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1 **Toxicity and inhibition assessment of ionic liquids by activated sludge**

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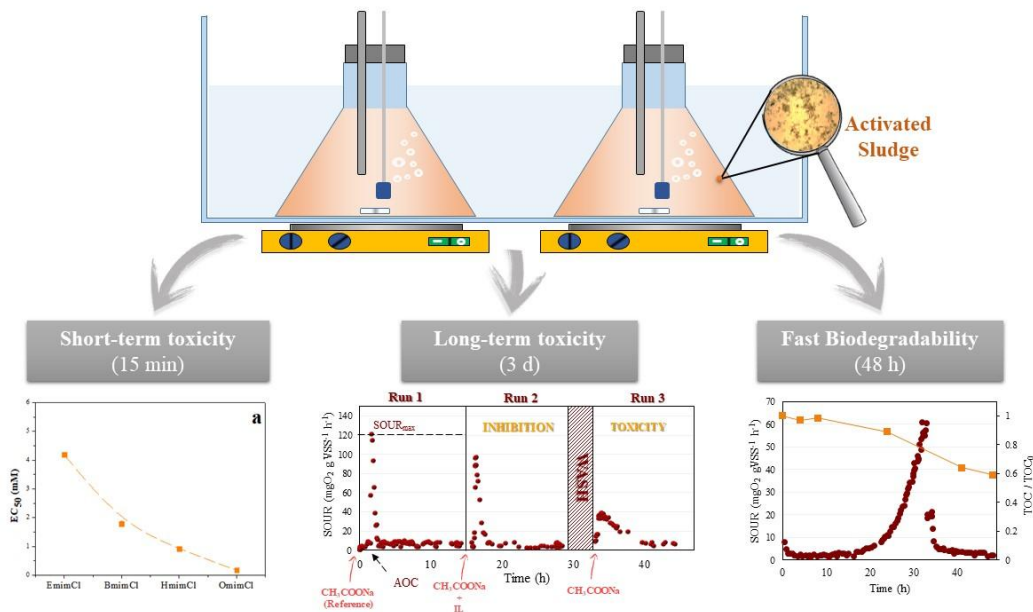
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1 Graphical Abstract



2 Keywords

Activated sludge; biodegradability; toxicity; inhibition; ionic liquid; respirometric measurements

3 Highlights

- Toxicity increases with the alkyl-chain length in imidazolium-based ILs
- Aromatic cations and [NTf₂]⁻ and [HSO₄]⁻ anions presents high toxicity due to the hydrophobic nature
- Most ILs increase their toxicity in long-term assays
- [Choline] cation shows high biodegradability regardless of the counterion

Abstract

Toxicity of 13 ionic liquids (ILs) corresponding to different families were studied by inhibition respiration assays (15 min) using activated sludge. Toxicity increased as increasing the number of carbons in the alkyl-chain of imidazolium-based ILs, with EC₅₀ values from 4.19 to 0.17 for 1-ethyl-3-methylimidazolium chloride ([Emim][Cl]) and 1-octyl-3-methylimidazolium chloride ([Omim][Cl]), respectively. An increase in toxicity was observed for aromatic-based ILs (pyridinium- and imidazolium-based ILs) due to the hydrophobic character of the head groups in comparison with linear structures as phosphonium and ammonium cations. Among to the anions studied fixing [Emim]⁺ as cation, [HSO₄]⁻ and [NTf₂]⁻ presented low EC₅₀ values (0.34 mM and 1.69 mM, respectively) while [Cl]⁻ and [EtSO₄]⁻ were considered harmless anions due to the hydrophilic character of chloride and the organic nature of [EtSO₄]⁻. ILs toxicity/inhibition was determined by adding a biodegradable compound and measuring the sludge response after being in contact with the ILs for at least 15 h. The exposure of sewage sludge to ILs for more than 15 min used in short inhibition assays caused more toxic effect on microorganisms, even for [Choline][NTf₂], previously defined as practically harmless (EC₅₀ = 2.79 mM). Biodegradability assays confirmed the biodegradable nature of choline cation, related with TOC conversion of 40%, only due to cation consumption. No oxygen consumption or even lysis of microbial cells was observed for Tetrabutylammonium bis(trifluoromethylsulfonyl)imide and for 1-Ethyl-3-methylimidazolium hydrogensulphate due to the presence of anions previously defined as hazardous ([NTf₂]⁻ and [HSO₄]⁻), maintaining their recalcitrant character to sewage systems.

1. Introduction

1 Ionic Liquids (ILs) are a relatively new promising class of organic salts, with a large
2 variety of structures and applications. ILs are formed by an organic cation (being
3 imidazolium, pyridinium, quaternary ammonium and quaternary phosphate the most
4 common) and an organic or inorganic anion. Therefore, ILs present a wide range of
5 combinations, giving rise to “designed products” (Plechko and Seddon 2007) with the
6 possibility of altering their properties, and favoring their applications in catalysis
7 (Olivier-Bourbigou et al. 2010), separation processes (Han and Armstrong 2007),
8 biotechnology (Gorke et al. 2010), materials and nanotechnology (Fukushima and Aida
9 2007), and polymers (Lu et al. 2009). The unique properties of ILs (negligible vapor
10 pressure, high thermal and chemical stability and low flammability) (Welton 1999),
11 allow considering these compounds as “green solvents” and a suitable alternative to
12 volatile conventional solvents (Ventura et al. 2013).

13 The significant increase of ILs application in the last decade demands the assessment of
14 their potential hazardous effect and environmental impact. Indeed, the large-scale ILs
15 processes may induce wastewater streams and pollute the aquatic media, eliminating
16 their environmental image and generating the need to find suitable solutions for the
17 removal of these compounds.

18 ILs are mainly considered as toxic and poor biodegradable compounds (Ranke et al.
19 2007; Coleman and Gathergood 2010), which implies that their removal by biological
20 treatments is restrictive or excessively slow (Wells and Coombe 2006). In this context,
21 physical and chemical treatments have been proposed for their abatement, making
22 possible partial mineralization and obtaining effluents with less toxic intermediates that
23 can be treated in a subsequent conventional biological treatment (Mena et al. 2018;
24 Gomez-Herrero et al. 2019).

1 The development of new approaches for the treatment of ILs effluents by biological
2 treatments should consider the potential toxic impact of these compounds on the
3 biomass. It is well known that low toxicity is not linked necessarily with high
4 biodegradability. Thus, exhaustive evaluation of toxicity and biodegradability are
5 required to avoid the possible destabilization of the biological system by the
6 accumulation of non-biodegradable compounds (Oller et al. 2007).

