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Pyrrolidinium-based Ionic Liquids: Aquatic Ecotoxicity, Biodegradability, and Algal Subinhibitory Stimulation

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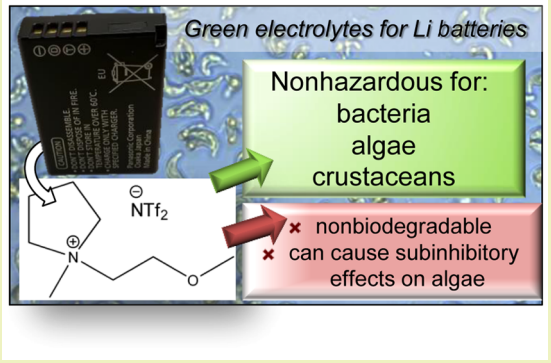
ABSTRACT: Pyrrolidinium imides are considered among the most promising electrolytes for the development of novel and sustainable portable energy devices. Because of this widespread potentiality, a risk scenario of an erroneous disposal of ionic liquids-based batteries in the environment has to be taken into account. In the present study, some of the best energy-performing pyrrolidinium-based ionic liquids were evaluated in terms of persistence in aquatic environments and hazard toward freshwater organisms (crustacean *Daphnia magna* and unicellular green alga *Raphidocelis subcapitata*). The examined ionic liquids were not aerobically biodegradable (biodegradation less than 5% in 28 days), but they demonstrated low toxicity toward algae and crustaceans, according to the standard bioassay end points (EC_{50} > 100 mg L^{-1}). However, ionic liquids were able to alter the cellular morphology of *R. subcapitata* and an increased amount of proteins (30%) was observed in the exposed cells, suggesting an inhibition of cellular division.



KEYWORDS: *Ionic liquids, Aquatic ecotoxicity, Biodegradability, Subinhibitory effects*

INTRODUCTION

Rechargeable Li batteries are ubiquitous energy devices used in many types of portable electronic equipments, such as cellular phones, laptop computers, and digital cameras. In the state-of-the-art technology of 4 V-class rechargeable Li batteries, a mixture of organic aprotic solvents (e.g., ethylene carbonate and diethyl carbonate) and the conducting lithium hexafluorophosphate salt ($LiPF_6$) is generally used as nonaqueous electrolyte.[1] However, the high vapor pressure and low flash point of organic solvents and the low thermal stability of $LiPF_6$ represent non-negligible safety issues.[2] In recent years, ionic liquids (ILs) have been brought to the forefront in the field of new safe electrolyte systems thanks to their undeniable advantages: nonvolatility, nonflammability, and good thermal stability.[3] In particular, cyclic saturated ammonium cations (e.g., pyrrolidinium), paired with electrochemically stable and weakly coordinating anions (e.g., bis(trifluoromethanesulfonyl)imide, $(CF_3SO_2)_2N^-$ or NTf_2), have (i) high conductivity values, exceeding 1 mS cm^{-1} at room temperature, (ii) wide electrochemical windows, (iii) high thermal stability, and (iv) relatively low viscosity.[4] In particular, ether-functionalized methylpyrrolidinium NTf_2 salts are amorphous over a wide molality range, have very low viscosities (down to 25 cP), and their room temperature conductivity remains constant at about 1 mS cm^{-1} for a wide range of salt content.[4,5]

In view of the potential widespread applicability of this class of ILs in the field of portable electronic devices and their consequent disposal scenarios, a major concern is the substantial lack of information about the environmental fate and ecotoxicological behavior of these compounds. In the past decade, extensive studies have been performed to evaluate the toxicity of many ILs against various biological targets, most of them being devoted to the ecotoxicity of imidazolium or pyridinium salts.[6] However, to the best of our knowledge, no information about the toxicity and the biodegradability of ether-functionalized pyrrolidinium imide salts is available in the literature; likewise, only a few data have been reported for the alkyl substituted analogues, in spite of the larger widespread diffusion expected for these ILs in comparison to imidazolium or pyridinium salts.

The aim of the present paper is to elucidate the toxicity and biodegradability of three of the most promising pyrrolidinium imide salts, providing the ecotoxicological information required by the REACH regulation for the registration of chemical compounds (first tonnage band). The ILs used for this study were chosen on the basis of their excellent performances as

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electrolytes: *N*-butyl-*N*-methylpyrrolidinium bis(trifluoromethanesulfonyl)imide (**1**, bmpyr NTf_2), *N*-methoxyethyl-*N*-methylpyrrolidinium bis(trifluoromethanesulfonyl)imide (**2**, moempyr NTf_2), and *N*-methoxyethoxymethyl-*N*-methylpyrrolidinium bis(trifluoromethanesulfonyl)imide (**3**, moeommpyr NTf_2) (Figure 1). The role of the anion on the environmental impact of pyrrolidinium salts was also investigated by evaluating *N*-methoxyethyl-*N*-methylpyrrolidinium tetrafluoroborate (**4**, moempyr BF_4) as comparison. Ready biodegradability in water, acute toxicity toward the crustacean *Daphnia magna*, and inhibition of the growth of the alga *Raphidocelis subcapitata* were determined. Additionally, a detailed analysis of the morphological and cellular effects that low concentrations of these ILs have on algae has been performed.

