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Comprehensive Analysis of the Acute Toxicity of Ionic Liquids Using Microtox® Bioassays

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Abstract: The ecotoxicity of a set of 30 ionic liquids, namely 23 aprotic compounds (APILs) and 7 protic compounds (PILs), was analyzed in this work by monitoring the inhibition of the bioluminescence of the bacteria *Aliivibrio fischeri* with varying concentrations of ILs utilizing the Microtox® standard toxicity test. The study covered ILs that have various synthetic natures, PILs and APILs, with a common anion or cation, and different alkyl chain lengths. The results indicate that both moieties, anion and cation, have an influence on toxicity, these being the ILs with the bis((trifluoromethyl)sulfonyl)imide (TFSI) anion and imidazolium cation, which are the most harmful, while those less toxic correspond to ammonium-based ILs. The alkyl chain length seems to have the most significant impact on toxicity, except for tris(pentafluoroethyl) trifluorophosphate (FAP) anion-based ILs, which, interestingly, showed the opposite behavior. A critical alkyl size (CAS) at C = 6 was observed for the rest of the families, resulting in a significant reduction in the effective concentration (EC) values: the connection between this CAS and toxicity has never been observed before, and it indicates a threshold that marks the end of harmlessness (C < 6) and the start of toxicity (C > 6).

Keywords: ionic liquids; Microtox® test; anion effect; cation effect; alkyl chain length effect

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1. Introduction

The term "ionic liquid" (IL) encompasses a broad category, which generally consists of the combination of an organic cation and organic or inorganic anion, with a low melting point, i.e., lower than 100 °C, being one of its main characteristics. A wide variety of ions can be found, ranging from inorganic to organic, chiral or achiral, and including fully or partially ionized acids or bases, charged bridging ligands, metalate coordination polymers, and organic polymeric metal ions, among others [1–3]. The high anisotropic character of these compounds is mainly due to their characteristic electrostatic and dispersive interactions.

These compounds are commonly labelled as green solvents, which has resulted in an increase in their potential applications in recent decades. Since the first IL, ethylammonium nitrate (EAN), was synthesized in 1914 by Paul Walden, ILs have been used in a wide variety of fields, including synthesis, coordination chemistry, nanotechnology, polymer materials, and electrolytes [4–7]. ILs can be classified in two different groups based on their chemical behavior: aprotic and protic ionic liquids. Protic ionic liquids (PILs) can be easily synthesized by combining a Brönsted acid and a Brönsted base, and they are characterized by the presence of a labile proton. Aprotic ionic liquids (APILs) are typically formed through Menshutkin-type reactions [8].

These fascinating compounds possess unique attributes, including the widely acknowledged low vapor pressure, high thermal and chemical stability, a broad electrochemical window, and low toxicity. However, their most noteworthy characteristic is their flexibility

of design or tunability, which allows the modification of physicochemical properties by adjusting anions and cations or introducing functional groups into their alkyl chains. This tunable nature has significantly increased the number of scientific studies analyzing their properties and the number of applications in recent decades. According to the Web of Knowledge (WOS) database, more than 9 thousand review papers had been published, which nearly 1000 corresponding to last past year, evidencing their interest and applicability.

Three generations of ILs have been considered by some authors [9,10]. The first generation is linked to halogenated anions, featuring high thermal stability and large liquid ranges but sensitivity to air and water. The second generation is stable to air and water but exhibits higher toxicity and reduced biodegradability, primarily associated with phosphonium and nitrogen-based anions. The third generation displays lower toxicity, high biodegradability, and biological activity, but is less suitable as a solvent.

In addition to its optimal physico-chemical properties, the use of ILs has also been highlighted by the introduction of European Union environmental laws, specifically REACH (Regulation concerning Registration, Evaluation, Authorization, and Restriction of Chemicals), that emphasize the importance of using safe materials in the industrial applications. This underscores the principles of Green Chemistry, encompassing prevention, economic considerations, less hazardous chemical synthesis, efficient energy use, utilization of renewable raw and biodegradable materials, and the assurance of an adequate level of chemical safety.

