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Simple screening method to identify toxic/non-toxic ionic liquids: Agar diffusion test adaptation

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ABSTRACT

A wide range of ionic liquids (ILs), containing a diverse set of cations, anions and alkyl chain lengths, was screened for their antimicrobial activity toward four microorganisms, *Escherichia coli* CCT-0355, *Staphylococcus aureus* ATCC-6533, *Fusarium* sp. LM03 and *Candida albicans* ATCC-76645. For that purpose an adaptation of the Agar Diffusion test was validated and successfully applied as a rapid screen method to identify toxic ILs, avoiding the use of more complex and expensive techniques. The effects of the cation alkyl chain length were studied, being observed both the "*alkyl side chain*" effect (increase in antimicrobial activity with the elongation of the alkyl chain) and "*cut-off*" effect (beyond a given chain length, the toxicity cannot be increased any further). Imidazolium-based ILs have in general, negative effects on the growth of these microorganisms dependent on the anion and alkyl chain length (growth inhibition halo from 1.98 ± 0.04 mm for $[C_2mim]Cl$ to 39.53 ± 0.81 mm for $[C_{10}mim]Cl$). On the opposite, the phosphonium-based ILs do not seem to have negative effects for the longest alkyl chains (growth inhibition halos between 0.00 ± 0.00 and 7.30 ± 0.42 mm). It was also observed that the alkyl chain, cation family, and anion moiety all have significant effects on the antimicrobial activity these effects being well correlated with the lipophilicity of the ILs tested. The results also show that the microorganisms responses to the diverse ILs tested are dependent on their morphologic differences.

1. Introduction

The design of safe and environmentally benign solvents has become increasingly important in the development of clean manufacturing processes. Conventional organic solvents are often toxic, flammable and volatile, causing potentially devastating effects when released into the environment. For this reason, there is considerable interest in using ionic liquids (ILs) as alternative solvents. ILs are a focus of interest due to their capacity to improve the solubility of proteins and enzymes, tailoring the reaction rate and extracting different compounds from the fermentation media (de los Rios et al., 2007; Hernández-Fernández et al., 2007). The use of ILs as alternative solvents is only industrially viable if (*i*) they are able of to be recycled and/or reused as many times as possible (Dennewald et al., 2011), (*ii*) they are inert in the presence of the products (Zhao, 2005), and (*iii*) they are environmentally safe for humans and microorganisms (Frade and Afonso, 2010; Matzke et al., 2010; Ranke et al., 2007a).

The toxicity evaluation and control of ILs have been carried out, showing that some of these ionic compounds have similar properties to (if not better than) the organic solvents they could potentially replace (Visser et al., 2000), whilst having negligible vapor pressures thus eliminating the potential risk of air pollution (Fredlake et al., 2004). Their operational safety advantages, including non-flammability and non-explosiveness, gave them the label of "green" solvents. However, without a sound knowledge about their synthesis, biodegradability and toxicological behavior, among others, there may be no justification for this classification. In what concerns the toxicologicology issue, it has been demonstrated that many commonly used ILs have a certain level of toxicity, which could be a major concern in their application in diverse fields. This explains the crescent number of publications in the area focused on the toxic effect of diverse hydrophobic and hydrophilic ILs. Different approaches were considered by the authors, from the aquatic and terrestrial microorganisms, passing by plants and including the effect of ILs in humans (Alvarez-Guerra and Irabien, 2011; Gilmore, 2011;

