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Ph.D. THESIS

to attain the academic degree of
Doctor of Philosophy

Environmental impact of ionic liquids with herbicidal activity

submitted by

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Table of contents

Abstract

This doctoral dissertation investigates the biological activity of ionic liquids (ILs) containing herbicidal anions, with a focus on their potential as eco-friendly alternatives to traditional herbicides. Herbicides, as a part of pesticides group, are designed to eliminate unwanted plants, but their formulations require the addition of various adjuvants to reduce the volatility of active ingredients and enhance both their effectiveness and environmental stability. Hence, ILs with herbicidal activity have gained scientific attention due to their unique ionic structures, which offer tuneable properties and low volatility, eliminating the need for additives before application. Although their biological properties are currently being researched, their environmental impact, particularly in agriculture, remains relatively unexplored.

In this study, a number of ionic liquids with herbicidal anions (*i.e.*, dicamba, MCPP, MCPA, and iodosulfuron-methyl) were synthesised and characterised to evaluate their biological activity and potential behaviour in the environment. The research explores how different cationic structures paired with these herbicidal anions affect the toxicity and biodegradability of the ILs. A comprehensive assessment was carried out through a wide array of bioassays on both target and non-target plant species, as well as microbiomes.

Studies on herbicide-resistant and susceptible plants revealed that the transformation of MCPP, MCPA, and iodosulfuron-methyl into ionic liquid forms resulted in highly effective weed-killing compounds, though they were unable to overcome resistance in weeds. On the other hand, microbial studies indicated increased toxicity to bacteria due to the presence of long, hydrophobic alkyl substituents in cationic structures of all studied herbicidal ionic liquids. Biodegradation assays further demonstrated that the presence of hydrophobic cations limited biodegradation, slowing the biotransformation of herbicides by bacteria and fungi. However, cations of natural origin did not exhibit such adverse effects. Despite this, residue analysis showed that naturally-derived cations were poorly degraded by microbes, raising concerns about their persistence in soil. In addition, molecular analyses revealed that ILs with herbicidal activity negatively impact microbial communities, reducing the diversity of microbiomes in both soil and plant tissues. Once again, the cation structure was identified as the driving factor, highlighting the importance of selecting appropriate cations in the design of herbicidal ionic liquids.

This doctoral dissertation contributes to the emerging field of ionic liquids in agriculture, offering insights into the development of greener, more efficient herbicides that could reduce the ecological impact of current chemical weed control methods. Ultimately, this research paves the way for more sustainable practices in crop management and environmental protection.

Streszczenie

Niniejsza rozprawa doktorska bada aktywność biologiczną cieczy jonowych (ILs) zawierających aniony herbicydowe, ze szczególnym uwzględnieniem ich potencjału jako ekologicznych alternatyw dla tradycyjnych związków chwastobójczych. Użycie herbicydów (związków należących do grupy pestycydów) jest przeznaczone do zwalczania niepożądanych roślin, jednak ich formulacje wymagają dodatku różnych adiuwantów w celu zmniejszenia lotności składników aktywnych oraz poprawy zarówno ich skuteczności, jak i stabilności środowiskowej. Dlatego też ILs o działaniu herbicydowym zyskały zainteresowanie naukowców ze względu na swoją unikalną strukturę jonową, która oferuje regulowane właściwości i niską lotność, eliminując tym samym konieczność stosowania dodatków przed aplikacją. Choć ich właściwości biologiczne są obecnie badane, ich wpływ na środowisko, szczególnie w kontekście rolnictwa, pozostaje stosunkowo niezbadany.

W niniejszej pracy zsyntetyzowano i scharakteryzowano szereg cieczy jonowych zawierających z aniony herbicydowe (tj. dikambę, MCPP, MCPA i jodosulfuron metylowy), w celu oceny ich aktywności biologicznej i potencjalnego zachowania w środowisku. Badania koncentrowały się na tym, w jaki sposób różne struktury kationowe w połączeniu z tymi anionami chwastobójczymi wpływają na toksyczność i biodegradowalność ILs. Przeprowadzono kompleksową ocenę tego zagadnienia poprzez szeroki zakres bioanaliz zarówno na gatunkach roślin docelowych, jak i niedocelowych, a także na całych społecznościach mikroorganizmów.

Badania na roślinach odpornych i podatnych na herbicydy wykazały, że przekształcenie MCPP, MCPA i jodosulfuronu metylowego w formę cieczy jonowych doprowadziło do powstania bardzo skutecznych związków herbicydowych, choć nie były one w stanie przełamać odporności chwastów. Z drugiej strony, badania mikrobiologiczne wykazały zwiększoną toksyczność względem bakterii z powodu obecności długich, hydrofobowych podstawników alkilowych w strukturze kationów wszystkich badanych cieczy jonowych o właściwościach herbicydowych. Dalsze badania biodegradacji wykazały, że obecność hydrofobowych kationów była czynnikiem ograniczającym biodegradację, spowalniając biotransformację herbicydów przez bakterie i grzyby. Pomimo tego, analiza pozostałości wykazała,

że kationy pochodzenia naturalnego były słabo degradowane przez mikroorganizmy, co rodzi obawy dotyczące ich zalegania w glebie.

Dodatkowo, analizy molekularne wykazały, że ciecze jonowe o działaniu herbicydowym negatywnie wpływają na społeczności mikroorganizmów, zmniejszając bioróżnorodność zarówno w glebie, jak i w tkankach roślin. Po raz kolejny struktura kationu została zidentyfikowana jako czynnik warunkujący to zjawisko, co wyraźnie podkreśla znaczenie odpowiedniego doboru kationów w projektowaniu cieczy jonowych o właściwościach chwastobójczych.

Niniejsza rozprawa doktorska wnosi wkład w rozwijające się pole badań nad cieczami jonowymi w rolnictwie, oferując wgląd w projektowanie bardziej ekologicznych i skuteczniejszych herbicydów, które mogą złagodzić wpływ na środowisko obecnych metod chemicznej ochrony roślin. Ostatecznie, badania te torują drogę do bardziej zrównoważonych praktyk w zarządzaniu uprawami i ochronie środowiska.

Abbreviations

2,4-D	2,4-dichlorophenoxyacetic acid
a.i.	Active Ingredient
ALS	Acetolactate Synthase
ARB	Antibiotic Resistant Bactyeria
ASV	Aplicon Sequence Variants
B	bioaugmented samples
CASO	Casein Soy Bean Digest Broth
CFU	Colony Forming Unit
Chol; choline	(2-hydroxyethyl)trimethylammonium
Dic; dicamba	3,6-dichloro-2-metoxybenzoate
DMAE	dimethylethanolamine
DMSO	Dimethylsulfoxide
EC_{50}	Half Maximal Effective Concentration
ED_{50}	Median Effective Dose
EUCAST	European Committee on Antimicrobial Susceptibility Testing
GI	Germination Index
Glyph; glyphosate	N-(Phosphonomethyl)glycine
HIL	Herbicidal Ionic Liquid
HPLC	High-Performance Liquids Chromatography
HPLC-MS	High-Performance Liquid Chromatography-Mass Spectrometry
IL	Ionic Liquid
IPM	Integrated Pest Management
ISM	Iodosulfuron-Methyl Sodium Salt
ISO	International Organisation for Standardization
ITS	Internal Transcribed Spacer
Kf	Filtration Coefficient
LC-MS/MS	Liquid Chromatography with tandem Mass Spectroscopy
MBC	Minimum Bactericidal Concentration
MCPA	(2-methyl-4-chlorophenoxy)acetic acid
MCPP	(±)-2-(4-chloro-2-methylphenoxy)propionic acid
MFC	Minimum Fungicidal Concentration
MIC	Minimum Inhibitory Concentration
NB	non-bioaugmented samples
NGS	Next-Generation Sequencing
OD	Optical Density
OECD	Organisation for Economic Co-operation and Development, Europe
OTU	Operational Taxonomic Units
PD	Potato Dextrose
PDA	Potato Dextrose Agar
PPA	Plant Protection Agrochemicals
QAS	Quaternary Ammonium Salts
RI	Resistance Index
s.d.w.	Soil Dry Weight
SEM	Standard Error of the Mean
ThOD	Theoretical Oxygen Demand
TMS	Tetramethylsilane
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth

1. Introduction

Humanity's greatness lies in its capacity to innovate, empathize, and collaborate, leading to remarkable achievements in science, art, and society. Theability to adapt and overcome challenges, whether natural disasters or global crises, showcases the resilience and determination to thrive in the face of adversity. The unbreakable will to persevere, deeply ingrained in the human spirit, fuels relentless pursuit of progress, offering hope for a brighter future even in the most trying circumstances.

The human population has been rapidly expanding since the 1800s, when it first reached one billion [1]. By 2013, it had surged to 7 billion, with projections estimating it will surpass 9.2 billion by 2050 and likely peak in 22nd century [2]. Notably, the most significant population growth occurred after 1950, with an increase of 4 billion people since then (**Fig. 1**) [1]. Such an excessive rise in number of individuals has intensified the demand for food crops, animal fodder and other products derived from natural sources, including biofuels [2]. Consequently, as the amount of land available for agricultural purposes is finite, it is essential to develop new and more efficient farming techniques, along with environmentally-friendly fertilizers and pesticides in order to ensure the long-term productivity and sustainability of farmlands.

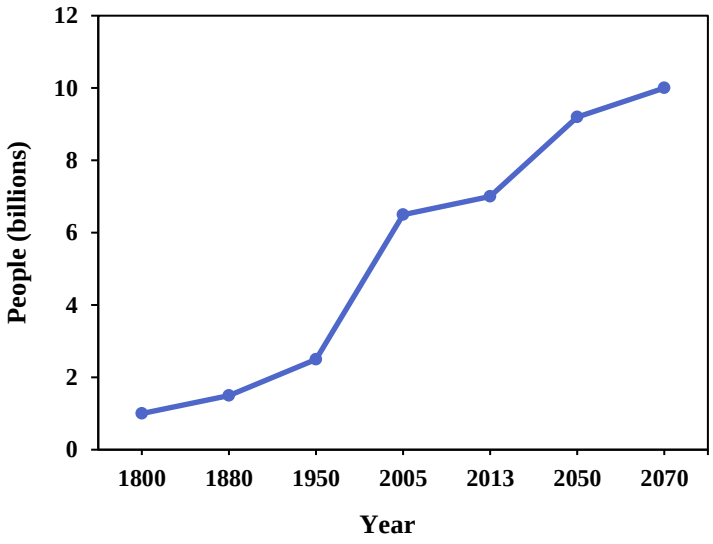


Figure 1. Projected growth of human population over time [1,2].

1.1. Modern agriculture

The beginnings of modern agriculture are inextricably intertwined with the Industrial Revolution, a period when mechanisation was first introduced into farming, initiating a gradual shift from extensive to intensive agricultural practices [3]. This transition was further accelerated by key discoveries, including the identification of nitrogen, phosphorus, and potassium (NPK) as essential nutrients for plant growth, which led to the development of synthetic fertilizers, greatly boosting crop yields [4]. Additional major breakthrough occurred in the post-war era with the discovery and commercialization of a new generation of chemical plant protection agrochemicals (PPA) [2]. These events marked the onset of the Green Revolution, a transformative period in agriculture characterized by the development of new, more resistant and high yield crop cultivars, the widespread use of agrochemicals, and the refinement of farming practices [5,6]. As a result, the availability of plant biomass yields per hectare increased significantly [7]. For example, between 1960 and 2000, yield percentages rose substantially in developing countries: 208% for wheat, 109% for rice, 157% for maize, 78% for potatoes, and 36% for cassava [7]. Therefore, chemical PPAs, including herbicides, fungicides and insecticides, became indispensable for maintaining high crop yields and ensuring the quality of produce. It is estimated that without the use of pesticides, global losses in crop production could reach 78% for fruits, 54% for vegetables and 32% for cereal, due to various pests [8]. Among these pests, weeds pose the greatest threat to crops, as they compete for essential nutrients [2] and have the highest potential to cause significant losses for farmers [9,10].

The consequences of pesticide overuse became evident as early as the 1950s, when such practices led to several ecological disasters and inability to control outbreaks of pests due to their rising resistance [11]. A striking example occurred in Peru, where despite more than 15 pesticidal treatments per season, cotton plantations were destroyed. In another instance, 90 applications over three months proved ineffective in controlling vermin on cotton farms. This trend extends to weed control, where the oversimplification of crop systems (*e.g.*, the abandonment of crop rotation) and an overreliance on chemical methods (**Fig. 2**), particularly when using a limited range of weed control strategies repeatedly, have spurred the rapid emergence of herbicide resistance [12].

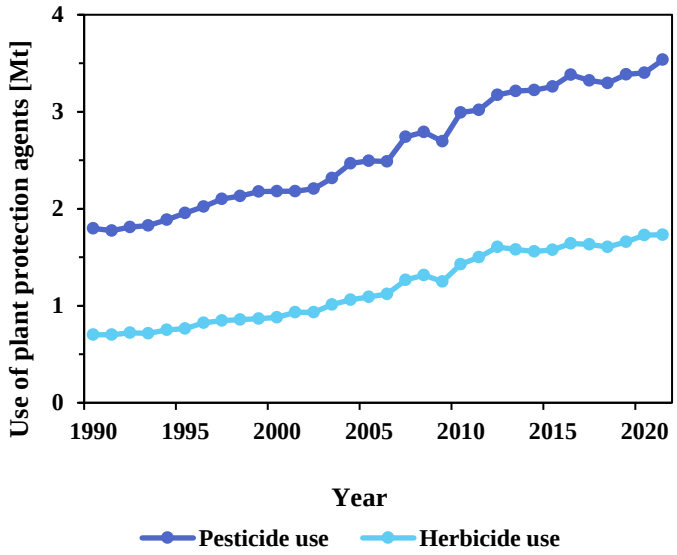


Figure 2. Global use of chemical plant protection agents in total and herbicides [13].

The scope of the problem is highlighted by the data regarding the number of active substances registered for use [14]. In the 2001, more than 1,000 of such compounds were approved for use in commercial formulations in the European Union, but by 2009, only 250 remained authorised. Moreover, while 70 new compounds were under development in the early 2000s, by 2012, only 28 active substances were still being actively researched [14]. This shrinking pool of pest control options, with fewer compounds offering different modes of action, exacerbates the challenge of resistance in weeds, pathogens, and pests, leaving farmers with increasingly limited tools to protect their crops effectively.

Such rapid emergence of weed resistance, coupled with the significant tightening of chemical regulations [15], has reignited interest in the concept of integrated pest management (IPM) among governing bodies. Originally proposed by the scientific community nearly 70 years ago [11], IPM emphasizes the long-term sustainability of agricultural systems by integrating various pest control methods that minimize environmental impact. After almost a century since its introduction, IPM has begun to be incorporated into the legislation of several countries [12], reflecting a shift towards more holistic and sustainable approaches in agriculture.

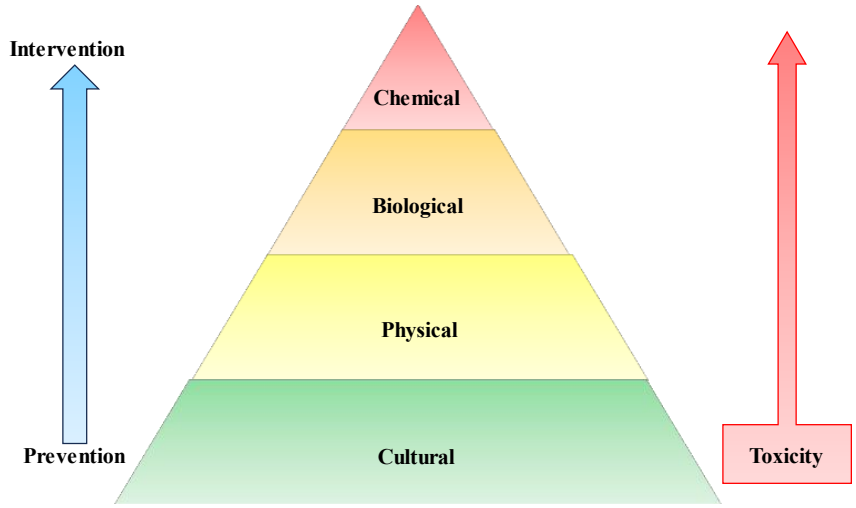


Figure 3. Integrated pest management rules for pest control in the agriculture [14].

The principles of integrated pest management are outlined in Annex III of Directive 2009/128/EC of the European Parliament and of the Council of 21 October 2009, establishing a framework for Community action to achieve the sustainable use of pesticides (OJ EU 24.11.2099 L 309/71). In line with this, the mandatory implementation of IPM practices from 1 January 2014 was stipulated by Regulation (EC) No 1107/2009 of the European Parliament and of the Council of 21 October 2009, repealing Council Directives 79/117/EEC and 91/414/EEC (OJ EU 24.11.2009 L 309/1). The core objective of this regulatory framework is to minimize the environmental impact of human activities and mitigate risks to human and animal health (**Fig. 3)**. This is why agrotechnical and breeding methods of crop protection are prioritized within the IPM approach. While chemical methods are permitted, their application have to be preceded by an economic analysis of potential yield losses. Notably, the goal of using chemical sprays is not to eradicate pathogens completely, but rather to reduce their presence to levels that are economically acceptable [12]. Therefore, herbicides will continue to be a crucial component of agricultural systems.

1.1.1. Herbicides

Herbicides are a chemically diverse group of compounds that disrupt and retard the growth of plants. They are applied on a wide scale in agriculture to control weeds, but they also find an application in the upkeep of railways, landscaping, and personal use [16]. The concept of using chemicals to control weeds dates back to early attempts that included the use of inorganic salts, waste oil or even sulfuric acid [17]. In 1896, the first organic herbicide, Sinox (sodium dinitrocresylate), was developed in France,

marking a shift towards more targeted chemical solutions [16]. This progress accelerated in the 1920s and 1930s with the discovery of plant growth hormones, which paved the way for development of new generation of herbicides based on organic compounds mimicking plants' hormones [18]. The first artificial auxin to be synthesized chemically was 2,4-D in 1940 in United Kingdom and in parallel in 1941 in United States of America. After World War II, 2,4-D and MCPA were commercialised and remain widely available to this day. These events manifest a start of the agrochemical era. This shift can be illustrated by the increase in pesticide usage: in 1940, only 140 metric tons of various pesticides were employed, while by 2016, global pesticide use had surged to over 4 million metric tons (**Fig. 2)** [19,20].

However, despite their widespread use, it is estimated that only 1% of pesticides reach their intended targets. The substantial quantities of remaining pesticides may in turn come in contact with unintended organisms in the biosphere and persist in the environment. Consequently, this pesticide contamination has led to the environmental pollution and negative impact on human health [8]. These adverse effects are further intensified by the fact that due to regulatory issues, herbicidally active compounds are held to different and more stringent toxicological safety standards than adjuvants [21–23].

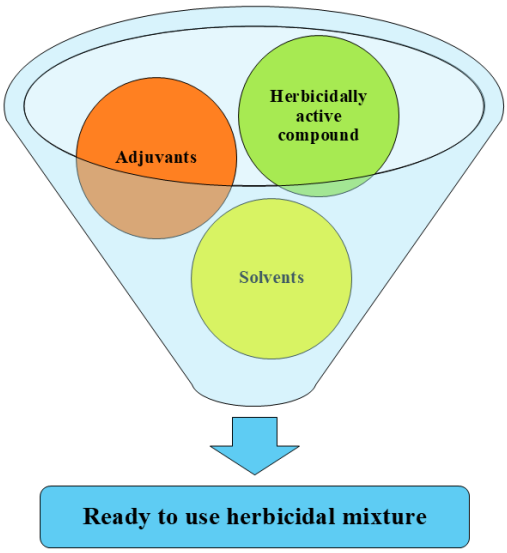


Figure 4. Simplified composition of typical herbicidal mixture for field use [24].

Adjuvants are additives included in herbicidal formulations to improve the performance and effectiveness of herbicides (**Fig. 4)** [25]. These compounds can include a wide range of compounds, and their specific roles in herbicidal formulations can vary greatly, depending on the herbicide, the targeted weed or crop, and the environmental conditions. Adjuvants play a crucial role in enhancing herbicidal efficacy, minimizing off-target effects, and maximizing cost-effectiveness of herbicide applications. However, their toxicity has become a growing concern because these additives, although not herbicides themselves, can significantly contribute to the overall environmental impact of herbicide applications.

In fact, some commercial spray mixtures exhibit high toxicity not because of the active herbicidal ingredient, but due to the presence of adjuvants [26]. A striking example comes from a study where 8 out of 9 commercially available pesticide formulations (comprising 3 fungicides, 3 herbicides, and 3 insecticides) demonstrated significantly higher levels of cytotoxicity across all tested cell lines compared to the individual active ingredients [21]. Additionally, it was established that polyoxyethylene tallow amine (POEA), a previously common, non-ionic surfactant used in glyphosate-based herbicides, emerged as a key contributor to the cytotoxicity of these products. Research showed that POE (15) tallow amine (POE-15) was found to be 10,000 times more toxic to mitochondrial activity in human cells than glyphosate itself. Moreover, the toxicity of the commercial formulations tested directly correlated with the amount of POE-15 they contained [27]. Further studies confirmed similar results regarding adjuvant toxicity *in vivo* across various animals and several pesticide formulations [28–32]. In response to these alarming findings, the concept of ionic liquids with herbicidal activity was proposed in 2011. This approach aimed to eliminate the need for adjuvants in herbicidal formulations, thereby reducing the environmental impact of conventional weed killers and addressing toxicity concerns associated with commercially available products [33].

1.2. Ionic liquids with herbicidal activity

The concept of herbicidal ionic liquids (HILs) represents an innovative approach in weed control, addressing some of the most persistent issues associated with conventional herbicides [34]. This novel class of compounds combines the unique characteristics of ionic liquids with herbicidal properties, potentially offering a solution to several problems inherent in traditional herbicides.

One of the most significant challenges with classic herbicidally active compounds, such as 2,4-D, dicamba or MCPA, is their high vapour pressure [35]. This characteristic leads to a phenomenon known as vapour drift, where herbicidal particles evaporate from already treated fields or are carried by wind during spraying operations to unintended locations [34]. This drift can result in serious environmental damage and harm to neighbouring crops. For instance, in the USA, allegedly over 1.45 million hectares of non-tolerant soybean cultivations were damaged by dicamba vapour drift in 2017 alone [36]. Even with the introduction of newer, lower-volatility formulations like XtenidMax [37] and Engenia [38] in 2016, significant damage continued, with allegedly over 404,000 ha of non-tolerant soybean crops damaged in 2021 and almost 65,000 ha of non-agricultural wildlife refuge injured in that same year [39]. Additionally, vapour drift of pesticides exposes individuals to health risks, particularly farmworkers, nearby residents, and others exposed to the drifting vapor or settled residues [40]. Finally, it can lead to legal and regulatory challenges, including fines, penalties, and damage claims from affected parties [41].

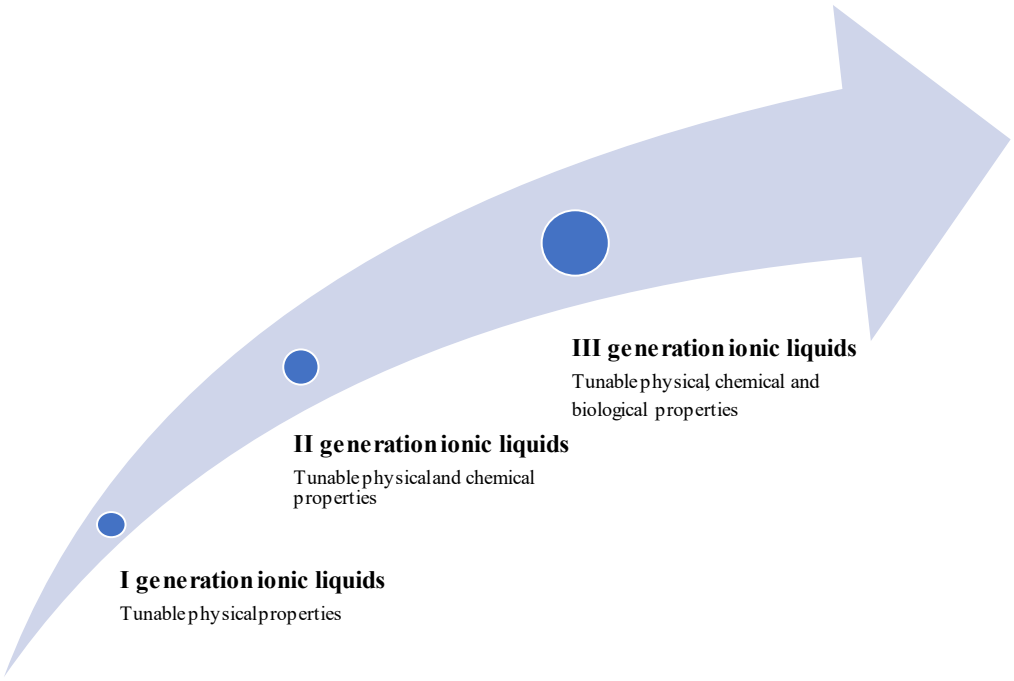


Figure 5. Development of ionic liquids properties [42].

On the other hand, ionic liquids, which are organic salts that remain in a liquid state below 100°C, behave quite differently from traditional salts, most notably in their reported very low vapour pressure [43]. Ionic liquids are renowned for their unique

properties, including high thermal stability, solvating capabilities and abovementioned low volatility. Additionally, due to a structure comprising large, asymmetrical organic cations paired with organic or inorganic anions, these compounds exhibit extraordinary potential to modify the structure and thus manipulate physicochemical and biological properties. These features make them valuable across various fields, such as green chemistry, catalysis, and materials science.

Ionic liquids with herbicidal activity aim to combine the strengths of ionic liquids with the herbicidal efficacy of specific chemical compounds. Designed to function as effective herbicides while retaining the advantageous properties of ionic liquids, they promise a range of significant benefits. By minimizing issues like vapour drift and optimizing herbicidal performance, HILs could potentially revolutionize weed management, offering a more environmentally friendly and effective alternative to conventional herbicides.

1.2.1. Current state of knowledge on HILs

Herbicidal ionic liquids belong to the third generation of ionic liquids, specifically designed to exhibit certain biological activity (**Fig. 5**) [42]. Initially, this concept was primarily explored within the pharmaceutical industry, in order to create medicines with enhanced therapeutic effectiveness [44]. However, the versatility of ionic liquids was later recognized for its potential in agricultural protection, leading to the development of HILs by integrating additional surface-active and pesticidal properties resulting from the counterions present in their structures [34]. These newly developed compounds displayed significantly reduced volatility, thus lowering the risk of vapour-drift phenomena [45]. The ability to modify the counterion also allows for customizable water solubility, which can reduce the movement of these compounds within soil and water, thereby lowering the risk of environmental contamination. Furthermore, the inherent surface-active properties of HILs eliminate the need for additional surfactants in herbicidal formulations, streamlining their application and potentially reducing overall environmental toxicity [34]. Moreover, early studies indicate that HILs can be applied in lower quantities compared to traditional herbicides, which could lead to a reduction in environmental toxicity and health risks [34,45].

Figure 6. Current state of knowledge regarding HILs; research focused on synthesis and physicochemical properties causes knowledge of biological activity to remain relatively understudied.

Despite these promising findings, it is crucial to recognize that the development and application of herbicidal ionic liquids remain active areas of research, with no commercial uses so far [34]. Current efforts in this field are focused on refining their formulations and assessing their long-term environmental impact, while ensuring that safety and regulatory standards are met before they can be widely adopted in agriculture (**Fig. 6**).

1.2.1.1. Synthesis of HILs

The core concept behind HIL synthesis is the transformation of conventional herbicides into ionic formulations, where the active herbicidal compound is integrated into the ionic liquid structure, either as a cation, an anion, or both [34]. The synthesis of HILs typically begins with the selection of an appropriate herbicide, which often contains functional groups like carboxylates or phosphonates that enable it to act as an anion [33,34,46]. Many commonly used herbicides, such as glyphosate, dicamba, and 2,4-D (2,4-dichlorophenoxyacetic acid), are anionic in nature and can be directly incorporated into ionic liquids as the anionic component. The cation is typically a large, organic species chosen for its complementary properties, such as increased solubility in water or organic solvents, or surface activity contributing to enhancement in the uptake of herbicide by plants [34]. Cations frequently used in HIL synthesis include imidazolium, pyridinium, quaternary ammonium, and phosphonium ions, which are known for their chemical stability and structural tunability [34,45,47].

The most common method of synthesizing HILs is through ion exchange reactions [34,48,49], in which a quaternary ammonium or imidazolium halide

(*e.g.*, [Chol][Cl]) is reacted with a herbicidal salt (*e.g.*, sodium iodosulfuron-methyl, [Na][ISM]). This process is widely used due to its simplicity and effectiveness in producing HILs with minimal byproducts. Beside ion exchange between two salts, the alternative synthetic route for HILs is the neutralization method, where a herbicidal acid reacts with a basic organic cation precursor [34,50,51]. For example, glyphosate (commonly used in its acidic form) can be neutralized with a didecyldimethylammonium hydroxide to form a herbicidal ionic liquid with a glyphosate anion. In addition to these synthesis routes, quaternization reactions are employed to produce herbicidal cations [52,53]. For instance, quaternary ammonium herbicidal cations can be synthesized by reacting a tertiary amine with a functionalized alkyl halide. Once the herbicidal cation is formed, it can be paired with another appropriate herbicidal anion to form the desired ionic liquid.

The key advantage of herbicidal ionic liquids lies in their tunability, meaning their physicochemical properties can be easily modified by altering the cation or anion structure [34]. By adjusting the length of alkyl chains on the cation or introducing functional groups, researchers can optimize properties such as surface activity, water solubility, viscosity, thermal stability, and biodegradability. For example, longer alkyl chains on the cation can increase the hydrophobicity of the herbicidal ionic liquid, enhancing its ability to penetrate plant waxy cuticles and improve herbicide efficacy. Moreover, certain cations may promote the transport of the herbicidal ionic liquid into plant tissues, improving bioavailability and absorption rates. Therefore, the conversion of herbicides into ionic liquid form poses an interesting alternative as an application form of these plant protection agrochemicals. Since this form potentially allows to mitigate the adverse effects of commercial formulations, the research on herbicidal activity of HILs, along with the first insights into their impact on the environment is currently being performed.

1.2.1.2. Herbicidal activity of HILs

HILs are proven to be potent weed-killing agents, as their activity is usually comparable or higher than referential herbicidal mixtures. For example, during herbicidal activity tests done by Cococaru et al. (2013), dicamba-based HILs with didecyldimethylammonium cation proved to be more effective in field tests at eradicating both *Chenopodium album* and *Centaurea cyanus*, by 9% and 5%, respectively, than commercial dicamba formulation Banvel® [46]. Likewise, when

field fresh weight reduction of *Chenopodium album* was investigated, spraying operation with HILs with ammonium-, piperidinium-, and morpholinium-based cations resulted in 62.5%, 62.5% and 57.5% mass reduction, respectively, while spraying with Banvel® caused only 42.5% reduction. Niemczak et al. (2017) reported similar effects in greenhouse experiments, where HILs with modified betaine cations (*N*-dodecylbetaine and *N*-(3-cocoamidopropyl)betaine) were much more effective at controlling *Chenopodium album* and *Brassica napus* than dicamba in sodium salt form [54]. While reported herbicidal efficacy toward *Centaurea cyanus*, was comparable for HILs and reference herbicide, in the field experiments with [C_{12}Bet][Dic], HIL proved to be more effective at controlling *Chenopodium album* and *Brassica napus* in spring barley cultivation than reference dicamba herbicide. Interestingly, Pernak et al. (2020) prepared dicamba-based ILs, with two compounds being synthetic analogues of commercial products XtenidMax® and Engenia®, and examined them against novel HILs and reference herbicide [53]. In the case of volatility, the analogues of XtenidMax®, Engenia® and other HILs designed by Pernak's research team proved to be significantly less volatile then dicamba in free acid form. As for the herbicidal activity, all of proposed HILs, analogues of XtenidMax®, Engenia®, as well as the reference dimethylammonium salt of dicamba, proved to be highly effective against *Amaranthus retroflexus*, reaching over 60% of fresh weight reduction. However, in the case of *Centaurea cyanus*, HILs incorporating long alkyl substituents demonstrated to be more effective than reference mixture and other HILs, reaching efficiency of approx. 70%, whereas HIL with oleyl substituent had efficiency similar to reference mixture and analogues of commercial formulations (approx. 50%). On the other hand, efficiency of fresh weight reduction showed similar trend for *Chenopodium album,* where aforementioned HILs with alkyl substituents reached the efficiency of 60%, while the reference mixture – only 40%. Similar results were achieved by Stachowiak et al. (2021), in an experiment with iodosulfuron-methyl-based HILs, where only HIL with docosyl ($C_{22}H_{45}$) substituent in its structure was less effective than referential herbicidal mix [49]. Albeit, this was probably caused by the limited solubility of this compound in water, which resulted in concentration well below the field application dose. Many other studies have been conducted, with each yielding comparable results, clearly demonstrating that the transformation of herbicide into an ionic form might be beneficial, as such compounds either retain [55,56] or exceed herbicidal activity of commercial formulations when applied at comparable doses [57,58].

