Ecotoxicological study of bio-based Deep Eutectic Solvents formed by glycerol derivatives in two aquatic biomodels

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Abstract: The growing environmental impact of non-renewable solvents has generated an increasing interest in the development of more sustainable alternatives. Among these options, Deep Eutectic Solvents (DES) are attracting great interest. The favourable physicochemical properties of these solvents make them a potential green alternative for several applications. However, its toxicological impact has not been studied enough to assume the absence of environmental risk. With the main purpose of establishing an initial overview of the aquatic toxicity, an acute ecotoxicity test of different eutectic solvents, composed of glycerol or glycerol-derived ethers and choline chloride (ChCl) or *N*,*N*,*N*-triethyl-*N*-(2,3-dihydroxypropyl)ammonium chloride (N00Cl), has been carried out in two aquatic biomodels: *Aliivibrio fischeri* (bacteria) and *Raphidocelis subcapitata* (algae). Furthermore, the content of chlorophyll was measured to observe the disruption of the photosynthetic process by the tested compounds. A dose-effect correlation has been observed, although very high concentrations of the solvents were necessary for the onset of the toxic effect. The toxicity of the DES, within the ChCl case, turned out to be greatly related to the polarizability and hydrophobicity of the solvents. Whereas N00Cl-based DES have shown an even-odd trend, compounds with even carbon numbers in the ether radical show lower toxicity than odd ones. These preliminary results point out a favourable eco-toxicological behaviour of glycerol derived DES, although studies in other bioindicators, as well as in cells and biodegradability tests are recommended in order to have a complete overview of the toxicological profiles of these promising solvents.

Keywords: deep eutectic solvents (DES), *Aliivibrio fischeri*, *Raphidocelis subcapitata*, toxic effect, dose-response relationship, green solvents, glycerol

Abbreviations:

DES: Deep Eutectic solvents

ChCl: Choline Chloride

Chl: Chlorophyll

N00Cl: *N*,*N*,*N*-triethyl-*N*-(2,3-dihydroxypropyl)ammonium chloride

A.fischeri: Aliivibrio fischeri

R. subcapitata: Raphidocelis subcapitata

HBD/HBA: Hydrogen bond donor/ Hydrogen bond acceptor

INTRODUCTION

The environmental problem caused by the use of traditional solvents is one of the main concerns of the scientific community[1]. During the last decades, growing environmental preoccupations have led to new regulations in order to mitigate the impact of solvents on the environment [2]. The increase in consumption, close to 20 million metric tons per year [3], and the non-renewable origin of these solvents (most from fossil sources), have forced industries to reduce, eliminate or replace the organic solvents used during the manufacturing processes [4].

In recent years, many alternatives to traditional solvents have been proposed. These so-called neoteric solvents are increasingly being used in industrial processes as e.g. in the pharmaceutical industry [5]. Some of these new solvents are biomass derivatives[6], supercritical fluids[7], ionic liquids (ILs)[8] and Deep Eutectic Solvents (DES)[9]. According to the principles of Green Chemistry[10], solvents should present low vapour pressures and high boiling points, good recyclability, high solvating power, be environmentally and humanly safe and have renewable origin[1].

Among the different renewable solvents, deep eutectic solvents are attracting increasing interest. In general, DES are mixtures formed by a hydrogen bond donor (HBD) and a hydrogen bond acceptor (HBA) that present lower melting points than their components individually. This phenomenon is due to the charge delocalization between the salt anion and the HBD component through hydrogen bonding[11]. DES are currently considered a good green alternative to ionic liquids due to their easy preparation, favourable cost of their starting materials, lower energy consumption, lower waste generation, higher biodegradability, low vapour pressure, non-flammability and lower toxicity profile, in addition to interesting catalytic and solvating properties[12–14]. These properties have prompted their use in catalysis[15], biocatalysis[16], organic synthesis[17] and extraction processes[18]. In addition, eutectic solvents have shown interesting advantages reducing carbon dioxide emissions[19], improving the efficiency of biomass and drug dissolution[20–22], as well as in their use for clinical therapy[23]. All of this makes DES promising green solvents for industrial use.

Among the multiple applications of DES (Figure 1), their solubilising power and catalytic properties stand out.

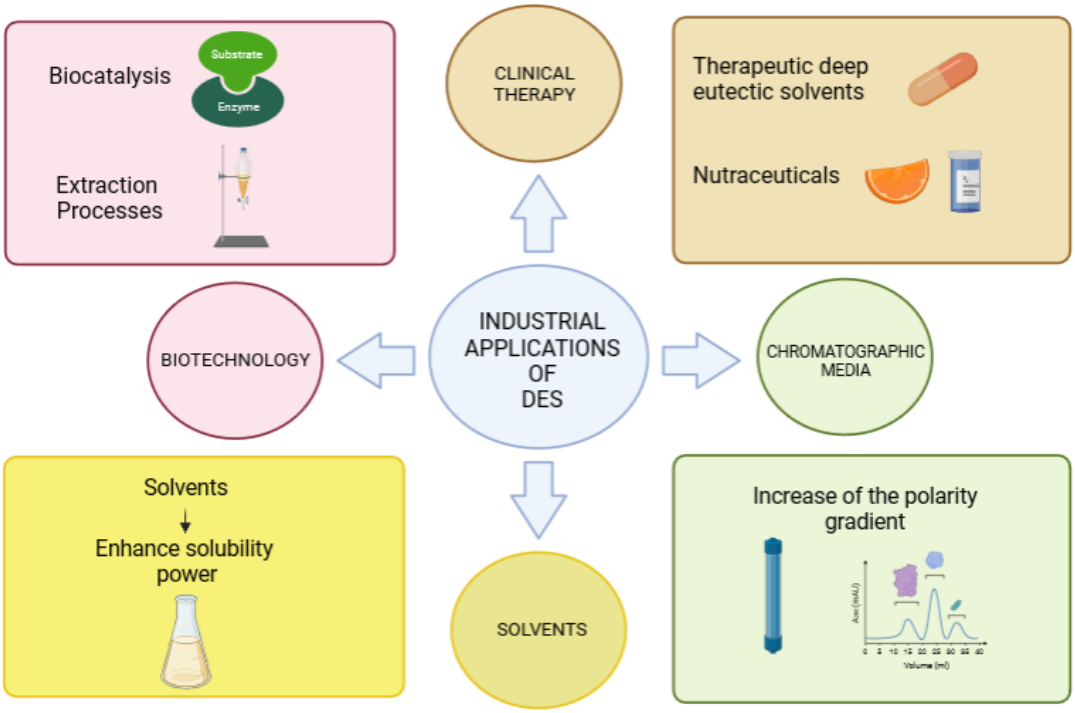

Figure 1. Industrial Application of DES

As DES arise as a more sustainable alternative to ionic liquids, their toxicity is being widely studied [24–27]. Ecotoxicity tests inform about the effect of a substance in the environment, determining whether a compound is in enough concentration to be or not harmful. Acute toxicity studies can be designed for a quick, easy and reproducible evaluation of the toxicological effect. To understand the toxic behaviour of a substance in a specific environment, studies in representative organisms along the trophic chain (bacteria, algae, crustaceans or fishes) are recommended [28,29]. This information allows to evaluate the bioaccumulation between species and aids to determine the aquatic impact of the studied substance.

Recently, the preparation and physicochemical properties of new glycerol-derived DES have been described[30]. These bio-based DES have shown interesting solvent properties for nanoparticle synthesis and catalysis[12,31] and are showing very promising solubilizing properties of hydroxycinnamic acids (unpublished results). In order to complete the study of these promising solvents, the ecotoxicity of 12 bio-based glycerol-derived DES (Figure 2) has been evaluated against the aquatic biomodel *Aliivibrio fischeri* (*A. fischeri)* a marine bacterium whose metabolism causes the emission of luminescence [32] and against *Raphidocelis subcapitata (R.subcapitata),* an algae specie present in the aquatic environment. Additionally, the structure-toxicity relationship has

also been established, as well as a discussion in terms of the structure of the DES components.

EXPERIMENTAL

Chemicals and synthesis of DES

The chemical structures and main physicochemical properties of the studied DES are respectively shown in Table 1 and Figure 2.

