

1 **Deep Eutectic Solvents Microbial Toxicity: Current State** 2 **of Art and Critical Evaluation of Testing Methods**

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26 **Abstract**

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

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

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51 **1. Introduction**

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

69 All the above-mentioned remarkable properties of DESs make them an ideal alternative to both
70 commonly used organic solvents and ILs[5, 14-16]. That is why, since their discovery, they have
71 been widely studied and applied in diverse fields, including biocatalysis[17-19],
72 electrochemistry[20-22], CO₂ capture[23, 24], separation and extraction techniques[25-31],
73 among others. Furthermore, beside the fact that up to now the most works focus on their

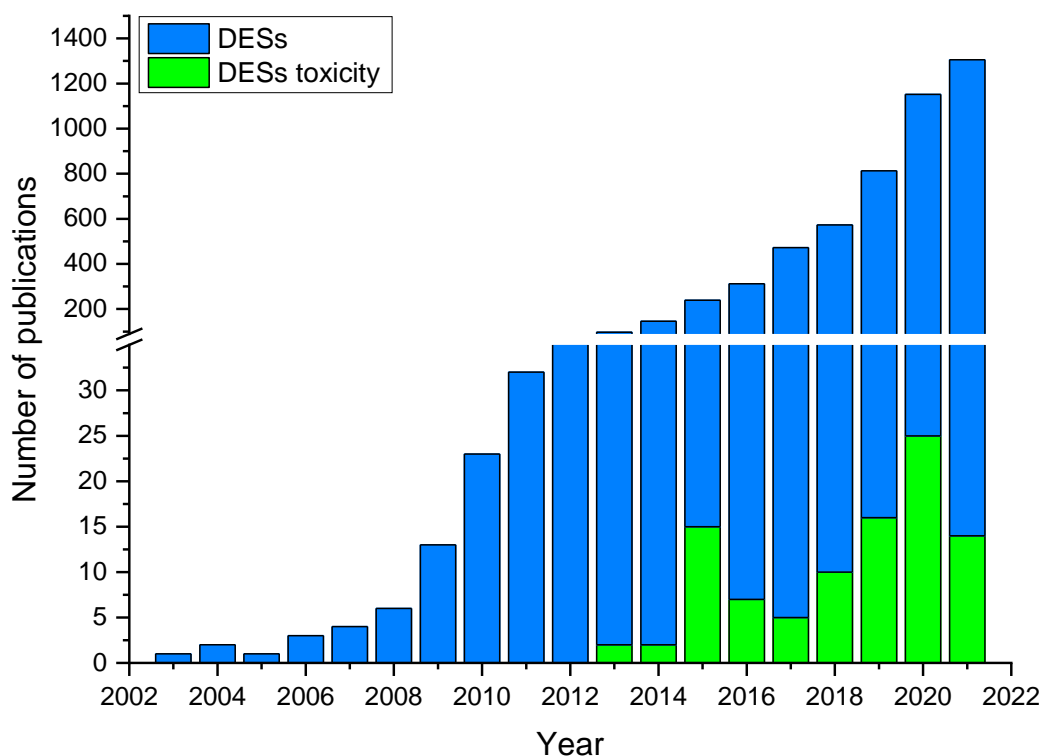


74 applications as green solvents for different chemical industries, more recently they started to be
75 also considered as promising fluids for cosmetic, food, pharmacological, biotechnological and
76 biomedical industries[32-36]. It is mainly related to the fact that DESs are considered as non-
77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

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



97 40, 48-52]. Therefore, in this work we decided to focus on reviewing the present state of art of
98 the DESs microbial toxicity against procaryotic and eukaryotic microorganisms and the critical
99 evaluation of usefulness of the microbiological methods used for this purpose.

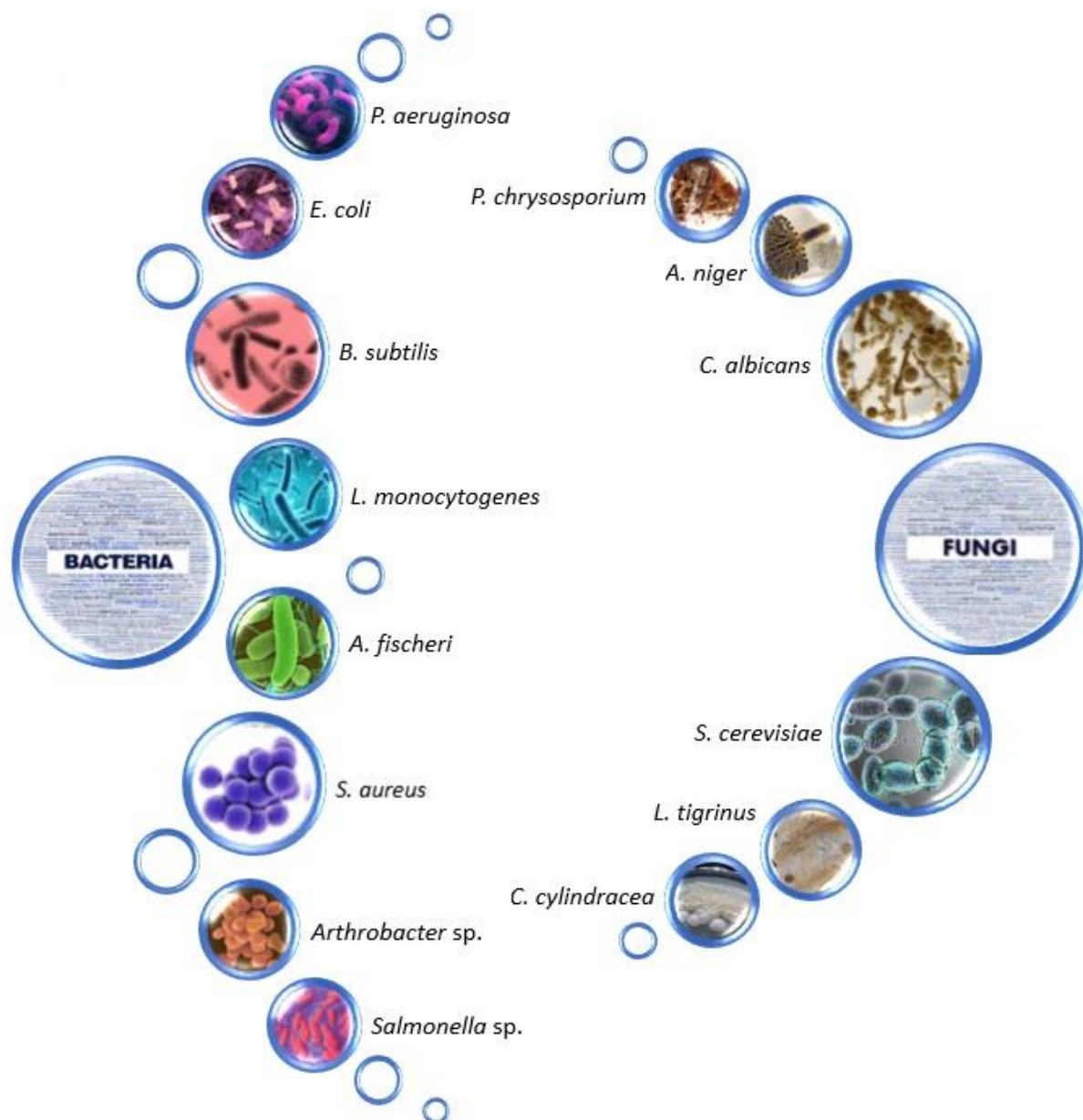


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101 Fig. 1: Evolution of the number of published papers in the field of DESs in general (blue) and
102 DESs toxicity (green) starting from 2003 that contained “deep eutectic solvents” or “deep
103 eutectic solvents and toxicity” in their titles, keywords,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

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



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



116

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.

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

127 **2. Methods used for DESs microbial toxicity assessment**

128 The analysis of the available literature showed that the following methods have been used to
129 assess the toxicity of DESs against prokaryotic and eukaryotic microorganisms: disk and well
130 diffusion method, broth dilution, Microtox assay for luminescence inhibition in *Aliivibrio*
131 *fischeri*, drop plate method and FTIR bioassay. Among these methods, for this purpose, the disk
132 or well diffusion method was most often used (16 studies, Table 1). Moreover, the broth dilution
133 method (macro- and micro-dilution) was also used relatively often (14 studies, Table 2). Methods
134 such as Microtox assay (Table 4), drop plate method (Table 5) or FTIR (Table 6) were used much
135 less frequently for this purpose. In addition, in view of an attempt to critically evaluate the
136 practical suitability of these methods to study DESs microbial toxicity (section 4), in sections 2.1-
137 2.3 besides the discussion of the results of toxicity studies with DESs using these methodologies,
138 each of these techniques is briefly presented and their major advantages and disadvantages are
139 listed.

140 **2.1. Diffusion methods**

141 **2.1.1. Disk diffusion method**

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



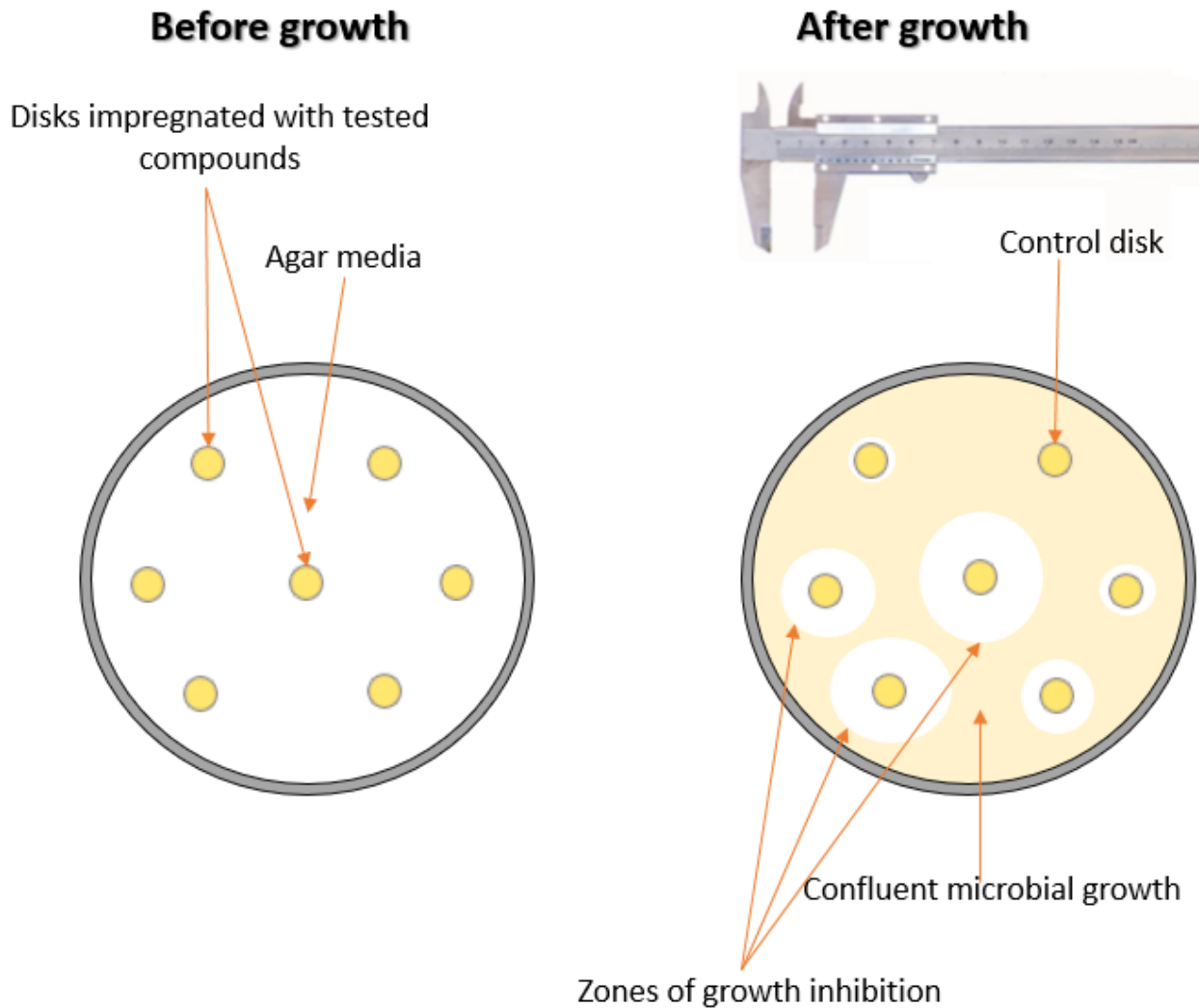
145 test, a filter-paper disk is impregnated with the compound to be tested and then placed on the
146 surface of the agar plate where microorganisms have been previously swabbed uniformly[54, 55].
147 After all the plate is left to grow the tested microorganisms (incubation at optimal growth
148 condition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

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



168 toxicity assessment and the results found in the literature for microbial toxicity of DESs using
169 disk diffusion method are presented in Table 1. On the other hand, the biggest drawback of this
170 method is the fact that it only allows us to assess whether the chemical agent is toxic, moderately
171 toxic, or non-toxic for the tested microorganism in question. That is why, in some cases, multiple
172 disks with different concentrations of the tested compound are used simultaneously on the same
173 agar plate. In that way, it is possible to estimate approximate minimum inhibitory concentration
174 (MIC) of compound. Nevertheless, for more precise toxicity assessment and MIC determination,
175 after disk test, the use of “dilution methods” for the same pair of tested compound and
176 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

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

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

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



209 various HBDs such as amines, alcohols, organic acids and sugars[49]. It was reported that ChCl-
210 based DESs formed with amines, alcohols, and sugars as HBDs did not have a significant toxic
211 effect on bacteria. These finding are in line with the study of Hayyan et al., where also no
212 inhibition of bacteria growth was observed for ChCl-based DESs[48]. On the other hand,
213 significant toxic effect was observed when organic acids were used as HBD of DES. The authors
214 suggested that the amine-, alcohol- and sugar-based DESs were used by bacteria as nitrogen or
215 carbon sources, while the organic acid-based DESs inhibited bacterial growth mainly as a result
216 of significant decrease of pH below the optimal values (pH=6.5–7.5) for bacterial growth of
217 tested microorganisms[49]. The obtained results revealed that gram-negative bacteria (*E. coli* and
218 *S. enteritidis*) were more sensitive than gram-positive (*S. aureus* and *L. monocytogenes*), most
219 likely due to the interaction of DESs components with the polysaccharide or peptide chains of the
220 cell wall through hydrogen bonding or electrostatic interactions, resulting in damage of cell
221 walls[49]. Moreover, the antibacterial activity of DESs based on saturated fatty acids, combining
222 capric acid with other saturated fatty acids with different chain size length (i.e., lauric acid,
223 myristic acid and stearic acid) was studied in the work of Silva et al.[60]. The disk test results
224 revealed that the DESs did not inhibit growth of gram-negative bacteria (*E. coli* and *P.*
225 *aeruginosa*) but showed antibacterial activity against the gram-positive bacteria (*S. aureus*,
226 Methicillin-resistant *S. aureus* (MRSA) and Methicillin-resistant *S. epidermis* (MRSE))[60]. As
227 an explanation, the authors suggested the differences in cell wall structure of gram-positive and -
228 negative bacteria[60]. According to previous reports gram-negative bacteria are usually resistant
229 to the antibacterial activity of fatty acids due to a presence of lipopolysaccharides on the cell wall
230 that prevents the fatty acids from reaching cell membrane[61-64], while the cell wall of gram-
231 positive bacteria readily absorbs fatty acids allowing their passage into the inner membrane[61,
232 63]. The same group also studied the antimicrobial properties of therapeutic DES (THEDES –



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

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



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



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

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

298



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)	<i>Bacillus subtilis</i> , <i>Staphylococcus aureus</i>	<i>Escherichia coli</i> , <i>Pseudomonas aeruginosa</i>		<ul style="list-style-type: none"> 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)	<i>Bacillus subtilis</i> , <i>Staphylococcus aureus</i>	<i>Escherichia coli</i> , <i>Pseudomonas aeruginosa</i>		<ul style="list-style-type: none"> 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)	<i>Staphylococcus aureus</i> , <i>Listeria monocytogenes</i>	<i>Escherichia coli</i> , <i>Salmonella enteritidis</i>		<ul style="list-style-type: none"> All the DESs except for acid containing DESs showed no toxic effect on tested genus of bacteria. The toxic effect of individual 	[49]

<p>ChCl:triethylene glycol (1:4)</p> <p>ChCl:xylitol (1:1)</p> <p>ChCl:D-sorbitol (1:1)</p> <p>ChCl:PTSA (1:1)</p> <p>ChCl:oxalic acid (1:1)</p> <p>ChCl:levulinic acid (1:2)</p> <p>ChCl:malonic acid (1:1)</p> <p>ChCl:malic acid (1:1)</p> <p>ChCl:citric acid (1:1)</p> <p>ChCl:tartaric acid (2:1)</p> <p>ChCl:xylose:water (1:1:1)</p> <p>ChCl:sucrose:water (5:2:5)</p> <p>ChCl:fructose:water (5:2:5)</p> <p>ChCl:glucose:water (5:2:5)</p> <p>ChCl:maltose:water (5:2:5)</p>				<p>components of DESs was not assayed.</p>	
<p>BC:acrylic acid (1:2)</p> <p>BC:methacrylic acid (1:2.5)</p>	<p><i>Staphylococcus aureus</i> NRS234</p>	<p><i>Escherichia coli</i> ATCC 25922</p>	<p><i>Candida albicans</i> ATCC 18804</p>	<ul style="list-style-type: none"> 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. 	<p>[69]</p>
<p>ChCl:1,2-</p>	<p><i>Staphylococcus</i></p>	<p><i>Escherichia</i></p>		<ul style="list-style-type: none"> All the DESs, but 	<p>[41]</p>

<p>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)</p>	<p><i>aureus</i>, <i>Listeria monocytogenes</i></p>	<p><i>coli</i>, <i>Salmonella enteritidis</i></p>		<p>glycerol:L-lysine (<i>E. coli</i>) and glycerol:L-arginine (all four bacterial strains), showed no toxic effect on tested genus of bacteria.</p> <ul style="list-style-type: none"> ChCl and glycerol individually showed no toxic effect on tested genus of bacteria. L-arginine showed relative toxic effect on <i>E. coli</i>. 	
<p>capric acid:lauric acid (2:1) capric acid:myristic acid (3:1) capric acid:stearic acid (4:1)</p>	<p><i>Staphylococcus aureus</i> ATCC 25923, <i>Staphylococcus aureus</i> ATCC 700698 (Methicillin-resistant strain, MRSA), <i>Staphylococcus epidermis</i> ATCC 35984 (Methicillin-resistant strain, MRSE)</p>	<p><i>Pseudomonas aeruginosa</i> ATCC 27853, <i>Escherichia coli</i> ATCC 25922</p>	<p><i>Candida albicans</i> ATCC 90029</p>	<ul style="list-style-type: none"> 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). 	<p>[60]</p>



menthol:stearic acid (8:1)	<i>Staphylococcus aureus</i> ATCC 25923, <i>Staphylococcus aureus</i> ATCC 700698 (MRSA), <i>Staphylococcus epidermis</i> ATCC 35984 (MRSE)	<i>Pseudomonas aeruginosa</i> ATCC 27853, <i>Escherichia coli</i> ATCC 25922		<ul style="list-style-type: none"> • 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)	<i>Staphylococcus epidermis</i>	<i>Pseudomonas aeruginosa</i> , <i>Escherichia coli</i>		<ul style="list-style-type: none"> • 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)	<i>Arthrobacter simplex</i> TCCC 11037			<ul style="list-style-type: none"> • 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 <i>A. simplex</i> at 60 % concentration. • Glycerol and urea individually showed no toxic effect on tested genus of bacteria, while toxic effect of ChCl toward 	[57]



				<i>A. simplex</i> was higher than for tested DESs.	
betaine:urea (1:1.5)		<i>Escherichia coli</i> ATCC 35218, <i>Pseudomonas aeruginosa</i> ATCC 27853		<ul style="list-style-type: none"> 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)		<i>Escherichia coli</i>		<ul style="list-style-type: none"> 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)	<i>Staphylococcus aureus</i> ATCC 9144	<i>Escherichia coli</i> ATCC 23564	<i>Candida albicans</i> ATCC 10231	<ul style="list-style-type: none"> 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)	<i>Staphylococcus aureus</i> 3048	<i>Escherichia coli</i> 3014, <i>Proteus mirabilis</i> 3008, <i>Salmonella typhimurium</i> 3064, <i>Pseudomonas aeruginosa</i>	<i>Candida albicans</i> 86	<ul style="list-style-type: none"> All acid containing DESs showed relative toxic effect on tested genus of bacteria. Only ChCl:oxalic acid DES inhibited growth of <i>C. albicans</i>. 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)	<i>Staphylococcus aureus</i> ATCC 25923, <i>Clostridium perfringens</i> ATCC 13124, <i>Listeria monocytogenes</i> ATCC 7644	<i>Escherichia coli</i> ATCC 25922, <i>Salmonella</i> spp. ATCC 13076		<ul style="list-style-type: none"> 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: <i>p</i> -toluene sulfonic acid (1:3) ChCl:malonic acid (1:1)			<i>Phanerochaete chrysosporium</i> , <i>Aspergillus niger</i> , <i>Lentinus tigrinus</i> , <i>Candida cylindracea</i>	<ul style="list-style-type: none"> Zinc salts and acid containing DESs showed toxic effect on all tested genus of fungi. The other DESs showed no toxic effect on <i>P. chrysosporium</i>, <i>A. niger</i>, <i>L. tigrinus</i>. ChCl:urea, ChCl:ethylene glycol, ChCl:diethylene glycol, ChCl:triethylene glycol DESs showed relative toxic effect on <i>C. cylindracea</i>. ZnCl₂, <i>p</i>-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 <i>C. cylindracea</i> .	
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300

301 Over the years there have also been reports, where the disk diffusion method was used to evaluate

302 DESs antifungal activity. Firstly, Hayyan's group tested ChCl-based DESs toxicity on four fungi

303 strains selected as a model of eukaryotic microorganisms (*Phanerochaete chrysosporium*,

304 *Aspergillus niger*, *Lentinus tigrinus* and *Candida cylindracea*)[52]. Among these DESs the

305 highest antifungal activity was observed for ChCl:ZnCl₂ DES for all tested fungi species,

306 followed by ChCl:malonic acid and ChCl:*p*-toluenesulfonic acid DES[52]. It was also noted that

307 the these three DESs were slightly less toxic to all tested fungi than their respective HBD

308 individually[52]. This phenomenon was assigned to the synergistic effect of forming DES

309 through hydrogen bonding[38, 48]. Furthermore, there have been several works where DESs and

310 NADESs antifungal activity towards *Candida albicans* yeast was studied[43, 60, 69, 72]. For

311 instance, Silva et al. reported that fatty acid-based DESs, namely capric acid:lauric acid, capric

312 acid:myristic acid, capric acid:stearic acid, exhibited antifungal activity towards *C. albicans*[60].