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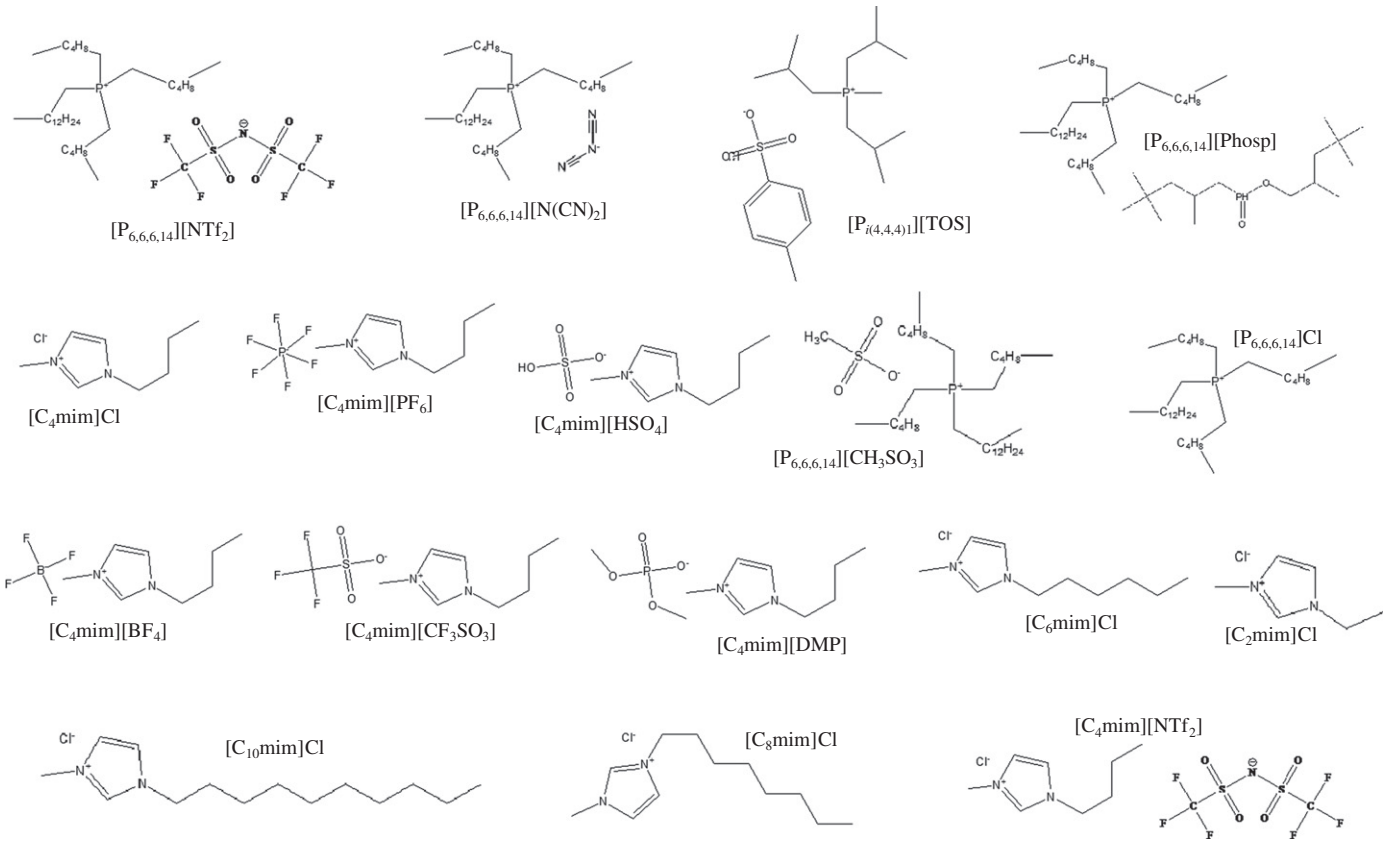

Fig. 1. Identification and chemical structure of all ILs studied.

Larson et al., 2008; Latala et al., 2010; Matzke et al., 2010; Matzke et al., 2008; Pham et al., 2009; Ranke et al., 2007b; Ranke et al., 2007a; Zhao et al., 2007). However, the majority of the results have an experimental basis and, unfortunately, these common (eco)toxicological tests are very time-consuming, costly and require significant operator expertise. It would be convenient to have simple and cheap techniques to allow a qualitative evaluation of the ILs' toxicity, so that unpromising structures can be rejected at the earliest possible stage. Beyond the simplicity required, these tests should be possible to carry out by non-experts (Wood et al., 2011). In general, microorganisms are used as indicator organisms because of their large environmental, ecological and industrial relevance, short generation times and quick growth.

The simplest methods are the antimicrobial activity assays, since they use simple and widely available equipment (Samorì, 2011). The majority of these tests have relied on basic planktonic susceptibility assays, described by the minimum inhibitory concentrations (MIC) (Busetti et al., 2010; Demberelnyamba et al., 2004; Luczak et al., 2010; Pernak et al., 2004; Pernak et al., 2003) or minimum bactericidal/fungicidal concentrations (MBC/MFC) (Busetti et al., 2010; Pernak et al., 2004; Pernak et al., 2003). In the use of these different methodologies, considerable microbiological expertise is needed to prepare the test cultures, the media, and the dilution series of the test chemical under sterile conditions. Further quantitative methodologies are described in literature (Wood and Stephens, 2010), namely the measure of effects on cell viability by measuring viable counts and the use of cell viability test kits. However, some of these techniques are considered as relatively complex and expensive procedures, also requiring microbiological expertise (Wood and Stephens, 2010). Despite the majority of the studies report the use of quantitative techniques, some authors are now starting to work with some qualitative methods, namely the Agar Diffusion test, used to evaluate the toxicity of ILs in solid media (Wood et al., 2011). This methodology (Bauer et al., 1966) is widely used in the determination of the microorganisms susceptibility through different chemical compounds, including several drugs (Vedel et al., 1996). It is simple, inexpensive, requires little preparation and no specialized equipment, uses small quantities of the test compound (Gilmore, 2011; Rebros et al., 2009; Wood and Stephens, 2010) and only basic microbiological skills are needed. Nevertheless, this methodology involves the soaking of the IL into filter paper disks. Their use raises some questions associated with possible reactions between the medium and the disk materials (Swatloski et al., 2002) when some ILs are used.

Aiming to eliminate some of these limitations (mainly the use of the disks), this work reports the use of an adaptation of the Agar Diffusion test, for the evaluation of the toxicity of several ILs. Four microorganisms are used: *Escherichia coli* CCT-0355 (Gram-positive bacterium), *Staphylococcus aureus* ATCC-6533 (Gram-negative bacterium), *Fusarium* sp. LM03 (fungus) and *Candida albicans* ATCC-76645 (yeast). They were chosen to cover a wide range of microorganisms that could have different responses to the toxicity of the compounds being tested. The antimicrobial activity is assessed through the diameter (halo) of the inhibition zones obtained by the addition of the hydrophobic and hydrophilic ILs (in pure state or high concentration solutions) in wells punched in the agar plate pre-cultured with the fresh microorganism (Santos et al., 2009; Silva et al., 2009). In this work, several imidazolium and phosphonium-based ILs were investigated. Besides the cation core (phosphonium and imidazolium), the effect of the cation alkyl chain length and anion moieties was also assessed. This work also intends to study the correlation

between the ILs action and the morphology of diverse microorganisms.

