Revealing fungal activity in the presence of ionic liquids

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“Our virtues and our failings are inseparable, like force and matter.
When they separate, man is no more.”

Nikola Tesla

Summary

Ionic liquids constitute a vast and heterogeneous group of chemicals, generally non-volatile and of high solvent quality. They are already used in industrial processes; future applications depend heavily on conscious design of ionic liquids. Given especially the global demand for sustainable chemicals, understanding environmental risks is a priority, necessitating a multidisciplinary research approach, covering a broad range of disciplines from biology to chemistry.

Ascomycota fungi are highly suitable model organisms, especially due to their environmental ubiquity and important role in the biotic decay of pollutants. This thesis reports the first ever use of Ascomycota fungi to investigate ionic liquids ecotoxicity and environmental persistence. Fungal strains of *Penicillium* and *Aspergillus* were in general found to be more tolerant to ionic liquids containing imidazolium, pyridinium, pyrrolidinium, cholinium or phosphonium cations, than any other microorganism tested to date (Chapters II, III and IV). The capacity of the strains to tolerate the ionic liquids tested was apparently correlated to their phylogeny. Ionic liquid toxicity was evaluated using common parameters, such as growth inhibition and death. Less frequently evaluated parameters were also analysed, including monitoring of the integrity of the cellular boundaries of fungal conidia by microscopy (Chapter IV) and determining the diffusible fungal metabolome by ESI-MS and LC (Chapter II and V). Overall, these data significantly contribute to current understanding of structure-activity relationships in ionic liquids. For example toxicity is apparently a function of alkyl chain length of both anion and cation (Chapters III and IV, respectively). A critical review of current understanding of toxicity and environmental impact of the principal ionic liquid groups made it clear that the common generalisation of ionic liquids being either “green” or “toxic”

solvents is misleading as neither of these statements is completely true (Chapter I, section 1.3.1).

Above issues have inspired conscious design of a novel group of ionic liquids - cholinium alkanoates, which display low toxicity and high biodegradability (Chapter III). They are amongst the most interesting groups of ionic liquids so far investigated, and have proven to be a remarkable breakthrough as efficient solvents for suberin in cork.

Under laboratory conditions the ability of filamentous fungi to produce secondary metabolites is often repressed. These natural compounds are of heightened interest, especially given their structural diversity and potential biological activity. Addition of sub-lethal concentrations of ionic liquids to the growth media altered the profile of the diffusible fungal metabolome (*i.e.* low molecular weight compounds secreted by the fungi). Three of five tested ionic liquids activated cryptic metabolites with demonstrable biological effects on HeLa cells and bacteria (Chapter V). These findings merit more detailed follow-up – the data presented here are preliminary but encouraging. In summary, ionic liquids potential to activate cryptic fungal metabolites is highly promising in the discovery of valuable novel natural products.

The work presented in this thesis constitutes a foundation for developing novel environmentally sustainable biotechnological processes.

Sumário

Os líquidos iónicos reúnem um numeroso e heterogéneo grupo de substâncias químicas, geralmente não-voláteis e de excelente desempenho como solventes. Apesar de serem já utilizados em inúmeros processos industriais, aplicações futuras dependem particularmente de uma formulação consciente de líquidos iónicos. Testemunhamos, globalmente, a procura de produtos químicos sustentáveis, pelo que compreender os riscos ambientais consequentes, é uma prioridade. É fundamental investigar estas questões de forma multidisciplinar, abrangendo um vasto leque de disciplinas, da biologia à química.

Os fungos Ascomycota são excelentes organismos modelo, especialmente dada a sua ubiquidade ambiental e proeminente capacidade de degradar inúmeros poluentes. Esta tese reúne o primeiro estudo que recorreu a fungos Ascomycota para investigar a ecotoxicidade e a persistência ambiental de líquidos iónicos. Estirpes de *Penicillium* e *Aspergillus* demonstraram ser, de uma forma geral, mais tolerantes aos líquidos iónicos contendo catiões do tipo imidazólio, piridínio, pirrolidínio, colínio ou fosfónio, do que qualquer outro microorganismo testado até à data (Capítulos II, III e IV). É muito provável que a capacidade das estripes fúngicas de tolerar os líquidos iónicos esteja correlacionada com a sua filogenia. A toxicidade dos líquidos iónicos foi estimada usando parâmetros comuns, tais como inibição do crescimento e morte. Os dados foram complementados estudando parâmetros raramente considerados, como a monitorização, por microscopia, da integridade dos conídios (Capítulo IV) e a determinação, por ESI-MS e LC, do metaboloma extracelular (Capítulo II e V). Estes resultados contribuem, de forma útil, para o estado actual da arte sobre a relação estrutura-actividade em líquidos iónicos. Por exemplo, a toxicidade é, aparentemente, função do comprimento da cadeia alquilo do anião e/ou catião (Capítulos III e IV, respectivamente). A revisão crítica do conhecimento actual sobre a toxicidade e o impacto ambiental dos principais grupos de

líquidos iónicos evidenciou que a sua vulgarização como solventes "verdes" ou "tóxicos" é fraudulenta, sendo que nenhuma destas declarações é completamente verdadeira (Capítulo I, secção 1.3.1.).

As questões acima descritas inspiraram a formulação consciente de um novo grupo de líquidos iónicos – os alcanoatos de colínio. Estes exibem baixa toxicidade e elevada biodegradabilidade (Capítulo III). Constituem entre os líquidos iónicos até agora estudados um dos grupos mais interessantes e permitiram um avanço notável: a solubilização eficiente de suberina em cortiça.

Em condições de laboratório, a capacidade dos fungos filamentosos de produzirem metabolitos secundários está reprimida. O elevado interesse nestes compostos naturais justifica-se pela sua diversidade estrutural e possível actividade biológica. Os líquidos iónicos, presentes em concentrações sub-letais no meio de crescimento do fungo, alteraram o perfil do metaboloma extracelular (ou seja, compostos de baixo peso molecular secretados pelo fungo). Três, entre os cinco líquidos iónicos aqui testados, activaram metabolitos crípticos com efeitos biológicos patenteáveis em células HeLa e bactérias (Capítulo V). Estes dados são preliminares, mas encorajadores e merecem estudos decorrentes. Em resumo, o potencial dos líquidos iónicos para activar a produção de metabolitos fúngicos crípticos é promissor na descoberta de produtos naturais de valor acrescentado.

O trabalho apresentado nesta tese é uma base sólida de conhecimento para o desenvolvimento de processos biotecnológicos ambientalmente sustentáveis.

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List of ionic liquids abbreviations

cations

$[\mathrm{C}_n\mathrm{mim}]^+$	1-alkyl-3-methylimidazolium
$[\mathrm{C}_n\mathrm{py}]^+$	1-alkylpyridinium
$[\mathrm{C}_n\mathrm{m}_\beta\mathrm{py}]^+$	1-alkyl-3-methylpyridinium
$[\mathrm{C}_n\mathrm{m}_\gamma\mathrm{py}]^+$	1-alkyl-4-methylpyridinium
$[\mathrm{C}_n\mathrm{OC}_1\mathrm{py}]^+$	1-alkoxymethylpyridinium cation
$[\mathrm{C}_n\mathrm{quin}]^+$	1-alkylquinolinium
$[\mathrm{C}_n\mathrm{mpyr}]^+$	1-alkyl-1-methylpyrrolidinium
$[\mathrm{C}_n\mathrm{mpip}]^+$	1-alkyl-1-methylpiperidinium
$[\mathrm{C}_n\mathrm{mmor}]^+$	4-alkyl-4-methylmorpholinium
$[\mathrm{C}_n\mathrm{emor}]^+$	4-alkyl-4-ethylmorpholinium
$[\mathrm{N}_{w\,x\,y\,z}]^+$	generic tetraalkylammonium
$[\mathrm{P}_{w\,x\,y\,z}]^+$	generic tetraalkylphosphonium
$[\mathrm{EtNH}_3][\mathrm{NO}_3]$	ethylammonium nitrate
$[\mathrm{N}_{1\,1\,1\,2\mathrm{OH}}]^+$	cholinium

anions

$[\mathrm{BF}_4]^-$	tetrafluoroborate
$[\mathrm{PF}_6]^-$	hexafluorophosphate
$[\mathrm{SbF}_6]^-$	hexafluoroantimonate
$[\mathrm{N(CN)}_2]^-$	dicyanamide
$[\mathrm{C}_1\mathrm{SO}_3]^-$ or $[\mathrm{CH}_3\mathrm{SO}_3]^-$	methanesulfonate
$[\mathrm{C}_2\mathrm{SO}_4]^-$	ethylsulfate
$[\mathrm{C}_8\mathrm{SO}_4]^-$	octylsulfate
$[\mathrm{O}_2\mathrm{CMe}]^-$ or $[\mathrm{O}_2\mathrm{CC}_1]^-$	ethanoate
$[\mathrm{C}_n\mathrm{H}_{2n+1}\mathrm{CO}_2]^-$	alkanoate
$[\mathrm{N(SO}_2\mathrm{CF}_3)_2]^-$ or $[\mathrm{NTf}_2]^-$	bis{(trifluoromethyl)sulfonyl}amide
$[\mathrm{N(CF}_3)_2]^-$	bis(trifluoromethyl)amide
$[(\mathrm{EtO})_2\mathrm{PO}_2]^-$ or $[\mathrm{C}_2\mathrm{PO}_2]^-$	diethylphosphate

$[CF_3SO_3]^-$ or $[OTf]^-$	1,1,1-trifluoromethanesulfonate
$[SCN]^-$	thiocyanate
$[lac]^-$	lactate

List of acronyms

APIs	active pharmaceutical ingredients
ATPases	a class of enzymes that catalyze the decomposition of adenosine triphosphate (ATP) into adenosine diphosphate (ADP)
ATR-FTIR	attenuated total reflectance fourier transform infrared spectroscopy
BAC	benzalkonium chloride
BASIL	Biphasic Acid Scavenging utilizing Ionic Liquids
BATIL	Biodegradability and Toxicity of Ionic Liquids meeting
DSC	differential scanning calorimetry
EC_{50}	effective concentration which causes 50 % of the maximal response
ESI-MS	electrospray ionisation mass spectrometry
fSM	fungal secondary metabolites
HCA	hierarchical cluster analysis
HF	hydrogen fluoride
ISO	International Organization for Standardization
ISO 11348	Water quality - Determination of the inhibitory effect of water samples on the light emission of *Vibrio fischeri* (Luminescent bacteria test)
K_{ow}	1-octanol/water partition coefficient
LC	liquid chromatography
MBC	minimal bactericidal concentration
MBEC	minimum biofilm eradication concentrations
MEA	malt extract agar
MIC	minimal inhibitory concentration
m.p.	melting point
NADPH	reduced form of nicotinamide adenine dinucleotide phosphate

NMR	nuclear magnetic resonance
OECD	Organisation for Economic Co-operation and Development
QSAR	quantitative structure-activity relationships
REACH	Registration, Evaluation, Authorisation and Restriction of CHemical substances
$scCO_2$	supercritical carbon dioxide
SDS	sodium dodecylsulphate
TGA	thermal gravimetric analysis
VOCs	volatile organic solvents

Chapter I

Introduction

Chapter I Introduction

1.1. Biology and ecology of filamentous fungi

1.2. Ionic liquids
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1.1. Biology and ecology of filamentous fungi

The following lines focus on general features of filamentous fungi. They do not provide a comprehensive review of the field. It contains, however, information essential for critical analysis of the ecological and biotechnological importance of filamentous fungi. Both concepts are significant for understanding of the chapters enclosed in the thesis.

Fungi are eukaryotic, heterotrophic, organisms. They were initially thought to be related to plants, in particular due to the presence of vacuoles. Knowledge of their protein-coding sequences,[1] and specific characteristics, *e.g.* chitin being a constituent of the cell wall and glycogen acting as a storage polymer, altered this view. In the early 1990's, the link between fungi and animals was finally accepted. Fungi were recognised by Whittaker in 1969 as one of the five kingdoms in a life-form classification system.[2] This concept was preserved in the six[3] or seven[4] kingdoms classification systems proposed afterwards. It includes an enormous number of fungal species, recently estimated to total 1.5 million, with only 5 % described to date.[5] They are divided into four phyla, namely Chytridiomycota, Zygomycota, Basidiomycota and Ascomycota. Ascomycota is the largest phylum and comprises more than 60000 species,[6] including *Aspergillus* and *Penicillium* genera which are of particular interest here (Figure 1). Knowledge on their complex physiology is being the subject of on-going research, including a continuously growing number of fully sequenced genomes of different species (more than ten at the moment of writing).[7-9]

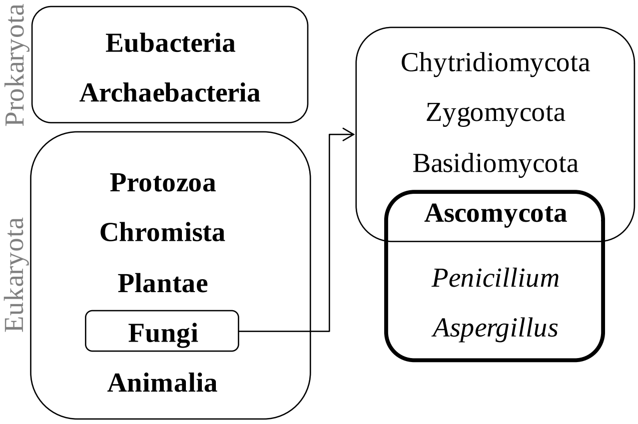

Figure 1. The seven kingdom life classification system according to Patterson and Sogin.[4] The four phyla in the fungal kingdom are listed, as are *Penicillium* and *Aspergillus* genera within Ascomycota.

Vegetative cells of filamentous fungi, hyphae, are 3-10 µm wide tubular filaments showing apical growth (Figure 2). They are interconnected and form branching networks, mycelium. It assembles differentiated hyphae, which are able to coordinate distinct roles in nutrient acquisition or development of reproductive structures.

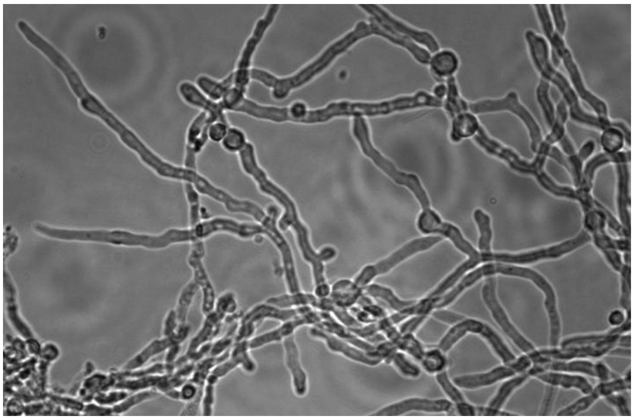

Figure 2. Microscopic image of 48 h culture of *Aspergillus nidulans* (63× magnification). The image was captured in Applied and Environmental Mycology Laboratory, ITQB, by Diego O. Hartmann.

Cells are separated by perforated septa which ensure that intracellular material can freely stream within the hyphae. When grown on solid substrate filamentous fungi form radially symmetrical colonies which grow at a constant rate (approximately 0.5 mm h^{-1} at 37 °C for *A. nidulans*[10]). Submerged in liquid culture they appear as dispersed filaments or mycelial masses. In response to different physical (*e.g.* agitation, rheology, light) and chemical factors (*e.g.* dissolved oxygen, carbon dioxide), hyphae can change morphology and physiology.[11]

The hyphal wall constitutes ~20 % of the cellular biomass and it is a remarkable element of the vegetative fungal cell.[12] It defines and maintains cell shape, and is composed of several cross-linked structural elements. The hyphal wall is extremely dynamic, at the level of both its composition/structure and function. The cell wall is a central trafficking element, controlling interactions with the environment, including cellular processes of uptake, secretion, recognition and communication. It comprises an inner layer of rigid fibrous polysaccharides and an outer layer of gel-like polymers (Figure 3).

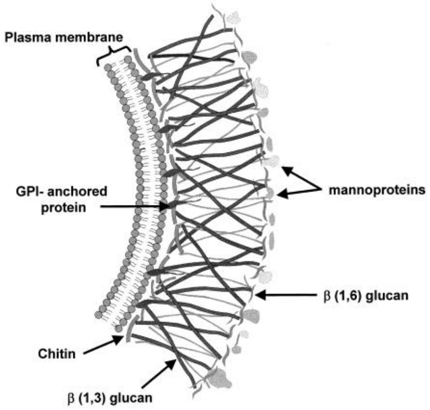

Figure 3. Schematic representation of the fungal cell wall and its major components: glucan, chitin and glycoproteins (adapted from Selitrennikoff[13]).

In Ascomycota fungi, the outer layer is essentially composed of glucans, mainly β-(1-3) and β-(1-6)-linked glucose units and chitin. The inner layer is a mechanically resistant polymer made of β(1-4)-linked N-acetylglucosamine units. Glycoproteins, containing mannose, galactose and xylose, and β-(1-3)-glucan are assembled as the embedding matrix. Some glycoproteins are covalently bound to the plasma membrane and/or the cell wall by glycosylphosphatidylinositol anchors.[14] Hydrophobins found to coat aerial hyphae, are hydrophobic proteins that reduce surface tension at the water-air interface. These proteins may also play a significant role in fungal pathogenesis, symbiosis and protection from environmental stress, *e.g.* desiccation.[15] The fungal cell wall may further contain pigments such as melanins, which increase its mechanical strength, resistance to lytic enzymes and afford radiation protection.[16] They might also act as virulence factors of plant pathogens.

The hyphal tip is generally considered as the most active and dynamic area of the hyphae, controlling nutrient uptake, secretion,[17] hyphal extension and cell wall synthesis.[18] There are two prevailing opinions regarding mechanisms involved in cell wall biosynthesis: steady-state growth and cell wall remodelling theories. Both assume turgor pressure as the driving force of the biosynthesis, and that components of the cell wall are transported in vesicles to the apex. In the steady-state growth theory, transported components are cross-linked and converted from a plastic to a rigid structure.[19] The cell wall remodelling theory suggests a balance between cell wall synthesis and lysis partially plasticises the apex of the hyphae (Figure 4).[14,20] Our understanding of the dynamics of cell wall biosynthesis and assembly is still incomplete.

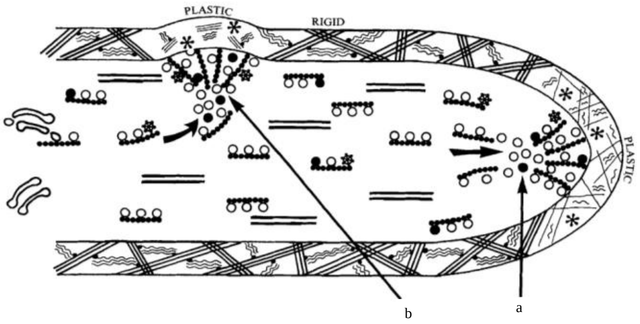

Figure 4. Schematic drawing of the cell wall remodelling theory, showing apical hyphal growth (a) and branching (b). Microvesicles (empty, solid and starred circles) are being transported to these two sites, and may contain cell wall biosynthetic enzymes and other materials necessary. They are associated with cytoskeleton - microfilaments (beaded lines) and microtubules (parallel lines). Apical and branch domes are depicted as plastic cell wall regions that become progressively rigid. Stars at the branching and apical site represent lytic enzymes. Model adapted from Gooday.[18]

Filamentous fungi use mycelial growth to colonise large areas, ramifying throughout the substrate. Aided by high turgor pressure they can also penetrate hard surfaces efficiently.[21] Owing to broad extracellular enzyme diversity, they can utilise a range of substrates, including recalcitrant polymers. Accordingly to the “bulk-flow theory” the degrading extracellular enzymes are exported from the cell together with apical cell wall components.[19] Generally, uptake of nutrients occurs through the plasma membrane, involving proton-motive force or specific transport proteins, such as permeases or carriers.

Ascomycota fungal life cycle can follow two distinct routes, producing either conidia (asexual stage) or ascospores (sexual stage). Figure 5 illustrates, as an example, the life cycle of *A. nidulans*. Morphological features of spore-bearing structures have traditionally been used for classification/identification purposes. The experimental work presented in this thesis is concerned with Ascomycota fungi in their asexual stage. Conidia of *Aspergillus* and *Penicillium* spp. are rounded, pigmented, hydrophobic, non-motile cells (see micrograph of *A. nidulans* conidia, Figure

5A in chapter IV).[22] They are produced in vast numbers and actively discharged or readily dispersed by raindrops, air currents or through contact with insects. The conidia are produced by fertile conidiogenous cells on specialised hyphae (conidiophores). During their maturation the composition of the cell wall is altered to form a four-layer wall, important for protection and facilitating dormancy of the conidium.[23] Conidiation is regulated by environmental conditions, *e.g.* generally enhanced by hyphal exposure to air or nutrient limitation.[5] Light is also an important factor, especially because the circadian rhythm regulates conidiation. Depending on environmental conditions, deposited conidia might germinate or remain dormant. To germinate, it first absorbs water and swells, a germ tube is formed and, at the same time, the metabolism is accelerated, *i.e.* respiration rate, enzyme activation and biosynthesis increase.[5]

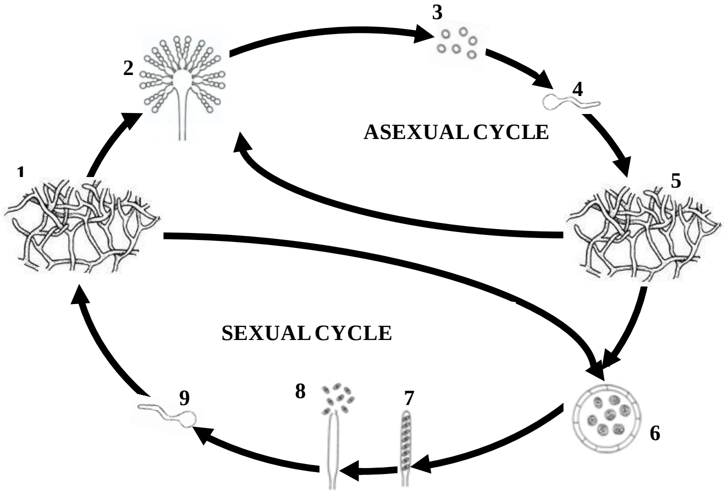

Figure 5. Schematic representation of the life cycle of Ascomycota fungi. As an example, *Aspergillus nidulans* asexual and sexual stages are shown: (1) and (5) vegetative mycelia; (2) conidiophores; (3) conidia; (4) germinating conidia; (6) ascocarp; (7) ascus; (8) ascospores; (9) germinating ascospore.

Fungi occupy critical ecological niches. For example they ensure degradation of lignocellulosic material,[24] generally through lignin peroxidise, manganese peroxidises and laccase,[24] and by endo- and exoglucanase activity.[25] Filamentous fungi are increasingly attracting interest as bioremediation agents, especially due to their catabolic potential and high surface area.[26] They are ubiquitous in all environments and able to survive even under extreme conditions. For example they can often be isolated from environments with high heavy metals,[27] dyes,[28] polycyclic aromatic hydrocarbons[29] or chlorinated phenols concentrations.[30] Their presence in Dead Sea[31] or at polar locations[32] is illustrative. *Penicillium* and *Aspergillus* spp. isolated from marine environments can be regarded as xerophiles, since they were able to survive at water activity below 0.85.[33] In these conditions, strains accumulate 'compatible solutes', *e.g.* glycerol and erythritol, to prevent loss of water.[34] Others, *e.g. Penicillium fellutanum*, accumulate glycine betaine and choline-O-sulfate.[35] These compounds, as well as choline chloride, when added to growth media (2 or 10 mM), act as osmoprotectors. Under low water activity conditions, the plasma membrane alters its fluidity; this is thought to be related with alterations in the sterol/phospholipids ratio and fatty acid unsaturation.[36]

The above illustrates that filamentous fungi are environmentally ubiquitous, have complex physiology, high biological robustness and potentially useful catabolism. In the following chapters of this thesis, *Penicillium* and *Aspergillus* strains high capacity to grow in medium supplemented with an ionic liquid will be demonstrated. The high relevance of this feature to biotechnological applications will be analysed and discussed.

1.2. Ionic liquids

1.2.1. Green solvents

The term "green chemistry" has evolved significantly since being proposed by Anastas in 1998 "*…the utilisation of a set of principles that reduces or eliminates the use or generation of hazardous substances in the design, manufacture and application of chemical products.*"[37] Behind this idea lies the awareness that many man-made chemicals are hazardous and adversely affect the environment. In recent decades, the scientific and industrial communities have become more engaged in green chemistry, especially evident by the foundation of the scientific journal *Green Chemistry* in 1999. Preventing pollution from occurring in the first place is clearly preferable to polluting and subsequently treating, and now recognised as crucial for both social and economic growth. At the same time, industrial processes now face many economical, technical and regulatory barriers,[38] being challenged, as never before, to achieve environmental and economic sustainability.

Green chemistry advances are permitting the replacement of petrochemical feedstocks by carbohydrate-based ones, the use of novel solvents and synthetic pathways, *e.g.* highly selective catalysis and/or biocatalysis. Solvents are of particular interest due to their widespread use in cleaning, coating, synthesis, separation and other applications. They control heat and mass transfer in most processes and influence reaction rates and selectivity. Annually, 4×10^6 tons of solvents are used in Europe[39] and as much again in the U.S..[38] Many conventional organic solvents are petroleum-based, volatile, flammable, corrosive, toxic and environmentally persistent. They contribute heavily to increase the Environmental Factor of a process, which, as proposed by Sheldon, expresses the amount of waste produced over the amount of product generated.[40] Alternative solvent formulations, their reduction and reuse or even development of solvent-free processes, should be a global priority.

Amongst alternative solvents, water, carbon dioxide, fluorous compounds and ionic liquids are the most widely studied.[41] Other potentially applicable groups are the alcohols, *viz.* methanol, ethanol and glycerol; and the esters, *e.g.* ethyl lactate. Fluorous compounds, despite some advantageous properties, carry a high risk of environmental persistence.[41] In contrast, water and carbon dioxide are naturally abundant, inexpensive and non-toxic. Used as liquid or supercritical (sc) fluids, both are regarded as highly promising and efficient yet environmentally friendly solvents.[42] For example $scCO_2$ was successfully used in synthesis[43] and extraction.[44-46] In the last decades, ionic liquids were often proposed as "revolutionary"[47] and as "green solvents of the future"[48] (for further details see section 1.2.2). Numerous questions were, and are still being raised regarding ecotoxicity and environmental persistence (see section 1.3.1.).

1.2.2. Ionic liquids: Properties and applications

The history of liquid salts started in the early 20th century, when the physical properties of ethylammonium nitrate ($[EtNH_3][NO_3]$; m.p. 13-14 °C) were first reported. However, only since the first reports of dialkylimidazolium ionic liquids, especially chemically stable variants thereof, has academia eagerly revisited the field.[49,50]

Ionic liquids are salts, therefore composed of ions, which are liquid below the conventional temperature of 100 °C. This definition, as pointed out by MacFarlane and Seddon,[51] contains their only two truly generic properties. The temperature threshold is, however, subjective and has no physical meaning.

> "*There are not many common characteristics of ionic liquids but lots of exceptions*.", Hiroyuki Ohno[52]

In the literature it is frequently stated that ionic liquids are green solvents, non-volatile, chemically and thermally stable, non-flammable, recyclable *etc*. However, none of these definitions can be used indiscriminately to every ionic liquid. Generally, their non-volatility is the main advantage over conventional volatile organic solvents (VOCs), yet some were recently shown to be distilled avoiding thermal degradation at 200-300 °C and low pressure (0.05 mbar).[53] Their non-flammability,[54] chemical[55] and thermal stability,[56] and recyclability[57] strongly depend on operational conditions. Ionic liquids should not be classified as green solvents when produced from petroleum feedstock, employing significant amounts of solvents and energy, and/or when they themselves are highly toxic and environmentally persistent (see section 1.3.1.). This means the whole "synthesis tree" needs to be considered when evaluating a solvents' greenness.[42] Microwave irradiation in a solvent-free route[58] and neutralisation are greener alternatives of synthesis than alkylation and metathesis.[59] Novel synthesis, purification and analytical methods are continuously being developed, guaranteeing advances towards safer ionic liquids of reproducible quality.[60]

The indisputable potential of ionic liquids lies in their enormous chemical diversity regarding both cation and anion. Consequently, their properties, both physical (melting point, density and viscosity) and chemical (polarity, hydrogen bonding ability) are highly tuneable. There are an estimated 10^{18} possible low-melting point salts[61] made of bulky, asymmetrical ions with delocalised charges and weak intermolecular forces. Their vast chemical diversity has been reviewed by Imperato *et al.*.[62] It is to a certain extent disappointing that, to date, the majority of studies focus on dialkylimidazolium cations combined with fluorinated anions. We are, however, witnessing growing interest in novel formulations, *e.g.* cations derived from chiral amino acids[63] or fructose,[64] and anions built from dicarboxilic acids.[65]

Ionic liquids excellent solvent quality for both polar and non-polar solutes can be explained by their ordered nanostructure which contains both high- and low- charge-density domains.[66,67] Hydrogen-bond donor and/or acceptor capacity and charge delocalisation also contribute to their quality as solvents.[68] They are commonly considered as “designer solvents” able to control thermodynamics, kinetics and reaction outcomes.[69] Understanding some structure-properties relationships is important for selection of an appropriate ionic liquid for a given application. Low-melting point salts can be obtained by combining bulky ions of low symmetry with long alkyl substituents.[70] These features generally reduce density[70] and possibly also conductivity due to reduced mobility of large ions and formation of ion pairs or aggregates.[71] Elongation of the alkyl substituent leads to higher hydrophobicity, viscosity,[55] and thermal stability.[72] Viscosity of ionic liquids is highly dependent on H-bonding and van der Waals interactions and generally is higher than that of other solvents.[71] This is a critical property in chemical engineering, but the system’s viscosity can be significantly reduced by adding co-solvents to the ionic liquid.[70]

Ionic liquids have already found application in many chemical reactions, both as solvents[73,74] and catalysts.[75] They show great potential in electrochemistry for batteries[76] and fuel cells.[77] Despite some existing industrial processes (*ca.* 10 as of 2008), applications of ionic liquids in the biological sciences are still limited. This will be discussed in the following chapters. Their biotechnological relevance is highlighted by their utility for extracting added-value natural compounds, *e.g.* erythromycin[78] or for optimising two-phase biotransformation processes.[78,79]

1.2.3. Ionic liquids as solutes

In the previous section properties and applications of neat ionic liquids were discussed. From an environmental perspective (as is the topic of this thesis) they need to be also considered as solutes. Once an ionic liquid accidently reaches an environmental compartment both, cation and anion, will interact with a dynamic and complex matrix. This includes numerous compounds, such as minerals, humic substances, other pollutants *etc.*, under variable conditions, *e.g.* pH, temperature and ionic strength. There are a range of physicochemical processes which a pollutant might undergo in the environment, such as oxidation, hydrolysis, photochemical reactions and complex formation.[80] One cannot accurately predict the type of interactions, if any, taking place between the ionic liquid and the molecular or ionic species in their vicinity. Under certain conditions, it is likely that ion pairs (between each other or with divergent ions) or aggregates might be formed. Ion pairs are held together by long-range, non-directional electrostatic forces. They may appear as solvent-separated, solvent-shared or contact ion pairs, having two, one or no solvent shells, respectively.[81] Ion pair formation depends greatly on the concentration and nature of both solute and solvent. In ionic liquids ecotoxicity this might be critical since formation of ion pairs has, under some conditions, been suggested to increase toxicity,[82,83] whilst in others to decrease bioavailability.[84] The critical aggregate/micelle concentrations in aqueous solution have been determined for some ionic liquids.[85,86] These concentrations are however much higher than expected to find in any environmental sphere. Further studies are necessary on ionic liquids behaviour in complex mixtures.

1.3. Ionic liquids and biological systems

Section 1.3.1. is the reprint of a published review article (176 references), complemented with recent literature findings (10 references):

Ionic liquids: a pathway to environmental acceptability

Marija Petkovic, Kenneth R. Seddon, Luís Paulo N. Rebelo, and Cristina Silva Pereira, Chemical Society Reviews, 2011, 40, 1383–1403.

1.3.1. Ecotoxicity and biodegradability of ionic liquids

Ionic liquids: A pathway to environmental acceptability

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Abstract

Ionic liquids were initially proposed as replacements for conventional organic solvents; however, their chemistry has developed remarkably and offers unexpected opportunities in numerous fields, ranging from electrochemistry to biology. As a consequence of ionic liquids advancing towards potential and actual applications, a comprehensive determination of their environmental, health and safety impact is now required. This critical review aims to present an overview of the current understanding of the toxicity and environmental impact of the principal ionic liquid groups, and highlights some emerging concerns. Each cation type is considered separately, examining the significance of the biological data, and identifying the most critical questions, some yet unresolved. The need for more, and more detailed, studies is highlighted.

Introduction

Ionic liquids are increasingly attracting interest in both the academic and the industrial *fora*, as demonstrated in the continually growing number of publications and patents (currently >10,000 and >2,000, respectively). Though their history effectively started in 1914 when the physical properties of ethylammonium nitrate ($[EtNH_3][NO_3]$; m.p. 13-14 °C) were first reported,[87] the accepted use of the term "ionic liquid" is quite recent, and as a major scientific discipline they have flourished only in the past decade.

Ionic liquids are salts, completely composed of ions, and generally are liquid below 100 °C.[61] Their negligible vapour pressure, conventional non-flammability, and outstanding solvation potential are the basis for them often being classified as "green" solvents.[88,89] Their potential is further emphasised by the fact that their physical and chemical properties may be finely tuned by varying both the cation and the anion. This dual nature – as well as that due to the existence of two (high and low) electrically charged nanodomains[66] – is, relative to conventional molecular organic solvents, a remarkable advantage. Their generic (but not universal) properties are enabling rapid advances in numerous applications, with some extant processes at an industrial scale, *e.g.* BASF (BASIL,[90] aluminium plating, cellulose dissolution),[74] Institut Français du Pétrole (Difasol),[91] Degussa (paint additives),[74] Linde (hydraulic ionic liquid compressor),[92] Pionics (batteries),[93] and G24i (solar cells).[94] Ionic liquids are also providing unexpected opportunities at the interface of chemistry with the life sciences, *e.g.* acting as solvents in enzymatic[95] and whole-cell biocatalysis,[96,97] and as protein stabilisation agents.[98-100] In addition, their potential use as active pharmaceutical ingredients, though still rather exploratory, further highlights their potential in biochemical studies.[101-104]

Globally, there is a growing awareness of the environmental impact of man-made chemicals, which consequently results in more severe legislation and restrictions. The conscious design of chemicals and the use of structure-activity relationships are essential tools to deliver safer chemicals with enhanced technical performance. These are embedded in the Twelve Principles of Green Chemistry,[105] which endeavour to prevent hazard generation, and challenge and encourage the development of creative solutions to improve old or to create novel processes.[106] Examples are spread across a broad diversity of fields, from catalysis and alternative solvents to renewable feedstocks.[38] The potential of ionic liquids to conceptually fulfil the requirements of environmental sustainability is remarkable. However, they are still fairly innovative (neoteric) solvents comprising a very heterogeneous group of fluids that cannot, *a priori*, be considered benign, especially because precise knowledge is still nebulous. Their lack of vapour pressure is, relative to traditional volatile molecular solvents, a significant advantage, yet true "greenness" should incorporate a sustainable synthesis,[107] low toxicity, and limited environmental persistence (Green Chemistry Principles 2, 3 and 10, respectively).[105] These aspects are yet to be comprehensively considered for the majority of ionic liquids.

The understanding of ionic liquids (their core chemistry, syntheses and purification methods) has advanced significantly over the past decade, and is currently set on solid ground, opening doors to the design of biocompatible ionic liquids,[62] incorporating (*inter alia*) amino acids,[108] carboxylic acids,[109] non-nutritive sweeteners,[110] or glucose.[111] Our current understanding of the issues of purity, and the influence of impurities on both physicochemical and toxicological studies, now allows the design of precise and reproducible syntheses, and the collection of viable and accurate data.[60,112,113]

Up to now, a broad range of testing models - bacteria, fungi, crustacean, *algae*, plants, mammalian cell lines and animals (representing the five Kingdoms in the classification of living organisms)[2,114] - has been used to evaluate the ecotoxicity of ionic liquids. The organisms within each Kingdom carry a set of general, yet specific, characteristics (*e.g.* multi or unicellular, carrying or not a cell wall), and in the presence of ionic liquids will show very distinct behaviours. The toxicity tests are often carried out under dissimilar conditions, *e.g.* incubation periods and end points, therefore providing uncorrelated information on lethal or sub-lethal effects.