7 Different bioassays have been developed to study the toxic effect of pollutants
8 (Kalčíková et al. 2011; Gomez-Herrero et al. 2018) selecting the appropriate type of
9 microorganism, the biomass to substrate ratio or the test length. Several organisms from
10 different trophic level have been proposed as tools for determining toxic effects,
11 including invertebrates (*Daphnia magna*, ISO 6341) (Stolte et al. 2012), algae
12 (*Selenastrum capricornutum*, ISO 8692) (Peric et al. 2013; Costa et al. 2015), plants
13 (*Lemna minor* ISO/CD 20079) (Ranke et al. 2007; Stolte et al. 2007) or mammalian
14 cells as *Rat leukemia cells* (IPC-81) (Ranke et al. 2007; Stolte et al. 2013). Microtox
15 assay, based on *Vibrio fischeri* activity, is one of the most used acute toxicity test due to
16 its simplicity, reproducibility and fast response. This test has been widely used to
17 evaluate ILs toxicity (Romero et al. 2008; Docherty et al. 2010; Domínguez et al. 2014;
18 Costa et al. 2015; Montalban et al. 2016; Gomez-Herrero et al. 2019), which allows to
19 obtain the EC₅₀ value, defined as the IL concentration (mM) that causes a decrease of
20 50% in *Vibrio fischeri* luminescence. However, one of the main drawbacks of the
21 aforementioned assay is the lack of representative behavior in the microbial community
22 used in bioprocesses, underestimating the ILs effect in wastewater streams. Therefore,
23 the use of activated sludge by respiration inhibition test (ISO 8192) is crucial to
24 evaluate the potential toxicity of ILs in [wastewater treatment plants](#) (Etxebarria et al.
25 2002). The IL impact on microorganisms could be determined registering the oxygen

uptake rate in presence of increasing concentrations of pollutant, due to the direct relationship between dissolved oxygen concentration and biomass activity. The study of Markiewicz et al. (2013) was the pioneer work in determining EC₅₀ values of ILs using activated sludge communities. Despite the sludge source could present different microorganisms population, results of respiration inhibition tests followed similar toxicity trends for other bioassays in the study of ILs toxicity. However, one of the main drawbacks of the toxicity test based on activated sludge respiration (15 min) was the short contact time with the sludge, underestimating the potential degradation in a sewage system. Guisasola et al. (1996) established a method that allows to measure the hazardous character of a toxic compound submitting the sludge with the pollutant for a longer period of time (approximately 3 hours), even if there is a biodegradable fraction, which may cause interferences in short-term toxicity assays since the consumption of the biodegradable fraction may lead to wrong EC₅₀ values.

Nevertheless, some pollutants which are regarded as toxic could be biodegraded at concentrations below their EC₅₀ (Polo et al. 2011). In this sense, a wide variety of biodegradability tests have been developed and are endorsed by the Organization for Economic Co-operation and Development (OECD) distinguishing between inherent biodegradability (OECD 1992), ready biodegradability test (OECD 1993a), and simulation tests (OECD 1993), based on the overall parameters TOC, COD and BOD₅. Pagga (1997) have compared these OECD bioassays with ISO tests, which differed not only in the operational conditions, but also in the inoculum concentration used. In order to establish a rapid evaluation of the biodegradability of xenobiotic compounds, a fast biodegradability test was proposed by Polo et al. (2011), based on the evolution of the oxygen uptake rate of the activated sludge in contact with the ILs for at least 24-48 h,

1 since the respirometric profile is directly related to the compound consumption (Gaudy
2 et al. 1988).

3 In the present work, the toxicity of 13 ionic liquids in aqueous phase was assessed by
4 different respirometric tests with activated sludge. Short-term toxicity test (15 min)
5 based on respiration inhibition assays allows to obtain EC_{50} values of the starting
6 compounds and to study the effect of cation and anion on toxicity. A long-term toxicity
7 test based on the method proposed by Guisasola et al. (1996) has been used to solve the
8 drawbacks of the short-term bioassays, as well as to determine and even to differentiate
9 between inhibition and toxicity caused by ILs in terms of accumulated oxygen
10 consumption (AOC) and maximum specific oxygen uptake rate ($SOUR_{max}$). The results
11 could help to predict the sludge behavior in a sewage system in the presence of these
12 potential hazardous compounds. Finally, a fast biodegradability test, which analyze
13 TOC evolution and SOUR profile by using activated sludge, was used for those ILs that
14 could present a biodegradable fraction.

16 **2. Materials and Methods**

17 *2.1 Chemicals*

18 Thirteen ionic liquids from different families were selected for this study (**Table 1**). ILs
19 were chosen in order to address the most common families and compare among their
20 structural properties, the length of the alkyl chain and the influence of different anions
21 on toxicity. ILs were purchased with a purity higher than 97 % (w/w) from Sigma-
22 Aldrich[®] and Iolitec. Sodium acetate, glucose and the micronutrients solution of the
23 culture media were analytical grade (Sigma-Aldrich[®]).

24 **Table 1.** Ionic liquids used in this work.

Ionic liquid	Abbreviation	Cation	Anion
1-Ethyl-3-methylimidazolium chloride	[Emim][Cl]		Cl^-
1-Butyl-3-methylimidazolium chloride	[Bmim][Cl]		Cl^-
1-Hexyl-3-methylimidazolium chloride	[Hmim][Cl]		Cl^-
1-Octyl-3-methylimidazolium chloride	[Omim][Cl]		Cl^-
1-Ethyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide	[Emim][NTf ₂]		
1-Ethyl-3-methylimidazolium hydrogensulphate	[Emim][HSO ₄]		
1-Ethyl-3-methylimidazolium ethylsulphate	[Emim][EtSO ₄]		
1-Butyl-4-methylpyridinium chloride	[Bmpyr][Cl]		Cl^-
1-Butyl-4-methylpyridinium bis(trifluoromethylsulfonyl)imide	[Bmpyr][NTf ₂]		
Choline bis(trifluoromethylsulfonyl)imide	[Choline][NTf ₂]		
Tetrabutylammonium bis(trifluoromethylsulfonyl)imide	[N4444][NTf ₂]		
Tetrabutylammonium chloride	[N4444][Cl]		Cl^-
Tetrabutylphosphonium chloride	[P4444][Cl]		Cl^-

1 2.2 Inoculum source

2 The activated sludge used as inoculum in respirometric assays was collected from a
3 municipal sewage treatment plant (Madrid, Spain). The activated sludge was maintained
4 with sodium acetate (150 mg COD L⁻¹) and glucose (150 mg COD L⁻¹) as carbon source
5 in a sequential batch reactor (SBR) at 25 °C. The culture medium was also

supplemented with nitrogen and phosphorous sources ((NH₄)₂SO₄ and H₃PO₄, respectively) and mineral salts as micronutrients (FeCl₃, CaCl₂, KCl and MgSO₄), fixing a COD:N:P:micronutrients ratio of 100:5:1:0.05 (w/w) . The sludge concentration was maintained at 3500 mg VSS L⁻¹, referring VSS to volatile suspended solids, without any acclimation process to the ILs studied.

2.3 Toxicity tests

2.3.1 Respiration inhibition test with activated sludge

Batch respirometric assays were performed in a Liquid-Static-Static (LSS) respirometer according to the method proposed by Polo et al. (2011). Two vessels equipped with oxygen probes were placed in a thermostatic bath (25 °C) and aerated. Reactors had no headspace so the external oxygen transfer could be neglected. Different short-term respirometric measurements were carried out using unacclimated activated sludge (350 mg VSS·L⁻¹) in contact with a reference easily-biodegradable compound (sodium acetate) alone or with IL at different concentrations. The activated sludge was maintained aerated and in starvation for 24 hours prior to the assays, in order to consume completely residual COD and to ensure that the effect on the sludge is only due to the reference compound and/or IL, avoiding possible interferences.

