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Ph.D. THESIS

to attain the academic degree of
Doctor of Philosophy

Introduction to bioaugmentation with microorganisms capable of degrading herbicides and herbicidal ionic liquids as a factor conducive to herbicide resistance spread

submitted by

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Abstract

The synthesis of herbicidal ionic liquids has been proposed as a new application form of commercially used herbicides, aiming to eliminate the need for adjuvants. These compounds most commonly comprise in their structure herbicidal anions and cations of surface-active properties. Hence, the proper selection of cations was believed to allow manipulation of the physicochemical properties of the resulting compounds, such as volatility or hydrophobicity. However, to date research is mainly focused on their synthesis methods, characterisation of physicochemical properties and herbicidal efficacy, while there is little research on the biological properties of these compounds. Yet, with the intention to introduce these compounds on the mass scale to the agricultural fields, these gaps of knowledge need to be filled.

The research presented in following Ph.D. thesis was focused on the environmental fate of HILs, their ionic integrity in the environment, as well as the introductory aspects concerning bioaugmentation efficiency and impact on possible acquirement of herbicidal resistance. With the use of ^{13}C-labelling approach, it has been proven that upon introduction to the environment, surface-active cations and herbicidal anions constituting HILs are degraded separately and differently. Their ionic integrity was compromised, as further studies revealed that cation's hydrophobic properties do not translate into reduced mobility of an anion. Additionally, large, hydrophobic cations of surface-active properties were toxic towards both soil microorganisms and plants. The high sorption and low bioavailability of cations was the limiting factor for bioaugmentation approach since these compounds were not available for microbial degradation. Yet, the positive impact of bioaugmentation was visible both in the HILs' increased mineralisation efficiencies and protective effect towards oxidative stress in plants. Furthermore, bioaugmentation was shown to significantly increased the genetic activity involved in the degradation of selected herbicides. Finally, further studies are in progress, evaluating whether stress conditions resulting from the use of cationic surfactants might promote unintentional gene transfer and herbicidal resistance acquisition.

Streszczenie

Synteza herbicydowych cieczy jonowych (HILs) została zaproponowana jako nowa forma aplikacyjna komercyjnie stosowanych herbicydów, mająca na celu wyeliminowanie konieczności stosowania adiuwantów. Związki te najczęściej zawierają w swojej strukturze aniony herbicydowe oraz kationy o właściwościach powierzchniowo-czynnych. Ponadto, odpowiedni dobór kationów w założeniu miał pozwolić na manipulowanie właściwościami fizykochemicznymi otrzymanych związków, takimi jak lotność czy hydrofobowość. Dotychczasowe badania koncentrują się jednak głównie na metodach syntezy herbicydowych cieczy jonowych, charakterystyce ich właściwości fizykochemicznych i skuteczności chwastobójczej, niewiele natomiast jest badań dotyczących właściwości biologicznych tych związków. Tymczasem, szczególnie mając na uwadze zamiar wprowadzenia tych związków na skalę masową na pola uprawne, należy zwrócić uwagę na braki w obecnym stanie wiedzy o HILs.

Badania przedstawione w niniejszej pracy doktorskiej dotyczyły losów środowiskowych HILs, ich integralności jonowej w środowisku, a także wstępnych kwestii dotyczących efektywności bioaugmentacji i wpływu na niezamierzone nabycie odporności herbicydowej. Wykorzystując metodę znakowania izotopowego ^{13}C udowodniono, że po wprowadzeniu do środowiska surfaktanty kationowe i aniony herbicydowe wchodzące w skład HILs ulegają odrębnej i odmiennej degradacji. Ich integralność jonowa została podważona, gdyż dalsze badania wykazały, że właściwości hydrofobowe kationu nie przekładają się na zmniejszenie mobilności anionu. Dodatkowo, duże, hydrofobowe kationy o właściwościach powierzchniowo-czynnych były toksyczne zarówno dla mikroorganizmów glebowych, jak i roślin. Wysoka sorpcja i niska biodostępność kationów była czynnikiem ograniczającym efektywność bioaugmentacji, ponieważ związki te były niedostępne dla mikroorganizmów. Jednakże, korzystny wpływ bioaugmentacji był widoczny zarówno w przypadku zwiększonej efektywności mineralizacji HILs, jak i w działaniu ochronnym na stres oksydacyjny w roślinach. Ponadto wykazano, że bioaugmentacja istotnie zwiększyła aktywność genetyczną związaną z degradacją wybranych herbicydów. Prowadzone obecnie badania mają na celu ocenę, czy warunki stresowe

wynikające ze stosowania kationowych środków powierzchniowo-czynnych mogą sprzyjać niezamierzonemu transferowi genów i nabywaniu odporności na herbicydy.

1. Overview

1.1. Plant protection products

Agrochemicals comprise a group of chemical substances used in agriculture, such as pesticides (plant protection agents, PPA), fertilisers and plant-growth hormones [5]. Their widespread use is currently a necessity both in terms of efficient food production for constantly growing global population and ensuring high quality of produce [6,7]. Namely, over the last seventy years the observed population increase was over 3-fold – from 2.5 billion people in 1950 to almost 7.8 billion in 2020 – and it is estimated to reach almost 11 billion by the year 2100 [8]. With this steady increase and limited agricultural space, the efficacy of crop cultivation must be kept at high level, both for nutritional purposes for people and as an animal feed, and production of plant-based products (*e.g.*, fibres, fuels). Yet, agricultural crops are susceptible to biotic and abiotic factors, both during growth and storage (**Fig. 1**) [6]. Hence, the use of various substances from pesticide group allows to obtain high yields, without simultaneous increase in costs and areal coverage [6,7].

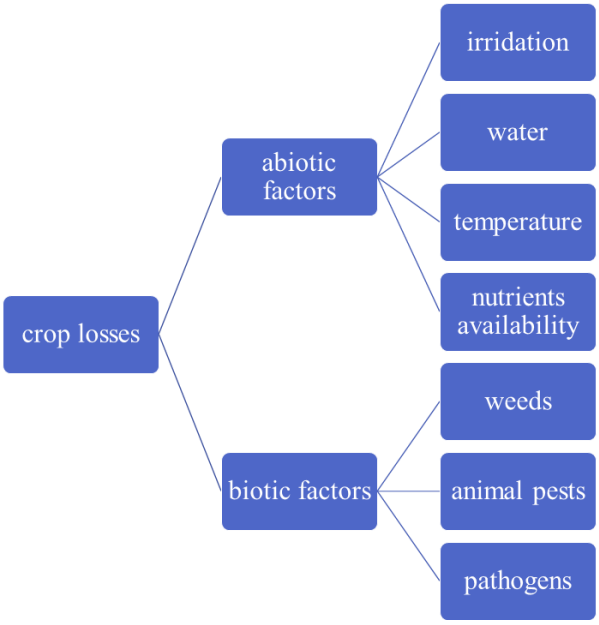

Fig. 1 Crop losses factors (adapted and modified from Oerke, 2006 [6]).

The amount of pesticides globally used increased considerably over the years. Starting from only 140 metric tons in 1940, it reached 1.7 million metric tons in 1990

and almost doubled in following 30 years – up to 2.7 million metric tons in 2020 [3,7,8]. The largest sub-groups within pesticides include herbicides, fungicides and bactericides, and insecticides, respectively, amounting for over 95 % of PPA used [8] (**Fig. 2**). Among all plant protection agrochemicals, herbicides are predominant pesticide type and make up for over 50 % of all pesticides utilized [8].

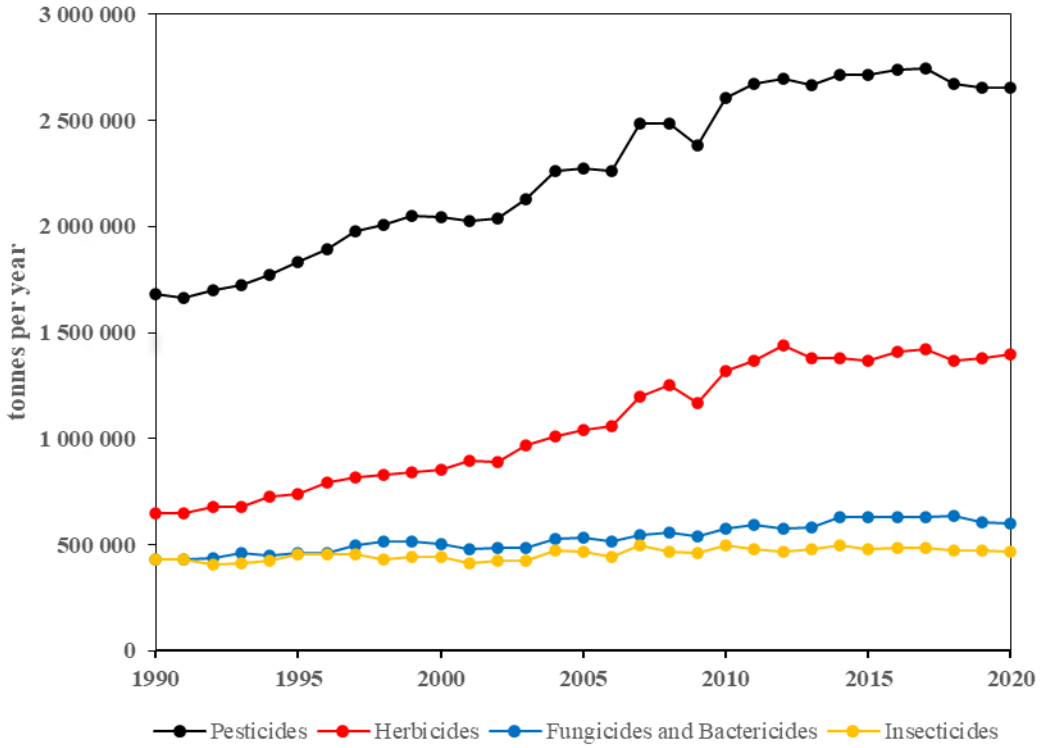

Fig. 2 Agricultural products worldwide use [8].

Herbicides are utilised in agricultural fields to control growth of various weeds competing with crops, mainly for sunlight and essential inorganic nutrients [7]. One of the most widely used active substance is glyphosate, broad-spectrum herbicide belonging to the organophosphorus group [9]. In European countries, sales of compounds from this group in 2020 amounted for over 13,000 metric tonnes in Spain only, followed by France, Italy and Poland [10]. However, researcher's attention is also focused on compounds from groups of phenoxy- (*e.g.*, 2,4-D, MCPA) and benzoic acid derivatives (*e.g.*, dicamba) herbicides (**Fig. 3**). Their sales in Europe are substantially lower than in the case of glyphosate, which might be attributed to numerous factors, such as high volatility or the fact that MCPA and 2,4-D present high

selectivity to weeds, contrary to glyphosate and dicamba. The latter one seems to be the least popular among farmers due to higher drift risk than in the case of glyphosate [11]. Nevertheless, this problem was a starting point for the research concerning herbicides' physicochemical properties adjustment [12–16].

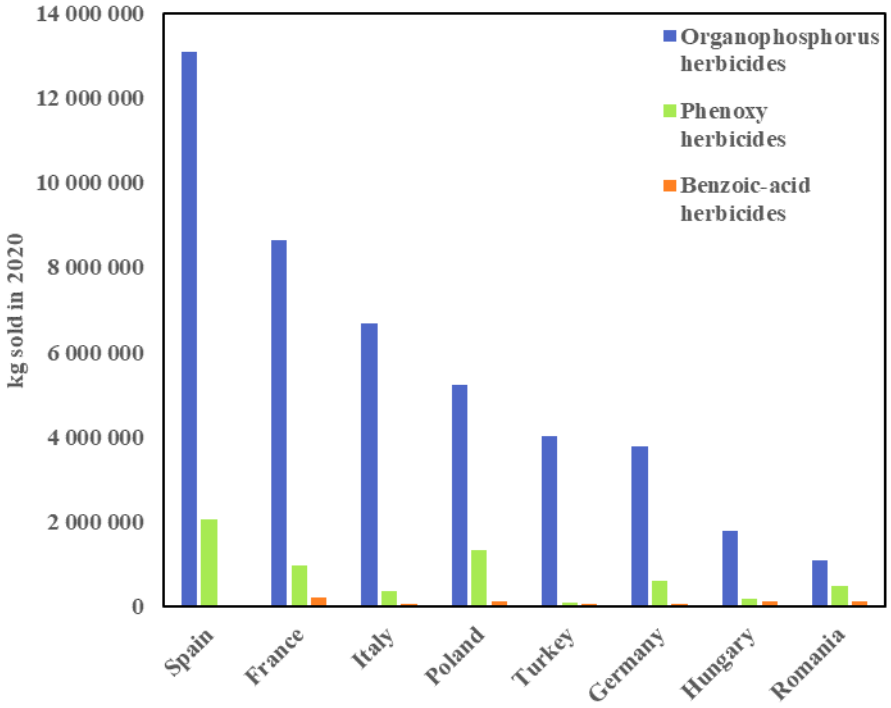

Fig. 3 Herbicides' sales in 2020 by top 8 bigger buyers in Europe [10].

Over the years, a number of disadvantages associated with the use of herbicides have been revealed (**Fig. 4**). Namely, these agrochemicals are known for their susceptibility to phenomena such as runoff, leaching and volatilisation, as well as present significant potential for accumulation in the environment [17–19]. The toxicity of herbicides to micro- and higher organisms is also reported in the literature, and is mostly related to their environmental persistence [17–22]. Additionally, due to the fact that modes of action of herbicidal formulations are based on limited number of molecular mechanisms, there is also a very real possibility for plants to acquire some level of resistance to them [20,23,24]. Another major concern is the addition of adjuvants to commercial herbicidal formulations. These substances are commonly used in order to adjust surface tension of obtained formulations [25]. This in turn allows to improve the solubility of the herbicides in water, increase their adhesion to

plant surface and, consequently, increase the penetration of the product into plant tissues, as well as to improve environmental stability of the active substance [25]. In addition, adjuvants are used in herbicidal formulations' volatility adjustment. For instance, dicamba-based herbicides were banned in some states in the US due to their high volatility and drift-induced losses to adjacent fields, and novel solutions and additives are being sought in order to reduce the volatility of resulting herbicidal mixtures [11,26]. Though, interestingly enough, the addition of adjuvants might increase the cytotoxicity of the formulation to up to 1000 times, as it was shown by the case of polyethoxylated amines in glyphosate formulations [27,28]. **However, unlike the active ingredients, adjuvants are subjected to less stringent registration control, thus final commercially available herbicidal formulation has the potential to present significantly higher environmental toxicity than the active herbicidal substance itself** [3,27–29]. Latest findings have already brought attention to the fact that the fate of herbicidal formulations is understudied, as only the active ingredient is tested [2,3,27–29]. Therefore, the impact of surface-active additives in commercial mixtures is omitted during environmental studies.

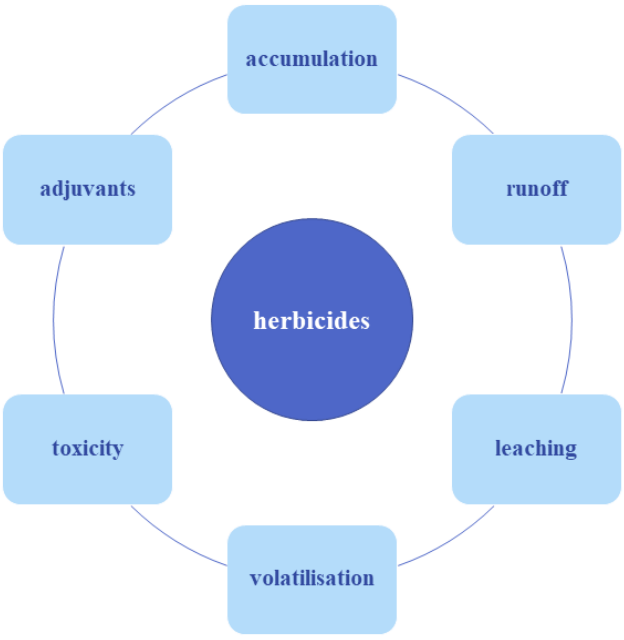

Fig. 4 Problems associated with herbicides use.

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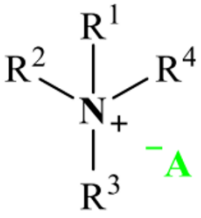
1.2. Herbicidal Ionic Liquids (HILs)

As a possible solution to the problem with toxic additives, the synthesis of herbicidal ionic liquids (HILs) has been proposed as a novel application form of commercially used herbicidal formulations [12]. The first manuscript that introduced the concept of HILs and kickstarted research on novel, adjuvants-free herbicidal application forms was published in 2011 and to date is cited 154 times (**Fig. 5**) [12]. These are included in the group of ionic liquids (ILs), defined as salts composed of discrete ions, occurring in liquid state in temperatures below 100 °C and possessing a number of unique properties and applications [30–32].

Fig. 5 The first manuscript introducing the concept of HILs [12].

Basing on their characteristics, HILs are divided into 1) first generation ILs, of tuneable physical properties, 2) second generation, of tuneable physical and chemical properties, 3) third generation, of tuneable physical, chemical and biological properties [12]. Hence, HILs are compounds belonging to the third generation of ILs, as their synthesis allows to include herbicidal properties paired with surface-active ones, thanks to careful selection of cations and anions (**Fig. 6)**.



A = herbicidal anion, *e.g.*, 2,4-D, MCPA, dicamba, glyphosate
R^1 – R^4 = aliphatic or aromatic alkyl substituents

Fig. 6 General structure of HILs based on quaternary ammonium salts [3].

As most of herbicidally active substances are occurring in anionic form, HILs-type compounds also usually incorporate herbicidal anion and cation that can be selected in order to obtain desired properties of the whole molecule. The most popular choice up to date is cation that exhibits surface-active properties that should improve adhesion and penetration of plants. Such a compound structure allows for elimination of the need for adjuvants. In addition, many researchers list such advantages as adjustable volatility, water solubility and toxicity, as well as other tuneable properties which originate from ions selection (*i.e.*, surface-active, herbicidal) [12,33–45]. Interestingly, the idea standing behind synthesis of this novel application form of herbicides seems to fall in line with the principles of integrated pest management, as well as green chemistry [46,47]. These principles aim at reduction of the detrimental impact on the environment caused by human activity and hence eliminating risks to human and animal health and life. This is the reason why particular emphasis is placed on agrotechnical and breeding methods of crop protection [46]. A point worth noting is that the integrated approach does not promote abandoning the use of chemical plant protection products. Rather, it aims at minimisation their use in order to achieve the best possible results with the least possible burden on the environment caused by PPA [47]. Therefore, the idea of compounds of low volatility and allowing for elimination of adjuvants use seems to fit perfectly the objectives of these principles.

Most commonly, the anions utilised in HILs synthesis exhibit herbicidal properties, and comprise of chemicals from various classes, *e.g.*, derivatives of phenoxyacids, benzoic acids, picolinic acids, sulfonylureas, glyphosate and bio-herbicides (*i.e.*, with anions of natural origin). It is also possible to synthesise formulations with more than one herbicidal anion, exhibiting either the same mode of action or different ones [3]. However, despite the fact that these anions exhibit herbicidal properties, these alone are not suitable for application due to the fact that they have insufficient wetting properties and might pose issues with lack of environmental stability. Consequently, to avoid the use of adjuvants, herbicidal anions are paired with cations of appropriate surface-active properties, as presented on **Fig. 7** below.

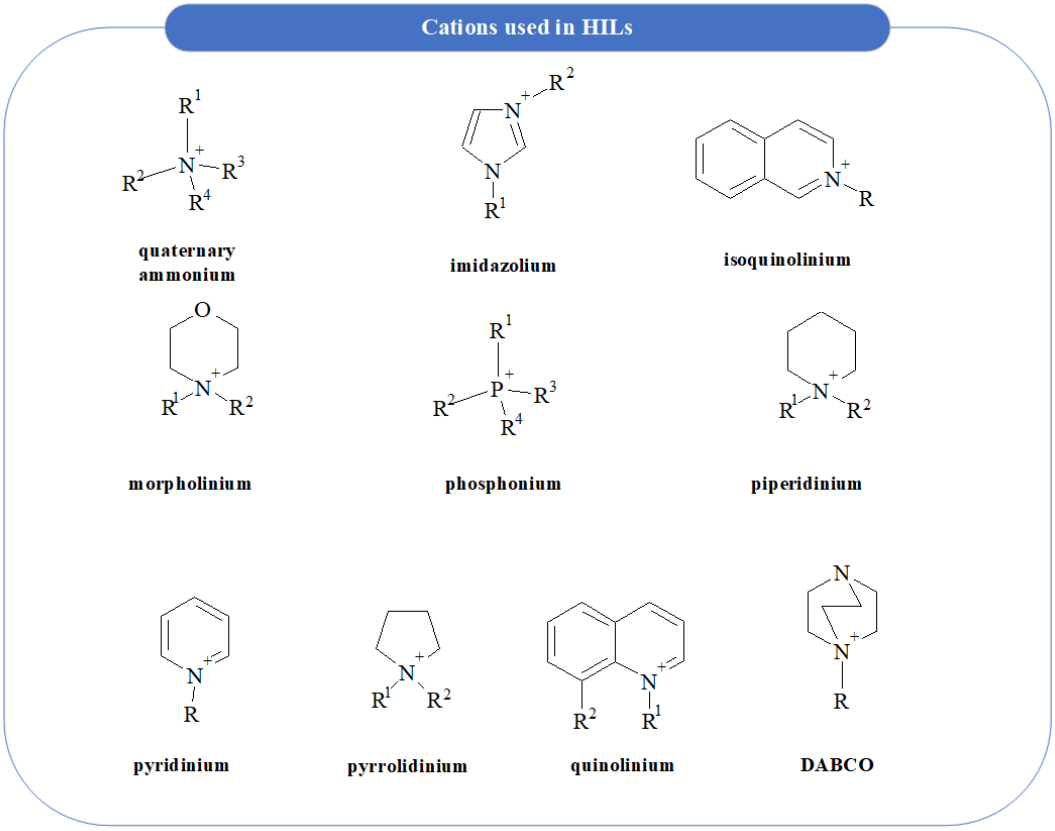


Fig. 7 Cations used in HILs [3].

Ammonium cations are the most commonly used, as they are relatively cheap and easily available, moreover are safe for human health as their commercial applications include disinfectants, fabric softeners, surfactants and even cosmetics products [3,48–56]. However, many other alternatives were analysed by researchers in an attempt to find the most advantageous pair for herbicidal anion [3]. For instance, phosphonium cations were tested, yet scarcely employed, as these are known to form toxic metabolites (phosphine oxides) which consequently inhibit degradation processes, leading to accumulation in the environment [57,58]. DABCO-derivatives with long hydrophobic chains were deemed not suitable for agricultural use due to poor degradation and high toxicity, causing possible environmental persistence [59,60]. Another example might be the use esterquats based on acrylane-derivatives, as it has been shown that these not only are toxic, but also characterised by poor surface activity and not improved herbicidal efficacy [61]. In addition, as a scientific curiosity, glucose-based cations were examined in terms of synthesis of compounds from renewable resources [62]. Another interesting approach was to synthesise double-salt

herbicidal ionic liquids (DSHILs) with the same cation, which resulted in synergistic effects when compared to single herbicidal salts [63].

With these findings in mind, the quaternary ammonium cations use seems to be the most promising attempt in the field of HILs synthesis. However, on the other hand, cationic surfactants are easily accumulating in the environment and tend to sorb in soils [64–66]. Moreover, the use of quaternary ammonium cations in the structure of HILs might have an impact on the degradation of anion. It is due to the fact that these are known for their ability to form complexes with anions, *i.e.*, to bind them to soil particles, which in turn might decrease degradation efficiency [67–69]. As not bioavailable for degradation, these stay in the environment and, with the intention of annual treatments, might accumulate in soils and groundwaters at high concentrations. In turn, these might pose a serious threat to biodiversity and overall health of microbiome and plants [48,69–76].

Quaternary ammonium compounds (QACs) are utilised as disinfectants for a reason – they are potent antimicrobial agents that disrupt microbial cells' membranes [48,73–76]. This is extremely important in agriculture for two reasons. Firstly, employing compounds that potentially act as germicides might lead to lowering biodiversity in the farmlands *via* destruction of bacteria less resistant to disinfectants. This in turn can potentially cause significant imbalance in microbiota of crop plants', which relay on complex plant-microbiome interactions to ensure their own health. Impoverished microbiome thus undermines plants' health and in turn limit the quality and quantity of the produce obtained [3,49–56]. That phenomena can be easily compared to the case of human gut microbiome: it is essential for digestion, immunity, and general health; barren might lead to serious consequences.

However, if the concentration of quaternary ammonium salt is too low to cause cell's death, it undergoes a series of events collectively known as oxidative stress and error prone DNA replications. These processes might lead to resistance acquisition *via in-situ* mutation of bacterial genetic material and promote horizontal transfer of genes thus spreading genes encoding resistance to herbicides [75]. Therefore, it is plausible that widespread use of HILs-type compounds incorporating QACs might lead to the activation of defence mechanisms in bacteria, leading to both emergence of herbicidal resistance and its further spread in the agricultural soils. It is an especially disturbing issue, since bacterial tolerance or resistance to cationic surfactants might result also in

antibiotic resistance spread over time [73,77–80]. It is attributed to the fact that bacteria employ extremely similar molecular mechanisms responsible for dealing with the presence of QACs and antibiotics from almost all families [81–83]. Namely, class 1 integrons (which harbour additional, non-essential genetic material) found in bacteria tolerant to quaternary ammonium compounds are also known to carry antibiotic resistance genes [73,81,82]. Hence, efficient removal of QAC from soils is a necessity in terms of reducing risks of herbicidal or pharmaceutical resistance spread. Unfortunately, to date, little is known on the subject of cationic surfactants combinations with herbicides, thus comprehensive studies are needed in the aspect of their ability to promote oxidative stress in microorganisms and resulting unintentional transfer of resistance genes from microorganisms.

1.3. Environmental research on HILs

Herbicidal ionic liquids, as all of the compounds intended for agricultural use, should be analysed not only in terms of physicochemical properties and herbicidal efficacy, but also examined for environmental effects and safety. After synthesis and following spectral and thermal analyses performed in order to ensure purity and proper structure, these compounds are subjected to research evaluating, *e.g.*, their volatility, solubility, surface tension, water and halide residues and octanol-water partition coefficient [3]. Less commonly, aspects such as mobility and sorption in soils are examined as well [41,84,85]. Mobility and resulting leaching and run-off of herbicides are the main ways of them entering groundwaters (**Fig. 8**). On the other hand, sorption leads to decreased bioavailability for microbial degradation and accumulation in the environment [3,5]. Yet, these vastly important analyses seem to be overlooked.

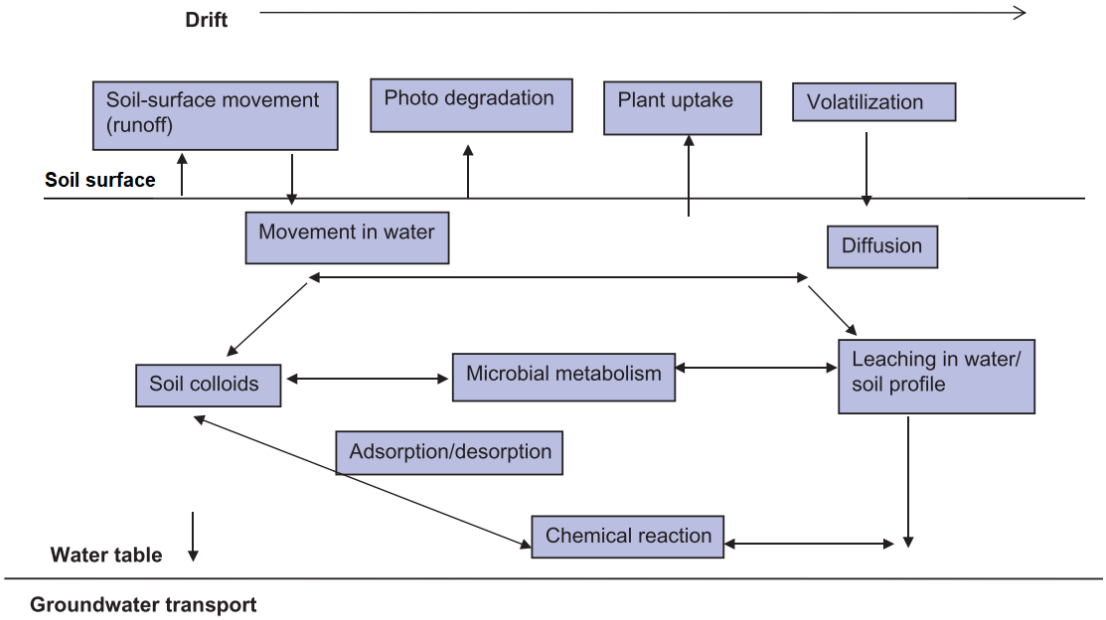


Fig. 8 Fates of herbicides in the environment (adapted and modified from Mandal, 2020 [5]).

When it comes to biological tests, the main point of interest are herbicidal efficacy studies, evaluated during both field and greenhouse experiments. Interestingly, despite the fact that scientific community acknowledge the problems associated with environmental challenges of HILs' application and environmental impact evaluation, research on novel formulations is still lacking routine degradation and toxicity studies [86]. As for the latter one, a few works examined the impact of selected HILs on animals, plants, algae and microorganisms, *via* standard protocols such as MIC (minimum inhibitory concentration) and MBC/MFC (minimum bactericidal/fungicidal concentration) [42,59], EC_{50} (half maximal effective concentration) [42,61,87,88] or LD_{50} (median lethal dose) [12,42,60,61,89,90]. More advanced techniques (*e.g.*, determination of changes in biodiversity and microbial membranes structures) are used even less frequently [58,91–93]. Yet, even currently available toxicity research is not one that is unified; data obtained so far is very scattered and hard to integrate into a full picture needed to assess potential environmental impact [3]. Hence, the environmental safety of HILs currently is often evaluated based on physicochemical parameters, such as low volatility (*i.e.*, reduced drift), water solubility (*i.e.*, reduced movement through soils and groundwaters), and high herbicidal efficiency, which allows for dose reduction, and consequently – lesser amount of xenobiotic in the environment.

Overview of biodegradation studies performed up to 2020 are presented below, in **Table 1** (adapted and modified from author's published work [3]). **Up to 2020, only nine publications covered the issue of environmental degradation of herbicidal ionic liquids** [42,61,88–90,94–97]. Interestingly, none of them evaluated degradation efficiencies for HILs with single glyphosate anion, which is one of the most popular active herbicidal substances. Most of the studies was based on standard OECD 301 F procedure in which the amount of carbon dioxide generated by the activated sludge is measured [98,99]. During the OECD procedure, the microorganisms present in the aqueous sample containing the test substance, which is their only carbon source, consume oxygen for their metabolic activity and produce a corresponding amount of carbon dioxide, proportional to the degradation of the compound. This in turn is absorbed by a strong base placed in the neck of the bottle, and the result of this process is a drop in gas pressure monitored by the measuring head [98,99]. Yet it has been already proven that this approach might not be the most suitable method for degradation assessment (**Fig. 9**) [2].

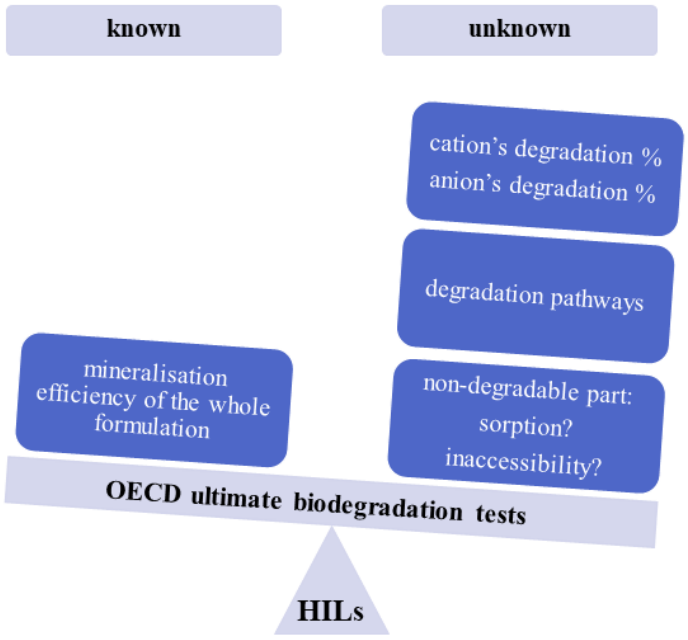


Fig. 9 Gaps of knowledge resulting from sole OECD ultimate biodegradation assessment.

First of all, the above-mentioned procedure does not reflect the actual environmental conditions, since microorganisms that might have had contact with

herbicides, and hence ability to degrade them, would rather be these present in the agricultural fields, and not activated sludge [5]. It is due to the fact that herbicides are expected to be found on farmlands, not urban sewage. At the same time, the activated sludge microorganisms are well-suited to surfactants' and disinfectants' degradation, as these enter wastewater treatment plants regularly, *e.g.*, with household and hospital effluents [65]. Furthermore, HILs are used in the form of aqueous solutions, so they can be expected to break down into cations and anions in solution, which may have different degradation potential in aqueous and soil systems. Meanwhile, OECD 301 tests only indicate general trends and do not provide data on the nature of mineralisation, *i.e.*, whether a cation or an anion or both are degraded and to which extent. It also does not provide any insights into degradation pathways of tested compounds, nor allows to distinguish between degradation, sorption or other physicochemical phenomena that might make the compounds inaccessible for microbes. Hence, even high mineralisation efficiencies do not mean that the whole compound was degraded, since this protocol lacks information as to whether the carbon dioxide is generated from the cation or the anion. **This in turn means that it is not known how efficiently the cation and the anion are degraded, and whether the compound is treated by bacteria as a singular entity or as a mixture of ions**. Namely, mineralisation efficiency of 50 % means that, for instance, only sole cation or sole anion was mineralised at 50 % or each one of them was mineralised at 25 % or other proportions summing up to 50 %. On the other hand, other analytical techniques allowing compound's degradation assessment (*e.g.*, high-performance liquid chromatography, HPLC) indicate certain metabolites or record the disappearance of the signal coming from the test substance, and therefore show only the overall degradation efficiency [58,100]. They also do not allow to distinguish whether signal disappearance is a result of actual degradation or sorption (inaccessibility for extraction and degradation) [64,65,70]. These gaps in knowledge were the basis for one of the works, which aimed in evaluation of environmental behaviour of HIL using ^{13}C-labelling [2]. However, ideally, mass balance with the use of ^{14}C-labelling would allow to gain insights into cations and anions behaviour in the environment, as this technique is easier and quicker than the use of ^{13}C [101]. However, for the time being, this is beyond the financial possibilities of standard studies [101].

Table 1 Overview of biodegradation studies on HILs with one herbicidal anion (adapted and modified from author's published work [3]).

