spectrometer from AB Sciex (Foster City, CA, USA). For the analysis the Gemini-NX C18 column (100 mm × 2.0 mm I.D.; 3 μm) from Phenomenex (Torrance, CA, USA) was used, which was held at a constant temperature of 35 °C. The sample was injected into the column in a quantity of 5 μL. Gradient elution was used in a mobile phase flow rate of 0.3 mL/min. The composition of phase A (5 mM CH_3COONH_4 in water) and phase B (methanol) eluents was different depending on the type of analyte. Anion [2,4-D] and cations [Car], [Chol], [Bet], [TMA], and [TBMA] were separated in the following gradient: 0 min – 50 % B; 1 min – 50 % B; 2 min – 100 % B; 3 min – 100 % B. For the rest of the cations, that is [C_{12}Chol], [C_{12}Bet], [CAPBet] and [TBA], the gradient of: 0 min – 80 % B; 2 min – 100 % B; 4 min – 100 % B was used. The column effluent was ionized in the electrospray ionization source (the Turbo Ion Spray) operated in negative or positive ion mode, depending on whether anions or cations were determined. The following settings of the source parameters were applied for all samples: curtain gas 10 psi, nebulizer gas 40 psi, auxiliary gas 45 psi, temperature 450 °C, ion spray voltage +/− 4500 V. Additional mass spectrometry parameters used for quantitative analysis are presented in **Table S2**.

Due to the complex matrix of the samples, the matrix effect was evaluated to verify whether the results obtained in the study are reliable. The calculations were based on the quotient of the slopes of two calibration curves. Two sets of curves were constructed at 5 to 7 concentration levels. The slope of the calibration curve constructed from the fortified sample was divided by the slope obtained for the standard calibration curve. The quotient of the fortified sample curve slope and the standard curve slope higher than 1 indicates the existence of a signal enhancement while the values lower than 1 show signal suppression. The quotients ranging between 0.8 and 1.2 indicate that no considerable matrix effect exists. Also, the limits of detection and quantitation were calculated based on the signal-to-noise ratio. A signal-to-noise ratio equal to 3 was employed for limits of detection. A signal-to-noise ratio equal to 10 was used for limits of quantitation. The obtained results are presented in the supplementary information (**Table S3**).

320 *2.6. Structural changes in the microbiome of bioaugmented and non-bioaugmented soil*
321 *treated with HILs*
322 The structure of soil bacterial community was assessed for all treatments. For this purpose,
323 DNA from samples was isolated in adherence to the procedure presented previously by Hornik
324 et al., 2021 [48] . Subsequently, PCR reactions were prepared with the use of the Ion 16S™
325 Metagenomics Kit (A26216, Life Technologies, Carlsbad, CA, USA), which amplifies the V2–
326 V9 region of the bacterial 16S rRNA gene. The reaction was prepared in accordance with the
327 manufacturer's protocol and consists of 15 µL of 2 × Environmental Master Mix, appropriate
328 primers (3 µL) and previously isolated DNA sample (12 µL). Reactions were conducted in a
329 Veriti thermal cycler (Life Technologies, Carlsbad, CA, USA) with program parameters as
330 follows: initial denaturation (10 min, 95 °C), 25 cycles of denaturation (30 s, 95 °C), annealing
331 (30 s, 58 °C), extension (20 s, 72 °C), final extension (7 min, 72 °C). The purification of the
332 reaction products was performed as described in Hornik et al., 2021 [48].
333 The library was then prepared using the Ion Plus Fragment Library Kit (4471252, Life
334 Technologies, Carlsbad, CA, USA) [48]. Thus prepared library was then applied to beads (used
335 for sequencing) in emulsion PCR with the use of the Ion PGM™ Hi-Q™ View OT2 Kit and
336 Ion One Touch 2 Instrument (A29900, Life Technologies, Carlsbad, CA, USA). The beads were
337 then purified with an Ion One Touch ES instrument (Life Technologies, Carlsbad, CA, USA)
338 and sequenced with the Ion PGM System (Life Technologies, Carlsbad, CA, USA) using the
339 Ion PGM™ Hi-Q™ View Sequencing Kit (A29900) on an Ion 316™ Chip Kit v2 BC.
340
341 *Bioinformatic analysis*
342 The sequence reads from Ion Torrent (Thermo Fisher Scientific, Waltham, MA, USA) were
343 imported into the CLC Genomics Workbench 20.0 software (Qiagen, Hilden, Germany) and
344 processed with CLC Microbial Genomics Module 20.1.1 (Qiagen, Hilden, Germany). Chimeras
345 and reads of low-quality were filtered and removed (quality limit = 0.05, ambiguity limit = 'N').
346 All reads were then clustered against SILVA v119 database at 97 % operational taxonomic unit
347 (OTU) similarity. A beta-biodiversity analysis was carried out to compare the biodiversity of
348 the analysed soil microbiomes with each other. The Bray-Curtis index applied in this study
349 measured the similarity of two populations based on quantitative and qualitative OTU analysis.
350
351 *2.7. tfdA genes abundance in bioaugmented and non-bioaugmented soil treated with HILs*
352 Genes level was analysed using a Power SYBR Green PCR Master Mix (Life Technologies,
353 Carlsbad, CA, USA) on ABI 7500 SDS (Applied Biosystems, Thermo Fischer Scientific,

Waltham, MA, USA). Primers used for real-time PCR are listed in **Table 1**. Total bacterial RNA was quantitated by real-time PCR amplification of fragment of bacterial 16S ribosomal RNA with universal bacterial primers and TaqMan MGB probe using TaqMan Universal Master Mix II (Life Technologies, Carlsbad, CA, USA) on ABI 7500 SDS (Applied Biosystems, Thermo Fischer Scientific, Waltham, MA, USA). Sequences of primers and probe used are listed in **Table 1**. All analyses were done in triplicate. In order to compare the gene expression in each sample, the mean expression index was calculated according to formula: C_T target/C_T 16S using data from 3 analyses. This parameter reflects the level of a specific gene compared to the level of the universal gene (16S RNA) in the whole metabiome.

TABLE 1

2.8. Statistical analysis

One-way ANOVA was employed in order to detect significance of statistical differences in all systems. Error bars correspond to standard errors of the mean (n = 3). All experiments were performed in triplicate.

