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Deep Eutectic Solvents Microbial Toxicity: Current State of Art and Critical Evaluation of Testing Methods

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Abstract

Deep eutectic solvents (DESs) were described at the beginning of 21st century and they consist of a mixture of two or more solid components, which gives rise to a lower melting point compared to the starting materials. Over the years, DESs have proved to be a promising alternative to traditional organic solvents and ionic liquids (ILs) due to their low volatility, low inflammability, easy preparation, and usually low cost of compounds used in their preparation. All these properties encouraged researchers to use them in diverse fields and applications e.g., as extractants for biomolecules and solvents in pharmaceutical and cosmetic industries. Nevertheless, despite undeniable potential of DESs, there is still controversy about their potential toxicity. Besides the low number of studies on this topic, there are also some contradicting reports on biocompatibility of these solvents. Such misleading reports could be mainly attributed to the lack of well design standard protocol for DESs toxicity determination or the use of out-off-purpose methodology. Thus, to better apply DESs in green and sustainable chemistry, more studies on their impact on organisms at different trophic levels and the use of proper techniques are required. This review focuses on DESs toxicity towards microorganisms and is divided into three parts: The first part provides a brief general introduction to DESs, the second part discusses the methodologies used for assessment of DESs microbial toxicity and the obtained results, and finally in the third part the critical evaluation of the methods is provided, as well as suggestions and guidelines for future research.

Keywords: Deep eutectic solvents, Toxicity, Pollutants; Antimicrobial activity, Disk diffusion, Broth dilution

1. Introduction

Deep eutectic solvents (DESs) emerged in 2003 and are a new class of solvents having liquid state around room temperature[1]. They are prepared by a simple mixing at certain molar ratio and heating of two or more chemicals often having a solid state at room temperature. In such mixture one of the compounds acts as a hydrogen-bond donor (HBD) and the other one as a hydrogen-bond acceptor (HBA). Consequently, a eutectic mixture for which the eutectic point temperature presents a deep depression to that of an ideal liquid mixture is formed. Lower melting point of the DES comparing to values for pure components is mainly assigned to the formation of hydrogen-bonds between the DES components[2, 3]. Nevertheless, also electrostatic interactions or Van der Waals forces were considered as possible factors that may also play an important role in this phenomenon[4-7]. Furthermore, DESs with ionic components are very often referred to as ionic liquids (ILs) analogues because they share some of their characteristic features such as low volatility, wide liquid temperature range, and high solvation ability for many compounds[7, 8]. On the other hand, compared to ILs, DESs have some advantageous characteristics, such as usually lower toxicity, higher biodegradability, easier preparation, and lower material cost[9]. Moreover, DESs similarly to ILs have highly tunable nature since through the manipulation of different types of HBAs, HBDs and molar ratios, it is possible to modify their biological and physicochemical properties to fit a specific application[10-13].

All the above-mentioned remarkable properties of DESs make them an ideal alternative to both commonly used organic solvents and ILs[5, 14-16]. That is why, since their discovery, they have been widely studied and applied in diverse fields, including biocatalysis[17-19], electrochemistry[20-22], CO_2 capture[23, 24], separation and extraction techniques[25-31], among others. Furthermore, beside the fact that up to now the most works focus on their

applications as green solvents for different chemical industries, more recently they started to be also considered as promising fluids for cosmetic, food, pharmacological, biotechnological and biomedical industries[32-36]. It is mainly related to the fact that DESs are considered as non-toxic, eco-friendly, biodegradable and benign solvents. Nevertheless, in order to make such conclusions and to use DESs in these areas, the more profound studies on DESs toxicity and biodegradability are essential.

There is a general assumption that DESs are non-toxic because usually their individual starting compounds are natural, biodegradable and low toxic. The lower toxicity and higher biodegradability of DESs were mainly assigned to the group of DESs composed of natural, low toxic compounds, such as cholinium chloride, natural carboxylic acids, sugars, amino acids, and, in some cases, water as a third component, the so-called *natural deep eutectic solvents* (NADESs)[37]. Nevertheless, it is not appropriate to assume that NADESs do not exhibit toxic effect on different organisms because after formation of hydrogen-bonds a new supramolecular structure is created[2, 3], making necessary to evaluate possible toxicity of NADESs as a result of this change. Notwithstanding, the number of works that studies toxicity of these compounds is rather limited. To the best of our knowledge, since DESs introduction around 96 papers have been published about toxicity of DESs (see Fig. 1). In most of these works, the toxicity of DESs was evaluated using prokaryotic microorganisms[38-43], however more recently also some eukaryotic organisms were used, including microorganisms (yeasts, molds), human and animal cell lines, and animal models (*Hydra sinensis*, *Cyprinus carpio* fish, *Artemia salina* brine shrimp)[6, 38, 39, 42-47]. Nevertheless, due to usually short generation time, easiness of culturing and possibility to use the same microbiological methods, most studies focus on both gram-positive and gram-negative bacterial strains, yeast and mold fungi strains (see Fig. 2)[38,

40, 48-52]. Therefore, in this work we decided to focus on reviewing the present state of art of the DESs microbial toxicity against procaryotic and eukaryotic microorganisms and the critical evaluation of usefulness of the microbiological methods used for this purpose.

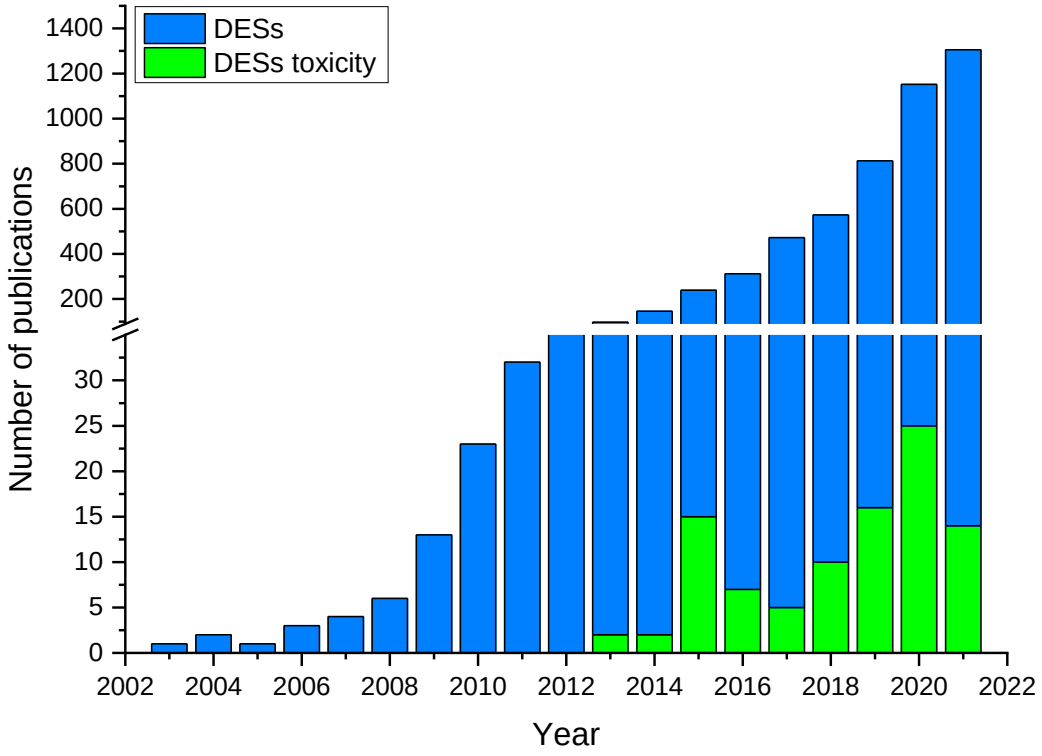
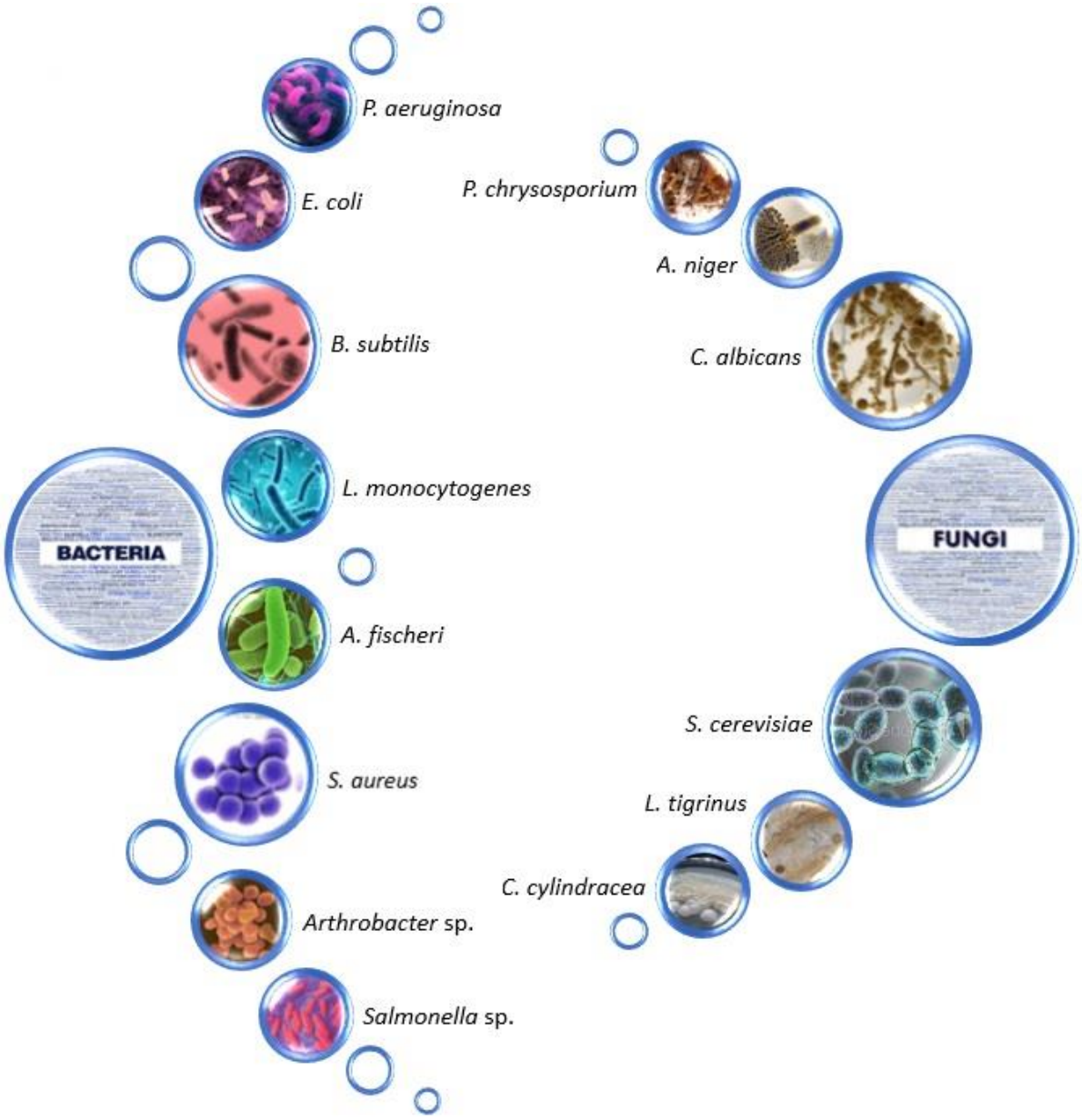


Fig. 1: Evolution of the number of published papers in the field of DESs in general (blue) and DESs toxicity (green) starting from 2003 that contained “deep eutectic solvents” or “deep eutectic solvents and toxicity” in their titles, keywords, or abstracts as obtained from Scopus. Data for 2021 included up to November.

Even though, in some of the reports the low toxic, eco-friendly and biodegradable nature of DESs is demonstrated, some other works claim exactly the opposite and toxicity of some DESs was shown[45, 53]. It leads to some confusion and confirm the need for toxicity studies for all DESs

present in literature. Such misleading reports can be also attributed to the lack of well design standard protocol for DESs toxicity determination. Having said that, the researchers planning their experiments on DESs toxicity should be aware what are the available methods and what are their advantages and disadvantages. Moreover, the researchers should be aware that not all the toxicity assessment methods are best suited for the DESs. For instance, the high viscosity, instability of aqueous solutions, among others, make some of the used methods not applicable. In other words, in many cases used protocols do not fit to the purpose. Thus, conclusions stated for such studies are simply not true.



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117 Fig. 2: Types of microorganisms mostly used in toxicological studies of DESs.

118 The selection of the test method always affects the results obtained. Thus, by proper planning and
119 use of correct methodology, the risk of misleading results will be minimized. Finally, it will
120 allow to compare the results obtained in different studies. This paper provides a review of the
121 procedures for the determination of toxicity of DESs. The available techniques are discussed
122 along with the advantages and general disadvantages related to the use of these methodologies.

Furthermore, the critical evaluation of the methods used for assessment of DESs toxicity, and the literature review of obtained results is presented. General discussion on DESs toxicity and possible mechanisms on how they promote toxicity are also included as well as suggestions and guidelines for future research are proposed.

2. Methods used for DESs microbial toxicity assessment

The analysis of the available literature showed that the following methods have been used to assess the toxicity of DESs against prokaryotic and eukaryotic microorganisms: disk and well diffusion method, broth dilution, Microtox assay for luminescence inhibition in *Aliivibrio fischeri*, drop plate method and FTIR bioassay. Among these methods, for this purpose, the disk or well diffusion method was most often used (16 studies, Table 1). Moreover, the broth dilution method (macro- and micro-dilution) was also used relatively often (14 studies, Table 2). Methods such as Microtox assay (Table 4), drop plate method (Table 5) or FTIR (Table 6) were used much less frequently for this purpose. In addition, in view of an attempt to critically evaluate the practical suitability of these methods to study DESs microbial toxicity (section 4), in sections 2.1-2.3 besides the discussion of the results of toxicity studies with DESs using these methodologies, each of these techniques is briefly presented and their major advantages and disadvantages are listed.

2.1. Diffusion methods

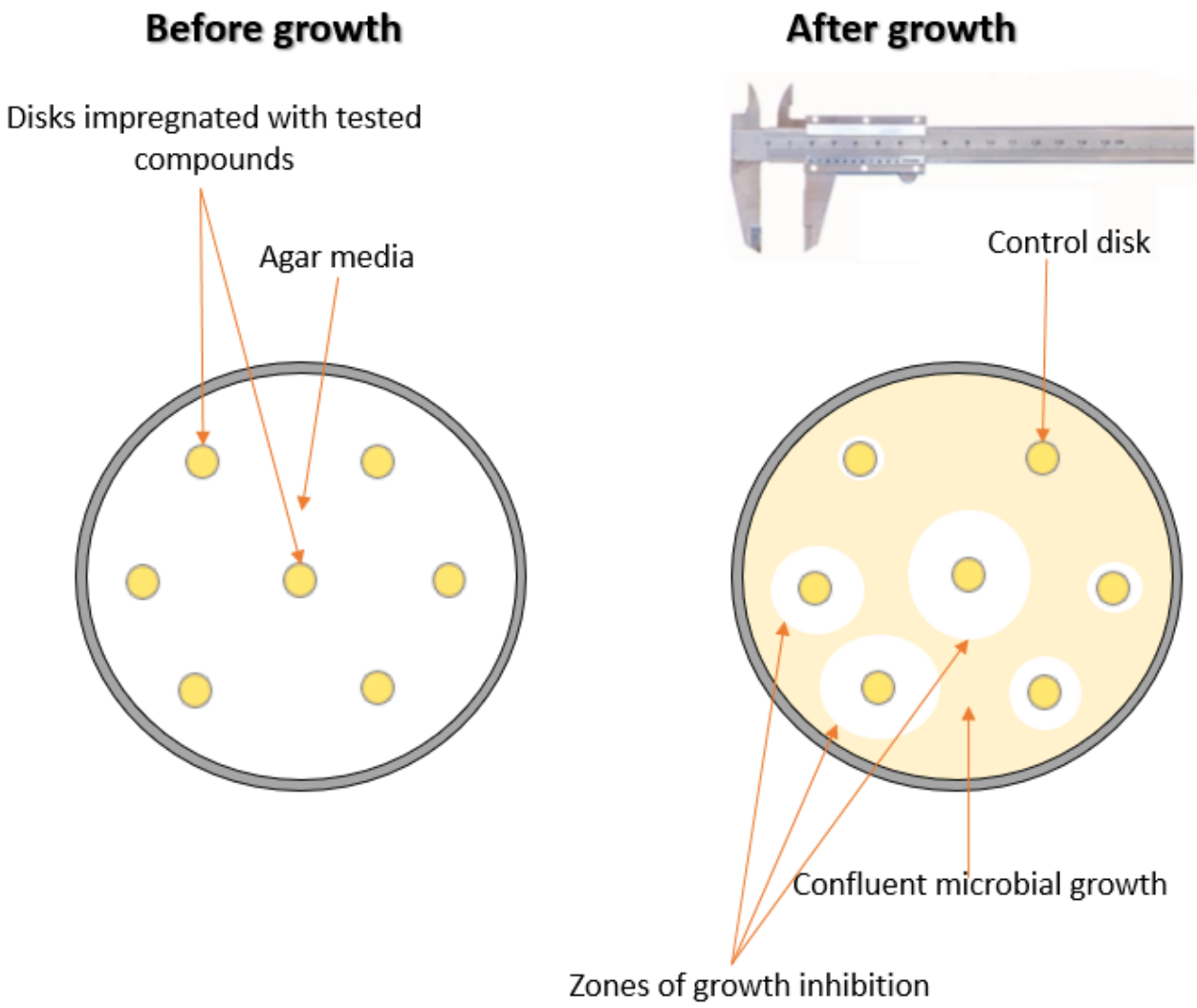
2.1.1. Disk diffusion method

Primarily, the disk diffusion method (agar diffusion test or Kirby–Bauer test) was used to test the susceptibility of microorganisms to antibiotics[54, 55], and later its application was also extended to test antimicrobial activity of different chemical compounds e.g., ILs[56] and DESs[48]. In this

test, a filter-paper disk is impregnated with the compound to be tested and then placed on the surface of the agar plate where microorganisms have been previously swabbed uniformly[54, 55]. Afterall the plate is left to grow the tested microorganisms (incubation at optimal growth condition e.g., temperature, time) and to allow the compound to diffuse from the disk into the agar. If the tested compound stops the microorganism growth, there will be an inhibition zone around the disk, where no colonies have grown[54, 55]. By measuring the size of the inhibition zone, the susceptibility of microorganism to chemical agent can be deducted. The size of the zone around the disk mainly depends on how effective the chemical compound is at stopping the growth of the microorganism and indicates where the concentration in the agar is greater than or equal to the effective concentration[54, 55]. Furthermore, another important factor that needs to be considered is the diffusion of the compound within the agar medium[54, 55]. The diffusion varies between different compounds based on their molecular structure and further on their hydrophobicity/hydrophilicity[54, 55]. Also, the viscosity of the tested solution has a great impact on the diffusion. Thus, while interpretating the results, it needs to be remembered that the size of inhibition zones is different for each compound not only because the different antimicrobial potency but also due to different diffusion and solubility of tested chemicals in agar medium. Having said that the disk with compound that produces the largest inhibition zone is not an indication of the real toxicity of the compound to the tested microorganism[54, 55]. The toxicity testing procedure using disk diffusion method is shown in Fig. 3.

The main advantages of the disk diffusion test are that it is a cost-efficient test that is easy to conduct and easy to evaluate. Furthermore, this method allows to test several antimicrobial agents simultaneously on the same plate. These characteristics, along with short period of time needed to obtain relevant information, made disk diffusion test most widespread method used for DESs

toxicity assessment and the results found in the literature for microbial toxicity of DESs using disk diffusion method are presented in Table 1. On the other hand, the biggest drawback of this method is the fact that it only allows us to assess whether the chemical agent is toxic, moderately toxic, or non-toxic for the tested microorganism in question. That is why, in some cases, multiple disks with different concentrations of the tested compound are used simultaneously on the same agar plate. In that way, it is possible to estimate approximate minimum inhibitory concentration (MIC) of compound. Nevertheless, for more precise toxicity assessment and MIC determination, after disk test, the use of "dilution methods" for the same pair of tested compound and microorganism (see section 2.2.) is recommended.



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178 Fig. 3: Toxicity testing using disk diffusion method.

179 The disk diffusion method was chosen in the first study on toxicity of DESs that was conducted
180 by Hayyan et al.[48]. In this work, DESs prepared using choline chloride (ChCl) as HBA and
181 glycerol, ethylene glycol, triethylene glycol, urea as HBDs were chosen and its toxicity to
182 different gram-positive (*Bacillus subtilis* and *Staphylococcus aureus*) and -negative (*Escherichia*
183 *coli* and *Pseudomonas aeruginosa*) bacteria was evaluated. The authors showed that all
184 investigated DESs had no inhibition on the studied bacterial strains[48]. Later, Mao et al.
185 extended this work and studied the effect of similar DESs (with exception of ChCl:triethylene

glycol) on toxicity of *Arthrobacter simplex*[57]. The authors found out that at 60% concentration these DESs (with exception of ChCl:urea) were toxic to *A. simplex* to some extent[57]. Interestingly, the obtained results revealed that the three tested DESs had much lower toxicity towards *A. simplex* than their individual components. This observation indicates that the toxic effects of DES individual components can be weakened by incorporating them into a DES. The authors hypothesized that hydrogen bonding network after DES formation prevented the salt anion from attacking the cellular membrane, thus resulting in lower toxicity of DESs towards *A. simplex*[57]. Considering these findings, the authors suggested that the toxicity of DESs may be species-dependent and associated with varied effects of DES components on the target microorganism[57].

In their second study, Hayyan et al. changed the HBA from ChCl to methyltriphenylphosphonium bromide (MTPB) and combined it with glycerol, ethylene glycol, triethylene glycol as HBDs[38]. All tested phosphonium-based DESs have been relatively toxic to gram-negative bacteria (*E. coli* and *P. aeruginosa*) and thus can be used as potential antibacterial agents[38]. On the other hand, only MTPB:ethylene glycol DES showed effective toxicity towards gram-positive bacteria (*B. subtilis* and *S. aureus)* indicating the HBD nature influences the antibacterial effect of DESs[38]. Furthermore, these results suggest that the HBA also affects toxicity of DESs since similar HBDs have been used in both studies. The contribution of HBA to DESs toxicity was attributed to the charge delocalization that occurs through hydrogen bonding since chemicals having delocalized charges are more toxic than chemicals with localized charges[58, 59].

Later, the disk test was also used to qualitatively evaluate the growth inhibition of bacteria (*E. coli*, *S. enteritidis*, *S. aureus* and *L. monocytogenes*) caused by ChCl-based DESs prepared using

various HBDs such as amines, alcohols, organic acids and sugars[49]. It was reported that ChCl-based DESs formed with amines, alcohols, and sugars as HBDs did not have a significant toxic effect on bacteria. These finding are in line with the study of Hayyan et al., where also no inhibition of bacteria growth was observed for ChCl-based DESs[48]. On the other hand, significant toxic effect was observed when organic acids were used as HBD of DES. The authors suggested that the amine-, alcohol- and sugar-based DESs were used by bacteria as nitrogen or carbon sources, while the organic acid−based DESs inhibited bacterial growth mainly as a result of significant decrease of pH below the optimal values (pH=6.5−7.5) for bacterial growth of tested microorganisms[49]. The obtained results revealed that gram-negative bacteria (*E. coli* and *S. enteritidis*) were more sensitive than gram-positive (*S. aureus* and *L. monocytogenes*), most likely due to the interaction of DESs components with the polysaccharide or peptide chains of the cell wall through hydrogen bonding or electrostatic interactions, resulting in damage of cell walls[49]. Moreover, the antibacterial activity of DESs based on saturated fatty acids, combining capric acid with other saturated fatty acids with different chain size length (i.e., lauric acid, myristic acid and stearic acid) was studied in the work of Silva et al.[60]. The disk test results revealed that the DESs did not inhibit growth of gram-negative bacteria (*E. coli* and *P. aeruginosa*) but showed antibacterial activity against the gram-positive bacteria (*S. aureus*, Methicillin-resistant *S. aureus* (MRSA) and Methicillin-resistant *S. epidermis* (MRSE))[60]. As an explanation, the authors suggested the differences in cell wall structure of gram-positive and -negative bacteria[60]. According to previous reports gram-negative bacteria are usually resistant to the antibacterial activity of fatty acids due to a presence of lipopolysaccharides on the cell wall that prevents the fatty acids from reaching cell membrane[61-64], while the cell wall of gram-positive bacteria readily absorbs fatty acids allowing their passage into the inner membrane[61, 63]. The same group also studied the antimicrobial properties of therapeutic DES (THEDES –

group of DESs for which one of the components of the eutectic mixture is an active pharmaceutical ingredient (API)[65, 66]) based on menthol and stearic acid[67]. It was observed that both, THEDES and its starting materials, did not inhibit the growth of gram-negative *E. coli* and *P. aeruginosa*, while growth of gram-positive bacteria (*S. aureus*, MRSA and MRSE) was only affected by the menthol[67]. Furthermore, the disk diffusion results showed the formation of deposit in all cases for menthol:stearic acid THEDES, which was assigned to fatty acid's low solubility and, consequently, low diffusion rate[67]. The presence of deposit prevented the authors from correct evaluation of inhibition zones for THEDES, but since it is majorly composed of menthol (molar ratio 8:1), which showed antimicrobial properties towards gram-positive bacteria, it was assumed that this THEDES is toxic to some degree and further toxicological studies using broth dilution were performed[67]. Recently, the antibacterial activity of menthol:lactic acid was also studied[68]. This DES can be classified as THEDES and furthermore as representant of hydrophobic DESs. In cited study, two gram-negative bacteria (*E. coli* and *P. aeruginosa*) and one gram-positive pathogen (*S. epidermis*) were selected and the antimicrobial activity evaluated using disk diffusion method[68]. It was shown that all the tested bacteria were susceptible to menthol:lactic acid DES and clear inhibition zones were observed[68]. Gram-positive *Staphylococcus epidermidis* was also found to be the most susceptible bacteria to the tested DES than gram-negative bacteria (*E.coli* and *P. aeruginosa*)[68]. The bactericidal activity of menthol:lactic DES was assigned to the use of lactic acid as a forming component thus higher toxicity of DES due to the additional hydroxyl group presence in its structure and the high acidity[68].

In another report Wang et al. evaluated the toxicity effect of benzalkonium chloride (BC):acrylic acid and benzalkonium chloride:methacrylic acid DESs, as well as their individual components,

towards *E. coli* and *S. aureus*[69]. The disk diffusion assay results revealed that DESs inhibited the growth of bacteria and that the inhibition potency of DESs mainly comes from benzalkonium chloride (BC) and not acrylic or methacrylic acid since DESs inhibition zone widths were slightly larger or close to that of BC and not acid[69]. It was also observed that the studied DESs were more toxic to the gram-positive bacteria (e.g., *S. aureus*) than gram-negative (e.g., *E. coli*). Furthermore, the introduction of methyl group within methacrylic acid resulted in decrease in DESs toxicity comparing to BC:acrylic acid DES[69]. The disk diffusion test was also applied to evaluate toxicity of DESs based on betaine[70, 71]. Firstly, it was shown that betaine:urea DESs is not toxic to *E. coli* and *P. aeruginosa* bacterial strains[70]. More recently, Jiang reported that betaine:malic acid DES has certain antibacterial activity towards *E. coli*[71]. Also, in the study of Jangir et al. antibacterial properties of ternary DESs were described[72]. The authors showed that ChCl:oxalic acid:ethylene glycol, ChCl:oxalic acid:glycerol, ChCl:citric acid:ethylene glycol and ChCl:citric acid:glycerol DESs inhibited the growth of *E. coli* and *S. aureus* strains[72]. In particular, ChCl:oxalic acid:ethylene glycol DES was the most toxic to the selected microbes, followed by ChCl:citric acid:ethylene glycol, ChCl:oxalic acid:glycerol and ChCl:oxalic acid:ethylene glycol, respectively[72]. Moreover, in the most recent work, the toxicity of ChCl:1,2-propanediol DES towards *S. aureus*, *E. coli*, *Clostridium perfringens*, *L. monocytogenes* and *Salmonella* sp. was studied[73]. According to the obtained results this DES was found relatively toxic to all tested bacterial strains[73]. It was concluded that part of this effect is due to the HBD - 1,2-propanediol - which was previously found effective against *E. coli* and *S. aureus*[74]. Among the studied bacteria, the lowest inhibition effect was observed for *E. coli* and it was hypothesized that their resistance could be related to the gram-negative status and the lower permeability of their surface for phenolic compounds[73]. On the other hand, this DES

showed intermediate inhibition effect on the other gram-negative (*Salmonella* sp.) and all gram-positive (*L. monocytogenes, S. aureus, C. perfringens*) bacteria[73].

Furthermore, the toxicities of NADESs were also evaluated using four bacteria (*S. aureus, L. monocytogenes, E. coli* and *S. enteritidis*)[41]. The obtained results agreed with the hypothesis that NADESs are non-toxic and biocompatible since most of the tested ChCl- and glycerol-based NADESs did not cause the inhibition of bacterial growth. The exception was NADES prepared from L-arginine and glycerol which showed high toxicity towards the four tested bacteria (*S. aureus, L. monocytogenes, E. coli* and *S. enteritidis*)[41]. This is an interesting result because separately both glycerol and L-arginine are recognized as non-toxic and FDA approved these compounds, but by forming NADES through hydrogen bonding, such eutectic mixture becomes toxic most likely due to charge delocalization[41]. In another report, Redovniković's group further studied the antibacterial activity of NADESs[43]. The disk diffusion assay was applied to evaluate toxicity of betaine-, choline-, citric acid-, sugar-, and sugar alcohol-based NADESs towards *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Staphylococcus aureus* and *E. coli*[43]. All the tested NADESs, except ChCl:xylitol, ChCl:sorbitol, and betaine:glucose were found toxic to the selected bacterial strains[43]. The antibacterial activity of NADESs was higher for the acid containing NADESs. Furthermore, contrary to some previous reports[38, 49, 60], the effect of NADESs was not related to whether the bacterial strain was gram- positive or gram- negative[43].