The environmental fate of ionic liquids is a complex situation which crosses numerous unknown abiotic and biotic factors. A better structure-based understanding of this is critical, and only recently have their major abiotic mechanisms been analysed, *e.g.* their sorption in soils.[115,116] Jastorff and co-workers have proposed a multidimensional risk analysis, correlating five distinct indicators, namely release, spatiotemporal range, bioaccumulation, biological activity, and uncertainty, which can be used for predicting the environmental impact of chemicals, *e.g.* antifouling biocides[117] and ionic liquids.[118] Though a multidimensional analysis is important for the risk assessment of any chemical, for most ionic liquids the proposed indicators are yet to be comprehensively addressed. With the expanding number of studies on the toxicity and biodegradability of ionic liquids, certain trends are becoming apparent and, though data interpretation and comparison should be made very cautiously, their predictive value is unquestionable. Nonetheless, there are yet major questions to be resolved, such as their modes of toxicity, biodegradation pathways, and behaviour concerning biosorption.

Some recent initiatives, such as the BATIL (Biodegradability and Toxicity of Ionic Liquids) meetings (Berlin, 2007 and Frankfurt, 2009)[119] and the online available UFT / Merck Ionic Liquids Biological Effects Database,[120] are valuable contributions to the exchange of multidisciplinary data, promoting a more articulate research strategy. The present review provides a critical outlook on the current understanding of the toxicity and environmental impact of the most commonly encountered ionic liquids, and highlights some emerging concerns. It examines the most relevant toxicity data, structured by the head group of the cations. Each bioassay has been explained so as to make it accessible to the reader who is not so familiar with the different tested organisms, the observed behaviours, and ultimately, their significance in the context of ecotoxicity. Though these subjects have been already reviewed,[121-123] we aim here to achieve overall a higher degree of systematisation of the data, and obviously higher clarity, especially for chemists. The major correlations between the chemical structure of the ions and their observed toxicities have been highlighted, despite the fact that, mostly due to their commercial availability, the great majority of the extant studies have focussed on the imidazolium family. It should be made clear, from the outset, that the common generalisations that ionic liquids are either "green" or "toxic" solvents should be avoided: both extremes are totally misleading.

Toxicity of ionic liquids containing aromatic head groups in the cation

Imidazolium-based ionic liquids

The imidazolium-based ionic liquids are the most commonly investigated group, for synthesis, in physical chemistry, and for environmental studies: they were one of the first to find application on an industrial scale.[74]

In the context of the toxicity of ionic liquids, there are numerous studies that may assist their advanced design, in order to deliver either biocompatible and/or biodegradable materials, or novel biocides. A pioneering study was performed more than ten years ago by Davis and co-workers, where for the first time imidazolium ionic liquids based on a biologically active molecule, namely miconazole ($C_{18}H_{14}Cl_4N_2O$) (Figure 1a) were synthesised and characterised.[124] Some years later, Pernak and co-workers developed new cationic surfactants: 1-alkoxymethyl-(3-nicotionylaminomethyl)benzimidazolium chlorides (m.p. 110 to 155 °C) (Figure 1b), and observed that their antimicrobial properties, defined by minimal inhibitory and bactericidal concentrations (MIC and MBC, distinguishing between growth inhibition and death, respectively) against microbial strains relevant for human health, increased with the length of the alkoxy chain (between 2 to 12 carbon atoms: 1.4 mM > MIC > 0.034 mM, respectively).[125] These data constituted one of the first systematic studies on the ecotoxicity of ionic liquids.

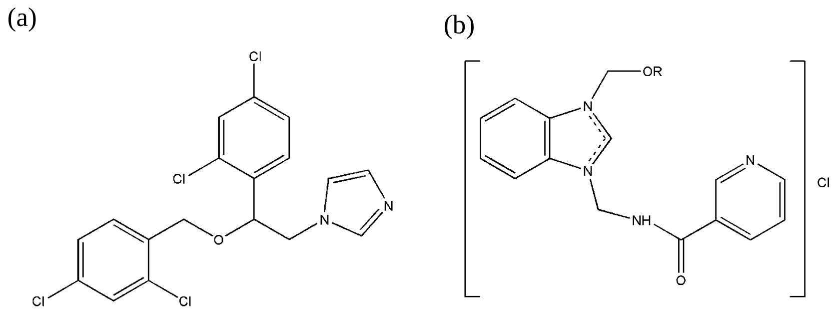
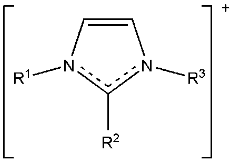


Figure 1. (a) The chemical structure of miconazole ($C_{18}H_{14}Cl_4N_2O$), a biologically active molecule used as a base for the synthesis of imidazolium ionic liquids by Davis *et al.*[124]; (b) the chemical structure of 1-alkoxymethyl-(3-nicotionylaminomethyl)benzimidazolium chlorides, cationic surfactants developed by Pernak and co workers.[125]

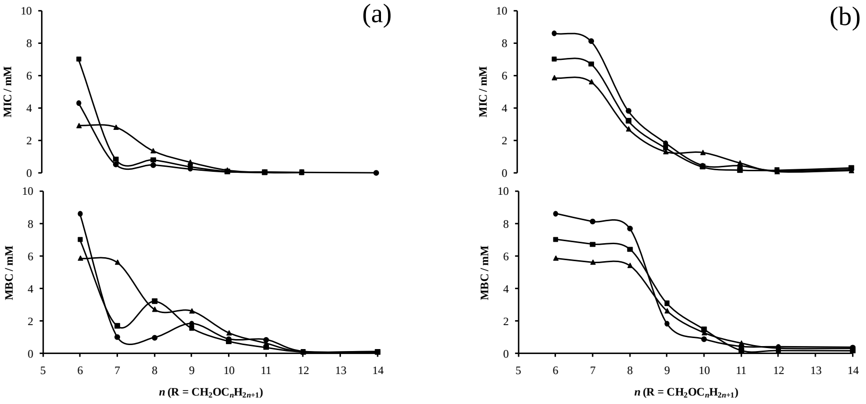
The same methodology was subsequently applied to investigate several novel imidazolium ionic liquids, comprising compounds with slight modifications in the substituted chain of the cation and different anions (Table 1), namely 1-alkyl-3-methylimidazolium chlorides and bromides; 1-alkyl-3-hydroxyethyl-2-methylimidazolium chlorides;[126] 1-alkoxymethyl-3-methylimidazolium chlorides, tetrafluoroborates ($[BF_4]^-$) and hexafluorophosphates ($[PF_6]^-$);[127] 1,3-dialkoxymethylimidazolium chlorides;[128] and 1-alkyl- and 1-alkoxymethylimidazolium lactates (DL and L).[129] The length of the alkyl or the alkoxy side chains varied between one and sixteen carbon atoms, as depicted in Table 1. These studies demonstrated high data consistency and a clear trend towards a stronger toxic effect with the increase in length of the side chain. This effect was constrained, however, since further elongation of the side chains, in position R^1 (>10 - 12)[127] or symmetrical chains in positions R^1 and R^3 (>7 - 9)[128] resulted in lower antimicrobial activities, probably due to steric effects which may limit interaction with the cell surface. In some cases, the growth media have shown eye-gauged turbidity, suggesting that the ionic liquid solubility limit was reached.[127]

Table 1. Structural formulations of imidazolium ionic liquids screened in the antimicrobial activity tests.



R^1	R^2	R^3	Anion	ref
C_nH_{2n+1}, n = 8, 10, 12, 14, 16	H	CH_3	Br^- or Cl^-	126
C_nH_{2n+1}, n = 14, 16	CH_3	C_2H_4OH	Cl^-	126
C_nH_{2n+1}, n = 1-12	H	H	DL- or L- lactate	129
$CH_2OC_nH_{2n+1}$, n = 4-12	H	H	DL- or L- lactate	129
$CH_2OC_nH_{2n+1}$, n = 3-12, 14, 16	H	CH_3	Cl^-, $[BF_4]^-$, or $[PF_6]^-$	127
$CH_2OC_nH_{2n+1}$, n = 3-12	H	$R^3 = R^1$	Cl^-	128

Overall, it becomes apparent that the effect of the tested anions was secondary to the effect of the cations on the observed toxicities (this was most evident for the less toxic cations), yet their broad diversity (often chemically unrelated) does not yet allow a conclusive analysis of their effect. In Figure 2, the MIC values of several 1-alkoxymethyl-3-methylimidazolium cations combined with Cl^-, $[BF_4]^-$ and $[PF_6]^-$ for three different microbial species are presented.[127] The L-lactate salts were more toxic than DL-lactate salts,[129] thus agreeing with previous observations.[130] These data have inspired some innovative applications, *viz.* 1-alkoxymethyl-3-methylimidazolium tetrafluoroborate, as a formalin substitute in embalming and tissue preservation,[131] and as a wood preservative.[132]



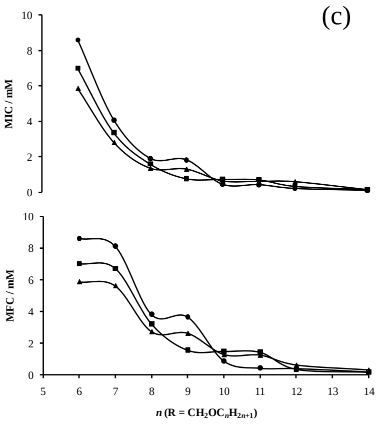


Figure 2. The influence of the length of the alkoxy side chain and of the anion on the antimicrobial activity of 1-alkoxymethyl-3-methylimidazolium ionic liquids {where R = $CH_2OC_nH_{2n+1}$, n = 6 - 12, and Cl^- (●), $[BF_4]^-$ (■) and $[PF_6]^-$ (▲) are the anions} towards (a) *Staphylococcus aureus* (Gram-positive bacteria), (b) *Escherichia coli* (Gram-negative bacteria), and (c) *Candida albicans* (yeast).
For each species, minimal inhibitory and bactericidal/fungicidal concentrations (MIC and MBC/MFC, respectively) were obtained from the study by Pernak *et al.*[127]

It was underlined that toxicity is correlated with the lipophilicity of the cation, suggesting that interaction with the surface of the microbial cells plays a major role. This assumption was validated by the observation that Gram-positive bacterial strains (*e.g. Staphylococcus aureus*) were more susceptible than Gram-negative strains (*e.g. Escherichia coli*). The classification of Gram-positive and Gram-negative bacteria is based on the chemical and physical properties of their cell walls (defined by an empirical staining method). Gram-positive bacteria have thicker and more hydrophobic cell walls,[133] and a much higher peptidoglycan content (~90 %); the cell walls of Gram-negative bacteria are chemically more complex and own an additional outer membrane mostly composed of lipopolysaccharides.[134] The latter is often associated with the higher resistance of Gram-negative bacteria to biocides.[135] Moreover, the methicillin-resistant *S. aureus* (MRSA) strains showed tolerance similar to Gram-negative bacteria,[127] probably due to their generally thickened cell walls and chemically altered peptidoglycan.[135] Docherty *et al.* noticed that while assessing the antimicrobial activity of $[C_n\text{mim}]Br$, which was correlated with the number of colony forming units (throughout 8 h, 2 h intervals), both the most and the least resistant strains were Gram-positive.[136] Based on this, the authors opposed the current opinion, suggesting that the structure of the bacterial cell wall was not determinant for the observed behaviour. The characteristics of the microbial strains could partially explain their atypical behaviour (*e.g.* antibiotic resistance); however, this information was not included, and their conclusions were probably confuted by the use of a single concentration and a shorter exposure time compared with those commonly used to determine MICs (*e.g.* 24 h[127]).

As initially proposed by Gathergood *et al.*,[137] incorporation of an oxygen atom in the side chains of the imidazolium cation reduced significantly toxicity and enhanced primary biodegradability.[138] In a recent study, potent antimicrobial agents were obtained by introducing selenium and aryl moiety as the side chain.[139] In addition, incorporation of a methyl group in the 1-position,[126,138] or a 2-hydroxyethyl group in 3-position of the ring,[126] has not consistently affected toxicity. This emphasises the complexity of comparing independent studies. Furthermore, amongst the tested anions, Br^-, $[BF_4]^-$, $[PF_6]^-$, $[N(CN)_2]^-$, $[NTf_2]^-$ and $[C_8SO_4]^-$, the last two were contributing more to the overall toxicity, but in most cases the inhibitory end points were not reached (MICs > 70 mM).[138]

More recently, the Agar Diffusion Test using a model Gram-positive bacteria (*Clostridium butyricum*) was suggested as a high throughput screening method for the biocompatibility of several $[C_n\text{mim}]^+$ ionic liquids.[140] Briefly, a disc soaked with the testing substance is placed on a lawn of a microbial culture, and after incubation the diameter of the inhibition zone is measured. However, no correlation between the inhibition zone diameters and the EC_{50} values (effective concentration scale based on a 50 % response) could be defined, suggesting that the former cannot be used quantitatively.[140] Agar Diffusion Tests assume that the chemical diffuses freely, and does not aggregate or interact with the solid nutrient medium.[141] Bearing in mind the high viscosities and densities of many ionic liquids, these assumptions are somewhat questionable, as highlighted by the authors.[142] While the selection of a single microbial species to determine endpoints can be extremely useful, generalisation and extrapolation of results should be strongly avoided. Generally, studies on biotransformations in ionic liquids are often based on a single species and the bioassay is adjusted to the purpose of the study. Though from an ecotoxicological perspective, they lack a quantitative significance, some might present valuable additional data, namely by defining lethal endpoints.[143,144] Some of these studies focussed on water-immiscible ionic liquids, yet (in our opinion) an optimal methodology for these is yet to be presented; the current approaches lead to contradictory observations, *e.g.* the cellular membrane of *E. coli* was, after exposure to $[C_4\text{mim}][\text{NTf}_2]$ (20 % v/v), undamaged[97,145,146] or severely disrupted.[96]

Microorganisms may form a biofilm where they are enclosed in a protective extracellular polymeric matrix, which usually confers higher resistance to antimicrobial agents. Though this is different from cell immobilisation, the latter was observed to increase *Saccharomyces cerevisiae* tolerance to $[C_n\text{mim}][\text{PF}_6]$, yet it has not altered the toxicity mode of action, since the longest alkyl chains ($n = 4, 5, 6$ or 8) were the most toxic.[147] Carson *et al*. evaluated for the first time the antimicrobial and antibiofilm activities of $[C_n\text{mim}]\text{Cl}$ ($n = 6, 8, 10, 12$ or 14) testing *e.g.* clinical MRSA strains and biofilm forming *S. epidermidis* strains.[148] The ionic liquids with alkyl chains of length 12 or 14 were proposed as surface biocides, reporting the lowest

Minimum Biofilm Eradication Concentrations (MBEC), *e.g.* varying for [C_{14}mim]Cl from 124 to 1984 µM for *S. aureus* and *Proteus mirabilis* biofilms, respectively: longer or shorter chains were less effective. Their potential is undeniable, especially regarding the increasing number of multi-antibiotic-resistant clinical strains, yet some critical questions, facing either regulatory demands (*e.g.* environmental persistence) or practical aspects (*e.g.* antibiofilm activity against mixed communities) need to be addressed. Based on current knowledge, especially our recent discovery of biocompatible ionic liquids able to dissolve complex biopolymers,[149] their use in combination with antibiotics should be encouraged.

Though some of the aforementioned ecotoxicological studies on ionic liquids have already included some microorganisms belonging to the Kingdom *Fungi* (unicellular yeasts), filamentous fungi were studied for the first time by our group.[150] Fungi are ubiquitous in all environmental compartments and are critical soil colonisers, playing a major role in the biotic decay of pollutants, especially by virtue of their high diversity of species, broad enzymatic capacities (playing a central role in the carbon cycle), extensive hyphae (*i.e.* long branching filamentous cells, collectively called a mycelium) reach, and high surface-to-cell ratio.[5] Ascomycota fungal strains were able to tolerate very high concentrations of ionic liquids (0.05 M) with a range of cations: the imidazolium ones were the most toxic, followed by the groups of pyridinium, pyrrolidinium, and piperidinium ionic liquids; cholinium salts were the most benign. The anion effect was less significant and, as often reported, less predictable.[150] Molecular 1-methylimidazole leads to complete inhibition of growth in all the tested fungal strains, thus exhibiting a more toxic effect than the imidazolium ionic liquids (*e.g.* [C_4mim]Cl inhibited only four of the ten tested fungal strains). This contradicts previous observations (two different studies: cell lines and *V. fischeri*) where the toxicity of the free base was lower than that of [C_4mim]Cl.[136,151] The reasons for this discrepancy are not clear: deviations may arise from the use of distant model organisms, different cultivation media, testing concentrations (higher in fungi by one order of magnitude) and alternate sources of 1-methylimidazole. One major breakthrough in the fungal study[150] was the suggestion of a high degree of correlation between the phylogenetic origin of the strains and their response to the ionic liquid environment, which may allow rationalisation of future toxicological assessments.[150] It also became obvious that sub-lethal concentrations of these ionic liquids have ubiquitously caused metabolic alterations (*i.e.* metabolomics) and that the $[C_2mim]^+$ cation, whilst being toxic and non-biodegradable, was the most effective.

Following the recommendations of regulatory agencies (*e.g.* Organisation for Economic Co-operation and Development, OECD), the cytotoxicity of novel chemicals is commonly analysed by measuring enzyme activities which may be correlated with cell proliferation and viability (period of exposure 24, 48 or 72 h).[152] Ranke *et al.* were the first to propose the use of rat cell lines, namely leukæmia IPC-81 and/or C6 glioma, to evaluate the cytotoxicity of ionic liquids.[83,153] Cellular sorption (*i.e.* adsorption to the membrane surface and uptake into the cell) was reported to be dose-dependent and amongst the tested ionic liquids, $[C_n mim][BF_4]$ (n = 4, 6, or 8), the longest alkyl chain showed, as expected, the highest affinity and cytotoxicity.[153] The use of gradient centrifugation of the lysated cells proved unsuitable to monitor the ionic liquid distribution in the membrane, nuclei and cytoplasm. Generally, the cytotoxicity of $[C_4mim]X$ was much higher than their corresponding Na^+ or Li^+ salts, indicating a major contribution from the cation.[82] However, as summarised in Table 2, the physical and chemical characteristics of the anion greatly influenced its intrinsic cytotoxicity, and very lipophilic and/or unstable anions (*e.g.* some fluorinated ones), were reported to play a major role in the cytotoxicity of the ionic liquids. The higher cytotoxicity of $[CF_3SO_3]^-$ (*i.e.* $[OTf]^-$), relative to $[CH_3SO_3]^-$, emphasised the major role of the anion lipophilicity; the higher cytotoxicity of $[SbF_6]^-$, relative to $[BF_4]^-$ or $[PF_6]^-$, could be due to its higher vulnerability to hydrolysis (*i.e.* low chemical stability), forming HF. This was partially substantiated in a later study where, after nine days, the hydrolysis rates of some ionic liquids containing $[PF_6]^-$, $[BF_4]^-$ and $[SbF_6]^-$ anions were reported to be null, moderate and extremely high, respectively.[154]

Table 2. Influence of the anion on the cytotoxicity of $[C_4mim]X$ (IPC-81 cell line). Values, given as EC_{50}, were adapted from the UFT/Merck database[156] (except for the methylpoly(oxy-1,2-ethanediyl)sulfates[82]) and $\log_{10}(K_{ow})$ of the anions were predicted using algorithms available on the ChemSpider website.[157]

anion structure	name	EC_{50} (μM)	$\log_{10}(K_{ow})$
Cl^-	chloride	3850	0.00
Br^-	bromide	2670	0.00
I^-	iodide	3030	0.00
$[Co(CO)_4]^-$	tetracarbonylcobaltate(-1)	277	-
[SCN]-	thiocyanate	2610	0.58
$[N(CN)_2]^-$	dicyanamide	1420	-0.67
$[HSO_4]^-$	hydrogensulfate	1940	-1.03
$[C_1SO_4]^-$	methylsulfate	1630	-0.595±0.4
$[C_8SO_4]^-$	octylsulfate	1680	3.27
$[H_3CO(CH_2)_2O(CH_2)_2OSO_3]^-$	2-(2-methoxyethoxy)ethylsulfate	1440	-0.80
$[H_3C(OCH_2CH_2)_nOSO_3]^-$	methylpoly(oxy-1,2-ethanediyl)sulfate	1100	-
$[H_3C(C_6H_4)SO_3]^-$	4-methylbenzenesulfonate	1950	0.93
$[CH_3SO_3]^-$	methanesulfonate	3250	-1.89
$[OTf]^-$ (*i.e.* $[CF_3SO_3]^-$)	trifluoromethanesulfonate	1050	-0.37
$[BF_4]^-$	tetrafluoroborate	1030	-
$[PF_6]^-$	hexafluorophosphate	1250	-
$[SbF_6]^-$	hexafluoroantimonate	180	-
$[N(CF_3)_2]^-$	bis(trifluoromethyl)amide	154	3.37
$[NTf_2]^-$ (*i.e.* $[N(SO_2CF_3)_2]^-$)	bis{(trifluoromethyl)sulfonyl}amide	481	1.49
$[(C_2F_5)_3PF_3]^-$	tris(pentafluoroethyl)trifluorophosphate	23.7	-

Despite the apparent high chemical stability of $Na[PF_6]$, it was suggested that the formation of ions pairs might explain its higher cytotoxicity, two and ten times more than $[C_4mim][PF_6]$ in IPC-81,[83] and

HeLa cells,[155] respectively. These observations clearly indicate that cytotoxicity of the ionic liquids may be influenced by side-reactions, strongly suggesting the need for integration of complementary chemical analyses.

Stolte *et al.* demonstrated that the model of concentration addition, which assumes that single substances of a mixture display a similar mode of toxic action and at the same target sites, could reasonably estimate the EC_{50} values of ionic liquids.[82] $[C_4mim][NTf_2]$ constituted an exception, exhibiting three times higher cytotoxicity than estimated from the EC_{50} values of the cations and the anions corresponding salts.

This deviant behaviour was suggested to involve the formation of ion pairs in the aqueous media of cation and anion moiety;[82] but no direct observation for ion pairing in water exists. The significant contribution of $[NTf_2]^-$ to the cytotoxicity of several imidazolium ionic liquids was reinforced in other studies, *e.g.* in IPC-81 cells[158] and MCF7 human breast cancer cells.[159] In the latter study, the authors have used saturated solutions of the hydrophobic $[C_nmim][NTf_2]$ (n = 3 or 6), thus avoiding the addition to the aqueous media of an organic solvent, *e.g.* dimethyl sulfoxide,[83] that despite being used below its toxicity threshold may lead to slight over-estimation of cytotoxicity.[159] The introduction of a terminal hydroxyl or nitrile group, or ether functions in the substituted chain of the imidazolium cation, decreased the ionic liquid cytotoxicity, independently of the anion ($[NTf_2]^-$ or halide).[158] The effect of ether functions has been previously reported in bacteria,[137] yet Stolte *et al.* demonstrated that its effect was strongly dependent on its position in the side chain.[158]

Despite the fact that generally elongation of the alkyl chain in the imidazolium ring leads to a regular increase of cytotoxicity, some exceptions have been reported, *e.g.* while increasing the length of the alkyl chain in position R^1 or R^3.[155] This cannot be attributed to the cell line (HeLa tumour cells), since in a later study with the same cells the effect of the substituted alkyl, allyl and benzyl chains exhibited the expected trends of cytotoxicity, with $[NTf_2]^-$ as the most toxic anion.[160] Ranke and co-authors collated their systematic data on ionic liquids cytotoxicity (IPC-81 cells)[121] which, when taken with a complementary study,[161] established that the cytotoxicity of the halides is strongly correlated with the lipophilicity of the imidazolium cation, a further characteristic of a mode of toxicity which involves disruption of the cell membrane. In addition, $[C_2mim][BF_4]$ was observed to increase the production of reactive oxygen species and the intracellular calcium concentration, while reducing the mitochondrial membrane potential, suggesting that its cytotoxicity involves membrane damage, correlated to cellular apoptosis (*i.e.* programmed cell death) and, to a less extent, necrosis (*i.e.* cell death due to injury or trauma).[160] The high consistency of these data is further highlighted by comparing the EC_{50} values after 48 h reported by Garcia-Lorenzo *et al.*[162] and Ranke *et al.*,[161] even though different cell lines were used (CaCo-2 and IPC-81, respectively). The most different source of data (human colon carcinoma cell lines, HT-29 and CaCo-2)[163,164] exhibited much higher EC_{50} values, most probably due to the very short exposure time imposed to the cell lines (4 h instead of the commonly used 24-72 h). Despite that, the typical toxicity trends were generally maintained, underlining the significant role of the exposure time in the assessment of dose response relationships.

In some of the aforementioned studies, several ionic liquids were observed to induce a sub-lethal stimulus for short exposure times,[155,158,165] a phenomenon (known as the hormetic effect), that is well described for toxic compounds,[166] though, up to now, its rationale remains unclear. In the studies by Frade *et al.*, some ionic liquids, *e.g.* 1-benzyl-3-methylimidazolium bis{(trifluoromethyl)sulfonyl}amide, have, at the selected temporal end point (4 h), significantly increased CaCo-2 cell viability (> 40 %) and were classified as “unsafe”.[164] The ranges of doses tested (~0.5 to 17 mM) have only covered the stimulatory effect of the ‘U’-shaped curves, though it is likely that this effect was hormesis: solely based on these data, the classification must be considered speculative.

Recently, some imidazolium ionic liquids were suggested to display a potential as future anticancer drugs, since they caused high cytostatic (*i.e.* inhibition of cell proliferation) and low cytotoxic (*i.e.* cell death) effects in several tumour cell lines, including MCF7 and HT-29.[167] These values were very heterogeneous, reinforcing the need of further standardisation while evaluating the cytotoxicity of ionic liquids.

It is unquestionable that, due to their general properties, the vast majority of ionic liquids do not present any risk of atmospheric contamination; however, considering their industrial exploitation,[74] they may present ecotoxicological risks to both aquatic and soil environments, *e.g.* due to accidental discharges. Consequently, it becomes critical to screen the ecotoxicity of ionic liquids on selected aquatic or terrestrial model organisms. One fast screening method, commonly used, is the standard ecotoxicological bioassay (ISO 11348),[168] which correlates the reduction of luminescence in cultures of *Vibrio fischeri* (Gram-negative bacteria) with cellular toxicity defining *e.g.* EC_{50} values. The EC_{50} data for $[C_n\text{mim}]^+$ (n = 3 to 10) ionic liquids (exposure time of 30 min) reinforced that their toxicity, higher than that of selected conventional organic solvents (*e.g.* methanol or propanone), was controlled by the cation lipophilicity (*i.e.* alkyl chain length), yet incorporating a minor contribution from the anion (Cl^-, Br^-, $[BF_4]^-$ and $[PF_6]^-$).[83] The indisputable correlation between the toxicity of $[C_n\text{mim}]^+$ ionic liquids and the alkyl chain length was reinforced afterwards in a study that also highlighted the consistency of the *V. fischeri* bioassay,[169] as judged by comparison of the EC_{50} values reported in different studies, even despite the use of divergent exposure periods.[83,136,170] *Daphnia magna*, an aquatic crustacean, is also commonly used in ecotoxicity tests (OECD chronic assays) and recognised as an adequate model organism because of its rapid reproduction rate and sensitivity to changing environmental conditions.[171] The crustacean's high susceptibility to $[C_4\text{mim}]^+$ ionic liquids was demonstrated by a dose-response analysis (lethal effect, LC_{50}), with the cation and the anions playing either a major (*i.e.* the corresponding simple salts of the anions showed lower toxicities) or a secondary role in toxicity, respectively.[172] Amongst the tested anions, namely Cl^-, $[BF_4]^-$, Br^- and $[PF_6]^-$, the last two were the most and the least toxic, respectively, and at sub-lethal concentrations, $[C_4mim]^+$ combined with either Br^- or $[BF_4]^-$ caused the most dramatic chronic effects, decreasing the number of first-brood neonates, total number of neonates, and average brood size.[171] A comparison between the acute effects of some imidazolium ionic liquids on *D. magna* (LC_{50} after 24 or 48 h) and *V. fischeri* (EC_{50} after 30 or 15 min) reinforced the higher susceptibility of the crustacean, by at least one order of magnitude, independent of the anions (Cl^-, Br^-, $[BF_4]^-$, $[PF_6]^-$, or $[N(CN)_2]^-$).[170,173] In addition, in both organisms, the alkyl chain length in the cation played a deciding role in toxicity, and the halides were the least toxic anions towards *V. fischeri*, yet in *D. magna*, the bromide was, as reported before,[172] the most toxic. The EC_{50} values of different studies were in good agreement; however, in the *V. fischeri* bioassay, different exposure periods lead to profound discrepancies, as noticed by comparison of EC_{50} values after 15,[173] and 30 minutes.[83,170] Interestingly, in *D. magna*, the activity of key antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxide and S-transferase) increased at ionic liquid concentrations close to the EC_{50} values, suggesting that oxidative stress plays an important role in their toxicity mechanism.[174]

Still in the context of ecotoxicological risks of ionic liquids in aquatic environments, *algae* appear as a large and diverse group of eukaryotic aquatic photosynthetic organisms classified in different phyla (groups), *e.g.* green *algae* and diatoms. The blue-green *algae* have been misplaced, but based on their characteristics, they have been re-grouped with other prokaryotes in the kingdom Monera, and renamed cyanobacteria.[175] These groups display very particular characteristics, some which will be discussed in correlation with their susceptibilities to ionic liquids. The acute effect of several imidazolium ionic liquids to green *algae* has been systematically investigated, focussing on the effect of the side chain length and substitution, and testing several anions, namely on *Oocystis submarina*,[176] *Pseudokirchneriella subcapitata*,[154,165,177-179] *Scenedesmus vacuolatus*,[180] *Scenedesmus quadricauda* and *Chlamydomonas reinhardtii*,[181] and *Scenedesmus obliquus* and *Chlorella ellipsoidea*.[182] A direct comparison between the EC_{50} values of several imidazolium halides on *S. vacuolatus*,[180] *S. quadricauda*,[181] *P. subcapitata*[165] (formerly known as *S. capricornutum*) and *C. reinhardtii*[181] is depicted in Table 3. The high data heterogeneity was probably due to the use of dissimilar methods, *e.g.* measures of cell density by electrical conductance, fluorometry, or optical density. This partially justifies the significant difference between the EC_{50} values defined by dioxygen evolution and growth measurements, after short (2 h)[178] or long (24-96 h)[165,180] exposure times, respectively.

Table 3. Comparison of toxicological data of three independent studies (different methods and incubation times) using green *algae*. The inhibitory effect of $[C_n\text{mim}]Br$ (n = 4, 6 and 8) is given as $\log_{10}(EC_{50})$ (EC_{50} in µM).

Algae	*Selenastrum capricornutum*[a]			*Scenedesmus vacuolatus*[b]	*Scenedesmus quadricauda*[c]	*Chlamydomonas reinhardtii*[c]
incubation time / h	48	72	96	24	96	96
				$\log_{10}(EC_{50})$		
$[C_4\text{mim}]Br$	3.46	3.36	3.02	2.25	1.34	3.69
$[C_6\text{mim}]Br$	2.57	2.54	2.46	0.08	-0.5	3.02
$[C_8\text{mim}]Br$	1.65	1.63	1.57	-2.67	-1.74	1.17

[a] Values obtained from Cho *et al*,[165] [b] Values obtained from Stolte *et al*,[180] [c] Values obtained from Kulacki *et al*.[181]

Amongst these variables, the exposure time played a major role in the dose-response behaviour, *e.g.* $[C_4\text{mim}]Br$ effect towards *P. subcapitata*[165] (Table 3) or $[C_n\text{mim}]Br$ (n = 4, 6, 8, 10 or 12) towards *S. obliquus*.[182] Their toxic effect is also affected by environmental conditions, *e.g.* $[C_n\text{mim}]Br$ (n = 4, 6, 8, 10 or 12) toxicity towards *C. ellipsoidea* was affected by the incubation temperature.[182] Nevertheless, the determining effect of the cation lipophilicity to the overall toxicity was, in all these studies, apparent, since the elongation of the side chain increased considerably the ionic liquids toxicities.

The imidazolium ionic liquids, especially those carrying long side-chains, may interact and disrupt the biological membranes, as demonstrated by the increased membrane/water partition coefficients,[180] strongly corroborating that their mode of toxic action is baseline toxicity.[183-185] In the very lipophilic compounds, *e.g.* alkyl chains carrying 14 or 16 carbons atoms, a cut-off effect has been reported, on *algae* and also on bacteria,[127,177,180] which may be related to their slow uptake due to steric effects. In addition, decreasing the cation lipophilicity by introducing hydroxyl, ether, nitrile, or chlorine in the side chains,[180,186,187] or by substitution of a methyl group with hydrogen in the 1-position of the ring,[186] has consistently reduced the ionic liquids toxicity to the green *algae* and diatoms. By combining $[C_4mim]^+$ with numerous anions (including some rarely studied), their individual contribution was systematically investigated as depicted in Table 4.[154,188]

Table 4. Comparison of the anion influence on the overall toxicity of $[C_4mim]X$ toward four different species of green *algae*. All values are given as EC_{50} (in µM).

X^-	*Scenedesmus vacuolatus*[a] (72 h)	*Selenastrum capricornutum*[b] (96 h)	*Chlorella vulgaris*[c] (72 h)	*Oocystis submarina*[c] (72 h)
		EC_{50} / µM		
Cl^-	140	2884	1026	2224
Br^-	-	2137	-	-
$[BF_4]^-$	130	2512	425	708
$[PF_6]^-$	-	1318	-	-
$[SbF_6]^-$	-	135	-	-
$[OTf]^-$ ($[CF_3SO_3]^-$)	-	2188	1417	1690
$[N(CF_3)_2]^-$	840	-	-	-
$[NTf_2]^-$ ($[N(SO_2CF_3)_2]^-$)	50	-	-	-
$[C_8SO_4]^-$	60	2239	-	-

[a] Values obtained from Matzke, *et al.*[188] [b] Values obtained from Cho *et al.*[154] [c] Values obtained from Latała *et al.*[189]

As reported before in cell lines,[83] low doses of some ionic liquids were observed to cause a stimulatory effect on *algae* growth (the hormetic effect).[165,180,181] Kulacki and co-workers suggested that the cell wall structure of *S. quadricauda* and *C. reinhardti,* mostly composed of cellulose and glycoprotein, respectively, could possibly explain the higher resistance of the latter (EC_{50} values higher by several orders of magnitude) to $[C_n mim]Br$ (n = 4, 6 or 8) (Table 3).[181] This hypothesis was not supported while comparing wild-type (having a cell wall) and mutant (lacking a cell wall) strains of *C. reinhardti,* which have demonstrated similar susceptibility to $[C_n mim]Br$ (n = 4 or 8) and $[C_4py]Br$. Even so, the significant role of the cell wall in the *algae* susceptibility to tetraalkylammonium ionic liquids was also evident, with the mutant strain presenting values of EC_{50} which are lower by two orders of magnitude.[190] The cell wall composition might also explain the higher susceptibility of diatoms (one of the most common types of phytoplankton), relative to green *algae, e.g. Cyclotella meneghiniana,*[176,189] *Bacillaria paxillifer*[191] and *Skeletonema marinoi,*[189] thought to be related to their silica coating,[192] and their more negatively charged cell walls. This observation was further supported by comparison of susceptibility of different diatoms, namely *Skeletonema marinoi* and *Phaeodactylum tricornutum.*[187] The former has a typical silica cell wall and was more susceptible than the latter, which does not require silica for its growth. Fourier transform infrared spectroscopy (FTIR) was used to access specific alterations in cell wall components, using a method adapted from Cornmell (discussed in more detail in "Quaternary ammonium ionic liquids" section).[193] When exposed to $[C_4mim]Cl$, *S. marinoi* and *P. tricornutum* showed cell wall alterations in the silica/protein or in protein/lipid spectral features, respectively. In addition, the obtained EC_{50} values of $[C_4mim]Cl$ for these two diatoms were one order of magnitude higher than previously observed by Latała *et al.*;[189,191] however, numerous differences in experimental methods could be noted.