Anion	Cation	Biodegradation	Ref.
MCPA	tebuconazole	OECD 301 F test: cation, 88 ± 4 %; anion, 0 ± 0 %	[94]
MCPA	propiconazole	OECD 301 F test: cation, 58 ± 3 %, anion, 3 ± 0 %	[94]
MCPA	tetraalkylammonium	BOD_5/COD ratio (t = 0–360 min, 0.4 = limit of biodegradability): electrochemical oxidation process, 0.25 – 0.35; electro-Fenton process, 0.35 – 0.65	[95]
		electrochemically treated, 28 – 57 %; non-treated, 0 – 8 %	[96]
MCPA	betainium	OECD 301 F test, 69 %	[90]
MCPA	*N*-alkylbetainium	OECD 301 F test, 59–62 %	[89]
MCPA	carnitinium	OECD 301 F test, 59 %	[90]
MCPA	acetylcholine	OECD 301 F test, 80 %	[97]
MCPA	[2-(methacryloyloxy)-ethyl]trimethylammonium [2-(acryloyloxy)-ethyl]trimethylammonium	OECD 301 F test, 29–37 %	[61]
MCPA	dialkanoyloxyethyl-dimethylammonium	OECD 301 F test, 63 %	[61]
2,4-D	tebuconazole	OECD 301 F test: cation, 94 ± 3 %; anion, 0 ± 0 %	[94]
2,4-D	propiconazole	OECD 301 F test: cation, 65 ± 3 %; anion, 0 ± 1 %	[94]
2,4-D	betainium	OECD 301 F test, 87 %	[90]
2,4-D	*N*-alkylbetainium	OECD 301 F test, 72 – 73 %	[89]
2,4-D	carnitinium	OECD 301 F test, 76 %	[90]
2,4-D	acetylcholine	OECD 301 F test, 70 %	[97]
2,4-D	4,4-dialkylmorpholinium	OECD 301 F test with microbiota isolated from different environmental niches: river sludge, 9–10 %; garden soil, 13–19 %; agricultural runoff stream, 18–25 %; agricultural soil, 14–24 %; waste repository, 20–31 %	[88]
		Primary biodegradation for microbiota isolated from different environmental niches: river sludge, cation 52–58 %, anion 9–11 %; garden soil, cation 74–77 %, anion 25–31 %; agricultural runoff stream, cation: 87–90 %, anion 60–61 %; agricultural soil, cation 88–92 %, anion 60 %; waste repository, cation 91–94 %, anion 51–55 %	
MCPP	tebuconazole	OECD 301 F test: cation, 89 ± 4 %; anion, 0 ± 1 %	[94]
MCPP	propiconazole	OECD 301 F test: cation, 68 ± 3 %; anion, 0.5 ± 0 %	[94]
MCPP	betainium	OECD 301 F test: 57 %	[90]
MCPP	*N*-alkylbetainium	OECD 301 F test: 51–55 %	[89]
MCPP	carnitinium	OECD 301 F test: 49 %	[90]
dicamba	tebuconazole	OECD 301 F test: cation, 100 ± 5 %; anion, 44 ± 2 %	[94]
dicamba	propiconazole	OECD 301 F test: cation, 56 ± 2 %; anion, 40 ± 2 %	[94]

dicamba	*N*-alkylbetainium	OECD 301 F test: 42–47 %	[89]
dicamba	acetylcholine	OECD 301 F test: 90 %	[97]
dicamba	4,4-dialkylmorpholinium	OECD 301 F test with microbiota isolated from different environmental niches: river sludge 0 %, garden soil 0 – 1 %, agricultural runoff stream 1–2 %, agricultural soil 2 %, waste repository: 2 % Primary biodegradation for microbiota isolated from different environmental niches: river sludge, cation 38–55 %, anion 0 %; garden soil, cation 56–77 %, anion 0 %; agricultural runoff stream, cation 77–81 %, anion 32 %; agricultural soil, cation 75–79 %, anion 29–35 %; waste repository, cation 83–86 %, anion 34–36 %	[88]
pelargonate	tetraalkylammonium	OECD 301 F test: 0–83 %	[42]
pelargonate	alkylbis(*n*-ethoxylated)-methylammonium	OECD 301 F test: 68 %	[42]
pelargonate	*N*-alkylbetainium	OECD 301 F test: 85 %	[42]
pelargonate	dialkanoyloxyethyl-dimethylammonium	OECD 301 F test: 52 %	[42]

Herbicides have been ordered in decreasing number of manuscripts; Table does not include salts with melting points greater than 100 °C; singular form – only one compound; Plural form – more than one compound; Presented data are up to year 2020. Adapted from Wilms et al., 2020 [3].

In view of problems with abovementioned environmental persistence of herbicides, biological approaches helpful in effective removal of xenobiotics are being sought after, *e.g.*, biostimulation, attenuation or bioaugmentation [22,102,103]. Among them, bioaugmentation is a process that involves inoculation of the environment with microorganisms capable of degrading a given xenobiotic (**Fig. 10**) [22,102–104]. In this process, additional microorganisms (single strains or mixed communities) are introduced into the contaminated field to improve the process of removing these compounds by enhancing field's catabolic potential [22,102,103]. This approach is recommended to use in sites lacking autochthonous microorganisms adapted to specific xenobiotic's degradation [19,22]. Furthermore, its usefulness in pesticidal removal has been confirmed by many researchers over the years, and new pesticide-degrading strains are being isolated [105–113].

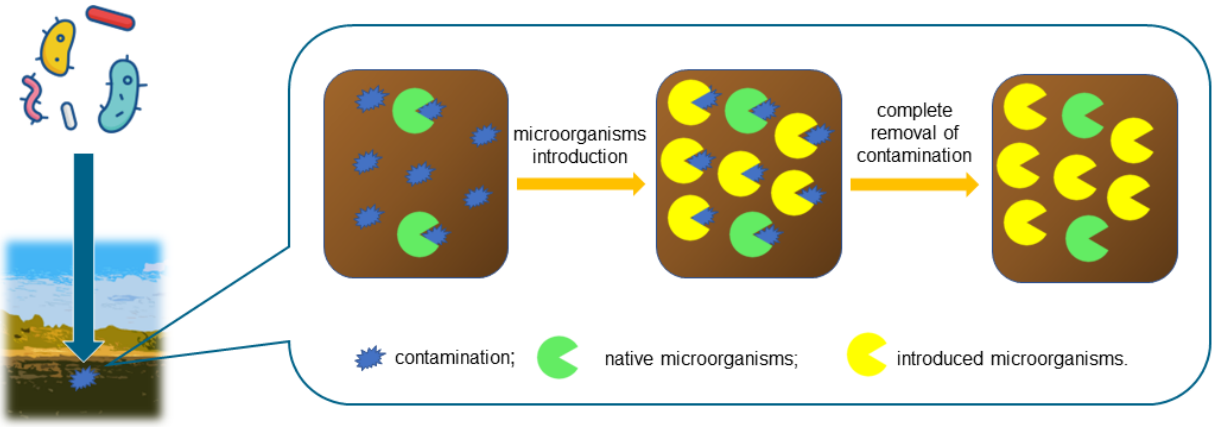


Fig. 10 Schematic presentation of bioaugmentation principle.

The most commonly applied approaches to select microorganisms for bioaugmentation include their isolation directly from contaminated sites or from sites of known contact with given xenobiotics, which is then followed by the reinoculation to the environment after culturing under laboratory conditions [103,114]. However, there are many factors which might have impact on the effectiveness of bioaugmentation approach [103,114–116]. Microorganisms introduced to the environment are susceptible to many abiotic factors affecting their survivability and adaptability, *e.g.*, pH, temperature, soil type, moisture and organic matter content, as well as nutrient content and aeration [103]. In addition, they are vulnerable also to biotic factors. Namely, the competition between autochthonous and introduced microorganisms might take place, as well as antagonistic and predatory interactions [103,114]. In general, microorganisms previously cultivated under laboratory conditions might not exhibit the same activity upon introduction to the environment which might translate into effectivity lower than expected [114].

On the other hand, a possible problem with bioaugmentation in herbicide-treated fields is that plants not only will have contact with these xenobiotics, but also will be subjected to the constant presence of microorganisms possessing genes related to the production of enzymes responsible for degrading herbicides [117]. This, in turn, might be a factor conducive to unintended resistance spread *via* two possible mechanisms. Namely, plant will utilise herbicide-resistant microorganisms that penetrated its tissues or these microorganisms themselves will pass on resistance genes directly to plants *via* horizontal gene transfer [23,24,117–122]. Hence, it is an issue of

the utmost importance to not only find appropriate techniques allowing to evaluate the real environmental fate of HILs, but also to test the environmental impact of microorganisms utilised in their degradation.

2. Objective of the thesis

The aims of following doctoral thesis are based on three main hypotheses:

H1) Herbicidal ionic liquids in the environment may act as individual cations and anions, hence their degradation may resemble the breakdown of herbicides in the presence of cationic surfactants – no specific action of HILs will be visible.

H2) The presence of surface-active cations in herbicidal formulations will result in increased toxicity, resulting mostly from QAC, which are known to promote microbial defence responses and stimulate behaviour that promotes the conditions suitable for transmission of resistance genes.

H3) The bioaugmentation with specialised bacteria with the ability to degrade herbicides into the environment will result in improved degradation efficiencies, providing the lack of cation's toxic effect.

These sub-hypotheses were later verified within OPUS 15 NCN Project titled "*Bioaugmentation with herbicide degrading bacteria as a potential factor in spreading resistance to herbicides among plants*".

3. Materials and methods

3.1. Synthesis and characterisation of HILs

For the purposes of studies performed as a part of this doctoral thesis, a number of herbicidal ionic liquids was synthesised, basing on anions of glyphosate, 2,4-D, MCPA and dicamba in collaboration with chemists from Faculty of Chemical Technology at Poznan University of Technology and Institute of the Bioorganic Chemistry at Polish Academy of Sciences. The list of synthesised compounds is presented in **Table 2**, along with their names, acronyms and references to synthesis details. Syntheses procedures are summarised below:

a) HILs based on glyphosate ([Chol][Glyph], [C_{12}Chol][Glyph], [DDA][Glyph], [C_{16}TMA][Glyph], [BA][Glyph]) were synthesised and characterised according to the procedures established during previous works [44,123–126].

b) HILs based on 2,4-D ([Chol][2,4-D], [Bet][2,4-D], [C_{12}Bet][2,4-D], [CAPBet][2,4-D], [Car][2,4-D]) were synthesised and characterised according to procedures established within the framework of previous works [1,89,90,127].

c) HILs based on 2,4-D ([C_{12}Chol][2,4-D], [TMA][2,4-D], [BTMA][2,4-D] and [TBA][2,4-D]) were synthesised as follow. Respective quaternary ammonium chlorides (0.01 mol) were dissolved in anhydrous methanol (10 mL) and mixed with 0.1 M potassium hydroxide dissolved in methanol (20 mL). Then, post-reaction mixture was cooled (to 0 °C) and the inorganic by-product (precipitate) was removed. Obtained quaternary ammonium hydroxides were then neutralised with 2,4-dichlorophenoxyacetic acid (at 25 °C). After that, solvents were evaporated, and products were dissolved in acetone for further purification. Precipitates were filtered off and solvent was again evaporated from final products which in the end were dried (40 °C, 24 h, 1–2 mbar) and stored over P_4O_{10} in a vacuum desiccator.

d) QTS based on MCPA and dicamba were synthesised according to following procedure [4]. Quaternary tropinium bromide [128] (6.5 g) was dissolved in a mixture of isopropanol and water (1:2, v/v). Herbicide sodium salts were prepared by mixing an aqueous solution of herbicide with an equimolar amount of 10 % NaOH (w/v), and heating until a clear solution was obtained. Then, the herbicide sodium salts were added to the previously prepared cations' bromide solutions and mixed. The reaction mixture was then extracted with chloroform, and the product was rinsed three times

with deionised water. Next, HILs solutions were transferred to a round-bottom flasks, the solvent was evaporated, and the final product was dried under vacuum to constant weight.

e) ^{13}C-labelled compounds [C_{12}-BA*][MCPA], [C_{12}-BA][MCPA*], and unlabeled [C_{12}-BA][MCPA] were synthesised according to procedure described in Wilms et al., 2020 [2] and presented on **Fig. 11** below. Shortly, respective labelled and unlabelled cation's bromides were obtained by the reaction of dodecyldimethylamine (0.005 mol) with benzyl bromide (^{13}C-labelled or unlabelled, 0.0058 mol) in acetonitrile (15 mL). The reaction was performed at 60 °C for 48 h, after which the solvent was evaporated. Next, water (5 mL) and hexane (5 mL) were added to isolate product *via* two-phase extraction. The aqueous phase was rinsed with hexane, followed by phases separation, water evaporation and drying (60 °C, 24 h). Final compounds were obtained in the ion exchange reaction according to Pernak et al., 2008 [129]. Namely, ^{13}C-labelled or unlabelled (4-chloro-2-methylphenoxy)acetic acid (0.005 mol) was combined with distilled water (5 mL) and potassium hydroxide (0.0051 mol), and heated (15 min, 50 °C) while stirring. Subsequently, stoichiometric amount of ^{13}C-labelled or unlabelled cation's bromides were added and stirred (30 min, 20 ± 2 °C). Finally, products were extracted with chloroform (5 mL) from aqueous phase and rinsed with distilled water until Cl^- and Br^- were washed away, and then dried under reduced pressure (60 °C, 24 h).

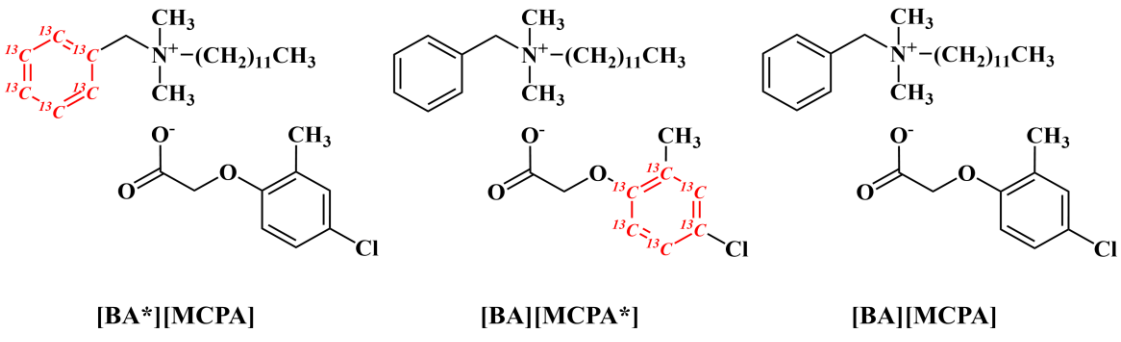
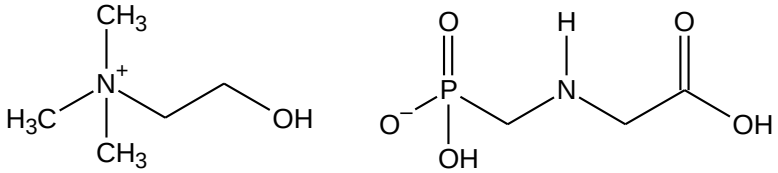
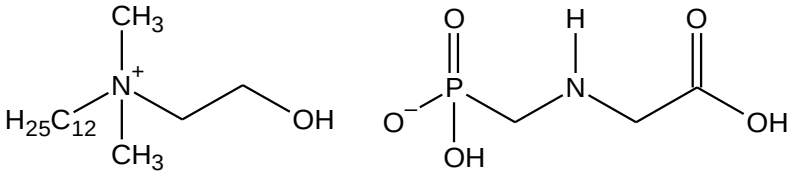
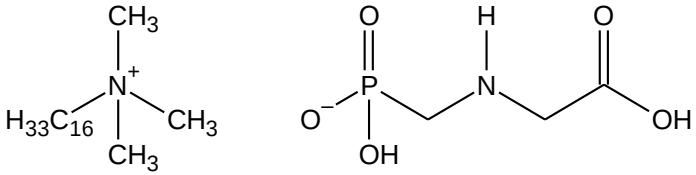
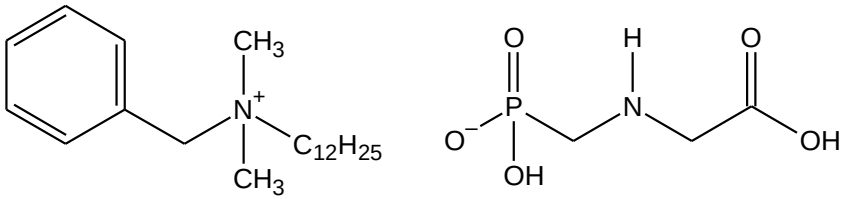
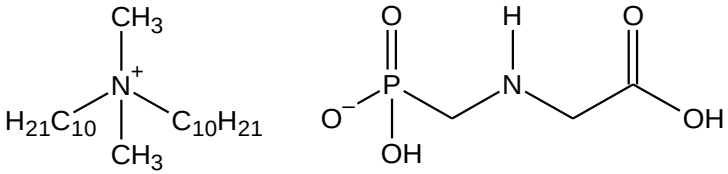
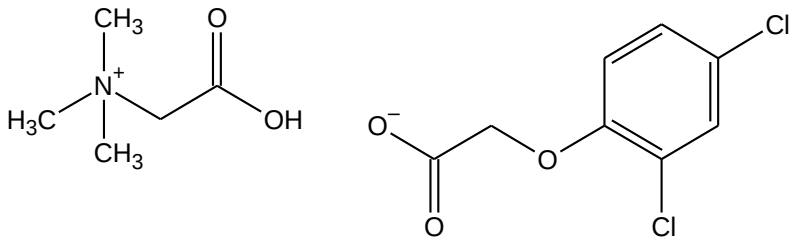
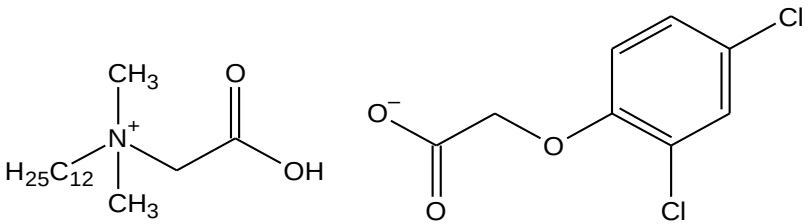
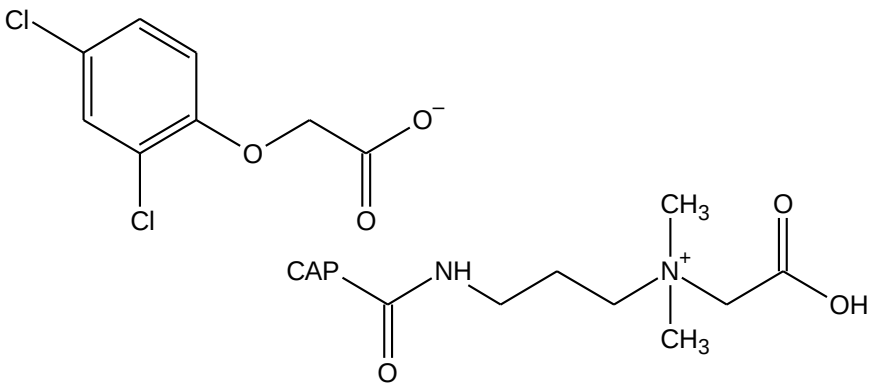


Fig. 11 Structures of ^{13}C-labelled ([C_{12}-BA*][MCPA], [C_{12}-BA][MCPA*]) and unlabelled ([C_{12}-BA][MCPA]) HILs (adapted from author's published work [2]).

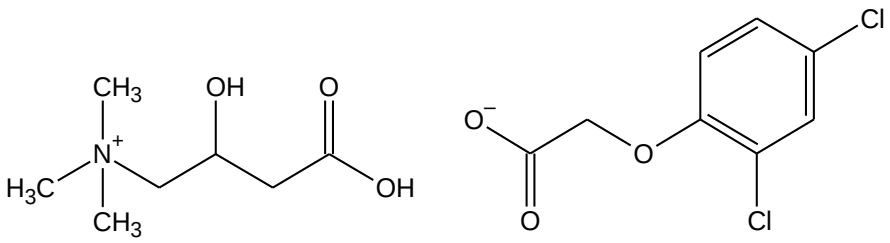
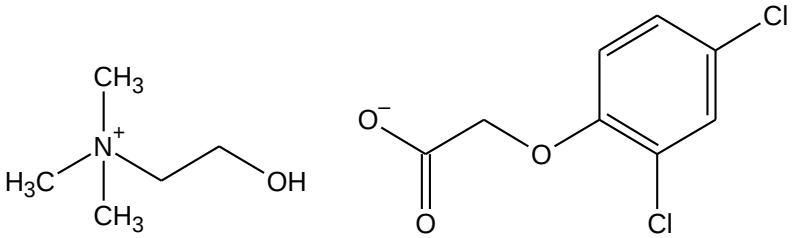
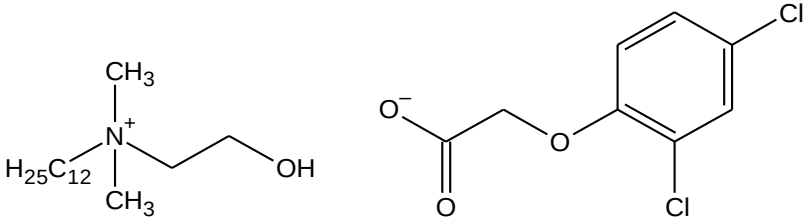
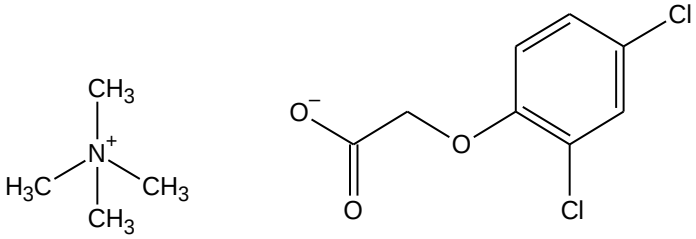
Structures of all obtained compounds were verified by analyses of ^{1}H NMR, ^{13}C NMR, ^{15}N NMR and ^{31}P NMR spectra (Bruker Ascend™ 400 MHz Nanobay spectrometer (Billerica, MA, USA) [4], Varian VNMR-S 400 MHz spectrometer (Crawley, UK), Mercury Gemini 300 spectrometer (Thermo Fischer Scientific, Waltham, MA, USA), Bruker Avance III 500 MHz (Billerica, MA, USA) spectrometer [1,2]). Tetramethylsilane (TMS) served as an internal standard. Additionally, NOESY experiments were performed for 2D homo- or heteronuclear correlation [1]. The spectra of all synthesised compounds are provided in the **Annex**.

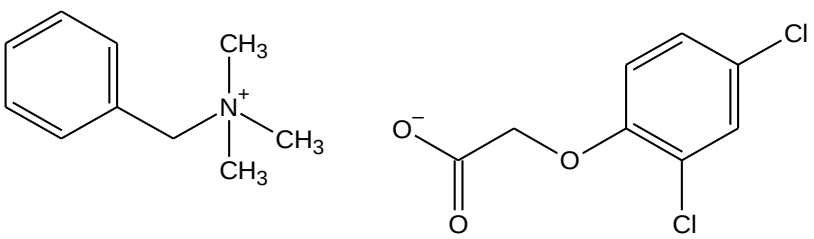
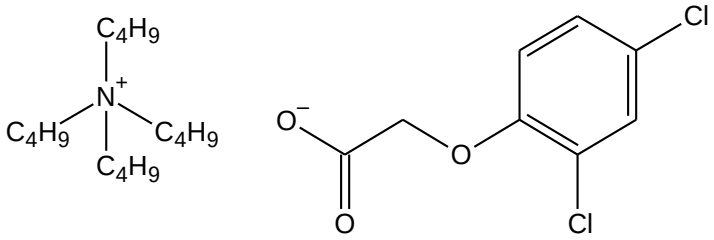
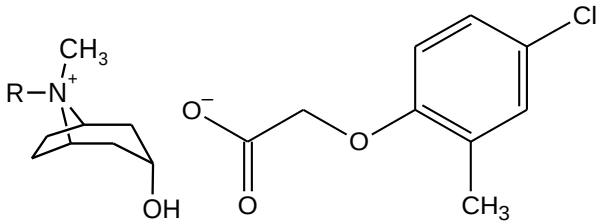
Table 2 Synthesised HILs used in experiments.

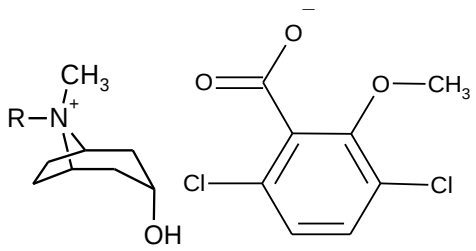
Acronym	Chemical structure	Full name	Synthesis procedure
[Chol][Glyph]		(2-hydroxyethyl)trimethylammonium glyphosate	[123]
[C_{12}Chol][Glyph]		dodecyl(2-hydroxyethyl)dimethylammonium glyphosate	[44]
[C_{16}TMA][Glyph]		hexadecyltrimethylammonium glyphosate	[124]
[BA][Glyph]		benzalkonium glyphosate	[125,126]
[DDA][Glyph]		didecyldimethylammonium glyphosate	[125,126]

[Bet][2,4-D]		betainium 2,4-dichlorophenoxyacetate	[1,90]
[C_{12}Bet][2,4-D]		dodecylbetainium 2,4-dichlorophenoxyacetate	[1,89]
[CAPBet][2,4-D]		cocamidopropylbetainium 2,4-dichlorophenoxyacetate	[1,89]

coco – mixture of saturated linear alkyl substituents:
C_8 (5 %), C_{10} (6 %), C_{12} (50 %), C_{14} (19 %), C_{16} (14 %), C_{18} (10 %)

[Car][2,4-D]		carnitinium 2,4-dichlorophenoxyacetate	[90]
[Chol][2,4-D]		(2-hydroxyethyl)trimethylammonium 2,4-dichlorophenoxyacetate	[127]
[C_{12}Chol][2,4-D]		dodecyl(2-hydroxyethyl)dimethylammonium 2,4-dichlorophenoxyacetate	[44]
[TMA][2,4-D]		tetramethylammonium 2,4-dichlorophenoxyacetate	[-]

[BTMA][2,4-D]		benzyltrimethylammonium 2,4-dichlorophenoxyacetate	[-]
[TBA][2,4-D]		tetrabutylammonium 2,4-dichlorophenoxyacetate	[-]
[QTS-C_8][MCPA]		N-octyltropinium 4-chloro-2-methylphenoxyacetate	[4]
[QTS-C_{10}][MCPA]		N-decyltropinium 4-chloro-2-methylphenoxyacetate	[4]
[QTS-C_{12}][MCPA]		N-dodecyltropinium 4-chloro-2-methylphenoxyacetate	[4]
[QTS-C_{14}][MCPA]		N-tetradecyltropinium 4-chloro-2-methylphenoxyacetate	[4]
[QTS-C_{16}][MCPA]	R = C_8H_{17}, $C_{10}H_{21}$, $C_{12}H_{25}$, $C_{14}H_{29}$, $C_{16}H_{33}$, $C_{18}H_{37}$	N-hexadecyltropinium 4-chloro-2-methylphenoxyacetate	[4]

[QTS-C_{18}][MCPA]		N-octadecyltropinium 4-chloro-2-methylphenoxyacetate	[4]
[QTS-C_8][dicamba]		N-octyltropinium 3,6-dichloro-2-methoxybenzoate	[4]
[QTS-C_{10}][dicamba]		N-decyltropinium 3,6-dichloro-2-methoxybenzoate	[4]
[QTS-C_{12}][dicamba]		N-dodecyltropinium 3,6-dichloro-2-methoxybenzoate	[4]
[QTS-C_{14}][dicamba]		N-tetradecyltropinium 3,6-dichloro-2-methoxybenzoate	[4]
[QTS-C_{16}][dicamba]		N-hexadecyltropinium 3,6-dichloro-2-methoxybenzoate	[4]
[QTS-C_{18}][dicamba]		N-octadecyltropinium 3,6-dichloro-2-methoxybenzoate	[4]

R = C_8H_{17}, $C_{10}H_{21}$, $C_{12}H_{25}$, $C_{14}H_{29}$, $C_{16}H_{33}$, $C_{18}H_{37}$

3.2. Soil characterisation

Agricultural soil samples utilised in the experiments were collected in Rzgow, Poland (N 52.151102, E 18.050041), from the depth of 10–20 cm to a sterile packages, and then sieved through a 1.6 or 2.0 mm sieve [130]. They were further subjected to characterisation according to United Soil Classification Systems (USCS) and described as fine-grained sandy loam (clay 1.9 %, silt 27.0 %, sand 71.0 %) [131]. Small variations in data results from the fact that analyses for the studies were performed individually, no longer than 3 days after sample collection. All assays were performed in triplicate and the symbol '±' represents the standard deviation from three independent replicates.

Detailed soil characteristics were as follow: organic carbon 1.0–1.5 %; total sulphur 276 mg/kg; nitrogen $N\text{-}NO_3$ 7.7–7.9 mg/kg d.w.s. (soil dry weight) and $N\text{-}NH_4$ 1.3–1.5 mg/kg d.w.s.; porosity 0.39 ± 0.03 m^3/m^3; bulk density 1.32 ± 0.06 Mg/m^3; field water capacity 0.22 ± 0.04 m^3/m^3; relative field capacity 0.564 ± 0.04 m^3/m^3; moisture during sampling 18 ± 1 %; cation exchange capacity 24.3 ± 0.5 $cmol_c/kg$; pH 5.92 (in KCl), 6.85 (in water); phosphorus 81.0 ± 1.1 mg/kg; potassium 88.0 ± 2.3 mg/kg; magnesium 69 ± 1.3 mg/kg.

3.3. HILs bioavailability in soil

Herbicides at a concentration 1000 mg/kg d.w.s. of active substance (glyphosate) were introduced to the soil (100 g), mixed thoroughly and dried (60 °C) to a constant weight. Subsequently, the first two steps of the Community Reference (BCR) sequential extraction [132,133] were conducted, in order to determine the amount of herbicides readily available/leachable with mild agents. In the first step, water-soluble herbicides were extracted. Briefly, 1 g of soil treated with each glyphosate-based HIL was introduced to 50 mL polyethylene centrifuge tubes with 40 mL of deionised water, followed by shaking (280 rpm, 16 h, 20 °C) and centrifugation (10,000 rpm, 10 min). The supernatants were then collected into new centrifuge tubes. In a second step, 40 mL of a 0.11 M acetic acid solution was added to the soil residue and treated in the same manner as in the first step. The supernatants were then filtered through a 0.22 μm PTFE filter (Whatman Puradisc, Sigma-Aldrich, Germany) and analysed for the content of herbicide anions and cations by LC-MS/MS.

3.4. HILs sorption in soil

Soil preparation and adsorption experiments were conducted in accordance with the OECD 106 guidelines [134]. Experiments were carried out for [K][2,4-D] and HILs with 2,4-D anion at concentrations of 1, 10 and 50 mg/L. First, test solution (5 mL) and dried sterile soil (1 g) were placed in PP centrifuge tubes, and then shaken in the dark (240 rpm, 24 h, 20 ± 1 °C) on an orbital shaker. Samples for analysis were collected after 1 min and then after 1, 2, 4, 6, 12 and 24 h. Once the equilibrium was reached, the tubes were centrifuged (10,000 rpm, 10 min). The resulting supernatant was filtered through a 0.22 μm PTFE filter (Whatman Puradisc, Sigma-Aldrich, Germany) and analysed by LC-MS/MS. In addition, blanks were performed by shaking the analysed compounds without substrate (5 mL), 1 g of soil with 5 mL of water and 5 mL of water (without analysed compounds and without soil). All experiments were performed in triplicate. The adsorption efficiency of herbicides in soil was determined using the following formula:

$$removal\ \% = \frac{c_0 - c_e}{c_0} \cdot 100\ \%$$

where: c_0 and c_e – initial concentration and the equilibrium concentration of the herbicide in the solution (mg/L), respectively.

The Langmuir and Freundlich isotherm models were employed in order to calculate the sorption parameters.

a) **The Langmuir model** is described according to following formulas [135]:

$$\frac{c_e}{q_e} = \frac{c_e}{q_{max}} + \frac{1}{bq_{max}}$$

where: q_e – concentration of adsorbed cations or anions in a unit mass of soil, determined by:

$$q_e = \frac{V \cdot (c_0 - c_e)}{m}$$

where: c_0 – initial concentration of cations or anions (mg/L), c_e – equilibrium concentration of cations or anions in solution (mg/L), V – volume of the solution (L), m – mass of the sorbent (kg), q_{max} (mg/kg) and b (L/mg) – maximum amounts of

cations or anions per sorbent's unit mass that forms a complete monolayer on its surface at c_e.

b) **The Freundlich model** describes c_e (mg/L) as the equilibrium concentration of cations or anions that stay unadsorbed and q_e (mg/kg) as the concentration of cations or anions adsorbed on the soil per unit mass. The Freundlich constants n and K_f ($mg^{1-1/n}$ $L^{1/n}/g$) describe the favourability of the adsorption process and adsorption capacity of the sorbent, respectively. The magnitude of the intercept and the slope of the log q_e plot are relative to log c_e:

$$logq_e = \frac{1}{n} logc_e + logK_f$$

3.5. Microorganisms' isolation procedure

The microorganisms isolated within the scope of this doctoral thesis originated from various sites, namely from plant's fyllosphere, endosphere and ryzosphere, as well as from agricultural soil (**Fig. 12**). These were further subjected to procedures aiming at adapting enrichment cultures and pure strains to the presence of herbicides (glyphosate, 2,4-D, dicamba, MCPA), with intention of selection of microorganisms with the ability to degrade selected herbicides. For bioaugmentation studies, the bacteria chosen were these utilising herbicides the most efficiently.

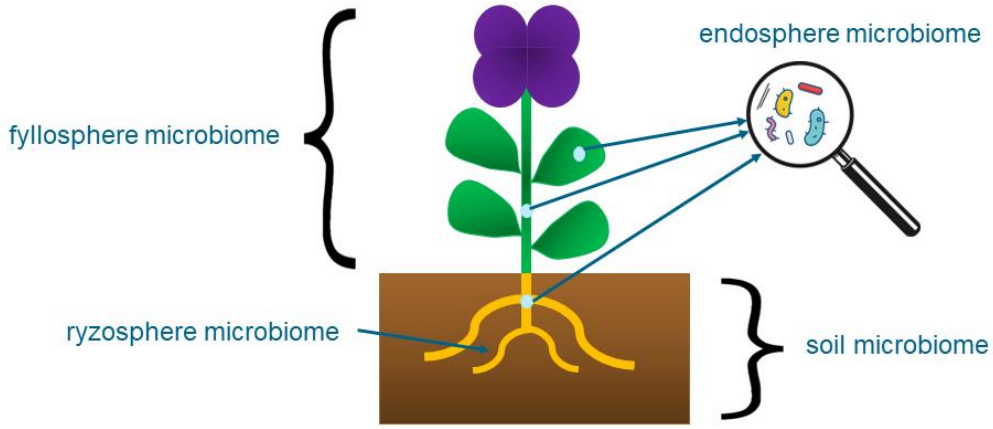


Fig. 12 The origin of microorganisms isolated within the scope of this Ph.D. study.

3.5.1. Microorganisms intended for soil bioaugmentation studies

For the isolation of microorganisms, capable of degrading herbicides and herbicidal ionic liquids, targeted for soil bioaugmentation studies, four agricultural soils with known history of contact with herbicides were used:

- Gorzow Wielkopolski, Poland (N 52.42337, E 15.17374),
- Kamionki_1, Poland (N 52.16467, E 16.59464),
- Kamionki_2, Poland (N 52.16489, E 16.59078),
- Grabienice, Poland (N 52.07309, E 18.03403).

First, soil samples were collected to sterile containers from a depth of 10–20 cm and then sieved through a 1.6 mm sieve [130]. Isolation procedure began within 24 h of sampling.