299 Table 1. The toxicity of DESs determined by disk diffusion method.

DES	Microorganisms			Toxicity results	Ref.
	Bacterium G(+)	**Bacterium G(-)**	**Fungi**		
ChCl:glycerol (1:3) ChCl:ethylene glycol (1:3) ChCl:triethylene glycol (1:3) ChCl:urea (1:3)	*Bacillus subtilis*, *Staphylococcus aureus*	*Escherichia coli*, *Pseudomonas aeruginosa*		• All the DESs showed no toxic effect on tested genus of bacteria. • The individual components of DESs showed no toxic effect on tested genus of bacteria.	[48]
MTPB:glycerol (1:3) MTPB:ethylene glycol (1:3) MTPB:triethylene glycol (1:3)	*Bacillus subtilis*, *Staphylococcus aureus*	*Escherichia coli*, *Pseudomonas aeruginosa*		• All the DESs showed relative toxic effect on gram-negative bacteria, while only MTPB:ethylene glycol DES showed effective toxicity towards gram-positive bacteria. • The toxic effect of individual components of DESs was not assayed.	[38]
ChCl:urea (1:2) ChCl:acetamide (1:2) ChCl:ethylene glycol (1:2) ChCl:glycerol (1:2) ChCl:1,4-butanediol (1:4)	*Staphylococcus aureus*, *Listeria monocytogenes*	*Escherichia coli*, *Salmonella enteritidis*		• All the DESs except for acid containing DESs showed no toxic effect on tested genus of bacteria. • The toxic effect of individual	[49]

ChCl:triethylene glycol (1:4) ChCl:xylitol (1:1) ChCl:D-sorbitol (1:1) ChCl:PTSA (1:1) ChCl:oxalic acid (1:1) ChCl:levulinic acid (1:2) ChCl:malonic acid (1:1) ChCl:malic acid (1:1) ChCl:citric acid (1:1) ChCl:tartaric acid (2:1) ChCl:xylose:water (1:1:1) ChCl:sucrose:water (5:2:5) ChCl:fructose:water (5:2:5) ChCl:glucose:water (5:2:5) ChCl:maltose:water (5:2:5)				components of DESs was not assayed.	
BC:acrylic acid (1:2) BC:methacrylic acid (1:2.5)	*Staphylococcus aureus* NRS234	*Escherichia coli* ATCC 25922	*Candida albicans* ATCC 18804	• All the DESs showed relative toxic effect on tested genus of bacteria and fungi. • The individual components of DESs showed relative toxic effect on tested genus of bacteria and fungi.	[69]
ChCl:1,2-	*Staphylococcus*	*Escherichia*		• All the DESs, but	[41]

propanediol (1:1) ChCl:glycerol (1:1) ChCl:glucose (2:5) ChCl:sucrose (1:1) ChCl:xylitol (1:2) ChCl:sorbitol (2:5) glycerol:L-proline (3:1) glycerol:L-alanine (3:1) glycerol:glycine (3:1) glycerol:L-histidine (3:1) glycerol:L-threonine (3:1) glycerol:L-lysine (4.5:1) glycerol:L-arginine (4.5:1)	*aureus*, *Listeria monocytogenes*	*coli*, *Salmonella enteritidis*		glycerol:L-lysine (*E. coli*) and glycerol:L-arginine (all four bacterial strains), showed no toxic effect on tested genus of bacteria. • ChCl and glycerol individually showed no toxic effect on tested genus of bacteria. L-arginine showed relative toxic effect on *E. coli*.	
capric acid:lauric acid (2:1) capric acid:myristic acid (3:1) capric acid:stearic acid (4:1)	*Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* ATCC 700698 (Methicillin-resistant strain, MRSA), *Staphylococcus epidermis* ATCC 35984 (Methicillin-resistant strain, MRSE)	*Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922	*Candida albicans* ATCC 90029	• All the DESs showed no toxic effect on tested genus of G(-) bacteria and showed relative toxic effect on tested genus of G(+) bacteria and fungi. • The individual components of DESs showed no toxic effect on tested genus of G(-) bacteria and showed relative toxic effect on tested genus of G(+) bacteria (except stearic acid) and fungi (except capric, lauric and myristic acid).	[60]

menthol:stearic acid (8:1)	*Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* ATCC 700698 (MRSA), *Staphylococcus epidermis* ATCC 35984 (MRSE)	*Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922		• This DES showed no toxic effect on tested genus of G(-) *and* showed relative toxic effect on tested genus of G(+) bacteria. • Stearic acid showed no toxic effect on tested genus of bacteria, while menthol showed relative toxic effect on tested genus of G(+) bacteria.	[67]
menthol:lactic acid (1:2)	*Staphylococcus epidermis*	*Pseudomonas aeruginosa*, *Escherichia coli*		• All the DESs showed toxic effect on tested genus of bacteria. • The toxic effect of individual components of DES was not assayed.	[68]
ChCl:urea (1:2) ChCl:ethylene glycol (1:2) ChCl:glycerol (1:2)	*Arthrobacter simplex* TCCC 11037			• All the DESs showed no toxic effect on tested genus of bacteria at 30 % concentration. • All the DESs, but ChCl:urea, showed relative toxic effect on *A. simplex* at 60 % concentration. • Glycerol and urea individually showed no toxic effect on tested genus of bacteria, while toxic effect of ChCl toward	[57]

				A. simplex was higher than for tested DESs.	
betaine:urea (1:1.5)		*Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853		• This DES showed no toxic effect on tested genus of bacteria. • The toxic effect of individual components of DES was not assayed.	[70]
betaine:malic acid (1:1)		*Escherichia coli*		• This DES showed relative toxic effect on tested genus of bacteria. • The toxic effect of individual components of DES was not assayed.	[71]
ChCl:oxalic acid:ethylene glycol (1:1:1) ChCl:oxalic acid:glycerol (1:1:1) ChCl:citric acid:ethylene glycol (1:1:1) ChCl:citric acid:glycerol (1:1:1)	*Staphylococcus aureus* ATCC 9144	*Escherichia coli* ATCC 23564	*Candida albicans* ATCC 10231	• All the DESs showed relative toxic effect on tested genus of bacteria and fungi. • The toxic effect of individual components of DESs was not assayed.	[72]
ChCl:oxalic acid (1:1) ChCl:urea (1:2) ChCl:xylitol (5:2) ChCl:sorbitol (2:3) betaine:glucose (5:2) betaine:malic acid:proline (1:1:1) betaine:malic acid:glucose (1:1:1)	*Staphylococcus aureus* 3048	*Escherichia coli* 3014, *Proteus mirabilis* 3008, *Salmonella typhimurium* 3064, *Pseudomonas aeruginosa*	*Candida albicans* 86	• All acid containing DESs showed relative toxic effect on tested genus of bacteria. • Only ChCl:oxalic acid DES inhibited growth of *C. albicans*. • The toxic effect of individual components of DESs	[43]

citric acid:proline (1:1) citric acid:glucose:glycerol (1:1:1) citric acid:fructose:glycerol (1:1:1)		3024		was not assayed.	
ChCl:1,2-propanediol (1:2)	*Staphylococcus aureus* ATCC 25923, *Clostridium perfringens* ATCC 13124, *Listeria monocytogenes* ATCC 7644	*Escherichia coli* ATCC 25922, *Salmonella* spp. ATCC 13076		• This DES showed relative toxic effect on tested genus of bacteria. • The toxic effect of individual components of DES was not assayed.	[73]
ChCl:$ZnCl_2$ (1:2) ChCl:urea (1:2) ChCl:glycerol (1:3) ChCl:ethylene glycol (1:3) ChCl:diethylene glycol (1:2) ChCl:triethylene glycol (1:3) ChCl:fructose (2:1) ChCl:glucose (2:1) ChCl:*p*-toluene sulfonic acid (1:3) ChCl:malonic acid (1:1)			*Phanerochaete chrysosporium, Aspergillus niger, Lentinus tigrinus, Candida cylindracea*	• Zinc salts and acid containing DESs showed toxic effect on all tested genus of fungi. • The other DESs showed no toxic effect on *P.chrysosporium, A.niger, L.tigrinus.* • ChCl:urea, ChCl:ethylene glycol, ChCl:diethylene glycol, ChCl:triethylene glycol DESs showed relative toxic effect on *C. cylindracea.* • $ZnCl_2$, *p*-toluene	[52]

				sulfonic acid and malonic acid individually showed relative toxic effect on all tested genus of fungi and ethylene glycol, diethylene glycol, triethylene glycol and fructose inhibited the growth of *C. cylindracea.*	

Over the years there have also been reports, where the disk diffusion method was used to evaluate DESs antifungal activity. Firstly, Hayyan's group tested ChCl-based DESs toxicity on four fungi strains selected as a model of eukaryotic microorganisms (*Phanerochaete chrysosporium*, *Aspergillus niger*, *Lentinus tigrinus* and *Candida cylindracea*)[52]. Among these DESs the highest antifungal activity was observed for ChCl:$ZnCl_2$ DES for all tested fungi species, followed by ChCl:malonic acid and ChCl:*p*-toluenesulfonic acid DES[52]. It was also noted that the these three DESs were slightly less toxic to all tested fungi than their respective HBD individually[52]. This phenomenon was assigned to the synergistic effect of forming DES through hydrogen bonding[38, 48]. Furthermore, there have been several works where DESs and NADESs antifungal activity towards *Candida albicans* yeast was studied[43, 60, 69, 72]. For instance, Silva et al. reported that fatty acid-based DESs, namely capric acid:lauric acid, capric acid:myristic acid, capric acid:stearic acid, exhibited antifungal activity towards *C. albicans*[60]. Furthermore, it was noted that studied yeast cells were overall less susceptible to DES formulations than gram-positive and -negative bacteria[60]. However, in the work of Wang et al. it was reported that inhibition zones widths caused by BC:acrylic acid and BC:methacrylic acid

DESs were slightly larger for *C. albicans* than these obtained for bacterial strains[69]. Moreover, in the study of Jangir and co-workers the antifungal activity of ternary DESs was reported[72]. From the studied DESs ChCl:oxalic acid:ethylene glycol and ChCl:citric acid:ethylene glycol inhibited the fungal growth, while for ChCl:oxalic acid:glycerol and ChCl:citric acid:glycerol no inhibition zones were observed[72]. These findings suggest that the toxicity of DESs is microbes type-dependent, since all four DESs were found toxic to bacteria[72]. The authors concluded that non-toxicity of ChCl:oxalic acid:glycerol and ChCl:citric acid:glycerol to *C. albicans* might be explained by highly acidic nature of these compounds thus easier penetration of the lipid layer of bacteria and not fungi[72]. Finally, Redovniković's group selected various betaine-, choline-, citric acid-, sugar-, and sugar alcohol-based NADESs and observed that *Candida albicans* was only inhibited by ChCl:oxalic acid NADES[43].

2.1.2. Well diffusion method

Another diffusion technique used to evaluate DESs toxicity was agar well diffusion method, which procedure is similar to that used in the disk diffusion test. It involves preparation of the agar plate culture of the strain of interest. This is followed by cutting a hole with a diameter of 6 to 8 mm using as a sterile cork borer or a tip, and then different volumes (20–100 µL) of the antimicrobial agent at desired concentration are deposited into the well. Afterall, agar plates are incubated under suitable conditions depending on the required conditions for the growth of tested microorganisms. During incubation the antimicrobial agent diffuses in the agar medium and if it is toxic to the cells, it inhibits the growth of the microbial strain tested. The size of the measured inhibition zone caused by tested compounds indicates antimicrobial potency.

So far, well diffusion method was only used in the work conducted by Hayyan's group in which the toxicity of ChCl-based DESs and N,N-diethyl ethanol ammonium chloride (EAC)-based

DESs towards *Aspergillus niger* was studied[51]. The authors showed that EAC:$ZnCl_2$ DES inhibited the fungal growth the most, already at the lowest DES dose tested (10 mg)[51]. This DES was followed by EAC:ZnN DES and EAC:malonic acid DES[51]. Furthermore, the obtained results indicated that ChCl-based DESs were less toxic to the mold since much higher concentration were needed to inhibit its growth[51].

2.2. Dilution methods

2.2.1. Agar and broth dilution technique

As it was mentioned earlier, one of the most used techniques for DESs microbial toxicity testing are agar or broth dilution method. These methods aim to determine the lowest concentration of the studied antimicrobial agent that, under defined test conditions, inhibits the visible growth of the microorganism under investigation. Hence, using broth or agar dilution such parameters as minimum inhibitory concentrations (MICs), or effective concentrations (EC_{50}) of antimicrobial agents can be determined. In agar dilution technique, inoculum of microbes with defined numbers of cells is applied directly onto the nutrient agar plates that have contained different concentrations of antimicrobial agent[75]. Then the plates are incubated at optimal conditions (e.g., temperature, incubation time) for growth of tested microorganism and after incubation the plates are visually inspected. The presence of colonies on the plates indicates growth of the microorganism and the plate with the lowest concertation of tested compound where microorganism did not grow indicates its MIC value[75]. The advantage of agar dilution is that it is a suitable method when testing large numbers of bacterial isolates against a limited number of antimicrobial agents in a limited number of concentrations[76]. However, when testing low concentrations, an even distribution within the agar must be assured[76]. The main drawback of agar dilution is the fact that it is time consuming method, which requires preparation of high

number of plates with different concentrations of antimicrobial agent[76]. For that reason, agar dilution is also not very cost-efficient technique[76]. What is more, it requires the availability of the antimicrobial agents to be tested as pure substances and individual mistakes in the preparation of stock concentrations or dilution series can occur, resulting in variability of results[76].

For comparison, in broth dilution method microorganisms are grown in liquid nutrient medium containing increasing concentrations (typically a two-fold dilution series) of the antimicrobial agent, which is then inoculated with a defined number of microbial cells[75, 77]. Depending on the final volume of the liquid medium in each analyzed sample, this method can be termed as macro-dilution for a total volume of 2 mL, or microdilution, if performed in microtiter plates format with total volume up to 500 µL per well[75, 77]. In broth dilution method, the growth is assessed after incubation of inoculated samples for a defined period of time (16–20 h) and the MIC or EC_{50} value is read. Moreover, for this purpose, antimicrobial agent-free test samples - which serve as growth controls - must be included in each assay. In broth dilution method the toxicity of compounds is determined by measuring the mortality or total number of viable cells after certain exposure time to specific concentrations of antimicrobial agents[75, 77]. The schematic representation of broth microdilution procedure is shown in Fig. 4. This technique can be used to test the susceptibility of microorganisms to multiple chemicals at once and quantitative data are obtained[76]. Another advantage of broth dilution is its high accuracy[76]. Other advantages include the possibility of performing this test in practically every laboratory, the easiness of testing and evaluating and the ability for the results of some tests to be read in automatic mode[76]. However, as in agar dilution, this method can be time consuming and individual mistakes in the preparation of stock concentrations or dilution series may take place especially when no automation equipment is available[76].

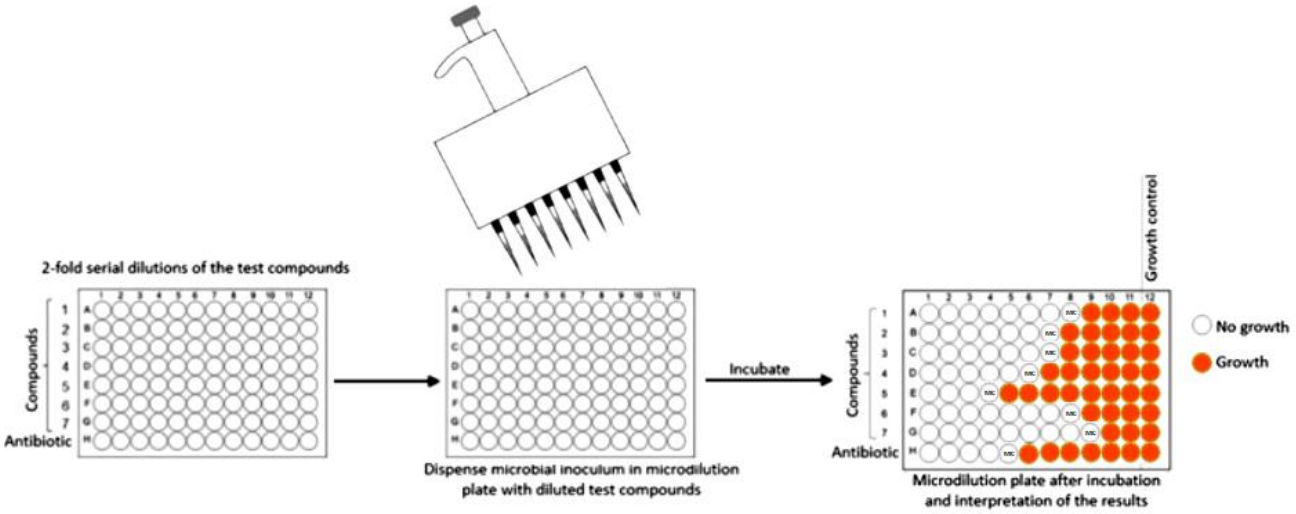

Fig. 4: Broth microdilution procedure for MIC determination.

Furthermore, there exist various methods for determination of the number of viable cells after incubation of tested microorganism with tested compounds. The cells viability can be evaluated using simple visual inspection or absorbance measurement of turbidity, and the obtained results that could be over- or underestimated due to, for example, turbidity of the compounds itself, can be further confirmed by subculturing of each tested concentration to agar plates that do not contain the test agent. By doing this it is possible to determine minimum bactericidal concentration (MBC) or minimum fungal concentration (MFC). MBC or MFC is complementary method to the MIC determination using broth dilution technique. MBC/MFC demonstrates the lowest concentration of antimicrobial agent that results in complete microbial death. This means that even if a particular MIC shows inhibition, plating the microbes onto agar might still result in organism proliferation because the antimicrobial agent did not cause death of all cells of tested microorganism. Moreover, for cells viability determination more accurate assays that employs colorimetric, or fluorescence dyes can be used. Such assays provide not only more accurate data but also the confirmation of the results by MBC/MFC determination could be avoided because

after staining it is possible to distinguish between living and dead cells. Therefore, the summary of literature results for DESs toxicity assayed by agar and broth dilution, with special respect to the cell viability determination methods used in each cited study, will be provided in the next subsections.

2.2.1.1. Visual or absorbance determination of cell viability based on turbidity

To date, in most of the published works, where the toxicity of DESs was examined with use of broth dilution method, the cells viability was determined either by visual inspection or by measuring the absorbance of the samples in the absence and presence of DESs. The summary of the results found in the literature for microbial toxicity of DESs determined by broth dilution technique and visual or absorbance determination of cell viability are presented in Table 2. In the first work conducted by Wen et al. broth macro-dilution was used to determine EC_{50} for series of ChCl- and cholinium acetate (ChAc)-based DESs against *E. coli* DH5α[39]. The bacterial growth was ascertained by measuring the absorbance of the samples at 550 nm. This study revealed that DES concentrations below 75 mM were almost non-toxic to the bacterial cells since the inhibition index was lower than 10%[39]. Furthermore, it was observed that 0.75 M DES inhibited the growth of 72.8–93.8%, indicating that at higher concentration DESs become significantly hazardous to *E. coli*[39]. The calculated EC_{50} values varied for different tested DESs and were mainly dependent on HBA used in DES formation. In general, DESs prepared with ChAc had lower EC_{50} values than respective ChCl-based DESs, indicating higher antibacterial activity of the former[39]. Moreover, the obtained results revealed that beside HBA also HBD has influence on DESs toxicity effect[39]. In particular, much higher EC_{50} values were obtained for DESs which have ethylene glycol (EG) in their composition (EC_{50} = 532.0 mM for ChCl:EG and EC_{50} = 281.1 mM for ChAc:EG)[39]. Overall, the most toxic compound was ChAc:glycerol

DES with EC_{50} of 58.0 mM, followed by ChAc:acetamide (EC_{50} = 97.2 mM)[39]. The obtained results also showed that bacterial cells of *E. coli* were more susceptible to the DESs than their individual components because the EC_{50} values following exposure to individual DES components were all much higher than 800 mM[39]. In this work, the authors hypothesized that DESs inhibited the bacterial growth by interacting with the cellular membrane. Furthermore, the fact that DES in aqueous solution may be partially dissociated was considered and the obtained results explained as a consequence of the possible interaction of the cholinium cation with the polysaccharide or peptide chains of peptidoglycan through hydrogen-bonding or electrostatic interaction, leading to cell wall distortion or disruption[39]. On the other hand, the higher toxicity of DESs than their individual components was assigned to charge delocalization through hydrogen bonding[39].

In another work, Lou's group used broth macro-dilution technique to quantitatively evaluate the toxicity of seven acid-based DESs, which were previously shown to inhibit bacterial growth as determined using disk diffusion assay[49]. In this study MIC values were obtained by measuring absorbance at 600 nm of the samples incubated with 8−52 mM (at 2 mM intervals) DESs solutions. The obtained results indicated that MIC values for gram-negative bacteria (*E. coli* and *S. enteritidis*) were generally lower than those for gram-positive bacteria (*S. aureus* and *L. monocytogenes*) and thus the studied DESs were more toxic to the tested gram-negative bacteria[49]. The ChCl:*p*-toluenesulfonic acid (PTSA) and the ChCl:malonic acid DESs had the highest MIC value from the studied DESs. Furthermore, it was observed that the MIC values increased with elongation of the carbon chain for ChCl:oxalic acid and ChCl:malonic acid DESs[49]. Moreover, DESs toxicity was related with the chemical structure of HBD used and introduction of an extra hydroxyl group in the HBD resulted in a slight increase in antibacterial

activity as observed for ChCl:malic acid and ChCl:tartaric acid DESs[49]. Overall, ChCl:oxalic acid, ChCl:levulinic acid, and ChCl:citric acid had the highest toxicity towards tested bacteria and the potency of antibacterial activity of the various ChCl-based DESs was associated with pH and to some extent to the chemical structure of HBDs[49]. After MIC determination, the bacterial suspension in the plate was cultured and MBC values for tested DESs were obtained. As it can be seen in Table 2, much higher concentrations of DESs were necessary to kill ≥99.9% of the test bacterium. In general, the obtained results confirmed that ChCl:PTSA and ChCl:malonic acid DESs exhibited the lowest toxicity towards tested genus of bacteria with MBC values ranging from 28.0-50.0 mM and 20.0-48.0 mM for ChCl:PTSA and ChCl:malonic acid, respectively[49].

Later, the broth microdilution technique was used to study the antibacterial activity of fatty acid-based DESs[60]. In this work, the results obtained from qualitative analysis done using disk diffusion assay were taken into account and MIC values were determined for 3 bacterial strains: *S. aureus* ATCC 25923, *S. aureus* ATCC 700698 (Methicillin-resistant strain, MRSA), *S. epidermis* ATCC 35984 (Methicillin-resistant strain, MRSE)[60]. The obtained MIC values for the DESs revealed that capric acid:lauric acid DES had the highest overall antimicrobial activity and was followed closely by capric acid:myristic acid and finally capric acid:stearic acid DES, which was the least toxic against studied bacteria[60]. Moreover, it was observed that DESs were usually less toxic than their individual components. Regarding DESs antibacterial activity for each of the tested bacteria, the MIC values indicated that these solvents were more toxic to the *S. aureus* than to the *S. aureus* MRSA and *S. epidermis* MRSE strains, which were, as expected, more competitive microorganisms due to their resistance to Methicillin[60]. The authors assumed that antimicrobial potential of DESs is derived from the non-specific antimicrobial action mechanism of fatty acids since they can lead to membrane destabilization/dissolution

causing a wide range of direct and indirect inhibitory effects[60]. Furthermore, it was also emphasized that for the studied DESs, and at the dilutions used, the vast network of intermolecular interactions was not weakened or disrupted, suggesting that the obtained MIC values are the effect of DESs interaction with bacterial cells and not mixture of their individual components[60]. The MBC study further confirmed that capric acid:lauric acid DES was the most toxic tested solvent and MBC values of 1250 µg/mL were obtained for all studied bacteria[60].

In another work of Silva et al., the authors further studied the antibacterial activity of DESs, and they selected THEDES composed of menthol and stearic acid[67]. After initial experiments using disk diffusion method, the MIC data for THEDES and its individual components against *S. aureus* ATCC 25923, *S. aureus* MRSA and *S. epidermis* MRSE using broth macro-dilution were gathered. According to the obtained results, the observations made from disk diffusion study were confirmed, and menthol was found toxic to the bacteria with MIC value of 4 and 8 mM for *S. aureus* ATCC 25923 and *S. aureus* MRSA, *S. epidermis* MRSE, respectively[67]. Furthermore, stearic acid did not exhibit any antibacterial activity[67]. THEDES showed antimicrobial activity against all the studied bacteria, being more efficient against *S. aureus* ATCC 25923 than Methicillin-resistant strains tested (*S. aureus* MRSA, *S. epidermis* MRSE)[67]. It was also observed that THEDES was more toxic to bacteria than menthol, even though the THEDES contains lower concentration of menthol than this needed to inhibit bacterial growth menthol itself[67]. This same was valid as far it comes to the anti-bactericidal properties of the studied THEDES and MBC values of 6.52 mM and 13.03 mM were obtained for *S. aureus* ATCC 25923 and both Methicillin-resistant strains tested, respectively. Therefore, it was concluded that it was an effect of a synergistic interaction between menthol and stearic acid that

493 increases antibacterial activity[67]. The toxicity of another THEDES (ChCl:mandelic acid) was
494 also studied by Mano and co-workers[78]. According to the MIC values obtained with broth
495 macro-dilution experiments, this THEDES was less toxic to *E. coli* and *S. aureus* than mandelic
496 acid with MIC of 5 and 2.5 mg/mL for both bacteria, respectively[78]. These results suggested
497 that the antibacterial activity of mandelic acid decreases when it is part of the supramolecular
498 THEDES structure with ChCl because of antagonistic effect[78].

Table 2. The toxicity of DESs determined by broth dilution method.

DES	Microorganism											
	Bacterium G(-)						Bacterium G(+)				Fungi	
	*Escherichia coli**		*Staphylococcus aureus**		*Listeria monocytogenes*		*Salmonella enteritidis*		*Salmonella typhimurium*	*Staphylo-coccus aureus* MRSA	*Staphylo-coccus epidermis* MRSE	*Aspergillus niger (filamentous fungus)*	*Candida albicans* (yeast)
	EC_{50} [mM]											
ChCl:urea (1:1),	295.9											
ChCl:acetamide (1:1),	275.2											
ChCl:glycerol (1:1),	532.0											
ChCl:ethylene glycol (1:1),	434.4											
ChAc:urea (1:1),	275.8											
ChAc:acetamide (1:1),	97.2											
ChAc:glycerol (1:1),	281.1											
ChAc:ethylene glycol (1:1)	58.0											
	MIC [mM]	MBC [mM]	MIC [mM]	MBC [mM]	MIC [mM]	MBC [mM]	MIC [mM]	MBC [mM]				
l:PTSA (1:1),	18	28	18	34	30	50	26	40				
l:oxalic acid (1:1),	12	18	12	26	14	30	12	22				
l:levulinic acid (1:2),	12	16	14	22	12	36	12	26				
l:malonic acid (1:1),	18	20	16	30	24	48	20	34				
l:malic acid (1:1),	14	20	14	24	22	48	18	42				
l:citric acid (1:1)	12	20	12	28	20	42	16	38				

ChCl:tartaric acid (2:1)	14	18	12	20	16	44	18	40								
			MIC [μg/mL]	MBC [μg/mL]						MIC [μg/mL]	MBC [μg/mL]	MIC [μg/mL]	MBC [μg/mL]		MIC [μg/mL]	MFC [μg/mL]
capric acid:lauric acid (2:1)			625	1250						625	1250	625	1250		625	1250
capric acid:myristic acid (3:1)			625	1250						625	1250	625	1250		1250	2500
capric acid:stearic acid (4:1)			1250	2500						1250	2500	1250	2500		1250	2500
	% of cell proliferation		% of cell proliferation						% of cell proliferation							
ChCl:glycerol (1:1.5)	100		54.92±2.72						100							
ChCl:glycerol (1:3)	100		53.49±3.14						100							
ChCl:formic acid (1:1.5)	100		47.65±2.84						97.78±1.71							
ChCl:formic acid (1:3)	100		44.75±4.95						98.55±1.88							
ChCl:lactic acid (1:1.5)	100		52.45±3.47						96.29±2.30							
ChCl:lactic acid (1:3)	100		50.73±2.63						100							
	MIC [mM]															
acetylcholine chloride:acetamide (1:2)	600															
														MIC [mg/mL]		
ChCl:ethylene glycol (1:2)														325.3±34		
l:glycerol (1:2)														550.4±51		
l:urea (1:2)														138.5±23		
:ethylene glycol (1:2)														314.8±44		
:glycerol (1:2)														495.4±63		
:malonic acid (1:1)														64.4±14		

EAC:ZnN (1:1) EAC:$ZnCl_2$ (1:2)										<2.2 <1.3	
		MIC [mM]	MBC [mM]				MIC [mM]	MBC [mM]	MIC [mM]	MBC [mM]		
menthol:stearic acid (8:1)		3.26	6.52				6.52	13.03	6.52	13.03		
	MIC [mg/mL]	MIC [mg/mL]										
ChCl:mandelic acid (1:2)	5	5										
	MIC [µL/mL]	MIC [µL/mL]										
perillyl alcohol:camphor (1:1)	31.25	31.25										
menthol:perillyl alcohol (1:1)	31.25	62.50										
menthol:camphor (1:1)	62.50	62.50										
menthol:eucalyptol (1:1)	62.50	62.50										
menthol:myristic acid (8:1)	62.50	62.50										
	MIC	MIC										
malic acid:sucrose:water (1:1:18)	1:1 (v/v)	1:1 (v/v)										
fructose:glucose:water 7)	Non-toxic	Non-toxic										
ose:sucrose:water 15)	Non-toxic	Non-toxic										

References in order of appearing in the table: [39], [49], [60], [79], [80], [51], [67], [78], [81], [82].

e that for *E. coli* and *S. aureus* bacterial species in some studies different strains were selected e.g. *E. coli* DH5α[39], *E. coli* ATCC 25922[79], *E. coli* BL21 (DE3)[80], *E. coli* K12 1498[78], *E. coli* ATCC 8739[81].

In the work of Teh et al., broth microdilution method was used to determine the toxicity of DESs prepared with ChCl as HBA and glycerol, formic acid, lactic acid as HBDs towards three bacterial species (*E. coli*, *S. aureus* and *Salmonella typhimurium*)[79]. Here, contrary to the most studies where MIC or EC values were obtained, the authors decided to determine the percentage of cell proliferation by measuring the absorbance at 600 nm of the samples incubated and not incubated with 1 mg/mL DESs solutions[79]. The obtained results showed that all studied DESs were almost non-toxic to both the gram-negative bacterial strains - *E. coli* and *S. typhimurium* - and more than 95% of cell viability after incubation was achieved[79]. These results were assigned to the structure of outer membrane of the gram-negative bacterial strains made up of lipopolysaccharide and protein[79]. It was assumed that *E. coli* and *S. typhimurium* formed a formidable barrier which restricted the attack of DESs from penetrating into the bacterial cell envelopes[79]. On the other hand, ChCl-based DESs were shown to be toxic to the gram-positive *S. aureus* at the same concentration because no barrier was established as its cell wall consists solely of a thick peptidoglycan layer, which seems to be more susceptible to DESs[79]. Additionally, all the studied DESs had comparable antibacterial activity against *E. coli* and *S. typhimurium* as their individual components, while for the *S. aureus* the lower toxicity was obtained for the DESs than for HBDs themselves[79]. In general, it was concluded that DESs toxicity is mainly dependent on the type of HBDs and very little on the HBA:HBD molar ratio used[79].

The toxicity of ChCl-based DESs towards *Kurthia gibsonii* was also assessed by broth macro-dilution in the work of Lou's group[83]. In this study, the bacterial growth was determined by measuring the absorbance at 600 nm and the results were expressed in terms of relative biomass, with the biomass in the DESs-free broth being defined as 100%[83]. The obtained results

498 revealed that the addition of DESs at 2% concentration did not significantly affected the bacterial
499 growth for all tested DESs except for ChCl:1,4-butanediol[83]. In case of ChCl:urea,
500 ChCl:glycerol and ChCl:triethylene glycol a slight decrease in the absorbance was observed
501 while for ChCl:ethanediol the absorbance increased slightly[83]. On the other hand, a visibly
502 higher absorbance was achieved in the system containing 2% ChCl:1,4-butanediol DES in
503 comparison to the control sample, thus the effect of other DES concentrations (4%, 8%, 12%,
504 16%, 20%) was further studied[83]. It was observed that the increase in the ChCl:1,4-butanediol
505 concentration decreased the growth of *K. gibsonii* and approximately 10% biomass of the control
506 at 20% of this DES was obtained[83]. Overall, it was concluded that the studied ChCl-based
507 DESs are non-toxic to *K. gibsonii*, and that a moderate concentration of adequate solvent can
508 increase the cellular growth[83]. Moreover, in order to further examined the effect of DESs on
509 these bacteria, the colorimetric determination of the damaged and dead cells was also performed,
510 as discussed in section 2.2.1.2.

511 In another study by Torregrosa-Crespo et al. the antimicrobial activity of acetylcholine
512 chloride:acetamide DES was examined[80]. The authors selected *Escherichia coli* BL21 (DE3)
513 as a model microorganism and used broth macro-dilution method to quantify potential toxicity of
514 the DES. Furthermore, in this work continuous monitoring of pH, temperature, shaking and
515 optical density of bacterial culture have been done to better understand the effect of DES on
516 bacterial cells survival[80]. Also, for the first time the degree of the cellular tolerance to the DES
517 was studied as experiments in preadapted and non-preadapted cells were conducted[80]. The
518 obtained results showed that at concentrations up to 300 mM the DES did not have toxic effect
519 towards *E. coli* and cellular preadaptation was crucial for the cells to grow[80]. Moreover, the
520 bacterial growth was still observed at concentrations between 300 mM and 450 mM, although

cellular growth and metabolic activities were slightly affected by such high DES concentrations as indicated with diauxic or triauxic growth curves and higher Lag times than those observed at lower DES concentrations[80]. However, the concentrations higher than 600 mM were found to be toxic, as complete inhibition of growth was observed[80]. The authors concluded that DES toxicity was a result of not only the chemical composition of the DES, but also the highly acidic pH of the growth medium supplemented with the DES[80].

In the most recent work, the toxicity on plant bacteria (*Xanthomonas campestris* CECT 97, *Erwinia amylovora* CECT 222, *Erwinia toletana* CECT 5263, *Clavibacter michiganensis* subsp. *michiganensis* CECT 790, *Clavibacter michiganensis* subsp. *insidious* CECT 5042, *Rhizobium radiobacter* CECT 4119, *Pseudomonas syringae* CECT 4429, *Pseudomonas savastanoi* CECT 5019) of six DESs namely ChCl:sucrose, ChCl:xylitol, fructose:glucose:sucrose (1:1:1), fructose:glucose:sucrose (2:3.6:1) betaine:sucrose (2:1), betaine:sucrose (4:1) was evaluated by broth microdilution method and the obtained results compared to the toxicity of classic solvents e.g. dimethylsulfoxide (DMSO), ethanol and glycerol[84]. It was revealed that most of the tested DESs were not toxic to the tested bacteria with MIC values 300-1200 $x10^3$ mg/L[84]. The biofriendly character of DESs composed of carbohydrates (fructose:glucose:sucrpose (1:1:1) and frucrose:glucose:sucrose (2:3.6:1) was assigned to the fact that their components e.g. glucose, fructose and sucrose are used as nutrition sources by these microorganisms[84]. Furthermore, betaine:sucrose (4:1) DES was the most toxic of DESs tested, with MIC values between 38-150 $x10^3$ mg/L[84]. In general, the following order of increasing toxicity of DESs was deducted: fructose:glucose:sucrose (1:1:1) = fructose:glucose:sucrose (2:3.6:1) < ChCl:sucrose (1:2) < ChCl:xylitol (2:1) < betaine:sucrose (2:1) < betaine:sucrose (4:1)[84]. Moreover, these DESs showed lower toxicity than glycerol or DMSO for most tested bacteria[84]. Even though, the

majority of the selected bacteria were gram-negative (except for the *Clavibacter* spp.), it was concluded that the toxic effects of DESs mainly depended on the type of compounds used in their preparations and on the susceptibility of the different bacteria strain and not on the cell membrane composition[84].

The toxicities of NADESs were also studied by broth microdilution in the work of Rodrigues and co-workers[81]. In this study, terpene-based NADESs, namely perillyl alcohol:camphor, menthol:perillyl alocohol, menthol:camphor, menthol:eucalyptol, menthol:myristic acid, were tested against *E. coli* and *S. aureus* bacterial strains. It was observed that all NADESs inhibited the growth of *E. coli* and *S. aureus*, with MICs ranging from 31.25 to 62.50 μL/mL[81]. Perilllyl acid:camphor NADES exhibited the highest antimicrobial activity from all studied NADESs[81]. Moreover, no significant differences in MICs were found for gram-positive and gram-negative bacteria[81]. The authors explained these results as a consequence of the antimicrobial effect of NADES starting materials – terpenes and fatty acids – which are well known antimicrobial agents against both gram-positive and -negative bacteria[81]. Later, Rachmaniah et al. studied the toxicity of malic acid:sucrose, fructose:glucose and fructose:sucrose NADESs towards *E. coli* and *S. aureus* bacterial strains[82]. In this work, broth macro-dilution method was used to determine MIC values and the obtained results revealed that malic acid:sucrose NADES had the highest toxicity of the studied solvents[82]. The high antimicrobial activity of this solvent was assigned to low pH of this NADES mainly derived from malic acid[82]. Meanwhile, both NADESs composed entirely of sugars, i.e. fructose:glucose and fructose:sucrose, were found non-toxic to bacterial strains used [82]. Beside higher pH of sugar-based NADESs, these results were also explained by the fact that carbohydrates (especially glucose and fructose) are the sources of carbon and energy for the growth of bacterial cells[82]. Furthermore, the MBC test

was applied to determine if studied NADESs possess ability to completely (>99.99 %) suppress bacterial growth. The obtained results showed the eradication of bacterial growth for malic acid:sucrose NADES, while the bacterial growth was not effected by fructose:glucose and fructose:sucrose NADESs[82].