1.2.2. Insights into biological activity towards target and non-target organisms

To date, most available data have focused on the herbicidal activity of novel compounds, with limited research addressing their antimicrobial properties and impact on non-target organisms [34]. Studies examining the impact of HILs on microbial soil communities are particularly scarce, with only a few articles published in this field, leaving much to be explored.

One of the earliest studies investigated the changes in microbial microcosms exposed to HILs based on 2,4-D and MCPA under anaerobic conditions [59]. However, further analysis revealed that the bacterial community lacked genes associated with 2,4-D biotransformation. This suggests that the observed changes may be due to the toxicity of the accumulated herbicide and the bacteria's inability to process this pollutant, rather than the inherent toxicity of the HILs themselves. Recently, attention has been given to the impact of cation structure on biodegradation efficiency [54,57,58,60]. For instance, Ławniczak et al. (2016) examined oligomeric ionic liquids with herbicidal activity based on MCPA and dicamba, finding that specific cations not only influence biodegradability but also alter the microbial structure of the samples [60]. Additionally, different cations were found to induce diverse changes in the microbiome's structure. Similar findings have been reported in studies on glyphosate- [61] and 2,4-D-based HILs [62]. These results suggest that the cation, rather than the herbicidal anion, is the main factor contributing to HIL toxicity. This is especially significant given that degradation pathways of many herbicides in the environment involves soil microbiome activity [63–65] whose disruption could lead to plant damage and reduced crop yields [66].

Moreover, it has been reported that the cationic and anionic components of HILs degrade at different rates, implying that ionic liquids may serve more as a delivery method for herbicides rather than exist as stable entities in the environment. For instance, Wilms et al. (2023) showed that sorption significantly reduces the bioavailability of cations in soil, potentially leading to their bioaccumulation and limited degradation, which could adversely affect the biotransformation of herbicidal anions [61,62]. These concerns about the integrity of HILs are supported by research of Woźniak-Karczewska et al. (2023), which demonstrated that the adsorption parameters for the cation and anion of 2,4-D-based HILs were entirely independent,

with the cations' adsorption K_f values correlated with their hydrophobicity [67]. Furthermore, studies on soil microcosms exposed to HILs indicate that cation selection is a key factor driving changes in microbial community composition [62,67]. These findings underscore the significant environmental implications of IL form and suggest crucial role of selection of cation in the HIL molecule structure. Additionally, growing awareness of the importance of diverse bacterial and fungal microcosms for plant health [66,68–70] further highlights the need to address the impact of HILs on soil-borne bacteria, which might be at elevated risk due to persistent contact with such xenobiotics (**Fig. 7)**.

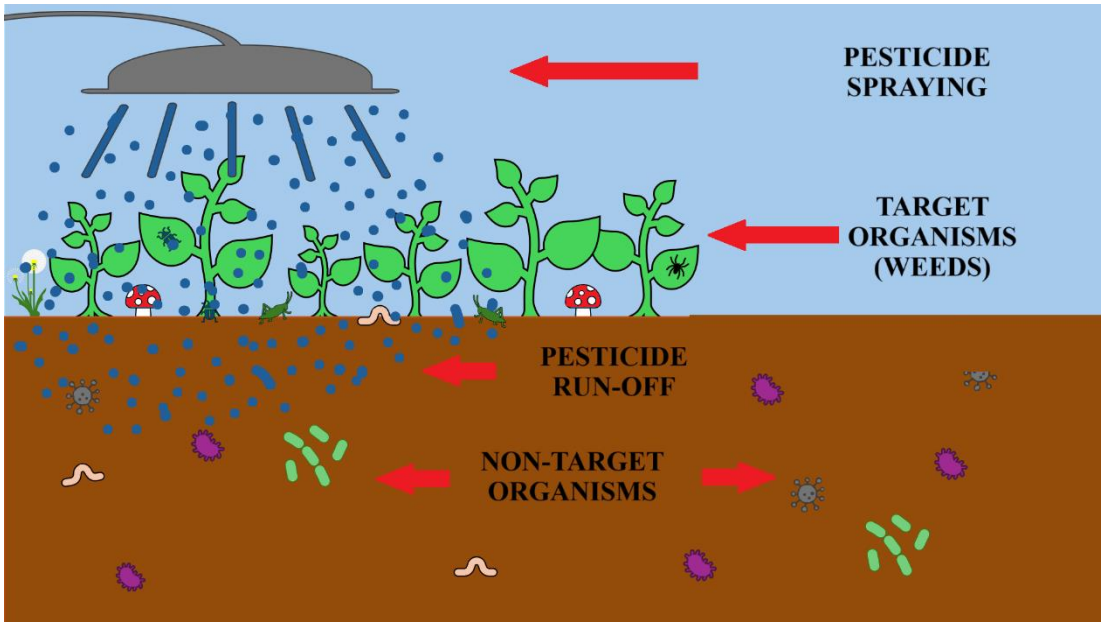
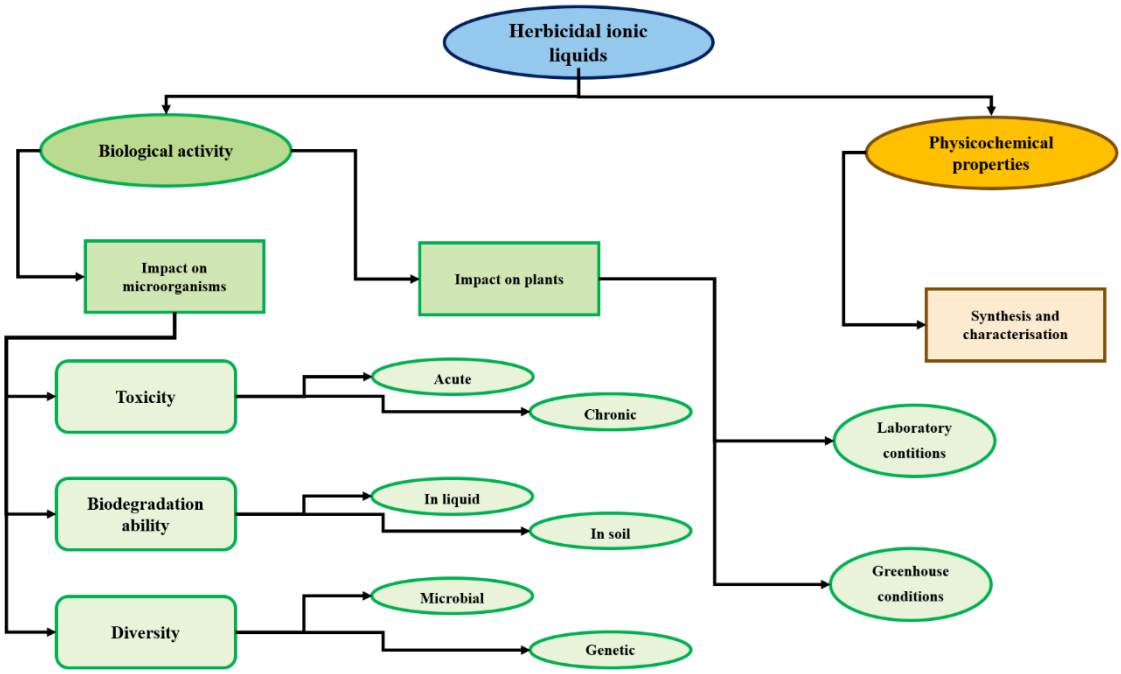


Figure 7. Herbicidal mixture spraying operation result in exposure of non-target organisms such as soil microbiome to herbicidal formulations.

Although cations utilized in HILs formulations are customizable, most incorporate quaternary ammonium structures modified with various substituents [34]. Quaternary ammonium salts, widely used in industry as potent surfactants, are also found in cosmetic products for their antibacterial properties, with more potent variants employed as medical disinfectants [71]. The antimicrobial effect of these compounds is linked to their ability to destabilize and disrupt bacterial cell membranes [71–73]. However, when quaternary ammonium compounds are present in insufficient concentrations to kill bacteria, the cells may experience oxidative stress [72], lose proton motive force [74], experience respiratory enzyme inhibition [75], and suffer

from error-prone DNA replication [75]. A critical aspect of oxidative stress is its promotion of the uptake of genetic material (*e.g.*, plasmids) from the environment [72,76]. This phenomenon is particularly concerning, as it could lead to the uncontrolled spread of various genes within agricultural soil [75,77], potentially turning soil bacteria into reservoirs of genes for resistance to plant protection chemicals or antibiotics (especially if manure is used as fertilizer). Moreover, this could include genes encoding virulence in certain bacterial species, possibly resulting in plant disease outbreaks. Such outcomes would ultimately contribute to the very issues herbicides are meant to prevent – decreased quantity and quality of plant biomass.

2. Objective of the thesis



1) Determination of biological activity of ionic liquids with herbicidal activity

Herbicidal ionic liquids are thought to be more potent than traditional herbicides, with research indicating their increased toxicity not only to weeds but also to non-target organisms. The tested hypothesis is that since HILs do not introduce a novel modes of action, but serve as a novel application form of known herbicides, they are ineffective against herbicide-resistant weeds. Another hypothesis tested within this task is that the presence of hydrophobic, surface-active cation in the structure of HILs contributes to increased toxicity (and hence biological activity) of tested compounds.

2) Determination of toxicity to microbes and microbial communities

Since highly hydrophobic cations have greater impact on bacteria compared to cations of natural origin, extending the length of the alkyl substituent should lead to increased toxicity of ILs toward various microorganisms. The analysed hypothesis is that the effects of HILs and reference herbicides on microorganisms will differ, depending heavily on the cation selection within the molecule. Among the tested microbes,

bacteria and fungi are genetically diverse groups, so their responses and tolerance to HILs are expected to vary.

3) **Determination of the impact of ionic liquid form on biotransformation of the herbicides by microbes**

The environmental persistence of xenobiotics is a particularly hazardous, as bacteria may activate cellular defence mechanisms to counteract the bactericidal effects, allowing them to persist in sublethal concentrations under conditions favourable for horizontal gene transfer. Hence, the tested hypothesis is that due to reduced vitality caused by the cation's toxicity, microorganisms will be less effective at biotransforming highly hydrophobic HILs, resulting in longer half-lives of tested compounds compared to reference herbicides.

4) **Determination of the influence of HILs on gene presence in the environment**

The known consequence on bactericidal activity of xenobiotics is their impact on biodiversity in the environment. Therefore, the final tested hypothesis is that the prolonged contact of microorganisms with HILs will result in enrichment of bacterial communities in various niches in highly resilient species, thus boosting the presence of genes taking part in breaking-up of herbicides.

3. Materials and methods

3.1. Synthesis and characterisation of HILs

3.1.1. Chemicals and Reagents

Herbicides (2-methyl-4-chlorophenoxy)acetic acid (MCPA) (97%) and (±)-2-(4-chloro-2-methylphenoxy)propionic acid (MCPP) (93%) were procured from Ciech-Sarzyna (Nowa Sarzyna, Poland). Iodosulfuron-methyl sodium salt (ISM, 96.6%) and 3,6-dichloro-2-methoxybenzoic acid (95%) was obtained from Pestinova (Jaworzno, Poland). Reagents for HIL synthesis, including 1-bromooctane (99%), 1-bromononane (98%), 1-bromodecane (98%), 1-bromoundecane (98%), 1-bromododecane (97%), 1-bromotetradecane (97%), 2-dimethylaminoethanol (≥99.5%), triethylamine (99.5%), thionyl chloride (97%), dimethylethanolamine (DMAE, 99%), betaine in zwitterionic form (99%), as well as an anionic resin AmberTec™ UP550 OH, were sourced from Sigma-Aldrich (Saint Louis, MO, USA). Didecyldimethylammonium chloride (50% v/v water:isopropanol solution) was obtained from Akzo-Nobel (Amsterdam, The Netherlands). Potassium hydroxide (99%), acetonitrile (99%), ethyl acetate (99%), ethanol (96%), methanol (99.8%), *n*-heksan (>95%), chloroform (99%), (2-hydroxyethyl)trimethylammonium (choline) chloride (99%) and hexyl bromide (98%) were obtained from Avantor (Gliwice, Poland) and International Enzymes Limited (Fareham, Hampshire, England). Deionized water with a conductivity below 0.1 μS/cm was obtained through a HLP Smart 1000 demineralizer (Poznań, Poland).

3.1.2. MCPA/MCPP-based HILs

HILs based on MCPA/MCPP herbicides were synthesised as described by Parus et al. (2021) [78]. The MCPA and MCPP herbicides were purified following Syguda et al. (2018) [57]. Briefly, MCPA chloride (80 g) was dissolved in chloroform (100 mL, 99%) and combined with equimolar DMAE-chloroform solution, and the product was filtered, rinsed with hexane (>95%), and dried. Then, the product (100 g) was mixed with chloroform (300 mL) and equimolar amount of triethylamine (99.5%), vigorously stirred and similarly processed. The resulting MCPA aminoester (5 g) was reacted with an appropriate alkyl bromide ($CH_3(CH_2)_7Br$, $CH_3(CH_2)_8Br$, $CH_3(CH_2)_9Br$, $CH_3(CH_2)_{10}Br$, $CH_3(CH_2)_{11}Br$, $CH_3(CH_2)_{13}Br$) at 55 °C for 24 h to yield (4-chloro-2-

methylphenoxy)-2-acetoxyethylalkyldimethylammonium bromides. These bromides (1 g) were then dissolved in a water-isopropanol solution (15 mL, 2:1, v/v). Separately, MCPP was reacted with an equimolar quantity of a sodium hydroxide (10% aqueous solution) to form sodium (±)-2-(4-chloro-2-methylphenoxy)propionate, which was then added to the esterquat bromides solution and vigorously stirred for 40 min. The mixture was phase-separated with chloroform, and the ionic liquid was rinsed with deionized water and dried at 60 °C until a constant mass was achieved.

Purity was assessed by two-phase titration [79,80]. The synthesized ionic liquids were characterized by ^{1}H NMR and ^{13}C NMR spectroscopy using a Bruker Avance 400 MHz Ultra Shield Plus spectrometer (Billerica, MA, USA), with tetramethylsilane (TMS) serving as the internal standard and DMSO-d_6 as the solvent. The instrument was utilized at 400 MHz for ^{1}H NMR and at 100 MHz for ^{13}C NMR spectra. The spectra are shown in the Anex (**Fig. S1-S10**).

3.1.3. Iodosulfuron-methyl-based HILs

Hexyl(2-hydroxyethyl)dimethyl bromide ([C_6Chol][Br]), dodecyl(2-hydroxyethyl)dimethyl chloride ([C_{12}Chol][Cl]) and *N*–tetradecylcholine bromide ([C_{14}Chol][Br]) were synthesized *via* quaternization reactions in acetonitrile, following established methodology [81]. After solvent removal, the product was precipitated with ethyl acetate (100 mL, 99%) and cooled (to 5 °C), and then carefully separated *via* vacuum filtration, washed with small portions of cooled ethyl acetate and dried under reduced pressure at 50 °C for 24 h.

For [C_{14}Chol][ISM] synthesis, an ion exchange reaction was employed, following a procedure from Niemczak et al. (2020) [82]. Initially, 0.01 mol of *N*-tetradecylcholine bromide was dissolved in methanol (15 mL, 99.8%) and mixed with 0.0102 mol of the sodium iodosulfuron-methyl in methanol (15 mL). The mixture was stirred at 50 °C for 15 min and then cooled to 0 °C. The resulting sodium bromide precipitate was filtered out, and the solvent was evaporated. The crude product was further purified by dissolving in chloroform (15 mL, >95%), then filtered off and dried under reduced pressure at 50 °C for 24 h.

For [Chol][ISM, [C_6Chol][ISM] and [C_{12}Chol][ISM] synthesis, 0.01 mol of sodium iodosulfuron-methyl was dissolved in ethanol (96% v/v, 15 mL). Then, equimolar quantities of the respective quaternary ammonium halides ((2-hydroxyethyltrimethyl

chloride for [Chol][ISM], hexyl(2-hydroxyethyl)dimethyl bromide for [C_6Chol][ISM], dodecyl(2-hydroxyethyl)dimethyl chloride for [C_{12}Chol][ISM]) were added, and the mixtures were vigorously stirred at 50°C for 15 min to ensure complete ion exchange. After cooling the solutions to room temperature, the by-product salts were filtered out, and the ethanol was evaporated. The final products were dried under reduced pressure at 25°C. The structures of the obtained salts were confirmed *via* spectroscopic methods. ^{1}H and ^{13}C NMR spectra were obtained using a Varian VNMR-S 400 MHz spectrometer (Crawley, UK), operating at a frequency of 400 and 100 MHz, respectively, with TMS as an internal standard. The IR spectrum was acquired with a ReactIR iC15 probe connected to an EasyMax 102 system (Mettler Toledo, Greifensee, Switzerland). Data processing was done using iCIR 4.3 software. The spectra are provided in the anex (**Fig. S11-S22**). The water content in all obtained products was measured with a TitroLine 7500 KF trace apparatus (SI Analytics, Germany) using the Karl Fischer titration method.

3.1.4. Dicamba-based HILs

(2-hydroxyethyl)trimethylammonium 3,6-dichloro-2-metoxybenzoate [Chol][Dic] and didecyldimethylammonium 3,6-dichloro-2-metoxybenzoate [DDA]Dic] were synthesized by neutralization of dicamba acid with appropriate hydroxide. The latter alkyl betainate salts (ethyl betainate 3,6-dichloro-2-metoxybenzoate [BetC_2][Dic], decyl betainate 3,6-dichloro-2-metoxybenzoate [BetC_{10}][Dic] and hexadecyl betainate 3,6-dichloro-2-metoxybenzoate [BetC_{16}][Dic]) were synthesized in an anion exchange reaction between dicamba potassium salt and alkyl betainates bromides according to works of Niemczak et al. (2024) and Stachowiak et al. (2022) [47,83]. The water content in all obtained products was measured with a TitroLine 7500 KF trace apparatus (SI Analytics, Germany) using the Karl Fischer titration method.

3.2. Biological material

3.2.1. Bacteria

3.2.2. Bacterial strains obtained from collections

Hanshlegiella zhihuiae S113 (DSM 18984), *Streptomyces griseolus* (ATCC 11796) (also identified as *Streptomyces halstedii* DSM 40854), and *Bacillus subtilis* 168

(DSM 23778) were obtained from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Berlin, Germany). *B. subtilis* 168 was utilized instead of *Bacillus subtilis* YB 1 due to its high sequence similarity: 99% overlap with the manganese ABC transporter over 306 amino acids; 100% match with vegetative catalase 1 over 476 amino acids, and 99% identity with acetoin dehydrogenase from the model *B. subtilis* [84,85]. Each bacterial strain was cultured following the protocols provided by the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Berlin, Germany).

3.2.3. Bacterial communities obtained from the environmental samples

Isolation of microorganisms from soil and rhizosphere

Approx. 2 g of soil (wet weight) was collected from the soil or root surface and placed into a sterile 150 mL Erlenmeyer flask. The samples were then mixed with 25 mL of 50% (w/v) Tryptic Soy Broth (TSB) medium (Sigma Aldrich, Poznań, Poland) and incubated on a rotary shaker at 30 °C for 48 h. After incubation, the enrichment cultures were separated from the soil sediments, centrifuged for 15 min at 4,500 rpm at 4 °C and stored in glycerol stocks (20%, v/v) at –80 °C until use. All assays were conducted in triplicate.

Isolation of epiphytes

The plants were thoroughly washed with tap water for approx. 10–15 minutes to remove excess soil particles, following Anjum et al. (2015) [86]. Then, approx. 2 g of plant material were placed under a sterile laminar flow cabinet in a clean breaker. The plant material was then immersed in sterile deionized water and stirred for 1 min. Next, 1 mL of the wash leachate was transferred to a sterile Erlenmeyer flask (150 mL) filled with 25 mL of 50% (w/v) TSB medium, placed on a rotary shaker, and incubated at 30 °C for 48 h. After incubation, the enrichment cultures were separated from the soil sediments, centrifuged for 15 min at 4,500 rpm at 4 °C and stored in glycerol stocks (20%, v/v) at –80 °C until use. All preparations were conducted in triplicate, following Hallman et al. (2007) [87].

Isolation of endophytes

After the epiphyte isolation procedure, plant material was drained of excess water, divided into three samples, and placed in a sterile beaker under a laminar flow cabinet.

Each sample was immersed in 70% (v/v) ethanol for 30 s, drained, and then submerged in a 1% (v/v) sodium hypochlorite solution with Triton X-100 surfactant addition for 2 min. Next, samples were again immersed in 70% (v/v) ethanol for 30 s and rinsed 5 times with sterile, deionized water. Then, plant tissues were gently crushed under the laminar flow cabinet using a sterile mortar with small amount of sterile, deionized water. Subsequently, 1 mL of the macerated mixture was added to 25 mL of 50% (w/v) TSB medium in a sterile Erlenmeyer flask (150 mL), placed on a rotary shaker, and incubated at 30 °C for 72 h, following Hallman et al. (2007) [87]. After incubation, the enrichment cultures were separated from the soil sediments, centrifuged for 15 min at 4,500 rpm at 4 °C and stored in glycerol stocks (20%, v/v) at –80 °C until use.

To verify sterility of the plant surfaces before endophyte isolation, 100 µL of water from the final rinse was placed on a 50% (w/v) TSA agar plate. If no growth was observed within 72 h of incubation at 30 °C, the sterilization process was considered successful. All preparations were conducted in triplicate.

3.2.4. Fungi

3.2.4.1. Isolation of fungi capable of herbicide biotransformation

Eight distinct soil samples were used in this study to identify fungi capable of degrading iodosulfuron-methyl. Soil 1 and Soil 2 were commercially available peat substrates (BIO Universal substrate) purchased from SIA Siluflora (Lapas, Latvia) in a 45L package. Soils 3–7 were collected from an agricultural field located in Rzgów, Poland (N 52.151102, E 18.050041), which had not been exposed to herbicides prior to the experiments. Soil 8 was sourced from a flowerbed in Poznań, Poland (N 52.393653, E 16.919016).

Soil samples (15 g) were enriched with iodosulfuron-methyl-sodium at a rate of 100 mg of active ingredient per 1 kg of dry soil. They were then mixed with 5 mL of sterile deionized water, transferred to a sterile Erlenmeyer flask (150 mL), and incubated for 7 days at 28 ± 2 °C. Following this incubation, the samples were transferred onto prepared PDA (Potato Dextrose Agar) agar plates (containing 200 g/L potato extract, 20 g/L glucose and 3.5 g/L agarose) using a sterile spatula. The samples on the agar plates were incubated for 5 days at 28 ± 2 °C. Subsequently, the fungal cultures that had developed were transferred onto fresh medium three times, with each incubation lasting 5 days at 28 ± 2 °C to obtain pure strains. The isolated strains were then

distributed onto media containing varying herbicide concentrations: 100, 500, 1000, and 2000 mg, respectively. These cultures were incubated for 5 days at 28 ± 2 °C and subsequently passaged onto fresh medium three times to ensure the cultures obtained were pure.

3.2.4.2. Identification of fungi

DNA extraction was carried out using the DNeasy Blood & Tissue Kit from Qiagen (Hilden, Germany). The ITS1 region was targeted for amplification using primers ITS1-F-KYO2 and ITS2-KYO2 [88] with added indels and adapters at the 5' ends for Ion Torrent sequencing (Life Technologies, Carlsbad, CA, USA). PCR amplification was conducted in two technical replicates, each in a 5 µL reaction containing Hot FIREPol DNA polymerase (Solis Bio-Dyne, Tartu, Estonia), 0.25 µM of each primer, and 1 µL of template DNA. The amplification process conditions were: initial denaturation at 95 °C for 12 min, followed by 35 cycles of 15 s at 95 °C, 30 s at 50 °C, and 45 s at 72 °C, with a final extension at 72 °C for 5 min.

The amplification efficiency was evaluated *via* electrophoresis on a 1.5% agarose gel. Amplicons were combined and purified using 2% E-Gel SizeSelect II Agarose Gels (Invitrogen, Waltham, MA, USA). DNA concentration and the fragment lengths distribution were evaluated using High Sensitivity D1000 Screen Tape on a 2200 Tape Station system (Agilent Technologies, Santa Clara, CA, USA). Clonal amplifications and sequencing were carried out with the Ion 520™ & Ion 530™ Kit-OT2 using the Ion 530 chip and the Ion S5 system (Life Technologies, Carlsbad, CA, USA), following the manufacturer's instructions.

Reads shorter than 200 base pairs and those with a quality score below 25 were excluded using the Ion Torrent Suite (Life Technologies, Carlsbad, CA, USA) [89–91]. Sequences were separated *via* Geneious Prime software R11.1.5 (Biomatters Ltd., Auckland, New Zealand) into various index combinations and trimmed at both the 5' and 3' ends. Amplicon sequence variants (ASVs) were grouped using the QIIME2 software [92,93]. ASVs representing ITS region sequences were identified and extracted using ITSx [94]. These ASVs were incorporated into the GenBank database at a 96% identity threshold [95].

To validate the taxonomic classification of the fungal species identified by NGS, additional genes suitable for fungal species identification (**Table 1)** were amplified. PCR products were purified using exonuclease I and alkaline phosphatase (Thermo

Scientific, Waltham, MA, USA) and then sequenced using BigDye Terminator v3.1 on an ABI Prism 3130XL Analyzer (Applied Biosystems, Thermo Fischer Scientific, Waltham, MA, USA), following the manufacturer's guidelines. Chromatograms were analysed using FinchTV (Geospiza Inc., Seattle, WA, USA) and Geneious Prime software.

Table 1. Primers used for taxonomic identification of isolated fungi.

Sample ID	Amplified region	Primers	Sequence	Ref.
Isolate I	ITS	ITS1	TCCGTAGGTGAACCTGCGG	[96]
		ITS4	TCCTCCGCTTATTGATATGC	
	Tef-1α	Ef728M	CATCGAGAAGTTCGAGAAGG	[97]
		TefR1	GCCATCCTTGGAGATACCAGC	
Isolate II	ITS	ITS1	TCCGTAGGTGAACCTGCGG	[96]
		ITS4	TCCTCCGCTTATTGATATGC	
	BenA	Bt2a	GGTAACCAAATCGGTGCTGCTTTC	[98]
		Bt2b	ACCCTCAGTGTAGTGACCCTTGGC	
	RPB2	RPB2-5F	GAYGAYMGWGATCAYTTYGG	[99]
		RPB2-7Cr	CCCATRGCTTGYTTRCCCAT	
	Tsr1	Tsr1-F1526Pc	GARTAYCCBCARTCNGAGATGT	[100]
		Tsr1-R2434	ASAGYTGVARDGCCTTRAACCA	
Isolate III	ITS	ITS1	TCCGTAGGTGAACCTGCGG	[96]
		ITS4	TCCTCCGCTTATTGATATGC	
	BenA	Bt2a	GGTAACCAAATCGGTGCTGCTTTC	[98]
		Bt2b	ACCCTCAGTGTAGTGACCCTTGGC	
	Tsr1	Tsr1-F1526Pc	GARTAYCCBCARTCNGAGATGT	[100]
		Tsr1-R2434	ASAGYTGVARDGCCTTRAACCA	
Isolate IV	ITS	ITS1	TCCGTAGGTGAACCTGCGG	[96]
		ITS4	TCCTCCGCTTATTGATATGC	
	Tef-1α	Ef728M	CATCGAGAAGTTCGAGAAGG	[97]
		TefR1	GCCATCCTTGGAGATACCAGC	
Isolate V	ITS	ITS1	TCCGTAGGTGAACCTGCGG	[96]
		ITS4	TCCTCCGCTTATTGATATGC	
	Tef-1α	Ef728M	CATCGAGAAGTTCGAGAAGG	[97]
		TefR1	GCCATCCTTGGAGATACCAGC	
Isolate VI	ITS	ITS1	TCCGTAGGTGAACCTGCGG	[96]
		ITS4	TCCTCCGCTTATTGATATGC	
	BenA	Bt2a	GGTAACCAAATCGGTGCTGCTTTC	[98]
		Bt2b	ACCCTCAGTGTAGTGACCCTTGGC	
	RPB2	RPB2-5F	GAYGAYMGWGATCAYTTYGG	[99]
		RPB2-7Cr	CCCATRGCTTGYTTRCCCAT	
	Tsr1	Tsr1-F1526Pc	GARTAYCCBCARTCNGAGATGT	[100]
		Tsr1-R2434	ASAGYTGVARDGCCTTRAACCA	

3.3. Impact of HILs on plants

3.3.1. Early development of plants

An evaluation was conducted to assess the influence of the selected HILs ([MCPA-DAE-C_8][MCPP], [MCPA-DAE-C_9][MCPP], [MCPA-DAE-C_{10}][MCPP], [MCPA-

DAE-C_{11}][MCPP], [MCPA-DAE-C_{12}][MCPP], [MCPA-DAE-C_{14}][MCPP]) on the germination and early growth of plants from various taxonomic groups. Model plants, namely maize (*Zea mays*) and cornflower (*Centaurea cyanus*), were chosen for this study. The soil utilized in these experiments exhibited the following elemental composition: 81 mg P/kg soil, 88 mg K/kg soil, 69 mg Mg/kg soil, with a pH of 5.92 and an organic carbon content of 1.01% (equivalent to 10.1 g/kg of soil). The examination of the HILs' impact was conducted using a phytotoxicity test based on the ISO-11269-2:2003 International Standard [101].

The assay was carried out in vertical plastic Phytotox containers (Phytotoxkit, Tigret company, Mariakerke, Belgium). Each container was filled with 100 g of soil, and 25 mL of the analysed substances were added to maintain soil moisture within field capacity limits. This resulted in effective concentrations of 0.0005, 0.001, 0.0024, and 0.02 mM per kilogram of soil dry weight (s.d.w.). A control sample was included, consisting of soil without the analysed compounds and rinsed with deionised water (25 mL). Additionally, reference samples were created using mixtures of commercial herbicides MCPA + MCPP at the same molar concentration as the test samples.

For each concentration of the test compounds, 10 seeds of maize or cornflower were individually planted in soil prepared according to the aforementioned protocol, with three containers for each concentration. These Phytotoxkit plastic containers were placed in the dark at 25 ± 1 °C. After 7 days, the number of germinated seeds was counted, and measurements were taken for both root length and shoot height. The impact of HILs on root growth inhibition of the plants was determined by calculating the germination index (GI) according to the equation below as described by Graj et al. (2013) [102]:

$$GI = \frac{Gs}{Gc} \cdot \frac{Ls}{Lc} \cdot 100\ [\%]$$

where: G_s and G_c are numbers of seeds germinated in the sample and control, respectively, L_s and L_c are the shoot lengths in the sample and control, respectively.

3.3.2. Herbicidal activity

3.3.2.1. MCPA/MCPP based HILs

To evaluate the phytotoxic effects of all tested compounds, common maize (*Zea mays*) and cornflower (*Centaurea cyanus*) were used. The seeds of these plants were placed

within a germination box and watered twice a day for a period of 4 days. Following this germination phase, 10 seedlings from each species were planted in separate 1 dm^3 plastic pots, each filled with the previously described soil. The application of the selected compounds was done by spraying the leaves when the plants were at the 4–6 leaf stage (BBCH 14–16) using a Lurmark 02 110 nozzle (TeeJet Technologies, Wheaton, IL, USA), capable of delivering up to 200 L of the spray solution at 220 kPa. All the compounds tested ([MCPA-DAE-C_8][MCPP], [MCPA-DAE-C_9][MCPP], [MCPA-DAE-C_{10}][MCPP], [MCPA-DAE-C_{11}][MCPP], [MCPA-DAE-C_{12}][MCPP], [MCPA-DAE-C_{14}][MCPP]) were dissolved in a mixture of water and isopropanol (2:1, v/v), with concentrations corresponding to a dosage of 300–400 g/ha. The typical agricultural dosage for these compounds ranges from 600 to 1000 g/ha, and this approach was selected in order to assess whether HILs are more effective than herbicides in their non-ionic liquid form. As a reference, a mixture of sodium salts of MCPA and MCPP at an identical dose to the compounds was used.

After completing the spraying treatment, all the plants were transferred to a greenhouse with controlled conditions, maintaining a constant photoperiod (16/8 h day/night), temperature (20 ± 2 °C), and humidity (60%). The impact on weed control was assessed 3 weeks after the application of the herbicidal mixture. The first plants in each pot were harvested individually, and their fresh weight was measured (Sartorius BP 2000 S, Sartorius Göttingen, Germany) with an accuracy of 0.01 g. The effects of the herbicidal mixtures were expressed as a reduction in fresh weight compared to the non-treated control group.