Short-term toxicity assays. Toxicity was evaluated by a modification of the method proposed by Ricco et al. (2004). The activated sludge was aerated to a maximum (oxygen saturation) followed by a decay, registering the endogenous oxygen consumption rate of the sludge (15 min). Sodium acetate was added as biodegradable substrate and the biomass activity was measured in terms of exogenous specific oxygen uptake rate of the reference substrate (SOUR_{exR}). ILs solutions were prepared with distilled water and increasing concentrations of the compound (0.25-2.5 mM) were

added together with sodium acetate, obtaining different SOUR values for each contaminant concentration ($SOUR_{exT}$).

The sludge was replaced in each assay to avoid acclimation to the studied compounds. EC_{50} was defined as the IL concentration that causes a 50% of reduction of the $SOUR_{exT}$.

Once $SOUR_{ex}$ values have been corrected by endogenous SOUR, γ parameter is calculated as equation [1]. The relationship between γ and the IL concentration, C, can be expressed as equation [2] where K and P were defined as the y-intercept and the slope obtained after linearization. Thus, when the IL causes a 50% of reduction of $SOUR_{exT}$, γ is equal to 1, and EC_{50} can be defined as equation [3]:

$$\gamma = \frac{SOUR_{exT} - SOUR_{ex}}{SOUR_{exT} - SOUR_{end}} \quad [1]$$

$$C = \frac{K}{P(\gamma - 1)} \quad [2]$$

$$EC_{50} = \frac{K}{P} \quad [3]$$

EC_{50} values were reported as the means of three replicate determinations \pm standard deviation (lower than 10% for all ILs studied). All the ILs depicted in Table 1 were studied with the aim of evaluating the effect of structural properties on toxicity. In IL assays where the results obtained need to extrapolate the linearization obtained by equation [2], the initial range of concentration of ILs was increased up to 5 mM.

Long term toxicity test. Toxicity and inhibition effect caused by IL after contact with the sludge for a long period of time (15 h) was evaluated using a modification the method proposed by Guisasola et al. (1996). ILs studied were [Emim][Cl], [Bmim][Cl], [Omim][Cl], [Emim][NTf₂], [N4444][NTf₂], [Choline][NTf₂] and [Emim][HSO₄]. The specific oxygen uptake rate (SOUR) profile was depicted interrupting the air supply and

registering the dissolved oxygen decay within a range of $0.2 \text{ mg O}_2 \text{ L}^{-1}$. The aeration pump was reconnected obtaining SOUR values calculated as the slope of the dissolved oxygen measured versus time related with the biomass concentration. Different operation conditions lead to three different SOUR profiles which are used to establish the toxicity and inhibition of the IL. In each profile, the maximum value of SOUR obtained and AOC were selected as target parameters for inhibition/toxicity evaluation. The complete assay is summarized in three different stages and depicted in **Figure 1** as example:

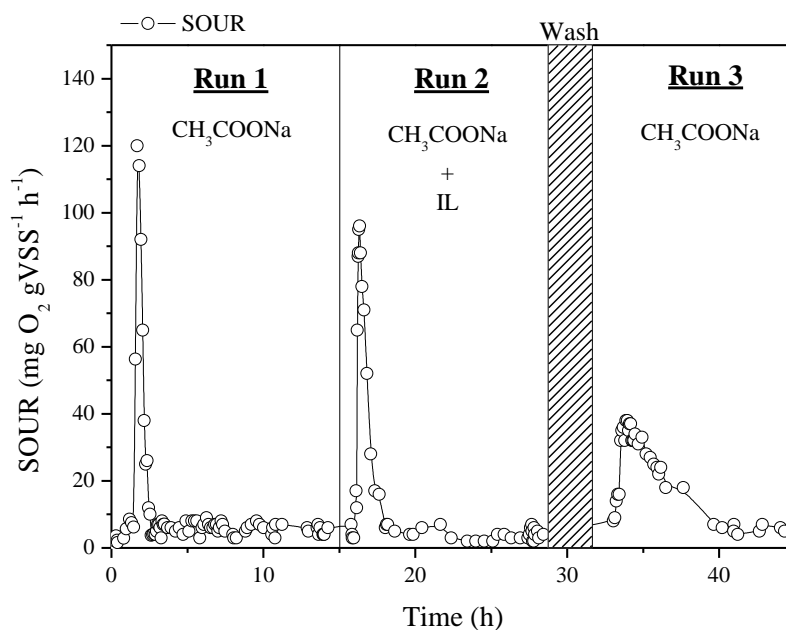


Figure 1. SOUR profile for the different runs in long-term toxicity assays.

- Run 1: the first run was performed by adding a pulse of a reference biodegradable substrate (sodium acetate, $900 \text{ mg COD L}^{-1}$). The SOUR increase was only related with the consumption of the biodegradable compound.
- “Run 2: Once the reference substrate was completely consumed, a mixture of sodium acetate ($900 \text{ mg COD L}^{-1}$) and IL (concentration corresponding to EC_{50} value with activated sludge) was added. In this stage, inhibition could be defined

as the negative effect observed on the sludge after addition of toxic and reference substrate and can be quantified according to equation [4] using $SOUR_{max}$ as model:

$$\frac{SOUR_{max} - SOUR_{max} \cdot \frac{AOC_1}{AOC_2}}{1 - \frac{AOC_1}{AOC_2}} \quad [4]$$

Where 1 and 2 are referring to the values obtained in Run 1 (only sodium acetate) and Run 2 (sodium acetate + IL), respectively. AOC values obtained in Run 1 and 2 allowed determining Inhibition related to AOC ($Inhibition_{AOC}$).

- Run 3: the wash of sludge with phosphate buffer removed the residual IL of run 2 and the biomass came into contact again with the biodegradable substrate, sodium acetate. Toxicity was regarded as the negative effect observed on biomass once the sludge has already been in contact with the toxic compound as described in run 2 and can be quantified according to equation [5]:

$$\frac{AOC_1 - AOC_3}{AOC_1} \quad [5]$$

Where 1 and 3 are referring to the values obtained in Run 1 (only sodium acetate) and Run 3 (sodium acetate after washing), respectively. AOC values obtained in Run 1 and 2 allowed calculating toxicity related to AOC ($Toxicity_{AOC}$).

The endogenous oxygen uptake rate was below $5 \text{ mg O}_2 \text{ mg}^{-1} \text{ VSS h}^{-1}$ for all the ILs studied, allowing to neglect it for the aforementioned assays. The results reported were the average values from duplicate measurements.

2.3.2 Luminescence inhibition test.

Toxicity assays were also performed using a standard Microtox test (ISO, 11348-3, 1998). Samples were neutralized prior the study of the inhibition effect on the

luminescence of by the marine bacteria *Vibrio fischeri*, detected by a Microtox 500 Analyzer (Azur Environmental) after 15 min of exposure.