2. Material and methods

2.1. Materials

The phosphonium based-ILs were kindly provided by Cytec Industries Inc. The imidazolium-based ILs used in this work were supplied by IoLiTec (Ionic Liquid Technologies, Heilbronn, Germany). Their mass fraction purities were further confirm by ^{1}H NMR and ^{13}C NMR. The molecular structures, full names, abbreviations and purity levels of the ILs used in this work are provided in Fig. 1 and Table 1.

Tetracycline (mass fraction purity 95%–100%), miconazole (mass fraction purity 99.77%) and NaCl (mass fraction purity 99%) were used in this study to prepare the positive and negative control solutions. Tetracycline, miconazole and NaCl were purchased at DEG Farmacêutica (Aracaju, Sergipe, Brasil), Genix Farmacêutica (Aracaju, Sergipe, Brasil) and Quimex (Aracaju, Sergipe, Brasil), respectively.

2.2. Methods

Miconazole (50 μg L^{-1}) and tetracycline (50 μg L^{-1}) were employed as antimicrobial compounds for fungi and bacteria (positive control), respectively. The negative control was based in an aqueous solution of NaCl 0.9% (w/v).

The target microorganisms, *Escherichia coli* CCT-0355 (*E. coli* CCT-0355), *Staphylococcus aureus* ATCC-6533 (*S. aureus* ATCC-6533), *Fusarium* sp. LM03 and *Candida albicans* ATCC-76645 (*C. albicans* ATCC-76645) were chosen based on their distinct morphologies. Stock cultures of each one of the microorganisms were maintained in their optimum conditions of temperature (4 °C) and medium (Müller-Hinton and Sabouraud for bacteria, molds and yeasts, respectively).

The target microorganisms were grown in Bushell-Hass medium (total composition, g L^{-1}: $MgSO_4$, 0.2; $CaCl_2$, 0.02; KH_2PO_4, 1.0; $(NH_4)_2HPO_4$, 1.0; KNO_3, 1.0; and $FeCl_3$, 0.05), aiming to reach the optical density of 1.0 of the MacFallen scale. Suspensions of 1 mL of the microorganisms were uniformly spread on the sterilized glass plates (by autoclaving) (Müller-Hinton medium for bacteria and Sabouraud medium for mold and yeast), and wells of 6 mm (diameter) were punched under a sterilized conditions (by heating with a Bunsen burner in a laminar flow chamber), with a sterile glass tube. Samples of each one of the ILs (50 μL of total volume in its pure form) were then placed into the wells (Ismail Hossain et al., 2011; Silva et al., 2009). The glass plates were then incubated at 37 °C. The growth inhibition halo was measured after 24 h (*E. coli* CCT-0355, *S. aureus* ATCC-6533, and *C. albicans* ATCC-76645) or 48 h (*Fusarium* sp. LM03), depending of the microorganism (Silva et al., 2009). Each IL was tested in triplicate and the halo of the inhibition zones measured using a vernier caliper rule is the average of the three replicates, being each growth inhibition halo associated with the respective standard deviation.

Table 1
Representation of the ILs full name, respective abbreviations and purity levels (% w/w).

Ionic liquid	Abbreviation	Purity % w/w
tetradecyltrihexylphosphonium bis(trifluoromethylsulfonyl)imide	$[P_{6,6,6,14}][NTf_2]$	> 98
tetradecyltrihexylphosphonium chloride	$[P_{6,6,6,14}]Cl$	< 95
tetradecyltrihexylphosphonium dicyanamide	$[P_{6,6,6,14}][N(CN)_2]$	≈ 97
tetradecyltrihexylphosphonium bis(2,4,4-trimethylpentyl)phosphinate	$[P_{6,6,6,14}]$[Phosph]	≈ 93
tributyl(methyl)phosphonium methylsulfate	$[P_{4,4,4,1}][CH_3SO_4]$	> 99
tri-iso-butyl(methyl)phosphonium tosylate	$[P_{i(4,4,4,4)1}]$[TOS]	> 99
1-butyl-3-methylimidazolium chloride	$[C_4mim]Cl$	99
1-butyl-3-methylimidazolium hexafluorophosphate	$[C_4mim][PF_6]$	99
1-butyl-3-methylimidazolium hydrogensulfate	$[C_4mim][HSO_4]$	98
1-butyl-3-methylimidazolium tetrafluoroborate	$[C_4mim][BF_4]$	99
1-butyl-3-methylimidazolium triflate	$[C_4mim][CF_3SO_3]$	99
1-butyl-3-methylimidazolium dimethylphosphate	$[C_4mim]$[DMP]	99
1-butyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide	$[C_4mim][NTf_2]$	99
1-decyl-3-methylimidazolium chloride	$[C_{10}mim]Cl$	98
1-methyl-3-octylimidazolium chloride	$[C_8mim]Cl$	99
1-hexyl-3-methylimidazolium chloride	$[C_6mim]Cl$	99
1-ethyl-3-methylimidazolium chloride	$[C_2mim]Cl$	98

3. Results and discussion

The experimental method used represents an adaptation of the commonly used Agar Diffusion (or Kirkby-Bauer) test (Bauer et al., 1966).