The media composition was found to strongly influence the cultures' susceptibility to some imidazolium halides, *e.g.* nutrient availability[181] or media salinity,[84,176] reinforcing the importance of testing under realistic environmental conditions. Concerning the influence of salinity, some discrepancy was noticed between studies, probably derived from different species and methods used. Latała *et al.* associated loss of toxicity in an high saline regime, with ion pairing or complexation of the imidazolium cations.[84] On the contrary, in a recent study by Samorì *et al.*, salinity was not altering their toxicity towards pre-adapted diatom culture.[187]

Additionally, ecotoxicity tests on monocotyledonous and dicotyledonous plants are often recommended in regulation of hazard assessments, such as lesser duckweed (*Lemna minor*) and wheat (*Triticum aestivum*), and cress (*Lepidium sativum*), respectively. Addition of $[C_n\text{mim}][BF_4]$ (n = 4 or 8)[151,194] or $[C_n\text{mim}]Cl$ (n = 2, 4 or 8)[195] affected the growth of these plants, and as expected, the imidazolium cations with the longest alkyl chains displayed the highest toxicities (doses tested varied between 10-100 mg l^{-1} and 10-1000 mg kg^{-1} in aqueous and soil media, respectively). Apparently $[C_2\text{mim}]Cl$ was similarly[194] or more toxic[195] than $[C_4\text{mim}]Cl$, thought to be related to its weaker sorption to soils. Addition of organic matter or smectite (with high cation exchange capacity) reduced the bioavailability of the ionic liquids, since lower toxicities (and in some cases even hormesis) were observed.[194] Amongst the tested anions, $[NTf_2]^-$ was shown to be the most toxic to wheat, independent of the soil composition.[196] Exposure of wheat seedlings to increasing concentrations of $[C_4\text{mim}][BF_4]$ caused major alterations in several growth parameters, perceived by an increased soluble proteins content and peroxidase activities, and a decrease of amylase activities in seeds and chlorophyll content (also reported in cress[195]).[197]

Some studies have ascertained that different organisms exhibit fairly diverse susceptibilities to ionic liquids, yet the high consistency of the toxicity trends defined suggest a similar mode of toxic action. Stolte *et al.* demonstrated a very consistent response of *V. fischeri* (bacteria) and *S. vacuolatus* (green *algae*), both for the cation (following several structural alterations, alkyl side chain length, and substitution) and the anion, even though the former is generally more resistant. The water plant *L. minor* was more susceptible to $[C_4mim]Cl$ than *V. fischeri*; however, $[C_4mim][NTf_2]$, which was significantly more toxic to the luminescent bacteria and green *algae*, at the doses tested have stimulated plant growth (hormetic effect).[180] *Daphnia magna* (crustacean) was reported to be more susceptible to the tested ionic liquids than *P. subcapitata* (green *algae*),[177] and both were much more susceptible than *Danio rerio* (fish).[186] Matzke *et al*. reported a rather thorough study on the toxicity of some imidazolium ionic liquids, focussing on very different trophic levels (*i.e.* position that an organism occupies in the food chain), including marine bacteria, green *algae*, plants, and a soil invertebrate.[188] The contribution of the cation to the overall toxicity was consistent in all organisms, with longer alkyl side chains exhibiting higher toxicities, while the anion effect varied in different species. Nevertheless, an overview of the anion effects suggest that Cl^- and $[BF_4]^-$ contributed in a similar way to the overall toxicity; $[C_8SO_4]^-$ was more toxic to marine bacterium and *algae*, and the fluorine-based anions: $[NTf_2]^-$ (*i.e.* $[N(SO_2CF_3)_2]^-$) and $[N(CF_3)_2]^-$ led, despite the differences in the organisms, to higher toxicities. The significantly higher toxicity of $[C_{14}mim][NTf_2]$ compared with both $[C_{14}mim]Cl$ and $Li[NTf_2]$ (for an inferred mixture toxicity),[188] imply that the toxicity mode of some ionic liquids cannot be solely explained by the model of concentration addition (as previously observed for $[C_4mim][NTf_2]$[82]), and may reinforce that ion-pairing and/or clustering, or some other synergistic effect, may be playing a major role.

Though not very frequently, other organisms have also been used to better understand the toxicity of ionic liquids, yet, despite the fact their insight relies predominantly on the type of bioassay used, a rapid helicopter view is proposed in this review. The dose-response curves of several $[C_nmim]^+$ (n = 4, 8 or 14) ionic liquids on *Caenorhabditis elegans* (nematode),[198] on *Physa acuta* (freshwater snail),[199] and on *Dreissena polymorpha* (zebra mussel)[200] reinforced the determining role of the alkyl chain length in toxicity. The snail feeding rates were significantly reduced when exposed to sub-lethal concentrations of the ionic liquids, *e.g.* close to the LC_{50} for $[C_4mim]Br$ and $[C_6mim]Br$ (1 and 0.22 mM, respectively).[199] This should emphasise that though mortality data are valuable, they project a one-sided aspect that often underestimates the severe effects caused by sub-lethal concentrations of the toxic, *e.g.* as those observed for fungi,[150] further highlighting the importance of measuring different endpoints in toxicity assessment.

Exposure of rats and/or mice to $[C_4mim]Cl$ led to slight irritation of the skin and the eye, and an acute oral LD_{50} of 550 mg kg^{-1} of body weight.[201] Moreover, after intravenous or oral administration of sub-lethal concentrations, the ionic liquid was readily absorbed and ultimately eliminated in urine, and hence no direct evidence supports its metabolism.[202] In addition, under a sub-lethal dose (225 mg kg^{-1} d^{-1}), $[C_4mim]Cl$ and $[C_{10}mim]Cl$ have apparently caused, in mice, maternal mortality and despite an unaltered number of viable fœtuses, their average weight was lower and the number of total malformations increased (although not statistically significant, it *might* suggest a teratogenic potential).[203-205] Interestingly, decreasing the length of the alkyl side chain, from 4 to 2 carbon atoms reduced substantially the toxicity (fœtal weight was conserved). In addition, the developmental toxicity of some imidazolium ionic liquids depends on the concentration (dose) and is stage-sensitive, *e.g.* $[C_8mim]Br$ exhibits increased embryonic mortality and caused morphological malformations in the frog *Rana nigromaculata*[206] and in the goldfish *Carassius auratus*.[207]

Toxic compounds can have a deleterious action on the genetic material of the cells, affecting their integrity (genotoxicity) and increasing the frequency of mutation above the natural background level (mutagenicity). No genotoxicity (Sister Chromatid Exchange test) was detected at $[C_4mim][BF_4]$ doses up to 20 µM, while $[C_{10}mim][BF_4]$ showed a dose dependent trend at a much lower dose (10 µM).[151] In addition $[C_nmim]Br$ (n = 4, 6 or 8) {and also $[C_nm_\beta py]Br$ (n = 4, 6 or 8) and $[N_{n\,n\,n\,n}]Br$ (n = 1, 2, 4 or 6)} cannot be classified as mutagenic (Ames Test evaluates the ability of defective mutants of *Salmonella typhimurium* to reverse back the mutation).[208] Obviously any extrapolation of these initial results to higher organisms is questionable.

Importantly, the model of mixture toxicity (*i.e.* the individual toxics will exhibit similar or different modes of action, leading to a concentration addition or an independent effect, respectively) has again underestimated the toxic effect of different ionic liquid mixtures ([C_4mim][BF_4], [C_8mim][BF_4] and [C_{14}mim][NTf_2]) on green *algae* (*S. vacuolatus*) and wheat (*T. aestivum*), further suggesting that interactions between the compounds or the environment have occurred.[209] The anion [NTf_2]$^-$ may initiate the observed divergence, since as previously reported,[82,188] it significantly contributes to the ionic liquid toxicity. Recently, Zhang *et al*. have observed that various four and two-component mixtures of ionic liquids showed mainly concentration addition effect on *V. quinghaiensis*.[210] Certain compositions were resulting in both synergistic and antagonistic toxic effects. Interestingly, cadmium[209] and pesticides[211] acted either antagonistically or synergistically on the ionic liquid toxicity, respectively. These findings imply that to better understand ionic liquids ecotoxicological risks, complex scenarios of mixture toxicity and pre-pollution need to be accounted for.

Pyridinium-based ionic liquids

Ionic liquids containing a pyridinium head group constitute a rather important and well-studied group, as underlined recently by Madaan *et al*. in a review on their synthesis, accenting the limitless pool of structural variations, properties and applications, especially as surfactants and antimicrobial agents.[212] Their usability as biocides was initially considered by Pernak *et al.* in studies focussing on the antimicrobial properties of some very uncommon and structurally interesting salts (m.p. ranging from 65 to 175 °C), as depicted in Figure 3, namely 1-alkoxymethyl-3-carbamoylpyridinium, 1-alkoxymethyl-3-(1-benzimidazolmethylamino) pyridinium, 1-alkoxymethyl-3-[1-(benzotriazol-1-yl)methylamino] pyridinium and 1,3-bis[3-(1-alkoxymethyl)pyridinyl]-1,3-diazapropane dichloride.[125,213]

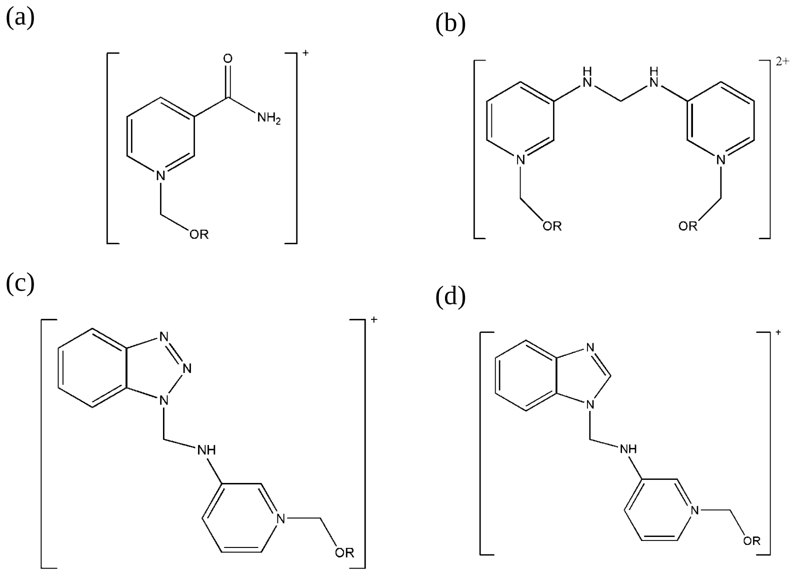


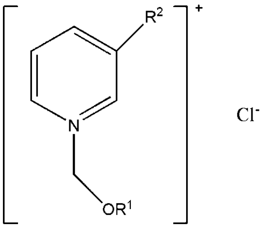
Figure 3. Chemical structures of some interesting pyridinium-based cations investigated by Pernak *et al.*: (a) 1-alkoxymethyl-3-carbamoylpyridinium, (b) 1,3-bis[3-(1-alkoxymethyl)pyridinyl]-1,3-diazapropane dichloride, (c) 1-alkoxymethyl-3-[1-(benzotriazol-1-yl)methylamino]pyridinium and (d) 1-alkoxymethyl-3-(1-benzimidazolmethylamino)pyridinium.[125,213]

The latter group, with alkoxy chain lengths of 9 to 12 carbon atoms and a melting point ranging from 68-73 °C, showed high biocidal potential and was generally more efficient against Gram-positive bacteria than Gram-negative.[125] As was found for the imidazolium ionic liquids, the length of the alkoxymethyl substituent in the pyridinium cation played a major role in toxicity.

In a subsequent study, these authors investigated 1-alkoxymethyl-3-hydroxypyridinium and 1-alkoxymethyl-3-dimethylaminopyridinium chlorides (Table 5), which reported null/weak and strong biocidal effects, respectively.[214] In both groups, the alkoxy chain lengths (3 to 18 carbon atoms) played a major role, following the typical trend of increased toxicity with higher lipophilicity, yet in the latter group this effect was not unlimited and a threshold was detected above fourteen carbons atoms. Incorporating different substituents in position R^3 in the pyridine ring (1-dodecyloxymethyl-

3-R-pyridinium chloride), strongly influenced the toxicity, *e.g.* hydroxyl and bromide lead to the lowest and highest antimicrobial activity, respectively.[214,215] Interestingly, the size of the anion also played a role in toxicity;[213] however no explanation was given.

Table 5. Structural variations of pyridinium salts, 1-alkoxymethyl-3-hydroxypyridinium and 1-alkoxymethyl-3-(dimethylamino)pyridinium chlorides screened in antimicrobial activity tests by Pernak *et al.*[214]



$R^1 = C_nH_{2n+1}$	R^2
n = 3-12, 14, 16, or 18	OH
n = 3-12, 14, 16, or 18	$N(CH_3)_2$

The ecotoxicity of [$C_n m_\beta py$]Br has been analysed using several organisms, namely the luminescent bacteria *V. fischeri*,[136] the freshwater snail *P. acuta*,[199] the zebra mussel *Dreissena polymorpha*,[200] and the green *algae P. subcapitata*,[178] focussing on the effect of the alkyl side chain on the pyridinium cation (n = 4, 6 or 8). As for the imidazolium-based salts, toxicity was observed to increase with the length of the chain, and a correlation between toxicity and $\log_{10}(K_{ow})$ (where K_{ow} is the 1-octanol/water partition coefficient) of the cation was proposed.[136] Docherty *et al.* have noted that methylation of the pyridinium ring (R^3/R^5) increased toxicity, possibly due to the more hydrophobic character of the cation.[136] The toxicity of methylpyridine was slightly higher than [$C_4m_\beta py$]Br, suggesting that even though the alkyl chain is contributing to toxicity, the ionic liquid may have a *greener* character than its synthetic precursors.[136]

The cytotoxicity of pyridinium ionic liquids to several cell lines was analysed mostly focussing on the effect of alkyl[160] or alkoxymethyl[216] side chains, or methylation[159] of the pyridinium ring, and, though less significantly, most of these studies have also covered the anion effect. As expected, increasing the length of the side chain, both alkyl and alkoxymethyl, increased toxicity, *e.g.* $[C_n py]^+$ (n = 4, 6, or 8) (HeLa)[160] and $[C_n OC_1 py]^+$ (1-alkoxymethylpyridinium cation) (n = 3 to 11) (IPC-81).[216] The incorporation of methyl groups in the pyridinium ring alters their toxicity,[215,216] but as previously observed in the imidazolium ionic liquids, the data do not allow a conclusive discussion. For example, Ranke *et al.* reported that an additional methyl group in positions (R^2 or R^3) or R^4 resulted in reduced and increased toxicity (IPC-81 cells), respectively,[161] whereas Kumar *et al.* observed that methylation in any position lead to a minor reduction of toxicity (MCF7 cells).[159] In agreement with previous observations, the effect of the anion becomes more pronounced when combined with less toxic cations, *e.g.* Br^- and $[NTf_2]^-$, exhibited apparently the same effect when combined with $[C_8 m_\gamma py]^+$,[159] but the latter was significantly more toxic with $[C_n py]^+$ (n = 2 or 4).[160] Decisively, the selection of benign anions such as saccharinate or acesulfamate (chemical structures presented in Figure 4) may constitute an advance towards a more conscious design of pyridinium ionic liquids.[215,216]

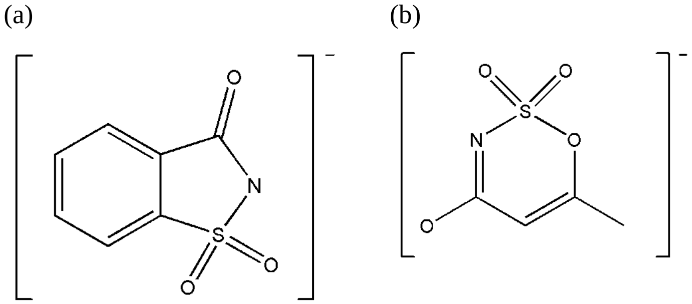


Figure 4. Chemical structures of “benign” anions used in ionic liquid chemistry: (a) saccharinate (1,1-dioxo-1,2-benzothiazol-3-one) and (b) acesulfamate (6-methyl-2,2-dioxo-1,2,3-oxathiazin-4-olate).[215,216]

Their cytotoxicity was much lower than that of chlorides with the same alkoxymethyl chain length (n = 3), yet for longer chains ($n \geq 7$) their effect was less obvious. Interestingly, the anions were demonstrated to play a minor effect on the ionic liquid molecular toxicity (inhibition of acetylcholinesterase activity).[216]

Comparison of the toxicities displayed by the pyridinium and the imidazolium cations carrying same-length alkyl side chains does not allow a consistent conclusion, *e.g.* $[C_4m_{\beta}py]Br$ was, relative to $[C_4mim]Br$, observed to be more toxic against *V. fischeri,*[136] while less toxic to *P. acuta*.[199] There are numerous examples substantiating these discrepancies within several model organisms, *e.g.* in *C. vulgaris* and *O. submarina*,[189] *P. subcapitata*,[178,217] and *D. polymorpha*.[200] $[C_4py]Cl$ was, relative to $[C_4mim]Cl$, less toxic (*D. magna*),[177] but exchanging Cl^- by $[NTf_2]^-$[186] inverted the toxicity ranking of the two groups. The anion has also been observed to influence toxicity even in the very resistant *Penicillium* strains (fungi), *e.g.* 50 mM of $[C_4py]Cl$, $[C_2py][O_2CMe]$ and $[C_2py][lactate]$ inhibited growth in 30 %, 20 % and 0 % of the tested strains, respectively,[150] while in bacteria $[C_4py]^+$ combined with Cl^-, Br^- or $[N(CN)_2]^-$ exhibited toxicities between 2.6 to 2.0 mM.[136] Couling *et al.* proposed a theoretical model for toxicity prediction which correlates several molecular descriptors with the EC_{50} values of several ionic liquids (*V. fischeri* and *D. magna* bioassays).[173] Despite the fact that lower toxicity of pyridinium salts was proposed, the deviant behaviours observed experimentally exposed some of the model weaknesses, yet to be resolved. Nevertheless, it highlighted the importance of considering multiple molecular descriptors while predicting toxicity, such as the number of nitrogen or electronegative atoms in the cation.

The effect of the head group on the toxicity/cytotoxicity of ionic liquids has been well addressed by Stolte and co-workers, since comparison of seven different cationic cores against *V. fischeri* (bacteria), *L. minor* (plant) and *S. vacuolatus* (algae) clearly demonstrated the higher toxicity of the aromatic cations (containing imidazolium and pyridinium rings), relative to the alicyclic (containing morpholinium, piperidinium, or pyrrolidinium) and quaternary ammonium cations.[180] Their toxicity could be reasonably explained by the lipophilicity of the cations (measured by chromatography, $\log_{10}(K_{ow})$ values between 0.18 to 0.68). Irrespective of the head group, these ionic liquids (carrying a butyl side chain) showed relatively low cytotoxicity to IPC-81 cells, with the exception of 1-alkyl-4-(dimethylamino)pyridinium salts. The latter exhibited much higher cytotoxicity than that predicted by its lipophilicity, probably suggesting a specific mode of toxic action in this cells.[158]

Quinolinium-based ionic liquids

Quinolinium ionic liquids are still rarely studied (Figure 5); even so their cytotoxicity potential (IPC-81) and molecular toxicity (inhibition of acetylcholinesterase) was observed to be extremely high, and to increase with the elongation of the substituted alkyl chain.[121]

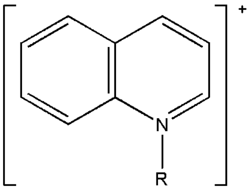


Figure 5. The structure of the 1-alkylquinolinium cation.

For example $[C_n\text{quin}]^+$ was more toxic than $[C_n\text{mim}]^+$ ($n \geq 4$) by at least one order of magnitude. Therefore, it is not surprising that they exhibited lower MBEC values, demonstrating higher potential than $[C_n\text{mim}]\text{Cl}$,[148] to eradicate bacterial biofilms.[218]

Toxicity of ionic liquids containing alicyclic head groups in the cation

Pyrrolidinium-, piperidinium- and morpholinium-based ionic liquids

The ecotoxicity of ionic liquids with nitrogen-containing alicyclic cations, namely pyrrolidinium, piperidinium and morpholinium (Figure 6) is yet to be comprehensively investigated. Pretti and co-workers focussed for the first time on their aquatic toxicity, testing, *inter alia*, several cations and anions, and reported that *e.g.* exposure to 0.24 µM of [C_4mpyr][NTf_2] did not cause, after 96 h, lethal effects in the zebrafish (*D. rerio*).[219] More recently, their cytotoxicity in IPC-81[158] and MCF7[159] cell lines was investigated, focussing on the effect of the substituted chains on the cation, and of the anions. That extensive set of data can be found in the review by Ranke *et al.*,[121] and in the UFT / Merck Ionic Liquids Biological Effects Database.[156]

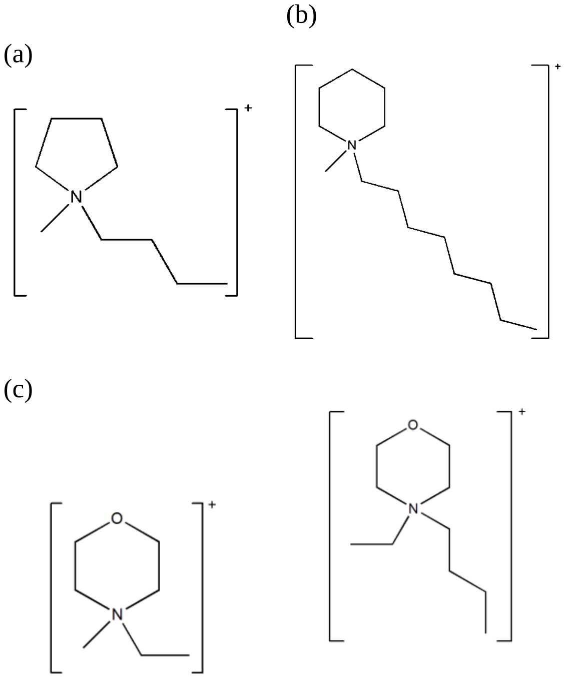


Figure 6. Structures of common alicyclic cations used to form ionic liquids: (a) 1-butyl-1-methylpyrrolidinium ([C_4mpyr]$^+$), (b) 1-methyl-1-octylpiperidinium ([C_8mpip]$^+$), and (c) 4-ethyl-4-methylmorpholinium ([C_2mmor]$^+$) and 4-butyl-4-ethylmorpholinium ([C_4emor]$^+$).

The data suggested that increasing the number of carbon atoms in the alicyclic ring generally increases toxicity, *e.g.* the piperidinium cation was more toxic than the pyrrolidinium cation (six and five member rings, respectively).[159] Moreover, these non-aromatic head groups were generally less toxic than their aromatic analogues.[158] This is highlighted by comparing the toxicities of ionic liquids with aromatic pyridinium and non-aromatic piperidinium rings, *e.g.* [C_8m_γpy]Br was 23-fold more toxic than [C_8mpip]Br, though the most toxic anions may lead to an exception, *e.g.* [C_8m_γpy][NTf_2] was slightly less toxic than [C_8mpip][NTf_2].[159]

As reported before, the length of the alkyl chains played a major role, as cytotoxicity increased with lipophilicity, *e.g.* [$C_n$mpyr]$^+$ ($n = 3$, 4, 6 or 8)[159,161] and [$C_n$mpip]$^+$ ($n = 3$, 4, or 8).[159] Also as expected, incorporation of two hexyl chains instead of one on the pyrrolidinium ring increased cytotoxicity significantly,[161] and the incorporation of an oxygen atom in the alkyl side chain reduced toxicity.[158] The anion effect on the ionic liquids' cytotoxicity, even accounting for their lipophilicity and chemical stability, was unpredictable, as observed while ranking their contribution, *e.g.* in [C_4mpyr]X, $Cl^- < Br^- < [NTf_2]^- < [BF_4]^-$,[121] and in [$C_8$mpip]X, $[N(CN)_2]^- < [BF_4]^- < [OTf]^- < Br^- < [NTf_2]^-$.[159]

To date, the ecotoxicity of these alicyclic rings has seldom been studied; nevertheless, their lower toxicity, relative to the corresponding aromatic rings, was generally evident in the bioassays with *V. fischeri*, *S. vacuolatus* and *L. minor.*[180] Further evidence for this can be highlighted, *e.g.* [C_4mpyr]$^+$ combined with different anions ($[N(CN)_2]^-$ and lactate)[140,150] and [C_4mpip][O_2CMe][150] were less toxic than the corresponding imidazolium ionic liquids. However, other organisms, namely the green alga *P. subcapitata*, showed quite similar susceptibilities with [C_4mpyr]Br and [C_4mim]Br.[217] The morpholinium ionic liquids, due to the incorporation of a oxygen atom in the ring, were the least toxic, *e.g.* [C_2mmor]Br and [C_4emor]Br were apparently nontoxic to *V. fischeri*[220] or *P. subcapitata* and *D. magna*.[186] The significant effect of the $[NTf_2]^-$ anion was again observed, since substituting the halide in [C_4mmor]X by $[NTf_2]^-$ increased, up to one hundred times, its toxicity against *V. fischeri* and *S. vacuolatus*.[180]

Due to the apparent low toxicity of ionic liquids with nitrogen-containing alicyclic cations, there are no doubts that they will be in the heart of a more conscious design, and will certainly be harnessed more in the near future.

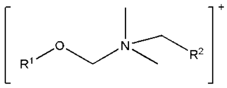
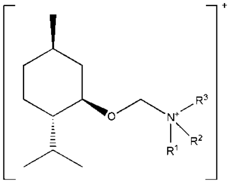
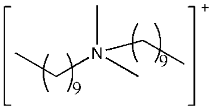
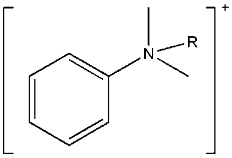
Quaternary ammonium ionic liquids

Quaternary ammonium salts (often referred to just as quats) are well known and widely used chemicals in numerous applications, as disinfectants, surfactants, antistatic agents, catalysts, *etc.*[221] Their properties depend on the chain length and functional groups, and on the anion.[222] Pernak and co-authors were the firsts to consider their antimicrobial activity, focussing on $[N_{1\,1}(CH_2OC_nH_{2n+1})(CH_2R)]Cl$ ($R = CH_2OH$, CH_2CH_2OH, $CH(CH_3)OH$, $CH_2OC(O)C_9H_{19}$, or $CH_2OC(O)Ph$)[223] and some very uncommon chiral ionic liquids, namely trialkyl[(1R,2S,5R)-(-)-menthoxymethyl]ammonium chlorides,[224] as depicted in Table 6.

Their antimicrobial activity was, as expected, governed by the length of the alkoxy and alkyl side chain (R^1), and was generally higher against Gram-positive bacteria, with *Pseudomonas aeruginosa* (Gram-negative) as the most resistant one (able to tolerate benzalkonium chloride (BAC) up to 23 µM).[223] Usually, the most toxic ionic liquids carried the longest alkoxymethyl chains, yet this effect was not monotonic and a threshold (as previously discussed for the imidazolium[127] and pyridinium[214] ionic liquids) was detected above 10, 14 or 16 carbon atoms, depending on the functional group. The alcohol derivatives $[N_{1\,1}(CH_2OC_nH_{2n+1})(CH_2R)]Cl$ ($R = CH_2OH$, CH_2CH_2OH, or $CH(CH_3)OH$) were slightly less toxic than those of the esters $[N_{1\,1}(CH_2OC_nH_{2n+1})(CH_2R)]Cl$ ($R = CH_2OC(O)C_9H_{19}$, or $CH_2OC(O)Ph$).[223]
A high biocompatibility was observed for the $[N_{2\,4}(C_2OH)_2]^+$ {butylethyldi(hydroxyethyl)ammonium} and $[N_{1\,1\,2\,4}]^+$ (butylethyldimethylammonium) cations, combined with $[C_2SO_4]^-$ and $[N(CN)_2]^-$ anions, against *C. sporogenes*, either by determining EC_{50} or inhibition zone diameters.[140] The antimicrobial activity of several $[N_{4\,4\,4\,4}]^+$ carboxylates was screened solely by using the Agar diffusion test, however

(mostly due to the method weaknesses), the uneven data cannot be compared with other studies.[225]

Table 6. Structural variations of substituted tetraalkylammonium ionic liquids screened in antimicrobial activity tests.

cation structure	R^1	R^2	R^3	anion	ref
	C_nH_{2n+1} n = 3-18	$HOCH_2$ HOC_2H_4 $HO(CH_3)CH$ $C_9H_{19}COOCH_2$ $PhCOOCH_2$	-	Cl^-	223
	C_nH_{2n+1} n = 2-12 CH_2Ph	CH_3 C_2H_5	CH_3 C_2H_5	Cl^-	224
	-	-	-	Cl^- $[NO_3]^-$ $[NO_2]^-$ $[BF_4]^-$ $[NTf_2]^-$	226
				DL- or L-lactate	227
				acesulfamate saccharinate (see Figure 4)	215
	mixture of R = C_nH_{2n+1} n = 8-18 (mostly n = 12)	-	-	Cl^- $[NO_3]^-$ $[NO_2]^-$ $[BF_4]^-$	226
				DL- or L-lactate	227
				acesulfamate saccharinate	215

As previously reported for the pyridinium ionic liquids,[215,216] the selection of the benign anion acesulfamate (Figure 4b) resulted in potent antimicrobial activity, *e.g.* alkoxymethyl(2-hydroxyethyl) dimethylammonium acesulfamates carrying long alkoxymethyl chains (but not too long).[228] The effect of other anions on the antimicrobial activity of several $[N_{1\,1\,10\,10}]^+$ or benzalkonium (predominantly $[N_{1\,1\,12}(CH_2C_6H_5)]^+$, but also contains C_{14} and C_{16} homologues) salts, further highlighted their potential use as disinfectants and wood preservatives, especially of nitrites and nitrates,[226] lactates (DL-lactate and L-lactate),[227] acesulfamates, and saccharinates.[215] On the other hand, some of these ionic liquids should be handled carefully. For example, didecyldimethylammonium saccharinate may be regarded as harmful; it displayed EC_{50} values < 10 μM in six human cancer cell lines,[229] a high potential for skin irritation,[215] acute oral toxicity in rats at 300 to 2000 mg kg^{-1} of body weight[215,226] and lowest-observed-adverse-effect-level (LOAEL) at 10 mg kg^{-1} of body weight.[229]

The toxicity of ammonium ionic liquids against *V. fischeri* (bacteria);[173] *D. magna* (crustacean) and *P. subcapitata* (*algae*),[177,186] *D. rerio* (fish),[186] and IPC-81 cells (leukæmia cells)[161] has been also considered. Previously described toxicity trends were generally demonstrated by the data, and hence the highest toxicity was observed for those carrying the longest alkyl chains, namely of $[N_{1\,8\,8\,8}][NTf_2]$ and EcoEng 500 (Peg-5 cocomonium methosulfate; see Figure 7),[177] and of AmmoEng 100 and 130 (Figure 7).[186] The tetraalkylammonium ionic liquids have shown low potential as anti-tumour drugs, since longer alkyl chains lead simultaneously to higher cytostatic and cytotoxic activity towards several human tumour cell lines ($[N_{1\,8\,8\,8}]^+$ cation), while shorter ones (up to 6 carbon atoms) were observed to be rather inactive.[159]

The cytotoxicity of $[N_{1\,1\,2\,4}]^+$ ionic liquids (IPC-81 cells) reinforced the lower toxicity of halides when compared to $[NTf_2]^-$.[158] Despite the lower cytotoxicity of $[N_{1\,1\,2\,4}]Cl$[121,158] and its “safeness” towards bacteria (*V. fischeri*) and *algae* (*S. vacuolatus*), it was highly toxic toward the duckweed (*L. minor*).[180] In addition, the higher antimicrobial activity of $[N_{1\,8\,8\,8}]Cl$ against bacteria, relative to $[N_{1\,8\,8\,8}][NTf_2]$, was attributed to its faster absorption by the cells, which rapidly caused cell death and lysis.[193]

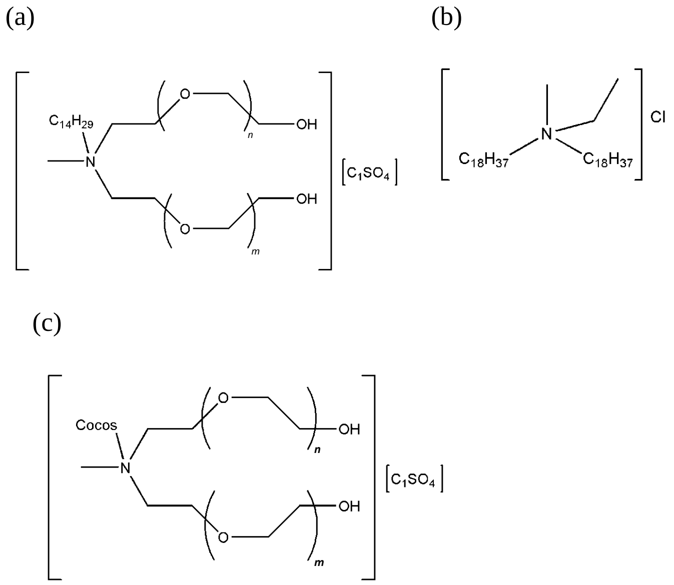


Figure 7. Structures of (a) EcoEng 500 (Peg-5 cocomonium methosulfate), (b) AmmoEng 130 and (c) AmmoEng 100.

The accumulation of the cation in the cells was detected after 3 h of exposure to $[N_{1\,8\,8\,8}]Cl$ by FTIR; however, the analysis was strongly influenced by the degree of cell lysis, and the bioaccumulated and the bioadsorbed fractions were equally accounted for. In addition, the ability to disrupt liposomes (model for a cell membrane) and toxicity/cytotoxicity of some potassium and lithium *N,N,N*-trialkylammoniododecaborates could be well correlated with the anion lipophilicity (alkyl chains carrying 1 to 6 carbon atoms).[230]

In the search for ammonium ionic liquids of higher biocompatibility (and potential biodegradability), some of the most interesting groups are those containing the 2-hydroxyethyltrimethylammonium cation (henceforward described as cholinium). The selection of a benign cation, such as cholinium (cholinium chloride is known as choline, and is part of the vitamin B complex), combined with benign anions constituted a major breakthrough in the conscious design of ionic liquids.[65,228,231-236] The low toxicity of several cholinium ionic liquids has been widely demonstrated, *e.g.* saccharinate and acesulfamate (*D. magna*)[237]; dimethylphosphate (*C. sporogenes*)[143]; several phosphate-based anions (mouse macrophage cells)[238]; lactates (DL-lactate)[150] and alkanoates (*Penicillium sp.*).[109] The last of these were analysed by our group for the first time, and included a range of linear alkanoate anions ($[C_nH_{2n+1}CO_2]^-$, $n = 1$ to 9), as well as two structural isomers (for $n = 3$ or 4).[109] From the data, it became apparent that the toxicity increased with the chain length of the anion and that the branched isomers were of lower toxicity than the corresponding linear ones with the same number of carbon atoms. Additionally, the high biodegradability potential of cholinium alkanoates[109] and the tremendous solvent ability of some of them, either towards suberin[149] or stearic acid,[239,240] has been proven, reinforcing their major utility and interest.

The lower toxicity of quaternary ammonium ionic liquids was predicted by computational modelling of the toxicity of ionic liquids, using quantitative structure-activity relationships (QSAR) defined by the data retrieved in two bioassays (*V. fischeri* and *D. magna*), which have correlated low toxicity with the lack of a nitrogen-containing ring and, in the case of cholinium, with the presence of the oxygen atom.[173] This is supported by the extant data, *e.g.* in *P. acuta*[199] and in HeLa cells.[160]

Quaternary phosphonium ionic liquids

The ecotoxicity of phosphonium ionic liquids, despite their high interest, is still seldom investigated. Some halides, *e.g.* $[P_{4\,4\,4\,4}]^+$ (tetrabutylphosphonium cation), exhibited similar levels of acute toxicity to the freshwater snail *P. acuta* as those of the imidazolium salts carrying the same chain length.[199] The antimicrobial properties of several $[P_{6\,6\,6\,n}]^+$ ($n = 2$ to 16, even numbers) halides (Figure 8) demonstrated that their toxicity was very selective (*e.g.* MIC of $[P_{6\,6\,6\,6}]$Cl was 2.5 µM in *E. coli* and 152 µM in *Candida albicans*).[241] The authors suggested that the structure of the cation plays a significant role in toxicity since the antimicrobial activity of the phosphonium chlorides was observed to decrease for the longest alkyl chains (carrying 8 to 14 carbon atoms). Interestingly, exchange of the halide by other anions, *e.g.* $[NTf_2]^-$, $[OTf]^-$, $[NO_3]^-$, $[N(CN)_2]^-$, $[BF_4]^-$, or $[PF_6]^-$, has resulted in a loss of antimicrobial activity and electrostatic properties,[241] thus rendering higher interest in these phosphonium ionic liquids.

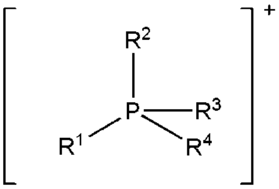


Figure 8. Structure of quaternary phosphonium cation.

