Ecotoxicological study of Glucose:Choline chloride and Sorbitol:Choline chloride at different content of water

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Abstract: The search of new solvents is currently focused on Deep Eutectic Solvents (DES). However, there are not many ecotoxicological studies in different biomodels of DES that allow knowing how these chemicals affect to the environment along the trophic chain. In this manuscript, two DES at different proportion of water have been prepared and characterized from the ecotoxicological point of view. These solvents are: glucose:chloride choline (2:5) and sorbitol:chloride choline (3:2) at different content of water. To carry out the ecotoxicological study, three biomodels have been used: bacteria *Allivibrio fisheri* (*A. fisheri*), crustacean *Daphnia magna* (*D. magna*) and algae *Raphidocelis subcapitata* (*R. subcapitata*). The obtained results show that the ecotoxicity of these chemicals depends on the biomodel used and the amount of water, being toxicity values lower for chemicals with higher water content. However, it is important to highlight that the ecotoxicity for all chemicals is quite low with effective concentrations, EC_{50} values above 1000 mg/L in all the studied cases.

Keywords: ecotoxicity, DES, glucose sorbitol, *Aliivibrio fischeri*, *Daphnia magna*, *Raphidocelis subcapitata*.

1. Introduction

Deep Eutectic Solvents (DES) and Natural Deep Eutectic Solvents (NADES) are mixtures formed by two or more chemicals at solid state at room temperature but, when combined in a specific molar ratio, form a liquid solution. This is mainly due the strong and complex network of intermolecular forces stablished that cause a sharp diminution of the melting point (Laura Lomba 2021a).

DES can be considered as "design solvents" since the possibility of combinations of different components and compositions is almost unlimited. The goal is setting the right mixture for obtaining the physicochemical properties required for a specific chemical processes(Mitar et al. 2019).

DES are formed by a hydrogen bond acceptor (HBA) and a hydrogen bond donor (HBD) and the typical components are natural chemicals such as: organic acids, alcohols, sugars, amino acids, urea, small hydrophilic molecules or natural metabolites among others(Nystedt et al. 2021).

DES are usually low cost, are also easy to prepare (Benvenutti et al. 2020, Benvenutti et al. 2019), present low volatility, are biodegradable (Wils et al. 2021) and normally non-reactive with water. The label of non-toxic mixtures has to be reviewed and lately there is some controversy in the scientific community in this regard; there are an important number of studies that show low toxicity of eutectic mixtures for the environment or human both *in vitro* and *in vivo* studies (Laura Lomba 2021b), while some others studies demonstrate the toxicity of DES above a certain limit of concentration (Torregrosa-Crespo et al. 2020). Nevertheless, the toxicity of DES depends on several factors that include not only the nature of the components of the mixture but also the organisms on which toxicity is studied, their pre-adaptation periods or even the sterilization processes carried out during the measurement of toxicity. This may be caused by the acidification of the medium caused by the DES hydrolysis (Torregrosa-Crespo et al. 2020). Thus, it is required more specific studies about the effect of DES on different endpoints, covering several trophic levels, organisms, cells or tissues and the use of more appropriate toxicological techniques (Marchel et al. 2022b, Ruesgas-Ramon et al. 2017).

It is also said that present good physicochemical properties for several chemical processes thanks to their wide polarity range and low vapour pressure or chemical stability, among others(Benvenutti et al. 2020, Laura Lomba 2021a, Laura Lomba 2021b, Silva et al. 2019). For these reasons DES can be used as stationary phases in chromatography (Momotko et al. 2021, 2022) and membranes (Castro-Munoz et al. 2022, Khajavian et al. 2022) and additionally, they are attractive for several industrial processes such as biocatalysis (Zhou et al. 2021), cryopreservation(Craveiro et al. 2021), biochemical applications(Silva et al. 2019, Yang 2019), extraction medium(Ruesgas-Ramon et al. 2017, Rukavina et al. 2021), biological assays (Liu et al. 2018), pharmaceutical industry(Liu et al. 2021, Morrison et al. 2009) or cosmetic industry(Benoit et al. 2021).

However, the main barrier to the widespread use of DES is the lack of information regarding various physiochemical and toxic properties. This limitation arises from the very nature of the DES; the multiple combinations between components and compositions (AlOmar et al. 2016, Lapena et al. 2020, Lapena et al. 2019b, a) makes difficult to have a complete set of properties that allow knowing the specific characteristics of a mixture (components and concentration) (Li et al. 2022) or even to predict the behaviour.