However, one of the characteristics of ILs most repeated in the papers is its low toxicity, as mentioned above; this recurrent statement is based on the low volatility of these compounds, without rigorous studies of the effects of these compounds on the different trophic levels. Unlike other physical and thermophysical properties, the influence of the two moieties of IL and substituents on toxicity is not yet clarified. Thus, the effect of the anion on the overall IL toxicity is the subject of debate in the literature, with different reports ranging from it having no or weak influence, to having a very important role in toxicity. On the other hand, IL toxicity is more extensively studied with respect to the cation, and therefore, toxicity is primarily attributed to this moiety. The effect of cationic alkyl side chains is one of the most thoroughly investigated aspects of IL toxicity, and numerous studies have established a direct correlation between toxicity and longer alkyl side chain lengths through various trophic levels [11–13].

Various techniques are currently employed to determine the toxicity of ILs, and the choice depends on the trophic level under consideration. Aquatic ecosystems (algal assays, *Daphnia magna*, *Aliivibrio fischeri*), microorganisms (mainly bacteria due to their short generation time), cytotoxicology (cell lines), enzyme inhibition, and animal tests are some of the most used microorganisms to determine the toxicity of ILs [14]. In addition to the most common methods, new methodologies are being developed to assess the toxicity of ILs as, for example, activated sludge response [15] or soil microbial activity and seed germination [16]. Among all the mentioned techniques, the acute toxicity test towards bioluminescent bacterium *Aliivibrio Fischeri* stands out as one of the less time-consuming, cost-effective methods, and most sensitive and susceptive bacteria to ILs [11,14,17,18].

To advance the understanding and the deep comprehension of the relationship between toxicity and structure and broadening the database concerning the toxic effects of ionic liquids (ILs), ecotoxicity assessments were carried out on a diverse large set of ILs in this work. The study covered ILs that have various synthetic natures, i.e., PILs and APILs, that have a common anion or cation, and different alkyl chain lengths. The tests were performed by monitoring changes in the bioluminescence of the bacteria *A. fischeri*, utilizing the Microtox® standard toxicity test. The effective concentration (EC_{50}) of these mixtures was determined over three standard periods of time, namely 5, 15, and 30 min, and compared with the corresponding values for pure ILs.

2. Materials and Methods

A set of 30 ILs were chosen for this work, corresponding to 23 aprotic compounds (APILs), and 7 protic compounds (PILs) with the common nitrate anion (NO_3). The choice of the following anions was due to their presence in numerous studies: TFSI related to energy, mostly in electrolytes for lithium-ion batteries [19]; FAP is proposed as a lubricant [20]; and NO_3 is the most used anion on PILs [21,22]. Regarding the cations, different cationic natures have been studied, mainly imidazolium, pyrrolidinium, piperidinium, pyridinium and ammonium. Table 1 provides a summary of the selected ILs, with their CAS number and initial purity. In addition, Figures 1 and 2 also display the set of study ILs, and the chemical structures, separating anion and cation for a better understanding and visualization of the proposed work. All the compounds were previously dried into high vacuum under constant stirring for at least 24 h, and the water content, measured by Karl Fischer titration, for all of them was below 100 ppm.

Table 1. Structure, abbreviations, and purity of the selected ILs.