2.4. Biodegradability assays.

A respirometric-based biodegradability test was carried out for checking in an easy and rapid way, the effect of ILs in a sewage system. [Choline][NTf₂], [N4444][NTf₂] and [Emim][HSO₄] were used to evaluate the biodegradability of different cation-anion pairs. The SOUR profile was obtained as previously described, interrupting the air supply and registering the dissolved oxygen concentration decay between two selected values (Tobajas et al. 2016) Biomass concentration was 350 mg VSS L⁻¹ and EC₅₀/2 was selected as the initial concentration of each IL, ensuring the sludge survival. The evolution of TOC reduction and IL removal was measured along the assay (48 h, 25 °C).

2.5 Analytical methods

Total Organic Carbon (TOC) was measured using a TOC-VCSH apparatus (Shimadzu). Biomass concentration was determined following the APHA procedure 2540E. ILs concentration was quantified by HPLC (Varian Prostar 325) with a UV-vis detector at 218 nm. A Synergy 4 mm Polar-RP 80 A column (15 cm length. 4.6 mm diameter, Phenomenex) was selected as the stationary phase and phosphate buffer with an acetonitrile gradient as the mobile phase with a constant 0.75 mL min⁻¹ flow.

3. Results and discussion

Toxicity tests

Table 2 shows the EC₅₀ values (mM) of selected ILs obtained by inhibition respiration assays (activated sludge, 15 min), as well as data found in the literature. For comparison

1 purpose the EC₅₀ values from Microtox test (*Vibrio fischeri*) have been also included. In
2 general, the marine bacteria *Vibrio fischeri* presented higher sensibility to most of the
3 ILs than using activated sludge. Abbas et al. (2018) performed a comparative study of
4 Microtox test with other reference toxicity bioassays and confirmed that *Vibrio fischeri*
5 inhibition assays was the more sensitive test for toxicity evaluation. The ecotoxicity of
6 imidazolium-based ILs, one of the most common ILs families, was higher than
7 conventional volatile organic solvents, discarding the green image of these compounds
8 in water solution. Even the less ecotoxic imidazolium-based IL studied, [Emim][Cl],
9 yielded EC₅₀ values significantly lower (higher ecotoxicity) than methanol, acetone or
10 acetonitrile, which showed EC₅₀ values above 10³ mM (Kaiser and Palabrica, 1991),
11 especially in this work, where [Emim][Cl] presented greater than EC₅₀ values reported
12 in the literature, up to an order of magnitude between both values.

1 **Table 2:** EC₅₀ values for different ILs towards activated sludge and *Vibrio fischeri*.

2

Ionic Liquid	Activated sludge		<i>Vibrio fischeri</i>	
	EC ₅₀ (mM)	Other authors (EC ₅₀)	EC ₅₀ (mM)	Other authors (EC ₅₀)
[Emim][Cl]	4.19 ± 0.04	>50 (Markiewicz et al. 2013)	2.84 ± 0.08	35.48 (Luis et al. 2007) 27.54 (Munoz et al. 2015)
[Bmim][Cl]	1.79 ± 0.08	2.34 (Diaz et al. 2018)	0.91 ± 0.04	1.62 (Peric et al. 2013) 5.12 (Docherty et al. 2010) 2.45 (Romero et al. 2008) 0.89 (Luis et al. 2007)
[Hmim][Cl]	0.91 ± 0.05	0.93 (Markiewicz et al. 2013) 0.33 (Diaz et al. 2018)	0.17 ± 0.01	0.93 (Ranke et al. 2007) 0.23 (Montalban et al. 2016) 0.15 (Romero et al. 2008) 0.08 (Luis et al. 2007)
[Omim][Cl]	0.17 ± 0.01	0.21 (Markiewicz et al. 2013) 0.02 (Diaz et al. 2018)	0.03 ± 0.0	0.01 (Peric et al. 2013) 0.10 (Ranke et al. 2004) 0.01 (Romero et al. 2008) 1.81 (Luis et al. 2007)
[Emim][NTf ₂]	1.69 ± 0.11	N.A.	1.11 ± 0.04	4.16 (Montalban et al. 2016) 2.39 (Costa et al. 2015)
[Emim][HSO ₄]	0.34 ± 0.01	N.A.	0.12 ± 0.01	N.A.
[Emim][EtSO ₄]	4.04 ± 0.26	N.A.	3.60 ± 0.14	10.47 (Luis et al. 2007)
[Bmpyr][Cl]	0.85 ± 0.05	N.A.	0.35 ± 0.02	1.73 (Peric et al. 2013) 1.28 (Costa et al. 2015) 0.43 (Luis et al. 2007)
[N4444][Cl]	5.21 ± 0.17	N.A.	3.13 ± 0.19	1.94 (Costa et al. 2015)
[P4444][Cl]	4.84 ± 0.25	N.A.	3.08 ± 0.20	N.A.
[Bmpyr][NTf ₂]	0.31 ± 0.02	N.A.	0.12 ± 0.01	N.A.
[N4444][NTf ₂]	1.66 ± 0.09	N.A.	1.03 ± 0.04	N.A.
[Choline][NTf ₂]	2.79 ± 0.13	N.A.	2.15 ± 0.14	N.A.

3 N.A. = not available

Despite the EC₅₀ values obtained in this work depicted the same trend in comparison with values calculated by other authors, the difference in the results lied on the inoculum source used, depending on which, a pollutant may cause changes in the adaptability of the microorganism population present in sewage sludge source (Markiewicz et al. 2013).

Figure 2 depicts the toxic behavior for several groups of ILs on activated sludge. In **Figure 2a**, four imidazolium-based ILs were selected varying the number of carbons of the alkyl chain from 2 ([Emim][Cl]) to 8 ([Omim][Cl]) with [Cl]⁻ as anion. The inhibition of the microorganism respiration increased as the number of carbons of the alkyl-chain length became higher, being the EC₅₀ value 24-fold lower for [Omim]⁺ than [Emim]⁺ cation. Results described a linear regression fit according to the expression: $\text{Log EC}_{50} = 1.47 - 0.27 C$ (being C number of carbons, $r^2 = 0.987$), similar to that obtained by Diaz et al. (2018) confirming the relationship observed in this study. In addition, Markiewicz et al. (2013) described an elevated toxicity in activated sludge communities with the elongation of the alkyl-chain length, at least 3 orders of magnitude between ILs with higher difference in number of carbons, from 8 ([Omim]⁺) to 2 ([Emim]⁺). The increase in toxicity as increasing the number of carbons in alkyl chain could be related with a loss of polarity and the hydrophobicity character of the ILs (Deng et al. 2011; Montalban et al. 2015). Non-polar nature is associated with a greater ability of the accumulation of IL on cellular membranes (Garcia et al. 2005), the main cause of the decrease in microbial activity. In addition, ILs with long alkyl-chains (imidazolium-based ILs) may interact not only with membrane lipid bilayers, but also with membrane proteins, altering the membrane integrity and the native state of the proteins causing oxidative stress or DNA damage among other cellular injuries. Tsarpali and Dailianis (2018) observed this effect of the length of alkyl-chains in mussel

hemocytes, although further studies were needed to establish the mechanism of action in activated sludge and the effect of ILs on biomembranes has to be studied case-by-case (Benedetto 2017).