We intend to eliminate some of the limitations of the common Agar Diffusion test. Thus, in this study the use of paper disks soaked with the target compound is eliminated, being the ILs placed directly into wells previously punched in the agar plates. Adopting this methodology it is possible to eliminate the difficulty on the determination of the extension of the disks soak normally used (Rebros et al., 2009) and the possibility of reaction between the material of the disks and the test compound (Swatloski et al., 2002; Wood et al., 2011). Also in our case, the parameter of toxicity is represented by the halo (diameter) of the growth inhibition zone formed around the wells.

The main goal of this work is the comparison between the effects of several IL structural features towards different microorganisms. Tetracycline and miconazole were used as reference compounds (positive control), as a comparison and a check of reliability of the methodology and biocompatibility of the ILs here used. The biocompatibility of ILs is here tested by comparison between the growth inhibition zones obtained by the presence of both the reference compounds and ILs, meaning that biocompatible ILs have lower growth inhibition halos when compared with the halos obtained for the positive controls. Thus, a biocompatible IL is described by us as a compound whose diameter of the inhibition zone is inferior to the halo obtained by the presence of the respective reference compound (tetracycline for bacteria and miconazole for fungus and yeast). The results obtained for the bacterium *E. coli* CCT-0355 were photographed aiming to validate the experimental methodology (Fig. S1 from Supporting Information). Some of the most descriptive pictures were collected and are shown in Fig. S2A (in Supporting Information). The four different inhibition zones observed are here exemplified: symmetric inhibition zone (tetracycline-T- and $[C_4mim][HSO_4]$-9-), asymmetric (non-circular) inhibition zone ($[C_4mim][NTf_2]$-11-), growth gradient inhibition zone ($[P_{4,4,4,1}][CH_3SO_4]$-8- and $[P_{i(4,4,4)1}]$[TOS]-7-), and an inhibition zone with an adaptation behavior of the bacteria ($[C_4mim][CF_3SO_3]$-3-). It was not possible to relate these symmetries/asymmetries with the IL natures or properties, namely the viscosity. The symmetric and asymmetric inhibition zones were previously described in literature (Wood et al., 2011). Thus, we believe that the asymmetric zones can be obtained due to the non uniformity of the agar gel and by the consequent diffusion problems (Wood et al., 2011). Despite these differences in the microorganism growth, and because these tests provide only a qualitative evaluation, the antimicrobial activity was assessed from the halo of the total growth inhibition zone, which includes the sum of the two inhibition zones described by the gradient growth observed or, in the case of asymmetric inhibition zones, the halo is given by the larger dimension of the halo observed (see Fig. S2B in Supporting Information).

3.1. Effect of the cation alkyl chain length

The effect of the cation alkyl chain length was studied using the imidazolium-based ILs $[C_n mim]Cl$, with $n=2$–10, and the respective results presented in Table 2. The results show that $[C_2mim]Cl$ has no effect against the fungus and yeast species (diameter of the growth inhibition zone is zero) and has a slightly effect towards the bacteria strains (growth inhibition halo $= 1.98 \pm 0.04$ mm). The elongation of the alkyl chain is responsible for the increase of the growth inhibition halo, which is commonly designated in literature by the "*alkyl side chain*" effect. The $[C_{10}mim]Cl$ is responsible for the strongest negative effects toward all the microorganisms (growth inhibition halos from 29.45 ± 0.42 to 39.53 ± 0.81 mm).

Table 2
Growth inhibition halo (mm) for the [$C_n$mim] Cl-based ILs studied plus the positive and negative controls against the four microorganisms.

Compound	Growth inhibition halo (mm)			
	Gram-negative bacteria *E. coli* CCT-0355	**Gram-positive bacteria** *S. aureus* ATCC-6533	**Mold** *Fusarium* sp. LM03	**Yeast** *C. albicans* ATCC-76645
Tetracycline (positive control)	21.70 ± 2.12	36.45 ± 0.71	–	–
Miconazole (positive control)	–	–	21.32 ± 0.03	19.60 ± 0.57
NaCl 0.9% w/w (negative control)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
[C_2mim]Cl	1.98 ± 0.04	1.98 ± 0.04	0.00 ± 0.00	0.00 ± 0.00
[C_4mim]Cl (71.0% w/w)	2.25 ± 0.28	1.98 ± 0.04	3.90 ± 1.84	5.58 ± 0.18
[C_6mim]Cl	20.98 ± 1.52	30.48 ± 2.30	26.75 ± 0.64	14.18 ± 0.74
[C_8mim]Cl	30.83 ± 0.32	30.68 ± 0.53	23.93 ± 0.04	22.00 ± 0.14
[C_{10}mim]Cl (44.6% w/w)	39.53 ± 0.81	29.45 ± 0.42	33.08 ± 0.18	27.25 ± 0.07

