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## Novel esterquat-based herbicidal ionic liquids incorporating MCPA and MCPP for simultaneous stimulation of maize growth and fighting cornflower

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## ABSTRACT

Modern agricultural practices are often based on the use of mixtures of specific herbicides to achieve efficient crop protection. The major drawbacks of commercial herbicidal formulations include the necessity to incorporate toxic surfactants and high volatility of active substances. Transformation of herbicides into herbicidal ionic liquids (HILs) seems to be a promising alternative which allows to almost completely reduce volatility due to ionic interactions. In the scope of this research, we transformed (2-methyl-4-chlorophenoxy)acetic acid (MCPA) into a quaternary ester (esterquat) with the use of derivatives of 2-dimethylaminoethanol. The obtained esterquats were later coupled with  $(\pm)$ -2-(4-chloro-2-methylphenoxy)propionic acid (MCPP) in the form of an anion. The combination of MCPA and MCPA is commonly applied in the UK, EU countries and also in the USA to increase the spectrum of targeted weed species. In the framework of this study, novel HILs with an esterquat moiety incorporating a long alkyl chain (C<sub>8</sub>, C<sub>9</sub>, C<sub>10</sub>, C<sub>11</sub>, C<sub>12</sub>, C<sub>14</sub>) were prepared and characterized in terms of basic physicochemical properties (solubility and volatility) as well as biodegradability. Their phytotoxicity was assessed towards cornflower (Centaurea cyanus) as a model weed and maize (Zea mays) as a crop plant. The presence of the esterquat cation contributed to satisfactory solubility in water and other low polar solvents, which eliminates the need to add exogenous adjuvants. Further experiments indicated that the tested HILs stimulated the germination stage of maize and maintained high herbicidal activity towards cornflower. No significant differences in terms of properties were observed in case of HILs which included alkyl substituents with an odd number of carbon atoms. Future studies should be focused on structural modifications in order to improve the biodegradability as well as field studies for evaluation of commercial applications.

### 1. Introduction

It is estimated that the human population will continue to increase for the next years (Bongaarts, 2009; Hopfenberg and Pimentel, 2001). Many different approaches have been employed in order to ensure sustainability, steady nourishment and supply of food sources. Among them, chemical methods (e.g. the use herbicides) are the most popular and cost-effective solution (Ferron and Deguine, 2005; Popp et al., 2013). Herbicides allow for effective control of weeds, which hinder the optimal growth of crop plants due to competition for nutrients and light (Oerke, 2006). However, the consistent use of classic herbicidal forms, such as salts, pure acids or esters, is often limited by their toxicity, high volatility, mobility due to drift and run-off as well as general public expectations for stricter environmental standards in agriculture (Gavrilescu, 2005). Moreover, typical herbicidal formulations require the addition of adjuvants, which may exhibit higher toxicity than the active substance (Defarge et al., 2018; Mesnage et al., 2013; Mesnage and Antoniou, 2018). As a result, there is a need to develop novel, more efficient agrochemicals in order meet the tightening environmental regulations.

Herbicidal ionic liquids (HILs) have been introduced as a potential method to overcome the limitations of classic herbicides (Pernak et al., 2011). The concept is based on the transformation of the active substance into an ionic form in order to decrease its volatility (Rogers and

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Seddon, 2003) and combine it with a counter-ion to introduce additional functionality (Hough et al., 2007; Pernak et al., 2011). As such, the herbicidal anion may be paired with almost any cation which results in improved properties, e.g. decreased dose of herbicide per hectare (Syguda et al., 2018), high wetting properties (Cojocaru et al., 2013; Lawniczak et al., 2015; Pernak et al., 2011), low toxicity (Pernak et al., 2018, 2016, 2015) and improved work safety (Rogers and Seddon, 2003). Additionally, the use of a cation of natural origin, such as choline or nicotinic acid, may possibly increase the biodegradability (Jordan and Gathergood, 2015, Niemczak et al., 2017b). Another important advantage of HILs is the possibility to adjust their surface activity by using various cation structures, such as morpholinium or quaternary ammonium. This may considerably limit or completely eliminate the use of adjuvants in commercially available herbicidal mixtures (Cojocaru et al., 2013; Lawniczak et al., 2015; Pernak et al., 2011).

A new group of herbicidal ionic liquids which include two herbicides has been introduced recently (Syguda et al., 2018). The concept behind such HILs is to combine one herbicide in the anionic form with a second herbicide, which is incorporated in the structure of the cation. The number of literature reports focused on the combination of herbicides such as 2,4-D, MCPA, MCPP, 4-CPA, chloropicolinic-acid, chlorobenzoic-acid, glyphosate or dicamba in order to limit the frequency of spraying or to increase the spectrum of targeted weed species is very limited (Choudhary et al., 2017; Niemczak et al., 2018; Syguda et al., 2018). Currently, there are no reports focused on HILs which incorporate both MCPA and MCPP, even though this combination of herbicides is often used in commercial formulations. Despite structural similarities, MCPA is characterized by a lower spectrum of controlled weed compared to 2,4-D, hence MCPP is supplemented to extend the activity (e.g. in case of chickweed or white clover). The combination of MCPA and MCPP is commonly used in the UK and EU countries, since MCPA is more popular in Europe than 2,4-D. Moreover, it is also used in the USA in case of certain crop plants, since 2,4-D may cause damage to peas and flax cultivars (Zimdahl, 2015).

In the framework of this study, 5 novel HILs were prepared, which incorporated a cation composed of MCPA in the form of a quaternary ester (esterquat) and MCPP as a herbicidal anion. It was assumed that this structural design will provide additional functionality, namely: i) enhanced efficiency and decreased volatility, as a result of transformation of herbicides into their respective ionic forms; ii) easier preparation of the spray solution due to superior solubility of the obtained cation-anion pairs; iii) displacement of surface active adjuvants due to the presence of the esterquat moiety with a long alkyl chain ( $C_8$ ,  $C_9$ ,  $C_{10}$ ,  $C_{11}$ ,  $C_{12}$ ,  $C_{14}$ ) as a substituent, which may contribute to lower toxicity. The aim of the study was to confirm the above-mentioned assumptions based on the evaluation of surface, herbicidal and antimicrobial activity. The obtained HILs were characterized in terms of basic physicochemical properties (solubility and volatility) as well as biodegradability. Additionally, their phytotoxicity was assessed towards both a model weed (cornflower (Centaurea cyanus)) and crop plant (maize (Zea mays)).