3.3.2.2. Iodosulfuron-methyl based HILs

Efficacy trials were carried out on cornflower (*Centaurea cyanus*) populations both susceptible and resistant to herbicides belonging to the ALS (acetolactate synthase) inhibitor group, at the Institute of Plant Protection – National Research Institute (Poznań, Poland). The selected cornflower population had previously demonstrated resistance in tests involving herbicides inhibiting ALS, with a resistance index surpassing 71.4 (very high resistance) in Beckie and Tardif's modified scale for ALS inhibitors [103].

Weed seeds were planted in pots filled with a commercially available slightly acidic medium (Kronen, Cerekwica, Poland) at a depth of 1 cm and were cultivated in a greenhouse with optimal conditions for plant growth (20 ± 2 °C, air humidity 60%,

photoperiod of 16/8 h day/night). Plants were appropriately watered and thinned to four plants per pot within 16 days of emergence. At the 4-leaf stage (BBCH 14), plants were treated with a aqueous solution of the tested compounds. The applied doses of HIL and reference herbicide (calculated per active substance) were 10 g/ha iodosulfuron-methyl sodium salt. The commercial herbicide used was Autumn 10 WG (10% iodosulfuron-methyl-sodium; Bayer, Leverkusen, Germany). Application was carried out using a mobile nozzle sprayer delivering 200 L/ha of spray solution through a flat-fan TeeJet 110/02 VP nozzle (TeeJet Technologies, Wheaton, IL, USA) at an operating pressure of 0.2 MPa. The nozzle was positioned 40 cm from the tops of plants and moved at a constant speed of 3.1 m/s. After three weeks, the aboveground part of the plants, along with the roots and soil, were collected for subsequent studies focused on isolating bacterial communities. The experiment was conducted with four replicates in a randomized setup. The error margin range is indicative of standard errors of the mean (SEM) and determined using the equation:

$$SEM = \frac{s}{\sqrt{n}}$$

where: s is the sample standard deviation, and n is the number of samples.

3.3.3. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal/Fungicidal Concentration (MBC/MFC)

The obtained MCPP/MCPA-based HILs ([MCPA-DAE-C_8][MCPP], [MCPA-DAE-C_9][MCPP], [MCPA-DAE-C_{10}][MCPP], [MCPA-DAE-C_{11}][MCPP], [MCPA-DAE-C_{12}][MCPP], [MCPA-DAE-C_{14}][MCPP]) were assessed for their toxicity against two Gram-negative bacterial strains (*Escherichia coli* and *Pseudomonas putida*) and one fungi (*Candida albicans*). The ISM-based HILs ([Chol][ISM], [C_6Chol][ISM] and [C_{12}Chol][ISM] were tested against *H. zhihuiae* S113 (DSM 18984), *S. griseolus* ATCC 11796 (*Streptomyces halstedii* DSM 40854) and *B. subtilis* 168 (DSM 23778) as well as their precursors ([Chol][Cl], [C_6Chol][Cl] and [C_{12}Chol][Cl]. Pure herbicides were used as controls. Each species was initially transferred from agar plates into 20 mL of a 50% (w/v) TSB broth (Sigma Aldrich, Saint Louis, MO, USA) and incubated for 24 h at 30 °C until reaching an optical density of OD_{600} = 0.10 ± 0.01, corresponding to 10^6 CFU/mL. The biomass was then washed three times with sterile 0.85% (w/v) NaCl and diluted 1:50 in 50% (w/v) TSB, resulting in a concentration of 2×10^4 CFU/mL.

The antimicrobial activity test followed the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines using the micro-dilution method [104]. In a sterile 96-well plate, 100 µL of each tested compound was placed in the first row, while the other wells received 50 µL of a 50% (w/v) TSB medium. Serial dilutions of the tested compounds were then performed. Subsequently, 200 µL of a microorganism suspension with resazurin (0.5 mg/mL) was added to each well. The concentrations of MCPA/MCPP-based ILs ranged from 0.00078 mM to 2 mM, while for ISM-based compounds, from 25 to 1000 mg/L for [Na][ISM], [Chol][Cl], [Chol][ISM], [C_6Chol][Cl], [C_6Chol][ISM] and 5 to 500 mg/L for [C_{12}Chol][Cl] and [C_{12}Chol][ISM] (due to their limited solubility in water). Biotic controls (microorganisms with resazurin but without the analysed compounds) and abiotic controls (tested compounds with resazurin but lacking microorganisms) were used for reference. The plates were incubated at 30 °C for 24 h, after which the results for MIC (minimum inhibition concentration) and MBC (minimum bacterial concentration) or MFC (minimum fungal concentration) were determined.

3.3.4. Half maximal effective concentration (EC_{50}) assay

This study aimed to assess the antibacterial activity of dicamba and dicamba-based HILs ([Chol][Dic], [DDA][Dic], [$BetC_2$][Dic], [$BetC_{10}$][Dic] and [$BetC_{16}$][Dic]) against three bacterial strains: *P. putida*, *E. coli* (Gram-negative) and *B. subtilis* 168 DSM 23778 (Gram-positive). Bacterial cultures were prepared and grown for 48 h at 30 °C with continuous shaking in liquid sterile 50% (w/v) TSB medium (Sigma Aldrich, Saint Louis, MO, USA). Dicamba and HILs were dissolved in 50% (w/v) TSB medium to achieve concentrations of 2500, 1250, 625, 312.5, 156.25, 78.125, 39.063, 19.533, 9.766, 4.883, 2.441, 1.221, and 0.610 mg/L. All prepared solutions were stored at 4 °C until use for up to 3 h.

After culturing, the bacterial strains were transferred to sterile 50% (w/v) TSB medium and diluted to an optical density OD_{600} of 0.100 ± 0.015. Cultures of 0.2 mL each were placed in sterile 96-well plates in triplicate and incubated at 30 °C with continuous shaking using a Synergy™ HTX Multi-Mode Microplate Reader (Thermo Fischer Scientific, Waltham, MA, USA). When the bacterial suspension reached an OD_{600} of 0.150 ± 0.015, 0.05 mL of each prepared solution was added, resulting in final test concentrations of 1000; 500; 250; 125; 62.5; 31.25; 15.63; 7.81; 3.91; 1.95; 0.98; 0.49;

0.24 and 0.12 mg/L. Control samples included abiotic (medium without microorganisms and chemical compounds) and biotic (medium with microorganisms but without chemical compounds), prepared in triplicates.

Bacterial growth was observed for 16 hours, and growth curves were generated once the bacteria reached the stationary phase. The rate of inhibition of microbial growth was calculated using the following formula:

$$\mu = \frac{\ln(\mathrm{OD}_{t_2}) - \ln(\mathrm{OD}_{t_1})}{t_2 - t_1}$$

where: OD_{t_1} and OD_{t_2} are the absorbance at times t_1 and t_2, respectively. Growth rate inhibition was then calculated relative to the control mean:

$$\%inhibition = 100 \cdot \left(1 - \frac{\mu_{sample}}{\mu_{control}}\right)$$

The relationship between the effect and the concentration of the tested compounds was plotted, and the EC_{50} was determined following the method provided previously [105–107].

3.4. Bacterial biodegradation potential

3.4.1. ISM based HILs

The assay was adapted from the method developed by Belaze et al. (2014) [108] and modified to evaluate the growth of bacteria in an environment contaminated with HILs. To accomplish this, the bacteria were cultivated in their respective dedicated media, namely FLB (BTL sp. z o.o., Łódź, Poland) for *H. zhihuiae* S113 (DSM 18984) and CASO (BTL sp. z o.o., Łódź, Poland) for *B. subtilis* 168 (DSM 23778). In 250 mL Erlenmeyer flasks, 50 mL of the respective culture medium was dispensed and the bacteria were introduced aseptically (in triplicate). Cultivation was conducted in the dark, at 30 °C, with continuous shaking (120 rpm). ISM or selected HILs ([Chol][ISM], [C_6Chol][ISM], [C_{12}Chol][ISM]) were solubilized in a sterile water/acetone mixture (8:2, v/v) and aseptically added to the cultures. Both biotic (cultivation medium without the analysed compounds) and abiotic controls (sterile cultivation medium with HILs or herbicides) were prepared. Optical density of the culture medium were monitored at 24-hour intervals. Additionally, separate samples

were collected every 24 h for residue analysis using a high-performance liquids chromatography (LC- MS/MS) (please see **Section 3.8.1.1.**).

3.5. Fungal biodegradation potential

The procedure for evaluating the capacity of isolates to transform model herbicide compounds comprised several steps. Initially, liquid PD (potato dextrose) media (containing 200 g/L of potato extract and 20 g/L of glucose) were prepared and supplemented with herbicide or selected HILs ([Chol][ISM] and [C_{12}Chol][ISM]) at a concentration of 500 mg/L of the active herbicidal component, and the pH was adjusted to a neutral range of 6.5–7.0. Following this, plates containing the chosen isolated strains (*Fusarium, Penicillium, Talaromyces* and *Aspergillus*) were rinsed with 5 mL of PD medium, and a 100 µL portion of this liquid was used to inoculate the culturing media (25 mL) in Erlenmeyer flasks (150 mL) and incubated in the dark for 2 weeks at 30 °C with continuous shaking (120 rpm). At intervals of 48 h, a 2 mL sample of the medium was collected into sterile centrifuge tube and stored at –20 °C until further analyses. Additionally, abiotic controls (medium without any microorganisms) were prepared to ensure that there was no spontaneous degradation.

3.5.1. Degradation dynamics

The degradation kinetics equation was calculated adhering to the following formula:

$$C_t = C_0 e^{-Kt}$$

The half-life ($t_{1/2}$) of the herbicide is given by:

$$t_{1/2} = \frac{\ln (2)}{K}$$

where: K is the degradation constant; t is reaction time; C_0 is the initial concentration of the herbicide at the start of the experiment; C_t is the concentration of herbicide solution at time t.

3.6. Sorption and bioavability of ISM based HILS

Sorption studies were carried out according to the methodology described in our earlier works [109,110]. Tests were performed for two concentrations of 50 and 100 mg/L

(concentrations converted to herbicide active ingredient content), which were used in the other studies. The analysis of the bioavailability of ILs with the ISM anion was performed in the same way as in the previously published works [61,62,111] for soil samples containing compounds with concentrations of 100 mg/kg per herbicide active ingredient.

3.7. Mineralization of HILs

3.7.1. MCPP/MCPA based HILs

The evaluation of HILs' mineralization potential was conducted following the OECD 301 F protocol [112]. Experimental samples were prepared in brown glass bottles, which were filled with sterile mineral medium as described by Ławniczak et al. (2016) [60]. The inoculum, derived from herbicide-exposed soil, was concentrated to achieve a cell density of approx. 10^6 CFU/mL. The tested compounds were added to each bottle to reach a concentration equivalent to 100 mg/L of Theoretical Oxygen Demand (ThOD). Mineralization efficiency was assessed by measuring oxygen consumption and carbon dioxide production using the Micro-Oxymax Respirometer (Columbus Instruments, Columbus, OH, USA). The ultimate biodegradation test was carried out at a temperature of 23 ± 2 °C over a 28-day period, with measurements taken every 5 h. Biotic (mineral medium without the tested compounds) and abiotic controls (sterile mineral medium with HILs) were also prepared in triplicate.

3.7.2. ISM based HILs

In order to assess the mineralization potential of HILs, a modified OECD 301 F protocol was employed. The bacteria *B. subtilis* 168 (DSM 23778) were introduced into a liquid 50% (w/v) TSB medium and incubated for 48 h. Subsequently, cell suspension was concentrated to obtain a cell density of approx. 10^6 CFU/mL each. The experimental samples were prepared in SIMAX glass bottles filled with 100 g of soil with no previous known contact with any herbicides. Prior to filling, the soil was mixed with the bacterial inoculum and the tested compound (at a concentration of 1 g per 1 kg of soil based on the concentration of active substance). The mineralization efficiency was determined by Warder respirometric method [113]. Carbon dioxide production was measured every 24 h for 28 days at 23 ± 2 °C. Biotic (soil without the analysed compounds) and abiotic (sterile soil with HILs) controls were also prepared.

3.8. Analysis of residues

3.8.1. MCPP/MCPA based HILs

After the mineralization experiment, biomass was separated by centrifugation at 10,000 × g for 15 min at 4 °C, and washed three times with the mineral medium (K_2HPO_4 1.73 g/L, KH_2PO_4 0.68 g/L, $MgSO_4 \cdot 7H_2O$ 0.1 g/L, $FeSO_4 \cdot 7H_2O$ 0.03 g/L, NH_4NO_3 1.0 g, $CaCl_2 \cdot 2H_2O$ 0.02 g/L, and NaCl 4.0 g/L). The combined portions of biomass and cell-free supernatant (10 mL) underwent ultrasound-assisted extraction using chloroform (1 mL, repeated three times). The extracts were filtered through a 0.2 μm PTFE syringe filter and diluted in 80% methanol (v/v). Prepared samples were analysed using HPLC-MS/MS with an UltiMate 3000 RSLC system (Dionex, Sunnyvale, CA, USA), equipped with a Hypersil GOLD column (100 mm × 2.1 mm I.D.; 1.9 μm) (Thermo Fischer Scientific, Waltham, MA, USA), and an API 4000 QTRAP triple quadrupole mass spectrometer (AB Sciex, Foster City, CA, USA). This analysis determined the concentration of remaining HILs.

3.8.1.1. Iodosulfuron based HILs

Recovery of ISM anion and [C_{12}Chol] [C_6Chol] cations

Soil sample (5 g) was transferred into a 50 mL sterile centrifuge tube, followed by the addition of 3 mL of ultra-pure water. The mixture was vortexed for 30 s, then 5 mL of acetonitrile with 0.1% (v/v) acetic acid was added and vortexed for 60 s. The tubes were placed in an ultrasonic bath for 10 min. Subsequently, 2 g of anhydrous $MgSO_4$ and 0.5 g of NaCl were added and vortexed for 1 min, and then the solution was centrifuged at 10,000 rpm for 10 min. The upper layer was decanted and filtered through a hydrophilic PTFE syringe filter.

Recovery of [Chol] cation

Soil sample (2 g) were combined with 5 mL of methanol (99.8%) and 0.5 mL of 0.1 M HCl (35%) in a 50 mL centrifuge tube, then vortexed for 30 s. The tubes were placed in an ultrasonic bath for 30 min, followed by centrifugation for 10 min at 10,000 rpm. The upper layer was decanted and filtered through a hydrophilic PTFE syringe filter.

HPLC-MS/MS

HPLC-MS/MS analysis was performed using an UltiMate 3000 RSLC (Dionex, Sunnyvale, CA, USA), and an API 4000 QTRAP triple quadrupole mass spectrometer (AB Sciex, Foster City, CA, USA). Samples (5 µL) were introduced into a Gemini-NX C18 column (100 mm × 2.0 mm I.D.; 3 µm) (Phenomenex, Torrance, CA, USA), and maintained at 35 °C. The elution used a gradient with phase A (5 mM aqueous solution of ammonium acetate) and phase B (methanol), at 0.3 mL/min flow rate. For determination of ISM in its sodium salt form and ISM incorporated into HILs with [Chol] and [C_6Chol], the following gradient was applied: 0 min – 50% B; 1 min – 50% B; 2 min – 100% B; 3 min – 100% B. In the case of ISM in [C_{12}Chol], an additional 4-minute final step with 100% B was added. For [Chol] and [C_6Chol], the gradient was as follows: 0 min – 50% B; 1 min – 50% B; 2 min – 100% B; 3 min – 100% B. In the case of [C_{12}Chol], the gradient was: 0 min – 80% B; 2 min – 100% B; 4 min – 100% B. The effluent was directed to the electrospray ionization source (Turbo Ion Spray), operating in negative ion mode for anions and positive ion mode for cations, with a curtain gas pressure of 10 psi, a nebulizer gas pressure of 40 psi, an auxiliary gas pressure of 45 psi, a temperature of 450 °C, and an ion spray voltage of +/– 4500 V.

3.9. Molecular analysis of effects of ISM based HILs on the microbiome

3.9.1.1. Impact on soil microbiome in laboratory conditions

Total genomic DNA was extracted from 200 mg of soil using DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Before PCR amplification, DNA extracts were normalized with sterile water to a concentration of 10 ng/µL. Blank DNA extraction was prepared and sequenced as a negative control. For microbial community profiling the V4 16S rRNA gene fragment was used, as previously described [67]. Sequencing was carried out using the Ion Torrent S5 system (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions to get at least 200,000 reads per sample.

Raw sequence data were pre-filtered by Ion Torrent Suite software version 5.18.1 (Life Technologies, Carlsbad, CA, USA) to remove polyclonal and low-quality sequences. Sequence reads shorter than 180-bp were removed from the dataset using Geneious Prime version 2023.1.2 (Biomatters Ltd.). FastX-Toolkit [90] was used to extract sequences with the minimum of 50% of bases with a quality score ≥25. Quality filtered

sequences were separated into individual combinations of indexes in Geneious Prime. Next, the sequences were trimmed at 5' and 3' ends to exclude PCR primers. Sequences were then denoised to generate amplicon sequencing variants (ASVs) using DADA2 denoise-pyro method implemented in QIIME2 version 2023.5 [92,93]. The UNCROSS2 algorithm was used to remove ASVs detected in control samples from the dataset [114]. The ASVs were compared against the SILVA database for ARB for small subunit ribosomal RNAs version 138.1 [115,116].

3.9.2. Impact on plants' microbiome

3.9.2.1. Isolation of DNA

DNA extraction from biological specimens was carried out utilizing the Genomic Mini Spin kit (060-100S, A&A Biotechnology, Gdańsk, Poland) following the manufacturer's protocol. Purified DNA was eluted and preserved at –80 °C following neutralization to minimize matrix degradation. The efficacy of the extraction process was regularly assessed using the fluorometric method with the Qbit 3.0 instrument and the Qubit™ dsDNA HS Assay Kit (Q32851, Thermo Fisher Scientific, Waltham, MA, USA). Each sample underwent three separate DNA extractions, and the results were combined after positive quantification.

3.9.2.2. PCR amplification and Next-Generation Sequencing (NGS)

The PCR amplification was conducted employing the Ion 16S™ Metagenomics Kit (A26216, Life Technologies, Carlsbad, CA, USA), facilitating the amplification of the V2–V9 segments of the bacterial 16S rRNA gene. The PCR reaction setup followed the manufacturer's guidelines, comprising 15 µL of 2 × Environmental Master Mix, 3 µL of the appropriate primer, and 12 µL of the DNA sample. Thermal cycling was carried out in a Veriti Thermal Cycler (Life Technologies, Carlsbad, CA, USA), with the following temperature program: initial denaturation (10 min at 95 °C), 25 cycles of denaturation (30 s at 95 °C), annealing (30 s at 58 °C), extension (20 s at 72 °C) and a final extension (7 min at 72 °C).

The reaction products were purified using the Agencourt AMPure XP Reagent (A63880, Beckman Coulter, Pasadena, CA, USA), as per manufacturer's instructions. This purification method relies on the binding of DNA to magnetic beads and the

removal of contaminants *via* ethanol washing. The DNA was eluted from the beads using nuclease-free water or low-TE buffer (10 mM Tris-HCl (pH 8.0) + 0.1 mM EDTA). A library was prepared using the Ion Plus Fragment Library Kit (4471252, Life Technologies, Carlsbad, CA, USA) and purified using the Agencourt AMPure XP Reagent (A63880, Beckman Coulter, Pasadena, CA, USA). The library concentration was evaluated using the Ion Universal Library Quantitation Kit (Thermo Fisher Scientific, Waltham, MA,USA) and a real-time PCR instrument Quant Studio 5 (A26217, Life Technologies, Carlsbad, CA, USA). The library was then diluted to a concentration of 10 pM and coated onto beads for sequencing, in emulsion PCR, using the Ion PGM™ Hi-Q™ View OT2 Kit reagent kit (Thermo Fisher Scientific, Waltham, MA,USA) and an Ion One Touch 2 Instrument (A29900, Life Technologies, Carlsbad, CA, USA). The library-coated beads were further purified employing an Ion One Touch ES Instrument (Life Technologies, Carlsbad, CA, USA).

The sequencing of the library-coated beads was performed using an Ion PGM System (Life Technologies, Carlsbad, CA, USA) along with the Ion PGM™ Hi-Q™ View Sequencing Kit (A29900) (Thermo Fisher Scientific, Waltham, MA,USA) on an Ion 316™ Chip Kit v2 BC(Thermo Fisher Scientific, Waltham, MA,USA).

3.9.2.3. Bioinformatic analysis

Sequence reads from the Ion Torrent (Thermo Fisher Scientific, Waltham, MA, USA) in BAM format 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). Chimeric and low-quality reads (quality limit = 0.05, ambiguous limit = 'N') were filtered and removed. Then, the sequence reads were clustered against the SILVA v119 database at 97% similarity of operational taxonomic units (OTUs). Significance of statistical differences was confirmed with one-way ANOVA.

3.10. Impact of ISM based HILs on gene presence and abundance in the environment

3.10.1. Genes and primer sequences for PCR analysis

The genes and their corresponding primer sequences used for PCR analysis are presented in **Table 2.**

Table 2. Genes, primer names, and primer sequences used for PCR analysis.

Gene name	Primer name	Primer Sequence	Ref.
sulE	sulEF1	5'-CTGATTGCATATGGAAACTGACAACGTGGAGCT-3'	[117]
	sulER1	5'-TACAAGCTTGCTTTCGTTCTGATCTAAGC-3'	
vegetative catalase 1	veg2F	5'-GGAATTCATGAGTTCAAATAAACTGACAAC-3'	
	veg2R	5'-CCGCTCGAGTTAAGAATCTTTTTTAATCGGC-3';	
Acetoin dehydrogenase E1	adE2F	5'-CGGGATCCATGGCGAGAGTCATAAGCATGTC-3'	
	adE2R	5'-CCGCTCGAGTTAATTCAATGCCGGCTCGC-3'	
manganese ABC transporter	mABCtF	5'-CATGCATATGAAAAGCGCTGATCAGCAA-3'	[84]
	mABCtR	5'-CATGCTCGAGTTATTTAGTAATCG-3'	
Vegetative catalase 1	veg1F	5'-CATGCATATGAGTTCAAATAAACTGACA-3'	
	veg1R	5'-CATGCTCGAGTTAAGAATCTTTTTTAATCGGCAA-3'	
acetoin dehydrogenase E1	adE1F	5'-CATGCATATGGCGAGAGTCATAAGC-3'	
	adE1R	5'-CATGCTCGAGTTAGGCTCGC-3'	
P450SU1 and its adjacent ferredoxin, Fd1 (CYP 105A1 gene)	P450SU1-Fd1F	5'-GTCATATGACCGATACCGCCACGACG-3'	[118]
	P450SU1-Fd1R	5'-CTGGATCCTATTCCGTGTCCTCGACG-3'	

3.10.2. PCR reaction

To ensure the proper optimization of PCR reaction parameters, positive controls were employed, including *H. zhihuiae* S113 (DSM 18984), *S. griseolus* 14576-4 (*S. halstedii* DSM 40854), and *B. subtilis* 168 (DSM 23778). The primers underwent temperature optimization, consistently generating only the desired product when applied to positive templates during the reaction. The appropriate annealing temperatures are detailed in **Table 3.** No visible products were observed on the electrophoretic gel when tested against negative templates at the specified temperatures. Each reaction used 25 ng of DNA from the corresponding biological sample as the template, following these parameters: initial denaturation (1 cycle; 4 min at 95 °C), 40 cycles of denaturation (30s at 95 °C), primer annealing (45 s at the temperature listed in **Table 3**), and extension (1 min 45 s at 72 °C), concluding with a final extension step (5 min at 72 °C) and a storage step (∞ min at 4 °C). Additionally, a control using deionized sterile water as the template was prepared.

Table 3. Primers and corresponding temperatures optimised for PCR use.

Primer pair name	Temperature [°C]
Sul E1	56
mABCt-1	65
Veg-1	58
Veg-2	56
AdE1	58
adE2	65
P450 SU-1	65

The PCR reaction products were resolved on a 1.5% agarose gel (Prona Agarose, Basica Le, Burgos, Spain) stained with Midori Green Advance DNA Stain (Nippon Genetics Europe, Düren, Germany) for 70 min at 120 V, employing the MultiSub Midi apparatus (CleaverScientific, Rugby, UK). The gels were visualized using a microDOC apparatus (CleaverScientific, Rugby, UK).

3.10.3. Genes and primer sequences for qPCR analysis

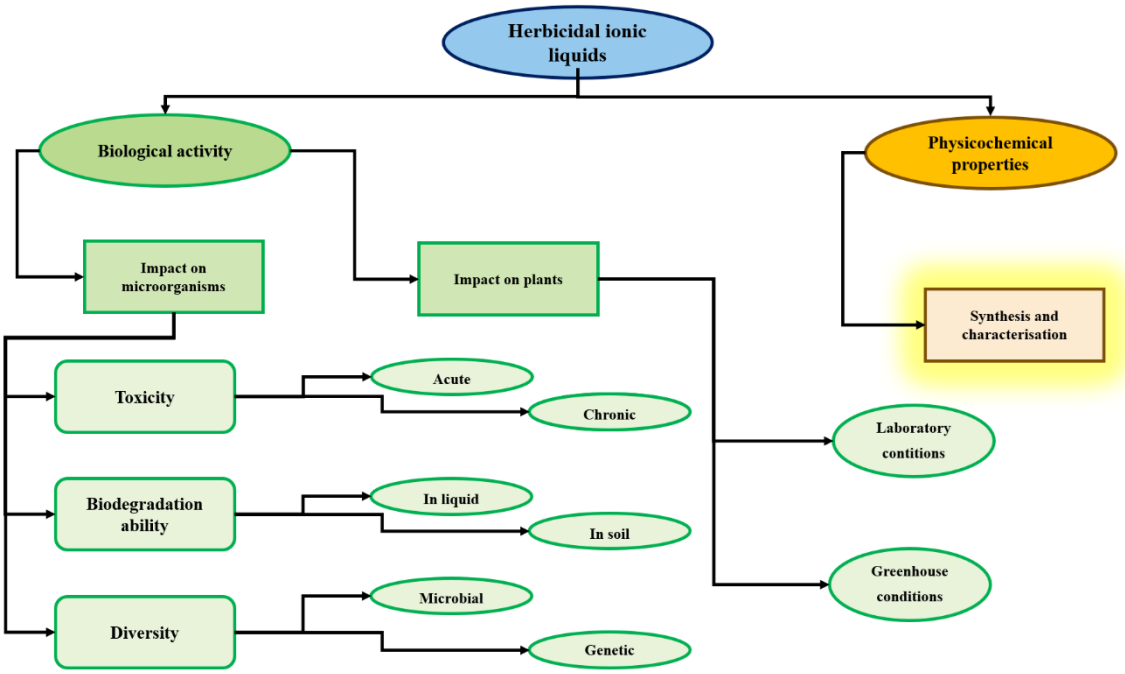
The analysis of gene expression involved the use of Power SYBR Green PCR Master Mix (Life Technologies, Carlsbad, CA, USA) on an ABI 7500 SDS (Applied Biosystems, Thermo Fischer Scientific, Waltham, MA, USA) for real-time PCR, using primers specified in **Table 4.** To quantify total bacterial RNA, real-time PCR amplification of a bacterial 16S ribosomal RNA fragment was conducted using universal bacterial primers and a TaqMan MGB probe (Thermo Fischer Scientific, Waltham, MA, USA). This procedure utilized TaqMan Universal Master Mix II (Life Technologies, Carlsbad, CA, USA) on ABI 7500 SDS (Applied Biosystems, Thermo Fischer Scientific, Waltham, MA, USA). All analyses were performed in triplicate. Gene expression in each sample was assessed by calculating the mean expression index using the formula C_T target/C_T 16S, based on data from three analyses. This parameter provides insight into the specific gene's expression level relative to the universal gene (16S RNA) within the entire metabiome.

Table 4. Genes, primer names, and primer sequences used for qPCR analysis.

Target Gene	Primer name	Forward sequence	Reverse sequence
sulE	Hz-sulE	5'-CGACGGCCTGAAAAGAGGAT-3'	5'-AGGAACAGAGGGCCGATACT-3'
manganese ABC transporter	Bs-ABC	5'-CTGCTGCATCGGCAATTTGA-3'	5'-TGGCAGCAGTACTTTTTGCG-3'
Vegetative catalase 1	Bs-vegcat	5'-CGATTTGGCGCAGCTTGATT-3'	5'-TCAAGCCGGCGATCTGTATC-3'
acetoin dehydrogenase E1	Bs-Actdh	5'-GAAAGAGCAAGAAACGGCGG-3'	5'-AGGTGCTCAACTCTTTCATCC-3'
16 S rRNA	F968 Forward R1401 Reverse	5'-AACGCGAAGAACCTTAC-3'	5'-CGGTGTGTACAAGACCC-3'

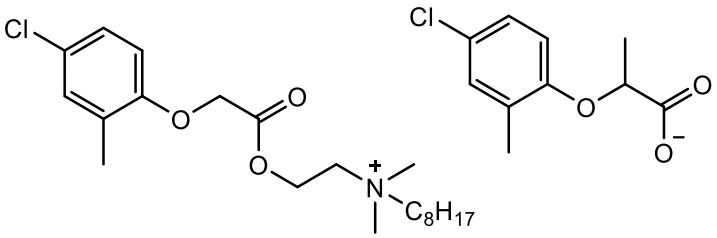
4. Results and discussion

4.1. Synthesis and characterisation of HILs

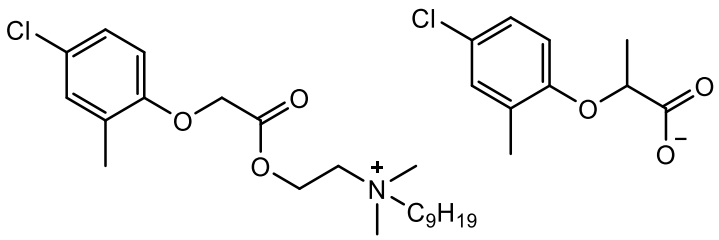


The synthesis reactions yielded herbicidal ionic liquids based on MCPA/MCPP, ISM and dicamba **(Tabe 5)**, which were utilized in all subsequent biological experiments. Their structures were confirmed *via* spectroscopic methods (**Fig S1-S22).** All of these compounds had their physicochemical properties examined, and were proven to possess characteristics typical of HILs. For specifics, please refer to [47,78,83,119].

Table 5. Synthetized HILs utilized in the course of this study.