313 Furthermore, it was noted that studied yeast cells were overall less susceptible to DES

314 formulations than gram-positive and -negative bacteria[60]. However, in the work of Wang et al.

315 it was reported that inhibition zones widths caused by BC:acrylic acid and BC:methacrylic acid



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

327 **2.1.2. Well diffusion method**

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

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



339 DESs towards *Aspergillus niger* was studied[51]. The authors showed that EAC:ZnCl₂ DES
340 inhibited the fungal growth the most, already at the lowest DES dose tested (10 mg)[51]. This
341 DES was followed by EAC:ZnN DES and EAC:malonic acid DES[51]. Furthermore, the
342 obtained results indicated that ChCl-based DESs were less toxic to the mold since much higher
343 concentration were needed to inhibit its growth[51].

344 **2.2. Dilution methods**

345 **2.2.1. Agar and broth dilution technique**

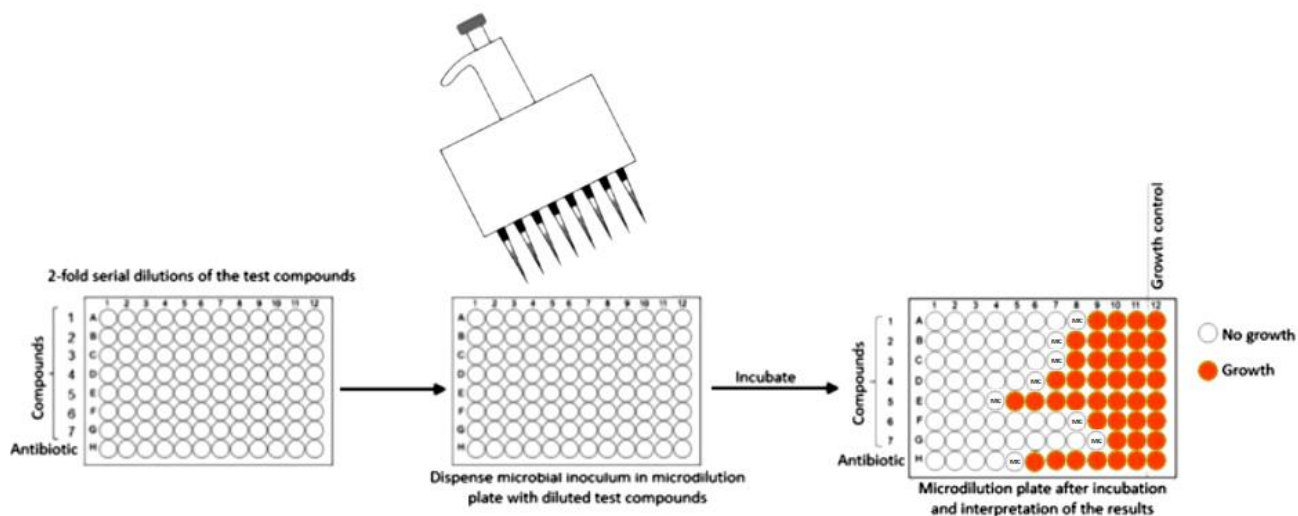
346 As it was mentioned earlier, one of the most used techniques for DESs microbial toxicity testing
347 are agar or broth dilution method. These methods aim to determine the lowest concentration of
348 the studied antimicrobial agent that, under defined test conditions, inhibits the visible growth of
349 the microorganism under investigation. Hence, using broth or agar dilution such parameters as
350 minimum inhibitory concentrations (MICs), or effective concentrations (EC₅₀) of antimicrobial
351 agents can be determined. In agar dilution technique, inoculum of microbes with defined numbers
352 of cells is applied directly onto the nutrient agar plates that have contained different
353 concentrations of antimicrobial agent[75]. Then the plates are incubated at optimal conditions
354 (e.g., temperature, incubation time) for growth of tested microorganism and after incubation the
355 plates are visually inspected. The presence of colonies on the plates indicates growth of the
356 microorganism and the plate with the lowest concentration of tested compound where
357 microorganism did not grow indicates its MIC value[75]. The advantage of agar dilution is that it
358 is a suitable method when testing large numbers of bacterial isolates against a limited number of
359 antimicrobial agents in a limited number of concentrations[76]. However, when testing low
360 concentrations, an even distribution within the agar must be assured[76]. The main drawback of
361 agar dilution is the fact that it is time consuming method, which requires preparation of high



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

366 For comparison, in broth dilution method microorganisms are grown in liquid nutrient medium
367 containing increasing concentrations (typically a two-fold dilution series) of the antimicrobial
368 agent, which is then inoculated with a defined number of microbial cells[75, 77]. Depending on
369 the final volume of the liquid medium in each analyzed sample, this method can be termed as
370 macro-dilution for a total volume of 2 mL, or microdilution, if performed in microtiter plates
371 format with total volume up to 500 μ L per well[75, 77]. In broth dilution method, the growth is
372 assessed after incubation of inoculated samples for a defined period of time (16–20 h) and the
373 MIC or EC₅₀ value is read. Moreover, for this purpose, antimicrobial agent-free test samples -
374 which serve as growth controls - must be included in each assay. In broth dilution method the
375 toxicity of compounds is determined by measuring the mortality or total number of viable cells
376 after certain exposure time to specific concentrations of antimicrobial agents[75, 77]. The
377 schematic representation of broth microdilution procedure is shown in Fig. 4. This technique can
378 be used to test the susceptibility of microorganisms to multiple chemicals at once and quantitative
379 data are obtained[76]. Another advantage of broth dilution is its high accuracy[76]. Other
380 advantages include the possibility of performing this test in practically every laboratory, the
381 easiness of testing and evaluating and the ability for the results of some tests to be read in
382 automatic mode[76]. However, as in agar dilution, this method can be time consuming and
383 individual mistakes in the preparation of stock concentrations or dilution series may take place
384 especially when no automation equipment is available[76].





385

386 Fig. 4: Broth microdilution procedure for MIC determination.

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



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

405 **2.2.1.1. Visual or absorbance determination of cell viability based on turbidity**

406 To date, in most of the published works, where the toxicity of DESs was examined with use of
407 broth dilution method, the cells viability was determined either by visual inspection or by
408 measuring the absorbance of the samples in the absence and presence of DESs. The summary of
409 the results found in the literature for microbial toxicity of DESs determined by broth dilution
410 technique and visual or absorbance determination of cell viability are presented in Table 2. In the
411 first work conducted by Wen et al. broth macro-dilution was used to determine EC_{50} for series of
412 ChCl- and cholinium acetate (ChAc)-based DESs against *E. coli* DH5 α [39]. The bacterial growth
413 was ascertained by measuring the absorbance of the samples at 550 nm. This study revealed that
414 DES concentrations below 75 mM were almost non-toxic to the bacterial cells since the
415 inhibition index was lower than 10% [39]. Furthermore, it was observed that 0.75 M DES
416 inhibited the growth of 72.8–93.8%, indicating that at higher concentration DESs become
417 significantly hazardous to *E. coli* [39]. The calculated EC_{50} values varied for different tested DESs
418 and were mainly dependent on HBA used in DES formation. In general, DESs prepared with
419 ChAc had lower EC_{50} values than respective ChCl-based DESs, indicating higher antibacterial
420 activity of the former [39]. Moreover, the obtained results revealed that beside HBA also HBD
421 has influence on DESs toxicity effect [39]. In particular, much higher EC_{50} values were obtained
422 for DESs which have ethylene glycol (EG) in their composition ($EC_{50} = 532.0$ mM for ChCl:EG
423 and $EC_{50} = 281.1$ mM for ChAc:EG) [39]. Overall, the most toxic compound was ChAc:glycerol



424 DES with EC₅₀ of 58.0 mM, followed by ChAc:acetamide (EC₅₀ = 97.2 mM)[39]. The obtained
425 results also showed that bacterial cells of *E. coli* were more susceptible to the DESs than their
426 individual components because the EC₅₀ values following exposure to individual DES
427 components were all much higher than 800 mM[39]. In this work, the authors hypothesized that
428 DESs inhibited the bacterial growth by interacting with the cellular membrane. Furthermore, the
429 fact that DES in aqueous solution may be partially dissociated was considered and the obtained
430 results explained as a consequence of the possible interaction of the cholinium cation with the
431 polysaccharide or peptide chains of peptidoglycan through hydrogen-bonding or electrostatic
432 interaction, leading to cell wall distortion or disruption[39]. On the other hand, the higher toxicity
433 of DESs than their individual components was assigned to charge delocalization through
434 hydrogen bonding[39].

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



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

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



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

477 In another work of Silva et al., the authors further studied the antibacterial activity of DESs, and
478 they selected THEDES composed of menthol and stearic acid[67]. After initial experiments using
479 disk diffusion method, the MIC data for THEDES and its individual components against *S.*
480 *aureus* ATCC 25923, *S. aureus* MRSA and *S. epidermis* MRSE using broth macro-dilution were
481 gathered. According to the obtained results, the observations made from disk diffusion study
482 were confirmed, and menthol was found toxic to the bacteria with MIC value of 4 and 8 mM for
483 *S. aureus* ATCC 25923 and *S. aureus* MRSA, *S. epidermis* MRSE, respectively[67].
484 Furthermore, stearic acid did not exhibit any antibacterial activity[67]. THEDES showed
485 antimicrobial activity against all the studied bacteria, being more efficient against *S. aureus*
486 ATCC 25923 than Methicillin-resistant strains tested (*S. aureus* MRSA, *S. epidermis*
487 MRSE)[67]. It was also observed that THEDES was more toxic to bacteria than menthol, even
488 though the THEDES contains lower concentration of menthol than this needed to inhibit bacterial
489 growth menthol itself[67]. This same was valid as far it comes to the anti-bactericidal properties
490 of the studied THEDES and MBC values of 6.52 mM and 13.03 mM were obtained for *S. aureus*
491 ATCC 25923 and both Methicillin-resistant strains tested, respectively. Therefore, it was
492 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	
	<i>Escherichia coli*</i>	<i>Staphylococcus aureus*</i>	<i>Listeria monocytogenes</i>	<i>Salmonella enteritidis</i>	<i>Salmonella typhimurium</i>	<i>Staphylococcus aureus</i> MRSA	<i>Staphylococcus epidermis</i> MRSE	<i>Aspergillus niger</i> (filamentous fungus)	<i>Candida albicans</i> (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			

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ChCl:tartaric acid (2:1)	14	18	12	20	16	44	18	40						
capric acid:lauric acid (2:1)			MIC [µg/ mL] 625	MBC [µg/ mL] 1250					MIC [µg/ mL] 625	MBC [µg/ mL] 1250	MIC [µg/ mL] 625	MBC [µg/ mL] 1250	MIC [µg/ mL] 625	MFC [µg/ mL] 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							
acetylcholine chloride:acetamide (1:2)	MIC [mM] 600													
ChCl:ethylene glycol (1:2)												MIC [mg/mL] 325.3±34		
:glycerol (1:2)												550.4±51		
: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)										<2.2	
EAC:ZnCl ₂ (1:2)										<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									
rose: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].

It is noted 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].

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

494 The toxicity of ChCl-based DESs towards *Kurthia gibsonii* was also assessed by broth macro-
495 dilution in the work of Lou's group[83]. In this study, the bacterial growth was determined by
496 measuring the absorbance at 600 nm and the results were expressed in terms of relative biomass,
497 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



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

527 In the most recent work, the toxicity on plant bacteria (*Xanthomonas campestris* CECT 97,
528 *Erwinia amylovora* CECT 222, *Erwinia toletana* CECT 5263, *Clavibacter michiganensis* subsp.
529 *michiganensis* CECT 790, *Clavibacter michiganensis* subsp. *insidious* CECT 5042, *Rhizobium*
530 *radiobacter* CECT 4119, *Pseudomonas syringae* CECT 4429, *Pseudomonas savastanoi* CECT
531 5019) of six DESs namely ChCl:sucrose, ChCl:xylitol, fructose:glucose:sucrose (1:1:1),
532 fructose:glucose:sucrose (2:3.6:1) betaine:sucrose (2:1), betaine:sucrose (4:1) was evaluated by
533 broth microdilution method and the obtained results compared to the toxicity of classic solvents
534 e.g. dimethylsulfoxide (DMSO), ethanol and glycerol[84]. It was revealed that most of the tested
535 DESs were not toxic to the tested bacteria with MIC values $300-1200 \times 10^3$ mg/L[84]. The
536 biofriendly character of DESs composed of carbohydrates (fructose:glucose:sucrose (1:1:1) and
537 fructose:glucose:sucrose (2:3.6:1) was assigned to the fact that their components e.g. glucose,
538 fructose and sucrose are used as nutrition sources by these microorganisms[84]. Furthermore,
539 betaine:sucrose (4:1) DES was the most toxic of DESs tested, with MIC values between 38-150
540 $\times 10^3$ mg/L[84]. In general, the following order of increasing toxicity of DESs was deduced:
541 fructose:glucose:sucrose (1:1:1) = fructose:glucose:sucrose (2:3.6:1) < ChCl:sucrose (1:2) <
542 ChCl:xylitol (2:1) < betaine:sucrose (2:1) < betaine:sucrose (4:1)[84]. Moreover, these DESs
543 showed lower toxicity than glycerol or DMSO for most tested bacteria[84]. Even though, the



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

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



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

571 Both agar and broth dilution methods were also used to study DESs antifungal activity[51, 60,
572 84-86]. Firstly, Hayyan's group examined the toxicity of eight different DESs using ChCl and
573 EAC as the HBAs and ethylene glycol, glycerol, urea, malonic acid, zinc chloride ($ZnCl_2$), and
574 zinc nitrate hexahydrate (ZnN) as the HBDs towards *Aspergillus niger*[51]. According to the
575 MIC data obtained by using broth macro-dilution method all the DESs were shown to be toxic to
576 the examined fungi and the antifungal activity of EAC- based DESs was higher than ChCl-
577 based DESs[51]. Furthermore, it was observed that EAC-based DESs that were prepared using
578 $ZnCl_2$, ZnN and malonic acid as HBDs were way more toxic than these prepared with ethylene
579 glycol and glycerol[51]. The obtained MIC data also revealed that both HBAs (ChCl and EAC)
580 were less toxic to *A. niger* than their respective DESs, while antifungal activities were slightly
581 higher (for the EAC- based DESs) or lower (for the ChCl- based DESs) than those of their
582 corresponding HBDs[51]. Overall, it was concluded that DES individual components play an
583 important role in the toxicity profile of these solvents, as well as their concentration and specific
584 interactions with microorganisms[51]. Later, Silva et al. determined the MIC and MFC values for
585 DESs based on fatty acids, which according to disk diffusion assay inhibited the growth of
586 *Candida albicans* yeast cells[60]. The obtained MIC/MFC data acquired by using broth
587 microdilution method revealed that capric acid:lauric acid DES had the highest antifungal activity
588 from all studied DESs[60]. The following order of the DESs toxicity against examined yeast was
589 deducted: capric acid:lauric acid > capric acid:myristic acid \approx 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 cerevisiae* 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 $\times 10^3$ mg/L[84]. Astonishingly, betaine:sucrose DES had the same MIC value of 600×10^3 mg/L as



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

619 **2.2.1.2. Colorimetric determination of cells viability**

620 Until now there are only five published works (see Table 3) where cells viability after incubation
621 with DES solutions using colorimetric techniques was performed[50, 83, 87-89]. In first report
622 baker's yeast (*Saccharomyces cerevisiae*) viability in different cholinium-based DESs containing
623 50% of water (w/w) and potassium phosphate buffer (100 mM, pH 7.4) was determined at 3 and
624 24 h after inoculation[50]. For that the cell suspension was mixed with an equal volume of
625 methylene blue and incubated for 5 min at room temperature. Here, methylene blue dye was used
626 to stain the yeast cells, however this dye can be applied to all aerobic microorganisms[90].
627 Methylene blue in a presence of living cells gets enzymatically reduced to a colorless product and
628 living cells become unstained, whereas dead cells are stained blue[90]. Therefore, after staining
629 with methylene blue, blue-colored cells can be easily visualized and counted as dead cells. In the
630 work of Redovniković's group, it was observed that ChCl:malic acid, ChCl:oxalic acid and
631 ChCl:urea DESs were toxic to the yeast cells[50]. Already after 3 hours of incubation yeast cells
632 viability decreased tremendously for these solvents and the most detrimental toxic effect was
633 observed for ChCl:oxalic acid DES with only 19% and 4% of living cells after 3 h and 24 h,
634 respectively[50]. On the other hand, no significant toxic effect was observed for DESs formed
635 using sugars, glycerol and ethylene glycol as HBDs with yeast viability of 76–99% and 62–98%
636 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)			<i>Saccharomyces cerevisiae</i> (yeast)	<ul style="list-style-type: none"> • 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)	<i>Kurthia gibsonii</i> SC0312			<ul style="list-style-type: none"> 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)	<i>Arthrobacter simplex</i> TCCC 11037			<ul style="list-style-type: none"> 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)	<i>Staphylococcus aureus</i> ATCC 6538	<i>Escherichia coli</i> ATCC 8739		<ul style="list-style-type: none"> This DES showed no toxic effect on tested genus of <i>E. coli</i> and was found toxic to <i>S. aureus</i>. DES individual components showed no toxic effect on tested genus of <i>E. coli</i>. DES individual components showed higher 	[88]



				antibacterial activity against <i>S. aureus</i> than tested DES.	
ChCl:ethylene glycol (1:2) ChCl:malonic acid (1:2)	<i>Bacillus cereus</i> EMB20			<ul style="list-style-type: none"> • 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]

649

650 In another work, the kit that consists of two dyes, propidium iodide (PI) and SYTO9, was used to
651 evaluate the viability of cells after incubation with ChCl-based DESs[87]. These two dyes are
652 able to stain nucleic acids, and green fluorescing SYTO9 can enter all cells of tested
653 microorganism and is used to determine total number of its cells in the assayed sample, whereas
654 red fluorescing PI enters only into the cells with damaged cytoplasmic membranes[91]. Even
655 though this kit only enables differentiation between cells with intact and damaged cytoplasmic
656 membranes, it is often used to distinguish viable and dead cells because it is accurate to assume
657 that membrane-compromised cells are dead[91]. In this study, gram-positive *Arthrobacter*
658 *simplex* TCCC 11037 was selected as model microorganism. The obtained results showed that
659 the effect of ChCl-based DESs on the *A. simplex* cell membrane was different depending on the
660 type of HBDs used[87]. For instance, the cells tolerated ChCl:glycerol DES better than ethanol
661 (positive control), and the membrane integrity decreased to 70% compared with that in water
662 (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



686 blue[92]. Since resorufin absorbs only weakly at the wavelength giving the maximum absorbance
687 for resazurin, the decrease in resazurin concentration may be measured using a
688 spectrophotometer, and, by varying the concentration of the test chemical, the EC₅₀ value for that
689 chemical may then be estimated[92]. This approach was used to test toxicity of DES composed of
690 menthol and decanoic acid towards *E. coli* and *S. aureus*[88]. Here, the resazurin dye was used
691 for the cell viability determination and the MIC and MBC value reading due to the white and
692 opaque nature of the samples. According to the results of experiments, neither DES starting
693 materials or DES itself had an inhibitory effect on gram-negative *E. coli* at concentrations used
694 in the assay (MIC and MBC > 500 µL/mL)[88]. On the other hand, for *S. aureus* the DES and its
695 individual components exhibited high antimicrobial properties with MIC and MBC values
696 ranging between 3.91-15.63 µL/mL and 7.81-31.25 µL/mL, respectively[88]. This higher
697 antibacterial and -bactericidal efficacy of these compounds against gram-positive *S. aureus* was
698 attributed to the hydrophobic nature of the DES starting materials and explained by the fact that
699 usually gram-positive bacteria are more susceptible to hydrophobic compounds, whereas gram-
700 negative to hydrophilic compounds taking advantage of the hydrophilic character of their
701 membrane porins[88]. Furthermore, it was also observed that for *S. aureus* ATCC 6538 strain the
702 MIC and MBC values for DES (MIC=15.63 µL/mL, MBC=31.25 µL/mL) were higher than the
703 MIC and MBC values for menthol (MIC/MBC=7.81 µL/mL) and for decanoic acid (MIC=3.91
704 µL/mL, MBC=15.63 µL/mL), indicating that tested DES has a lower antibacterial and -
705 bactericidal activity per volume of the mixture used when compared to its individual
706 components[88].