The influence of the anion on toxicity towards activated sludge was tested (**Figure 2b**) for the lower toxic cation ($[\text{Emim}]^+$) of the imidazolium family with four different anions ($[\text{Cl}]^-$, $[\text{NTf}_2]^-$, $[\text{HSO}_4]^-$ and $[\text{EtSO}_4]^-$). The following toxicity sequence can be established: $[\text{HSO}_4]^- > [\text{NTf}_2]^- > [\text{Cl}]^- > [\text{EtSO}_4]^-$. Great toxicity was observed for $[\text{NTf}_2]^-$ and $[\text{HSO}_4]^-$ anions, for both Microtox and activated sludge tests. $[\text{NTf}_2]^-$ is considered a toxic anion due to its capability to form $[\text{HF}]$, which is a highly hazardous compound (Matzke et al. 2007), together with the hydrophobic nature of this anion, increasing the toxicity towards activated sludge. On the other hand, $[\text{Cl}]^-$ and $[\text{EtSO}_4]^-$ anions presented lower toxicity, with EC_{50} values above 4 mM, since $[\text{Cl}]^-$ is recognized as a hydrophilic compound that interact strongly with water and decreased the toxic effect on the microorganisms. In the case of $[\text{EtSO}_4]^-$ anion, the organic part in its structure can be biodegraded by the sludge.

Figure 2c shows the cation toxicity for four different ILs families, $[\text{Bmim}]^+$ and $[\text{Bmpyr}]^+$ for imidazolium- and pyridinium-based ILs, as well as tetrabutylammonium and tetrabutylphosphonium ILs, with $[\text{Cl}]^-$ as counterion due to its low toxicity. Pyridinium cation resulted as more toxic than imidazolium one, which could be related to the number of atoms in the core cation (Freire et al. 2008). On the other hand, according to the EC_{50} values obtained towards the respirometric inhibition assay, both tetrabutylammonium and tetrabutylphosphonium were three times less toxic than those ILs with heteroatoms in their structure. This statement confirmed that sewage microorganisms were affected in a greater way by aromatic compounds than by linear structures, although the head group presented minor relevance to alter

1 toxicity/lipophilicity than the effect of the elongation of the alkyl-chain (Stolte et al.
2 2007). The influence of a more ecotoxic anion was studied fixing $[\text{NTf}_2]^-$ anion and
3 varying the cation group (**Figure 2d**). In this case, $[\text{Choline}]^+$, a biodegradable cation
4 was introduced in the comparative study. According to these results, $[\text{NTf}_2]^-$ anion
5 showed a more hazardous effect than chloride, due to its high hydrophobicity and low-
6 polar character (Stolte et al. 2006). $[\text{Bmpyr}][\text{NTf}_2]$ appeared to be as the most toxic IL
7 due to the hydrophobic character of both cation and anion. However, the toxicity
8 sequence remained similar to that obtained with $[\text{Cl}]^-$ anion, which reflected that despite
9 the anion used, the toxicity nature of the head groups was comparable. $[\text{Choline}][\text{NTf}_2]$
10 was regarded as the less toxic IL due to the biodegradable character of the cation
11 (Petkovic et al. 2011; Mena et al, 2019). Despite Choline cation belongs to quaternary-
12 ammonium IL family, the incorporation of an alcohol group in the side-chain, reduced
13 the toxic effect (Deng et al. 2011).

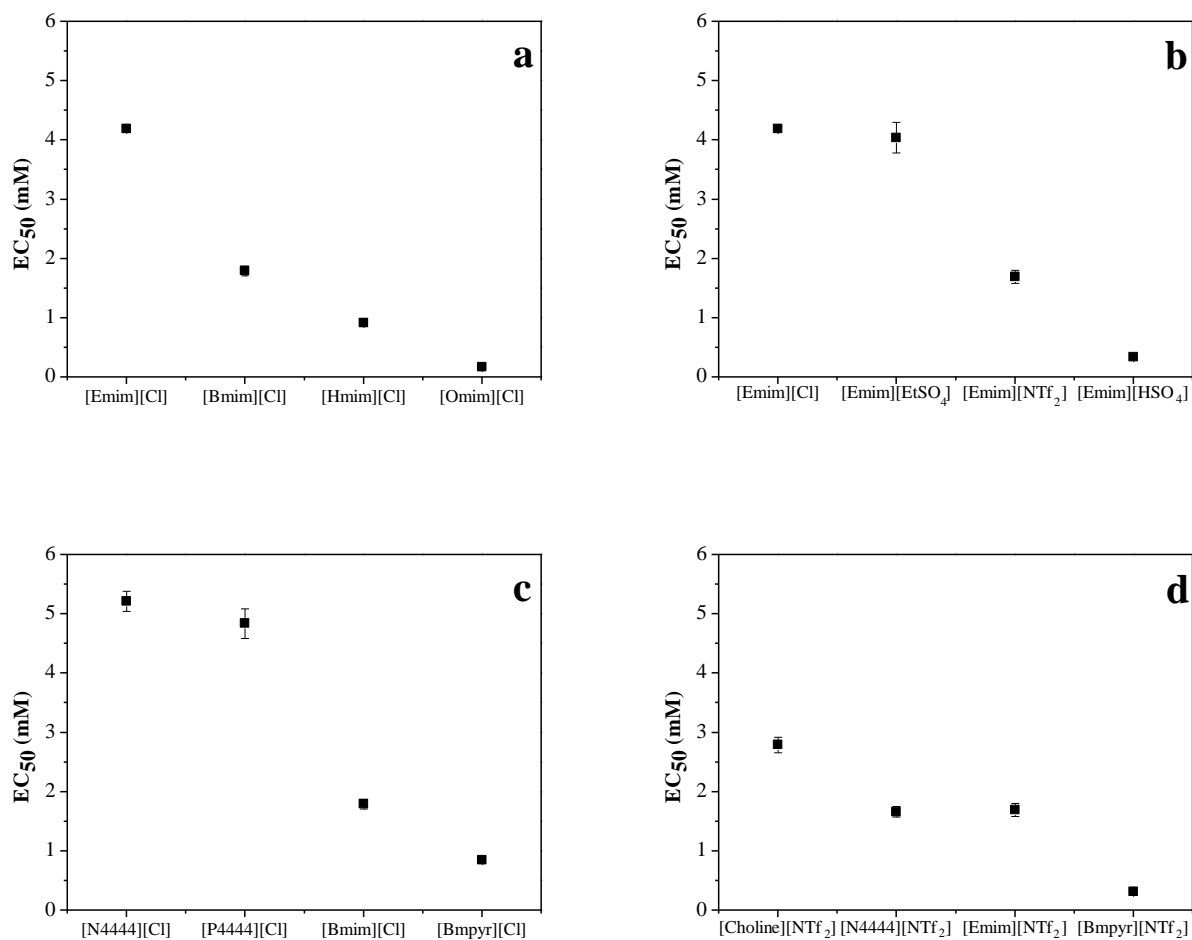


Figure 2. Relationship between EC_{50} values using inhibition respiration test with activated sludge as a function of the length of the alkyl chain (a), the anion in imidazolium ILs (b), IL family with Cl^- anion (c) and IL family with NTf₂⁻ anion (d). Error bars represent \pm standard deviation.