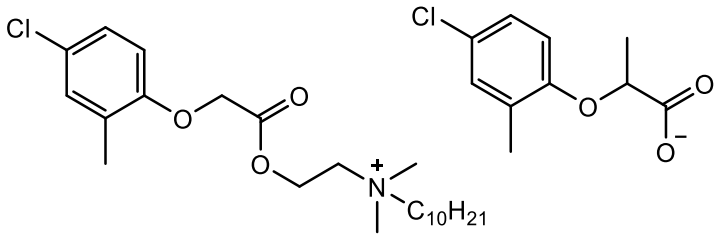
MCPA/MCPP based HILs	
Abbreviation of ionic liquid	**Structure**
[MCPA-DAE-C_8][MCPP]	 *(4-chloro-2-methylphenoxy)-2-acetoxyethyldimethyloctyl-ammonium (±)-2-(4-chloro-2-methylphenoxy)propionate*

[MCPA-DAE-C_9][MCPP]



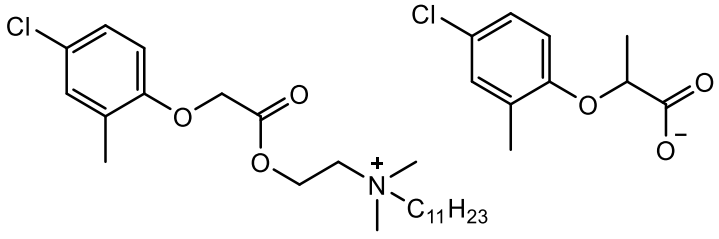
(4-chloro-2-methylphenoxy)-2-acetoxyethyldimethylnonyl-ammonium (±)-2-(4-chloro-2-methylphenoxy)propionate

[MCPA-DAE-C_{10}][MCPP]



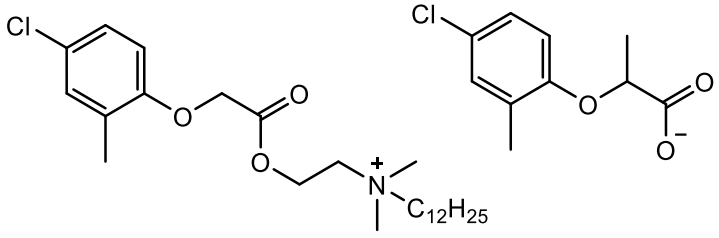
(4-chloro-2-methylphenoxy)-2-acetoxyethydecyl-ammonium (±)-2-(4-chloro-2-methylphenoxy)propionate

[MCPA-DAE-C_{11}][MCPP]



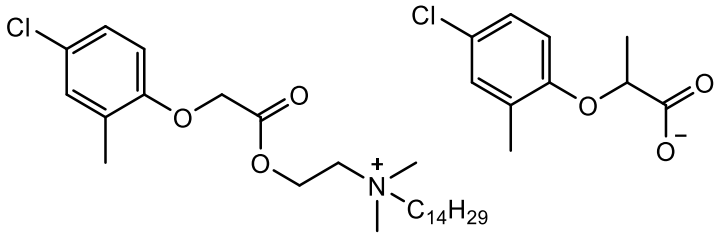
(4-chloro-2-methylphenoxy)-2-acetoxyethyludimethylndecyl-ammonium (±)-2-(4-chloro-2-methylphenoxy)propionate

[MCPA-DAE-C_{12}][MCPP]

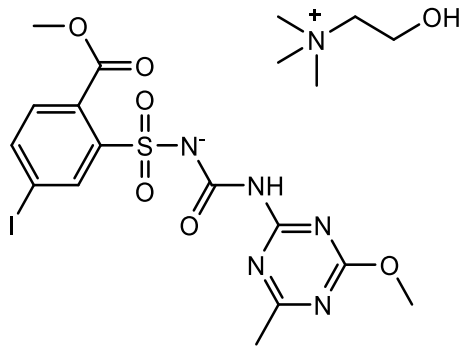
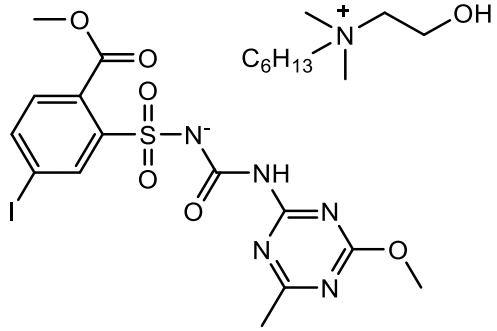
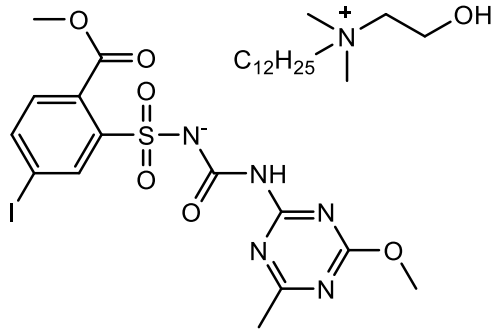


(4-chloro-2-methylphenoxy)-2-acetoxyethyldodecyldimethyl-ammonium (±)-2-(4-chloro-2-methylphenoxy)propionate

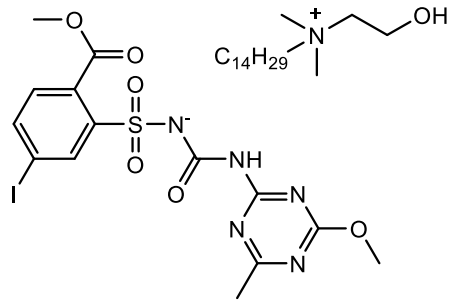
[MCPA-DAE-C_{14}][MCPP]



(4-chloro-2-methylphenoxy)-2-acetoxyethyldimethyltetradecyl-ammonium (±)-2-(4-chloro-2-methylphenoxy)propionate

ISM based HILs	
Abbreviation of ionic liquid	**Structure**
[Chol][ISM]	
	choline (5-iodo-2-methoxycarbonylphenyl)sulfonyl-[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)carbamoyl]azanide
[CholC_6][ISM]	
	N-hexylcholine (5-iodo-2-methoxycarbonylphenyl)sulfonyl-[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)carbamoyl]azanide
[CholC_{12}][ISM]	
	N-dodecylcholine (5-iodo-2-methoxycarbonylphenyl)sulfonyl-[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)carbamoyl]azanide

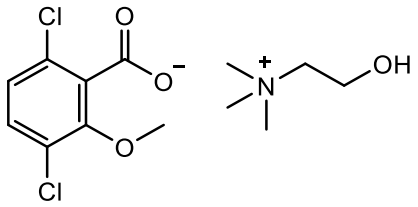
[$CholC_{14}$][ISM]



N-tetradecylcholine (5-iodo-2-methoxycarbonylphenyl)sulfonyl-[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)carbamoyl]azanide

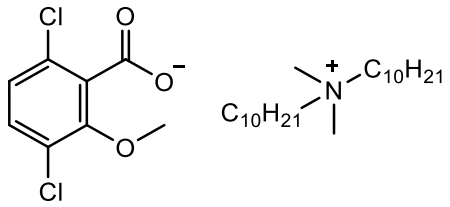
Dicamba based HILs	
Abbreviation of ionic liquid	**Structure**

[Chol][Dic]



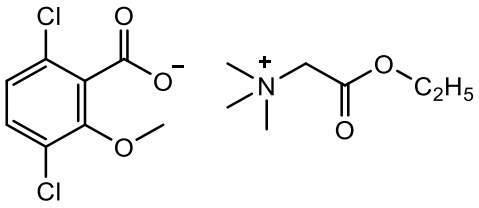
choline 3,6-dichloro-2-metoxybenzoate

[DDA][Dic]



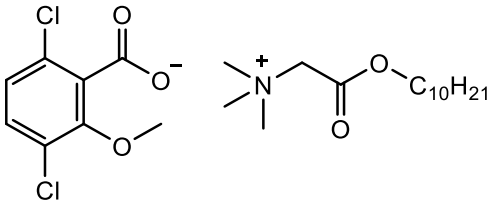
didecyldimethylammonium 3,6-dichloro-2-metoxybenzoate

[$BetC_2$][Dic]

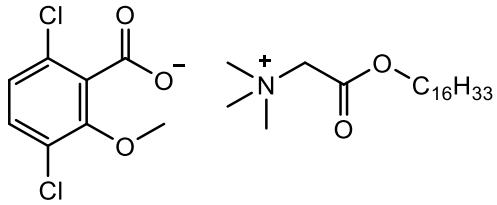


ethyl betainate 3,6-dichloro-2-metoxybenzoate

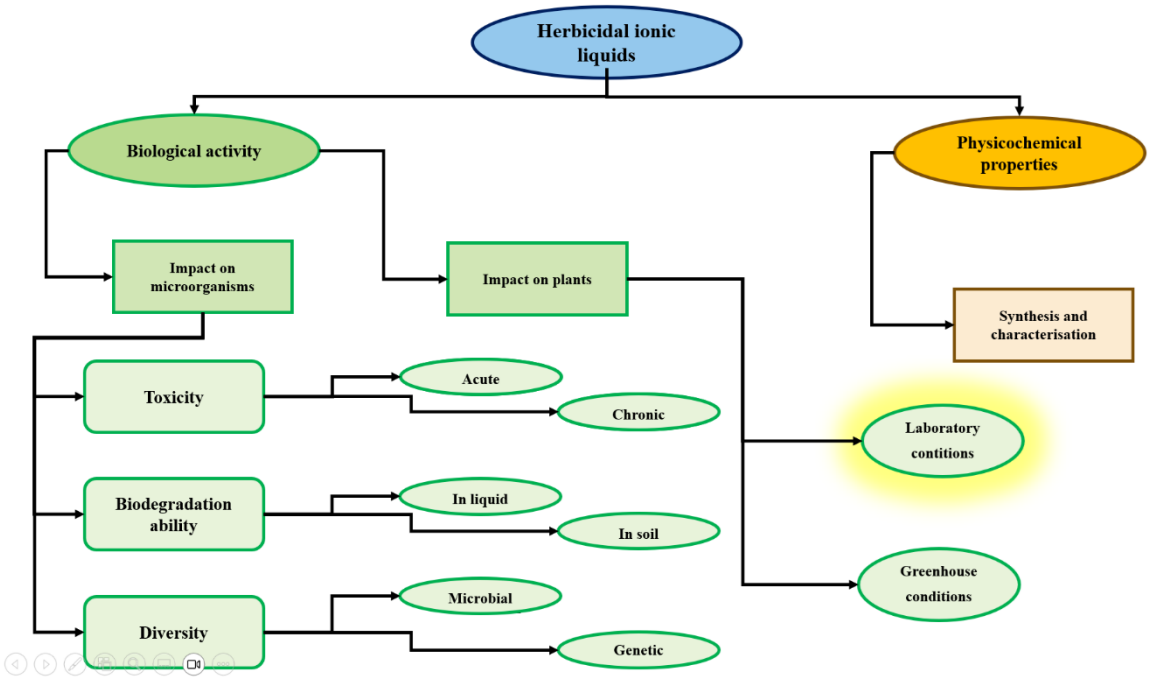
[$BetC_{10}$][Dic]



decyl betainate 3,6-dichloro-2-metoxybenzoate

[BetC$_{16}$][Dic]		*hexadecyl betainate 3,6-dichloro-2-metoxybenzoate*

4.2. Impact on early development of plants



4.2.1. MCPA/MCPP based HILs

This phase of the experiment focused on assessing the impact of the acquired HILs on the growth of roots and shoots in a model weed (*Centaurea cyanus*) and a crop plant (*Zea mays*), in reference to a model herbicidal mixture (sodium salts of MCPA and MCPP). The germination test conducted for maize revealed two distinct trends for the examined HILs: growth stimulation at low concentrations and inhibition at high concentrations, confirming the synthetic auxin properties of the compounds. Notably, the effects of HILs on maize germination were consistently more favourable compared to the model herbicidal mixture. At a concentration of 0.0005 mM/kg of soil dry weight

(s.d.w.), all studied HILs stimulated seed germination, with GI ranging from 120 to 142%, while the model mixture inhibited it (GI of 72%). Even at higher concentrations, the HILs reduced the germination index, but this effect was consistently milder compared to sodium salts of MCPA and MCPP. Overall, the highest stimulation occurred for [MCPA-DAE-C_{12}][MCPP] at the lowest studied concentration (0.0005 mM/kg s.d.w.), whereas the presence of [MCPA-DAE-C_{14}][MCPP] led to the most significant inhibition of germination (GI of approx. 19%) at the highest concentration (0.02 mM/kg s.d.w.).

Subsequent measurements of root and shoot length in maize seedlings confirmed the stimulating effects of the studied HILs (**Fig. 8**). Both root and shoot lengths were 50 to 100% higher compared to the model mixture of herbicides, particularly pronounced at lower concentrations (0.0005 and 0.001 mM s.d.w.) and diminishing with increased doses. However, even at the highest studied concentration (0.02 mM), maize seedlings exhibited root and shoot growth in the presence of HILs, in contrast to the model mixture of MCPA and MCPP, which completely inhibited plant growth.

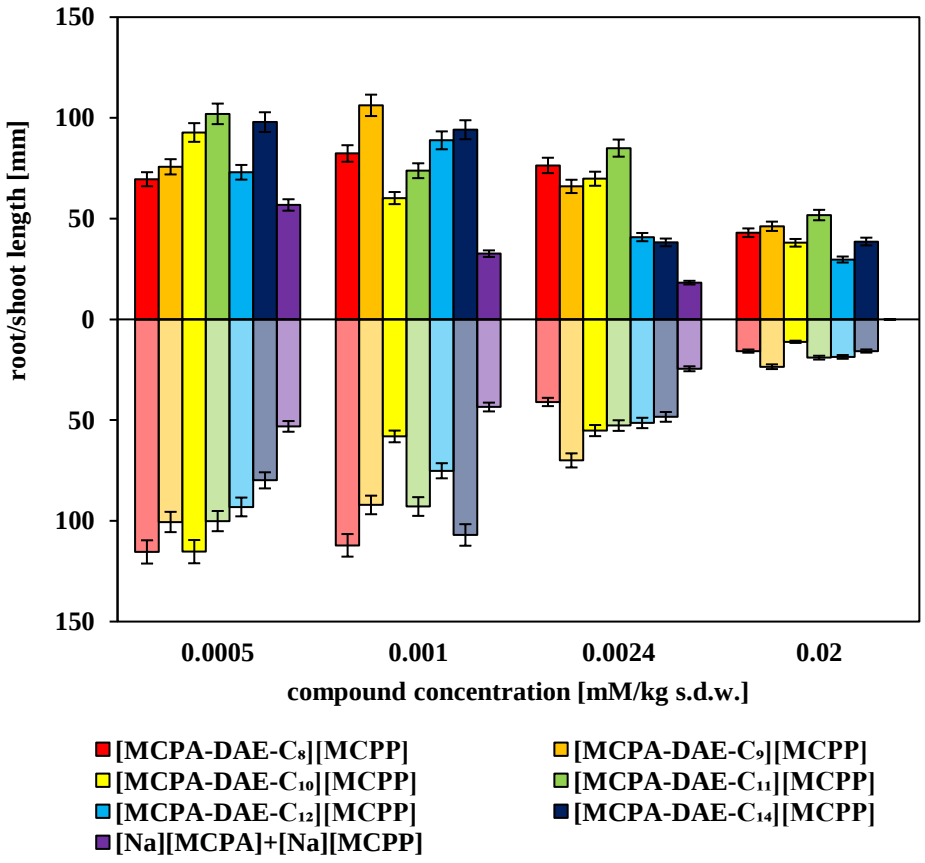


Figure 8. Effects of HILs esterquats on shoot and root length of maize seedlings. Adapted and modified from author's published work [78].

Assessing the impact of HILs on cornflower germination revealed that their efficacy is contingent on both concentration and the length of the alkyl substituent in the cation. In general, each of the examined HILs displayed greater effectiveness in hindering cornflower seed germination at the lowest concentration studied, with variable effects at higher doses and no discernible trends. However, [MCPA-DAE-C_{12}][MCPP] and [MCPA-DAE-C_{14}][MCPP] demonstrated a similar or even enhanced inhibitory effect compared to the model mixture of MCPA and MCPP.

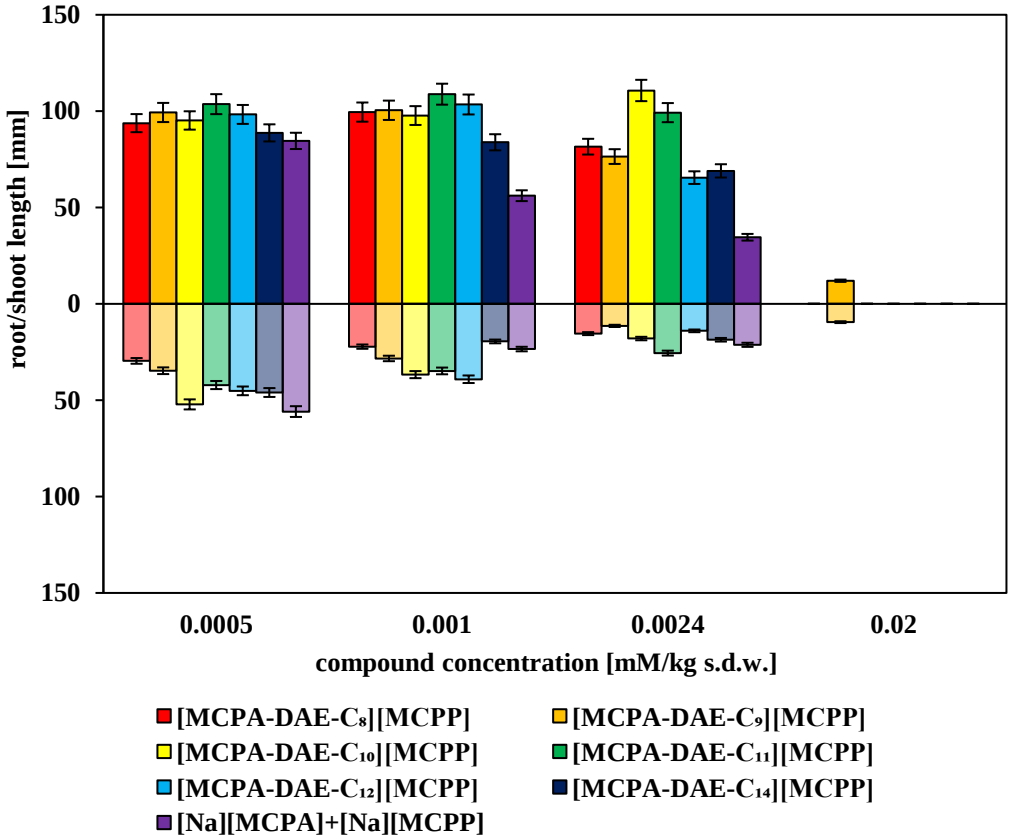


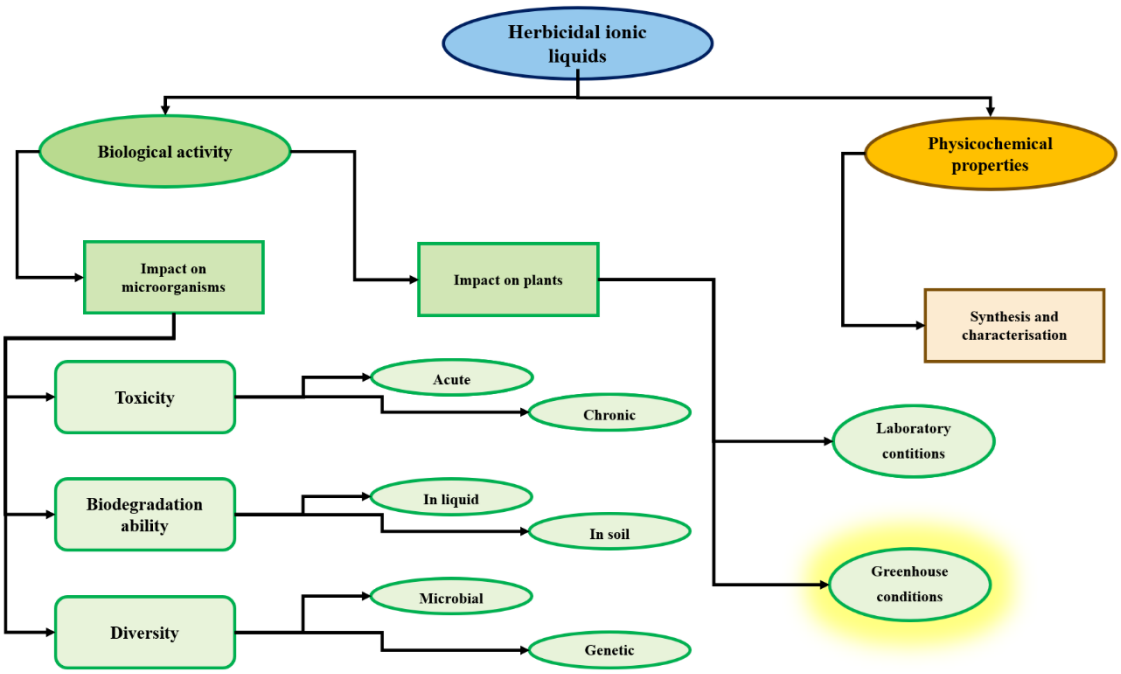
Figure 9. Effects of HILs esterquats on shoot and root length of cornflower seedlings. Adapted and modified from author's published work [78].

Analysis of root and shoot length in cornflower (**Fig. 9**) indicated that effective inhibition of plant growth by HILs was only achieved at the highest tested concentration (0.02 mM s.d.w.). At the lowest concentration, the impact of HILs was comparable to the model mixture of herbicides, while at middle doses (0.001 and 0.0024 mM s.d.w.), the inhibitory effect of the synthesized compounds was less pronounced compared to the model mixture. Consistent with other findings on HILs [46,57], the herbicidal activity of the compounds examined in this study against

cornflower was either similar to or greater than that of the reference mixture of model herbicides.

The obtained results validate earlier observations that compounds with alkyl chains ranging from C_{10} to C_{14} exhibit the highest herbicidal activity [120], although the exact effect is strongly influenced by the dose. Following the implications of Choudhary et al. (2017) [121], the structural modifications of the compounds described in this study may contribute to their efficacy against herbicide-resistant weed species, but this aspect requires further clarification in future investigations. Namely, since cations with long alkyl chains were proven to be highly effective in hindering growth of weeds, it is most possibly due to their known toxicity [122]. This, in turn, raise questions on their influence on other organisms, as the well-being of soil bacteria and fungi is directly linked to the biodegradation of xenobiotics. Consequently, the results on the effects on early development of crop and weed were a starting point in the evaluation of the environmental impact of herbicidal treatments, such as their further herbicidal activity, biodegradation and toxicity towards microbiota.

4.3. Herbicidal activity



4.3.1. MCPA/MCPP based HILs

The herbicidal activity of the synthesized HILs was assessed based on the reduction of cornflower's fresh weight (**Fig. 10)**. These findings confirmed that the herbicidal properties remained intact following the transformation of MCPA into an esterquat cation and MCPP into an anion. Notably, the herbicidal effect of HILs was achieved at a substantially lower dose (approx. 300–400 g a.i./ha) compared to the recommended dosage for conventional herbicidal mixtures (600–1000 g a.i./ha) [34]. Moreover, the efficacy of these compounds in reducing the fresh weight of cornflower was comparable or even superior to the model herbicidal mixture. This is particularly evident for HILs containing C_{10}, C_{11}, and C_{12} alkyl substituents in the cation. Given that the same anion was used in all tested compounds, the observed differences can be attributed to the cation's structure. The significant impact of the cation on the overall herbicidal action of HILs has been documented previously [50,121], and possibly linked to the surface-active properties of cations [120].

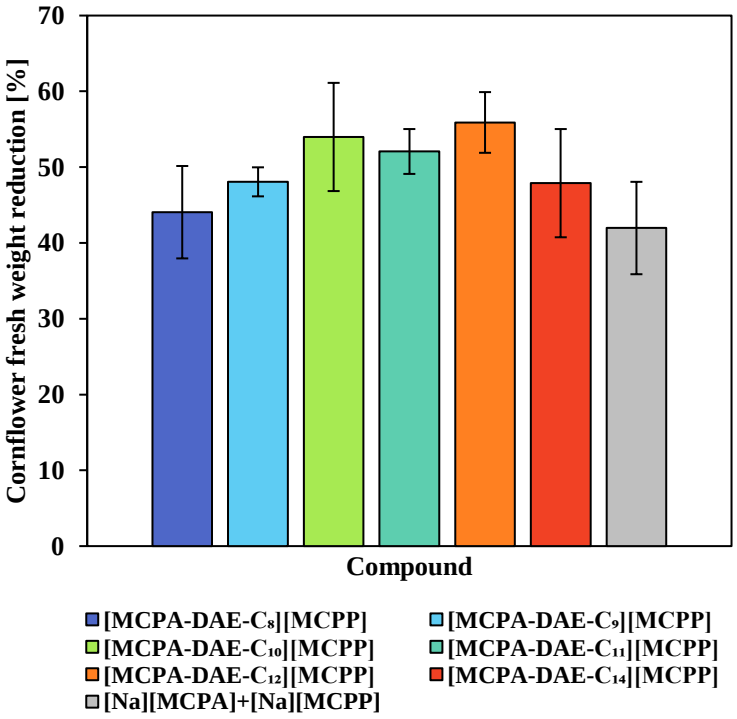


Figure 10. Herbicidal efficiency of HILs based on MCPA and MCPP. . Adapted and modified from author's published work [78].

4.3.2. Iodosulfuron-methyl based HILs

To impede the emergence of weed resistance to herbicides, and especially ALS inhibitors, it is imperative to deploy herbicides featuring diverse modes of action [123]. Recent scientific reports have delved into ionic liquids with two or three active ingredients possessing distinct modes of action [124,125]. HILs containing tribenuron-methyl and herbicides from the synthetic auxins group were analysed, and it was suggested that ionic liquids containing an anion from the phenoxy acid group can impede cornflower resistance to ALS [125]. Similarly, the impact of HILs with double or triple anions (sulfonylurea and auxin-like herbicides) on controlling herbicide-resistant cornflower was explored, demonstrating efficient reduction of the resistant biotype [124].

In this study, *N*-tetradecylcholine iodosulfuron-methyl ([C_{14}Chol][ISM]) was utilised to manage cornflower biotypes susceptible (S) and resistant (R) to ALS inhibitors. The tested HIL and commercial product displayed herbicidal activity against the susceptible cornflower but were ineffective in controlling the sulfonylurea herbicide-resistant biotype (**Fig. 11**). Fresh weight reduction for the susceptible population reached 87% and 94% for HIL and the reference product, respectively. Comparable trends were noted for the resistant population.

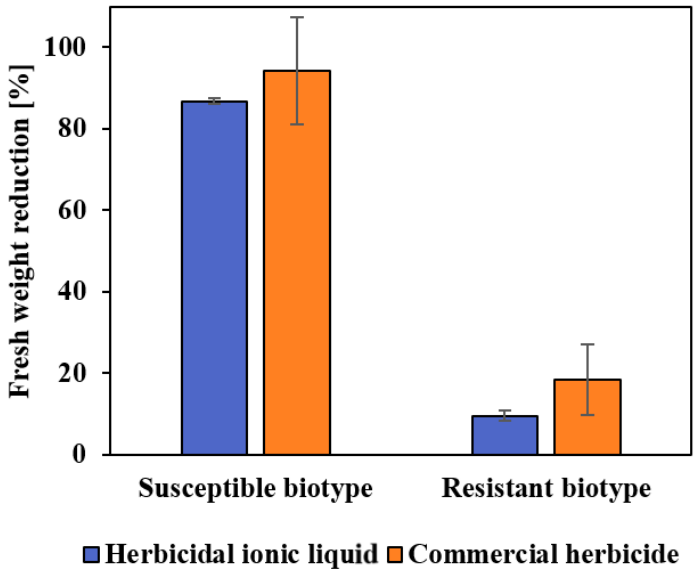


Figure 11. The impact of the examined HILs and the reference herbicide on cornflower biotypes resistant (R) and susceptible (S) to ALS inhibitors. Adapted and modified from author's published work [119].

Although the obtained results show herbicidal efficacy of HILs generally higher than for commercial products [78], the use of surface-active cations in their structure poses a concern on their environmental toxicity. Namely, ILs with herbicidal activity were proposed as a novel solution to the problem of growing resistance toward currently employed herbicides [55,121,126]. Yet, the addition of hydrophobic cations to the structure of known compounds might not be an answer to this issue. Specifically, cationic surfactants themselves do not seem to cause significant plant damage (both tested compounds induced only mild symptoms, with effectiveness below 15%), which allows for their use in agriculture. At the same time, their presence allows to minimize the dose of active ingredients, which appears as an advantage. However, cationic surfactants are commonly used as biocides [77,127,128] and contribute significantly to the phenomenon of microorganisms acquiring resistance to xenobiotics [75,76]. Hence, the next step of this research was to investigate the impact of HILs on soil microbiome, both on the isolated and referential microorganisms.

4.4. Identification of isolated fungi

The isolated fungi strains were cultivated on PD agar plates and subjected to preliminary identification based on mycelium morphology. Isolates I, IV and V exhibited filamentous, dense, wattle-like mycelium, forming rapidly growing flocculent colonies with a light blueish-grey colour. Isolate II initially displayed dense, putrid green mycelium, which later become fluffy. Isolate III showed yellowish, cottony mycelium, whereas Isolate VI had rapidly growing white, fluffy mycelium which turned black on the oldest parts.

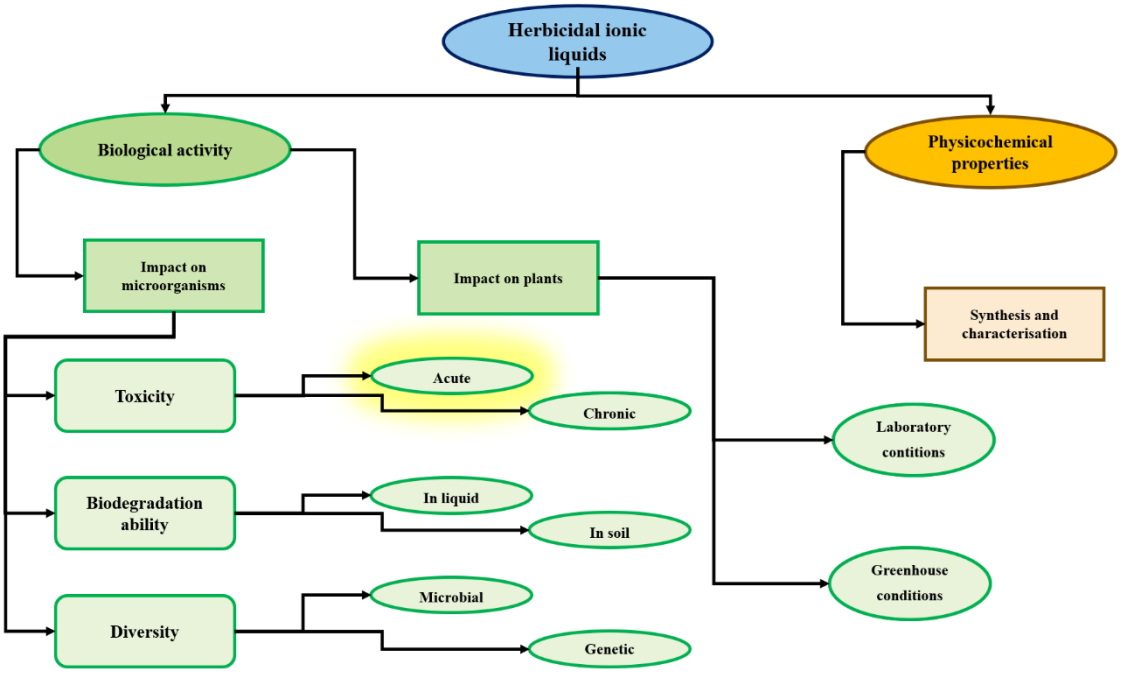
Following ITS (internal transcribed spacer) fragment sequencing, the isolates were identified as follows: Isolates I, IV and V as *Fusarum solani*, Isolate II as *Penicillium ochrochloron* or *Penicillium pulvillorum*, Isolate III as *Talaromyces muroii* and Isolate VI as *Aspergillus niger* or *Aspergillus welwitschiae*. The divergences in the case of Isolates II and VI are due to molecular markers indicating either of the potential species.

Taking into account the fact that during isolation step fungi were subjected to very high doses of ISM (compared to the doses used in herbicidal treatments, for details please see **Section 3.2.4.1.**), the acquired organisms likely possess very efficient system of detoxification of ALS inhibitors [129]. Consequently, they potentially

constitute an important element of agricultural soils' self-detoxification after herbicidal treatments. Therefore, they were selected to further study HILs – to answer a question whether their ionic form affects the ability of fungi to biodegrade herbicides.

4.5. Antimicrobial activity

4.5.1. Acute toxicity



4.5.1.1. Toxicity of ISM based HILs

The study of the toxicity of ISM-based compounds started with model representatives of soil microbiome, namely Gram-negative (*H. zhihuaiae*) and Gram-positive bacteria (*B. subtilis* and *S. halstedii*). The majority of the tested compounds did not exhibit inhibitory or bactericidal effects within the studied concentration range (**Table 6**). However, it is important to note that the concentration of 1000 mg/L is significantly higher than the doses typically applied in agriculture, which usually range from 10 to 40 g per hectare in case of sulfonylurea herbicides [130]. Hence, according to U.S. Fish and Wildlife Service scale, these compounds could be regarded as Relatively Harmless [131].

Among all tested compounds, only [C_{12}Chol][Cl] and [C_{12}Chol][ISM] displayed antimicrobial activity. In the case of *H. zhihuaiae* and *B. subtilis*, the characteristic growth inhibition parameters of both compounds were at the same level (MIC of

25 mg/L and MBC of 75 mg/L), while for *S. halstedii* these values were obtained only for [C_{12}Chol][ISM]. Interestingly, slightly lower values of MIC and MBC (10 and 50 mg/L, respectively) were obtained for *S. halstedii* exposed to [C_{12}Chol][[Cl].

Table 6. Antimicrobal activity of the ISM-based HILs towards selected soil bacteria. MIC/MBC values are calculated per active ingredient of referential sodium salt of iodosulphuron-methyl herbicide and iodosulphuron-methyl based HILs.