3. Results and discussion

3.1. Synthesis

In the framework of the following research nine 2,4-D-based quaternary ammonium salts (**Fig. 1**) were synthesized with yields exceeding 85 %. Four compounds from this group possessed a betaine-type cation: [Bet], [C_{12}Bet], [CAPBet] as well as [Car] in which a protonated carboxylic group is present. Their amino acid-like structure is responsible for different physicochemical and biological properties compared to known and widely applied classical tetraalkylamomnium cations [49,50]. The latter five cations: [Chol], [C_{12}Chol], [TMA], [BTMA] and [TBA] are substituted with conventional functional groups like straight alkyl, hydroxylalkyl or benzyl. It should be also noted that three cations ([Bet], [Car] and [Chol]) are widely present in nature and play important biological roles in various living organisms. In the group of products, [Bet][2,4-D], [C_{12}Bet][2,4-D], [CAPBet][2,4-D], [Car][2,4-D] and [Chol][2,4-D] were reported and characterized previously [6,26,27], whereas [C_{12}Chol][2,4-D], [TMA][2,4-D], [BTMA][2,4-D] and [TBA][2,4-D] are novel compounds. A thorough analysis revealed that the structure of the substituents attached to the nitrogen atom in the cation has a significant impact on the melting point of the obtained salts. In effect, four products containing [CAPBet], [Car], [C_{12}Chol] and [TBA] were found to be greasy waxes at

388 room temperature. Moreover, [Bet][2,4-D], [C_{12}Bet][2,4-D] and [BTMA][2,4-D] melted in a
389 temperature below 100 °C (in a range from 63 to 81 °C), while the salt with the smallest
390 tetramethylammonium cation [TMA][2,4-D] exhibited the highest value of melting point (184
391 °C), which was additionally accompanied with simultaneous decomposition. Nonetheless,
392 except [TMA][2,4-D] and [Chol][2,4-D], all the synthesized salts possess a melting point below
393 the established threshold (100 °C) and can be classified as ionic liquids (ILs). Analysis of the
394 water content in the obtained salts *via* Karl Fischer titration showed that they contain approx.
395 0.5–1.5 % water. The greatest values were noted for [Chol][2,4-D] and [TMA][2,4-D] that
396 contain cations exhibiting the most significant hygroscopicity. Analysis of the available data
397 provided in **Table S4** also indicate that, due to the presence of the ionic bond, 2,4-D-based
398 quaternary ammonium salts are thermally stable to the temperatures exceeding even 150 °C.
399 The collected results are typical for majority of HILs, nonetheless the synthesised compounds
400 can be considered as non-volatile, thus they exhibit extremely low risk of unintended drift *via*
401 vaporization [30].
402
403 **FIGURE 1**
404
405 *3.2. Toxicity*
406 In order to use a herbicide in the European Union, it is necessary to register selected
407 compound in accordance with Regulation (EU) No 528/2012 on the availability and use of
408 biocidal products (BPR Regulation) [51]. The registration of compounds based on the
409 provisions of the REACH and BPR regulations must be carried out according to the guidelines
410 described in Regulation (EC) No. 1272/2008, which is a regulation on classification, labeling
411 and packaging of substances and mixtures (CLP Regulation) [52]. Tests specified in the
412 REACH, BPR and CLP regulations should be carried out based on OECD guidelines [53].
413 The legislation regarding adjuvants in the EU is far different from herbicides, which
414 originates from the fact that they are considered as completely non-biologically active
415 substances. Therefore, such additives to plant protection products fall within the scope of other
416 regulation – (EC) No 1107/2009 [54]. In consequence, currently there are no specific
417 requirements (regarding, *e.g.*, recommended protocols, data acquisition and evaluation) for the
418 authorization of adjuvants within EU members. Nonetheless, the obligation to authorize an
419 adjuvant before it can be placed on the market is mentioned in the Regulation on Plant
420 Protection Products 1107/2009. However, the requirements for obtaining the authorization are
421 elaborated by each country individually and depend only on domestic legislation [54]. As a

result, the problem of the release of potentially toxic compounds into the environment in the European Union Member States has not been finally resolved.

Thus, it is the issue of the utmost importance to evaluate the toxicity of newly obtained herbicidal compounds, which are intended for commercial, agricultural use [55,56]. In **Table 2**, the results of half-maximal effective concentration (EC_{50}) analysis of HILs and respective cations used in their synthesis are presented. So far, the toxicity response of HILs were mainly evaluated toward model axenic strains [35,36,57], while here we determine their effect on enrichment culture to mimic conditions closer to the environment.

In accordance with literature data, sole 2,4-D anion is harmless to microorganisms, similarly to choline, carnitine and betaine (cations of natural origin) [8,58,59]. The addition of C_{12} alkyl modified chain or CAP to these cations resulted in increased toxicity against microorganisms. It is an effect observed in previous research, where modification of otherwise hydrophilic cations led to increased toxicity towards microbial community utilized in the experiment, and the toxicity of cationic precursors reflected the toxicity of respective HILs [60]. In fact, an increase hydrophobicity of cations in ILs formulations was often positively correlated with their higher toxicity to microorganisms, due to the disruption of bacterial cellular membranes associated with surfactant's properties [61–63]. Moreover, the previous research on HILs microbial toxicity has proven that cations are mainly responsible for the toxicity of the whole formulation [35,36,60]. The results of Piotrowska et al. have shown that increasing hydrophobicity of cation results in increased toxicity of the whole formulation, while dicamba and MCPP anions had only minor impact [35]. Similar observations were noted for double action HILs based on the esterquats, where the length of alkyl substituents was also factor greatly influencing the toxicity of the HIL [36]. However, as we used a bacterial community in this assay, the mutual synergistic effect reducing toxicity of HILs and cations' chlorides was observed in comparison to our previous research with axenic strains [32]. Thus the rest of the compounds were harmless to cultivable microbial community used in bioaugmentation approach, which increases their potential for use as crop protection products.

TABLE 2

3.3. Biodegradation of HILs in soil environment

3.3.1. Primary biodegradation

When considering primary degradation results (**Table 3**), the herbicidal anion (NB) was degraded in approx. 60 % within 90 days when it was not paired with any organic cation.

However, the presence of organic cation, either of natural origin or not, resulted in the significant decrease in degradation efficiencies of the anion. This might be attributed to the fact that easily degraded, non-toxic carbon source, such as choline, betaine and carnitine would be degraded preferentially, at the same time limiting or delaying the decomposition of herbicidal anion serving a less attractive carbon source for microorganisms. The impact of choline and carnitine cations was the smallest, yet still 2,4-D degradation was decreased (up to approx. 40 and 50 %, respectively). The introduction of other cations ([C_{12}Chol], [Bet], [C_{12}Bet], [CAPBet], [TBA], [BTMA]) caused the anion to be practically not degraded at all. It is phenomenon mentioned previously in the literature concerning HILs, yet not attributed to any specific factor by the authors [21,24,30]. In fact, this issue may be overlapped by several factors: (i) differences in cations' sorption and desorption processes which disrupts the balance in microcosms and affects 2,4-D biodegradation kinetics [64]; (ii) negative effect of cationic surfactants on herbicide-degrading microbial community during first step of biodegradation when the growth of 2,4-D degraders is still slow in pristine soil [65]; (iii) toxicity of quaternary surface-active cations and their metabolites [66]; and (iv) increase of the bioavailability of compounds harmful to 2,4-D degraders through the formation of cationic surfactant micelles [5,67].

When it comes to the impact of bioaugmentation, the addition of 2,4-D degraders resulted in generally higher degradation efficiencies of obtained herbicide. However, in the case of highly hydrophobic HILs with cations of [C_{12}Bet], [CAPBet] and [BTMA], 2,4-D degradation was not improved, yet stayed at the same level. The presences of co-occurrences of cationic surfactants might have adverse effect on the breakdown of chlorophenoxyacetic acid herbicides even though the conditions should favourable the degradation processes. Such negative impact on biodegradation kinetics of pesticides was previously demonstrated in the presence of other fungicides [68], heavy metals or even surfactants [69].