The important contribution of the anion has been demonstrated in other studies, *e.g.* $[P_{6\,6\,6\,14}]^+$ combined with chloride and $[NTf_2]^-$ led to high and low toxicity against *E. coli*.[193] Based on the ATR-FTIR analyses of the cells, the halide was suggested to be more rapidly biosorbed (*i.e.* adsorption onto the cell surface plus bioaccumulation); nevertheless, the data (as discussed above) were strongly influenced by the degree of cell lysis. Despite the fact that cross-contamination of the cytoplasmatic and the membrane sub-cellular fractions may have occurred, $[P_{6\,6\,6\,14}][NTf_2]$ was found mainly in the cell membrane;[193] unfortunately, the authors have not also monitored $[P_{6\,6\,6\,14}]Cl$ distribution in the cells. As previously described in the imidazolium ionic liquids section, some water-immiscible phosphonium ionic liquids have generated high interest for developing novel biotransformation processes, *e.g.* xenobiotics-degradation.[79]

The apparently high toxicity of other phosphonium halides (namely $[P_{4\,4\,4\,4}]Br$, $[P_{6\,6\,6\,14}]Br$ and $[P_{6\,6\,6\,14}]Cl$) against *V. fischeri* and *D. magna*,[173,177] and *P. subcapitata*,[177,217] was also demonstrated. Interestingly, though substantially less effective than the halides, $[P_{2\,4\,4\,4}][(EtO)_2PO_2]$ was reported to be toxic to *V. fischeri* (0.07 mM),[173] and *P. subcapitata* (0.016 mM).[177]

In addition, their usually high cytotoxicity, despite the anions, was demonstrated (HeLa and IPC-81 cells) after analysing $[P_{4\,4\,4\,14}]^+$ and $[P_{6\,6\,6\,14}]^+$ combined with $[NTf_2]^-$[160] or $[BF_4]^-$,[161] and $[P_{4\,4\,4\,4}]Br$.[161] The lack of systematisation in these studies does not permit a conclusive rationalisation. Nonetheless, $[P_{6\,6\,6\,14}]^+$ combined with different anions, namely $[PF_6]^-$ and $[NTf_2]^-$, displayed high and low cytotoxic activities against a broad diversity of human cancer cell lines, respectively.[159] The same cation combined with $[BF_4]^-$ exhibited very inconsistent behaviour, thought to be related to its low solubility in water (and therefore lower membrane permeability potential).

Biodegradability of ionic liquids

The biodegradability of ionic liquids plays an important role in evaluating their environmental impact. This subject has been recently discussed in an excellent review by Coleman and Gathergood;[242] hence only selected key relevant facts will be discussed here. Most biodegradability assays are defined under static laboratory conditions and, despite their importance, are usually unrealistic, failing to reproduce the numerous abiotic and biotic processes occurring in the environment. The biodegradability potential of a chemical is often discussed accordingly to the OECD guidelines, meaning that its rapid and complete mineralisation indicates that the chemical is readily biodegradable, while its weak or extensive molecular cleavage suggests primary or ultimate biodegradability potential, respectively.

The conscious design of chemicals and the rules of thumb for delivering biodegradable chemicals, including ionic liquids, were excellently reviewed by Boethling *et al.*[243] Generalisations should be made cautiously, yet it is generally accepted that several molecular features strongly enhance biodegradability (not necessarily resulting in intermediate chemicals of lower toxicity), such as the presence of esters, amides, hydroxyl, aldehyde, carboxylic acid groups, or linear alkyl chains. Some examples can be found in the available literature on ionic liquids, *e.g.* the incorporation of an ester group in the side chain of several imidazolium[170,244] or pyridinium[245] ionic liquids has significantly improved the primary biodegradability of the cation (*i.e.* weak molecular cleavage). Those carrying alkyl chains of four carbon atoms have been observed to be poorly biodegradable,[169,170,246,247] even when unsaturated groups (allyl or vinyl) or a sulfonate group were incorporated.[248] Pham *et al.* proposed a degradation pathway for [C_4m_βpy]Br through hydroxylation of the side chain, even though that the intermediate chemical product (3-methylpyridine) could not be conclusively identified.[249] Nevertheless, pyridinium ionic liquids have, relative to the corresponding imidazolium ones, higher biodegradability potential.[250] For example, [$C_n$mim]Br and [$C_nm_\beta$py]Br (n = 6 or 8) were both degraded by an activated sludge microbial community, with the longer alkyl chains reaching, after over 40 days, partial and total mineralisation, respectively; however, after 25 days only [C_8m_βpy]Br was shown to be fully mineralised (*i.e.* readily biodegradable).[247] More recently, the degradation pathway of [$C_nm_\beta$py]Br (n = 4, 6 or 8) was reported to involve unsaturation of the alkyl side chain and hydroxylation of the aromatic ring,[251] thus opposing that previously suggested by Pham *et al.*[249] Taken together, these studies suggested that there are different possible degradation pathways that need to be considered, which are ultimately defined by the metabolic capacities of the microbial community and stand for a "regional" impact of the data.

Additionally, some phosphonium ionic liquids, namely tricyclohexylphosphine- and trihexylphosphine-derived cations with various ester side chains, and combined with different anions, were observed to be very resistant to microbial attack.[252] On the other hand, the cholinium cation was reported to be readily biodegradable,[243] and more recently, our group demonstrated the high biodegradability potential of several cholinium alkanoates.[109]

The complexity in designing more reliable bioassays for determining the biodegradability potential of ionic liquids is partially due to critical knowledge gaps on their potential uses and disposal methodologies, their contamination levels and fate, and/or bioaccumulation factors in the environment. Ionic liquids sorption onto sediments and soil will have a major influence on their transport, reactivity (*e.g.* oxidation, hydrolysis and photolysis), bioavailability and ultimately, biodegradability. Even if still poorly investigated, there is an increasing interest in the behaviour of ionic liquids in soils; however, up to now, most studies have focussed on the imidazolium family, further demonstrating their high persistence and ecotoxicological risk.[253,254] Their sorption and desorption in soil were observed to be ruled by ionic interactions;[115,255,256] nonetheless, their lipophilicity, the length of the side chains, and Coulombic interactions may greatly influence their mobility.[116] The first biodegradability assessment in soil was conducted using several imidazolium-based ionic liquids, monitoring solely CO_2 evolution, which is not sufficiently informative to judge toxicity and biodegradability.[257] Nevertheless, mostly based on the high capacity of several environmental fungal strains (which are commonly found in soil) to tolerate high concentrations of ionic liquids,[109,150,258,259] the role of filamentous fungi in their biodegradation in soil is expected to be high.

The prevailing opinion is that the cation and anion will undergo completely distinct fate mechanisms; however ion pairs possibly formed between them and/or with other ions ubiquitously present in that environment may be involved. The faster biodegradability of certain anions relative to the cations has been demonstrated, *e.g.* alkylsulfates,[137,170] saccharinate and acesulfamates,[216] and alkanoates,[109] further suggesting that their degradation followed distinctive pathways. This also stresses the importance of considering both the cation and the anion for successful conscious design of ionic liquids.

Molecular toxicity of ionic liquids

Some general considerations on molecular toxicity of ionic liquids will now be analysed. While molecular toxicity falls under the umbrella of toxicity, a direct extrapolation of any toxic behaviour based on *in vitro* analyses of single molecules/reactions may lead to an erroneous conclusion. Chemicals (or their biotransformation products) can display many modes of molecular toxicity (*i.e.* molecular mechanisms whereby chemicals cause toxicity) through interaction with specific biologic macromolecules, such as proteins and DNA.[260] Encouraged by this, Jastorff and co-workers have included the concept of molecular toxicity as a standard component of their ecotoxicity test battery, selecting the acetylcholinesterase assay.[188,202,261,262] Preference was given to this enzyme (already mentioned in the sections for pyridinium[216] and quinolinium[121] ionic liquids) because it plays an essential role in the nervous system of almost all higher organisms, and the enzyme-active site is highly conserved amongst organisms. By the extensive set of IC_{50} data based on this assay (effective concentration scale based on a 50 % inhibition response) presented by Ranke *et al.*, it became apparent that the chain length of the cation strongly influenced the activity of the enzyme, probably due to the binding to the lipophilic active site; however, the effects of additional methyl groups in the ring and of the anions were unclear.[121] Even so, the fluorinated anions displayed consistently higher molecular toxicity,[121] probably due to their higher chemical instability. In addition, the introduction of polar hydroxyl or ether functions in the side chains induces reduced toxicity, relative to the alkyl chain.[262] The introduction of an additional hydroxyl group in position R^3 in the pyridinium ring may have prevented the interaction of the quaternary nitrogen with the active site of the enzyme,[216] since a reduction, by two orders of magnitude, was observed, *e.g.* while comparing 1-alkoxymethyl-3-hydroxypyridinium[216] and 1-alkyl-3-methylpyridinium ionic liquids.[261] The head groups in the cation played a deciding role, mostly due to their ability to bind to the active site of the enzyme or its gorge.[121,262] Essentially, the large aromatic systems of 1-alkyl-4-(dimethylamino)pyridinium and quinolinium, and the non-aromatic morpholinium and tetrabutylammonium cations, were responsible for leading to strong and weak inhibitions, respectively.[262] In addition, the sterically bulky structure and positive charged moiety shielded by the long alkyl chains of the tetraalkylphosphonium and tetraalkylammonium ionic liquids have probably reduced the interaction with the active centre of the enzyme, leading to weak inhibition, yet the benzyl aromatic residue in the benzyldecyldimethylammonium cation rationalises the deviant behaviour of high toxicity. In two recent studies, imidazolium-based ionic liquids were observed to inhibit acetylcholinesterase activity in living systems, *i.e.* rat pheochromocytoma PC12 cells[263] and earthworm.[264]

There are only a few studies on the molecular toxicity of ionic liquids focussing on, instead of the acetylcholinesterase, other systems such as the adenosine monophosphate (AMP) deaminase,[265] or the cytochrome P_{450} assay.[266,267] The activity of AMP deaminase in eukaryotes constitutes a primary step in the regulation of intracellular adenine nucleotide pools,[268] and has therefore been proposed as a model enzyme for assessment of chemical risk.[269] Likewise, cytochrome P_{450}, a family of monooxygenases ubiquitously present in organisms, uses a wide spectrum of substrates, and are known to play an important role in xenobiotic metabolism, catalysing hydroxylation of C-H bonds.[270] The activity of AMP deaminase was inhibited by several $[C_4mim]^+$ ionic liquids and, amongst the tested anions, the chloride and 4-tosylate exhibited slightly lower molecular toxicity than $[BF_4]^-$ and $[PF_6]^-$, with IC_{50} values of 10 and 5 µM, respectively.[265] Likewise, inhibition of cytochrome P_{450} BM-3 activity in the presence of $[C_n mim]Cl$ (n = 4, 6 or 8) was stronger for the longer alkyl side chains.[266] This agrees with that previously observed while using the acetylcholinesterase assay,[121] though higher resistance of cytochrome P_{450} was noticed with IC_{50} values higher by at least one order of magnitude. Surprisingly, the head group of the cation, and especially its aromaticity, apparently has played a minor role in the ionic liquid molecular toxicity against cytochrome P_{450}, since imidazolium, pyridinium and pyrrolidinium chlorides exhibited IC_{50} values of 148, 195 and 175 mM, respectively.[266] In addition, the activity of the purified human enzyme cytochrome P_{450} 3A4 was significantly inhibited in the presence of $[C_n mim][BF_4]$ (n = 1 or 4) and $[C_4py][BF_4]$.[267] On the other hand, imidazolium ionic liquids apparently cannot be used as substrates by cytochrome P_{450} BM-3, since the enzyme could not hydrolyse any of the tested $[C_n mim]Cl$ (monitored by NADPH consumption).[266]

Modes of toxicity of ionic liquids

The toxic effect of any chemical is an expression of the disorder of particular metabolic pathways, and their potential modes of action include disruption of membranes, mostly affecting membrane permeability and transport proteins, enzyme inhibition, and DNA damage. A better knowledge of chemical toxicity, *e.g.* in biocidal and drug design, is essential to identify their modes of action, and ultimately to further advance towards their conscious design.

The hydrophobic interaction between chemicals and biological membranes results in non-specific toxicity, called baseline toxicity or narcosis, which is mainly governed by solubility and partitioning;[185] and, though argued, there are possibly both polar and non-polar narcosis mechanisms.[184] However, chemicals can also interact with biological systems through hydrogen or covalent bonding and charge transfer, with higher (relative to baseline toxicity) effectiveness. Baseline toxicity has been mentioned often in this review, by suggestion if not implicitly, as the basis for the observed correlation between the ionic liquid lipophilicity (at the level of both the cation and the anion) and toxicity. It becomes important to select suitable descriptors for the lipophilicity of ionic liquids, such as the K_{ow} and the membrane/water partition coefficients.[180] Even though the significance of the K_{ow} coefficients is still under debate, they are often used, mostly because they can be retrieved by direct measurement, by reversed phase liquid chromatography, or predicted by computational methods.[161] Jastorff and co-workers observed a good correlation between the cations' lipophilicity (defined chromatographically) and the toxicity of the corresponding ionic liquid (focussing on hydrophilic and chemically stable anions, such as the halides).[158,180] Likewise, the high correlation observed by us between the antimicrobial activity of several cholinium alkanoates and the calculated anion hydrophobicity $\log_{10}(K_{ow})$,[109] may be explained by the cation hydrophilicity and benign nature. Significantly, even when a good correlation between lipophilicity and toxicity was observed, this effect, probably due to limited solubility and/or steric limitations, was not universal. There are several examples where very lipophilic cations[127-129,214,228] or anions,[109] commonly carrying side chains longer than 10-12 carbon atoms, exhibited lower toxicity than that predicted. In the latest study, the cholinium dodecanoate exhibits lower toxicity than expected, probably due to its limited solubility. In addition, in a very recent study focussing on imidazolium ionic liquids, a strong relationship between antimicrobial efficacy, structure of the cation and surface activity of the aqueous solutions containing $[C_n\text{mim}]^+$ ($n = 8$ to 18) was reported.[271]

Liposomes (phospholipid vesicles) constitute a simple and attractive methodology to analyse interactions of ionic liquids with cellular membrane.[230] Both $[C_8\text{mim}]^+$ and $[\text{NTf}_2]^-$ were demonstrated to cause severe disruptions in a supported phospholipid bilayer, with $[\text{NTf}_2]^-$ leading to the most extensive loss of lipids.[272] The higher toxicity of the latter was tentatively explained using molecular simulations, predicting that $[\text{NTf}_2]^-$ was readily incorporated into the cholesterol bilayer, strongly limiting the adsorption of the cation and, consequently, the insertion of the butyl chain of $[C_4\text{mim}]^+$ into the bilayer.[273]

As an attempt to rationalise the toxicity mode of ionic liquids, some processes have been proposed to play a key role. Nevertheless, one should bear in mind the high complexity of living organisms and the numerous physical and biochemical processes occurring simultaneously. The important observations on the critical alterations detected in fungal metabolism after exposure to sub-lethal concentrations of some hydrophilic ionic liquids[150] may suggest a specific mode of toxicity.

Quantitative structure-activity relationships (QSAR) for ionic liquids

Noteworthy efforts are increasingly being reported in order to define computational methods which can be used to reasonably predict ionic liquid toxicity. There is no doubt that computer modelling will, as more ecotoxicity data becomes available, develop further. Couling *et al.* applied QSAR modelling to their experimental data on the aquatic toxicity of twenty-five ionic liquids (containing imidazolium, pyridinium, tetraalkylammonium, and tetraalkylphosphonium cations) (correlation with R^2 = 0.78-0.88).[173] Based on the derived descriptors, calculated at a low semi-empirical computational level, certain generalised predictions could be raised: toxicity is mainly governed by the cation, with the aromatics displaying higher toxicity than non-aromatic ones, and increasing with longer substituted alkyl chains. Additionally, in the aromatic ring, an increased number of nitrogen atoms leads to higher toxicity, while its methylation decreases it. A different approach was used by Luis *et al.*, since they designed an algorithm based on group contribution methods to estimate the aquatic toxicity of forty-three imidazolium, pyridinium and pyrrolidinium ionic liquids (correlation with R^2 = 0.92).[274] Based on this modelling, some new generalisations were suggested, such as a toxic ranking of the head groups (pyrrolidinium < imidazolium < pyridinium), while the anion effect remained less predictable, though some (bromide, dicyanamide and ethylsulfate) were considered highly toxic. Garcia-Lorenzo *et al.* built a QSAR model, according to the Topological Sub-Structural Molecular Design (TOSS-MODE) approach, which uses graph-based molecular descriptors (based on spectral moments) to predict the cytotoxicity of fifteen imidazolium-derived ionic liquids in CaCo-2 cells (correlation with R^2 = 0.98). Different mathematical models were proposed by Torrecilla *et al.*,[275] using empirical formulae (elemental composition) and molecular weights of 153 ionic liquids (ammonium, imidazolium, morpholinium, phosphonium, piperidinium, pyridinium, pyrrolidinium and quinolinium salts) available in the open literature[121] to estimate their cytotoxicity (IPC-81 cells) and molecular toxicity (acetylcholinesterase) by neural network (NN) models (correlation with R^2 = 0.98 and 0.97, respectively). Recently, COSMO-RS (Conductor like Screening Model for Realistic Solvents) molecular descriptors, which related cytotoxicity to the polar charge distribution of the cations and the anions, were used (non-linear neural network) (96 formulations in IPC-81 cells,[121] correlation with $R^2 = 0.98$): this leads to the highly significant conclusion that the cytotoxicity of ionic liquids cannot be systematically estimated by a summation of the independent contributions of the intrinsic toxicity of the cation and anion.[275,276] More recently, Alvarez-Guerra et al. applied PLS-DA (Partial Least Squares-Discriminant Analysis) model on the set of EC_{50} values for 131 ionic liquids in *V. fischeri*.[277] Toxicity of ionic liquids was classified as positive (high) or negative (low) relatively to toluene or chloroform, while the contribution of each ion to this classification was calculated.

General considerations

In the light of the studies referred to above, it is clear that the numerous formulations of ionic liquids available provide a great pool of, and impetus for, many commercial applications, but not without significant toxicological and environmental concerns. The vast majority of toxicological studies on ionic liquids, available up to the present date, have focussed on imidazolium ionic liquids. In addition, frequently, the ionic liquids “selected” for study tackled under a common assay were randomly chosen. Despite the scientific weight of these studies, the lack of systematisation (*e.g.* monitoring the effect of defined structural alterations in a specific head group) means that it is impossible at the moment to achieve a holistic analysis, which weakens conclusions and devalues the predictive algorithms under development.

Furthermore, the selected bioassay, despite its relevance, appears to be sometimes subjective. Ecotoxicity examination should be based on a realistic analysis of the risk of exposure, accounting predicatively for which environmental compartments are most likely to be involved, yet it is necessarily regionally-oriented. Legislation demands and standardised tests should be kept as a priority, engaging models of different complexity. However, one may doubt their suitability, since testing a vast number of organisms and the diversity of all available formulations is costly and irrational. Indeed, testing the most sensitive species and determining the chemical lethal concentration, will provide useful information, but this may lack a real ecological meaning, as sub-lethal concentrations significantly affect the ecology of the exposed niche (*e.g.* species interactions and food web balance). In addition, the environmental persistence of any chemical should be taken as one of the most critical ecotoxicological parameters (advancing their conscious design), and putatively their mitigation will involve the tolerant species able to metabolise it. The most frequently used toxicity tests establish median effective concentrations (inhibitory or lethal), which are useful parameters for comparison of different chemicals, but it is obvious that further standardisation should be pursued. The conditions selected, *e.g.* media composition, temperature, light/dark cycle, and more importantly the exposure time, have generated a considerable volume of data on ionic liquid toxicity that cannot be directly compared. Furthermore, straightforward comparisons were not easy because of the use of diverse end-points and units.

The European Community regulation on chemicals and their safe use - REACH (Registration, Evaluation, Authorisation and Restriction of CHemical substances)[278] - aims to increase the awareness of the industry on hazards and risk management. REACH registration, in force since 2007, is mandatory for any chemical produced in the quantity over one tonne per year. Although it is being criticised for its ever-increasing cost and the number of animals employed in testing,[279] it undoubtedly provides a meaningful, and necessary, framework to raise human and environmental safety. Currently, only the ionic liquids which have already found application on industrial scale are undergoing REACH registration, such as $[C_2mim]X$ ($X = Cl^-$, $[C_2SO_4]^-$, $[C_1SO_3]^-$, $[O_2CMe]^-$ and $[NTf_2]^-$) and $[C_4mim]Cl$,[274] but there are no doubts that this number will continuously increase. They have received the classification of substances, *i.e.* as salts, which assumes that in the water/soil matrix, the cation and the anion will behave as independent chemical entities, displaying distinct toxicity and biodegradability potential. But, as noted above, it appears (irrespective of speculation as to the cause) that the cytotoxicity of ionic liquids cannot be systematically estimated by a summation of the independent effects of the cation and anion.[275,276]

Moreover, it should also be noted that, to date, mixtures (binary or ternary) of ionic liquids have been rarely investigated.[209]

In conclusion, one should recognise that the ionic liquid scientific community needs to increase the public awareness of the immense diversity of possible formulations and properties included in such a classification. For example, despite the significance of the data, especially as the first study reporting aquatic toxicity,[219] the attention attracted by the headline "Warning shot for green chemistry" in *Nature*,[280] or "Ionic liquids toxic to fish" in *Dalton Transactions*,[281] was almost certainly counterproductive and misleading, distorting as it did the excellent data reported by the original authors, where thirteen out of fifteen tested ionic liquids were shown to have LC_{50} values above 100 mg l^{-1} after 96 h of exposure. In contrast, unwarranted claims that ionic liquids, as a class of materials, are "green" (and many papers erroneously claim this in their introductory sentences) are just as damaging to the field as claims that ionic liquids are "toxic". With well over one million simple ionic liquids,[282] arguing from the specific to the generic is both misguided and intellectually dishonest. It should be recognised that sweeping generalisations do not favour our community, and it is the community itself that needs to protect ionic liquids indubitable broad utility, obviously by continuously following our duty to attain higher safety by their conscious design.

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1.3.2. Whole-cell biocatalysis with ionic liquids

Biocatalysis is an elegant strategy for the highly selective and efficient production of fine chemicals. Ionic liquids' application in biocatalysis has been studied widely, and is excellently reviewed by van Rantwijk and Sheldon.[95] Most of these studies exploited ionic liquids containing $[BF_4]^-$ and $[PF_6]^-$ anions, although their hydrolysis[55] was previously shown to drastically affect chemical and biochemical reactions. The majority of studies to date have considered enzymatic reactions, with some notable exceptions on whole-cell biocatalysis. So far analysis of ionic liquid biocompatibility is concerned exclusively with the damage caused to the plasma membrane of the whole-cell biocatalysts.[283] Single phase or biphasic systems can be considered. Whilst in single phase cells are grown in contact with water-miscible ionic liquids,[284] in biphasic systems they grow in one of the phases whilst the cell free layer contains water-immiscible ionic liquids. Representative examples of biphasic systems have permitted asymmetric reduction of 4-chloroacetophenone by *Lactobacillus kefir*[146] or *Escherichia coli*,[96] and phenol degradation by xenobiotic-degrading bacteria.[79] In these systems, partition of the ionic liquid phase prevents accumulation of a toxic product,[97] or substrate,[79,96,146] in the aqueous growth medium phase. Low amounts of the ionic liquid will also be solubilised in the aqueous phase, hence biocompatibility of the biocatalyst must be ensured.

The most recent advances on ionic liquids conscious design (*e.g.* Chapter III) strongly indicate a growing interest in their application in novel biotechnological processes for industrial, pharmacological and environmental usage.

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Chapter II

Exploring fungal activity in the presence of ionic liquids

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Abstract

In this work, the toxicological assessment towards filamentous fungi (*Penicillium* sp.) as model eukaryotic organisms of sixteen ionic liquids (containing an imidazolium, pyridinium, or cholinium cation) is presented. Amongst these fungi are members which show much higher tolerance towards ionic liquids than any other microorganism so far studied. Furthermore, guided by the paradigm that the choice of an ionic liquid as catalyst can alter the outcome of a given chemical reaction, the ability of ionic liquids to alter the metabolic profile in fungi was studied. The metabolic footprint, as investigated by electrospray ionisation mass spectrometry, revealed that fungal cultures respond to specific ionic liquids by changing their cell biochemistry, resulting in an altered pattern of secondary metabolites.

Introduction

In the last two decades, ionic liquids attracted a lot of scientific and commercial interest, as demonstrated in numerous publications and patents. They are, by definition, salts that are liquid at, or near, room temperature, completely composed of ions.[1,2] Their negligible vapour pressure and usual non-flammability are the basis for them sometimes being classified as “green” solvents.[3] In addition, ionic liquids can be, by design, chemically and thermally stable, recyclable, and with tuneable physical and chemical properties. Integration with their outstanding solvation ability opens doors for numerous industrial applications as replacements for conventional organic solvents.[1] Further to remarkable developments in their core chemical properties and applications, ionic liquids are now providing unexpected opportunities at the interface of chemistry with the life sciences, *e.g.* acting as solvents in enzymatic[4] and whole-cell[5] biocatalysis. In order to move ionic liquids beyond being an academic curiosity, their environmental, health, and safety impacts must be fully determined.[6] Although this field is still in its infancy, significant efforts are being made to obtain ecotoxicological data and design ionic liquids composed of naturally-derived materials for reduced toxicity and increased biodegradability.[7] Reports that ionic liquids can be used as active pharmaceutical ingredients (APIs) serve to further emphasise their potential for *in vitro* biochemical studies.[8,9]

Filamentous fungi are significant members of the Mycota kingdom, widespread in nature, especially in soil.[10] They play an important role in the carbon cycle on Earth, food spoilage, and as pathogens. On the other hand, due to the enormous diversity of species and their rich enzymatic systems, resulting in a broad range of secondary metabolites, they are widely used in biotechnological processes for production of chemicals, pharmaceuticals, food ingredients, and enzymes.[11] Many secondary metabolites of filamentous fungi have found application as therapeutic and/or bioactive compounds (*e.g.* antibiotics, cholesterol-lowering agents, anti-tumour agents, and immunosuppressors).[12,13] Their ability to adapt to extreme environmental conditions and to degrade some of the most recalcitrant materials (such as lignin and aromatic xenobiotics) makes them a very attractive model for screening the toxicity of chemicals with an unknown risk factor. In this work, for the first time, filamentous fungi were used as model eukaryotic organisms to assess the possible impact of ionic liquids whenever they are released into the environment.

Metabolic profiling using mass spectrometry (MS) became one of the most important techniques in fields ranging from diagnostics to functional genomics,[14] from taxonomy of filamentous fungi,[15] to screening of new compounds for biological activity. Here, metabolic footprinting is focussed on qualitative scanning of metabolites secreted by the cells. Components of the substrate that were transformed by the organism are also a part of the footprint.[14]

The cell chemodiversity strongly depends on inherited phylogenetic information (genomics), as well as on environmental conditions. Here, a relationship is proposed between the phylogenetic origin of fungal strains, their specific response to the presence of ionic liquids, and the modification of the metabolic profile caused by it.

Experimental

Chemicals

All ionic liquids used in this study (see Figure 1) were prepared by QUILL (The Queen´s University Ionic Liquids Laboratories, Belfast, UK), except for cholinium chloride (Sigma, Germany), 1-ethyl-3-methylimidazolium thiocyanate, $[C_2mim][SCN]$ (Fluka, Switzerland) and 1-ethyl-3-methylimidazolium ethyl sulfate, $[C_2mim][C_2SO_4]$ (Solvent Innovation, Germany). D(+)-glucose, K_2HPO_4, $ZnSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, $FeSO_4 \cdot 7H_2O$, KCl, glycerol (≥ 99.5 %), Tween 20 and ethyl ethanoate were purchased from Sigma Aldrich (Germany); CsCl, $MgSO_4 \cdot 7H_2O$, $NaNO_3$, 1-methylimidazole, and pyridine from Fluka (Switzerland); NaCl from Panreac (Spain), and ethanenitrile from Merck (Germany).

Ionic liquids prepared at QUILL were characterised by a combination of ^1H NMR, ^{13}C NMR, electrospray ionisation mass spectrometry, halide content, CHN elemental analysis, and water content. Typical halide content was < 0.1 %, and the maximum amount in one sample was 1.2 %.

Fungal strains

The following fungal strains were used, all belonging to the *Instituto de Biologia Experimental e Tecnológica* (IBET) culture collection: *Penicillium brevicompactum* Dierckx (IBETPeA), *P. olsonii* Bainier and Sartory (DSM 16515), *P. janczewskii* K.M. Zalessky (IBETF5), *P. glandicola* (Oudem) Seifert and Samson (IBETPeB), *P. corylophilum* Dierckx (IBETF6), *P. glabrum* (Wehmer) Westling (DSM 16516), *P. restrictum* J.C. Gilman and E.V. Abbot (IBETF4), *P. adametzii* Zaleski (IBETF2), *P. variabile* Sopp (IBETD2) and *P. diversum* Raper and Fennell (IBETPeE). They had previously been isolated from cork samples purchased from several Portuguese cork industries.[16,17]

Fungi were grown on MEA (malt extract agar) medium for one week at 27 °C. Fungal conidia were harvested using 0.1 % v/v Tween 20 (polyoxyethylene (20) sorbitan monolaurate) and filtered through glass wool. The suspension obtained was centrifuged (18500 *g*, 4 °C) and washed three times with 0.85 wt.% NaCl. The fungal conidia pellet was resuspended in 10 % v/v glycerol and stored at -80 °C.

Toxicity tests

The toxicity of ionic liquids towards filamentous fungi was determined by measuring the culture medium absorbance at 600 nm.

The minimal culture medium containing D(+)-glucose (1.0 g l^{-1}) and K_2HPO_4 (1.0 g l^{-1}) was dissolved in distilled water, pH of the solution was adjusted to 7.0 using phosphoric acid, sterilised in an autoclave (20 min; 121 °C), and supplemented with the mixture of essential salts, previously sterilised by filtration: $NaNO_3$ (3.0 g l^{-1}), $ZnSO_4{\cdot}7H_2O$, (0.01 g l^{-1}), $CuSO_4{\cdot}5H_2O$ (0.005 g l^{-1}), $MgSO_4{\cdot}7H_2O$ (0.5 g l^{-1}), $FeSO_4{\cdot}7H_2O$ (0.01 g l^{-1}), and KCl (0.5 g l^{-1}). Ionic liquids were added in order to obtain a final concentration of 50 mM (corresponding to 7-20 g l^{-1}, depending on the molecular weight of the ionic liquid).

Each liquid medium (2 ml) was inoculated with a suspension of fungal conidia in order to obtain the final concentration of 10^5 conidia per ml, and divided into eight wells (0.25 ml each) of a 96 well microtitre plate. Cultures were incubated in the dark at 27 °C.

The control samples (inoculated) and the blanks (non-inoculated) were produced and incubated under the same conditions: negative control (an ionic liquid free medium), osmotic stress controls (media supplemented with either aqueous NaCl (50 mM) or aqueous CsCl (50 mM): high charge density and low polarisability, or low charge density and high polarisability, respectively), organic function controls (media with aqueous 1-methylimidazole (50 mM) or aqueous pyridine (50 mM)), and blank samples (media with addition of the selected substances).

Fungal growth (or lack thereof) was followed daily by measuring the absorbance (600 nm) of the medium. Increase of absorbance was taken as indication of growth. Conidia formation (gauged by eye) and/or the absorbance approaching a constant value indicated that the culture entered a stationary growth phase. The culture supernatant liquid was separated from the mycelium by centrifuging (4 °C) and filtration (0.2 µm nylon membrane), and stored at -20 °C awaiting further analysis.

Toxicity data analysis

The toxicity data were analysed using scripts written in the R language, version 2.7.1 (for revision see reference[18,19]). The relation between any two toxicity profiles was quantified by the Jaccard distance, which measures dissimilarity between sample sets consisting of binary data. The obtained distance matrix was used to assess the existence of groups of fungal strains with similar toxicity profiles by means of hierarchical cluster analysis (HCA). Results are presented based on Ward's method for cluster linkage, which is designed to minimise the variance of distances within each cluster.[19] The robustness of the resulting dendrogram was validated using several other linkage methods as well. Given the binary nature of the data (growth or its inhibition), the distance measured between strains is equivalent to the number of different cases between two fungal strains.

Metabolites extraction

Fungal secondary metabolites (fSM, ionic liquid free) were extracted from the fungal cultures. The mycelium-free cultures (1 ml) were lyophilised (freeze-dried) in order to remove water, and ethyl ethanoate (1 ml) was added to the residue. Ethyl ethanoate was selected, due to its common use for extraction of fSM and to its limited miscibility with the ionic liquids studied. The upper layer (ethyl ethanoate) contains the fSM. This extraction was repeated once more and the combined ethyl ethanoate extracts were evaporated in a vacuum concentrator. The residue was dissolved in ethanenitrile (0.4 ml) and ultrasonicated (10 min). The same procedure was applied on blank samples previously prepared (see *Toxicity Tests*).

ESI-MS analysis

The electrospray mass spectra were recorded on a Bruker Esquire 3000 plus ion trap mass spectrometer in the positive and negative polarity modes (Bruker Daltonics, Billerica, MA). The samples (fSM in ethanenitrile) were injected at a rate of 0.1 ml h^{-1} into the electrospray ionisation (ESI) probe. The capillary temperature was set to 250 °C. All spectra were acquired using the Esquire Control Programme (Bruker Daltonics, Billerica, MA) and then converted to ASCII file format for computational interpretation.

Computational interpretation of MS data

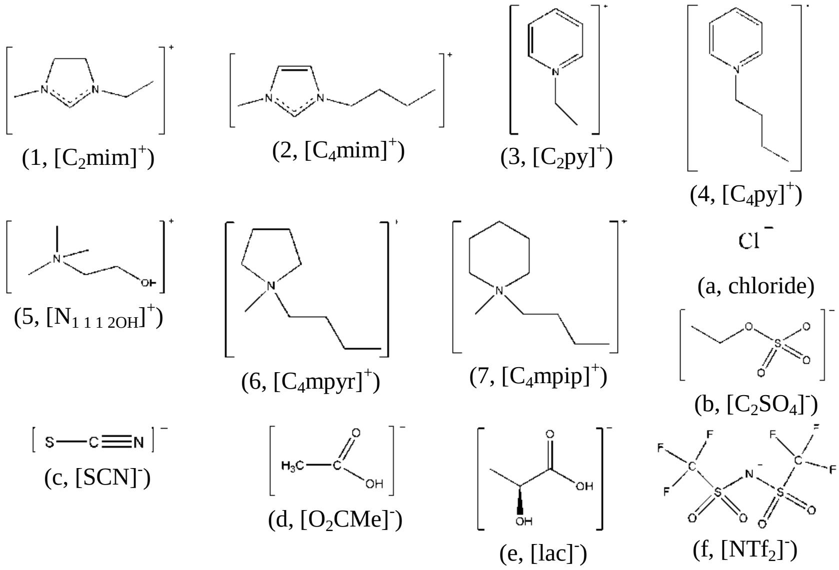
Mass spectrometry data were analysed with the same software as the toxicity data. After binning the intensities of mass spectra to integral *m/z* values, spectra pre-processing, peak detection and alignment of the spectra were performed using the msProcess package.[20] Peak intensities were normalised by their total, and values below 0.001 were set to zero. Peaks of the sample spectra were compared with the spectra from corresponding solvents and blanks, and coinciding peaks were eliminated from the peak matrix. To eliminate ionic liquid cations or anions with z=1, only *m/z* values between 150 and 1100 were considered in the analysis. Ionic liquid solubility in ethyl ethanoate (see *Metabolites Extraction*) is very limited but its nature may lead to an intense *m/z* peak even at a negligible concentration. The resulting binary matrices, peaks present or absent, were further analysed by HCA in an analogous manner to the toxicity data.

Results and discussion

Toxicological assessment

In this work, an examination of the toxicity of ionic liquids to filamentous fungi, belonging to the *Penicillium* genus, is presented. The selection of sixteen ionic liquids as test compounds was made by combining seven different cations and six different anions (see Figure 1).

This approach enabled us to investigate *inter alia* the toxic effect of the head group in the cation. Focussing on chloride, ethanoate, and DL-lactate anions (herein depicted as lactate) enabled the toxic effect of the anion to be studied.