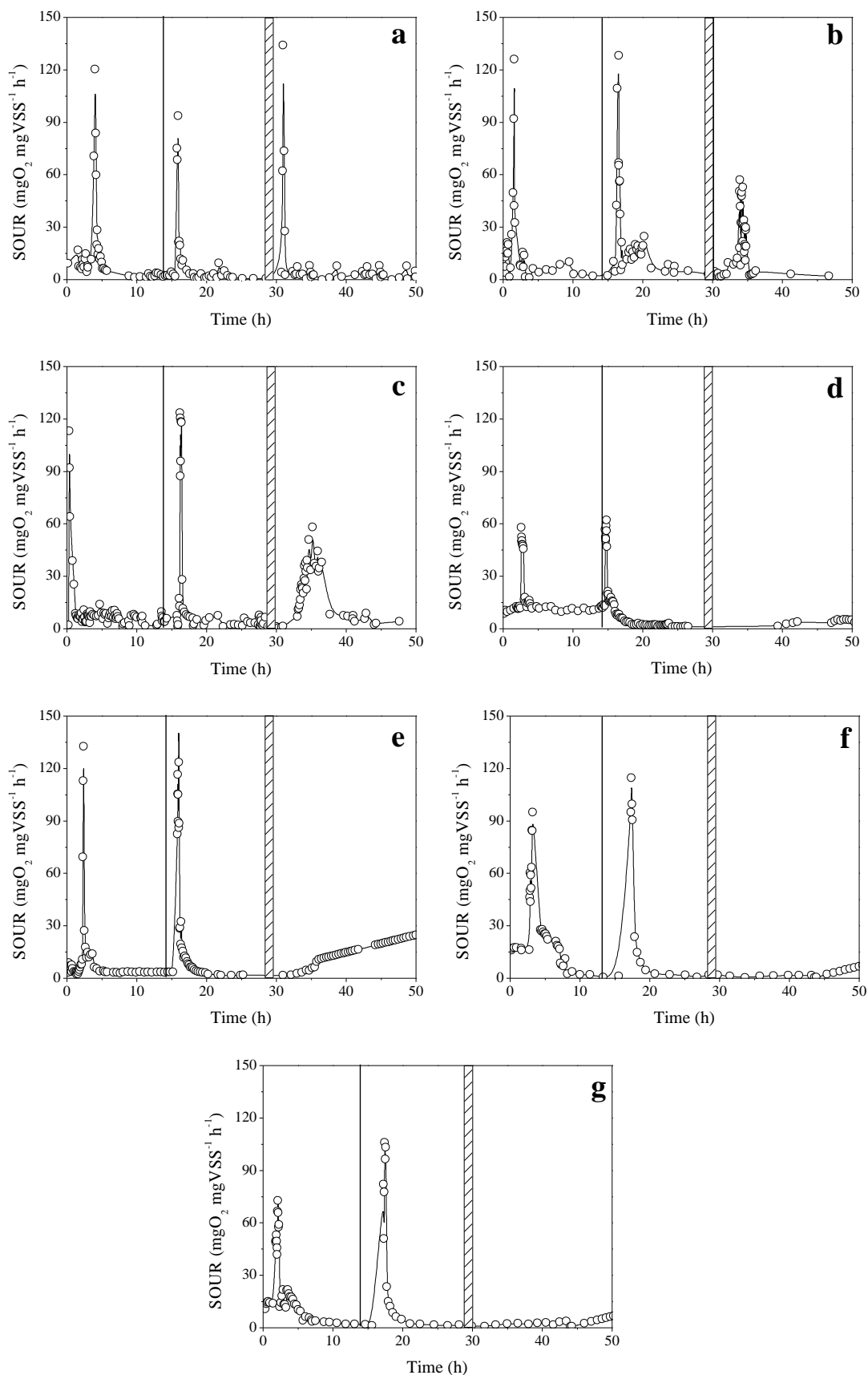


Figure 3. SOUR profile for long-term respirometric assays of [Emim][Cl] (a), [Bmim][Cl] (b), [Omim][Cl] (c), [Emim][HSO₄] (d), [Emim][NTf₂] (e), [N4444][NTf₂] (f) and [Choline][NTf₂] (g).

In order to study the inhibitory and toxic effect of the ILs on the sludge, long term respirometric assays were performed. Although these assays are not standardized, they can provide information to know the behavior of the sewage microorganisms in wastewater with presence of ILs, with hydraulic retention time considerably longer than in short-term experiments (15 min). Seven ILs were used to study the effect of the anion and cation structure on inhibition and toxicity of the sewage sludge (Figure 3). ILs were chosen according to the results obtained in short-term toxicity test: [Emim][Cl], [Bmim][Cl] and [Omim][Cl] allowed to determine the effect of the alkyl chain length and compare the results with those obtained in 15 minutes toxicity assay. As [NTf₂]⁻ was established as a high toxic anion, the effect on the sludge after the exposure to different ILs' families containing [NTf₂]⁻ was also evaluated ([Emim][NTf₂], [N4444][NTf₂] and [Choline][NTf₂]). Finally, [HSO₄]⁻ was described as the highest toxic anion according to previous short assays, so it was also considered necessary to measure the sludge activity at [Emim][HSO₄] exposure for longer time.

Table 3. Maximum specific oxygen uptake rate (SOUR_{max}) and accumulated oxygen consumption (AOC) in each Run for long-term respirometric assays.

IL	Parameter	Run 1	Run 2	Run 3	Inhibition	Toxicity
[Emim][Cl]	SOUR _{max}	120.3	93.8	134.1	0.22	--
	AOC	24.5	19.0	23.8	0.23	0.03
[Bmim][Cl]	SOUR _{max}	126.0	128.1	49.9	--	0.60
	AOC	23.5	24.7	15.7		0.33
[Omim][Cl]	SOUR _{max}	113.1	118.1	58.1	--	0.49
	AOC	23.3	25.4	19.4	--	0.17
[Emim][HSO ₄]	SOUR _{max}	58.0	62.1	0	--	1
	AOC	22.3	20.1	0	0.1	1

[Emim][NTf₂]	SOUR _{max}	152.2	132.3	0	0.13	1
	AOC	25.7	26.1	0	--	1
[N4444][NTf₂]	SOUR _{max}	95.6	114.7	0	--	1
	AOC	26.3	34.1	0	--	1
[Choline][NTf₂]	SOUR _{max}	72.8	106.1	0	--	1
	AOC	23.0	25.9	0	--	1