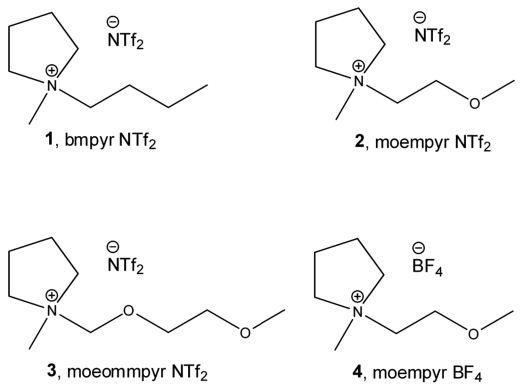

Figure 1. Structures of the ILs tested in the present study.

RESULTS AND DISCUSSION

Biodegradability in Water. The biodegradability of all ILs did not exceed 5% in 28 days (Figure 2). The OECD guidelines state that a compound can be defined as "readily biodegradable" if its level of biodegradation reaches 60% of ThOD, achieved within 10 days after starting the degradation (the start of the degradation is taken as the time when 10% of the substance has been degraded).[7] Thus, all ILs tested here were "not readily biodegradable", independently of the anion (NTf_2 or BF_4) or the chain on the cationic ring (alkyl or alkoxyl). The simultaneous toxicity test on **2** (as representative for the other ILs) and glucose reached a cumulative biodegradation value of 25% in 3 days. Thus, according to OECD,[7] IL **2** is not an inhibitory compound for bacteria.

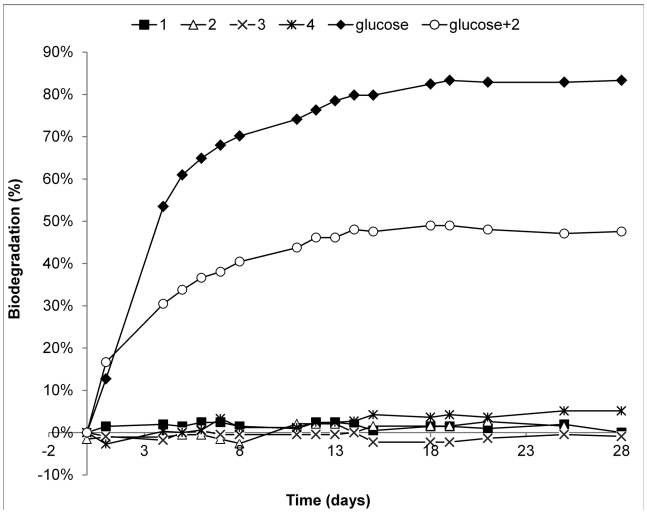

Figure 2. Biodegradability in water of ILs **1**, **2**, **3**, and **4**, and glucose as reference compound for test validation.

Neumann et al. has recently reported a degradation rate lower than 25% over 28 days for *N*-butyl-*N*-methylpyrrolidinium bromide (bmpyr Br).[8] The authors reported also a trend of increasing biodegradability by increasing the alkyl chain length on the cation; *N*-octyl-*N*-methylpyrrolidinium bromide was readily biodegradable, whereas *N*-ethyl-*N*-methylpyrrolidinium bromide was not biodegradable at all. Similarly, Deng et al. found no change in the concentration of IL **1** over the 28 d incubation period, confirming that an activated sludge is not able to metabolize this compound.[9] The anions NTf_2 and BF_4 are known to be not biodegradable due to the absence of oxidizable carbons in their structure.[10] Specifically, the high chemical stability of the NTf_2 anion leads to a high resistance against biological and abiotic degradation. However, although modifications of the anion are known to influence the physical and chemical properties of the ionic liquids, the effect of the counterion on biodegradability is generally not pronounced. The only exception is the anion octyl sulfate, which is able to provide a good biodegradability to its ion pairings.[10] In the case of the pyrrolidinium salts tested here, the ion pairing of recalcitrant anions with poorly degradable cations resulted in very persistent ILs, which could remain in aquatic environments for a long time if an erroneous disposal of the electronic devices containing these ILs would occur.

As predictable, the effect on the biodegradability that the introduction of oxygen atoms in the alkyl chain of ILs can have depends on the position. The presence of a terminal alcohol moiety facilitates the oxidation pathway since the initial ϖ-oxidation catalyzed by cytochrome P450 to obtain a terminal OH group is no longer necessary and β-oxidation can occur faster.[11] On the other hand, the presence of one or more alkoxy units does not significantly improve the primary biodegradation. This finding has been already observed for ethoxylated imidazolium ILs, for which the biodegradation in soil was lower than that of the alkyl counterparts.[12]

Acute Toxicity toward *Daphnia magna*. The EC_{50} values (mM) of the tested ILs for *D. magna* ranged from 0.17 mM for **1** to 0.72 mM for **4** (Table 1). Differences among EC_{50} values were statistically significant ($p < 0.05$), and it resulted clear that the contribution of the anion was relevant. Compounds **1**–**3**, in fact, were more toxic than **4** having a BF_4 anion (all differences were significant).