(1, $[C_2mim]^+$)
(2, $[C_4mim]^+$)
(3, $[C_2py]^+$)
(4, $[C_4py]^+$)
(5, $[N_{1\,1\,1\,2OH}]^+$)
(6, $[C_4mpyr]^+$)
(7, $[C_4mpip]^+$)
Cl^-
(a, chloride)
(b, $[C_2SO_4]^-$)
(c, $[SCN]^-$)
(d, $[O_2CMe]^-$)
(e, $[lac]^-$)
(f, $[NTf_2]^-$)

Figure 1. Chemical structures of all ionic liquids used (cations and anions, numbers and letters, respectively). (1a) 1-ethyl-3-methylimidazolium chloride ($[C_2mim]Cl$); (1b) 1-ethyl-3-methylimidazolium ethyl sulfate $[C_2mim][C_2SO_4]$); (1c) 1-ethyl-3-methylimidazolium thiocyanate ($[C_2mim][SCN]$); (1d) 1-ethyl-3-methylimidazolium ethanoate ($[C_2mim][O_2CC_1]$); (1e) 1-ethyl-3-methylimidazolium DL-lactate ($[C_2mim][lac]$); (2a) 1-butyl-3-methylimidazolium chloride ($[C_4mim]Cl$); (3a) 1-butylpyridinium chloride ($[C_4py]Cl$); (4d) 1-ethylpyridinium ethanoate ($[C_2py][O_2CC_1]$); (4e) 1-ethylpyridinium DL-lactate ($[C_2py][lac]$); (5a) cholinium chloride ($[N_{1\,1\,1\,2OH}]Cl$); (5d) cholinium ethanoate ($[N_{1\,1\,1\,2OH}][O_2CC_1]$); (5e) cholinium DL-lactate ($[N_{1\,1\,1\,2OH}][lac]$); (5f) cholinium bis((trifluoromethyl)sulfonyl)amide ($[N_{1\,1\,1\,2OH}][NTf_2]$); (6a) 1-butyl-1-methylpyrrolidinium chloride ($[C_4mpyr]Cl$); (6e) 1-butyl-1-methylpyrrolidinium DL-lactate ($[C_4mpyr][lac]$); (7d) 1-butyl-1-methylpiperidinium ethanoate ($[C_4mpip][O_2CC_1]$).

In order to prove that the ionic liquids are not causing a toxic effect solely due to osmotic stress, the effects of sodium chloride and caesium chloride were tested. The latter loosely approximates the low-charge density and high polarisability of ionic liquids. Its addition to the growth medium has inhibited fungal growth (except for the case of *P. adametzii*), causing a stronger growth inhibitory effect than all the tested ionic liquids. These data were not used in the hierarchical clusters presented (see Figures 2 and 3), but their inclusion does not alter the cluster profile. 1-Methylimidazole and pyridine, as commonly used building blocks in ionic liquid chemistry, were also tested to assess whether their unsaturated nature had a deciding role to play in the antifungal activity of the ionic liquids.

The hierarchical cluster analysis (HCA) of the inhibitory effect of twenty selected compounds at 50 mM upon the growth of ten *Penicillium* strains is illustrated in Figure 2. The ionic liquid growth inhibitory effect was simplified to a binary matrix of fungal growth inhibition or no inhibition. It became evident that the chemical nature of the cationic head group influenced the overall toxicity of the ionic liquid, which has been demonstrated in numerous other studies.[21-25]

The imidazolium-based ionic liquids have the highest toxicity rankings of those studied, with an average of three toxic cases. Comparison of [C_2mim]Cl and [C_4mim]Cl showed the expected tendency[24] of increased toxicity with increasing alkyl side chain length. The $[N_{1\,1\,1\,2OH}]^+$ cation elicited the lowest toxic effect, and choline chloride, $[N_{1\,1\,1\,2OH}]$Cl, failed to inhibit growth in any of the fungal strains. With the exception of [C_2py][lac], which showed no toxicity, the remaining ionic liquids (containing pyridinium, pyrrolidinium, or piperidinium cations) lay in between these extremes.

The control compounds, 1-methylimidazole and pyridine, inhibited growth in 100% and 60% of the cases, respectively, proving to be significantly more toxic to fungi than any of their derived ionic liquids (studied herein).

Within the two groups of ionic liquids containing $[C_2\text{mim}]^+$ or $[N_{1\,1\,1\,2OH}]^+$ combined with Cl^-, $[O_2CMe]^-$ or $[\text{lac}]^-$, the $[O_2CMe]^-$ anion was the most toxic. None of the other ionic liquids tested showed any significant correlation with the nature of the selected anion. This suggests that a more extensive study would be of great value. Here, it is noted that, while the cation has the more predictable effect, the anion also contributes toward the antifungal activity (*cf.* with rat leukaemia cell line).[23]

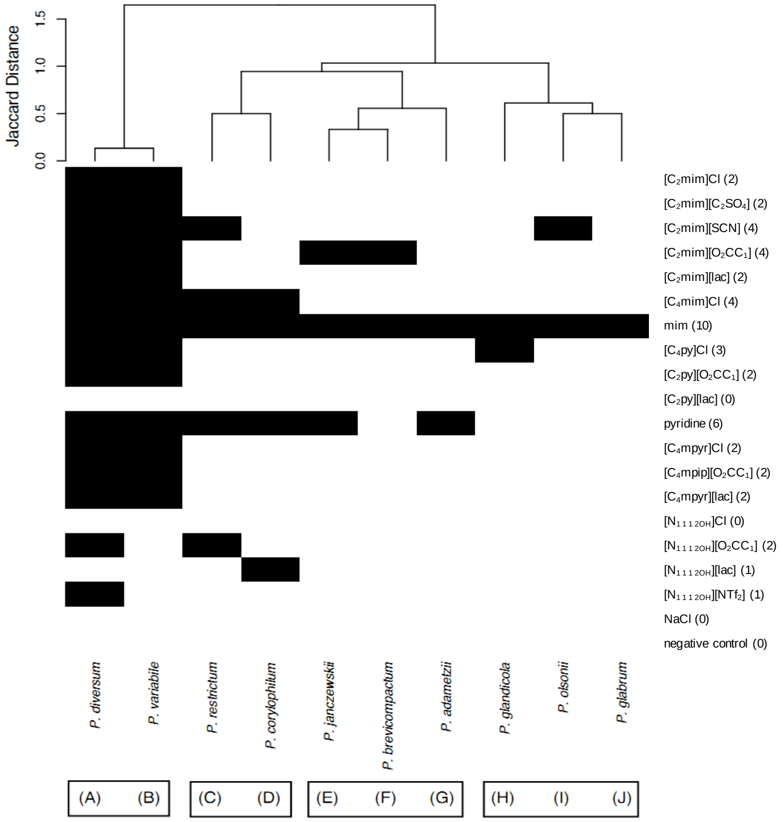


Figure 2. Hierarchical cluster analysis of the growth behaviour of *Penicillium* strains in the ionic liquid containing media and controls (see *Experimental* section for details). Black fields show cases of growth inhibition; row labels indicate the tested ionic liquids (ordered by common cation group) and control substances; the number in brackets corresponds to the toxicity ranking (from 0 to 10).

The HCA of the toxicity profiles (Figure 2) revealed four groups of fungal strains with similar growth behaviours. The group {A, B} showed the highest ionic liquid susceptibility. The group {C, D} showed intermediate susceptibility, and the remaining groups {E, F, G} and {H, I, J} low susceptibility. It was interesting to compare and correlate these data with the clusters in a phylogenetic study on *Penicillium* strains by Wang and Zhuang, which they based on calmodulin gene partial sequences (about 600 nucleotides).[26] Their study distinguished eleven clusters and covered all the strains featured in our work, except {A} and {I}, and a high degree of correlation between the phylogenetic background of the strains and their response to the ionic liquid environment was observed (see Table 1).

Table 1. Comparison of the results of this work with those of Wang and Zhuang[26].

Toxicity study (this work)	Phylogenetic study[26]
{B}	{XI}
{C, D}	{VIII}
{E, F, G}, {H, J}	{I, II}

This detected correlation between the genetic proximity of the strains and their susceptibility to different environmental conditions can be used in the rationalisation of toxicological studies, and in the prediction of the behaviour of different strains.

Metabolic footprinting

In order to assess the ability of ionic liquids to alter the metabolism of fungi, fungal culture extracts (fSM extracts, ionic liquid free) were analysed by ESI-MS, the spectra being interpreted qualitatively according to the presence or absence of peaks.

The selection of samples (combinations of fungal strains and ionic liquids) was made based on the analysis of the toxicological data presented above. The strains {B}-{H} were used, including one or more strains from each cluster. The ionic liquids, [C_2mim]Cl, [C_2mim][lac], [C_4py]Cl, [C_2py][lac], [$N_{1\,1\,1\,2OH}$]Cl and [$N_{1\,1\,1\,2OH}$][lac], were selected according to Figure 2, comprising all cationic groups which have shown distinct effects, imidazolium and cholinium, as the most and the least toxic, respectively, and pyridinium, with intermediate toxic effect. In addition, negative and sodium chloride controls were included.

After growth, extraction of fungal metabolites, and collection of their ±ESI-MS spectra, the HCA of these "metabolic footprints" for all ionic liquids tested per strain showed no discernable pattern (data not shown). However, the metabolic footprints induced by [C_2mim]Cl and [C_2mim][lac] were clustered together for all fungal strains, and were disassociated from the metabolic footprints induced by the other ionic liquids (data not shown). This observation is confirmed in the integrated assessment of the joint peak lists, uniting all individual peak matrices (depicted in Figure 3), and this result was robust against variations of the clustering algorithm.

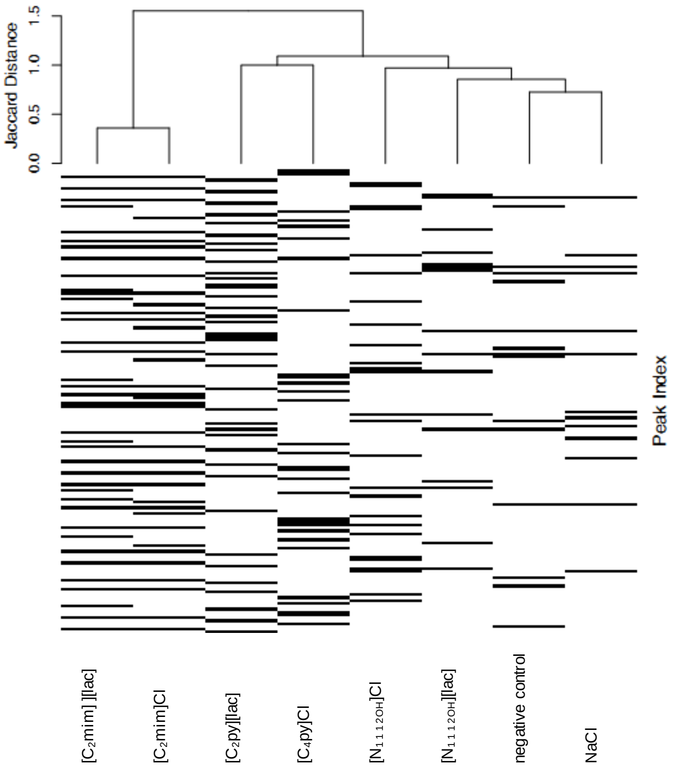


Figure 3. Hierarchical cluster analysis of the joint peak lists, uniting all individual peak matrices of positive and negative mode, detected by ESI-MS after fungal growth in the ionic liquid containing media and controls (see *Experimental* section for details). Black fields indicate the presence of a peak at a given *m/z* value.

The remaining metabolic footprints produced a single cluster divided into two subclusters: those induced by [C_4py]Cl and [C_2py][lac], and those induced by [$N_{1\,1\,1\,2OH}$]Cl, [$N_{1\,1\,1\,2OH}$][lac] and controls. In the latter, the metabolic footprints induced by the cholinium-based ionic liquids showed weak correlation with controls and between themselves.

Distinct groups are also revealed when one merely accounts for the number of the peaks in the metabolic profiles. The correlation between the number of detectable mass species in the extracts and the toxicity of the corresponding ionic liquids is apparent. [C_2mim]Cl and [C_2mim][lac] induced the highest number of peaks (≥38), significantly different from the controls (≤15). Interestingly, despite the fact that [C_2py][lac] and [$N_{1\,1\,1\,2OH}$]Cl showed no inhibition to fungal growth, both have still induced significant metabolic alterations.

In order to provide a more complete picture of the specific events in the metabolic network (strains and ionic liquid dependency), quantitative analysis will be attempted in the future.

Conclusions

This is the first time that filamentous fungi have been used in a toxicological study on ionic liquids. The most significant observation is the very high tolerance of *Penicillium* strains towards the ionic liquids. Complete inhibition of growth was noted in only 20 % of ionic liquids/strains cases. The concentration of ionic liquids that was tested in this study (50 mM) is much higher, by several orders of magnitude, than in any other testing system reported so far.[21,25]

In addition, we have observed that fungi can tolerate, in some cases, even higher ionic liquid concentrations, *e.g. P. olsonii* can grow in the presence of 0.375 M of [C_2mim]Cl (data not shown).

Testing a vast number of ionic liquids and organisms is rather time-consuming and costly, but the cluster strategy presented here, along with the concept of QSAR (quantitative structure-activity relationship), which refers to estimation of the hazard potential of ionic liquids based on structure, simplifies their risk evaluation.

These data show that the ionic liquid induced effects on fungal metabolism cannot be simply explained by the ionic liquid toxicity. Moreover, the metabolic footprints induced by the ionic liquids do not correlate with those induced by common salts, such as sodium chloride. Even caesium chloride, in the specific case of *P. adametzii*, clustered closer to the controls than to the ionic liquids. This is supported by preliminary data on the fungal secretome (bidimensional electrophoresis analysis) induced by the different ionic liquids (unpublished data). Results suggest that some ionic liquids, *e.g.* $[N_{1\,1\,1\,2OH}]Cl$ and $[C_4mpyrr]Cl$, have induced the expression of a distinct set of fungal extracellular proteins, which could be grouped into ionic liquid responsive, ionic liquid specific or non-specific, and inorganic salt responsive proteins. In addition, these metabolic alterations cannot be, in the case of all ionic liquids, a result of their co- or direct metabolism by the fungi. That would certainly not be the case of imidazolium cation, which is resistant to microbial degradation.[27,28]

The behaviour of fungi, and any other organisms, is highly linked to different omes (genome, transcriptome, proteome and metabolome) and influenced by environmental conditions. Transcriptional profiling and proteomic analysis are becoming routine techniques and, joined with metabolite analysis, could build a platform applicable in many areas. Going beyond ecotoxicological studies to describe the effect of toxics on living cells, it could be useful in the discovery of novel natural compounds and in the development of efficient and environmentally friendly bioprocesses.

The test systems described in this work are highly promising, especially considering that filamentous fungi have the ability to produce biologically active secondary metabolites.[12,13] Further studies are necessary to prove whether or not ionic liquids have the ability to induce a tailored metabolic alteration. Should this be observed, it would be a significant breakthrough in whole-cell biocatalysis, creating a new concept: an ionic liquid controlled bio-tool for designing targeted end products.

Acknowledgments

M. P. has done most of the experimental work and data interpretation (except computational analysis). The spectrometry analyses were done in collaboration with experts from MS unit at ITQB. She has also prepared the draft of the manuscript.

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Chapter III

Novel biocompatible cholinium-based ionic liquids - toxicity and biodegradability

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Novel biocompatible cholinium-based ionic liquids - toxicity and biodegradability

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Novel biocompatible cholinium-based ionic liquids - toxicity and biodegradability

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Abstract

The synthesis, characterisation and toxicological assessment of a new group of environmentally friendly ionic liquids are presented. Focussing on the toxic effect of the anion, the ionic liquids were designed by combining the benign cholinium cation, $[N_{1\,1\,1\,2OH}]^+$, with a range of linear alkanoate anions ($[C_nH_{2n+1}CO_2]^-$, $n = 1 - 9$), as well as two structural isomers ($n = 3$ or 4). The toxicity of these ionic liquids was evaluated using filamentous fungi as model eukaryotic organisms. Surprisingly, most of the tested strains showed active growth in media containing extremely high ionic liquid concentrations, up to molar ranges in some cases. The biodegradability of these ionic liquids was assessed, and new biotechnological applications for them are proposed, *e.g.* as solvents for biopolymers. This study leads to the better understanding of the anion influence on the ionic liquid toxicity, but its core is the recognition that conscious design of ionic liquids can be used to deliver truly biocompatible salts without adversely affecting one of the most striking of their properties – their outstanding solvent ability.

Introduction

The chemistry of ionic liquids has developed dramatically during the last decade. Their generic, yet not universal, properties, such as negligible vapour pressure, non-flammability, chemical and thermal stability, and outstanding solvation ability, enabled rapid advance in numerous applications.[1] The potential of ionic liquids is further emphasised because their physical and chemical properties may be finely tuned by varying the cation and the anion.[2] Their dual nature is, relative to conventional molecular organic solvents, a clear advantage.

In order to improve old, or to create novel, ionic liquid based processes, aiming at cost-efficiency and sustainability, an interdisciplinary approach is essential. So-called risk-conscious design and "thinking in terms of structure-activity relationships" should be applied before implementing any new materials on a large scale. Thus, a better, structure-based, understanding of the environmental fate of ionic liquids is critical.[3] Unfortunately, this is a complex equation which crosses numerous unknown abiotic and biotic factors. The enormous diversity of ionic liquids is also a major concern from the ecotoxicological point of view, since testing such a vast number of ionic liquids and organisms is inconceivable.

In the expanding studies on the toxicity and biodegradability of ionic liquids, they were not *a priori* accepted as environmentally benign.[4] It is widely accepted that the head group of the cation has a deciding role in toxicity,[5,6] that longer side chains have a more severe impact on living cells,[7] and that incorporation of an ester group significantly increases their biodegradability.[8] The anion plays an essential role in the ionic liquid physicochemical properties,[2] and it is known to contribute to the overall toxicity,[9] but its effect is usually neglected. The predictive value of certain trends, both for the cation and the anion, is unquestionable; however, generalisations should be taken cautiously, given the current limited understanding of the modes of toxicity of ionic liquids, their biodegradation pathways, and their behaviour concerning biosorption, bioaccumulation, *etc*. This complexity can be simplified if ionic liquids are synthesised from carefully selected naturally-derived materials, which are safer, carrying reduced toxicity and enhanced biodegradability.[10]

Although a wide range of cholinium ionic liquids have been known for some time,[11-17] the alkanoates have been largely ignored. We present here a new group of cholinium ionic liquids. Cholinium, a quaternary ammonium cation, $[N_{1\,1\,1\,2OH}]^+$, which is an essential micronutrient,[18] was chosen as the benign cation and combined with alkanoates of systematically elongated chains. In order to better understand the anion influence on ionic liquid toxicity, the toxicities of the obtained ionic liquids were evaluated using filamentous fungi – previously demonstrated to be an excellent test system.[19]

Experimental section

Chemicals

The materials used in the ionic liquid syntheses, including source and grade, were as follows: cholinium hydrogencarbonate (Aldrich, 75 wt.% in H_2O), ethanoic acid (Aldrich, 99-100 % puriss), propanoic acid (Aldrich, 99.5 %), butanoic acid (Lancaster, 99+ %), 2-methylpropanoic acid (Aldrich, 99 %), pentanoic acid (Aldrich, 99 %), 2,2-dimethylpropanoic acid (Aldrich, 99 %), hexanoic acid (Alfa Aesar, 99 %), octanoic acid (Sigma, 99 %) and decanoic acid (Aldrich, 96 %). Sodium salts of the corresponding carboxylic acids were prepared *via* addition of the acid to sodium methoxide (Sigma Aldrich 25 wt.% in methanol), vacuum-filtration, and washing the precipitated salt with cold methanol, followed by drying under high vacuum (80 °C, >14 h).

Other chemicals used in the study were: cholinium chloride (choline; Sigma, ≥98 %), sodium dodecyl sulfate (SDS, Amersham Biosciences, >99 %), benzalkonium chloride (BAC, predominantly $[C_{12}H_{25}N(CH_3)_2(CH_2C_6H_5)]Cl$, but also contains C_{14} and C_{16} homologues, Aldrich), ethanol (Panreac, absolute PA) and propanone (Fisher Scientific, analytical grade). D(+)-glucose, K_2HPO_4, $ZnSO_4·7H_2O$, $CuSO_4·5H_2O$, $FeSO_4·7H_2O$, KCl, glycerol (≥ 99.5 %), Tween 20 and were purchased from Sigma Aldrich (Germany). $MgSO_4·7H_2O$ and $NaNO_3$ were purchased from Fluka (Switzerland).

Ionic liquids

The ionic liquids were prepared by dropwise addition of the corresponding acid (1:1) to aqueous cholinium hydrogencarbonate, stirring at ambient temperature and pressure. Water was then removed under reduced pressure, first using a rotary evaporator (*e.g.* 70 °C, 30 min), and then stir-heating *in vacuo* (65-70 °C, 24 h, *ca.* 0.01 mbar).

The ionic liquids were characterised by ^1H and ^{13}C NMR (Bruker Avance III spectrometer, 400 MHz) spectroscopy at 25 °C. Their purity was confirmed by electrospray ionisation mass spectrometry (ESI-MS) (Waters LCT Premier fitted with electrospray). The spectroscopic analyses are provided below. The water contents, determined by Karl-Fischer titration, were below 0.1 wt.%. The obtained salts fulfilled the requirements of the present study.

Cholinium ethanoate: ^1H NMR (D_2O, 400 MHz): δ/ppm = 1.86 (s, 3H, $C\mathbf{H_3}COO$); 3.15 (s, 9H, $N(C\mathbf{H}_3)_3$); 3.46 (t, J = 4.8 Hz, 2H, $C\mathbf{H}_2OH$); 4.01 (m, 2H, $NC\mathbf{H_2}$). ^{13}C NMR (D_2O, 100 MHz): δ/ppm = 23.591 ($\mathbf{C}H_3COO$); 54.404 (t, $N(\mathbf{C}H_3)_\mathbf{3}$); 56.038 ($\mathbf{C}H_2OH$); 67.839 (t, $N\mathbf{C}H_2$); 181.714 ($\mathbf{C}OO$). MS ES+ *m/z* (% rel. Intensity): 104 M^+ (100). Calc. 104.1075, found 104.1078.

Cholinium propanoate: ^1H NMR (D_2O, 400 MHz): δ/ppm = 0.88 (t, J = 7.7 Hz, 3H, $C\mathbf{H}_3CH_2$); 1.99 (q, J = 7.5 Hz, 2H, $C\mathbf{H}_2COO$); 3.02 (s, 9H, $N(C\mathbf{H}_3)_3$); 3.34 (t, J = 5.0 Hz, 2H, $C\mathbf{H}_2OH$); 3.87 (m, 2H, $NC\mathbf{H}_2$). ^{13}C NMR (D_2O, 100 MHz): δ/ppm = 10.21($\mathbf{C}H_3CH_2$); 30.65 ($\mathbf{C}H_2COO$); 53.77 (t, $N(\mathbf{C}H_3)_\mathbf{3}$); 55.47 ($\mathbf{C}H_2OH$); 57.33 (t, $N\mathbf{C}H_2$); 184.21 ($\mathbf{C}OO$). MS ES+ *m/z* (% rel. Intensity): 104 M^+ (100). Calc. for $C_5H_{14}NO$ 104.1075, found 104.1079.

MS ES- *m/z* (% rel. Intensity): 73 M$^-$ (100). Calc. for $C_3H_5O_2$ 73.0710, found 73.0290.

Cholinium butanoate: ^{1}H NMR (D_2O, 400 MHz): δ/ppm = 0.76 (t, *J* = 7.5 Hz, 3H, C**H**$_3$CH$_2$); 1.42 (m, 2H, CH$_3$C**H**$_2$); 2.02 (t, *J* = 7.1 Hz, 2H, C**H**$_2$COO); 3.07 (s, 9H, N(C**H**$_3$)$_3$); 3.38 (t, *J* = 4.7 Hz, 2H, C**H**$_2$OH); 3.92 (m, 2H, NC**H**$_2$). ^{13}C NMR (D_2O, 100 MHz): δ/ppm = 13.26 (**C**H$_3$CH$_2$); 19.34 (CH$_3$**C**H$_2$); 39.62 (**C**H$_2$COO); 53.81 (t, N(**C**H$_3$)$_3$); 55.52 (**C**H$_2$OH); 67.6 (t, N**C**H$_2$); 183.79 (**C**OO). MS ES+ *m/z* (% rel. Intensity): 104 M$^+$ (100). Calc. for $C_5H_{14}NO$ 104.1075, found 104.1076. MS ES$^-$ *m/z* (% rel. Intensity): 87 M$^-$ (100). Calc. for $C_4H_7O_2$ 87.0446, found 87.0443.

*Cholinium pentanoate***:** ^{1}H NMR (D_2O, 400 MHz): δ/ppm = 0.86 (t, *J* = 6.0 Hz, 3H, C**H**$_3$CH$_2$); 1.27 (m, 2H, CH$_3$C**H**$_2$); 1.50 (m, 2H, CH$_3$CH$_2$C**H**$_2$); 2.15 (t, *J* = 6.0 Hz, 2H, C**H**$_2$COO); 3.17 (s, 9H, N(C**H**$_3$)$_3$); 3.49 (t, *J* = 6.0 Hz, 2H, C**H**$_2$OH); 4.02 (m, 2H, NC**H**$_2$). ^{13}C NMR (D_2O, 100 MHz): δ/ppm = 13.73 (**C**H$_3$CH$_2$); 22.55 (CH$_3$**C**H$_2$); 28.74 (CH$_3$CH$_2$**C**H$_2$); 37.66 (**C**H$_2$COO); 54.26 (t, N(**C**H$_3$)$_3$); 55.98 (**C**H$_2$OH); 67.77 (t, N**C**H$_2$); 184.24 (**C**OO). MS ES+ *m/z* (% rel. Intensity): 104 M$^+$ (100). Calc. for $C_5H_{14}NO$ 104.1075, found 104.1079. MS ES- *m/z* (% rel. Intensity): 115 M$^-$ (100). Calc. for $C_5H_9O_2$ 101.0603, found 101.0622.

Cholinium hexanoate: ^{1}H NMR (D_2O, 400 MHz): δ/ppm = 0.76 (t, *J* = 7.2 Hz, 3H, C**H**$_3$CH$_2$); 1.17 (m, 4H, CH$_3$(C**H**$_2$)$_2$); 1.43 (m, 2H, C**H**$_2$CH$_2$COO); 2.04 (t, *J* = 7.6 Hz, 2H, C**H**$_2$COO); 3.08 (s, 9H, N(C**H**$_3$)$_3$); 3.40 (t, *J* = 5.0 Hz, 2H, C**H**$_2$OH); 3.94 (m, 2H, NC**H**$_2$). ^{13}C NMR (D_2O, 100 MHz): δ/ppm = 13.30 (**C**H$_3$CH$_2$); 21.77 (CH$_3$**C**H$_2$); 25.58 (CH$_3$CH$_2$**C**H$_2$); 31.01 (**C**H$_2$CH$_2$COO); 37.63 (**C**H$_2$COO); 53.82 (t, N(**C**H$_3$)$_3$); 55.53 (**C**H$_2$OH); 67.37 (t, N**C**H$_2$);184.00 (**C**OO). MS ES+ *m/z* (% rel. Intensity): 104 M$^+$ (100). Calc. for $C_5H_{14}NO$ 104.1075, found 104.1077. MS ES- *m/z* (% rel. Intensity): 115 M$^-$ (100). Calc. for $C_6H_{11}O_2$ 115.0759, found 115.0758.

Cholinium octanoate: ^{1}H NMR (D_2O, 400 MHz): δ/ppm = 0.76 (t, *J* = 7.0 Hz, 3H, C**H**$_3$CH$_2$); 1.18 (m, 8H, CH$_3$(C**H**$_2$)$_4$); 1.43 (m, 2H, C**H**$_2$CH$_2$COO); 2.05 (t, *J* = 7.7 Hz, 2H, C**H**$_2$COO); 3.09 (s, 9H, N(C**H**$_3$)$_3$); 3.40 (t, *J* = 4.9 Hz, 2H, C**H**$_2$OH); 3.94 (m, 2H, NC**H**$_2$). ^{13}C NMR (D_2O, 100 MHz): δ/ppm = 13.55 (**C**H$_3$CH$_2$); 22.12 (CH$_3$**C**H$_2$); 26.02 (CH$_3$CH$_2$**C**H$_2$); 28.45 (CH$_3$(CH$_2$)$_2$**C**H$_2$); 28.91 (CH$_3$(CH$_2$)$_3$**C**H$_2$); 31.22 (**C**H$_2$CH$_2$COO); 37.74 (**C**H$_2$COO); 53.85 (t, N(**C**H$_3$)$_3$); 55.54 (**C**H$_2$OH); 67.40 (t, N**C**H$_2$); 183.65 (**C**OO). MS ES+ *m/z* (% rel. Intensity): 104 M^+ (100). Calc. for $C_5H_{14}NO$ 104.1075, found 104.1077. MS ES- *m/z* (% rel. Intensity): 143 M^- (100). Calc. for $C_8H_{15}O_2$ 143.1072, found 143.1060.

Cholinium decanoate: ^{1}H NMR (D_2O, 400 MHz): δ/ppm = 0.78 (t, *J* = 7.0 Hz, 3H, C**H**$_3$CH$_2$); 1.19 (m, 12H, CH$_3$(C**H**$_2$)$_6$); 1.44 (m, 2H, C**H**$_2$CH$_2$COO); 2.05 (t, *J* = 7.5 Hz, 2H, C**H**$_2$COO); 3.10 (s, 9H, N(C**H**$_3$)$_3$); 3.41 (t, *J* = 4.9 Hz, 2H, C**H**$_2$OH); 3.95 (m, 2H, NC**H**$_2$). ^{13}C NMR (D_2O, 100 MHz): δ/ppm = 13.73 (**C**H$_3$CH$_2$); 22.45 (CH$_3$**C**H$_2$); 26.25 (CH$_3$CH$_2$**C**H$_2$); 29.16 (CH$_3$(CH$_2$)$_2$**C**H$_2$); 29.29 (CH$_3$(CH$_2$)$_3$**C**H$_2$); 29.43 (CH$_3$(CH$_2$)$_4$CH$_2$); 29.44 (CH$_3$(CH$_2$)$_5$**C**H$_2$); 31.74 (**C**H$_2$CH$_2$COO); 37.85 (**C**H$_2$COO); 53.86 (t, N(**C**H$_3$)$_3$); 55.56 (**C**H$_2$OH); 67.44 (t, N**C**H$_2$); 183.03 (**C**OO). MS ES+ *m/z* (% rel. Intensity): 104 M^+ (100). Calc. for $C_5H_{14}NO$ 104.1075, found 104.1075. MS ES- *m/z* (% rel. Intensity): 171 M^- (100). Calc. for $C_{10}H_{19}O_2$ 171.1385, found 171.1384.

Cholinium 2-methylpropanoate: ^{1}H NMR (D_2O, 400 MHz): δ/ppm = 0.9 (d, *J* = 7.0 Hz, 6H, (C**H**$_3$)$_2$CH); 2.35 (m, H, C**H**(CH$_3$)$_2$); 3.17 (s, 9H, N(C**H**$_3$)$_3$); 3.48 (t, *J* = 3.0 Hz, 2H, C**H**$_2$OH); 4.03 (m, 2H, NC**H**$_2$). ^{13}C NMR (D_2O, 100 MHz): δ/ppm = 19.38 ((**C**H$_3$)$_2$CH); 35.89 (**C**HCOO); 53.81 (t, N(**C**H$_3$)$_3$); 55.53 (**C**H$_2$OH); 57.36 (t, N**C**H$_2$); 187.58 (**C**OO). MS ES+ *m/z* (% rel. Intensity): 104 M^+ (100). Calc. for $C_5H_{14}NO$ 104.1075, found 104.1078. MS ES- *m/z* (% rel. Intensity): 87 M^- (100). Calc. for $C_4H_7O_2$ 87.0446, found 87.0448.

Cholinium 2,2-dimethylpropanoate: ^{1}H NMR (D_2O, 400 MHz): δ/ppm = 1.00 (s, 9H, C(C**H**$_3$)$_3$); 3.10 (s, 9H, N(C**H**$_3$)$_3$); 3.41 (t, *J* = 5.0 Hz, 2H, C**H**$_2$OH); 3.96 (m, 2H, NC**H**$_2$). ^{13}C NMR (D_2O, 100 MHz): δ/ppm=27.47 (C(**C**H$_3$)$_3$); 39.76 (**C**(CH$_3$)$_3$); 53.82 (t, N(**C**H$_3$)$_3$); 55.54 (**C**H$_2$OH); 67.37 (t, N**C**H$_2$); 188.83 (**C**OO). MS ES+ *m/z* (% rel. Intensity): 104 M^+ (100). Calc. for

$C_5H_{14}NO$ 104.1075, found 104.1079. MS ES- *m/z* (% rel. Intensity): 101 M^- (100). Calc. for $C_5H_9O_2$ 101.0603, found 101.0603.

Thermal properties analysis

The thermal stabilities and decomposition temperatures of the ionic liquids were measured using a thermal gravimetric analyser (TGA Q5000 V3.10 Build 258). All samples were recorded in aluminium pans under a dinitrogen atmosphere. Samples were heated up to 300 °C at a heating rate of 5 °C min^{-1} until complete thermal degradation was achieved. Universal Analysis, version 4.4A software, was used to determine the onset (T_{onset}) and the decomposition (T_{dec}) temperatures, as the temperatures at which the baseline slope changes during the heating, and at which 50 % of weight loss was observed, respectively. Additionally, their melting (T_m) and glass transition temperatures (T_g) were determined by differential scanning calorimetry (DSC) (DSC Q2000 V24.2 Build 107). Heating and cooling cycles were conducted at the rate of 5 °C min^{-1}. Results are presented in Table 1.

Table 1. The thermal properties of the ionic liquids: onset (T_{onset}), decomposition (T_{dec}), melting (T_m) and glass transition (T_g) temperatures.

Ionic liquid	T_{onset}*/ °C	T_{dec}*/ °C	T_m/ °C	T_g/ °C
cholinium ethanoate	169	210	80	
cholinium propanoate	113	172	n.d.	-74
cholinium butanoate	97	166	45	
cholinium 2-methylpropanoate	110	172	35	
cholinium pentanoate	165	203	31	
cholinium 2,2-dimethylpropanoate	112	177	57	
cholinium hexanoate	106	169	52	
cholinium octanoate	107	166	26	
cholinium decanoate	116	168	50	

*T_{onset} and T_{dec} defined as the temperatures at which the baseline slope changes during the heating, and at 50 % weight loss, respectively. Please note these are from scanning TGA, and do not represent isothermal stabilities.

Fungal strains

The following fungal strains were used, all belonging to the Instituto de Biologia Experimental e Tecnológica (IBET) culture collection: *Penicillium brevicompactum* Dierckx (IBETPeA), *P. glandicola* (Oudem) Seifert and Samson (IBETPeB), *P. corylophilum* Dierckx (IBETF6) and *P. diversum* Raper and Fennell (IBETPeE). They had previously been isolated from cork samples purchased from several Portuguese cork industries.[20,21]

Fungi were grown on MEA (malt extract agar) medium for one week at 27 °C. Fungal conidia were harvested using 0.1 % v/v Tween 20 (polyoxyethylene (20) sorbitan monolaurate) and filtered through glass wool. The suspension obtained was centrifuged (18500 *g*, 4 °C) and washed three times with 0.85 wt.% NaCl. The fungal conidia pellet was resuspended in 10 % v/v glycerol and stored at -80 °C.

Toxicity tests

The toxicity of ionic liquids to the fungal strains was evaluated by determining their minimal inhibitory concentrations (MIC) and minimal fungicidal concentrations (MFC), distinguishing between growth inhibition and death, respectively.

The minimal culture medium containing D(+)-glucose (1.0 g l^{-1}) and K_2HPO_4 (1.0 g l^{-1}) was dissolved in distilled water, pH of the solution was adjusted to 7.0 using phosphoric acid, sterilised in an autoclave (20 min; 121 °C), and supplemented with the mixture of essential salts, previously sterilised by filtration: $NaNO_3$ (3.0 g l^{-1}), $ZnSO_4{\cdot}7H_2O$, (0.01 g l^{-1}), $CuSO_4{\cdot}5H_2O$ (0.005 g l^{-1}), $MgSO_4{\cdot}7H_2O$ (0.5 g l^{-1}), $FeSO_4{\cdot}7H_2O$ (0.01 g l^{-1}), and KCl (0.5 g l^{-1}). The testing compounds (ionic liquids and control compounds) were added to obtain final concentrations from 2.5 mM up to 2 M (distributed stepwise from 0.5 mM to 0.1 M) in growth media.

Each liquid medium (1 ml) was inoculated with a suspension of fungal conidia in order to obtain the final concentration of 10^5 conidia per cm^3, and divided into four wells (0.25 ml each) of a 96-well microtitre plate.

Cultures were incubated in the dark, at 27 °C, for 14 days. Fungal growth (or lack thereof) was followed by measuring the absorbance (600 nm) of the medium and, when necessary, at the end of incubation gauging by eye the formation of mycelium (turbidity) and/or conidia. The lowest concentration that inhibited the formation of mycelium was taken as the MIC.