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



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

719 **2.2.2. Microtox assay for luminescence inhibition**

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



731 For the first time, the DESs ecotoxicity was assessed using the Microtox test in the work of de
732 Morais et al.[40]. In this study, the toxicity of DESs based on the HBA - ChCl - and different
733 organic acids (acetic acid (AA), lactic acid (LA), citric acid (CA), and glycolic acid (GA)) as
734 HBDs was examined[40]. The obtained EC₅₀ values indicated that all studied DESs were
735 relatively toxic to *A. fischeri*, which is contrary to the generalized idea that DESs are of low
736 toxicity[40]. The following order of toxicity for DESs with different molar ratios and their
737 individual components was deducted: ChCl \ll ChCl/acid (2:1) < ChCl/acid (1:1) < ChCl/acid
738 (1:2) < acid, indicating that DESs had an intermediate value of toxicity when compared to the
739 starting materials (acids and ChCl)[40]. Furthermore, it was observed that DES toxicity increased
740 with an increase in concentration of the acid (the mole ratio of ChCl:acid)[40]. As far it comes to
741 the HBD used in DES preparation, the following antibacterial activity order was obtained:
742 ChCl/AA < ChCl/LA < ChCl/GA < ChCl/CA, which is in agreement with the decreasing order of
743 the lipophilicity of the acid[40]. The obtained EC₅₀ values showed that the effect of the acid used
744 in DES preparation is preponderant in the toxicity because the toxic effect for the various DESs
745 was similar to that of their corresponding organic acids separately[40]. The authors explained
746 these results as a consequence of low pH values of the DESs containing organic acids and thus
747 having a negative effect on the cell activity, through denaturation of proteins[40]. Furthermore,
748 these DESs were more toxic than the respective ILs, namely, choline acetate (ChAc), choline
749 lactate (ChLa), choline citrate (ChCit), and choline glycolate (ChGly) and it was hypothesized
750 that it is a consequence of hydrogen bonding between the mixture compounds and the respective
751 charge delocalization, since chemicals having delocalized charges are more toxic than chemicals
752 with localized charges[40]. Overall, it was concluded that DESs might not be as “green” as
753 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	
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-propanediol (1:1)	73492	
ChCl:1,2-propanediol (2:1)	61342	
ChCl:1,2-propanediol (1:2)	44048	
ChCl:1,2-propanediol (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	

755

756 In the following work, for the first time the mixtures toxicity theory was used to analyze the
757 results obtained from Microtox test for ChCl-based DESs[94]. The Concentration Addition (CA)
758 model of mixtures toxicity was applied since the dissociation of DESs in water was
759 considered[94]. For that purpose, the EC₅₀ values for both individual DES components and series
760 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 EC₅₀ 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 EC₅₀
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 [N₁₁₁₁]Cl, [N₂₂₂₂]Cl and [N₃₃₃₃]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 EC₅₀ 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 ([N₁₁₁₁]Cl-based DESs < [N₂₂₂₂]Cl-based



784 DESs < [N₃₃₃₃]Cl-based DESs)[95]. Accordingly, [N₃₃₃₃]Cl-based DESs exhibited high overall
785 toxicity towards *A. fischeri* compared to the other DESs under study[95]. This increased toxicity
786 was most likely a consequence of decrease in hydrophilicity of the HBA from [N₁₁₁₁]Cl to
787 [N₃₃₃]Cl[95]. Furthermore, antagonism between HBA and HBD was observed for [N₁₁₁₁]Cl-
788 based DESs, while synergism for [N₃₃₃₃]Cl-based DESs and for [N₂₂₂₂]Cl:1-propanol[95]. It
789 shows that DESs toxicity cannot be predicted based solely on the toxicity of the starting
790 materials. The obtained results further highlighted that for these solvents both the HBD and HBA
791 have an impact on DESs toxicity, agreeing with the study of Wen et al.[39].

792 The latest study carried out by Lapeña et al. was an attempt to further explore toxicity of ChCl-
793 based DESs towards *A. fischeri*[96]. Similarly, to the work of Macario et al.[94] the authors
794 selected DESs prepared using ChCl as HBA combined with urea, glycerol, and ethylene glycol as
795 HBDs. Furthermore, DESs that contained water as third component were also prepared. The
796 obtained EC₅₀ values from the *A. fischeri* ecotoxicity test showed that the most toxic DES was
797 ChCl:urea, followed by ChCl:glycerol, ChCl:urea:H₂O, ChCl:ethylene glycol, ChCl:ethylene
798 glycol:H₂O and ChCl:glycerol:H₂O[96]. Nevertheless, for all DESs under study the EC₅₀ values
799 were higher than 25000 mg/L and for some higher than 100000 mg/L, indicating non-hazardous
800 nature of the tested DESs to this species[96]. In the case of *A. fischeri*, the presence of water
801 decreased the toxicity with respect to the three pure DESs studied[96]. Even though, there is one
802 previous work in which the ecotoxicity of such DESs towards *A. fischeri* was evaluated, the
803 direct comparison of the results is not possible. The dissimilarities in the obtained EC₅₀ values are
804 the outcome of differences in the experimental methodology used in both works. In the study of
805 Lapeña et al. pH of the samples was controlled and adjusted to be in optimal range for the
806 culturing of these bacteria (pH of 6–8.5)[96], while in the work of Macario et al. pH was not



807 controlled[94]. Thus, it could be hypothesized that usually lower EC₅₀ values were obtained in
808 the study of Macario et al.[94] because the severe effect of pH on the toxicity towards *A. fischeri*
809 bacteria has been previously observed[97].

810 **2.2.3. Drop plate method**

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

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



830 acid:fructose:glucose and citric acid:sucrose NADESs against *E. coli*, *E. faecalis* and *S.*
831 *aureus*[100]. Here, the results obtained in the first work of Wikene et al.[98] were confirmed, and
832 citric acid:sucrose NADES was found non-toxic to all three bacterial strains[100]. The same was
833 valid for the other two NADESs under evaluation. At dilutions used in the experiments (400-fold
834 and 200-fold for malic acid:fructose:glucose and ChCl:xylitol, respectively), these NADESs did
835 not reduce significantly the number of viable bacteria as compared to the control samples
836 prepared in PBS[100]. Lastly, the effect of citric acid:sucrose and malic acid:fructose:glucose
837 NADESs on the viability of *E. coli*, *Klebsiella pneumoniae*, *S. epidermis*, *P. aeruginosa* bacteria
838 and *C. albicans* yeast was studied[101]. The obtained results revealed that both NADES diluted
839 100 times reduced the survival of *E. coli* by 96% and 24% for citric acid:sucrose and malic
840 acid:fructose:glucose, respectively[101]. Furthermore, it was observed that *E. coli* tolerated better
841 citric acid-based NADES than an equimolar concentration of citric acid[101]. On the other hand,
842 for malic acid-based NADES no significant differences in cell viability were seen compared to an
843 equimolar concentration of malic acid[101]. Regarding sugar components of NADES, neither
844 fructose, glucose nor sucrose showed effect on *E. coli* survival[101]. Both NADESs were also
845 found toxic to *P. aeruginosa*, and no bacterial survival was observed for 200 times dilution. The
846 toxic effect was further observed for *S. epidermidis*, however, these NADESs exhibited lower
847 antibacterial potency than against *P. aeruginosa*, and 3-9% of cells survived the exposure to
848 NADESs[101]. Moreover, citric acid:sucrose NADES reduced by 37% the bacterial survival of
849 *K. pneumoniae* compared to the control, while malic acid:fructose:glucose NADES did not
850 significantly affected the number of viable bacteria[101]. Finally, these NADESs did not show
851 antifungal activity and no reduction in survival of *C. albicans* yeast was observed[101].

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



NADES	Microorganisms			Toxicity results	Ref.
	Bacterium	Bacterium	Fungi		
	G(+)	G(-)			
citric acid:sucrose (1:1) glucose:malic acid (1:1)	<i>Enterococcus faecalis</i> ATCC 19433	<i>Escherichia coli</i> ATCC 25922		<ul style="list-style-type: none"> 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:malic acid (3:1)		<i>Escherichia coli</i> ATCC 25922		<ul style="list-style-type: none"> Glucose:sucrose NADES showed no toxic effect on tested genus of <i>E. coli</i>. ChCl:malic acid NADES showed relative toxic effect on tested genus of <i>E. coli</i>. 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)	<i>Enterococcus faecalis</i> ATCC 19434, <i>Staphylococcus aureus</i> (strain Newman)	<i>Escherichia coli</i> ATCC 25922		<ul style="list-style-type: none"> 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	<i>Staphylococcus</i>	<i>Escherichia coli</i>	<i>Candida</i>	<ul style="list-style-type: none"> Citric acid:sucrose 	[101]



(1:1) malic acid:fructose:glucose (1:1:1)	<i>epidermis</i> ATCC 35984	BW25113, <i>Klebsiella</i> <i>pneumoniae</i> ATCC 31488, <i>Pseudomonas</i> <i>aeruginosa</i> ATCC 9027	<i>albicans</i> ATCC CRM- 10231	NADES showed relative toxic effect on tested genus of bacteria. <ul style="list-style-type: none"> • Malic acid:fructose:glucose NADES showed relative toxic effect on bacteria except <i>K.</i> <i>pneumoniae</i>. • Both NADESs showed no toxic effect on tested genus of yeast. • The toxic effect of individual components of DESs was not assayed.
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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



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

868 For the first time FTIR-based bioassay was applied to study DESs toxicity in the work of
 869 Cardellini and co-workers, where the authors evaluated the antifungal activity of novel DESs
 870 formed by zwitterionic trimethylglycine and high melting point carboxylic acids[102]. In this
 871 work the yeast strain *Saccharomyces cerevisiae* CBS 13873 was employed as target and model
 872 eukaryotic microorganisms. Preliminary studies showed that these DESs caused a very rapid
 873 decrease of cell viability after a short exposure times to the tested DESs, suggesting that these
 874 DESs are highly toxic to the cells[102]. Basing on these results, it was hypothesized that the high
 875 concentration of these solvents caused a very rapid exit of the cell water and consequently led to
 876 their inactivation[102]. In fact, this hypothesis was confirmed via FTIR-based assay since the
 877 normalized FTIR spectra from the yeast cells treated with DESs and CaCl₂ (a well- known non-
 878 toxic dehydrating agent) were almost identical[102]. This observation led to a conclusion that
 879 these DESs act as dehydrating agents on the model cells.

880 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)	<i>Saccharomyces cerevisiae</i> CBS 13873	<ul style="list-style-type: none"> 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 <i>S</i>)-(+)-10-camphorsulfonic acid aromatic sulfobetaines:(1 <i>S</i>)-(+)-10-camphorsulfonic acid amphiphilic sulfobetaines:(1 <i>S</i>)-(+)-10-camphorsulfonic acid	<i>Saccharomyces cerevisiae</i> CBS 13873	<ul style="list-style-type: none"> All the DESs showed relative toxic effect on yeast cells and exerted a stronger dehydration effect than CaCl₂. The toxic effect of individual components of DESs was not assayed. 	[103]

881

882 In their following work, Cardellini et al. extended DESs toxicity studies for DESs prepared using
883 differently structured sulfobetaines (SBs) with aliphatic, aromatic and amphiphilic moieties and
884 (1*S*)-(+)-10-camphorsulfonic acid[103]. As it was observed for zwitterionic
885 trimethylglycine:carboxylic acids DESs, these DESs exert a dehydration effect on the
886 *Saccharomyces cerevisiae* CBS 13873 cells as this observed for CaCl₂[103]. Furthermore, it was
887 noted that the DESs were stronger dehydrating agents than calcium chloride salt, indicating more
888 affinity of these compounds to water[103]. In general, these results highlight these DESs as
889 promising green media since the presence of water can inactivate the effect of these mixtures on
890 the cells[103].

891 3. General discussion about DES microbial toxicity

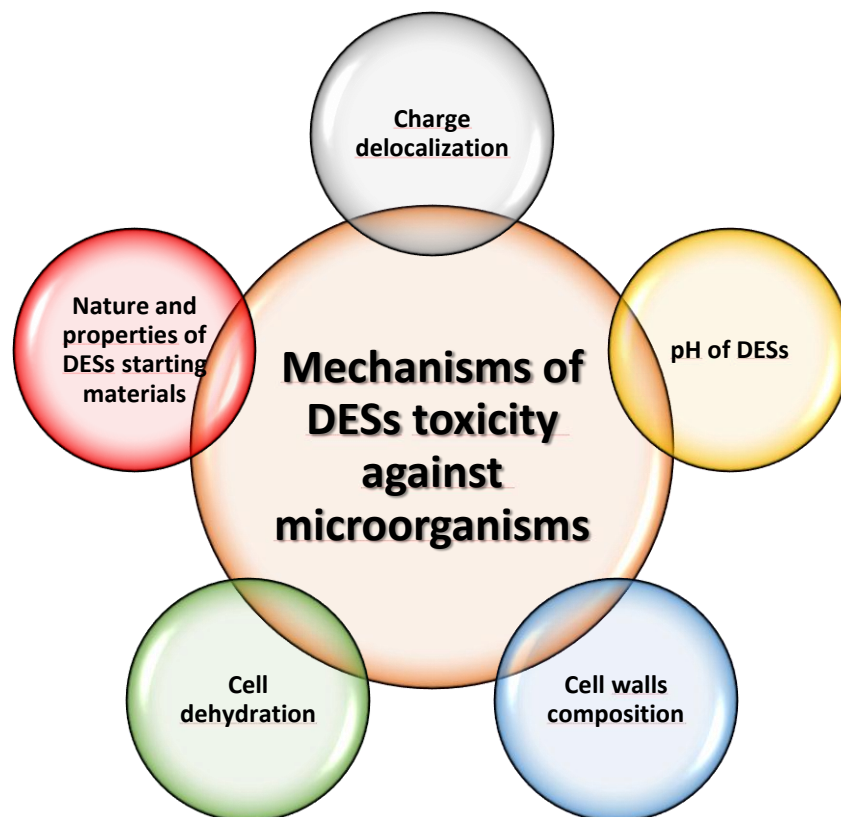
892 A good question was asked in the first work where the toxicity of DESs was studied: “Are deep
893 eutectic solvents benign or toxic?”[48]. Examining the results presented in around 96 works in
894 which the authors looked for the answer on this question, it is still not possible to give a direct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



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

904 There are several factors that were proposed to explain DESs' toxicity mechanism against tested
905 prokaryotic and eukaryotic microorganisms such as negative impact of their pH on the growth of
906 examined microorganisms[40, 49, 50, 80, 82], charge delocalization occurring during DES
907 formation[38-42, 49], and cell dehydration in presence of DESs in growth medium[50, 102, 103],
908 among others (see Fig. 5). Obviously, the impact of each of this factor differs for different DESs,
909 depending on the nature and properties of starting materials used in solvent preparation. For
910 instance, several studies have concluded that DESs possess higher toxicity than their individual
911 components[39, 44, 67, 94, 95], however, other studies reported the opposite[52, 57, 60, 79, 88,
912 94, 95]. All these observations further highlight the need to elucidate DESs' toxicity mechanisms
913 and in this section an attempt to summarize and systematized what have been discovered in
914 regards on how DESs promote toxicity towards prokaryotic and eukaryotic microorganisms will
915 be made.





916

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

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

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

936 Another factor that was proposed to explain DESs' toxicity mechanism is the acidity or alkalinity
937 (pH) of the DESs[40, 49, 50, 80, 82]. Since the optimal pH for bacterial and fungal growth is
938 6.5–7.5[105] and 5.0-9.0[106, 107], respectively; if the DESs had a higher or lower pH value
939 than optimal ones, it influenced the antimicrobial effect of these solvents. This is because the pH
940 value besides theirs optimal ranges for microorganisms growth, has a negative effect on the cell
941 activity, due to denaturation of proteins located on the microorganism cell wall. Consequently,
942 the pH values far from those optimal for microbial growth may alter cellular proliferation and
943 metabolic properties. For instance, de Morais et al. observed that the pH values of DESs
944 composed of ChCl and organic acids were lower than 3 and as a result, the denaturation of
945 proteins and decreased *A. fischeri* cell activity was discovered[40]. Moreover, it was noted that
946 this effect was more pronounced when the acid content was higher further confirming that pH has
947 a great influence on DESs' toxicity[40]. The same phenomenon was also observed for organic
948 acid-based DESs against both gram negative and -positive bacterial strains[49]. Furthermore, the
949 low pH was assumed to be the reason of increased toxicity towards bacteria for malic
950 acid:sucrose[82] and acetylcholine chloride:acetamide DESs[80]. The negative impact of pH on



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

960 Moreover, another factor that may be involved in mechanism of DESs toxicity is cell
961 dehydration[50, 102, 103]. In the studies of Cardellini et al., in which the mechanism of DESs
962 toxicity towards yeast *S. cerevisiae* using FTIR-based assay was evaluated, the authors
963 hypothesized that DESs might cause a very rapid exit of water from the cells[102, 103]. The
964 obtained results confirmed this hypothesis as similar effect to that caused by CaCl₂ (well-known
965 dehydrating agent) was observed[102, 103]. In the case of DESs, high concentrations generate
966 high osmotic pressure to the cells and the cell water leakage, resulting in the yeast cells death.
967 Furthermore, it was assumed that this dehydrating effect of DESs is rather independent of the
968 chemical structure of these solvents, because all tested DESs challenged the yeast cells in the
969 same way[102, 103]. Similar observations were made in the work of Redovniković's group,
970 where high concentrations of ChCl:ethylene glycol and ChCl:glucose caused high osmotic
971 pressure and decreased viability of baker's yeast cells[50].

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



974 40, 60, 72, 79]. For instance, in some studies it was proposed that the bacterial cell wall, which is
975 composed of peptidoglycan, is permeable for small substrates because of its high porosity.
976 Consequently, various DESs can diffuse across cellular membranes and exert their toxic effects
977 inside the cytoplasm by denaturation of enzymes, oxidative stress, among others. In the work of
978 de Morais et al., the authors hypothesized that organic acids containing DESs diffused through
979 the cell membrane and therefore exerted toxic effect on cells of *A. fischeri* bacteria[40].
980 Furthermore, in the study conducted by Wen and co-workers it was assumed that DESs inhibited
981 the bacterial growth of *E. coli* DH5 α by interacting with the cellular membrane[39]. According to
982 their revelations DESs components may interact with the polysaccharide or peptide chains of
983 peptidoglycan through hydrogen-bonding or electrostatic interaction, leading to cell wall
984 distortion or disruption[39]. Moreover, in some reports the different antibacterial potency of
985 DESs towards gram-negative and -positive bacteria was explained by differences in their cell
986 wall structure[60, 79]. Silva et al. concluded that for fatty acid-based DESs, their lower toxicity
987 towards gram-negative bacteria was due to a presence of lipopolysaccharides (LPS) on the outer
988 membrane that prevented the fatty acids DESs from reaching cell membrane[60]. On the other
989 hand, because of the lack outer cell membrane with LPSs, the cell wall of gram-positive bacteria
990 absorbed more easily the fatty acids composed solvents and thus they passed through the inner
991 membrane and exerted the toxic effect[60]. Similar observations were made by Teh and co-
992 workers for ChCl-based DESs where it was assumed that gram-negative bacteria formed a
993 formidable barrier which restricted the attack of DESs from penetrating into the bacterial cell
994 envelopes, while gram-positive *S. aureus* was not able to do that because its cell wall solely
995 consists of thick peptidoglycan layer[79]. Furthermore, the differences in cell wall composition
996 were also suggested as the reason why ChCl:oxalic acid:glycerol and ChCl:citric acid:glycerol
997 were found toxic to bacteria and no to yeast *C. albicans*[72]. According to this report, it is a



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

1000 As mentioned earlier the toxicity profiles of DESs are also influenced by the nature and
1001 properties of starting materials used in solvent preparation[38, 39, 48-50, 79, 87, 94, 95]. In most
1002 of these studies, the negative impact of HBD was discovered. It was mainly observed that the
1003 DESs having organic acids in their compositions exhibited increased antimicrobial properties.
1004 However, enhanced toxicity of such fluids was assigned to not only acidity of DESs (negative pH
1005 effect, see above please) but also their higher viscosity. In addition, the highly viscous nature of
1006 carbohydrates containing DESs, as well as osmotic pressure (negative dehydration effect, see
1007 above please), might also be the reason of increased toxicity of some of these solvents.
1008 Nonetheless, some of the researchers claimed that beside HBD also HBA has an impact on
1009 overall toxicity of DESs[38, 39, 95]. For instance, DESs prepared using the same HBDs were
1010 found toxic to bacteria when MTPB was used as HBA and the opposite was observed for DESs
1011 formed with ChCl[38, 48]. Also, increased toxicity of ChAc-based DESs compared to ChCl-
1012 based ones was observed in the work of Wen et al.[39]. The influence of HBA on DESs toxicity
1013 was further reported by Macario et al. and solvents based on different quaternary ammonium salts
1014 exhibited different ecotoxicity towards *A. fischeri*[95]. Moreover, depending on DESs starting
1015 material and the method used in DESs preparation, the obtained solvents may possess different
1016 toxicities. For example, very often while using the heating method, the formation of impurities is
1017 observed[108]. The presence of impurities can change some of the mixture properties (e.g., by
1018 increasing their viscosities) and indirectly intensifying toxic effect of these DESs.

1019 As discussed in this section, there are proposed various mechanisms regarding DESs toxicology,
1020 nevertheless the knowledge on this topic is still very limited. An interesting idea in the search for



1021 other mechanisms of toxicity towards microbial cells would be to perform studies on the toxic
1022 effect of DESs on the metabolism of microorganisms used in the discussed works (Table 1-6),
1023 e.g. *E. coli* bacteria or *S. cerevisiae* yeast. This would be an analogous approach to that used in
1024 the metabolomic cytotoxicity studies of selected DESs that were performed on HepG2 and HEK
1025 293T mammalian cells (in vitro) and in ICR mice (in vivo)[109]. To the best of our knowledge,
1026 there are no reports on the study of DESs toxicity mechanisms based on the generation of e.g.
1027 oxidative stress or the influence of DESs on the metabolism of basic carbon or nitrogen sources
1028 in microbial cells. Hence, with more studies on DESs toxicity towards various organism, not
1029 mainly focused on prokaryotic and eukaryotic microorganisms, it will be possible to create a
1030 database of truly green and biocompatible DESs and further extend their applications in food,
1031 pharmaceutical, biotechnological, or biomedical sectors. Overall, most of the studies on the
1032 toxicity of DESs revealed that solvents prepared with ChCl as HBA and HBDs from natural
1033 sources such as amines, alcohols, and carbohydrates are generally low toxic to different
1034 microorganisms. On the other hand, acid containing DESs exhibited strong antimicrobial
1035 properties. Furthermore, also the DESs based on quaternary ammonium salts, such as [N₁₁₁₁]Cl,
1036 [N₂₂₂₂]Cl or [N₃₃₃₃]Cl were found more toxic than these prepared using ChCl. All of this proves
1037 once again, that biocompatibility of DESs is mainly dependent on their composition.
1038 Nevertheless, most of the DESs are usually less toxic than conventional organic solvents or ILs
1039 therefore the use of DESs is encouraged.