#### 2. Materials and methods

#### 2.1. Chemicals

Reagents such as  $(\pm)-2$ -(4-chloro-2-methylphenoxy)propionic acid (MCPP) (93%), (2-methyl-4-chlorophenoxy)acetic acid (MCPA) (97%) were purchased from Ciech-Sarzyna S.A. Thionyl chloride (97%), deanol (DAE) (99%), triethylamine (99.5%), 1-bromooctane (99%), 1-bromononane (98%), 1-bromodecane (98%), 1-bromoundecane (98%), 1-bromodecane (98%), 1-bromodecane (97%) were obtained from Sigma-Aldrich (Germany). Other chemical reagents and solvents were obtained from Sigma-Aldrich (Germany), Merck (Germany), Fluka (Switzerland), POCH (Poland), Chempur (Poland) and International Enzymes Limited (USA).

Herbicides used for synthesis (MCPA; MCPP) were additionally purified prior to their use according to the procedure provided by Syguda et al. (2018). Briefly, MCPA and MCPP were dissolved in hot toluene with activated carbon and stirred intensely. Then, the solution was subjected to filtration in order to remove any contaminants using activated carbon. Finally, the herbicides were recrystallized from cold toluene.

## 2.2. Synthesis of studied compounds

The synthesis was conducted in accordance with the procedure provided by Syguda et al. (2018). Each step was described below.

## 2.2.1. (2-methyl-4-chlorophenoxy)acetyl chloride

In order to prepare the (2-methyl-4-chlorophenoxy)acetyl chloride, 100 g of (2-methyl-4-chlorophenoxy)acetic acid was placed in a round bottom flask (500 mL) which was supplied with a reflux condenser, a dropping funnel and a magnetic stirrer. Thionyl chloride (with a 3-fold excess) was slowly added with continuous mixing and the mixture was heated at 75 °C for two hours. Then, the excess of thionyl chloride was removed via evaporation and a colourless liquid was obtained (boiling point at 145–146 °C, 17–18 hPa). The reaction yield of (2-methyl-4-chlorophenoxy)acetyl chloride was equal to 95%.

## 2.2.2. (2-dimethylamine)ethyl (2-methyl-4-chlorophenoxy)acetate hydrochloride

First, 80 g of (2-methyl-4-chlorophenoxy)acetyl chloride was dissolved in 100 mL of chloroform and a solution of an equimolar amount of DAE in 100 mL of chloroform was prepared. Then, the DAE – chloroform solution was introduced to a reaction flask (500 mL) supplied with a funnel, a magnetic stirrer and an ice-water bath. Next, the (2methyl-4-chlorophenoxy)acetyl chloride solution was added dropwise to the DEA solution. The reaction was conducted for 20 min with continuous mixing. Afterwards, chloroform was completely evaporated and the flask with the product was placed in a refrigerator. After solidification, the product was filtered under vacuum. Next, hexane was added to the flask and the solution was filtered using a Büchner funnel, additionally rinsed with hexane and dried. The reaction yield was equal to 96%.

## 2.2.3. (2-dimethylamine)ethyl (2-methyl-4-chlorophenoxy)acetate

In order to prepare the (2-dimethylamine)ethyl (2-methyl-4-chlorophenoxy)acetate, 100 g of aminoester hydrochloride and 300 mL of chloroform were placed in a flask (1000 mL), an equimolar amount of triethylamine was added and the solution was mixed intensely. After 15 min, the reaction mixture was transferred to an evaporator and the solvent was completely removed. After solidification, the product was rinsed and dried similarly to the previous step. A colourless liquid was obtained. The reaction yield was equal to 88%.

#### 2.2.4. (4-chloro-2-methylphenoxy)-2-

#### acetoxyethylalkyldimethylammonium bromides

At first, 5 g of (2-dimethylamine)ethyl (2-methyl-4-chlorophenoxy) acetate was dissolved in 5 mL of acetone in a flask (100 mL). Next, an appropriate alkyl bromide  $(CH_3(CH_2)_7Br, CH_3(CH_2)_8Br, CH_3(CH_2)_9Br, CH_3(CH_2)_{11}Br, CH_3(CH_2)_{13}Br)$  was added to the flask with a 5% excess. The flasks were equipped with a reflux condenser and heated for 24 h up to 55 °C. Afterwards, the flasks were placed in a refrigerator in order to crystallize the product. The product was then filtered using the Büchner funnel, rinsed thoroughly with hexane and cold acetone, and finally dried. The reaction was presented in Fig. S1 (ESI) and the product yields were listed in Table S1 (ESI).

#### 2.3. Esterquats with herbicidal anions

Approx. 1 g of an appropriate (4-chloro-2-methylphenoxy)-2-

acetoxyethylalkyldimethylammonium bromide was introduced into a 100 mL beaker and then dissolved in 15 mL of water-isopropanol solution (2:1, v/v). In a separate beaker,  $(\pm)$ -2-(4-chloro-2-methylphenoxy) propionic acid was introduced to an equimolar amount of 10% aqueous solution of sodium hydroxide and approx. 20 mL of deionized water was added. The mixture was heated until a clear solution was obtained. The solution of sodium  $(\pm)-2$ -(4-chloro-2-methylphenoxy)propionate was added to the water-isopropanol solution of esterguat bromides and stirred vigorously. After 40 min, the reaction mixture was transferred to a separator, then 30 mL of chloroform were also added and shaken vigorously. Afterwards, the organic and inorganic phases of the mixture were separated. The organic layer, which contained the ionic liquid, was washed four times with deionized water (50 mL) in order to remove the resulting sodium bromide and the excess of sodium salt of  $(\pm)-2$ -(4chloro-2-methylphenoxy)propionic acid (MCPP). Subsequently, the inorganic layer was checked to determine the presence of bromide ions using a silver nitrate solution. The organic phase was then poured into a 100 mL flask and the solvent was evaporated completely. Afterwards, the flask with the product was dried at 60 °C until constant mass was obtained. The reaction was presented in Fig. 1.