However, it is important notice that the thermal stability of DES can be diminished at high temperature. Most of the studies about this issue analyse the thermal stability of DES using the onset decomposition temperatures (Tonset). These temperatures, that were normally obtained from dynamic thermogravimetry (TGA) under different experimental conditions that, in general, lead to the overestimation of the onset decomposition temperature. It is important to bear in mind that there are some scientific studies related to the thermal stability of DES that have found, in some cases, that DES can decompose into HBA and HBD due to the weaking of the hydrogen bonds. This process can occur in two steps: the first one, the DES component with a lower boiling point or little stability undergoes volatilization or decomposition (normally HBD) and the second one, the decomposition of the other DES at a higher temperature (normally the HBA). Thus, the role of the hydrogen bonds in the thermal stability seems to be determinant. (Gutierrez et al. 2010, Marchel et al. 2022a). Additionally, it is important to note that the toxicity of DES may vary when the hydrogen bond net is modified; new supramolecular structures can be created, and thus, the toxicological activity may be also different.(Gutierrez et al. 2010, Marchel et al. 2022b, a)

With the aim of increasing the information available on these substances, we have previously explored the ecotoxicity of some of them: reline, ethaline and glyceline towards several aquatic biomodels (algae, crustaceans and bacteria)(Lapena et al. 2021). We founded that the ecotoxic effect clearly depended on the studied biomodel and the type of test used as a tool to monitor toxicity. Furthermore, the water content did not follow the expected trend and studied DES behaved erratically with regards to the water composition(Lapena et al. 2021).

Thus, with the objective of deepening the knowledge of the ecotoxic behaviour of DES, in this work we have studied the ecotoxicity of several eutectic mixtures containing sugars and chloride choline at different concentrations of water: glucose:chloride choline (2:5) and sorbitol:chloride choline (3:2) with different water content. Concretely, we have obtained the toxic effect that these mixtures produce in various aquatic organisms, covering the trophic chain: bacteria *Allivibrio fisheri* (*A. fisheri*), crustacean *Daphnia magna* (*D. magna*) and algae *Raphidocelis subcapitata* (*R. subcapitata*).

Besides, it has been previously reported that viscosity is an important properties key to explain toxicity in some cells. An increase of toxicity is normally associated with higher cell lethality rates(Hayyan et al. 2016). For that reason, we have also measured the kinematic viscosity of the studied DES. Toxicity results have first been analysed in an independent way, considering both the DES and the biomodel. They were then considered together and the final conclusions about the ecotoxic behavior have been reached.

2. Material and methods

2.1 Chemicals and preparation of Deep Eutectic Solvents

In Table 1, the used chemicals for the DES preparation are gathered. Choline chloride has been dried under vacuum for 24 h prior to use. Mixtures with water have been prepared considering the previous amount of water of each of the components of the mixtures with Milli-Q water (resistivity less than 18.2 MΩ·cm) using a Sartorius Entris 5201-1S balance (uncertainty ±10^{-1} g). Once each substance has been weighed, has been transferred to a closed flask (250 ml) with a magnetic nucleus inside. The flask has been placed on a heating plate and heated to a temperature of approximately 80ºC with continuous stirring. The process has been completed within one hour and a homogeneous liquid has been observed. In Table 2, studies DES, composition, and abbreviation are shown.

Table 1. Characteristics of the used chemicals

Name	CAS	Supplier	Mass fraction purity	Formula	Molar mass (g/mol)
Glucose anhydrous	50-99-77	Panreac	0.975	$C_6H_{12}O_6$	180.16
Sorbitol	50-70-4	Sigma-Adrich	0.980	$C_6H_{14}O_6$	182.17
Choline Chloride	67-48-1	Sigma-Aldrich	0.993	$C_5H_{14}ClNO$	139.63

Table 2. Studied DES: composition and abbreviation

HBD	HBA	Add-on	Molar ratio	Abbreviation
Glucose	Chloline chloride	Water	5:2:5	Glu5
			5:2:7.5	Glu7.5
			5:2:10	Glu10
Sorbitol	Chloline chloride	water	2:3:5	Sor5
			2:3:7.5	Sor7.5
			2:3:10	Sor10

2.2 Viscosity measurements

Kinematic viscosities, *v*, have been obtained using a Schoot-GeräteAVS-440 automatic measuring unit along with several Ubbelohde capillary viscosimeters. The uncertainty of the time flow measurements is 0.01 s, and kinetic energy corrections have been applied to the experimental data. The temperature has been controlled at 25ºC by means of a Schoot-Geräte CT 1150/2 thermostat, with a temperature uncertainty of 0.01K.