Herbicides used in the microorganisms isolation along with their concentrations were selected based on the literature data [136–150]. They served as the only source of carbon and energy for the isolated microorganisms. In the course of the experiment, different concentrations (in accordance with literature data [136–150]) were used to increase the chance of obtaining herbicide-degrading strains:

- Glyphosate: 1.00, 0.50, 0.25 g/L,
- 2,4-D: 0.50, 0.25 g/L,
- MCPA: 0.20, 0.10 g/L
- Dicamba: 0.50, 0.25 g/L

The isolation of enrichment cultures was schematically shown in the **Fig. 13**. Briefly, approx. 5 g of soil (wet weight) was placed in a 150 mL sterile Erlenmeyer flask filled with 25 mL of sterile mineral medium (MM) [151] amended with herbicidal substances (H, herbicide) and supplemented with 100 µL of vitamin solution. The microelements solution was sterilised using a 0.22 µm membrane filter (MCE, Mixed Cellulose Ester, Sigma Aldrich, Germany) and added to a cold, sterile MM+H (sterilised in an autoclave, 15 min, 121 °C). The compositions of the solutions used are as follows:

- Mineral medium: 7.0 g/L $Na_2HPO_4 \times 2H_2O$, 2.8 g/L KH_2PO_4, 0.5 g/L NaCl, 1.0 g/L NH_4Cl,
- Microelements solution: 200 mg/L $MgSO_4 \times 7\ H_2O$, 20 mg/L $FeSO_4 \times 7\ H_2O$, 10 mg/L $MnSO_4 \times 4\ H_2O$, 12.8 mg/L ZnCl, 2 mg/L $CaCl_2 \times 6\ H_2O$,

1.2 mg/L $BaCl_2$, 0.72 mg/L $CoSO_4$ ×7 H_2O, 0.072 mg/L $CuSO_4$ × 5 H_2O, 13 mg/L H_3BO_3, 20 mg/L EDTA, and HCl 37 % 0.292 mL/L.

After 7 days of incubation (28 °C, 120 rpm), 1 mL of inoculum was transferred to fresh MM+H. After third transfer, the enriched cultures were stored (–80 °C) in 20 % (v/v) glycerol stocks until used. Additional information are provided in the **Annex**.

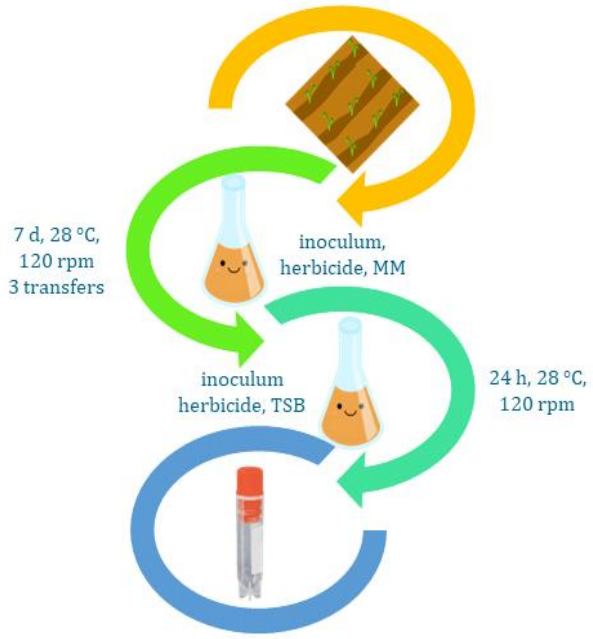


Fig. 13 The schematic procedure of the microorganisms' isolation performed within the scope of this Ph.D. study.

3.5.2. Microorganisms' isolation form plants tissues and rhizosphere

a) Isolation of microorganisms from the rhizosphere

Ten random samples of the soil form plants' rhizosphere were collected with the use of sterile tweezers and homogenised. Next, to a sterile Erlenmeyer flask with 50 % tryptic soy broth (TSB) medium (25 mL), approx. 2 g of soil (wet weight) was added, and then incubated (48 h, 30 °C,). Subsequently, the enrichment cultures were separated from the sediments, centrifuged (4500 rpm, 15 min, 4 °C) and stored in 20 % (v/v) glycerol stocks (–80 °C) until molecular analyses.

b) Isolation of microorganisms from plant surface (epiphytes) and interior (endophytes)

First, plants were washed thoroughly with tap water for 10–15 min in order to remove soil particles [152], and then cut into pieces of 2–4 cm (leaves, shoots and roots separately). From there, all operations were carried out under sterile conditions. Plant material of approx. 1 g was placed in a sterile crystalliser filled with sterile deionised water, and stirred for 1 min. In order to secure epiphytes, 1 mL of leachate was placed in a sterile Erlenmeyer flask with 50 % TSB medium (25 mL), incubated (48 h, 30 °C), centrifuged (4500 rpm, 15 min, 4 °C) and stored in 20 % (v/v) glycerol stocks (–80 °C) until molecular analyses.

Subsequently, the washed plant material from crystalliser was divided into three equal parts of approx. 0.3 g. Each part (separately) was then immersed in 70 % ethanol (30 s), sodium hypochlorite solution (1 %, v/v) with the addition of Triton X-100 surfactant (2 min), and again in 70 % ethanol (30 s). Then, plants were washed five times with sterile, deionised water. Next, plant tissues were grinded in a sterile mortar filled with small amount of sterile, deionised water. Finally, 1 mL of mixture after maceration was placed in a sterile Erlenmeyer flask with 50 % TSB medium (25 mL), incubated (72 h, 30 °C), centrifuged (4500 rpm, 15 min, 4 °C) and thus isolated endophytes were stored in 20 % (v/v) glycerol stocks (–80 °C) until molecular analyses.

Before endophytes isolation, sterility of plant's surface was evaluated. In order to do that, 100 μL of water from the last wash was inoculated on a tryptic soy agar plate (TSA). If the plates were free of microorganisms after 72 h, the plant surface was deemed properly sterilised. All assays were performed in triplicates.

3.6. Mineralisation experiments

3.6.1. Modified OECD 301 F procedure

The modified OECD 301 F procedure was applied in experiments evaluating the degradation of HILs based on glyphosate and 2,4-D anions in variants with bioaugmentation (B) and without bioaugmentation (NB). Shortly, previously isolated enrichment cultures were incubated (120 rpm, 28 °C). After three transfers to a fresh medium (MM+H), precultures were transferred to a sterile TSB medium (1 L)

amended with glyphosate (1 g/L) or 2,4-D (0.5 g/L) (TSB+H). After incubation (120 rpm, 72 h, 28 °C), the biomass was washed with sterile 0.85 % (v/v) NaCl solution three times, centrifuged (4500 rpm, 15 min, 4 °C) and resuspended in 0.85 % NaCl solution.

The mineralisation experiments were conducted in sealed glass bottles (volume of 1 L) containing non-sterile agricultural soil (100 g). The soil prior to experiment was sieved through a 1.6 mm sieve and mixed with either 15 or 20 mL of aqueous solution to ensure optimum moisture content, volumes depending on the HILs examined. The solution added to soil were as follow:

a) HILs based on glyphosate: 18 mL of HIL* and 2 mL of sterile 0.85 % (v/v) NaCl (non-bioaugmented approach, NB), 18 mL of HIL and 2 mL of inoculum suspended in sterile 0.85 % (v/v) NaCl (bioaugmented approach, B), 18 mL of deionised water and 2 mL of sterile 0.85 % (v/v) NaCl (control NB), 18 mL of deionised water and 2 mL of inoculum suspended in sterile 0.85 % (v/v) NaCl (control B).

b) HILs based on 2,4-D: 10 mL of HIL*, 3 mL of sterile 0.85 % (v/v) NaCl and 2 mL of N/P solution (approach NB), 10 mL of HIL, 3 mL of inoculum suspended in sterile 0.85 % (v/v) NaCl and 2 mL of N/P solution (approach B), 10 mL of deionised water, 3 mL of sterile 0.85 % (v/v) NaCl and 2 mL of N/P solution (control NB), 10 mL of deionised water, 3 mL of inoculum suspended in sterile 0.85 % (v/v) NaCl and 2 mL of N/P solution (control NB). The N/P solution served as solution biostimulating microbial growth and its concentration was established experimentally: 95.75 g/L NH_4NO_3, 119.15 g/L KNO_3, 28.12 g/L K_2HPO_4.

*The concentration of HILs in the soil were set as 1 g of active ingredient/1 kg soil for glyphosate and 0.5 of active ingredient/1 kg soil for 2,4-D. HILs active ingredient concentrations were significantly higher than the field concentrations of respective herbicides (up to 1080 g/ha for glyphosate and up to 400 g/ha for 2,4-D), as these were established experimentally to be sufficient to detect differences in their degradation.

The CO_2 traps filled with 0.75 M NaOH solution (10 mL in 20 mL vials) were then positioned inside each bottle to study the evolution of CO_2. The mineralisation extent was determined in accordance with Wader titration of NaOH and Na_2CO_3

solutions from CO_2 traps with HCl (0.1 M), with the use of an automatic titrator (Metrohm titroprocessor 686, Herisau, Switzerland). After each measurement, the vials were rinsed with deionised water, dried and refilled with fresh NaOH solution. The control samples were used to investigate background soil respiration (without added compounds). The microcosms were incubated at 20 ± 2 °C for 12 weeks. All assays were performed in triplicate.

3.6.2. ^{13}C-labelling approach

a) Liquid microcosms

Liquid microcosms were set up in sterile 200 mL flasks (Glasgerätebau Ochs, Germany). Each flask was filled with 55 mL of sterile mineral medium. The activated sludge used for biodegradation tests was obtained from the municipal wastewater treatment plant in Szamotuly, Poland (N 52.37415, E 16.35132) and was washed three times in MM before use. The initial cell density in samples was set at approx. 10^6 cells/mL. Prior to experiment, rubber butyl stoppers (Glasgerätebau Ochs, Germany) and aluminium crimps (VWR, Germany) were autoclaved, and were then used to hermetically seal the flasks. The oxygen content was determined with the use of Fibox 3 Trace oxygen meter (PreSens Precision Sensing GmbH, Germany) with an oxygen-sensitive optode spots (SP-PSt3, PreSens Precision Sensing GmbH, Germany).

Microcosms were supplemented with tested compounds with the use of a glass chromatographic syringe. The following setups were analysed: [C_{12}-BA][MCPA], [C_{12}-BA*][MCPA], [C_{12}-BA][MCPA*] (approx. 1.7 mg/mL) and [C_{12}-BA][Br] and [C_{12}-BA*][Br] (approx. 1.3 mg/mL). Supplementary information provided in the **Annex**. Liquid microcosms were then incubated in semi-dark room (150 rpm, 20 °C). Flasks containing MM and 1) activated sludge without tested compounds and 2) tested compound and without activated sludge, served as biotic and abiotic controls, respectively. All assays were performed in triplicate. Samples were then analysed for CO_2 carbon isotope ratio after 0, 5, 10, 20, 30, 40 and 73 days of incubation. Additionally, mineralization of [C_{12}-BA*][MCPA] and [C_{12}-BA][MCPA*] at lower ^{13}C concentrations were investigated within short-term experiment (100 h). Spiking was performed at the same concentrations, but ^{13}C-labelled compounds were diluted 1:9 (v/v) with unlabelled compounds.

b) Soil microcosms

Microcosms with agricultural soil (40 g) were set up in sterile 200 mL flasks (Glasgerätebau Ochs, Germany). Agricultural soil with known history of contact with herbicides was collected from a seasonally cultivated field in the Agricultural Experimental Station of the Plant Protection Institute in Winna Gora, Poland (N 52.12295, E 17.26102). The soil was then analysed (70 % sand, 30 % silt) and classified as sandy loam or silty loam. Other determined parameters include: soil organic matter (loss on ignition 19.6 ± 1.1 mg/g dry matter d.m.); porosity (317 ± 19 µL/g d.m.); field moisture (water/dry soil 103.6 ± 1.8 µL/g d.m.; water/wet soil 93.8 ± 1.4 µL/g wet matter w.m.); water holding capacity (water/dry soil 192.9 ± 9.3 µL/g d.m.). After filling the flasks with soil, they were hermetically sealed, and oxygen concentrations were determined as described for liquid microcosms.

Microcosms were supplemented with tested compounds with the use of a glass chromatographic syringe. The following setups were analysed: [C_{12}-BA][Br], [C_{12}-BA*][Br], [C_{12}-BA][MCPA], [C_{12}-BA*][MCPA], [C_{12}-BA][MCPA*] (approx. 100 µmol/kg). Supplementary information provided in the **Annex**. Soil microcosms were then incubated in semi-dark room (20 °C). Flasks containing 1) agricultural soil without tested compounds and 2) oven-sterilised agricultural soil (105 °C, 6 h) with tested compounds (sterility verified by plating) served as biotic and abiotic controls, respectively. All assays were performed in triplicate. Samples were then analysed for CO_2 carbon isotope ratio after 0, 5, 10, 20, 30, 40 and 73 days of incubation. Additionally, mineralization of [C_{12}-BA*][MCPA] and [C_{12}-BA][MCPA*] at lower ^{13}C concentrations were investigated within short-term experiment (260 h). Spiking was performed at the same concentrations, but ^{13}C-labelled compounds were diluted 1:9 (v/v) with unlabelled compounds.

c) Measurements of CO_2 carbon isotope ratios ($^{13}C/^{12}C$)

The protocol used for sampling during the experiment was as follow. First, a 10 mL headspace vial was flushed with nitrogen and instantly sealed with an aluminium crimp with a PTFE-coated septum. Next, with the use of 10 mL syringe, gas volume of 10 mL was withdrawn from the vial. Then, 10 mL of microcosms' headspace was withdrawn in the same manner, transferred to a headspace vial, and stored (5 °C) until analysis. Subsequently, the microcosms after sampling were filled

with 10 mL of CO_2-free air passed through a sterile 0.2 μm PTFE syringe filter. If necessary, pure oxygen was used to resupply the cultures. Samples were collected after 0, 5, 10, 20, 30, 40 and 73 days of incubation. On day 0, samples were collected immediately after microcosms' preparation (*i.e.*, approx. 2 h after spiking). In the case of short-term experiments, CO_2 carbon isotope ratios $^{13}C/^{12}C$ were measured by injecting headspace directly into the instrument. Samples were collected every 4 h on the first day of incubation and then once a day until the end of the experiment.

Carbon isotope ratios $^{13}C/^{12}C$ of CO_2 evolved from the samples were determined using a gas chromatography–combustion–isotope ratio mass spectrometry (GC-C-IRMS) instrument which consisted of a gas chromatograph (GC; Agilent 7890, CA, USA) connected to an isotope ratio mass spectrometer (IRMS; Thermo Fisher Scientific MAT 253, Waltham, MA, USA) *via* combustion unit (C; Thermo Fisher Scientific, Waltham, MA, USA). Depending on the expected CO_2 concentration, 100 to 1000 μL samples were manually injected into a GC (250 °C, split ratio 1:5) using a gas-tight syringe (Hamilton, USA). Carbon dioxide chromatographic separation was performed isothermally at 45 °C using a PoraPLOT Q column (25 m, inner diameter 0.32 mm, 10 μm film) at a flow rate of 2 mL/min with helium as carrier gas. The carbon isotope ratios ($^{13}C/^{12}C$) expressed in the $\delta^{13}C$ notation relative to the Vienna Pee Dee Belemnite (V-PDB) standard [153] (**Eq 1**):

$$\delta^{13}C[‰] = \frac{R_S}{R_{Std}} - 1 \quad (1)$$

where: R_S and R_{Std} – $^{13}C/^{12}C$ ratios in the sample and in the international standard, respectively. Values were reported mostly in per mil (‰) due to small variations in natural isotope abundance. Samples were measured in triplicate.

d) Mineralisation determination

The total headspace CO_2 concentration [ppm] was calculated based on the peak area at 0 h and an assumption that CO_2 concentration was 402.8 ppm at that time (http://www.esrl.noaa.gov/gmd/ccgg/trends/). Based on the $\delta^{13}C_{CO_2}$ value, the percentage of $^{13}CO_2$ was calculated [154] (**Eq 2**):

$$atom\ \%\ ^{13}C = \frac{[^{13}CO_2]}{[^{12}CO_2]+[^{13}CO_2]} \cdot 100 = \frac{I_{45}}{I_{44}+I_{45}} \cdot 100 \quad (2)$$

where: I_{44} and I_{45} – peaks intensities at *m/z* 44 and 45, respectively.

The abundance of ^{13}C in CO_2 was determined by measuring the ionic intensity at m/z 44 and 45. If R' = I_{44}/I_{45} then (**Eq 3**):

$$atom\ \%\ ^{13}C = \frac{100}{R\prime+1} \quad (3)$$

To convert $\delta^{13}C_{V\text{-}PDB}$ to atom % (AP) ^{13}C (**Eq 4**):

$$AP^{13}C = \frac{100}{\frac{1}{\left(\frac{\delta^{13}C}{1000}+1\right)\cdot R_{V-PDB}}+1} \quad (4)$$

where: $\delta^{13}C$ – measured $\delta^{13}C_{CO_2}$-value, $R_{V\text{-}PDB}$ – carbon isotope ratio ($^{13}C/^{12}C$) of V-PDB with 0.0111802 [155].

The headspace concentration of $^{13}CO_2$ [ppm] derived from the tested compounds was calculated based on the multiplication of the atomic %^{13}C at 0 h (considered as the background level of ^{13}C) and the total CO_2 concentration (**Eq 4**). The concentration of $^{13}CO_2$ from HIL dissolved in the aqueous phase [nmol/mL] was then calculated using Henry's law (**Eq 5**):

$$p = k_H c \quad (5)$$

where: p – the content of $^{13}CO_2$ derived from HIL dissolved in water phase [nmol/mL], k_H = 0.0345 [mol/m^3 Pa] [156], c – mole fraction.

The $[H^{13}CO_3]^-$ derived from the HIL concentration [nmol/mL] was then calculated from the dissolved $^{13}CO_2$ derived from the HIL concentration in water (pK_a 6.37) (**Eq 6**). The concentration of $[CO_3]^{2-}$ (pH 7) was very low and therefore neglected.

$$[H^{13}CO_3]^- = \frac{pK_a}{[H^+]} \quad (6)$$

In order to calculate the amount of ^{13}C obtained from HIL in the headspace [nmol], the concentration of ^{13}C obtained from HIL in the headspace was divided by the headspace volume (140 mL = 6.25 mmol). To calculate the total amount of $^{13}CO_2$ and $[H^{13}CO_3]$ derived from HIL dissolved in the aqueous phase, their concentrations were multiplied by the volume of the aqueous phase (60 mL). Thus, the total amount of ^{13}C mineralisation products of HILs could be calculated as the sum of all of the mentioned above [nmol]. Lastly, it was then possible to calculate the mineralisation extent [%] based on the initial amount of ^{13}C-labelled compound [157,158] (**Eq 7**):

$$mineralization\ [\%] = \frac{total\ amount\ of\ ^{13}C\ \text{products of HIL}}{initial\ ^{13}C\ content} \quad (7)$$

where the initial content of ^{13}C was 3600 nmol.

To compare kinetics of mineralisation with published values, a non-linear regression analysis of cumulative mineralisation curves was performed using the SigmaPlot version 11 (Systat Software, Inc., San Jose, CA, USA). Mineralisation data with values greater than zero were fitted using a first-order exponential growth model, which was chosen due to the fact that it showed better F-test results ($p < 0.05$) compared to the zero-order or logarithmic kinetic models (data not shown).

3.7. Primary biodegradation experiments

a) QTS based on MCPA and dicamba

The microorganisms for the biodegradation experiments [4] were isolated from agricultural soil with known history of herbicidal applications, used in other study [2]. Biodegradation studies were conducted in glass bottles (1 L) filled with microorganisms suspension in mineral medium, MM (100 mL, 10^6 CFU/mL, pH 7), in accordance with procedure described previously [128]. Briefly, approximately 50 mg/L of QTS were added to MM and incubated (120 rpm, 28 days, 25 ± 3 °C). Suitable abiotic (MM with the QTS, without microorganisms), biotic (MM with microorganisms, without the QTS) and inactivated (autoclaved MM with microorganisms and the QTS) control samples were prepared. All assays were performed in triplicate. Biodegradation was assessed using UltiMate 3000 Dionex liquid chromatography coupled with API 4000 QTRAP Applied Biosystems mass spectrometer (Thermo Fischer Scientific, Waltham, MA, USA).

b) HILs based on glyphosate

After 28 and 90 days of mineralisation experiment, the soil samples were analysed for the content of HILs' cations and anions. The samples for analyses were obtained *via* two-step extraction. First, soil samples were thoroughly mixed in bottles, and then masses of approx. 5 g were weighed into a 50 mL centrifuge tubes. Then, 10 mL of deionised water was added and vortexed (10 s), followed by shaking (320 rpm, 30 min) and centrifugation (10,000 rpm, 15 min). Subsequently, the extracts were filtered through quantitative strainers and transferred to a new centrifuge tube

(50 mL). Next, the aliquots of 10 mL of deionised water were added to respective soil sediments, followed by shaking (320 rpm, 15 min), centrifugation (5,000 rpm, 15 min) and decantation through quantitative filter. Next, for cation's extraction 10 mL of methanol (99.9 %) with 1 mL of 0.1 M hydrochloric acid were added to soil sediments and extraction procedure was performed as described above. Then, the extracts were combined, filtered through a 0.22 μm PTFE filter (Advantec, Tokyo, Japan) and stored (4 °C) prior to chromatographic analysis. The LC-MS/MS analysis was conducted using an UltiMate 3000 RSLC chromatography system (Dionex, Thermo Fischer Scientific, Waltham, MA, USA) coupled with an API 4000 QTRAP triple quadrupole mass spectrometer with electrospray ionisation (ESI) (AB Sciex, Foster City, CA, USA) in the positive mode (LC-MS/MS). The extraction method was previously validated by extracting the whole samples in order to verify the representativeness of a 5 g sample. The ions recoveries were as follow (**Table 3**). All assays were performed in triplicate.

Table 3 Ions' recoveries after extraction from soil.

Compound	Recovery [%]	
	Cation	Anion
[K][Glyph]	[-]	101.5 ± 0.85
[Chol][Glyph]	101.60 ± 1.51	97.55 ± 0.93
[C_{12}Chol][Glyph]	99.65 ± 2.62	97.95 ± 1.49
[C_{16}TMA][Glyph]	100.15 ± 0.64	100.05 ± 0.34
[BA][Glyph]	98.14 ± 0.76	100.4 ± 0.43
[DDA][Glyph]	98.75 ± 0.92	98.55 ± 0.49

c) HILs based on 2,4-D

After 28 and 90 days of mineralisation experiment, the soil samples were analysed for the content of HILs' cations and anions. First, soil samples from the test bottles were thoroughly mixed and then masses of approx. 2 g were weighed to centrifuge tubes (15 mL). Next, hydrochloric acid (0.5 mL, 0,1 M) and acetonitrile (5 mL) were added to the respective samples, followed by vortexing (10 s), homogenisation (30 min, ultrasound bath) while cooling, and centrifugation (10,000 rpm, 5 min). The extracts were then filtered through a 0.22 μm PTFE filter (Advantec, Tokyo, Japan) into new centrifuge tubes (15 mL). Next, the solutions of HCl (0.5 mL, 0.1 M) and acetonitrile (5 mL) were added to the soil sediments and the process was repeated. Both extracts were then combined and stored (4 °C) prior to chromatographic

analysis. The LC-MS/MS analysis was conducted using an UltiMate 3000 RSLC chromatography system (Dionex, Thermo Fischer Scientific, Waltham, MA, USA) coupled with an API 4000 QTRAP triple quadrupole mass spectrometer with electrospray ionisation (ESI) (AB Sciex, Foster City, CA, USA) in the positive mode (LC-MS/MS). The extraction method was previously validated by extracting the whole samples in order to verify the representativeness of a 2 g sample. The ions recoveries were as follow (**Table 4**). All assays were performed in triplicate.

Table 4 Ions' recoveries after extraction from soil.

Compound	Recovery [%]	
	Cation	Anion
[K][2,4-D]	[-]	103.98 ± 2.03
[Chol][2,4-D]	98.36 ± 0.99	104.15 ± 4.83
[C_{12}Chol][2,4-D]	100.08 ± 0.98	107.09 ± 7.17
[Bet][2,4-D]	105.79 ± 5.44	103.38 ± 2.54
[C_{12}Bet][2,4-D]	100.54 ± 2.08	99.34 ± 0.45
[CAPBet][2,4-D]	102.15 ± 0.85	99.53 ± 1.02
[Car][2,4-D]	102.69 ± 1.73	99.24 ± 1.43
[TMA][2,4-D]	101.34 ± 1.76	98.23 ± 0.69
[BTMA][2,4-D]	101.96 ± 0.57	100.42 ± 0.92
[TBA][2,4-D]	98.47 ± 0.96	97.86 ± 0.74

3.7.1. ^{13}C-labelling approach

a) Liquid microcosms – ion removal assessment

Immediately after each headspace sample collection for mineralisation measurement, a liquid phase subsample (1 mL) was collected using a disposable syringe and transferred to a 1.5 mL centrifuge tube or glass vial in the case of a sterile controls without biomass. The samples were then centrifuged (4500 rpm, 15 min, 4 °C) stored frozen before analysis. After thawing, samples were diluted 50-fold with water and then 10-fold with a mixture of water and methanol (1:4, v/v). This dilution resulted in a final cation and anion concentrations of 0.2 nmol/mL. Samples were then analysed by HPLC-MS/MS in order to determine cations and anions. For quantification, the same solutions that were used for the microcosms' spiking were diluted accordingly and used as external standards. The recoveries of cations and anions from the sterile controls were quantitative so it was confirmed that no abiotic degradation had occurred.

b) Soil microcosms – ion removal assessment

Since soil subsamples collection was not possible in a way that does not disturb the microcosm, parallel microcosms were prepared and frozen on sampling days. The following procedure was used to extract the test compounds from the soil. The samples were thawed, anhydrous ethanol was added (35 mL), and then the samples were shaken vigorously and sonicated in a water bath (10 min, 40 °C). Next, the samples were cooled to ambient temperature and allowed for sedimentation. Extracts were transferred to centrifuge tubes (50 mL) and centrifuged (4500 rpm, 5 min) and the supernatants were transferred to 100 mL volumetric flasks. The process was performed in triplicate and the extracts were then combined. The remaining soil sediments in the centrifuge tubes were extracted with anhydrous ethanol (2.5 mL). Finally, all extracts were combined, and the volume was brought to 100 mL with anhydrous ethanol. An aliquot of the extract was passed through a 0.45 μm PTFE syringe filter (Whatman International Ltd., Maidstone, U.K.) and collected in a 10 mL glass vial. For the determination of residual anions and cations, an aliquot of the extract was diluted 40-fold with anhydrous ethanol and then 5-fold with a mixture of water and anhydrous methanol (1:4, v/v), resulting in final cation and anion concentrations of 0.2 nmol/mL. For quantitative analysis, the experimental [C_{12}-BA][MCPA] solution at 10 %, 50 % and 100 % of the original content (volumes of 12, 60 and 120 μL, respectively) was added to six standard samples (4 g of field-moist soil). These samples were then subjected to the extraction protocol and analysed. Standard solutions prepared by appropriate dilution were used to compare and calculate extraction efficiencies. After 73 days, the recoveries of cations and anions from the sterile soil microcosms were quantitative, confirming the sterility of the microcosms and the suitability of the developed sample preparation protocol. Supplementary information provided in the **Annex**.

c) HPLC-MS/MS characteristics

The UltiMate 3000 RSLC chromatographic system from Dionex (Sunnyvale, CA, USA) was used. The sample of 2 μL were injected onto an octadecyl Hypersil GOLD column (100 mm × 2.1 mm inner diameter, 1.9 μm) from Thermo Fischer Scientific (Waltham, MA, USA) using a 2.1 mm inner diameter, filter cartridge (0.2 μm) from the same supplier. The mobile phase used in the analysis consisted of

5 mM ammonium acetate in water and methanol at a flow rate of 0.2 mL/min. The analysis was carried out using a gradient elution where the percentage of organic modifier was increasing from 85 % to 100 % over 2 min and then held at 100 % for 2 min. The eluent from the liquid chromatography column was delivered to an API 4000 QTRAP triple quadrupole mass spectrometer from AB Sciex (Foster City, CA, USA) *via* an electrospray ionisation source. The source operated in positive ion mode for cation detection and in negative ion mode for anion detection. The dwell time was set to 200 ms for each mass transition detected in MS/MS multiple reaction monitoring mode. The mass spectrometer settings were as follow: curtain gas 10 psi; nebuliser gas 40 psi; auxiliary gas 40 psi; temperature 400 °C and collision gas medium; atomisation voltage of 4500 V for cations and -4500 V for anions; inclination potential of 50 V for cations and -50 V for anions.

3.7.2. ^{13}C – determination of metabolites

A protocol for the extraction and derivatisation of the selected analytes (as shown in **Fig. 20**, **Section 4**) was proposed [2]. The experiments carried out with the standard solutions of the selected analytes (Sigma Aldrich, Germany) are briefly described below. All analytes were converted to trimethylsilyl (TMS) derivatives, with the sole exception of benzaldehyde (99 %), which was first converted to oxime by reaction with hydroxylamine (99 %). In order to examine the derivatization reactions and to obtain derivatives' mass spectrometry (MS) spectra, each analyte was dissolved in dichloromethane (99.5 %). Then, solution's aliquot of 10 µL was evaporated to dryness in a glass insert under a gentle stream of nitrogen. In the case of benzaldehyde, the oxime derivative was prepared by reacting benzaldehyde with hydroxylamine in a water-ethanol mixture (60 min, 60 °C) under alkaline conditions, after which the product was extracted with dichloromethane (99.5 %). Subsequently, 50 µL of BSTFA (*N*,*O*-bis(trimethylsilyl)trifluoroacetamide (99 %) was added prior to sample incubation (60 min, 60 °C). Finally, prepared samples were analysed by GC×GC-TOF-MS.

a) Liquid microcosms

The previously frozen samples were thawed and 800 µL of supernatant was transferred to a 1.5 mL GC vial. The sample was alkalised (pH 11) by adding 10 µL

of NaOH (5 M). Then, 20 µL of aqueous hydroxylamine hydrochloride solution (50 mg/mL) was added, vortexed and incubated (1 h, 60 °C). Amines and neutrals were extracted according to following procedure. Samples were extracted with three 500 µL aliquots of diethyl ether (99 %), followed by extraction with 500 µL of *n*-pentane (99 %). The fractions were then collected in a 4 mL glass vials. Acids and phenols were extracted according to following procedure. The sample was acidified (pH 2) by the addition of 12.5 µL of concentrated hydrochloric acid (36.5 %), followed by sample extraction with three 500 µL aliquots of diethyl ether (99 %) followed by extraction with 500 µL of *n*-pentane (99 %). These fractions were combined with the previously collected fractions in a vial, the extracts were dried with a small amount of anhydrous Na_2SO_4 and then concentrated to a volume of approx. 200 µL under a gentle stream of nitrogen (ambient temperature). The extract was collected using a Pasteur pipette and transferred to a 400 µL glass insert placed in a 1.5 mL GC vial, and then combined with the washings from remaining desiccant's rinsing with two 100 µL aliquots of diethyl ether (99 %). The extract was then evaporated to dryness under a gentle stream of nitrogen (at ambient temperature). Next, silylation of amines, oximes, acidic and phenolic groups was performed by 50 µL of BSTFA addition, followed by samples' shaking and incubation (30 min, 60 °C) and direct analyses by GC×GC-TOF-MS.

b) Soil microcosms

Volumes of 1.5 mL of the extracts from the previously prepared samples were transferred to 1.5 mL GC vials. The carbonyl compounds conversion into oximes was conducted. The sample was alkalised (pH 11) by adding 30 µL of NaOH (1 M) solution in ethanol, followed by addition of 50 µL of hydroxylamine hydrochloride (50 mg/mL) solution in ethanol, vortexing and incubation (1 h, 60 °C). The extract was concentrated to approx. 200 µL under a gentle stream of nitrogen (at ambient temperature), then collected with a Pasteur pipette and transferred to a 400 µL glass insert placed in a 1.5 mL GC vial, and then combined with the washings from remaining vial's rinsing with two 100 µL aliquots of diethyl ether. The extract was then evaporated to dryness under a gentle stream of nitrogen (at ambient temperature). Finally, silylation of amines, oximes, acidic and phenolic groups was performed by

50 µL of BSTFA addition, followed by samples' shaking and incubation (30 min, 60 °C) and direct analyses by GC×GC-TOF-MS.

c) GC×GC-TOF-MS analysis

Comprehensive two-dimensional gas chromatography coupled with time-of-flight mass spectrometry (GC×GC-TOF-MS) using an Agilent 7890 gas chromatograph and LECO Pegasus 4D TOF-MS mass spectrometer (CA, USA) was performed in order to analyse the samples.

Column setup: first dimension SGE BPX5, 30 m, 250 µm, 0.25 µm; second dimension Restek RXI-17, 0.95 m (10 cm in modulator, 74 cm in secondary oven, 21 cm in the transfer line), 100 µm, 0.1 µm, split/splitless injector operating at 250 °C in splitless mode, 1 µL injected, split vent open after 40 s.

Separation conditions: carrier gas (helium) flow constant at 1 mL/min; main oven program: 60 °C for 1 min, ramped up to 280 °C (4 °C/min) and held for 20 min; secondary oven program: 65 °C for 4 min, ramped up to 290 °C (4 °C/min), modulator temperature set to 25 °C above secondary oven temperature, modulation period 3 s.

Detection conditions: ion source temperature 250 °C, ion source voltage 70 eV, solvent delay 400 s, acquisition range 25–500 m/z, acquisition rate 150 spectra/s.

3.8. Evaluation of HILs' toxicity towards microorganisms and plants

3.8.1. Half maximal effective concentration (EC_{50})

The half maximal effective concentration (EC_{50}) tests were performed for the enrichment cultures used in degradations studies. The respective enrichments cultures were transferred from glycerol (20 %, v/v) to tryptic soy broth (TSB, 50 %) medium (Sigma Aldrich, Poland), and incubated (24 h, 28 °C). After third transfer of biomass, a cell suspension was prepared to obtain optical density of $OD_{600} = 0.100 \pm 0.010$. Next, 200 µL of microbial suspension was placed in a sterile 96-well plate and incubated (120 rpm, 3 h) to reach exponential growth stage. Subsequently, a volume of 50 µL of tested compounds was added to respective wells and incubation was continued for 5.5 h. The concentrations were calculated for active substance (glyphosate or 2,4-D anions) content of 1, 5, 10, 50, 100, 250, 500, 1000 mg/L. The solutions of compounds lacking microorganisms served as abiotic controls, while

microorganisms without the analysed compounds were used as biotic controls. Finally, EC_{50} values were determined [159]. All assays were performed in triplicate.

3.8.2. Minimum inhibitory concentration (MIC) and minimum bactericidal/fungicidal concentration (MBC/MFC)

Antimicrobial activity tests were performed using the following model organisms:

a) Gram-negative bacteria: *Pseudomonas putida* KT2440 [1,4], *Pseudomonas aeruginosa* PAO1 [1], *Escherichia coli* K-12 [1],
b) Gram-positive bacteria: *Staphylococcus aureus* ATCC 6538 [1], *Bacillus cereus* ATCC 11778 [1,4],
c) Fungal species: *Candida albicans* ATCC 10231 [1].