1

2 **Figure 3a-c** shows SOUR profile for imidazolium-based ILs with different alkyl-chain
3 length ([Emim][Cl], [Bmim][Cl] and [Omim][Cl], respectively), with the aim to analyze
4 the recovery of sewage sludge activity (run 3). After a pulse of sodium acetate,
5 SOUR_{max} was nearly 120 mgO₂ gVSS⁻¹ h⁻¹ and experimented a fast decay due to the
6 immediate consumption of the biodegradable substrate by microbial population. Once
7 the SOUR reached the endogenous oxygen respiration, a second pulse of a
8 biodegradable substrate (sodium acetate) mixed with IL was added. Similar SOUR_{max}
9 results as run 1 were obtained. Maximum SOUR values and AOC obtained in each
10 stage are summarized in **Table 3**. Accumulated oxygen consumption values remained
11 invariable in stages 1 and 2, concluding that there was no inhibition effect of
12 [Bmim][Cl] and [Omim][Cl] on the sludge. However, a slight inhibition was observed
13 in case of [Emim][Cl] (22%). Once the ILs have been in contact with the
14 microorganisms for a long period of time (endogenous consumption), the sludge was
15 washed to remove residual ILs and was submitted again for a new pulse with sodium
16 acetate (Run 3) leading to different answers. [Emim][Cl] showed no toxic effect on
17 activated sludge because reached similar AOC and SOUR_{max} values (24 mgO₂ L⁻¹ and
18 120 mgO₂ mgVSS⁻¹ h⁻¹, respectively) to those obtained in the first stage. However, toxic
19 character of [Bmim][Cl] and [Omim][Cl] was found in run 3: the sludge was affected
20 by the previous contact with IL, which caused a decrease in SOUR_{max}, despite the

1 absence of inhibition in run 2. The results of AOC values together with wider SOUR
2 peaks reflected that the degradation capacity of the sludge decreased as increasing the
3 length of the alkyl-chain and microorganisms required more time for total consumption
4 of the biodegradable compound. With the aim to study the capability of the
5 microorganisms to return to initial degradation rates, an additional pulse with sodium
6 acetate (run 4) was performed. Comparable SOUR results of those found in run 1 were
7 obtained, indicating that although the sludge was previously affected by the IL presence,
8 after a certain period of time it can be recovered from the toxic effect and return to
9 initial biodegradation capacity. Comparing these results with those shown in **Table 2**,
10 the toxic effect of imidazolium-based ILs increased with the number of carbons in the
11 alkyl-chain length, confirmed by a lower degradation activity of easily biodegradable
12 substrate in case of [Omim][Cl] in comparison with smaller imidazolium-based ILs.

13 The effect of a well-known toxic anion, $[\text{NTf}_2]^-$ was also studied in long-term
14 respirometric assays for [Emim][NTf₂], [N4444][NTf₂] and [Choline][NTF₂] (**Figure 3**
15 **e-g**). As shown in assays with imidazolium-based ILs, run 1 and 2 described
16 comparable SOUR profiles, with similar SOUR_{max} and time required for total
17 consumption of sodium acetate. However, in the last stage, the absence of sludge
18 recovery described a great toxicity effect for [NTf₂]-based ILs. The results of EC₅₀ in
19 **Table 2** indicated that the ILs studied presented low toxic values, so it was expected
20 that the contact of the IL with the sludge should affect in a similar way than
21 imidazolium-based ILs with [Cl]⁻ anion. However, none of the cases led to a sludge
22 recovery in run 3, exhibiting [NTf₂]⁻ anion as a very toxic compound that can affect
23 activated sludge to a greater extent, especially for extended contact times. On the other
24 hand, accumulated oxygen consumption and SOUR_{max} in run 2 for [N4444][NTf₂] and
25 [Choline][NTf₂] was higher than the values obtained in the first stage, reflecting a

possible consumption of this cation despite the toxic character of the anion. In order to confirm this statement, further biodegradable assays would be required. An additional experiment using EmimHSO₄ was performed in order to compare the anion effect ([Cl]⁻, [HSO₄]⁻ and [NTf₂]⁻ depicted in **Figure 3 a, d, e**, respectively) on the activated sludge fixing a harmless cation as Emim⁺. Following the trend obtained in short-term assays, [HSO₄]⁻ anion was found to show the highest toxicity. No recovery of the sludge was appreciated, even with an additional pulse of biodegradable substrate (run 4) where the biomass remained unable to biodegrade sodium acetate showing similar SOUR profile that the depicted in run 3.

Biodegradability assays

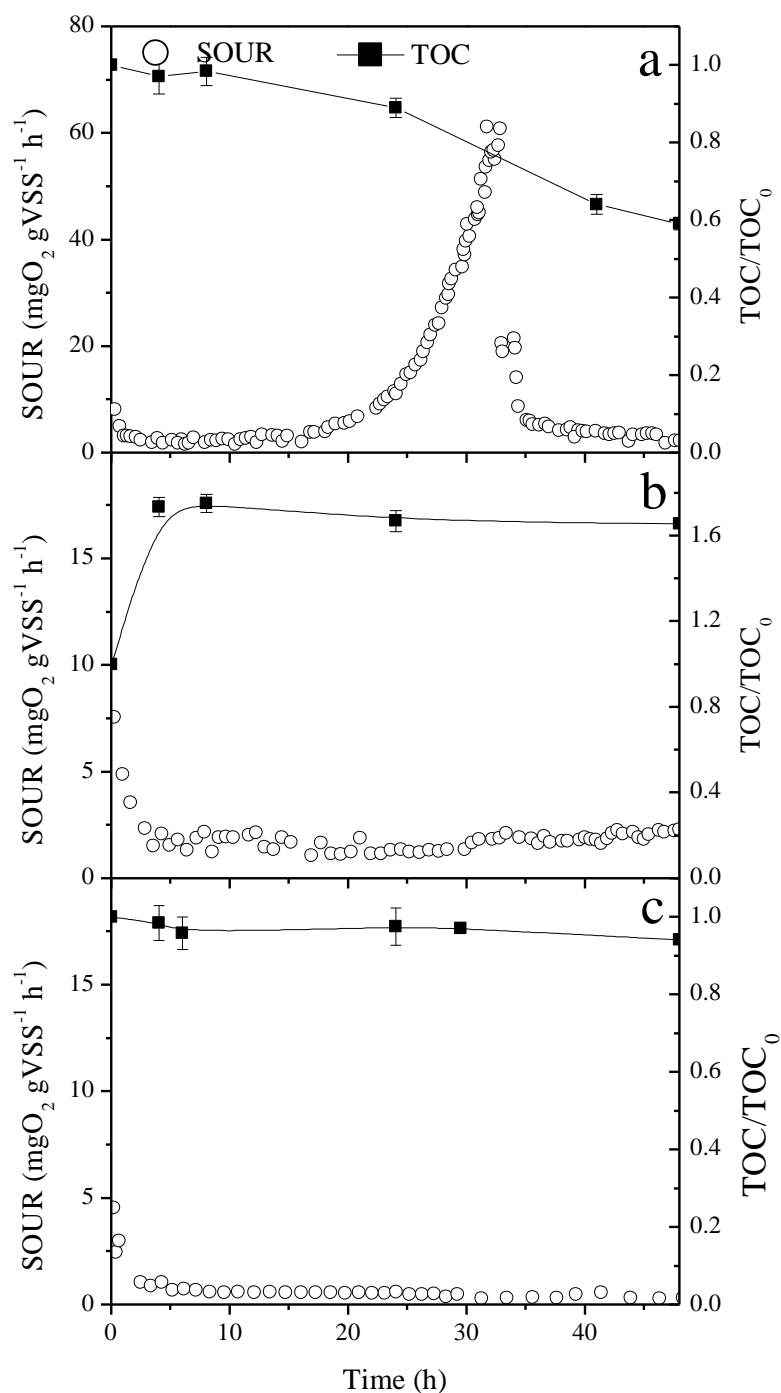


Figure 4. Sour profile and normalized TOC evolution for [Choline][NTf₂] (a), [N4444][NTf₂] (b) and [Emim][HSO₄] (c) during fast biodegradability test. Error bars represent \pm standard deviation