## 3. Determination of cationic active substance content by twophase titration

The purity of the ionic liquids was determined on the basis of twophase titration carried out according to a standard procedure (PN-EN ISO, 2871-1; PN-EN ISO, 2871-2).

First, 100.0  $\pm$  0.1 mg of the test compound was dissolved in a 2:1 (v/ v) mixture of deionised water and isopropanol in a 50 mL volumetric flask. Then, a 10 mL sample was collected and placed into a conical flask. Afterwards, 15 mL of chloroform and 5 mL of working solution of mixed indicator (mixture of water-isopropanol solutions of dimidium bromide, disulfinic blue and sulphuric(VI) acid) were added. The obtained two-phase system was titrated using a 0.4 mmol/L sodium dodecyl sulphate(VI) solution. After addition of the titrant, the conical flask was closed with a ground stopper and the sample was stirred vigorously. The titrations were carried out until a colour change from turquoise to heather occurred. The percentage of cationic active substance was calculated using the following formula:

$$\mathbf{X} = \frac{\mathbf{V}_1 \cdot \mathbf{C} \cdot \mathbf{V} \cdot \mathbf{M}}{\mathbf{V}_2 \cdot \mathbf{m} \cdot 1000} \cdot 100\%$$

X - percentage of cationic active substance in the sample [%].

V1 - total volume of the prepared solution of analysed substance [mL].

C - concentration of sodium dodecyl sulphate(VI) [mol/L].

V - volume of sodium dodecyl sulphate(VI) [mL].

M - molar mass of the cationic active compound under investigation

[g/mol].

V<sub>2</sub> - volume of titrated analyte [mL].

m - mass of the test substance [g].

## 4. Analysis

The synthesized ionic liquids were characterized by <sup>1</sup>H NMR and <sup>13</sup>C NMR spectroscopy in order to verify their chemical structures and purity. <sup>1</sup>H NMR spectra were recorded using a Bruker Avancell 400 MHz Ultra Shield Plus spectrometer. The device was operating at 400 MHz, tetramethylsilane was used as the internal standard and DMSO-d<sub>6</sub> as a solvent. <sup>13</sup>C NMR spectra were obtained using the same instrument at 100 MHz. The spectra were presented in the ESI (Fig. S2-S11).

## 5. Volatility

The volatility of herbicidal esterguats and a mixture of MCPA and MCPP sodium salts was determined based on the thermal gravimetric analysis (Cojocaru et al., 2013; Ding et al., 2019; Niu et al., 2018). During the assay,  $100.0 \pm 0.1$  mg of the corresponding HILs and an equivalent amount of the MCPA and MCPP mixture were placed in aluminium pants at isotheral conditions, at 75 °C with constant nitrogen flow. The sample was weighted again after 24 h. The volatility was determined based on mass loss of the sample, which was calculated using the following formula (1):

$$Mass \, loss(\%) = \frac{W_1 - W_2}{W_1} \times 100\% \tag{1}$$

where:

 $W_1$  is the initial sample weight before heating [g].  $W_2$  is the weight of sample after heating [g].

All experiments were replicated three time.

#### 6. Solubility

In order to determine the solubility,  $0.100 \pm 0.005$  g of each herbicidal esterquat was added to glass vessels with water, methanol, acetone, chloroform, toluene, acetonitrile, isopropanol or hexane, according to the procedure described by Vogel et al. (1989). The solubility in different solvents was classified using three categories: "soluble" -0.1 g of the sample fully dissolved in 1 mL of the solvent (marked as "+"), "limited solubility" – 0.1 g of the sample fully dissolved in 3 mL of the solvent (marked as " $\pm$ "), "insoluble" – 0.1 g of the sample did not dissolve in 3 mL of the solvent (marked as "-").

### 7. Surface activity

The measurement of contact angle and surface tension was carried out using a Krűss Drop Analyzer (Germany) (accuracy  $\pm$  0.01 mN/m).



where:  $R = C_8 H_{17} - C_{14} H_{29}$ 

Fig. 1. Synthesis of herbicidal esterquats with the MCPP anion (HILs).

where:

The test was conducted at a constant temperature of 25 °C in a thermostatic bath. Single measurement method was employed to determine the surface tension and calculations were based on the analysis of the drop profile in accordance with the Laplace equation. Critical micelle concentration (CMC) and the surface tension at the CMC ( $\gamma$ CMC) were investigated with the use of linear regression analysis method. The pC20 is defined as the negative logarithm of the surface tension of water by 20 mN m<sup>-1</sup>, which represents the efficiency of surface adsorption on the air–water interface. The contact angle (CA) was measured based on the sessile drop method using the Young-Laplace equation. Moreover, additional droplets of herbicidal esterquats were placed on a piece of solid paraffin. The CA was measured on the basis of the tangent slope at the 3-phase point (solid paraffin surface-liquid, liquid-air and air-solid paraffin surface).

## 8. Determination of antimicrobial activity

The toxicity of the obtained herbicidal esterquats was tested against two bacterial species (*Escherichia coli* and *Pseudomonas putida*) and a single fungal species (*Candida albicans*). The culture of each species was transferred from agar plates into 20 mL of a 50% TSB broth (Sigma Aldrich, Poland). Then, each culture was incubated for 24 h at 30 °C. When the cell suspension reached the optical density equal to approx.  $OD_{600} = 0.10 \pm 0.01$  (which corresponds to  $10^6$  cells per millilitre), the biomass was washed three times with sterile NaCl (0.85%, w/v) and diluted (1:50) in 50% TSB to achieve  $2 \times 10^4$  cells per millilitre.