Few comparisons are possible between the data presented here and literature toxicity values; EC_{50} values of 0.09 and 0.35

Table 1. 50% Effect Concentrations (EC_{50}, mM) of Tested Ionic Liquids to *D. magna* in a 48 h Immobilization Test and to *R. subcapitata* in a 72 h Growth Inhibition Test[a]

	EC_{50} (mM)	
ILs	*D. magna*	*R. subcapitata*
1	0.17 (0.13–0.24)	1.7 (1.4–2.1)
2	0.39 (0.35–0.43)	1.3 (1.0–1.6)
3	0.26 (0.24–0.28)	0.9 (0.7–1.2)
4	0.72 (0.56–0.94)	2.4 (2.1–2.6)

[a]Values are reported with 95% confidence limits in bracket.

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mM have been reported for compound **1** toward *D. magna*,[13,14] and the value found here (0.17 mM) is in the middle of this range. According to the UN criteria,[15] **1** would be classified as *hazardous for the aquatic environment* in Category Acute 3 since its EC_{50} (73 mg L^{-1}) is between 10 and 100 mg L^{-1}. The introduction of oxygenated chains in **2**, **3**, and **4** significantly reduces the toxicity and all of them can be classified as *nonhazardous*. However, the toxicity reduction was not as large as that observed in the corresponding imidazolium ILs bearing tetrafluoroborate (BF_4) and dicyanamide ($N(CN)_2$) anions.[16,17] We could presumably assume that this result is due to the presence of NTf_2 anion, known to be intrinsically toxic toward many target species.[18]

Growth Inhibition of *Raphidocelis subcapitata* and Subinhibitory Effects. The ecotoxicity of various ILs against many algal species has been largely deepened in the past decades, elucidating in particular the ILs mechanism of action and demonstrating a peculiar algal species-specific sensitivity to different ILs.[19−22]

The EC_{50} values (mM) of the tested ILs for *R. subcapitata* ranged from 0.9 mM for **3** to 2.4 mM for **4** (Table 1). Compound **4** resulted significantly less toxic ($p < 0.05$) than all other ILs, thus indicating that the contribution of the anion NTf_2 to the toxicity was relevant also in this case. This finding has been confirmed also from literature data; bmpyr Br, for example, is 5 times less toxic than **1** (EC_{50} of 9.3[23] vs 1.7 mM, respectively). The EC_{50} found here for **1** was around 2 orders of magnitude higher than the value reported by Ventura et al. (0.034 mM)[14] but in agreement with Pretti et al. (>0.3 mM).[13] Again, no clear toxicity reduction was observed by introducing oxygen atoms in the lateral chain of the cation, in disagreement with what was previously reported for ether-functionalized imidazolium ILs toward algae.[24] However, alkoxy pyrrolidinium imides are generally less toxic than the imide salts of other cations (e.g., imidazolium or pyridinium); 1-methoxyethyl-3-methylimidazolium bis(trifluoromethanesulfonyl)imide (moemim NTf_2), for example, is 15 times more toxic than **2** (EC_{50} of 0.09 mM[10] vs 1.3 mM, respectively).

According to the algal growth inhibition test, all ILs would be classified as *nonhazardous,* being their EC_{50} values above the limit of 100 mg L^{-1}.[15] A comparison with the values found for *D. magna* shows that the EC_{50} values for the crustacean were distinctly lower than those for *R. subcapitata* (1 order of magnitude for **1**), indicating that *D. magna* is the most sensitive among these two organisms. On the other hand, for both species the NTf_2 anion resulted more toxic than BF_4.

Microscope evaluation of algal cells exposed to concentrations below the EC_{50} revealed that all NTf_2 salts were able to cause an alteration of the cellular morphology (Figure 3), inducing a thicker and more rounded shape that suggests a reduced cell division. The evidence of subtoxic effects at low concentrations of ILs has been already described in the literature after an exposure to bmpyr Br able to stimulate an hormesis effect.[23] In the present study, an actual increase of the algal cell biovolume was observed with all NTf_2 salts; for example, the biovolume increased from 16.6 ± 4.8 μm^3 in the control to 62.0 ± 23.4 μm^3 after treatment with **2**. This finding has been already described for algal cells exposed to subinhibitory growth concentrations of Cd(II), Cr(VI), and Cu(II), being that these metals are able to alter algal cell cycle progression (proliferative capacity) as well as their morphology (cell volume).[25,26]

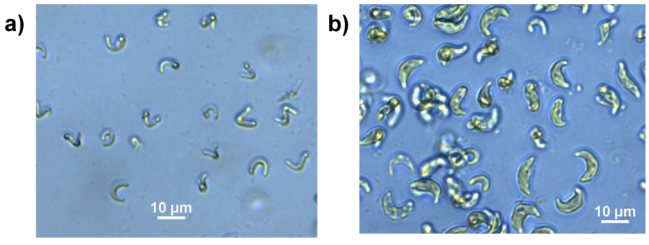

Figure 3. *R. subcapitata* cells after 72 h of exposure to a concentration of 0.09 mM of **2**: (a) control and (b) treatment with **2**.