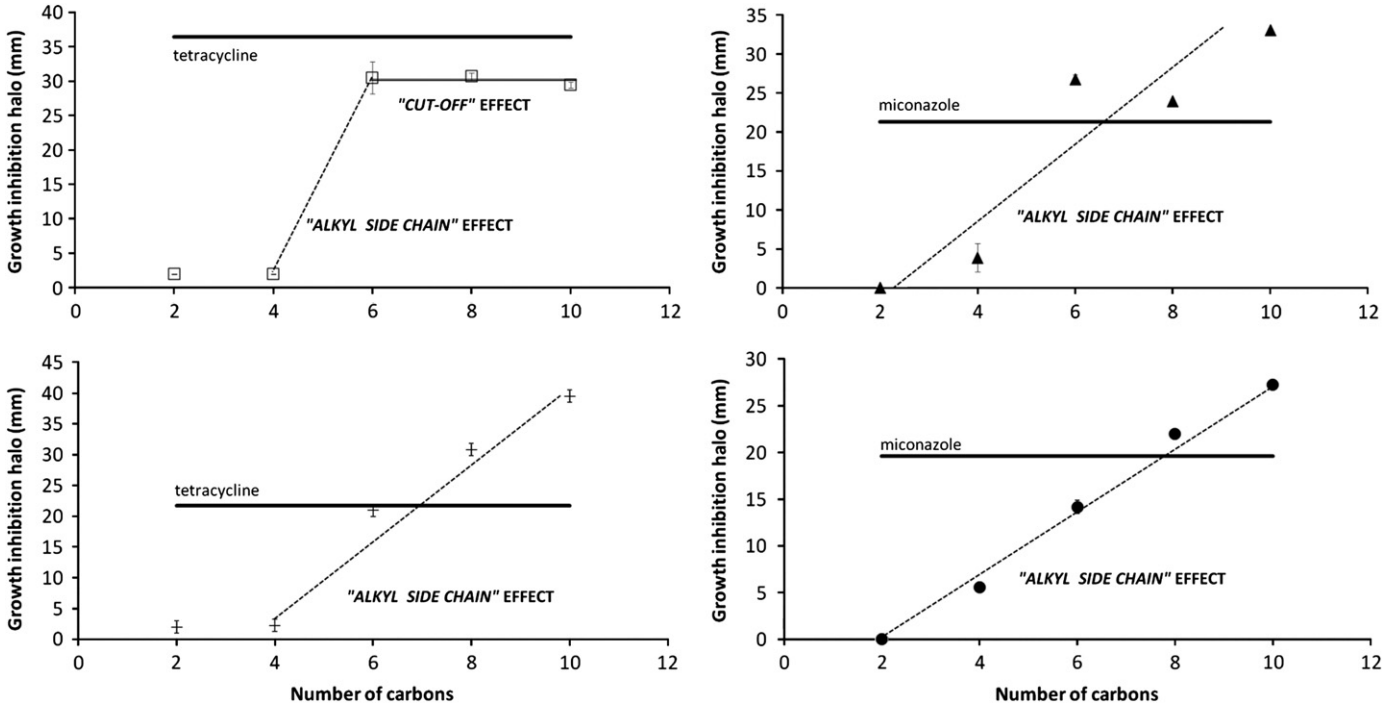

Fig. 2. Growth inhibition halo versus number of carbons in the alkyl chain and respective positive controls (tetracycline and miconazole—bold line): (□) *S. aureus* ATCC-6533, (+) *E. coli* CCT-0355, (▲) *Fusarium* sp. LM03, (●) *C. albicans* ATCC-76645.

The effects observed for the variation of the alkyl chain differ with the morphologic aspects of the microorganisms studied. It is possible to recognize the higher sensitivity of the bacteria when compared with the remaining microorganisms. In particular, the Gram-negative bacterium shows the higher resistance to the ILs' effect, when compared with the Gram-positive. This behavior is explained by the differences in the external structures of these two bacteria strains (Salyers and Whitt, 2001). Both have similar internal, but distinct external structures, being the Gram-negative the most morphologically complex. While the Gram-positive bacterium has a thick, multilayered cell wall consisting mainly of peptidoglycan surrounding the cytoplasmatic membrane, the Gram-negative cell wall contains two layers, external to the cytoplasmatic membrane and the same peptidoglycan surrounding the cytoplasmatic membrane (Salyers and Whitt, 2001). In what concerns the comparison between the yeast and the mold, in general, it is observed that the latest microorganism is the most sensitive. In respect to their morphologic characteristics, it is here highlighted the protector capsule of the yeast (Walke, 1998), which is responsible for the protection of the microorganism against the attack of the ILs. Summing up, the crescent level of sensitivity of the microorganisms is represented by the following trend: yeast < mold < Gram-negative bacterium < Gram-positive bacterium.