Both agar and broth dilution methods were also used to study DESs antifungal activity[51, 60, 84-86]. Firstly, Hayyan's group examined the toxicity of eight different DESs using ChCl and EAC as the HBAs and ethylene glycol, glycerol, urea, malonic acid, zinc chloride ($ZnCl_2$), and zinc nitrate hexahydrate (ZnN) as the HBDs towards *Aspergillus niger*[51]. According to the MIC data obtained by using broth macro-dilution method all the DESs were shown to be toxic to the examined fungi and the antifungal activity of EAC- based DESs was higher than ChCl-based DESs[51]. Furthermore, it was observed that EAC-based DESs that were prepared using $ZnCl_2$, ZnN and malonic acid as HBDs were way more toxic than these prepared with ethylene glycol and glycerol[51]. The obtained MIC data also revealed that both HBAs (ChCl and EAC) were less toxic to *A. niger* than their respective DESs, while antifungal activities were slightly higher (for the EAC- based DESs) or lower (for the ChCl- based DESs) than those of their corresponding HBDs[51]. Overall, it was concluded that DES individual components play an important role in the toxicity profile of these solvents, as well as their concentration and specific interactions with microorganisms[51]. Later, Silva et al. determined the MIC and MFC values for DESs based on fatty acids, which according to disk diffusion assay inhibited the growth of *Candida albicans* yeast cells[60]. The obtained MIC/MFC data acquired by using broth microdilution method revealed that capric acid:lauric acid DES had the highest antifungal activity from all studied DESs[60]. The following order of the DESs toxicity against examined yeast was deducted: capric acid:lauric acid > capric acid:myristic acid ≈ capric acid:stearic acid[60].

590 Interestingly, this is not the same order as this obtained using disk diffusion assay (capric
591 acid:stearic acid > capric acid:lauric acid > capric acid:myristic acid)[60]. Furthermore, also the
592 DESs individual components possessed significant MIC values, while these fatty acids displayed
593 no activity during the disk diffusion assay[60]. This observation clearly indicates that a negative
594 result in the disk diffusion assay does not necessarily exclude toxicity of some compounds and
595 highlight the need of further analysis by broth dilution method[60]. The broth macro-dilution
596 method was also used to evaluate toxicity of NADES composed of lactic acid:glucose towards *C.*
597 *albicans*[85]. It was shown that this solvent is non-toxic to yeast cells, because at the dilutions
598 used, the growth of *C. albicans* was not inhibited[85]. Furthermore, in the work of Boiteux et al.
599 the toxicity of this same NADES towards *Botrytis cinerea* was evaluated using agar dilution
600 method[86]. Once again, the obtained results showed that all seven tested dilutions of NADES
601 did not present antifungal effect and thus this NADES can be considered as non-toxic to *B.*
602 *cinerea*[86]. Recently, Rodriguez-Juan et al. also studied the toxicity of DESs against seven
603 yeasts present in wine fermentation, namely *Saccharomyces paradoxus* CECT 1939,
604 *Hanseniaspora guillermondi* CECT11102, *Hanseniaspora uvarum* CECT 10389, *Metschnikowia*
605 *pulcherrima* CECT12890, *Torulaspora delbrueckii* CECT 10589, *Saccharomyces cerevisae* EC
606 1118 and *Starmerella bombicola* CBS 268[84]. Here, various DESs combining ChCl,
607 carbohydrates, betaine, alcohols as HBAs and HBDs were selected and MICs determined using
608 broth microdilution[84]. The obtained results can be summarized to the following order of
609 increasing toxicity: fructose:glucose:sucrose (1:1:1) = fructose:glucose:sucrose (2:3.6:1) =
610 betaine:sucrose (2:1) < ChCl:sucrose (1:2) < ChCl:1,2-propanediol (1:1) < ChCl:xylitol (2:1) <
611 ChCl:1,4-butanediol (1:5)[84]. As expected, all tested DESs that contained carbohydrates in their
612 composition were found to be practically not toxic to the tested yeasts with MIC values of 600
613 $x10^3$ mg/L[84]. Astonishingly, betaine:sucrose DES had the same MIC value of $600x10^3$ mg/L as

fructose:glucose:sucrose (1:1:1) and fructose:glucose:sucrose (2:3.6:1) and thus did did not show any toxic effect on tested yeast, while it the same DES was found moderately toxic to the plant bacteria, as discussed earlier[84]. Overall, it was observed that the tested yeasts were usually less susceptible to DESs than conventional solvents such as DMSO and glycerol, making these solvents an interesting candidates for use for example in cryoprotection[84].

2.2.1.2. Colorimetric determination of cells viability

Until now there are only five published works (see Table 3) where cells viability after incubation with DES solutions using colorimetric techniques was performed[50, 83, 87-89]. In first report baker's yeast (*Saccharomyces cerevisiae*) viability in different cholinium-based DESs containing 50% of water (w/w) and potassium phosphate buffer (100 mM, pH 7.4) was determined at 3 and 24 h after inoculation[50]. For that the cell suspension was mixed with an equal volume of methylene blue and incubated for 5 min at room temperature. Here, methylene blue dye was used to stain the yeast cells, however this dye can be applied to all aerobic microorganisms[90]. Methylene blue in a presence of living cells gets enzymatically reduced to a colorless product and living cells become unstained, whereas dead cells are stained blue[90]. Therefore, after staining with methylene bleu, blue-colored cells can be easily visualized and counted as dead cells. In the work of Redovniković's group, it was observed that ChCl:malic acid, ChCl:oxalic acid and ChCl:urea DESs were toxic to the yeast cells[50]. Already after 3 hours of incubation yeast cells viability decreased tremendously for these solvents and the most detrimental toxic effect was observed for ChCl:oxalic acid DES with only 19% and 4% of living cells after 3 h and 24 h, respectively[50]. On the other hand, no significant toxic effect was observed for DESs formed using sugars, glycerol and ethylene glycol as HBDs with yeast viability of 76–99% and 62–98% after 3 and 24 h incubation, respectively[50]. Furthermore, the comparable viability of yeast in

637 ChCl:ethylene glycol and ChCl:glucose after 24 h, as in control samples in potassium phosphate
638 buffer (100 mM, pH 7.4), was observed[50]. The toxicity of DESs was assigned to the high
639 osmotic pressure imposed on the yeast cells by such high concentrations of these solvents,
640 resulting in diffusion of water out of the cells[50]. Furthermore, the differences in the potency of
641 antifungal activity for different DESs was explained by differences in the pH values of the
642 solvents[50]. Consequently, DESs prepared with organic acids as HBDs were the most toxic to
643 yeast cells due to their pH values (pH < 3) lower than the optimum pH range for *S. cerevisiae*
644 growth (between 4 and 6)[50]. Contrastingly, the pH values for DESs containing carbohydrate
645 and glycerol were around 4.5 thus resulting in lower toxicity of these DESs[50]. Moreover, non-
646 toxicity of these DESs was further explained by the fact that sugar and glycerol could be used as
647 a nutrition source for growth of yeast cells[50].

648 Table 3. The toxicity of DESs obtained using colorimetric assays for cell viability determination.

DES	**Microorganisms**			**Toxicity results**	**Ref.**
	Bacterium G(+)	**Bacterium G(-)**	**Fungi**		
ChCl:glycerol (1:2) ChCl:ethylene glycol (1:2) ChCl:oxalic acid (1:1) ChCl:malic acid (1:1) ChCl:glucose (2:1) ChCl:fructose (3:2) ChCl:xylose (2:1) ChCl:urea (1:2)			*Saccharomyces cerevisiae* (yeast)	• Acid and urea containing DESs highly decreased yeast cell viability and thus showed toxic effect on tested genus of yeast. • Carbohydrate, glycerol, and ethylene glycol containing DES showed good biocompatibility and 62–98% cell viability after 24 h was obtained. • The toxic effect of	[50]

				individual components of DESs was not assayed.	
ChCl:urea (1:2) ChCl:glycerol (1:2) ChCl:ethanediol (1:2) ChCl:triethylene glycol (1:4) ChCl:1,4-butanediol (1:4)	*Kurthia gibsonii* SC0312			• ChCl:urea, ChCl:triethylene glycol and ChCl:1,4-butanediol DESs slightly increased the number of damaged cells at 2% concentration. • ChCl:ethanediol and especially ChCl:glycerol highly decreased the bacterial cell viability at 2% concentration. • The toxic effect of individual components of DESs was not assayed.	[83]
ChCl:urea (1:2) ChCl:glycerol (1:2) ChCl:ethylene glycol (1:2)	*Arthrobacter simplex* TCCC 11037			• All the DESs showed relative toxic effect on tested genus of bacteria, and membrane integrity decreased to 70, 51, 39% for ChCl:glycerol, ChCl:ethylene glycol, ChCl:urea, respectively. • The toxic effect of individual components of DESs was not assayed.	[87]
menthol:decanoic acid (1:2)	*Staphylococcus aureus* ATCC 6538	*Escherichia coli* ATCC 8739		• This DES showed no toxic effect on tested genus of *E. coli* and was found toxic to *S. aureus*. • DES individual components showed no toxic effect on tested genus of *E. coli.* • DES individual components showed higher	[88]

				antibacterial activity against *S. aureus* than tested DES.	
ChCl:ethylene glycol (1:2) ChCl:malonic acid (1:2)	*Bacillus cereus* EMB20			• ChCl:ethylene glycol showed relative toxic effect on tested genus of bacteria, and 54% growth inhibition was observed. • ChCl:malonic acid was highly toxic and caused the death of all cells. • The toxic effect of individual components of DESs was not assayed.	[89]

In another work, the kit that consists of two dyes, propidium iodide (PI) and SYTO9, was used to evaluate the viability of cells after incubation with ChCl-based DESs[87]. These two dyes are able to stain nucleic acids, and green fluorescing SYTO9 can enter all cells of tested microorganism and is used to determine total number of its cells in the assayed sample, whereas red fluorescing PI enters only into the cells with damaged cytoplasmic membranes[91]. Even though this kit only enables differentiation between cells with intact and damaged cytoplasmic membranes, it is often used to distinguish viable and dead cells because it is accurate to assume that membrane-compromised cells are dead[91]. In this study, gram-positive *Arthrobacter simplex* TCCC 11037 was selected as model microorganism. The obtained results showed that the effect of ChCl-based DESs on the *A. simplex* cell membrane was different depending on the type of HBDs used[87]. For instance, the cells tolerated ChCl:glycerol DES better than ethanol (positive control), and the membrane integrity decreased to 70% compared with that in water (control sample)[87]. On the other hand, for DESs containing urea and ethylene glycol as HBDs,

the cell viability decreased to 39% and 51%, respectively[87]. Furthermore, these DESs were more toxic to bacteria than ethanol[87]. In general, the toxic effect of three ChCl-based DESs on *A. simplex* was found in this study and degree to which each solvent promoted toxicity was mainly dependent on the nature of the HBDs used in DESs preparation[87].

Furthermore, PI fluorescein dye was also used to evaluate the effect of ChCl-based DESs on the number of dead cells of *K. gibsonii*[83]. It was observed that compared with the control cells there was a slight increase in the number of damaged/dead cells for 2% of ChCl:triethylene glycol, ChCl:urea and ChCl:1,4-butanediol DESs[83]. On the other hand, more significant increase in the number of dead cells was observed for ChCl:ethanediol and ChCl:glycerol, suggesting that these two solvents are relatively toxic to this bacterium[83]. Moreover, it was shown that the effect of DESs on the cell viability is concentration dependent[83]. According to the experiments using different concentrations of ChCl:1,4-butanediol, the number of damaged cells increased with the increased DES concentration, achieving its maximum value at 16% of DES[83]. Based on these data, it was suggested that the lower viability of cells in the presence of higher DESs concentrations was the result of the changed osmotic pressure in buffer[83].

Moreover, there also exist the test to study chemical toxicity that employs an electron acceptor dye, resazurin, which changes color in the presence of dehydrogenase enzyme activity resulting from procaryotic and eucaryotic cells actively growing in a culture medium[92]. Resazurin in the presence of an active viable cells of examined organisms, is oxidized by cell dehydrogenases to the resofurin[92]. Therefore, in such condition the analyzed samples changes color from blue (the color of resazurin) to pink (the color of resofurin)[92]. Thus, if the cells growth is inhibited by the presence in culture medium of chemical compound which toxicity is examined against selected organism, no reduction of the resazurin occurs, and such a sample would remain

blue[92]. Since resorufin absorbs only weakly at the wavelength giving the maximum absorbance for resazurin, the decrease in resazurin concentration may be measured using a spectrophotometer, and, by varying the concentration of the test chemical, the EC_{50} value for that chemical may then be estimated[92]. This approach was used to test toxicity of DES composed of menthol and decanoic acid towards *E. coli* and *S. aureus*[88]. Here, the resazurin dye was used for the cell viability determination and the MIC and MBC value reading due to the white and opaque nature of the samples. According to the results of experiments, neither DES starting materials or DES itself had and inhibitory effect on gram-negative *E. coli* at concentrations used in the assay (MIC and MBC > 500 μL/mL)[88]. On the other hand, for *S. aureus* the DES and its individual components exhibited high antimicrobial properties with MIC and MBC values ranging between 3.91-15.63 μL/mL and 7.81-31.25 μL/mL, respectively[88]. This higher antibacterial and -bactericidal efficacy of these compounds against gram-positive *S. aureus* was attributed to the hydrophobic nature of the DES starting materials and explained by the fact that usually gram-positive bacteria are more susceptible to hydrophobic compounds, whereas gram-negative to hydrophilic compounds taking advantage of the hydrophilic character of their membrane porins[88]. Furthermore, it was also observed that for *S. aureus* ATCC 6538 strain the MIC and MBC values for DES (MIC=15.63 μL/mL, MBC=31.25 μL/mL) were higher than the MIC and MBC values for menthol (MIC/MBC=7.81 μL/mL) and for decanoic acid (MIC=3.91 μL/mL, MBC=15.63 μL/mL), indicating that tested DES has a lower antibacterial and -bactericidal activity per volume of the mixture used when compared to its individual components[88].

In another work, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay was used to assess viability of bacterial cells growing in the presence or absence of DESs at a final

concentration of 0.5 mg/mL[89]. In this assay, MTT is reduced by actively respiring cells to water-insoluble purple formazan. The formazan is then solubilized, and its concentration determined by reading absorbance of prepared samples at 570 nm. Since activity of respiring cells is constant, an increase or decrease in the number of viable cells has a direct correlation with the number of formazan crystals. Here, two ChCl-based DESs, namely ChCl:ethylene glycol and ChCl:malonic acid, were selected and its effect on the inhibition of *Bacillus cereus* growth was studied[89]. The obtained results revealed that ChCl:ethylene glycol DES was moderately toxic and approximately 54% growth inhibition of *B. cereus* cells compared to control sample was observed[89]. On the other hand, in the case of ChCl:malonic acid DES, cellular growth was not observed thus this DES was considered highly toxic to *B. cereus* cells[89].

2.2.2. Microtox assay for luminescence inhibition

Microtox assay is an *in vitro* testing method which employs bioluminescent bacteria *Aliivibrio fischeri* to determine the toxicity of different substances[93]. *A. fischeri* are non-pathogenic, marine bacteria that luminesce as a natural part of their metabolism[93]. Since toxic chemicals disrupt the respiratory process of these bacteria, resulting in decrease in the light output, the change in luminescence compared to control untreated bacterial cells with tested chemicals can be used to calculate a percent inhibition of *A. fischeri* growth[93]. This approach is rapid, simple, and sensitive method. Furthermore, it uses a specific clonal strain of bioluminescent bacteria prepared in a lyophilized vial format, increasing their shelf life and usability[93]. *A. fischeri* have demonstrated high sensitivity across a wide variety of substances, including DESs[40, 94-96]. The summary of the results found in the literature for toxicity of DESs towards *A. fischeri* determined by Microtox assay are presented in Table 4.

For the first time, the DESs ecotoxicity was assessed using the Microtox test in the work of de Morais et al.[40]. In this study, the toxicity of DESs based on the HBA - ChCl - and different organic acids (acetic acid (AA), lactic acid (LA), citric acid (CA), and glycolic acid (GA)) as HBDs was examined[40]. The obtained EC_{50} values indicated that all studied DESs were relatively toxic to *A. fischeri*, which is contrary to the generalized idea that DESs are of low toxicity[40]. The following order of toxicity for DESs with different molar ratios and their individual components was deducted: ChCl ≪ ChCl/acid (2:1) < ChCl/acid (1:1) < ChCl/acid (1:2) < acid, indicating that DESs had an intermediate value of toxicity when compared to the starting materials (acids and ChCl)[40]. Furthermore, it was observed that DES toxicity increased with an increase in concentration of the acid (the mole ratio of ChCl:acid)[40]. As far it comes to the HBD used in DES preparation, the following antibacterial activity order was obtained: ChCl/AA < ChCl/LA < ChCl/GA < ChCl/CA, which is in agreement with the decreasing order of the lipophilicity of the acid[40]. The obtained EC_{50} values showed that the effect of the acid used in DES preparation is preponderant in the toxicity because the toxic effect for the various DESs was similar to that of their corresponding organic acids separately[40]. The authors explained these results as a consequence of low pH values of the DESs containing organic acids and thus having a negative effect on the cell activity, through denaturation of proteins[40]. Furthermore, these DESs were more toxic than the respective ILs, namely, choline acetate (ChAc), choline lactate (ChLa), choline citrate (ChCit), and choline glycolate (ChGly) and it was hypothesized that it is a consequence of hydrogen bonding between the mixture compounds and the respective charge delocalization, since chemicals having delocalized charges are more toxic than chemicals with localized charges[40]. Overall, it was concluded that DESs might not be as “green” as generally it was assumed.

754 Table 4. The toxicity of DESs towards *Aliivibrio fischeri*.

DES	EC_{50} [mg/L] 30 min	Ref.
ChCl:acetic acid (1:2)	130	[40]
ChCl:lactic acid (1:2)	34	
ChCl:glycolic acid (1:2)	30	
ChCl:citric acid (1:2)	16	
ChCl:acetic acid (1:1)	197	
ChCl:lactic acid (1:1)	62	
ChCl:glycolic acid (1:1)	33	
ChCl:citric acid (1:1)	22	
ChCl:acetic acid (2:1)	337	
ChCl:lactic acid (2:1)	67	
ChCl:glycolic acid (2:1)	62	
ChCl:citric acid (2:1)	32	
ChCl:ethylene glycol (1:1)	67806	[94]
ChCl:ethylene glycol (2:1)	90343	
ChCl:ethylene glycol (1:2)	41821	
ChCl:ethylene glycol (1:4)	48653	
ChCl:glycerol (1:1)	76726	
ChCl:glycerol (2:1)	90156	
ChCl:glycerol (1:2)	104612	
ChCl:propionic acid (1:1)	20	
ChCl:propionic acid (2:1)	8	
ChCl:propionic acid (1:2)	12	
ChCl:propionic acid (1:4)	6	
ChCl:1,2-propanodiol (1:1)	73492	
ChCl:1,2-propanodiol (2:1)	61342	
ChCl:1,2-propanodiol (1:2)	44048	
ChCl:1,2-propanodiol (1:4)	74309	
ChCl:urea (1:1)	59825	
ChCl:urea (2:1)	69924	
ChCl:urea (1:2)	41693	
ChCl:urea (1:4)	39810	
ChCl:1-propanol (1:1)	34708	
ChCl:1-propanol (2:1)	44487	

ChCl:1-propanol (1:2)	21271	
ChCl:1-propanol (1:4)	17352	
$[N_{1111}]$Cl:1-propanol (1:1)	20870	
$[N_{1111}]$Cl:1-propanol (1:2)	16150	
$[N_{1111}]$Cl:1-propanol (1:4)	15360	
$[N_{2222}]$Cl:1-propanol (1:1)	18090	
$[N_{2222}]$Cl:1-propanol (2:1)	22260	
$[N_{2222}]$Cl:1-propanol (1:2)	15550	
$[N_{2222}]$Cl:1-propanol (1:4)	9500	
$[N_{3333}]$Cl:1-propanol (1:1)	4981	
$[N_{3333}]$Cl:1-propanol (2:1)	1555	
$[N_{3333}]$Cl:1-propanol (1:2)	1845	
$[N_{3333}]$Cl:1-propanol (1:4)	1120	
$[N_{1111}]$Cl:ethylene glycol (1:1)	53990	[95]
$[N_{1111}]$Cl:ethylene glycol (2:1)	30200	
$[N_{1111}]$Cl:ethylene glycol (1:2)	49250	
$[N_{1111}]$Cl:ethylene glycol (1:4)	65620	
$[N_{2222}]$Cl:ethylene glycol (1:1)	23940	
$[N_{2222}]$Cl:ethylene glycol (2:1)	18930	
$[N_{2222}]$Cl:ethylene glycol (1:2)	18610	
$[N_{2222}]$Cl:ethylene glycol (1:4)	36390	
$[N_{3333}]$Cl:ethylene glycol (1:1)	3665	
$[N_{3333}]$Cl:ethylene glycol (2:1)	971	
$[N_{3333}]$Cl:ethylene glycol (1:2)	945	
$[N_{3333}]$Cl:ethylene glycol (1:4)	1285	
ChCl:glycerol (1:2)	86726	
ChCl:urea (1:2)	26346	[96]
ChCl:ethylene glycol (1:2)	108526	

In the following work, for the first time the mixtures toxicity theory was used to analyze the results obtained from Microtox test for ChCl-based DESs[94]. The Concentration Addition (CA) model of mixtures toxicity was applied since the dissociation of DESs in water was considered[94]. For that purpose, the EC_{50} values for both individual DES components and series combining them in different proportions to establish different DESs were acquired. The

761 performed analysis indicated that all DESs with the exception of ChCl:propionic acid (2:1 and
762 1:4 molar ratio) had antagonistic effect (regardless molar ratios involved), which means that DES
763 can be less toxic than either of their starting materials dosed separately[94]. This observation is
764 opposite to the most previously published works, where synergistic effect for DESs was mainly
765 reported. Furthermore, for some DESs mixtures the EC_{50} values were found to be between the
766 values for corresponding HBA and HBD (e.g., ChCl:ethylene glycol, ChCl:glycerol,
767 ChCl:propionic acid and ChCl:1,2 propanediol)[94], which is consistent with the work of de
768 Morais et al.[40]. On the other hand, for ChCl:urea and ChCl:1-propanol much higher
769 concentrations, than those found for both DESs individual components, were needed to induce
770 50% *A. fischeri* luminescence inhibition, making these DESs very promising and biocompatible
771 alternative solvents[94]. In general, it was concluded that the toxicity was mainly dependent on
772 DES composition, as well as on molar ratios of the starting materials[94]. It was also suggested
773 that the HBD may have a role in modulating the ecotoxicity of the DES, because different EC_{50}
774 values were obtained for different HBDs joined to ChCl. Moreover, lower concentrations were
775 necessary to induce 50% *A. fischeri* luminescence inhibition as HBD molar proportion increases
776 within each DES[94].

777 In their following study, Macario et al. further evaluated the ecotoxicological profile of DESs
778 based on $[N_{1111}]Cl$, $[N_{2222}]Cl$ and $[N_{3333}]Cl$ as HBAs combined with ethylene glycol and 1-
779 propanol as HBDs, through the Microtox test[95]. The gathered results showed that DESs were
780 not hazardous to *Aliivibrio fischeri*, as the EC_{50} values were above 100 mg/L[95]. Therefore,
781 these DESs can be considered as green solvents. Moreover, DESs toxicity followed the same
782 trend as observed for HBAs individually and an increase in the alkyl chain length of quaternary
783 ammonium salt resulted in increased toxicity of DESs ($[N_{1111}]Cl$-based DESs < $[N_{2222}]Cl$-based

DESs < [N_{3333}]Cl-based DESs)[95]. Accordingly, [N_{3333}]Cl-based DESs exhibited high overall toxicity towards *A. fischeri* compared to the other DESs under study[95]. This increased toxicity was most likely a consequence of decrease in hydrophilicity of the HBA from [N_{1111}]Cl to [N_{333}]Cl[95]. Furthermore, antagonism between HBA and HBD was observed for [N_{1111}]Cl-based DESs, while synergism for [N_{3333}]Cl-based DESs and for [N_{2222}]Cl:1-propanol[95]. It shows that DESs toxicity cannot be predicted based solely on the toxicity of the starting materials. The obtained results further highlighted that for these solvents both the HBD and HBA have an impact on DESs toxicity, agreeing with the study of Wen et al.[39].

The latest study carried out by Lapeña et al. was an attempt to further explore toxicity of ChCl-based DESs towards *A. fischeri*[96]. Similarly, to the work of Macario et al.[94] the authors selected DESs prepared using ChCl as HBA combined with urea, glycerol, and ethylene glycol as HBDs. Furthermore, DESs that contained water as third component were also prepared. The obtained EC_{50} values from the *A. fischeri* ecotoxicity test showed that the most toxic DES was ChCl:urea, followed by ChCl:glycerol, ChCl:urea:H_2O, ChCl:ethylene glycol, ChCl:ethylene glycol:H_2O and ChCl:glycerol:H_2O[96]. Nevertheless, for all DESs under study the EC_{50} values were higher than 25000 mg/L and for some higher than 100000 mg/L, indicating non-hazardous nature of the tested DESs to this species[96]. In the case of *A. fischeri*, the presence of water decreased the toxicity with respect to the three pure DESs studied[96]. Even though, there is one previous work in which the ecotoxicity of such DESs towards *A. fischeri* was evaluated, the direct comparison of the results is not possible. The dissimilarities in the obtained EC_{50} values are the outcome of differences in the experimental methodology used in both works. In the study of Lapeña et al. pH of the samples was controlled and adjusted to be in optimal range for the culturing of these bacteria (pH of 6–8.5)[96], while in the work of Macario et al. pH was not

controlled[94]. Thus, it could be hypothesized that usually lower EC_{50} values were obtained in the study of Macario et al.[94] because the severe effect of pH on the toxicity towards *A. fischeri* bacteria has been previously observed[97].

2.2.3. Drop plate method

Moreover, Wikene and co-workers for DESs' toxicity testing used a modified drop plate method (Table 5), which combines 24-well plates for serial dilutions, followed by drop plating on agar in a 4×4 format using an automatic spiral plater[98-101]. Afterwards, plates are left to dry for a few minutes and then placed into an incubator for 18–20 h (37°C). After incubation viable colony forming units (CFUs) are counted and numbers compared to control samples.

At first, bacterial toxicity of two NADESs, citric acid:sucrose and glucose:malic acid, was studied[98]. Here, bacterial strains of *E. coli* and *Enterococcus faecalis* were selected as model microorganisms. The obtained results showed that 100 times dilutions of these two NADESs were practically not toxic to bacteria and non-significant reduction in CFUs as compared to untreated control samples was observed[98]. Furthermore, it was noted that non-toxic effect of NADESs was not dependent on whether the aliquots from bacterial cultures used in the assay were in stationary or exponential phase of growth[98]. Later, the database for NADESs toxicity determined by drop plate method was further extended and toxic effect of glucose:sucrose and ChCl:maleic acid NADESs on *E. coli* was evaluated[99]. Carbohydrates-based NADES was found non-toxic to *E. coli* and no significant reduction in viable bacteria was observed[99]. On the other hand, the toxic effect of ChCl:maleic acid NADES was detected for solvent diluted 100 times[99]. Nevertheless, the bacterial cells tolerated well this NADES when treated with 200-fold dilution, suggesting that the antibacterial effect is concentration dependent[99]. In the following year, the drop plate method was used to study the antibacterial effect of ChCl:xylitol, malic

acid:fructose:glucose and citric acid:sucrose NADESs against *E. coli*, *E. faecalis* and *S. aureus*[100]. Here, the results obtained in the first work of Wikene et al.[98] were confirmed, and citric acid:sucrose NADES was found non-toxic to all three bacterial strains[100]. The same was valid for the other two NADESs under evaluation. At dilutions used in the experiments (400-fold and 200-fold for malic acid:fructose:glucose and ChCl:xylitol, respectively), these NADESs did not reduce significantly the number of viable bacteria as compared to the control samples prepared in PBS[100]. Lastly, the effect of citric acid:sucrose and malic acid:fructose:glucose NADESs on the viability of *E. coli*, *Klebsiella pneumoniae, S. epidermis*, *P. aeruginosa* bacteria and *C. albicans* yeast was studied[101]. The obtained results revealed that both NADES diluted 100 times reduced the survival of *E. coli* by 96% and 24% for citric acid:surcrose and malic acid:fructose:glucose, respectively[101]. Furthermore, it was observed that *E. coli* tolerated better citric acid-based NADES than an equimolar concentration of citric acid[101]. On the other hand, for malic acid-based NADES no significant differences in cell viability were seen compared to an equimolar concentration of malic acid[101]. Regarding sugar components of NADES, neither fructose, glucose nor sucrose showed effect on *E. coli* survival[101]. Both NADESs were also found toxic to *P. aeruginosa*, and no bacterial survival was observed for 200 times dilution. The toxic effect was further observed for *S. epidermidis*, however, these NADESs exhibited lower antibacterial potency than against *P. aeruginosa*, and 3-9% of cells survived the exposure to NADESs[101]. Moreover, citric acid:sucrose NADES reduced by 37% the bacterial survival of *K. pneumoniae* compared to the control, while malic acid:fructose:glucose NADES did not significantly affected the number of viable bacteria[101]. Finally, these NADESs did not show antifungal activity and no reduction in survival of *C. albicans* yeast was observed[101].

Table 5. The toxicity of NADESs determined using drop plate method.

NADES	Microorganisms			Toxicity results	Ref.
	Bacterium G(+)	Bacterium G(-)	Fungi		
citric acid:sucrose (1:1) glucose:malic acid (1:1)	*Enterococcus faecalis* ATCC 19433	*Escherichia coli* ATCC 25922		• All the NADESs showed no toxic effect on tested genus of bacteria. • The toxic effect of individual components of DESs was not assayed.	[98]
glucose:sucrose (1:1) ChCl:maleic acid (3:1)		*Escherichia coli* ATCC 25922		• Glucose:sucrose NADES showed no toxic effect on tested genus of *E. coli*. • ChCl:maleic acid NADES showed relative toxic effect on tested genus of *E. coli*. • The toxic effect of individual components of DESs was not assayed.	[99]
citric acid:sucrose (1:1) ChCl:xylitol (5:2) malic acid:fructose:glucose (1:1:1)	*Enterococcus faecalis* ATCC 19434, *Staphylococcus aureus* (strain Newman)	*Escherichia coli* ATCC 25922		• All the NADESs showed no toxic effect on tested genus of bacteria. • The toxic effect of individual components of DESs was not assayed.	[100]
citric acid:sucrose	*Staphylococcus*	*Escherichia coli*	*Candida*	• Citric acid:sucrose	[101]

(1:1) malic acid:fructose:glucose (1:1:1)	*epidermis* ATCC 35984	BW25113, *Klebsiella pneumoniae* ATCC 31488, *Pseudomonas aeruginosa* ATCC 9027	*albicans* ATCC CRM-10231	NADES showed relative toxic effect on tested genus of bacteria. • Malic acid:fructose:glucose NADES showed relative toxic effect on bacteria except *K. pneumoniae.* • Both NADESs showed no toxic effect on tested genus of yeast. • The toxic effect of individual components of DESs was not assayed.	

853

854 **2.3. FTIR-based biological assay**

855 Another method used for DESs toxicity testing is FTIR-based bioassay (see Table 6)[102, 103].
856 This assay was primarily based on *Saccharomyces cerevisiae* cells however it offers the
857 possibility to also use as biosensor the cells from different organisms, including different
858 microbial cells or mammal cell cultures[104]. The principles of this method are based on the fact
859 that cells under stress exhibit very fast changes in terms of cell metabolites and thus a
860 metabolomic analysis, using FTIR, may be capable of detecting these variations as early as in the
861 first hours of exposure[104]. This bioassay estimates the toxicity level as function of the FTIR
862 spectra variation of the cells upon exposition to the chemicals and provides metabolic indexes
863 which can be used for the classification and the relative quantification of the toxicity[104]. The
864 major benefit of FTIR-based assay is that it is a fast and reproducible procedure, which besides

the information whether chemical agent is toxic also provides more detailed metabolomic analyses necessary to elucidate the mechanisms on how the studied compounds promote toxicity towards selected microorganisms[104].

For the first time FTIR-based bioassay was applied to study DESs toxicity in the work of Cardellini and co-workers, where the authors evaluated the antifungal activity of novel DESs formed by zwitterionic trimethylglycine and high melting point carboxylic acids[102]. In this work the yeast strain *Saccharomyces cerevisiae* CBS 13873 was employed as target and model eukaryotic microorganisms. Preliminary studies showed that these DESs caused a very rapid decrease of cell viability after a short exposure times to the tested DESs, suggesting that these DESs are highly toxic to the cells[102]. Basing on these results, it was hypothesized that the high concentration of these solvents caused a very rapid exit of the cell water and consequently led to their inactivation[102]. In fact, this hypothesis was confirmed via FTIR-based assay since the normalized FTIR spectra from the yeast cells treated with DESs and $CaCl_2$ (a well- known non-toxic dehydrating agent) were almost identical[102]. This observation led to a conclusion that these DESs act as dehydrating agents on the model cells.

Table 6. The toxicity of DESs towards yeast cells determined using FTIR-based bioassay.

DES	Microorganisms	Toxicity results	Ref.
benzoic acid:betaine (1.5:1) salicylic acid:betaine (1.5:1) 4-chlorobenzoic acid:betaine (1.5:1) 2-chlorobenzoic acid:betaine (1.5:1) 3-chlorobenzoic acid:betaine (1.5:1) 2-furoic acid:betaine (2:1)	*Saccharomyces cerevisiae* CBS 13873	• All the DESs showed relative toxic effect on tested genus of yeast cells and acted as dehydrating agents. • The toxic effect of individual components of DESs was not assayed.	[102]

phenylacetic acid:betaine (2:1) D-(+)-mandelic acid:betaine (1:1) glycolic acid:betaine (2:1) oxalic acid:betaine (2:1) citric acid:betaine (1.5:1)			
aliphatic sulfobetaines:(1*S*)-(+)-10-camphorsulfonic acid aromatic sulfobetaines:(1*S*)-(+)-10-camphorsulfonic acid amphiphilic sulfobetaines:(1*S*)-(+)-10-camphorsulfonic acid	*Saccharomyces cerevisiae* CBS 13873	• All the DESs showed relative toxic effect on yeast cells and exerted a stronger dehydration effect than $CaCl_2$. • The toxic effect of individual components of DESs was not assayed.	[103]

In their following work, Cardellini et al. extended DESs toxicity studies for DESs prepared using differently structured sulfobetaines (SBs) with aliphatic, aromatic and amphiphilic moieties and (1*S*)-(+)-10-camphorsulfonic acid[103]. As it was observed for zwitterionic trimethylglycine:carboxylic acids DESs, these DESs exert a dehydration effect on the *Saccharomyces cerevisiae* CBS 13873 cells as this observed for $CaCl_2$[103]. Furthermore, it was noted that the DESs were stronger dehydrating agents than calcium chloride salt, indicating more affinity of these compounds to water[103]. In general, these results highlight these DESs as promising green media since the presence of water can inactivate the effect of these mixtures on the cells[103].