Salt	*H. zhihuaiae*		*B. subtilis*		*S. halstedii*	
	MIC [mg/L]	**MBC [mg/L]**	**MIC [mg/L]**	**MBC [mg/L]**	**MIC [mg/L]**	**MBC [mg/L]**
[Na][ISM]	>1000	>1000	>1000	>1000	>1000	>1000
[Chol][Cl]	>1000	>1000	>1000	>1000	>1000	>1000
[Chol][ISM]	>1000	>1000	>1000	>1000	>1000	>1000
[C_6Chol][Cl]	>1000	>1000	>1000	>1000	>1000	>1000
[C_6Chol][ISM]	>1000	>1000	>1000	>1000	>1000	>1000
[C_{12}Chol][Cl]	25	75	25	75	10	50
[C_{12}Chol][ISM]	25	75	25	75	25	75

The results of toxicity study hint at the fact that the toxic effect is associated not only with the structure of the cation, but also bacterial predispositions. Many studies have already evidenced a phenomenon where the structure of bacterial cell wall, as well as its species specific defence mechanisms, impacts the cell's susceptibility to examined IL type compound [48,49,67,78,105]. Moreover, one must consider that the anion possesses a significant mass, such that within 25 mg of [C_{12}Chol][ISM] only approximately 10 mg of cation is present. Consequently, when both chloride and HIL yield identical results, such as 75 mg/L, it implies that the cation constitutes a lower percentage of the total weight in the ionic liquid, with the anion bearing the majority of the mass. Nonetheless, the toxicity of [C_{12}Chol][ISM] remains consistent in cases of *Hansschlegelia* and *Bacillus*, which hints at the fact that cations in addition to being toxic, seem to enhance the toxicity of ISM. Though at the same time, successful application of sulfonylurea-based herbicides relies on use of surface activity enhancers (adjuvants). In this context, surface active amphiphilic cations, like [C_{12}Chol], may not be the most suitable choice as adjuvants to iodosulfuron-methyl due to abovementioned observed toxicity increase.

4.5.1.2. Toxicity of MCPA/MCPP based HILs

To assess the toxicity of MCPA/MCPP-based HILs, microorganisms commonly found in the environment and frequently isolated from soils were used (*B. cereus*, *P. putida* and a single fungal species *Candida albicans*). Certain strains of *B. cereus* and *C. albicans* also act as opportunistic pathogens in the gastrointestinal tracts of humans and animals [132,133]. The antibacterial and antifungal characteristics of the examined HILs are detailed in **Table 7**, with values presented in mM (contrary to the previous results) due to the fact that compounds used in this analysis comprises two herbicidally active substances with different molar masses.

Table 7. Antimicrobial activity of the MCPA/MCPP based HILs towards selected soil bacteria and fungi. Adapted and modified from author's published work [84].

Salt	*B. cereus*		*P. putida*		*C. albicans*	
	MIC [mM]	**MBC [mM]**	**MIC [mM]**	**MBC [mM]**	**MIC [mM]**	**MFC [mM]**
[MCPA-DAE-C_8][MCPP]	0.125	0.5	0.5	1	0.250	0.5
[MCPA-DAE-C_9][MCPP]	0.125	0.5	0.5	1	0.250	0.5
[MCPA-DAE-C_{10}][MCPP]	0.125	0.5	0.5	1	0.125	0.25
[MCPA-DAE-C_{11}][MCPP]	0.125	0.5	0.125	1	0.125	0.25
[MCPA-DAE-C_{12}][MCPP]	0.0313	0.25	0.125	0.5	0.125	0.25
[MCPA-DAE-C_{14}][MCPP]	0.0078	0.0313	0.05	0.125	0.0125	0.025
[MCPA][Na] + [MCPP][Na]	2.5	10	10	> 10	5	10

The results revealed that all compounds displayed antimicrobial activity, with MIC ranging from 0.0078 to 1.00 mM and MBC/MFC ranging from 0.0313 to 1.00 mM. Regarding species-specific effects, *P. putida* displayed the highest resistance, while *B. cereus* was the most susceptible microorganism, exhibiting the lowest MIC and MBC values for all of the tested compounds. Across all species, [MCPA-DAE-C_{14}][MCPP] exhibited the most potent effect, followed by [MCPA-DAE-C_{12}][MCPP], while [MCPA-DAE-C_8][MCPP] and [MCPA-DAE-C_9][MCPP] showed the lowest antimicrobial activity. This underscores the significance of the alkyl chain length in the cation in determining the biological activity of HILs.

All tested HILs demonstrated significantly higher antimicrobial activity compared to the MCPA and MCPP mixture, differing by one to three orders of magnitude. Albeit, the MIC and MBC/MFC values surpass the doses of MCPA and MCPP mixtures in spray solutions. This heightened activity is attributed to the presence of the esterquat

cation. Quaternary ammonium cations, known for their affinity to microbial cells and surface activity, cause phospholipid bilayer disruption. Previous research on HILs' antimicrobial activity indicates substantial variations, with the type of cation significantly influencing MIC and MBC/MFC values [50]. Notably, the MIC values obtained here are higher than those reported for cetyltrimethylammonium bromide (CTAB) against *E. coli* and glyphosate against *E. coli* [134]. This aligns with the idea that the surface-active nature of the cation in HILs is responsible for heightened antimicrobial activity of studied compounds.

4.5.1.3. Toxicity of dicamba based HILs

The commonly found soil microorganisms *E. coli*, *P. putida*, and *B. subtilis* play a significant role in herbicide degradation. Therefore, understanding the impact of surface-active cations on the growth of microorganisms is crucial for developing environmentally friendly agrochemicals. In this study, model organisms [135–137] were employed to assess the toxicity of tested compounds using the half maximal effective concentration (EC_{50}) method. The results, illustrated in **Table 8**, unequivocally highlight the cation as the decisive factor in microbial toxicity. For instance, [Chol][Dic] and [C_2Bet][Dic] exhibited no harmful effects on microorganisms, similar to the reference dicamba herbicide. Conversely, [DDA][Dic] was over 100 times more toxic to all examined strains. This toxicity is attributed to increased hydrophobicity (and consequently, lipophilicity), a key factor in the bactericidal properties of quaternary ammonium salts (QASs) and ILs [138]. Namely, as hydrophobicity increases with the length of the nonpolar alkyl chain, the affinity of these compounds to cell membranes also increases [139,140]. Consequently, QASs permeate the membrane, interact with its components, leading to pore formation, intracellular compound leakage, and rapid cell lysis [75]. The most significant biological activity is estimated for alkyl chain lengths ranging from C_{10} to C_{14} [141,142], with a further increase in chain length reducing the toxic effect, primarily due to hindered solubility [143].

Table 8. The results of antimicrobial assay of dicamba-based salts towards selected soil bacteria. EC_{50} values are calculated per active ingredient of referential potassium salt of dicamba herbicide and dicamba based HILs. The classes were assessed according to acute toxicity rating scale by Fish and Wildlife Service. Adapted and modified from author's published work [144].

Salt	*B. subtilis*		*P. putida*		*E. coli*	
	EC_{50} [mg/L]	**Class**	**EC_{50} [mg/L]**	**Class**	**EC_{50} [mg/L]**	**Class**
[K][Dic]	>1000	Relatively Harmless	>1000	Relatively Harmless	>1000	Relatively Harmless
[Chol][Dic]	>1000	Relatively Harmless	>1000	Relatively Harmless	>1000	Relatively Harmless
[C_2Bet][Dic]	>1000	Relatively Harmless	>1000	Relatively Harmless	>1000	Relatively Harmless
[C_{10}Bet][Dic]	75.18 ± 0.18	Practically Nontoxic	104.52 ± 3.94	Practically Nontoxic	166.53 ± 0.34	Practically Nontoxic
[C_{16}Bet][Dic]	4.12 ± 0.036	moderately toxic	26.03 ± 0.84	Slightly Toxic	42.16 ± 0.01	Slightly Toxic
[DDA][Dic]	2.98 ± 0.025	Moderately Toxic	4.57 ± 0.32	Moderately Toxic	2.38 ± 0.08	Moderately Toxic

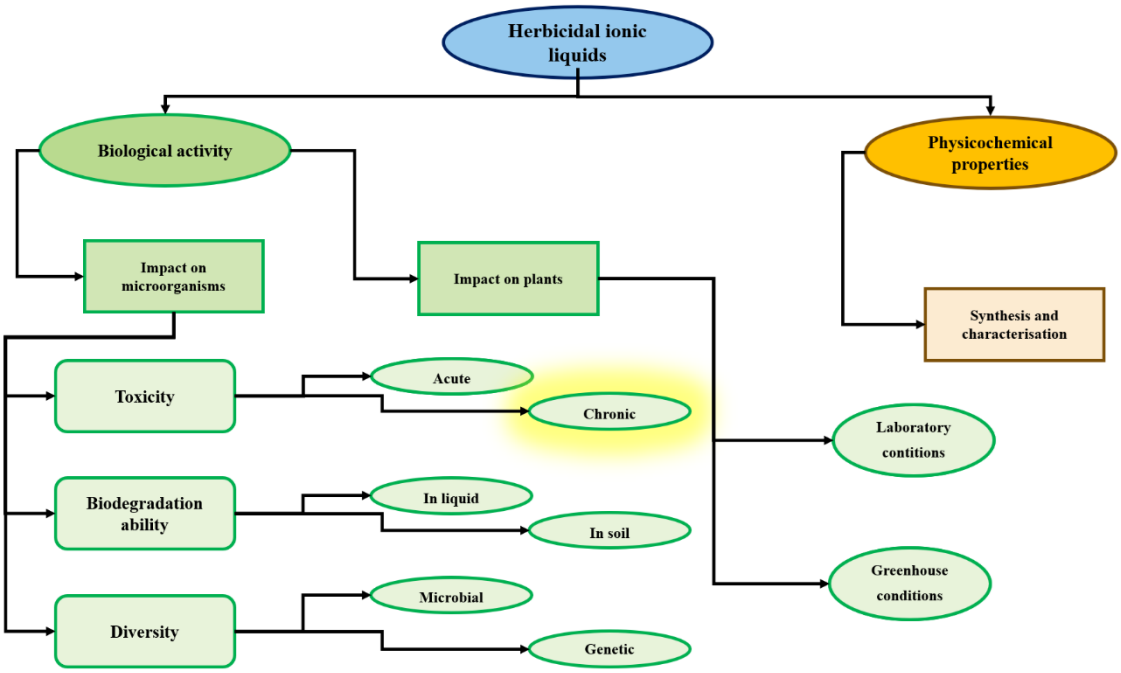
The introduction of diverse substituents to the cation, along with subsequent modifications like elongating the alkyl chain, significantly impacted the overall toxicity of QASs [127]. All examined strains exhibited high resistance to [C_2Bet][Dic], and toxicity notably increased with the elongation of the alkyl chain for [C_{10}Bet][Dic] and [C_{16}Bet][Dic]. These findings align well with previous reports, not only in the context of dicamba-based herbicidal ionic liquids [48,105,122] but also for synthetic analogues of auxins [48,78,122] and other herbicides [109,110].

Notably, the most toxic HIL with a betaine-based cation, [C_{16}Bet][Dic], still exhibited lower toxicity than [DDA][Dic], which contains a fully synthetic cation (approx. 2-fold lower for *B. subtilis*, 5-fold for *P. putida*, and 20-fold for *E. coli*). The observed variations may be attributed to the fact that *B. subtilis* is a Gram-positive bacterium, while both *P. putida* and *E. coli* are Gram-negative, leading to substantial differences in the construction of their cell walls. Slight variations in toxicity values for *P. putida* and *E. coli* may arise from their belonging to different genera, *Pseudomonas* and *Enterobacteriaceae*, respectively, resulting in distinctions not only in cell wall compositions but also in the metabolic apparatus used to counter the toxic effects of the tested compounds [48,49].

After the acute toxicity testing, it seems evident that cation's presence is a factor contributing to high toxicity towards bacteria, even with low doses of herbicidal ionic liquid [78,144]. At the same time, the studies with ISM-based HILs showed that the toxicity might result from synergistic interaction of QASs and herbicide. Therefore,

the next step of research was to study whether the prolonged contact of microorganisms with herbicides in ionic form might be comparable to the QASs' impact on bacteria and carry similar threats.

4.5.2. Chronic toxicity



4.5.2.1. Dicamba based HILs

Understanding the prolonged toxicity of herbicides towards soil bacteria is a crucial issue in herbicide development. These microorganisms play a fundamental role in maintaining soil health and fertility over extended periods. Adverse effects on these bacteria can disrupt essential ecosystem functions, including nutrient cycling, organic matter decomposition, and the breakdown of xenobiotics, ultimately leading to soil degradation. Hence, following acute toxicity tests, a long-term assessment of the impact of selected herbicidal ionic liquids ([DDA][Dic], [Chol][Dic], [$BetC_2$][Dic], [$BetC_{10}$][Dic], [$BetC_{16}$][Dic]), on microorganisms was conducted. The influence of the cation's presence on the optical density of selected bacterial cultures is depicted in **Fig. 12** for Gram-negative (*E. coli*, *P. putida*) and Gram-positive (*B. subtilis*) species.

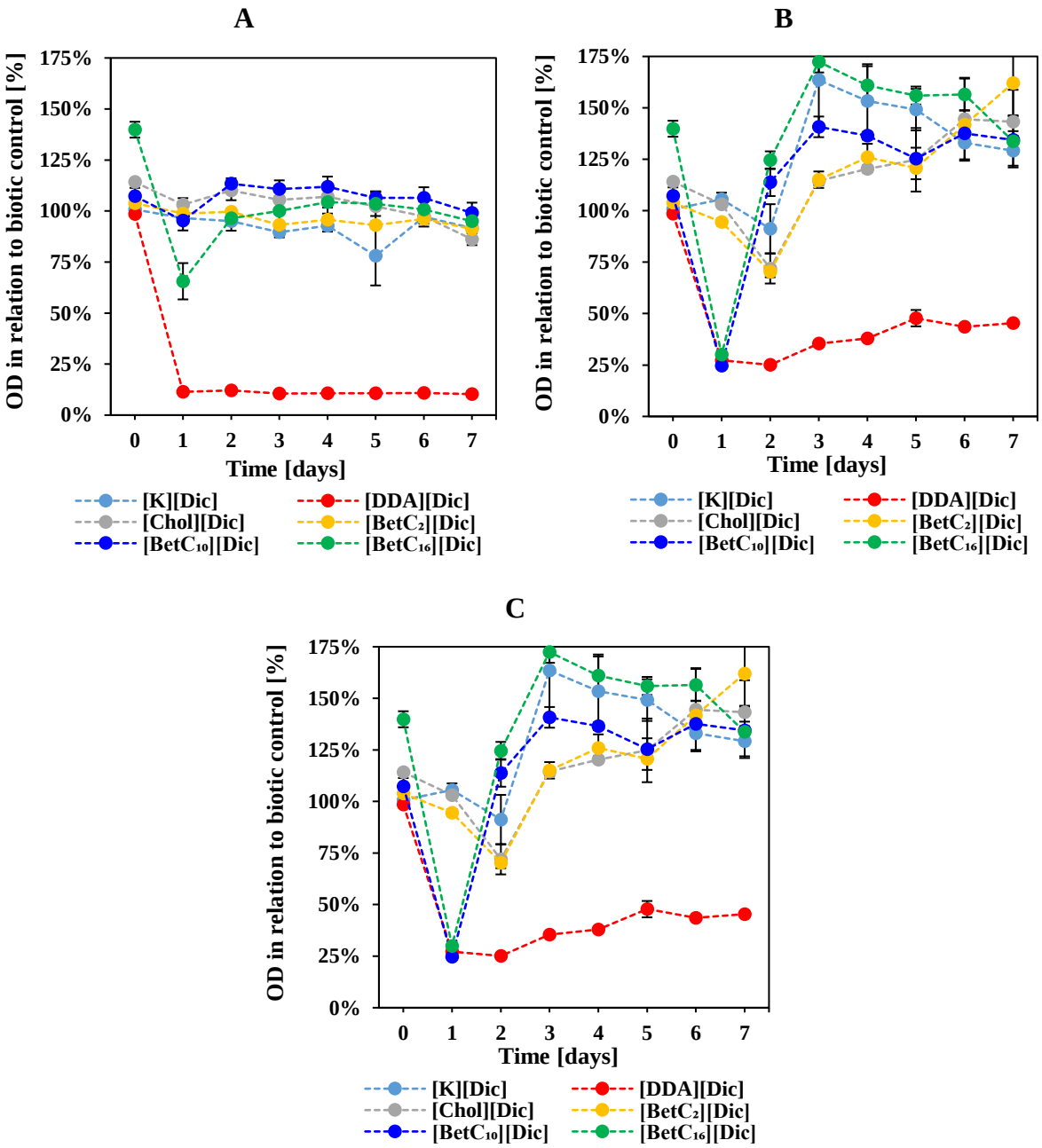


Figure 12. Optical density of liquid cultures of Gram-negative (**A** – *E. coli*, **B** – *P. putida*) and Gram-positive (**C** – *B. subtilis*) soil bacteria treated with dicamba-based salts in a concentration corresponding to 20 mg of anion/L in relation to optical density of biotic controls. Adapted and modified from author's published work [144].

In general, the addition of ionic liquids with herbicidal activity at the start of experiment led to decreased optical density compared to reference herbicide ([K][Dic]), with [BetC$_{16}$][Dic] and [DDA][Dic] having the most pronounced negative impact. Consistent with previous assessments, the [DDA] cation completely arrested the growth of *E. coli* and *P. putida* and significantly inhibited the growth of *B. subtilis*. In the case of [BetC$_{16}$][Dic], the initial opacity in wells containing this compound was

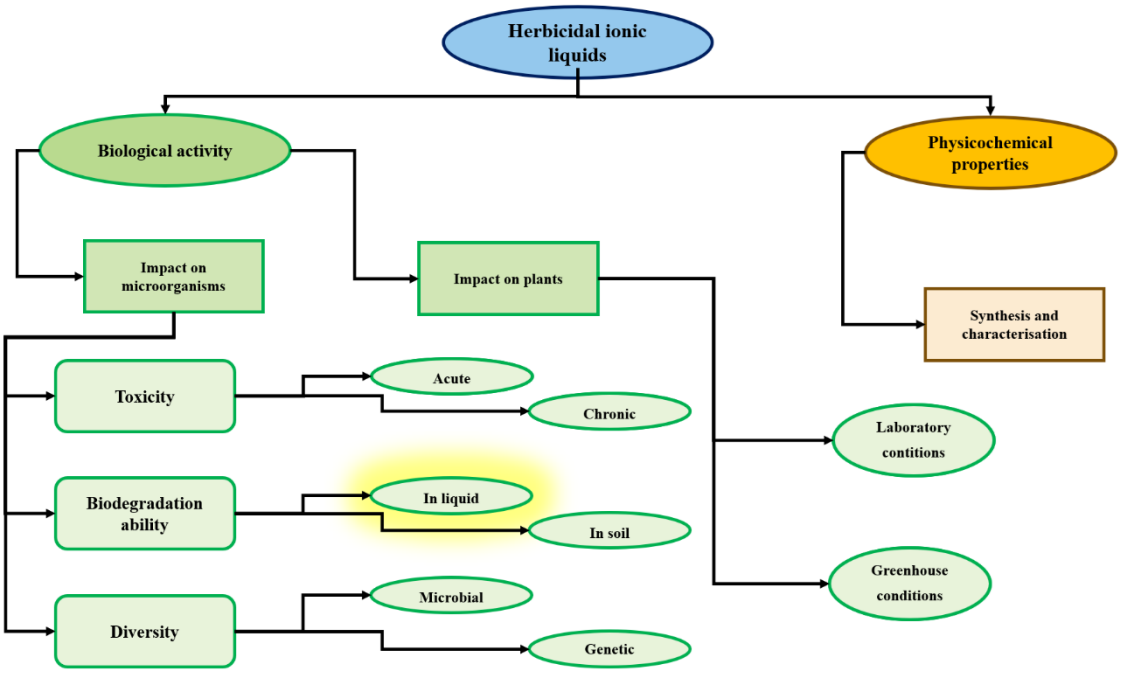
likely due to its micellar solubilization, as the growth medium was sterilized through autoclaving prior to experiment, and the HILs solution was introduced *via* a syringe with a microbial filter to avoid the decomposition of tested compounds. Though, in the *E. coli* assay, [$BetC_{16}$][Dic] surpassed the OD of the reference herbicide and continued to increase, reaching almost 125% of the biotic control's optical density by the experiment's endpoint. As for the *P. putida*, the presence of dicamba potassium salt led to only a slight decrease in OD compared to the biotic control. However, [$BetC_2$][Dic] had no significant impact on the optical density of the culture, while [Chol][Dic] and [$BetC_{10}$][Dic] showed a slight stimulating effect on the growth of the tested bacteria. Ultimately, at the end of the experiment, no significant variability in OD was noted for all forms of dicamba except [DDA][Dic]. Finally, for *B. subtilis*, at the beginning of the experiment, HILs with [$BetC_{10}$] and [$BetC_{16}$] cations had a similar to [DDA] effect on bacteria, significantly reducing the growth rate of *B. subtilis*. Yet, by the next day, [$BetC_{10}$][Dic] and [$BetC_{16}$][Dic] achieved higher OD compared to the reference herbicide, while the bacteria supplemented with [DDA][Dic] adapted to its presence by the third day when the OD started to increase gradually. Ultimately, cultures supplemented with HILs (except for [DDA][Dic]) exceeded 125% of OD, with the highest value observed for [$BetC_2$][Dic]. This indicates that at the end of the experiment, all compounds, except [DDA][Dic], showed a notable stimulating effect compared to the biotic control.

The findings align with the mechanisms underlying the long-term toxicity of quaternary ammonium salts (QASs) to bacteria [75,127]. The acute toxicity assay revealed that, with the exception of [DDA][Dic], bactericidal concentrations of herbicidal ionic liquids (HILs) were not reached in the long-term exposure assay. Bacterial cells might initiate defence mechanisms against sub-lethal concentrations, involving alterations in the composition of bacterial cells [75,77,138], the assembly of additional trans-membrane efflux pumps [145–147], and shifts in the expression patterns of numerous other genes associated with the stress response [146,148]. For instance, Forbes et al. (2019) demonstrated the extent of bacterial cells adaptations to long-term contact with benzalkonium chloride using *E. coli* MG1655, with expression patterns of 680 genes changing [146]. Most upregulated genes were correlated with efflux pumps, while downregulated genes were involved in cell wall properties control. Additionally, QASs have been shown to induce oxidative stress in various bacteria [149,150] and cause damage to numerous cellular structures beyond

the cell wall [138,150]. Under such conditions, another bacterial defence mechanism is triggered, namely the promotion of horizontal gene transfer [76,151]. Consequently, the application of HILs in agriculture may facilitate the spread of genes responsible for herbicide resistance. This phenomenon has already been demonstrated for glyphosate, glufosinate, and dicamba herbicides, known to promote the dissemination of antibiotic resistance genes [152]. Therefore, it is plausible that exerting more pressure on bacteria through the combination of organic cations with herbicides may lead to an undesirable acceleration of horizontal gene transfer.

Assuming that herbicides in the form of ILs are treated by bacteria similar to QASs and the prolonged contact of microorganisms with these compounds results in activation of defence mechanisms [144], further study on the environmental impact of these compounds is therefore required. Namely, cations' presence has a vast impact on bacteria [78,144] but at the same time chronic toxicity tests revealed that bacteria themselves are able to counteract these adverse effects [144]. As a consequence, study on bacterial biodegradation potential of herbicides in ionic form is required to assess the risks associated with the bioaccumulation of xenobiotics.

4.6. Biodegradation potential



4.6.1. Bacterial ability to degrade HILs in liquid medium

The increase of OD of culture of *H. zhihuaiae S113* and *B. subtilis 168* were investigated, primary biodegradation was explored and half-lives were calculated in order to access impact of HILs structure on bacterial ability to transform sulfonylurea herbicide in aquatic medium. The results for *H. zhihuaiae* are presented in **Table 9**, **Fig. 13** and **Fig. S23,** while results for *B. subtilis* are displayed in **Table 10**, **Fig. 14** and **Fig. S24.**

The growth curves obtained for *H. zhihuaiae S113* and *B. subtilis 168* correspond well with the results of antimicrobial assays. In both cases the bacterial cells were capable of growth in the presence of the studied compounds regardless of their concentration, with the exception of [C_{12}Chol][ISM]. In case of this compound microbial growth was notably inhibited (50 mg/L) after a lag-phase that lasted almost 5 days. This finding might suggest that the toxicity mechanisms of HILs cations are countered by bacterial defence mechanisms that require time to be activated. However, [C_{12}Chol][ISM] at a concentration of 100 mg/L completely hindered microbial proliferation (it is at the level of abiotic control in **Fig 13B** and **Fig 14B**).

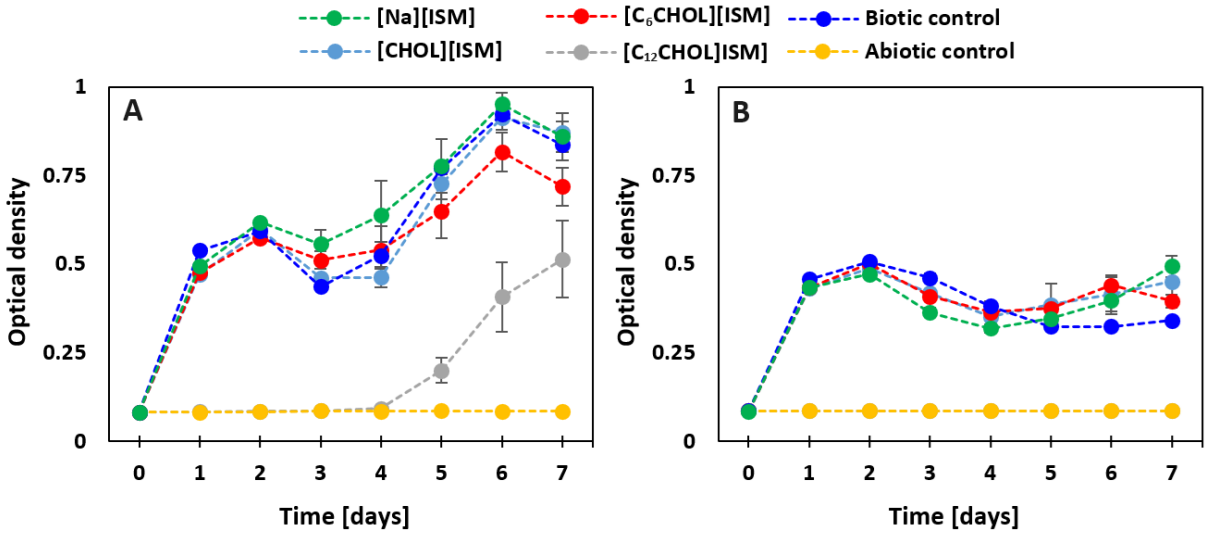


Figure 13. Growth characteristics of *H. zhihuaiae* S113 exposed to herbicide and HILs with different cations. The concentration (A – 50 mg/L; B – 100 mg/L) was calculated based on the amount of the active substance.

Interestingly, with the exception of [C_{12}Chol], the cations seem to have little impact on biotransformation efficiency of iodosulfuron-methyl, as in both concentrations the differences between biodegradation extents of anions of [Chol][ISM], [C_6Chol][ISM] and referential herbicide sodium salt did not exceed 5% (**Table 9**). Conversely, in the

case of salt with [C_{12}Chol] cation, clear correlation between the toxic effect and concentration could be observed. At 100 mg/L of supplemented active compound, no bacterial growth could be observed and thus no biodegradation occurred. However, at 50 mg/L of supplemented active compound (which corresponds to the field dose of certain sulfonylurea herbicides [130]), approx. 7% of ISM was degraded. Though, compared to other compounds, the half-life time quadrupled, which yet again underscores the crucial role of cation incorporated in the structure of HIL molecule. Furthermore, contrary to the ISM anions, no significant degradation of any cation could be observed in any of the investigated samples (**Fig. S23**). Such phenomena hint at the possibility that in carbon rich environment such as soil, bacteria might not be inclined to deteriorate cations, which in turn would lead to their accumulation, and thus increase the toxicity of the soil itself.

Table 9. Primary biodegradation extent and biodegradation rates of anions for ISM sodium salt and ISM based HILs in aquatic matrix by *H. zhihuaiae*.

Compound	Biodegradation extent [%]	1st order rate constants k [d^{-1}]	$t_{1/2}$ [days]
50 mg/L of herbicide active substance			
[Na][ISM]	27.6	0.046	15.0
[Chol][ISM]	26.5	0.044	15.8
[C_6Chol][ISM]	30.6	0.052	13.3
[C_{12}Chol][ISM]	7.0	0.010	66.5
100 mg/L of herbicide active substance			
[Na][ISM]	15.5	0.024	28.8
[Chol][ISM]	16.1	0.025	27.6
[C_6Chol][ISM]	17.3	0.027	25.6
[C_{12}Chol][ISM]	No biodegradation occured	[–]	[–]

In contrast, *B. subtilis 168* was characterized by higher tolerance to the examined compounds as significantly shorter lag phase in case of medium supplemented with 50 mg/L of [C_{12}Chol][ISM] could be observed; it lasted not even a day, compared to almost 5 days in case of *H. zhihuaiae.* Moreover, after just 3 days, *B. subtilis* cultivated in [C_{12}Chol][ISM] managed to grow to OD comparable to OD of bacteria exposed to referential herbicide. These findings not only underscore the relationship between HIL structure and toxicity, but also demonstrate that different bacteria species react differently to the exposure of HILs. Hence, it can be speculated that distinct molecular mechanisms are employed by bacteria to mitigate the effects of HIL-type compounds.

However, higher concentration (100 mg/L) of [C_{12}Chol][ISM] compound completely inhibited the ability of *B. subtilis* to grow, based on OD analysis.

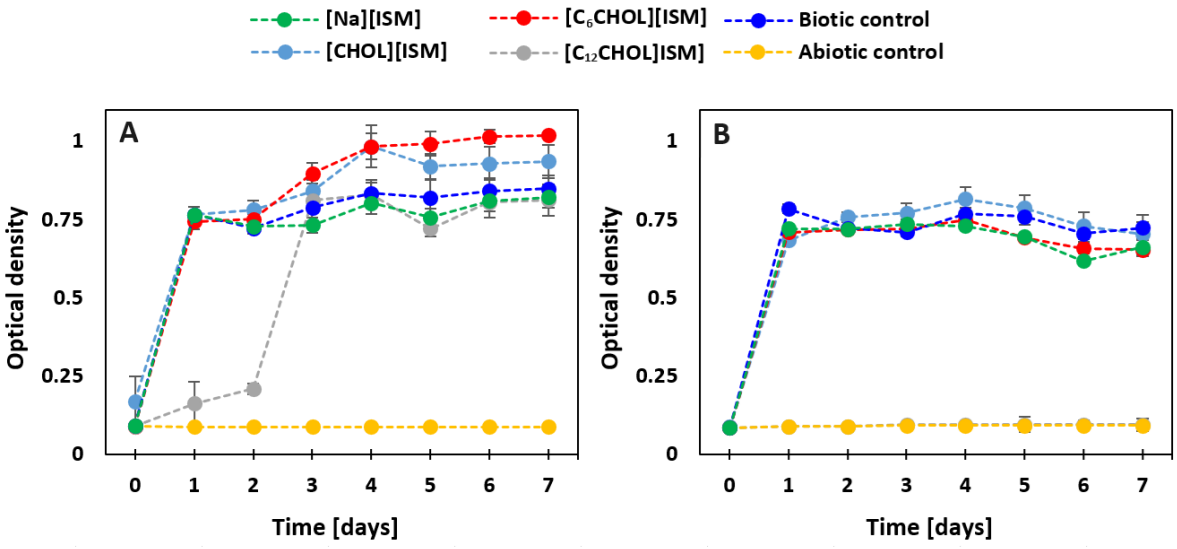


Figure 14. Growth characteristics of *B. subtilis* 168 exposed to herbicide and HILs with different cations. The concentration (A – 50 mg/L; B – 100 mg/L) was calculated based on the amount of the active substance.

Interestingly, the biodegradation efficiency of iodosulfuron methyl sodium salt was similar in each of two concentrations, at approximately 19% and 18% respectively, but, unexpectedly, the degradation of ISM anion varied among HILs compounds and concentrations (**Table 10**). Namely, in lower concentration range, the ISM reference salt was degraded the most efficiently, while in higher concentration range it was degraded less effectively compared to HILs with [Chol] and [C_6Chol] cations. Interestingly, the rate of ISM referential salt has not changed, rather it seems that higher concentrations of practically harmless compounds seem to have stimulated the degradation process. For example, the half-lives of ISM in [Chol][ISM] and [C_{12}Chol][ISM] have decreased by over 10 days and this phenomenon might be in fact caused by incorporation of choline in the structure of these compounds which is generally well known as an osmoprotectant. Consequently, this may have facilitated *B. subtilis* in more effective management of the stress caused by the ISM itself. As for [C_6Chol][ISM], in lower concentration range it was biotransformed almost as efficiently as other HILs, with half-live of only 2 day longer when compared to [Chol][ISM]. On the contrary to [Chol][ISM], in higher concentrations range the half-lives of [C_{12}Chol][ISM] anion increased substantially up to 50 days, which is

particularly noteworthy due to the fact that no growth could be observed based on OD assay. This phenomenon might be partly explained by the fact that enzymes produced by *B. subtilis* are excreted from the cell into the medium [85], thus either some from the initial culture inoculated into the examined sample might have stayed active for some time, or [C_{12}Chol] cation enabled for slow bacterial growth so much that it was undetectable but some bacteria managed to stay alive and active. Similarly to *H. zhihuaiae*, no significant biotransformation of cations occurred in carbon rich medium, in which *B. subtilis* bacteria were cultivated (**Fig S24**).