Table 1. Some physicochemical properties of the studied DES

DES code	T_c (°C)	Density (g/mL) [a]	Viscosity (cP) [a]	Polarizability (Å^3) [b]	HBD LogP [c]
ChCl-Glycerol	<0	1.191	368	10.50	–1.4080
ChCl-100	33	1.122	132	11.57	–0.9996
ChCl-200	52	1.085	148	12.84	–0.6508
ChCl-3F00	20	1.285	159	12.89	0.0078
ChCl-300	67	1.065	150	14.15	–0.1271
ChCl-3i00	60	1.060	162	13.86	–0.2733
ChCl-400	74	1.045	152	15.46	0.3290
N00Cl-Glycerol	<0	1.183	2693	12.89	–1.4080
N00Cl-100	<0	1.125	453	14.08	–0.9996
N00Cl-200	<0	1.095	450	15.32	–0.6508
N00Cl-3F00	<0	1.263	553	15.44	0.0078
N00Cl-300	30	1.072	447	16.56	–0.1271
N00Cl-3i00	40	1.069	552	16.54	–0.2733
N00Cl-400	40	1.054	443	17.77	0.3290

[a] Determined at 25 ºC. [b] Calculated according to Marcus [65]. [c] Calculated using the T.E.S.T. EPA version 4.2.1 software.

For DES preparation, the HBA (**ChCl** or **N00Cl)** and the HBD (**100**, **200**, **3F00**, **300**, **3i00** and **400** glycerol ethers) have been mixed in a 1:2 molar ratio and stirred in a closed glass vial at 70 ºC. As a transparent liquid has been formed, the eutectic mixture has been cooled down to room temperature and kept under argon.

All the glycerol derivatives, including the glycerol monoethers and the N00Cl salt, have been synthesized according to our previously described methodologies [30,66]. All the chemicals have been dried under vacuum for 24 h prior to use.

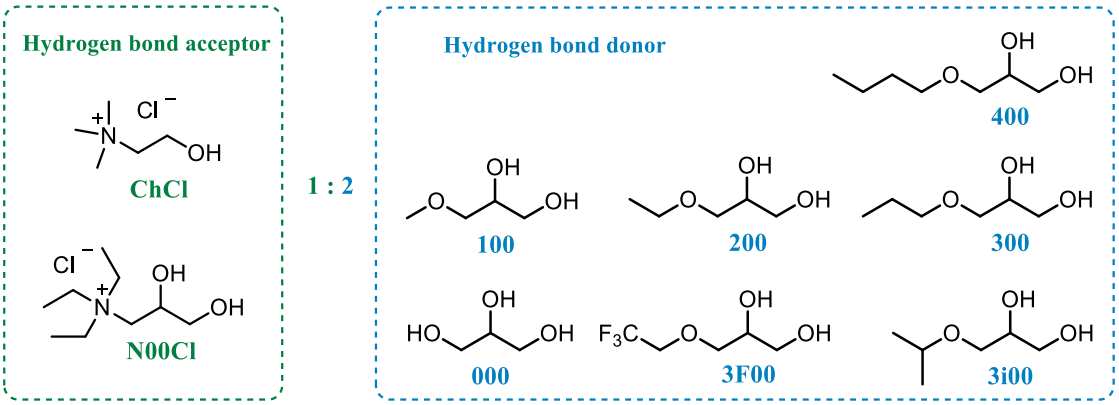

Figure 2. Chemical structure of HBA and HBD components of the studied solvents.

Luminescence inhibition assay on *A. fischeri* bioindicator

The employed biomodel *A.fischeri* is a Gram-negative, flagellated bacteria, present in the marine environment and widely used in ecotoxicological essays due to his easy reproducibility and high sensitivity to toxic compounds. The measurable endpoint in the biomodel *A. fischeri* is the bioluminescence emission caused by the enzymatic mechanism of luciferase. The two substrates involved in the reaction are flavin mononucleotides in their reduced form ($FMNH_2$), called luciferin and long-chain aldehydes. Through the enzymatic action of luciferase and the presence of oxygen, the reduced form of the flavin mononucleotide is converted to its oxidized form (FMN) and the aldehyde turns into a long-chain acid. The oxidation reaction produced by this enzyme releases light at a wavelength of 490 nm[33]. This process is related to electron transport chain and therefore to respiration and gives an idea about the metabolic status as a chemical toxicity. The toxic compounds inhibit the bacterial metabolism, this is reflected in a decrease of light emission[32–34].

The employed methodology follows all the conditions and protocols established on the standardized tests for the determination of ecotoxicity in *A. fischeri* (UNE-EN ISO 2009) [67]. The experiments have been carried out in triplicate for each tested solvent to ensure the reproducibility of the test.

To establish the toxicity range of concentration for tested substances, a pre-essay was carried out. In the case of ChCl mixtures: [300000-1000 mg/L] for **ChCl-000**, [300000-500 mg/L] for **ChCl-100**, [150000-3500 mg/L] for **ChCl-200**, [40000-5 mg/L] for **ChCl-300**, [90000-500 mg/L] for **ChCl-3F00**, [50000-500 mg/L] **ChCl-3i00** and [2500-10 mg/L] for **ChCl-400**. In the case of N00Cl DES series the range of concentrations of each mixture were: [400000-5000 mg/L] for **N00Cl-000**, [400000-500

mg/L] for **N00Cl-100**, [300000-1000 mg/L] for **N00Cl-200**, [90000-500 mg/L] for **N00Cl-300**, [100000-5000 mg/L] for **N00Cl-3F00**, [80000-5000 mg/L] for **N00Cl-3i00** and [20000-500 mg/L] for **N00Cl-400**.

Lyophilized vials of *A. fischeri* used in this test have been purchased from the supplier Macherey-Nagel (ref. 945 006). First, bacteria have been rehydrated and stored in the refrigerator at 2-8 °C for 5 min using the reactivation solution provided by the manufacturer.

Serial dilutions of each tested solvent have been prepared using a 2% NaCl solution as culture medium. Solution pH has been adjusted to 7-7.5 using 0.1 M HCl and 0.1 M NaOH solutions. For the correct development of the essay, a negative (culture medium) and positive (phenol 42.5 mg/L) control have been used[68]. Aliquots of 500 µL of reactivated bacterial suspension have been transferred to cuvettes and cooled in a bath at 15 ºC for 10 min. Then, an initial luminescence measurement has been carried out using a BioFix® Lumi-10 luminometer (Macherey-Nagel) equipped with an ultrafast photonic detector covering a wavelength range of 380-630 nm. After the first measurements, 500 µL of the solution to be tested have been added. Throughout the essay, the bacteria have been exposed to different solvent concentrations for 30 min at 15°C. Then, the second luminescence measurement has been performed. Obtained values reflect the difference between emitted luminescence without exposure to DES after 30 minutes of exposure. The toxic effect is detected due to a decrease in bacterial light production.

Algal culture

R. subcapitata is a freshwater alga with, usually, a 15-50 μm^2 of surface area. When they are healthy, they present a sickle shape that they usually can change when they suffer damage or physiological changes [69,70].

Algae were provided by ECOTEST, Valencia, (SC2B1214). The culture medium pH was adjusted at 8.1 ± 0.2 and prepared according to supplier specifications. The algae cells were stored at 23 °C in a 100 mL beaker in the incubator with an illumination of 10000 lux. The starting algal concentration for each of the tested solutions was $3 \cdot 10^5$ cells/mL.

Algal growth inhibition test

The employed methodology for the algal growth inhibition test was carried out according to the OECD 201 test condition and following the standardised methodology and protocol [71]. To ensure the repeatability, the test was conducted in triplicate.

Before starting the test, it was necessary to carry out a pre-essay in order to determine the concentration range for each of the tested DES. In the case of the ChCl mixtures the range-concentrations were: [200000-100 mg/L] for **ChCl-000**, [100000-3125 mg/L] for **ChCl-100**, [80000-500 mg/L] for **ChCl-200**, [50000-1000 mg/L] for **ChCl-300**, [100000-1000 mg/L] for **ChCl-3F00**, [50000-500 mg/L] **ChCl-3i00** and [30000-500 mg/L] for **ChCl-400**. In the case of N00Cl DES series the range of concentrations for each mixture were: [200000-10 mg/L] for **N00Cl-000**, [100000-500 mg/L] for **N00Cl-100** as well as for **N00Cl-200**, [90000-500 mg/L] for **N00Cl-300**, [100000-500 mg/L] for **N00Cl-3F00** and [50000-500 mg/L] for **N00Cl-3i00** as well as for **N00Cl-400**.

Dilutions of the tested mixtures were prepared in a culture medium with an adjusted pH range between 7.9 and 8.3 using a 0.1M NaOH or 0.1M HCl solutions, and a 0 mg/L solution was used as a negative control. The initial OD was measured at 670 nm with a BioTek (Synergy H1) absorbance-luminescence-fluorescence microplate reader. Then, the well plate was incubated in a CIR-DBO/180 incubator at 23 °C for 72 h. Before the final measurement of the OD, all plates were resuspended to ensure the homogeneity of the optical density measurement and to prevent the algae from settling. Obtained values show the inhibition of the algal growth after 72 h of DES exposure.