2.3 A. fischeri ecotoxicity test

The lyophilized *A. fischeri* (strain NRRL-B-11177 and reference 945006) have been supplied by Macharey-Nagel. This test has been based on UNE-11348-3(UNE-11348-3 2009). All solutions have been prepared with 2% of NaCl and the pH has been adjusted using 0.1 M HCl or 0.1 M NaOH in 2 % NaCl. Additionally, positive controls such as phenol and zinc sulfate have been used(Jennings et al. 2001) and negative control has been culturing medium (Biofix Lumi medium for freeze-dried luminous bacteria by DIN EN USO 11348–3, Macherey-Nagel, Duren, Germany).

Luminescence has been measured in acute mode (Biotox B) using a Biofix Lumi-10 luminometer (MachereyNagel) after 30 minutes of exposure and at 15ºC. Three replicates for each control have been tested. More details of this test can be found in our previous works(Garcia et al. 2015, Lomba et al. 2014).

2.4 D. magna ecotoxicity test

D. magna ecotoxicity test have been based on the guidelines OECD 202(202 2004). The *D. magna* ephippia (Toxkit, Daphtokit F Magna, ref. DM090812) have been stored at 4ºC and purchased from Vidrafoc. The eggs have been incubated with culturing medium considering the specifications of the supplier, during 72 h at 22ºC with 6000 lux in a Toxkit CH-0120D-AC/DC incubator (supplied by Ecotest) and fed with Spirulina algae 2 h before starting the experiment. The pH has been adjusted 7-7.5 before exposure. Furthermore, positive control (sodium dichromate) and negative controls have been tested.

A total of 20 newborn daphnids (aged less than 24 h) have been exposed to the different dilutions of the DES in dark for 24 h at 20ºC per compound and concentration. The crustaceans have been separated into four groups of five organisms, four replicates per concentration exposure. The test has been repeated in triplicate. Daphnids have been considered immobilized if they have been not able to swim for 15 s after agitation. More details of this protocol can be found in bibliography(Perales et al. 2017).

2.5 R. subcapitata ecotoxicity test

R. subcapitata test have been carried out according to OECD 201(OECD 1984). Algae have been purchased from ECOTEST (SC2B1214). All the experiments have been repeated in triplicate. Moreover, culturing medium and de-mobilization of algae have been prepared using the supplier specifications.

The algae cells have been incubated at 23ºC in 100 mL beaker containing 50 mL of culturing medium with 10000 lux of illumination. Cells densities of have been correlated with optical density (OD) at 670 nm basing on the supplier specifications. The algae initial concentration has been $3 \cdot 10^5$ cells/ml.

The pH of the solutions has been adjusted in the range of 7.9 and 8.2 using 0.1 M NaOH or 0.1 M HCl solutions. The assay has been carried out in a 96 well plate. To minimize evaporation, the outer wells of the plate have been filled with 0.3 mL of distilled water and only the inner wells have been filled with controls and test solutions. The initial OD has been measured at 670 nm. The well plate has been incubated at 23ºC for 72 hours. Next, all plates have been re-suspended to avoid the settling of algae and the final OD has been measured.

2.6 Statistics treatment

Data have been analysed using a non-linear regression-representing log (inhibitor) versus inhibition.

To obtain the EC_{50} values of each compound, the experimental results have been fitted to the following function:

$$\%\mathrm{I} = \frac{100}{\left(1+10^{(a-\log C)b}\right)} \qquad (4)$$

where %I denotes % inhibition of luminescence for *A. fischeri*, % of immobilization for *D. magna* and % of inhibition of growth for *R. subcapitata*; C is the concentration in mg/L and Log EC_{50} and *a* are the adjustable parameters.

