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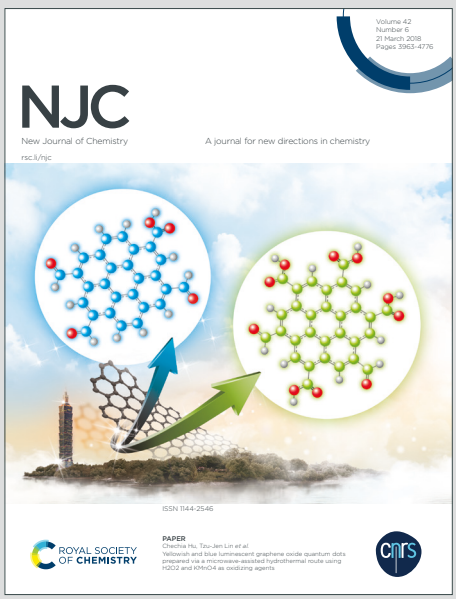

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Insights into the levulinate-based ionic liquid class: synthesis, cellulose dissolution evaluation and ecotoxicity assessment

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Novel ammonium, phosphonium and imidazolium ionic liquids (ILs) based on levulinic acid as the anion were prepared and characterized (NMR, FT-IR, TGA, viscosity). Commercial methyl carbonate precursors were employed to access the target levulinate ILs through a quick, one-step synthetic procedure that does not require the use of halogenated solvents or produce any waste water. The ability of the levulinate ILs to dissolve cellulose was investigated in detail. Provided the right conditions are employed, these ILs display good to remarkable dissolution ability toward their parent polysaccharide. In particular, [EMIM][Lev] is able to dissolve a 29 wt% amount of cellulose at 100 °C, which increases to 38 wt% under reduced pressure. For the best performing IL, several variations of the dissolution conditions, which are known to prevent potential side reactions of the imidazolium cation, have also been tested. The ecotoxicity of the prepared ILs as well as of the previously reported protic levulinate ILs have also been assessed by means of both freshwater and seawater model organisms belonging to different steps of the trophic chain.

Introduction

In the last ten years, biomasses have been regarded as the ultimate option for addressing the challenge of replacing the fast depleting fossil fuels as source of materials and bulk chemicals.[1,2] In particular, cellulose, which is the most abundant biopolymer on earth, has been studied for instance as a source of new polymeric materials (either for packaging or for the production of fabrics),[3] for the obtainment of new fuels (as for instance bio-ethanol)[4] or for the synthesis of useful building blocks (eg levulinic acid, HMF).[5,6] One of the most difficult aspects of cellulose processing is its low (if any) solubility in most solvents due to the inter- and intramolecular hydrogen bonding pattern present in its native structure. Non-derivatives solvents for cellulose have been studied since the late 1800s, and *N,N*-dimethylacetamide/lithium chloride (DMAc/LiCl)[7] and *N*-methylmorpholine-*N*-oxide, which is used in the so-called Lyocell process,[8] achieved a certain notoriety. In 2002, a seminal paper by Rogers *et al.*[9] illustrated for the first time how the ionic liquids (ILs) research area could be applied to the field of cellulose dissolution and thus modification under homogenous conditions. ILs are organic salts that are liquid at or below 100 °C. They are composed by an organic cation and an organic or inorganic anion. The main properties of ILs which attracted the interest of the research community from the beginning are their low flammability, the absence of vapor pressure, and their wide electrochemical window.[10] Nowadays, far beyond these initial studies aimed at finding alternative to volatile organic solvents, ILs are investigated in a great range of applications such as electrochemical[11] or analytical[12] applications, transformation of biopolymers,[13-16] or development of high heat storage materials (in this case often dicationic ILs[17] are employed). It has been estimated that up to 10^{18} different ILs are possible by combining known anions and cations, which allows for tailoring their properties to a specific process and gave them the reputation of "designer solvents".[18]

One of the recent trends in the ILs field is to replace at least one of the traditional petroleum-derived constituent ions with natural or bio-based ions.[19] In this regards, surprisingly few studies have been performed in which levulinic acid (LA) is used as the anion constituent of ILs ([BMIM][Lev], ammonium Lev, and cholinium Lev).[20-23] LA can be prepared from cellulose through an acid-catalyzed one-pot reaction (Figure 1)[24] and has been recognized by the US Department of Energy as one of the top 10 carbohydrate-derived target chemicals.[25] Acid catalysts able to further optimize the conversion of cellulose into LA are continuously developed.

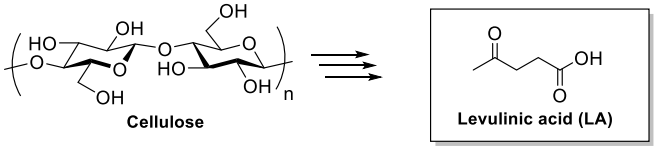

Figure 1. One-pot acid catalyzed conversion of cellulose into levulinic acid (LA).

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Apart from its cellulose-derived nature, LA bears a ketone functional group in addition to the carboxylic group. Ketone functionalized ILs have been recently exploited as alcohol "capture and release" systems with possible fragrance delivery applications,[26] and for selective tantalum extractions.[27] These applications are both striking examples of task specific ionic liquids (TSILs).[28] Recently, we reported the preparation of protic levulinate ILs (Lev PILs) carried out by adding levulinic acid to DBU or DBN, and we tested them as cellulose dissolution and functionalization media.[29] Interestingly enough, almost at the same time, a paper by Buxing Han *et al.* was published which showed instead the promising catalytic activity of a levulinate-based phosphonium ionic liquid in transforming CO_2 into alkylidene cyclic carbonates,[30] thus confirming the potential and the interest towards this kind of biomass-derived ILs.