1040 **4. Critical evaluation of the methods used for DES microbial toxicity determination**

1041 a) Disk and well diffusion method as DES microbial toxicity assay

1042 Due to the simplicity of execution, the disk or well diffusion method is well suited technique for
1043 testing the toxicity of a large number of DESs, differing in terms of composition and molar ratios



1044 of HBA and HBD used in their preparation (see examples in Table 1). However, the obtained
1045 results allow, first of all, to assess whether the tested DES or its solution exhibits toxicity.
1046 Nevertheless, this method does not allow to estimate the toxicity of tested DES against selected
1047 microorganisms by determining the MIC or EC₅₀ value. On the other hand, by selecting strictly
1048 defined strains of gram-negative bacteria, gram-positive bacteria, and fungi (both yeasts and
1049 molds) derivate from certified microbial collection (e.g., ATCC, DSMZ, JCM or CBS-KNAW)
1050 which were previously used for toxicity examination of antibiotics and other natural or synthetic
1051 antimicrobial agents, commercially available microbiological growth media and sterile disks used
1052 in assay, it is possible to normalize this method for DESs toxicity studies and use it in various
1053 laboratories, allowing the comparison of the obtained results. Unfortunately, so far researchers
1054 have approached these issues very freely, using various species of bacteria and yeast in their
1055 research (Table 1). For example, when the same bacterial species, e.g., *S. aureus* was used,
1056 different strains were selected, e.g., *S. aureus* NRS234[69] and *S. aureus* ATCC 25923[60, 67].

1057 What is important to note, due to the key role of the DES diffusion process from a soaked sterile
1058 disk to the growth medium, this method is not suitable for high viscosity DESs. DESs with high
1059 viscosity are those where, for example, carbohydrates or organic acids were used as HBD for
1060 their preparation. The high viscosity also limits the precise application of the same amount of
1061 DES to the sterile disk in repetitions, which may affect the reproducibility of the results. For
1062 instance, in the work of Zhao et al. it was observed that ChCl:urea, ChCl:acetamide,
1063 ChCl:glycerol, ChCl:ethylene glycol did not inhibited *E. coli* growth according to the results
1064 obtained using disk test[49]. However, the exact same DESs have shown the antibacterial activity
1065 and the EC₅₀ values between 275.2-532.0 mM were obtained using broth dilution[39]. The false
1066 results obtained using disk diffusion assay seemed to lead Lou's group to conclude that these



1067 DESs are not toxic towards *E. coli* and thus their toxicity was not further examined using broth
1068 dilution method. These examples highlight the need for careful analysis of DES density and
1069 viscosity before using diffusion methods.

1070 On the other hand, due to the hydrophilic nature of agar medium, diffusion of DES with high
1071 hydrophobicity into agar will be rather difficult and not such effective as for hydrophilic ones.
1072 Hence, it may seem that this physicochemical DES parameter may have also impact on DES
1073 toxicity estimated by disk diffusion method.

1074 Summing up, due to above mentioned disadvantages, it seems too simple and insufficient to
1075 withdraw conclusions about DES toxicity basing exclusively on the results of the tests performed
1076 using disk or well diffusion method. The DES toxicity results obtained with these methods
1077 should be compared with those obtained with one of the alternative techniques. On the other
1078 hand, due to the simplicity and the possibility of standardization of disk diffusion method (under
1079 conditions of using commercially available sterile disks with the same size and made from the
1080 same material), this method seems to be the best of all discussed methods to perform the
1081 preliminary studies on toxicity of DESs (Table 1). Hence, in our opinion, apart from the
1082 mentioned exceptions, e.g., highly viscous DESs, disk diffusion method should be used as one of
1083 the DESs toxicity testing techniques.

1084 b) Broth dilution method as DES microbial toxicity assay

1085 Among the different dilution methods (macro- or microdilution) used so far, the microdilution
1086 method seems to be the best in terms of its reproducibility, validity of obtained results and
1087 application for DESs toxicity assessment. However, when analyzing the published results for
1088 DESs toxicity using broth dilution methods (Table 2), it can be concluded that the researchers



1089 selected the species and strains of microorganisms used in these studies in a very arbitrary and
1090 independent manner from previously published DESs toxicity results. For instance, in one of the
1091 studies only gram-negative *E. coli* strain was used[39], and in another work when the same *E.*
1092 *coli* species was used, different strain was selected - the *E. coli* BL21 (DE3) strain dedicated for
1093 recombinant protein production in pET expression system (Novagen, Merck Millipore)[80].

1094 Furthermore, as in the disk diffusion method, also in broth dilution methods, by selecting the
1095 appropriate microbiological growth media and culture conditions, it is possible to carry out
1096 toxicity tests against gram-negative and gram-positive bacteria, yeasts, and filamentous fungi.
1097 However, contrary to the previously discussed disk diffusion method, broth dilution methods
1098 allow the determination of MIC and EC₅₀ parameters, which, in the case of method
1099 standardization, will allow the comparison of the results obtained by various research groups.
1100 Moreover, since in broth dilution methods serial dilutions of tested DESs are used, the negative
1101 effect of high viscosity of some DESs can be reduced. On the other hand, for broth dilution
1102 technique stability of DESs solutions should be controlled before toxicological analysis. It is
1103 known that high amounts of water are responsible for breaking of hydrogen bonds between HBA
1104 and HBD of DES[110]. Also, DESs or their hydrolyzed individual components may interact with
1105 the salts or nutrients in growth medium and it may be expressed in higher toxicity than the
1106 toxicity of DES itself without the presence of these interactions[80]. Consequently, for lower
1107 concentrations instead of DES toxicity, the toxicity of an aqueous solution of DES components is
1108 determined.

1109 Moreover, the determination of toxicity by broth dilution methods, and in particular the most
1110 popular microdilution method, is not as easy to perform as the disk diffusion method. In the case
1111 of determining the MIC value using the microdilution method, to increase the precision of the



1112 assay and the obtained results, it is sometimes necessary to use spectrophotometric measurements
1113 to assess the viability of the cells of the tested microorganisms (assessment of the turbidity of the
1114 culture). In addition, it is also possible to use resazurin (see section 5) to assess the cell viability
1115 of a cultured microorganism after treatment with DES, which is independent of the turbidity of
1116 the culture, increasing the precision of determination of the MIC and EC₅₀ values. Interestingly,
1117 to the best of our knowledge, there is only one study where resazurin was used for this purpose in
1118 the DESs toxicity studies performed using broth dilution methods (Table 3,[88]). Moreover, after
1119 performing DES toxicity measurements with the broth microdilution method, the minimum
1120 bactericidal concentration (MBC), can be determined for the tested microorganism. In summary,
1121 due to the possibility of quantifying the toxicity of DESs by determining the MIC and EC₅₀ or
1122 MBC, the possibility of selecting a wide range of microorganisms (bacteria, filamentous fungi,
1123 yeasts), the possibility of assessing the viability of cells of the tested microorganism using
1124 resazurin or indirectly by determining the MBC value - the method of microdilution seems to be
1125 the optimal method to assess the toxicity of DES against wide spectrum of both bacteria and
1126 fungi.

1127 c) Microtox assay as DES microbial toxicity testing method

1128 In four out of 96 studies in which the toxicity of DESs was evaluated, the commercially available
1129 Microtox kit was selected for this purpose (Table 4). Thanks to the use of uniform conditions in
1130 this kit for the toxicity assessment against the bioluminescent bacteria *Aliivibrio fischeri*, it is
1131 possible to determine and compare the EC₅₀ values for several different DESs differing in their
1132 composition and molar ratio of HBA and HBD used for their preparation (Table 4). Moreover,
1133 due to the use of one strictly defined *Aliivibrio fischeri* strain, it is possible to compare the results
1134 obtained by different researchers. Contrary to the two previously discussed methods, due to the



1135 fact that we use a commercially standardized test, the method does not need to be validated.
1136 However, since the test is based solely on testing toxicity towards *Aliivibrio fischeri*, the obtained
1137 results are limited to only one type of microorganism – gram-negative bacteria. As shown in the
1138 studies cited in this review, the mechanism of action and susceptibility of gram-negative and
1139 gram-positive bacteria may differ significantly from each other for the same DESs due to the
1140 different structure of the cell wall, and it is mostly depending on the chemical nature of HBA and
1141 HBD used for solvent preparation[39, 40, 60, 72, 79]. This also applies to the differences in the
1142 toxicity of DESs against bacteria and fungi resulting from chemical and structural differences in
1143 the structure of the cell walls of both groups of microorganisms. Hence, this method, despite
1144 many advantages resulting from the use of standardized commercial kit, should be a
1145 complementary method to another more universal technique, e.g., broth microdilution.

1146 d) Other methods as DES microbial toxicity assay

1147 In two analyzed and cited studies in this review, the toxicity of the examined DESs was assessed
1148 using a method based on the analysis of FTIR spectra variation of the cells upon exposition to the
1149 chemicals. In both studies, this method was used to assess DESs toxicity towards *S. cerevisiae*
1150 yeast (Table 6), however, as previously mentioned, this method can be used to evaluate the
1151 toxicity of DESs against different microbial cells[104]. This assay seems to be interesting
1152 because, compared to the previously discussed methods, it allowed to elucidate the mechanism
1153 on how DESs exert their toxic effect (yeast cells dehydration). Hence, FTIR-based bioassay is
1154 worth considering in all studies that aim at determining the possible toxicity mechanisms of
1155 selected DESs in relation to various groups of tested microorganisms.

1156 e) pH of DESs as an important factor in described microbial toxicity methods



1157 Since pH of some DESs is the important parameter that affect the applicability of basically each
1158 of the methods discussed above, it is important to consider this factor before testing DESs
1159 toxicity. Some studies about the toxicity of DESs suggest that the pH of growth media after
1160 preparation of DESs serial dilutions changes significantly[49, 51, 80]. As a result, the pH
1161 decreases below or increases above the optimal values for microbial growth (6.5–7.5[105] and
1162 5.0-9.0[106, 107] for growth of not acidophilic or basophilic bacterial and fungal
1163 microorganisms, respectively), consequently increasing the cells mortality in the tested samples.
1164 It is mostly observed when one of the DESs components are acids. For this reason, it is necessary
1165 to firstly analyze the pH of DESs solutions and if the values are far from those optimal for
1166 microorganisms growth (e.g. for the most often used microorganisms in DESs toxicity studies -
1167 *E. coli* - optimal pH growth range is between 6.5 and 7.5[49]), the DESs solutions should be
1168 prepared in the buffered media. For example, the dissimilarities in the obtained EC₅₀ values for
1169 ChCl-based DESs were noted in the work of Lapeña et al., where pH of the samples was
1170 controlled and adjusted to be in optimal range for the culturing of *A. fischeri*[96] and in the study
1171 of Macario et al. where pH was not controlled[94]. Consequently, lower EC₅₀ values were
1172 obtained in the study of Macario et al. which seems to be due to the pH effect on bacterial
1173 growth, leading to overestimated toxicity of ChCl-based DESs towards *A. fischeri*. In our
1174 opinion, these examples clearly show the need of buffering of DESs before testing their toxicity.

1175 Overall, for proper hazard and risk assessment of DESs, the toxicity data from diffusion method
1176 and broth dilution should be evaluated together for both DESs and their separate individual
1177 components. Since currently there are no standard protocols for testing toxicity of DESs, it makes
1178 difficult to draw conclusions across different studies due to discrepancies in experimental
1179 conditions and lack of test standardization. Nevertheless, we believe that following the



1180 suggestions and guidelines pointed out in subsequent section more precise and comparable data
1181 could be obtained.

1182 **5. Suggestions and guidelines for future research**

1183 The literature review and experience of the authors of this paper acquired during our recent
1184 toxicological studies against selected microorganisms and previous experience in using of some
1185 of above-described methods for testing of others antimicrobial agents, incline us to propose a few
1186 general rules for the future investigation of DESs toxicity. When applying well-established
1187 microbial toxicity testing methods (e.g., disk diffusion assay, broth dilution) for DESs, one
1188 should keep in mind that these methods may need methodological modifications to be applied to
1189 these compounds. We believe that by following the proposed suggestions and guidelines will
1190 enable to get accurate results and facilitate a comparison with the results of other researchers.
1191 Furthermore, with comparable results of investigations of various groups, it will be possible to
1192 further understand the mechanisms on which these solvents exert their toxic effect. The
1193 suggestions and guidelines for future research on toxicity of DESs are outlined below.

1194 i) The description of the methodology used to evaluate DESs toxicity should include all
1195 the details such as the detailed description of strain of microorganism used, detailed
1196 description of inoculum preparation (defined optical density of bacterial cells or CFU
1197 in inoculum), type and composition of growth medium, incubation conditions and
1198 endpoints determination, as well as details on the DES solutions preparation (initial
1199 molar ratio, dilutions) before analysis. The availability of this information will allow
1200 other researchers to better plan their own investigations and compare their results with
1201 different studies. For instance, for DESs toxicity assay using broth dilution method we
1202 encourage to use Mueller-Hinton broth culture media. Mueller-Hinton broth is



1203 recommended by FDA, NCCLS and WHO for testing MICs of for example,
1204 antibiotics against most encountered aerobic and facultative anaerobic bacteria in food
1205 and clinical material. This is excellent medium for cultivation *Escherichia coli*,
1206 *Staphylococcus aureus* and *Pseudomonas aeruginosa* strains previously used in DESs
1207 toxicity studies (Tables 1-3, 5).

1208 ii) Pure DESs should be characterized as much as possible, in particular their
1209 physicochemical properties, such as color/clearness, density, viscosity and pH (or pH
1210 of its solution in water). Disregarding these parameters may lead to the selection of
1211 the assessment method and model microorganism that will not be best suited and
1212 consequently will diminish the validity of the results and conclusions.

1213 a) Both viscosity and density were shown to have a large effect on the obtained
1214 toxicity results. For instance, the viscosity of DESs may have great impact on the
1215 results obtained using disk diffusion assay due to low diffusion of highly viscous
1216 compounds in agar medium.

1217 b) pH mostly influences the results obtained using broth dilution method, especially
1218 when pH of growth medium supplemented with DES is lower or higher than
1219 optimal for microbial growth. Due to pH changes caused by DESs, it is
1220 recommended to use buffered culture media instead of unbuffered cultures or to
1221 prepare DESs solutions in buffers. It will allow to diminish the negative impact of
1222 pH on the microbial growth, obtain more valid results and conclusions.

1223 c) Some DESs may not be transparent liquids and cause some turbidity of the
1224 samples [88], resulting in the increased absorbance readings and thus leading to
1225 lower accuracy of the obtained results in broth macro- or microdilution methods.



1226 d) Crossed reactions between DESs and the salts or nutrients of the culture media
1227 could also take place and influence both the pH and growth[80]. Moreover, such
1228 crossed reactions may be increased in the case of DESs hydrolysis that could
1229 occur in the presence of significant amount of water. Consequently, free HBA and
1230 HBD may react with the salts, amino acids, carbohydrates present in culture
1231 media, changing the pH and decreasing the nutrition sources.

1232 iii) Beside determination of DESs toxicity, it should be mandatory to also evaluate the
1233 toxicity of DES individual components (HBA and HBD) at the same concentrations as
1234 these used for DES preparation. It will allow to better understand the results obtained
1235 in toxicological studies of DESs and withdraw more proper conclusions.

1236 iv) As discussed throughout this paper there are various methods used to evaluate toxicity
1237 of DESs. Our literature study revealed that disk diffusion assay was the most
1238 commonly used method for this purpose (Table 1). The second most frequent used
1239 method was broth dilution method (Table 2). However, other microbiological methods
1240 dedicated for assaying antimicrobial activity of natural or synthetic chemical
1241 compounds were used much more rarely for assaying DESs toxicity against bacteria
1242 and fungi (Tables 3-6). In the light of presented data, although the disk diffusion
1243 method is the most commonly used method for assaying DESs toxicity against
1244 microorganisms, our recommendation is to use broth dilution technique instead of
1245 disk diffusion assay for this purpose. Broth dilution method offers more versatility
1246 and precision than mostly used disk test. It is undeniable that in most of the studies in
1247 which DESs toxicity was evaluated using sterile disks soaked with DESs and placed
1248 on agar plates, the obtained results were less accurate and may not reflect real
1249 interaction between DESs and cells. It is related with high density and viscosity of



1250 most of the DESs which leads to decreased DESs diffusion from the disk into agar
1251 medium. On the other hand, using broth dilution technique the negative impact of
1252 density and viscosity is minimized and quantitative results could be obtained.
1253 Nevertheless, it must be remembered that in high amounts of water DESs hydrolysis
1254 takes place, which may also have an impact on toxicity data obtained. Therefore,
1255 taking all of this into consideration, and if possible, it would be beneficial to firstly
1256 perform analysis using disk diffusion assay with pure DESs and then obtain more
1257 details with broth dilution technique. However, it is important to note, that disk
1258 diffusion method has one important advantage. With this method we can quickly and
1259 cheaply estimate the toxicity of a range of DESs differing in a) the HBA used, b) the
1260 HBD used, or c) the molar ratios of HBA and HBD used to obtain a given type of
1261 DES. Hence, in our opinion, for such DESs toxicity studies, the results of disk test
1262 provide valuable data which can support the analysis of DESs toxicity based on the
1263 results of broth dilution method or other alternative method.

1264 On the other hand, from other methods reported in the literature for DESs microbial
1265 toxicity studies, the methods based on i) analysis of FTIR spectra variation of the
1266 microorganism's cells upon exposition or not to the DESs; ii) the use of commercial
1267 kit that consists of two dyes, propidium iodide (PI) and SYTO9 for staining microbial
1268 cells exposed for DES seem to be interesting solution. They allow to compare DESs
1269 toxicity results obtained with these methods with results of DESs toxicity obtained
1270 with broth dilution method. In contrast to Microtox assay, both these methods give the
1271 possibility of selection of the same microorganism (bacteria or fungi) as used in broth
1272 dilution method. Moreover, the second of above-mentioned methods seem to be quite
1273 easy for validation, because of employing the commercially available kit.



1274 v) If possible, we advise to use the assays based on colorimetric dyes (e.g., cell
1275 incubation with resazurin) for cell viability and vitality determination, which not only
1276 provide more precise values than these obtained by simple visual inspection or
1277 spectrophotometric measurements of turbidity (especially during MIC evaluation by
1278 broth microdilution method), but also higher quality data. Using this method there is
1279 no need of confirmation of the results by subculturing of each concentration onto agar
1280 for 24 h (MBC evaluation). Furthermore, the influence of DESs turbidity on the
1281 absorbance of the samples is reduced for these methods.

1282 vi) The use of preadapted cells of microorganisms selected for study of DESs toxicity is
1283 encouraged. Until now there is one work where the preadaptation of cells to the DESs
1284 was performed[80]. It was demonstrated that non-preadapted cells did not grow in the
1285 presence of 600 mM acetylcholine chloride:acetamide DES, however, when they were
1286 pre-adapted to this concentration, cellular growth was observed[80]. By including the
1287 cellular pre-adaptation in future studies, it will be possible to gain insights on the
1288 capability of the cells to tolerate or assimilate DESs and to obtain more accurate data
1289 on the antimicrobial properties of DESs.

1290 vii) In case of studies where DESs are applied in the processes (such as extraction,
1291 chemical reaction etc.), the toxicity should be controlled for primary DES as well as
1292 for DES recovered after the process. In many cases, elevated temperatures as well as
1293 other factors, such as ultrasounds or microwaves used during the process, can cause
1294 DES chemical instability. As a result, harmful byproducts can be formed and strongly
1295 affect the eco-friendly character of primary DES. Recycled DES can introduce these
1296 byproducts to extracted fraction or product of reaction. On the other hand,



1297 accumulation of toxic byproducts will strongly affect methods available for its safe
1298 disposal after usage.

1299 **6. Conclusions and outlook**

1300 Deep eutectic solvents (DESs) are one of the most interesting classes of alternative solvents,
1301 mainly because of their simple preparation, usually low cost, and versatility due to possibility of
1302 their task- specific design to meet the needs of a specific process. Furthermore, they can be
1303 prepared using all- natural substances which opened exciting new perspectives to design truly
1304 green compounds that will meet with the requirements of green and sustainable chemistry. All
1305 these characteristics confer DESs as an ideal alternative to both organic solvents and ILs. Since
1306 their discovery DESs have been used in a myriad of applications as solvents, reaction media,
1307 catalysts, additives, lubricants, or materials for a wide range of fields from pharmaceutical to
1308 energy. Nevertheless, new studies are constantly conducted in order to learn as much as possible
1309 about the properties of DESs and further increase their applications in new fields important for
1310 the quality of life such as cosmetic, food, drug production and medicine. However, before the
1311 implementation of DESs in these areas will be possible, it is essential to study their toxicity and
1312 gain knowledge on their possible modes of interaction with living beings. Even though, DESs are
1313 considered as green, benign, and non-toxic compounds, a literature review conducted in this
1314 paper indicated that this statement is not entirely true and such generalization should be avoided.
1315 In fact, several examples proved that often out-off-purpose methodology was used, resulting in
1316 false conclusions. Secondly, more than 5200 studies were published about DESs after their
1317 discovery and only around 96 evaluate and discuss the toxicity of these compounds (mainly
1318 against selected microorganisms). It highlights the need for more studies in this topic, which will



1319 allow to gain sufficient insights on DESs toxicity towards different organisms at various trophic
1320 levels and on how they exert their toxic effect.