Determination of the chlorophyll a, chlorophyll b and total chlorophyll

This experiment was carried out according to the Lichtenthaler protocol[71]. After 72 h of the algae's exposition to NADES, 5 mL of each algal dilution were centrifuged at 1000 g for 15 min. The obtained pellet was dissolved in 5 mL of methanol and vigorously vortexed. The samples were refrigerated at 4°C in the dark and then centrifuged for 5 min at 10.000 g. After 24h the supernatant was analysed spectrophotometrically at 750, 665.2 and 652 nm using methanol as a blank. Then, the following equations were used to calculate the concentration (mg/L) of chlorophyll a (Chl a), chlorophyll b (Chl b) and total chlorophyll (total Chl):

$$Chl_a = 16.72\ (A_{665,2} - A_{750}) - 9.16\ (A_{652,4} - A_{750}) \quad \text{(eq. 1)}$$

$$Chl_b = 34.09\ (A_{652,4} - A_{750}) - 15.78(A_{665,2} - A_{750}) \quad \text{(eq. 2)}$$

$$Chl_{total} = 1.44\left(A_{665,2} - A_{750}\right) + 24.93\ (A_{652,4} - A_{750}) \quad \text{(eq. 3)}$$

Ecotoxicity mathematical treatment and statistics

For the statistical analysis, data from the logarithm of concentration against the percentage of luminescence for *A.fischeri* and the growth inhibition for *R.subcapitata* have been represented by means of a non-linear regression using GraphPad Prism version 9.0 program. Results have been adjusted by applying the least squares method to the following formula:

$$\%I = 100/\ (1 + 10^{(\log EC_{50} - \log C)a}) \quad \text{(eq. 4.)}$$

where %I is the inhibition percentage of luminescence in *A. fischeri* biomodel and the percentage of growth inhibition in *R. subcapitata* biomodel, *C* is the concentration expressed in mg/L, while log EC_{50} and *a* are adjustable parameters obtained after the correlation of experimental values.

A comparison between each one of the solvents was performed using the statistical ANOVA test with a single pooled variance. The null hypothesis was that the ratio obtained by dividing the EC_{50} values was 1; if it significantly differed ($p < 0.05$) from 1, the null hypothesis was rejected.

RESULTS AND DISCUSSION

The use of glycerol and its derivatives for DES preparation guarantees the renewable origin of the solvents. The fine tuning of the physical-chemical properties of these DES can be achieved by varying the nature of glycerol ethers substituents or the ammonium salt [30]. The variation in the structure of the DES components can also provide different ecotoxicity profiles, this fact motivating the present study. Two groups of bio-based DES were prepared using two different HBAs, choline chloride (ChCl) and *N*,*N*,*N*-triethyl-*N*-(2,3-dihydroxypropyl)ammonium chloride (N00Cl), in combination with glycerol (**000**) and glycerol-derived monoethers (**R00**) with R = methyl (**100**), ethyl (**200**), 2,2,2-trifluoroethyl (**3F00**), propyl (**300**), isopropyl (**3i00**), and butyl (**400**).

A.fischeri ecotoxicity test

The EC_{50} and standard deviation values obtained from Eq.4 for the studied substances in the bacteria and the toxicity of pure glycerol monoethers[35] are shown in

Table 2. Additionally, the results obtained in the statistical study previously described are gathered in Table S1 in the supplementary information.

Table 2. EC_{50} and standard deviation for studied DES and their HBD precursors in *A. fischeri*.

ChCl-DES	EC_{50} (mg/L)	N00Cl-DES	EC_{50} (mg/L)	HBD	EC_{50} (mg/L)
ChCl-000	141380 ± 6430	**N00Cl-000**	83277 ± 4282	**Glycerol**	108421 [35]
ChCl-100	81817 ± 15458	**N00Cl-100**	93192 ± 4487	**100**	21052 [35]
ChCl-200	30292 ± 825	**N00Cl-200**	8089 ± 128	**200**	4240 [35]
ChCl-3F00	19181 ± 654	**N00Cl-3F00**	34957 ± 4525	**3F00**	16669
ChCl-300	6249 ± 317	**N00Cl-300**	16976 ± 2766	**300**	11939
ChCl-3i00	8648 ± 416	**N00Cl-3i00**	24754 ± 1205	**3i00**	11614
ChCl-400	550 ± 9	**N00Cl-400**	3446 ± 1132	**400**	941 [35]

For both groups of mixtures, the increase in the concentration causes a greater toxic effect (Figure 3). First, in the case of ChCl mixtures, the following increasing toxicity trend was observed: **ChCl-000** < **ChCl-100** < **ChCl-200** < **ChCl-3F00** < **ChCl-3i00** < **ChCl-300** < **ChCl-400**. These results show an increase in DES toxicity by lengthening the alkyl chain of the HBD in **ChCl-100**, **ChCl-200**, **ChCl-300** and **ChCl-400** (Figure 3). It has been reported that ionic liquids with a longer alkyl chain are able to cross the cell membrane more easily, presenting greater toxicity[36,37]. The same trend has been observed in previous works for quaternary ammonium-based DES, as the DES toxicity increased with the length of the alkyl chains [26]. In addition, other ecotoxicity tests carried out on the same biomodel but with glycerol derivatives[35] and levulinate derivatives [38] also showed the same trend.

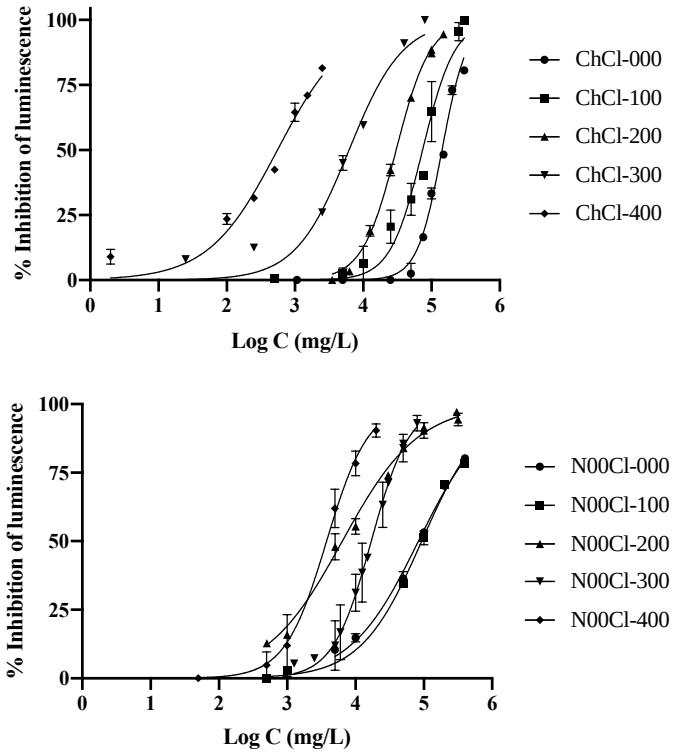

Figure 3. Dose–response curves for ChCl (**ChCl-000**, **ChCl-100**, **ChCl-200**, **ChCl-300** and **ChCl-400)** and N00Cl (**N00Cl-000**, **N00Cl-100**, **N00Cl-200**, **N00Cl-300** and **N00Cl-400**) solvents in *A. fischeri.*

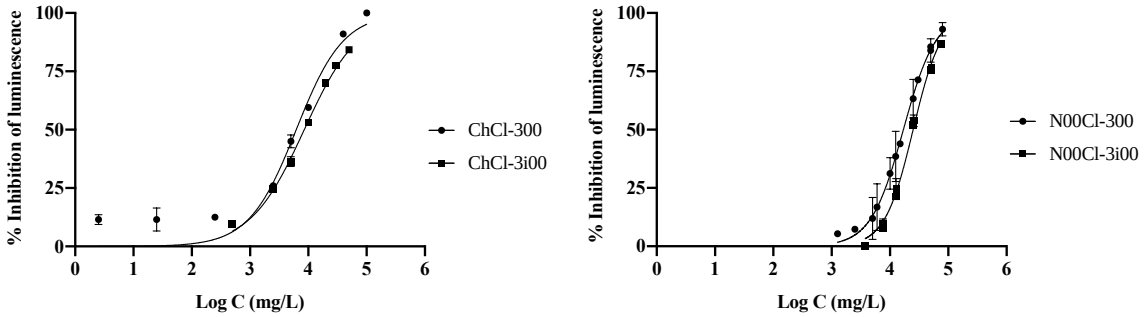

Figure 4. Dose–response curves for the DES composed of **3i00** and **300** glycerol ethers in *A. fischeri.*

Moreover, a very high correlation between LogP (hydrophobicity) values of **R00** (HBD) component of the studied ChCl-DES and the DES logEC_{50} values was observed (Figure 5), thus demonstrating the great influence of the nature of the HBD component in the ecotoxicity of these mixtures in this case.