TABLE 3

Comparison of the degradation efficiencies of 2,4-D anion in our experiment in bioaugmented and non-bioaugmented systems with the respect to different cations is presented in **Fig. 2A**. As it can be observed by points present above trend-line, the bioaugmentation approach mitigates the adverse effects of cations on anion degradation. Additionally, two groups of compounds can be distinguished – well-degradable group consisting of [Na][2,4-D] and compounds with choline and carnitine cations (1), and poorly degraded compounds, where anion is paired with

hydrophobic cations (2). In the case of latter one, bioaugmentation improved their degradation, yet no to the level comparable to salts with cations of no negative impact such as [Car][2,4-D]. This might indicate that introduction of cations, whether of natural or synthetic origin, to the 2,4-D anion might be a factor determining the degradability potential of such structures.

FIGURE 2

To provide better insight into 2,4-D degradation in soil, we compiled its half-lives ($\tau_{1/2}$) with respect to the soil type used in the experiment (**Fig 3, Table S5**). These degradation half-lives are within the range of approx. 10 – 60 days [8,9,70–72], while the estimated $\tau_{1/2}$ of 2,4-D for the bioaugmented soil utilized in current experiment was approx. 32 days. However, the estimated $\tau_{1/2}$ of 2,4-D for the non-bioaugmented soil was longer (approx. 50 days) compared with bioaugmented soil, which is consistent with several studies highlighting low degradation kinetics of 2,4-D in previously untreated soils [65,73]. Additionally, we presented in **Fig. 2B** estimated half-lives of 2,4-D anion in four salts with the highest degradation extent (based on **Table 3**) in bioaugmented and non-bioaugmented systems. As it can be clearly seen, the addition of any cation contributed to longer half-lives of 2,4-D anion. Even with the introduction of cations of natural origin, such as choline and carnitine, a vast impact on half-lives can be observed – from less than approx. 40 days for 2,4-D in a form of sodium salt to approx. 120 days for [Chol][2,4-D] or more than 300 days for [TMA][2,4-D]. In general, all compounds apart from [BTMA][2,4-D] revealed higher values of estimated half-lives in non-bioaugmented soil in comparison to bioaugmented soil; however, these times for bioaugmented soil still reaching values ranging from a few months to even more than 100 years.

FIGURE 3

3.3.2. Mineralisation of HILs in bioaugmented and non-bioaugmented soil

It should be noted that primary degradation, described in the previous section, only indicates the disappearance of the analytical signal from the main compound and does not allow a deeper analysis of what is happening in the environment. Thus we performed the mineralization experiment to illustrate CO_2 evolution in bioaugmented and non-bioaugmented soils treated with HILs. The main thing observed was stimulating effect of bioaugmentation on degradation (**Fig. 4**). This means that the soil-adapted microbial community has revealed the high survival rate, persistence as well as proliferation in soil contaminated with HILs [74]. However,

supplementation with 2,4-D degraders accelerated mineralisation only within the first 5-28 days, depending on the compound, which is related to the most intensive biodegradation processes after introduction of xenobiotics to soil [75].

FIGURE 4

Extent of mineralization correlates well with primary degradation of HILs. As presented in the **Table 4**, bioaugmentation with 2,4-D degraders resulted in the increase in mineralisation efficiencies in almost each case. Sole herbicide ([Na][2,4-D]) by the end of experiment was however mineralised only in approx. 3 and 33 % (non-bioaugmented and bioaugmented samples, respectively). As mentioned before, this is connected to the fact that pristine soil utilised in the experiment had no previous contact with 2,4-D herbicide, and former research has already proven that xenobiotics are efficiently degraded in the environment adapted to their presence [31]. Additionally, 2,4-D is considered a mobile compound in soils, thus microorganisms and genes responsible for its degradation might not be abundant in soils [9].
The introduction of cations of natural origin (choline, carnitine, betaine) resulted in vastly greater mineralisation efficiencies at the end of the experiment, most probably due to the presence of easily degraded cation, rather than better mineralisation of an anion. On the other hand, introduction of hydrophobic aliphatic chains (such as C_{12}), as well as synthetic quaternary amines ([TBA], [TMA], [BTMA]) resulted in substantially lower mineralisation efficiencies. Most of the cations applied in this study were cationic surfactants, either synthetic or modified natural ones, thus the surfactants' concentration would be an important factor affecting degradation of co-occurring herbicides. On the one hand, recent studies have shown that surfactants applied to soil may inhibit microbial activity by inducing changes in bacterial membrane integrity and permeability [67]. Certain concentration of surfactants, commonly used in soil remediation, may pose toxic effects on soil microbiome, as presented on the example of less toxic sodium dihexylsulfosuccinate, which stopped degradation of tributyltin biocide [76]. On the other hand, at some conditions, cationic surfactants may tend to increase the adsorption coefficient value (K_f) of herbicides by partitioning them into the hydrophobic part of surfactants [77], resulting in reduced bioavailability of those herbicides. However, when considering HILs based on 2,4-D, it has been proven that cations and anions are sorbed independently of each other, but their biodegradation potential, considering the desorption of surfactants even in low quantities, is still unknown [32,78,79].

TABLE 4

3.4. Structural changes in the microbiome of bioaugmented and non-bioaugmented soil treated with HILs

Next, a sequencing analysis of a highly variable 16S rRNA region was performed in order to determine changes in the structure of the bacterial community in soil microcosms (**Fig. S7**). Pristine soil (non‑bioaugmented, with no addition of HILs), consisting of native microorganisms solely, was dominated by two types of bacteria: *Proteobacteria* (41.0 %) and *Firmicures* (20.0 %). The proportion of bacteria belonging to the *Bacterioidetes*, *Actinomycetes* and *Planctomycetes* phyla ranged from 6.3–9.0 %, while the proportion of other bacterial types did not exceed 5.0 %. In contrast, bioaugmented samples were dominated by three types of bacteria, *i.e.*, *Proteobacteria* (51.0 %), *Bacterioidetes* (22.3 %) and *Firmicutes* (18.7 %). The proportion of the other types did not exceed 2.7 %. Vast majority of 2,4-D-degrading microorganisms, isolated from agricultural soil environments, belong to both *Proteobacteria* and *Bacterioidetes* phyla [80,81]. Based on beta-biodiversity analysis, untreated soil and soil supplemented with 2,4-D degrading enrichment culture lie at a considerable distance from each other, indicating that after 90 days the microbiome of the bioaugmented soil has changed significantly with respect to the non-bioaugmented soil (**Fig. 5**).

The addition of 2,4-D based compounds with inorganic as well as organic, naturally originated cations (carnitine and choline) to soil caused the initial significant enhancement in *Proteobacteria* within the first 28 days, which was not a case for other herbicidal compounds (**Fig. S7)**. Taking into consideration that 2,4-D anions of those salts revealed the highest degradation extent, it was not a surprise that well-known 2,4-D degrading genera from *Proteobacteria* will adapt effectively to those microsomes [82]. However, it should be also noted that after 90 days, the proportion of *Bacterioidetes* (20.9–23.5 %) and *Firmicutes* (8.7–17.6 %) increased, while the proportion of bacteria belonging to the *Proteobacteria* type returned to its original level (39.4–44.3 %). These findings correspond well with the results obtained by Nguyen et al. (2021) [83], where the increase in *Bacteroidetes* and some genera from *Firmicutes* were observed in soil microcosms treated with 2,4-D and 2,4,5-T; or those described by Pan et al. (2022) [84] who identified *Firmicutes* as one of the dominant phyla in a soil community treated with MCPA (which bears structural similarity to 2,4-D). Our results indicate that the observed shifts in the abundance of specific phyla may be related to their ability to degrade chlorophenoxyacetic acid herbicides. In the bioaugmented samples ([Na][2,4-D], [Car][2,4-D] and [Chol][2,4-D]) the proportion of *Proteobacteria* and *Bacterioidetes* decreased

(to 43.1–46.5 % and 16.8–19.8 %) after 90 days, respectively, while *Firmicutes* ranged from 16.0–22.2 %, meaning that the soil microbiome begins to recover to its original state of equilibrium. Other studies have also indicated that long-term herbicide application had no significant effect on changes in microbial community or soil biochemical processes in both laboratory and field experiments [85–87].