The antimicrobial activity of the test substances was determined by the microdilution method according to EUCAST (European Committee on Antimicrobial Susceptibility Testing) guidelines [160], according to the following procedure. Respective microorganisms were transferred from glycerol (20 %, v/v) to TSB medium (50 %) and incubated (24 h, 30 °C). After three biomass transfers, a cell suspension was prepared to obtain an optical density of OD_{600} = 0.100 ± 0.010, corresponding to 10^6 CFU/mL. The suspension was then diluted 1:50, yielding 2×10^4 CFU/mL. Then, in a sterile 96-well plate, 100 µL of tested compound water solutions was placed in the first row. Subsequently, 50 µL of 50 % TSB was added to all other wells in order to perform serial dilution. Next, 200 µL of microbial suspension in medium with resazurin solution was added.

Tested compounds concentrations were as follow:

a) **QTS based on dicamba and MCPA**: 0.00078, 0.00156, 0.00313, 0.00625, 0.0125, 0.025, 0.05, 0.1, 0.0156, 0.0313, 0.0625, 0.125, 0.25, 0.5, 1, 2 mM [4],
b) **[Bet][2,4-D], [C_{12}Bet][2,4-D], [CAPBet][2,4-D]:** 1, 5, 10, 50, 100, 250, 500, 1000 mg/L [1].

The solutions of compounds lacking microorganisms and with resazurin addition served as abiotic controls, while microorganisms with resazurin but without the analysed compounds were used as biotic controls. Plates were when incubated at

30 °C for 24 h. After incubation, MIC, MBC and MFC parameters were determined. All assays were performed in triplicate.

3.8.3. Effect on the germination and early development of plants

The effect of selected HILs on germination and early plants' development was determined for corn (*Zea mays*), cornflower (*Centaurea cyanus*) and spring barley (*Hordeum vulgare*). Studies were performed using a phytotoxicity test based on the international standard ISO-11269-2:2003 [161]. Shortly, 100 g of soil was applied to Phytotoxkit containers (Tigret, Belgium), and then appropriate amounts of tested compounds dissolved in water (25 mL) were added so that their effective concentrations were:

a) QTS based on dicamba and MCPA: 0.001, 0.0024, 0.02 and 0.1 mmol/kg d.w.s. [4],

b) [Bet][2,4-D], [C_{12}Bet][2,4-D], [CAPBet][2,4-D]: 1, 10, 50 and 100 mg/kg d.w.s [1].

Solutions consisting of the appropriate sodium salt of the herbicide (MCPA, dicamba, 2,4-D) at the same concentrations were used as reference samples. Ten seeds of corn, cornflower or spring barley were then placed on such prepared soil in Phytotoxkits containers and kept in the dark at 25 ± 1 °C for 7 days. At the end of the experiment, the number of germinated seeds was counted and root length and shoot height were measured. All experiments were conducted in triplicate.

After the germination tests were completed, the effect of herbicides on the inhibition of plant root growth was evaluated. Based on the results, the germination index (GI) was calculated according to the equation [45]:

$$GI = \frac{G_S}{G_C} \cdot \frac{L_S}{L_C} \cdot 100\ [\%]$$

where: G_S, G_C – numbers of seeds germinated in the sample and control, respectively; L_S, L_C – roots lengths in the sample and control, respectively.

3.9. Assimilation of ^{13}C into the biomass

a) Assimilation of ^{13}C-labelled $[C_{12}\text{-BA*}]^+$ and $[MCPA*]^-$ into the biomass

Upon the end of the mineralisation experiments, the assimilation of the tested compounds in the biomass was assessed. Therefore, phospholipid fatty acids (PLFAs), which constitute cell membranes, were extracted, derivatised by alkaline methanolysis and analysed by GC-C-IRMS. The sample preparation protocol was based on the modified works of Bombach et al., 2010 [162] and Bligh and Dyer, 1959 [163]. All glassware was washed with acetone (99.5 %), dried under a fume hood, wrapped in aluminium foil and heated (4 h, 450 °C). Only PTFE screw caps were used, prior to use washed with acetone, dried and stored wrapped in aluminium foil. Acetone-cleaning step was omitted in case of disposable materials (Pasteur pipettes, GC vials). The phosphate buffer (KH_2PO_4/Na_2HPO_4, 0.05 M) was prepared freshly before use as follow. A solution of Na_2HPO_4 (8.7 g/L of bidistilled water) was adjusted to pH 7.4 with a few drops of concentrated hydrochloric acid (36.5 %), passed through a 0.2 µm filter (Whatman International Ltd., Maidstone, UK) and collected in a screw-cap flask. Then, $CHCl_3$ (50 mL) was added to the flask and the solution (after gentle shaking) was left overnight (4 °C) for phase separation.

b) Liquid microcosms

In order to suspend biomass, flasks containing liquid microcosms were vortexed, then stoppers were removed, the cultures (approx. 30 mL) were centrifuged in a 50 mL centrifuge tubes (6000 rpm, 15 min), and the supernatants were discarded. The same procedure was repeated for the cultures' remaining volumes. Both biomass sediments were vortexed with phosphate buffer (2 mL), combined in a 45 mL glass vial with a PTFE cap, followed by addition of methanol (10 mL), chloroform (5 mL) and vortexing (5 min). Then, the vials were sonicated (6 min) and shaken overnight (100 rpm, 30 °C). Next, chloroform (98 %, 5 mL) and nanopure water (5 mL) were added, the samples were vortexed (2 min) and centrifuged (2000 rpm, 20 min, 4 °C). The organic layer was collected using a Pasteur pipette and transferred to a 10 mL glass vial with a PTFE cap. The solvent was then evaporated to dryness under a gentle stream of nitrogen and derivatisation was conducted.

c) Soil microcosms

A modified protocol proposed by Bossio and Scow, 1998 [164] was conducted as follow. Moist soil samples (4 g) from the microcosms were transferred to 10 mL glass vials, followed by phosphate buffer (1.5 mL, 0.05 M) addition and vortexing (1 min). Then, anhydrous methanol (2.5 mL) and anhydrous chloroform (2.5 mL) were added, followed by vortexing (1 min). The samples were then sonicated (6 min), shaken (100 rpm, 2 h, 30 °C) while vortexing for 30 s every 30 min and centrifuged (2000 rpm, 15 min, 4 °C). The resulting extracts were then collected with a Pasteur pipette and transferred to 30 mL glass vials with a PTFE caps. The remaining sediments were mixed with phosphate buffer (2 mL), chloroform (2.5 mL) and methanol (5 mL), vortexed, shaken overnight (100 rpm, 30 °C), centrifuged again (2000 rpm, 15 min, 4 °C). The extracts were then collected and combined with the previous ones. The extracts were then separated into two phases overnight by adding water (5 mL) and chloroform (5 mL). The chloroform layer was collected using a Pasteur pipette and transferred to a 10 mL glass vial. The solvent was then evaporated to dryness under a gentle stream of nitrogen and derivatisation was conducted.

d) Derivatization

For gas chromatographic analysis, the extracted lipids were converted to the methyl ester derivatives (which were less polar and more volatile) *via* mild alkaline methanolysis [165]. Briefly, freshly prepared 0.2 M KOH solution in methanol (2 mL) was added to the sample, which was then vortexed and incubated (60 min, 37 °C). After cooling to ambient temperature, 200 µL of a mixture of methanol:glacial acetic acid (9:1, v/v) was added. Subsequently, fatty acid methyl esters (FAMEs) were extracted with hexane (3 mL) by shaking on a vortex mixer (10 min). Samples were then centrifuged (2000 rpm, 10 min, 4 °C) and the organic layers were collected with Pasteur pipettes and transferred to a 10 mL glass vial with a PTFE cap. FAMEs extraction precure was repeated twice, resulting in approx. 9 mL of FAMEs-containing hexane extracts, which were then concentrated to approx. 100 µL under a gentle nitrogen stream, followed by addition of 21:0 solution of FAMEs as an internal standard. The extracts were transferred to a glass inserts placed in GC vials using a Pasteur pipette. The vials were washed twice with *n*-hexane (99 %, 75 µL) and then

transferred to glass inserts. Lastly, the extracts were concentrated to approx. 50 μL under a gentle stream of nitrogen, the vials were sealed with PTFE caps and stored (– 20 °C) until analysed. A solution of the internal standard was added prior to GC analysis.

e) GC-C-IRMS analysis

The GC-C-IRMS analysis was applied in order to determine carbon isotope ratios in FAMEs extracted from liquid and soil microcosms. The GC-C-IRMS system was the same as for the previously described carbon isotope analysis, the only difference being a SGE BPX-5 column (30 m × 0.32 m × 0.25 μm). The extract volumes of 5 mL were injected into a GC injector, which was kept at 250 °C and operated in splitless mode with constant carrier gas (helium) flow (2 mL/min). The temperature program used: starting temperature 70 °C (held for 1 min), ramped up to 130 °C at 20 °C/min, to 150 °C at 2 °C/min to (held for 5 min), to 165 °C at 2 °C/min (held for 5 min), to 230 °C at 2 °C/min and to 300 °C at 20 °C/min (held for 10 min). The FAMEs standard solutions were analysed under the same conditions and identified according to retention times.

3.10. Assessment of bacterial community structure in soils *via* 16S rRNA barcoding

3.10.1. Bioaugmentation experiment with HILs based on glyphosate and field studies with HILs based on 2,4-D

The structure of bacterial community was assessed in collaboration with scientists from Faculty of Biology at Adam Mickiewicz University. The DNA isolation and NGS sequencing were conducted in accordance with manufacturer's protocols. Read processing and data analyses were performed according to Trzebny et al., 2021 [166].

a) DNA extraction, library construction, NGS sequencing

Biological material was obtained by centrifugation (14,100 g, 10 min) of bacterial glycerol stock (100 μL). Then, obtained pellets were dissolved in 50 μL of 10 mM Tris-EDTA buffer. Next, lysozyme (A&A Biotechnology, Gdansk, Poland)

was added to the final concentration of 0.1 mg/mL, and the samples were incubated (30 min, 37 °C). Subsequently, 360 µL of ATL lysis buffer (Qiagen, Hilden, Germany) and 40 µL of 2 mg/mL Proteinase K (Bio Basic, Markham, ON, Canada) were added, followed by incubation (30 min, 56 °C). Next, lysate from each sample (200 µL) was used to isolate total genomic DNA employing the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany), in accordance with the manufacturer's protocol for animal tissue. All of the obtained DNA extracts were standardised to a concentration of 10 ng/µL with sterile, DNase-free water. A blank DNA extract used as a negative control.

Fragments of the bacterial 16S rRNA gene (16S) were amplified with primers V4F (CGATCAGCAGCCGCGGTAATA) and V4R (ATGGGACTACCAGGGTAT CTAA), which target the V4 region [167]. Primers were tailed at 5'-ends with dual-indexed Ion Torrent adapters for sequencing with the Ion Torrent system (Life Technologies, Carlsbad, CA, USA). PCRs were performed in two technical replications, each in a total volume of 10 µL with HOT FIREPol DNA Polymerase (Solis BioDyne, Tartu, Estonia), 0.25 µM of each primer and 1 µL of template DNA. The PCR program parameters were as following: initial denaturation (12 min, 95 °C), 30 cycles of denaturation (15 s, 95 °C), annealing (1 min, 50 °C) and DNA synthesis (45 s, 72 °C), with a final extension step for 5 min at 72 °C. Blank PCRs were used as negative controls. After PCR, technical replicates were pooled and 3 µL of each sample was electrophoresed on a 2 % agarose gel to verify amplification efficiency. All samples were then pooled in equal amounts and purified with the 2 % E-Gel SizeSelect II Agarose Gels system (Invitrogen, Waltham, MA, USA) in accordance with the manufacturer's protocol.

DNA concentration and fragment length distribution of the library were determined with the use of the High Sensitivity D1000 Screen Tape Assay on the 2200 Tape Station system (Agilent, Santa Clara, CA USA). Clone template amplification was performed with the Ion Torrent One Touch System II and the Ion Torrent OT2 Kit in accordance with the manufacturer's protocol. Sequencing was performed with the Ion 540 Kit-OT2 and the Ion Torrent S5 System in accordance with the manufacturer's protocol.

b) Read processing and data analysis

Raw sequence data were pre-filtered using Ion Torrent Suite software version 5.12.2 (Life Technologies, Carlsbad, CA, USA) in order to remove polyclonal and poor-quality sequences. Further bioinformatic analyses were performed with the use of fastq data and custom workflow. Sequences shorter than 200-bp were removed from the dataset using Geneious R11.1.5 (Biomatters Ltd., Auckland, New Zealand). Leading and trailing low-quality bases were removed with the use of Trimmomatic version 0.39 [168]. The FASTX-Toolkit [169] was utilised in order to extract sequences with at least 50 % of bases with a quality score ≥ 25. Quality-filtered sequences were separated by barcodes and trimmed at the 5' and 3' ends to exclude PCR primers in Geneious R11.1.5. The singletons (<10 reads) were removed with the use of the FASTX_UNIQUES and SORTBYSIZE algorithms [170]. Chimeras were removed using the default settings in UCHIME2 version 4.2.40 [171].

Operational taxonomic unit (OTU) clustering with 97 % similarity was performed in USEARCH version 11.0.667 [170]. Sequences were de-noised into zero-radius operational taxonomic units (ZOTUs), and then a ZOTU table was created in accordance with the DENOISING STEPS [171]. The ZOTU table was then corrected to a copy number of 16S using the UNIBAS algorithm. Phylogenetic affiliations were analysed using the USEARCH SINTAX algorithm with a confidence threshold of 0.8 [172–175]. ZOTUs were compared against the SILVA database for ARB for small subunit ribosomal RNAs version 138 [176–178]. The UNCROSS2 algorithm was applied in order to remove from the dataset ZOTUs detected in control samples [179]. Furthermore, the values were normalized using the OTUTAB_RARE algorithm [172] to compare the diversities of the samples.

- **HILs based on glyphosate**

The potential orthologs of prokaryotic communities in all samples were predicted with the use of the software package Phylogenetic Investigation of Communities by Reconstruction of Unobserved States version 2.4.1 (PICRUSt2) [180]. The ZOTU table normalised by the 16S rRNA gene copy number was used to predict orthologs and create the Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthologs (KOs) table [181–183]. The predictions were categorized by KEGG Orthology. As an indicator of the accuracy of the PICRUST2 prediction, the Nearest

Sequenced Taxon Index (NSTI) was estimated and calculated for each sample [184]. Comparison of the ratios between *soxA* and *phnJ* orthologs was performed in STAMP (Statistical Analysis of Metagenomic Profiles) version 2.1.3 [185]. Visualization of bacterial microbiome composition profiles at the family-level and cluster tree based on the unweighted pair-group method with arithmetic median (UPGMA) were performed in STAMP version 2.1.3. The MetacodeR package version 0.3.5 was used to visualise bacterial diversity in the form of heat trees, and the Wilcoxon Rank Sum test was applied to compare differences in taxon abundances between samples [186]. Diagrams were edited in Corel Draw Graphics Suite 2021.

- **HILs based on 2,4-D**

Venn package version 1.10 was utilised in order to compare differences between the taxa presence among samples. The relative abundance of major bacterial families and classes visualisation was prepared in Corel Draw Graphics Suite 2021. In order to reveal the variation in structure of bacterial community, the PCA analysis (principal component analysis) biplot generated in Canoco version 5.12 was used.

3.10.2. Bioaugmentation experiments for HILs based on 2,4-D

The structure of bacterial community was assessed in collaboration with scientists from Poznan University of Life Sciences. The DNA isolation and NGS sequencing were conducted in accordance with manufacturer's protocols. Read processing and data analyses were performed according to Hornik et al., 2021 [187].

a) Isolation of DNA, PCR amplification and NGS sequencing

DNA from samples was isolated with the use of the Genomic Mini AX Soil Spin Kit (060–100S, A&A Biotechnology, Gdansk, Poland) in accordance with manufacturer's protocol. The purified DNA was eluted, and the isolates were stored at –80 °C after neutralisation to minimise matrix degradation. The isolation efficiency was tested *via* fluorometric method, using a Qbit 3.0 instrument and a Qubit™ dsDNA HS Assay Kit (Q32851, Thermo Fisher Scientific, Waltham, MA, USA). Each sample was subjected to three DNA extractions and combined after positive quantification.

PCR reactions were prepared with the use of the Ion 16S™ Metagenomics Kit (A26216, Life Technologies, Carlsbad, CA, USA), which amplifies the V2–V9 region

of the bacterial 16S rRNA gene. The reaction was prepared in accordance with the manufacturer's protocol and consists of 15 μL of 2 × Environmental Master Mix, appropriate primers (3 μL) and previously isolated DNA sample (12 μL). Reactions were conducted in a Veriti thermal cycler (Life Technologies, Carlsbad, CA, USA) with program parameters as follow: initial denaturation (10 min, 95 °C), 25 cycles of denaturation (30 s, 95 °C,), annealing (30 s, 58 °C), extension (20 s, 72 °C,), final extension (7 min, 72 °C). The purification of the reaction products was performed with the use of Agencourt AMPure XP reagent (A63880, Beckman Coulter, Pasadena, CA, USA) in accordance with manufacturer's protocol basing on the DNA's binding to magnetic beads and washing off contaminants with ethanol. The DNA was washed off the beads with nuclease-free water or low-TE buffer.

The library was prepared using the Ion Plus Fragment Library Kit (4471252, Life Technologies, Carlsbad, CA, USA) and purified with the use of Agencourt AMPure XP Reagent (A63880, Beckman Coulter, Pasadena, CA, USA), both in accordance with the manufacturers' protocols. Library concentration was determined with the use of the Ion Universal Library Quantification Kit and the real-time PCR instrument Quant Studio 5 (A26217, Life Technologies, Carlsbad, CA, USA), followed by library's dilution (10 pM). Thus prepared library was then applied to beads (used for sequencing) in emulsion PCR with the use of the Ion PGM™ Hi-Q™ View OT2 Kit and Ion One Touch 2 Instrument (A29900, Life Technologies, Carlsbad, CA, USA). The beads were then purified with an Ion One Touch ES instrument (Life Technologies, Carlsbad, CA, USA) and sequenced with the Ion PGM System (Life Technologies, Carlsbad, CA, USA) using the Ion PGM™ Hi-Q™ View Sequencing Kit (A29900) on an Ion 316™ Chip Kit v2 BC.

b) Bioinformatic analysis

The sequence reads from Ion Torrent (Thermo Fisher Scientific, Waltham, MA, USA) were imported into the CLC Genomics Workbench 20.0 software (Qiagen, Hilden, Germany) and processed with CLC Microbial Genomics Module 20.1.1 (Qiagen, Hilden, Germany) for processing. Chimeras and reads of low-quality were filtered and removed (quality limit = 0.05, ambiguity limit = 'N'). All reads were then clustered against SILVA v119 database at 97 % operational taxonomic unit (OTU) similarity.

c) Genetic activity of bacteria exposed to HILs

Genes level was analysed using a Power SYBR Green PCR Master Mix (Life Technologies, 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 5**. 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 5**. All analysis was 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 5 Primers used for real-time PCR.

Primers	Sequence (5' to 3')	References
tfdA (CI)-class I	F: GTGAGCGTCGTCGCAAAT	[188]
	R: GCATCGTCCAGGGTGGTC	
tfdA (CII)-class II	F: TGAGCATCAATTCCGAATACC882	[188]
	R: AAGACTGACCCCGTGGACT	
tfdA (CII)-class III	F: TGAGCATCACTTCCGAATACC856	[188]
	R: ACAGCGTCGTCCAACGTC	
16S rDNA		[189]
968 Forward	AACGCGAAGAACCTTAC	
1401 Reverse	CGGTGTGTACAAGACCC	

3.11. Greenhouse experiment

Greenhouse experiment was conducted in collaboration with Institute of Plant Protection, Poznan, Poland, in order to verify differences in behaviour of HILs with glyphosate anion and modified choline cation towards rapeseed (*Brassica napus*). In addition, the impact of bioaugmentation with isolated microorganisms specialised in glyphosate degradation on herbicidal efficiency and microbial community composition within and near plants were evaluated. For the sake of comparisons, fresh weight reduction was determined, along with herbicidal residues in plants' tissues and

oxidative stress. Moreover, biodiversity of samples was examined *via* 16S rRNA gene hypervariable fragment MiSeq sequencing analysis.

The composition of enrichment culture utilised in the bioaugmentation approach was dominated by the bacteria from families of Planococcaceae (22.91 %), Clostridiaceae (21.25 %), Moraxellaceae (18.27 %), Bacillales (15.09 %), Peptostreptococcaceae (5.25 %), Pseudomonadaceae (4.17 %), Bacillaceae (4.01 %), Enterobacteriaceae (2.69 %), Paenibacillaceae (2.22 %), Aeromonadaceae (1.34 %). Other families amounted for less than 1 %.

The experimental setup consisted of two sets of plants, bioaugmented with microbial community and non-bioaugmented. Biomass for the experiment was obtained as following. Previously isolated glyphosate-degrading enrichment culture (used in mineralisation study) were incubated (120 rpm, 48 h, 28 °C) in TSB medium (50 mL) amended with glyphosate (1 g/L). After three transfers to a fresh medium (TSB+H), they were transferred to a fresh TSB+H medium (500 mL). After incubation (120 rpm, 72 h, 28 °C), the biomass was introduced to a bioreactor (New Brunswick Scientific Bioflo III Batch Continuous Fermentor, Eppendorf, Germany) with a capacity of 3 L, operating for 6 days at pH 7 (adjusted by 2 % (v/v) NaOH and 10 % (v/v) H_3PO_4), temperature of 28 °C, 150 rpm, air flow 2.5 L/min, supplemented daily with aqueous glucose solution (100 mL, 150 g/L). After cultivation, the biomass was washed with sterile 0.85 % (v/v) NaCl solution three times, centrifuged (4500 rpm, 15 min, 4 °C) and resuspended in mineral medium ($OD_{600} \approx 6.2$).

The plants were cultivated in plastic pots (0.5 L) filled with commercial peat-based potting material (pH 6). Every 6 days, starting at the day of sowing, half of experimental samples was inoculated with cultivated microorganisms (bioaugmentation approach), while the other half was watered with mineral medium without microorganisms' addition (approach without bioaugmentation). Aqueous solutions of herbicidal salts ([Chol][Glyph], [C_{12}Chol][Glyph]) were prepared in such a way to reach active substance amount corresponding to commercial herbicidal formulation dose (1080 g/ha). Potassium glyphosate salt and Roundup 360 SL were used as reference products, while plants untreated served as controls. The herbicidal treatment was applied at 4–6 leaf growth stage. The plants were sprayed *via* moving sprayer (APORO, Poznan, Poland) with TeeJet110/02 flat-fan nozzle (TeeJet Technologies, Wheaton, IL, USA) delivering 200 L/ha of spray solution

at 0.2 MPa operating pressure. After treatment, the plants were kept in a greenhouse (20 °C, humidity 60 %, photoperiod 16/8 day/night h).

The fresh weight reduction of plants was performed 3, 7 and 14 days after herbicidal treatment. Briefly, plants' overground parts were cut to the level of soil and weighed (Sartorius BP 2000 S, Sartorius Göttingen, Germany) at 0.01 g accuracy. The obtained values were compared to untreated controls (plants bioaugmented or non-bioaugmented, respectively) *via* ANOVA one-way analyses of variance ($\alpha = 0.05$). All assays were performed in triplicate.

Additionally, isolation of microorganisms from plants' rhizosphere, shoots and roots (both endophytes and epiphytes) was performed 3, 7 and 14 days after herbicidal treatment, according to isolation procedure described in the **Section 3.5.2**. Isolates from day 0 served as controls. Thus prepared enrichment cultures were sent for molecular analyses.

3.11.1. Herbicidal residues in plants' tissues

For herbicidal residues in plants assessment, the plants were cut off and stored (–80 °C) before analysis. The sample was prepared by weighing 5 g of thawed and homogenised plant material to a centrifuge tube (50 mL), followed by addition of deionised water, 1 % formic acid solution in methanol, concentrated formic acid (100 µL) and internal standard (IS, 100 µL ^{13}C- and ^{15}N-isotopic glyphosate). Samples were then shaken manually. Next, 10 % EDTA aqueous solution was added, and samples were shaken on an orbital shaker (15 min), placed in freezer (1.5 h, –80 °C) and centrifuged (10,000 rpm, 5 min). Then, 400 µL of supernatant was collected to test tube, combined with 1600 µL of LC mobile phase (95 mL of deionised water, 5 mL of acetonitrile, 100 µL of concentrated formic acid) and filtered through syringe filter to the autosampler's glass vial (dilution factor = 20). Thus prepared samples were then analysed *via* Sciex 6500 LC-MS/MS (Framingham, MA, USA).

3.11.2. Oxidative stress

The oxidative stress determination experiment was conducted in collaboration with scientists from Department of Plant Physiology at the University of Life Science in Poznan, Poland.

a) Lipid Peroxidation

The degree of lipid peroxidation was determined according to the method of Heath and Packer, 1965 [190], which involves the testing of the level of MDA as a lipid peroxidation product by means of a coloured reaction with TBA. Leaves samples (0.2 g) were homogenized in a cooled mortar with 2 mL of P-K buffer (0.1 M, pH 7). As a result, a colourful product was formed whose concentration in the sample can be determined by a spectrophotometric method. The tubes containing 500 μL of the supernatant were filled with 2.0 mL of 0.5 % TBA in 20 % TCA and placed in a boiling water bath for 30 min. After this time, the tubes were quickly cooled and then centrifuged (12,000 × g, 10 min). The absorbance of the supernatant was measured at wavelength λ = 532 nm (A_{532}) and 600 nm (A_{600}). The result was corrected by subtracting from A_{532} the A_{600} value resulting from the reaction of non-specific products with TBA (UV-Vis Jasco spectrophotometer with V-530 software for Windows). The concentration of MDA was calculated from the molar absorption coefficient (155 m/(M·cm)) according to the formula: C = A_{532}–A_{600} 155 mM. All assays were performed in triplicate.

b) Superoxide Anion ($O_2^{\bullet-}$)

The $O_2^{\bullet-}$ was determined on the basis of its ability to reduce nitroblue tetrazolium (NBT, Lab Empire, Poland) [191]. Leaves samples (0.2 g) were placed in 3.0 mL of potassium phosphate buffer (0.01 M, pH 7.8) with the addition of 0.05 % NBT and 0.5 mM NaN_3. The reference sample was an incubation mixture without roots. The samples were incubated for one hour at room temperature and shook every 15 min. For measurement, 2.0 mL of the incubation mixture was collected and heated in a water bath (15 min, 80 °C). After cooling in ice to room temperature, the absorbance of the solution was measured at 580 nm. $O_2^{\bullet-}$ content was expressed in absorbance units per 1 g of fresh weight. All assays were performed in triplicate.

c) Hydrogen peroxide (H_2O_2)

Hydrogen peroxide concentration was assayed using the method of Messner and Boll, 1994 [192]. Leaves samples (200 mg) were ground in 2 mL cold K phosphate buffer (pH 7) containing 0.02 g Polyclar AT. The homogenate was centrifuged (15,000 × g, 25 min, 4 °C). The reaction mixture contained 1.5 mL extract, 0.15 mL

K phosphate buffer (pH 7), 50 mL horseradish peroxidase (1 mg/1 mL 100 mM K phosphate buffer, 60 units/mg) and 0.05 mL 0.05 M ABTS (2,2'-azino-bis(3-ethylobenzothiazoline-6-sulfonic acid)diammonium salt). Absorbance increase according to the H_2O_2 level at 415 nm was measured after 3 min and compared to the standard curve of freshly prepared 0-30 nM H_2O_2 solutions in 100 mM K-phosphate buffer (pH 7). The results are given in nmol hydrogen peroxide (H_2O_2) nmol per gram fresh weight. All assays were performed in triplicate.

3.12. Field experiments

Field experiments 2,4-D-based HILs were performed in Rzgow, Poland in 2020 and 2021 in accordance with the modified OECD 208 guideline [193]. Shortly, agricultural soil (approx. 3.5 kg) was placed in plastic pots (d = 30 cm), and ten seeds of cornflower (*Centaurea cyanus*) and spring barley (*Hordeum vulgare*) were then sown and covered with thin soil layer. All samples were prepared in four replicates. Thus prepared pots were then placed in the ground in a randomised design, approx. 1 m apart. When plants reached the 4–6 leaf growth stage (after two weeks growth), 2,4-D-HILs treatment was carried out with the use of commercial agricultural pressure sprayer. Aqueous solutions of [Bet][2,4-D], [C_{12}Bet][2,4-D] and [CAPBet][2,4-D] were prepared at the concentration of 400 g of active ingredient per hectare. Potassium salt of 2,4-D was used as a model compound. The herbicidal efficacy of the tested salts was evaluated four weeks after treatment, and the pots were collected to sterile bags and transported to the laboratory for further treatments. The plants' stems and leaves were cut and weighed separately.

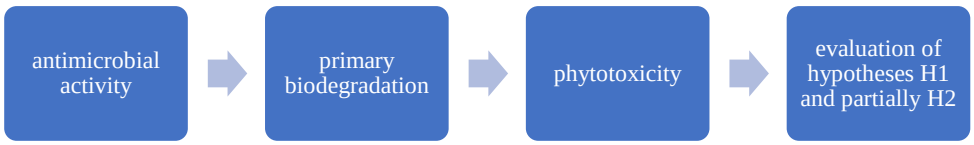
3.13. Statistical analysis

All controls and samples were performed in triplicates. Error bars reflect the standard errors of the mean (n = 3). ANOVA one-way analyses of variance ($\alpha = 0.05$) were conducted in order to detect significant differences between the samples and controls.

4. Results and discussion

The herbicidal ionic liquids are thoroughly analysed in terms of their synthesis methods and physicochemical characteristics. However, with limited biological studies, scientific questions concerning their environmental behaviour and stability arose. The initial studies aimed in the synthesis of dual-function herbicidal salts with tropinium-based cations of natural origin as an approach to obtain compounds with low environmental toxicity and good degradation efficiencies [4]. Hence, in order to do that, beside physicochemical tests (data not shown), synthesised salts (**Section 3.1**, **Table 2**) were evaluated for their toxicity towards plants and microorganisms, as well as degradation.

Block diagram presenting experiments performed within the scientific research



Antimicrobial activity

The results of antimicrobial tests (**Section 3.8.2**) of QTS towards model soil microorganisms (*Pseudomonas putida*, *Bacillus cereus*) are presented in **Table 6**. Generally, synthesised compounds were less toxic towards *Pseudomonas putida*. However, interestingly, the toxicity of formulations seemed to be only cation dependent. MCPA and dicamba sodium salts were deemed non-toxic, while introduction of quaternary tropinium cations into the structure resulted in significantly increased toxicity. Moreover, cation chain length has a vast impact on the toxicity of resulting formulations. Namely, in the case of *Bacillus cereus*, the toxicity increased with the chain length increase, while in the case of *Pseudomonas putida* – increased up to C_{14} and decreased with further chain elongation. This phenomena is known as 'cut-off effect' reported by other authors studying HILs' toxicity and its correlation to

carbon chain length [87,88,194]. The obtained toxicity results brought attention to the fact that even cations of natural origin might have adverse effects on environmental fate of HILs. After all, these cations are surface active compounds, which have the ability to disrupt cellular membranes as was discussed earlier (**Section 1.2**). Moreover, long aliphatic chains even enhance this undesired influence, as their introduction results in increased hydrophobicity and might further promote cell disruption [88,195]. Consequently, such behaviour of cations might have an adverse effect also on microbial degradation of these compounds.

Table 6 Determination of MIC and MBC values for QTS with MCPA and dicamba anions (adapted from author's published work [4]).

Quaternary tropinium salt	***Pseudomonas putida***		***Bacillus cereus***	
	MIC [mmol/L]	**MBC [mmol/L]**	**MIC [mmol/L]**	**MBC [mmol/L]**
[Na][MCPA]	>10	>10	10	>10
[QTS-C_8][MCPA]	1.0	2.0	0.25	0.5
[QTS-C_{10}][MCPA]	1.0	2.0	0.025	0.5
[QTS-C_{12}][MCPA]	0.5	1.0	0.0063	0.05
[QTS-C_{14}][MCPA]	0.063	0.125	0.00313	0.0125
[QTS-C_{16}][MCPA]	0.5	1.0	0.00156	0.00313
[QTS-C_{18}][MCPA]	1.0	2.0	0.00156	0.00313
[Na][dicamba]	>10	>10	10	>10
[QTS-C_8][dicamba]	1.0	2.0	0.5	1.0
[QTS-C_{10}][dicamba]	1.0	2.0	0.5	1.0
[QTS-C_{12}][dicamba]	0.5	1.0	0.025	0.5
[QTS-C_{14}][dicamba]	0.063	0.25	0.0063	0.0125
[QTS-C_{16}][dicamba]	0.5	1.0	0.00313	0.00625
[QTS-C_{18}][dicamba]	1.0	2.0	0.00313	0.00625

Primary biodegradation

Degradation studies (**Section 3.7**) on QTS (**Table 7**) brought attention to the issue of structural integrity of HILs in the environment, though applied measurement technique did not allow to prove the theory that cations and anions are separately degraded in the environment. However, it can be clearly seen that cation's addition has worsen the degradation of synthesised salts, in the case of both dicamba and MCPA. Especially quaternary tropinium cations, which were proven to be toxic towards microorganisms [4,128], were characterised by limited susceptibility to degradation.

In addition, their introduction to compounds' structure reflects also in decreased degradability of anion. It is an issue previously mentioned in the literature on HILs [88]. Namely, the introduction of morpholine-based cations into herbicidal structure resulted in decreased anion's degradation [88]. Curiously, even though cations were readily biodegradable, the decline in degradation efficiencies for anions was still observed, leading to the conclusion that perhaps natural origin of cations will not surpass the fact that these are still cationic surfactants.

Table 7 Biodegradation efficiencies for QTS with MCPA and dicamba anions (adapted from author's published work [4]).

Quaternary tropinium salt	Biodegradation efficiency [%]	
	Cation	Anions
[Na][MCPA]	---	61.0 ± 9.12
[QTS-C_8][MCPA]	1.03 ± 0.15	34.45 ± 6.12
[QTS-C_{10}][MCPA]	0.96 ± 0.27	35.12 ± 5.45
[QTS-C_{12}][MCPA]	3.42 ± 0.33	35.03 ± 6.47
[QTS-C_{14}][MCPA]	2.34 ± 0.12	34.12 ± 6.32
[QTS-C_{16}][MCPA]	3.12 ± 0.24	34.34 ± 4.23
[QTS-C_{18}][MCPA]	4.08 ± 0.30	33.34 ± 5.12
[Na][dicamba]	---	42.0 ± 8.75
[QTS-C_8][dicamba]	0.97 ± 0.14	29.0 ± 5.64
[QTS-C_{12}][dicamba]	1.56 ± 0.25	30.42 ± 7.18
[QTS-C_{14}][dicamba]	3.15 ± 0.36	32.02 ± 6.98
[QTS-C_{16}][dicamba]	2.96 ± 0.45	31.23 ± 6.14
[QTS-C_{18}][dicamba]	3.98 ± 0.23	32.18 ± 5.98

± represents SEM values from three independent samples.