3. Results and discussion

In this section, the obtained results for the studies compounds are shown. Regarding to the prepared DES, it is important to note that all DES formed by sorbitol (Sor5, Sor7.5 and Sor10) and glucose (Glu10) have been obtained in a unique a clear phase at room temperature (25ºC), however, in the case of glucose DES (Glu5, Glu7) it has been necessary to slightly heat (30ºC) to obtain that liquid phase before the tests.

In this section, results have been analyzed from the perspective of the DES (or DES + water mixtures) itself, as a whole. However, it is important to revise the nature of the components of the eutectic mixtures from the toxicological point of view. For instance, choline chloride is a known component of Vitamin B, and plays important metabolic functions; choline is usually the chosen cellular raw material for the synthesis of cellular phospholipidic membranes (phosphatidylcholine and sphingomyelin) (Hayyan et al. 2016). On the other hand, from a cellular perspective glucose and sorbitol are essential carbohydrates; their metabolism provides the required energy for different cellular functions. After adsorption, glucose undergo glycolysis when energy is required in the cell, otherwise is stored as glycogen. The sorbitol metabolism is an accessory pathway in the glucose route. Through a chain of reactions, the sorbitol is an intermediate that converts glucose into fructose. The glycolytic pathway for glucose and fructose leads to synthesis of nucleic acids through the pentose phosphate pathway, the energy production in the mitochondria through tricarboxylic acid pathway and the fatty acid synthesis through the lipogenesis process. For these reasons, it is expected high cellular tolerance of the studied DES formed by choline and these sugars. However, during the eutectic mixture formation process and the establishment of the hydrogen bond network, an important modification of the chemical and physical properties is made, and this includes the toxicological behavior of the eutectic mixtures.

Returning to the analysis of the results obtained by the DES studied in the biomodels, values of EC_{50} towards *A. fischeri*, *D. magna* and *R. subcapitata* are shown in Table 3. For all the studied chemicals, there is a dependence relationship between the toxicity and the concentration; *i.e*, the toxic effect increases as the concentration does. The general toxicity trend observed for all biomodels is the following: Glu 5 > Glu 7.5 > Glu10 > Sor5 > Sor 7.5 > Sor10. In Figure 1, the toxicity of obtained values for all the studied biomodels are presented. The results indicate that the most sensitive species was *D. magna* followed by *R. subcapitata* and *A. fischeri*.

It is important to highlight that none of the studies mixtures can be considered toxic to the environment (values of EC_{50} do not exceed the general limit of 1000 mg/L). The Globally Harmonized System of classification And labelling of Chemicals (GHS) (Nations 2021) has been used to categorize the toxicity for substances hazardous to the aquatic environment, Short-term (acute) as follow: Acute 1 (EC_{50} $\leq$ 0.1 mg/L), acute 2 (EC_{50} > 1 but $\leq$ 10 mg/L), acute 3 (EC_{50} > 10 but $\leq$ 100 mg/L). For this GHS classification, algae, crustacea and fish, are considered surrogate species. However, data on other organisms may also be considered and the classification can be applied too. The ecotoxicity results obtained int this work indicates that for all studied mixtures there is no need to classify for short-term (acute) hazard. It is worth mentioning the case *of A. fischeri* where some mixtures exhibited values of EC_{50} higher than 100000 mg/L indicating very low toxicity to this biomodel (Figure 1).

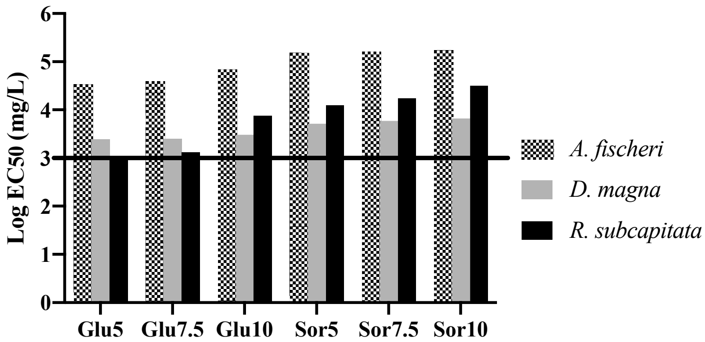

Figure 1. Comparison of the obtained results for each biomodel. The solid line at Log EC_{50} = 3 mg/L correspond to the general toxicity limit 1000 mg/L.

