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Evaluation of toxicity and biodegradability of choline chloride based deep eutectic solvents

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ABSTRACT

Deep eutectic solvents (DESs) have been dramatically expanding in popularity as a new generation of environmentally friendly solvents with possible applications in various industrial fields, but their ecological footprint has not yet been thoroughly investigated. In the present study, three choline chloride-based DESs with glucose, glycerol and oxalic acid as hydrogen bond donors were evaluated for in vitro toxicity using fish and human cell line, phytotoxicity using wheat and biodegradability using wastewater microorganisms through closed bottle test. Obtained in vitro toxicity data on cell lines indicate that choline chloride: glucose and choline chloride:glycerol possess low cytotoxicity ($EC_{50} > 10$ mM for both cell lines) while choline chloride:oxalic acid possess moderate cytotoxicity (EC_{50} value 1.64 mM and 4.19 mM for fish and human cell line, respectively). Results on phytotoxicity imply that tested DESs are non-toxic with seed germination EC_{50} values higher than 5000 mg L^{-1}. All tested DESs were classified as'readily biodegradable' based on their high levels of mineralization (68–96%). These findings indicate that DESs have a *green* profile and a good prospect for a wider use in the field of *green* technologies.

1. Introduction

The design of environmentally friendly solvents in recent years finds its strategic place within the framework of *green* technologies (Anastas and Warner, 1998; Anastas and Eghbali, 2010; EEA, 2013). In the past 20 years, ionic liquids (ILs) have attracted attention as a new generation of *green* designer solvents with potential uses in various industrial fields (Petkovic et al., 2011). However, regardless of the ILs' declarative *green* properties (non-volatility, non-flammability, reusability), the limitations of conventional ILs, such as imidazolium- and pyridinium-based ones, are high cost (5–20 times higher than the cost of conventional organic solvents), toxicity similar to or even higher than organic solvents and generally poor biodegradability (Cvjetko Bubalo et al., 2014a). These facts have mobilized scientists to develop solvents that would retain excellent technological properties of ILs while being low-cost and exerting minimal environmental effects. In that manner, a type of solvents with similar physical properties and phase behavior to ILs, called deep eutectic solvents (DESs), have emerged (Carriazo et al., 2012; Ruß and König, 2012; Zhang et al., 2012a; Francisco et al., 2013). In the literature, DESs are sometimes referred to as the fourth-generation of ILs, even though they could not be considered ILs as they are not entirely composed of ionic species. The DESs are easily prepared by mixing two or three low-cost components (e.g., quaternary ammonium salts, amides, organic acids, polyalcohols), forming an eutectic mixture based on hydrogen bonding interactions with a melting point much lower than either of the individual components (Carriazo et al., 2012; Ruß and König, 2012; Zhang et al., 2012a; Dai et al., 2013a; Francisco et al., 2013). One of the most popular components used for the formation of these DESs is choline chloride (ChCl), a cheap, biodegradable and non-toxic salt, which is approved without a time limit under Council Directive 70/524/EEC8 for use as a nutritional additive in all species (EFSA Panel on Additives and Products or Substances used in Animal Feed, 2011). Since their emergence, DESs as a *greener* version of ILs have attracted attention in synthesis, metal-catalyzed organic reactions, electrochemistry, nanomaterials, biochemistry, separation, and analysis. Accordingly, the number of DESs-related references has

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increased rapidly in recent years (almost 300 papers in this period) (Carriazo et al., 2012; Ruß and König, 2012; Zhang et al., 2012a; Francisco et al., 2013; Tang and Row, 2013; Vidal et al., 2014).

The industrial use can be expected based on numerous potential DESs applications in the *green* technologies, meaning that environmental impact and fate (e.g., biodegradation and ecotoxicity) of DESs has to be extensively and critically evaluated before their large-scale production (Tanneberger, 2010). So far, the assumption that DESs are benign is based on toxicity data for the components that make up DESs, which are biomaterial-derived and pharmaceutically acceptable. However, this theory does not take into account the possibility of synergetic effect of combining the compounds in the DESs (Hayyan et al., 2013a) which could have significant impact on biological properties of such mixtures. To date, three publications dealing with the toxicity of DESs have been published (Hayyan et al., 2013a, 2013b; Paiva et al., 2014). Hayyan et al. (2013a, 2013b) assessed toxicity of several ChCl- and phosphonium-based DESs toward brine shrimp and bacteria. For instance, it was shown that although ChCl based DESs were completely harmless for the tested bacteria, the phosphonium-based DESs exhibited slight antibacterial activity. However, the cytotoxicity of the DESs tested was much higher than that of their individual components, indicating a noticeable synergistic effect after forming DESs. Paiva et al. (2014) were the first ones who tested the effect of 11 different DESs on L929 fibroblast-like cell line and compared it to two ILs. However, the obtained results did not indicate a clear trend between the cytotoxic effect and the constitution of DES. Therefore, the use of terminology *green* for DESs should still be used with caution whereby DESs toxicity toward organisms at different trophic levels should be proactively assessed prior to their large-scale use. Also, since there are no available data on DESs biodegradability it is necessary to determine their biodegradation potential by wastewater organisms.

Based on the aforementioned, the aim of this work was to evaluate three commonly used ChCl-based DESs containing sugar (glucose), alcohol (glycerol), and organic acid (oxalic acid) as hydrogen donor for in vitro toxicity using fish and human cell line, phytotoxicity on wheat, and their biodegradability using wastewater microorganisms in the closed bottle test. The data obtained would serve to fill the existing gaps in the knowledge about environmental fate of DESs and could be used to predict their effects on human health and environment.

2. Experimental

2.1. Biological and chemical materials

All chemicals for DESs syntheses (choline chloride, glucose, oxalic acid and glycerol) were purchased from Sigma Aldrich (purity of $\geq 99\%$) and used without further purification. The CCO fish cell line (ATCC no. CRL-2772) and MCF-7 human tumor cell line (ATCC no. HTB-22) were used in this work. WST-1 assay was purchased from Roche, Germany. Dulbecco's Modified Eagle's Medium and fetal bovine serum were purchased from Gibco, UK. Wheat seeds (*Triticum aestivum*) were obtained from the Mladen Commerce d.o.o. market, Croatia. All other chemicals were from commercial sources (Sigma Aldrich) and were of the highest purity available.