3. General discussion about DES microbial toxicity

A good question was asked in the first work where the toxicity of DESs was studied: "Are deep eutectic solvents benign or toxic?"[48]. Examining the results presented in around 96 works in which the authors looked for the answer on this question, it is still not possible to give a direct response. In general, although DESs have been considered as the green solvents, with low or no

toxicity, there are numerous studies that show that depending on the choice of the starting materials (which very often are non-toxic) used for their preparation, the respective DESs possess a certain degree of toxicity. This calls for in-depth studies on DES toxicity toward different organisms at various trophic levels in order to take full advantage of these new types of solvents and to broaden their applications. Furthermore, in various works different toxic effects were observed for the same DESs depending on the toxicity assessment method and model organisms used. Thus, the toxicity results cannot be generalized to all DESs, or different organisms and it is essential to elucidate mechanisms on how DESs promote toxicity.

There are several factors that were proposed to explain DESs' toxicity mechanism against tested prokaryotic and eukaryotic microorganisms such as negative impact of their pH on the growth of examined microorganisms[40, 49, 50, 80, 82], charge delocalization occurring during DES formation[38-42, 49], and cell dehydration in presence of DESs in growth medium[50, 102, 103], among others (see Fig. 5). Obviously, the impact of each of this factor differs for different DESs, depending on the nature and properties of starting materials used in solvent preparation. For instance, several studies have concluded that DESs possess higher toxicity than their individual components[39, 44, 67, 94, 95], however, other studies reported the opposite[52, 57, 60, 79, 88, 94, 95]. All these observations further highlight the need to elucidate DESs' toxicity mechanisms and in this section an attempt to summarize and systematized what have been discovered in regards on how DESs promote toxicity towards prokaryotic and eukaryotic microorganisms will be made.

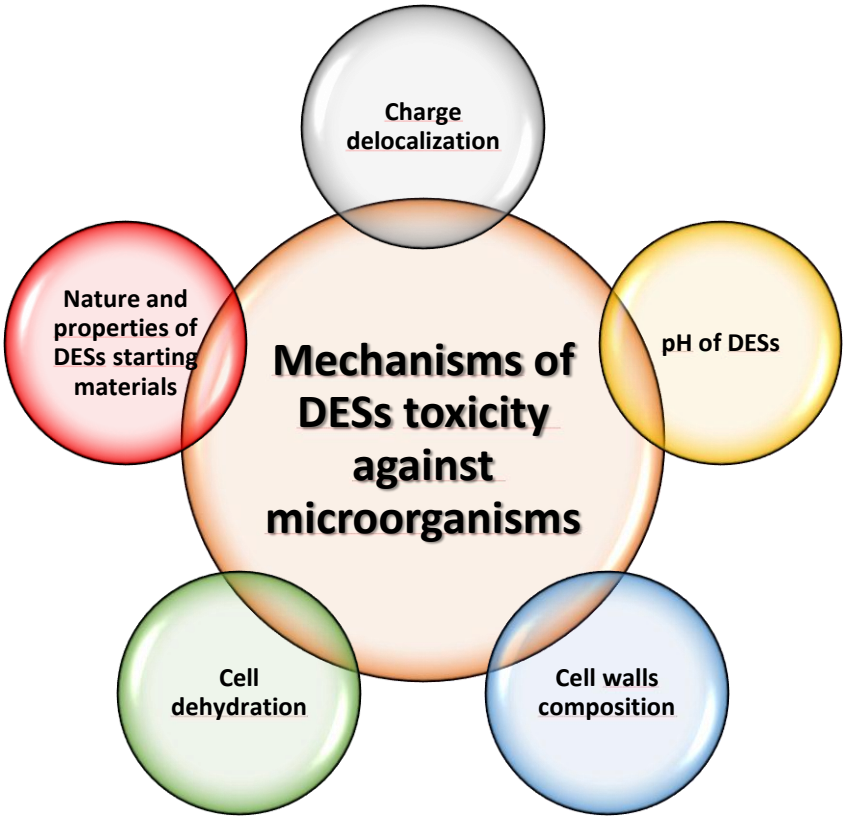

Fig. 5: Overview of factors proposed to explain the mechanisms of DESs toxicity against prokaryotic and eukaryotic microorganisms.

According to some reports higher toxicity of DESs than their individual components is a results of charge delocalization that occurs during the formation of DESs[38-42, 49]. This enhance in toxicity is explained by the observation that chemicals which contain delocalized charges express higher toxicity than those with localized ones. For instance, one of the most commonly used salts in DESs preparation - ChCl - has delocalized cation, thus very often higher toxicity of ChCl-based DESs is explained, as a result of interaction of cholinium cation side chains and head groups with cellular membrane groups[39, 59]. Furthermore, it was suggested that accumulation of positively charged cations, as cholinium, enhances the electrostatic interactions with negatively charged bilayer on the surface of cell's membranes, leading to cell wall distortion or

disruption[39]. It is also assumed that it causes proteins denaturation and enzymatic reactions inhibition, which may lead to cell collapse and death[42]. Moreover, it was also shown that the salt's counter anion contributes to the charge delocalization and thus affect DESs' toxicity. In the study of Wen et al. it was reported that DESs prepared using ChAc and ChCl as HBA had different antibacterial potency against *E. coli*, and the ChAc-based DESs had a greater detrimental effect than the ChCl-based DESs[39]. Additionally, according to Zhao et al. higher toxicity of acid-based DESs can be explained by the fact that the hydrogen bond network is more dense and compact, further increasing the charge delocalization effect on DESs toxicity[49].

Another factor that was proposed to explain DESs' toxicity mechanism is the acidity or alkalinity (pH) of the DESs[40, 49, 50, 80, 82]. Since the optimal pH for bacterial and fungal growth is 6.5–7.5[105] and 5.0-9.0[106, 107], respectively; if the DESs had a higher or lower pH value than optimal ones, it influenced the antimicrobial effect of these solvents. This is because the pH value besides theirs optimal ranges for microorganisms growth, has a negative effect on the cell activity, due to denaturation of proteins located on the microorganism cell wall. Consequently, the pH values far from those optimal for microbial growth may alter cellular proliferation and metabolic properties. For instance, de Morais et al. observed that the pH values of DESs composed of ChCl and organic acids were lower than 3 and as a result, the denaturation of proteins and decreased *A. fischeri* cell activity was discovered[40]. Moreover, it was noted that this effect was more pronounced when the acid content was higher further confirming that pH has a great influence on DESs' toxicity[40]. The same phenomenon was also observed for organic acid-based DESs against both gram negative and -positive bacterial strains[49]. Furthermore, the low pH was assumed to be the reason of increased toxicity towards bacteria for malic acid:sucrose[82] and acetycholine chloride:acetamide DESs[80]. The negative impact of pH on

DESs' toxicity towards yeast *S. cerevisiae* was observed in the work of Redovniković's group, where it was found out that solvents prepared with organic acids (pH < 3) and urea (pH > 8) as HBDs were the most toxic to the tested yeast cells[50]. Similar negative impact of basic urea-based DESs was observed in the studies of Hayyan's group, where ChCl:urea DES showed relative toxic effect on the tested genus of *Aspergillus niger* filamentous fungi[51] and *Candida cylindracea* yeast[52]. Nevertheless, it is worth mentioning that so far increased toxicity due to basic pH of DESs was only observed for the fungi, which have much narrower optimal pH growth range than bacteria (see above). Thus, in other studies where toxicity of urea-based DESs was studied usually no toxic effect towards various bacteria was found[43, 48, 49, 57, 70].

Moreover, another factor that may be involved in mechanism of DESs toxicity is cell dehydration[50, 102, 103]. In the studies of Cardellini et al., in which the mechanism of DESs toxicity towards yeast *S. cerevisiae* using FTIR-based assay was evaluated, the authors hypothesized that DESs might cause a very rapid exit of water from the cells[102, 103]. The obtained results confirmed this hypothesis as similar effect to that caused by $CaCl_2$ (well-known dehydrating agent) was observed[102, 103]. In the case of DESs, high concentrations generate high osmotic pressure to the cells and the cell water leakage, resulting in the yeast cells death. Furthermore, it was assumed that this dehydrating effect of DESs is rather independent of the chemical structure of these solvents, because all tested DESs challenged the yeast cells in the same way[102, 103]. Similar observations were made in the work of Redovniković's group, where high concentrations of ChCl:ethylene glycol and ChCl:glucose caused high osmotic pressure and decreased viability of baker's yeast cells[50].

Findings in other reports suggest that DESs' toxicity mechanism may also be related to the cellular organization of the organisms, in particular to the differences in cell wall composition[39,

40, 60, 72, 79]. For instance, in some studies it was proposed that the bacterial cell wall, which is composed of peptidoglycan, is permeable for small substrates because of its high porosity. Consequently, various DESs can diffuse across cellular membranes and exert their toxic effects inside the cytoplasm by denaturation of enzymes, oxidative stress, among others. In the work of de Morais et al., the authors hypothesized that organic acids containing DESs diffused through the cell membrane and therefore exerted toxic effect on cells of *A. fischeri* bacteria[40]. Furthermore, in the study conducted by Wen and co-workers it was assumed that DESs inhibited the bacterial growth of *E. coli* DH5α by interacting with the cellular membrane[39]. According to their revelations DESs components may interact with the polysaccharide or peptide chains of peptidoglycan through hydrogen-bonding or electrostatic interaction, leading to cell wall distortion or disruption[39]. Moreover, in some reports the different antibacterial potency of DESs towards gram-negative and -positive bacteria was explained by differences in their cell wall structure[60, 79]. Silva et al. concluded that for fatty acid-based DESs, their lower toxicity towards gram-negative bacteria was due to a presence of lipopolysaccharides (LPS) on the outer membrane that prevented the fatty acids DESs from reaching cell membrane[60]. On the other hand, because of the lack outer cell membrane with LPSs, the cell wall of gram-positive bacteria absorbed more easily the fatty acids composed solvents and thus they passed through the inner membrane and exerted the toxic effect[60]. Similar observations were made by Teh and co-workers for ChCl-based DESs where it was assumed that gram-negative bacteria formed a formidable barrier which restricted the attack of DESs from penetrating into the bacterial cell envelopes, while gram-positive *S. aureus* was not able to do that because its cell wall solely consists of thick peptidoglycan layer[79]. Furthermore, the differences in cell wall composition were also suggested as the reason why ChCl:oxalic acid:glycerol and ChCl:citric acid:glycerol were found toxic to bacteria and no to yeast *C. albicans*[72]. According to this report, it is a

result of easier penetration of the lipid layer of bacteria and not fungus which have two-layered cell wall mainly composed of chitin and glucans[72].

As mentioned earlier the toxicity profiles of DESs are also influenced by the nature and properties of starting materials used in solvent preparation[38, 39, 48-50, 79, 87, 94, 95]. In most of these studies, the negative impact of HBD was discovered. It was mainly observed that the DESs having organic acids in their compositions exhibited increased antimicrobial properties. However, enhanced toxicity of such fluids was assigned to not only acidity of DESs (negative pH effect, see above please) but also their higher viscosity. In addition, the highly viscous nature of carbohydrates containing DESs, as well as osmotic pressure (negative dehydration effect, see above please), might also be the reason of increased toxicity of some of these solvents. Nonetheless, some of the researchers claimed that beside HBD also HBA has an impact on overall toxicity of DESs[38, 39, 95]. For instance, DESs prepared using the same HBDs were found toxic to bacteria when MTPB was used as HBA and the opposite was observed for DESs formed with ChCl[38, 48]. Also, increased toxicity of ChAc-based DESs compared to ChCl-based ones was observed in the work of Wen et al.[39]. The influence of HBA on DESs toxicity was further reported by Macario et al. and solvents based on different quaternary ammonium salts exhibited different ecotoxicity towards *A. fischeri*[95]. Moreover, depending on DESs staring material and the method used in DESs preparation, the obtained solvents may possess different toxicities. For example, very often while using the heating method, the formation of impurities is observed[108]. The presence of impurities can change some of the mixture properties (e.g., by increasing their viscosities) and indirectly intensifying toxic effect of these DESs.

As discussed in this section, there are proposed various mechanisms regarding DESs toxicology, nevertheless the knowledge on this topic is still very limited. An interesting idea in the search for

other mechanisms of toxicity towards microbial cells would be to perform studies on the toxic effect of DESs on the metabolism of microorganisms used in the discussed works (Table 1-6), e.g. *E. coli* bacteria or *S. cerevisiae* yeast. This would be an analogous approach to that used in the metabolomic cytotoxicity studies of selected DESs that were performed on HepG2 and HEK 293T mammalian cells (in vitro) and in ICR mice (in vivo)[109]. To the best of our knowledge, there are no reports on the study of DESs toxicity mechanisms based on the generation of e.g. oxidative stress or the influence of DESs on the metabolism of basic carbon or nitrogen sources in microbial cells. Hence, with more studies on DESs toxicity towards various organism, not mainly focused on prokaryotic and eukaryotic microorganisms, it will be possible to create a database of truly green and biocompatible DESs and further extend their applications in food, pharmaceutical, biotechnological, or biomedical sectors. Overall, most of the studies on the toxicity of DESs revealed that solvents prepared with ChCl as HBA and HBDs from natural sources such as amines, alcohols, and carbohydrates are generally low toxic to different microorganisms. On the other hand, acid containing DESs exhibited strong antimicrobial properties. Furthermore, also the DESs based on quaternary ammonium salts, such as $[N_{1111}]Cl$, $[N_{2222}]Cl$ or $[N_{3333}]Cl$ were found more toxic than these prepared using ChCl. All of this proves once again, that biocompatibility of DESs is mainly dependent on their composition. Nevertheless, most of the DESs are usually less toxic than conventional organic solvents or ILs therefore the use of DESs is encouraged.

4. Critical evaluation of the methods used for DES microbial toxicity determination

a) Disk and well diffusion method as DES microbial toxicity assay

Due to the simplicity of execution, the disk or well diffusion method is well suited technique for testing the toxicity of a large number of DESs, differing in terms of composition and molar ratios

of HBA and HBD used in their preparation (see examples in Table 1). However, the obtained results allow, first of all, to assess whether the tested DES or its solution exhibits toxicity. Nevertheless, this method does not allow to estimate the toxicity of tested DES against selected microorganisms by determining the MIC or EC_{50} value. On the other hand, by selecting strictly defined strains of gram-negative bacteria, gram-positive bacteria, and fungi (both yeasts and molds) derivate from certified microbial collection (e.g., ATCC, DSMZ, JCM or CBS-KNAW) which were previously used for toxicity examination of antibiotics and other natural or synthetic antimicrobial agents, commercially available microbiological growth media and sterile disks used in assay, it is possible to normalize this method for DESs toxicity studies and use it in various laboratories, allowing the comparison of the obtained results. Unfortunately, so far researchers have approached these issues very freely, using various species of bacteria and yeast in their research (Table 1). For example, when the same bacterial species, e.g., *S. aureus* was used, different strains were selected, e.g., *S. aureus* NRS234[69] and *S. aureus* ATCC 25923[60, 67].

What is important to note, due to the key role of the DES diffusion process from a soaked sterile disk to the growth medium, this method is not suitable for high viscosity DESs. DESs with high viscosity are those where, for example, carbohydrates or organic acids were used as HBD for their preparation. The high viscosity also limits the precise application of the same amount of DES to the sterile disk in repetitions, which may affect the reproducibility of the results. For instance, in the work of Zhao et al. it was observed that ChCl:urea, ChCl:acetamide, ChCl:glycerol, ChCl:ethylene glycol did not inhibited *E. coli* growth according to the results obtained using disk test[49]. However, the exact same DESs have shown the antibacterial activity and the EC_{50} values between 275.2-532.0 mM were obtained using broth dilution[39]. The false results obtained using disk diffusion assay seemed to lead Lou's group to conclude that these

DESs are not toxic towards *E. coli* and thus their toxicity was not further examined using broth dilution method. These examples highlight the need for careful analysis of DES density and viscosity before using diffusion methods.

On the other hand, due to the hydrophilic nature of agar medium, diffusion of DES with high hydrophobicity into agar will be rather difficult and not such effective as for hydrophilic ones. Hence, it may seem that this physicochemical DES parameter may have also impact on DES toxicity estimated by disk diffusion method.

Summing up, due to above mentioned disadvantages, it seems too simple and insufficient to withdraw conclusions about DES toxicity basing exclusively on the results of the tests performed using disk or well diffusion method. The DES toxicity results obtained with these methods should be compared with those obtained with one of the alternative techniques. On the other hand, due to the simplicity and the possibility of standardization of disk diffusion method (under conditions of using commercially available sterile disks with the same size and made from the same material), this method seems to be the best of all discussed methods to perform the preliminary studies on toxicity of DESs (Table 1). Hence, in our opinion, apart from the mentioned exceptions, e.g., highly viscous DESs, disk diffusion method should be used as one of the DESs toxicity testing techniques.

b) Broth dilution method as DES microbial toxicity assay

Among the different dilution methods (macro- or microdilution) used so far, the microdilution method seems to be the best in terms of its reproducibility, validity of obtained results and application for DESs toxicity assessment. However, when analyzing the published results for DESs toxicity using broth dilution methods (Table 2), it can be concluded that the researchers

selected the species and strains of microorganisms used in these studies in a very arbitrary and independent manner from previously published DESs toxicity results. For instance, in one of the studies only gram-negative *E. coli* strain was used[39], and in another work when the same *E. coli* species was used, different strain was selected - the *E. coli* BL21 (DE3) strain dedicated for recombinant protein production in pET expression system (Novagen, Merck Millipore)[80].

Furthermore, as in the disk diffusion method, also in broth dilution methods, by selecting the appropriate microbiological growth media and culture conditions, it is possible to carry out toxicity tests against gram-negative and gram-positive bacteria, yeasts, and filamentous fungi. However, contrary to the previously discussed disk diffusion method, broth dilution methods allow the determination of MIC and EC_{50} parameters, which, in the case of method standardization, will allow the comparison of the results obtained by various research groups. Moreover, since in broth dilution methods serial dilutions of tested DESs are used, the negative effect of high viscosity of some DESs can be reduced. On the other hand, for broth dilution technique stability of DESs solutions should be controlled before toxicological analysis. It is known that high amounts of water are responsible for breaking of hydrogen bonds between HBA and HBD of DES[110]. Also, DESs or their hydrolyzed individual components may interact with the salts or nutrients in growth medium and it may be expressed in higher toxicity than the toxicity of DES itself without the presence of these interactions[80]. Consequently, for lower concentrations instead of DES toxicity, the toxicity of an aqueous solution of DES components is determined.

Moreover, the determination of toxicity by broth dilution methods, and in particular the most popular microdilution method, is not as easy to perform as the disk diffusion method. In the case of determining the MIC value using the microdilution method, to increase the precision of the

assay and the obtained results, it is sometimes necessary to use spectrophotometric measurements to assess the viability of the cells of the tested microorganisms (assessment of the turbidity of the culture). In addition, it is also possible to use resazurin (see section 5) to assess the cell viability of a cultured microorganism after treatment with DES, which is independent of the turbidity of the culture, increasing the precision of determination of the MIC and EC_{50} values. Interestingly, to the best of our knowledge, there is only one study where resazurin was used for this purpose in the DESs toxicity studies performed using broth dilution methods (Table 3,[88]). Moreover, after performing DES toxicity measurements with the broth microdilution method, the minimum bactericidal concentration (MBC), can be determined for the tested microorganism. In summary, due to the possibility of quantifying the toxicity of DESs by determining the MIC and EC_{50} or MBC, the possibility of selecting a wide range of microorganisms (bacteria, filamentous fungi, yeasts), the possibility of assessing the viability of cells of the tested microorganism using resazurin or indirectly by determining the MBC value - the method of microdilution seems to be the optimal method to assess the toxicity of DES against wide spectrum of both bacteria and fungi.

c) Microtox assay as DES microbial toxicity testing method

In four out of 96 studies in which the toxicity of DESs was evaluated, the commercially available Microtox kit was selected for this purpose (Table 4). Thanks to the use of uniform conditions in this kit for the toxicity assessment against the bioluminescent bacteria *Aliivibrio fischeri*, it is possible to determine and compare the EC_{50} values for several different DESs differing in their composition and molar ratio of HBA and HBD used for their preparation (Table 4). Moreover, due to the use of one strictly defined *Aliivibrio fischeri* strain, it is possible to compare the results obtained by different researchers. Contrary to the two previously discussed methods, due to the

fact that we use a commercially standardized test, the method does not need to be validated. However, since the test is based solely on testing toxicity towards *Aliivibrio fischeri*, the obtained results are limited to only one type of microorganism – gram-negative bacteria. As shown in the studies cited in this review, the mechanism of action and susceptibility of gram-negative and gram-positive bacteria may differ significantly from each other for the same DESs due to the different structure of the cell wall, and it is mostly depending on the chemical nature of HBA and HBD used for solvent preparation[39, 40, 60, 72, 79]. This also applies to the differences in the toxicity of DESs against bacteria and fungi resulting from chemical and structural differences in the structure of the cell walls of both groups of microorganisms. Hence, this method, despite many advantages resulting from the use of standardized commercial kit, should be a complementary method to another more universal technique, e.g., broth microdilution.

d) Other methods as DES microbial toxicity assay

In two analyzed and cited studies in this review, the toxicity of the examined DESs was assessed using a method based on the analysis of FTIR spectra variation of the cells upon exposition to the chemicals. In both studies, this method was used to assess DESs toxicity towards *S. cerevisiae* yeast (Table 6), however, as previously mentioned, this method can be used to evaluate the toxicity of DESs against different microbial cells[104]. This assay seems to be interesting because, compared to the previously discussed methods, it allowed to elucidate the mechanism on how DESs exert their toxic effect (yeast cells dehydration). Hence, FTIR-based bioassay is worth considering in all studies that aim at determining the possible toxicity mechanisms of selected DESs in relation to various groups of tested microorganisms.

e) pH of DESs as an important factor in described microbial toxicity methods

Since pH of some DESs is the important parameter that affect the applicability of basically each of the methods discussed above, it is important to consider this factor before testing DESs toxicity. Some studies about the toxicity of DESs suggest that the pH of growth media after preparation of DESs serial dilutions changes significantly[49, 51, 80]. As a result, the pH decreases below or increases above the optimal values for microbial growth (6.5–7.5[105] and 5.0-9.0[106, 107] for growth of not acidophilic or basophilic bacterial and fungal microorganisms, respectively), consequently increasing the cells mortality in the tested samples. It is mostly observed when one of the DESs components are acids. For this reason, it is necessary to firstly analyze the pH of DESs solutions and if the values are far from those optimal for microorganisms growth (e.g. for the most often used microorganisms in DESs toxicity studies - *E. coli* - optimal pH growth range is between 6.5 and 7.5[49]), the DESs solutions should be prepared in the buffered media. For example, the dissimilarities in the obtained EC_{50} values for ChCl-based DESs were noted in the work of Lapeña et al., where pH of the samples was controlled and adjusted to be in optimal range for the culturing of *A. fischeri*[96] and in the study of Macario et al. where pH was not controlled[94]. Consequently, lower EC_{50} values were obtained in the study of Macario et al. which seems to be due to the pH effect on bacterial growth, leading to overestimated toxicity of ChCl-based DESs towards *A. fischeri*. In our opinion, these examples clearly show the need of buffering of DESs before testing their toxicity.

Overall, for proper hazard and risk assessment of DESs, the toxicity data from diffusion method and broth dilution should be evaluated together for both DESs and their separate individual components. Since currently there are no standard protocols for testing toxicity of DESs, it makes difficult to draw conclusions across different studies due to discrepancies in experimental conditions and lack of test standardization. Nevertheless, we believe that following the

suggestions and guidelines pointed out in subsequent section more precise and comparable data could be obtained.

5. Suggestions and guidelines for future research

The literature review and experience of the authors of this paper acquired during our recent toxicological studies against selected microorganisms and previous experience in using of some of above-described methods for testing of others antimicrobial agents, incline us to propose a few general rules for the future investigation of DESs toxicity. When applying well-established microbial toxicity testing methods (e.g., disk diffusion assay, broth dilution) for DESs, one should keep in mind that these methods may need methodological modifications to be applied to these compounds. We believe that by following the proposed suggestions and guidelines will enable to get accurate results and facilitate a comparison with the results of other researchers. Furthermore, with comparable results of investigations of various groups, it will be possible to further understand the mechanisms on which these solvents exert their toxic effect. The suggestions and guidelines for future research on toxicity of DESs are outlined below.

i) The description of the methodology used to evaluate DESs toxicity should include all the details such as the detailed description of strain of microorganism used, detailed description of inoculum preparation (defined optical density of bacterial cells or CFU in inoculum), type and composition of growth medium, incubation conditions and endpoints determination, as well as details on the DES solutions preparation (initial molar ratio, dilutions) before analysis. The availability of this information will allow other researchers to better plan their own investigations and compare their results with different studies. For instance, for DESs toxicity assay using broth dilution method we encourage to use Mueller-Hinton broth culture media. Mueller-Hinton broth is

recommended by FDA, NCCLS and WHO for testing MICs of for example, antibiotics against most encountered aerobic and facultative anaerobic bacteria in food and clinical material. This is excellent medium for cultivation *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* strains previously used in DESs toxicity studies (Tables 1-3, 5).

ii) Pure DESs should be characterized as much as possible, in particular their physicochemical properties, such as color/clearness, density, viscosity and pH (or pH of its solution in water). Disregarding these parameters may lead to the selection of the assessment method and model microorganism that will not be best suited and consequently will diminish the validity of the results and conclusions.

a) Both viscosity and density were shown to have a large effect on the obtained toxicity results. For instance, the viscosity of DESs may have great impact on the results obtained using disk diffusion assay due to low diffusion of highly viscous compounds in agar medium.

b) pH mostly influences the results obtained using broth dilution method, especially when pH of growth medium supplemented with DES is lower or higher than optimal for microbial growth. Due to pH changes caused by DESs, it is recommended to use buffered culture media instead of unbuffered cultures or to prepare DESs solutions in buffers. It will allow to diminish the negative impact of pH on the microbial growth, obtain more valid results and conclusions.

c) Some DESs may not be transparent liquids and cause some turbidity of the samples [88], resulting in the increased absorbance readings and thus leading to lower accuracy of the obtained results in broth macro- or microdilution methods.

d) Crossed reactions between DESs and the salts or nutrients of the culture media could also take place and influence both the pH and growth[80]. Moreover, such crossed reactions may be increased in the case of DESs hydrolysis that could occur in the presence of significant amount of water. Consequently, free HBA and HBD may react with the salts, amino acids, carbohydrates present in culture media, changing the pH and decreasing the nutrition sources.

iii) Beside determination of DESs toxicity, it should be mandatory to also evaluate the toxicity of DES individual components (HBA and HBD) at the same concentrations as these used for DES preparation. It will allow to better understand the results obtained in toxicological studies of DESs and withdraw more proper conclusions.

iv) As discussed throughout this paper there are various methods used to evaluate toxicity of DESs. Our literature study revealed that disk diffusion assay was the most commonly used method for this purpose (Table 1). The second most frequent used method was broth dilution method (Table 2). However, other microbiological methods dedicated for assaying antimicrobial activity of natural or synthetic chemical compounds were used much more rarely for assaying DESs toxicity against bacteria and fungi (Tables 3-6). In the light of presented data, although the disk diffusion method is the most commonly used method for assaying DESs toxicity against microorganisms, our recommendation is to use broth dilution technique instead of disk diffusion assay for this purpose. Broth dilution method offers more versatility and precision than mostly used disk test. It is undeniable that in most of the studies in which DESs toxicity was evaluated using sterile disks soaked with DESs and placed on agar plates, the obtained results were less accurate and may not reflect real interaction between DESs and cells. It is related with high density and viscosity of

most of the DESs which leads to decreased DESs diffusion from the disk into agar medium. On the other hand, using broth dilution technique the negative impact of density and viscosity is minimized and quantitative results could be obtained. Nevertheless, it must be remembered that in high amounts of water DESs hydrolysis takes place, which may also have an impact on toxicity data obtained. Therefore, taking all of this into consideration, and if possible, it would be beneficial to firstly perform analysis using disk diffusion assay with pure DESs and then obtain more details with broth dilution technique. However, it is important to note, that disk diffusion method has one important advantage. With this method we can quickly and cheaply estimate the toxicity of a range of DESs differing in a) the HBA used, b) the HBD used, or c) the molar ratios of HBA and HBD used to obtain a given type of DES. Hence, in our opinion, for such DESs toxicity studies, the results of disk test provide valuable data which can support the analysis of DESs toxicity based on the results of broth dilution method or other alternative method.

On the other hand, from other methods reported in the literature for DESs microbial toxicity studies, the methods based on i) analysis of FTIR spectra variation of the microorganism's cells upon exposition or not to the DESs; ii) the use of commercial kit that consists of two dyes, propidium iodide (PI) and SYTO9 for staining microbial cells exposed for DES seem to be interesting solution. They allow to compare DESs toxicity results obtained with these methods with results of DESs toxicity obtained with broth dilution method. In contrast to Microtox assay, both these methods give the possibility of selection of the same microorganism (bacteria or fungi) as used in broth dilution method. Moreover, the second of above-mentioned methods seem to be quite easy for validation, because of employing the commercially available kit.

v) If possible, we advise to use the assays based on colorimetric dyes (e.g., cell incubation with resazurin) for cell viability and vitality determination, which not only provide more precise values than these obtained by simple visual inspection or spectrophotometric measurements of turbidity (especially during MIC evaluation by broth microdilution method), but also higher quality data. Using this method there is no need of confirmation of the results by subculturing of each concentration onto agar for 24 h (MBC evaluation). Furthermore, the influence of DESs turbidity on the absorbance of the samples is reduced for these methods.

vi) The use of preadapted cells of microorganisms selected for study of DESs toxicity is encouraged. Until now there is one work where the preadaptation of cells to the DESs was performed[80]. It was demonstrated that non-preadapted cells did not grow in the presence of 600 mM acetylcholine chloride:acetamide DES, however, when they were pre-adapted to this concentration, cellular growth was observed[80]. By including the cellular pre-adaptation in future studies, it will be possible to gain insights on the capability of the cells to tolerate or assimilate DESs and to obtain more accurate data on the antimicrobial properties of DESs.

vii) In case of studies where DESs are applied in the processes (such as extraction, chemical reaction etc.), the toxicity should be controlled for primary DES as well as for DES recovered after the process. In many cases, elevated temperatures as well as other factors, such as ultrasounds or microwaves used during the process, can cause DES chemical instability. As a result, harmful byproducts can be formed and strongly affect the eco-friendly character of primary DES. Recycled DES can introduce these byproducts to extracted fraction or product of reaction. On the other hand,

accumulation of toxic byproducts will strongly affect methods available for its safe disposal after usage.

6. Conclusions and outlook

Deep eutectic solvents (DESs) are one of the most interesting classes of alternative solvents, mainly because of their simple preparation, usually low cost, and versatility due to possibility of their task- specific design to meet the needs of a specific process. Furthermore, they can be prepared using all- natural substances which opened exciting new perspectives to design truly green compounds that will meet with the requirements of green and sustainable chemistry. All these characteristics confer DESs as an ideal alternative to both organic solvents and ILs. Since their discovery DESs have been used in a myriad of applications as solvents, reaction media, catalysts, additives, lubricants, or materials for a wide range of fields from pharmaceutical to energy. Nevertheless, new studies are constantly conducted in order to learn as much as possible about the properties of DESs and further increase their applications in new fields important for the quality of life such as cosmetic, food, drug production and medicine. However, before the implementation of DESs in these areas will be possible, it is essential to study their toxicity and gain knowledge on their possible modes of interaction with living beings. Even though, DESs are considered as green, benign, and non-toxic compounds, a literature review conducted in this paper indicated that this statement is not entirely true and such generalization should be avoided. In fact, several examples proved that often out-off-purpose methodology was used, resulting in false conclusions. Secondly, more than 5200 studies were published about DESs after their discovery and only around 96 evaluate and discuss the toxicity of these compounds (mainly against selected microorganisms). It highlights the need for more studies in this topic, which will

allow to gain sufficient insights on DESs toxicity towards different organisms at various trophic levels and on how they exert their toxic effect.

Throughout this review, we show the advantages and disadvantages of methods used for DESs toxicity determination. Our analysis indicated that it is necessary to have an improved, standard protocol for determination of DESs toxicity. In this way, it will be possible to create a database, compare the results obtained in different studies and for various solvents. In our opinion, in order to obtain valuable results, it would be beneficial to use both disk diffusion assay and broth dilution technique in future studies on toxicity of DESs. We believe that the negative impact of pH may be overcome by using extremophilic microorganisms instead of standard microbial strains. Hence, it is essential to improve, for example, the broth dilution technique by always using buffered medium or by preparing DESs solutions in buffer. Furthermore, another aspect that should be considered while using standard microorganisms is cellular preadaptation with DESs which was shown to be a viable approach allowing to gain insights on the capability of the cells to tolerate or assimilate DESs and to obtain more accurate data on the antimicrobial properties of DESs for which growth for some concentrations was not observed for non-adapted cells.

It is expected that, in a future, by using the standardized and validated above-mentioned methods, the theoretical and experimental knowledge about toxicity of DESs will evolve rapidly. It will allow to further explore these solvents in different applications such as biomedical and pharmaceutical. Furthermore, it will be possible to address once for all the DESs biosafety issue and answer with conviction if deep eutectic solvents are benign or toxic.

Conflicts of interest

There are no conflicts to declare.

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Deep Eutectic Solvents Microbial Toxicity: Current State of Art and Critical Evaluation of Testing Methods

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Abstract

Deep eutectic solvents (DESs) were described at the beginning of 21st century and they consist of a mixture of two or more solid components, which gives rise to a lower melting point compared to the starting materials. Over the years, DESs have proved to be a promising alternative to traditional organic solvents and ionic liquids (ILs) due to their low volatility, low inflammability, easy preparation, and usually low cost of compounds used in their preparation. All these properties encouraged researchers to use them in diverse fields and applications e.g., as extractants for biomolecules and solvents in pharmaceutical and cosmetic industries. Nevertheless, despite undeniable potential of DESs, there is still controversy about their potential toxicity. Besides the low number of studies on this topic, there are also some contradicting reports on biocompatibility of these solvents. Such misleading reports could be mainly attributed to the lack of well design standard protocol for DESs toxicity determination or the use of out-off-purpose methodology. Thus, to better apply DESs in green and sustainable chemistry, more studies on their impact on organisms at different trophic levels and the use of proper techniques are required. This review focuses on DESs toxicity towards microorganisms and is divided into three parts: The first part provides a brief general introduction to DESs, the second part discusses the methodologies used for assessment of DESs microbial toxicity and the obtained results, and finally in the third part the critical evaluation of the methods is provided, as well as suggestions and guidelines for future research.