1321 Throughout this review, we show the advantages and disadvantages of methods used for DESs
1322 toxicity determination. Our analysis indicated that it is necessary to have an improved, standard
1323 protocol for determination of DESs toxicity. In this way, it will be possible to create a database,
1324 compare the results obtained in different studies and for various solvents. In our opinion, in order
1325 to obtain valuable results, it would be beneficial to use both disk diffusion assay and broth
1326 dilution technique in future studies on toxicity of DESs. We believe that the negative impact of
1327 pH may be overcome by using extremophilic microorganisms instead of standard microbial
1328 strains. Hence, it is essential to improve, for example, the broth dilution technique by always
1329 using buffered medium or by preparing DESs solutions in buffer. Furthermore, another aspect
1330 that should be considered while using standard microorganisms is cellular preadaptation with
1331 DESs which was shown to be a viable approach allowing to gain insights on the capability of the
1332 cells to tolerate or assimilate DESs and to obtain more accurate data on the antimicrobial
1333 properties of DESs for which growth for some concentrations was not observed for non-adapted
1334 cells.

1335 It is expected that, in a future, by using the standardized and validated above-mentioned methods,
1336 the theoretical and experimental knowledge about toxicity of DESs will evolve rapidly. It will
1337 allow to further explore these solvents in different applications such as biomedical and
1338 pharmaceutical. Furthermore, it will be possible to address once for all the DESs biosafety issue
1339 and answer with conviction if deep eutectic solvents are benign or toxic.

1340 **Conflicts of interest**



1341 There are no conflicts to declare.

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1 **Deep Eutectic Solvents Microbial Toxicity: Current State** 2 **of Art and Critical Evaluation of Testing Methods**

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26 **Abstract**

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

45

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

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51 **1. Introduction**

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

69 All the above-mentioned remarkable properties of DESs make them an ideal alternative to both
70 commonly used organic solvents and ILs[5, 14-16]. That is why, since their discovery, they have
71 been widely studied and applied in diverse fields, including biocatalysis[17-19],
72 electrochemistry[20-22], CO₂ capture[23, 24], separation and extraction techniques[25-31],
73 among others. Furthermore, beside the fact that up to now the most works focus on their

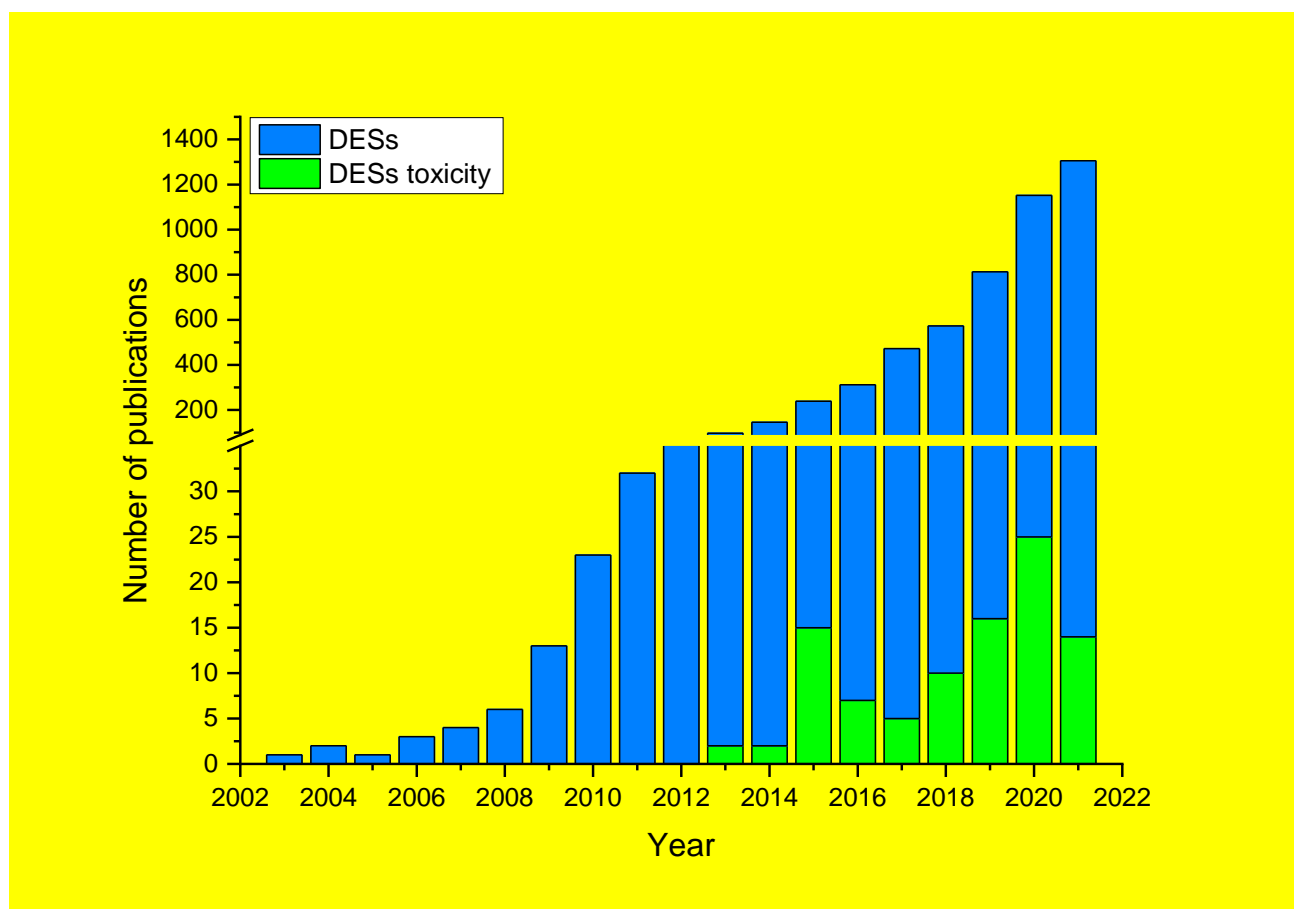


74 applications as green solvents for different chemical industries, more recently they started to be
75 also considered as promising fluids for cosmetic, food, pharmacological, biotechnological and
76 biomedical industries[32-36]. It is mainly related to the fact that DESs are considered as non-
77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

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



97 40, 48-52]. Therefore, in this work we decided to focus on reviewing the present state of art of
98 the DESs microbial toxicity against procaryotic and eukaryotic microorganisms and the critical
99 evaluation of usefulness of the microbiological methods used for this purpose.

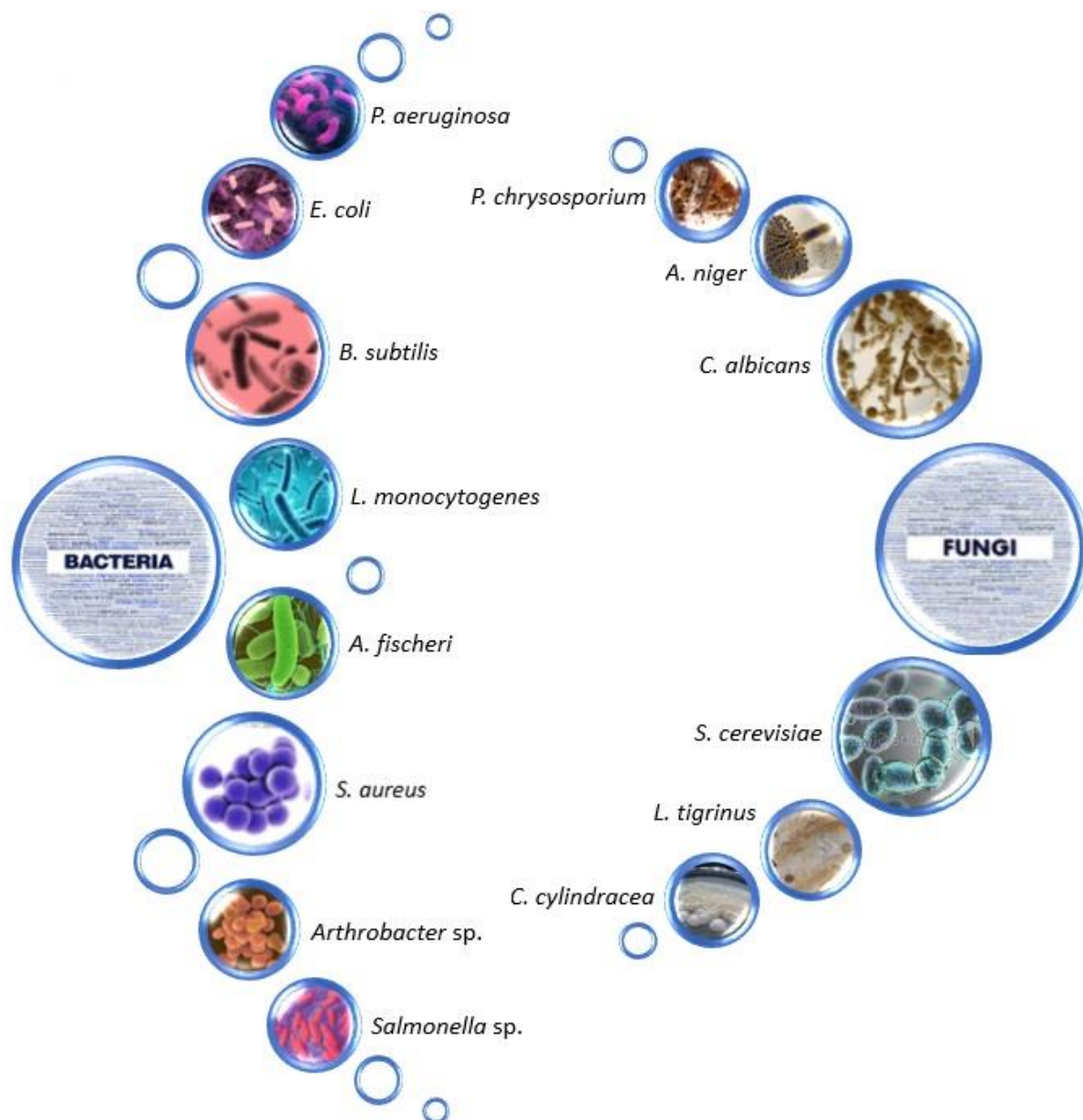


100

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

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

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



116

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.



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

127 **2. Methods used for DESs microbial toxicity assessment**

128 The analysis of the available literature showed that the following methods have been used to
129 assess the toxicity of DESs against prokaryotic and eukaryotic microorganisms: disk and well
130 diffusion method, broth dilution, Microtox assay for luminescence inhibition in *Aliivibrio*
131 *fischeri*, drop plate method and FTIR bioassay. Among these methods, for this purpose, the disk
132 or well diffusion method was most often used (16 studies, Table 1). Moreover, the broth dilution
133 method (macro- and micro-dilution) was also used relatively often (14 studies, Table 2). Methods
134 such as Microtox assay (Table 4), drop plate method (Table 5) or FTIR (Table 6) were used much
135 less frequently for this purpose. In addition, in view of an attempt to critically evaluate the
136 practical suitability of these methods to study DESs microbial toxicity (section 4), in sections 2.1-
137 2.3 besides the discussion of the results of toxicity studies with DESs using these methodologies,
138 each of these techniques is briefly presented and their major advantages and disadvantages are
139 listed.

140 **2.1. Diffusion methods**

141 **2.1.1. Disk diffusion method**

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

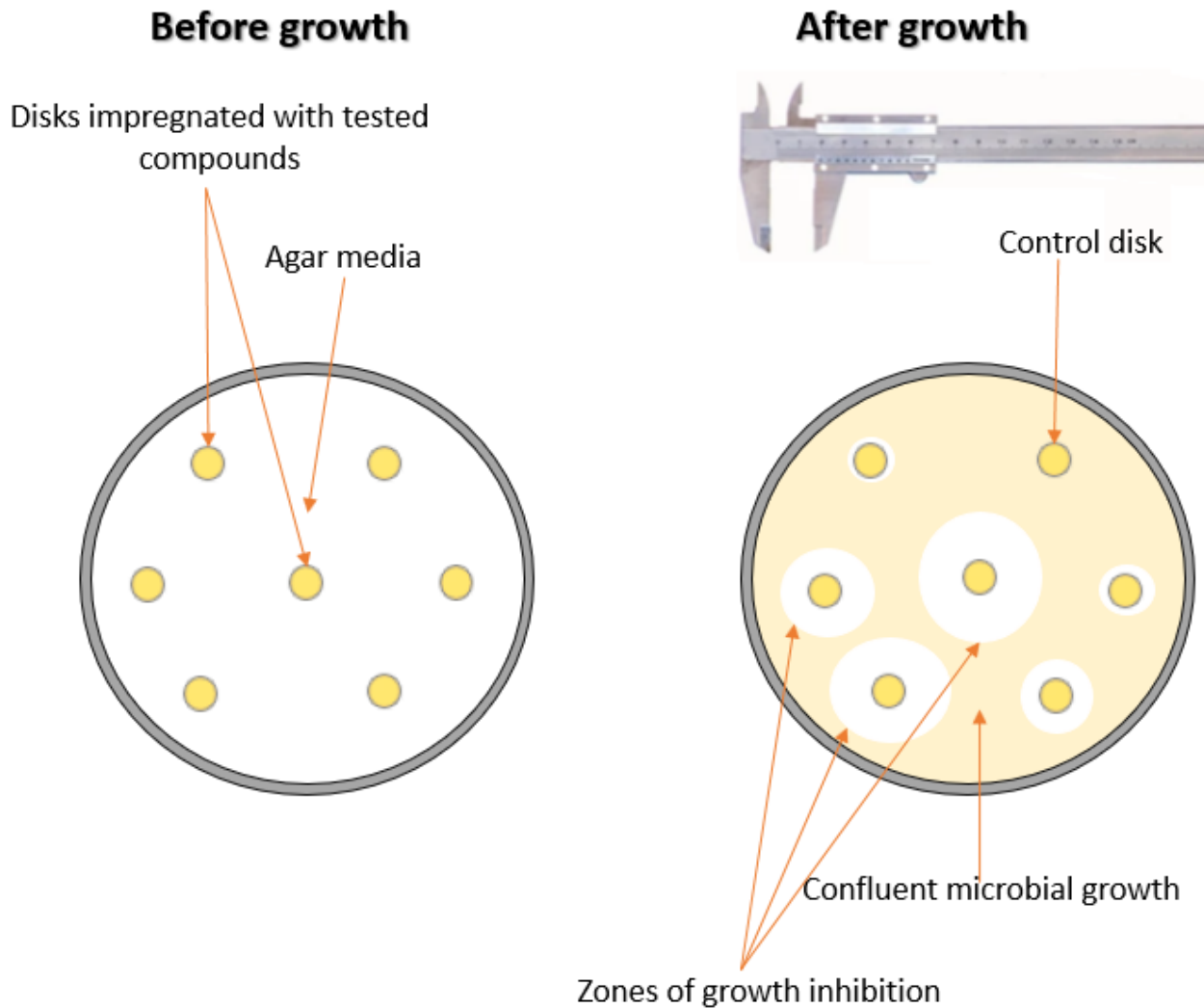


145 test, a filter-paper disk is impregnated with the compound to be tested and then placed on the
146 surface of the agar plate where microorganisms have been previously swabbed uniformly[54, 55].
147 After all the plate is left to grow the tested microorganisms (incubation at optimal growth
148 condition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

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



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



177

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

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

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

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



209 various HBDs such as amines, alcohols, organic acids and sugars[49]. It was reported that ChCl-
210 based DESs formed with amines, alcohols, and sugars as HBDs did not have a significant toxic
211 effect on bacteria. These finding are in line with the study of Hayyan et al., where also no
212 inhibition of bacteria growth was observed for ChCl-based DESs[48]. On the other hand,
213 significant toxic effect was observed when organic acids were used as HBD of DES. The authors
214 suggested that the amine-, alcohol- and sugar-based DESs were used by bacteria as nitrogen or
215 carbon sources, while the organic acid-based DESs inhibited bacterial growth mainly as a result
216 of significant decrease of pH below the optimal values (pH=6.5–7.5) for bacterial growth of
217 tested microorganisms[49]. The obtained results revealed that gram-negative bacteria (*E. coli* and
218 *S. enteritidis*) were more sensitive than gram-positive (*S. aureus* and *L. monocytogenes*), most
219 likely due to the interaction of DESs components with the polysaccharide or peptide chains of the
220 cell wall through hydrogen bonding or electrostatic interactions, resulting in damage of cell
221 walls[49]. Moreover, the antibacterial activity of DESs based on saturated fatty acids, combining
222 capric acid with other saturated fatty acids with different chain size length (i.e., lauric acid,
223 myristic acid and stearic acid) was studied in the work of Silva et al.[60]. The disk test results
224 revealed that the DESs did not inhibit growth of gram-negative bacteria (*E. coli* and *P.*
225 *aeruginosa*) but showed antibacterial activity against the gram-positive bacteria (*S. aureus*,
226 Methicillin-resistant *S. aureus* (MRSA) and Methicillin-resistant *S. epidermis* (MRSE))[60]. As
227 an explanation, the authors suggested the differences in cell wall structure of gram-positive and -
228 negative bacteria[60]. According to previous reports gram-negative bacteria are usually resistant
229 to the antibacterial activity of fatty acids due to a presence of lipopolysaccharides on the cell wall
230 that prevents the fatty acids from reaching cell membrane[61-64], while the cell wall of gram-
231 positive bacteria readily absorbs fatty acids allowing their passage into the inner membrane[61,
232 63]. The same group also studied the antimicrobial properties of therapeutic DES (THEDES –



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

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



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



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

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

298

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)	<i>Bacillus subtilis</i> , <i>Staphylococcus aureus</i>	<i>Escherichia coli</i> , <i>Pseudomonas aeruginosa</i>		<ul style="list-style-type: none"> 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)	<i>Bacillus subtilis</i> , <i>Staphylococcus aureus</i>	<i>Escherichia coli</i> , <i>Pseudomonas aeruginosa</i>		<ul style="list-style-type: none"> 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)	<i>Staphylococcus aureus</i> , <i>Listeria monocytogenes</i>	<i>Escherichia coli</i> , <i>Salmonella enteritidis</i>		<ul style="list-style-type: none"> All the DESs except for acid containing DESs showed no toxic effect on tested genus of bacteria. The toxic effect of individual 	[49]

<p>ChCl:triethylene glycol (1:4)</p> <p>ChCl:xylitol (1:1)</p> <p>ChCl:D-sorbitol (1:1)</p> <p>ChCl:PTSA (1:1)</p> <p>ChCl:oxalic acid (1:1)</p> <p>ChCl:levulinic acid (1:2)</p> <p>ChCl:malonic acid (1:1)</p> <p>ChCl:malic acid (1:1)</p> <p>ChCl:citric acid (1:1)</p> <p>ChCl:tartaric acid (2:1)</p> <p>ChCl:xylose:water (1:1:1)</p> <p>ChCl:sucrose:water (5:2:5)</p> <p>ChCl:fructose:water (5:2:5)</p> <p>ChCl:glucose:water (5:2:5)</p> <p>ChCl:maltose:water (5:2:5)</p>				<p>components of DESs was not assayed.</p>	
<p>BC:acrylic acid (1:2)</p> <p>BC:methacrylic acid (1:2.5)</p>	<p><i>Staphylococcus aureus</i> NRS234</p>	<p><i>Escherichia coli</i> ATCC 25922</p>	<p><i>Candida albicans</i> ATCC 18804</p>	<ul style="list-style-type: none"> 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. 	<p>[69]</p>
<p>ChCl:1,2-</p>	<p><i>Staphylococcus</i></p>	<p><i>Escherichia</i></p>		<ul style="list-style-type: none"> All the DESs, but 	<p>[41]</p>

<p>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)</p>	<p><i>aureus</i>, <i>Listeria monocytogenes</i></p>	<p><i>coli</i>, <i>Salmonella enteritidis</i></p>		<p>glycerol:L-lysine (<i>E. coli</i>) and glycerol:L-arginine (all four bacterial strains), showed no toxic effect on tested genus of bacteria.</p> <ul style="list-style-type: none"> ChCl and glycerol individually showed no toxic effect on tested genus of bacteria. L-arginine showed relative toxic effect on <i>E. coli</i>. 	
<p>capric acid:lauric acid (2:1) capric acid:myristic acid (3:1) capric acid:stearic acid (4:1)</p>	<p><i>Staphylococcus aureus</i> ATCC 25923, <i>Staphylococcus aureus</i> ATCC 700698 (Methicillin-resistant strain, MRSA), <i>Staphylococcus epidermis</i> ATCC 35984 (Methicillin-resistant strain, MRSE)</p>	<p><i>Pseudomonas aeruginosa</i> ATCC 27853, <i>Escherichia coli</i> ATCC 25922</p>	<p><i>Candida albicans</i> ATCC 90029</p>	<ul style="list-style-type: none"> 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). 	<p>[60]</p>



menthol:stearic acid (8:1)	<i>Staphylococcus aureus</i> ATCC 25923, <i>Staphylococcus aureus</i> ATCC 700698 (MRSA), <i>Staphylococcus epidermis</i> ATCC 35984 (MRSE)	<i>Pseudomonas aeruginosa</i> ATCC 27853, <i>Escherichia coli</i> ATCC 25922		<ul style="list-style-type: none"> 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)	<i>Staphylococcus epidermis</i>	<i>Pseudomonas aeruginosa</i> , <i>Escherichia coli</i>		<ul style="list-style-type: none"> 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)	<i>Arthrobacter simplex</i> TCCC 11037			<ul style="list-style-type: none"> 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 <i>A. simplex</i> at 60 % concentration. Glycerol and urea individually showed no toxic effect on tested genus of bacteria, while toxic effect of ChCl toward 	[57]



				<i>A. simplex</i> was higher than for tested DESs.	
betaine:urea (1:1.5)		<i>Escherichia coli</i> ATCC 35218, <i>Pseudomonas aeruginosa</i> ATCC 27853		<ul style="list-style-type: none"> 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)		<i>Escherichia coli</i>		<ul style="list-style-type: none"> 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)	<i>Staphylococcus aureus</i> ATCC 9144	<i>Escherichia coli</i> ATCC 23564	<i>Candida albicans</i> ATCC 10231	<ul style="list-style-type: none"> 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)	<i>Staphylococcus aureus</i> 3048	<i>Escherichia coli</i> 3014, <i>Proteus mirabilis</i> 3008, <i>Salmonella typhimurium</i> 3064, <i>Pseudomonas aeruginosa</i>	<i>Candida albicans</i> 86	<ul style="list-style-type: none"> All acid containing DESs showed relative toxic effect on tested genus of bacteria. Only ChCl:oxalic acid DES inhibited growth of <i>C. albicans</i>. 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)	<i>Staphylococcus aureus</i> ATCC 25923, <i>Clostridium perfringens</i> ATCC 13124, <i>Listeria monocytogenes</i> ATCC 7644	<i>Escherichia coli</i> ATCC 25922, <i>Salmonella</i> spp. ATCC 13076		<ul style="list-style-type: none"> 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: <i>p</i> -toluene sulfonic acid (1:3) ChCl:malonic acid (1:1)			<i>Phanerochaete chrysosporium</i> , <i>Aspergillus niger</i> , <i>Lentinus tigrinus</i> , <i>Candida cylindracea</i>	<ul style="list-style-type: none"> Zinc salts and acid containing DESs showed toxic effect on all tested genus of fungi. The other DESs showed no toxic effect on <i>P. chrysosporium</i>, <i>A. niger</i>, <i>L. tigrinus</i>. ChCl:urea, ChCl:ethylene glycol, ChCl:diethylene glycol, ChCl:triethylene glycol DESs showed relative toxic effect on <i>C. cylindracea</i>. ZnCl₂, <i>p</i>-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 <i>C. cylindracea</i> .	
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300

301 Over the years there have also been reports, where the disk diffusion method was used to evaluate

302 DESs antifungal activity. Firstly, Hayyan's group tested ChCl-based DESs toxicity on four fungi

303 strains selected as a model of eukaryotic microorganisms (*Phanerochaete chrysosporium*,

304 *Aspergillus niger*, *Lentinus tigrinus* and *Candida cylindracea*)[52]. Among these DESs the

305 highest antifungal activity was observed for ChCl:ZnCl₂ DES for all tested fungi species,

306 followed by ChCl:malonic acid and ChCl:*p*-toluenesulfonic acid DES[52]. It was also noted that

307 the these three DESs were slightly less toxic to all tested fungi than their respective HBD

308 individually[52]. This phenomenon was assigned to the synergistic effect of forming DES

309 through hydrogen bonding[38, 48]. Furthermore, there have been several works where DESs and

310 NADESs antifungal activity towards *Candida albicans* yeast was studied[43, 60, 69, 72]. For

311 instance, Silva et al. reported that fatty acid-based DESs, namely capric acid:lauric acid, capric

312 acid:myristic acid, capric acid:stearic acid, exhibited antifungal activity towards *C. albicans*[60].