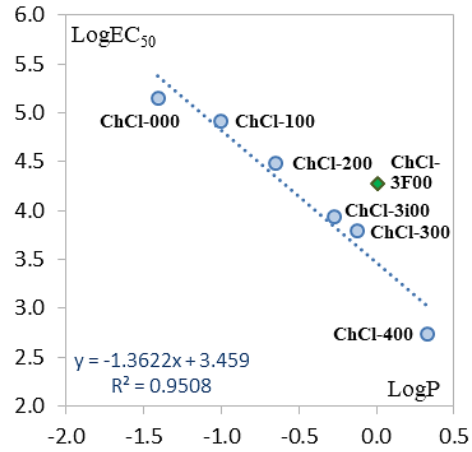

Figure 5. Plot of the ecotoxicity in *A. fischeri* of the studied ChCl-based DES vs. LogP of the HBD component.

The comparison between the toxicity of pure glycerol monoethers and their derived DES both with ChCl and N00Cl in the *A. fischeri* biomodel showed that, in general, the studied DES present higher EC_{50} values than their corresponding glycerol monoethers[35]. This is a consequence of the hydrogen bonding interactions between glycerol ether with the ammonium salt upon DES formation, which strongly seems to contribute to the toxicity reduction of DES with regard to the pure HBD components.

In addition, in less stable ChCl-DES, that is **ChCl-300**, **ChCl-3i00** and **ChCl-400,** [30] a synergetic effect is observed when evaluating EC_{50} values. In these cases, the ecotoxicity of the mixtures increased compared to the pure components values(Table 2), thus suggesting that the stability of the DES influences ecotoxicity and proving that more stable DES are less ecotoxic.

This conclusion is reinforced by the fact that EC_{50} values of N00Cl-DES are higher than their ChCl counterparts. Although ChCl (EC_{50}: 202897 ± 12519 mg/L) has a slightly better ecotoxicological profile than N00Cl (EC_{50}: 183691 ± 23281 mg/L), the **N00Cl-R00** mixtures presented higher EC_{50} values (Figure 3), in agreement to the higher stability and hydrogen-bond capacity of N00Cl-derived DES[30]**.**

However, for N00Cl-composed solvents, no correlation of EC_{50} values neither with the LogP nor with the polarizability or hydrogen bond formation ability (α parameter) (see table xx and figures SX and SXX) of the DES is observed. Although the general trend of the increase of eco-toxicity with the increase of the alkyl chain of HBD component is observed, in this case, an even-odd effect on toxicity appears (Figures 3-4). Thus, in mixtures of HBD component with an even carbon number in the substituent, a lower EC_{50} is observed than in the odd ones, and therefore a higher toxicity (Table 2). As

this kind of solvents has not been studied before, there are no trials to support this structure-relationship or to explain the mechanism of toxicity affecting the bacteria.

In both groups of DES, regardless HBA (N00Cl or ChCl), mixtures containing **3i00** glycerol ether seem to be less toxic than in the ones containing **300** ether (Table 2 and Figure 4). Thus, the presence of ramifications in the HBD alkyl chain seems to produce an increase in the EC_{50} value and therefore a decrease in DES toxicity. This phenomenon seems to be related to the difficulty of the substance to cross the cellular barrier, as the molecular size of the radicals increases[35].

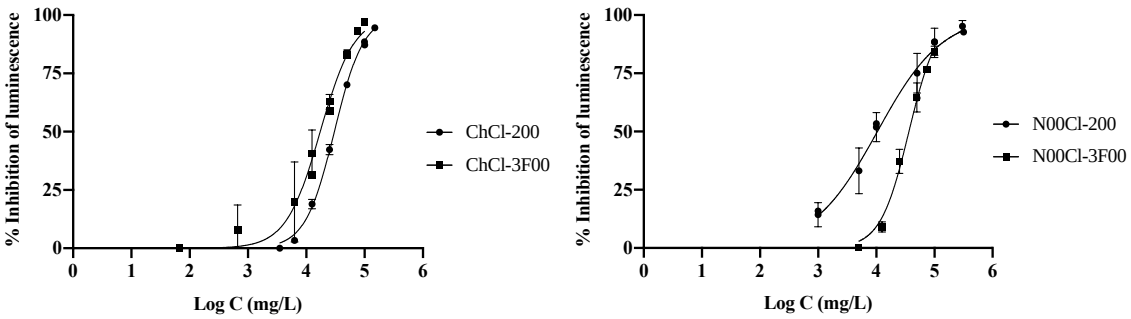

Figure 6. Dose–response curves for the DES composed of **200** and **3F00** glycerol ethers for *A. fischeri.*

The effect of the presence of fluorine atoms on HBD component of DES on EC50 values has also been evaluated and an opposite trend is observed with both HBA. Thus, comparing **ChCl-200** and **ChCl-3F00** solvents (Figure 6), the presence of fluorine atoms in the HBD component seems to increase toxicity. However, the opposite trend is observed both in the N00Cl-derived DES (Figure 6) and in pure glycerol ethers. In these cases, **3F00 and N00Cl-3F00** show a lower eco-toxicity than **200 and N00Cl-200**, respectively (Figure 6, Table 2).

The *vibrio* cell wall is composed of peptidoglycan molecules, responsible for the rigidity of the structure. In addition, some studies support that **ChCl** interacts with these polysaccharides through hydrogen bonds, causing cell disruption[39]. The full mechanism of action is still unknown. It is also known that compounds that exhibit charge delocalisation are more toxic[39]. Thus, it is suspected that eutectic mixtures presenting more charge delocalization in their structure will interact more with the membrane, causing its disruption. In the case of the **ChCl** mixtures studied, this behavior is directly

related to the high correlation found between electronic polarizability and EC_{50} values (Figure 7).

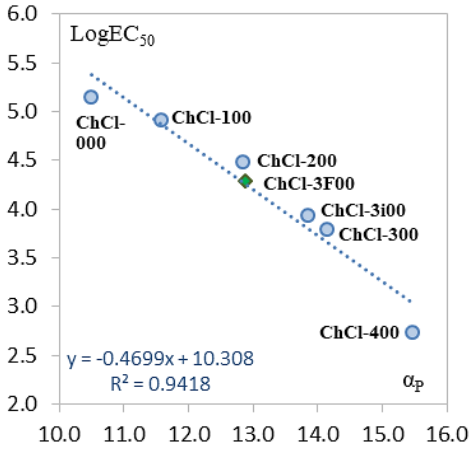

Figure 7. Plot of the ecotoxicity of the ChCl-DES vs. their electronic polarizabilities (in $Å^3$).

On the other hand, more stable DES formed with N00Cl show lower toxicity. The higher stability of N00Cl versus ChCl derived DES has been previously described (referencia 30) and it is closely related to the formation of hydrogen bonds between the components of the eutectic mixtures. This fact seems to prevent the formation of these bonds with elements of the plasma membrane [26]. This seems also to be the reason why N00Cl-R00 show a different trend in toxicity. Thus, in the case of N00Cl-DES and glycerol derived ethers the even-odd tendency seems to be the driving force and no correlation of EC_{50} neither with polarity nor with polarizability or the hydrogen bond formation ability (α parameter) is observed as above mentioned. (see figures Sxx and SXXX)

As it has been described above, when comparing the DES formed with the same hydrogen bond donor and different ammonium salt (ChCl vs N00Cl), the toxicity of the mixtures changes with the variation of the hydrogen bond acceptor. Thus, both the modification of the HBA and the HBD directly affects the ecotoxicity of resulting DES. This variation in the toxicity of DES after the change in the HBD has been previously reported in other ecotoxicological studies[39–42].

It is also interesting to analyse the ecotoxicity of the studied DES by comparing with their components separately, and specially to the corresponding glycerol-derived ethers, also used as green solvents [43]. It has been described that the toxicity of the starting materials of DES varies comparing with their derived eutectic mixture. Mácario et al.

carried out a predictive test for ChCl mixtures in which DES showed less toxicity than their starting materials individually [44]. This supports the results obtained with the studied glycerol-derived DES in this work, which in general are less toxic than their components individually, except in some cases in which a synergic effect is observed (**ChCl-300**, **ChCl-3i00** and **ChCl-400**). Nevertheless, the study of the synergic and antagonistic effect of DES has been performed coming to the conclusion that predictive models cannot be used to determine the behaviour of DES toxicity[44].

R. subcapitata ecotoxicity test and chlorophyll concentration measurements

R. subcapitata is a very common specie employed for the evaluation of the aquatic toxicity. This alga, as a primary producer, helps the maintenance of the structure of aquatic ecosystems, taking part in the trophic chain. The Organization for Economic Cooperation and Development (OECD) recommends the use of this alga as a biomodel because of its wide distribution, fast growth, and great sensitivity[45].