Aiming to facilitate the analysis of the results for [$C_n$mim]Cl ILs, the corresponding results of the growth inhibition halo are presented in Fig. 2, for each one of the microorganisms tested, as well as the respective values obtained for the positive controls (tetracycline or miconazole, depending of the microorganism—bold lines). From Fig. 2, it is clear the increase in the toxic effects of the ILs with the elongation of the alkyl chain. This behavior is in close agreement with literature, and is independent of the experimental technique and organism (Busetti et al., 2010; Carson et al., 2009; Lee et al., 2005; Luczak et al., 2010; Pernak et al., 2003; Rebros et al., 2009). Also in this figure, the importance of the morphology and/or composition of the cell walls is observed (Samorì, 2011). The "*alkyl side chain*" effect, described by the dashed lines in Fig. 2 is observed for all the microorganisms, but only for the *S. aureus* ATCC-6533 the "*cut-off*" effect is reached (solid line in Fig. 2). Different explanations were used to justify the latter effect, (*i*) insufficient solubility of the compound (which is not the case in this work, since the ILs here studied are completely soluble in water (Domanska et al., 2003)); (*ii*) steric effects

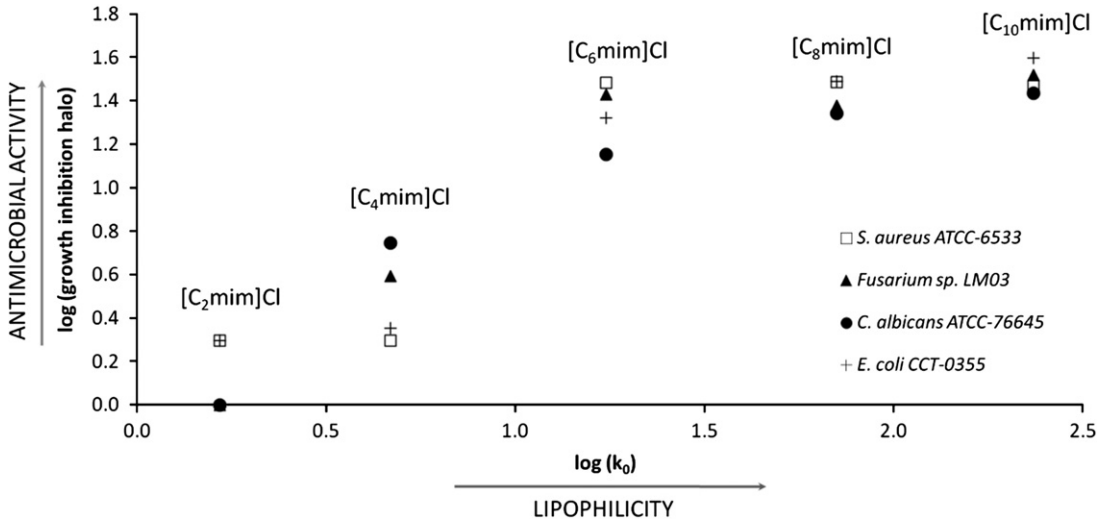

Fig. 3. Relation between the log(growth inhibition halo) and log(k_0) results for the [$C_n$mim]Cl-based ILs studied and the different microorganisms: (□) *S. aureus* ATCC-6533, (▲) *Fusarium* sp. LM03, (+) *E. coli* CCT-0355, and (●) *C. albicans* ATCC-76645.

associated to the increase in the ILs volume, caused by the elongation of the alkyl chain; or (*iii*) the formation of IL aggregates (Matzke et al., 2010). Furthermore, this phenomenon must also be related with the different morphologies of the microorganisms' cells, which is certainly a regulator of the ILs penetration into the cell (Samorì, 2011). By a careful analysis of Fig. 2, and comparing the halos formed for each of the ILs with the positive control, it should be highlighted that some of these ILs have lower toxic effects in comparison with the positive reference control, which means that, despite their negative effects, they were not as severe as the toxic effects of the positive controls. However, it is evident that this is organism-dependent. If for *S. aureus* ATCC-6533, all the ILs can be considered as biocompatible (meaning ILs with lower growth inhibition halos when compared with the respective positive control) and for *C. albicans* ATCC-76645 (yeast), ILs with alkyl chains until 6 carbons were considered as biocompatible, for *Fusarium* sp. LM03 (mold) and the bacteria strains, the biocompatibility is observed only for alkyl chains with 2 and until 4 carbons, respectively.

The ability of an IL to be absorbed into the cells is normally described by their lipophilicity (Ranke et al., 2007b; Stepnowski and Storoniak, 2005). Normally, the increase in the negative effects of the ILs, is attributed to the increase of the hydrophobic nature of the chemical compound or, alternatively, of its lipophilic character (Ranke et al., 2007b; Stepnowski and Storoniak, 2005). To confirm this theory, the correlation between the lipophilicity of the [$C_n$mim]Cl ILs (determined by the reversed-phase and immobilized artificial membrane chromatography technique) described by the parameter log(k_0) (Stepnowski and Storoniak, 2005) and the logarithm function of the growth inhibition halo is presented in Fig. 3. The results show that the lipophilicity is a key parameter on the toxicity observed for the ILs here demonstrated by the linear correlation between these two parameters. However, it seems that this parameter is not enough for the full understanding of the toxicity as this linearity is not maintained for all the alkyl chains (e.g. "*cut-off*" effect).