Additionally, all the samples where no active growth was detected were used as inocula and spread, with a ~1 µl loop, onto malt extract agar medium (Oxoid, UK). The plates were incubated in the dark, at 27 °C, for 7 days. The lowest concentration of the test compound which results in unviable conidia was taken as MFC. MIC and MFC values should not be interpreted as absolute ones, but rather as an indication of the inhibitory and the fungicidal upper concentrations limits.

The control samples were incubated under the same conditions: negative control (ionic liquid free medium), cationic surfactant control (BAC), anionic surfactant control (SDS), anion effect control (sodium salts), common organic solvents (ethanol and propanone) and blank samples (non-inoculated). Cholinium chloride (choline) was also tested to measure the cation effect.

Biodegradability assessment of the ionic liquids

Penicillium corylophilum was chosen for studies on ionic liquid biodegradability. Concentrations of tested compounds used in this assay were below the previously determined MICs. Fungal cultures of 20 ml were inoculated as described above, and incubated in the dark, at 27 °C, under agitation (90 rpm), for 28 days. The aliquot of 1 cm^3 was taken from the cultures, filtered (0.2 µm), freeze-dried to remove water, dissolved in 0.7 ml of deuteriated water, and analysed by ^{1}H NMR spectroscopy. Ionic liquid biodegradability in the static fungal cultures (toxicity tests) was analysed by ^{1}H NMR spectroscopy, and in some cases the anion biodegradability confirmed by liquid chromatography.[22]

Results and Discussion

In the present work, a new group of ionic liquids, composed by the benign cholinium cation[18] and alkanoate anions, was investigated (Figure 1). The physical and thermal properties of cholinium ethanoate and propanoate have been reported previously,[23] but these salts are seldom studied. The linear chains in the alkanoate anions were systematically elongated, from ethanoate to decanoate, and two branched isomers, 2-methylpropanoate and 2,2-dimethylpropanoate, were also analysed. This constitutes, *pace* cholinium ethanoate,[19] the very first systematic study on their toxicity.

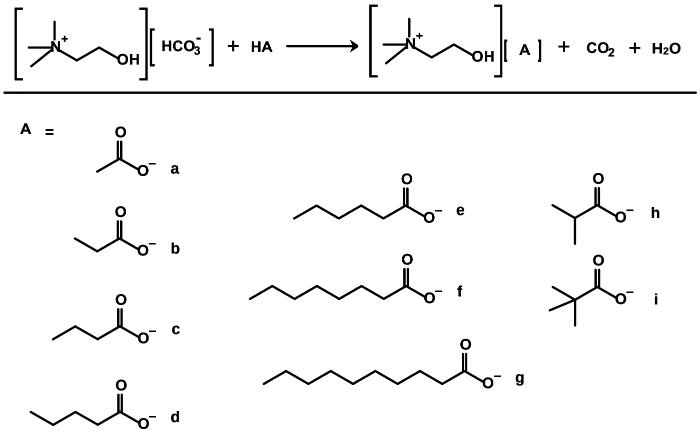


Figure 1. Chemical route for obtaining the cholinium alkanoate ionic liquids, $[N_{1\,1\,1\,2OH}][C_nH_{2n+1}CO_2]$ (n = 1-9) and chemical structures of the anions used: (a) ethanoate, (b) propanoate, (c) butanoate, (d) pentanoate, (e) hexanoate, (f) octanoate, (g) decanoate, (h) 2-methylpropanoate, (i) 2,2-dimethylpropanoate.

Previously, it was demonstrated that several phylogenetically closely related fungal strains, all belonging to the *Penicillium* genus, were distinctively grouped according to their growth susceptibility in media containing ionic liquids.[19] Importantly, a high degree of correlation between the strains genetic proximity and their response to the ionic liquid environment was also inferred.

One representative fungal strain from each susceptibility group was therefore selected for this work. They were, set by order of tolerance: *P. brevicompactum*, *P. glandicola*, *P. corylophilum* and *P. diversum*. These fungal strains were able to grow in media containing relatively high concentrations of cholinium alkanoates (Table 2), and yet showed very distinct behaviours, *e.g.* MIC values varied from 2.5 mM to 1.5 M (Figure 2). The fungi tolerance ranking defined here is consistent with that previously reported,[19] further highlighting the robustness and the predictive value of the testing system, indifferent to the chemical nature of the salt.

The selection of tested compounds enabled us to investigate the specific influence of the anion on the overall toxicity of the ionic liquid. It becomes apparent that the anion toxicity is defined by the length of the linear chain and can be ranked as follows:

ethanoate < propanoate < butanoate < pentanoate < hexanoate < octanoate < d ecanoate

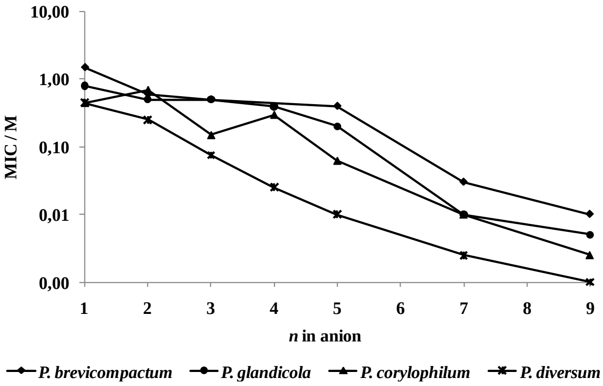


Figure 2. Minimal inhibitory concentrations (MIC) of the cholinium alkanoates ($[N_{1\,1\,1\,2OH}][C_nH_{2n+1}CO_2]$, n = 1-9 linear chain length): comparison of MIC values of cholinium alkanoates in four fungal strains, *Penicillium brevicompactum*, *P. glandicola*, *P. corylophilum* and *P. diversum*. MIC values were plotted in logarithmic scale.

Table 2. Minimal inhibitory and fungicidal concentrations (MIC and MFC, respectively) of cholinium alkanoates (mM) in four fungal strains: *Penicillium brevicompactum (P. b.), P. glandicola (P. g.), P. corylophilum (P. c.)* and *P. diversum (P. d.*). Results obtained for control compounds (sodium salts of corresponding carboxylic acids, cholinium chloride, SDS, BAC, ethanol and propanone) are also presented.

Fungal strains Test compounds	*P. b.*		*P. g.*		*P. c.*		*P. d.*	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
cholinium ethanoate	>1500	>2000	800	900	450	500	450	500
cholinium propanoate	600	600	500	700	700	700	250	375
cholinium butanoate	500	1000	500	750	150	150	75	75
cholinium 2-methylpropanoate	500	1500	600	750	250	250	100	200
cholinium pentanoate	n.d.	n.d.	400	600	300	300	25	50
cholinium 2,2-dimethylpropanoate	750	1500	400	700	750	1500	75	150
cholinium hexanoate	400	500	200	200	62.5	70	10	25
cholinium octanoate	30	30	10	20	10	30	2.5	5
cholinium decanoate	10	10	5	7.5	2.5	5	1	2.5
sodium ethanoate	1250	1500	750	750	400	500	200	500
sodium propanoate	1000	1250	600	750	300	400	75	150
sodium butanoate	250	>1000	400	600	400	500	50	75
sodium 2-methylpropanoate	750	>1000	400	600	400	400	75	150
sodium pentanoate	n.d.	n.d.	n.d.	n.d.	100	100	30	50
sodium 2,2-dimethylpropanoate	500	1000	250	400	250	250	50	75
sodium hexanoate	100	150	50	50	50	50	10	25
sodium octanoate	20	25	7.5	20	20	30	2.5	5
sodium decanoate	15	20	1	5	10	15	5	5
cholinium chloride	>2000	>2000	1250	>2000	1250	>2000	800	>2000
SDS	<1	<1	<0.1	<0.1	<0.1	125	<0.1	<0.1
BAC	<2.5	<2.5	<0.25	<0.25	2.5	5	<1	<1
ethanol	1000	1000	n.d.	n.d.	2000	2000	500	1000
propanone	>4000	>4000	n.d.	n.d.	>4000	>4000	>4000	>4000

n.d. not determined. MIC and MFC concentrations (M) were reproducible in the four replicates. Data consistency was further confirmed re-testing, randomly, ~20 % of the fungal cultures. The concentrations ranged from 2.5 mM to 2 M and were distributed stepwise increasing from 0.5 mM to 0.1 M.

A similar effect was reported for the cation, where high correlation between toxicity and the side chain in the cation has been observed.[24] The dodecanoate anion was apparently slightly less toxic than decanoate (data not shown), but was not considered in the present study because it has induced the formation of a precipitate.

MIC and MFC values followed the same trend (Table 2). Fungal ionic liquid susceptibility *versus* the length of the linear chain in the anion is depicted in Figure 3 for *P. brevicompactum* and *P. diversum* (the most and least tolerant fungal strains, respectively).

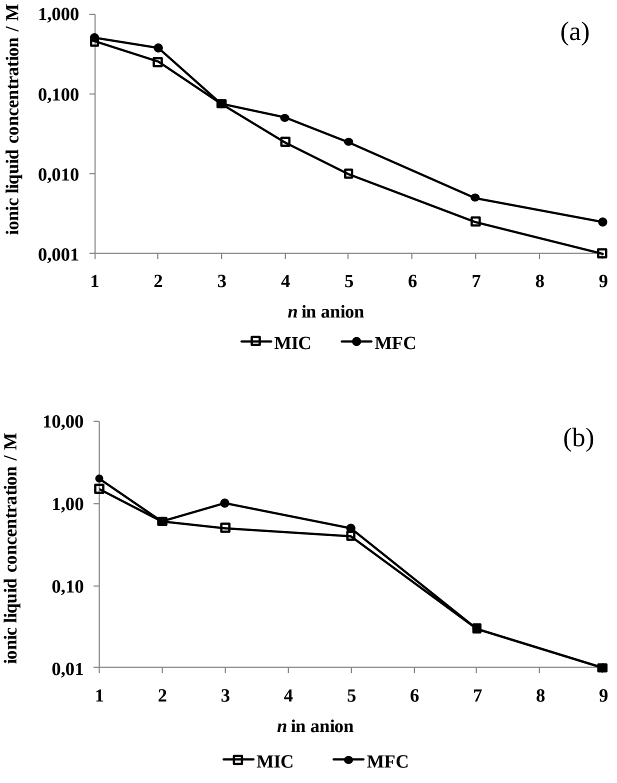


Figure 3. Minimal inhibitory and fungicidal concentrations (MIC and MFC, respectively) of the cholinium alkanoates $[N_{1\,1\,1\,2OH}][C_nH_{2n+1}CO_2]$, n = 1-9 linear chain length): comparison of MIC and MFC values of cholinium alkanoates for the least (a) and the most (b) tolerant fungal strains, *P. diversum* and *P. brevicompactum*, respectively. MIC and MFC values were plotted in logarithmic scale.

In more than 40 % of the cases, the reported MIC and MFC values were very similar, suggesting that growth inhibition and the conidia viability are somehow correlated events.

2-Methylpropanoate and 2,2-dimethylpropanoate are branched isomers of butanoate and pentanoate, respectively. They exhibited, relative to the linear isomers, lower inhibitory and fungicidal effects, especially evident in the latter isomer (Table 2). Although MIC and MFC values in the non-linear isomers were in good agreement for the same strains, no trend could be defined. Likewise, no correlation can be observed between their toxicities and that of propanoate (equal linear chain length).

The toxicity of cholinium alkanoates, as a function of the anion, may be partially understood by QSAR analyses using different molecular descriptors, such as the 1-octanol/water partition coefficient, $\log_{10}(K_{ow})$.[25] In studies with ionic liquids which are not hydrophobic, $\log_{10}(K_{ow})$ can be measured from thermodynamic data[26] or indirectly determined by chromatographic methods if the anion is a constant (the anion governs miscibility with the HPLC mobile phase).[27] Most studies have focussed on the cation, leaving the effect of the anion neglected. Nevertheless, a correlation between toxicity and lipophilicity of the cation or the anion has been observed elsewhere.[28] In the present study, the $\log_{10}(K_{ow})$ values of the isolated anions were predicted using algorithms[29] available on the ChemSpider website, www.chemspider.com. These values established a relative scale of lipophilicity for the anions studied, increasing linearly, from -0.285 ± 0.184 to 3.965 ± 0.185, with the increase of the number of carbon atoms in the linear anions. The branched isomers constitute an outlier group, reporting, relative to their linear isomers, slightly lower $\log_{10}(K_{ow})$ values. The lipophilic trend shown by this set of data describes perfectly the observation that toxicity increases with the elongation of the linear chain in the anion. In addition, branching, which generally reduces lipophilicity,[30] resulted in lower toxicity. This interpretation thus constitutes a reasonable view of the ionic liquids' toxicity, but experimental standardised methods, and probably other molecular descriptors, are necessary for better understanding the specificity of their toxicity mechanisms.

The cationic and anionic surfactant controls, SDS and BAC, and the common organic solvents, ethanol and propanone, were either extremely more toxic or less toxic, respectively (Table 2). The sodium salts used as controls showed the same trend, but were slightly more toxic than the ionic liquids, reiterating the biocompatible nature of cholinium in this test system (Table 2). Fungal susceptibility to the ionic liquids and the salt controls *versus* the length of the linear chain in the anion is depicted in Figure 4 for *P. glandicola*. In comparison, cholinium chloride, which measured individually the cation contribution, showed the lowest ionic liquid toxicity. This reinforces the statement that ionic liquid toxicity is a very complex equation that cannot be explained exclusively by the properties of the individual counter ions.

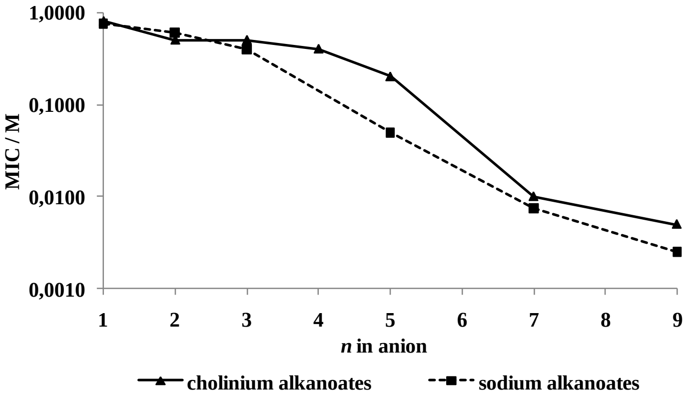


Figure 4. Comparison of the toxic effects (minimal inhibitory concentration, MIC) of the tested cholinium alkanoates $[N_{1\,1\,1\,2OH}][C_nH_{2n+1}CO_2]$, n = 1-9 linear chain length) and the sodium alkanoates in *Penicillium glandicola.* MIC values were plotted in logarithmic scale.

The biodegradation of ionic liquids in *P. corylophilum* cultures, after four weeks of incubation, was monitored by NMR spectroscopy (Table 3). The peaks attributed to the cholinium cation were conserved in the spectral analyses, thus suggesting that it was only partially degraded (Figure 5).

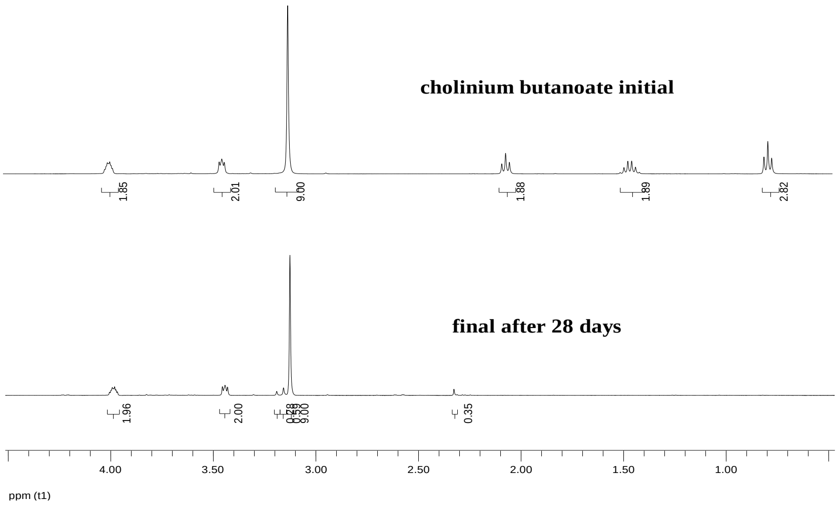
Under aerobic conditions, choline has been previously reported to undergo almost complete biodegradation (93 %).[31] The anions demonstrated distinct biodegradability potentials, which were confirmed by liquid chromatography (data not shown).

Table 3. Biodegradation assessment of ionic liquids using fungal strain *Penicillium corylophilum*. Concentrations of tested compounds used in this assay were below the previously determined minimal inhibitory concentrations (MIC) of ionic liquids.

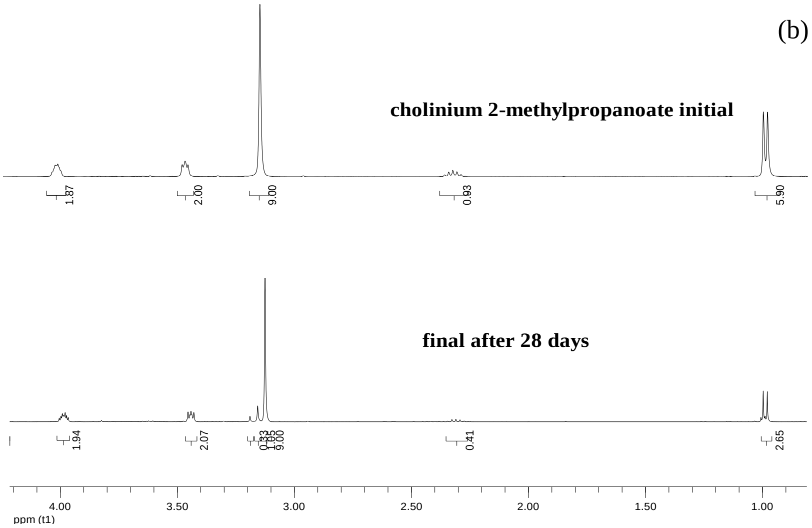
ionic liquid	MIC / M	tested conc / M	anion degradation (^{1}H NMR spectral analyses)
cholinium ethanoate	0.45	0.375	not detected
cholinium propanoate	0.7	0.5	not detected
cholinium butanoate	0.15	0.1	complete degradation
cholinium 2-methylpropanoate	0.25	0.1	partial degradation
cholinium pentanoate	0.3	0.1	complete degradation
cholinium 2,2-dimethylpropanoate	0.75	0.5	not detected
cholinium hexanoate	0.0625	0.05	complete degradation
cholinium octanoate	0.01	0.01	complete degradation

Very efficient degradation was observed in the longer linear chain anions, butanoate, pentanoate, hexanoate and octanoate, shown by the disappearance of the anion peaks in the spectra. In contrast, the short linear chain anions (ethanoate and propanoate) were not fully degraded, most probably due to their high concentration in media (0.375 and 0.5 M). This was supported by the chromatographic analyses of *P. corylophilum* static cultures (toxicity tests). The anion biodegradability was observed to be highly concentration dependent, *e.g.* ethanoate, 0.125 and 0.25 M, was degraded after two weeks up to 52 % and 16 %, respectively. The branched chain anions, 2,2-dimethylpropanoate and 2-methylpropanoate, both less toxic than their corresponding linear isomers, were more resistant to fungal attack, leading to null or partial degradation. Chain branching is generally considered to increase resistance to aerobic biodegradation.[31]

(a)



(b)



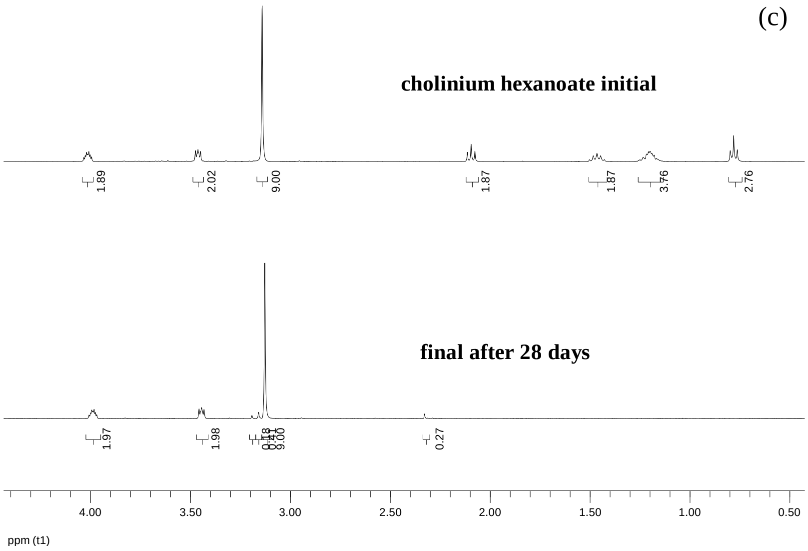


Figure 5. Biodegradability assessment using *Penicillium corylophilum*. ^{1}H NMR spectra at the beginning and the end of the incubation period (28 days) for (a) cholinium butanoate (0.1 M), (b) cholinium 2-methylpropanoate (0.1 M), and (c) cholinium hexanoate (0.05 M).

The distinctive biodegradability potential of isomers is well proven while comparing the biodegradation levels of butanoate and 2-methylpropanoate in media containing the same ionic liquid concentration (0.1 M) (Figure 5 a, b). In the static cultures (toxicity tests), only partial biodegradation of the ionic liquids was detected (data not shown). This suggests that the cultivation conditions (*e.g.* agitation, dioxygen availability and incubation time) play a critical role, influencing biodegradability.

Conclusions

The benign cholinium cation, combined with a range of alkanoate anions, allowed the synthesis of a novel set of highly promising biocompatible ionic liquids, $[N_{1\,1\,1\,2OH}][C_nH_{2n+1}CO_2]$ ($n = 1 - 9$). Their toxicity evaluation, using filamentous fungi as model eukaryotic organisms, demonstrated that fungi can actively grow in media containing, in some cases, concentrations up to molar range. The cholinium alkanoates were less toxic than their corresponding sodium salts. It becomes apparent that the anion toxicity is defined by its lipophilicity, as a consequence of the length of the linear chain and its branching. The cation was not completely mineralised by the fungi, and the isomers demonstrated also distinct biodegradability potentials, with branched isomers being more resistant to *P. corylophilum* attack. The methodology used for toxicity assessment has proven to be a rather robust one. Nevertheless, advanced studies on the ionic liquids' interaction with the conidial coats and other cellular boundary structures are necessary to further predict their environmental risks. Cholinium alkanoates are not only environmentally benign and biodegradable, but also extraordinarily good solvents for some very recalcitrant plant biocomposites, in some cases reaching dissolution yields of 60 wt.%.[22] There are no doubts that this family of ionic liquids, which promises high value for future biotechnological applications, will be harnessed further in future.

Acknowledgments

M. P. has performed most of the experiments, including biological assays and organic synthesis. Some of the chemical characterisation tests were done in collaboration with QUILL staff members. She analysed the data and prepared the draft of the manuscript.

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Chapter IV

Unravelling the mechanism of toxicity of alkyltributylphosphonium chlorides in *Aspergillus nidulans* conidia

This chapter is the reprint of the manuscript:
Unravelling the mechanism of toxicity of alkyltributylphosphonium chlorides in Aspergillus nidulans *conidia*
Marija Petkovic, Diego O. Hartmann, Gabriela Adamová, Kenneth R. Seddon, Luís Paulo N. Rebelo and Cristina Silva Pereira, New Journal of Chemistry, DOI: 10.1039/c1nj20470j.

Unravelling the mechanism of toxicity of alkyltributylphosphonium chlorides in *Aspergillus nidulans* conidia

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Abstract

The mechanism of toxicity of alkyltributylphosphonium chlorides $[P_{4\,4\,4\,n}]Cl$ (n = 1, 3-8, 10, 12 or 14) in conidia of the filamentous fungus *Aspergillus nidulans* is reported. Systematic elongation of one of the alkyl substituents resulted generally in higher toxicity, as defined by their inhibitory and lethal effects. In this study, fluorescence microscopy is proposed as a direct method for assessing the impact of ionic liquids on the plasma membrane integrity. Data was complemented by microscopic evaluation of the conidia cell wall and morphology. The higher toxicity of phosphonium ionic liquids carrying long alkyl substituents is most likely due to their strong interaction with the conidia cellular boundaries.

Introduction

The potential of ionic liquids has been thoroughly explored over the last two decades. From estimated millions of possible formulations,[1] several hundred ionic liquids are already well known and characterised.[2] Ionic liquids are generally defined as salts that are liquid below 100 °C,[1] and some are regarded as green solvents due to their excellent solvation capacity, negligible vapour pressure, and bulk non-flammability.[3] Their inherent potential comes also from their structural diversity and tuneable physical and chemical properties. However, ionic liquids comprise a very heterogeneous group of fluids that are not intrinsically green: some are toxic and non-biodegradable, as seen in recent reviews on their biodegradability and environmental impact.[4,5]

A considerable amount of data is now available on the chemistry[1] and physical properties[2] of ionic liquids, and numerous applications have been already proposed.[6] Imidazolium ionic liquids are still at the heart of most of these studies, but the focus of interest is moving towards other cationic groups. These include quaternary phosphonium ionic liquids, some of which are currently produced in tonne quantities, particularly by Cytec Industries Inc.[7] They are generally considered thermally and chemically more stable (the latter due to the absence of an acidic proton) than the quaternary ammonium or imidazolium salts.[8,9] Their properties, including biological activity, have inspired numerous patents for a broad range of applications, *e.g.* intermediates in the chemical synthesis of terpenes,[10] antistatic agents,[11] biocides, either alone[12] or combined with other compounds,[13,14] plant growth regulators[15] and anti-cancer agents.[16]

Amongst the quaternary phosphonium ionic liquids, the tetraalkylphosphonium ones are, to present, the most studied. They have been already investigated for diverse applications, *e.g.* solvents in separation processes[17,18] and phenol bioremediation,[19] media for chemical reactions,[20,21] lubricants,[22] and antimicrobial[23] and anti-cancer agents.[24] In the search for benign counterions, tetraalkylphosphonium cations were combined with various amino acids[25,26] or acesulfamate.[27]

Up to now, there are only few studies regarding the toxicity of phosphonium ionic liquids. Their apparent high toxicity was observed in various aquatic organisms, *i.e*. *Vibrio fischeri* and *Daphnia magna*,[28,29] and *Pseudokirchneriella subcapitata*.[29,30] To the best of our knowledge, the only systematic study on tetraalkylphosphonium ionic liquids was performed with $[P_{6\,6\,6\,n}]Cl$ ($n = 6$ to 16).[31] Toxicity against several bacterial and yeast strains was shown to generally increase with the elongation of the alkyl substituent. This toxicity trend has also been observed for different ionic liquids cations,[32,33] and correlates well with the cation lipophilicity.[34]

In this work, we present a toxicity and biodegradability assessment of $[P_{4\,4\,4\,n}]Cl$, where $n = 1$, 3-8, 10, 12 or 14, towards *Aspergillus nidulans*. Their inhibitory and lethal effects against fungal conidia were, as expected, determined by the length of the alkyl substituent in the cation. To better understand their mechanism of toxicity, we have also observed their effect on the integrity of conidia boundaries and morphology. Fluorescence microscopy has been used previously to illustrate the effect of a single imidazolium-based ionic liquid on eukaryotic cells (HeLa cells).[35] However, this is the first systematic study where the interaction of ionic liquids with cellular boundaries is being accounted for with fluorescence microscopy. This technique allowed a direct measure of their effect, strongly suggesting that narcosis[36-38] is the basis of their toxic action.

Experimental

Chemicals

All compounds used in the preparation of minimal media and in plasma membrane and cell wall integrity assays, with the exception of NaCl (Panreac, 99.5%), were purchased from Sigma Aldrich: D(+)-glucose, K_2HPO_4, $ZnSO_4{\cdot}7H_2O$, $CuSO_4{\cdot}5H_2O$, $FeSO_4{\cdot}7H_2O$, $MgSO_4{\cdot}7H_2O$, $NaNO_3$ and KCl;

and dimethylsulfoxide (dmso; 99.5%), propidium iodide (≥ 94%, stock solution 20 mM in dmso), Calcofluor White M2R (Fluorescent Brightener 28, stock solution 5 mM in water) and glycerol (≥ 99.5%), respectively. Deuteriated propanone used in the biodegradability assessment was purchased from EURISO-TOP (France).

Ionic liquids

All ionic liquids used in this study were prepared by QUILL (Queen´s University Ionic Liquids Laboratory, Belfast, UK), except for $[P_{4\,4\,4\,4}]Cl$ and $[P_{4\,4\,4\,14}]Cl$ which were supplied by Cytec Industries, Canada. The comprehensive study on synthesis and physicochemical properties of these ionic liquids will be published elsewhere.[39] $[P_{4\,4\,4\,n}]Cl$, where $n = 3$, 5-8, 10 or 12, were prepared by nucleophilic (S_N2) addition of tributylphosphine to the respective 1-chloroalkane. $[P_{4\,4\,4\,1}]Cl$ was synthesised by a neutralisation reaction of methanolic tributylmethylphosphonium methylcarbonate with concentrated aqueous hydrochloric acid. Ionic liquids were characterised by ^{1}H, ^{13}C and ^{31}P NMR spectroscopy, mass spectrometry, CHN elemental analysis, and halide and water content analyses.

Fungal strain

Aspergillus nidulans strain FGSC A4 was cultivated on DG18 agar, for one week at 27 °C. Fungal conidia were harvested using 0.1 % v/v Tween 20 (polyoxyethylene (20) sorbitan monolaurate) and filtered through glass wool. The suspension obtained was centrifuged (18500 *g*, 4 °C) and washed three times with 0.85 wt.% NaCl. The fungal conidia pellet was resuspended in 10 % v/v glycerol and stored at -80 °C.

Toxicity tests

The toxicity of ionic liquids to *A. nidulans* was evaluated by determining their minimal inhibitory (MIC) and fungicidal concentrations (MFC), distinguishing between growth inhibition and death, respectively.

The minimal culture medium containing glucose (1.0 g l^{-1}) and $K_2[HPO_4]$ (1.0 g l^{-1}) was dissolved in distilled water, sterilised in an autoclave (20 min; 121 °C), and supplemented with the mixture of essential salts, previously sterilised by filtration: $NaNO_3$ (3.0 g l^{-1}), $ZnSO_4\cdot7H_2O$, (0.01 g l^{-1}), $CuSO_4\cdot5H_2$ O(0.005 g l^{-1}), $MgSO_4\cdot7H_2O$ (0.5 g l^{-1}), $FeSO_4\cdot7H_2O$ (0.01 g l^{-1}) and KCl (0.5 g l^{-1}). The ionic liquids were added to the minimal culture media with final concentrations from 10 μM to 95 mM (distributed stepwise from 1.25 μM to 2.5 mM). Each liquid medium (1 cm^3) was inoculated with a suspension of fungal conidia, in order to obtain the final concentration of 10^5 conidia per cm^3, and divided into four wells (0.25 cm^3 each) of a 96-well microtitre plate. Cultures were incubated in the dark, at 27 °C, for seven days. Fungal growth (or lack thereof) was evaluated at the end of incubation, gauging by eye the formation of mycelium (turbidity) and/or conidia. The lowest concentration that inhibited growth was taken as the MIC. Additionally, all the samples where no active growth was detected were used as inocula and spread, with a 1 µl loop, onto malt extract agar medium (Oxoid). The plates were incubated in the dark, at 27 °C, for seven days. The lowest concentration of the testing compound which resulted in unviable conidia was taken as the MFC. MIC and MFC values should not be interpreted as absolute ones, but rather as an indication of the inhibitory and the fungicidal upper concentration limits.

Membrane and cell wall integrity assays

Four ionic liquids were chosen for the plasma membrane and cell wall integrity assays, namely $[P_{4\,4\,4\,n}]Cl$, where $n = 1$, 4, 8 or 12. The selected testing concentrations (0.01, 0.1, 1, 10 and 100 mM) range below and above the previously obtained MIC and MFC values in order to facilitate the comparison of ionic liquids effects. Sodium chloride solutions (0.05, 0.5, 1 and 2 M) were included as osmotic stress control (membrane integrity assays).

Aspergillus nidulans was cultivated on DG18 agar, at 27 °C, in the dark, for 5-6 days prior to harvest. Conidia were harvested with a saline solution (0.85% w/v NaCl) and used immediately. A suspension of 10^6 conidia per cm^3 of the testing ionic liquid solution was incubated for one hour at 27 °C under agitation (90 rpm). The incubation time for cell wall integrity assay was prolonged to two and four hours, since within the first hour no significant alterations were detected for $[P_{4\,4\,4\,1}]Cl$ and $[P_{4\,4\,4\,4}]Cl$. In both assays, conidia suspension was centrifuged (15 000 rpm, 4 °C) and washed three times with a saline solution in order to remove the ionic liquid.

For the membrane integrity assay, conidia were incubated afterwards with propidium iodide (PI) (λ_{ex} = 538 nm, λ_{em} = 617 nm, red), at the final concentration of 20 μM, for 15 min, at 27 °C in the dark and under agitation. Residual dye was removed by centrifugation (15 000 rpm, 4 °C) and washing (3×) and conidia were resuspended in 100 μl of saline solution with 10% v/v glycerol. Slides were mounted with 10 μl of the obtained suspension, in triplicates (technical replicates). Conidia were observed with an Axio Imager.M1 fluorescence microscope (Zeiss) using a 15 AlexaFluor 546 filter set (PI-stained conidia) and differential interference contrast (DIC, total number of conidia). The objective was an EC Plan-Neofluar with 40× magnification and images were captured with an ORCA-ER digital camera (Hamamatsu). Three fields of view from each slide were chosen randomly. Counting of conidia was carried out manually in JMicroVision v1.27. The percentage of membrane-damaged cells was obtained as (number of PI-stained conidia / total number of conidia) × 100. The experiment was repeated three times. When membrane damage was higher than 50%, the assay was repeated with shorter exposure periods (15, 30 and 45 minutes).

In the cell wall integrity assay, conidia were stained with Calcofluor White (CFW) (λ_{ex} = 346 nm, λ_{em} = 460 nm, blue), at the final concentration of 25 μM, incubated for 30 min at 27 °C, in the dark, under agitation and further processed as described above. In this assay, a 49 DAPI filter set (CFW-staining) and DIC were used with a Plan-Apochromat 63× oil immersion objective. Three fields of view, chosen randomly from each slide, were imaged. The experiment was repeated twice. This assay provided only qualitative analysis of the alterations of the cell wall.

The water activity (a_w) of some testing solutions was determined with a portable water activity indicator (HydroPalm AW1) following the manufacturer's instructions at 27ºC.

Scanning Electron Microscopy

Freshly harvested conidia of *A. nidulans* (as described in the previous subsection) were incubated with a 100 mM solution of $[P_{4\,4\,4\,n}]Cl$, where $n = 1$, 8 or 12, at a final concentration of 10^6 conidia per cm^3. After two hours at 27 °C under agitation, conidia were centrifuged and washed (4×) with saline solution in order to remove the ionic liquid. Water was removed from the samples by lyophilisation and coated with a 15 nm layer of Au/Pd using a sputter coater (ex-Polaron E5100). Electron micrographs were recorded using an analytical field emission gun-scanning electron microscope (FEG-SEM: JEOL JSM7001F with Oxford light elements EDS detector) operated at 2 kV. The selected micrographs, from the biological triplicates, are regarded as representative ones of the overall sample (5000×) and individual conidia (15000×).

Biodegradability assessment

Concentrations of ionic liquids used in the biodegradability assay were approximately one half of the previously determined MIC values. Fungal cultures of 20 cm^3 were inoculated as described in the subsection Toxicity tests, and incubated in the dark at 27 °C under agitation (90 rpm) for 21 days. The culture extracts were filtered (glass fibre prefilters) and lyophilised to remove water. Solid remains were dissolved in 1 cm^3 of deuteriated propanone, filtered and analysed by ^1H and ^{31}P NMR spectroscopy.

Results and discussion

In the present work, the toxicological assessment of a series of quaternary phosphonium ionic liquids, namely $[P_{4\,4\,4\,n}]Cl$, where $n = 1$, 3-8, 10, 12, or 14 (Figure 1), was performed. While the $[P_{6\,6\,6\,n}]^+$ series of ionic liquids,[31] and some based on the $[P_{4\,4\,4\,n}]^+$ cation,[40] have already been investigated for their ecotoxicity, this is the first systematic study on $[P_{4\,4\,4\,n}]Cl$.

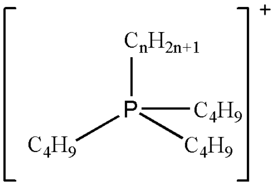

Figure 1. The structure of the alkyltributylphosphonium cation, $[P_{4\,4\,4\,n}]^+$, where $n = 1$, 3 - 8, 10, 12 or 14.