The tested herbicidal esterquats ([MCPA-DAE-C<sub>8</sub>][MCPP], [MCPA-DAE-C<sub>9</sub>][MCPP], [MCPA-DAE-C<sub>10</sub>][MCPP], [MCPA-DAE-C<sub>11</sub>][MCPP], [MCPA-DAE-C<sub>12</sub>][MCPP], [MCPA-DAE-C<sub>14</sub>][MCPP] at 0.01 M) were placed in 100 mL volumetric flasks and dissolved in a water:isopropanol solution (2:1, v/v). A mixture of 0.01 M MCPA and 0.01 M MCPP was prepared according to the same procedure and used as reference. All solutions were stored in a refrigerator at 4 °C for a period no longer than a week.

The antimicrobial activity test was carried out in accordance with the European Committee on Antimicrobial Susceptibility Testing using the micro-dilution method and the EUCAST guidelines (Clinical and Laboratory Standard Institute, 2016). In a sterile 96-well plate, 100 µL of each herbicidal esterquat solution were placed into the first row of the plate. Then, 50  $\mu$ L of a 50% TSB medium was added to all other wells and serial dilution of the tested compounds was performed. Subsequently, 200 µL of microorganisms suspension with resazurin (0.5 mg/mL) were added to each well. The analysis of herbicidal esterquats was carried out using the following 16 concentrations: 2.0, 1.0, 0.5, 0.25, 0.125, 0.0625, 0.0313, 0.0156, 0.02, 0.01, 0.005, 0.00125, 0.00625, 0.00313, 0.00156 and 0.00078 mM. The microorganisms with resazurin (0.5 mg/mL) but without the analyzed compounds (biotic control) as well as the solutions of tested compounds with resazurin (0.5 mg/mL) lacking microorganisms (abiotic control) were also prepared.

The plates were then incubated at 30  $^{\circ}$ C for 24 h, and afterwards the results of MIC (the minimal inhibition concentration) and MBC (the minimal bacterial concentration) or MFC (the minimal fungal concentration) were established. The values were determined on the basis of colour change of the indicator added to the culture. The MIC value was selected as the lowest concentration at which the indicator changed its colour from navy blue to red, while the MBC or MFC values were established as the lowest concentrations at which no discolouration of the dye was observed (the dark blue colour remained). Complete discolouration of the indicator reflects a lack of effect of the tested concentration on the microorganisms.

## 9. Determination of the impact of herbicidal esterquats on the early development of plants

Analysis of the impact of the obtained herbicidal esterquats on the germination and early development of plants belonging to different taxonomic units was carried out. Maize (*Zea mays*) and cornflower (*Centaurea cyanus*) were selected as the model plants due to their common prevalence and for comparative purposes. The soil used in the experiments was characterized by the following elemental composition: 81 mg P/kg soil, 88 mg K/kg soil, 69 mg Mg/kg soil, pH of 5.92, C organic content of 1.01% (10.1 g/kg soil). The influence of HILs was investigated using the phytotoxicity test based on the ISO-11269–2:2003 International Standard (Van Reeuwijk, 1995).

The assay was carried out in vertical plastic Phytotox containers (Phytotoxkit, Tigret company, Belgium). The amount of water added to the soil was determined in accordance with the instructions provided by the manufacturer, i.e. after determining the sorption capacity of the soil. Then, stock solutions of herbicidal esterquats with concentrations of 0.002, 0.004, 0.0096, 0.08 mM/L were prepared and introduced (25 mL) into 100 g of soil, which resulted in effective concentrations of 0.0005, 0.001, 0.0024 and 0.02 mM/kg of soil dry weight (d.w.).

Soil without the analysed compounds and rinsed with deionized water (25 mL) was used as the control sample. Solutions consisting of a mixture of respective commercial herbicides MCPA + MCPP (at the same molar concentration as test samples) were used as reference samples. Afterwards, 10 seeds of maize or cornflower were planted separately (3 plates for each concentration of the test compounds) in soil prepared according to the protocol described above. Then, the phytotoxkit plastic containers were placed in the dark and kept at constant temperature ( $25 \pm 1$  °C). After the termination of the experiment (after 7 days), the number of germinated seeds was counted and measurements of the root length and shoot height were carried out.

The impact of herbicidal esterquats on root growth inhibition of plants was assessed based on the germination index (GI) calculated according to Eq. (2) (Graj et al., 2013):

$$GI = \frac{Gs}{Gc} \cdot \frac{Ls}{Lc} \cdot 100[\%].$$
<sup>(2)</sup>

where:

Gs and Gc are numbers of seeds germinated in the sample and control, respectively,

Ls and Lc are the shoot lengths in the sample and control, respectively.

## 10. Determination of herbicidal efficacy of the tested herbicidal esterquats

Common maize (Zea mays) and cornflower (Centaurea cyanus) plants were used to assess the phytotoxic activity of all examined compounds. The seeds of selected plants were placed in a germination box and watered two times a day for 4 days. Then, 10 seedlings were sown in 1 dm<sup>3</sup> plastic pots. Each of the pots was filled with the previously described soil. Selected compounds were applied to the leafs when plants were at the 4-6 leaf stage (BBCH 14-16) using a Lurmark 02 110 nozzle (TeeJet Technologies, Wheaton, IL, USA), which is capable of delivering up to 200 dm<sup>3</sup> of spray solution at 220 kPa. All tested compounds ([MCPA-DAE-C<sub>8</sub>][MCPP], [MCPA-DAE-C<sub>9</sub>][MCPP], [MCPA-DAE-C<sub>10</sub>][MCPP], [MCPA-DAE-C<sub>11</sub>][MCPP], [MCPA-DAE-C<sub>12</sub>][MCPP], [MCPA-DAE-C<sub>14</sub>][MCPP]) were dissolved in a 2:1 (v/v) water:isopropanol mixture, and the concentrations corresponded to a dose of 300-400 g/ha, whereas the dose commonly used in agriculture is typically in the range of 600-1000 g/ha. A mixture of sodium salts of 1 M MCPA and 1 M MCPP was used as a reference sample at an identical dose to that of the examined compounds. When the spraying treatment was complete, all plants were placed in a greenhouse with a constant

photoperiod (16 h night/ 8 h day), temperature (20  $\pm$  2 °C) and humidity (60%). The effect of weed control was inspected 3 weeks after application. First plants growing in each of the pots were cut separately and then weighted in order to access the fresh weight (Sartorius BP 2000 S, Sartorius Göttingnen, Germany, 0.01 g accuracy). Effects of herbicidal mixtures were presented as fresh weight reduction in comparison to non-treated plants (control group).