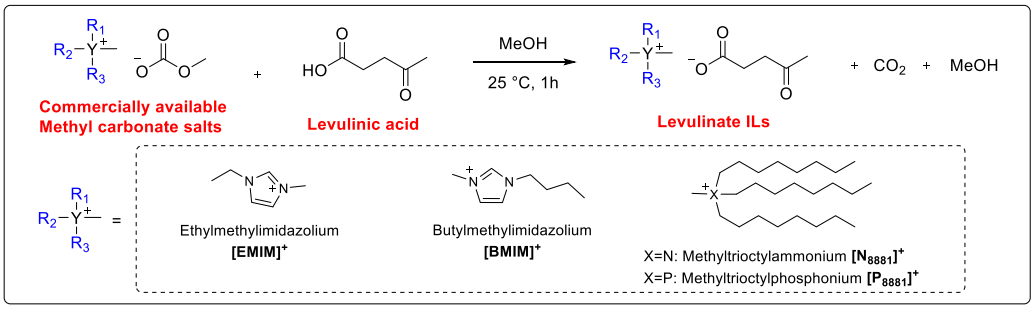
As part of an ongoing project aimed at the development of bio-based ionic liquids through the exploitation of environmentally friendly synthetic routes, we present here the synthesis of novel levulinate ILs. These compounds were prepared *via* a synthetic approach[31] that overcomes the drawbacks of previous strategies and has the potential to be scaled-up. It is worth pointing out that these latter aspects are often overlooked when new ILs are proposed. Levulinate ILs were characterized and studied as potential media for the dissolution of cellulose in view of gauging the compatibility of the levulinate anion in this process as a partner for the most investigated cation types (ammonium, phosphonium and imidazolium). [EMIM][Lev] displayed remarkable cellulose dissolution capacity. Hence, a few critical aspects of the process (eg recyclability of the IL, performances in the presence of potential pollutants) have been further studied for this IL. Finally, the ecotoxicity profiles of the proposed Lev ILs as well as of the Lev PILs recently prepared have been assessed. Taken together, our findings indicate new avenues in the field of novel biomass-based ILs (synthesis and ecotoxicity profiles) and anticipate their potential application in cellulose dissolution processes.

Results and discussion

Synthesis of levulinate ILs - Apart from having been barely studied, not a great deal of attention had been previously paid to the synthesis of levulinate ILs. This aspect is one of the emerging critical issues of the life-cycle of an IL, which is often overlooked.[32-34] A potential easy alternative to the traditionally-used exchange resin method or silver salts-based method for the preparation of carboxylate ILs is represented by using methyl carbonate ILs. A variety of methyl carbonate salts, as methanol or aqueous solutions, are nowadays commercially available and produced in ton scale by Proionic GmbH. The synthetic step that from these material leads to the desired carboxylate IL is quite simple and consists of the combination of such salts with an equimolar amount of an acid. A neutralization reaction easily occurs, which results into the decomposition of the methyl carbonate (into CO_2 and methanol) with the concomitant formation of the target IL. Following this procedure, [EMIM][Lev], [BMIM][Lev], [N_{8881}][Lev] and [P_{8881}][Lev] (Scheme 1) were prepared in quantitative yields by removing methanol under reduced pressure. The clear viscous liquids obtained were further dried at 50 °C for at least 8 h to ensure that no reaction solvent was left behind. The only operational precaution required for the correct implementation of this method is the careful determination of the amount of the methyl carbonate in the solution by titration before use. This allows for avoiding the addition of sub- or super stoichiometric carboxylic acid amounts which would affect the final IL purity.

Apart from their easy conversion into carboxylate ILs and the potential to scale up the reaction, methyl carbonate ILs are interesting also for other aspects. The absence of residual halides (chlorine or bromine) in the final IL, potentially arising from the metathesis reaction and having a well-known negative impact on rheological properties of ILs, can be ensured. Furthermore, tedious and not easily scalable procedures based on the use of silver salts or exchange resins, which have been followed for instance in the previous preparation of [BMIM][Lev],[20] can be avoided.

Characterization – FT-IR and NMR analyses (please refer to the Supporting Information file) confirmed the structures of the Lev ILs. In particular, the ^{1}H-NMR spectra ascertained the purity of the prepared ILs and the 1:1 ratio between cation and anion. As for the previous imidazolium long chain fatty acids ILs, which were prepared following the methyl carbonate procedure,[31] signals arising from carbene side products were not detected. The FT-IR spectra for Lev ILs (Fig S11-S14) all show the characteristic bands of the levulinate anion, namely the asymmetric and the symmetric stretching of the carboxylate group (1582-1566 cm^{-1} and 1378-1360 cm^{-1} respectively) and the C=O stretching (1712-1704 cm^{-1}).



Scheme 1. Syntheses of levulinate ILs

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As for the cation, [EMIM][Lev] and [BMIM][Lev] present the methyl bending at 1378 cm^{-1} and 1374 cm^{-1}, which overlap with the symmetric stretching band of the carboxylate, and the in-plane C_{Im} bending at 1165 cm^{-1} and 1163 cm^{-1}, respectively. $[N_{8881}][Lev]$ and $[P_{8881}][Lev]$, due to the long alkyl chains, present the well distinguishable methyl and methylene symmetric and asymmetric stretching at 2955-2855 cm^{-1}.

It is of primary importance to determine the safe and correct operating temperature range for each IL to understand its suitability for a given application.[35-37] Therefore, the thermal stabilities of the synthesized Lev ILs were investigated and compared, when possible, with their OAc analogues. It is well-known that the experimental conditions (eg sample mass, heating rate, nature of the atmosphere and atmosphere flow rate) can affect the results obtained with this technique.[38] The following experimental conditions were used: mass set at 15−18 mg, N_2 atmosphere with flow rate 100 mL/min, platinum pans, and a 10 °C/min heating rate. The T_{peak} values, which refer to the highest peak found in DTG curves, are calculated based on the average values of three distinct temperature-ramped TGA experiments, which were performed for each IL. The two