2.2. Preparation of DESs

The DESs samples were synthesized as reported previously (Dai et al., 2013b; Hayyan et al., 2013c). Briefly, the mixture of choline chloride (ChCl) and hydrogen bond donor was stirred in a flask at 80 °C for 2–6 h until a homogeneous transparent colorless liquid was formed. The hydrogen donors used were glucose, oxalic acid and glycerol which were mixed with choline chloride in molar ratios 2:1, 1:1, and 1:2, respectively. DESs samples were vacuum dried prior to toxicity and biodegradability assessment. Average molecular weights of synthesized DESs were calculated as the sum of the molecular weight of each compound forming the mixture multiplied by its mass fraction. For DESs cholin chloride:glucose (ChCl:Glc), cholin chloride:oxalic acid (ChCl:OA) and cholin chloride:glycerol (ChCl:Gly) average molecular weights were as follows: 156.80 g mol^{-1}, 133.30 g mol^{-1} and 112.67 g mol^{-1}.

2.3. Cytotoxicity assay

Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM,) supplemented with 10% (v/v) fetal bovine serum (FBS) in the incubator with 5% CO_2 and humidified atmosphere at 30 °C for CCO cells and 37 °C for MCF-7 cells.

The effect of synthesized DESs on cell proliferation was examined by the WST-1 assay, which is a modification of the classical MTT test (Mosmann, 1983), according to the manufacturer's instructions. Briefly, CCO and MCF-7 cells were seeded in 96-well plates at a density of 5×104 cells per well in 100 μL of media. After overnight incubation, cells were treated with tested compounds (DESs, DESs′ components or organic solvents) and incubated for 72 h, wherein the nominal tested concentrations were from 1 mg L^{-1} to 2000 mg L^{-1}. Following exposure, 10 μL of tetrazolium salt WST-1 was added to each well and cells were incubated for another 4 h, after which absorbance at 450 nm was measured on the microplate reader (Tecan, Switzerland). Cell viability was expressed as percentage of treated cells versus control cells, and corresponding EC_{50} values, defined as the concentration of tested compounds that resulted in 50% growth inhibition, were calculated from the dose–response curves using equations of best-fitted trend-lines. The pH values of DES solutions, as well as the solutions of forming compounds, in DMEM were measured by digital pH meter (Mettler Toledo, Switzerland).

Light microscopy was used to observe morphological changes during the exposure. Therefore, CCO cell were seeded (1×105 cells mL^{-1}) in 6-well plate and exposed to EC_{50} concentration, if determined, or to the highest tested concentration of DESs (2000 mg L^{-1}). Images of CCO cells were taken using an inverted microscope (Carl Zeiss, Germany) and Dino-Eye digital camera (AnMo Electronics Co., Taiwan).

2.4. Phytotoxicity assay

Prior to germination, wheat seeds (*T. aestivum*) were sterilized in 1% NaOCl (v/v) for 30 min and then thoroughly washed 3 times with distilled water. Seeds were incubated for 24 h in the darkness at 24 °C. After that, 30 seeds were placed in a Petri plate ($d=15$ cm) on a piece of filter paper covered with a thin layer of cotton wool and moistened with 30 mL of DESs′ solutions. The treatment concentrations of DESs′ were set to 100, 500, 1000, 5000, 10,000 and 20,000 mg L^{-1}. The pH values of prepared DESs aqueous solutions were also measured by digital pH meter. The control was maintained with 30 mL of distilled water. Wheat seedlings were grown under controlled conditions at 24 ± 1 °C with a shift cycle of 14 h/day and 10 h/night. The solutions were daily renewed to keep DES concentration stable. All treatments were replicated 3 times. After 7 days of exposure, seedlings were harvested and the effect of DESs on germination and early growth of wheat was determined. Wheat seeds were considered germinated when both the plumule and radicle extended to more than 3 mm from their junction. Results were expressed as germination

inhibition, shoot height and root length inhibition in comparison to control and the corresponding EC_{50} values were calculated. In order to get an insight into oxidative stresses and antioxidative enzyme responses to DES treatments, the degree of membrane peroxidation in leaves, photosynthetic pigments content and antioxidant enzyme activities were measured spectrophotometrically (Thermo Scientific, USA) after ChCl:OA treatment.

2.4.1. Estimation of lipid peroxidation

Fresh tissue of wheat leaves (200 mg) was homogenized in 5 mL of 0.1% trichloroacetic acid (TCA) (w/v). The supernatant was used for malondialdehyde (MDA) content assays. The levels of lipid peroxidation in the wheat seedling were evaluated by measuring MDA using the thiobarbituric acid method (Heath and Packer, 1968).The MDA content was calculated according to its extinction coefficient of 155 mM^{-1} cm^{-1} and expressed as mmol g^{-1} FW.

2.4.2. Determination of chlorophyll content

Chlorophyll content was determined according to the method reported by Arnon (1949). The content of chlorophyll was determined by measuring the absorbance at 663 nm and 645 nm. The contents of chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*) and total chlorophyll were expressed as μg chlorophyll g^{-1} FW.

2.4.3. Antioxidant enzyme extraction and assay

For enzyme analysis, fresh seedlings of wheat were homogenized with 0.1 g hydrated polyvinylpolypyrrolidone in 100 mM potassium phosphate buffer solution (PBS, pH=7.0) that included 1 mM ethylenediaminetetraacetic acid using pre-chilled mortar and pestle. The supernatant was used for enzyme activity and protein content assays. Total soluble protein contents of the enzyme extracts were estimated according to Lowry et al. (1951) using bovine serum albumin as a standard.

The superoxide dismutase (SOD) activity was determined by measuring the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT) using the method of Beauchamp and Fridovich (1971). The increase in absorbance due to formazan formation was measured at 560 nm. One unit of SOD activity was defined as the amount of enzyme that inhibits the NBT photoreduction by 50%. The activity of SOD was expressed as unit mg^{-1} protein.

The guaiacol peroxidase (GPX) activity was measured following the method of Chance and Maehly (1955). GPX activity was estimated by the increase in absorbance of oxiguaiacol at 470 nm (ε=26.6 mM^{-1} cm^{-1}) and was expressed as nmol of guaiacol oxidized mg^{-1} protein min^{-1}.

The catalase (CAT) activity was determined by decomposition of H_2O_2 and was measured spectrophotometrically by assessing the decrease in absorbance at 240 nm (Aebi, 1984). The activity was calculated using the extinction coefficient (ε=40 mM^{-1} cm^{-1}) and was expressed as mmol of H_2O_2 decomposed mg^{-1} protein min^{-1}.

The ascorbate peroxidase (APX) activity was measured according to Nakano and Asada (1981). APX activity was determined by following the decrease in absorbance of ascorbate at 290 nm (ε=2.8 mM^{-1} cm^{-1}) and was expressed as nmol of ascorbate oxidized mg^{-1} protein min^{-1}.