## 11. Evaluation of mineralization and primary biodegradation of herbicidal esterquats mineralization test

OECD 301F protocol was applied in order to determine the mineralization potential of herbicidal esterquats. The experimental samples were prepared in brown glass bottles filled with sterile mineral medium as described by Ławniczak et al. (2016). The inoculum was previously isolated from soil treated with herbicides and concentrated to obtain a cell density of approx. 10<sup>6</sup> CFU/mL. The tested compounds were added to obtain a concentration equal to 100 mg/L of Theoretical Oxygen Demand. The mineralization efficiency was determined based on oxygen uptake and carbon dioxide production by means of the Micro-Oxymax Respirometer (Columbus Instruments, USA). The ultimate biodegradation test was carried out at the temperature 23  $^\circ$ C  $\pm$  2  $^\circ$ C for 28 days with measurements taken every 5 h. Biotic (mineral medium without the analysed compounds) and abiotic (sterile mineral medium with HILs) controls were also prepared.

#### 12. HPLC-MS analysis of residues - primary biodegradation

Upon termination of the mineralization experiment, the biomass was centrifuged (10.000g, 15 min, 4 °C) and rinsed three times with the mineral medium (3  $\times$  1 mL). Subsequently, the obtained aliquots were combined with a cell-free supernatant and 10 mL of this solution were subjected to ultrasound-assisted extraction with chloroform (3  $\times$ 1 mL). The extracts were collected, filtered through a 0.2 µm PTFE syringe filter and diluted in 80% methanol (v/v). The prepared samples were analyzed by means of HPLC-MS (UltiMate 3000 RSLC, Dionex, with a Hypersil GOLD column 100 mm  $\times$  2.1 mm I.D.; 1.9 µm coupled with an API 4000 OTRAP triple quadrupole mass spectrometer, AB Sciex) in order to determine the concentration of residual HILs.

#### 13. Statistical analysis

Abbreviation of Honic liquid

All samples and controls were prepared in triplicates. The obtained results were presented as values with standard errors of the mean (SEM).

## Table 1

C]

 $\cap$ 

The SEM values were calculated according to Eq. (3):

$$SEM = \frac{s}{n^{0.5}}$$
(3)

where:

SEM - standard error of the mean,

s - sample standard deviation,

n – number of samples.

Additionally analysis of variance (one way ANOVA) was performed for all results obtained during phytotoxicity tests in order to ensure that the differences of root and shoot length obtained at different concentrations are statistically significant. The corresponding results were included in the ESI (Tables S12-S35).

## 14. Results and discussion

### 14.1. Synthesis

The synthesized herbicidal esterquats were presented in Table 1 along with the reaction yield and the surfactant content. All of the obtained compounds were liquids at room temperature, which allows to classify them as ionic liquids. The obtained compounds are novel HILs, with the sole exception of [MCPA-DAE-C10][MCPP], which has been synthesised previously (Syguda et al., 2018). The design of the research assumed that the alkyl substituents in the cation will include from 8 to 14 carbon atoms since such groups usually contribute to the highest biological activity (Syguda et al., 2018). Furthermore, the majority of studies regarding cation structures was focused solely on substituents with an even number of carbon atoms, therefore this study also included C<sub>9</sub> and C<sub>11</sub> groups, in order to elucidate the possible influence of odd number of carbon atoms.

The efficiency of the employed synthesis protocol was high and ranged from 81% to 92%. The determined surfactant content was within the range of 90.5–99.5 + %. These results initially confirmed the hypothesis that incorporation of an esterguat structure will contribute to surface activity of the synthesized compounds. The findings also indicate that the length of the alkyl substituent in the cation structure influenced the reaction yield and surfactant content. In both cases, the best results were obtained for the HIL with the dodecyl substituent ([MCPA-DAE-C<sub>12</sub>][MCPP]), whereas compounds with the octyl group ([MCPA-DAE-C<sub>8</sub>][MCPP]) exhibited the lowest values. Subsequent <sup>1</sup>H and <sup>13</sup>C NMR analyses (ESI, Fig. S2-S11) confirmed the correctness of the employed synthesis pathway (i.e. the intended structures of the products were obtained) and the purity of the compounds (i.e. no



CH<sub>3</sub>

<sup>a</sup> Indicates that the compound has been previously synthesized ((Syguda et al., 2018)).

C1

CH<sub>2</sub>

R

Yield and surfactant content of the synthesized herbicidal esterquats.

( )

significant impurities were detected).

## 15. Solubility

The next experimental step was focused on the evaluation of solubility of the obtained herbicidal esterquats in common polar and nonpolar solvents. This evaluation was crucial, as the solubility of the active compound directly affects the composition of the final product. The results were presented in Table S6 (ESI). The synthesized herbicidal esterquats were characterized by very good solubility in the majority of solvents. The exceptions included water, in case of which the compounds exhibited limited solubility, as well as hexane, in which the compounds were insoluble. The former effect may be associated with the presence of relatively long alkyl chains as well as bulky, aromatic structures in case of both the cation and the anion, which may contribute to hydrophobicity. Nevertheless, the studied aqueous solubility of the obtained HILs is satisfactory in terms of achieving the appropriate concentration of the active compound in the spray solution. The fact that the compounds were completely insoluble in hexane may be primarily associated with the presence of quaternary ammonium cation, which facilitates their hydrophilic properties. In this case no significant influence of the alkyl chain length in the cation was observed.