Phytotoxicty

Bearing in mind results from QTS toxicity and degradation, their effects on germination and early development of maize and cornflower as model crop plant and weed, respectively, were also evaluated (**Section 3.8.3**). As presented in **Table 8**, the impact of cations chain length could not be observed, as results were mostly depended on herbicidal concentration used. In case of crop plant, tested QTS exhibited stimulating effect on seed germination (**Table 8**), while plant growth promotion results were comparable with controls, with few exceptions in case of dicamba anion (**Fig. 14)**. When it comes to herbicidal activity towards weeds (**Fig. 15)**, synthesised salts inhibited cornflower's growth and germination (**Table 8**), and inhibitory effect

was increasing with the concentration of herbicidal salts. Interestingly, however, the mode of action of MCPA and dicamba, *i.e.*, root growth inhibition, was exhibited also by newly synthesised formulations, while their herbicidal efficacy was similar to controls. This might suggest that the observed HILs' behaviour was that of herbicidal anion acting in the presence of surface-active cation. Admittedly, the use of quaternary tropinium cations allowed for volatility reduction (data not shown) and hence, the lack of the need for adjuvants use. However, despite the use of substances of natural origin in synthesis, the resulting compounds were proven to be toxic and poorly degraded, which was most probably attributed to the presence of surface-active cations. This in turn allowed conclusion that their use might not be beneficial for HILs synthesis, as negative biological aspects surpass advantages in physicochemical properties.

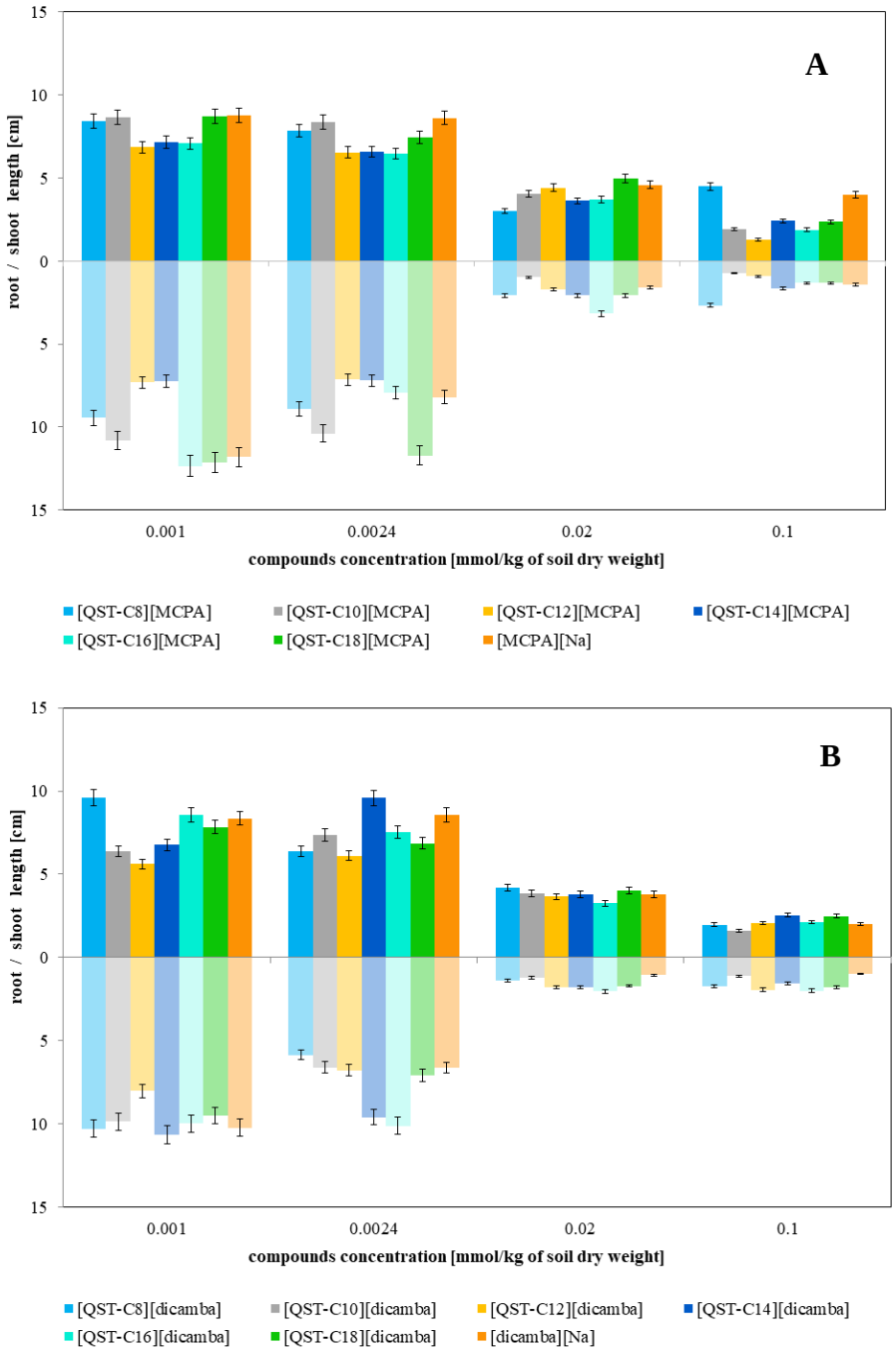


Fig. 14 The effect of *N*-alkyltropinium MCPA (a) and dicamba (b) salts concentration on the shoots and roots length of maize (adapted from author's published work [4]).

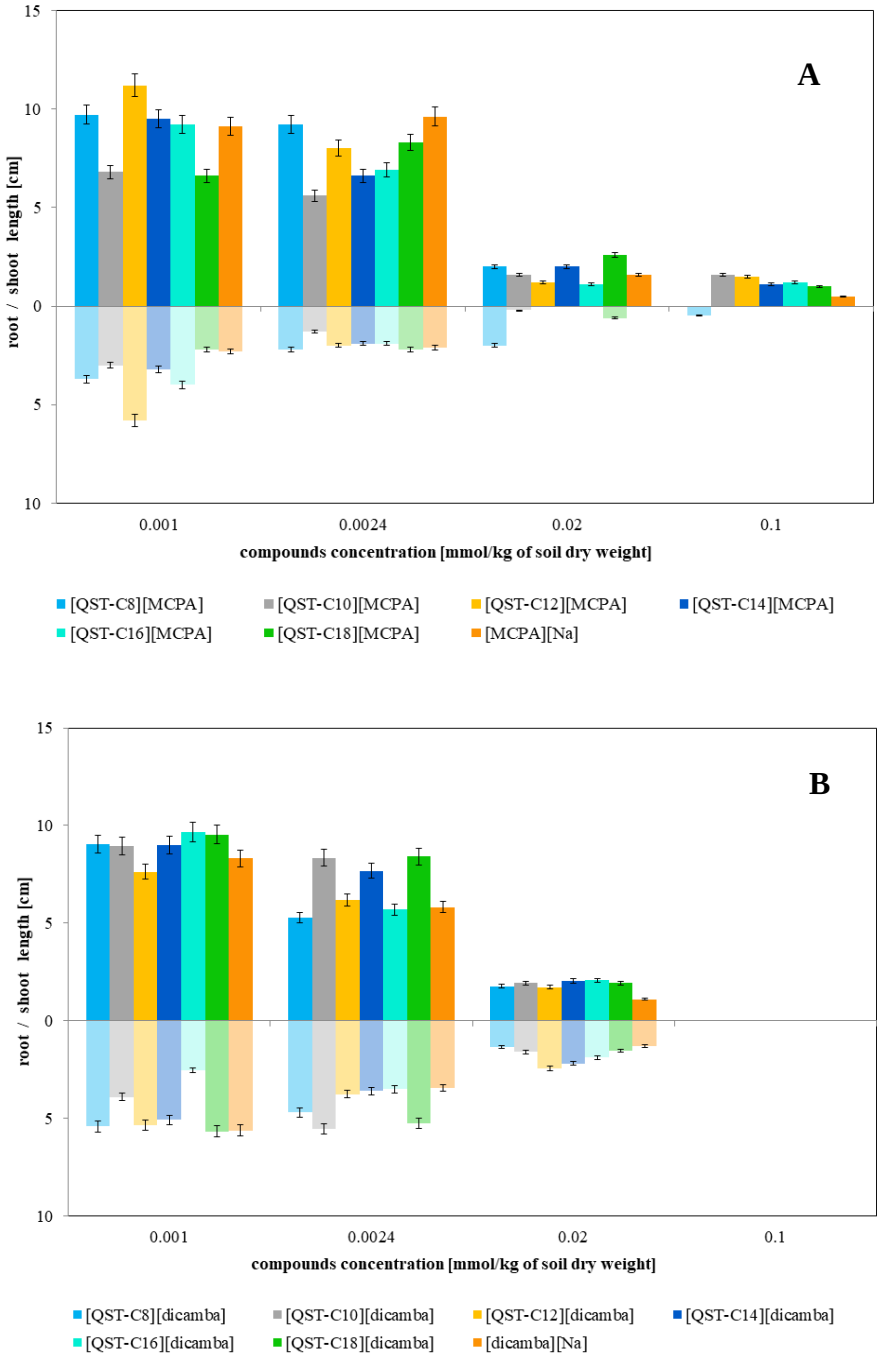


Fig. 15 The effect of *N*-alkyltropinium MCPA (a) and dicamba (b) salts concentration on the shoots and roots length of cornflower (adapted from author's published work [4]).

Table 8 The effect of *N*-alkyltropinium MCPA and dicamba salts concentration on the germination index of maize (adapted from author's published work [4]).

	Germination index [%]							
	maize				**cornflower**			
Concentration [mmol/kg of soil d. w.] / **Quaternary tropinium salt**	**0.001**	**0.0024**	**0.02**	**0.1**	**0.001**	**0.0024**	**0.02**	**0.1**
[MCPA][Na]	140.64 ± 5.01	65.67 ± 2.06	12.91 ± 1.15	14.64 ± 1.45	39.91 ± 2.54	41.00 ± 1.57	0.00 ± 0.00	0.00
[QTS-C_8][MCPA]	97.32 ± 3.12	91.96 ± 2.53	23.71 ± 1.88	24.47 ± 2.51	38.61 ± 2.77	34.71 ± 1.23	8.10 ± 1.87	0.00
[QTS-C_{10}][MCPA]	98.24 ± 1.89	119.01 ± 3.87	5.61 ± 1.45	2.58 ± 3.16	38.83 ± 2.89	19.31 ± 2.36	3.04 ± 2.36	0.00
[QTS-C_{12}][MCPA]	67.08 ± 2.56	60.46 ± 1.15	13.79 ± 2.31	5.27 ± 1.85	112.58 ± 4.56	64.49 ± 1.47	0.00 ± 0.00	0.00
[QTS-C_{14}][MCPA]	68.13 ± 2.45	65.98 ± 2.14	18.97 ± 1.05	15.03 ± 1.81	41.65 ± 1.87	20.39 ± 2.34	0.00 ± 0.00	0.00
[QTS-C_{16}][MCPA]	141.35 ± 3.49	72.67 ± 1.88	36.31 ± 3.24	9.07 ± 2.09	52.28 ± 2.13	20.61 ± 2.14	0.00 ± 0.00	0.00
[QTS-C_{18}][MCPA]	122.58 ± 2.97	134.14 ± 1.66	23.71 ± 1.96	12.19 ± 1.56	68.02 ± 3.12	42.30 ± 1.12	8.13 ± 1.86	0.00
[dicamba][Na]	98.52 ± 1.23	54.76 ± 1.54	11.69 ± 1.56	6.75 ± 1.39	113.08 ± 4.63	43.08 ± 2.45	13.10 ± 2.86	0.00
[QTS-C_8][dicamba]	113.37 ± 4.23	40.39 ± 2.78	9.51 ± 1.06	14.47 ± 1.29	95.41 ± 3.42	106.35 ± 3.45	20.11 ± 3.15	0.00
[QTS-C_{10}][dicamba]	108.74 ± 3.85	90.98 ± 2.36	15.01 ± 2.19	10.61 ± 1.34	68.61 ± 2.74	55.73 ± 2.42	12.09 ± 2.56	0.00
[QTS-C_{12}][dicamba]	99.26 ± 2.45	74.77 ± 2.48	21.84 ± 2.46	23.95 ± 1.25	67.27 ± 1.23	75.59 ± 1.89	18.37 ± 1.32	0.00
[QTS-C_{14}][dicamba]	146.82 ± 3.68	118.86 ± 1.98	22.33 ± 2.56	19.11 ± 1.47	89.42 ± 2.42	72.56 ± 3.48	27.46 ± 2.47	0.00
[QTS-C_{16}][dicamba]	123.70 ± 2.15	83.54 ± 5.05	25.31 ± 2.37	24.81 ± 2.23	57.60 ± 1.56	61.73 ± 1.56	42.63 ± 1.85	0.00
[QTS-C_{18}][dicamba]	104.77 ± 1.96	87.72 ± 2.38	23.44 ± 1.88	19.96 ± 1.09	114.08 ± 5.28	132.53 ± 4.02	26.98 ± 1.26	0.00

± represents SEM values from three independent samples

The gaps in knowledge on HILs' behaviour and stability in the environment as ionic compounds (**Fig. 16**) were starting point for next research [2]. Namely, the use of ^{13}C-labelled compounds was proposed as a way to determine fate of cations and anions constituting HILs in aqueous and terrestrial systems – their degradation pathways, mineralisation efficiencies and impact of microbiota.

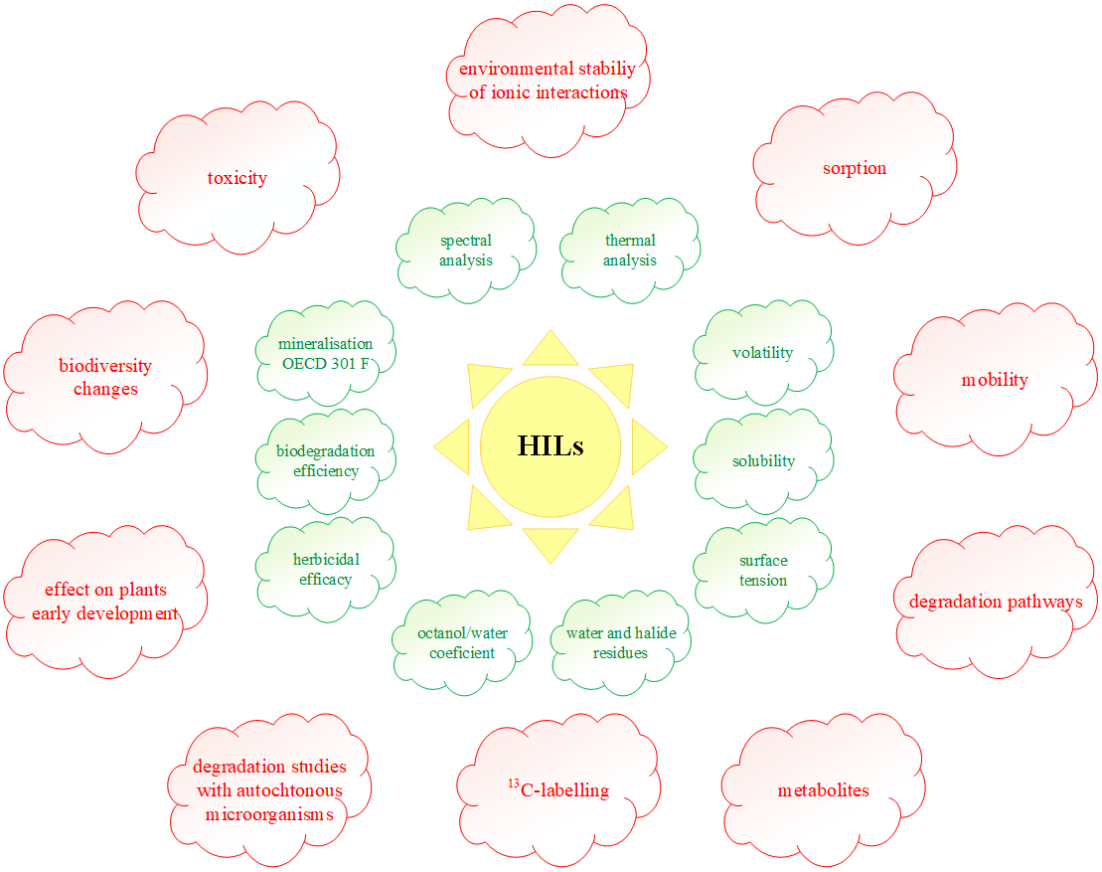


Fig. 16 Studies performed on HILs (**green**) along with gaps of knowledge (**red**) evaluated within the scope of this Ph.D. thesis.

Block diagram presenting experiments performed within the scientific research

Mineralisation

The standard mineralisation assessment procedures for liquid and soil microcosms have been modified (**Section 3.6.2**) to allow detection of $^{13}C/^{12}C$ isotope ratio in emitted CO_2. Namely, since phenyl ring was ^{13}C-labelled, the $^{13}CO_2$ evolution indicated full mineralisation of aromatic ring, while lack of it – no mineralisation at all or limited biotransformation. Results presented in **Table 9** show that cations and anions are mineralised differently, depending on environment (aqueous or terrestrial). Values highlighted by red frames indicate which ions were mineralised, *i.e.*, cations in aqueous matrix with activated sludge, anions in terrestrial matrix with autochthonous soil microorganisms. Cations in soil microcosms were subjected only to trace mineralisation (biotransformation had occurred). Unfortunately, the values exceeding 10,000 did not allow to determine the exact mineralisation kinetics, indicating that the process was too fast, and hence – short term experiment was proposed, targeted at monitoring mineralisation kinetics within the first few days of experiment (**Section 3.6.2**).

Table 9 $\delta^{13}CO_2$-values [‰] emitted in liquid and soil samples (adapted and modified from author's published work [2]).

Compound \ Days	0	5	10	20	30	40	73
	liquid microcosm						
[C_{12}-BA*][MCPA]	-7.2	9022.2[a]	9265.3	>10,000	>10,000	>10,000	>10,000
[C_{12}-BA][MCPA*]	-17.2	-14.5	-22.7	-17.3	-18.1	-17.2	-10.8
[C_{12}-BA][MCPA]	-19.5	-27.4	-30.7	-28.6	-30.7	-30.0	-27.6
[C_{12}-BA*][Br]	-18.8	6545.2	>10,000	>10,000	>10,000	>10,000	>10,000
[C_{12}-BA][Br]	-19.6	-24.1	-31.6	-30.1	-28.8	-28.3	-26.3
	soil microcosm						
[C_{12}-BA*][MCPA]	6.2	45.8	86.8	40.3	32.7	37.0	83.5
[C_{12}-BA][MCPA*]	-2.8	453.7	5914.7	>10,000	>10,000	>10,000	>10,000
[C_{12}-BA][MCPA]	-18.3	-21.8	-24.9	-24.2	-24.9	-23.9	-24.9
[C_{12}-BA*][Br]	-16.8	24.8	9.4	2.4	4.6	10.5	47.3
[C_{12}-BA][Br]	-16.7	-32.2	-26.6	-26.7	-26.6	-25.3	-24.0

[a] Values higher than 0 indicate that mineralization occurred.

Short term experiment with lower ^{13}C enrichment allowed to monitor mineralisation kinetics within the first 100 (liquid microcosms) or 260 h (soil microcosms). The results presented in **Fig. 17** show cation's rapid mineralisation in liquid microcosms (45 h lag phase, half-life 6.5 days), while anion was mineralised in soil (7 days lag phase, half-life 32 days). The calculated half-lives were consistent with literature data for [BA][Cl] (half-life 12 days), [HMCPA] (half-life 28 days) and [Na][MCPA] (full degradation within 46-82 days) [66,196,197].

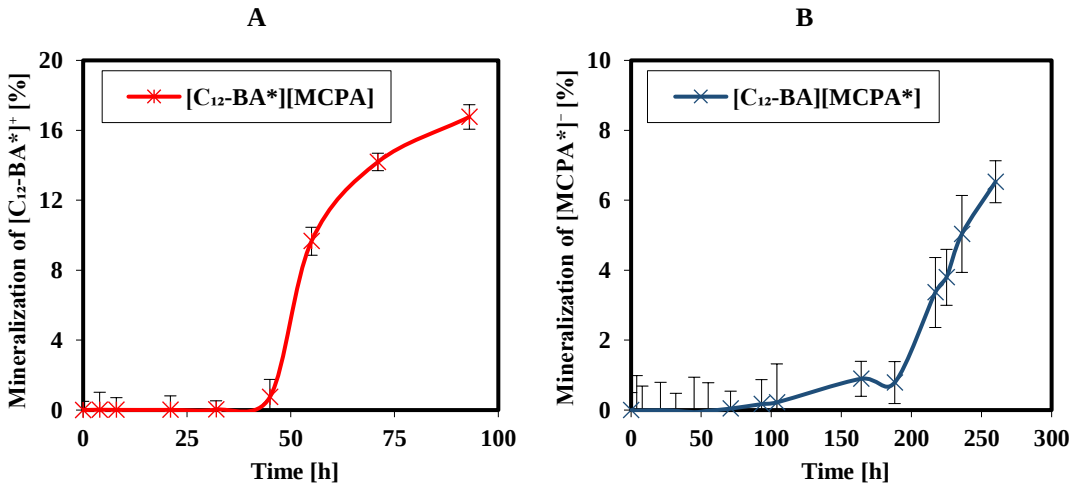


Fig. 17 Mineralization kinetics of ^{13}C-labeled $[C_{12}\text{-BA*}]^+$ in liquid microcosms amended with [C_{12}-BA*][MCPA] **(A)** and ^{13}C-labeled $[MCPA*]^-$ in soil microcosms amended with [C_{12}-BA][MCPA*] **(B)** (adapted and modified from author's published work [2]).

primary biodegradation

Primary biodegradation

The behaviour of ions constituting HILs was further tested *via* primary degradation study (**Section 3.7.1**). It provided additional evidence that cations and anions are degraded separately and differently (**Fig. 18**, **19**). Namely, in liquid microcosm, cation was quantitatively transformed within 2 days, while no MCPA anion biotransformation occurred within 73 days of the experiment. On the other hand, in soil microcosm, both cation and anion were degraded (40 and 20 days, respectively). However, in accordance with data from mineralisation experiment it can be concluded that anion was degraded and then mineralised, while in the case of cation, only biotransformation with persistent metabolites formation.

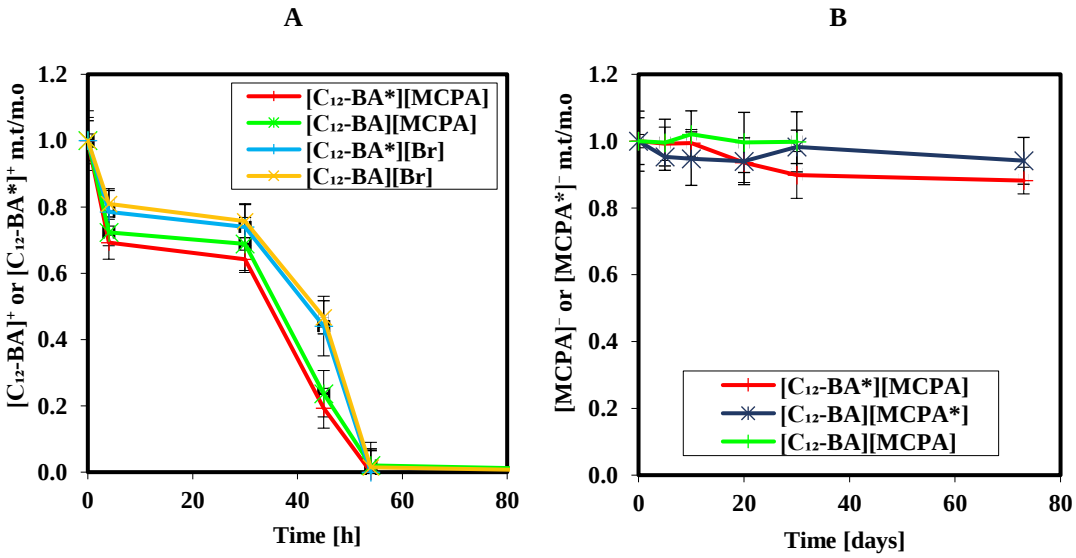


Fig. 18 Biotransformation kinetics for $[C_{12}\text{-BA}]^+$ or $[C_{12}\text{-BA*}]^+$ **(A)**; and $[MCPA]^-$ or $[MCPA*]^-$ **(B)** (liquid microcosms) (adapted and modified from author's published work [2]).

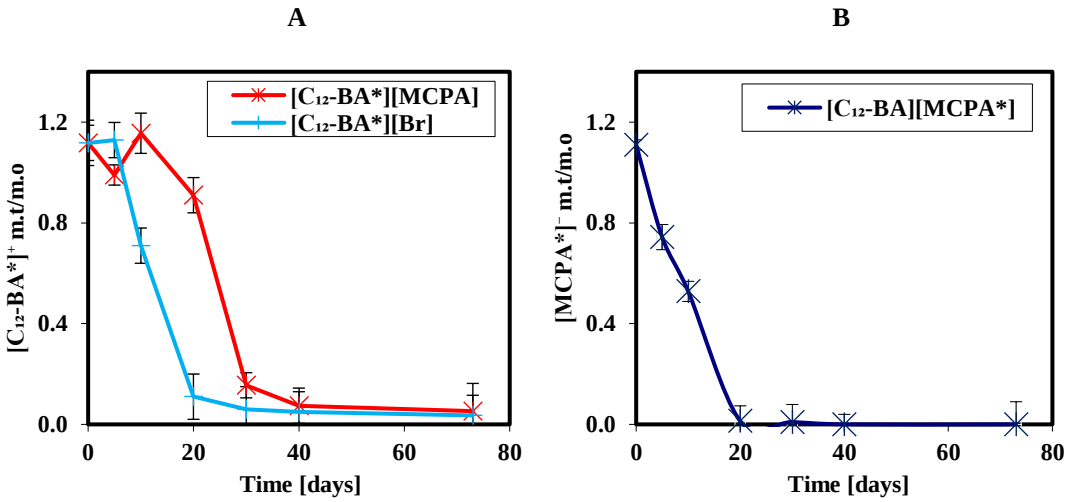


Fig. 19 Kinetics of biotransformation for $[C_{12}\text{-BA*}]^+$ cation **(A)**; and $[MCPA*]^-$ anion **(B)** (soil microcosms) (adapted and modified from author's published work [2]).

metabolites determination

Determination of metabolites

The cation's fate in soils was then further investigated by the search for $[C_{12}\text{-BA}]^+$ metabolites (**Section 3.7.2**). Hence, the proposed on the basis of literature data biotransformation pathway was studied (**Fig. 20**) [2,198]. The results revealed that the process of dealkylation took place, as the presence of benzyldimethylamine (1) and decanoic acid (2) was detected. Interestingly, however, no further biotransformation of benzyldimethylamine occurred. Taking into account initial rapid biotransformation of $[C_{12}\text{-BA}]^+$, it can be concluded that detected metabolites were toxic towards microorganisms and hence, the further biodegradation process was inhibited [199].

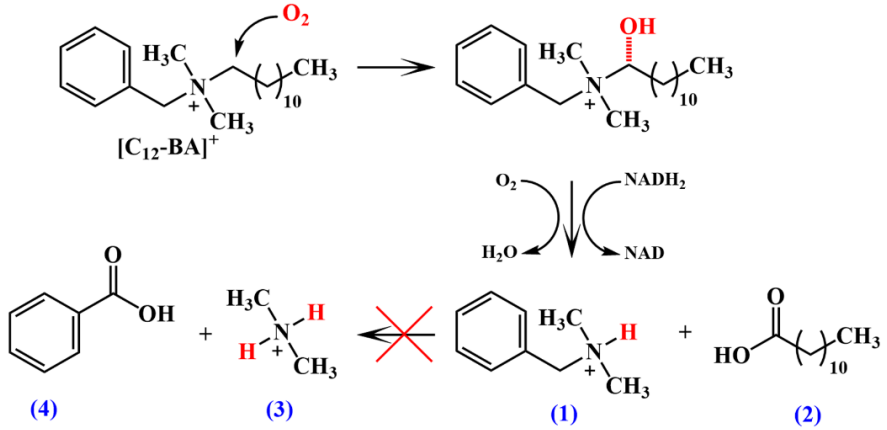
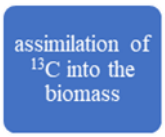


Fig. 20 Proposed pathway of $[C_{12}\text{-BA}]^+$ biotransformation (adapted from author's published work [2]).



Assimilation of ^{13}C into the biomass

Finally, as an ultimate proof of microbial utilisation of tested compounds, the assimilation of ^{13}C by the biomass was assessed (**Section 3.9**). Namely, if microorganisms are capable of degradation of xenobiotic, carbon resulting from xenobiotic breakdown will be incorporated into cells building blocks, *e.g.*, fatty acids or amino acids. In the case of ^{13}C-labelled compounds, the ^{13}C should be found in phospholipid fatty acids (PLFAs) which constitute cell membranes [162,200]. Therefore, monitoring PLFAs ^{13}C-enrichment should give a definite confirmation whether tested compounds were mineralised. According to obtained results, the fatty acids specific for Gram negative (cy17:0, cy19:0, 16:1ω7c, 16:1ω7t, 18:1ω9c and 18:1ω9t) and Gram-positive bacteria (i15:0, a15:0, i16:0, and i17:0) [201] were enriched in ^{13}C (**Fig. 21, 22**). One should note that the extraction from aqueous sample is an easier process than in the case of terrestrial one, which explains why the recorded results from liquid microcosms are of higher intensity than from soil microcosms. These results stand in perfect agreement with previously described mineralisation and degradation studies of [C_{12}-BA][MCPA]. They confirm assimilation of ^{13}C resulting from cation in liquid microcosms, thus proving its ultimate degradation. At the same time, the ^{13}C-enrichment for anion was not detected, which corresponds well with lack of anion's mineralisation nor biotransformation in liquid microcosms with activated sludge. On the other hand, in soil microcosms, PLFAs ^{13}C-enrichment resulted only from anion. This also stands in agreement with the statement that [C_{12}-BA] cation in soil was only subjected to biotransformation, with creation of metabolites which were no further degraded.

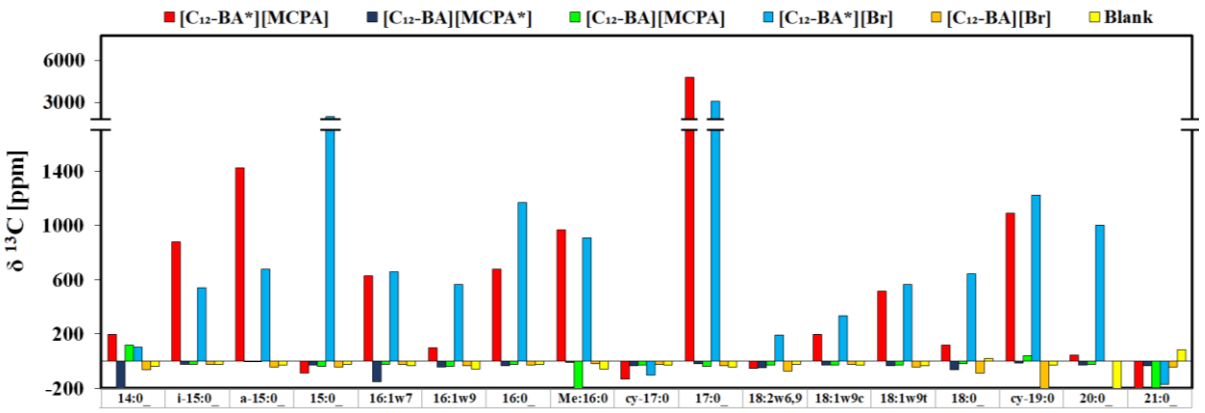


Fig. 21 δ^{13}C-values [‰] of PLFAs extracted from liquid microcosms.

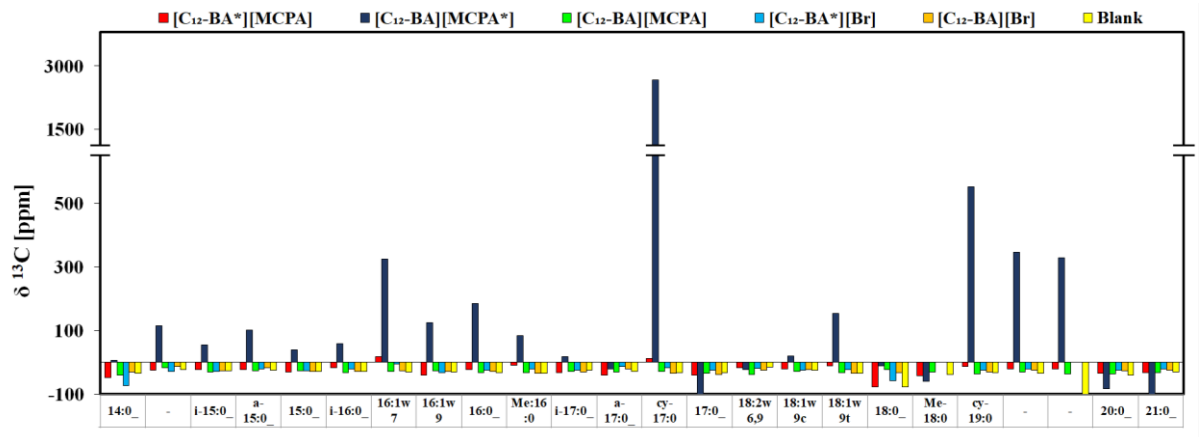
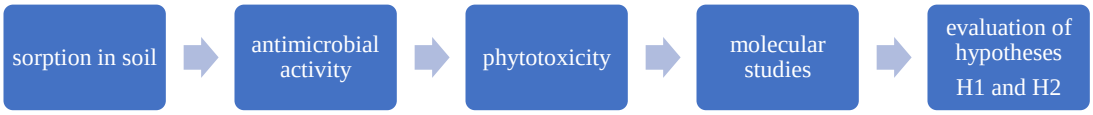


Fig. 22 δ^{13}C-values [‰] of PLFAs extracted from soil microcosms.

All of the results obtained on the basis of degradation studies of ^{13}C-labelled compounds seem to prove that HILs in the environment are degraded separately and differently, depending on the environments (aqueous, terrestrial) and microorganisms (activated sludge, soil microorganisms). This, in turn, might mean that upon introduction to the environment, these compounds might be only mixtures of cations and anions, as hypothesised in **H1**. Moreover, comprehensive analysis on HILs behaviour in the environment leads to a conclusion that currently used standards (*i.e.*, OECD 301 F) are insufficient in terms of their environmental fate assessment. First of all, the use of aqueous media with activated sludge is not suitable for the degradation studies of compounds that are intended for introduction to agricultural soils, as the half-lives might be falsified by improper degrader's choice. Activated

sludge microorganisms are well-suited for surfactants degradation, as these often enter wastewater treatment plants (*e.g.*, from households), while autochthonous soil microbiota – to herbicides which are commonly present on agricultural soils. Secondly, the use of standard mineralisation assessment does not allow for determination whether CO_2 is a result of degradation of cation, anion, or both. At the same time, the use of only primary degradation assessment does not give insights into compounds metabolites and formation of possibly toxic and persistent metabolites, as it only indicates the disappearance of the signal from main compound. Finally, only the use of appropriate degradation assessment method allows to obtain the full picture of HILs' behaviour in the environment, which is especially important as these compounds are intended for agricultural use.