2.5. Biodegradability in water

The Closed Bottle Test described in the OECD Test Guidelines (OECD 301D, 1992) was used to determine the ready biodegradability of investigated DESs. The Closed Bottle Test described in the OECD Test Guidelines (OECD 301D, 1992) was used to determine the ready biodegradability of investigated DESs. The Closed Bottle Test described in the OECD Test Guidelines (OECD 301D, 1992) was used to determine the ready biodegradability of investigated DESs. The pass level for ready biodegradability for this method (Clossed Bottle Test) is 60% removal of theoretical oxygen demand (ThOD) in a 10-d window within the 28-d period of the test. The solution of each DES individually in mineral medium, at 100 mg L^{-1}, was inoculated with effluent from an urban wastewater treatment plant. A control with inoculums, without DES addition, was conducted as the oxygen blank, while as a reference substance sodium acetate was used. During the standard test period of 28 days, the test bottles were kept in the dark at constant temperature of 20 °C. Periodically, every 7 days, the biological oxygen demand (BOD), was determined. The mineral medium was prepared of 8.5 mg L^{-1} KH_2PO_4, 21.75 mg L^{-1} K_2HPO_4, 26.65 mg L^{-1} Na_2HPO_4, 0.5 mg L^{-1} NH_4Cl, 36.4 mg L^{-1} $CaCl_2 \times 2H_2O$, 22.5 mg L^{-1} MgSO4 × $7H_2O$, and 0.25 mg L^{-1} FeCl3 × $6H_2O$, dissolved in water. The biodegradability of investigated DESs was conducted according OECD 301 D guidelines in manometric respirometers (WTW, OxiTop, Weilheim, Germany).

2.6. Statistical analysis

All experimental results were statistically analyzed using the Statistica 9.1 software. All the experiments were conducted at least in triplicate. Data in the text and tables were expressed as mean ± standard deviation (± SD), and error bars in the figures indicate standard deviation. Differences between means were analyzed by the ANOVA test followed by the post hoc Tukey's test. A significant difference was considered at the level of $p < 0.05$.

Table 1
The EC_{50} values for CCO and MCF-7 cells following exposure to choline chloride based DESs, individual components used for DESs preparation and organic solvents. Values are mean (n=3) ± SD.

Tested compound		CCO cells		MCF-7 cells	
		EC_{50} (mg L^{-1})	EC_{50} (mM)	EC_{50} (mg L^{-1})	EC_{50} (mM)
DES	ChCl:Glc	> 2000	> 10	> 2000	> 10
	ChCl:Gly	> 2000	> 10	> 2000	> 10
	ChCl:OA	218.7 ± 18.23	1.64 ± 0.14	558.98 ± 54.32	4.19 ± 0.41
Individual components	Choline chloride	> 2000	> 10	> 2000	> 10
	Glycerol	> 2000	> 10	> 2000	> 10
	Glucose	> 2000	> 10	> 2000	> 10
	Oxalic acid	633.02 ± 58.22	5.02 ± 0.46	1668.33 ± 121.54	13.23 ± 0.96
Organic solvents	Ethanol	> 2000	> 10	> 2000	> 10
	Dichloromethane	> 2000	> 10	> 2000	> 10
	Dimethyl sulfoxide	> 2000	> 10	> 2000	> 10
	Toluene	> 2000	> 10	> 2000	> 10

3. Results and discussion

3.1. Cytotoxicity assessment of DESs

Within this work the ChCl-based DESs were assayed for cytotoxicity in fish (CCO) and human (MCF-7) cell line by the WST-1 cell proliferation assay after 72 h of treatment. The cytotoxicity results were interpreted and presented as EC_{50} values (Table 1). According toUFT Merck ILs Biological Effects Database (http://www.il-eco.uft.uni-bremen.de) tested ChCl-based DESs show low ($EC_{50} > 5$ mM) to moderate (0.1 mM $< EC_{50} <$ 5 mM) cytotoxicity for CCO and MCF-7 cells. The lowest EC_{50} values were determined for ChCl:OA and were 1.64 mM (218.7 mg L^{-1}) and 4.19 mM (558.98 mg L^{-1}) in CCO and MCF-7 cells, respectively, meaning that ChCl:OA exhibited moderate cytotoxicity. In both cell lines ChCl:Glc and ChCl:Gly did not cause 50% of inhibition in the range of tested concentrations (1–2000 mg L^{-1}). Therefore, we declared their EC_{50} values to be >2000 mg L^{-1} i.e. >5 mM representing the low cytotoxicity for those DESs.

CCO cells are slightly more sensitive to ChCl:OA than MCF-7 cells although the obtained EC_{50} values are in the same range of cytotoxicity, which was consistent to our previous work on cytotoxic effects of imidazolium ILs where EC_{50} did not differ much between fish CCO and human HeLa cells (Cvjetko et al., 2012). Nevertheless, it should be bared in mind that specific mechanisms of action can depend on cell type or origin of cell line and for the more comprehensive study on DESs cytotoxicity evaluation, bigger panel of cell lines would be more than desirable. Since ILs, whose toxic effects are well investigated in different human and mammalian cell lines (Ranke et al., 2004; Stepnowski et al., 2004; Frade et al., 2009; Samori et al., 2010; Peric et al., 2013) cytotoxicity data on DESs with literature data on ILs was compared. In our previous work on imidazolium-based ILs (Radošević et al., 2013) most of the tested ILs possessed moderate or low cytotoxicity toward CCO cells, whereas a high level of cytotoxicity was only observed for 1-heptyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide and 1-decyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide. Comparing the EC_{50} values obtained in CCO cells for previously mentioned ILs and herein presented EC_{50} values, we can conclude that the cytotoxicity of ChCl-based DESs is in overall lower than that of imidazolium-based ILs. Recently, Peric et al. (2013) conducted initial comparative hazard assessment of protic ILs derived from aliphatic amines and organic acids, showing that they are, in terms of cellular toxicity toward IPC-81 cells, much favorable than the classical substituted imidazolium and pyridinium chlorides. Namely, EC_{50} values for protic ILs ranged between 339 and 3311 mg L^{-1}. These values are in a similar range as EC_{50} obtained for DESs in this study, confirming that the toxicity of DESs is by several orders of magnitude lower than that of imidazolium ILs. Similar conclusion can be derived from work of Paiva et al. (2014) who reported about the cytotoxicity of 11 different DESs on L929 cell line. The ChCl-based DESs with tartaric and citric acid were the most deterimental for cell viability, while other were less toxicy than two ILs also evaluated in this work.