3.2. Effect of the anion and cation core

The antimicrobial activity of several short-chained imidazolium ILs based in the [C_4mim] cation core and different anions was assessed (Table 3). The exchange of the halide by other anions results in an increase of their antimicrobial activities, mainly when the chloride is replaced by the anion [HSO_4] (growth inhibition halo around 21.13 ± 1.17 and 34.27 ± 2.30 mm). The decrease in the antimicrobial activity is represented by the following trends:

E. coli CCT-0355

$[HSO_4] > [NTf_2] > [CF_3SO_3] > [BF_4] > [PF_6] > [DMP] > Cl$

S. aureus ATCC-6533

$[HSO_4] > [NTf_2] > [DMP] > [CF_3SO_3] > [PF_6] > [BF_4] > Cl$

C. albicans ATCC-76645

$[HSO_4] > [BF_4] > [NTf_2] \approx [CF_3SO_3] > [DMP] > [PF_6] \approx Cl$

Fusarium sp. LM03

$[HSO_4] > [BF_4] > [PF_6] > [NTf_2] > [CF_3SO_3] > [DMP] \approx Cl$

As observed for the alkyl chain length the influence of the anions is also different for each microorganism. The Gram-negative bacterium again appears as the most tolerant bacterium due to the different bacteria morphologies (Salyers and Whitt, 2001). The Gram-positive bacterium is affected by all the ILs tested, while the Gram-negative bacterium growth is only significantly affect by the more hydrophobic anion, as observed for the bis(trifluoromethylsulfonyl)imide [NTf_2]. Also, it is observed that *Fusarium* sp. LM03 is more sensitive to the hydrophobic anions ([PF_6], [NTf_2], and [BF_4]) while *C. albicans* ATCC-76645 is negatively affected by all the ILs, although the effect of [PF_6] was less pronounced. Furthermore, the effect of the [HSO_4] anion stands out for all the microorganisms, which may also be related with the acidity of this IL. Aiming to determine the biocompatibility of these ILs with distinct anions, the growth inhibition halo for the representative positive controls was also assessed and compared with the results obtained for the ILs studied. The results suggest that, for all the microorganisms studied, the [C_4mim]X ILs investigated show lower effects in the growth by comparison with the reference compounds, tetracycline and miconazole.

The results here reported are in good agreement with previous observations that the anion toxic effect is comparatively small when compared to the alkyl chain length effect (Pernak et al., 2003). The anion [HSO_4] seems to be an exception with an atypical increase of the antimicrobial activity which is microorganism-independent. Aiming to find the explanation for the trends described before and the different responses of each one of the microorganisms, the

Table 3
Growth inhibition halo (mm) for [C_4mim]X ILs plus the positive and negative controls against the four microorganisms. The octanol–water partition coefficients (P_{ow}) for some of the ILs investigated are also given.

Compound	Growth inhibition halo (mm)				
	Gram-negative bacteria *E. coli* CCT-0355	**Gram-positive bacteria** *S. aureus* ATCC-6533	**mold** *Fusarium* sp. LM03	**yeast** *C. albicans* ATCC-76645	P_{ow}
Tetracycline (positive control)	21.70 ± 2.12	36.45 ± 0.71	–	–	–
Miconazole (positive control)	–	–	21.32 ± 0.03	19.60 ± 0.57	
NaCl 0.9% w/w (negative control)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	–
[C_4mim][CF_3SO_3]	6.70 ± 0.57	13.98 ± 0.39	5.95 ± 0.21	9.95 ± 0.52	–
[C_4mim][HSO_4]	21.13 ± 1.17	23.33 ± 2.30	30.47 ± 1.02	34.27 ± 2.30	1.995[a]
[C_4mim][DMP]	3.75 ± 0.21	14.33 ± 0.81	2.60 ± 0.28	7.07 ± 0.18	–
[C_4mim]Cl (71.0% w/w)	2.25 ± 0.28	1.98 ± 0.04	3.90 ± 1.84	5.58 ± 0.18	0.003[b]
[C_4mim][PF_6]	4.48 ± 0.39	12.47 ± 1.35	13.05 ± 0.14	5.10 ± 0.07	0.022[b]
[C_4mim][BF_4]	5.53 ± 0.39	10.95 ± 0.64	14.05 ± 0.50	10.42 ± 0.67	0.003[b]
[C_4mim][NTf_2]	10.85 ± 1.13	19.93 ± 1.38	12.00 ± 0.07	9.90 ± 0.92	0.112[c]

[a] data from (Sigma Aldrich MSDS, 2010).
[b] data from (Ropel et al., 2005).
[c] data from (Belvèze, 2004).

Table 4
Growth inhibition halo (mm) for phosphonium-based ILs plus the positive and negative controls against the four microorganisms.

Compound	Growth inhibition halo (mm)			
	Gram-negative bacteria *E. coli* CCT-0355	**Gram-positive bacteria** *S. aureus* ATCC-6533	**Mold** *Fusarium* sp. LM03	**Yeast** *C. albicans* ATCC-76645
Tetracycline (positive control)	21.70 ± 2.12	36.45 ± 0.71	–	–
Miconazole (positive control)	–	–	21.32 ± 0.03	19.60 ± 0.57
NaCl 0.9% w/w (negative control)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
[$P_{6,6,6,14}$][Phosph]	5.58 ± 0.04	22.55 ± 1.70	0.00 ± 0.00	0.00 ± 0.00
[$P_{6,6,6,14}$][NTf_2]	1.33 ± 0.11	2.83 ± 0.11	0.00 ± 0.00	3.08 ± 0.18
[$P_{6,6,6,14}$][$N(CN)_2$]	2.78 ± 0.04	4.40 ± 0.21	0.00 ± 0.00	7.30 ± 0.42
[$P_{6,6,6,14}$]Cl	13.95 ± 0.14	6.90 ± 0.49	0.00 ± 0.00	0.00 ± 0.00
[$P_{i(4,4,4)1}$][TOS]	21.05 ± 1.34	18.90 ± 1.20	13.03 ± 0.35	18.47 ± 0.53
[$P_{4,4,4,1}$][CH_3SO_4]	20.90 ± 1.56	21.03 ± 2.23	12.05 ± 0.64	15.40 ± 0.21