Filamentous fungi of the *Penicillium* genus show high tolerance and biodegradation ability towards different groups of ionic liquids.[41,42] *Aspergillus nidulans* (whose genome is fully sequenced), due to its high phylogenetic correlation with *Penicillium* spp.,[43] was therefore selected for this study.

The toxic effect of the $[P_{4\,4\,4\,n}]Cl$ ($n = 1$, 3-8, 10, 12 or 14) was defined solely by the cation structure, and for $n \geq 4$ increased, almost exponentially, with the elongation of the alkyl chain substituent (Table 1 and Figure 2A). MIC and MFC values were distributed over a broad range (Table 1), *e.g.* MICs of $[P_{4\,4\,4\,4}]Cl$ and $[P_{4\,4\,4\,14}]Cl$ were 37.6 and 0.011 mM, respectively.

The toxicity of $[P_{4\,4\,4\,n}]Cl$ ($n = 4$-8, 10, 12 or 14) as a function of the cation might be explainable, despite its limitations, by the 1-octanol/water partition coefficient, $\log_{10}(k_0)$. For $[P_{4\,4\,4\,4}]^+$ and $[P_{4\,4\,4\,14}]^+$, $\log_{10}(k_o)$ values were 2.5 and 6.9, respectively.[34] They suggest a relative scale of hydrophobicity for $[P_{4\,4\,4\,n}]^+$ when $n \geq 4$, which is expected to increase linearly with the increase of the number of carbon atoms in the alkyl substituent.

Table 1. Numerical values of minimal inhibitory and fungicidal concentrations (MIC and MFC, respectively) of the alkyltributylphosphonium chlorides, $[P_{4\,4\,4\,n}]Cl$, defined for *Aspergillus nidulans*.

n	MIC / mM	MFC / mM
1	32.5	92.5
3	77.6	87.6
4	37.6	85.23
5	7.53	17.56
6	4.52	7.03
7	1.91	2.41
8	0.8	0.9
10	0.24	0.24
12	0.021	0.021
14	0.011	0.011

While considering the MFC values, $[P_{4\,4\,4\,n}]Cl$, where $n = 1$, 3 or 4, reported similar lethal effects. Their inhibitory effects showed that $[P_{4\,4\,4\,3}]Cl$ was an exception, since the MIC value was higher than expected, when compared with $[P_{4\,4\,4\,1}]Cl$ and $[P_{4\,4\,4\,4}]Cl$. No clear explanation exists for this observation. It might be hypothesised that the longer alkyl substituents will lead to toxicity, probably by interaction with the cellular boundaries. Therefore, for $[P_{4\,4\,4\,n}]Cl$, where $n = 1$, 3 or 4, the effect will be reasonably similar, *i.e.* controlled by the three butyl substituents.

The toxicity trend observed herein has been reported before in numerous studies focussing on other common groups of ionic liquids, such as the imidazolium and ammonium derivatives.[32,33] This trend, as aforementioned, is usually correlated with increased lipophilicity of the ionic liquids.[34] Through interaction with biological membranes, chemicals may cause a loss of membrane integrity, leakage of intracellular material and, ultimately, cell death. Apart from studies on artificial models of the cell membrane (*i.e.* supported phospholipid bilayers[44] or liposomes[45]), to the best of our knowledge, hitherto no direct evidence of this effect has been reported in the ionic liquid literature. Infrared spectroscopy has been used as an attempt to directly detect the fraction of ionic liquid bound to the bacterial cells.[46] When applied to whole cells the bioaccumulated and the bioadsorbed fractions are, however, equally accounted for. Interestingly, using a similar method it was possible to detect compositional alterations in the cell wall of diatoms after exposure to $[C_4mim]Cl$.[47]

In this study, we propose a rapid method to detect the effects of ionic liquids in cellular boundaries, instead of determining their fate *in loco*. Alterations of the plasma membrane integrity are commonly identified by microscopy or detection of intracellular components leakage.[48] While considering the former, fluorescence microscopy is a reliable technique for observation of cellular structures of filamentous fungi.[49]

Propidium iodide (PI) and Calcofluor White (CFW) fluorescent probes were successfully used in fungi for a rapid assessment of viability and membrane integrity[50] and cell wall detection,[51,52] respectively. PI can only enter membrane-damaged cells where it binds to nucleic acids (detected as red fluorescence).

Aspergillus nidulans conidia were exposed to distinct concentrations of $[P_{4\,4\,4\,n}]Cl$ ($n = 1$, 4, 8 or 12) for one hour and stained with PI. The concentrations selected were 0.01, 0.1, 1, 10 and 100 mM, covering values below and above the MFC for all tested ionic liquids. For each testing condition a percentage of cells with membrane damage was determined. The saline solution control (0.85% w/v NaCl) showed approximately 5% of conidia with membrane damage, considered as a basal level of injured cells or a consequence of the harvesting procedure. The water activity (a_w) of the ionic liquid solutions was equal to that of distilled water ($a_w = 1$). In addition, after exposing conidia to sodium chloride solutions up to 2 M ($a_w \geq 0.937$), the level of membrane damage was similar to that observed in the saline solution control. Both observations, suggest that membrane damage observed after exposure to ionic liquids was not due to osmotic stress.

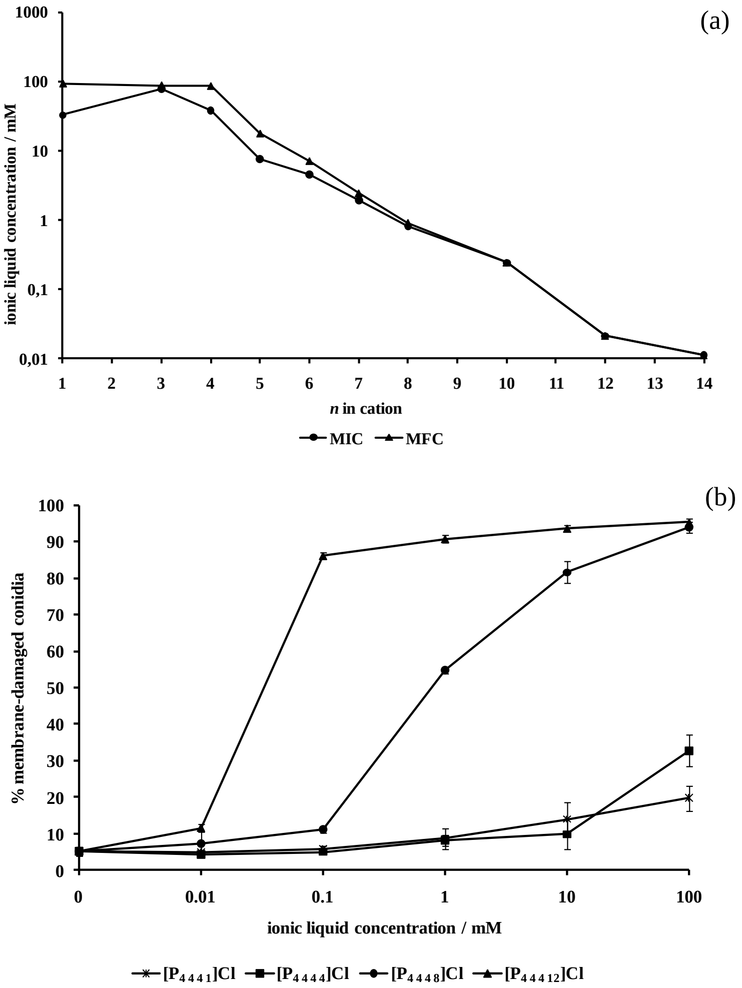

Figure 2. (A) Minimal inhibitory and fungicidal concentrations (MIC and MFC, respectively) of the alkyltributylphosphonium chlorides, $[P_{4\,4\,4\,n}]Cl$, where $n = 1, 3 - 8, 10, 12$ or 14, defined for *Aspergillus nidulans*. MIC and MFC values are plotted on a logarithmic scale. (B) Percentage of membrane-damaged conidia after one hour of incubation with alkyltributylphosphonium chlorides, $[P_{4\,4\,4\,n}]Cl$ ($n = 1$, 4, 8 or 12), obtained as (number of propidium iodide-stained conidia / total number of conidia) × 100.

Percentages of membrane-damaged conidia after exposure to concentrations below MFC values of [$P_{4\,4\,4\,1}$]Cl and [$P_{4\,4\,4\,4}$]Cl were, with some exceptions, reasonably similar to the control (Table 2, Figure 2B). Obviously, the increase in concentration, still below the MFC, was followed by a slight increase in the number of membrane-damaged cells. The same trend was observed for [$P_{4\,4\,4\,8}$]Cl and [$P_{4\,4\,4\,12}$]Cl (~ 4 to 14%).

Table 2. Percentage of membrane-damaged conidia after one hour of incubation with alkyltributylphosphonium chlorides, [$P_{4\,4\,4\,n}$]Cl ($n = 1$, 4, 8 or 12), obtained as (number of propidium iodide-stained conidia / total number of conidia) × 100. Percentage of membrane-damaged conidia in the saline solution control was 5.11 %. Emboldened values were obtained with concentrations above the MFC. The standard deviation of biological triplicates is shown in parenthesis.

n	Ionic liquid concentration / mM				
	0.01	0.1	1	10	100
1	4.9 (0.7)	5.7 (1.0)	8.7 (2.9)	14.0(4.5)	**19.7 (3.4)**
4	4.3 (0.4)	5.0 (0.4)	8.2 (1.5)	9.8 (4.2)	**32.7 (4.3)**
8	7.3 (3.2)	11.1 (1.0)	**54.8 (0.9)**	**81.6 (3.0)**	**93.9 (1.5)**
12	11.6 (2.4)	**86.2 (4.2)**	**90.8 (4.6)**	**93.6 (2.3)**	**95.3 (0.2)**

While testing ionic liquids concentrations above the MFC value (*i.e.* leading to cell death), the [$P_{4\,4\,4\,n}$]Cl with shorter ($n = 1$ or 4) and longer ($n = 8$ or 12) alkyl substituents induced moderate and severe membrane damage, respectively. [$P_{4\,4\,4\,8}$]Cl and [$P_{4\,4\,4\,12}$]Cl at the lowest ionic liquid concentrations causing death, show membrane damage in 55% and 86% of the conidia, respectively. The values for [$P_{4\,4\,4\,1}$]Cl and [$P_{4\,4\,4\,4}$]Cl, however, did not exceed 20% and 33%, respectively. This is illustrated in Figure 3, where the total number of conidia in each field of view obtained at the DIC (A - E) can be compared with the number of conidia stained by PI (A´ - E´) in the control or in 100 mM solution of the ionic liquid. An extremely high degree of plasma membrane damage could be observed for [$P_{4\,4\,4\,n}$]Cl, where $n = 8$ or 12.

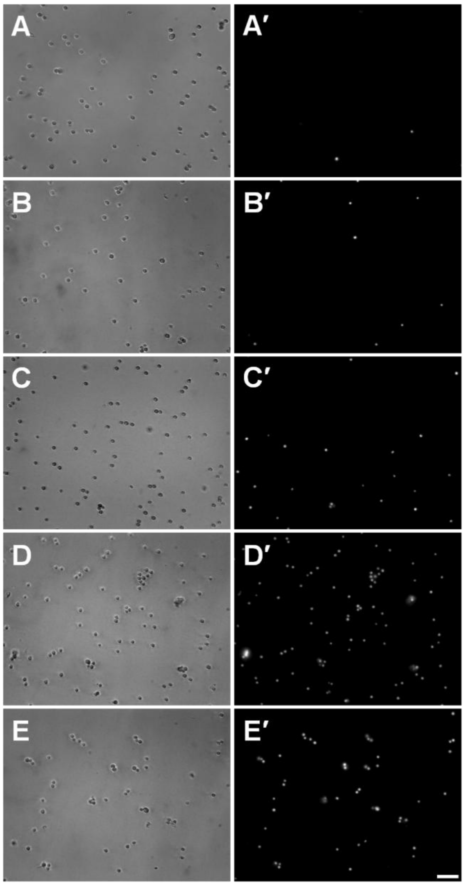

Figure 3. Membrane integrity assay. Conidia treated with 100 mM of alkyltributylphosphonium chlorides, [$P_{4\,4\,4\,n}$]Cl (n = 1, 4, 8 or 12), during one hour of incubation, and stained with propidium iodide, PI. Left column (A - E) shows totality of conidia in the differential interference contrast (DIC); right column shows PI-stained conidia (A´ - E´). Observed fluorescence indicates membrane-damaged conidia in: A, A´ saline solution control; B, B´ n = 1; C, C´ n = 4; D, D´ n = 8 and E, E´ n = 12. Scale bar (E'): 20 µm.

Disruption of membrane permeability, apparently ruling the toxicity of the alkyltributylphosphonium chlorides carrying the longer alkyl substituents (n > 4), is not the main mode of toxicity for the shorter ones. High percentages of membrane damage within a short exposure time suggest that toxicity is probably due to loss of plasma membrane integrity, as reported before for other compounds.[50] Permeabilisation of the plasma membrane might also be associated with the late stage of apoptosis (programmed cell death), however, even after two hours, apoptotic cells of *A. nidulans* show intact plasma membranes.[53] In our study, when the percentage of membrane-damaged conidia was higher than 50% ([$\mathrm{P}_{4\,4\,4\,n}$]Cl, where n = 8 or 12) the assay was repeated with shorter exposure periods (15, 30 and 45 minutes). The percentages of membrane-damaged conidia were similar, regardless of the exposure time (data not shown). The data makes apparent that the observed membrane damage is a direct effect of the ionic liquids. Severe membrane disruption also explains the similarity between the observed MIC and MFC values, indicating that inhibition of fungal growth is accomplished by cell death.

In order to better understand the interactions of these ionic liquids with the conidia boundaries, the cell wall was also investigated. It is a well-organised complex of glucans, mannoproteins and chitin (constituting ~20% of cellular biomass),[54] crucial for maintaining cell morphology and acting as a barrier against mechanical and environmental stress. CFW is a fluorescent probe that binds to chitin and glucans of the cell wall. When stained with CFW, conidia with intact cell walls show a homogenous distribution of fluorescence, when observed under the microscope (see Figure 4A). If the continuity of the conidia cell wall is disrupted, heterogeneous binding of CFW and irregular distribution of the fluorescence is expected. This fluorescent dye was successfully used to detect cell wall alterations provoked by chemical[52] or environmental stress.[55] *Aspergillus nidulans* conidia were treated with [$\mathrm{P}_{4\,4\,4\,n}$]Cl (n = 1, 4, 8 or 12) for one hour, at the concentrations used in the previously described membrane damage assessment.

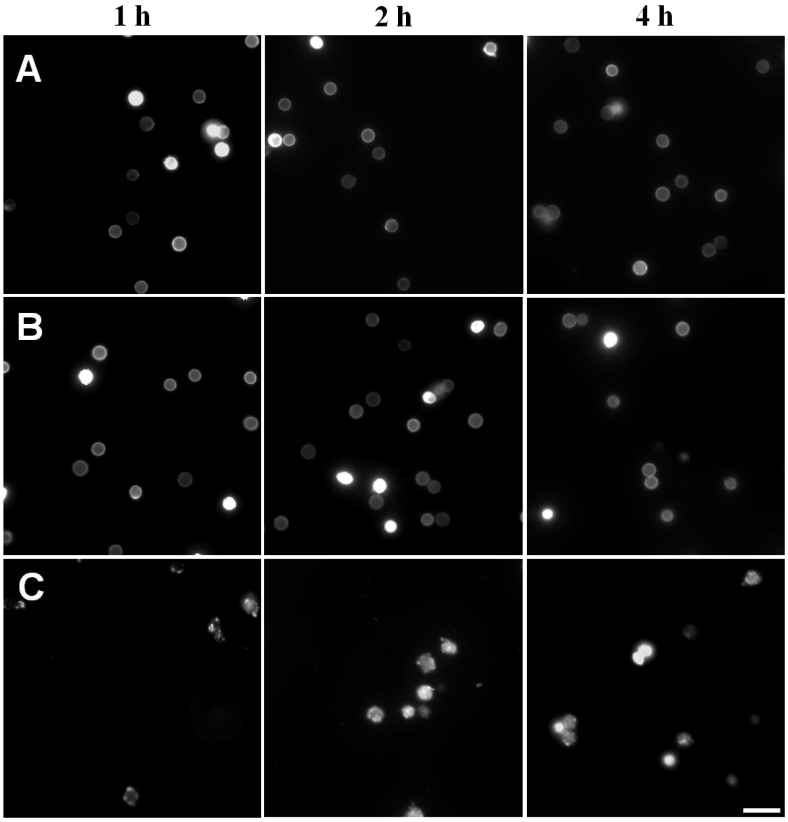

Figure 4. Cell wall damage assay. Conidia treated with 100 mM of alkyltributylphosphonium chlorides, $[P_{4\,4\,4\,n}]Cl$ ($n = 1$ or 12), during one, two or four hours of incubation and stained with Calcofluor White M2R (CFW). A: saline solution control; B: no obvious alteration of the cell wall in the presence of $[P_{4\,4\,4\,1}]Cl$ (even distribution of fluorescence); C: significant cell wall damage in the presence of $[P_{4\,4\,4\,12}]Cl$. Scale bar (C, 4 h): 10 µm.

$[P_{4\,4\,4\,n}]Cl$ with concentrations above the MFC, where $n = 8$ or 12, were leading to alterations in the fluorescence distribution on the conidia cell wall classified as moderate and strong, respectively. However, in the case of $n = 1$ or 4, no alterations were observed. The influence of exposure time, namely one, two or four hours, is exemplified for $[P_{4\,4\,4\,n}]Cl$ ($n = 1$ or 12) in Figures 4B and 4C. After two and four hours, concentrations above the MFC of all tested ionic liquids caused a greater effect in the fluorescence distribution compared with one hour exposure, with the exception of $[P_{4\,4\,4\,1}]Cl$.

Excluding the latter, concentrations above the MFC values appeared to cause cell wall damage. This suggests that, along with membrane damage, cell wall damage plays an important role in the toxicity of [$P_{4\,4\,4\,n}$]Cl. Sena *et al.* also suggested this, based on the observation that algae mutants lacking cell walls showed, relatively to the wild type, higher and similar susceptibility to tetrabutylammonium bromide and 1-butyl-3-methylimidazolium bromide, respectively.[56]

Fungal cell wall damage is also expected to cause alterations in the overall morphology of conidia. Some of these changes were noted in the DIC of cells treated with ionic liquids for two hours or more (data not shown) and confirmed by FEG-SEM. The *A. nidulans* conidia from control samples showed a typical spherical morphology, with a convoluted surface.[57] Amongst the selected ionic liquids for this analysis, only [$P_{4\,4\,4\,1}$]Cl treatment presented conidia with morphology similar to the control. [$P_{4\,4\,4\,8}$]Cl and [$P_{4\,4\,4\,12}$]Cl caused major alterations resulting in an irregular shape (Figure 5). The conidia of the control sample and treated with [$P_{4\,4\,4\,1}$]Cl had a characteristic flat-ball shape (Figures 5A and 5B) which was not observed in the case of [$P_{4\,4\,4\,8}$]Cl and [$P_{4\,4\,4\,12}$]Cl (Figures 5C and 5D). Conidia with intact membranes collapse during dehydration under high-vacuum conditions (sample preparation), due to a loss of turgor pressure.[58] Membrane damage, on the contrary, circumvents this effect.

Quaternary phosphonium ionic liquids were observed to be very resistant to microbial attack, *e.g.* tricyclohexylphosphine- and trihexylphosphine-derived cations.[59] The capacity of *A. nidulans* to biodegrade aerobically [$P_{4\,4\,4\,n}$]Cl ($n = 1$, 3-8, 10, 12 or 14) was also assessed. After 21 days, the extracellular extract was collected and analysed by ^{31}P and ^{1}H NMR spectroscopy. Under the testing conditions, no alterations of the spectral peaks of the cations could be observed (data not shown), strongly suggesting null or very weak degradation. Recently, rapid mineralisation of [$P_{4\,4\,4\,4}$]$^+$ and [$P_{4\,4\,4\,6}$]$^+$ by *Sphingomonas paucimobilis* bacterium was inferred by an indirect impedance test.[60] These data, despite the lack of direct proof of cation depletion from the cultivation media, should stimulate further exploration of the potential of phosphonium ionic liquids.

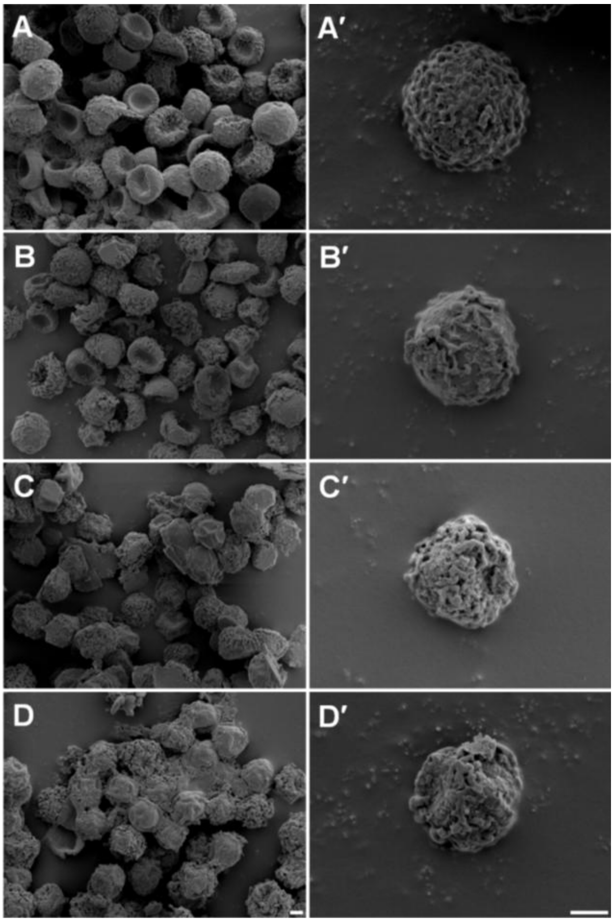

Figure 5. Scanning Electron Microscopy (SEM) analysis of conidia treated with 100 mM of alkyltributylphosphonium chlorides, $[P_{4\,4\,4\,n}]Cl$ ($n = 1$, 8 or 12), during two hours of incubation. Micrographs A - D present conidia at 5000× magnification, and A´ - D´ show individualised conidium at 15000×. A, A´: saline solution control; B, B´: $n = 1$; C, C´: $n = 8$; D, D´: $n = 12$. Scale bars (D, D´): 1 µm.

Conclusions

Toxicity of alkyltributylphosphonium chlorides $[P_{4\,4\,4\,n}]Cl$ ($n = 4$-8, 10, 12 or 14) towards *A. nidulans* conidia was observed to increase with the systematic elongation of one alkyl substituent. Analyses of conidia plasma membrane damage and cell wall integrity clearly suggest that the toxicity of [$P_{4\,4\,4\,n}$]Cl, where $n \geq 4$, is being ruled by direct interaction with both cellular structures. This behaviour may also explain the high similarity of their MIC and MFC values. For the shorter alkyl substituents ($n = 1$ or 4), despite provoking membrane damage in ~ 20% of the conidia, inhibition of growth and death are more separated events. [$P_{4\,4\,4\,1}$]Cl, even after four hours of exposure, had no perceived effects on the cell wall, suggesting that its ability to protect the membrane from damage depends on the ionic liquid structure.

The high potential of fluorescence microscopy to screen membrane damage provoked by other groups of ionic liquids has been demonstrated here. The selected probe is adequate for a broad range of organisms, such as bacteria[61] and mammalian cells.[62] Screening membrane damage at lethal concentrations of ionic liquids will rapidly inform if narcosis is ruling their toxicity. This robust toxicity screening method should stimulate ionic liquids research towards their conscious design.

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Chapter V

Preliminary evaluation of the biological potential of diffusible fungal metabolites induced by an ionic liquid

The study reported here forms part of ongoing work, and includes unpublished data (manuscript in preparation).

Preliminary evaluation of the biological potential of diffusible fungal metabolites induced by an ionic liquid

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Abstract

The ability of filamentous fungi to tolerate ionic liquids is remarkably high compared to that seen in other microorganisms. When exposed to sub-lethal concentrations of ionic liquids, they respond by altering their cellular biochemistry. This leads to an altered pattern of diffusible secondary metabolites. Here we present a preliminary evaluation of the biological activity of the diffusible fungal metabolome by testing on HeLa cells and bacteria. Further studies are necessary to profile the identity of divergent metabolites, and their pharmacological potential. Ionic liquids constitute a virtually unlimited source of stimuli for activating secondary metabolite biosynthetic pathways, raising the question whether they can be used for tailored alterations. This constitutes a fascinating advance toward biological applications for ionic liquids, and may pave the way to important biotechnological breakthroughs.

Introduction

Filamentous fungi fully deserve being referred to as "creative" microorganisms[1] or even "metabolic artists"[2] due to their remarkable potential to produce a variety of metabolites. Fungal secondary metabolites (fSM) are known and have been used as pharmaceuticals for decades. The best-known are the antibiotics (*e.g.* penicillin[3]), immunosuppressive agents (*e.g.* cyclosporin A[4] and mycophenolic acid[5]) and cholesterol-lowering agents (*e.g.* lovastatin[6]) produced by fungi belonging to the Ascomycota phylum. According to Pelãez *et al.* more than half the fSM described between 1993 and 2001 (1500 compounds) demonstrated antibacterial, anti-fungal or anti-tumour activity.[7] They constitute a largely unexplored source of valuable natural products and/or chemical functionalities that can be used in drug design.[8]

Fungal secondary metabolites are chemically diverse and complex low molecular weight molecules, generally not essential for growth of the producer fungi.[9] The biosynthesis of fSM strongly depends on inherited phylogenetic information (genomics), morphological stage, and environmental conditions.[10] Many compounds possess biological activity and are thought to be associated with intra- and inter-species communication, defence or inhibition of competitors.[11]

Major disadvantages in the finding of novel natural compounds, so far, has been associated with the need to accurately reproduce conditions which support growth or metabolic capacities found in nature.[12] Identification of numerous secondary metabolite biosynthetic and regulatory genes has opened new ways to circumvent these difficulties (Brakhage *et al.*[13] and references herein). Numerous silent gene clusters, associated with "cryptic" or "orphan" metabolites, can be activated, using cultivation-dependent approaches and/or molecular techniques.[13] Bode *et al.* suggested the 'OSMAC (One Strain-Many Compounds) approach where numerous variables can be tested to diversify the production of fSM, such as media composition, pH value, temperature, oxygen supply, light, incubation time, enzyme inhibitors and co-cultivation.[2] Recently the latter has been attracting significant attention.[14,15] Williams *et al*. identified novel fSM after exposing growing fungal cultures to chemical inhibitors of histone deacetylation and DNA methylation.[16] These reactions are associated with chromatin remodelling and are thought to control the expression of some fSM gene clusters.[13]

In this study, ionic liquids, salts containing various chemical functions, are used as a novel class of chemical stimuli to induce cryptic fSM production. *Penicillium brevicompactum* diffusible fSM produced during growth in media supplemented with selected ionic liquids were qualitatively profiled by liquid chromatography (LC). We further tested their cytotoxicity to human epithelial cervix carcinoma cell line HeLa and antibacterial activity against Gram-positive *Bacillus subtilis* and Gram-negative *Escherichia coli*. The potential for use of ionic liquids to induce production of novel fSM with pharmacological activity is discussed.

Experimental

Chemicals

All ionic liquids used in this study were prepared by QUILL (The Queen's University Ionic Liquid Laboratories, Belfast, UK), except for cholinium chloride (purchased from Sigma, Germany). For ionic liquid structures see Figure 1. Ionic liquids were characterised using a combination of ^{1}H NMR, ^{13}C NMR, electrospray ionisation mass spectrometry, halide content, CHN elemental and water content analysis.

D(+)glucose, K_2HPO_4, $ZnSO_4{\cdot}7H_2O$, $CuSO_4{\cdot}5H_2O$, $FeSO_4{\cdot}7H_2O$, KCl, glycerol ($\geq$ 99.5 %), Tween 20 and ethyl ethanoate were purchased from Sigma Aldrich (Germany), $MgSO_4{\cdot}7H_2O$ $NaNO_3$, and mycophenolic acid (MPA) from Fluka (Switzerland), NaCl from Panreac (Spain), ethanenitrile from Merck (Germany) and chloroform from Fischer Scientific Inc (UK).

Cell culture reagents were purchased from Biochrom (Germany), Lysogeny Broth (LB) medium (formulation: 10 g l^{-1} tryptone, 10 g l^{-1}, NaCl, 5 g l^{-1} yeast extract) from NZYTech Lda. (Portugal).

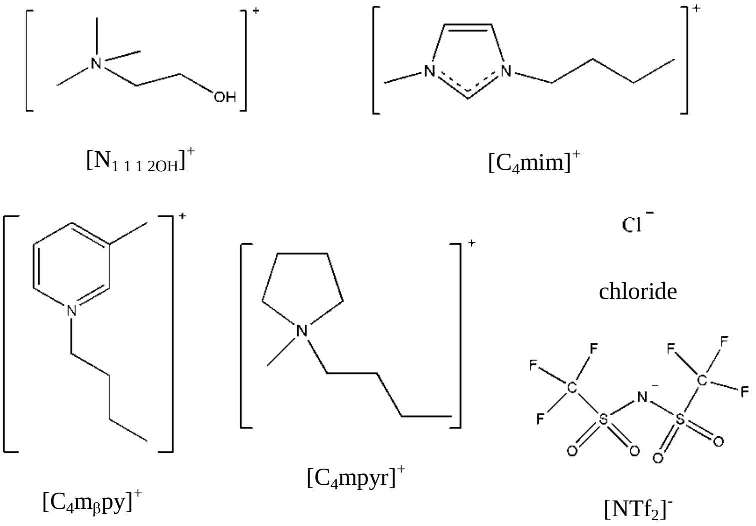


Figure 1. Chemical structures of the cations and anions used in this study: cholinium chloride ($[N_{1\,1\,1\,2OH}]Cl$); cholinium bis((trifluoromethyl)sulfonyl)amide ($[N_{1\,1\,1\,2OH}][NTf_2]$); 1-butyl-3-methylimidazolium chloride ($[C_4mim]Cl$); 1-butyl-3-methylpyridinium chloride ($[C_4m_\beta py]Cl$); 1-butyl-1-methylpyrrolidinium chloride ($[C_4mpyr]Cl$).

Microorganisms

Fungi. *Penicillium brevicompactum* strain Dierckx (IBETPeA) was used in this study. It had previously been isolated from several separate commercially obtained Portuguese cork samples.[17,18] The strain belongs to the *Instituto de Biologia Experimental e Tecnológica* (IBET) culture collection.

Fungi were grown on MEA (malt extract agar) medium for one week at 27 °C. Fungal spores were harvested using 0.1 % v/v Tween 20 (polyoxyethylene (20) sorbitan monolaurate) and filtered through glass wool. The suspension obtained was centrifuged (18500 *g*, 4 °C) and washed three times with 0.85 % w/v NaCl. The fungal spore pellet was resuspended in 10 % v/v glycerol and stored at -80 °C.

Bacteria. *Bacillus subtilis* (*trpC2 metC3*; wild type) belongs to the Microbial Development Laboratory (ITQB). *Escherichia coli* (One Shot®) TOP10 was purchased from Invitrogen. Bacterial cultures were grown in LB media for 24 h at 37 °C under agitation (120 rpm).

Fungal cultures

The minimal culture medium containing glucose (1.0 g l^{-1}) and K_2HPO_4 (1.0 g l^{-1}) was dissolved in distilled water, pH adjusted to 7.0 using phosphoric acid, sterilised in an autoclave (20 min; 121 °C), and supplemented with the mixture of essential salts previously sterilised by filtration: $NaNO_3$ (3.0 g l^{-1}), $ZnSO_4{\cdot}7H_2O$, (0.01 g l^{-1}), $CuSO_4{\cdot}5H_2O$ (0.005 g l^{-1}), $MgSO_4{\cdot}7H_2O$ (0.5 g l^{-1}), $FeSO_4{\cdot}7H_2O$ (0.01 g l^{-1}), and KCl (0.5 g l^{-1}). Ionic liquids were added to a final concentration of 50 mM (corresponding to 6-20 g l^{-1}, depending on the molecular weight of the ionic liquid).

Fungal cultures, inoculated with a suspension of *P. brevicompactum* spores (10^5 spores per ml), were cultivated in 100 ml Erlenmeyer flasks, containing 50 ml growth medium supplemented with 50 mM of an ionic liquid. Cultures were incubated for a fortnight in the dark, at 27 °C, under agitation (90 rpm). Mycelia were separated from the medium by filtration through a glass fibre filter. Corresponding negative controls (fungi grown without addition of ionic liquid) and blanks (non-inoculated media containing ionic liquid) were prepared and treated in the same manner. All cultures were prepared in triplicate.

Fungal metabolites extraction (fSM extracts)

Metabolite containing culture media (filtrates) were dried under vacuum (lyophilised) and extracted twice using 10 ml ethyl ethanoate. In the case of cultures grown in media supplemented with $[N_{1\,1\,1\,2OH}][NTf_2]$, trichloromethane was instead used for extraction. Solvents were chosen because of their limited miscibility with the ionic liquids tested. Solvents were removed from the culture extracts under a gentle nitrogen stream, and dried pellets dissolved (dissolution aided by ultrasonication for 10 minutes) in 200 µl methanol (for fSM qualitative profiling) or 25 µl of ethanol (for biological activity testing). Throughout the text these samples are referred to as fSM extracts. The same procedure was used for control samples (blanks and negative controls).

Liquid chromatography analyses of fSM extracts

Chromatographic profile of diffusible fungal metabolites. Diffusible fungal metabolites in fSM extracts were chromatographically separated using a Waters (Waters Corporation, Milford, USA) Acquity chromatographer with Photodiode Array (PDA) detector, cooling auto-sampler, and column oven. A Symmetry® C18 column (250 × 4.6 mm), packed with end-capped particles (5 µm, pore size 100 Å) (Waters Corporation) was used at 26 °C. Data were acquired using Empower 2 software, 2006 (Waters Corporation). All solvents were of the highest analytical grade, and water obtained from a Milli-Q system (Millipore). Injections of the fSM extracts in methanol were made using a 10 µl loop operated in full-loop mode. The mobile phase, at a flow rate of 0.9 ml min^{-1}, consisted of a solution of 0.1 % trifluoroacetic acid (solvent A) and ethanenitrile (solvent B), set to: a linear gradient of 99.5 % to 0 % of solvent A in 30 min, maintaining 100 % of solvent B for 10 min, 2 min to return to the initial conditions, and additional 10 min to re-equilibrate the column. Detection wavelengths were 200, 245, 254 and 280 nm. MPA was detected at 200 nm, at retention time 18.84 min. The external standard method was used for quantifying MPA in samples, within the calibration range 20-325 µg ml^{-1}.

Chromatographic quantification of residual ionic liquid in the fSM extracts. Contamination of the fSM extract with ionic liquid, even at low level, might influence biological activity. Residual levels of each ionic liquid were determined by quantifying the amount of the cation. Measured ionic liquid concentrations were used to normalise each fSM extract and corresponding blanks.

Waters Corporation equipment and software used are described above. All solvents were analytical grade, and water was obtained from a Milli-Q system (Millipore). For ionic liquid analysis a Synergi Polar-RP column (150 × 4.6 mm) was used, packed with polar end-capped particles (4 µm, pore size 80 Å) (Phenomenex, Torrance, USA) and set at 26 °C. Injections of fSM extracts were made using a 10 µl loop operated in full-loop mode. Chromatographic separations were operated isocratically and the external standard method was used for cation quantification. Conditions used for the chromatographic separations are listed in Table 1.

Table 1. Chromatographic conditions used for quantifying residual levels of ionic liquid cation in culture extracts.

cation	WL (nm)	mobile phase	flow rate (ml min^{-1})	RT (min)
$[N_{1\,1\,1\,2OH}]^+$	215	2% (v/v) methanol and 98% (v/v) of an aqueous solution of 0.1% (v/v) HFB acid adjusted to pH 6	1.0	4.37
$[C_4mim]^+$	218	2% (v/v) acetonitrile and 98% (v/v) of an aqueous solution of 5 mM phosphate buffer, adjusted to pH 3	1.2	7.30
$[C_4m_\beta py]^+$				11.86
$[C_4mpyr]^+$				6.23

WL – detection wavelength, RT – retention time, HFB acid - heptafluorobutyric acid

Biological activity tests

Cytotoxicity test. The cytotoxicity of fSM extracts and ionic liquids was determined using the MTT assay,[19] monitoring reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, a yellow salt, to a blue product, by mitochondrial dehydrogenases. HeLa cells (human epithelial cervix carcinoma cells provided by the American Type Culture Collection, ATCC) with a passage number between 25 and 35 were used. Cells were cultured in Earle's minimum essential medium containing 0.85 g l^{-1} $NaHCO_3$, supplemented with foetal calf serum (10 % v/v), L-glutamine (2 mM) and non-essential amino acids (1 % v/v). Ethanolic test solutions, namely extracts of fSM and blanks, contained 40-88 mg ml^{-1} and 30-100 mg ml^{-1} of dry material, respectively. These were diluted 20 fold in culture media prior to application, to ensure that ethanol concentration did not affect cell viability (≤ 0.5 % v/v).