Besides biochemical evidence of DESs impact on the growth of CCO and MCF-7 cells, morphological changes were observed in the culture during the treatment. Examination of CCO cells by light microscopy is in good agreement with cytotoxicity results and showed no inhibitory effect on CCO cells treated with ChCl:Glc and ChCl:Gly (Fig. 1b and c), which were well attached and showed typical fibroblastic morphology as control CCO cells (Fig. 1a). Exposure to EC_{50} concentration of ChCl:OA for 72 h resulted in obvious reduction of cell number and decreased monolayer density (Fig. 1d). Furthermore, during the treatment with ChCl:OA at a concentration of ≥ 200 mg L^{-1}, the formation of calcium-oxalate crystals was observed, as seen in Fig. 1d, what can be explained by the fact that DME medium, used for culturing of CCO cells, contains Ca^{2+} ions. The same was visualized when an individual component, i.e. oxalic acid was examined, yielding an interesting fact that the formation of calcium oxalate crystals occurs around EC_{50} concentration. Therefore, we can assume that

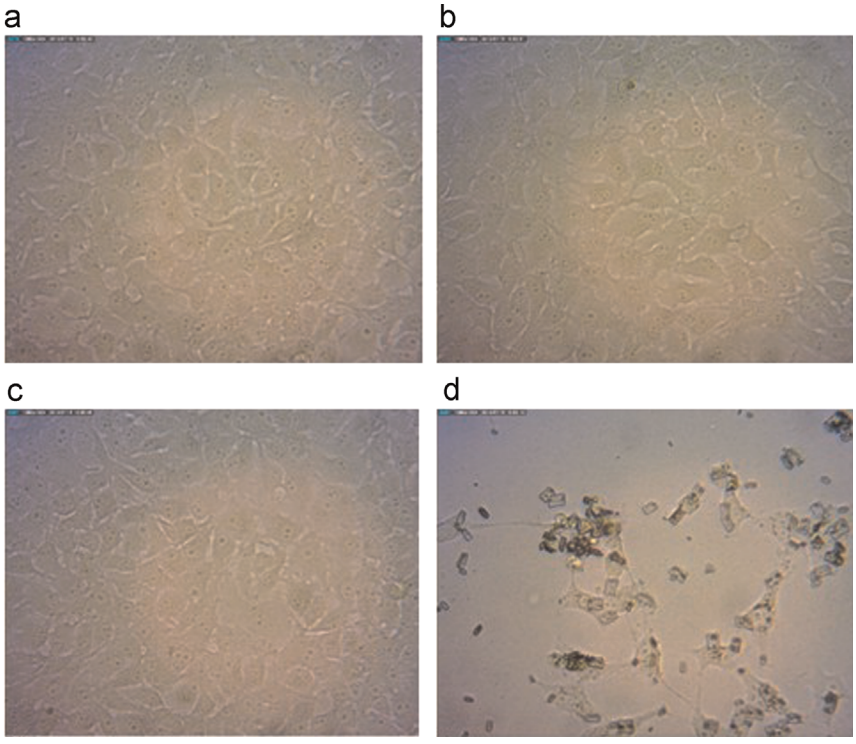

Fig. 1. Morphology of CCO cells photographed under a light microscope with the magnification 400 ×. Control CCO cells (a), CCO cells treated for 72 h with the highest tested concentration (2000 mg L^{-1}) of ChCl:Gly (b), and ChCl:Glc (c), and with EC_{50} concentration of ChCl:OA (d).

the inhibitory properties of ChCl:OA can be related to the formation of cell damaging calcium-oxalate crystals. Besides that, the cell proliferation might be affected by the sudden drop of pH in the culture medium following the ChCl:OA addition. Decrease in pH value from 7.4 to 6.5 was observed after addition of ChCl:OA aqueous solution in DMEM in order to obtain 2000 mg L^{-1} DES solutions. Although most cell lines are tolerant of mild acidic conditions in terms of survival, it is well known that environmental pH change can modify cellular proliferation and metabolic properties.

To explore the difference in inhibitory effect between ChCl-based DESs and individual components, the cytotoxicity of ChCl, glucose, glycerol and oxalic acid was also assayed and is illustrated in Table 1. EC_{50} values for all compounds were found to be higher than 2000 mg L^{-1}, except for oxalic acid (EC_{50} was 633.02 and 1668.33 mg L^{-1} for CCO and MCF-7 cells, respectively). If we express those mass concentrations as their molar equivalents (mM), all EC_{50} values are higher than 5 mM which means that DES forming compounds possess low cytotoxicity to CCO and MCF-7 cells. Comparison of the EC_{50} values determined for ChCl:OA with those of individual compounds (ChCl and oxalic acid) confirms the results of Hayyan et al. (2013a, 2013b) who reported that DESs cytotoxicity is higher than their individual components. Also, they conducted an experiment in order to determine if the increase in cytotoxicity is due to the occurring synergetic effect of salt and hydrogen bond donor, or due to the formation of the DES. It was very clear that there is no significant synergetic effect due to the presence of salt and hydrogen bond donor in aqueous solution indicating that toxicity effects is due to DES formation. The authors explain this difference by the charge delocalization occurring through hydrogen bonding between organic salt and hydrogen bond donors, since it is well known that chemicals having delocalized charges are more toxic than chemicals with localized charges (Hayyan et al., 2013b).

Since DESs are considered to be the *green* replacement for currently used organic solvents it was worth to assess the cytotoxicity of some conventional solvents in CCO and MCF-7 cells (Table 1). Therefore, the EC_{50} values for ethanol, dichloromethane, dimethyl sulfoxide and toluene were determined in the same way as described previously for DESs, EC_{50} values of all five conventional solvents were found to be higher than 2000 mg L^{-1} (>5 mM) in both cell lines indicating low cytotoxicity. Obtained EC_{50} values are in agreement with the reports from other authors (Stepnowski et al., 2004; Ranke et al., 2004) indicating that the tested DESs appear to have similar inhibitory effect in vitro than some commonly used industrial solvents. However, the most significant environmental benefit of using DESs as solvents results from their low vapor pressure and non-flammability, which surely makes them preferable to volatile and flammable organic solvents.