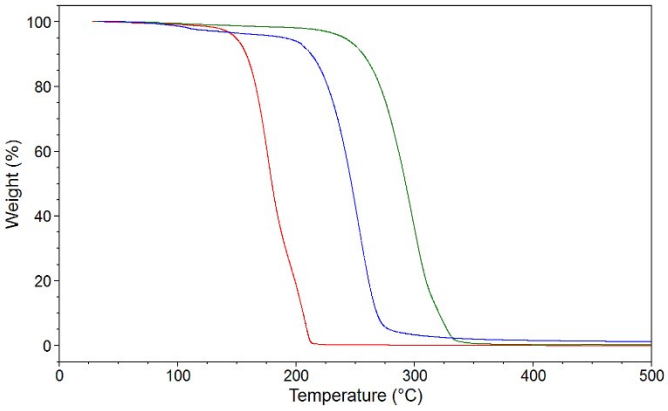

Figure 2. Temperature-ramped TGA thermographs with heating rate of 10 °C/min of [EMIM][Lev] in blue, $[N_{8881}][Lev]$ in red, $[P_{8881}][Lev]$ in green

imidazolium ILs showed a similar thermal stability (T_{peak} 252,6 °C for [EMIM][Lev] and 249,2 °C for [BMIM][Lev]) which was practically the same of their respective OAc analogues (T_{peak} 244 °C for [EMIM][OAC] and 242 °C for [BMIM][OAc]).[39] As expected, the least and the most stable ILs were found to be respectively $[N_{8881}][Lev]$ (T_{peak} 182,3 °C) and $[P_{8881}][Lev]$ (T_{peak} 298,5 °C), in agreement with our previous findings.[31] A comparison of the temperature-ramped TGA thermographs of the Lev ILs is reported in Figure 2 (the TGA thermograph of [BMIM][Lev] is omitted for clarity as it is very similar to that of [EMIM][Lev], please check the Supporting Information file).The viscosity values as a function of temperature for the prepared ILs (and for mixed systems with DMSO in the case of $[N_{8881}][Lev]$ and $[P_{8881}][Lev]$) are reported in Table 1. Viscosity was determined in a temperature range of 20 to 80 °C by applying 15 different shearing rates between 1 s^{-1} and 100 s^{-1}.

As expected, [BMIM][Lev] displayed higher viscosity values when compared with [EMIM][Lev] at all temperatures, which can be explained by considering the longer alkyl substituent on the imidazolium cation. For $[N_{8881}][Lev]$ and $[P_{8881}][Lev]$, the viscosity values are in line with what observed before by us.[31] The latter IL displays a lower viscosity at all temperatures when compared to the ammonium IL as a consequence of the different size of the two cations. As expected, the addition of DMSO (50 wt%) drastically reduced the viscosity of both systems.

Dissolution of cellulose – The dissolution of microcrystalline cellulose (MCC) was studied as a potential application of the prepared levulinate ILs. As said in the introduction, there is a great interest in understanding the scope of IL media for the dissolution and modification of cellulose as well as other biopolymers. Several ILs and different dissolution conditions have been tested in the last two decades.[40-46] It is also worth mentioning that together with the cellulose dissolution limit, other parameters are of great interest for understanding the suitability of an IL for a given application such as for instance the recyclability of the system, its (eco)toxicity, the rheological

Tab 1. Viscosity data for ILs (mPa·s) measured at temperatures ranging from 20 to 80 °C.

IL	Temperature (°C)			
	20	40	60	80
[EMIM][Lev]	244,5 ±1,21	68,64 ±0,27	27,27 ±0,31	13,75 ±0,15
[BMIM][Lev]	396,8 ±5,32	94,66 ±0,63	32,78 ±0,20	17,00 ±0,03
$[N_{8881}][Lev]$	1471 ±8,90	352,2 ±2,83	107,1±0,38	48,46 ±0,25
$[N_{8881}][Lev]$/ DMSO 1:1	20,68 ±0,41	9,50 ±0,06	5,67 ±0,06	<5
$[P_{8881}][Lev]$	507,1 ±1,36	156,3 ±0,56	61,74 ±0,38	29,23 ±0,16
$[P_{8881}][Lev]$/ DMSO 1:1	7,28 ±0,09	<5	<5	<5

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properties of IL-cellulose solutions at different loadings and the crystallinity of regenerated cellulose. For what concern levulinate ILs, previous studies showed the significant dissolution power toward MCC of short-chain ammonium ILs,[22] and protic ILs.[29] The new compounds proposed here represent instead the levulinate version of some of the mostly investigated systems in this area (imidazolium based ILs and long chain phosphonium based ILs). Therefore, by performing the cellulose dissolution study, a broader picture of the suitability of the levulinate anion for this application would be gained.

To determine the MCC dissolution limit of each IL, we followed a previously reported procedure.[29] Briefly, a rough evaluation of this limit was first obtained by adding consecutive 2 wt% amounts of MCC to the selected IL, which was kept under magnetic stirring at a certain temperature. Each new portion of MCC was added only after complete dissolution (visual inspection). The precise dissolution limit was then identified by repeating the dissolution process and adding 0,5 wt% of MCC when approaching the previously roughly estimated dissolution limit. This second part of the procedure was repeated in triplicate and was confirmed by visual inspection and optical microscopy (please refer to the Supporting Information file). The dissolution data for the Lev ILs are summarized in Table 2 [wt% dissolution capacity refers to grams of MCC per grams of IL (or IL/DMSO solution), multiplied by 100; molar dissolution capacity refers to moles of anhydroglucose unit (AGU) per moles of IL]. Taking into account that the levulinate anion is common to all ILs, the cellulose dissolution capacity of the four Lev ILs can be discussed by dividing them into two groups on the basis of the cation structure: imidazolium Lev ILs and long alkyl chain Lev ILs. As expected (Table 2, wt% column), the best solubilization power was observed for the imidazolium series with the [EMIM] cation (entry 1) furnishing better results than the [BMIM] cation (entry 5) at all temperatures. This trend is in good agreement with what reported for [EMIM][OAc] and [BMIM][OAc].[40] It is worth stressing that [EMIM][Lev] is able to dissolve 6 wt% of MCC even at 25 °C. The lower viscosity observed for [EMIM][Lev] compared to [BMIM][Lev] in the 20°C-80°C temperature range (Table 1) could account for its (slightly) higher cellulose dissolution capacity. Overall, both ILs are able to dissolve remarkable amounts of MCC, comparable with that of the benchmark imidazolium OAc ILs. Conversely, long chain cation ILs $[P_{8881}]$[Lev] and $[N_{8881}]$[Lev] are not able to solubilize cellulose as pure salts. It was reported first by Rinaldi[47] that solutions of ionic liquids in some organic solvents are instead able to dissolve polysaccharides. These systems are known as organic electrolyte solutions. The $[P_{8881}]$[Lev]/DMSO 50 wt% and $[N_{8881}]$[Lev]/DMSO 50 wt% mixtures were thus tested. The amount of organic cosolvent was chosen based on previous papers by King *et al.*,[48,49] who studied analogous phosphonium OAc ILs. In our case, the dissolution capacity was higher for the ammonium Lev IL at all temperatures. Long chain ammonium ILs, which are less expensive than phosphonium ILs, have never been studied before as organic electrolytes in DMSO in the cellulose dissolution process. It has also to be highlighted that the $[P_{8881}]$[Lev]/DMSO 50 wt% solution afforded particularly low results in comparison to its OAc analogue, which is reported to dissolve up to 19 wt% of cellulose at 60 °C.[48] However, a complete study of different IL-DMSO ratios for each IL would be required to ascertain the best organic electrolyte solution composition. Small shifts from the optimal composition can produce substantial decrease in the cellulose solubilization power.[49]