Keywords: Deep eutectic solvents, Toxicity, Pollutants, Antimicrobial activity, Disk diffusion, Broth dilution

1. Introduction

Deep eutectic solvents (DESs) emerged in 2003 and are a new class of solvents having liquid state around room temperature[1]. They are prepared by a simple mixing at certain molar ratio and heating of two or more chemicals often having a solid state at room temperature. In such mixture one of the compounds acts as a hydrogen-bond donor (HBD) and the other one as a hydrogen-bond acceptor (HBA). Consequently, a eutectic mixture for which the eutectic point temperature presents a deep depression to that of an ideal liquid mixture is formed. Lower melting point of the DES comparing to values for pure components is mainly assigned to the formation of hydrogen-bonds between the DES components[2, 3]. Nevertheless, also electrostatic interactions or Van der Waals forces were considered as possible factors that may also play an important role in this phenomenon[4-7]. Furthermore, DESs with ionic components are very often referred to as ionic liquids (ILs) analogues because they share some of their characteristic features such as low volatility, wide liquid temperature range, and high solvation ability for many compounds[7, 8]. On the other hand, compared to ILs, DESs have some advantageous characteristics, such as usually lower toxicity, higher biodegradability, easier preparation, and lower material cost[9]. Moreover, DESs similarly to ILs have highly tunable nature since through the manipulation of different types of HBAs, HBDs and molar ratios, it is possible to modify their biological and physicochemical properties to fit a specific application[10-13].

All the above-mentioned remarkable properties of DESs make them an ideal alternative to both commonly used organic solvents and ILs[5, 14-16]. That is why, since their discovery, they have been widely studied and applied in diverse fields, including biocatalysis[17-19], electrochemistry[20-22], CO_2 capture[23, 24], separation and extraction techniques[25-31], among others. Furthermore, beside the fact that up to now the most works focus on their

applications as green solvents for different chemical industries, more recently they started to be also considered as promising fluids for cosmetic, food, pharmacological, biotechnological and biomedical industries[32-36]. It is mainly related to the fact that DESs are considered as non-toxic, eco-friendly, biodegradable and benign solvents. Nevertheless, in order to make such conclusions and to use DESs in these areas, the more profound studies on DESs toxicity and biodegradability are essential.

There is a general assumption that DESs are non-toxic because usually their individual starting compounds are natural, biodegradable and low toxic. The lower toxicity and higher biodegradability of DESs were mainly assigned to the group of DESs composed of natural, low toxic compounds, such as cholinium chloride, natural carboxylic acids, sugars, amino acids, and, in some cases, water as a third component, the so-called *natural deep eutectic solvents* (NADESs)[37]. Nevertheless, it is not appropriate to assume that NADESs do not exhibit toxic effect on different organisms because after formation of hydrogen-bonds a new supramolecular structure is created[2, 3], making necessary to evaluate possible toxicity of NADESs as a result of this change. Notwithstanding, the number of works that studies toxicity of these compounds is rather limited. To the best of our knowledge, since DESs introduction around 96 papers have been published about toxicity of DESs (see Fig. 1). In most of these works, the toxicity of DESs was evaluated using prokaryotic microorganisms[38-43], however more recently also some eukaryotic organisms were used, including microorganisms (yeasts, molds), human and animal cell lines, and animal models (*Hydra sinensis*, *Cyprinus carpio* fish, *Artemia salina* brine shrimp)[6, 38, 39, 42-47]. Nevertheless, due to usually short generation time, easiness of culturing and possibility to use the same microbiological methods, most studies focus on both gram-positive and gram-negative bacterial strains, yeast and mold fungi strains (see Fig. 2)[38,

40, 48-52]. Therefore, in this work we decided to focus on reviewing the present state of art of the DESs microbial toxicity against procaryotic and eukaryotic microorganisms and the critical evaluation of usefulness of the microbiological methods used for this purpose.

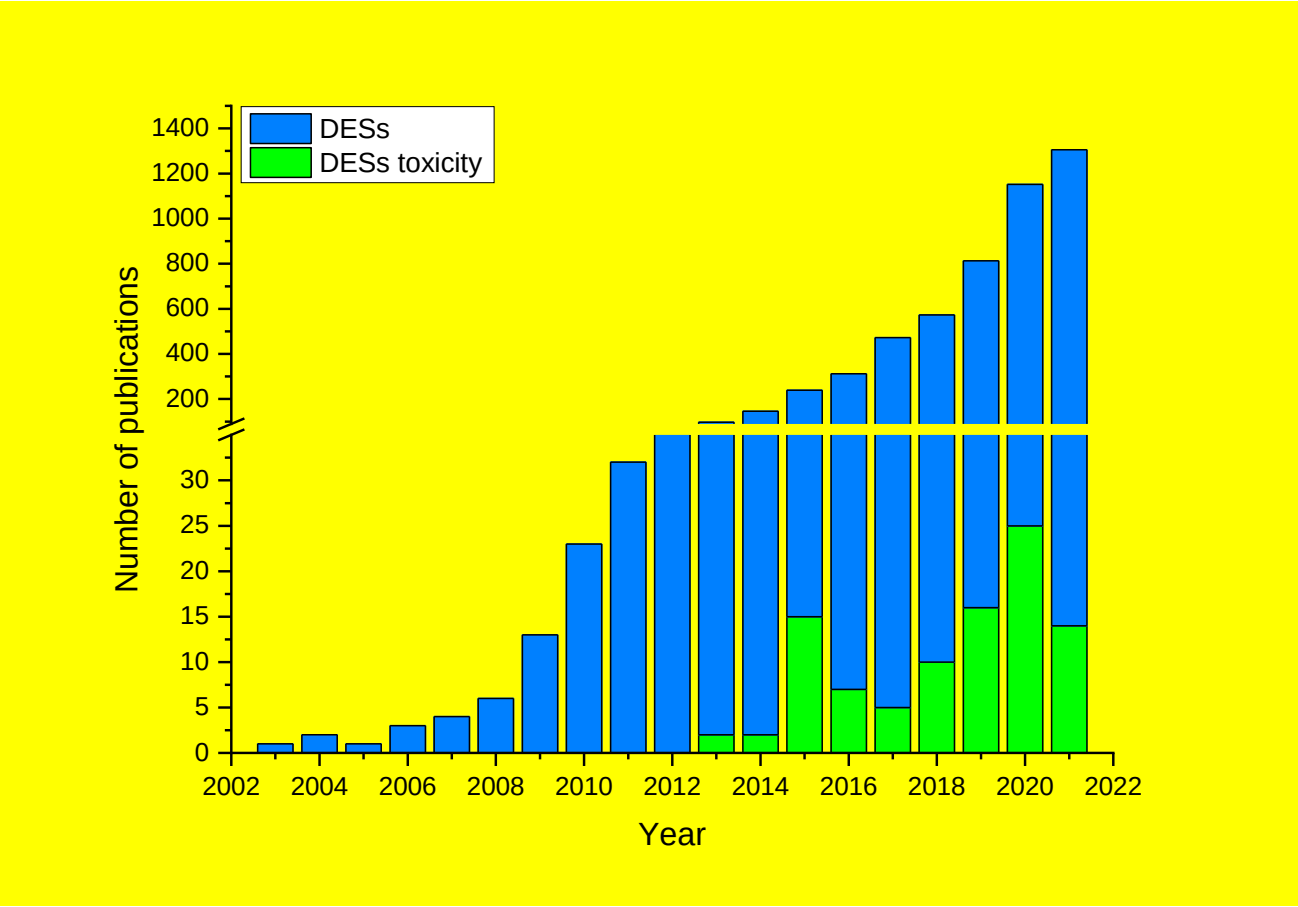
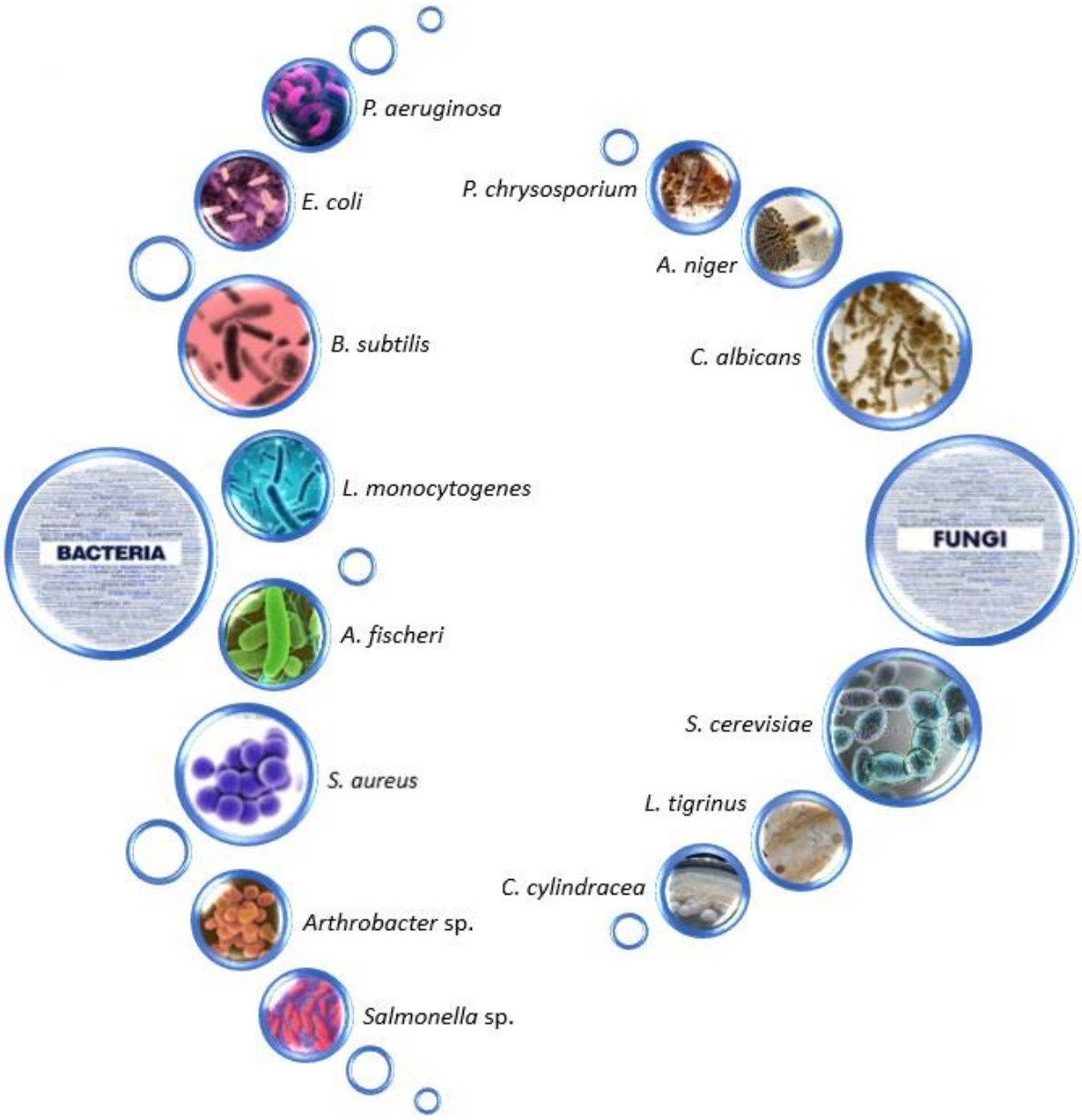


Fig. 1: Evolution of the number of published papers in the field of DESs in general (blue) and DESs toxicity (green) starting from 2003 that contained "deep eutectic solvents" or "deep eutectic solvents and toxicity" in their titles, keywords, or abstracts as obtained from Scopus. Data for 2021 included up to November.

Even though, in some of the reports the low toxic, eco-friendly and biodegradable nature of DESs is demonstrated, some other works claim exactly the opposite and toxicity of some DESs was shown[45, 53]. It leads to some confusion and confirm the need for toxicity studies for all DESs

present in literature. Such misleading reports can be also attributed to the lack of well design standard protocol for DESs toxicity determination. Having said that, the researchers planning their experiments on DESs toxicity should be aware what are the available methods and what are their advantages and disadvantages. Moreover, the researchers should be aware that not all the toxicity assessment methods are best suited for the DESs. For instance, the high viscosity, instability of aqueous solutions, among others, make some of the used methods not applicable. In other words, in many cases used protocols do not fit to the purpose. Thus, conclusions stated for such studies are simply not true.



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117 Fig. 2: Types of microorganisms mostly used in toxicological studies of DESs.

118 The selection of the test method always affects the results obtained. Thus, by proper planning and
119 use of correct methodology, the risk of misleading results will be minimized. Finally, it will
120 allow to compare the results obtained in different studies. This paper provides a review of the
121 procedures for the determination of toxicity of DESs. The available techniques are discussed
122 along with the advantages and general disadvantages related to the use of these methodologies.

Furthermore, the critical evaluation of the methods used for assessment of DESs toxicity, and the literature review of obtained results is presented. General discussion on DESs toxicity and possible mechanisms on how they promote toxicity are also included as well as suggestions and guidelines for future research are proposed.

2. Methods used for DESs microbial toxicity assessment

The analysis of the available literature showed that the following methods have been used to assess the toxicity of DESs against prokaryotic and eukaryotic microorganisms: disk and well diffusion method, broth dilution, Microtox assay for luminescence inhibition in *Aliivibrio fischeri*, drop plate method and FTIR bioassay. Among these methods, for this purpose, the disk or well diffusion method was most often used (16 studies, Table 1). Moreover, the broth dilution method (macro- and micro-dilution) was also used relatively often (14 studies, Table 2). Methods such as Microtox assay (Table 4), drop plate method (Table 5) or FTIR (Table 6) were used much less frequently for this purpose. In addition, in view of an attempt to critically evaluate the practical suitability of these methods to study DESs microbial toxicity (section 4), in sections 2.1-2.3 besides the discussion of the results of toxicity studies with DESs using these methodologies, each of these techniques is briefly presented and their major advantages and disadvantages are listed.

2.1. Diffusion methods

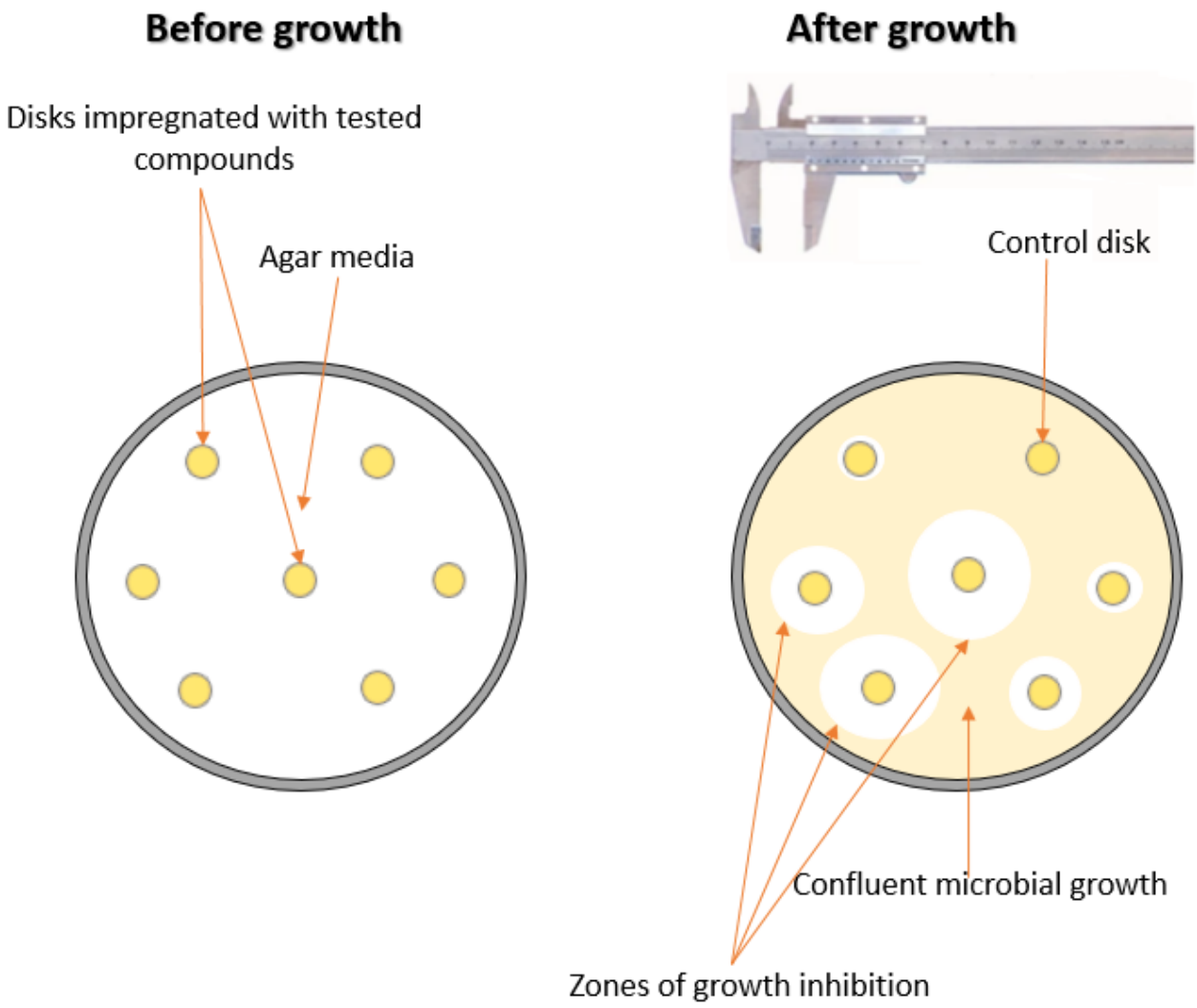
2.1.1. Disk diffusion method

Primarily, the disk diffusion method (agar diffusion test or Kirby–Bauer test) was used to test the susceptibility of microorganisms to antibiotics[54, 55], and later its application was also extended to test antimicrobial activity of different chemical compounds e.g., ILs[56] and DESs[48]. In this

test, a filter-paper disk is impregnated with the compound to be tested and then placed on the surface of the agar plate where microorganisms have been previously swabbed uniformly[54, 55]. Afterall the plate is left to grow the tested microorganisms (incubation at optimal growth condition e.g., temperature, time) and to allow the compound to diffuse from the disk into the agar. If the tested compound stops the microorganism growth, there will be an inhibition zone around the disk, where no colonies have grown[54, 55]. By measuring the size of the inhibition zone, the susceptibility of microorganism to chemical agent can be deducted. The size of the zone around the disk mainly depends on how effective the chemical compound is at stopping the growth of the microorganism and indicates where the concentration in the agar is greater than or equal to the effective concentration[54, 55]. Furthermore, another important factor that needs to be considered is the diffusion of the compound within the agar medium[54, 55]. The diffusion varies between different compounds based on their molecular structure and further on their hydrophobicity/hydrophilicity[54, 55]. Also, the viscosity of the tested solution has a great impact on the diffusion. Thus, while interpretating the results, it needs to be remembered that the size of inhibition zones is different for each compound not only because the different antimicrobial potency but also due to different diffusion and solubility of tested chemicals in agar medium. Having said that the disk with compound that produces the largest inhibition zone is not an indication of the real toxicity of the compound to the tested microorganism[54, 55]. The toxicity testing procedure using disk diffusion method is shown in Fig. 3.

The main advantages of the disk diffusion test are that it is a cost-efficient test that is easy to conduct and easy to evaluate. Furthermore, this method allows to test several antimicrobial agents simultaneously on the same plate. These characteristics, along with short period of time needed to obtain relevant information, made disk diffusion test most widespread method used for DESs

toxicity assessment and the results found in the literature for microbial toxicity of DESs using disk diffusion method are presented in Table 1. On the other hand, the biggest drawback of this method is the fact that it only allows us to assess whether the chemical agent is toxic, moderately toxic, or non-toxic for the tested microorganism in question. That is why, in some cases, multiple disks with different concentrations of the tested compound are used simultaneously on the same agar plate. In that way, it is possible to estimate approximate minimum inhibitory concentration (MIC) of compound. Nevertheless, for more precise toxicity assessment and MIC determination, after disk test, the use of "dilution methods" for the same pair of tested compound and microorganism (see section 2.2.) is recommended.



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178 Fig. 3: Toxicity testing using disk diffusion method.

179 The disk diffusion method was chosen in the first study on toxicity of DESs that was conducted
180 by Hayyan et al.[48]. In this work, DESs prepared using choline chloride (ChCl) as HBA and
181 glycerol, ethylene glycol, triethylene glycol, urea as HBDs were chosen and its toxicity to
182 different gram-positive (*Bacillus subtilis* and *Staphylococcus aureus*) and -negative (*Escherichia*
183 *coli* and *Pseudomonas aeruginosa*) bacteria was evaluated. The authors showed that all
184 investigated DESs had no inhibition on the studied bacterial strains[48]. Later, Mao et al.
185 extended this work and studied the effect of similar DESs (with exception of ChCl:triethylene

glycol) on toxicity of *Arthrobacter simplex*[57]. The authors found out that at 60% concentration these DESs (with exception of ChCl:urea) were toxic to *A. simplex* to some extent[57]. Interestingly, the obtained results revealed that the three tested DESs had much lower toxicity towards *A. simplex* than their individual components. This observation indicates that the toxic effects of DES individual components can be weakened by incorporating them into a DES. The authors hypothesized that hydrogen bonding network after DES formation prevented the salt anion from attacking the cellular membrane, thus resulting in lower toxicity of DESs towards *A. simplex*[57]. Considering these findings, the authors suggested that the toxicity of DESs may be species-dependent and associated with varied effects of DES components on the target microorganism[57].

In their second study, Hayyan et al. changed the HBA from ChCl to methyltriphenylphosphonium bromide (MTPB) and combined it with glycerol, ethylene glycol, triethylene glycol as HBDs[38]. All tested phosphonium-based DESs have been relatively toxic to gram-negative bacteria (*E. coli* and *P. aeruginosa*) and thus can be used as potential antibacterial agents[38]. On the other hand, only MTPB:ethylene glycol DES showed effective toxicity towards gram-positive bacteria (*B. subtilis* and *S. aureus)* indicating the HBD nature influences the antibacterial effect of DESs[38]. Furthermore, these results suggest that the HBA also affects toxicity of DESs since similar HBDs have been used in both studies. The contribution of HBA to DESs toxicity was attributed to the charge delocalization that occurs through hydrogen bonding since chemicals having delocalized charges are more toxic than chemicals with localized charges[58, 59].

Later, the disk test was also used to qualitatively evaluate the growth inhibition of bacteria (*E. coli*, *S. enteritidis*, *S. aureus* and *L. monocytogenes*) caused by ChCl-based DESs prepared using

various HBDs such as amines, alcohols, organic acids and sugars[49]. It was reported that ChCl-based DESs formed with amines, alcohols, and sugars as HBDs did not have a significant toxic effect on bacteria. These finding are in line with the study of Hayyan et al., where also no inhibition of bacteria growth was observed for ChCl-based DESs[48]. On the other hand, significant toxic effect was observed when organic acids were used as HBD of DES. The authors suggested that the amine-, alcohol- and sugar-based DESs were used by bacteria as nitrogen or carbon sources, while the organic acid−based DESs inhibited bacterial growth mainly as a result of significant decrease of pH below the optimal values (pH=6.5−7.5) for bacterial growth of tested microorganisms[49]. The obtained results revealed that gram-negative bacteria (*E. coli* and *S. enteritidis*) were more sensitive than gram-positive (*S. aureus* and *L. monocytogenes*), most likely due to the interaction of DESs components with the polysaccharide or peptide chains of the cell wall through hydrogen bonding or electrostatic interactions, resulting in damage of cell walls[49]. Moreover, the antibacterial activity of DESs based on saturated fatty acids, combining capric acid with other saturated fatty acids with different chain size length (i.e., lauric acid, myristic acid and stearic acid) was studied in the work of Silva et al.[60]. The disk test results revealed that the DESs did not inhibit growth of gram-negative bacteria (*E. coli* and *P. aeruginosa*) but showed antibacterial activity against the gram-positive bacteria (*S. aureus*, Methicillin-resistant *S. aureus* (MRSA) and Methicillin-resistant *S. epidermis* (MRSE))[60]. As an explanation, the authors suggested the differences in cell wall structure of gram-positive and -negative bacteria[60]. According to previous reports gram-negative bacteria are usually resistant to the antibacterial activity of fatty acids due to a presence of lipopolysaccharides on the cell wall that prevents the fatty acids from reaching cell membrane[61-64], while the cell wall of gram-positive bacteria readily absorbs fatty acids allowing their passage into the inner membrane[61, 63]. The same group also studied the antimicrobial properties of therapeutic DES (THEDES –

group of DESs for which one of the components of the eutectic mixture is an active pharmaceutical ingredient (API)[65, 66]) based on menthol and stearic acid[67]. It was observed that both, THEDES and its starting materials, did not inhibit the growth of gram-negative *E. coli* and *P. aeruginosa*, while growth of gram-positive bacteria (*S. aureus*, MRSA and MRSE) was only affected by the menthol[67]. Furthermore, the disk diffusion results showed the formation of deposit in all cases for menthol:stearic acid THEDES, which was assigned to fatty acid's low solubility and, consequently, low diffusion rate[67]. The presence of deposit prevented the authors from correct evaluation of inhibition zones for THEDES, but since it is majorly composed of menthol (molar ratio 8:1), which showed antimicrobial properties towards gram-positive bacteria, it was assumed that this THEDES is toxic to some degree and further toxicological studies using broth dilution were performed[67]. Recently, the antibacterial activity of menthol:lactic acid was also studied[68]. This DES can be classified as THEDES and furthermore as representant of hydrophobic DESs. In cited study, two gram-negative bacteria (*E. coli* and *P. aeruginosa*) and one gram-positive pathogen (*S. epidermis*) were selected and the antimicrobial activity evaluated using disk diffusion method[68]. It was shown that all the tested bacteria were susceptible to menthol:lactic acid DES and clear inhibition zones were observed[68]. Gram-positive *Staphylococcus epidermidis* was also found to be the most susceptible bacteria to the tested DES than gram-negative bacteria (*E.coli* and *P. aeruginosa*)[68]. The bactericidal activity of menthol:lactic DES was assigned to the use of lactic acid as a forming component thus higher toxicity of DES due to the additional hydroxyl group presence in its structure and the high acidity[68].

In another report Wang et al. evaluated the toxicity effect of benzalkonium chloride (BC):acrylic acid and benzalkonium chloride:methacrylic acid DESs, as well as their individual components,

towards *E. coli* and *S. aureus*[69]. The disk diffusion assay results revealed that DESs inhibited the growth of bacteria and that the inhibition potency of DESs mainly comes from benzalkonium chloride (BC) and not acrylic or methacrylic acid since DESs inhibition zone widths were slightly larger or close to that of BC and not acid[69]. It was also observed that the studied DESs were more toxic to the gram-positive bacteria (e.g., *S. aureus*) than gram-negative (e.g., *E. coli*). Furthermore, the introduction of methyl group within methacrylic acid resulted in decrease in DESs toxicity comparing to BC:acrylic acid DES[69]. The disk diffusion test was also applied to evaluate toxicity of DESs based on betaine[70, 71]. Firstly, it was shown that betaine:urea DESs is not toxic to *E. coli* and *P. aeruginosa* bacterial strains[70]. More recently, Jiang reported that betaine:malic acid DES has certain antibacterial activity towards *E. coli*[71]. Also, in the study of Jangir et al. antibacterial properties of ternary DESs were described[72]. The authors showed that ChCl:oxalic acid:ethylene glycol, ChCl:oxalic acid:glycerol, ChCl:citric acid:ethylene glycol and ChCl:citric acid:glycerol DESs inhibited the growth of *E. coli* and *S. aureus* strains[72]. In particular, ChCl:oxalic acid:ethylene glycol DES was the most toxic to the selected microbes, followed by ChCl:citric acid:ethylene glycol, ChCl:oxalic acid:glycerol and ChCl:oxalic acid:ethylene glycol, respectively[72]. Moreover, in the most recent work, the toxicity of ChCl:1,2-propanediol DES towards *S. aureus*, *E. coli*, *Clostridium perfringens*, *L. monocytogenes* and *Salmonella* sp. was studied[73]. According to the obtained results this DES was found relatively toxic to all tested bacterial strains[73]. It was concluded that part of this effect is due to the HBD - 1,2-propanediol - which was previously found effective against *E. coli* and *S. aureus*[74]. Among the studied bacteria, the lowest inhibition effect was observed for *E. coli* and it was hypothesized that their resistance could be related to the gram-negative status and the lower permeability of their surface for phenolic compounds[73]. On the other hand, this DES

showed intermediate inhibition effect on the other gram-negative (*Salmonella* sp.) and all gram-positive (*L. monocytogenes, S. aureus, C. perfringens*) bacteria[73].

Furthermore, the toxicities of NADESs were also evaluated using four bacteria (*S. aureus, L. monocytogenes, E. coli* and *S. enteritidis*)[41]. The obtained results agreed with the hypothesis that NADESs are non-toxic and biocompatible since most of the tested ChCl- and glycerol-based NADESs did not cause the inhibition of bacterial growth. The exception was NADES prepared from L-arginine and glycerol which showed high toxicity towards the four tested bacteria (*S. aureus, L. monocytogenes, E. coli* and *S. enteritidis*)[41]. This is an interesting result because separately both glycerol and L-arginine are recognized as non-toxic and FDA approved these compounds, but by forming NADES through hydrogen bonding, such eutectic mixture becomes toxic most likely due to charge delocalization[41]. In another report, Redovniković's group further studied the antibacterial activity of NADESs[43]. The disk diffusion assay was applied to evaluate toxicity of betaine-, choline-, citric acid-, sugar-, and sugar alcohol-based NADESs towards *Proteus mirabilis, Pseudomonas aeruginosa, Salmonella typhimurium, Staphylococcus aureus* and *E. coli*[43]. All the tested NADESs, except ChCl:xylitol, ChCl:sorbitol, and betaine:glucose were found toxic to the selected bacterial strains[43]. The antibacterial activity of NADESs was higher for the acid containing NADESs. Furthermore, contrary to some previous reports[38, 49, 60], the effect of NADESs was not related to whether the bacterial strain was gram- positive or gram- negative[43].

