Green profiling of Aprotic *versus* Protic Ionic Liquids: Synthesis and Microbial toxicity of analogous structures

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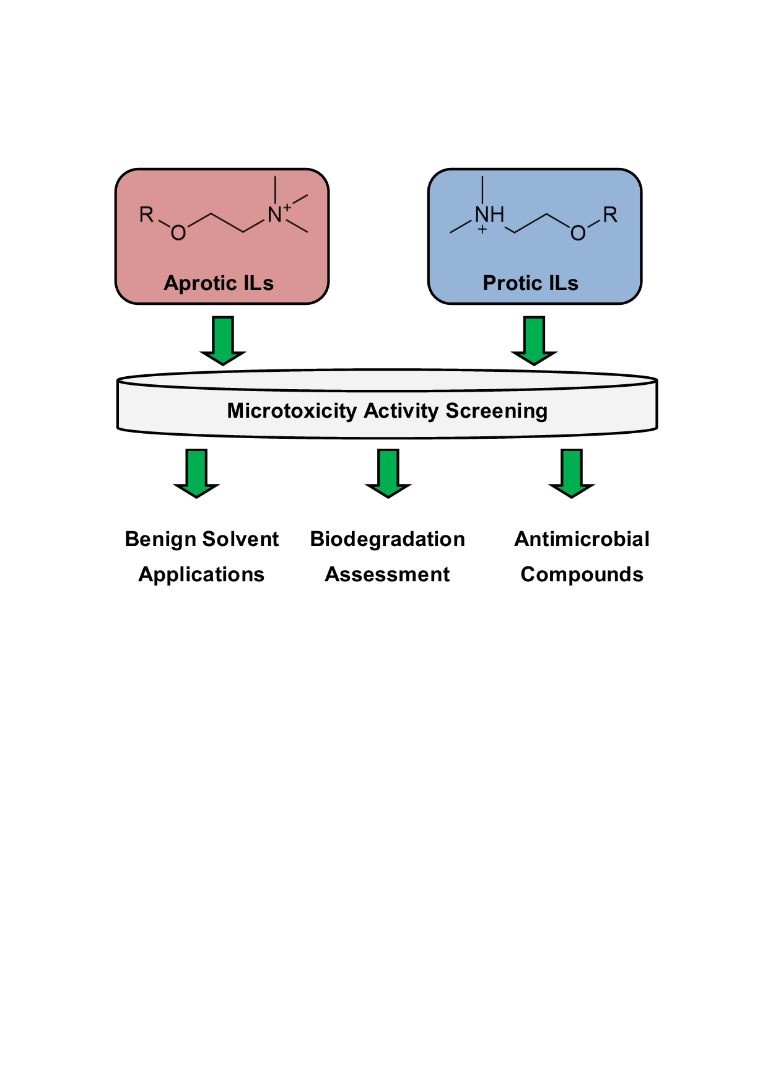
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# Abstract

How does the variation in the ionic nature of ionic liquids (ILs) affect their antimicrobial properties? To answer this question with a direct connection to the molecular structure of ILs is integral for the design of new task specific ILs. The effect of ionic nature can be investigated through a comparison between analogous aprotic and protic ILs. However, while there have been extensive studies on the toxicology of both aprotic and protic ILs, the number of different structures and procedures employed makes quantitative comparison impossible. To address this, a series of analogous *N,N,N-*trimethylethanolammonium (cholinium) derived aprotic ILs (AILs) and *N,N-*dimethylethanolammonium derived protic ILs (PILs) with acetate, hexanoate, D,L-mandelate and 3-ethoxypropionate anions were prepared and characterised. All ILs were subsequently screened for antimicrobial activity against eight bacterial and twelve fungi strains. From the antimicrobial activity screening, little difference was found between the toxicities of AILs and PILs with shorter chains terminating in hydroxyl functional groups (e.g cholinium hexanoate and *N,N*-dimethylethanolammonium hexanoate). Variations between anion structure demonstrated slightly higher toxicities for more lipophilic anions. Antimicrobial activities were found to significantly increase for ILs with a long ether chain functional groups in the cation, due to the enhanced surfactant properties of these long chain cations. The importance of toxicity screening of analogous series of AILs and PILs as part of a future comprehensive biodegradation analysis has also been proposed based on postulated IL breakdown pathways.

**Abstract Graphic**

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**Synopsis**

The antimicrobial activity of an analogous series of aprotic and protic ionic liquids have been analysed with the aim of understanding how the variation of their ionic nature relates to their molecular structure. This information is integral for the design of new safer task specific ILs.

**Keywords**

Toxicology; Antimicrobial activity; Protic Ionic Liquids; Aprotic Ionic Liquids; Choline analogues;

# Introduction

Ionic liquids (ILs), chemicals composed of ionic species which are most often liquid below 100°C (MacFarlane et al., 2017), continue to be widely researched (Austen Angell et al., 2012; Greaves and Drummond, 2015; Hallett and Welton, 2011). The classifications of ILs can be refined further by considering their ionic nature, such as aprotic ionic liquids (AILs) typically formed through the Menshutkin type reactions (Wang, 2010) and protic ionic liquids (PILs) formed from the proton transfer reaction between a Brønsted acid and a suitable base. Particular properties of both aprotic and protic ILs can be altered, such as solvation environment and thermal stability, by modification of either anion or cation. ILs have been showcased in numerous applications to date, including biomass processing and extraction (Idris et al., 2014; Kilpeläinen et al., 2007; Verdía et al., 2014; Wang et al., 2012), catalysis(Parvulescu and Hardacre, 2007; Sheldon, 2001, 2016, Walker and Bruce, 2004a, 2004b), pharmacology (Smiglak et al., 2014; Stoimenovski et al., 2012) and electrochemistry (Armand et al., 2009; Macfarlane et al., 2014, 2007; Xu and Angell, 2003).