Name Molecular Mass (g/mol)	Abbreviation CAS Number	Purity Molecular Mass (g/mol)
1-ethyl-3-methylimidazolium bis((trifluoromethyl)sulfonyl)imide	C_2C_1Im TFSI 174899-82-2	>0.99 [1] 391.3
1-butyl-3- methylimidazolium bis((trifluoromethyl)sulfonyl)imide	C_4C_1Im TFSI 174899-83-3	>0.99 [1] 419.4
1-butyl-2,3-dimethyl imidazolium bis(trifluoromethylsulfonyl)imide	$C_4C_1C_1$Im TFSI 350493-08-2	>0.99 [1] 433.39
1-hexyl-3- methylimidazolium bis((trifluoromethyl)sulfonyl)imide	C_6C_1Im TFSI 382150-50-7	>0.99 [1] 447.4
1-octyl-3- methylimidazolium bis((trifluoromethyl)sulfonyl)imide	C_8C_1Im TFSI 178631-04-4	>0.99 [1] 475.5
1-allyl-3-methylimidazolium bis((trifluoromethyl)sulfonyl)amide	AC_1Im TFSI 655249-87-9	>0.99 [1] 403.3
1-allyl-3-methylimidazolium chloride	AC_1Im Cl 65039-10-3	>0.98 [1] 158.6
1-allyl-3-methylimidazolium dicyanamide	AC_1Im DCA 917956-73-1	>0.99 [1] 190.2
1-butyl-3-methyl imidazolium tris(pentafluoroethyl) trifluorophosphate	C_4C_1Im FAP 917762-91-5	>0.99 [2] 584.23
1,3-dimethylimidazolium dimethylphosphate	C_1C_1Im DMP 945611-27-8	>0.99 [1] 222.18
1-ethylpyridinium bis((trifluoromethyl)sulfonyl)imide	C_2Py TFSI 712354-97-7	>0.99 [1] 388.3
1-butylpyridinium bis((trifluoromethyl)sulfonyl)imide	C_4Py TFSI 187863-42-9	>0.99 [1] 416.4
1-hexylpyridinium bis((trifluoromethyl)sulfonyl)imide	C_6Py TFSI 460983-97-5	>0.99 [1] 444.4
1-methyl-1-propylpiperidinium bis((trifluoromethyl)sulfonyl)imide	C_3C_1Pip TFSI 608140-12-1	>0.99 [1] 422.4
1-methyl-1-butylpiperidinium bis((trifluoromethyl)sulfonyl)imide	C_4C_1Pip TFSI 623580-02-9	>0.99 [1] 436.4
1-butyl-1-methylpyrrolidinium bis(trifluoromethylsulfonyl)imide	C_4C_1Pyrr TFSI 223437-11-4	>0.99 [1] 422.41

Table 1. *Cont.*

Name Molecular Mass (g/mol)	Abbreviation CAS Number	Purity Molecular Mass (g/mol)
1-(2-methoxyethyl)-1-methylpyrrolidinium tris(pentafluoroethyl)trifluorophosphate	$C_1OC_2C_1$PyrrFAP 1195983-48-2	>0.98 [2] 589.24
1-hexyl-1-methylpyrrolidinium bis((trifluoromethyl)sulfonyl)imide	C_6C_1Pyrr TFSI 380497-19-8	>0.99 [1] 450.5
1-octyl-1-methylpyrrolidinium bis((trifluoromethyl)sulfonyl)imide	C_8C_1Pyrr TFSI 927021-43-0	>0.99 [1] 478.5
1-butyl-1-methylpyrrolidinium tris(pentafluoroethyl) trifluorophosphate	C_4C_1Pyrr FAP 851856-47-8	>0.98 [2] 587.28
1-butyl-1-methylpyrrolidinium tris(nonafluorobutyl) trifluorophosphate	C_4C_1Pyrr C_4FAP 851856-47-8	>0.99 [2] 830.32
1-butyl-1-methylpyrrolidinium tris(perfluorooctyl) trifluorophosphate	C_4C_1Pyrr C_8FAP ---	>0.98 [2] 1430.41
Tetrabutylphosphonium tris(pentafluoroethyl) trifluorophosphate	Ph_3t FAP 482635-81-4	>0.98 [2] 704.36
Propylammonium Nitrate	EAN 22113-86-6	>0.97 [1] 108.10
Butylammonium Nitrate	PAN 22113-88-8	>0.97 [1] 122.12
Ethylammonium Nitrate	BAN 58888-50-9	>0.97 [1] 136.15
Penthylammonium nitrate	PEAN ---	≥99 [3] 150.18
Hexylammonium nitrate	HEAN ---	≥99 [3] 164.20
Octylammonium nitrate	OAN ---	≥99 [3] 192.25
Ethylimidazolium nitrate	C_2Im NO_3 501693-38-5	>0.98 [1] 159.14

[1] Iolitec; [2] Merck KGaA; [3] Synthesized [23].