The addition of the other ionic liquids with more hydrophobic cations such as [C_{12}Chol][2,4-D], [C_{12}Bet][2,4-D], [TBA][2,4-D] or [TMA][2,4-D] reduced the proportion of *Proteobacteria* in the soil microbiome to the range of 25.0–30.0 %, *Bacterioidetes* to 7.5–14.0 %, and *Firmicutes* to 11.3–26.7 %. Additionally, in the bioaugmented soil, the same compounds also caused a significant reduction in the proportion of bacteria belonging to the *Proteobacteria* (less than 31 %), *Bacterioidetes* 5.8–17.6 % and *Firmicutes* (15.0–29.8 %) (**Fig. S8**). However, the growing abundance of bacteria belonging to *Planctomycetes* (by 5.4–19.3 %), *Actinobacteria* (by 6.9–16.5 %) and *Acidobacteria* (by 7.2–10.4 %) was determined. An increase in the number of *Actinobacteria* members was also detected in the presence of oligomeric herbicidal ionic liquids with MCPA and dicamba anion [88]. It is worth noting that, as in the present study, an enhancement of *Actinobacteria* abundance was observed in the rhizosphere bacterial community structure treated with [Bet][2,4-D] during the field experiment [32].

The above-mentioned differences were also reflected in beta-biodiversity analysis, where three clusters of microbiomes were located between microbiomes of soils nontreated with HILs, each located in a different plane (**Fig. 5**). The points closest to the non-bioaugmented soil microbiome represent the microbiome of soils to which [Na][2,4-D], [Car][2,4-D] and [Chol][2,4‑D] have been added, both with and without supplementation of enrichment culture. In contrast, the microbiomes of [C_{12}Chol][2,4-D], [TBA][2,4-D], [TMA][2,4-D] and [Bet][2,4-D], in both soil with and without bioaugmentation, had microbiome structure more similar to soil supplemented 2,4-D degraders, while the microbiomes of [C_{12}Bet][2,4-D], [CAPBet][2,4-D] and [BTMA][2,4-D] were equidistant from both soils. Based on beta-biodiversity analysis, it can be concluded that bioaugmentation was successful and that the changes in structure of the soil microbiome depend mainly on the cationic structure in herbicidal ionic liquids. However, it should be emphasised that differences in sorption and cation exchange capacity between soil types can affect both the behaviour of HILs as well as their impact on the soil microbial community.

FIGURE 5

3.5. tfdA genes abundance in bioaugmented and non-bioaugmented soil treated with HILs

To correlate changes in the microorganisms found in the soil treated with HILs, we monitored the abundance of 2,4-D degradation genes compared to their presence in the microbiome of untreated soil. Three subgroups of the oxygenase gene (*tfdA*) responsible for the initial transformation of 2,4-dichlorophenoxyacetic acid to 2,4-dichlorophenol were analysed [82]. The addition of herbicidal ionic liquids to non-bioaugmented soil did not notably change the number of *tfdA* genes in the environmental gene pool, yet a higher amount of these genes was determined within the first 28 days (**Fig. S9**). However, a significant effect of bioaugmentation on the enhancement in the number of *tfdA* genes in the gene pool of soil microorganisms was evident (**Fig. 6**). Bioaugmentation caused an increase in the abundance of all analysed *tfdA* genes (class I, II and III) visible at day 28 of the process, and at day 90 the number of genes responsible for 2,4-D biodegradation decreased. Overall, the number of copies of the gene encoding TFD A class III was the highest, followed by class II and class I enzymes. It was found out that during degradation of phenoxyalkanoic acid herbicide in treated and untreated soils, mainly class III *tfdA* genes were involved in mineralization of compounds such as MCPP [89], MCPA [90] or 2,4-D [91,92]. Class III *tfdA* genes includes oligotrophic, slowly growing α-*Proteobacteria*, class II *tfdA* genes is composed of strains from α subdivision of *Proteobacteria* and class I comprise fast-growing genera in the β- and γ-subdivisions of the *Proteobacteria* [93,94]. The higher abundance of *tfdA* genes correspond well with the structural changes of microbiomes treated with HILs, where the dominant phyla of those soil communities was *Proteobacteria.* The observed shifts in the abundance of specific classes of *tfdA* genes were associated with 2,4-D degradation in the short term. Gonod et al. (2006) showed that microbial community structure and variation in the number of these genes were significantly modified in response to soil treatment with ^{14}C-ring labelled 2,4-D only shortly after application of the herbicide [95]. Results of this study suggested that the impact of 2,4-D on the soil microbial community is transient and does not cause permanent changes in the terrestrial environment. Thus, in the long term, soil microbial structure and gene abundance variability would regain their original equilibrium balance when toxicity of analysed compounds can be omitted [96,97]. It should be noted that the introduction of cations, whether of natural origin or not, into 2,4-D-based HILs resulted in a decrease in the total number of *tfdA* genes in bioaugmented soil after 90 days compared to 2,4-D in the form of sodium salt (**Fig. S9**). However, higher total number of genes was observed for [TBA][2,4-D] and [BTMA][2,4-D], which also reveal the lowest mineralization efficiency. Bearing in mind the recent reports that in the environment HILs no longer form ionic pairs and undergo physicochemical and biological processes separately

660 [31,97], the above-mentioned results are clear evidence that the degradation of herbicidal ionic
661 liquids in soil may be shaped by various factors.
662
663 **FIGURE 6**
664
665 **4. Conclusions**
666 The study evaluated the effect of cations in HILs on the degradation of 2,4-D anion in soil.
667 Although the primary biodegradation of cations was high, their presence in the structure of
668 HILs was proven to exhibit inhibitory effects on 2,4-D degraders and resulted in limiting or
669 delaying the decomposition of herbicidal anion. Moreover, molecular studies confirmed the
670 negative impact of hydrophobic cationic surfactants on the microbial biodiversity.
671 Bioaugmentation with 2,4-D-degrading strains improved herbicides' degradation, as reflected
672 in mineralization efficiencies, soil microbiome structure and higher abundance of *tfdA* genes.
673 However, significant differences in 2,4-D degradation, herbicides' half-life and *tfdA* gene
674 abundance were evident only for cations of natural origin. In the case of transformation of
675 naturally-derived cations, no matter whether synthetic or modified cations were used, those
676 differences were suppressed.
677 The multidimensional approach to study biodegradation and mineralisation of HILs, their
678 toxicity towards microorganisms, structure of soil microbiome as well as the gene abundance
679 provide an important guidelines for future research on new generation of plant protection
680 agrochemicals. The influence of these auxiliary surface-active substances can be crucial and
681 might determine the environmental fate of the active substance, as can be seen from our
682 experiment. Nowadays, the degradation studies focused on single pollutants are insufficient, as
683 these xenobiotics occur in the environment in complex mixtures. The fate of herbicidal ionic
684 liquids is currently understudied, as their ionic integrity upon their introduction to the
685 environment is starting to be questioned. In fact, the current state of knowledge lacks a
686 comprehensive approach to monitor the degradation of such co-contaminants at the molecular
687 level. Therefore, understanding of changes in the structure of the soil microbiome and shifts in
688 the abundance of *tfdA* genes in the presence of 2,4-D-based quaternary ammonium salts is
689 essential in order to develop an effective removal protocol for surfactant-herbicide
690 contaminations. Moreover, since the registration regulations refer only to active substances and
691 not to adjuvants themselves, the fast reconsideration of the currently prevailing requirements
692 and their modification in order to seal the bureaucratic system are crucial to avoid irreparable
693 losses, such as those in recent years.