Table 10. Primary biodegradation extent and biodegradation rates of anions for ISM sodium salt and ISM based HILS in aquatic matrix by *B. subtilis*.

Compound	Biodegradation extent [%]	1st order rate constants k [d^{-1}]	$t_{1/2}$ [days]
50 mg/L of herbicidally active substance			
[Na][ISM]	18.9	0.030	23.1
[Chol][ISM]	15.4	0.024	29.0
[C_6Chol][ISM]	14.4	0.022	31.2
[C_{12}Chol][ISM]	13.7	0.021	32.8
100 mg/L of herbicidally active substance			
[Na][ISM]	18.3	0.029	23.9
[Chol][ISM]	25.5	0.042	16.5
[C_6Chol][ISM]	21.8	0.035	19.7
[C_{12}Chol][ISM]	9.2	0.014	50.0

Overall, the primary biodegradation assay in aquatic medium indicated that both *B. subtilis* and *H. zhihuaiae* were capable of surviving the impact of iodosulfuron and iodosulfuron-based HILs applied at a concentration of 100 mg/L, apart from [C_{12}Chol][ISM] which seemed to be lethal to both species of microorganisms. Moreover, both bacterial species exhibited ability to degrade iodosulfuron, which corresponds well with the previous reports regarding ability of these strains to handle herbicides from the sulfonylurea group [117,153,154]. Additionally, it was demonstrated that the structure of the cation used in HIL molecule impacts bacterial ability to biotransform parent herbicide in addition to its overall toxicity. Similar results underscoring the critical role of cation have been obtained previously, albeit for bacterial consortia in soil matrix [61,62]. Furthermore, the ions were found to be degraded separately as opposed to the HIL being decomposed as a whole uniform molecule. This in turn might suggest, that HILs molecules are unstable in the environment when exposed to a mixture of enzymes produced by native microbiota.

In fact, the structural integrity of HILs is already in question, but its loss is discussed in the context of physicochemical phenomena such as dissociation and solvation, which then leads to the sorption of separate ions into soil particles [67,109,110], rather than as an action of a microbial enzymatic apparatus.

4.6.2. Fungal ability to degrade HILs in liquid medium

Each of the fungal isolates demonstrated the ability to biotransform iodosulfuron-methyl. Subsequently, the half-lives ($t_{1/2}$) were calculated and are presented below in **Table 11**. The half-life values show clear variations based on the fungal genus, with even strain-specific differences observed among the *Fusarium solani* isolates. Consequently, genus-specific subsections present and discuss specific results and the influence of cations on fungi.

Table 11. Half-lives ($t_{1/2}$) for iodosulfuron-methyl anion in examined herbicidal ionic liquids and referential sodium salt form. Adapted and modified from author's published work [129].

Fungi species / **Compound**	*Fusarium solani* (Isolate I)	*Fusarium solani* (Isolate IV)	*Fusarium solani* (Isolate IV)	*Penicillium* sp.	*Talaromyces muroii*	*Aspergillus* sp.
[Na][ISM]	3.67	2.33	1.25	4.92	4.76	2.15
[Chol][ISM]	3.09	2.01	1.40	4.39	1.43	2.14
[C_{12}Chol][ISM]	40.9	[–]	[–]	1310	19.10	1.58

4.6.2.1. *Fusarium* sp.

Fusarium solani, a commonly found soil fungus [155–157], has been isolated in three strains for this study. *Fusarium* is widely recognized for causing diseases in arable crops [155–161] and producing mycotoxins [155,157,161], resulting in significant economic losses [157–160,162]. Despite its pathogenic nature, *Fusarium* demonstrates the ability to degrade various xenobiotics [163–168], including herbicidally active substances like glyphosate [169,170] and atrazine [166].

Notably, there were slight variations among the isolated strains in the degradation of the pure referential iodosulfuron-methyl and [Chol][ISM]. However, the adverse impact of the [C_{12}Chol] cation on the anion biotransformation rate was evident in all cases. Isolate I (**Fig. 15A**), IV (**Fig. 15B**), and V (**Fig. 15C**) transformed 92.9%, 98.5%, and 99.9% of the herbicidally active compound, respectively. Moreover, the choline

cation did not negatively affect the biodegradation dynamic and even slightly enhanced it. This enhancement aligns well with the role of choline as a component of the major membrane phospholipid lecithin, regulating sulphate metabolism, mycelial growth, and hyphal growth in fungi [171]. This stimulating effect is evident in the observed decrease in half-lives from 3.67 to 3.09 days for Isolate I and from 2.33 to 2.01 days for Isolate IV. However, Isolate V showed a slight increase, from 1.25 to 1.40 days. Interestingly, only Isolate I could survive in the presence of [C_{12}Chol][ISM], but the herbicide's half-life increased over 10-fold, up to 41.84 days, with only 20.7% of ISM dissipated by the experiment's end. The choline cation appears to have no negative effect on *F. solani*'s ability to dissipate ISM. In fact, in the case of Isolate I, this ion seems to slightly enhance ISM degradation and might serve as an additional carbon source for the fungus. However, the [C_{12}Chol] cation, with one long alkyl chain, significantly limits fungal growth and herbicide metabolism, as it completely inhibits the development of Isolate IV and V and substantially reduces the degradation rate of Isolate I.

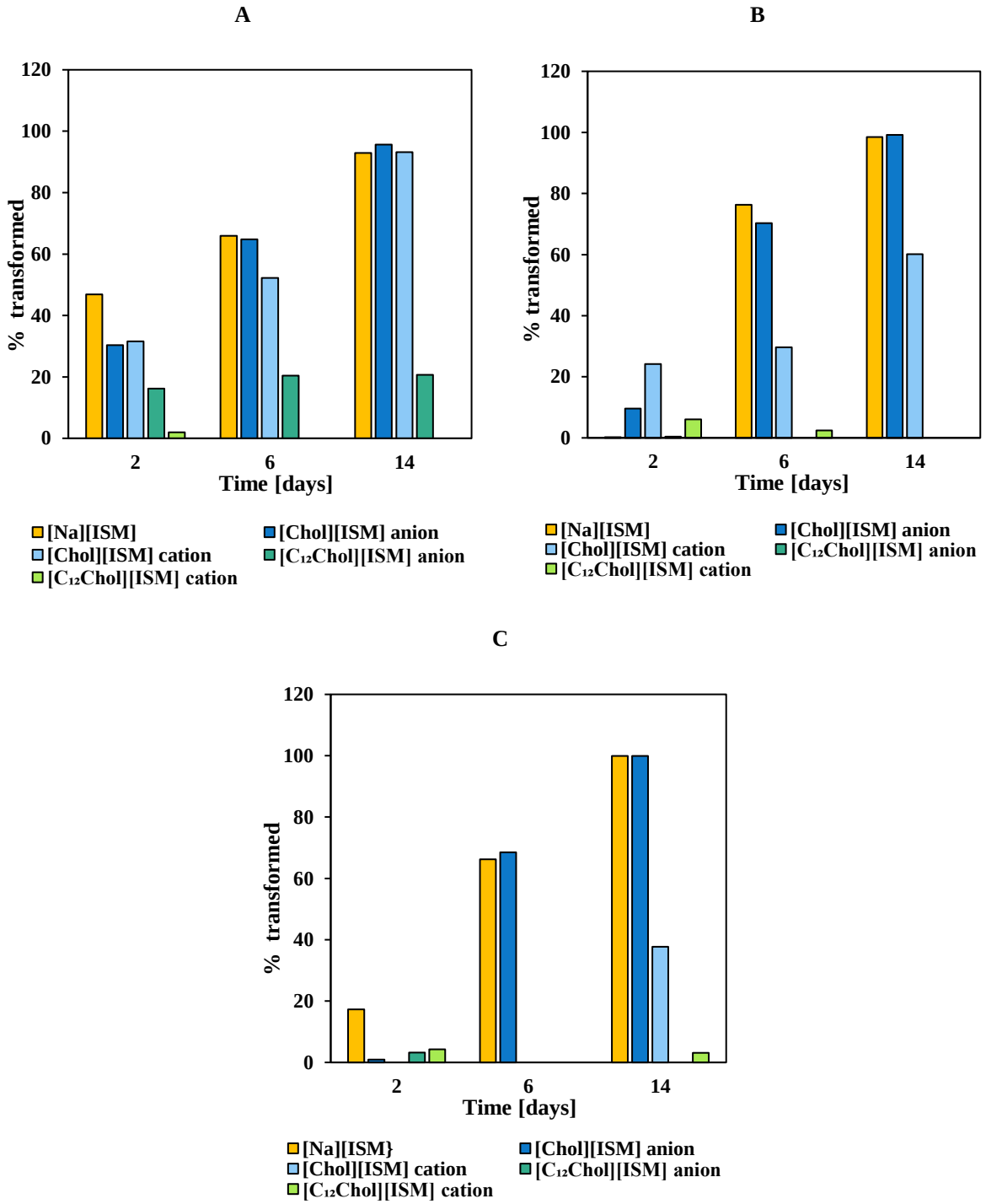


Figure 15. *Fusarium solani* (A – Isolate I, B – Isolate IV, C – Isolate V) ability to biotransform ILs based on iodosulfuron-methyl. Adapted and modified from author's published work [129].

The disparities in herbicide degradation in this study can be attributed to genetic differences among the isolated strains. Previous research has shown that *Fusarium* sp. can carry plasmids [172–176] and vary significantly in genetic composition, with genes located on lineage-specific chromosomes associated with niche adaptation and

pathogenicity [158,177–179]. This diversity can be observed between species or even within a single species [161]. Additionally, *Fusarium* sp., like most fungi, produce numerous secondary metabolites, where only the chemical structure is known, but information about the mechanisms regulating their production is lacking [155,157]. This information is crucial as these compounds affect factors such as the virulence of specific strains and the progression of disease in plant tissues [157]. Consequently, the cation's toxicity is the limiting factor for the efficient dissipation of iodosulfuron-methyl in isolated strains of *F. solani*, with the specific details tightly linked to the genetic composition of each isolate.

4.6.2.2. *Penicillium* sp.

In this investigation, *Penicillium ochrochloron* showcased a remarkable capacity for iodosulfuron-methyl degradation (**Fig. 16**). Within a 14-day period, more than 86% of the initial herbicide dose was transformed, although only 30% had dissipated by the end of the first week ($t_{1/2}$ of 4.92 days for the reference herbicide). Previous studies have established that fungi from the *Penicillium* genus are effective herbicide degraders [180–188] . Moreover, it has been shown that while many strains can utilize sulfonylureas as a sole source of carbon [117,184,186] or nitrogen [180], degradation rates are higher under co-metabolic conditions in an enriched medium [184]. Additionally, it was proven that the type of carbon source is crucial; *Penicillium oxalicum* achieved over 97% degradation of nicosulfuron when supplemented with glucose, sucrose, or starch, whereas the addition of glycerine or lactose resulted in almost no degradation [180].

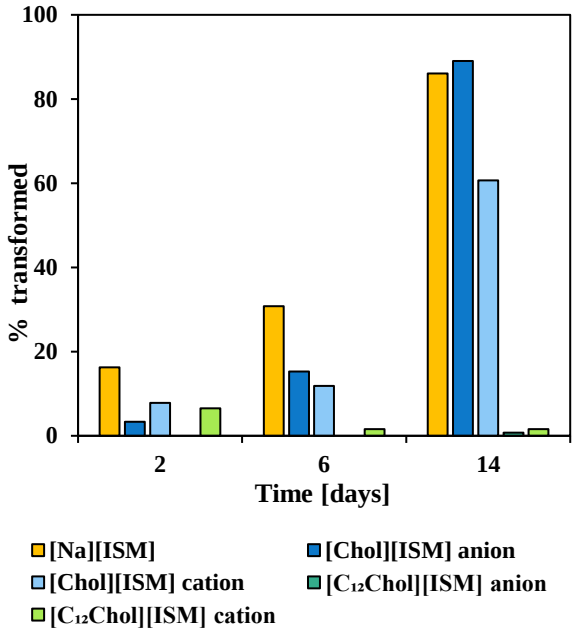


Figure 16. *Penicillium ochrochloron* ability to biotransform ILs based on iodosulfuron-methyl. Adapted and modified from author's published work [129].

Interestingly, the half-life of the herbicide in [Chol][ISM] decreased to 4.39 days from 4.92, likely due to choline stimulating the growth of *Penicillium ochrohloron*. In the first week, 11.9% of the choline cation itself was degraded, and by the end of the experiment, 60% of the initial cation dissipated. Conversely, the [C_{12}Chol] cation significantly hindered the fungus' ability to grow and biotransform the xenobiotic. The herbicidal anion's half-life increased to 1310 days, with less than 1% of the anion and 4.4% of the cation dissipated by the end of the experiment. These findings clearly indicate that the hydrophobic nature of the cation is the primary factor influencing *Penicillium ochrohloron*'s ability to metabolize ISM. Namely, while choline serves as a precursor for cell membrane components [189,190] the C_{12} alkyl is a highly hydrophobic compound that entirely nullifies the stimulating effects of choline.

4.6.2.3. *Talaromyces* sp.

Talaromyces sp. comprises a diverse group of fungi known for producing a wide array of secondary metabolites [191,192]. *Talaromyces muroii*, isolated from soil during experiments, displayed a remarkable capability for biodegrading the reference iodosulfuron-methyl herbicide in a liquid culture (**Fig. 17)**. Initially, during a short adaptation period, only approx. 20% of the reference compound was removed from

the solution. However, degradation accelerated in the subsequent days, reaching 80.5% after 7 days and 87% by the end of the experiment.

Notably, the type of cation in the structure of HILs appeared to significantly influence biotransformation dynamics. In the case of [Chol][ISM], the choline cation initially exhibited an inhibitory effect, but after one week, upon the end of the adaptation phase, the final amount of the herbicidal anion was lower than 1%, compared to 13% for [Na][ISM], showing stimulatory effect of this cation on the anion's biotransformation. Though, the cation itself underwent limited transformation; after 7 days, only 9% was degraded, and by the end of the experiment, 22.3% dissipated. The [C_{12}Chol] cation exhibited even lower degradation rates, with only 8.9% and 17.7% dissipated after 7 and 14 days, respectively. At the same time, its presence negatively impacted the biotransformation of the herbicidal anion, with only 39.9% degraded by the end of the experiment. This is further supported by the comparison of half-lives of tested iodosulfuron-methyl salts (**Table 11**): 4.76 days for [Na][ISM], 1.43 days for [Chol][ISM], and 19.1 days for [C_{12}Chol].

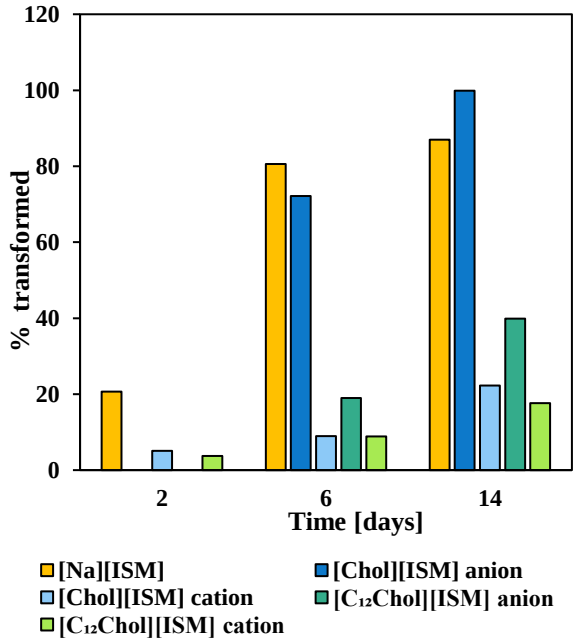


Figure 17. *Talaromyces muroii* ability to biotransform ILs based on iodosulfuron-methyl. Adapted and modified from author's published work [129].

While information on the ability of *Talaromyces* sp. to handle herbicidal contamination in the environment is relatively limited, past reports have demonstrated their capacity for herbicide degradation [193–196]. For instance, *Talaromyces flavus* LZM1, isolated from activated sludge, has been shown to efficiently biotransform four sulfonylurea herbicides, achieving over 90% reduction of nicosulfuron, tribenuron methyl, chlorsulfuron, and bensulfuron methyl over 5 days [195]. Additionally, *Talaromyces helicus* was proven to use isoproturon herbicide as a sole carbon source, removing approx. 80% of the initial herbicide over 30 days [194]. These findings support the notion that *Talaromyces* sp. is proficient in degrading sulfonylurea herbicides such as iodosulfuron-methyl. Consequently, the results from this study underscore the critical influence of the molecular structure of cations on *Talaromyces muroii*'s ability to degrade HILs. Specifically, the incorporation of highly hydrophobic C_{12} alkyl into the cation structure significantly reduces *Talaromyces muroii*'s capacity to degrade iodosulfuron-methyl in a liquid medium.

4.6.2.4. *Asperegillus* sp.

An isolated strain of *Aspergillus* has demonstrated considerable proficiency in degrading iodosulfuron-methyl, as indicated by the results of the biotransformation assay (**Fig. 18)**. Within 2 days of fungal colony growth, 32% of the reference herbicide was biotransformed, increasing to over 93% after 6 days and reaching 99% after 14 days. Similar efficiency was observed for the ionic liquids based on [Chol] and [C_{12}Chol], with final biodegradation reaching 99% in both cases. However, the cations were less effectively degraded by the fungi. In the case of [Chol][ISM], only 40% of the cation was degraded after 7 days, reaching about 60% over the 2-week period. Conversely, only 5% of the [C_{12}Chol] cation dissipated within the given time. Despite variations in cation dissipation, they seemed to have no substantial impact on anion biotransformation, as evidenced by the half-lives of these compounds (**Table 11)**: 2.15 days for the reference herbicide, 2.14 days for [Chol][ISM], and 1.58 days for [C_{12}Chol][ISM].

Numerous representatives of the *Aspergillus* genus have previously been isolated from herbicide-contaminated soil [197–205]. These fungi have been shown to be potent degraders of various herbicidal compounds, including atrazine [202,206], glyphosate [207], clodinafop-propargyl [198], penoxsulam [201], thiobencarb, butachlor,

molinate [205], and notably, many sulfonurea ALS inhibitors [199,200,203,208–210]. Notably, fungi from this genus are recognized for producing a diverse group of extracellular metabolites, including citric acid [211–215] which could lower the pH in the culture medium. This is particularly significant because some weak acid sulfonylureas are prone to chemical degradation in acidified cultures rather than biotransformation [216].

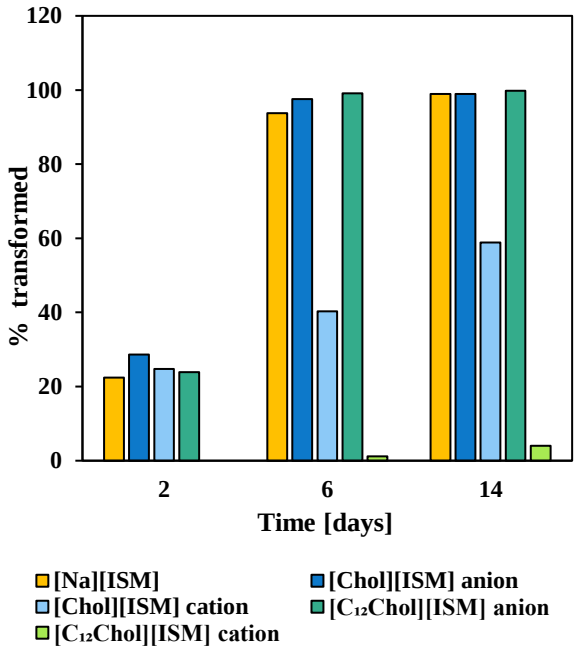
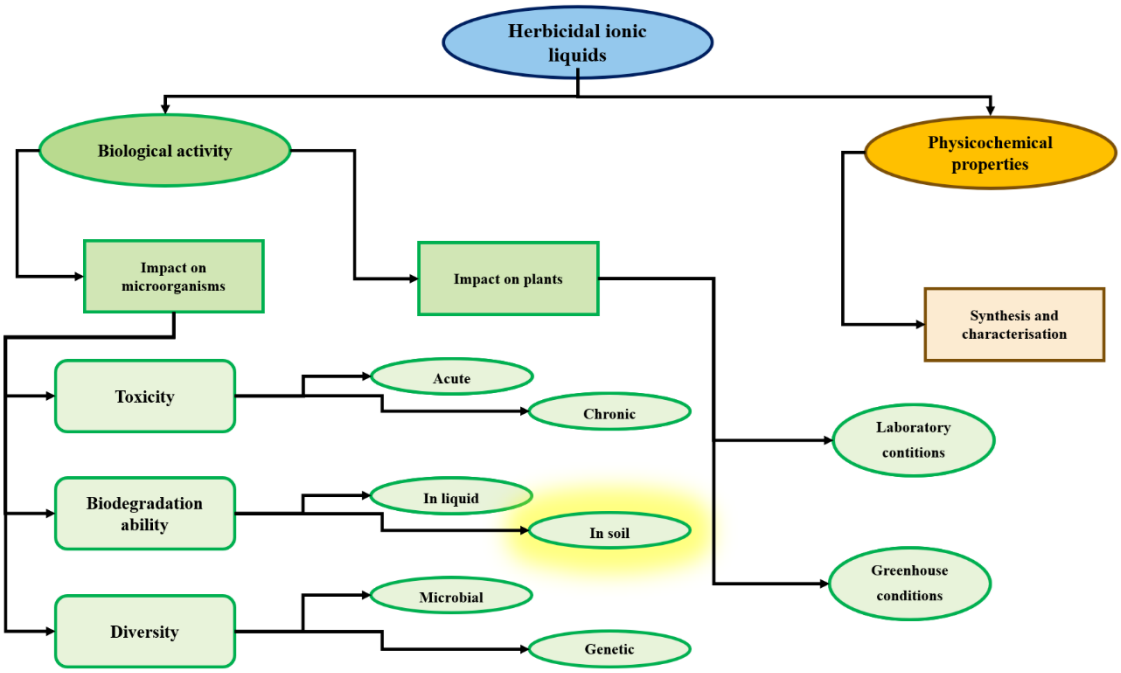


Figure 18. *Asperegillus* sp. ability to biotransform ILs based on iodosulfuron-methyl. Adapted and modified from author's published work [129].

Conversely, it has been established that the biodegradation of herbicides by *Aspergillus* occurs preferentially under co-metabolic conditions in a rich medium, rather than in a minimal mineral medium, and degradation rates are significant in buffered media [209,210], affirming the important role of *Aspergillus* in the degradation of sulfonylureas. However, it is noteworthy that *Aspergillus niger* can use chlorimuron-ethyl, a sister compound of iodosulfuron-methyl sodium, as a sole carbon source [203]. Similar reports on *Aspergillus*' capability to utilize other sulfonylureas from the ALS inhibitor herbicides group as a sole carbon source are also available [199]. Nevertheless, these findings indicate that, while cations have no significant impact on iodosulfuron-methyl anion degradation by *Aspergillus* sp., the cations themselves are poorly utilized. This is crucial, as *Aspergillus* sp. has proven to be the most resistant strain to cation influence in this study, potentially indicating its ability to dominate

other fungi in agrarian soil exposed to HILs with highly hydrophobic moieties. This dominance, in turn, could lead to contamination of crops, subsequent damage to produce in storage, and an increase in post-harvest losses.

4.7. Mineralisation and primary biodegradation in soil



4.7.1. MCPA/MCPP based HILs

The final phase of the experiment concentrated on evaluating the mineralisation efficiency of the obtained HILs (**Table 12**). According to the OECD 301F test criteria, a substance is considered 'biodegradable' if its mineralisation exceeds the 60% within a 10-day window of the 28-day testing period. The results revealed that none of the investigated compounds met this criterion, as their mineralisation efficiency was below 20%. Specifically, the mineralisation of HILs ranged between 2–12%, whereas the reference model herbicide mixture showed a higher mineralisation efficiency od 18%. Additionally, the susceptibility to biodegradation decreased with the lengthening of the alkyl chain in the cation, further indicating that longer alkyl chains in HILs contribute to reduced biodegradability.

Table 12. Mineralization of the studied HILs. Adapted and modified from author's published work [78].

HILs	Mineralization efficiency after 28 days [%]	Mineralization efficiency after 28 days [%]	Classification
[MCPA-DAE-C_8] [MCPP]	12.0 ± 2.0	-	Not readily biodegradable
[MCPA-DAE-C_9] [MCPP]	11.0 ± 3.0	-	Not readily biodegradable
[MCPA-DAE-C_{10}] [MCPP]	9.0 ± 1.0	-	Not readily biodegradable
[MCPA-DAE-C_{11}] [MCPP]	6.0 ± 2.0	-	Not readily biodegradable
[MCPA-DAE-C_{12}] [MCPP]	4.0 ± 1.0	-	Not readily biodegradable
[MCPA-DAE-C_{14}] [MCPP]	2.0 ± 1.0	-	Not readily biodegradable
[MCPA][Na]+[MCPP][Na]	18.0 ± 4.0	-	Not readily biodegradable

Following the assessment of mineralisation efficiency, further examination of the decomposition of HILs' cations and anions initial structures (primary biodegradation), as well as reference herbicidal mixture, was conducted (**Table 13**). The analysis of residues revealed that for all studied HILs, the anion dissipated more efficiently than the cation. The primary biodegradation values of HILs decreased with the increasing alkyl chain length in the cation, and the model mixture of MCPA and MCPP exhibited twice the biodegradation efficiency compared to the corresponding HILs.

Table 13. Primary biodegradation of DSHILs. Adapted and modified from author's published work [78].

HILs	Biodegradation efficiency [%]	
	Cation	Anion
[MCPA-DAE-C_8][MCPP]	8.6 ± 2.0	32.2 ± 3.1
[MCPA-DAE-C_9][MCPP]	7.2 ± 1.7	30.5 ± 4.7
[MCPA-DAE-C_{10}][MCPP]	7.9 ± 2.3	27.1 ± 2.9
[MCPA-DAE-C_{11}][MCPP]	6.1 ± 1.9	28.9 ± 3.2
[MCPA-DAE-C_{12}][MCPP]	5.4 ± 0.9	25.5 ± 4.9
[MCPA-DAE-C_{14}][MCPP]	5.9 ± 1.3	26.4 ± 3.5
[MCPA][Na]+[MCPP][Na]	[–]	60.2 ± 5.4 [MCPA] 58.9 ± 4.9 [MCPP]

Combining the outcomes of both primary biodegradation and mineralisation assessments reveals that the examined compounds undergo initial decomposition to varying degrees, but they are not effectively mineralized, suggesting a likelihood of biotransformation into stable metabolites. For HILs, the biodegradation of the anion is likely the primary contributor to the observed mineralisation; the low mineralization

efficiency may be attributed to the antimicrobial properties of HILs, particularly due to the surface activity of the quaternary ammonium cation.

4.7.2. ISM based HILs

The biodegradation experiment in soil matrix was focused on establishing the carbon dioxide evolution of the studied compounds and examining the potential impact of bioaugmentation with bacteria specialised in degradation of sulfonylurea herbicide on HILs environmental behaviour. To this extent, the amount of CO_2 emitted by the soil microbiome was measured within a period of 3 months, subsequently the residues of the compounds in the soil were determined and half-life values were calculated. The summary results are presented in **Fig. 19** and **Table 14.**

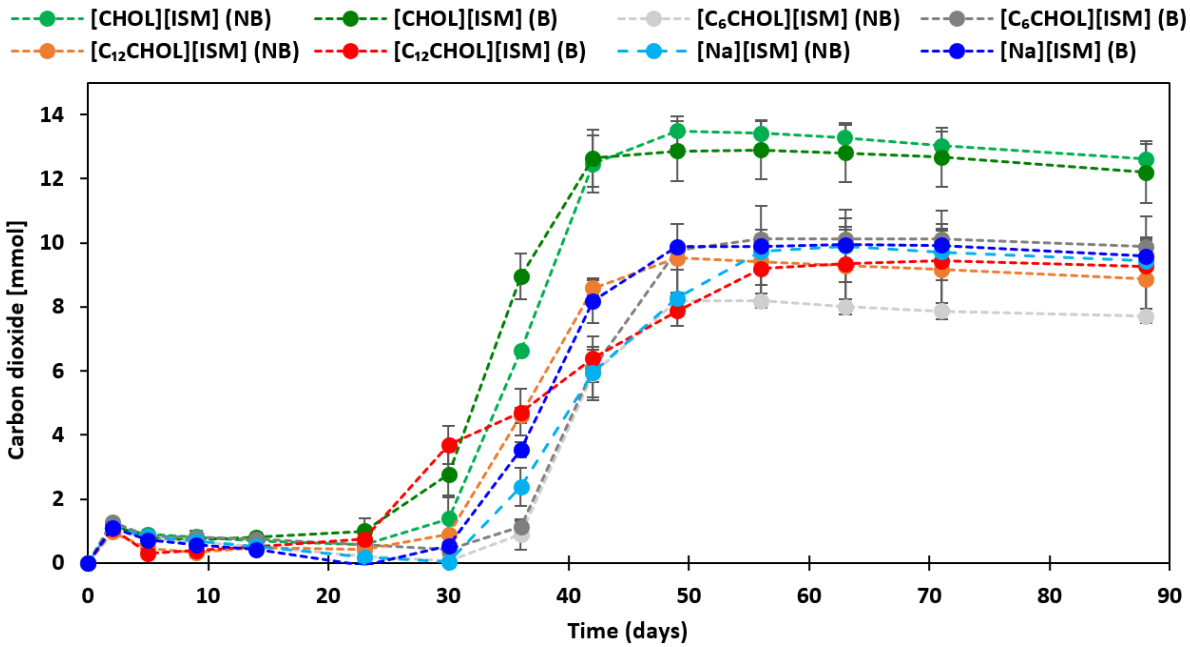


Figure 19. Ability of soil microbiome to mineralize the ISM herbicide and HILs with different cations: [Chol][ISM], [C_6Chol][ISM] and [C_{12}Chol][ISM] in non-bioaugmented (NB) soil and soil bioaugmented (B) with *B. subtilis 168*.

The obtained CO_2 emission curves, which are a representation of microbial activity in soil, are demonstrating similar trends for all of the studied systems. Namely, in all cases bioaugmentation resulted in higher production of CO_2, after initial adaptation period. However, the initial enhancement observed in bioaugmented samples did not contribute to long-term effects, as in most cases the final amount of emitted carbon

dioxide was very similar between the samples that were bioaugmented and those that were not. Interestingly, this phenomenon could not be observed only in the case of [C_6Chol][ISM], where the difference in favour of bioaugmented systems was notable. In case of samples which contained [Na][ISM], the final amount of measured carbon dioxide was of approx. 10 mmol. The highest value, equal to approx. 12 mmol, was observed in the case of samples containing [Chol][ISM]. For [C_6Chol][ISM], the amount of measured carbon dioxide was equal to 10 mmol in case of bioaugmented system and 8 mmol for the non-bioaugmented system, which is the lowest value among all the samples. Interestingly, the most toxic HIL ([C_{12}Chol][ISM]) was efficiently degraded in the long-term, as the CO_2 emission was at 9 mmol. These results hint at the fact that the ability to biodegrade herbicides from the sulfonylurea group might be more widespread than initially suspected, yet bioaugmentation seem to be a viable strategy to jump-start biodegradation of these compounds, especially in soil exhausted by excessive use of agrochemicals. In this context the results obtained for [Chol][ISM] are particularly interesting, as addition of this compound, both bioaugmented and non-bioaugmented, resulted in CO_2 evolution even higher than ISM referential herbicide. Consequently, this might mean that osmoprotective choline decrease the toxicity of iodosulfuron to vulnerable native soil bacteria, thus boosting microbiome soil activity.

Table 14. Biodegradation efficiency of [Na][ISM] and ISM based HILs in bioaugmented (B) and non-bioaugmented (NB) soil systems after 3 months.