Block diagram presenting experiments performed within the scientific research



Sorption in soil

The concept of ionic integrity of HILs was further evaluated in another study [1]. Namely, according to the ionic liquids definition, the synthesis should result in obtaining new compound of in the form of close ionic pairs, of unique, designable properties, and not a simple sum of cations and anions properties [12,202–204]. It is promising assumption since designable hydrophobicity of synthesised compounds should serve as possible solution to the problem of volatile herbicidal anions. Namely, increasing cation's hydrophobicity (and in theory also that of an anion) should result in synthesis of compound of reduced mobility in the environment [1]. However, the ionic interactions in HILs have already been discussed, as their stability in pure forms and aqueous solution was discovered to be lower than expected – up to 80 and 60 % was proven to dissociate, respectively [205]. Hence, the impact of introduction of hydrophobic cation into the HILs structure on anion's mobility in soil was studied

(**Section 3.4**), along with cations' effect on the environmental behaviour of HILs [1]. The obtained results indicated that, as expected, with increasing cation's hydrophobicity, the sorption in soil increases (**Table 10**). Though, at the same time, the adsorption of a 2,4-D anion stayed at the same level, irrespective of cation's addition and reflecting sorption parameters of the potassium salt of the herbicide. It is an issue reported earlier [206,207], and seems to confirm the hypothesis **H1**, that HILs act as mixture of separate cations and anions of different characteristics upon introduction to environment. Namely, if they act as ionic liquids and remain strong ionic interactions, the hydrophobic effect of cation should translate also into anion properties.

Table 10 Average sorption values for betaine-based cations of increasing hydrophobicity and 2,4-D anion (adapted and modified from author's published work [1]).

HIL	Average cation sorption [%]	Average anion sorption [%]
[Bet][2,4-D]	3.5 ± 2.5	10.5 ± 2.6
[C_{12}Bet][2,4-D]	94.6 ± 2.1	9.2 ± 2.2
[CAPBet][2,4-D]	86.5 ± 1.8	6.5 ± 2.1

The findings are further confirmed by Freundlich isotherms (**Fig. 23**), where cations and anions are clearly characterised by separate properties. In the case of cations, the changes in hydrophobicity translating into sorption potential are easily observable (**Fig. 23 A**), while at the same time, no significant differences in adsorption were noted for 2,4-D anion, irrespective of its cationic pair (**Fig. 23 B**). This in turn allows the conclusion that cations in HILs do not have a statistically significant impact on anion's sorption in soil, and they are in fact sorbed independently. It is a proof that HILs might not actually remain ionic pairs upon introduction to the environment but rather act as mixtures of separate ions.

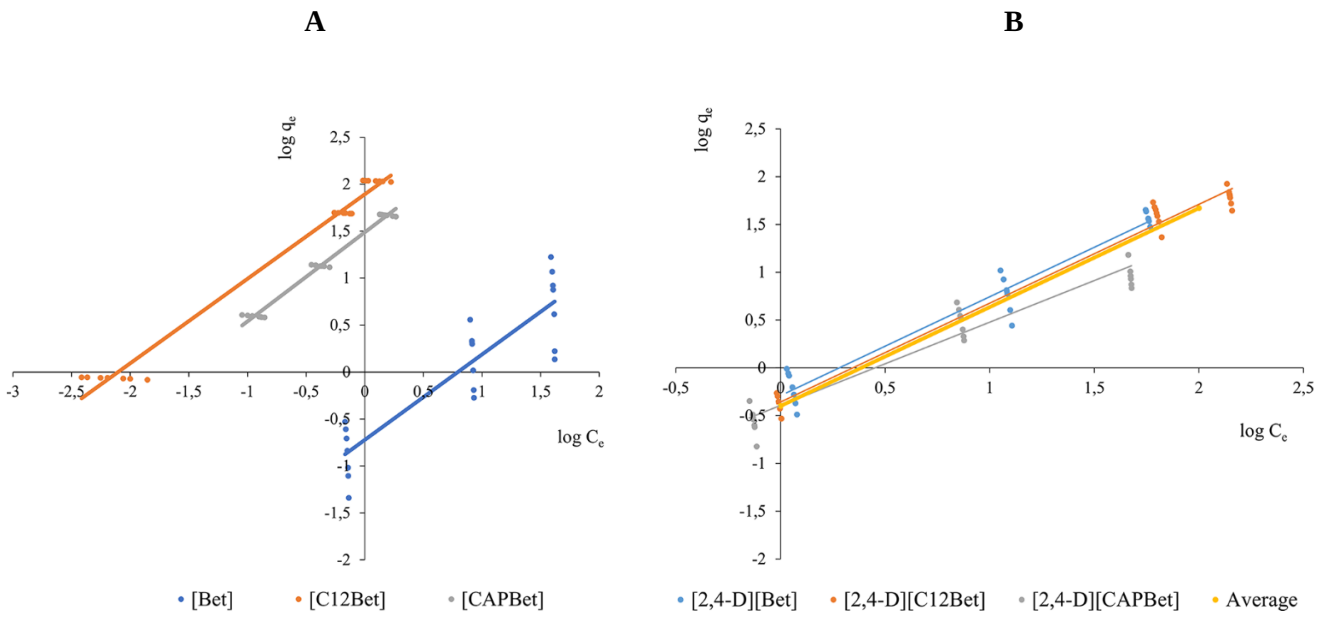


Fig. 23 Freundlich isotherms for betaine-based cations **(A)** and 2,4-D anions in corresponding salts **(B)** **(**adapted from author's published work [1]).

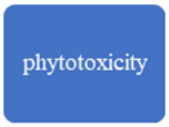
antimicrobial activity

Antimicrobial activity

In addition, the impact of synthesised salts towards microorganisms and plants was evaluated (**Section 3.8.2**). The results of microbial toxicity towards model organisms are presented in **Table 11**. The analysed compounds were deemed non-toxic, which was expected in the case of 2,4-D anion [208–211], as well as hydrophilic betaine (present in cells) [212,213]. Additionally, the alkyl betaines are often used in cosmetics and hence also described as non-toxic [214]; yet their toxicities are slightly higher than in the case of betaine, most probably due to the presence of hydrophobic alkyl chain addition [194,215].

Table 11 Determination of MIC and MBC/MFC values for HILs with betainium-based cations and 2,4-D anions (adapted and modified from author's published work [1]).

		[K] [2,4-D]	**[Bet] [Cl]**	**[CAPBet] [Cl]**	**[C_{12}Bet] [Cl]**	**[Bet] [2,4-D]**	**[CAPBet] [2,4-D]**	**[C_{12}Bet] [2,4-D]**
	MIC	>1000	>1000	1000	1000	>1000	1000	1000
P. putida	MBC	>1000	>1000	>1000	500	>1000	>1000	1000
	MIC	>1000	>1000	>1000	1000	>1000	750	1000
P. aeruginosa	MBC	>1000	>1000	>1000	500	>1000	>1000	>1000
	MIC	>1000	>1000	>1000	1000	>1000	1000	750
E. coli	MBC	>1000	>1000	>1000	500	>1000	>1000	>1000
	MIC	>1000	>1000	750	1000	1000	500	750
S. aureus	MBC	>1000	>1000	>1000	500	>1000	>1000	>1000
	MIC	>1000	>1000	500	500	750	500	500
B. cereus	MBC	>1000	>1000	500	750	1000	500	750
	MIC	>1000	>1000	750	1000	>1000	750	1000
C. albicans	MFC	>1000	>1000	>1000	>1000	>1000	>1000	>1000



Phytotoxicty

The impact of HILs on plants was evaluated *via* effect on plants germination (spring barley and cornflower as model crop plant and weed) (**Fig. 24**) and herbicidal activity of used formulations (**Sections 3.8.3** and **3.12**). In general, no effect of cation selection on herbicidal anion efficacy was observed – only the increasing concentration of herbicidal salts had an impact on phytotoxic effect (seen above 10 mg/kg d.w.s.). This further supports the hypothesis that HILs' behaviour reflects that of a herbicidal anion and surface-active cation. In such a case, when considering non-toxic cations, the phytotoxicity studies should reflect only the impact of herbicidal anion. In fact, within the scope of herbicidal efficacy study (**Table 12**), it has been established that in the case of both plants, salts with betainium cation had stimulatory effect on their growth. For HILs with hydrophobic cations, they had no impact on monocotyledonous spring barley, while for dicotyledonous cornflower, toxic effects were observed (fresh weight reduction of 30 % relative to reference for [C_{12}Bet] and complete plant destruction for [CAPBet].

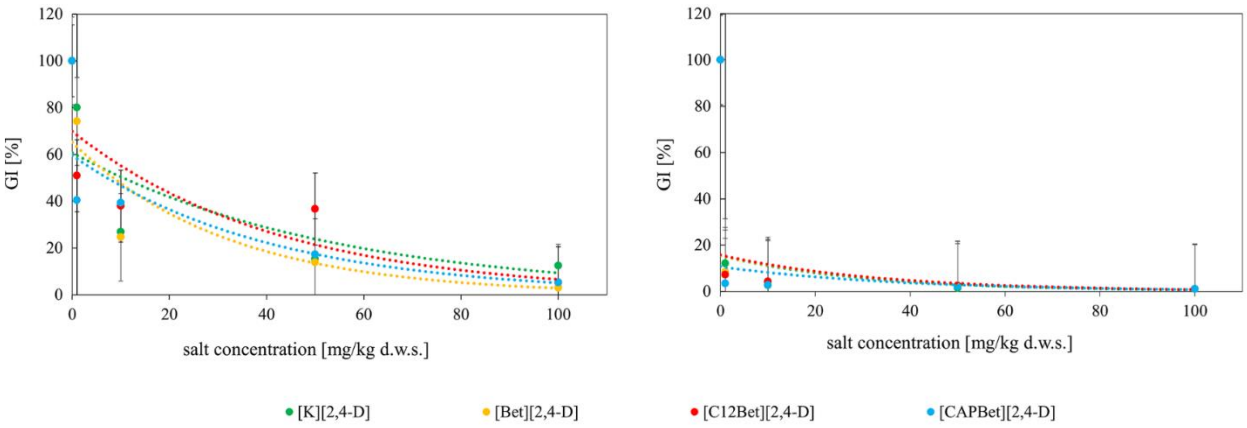


Fig. 24 Effect of concentration of 2,4-D-based HILs on germination index (GI) of spring barley (left) and cornflower (right) (adapted from author's published work [1]).

Table 12 The fresh weight of spring barley and cornflower at the end of field experiment (adapted from author's published work [1]).

treatment	fresh weight [g]	
	spring barley	cornflower
untreated soil	8.25 ± 0.25	1.52 ± 0.09
[K][2,4-D]	8.71 ± 0.11	0.17 ± 0.05
[Bet][2,4-D]	9.39 ± 0.23	0.38 ± 0.02
[CAPBet][2,4-D]	7.94 ± 0.15	[-]
[C_{12}Bet][2,4-D]	7.42 ± 0.3	0.12 ± 0.04

molecular studies

Assessment of bacterial community structure

Finally, the impact of HILs on rhizosphere microbial community was evaluated (**Section 3.10.1**). Here, the differences in the microbiome structure depending on cation's used were clearly visible (**Fig. 25**). In the untreated soil, classes of *Gammaproteobacteria* (43.4 %) and *Bacilli* (41.7 %) dominated. At a family level, *Bacillaceae* (41.0 %) were predominant, followed by *Pseudomonadaceae* (16.6 %), *Moraxellaceae* (14.7 %) and *Enterobacteriaceae* (12.0 %). The herbicidal treatment with [K][2,4-D] caused the predominance of *Bacillaceae* (86.5 %), with simultaneous decrease in the abundance of bacteria from other families. The impact on microbial community was the smallest in the case of [Bet][2,4-D], correlating well with the observed stimulatory effect on plants growth. However, when betaine structure was

modified with hydrophobic alkyl chain addition, the impact on microbial community structure was significant and differ from observations made in the case of anion's potassium salt. Namely, the *Pseudomonadaceae* family members were significantly more abundant, reaching 50 % for [CAPBet] and 75 % for [C_{12}Bet]. It is an interesting observation, since some of the *Pseudomonas* species are known as 2,4-D degraders and also are capable of functioning in surfactant-rich environmental niches or even producing biosurfactants themselves [216].

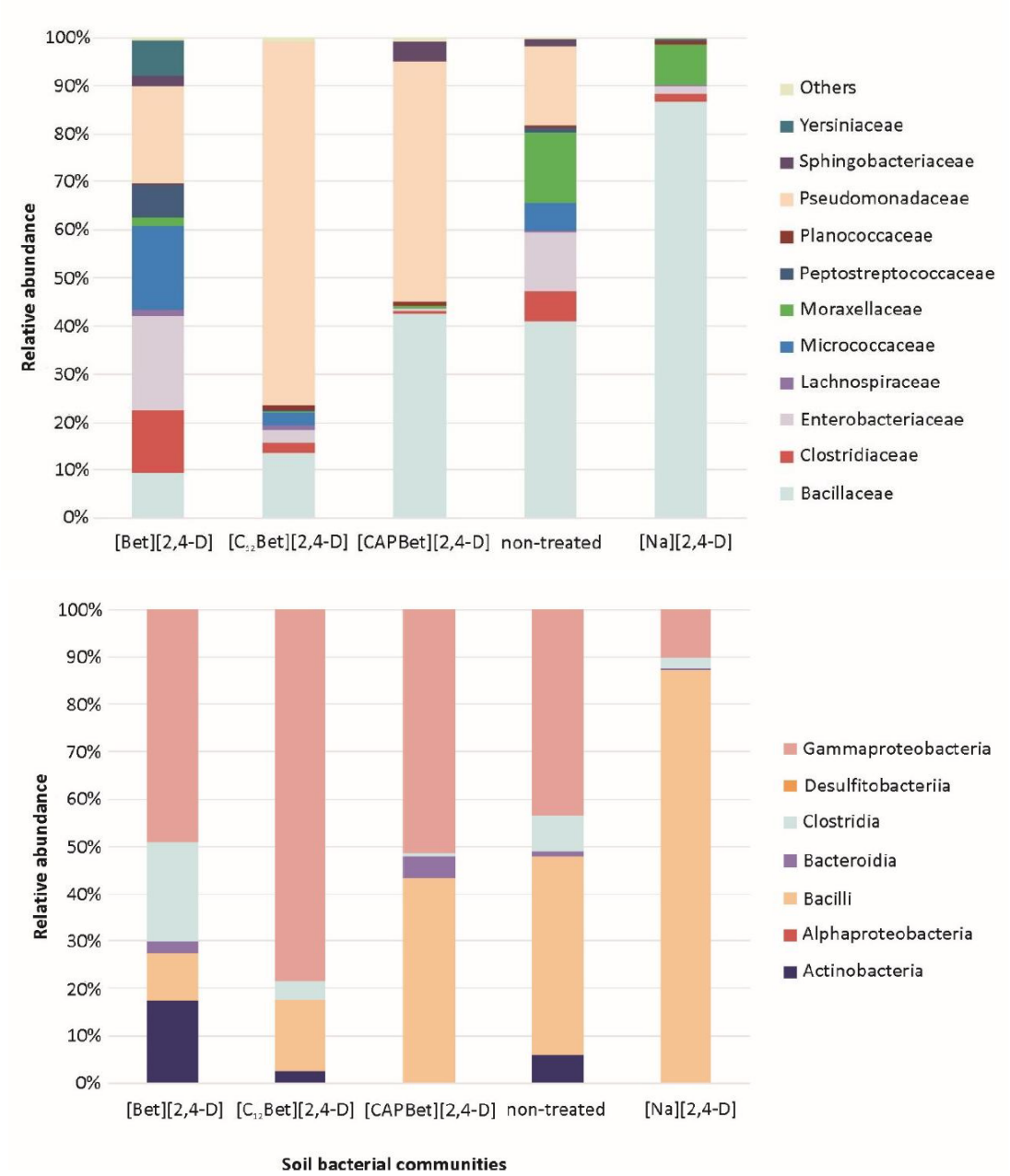


Fig. 25 Relative abundance of major bacterial families (**A**) and classes (**B**) in the rhizosphere bacterial communities treated with analysed HILs (adapted from author's published work [1]).

Those results prove the validity of hypothesis **H1**, as they clearly indicate that herbicidal ionic liquids upon introduction to the environment do not act as ionic compounds but rather as separate moieties – surface-active cations and herbicidal anions. Their degradation is also ion-depended, as it has been proven that cations are degraded preferentially in aqueous environment by activated sludge microorganisms, while herbicidal anions are degraded preferentially in agricultural soils with native microbiota. These claims were further confirmed by sorption studies of herbicidal anion paired with non-toxic cations of different sorption potential, where it has been proven that these ions in fact act separately in soils and cation has no impact whatsoever on anion's properties.

Bearing in mind these findings, additional experiments were performed within the scope of following Ph.D. thesis (results are in the publishing process), aiming in development of cheap degradation assessment technique for HILs and gaining further insight into cation's role and effects in these herbicidal application forms (**H2**). Moreover, basing on poor degradation efficiencies of previously tested HILs, the relevance of bioaugmentation approach was discussed (**H3**).

Block diagram presenting experiments performed within the scientific research

Primary degradation vs bioavailability in soil

The degradation of HILs with glyphosate anion has been evaluated *via* modified OECD 301 F mineralisation test (**Section 3.6.1**) combined with chromatographic determination of primary degradation efficiencies (**Section 3.7**). However, differently from previous approaches, degradation tests were also compared to the HILs' bioavailability for microbial degradation (**Section 3.3**), as presented in **Table 13**. Interestingly, despite the fact that recorded primary degradation efficiencies showed almost complete degradation of ions constituting glyphosate-based HILs, bioavailability fractions are disturbing. Namely, the herbicidal anion was highly available for microorganisms, irrespective of its cationic pair, which is consistent with

previous studies showing their independence of each other [1,2]. At the same time, hydrophobic cations (*i.e.*, all except hydrophilic choline) were characterised by low bioavailability of 13–20 % (**Table 13**). The high sorption of hydrophobic surfactants to various materials (*e.g.*, minerals, biomass, natural sediments, proteins) was discussed by many researchers [64,65,217], so these results are not surprising. However, they brought attention to the fact that primary degradation values, often used for HILs' degradation potential assessment, might not actually reflect their degradation but rather recovery of bioavailable part, *i.e.*, the part that is accessible for microorganisms and hence susceptible to microbial degradation.

Table 13 Primary degradation of examined HILs in comparison with their bioavailability (28 days).

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

In terms of removing contaminants from the environment, many authors have discussed sorption of xenobiotics onto soils [218–222]. Specifically, the addition of surface-active chemicals can stimulate the desorption of matrix-bound xenobiotics [218–222]. Only then are the contaminants bioavailable and readily degraded by microorganisms. Interestingly, the structure of HILs includes cationic surfactants that are known for their high adsorption potential. Additionally, it has been proven by previously performed studies that hydrophobic cations are subjected to higher sorption than hydrophilic ones [1]. In turn, their use in HILs structure should lead not only to an almost complete lack of effect in stimulating herbicide desorption from the soil, but also to an increase in the adsorption of cations in the soil, leading to an accumulation of contamination, especially when compared to commercially used herbicidal formulations with anionic or non-ionic adjuvants.

Even considering cations and anions constituting HILs as mixtures of separate ions, the cations' presence might have a vast impact on the anion's degradation.

Namely, since QACs are known to have ability to form complexes with anions in soils [67–69], they might bind herbicidal anions to soil, in turn decreasing their degradation, especially in the case of anions that tend to sorb to soils [223]. Moreover, the toxic effect of cations towards microorganisms might also influence the ions' degradation efficiencies in the environment [69–72]. Taking into account these considerations, the conclusion arose that the primary degradation studies alone do not allow to distinguish between sorption, complexation or other physical phenomena that might take place in soils from the actual xenobiotic's degradation [64,65,69].

Mineralisation and bioaugmentation

With these results in mind, one should expect ultimate biodegradation (mineralisation) values to be significantly lower than primary biodegradation. Indeed, in contrast to data close to 100 %, presented mineralisation efficiencies (**Table 14**) of glyphosate-based HILs were within the range of 15–53 %.

Table 14 Mineralization efficiencies of glyphosate-based HILs (90 days).

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

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

Since one of the tested hypotheses (H3) states that bioaugmentation should lead to improved degradation, providing lack of cation's toxic effect, bioaugmentation approach has been proposed in mineralisation studies. Within the scope of this experiment, also the addition of previously isolated microbial community capable of glyphosate degradation was studied in terms their effect on mineralisation efficiencies (**Sections 3.5.1** and **3.6.1**). It has been proven that bioaugmentation improves degradation in systems with QACs [71]. In the mineralisation study with glyphosate-based HILs, the addition of enrichment cultures indeed allowed to reach higher

degradation efficiencies, but, at the same time, not as significant as expected (**Fig. 26**). Overall, the only factor affecting CO_2 emissions was the initial carbon content of the samples, *i.e.*, samples containing [K][Glyph], which has only three carbon atoms in its structure, were characterised by the lowest CO_2 emissions. The approach to introduce specialised microorganisms into soil containing compounds with high bioavailability characteristics ([K][Glyph] and [Chol][Glyph]) showed increased mineralisation. However, in the case of poorly bioavailable compounds (HILs with hydrophobic cations), the bioaugmentation approach did not result in a significant increase in mineralisation efficiencies, as the contaminants were simply not accessible for the microorganisms.

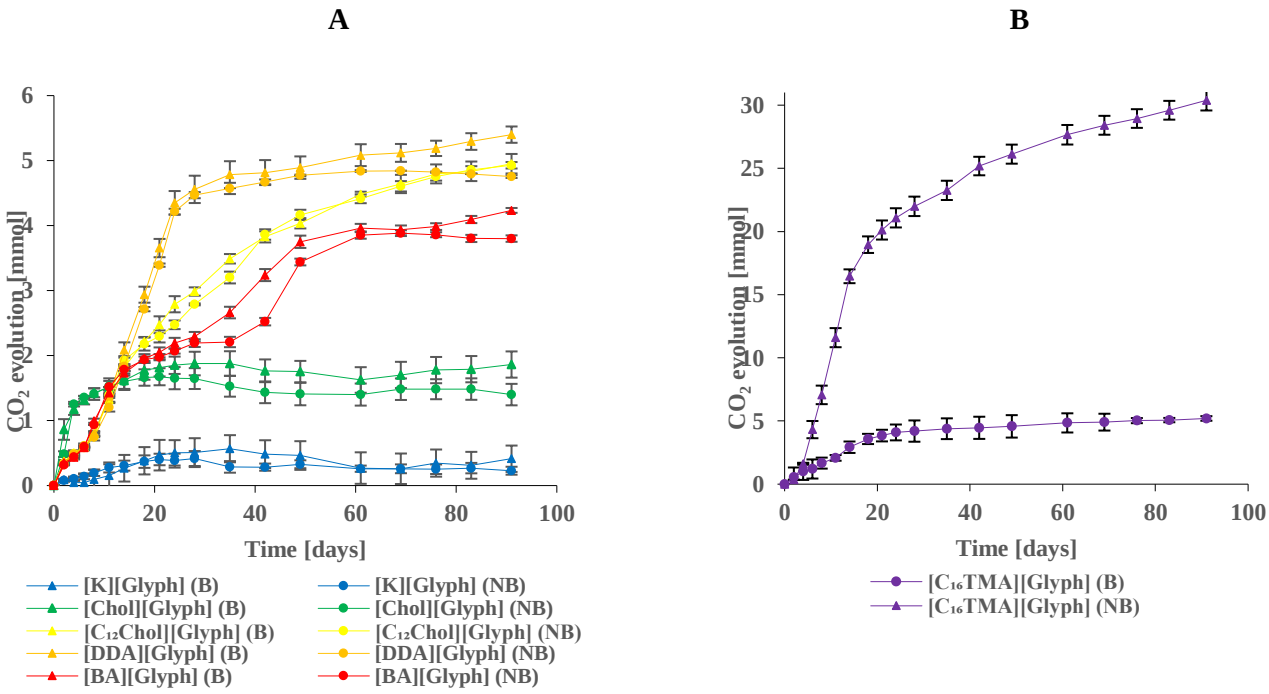


Fig. 26 CO_2 evolution curves for tested HILs **(A)**. The [C_{16}TMA][Glyph] mineralization curves are presented as a unique example **(B)**. NB – non-bioaugmented samples, B – bioaugmented samples. The CO_2 evolution values of respective bioaugmented and non-bioaugmented controls were subtracted from curves in order to illustrate signals resulting only from compounds' degradation.

antimicrobial activity

Antimicrobial activity

The results of antimicrobial activity of tested HILs towards enrichment culture used in bioaugmentation study (**Section 3.8.1**) are presented in **Table 15**. Glyphosate in the form of potassium salt was harmless to microorganisms, which is consistent with other reports of its toxicity [224,225]. The addition of choline, a naturally occurring hydrophilic cation, to the formulation does not result in increased toxicity [226]. As it has been established, choline is a precursor to the neurotransmitter acetylcholine and, as a compound present in metabolic pathways, is not considered to be toxic per se [226–228]. However, the introduction of hydrophobic cationic surfactants leads to a significant increase in the toxicity of the resulting compounds. Even the simple addition of a hydrophobic aliphatic chain to choline (C_{12}Chol) leads to a substantial decrease in EC_{50} values. Further modifications and formation of hydrophobic compounds translates into an even greater increase in toxicity. In addition, the toxicity of HILs appears to reflect the toxicity of cations only. That is, glyphosate alone proved to be non-toxic, while the cations with chloride anions and their corresponding HILs (with glyphosate anion) were very similar in toxicity. This in turn may further support the hypothesis that the cations and anions in HILs act as separate moieties. In any other case, the toxicity of the whole compound would be different from the toxicity of the cations and anions, rather than a simple sum of their toxicity.

Table 15 Antimicrobial activity of HILs with glyphosate anion and their respective precursors towards enrichment culture used in the mineralisation experiment.

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

[a] The tested concentrations were set by active substance (Glyph); and the amounts of cations in precursors are equal to those in HILs. [b] Classification of toxicity according to Passino and Smith, 1987 [229]; >1000 mg/L – harmless, 100–1000 mg/L – practically harmless, 10–100 mg/L – slightly toxic, 1–10 mg/L – moderately toxic, <1 mg/L – toxic.

These results are consistent with the observations of some authors [12,90,95,230] who consider the ability to apply lower doses of herbicides on fields as an advantage of HILs. This is a reasonable argument, as the high toxicity of the cations combined with the toxicity of the herbicide anions would result in formulations that are destructive to weeds. However, as discussed earlier, these substances may be strongly adsorbed to the soil when released into the environment, thus reducing their biodegradation potential. This in turn raises the question whether combinations of herbicide anions with cationic surfactants are desirable in terms of environmental fate.

Assessment of bacterial community structure

The next study carried out to determine changes in the structure of the bacterial community isolated from the experimental samples was a sequencing analysis of highly variable 16S rRNA regions (**Section 3.10.1**). At the class level, the soil bacterial community was dominated by members of the *Bacilli* and *Gammaproteobacteria*, followed by *Clostridia*. No trends could be clearly observed when it comes to the impact of bioaugmentation and cation's choice on the bacterial community structure. However, the structure of microbial community in samples treated with herbicides differ from untreated controls (**Fig. 27, 28**).

Samples non bioaugmented

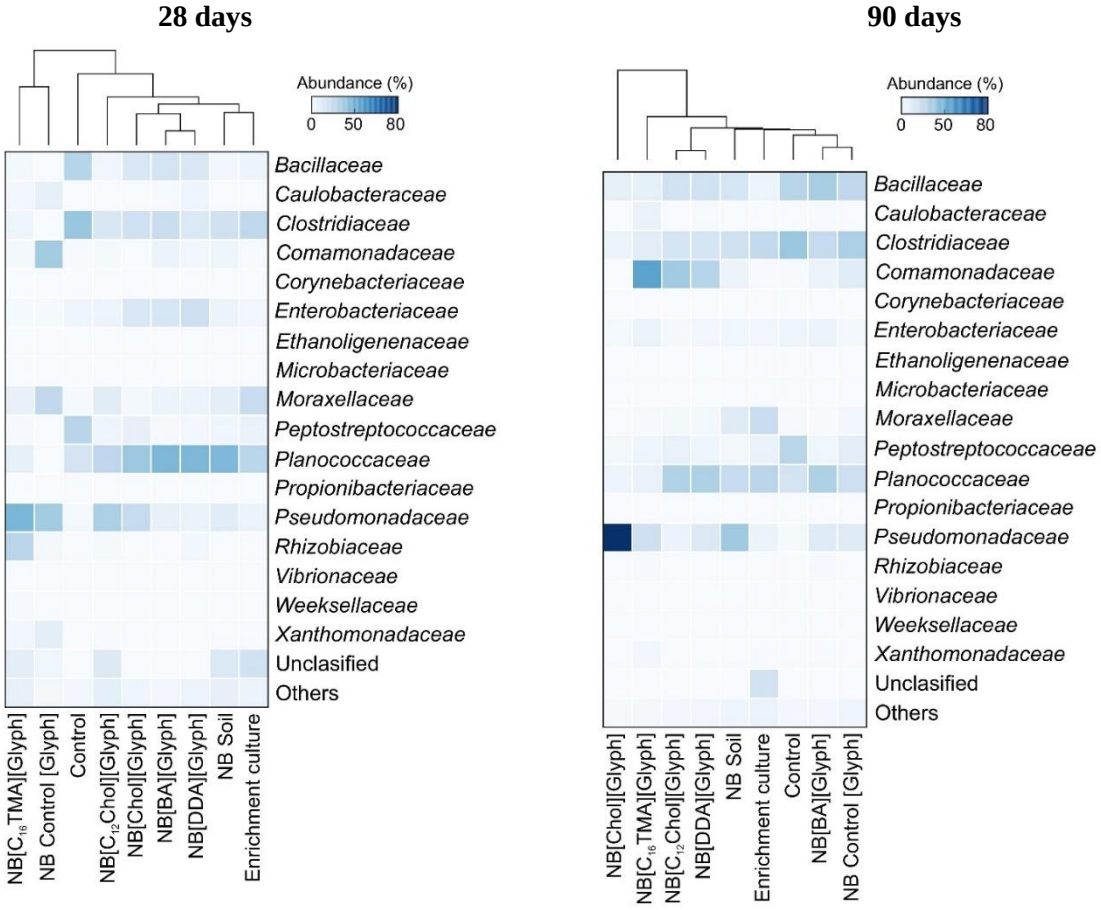


Fig. 27 Heatmap of hierarchical clustering of dominant (>5 % in at least one sample) family-level bacterial microbiome composition profiles. ZOTUs not assigned to families were grouped as Unclassified, while ZOTUS with <5 % abundance was grouped as Others. Darker colour represents higher abundance in the samples.

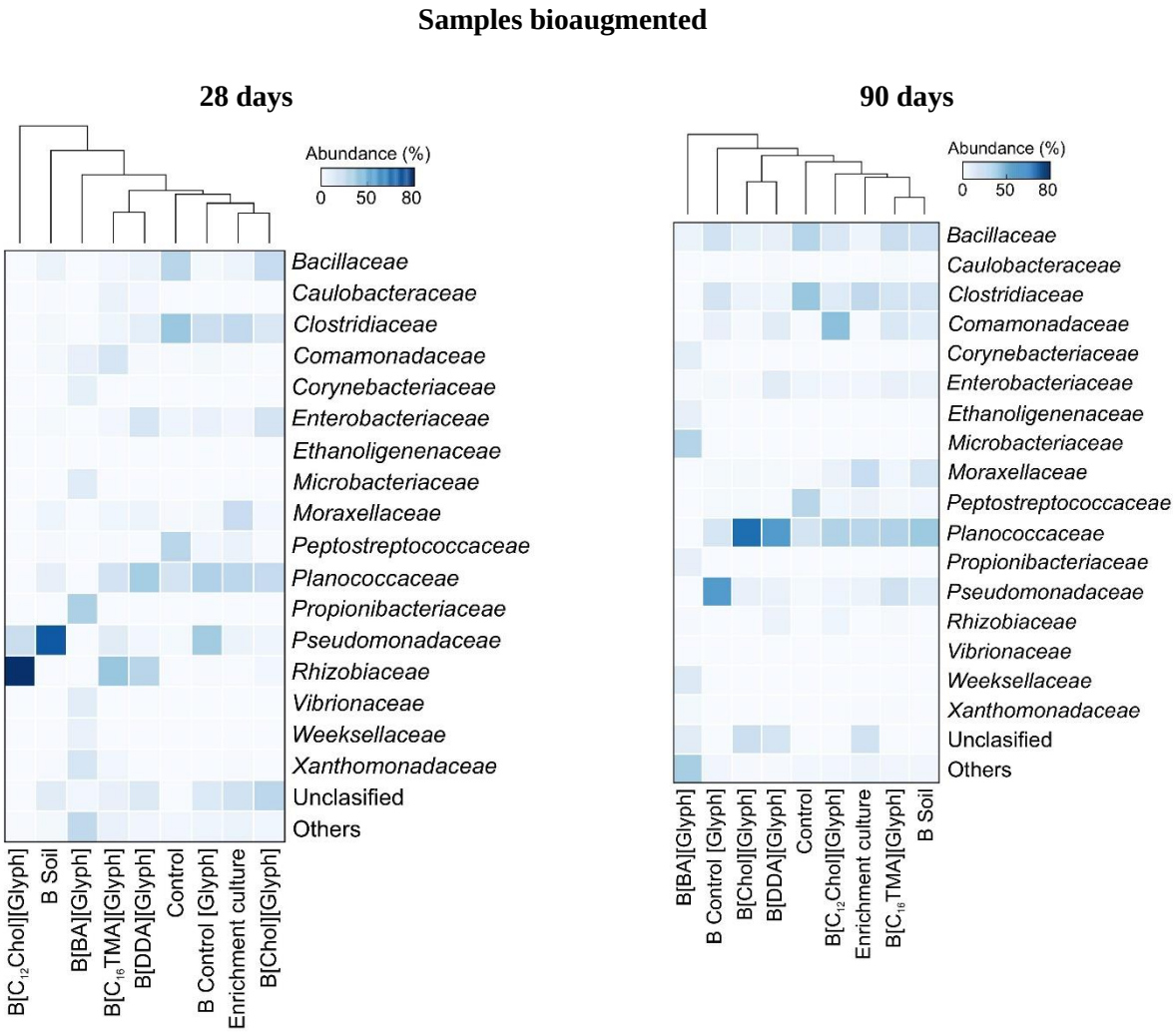


Fig. 28 Heatmap of hierarchical clustering of dominant (> 5 % in at least one sample) family-level bacterial microbiome composition profiles. ZOTUs not assigned to families were grouped as Unclassified, while ZOTUS with < 5 % abundance were grouped as Others. Darker colour represents higher abundance in the samples.

In addition, an attempt has been made to estimate the abundance of key glyphosate biodegradation genes (*soxA* and *phnJ*) in the bacterial communities studied using PICRUSt2 (**Section 3.10.1**). The relative abundance of the gene *phnJ* varied mainly between 0.001 and 0.005 %. The peak abundance (0.029 %) was observed after 28 days in soils treated with [C_{12}Chol][Glyph] in which the bacterial community was dominated by *Rhizobiaceae*. Overall, relative abundance of the *phnJ* gene was observed to be >0.005 % in those bacterial communities with relatively high abundance (20–30 %) of *Rhizobiaceae*. This observation is not surprising due to the fact that the *phnJ* gene encodes an alphaproteobacterial C-P bond lyases [231]. As for *soxA*, its relative abundance in most samples was approx. 0.05 %. The highest

predicted levels were found in non-bioaugmented soils treated with [C_{12}Chol][Glyph] (28 days), while the lowest predicted values – for soils bioaugmented and treated with [Chol][Glyph], [DDA][Glyph] and [BA][Glyph] (28 and 90 days). The detrimental effects of these treatments on *soxA* abundance can be clearly seen in **Fig. 29**. However, the reason for the significant decrease in *soxA* abundance remains unclear, as the treatment-specific effect could not be determined.