The same effect on the morphology was also observed in the experiments performed in the dark whenever any NTf_2 salt was added. Instead, in the presence of **4** (anion without N), the classic cell morphology was observed. Therefore, a more detailed characterization of algal cells was performed by studying the cellular effect caused by an exposure to **2** (chosen as representative of the NTf_2 salts) and **4** (same cation of **2** but different anion), both at concentrations below the EC_{50} values (0.9 mM in the case of **2** and 1.7 mM for **4**). Table 2 reports the ammonium consumption, algal cell numbers, and growth rate in the samples treated with **2** in comparison to the control (light and dark conditions). The data related to the exposure of algal cells to compound **4** under light conditions are also reported.

Table 2. Ammonium Concentration in the Test Medium (mg L^{-1}), Algal Growth Rate, and Cell Number After 72 h of Exposure to 2 (0.9 mM) and 4 (1.7 mM), Under Light and Dark Conditions

		NH_3−N (mg L^{-1})		algal cell number (10^4 cell mL^{-1})		growth rate (μ) (d^{-1})	
entry	treatment	light	dark	light	dark	light	dark
1	control[a]	1.2	3.9	50.9	1.9	1.5	0.08
2	**2**[b]	3.5	3.5	1	1	0	0
3	**2**[c]	6.3	7.1	41.7	2.4	1.3	0.11
4	**4**	nd	nd	41.3	nd	1.1	nd

[a]N-source: NH_4Cl, initial ammonium level of 4.3 mg L^{-1}. [b]N-source: **2**, initial ammonium level of 3.5 mg L^{-1}. [c]N-source: NH_4Cl and **2**, initial ammonium level of 7.8 mg L^{-1}.

After 72 h, the ammonium consumption of algae in the control medium (NH_4Cl as N-source) under light and dark conditions was 71% and 10%, respectively (entry 1, Table 2). When cells were cultivated in a medium without NH_4Cl but just **2** (entry 2, Table 2), they were unable to grow (number of cells at the beginning of the test and after 72 h was 1×10^4 cell mL^{-1}) and, therefore, to consume nitrogen; this result led to the conclusion that algae were not able to use **2** as N-source, presumably because its size could hamper the passage through the membranes. In a medium containing both NH_4Cl and **2** (entry 3, Table 2), algae consumed a certain percentage of ammonium (19% and 9% under light and dark conditions, respectively), but it is reasonable to assume that just NH_4Cl, and not **2**, was partially used as N-source. In this case, in fact, the growth rates were 1.3 d^{-1} and 0.11 d^{-1} under light and dark conditions, respectively. Therefore, a reduction of about 20% of the algal cell number was observed in the light-condition experiment, but the algal cell number was 1.3 times higher than the control in the dark. The results obtained after an exposure

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Table 3. Biochemical Composition of Algae Harvested after 72 h of Exposure to 2 (0.9 mM) and 4 (1.7 mM) under Light Conditions

treatment	N	C	H	S	proteins (wt %)	FAMEs (wt %)
control	4.4 ± 0.4	52.9 ± 1.0	7.9 ± 0.2	0.3 ± 0.1	53.2 ± 0.8	16.2 ± 3.2
2	7.3 ± 0.2	52.1 ± 0.9	7.7 ± 0.2	0.5 ± 0.2	70.5 ± 1.1	13.9 ± 1.1
4	6.4 ± 0.5	52.7 ± 0.7	7.6 ± 0.5	0.4 ± 0.1	64.8 ± 0.8	13.3 ± 0.3

to a concentration of 1.7 mM of **4** were very similar to those observed with **2**, both in terms of algal cell number and growth rate.

The evaluation of the biochemical composition (in terms of proteins and lipids) of algal cells harvested after such subtoxic stimuli can help in better understanding the macroscopic effects observed on the morphology. To the best of our knowledge, the present study reports for the first time this kind of correlation on algae. Table 3 shows the elemental analysis (determined by CHNS elemental analyzer), protein amount (measured by Lowry protocol[27]), and lipid content (calculated as fatty acid methyl esters, FAMEs, after transesterification) of algae collected after 72 h of incubation with 0.9 and 1.7 mM concentrations of **2** and **4**, respectively, in comparison to the control.

The major effect observable at biochemical level was the increase in the protein amount, associated with a statistically significant increase of N content in the treated algal cells. The protein content of the control was 53.2 ± 0.8 wt %; after treatments with **2** and **4**, this value increased to 70.5 ± 1.1 and 64.8 ± 0.8 wt %, respectively, values 32% and 22% higher than the control. The N-amount was found 4.4 ± 0.4% in the control, 7.3 ± 0.2% in algae treated with **2**, and 6.4 ± 0.5% in the treatment with **4**, corresponding to a C/N ratio of 14.2, 8.3, and 9.7, respectively. Therefore, in spite of not having altered the cellular biovolume, the treatment with **4** contributed to significantly increase the protein content but to an extent that was lower than that obtained with **2**. Considering the increased volume of the cells and the fact that the cell division can be arrested at different stages when exposed to toxicants,[25] we could hypothesize that the growth of cells exposed to **2** and **4** was stopped during the cell cycle after the production and accumulation of proteins; thus, protein concentration resulted much higher than in the control cells.

Thus, in spite of alkoxyl pyrrolidinium ILs can be classified as "*nonhazardous*" according to the international guidelines, they are able to cause relevant sublethal effects to algae, altering their natural growth cycles.