With the increased popularity of IL research, many questions have been raised about the wider implications of using these materials, particularly for the replacement of traditional solvents with ILs in chemical and biological processes. Understanding the hazards that these materials pose to both humans and the environment has become a topic of considerable research. To date, there has been extensive research on IL toxicity (Amde et al., 2015; Egorova and Ananikov, 2014; Petkovic et al., 2011; Zhao et al., 2007), biodegradability (Coleman and Gathergood, 2010; Jordan and Gathergood, 2015) and to a lesser extent mutagenicity (Docherty et al., 2006; Reid et al., 2015), with much more attention focused on aprotic ILs rather than protic ILs. It has been made apparent that numerous IL structures commonly designed as potential candidates as replacement solvents are highly toxic and/or do not pass standardised ISO and OECD biodegradation tests (Bernot et al., 2005; Docherty and Kulpa, Jr., 2005; Romero et al., 2008; Wells and Coombe, 2006). In response to this, a selection of new IL structures which have low microbial toxicity and/or are readily biodegradable have been successfully applied as catalysts (Ferlin et al., 2013b; Myles et al., 2013a, 2013b), electrolytes for dye-sensitised solar cells (Ghavre et al., 2014) and solvents for biomass processing (An et al., 2015; Garcia et al., 2010; Hou et al., 2013a; Liu et al., 2015). IL toxicity is intrinsically linked to biodegradation and undertaking preliminary toxicity screening of ILs before biodegradation studies has been recommended by our group previously (Ferlin et al., 2013b; Haiß et al., 2016; Jordan et al., 2016; Myles et al., 2013a, 2013b; Prydderch et al., 2017). Thus, understanding how molecular structure influences IL microtoxicity can be considered the first step in assessing their potential hazards.

A feature of ILs that exhibit low environmental hazard was the presence of non-heterocyclic ammonium cations. Common structures for ammonium AILs screened include tetrabutylammonium (Ferlin et al., 2013a, 2013b) and cholinium (*N,N,N-*trimethylethanolammonium) (Hou et al., 2013b), the latter being of particular interest due to its occurrence in many natural systems. For example, the cytotoxicities of cholinium acetate and 1-ethyl-3-methylimidazolium acetate were assessed by evaluating the effective concentration at 50% population depletion (EC50) towards *Saccharomyces cerevisiae* MT8-1 at different doses (Ninomiya et al., 2013). The EC50 value for cholinium acetate (510 mM) was almost an order of magnitude greater than that of 1-ethyl-3-methylimidazolium acetate (73 mM), showcasing the stark contrast in biocompatibility of the two cation structures.

The effect of microbial toxicity on the resulting biodegradability of cholinium ILs has been shown elsewhere. Three ILs featuring the methylsulfonate anion and the quaternary ammonium cations cholinium, *N,N,N*-trimethylbutylammonium and *N,N,N*-trimethyl-2-methoxyethylammonium were assessed for their toxicity and biodegradability (Stolte et al., 2012). The biodegradability testing, carried out by CO2 evolution (OECD 301B) and O2 consumption (OECD 301F), found that the cholinium and *N,N,N*-trimethylbutylammonium ILs were readily biodegradable (> 60% degradation over 28 days), whereas the *N,N,N*-trimethyl-2-methoxyethylammonium IL was not readily biodegradable. This could be considered consistent with the decreased EC50 value towards *Daphnia magna* of the *N,N,N*-trimethyl-2-methoxyethylammonium IL (EC50 8 mg L-1), in comparison to the cholinium and *N,N,N*-trimethylbutylammonium ILs which presented with higher EC50 values (EC50 >100 mg L-1 and EC50 14 mg L-1, respectively). However, all three ILs shared EC50 values of the maximum tested concentrations towards IPC-81, *Vibrio fischeri* and *Selenastrum capricornutum*, highlighting that toxicities of ILs vary significantly between different microbial strains and that the connection between IL toxicity and biodegradability is complex and requires comprehensive independent assessment of both characteristics. The cytotoxicity, as quantified by the concentration required for 50% inhibition (IC50), of a series of cholinium carboxylate ILs were determined using the human breast cancer cell line, MCF-7. These results included cholinium acetate and cholinium hexanoate, which were both found to have similar IC50 values of 10.5 mM and 14.6 mM, respectively (Muhammad et al., 2012). In a separate study, the same two cholinium carboxylate ILs were part of a series of ILs screened for their biodegradability in the presence of *Penicillium* *corylophilum* cultures (Petkovic et al., 2009).The results suggested that cholinium ILs featuring longer alkyl chains (e.g. butanoate, hexanoate) on the anion were more readily biodegradable than shorter alkyl chains (e.g. acetate, formate) on the anion. This could be rationalised by the observed increased toxicity towards filamentous fungi of the cholinium acetate IL with respect to the cholinium hexanoate IL. However, the dose variations between the biodegradation and toxicity test procedures means quantitative comparison of the effect of alkyl chain length is not possible. A recent article has brought into question the benign aspects of Choline ILs, where a number of ILs containing the choline cation where shown to exhibit greater toxicities than molecular solvents (Santos et al., 2015). The same study also questions the suitability of using the conclusions drawn from the results of single tests to define potential ecological hazards, and cautions the use of quantitative structure activity relationships (QSARs) with IL toxicity.

As for PILs, cation structures vary between primary, secondary and tertiary ammonium cations typically with either alkyl or hydroxyl functional groups. While there was a wide selection of anions paired with aprotic cations in these studies (Petkovic et al., 2009), typically only carboxylate anions were paired with protic cations for the study of PILs. Numerous different procedures for screening the microbial toxicity of ammonium PILs have been undertaken in recent years (vide infra).