313 Furthermore, it was noted that studied yeast cells were overall less susceptible to DES

314 formulations than gram-positive and -negative bacteria[60]. However, in the work of Wang et al.

315 it was reported that inhibition zones widths caused by BC:acrylic acid and BC:methacrylic acid

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

327 2.1.2. Well diffusion method

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

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



339 DESs towards *Aspergillus niger* was studied[51]. The authors showed that EAC:ZnCl₂ DES
340 inhibited the fungal growth the most, already at the lowest DES dose tested (10 mg)[51]. This
341 DES was followed by EAC:ZnN DES and EAC:malonic acid DES[51]. Furthermore, the
342 obtained results indicated that ChCl-based DESs were less toxic to the mold since much higher
343 concentration were needed to inhibit its growth[51].

344 **2.2. Dilution methods**

345 **2.2.1. Agar and broth dilution technique**

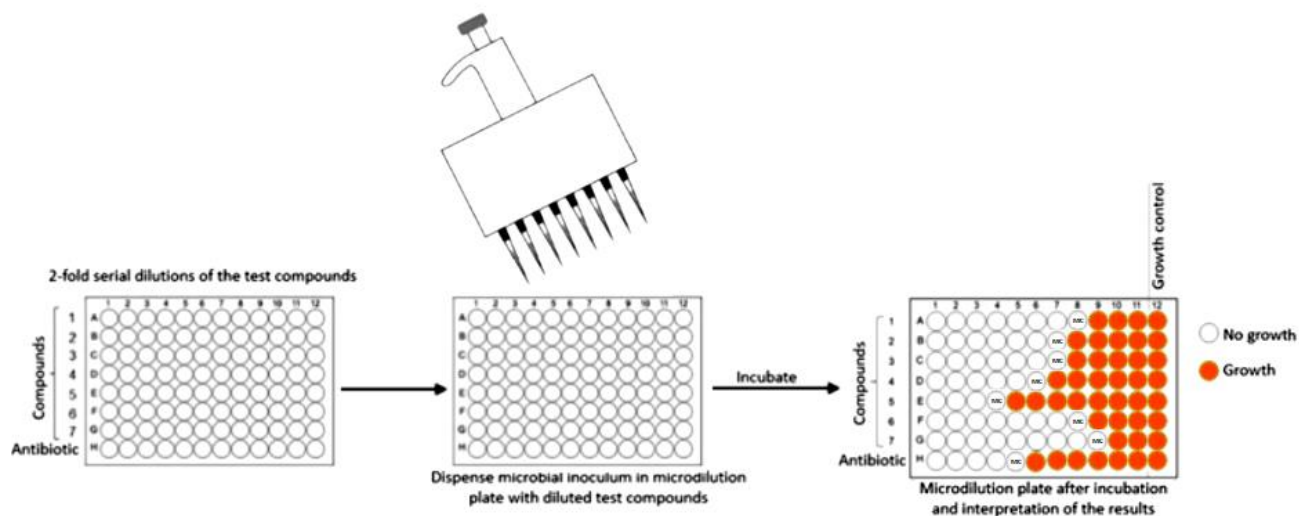
346 As it was mentioned earlier, one of the most used techniques for DESs microbial toxicity testing
347 are agar or broth dilution method. These methods aim to determine the lowest concentration of
348 the studied antimicrobial agent that, under defined test conditions, inhibits the visible growth of
349 the microorganism under investigation. Hence, using broth or agar dilution such parameters as
350 minimum inhibitory concentrations (MICs), or effective concentrations (EC₅₀) of antimicrobial
351 agents can be determined. In agar dilution technique, inoculum of microbes with defined numbers
352 of cells is applied directly onto the nutrient agar plates that have contained different
353 concentrations of antimicrobial agent[75]. Then the plates are incubated at optimal conditions
354 (e.g., temperature, incubation time) for growth of tested microorganism and after incubation the
355 plates are visually inspected. The presence of colonies on the plates indicates growth of the
356 microorganism and the plate with the lowest concentration of tested compound where
357 microorganism did not grow indicates its MIC value[75]. The advantage of agar dilution is that it
358 is a suitable method when testing large numbers of bacterial isolates against a limited number of
359 antimicrobial agents in a limited number of concentrations[76]. However, when testing low
360 concentrations, an even distribution within the agar must be assured[76]. The main drawback of
361 agar dilution is the fact that it is time consuming method, which requires preparation of high



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

366 For comparison, in broth dilution method microorganisms are grown in liquid nutrient medium
367 containing increasing concentrations (typically a two-fold dilution series) of the antimicrobial
368 agent, which is then inoculated with a defined number of microbial cells[75, 77]. Depending on
369 the final volume of the liquid medium in each analyzed sample, this method can be termed as
370 macro-dilution for a total volume of 2 mL, or microdilution, if performed in microtiter plates
371 format with total volume up to 500 μ L per well[75, 77]. In broth dilution method, the growth is
372 assessed after incubation of inoculated samples for a defined period of time (16–20 h) and the
373 MIC or EC₅₀ value is read. Moreover, for this purpose, antimicrobial agent-free test samples -
374 which serve as growth controls - must be included in each assay. In broth dilution method the
375 toxicity of compounds is determined by measuring the mortality or total number of viable cells
376 after certain exposure time to specific concentrations of antimicrobial agents[75, 77]. The
377 schematic representation of broth microdilution procedure is shown in Fig. 4. This technique can
378 be used to test the susceptibility of microorganisms to multiple chemicals at once and quantitative
379 data are obtained[76]. Another advantage of broth dilution is its high accuracy[76]. Other
380 advantages include the possibility of performing this test in practically every laboratory, the
381 easiness of testing and evaluating and the ability for the results of some tests to be read in
382 automatic mode[76]. However, as in agar dilution, this method can be time consuming and
383 individual mistakes in the preparation of stock concentrations or dilution series may take place
384 especially when no automation equipment is available[76].





385

386 Fig. 4: Broth microdilution procedure for MIC determination.

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



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

405 **2.2.1.1. Visual or absorbance determination of cell viability based on turbidity**

406 To date, in most of the published works, where the toxicity of DESs was examined with use of
407 broth dilution method, the cells viability was determined either by visual inspection or by
408 measuring the absorbance of the samples in the absence and presence of DESs. The summary of
409 the results found in the literature for microbial toxicity of DESs determined by broth dilution
410 technique and visual or absorbance determination of cell viability are presented in Table 2. In the
411 first work conducted by Wen et al. broth macro-dilution was used to determine EC_{50} for series of
412 ChCl- and cholinium acetate (ChAc)-based DESs against *E. coli* DH5 α [39]. The bacterial growth
413 was ascertained by measuring the absorbance of the samples at 550 nm. This study revealed that
414 DES concentrations below 75 mM were almost non-toxic to the bacterial cells since the
415 inhibition index was lower than 10%[39]. Furthermore, it was observed that 0.75 M DES
416 inhibited the growth of 72.8–93.8%, indicating that at higher concentration DESs become
417 significantly hazardous to *E. coli*[39]. The calculated EC_{50} values varied for different tested DESs
418 and were mainly dependent on HBA used in DES formation. In general, DESs prepared with
419 ChAc had lower EC_{50} values than respective ChCl-based DESs, indicating higher antibacterial
420 activity of the former[39]. Moreover, the obtained results revealed that beside HBA also HBD
421 has influence on DESs toxicity effect[39]. In particular, much higher EC_{50} values were obtained
422 for DESs which have ethylene glycol (EG) in their composition ($EC_{50} = 532.0$ mM for ChCl:EG
423 and $EC_{50} = 281.1$ mM for ChAc:EG)[39]. Overall, the most toxic compound was ChAc:glycerol



424 DES with EC₅₀ of 58.0 mM, followed by ChAc:acetamide (EC₅₀ = 97.2 mM)[39]. The obtained
425 results also showed that bacterial cells of *E. coli* were more susceptible to the DESs than their
426 individual components because the EC₅₀ values following exposure to individual DES
427 components were all much higher than 800 mM[39]. In this work, the authors hypothesized that
428 DESs inhibited the bacterial growth by interacting with the cellular membrane. Furthermore, the
429 fact that DES in aqueous solution may be partially dissociated was considered and the obtained
430 results explained as a consequence of the possible interaction of the cholinium cation with the
431 polysaccharide or peptide chains of peptidoglycan through hydrogen-bonding or electrostatic
432 interaction, leading to cell wall distortion or disruption[39]. On the other hand, the higher toxicity
433 of DESs than their individual components was assigned to charge delocalization through
434 hydrogen bonding[39].

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



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

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



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

477 In another work of Silva et al., the authors further studied the antibacterial activity of DESs, and
478 they selected THEDES composed of menthol and stearic acid[67]. After initial experiments using
479 disk diffusion method, the MIC data for THEDES and its individual components against *S.*
480 *aureus* ATCC 25923, *S. aureus* MRSA and *S. epidermis* MRSE using broth macro-dilution were
481 gathered. According to the obtained results, the observations made from disk diffusion study
482 were confirmed, and menthol was found toxic to the bacteria with MIC value of 4 and 8 mM for
483 *S. aureus* ATCC 25923 and *S. aureus* MRSA, *S. epidermis* MRSE, respectively[67].
484 Furthermore, stearic acid did not exhibit any antibacterial activity[67]. THEDES showed
485 antimicrobial activity against all the studied bacteria, being more efficient against *S. aureus*
486 ATCC 25923 than Methicillin-resistant strains tested (*S. aureus* MRSA, *S. epidermis*
487 MRSE)[67]. It was also observed that THEDES was more toxic to bacteria than menthol, even
488 though the THEDES contains lower concentration of menthol than this needed to inhibit bacterial
489 growth menthol itself[67]. This same was valid as far it comes to the anti-bactericidal properties
490 of the studied THEDES and MBC values of 6.52 mM and 13.03 mM were obtained for *S. aureus*
491 ATCC 25923 and both Methicillin-resistant strains tested, respectively. Therefore, it was
492 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	
	<i>Escherichia coli</i> *	<i>Staphylococcus aureus</i> *	<i>Listeria monocytogenes</i>	<i>Salmonella enteritidis</i>	<i>Salmonella typhimurium</i>	<i>Staphylococcus aureus</i> MRSA	<i>Staphylococcus epidermis</i> MRSE	<i>Aspergillus niger</i> (filamentous fungus)	<i>Candida albicans</i> (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			

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ChCl:tartaric acid (2:1)	14	18	12	20	16	44	18	40							
capric acid:lauric acid (2:1)			MIC [µg/ mL] 625	MBC [µg/ mL] 1250						MIC [µg/ mL] 625	MBC [µg/ mL] 1250	MIC [µg/ mL] 625	MBC [µg/ mL] 1250	MIC [µg/ mL] 625	MFC [µg/ mL] 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							
acetylcholine chloride:acetamide (1:2)		MIC [mM] 600													
ChCl:ethylene glycol (1:2)													MIC [mg/mL] 325.3±34		
:glycerol (1:2)													550.4±51		
: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)										<2.2	
EAC:ZnCl ₂ (1:2)										<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									
rose: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].

It is noted 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].



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

494 The toxicity of ChCl-based DESs towards *Kurthia gibsonii* was also assessed by broth macro-
495 dilution in the work of Lou's group[83]. In this study, the bacterial growth was determined by
496 measuring the absorbance at 600 nm and the results were expressed in terms of relative biomass,
497 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



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

527 In the most recent work, the toxicity on plant bacteria (*Xanthomonas campestris* CECT 97,
528 *Erwinia amylovora* CECT 222, *Erwinia toletana* CECT 5263, *Clavibacter michiganensis* subsp.
529 *michiganensis* CECT 790, *Clavibacter michiganensis* subsp. *insidious* CECT 5042, *Rhizobium*
530 *radiobacter* CECT 4119, *Pseudomonas syringae* CECT 4429, *Pseudomonas savastanoi* CECT
531 5019) of six DESs namely ChCl:sucrose, ChCl:xylitol, fructose:glucose:sucrose (1:1:1),
532 fructose:glucose:sucrose (2:3.6:1) betaine:sucrose (2:1), betaine:sucrose (4:1) was evaluated by
533 broth microdilution method and the obtained results compared to the toxicity of classic solvents
534 e.g. dimethylsulfoxide (DMSO), ethanol and glycerol[84]. It was revealed that most of the tested
535 DESs were not toxic to the tested bacteria with MIC values $300-1200 \times 10^3$ mg/L[84]. The
536 biofriendly character of DESs composed of carbohydrates (fructose:glucose:sucrose (1:1:1) and
537 fructose:glucose:sucrose (2:3.6:1) was assigned to the fact that their components e.g. glucose,
538 fructose and sucrose are used as nutrition sources by these microorganisms[84]. Furthermore,
539 betaine:sucrose (4:1) DES was the most toxic of DESs tested, with MIC values between 38-150
540 $\times 10^3$ mg/L[84]. In general, the following order of increasing toxicity of DESs was deducted:
541 fructose:glucose:sucrose (1:1:1) = fructose:glucose:sucrose (2:3.6:1) < ChCl:sucrose (1:2) <
542 ChCl:xylitol (2:1) < betaine:sucrose (2:1) < betaine:sucrose (4:1)[84]. Moreover, these DESs
543 showed lower toxicity than glycerol or DMSO for most tested bacteria[84]. Even though, the



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

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



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

571 Both agar and broth dilution methods were also used to study DESs antifungal activity[51, 60,
572 84-86]. Firstly, Hayyan's group examined the toxicity of eight different DESs using ChCl and
573 EAC as the HBAs and ethylene glycol, glycerol, urea, malonic acid, zinc chloride ($ZnCl_2$), and
574 zinc nitrate hexahydrate (ZnN) as the HBDs towards *Aspergillus niger*[51]. According to the
575 MIC data obtained by using broth macro-dilution method all the DESs were shown to be toxic to
576 the examined fungi and the antifungal activity of EAC- based DESs was higher than ChCl-
577 based DESs[51]. Furthermore, it was observed that EAC-based DESs that were prepared using
578 $ZnCl_2$, ZnN and malonic acid as HBDs were way more toxic than these prepared with ethylene
579 glycol and glycerol[51]. The obtained MIC data also revealed that both HBAs (ChCl and EAC)
580 were less toxic to *A. niger* than their respective DESs, while antifungal activities were slightly
581 higher (for the EAC- based DESs) or lower (for the ChCl- based DESs) than those of their
582 corresponding HBDs[51]. Overall, it was concluded that DES individual components play an
583 important role in the toxicity profile of these solvents, as well as their concentration and specific
584 interactions with microorganisms[51]. Later, Silva et al. determined the MIC and MFC values for
585 DESs based on fatty acids, which according to disk diffusion assay inhibited the growth of
586 *Candida albicans* yeast cells[60]. The obtained MIC/MFC data acquired by using broth
587 microdilution method revealed that capric acid:lauric acid DES had the highest antifungal activity
588 from all studied DESs[60]. The following order of the DESs toxicity against examined yeast was
589 deducted: capric acid:lauric acid > capric acid:myristic acid \approx 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 cerevisiae* 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 $\times 10^3$ mg/L[84]. Astonishingly, betaine:sucrose DES had the same MIC value of 600×10^3 mg/L as



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

619 2.2.1.2. Colorimetric determination of cells viability

620 Until now there are only five published works (see Table 3) where cells viability after incubation
621 with DES solutions using colorimetric techniques was performed[50, 83, 87-89]. In first report
622 baker's yeast (*Saccharomyces cerevisiae*) viability in different cholinium-based DESs containing
623 50% of water (w/w) and potassium phosphate buffer (100 mM, pH 7.4) was determined at 3 and
624 24 h after inoculation[50]. For that the cell suspension was mixed with an equal volume of
625 methylene blue and incubated for 5 min at room temperature. Here, methylene blue dye was used
626 to stain the yeast cells, however this dye can be applied to all aerobic microorganisms[90].
627 Methylene blue in a presence of living cells gets enzymatically reduced to a colorless product and
628 living cells become unstained, whereas dead cells are stained blue[90]. Therefore, after staining
629 with methylene blue, blue-colored cells can be easily visualized and counted as dead cells. In the
630 work of Redovniković's group, it was observed that ChCl:malic acid, ChCl:oxalic acid and
631 ChCl:urea DESs were toxic to the yeast cells[50]. Already after 3 hours of incubation yeast cells
632 viability decreased tremendously for these solvents and the most detrimental toxic effect was
633 observed for ChCl:oxalic acid DES with only 19% and 4% of living cells after 3 h and 24 h,
634 respectively[50]. On the other hand, no significant toxic effect was observed for DESs formed
635 using sugars, glycerol and ethylene glycol as HBDs with yeast viability of 76–99% and 62–98%
636 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)			<i>Saccharomyces cerevisiae</i> (yeast)	<ul style="list-style-type: none"> • 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)	<i>Kurthia gibsonii</i> SC0312			<ul style="list-style-type: none"> 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)	<i>Arthrobacter simplex</i> TCCC 11037			<ul style="list-style-type: none"> 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)	<i>Staphylococcus aureus</i> ATCC 6538	<i>Escherichia coli</i> ATCC 8739		<ul style="list-style-type: none"> This DES showed no toxic effect on tested genus of <i>E. coli</i> and was found toxic to <i>S. aureus</i>. DES individual components showed no toxic effect on tested genus of <i>E. coli</i>. DES individual components showed higher 	[88]



				antibacterial activity against <i>S. aureus</i> than tested DES.	
ChCl:ethylene glycol (1:2) ChCl:malonic acid (1:2)	<i>Bacillus cereus</i> EMB20			<ul style="list-style-type: none"> • 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]

649

650 In another work, the kit that consists of two dyes, propidium iodide (PI) and SYTO9, was used to
651 evaluate the viability of cells after incubation with ChCl-based DESs[87]. These two dyes are
652 able to stain nucleic acids, and green fluorescing SYTO9 can enter all cells of tested
653 microorganism and is used to determine total number of its cells in the assayed sample, whereas
654 red fluorescing PI enters only into the cells with damaged cytoplasmic membranes[91]. Even
655 though this kit only enables differentiation between cells with intact and damaged cytoplasmic
656 membranes, it is often used to distinguish viable and dead cells because it is accurate to assume
657 that membrane-compromised cells are dead[91]. In this study, gram-positive *Arthrobacter*
658 *simplex* TCCC 11037 was selected as model microorganism. The obtained results showed that
659 the effect of ChCl-based DESs on the *A. simplex* cell membrane was different depending on the
660 type of HBDs used[87]. For instance, the cells tolerated ChCl:glycerol DES better than ethanol
661 (positive control), and the membrane integrity decreased to 70% compared with that in water
662 (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



686 blue[92]. Since resorufin absorbs only weakly at the wavelength giving the maximum absorbance
687 for resazurin, the decrease in resazurin concentration may be measured using a
688 spectrophotometer, and, by varying the concentration of the test chemical, the EC₅₀ value for that
689 chemical may then be estimated[92]. This approach was used to test toxicity of DES composed of
690 menthol and decanoic acid towards *E. coli* and *S. aureus*[88]. Here, the resazurin dye was used
691 for the cell viability determination and the MIC and MBC value reading due to the white and
692 opaque nature of the samples. According to the results of experiments, neither DES starting
693 materials or DES itself had an inhibitory effect on gram-negative *E. coli* at concentrations used
694 in the assay (MIC and MBC > 500 µL/mL)[88]. On the other hand, for *S. aureus* the DES and its
695 individual components exhibited high antimicrobial properties with MIC and MBC values
696 ranging between 3.91-15.63 µL/mL and 7.81-31.25 µL/mL, respectively[88]. This higher
697 antibacterial and -bactericidal efficacy of these compounds against gram-positive *S. aureus* was
698 attributed to the hydrophobic nature of the DES starting materials and explained by the fact that
699 usually gram-positive bacteria are more susceptible to hydrophobic compounds, whereas gram-
700 negative to hydrophilic compounds taking advantage of the hydrophilic character of their
701 membrane porins[88]. Furthermore, it was also observed that for *S. aureus* ATCC 6538 strain the
702 MIC and MBC values for DES (MIC=15.63 µL/mL, MBC=31.25 µL/mL) were higher than the
703 MIC and MBC values for menthol (MIC/MBC=7.81 µL/mL) and for decanoic acid (MIC=3.91
704 µL/mL, MBC=15.63 µL/mL), indicating that tested DES has a lower antibacterial and -
705 bactericidal activity per volume of the mixture used when compared to its individual
706 components[88].