Tab 2. Cellulose-dissolving capacity of different Lev ILs at selected temperatures, expressed in weight percent (wt%) (left side), and in molar ratio of anhydroglucose unit (AGU) moles over moles of IL (right side).

Entry	Cation	Dissolution capacity (wt%) Temperature (°C)					Molar dissolution capacity (AGU: IL) Temperature (°C)				
		100	80	60	40	25	100	80	60	40	25
1	[EMIM]	29	26	18	8	6	0,41	0,36	0,25	0,11	0,08
2	[EMIM] (vacuum)	38	33	20	12	8	0,53	0,46	0,28	0,17	0,11
3	[EMIM] (1° recycle)	37	--	--	--	--	0,52	--	--	--	--
4	[EMIM] (2° recycle)	37	--	--	--	--	0,52	--	--	--	--
5	[BMIM]	24	22	16	7	2	0,38	0,35	0,25	0,11	0,03
6	[BMIM] (vacuum)	34	31	25	12	3	0,53	0,49	0,39	0,19	0,05
7	$[N_{8881}]$/ DMSO 1:1	12	10	9	--	13 (vacuum)	0,72	0,60	0,54	--	0,78 (vacuum)
8	$[P_{8881}]$/ DMSO 1:1	10	8	7	--	11 (vacuum)	0,62	0,49	0,43	--	0,68 (vacuum)

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Indeed, the highly polar co-solvent (DMSO) determines the separation of the ion pair facilitating the hydrogen bond formation between the levulinate basic anion and the hydroxyl groups of the polysaccharide. For what concerns the long chain cation, it has been reported[48] that it plays an active role in solvating cellulose, thanks to its hydrophobic nature which favors the establishment of van der Waals interactions with the amphiphilic polysaccharide.

On the right side of Table 2, the dissolution data are expressed as molar ratio between the dissolved cellulose and the employed IL. It is evident that high molecular weight ILs result as the best performing systems even if they practically dissolve low amounts of cellulose. Although this different analysis has a limited value when particularly disparate IL structures or ILs containing systems are compared, it allows in some cases for further interesting considerations. For instance, for equal wt% solution of cellulose in [EMIM][OAc] and [EMIM][Lev], a lower amount of the imidazolium cation, which is certainly the most expensive part of both ionic liquids, is present in the latter solution thanks to the higher molecular weight of the anion. Therefore, [EMIM][Lev] could be used as a cheaper alternative to [EMIM][OAc] in several applications considering the wide cellulose dissolution range found. For all the systems studied (Figure 3 and Figure S19-S28), a slight darkening of the solution was observed while working at high temperatures and high cellulose loading.

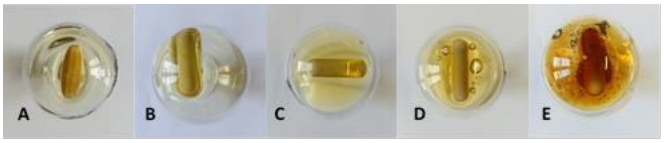

Figure 3. Pictures of MCC dissolved in [EMIM][Lev]: 25 °C, 6 wt% (**A**); 40 °C, 8 wt% (**B**); 60 °C, 18 wt%(**C**); 80 °C, 26 wt%(**D**); 100 °C, 29 wt%(**E**)

A variation of the classical solubilization conditions was also studied, on the basis of previous literature findings[50]: for dissolution systems which are not easily affected by evaporation ([EMIM][Lev], [BMIM][Lev], [P_{8881}][Lev]/DMSO 50 wt% at 25 °C, and [N_{8881}][Lev]/DMSO 50 wt% at 25 °C), the cellulose dissolution process was performed under vacuum. Reduced pressure was made after each cellulose addition until bubble formation was observed. It is envisaged that this would reduce the water content, which is present even on dried cellulose or is introduced during each cellulose addition, and will favor the solution mixing through bubble formation, especially for highly viscous solutions. Indeed, higher MCC loadings were possible at each temperature. For [EMIM][Lev] a 38 wt% amount of dissolved MCC at 100 °C was obtained. Even if these conditions require higher energy consumption and the feasibility on a large scale needs to be proved, they could be of interest when applied to the treatment and fractionation of lignocellulosic biomasses.

[EMIM][Lev] was taken as the model IL to study the scope of these systems, and some known critical aspects were further investigated. The thermal stability of [EMIM][Lev] was checked by keeping it at 120 °C for 12 h. The ^{1}H-NMR spectrum did not show significant changes if compared to the freshly prepared IL (please refer to the Supporting Information file). After the highest loading of MCC (38 wt% at 100 °C) was obtained, cellulose was regenerated by adding ethanol as the anti-solvent. The polysaccharide was filtered off, and [EMIM][Lev] was recovered (98%) by removing the alcohol under vacuum. Without further purification, the IL was used again in the dissolution process for two times with only very minor decreases in the dissolution power (37 wt%, entry 3-4, Table 2). Again the ^{1}H-NMR spectrum after two cycles confirmed the high purity of the recovered [EMIM][Lev] (Figure S3), thus demonstrating the feasibility of the recycle process.