299 Table 1. The toxicity of DESs determined by disk diffusion method.

DES	Microorganisms			Toxicity results	Ref.
	Bacterium G(+)	**Bacterium G(-)**	**Fungi**		
ChCl:glycerol (1:3) ChCl:ethylene glycol (1:3) ChCl:triethylene glycol (1:3) ChCl:urea (1:3)	*Bacillus subtilis*, *Staphylococcus aureus*	*Escherichia coli*, *Pseudomonas aeruginosa*		• All the DESs showed no toxic effect on tested genus of bacteria. • The individual components of DESs showed no toxic effect on tested genus of bacteria.	[48]
MTPB:glycerol (1:3) MTPB:ethylene glycol (1:3) MTPB:triethylene glycol (1:3)	*Bacillus subtilis*, *Staphylococcus aureus*	*Escherichia coli*, *Pseudomonas aeruginosa*		• All the DESs showed relative toxic effect on gram-negative bacteria, while only MTPB:ethylene glycol DES showed effective toxicity towards gram-positive bacteria. • The toxic effect of individual components of DESs was not assayed.	[38]
ChCl:urea (1:2) ChCl:acetamide (1:2) ChCl:ethylene glycol (1:2) ChCl:glycerol (1:2) ChCl:1,4-butanediol (1:4)	*Staphylococcus aureus*, *Listeria monocytogenes*	*Escherichia coli*, *Salmonella enteritidis*		• All the DESs except for acid containing DESs showed no toxic effect on tested genus of bacteria. • The toxic effect of individual	[49]

ChCl:triethylene glycol (1:4) ChCl:xylitol (1:1) ChCl:D-sorbitol (1:1) ChCl:PTSA (1:1) ChCl:oxalic acid (1:1) ChCl:levulinic acid (1:2) ChCl:malonic acid (1:1) ChCl:malic acid (1:1) ChCl:citric acid (1:1) ChCl:tartaric acid (2:1) ChCl:xylose:water (1:1:1) ChCl:sucrose:water (5:2:5) ChCl:fructose:water (5:2:5) ChCl:glucose:water (5:2:5) ChCl:maltose:water (5:2:5)				components of DESs was not assayed.	
BC:acrylic acid (1:2) BC:methacrylic acid (1:2.5)	*Staphylococcus aureus* NRS234	*Escherichia coli* ATCC 25922	*Candida albicans* ATCC 18804	• All the DESs showed relative toxic effect on tested genus of bacteria and fungi. • The individual components of DESs showed relative toxic effect on tested genus of bacteria and fungi.	[69]
ChCl:1,2-	*Staphylococcus*	*Escherichia*		• All the DESs, but	[41]

propanediol (1:1) ChCl:glycerol (1:1) ChCl:glucose (2:5) ChCl:sucrose (1:1) ChCl:xylitol (1:2) ChCl:sorbitol (2:5) glycerol:L-proline (3:1) glycerol:L-alanine (3:1) glycerol:glycine (3:1) glycerol:L-histidine (3:1) glycerol:L-threonine (3:1) glycerol:L-lysine (4.5:1) glycerol:L-arginine (4.5:1)	*aureus*, *Listeria monocytogenes*	*coli*, *Salmonella enteritidis*		glycerol:L-lysine (*E. coli*) and glycerol:L-arginine (all four bacterial strains), showed no toxic effect on tested genus of bacteria. • ChCl and glycerol individually showed no toxic effect on tested genus of bacteria. L-arginine showed relative toxic effect on *E. coli*.	
capric acid:lauric acid (2:1) capric acid:myristic acid (3:1) capric acid:stearic acid (4:1)	*Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* ATCC 700698 (Methicillin-resistant strain, MRSA), *Staphylococcus epidermis* ATCC 35984 (Methicillin-resistant strain, MRSE)	*Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922	*Candida albicans* ATCC 90029	• All the DESs showed no toxic effect on tested genus of G(-) bacteria and showed relative toxic effect on tested genus of G(+) bacteria and fungi. • The individual components of DESs showed no toxic effect on tested genus of G(-) bacteria and showed relative toxic effect on tested genus of G(+) bacteria (except stearic acid) and fungi (except capric, lauric and myristic acid).	[60]

menthol:stearic acid (8:1)	*Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* ATCC 700698 (MRSA), *Staphylococcus epidermis* ATCC 35984 (MRSE)	*Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922		• This DES showed no toxic effect on tested genus of G(-) *and* showed relative toxic effect on tested genus of G(+) bacteria. • Stearic acid showed no toxic effect on tested genus of bacteria, while menthol showed relative toxic effect on tested genus of G(+) bacteria.	[67]
menthol:lactic acid (1:2)	*Staphylococcus epidermis*	*Pseudomonas aeruginosa*, *Escherichia coli*		• All the DESs showed toxic effect on tested genus of bacteria. • The toxic effect of individual components of DES was not assayed.	[68]
ChCl:urea (1:2) ChCl:ethylene glycol (1:2) ChCl:glycerol (1:2)	*Arthrobacter simplex* TCCC 11037			• All the DESs showed no toxic effect on tested genus of bacteria at 30 % concentration. • All the DESs, but ChCl:urea, showed relative toxic effect on *A. simplex* at 60 % concentration. • Glycerol and urea individually showed no toxic effect on tested genus of bacteria, while toxic effect of ChCl toward	[57]

				A. simplex was higher than for tested DESs.	
betaine:urea (1:1.5)		*Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853		• This DES showed no toxic effect on tested genus of bacteria. • The toxic effect of individual components of DES was not assayed.	[70]
betaine:malic acid (1:1)		*Escherichia coli*		• This DES showed relative toxic effect on tested genus of bacteria. • The toxic effect of individual components of DES was not assayed.	[71]
ChCl:oxalic acid:ethylene glycol (1:1:1) ChCl:oxalic acid:glycerol (1:1:1) ChCl:citric acid:ethylene glycol (1:1:1) ChCl:citric acid:glycerol (1:1:1)	*Staphylococcus aureus* ATCC 9144	*Escherichia coli* ATCC 23564	*Candida albicans* ATCC 10231	• All the DESs showed relative toxic effect on tested genus of bacteria and fungi. • The toxic effect of individual components of DESs was not assayed.	[72]
ChCl:oxalic acid (1:1) ChCl:urea (1:2) ChCl:xylitol (5:2) ChCl:sorbitol (2:3) betaine:glucose (5:2) betaine:malic acid:proline (1:1:1) betaine:malic acid:glucose (1:1:1)	*Staphylococcus aureus* 3048	*Escherichia coli* 3014, *Proteus mirabilis* 3008, *Salmonella typhimurium* 3064, *Pseudomonas aeruginosa*	*Candida albicans* 86	• All acid containing DESs showed relative toxic effect on tested genus of bacteria. • Only ChCl:oxalic acid DES inhibited growth of *C. albicans*. • The toxic effect of individual components of DESs	[43]

citric acid:proline (1:1) citric acid:glucose:glycerol (1:1:1) citric acid:fructose:glycerol (1:1:1)		3024		was not assayed.	
ChCl:1,2-propanediol (1:2)	*Staphylococcus aureus* ATCC 25923, *Clostridium perfringens* ATCC 13124, *Listeria monocytogenes* ATCC 7644	*Escherichia coli* ATCC 25922, *Salmonella* spp. ATCC 13076		• This DES showed relative toxic effect on tested genus of bacteria. • The toxic effect of individual components of DES was not assayed.	[73]
ChCl:$ZnCl_2$ (1:2) ChCl:urea (1:2) ChCl:glycerol (1:3) ChCl:ethylene glycol (1:3) ChCl:diethylene glycol (1:2) ChCl:triethylene glycol (1:3) ChCl:fructose (2:1) ChCl:glucose (2:1) ChCl:*p*-toluene sulfonic acid (1:3) ChCl:malonic acid (1:1)			*Phanerochaete chrysosporium, Aspergillus niger, Lentinus tigrinus, Candida cylindracea*	• Zinc salts and acid containing DESs showed toxic effect on all tested genus of fungi. • The other DESs showed no toxic effect on *P.chrysosporium, A.niger, L.tigrinus.* • ChCl:urea, ChCl:ethylene glycol, ChCl:diethylene glycol, ChCl:triethylene glycol DESs showed relative toxic effect on *C. cylindracea.* • $ZnCl_2$, *p*-toluene	[52]

				sulfonic acid and malonic acid individually showed relative toxic effect on all tested genus of fungi and ethylene glycol, diethylene glycol, triethylene glycol and fructose inhibited the growth of *C. cylindracea.*	

Over the years there have also been reports, where the disk diffusion method was used to evaluate DESs antifungal activity. Firstly, Hayyan's group tested ChCl-based DESs toxicity on four fungi strains selected as a model of eukaryotic microorganisms (*Phanerochaete chrysosporium*, *Aspergillus niger*, *Lentinus tigrinus* and *Candida cylindracea*)[52]. Among these DESs the highest antifungal activity was observed for ChCl:$ZnCl_2$ DES for all tested fungi species, followed by ChCl:malonic acid and ChCl:*p*-toluenesulfonic acid DES[52]. It was also noted that the these three DESs were slightly less toxic to all tested fungi than their respective HBD individually[52]. This phenomenon was assigned to the synergistic effect of forming DES through hydrogen bonding[38, 48]. Furthermore, there have been several works where DESs and NADESs antifungal activity towards *Candida albicans* yeast was studied[43, 60, 69, 72]. For instance, Silva et al. reported that fatty acid-based DESs, namely capric acid:lauric acid, capric acid:myristic acid, capric acid:stearic acid, exhibited antifungal activity towards *C. albicans*[60]. Furthermore, it was noted that studied yeast cells were overall less susceptible to DES formulations than gram-positive and -negative bacteria[60]. However, in the work of Wang et al. it was reported that inhibition zones widths caused by BC:acrylic acid and BC:methacrylic acid

DESs were slightly larger for *C. albicans* than these obtained for bacterial strains[69]. Moreover, in the study of Jangir and co-workers the antifungal activity of ternary DESs was reported[72]. From the studied DESs ChCl:oxalic acid:ethylene glycol and ChCl:citric acid:ethylene glycol inhibited the fungal growth, while for ChCl:oxalic acid:glycerol and ChCl:citric acid:glycerol no inhibition zones were observed[72]. These findings suggest that the toxicity of DESs is microbes type-dependent, since all four DESs were found toxic to bacteria[72]. The authors concluded that non-toxicity of ChCl:oxalic acid:glycerol and ChCl:citric acid:glycerol to *C. albicans* might be explained by highly acidic nature of these compounds thus easier penetration of the lipid layer of bacteria and not fungi[72]. Finally, Redovniković's group selected various betaine-, choline-, citric acid-, sugar-, and sugar alcohol-based NADESs and observed that *Candida albicans* was only inhibited by ChCl:oxalic acid NADES[43].

2.1.2. Well diffusion method

Another diffusion technique used to evaluate DESs toxicity was agar well diffusion method, which procedure is similar to that used in the disk diffusion test. It involves preparation of the agar plate culture of the strain of interest. This is followed by cutting a hole with a diameter of 6 to 8 mm using as a sterile cork borer or a tip, and then different volumes (20–100 µL) of the antimicrobial agent at desired concentration are deposited into the well. Afterall, agar plates are incubated under suitable conditions depending on the required conditions for the growth of tested microorganisms. During incubation the antimicrobial agent diffuses in the agar medium and if it is toxic to the cells, it inhibits the growth of the microbial strain tested. The size of the measured inhibition zone caused by tested compounds indicates antimicrobial potency.

So far, well diffusion method was only used in the work conducted by Hayyan's group in which the toxicity of ChCl-based DESs and N,N-diethyl ethanol ammonium chloride (EAC)-based

DESs towards *Aspergillus niger* was studied[51]. The authors showed that EAC:$ZnCl_2$ DES inhibited the fungal growth the most, already at the lowest DES dose tested (10 mg)[51]. This DES was followed by EAC:ZnN DES and EAC:malonic acid DES[51]. Furthermore, the obtained results indicated that ChCl-based DESs were less toxic to the mold since much higher concentration were needed to inhibit its growth[51].

2.2. Dilution methods

2.2.1. Agar and broth dilution technique

As it was mentioned earlier, one of the most used techniques for DESs microbial toxicity testing are agar or broth dilution method. These methods aim to determine the lowest concentration of the studied antimicrobial agent that, under defined test conditions, inhibits the visible growth of the microorganism under investigation. Hence, using broth or agar dilution such parameters as minimum inhibitory concentrations (MICs), or effective concentrations (EC_{50}) of antimicrobial agents can be determined. In agar dilution technique, inoculum of microbes with defined numbers of cells is applied directly onto the nutrient agar plates that have contained different concentrations of antimicrobial agent[75]. Then the plates are incubated at optimal conditions (e.g., temperature, incubation time) for growth of tested microorganism and after incubation the plates are visually inspected. The presence of colonies on the plates indicates growth of the microorganism and the plate with the lowest concertation of tested compound where microorganism did not grow indicates its MIC value[75]. The advantage of agar dilution is that it is a suitable method when testing large numbers of bacterial isolates against a limited number of antimicrobial agents in a limited number of concentrations[76]. However, when testing low concentrations, an even distribution within the agar must be assured[76]. The main drawback of agar dilution is the fact that it is time consuming method, which requires preparation of high

number of plates with different concentrations of antimicrobial agent[76]. For that reason, agar dilution is also not very cost-efficient technique[76]. What is more, it requires the availability of the antimicrobial agents to be tested as pure substances and individual mistakes in the preparation of stock concentrations or dilution series can occur, resulting in variability of results[76].

For comparison, in broth dilution method microorganisms are grown in liquid nutrient medium containing increasing concentrations (typically a two-fold dilution series) of the antimicrobial agent, which is then inoculated with a defined number of microbial cells[75, 77]. Depending on the final volume of the liquid medium in each analyzed sample, this method can be termed as macro-dilution for a total volume of 2 mL, or microdilution, if performed in microtiter plates format with total volume up to 500 µL per well[75, 77]. In broth dilution method, the growth is assessed after incubation of inoculated samples for a defined period of time (16–20 h) and the MIC or EC_{50} value is read. Moreover, for this purpose, antimicrobial agent-free test samples - which serve as growth controls - must be included in each assay. In broth dilution method the toxicity of compounds is determined by measuring the mortality or total number of viable cells after certain exposure time to specific concentrations of antimicrobial agents[75, 77]. The schematic representation of broth microdilution procedure is shown in Fig. 4. This technique can be used to test the susceptibility of microorganisms to multiple chemicals at once and quantitative data are obtained[76]. Another advantage of broth dilution is its high accuracy[76]. Other advantages include the possibility of performing this test in practically every laboratory, the easiness of testing and evaluating and the ability for the results of some tests to be read in automatic mode[76]. However, as in agar dilution, this method can be time consuming and individual mistakes in the preparation of stock concentrations or dilution series may take place especially when no automation equipment is available[76].

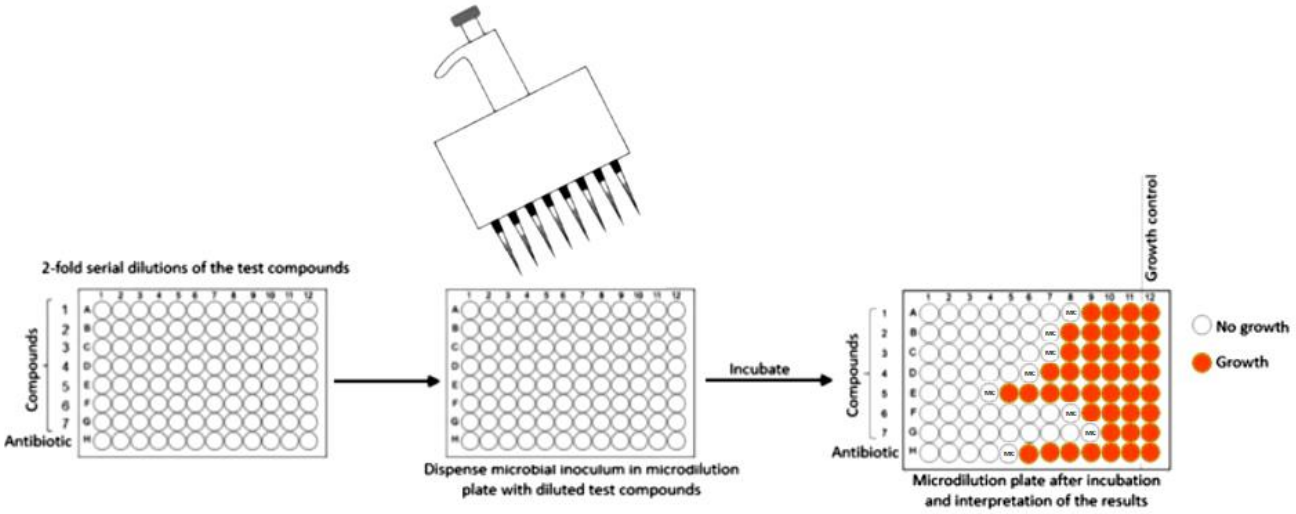

Fig. 4: Broth microdilution procedure for MIC determination.

Furthermore, there exist various methods for determination of the number of viable cells after incubation of tested microorganism with tested compounds. The cells viability can be evaluated using simple visual inspection or absorbance measurement of turbidity, and the obtained results that could be over- or underestimated due to, for example, turbidity of the compounds itself, can be further confirmed by subculturing of each tested concentration to agar plates that do not contain the test agent. By doing this it is possible to determine minimum bactericidal concentration (MBC) or minimum fungal concentration (MFC). MBC or MFC is complementary method to the MIC determination using broth dilution technique. MBC/MFC demonstrates the lowest concentration of antimicrobial agent that results in complete microbial death. This means that even if a particular MIC shows inhibition, plating the microbes onto agar might still result in organism proliferation because the antimicrobial agent did not cause death of all cells of tested microorganism. Moreover, for cells viability determination more accurate assays that employs colorimetric, or fluorescence dyes can be used. Such assays provide not only more accurate data but also the confirmation of the results by MBC/MFC determination could be avoided because

after staining it is possible to distinguish between living and dead cells. Therefore, the summary of literature results for DESs toxicity assayed by agar and broth dilution, with special respect to the cell viability determination methods used in each cited study, will be provided in the next subsections.

2.2.1.1. Visual or absorbance determination of cell viability based on turbidity

To date, in most of the published works, where the toxicity of DESs was examined with use of broth dilution method, the cells viability was determined either by visual inspection or by measuring the absorbance of the samples in the absence and presence of DESs. The summary of the results found in the literature for microbial toxicity of DESs determined by broth dilution technique and visual or absorbance determination of cell viability are presented in Table 2. In the first work conducted by Wen et al. broth macro-dilution was used to determine EC_{50} for series of ChCl- and cholinium acetate (ChAc)-based DESs against *E. coli* DH5α[39]. The bacterial growth was ascertained by measuring the absorbance of the samples at 550 nm. This study revealed that DES concentrations below 75 mM were almost non-toxic to the bacterial cells since the inhibition index was lower than 10%[39]. Furthermore, it was observed that 0.75 M DES inhibited the growth of 72.8–93.8%, indicating that at higher concentration DESs become significantly hazardous to *E. coli*[39]. The calculated EC_{50} values varied for different tested DESs and were mainly dependent on HBA used in DES formation. In general, DESs prepared with ChAc had lower EC_{50} values than respective ChCl-based DESs, indicating higher antibacterial activity of the former[39]. Moreover, the obtained results revealed that beside HBA also HBD has influence on DESs toxicity effect[39]. In particular, much higher EC_{50} values were obtained for DESs which have ethylene glycol (EG) in their composition (EC_{50} = 532.0 mM for ChCl:EG and EC_{50} = 281.1 mM for ChAc:EG)[39]. Overall, the most toxic compound was ChAc:glycerol

DES with EC_{50} of 58.0 mM, followed by ChAc:acetamide (EC_{50} = 97.2 mM)[39]. The obtained results also showed that bacterial cells of *E. coli* were more susceptible to the DESs than their individual components because the EC_{50} values following exposure to individual DES components were all much higher than 800 mM[39]. In this work, the authors hypothesized that DESs inhibited the bacterial growth by interacting with the cellular membrane. Furthermore, the fact that DES in aqueous solution may be partially dissociated was considered and the obtained results explained as a consequence of the possible interaction of the cholinium cation with the polysaccharide or peptide chains of peptidoglycan through hydrogen-bonding or electrostatic interaction, leading to cell wall distortion or disruption[39]. On the other hand, the higher toxicity of DESs than their individual components was assigned to charge delocalization through hydrogen bonding[39].

In another work, Lou's group used broth macro-dilution technique to quantitatively evaluate the toxicity of seven acid-based DESs, which were previously shown to inhibit bacterial growth as determined using disk diffusion assay[49]. In this study MIC values were obtained by measuring absorbance at 600 nm of the samples incubated with 8−52 mM (at 2 mM intervals) DESs solutions. The obtained results indicated that MIC values for gram-negative bacteria (*E. coli* and *S. enteritidis*) were generally lower than those for gram-positive bacteria (*S. aureus* and *L. monocytogenes*) and thus the studied DESs were more toxic to the tested gram-negative bacteria[49]. The ChCl:*p*-toluenesulfonic acid (PTSA) and the ChCl:malonic acid DESs had the highest MIC value from the studied DESs. Furthermore, it was observed that the MIC values increased with elongation of the carbon chain for ChCl:oxalic acid and ChCl:malonic acid DESs[49]. Moreover, DESs toxicity was related with the chemical structure of HBD used and introduction of an extra hydroxyl group in the HBD resulted in a slight increase in antibacterial

activity as observed for ChCl:malic acid and ChCl:tartaric acid DESs[49]. Overall, ChCl:oxalic acid, ChCl:levulinic acid, and ChCl:citric acid had the highest toxicity towards tested bacteria and the potency of antibacterial activity of the various ChCl-based DESs was associated with pH and to some extent to the chemical structure of HBDs[49]. After MIC determination, the bacterial suspension in the plate was cultured and MBC values for tested DESs were obtained. As it can be seen in Table 2, much higher concentrations of DESs were necessary to kill ≥99.9% of the test bacterium. In general, the obtained results confirmed that ChCl:PTSA and ChCl:malonic acid DESs exhibited the lowest toxicity towards tested genus of bacteria with MBC values ranging from 28.0-50.0 mM and 20.0-48.0 mM for ChCl:PTSA and ChCl:malonic acid, respectively[49].

Later, the broth microdilution technique was used to study the antibacterial activity of fatty acid-based DESs[60]. In this work, the results obtained from qualitative analysis done using disk diffusion assay were taken into account and MIC values were determined for 3 bacterial strains: *S. aureus* ATCC 25923, *S. aureus* ATCC 700698 (Methicillin-resistant strain, MRSA), *S. epidermis* ATCC 35984 (Methicillin-resistant strain, MRSE)[60]. The obtained MIC values for the DESs revealed that capric acid:lauric acid DES had the highest overall antimicrobial activity and was followed closely by capric acid:myristic acid and finally capric acid:stearic acid DES, which was the least toxic against studied bacteria[60]. Moreover, it was observed that DESs were usually less toxic than their individual components. Regarding DESs antibacterial activity for each of the tested bacteria, the MIC values indicated that these solvents were more toxic to the *S. aureus* than to the *S. aureus* MRSA and *S. epidermis* MRSE strains, which were, as expected, more competitive microorganisms due to their resistance to Methicillin[60]. The authors assumed that antimicrobial potential of DESs is derived from the non-specific antimicrobial action mechanism of fatty acids since they can lead to membrane destabilization/dissolution

causing a wide range of direct and indirect inhibitory effects[60]. Furthermore, it was also emphasized that for the studied DESs, and at the dilutions used, the vast network of intermolecular interactions was not weakened or disrupted, suggesting that the obtained MIC values are the effect of DESs interaction with bacterial cells and not mixture of their individual components[60]. The MBC study further confirmed that capric acid:lauric acid DES was the most toxic tested solvent and MBC values of 1250 µg/mL were obtained for all studied bacteria[60].

In another work of Silva et al., the authors further studied the antibacterial activity of DESs, and they selected THEDES composed of menthol and stearic acid[67]. After initial experiments using disk diffusion method, the MIC data for THEDES and its individual components against *S. aureus* ATCC 25923, *S. aureus* MRSA and *S. epidermis* MRSE using broth macro-dilution were gathered. According to the obtained results, the observations made from disk diffusion study were confirmed, and menthol was found toxic to the bacteria with MIC value of 4 and 8 mM for *S. aureus* ATCC 25923 and *S. aureus* MRSA, *S. epidermis* MRSE, respectively[67]. Furthermore, stearic acid did not exhibit any antibacterial activity[67]. THEDES showed antimicrobial activity against all the studied bacteria, being more efficient against *S. aureus* ATCC 25923 than Methicillin-resistant strains tested (*S. aureus* MRSA, *S. epidermis* MRSE)[67]. It was also observed that THEDES was more toxic to bacteria than menthol, even though the THEDES contains lower concentration of menthol than this needed to inhibit bacterial growth menthol itself[67]. This same was valid as far it comes to the anti-bactericidal properties of the studied THEDES and MBC values of 6.52 mM and 13.03 mM were obtained for *S. aureus* ATCC 25923 and both Methicillin-resistant strains tested, respectively. Therefore, it was concluded that it was an effect of a synergistic interaction between menthol and stearic acid that

493 increases antibacterial activity[67]. The toxicity of another THEDES (ChCl:mandelic acid) was
494 also studied by Mano and co-workers[78]. According to the MIC values obtained with broth
495 macro-dilution experiments, this THEDES was less toxic to *E. coli* and *S. aureus* than mandelic
496 acid with MIC of 5 and 2.5 mg/mL for both bacteria, respectively[78]. These results suggested
497 that the antibacterial activity of mandelic acid decreases when it is part of the supramolecular
498 THEDES structure with ChCl because of antagonistic effect[78].

Table 2. The toxicity of DESs determined by broth dilution method.

DES	Microorganism											
	Bacterium G(-)						Bacterium G(+)				Fungi	
	*Escherichia coli**		*Staphylococcus aureus**		*Listeria monocytogenes*		*Salmonella enteritidis*		*Salmonella typhimurium*	*Staphylo-coccus aureus* MRSA	*Staphylo-coccus epidermis* MRSE	*Aspergillus niger (filamentous fungus)*	*Candida albicans* (yeast)
	EC_{50} [mM]											
ChCl:urea (1:1),	295.9											
ChCl:acetamide (1:1),	275.2											
ChCl:glycerol (1:1),	532.0											
ChCl:ethylene glycol (1:1),	434.4											
ChAc:urea (1:1),	275.8											
ChAc:acetamide (1:1),	97.2											
ChAc:glycerol (1:1),	281.1											
ChAc:ethylene glycol (1:1)	58.0											
	MIC [mM]	MBC [mM]	MIC [mM]	MBC [mM]	MIC [mM]	MBC [mM]	MIC [mM]	MBC [mM]				
l:PTSA (1:1),	18	28	18	34	30	50	26	40				
l:oxalic acid (1:1),	12	18	12	26	14	30	12	22				
l:levulinic acid (1:2),	12	16	14	22	12	36	12	26				
l:malonic acid (1:1),	18	20	16	30	24	48	20	34				
l:malic acid (1:1),	14	20	14	24	22	48	18	42				
l:citric acid (1:1)	12	20	12	28	20	42	16	38				

ChCl:tartaric acid (2:1)	14	18	12	20	16	44	18	40								
			MIC [μg/mL]	MBC [μg/mL]						MIC [μg/mL]	MBC [μg/mL]	MIC [μg/mL]	MBC [μg/mL]		MIC [μg/mL]	MFC [μg/mL]
capric acid:lauric acid (2:1)			625	1250						625	1250	625	1250		625	1250
capric acid:myristic acid (3:1)			625	1250						625	1250	625	1250		1250	2500
capric acid:stearic acid (4:1)			1250	2500						1250	2500	1250	2500		1250	2500
	% of cell proliferation		% of cell proliferation						% of cell proliferation							
ChCl:glycerol (1:1.5)	100		54.92±2.72						100							
ChCl:glycerol (1:3)	100		53.49±3.14						100							
ChCl:formic acid (1:1.5)	100		47.65±2.84						97.78±1.71							
ChCl:formic acid (1:3)	100		44.75±4.95						98.55±1.88							
ChCl:lactic acid (1:1.5)	100		52.45±3.47						96.29±2.30							
ChCl:lactic acid (1:3)	100		50.73±2.63						100							
	MIC [mM]															
acetylcholine chloride:acetamide (1:2)	600															
														MIC [mg/mL]		
ChCl:ethylene glycol (1:2)														325.3±34		
l:glycerol (1:2)														550.4±51		
l:urea (1:2)														138.5±23		
:ethylene glycol (1:2)														314.8±44		
:glycerol (1:2)														495.4±63		
:malonic acid (1:1)														64.4±14		

EAC:ZnN (1:1) EAC:$ZnCl_2$ (1:2)										<2.2 <1.3	
		MIC [mM]	MBC [mM]				MIC [mM]	MBC [mM]	MIC [mM]	MBC [mM]		
menthol:stearic acid (8:1)		3.26	6.52				6.52	13.03	6.52	13.03		
	MIC [mg/mL]	MIC [mg/mL]										
ChCl:mandelic acid (1:2)	5	5										
	MIC [µL/mL]	MIC [µL/mL]										
perillyl alcohol:camphor (1:1)	31.25	31.25										
menthol:perillyl alcohol (1:1)	31.25	62.50										
menthol:camphor (1:1)	62.50	62.50										
menthol:eucalyptol (1:1)	62.50	62.50										
menthol:myristic acid (8:1)	62.50	62.50										
	MIC	MIC										
malic acid:sucrose:water (1:1:18)	1:1 (v/v)	1:1 (v/v)										
fructose:glucose:water 7)	Non-toxic	Non-toxic										
ose:sucrose:water 15)	Non-toxic	Non-toxic										

References in order of appearing in the table: [39], [49], [60], [79], [80], [51], [67], [78], [81], [82].

e that for *E. coli* and *S. aureus* bacterial species in some studies different strains were selected e.g. *E. coli* DH5α[39], *E. coli* ATCC 25922[79], *E. coli* BL21 (DE3)[80], *E. coli* K12 1498[78], *E. coli* ATCC 8739[81].

In the work of Teh et al., broth microdilution method was used to determine the toxicity of DESs prepared with ChCl as HBA and glycerol, formic acid, lactic acid as HBDs towards three bacterial species (*E. coli*, *S. aureus* and *Salmonella typhimurium*)[79]. Here, contrary to the most studies where MIC or EC values were obtained, the authors decided to determine the percentage of cell proliferation by measuring the absorbance at 600 nm of the samples incubated and not incubated with 1 mg/mL DESs solutions[79]. The obtained results showed that all studied DESs were almost non-toxic to both the gram-negative bacterial strains - *E. coli* and *S. typhimurium* - and more than 95% of cell viability after incubation was achieved[79]. These results were assigned to the structure of outer membrane of the gram-negative bacterial strains made up of lipopolysaccharide and protein[79]. It was assumed that *E. coli* and *S. typhimurium* formed a formidable barrier which restricted the attack of DESs from penetrating into the bacterial cell envelopes[79]. On the other hand, ChCl-based DESs were shown to be toxic to the gram-positive *S. aureus* at the same concentration because no barrier was established as its cell wall consists solely of a thick peptidoglycan layer, which seems to be more susceptible to DESs[79]. Additionally, all the studied DESs had comparable antibacterial activity against *E. coli* and *S. typhimurium* as their individual components, while for the *S. aureus* the lower toxicity was obtained for the DESs than for HBDs themselves[79]. In general, it was concluded that DESs toxicity is mainly dependent on the type of HBDs and very little on the HBA:HBD molar ratio used[79].

The toxicity of ChCl-based DESs towards *Kurthia gibsonii* was also assessed by broth macro-dilution in the work of Lou's group[83]. In this study, the bacterial growth was determined by measuring the absorbance at 600 nm and the results were expressed in terms of relative biomass, with the biomass in the DESs-free broth being defined as 100%[83]. The obtained results

revealed that the addition of DESs at 2% concentration did not significantly affected the bacterial growth for all tested DESs except for ChCl:1,4-butanediol[83]. In case of ChCl:urea, ChCl:glycerol and ChCl:triethylene glycol a slight decrease in the absorbance was observed while for ChCl:ethanediol the absorbance increased slightly[83]. On the other hand, a visibly higher absorbance was achieved in the system containing 2% ChCl:1,4-butanediol DES in comparison to the control sample, thus the effect of other DES concentrations (4%, 8%, 12%, 16%, 20%) was further studied[83]. It was observed that the increase in the ChCl:1,4-butanediol concentration decreased the growth of *K. gibsonii* and approximately 10% biomass of the control at 20% of this DES was obtained[83]. Overall, it was concluded that the studied ChCl-based DESs are non-toxic to *K. gibsonii*, and that a moderate concentration of adequate solvent can increase the cellular growth[83]. Moreover, in order to further examined the effect of DESs on these bacteria, the colorimetric determination of the damaged and dead cells was also performed, as discussed in section 2.2.1.2.

In another study by Torregrosa-Crespo et al. the antimicrobial activity of acetylcholine chloride:acetamide DES was examined[80]. The authors selected *Escherichia coli* BL21 (DE3) as a model microorganism and used broth macro-dilution method to quantify potential toxicity of the DES. Furthermore, in this work continuous monitoring of pH, temperature, shaking and optical density of bacterial culture have been done to better understand the effect of DES on bacterial cells survival[80]. Also, for the first time the degree of the cellular tolerance to the DES was studied as experiments in preadapted and non-preadapted cells were conducted[80]. The obtained results showed that at concentrations up to 300 mM the DES did not have toxic effect towards *E. coli* and cellular preadaptation was crucial for the cells to grow[80]. Moreover, the bacterial growth was still observed at concentrations between 300 mM and 450 mM, although

cellular growth and metabolic activities were slightly affected by such high DES concentrations as indicated with diauxic or triauxic growth curves and higher Lag times than those observed at lower DES concentrations[80]. However, the concentrations higher than 600 mM were found to be toxic, as complete inhibition of growth was observed[80]. The authors concluded that DES toxicity was a result of not only the chemical composition of the DES, but also the highly acidic pH of the growth medium supplemented with the DES[80].

In the most recent work, the toxicity on plant bacteria (*Xanthomonas campestris* CECT 97, *Erwinia amylovora* CECT 222, *Erwinia toletana* CECT 5263, *Clavibacter michiganensis* subsp. *michiganensis* CECT 790, *Clavibacter michiganensis* subsp. *insidious* CECT 5042, *Rhizobium radiobacter* CECT 4119, *Pseudomonas syringae* CECT 4429, *Pseudomonas savastanoi* CECT 5019) of six DESs namely ChCl:sucrose, ChCl:xylitol, fructose:glucose:sucrose (1:1:1), fructose:glucose:sucrose (2:3.6:1) betaine:sucrose (2:1), betaine:sucrose (4:1) was evaluated by broth microdilution method and the obtained results compared to the toxicity of classic solvents e.g. dimethylsulfoxide (DMSO), ethanol and glycerol[84]. It was revealed that most of the tested DESs were not toxic to the tested bacteria with MIC values 300-1200 $x10^3$ mg/L[84]. The biofriendly character of DESs composed of carbohydrates (fructose:glucose:sucrpose (1:1:1) and frucrose:glucose:sucrose (2:3.6:1) was assigned to the fact that their components e.g. glucose, fructose and sucrose are used as nutrition sources by these microorganisms[84]. Furthermore, betaine:sucrose (4:1) DES was the most toxic of DESs tested, with MIC values between 38-150 $x10^3$ mg/L[84]. In general, the following order of increasing toxicity of DESs was deducted: fructose:glucose:sucrose (1:1:1) = fructose:glucose:sucrose (2:3.6:1) < ChCl:sucrose (1:2) < ChCl:xylitol (2:1) < betaine:sucrose (2:1) < betaine:sucrose (4:1)[84]. Moreover, these DESs showed lower toxicity than glycerol or DMSO for most tested bacteria[84]. Even though, the

majority of the selected bacteria were gram-negative (except for the *Clavibacter* spp.), it was concluded that the toxic effects of DESs mainly depended on the type of compounds used in their preparations and on the susceptibility of the different bacteria strain and not on the cell membrane composition[84].

The toxicities of NADESs were also studied by broth microdilution in the work of Rodrigues and co-workers[81]. In this study, terpene-based NADESs, namely perillyl alcohol:camphor, menthol:perillyl alocohol, menthol:camphor, menthol:eucalyptol, menthol:myristic acid, were tested against *E. coli* and *S. aureus* bacterial strains. It was observed that all NADESs inhibited the growth of *E. coli* and *S. aureus*, with MICs ranging from 31.25 to 62.50 μL/mL[81]. Perilllyl acid:camphor NADES exhibited the highest antimicrobial activity from all studied NADESs[81]. Moreover, no significant differences in MICs were found for gram-positive and gram-negative bacteria[81]. The authors explained these results as a consequence of the antimicrobial effect of NADES starting materials – terpenes and fatty acids – which are well known antimicrobial agents against both gram-positive and -negative bacteria[81]. Later, Rachmaniah et al. studied the toxicity of malic acid:sucrose, fructose:glucose and fructose:sucrose NADESs towards *E. coli* and *S. aureus* bacterial strains[82]. In this work, broth macro-dilution method was used to determine MIC values and the obtained results revealed that malic acid:sucrose NADES had the highest toxicity of the studied solvents[82]. The high antimicrobial activity of this solvent was assigned to low pH of this NADES mainly derived from malic acid[82]. Meanwhile, both NADESs composed entirely of sugars, i.e. fructose:glucose and fructose:sucrose, were found non-toxic to bacterial strains used [82]. Beside higher pH of sugar-based NADESs, these results were also explained by the fact that carbohydrates (especially glucose and fructose) are the sources of carbon and energy for the growth of bacterial cells[82]. Furthermore, the MBC test

was applied to determine if studied NADESs possess ability to completely (>99.99 %) suppress bacterial growth. The obtained results showed the eradication of bacterial growth for malic acid:sucrose NADES, while the bacterial growth was not effected by fructose:glucose and fructose:sucrose NADESs[82].