According to the results obtained in toxicity assays, the IL addition (Run 2) caused an increase in the AOC in some cases, which suggests that those ILs may show certain biodegradability. This fact was only observed in tetrabutylammonium and choline

families ([N4444][NTf₂] and [Choline][NTf₂]), despite the toxic behavior of [NTf₂]⁻ anion. With the aim of evaluating the biodegradability of a harmless cation combined with a potential hazardous anion, an additional IL was carefully chosen ([Emim][HSO₄]), which was previously described as the most toxic IL by the short-term toxicity test (15 min). Besides the well-known refractory character to biological treatments of the imidazolium cation [Emim]⁺ (Garcia et al. 2005) the biodegradability of the hazardous anion [HSO₄]⁻ was checked. A respirometric test capable of providing a rapid evaluation of the biodegradability of pollutants is depicted in **Figure 4** for [Choline][NTf₂], [N4444][NTf₂] and [Emim][HSO₄] in terms of SOUR profile and TOC evolution.

The consumption of the cation ([Choline]⁺) caused a decrease in TOC value as the SOUR increased after an acclimation period of 20 h (**Figure 4a**). For the biodegradation of the cation, an acclimation period was required, from which was possible to establish a direct relationship between TOC decrease and cation removal. The amount of Choline was reduced from 95 to 28 mg L⁻¹, corresponding to the mineralized carbon measured in the SOUR increase. However, the [NTf₂]⁻ anion remained invariable along the respirometric assay, which was related to its non-biodegradable character (Mena et al. 2019), concluding that SOUR increase was only caused by the choline cation removal. Some authors described the presence of polar functional groups (ether, hydroxyl and nitrile groups) as a solution for reaching a better ILs biodegradation ratio (Petkovic et al. 2010, Krishnan et al. 2016) as occurred in Choline-based ILs with the presence of an alcohol substituent. In addition, linear structures with four or more carbons in their alkyl-chains could be more accessible to bacteria and the attack by oxygenases (Docherty et al. 2007, Gathergood et al. 2004).

On the other hand, the biodegradability of Choline cation and the toxicity of $[\text{NTf}_2]^-$ was corroborated in the long-term assays of $[\text{Choline}][\text{NTf}_2]$ (**Figure 3g**). The increase of SOUR_{max} in presence of the mixture sodium acetate-IL compared to SOUR_{max} due to sodium acetate alone (run 1), was due to the biodegradability of the $[\text{Choline}]^+$ cation. However, the $[\text{NTf}_2]^-$ anion caused toxic effect on the sludge after washing (run 3), where microorganisms were only capable of registering endogenous SOUR.

In the case of $[\text{N4444}][\text{NTf}_2]$ (**Figure 4b**), it was observed a decrease in SOUR in the first hours of reaction, coinciding with an increase of TOC value. The decrease in oxygen consumption of the sludge is consequence of the toxic effect of $[\text{NTf}_2]^-$ anion, which caused microbial death, cell lysis and the release of intracellular content to the reaction medium. With the aim to establish a linear relationship between microorganism death and TOC from cell lysis, increasing concentrations of microorganisms ($50 - 500 \text{ mgVSS} \cdot \text{L}^{-1}$) were submitted to successive heat shocks, where the drastic change in temperature (from -20°C to 80°C) caused cell lysis and allowed to relate the increase in the available carbon with the reduction of biomass concentration ($\text{mg VSS L}^{-1} = 6.092 \cdot \text{mg TOC L}^{-1}$; $R^2 = 0.99$). An increase of TOC values around 30 mg L^{-1} caused cell lysis of 180 mgVSS L^{-1} of activated sludge, concluding that the increase of TOC was only due to the breakage of the microorganisms, reducing the activated sludge present in the reaction on 50% of the initial concentration ($350 \text{ mg VSS L}^{-1}$). After the first 5 h, the sludge maintained an endogenous respiration with no increase in SOUR value, concluding that $[\text{N4444}][\text{NTf}_2]$ was no biodegradable. Despite the ionic liquids can combine multiple choices of cation and anion and create “designed ILs”, the toxic or biodegradable effect depends not only on the components that constitute the IL, but also on the way they interact, since the same anion, in this case $[\text{NTf}_2]^-$, can cause different

toxic-lethal or inhibitory effects in the sludge when combined with [N4444]⁺ or [Choline]⁺ cations, respectively.

The biodegradability of [Emim][HSO₄] was also depicted in **Figure 4c**. A decay in SOUR was detected once the toxic was put together with the activated sludge. After that, SOUR evolution depicted a plateau until the end of the assay, corresponding to the endogenous respiration. However, there was no increase in TOC values, remaining constant throughout the 48 h assay. [Emim][HSO₄] showed no biodegradable character and exhibited an inhibition of the sludge respiration. However, the toxic effect was not as drastic as for [N4444][NTf₂], no cell lysis occurred, matching with the results obtained in long-term inhibition assays for [Emim][HSO₄]. The main difference between both toxic ILs to explain the effect on the sludge lied in the physico-chemical properties of the anion. [NTf₂]⁻ presented hydrophobic character while [HSO₄]⁻ was considered hydrophilic (Jain and Kumar 2016). Hereby, the hydrophobic IL tended to be adsorbed on the microorganisms, while the hydrophilic one remained on the aqueous phase, causing less toxic effect than [NTf₂]-containing ILs.

4. Conclusions

Respiration inhibition tests using activated sludge of 13 different ILs showed higher EC₅₀ values (lower toxicity) than those obtained by *Vibrio fischeri* response, despite similar toxicity trends were observed for both assays. The effect of structural IL characteristics indicated that the increase in number of carbons of alkyl-chain length in imidazolium-based ILs presented EC₅₀ values 24-fold lower from [Emim][Cl] than for [Omim][Cl] (from 2 to 8 carbons, respectively). Among to the anions studied, the following toxicity sequence was established: [HSO₄]⁻ > [NTf₂]⁻ > [Cl]⁻ > [EtSO₄]⁻. EC₅₀

values were also lower for aromatic-based IL, more perceptible in presence of anions with hydrophobic character ([NTf₂]⁻).

Long-term toxicity assays showed that higher contact of the pollutant with the sludge (15 h) caused more toxic effect than short-term toxicity assays for all ILs studied. The recovery of the sludge activity was affected by apparently harmless ILs in 15 min tests, which induced a decrease of the sludge respiration when exposed to a biodegradable compound.

Most of the studied ILs showed high toxicity and poor biodegradability which would be recalcitrant in sewage systems. Only [Choline]⁺ was totally biodegraded and a fairly high TOC reduction was observed along the biodegradability test. Biodegradability experiments performed with ILs containing anions described as toxic ([NTf₂]⁻ and [HSO₄]⁻) showed no oxygen consumption ([Emim][HSO₄]) or lysis of microbial cells ([N4444][NTf₂]).

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