Eight PILs featuring either formate or acetate anions paired with primary, secondary or tertiary ammonium cations with 2-hydroxyethyl functional groups were screened for antimicrobial activity based on the well diffusion method (Ismail Hossain et al., 2011). All eight PILs from this study displayed some degree of antimicrobial behaviour, in particular with the *Staphylococcus aureus* strain; however, all showed either equal or less inhibition than the control antibiotic Gentamicin. Around the same time, a separate study investigated the antimicrobial activity of seven PILs, including three from the previously mentioned study, with *S. aureus* and *Escherichia coli*. (Ding et al., 2011). Microcalorimetry was utilised to measure the growth rate constants for systems with varying concentrations of PILs. The minimum biocidal concentrations (MBC) calculated for each PIL showed similar trends to the aforementioned study. In addition, four PILs based on combinations of 2-hydroxyethyl functionalised amines (including the tertiary amine *N,N*-dimethylethanolamine) with 2-(acetyloxy)benzoic acid were screened as potential water soluble liquid forms of aspirin (Adamovich et al., 2012). All four PILs were found to have low toxicity compared to peritoneal LD50,the lethal dose of a substance to kill 50% of a test population, of albino mice (2000 mg kg-1 for all PILs compared to 1430 mg kg-1 for aspirin). A recent study showed that four secondary ammonium carboxylate PILs exhibited some degree of antimicrobial and antifungal properties, as determined using growth inhibition analysis with a variety of test cultures (e.g. *S. aureus, E. coli,* and *C. albicans*) (Oliveira et al., 2016). In the same study the biodegradability of the studied PILs, as determined from oxygen consumption, found none of the PILs to be readily biodegradable. These results so far suggest that PILs prepared from primary or secondary amines and aliphatic carboxylic acids can exhibit varying degrees of microbial and ecological toxicity, depending on the anions they were paired with and the microbial strains that were studied (Tzani et al., 2016).

Of these two classes of ionic liquid, AILs and PILs, is it possible to state that either be considered more environmentally benign than the other? Despite the extensive research on AILs and PILs, there is little data available to quantitatively compare these characteristics between these ILs (Peric et al., 2014). By not comparing the toxicology (and biodegradation) of PILs and AILs with analogous structures, we lack systematic insight into how the ionic nature of an IL affects the toxicology. We hypothesise that the variation in ionic nature will influence the biocompatibility of the resulting ILs. In general, ionic species will travel slower through cell walls in the absence of active uptake mechanisms than neutral species of similar sizes (Seward and Schultz, 1999). Protic ILs are often regarded as having less ionic character than their aprotic counterparts, due to the proton transfer equilibrium that results in neutral species, as well as strong ion-ion interactions through anion-cation hydrogen bonding interactions (Austen Angell et al., 2012; Greaves and Drummond, 2015; Reid et al., 2017b). This fundamental difference in the molecular interactions between aprotic and protic ILs (Reid et al., 2017c) has significant implications on the use of QSARs for PILs and AILs by not compensating for the difference in ionic nature.

To address this, twelve aprotic and twelve protic ILs (**1a-6d**) with analogous cation and anion structure were synthesised for identical microtoxicity and biodegradation screening (Table 1). By selecting AILs derived from cholinium cations ([Ch] **1a-d**) and PILs derived from *N,N*-dimethylethanolammonium cations ([DMEtAH] **4a-d**), the structures can be considered analogous and allow for the assessment of ionic nature and cation functionality in parallel. We also further explore the effects of cation functionality by modifying the hydroxyl functional group to either a 2-(2-hydroxyethoxy)ethyl- (cations **2a-d** and **5a-d**) or a 2-(2-ethoxyethoxy)ethyl- (cations **3a-d** and **6a-d)** functional group. A selection of carboxylate anions with varying functionality have been used to study the effects of anion structure; acetate (**1a**-**6a**), hexanoate, (**1b**-**6b**) d,l-mandelate (**1c**-**6c**) and 3-ethoxypropionate (**1d**-**6d**) anions.

**Table 1** Structures of the various cation and anion structures of the APILs (**1a–3d**) and PILs (**4a–6d**) in this study.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | **[OAc],a** | **[Hx],b** | **[Md],c** | **[EtOPr],d** |
| **[Ch], 1** | **1a** | **1b** | **1c** | **1d** |
| **[TMHEEA], 2** | **2a** | **2b** | **2c** | **2d** |
| **[TMEEEA], 3** | **3a** | **3b** | **3c** | **3d** |
| **[DMEtAH], 4** | **4a** | **4b** | **4c** | **4d** |
| **[DMHEEAH], 5** | **5a** | **5b** | **5c** | **5d** |
| **[DMEEEAH], 6** | **6a** | **6b** | **6c** | **6d** |

# Materials and Methods

## Synthesis

All AILs (**1a-3d**)were prepared according to a modified version of the literature procedures (Alcalde et al., 2012; Fukaya et al., 2007). All PILs (**4a-6d**) were prepared according to the synthesis procedure of Walker (Walker, 2004). The precursor amine to the ILs with either cation [TMEEEA]or [DMEEEAH], 2-(2-ethoxyethoxy)-*N,N*-dimethylethan-1-amine, was prepared according to a modified version of the literature procedure (Bodmann and Manuela, 2005). Precursor iodide ILs 2-(2-hydroxyethoxy)-*N,N,N*-trimethylethan-1-aminium iodide and 2-(2-ethoxyethoxy)-*N,N,N*-trimethylethan-1-aminium iodide were prepared according to a modified version of the literature procedures (Hallett and Welton, 2011; Prydderch et al., 2017). See supporting information for full synthetic procedures and compound characterisation.

# Microbial toxicology assessment

The *in vitro* microtoxicity of the 24 ILs (**1a–6d**) was assessed against eight bacterial strains (SA: *Staphylococcus aureus* ATTC 6538, MRSA: *Staphylococcus aureus* MRSA HK5996/08, SE: *Staphylococcus epidermidis* HK6966/08, EF: *Enterococcus sp.* HK14365/08, EC: *Escherichia coli* ATTC 8739, KP: *Klebsiella pneumoniae* HK11750/08, KP-E: *Klebsiella pneumoniae ESBL* HK14368/08, and PA: *Pseudomonas aeruginosa* ATTC 9027) and twelve fungal strains(CA1: *Candida albicans* ATCC 44859, CA2: *Candida albicans* ATCC 90028, CP: *Candida parapsilosis* ATCC 22019, CK1: *Candida krusei* ATCC 6258, CK2: *Candida krusei* E28, CT: *Candida tropicalis* 156, CG: *Candida glabrata* 20/I, CL: *Candida lusitaniae* 2446/I, TA: *Trichosporon asahii* 1188, AF: *Aspergillus fumigatus* 231, AC: *Absidia corymbifera* 272, TM: *Trichophyton mentagrophytes* 445) using the procedure followed by Jordan *et al* (Jordan et al., 2016). Full procedural details of the bacterial and fungal microtoxicity assessment can be found in the supporting information of this manuscript.