96-well micro-plates were used for MTT assays, using the two fold dilution method. Equal volumes of cell suspension and test solution in culture media were added to each well, to obtain final volume of 150 μl with 1.125×10^4 cells. The plates were incubated for 68 h at 37 °C in a 5 % CO_2 atmosphere. A solution of MTT was then added to each well to reach a final concentration of 3.6 mg ml^{-1}, and then incubated as before for further 4 h. Afterwards, the supernatant media was removed and substituted by a 10 % v/v SDS solution, followed by overnight incubation in the dark at room temperature. At the end of the experiment, MTT reduction was monitored with a micro-plate reader scoring absorbance at 560 nm. $[N_{1\,1\,1\,2OH}][NTf_2]$ itself forms a bright yellow precipitate with MTT; therefore the method was modified for testing relevant samples. Briefly, cells were incubated with the testing solutions for 72 h, supernatant removed, cells washed with phosphate buffer solution, and fresh culture media and MTT then added.

IC_{50} values, *i.e.* the concentration of test substance that lowers MTT reduction by 50% compared to an untreated control, were calculated from the concentration–response curves. Each experiment was repeated at least three times (five times in the case of ionic liquids). The average IC_{50} reported showed standard deviations below 15 %, except for the modified method (~30 %).

Antibacterial test. Antibacterial activity of the fSM extracts and corresponding blanks, was measured monitoring growth of *Bacillus subtilis* and *Escherichia coli* in LB media. Assays were done in 96-well micro-plates using the two fold dilution method. Ethanolic fSM extracts and blanks were diluted 20 fold in LB media to ensure the ethanol concentration did not affect bacterial viability ($\leq$ 1% v/v).

The final concentration of bacterial cells in each well was 10^6 cfu per ml (colony forming units per ml). Micro-plates were incubated at 37 °C under agitation (120 rpm), and growth followed by measuring absorbance (600 nm) hourly during the first 10 h and at the end of 24 h. Assays were repeated three times.

Results and discussion

Metabolic footprinting by LC

Supplementation of growth media with ionic liquids was previously observed to alter the ESI-MS profile of fSM extracts (see Figure 3 in Chapter II). Metabolic footprints were not correlated with those induced by common salts, such as sodium or caesium chloride. Amongst previously tested fungal strains, *P. brevicompactum* consistently showed the highest tolerance toward ionic liquids (see Chapter II and III), and is a well-known producer of important secondary metabolites, such as mycophenolic acid and adenophostins.[5,20] This fungal strain was selected here for a preliminary study of the fSM induced by specific ionic liquids. The selection of ionic liquids was mainly focused on different head groups, whilst keeping the substituted alkyl chain and anion constant. Growth medium was supplemented with 50 mM of each ionic liquid, obtained through combination of the following cations: $[C_4mim]^+$, $[C_4mpy]^+$, $[C_4mpyr]^+$ and $[N_{1\,1\,1\,2OH}]^+$ with chloride anion. Additionally, $[N_{1\,1\,1\,2OH}]^+$ was also combined with $[NTf_2]^-$ anion.

Significant differences were detected in the LC profiles of fSM extracts from cultures grown in media supplemented with the various ionic liquids (Figure 2). Taking into account all chromatographic peaks in each LC profile, the highest diversity was detected in cultures grown in ionic liquid-free media (negative control), or media supplemented with the least toxic ionic liquids, namely [$N_{1\,1\,1\,2OH}$]Cl and [C_4mpyr]Cl. This might be a direct result of the larger amount of fungal biomass formed in these cultures.

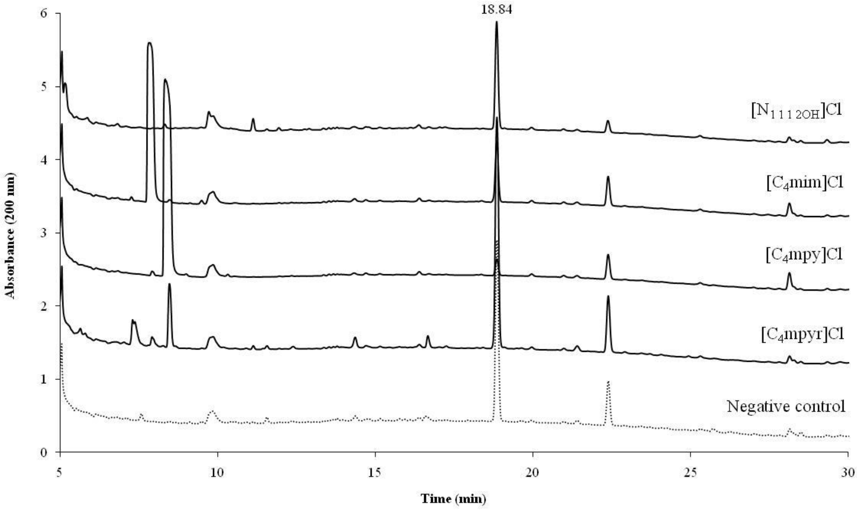


Figure 2. Chromatographic profiles of *Penicillium brevicompactum* culture extracts after growth in media supplemented with ionic liquids (from the top: [$N_{1\,1\,1\,2OH}$]Cl, [C_4mim]Cl, [C_4mpy]Cl and [C_4mpyr]Cl) and negative control (dashed line) at detection wavelength 200 nm. Mycophenolic acid was eluted at 18.84 min.

The finding that the number of detectable mass species (ESI-MS) was higher in cultures grown in an ionic liquid containing media than in the controls (as reported in Chapter II) differs from findings of the present study. There are, however, numerous differences between the two studies, especially considering sensitivity/resolution of analytical methods used. In the study reported in this chapter the aim was to increase production scale, implement straightforward analyses, and ultimately, obtain a simpler purification system. Despite the higher sophistication of MS techniques, liquid chromatographic methods are state of the art as regards metabolomic screens for taxonomic purposes.[21]

Taking into account only the chromatographic peaks absent in the LC profile of the negative control (culture grown in ionic liquid free media), the ability of each ionic liquid to induce novel diffusible metabolites was apparent (Table 2). This is also illustrated by the chromatographic peak matrix at the detection wavelength 200 nm, presented in Figure 3.

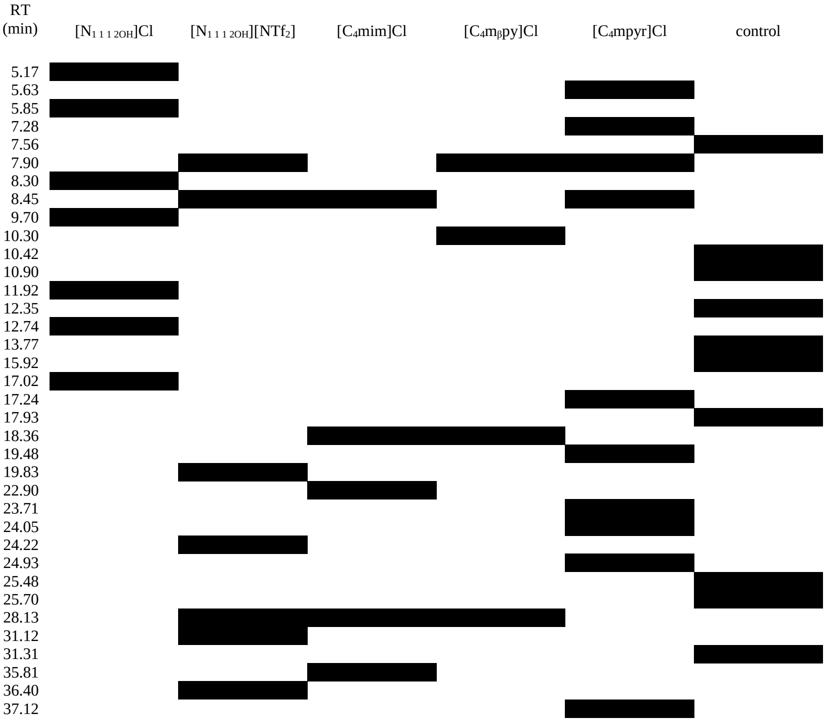
Table 2. Number of differentially detected chromatographic peaks in *Penicillium brevicompactum* culture extracts after growth in media supplemented with ionic liquids, relative to the negative control. As an example four detection wavelengths were selected.

fSM extracts	detection wavelength (nm)			
	200	245	254	280
$[N_{1\,1\,1\,2OH}]Cl$	7	21	13	15
$[N_{1\,1\,1\,2OH}][NTf_2]$	7	6	7	10
$[C_4mim]Cl$	5	4	3	4
$[C_4m_{\beta}py]Cl$	4	4	6	3
$[C_4mpyr]Cl$	10	13	11	11

The number of chromatographic peaks, specific for the negative control, was: 10, 17, 25 and 21 detected at 200, 245, 254 and 280 nm, respectively.

The metabolic alterations observed cannot be solely attributed to biodegradation of the ionic liquid by the fungi. *Penicillium brevicompactum* grown at a micro-culture scale failed to degrade $[C_4mim]^+$ and $[C_4mpy]^+$ cations (data not shown).

The data strongly suggest that the addition of sub-lethal concentrations of ionic liquids in growth media activate the biosynthesis of some new metabolites. Although a more in depth analysis of their chemical identity needs to be carried out, evaluation of the biological activity of crude extracts was taken as a starting point for identifying products with pharmacological potential.



RT (min)	[N$_{1\,1\,1\,2OH}$]Cl	[N$_{1\,1\,1\,2OH}$][NTf$_2$]	[C$_4$mim]Cl	[C$_4$m$_\beta$py]Cl	[C$_4$mpyr]Cl	control
5.17	■					
5.63					■	
5.85	■					
7.28					■	
7.56						■
7.90		■		■	■	
8.30	■					
8.45		■	■		■	
9.70	■					
10.30				■		
10.42						■
10.90						■
11.92	■					
12.35						■
12.74	■					
13.77						■
15.92						■
17.02	■					
17.24					■	
17.93						■
18.36			■	■		
19.48					■	
19.83		■				
22.90			■			
23.71					■	
24.05					■	
24.22		■				
24.93					■	
25.48						■
25.70						■
28.13		■	■	■		
31.12		■				
31.31						■
35.81			■			
36.40		■				
37.12					■	

Figure 3. Differential chromatographic peak matrix of *Penicillium brevicompactum* cultures after growth in media supplemented with ionic liquids, relative to the negative control (*i.e.* excluding peaks detected also in the negative control) at the detection wavelength 200 nm.

Biological activity of ionic liquid-induced fungal metabolites

HeLa cells are a widely used in cytotoxicity testing (*e.g.* ionic liquids[22,23] or natural products[24]). Here, the well-established MTT assay was used to evaluate cell viability[19] after a 72 hour exposure to compounds under evaluation. These included pure ionic liquids and fSM extracts from *P. brevicompactum* cultures grown in the presence of an ionic liquid, as well as corresponding blanks. IC$_{50}$ values (average of five or three independent experiments) are summarised in the Table 3.

Table 3. Inhibitory concentrations (IC_{50}) of fungal metabolite extracts, after growth of *P. brevicompactum* in media supplemented with the ionic liquids, ionic liquids themselves and blanks, in HeLa cells after 72 h of incubation. Standard deviation was below 15%, except in $[N_{1\,1\,1\,2OH}][NTf_2]$ experiments. Mycophenolic acid (MPA) weight percentage is also indicated (estimated by LC).

	IC_{50} (µg ml^{-1})			% (w/w)
Ionic liquid	compound	blank sample	fSM extract	MPA
$[N_{1\,1\,1\,2OH}]Cl$	>23732	n.d.	31.52	0.35
$[N_{1\,1\,1\,2OH}][NTf_2]$	1403	n.d.	6.69	0.12
$[C_4mim]Cl$	128	125	75.33	0.18
$[C_4m_\beta py]Cl$	130	110	174.67	0.03
$[C_4mpyr]Cl$	377	n.d.	37.73	1.86
fungal growth medium	-	n.d.	10.47	0.49

The cytotoxicity of pure ionic liquids followed the general trend described in literature,[25] reinforcing the robustness of the testing model. The most toxic cations were those containing aromatic rings, namely $[C_4mim]^+$ and $[C_4m_\beta py]^+$, followed by the non-aromatic $[C_4mpyr]^+$ and ultimately, the $[N_{1\,1\,1\,2OH}]^+$. $[N_{1\,1\,1\,2OH}]Cl$, known to be an essential nutrient for humans,[26] showed no significant toxicity, *i.e.* the reduction of viability to 61.3 % was observed with the highest tested concentration (>24 mg ml^{-1}). With this exception, all ionic liquids tested can be classified as moderately cytotoxic according to the classification proposed by Stolte *et al.*[27] The observed effect of $[N_{1\,1\,1\,2OH}][NTf_2]$ needs reassessment by a method other than MTT assay as despite use of a modified bioassay, very high standard deviations were observed (see Experimental section). In previous studies using HeLa cells, the IC_{50} value for $[N_{1\,1\,1\,2OH}]Cl$ was lower (approximately 10 mg ml^{-1}),[22] whilst for $[C_4mim]Cl$ it was one order of magnitude higher (approximately 2 mg ml^{-1})[23] than the values reported here. Reasons for this discrepancy are not clear; different media composition, concentrations of cells, and incubation times were used and ultimately different enzyme assays were explored. This may contribute to the discrepancy with previous reports.

The most potent biological activity was observed for the fSM extracts collected from *P. brevicompactum* cultures grown in negative control media and in media supplemented with $[N_{1\,1\,1\,2OH}][NTf_2]$, followed by $[N_{1\,1\,1\,2OH}]Cl$ and $[C_4mpyr]Cl$ (Table 3). Corresponding blanks, *i.e.* extracted non-inoculated media containing ionic liquid, revealed no cytotoxicity. Effects observed for these three fSM extracts can, therefore, be clearly attributed to the natural compounds. Examples of dose-response curves are depicted in Figure 4 for $[C_4mpyr]Cl$, its blank and the induced fungal metabolites.

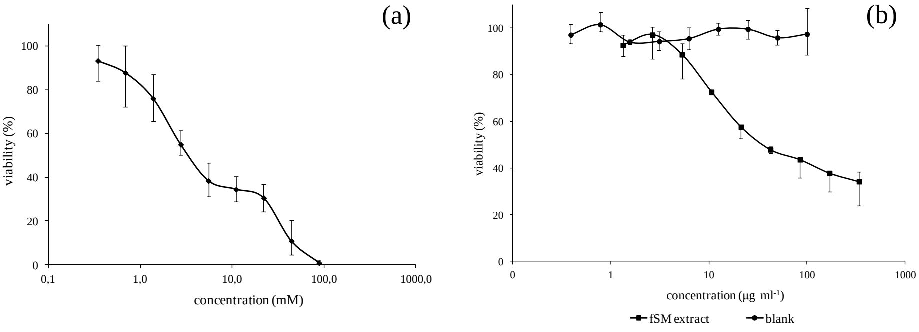


Figure 4. Dose-response curves for (a) $[C_4mpyr]Cl$, and (b) $[C_4mpyr]Cl$ blank and fungal metabolites produced in its presence.

The observed IC_{50} values for $[C_4mim]Cl$ and $[C_4mpy]Cl$ were 128 and 130 μg ml^{-1} ,respectively (Table 3). Estimates based on the residual weight percentage of these ionic liquids in their corresponding fSM extracts (Table 4) rule out an ionic liquid contribution to the measured effects. The low percentage of ionic liquid in these samples was insufficient to provoke a measurable effect on cell viability.

Table 4. Quantification of the residual amount of ionic liquid in the fSM extracts and the corresponding blanks.

	residual ionic liquid content (%)	
ionic liquid	blank	fSM extract
$[N_{1\,1\,1\,2OH}]Cl$	n.d.	0.09
$[N_{1\,1\,1\,2OH}][NTf_2]$	13.78	3.35
$[C_4mim]Cl$	12.58	5.92
$[C_4m_{\beta}py]Cl$	21.90	7.83
$[C_4mpyr]Cl$	-	n.d.

Likewise, estimating cytotoxicity of $[C_4mim]Cl$ and $[C_4m_{\beta}py]Cl$ blank samples based on the ionic liquid content, suggests that unidentified compound(s) other than ionic liquids were responsible for the cytotoxicity observed. The chance that these ionic liquids were transformed into toxic compounds by the HeLa cells themselves is very remote.[28] The concentration of ionic liquids in the blank samples was always higher than in the corresponding fSM extracts. This might result from particular partition phenomena. Therefore, the cytotoxicity of blank samples should be evaluated individually.

IC_{50} values of the *P. brevicompactum* fSM extracts produced by growth in the presence of ionic liquids were > 30 $\mu g\ ml^{-1}$, with the exception of $[N_{1\,1\,1\,2OH}][NTf_2]$. Regarding the cytotoxic activity of crude extracts of natural products, there are some commonly accepted threshold values, *e.g.* plant extracts are considered to be active when $IC_{50} \leq 20\ \mu g\ ml^{-1}$.[29] Despite their utility, such thresholds do not account for the total diversity of compounds in crude extracts. In the present study, compounds in the extracts are highly diverse, making it impossible to directly compare their cytotoxicity. The most biologically potent compounds might be present in the crude extract at low or even trace amounts only.

The antibacterial activity of *P. brevicompactum* fSM extracts, as well as their corresponding blanks, was assessed using Gram-negative *Escherichia coli* and Gram-positive *Bacillus subtilis* (Figure 4, a and b). Under test conditions, none of these samples showed rapid bactericidal effects; however, in some cases obvious growth inhibition, *i.e.* bacteriostatic effect, was noted. The fSM extracts collected from cultures grown in media supplemented with [$N_{1\,1\,1\,2OH}$]Cl and [C_4mpyr]Cl caused a 45 and 60 % growth reduction in *B. subtilis* , respectively. These extracts also affected *E. coli* cultures, both reducing growth by approximately 30 %. Growth reduction in *E. coli* cultures was estimated in blank samples (~10 %) with a similar [C_4mpyr]Cl concentration to that estimated in the fSM extracts. As discussed above, blank samples might contain unidentified antibacterial compounds other than ionic liquids. [$N_{1\,1\,1\,2OH}$]Cl blank sample had no detectable ionic liquid concentration (Table 4), yet displayed some growth inhibitory activity (Figure 4 a), further reinforcing this possibility.

The differences in the cell wall structures of *E. coli* and *B. subtilis* might contribute to their distinct susceptibilities. Gram-positive bacteria have thicker and more hydrophobic cell walls,[30] and a much higher peptidoglycan content (~90 %); the cell walls of Gram-negative bacteria are chemically more complex and possess an additional outer membrane composed principally of lipopolysaccharides.[31] The latter is often associated with the higher resistance of Gram-negative bacteria to biocides.[32]

(a)

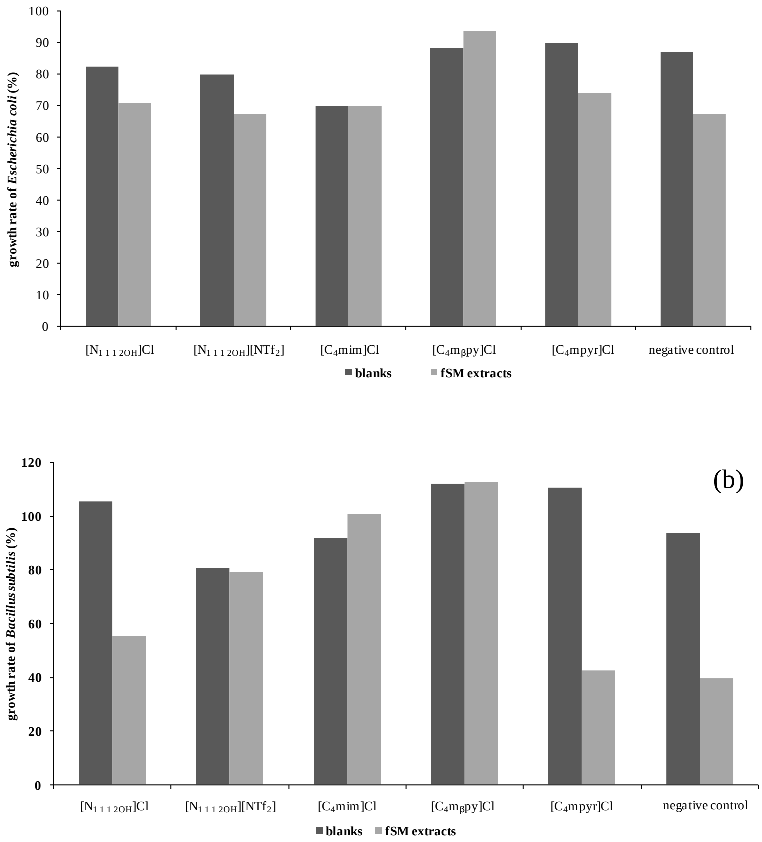


Figure 5. Growth rates of (a) *Escherichia coli* and (b) *Bacillus subtilis* after 24 h incubation with fSM extracts and corresponding blanks. Growth rate values (%) were calculated by comparison with the control sample.

Mycophenolic acid is a natural compound known to be cytotoxic[33] and have limited antibacterial activity.[34] This compound is known to be produced by *P. brevicompactum* and was detected in all fSM extracts tested (Table 3). The levels of MPA detected suggest that other cytotoxic compounds are present in fSM extracts. This could explain why, for example fSM extracts from cultures grown in media with [N$_{1\,1\,1\,2OH}$]Cl are similarly cytotoxic as those derived from [C$_4$mpyr]Cl supplemented media. Using the same cell line the EC$_{50}$ value for MPA was 0.256 µg ml^{-1} (48 h exposure). This serves as a preliminary indication that the MPA abundance in fSM does not correlate with the samples EC$_{50}$ values. Another hypothesis is that some compounds in the fSM mixture might, antagonistically or synergistically, alter the cytotoxicity of a particular compound. At the level of compound diversity, here inferred in a preliminary fashion from the chromatographic peaks detected *e.g.* at 200 nm, only one peak (different from MPA) was found to be exclusively present in fSM from control media, and media with $[N_{1\,1\,1\,2OH}]Cl$ or $[C_4mpyr]Cl$. This might suggest that the observed biological activities could be, at least in part, be accounted for by similar compounds in divergent samples.

fSM extracts from cultures grown in media with $[N_{1\,1\,1\,2OH}]Cl$ or $[C_4mpyr]Cl$ or $[N_{1\,1\,1\,2OH}][NTf_2]$, showed the highest cytotoxicity and, with the exception of $[N_{1\,1\,1\,2OH}][NTf_2]$, also the highest antibacterial activity. Any extrapolation between such distinctive bioassays is purely speculative. Three of five tested ionic liquids activate the production of new metabolites with demonstrable biological effects. These findings merit a more detailed follow-up.

Conclusions

It was observed that addition of sub-lethal concentrations of ionic liquids to the growth media apparently activates biosynthesis of new metabolites, relative to the minimal culture medium. In other Ascomycota fungi, ionic liquids increase expression levels of core genes in some fSM biosynthetic clusters (unpublished data). This might be, at least in part, related to the mode of toxicity of ionic liquids tested, a hypothesis that needs further investigation. Data on biological activity of fSM extracts suggested that some compounds, other than MPA, in these mixtures were active towards HeLa cells and bacteria (more evident in Gram-positive ones). Similar extracts have caused read-outs suggestive of their anti-cancer activity (work performed in cooperation with Thelial Technologies S.A., unpublished data), reinforcing ionic liquids' potential in discovery of novel pharmaceutically valuable natural compounds.

Ionic liquids constitute a virtually unlimited source of stimuli for induction of secondary metabolite production. Currently, our major challenge is to identify induced fungal metabolites, and to perform advanced studies on their bioactivity.

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M. P. has performed all the experimental work (except chromatographic analysis), data analysis and prepared the draft of the manuscript.
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Chapter VI

Final discussion

Final discussion

The following discussion focuses essentially on data presented in the previous research chapters and on future perspectives. The importance of the studies herein discussed is reinforced through a brief analysis of succeeding studies. I would like to thank the members of Applied and Environmental Mycology Laboratory, namely Diego O. Hartmann, Paula Cristina Alves and Isabel Martins for sharing their unpublished data.

There are no doubts that ionic liquids are promising alternative solvents, often improving process efficiency and sustainability. Concomitantly with the growing interest in their applications, numerous questions regarding their environmental impact are being analysed. Progress on understanding ionic liquids ecotoxicity, despite existing knowledge gaps, is already substantial and provides initial guidelines for their conscious design. We anticipate a rising interest of industry in environmentally sustainable ionic liquids. The work presented in this thesis contributed to the aforementioned and is certainly paving the way for novel applications.

Filamentous fungi are ubiquitous, easily adaptable microorganisms, which play important ecological roles.[1,2] They were used in ecotoxicological studies on ionic liquids for the first time by our group. Ascomycota fungal strains are, in general, able to tolerate very high concentrations of ionic liquids, *e.g. Penicillium olsonii* can grow in a minimal growth medium supplemented with 375 mM of [C_2mim]Cl (Chapter II) and *Aspergillus nidulans* tolerates concentrations above 1 M (unpublished data). The supremacy of fungal over bacterial strains isolated from soils with high saline or hydrocarbon level, with regard to their ability to grow in molar solutions of ionic liquids, was also reported by our group.[3]

Amongst ten tested *Penicillium* strains there were four groups of different tolerance levels, regardless of the chemical nature of the ionic liquid. This distinction appeared to correlate well with the phylogenetic proximity of the fungal strains (Chapter II), and allowed us to select representative ones for further evaluations (Chapter III). The predictive value of this correlation can be enhanced by providing more inputs and including phylogenetically distant fungal strains. This approach can be used in rationalisation of toxicological studies.

The risk analysis of each ionic liquid needs to be considered in the context of its application, which directly influences possible exposure routes. Predicting their fate in the environment is complex and implicates high uncertainty. In any environmental compartment, numerous abiotic and biotic processes and interactions with neighbouring molecular or ionic species will influence the ionic liquid fate. Physicochemical properties of ionic liquids will greatly determine their mobility, partition and transport within and between environmental compartments. Furthermore, ionic liquids can undergo various physical, chemical or biological transformations, which might result in products of higher toxicity or persistence. Systematic studies on abiotic degradation of ionic liquids are, so far, scarce. Some ionic liquids might be more recalcitrant and act as persistent and/or bioaccumulative toxicants, which ultimately will depend on their bioavailability. It is also possible that some toxic ionic liquids enter the food chain through multiple routes.

The most probable sources of environmental contamination with ionic liquids are accidental spills and waste disposal. Those of high hydrophobicity will strongly interact with the soil, due to sorption affinity and reduced mobility. Their initial concentrations in the polluted soil might therefore be very high, especially in case of an accidental spill. In this scenario, filamentous fungi showing high catabolic potential and resistance will play a deciding role in the mitigation of the ionic liquid. Predicting tolerance level of fungi through analysis of their phylogenetic classification, as discussed above, can be used to select possible candidates for bioremediation of ionic liquids.

Biodegradability is certainly one of the most critical parameters for the evaluation of ionic liquids environmental impact. It is expected that the cation and the anion will undergo different metabolic routes, as observed in our studies on their biodegradability. Namely, complete degradation of alkanoate anions, $[C_nH_{2n+1}CO_2]^-$ where $n = 3, 4, 5$ or 7, was observed, while cholinium cation was only partially degraded by *P. corylophilum* (Chapter III). Introducing chemical functions such as esters, carboxylic groups or linear alkyl chains, generally enhance primary biodegradability. The long linear alkyl chains certainly contribute to the high toxicity of $[P_{4\,4\,4\,n}]^+$ cation towards *A. nidulans* (Chapter IV). They impaired plasma membrane damage in conidia and despite vegetative growth no biodegradation was observed under any of the tested conditions. For imidazolium-based ionic liquids, the most studied and currently the only group that is under REACH evaluation, no evidence of aromatic ring biotransformation was observed in our studies. Recently, an indirect impedance method was used to measure CO_2 produced during *Sphingomonas paucimobilis* growth in media supplemented with ionic liquids. For example, 39 % of biodegradation for $[C_4mim]Br$ was reported; however, a direct proof of the cation degradation was not provided, which in my opinion weakens the authors' conclusions. Finding optimal conditions for biodegradation of ionic liquids should be of high importance in future. This must include various cultivation conditions, *e.g.* light, pH, nutrients, and different inocula, such as pre-adapted and mixed cultures ones. Community approaches are key to understanding ionic liquids biodegradation in the environment. In addition, the catabolic potential of uncommon strains should be further explored, including those from extreme environments or polluted sites.[3]

The toxicological data presented in previous chapters, indisputably recognises filamentous fungi as relevant model organisms. We started with a rapid evaluation of a broad set of ionic liquids by scoring the effect of a single concentration (Chapter II). Certain toxicity trends became immediately apparent, and in the subsequent studies we focussed on relevant families of ionic liquids. By changing systematically the structure of either the anion (Chapter III) or the cation (Chapter IV) and determining minimal inhibitory and fungicidal concentrations (MIC and MFC, respectively), specific structure-activity trends were defined. Some aspects of the specific mode of toxicity were herein investigated for the first time, namely the alterations of fungal diffusible metabolome in the presence of sub-lethal concentrations of ionic liquids.

From the enormous pool of possible cation/anion combinations, we have tested *ca.* 40 different ionic liquids. The integrated analysis of the data reveals the following ranking for the influence of cation head groups on observed toxicity:

$$\text{cholinium} < \text{piperidinium} \approx \text{pyrrolidinium} < \text{pyridinium} \\ \leq \text{imidazolium} < \text{quaternary phosphonium}$$

Cholinium elicited the lowest toxicity, regardless of the choice of anion, while the strongest inhibitory and fungicidal effect was observed for $[P_{4\,4\,4\,n}]Cl$. Quaternary phosphonium ionic liquids are produced at industrial scale and yet scarcely considered in ecotoxicological studies. These initial findings accentuate that further risk analysis, including exposure and possible biodegradation routes, is necessary. Aromatic imidazolium and pyridinium-based ionic liquids were more toxic than those containing an alicyclic pyrrolidinium or piperidinium ring. This general trend was observed also in cytotoxicity assessment using human epithelial cervix carcinoma HeLa cells (Chapter V) and, with some exceptions, in numerous studies with different testing models (Chapter I, section 1.3.1. with more than 150 references). This information becomes critical to understand ionic liquid interactions with organisms located at different trophic levels.

The elongation of the alkyl chain in the cation severely increased the ionic liquids toxicity, the most evident in the systematic study on $[P_{4\,4\,4\,n}]Cl$, where $n = 1$, $3 - 8$, 10, 12 or 14 (Chapter IV). Minimal inhibitory concentrations of $[P_{4\,4\,4\,1}]Cl$ and $[P_{4\,4\,4\,14}]Cl$ were 32.5 and 0.011 mM, respectively, for *A. nidulans*.

The anion contribution to the overall toxicity of ionic liquids was often considered limited or less predictable, mainly due to the comparison of very different chemical structures. With combination of the benign cholinium cation with a range of alkanoates we made a step forward in conscious design of ionic liquids (Chapter III). Toxicity of $[N_{1\,1\,1\,2OH}][C_nH_{2n+1}CO_2]$, where n = 1 - 9, although low, was defined by the length of the linear chain in the anion. For example, active growth of the most susceptible fungal strain *P. diversum* was inhibited in medium containing 450 mM cholinium ethanoate or 1 mM cholinium decanoate. Non-linear alkanoates, 2,2-dimethylpropanoate and 2-methylpropanoate, showed lower inhibitory and fungicidal effects relative to the linear isomers. Our conclusions further support the need of considering both cation and anion in optimisation of ionic liquids formulations.

The potential modes of toxicity of chemicals in general include disruption of membranes, mostly affecting membrane permeability and transport proteins, enzyme inhibition, and DNA damage. Elongation of the alkyl chain in cation or anion leads to increased lipophilicity and, therefore, increased toxicity. This effect, considered as non-specific or baseline toxicity,[4] is widely documented for ionic liquids and other compounds. The extent of damage of the plasma membrane of *A. nidulans* conidia increased with the lipophilicity of $[P_{4\,4\,4\,n}]^+$ cation, where $n > 4$, as directly assessed by fluorescence microscopy. This also explains the high similarity of the observed MIC and MFC values; *i.e.* severe membrane damage resulted in cell death. Predicted lipophilicity parameters were used to explain the toxicity trend for linear and non-linear alkanoate anions (Chapter III). On the contrary, most recent findings obtained in our group suggest that plasma membrane damage is unlikely the basis of cholinium alkanoates toxicity (unpublished data). It seems that other toxicity mechanisms might therefore be involved. For example, membrane damage was observed in only 9.7 % of *A. nidulans* conidia in 100 mM cholinium octanoate solution

(MFC = 22.75 mM). A possible explanation is repulsion between an overall negatively charged cell surface and the carboxylic group, which prevents an interaction with the linear chain of the alkanoate anions.

The aforementioned ecotoxicological data paved the way for some promising biotechnological applications. The demonstration that $[N_{1\,1\,1\,2OH}][C_nH_{2n+1}CO_2]$, $n = 5$, 7 or 9, are remarkable solvents, was one of the most important achievements of the multidisciplinary approach undertaken in our team.[5] These ionic liquids, biodegradable and of low toxicity, can selectively dissolve suberin in cork. New efficient environmentally sustainable processes for selective extraction of plant biopolyesters, aiming at developing new biomaterials, will be explored. The excellent solvation ability of these benign ionic liquids suggests that they might also be used to augment bioremediation/biodegradation efficiency. Consciously designed ionic liquids of good solvent quality, integrated with catabolic potential and unique metabolic response of fungi to ionic liquids, should inspire innovative biorefinery concepts.

Ionic liquids carrying short alkyl chains in the cation were observed to have a specific mode of toxic action by interfering with metabolic pathways in fungi. Comparative profiling of fungal metabolites revealed that sub-lethal concentrations of ionic liquids alter the cell biochemistry (Chapter II and V). The observed responses were significantly different from that caused by inorganic salts, such as sodium or caesium chloride, ruling out osmotic stress effect as a key factor. In addition, they cannot be a result of co-metabolism of ionic liquids, at least in the case of imidazolium and pyridinium-based ones which were not biodegraded under the testing conditions. In a subsequent study, it was observed that ionic liquids provoked major alterations in transcript levels of genes related with growth and development processes in *A. nidulans* (unpublished data). Major changes in the fungal proteome were also observed, with ~ 10 % of the mycelial polypeptides being differentially expressed (unpublished data). These observations reveal the complexity of the ionic liquids mode of toxicity in fungi at a molecular level. Future studies will include time-course analysis of expression level of selected genes. The target events to be followed are asexual and sexual reproduction and biosynthesis of membranes and cell wall. These will provide more detailed information on specific response of fungi to ionic liquids.

Only a limited fraction of fungal secondary metabolites are currently known, and progress is hampered because traditional cultivation methods fail to reveal their diversity. Discovery that biosynthesis of new fungal metabolites can be activated simply by adding sub-lethal concentrations of ionic liquids to growth media is, in my opinion, the most enthusiastic for the future investigations. Transcriptomics data show that ionic liquids provoked in *A. nidulans* major alterations in the expression level of genes coding for secondary metabolite biosynthetic enzymes (unpublished data). Some of the up-regulated genes are thought to be involved in yet uncharacterised biosynthetic pathways. In the study herein presented, three of five tested ionic liquids activated biosynthesis of metabolites with demonstrable cytotoxic and moderate antibacterial effects (Chapter V). This data is highly promising especially considering the vast number of possible ionic liquids formulations. A more in depth analysis of this effect is necessary to validate whether ionic liquids, in general, can be used to specifically induce or enhance the production of fungal metabolites with pharmacological value. Data on biological activity of *P. brevicompactum* culture extracts after growth in media supplemented with ionic liquids suggested that bioactive compounds, other than mycophenolic acid, were present. Although of a preliminary nature, this study provides a proof of concept. At present, our major challenge is to identify these novel metabolites, and to perform advanced studies on their bioactivity.

If the specific mode of toxicity of ionic liquids can be advantageously exploited in the discovery of natural compounds, it would constitute a remarkable advance for ionic liquids in biological sciences.

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