The overall solubility of compounds obtained in the framework of this study is higher compared to other HILs which incorporated two herbicidal anions (glyphosate, MCPA or dicamba) (Niemczak et al., 2018), as the herbicidal esterquats can be dissolved more efficiently in various solvents. Moderate aqueous solubility seems to be a common trait for the majority of HILs, nevertheless it should be emphasized that in case of the described herbicidal esterquats, their solubility is higher by one order of magnitude compared to the maximum recommended dose for commercial herbicidal formulations (i.e. 0.1 g of the active substance is dissolved in approx. 27 mL of water).

## 16. Volatility

The next experimental step involved the analysis of volatility. The obtained results were presented in Table S7 (ESI). The results obtained in the framework of this assay justified the assumption that ionic forms of herbicides will exhibit considerably lower volatility compared to classic herbicides. The volatility values of the obtained herbicidal esterquats were lower by one order of magnitude compared to the mixture of MCPA and MCPP used as a reference sample. This is a significant improvement, as the reduction of volatility limits the risk of unintentional contamination and allows for a more precise application of the active substance at target location. Furthermore, the volatility of the synthesized herbicidal esterguats decreases with the increase of the alkyl chain length, although the effects are diminishing (the difference between C<sub>12</sub> and C<sub>14</sub> is not significant). This is consistent with the findings of a previous report regarding HILs, which also indicates that higher length of the alkyl substituent is also advantageous in terms of adherence to the leaves of weeds and their long-term herbicidal effect (Niemczak et al., 2019).

## 17. Surface activity

Subsequent experimental steps involved the evaluation of the following surface activity parameters: critical micelle concentration (CMC) and surface tension at CMC ( $\gamma$ CMC). The results were presented in Table S8 (ESI). The surface tension values decreased from the value determined for water (72.2 mN/m) to values in the range of 26 – 33 mN/m. The CMC values were obtained in the range from 0.4 to 1.0 mmol. The best results (corresponding to the highest decrease of surface tension and lowest CMC) were achieved in case of [MCPA-DAE-C<sub>14</sub>][MCPP], followed by [MCPA-DAE-C<sub>12</sub>][MCPP]. In general, the  $\gamma$ CMC values decreased with the elongation of the alkyl chain in the cation. The same trend was reported previously for other herbicidal ionic liquids

(Kaczmarek et al., 2020; Niemczak et al., 2017a). The results confirmed the assumption that the length of the alkyl substituent in the cation has a significant influence on surface activity in water (Romanowski et al., 2012). Furthermore, the evaluation of surface activity justifies the hypothesis that the synthesized HILs act as effective surfactants which may eliminate the necessity to introduce additional adjuvants.

### 18. Antimicrobial activity

Due to the fact that cationic surfactants often exhibit antimicrobial activity, the next step was focused on the antibacterial and antifungal properties of the studied herbicidal esterquats. The results were presented in Table 2.

The relative standard deviation for each measurement was in a range of 0.9–6.5%.

The test was carried out using model, representative species of Grampositive (*Bacillus cereus*) and Gram-negative (*Pseudomonas putida*) bacteria as well as a single species of fungi (*Candida albicans*). The tested microorganisms are widely distributed in the environment and commonly isolated from soil. Some strains of *B. cereus* and *C. albicans* are also opportunistic pathogens of gastrointestinal tracts in humans and animals (Berthold-Pluta et al., 2015; Knoche, 1994).

The obtained results indicate that all of the compounds exhibit antimicrobial activity, which ranged from 0.008 to 1.000 mM in terms of minimal inhibitory concentration (MIC) and from 0.031 to 1.000 mM in terms of minimal bactericidal/fungicidal concentration (MBC/MFC). The results were influenced by both the structure and tested microorganism. In case of all tested species, the highest efficiency was observed for [MCPA-DAE-C<sub>14</sub>][MCPP] followed by [MCPA-DAE-C<sub>12</sub>][MCPP], whereas [MCPA-DAE-C<sub>8</sub>][MCPP] and [MCPA-DAE-C<sub>9</sub>][MCPP] exhibited the lowest antimicrobial activity. This further confirms that the length of the alkyl chain in the cation has a significant impact on the biological activity of HILs. In terms of species-specific influence, *P. putida* cells exhibited the highest resistance to the studied herbicidal esterquats (as indicated by the highest MIC and MBC values), whereas *B. cereus* was characterized by the highest susceptibility (lowest MIC and MBC values).

All the studied herbicidal esterquats exhibited notably higher antimicrobial activity compared to the mixture of MCPA and MCPP (the difference ranged from one to three orders of magnitude). This is associated with the presence of the esterguat cation. Quaternary ammonium cations exhibit high toxicity due to their affinity to microbial cells (which are usually negatively charged) and surface activity, which results in the disruption of the phospholipid bilayer. For example, the reported MIC values for a model cationic surfactant, cetyltrimethylammonium bromide (CTAB), determined towards E. coli ranged from 10 to 20 µM (Nakata et al., 2011) [38]. It should be highlighted, that the MIC values obtained in the framework of this manuscript are notably higher compared to CTAB, and are within the MIC range established for glyphosate towards E. coli (approx. 0.030 -0.120 mM) (Bote et al., 2019). Furthermore, the MIC and MBC/MFC values are notably higher compared to the dose of MCPA and MCPP mixtures used in spray solutions. As such, the application of the studied HILs may potentially limit the activity of harmful pathogens towards crop plants during the initial stages, however it should not contribute to significant toxic effects in comparison to the commonly used herbicidal active substances.

Previous studies regarding the antimicrobial activity of HILs indicate that this parameter may significantly differ and that the type of the cation has a significant influence on the range of MIC as well as MBC/MFC values (Pernak et al., 2018). This corresponds well with the assumption that the surface-active nature of the cation used in herbicidal esterquats tested in the framework of this study was responsible for their high antimicrobial activity.

#### Table 2

Antimicrobial activity of the obtained herbicidal esterquats.