The EC_{50} and standard deviation values obtained from Eq.4 for the studied mixtures in the algal biomodel are shown in Table 4. In addition, the results obtained in the statistical study previously described are gathered in Table S2 in the supplementary information.

Table 4. EC_{50} and standard deviation values for ChCl and N00Cl DES in *R. subcapitata*.

ChCl-DES	EC_{50} (mg/L)	N00Cl-DES	EC_{50} (mg/L)
ChCl-000	20854 ± 558	**N00Cl-000**	7015 ± 170
ChCl-100	15516 ± 362	**N00Cl-100**	11423 ± 924
ChCl-200	12331 ± 155	**N00Cl-200**	7343 ± 567
ChCl-3F00	13289 ± 942	**N00Cl-3F00**	12560 ± 196
ChCl-300	10521 ± 634	**N00Cl-300**	8087 ± 523
ChCl-3i00	14039 ± 161	**N00Cl-3i00**	9597 ± 1205
ChCl-400	4758 ± 141	**N00Cl-400**	5828 ± 666

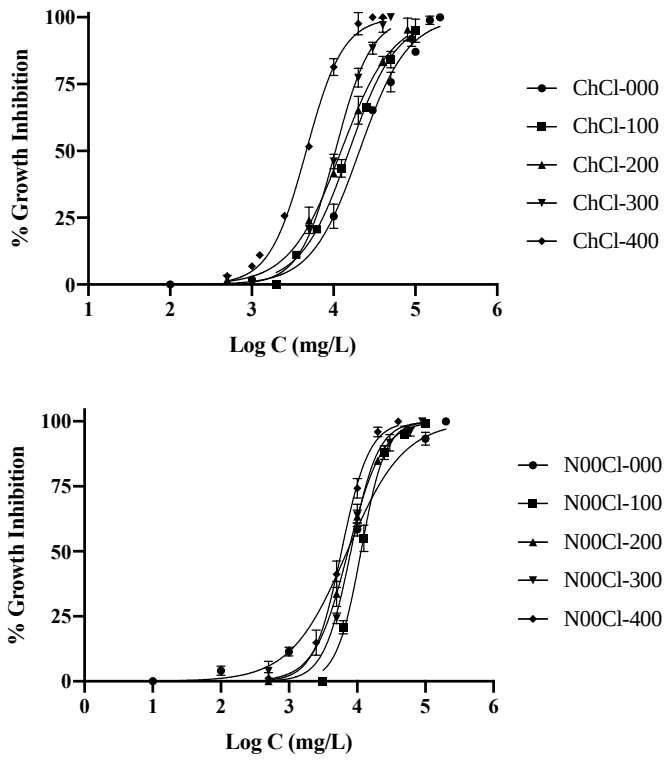

Figure 8. Dose–response curves for ChCl (**ChCl-000**, **ChCl-100**, **ChCl-200**, **ChCl-300** and **ChCl-400)** and N00Cl derivatives (**N00Cl-000**, **N00Cl-100**, **N00Cl-200**, **N00Cl-300** and **N00Cl-400**) solvents in *R. subcapitata.*

In *R. subcapitata,* an increase in toxicity was observed related to the increase in concentration, so a correlation between DES concentration and the toxic effect could be established. As mentioned above, the structure-toxicity pattern of the compounds is very similar for both biomodels. In this case, we observed the same trends as for *A. fisheri.* For **ChCl** (mixtures with **000** to **400**) we observed an increase in toxicity related to the increase of the alkyl chain length. Previous studies have already revealed the correlation between aquatic toxicity and the molecular structure of the compounds[46]. Thus, it was shown that the increase in the alkyl chain in hydroxyl radicals decreased the length of the C–H bonds, thus modifying physicochemical properties such as lipophilicity and toxicity, which increased in these cases.

Perales et al.[47] established the same hypothesis in their toxicological study of glycerol derivatives. Although their results were performed on *Chlamydomonas reinhardtii* instead of *R.subcapitata,* the trend was the same: lipophilicity related to the number of carbons on the ether substituent favoured toxicity in algal biomodel. Furthermore, the correlation alkyl chain length-toxicity has been already established for ammonium and phosphonium ionic liquids[48].

Again, as in the study with *A. fischeri* biomodel a good correlation between EC_{50} and LogP of the HBD component is observed (figure 9).

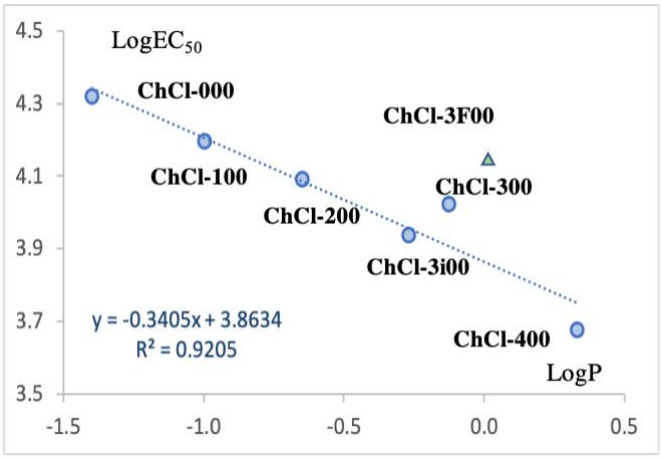

Figure 9. Plot of the ecotoxicity in *R.subcapitata* biomodel for the studied ChCl- DES vs LogP of the HBD component.

On the other hand, the EC_{50} results for **N00Cl** mixtures are lower when the number of carbons in the HBD radical is even, these compounds are more toxic in *R.subcapitata*. As in the *A.fischeri* case, as **N00Cl** mixtures have not been studied before, it is not possible to establish a cause for the structural toxicity mechanism. However, comparing both groups it is observed that changes in the HBA not only modify the EC_{50} values but also influences the structure-toxicity relationship. In this case, the toxic effect of the **N00Cl** mixtures seems not to be related to the lipophilicity of the HBD.

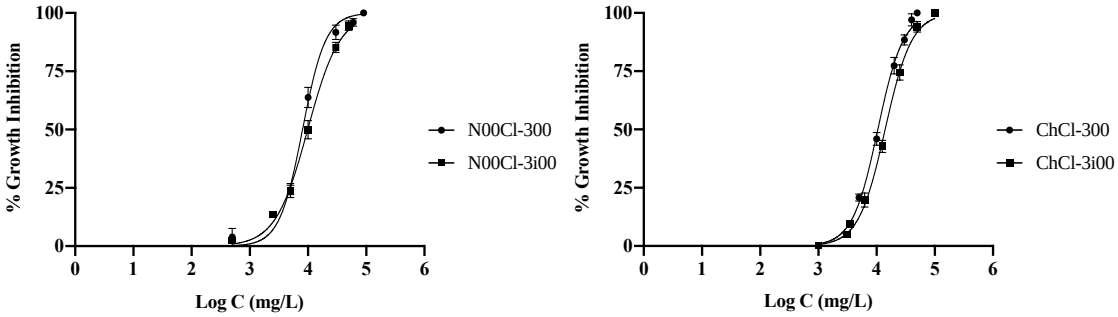

Figure 10. Dose–response curves for the DES composed of **3i00** and **300** glycerol ethers in *R. subcapitata.*

A difference in the toxicity in the algal biomodel is observed related to **3i00** and **300** compounds (Figure 10). DES **ChCl-300** (EC_{50}: 10521 ± 634 mg/L) shows higher toxicity than **ChCl-3i00** (EC_{50}:14039 ± 161 mg/L). The same trend is observed for **N00Cl** mixtures, **N00Cl-300** (EC_{50}:8087 ± 523 mg/L) shows lower EC_{50} than **N00Cl-3i00** (9597 ± 1205 mg/L). Therefore, in both cases, the presence of ramifications in the DES structure leads to a lower toxicity.