# Results and Discussion

To assist with visualising the microtoxicity data, we have used a colour scheme in all results tables which corresponds to the test results in general. Results shaded green indicate minimum inhibition concentrations (MICs) greater than the maximum screening concentration of 2000 μM, results shaded yellow indicate a moderate toxicity MIC which was in the range 500–2000 μM, and results shaded red indicate high microbial toxicities as inferred from low MICs in the range 7.8–250 μM. ILs with high antimicrobial or antifungal activity may fail future biodegradation studies due to this adverse biocidal property. For readers who are colour blind, versions of the data tables featuring a greyscale highlighting scheme can be found in the supporting information of this manuscript. Of the 24 ILs studied, eight exhibited MICs greater than the maximum screening concentration towards all microbial strains studied. This result does not classify these ILs as non-toxic, but rather highlights that they do not exhibit acute microtroxicity.

## Antibacterial activity screening

For most of the ILs derived from either the [Ch] (**1a-d**) or [DMEtAH] cation (**4a-d**), high toxicities were not observed with MICs greater than the maximum screening concentration of 2000 μM for all eight strains (Table 2). The only exceptions were for **1a** (IC95 1000 μM, SE, 48 h; IC95 2000 μM, EF, 48 h) and **4d** (IC95 1000 μM, SE, 48 h). In addition, four ILs (**2c**, **2d**, **5b** and **5d**) from the [TMHEEA] (**2a-d**) and [DMHEEAH] (**5a-d**) cation series, were also found to have MICs greater than the maximum screening concentration (Table 3).

However, there were four ILs with MICs equal to or less than 2000 µM in the antibacterial activity screening. This was slightly more toxic when compared to ILs **1a-d**, and **4a-d** with [Ch] or [DMEtAH] cations. Specifically, this includes **5a** (IC95 = 1000 μM, SE, 48 h), **5d** (IC95 = 1000 μM, SE, 48 h), **2a** (IC95 = 250 μM, SE, 48 h; IC95 = 2000 μM, EC, 48 h) and **2b** (IC95 = 2000 μM, MRSA, 48 h; IC95 = 250 μM, SE, 48 h; IC95 = 250 μM, EC, 48 h). While there were isolated instances where antibacterial activity was greater, i.e. for one class of IL with the 2-(2-hydroxyethoxy)ethyl functional group over another, there were no extended trends of toxicity with cation ionic nature.

**Table 2** Antibacterial activity screening results for the [Ch] AILs (**1a–1d**) and the [DMEtAH] PILs (**4a–4d**) (MIC, IC95)

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Straina** | **Time (h)** | **IL MIC (IC95,****μM)** | | |
| **1a** | **1b-1d,**  **4a-4c** | **4d** |
| **SA** | 24 | >2000 | >2000 | >2000 |
| 48 | >2000 | >2000 | >2000 |
| **MRSA** | 24 | >2000 | >2000 | >2000 |
| 48 | >2000 | >2000 | >2000 |
| **SE** | 24 | 1000 | >2000 | 1000 |
| 48 | 1000 | >2000 | 1000 |
| **EF** | 24 | 2000 | >2000 | >2000 |
| 48 | 2000 | >2000 | >2000 |
| **EC** | 24 | >2000 | >2000 | >2000 |
| 48 | >2000 | >2000 | >2000 |
| **KP** | 24 | >2000 | >2000 | >2000 |
| 48 | >2000 | >2000 | >2000 |
| **KP-E** | 24 | >2000 | >2000 | >2000 |
| 48 | >2000 | >2000 | >2000 |
| **PA** | 24 | >2000 | >2000 | >2000 |
| 48 | >2000 | >2000 | >2000 |

aSA: *Staphylococcus aureus* ATTC 6538, MRSA: *Staphylococcus aureus* MRSA HK5996/08, SE: *Staphylococcus epidermidis* HK6966/08, EF: *Enterococcus sp.* HK14365/08, EC: *Escherichia coli* ATTC 8739, KP: *Klebsiella pneumoniae* HK11750/08, KP-E: *Klebsiella pneumoniae ESBL* HK14368/08, PA: *Pseudomonas aeruginosa* ATTC 9027.

**Table 3** Antibacterial activity results for the [TMHEEA] AILs (**2a–2d**) and the [DMHEEAH] PILs (**5a–5d**) (MIC, IC95)

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Straina** | **Time (h)** |  | **IL MIC (μM)** | | | | |  |
| **2a** | **5a** | **2b** | **5b** | **2c** | **5c** | **2d, 5d** |
| **SA** | 24 | >2000 | >2000 | >2000 | >2000 | >2000 | >2000 | >2000 |
| 48 | >2000 | >2000 | >2000 | >2000 | >2000 | >2000 | >2000 |
| **MRSA** | 24 | >2000 | >2000 | 2000 | >2000 | >2000 | >2000 | >2000 |
| 48 | >2000 | >2000 | 2000 | >2000 | >2000 | >2000 | >2000 |
| **SE** | 24 | 250 | 1000 | 250 | >2000 | >2000 | 500 | >2000 |
| 48 | 250 | 1000 | 250 | >2000 | >2000 | 500 | >2000 |
| **EF** | 24 | >2000 | >2000 | >2000 | >2000 | >2000 | >2000 | >2000 |
| 48 | >2000 | >2000 | >2000 | >2000 | >2000 | >2000 | >2000 |
| **EC** | 24 | 2000 | >2000 | 250 | >2000 | >2000 | >2000 | >2000 |
| 48 | 2000 | >2000 | 250 | >2000 | >2000 | >2000 | >2000 |
| **KP** | 24 | >2000 | >2000 | >2000 | >2000 | >2000 | >2000 | >2000 |
| 48 | >2000 | >2000 | >2000 | >2000 | >2000 | >2000 | >2000 |
| **KP-E** | 24 | >2000 | >2000 | >2000 | >2000 | >2000 | >2000 | >2000 |
| 48 | >2000 | >2000 | >2000 | >2000 | >2000 | >2000 | >2000 |
| **PA** | 24 | >2000 | >2000 | >2000 | >2000 | >2000 | >2000 | >2000 |
| 48 | >2000 | >2000 | >2000 | >2000 | >2000 | >2000 | >2000 |

aSA: *S. aureus* ATTC 6538, MRSA: *S. aureus* MRSA HK5996/08, SE: *S. epidermidis* HK6966/08, EF: *E. sp.* HK14365/08, EC: *E. coli* ATTC 8739, KP: *K. pneumoniae* HK11750/08, KP-E: *K. pneumoniae ESBL* HK14368/08, PA: *P. aeruginosa* ATTC 9027.