Compound	Microorganism					
	Bacillus cereus		Pseudomonas putida		Candida albicans	
	MIC [mM]	MBC [mM]	MIC [mM]	MBC [mM]	MIC [mM]	MFC [mM]
[MCPA-DAE-C8][MCPP]	0.1250	0.5000	0.5000	1.0000	0.2500	0.5000
[MCPA-DAE-C <sub>9</sub> ][MCPP]	0.1250	0.5000	0.5000	1.0000	0.2500	0.5000
[MCPA-DAE-C <sub>10</sub> ][MCPP]	0.1250	0.5000	0.5000	1.0000	0.1250	0.2500
[MCPA-DAE-C <sub>11</sub> ][MCPP]	0.1250	0.5000	0.1250	1.0000	0.1250	0.2500
[MCPA-DAE-C <sub>12</sub> ][MCPP]	0.0313	0.2500	0.1250	0.5000	0.1250	0.2500
[MCPA-DAE-C14][MCPP]	0.0078	0.0313	0.0500	0.1250	0.0125	0.0250
$MCPA\;Na + MCPP\;Na$	2.5000	10.0000	10.0000	>10.0000	5.0000	10.0000

## 19. Effects of herbicidal esterquats on the development of roots and shoot in maize and cornflower

The next experimental stage was focused on the effect of the obtained HILs on root and shoot length in a model weed (*Centaurea cyanus*) and crop plant (*Zea mays*). This parameter was compared with a mixture of sodium salts of MCPA and MCPP applied at the same dose as the studied herbicidal esterquats. The results were presented in Figs. 2 and 3 as well as in Tables S10 and S11 (ESI).

The germination test carried out for maize indicated two distinct trends for the studied herbicidal esterquats: stimulation at low concentration and inhibition at high concentration, which confirms that the compounds retained their properties as synthetic auxins (Table S10 ESI). The effects of herbicidal esterquats on the germination of maize were always more beneficial compared to the model mixture of MCPA and MCPP. At the concentration of 0.0005 mM/kg of soil (d.w.), all herbicidal esterquats stimulated the germination of seeds (GI ranged from 120% to 142%), while the model mixture inhibited the germination process (GI equal to 72%). At higher concentrations the HILs also decreased the GI, nevertheless, this effect was always lower compared to sodium salts of MCPA and MCPP. Overall, the highest stimulation was achieved in case of [MCPA-DAE-C12][MCPP] at the lowest studied concentration, whereas [MCPA-DAE-C14][MCPP] contributed to the highest inhibition of germination (GI equal to approx. 19%) at the highest concentration.

Subsequent measurements of root and shoot length in maize seedlings further confirmed the stimulating effects of the studied HILs (Fig. 2). In this case, both the root and shoot values were higher by 50–100% compared to the model mixture of MCPA and MCPP. This effect was most notable at lower concentrations (0.0005 and 0.001 mM) and diminished with the increase of the dose. Nevertheless, even at the highest studied concentration (0.02 mM) maize seedlings retained roots and shoots in the presence of HILs, in contrast to the model mixture of MCPA and MCPP which completely inhibited plant growth.

Evaluation of the influence of herbicidal esterquats on the germination of cornflower as the model weed suggested that their efficiency depends on the concentration as well as the length of the alkyl substituent in the cation (Table S11 ESI). Overall, each of the studied HILs was more effective in terms of inhibition of cornflower seed germination at the lowest studied concentration, whereas no distinct trends could be established at higher doses. Nevertheless, [MCPA-DAE-C<sub>12</sub>][MCPP] or [MCPA-DAE-C<sub>14</sub>][MCPP] exhibited a similar or even improved inhibitory effect compared to the model mixture of MCPA and MCPP.

In case of cornflower (Fig. 3), the measurement of root and shoot length revealed that satisfactory plant growth inhibition by the herbicidal esterquats was achieved only at the highest tested concentration (0.02 mM). At the lowest concentration, the effects of herbicidal esterquats were similar to the model mixture of MCPA and MCPP, whereas in case of the middle doses (0.001 and 0.0024 mM), the inhibitory effect of the synthesized compounds was lower compared to the model mixture,



Fig. 2. Effects of herbicidal esterquats on shoot and root length of maize seedlings.



Fig. 3. Effects of herbicidal esterquats on shoot and root length of cornflower seedlings.

Tab

especially in terms of shoot length.

The obtained results confirm the previous observations that the compounds which comprise alkyl chains ranging from  $C_{10}$  to  $C_{14}$  are characterized by the highest herbicidal activity (Niemczak et al., 2018), although the exact effect is strongly influenced by the dose. In accordance with the implications of Choudhary et al. (2017), the structural modifications of compounds described in the framework of this study may also contribute to their efficacy in case of herbicide-resistant weed species, however this aspect should be further elucidated during future investigations.

#### 20. Herbicidal efficacy of the tested herbicidal esterquats

The herbicidal efficacy of the synthesized herbicidal esterguats was evaluated based on the reduction of fresh weight of cornflower. The results were presented in Table S11 (ESI). The obtained results confirmed that the herbicidal properties were preserved after the transformation of MCPA into an esterquat cation and MCPP into an anion. Furthermore, their efficacy was similar or better compared to the model mixture of MCPA and MCPP. This is particularly visible in case of herbicidal esterquats which incorporate C10, C11 and C12 alkyl substituents in the cation. Since the same anion was used in all tested compounds, the differences are associated with the structure of the cation. High influence of the cation on the overall herbicidal activity of HILs was also reported previously (Choudhary et al., 2017; Pernak et al., 2018). This effect may be associated with the surface active properties of cations, in accordance with the findings of Niemczak et al. 2018. It should be highlighted that the herbicidal effect in case of herbicidal esterquats was achieved at a much lower dose (approx. 300-400 g/ha) compared to that recommended for classic herbicidal mixtures (600-1000 g/ha), which is an additional benefit.