A few studies[51-53] reported that imidazolium carboxylate ILs are not innocent solvents toward cellulose during the dissolution process. In fact, especially at high temperatures, the aromatic ring can give rise to the formation of carbenes, which are able to react with the aldehyde group at the polymer chains reducing end, giving a stable adduct and further degradation of the sugar frame.[51] Therefore, the ability of [EMIM][Lev] to work in the presence of additives (glycerol 10% and acetic acid 1%), which have been reported to suppress the carbene formation,[53] was tested. At 100 °C, this IL was still able to solubilize 26 wt% of MCC. Finally, the effect of contaminants, potentially arising from the synthetic strategy (residual MeOH), the regeneration step (residual EtOH), or cellulose (residual water), was considered. Three additional tests were then performed by adding a 10 mol % of MeOH, EtOH, and H_2O. In each case, a 15 wt% of MCC was dissolved at 60 °C without significant differences to the dried [EMIM][Lev]. These results are of practical importance and confirm the versatility of this IL in the process under investigation.

Regeneration of cellulose was obtained by adding ethanol as anti-solvent to the cellulose-Lev ILs solutions. In general, a change of crystallinity is observed in the regenerated cellulose.[54-56] The FT-IR confirmed also in the present case the decrease of the typical cellulose I absorption bands (898 cm^{-1}, 1105 cm^{-1}, 1158 cm^{-1} and 1425 cm^{-1})[57] and the concomitant appearance of the absorption band at 1260 cm^{-1}, which is attributed to the CH bending vibration in cellulose II. Furthermore, no diagnostic absorption bands of the ILs (either of the levulinate anions or of the three different cations) were found in the FT-IR spectra (Supporting Information file). Again for [EMIM][Lev] which was able to dissolve the highest amount

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of cellulose, the crystallinity of regenerated cellulose was further analyzed by XRD. In Figure 4 the XRD interferograms for pristine cellulose and regenerated cellulose at the maximum loading at 100 °C are reported. The conversion of the Cellulose I (peaks found at 2θ=14.8, 16.3, 22.6, and 34.6°) to the Cellulose II polymorph (peaks found at 2θ=12.3, 20.1, and 21.5°) is clearly detectable. The same outcome has been obtained for the other regenerated celluloses obtained from [EMIM][Lev] at the maximum loading and at the different temperatures (25, 40, 60, and 80 °C, please refer to the Supporting Information file).

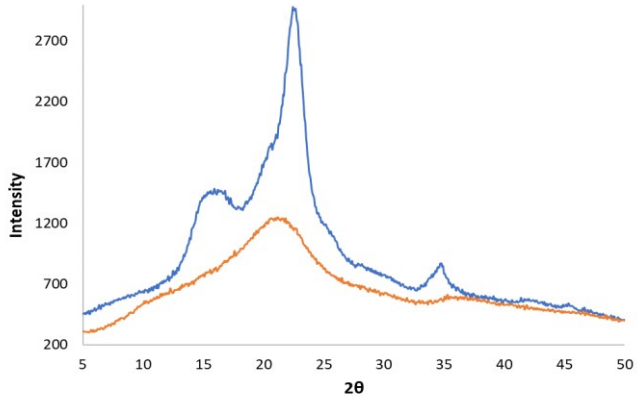

Figure 4: XRD analysis of MCC (blu line) and cellulose regenerated from [EMIM][Lev] at 100 °C and maximum loading (orange line).

Ecotoxicity evaluation – In the last decade the aquatic toxicity of ILs has been investigated from different points of view. In particular, the trends in toxicity of model organisms belonging to different taxa and toxic effects were found to correlate to the chemical structure of the ILs. Indeed, several different factors have been reported to influence the toxicity of an IL such as the type of cation and anion together with their interaction, the alkyl chain length and the presence of functional groups in the ILs.[58-60] As an example, Docherty and Kulpa[60] compared the toxicity of some ILs characterized by different alkyl chain length; toxicity of ILs with the octyl and hexyl alkyl chains was higher than benzene and toluene. This means that toxicity of ionic liquids could be more than that of conventional organic solvents, depending of their chemical structure.

The ecotoxicity of the Lev ILs here proposed (imidazolium-, ammonium-, phosphonium-) as well as of the Lev protic ILs recently prepared[29] (Figure 5) have been evaluated using both freshwater and seawater model organisms belonging to different steps of the trophic chain.

Table 3 summarizes the results of the limit test (concentration set at 100 mg/L). As a general consideration, ILs exhibited a higher degree of toxicity towards freshwater organisms compared to marine organisms; it can be speculated that the presence of high concentrations of salts (mainly sodium chloride) found in marine waters could form complexes with ILs thus reducing their bioavailability.

In the freshwater limit test, only $[N_{8881}][Lev]$ and $[P_{8881}][Lev]$ revealed a percent of maximal effect > 20% in all the performed bioassays, while [EMIM][Lev] and [BMIM][Lev] were submitted to a full test limiting to unicellular *R. subcapitata* and *D. magna*. No toxicity was detected after the exposure to protic ILs. Regarding the seawater limit test, $[N_{8881}][Lev]$ and $[P_{8881}][Lev]$ showed a percent of maximal effect > 20% in all the performed bioassays. All ILs, except $[N_{8881}][Lev]$ and $[P_{8881}][Lev]$, showed a consistent hormetic effect (biostimulation) in the assay with the marine unicellular alga *D. tertiolecta*; the hormetic effects of mixtures of different ILs have been observed by Ge et al.[61] in a luciferase assay. Limiting to the sperm toxicity test, a percent of effect >20% was detected after the exposure to all ILs. For this reason, all ILs were submitted to the full test. In Table 4 the results of the full test are reported and the ecotoxicological parameter EC_{50} was calculated.

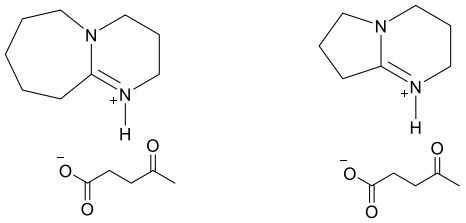

Figure 5: Structures of Lev PILs studied in the ecotoxicity assays

Tab 3. Limit test performed with freshwater and seawater organisms at the concentration of 100 mg of IL/L. In bold ionic liquids that showed an effect >20% and that were then submitted to a full test.