With the 2-(2-ethoxyethoxy)ethyl functionalised ILs (AILs **3a–d** and PILs **6a–d**) a significant decrease in the MICs to all bacterial strains for both protic and aprotic ILs was identified (Table 4). These eight ILs all exhibited high bacterial toxicity towards the *S. epidermidis* strain (IC95 ≤ 250 μM), with the PIL **6d** showing the lowest MIC (IC95 = 7.8 μM). In contrast, the strain most resilient to the presence of added ILs was *P. aeruginosa*, which gave MICs above the testing concentration for the AILs **3a-d** and MICs indicating moderate antibacterial activity for the PILs **6a-d**. For the AILs, comparing between AIL and PIL antibacterial activity, the PILs **6a-d** exhibit lower MICs than their aprotic analogues **3a-d**, based on a greater number of bacterial strains that exhibited MICs above screening concentrations.

**Table 4** Antibacterial screening results for the [TMEEEA] AILs (**3a–3d**) and the [DMEEEAH] PILs (**6a–6d**) (MIC, IC95)

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Straina | Time (h) | IL MIC (μM) | | | | | | | |
| **3a** | **6a** | **3b** | **6b** | **3c** | **6c** | **3d** | **6d** |
| **SA** | 24 | 125 | 62.5 | 500 | 31.3 | 250 | 31.3 | 1000 | 15.6 |
| 48 | 125 | 62.5 | 500 | 31.3 | 250 | 31.3 | 1000 | 15.6 |
| **MRSA** | 24 | 500 | 62.5 | 1000 | 62.5 | 2000 | 62.5 | 2000 | 62.5 |
| 48 | 500 | 62.5 | 1000 | 62.5 | 2000 | 62.5 | 2000 | 62.5 |
| **SE** | 24 | 250 | 125 | 125 | 15.6 | 250 | 62.5 | 125 | 7.8 |
| 48 | 250 | 125 | 125 | 15.6 | 250 | 62.5 | 125 | 7.8 |
| **EF** | 24 | 2000 | 250 | 2000 | 125 | 2000 | 125 | >2000 | 125 |
| 48 | 2000 | 250 | 2000 | 125 | 2000 | 125 | >2000 | 125 |
| **EC** | 24 | 1000 | 500 | 2000 | 125 | 2000 | 125 | >2000 | 250 |
| 48 | 1000 | 500 | 2000 | 125 | 2000 | 125 | >2000 | 250 |
| **KP** | 24 | >2000 | 500 | 2000 | 500 | >2000 | 250 | >2000 | 500 |
| 48 | >2000 | 500 | 2000 | 500 | >2000 | 250 | >2000 | 500 |
| **KP-E** | 24 | 2000 | 250 | 2000 | 125 | >2000 | 250 | >2000 | 250 |
| 48 | 2000 | 250 | 2000 | 125 | >2000 | 250 | >2000 | 250 |
| **PA** | 24 | >2000 | 2000 | >2000 | 2000 | >2000 | 1000 | >2000 | 1000 |
| 48 | >2000 | 2000 | >2000 | 2000 | >2000 | 1000 | >2000 | 1000 |

aSA: *S. aureus* ATTC 6538, MRSA: *S. aureus* MRSA HK5996/08, SE: *S. epidermidis* HK6966/08, EF: *E. sp.* HK14365/08, EC: *E. coli* ATTC 8739, KP: *K. pneumoniae* HK11750/08, KP-E: *K. pneumoniae ESBL* HK14368/08, PA: *P. aeruginosa* ATTC 9027.

In each class of IL, the anion was found to influence the antibacterial activity of the ILs in Table 4 differently. For AILs, the trend of observed microtoxicity follows [EtOPr] < [Md] < [Hx] ≈ [OAc], and for PILs the trend follows [OAc] < [Hx] ≈ [Md] < [EtOPr]. Although, the ILs **3a** and **6a**, featuring the [OAc] anion, have the most similar IC95 values when considering all bacteria strains studied, whereas the MICs of the PILs **3b-d** were much lower than the MICs of their analogous aprotic structures (**6a–6d**).

In terms of relative bacterial toxicity towards the strains used in this screening, both AILs and PILs classes of ILs show similar responses; in general higher toxicities were observed for the four Gram-positive bacterial strains (*S. aureus***,** *S. aureus* MRSA**,** *S. epidermidis* and *Enterococcus* sp.) compared to the four Gram- negative bacterial strains (*E. coli*, *K. pneumoniae,* *K. pneumoniae* ESBL and *P. aeruginosa*). Similar observations have been made previously for other ILs (Amde et al., 2015), as well as for molecular solvent toxicity towards Gram- positive and Gram- negative bacterial strains (Vermuë et al., 1993), which has been rationalised by the variation of cell wall composition between these two classes of bacteria (Russell and Gould, 1988). Of note was the susceptibility of the *S, aureus* strain to the ILs tested, with IC95 values ranging from >2000 μM to as low as 7.8 μM. This sensitivity of this bacteria to the IL structures could make it a useful guide to the antibacterial properties of the ILs screened here. However, we caution the use of a single bacterial strain to characterise antimicrobial activity of an IL – the MICs for the *S. epidermidis* do not correlate well with the MICs towards all other bacterial strains for the ILs **1a, 2a, 2b, 4d, 5a** or **5c**.