## 21. Evaluation of mineralization and primary biodegradation of HILs

The final experimental stage was focused on the assessment of mineralization efficiency of the obtained herbicidal esterquats. The obtained results were presented in Table 3. Based on the

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Mineralization of the studied herbicidal e	esterquats.
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HILS	Biodegradation efficiency after 28 days [%]	Time window between 10% and 60% [days]	Classification
[MCPA-DAE- C <sub>8</sub> ] [MCPP]	$12.0\pm2.0$	_	Not readily biodegradable
[MCPA-DAE- C <sub>9</sub> ] [MCPP]	$11.0\pm3.0$	_	Not readily biodegradable
[MCPA-DAE- C <sub>10</sub> ] [MCPP]	$9.0\pm1.0$	_	Not readily biodegradable
[MCPA-DAE- C <sub>11</sub> ] [MCPP]	$6.0\pm2.0$	_	Not readily biodegradable
[MCPA-DAE- C <sub>12</sub> ] [MCPP]	$\textbf{4.0} \pm \textbf{1.0}$	_	Not readily biodegradable
[MCPA-DAE- C <sub>14</sub> ] [MCPP]	$2.0\pm1.0$	-	Not readily biodegradable
[MCPA] [Na]+ [MCPP] [Na]	$18.0\pm4.0$	-	Not readily biodegradable

recommendations of the OECD 301F test results, a compound may be classified as "biodegradable" when its mineralization exceeds the 60% threshold within a 10 days window of the 28-days period. Since the mineralization efficiency of all studied compounds was lower than 20%, none of them passed the test threshold. The mineralization of herbicidal esterquats (2–12%) was lower compared to that of the model mixture of herbicides (18%) and their susceptibility to biodegradation decreased with the increase of the alkyl chain length.

After evaluation of mineralization efficiency, an in-depth investigation regarding the decomposition of initial structures (primary biodegradation) of cations and anions in herbicidal esterquats as well as sodium salts of MCPA and MCPP in the reference herbicidal mixture was carried out (Table 4). Subsequent analysis of residues indicated that the

#### Table 4

Primary biodegradation of herbicidal esterquats.

DSHILs	Biodegradation efficiency [%]		
	Cation	Anion	
[MCPA-DAE-C <sub>8</sub> ][MCPP]	$\textbf{8.6} \pm \textbf{2.0}$	$32.2\pm3.1$	
[MCPA-DAE-C <sub>9</sub> ][MCPP]	$\textbf{7.2} \pm \textbf{1.7}$	$\textbf{30.5} \pm \textbf{4.7}$	
[MCPA-DAE-C <sub>10</sub> ][MCPP]	$\textbf{7.9} \pm \textbf{2.3}$	$\textbf{27.1} \pm \textbf{2.9}$	
[MCPA-DAE-C <sub>11</sub> ][MCPP]	$6.1\pm1.9$	$\textbf{28.9} \pm \textbf{3.2}$	
[MCPA-DAE-C <sub>12</sub> ][MCPP]	$\textbf{5.4} \pm \textbf{0.9}$	$25.5\pm4.9$	
[MCPA-DAE-C14][MCPP]	$5.9 \pm 1.3$	$\textbf{26.4} \pm \textbf{3.5}$	
[MCPA][Na]+MCPP][Na]	-	$60.2\pm5.4$ [MCPA]	
		$58.9 \pm 4.9 \; [\text{MCPP}]$	

anion was dissipated more efficiently compared to the cation in all studied herbicidal esterquats. The susceptibility of herbicidal esterquats to primary biodegradation decreased with increasing length of the alkyl chain in the cation. It should also be emphasized that the biodegradation efficiency in case of the model mixture of MCPA and MCPP was twotimes higher compared to the corresponding herbicidal esterquats.

Compilation of both primary biodegradation and mineralization results indicates that: i) the studied compounds are initially decomposed to a various extent, however they are not efficiently mineralized, which suggests that biotransformation into stable metabolites is possible; ii) in case of herbicidal esterquats, the biodegradation of the anion most likely accounts for the majority of observed mineralization; iii) the low mineralization efficiency of herbicidal esterquats may result from their antimicrobial properties and, more specifically, from the surface activity of the esterquat cation.

#### 22. Conclusions

This study presents the synthesis pathway and detailed characterization of novel HILs which incorporate MCPA as an esterquat cation and MCPP as an anion. Transformation of both herbicides into their respective ionic forms allowed to notably reduce their volatility and maintain their anti-weed activity. The presence of the esterquat cation contributed to satisfactory solubility in water (which is crucial in terms of preparation of spray solutions) as well as other solvents characterized by low polarity. Subsequent evaluation of surface activity revealed that the obtained HILs are effective surfactants and confirmed the hypothesis regarding the possible displacement of additional adjuvants. Further experiments indicated that the combination of MCPA and MCPP in the form of herbicidal esterquats resulted in an additional functionality, since stimulation of maize was observed during the germination stage. The results obtained throughout this research suggest that the use of alkyl substituents with an odd number of carbon atoms does not contribute to any significant differences in terms of the studied characteristics of herbicidal esterquats. Future studies should be focused on structural modifications of the obtained herbicidal esterguats that would allow to improve their low biodegradability (which is most likely associated with their antimicrobial properties) and include a broader range of model plant species to establish whether the observed effects also apply to other crop and weed species.

#### CRediT authorship contribution statement

Anna Parus: Methodology, Validation, Investigation, Data Curation, Writing - original draft, Writing - review & editing, Supervision. Jan Homa: Methodology, Validation, Formal analysis, Investigation, Data Curation, Writing - original draft. Dariusz Radoński: Investigation. Grzegorz Framski: Investigation, Resources. Marta Woźniak-Karczewska: Methodology, Validation, Investigation, Data Curation, Writing - original draft, Project administration. Anna Syguda: Methodology, Validation, Investigation, Resources, Data Curation, Writing original draft, Writing - review & editing. Łukasz Ławniczak: Conceptualization, Writing - original draft, Writing - review & editing. **Łukasz Chrzanowski:** Conceptualization, Project administration, Funding acquisition, Supervision.

#### **Declaration of Competing Interest**

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.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2020.111595.

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