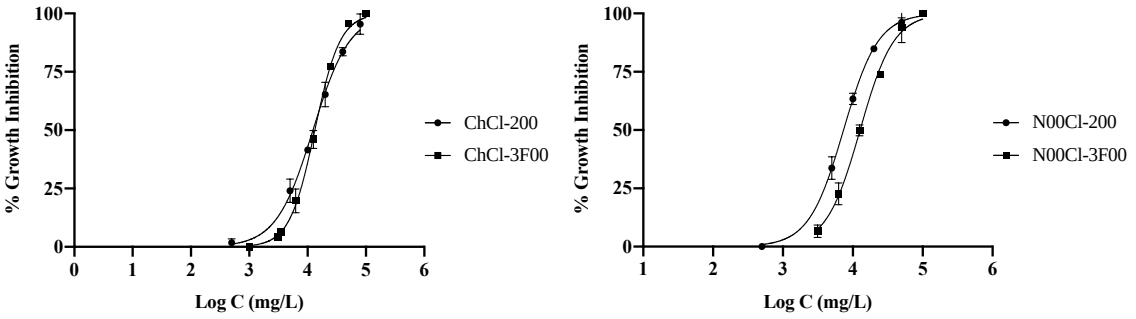

Figure 11. Dose–response curves for the DES composed of **200** and **3F00** glycerol ethers for *R. subcapitata.*

The influence of the presence of fluorine atoms can also be studied by comparing EC50 values for DES with **200** and **3F00** (Figure 10). In both cases, **ChCl** and **N00Cl** mixtures, the presence of fluorine atoms in the HBD structure leads to a decrease in toxicity. While in **ChCl** based DES there is very little difference between EC_{50} values ($EC_{50\ ChCl\text{-}200}$: 12331 ± 155 mg/L; $EC_{50\ ChCl\text{-}3F00}$: 13289 ± 942 mg/L), for DES containing **N00Cl**, the difference is more noticeable ($EC_{50\ N00Cl\text{-}200}$: 7343 ± 67mg/L; $EC_{50\ N00Cl\text{-}3F00}$: 12560 ± 196 mg/L). It has been seen that the presence of fluorine in the aquatic environment can enhance or inhibit of the algae population[49]. In this case, the incorporation of fluorine atoms may avoid the penetration of the compounds in the algae structure.

Chlorophyll is a pigment contained in higher plants and all other organisms capable of photosynthesis. It is closely involved in all stages of photosynthesis, including light harvesting, energy transfer, and light energy conversion. Therefore, changes in the growth of microalgae when exposed to toxic compounds are always related to chlorophyll biosynthesis[50]. The amount of chlorophyll serves as a protective mechanism to eliminate the accumulated ROS[51,52].

A reduction in photosynthetic pigments is also a common stress response in plants and microalgae that can be caused by the decreased biosynthesis and/or increased degradation of chlorophyll, both resulting in decreases in photosynthetic rates[50,53]. It is

widely accepted that chlorophyll degradation involves hydroxyl radicals produced by reactions between superoxide anion and H_2O_2[50]. Photosynthesis provides enough energy for algae growth and cell division. Chlorophyll is extremely crucial for photosynthesis, as a decrease in the chlorophyll content can be problematic for the algae[50].

Chemicals could enter in the cellular structure or could react with some part of the plasmatic membrane generating an information pathway[54]. There are many hypotheses related to toxicity in the algae biomodel. Normally, compounds can affect the electron flow in photosynthesis by a decrease of the yields in Photosystem II, Y (II)[55]. Another explanation is the effect of lipophilicity, causing damage to membranes and increasing the toxicity in the biomodel. An important endpoint on microalgae toxicity is related to alterations in the content of chlorophyll a. This main pigment provides information about photosynthesis efficiency. It plays an important role in ensuring photochemical reaction processes[56].

The amount of chlorophyll a (Chl *a*) and b (Chl *b*) was measured in the tested mixtures. In all cases, the concentration of Chl *a* was higher than the accessory pigment Chl *b*. Furthermore, all the tested DES show an increase in the amount of Chl *a* with the raise of the concentration. According to Lichtenhaler[57] the normal ratio of Chla/Chlb is 3/1. However environmental or growth conditions can affect this ratio. In Figures 12-14, the concentrations of Chl, Chlb and the total amount of Chl are represented in different concentrations.

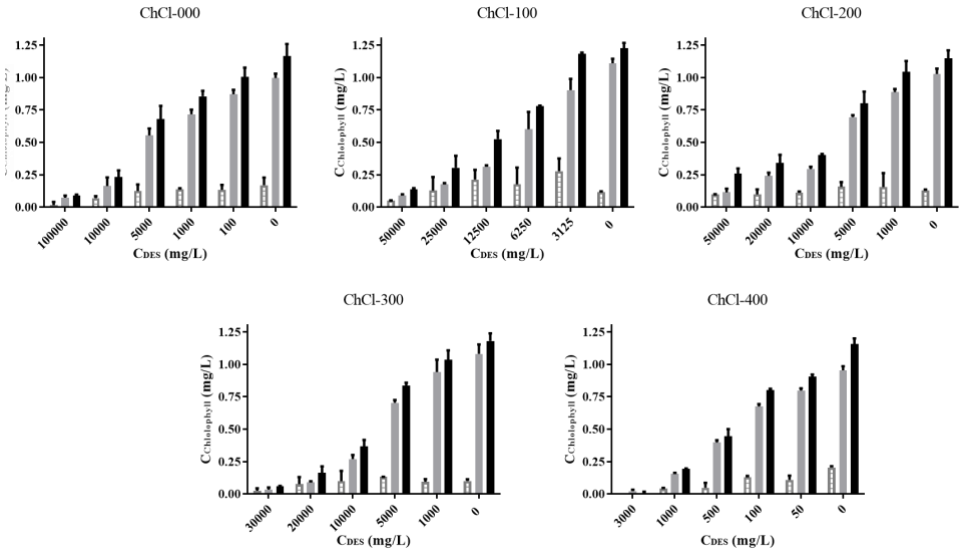

Figure 12. Concentration of chlorophyll a (grey bar), chlorophyll b (white bar) and total chlorophyll (black bar) versus the concentration of **ChCl-000**, **ChCl-100**, **ChCl-200**, **ChCl-300** and **ChCl-400** in *R. subcapitata.*

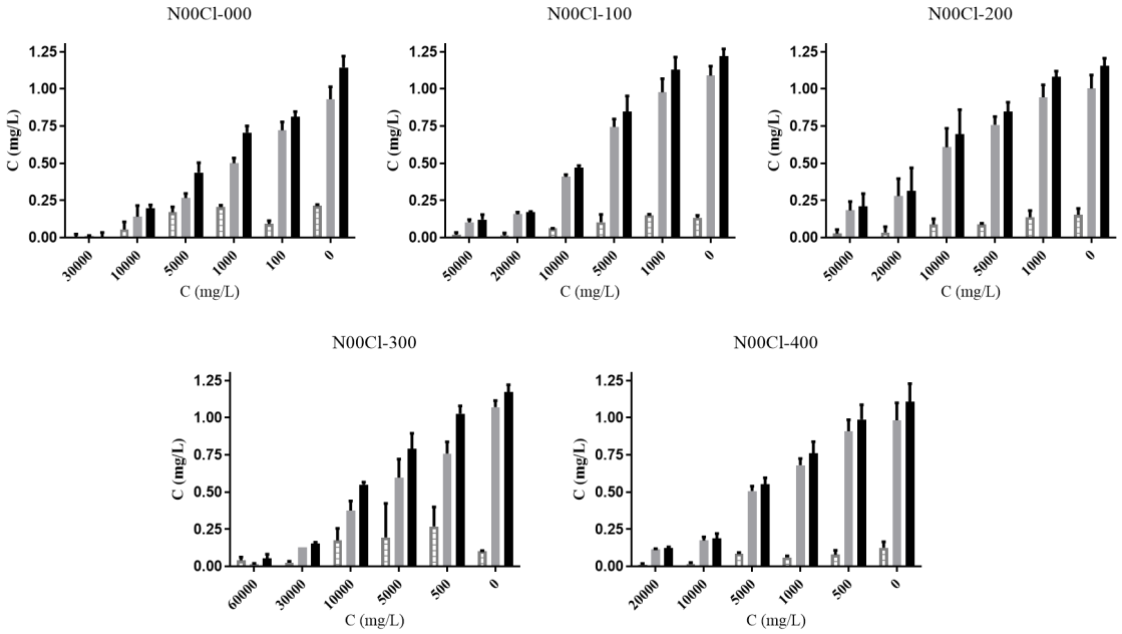

Figure 13. Concentration of chlorophyll a (grey bar), chlorophyll b (white bar) and total chlorophyll (black bar) versus the concentration of **N00Cl-000**, **N00Cl-100**, **N00Cl-200**, **N00Cl-300** and **N00Cl-400** in *R. subcapitata.*