The lipid amount of the control, expressed as FAMEs, was 16 ± 3 wt %; a not statistically different value was found for algae treated with **2** and **4** (14 ± 1 and 13.0 ± 0.3 wt %, respectively), confirming that green algae generally enhance lipid production when they are under nutrient limitation and/or high light conditions.

CONCLUSIONS

Rechargeable Li-based portable electronic devices can potentially release hazardous chemicals (metals or electrolytes) in the environment if not correctly disposed. To maximize their safety, these batteries should be designed for end-use applications, especially by projecting the use of nontoxic components and chemicals. Ionic liquids can represent a reliable key solution to the issue of safe electrolytes in Li-based devices, thanks to their good performances coupled with the claimed low environmental impact. More specifically, ILs have the great advantage to be "tunable", since an appropriate choice of the cation and anion structures can improve the physicochemical properties as well as influence the ecotoxicological profile. All pyrrolidinium imide salts evaluated in the present study, claimed to be the best performing among the various ILs options, were not readily biodegradable by microbial communities. However, they were not hazardous for algae, even if they were able to inhibit the cellular division, resulting in a perturbation of the cell physiological status. The tests performed to check the effects on crustaceans confirmed that the salts with alkoxy lateral chains had lower toxicity than alkyl counterparts and that the NTf_2 anion was more toxic than BF_4.

EXPERIMENTAL SECTION

Chemicals. All solvents and chemicals used in this study were obtained from Sigma-Aldrich (purities ≥98%) and were used without purification.

Synthesis and Characterization of ILs. All ILs were prepared from the corresponding halides (chloride, bromide, or iodide)[3] and purified following published procedures.[28] Briefly, the halide salts were obtained in almost quantitative yields by reacting *N*-methyl pyrrolidine with butyl iodide (IL **1**), 2-bromoethyl methyl ether (ILs **2** and **4**), or 2-methoxyethoxymethyl chloride (IL **3**) in ethyl acetate. The anion exchange was performed in water with $LiNTf_2$ in the case of ILs **1** (overall yield: 72% after purification), **2** (overall yield: 60% after purification), and **3** (overall yield: 71% after purification), or in acetone with $NaBF_4$ in the case of compound **4** (overall yield: 85% after purification). Purity was established to be at least 98% through proton nuclear magnetic resonance (^{1}H NMR) spectra by integration of proton signals with respect to an internal standard. To this purpose, spectral data have been acquired in $CDCl_3$ (in which the tested ILs are completely soluble) with a known amount of tetrakis(trimethylsilyl)-silane (TTMS) as an internal standard and a delay time between successive scans of 20 s to ensure complete proton relaxation and therefore quantitative integration. NMR spectra were recorded using a 5 mm probe on a Varian Inova 400 spectrometer. Spectroscopic data of ILs **1**, **2**, **3**, and **4** were in accordance with the literature.[3,29]

Biodegradability Test. Biodegradation was determined by a ready biodegradability test in an aerobic aqueous medium according to the OECD (Organization for Economic Cooperation and Development) guideline 301 F, "Manometric respirometry".[7] The test medium was prepared by adding to distilled water certain concentrations of mineral components from stock solutions (potassium and sodium phosphates plus ammonium chloride, calcium chloride, magnesium sulfate, and iron(III) chloride). The bacterial inoculum, derived from an activated sludge taken from a treatment plant receiving domestic sewage located in Ravenna, Italy, was aerated in mineral medium for 5 days at the test temperature. The biodegradability tests were carried out in bottles for 28 days at 20 ± 2 °C. ILs **1**, **2**, **3**, and **4** were tested in duplicate, run in parallel with glucose (reference compound), a blank (containing only inoculum), and a toxicity control (containing **2**, glucose, and inoculum). The concentrations of ILs (mM) and the corresponding Theoretical Oxygen Demand (ThOD, mg O_2 L^{-1}) are reported in Table 4; all ILs were completely solubilized at the tested concentrations. ThOD was calculated under the assumption that nitrogen was eliminated as ammonia. The consumption of oxygen was determined by measuring the change in pressure inside the apparatus. Evolved carbon dioxide was absorbed in a solution of potassium

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Table 4. ILs Concentration (mM) and ThOD (mg O_2 L^{-1}) for Biodegradability Test and Concentration Range (mM) Used in the Ecotoxicity Test

ILs	biodegradability test concentration (mM)	ThOD (mg O_2 L^{-1})	test range concentration (mM)	
			D. magna	*R. subcapitata*
1	0.2	103	0.04−0.6	0.9−2.6
2	0.2	96	0.1−0.8	0.8−4.7
3	0.2	111	0.1−0.6	0.6−2.1
4	0.4	166	0.2−1.1	1.5−8.6

hydroxide. The amount of oxygen taken up by the microbial population during the biodegradation of the test substance (corrected for uptake by blank inoculum) was expressed as a percentage of ThOD.