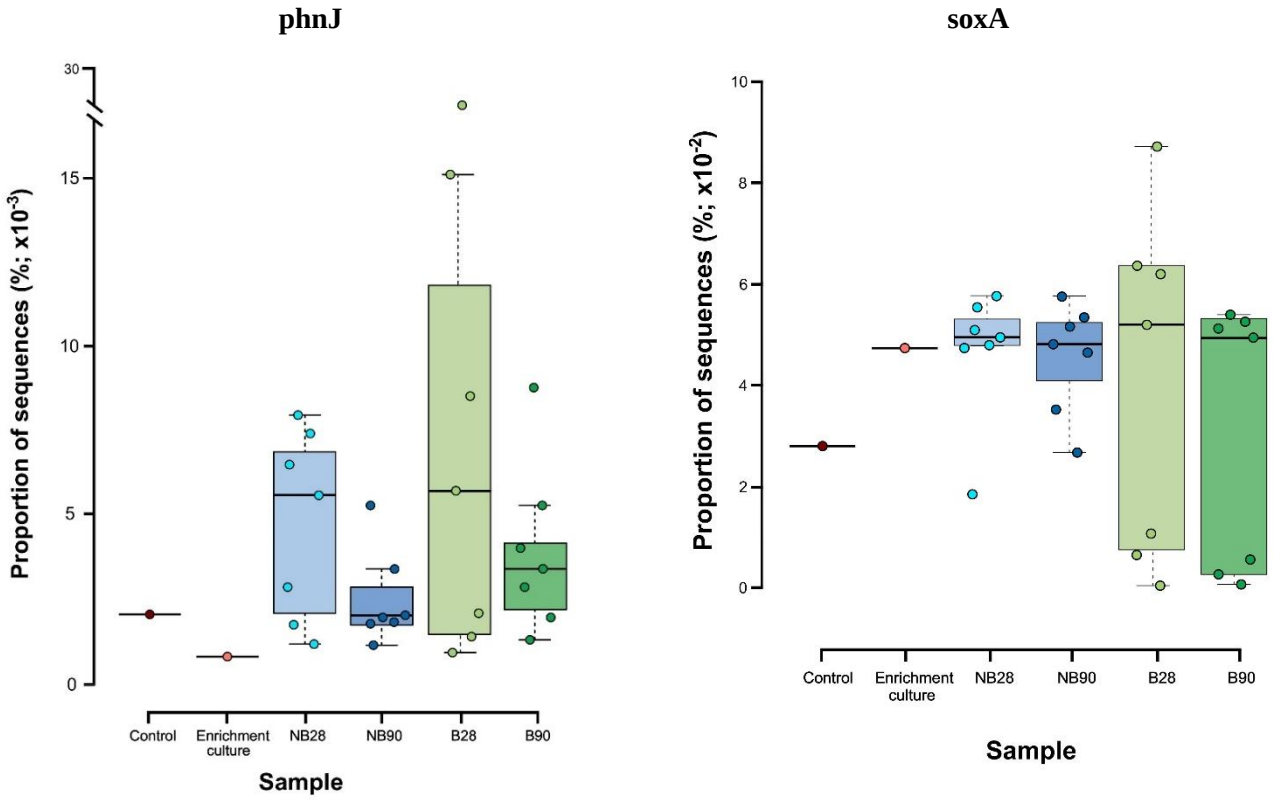


Fig. 29 Boxplot representation of relative abundance of the soxA/phnJ ortholog related to KEGG. B28: bioaugmented samples after 28 days, B90: bioaugmented samples after 90 days, NB28: non-bioaugmented samples after 28 days, NB90: non-bioaugmented samples after 90 days.

Block diagram presenting experiments performed within the scientific research

Primary degradation

Similar studies were performed for HILs with 2,4-D anion. When considering primary degradation (**Section 3.7**) results, the herbicidal anion in the form of sodium salt was degraded in approx. 60 % within 90 days (**Table 16**). However, pairing the anion with cation, either of natural origin or not, resulted in the decrease in degradation efficiencies of anion. The impact of choline and carnitine cations was the smallest, yet still degradation was decreased (approx. to 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 [3,88,94].

When it comes to the impact of bioaugmentation (**Sections 3.5** and **3.6.1**), the addition of isolated microorganisms resulted in generally higher degradation efficiencies of HILs. However, in the case of highly hydrophobic HILs with cations of C_{12}Bet, CAPBet and BTMA, their degradation was not improved. It is consistent with results obtained for glyphosate-based HILs, where the properties of cation were the bioaugmentation's limiting factor.

Table 16 Primary degradation of tested HILs with 2,4-D anion after 90 days.

HILs	Cation		Anion	
	non-bioaugmented	bioaugmented	non-bioaugmented	bioaugmented
[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 %

Mineralisation and bioaugmentation

The mineralisation experiment (**Section 3.6.1**) results were illustrated *via* CO_2 evolution curves (**Fig. 30**), where the main thing observed was stimulating effect of bioaugmentation on degradation. However, generally the highest CO_2 evolution values should be obtained for carbon rich HILs, *i.e.*, [CAPBet][2,4-D], [C_{12}Bet][2,4-D], [C_{12}Chol][2,4-D] and [TBA][2,4-D]. Interestingly enough, the highest CO_2 evolution was observed for HIL with CAPBet cation, followed by these with betaine, carnitine and choline cations. This might mean that the addition of hydrophobic modifications to cations structures, as well as introduction of quaternary amines might negatively affect degradation processes, which is consistent with previously discussed results.

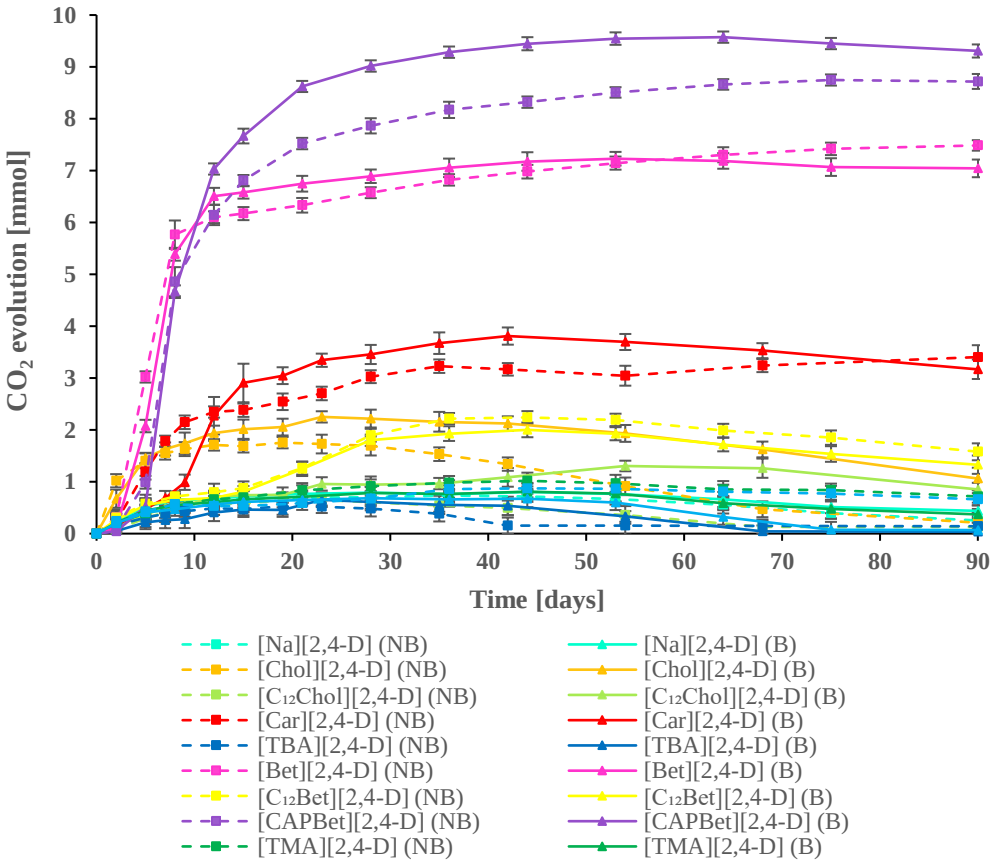


Fig. 30 Mineralisation curves (CO_2 evolution) for analysed HILs with 2,4-D anion during 90 days of experiment. The CO2 evolution values of respective bioaugmented and non-bioaugmented controls were subtracted from curves in order to illustrate signals resulting only from compounds' degradation.

As presented in the **Table 17**, bioaugmentation with specialised microorganisms (**Sections 3.5** and **3.6.1**) in each case resulted in the increase in mineralisation efficiencies. Sodium salt of herbicidal anion was however mineralised only in approx. 3 and 33 % (non-bioaugmented and bioaugmented samples, respectively) by the end of experiment. This might be linked to the fact that soil utilised in the experiment had no previous contact with 2,4-D herbicide, and former research has already proven that xenobiotics can be effectively degraded only in the environment adapted to their presence [2]. Additionally, 2,4-D is considered a mobile compound in soils, so microorganisms capable of its degradation simply might not be present in soils as frequently [135]. 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 (C_{12}) to cations of natural origin, as well as synthetic quaternary amines (TBA, TMA, BTMA, see **Section 3.1**, **Table 2**) resulted in substantially lower mineralisation efficiencies. It is an effect well described in the literature [65], and it confirms the toxic effect of cations to the overall well-being of microbiome, and consequently – lower degradation.

Table 17 Mineralisation efficiencies (after 28 days and 90 days).

HILs	28 days		90 days	
	non-bioaugmented	bioaugmented	non-bioaugmented	bioaugmented
[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]	7.1 ± 0.5 %	7.3 ± 0.8 %	6.6 ± 0.4 %	14.2 ± 0.9 %

The CO_2 evolution results correlate well with primary degradation of HILs. While primary degradation gives only information on the content of compound in its main form (be it cation or anion), mineralisation results allow to determine total amount of carbon utilised by microorganisms. Comparing results from mineralisation and biodegradation experiments, it can be observed that microorganisms' addition in each case resulted in higher CO_2 production, *i.e.*, higher degradation efficiency. Moreover, bioaugmentation with microorganisms specialised in 2,4-D degradation allowed to reduce the negative impact of cation on anion's degradation, which is visible both by an increase in mineralisation efficiencies and anion's primary degradation. This, in turn, might be attributed to the fact that, contrary to HILs with glyphosate anion, synthesised 2,4-D-based compounds were generally non-toxic towards enrichment culture capable of 2,4-D degradation (**Sections 3.5.1** and **3.8.1**) used in the bioaugmentation approach (**Table 18**).

antimicrobial activity

Toxicity

Table 18 Toxicity (EC_{50}) towards enrichment culture utilized in the experiment of HILs with 2,4-D anion.

HIL	EC_{50} [mg/L][a]	Toxicity[b]
[Na][2,4-D]	>1000	harmless
[Car][2,4-D]	>1000	harmless
[Chol][2,4-D]	>1000	harmless
[C_{12}Chol][2,4-D]	36.3 ± 0.8	slightly toxic
[Bet][2,4-D]	>1000	harmless
[C_{12}Bet][2,4-D]	321.7 ± 8.7	practically harmless
[CAPBet][2,4-D]	54.9 ± 0.1	slightly toxic
[TBA][2,4-D]	>1000	harmless
[TMA][2,4-D]	>1000	harmless
[BTMA][2,4-D]	>1000	harmless

[a] The tested concentrations were set by active substance (2,4-D). [b] Toxicity classification according to Passino and Smith, 1987 [229]; >1000 mg/L – harmless, 100–1000 mg/L – practically harmless, 10–100 mg/L – slightly toxic, 1–10 mg/L – moderately toxic, <1 mg/L – toxic.

molecular analyses

Assessment of bacterial community structure

Next, a sequencing analysis of a highly variable 16S rRNA region was performed (**Section 3.10.2**) in order to determine changes in the structure of the bacterial community isolated from the experimental samples (**Fig. 31**). Control (non-bioaugmented soil, nontreated) 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* families ranged from 6.3–9.0 %. The proportion of other bacterial types did not exceed 5.0 %. The application of the bioaugmentation resulted in changes in the composition of the soil microbiome. Namely, 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 %.

The addition of the [Na][2.4-D], [Car][2.4-D] and [Chol][2.4-D] to the soil increased the proportion of *Bacterioidetes* (20.9–23.5 %) and *Firmicutes* (8.7–17.6 %), while there was no significant change in the proportion of bacteria belonging to the *Proteobacteria* type (39.4–44.3 %). Though in the bioaugmented samples, the

proportion of *Proteobacteria* and *Bacterioidetes* decreased (to 43.1–46.5 % and 16.8–19.8 %), respectively, while *Firmicutes* ranged from 16.0–22.2 %. The addition of the other ionic liquids to the soil reduced the proportion of *Proteobacteria* in the 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 %).

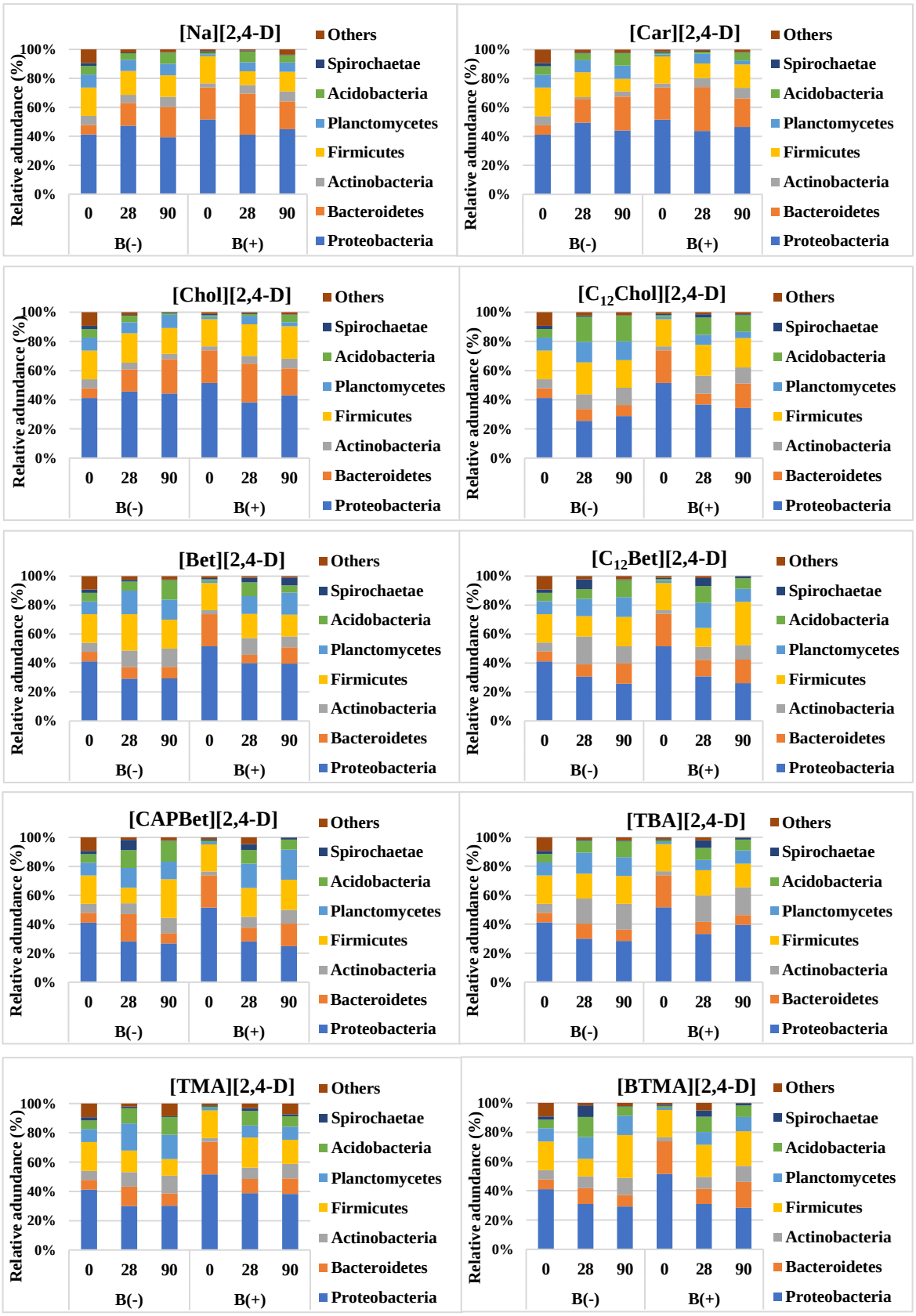


Fig. 31 The relative abundances of bacterial phylum in isolated soil samples. B(-) – non-bioaugmented samples, B(+) – bioaugmented samples.

Furthermore, a beta-biodiversity analysis was also carried out to compare the biodiversity of the analysed environments with each other (**Fig. 32**). This analysis was performed on the basis of the determined Bray-Curtis index. This index is a measure of the similarity of two populations based on quantitative and qualitative OTU analysis (**Section 3.10.2**). The points representing the soil microbiome ("Soil" – non-bioaugmented, "Soil+" – bioaugmented) lie at a considerable distance from each other, indicating that the microbiome of the bioaugmented soil has changed significantly with respect to the control soil. Three clusters of microbiomes are located between these points, each located in a different plane. The points closest to the 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 to the soil itself and to the bioaugmented soil. In contrast, the microbiomes of [C_{12}Chol][2,4-D], [TBA][2,4-D], [TMA][2,4-D] [Bet][2,4-D] in both soil with and without bioaugmentation had microbiome structure more similar to Soil+.

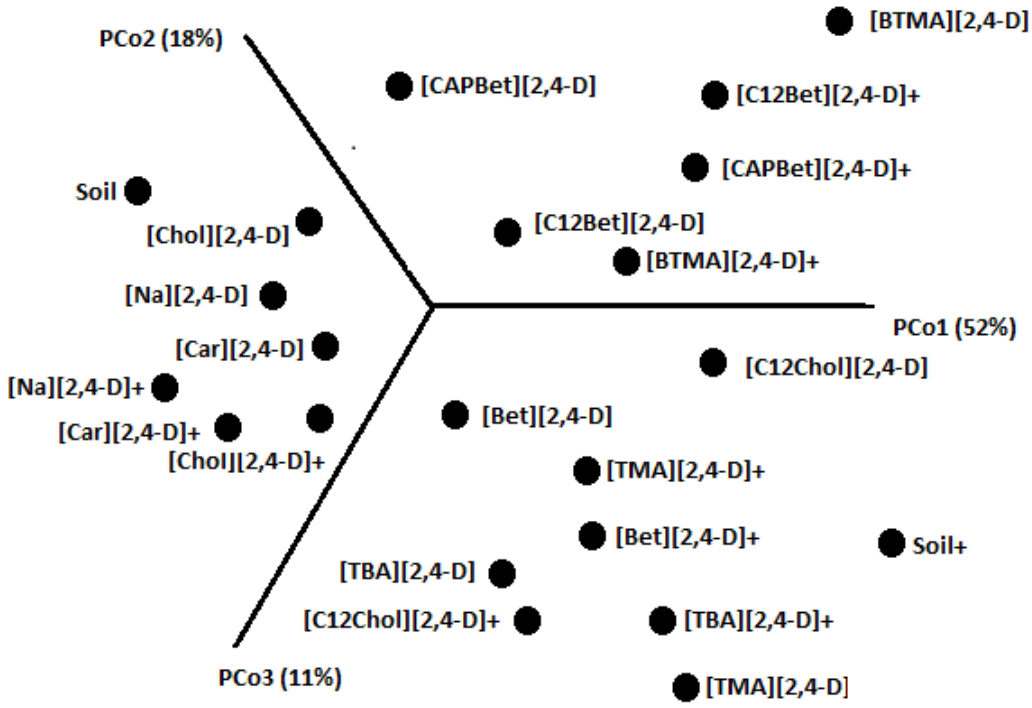


Fig. 32 Principal coordinate analysis (PCoA) based on the Bray–Curtis dissimilarity metrics showing the distance in the bacterial communities between analysed samples.

The gene potential of the microorganisms present in the soil having contact with respective herbicide ionic liquids and responsible for the first stage of 2,4-D biodegradation was compared with their presence in the control samples (soil untreated) (**Section 3.10.2**). Three classes of the *tfdA* gene were analysed. A significant effect of bioaugmentation on the increase in the number of *tfdA* genes in the gene pool of soil microorganisms was evident (**Fig. 33**). Bioaugmentation caused an increase in the number of all analysed genes visible at day 28 of the process. At day 90, the number of genes responsible for biodegradation decreased in all analysed samples and often approached the gene pool of the control sample. In the samples analysed, the number of copies of the gene encoding TFD A class III was the highest, followed by class II, and the number of genes encoding class I enzymes was the lowest. In addition, the addition of herbicide ionic liquids to non-bioaugmented soil did not change the number of *tfdA* genes in the environmental gene pool.

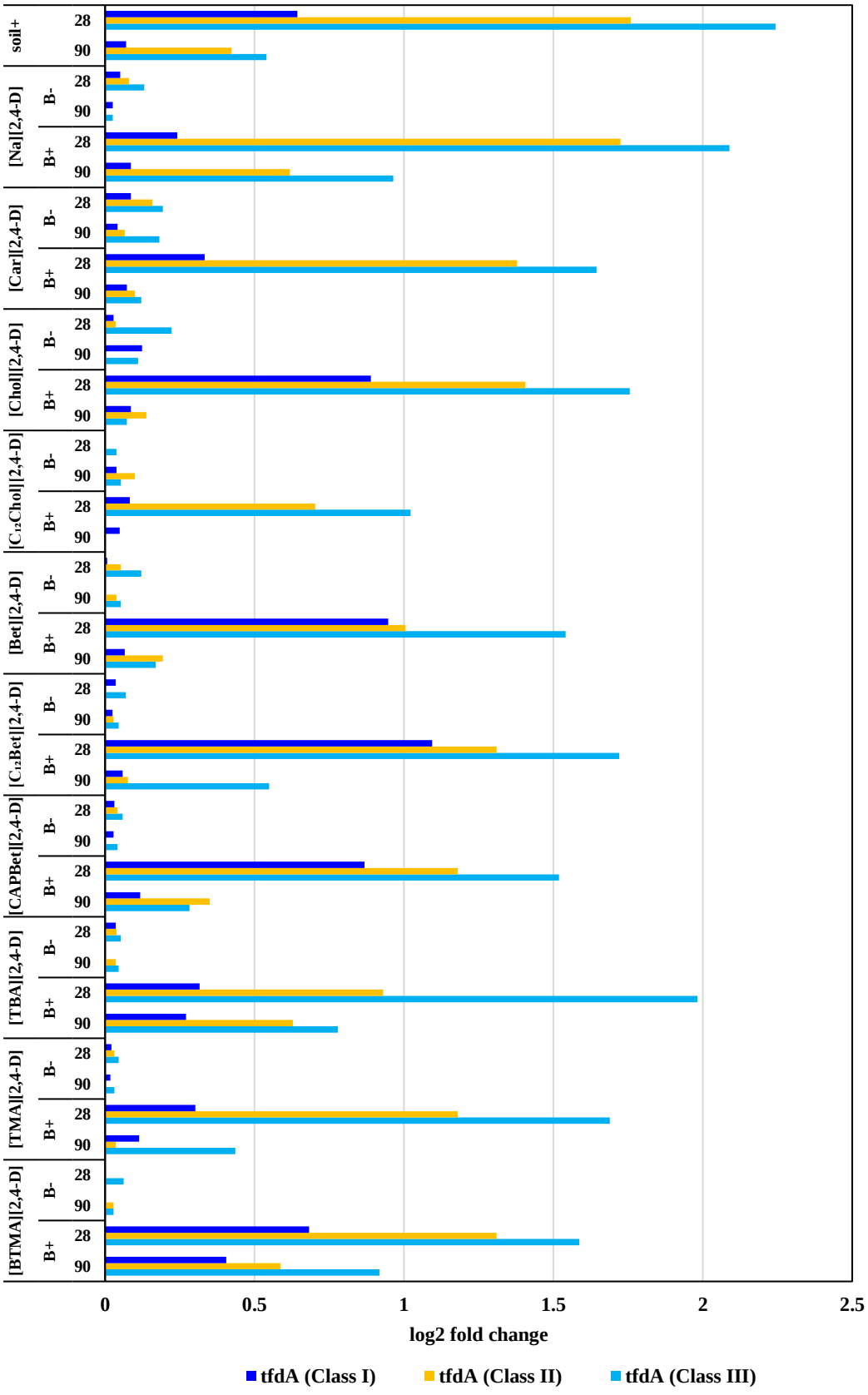
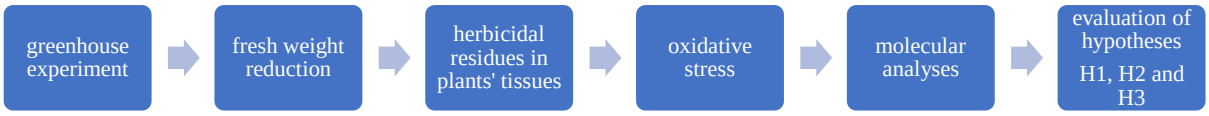


Fig. 33 Number of *tfdA* genes of microorganisms isolated within the scope of mineralisation experiment.

Block diagram presenting experiments performed within the scientific research



Greenhouse experiment

The final study performed within the scope of Ph.D. thesis was greenhouse experiment for rapeseed growing in bioaugmented and non-bioaugmented soils, treated with glyphosate-based HILs (**Section 3.11**). The aim of this study was to determine the effect of different HILs on plants, as well as the bioaugmentation impact on herbicidal efficiency and microbial community composition within and near plants. Unfortunately, the final sequencing analysis of 16S rRNA gene hypervariable fragment MiSeq is still ongoing and hence the results cannot be fully presented in the Ph.D. thesis.

Fresh weight reduction

The reduction in fresh weight (**Section 3.11**) was presented on **Fig. 34**. Here, it can be observed that bioaugmentation approach did not translate into lower fresh weight reduction than in the case of the samples non-bioaugmented. The growth promotion observed for [K][Glyph] NB (14 DAT) and [Chol][Glyph] NB (3 DAT), as well as other variations can be explained by the compounds' structure. Namely, [K][Glyph] did not comprise surfactant in its structure, hence its adherence to plants' surface might have been non-uniform, and resulting weight reduction might have been subjected to greater error between plant's replications. In the case of [Chol][Glyph], the salts with choline cation are known for their poor herbicidal efficacy or even plant growth promotion properties [44,127]. Generally, the [C_{12}Chol][Glyph] exhibited herbicidal efficacy at the same level as the commercial formulation, Roundup 360 SL, and generally higher than [K][Glyph] and [Chol][Glyph]. This, in turn, might be a

result of high phytotoxicity of hydrophobic QAC, which effect was similar to commercial formulation which incorporate adjuvants in its mixture.

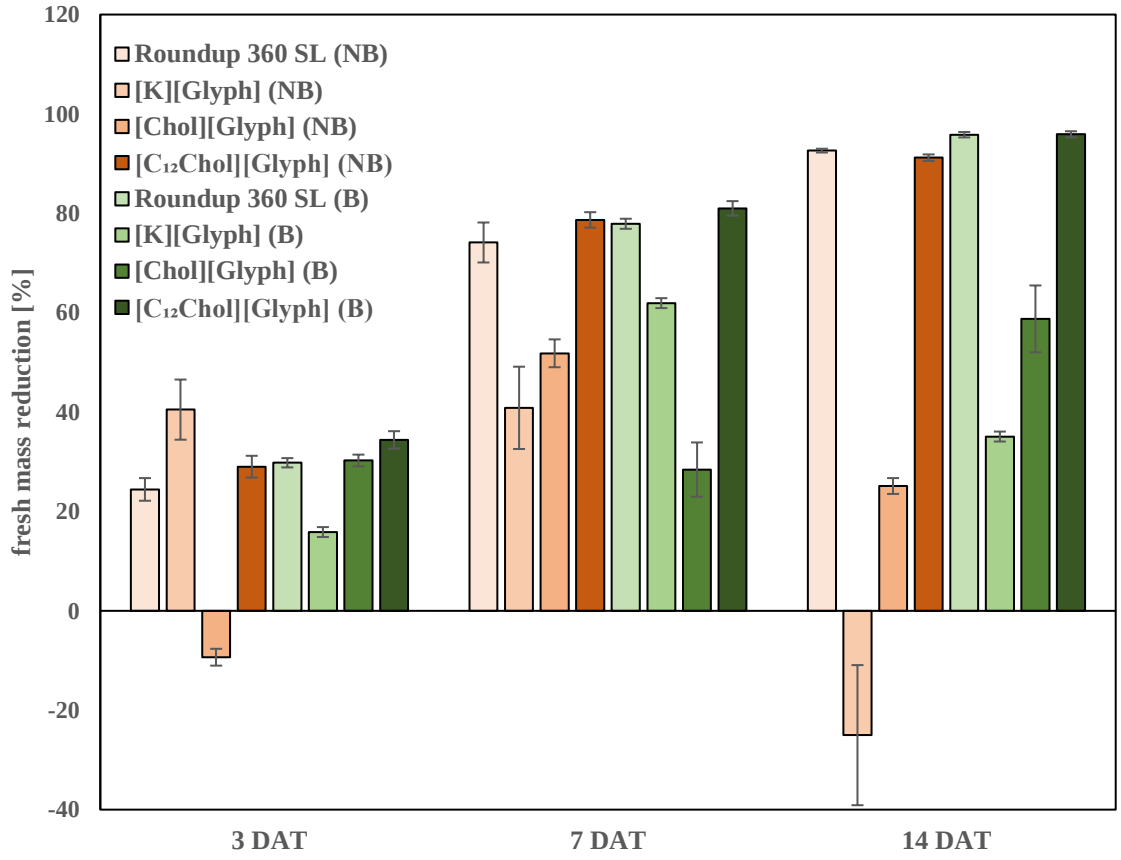


Fig. 34 Fresh weight reduction with regard to respective controls **(**B – bioaugmented, NB – non-bioaugmented**)**, DAT – days after treatment.

fresh weight reduction

Herbicidal residues in plant's tissues

The results of glyphosate residues in plants' tissues (**Section 3.11.1**) seem to correlate well with the fresh mass reduction (**Table 19)**. Namely, the xenobiotic content in plants' tissues was the highest in the case of commercial formulation (Roundup 360 SL) and [C_{12}Chol][Glyph], as these mixtures were able to effectively penetrate plant tissues due to the presence of either adjuvant or cationic surfactant. Statistically significant impact of bioaugmentation was also not observed here, which

might have indicated that the introduction of microorganisms able to degrade glyphosate does not translate into lower residual content of this herbicide in plants.

Table 19 Glyphosate residues in test samples.

Compound		glyphosate content [mg/kg]	
		3 days after treatment	7 days after treatment
Roundup 360 SL	NB[a]	54.31 ± 1.07	50.55 ± 2.02
	B[b]	55.17 ± 1.29	47.17 ± 1.87
[K][Glyph]	NB	4.77 ± 0.24	12.78 ± 0.19
	B	9.17 ± 0.29	6.84 ± 0.21
[Chol][Glyph]	NB	8.18 ± 0.32	8.99 ± 0.38
	B	6.08 ± 0.11	10.08 ± 0.43
[C_{12}Chol][Glyph]	NB	52.26 ± 2.05	34.43 ± 1.76
	B	52.63 ± 1.56	36.53 ± 1.48

[a] NB – non-bioaugmented samples, B – bioaugmented samples.

oxidative stress

Oxidative stress

Plants respond to environmental stresses by increasing the production of reactive oxygen species (ROS), leading to damage to chloroplasts, as well as to increased activity of antioxidant enzymes [232–234]. ROS include singlet oxygen ($^1O_2$), superoxide radical ($O_2^{\bullet-}$) hydroxyl radical ($^{\bullet}OH$) and hydrogen peroxide (H_2O_2) [235], and are present in plants constitutively at low levels (which are not harmful to plants) during homeostasis [235,236]. However, in the case of balance disruption, the stress in plants might be induced, leading to overproduction of oxygen radicals, peroxidation of proteins and lipids constituting cell membranes (responsible for the destruction of cells) and, finally, diseases, aging and even death [234,235,237].

The literature reports indicate that ILs might induce oxidative stress in animal [238–242] and plants, with response similar to this caused by herbicide presence [233,236,237,243–247]. The impact of ILs' concentration on the H_2O_2 accumulation in plants' tissues has also been described by Liu et al., 2014 [237] and Zhang et al., 2013 [248]. Additionally, it has been reported that the glyphosate treatment significantly inhibited the growth of barley, as it led to accumulation of hydrogen peroxide (82 % in leaves, 123 % in roots) and superoxide radical, which in turn

increased the lipid peroxidation (MDA accumulation of 45 % in leaves and 104 % in roots) [249]. Tomatoes after glyphosate application also were characterised by increased levels of H_2O_2 (40 %) and $O_2^{\bullet-}$ (100 %) [250].

The oxidative stress results (**Section 3.11.2**) indicate that the bacteria used in bioaugmentation might have had a protective effect, alleviating the stress caused by the herbicide or the liquid itself, which is especially visible in the case of [C_{12}Chol][Glyph] (**Fig. 35–37**). After the first 24 h, the H_2O_2 level was higher comparing to non-bioaugmented control for all salts except bioaugmented [C_{12}Chol][Glyph] (B). During the next measuring points, the higher plants' response has been recorded for commercial formulation (Roundup) and [K][Glyph]. The H_2O_2 levels for bioaugmented samples of [C_{12}Chol][Glyph] were always lower than in the case of samples non-bioaugmented. Bioaugmented [Chol][Glyph], interestingly, has higher levels of H_2O_2 than non-bioaugmented samples after 72 h and 7 days.

In the case of the $O_2^{\bullet-}$ levels, the differences were statistically unsignificant in most samples. However, at every measuring point, bioaugmented [C_{12}Chol][Glyph] samples were subjected to significantly lower stress than non-bioaugmented samples. The general trend here is that sample's $O_2^{\bullet-}$ levels stayed at the same level for at least 72 h after treatment. Then, at day 7 after treatment, the stress was decreased in all samples. This correlates well with MDA levels results. The malondialdehyde (MDA) is an end-product of lipid peroxidation, which increased level is an indicator of plants' oxidative stress [232–234,237,243]. Hence, during the first 72 h period, its values were at the similar level for all samples, with significant increase in values at day 7 after treatment. Similarly, like in the case of previously described results, both commercial formulation and [C_{12}Chol][Glyph] exhibited lower MDA content when subjected to bioaugmentation approach.

Generally, this indicates that bioaugmentation process might have had an effect on plants' reaction to the xenobiotic's introduction. It was especially visible on the example of [C_{12}Chol][Glyph], which was always at lower stress level if previously bioaugmented with microbial community. Additionally, MDA results indicate that its accumulation in plants' tissues was also lower for bioaugmented samples treated with commercial formulation.