The lipophilia is one of the key properties traditionally used to explain the toxic behavior: the higher lipophilic character, the higher ability of the molecules to cross through the biological membranes and produce a whatever effect on the activity. In this case, focusing of the sugars forming part of the DES, glucose and sorbitol, our toxicity results are in close agreement with lipophilic trend described by their corresponding octanol-water partition coefficients, log Kow = -3,17 and -4.67 respectively (Chemspider, accessed at April 13rd 2022).

In Table 3, values of kinematic viscosity are also gathered. For these mixtures, the viscosity has been higher than the viscosity of some typical molecular liquids such as water or some other low molecular weight organic solvents. In the case of glucose mixtures, the higher viscosity has been obtained for Glu5 followed by Glu7 and Glu10. In the case of DES containing sorbitol, the trend is the same as for glucose mixtures, being the most viscous Sor5 followed by Sor7.5 and Sor10. This property is clearly affected by the nature of its components, temperature, or molar ratio. The content of water is important too, not only in physicochemical properties but also in the integrity of DES changing their supramolecular interactions (El Achkar et al. 2021). As mentioned before, viscosity and content of water may explain the obtained results; it has been observed that high viscosity are associated with increased lethality(Hayyan et al. 2016).

Table 3. Values of EC_{50} (mg/L) for *A. fischeri*, *D. magna* and *R. subcapitata* and values of kinematic viscosity at 25ºC.

DES	***A. fischeri***	***D. magna***	***R. subcapitata***	**Kinematic Viscosities**
	30 min	24 h	72 h	ν, (mm/s)
Glu5	34196 ± 3965	2433 ± 31	1021 ± 122	787.69
Glu7.5	39955 ± 2455	2527 ± 22	1305 ±114	375.22
Glu10	69591 ± 3073	3048 ± 24	7544 ± 475	185.02
Sor5	149748 ± 1279	5153 ± 85	12496 ± 663	3333.9
Sor7.5	161857 ± 812	5942 ± 283	17545 ± 295	613.01
Sor10	171209 ± 789	6557 ± 242	31325 ± 131	503.51

A. fischeri bacteria is Gram-negative, flagellated, non-pathogenic and rod-shaped bacterium, which is distributed in marine environments(Abbas et al. 2018). The bioluminescence mechanism is based in two substrates: luciferin and a long chain fatty aldehyde. Exogenous agents (reduced) produce the reduction of FMN (flavin mononucleotide oxidated) to FMNH2 (flavin mononucleotide reduced) through luciferase (flavin mono-oxygenase oxidoreductase) action which reacts with oxygen forming an intermediate chemical called 4a-peroxy-flavin. This compound oxidizes the fatty aldehyde forming its corresponding acid and a luciferase–hydroxyflavin complex. This intermediate is decomposed slowly and emitting a blue-green light with its highest intensity at 490 nm(Meighen 1991). This process is completely linked to respiration, through the electron transport chain, and gives an idea about the metabolic status as a chemical toxicity. The presence of toxic substances diminishes the resultant luminescence. The inhibition of bacterial metabolism is manifested by attenuation of light emittance which corresponds to the toxicity level of the tested substance (Abbas et al. 2018, Bulich 1982).

For the studied mixtures, the curve dose-response for *A. fischeri* at 30 min are shown in Figure 2. For this biomodel, the values of EC_{50} are very high varying between 30000-172000 mg/L, indicating a low toxicity of the studied mixtures towards this biomodel; however, DES containing glucose are more toxic than DES containing sorbitol, concretely between 4.1 and 2.5 times more toxic, depending on the DES. In all cases, toxicity decreases with water content. In the case of DES with sorbitol, this decrease of toxicity is almost linear while for glucose DES, the the decrease is more pronounced if the water content increases.