## Antifungal activity screening

A general observation was that the MICs were greater for the fungal strains than they were for the bacterial strains. This was in part due to the difference in IC values determined for the bacterial screening (IC95) and the fungal screening (IC80 for yeasts and IC50 for filamentous fungi). The [Ch] AILs (**1a**-**d**) and [DMEtAH] PILs (**4a**-**d**) all exhibited MIC values greater than the maximum screening concentration (2000 µM) and were not highly toxic to the fungal strains (Table 5). The [TMHEEA] AILs (**2a-d**) all had MIC values greater than the maximum screening concentration (2000 µM) for all fungal strains, while for the analogous PIL series (**5a-d**) only **5a** and **5c** were consistent with this result (Table 5). The PIL **5b** had IC80/IC50 values to all strains above the maximum screening concentration, which was limited to 500 µM due to low IL solubility in the media required for the test assay. The PIL **5d** exhibited MIC values at the maximum test concentration towards two strains; *C. krusei* (both variants, IC80 = 2000 μM, 48 h) and *T. mentagrophytes* (IC50 = 2000 μM, 120 h) (Table 5).

**Table 5** Antifungal screening results for the [Ch] AILs (**1a-d)**, [TMHEEA] AILs (**2a-d**), [DMEtAH] PILs (**4a-d**) and the [DMHEEAH] PILs (**5a-d**) (MIC, IC80 or IC50).

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Straina** | **Time (h)** | **IL MIC (μM)b** | | |
| **1a-d,** | **5bc** | **5d** |
| **2a-d,** |
| **4a-4d,** |
| **5a, 5c** |
| **CA1** | 24 | >2000 | >500 | >2000 |
| 48 | >2000 | >500 | >2000 |
| **CA2** | 24 | >2000 | >500 | >2000 |
| 48 | >2000 | >500 | >2000 |
| **CP** | 24 | >2000 | >500 | >2000 |
| 48 | >2000 | >500 | >2000 |
| **CK1** | 24 | >2000 | >500 | 2000 |
| 48 | >2000 | >500 | 2000 |
| **CK2** | 24 | >2000 | >500 | 2000 |
| 48 | >2000 | >500 | 2000 |
| **CT** | 24 | >2000 | >500 | >2000 |
| 48 | >2000 | >500 | >2000 |
| **CG** | 24 | >2000 | >500 | >2000 |
| 48 | >2000 | >500 | >2000 |
| **CL** | 24 | >2000 | >500 | >2000 |
| 48 | >2000 | >500 | >2000 |
| **TA** | 24 | >2000 | >500 | >2000 |
| 48 | >2000 | >500 | >2000 |
| **AF** | 24 | >2000 | >500 | >2000 |
| 48 | >2000 | >500 | >2000 |
| **AC** | 24 | >2000 | >500 | >2000 |
| 48 | >2000 | >500 | >2000 |
| **TM** | 72 | >2000 | >500 | 2000 |
| 120 | >2000 | 500 | 2000 |

aCA1: *Candida albicans* ATCC 44859, CA2: *Candida albicans* ATCC 90028, CP: *Candida parapsilosis* ATCC 22019, CK1: *Candida krusei* ATCC 6258, CK2: *Candida krusei* E28, CT: *Candida tropicalis* 156, CG: *Candida glabrata* 20/I, CL: *Candida lusitaniae* 2446/I, TA: *Trichosporon asahii* 1188, AF: *Aspergillus fumigatus* 231, AC: *Absidia corymbifera* 272, TM: *Trichophyton mentagrophytes* 445. bIC50 values were assessed for AF, AC and TM and IC80 for all other strains. **c**Maximum screening concentration was limited to 500 μM due to low solubility of **5b** in test media.

**Table 6** Antifungal screening results for the [TMEEEA] AILs (**3a–3d**) and the [DMEEEAH] PILs (**6a–6d**) (MIC, IC80 or IC50).

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Straina** | **Time (h)** | **IL MIC (μM)b** | | | | | | | |
| **3a** | **6a** | **3b** | **6b** | **3c** | **6c** | **3d** | **6d** |
| **CA1** | 24 | >2000 | 64.5 | >2000 | 125 | >2000 | 62.5 | >2000 | 125 |
| 48 | >2000 | 125 | >2000 | 125 | >2000 | 62.5 | >2000 | 125 |
| **CA2** | 24 | >2000 | 125 | >2000 | 125 | >2000 | 62.5 | >2000 | 125 |
| 48 | >2000 | 125 | >2000 | 125 | >2000 | 62.5 | >2000 | 125 |
| **CP** | 24 | 1000 | 62.5 | 2000 | 62.5 | 2000 | 62.5 | 2000 | 125 |
| 48 | 1000 | 62.5 | 2000 | 125 | 2000 | 62.5 | 2000 | 125 |
| **CK1** | 24 | 1000 | 31.3 | 250 | 31.3 | 500 | 31.3 | 2000 | 62.5 |
| 48 | 1000 | 62.5 | 250 | 62.5 | 500 | 31.3 | 2000 | 62.5 |
| **CK2** | 24 | 1000 | 31.3 | 250 | 31.3 | 500 | 31.3 | 2000 | 62.5 |
| 48 | 1000 | 62.5 | 250 | 62.5 | 500 | 31.3 | 2000 | 62.5 |
| **CT** | 24 | 1000 | 62.5 | 1000 | 125 | 500 | 62.5 | >2000 | 62.5 |
| 48 | 1000 | 62.5 | 1000 | 125 | 500 | 62.5 | >2000 | 62.5 |
| **CG** | 24 | >2000 | 500 | >2000 | 500 | >2000 | 250 | >2000 | 250 |
| 48 | >2000 | 500 | >2000 | 500 | >2000 | 250 | >2000 | 250 |
| **CL** | 24 | >2000 | 500 | >2000 | 500 | >2000 | 500 | >2000 | 500 |
| 48 | >2000 | 500 | >2000 | 500 | >2000 | 500 | >2000 | 500 |
| **TA** | 24 | >2000 | 250 | >2000 | 500 | >2000 | 250 | >2000 | 500 |
| 48 | >2000 | 250 | >2000 | 500 | >2000 | 250 | >2000 | 500 |
| **AF\*** | 24 | >2000 | 250 | >2000 | 500 | >2000 | 500 | >2000 | 500 |
| 48 | >2000 | 500 | >2000 | 1000 | >2000 | 500 | >2000 | 500 |
| **AC\*** | 24 | >2000 | 250 | >2000 | 1000 | >2000 | 500 | >2000 | 1000 |
| 48 | >2000 | 500 | >2000 | 1000 | >2000 | 500 | >2000 | 1000 |
| **TM\*** | 72h | >2000 | 62.5 | >2000 | 125 | >2000 | 125 | >2000 | 125 |
| 120h | >2000 | 62.5 | >2000 | 125 | >2000 | 125 | >2000 | 125 |

aCA1: *C. albicans* ATCC 44859, CA2: *C. albicans* ATCC 90028, CP: *C. parapsilosis* ATCC 22019, CK1: *C. krusei* ATCC 6258, CK2: *C. krusei* E28, CT: *C. tropicalis* 156, CG: *C. glabrata* 20/I, CL: *C. lusitaniae* 2446/I, TA: *T. asahii* 1188, AF: *A. fumigatus* 231, AC: *A. corymbifera* 272, TM: *T. mentagrophytes* 445. bIC50 values were assessed for AF, AC and TM and IC80 for all other strains.