Freshwater assays

IL	*V. fischeri* I%	*P. subcapitata* % (+=inhibition; -biostimulation)	*D. magna* % mortality
[EMIM][Lev]	-2,842	**60,41**	**100**
[BMIM][Lev]	4,371	**63,59**	**100**
$[N_{8881}][Lev]$	**99,91**	**99,85**	**100**
$[P_{8881}][Lev]$	**99,92**	**99,85**	**100**
[DBUH][Lev]	-17,65	4,77	8,33
[DBNH][Lev]	-7,943	15,90	20

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Seawater assays

IL	*V. fischeri* I%	*D. tertiolecta* % (+=inhibition; -biostimulation)	*A. franciscana* % mortality	*F. enigmaticus* % unfertilized eggs
[EMIM][Lev]	-4,831	-90,02	0	**61,85**
[BMIM][Lev]	15,32	-24,06	4	**67,22**
[N_{8881}][Lev]	**99,85**	**99,57**	**100**	**100,00**
[P_{8881}][Lev]	**99,83**	**99,12**	**100**	**100,00**
[DBUH][Lev]	4,011	-122,10	0	**49,49**
[DBNH][Lev]	-2,039	-49,02	0	**60,13**

Tab 4. EC_{50} values (expressed as mg of IL/L) for those Lev (P)ILs which showed a percent of maximal effect > 20% in the limit test. In parentheses the 95% confidence limits (lower and upper).

Freshwater assays

IL	*V. fischeri*	*R. subcapitata*	*D. magna*
[EMIM][Lev]	-	15.81 (2.64-59.72)	68.15 (12.42-96.54)
[BMIM][Lev]	-	41.07 (1.25-69.73)	11.568 (9.12-15.75)
[N_{8881}][Lev]	0.41 (0.09-1.89)	0.25 (0.04-0.30)	0.177 (0.14- 0.22)
[P_{8881}][Lev]	1.27 (0.93-1.73)	0.10 (0.04-0.15)	0.241 (0.18 -0.31)

Seawater assays

IL	*V. fischeri*	*D. tertiolecta*	*A. franciscana*	*F. enigmaticus*
[EMIM][Lev]	-	-	-	33.11 (0.70-218.41)
[BMIM][Lev]	-	-	-	45.21 (25.56-75.89)
[N_{8881}][Lev]	1.03 (0.41-2.61)	0.49 (0.43-0.57)	5.02 (3.22-6.77)	0.13 (0.11-0.16)
[P_{8881}][Lev]	11.28 (3.98-31.95)	0.162 (0.15-0.17)	7.20 (5.32-9.17)	0.11 (0.09-0.13)
[DBUH][Lev]	-	-	-	78.57 (35.20-258.31)
[DBNH][Lev]	-	-	-	57.33 (33.05-98.12)

In the freshwater full test assays, the organism sensitivity order for ammonium-based IL was *D. magna*> *R. subcapitata*> *V. fischeri*, while the pattern of toxicity was slightly different for phosphonium-based IL (*R. subcapitata*> *D. magna*> *V. fischeri*). In the seawater full test assays, the phosphonium-based IL exhibited the highest degree of toxicity starting from the sperm toxicity assay with the serpulid *F. enigmaticus* (EC_{50}=0.11 mg/L, quite similar to that observed for [N_{8881}][Lev]). The unicellular alga *D. tertiolecta* showed a lower EC_{50} value when exposed to [P_{8881}][Lev] respect to the cells exposed to [N_{8881}][Lev]. Regarding to the *F. enigmaticus* assay, it should be noted that even if for all tested ILs an EC_{50} value was calculable, all ILs (with the exception of [N_{8881}][Lev] and [P_{8881}][Lev]) exhibited values very far from environmental relevant concentrations. The obtained results indicated that among all tested ILs sharing the levulinic acid as anion, only [N_{8881}][Lev] and [P_{8881}][Lev] showed high degree of ecotoxicity both in fresh- and seawater. The longer alkyl chain length probably played a key role in the mechanisms of toxicity, together with their nature of quaternary salts.

High toxicity of phosphonium halides against *V. fischeri* and *D. magna*, and *P. subcapitata*[62,63] was already reported. Interestingly, in the same work, though substantially less effective than the halides, [P_{2444}][$(EtO)_2PO_2$] was reported to be toxic to *V. fischeri* and *P. subcapitata*.

To our knowledge, limited data is available on the aquatic toxicity of levulinate (P)ILs. Markiewicz *et al.* [64] observed that [BMIM][Lev] exhibited low toxicity towards *V. fischeri* and *D. magna*: these results are consistent with those obtained in this work, where for the same IL a concentration of 100 mg/L was not enough to produce effects in the same organisms.

Experimental

Materials

1-Ethyl-3-methylimidazolium [EMIM] (98%), 1-Butyl-3-methylimidazolium [BMIM] (98%), trioctylmethylammonium [N_{8881}] (98%) and trioctylmethylphosphonium [P_{8881}] (98%) methylcarbonate methanol solutions were purchased from Proionic GmbH. Dry DMSO (99.9%) and ethanol (99.8%) were purchased from Sigma-Aldrich. Levulinic Acid (98%) and microcrystalline cellulose (MCC) were obtained from Alfa Aesar. All reagents were used as received, without further purification.

Methods

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Preparation of [EMIM][Lev], [BMIM][Lev], [N_{8881}][Lev] and [P_{8881}][Lev]

Accurate concentrations of methylcarbonate ILs in the commercial methanol solutions were determined by titration using a 1M HCl solution (pH Meter EUTECH pH 700, calibrated with three standard buffer solutions at pH 4.01, 7.00, and 10.00). An equimolar amount of levulinic acid was added to the commercial methylcarbonate IL (1-Ethyl-3-methylimidazolium [EMIM], 1-Butyl-3-methylimidazolium [BMIM], trioctylmethylammonium [N_{8881}] or trioctylmethylphosphonium [P_{8881}]) methanol solution at room temperature. Immediately after the levulinic acid addition, CO_2 evolution was observed. After 1h, the mixture was concentrated in vacuo at 50 °C to remove methanol and was dried under high vacuum and continuous stirring at 50 °C for 8 h.