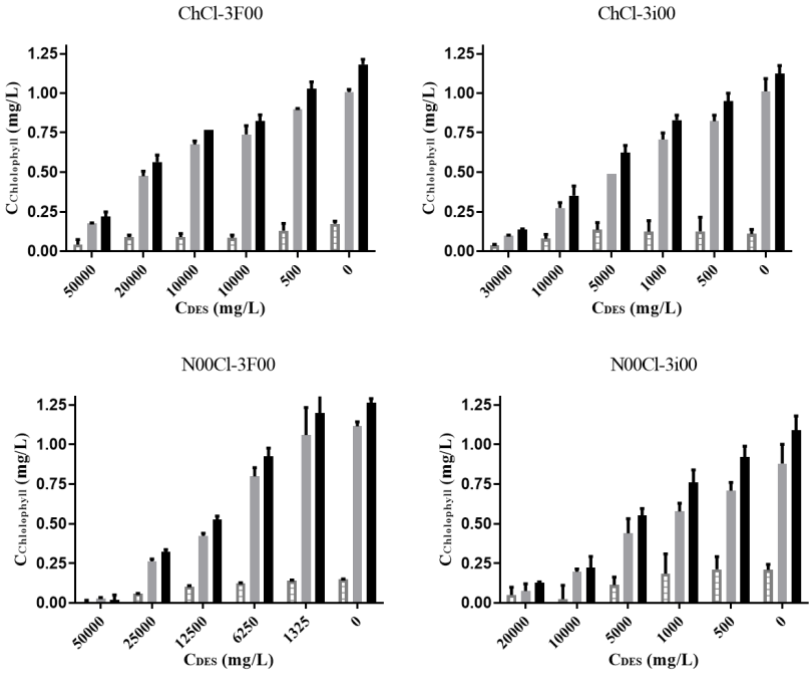

Figure 14. Concentration of chlorophyll a (grey bar), chlorophyll b (white bar) and total chlorophyll (black bar) versus the concentration of **3i00** and **3F00** DES in *R. subcapitata.*

Results show a decrease in the total chlorophyll amount with the increase in DES concentration. So, a correlation concentration-toxic effect can also be established in the measurement of chlorophyll for all the mixtures. Furthermore, the amount of Chl *a* was higher than Chl *b* in both groups of tested DES. The tested mixtures show in most cases a range between 0-1 mg/L in total Chl. Previous studies demonstrate the effect of the hydrophobicity of compounds in the photosynthetic activity of algae. Cho et al. indicate that some of the traditional solvents tested in their essay showed lower photosynthetic activity as increasing the hydrophobicity of the solvent[58].

The highest Chl a/ Chl b ratio was approximately 10 for **ChCl-400** and **N00Cl-400** mixtures. The highest content of chlorophyll has been found in the DES **ChCl-000**. However, there was no clear correlation between the DES toxicity found for algae and chlorophyll contents. This may be related to the different adaptative mechanism that prevents chloroplast light-harvesting[59].

Comparative between both biomodels

A comparison of the EC_{50} between algae and bacterial biomodels has been carried out. In both cases the same structure-toxicity trend is observed. However, for almost all the mixtures, the algae show higher sensitivity than the bacterial biomodel. Even so, none of the studied DES reaches the toxicity threshold in algae, showing in all cases EC_{50}> 1000 mg/L. Only one of the solvents present values of EC_{50}< 1000 mg/L, **ChCl-400** (EC_{50}= 550 ± 9 mg/L) is classified as practically nontoxic instead of relatively harmless in *A. fischeri.*

In order to determine the environmental toxic potential of these solvents, the Passino and Smith classification (PSC) has been used (Figure 15)[60]. This method classifies substances according to their toxicological potential into very toxic compounds (EC_{50} < 10 mg/L), moderately toxic (EC_{50}: 10–100 mg/L), slightly toxic (EC_{50}: 100–1000 mg/L) and not toxic at all (EC_{50} > 1000 mg/L). In all studied solvents, the concentration-toxicity dependence is observed. In most the cases, the toxic effect is manifested at very high concentrations.

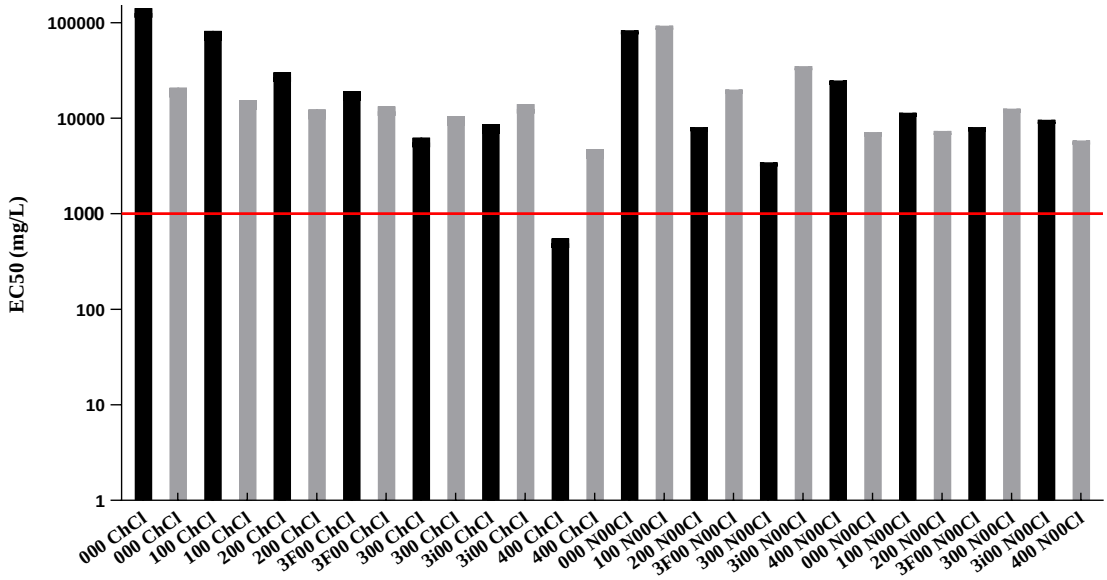

Figure 15. Classification of the studied DES using the Passino and Smith Classification [60]. Black bars shows *A.fischeri* results and grey ones correspond with *R. subcapitata* EC_{50}. The line shows the limit between slightly toxic and harmless substances.

Finally, in sake of comparison, table 5 gathers the values of ecotoxicity of different groups of solvents (traditional organic solvents, ILs and DES). In general, the algae biomodel is more sensitive than the bacterial in all the cases (DES, Traditional organic solvents and ILs). As it can be seen, DES compiled in the table show much higher EC_{50} values and therefore less toxicity than common organic solvents and ILs[29,61]. Comparing the ecotoxicity values of the DES studied in this work with the values compiled in table 5, it can be observed that some of the glycerol ether derived DES present favourable eco-toxicities in these two biomodels, comparable to traditional DES, but with the advantage of the tunability of physico-chemical and eco-toxicological properties by adjusting the nature of the HBD alkyl chain.

Table 5. EC_{50} (mg/L) values for different solvent groups in *A. fischeri* biomodel

Solvent		*A. fischeri*	*R. subcapitata*
Deep Eutectic Solvents	1:2 ChCl:glycerol	86726 [29]	7080 [29]
	1:2 ChCl:urea	26346 [29]	8532 [29]
	1:2 ChCl:ethylene glycol	108526 [29]	9196 [29]
	1:2:1 ChCl:glycerol:water	143686 [29]	6617 [29]
	1:2:1 ChCl:urea:water	98409 [29]	2896 [29]
	1:2:1 ChCl:ethylene glycol:water	115450 [29]	3536 [29]
Tradicional organic solvents	Methanol	101068 [62]	-
	Acetone	19311 [62]	7270 [63]
	Benzene	108 [62]	26.3 [63]
	Phenol	30.8 [62]	61.41 [64]
	Toluene	31.7 [62]	28.7 [63]
	Chloroform	1199 [62]	-
	Dichloromethane	2532 [62]	-
Ionic Liquids	1-Decyl-3-methylimidazolium tetrafluoborate	0.204 [61]	-
	1-Nonyl-3-methylimidazolium tetrafluoborate	1.55 [61]	-
	1-Octyl-3-methylimidazolium tetrafluoborate	7.25 [61]	-
	1-Hexyl-3-ethylimidazolium tetrafluoborate	37.8 [61]	-
	1-Hexyl-3-methylimidazolium tetrafluoborate	385 [61]	-
	1-Heptyl-3-methylimidazolium tetrafluoborate	73.8 [61]	-
	1-Butyl-3-ethylimidazolium tetrafluoborate	151 [61]	-
	1-Butyl-3-methylimidazolium tetrafluoborate	284 [61]	-
	1-Pentyl-3-methylimidazolium tetrafluoborate	331 [61]	-
	1-Pentyl-3-ethylimidazolium tetrafluoborate	350 [61]	-
	1-Propyl-3-ethylimidazolium tetrafluoborate	1850 [61]	-
	1-Propyl-3-methylimidazolium bromide		399.7 [63]
	1-Butyl-3-methylpyridinium bromide		1200 [63]
	1-Butyl-1-methylpyrrolidinium bromide		2100 [63]
	1-Hexyl-3-methylimidazolium bromide		85.69 [63]
	1-Octyl-3-methylimidazolium bromide		13.17 [63]