Consistent with the data from the bacteria toxicity screening, there was a distinct decrease in the MIC towards fungal strains when changing from the hydroxyl functional end group to the ethoxy end group in the IL cation. (Table 6) The AILs (**3a-d**) all demonstrated moderate toxicity towards *C. krusei*, *C. parapsilosis* and *C. tropicalis* strains, with **3b** exhibiting high antifungal activity towards some strains (IC80 = 250 μM, CK1 and CK2, 48 h). However towards all other strains, including all three filamentous fungal strains, the AILs **3a-d** exhibited IC80 and IC50 values above the maximum tested concentrations.

In contrast, the [DMEEEAH] PILs (**6a-d**) all showed high antifungal activity to a majority of the fungal strains. Moderate antifungal activity for the PILs **6a-d** after 24 and 48 hours was found only for the *C. lusitaniae* strain, and none of the PILs exhibited IC80 or IC50 values above the maximum tested concentrations. As with the AILs **3a-d**, the PILs **6a-d** exhibited higher antifungal activities towards the *C. krusei*, *C. parapsilosis* and *C. tropicalis* strains, in addition to the *C. Albicans* and *T. mentagrophytes* strains. Varying the anion appears to influence the fungal microtoxicities of each class of IL differently, but not in a consistent manner as found with the bacterial microtoxicities. For AILs (**3a–3d**), the trend of increasing fungal microtoxicity follows [EtOPr] < [OAc] ≈ [Md] < [Hx], while for PILs (**6a–6d**) the trend follows [EtOPr] < [Hx] ≈ [OAc] < [Md].

## Effect of anion and cation structure on IL microtoxicity results

Considering first the cations, almost identical MICs were demonstrated towards the bacterial (Table 2) and fungal (Table 5) strains for the ILs with [Ch], (**1a-d**) or [DMEtAH], (**4a-d**) cations. Similar results were also seen between the ILs with the [TMHEEA], (**2a-d**) and the [DMHEEAH], (**5a-d**) cations (Tables 3 and 5). However, some MICs for the AILs **2a** and **2b** were as low as 250 μM for particular bacterial strains (Table 3), while there were no MICs below 1000 μM for the corresponding PILs **5a** and **5b**. Additionally, the PILs **5c** and **5d** showed slight reductions in MICs for certain strains screened, whereas the aprotic analogues exhibited MICs at the maximum concentration screened for all strains studied. While there was some variation in microtoxicity towards particular strains for these analogous AILs and PILs, there were no consistent trends indicating that either class of IL can be considered to have greater microtoxicity.

For the ILs with the [TMEEEA], (**3a-d**), or the [DMEEEAH], (**6a-d**), cation we observed low MICs towards a number of bacterial and fungal strains (Tables 4 and 6). This decrease in MICs for all ILs featuring the 2-(2-ethoxyethoxy)ethyl functional group, compared to the hydroxyl and the hydroxyl-ether functionalised ILs, supports the finding that it was not simply sufficient to introduce oxygen atoms into a cation structure to reduce the microtoxicity (Morrissey et al., 2009). In this study, the ether functionality alone has a very minor impact on reducing the microtoxicity of alkyl functionalised ILs. While there were no ILs screened in this study with purely alkyl functional groups on the cation, a recent study on the effect of increasing the alkyl chain length of cholinium-derivative ILs highlights the significance of increased alkyl chain length on observed toxicity (e Silva et al., 2014). The absence of a hydroxyl terminal groups and the increased carbon content on the cation of these ILs would increase the lipophilic nature of these materials. This lipophilic structure was similar to common ionic surfactants, rationalising the high antimicrobial activity observed for these molecules (Dolezal et al., 2016; El Hage et al., 2014). Additionally, the connection between lipophilicity of an IL and the compounds resultant toxicity has been shown previously (Matzke et al., 2007; Ranke et al., 2007). In these studies, the introduction of oxygen into the cation resulted in reducing the toxicity of the IL analogue, regardless of the oxygen containing functional group. This was contrary to our results, which suggest that the nature of the oxygen containing functional group plays a significant role in the resulting microtoxicity of the IL. The presence of a hydroxyl functional group appears to have a more dominant effect on the microtoxicities of the ILs in this study than the presence of an ether functional group. Further work that performs a more systematic comparison of the effects of oxygen containing functional groups is required before any generalizations can be made.

In general, AILs with cations with the 2-(2-ethoxyethoxy)ethyl functional group (**3**) appear to exhibit higher MICs for all of the bacterial or fungal strains tested in comparison to their protic analogues. This difference in observed toxicity between AILs and PILs was difficult to rationalise, as there were many parameters that govern physiochemical and phase toxicity. It was possible that the differences observed in MICs between these two classes of ILs were due to the differences in charge contributions to the lipophilicity of the resultant IL, which as discussed above has been shown to relate to microtoxicity. This could likely be a result of the proton transfer equilibrium between the cation and anion in PILs, which will always produce some neutral precursor species (Reid et al., 2017b). While the anion structures of the APILs and PILs will appear identical as drawn in Table 1, the dynamic equilibria associated with the ionic nature of PILs results in a significant difference in the chemistry of these two classes of ILs, particularly in aqueous systems such as is used in the antimicrobial activity screening. The proton equilibrium was particularly important, as the ILs were screened in dilute aqueous buffer solutions, meaning that the PIL ions were also in equilibrium with water (Reid et al., 2017c).