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



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

719 **2.2.2. Microtox assay for luminescence inhibition**

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



731 For the first time, the DESs ecotoxicity was assessed using the Microtox test in the work of de
732 Morais et al.[40]. In this study, the toxicity of DESs based on the HBA - ChCl - and different
733 organic acids (acetic acid (AA), lactic acid (LA), citric acid (CA), and glycolic acid (GA)) as
734 HBDs was examined[40]. The obtained EC₅₀ values indicated that all studied DESs were
735 relatively toxic to *A. fischeri*, which is contrary to the generalized idea that DESs are of low
736 toxicity[40]. The following order of toxicity for DESs with different molar ratios and their
737 individual components was deducted: ChCl \ll ChCl/acid (2:1) < ChCl/acid (1:1) < ChCl/acid
738 (1:2) < acid, indicating that DESs had an intermediate value of toxicity when compared to the
739 starting materials (acids and ChCl)[40]. Furthermore, it was observed that DES toxicity increased
740 with an increase in concentration of the acid (the mole ratio of ChCl:acid)[40]. As far it comes to
741 the HBD used in DES preparation, the following antibacterial activity order was obtained:
742 ChCl/AA < ChCl/LA < ChCl/GA < ChCl/CA, which is in agreement with the decreasing order of
743 the lipophilicity of the acid[40]. The obtained EC₅₀ values showed that the effect of the acid used
744 in DES preparation is preponderant in the toxicity because the toxic effect for the various DESs
745 was similar to that of their corresponding organic acids separately[40]. The authors explained
746 these results as a consequence of low pH values of the DESs containing organic acids and thus
747 having a negative effect on the cell activity, through denaturation of proteins[40]. Furthermore,
748 these DESs were more toxic than the respective ILs, namely, choline acetate (ChAc), choline
749 lactate (ChLa), choline citrate (ChCit), and choline glycolate (ChGly) and it was hypothesized
750 that it is a consequence of hydrogen bonding between the mixture compounds and the respective
751 charge delocalization, since chemicals having delocalized charges are more toxic than chemicals
752 with localized charges[40]. Overall, it was concluded that DESs might not be as “green” as
753 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	
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-propanediol (1:1)	73492	
ChCl:1,2-propanediol (2:1)	61342	
ChCl:1,2-propanediol (1:2)	44048	
ChCl:1,2-propanediol (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	

755

756 In the following work, for the first time the mixtures toxicity theory was used to analyze the
757 results obtained from Microtox test for ChCl-based DESs[94]. The Concentration Addition (CA)
758 model of mixtures toxicity was applied since the dissociation of DESs in water was
759 considered[94]. For that purpose, the EC₅₀ values for both individual DES components and series
760 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 EC₅₀ 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 EC₅₀
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 [N₁₁₁₁]Cl, [N₂₂₂₂]Cl and [N₃₃₃₃]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 EC₅₀ 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 ([N₁₁₁₁]Cl-based DESs < [N₂₂₂₂]Cl-based



784 DESs < [N₃₃₃₃]Cl-based DESs)[95]. Accordingly, [N₃₃₃₃]Cl-based DESs exhibited high overall
785 toxicity towards *A. fischeri* compared to the other DESs under study[95]. This increased toxicity
786 was most likely a consequence of decrease in hydrophilicity of the HBA from [N₁₁₁₁]Cl to
787 [N₃₃₃]Cl[95]. Furthermore, antagonism between HBA and HBD was observed for [N₁₁₁₁]Cl-
788 based DESs, while synergism for [N₃₃₃₃]Cl-based DESs and for [N₂₂₂₂]Cl:1-propanol[95]. It
789 shows that DESs toxicity cannot be predicted based solely on the toxicity of the starting
790 materials. The obtained results further highlighted that for these solvents both the HBD and HBA
791 have an impact on DESs toxicity, agreeing with the study of Wen et al.[39].

792 The latest study carried out by Lapeña et al. was an attempt to further explore toxicity of ChCl-
793 based DESs towards *A. fischeri*[96]. Similarly, to the work of Macario et al.[94] the authors
794 selected DESs prepared using ChCl as HBA combined with urea, glycerol, and ethylene glycol as
795 HBDs. Furthermore, DESs that contained water as third component were also prepared. The
796 obtained EC₅₀ values from the *A. fischeri* ecotoxicity test showed that the most toxic DES was
797 ChCl:urea, followed by ChCl:glycerol, ChCl:urea:H₂O, ChCl:ethylene glycol, ChCl:ethylene
798 glycol:H₂O and ChCl:glycerol:H₂O[96]. Nevertheless, for all DESs under study the EC₅₀ values
799 were higher than 25000 mg/L and for some higher than 100000 mg/L, indicating non-hazardous
800 nature of the tested DESs to this species[96]. In the case of *A. fischeri*, the presence of water
801 decreased the toxicity with respect to the three pure DESs studied[96]. Even though, there is one
802 previous work in which the ecotoxicity of such DESs towards *A. fischeri* was evaluated, the
803 direct comparison of the results is not possible. The dissimilarities in the obtained EC₅₀ values are
804 the outcome of differences in the experimental methodology used in both works. In the study of
805 Lapeña et al. pH of the samples was controlled and adjusted to be in optimal range for the
806 culturing of these bacteria (pH of 6–8.5)[96], while in the work of Macario et al. pH was not



807 controlled[94]. Thus, it could be hypothesized that usually lower EC₅₀ values were obtained in
808 the study of Macario et al.[94] because the severe effect of pH on the toxicity towards *A. fischeri*
809 bacteria has been previously observed[97].

810 **2.2.3. Drop plate method**

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

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



830 acid:fructose:glucose and citric acid:sucrose NADESs against *E. coli*, *E. faecalis* and *S.*
831 *aureus*[100]. Here, the results obtained in the first work of Wikene et al.[98] were confirmed, and
832 citric acid:sucrose NADES was found non-toxic to all three bacterial strains[100]. The same was
833 valid for the other two NADESs under evaluation. At dilutions used in the experiments (400-fold
834 and 200-fold for malic acid:fructose:glucose and ChCl:xylitol, respectively), these NADESs did
835 not reduce significantly the number of viable bacteria as compared to the control samples
836 prepared in PBS[100]. Lastly, the effect of citric acid:sucrose and malic acid:fructose:glucose
837 NADESs on the viability of *E. coli*, *Klebsiella pneumoniae*, *S. epidermis*, *P. aeruginosa* bacteria
838 and *C. albicans* yeast was studied[101]. The obtained results revealed that both NADES diluted
839 100 times reduced the survival of *E. coli* by 96% and 24% for citric acid:sucrose and malic
840 acid:fructose:glucose, respectively[101]. Furthermore, it was observed that *E. coli* tolerated better
841 citric acid-based NADES than an equimolar concentration of citric acid[101]. On the other hand,
842 for malic acid-based NADES no significant differences in cell viability were seen compared to an
843 equimolar concentration of malic acid[101]. Regarding sugar components of NADES, neither
844 fructose, glucose nor sucrose showed effect on *E. coli* survival[101]. Both NADESs were also
845 found toxic to *P. aeruginosa*, and no bacterial survival was observed for 200 times dilution. The
846 toxic effect was further observed for *S. epidermidis*, however, these NADESs exhibited lower
847 antibacterial potency than against *P. aeruginosa*, and 3-9% of cells survived the exposure to
848 NADESs[101]. Moreover, citric acid:sucrose NADES reduced by 37% the bacterial survival of
849 *K. pneumoniae* compared to the control, while malic acid:fructose:glucose NADES did not
850 significantly affected the number of viable bacteria[101]. Finally, these NADESs did not show
851 antifungal activity and no reduction in survival of *C. albicans* yeast was observed[101].

852



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)	<i>Enterococcus faecalis</i> ATCC 19433	<i>Escherichia coli</i> ATCC 25922		<ul style="list-style-type: none"> 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:malic acid (3:1)		<i>Escherichia coli</i> ATCC 25922		<ul style="list-style-type: none"> Glucose:sucrose NADES showed no toxic effect on tested genus of <i>E. coli</i>. ChCl:malic acid NADES showed relative toxic effect on tested genus of <i>E. coli</i>. 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)	<i>Enterococcus faecalis</i> ATCC 19434, <i>Staphylococcus aureus</i> (strain Newman)	<i>Escherichia coli</i> ATCC 25922		<ul style="list-style-type: none"> 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)	<i>Staphylococcus epidermis</i> ATCC 35984	<i>Escherichia coli</i> BW25113, <i>Klebsiella pneumoniae</i> ATCC 31488, <i>Pseudomonas aeruginosa</i> ATCC 9027	<i>Candida albicans</i> ATCC CRM-10231	<ul style="list-style-type: none"> • 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 <i>K. pneumoniae</i>. • Both NADESs showed no toxic effect on tested genus of yeast. • The toxic effect of individual components of DESs was not assayed. 	[101]

854

855 **2.3. FTIR-based biological assay**

856 Another method used for DESs toxicity testing is FTIR-based bioassay (see Table 6)[102, 103].

857 This assay was primarily based on *Saccharomyces cerevisiae* cells however it offers the
858 possibility to also use as biosensor the cells from different organisms, including different
859 microbial cells or mammal cell cultures[104]. The principles of this method are based on the fact
860 that cells under stress exhibit very fast changes in terms of cell metabolites and thus a
861 metabolomic analysis, using FTIR, may be capable of detecting these variations as early as in the
862 first hours of exposure[104]. This bioassay estimates the toxicity level as function of the FTIR
863 spectra variation of the cells upon exposition to the chemicals and provides metabolic indexes



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

869 For the first time FTIR-based bioassay was applied to study DESs toxicity in the work of
870 Cardellini and co-workers, where the authors evaluated the antifungal activity of novel DESs
871 formed by zwitterionic trimethylglycine and high melting point carboxylic acids[102]. In this
872 work the yeast strain *Saccharomyces cerevisiae* CBS 13873 was employed as target and model
873 eukaryotic microorganisms. Preliminary studies showed that these DESs caused a very rapid
874 decrease of cell viability after a short exposure times to the tested DESs, suggesting that these
875 DESs are highly toxic to the cells[102]. Basing on these results, it was hypothesized that the high
876 concentration of these solvents caused a very rapid exit of the cell water and consequently led to
877 their inactivation[102]. In fact, this hypothesis was confirmed via FTIR-based assay since the
878 normalized FTIR spectra from the yeast cells treated with DESs and CaCl₂ (a well- known non-
879 toxic dehydrating agent) were almost identical[102]. This observation led to a conclusion that
880 these DESs act as dehydrating agents on the model cells.

881



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)	<i>Saccharomyces cerevisiae</i> CBS 13873	<ul style="list-style-type: none"> 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:(1S)-(+)-10-camphorsulfonic acid aromatic sulfobetaines:(1S)-(+)-10-camphorsulfonic acid amphiphilic sulfobetaines:(1S)-(+)-10-camphorsulfonic acid	<i>Saccharomyces cerevisiae</i> CBS 13873	<ul style="list-style-type: none"> All the DESs showed relative toxic effect on yeast cells and exerted a stronger dehydration effect than CaCl₂. 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 (1S)-(+)-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 CaCl₂[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



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

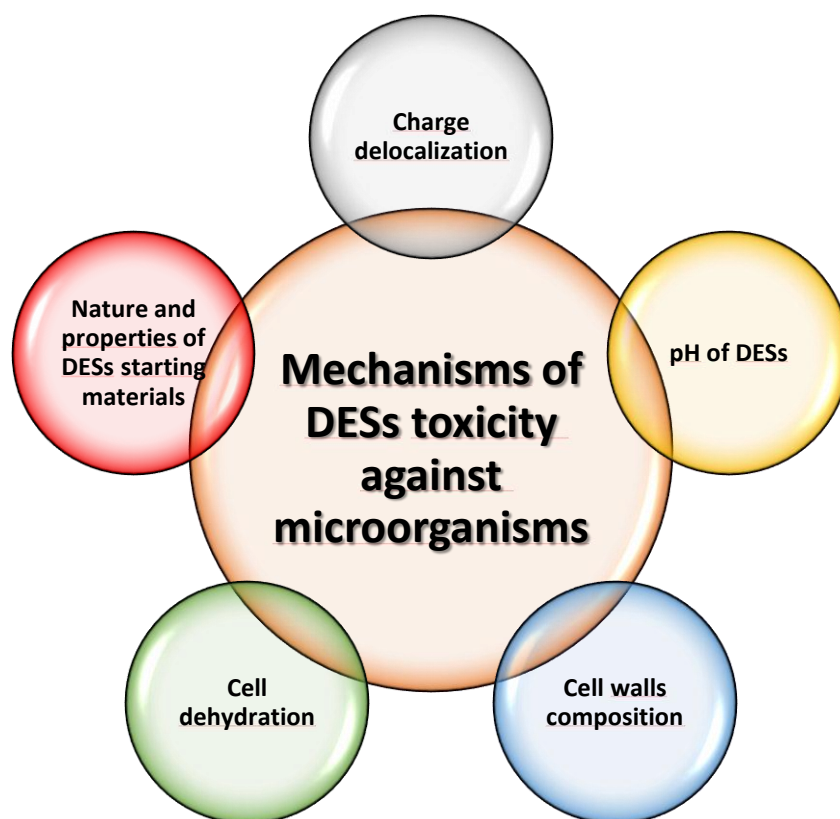
893 **3. General discussion about DES microbial toxicity**

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

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



914 94, 95]. All these observations further highlight the need to elucidate DESs' toxicity mechanisms
915 and in this section an attempt to summarize and systematized what have been discovered in
916 regards on how DESs promote toxicity towards prokaryotic and eukaryotic microorganisms will
917 be made.



918
919 Fig. 5: Overview of factors proposed to explain the mechanisms of DESs toxicity against
920 prokaryotic and eukaryotic microorganisms.

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

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

938 Another factor that was proposed to explain DESs' toxicity mechanism is the acidity or alkalinity
939 (pH) of the DESs[40, 49, 50, 80, 82]. Since the optimal pH for bacterial and fungal growth is
940 6.5–7.5[105] and 5.0-9.0[106, 107], respectively; if the DESs had a higher or lower pH value
941 than optimal ones, it influenced the antimicrobial effect of these solvents. This is because the pH
942 value besides theirs optimal ranges for microorganisms growth, has a negative effect on the cell
943 activity, due to denaturation of proteins located on the microorganism cell wall. Consequently,
944 the pH values far from those optimal for microbial growth may alter cellular proliferation and
945 metabolic properties. For instance, de Morais et al. observed that the pH values of DESs
946 composed of ChCl and organic acids were lower than 3 and as a result, the denaturation of
947 proteins and decreased *A. fischeri* cell activity was discovered[40]. Moreover, it was noted that
948 this effect was more pronounced when the acid content was higher further confirming that pH has



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

962 Moreover, another factor that may be involved in mechanism of DESs toxicity is cell
963 dehydration[50, 102, 103]. In the studies of Cardellini et al., in which the mechanism of DESs
964 toxicity towards yeast *S. cerevisiae* using FTIR-based assay was evaluated, the authors
965 hypothesized that DESs might cause a very rapid exit of water from the cells[102, 103]. The
966 obtained results confirmed this hypothesis as similar effect to that caused by CaCl₂ (well-known
967 dehydrating agent) was observed[102, 103]. In the case of DESs, high concentrations generate
968 high osmotic pressure to the cells and the cell water leakage, resulting in the yeast cells death.
969 Furthermore, it was assumed that this dehydrating effect of DESs is rather independent of the
970 chemical structure of these solvents, because all tested DESs challenged the yeast cells in the
971 same way[102, 103]. Similar observations were made in the work of Redovniković's group,



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

974 Findings in other reports suggest that DESs' toxicity mechanism may also be related to the
975 cellular organization of the organisms, in particular to the differences in cell wall composition[39,
976 40, 60, 72, 79]. For instance, in some studies it was proposed that the bacterial cell wall, which is
977 composed of peptidoglycan, is permeable for small substrates because of its high porosity.
978 Consequently, various DESs can diffuse across cellular membranes and exert their toxic effects
979 inside the cytoplasm by denaturation of enzymes, oxidative stress, among others. In the work of
980 de Morais et al., the authors hypothesized that organic acids containing DESs diffused through
981 the cell membrane and therefore exerted toxic effect on cells of *A. fischeri* bacteria[40].
982 Furthermore, in the study conducted by Wen and co-workers it was assumed that DESs inhibited
983 the bacterial growth of *E. coli* DH5 α by interacting with the cellular membrane[39]. According to
984 their revelations DESs components may interact with the polysaccharide or peptide chains of
985 peptidoglycan through hydrogen-bonding or electrostatic interaction, leading to cell wall
986 distortion or disruption[39]. Moreover, in some reports the different antibacterial potency of
987 DESs towards gram-negative and -positive bacteria was explained by differences in their cell
988 wall structure[60, 79]. Silva et al. concluded that for fatty acid-based DESs, their lower toxicity
989 towards gram-negative bacteria was due to a presence of lipopolysaccharides (LPS) on the outer
990 membrane that prevented the fatty acids DESs from reaching cell membrane[60]. On the other
991 hand, because of the lack outer cell membrane with LPSs, the cell wall of gram-positive bacteria
992 absorbed more easily the fatty acids composed solvents and thus they passed through the inner
993 membrane and exerted the toxic effect[60]. Similar observations were made by Teh and co-
994 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 not 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
1015 was further reported by Macario et al. and solvents based on different quaternary ammonium salts
1016 exhibited different ecotoxicity towards *A. fischeri*[95]. Moreover, depending on DESs starting
1017 material and the method used in DESs preparation, the obtained solvents may possess different



1018 toxicities. For example, very often while using the heating method, the formation of impurities is
1019 observed[108]. The presence of impurities can change some of the mixture properties (e.g., by
1020 increasing their viscosities) and indirectly intensifying toxic effect of these DESs.

1021 As discussed in this section, there are proposed various mechanisms regarding DESs toxicology,
1022 nevertheless the knowledge on this topic is still very limited. An interesting idea in the search for
1023 other mechanisms of toxicity towards microbial cells would be to perform studies on the toxic
1024 effect of DESs on the metabolism of microorganisms used in the discussed works (Table 1-6),
1025 e.g. *E. coli* bacteria or *S. cerevisiae* yeast. This would be an analogous approach to that used in
1026 the metabolomic cytotoxicity studies of selected DESs that were performed on HepG2 and HEK
1027 293T mammalian cells (in vitro) and in ICR mice (in vivo)[109]. To the best of our knowledge,
1028 there are no reports on the study of DESs toxicity mechanisms based on the generation of e.g.
1029 oxidative stress or the influence of DESs on the metabolism of basic carbon or nitrogen sources
1030 in microbial cells. Hence, with more studies on DESs toxicity towards various organism, not
1031 mainly focused on prokaryotic and eukaryotic microorganisms, it will be possible to create a
1032 database of truly green and biocompatible DESs and further extend their applications in food,
1033 pharmaceutical, biotechnological, or biomedical sectors. Overall, most of the studies on the
1034 toxicity of DESs revealed that solvents prepared with ChCl as HBA and HBDs from natural
1035 sources such as amines, alcohols, and carbohydrates are generally low toxic to different
1036 microorganisms. On the other hand, acid containing DESs exhibited strong antimicrobial
1037 properties. Furthermore, also the DESs based on quaternary ammonium salts, such as [N₁₁₁₁]Cl,
1038 [N₂₂₂₂]Cl or [N₃₃₃₃]Cl were found more toxic than these prepared using ChCl. All of this proves
1039 once again, that biocompatibility of DESs is mainly dependent on their composition.



1040 Nevertheless, most of the DESs are usually less toxic than conventional organic solvents or ILs
1041 therefore the use of DESs is encouraged.

1042 **4. Critical evaluation of the methods used for DES microbial toxicity determination**

1043 a) Disk and well diffusion method as DES microbial toxicity assay

1044 Due to the simplicity of execution, the disk or well diffusion method is well suited technique for
1045 testing the toxicity of a large number of DESs, differing in terms of composition and molar ratios
1046 of HBA and HBD used in their preparation (see examples in Table 1). However, the obtained
1047 results allow, first of all, to assess whether the tested DES or its solution exhibits toxicity.
1048 Nevertheless, this method does not allow to estimate the toxicity of tested DES against selected
1049 microorganisms by determining the MIC or EC₅₀ value. On the other hand, by selecting strictly
1050 defined strains of gram-negative bacteria, gram-positive bacteria, and fungi (both yeasts and
1051 molds) derivate from certified microbial collection (e.g., ATCC, DSMZ, JCM or CBS-KNAW)
1052 which were previously used for toxicity examination of antibiotics and other natural or synthetic
1053 antimicrobial agents, commercially available microbiological growth media and sterile disks used
1054 in assay, it is possible to normalize this method for DESs toxicity studies and use it in various
1055 laboratories, allowing the comparison of the obtained results. Unfortunately, so far researchers
1056 have approached these issues very freely, using various species of bacteria and yeast in their
1057 research (Table 1). For example, when the same bacterial species, e.g., *S. aureus* was used,
1058 different strains were selected, e.g., *S. aureus* NRS234[69] and *S. aureus* ATCC 25923[60, 67].