antimicrobial activity results obtained for the different [C_4mim]X ILs were correlated with their lipophilicity. Since the parameter log(k_0) is anion-independent (Ranke et al., 2007b), in this analysis, it was replaced by the octanol–water partition coefficient (P_{ow}) reported in Table 3. The parameter P_{ow} is commonly used in the (eco)toxicological risk evaluation, since it displays similarities to the partition of biological compounds between water and living organisms (Sangster, 1997; Ventura et al., 2011). Despite the inaccuracies reported in our recent work (Ventura et al., 2011) in octanol–water partition coefficient (P_{ow}) results from literature, this parameter is used here only as a qualitative parameter to evaluate the ILs capacity to interact with cell tissues. In this context, a general tendency represented by the increase of both the anion lipophilicity and antimicrobial activity is observed, for all the microorganisms. In fact, it should be highlighted that [HSO_4] (P_{ow} = 1.99) and Cl (P_{ow} = 0.0029) are the anions with the higher and lower 1-octanol–water partition coefficients, and consequently, lipophilic nature, which corresponds perfectly to their position in the antimicrobial activity trends described above.

Table 4 presents the antimicrobial activity results for six phosphonium-based ILs, with distinct anions. In general, the anion influence in the toxicity of phosphonium-based ILs is lower than the observed for the imidazolium species here reported (Matzke et al., 2010; Ventura et al., 2012). These results are probably explained by the overlapping effect of the long alkyl chains substituted in the cation core (Ventura et al., 2012). Nevertheless, some exceptions represented by the phosphinate, [Phosph] and chloride, Cl anions are observed. In these two cases the halo obtained is significantly higher against *S. aureus* ATCC-6533 and the *E. coli* CTT-0355 bacteria, respectively. These six phosphonium-based ILs can be categorized in two different groups. The first is represented by the phosphonium-based ILs substituted with shorter alkyl chains ([$P_{i(4,4,4)1}$][TOS] and [$P_{4,4,4,1}$][CH_3SO_4]) and the second is composed of four phosphonium-based ILs with long hexyl and tetradecyl chains. Comparing the data of both groups, it seems that the higher toxic effects are observed for the ILs with shorter alkyl chains ([$P_{4,4,4,1}$] and [$P_{i(4,4,4)1}$]). These results do not fit into the general trend described by the "*alkyl chain length*" effect (Matzke et al., 2010; Ventura et al., 2012) observed for the imidazolium and other ILs based on cations with a single alkyl chain. The loss of antimicrobial activity observed for the phosphonium-based ILs substituted with the longest alkyl chains may be explained by the bulkier structure of these ILs due to the presence of four large alkyl side chains around the phosphorous atom (Cieniecka-Roslonkiewicz et al., 2005). Although the bacteria are the most sensitive organisms to the short chain phosphonium-based ILs action, the mold and yeast are also harmed, although the mold shows a slightly higher tolerance against these ILs, when compared with the yeast.

In what concerns the [$P_{6,6,6,14}$] ionic structures, the data suggests that their antimicrobial activity is selective: no effects are observed for the mold (*Fusarium* sp. LM03), and insignificant effects are obtained against yeast (*C. albicans* ATCC-76645). Moreover, these long chain ILs present some variable toxicity for both the bacteria strains. The Gram-positive bacterium is the most sensitive to the ILs action, when compared with the Gram-negative. This behavior may be explained by the larger volume of these phosphonium cations, which increases the difficulty of these ionic structures to penetrate into, or interact with, the cell wall and/or membranes of the microorganism.

Comparing the results obtained for the different phosphonium-based ILs and the respective reference compounds, it is

possible to conclude that all the $[P_{6,6,6,14}]$-based ILs may be considered as "benign" structures, since the growth inhibition halos obtained for these phosphonium-based ILs are, in general, significantly lower than those verified for the reference compounds. These results appear as a significant improvement in the search for non-toxic or more "benign" ILs' structures. The method here proposed can thus be a simple, quick, cheaper and valid screen methodology to test the toxicity of several ILs, considered as chemical compounds with an increased industrial potential.

4. Conclusions

The ability of an adapted methodology from the Agar Diffusion test, in the search for non-toxic IL structures was here demonstrated. Using this approach it is possible to select the most and less toxic ILs from a large set of ionic compounds, and understanding their particular effects towards the different microorganisms' morphologic structures. The importance of the lipophilic nature of the imidazolium-based ILs on their toxicity is here demonstrated. It is also shown that the hydrophobic phosphonium-based ILs with long alkyl chains are the less toxic (growth inhibition halo 0.00 ± 0.00 and 7.30 ± 0.42 mm), which is a good evidence of their industrial potential in chemical and biocatalytic processes.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ecoenv.2012.06.002.

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