Dissolution of cellulose

All ionic liquids were thoroughly dried under vacuum before use. Microcrystalline cellulose (MCC) was dried at 70 °C under vacuum for 24 h to reduce the presence of water before use. In a typical experiment, 2.0 g of IL (or a mixture of the IL with dry DMSO 1:1 in the case of [N_{8881}][Lev] and [P_{8881}][Lev]) were heated under stirring to the selected temperature (25, 40, 60, 80, 100 °C). Then, dry MCC (5% w/w) was added to the IL (or IL/DMSO 1:1 mixture) and the mixture was stirred at the selected temperature until complete dissolution of cellulose. When cellulose was completely dissolved, further dry MCC was added portion-wise (0,5-2 % w/w) until the solubility limit was reached. The dissolution process was repeated in triplicate for each temperature and each ionic liquid.

Dissolution of cellulose under vacuum

The above procedure was followed. After every addition of cellulose, the mixture was left under vacuum until bubble formation was observed.

Regeneration of cellulose

Cellulose was regenerated by addition of ethanol as the anti-solvent to the ionic liquid-cellulose solution. Before adding the anti-solvent, the IL-cellulose solution (or the IL/DMSO-cellulose solution) was cooled to 60 °C in case the dissolution process was performed at higher temperatures. In a typical procedure, ethanol (20mL) was then added and the mixture was stirred at 60 °C for 3h. The regenerated cellulose was then separated by centrifugation (centrifuge REMI R-8D operating at 6000 RMP max). The recovered cellulose was further washed with ethanol (20 ml) at 60 °C for 3h and again recovered by centrifugation. The washing procedure was repeated two times. After the final separation, cellulose was dried at 40 °C under vacuum for 8 h to eliminate traces of the anti-solvent.

[Emim][Lev] recycle

After removal of ethanol under vacuum, the recovered IL was directly reused in the dissolution experiment without any further purification, following the same procedure described above. The dissolution ability of recovered [EMIM][Lev] was tested in two subsequent cycles.

Characterization

NMR spectra were recorded at room temperature using a Bruker Instrument at 250 MHz (^{1}H) and 62.9 MHz (^{13}C) using deuterated chloroform as solvent. Infrared spectra were registered using an ATR-FTIR Agilent 660 (Agilent Technologies, Santa Clara, CA, USA). Optical microscopy images of cellulose in levulinate ionic liquids were acquired using a Cary 600 Series FTIR Microscope (Agilent Technologies, Santa Clara, CA, USA) with a magnification at 4x and at 15x/0.62 N.A. The thermal stability of the synthesized ILs was investigated by thermal gravimetric analysis (TGA), conducted in a TA Instruments Q500 TGA. The IL (15-18 mg) was heated in a platinum crucible. First, the heating mode was set to isothermal at 40 °C in N_2 (100 mL/min) for 5 min. Then, IL was heated from 40 to 700 °C with a heating rate of 10 °C min^{-1} under nitrogen (100 mL/min) and maintained at 700 °C for 2 min. Mass change was recorded as a function of temperature. TGA experiments were carried out in triplicate. Viscosities of ILs as a function of temperature were measured by Brookfield DV-II+Pro (Brookfield AMETEK, Inc., Middleboro, MA, USA) programmable viscometer, with an uncertainty of ±2%. The measurements were carried out in the temperature range from 20 to 80 °C which is controlled by Brookfield TC-502 thermostat with an accuracy of ± 0.1°C. X-ray diffraction (XRD) measurements were carried out with a Rigaku MiniFlex II diffractometer equipped with Ni-filtered Cu Kα radiation (1.54059 Å). The X-ray tube was operated at 30 kV and 15 mA. The samples were measured within the 2θ range of 5.0 - 50.0°. The scan rate was 2°/min. The program used to process the data package was Jade 8.

Ecotoxicity assessment

For the ecotoxicological evaluation of ILs, an ecotoxicological battery of freshwater and marine water bioassays was used. The model organisms were: the bioluminescent bacteria *Vibrio fischeri* (used in fresh- and marine water assay), the unicellular algae *Raphidocelis subcapitata* (freshwater) and *Dunaliella tertiolecta* (marine water), the crustaceans *Daphnia magna* (freshwater) and *Artemia franciscana* (marine water), the polychaete serpulid *Ficopomatus enigmaticus* (marine water). Prior to submit the samples to a full test (identification of ecotoxicological parameters such as $EC_{20}/_{50}$ limit test at maximum concentration of 100 mg/L was performed. Only substances that showed the maximum percent of effect %>20% (*Vibrio fischeri* in freshwater and marine water), the maximum inhibition of growth >20% (*R. subcapitata* and *D. tertiolecta*), the maximum mortality >20% (*D. magna* and *A. franciscana*) and the maximum percent of unfertilized eggs >20% (*F. enigmaticus*) were submitted to the full test.

***Vibrio fischeri*: inhibition of bioluminescence (freshwater and seawater assay)**

The inhibition of bioluminescence test was performed according to standard operating procedure based on the ISO

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procedures.[65] Bacteria were obtained from Ecotox LSD (Pregnana Milanese, Italy) as freeze-lyophilized cells. For the marine water assay natural seawater was used as diluent. Bacteria were exposed to a dilution series of the sample and their light emission was determined after incubation. The light emission of the bacteria in the samples was measured after 5, 15 and 30 min and compared to an aqueous control. The tests were performed at 15 °C within the pH operative range (6–8,) by the use of three replicates and four controls. All measurements were performed by using the M500 luminometer equipped with the appropriate cells. $ZnSO_4$ was used as reference toxicant.

***R. subcapitata* and *D. tertiolecta*: inhibition of growth of unicellular algae**

The inhibition of growth of *R. subcapitata* and *D. tertiolecta* was evaluated according to the protocol described in ASTM procedures,[66] with slight modifications. *D. tertiolecta* strain CCAP 19/27 and *R. subcapitata* CCAP 278/4 were purchased from the reference center CCAP (Culture Collection of Algae and Protozoa—Scottish Association for Marine Science/SAMS Research Services Ltd). Algae were cultured in the appropriate medium and the late logarithmic phase were inoculated in 25 mL fresh medium (50 mL conical flasks) to an initial concentration of 104 cells/mL and were grown at 20±2 °C under cool white fluorescent continuous light of 7000 lx under slow shaking (80 rpm) for 72 h. All cultures were aseptic and bacteria free. Experiments were performed in triplicate. The medium acted as control. Ionic liquids were suspended in medium at serial concentrations. Potassium dichromate was used as reference toxicant. The endpoint was the inhibition of growth (n cells/mL) at the end of 72 h. Cells were counted using a Bürker Counting Cell. EC_{50} values were calculated with the Linear Interpolation Method for Sublethal Toxicity software (U.S. EPA, 1993). Prior to performing both toxicity tests with samples, tests with the reference chemical potassium dichromate were carried out to check the reliability of the test procedures.