Conflicts of interest

There are no conflicts to declare.

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CAPTIONS FOR FIGURES

Fig. 1. Structures of the ions in the synthesized salts

Fig. 2. Comparison of degradation efficiencies of 2,4-D anion with the respect to different cations (A) and estimated half-lives for 2,4-D anions in [Na][2,4-D], [Chol][2,4-D], [Car][2,4-D] and [TMA][2,4-D] in bioaugmented and non-bioaugmented soils (B).

Fig. 3. Half-lives of 2,4-D reported in the literature for soils (1–10) of varying characteristics (Table S5). The dashed line (no. 6) indicates half-life of 2,4-D in the non-bioaugmented soil used in the current study.

Fig. 4. Mineralisation curves (CO_2 evolution) for analysed HILs with 2,4-D anion during 90 days. Values are presented with regard to appropriate controls (NB for the approach without bioaugmentation, B for bioaugmented samples).

Fig. 5. Principal coordinate analysis (PCoA) based on the Bray–Curtis dissimilarity metrics showing the distance in the bacterial communities between bioaugmented (circle) and non-bioaugmented (square) soils treated with HILs.

Fig. 6. Log2-fold change values determined by real-time PCR of *tfdA* genes from the subgroup: class I (A), class II (B) and class III (C). NB28 days means non-bioaugmented samples after 28 days, NB90 days means non-bioaugmented samples after 90 days, B28 days means bioaugmented samples after 28 days, B90 days means bioaugmented samples after 90 days.

Table 1. Primers used for real-time PCR.

Target genes	Primers	Sequence (5' to 3')	Ref.
tfdA	*tfdA* (CI)-class I	F: GTGAGCGTCGTCGCAAAT R: GCATCGTCCAGGGTGGTC	[42]
	tfdA (CII)-class II	F: TGAGCATCAATTCCGAATACC882 R: AAGACTGACCCCGTGGACT	[42]
	tfdA (CII)-class III	F: TGAGCATCACTTCCGAATACC856 R: ACAGCGTCGTCCAACGTC	[42]
16S rRNA	F968 Forward R1401 Reverse	F: AACGCGAAGAACCTTAC R: CGGTGTGTACAAGACCC	[43]

Table 2. Toxicity towards enrichment culture isolated within this study of salts with 2,4-D anion.

Precursor	EC_{50} [mg/L]*	Toxicity**	Compounds	EC_{50} [mg/L]*	Toxicity**
[Na][2,4-D]	>1000	H	[-]	[-]	[-]
[Car][Cl]	>1000	H	[Car][2,4-D]	>1000	H
[Chol][Cl]	>1000	H	[Chol][2,4-D]	>1000	H
[C_{12}Chol][Cl]	47.5 ± 0.9	ST	[C_{12}Chol][2,4-D]	36.3 ± 0.8	ST
[Bet][Cl]	>1000	H	[Bet][2,4-D]	>1000	H
[C_{12}Bet][Cl]	342.2 ± 1.4	PH	[C_{12}Bet][2,4-D]	321.7 ± 8.7	PH
[CAPBet][Cl]	61.5 ± 0.3	ST	[CAPBet][2,4-D]	54.9 ± 0.1	ST
[TBA][Cl]	>1000	H	[TBA][2,4-D]	>1000	H
[TMA][Cl]	>1000	H	[TMA][2,4-D]	>1000	H
[BTMA][Cl]	>1000	H	[BTMA][2,4-D]	>1000	H

* The concentrations were determined by active substance (2,4-D)
**Toxicity classification according to [98]; >1000 mg/L – harmless (H), 100–1000 mg/L – practically harmless (PH), 10–100 mg/L – slightly toxic (ST), 1–10 mg/L – moderately toxic (MT), <1 mg/L – toxic (T).

Table 3. Primary degradation of tested compounds after 90 days.

Compounds	Primary degradation [%]			
	cation NB*	cation B**	anion NB*	anion B**
[Na][2,4-D]	[-]	[-]	64.3 ± 1.2	61.5 ± 1.4
[Car][2,4-D]	99.7 ± 2.1	99.8 ± 2.7	50.8 ± 1.3	74.4 ± 0.9
[Chol][2,4-D]	98.4 ± 2.3	97.9 ± 2.2	40.4 ± 0.6	39.9 ± 0.7
[C_{12}Chol][2,4-D]	74.1 ± 1.1	78.7 ± 1.5	0.2 ± 0.4	21.5 ± 1.0
[Bet][2,4-D]	98.5 ± 2.4	99.2 ± 2.6	0.1 ± 0.3	10.0 ± 1.0
[C_{12}Bet][2,4-D]	98.5 ± 2.5	98.2 ± 2.5	0.2 ± 0.4	0.3 ± 0.4
[CAPBet][2,4-D]	98.6 ± 2.3	98.8 ± 2.2	0.3 ± 0.5	0.4 ± 0.3
[TBA][2,4-D]	0.2 ± 0.3	31.9 ± 0.9	0.3 ± 0.5	20.3 ± 1.1
[TMA][2,4-D]	0.1 ± 0.4	0.2 ± 0.5	11.2 ± 0.9	17.2 ± 0.9
[BTMA][2,4-D]	0.1 ± 0.2	0.3 ± 0.3	3.6 ± 0.7	0.1 ± 0.5

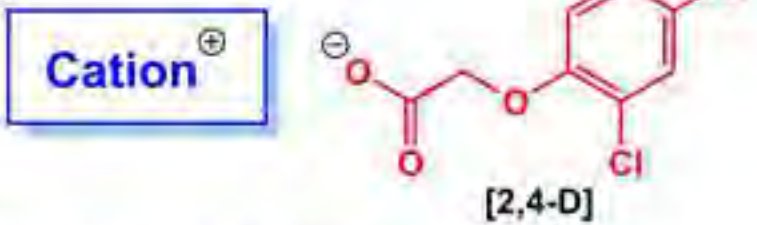
*NB – non-bioaugmented; **B – bioaugmented