Compound	Biodegradation extent [%]		ISM anion degradation parameters	
	Cation	Anion	1st order rate constants k [d^{-1}]	$t_{1/2}$ [days]
[Na][ISM] (NB)	-	90.9	0.027	26.1
[Na][ISM] (B)	-	89.4	0.025	27.8
[Chol][ISM] (NB)	99.0	94.0	0.031	22.2
[Chol][ISM] (B)	99.0	94.0	0.031	22.3
[C_6Chol][ISM] (NB)	90.3	90.9	0.027	26.0
[C_6Chol][ISM] (B)	89.9	90.2	0.026	26.9
[C_{12}Chol][ISM] (NB)	89.7	93.4	0.030	22.9
[C_{12}Chol][ISM] (B)	87.3	91.6	0.028	25.2

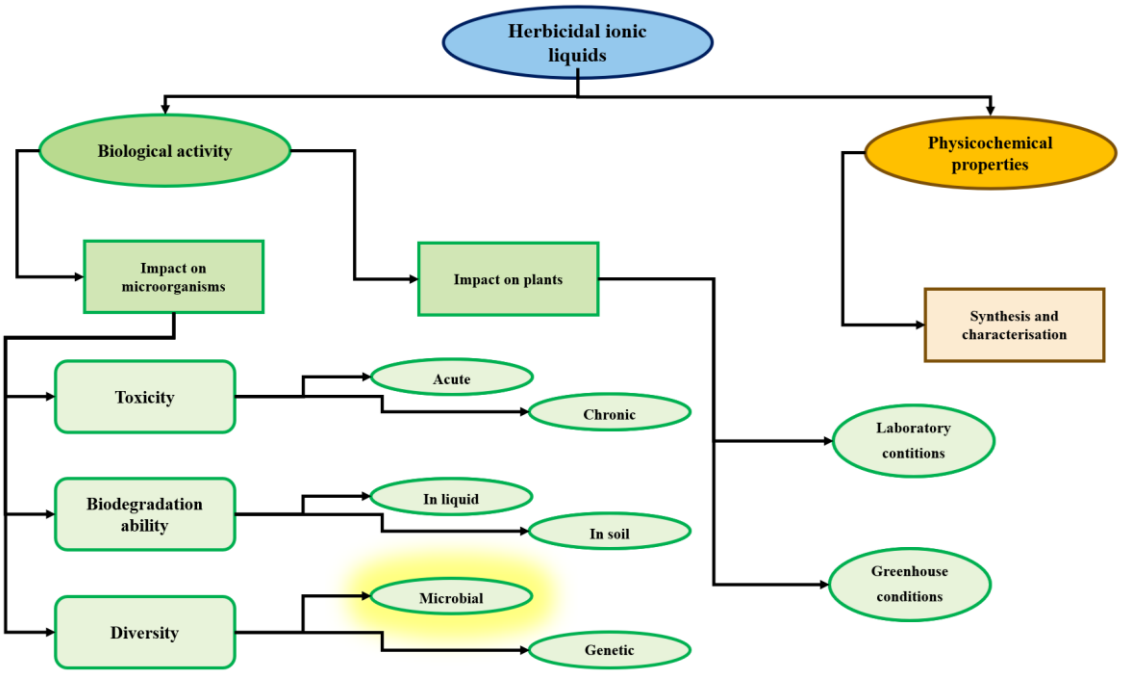
Subsequent evaluation of residues left after the biodegradation study showed that the herbicidal anion was efficiently degraded in all samples, with the lowest removal value of 89% for bioaugmented pure herbicide and the highest at almost 94% for bioaugmented [Chol][ISM]. Hence, bioaugmentation does not seem to have

a significant effect on the biodegradation extent of the herbicidal anion in the soil matrix in the long run. Interestingly, despite the fact that half-lives values recorded for the examined compounds are very similar to those obtained for *B. subtilis* in liquid matrix (tested against [Na][ISM]), both the negative and positive impacts of employed cations which was observed in liquid medium, disappeared in soil assay. These results stand in contrast to previous observations, where cations were generally crucial factor impacting negatively the ability of microbial community to biotransform herbicidal anions [61,62]. However, this might have been caused either by involvement of other microorganisms (such as fungi) that are more resilient in biodegradation process [217], or the fact that enzymes taking part in biodegradation of sulfonylureas are often extracellular [84,85,188,203,218,219]. Another possibility is that microbial degradation of ISM was enhanced by processes such as hydrolysis [216], which might resulted in similar efficiencies of deterioration in this study.

The degradation susceptibility of cations varied among tested HILs, but no significant impact of bioaugmentation could be observed. Data demonstrated that the degradation efficiency of the choline cation reached approx. 99% in bioaugmented and non-bioaugmented samples, while [C_{12}Chol] cation was degraded at 89% in non-bioaugmented samples and at 87% in bioaugmented samples. Although, this might suggest that native microbes present in soil are proficient in degrading cations employed in this study, contrary to the bacteria utilised in liquid medium degradation assay. Additionally, one must take into account that said cations belong to quaternary ammonium salts and thus are prone to sorption to soil particles [75,128]. The additional studies on sorption and bioavailability (**Tables S1-S2**) showed that the ISM anion was not sorbed regardless of the compound used. In contrast, the sorption of choline cations increased with increasing hydrophobicity and, as for other studied HILs [61,62,109,111], the presence of ISM in the structure had no effect. Consequently, this phenomenon is limiting the biological availability of this type of compounds and is leading to their bioaccumulation in soil, which was also observed for ILs with an ISM anion [75]. The bioavailability results presented in **Table S2** confirm the ease of desorption of the ISM anion and the decrease in bioavailability of choline cations with increasing hydrophobicity. In fact, these conjectures on the behaviour of HILs' cations in the soil environment have been already confirmed by other studies [67,110,111]. Hence, more research is needed to determine the full extent of negative effects of cations employed in HIL-type compounds on the microbiome

and in particular to examine the possibility of release into the environment of cations previously adsorbed to soil particles.

4.8. Impact of HILs on the microbiome



4.8.1. Impact of iodosulfuron-methyl based HILs on soil microbiome

The health of soil is fundamental to maintaining the health of farmed crops [66], and thus the impact of plant protection agents should be evaluated in respect of their impact on the soil microcosm. Hence, ISM based HILs were assessed in this regard, moreover not only initial changes were examined, but also the changes of soil microbiome over the course of 3 months after the application of tested compounds (**Fig. 20)**.

In all cases the core of microbiome was formed by *Actinobacteriota*, *Proteobacteria* and *Bacteroidota*, which is to be expected [220]. The impact of bioaugmentation on initial microcosm structure compared to the native soil microbiome (**Fig. S25**) can be clearly seen, but more importantly, it is still evident after 7 days, as *Firmicutes* abundance is significantly more pronounced in all of HILs and herbicide treated bioaugmented samples (1.95%, 1.21%, 10.43%, and 3.25% for [Chol][ISM], [C_6Chol][ISM], [C_{12}Chol][JS-M], [Na][ISM], respectively) than in those not supplemented with *B. subtilis* (1.11%, 0.92%, 2.60% and 1.54% for [Chol][ISM],

[C_6Chol][ISM], [C_{12}Chol][ISM], [Na][ISM], respectively). Interestingly, the addition of HILs and iodosulfuron-methyl plausibly have promoted the proliferation of *Firmicutes* in non-bioaugmented samples, as it can be observed that in biotic control this group of microbes amount to 0.51% of the total microbiome composition.

Remarkably, after 28 days the trend regarding *Firmicutes* remained the same, *i.e.*, these bacteria were more abundant in bioaugmented samples. Their occurrence in non-bioaugmented samples remained relatively stable, contrary to the samples bioaugmented, where their abundance in microbiome increased significantly in [C_6Chol][ISM] and [Na][ISM] up to 7.67% and 7.27% respectively, increased slightly up to 2.68% in [Chol][ISM] and decreased to 5.04% in case of [C_{12}Chol][ISM]. Yet, the more pronounced change was the significant increase of *Proteobacteria* in all samples treated with examined compounds, while the contribution of this bacteria to microbiome structure remained at similar levels in biotic controls. This might be caused by the fact that *Proteobacteria* phylum contain many species, such as *Pseudomonas putida*, which are well known as resilient and capable of degrading various xenobiotics [221,222].

The changes induced by HILs and ISM seem to be lasting, as after another 60 day period, microbiomes of bioaugmented and non-bioaugmented treated samples were more similar to each other than to either of biotic controls. The dominance of *Proteobacteria* was sustained in either of treatments similarly to *Actinobacteriota*. Intriguingly, in the case of non-bioaugmented samples elevated levels of *Firmicutes* could be observed when compared to previous measurements. Additionally, their highest abundance could be found in [C_{12}Chol][ISM], while lowest in [Chol][ISM], reaching values similar to bioaugmented samples after 7 days. Hence, it could be speculated, that the difference in the amount of carbon dioxide released in the mineralization experiment (or rather its faster release) in bioaugmented samples could be attributed to changes in the soil microbiome.

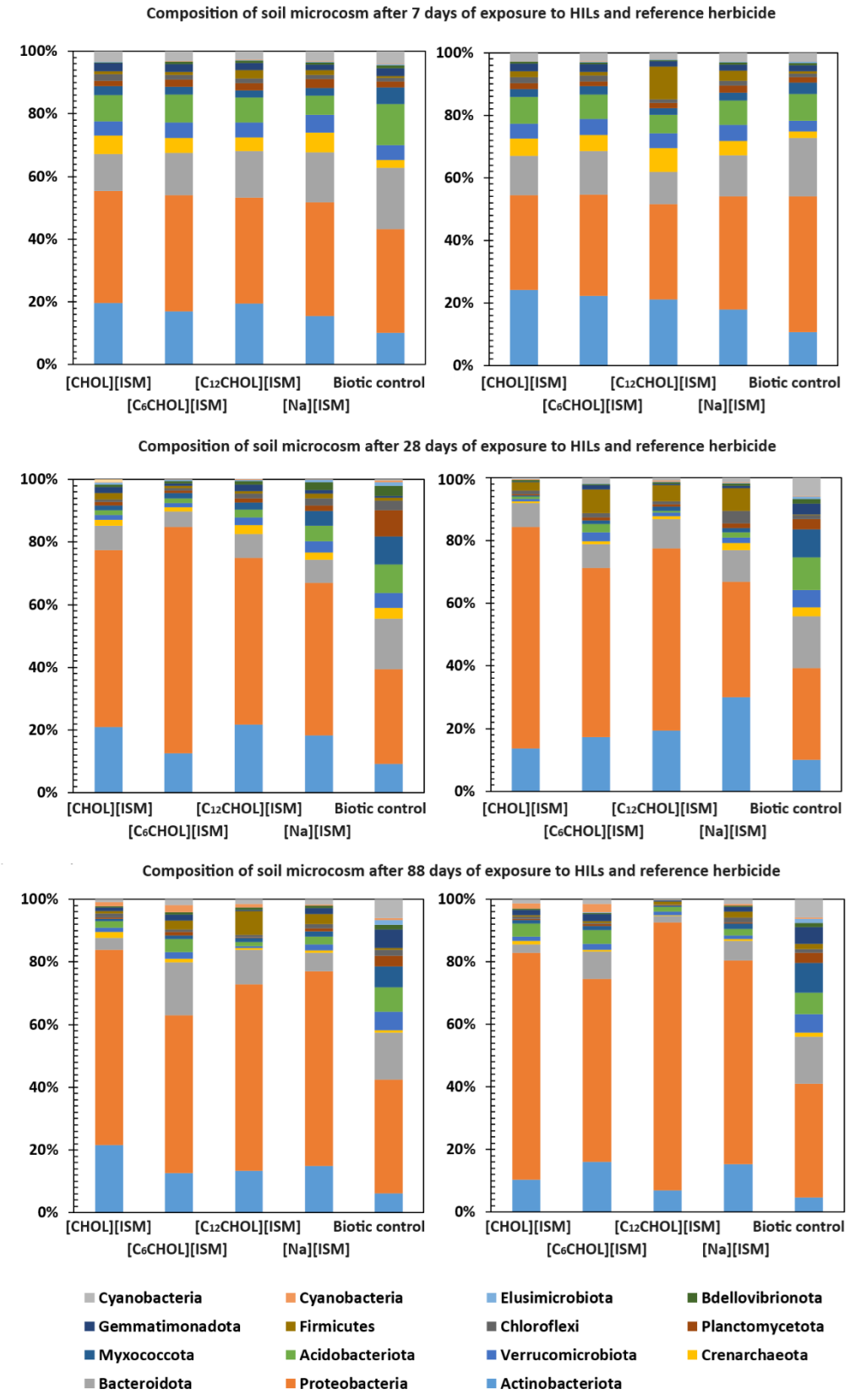


Figure 20. Shifts in microbiome composition over time in non-bioaugmented system (left column) and bioaugmented system (right column).

Analysis of biodiversity in the studied samples confirmed that the treatments reduced the diversity of microcosm. The values of the Shannon diversity indices can be arranged in the following order: Control sample > [Na][ISM] > [Chol][ISM] = [C_6Chol][ISM] > [C_{12}Chol][ISM] (**Fig. S26**). The following trend was established in case of Simpson's diversity index: Control sample > [Chol][ISM] > [Na][ISM] = [C_6Chol][ISM] > [C_{12}Chol][ISM] (**Fig. S27**). In both cases, the lowest biodiversity values were observed in systems treated with [C_{12}Chol][ISM], which further supports the hypothesis regarding its most notable impact on soil microorganisms. It should be highlighted that for all tested samples, bioaugmentation did not contributed to statistically significant differences relative to the respective non-bioaugmented systems.

4.8.2. Impact of iodosulfuron-methyl based HILs on the microbiome of *C. cyanus* L.

4.8.2.1. Root surfaces

The analysis of the root surface microbiome in cornflower (**Fig. 21**) revealed *Proteobacteria* as the dominant type in all examined plants, with a relative abundance exceeding 90% in both biotypes. This corresponds well with the fact that *Proteobacteria* is among the most abundant and diverse bacterial types [110]. Notably, certain members of this group, such as *Pseudomonas putida*, are known for their significant ability to break down xenobiotics [221,222], which might translate into their relatively high presence in soil treated with herbicides. The second most prevalent type, *Firmicutes*, varied from 0.2% to 9.0%, with its lowest relative abundance observed in the root epiphytes of susceptible cornflower treated with sterile water. Interestingly, comparable levels of abundance were noted in samples from both resistant (R) and susceptible (S) biotypes treated with herbicides and the HIL, similar to control samples from the resistant biotype. This finding is particularly interesting, considering the recognized significance of *Firmicutes* in agroecology [223]. Namely, their ability to regulate plant hormonal production or to produce analogues of plant hormones might aid plants in dealing with herbicidal stress, potentially bolstering their resistance [223].

Contributions from other bacterial types did not surpass 0.05%. The predominant bacterial classes in this environment were *Gammaproteobacteria*,

Alphaproteobacteria, *Betaproteobacteria*, and *Bacilli*, hinting at the potential presence of strains capable of degrading sulfonylurea, such as *Hanshlegiella zhihuiae* and *Bacillus subtilis* in the examined samples. The likelihood of such presence is increased by the fact that these strains were initially isolated from farmland soil contaminated with sulfonylurea herbicides, specifically chlorimuron-ethyl [153] and nicosulfuron [208].

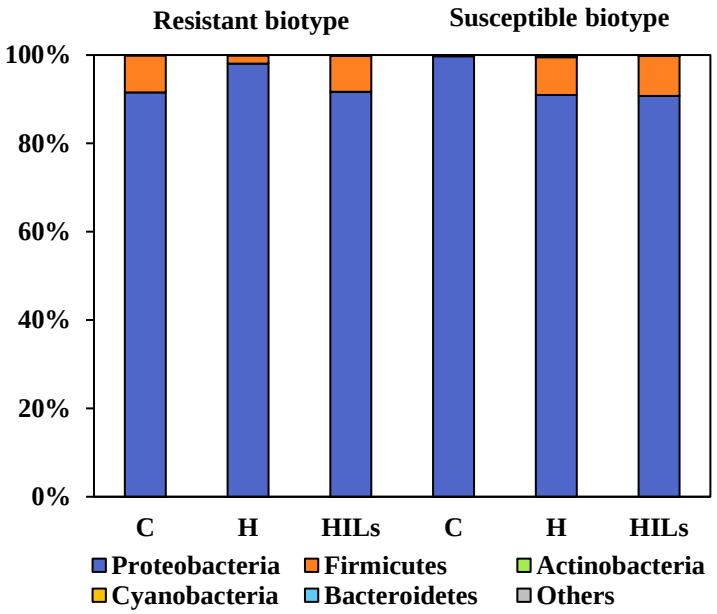


Figure 21. Analysis of the root surface microbiome composition of susceptible and herbicide-resistant cornflower. C – untreated control; H – herbicide treatment; HILs – herbicidal ionic liquid treatment. Adapted and modified from author's published work [119].

4.8.2.2. Root inner tissues

The examination of endophytes in cornflowers, both in untreated controls and plants subjected to herbicide treatment, did not reveal any significant differences (**Fig. 22**). *Proteobacteria* was the prevalent type in all examined plant tissues, contributing to between 92% and 99%. Herbicide-treated susceptible weeds displayed elevated levels of *Proteobacteria* in the internal root tissue (99.6%). However, in herbicide-resistant cornflowers treated with HIL, the contribution of *Proteobacteria* in endophytes decreased to 45%, with *Firmicutes* emerging as the dominant type at 54%. This shift is likely attributed to the toxicity of HILs, being dictated by the cation's toxicity, and the susceptibility of bacteria being influenced by their cell wall structure [49,61,62,78]. Consequently, it is probable that the endophyte population was more impacted by the

cation present in the HILs molecule than by the herbicide alone. Untreated plants microbiome exhibited also the presence of *Actinobacteria*, ranging from 3.8% to 5.4%, which was not observed in any of the herbicide-treated plants. This underscores the significant impact of modern agricultural practices on the native microbiome, as *Actinobacteria* are widely acknowledged for their substantial contributions to plant growth stimulation, plant survival, and soil management [224,225].

At the class level, the predominant classes included *Alphaproteobacteria*, *Gammaproteobacteria*, *Actinobacteria*, and *Bacilli*. The noticeable increase in the contribution of the *Bacilli* class was particularly evident in the internal root tissue of herbicide-resistant plants treated with HILs, comprising 55% of the overall population, which is particularly interesting in light of prior research by Stachowiak et al. (2021), which demonstrated that *Bacillus cereus* (a member of the *Bacilli* class) is generally more sensitive to ionic forms of iodosulphuron-methyl than *Pseudomonas putida* (a member of the *Gammaproteobacteria* class) [49].

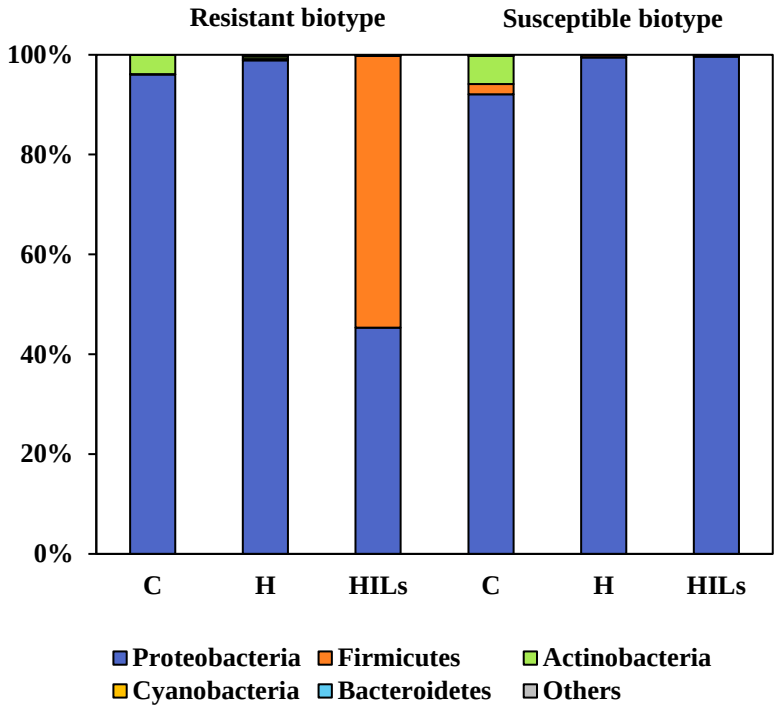


Figure 22. Analysis of the inner root tissue microbiome composition of susceptible and herbicide-resistant cornflower. C – untreated control; H – herbicide treatment; HIL – herbicidal ionic liquid treatment. Adapted and modified from author's published work [119].

4.8.2.3. Shoot surfaces

The analysis of the shoot surface microbiomes of untreated sensitive and resistant biotypes exhibited no significant distinctions between them (**Fig. 23**), with *Firmicutes* being predominant (90–92.5%). However, herbicide and HIL treatments caused substantial changes in the shoot surface microbiome for both plant types. These changes varied between S and R biotypes, suggesting a potential role of the microbiome in the plant's resistance to the herbicide. In resistant populations treated with the herbicide alone, the contribution of *Firmicutes* decreased to 61%, with *Proteobacteria* constituting the remaining 39%. Conversely, the shoot surface microbiome of susceptible cornflower treated with the herbicide was dominated by *Proteobacteria*, accounting for 99.7% of the microbiome. This dominance, surpassing 98%, was also observed in both S and R populations after the application of HILs.

It is important to note that the phyllosphere microbiome composition is more dynamic compared to rhizosphere and endosphere environments [226]. Microbial inhabitants in the phyllosphere experience fluctuations in temperature, moisture, and sunlight exposure throughout the day and seasons, especially due to herbicidal spraying. These environmental factors also influence plant functions such as photosynthesis, respiration, and water uptake, indirectly shaping the composition of the microbiome. Overall, these results indicate that the toxicity of HILs has a more pronounced impact on the microbiome compared to the reference herbicide, similar to the effects observed in the inner root tissue experiment.

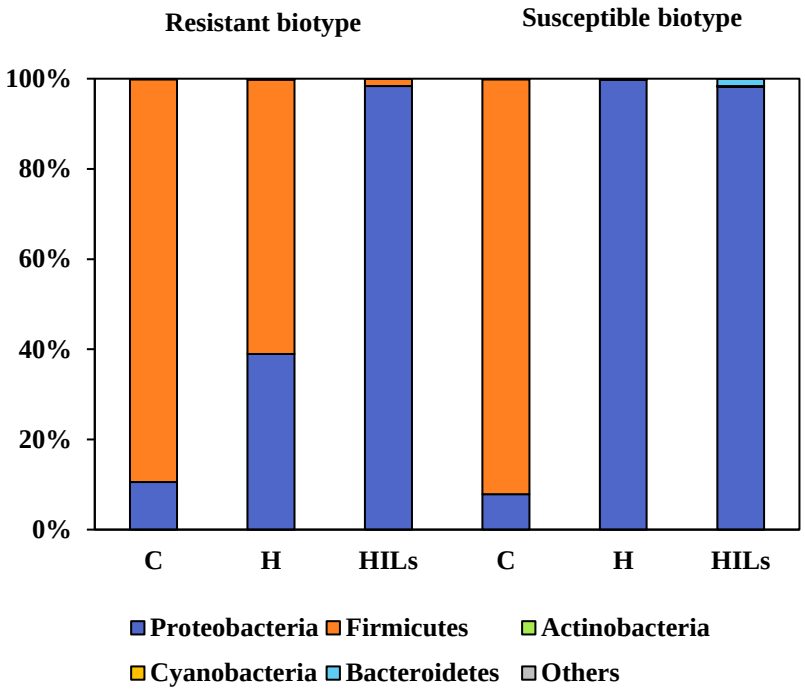


Figure 23. Analysis of the shoot surface microbiome composition of susceptible and herbicide-resistant cornflower. C– untreated control; H – herbicide treatment; HIL – herbicidal ionic liquid treatment. Adapted and modified from author's published work [119].

4.8.2.4. Shoot inner tissues

The analysis of the microbiome in both untreated sensitive and resistant biotypes, subjected to treatment with sterile water, revealed noteworthy alterations in their microbiomes (**Fig. 24**). Herbicide-resistant plants demonstrated a prevalence of *Firmicutes* bacteria, particularly the *Bacilli* class (99%), while herbicide-susceptible plants exhibited dominance by *Proteobacteria* (96%), with the *Gammaproteobacteria* class making up 91% of the microbial composition. Application of the herbicide did not significantly alter the microbiome of the sensitive populations. However, in resistant populations, the bacterial community structure shifted markedly, resembling that of herbicide-susceptible biotypes, with the exclusive presence of *Cyanobacteria* in the resistant biotypes. This observation further implies that in the studied plants, the microbiome may contribute to the resistance of weeds to herbicides, particularly given the known role of *Cyanobacteria* in enhancing stress tolerance [227].

The introduction of herbicide in the form of HIL led to distinct changes in the endophytes composition of both populations. *Actinobacteria* became dominant in both sensitive (56%) and resistant (73%) weeds. In the sensitive population, *Proteobacteria*

was the second dominant type (43%), while in the resistant population, it was *Firmicutes* (25%). At the class level, *Actinobacteria* (56%) and *Alphaproteobacteria* (43%) were predominant in sensitive plants, whereas in the resistant biotype, *Actinobacteria* accounted for 73%, and *Bacilli* for 25%. It is evident that the HIL significantly influenced the composition of the microbiome in the examined plants. Specifically, while the core microbiome remained consistent with untreated plants, the ionic liquid treatment notably increased the population of *Actinobacteria*. This indicates that HILs can cause substantial shifts in microbial communities, potentially affecting plant health and resistance mechanisms.

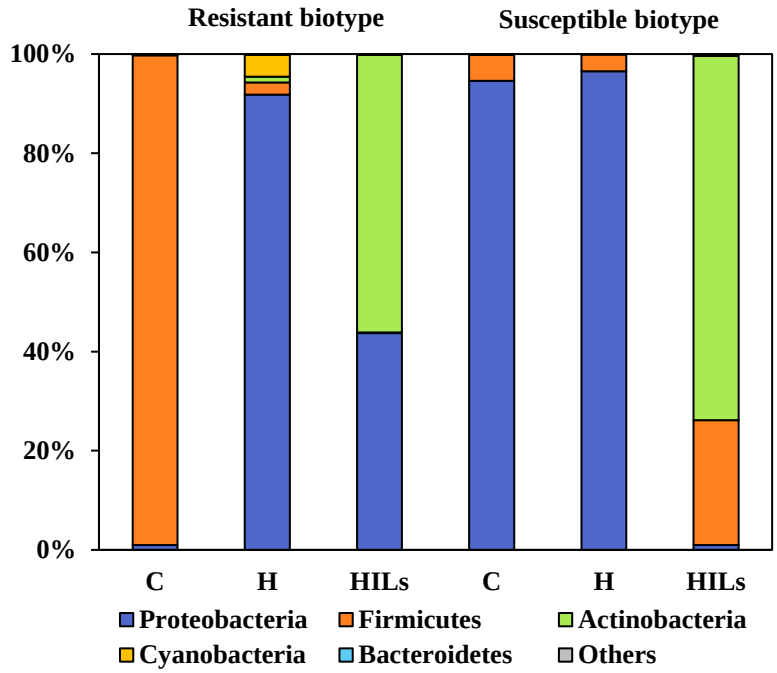


Figure 24. Analysis of the inner shoot tissue composition of microbiome of susceptible and herbicide-resistant cornflower. C – untreated control; H – herbicide treatment; HIL– herbicidal ionic liquid treatment. Adapted and modified from author's published work [119].

4.8.2.5. Statistical parameters of microbiomes

The examination of diversity parameters in bacterial communities revealed several significant patterns in plant microbiomes (**Table 15**). Firstly, it was noted that the root microbiome displayed higher biodiversity compared to the shoot microbiome. Moreover, the biodiversity of microorganisms on the surfaces of shoots and roots exceeded that within their respective tissues. In the case of the phyllosphere, this phenomenon rises from the relatively low nutrient content on leaves surfaces

compared to the rhizosphere and endosphere. Additionally, natural fluctuations in heat, moisture, and sunlight exposure throughout the day and seasons contribute to lower biodiversity [226]. Furthermore, it was demonstrated that host plant genetic control of the microbiome is evident in leaves but not in roots [228]. Consequently, in this study, root endophyte biodiversity was more pronounced than in the case of shoots.

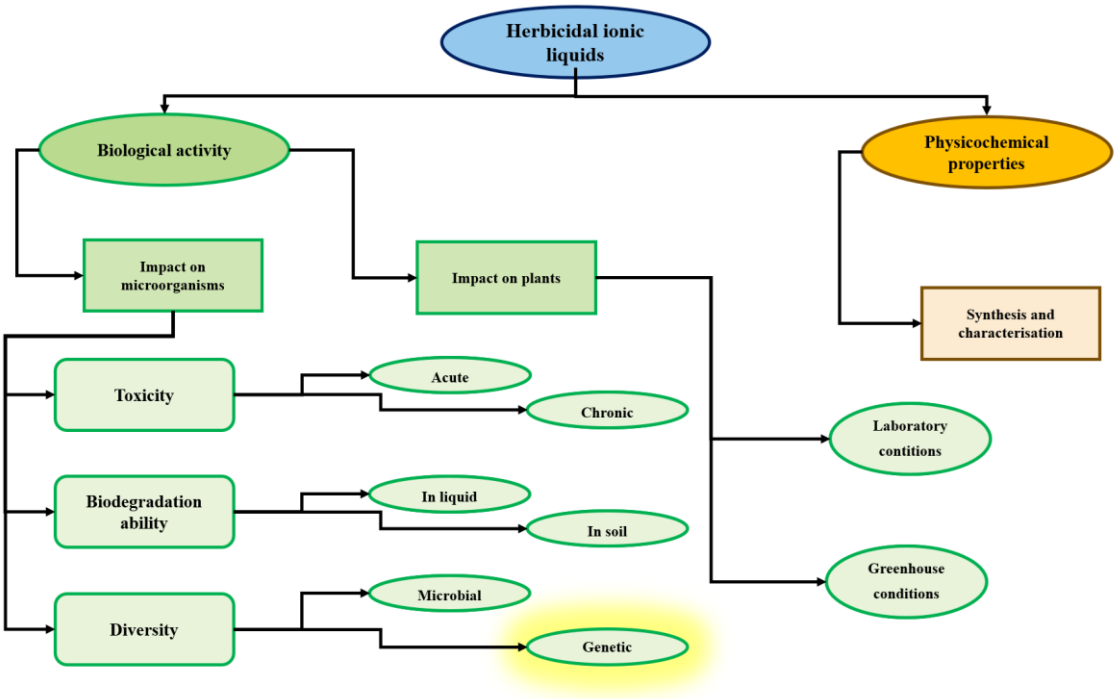
Generally, the microbiome of untreated plants exhibited greater biodiversity than that of plants subjected to herbicidal treatment. This outcome aligns with increasing awareness of the impact of herbicides on the soil microbiome, as alterations in the soil microbiome are known to affect crucial nutrient cycling and processes between plants and soil [229]. However, no significant differences were observed in the root epiphyte microbiome of both susceptible and resistant weeds. In contrast, distinct variations were noticed in the shoot epiphyte microbiome in both plant types, likely due to the specific properties of the phyllosphere mentioned above [226]. Overall, the study highlights the complex interactions between plant microbiomes and herbicide treatments, emphasizing the importance of understanding these dynamics for sustainable agricultural practices.

Table 15. Diversity metrics of the microbiome extracted from the roots, shoots, and foliage of cornflower plants belonging to both susceptible and resistant biotypes. Adapted and modified from author's published work [119].

		Biodiversity	Susceptible biotype			Resistant biotype		
			Control	Herbicide	HILs	Control	Herbicide	HILs
Roots	Epiphytes	OTU	2039	1211	1511	1816	1383	1509
		Chao1Bias	2639	1372	1613	2146	1284	2024
		Shannon Index	6.15	5.67	5.75	5.93	5.79	5.69
		Phylogenetic diversity	5.32	4.92	4.90	5.21	5.06	5.11
	Endophytes	OTU	1576	992	1087	1302	940	1312
		Chao1Bias	1860	1325	1422	1525	1284	1470
		Shannon Index	5.76	4.29	5.21	4.60	4.34	4.59
		Phylogenetic diversity	6.04	5.97	5.58	6.19	6.28	5.51

Shots and foliage	Epiphytes	OTU	1702	1740	1426	1562	994	1462
		Chao1Bias	1784	2217	1608	1320	1175	1845
		Shannon Index	5.99	5.82	5.23	5.87	3.33	5.23
		Phylogenetic diversity	4.24	4.78	4.42	4.92	4.96	5.04
	Endophytes	OTU	1445	938	813	1427	837	518
		Chao1Bias	1613	1029	899	1544	887	581
		Shannon Index	5.02	4.84	3.88	5.44	4.70	4.22
		Phylogenetic diversity	6.28	6.27	5.79	5.27	4.30	5.91

4.9. Impact of iodosulfuron-methyl based HILs on presence and abundance of catabolic genes in the environment



4.9.1. Gene presence in soil and rhizosphere

Microbial activity is playing very important role in degradation of sulfonylurea-based herbicides [130,216,230,231]. Some of the enzymes involved in catabolism of such xenobiotics are well established, such as cytochrome monooxygenases [232,233], some had their activity in herbicide transformation recently uncovered, such as vegetative catalase, acetoin dehydrogenase and manganese ABC transporter discovered in *B. subtilis* YB1 [84,85,208,234]. Yet, most fascinatingly, only one

enzyme in *H. zhihuaiae* was determined to be involved exclusively in degradation of sulfonylurea herbicides [117,153,235]. Therefore, due to the available information regarding genetic composition of said bacteria, these enzymes were selected in order to asses the impact of HILs on gene abundance of catabolic genes in the environment. All the examined genes that code the enzymes discussed, with the exception of the vegetative catalase from *B. subtilis*, were identified in samples obtained from the soil (**Table 16**). The genes encoding acetoin dehydrogenase showed no clear pattern, as both primers targeting this enzyme's gene were present at similar rates across soil samples from both susceptible and resistant cultivars. Notably, the manganese ABC transporter, the cytochrome P450 enzyme genes were more prevalent in the soil associated with the resistant biotype. For the gene that encodes the sulfonylurea deestrification estrase SulE, it was predominantly found in samples from both cultivars exposed to HIL, rather than those treated with conventional herbicide, although it was also detectable in the soil of untreated resistant weeds.