CONCLUSIONS

This study provides, for the first time, information on the ecotoxicity of a series of bio-based solvents formed from the combination of **ChCl** or **N00Cl** ammonium salts as hydrogen bond acceptors (HBA) and glycerol-derived ethers and glycerol as hydrogen bond donors (HBD). The ecotoxicological study of DES has been performed in the aquatic bioindicators *A.fischeri* and *R.subcapitata,* in order to get an initial overview of the aquatic ecotoxicity. Among these mixtures, only **ChCl-400** can be considered low

toxic (550 ± 9 mg/L) in the bacterial biomodel. The rest of the studied solvents show EC_{50} values much higher than 1000 mg/L, thus being classified as non-toxic substances (PSC). *R.subcapitata* shows in most of the cases a higher sensitivity than *A.fischeri*. For *A.fischeri*, it appears that stability and hydrogen bond ability of DES greatly influences their ecotoxicity, thus most of the DES showed less toxicity than their components separately. Additionally, in **ChCl** mixtures, good correlations between the HBD LogP and the DES polarizability with EC_{50} values have been observed, indicating the great influence of the nature of the HBD component on DES toxicity. However, in **N00Cl** mixtures an odd-even effect of the number of carbons on ether substituent on EC50 values is observed: mixtures with even carbon numbers in the ether chain show lower EC_{50} values.

Mixtures containing **3i00** and **3F00** glycerol ethers do not show the same toxicity trend in both biomodels. In the case of the bacterial biomodel, mixtures containing **3F00** and **3i00** are less toxic than the mixtures containing **300** derivatives but more toxic than **200** mixtures. However, in algae, the presence of ramifications and fluorine atoms decreases the toxicity, thus **3F00** and **3i00** show a better ecotoxicological profile than **200** and **300** compounds respectively.

In general, very high concentrations of these solvents are needed for a manifestation of the toxic effect in both biomodels, as well as in the measurement of the chlorophyll content. A concentration-toxicity correlation is present throughout the entire trials, these two parameters being directly proportional. A comparison with other green solvents, such as ionic liquids and biomass derivatives, indicates that the studied solvents show good ecotoxicological profiles comparable to traditional DES such as reline or glycine but with the advantage of the tunability of physico-chemical and eco-toxicological properties by adjusting the nature of the HBD alkyl chain.

Although the results are promising, additional tests in other aquatic bioindicators would be necessary to represent different trophic levels and obtain a full understanding of the aquatic toxicity of these new green solvents.

Conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Table S1. Ordinary one-way multiple comparisons ANOVA test with a single pooled variance for studied DES in *A. fischeri*.

p* values in *A. fischeri														
	ChCl-000	**ChCl-100**	**ChCl-200**	**ChCl-300**	**ChCl-3F00**	**ChCl-3i00**	**ChCl-400**	**N00Cl-000**	**N00Cl-100**	**N00Cl-200**	**N00Cl-300**	**N00Cl-3F00**	**N00Cl-3i00**	**N00Cl-400**
ChCl-000	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ChCl-000	p< 0.0001	-	-	-	-	-	-	-	-	-	-	-	-	-
ChCl-000	p< 0.0001	p< 0.0001	-	-	-	-	-	-	-	-	-	-	-	-
ChCl-200	p< 0.0001	p< 0.0001	p< 0.0001	-	-	-	-	-	-	-	-	-	-	-
ChCl-300	p< 0.0001	p< 0.0001	p= 0.0915	p= 0.0672	-	-	-	-	-	-	-	-	-	-
ChCl-3F00	p< 0.0001	p< 0.0001	p< 0.0001	p> 0.9999	p= 0.1294	-	-	-	-	-	-	-	-	-
ChCl-3i00	p< 0.0001	p< 0.0001	p< 0.0001	p= 0.9703	p= 0.0015	p= 0.6236	-	-	-	-	-	-	-	-
ChCl-400	p< 0.0001	p= 0.0215	p< 0.0001	p< 0.0001	p< 0.0001	p< 0.0001	p< 0.0001	-	-	-	-	-	-	-
N00Cl-000	p< 0.0001	p< 0.0001	p< 0.0001	p< 0.0001	p< 0.0001	p< 0.0001	p< 0.0001	p= 0.1960	-	-	-	-	-	-
N00Cl-100	p< 0.0001	p< 0.0001	p< 0.0001	p= 0.9999	p= 0.1735	p> 0.9999	p= 0.5368	p< 0.0001	p< 0.0001	-	-	-	-	-
N00Cl-200	p< 0.0001	p< 0.0001	p= 0.0099	p= 0.1691	p> 0.9999	p= 0.3222	p= 0.0039	p< 0.0001	p< 0.0001	p= 0.4107	-	-	-	-
N00Cl-300	p< 0.0001	p< 0.0001	p= 0.9673	p< 0.0001	p< 0.0001	p< 0.0001	p< 0.0001	p< 0.0001	p< 0.0001	p< 0.0001	p= 0.0002	-	-	-
N00Cl-3F00	p< 0.0001	p< 0.0001	p= 0.9034	p= 0.0017	p= 0.8960	p= 0.0024	p< 0.0001	p< 0.0001	p< 0.0001	p= 0.0035	p= 0.4105	p= 0.1562	-	-
N00Cl-3i00	p< 0.0001	p< 0.0001	p< 0.0001	p> 0.9999	p= 0.0060	p= 0.9816	p= 0.9985	p< 0.0001	p< 0.0001	p= 0.9580	p= 0.0165	p< 0.0001	p< 0.0001	-

Table S2. Ordinary one-way multiple comparisons ANOVA test with a single pooled variance for studied DES in *R. subcapitata*

p* values in *R.subcapitata														
	ChCl-000	**ChCl-100**	**ChCl-200**	**ChCl-300**	**ChCl-3F00**	**ChCl-3i00**	**ChCl-400**	**N00Cl-000**	**N00Cl-100**	**N00Cl-200**	**N00Cl-300**	**N00Cl-3F00**	**N00Cl-3i00**	**N00Cl-400**
ChCl:000	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ChCl:100	p< 0.0001	-	-	-	-	-	-	-	-	-	-	-	-	-
ChCl:200	p< 0.0001	p< 0.0001	-	-	-	-	-	-	-	-	-	-	-	-
ChCl:300	p< 0.0001	p=0.3033	p< 0.0001	-	-	-	-	-	-	-	-	-	-	-
ChCl:3F00	p< 0.0001	p< 0.0001	p>0.9999	p< 0.0001	-	-	-	-	-	-	-	-	-	-
ChCl:3i00	p< 0.0001	p< 0.0001	p< 0.0001	p< 0.0001	p< 0.0001	-	-	-	-	-	-	-	-	-
ChCl:400	p< 0.0001	p< 0.0001	p< 0.0001	p< 0.0001	p< 0.0001	p< 0.0001	-	-	-	-	-	-	-	-
N00Cl:000	p< 0.0001	p< 0.0001	p< 0.0001	p< 0.0001	p< 0.0001	p=0.0246	p< 0.0001	-	-	-	-	-	-	-
N00Cl:100	p< 0.0001	p< 0.0001	p=0.5183	p< 0.0001	p=0.8214	p< 0.0001	p=0.5368	p< 0.0001	-	-	-	-	-	-
N00Cl:200	p< 0.0001	p< 0.0001	p< 0.0001	p< 0.0001	p< 0.0001	p=0.0771	p=0.0039	p>0,9999	p< 0.0001	-	-	-	-	-
N00Cl:300	p< 0.0001	p< 0.0001	p< 0.0001	p< 0.0001	p< 0.0001	p=0.9526	p< 0.0001	p=0.4744	p< 0.0001	p=0.7852	-	-	-	-
N00Cl:3F00	p< 0.0001	p< 0.0001	p>0.9999	p< 0.0001	p=0.09968	p< 0.0001	p< 0.0001	p< 0.0001	p=0.2054	p< 0.0001	p< 0.0001	-	-	-
N00Cl:3i00	p< 0.0001	p< 0.0001	p< 0.0001	p< 0.0001	p< 0.0001	p=0.4798	p=0.9985	p< 0.0001	p=0.032	p=0.0002	p=0.025	p< 0.0001	-	-
N00Cl:400	p< 0.0001	p< 0.0001	p< 0.0001	p< 0.0001	p< 0.0001	p< 0.0001	p=0.2789	p=0.0763	p< 0.0001	p=0.0244	p=0.0002	p< 0.0001	p< 0.0001	-