Table 4. *Mineralisation efficiencies of HILs after 28 and 90 days.*

Compounds	Mineralisation efficiency [%]			
	28 days		90 days	
	NB*	B**	NB*	B**
[Na][2,4-D]	3.6 ± 0.2	20.9 ± 1.9	3.8 ± 0.4	32.5 ± 1.7
[Car][2,4-D]	55.7 ± 3.1	63.4 ± 4.8	82.3 ± 4.4	87.1 ± 4.3
[Chol][2,4-D]	30.5 ± 2.5	42.9 ± 4.4	36.8 ± 2.6	52.8 ± 4.1
[C_{12}Chol][2,4-D]	5.9 ± 0.5	10.4 ± 1.1	17.5 ± 0.9	26.5 ± 1.3
[Bet][2,4-D]	95.0 ± 2.2	97.6 ± 1.2	97.3 ± 1.6	98.2 ± 1.1
[C_{12}Bet][2,4-D]	14.1 ± 1.1	14.9 ± 1.3	16.7 ± 0.7	17.8 ± 0.8
[CAPBet][2,4-D]	50.8 ± 3.0	58.3 ± 3.5	60.9 ± 2.1	65.3 ± 2.4
[TBA][2,4-D]	4.1 ± 0.2	5.9 ± 0.4	8.1 ± 0.6	12.6 ± 0.7
[TMA][2,4-D]	12.4 ± 1.9	14.3 ± 1.5	18.4 ± 1.0	22.3 ± 1.7
[BTMA][2,4-D]	6.6 ± 0.5	7.3 ± 0.8	7.1 ± 0.4	14.2 ± 0.9

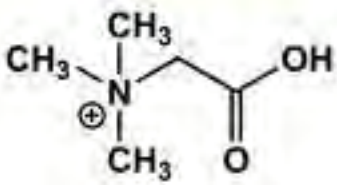
*NB – non-bioaugmented; **B – bioaugmented

General structure of synthesized salts:

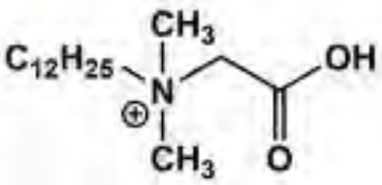


[2,4-D]

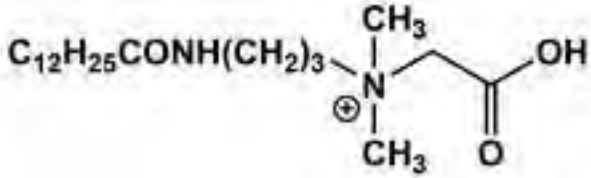
Structures of cations:



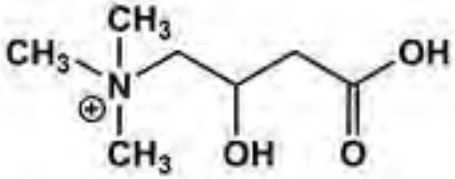
[Bet]



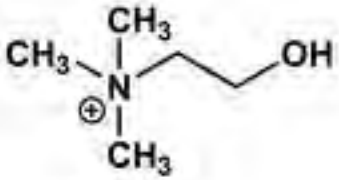
[C_{12}Bet]



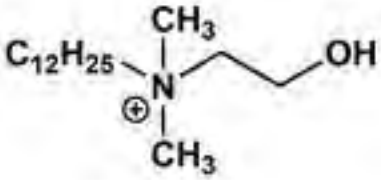
[CAPBet]



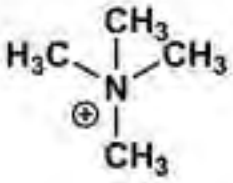
[Car]



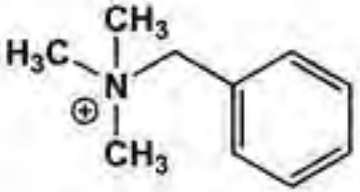
[Chol]



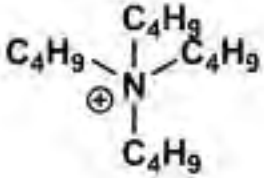
[C_{12}Chol]



[TMA]



[BTMA]



[TBA]