48 h Immobilization Assay with *Daphnia magna*. The acute (48 h) toxicity test with *Daphnia magna* (Crustacea, Cladocera) was performed in accordance with the OECD 202 guideline for testing chemicals.[30] Briefly, 20 neonates (<24 h old) were transferred to glass cups (5 per cup) containing 20 mL of a control solution (without toxicant) or test solutions with IL (4 replicates per treatment). Stock solutions of ILs **1**−**4** (4.7, 7.0, 8.2, and 2.7 mM, respectively) were prepared in the OECD medium (pH 7.8 ± 0.5) and tested simultaneously. The five test concentrations of each ionic liquid, identified through a preliminary range-finding test, were arranged in geometric series (Table 4); the control and test solutions were prepared in 100 mL borosilicate glass flasks in sterile conditions. The number of immobilized *D. magna* was recorded at 24 and 48 h. Animals that were not able to swim within 15 s, after gentle agitation of the test vessel, were considered to be immobilized (even if they could still move their antennae).

72 h Algal Growth Inhibition Assay with *Raphidocelis subcapitata*. The algal growth inhibition test was conducted in accordance with the OECD 201 algal growth inhibition test protocol,[31] with the unicellular green alga *Raphidocelis subcapitata* (Chlorphyta, Chlorophyceae), formerly named *Pseudokirchneriella subcapitata* or *Selenastrum capricornutum*. Stock solutions of IL **1**, **2**, **3**, and **4** (4.7, 7.0, 8.2, and 12.8 mM, respectively) were prepared in the OECD medium (pH 8.1 ± 0.5) and tested simultaneously using the same algae batch. The five test concentrations of each ionic liquid, identified through a preliminary range-finding test, were arranged in geometric series (Table 4); the control and test solutions were prepared in 100 mL borosilicate glass flasks in sterile conditions. The 50 mL samples were inoculated with an aliquot of exponentially growing algae to ensure an initial algal concentration of 10^4 cells mL^{-1}. The cells were then incubated at 23 °C on an orbital shaker at 100 rpm under continuous "cool white" fluorescent light (intensity of 6000 lx at the surface of the flask), verified using an illumination meter. The number of the algal cells in the flasks was determined by counting under microscope (Nikon Eclipse E600, 400x) in the Burker hemocytometer at the beginning and the end of the test. The average specific growth rate μ (d^{-1}) was calculated as the slope of a linear regression of the natural logarithm of the measured cell density (corrected for background) versus time.

Data Analysis for Ecotoxicity Tests. The 50% effect concentration (EC_{50}) of each substance for 72 h algal growth inhibition tests and for 48 h *D. magna* acute toxicity test was estimated by fitting the experimental concentration−response curves to a logistic model:

$$y = \frac{\max}{1 + \left(\frac{x}{\log_{10} EC_{50}}\right)^{\text{slope}}}$$

where y = end point value (algal growth rate or number of active *D. magna*); x = $\log_{10}$ of substance concentration; max = expected end point value when the concentration of the toxicant is zero; slope = slope parameter. The parameters of the equation, including the EC_{50} and their standard errors and confidence limits were estimated using the nonlinear regression procedures implemented in Statistica (Statsoft, Tulsa, OK, U.S.A.). Differences among EC_{50} values were tested using a Student's t test based on the standard errors and degrees of freedom provided by the regression analysis. Differences were considered significant if $p < 0.05$. Probability was corrected for multiple comparisons using a sequential Bonferroni procedure.[32]

Analysis of Algal Subinhibitory Stimulation. After an incubation time of 72 h at 0.9 and 1.7 mM of **2** and **4**, respectively (concentrations determined from dose−response curves, able to cause a detectable but limited growth inhibition), algal cells were collected by centrifugation at 3000 rpm for 10 min, washed with a physiological saline solution, and then centrifuged again; this procedure was repeated three times in order to ensure the removal of any eventual IL trace on algal surfaces. Then algal cells were freeze-dried and analyzed to determine their nitrogen (by elemental analysis), protein (by the Lowry protocol[27]), and lipid (by GC-MS) content. Ammonium (NH_3−N, mg L^{-1}) was measured at day 0 and after 72 h in the filtrate through test kit colorimetric analyses (DR/2010; Hach, Colorado, U.S.A.) by using the Nessler Method.[33]

Algae were also exposed to a concentration of 0.9 mM of **2** under the dark and using **2** as the sole N-source in the growth medium (OECD medium prepared with all nutrients besides NH_4Cl). Controls were run in parallel in each experiment. The biovolume determination was performed in accordance to the literature;[25] 20 cells were randomly selected and the sizes (length and width) were measured. Elemental analyses were performed by using an elemental analyzer (Thermo Scientific, Flash 2000, Organic Elemental Analyzer) by means of the flash combustion technique.

The fatty acid methyl esters (FAMEs) content of algal cells (indicator of the lipid amount) was determined as follows: Samples (about 2 mg) were suspended in dimethyl carbonate (0.4 mL). 2,2-Dimethoxypropane (0.1 mL) and 0.5 M NaOH in MeOH (0.1 mL) were then added; the samples were placed in an incubator at 90 °C for 30 min. After cooling for 5 min to room temperature, 1.3 M BF_3-methanol 10% (w/w) reagent (0.7 mL) was added before repeating the incubation for 30 min. After cooling for 5 min to room temperature, saturated NaCl aqueous solution (2 mL) and hexane (1 mL) containing methyl nonadecanoate (internal standard for GC-MS quantification, 0.02 mg) were added, and the samples were centrifuged at 4000 rpm for 1 min. The upper hexane-dimethylcarbonate layer, containing FAMEs, was transferred to vials for GC-MS analysis. Each analysis was repeated in duplicate. The relative response factors used for the quantitation were obtained by injecting solutions of known amounts of methyl nonadecanoate and commercial FAMEs mixture.