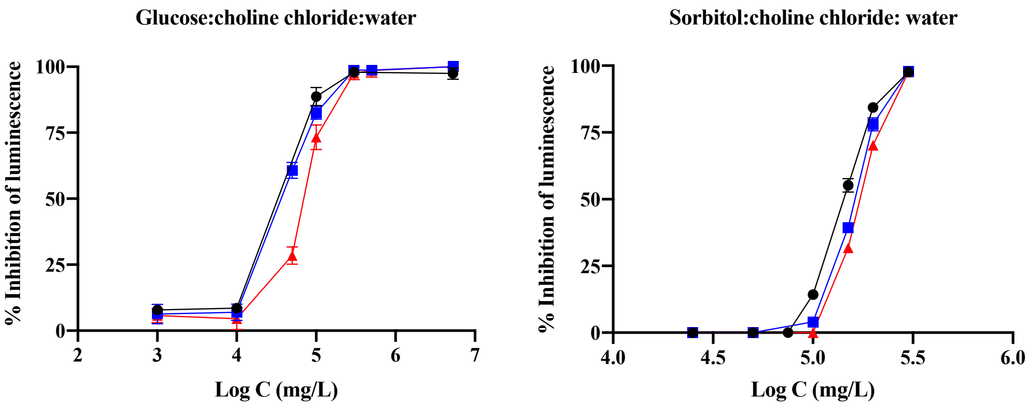

Figure 2. Curve dose-response in A. fischeri for Glu and Sor at 30 min. Content of: 5 of water (●), 7.5 of water (■) and 10 of water (▲).

D. magna is one of the oldest organisms used in biological research (Tkaczyk et al. 2021). They can filter bacteria, algae, protozoans or other small aqueous particles. *Daphnia* are quite important for the freshwater aquatic food chain, as a primary consumer for predatory invertebrates and fish. Additionally, daphnids consume algae and thus, improve water quality (Dietrich et al. 2010, Tkaczyk et al. 2021). The *Daphnia* motility is easy to observe and therefore, this feature is commonly used in immobilization, lethality and reproduction tests(Bownik 2020). In addition, swimming behavior parameters (swimming activity, swimming time, swimming speed, behavioral strength and hopping frequency) can be used as sensitive endpoints (Bownik 2017). However, it is important to control the assay conditions because variations in water temperature or medium culture can affect to the physiological and physical effects on these organisms(Loiterton et al. 2004).

For *D. magna*, the curves dose-response at 24 h for all the studied chemicals are shown in Figure 3. Once again, DES containing sorbitol are less toxic than glucose DES (nearly 2 times less toxic). Besides, toxicity decreases in both cases with water content; the behaviour of DES containing sorbitol is linear-like while, for glucose DES the decrease in toxicity is more pronounced.

Algae play an important role in the maintaining the structure and function of aquatic ecosystems since are primary producers. Algae can be considered as an important bioindicator in the evaluation of the water quality and ecotoxicity of substances(Guo et al. 2020). In particular, *R. subcapitata* is a typical species used in ecotoxicological studies, recommended by the Organization for Economic Cooperation and Development (OECD), because of its global distribution, fast growth, and great sensitivity(Mo et al. 2022).

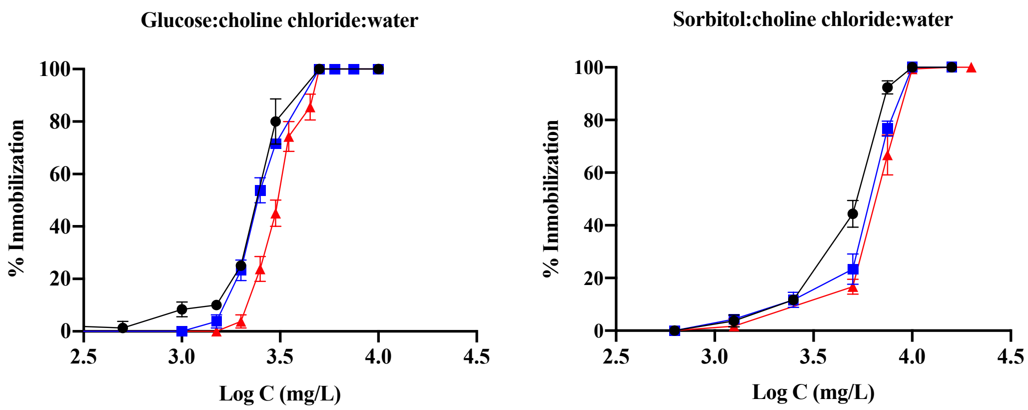

Figure 3. Curve dose-response in D. magna for Glu and Sor at 24 h. Content of: 5 of water (●), 7.5 of water (■) and 10 of water (▲).