***D. magna* and *A. franciscana* mortality test**

The hatching of *A. franciscana* cysts (Artemia Gold Argentemia) followed the procedure described in standardised short-term toxicity test (ARC-test) with nauplii.[67] The newly hatched nauplii were collected and twenty animals (divided into batches of 5) for each tested ILs concentration were used, and one control without the test substance was run. Test was carried out in Petri dishes containing 5 ml of artificial sea water. The plates were sealed, incubated at 25 °C in the darkness for 24 h under a gentle shaking (80 rpm). The endpoint (immobility/death) was assessed at the end of the test with a Zeiss stereomicroscope: a nauplium was considered to be immobile or dead, if it could not move its antennae after slight agitation of the water. Potassium dichromate was used as reference toxicant. Three independent experiments were performed.

The *D. magna* test was performed following the ISO (2012) protocol[68] using the Daphtoxkit F™ (Microbiotests, BE). Tests were performed at 20 °C, in darkness, for 48 h, after which immobility was recorded. Twenty animals (divided into batches of 5) for each tested concentration were used, and one control without the test substance was run. Nauplii (24 h old) were transferred in a multiwell plate system (10 mL/well; 5 animals/well). ILs were directly dissolved in test water. Potassium dichromate was used as reference toxicant. Three independent experiments were performed.

***F. enigmaticus* sperm toxicity test**

The test was performed according to Oliva *et al*.[69] After the emission of gametes, sperm from 4 to 5 males was collected and gently mixed in a 1.5 mL eppendorf. Sperms quantification was done using Bürker Counting Cell (HBG, Germany). A concentration of 8x106 sperms/mL was collected and exposed to a final volume of 5 mL of ASW containing dissolved ILs at different concentrations for 20 minutes (ASW without ILs acted as control); after this period, 1 mL of exposed sperms was added to a 5 mL vial containing 4 mL of an eggs suspension (250 cell/mL) and the fertilization process was carried out under gentle stirring, stopping it after 90 minutes by adding 50 µL of 37% formaldehyde. One hundred eggs per replicate were counted and fertilization rate was calculated. Only 2 or more-cell stages were considered as fertilized; unfertilized eggs appeared smaller than fertilized ones and completely clear. All experiments were run in triplicate. $CuSO_4*5H_2O$ was used as reference toxicant.

Data treatment

Data for *V. fischeri* test were obtained with the Microtox Omni 1.16 software, for acquisition, data handling and EC_{50} calculation (Least Square Method). EC_{50} values (mg/L) are expressed as means together with confidence limits (95 percent) of three replicate determinations.

The EC_{50} (48 h) and (24 h) values for the *D. magna* and *A. franciscana* respectively, reference tests and their 95% confidence limits were determined by Probit analysis using the software USEPA Probit analysis program, version 1.5.

The EC_{50} (72 h) values for the algal reference test were calculated according to the procedure outlined in ASTM (2012)[66] for the calculation of growth rates; the test endpoint was the inhibition of growth, expressed as logarithmic algal biomass increase (average growth rate) during the exposure period. Calculation was performed by the use of the software Prism, GraphPad Software (San Diego, CA, USA).

The assay with *F. enigmaticus* was evaluated as percentage of success (fertilization of eggs). EC_{50} calculations were normalized to the control mean percentage of success using Abbott's formula,[70,71] $P=(Pe-Pc/100-Pc)\times100$ where Pc and Pe are control and experimental percentage response, respectively. EC_{50} and their 95% confidence intervals were calculated according to Probit analysis.[72] Each experiment was run in triplicate and an EC_{50} mean was obtained.

Conclusions

Simple, clean, one-pot access to ILs based on LA has been established. The proposed synthetic pathways do not require

toxic VOCs, resins or water. An additional important advantage is the possibility to easily scale-up these syntheses in view of future industrial applications. These ILs, which contain a cellulose-derived anion, displayed a very promising ability to dissolve their parent polysaccharide. In particular, [EMIM][Lev] showed the highest dissolution power. Therefore, for this Lev IL several critical aspects of the cellulose dissolution process have been analyzed in detail proving the versatility of the system, which works well also in the presence of protic contaminants (water, methanol, and ethanol) or of additives which are known to prevent the occurrence of side reactions (acetic acid, glycerol).

The ecotoxicity profiles of the Lev ILs and Lev PILs were assessed by using both freshwater and seawater model organisms belonging to different steps of the trophic chain. Apart from $[N_{8881}][Lev]$ and $[P_{8881}][Lev]$, which would require extra care in their use and disposal, imidazolium based Lev ILs and Lev PILs are sufficiently harmless to the aquatic species tested, displaying EC_{50} values very far from environmental relevant concentrations.

The presented results outline the power of levulinate based ILs as media for biomass dissolution even if further studies are needed to assess their scope in this area. For instance, their potential in dissolving other biopolymers, in preparing composite materials, or in the cellulose spinning process is currently under investigation.

Also, the levulinate based ILs present a ketone functional group on the anion moiety which deserves to be investigated further for new task-specific purposes.

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Insights into the levulinate-based ionic liquid class: synthesis, cellulose dissolution evaluation and ecotoxicity assessment

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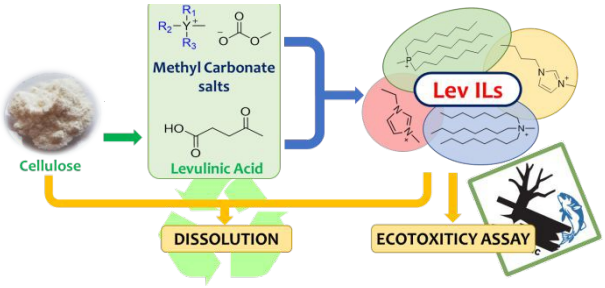

New levulinate ionic liquids (ILs) were able to dissolve cellulose in high amounts. The ecotoxicity profiles of these new ILs were also assessed.