3.2. Phytotoxicity of DESs

In order to evaluate the impact of synthesized DESs on wheat (*T. aestivum*) as a widely used agricultural plant, wheat seeds were treated with different concentrations of DES and growth parameters (germination inhibition, shoot height and root length inhibition in comparison to control) were recorded and the corresponding EC_{50} values were calculated (Table 2). Differences among tested DESs were pronounced. The EC_{50} values for germination inhibition of ChCl:Gly, ChCl:Glc were above 20,000 mg L^{-1}, while the values for ChCl:OA were about 4 times lower. In general, seed germination EC_{50} values for tested DESs were higher than 5000 mg L^{-1}, indicating their low toxicity (Studzińska and Buszewski, 2008; Cvjetko Bubalo et al., 2014b). Furthermore, early growth of seedling was more sensitive than the seed germination since the corresponding EC_{50} values for growth were at least 5.5 lower than those for germination inhibition. Also, once the seeds germinated the inhibition of root growth was more pronounced than the growth of shoots, which could be explained by direct contact between DES and root. Among tested DESs, the most pronounced inhibitory effect on early development of wheat seedlings was shown by ChCl:OA, followed by ChCl:Gly and ChCl:Glc. The highest growth inhibition of ChCl:OA on wheat could be due to the very low pH values of prepared solution. The pH value of aqueous solution containing 10,000 mg L^{-1} of ChCl:OA was 2.08. pH value of ChCl:Gly and ChCl:Glc aqueous solution were around 6.0, indicating that the degree of inhibition could be related to pH of DES solution, what we have already indicated in cytotoxicity assay with CCO and MCF-7 cells. In addition, as it was expected when comparing phytotoxicity of DESs and 'classic' ILs, such as imidazolium based ones (Wang et al., 2009; Cvjetko Bubalo et al., 2014b), DESs showed much lower phytotoxicity indicating their lower environmental impact.

Evidently, DESs influenced early development of wheat seedling causing growth inhibition and therefore we were interested in how the antioxidant defense machinery is coordinated with such a wheat response. We monitored changes in antioxidative enzyme activities after ChCl:OA treatment. Firstly, in order to estimate the extent of oxidative stress, the content of MDA as an indicator of lipid peroxidation caused by enhanced levels of ROS was measured (Fig. 2a). In general, the higher the DES toxicity, the increase in MDA content was greater and significantly increased MDA content was noticed for all treatments higher than 500 mg L^{-1} ($p<0.05$), indicating that antioxidant enzymes cannot fully remove ROS during DES exposure, leading to their accumulation. Furthermore, we measured photosynthetic pigments (chlorophyll *a*, chlorophyll *b* and total chlorophyll) as other biochemical stress indicators (Ashraf and Harris, 2013). We found a good correlation between chlorophyll content and growth inhibition, indicating that among a multitude of physiological processes, photosynthesis is one of the key phenomena substantially contributing to the plant growth and development under stress (Fig. 2b). Similarly, a decrease in chlorophyll content has been observed in other plants species after exposure to ILs, also indicating strong relationship between chlorophyll content and plant growth (Wang et al., 2009; Zhang et al., 2012b; Cvjetko Bubalo et al., 2014b). However, alteration in chlorophyll content could be due to biosynthesis inhibition and degradation of chlorophyll caused by ROS generation and chloroplast membrane disruption. Furthermore, the decrease in the photosynthetic pigment content was consistent with the increase of MDA content, indicating that MDA accumulation may contribute to the photosynthetic system damage (Ashraf and Harris, 2013).

Table 2
The EC_{50} values for wheat following exposure to choline chloride based DESs. Values are mean ($n=3$) ± SD.

DES	Germination		Shoot growth		Root growth	
	EC_{50} (mg L^{-1})	EC_{50} (mM)	EC_{50} (mg L^{-1})	EC_{50} (mM)	EC_{50} (mg L^{-1})	EC_{50} (mM)
ChCl:Glc	> 20,000	> 100	1545.45 ± 123.25	9.85 ± 0.78	848.99 ± 63.68	5.41 ± 0.40
ChCl:Gly	> 20,000	> 100	3697.57 ± 213.50	32.81 ± 1.89	3248.66 ± 153.77	28.83 ± 1.36
ChCl:OA	5325.26 ± 320.12	39.94 ± 2.66	489.64 ± 48.55	3.67 ± 0.40	408.48 ± 43.29	3.06 ± 0.45

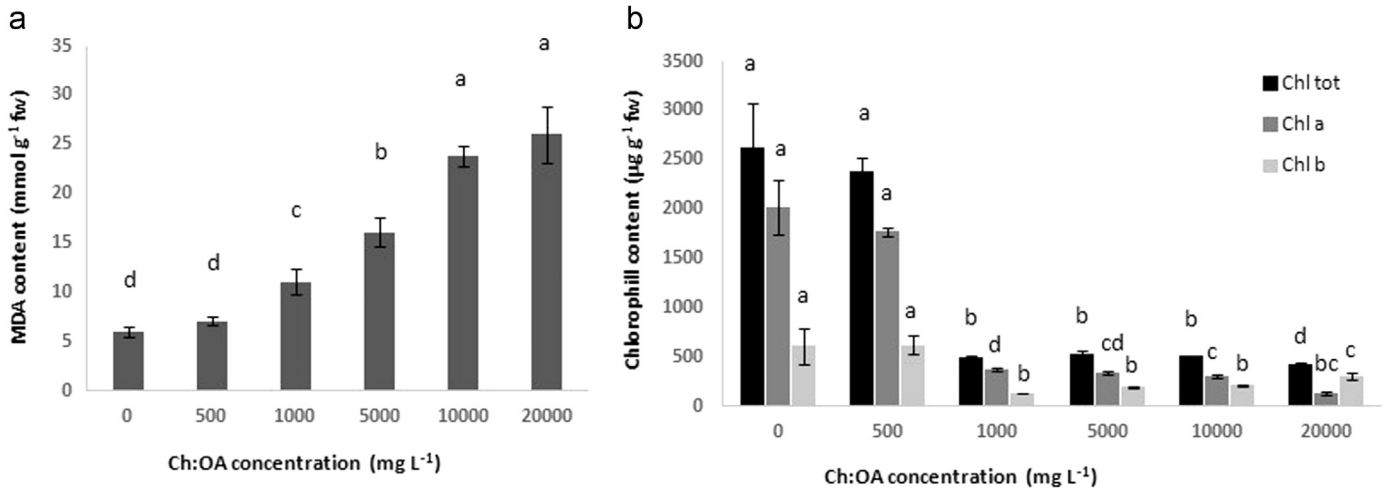

Fig. 2. Effect of ChCl:OA on malondialdehyde (MDA) content (a) and photosynthetic pigment (b). Mean values ($n=3$) ± SD in each column followed by different lower-case letters are significantly different ($P<0.05$) in comparison to control plants as measured by Tukey's HSD test. Chlorophyll *a*-Chl-*a*; Chlorophyll *b*-Chl-*b*, and total chlorophyll-Chl-tot.