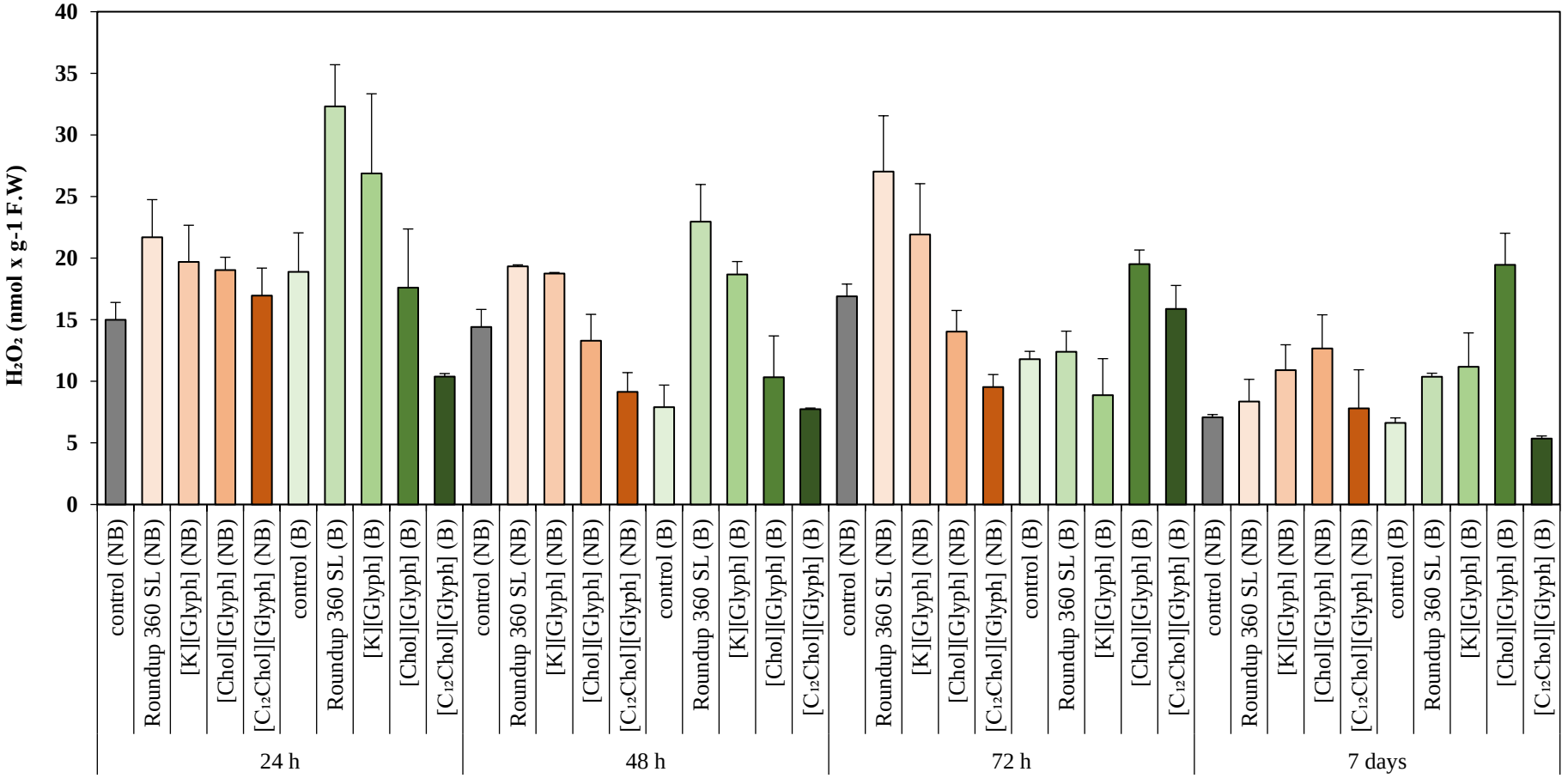


Fig. 35 Generation of hydrogen peroxide (H_2O_2) in *Brasicca napus* leaves.

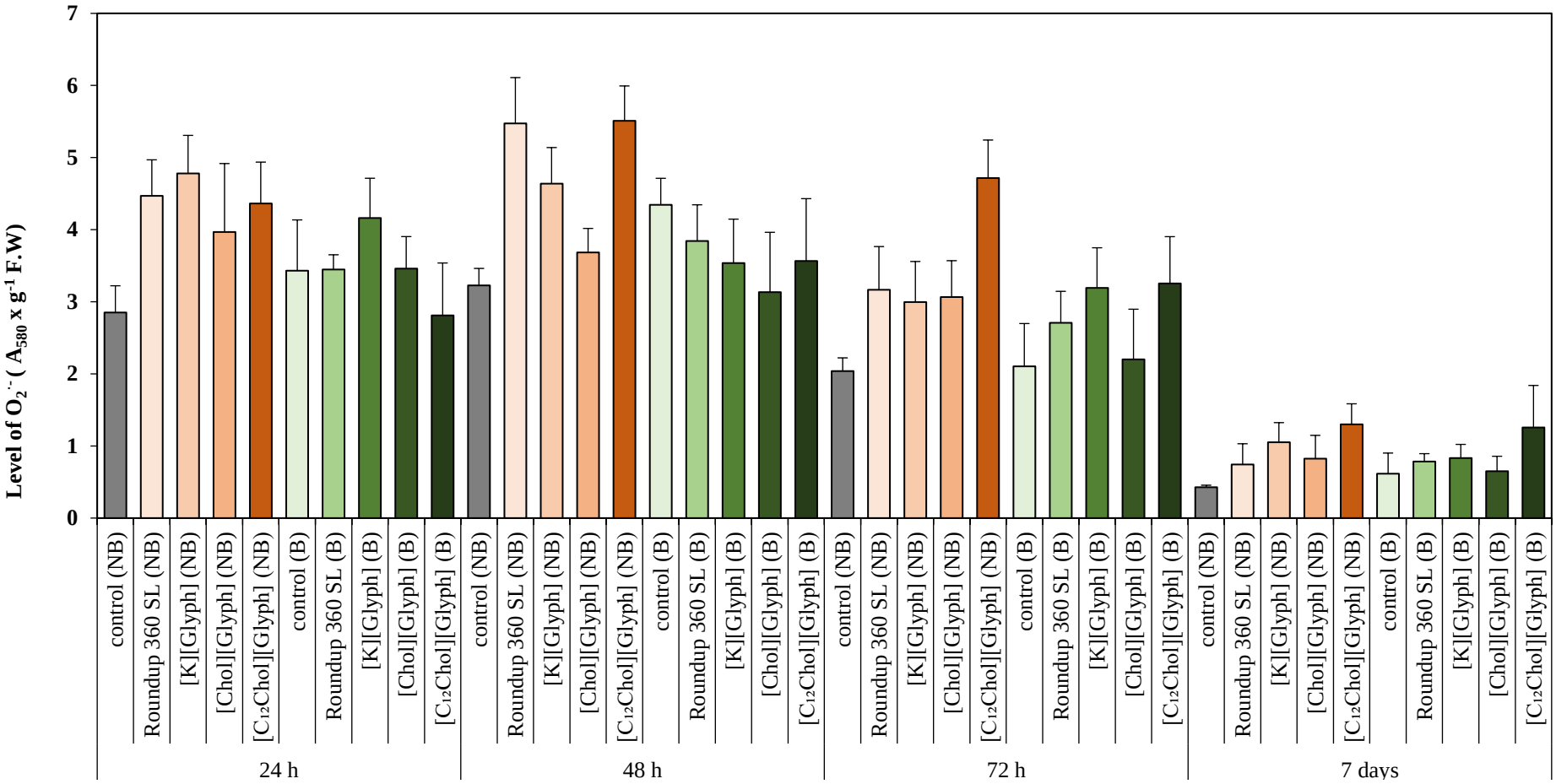


Fig. 36 Generation of superoxide radical $O_2^{\bullet-}$ in *Brasicca napus* leaves.

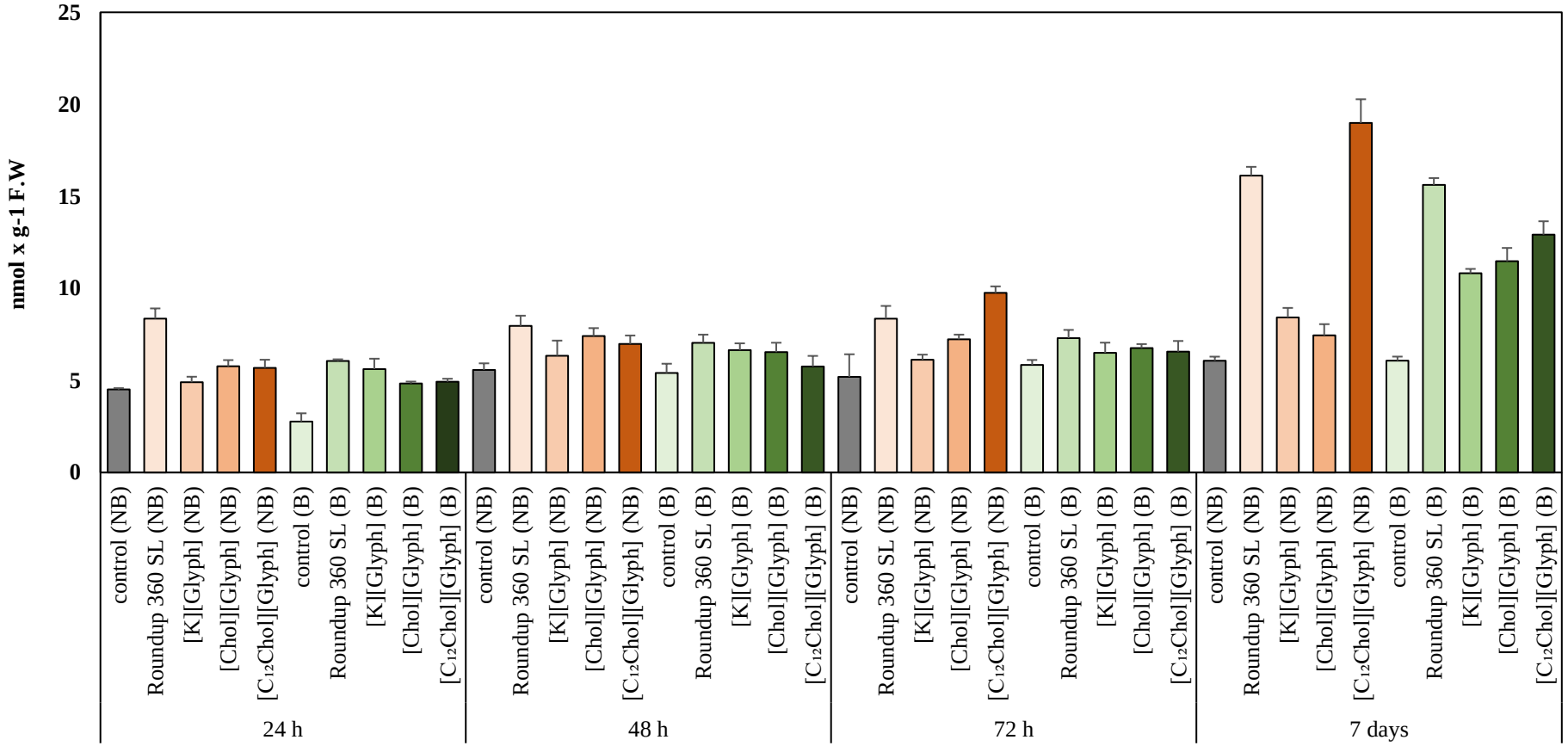


Fig. 37 Accumulation of malondialdehyde (MDA) in *Brasicca napus* leaves

During the greenhouse studies, isolation of microorganisms from plants' rhizosphere and tissues (shoots and roots) was conducted at day 3, 7 and 14 after treatment (**Section 3.5.2**). These samples are currently subjected to molecular studies, *i.e.*, assessment of bacterial community structure and determination of activity of genes associated with glyphosate degradation. The comparisons will be made to evaluate the impact of different herbicidal treatments as well as bioaugmentation with specialised microorganisms on microbial structure. Additionally, the effect of quaternary ammonium surfactants on the possibility of unintentional herbicidal resistance spread is planned for future studies.

5. Summary and conclusions

The synthesis of herbicidal ionic liquids is a recently discussed novel application form of herbicides, which in theory allow to eliminate adjuvants use. However, despite the fact that these compounds are intended for agricultural use, the knowledge on their environmental fate is severely lacking standardised degradation and toxicity studies. At the same time, proper evaluation of these qualities of HILs is a necessity for them to be commercialised successfully. Moreover, in the view of recent research, the issue of environmental stability of herbicidal ionic liquids has been brought, along with discussion on their ionic character beyond laboratory conditions. Additionally, the microbial toxicity of cationic surfactants that might be a factor conducive to herbicidal resistance spread is a concern that should not be tread lightly. Hence, this thesis aims in addressing gaps in the field and giving insights into HILs' environmental fate and impact.

The current stage of knowledge on herbicidal ionic liquids was summarised and analysed within the scope of the first review focused solely on HILs, their synthesis, advantages and shortcomings, physicochemical characterisation and biological aspects. **During literature studies, a question has arisen whether HILs are new chemical compounds or only mixtures of cations and anions of separate properties.** However, up to 2020, no study shed light on degradation pathways of these compounds, nor their environmental stability as ionic compounds. These gaps in knowledge inspired further studies constituting this Ph.D. thesis.

Bearing in mind limitations in degradation and toxicity studies, as well as dubious nature of ionic interactions, a more environmentally relevant approach has been proposed. The impact of cations of natural origin (tropane derivatives) on properties of synthesised compounds was evaluated *via* studies on their physicochemical properties (solubility, volatility, surface tension, foamability), effect on crops and weeds growth, microbial toxicity and degradation. The idea standing before quaternary tropinium cations' use was the fact that tropane alkaloids are naturally occurring in plants, and are known for their plant growth regulating properties [128,251–253]. Moreover, their quaternary salts exhibit surface-active properties [252]. Therefore, the combination of cation with such attractive properties with highly volatile herbicidal anions (MCPA and dicamba) should result in formation

of dual function herbicidal formulations, devoid of necessity for adjuvants use and of reduced volatility. However, obtained results have brought attention to issue of cations' selection in HILs. Namely, the proposed approach indeed allowed to omit adjuvants applications, yet the environmental studies showed that **addition of hydrophobic quaternary cations of surface-active properties to herbicidal anion, worsen the degradation efficiencies of anions and increased microbial toxicity of the formulations**. It is due to the effect of overlapping toxicity, *i.e.*, if cation presents toxic properties, it will have an impact on the whole compound, increasing its toxicity. Consequently, with higher microbial toxicity, the biodegradation efficiency will be lower.

The problem with the use of cationic surfactants is that these compounds are known to disrupt cellular membranes and hence – are toxic towards microorganisms. In the case of cations used in HILs, these not only are cations with surface-active properties, but also ions that incorporate long hydrophobic chains in their structures, which also facilitate cells' disruption [65,88,128,195]. The obtained results revealed that **otherwise non-toxic anions when paired with surface-active cations form compounds that are substantially more toxic towards microorganisms**. Admittedly, the herbicidal efficacy of quaternary tropinium salts was comparable to these of control samples with commercial herbicides with the same doses used, yet the environmental aspects of HILs' use made them inadequate for use on fields. It brings attention to the fact that widely discussed **elimination of adjuvants use, reduced doses and volatility of resulting formulation, as well as the use of cation of natural origin, do not mean that resulting salt will be environmental-friendly**. With reference to green chemistry rules, these compounds are not environmentally benign as they exhibit high toxicity and poor degradation efficiency. Hence, even other advantages do not compensate for serious drawbacks associated with their environmental risks.

Consequently, studies evaluating HILs environmental fate and nature of ionic interactions were performed. A novel approach was proposed, namely the use of ^{13}C-labelling in evaluation of biodegradation potential of HILs. **It was the first study ever conducted that uses ^{13}C-labelling and shows the environmental behaviour of HILs or, in general, ILs with organic cations and anions.** The experiment with isotope-labelled cations and anions, in different environments (aqueous, terrestrial)

and microorganisms used (activated sludge, autochthonic soil microbiota) allowed to observe the behaviour of HILs upon introduction to the environment. Namely, the separate data on cations' and anions' degradation could be obtained, along with determination of metabolites and amount of carbon assimilated by biomass. **The use of ^{13}C-labelling approach revealed that cation and anions in HILs are in fact degraded differently and separately upon introduction to the environment.** Specifically, surface-active cations were degraded efficiently in aqueous environment with activated sludge as the source of microorganisms, while herbicidal anions – in soil with native microbiota. It further supported the hypothesis that degradation assessment based only on standard OECD 301 tests are insufficient in terms of understanding the real environmental fate of tested formulations, as these tests only allow to determine total mineralisation efficiency, without considering individual degradation potential of cations and anions. **A proper choice of both microbial degrader's origin (activated sludge, autochthonic soil microbiota) and tested matrix (aqueous, terrestrial) was revealed to be crucial in terms of proper evaluation of HILs' degradation.** Without developing suitable technique, the obtained results will be not only incomparable with the others, but also will not give deep insights into herbicidal formulations behaviour in the environment. With that in mind, ^{13}C-labelling seems to be suitable solution in understanding degradation pathways of cations and anions comprising HILs, yet unfortunately, due to the high cost of such approach other, cheaper techniques needs to be found and validated.

The findings resulting from isotopic-labelling have put the integrity of HILs' ionic bond in question. This topic was further evaluated within the scope of further studies where, on the basis of sorption studies combined with toxicity assessment towards plants and microorganisms, it was further confirmed that ions constituting herbicidal ionic liquids behave independently in soil. Namely, the ionic interactions between ions were not strong enough and HILs' cations and anions acted separately in the environment. The cation's potential to soil adsorption was confirmed to be dependent on hydrophobic properties. At the same time, cation's properties had no impact whatsoever on anion's mobility, which is especially troubling when considering the fact that the idea standing behind HILs stated that cation's choice will have impact on anion's properties and hence would allow to adjust, *e.g.*, its hydrophobicity. Additionally, similarly to situation described in other works,

hydrophobic cations were solely responsible for the increase in HILs' toxicity. **Finally, it might be a proof that at least certain HILs may in fact act as mixture of independent surface-active cations and herbicidal anions in the environment, thus confirming the hypothesis H1.**

The additional degradation studies on HILs aimed in development of approach cheaper than isotope-labelling yet providing with information sufficient for environmental fate assessment. The role of cations in HILs was further evaluated there. It has been confirmed that the use of large, hydrophobic cations has led to HILs' high toxicity towards microorganisms, sorption to soil particles, low bioavailability and consequently – low degradation. It has also proven that primary degradation values allow to assess only degradation of bioavailable part, which in fact might be a really small fraction of the whole compound. The integrity of HILs in the environment has been compromised, since from ionic liquids definition, cations and anions in HILs should act as a whole chemical compound, of its own properties, different from these of cation and anion on their own. Yet, it has been established that cations and anions act as separate moieties upon introduction to the environment – the toxicity of tested compounds reflected cations' toxicities, and the degradation of anion was limited in the presence of cation.

These studies also aimed in verification of hypothesis **H3**, stating that bioaugmentation will improve degradation of xenobiotic providing the lack of cation's toxic effect. The approach of bioaugmentation with microorganisms specialised in glyphosate degradation allowed in fact to achieve higher degradation efficiencies, yet still cation's sorption was the limiting factor, as not bioavailable xenobiotics cannot be degraded even by specialised microorganisms. Interestingly, it is a common practice to use surfactants to free sorbed chemicals from the matrix and facilitate their utilisation by microorganisms. However, the problem with the presence of cationic surfactants in HILs is that QAC will not help with desorption, on the contrary, these will facilitate even higher accumulation of xenobiotics in soil [218–222]. Additionally, their toxicity might lead to the activation of defence mechanisms in bacteria, potentially leading to promotion of resistance spread, as stated in hypothesis **H2**.

Finally, the last experiment conducted within the scope of this Ph.D. study evaluated all three proposed hypotheses – structural integrity of HILs, possible detrimental effects of cations utilised in their structure, as well as impact of

bioaugmentation. The greenhouse studies were performed in order to analyse the impact of HILs on plants (*Brassica napus*), as well as the role of bioaugmentation on herbicidal phytotoxic effects and oxidative stress in plants. Here, the introduced bacteria have the protective effect on tested plants, as especially in the case of plants treated with HIL with toxic and hydrophobic cation, the levels of ROS were lower in samples bioaugmented. With regard to that observation, one might speculate that bioaugmentation has occurred at the level of penetration of plant tissues by bacteria, which in turn creates the conditions for horizontal gene transfer due to the proximity of bacterial and plant genetic material. However, with the current state of knowledge on the topic and still ongoing molecular research, this might be a hypothesis to be examined within the scope of the future studies.

6. Recommendations and future directions

On the basis of the results obtained within the scope of Ph.D. studies, recommendations and directions for future studies on HILs have been prepared.

- First of all, all studies that describe HILs are a new type of emerging environmental pollutants should consider cations and anions separately. Namely, since it has been proven that ionic bond in HILs is not strong enough to prevail beyond laboratory conditions, the further research should examine their environmental fate as that of mixtures of herbicidal anions and surface-active cations
- Secondly, the biological tests on HILs are a necessity should these compounds ever be introduced on commercial scale onto agricultural fields. Assessment basing mainly on their recorded physicochemical properties and model organisms are not enough to ensure their environmental safety. Studies on their mobility, sorption and bioavailability should be the foundation on which further degradation assays are performed. In addition, their toxicity towards autochthonous soil microorganisms should be evaluated in order to assess their impact on the overall microbiome health.
- As for the degradation studies themselves, these are recommended to be performed with regard to the suitable matrix and microbial degraders use. Furthermore, appropriate degradation assessment technique should be applied. In the future, a study with both ^{13}C- and ^{15}N-labelling would allow to determine whether degraded compounds are utilised in *de novo* synthesis of *e.g.*, fatty acids, amino acids, similarly as they have been proven to be used in the phospholipids' fatty acids formation.

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8. Abbreviations

2,4-D	2,4-dichlorophenoxyacetic acid
B	Bioaugmented samples
BCR	Community Bureau of Reference
CFU	Colony Forming Unit
d.m.	Dry matter
d.w.s.	Soil dry weight
dicamba	3,6-dichloro-2-methoxybenzoic acid
DSHIL	Double Salt Herbicidal Ionic Liquid
EC50	Half maximal effective concentration
EUCAST	European Committee on Antimicrobial Susceptibility Testing
GC×GC-TOF-MS	Two-dimensional Gas Chromatography coupled with Time-Of-Flight Mass Spectrometry
GC-C-IRMS	Gas Chromatography–Combustion–Isotope Ratio Mass Spectrometry
GI	Germination Index
glyphosate	*N*-(Phosphonomethyl)glycine
H1	Hypothesis 1
H2	Hypothesis 2
H3	Hypothesis 3
H	Herbicide
HIL	Herbicidal Ionic Liquid
HPLC	High-Performance Liquid Chromatography
IL	Ionic Liquid
ISO	International Organisation for Standardization
KEGG	Kyoto Encyclopedia of Genes and Genomes
LC-MS/MS	Liquid Chromatography with tandem mass spectrometry
LD50	Median Lethal Dose
MBC	Minimum Bactericidal Concentration
MCE	Mixed Cellulose Ester
MCPA	4-chloro-2-methylphenoxyacetic acid
MDA	Malondialdehyde
MFC	Minimum Fungicidal Concentration
MIC	Minimum Inhibitory Concentration
MM	Mineral Medium
MM+H	Mineral Medium amended with herbicide
NB	Non-bioaugmented samples
NGS	Next Generation Sequencing
NSTI	Nearest Sequenced Taxon Index
OD_{600}	Optical Density at 600 nm
OECD	Organisation for Economic Co-operation and Development, Europe

OTU	Operational Taxonomic Unit
PLFAs	Phospholipid Fatty Acids
PPA	Plant Protection Agrochemicals
QAC	Quaternary Ammonium Compounds
QTS	Quaternary Tropinium Salt
ROS	Reactive Oxygen Species
STAMP	Statistical Analysis of Metagenomic Profiles
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
TSB+H	Tryptic Soy Broth amended with herbicide
USCS	United Soil Classification Systems
w.m.	Wet matter

9. Figure captions

10. List of tables

11. Scientific activity

Publications resulting from OPUS 15 studies:

- **Wilms W.**, Woźniak-Karczewska M., Syguda A., Niemczak M., Ławniczak Ł., Pernak J., Rogers R.D., Chrzanowski Ł., *Herbicidal ionic liquids – a promising future for old herbicides? Review on synthesis, toxicity, biodegradation and efficacy studies*, J. Agric. Food Chem. 2020; 68, 39: 10456–10488, https://doi.org/10.1021/acs.jafc.0c02894, **IF_{2020} = 5.729**

- Parus A., **Wilms W.**, Verkhovetska V., Framski G., Woźniak-Karczewska M., Syguda A., Strzemiecka B., Borkowski A., Ławniczak Ł., Chrzanowski Ł., *Transformation of herbicides into dual function quaternary tropinium salts*, New J Chem 2020; 44: 8869–8877, https://doi.org/10.1039/D0NJ01597K, **IF_{2020} = 3.591**

- **Wilms W.**, Woźniak-Karczewska M., Niemczak M., Lisiecki P., Zgoła-Grześkowiak A., Ławniczak Ł., Framski G., Pernak J., Owsianiak M., Vogt C., Fischer A., Rogers R.D., Chrzanowski Ł., *Quantifying the Mineralization of ^{13}C-Labeled Cations and Anions Reveals Differences in Microbial Biodegradation of Herbicidal Ionic Liquids between Water and Soil*, ACS Sustain Chem Eng 2020; 8: 3412–3426, https://doi.org/10.1021/acssuschemeng.9b07598, **IF_{2020} = 7.362**

- Woźniak-Karczewska M., Parus A., Ciesielski T., Trzebny A., Szumski R., **Wilms W.**, Homa J., Framski G., Baranowski D., Frankowski R., Zgoła-Grzeskowiak A., Niemczak M., Dabert M., Táncsics A., Chrzanowski Ł., *Effect of Cation Sorption on 2,4-D Mobility of Herbicidal Ionic Liquids in Agricultural Soil Combined with Diversity of the Bacterial Community*, ACS Sustainable Chem. Eng 2022, https://doi.org/10.1021/acssuschemeng.2c02665, **IF_{2021} = 9.224**

Total Impact Factor: 25.906

Additional publication

- **Wilms W.**, Woźniak-Karczewska M., Corvini P. F.-X., Chrzanowski Ł., *Nootropic drugs: Methylphenidate, modafinil and piracetam - Population use trends, occurrence in the environment, ecotoxicity and removal methods – A review,* Chemosphere 2019; 233: 771–785, 10.1016/j.chemosphere.2019.06.016, **IF = 5.108**

Popular science publication

- **Wilms W.**, Woźniak-Karczewska M., *Bestia z kanałów – fikcja czy nieuchronna przyszłość?*, Eko i My, 2 (266), 02.2019, 21–25, ISSN 1232-4531.

Oral Presentations at National/International Conferences

2022

- **Wilms W.**, Homa J., Woźniak-Karczewska M., Parus A., *Wpływ doboru kationów na degradację HILs z anionem glifosatu*, VII Ogólnopolska Konferencja Doktorantów Nauk o Życiu BioOpen, 7-8.04.2022 **(Distinction in the 'Ecology and Environmental Protection' section)**.

2021

- Lisiecka N., **Wilms W.**, Woźniak-Karczewska M., Framski G., Syguda A., Parus A., *Potencjał aplikacyjny herbicydowych soli tropiniowych w uprawach rolnych*, Ogólnopolska Studencka Konferencja Naukowa "Bliżej Chemii", 09–10.01.2021.
- **Wilms W.**, Homa J., Woźniak-Karczewska M., Parus A., *Biodegradacja herbicydowych cieczy jonowych na bazie glifosatu w środowisku glebowym,* VI Ogólnopolska Konferencja Doktorantów Nauk o Życiu BioOpen, 15–16.04.2021 **(Distinction in the 'Ecology and Environmental Protection' section)**.
- Woźniak-Karczewska M., **Wilms W.**, Parus A., *Czy modyfikacje strukturalne klasycznych herbicydów na bazie 2,4-D są bardziej ekologicznym narzędziem do zwalczania chwastów?,* III Ogólnopolska Konferencja Naukowa „Ochrona środowiska – rozwiązania i perspektywy", 21.05.2021.

- Woźniak-Karczewska M., **Wilms W.**, Parus A., *Modyfikacje strukturalne herbicydów na bazie 2,4-D jako alternatywne narzędzie do zwalczania chwastów o zmniejszonej mobilności w glebie*, II EU Green Week w Lublinie – Spacer żywiołów: woda, ziemia, powietrze, 01.06.2021.
- Woźniak-Karczewska M., **Wilms W.**, Parus A., *Strukturalne modyfikacje 2,4-D jako alternatywny sposób zwalczania chwastów o kontrolowanej mobilności w glebie*, 63. Zjazd Naukowy Polskiego Towarzystwa Chemicznego, 13-17.09.2021.

2020

- **Wilms W.**, Parus A., Framski G., Woźniak-Karczewska M., Syguda A., Strzemiecka B., *Dwufunkcyjne czwartorzędowe sole tropiniowe jako nowa forma herbicydów*, III Edycja Studenckiej Konferencji Nauk Ścisłych im. Prof. Antoniego Hoborskiego, 14.11.2020, **(3rd place in the Chemical Sciences section)**.
- Lisiecka N., **Wilms W.**, Woźniak-Karczewska M., Framski G., Syguda A., Parus A., *Analiza fitotoksyczności dwufunkcyjnych herbicydowych czwartorzędowych soli względem wybranych organizmów,* III Ogólnopolska Konferencja „Biotechnologia niejedno ma imię", 21.11.2020.
- **Wilms W.**, Woźniak-Karczewska M., Niemczak M., Framski G., *Różnice w biodegradacji znakowanych izotopowo herbicydowych cieczy jonowych w układach wodnym i glebowym,* III Ogólnopolska Konferencja „Biotechnologia niejedno ma imię", 21.11.2020.
- Lisiecka N., **Wilms W.**, Woźniak-Karczewska M., Framski G., Syguda A., Parus A., *Czwartorzędowe sole tropiniowe - alternatywa wobec adiuwantów w preparatach herbicydowych?,* e-Zjazd Zimowy SSPTChem 2020, 19.12.2020.

2019

- **Wilms W.**, Woźniak-Karczewska M., Corvini P. F.-X., Chrzanowski Ł., *Zażywanie leków nootropowych a środowisko,* VI Ogólnopolska Konferencja Młodych Naukowców w Poznaniu „Nauka dla środowiska przyrodniczego ze szczególnym uwzględnieniem terenów chronionych", 24–27.04.2019, **(2nd place)**.

Posters at National/International Conferences

2022

- **Wilms W.**, Homa J., Woźniak-Karczewska M., Parus A., Chrzanowski Ł., *Impact of the cation on the degradation of glyphosate anion in herbicidal ionic liquids*, 8th Central European Congress of Life Sciences EUROBIOTECH, 20-22.06.2022.

2021

- Lisiecka N., **Wilms W.**, Woźniak-Karczewska M., Framski G., Parus A., *Ocena fitotoksyczności dwufunkcyjnych herbicydowych cieczy jonowych na bazie kwasu 3,6-dichloro-2-metoksybenzoesowego*, II Pomorskie Studenckie Sympozjum Chemiczne, 20–21.03.2021.
- **Wilms W.**, Homa J., Woźniak-Karczewska M., Parus A., *Mineralizacja herbicydowych cieczy jonowych z anionem glifosatu w matrycy glebowej*, II Pomorskie Studenckie Sympozjum Chemiczne, 20–21.03.2021.
- **Wilms W.**, Woźniak-Karczewska M., Parus A., *Biodegradacja herbicydowych cieczy jonowych z anionem glifosatu w środowisku glebowym*, 63. Zjazd Naukowy Polskiego Towarzystwa Chemicznego, 13-17.09.2021.

2020

- **Wilms W.**, Parus A., Framski G., Woźniak-Karczewska M., Syguda A., Strzemiecka A., *Transformacja herbicydów w dwufunkcyjne czwartorzędowe sole tropiniowe*, I Pomorskie Studenckie Sympozjum Chemiczne, 26–27.09.2020.

2019

- **Wilms W.**, Woźniak-Karczewska M., Corvini P. F.-X., Chrzanowski Ł., *Leki nootropowe – spożycie a środowisko*, IX Krajowa Konferencja Bioindykacyjna, 08–10.04.2019.
- **Wilms W.**, Woźniak-Karczewska M., *Bestia z kanałów – fikcja czy nieuchronna przyszłość?*, VI Ogólnopolska Konferencja Młodych Naukowców w Poznaniu „Nauka dla środowiska przyrodniczego ze szczególnym uwzględnieniem terenów chronionych", 24–27.04.2019.
- **Wilms W.**, Woźniak-Karczewska M., *Metylofenidat, modafinil, piracetam – leki nootropowe a środowisko,* III Ogólnopolskie Sympozjum Chemii Bioorganicznej, Organicznej i Biomateriałów w Poznaniu, 7.12.2019.

- Homa J., Mikołajczak K., Basińska-Barczak A., **Wilms W.**, Błaszczyk L., *Wpływ inokulacji grzybami z rodzaju Trichoderma na zmianę ekspresji genów związanych z odpornością u pszenicy zwyczajnej (Triticum aestivum L.)*, III Ogólnopolskie Sympozjum Chemii Bioorganicznej, Organicznej I Biomateriałów w Poznaniu, 7.12.2019.

Additional Scientific Acitivty

- Member of Student Scientific Society „BioInicjatywa" at Poznan Univeristy of Technology (2018-2022).
- Conducting a lecture titled „Oporność chwastów na herbicydy: przyczyny i sposoby ograniczania tego zjawiska" for members of Polish Allotment Gardens Association (2020).
- Training „How to Get Published with the IEEE" (2019).
- Participation in the national final stage of the XXII National English Language Olympiad for Technical University Students (24.04.2021).

Projects

- OPUS 15 funded by the National Science Centre in Poland, conferred on the basis of decision DEC-2018/29/B/NZ9/ 01136, titled "Bioaugmentation with herbicide degrading bacteria as a potential factor in spreading resistance to herbicides among plants", 10.2019–09.2022.

Research career

- **Ph.D. Student (01.10.2018 – present)**

  Ph.D. Supervisor: Prof. Łukasz Chrzanowski, Ph.D. Eng.

  Poznan University of Technology

  Faculty of Chemical Technology

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- **Master studies at Poznan University of Technology (27.02.2017-18.06.2018)**

  Master of Science, Engineer: 18.06.2018

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  Chemical Technology; Specialisation: Composites and Nanomaterials

  Supervisor: Ewa Stanisz, Ph.D. Eng.

  Thesis title: Nanomaterials and low cost adsorbents in dispersive micro solid-phase extraction for simultaneous determination of indium and nickel

- **Master studies at The Ignacy Jan Paderewski Academy of Music in Poznan (02.10.2018-17.06.2019)**

  Master of Arts: 17.06.2019

  Faculty of String Instruments, Guitar, Harp and Lutherie

  Instrumental Studies; Specialisation: Violin

  Supervisor: Jarosław Żołnierczyk, Ph.D.

  Thesis title: Twórczość skrzypcowa Camille Saint-Saënsa w kontekście dorobku wybranych kompozytorów francuskich XIX w. w oparciu o III koncert skrzypcowy h-moll, op. 61

- **Bachelor studies at Poznan Univeristy of Technology (30.09.2013-06.02.2017)**

  Bachelor of Science, Engineer: 06.02.2017

  Poznan University of Technology

  Faculty of Chemical Technology

  Chemical Technology

  Supervisor: Ewa Stanisz, Ph.D. Eng.

  Thesis title: Wybrane szkodliwe substancje chemiczne w odzieży (Harmful chemicals in clothing)

- **Bachelor studies at The Ignacy Jan Paderewski Academy of Music in Poznan (21.09.2013-12.06.2016)**

  Bachelor: 12.06.2016

  Faculty of String Instruments, Guitar, Harp and Lutherie

  Instrumental Studies; Specialisation: Violin

  Supervisor: Jarosław Żołnierczyk, Ph.D.