In the rhizosphere, a consistent trend was observed – a decrease in the presence of genes originating from *B. subtilis* in control samples and those subjected to herbicidal treatment. Despite this decrease, genes encoding *B. subtilis* enzymes and vegetative catalase were still present in the susceptible population. An increase in the presence of both P450 SU-1 and SulE encoding genes was noted, which is particularly intriguing for the latter, given that *H. zhihuaiae* can infiltrate plant tissues and persist as an endophyte [153].

Table 16. A heat map illustrates the frequency of genes linked to ISM biotransformation in microbiomes obtained from both soil and rhizosphere. Dark blue indicates the gene was present in all three biological replicates, blue denotes presence in two biological replicates, light blue signifies presence in one biological replicate, and the absence of colour indicates that no gene was detected. Adapted and modified from author's published work [119].

	Enzyme	**Primer**	**Resistant biotype**			**Susceptible biotype**		
			control	**herbicide**	**HILs**	**control**	**herbicide**	**HILs**
Soil	vegetative catalase 1	Veg-1						
		Veg-2						
	acetoin dehydrogenase E1	AdE-1						
		AdE-2						
	manganese ABC transporter	mABCt						
	Cytochrome P-450$_{SU-1}$ monooxygenases	P450-SU-1						
	SulE	SulE-1						

Rhizosphere	vegetative catalase 1	Veg-1						
		Veg-2						
	acetoin dehydrogenase E1	AdE-1						
		AdE-2						
	manganese ABC transporter	mABCt						
	Cytochrome P-450$_{SU-1}$ monooxygenases	P450-SU-1						
	SulE	SulE-1						

4.9.1.1. Gene presence in root epiphytes and endophytes

In general, the quantity of gene copies in the root samples of both cornflower populations is lower compared to the rhizosphere (**Table 17**). Notably, the susceptible cultivar shows a higher number of examined gene copies than the resistant one. The presence of SulE genes is particularly significant, as it was present in both epi- and endophytes of the susceptible cultivar treated with HILs, and in epiphytes when exposed to commercial herbicide treatment. Conversely, the SulE genes were only detected in the epiphytes of control samples from the resistant cultivar. This finding aligns with Zhang et al. (2018), who reported that *H. zhihuaiae* can biotransform and detoxify sulfonylurea herbicides in the plant rhizosphere [153]. Furthermore, primers targeting the SulE gene are used as a tool-marker for isolating new strains of *Hanschlegiella* bacteria [236], suggesting potential for discovering a novel strain capable of degrading sulfonylurea herbicides.

Interestingly, both the acetoin dehydrogenase-encoding gene and the gene encoding manganese ABC transporter were present in samples isolated from both epiphytes and endophytes in roots of both susceptible and resistant cornflower populations. However, vegetative catalase was found more frequently in the susceptible biotype than in resistant one. In addition, the gene encoding the cytochrome P450 enzyme was exclusively identified in the susceptible population treated with HILs. This specificity highlights the potential role of these genes in the differential response of cornflower populations to herbicide treatment and suggests that HILs may have a more pronounced impact on the microbial community associated with the susceptible biotype.

Table 17. A heat map illustrates the frequency of genes linked to ISM biotransformation in microbiomes obtained from root surface and root tissue. Dark blue indicates the gene was present in all three biological replicates, blue denotes presence in two biological replicates, light blue signifies presence in one biological replicate, and the absence of colour indicates that no gene was detected. Adapted and modified from author's published work [119].

	Enzyme	Primer	Resistant biotype			Susceptible biotype		
			control	herbicide	HILs	control	herbicide	HILs
Epiphytes	vegetative catalase 1	Veg-1						
		Veg-2						
	acetoin dehydrogenase E1	AdE-1						
		AdE-2						
	manganese ABC transporter	mABCt						
	Cytochrome P-450$_{SU-1}$ monooxygenases	P450-SU-1						
	SulE	SulE-1						
Endophytes	vegetative catalase 1	Veg-1						
		Veg-2						
	acetoin dehydrogenase E1	AdE-1						
		AdE-2						
	manganese ABC transporter	mABCt						
	Cytochrome P-450$_{SU-1}$ monooxygenases	P450-SU-1						
	SulE	SulE-1						

4.9.1.2. Gene presence in shoot epiphytes and endophytes

The analysis of samples obtained from shoots and foliage revealed notable patterns in the presence of genes involved in sulfonylurea degradation (**Table 18**). Specifically, these genes were less prevalent in the S biotype when compared to the R biotypes. Genes encoding vegetative catalase, acetoin dehydrogenase, manganese ABC transporter, and sulfonylurea esterase were detected in epiphytic samples from both susceptible and resistant populations treated with the herbicide. However, following treatment with HILs, these genes were exclusively detected in resistant plants.

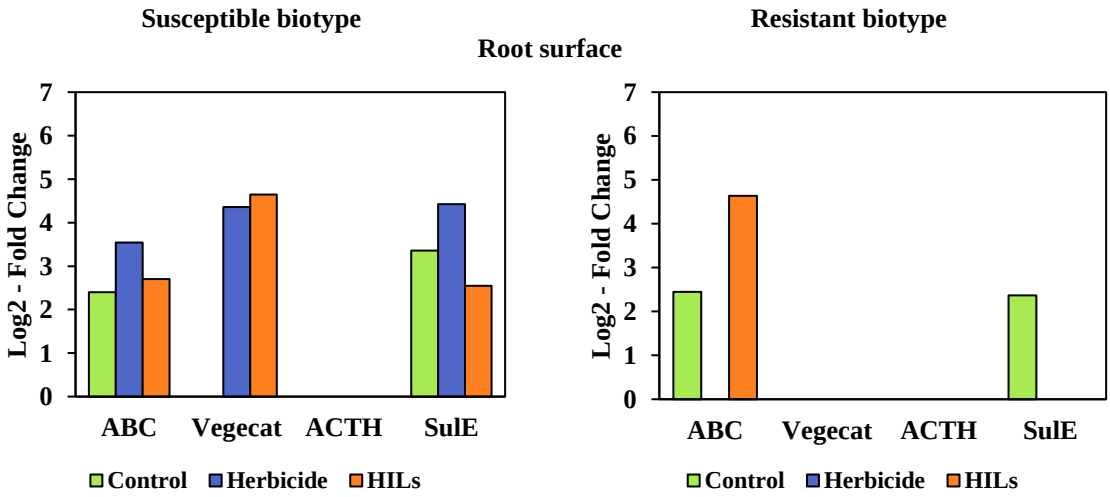
In the endophytic samples, all genes encoding enzymes providing resistance to sulfonylureas were identified in the resistant biotype, while only SulE and P450 genes were detected in the susceptible biotype. Notably, the acetoin dehydrogenase gene was present in the resistant cornflower treated with HILs but was absent in the same population treated solely with the herbicide. This suggests that the HILs treatment may influence the gene expression patterns related to sulfonylurea degradation differently than the herbicide alone, particularly in the resistant biotype.

Table 18. A heat map illustrates the frequency of genes linked to ISM biotransformation in microbiomes obtained from shoots and foliage. Dark blue indicates the gene was present in all three biological replicates, blue denotes presence in two biological replicates, light blue signifies presence in one biological replicate, and the absence of colour indicates that no gene was detected. Adapted and modified from author's published work [119].

	Enzyme	**Primer**	**Resistant biotype**			**Susceptible biotype**		
			control	**herbicide**	**HILs**	**control**	**herbicide**	**HILs**
Epiphytes	vegetative catalase 1	Veg-1						
		Veg-2						
	acetoin dehydrogenase E1	AdE-1						
		AdE-2						
	manganese ABC transporter	mABCt						
	Cytochrome P-450$_{SU-1}$ monooxygenases	P450-SU-1						
	SulE	SulE-1						
Endophytes	vegetative catalase 1	Veg-1						
		Veg-2						
	acetoin dehydrogenase E1	AdE-1						
		AdE-2						
	manganese ABC transporter	mABCt						
	Cytochrome P-450$_{SU-1}$ monooxygenases	P450-SU-1						
	SulE	SulE-1						

4.9.2. Gene abundance

The examination of gene presence in environmental samples has revealed a crucial insight regarding genes encoding enzymes that facilitate bacterial degradation of sulfonylureas. Namely, these genes were more prevalent than initially anticipated, particularly present in soil and the rhizosphere. Their occurrence was slightly higher in samples where the resistant biotype of cornflower was present. A similar pattern was observed in samples from shoots and foliage, whereas no clear trend was discernible in root samples. Furthermore, the impact of HILs and herbicides on gene frequency remains inconclusive. To address this, the RT-qPCR technique was employed to assess the abundance of these genes in the microbiome of specific niches, using the 16S RNA gene as a reference. Notably, certain genes identified in these samples could not be efficiently quantified *via* RT-qPCR (**Fig. 25**, **Table S3**). This suggests that while the genes were present, their abundance was extremely low. Consequently, their significant impact on the potential detoxification of ISM and ISM-based HILs in the environment is relatively low.



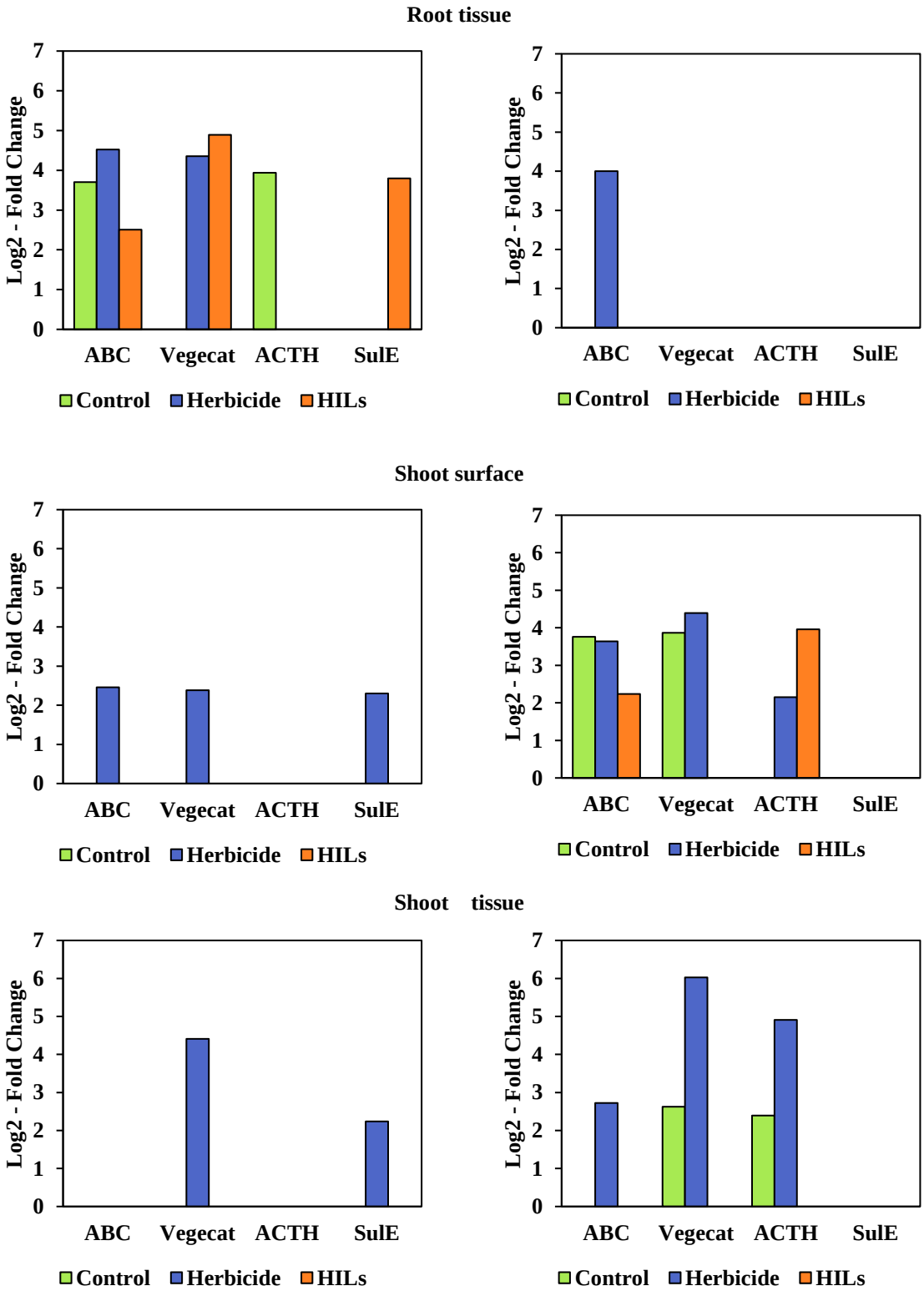


Figure 25. Log2-fold change values obtained through real-time PCR analysis of ABC, Vegecat, ACTH, and SulE genes in samples derived from both resistant and susceptible cultivars of cornflower. Adapted and modified from author's published work [119]

The analysis of gene enrichment on the root surface of cornflower biotypes revealed a notable difference between the susceptible and resistant plants. The susceptible biotype's root surface showed greater enrichment of genes involved in sulfonylurea degradation compared to the resistant biotype. This could be attributed to the resistant plants' inherent ability to metabolise sulfonylureas independently. In contrast, susceptible plants, which lack these molecular mechanisms, rely more on specialised bacteria with the necessary degradation genes to manage the presence of xenobiotics [237]. Unfortunately, the acetoin dehydrogenase gene was not detected in either weed biotype, and the vegetative catalase gene was absent in resistant cornflower. However, vegetative catalase was present in the susceptible population, with slightly higher gene numbers in HIL-treated plants compared to herbicide-treated plants. This could be due to the higher *Firmicutes* content in the microbiome of HIL-treated plants. Interestingly, the vegetative catalase gene could not be quantified in control plants despite a similar abundance of *Firmicutes* in HIL-treated plants. In addition, genes encoding the manganese ABC transporter were found in all treatments of sensitive cornflower, with up to a 2-fold higher abundance in HIL-treated resistant biotype. For the SulE deesterification esterase genes, they were present in all treatments of the sensitive biotype, with the highest abundance in herbicide-treated samples. These findings suggest that while determining the impact of herbicide form (ionic liquid or pure herbicide) on the abundance of genes encoding enzymes degrading sulfonylureas is challenging, a clear trend indicates that these genes are more frequent in the microbiome of treated plants compared to control plants.

In the inner parts of roots, only the manganese ABC transporter gene was quantifiable in the resistant population, suggesting that other genes were too low in abundance to be detected despite being identified in previous analyses. Interestingly, vegetative catalase was detected at similar levels in the roots of susceptible plants as in the rhizosphere, despite significant shifts in the microbial community composition. This was even more evident for manganese ABC transporter, which was least abundant in HIL-treated plants, regardless of *Firmicutes* making up 54% of the community. In contrast, it was 2-fold higher in herbicide-treated plants, where *Firmicutes* constituted less than 5% of the isolated microbiome.

In the shoot surface and inner tissues of the S biotype, gene quantification was similar to the root tissue of the R biotype, with quantifiable genes only in herbicidal treatment. This suggests an extreme scarcity of these genes in the microcosm, possibly due to

HIL toxicity impairing bacterial growth in these tissues. However, significantly higher copies number of genes encoding vegetative catalase and acetoin dehydrogenase were detected in resistant weeds after herbicide application compared to the untreated population, suggesting that bacteria may aid in detoxifying the herbicide. The absence of these genes in HIL-treated samples indicates that cation toxicity impacts the microbiome of the inner tissue as well, thus possibly aiding in the overcoming of herbicidal resistance in the short term. Yet, in scope of toxicity of examined cations and their poor biodegradability, their constant presence in the environment would certainly put additional pressure on the microbiome to attain features necessary for survival. Therefore, recent trends in using HILs as potential tools for controlling herbicide-resistant weeds [124,125], might encourage development of herbicidal resistance, rather than fight it.

5. Summary and discussion

Contemporary agriculture demands agrochemicals with high herbicidal efficacy, cost-efficiency, minimal environmental impact, short-term biodegradation, and novel modes of action to counter resistance development [238–241]. Hence, ILs with herbicidal properties have been explored as a potential solution, offering benefits such as reduced soil mobility of the active ingredient, lower application doses, and high herbicidal effectiveness [34]. **Though, despite their potential advantages, these compounds are still understudied when it comes to their behaviour in the environment [78,144]**, as shown in author's already published works. These observations were a starting point for the studies presented within this Ph.D. thesis. Therefore, a comprehensive assessment of the environmental impact of HILs on non-target organisms, including soil fungi and bacteria, as well as weeds and crop plants, was performed [119,129].

The synthesis of HILs incorporating MCPA and MCPP or ISM showed that transforming herbicides into ionic forms indeed reduced the volatility of active ingredients [78,119]. Hence, they were chosen for further experiments on plants, where it was proven that this application form allows for their high efficacy against weeds. Moreover, the combination of MCPA and MCPP into an esterquat HILs demonstrated beneficial effects on maize germination, highlighting the multifunctionality of HILs. However, **the studies revealed that the length of alkyl chain of the cation affects significantly the toxicity of these compounds toward plants [78].** Further research explored this issue during trials with plants susceptible and resistant to herbicides.

Herbicide resistance is a serious and growing problem, with over 250 weed species resistant to 23 of 26 herbicidal modes of action [238]. Addressing this issue requires either the development of new active compounds or the use of herbicide mixtures with different modes of action [242,243]. However, **HILs do not offer new mechanisms of action, meaning weeds resistant to traditional herbicides will also resist their HIL derivatives [119]**, as shown by the herbicidal efficiency test with susceptible and resistant cultivars of weed in this study. Even more troubling in the context of herbicide resistance is that compounds belonging to the QASs group, although not acutely toxic to mammals, present significant environmental challenges through chronic exposure [75], including cation-dependent issues associated with toxicity to non-target organisms, negative effects on the microbiome's biodegradation

ability [61,62], substantial changes in the microbiome's structure itself [61,62,67], or even increased horizontal gene transfer and resistance spread [75,77]. These phenomena have been confirmed to be caused by HILs as well, as it has been demonstrated by tests on chronic exposure to dicamba-based HILs [144]. This extend to aquatic ecosystems, where the toxicity of HILs to algae and crustaceans threatens the base of trophic chains [50,244], although no studies have yet assessed bioaccumulation in these organisms.

It was discovered that the chemical structure of the cation in ILs plays a critical role in determining acute and chronic toxicity, with longer alkyl chains correlating with increased harmful effects [144]. In a study where the acute toxicity of dicamba-based HILs was investigated, small, naturally-derived cations like [Chol] and [BetC_2] exhibited significantly lower toxicity, emphasizing the importance of hydrophobicity for the environmental impact of QAS-type compounds. In addition, time-dependent toxicity assays indicated that HILs containing ester bonds could be more environmentally friendly than their fully synthetic counterparts, such as [DDA][Dic], as they undergo hydrolysis into less harmful substances. For instance, [C_{16}Bet][Dic] demonstrated reduced toxicity after 96 h, suggesting that such compounds could decompose after serving as adjuvants, reducing their environmental impact, yet still QAS would be released in large quantities into the environment in such scenario [144].

Having in mind that **exposure of microorganisms to low concentrations of xenobiotics might promote activation of defence mechanisms and therefore increased possibility of horizontal gene transfer**, further studies on the degradation of these compounds, along with the impact assessment on native microbiota, were conducted. **All synthesised compounds demonstrated low biodegradability, likely due to the antimicrobial properties of cationic surfactants used as counterions [78,129].** This clearly demonstrated that the transformation of herbicides into ILs does not mitigate the toxicity of the active substances themselves [15], but might even increase the overall toxicity. Abovementioned adverse effects threaten the soil health and contradict the principles of Integrated Pest Management (IPM), which is critical for sustainable farming.

In degradation studies with iodosulfuron-methyl-based HILs, it was revealed that while bacteria such as *H. zhihuaiae* and *B. subtilis* are capable of effectively degrading ISM, the associated **cations in HIL molecules were resistant to primary**

degradation both in soil and liquid cultures [78,129]. Similarly, fungi isolated from soil exhibited significant potential in degrading pure herbicide, indicating their role in the bioremediation of this type of xenobiotic in the environment. However, cations in HILs limited fungal growth, and the length of the alkyl chain was identified as a primary driver of toxicity. The variability in cation toxicity among fungal species underscores the need to consider fungal tolerance when designing new formulations of weed killers, as well as when selecting fungal species for bioremediation operations [129].

The studies also demonstrated **that the biodiversity of agricultural fields, which is crucial for healthy microbiome, was negatively affected by the long-term exposure to HILs [119].** Moreover, this effect was more pronounced than when commercial herbicides were applied. It was shown that HILs decreased microbiome biodiversity in susceptible and resistant cornflower populations. At the same time, the presence of bacterial genes involved in herbicide degradation in both susceptible and resistant plant tissues indicates that bacteria may assist plants in coping with herbicide-induced stress. **These results suggest that HILs may be more toxic to bacteria than conventional herbicides, with the cation being the likely contributor [119].**

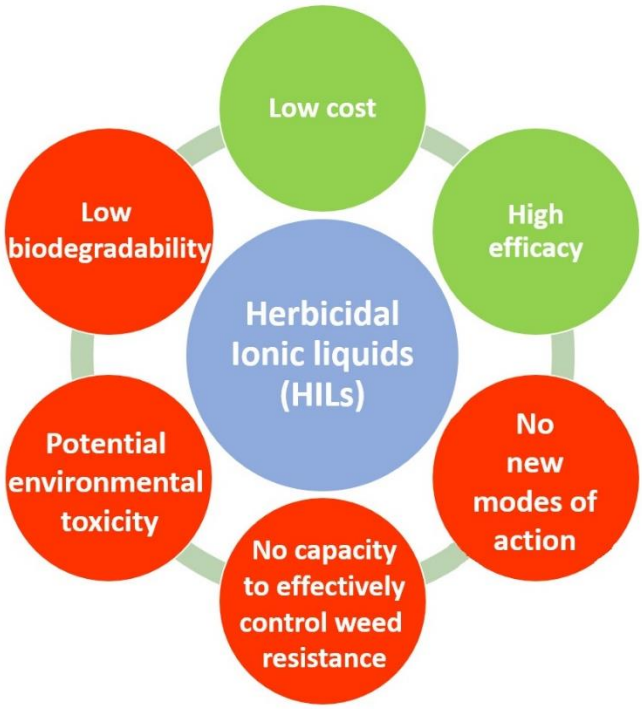


Figure 26. Actual state of completion of goals set for development of herbicides for modern agriculture by HILs. Adapted and modified from author's published work [144].

In conclusion, this study underscores the critical role of cation structure in HIL toxicity and the need for further research to improve the environmental safety of HILs. Although HILs offer potential as herbicidal alternatives incorporating surface-active cations in their structure, their environmental impact remains a concern, particularly regarding their low biodegradability and elevated toxicity. **Namely, the anticipated reduction in active substance doses is outweighed by the environmental risks posed by cationic surfactants currently utilized in HIL structures, including the increased risk of acceleration of herbicide resistance gain.** Future direction of the studies should focus on structural modifications of ionic application forms in order to enhance biodegradability, followed by exploration of the broader applicability of HILs across different crop species, and further investigation of the role of microorganisms in mitigating HIL-associated environmental risks.

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7. Figure captions

8. List of tables

9. Additional scientific activity

Publications resulting from OPUS 15 studies:

- **Jan Homa,** Kosma Konończuk, Robert Frankowski, Agnieszka Zgoła-Grześkowiak, Łukasz Ławniczak, Łukasz Chrzanowski, Witold Stachowiak, Michał Niemczak; *Cations impact the biodegradation of iodosulfuron-methyl herbicidal ionic liquids by fungi*; Environmental Technology; 2024; vol. 2024; s. 1-14; DOI: 10.1080/09593330.2024.2357696

  IF: 2.2 *(2023)*

- **Jan Homa,** Wiktoria Wilms, Katarzyna Marcinkowska, Paweł Cyplik, Łukasz Ławniczak, Marta Woźniak-Karczewska, Michał Niemczak, Łukasz Chrzanowski; *Comparative analysis of bacterial populations in sulfonylurea-sensitive and -resistant weeds: insights into community composition and catabolic gene dynamics*; Environmental Science and Pollution Research; 2024; vol. 31; iss. 38; DOI: 10.1007/s11356-024-34593-z

  IF: 5.8 *(2022)*

- **Jan Homa**, Witold Stachowiak , Adriana Olejniczak, Łukasz Chrzanowski, Michał Niemczak; *Ecotoxicity studies reveal that organic cations in dicamba-derived ionic liquids can pose a greater environmental risk than the herbicide itself*; Science of the Total Environment; 2024; vol. 922; 171062-1 - 171062-13; DOI: 10.1016/j.scitotenv.2024.171062

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- Wiktoria Wilms, Anna Parus, **Jan Homa**, Milena Batycka, Michał Niemczak, Marta Woźniak-Karczewska, Artur Trzebny, Joanna Zembrzuska, Mirosława Dabert, András Táncsics, Tomáš Cajthaml, Hermann J. Heipieper, Łukasz Chrzanowski; *Glyphosate versus glyphosate based ionic liquids: Effect of cation on glyphosate biodegradation, soxA and phnJ genes abundance and microbial populations changes during soil bioaugmentation*; Chemosphere; 2023; vol 316; 137717-1 - 137717-12; DOI: 10.1016/j.chemosphere.2022.137717

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- Marta Woźniak-Karczewska, Anna Parus, Tomasz Ciesielski, Artur Trzebny Radosław Szumski, Wiktoria Wilms, Jan Homa, Grzegorz Framski, Daniel Baranowski, Robert Frankowski, Agnieszka Zgoła-Grześkowiak, Michał Niemczak, Mirosława Dabert, András Táncsics, Łukasz Chrzanowski; *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 Chemistry & Engineering; 2022, vol. 10; no. 38; 12559 – 12568; DOI: 10.1021/acssuschemeng.2c02665

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- Wiktoria Wilms, **Jan Homa**, Marta Woźniak-Karczewska, Mikołaj Owsianiak, Łukasz Chrzanowski; *Biodegradation half-lives of biodiesel fuels in aquatic and terrestrial systems: A review*; Chemosphere; 2024; vol. 313; 137236-1 - 137236-30; DOI: 10.1016/j.chemosphere.2022.137236

- **IF: 8.1** *(2023)*

- Marcel Jakubowski, Aleksandra Domke, Maria Ratajczak, Tomasz Buchwald, Łukasz Ławniczak, **Jan Homa**, Adam Voelkel, Mariusz Sandomierski; *Chitosan hydrogel modified with lanthanum as a drug delivery system for epigallocatechin gallate: Investigation of hydrogel – drug interaction by FT-IR and Raman spectroscopy*; Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy; 2023; vol. 297; 122748-1 - 122748-12; DOI: 10.1016/j.saa.2023.122748

- **IF: 4.3** *(2023)*

Total Impact Factor: 12.4

Oral Presentations at National/International Conferences

- **J. Homa, W. Stachowiak, A. Parus, M. Woźniak Karczewska,** ***Aktywność biologiczna herbicydowych cieczy jonowych na bazie jodosulfuronu metylosodowego,*** **VII Ogólnopolska Konferencja Doktorantów Nauk o Życiu BioOpen, Łódź 7-8.04.2022**

- W. Wilms, **J. Homa**, M. Woźniak-Karczewska, A. Parus, Wpływ doboru kationów na degradację HILs z anionem glifosatu, VII Ogólnopolska Konferencja Doktorantów Nauk o Życiu BioOpen, Łódź 7-8.04.2022

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- **J. Homa,** A. Parus, G. Framski, M. Woźniak-Karczewska, A. Syguda, *Ciecze jonowe o podwójnej funkcji z herbicydami MCPA i MCPP*, Ogólnopolska Konferencja „Biotechnologia niejedno ma imię", Poznań 21.11.2020

- **J. Homa,** A. Parus, G. Framski, M.Wozniak-Karczewska, A. Syguda, *Dwufunkcyjne herbicydowe ciecze jonowe z MCPA i MCPP jako substancje do walki z chabrem i stymulacji wzrostu kukurydzy*, III edycja Studenckiej Konferencji Nauk Scisłych im. Prof. Antoniego Hoborskiego, Kraków 14.11.2020

Posters at National/International Conferences

- **J. Homa**, W. Stachowiak, A. Parus, M. Woźniak-Karczewska, Ł. Chrzanowski, *Biological activity of herbicidal ionic liquids based on iodosulfuron methyl sodium herbicide,* 8th central European congress of life sciences EUROBIOTECH, Kraków, 20-22.06.2022

- W. Wilms, **J. Homa**, M. Woźniak-Karczewska, A. Parus, Ł. Chrzanowski; *Impact of the cation on the degradation of glyphosate anion in herbicidal ionic liquids*, 8th central European congress of life sciences EUROBIOTECH, Kraków, 20-22.06.2022

- **J. Homa**, W. Stachowiak, M. Woźniak-Karczewska, A. Parus, *Toksyczność herbicydowych cieczy jonowych na bazie jodosulfuronu metylosodowego względem roślin i bakterii glebowych*, BioOrg 2022 IV Ogólnopolskie Sympozjum Chemii Bioorganicznej, Organicznej i Biomateriałów, Poznań, 3.12.022

- **J. Homa**, A. Parus, G. Framski, M. Woźniak-Karczewska, A. Syguda, Herbicydowe ciecze jonowe o podwójnej funkcji na bazie MCPP i MCPA, 63. Zjazd Naukowy Polskiego Towarzystwa Chemicznego, Łódź 13-17 września 2021

- **J. Homa**, A. Parus, G. Framski, M. Woźniak-Karczewska, A. Syguda, *Nowe herbicydowe ciecze jonowe na bazie esterquatów zawierające MCPA i MCPP jako substancje do jednoczesnej stymulacji wzrostu kukurydzy i walki z chabrem bławatkiem*, I Pomorskie Studenckie Sympozjum Chemiczne, Gdańsk 26-27.09.2020 r.

- **J. Homa**, K. Mikołajczak, A. Basińska-Barczak, W. Wilms, L. Błaszczyk, *Wpływ inokulacji grzybami z rodzaju Trichoderma na zmianę ekspresji genów związanych z odpornością u pszenicy zwyczajnej (Triticum aestivum L.,)*, BioOrg 2019 III Ogólnopolskie Sympozjum Chemii Bioorganicznej, Organicznej i Biomateriałów Poznań, 7.12.2019 r.

Additional Scientific Acitivty

- Member of Student Scientific Society „BioInicjatywa" at Poznan Univeristy of Technology (2019-2024).
- Member of Student Scientific Society „Sekcja Medycyny Regeneracyjnej I Badań and Nowotworami KNP" at Adam Mickiewicz University in Poznań (2017-2019).

Internships

- Institute of Biology, Biotechnology and the Environment, Department of natural sciences, University of Silesia, Katowice, 16.04-24.06.2023

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", 10.2019–09.2024.

Research career

- **Ph.D. Student**
  (01.10.2019 – present)
  Ph.D. Supervisor: Prof. Łukasz Chrzanowski, Ph.D. Eng.
  Ph.D. Auxiliary Supervisor: Prof. Paweł Cyplik, Ph.D.
  Poznan University of Technology
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- **Bachelor studies at Poznan University of Technology**
  Bachelor of Science, Engineer: 11.10.2019
  Poznan University of Technology
  Faculty of Chemical Technology
  Chemical Technology;
  Supervisor: Anna Parus, Ph.D., Eng.
  Thesis title: *Konflikty zbrojne a środowisko naturalne*

- **Master studies at Adam Mickiewicz University in Poznań**
  Master od Science: 31.07.2019
  Adam Mickiewicz University in Poznań
  Faculty of Biology
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  Supervisor: Lidia Błaszczyk, Ph.D.
  Thesis title: *Analiza ekspresji wybranych genów w roślinach pszenicy zwyczajnej (Triticum aestivum L,) w odpowiedzi na inokulację roślin grzybami z rodzaju Trichoderma*

- **Bachelor studies at Adam Mickiewicz University in Poznań**
  Bachelor of Science, Engineer: 13.07.2017
  Adam Mickiewicz University in Poznań
  Faculty of Biology
  Biotechnology
  Supervisor: Andrzej Woyda-Płoszczyca Ph.D.
  Thesis title: *Zastosowanie inhibitorów mitochondrialnej oksydazy alternatywnej (AOX) jako fungicydów w ochronie roślin*