Figure 2

Click here to access/download;Figure;Fig 2.pptx

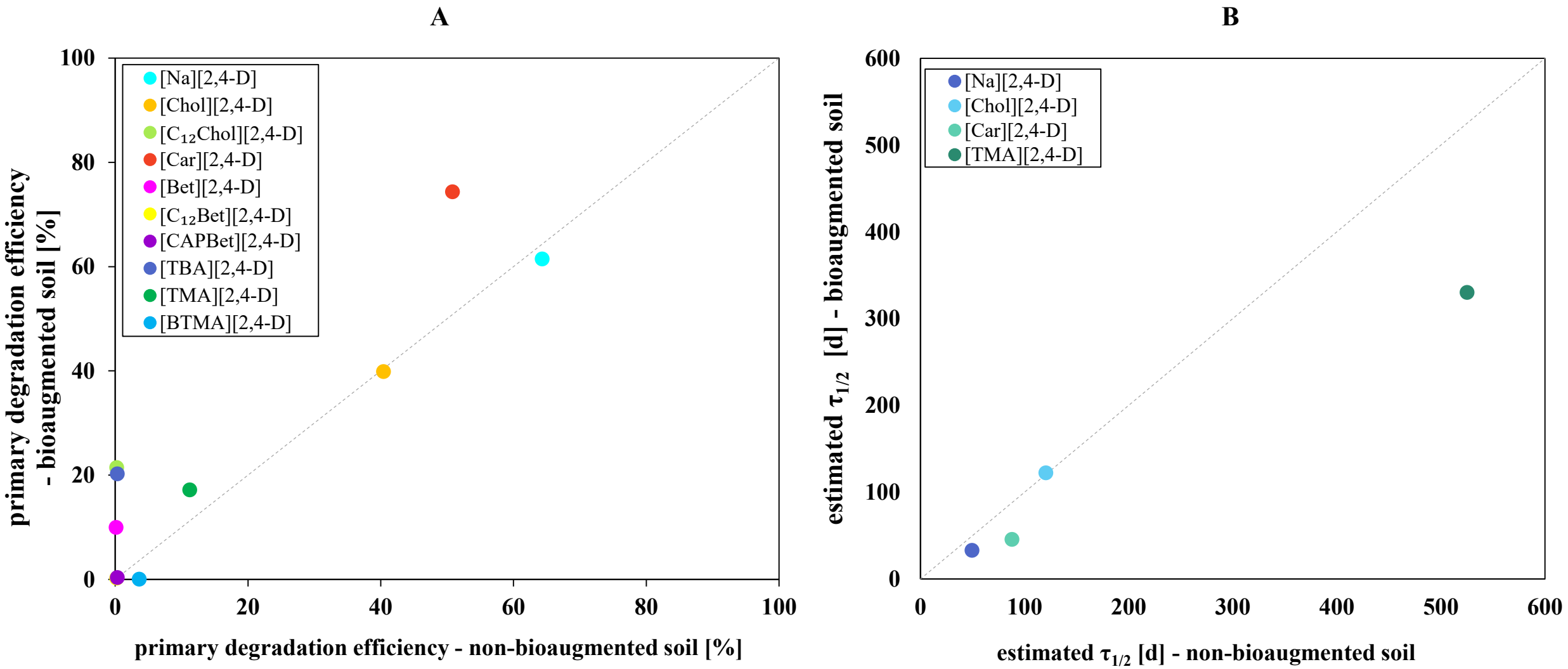


Figure 3

Click here to access/download;Figure;Fig 3.pptx

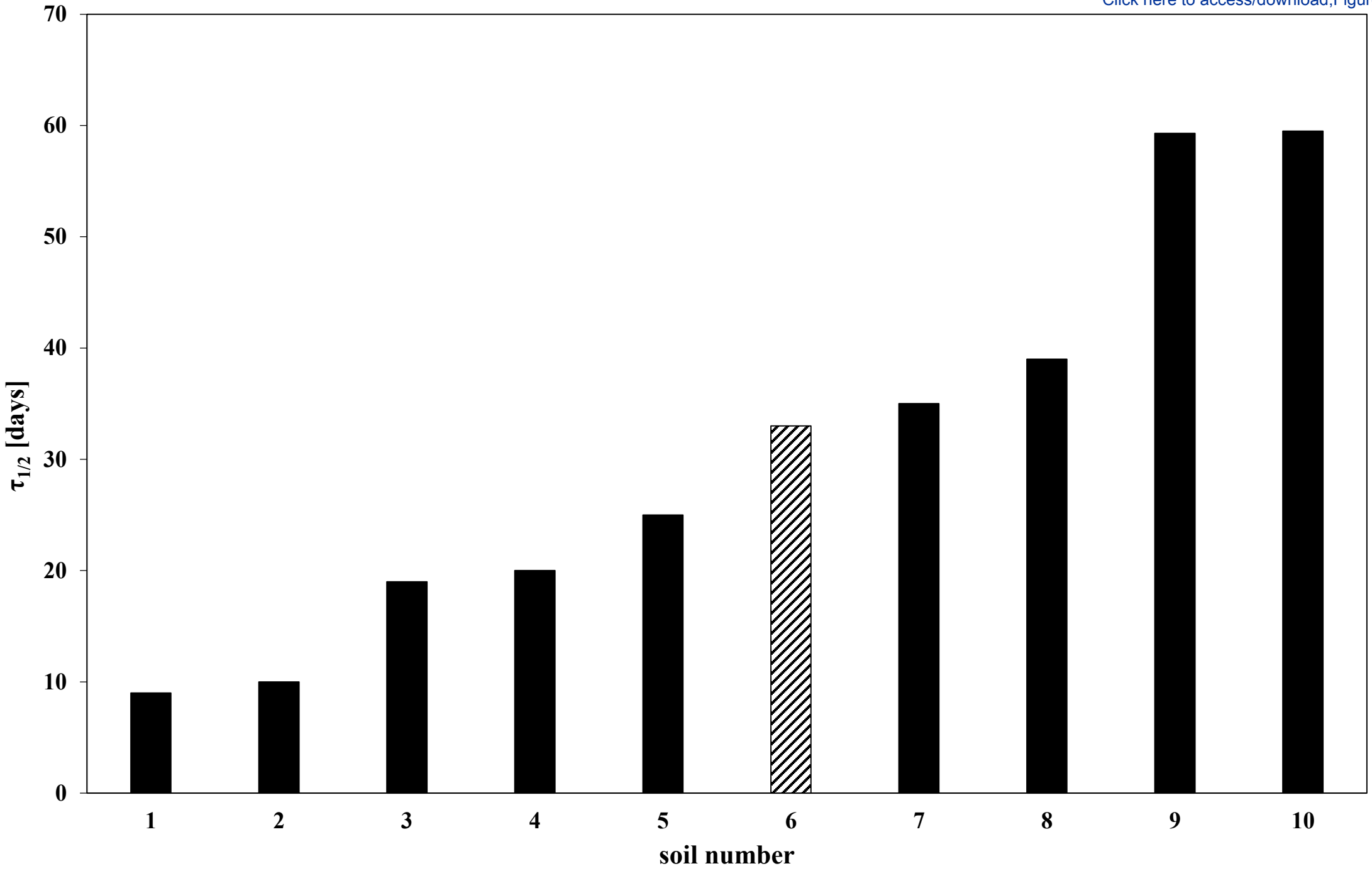


Figure 4

Click here to access/download;Figure;Fig 4.pptx

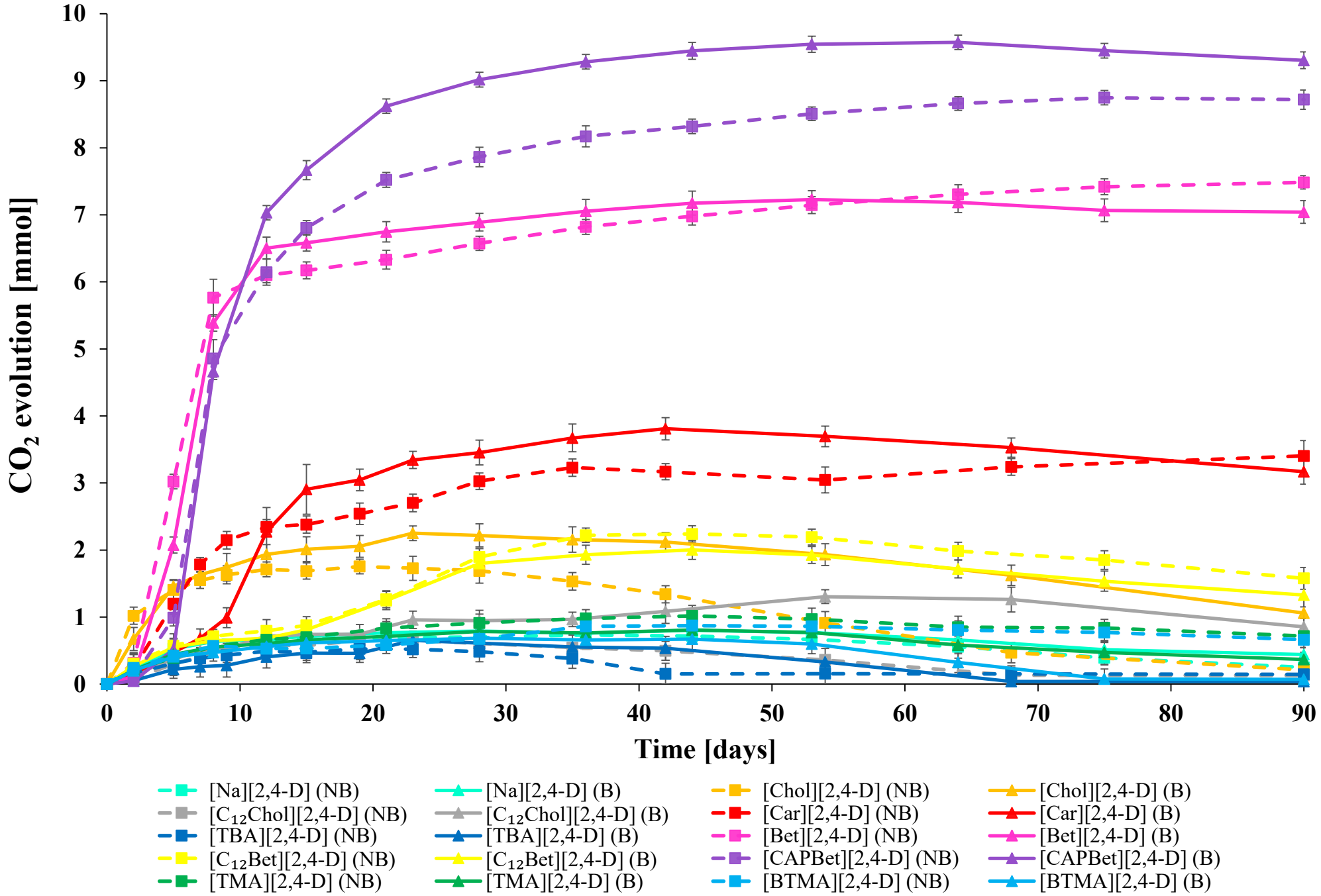


Figure 5

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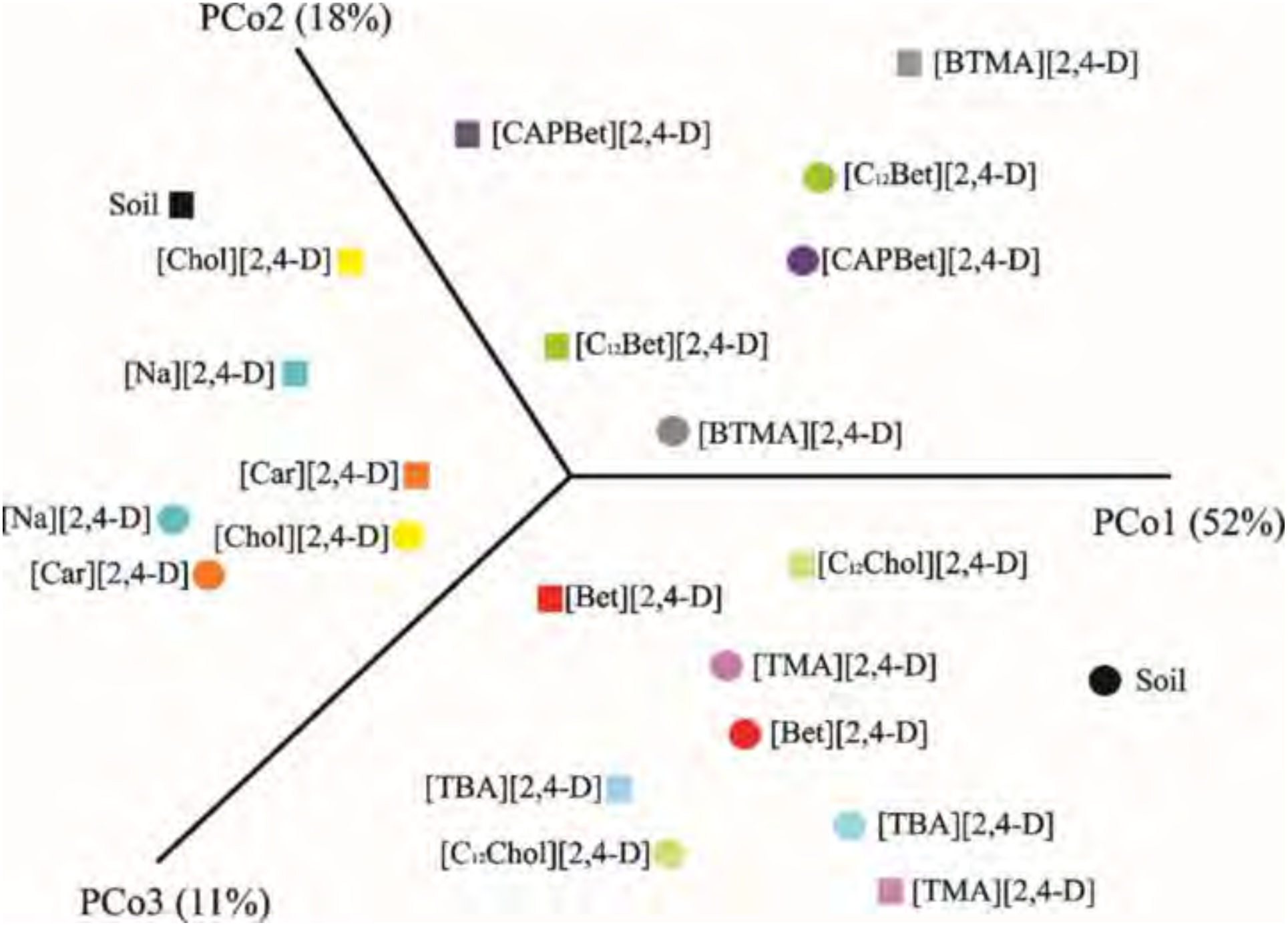
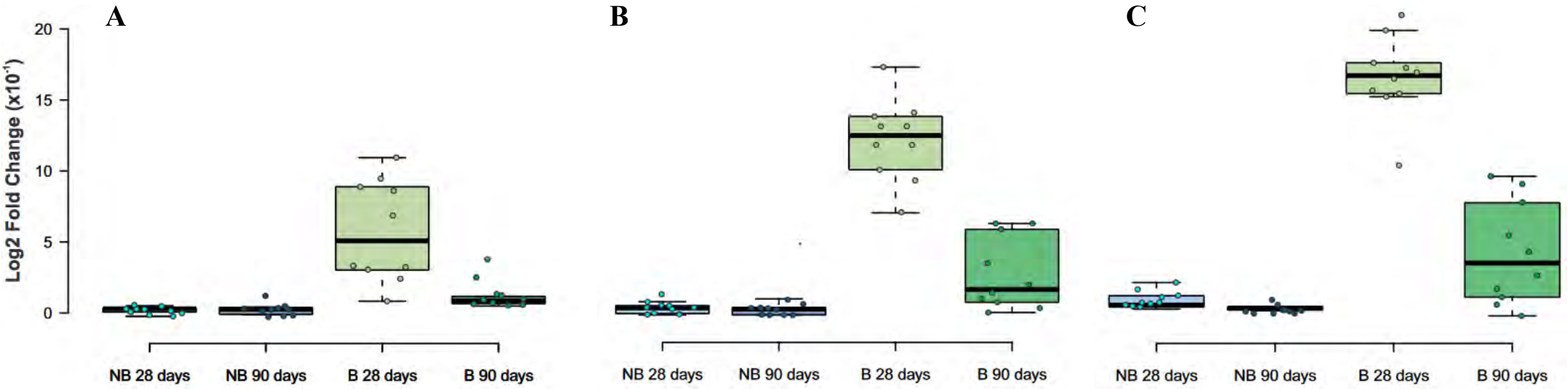


Figure 6

Click here to access/download;Figure;Fig 6.pptx



***tfd A* class I** ***tfd A* class II** ***tfd A* class III**

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Wiktoria Wilms: Investigation, Validation, Formal analysis, Visualization, Writing - Original Draft; **Marta Woźniak-Karczewska:** Conceptualization, Methodology, Investigation, Resources, Writing - Original Draft, Project administration; **Michał Niemczak**: Investigation; Writing - Review & Editing; **Anna Parus:** Investigation, Methodology, Formal analysis, Visualization; **Robert Frankowski:** Investigation, Formal analysis; **Łukasz Wolko:** Investigation; **Jakub Czarny:** Investigation; **Agnieszka Piotrowska-Cyplik:** Investigation, Formal analysis; **Agnieszka Zgoła-Grześkowiak**: Validation, Formal analysis; **Hermann J. Heipieper:** Writing - Review & Editing **Łukasz Chrzanowski:** Supervision, Conceptualization, Writing - Review & Editing, Funding acquisition, Project administration

Declaration of interests

☐The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☒The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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