In general, adaptive plant responses to stress indicate that the balance between the ROS produced and the oxidative system including fine tuning of antioxidative enzyme activities is crucial for plant survival and adaptation (Ranke et al., 2004; Sharma et al., 2012). As expected, differences were found in the antioxidant enzyme activities after treatments with various concentrations of ChCl:OA (Fig. 3). The activity of SOD in wheat seedling increased with the increased concentration up to 1000 mg L^{-1} of ChCl:OA

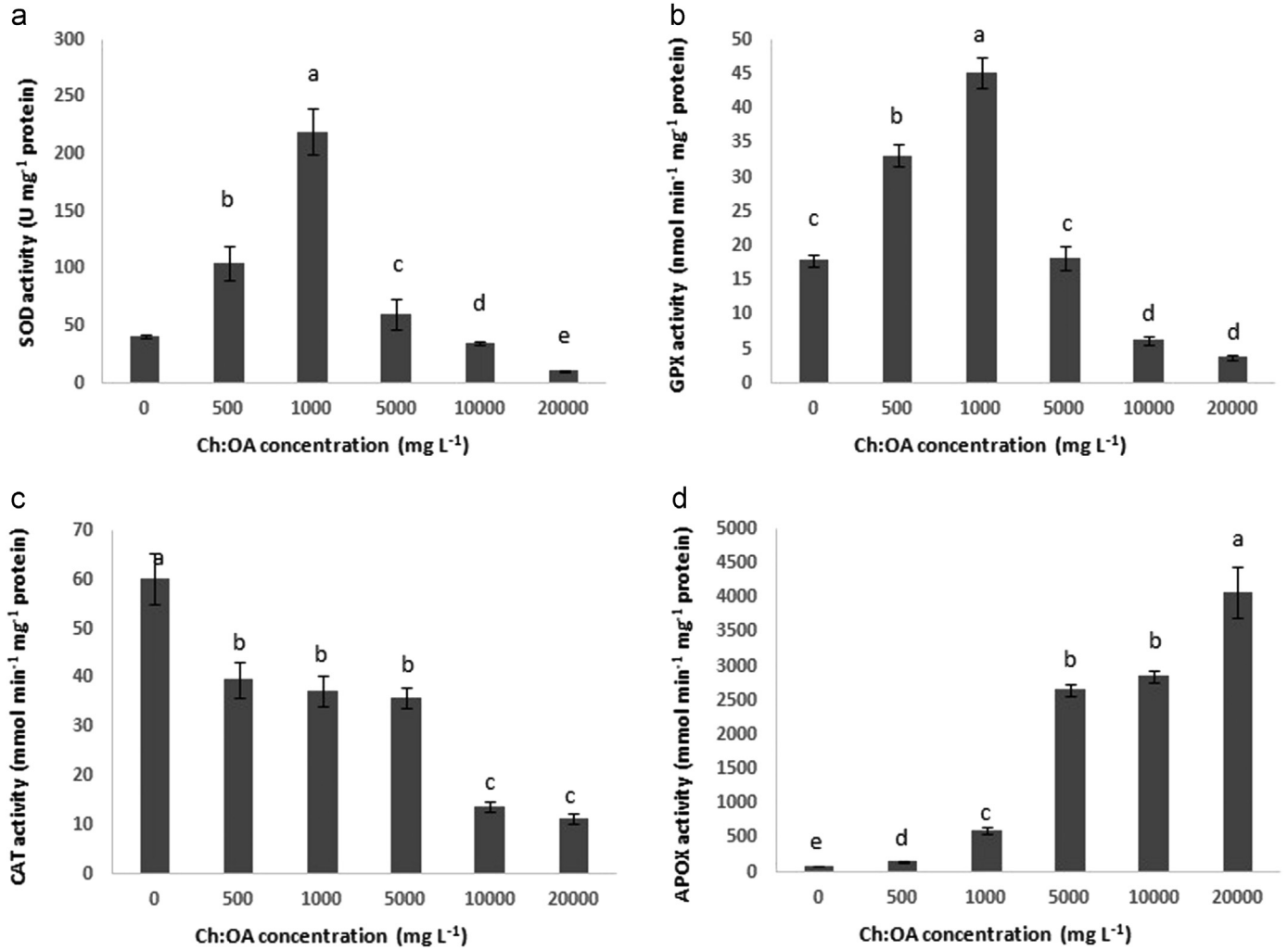

Fig. 3. Enzymatic activities of SOD (a), GPX (b), CAT (c), APX (d) in wheat seedlings treated with ChCl:OA. Mean values ($n=3$) ± SD in each column followed by different lower-case letters are significantly different ($P<0.05$) in comparison to control plants as measured by Tukey's HSD test.

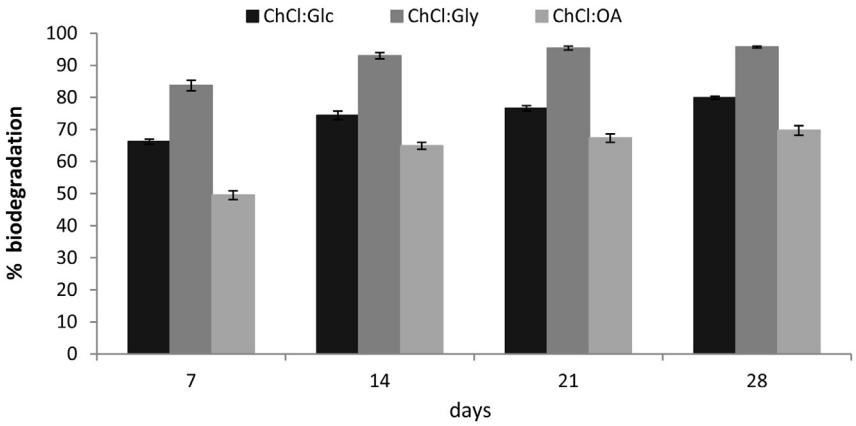

Fig. 4. Biodegradation of DESs determined by the Closed Bottle Test. Values are mean ($n=3$) ± SD.