Both agar and broth dilution methods were also used to study DESs antifungal activity[51, 60, 84-86]. Firstly, Hayyan's group examined the toxicity of eight different DESs using ChCl and EAC as the HBAs and ethylene glycol, glycerol, urea, malonic acid, zinc chloride ($ZnCl_2$), and zinc nitrate hexahydrate (ZnN) as the HBDs towards *Aspergillus niger*[51]. According to the MIC data obtained by using broth macro-dilution method all the DESs were shown to be toxic to the examined fungi and the antifungal activity of EAC- based DESs was higher than ChCl-based DESs[51]. Furthermore, it was observed that EAC-based DESs that were prepared using $ZnCl_2$, ZnN and malonic acid as HBDs were way more toxic than these prepared with ethylene glycol and glycerol[51]. The obtained MIC data also revealed that both HBAs (ChCl and EAC) were less toxic to *A. niger* than their respective DESs, while antifungal activities were slightly higher (for the EAC- based DESs) or lower (for the ChCl- based DESs) than those of their corresponding HBDs[51]. Overall, it was concluded that DES individual components play an important role in the toxicity profile of these solvents, as well as their concentration and specific interactions with microorganisms[51]. Later, Silva et al. determined the MIC and MFC values for DESs based on fatty acids, which according to disk diffusion assay inhibited the growth of *Candida albicans* yeast cells[60]. The obtained MIC/MFC data acquired by using broth microdilution method revealed that capric acid:lauric acid DES had the highest antifungal activity from all studied DESs[60]. The following order of the DESs toxicity against examined yeast was deducted: capric acid:lauric acid > capric acid:myristic acid ≈ capric acid:stearic acid[60].

590 Interestingly, this is not the same order as this obtained using disk diffusion assay (capric
591 acid:stearic acid > capric acid:lauric acid > capric acid:myristic acid)[60]. Furthermore, also the
592 DESs individual components possessed significant MIC values, while these fatty acids displayed
593 no activity during the disk diffusion assay[60]. This observation clearly indicates that a negative
594 result in the disk diffusion assay does not necessarily exclude toxicity of some compounds and
595 highlight the need of further analysis by broth dilution method[60]. The broth macro-dilution
596 method was also used to evaluate toxicity of NADES composed of lactic acid:glucose towards *C.*
597 *albicans*[85]. It was shown that this solvent is non-toxic to yeast cells, because at the dilutions
598 used, the growth of *C. albicans* was not inhibited[85]. Furthermore, in the work of Boiteux et al.
599 the toxicity of this same NADES towards *Botrytis cinerea* was evaluated using agar dilution
600 method[86]. Once again, the obtained results showed that all seven tested dilutions of NADES
601 did not present antifungal effect and thus this NADES can be considered as non-toxic to *B.*
602 *cinerea*[86]. Recently, Rodriguez-Juan et al. also studied the toxicity of DESs against seven
603 yeasts present in wine fermentation, namely *Saccharomyces paradoxus* CECT 1939,
604 *Hanseniaspora guillermondi* CECT11102, *Hanseniaspora uvarum* CECT 10389, *Metschnikowia*
605 *pulcherrima* CECT12890, *Torulaspora delbrueckii* CECT 10589, *Saccharomyces cerevisae* EC
606 1118 and *Starmerella bombicola* CBS 268[84]. Here, various DESs combining ChCl,
607 carbohydrates, betaine, alcohols as HBAs and HBDs were selected and MICs determined using
608 broth microdilution[84]. The obtained results can be summarized to the following order of
609 increasing toxicity: fructose:glucose:sucrose (1:1:1) = fructose:glucose:sucrose (2:3.6:1) =
610 betaine:sucrose (2:1) < ChCl:sucrose (1:2) < ChCl:1,2-propanediol (1:1) < ChCl:xylitol (2:1) <
611 ChCl:1,4-butanediol (1:5)[84]. As expected, all tested DESs that contained carbohydrates in their
612 composition were found to be practically not toxic to the tested yeasts with MIC values of 600
613 $x10^3$ mg/L[84]. Astonishingly, betaine:sucrose DES had the same MIC value of $600x10^3$ mg/L as

fructose:glucose:sucrose (1:1:1) and fructose:glucose:sucrose (2:3.6:1) and thus did did not show any toxic effect on tested yeast, while it the same DES was found moderately toxic to the plant bacteria, as discussed earlier[84]. Overall, it was observed that the tested yeasts were usually less susceptible to DESs than conventional solvents such as DMSO and glycerol, making these solvents an interesting candidates for use for example in cryoprotection[84].

2.2.1.2. Colorimetric determination of cells viability

Until now there are only five published works (see Table 3) where cells viability after incubation with DES solutions using colorimetric techniques was performed[50, 83, 87-89]. In first report baker's yeast (*Saccharomyces cerevisiae*) viability in different cholinium-based DESs containing 50% of water (w/w) and potassium phosphate buffer (100 mM, pH 7.4) was determined at 3 and 24 h after inoculation[50]. For that the cell suspension was mixed with an equal volume of methylene blue and incubated for 5 min at room temperature. Here, methylene blue dye was used to stain the yeast cells, however this dye can be applied to all aerobic microorganisms[90]. Methylene blue in a presence of living cells gets enzymatically reduced to a colorless product and living cells become unstained, whereas dead cells are stained blue[90]. Therefore, after staining with methylene bleu, blue-colored cells can be easily visualized and counted as dead cells. In the work of Redovniković's group, it was observed that ChCl:malic acid, ChCl:oxalic acid and ChCl:urea DESs were toxic to the yeast cells[50]. Already after 3 hours of incubation yeast cells viability decreased tremendously for these solvents and the most detrimental toxic effect was observed for ChCl:oxalic acid DES with only 19% and 4% of living cells after 3 h and 24 h, respectively[50]. On the other hand, no significant toxic effect was observed for DESs formed using sugars, glycerol and ethylene glycol as HBDs with yeast viability of 76–99% and 62–98% after 3 and 24 h incubation, respectively[50]. Furthermore, the comparable viability of yeast in

637 ChCl:ethylene glycol and ChCl:glucose after 24 h, as in control samples in potassium phosphate
638 buffer (100 mM, pH 7.4), was observed[50]. The toxicity of DESs was assigned to the high
639 osmotic pressure imposed on the yeast cells by such high concentrations of these solvents,
640 resulting in diffusion of water out of the cells[50]. Furthermore, the differences in the potency of
641 antifungal activity for different DESs was explained by differences in the pH values of the
642 solvents[50]. Consequently, DESs prepared with organic acids as HBDs were the most toxic to
643 yeast cells due to their pH values (pH < 3) lower than the optimum pH range for *S. cerevisiae*
644 growth (between 4 and 6)[50]. Contrastingly, the pH values for DESs containing carbohydrate
645 and glycerol were around 4.5 thus resulting in lower toxicity of these DESs[50]. Moreover, non-
646 toxicity of these DESs was further explained by the fact that sugar and glycerol could be used as
647 a nutrition source for growth of yeast cells[50].

648 Table 3. The toxicity of DESs obtained using colorimetric assays for cell viability determination.

DES	**Microorganisms**			**Toxicity results**	**Ref.**
	Bacterium G(+)	**Bacterium G(-)**	**Fungi**		
ChCl:glycerol (1:2) ChCl:ethylene glycol (1:2) ChCl:oxalic acid (1:1) ChCl:malic acid (1:1) ChCl:glucose (2:1) ChCl:fructose (3:2) ChCl:xylose (2:1) ChCl:urea (1:2)			*Saccharomyces cerevisiae* (yeast)	• Acid and urea containing DESs highly decreased yeast cell viability and thus showed toxic effect on tested genus of yeast. • Carbohydrate, glycerol, and ethylene glycol containing DES showed good biocompatibility and 62–98% cell viability after 24 h was obtained. • The toxic effect of	[50]

				individual components of DESs was not assayed.	
ChCl:urea (1:2) ChCl:glycerol (1:2) ChCl:ethanediol (1:2) ChCl:triethylene glycol (1:4) ChCl:1,4-butanediol (1:4)	*Kurthia gibsonii* SC0312			• ChCl:urea, ChCl:triethylene glycol and ChCl:1,4-butanediol DESs slightly increased the number of damaged cells at 2% concentration. • ChCl:ethanediol and especially ChCl:glycerol highly decreased the bacterial cell viability at 2% concentration. • The toxic effect of individual components of DESs was not assayed.	[83]
ChCl:urea (1:2) ChCl:glycerol (1:2) ChCl:ethylene glycol (1:2)	*Arthrobacter simplex* TCCC 11037			• All the DESs showed relative toxic effect on tested genus of bacteria, and membrane integrity decreased to 70, 51, 39% for ChCl:glycerol, ChCl:ethylene glycol, ChCl:urea, respectively. • The toxic effect of individual components of DESs was not assayed.	[87]
menthol:decanoic acid (1:2)	*Staphylococcus aureus* ATCC 6538	*Escherichia coli* ATCC 8739		• This DES showed no toxic effect on tested genus of *E. coli* and was found toxic to *S. aureus*. • DES individual components showed no toxic effect on tested genus of *E. coli.* • DES individual components showed higher	[88]

				antibacterial activity against *S. aureus* than tested DES.	
ChCl:ethylene glycol (1:2) ChCl:malonic acid (1:2)	*Bacillus cereus* EMB20			• ChCl:ethylene glycol showed relative toxic effect on tested genus of bacteria, and 54% growth inhibition was observed. • ChCl:malonic acid was highly toxic and caused the death of all cells. • The toxic effect of individual components of DESs was not assayed.	[89]

In another work, the kit that consists of two dyes, propidium iodide (PI) and SYTO9, was used to evaluate the viability of cells after incubation with ChCl-based DESs[87]. These two dyes are able to stain nucleic acids, and green fluorescing SYTO9 can enter all cells of tested microorganism and is used to determine total number of its cells in the assayed sample, whereas red fluorescing PI enters only into the cells with damaged cytoplasmic membranes[91]. Even though this kit only enables differentiation between cells with intact and damaged cytoplasmic membranes, it is often used to distinguish viable and dead cells because it is accurate to assume that membrane-compromised cells are dead[91]. In this study, gram-positive *Arthrobacter simplex* TCCC 11037 was selected as model microorganism. The obtained results showed that the effect of ChCl-based DESs on the *A. simplex* cell membrane was different depending on the type of HBDs used[87]. For instance, the cells tolerated ChCl:glycerol DES better than ethanol (positive control), and the membrane integrity decreased to 70% compared with that in water (control sample)[87]. On the other hand, for DESs containing urea and ethylene glycol as HBDs,

663 the cell viability decreased to 39% and 51%, respectively[87]. Furthermore, these DESs were
664 more toxic to bacteria than ethanol[87]. In general, the toxic effect of three ChCl-based DESs on
665 *A. simplex* was found in this study and degree to which each solvent promoted toxicity was
666 mainly dependent on the nature of the HBDs used in DESs preparation[87].

667 Furthermore, PI fluorescein dye was also used to evaluate the effect of ChCl-based DESs on the
668 number of dead cells of *K. gibsonii*[83]. It was observed that compared with the control cells
669 there was a slight increase in the number of damaged/dead cells for 2% of ChCl:triethylene
670 glycol, ChCl:urea and ChCl:1,4-butanediol DESs[83]. On the other hand, more significant
671 increase in the number of dead cells was observed for ChCl:ethanediol and ChCl:glycerol,
672 suggesting that these two solvents are relatively toxic to this bacterium[83]. Moreover, it was
673 shown that the effect of DESs on the cell viability is concentration dependent[83]. According to
674 the experiments using different concentrations of ChCl:1,4-butanediol, the number of damaged
675 cells increased with the increased DES concentration, achieving its maximum value at 16% of
676 DES[83]. Based on these data, it was suggested that the lower viability of cells in the presence of
677 higher DESs concentrations was the result of the changed osmotic pressure in buffer[83].

678 Moreover, there also exist the test to study chemical toxicity that employs an electron acceptor
679 dye, resazurin, which changes color in the presence of dehydrogenase enzyme activity resulting
680 from procaryotic and eucaryotic cells actively growing in a culture medium[92]. Resazurin in the
681 presence of an active viable cells of examined organisms, is oxidized by cell dehydrogenases to
682 the resofurin[92]. Therefore, in such condition the analyzed samples changes color from blue (the
683 color of resazurin) to pink (the color of resofurin)[92]. Thus, if the cells growth is inhibited by
684 the presence in culture medium of chemical compound which toxicity is examined against
685 selected organism, no reduction of the resazurin occurs, and such a sample would remain

blue[92]. Since resorufin absorbs only weakly at the wavelength giving the maximum absorbance for resazurin, the decrease in resazurin concentration may be measured using a spectrophotometer, and, by varying the concentration of the test chemical, the EC_{50} value for that chemical may then be estimated[92]. This approach was used to test toxicity of DES composed of menthol and decanoic acid towards *E. coli* and *S. aureus*[88]. Here, the resazurin dye was used for the cell viability determination and the MIC and MBC value reading due to the white and opaque nature of the samples. According to the results of experiments, neither DES starting materials or DES itself had and inhibitory effect on gram-negative *E. coli* at concentrations used in the assay (MIC and MBC > 500 μL/mL)[88]. On the other hand, for *S. aureus* the DES and its individual components exhibited high antimicrobial properties with MIC and MBC values ranging between 3.91-15.63 μL/mL and 7.81-31.25 μL/mL, respectively[88]. This higher antibacterial and -bactericidal efficacy of these compounds against gram-positive *S. aureus* was attributed to the hydrophobic nature of the DES starting materials and explained by the fact that usually gram-positive bacteria are more susceptible to hydrophobic compounds, whereas gram-negative to hydrophilic compounds taking advantage of the hydrophilic character of their membrane porins[88]. Furthermore, it was also observed that for *S. aureus* ATCC 6538 strain the MIC and MBC values for DES (MIC=15.63 μL/mL, MBC=31.25 μL/mL) were higher than the MIC and MBC values for menthol (MIC/MBC=7.81 μL/mL) and for decanoic acid (MIC=3.91 μL/mL, MBC=15.63 μL/mL), indicating that tested DES has a lower antibacterial and -bactericidal activity per volume of the mixture used when compared to its individual components[88].

In another work, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay was used to assess viability of bacterial cells growing in the presence or absence of DESs at a final

concentration of 0.5 mg/mL[89]. In this assay, MTT is reduced by actively respiring cells to water-insoluble purple formazan. The formazan is then solubilized, and its concentration determined by reading absorbance of prepared samples at 570 nm. Since activity of respiring cells is constant, an increase or decrease in the number of viable cells has a direct correlation with the number of formazan crystals. Here, two ChCl-based DESs, namely ChCl:ethylene glycol and ChCl:malonic acid, were selected and its effect on the inhibition of *Bacillus cereus* growth was studied[89]. The obtained results revealed that ChCl:ethylene glycol DES was moderately toxic and approximately 54% growth inhibition of *B. cereus* cells compared to control sample was observed[89]. On the other hand, in the case of ChCl:malonic acid DES, cellular growth was not observed thus this DES was considered highly toxic to *B. cereus* cells[89].

2.2.2. Microtox assay for luminescence inhibition

Microtox assay is an *in vitro* testing method which employs bioluminescent bacteria *Aliivibrio fischeri* to determine the toxicity of different substances[93]. *A. fischeri* are non-pathogenic, marine bacteria that luminesce as a natural part of their metabolism[93]. Since toxic chemicals disrupt the respiratory process of these bacteria, resulting in decrease in the light output, the change in luminescence compared to control untreated bacterial cells with tested chemicals can be used to calculate a percent inhibition of *A. fischeri* growth[93]. This approach is rapid, simple, and sensitive method. Furthermore, it uses a specific clonal strain of bioluminescent bacteria prepared in a lyophilized vial format, increasing their shelf life and usability[93]. *A. fischeri* have demonstrated high sensitivity across a wide variety of substances, including DESs[40, 94-96]. The summary of the results found in the literature for toxicity of DESs towards *A. fischeri* determined by Microtox assay are presented in Table 4.

For the first time, the DESs ecotoxicity was assessed using the Microtox test in the work of de Morais et al.[40]. In this study, the toxicity of DESs based on the HBA - ChCl - and different organic acids (acetic acid (AA), lactic acid (LA), citric acid (CA), and glycolic acid (GA)) as HBDs was examined[40]. The obtained EC_{50} values indicated that all studied DESs were relatively toxic to *A. fischeri*, which is contrary to the generalized idea that DESs are of low toxicity[40]. The following order of toxicity for DESs with different molar ratios and their individual components was deducted: ChCl ≪ ChCl/acid (2:1) < ChCl/acid (1:1) < ChCl/acid (1:2) < acid, indicating that DESs had an intermediate value of toxicity when compared to the starting materials (acids and ChCl)[40]. Furthermore, it was observed that DES toxicity increased with an increase in concentration of the acid (the mole ratio of ChCl:acid)[40]. As far it comes to the HBD used in DES preparation, the following antibacterial activity order was obtained: ChCl/AA < ChCl/LA < ChCl/GA < ChCl/CA, which is in agreement with the decreasing order of the lipophilicity of the acid[40]. The obtained EC_{50} values showed that the effect of the acid used in DES preparation is preponderant in the toxicity because the toxic effect for the various DESs was similar to that of their corresponding organic acids separately[40]. The authors explained these results as a consequence of low pH values of the DESs containing organic acids and thus having a negative effect on the cell activity, through denaturation of proteins[40]. Furthermore, these DESs were more toxic than the respective ILs, namely, choline acetate (ChAc), choline lactate (ChLa), choline citrate (ChCit), and choline glycolate (ChGly) and it was hypothesized that it is a consequence of hydrogen bonding between the mixture compounds and the respective charge delocalization, since chemicals having delocalized charges are more toxic than chemicals with localized charges[40]. Overall, it was concluded that DESs might not be as “green” as generally it was assumed.

754 Table 4. The toxicity of DESs towards *Aliivibrio fischeri*.

DES	EC_{50} [mg/L] 30 min	Ref.
ChCl:acetic acid (1:2)	130	[40]
ChCl:lactic acid (1:2)	34	
ChCl:glycolic acid (1:2)	30	
ChCl:citric acid (1:2)	16	
ChCl:acetic acid (1:1)	197	
ChCl:lactic acid (1:1)	62	
ChCl:glycolic acid (1:1)	33	
ChCl:citric acid (1:1)	22	
ChCl:acetic acid (2:1)	337	
ChCl:lactic acid (2:1)	67	
ChCl:glycolic acid (2:1)	62	
ChCl:citric acid (2:1)	32	
ChCl:ethylene glycol (1:1)	67806	[94]
ChCl:ethylene glycol (2:1)	90343	
ChCl:ethylene glycol (1:2)	41821	
ChCl:ethylene glycol (1:4)	48653	
ChCl:glycerol (1:1)	76726	
ChCl:glycerol (2:1)	90156	
ChCl:glycerol (1:2)	104612	
ChCl:propionic acid (1:1)	20	
ChCl:propionic acid (2:1)	8	
ChCl:propionic acid (1:2)	12	
ChCl:propionic acid (1:4)	6	
ChCl:1,2-propanodiol (1:1)	73492	
ChCl:1,2-propanodiol (2:1)	61342	
ChCl:1,2-propanodiol (1:2)	44048	
ChCl:1,2-propanodiol (1:4)	74309	
ChCl:urea (1:1)	59825	
ChCl:urea (2:1)	69924	
ChCl:urea (1:2)	41693	
ChCl:urea (1:4)	39810	
ChCl:1-propanol (1:1)	34708	
ChCl:1-propanol (2:1)	44487	

ChCl:1-propanol (1:2)	21271	
ChCl:1-propanol (1:4)	17352	
$[N_{1111}]$Cl:1-propanol (1:1)	20870	
$[N_{1111}]$Cl:1-propanol (1:2)	16150	
$[N_{1111}]$Cl:1-propanol (1:4)	15360	
$[N_{2222}]$Cl:1-propanol (1:1)	18090	
$[N_{2222}]$Cl:1-propanol (2:1)	22260	
$[N_{2222}]$Cl:1-propanol (1:2)	15550	
$[N_{2222}]$Cl:1-propanol (1:4)	9500	
$[N_{3333}]$Cl:1-propanol (1:1)	4981	
$[N_{3333}]$Cl:1-propanol (2:1)	1555	
$[N_{3333}]$Cl:1-propanol (1:2)	1845	
$[N_{3333}]$Cl:1-propanol (1:4)	1120	
$[N_{1111}]$Cl:ethylene glycol (1:1)	53990	[95]
$[N_{1111}]$Cl:ethylene glycol (2:1)	30200	
$[N_{1111}]$Cl:ethylene glycol (1:2)	49250	
$[N_{1111}]$Cl:ethylene glycol (1:4)	65620	
$[N_{2222}]$Cl:ethylene glycol (1:1)	23940	
$[N_{2222}]$Cl:ethylene glycol (2:1)	18930	
$[N_{2222}]$Cl:ethylene glycol (1:2)	18610	
$[N_{2222}]$Cl:ethylene glycol (1:4)	36390	
$[N_{3333}]$Cl:ethylene glycol (1:1)	3665	
$[N_{3333}]$Cl:ethylene glycol (2:1)	971	
$[N_{3333}]$Cl:ethylene glycol (1:2)	945	
$[N_{3333}]$Cl:ethylene glycol (1:4)	1285	
ChCl:glycerol (1:2)	86726	
ChCl:urea (1:2)	26346	[96]
ChCl:ethylene glycol (1:2)	108526	

In the following work, for the first time the mixtures toxicity theory was used to analyze the results obtained from Microtox test for ChCl-based DESs[94]. The Concentration Addition (CA) model of mixtures toxicity was applied since the dissociation of DESs in water was considered[94]. For that purpose, the EC_{50} values for both individual DES components and series combining them in different proportions to establish different DESs were acquired. The

761 performed analysis indicated that all DESs with the exception of ChCl:propionic acid (2:1 and
762 1:4 molar ratio) had antagonistic effect (regardless molar ratios involved), which means that DES
763 can be less toxic than either of their starting materials dosed separately[94]. This observation is
764 opposite to the most previously published works, where synergistic effect for DESs was mainly
765 reported. Furthermore, for some DESs mixtures the EC_{50} values were found to be between the
766 values for corresponding HBA and HBD (e.g., ChCl:ethylene glycol, ChCl:glycerol,
767 ChCl:propionic acid and ChCl:1,2 propanediol)[94], which is consistent with the work of de
768 Morais et al.[40]. On the other hand, for ChCl:urea and ChCl:1-propanol much higher
769 concentrations, than those found for both DESs individual components, were needed to induce
770 50% *A. fischeri* luminescence inhibition, making these DESs very promising and biocompatible
771 alternative solvents[94]. In general, it was concluded that the toxicity was mainly dependent on
772 DES composition, as well as on molar ratios of the starting materials[94]. It was also suggested
773 that the HBD may have a role in modulating the ecotoxicity of the DES, because different EC_{50}
774 values were obtained for different HBDs joined to ChCl. Moreover, lower concentrations were
775 necessary to induce 50% *A. fischeri* luminescence inhibition as HBD molar proportion increases
776 within each DES[94].

777 In their following study, Macario et al. further evaluated the ecotoxicological profile of DESs
778 based on $[N_{1111}]Cl$, $[N_{2222}]Cl$ and $[N_{3333}]Cl$ as HBAs combined with ethylene glycol and 1-
779 propanol as HBDs, through the Microtox test[95]. The gathered results showed that DESs were
780 not hazardous to *Aliivibrio fischeri*, as the EC_{50} values were above 100 mg/L[95]. Therefore,
781 these DESs can be considered as green solvents. Moreover, DESs toxicity followed the same
782 trend as observed for HBAs individually and an increase in the alkyl chain length of quaternary
783 ammonium salt resulted in increased toxicity of DESs ($[N_{1111}]Cl$-based DESs < $[N_{2222}]Cl$-based

DESs < [N_{3333}]Cl-based DESs)[95]. Accordingly, [N_{3333}]Cl-based DESs exhibited high overall toxicity towards *A. fischeri* compared to the other DESs under study[95]. This increased toxicity was most likely a consequence of decrease in hydrophilicity of the HBA from [N_{1111}]Cl to [N_{333}]Cl[95]. Furthermore, antagonism between HBA and HBD was observed for [N_{1111}]Cl-based DESs, while synergism for [N_{3333}]Cl-based DESs and for [N_{2222}]Cl:1-propanol[95]. It shows that DESs toxicity cannot be predicted based solely on the toxicity of the starting materials. The obtained results further highlighted that for these solvents both the HBD and HBA have an impact on DESs toxicity, agreeing with the study of Wen et al.[39].

The latest study carried out by Lapeña et al. was an attempt to further explore toxicity of ChCl-based DESs towards *A. fischeri*[96]. Similarly, to the work of Macario et al.[94] the authors selected DESs prepared using ChCl as HBA combined with urea, glycerol, and ethylene glycol as HBDs. Furthermore, DESs that contained water as third component were also prepared. The obtained EC_{50} values from the *A. fischeri* ecotoxicity test showed that the most toxic DES was ChCl:urea, followed by ChCl:glycerol, ChCl:urea:H_2O, ChCl:ethylene glycol, ChCl:ethylene glycol:H_2O and ChCl:glycerol:H_2O[96]. Nevertheless, for all DESs under study the EC_{50} values were higher than 25000 mg/L and for some higher than 100000 mg/L, indicating non-hazardous nature of the tested DESs to this species[96]. In the case of *A. fischeri*, the presence of water decreased the toxicity with respect to the three pure DESs studied[96]. Even though, there is one previous work in which the ecotoxicity of such DESs towards *A. fischeri* was evaluated, the direct comparison of the results is not possible. The dissimilarities in the obtained EC_{50} values are the outcome of differences in the experimental methodology used in both works. In the study of Lapeña et al. pH of the samples was controlled and adjusted to be in optimal range for the culturing of these bacteria (pH of 6–8.5)[96], while in the work of Macario et al. pH was not

controlled[94]. Thus, it could be hypothesized that usually lower EC_{50} values were obtained in the study of Macario et al.[94] because the severe effect of pH on the toxicity towards *A. fischeri* bacteria has been previously observed[97].

2.2.3. Drop plate method

Moreover, Wikene and co-workers for DESs' toxicity testing used a modified drop plate method (Table 5), which combines 24-well plates for serial dilutions, followed by drop plating on agar in a 4×4 format using an automatic spiral plater[98-101]. Afterwards, plates are left to dry for a few minutes and then placed into an incubator for 18–20 h (37°C). After incubation viable colony forming units (CFUs) are counted and numbers compared to control samples.

At first, bacterial toxicity of two NADESs, citric acid:sucrose and glucose:malic acid, was studied[98]. Here, bacterial strains of *E. coli* and *Enterococcus faecalis* were selected as model microorganisms. The obtained results showed that 100 times dilutions of these two NADESs were practically not toxic to bacteria and non-significant reduction in CFUs as compared to untreated control samples was observed[98]. Furthermore, it was noted that non-toxic effect of NADESs was not dependent on whether the aliquots from bacterial cultures used in the assay were in stationary or exponential phase of growth[98]. Later, the database for NADESs toxicity determined by drop plate method was further extended and toxic effect of glucose:sucrose and ChCl:maleic acid NADESs on *E. coli* was evaluated[99]. Carbohydrates-based NADES was found non-toxic to *E. coli* and no significant reduction in viable bacteria was observed[99]. On the other hand, the toxic effect of ChCl:maleic acid NADES was detected for solvent diluted 100 times[99]. Nevertheless, the bacterial cells tolerated well this NADES when treated with 200-fold dilution, suggesting that the antibacterial effect is concentration dependent[99]. In the following year, the drop plate method was used to study the antibacterial effect of ChCl:xylitol, malic

acid:fructose:glucose and citric acid:sucrose NADESs against *E. coli*, *E. faecalis* and *S. aureus*[100]. Here, the results obtained in the first work of Wikene et al.[98] were confirmed, and citric acid:sucrose NADES was found non-toxic to all three bacterial strains[100]. The same was valid for the other two NADESs under evaluation. At dilutions used in the experiments (400-fold and 200-fold for malic acid:fructose:glucose and ChCl:xylitol, respectively), these NADESs did not reduce significantly the number of viable bacteria as compared to the control samples prepared in PBS[100]. Lastly, the effect of citric acid:sucrose and malic acid:fructose:glucose NADESs on the viability of *E. coli*, *Klebsiella pneumoniae, S. epidermis*, *P. aeruginosa* bacteria and *C. albicans* yeast was studied[101]. The obtained results revealed that both NADES diluted 100 times reduced the survival of *E. coli* by 96% and 24% for citric acid:surcrose and malic acid:fructose:glucose, respectively[101]. Furthermore, it was observed that *E. coli* tolerated better citric acid-based NADES than an equimolar concentration of citric acid[101]. On the other hand, for malic acid-based NADES no significant differences in cell viability were seen compared to an equimolar concentration of malic acid[101]. Regarding sugar components of NADES, neither fructose, glucose nor sucrose showed effect on *E. coli* survival[101]. Both NADESs were also found toxic to *P. aeruginosa*, and no bacterial survival was observed for 200 times dilution. The toxic effect was further observed for *S. epidermidis*, however, these NADESs exhibited lower antibacterial potency than against *P. aeruginosa*, and 3-9% of cells survived the exposure to NADESs[101]. Moreover, citric acid:sucrose NADES reduced by 37% the bacterial survival of *K. pneumoniae* compared to the control, while malic acid:fructose:glucose NADES did not significantly affected the number of viable bacteria[101]. Finally, these NADESs did not show antifungal activity and no reduction in survival of *C. albicans* yeast was observed[101].

853 Table 5. The toxicity of NADESs determined using drop plate method.

NADES	Microorganisms			Toxicity results	Ref.
	Bacterium G(+)	**Bacterium G(-)**	**Fungi**		
citric acid:sucrose (1:1) glucose:malic acid (1:1)	*Enterococcus faecalis* ATCC 19433	*Escherichia coli* ATCC 25922		• All the NADESs showed no toxic effect on tested genus of bacteria. • The toxic effect of individual components of DESs was not assayed.	[98]
glucose:sucrose (1:1) ChCl:maleic acid (3:1)		*Escherichia coli* ATCC 25922		• Glucose:sucrose NADES showed no toxic effect on tested genus of *E. coli*. • ChCl:maleic acid NADES showed relative toxic effect on tested genus of *E. coli*. • The toxic effect of individual components of DESs was not assayed.	[99]
citric acid:sucrose (1:1) ChCl:xylitol (5:2) malic acid:fructose:glucose (1:1:1)	*Enterococcus faecalis* ATCC 19434, *Staphylococcus aureus* (strain Newman)	*Escherichia coli* ATCC 25922		• All the NADESs showed no toxic effect on tested genus of bacteria. • The toxic effect of individual components of DESs	[100]

				was not assayed.	
citric acid:sucrose (1:1) malic acid:fructose:glucose (1:1:1)	*Staphylococcus epidermis* ATCC 35984	*Escherichia coli* BW25113, *Klebsiella pneumoniae* ATCC 31488, *Pseudomonas aeruginosa* ATCC 9027	*Candida albicans* ATCC CRM-10231	• Citric acid:sucrose NADES showed relative toxic effect on tested genus of bacteria. • Malic acid:fructose:glucose NADES showed relative toxic effect on bacteria except *K. pneumoniae.* • Both NADESs showed no toxic effect on tested genus of yeast. • The toxic effect of individual components of DESs was not assayed.	[101]

2.3. FTIR-based biological assay

Another method used for DESs toxicity testing is FTIR-based bioassay (see Table 6)[102, 103]. This assay was primarily based on *Saccharomyces cerevisiae* cells however it offers the possibility to also use as biosensor the cells from different organisms, including different microbial cells or mammal cell cultures[104]. The principles of this method are based on the fact that cells under stress exhibit very fast changes in terms of cell metabolites and thus a metabolomic analysis, using FTIR, may be capable of detecting these variations as early as in the first hours of exposure[104]. This bioassay estimates the toxicity level as function of the FTIR spectra variation of the cells upon exposition to the chemicals and provides metabolic indexes

which can be used for the classification and the relative quantification of the toxicity[104]. The major benefit of FTIR-based assay is that it is a fast and reproducible procedure, which besides the information whether chemical agent is toxic also provides more detailed metabolomic analyses necessary to elucidate the mechanisms on how the studied compounds promote toxicity towards selected microorganisms[104].

For the first time FTIR-based bioassay was applied to study DESs toxicity in the work of Cardellini and co-workers, where the authors evaluated the antifungal activity of novel DESs formed by zwitterionic trimethylglycine and high melting point carboxylic acids[102]. In this work the yeast strain *Saccharomyces cerevisiae* CBS 13873 was employed as target and model eukaryotic microorganisms. Preliminary studies showed that these DESs caused a very rapid decrease of cell viability after a short exposure times to the tested DESs, suggesting that these DESs are highly toxic to the cells[102]. Basing on these results, it was hypothesized that the high concentration of these solvents caused a very rapid exit of the cell water and consequently led to their inactivation[102]. In fact, this hypothesis was confirmed via FTIR-based assay since the normalized FTIR spectra from the yeast cells treated with DESs and $CaCl_2$ (a well- known non-toxic dehydrating agent) were almost identical[102]. This observation led to a conclusion that these DESs act as dehydrating agents on the model cells.

882 Table 6. The toxicity of DESs towards yeast cells determined using FTIR-based bioassay.

DES	Microorganisms	Toxicity results	Ref.
benzoic acid:betaine (1.5:1) salicylic acid:betaine (1.5:1) 4-chlorobenzoic acid:betaine (1.5:1) 2-chlorobenzoic acid:betaine (1.5:1) 3-chlorobenzoic acid:betaine (1.5:1) 2-furoic acid:betaine (2:1) phenylacetic acid:betaine (2:1) D-(+)-mandelic acid:betaine (1:1) glycolic acid:betaine (2:1) oxalic acid:betaine (2:1) citric acid:betaine (1.5:1)	*Saccharomyces cerevisiae* CBS 13873	• All the DESs showed relative toxic effect on tested genus of yeast cells and acted as dehydrating agents. • The toxic effect of individual components of DESs was not assayed.	[102]
aliphatic sulfobetaines:(1*S*)-(+)-10-camphorsulfonic acid aromatic sulfobetaines:(1*S*)-(+)-10-camphorsulfonic acid amphiphilic sulfobetaines:(1*S*)-(+)-10-camphorsulfonic acid	*Saccharomyces cerevisiae* CBS 13873	• All the DESs showed relative toxic effect on yeast cells and exerted a stronger dehydration effect than $CaCl_2$. • The toxic effect of individual components of DESs was not assayed.	[103]

883

884 In their following work, Cardellini et al. extended DESs toxicity studies for DESs prepared using
885 differently structured sulfobetaines (SBs) with aliphatic, aromatic and amphiphilic moieties and
886 (1*S*)-(+)-10-camphorsulfonic acid[103]. As it was observed for zwitterionic
887 trimethylglycine:carboxylic acids DESs, these DESs exert a dehydration effect on the
888 *Saccharomyces cerevisiae* CBS 13873 cells as this observed for $CaCl_2$[103]. Furthermore, it was
889 noted that the DESs were stronger dehydrating agents than calcium chloride salt, indicating more
890 affinity of these compounds to water[103]. In general, these results highlight these DESs as

promising green media since the presence of water can inactivate the effect of these mixtures on the cells[103].