GC-MS analyses were performed by using a 6850 Agilent HP gas chromatograph connected to a 5975 Agilent HP quadrupole mass spectrometer. The injection port temperature was 280 °C. Analytes were separated by a HP-5 fused-silica capillary column (stationary phase poly[5% diphenyl/95% dimethyl]siloxane, 30 m, 0.25 mm i.d., 0.25 μm film thickness), with helium as the carrier gas (at constant pressure, 33 cm s^{-1} linear velocity at 200 °C). Mass spectra were recorded under electron ionization (70 eV) at a frequency of 1 scan s^{-1} within the 12−600 m/z range. The temperature of the column was increased from 50 °C up to 180 °C at 50 °C min^{-1} and then from 180 °C up to 300 °C at 5 °C min^{-1}.

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Yoshio, M.; Brodd, R. J.; Kozawa, A. *Lithium-Ion Batteries: Science and Technologies*; Springer Science & Business Media, 2010.

(2) Ravdel, B.; Abraham, K. M.; Gitzendanner, R.; Di Carlo, J.; Lucht, B.; Campion, C. Thermal stability of lithium-ion battery electrolytes. *J. Power Sources* **2003**, *805*, 119−121.

(3) Zhou, Z. B.; Matsumoto, H.; Tatsumi, K. Cyclic quaternary ammonium ionic liquids with perfluoroalkyltrifluoroborates: synthesis, characterization, and properties. *Chem.—Eur. J.* **2006**, *12*, 2196−2212.

(4) Ferrari, S.; Quartarone, E.; Mustarelli, P.; Magistris, A.; Fagnoni, M.; Protti, S.; Gerbaldi, C.; Spinella, A. Lithium ion conducting PVdF-HFP composite gel electrolytes based on N-methoxyethyl-N-methylpyrrolidinium bis(trifluoromethanesulfonyl)-imide ionic liquid. *J. Power Sources* **2010**, *195*, 559−566.

(5) Ferrari, S.; Quartarone, E.; Mustarelli, P.; Magistris, A.; Protti, S.; Lazzaroni, S.; Fagnoni, M.; Albini, A. A binary ionic liquid system composed of N-methoxyethyl-N-methylpyrrolidinium bis-(trifluoromethanesulfonyl)-imide and lithium bis-(trifluoromethanesulfonyl)imide: A new promising electrolyte for lithium batteries. *J. Power Sources* **2009**, *194*, 45−50.

(6) Egorova, K. S.; Ananikov, V. P. Toxicity of ionic liquids: eco(cyto)activity as complicated, but unavoidable parameter for task-specific optimization. *ChemSusChem* **2014**, *7*, 336−360.

(7) *Guideline for Testing of Chemicals: Ready Biodegradability*; OECD Guideline 301; Organization for Economic Cooperation and Development: Paris, 1992.

(8) Neumann, J.; Steudte, S.; Cho, C. W.; Thöming, J.; Stolte, S. Biodegradability of 27 pyrrolidinium, morpholinium, piperidinium, imidazolium and pyridinium ionic liquid cations under aerobic conditions. *Green Chem.* **2014**, *16*, 2174−2184.

(9) Deng, Y.; Beadham, I.; Ghavre, M.; Costa Gomes, M. F.; Gathergood, N.; Husson, P.; Légeret, B.; Quilty, B.; Sancelme, M.; Besse-Hoggan, P. When can ionic liquids be considered readily biodegradable? Biodegradation pathways of pyridinium, pyrrolidinium and ammonium-based ionic liquids. *Green Chem.* **2015**, *17*, 1479−1491.

(10) Garcia, M. T.; Gathergood, N.; Scammells, P. J. Biodegradable ionic liquids Part II. Effect of the anion and toxicology. *Green Chem.* **2005**, *7*, 9−14.

(11) Stolte, S.; Abdulkarim, S.; Arning, J.; Blomeyer-Nienstedt, A. K.; Bottin-Weber, U.; Matzke, M.; Ranke, J.; Jastorff, B.; Thöming, J. Primary biodegradation of ionic liquid cations, identification of degradation products of 1-methyl-3-octyl-imidazolium chloride and electrochemical waste water treatment of poorly biodegradable compounds. *Green Chem.* **2008**, *10*, 214−224.

(12) Modelli, A.; Sali, A.; Galletti, P.; Samorì, C. Introduction of oxygenated side chain into imidazolium ionic liquids: Evaluation of the effects at different biological organization levels. *Chemosphere* **2008**, *73*, 1322−1327.

(13) Pretti, C.; Chiappe, C.; Baldetti, I.; Brunini, S.; Monni, G.; Intorre, L. Acute toxicity of ionic liquids for three freshwater organisms: *Pseudokirchneriella subcapitata*, Daphnia magna and Danio rerio. *Ecotox. Environ. Safe.* **2009**, *72*, 1170−1176.

(14) Ventura, S. P. M.; Goncalves, A. M. M.; Goncalves, F.; Coutinho, J. A. P. Assessing the toxicity on $[C_3mim][Tf_2N]$ to aquatic organisms of different trophic levels. *Aquat. Toxicol.* **2010**, *96*, 290−297.