($p<0.05$) in comparison with control. Later, a decline in SOD activity was noticed, with a value significantly lower ($p<0.05$) compared to control after treatment with 20,000 mg L^{-1} of ChCl:OA. Among the enzyme activities measured, similar behavior was recorded for GPX activity indicating that a concentration higher than the corresponding EC_{50} value for germination inhibition caused a decline of SOD and GPX activity. Furthermore, with increased concentration of ChCl:OA CAT activity decreased while APX activity showed reverse regulation. However, data indicated that treatment with a higher concentration of ChCl:OA caused oxidative stress due to perturbation of antioxidative enzyme activities triggering inhibition of SOD, GPX and CAT activity. This plant response along with poorly seedling performance suggests inefficiencyof wheat defense against treatment with higher ChCl:OA concentrations.

3.3. Biodegradability of DESs

Biodegradation of the substance also plays a very important role in their environmental impact and fate, thus we evaluated aerobic biodegradability of the selected DESs by a standard method according to OECD 301 D guidelines. The DESs were added to aerobic aqueous media inoculated with wastewater microorganisms, and the BOD value was determined periodically (Fig. 4). According to the OECD, the compounds reaching a biodegradation level of 60% in a 10-d window within the 28-d period of the test are considered to pass the biodegradation test (OECD 301D, 1992). In this study, biodegradability of DESs tested was more than 60% and therefore all of them can be referred to as 'readily biodegradable', showing level of biodegradation as follows: ChCl:Gly > ChCl:Glc > ChCl:OA. The highest and the lowest level of biodegradation of 96% and 68% was observed with the ChCl:Gly, and ChCl:OA, respectively. Furthermore, ChCl:Gly and ChCl:Glc already reached 84% and 66% biodegradation level after 7 days. Moreover, biodegradation of ChCl:OA reached over 60% after 14 days.

The high degrees of biodegradation of the tested DESs could be attributed to the components forming them. Namely, ChCl is 'readily biodegradable' according to OECD-criteria (MITI-I Test; BOD measurements) reaching 93% degradation within 14 days (OECD 301D, 1992), while according to safety data sheets hydrogen bond donors used in this study (glucose, glycerol, urea and oxalic acid) are also 'readily biodegradable' in the aquatic environment. The potential of cholinium-based solvents as biodegradable was recently confirmed by Hou et al. (2013) who evaluated the biodegradability of 18 cholinium amino acid ILs via the closed bottle and CO_2 headspace tests using wastewater microorganisms and concluded that all the ILs can be classified as 'readily biodegradable' based on their high levels of mineralization (62–87%). Our results, together with results of Hou et al. (2013) strongly indicate that changing the focus of interest from classical imidazolium and pyridinium ILs toward ILs and DESs from natural sources (e.g. choline, amino acids, sugars, alcohols and organic acids) is a promising approach for development of biodegradable and eco-friendly solvents. Namely, according to literature poor degradation of imidazolium and pyridinium ILs was confirmed by numerous authors (Pham et al., 2010; Cvjetko Bubalo et al., 2014a). Besides, a matter of concern is that some 'readily biodegradable' classical ILs, such as those with long alkyl chains, were simultaneously reported to be highly toxic due to their lipophilic character, while ILs with a shorter alkyl side-chain are safer with respect to (eco)toxicity issues, but pose a higher risk of persistency and mobility due their lack of biodegradability and reduced sorption to organic matter and clay minerals (Cvjetko Bubalo et al., 2014a). This conflict between the toxicity and biodegradability did not occur for most of the cholinium based ILs since low toxicity correlated with good biodegradability (Hou et al., 2013), as well as for DESs tested in this study.

4. Conclusion

The obtained data on cytotoxicity and phytotoxicity, indicate that the three studied ChCl-based DESs possess low cytotoxicity to moderate cytotoxicity and do not inhibit wheat seed germination. Additionally, all DESs were classified as 'readily biodegradable' and a good correlation between toxicity and biodegradability was found, suggesting that ChCl-based DESs have a potential *green* profile and a good prospect for a wider use. However, we encourage further studies in order to provide more comprehensive information about environmental impact of DESs prior to their application at industrial scale. A systematic design of DESs by reasonable selection of their building components might result in even better ecotoxicological profile, while still keeping desired physicochemical properties of the solvents.

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References

Aebi, H., 1984. Catalase in vitro. Methods Enzymol. 105, 121–126.

Anastas, P.T., Warner, J.C., 1998. Green Chemistry: Theory and Practice. Oxford University Press, New York.

Anastas, P.T., Eghbali, N., 2010. Green chemistry: principles and practice. Chem. Soc. Rev. 39, 301–312.

Arnon, D.I., 1949. Copper enzyme in isolated chloroplasts: polyphenol oxidase in *Beta vulgaris*. Plant Physiol. 24, 1–15.

Ashraf, M., Harris, P.J.C., 2013. Photosynthesis under stressful environments: an overview. Photosynthetica 51, 163–190.

Beauchamp, C., Fridovich, I., 1971. Superoxide dismutase: improved assay and an assay applicable to PAGE. Anal. Biochem. 44, 276–287.

Carriazo, D., Serrano, M.C., Gutiérrez, M.C., Ferrer, M.L., del Monte, F., 2012. Deep-eutectic solvents playing multiple roles in the synthesis of polymers and related materials. Chem. Soc. Rev. 41, 4996–5014.

Chance, B., Maehly, A.C., 1955. Assay of catalases and peroxidases. In: Colowick, S.P., Kaplan, N.O. (Eds.), Methods in Enzymology. Academic Press, New York.

Cvjetko Bubalo, M., Radošević, K., Radojčić Redovniković, I., Halambek, J., Gaurina Srček, V., 2014a. A brief overview of the potential environmental hazards of ionic liquids. Ecotoxicol. Environ. Saf. 99, 1–12.

Cvjetko Bubalo, M., Hanousek, K., Radošević, K., Gaurina Srček, V., Jakovljević, T., Radojčić Redovniković, I., 2014b. Imidiazolium based ionic liquids: effects of different anions and alkyl chains lengths on the barley seedlings. Ecotoxicol. Environ. Saf. 101, 116–123.

Cvjetko, M., Radošević, K., Tomica, A., Slivac, I., Vorkapić-Furač, J., Gaurina Srček, V., 2012. Cytotoxic effects of imidazolium ionic liquids on fish and human cell lines. Arh. Hig. Rada. Toksikol. 63, 15–19.

Dai, Y., Witkamp, G.J., Verpoorte, R., Choi, Y.H., 2013a. Natural deep eutectic solvents as a new extraction media for phenolic metabolites in *Carthamus tinctorius* L. Anal. Chem. 85, 6272–6278.

Dai, Y., van Spronsen, J., Witkamp, G.J., Verpoorte, R., Choi, Y.H., 2013b. Ionic liquids and deep eutectic solvents in natural products research: mixtures of solids as extraction solvents. J. Nat. Prod. 22, 2162–2173.