1059 What is important to note, due to the key role of the DES diffusion process from a soaked sterile
1060 disk to the growth medium, this method is not suitable for high viscosity DESs. DESs with high
1061 viscosity are those where, for example, carbohydrates or organic acids were used as HBD for



1062 their preparation. The high viscosity also limits the precise application of the same amount of
1063 DES to the sterile disk in repetitions, which may affect the reproducibility of the results. For
1064 instance, in the work of Zhao et al. it was observed that ChCl:urea, ChCl:acetamide,
1065 ChCl:glycerol, ChCl:ethylene glycol did not inhibited *E. coli* growth according to the results
1066 obtained using disk test[49]. However, the exact same DESs have shown the antibacterial activity
1067 and the EC₅₀ values between 275.2-532.0 mM were obtained using broth dilution[39]. The false
1068 results obtained using disk diffusion assay seemed to lead Lou's group to conclude that these
1069 DESs are not toxic towards *E. coli* and thus their toxicity was not further examined using broth
1070 dilution method. These examples highlight the need for careful analysis of DES density and
1071 viscosity before using diffusion methods.

1072 On the other hand, due to the hydrophilic nature of agar medium, diffusion of DES with high
1073 hydrophobicity into agar will be rather difficult and not such effective as for hydrophilic ones.
1074 Hence, it may seem that this physicochemical DES parameter may have also impact on DES
1075 toxicity estimated by disk diffusion method.

1076 Summing up, due to above mentioned disadvantages, it seems too simple and insufficient to
1077 withdraw conclusions about DES toxicity basing exclusively on the results of the tests performed
1078 using disk or well diffusion method. The DES toxicity results obtained with these methods
1079 should be compared with those obtained with one of the alternative techniques. On the other
1080 hand, due to the simplicity and the possibility of standardization of disk diffusion method (under
1081 conditions of using commercially available sterile disks with the same size and made from the
1082 same material), this method seems to be the best of all discussed methods to perform the
1083 preliminary studies on toxicity of DESs (Table 1). Hence, in our opinion, apart from the



1084 mentioned exceptions, e.g., highly viscous DESs, disk diffusion method should be used as one of
1085 the DESs toxicity testing techniques.

1086 b) Broth dilution method as DES microbial toxicity assay

1087 Among the different dilution methods (macro- or microdilution) used so far, the microdilution
1088 method seems to be the best in terms of its reproducibility, validity of obtained results and
1089 application for DESs toxicity assessment. However, when analyzing the published results for
1090 DESs toxicity using broth dilution methods (Table 2), it can be concluded that the researchers
1091 selected the species and strains of microorganisms used in these studies in a very arbitrary and
1092 independent manner from previously published DESs toxicity results. For instance, in one of the
1093 studies only gram-negative *E. coli* strain was used[39], and in another work when the same *E.*
1094 *coli* species was used, different strain was selected - the *E. coli* BL21 (DE3) strain dedicated for
1095 recombinant protein production in pET expression system (Novagen, Merck Millipore)[80].

1096 Furthermore, as in the disk diffusion method, also in broth dilution methods, by selecting the
1097 appropriate microbiological growth media and culture conditions, it is possible to carry out
1098 toxicity tests against gram-negative and gram-positive bacteria, yeasts, and filamentous fungi.
1099 However, contrary to the previously discussed disk diffusion method, broth dilution methods
1100 allow the determination of MIC and EC₅₀ parameters, which, in the case of method
1101 standardization, will allow the comparison of the results obtained by various research groups.
1102 Moreover, since in broth dilution methods serial dilutions of tested DESs are used, the negative
1103 effect of high viscosity of some DESs can be reduced. On the other hand, for broth dilution
1104 technique stability of DESs solutions should be controlled before toxicological analysis. It is
1105 known that high amounts of water are responsible for breaking of hydrogen bonds between HBA
1106 and HBD of DES[110]. Also, DESs or their hydrolyzed individual components may interact with



1107 the salts or nutrients in growth medium and it may be expressed in higher toxicity than the
1108 toxicity of DES itself without the presence of these interactions[80]. Consequently, for lower
1109 concentrations instead of DES toxicity, the toxicity of an aqueous solution of DES components is
1110 determined.

1111 Moreover, the determination of toxicity by broth dilution methods, and in particular the most
1112 popular microdilution method, is not as easy to perform as the disk diffusion method. In the case
1113 of determining the MIC value using the microdilution method, to increase the precision of the
1114 assay and the obtained results, it is sometimes necessary to use spectrophotometric measurements
1115 to assess the viability of the cells of the tested microorganisms (assessment of the turbidity of the
1116 culture). In addition, it is also possible to use resazurin (see section 5) to assess the cell viability
1117 of a cultured microorganism after treatment with DES, which is independent of the turbidity of
1118 the culture, increasing the precision of determination of the MIC and EC₅₀ values. Interestingly,
1119 to the best of our knowledge, there is only one study where resazurin was used for this purpose in
1120 the DESs toxicity studies performed using broth dilution methods (Table 3,[88]). Moreover, after
1121 performing DES toxicity measurements with the broth microdilution method, the minimum
1122 bactericidal concentration (MBC), can be determined for the tested microorganism. In summary,
1123 due to the possibility of quantifying the toxicity of DESs by determining the MIC and EC₅₀ or
1124 MBC, the possibility of selecting a wide range of microorganisms (bacteria, filamentous fungi,
1125 yeasts), the possibility of assessing the viability of cells of the tested microorganism using
1126 resazurin or indirectly by determining the MBC value - the method of microdilution seems to be
1127 the optimal method to assess the toxicity of DES against wide spectrum of both bacteria and
1128 fungi.

1129 c) Microtox assay as DES microbial toxicity testing method



1130 In four out of 96 studies in which the toxicity of DESs was evaluated, the commercially available
1131 Microtox kit was selected for this purpose (Table 4). Thanks to the use of uniform conditions in
1132 this kit for the toxicity assessment against the bioluminescent bacteria *Aliivibrio fischeri*, it is
1133 possible to determine and compare the EC₅₀ values for several different DESs differing in their
1134 composition and molar ratio of HBA and HBD used for their preparation (Table 4). Moreover,
1135 due to the use of one strictly defined *Aliivibrio fischeri* strain, it is possible to compare the results
1136 obtained by different researchers. Contrary to the two previously discussed methods, due to the
1137 fact that we use a commercially standardized test, the method does not need to be validated.
1138 However, since the test is based solely on testing toxicity towards *Aliivibrio fischeri*, the obtained
1139 results are limited to only one type of microorganism – gram-negative bacteria. As shown in the
1140 studies cited in this review, the mechanism of action and susceptibility of gram-negative and
1141 gram-positive bacteria may differ significantly from each other for the same DESs due to the
1142 different structure of the cell wall, and it is mostly depending on the chemical nature of HBA and
1143 HBD used for solvent preparation[39, 40, 60, 72, 79]. This also applies to the differences in the
1144 toxicity of DESs against bacteria and fungi resulting from chemical and structural differences in
1145 the structure of the cell walls of both groups of microorganisms. Hence, this method, despite
1146 many advantages resulting from the use of standardized commercial kit, should be a
1147 complementary method to another more universal technique, e.g., broth microdilution.

1148 d) Other methods as DES microbial toxicity assay

1149 In two analyzed and cited studies in this review, the toxicity of the examined DESs was assessed
1150 using a method based on the analysis of FTIR spectra variation of the cells upon exposition to the
1151 chemicals. In both studies, this method was used to assess DESs toxicity towards *S. cerevisiae*
1152 yeast (Table 6), however, as previously mentioned, this method can be used to evaluate the



1153 toxicity of DESs against different microbial cells[104]. This assay seems to be interesting
1154 because, compared to the previously discussed methods, it allowed to elucidate the mechanism
1155 on how DESs exert their toxic effect (yeast cells dehydration). Hence, FTIR-based bioassay is
1156 worth considering in all studies that aim at determining the possible toxicity mechanisms of
1157 selected DESs in relation to various groups of tested microorganisms.

1158 e) pH of DESs as an important factor in described microbial toxicity methods

1159 Since pH of some DESs is the important parameter that affect the applicability of basically each
1160 of the methods discussed above, it is important to consider this factor before testing DESs
1161 toxicity. Some studies about the toxicity of DESs suggest that the pH of growth media after
1162 preparation of DESs serial dilutions changes significantly[49, 51, 80]. As a result, the pH
1163 decreases below or increases above the optimal values for microbial growth (6.5–7.5[105] and
1164 5.0-9.0[106, 107] for growth of not acidophilic or basophilic bacterial and fungal
1165 microorganisms, respectively), consequently increasing the cells mortality in the tested samples.
1166 It is mostly observed when one of the DESs components are acids. For this reason, it is necessary
1167 to firstly analyze the pH of DESs solutions and if the values are far from those optimal for
1168 microorganisms growth (e.g. for the most often used microorganisms in DESs toxicity studies -
1169 *E. coli* - optimal pH growth range is between 6.5 and 7.5[49]), the DESs solutions should be
1170 prepared in the buffered media. For example, the dissimilarities in the obtained EC₅₀ values for
1171 ChCl-based DESs were noted in the work of Lapeña et al., where pH of the samples was
1172 controlled and adjusted to be in optimal range for the culturing of *A. fischeri*[96] and in the study
1173 of Macario et al. where pH was not controlled[94]. Consequently, lower EC₅₀ values were
1174 obtained in the study of Macario et al. which seems to be due to the pH effect on bacterial



1175 growth, leading to overestimated toxicity of ChCl-based DESs towards *A. fischeri*. In our
1176 opinion, these examples clearly show the need of buffering of DESs before testing their toxicity.

1177 Overall, for proper hazard and risk assessment of DESs, the toxicity data from diffusion method
1178 and broth dilution should be evaluated together for both DESs and their separate individual
1179 components. Since currently there are no standard protocols for testing toxicity of DESs, it makes
1180 difficult to draw conclusions across different studies due to discrepancies in experimental
1181 conditions and lack of test standardization. Nevertheless, we believe that following the
1182 suggestions and guidelines pointed out in subsequent section more precise and comparable data
1183 could be obtained.

1184 **5. Suggestions and guidelines for future research**

1185 The literature review and experience of the authors of this paper acquired during our recent
1186 toxicological studies against selected microorganisms and previous experience in using of some
1187 of above-described methods for testing of others antimicrobial agents, incline us to propose a few
1188 general rules for the future investigation of DESs toxicity. When applying well-established
1189 microbial toxicity testing methods (e.g., disk diffusion assay, broth dilution) for DESs, one
1190 should keep in mind that these methods may need methodological modifications to be applied to
1191 these compounds. We believe that by following the proposed suggestions and guidelines will
1192 enable to get accurate results and facilitate a comparison with the results of other researchers.
1193 Furthermore, with comparable results of investigations of various groups, it will be possible to
1194 further understand the mechanisms on which these solvents exert their toxic effect. The
1195 suggestions and guidelines for future research on toxicity of DESs are outlined below.



1196 i) The description of the methodology used to evaluate DESs toxicity should include all
1197 the details such as the detailed description of strain of microorganism used, detailed
1198 description of inoculum preparation (defined optical density of bacterial cells or CFU
1199 in inoculum), type and composition of growth medium, incubation conditions and
1200 endpoints determination, as well as details on the DES solutions preparation (initial
1201 molar ratio, dilutions) before analysis. The availability of this information will allow
1202 other researchers to better plan their own investigations and compare their results with
1203 different studies. For instance, for DESs toxicity assay using broth dilution method we
1204 encourage to use Mueller-Hinton broth culture media. Mueller-Hinton broth is
1205 recommended by FDA, NCCLS and WHO for testing MICs of for example,
1206 antibiotics against most encountered aerobic and facultative anaerobic bacteria in food
1207 and clinical material. This is excellent medium for cultivation *Escherichia coli*,
1208 *Staphylococcus aureus* and *Pseudomonas aeruginosa* strains previously used in DESs
1209 toxicity studies (Tables 1-3, 5).

1210 ii) Pure DESs should be characterized as much as possible, in particular their
1211 physicochemical properties, such as color/clearness, density, viscosity and pH (or pH
1212 of its solution in water). Disregarding these parameters may lead to the selection of
1213 the assessment method and model microorganism that will not be best suited and
1214 consequently will diminish the validity of the results and conclusions.

1215 a) Both viscosity and density were shown to have a large effect on the obtained
1216 toxicity results. For instance, the viscosity of DESs may have great impact on the
1217 results obtained using disk diffusion assay due to low diffusion of highly viscous
1218 compounds in agar medium.



- 1219 b) pH mostly influences the results obtained using broth dilution method, especially
1220 when pH of growth medium supplemented with DES is lower or higher than
1221 optimal for microbial growth. Due to pH changes caused by DESs, it is
1222 recommended to use buffered culture media instead of unbuffered cultures or to
1223 prepare DESs solutions in buffers. It will allow to diminish the negative impact of
1224 pH on the microbial growth, obtain more valid results and conclusions.
- 1225 c) Some DESs may not be transparent liquids and cause some turbidity of the
1226 samples [88], resulting in the increased absorbance readings and thus leading to
1227 lower accuracy of the obtained results in broth macro- or microdilution methods.
- 1228 d) Crossed reactions between DESs and the salts or nutrients of the culture media
1229 could also take place and influence both the pH and growth[80]. Moreover, such
1230 crossed reactions may be increased in the case of DESs hydrolysis that could
1231 occur in the presence of significant amount of water. Consequently, free HBA and
1232 HBD may react with the salts, amino acids, carbohydrates present in culture
1233 media, changing the pH and decreasing the nutrition sources.
- 1234 iii) Beside determination of DESs toxicity, it should be mandatory to also evaluate the
1235 toxicity of DES individual components (HBA and HBD) at the same concentrations as
1236 these used for DES preparation. It will allow to better understand the results obtained
1237 in toxicological studies of DESs and withdraw more proper conclusions.
- 1238 iv) As discussed throughout this paper there are various methods used to evaluate toxicity
1239 of DESs. Our literature study revealed that disk diffusion assay was the most
1240 commonly used method for this purpose (Table 1). The second most frequent used
1241 method was broth dilution method (Table 2). However, other microbiological methods
1242 dedicated for assaying antimicrobial activity of natural or synthetic chemical



1243 compounds were used much more rarely for assaying DESs toxicity against bacteria
1244 and fungi (Tables 3-6). In the light of presented data, although the disk diffusion
1245 method is the most commonly used method for assaying DESs toxicity against
1246 microorganisms, our recommendation is to use broth dilution technique instead of
1247 disk diffusion assay for this purpose. Broth dilution method offers more versatility
1248 and precision than mostly used disk test. It is undeniable that in most of the studies in
1249 which DESs toxicity was evaluated using sterile disks soaked with DESs and placed
1250 on agar plates, the obtained results were less accurate and may not reflect real
1251 interaction between DESs and cells. It is related with high density and viscosity of
1252 most of the DESs which leads to decreased DESs diffusion from the disk into agar
1253 medium. On the other hand, using broth dilution technique the negative impact of
1254 density and viscosity is minimized and quantitative results could be obtained.
1255 Nevertheless, it must be remembered that in high amounts of water DESs hydrolysis
1256 takes place, which may also have an impact on toxicity data obtained. Therefore,
1257 taking all of this into consideration, and if possible, it would be beneficial to firstly
1258 perform analysis using disk diffusion assay with pure DESs and then obtain more
1259 details with broth dilution technique. However, it is important to note, that disk
1260 diffusion method has one important advantage. With this method we can quickly and
1261 cheaply estimate the toxicity of a range of DESs differing in a) the HBA used, b) the
1262 HBD used, or c) the molar ratios of HBA and HBD used to obtain a given type of
1263 DES. Hence, in our opinion, for such DESs toxicity studies, the results of disk test
1264 provide valuable data which can support the analysis of DESs toxicity based on the
1265 results of broth dilution method or other alternative method.



1266 On the other hand, from other methods reported in the literature for DESs microbial
1267 toxicity studies, the methods based on i) analysis of FTIR spectra variation of the
1268 microorganism's cells upon exposition or not to the DESs; ii) the use of commercial
1269 kit that consists of two dyes, propidium iodide (PI) and SYTO9 for staining microbial
1270 cells exposed for DES seem to be interesting solution. They allow to compare DESs
1271 toxicity results obtained with these methods with results of DESs toxicity obtained
1272 with broth dilution method. In contrast to Microtox assay, both these methods give the
1273 possibility of selection of the same microorganism (bacteria or fungi) as used in broth
1274 dilution method. Moreover, the second of above-mentioned methods seem to be quite
1275 easy for validation, because of employing the commercially available kit.

1276 v) If possible, we advise to use the assays based on colorimetric dyes (e.g., cell
1277 incubation with resazurin) for cell viability and vitality determination, which not only
1278 provide more precise values than these obtained by simple visual inspection or
1279 spectrophotometric measurements of turbidity (especially during MIC evaluation by
1280 broth microdilution method), but also higher quality data. Using this method there is
1281 no need of confirmation of the results by subculturing of each concentration onto agar
1282 for 24 h (MBC evaluation). Furthermore, the influence of DESs turbidity on the
1283 absorbance of the samples is reduced for these methods.

1284 vi) The use of preadapted cells of microorganisms selected for study of DESs toxicity is
1285 encouraged. Until now there is one work where the preadaptation of cells to the DESs
1286 was performed[80]. It was demonstrated that non-preadapted cells did not grow in the
1287 presence of 600 mM acetylcholine chloride:acetamide DES, however, when they were
1288 pre-adapted to this concentration, cellular growth was observed[80]. By including the
1289 cellular pre-adaptation in future studies, it will be possible to gain insights on the



1290 capability of the cells to tolerate or assimilate DESs and to obtain more accurate data
1291 on the antimicrobial properties of DESs.

1292 vii) In case of studies where DESs are applied in the processes (such as extraction,
1293 chemical reaction etc.), the toxicity should be controlled for primary DES as well as
1294 for DES recovered after the process. In many cases, elevated temperatures as well as
1295 other factors, such as ultrasounds or microwaves used during the process, can cause
1296 DES chemical instability. As a result, harmful byproducts can be formed and strongly
1297 affect the eco-friendly character of primary DES. Recycled DES can introduce these
1298 byproducts to extracted fraction or product of reaction. On the other hand,
1299 accumulation of toxic byproducts will strongly affect methods available for its safe
1300 disposal after usage.

1301 **6. Conclusions and outlook**

1302 Deep eutectic solvents (DESs) are one of the most interesting classes of alternative solvents,
1303 mainly because of their simple preparation, usually low cost, and versatility due to possibility of
1304 their task- specific design to meet the needs of a specific process. Furthermore, they can be
1305 prepared using all- natural substances which opened exciting new perspectives to design truly
1306 green compounds that will meet with the requirements of green and sustainable chemistry. All
1307 these characteristics confer DESs as an ideal alternative to both organic solvents and ILs. Since
1308 their discovery DESs have been used in a myriad of applications as solvents, reaction media,
1309 catalysts, additives, lubricants, or materials for a wide range of fields from pharmaceutical to
1310 energy. Nevertheless, new studies are constantly conducted in order to learn as much as possible
1311 about the properties of DESs and further increase their applications in new fields important for
1312 the quality of life such as cosmetic, food, drug production and medicine. However, before the



1313 implementation of DESs in these areas will be possible, it is essential to study their toxicity and
1314 gain knowledge on their possible modes of interaction with living beings. Even though, DESs are
1315 considered as green, benign, and non-toxic compounds, a literature review conducted in this
1316 paper indicated that this statement is not entirely true and such generalization should be avoided.
1317 In fact, several examples proved that often out-of-purpose methodology was used, resulting in
1318 false conclusions. Secondly, more than 5200 studies were published about DESs after their
1319 discovery and only around 96 evaluate and discuss the toxicity of these compounds (mainly
1320 against selected microorganisms). It highlights the need for more studies in this topic, which will
1321 allow to gain sufficient insights on DESs toxicity towards different organisms at various trophic
1322 levels and on how they exert their toxic effect.

1323 Throughout this review, we show the advantages and disadvantages of methods used for DESs
1324 toxicity determination. Our analysis indicated that it is necessary to have an improved, standard
1325 protocol for determination of DESs toxicity. In this way, it will be possible to create a database,
1326 compare the results obtained in different studies and for various solvents. In our opinion, in order
1327 to obtain valuable results, it would be beneficial to use both disk diffusion assay and broth
1328 dilution technique in future studies on toxicity of DESs. We believe that the negative impact of
1329 pH may be overcome by using extremophilic microorganisms instead of standard microbial
1330 strains. Hence, it is essential to improve, for example, the broth dilution technique by always
1331 using buffered medium or by preparing DESs solutions in buffer. Furthermore, another aspect
1332 that should be considered while using standard microorganisms is cellular preadaptation with
1333 DESs which was shown to be a viable approach allowing to gain insights on the capability of the
1334 cells to tolerate or assimilate DESs and to obtain more accurate data on the antimicrobial



1335 properties of DESs for which growth for some concentrations was not observed for non-adapted
1336 cells.

1337 It is expected that, in a future, by using the standardized and validated above-mentioned methods,
1338 the theoretical and experimental knowledge about toxicity of DESs will evolve rapidly. It will
1339 allow to further explore these solvents in different applications such as biomedical and
1340 pharmaceutical. Furthermore, it will be possible to address once for all the DESs biosafety issue
1341 and answer with conviction if deep eutectic solvents are benign or toxic.

1342 **Conflicts of interest**

1343 There are no conflicts to declare.

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