3. General discussion about DES microbial toxicity

A good question was asked in the first work where the toxicity of DESs was studied: "Are deep eutectic solvents benign or toxic?"[48]. Examining the results presented in around 96 works in which the authors looked for the answer on this question, it is still not possible to give a direct response. In general, although DESs have been considered as the green solvents, with low or no toxicity, there are numerous studies that show that depending on the choice of the starting materials (which very often are non-toxic) used for their preparation, the respective DESs possess a certain degree of toxicity. This calls for in-depth studies on DES toxicity toward different organisms at various trophic levels in order to take full advantage of these new types of solvents and to broaden their applications. Furthermore, in various works different toxic effects were observed for the same DESs depending on the toxicity assessment method and model organisms used. Thus, the toxicity results cannot be generalized to all DESs, or different organisms and it is essential to elucidate mechanisms on how DESs promote toxicity.

There are several factors that were proposed to explain DESs' toxicity mechanism against tested prokaryotic and eukaryotic microorganisms such as negative impact of their pH on the growth of examined microorganisms[40, 49, 50, 80, 82], charge delocalization occurring during DES formation[38-42, 49], and cell dehydration in presence of DESs in growth medium[50, 102, 103], among others (see Fig. 5). Obviously, the impact of each of this factor differs for different DESs, depending on the nature and properties of starting materials used in solvent preparation. For instance, several studies have concluded that DESs possess higher toxicity than their individual components[39, 44, 67, 94, 95], however, other studies reported the opposite[52, 57, 60, 79, 88,

94, 95]. All these observations further highlight the need to elucidate DESs' toxicity mechanisms and in this section an attempt to summarize and systematized what have been discovered in regards on how DESs promote toxicity towards prokaryotic and eukaryotic microorganisms will be made.

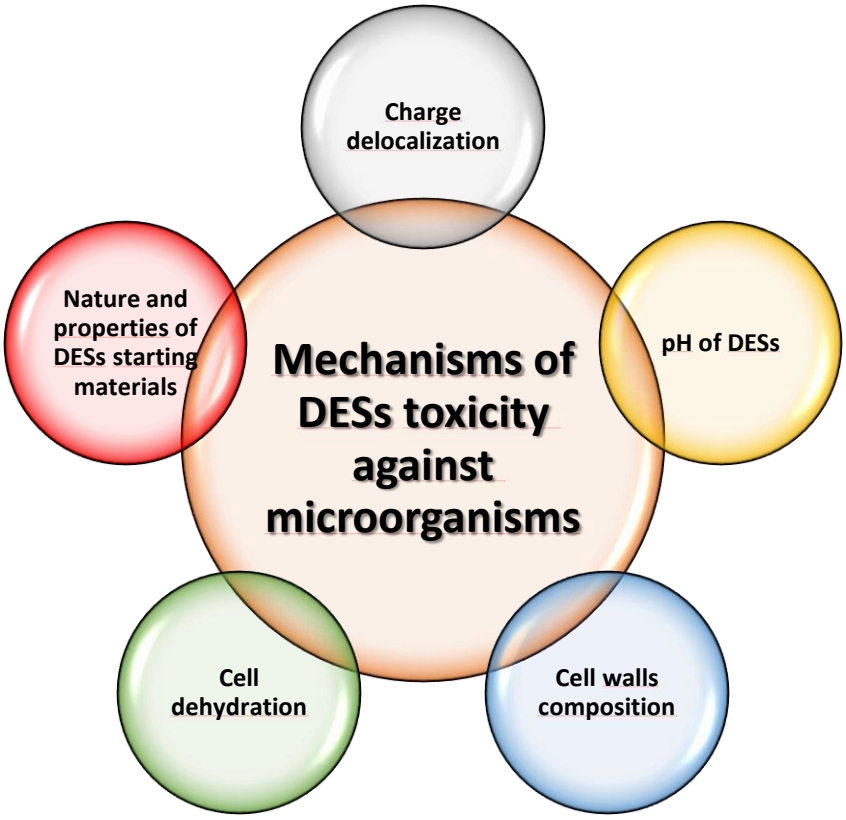

Fig. 5: Overview of factors proposed to explain the mechanisms of DESs toxicity against prokaryotic and eukaryotic microorganisms.

According to some reports higher toxicity of DESs than their individual components is a results of charge delocalization that occurs during the formation of DESs[38-42, 49]. This enhance in toxicity is explained by the observation that chemicals which contain delocalized charges express higher toxicity than those with localized ones. For instance, one of the most commonly used salts in DESs preparation - ChCl - has delocalized cation, thus very often higher toxicity of ChCl-

based DESs is explained, as a result of interaction of cholinium cation side chains and head groups with cellular membrane groups[39, 59]. Furthermore, it was suggested that accumulation of positively charged cations, as cholinium, enhances the electrostatic interactions with negatively charged bilayer on the surface of cell's membranes, leading to cell wall distortion or disruption[39]. It is also assumed that it causes proteins denaturation and enzymatic reactions inhibition, which may lead to cell collapse and death[42]. Moreover, it was also shown that the salt's counter anion contributes to the charge delocalization and thus affect DESs' toxicity. In the study of Wen et al. it was reported that DESs prepared using ChAc and ChCl as HBA had different antibacterial potency against *E. coli*, and the ChAc-based DESs had a greater detrimental effect than the ChCl-based DESs[39]. Additionally, according to Zhao et al. higher toxicity of acid-based DESs can be explained by the fact that the hydrogen bond network is more dense and compact, further increasing the charge delocalization effect on DESs toxicity[49].

Another factor that was proposed to explain DESs' toxicity mechanism is the acidity or alkalinity (pH) of the DESs[40, 49, 50, 80, 82]. Since the optimal pH for bacterial and fungal growth is 6.5–7.5[105] and 5.0-9.0[106, 107], respectively; if the DESs had a higher or lower pH value than optimal ones, it influenced the antimicrobial effect of these solvents. This is because the pH value besides theirs optimal ranges for microorganisms growth, has a negative effect on the cell activity, due to denaturation of proteins located on the microorganism cell wall. Consequently, the pH values far from those optimal for microbial growth may alter cellular proliferation and metabolic properties. For instance, de Morais et al. observed that the pH values of DESs composed of ChCl and organic acids were lower than 3 and as a result, the denaturation of proteins and decreased *A. fischeri* cell activity was discovered[40]. Moreover, it was noted that this effect was more pronounced when the acid content was higher further confirming that pH has

a great influence on DESs' toxicity[40]. The same phenomenon was also observed for organic acid-based DESs against both gram negative and -positive bacterial strains[49]. Furthermore, the low pH was assumed to be the reason of increased toxicity towards bacteria for malic acid:sucrose[82] and acetycholine chloride:acetamide DESs[80]. The negative impact of pH on DESs' toxicity towards yeast *S. cerevisiae* was observed in the work of Redovniković's group, where it was found out that solvents prepared with organic acids (pH < 3) and urea (pH > 8) as HBDs were the most toxic to the tested yeast cells[50]. Similar negative impact of basic urea-based DESs was observed in the studies of Hayyan's group, where ChCl:urea DES showed relative toxic effect on the tested genus of *Aspergillus niger* filamentous fungi[51] and *Candida cylindracea* yeast[52]. Nevertheless, it is worth mentioning that so far increased toxicity due to basic pH of DESs was only observed for the fungi, which have much narrower optimal pH growth range than bacteria (see above). Thus, in other studies where toxicity of urea-based DESs was studied usually no toxic effect towards various bacteria was found[43, 48, 49, 57, 70].

Moreover, another factor that may be involved in mechanism of DESs toxicity is cell dehydration[50, 102, 103]. In the studies of Cardellini et al., in which the mechanism of DESs toxicity towards yeast *S. cerevisiae* using FTIR-based assay was evaluated, the authors hypothesized that DESs might cause a very rapid exit of water from the cells[102, 103]. The obtained results confirmed this hypothesis as similar effect to that caused by $CaCl_2$ (well-known dehydrating agent) was observed[102, 103]. In the case of DESs, high concentrations generate high osmotic pressure to the cells and the cell water leakage, resulting in the yeast cells death. Furthermore, it was assumed that this dehydrating effect of DESs is rather independent of the chemical structure of these solvents, because all tested DESs challenged the yeast cells in the same way[102, 103]. Similar observations were made in the work of Redovniković's group,

where high concentrations of ChCl:ethylene glycol and ChCl:glucose caused high osmotic pressure and decreased viability of baker's yeast cells[50].

Findings in other reports suggest that DESs' toxicity mechanism may also be related to the cellular organization of the organisms, in particular to the differences in cell wall composition[39, 40, 60, 72, 79]. For instance, in some studies it was proposed that the bacterial cell wall, which is composed of peptidoglycan, is permeable for small substrates because of its high porosity. Consequently, various DESs can diffuse across cellular membranes and exert their toxic effects inside the cytoplasm by denaturation of enzymes, oxidative stress, among others. In the work of de Morais et al., the authors hypothesized that organic acids containing DESs diffused through the cell membrane and therefore exerted toxic effect on cells of *A. fischeri* bacteria[40]. Furthermore, in the study conducted by Wen and co-workers it was assumed that DESs inhibited the bacterial growth of *E. coli* DH5α by interacting with the cellular membrane[39]. According to their revelations DESs components may interact with the polysaccharide or peptide chains of peptidoglycan through hydrogen-bonding or electrostatic interaction, leading to cell wall distortion or disruption[39]. Moreover, in some reports the different antibacterial potency of DESs towards gram-negative and -positive bacteria was explained by differences in their cell wall structure[60, 79]. Silva et al. concluded that for fatty acid-based DESs, their lower toxicity towards gram-negative bacteria was due to a presence of lipopolysaccharides (LPS) on the outer membrane that prevented the fatty acids DESs from reaching cell membrane[60]. On the other hand, because of the lack outer cell membrane with LPSs, the cell wall of gram-positive bacteria absorbed more easily the fatty acids composed solvents and thus they passed through the inner membrane and exerted the toxic effect[60]. Similar observations were made by Teh and co-workers for ChCl-based DESs where it was assumed that gram-negative bacteria formed a

995 formidable barrier which restricted the attack of DESs from penetrating into the bacterial cell
996 envelopes, while gram-positive *S. aureus* was not able to do that because its cell wall solely
997 consists of thick peptidoglycan layer[79]. Furthermore, the differences in cell wall composition
998 were also suggested as the reason why ChCl:oxalic acid:glycerol and ChCl:citric acid:glycerol
999 were found toxic to bacteria and no to yeast *C. albicans*[72]. According to this report, it is a
1000 result of easier penetration of the lipid layer of bacteria and not fungus which have two-layered
1001 cell wall mainly composed of chitin and glucans[72].

1002 As mentioned earlier the toxicity profiles of DESs are also influenced by the nature and
1003 properties of starting materials used in solvent preparation[38, 39, 48-50, 79, 87, 94, 95]. In most
1004 of these studies, the negative impact of HBD was discovered. It was mainly observed that the
1005 DESs having organic acids in their compositions exhibited increased antimicrobial properties.
1006 However, enhanced toxicity of such fluids was assigned to not only acidity of DESs (negative pH
1007 effect, see above please) but also their higher viscosity. In addition, the highly viscous nature of
1008 carbohydrates containing DESs, as well as osmotic pressure (negative dehydration effect, see
1009 above please), might also be the reason of increased toxicity of some of these solvents.
1010 Nonetheless, some of the researchers claimed that beside HBD also HBA has an impact on
1011 overall toxicity of DESs[38, 39, 95]. For instance, DESs prepared using the same HBDs were
1012 found toxic to bacteria when MTPB was used as HBA and the opposite was observed for DESs
1013 formed with ChCl[38, 48]. Also, increased toxicity of ChAc-based DESs compared to ChCl-
1014 based ones was observed in the work of Wen et al.[39]. The influence of HBA on DESs toxicity
.015 was further reported by Macario et al. and solvents based on different quaternary ammonium salts
.016 exhibited different ecotoxicity towards *A. fischeri*[95]. Moreover, depending on DESs staring
.017 material and the method used in DESs preparation, the obtained solvents may possess different

toxicities. For example, very often while using the heating method, the formation of impurities is observed[108]. The presence of impurities can change some of the mixture properties (e.g., by increasing their viscosities) and indirectly intensifying toxic effect of these DESs.

As discussed in this section, there are proposed various mechanisms regarding DESs toxicology, nevertheless the knowledge on this topic is still very limited. An interesting idea in the search for other mechanisms of toxicity towards microbial cells would be to perform studies on the toxic effect of DESs on the metabolism of microorganisms used in the discussed works (Table 1-6), e.g. *E. coli* bacteria or *S. cerevisiae* yeast. This would be an analogous approach to that used in the metabolomic cytotoxicity studies of selected DESs that were performed on HepG2 and HEK 293T mammalian cells (in vitro) and in ICR mice (in vivo)[109]. To the best of our knowledge, there are no reports on the study of DESs toxicity mechanisms based on the generation of e.g. oxidative stress or the influence of DESs on the metabolism of basic carbon or nitrogen sources in microbial cells. Hence, with more studies on DESs toxicity towards various organism, not mainly focused on prokaryotic and eukaryotic microorganisms, it will be possible to create a database of truly green and biocompatible DESs and further extend their applications in food, pharmaceutical, biotechnological, or biomedical sectors. Overall, most of the studies on the toxicity of DESs revealed that solvents prepared with ChCl as HBA and HBDs from natural sources such as amines, alcohols, and carbohydrates are generally low toxic to different microorganisms. On the other hand, acid containing DESs exhibited strong antimicrobial properties. Furthermore, also the DESs based on quaternary ammonium salts, such as $[N_{1111}]Cl$, $[N_{2222}]Cl$ or $[N_{3333}]Cl$ were found more toxic than these prepared using ChCl. All of this proves once again, that biocompatibility of DESs is mainly dependent on their composition.

Nevertheless, most of the DESs are usually less toxic than conventional organic solvents or ILs therefore the use of DESs is encouraged.

4. Critical evaluation of the methods used for DES microbial toxicity determination

a) Disk and well diffusion method as DES microbial toxicity assay

Due to the simplicity of execution, the disk or well diffusion method is well suited technique for testing the toxicity of a large number of DESs, differing in terms of composition and molar ratios of HBA and HBD used in their preparation (see examples in Table 1). However, the obtained results allow, first of all, to assess whether the tested DES or its solution exhibits toxicity. Nevertheless, this method does not allow to estimate the toxicity of tested DES against selected microorganisms by determining the MIC or EC_{50} value. On the other hand, by selecting strictly defined strains of gram-negative bacteria, gram-positive bacteria, and fungi (both yeasts and molds) derivate from certified microbial collection (e.g., ATCC, DSMZ, JCM or CBS-KNAW) which were previously used for toxicity examination of antibiotics and other natural or synthetic antimicrobial agents, commercially available microbiological growth media and sterile disks used in assay, it is possible to normalize this method for DESs toxicity studies and use it in various laboratories, allowing the comparison of the obtained results. Unfortunately, so far researchers have approached these issues very freely, using various species of bacteria and yeast in their research (Table 1). For example, when the same bacterial species, e.g., *S. aureus* was used, different strains were selected, e.g., *S. aureus* NRS234[69] and *S. aureus* ATCC 25923[60, 67].

What is important to note, due to the key role of the DES diffusion process from a soaked sterile disk to the growth medium, this method is not suitable for high viscosity DESs. DESs with high viscosity are those where, for example, carbohydrates or organic acids were used as HBD for

their preparation. The high viscosity also limits the precise application of the same amount of DES to the sterile disk in repetitions, which may affect the reproducibility of the results. For instance, in the work of Zhao et al. it was observed that ChCl:urea, ChCl:acetamide, ChCl:glycerol, ChCl:ethylene glycol did not inhibited *E. coli* growth according to the results obtained using disk test[49]. However, the exact same DESs have shown the antibacterial activity and the EC_{50} values between 275.2-532.0 mM were obtained using broth dilution[39]. The false results obtained using disk diffusion assay seemed to lead Lou's group to conclude that these DESs are not toxic towards *E. coli* and thus their toxicity was not further examined using broth dilution method. These examples highlight the need for careful analysis of DES density and viscosity before using diffusion methods.

On the other hand, due to the hydrophilic nature of agar medium, diffusion of DES with high hydrophobicity into agar will be rather difficult and not such effective as for hydrophilic ones. Hence, it may seem that this physicochemical DES parameter may have also impact on DES toxicity estimated by disk diffusion method.

Summing up, due to above mentioned disadvantages, it seems too simple and insufficient to withdraw conclusions about DES toxicity basing exclusively on the results of the tests performed using disk or well diffusion method. The DES toxicity results obtained with these methods should be compared with those obtained with one of the alternative techniques. On the other hand, due to the simplicity and the possibility of standardization of disk diffusion method (under conditions of using commercially available sterile disks with the same size and made from the same material), this method seems to be the best of all discussed methods to perform the preliminary studies on toxicity of DESs (Table 1). Hence, in our opinion, apart from the

mentioned exceptions, e.g., highly viscous DESs, disk diffusion method should be used as one of the DESs toxicity testing techniques.

b) Broth dilution method as DES microbial toxicity assay

Among the different dilution methods (macro- or microdilution) used so far, the microdilution method seems to be the best in terms of its reproducibility, validity of obtained results and application for DESs toxicity assessment. However, when analyzing the published results for DESs toxicity using broth dilution methods (Table 2), it can be concluded that the researchers selected the species and strains of microorganisms used in these studies in a very arbitrary and independent manner from previously published DESs toxicity results. For instance, in one of the studies only gram-negative *E. coli* strain was used[39], and in another work when the same *E. coli* species was used, different strain was selected - the *E. coli* BL21 (DE3) strain dedicated for recombinant protein production in pET expression system (Novagen, Merck Millipore)[80].

Furthermore, as in the disk diffusion method, also in broth dilution methods, by selecting the appropriate microbiological growth media and culture conditions, it is possible to carry out toxicity tests against gram-negative and gram-positive bacteria, yeasts, and filamentous fungi. However, contrary to the previously discussed disk diffusion method, broth dilution methods allow the determination of MIC and EC_{50} parameters, which, in the case of method standardization, will allow the comparison of the results obtained by various research groups. Moreover, since in broth dilution methods serial dilutions of tested DESs are used, the negative effect of high viscosity of some DESs can be reduced. On the other hand, for broth dilution technique stability of DESs solutions should be controlled before toxicological analysis. It is known that high amounts of water are responsible for breaking of hydrogen bonds between HBA and HBD of DES[110]. Also, DESs or their hydrolyzed individual components may interact with

the salts or nutrients in growth medium and it may be expressed in higher toxicity than the toxicity of DES itself without the presence of these interactions[80]. Consequently, for lower concentrations instead of DES toxicity, the toxicity of an aqueous solution of DES components is determined.

Moreover, the determination of toxicity by broth dilution methods, and in particular the most popular microdilution method, is not as easy to perform as the disk diffusion method. In the case of determining the MIC value using the microdilution method, to increase the precision of the assay and the obtained results, it is sometimes necessary to use spectrophotometric measurements to assess the viability of the cells of the tested microorganisms (assessment of the turbidity of the culture). In addition, it is also possible to use resazurin (see section 5) to assess the cell viability of a cultured microorganism after treatment with DES, which is independent of the turbidity of the culture, increasing the precision of determination of the MIC and EC_{50} values. Interestingly, to the best of our knowledge, there is only one study where resazurin was used for this purpose in the DESs toxicity studies performed using broth dilution methods (Table 3,[88]). Moreover, after performing DES toxicity measurements with the broth microdilution method, the minimum bactericidal concentration (MBC), can be determined for the tested microorganism. In summary, due to the possibility of quantifying the toxicity of DESs by determining the MIC and EC_{50} or MBC, the possibility of selecting a wide range of microorganisms (bacteria, filamentous fungi, yeasts), the possibility of assessing the viability of cells of the tested microorganism using resazurin or indirectly by determining the MBC value - the method of microdilution seems to be the optimal method to assess the toxicity of DES against wide spectrum of both bacteria and fungi.

c) Microtox assay as DES microbial toxicity testing method

In four out of 96 studies in which the toxicity of DESs was evaluated, the commercially available Microtox kit was selected for this purpose (Table 4). Thanks to the use of uniform conditions in this kit for the toxicity assessment against the bioluminescent bacteria *Aliivibrio fischeri*, it is possible to determine and compare the EC_{50} values for several different DESs differing in their composition and molar ratio of HBA and HBD used for their preparation (Table 4). Moreover, due to the use of one strictly defined *Aliivibrio fischeri* strain, it is possible to compare the results obtained by different researchers. Contrary to the two previously discussed methods, due to the fact that we use a commercially standardized test, the method does not need to be validated. However, since the test is based solely on testing toxicity towards *Aliivibrio fischeri*, the obtained results are limited to only one type of microorganism – gram-negative bacteria. As shown in the studies cited in this review, the mechanism of action and susceptibility of gram-negative and gram-positive bacteria may differ significantly from each other for the same DESs due to the different structure of the cell wall, and it is mostly depending on the chemical nature of HBA and HBD used for solvent preparation[39, 40, 60, 72, 79]. This also applies to the differences in the toxicity of DESs against bacteria and fungi resulting from chemical and structural differences in the structure of the cell walls of both groups of microorganisms. Hence, this method, despite many advantages resulting from the use of standardized commercial kit, should be a complementary method to another more universal technique, e.g., broth microdilution.

d) Other methods as DES microbial toxicity assay

In two analyzed and cited studies in this review, the toxicity of the examined DESs was assessed using a method based on the analysis of FTIR spectra variation of the cells upon exposition to the chemicals. In both studies, this method was used to assess DESs toxicity towards *S. cerevisiae* yeast (Table 6), however, as previously mentioned, this method can be used to evaluate the

toxicity of DESs against different microbial cells[104]. This assay seems to be interesting because, compared to the previously discussed methods, it allowed to elucidate the mechanism on how DESs exert their toxic effect (yeast cells dehydration). Hence, FTIR-based bioassay is worth considering in all studies that aim at determining the possible toxicity mechanisms of selected DESs in relation to various groups of tested microorganisms.

e) pH of DESs as an important factor in described microbial toxicity methods

Since pH of some DESs is the important parameter that affect the applicability of basically each of the methods discussed above, it is important to consider this factor before testing DESs toxicity. Some studies about the toxicity of DESs suggest that the pH of growth media after preparation of DESs serial dilutions changes significantly[49, 51, 80]. As a result, the pH decreases below or increases above the optimal values for microbial growth (6.5–7.5[105] and 5.0-9.0[106, 107] for growth of not acidophilic or basophilic bacterial and fungal microorganisms, respectively), consequently increasing the cells mortality in the tested samples. It is mostly observed when one of the DESs components are acids. For this reason, it is necessary to firstly analyze the pH of DESs solutions and if the values are far from those optimal for microorganisms growth (e.g. for the most often used microorganisms in DESs toxicity studies - *E. coli* - optimal pH growth range is between 6.5 and 7.5[49]), the DESs solutions should be prepared in the buffered media. For example, the dissimilarities in the obtained EC_{50} values for ChCl-based DESs were noted in the work of Lapeña et al., where pH of the samples was controlled and adjusted to be in optimal range for the culturing of *A. fischeri*[96] and in the study of Macario et al. where pH was not controlled[94]. Consequently, lower EC_{50} values were obtained in the study of Macario et al. which seems to be due to the pH effect on bacterial

growth, leading to overestimated toxicity of ChCl-based DESs towards *A. fischeri*. In our opinion, these examples clearly show the need of buffering of DESs before testing their toxicity.

Overall, for proper hazard and risk assessment of DESs, the toxicity data from diffusion method and broth dilution should be evaluated together for both DESs and their separate individual components. Since currently there are no standard protocols for testing toxicity of DESs, it makes difficult to draw conclusions across different studies due to discrepancies in experimental conditions and lack of test standardization. Nevertheless, we believe that following the suggestions and guidelines pointed out in subsequent section more precise and comparable data could be obtained.

5. Suggestions and guidelines for future research

The literature review and experience of the authors of this paper acquired during our recent toxicological studies against selected microorganisms and previous experience in using of some of above-described methods for testing of others antimicrobial agents, incline us to propose a few general rules for the future investigation of DESs toxicity. When applying well-established microbial toxicity testing methods (e.g., disk diffusion assay, broth dilution) for DESs, one should keep in mind that these methods may need methodological modifications to be applied to these compounds. We believe that by following the proposed suggestions and guidelines will enable to get accurate results and facilitate a comparison with the results of other researchers. Furthermore, with comparable results of investigations of various groups, it will be possible to further understand the mechanisms on which these solvents exert their toxic effect. The suggestions and guidelines for future research on toxicity of DESs are outlined below.

i) The description of the methodology used to evaluate DESs toxicity should include all the details such as the detailed description of strain of microorganism used, detailed description of inoculum preparation (defined optical density of bacterial cells or CFU in inoculum), type and composition of growth medium, incubation conditions and endpoints determination, as well as details on the DES solutions preparation (initial molar ratio, dilutions) before analysis. The availability of this information will allow other researchers to better plan their own investigations and compare their results with different studies. For instance, for DESs toxicity assay using broth dilution method we encourage to use Mueller-Hinton broth culture media. Mueller-Hinton broth is recommended by FDA, NCCLS and WHO for testing MICs of for example, antibiotics against most encountered aerobic and facultative anaerobic bacteria in food and clinical material. This is excellent medium for cultivation *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* strains previously used in DESs toxicity studies (Tables 1-3, 5).

ii) Pure DESs should be characterized as much as possible, in particular their physicochemical properties, such as color/clearness, density, viscosity and pH (or pH of its solution in water). Disregarding these parameters may lead to the selection of the assessment method and model microorganism that will not be best suited and consequently will diminish the validity of the results and conclusions.

a) Both viscosity and density were shown to have a large effect on the obtained toxicity results. For instance, the viscosity of DESs may have great impact on the results obtained using disk diffusion assay due to low diffusion of highly viscous compounds in agar medium.

b) pH mostly influences the results obtained using broth dilution method, especially when pH of growth medium supplemented with DES is lower or higher than optimal for microbial growth. Due to pH changes caused by DESs, it is recommended to use buffered culture media instead of unbuffered cultures or to prepare DESs solutions in buffers. It will allow to diminish the negative impact of pH on the microbial growth, obtain more valid results and conclusions.

c) Some DESs may not be transparent liquids and cause some turbidity of the samples [88], resulting in the increased absorbance readings and thus leading to lower accuracy of the obtained results in broth macro- or microdilution methods.

d) Crossed reactions between DESs and the salts or nutrients of the culture media could also take place and influence both the pH and growth[80]. Moreover, such crossed reactions may be increased in the case of DESs hydrolysis that could occur in the presence of significant amount of water. Consequently, free HBA and HBD may react with the salts, amino acids, carbohydrates present in culture media, changing the pH and decreasing the nutrition sources.

iii) Beside determination of DESs toxicity, it should be mandatory to also evaluate the toxicity of DES individual components (HBA and HBD) at the same concentrations as these used for DES preparation. It will allow to better understand the results obtained in toxicological studies of DESs and withdraw more proper conclusions.

iv) As discussed throughout this paper there are various methods used to evaluate toxicity of DESs. Our literature study revealed that disk diffusion assay was the most commonly used method for this purpose (Table 1). The second most frequent used method was broth dilution method (Table 2). However, other microbiological methods dedicated for assaying antimicrobial activity of natural or synthetic chemical

compounds were used much more rarely for assaying DESs toxicity against bacteria and fungi (Tables 3-6). In the light of presented data, although the disk diffusion method is the most commonly used method for assaying DESs toxicity against microorganisms, our recommendation is to use broth dilution technique instead of disk diffusion assay for this purpose. Broth dilution method offers more versatility and precision than mostly used disk test. It is undeniable that in most of the studies in which DESs toxicity was evaluated using sterile disks soaked with DESs and placed on agar plates, the obtained results were less accurate and may not reflect real interaction between DESs and cells. It is related with high density and viscosity of most of the DESs which leads to decreased DESs diffusion from the disk into agar medium. On the other hand, using broth dilution technique the negative impact of density and viscosity is minimized and quantitative results could be obtained. Nevertheless, it must be remembered that in high amounts of water DESs hydrolysis takes place, which may also have an impact on toxicity data obtained. Therefore, taking all of this into consideration, and if possible, it would be beneficial to firstly perform analysis using disk diffusion assay with pure DESs and then obtain more details with broth dilution technique. However, it is important to note, that disk diffusion method has one important advantage. With this method we can quickly and cheaply estimate the toxicity of a range of DESs differing in a) the HBA used, b) the HBD used, or c) the molar ratios of HBA and HBD used to obtain a given type of DES. Hence, in our opinion, for such DESs toxicity studies, the results of disk test provide valuable data which can support the analysis of DESs toxicity based on the results of broth dilution method or other alternative method.

On the other hand, from other methods reported in the literature for DESs microbial toxicity studies, the methods based on i) analysis of FTIR spectra variation of the microorganism's cells upon exposition or not to the DESs; ii) the use of commercial kit that consists of two dyes, propidium iodide (PI) and SYTO9 for staining microbial cells exposed for DES seem to be interesting solution. They allow to compare DESs toxicity results obtained with these methods with results of DESs toxicity obtained with broth dilution method. In contrast to Microtox assay, both these methods give the possibility of selection of the same microorganism (bacteria or fungi) as used in broth dilution method. Moreover, the second of above-mentioned methods seem to be quite easy for validation, because of employing the commercially available kit.

v) If possible, we advise to use the assays based on colorimetric dyes (e.g., cell incubation with resazurin) for cell viability and vitality determination, which not only provide more precise values than these obtained by simple visual inspection or spectrophotometric measurements of turbidity (especially during MIC evaluation by broth microdilution method), but also higher quality data. Using this method there is no need of confirmation of the results by subculturing of each concentration onto agar for 24 h (MBC evaluation). Furthermore, the influence of DESs turbidity on the absorbance of the samples is reduced for these methods.

vi) The use of preadapted cells of microorganisms selected for study of DESs toxicity is encouraged. Until now there is one work where the preadaptation of cells to the DESs was performed[80]. It was demonstrated that non-preadapted cells did not grow in the presence of 600 mM acetylcholine chloride:acetamide DES, however, when they were pre-adapted to this concentration, cellular growth was observed[80]. By including the cellular pre-adaptation in future studies, it will be possible to gain insights on the

capability of the cells to tolerate or assimilate DESs and to obtain more accurate data on the antimicrobial properties of DESs.

vii) In case of studies where DESs are applied in the processes (such as extraction, chemical reaction etc.), the toxicity should be controlled for primary DES as well as for DES recovered after the process. In many cases, elevated temperatures as well as other factors, such as ultrasounds or microwaves used during the process, can cause DES chemical instability. As a result, harmful byproducts can be formed and strongly affect the eco-friendly character of primary DES. Recycled DES can introduce these byproducts to extracted fraction or product of reaction. On the other hand, accumulation of toxic byproducts will strongly affect methods available for its safe disposal after usage.

6. Conclusions and outlook

Deep eutectic solvents (DESs) are one of the most interesting classes of alternative solvents, mainly because of their simple preparation, usually low cost, and versatility due to possibility of their task- specific design to meet the needs of a specific process. Furthermore, they can be prepared using all- natural substances which opened exciting new perspectives to design truly green compounds that will meet with the requirements of green and sustainable chemistry. All these characteristics confer DESs as an ideal alternative to both organic solvents and ILs. Since their discovery DESs have been used in a myriad of applications as solvents, reaction media, catalysts, additives, lubricants, or materials for a wide range of fields from pharmaceutical to energy. Nevertheless, new studies are constantly conducted in order to learn as much as possible about the properties of DESs and further increase their applications in new fields important for the quality of life such as cosmetic, food, drug production and medicine. However, before the

implementation of DESs in these areas will be possible, it is essential to study their toxicity and gain knowledge on their possible modes of interaction with living beings. Even though, DESs are considered as green, benign, and non-toxic compounds, a literature review conducted in this paper indicated that this statement is not entirely true and such generalization should be avoided. In fact, several examples proved that often out-off-purpose methodology was used, resulting in false conclusions. Secondly, more than 5200 studies were published about DESs after their discovery and only around 96 evaluate and discuss the toxicity of these compounds (mainly against selected microorganisms). It highlights the need for more studies in this topic, which will allow to gain sufficient insights on DESs toxicity towards different organisms at various trophic levels and on how they exert their toxic effect.

Throughout this review, we show the advantages and disadvantages of methods used for DESs toxicity determination. Our analysis indicated that it is necessary to have an improved, standard protocol for determination of DESs toxicity. In this way, it will be possible to create a database, compare the results obtained in different studies and for various solvents. In our opinion, in order to obtain valuable results, it would be beneficial to use both disk diffusion assay and broth dilution technique in future studies on toxicity of DESs. We believe that the negative impact of pH may be overcome by using extremophilic microorganisms instead of standard microbial strains. Hence, it is essential to improve, for example, the broth dilution technique by always using buffered medium or by preparing DESs solutions in buffer. Furthermore, another aspect that should be considered while using standard microorganisms is cellular preadaptation with DESs which was shown to be a viable approach allowing to gain insights on the capability of the cells to tolerate or assimilate DESs and to obtain more accurate data on the antimicrobial

properties of DESs for which growth for some concentrations was not observed for non-adapted cells.

It is expected that, in a future, by using the standardized and validated above-mentioned methods, the theoretical and experimental knowledge about toxicity of DESs will evolve rapidly. It will allow to further explore these solvents in different applications such as biomedical and pharmaceutical. Furthermore, it will be possible to address once for all the DESs biosafety issue and answer with conviction if deep eutectic solvents are benign or toxic.

Conflicts of interest

There are no conflicts to declare.

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