The effect of varying the IL anion structure on IL microtoxicity appears to be more subtle than the effect of varying the IL cation structure. For the AILs **1a-2d** and PILs **4a-5d**, there were no consistent trends in variation of either antibacterial or antifungal activity with variation of anion structure. This alone would suggest that none of the four anions studied significantly contributed to the antimicrobial activity of the IL. For the AILs **3a-d** and PILs **6a-d** we actually observed different trends between anion structure and microtoxicity. The trend of increasing AIL toxicity follows [EtOPr] < [Md] < [OAc] ≈ [Hx], while for PILs the trend followed [OAc] ≈ [Hx] < [EtOPr] < [Md]. The cause for this difference was most likely a result of the dynamic proton equilibrium between species in the neat PILs, as well as in the dilute aqueous buffer systems as discussed above. This difference in trends with anion structure suggests that the nature of the IL, i.e. whether it was an AIL or a PIL, can have a minor influence on the microtoxicity of an IL.

Despite these minor trends in antimicrobial activity of the ILs in relation to their anion, the absolute microtoxicities for each IL with the same cation functionality were found to be fairly comparable. This suggests that the variation of anion structure was not at the detriment of IL microtoxicity. Considering ILs with the [Hx] or [EtOPr] anion, which were alkyl and ether functional analogues of one another, no consistent trend was observed, indicating that simply including additional oxygen atoms into the molecular structure of an IL was not sufficient to reduce IL microtoxicity. While there were more ILs with the [EtOPr] anion than the [Hx] anion that had an MIC greater than the maximum screening concentration when combining the results of the bacterial and fungal toxicity screening, the lowest observed MICs in this study were that of the PIL [DMEEEAH][EtOPr] (**6d**), (SE, 24 h, 7.81 μM) (Table 4). This further supports the finding that the effect of substituting ether functionality onto a carboxylate anion of an IL has little to no influence on the net antimicrobial activity of the IL.

The results shown herein offer valuable discussion as to how variation of cation and anion structure, as well as the ionic nature between aprotic and protic ILs, play a role in the microtoxicity of an IL. However, the data provided in this manuscript does not show definitively if the change in microtoxicity with respect to change in ion structure is independent of any change in the IL chemistry as a result of varying anion or cation structure. Changing the structures of both aprotic (Hallett and Welton, 2011) and protic (Greaves and Drummond, 2015; Reid et al., 2017a, 2017b) ILs can have a significant impact on the intermolecular interactions between species in ILs. Establishing whether these intermolecular interactions have a significant impact on an ILs microtoxicity requires a comparative study of the microtoxicity of ILs and their corresponding precursor materials. While beyond the scope of this initial investigation, we believe that establishing connections between the microtoxicity of ILs and their precursor materials will prove invaluable in the future for the design of ILs with low microtoxicities.

## Connection between biodegradation and microtoxicity

There is growing evidence suggesting a correlation between the microtoxicity and the biodegradability of an IL(Prydderch et al., 2017). In particular, a low toxicity IL would be expected to have a greater chance at undergoing biodegradation because it may be less toxic to the biodegradation enzymes. Additionally, if an IL breaks down to produce metabolites that were of low microtoxicity, it will increase the likelihood that the IL will be fully mineralizable (due to a decreased toxicity to enzymes responsible for biodegradation). For example, the PIL *N,N*-dimethylethanolammonium acetate (**4a**), has been shown to undergoes complete decomposition in biotic conditions with high kinetics of biodegradation observed (Deng et al., 2015). The same PIL was found to increase the growth rate of *Clostridium sporogenes*, suggesting that the PIL either metabolised or increased the availability of nutrients (Dipeolu et al., 2009). This correlates well with the high MICs for both microbial and fungal strains observed for this same PIL (**4a**) in our study. Understanding if an IL could potentially breakdown to a metabolite with known high microtoxicity will highlight how ILs can be better designed to be readily biodegradable. The ILs in this study were an ideal selection for addressing this in future work. There were systematic variations of the cation structures in the ILs presented in this work, either through the nitrogen head (quaternary versus tertiary ammonium cation functionality) or through the functional group modification. Starting from the [TMEEEA] cation, it was possible to propose multiple possible biodegradation pathways to smaller molecules (Scheme 1). Based on our antimicrobial activity screening, it could be suggested that the nature of the low MIC’s observed for the ILs with either cation **3** or **6** was a result of slow breakdown kinetics of the ether functional chain to a shorter functional chain with a hydroxyl terminal group. As the ILs with cations **1,** **2, 4** and **5** have much larger MIC values, it was expected that these ILs will be readily biodegradable.



**Scheme 1** Cations (**1**-**6**) of ILs (**1a**-**6d**) related by a proposed biodegradation pathway.

# Conclusion

The effects of cation nature (AIL vs PIL) on microtoxicity for the ILs screened in this investigation appear to be secondary to the effect of the cation functional groups. Hydroxyl functionalised cations presented low toxicities regardless of anion structure, whereas cations with only ether functionality exhibited greater toxicities which the authors propose to be due to an assumed greater lipophilicity resulting from the surfactant-like structures of these ILs. . While lower MIC values for PILs with only ether functionalities with respect to their AIL analogues were observed, the effect of varying the ionic nature of the cation (AIL vs PIL) had a less pronounced effect on resulting toxicity than the variation of cation functional group. Variation of the anion functionality appeared to have a minor influence on the observed microtoxicity of either AILs of PILs, despite the variation in ionic nature of the anion. From the microtoxicity results, it was found that all ILs in this study were suitable for subsequent biodegradation analysis. No potent broad spectrum microbial biocides were demonstrated in this study. The ILs with the lowest microbial toxicity (MIC >2000 mM to all 20 strains screened) were **1b, 1c, 1d**, **2c**, **2d**, **4a, 4b and 4c**. The cation structures of the ILs utilised in this study offer an opportunity to further investigate the effect of the microtoxicity of potential IL biodegradation metabolites on ILs biodegradability.

**Supporting Information**

Includes details of the microbial strains used for antimicrobial activity screening, experimental procedures for the synthesis of IL precursors and ILs with characterization data (NMR, MS) and copies of 1H and 13C NMR spectra all ILs.

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