EEA, 2013. Towards a green economy in Europe. EU environmental policy targets and objectives 2010–2050, EEA Report no. 8/2013, pp. 1–48.

EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP), 2011. Scientific Opinion on safety and efficacy of choline chloride as a feed additive for all animal species. EFSA J. 9, 2353 ([15 pp.]).

Frade, R., Rosatella, A., Marques, C., Branco, L., Kulkarni, P., Mateus, N., Afonso, C., Duarte, C., 2009. Toxicological evaluation on human colon carcinoma cell line (CaCo-2) of ionic liquids based on imidazolium, guanidinium, ammonium, phosphonium, pyridinium and pyrrolidinium cations. Green Chem. 11, 1160–1165.

Francisco, M., van den Bruinhorst, A., Kroon, M.C., 2013. Low-transition-temperature mixtures (LTTMs): a new generation of designer solvents. Angew. Chem. Int. Ed. 52, 3074–3085.

Hayyan, M., Hashim, M.A., Hayyan, A., Al-Saadi, M.A., Alnashef, I.M., Mirghani, M.E., Saheed, O.K., 2013a. Are deep eutectic solvents benign or toxic? Chemosphere 90, 2193–2195.

Hayyan, M., Hashim, M.A., Al-Saadi, M.A., Hayyan, A., Alnashef, I.M., Mirghani, M.E., 2013b. Assessment of cytotoxicity and toxicity for phosphonium-based deep eutectic solvents. Chemosphere 93, 455–459.

Hayyan, A., Mjalli, F.S., Alnashef, I.M., Al-Wahaibi, Y.M., Al-Wahaibi, T., Hashim, M.A., 2013c. Glucose-based deep eutectic solvents: physical properties. J. Mol. Liq. 178, 137–141.

Heath, R.L., Packer, L., 1968. Photoperoxidation in isolated chloroplasts. I-kinetics and stoichiometry of fatty acid peroxidation. Arch. Biochem. Biophys. 125, 189–198.

Hou, X.D., Liu, Q.P., Smith, T.J., Li, N., Zong, M.H., 2013. Evaluation of toxicity and biodegradability of cholinium amino acids ionic liquids. PLoS One 8, e59145.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55–63.

Nakano, Y., Asada, K., 1981. Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiol. 22, 867–880.

OECD 301D, 1992D. Guidelines for Testing of Chemicals. Closed Bottle Test. Organisation of Economic Cooperation and Development, Paris.

Paiva, A., Craveiro, R., Arosso, I., Martins, M., Reis, R.L., Duarte, A.R.C., 2014. Natural deep eutectic solvents – solvents for the 21st century. ACS Sustain. Chem. Eng. 2, 1063–1071.

Pham, T.P., Cho, C.W., Yun, Y.S., 2010. Environmental fate and toxicity of ionic liquids: a review. Water Res. 44, 352–372.

Peric, B., Sierra, J., Martí, E., Cruañas, R., Garau, M.A., Arning, J., Bottin-Weber, U., Stolte, S., 2013. (Eco)toxicity and biodegradability of selected protic and aprotic ionic liquids. J. Hazard. Mater. 261, 99–105.

Petkovic, M., Seddon, K.R., Rebelo, L.P., Silva Pereira, C., 2011. Ionic liquids: a pathway to environmental acceptability. Chem. Soc. Rev. 40, 1383–1403.

Radošević, K., Cvjetko, M., Kopjar, N., Novak, R., Dumić, J., Gaurina Srček, V., 2013. In vitro cytotoxicity assessment of imidazolium ionic liquids: biological effects in fish Channel Catfish Ovary (CCO) cell line. Ecotoxicol. Environ. Saf. 92, 112–118.

Ranke, J., Molter, K., Stock, F., Bottin-Weber, U., Poczobutt, J., Hoffmann, J., Ondruschka, B., Filser, J., Jastorff, B., 2004. Biological effects of imidazolium ionic liquids with varying chain lengths in acute *Vibrio fischeri* and WST-1 cell viability assays. Ecotoxicol. Environ. Saf. 58, 396–404.

Ruß, C., König, B., 2012. Low melting mixtures in organic synthesis – an alternative to ionic liquids? Green Chem. 14, 2969–2982.

Samori, C., Malferrari, D., Valbonesi, P., Montecavalli, A., Moretti, F., Galletti, P., Sartor, G., Tagliavini, E., Fabbri, E., Pasteris, A., 2010. Introduction of oxygenated side chain into imidazolium ionic liquids: evaluation of the effects at different biological organization levels. Ecotoxicol. Environ. Saf. 73, 1456–1464.

Sharma, P., Jha, A.B., Shanker Dubey, R., Pessarakli, M., 2012. Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. J. Bot 2012, 1–26.

Stepnowski, P., Skladanowski, A.S., Ludwiczak, A., Laczyńska, E., 2004. Evaluating the cytotoxicity of ionic liquids using human cell line HeLa. Hum. Exp. Toxicol. 23, 513–517.

Studzińska, S., Buszewski, B., 2008. Study of toxicity of imidazolium ionic liquids to watercress (*Lepidium sativum* L.). Anal. Bioanal. Chem. 393, 983–990.

Tang, B., Row, K.H., 2013. Recent developments in deep eutectic solvents in chemical sciences. Mon. Chem. 144, 1427–1454.

Tanneberger, K., 2010. Assessment of chemicals: fish cells as an alternative to whole fish. Eawag. News 68e, 25–27.

Vidal, C., Súarez, F.J., García-Álvarez, J., 2014. Deep Eutectic Solvents (DES) as green reaction media for the redox isomerization of allylic alcohols into carbonyl compounds catalyzed by the ruthenium complex [Ru(η3:η3-C10H16)Cl2)Cl2 (benzimidazole)]. Catal. Commun. 44, 76–79.

Wang, S., Wang, L., Wang, L., Wang, G., Li, Z.H., Wang, J.J., 2009. Effect of 1-butyl-3-methylimidazolium tetrafluoroborate on the wheat (*Triticum aestivum* L.) seedlings. Environ. Toxicol. 24, 296–303.

Zhang, Q., De Oliveira Vigier, K., Royer, S., Jérôme, F., 2012a. Deep eutectic solvents: syntheses, properties and applications. Chem. Soc. Rev. 41, 108–7146.

Zhang, B., Li, X., Chen, D., Wang, J., 2012b. Effect of 1-octyl-3-methylimidazolium bromide on the antioxidant system of Lemna minor. Protoplasma 250, 103–110.