For *R. subcapitata,* the curves dose-response at 72 h for all studied chemicals are shown in Figure 4. As we can observed, the DES formed by glucose and choline chloride show a non-typical sigmoidal curve, especially the case of Glu5 and Glu7.5. Although the effect of a certain DES concentration with exposure time has not been studied, this characteristic profile could be indicative of a diauxic effect in the inhibition growth process (Blaiseau &Holmes 2021). The slope in growth inhibition is very steep until the concentration of DES reaches a value of approximately 1000 mg/L. From there, the inhibition remains constant around 75% in a very wide range of concentrations (from 1,000 to 100,000 mg/L). This behaviour can be related to the viscosity of these mixtures because they are more viscous than Glu10. For this biomodel, toxicity decreases with the water content in the moiety, being the most pronounced decrease in the case of Glu10 DES. DES containing sorbitol are clearly less toxic than glucose DES. These results can be explained in terms of viscosity; Qin *et al*. showed that the motility of algae (*Chlamydomonas reinhardtii*) is influenced by the viscosity of the environment, *i.e,* the motility of algae is related to a complex interaction between swimmers kinematics and the properties of that material(Qin et al. 2015).

Additionally, Coppola *et al*. showed that the velocity and angular diffusion of the algae decreases when the viscosity of the surrounding medium is increased. They also observed that algae do not concentrate in the region of high viscosity despite them swimming slower there(Coppola &Kantsler 2021).

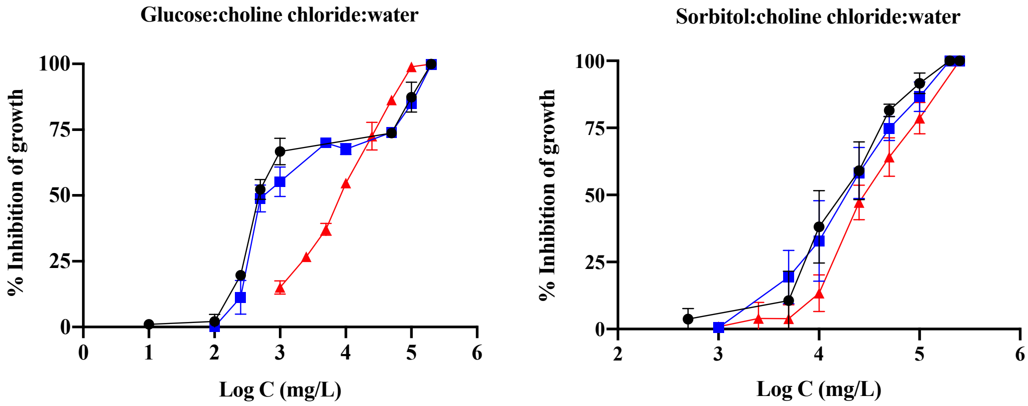

Figure 4. Curve dose-response in R. subcapitata for Glu and Sor at 72 h. Content of: 5 of water (●), 7.5 of water (■) and 10 of water (▲).

4. Conclusions

In this paper, the ecotoxicity of several DES (Glu5, Glu7.5, Glu10, Sor5, Sor7.5 and Sor10) has been evaluated in three aquatic biomodels (*A. fischeri*, *D. magna* and *R. subcapitata*). Results indicate a low ecotoxicty of the studied solvents. For glucose DES, the most sensitive biomodel is *R. subcapitata*, while for DES containing sorbitol, the most sensitive species is *D. magna*. The highest values of EC_{50} are found for the biodel *A. fischeri* for all the studied DES.

To explain the results, the kinematic viscosity of the mixtures has been also measured at 25ºC. The toxicity of the DES decreases as the water content increases. This result correlates with the kinematic viscosity values found for the studied DES. Regarding to the GHS classification, there is no need to classify for short-term (acute) hazard for all mixtures under study.

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Compliance with Ethical Standards

Competing Interests: the authors have no relevant financial or non-financial interests to disclose.

Research involving Human Participants and/or Animals: no human or superior animals have been used in this work.

Informed consent: does not apply in this work.

Availability of data and materials: does not apply in this work.

Author Contribution

Conceptualization: Laura Lomba and Beatriz Giner; Formal analysis: Laura Lomba and Beatriz Giner; Investigation: Diego Errazquin, Mª Pilar Garralaga, and Noelia López; Methodology: Laura Lomba; Project administration: Beatriz Giner.; Resources: Laura Lomba.; Supervision: Beatriz Giner.

Consent to participate

All authors agreed with the content and that all gave explicit consent to submit.

Consent to publish

All authors agreed with the publication of the manuscript in Environmental Science and Pollution Research.

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