(15) *Globally Harmonized System of Classification and Labelling of Chemicals (GHS)*, 5th ed.; United Nations: New York and Geneva, 2013.

(16) Samorì, C.; Pasteris, A.; Galletti, P.; Tagliavini, E. Acute toxicity of oxygenated and nonoxygenated imidazolium-based ionic liquids to *Daphnia magna* and *Vibrio fischeri*. *Environ. Toxicol. Chem.* **2007**, *26*, 2379−2382.

(17) Samorì, C.; Malferrari, D.; Valbonesi, P.; Montecavalli, A.; Moretti, F.; Galletti, P.; Sartor, G.; Tagliavini, E.; Fabbri, E.; Pasteris, A. Introduction of oxygenated side chain into imidazolium ionic liquids: Evaluation of the effects at different biological organization levels. *Ecotox. Environ. Safe.* **2010**, *73*, 1456−1464.

(18) Wang, H.; Malhotra, S. V.; Francis, A. J. Toxicity of various anions associated with methoxyethyl methyl imidazolium-based ionic liquids on Clostridium sp. *Chemosphere* **2011**, *82*, 1597−1603.

(19) Latała, A.; Stepnowski, P.; Nedzi, M.; Mrozik, W. Marine toxicity assessment of imidazolium ionic liquids: Acute effects on the Baltic algae *Oocystis submarina* and *Cyclotella meneghiniana*. *Aquat. Toxicol.* **2005**, *73*, 91−98.

(20) Latała, A.; Nedzi, M.; Stepnowski, P. Toxicity of imidazolium and pyridinium based ionic liquids towards algae. *Chlorella vulgaris*, *Oocystis submarina* (green algae) and *Cyclotella meneghiniana*, *Skeletonema marinoi* (diatoms). *Green Chem.* **2009**, *11*, 580−588.

(21) Latała, A.; Nedzi, M.; Stepnowski, P. Toxicity of imidazolium and pyridinium based ionic liquids towards algae. *Bacillaria paxillifer* (a microphytobenthic diatom) and *Geitlerinema amphibium* (a microphytobenthic blue green alga). *Green Chem.* **2009**, *11*, 1371−1376.

(22) Latała, A.; Nedzi, M.; Stepnowski, P. Toxicity of imidazolium ionic liquids towards algae. Influence of salinity variations. *Green Chem.* **2010**, *12*, 60−64.

(23) Cho, C. W.; Jeon, Y. C.; Pham, T. P. T.; Vijayaraghavan, K.; Yun, Y. S. The ecotoxicity of ionic liquids and traditional organic solvents on microalga *Selenastrum capricornutum*. *Ecotox. Environ. Safe.* **2008**, *71*, 166−171.

(24) Samorì, C.; Sciutto, G.; Pezzolesi, L.; Galletti, P.; Guerrini, F.; Mazzeo, R.; Pistocchi, R.; Prati, S.; Tagliavini, E. Effects of imidazolium ionic liquids on growth, photosynthetic efficiency, and cellular components of the diatoms *Skeletonema marinoi* and *Phaeodactylum tricornutum*. *Chem. Res. Toxicol.* **2011**, *24*, 392−401.

(25) Machado, M. D.; Soares, E. V. Modification of cell volume and proliferative capacity of *Pseudokirchneriella subcapitata* cells exposed to heavy metal stress. *Aquat. Toxicol.* **2014**, *147*, 1−6.

(26) Machado, M. D.; Lopes, A. R.; Soares, E. V. Responses of the alga *Pseudokirchneriella subcapitata* to long-term exposure to metal stress. *J. Hazard. Mater.* **2015**, *296*, 82−92.

(27) Lowry, O. H.; Rosebrough, N. J.; Lewis Farr, A.; Randall, R. J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265−275.

(28) Merli, D.; Protti, S.; Petracca, P.; Fagnoni, M.; Profumo, A. A detailed study of the (electro)chemical behavior of bis-(trifluoromethanesulfonyl)imide based ionic liquids at different purification steps. *Electroanalysis* **2013**, *25*, 1453−1460.

(29) Nokami, T.; Matsumoto, K.; Itoh, T.; Fukaya, Y.; Itoh, T. Synthesis of ionic liquids equipped with 2-methoxyethoxymethyl/methoxymethyl groups using a simple microreactor system. *Org. Process Res. Dev.* **2014**, *18*, 1367−1371.

(30) *Guideline for Testing of Chemicals: Daphnia sp. Acute Immobilization Test*; OECD Guideline 202; Organization for Economic Cooperation and Development: Paris, 2004.

(31) *Guideline for Testing of Chemicals: Freshwater Alga and Cyanobacteria, Growth Inhibition Test*; OECD Guideline 201; Organization for Economic Cooperation and Development: Paris, 2011.

(32) Hochberg, Y. A sharper Bonferroni procedure for multiple tests of significance. *Biometrika* **1988**, *75*, 800−802.

(33) Koch, F. C.; McMeekin, T. L. A new direct nesslerization micro-Kjeldahl method and a modification of the Nessler-Folin reagent for ammonia. *J. Am. Chem. Soc.* **1924**, *46*, 2066−2069.

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