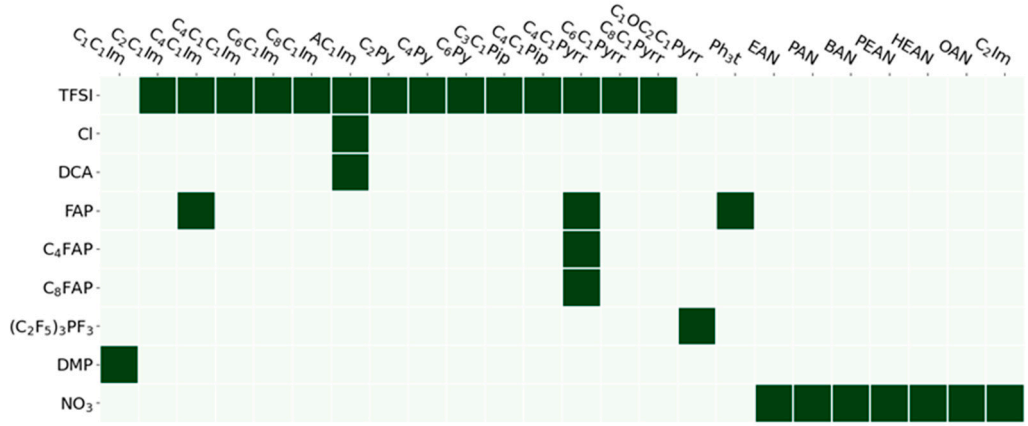

Figure 1. Ionic liquids selected for this work.

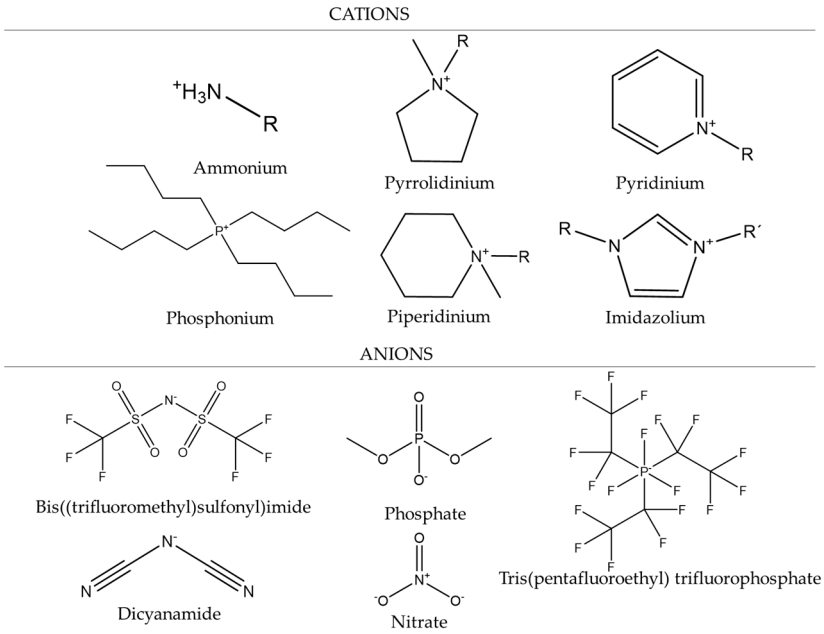

Figure 2. Chemical structure of the selected ions. R represents the abbreviation for the different selected radicals of Table 1.

Standard Microtox® liquid phase assays (M500 Analyzer—Modern water, Rema Tek LLC, Bonsall, USA) were employed for the evaluation of the acute toxicity by measuring the luminescence inhibition of the rod-shaped Gram-negative marine bacteria *Aliivibrio fischeri* (*A. fischeri*), the bioluminescence of which, through a population-dependent mechanism known as quorum sensing, is sensitive to various toxic substances [24,25]. The light output was measured after being exposed to different IL aqueous serial solutions (ranging from 0 to 81.9%); 100% of the IL corresponds to the known concentration of a stock solution previously prepared, at 15 °C, and by comparing it with a blank control sample. EC_{50} is commonly used as the primary parameter, representing the concentration causing a 50% reduction in bacterial luminescence. In addition, EC_{10} and EC_{20} (concentrations resulting in 10% and 20% reduction in initial luminescence, respectively) also offer valuable intermediate toxicity references. These concentrations are calculated, along with the corresponding 95% confidence intervals, through a non-linear regression using the least-squares method to fit the data to the logistic equation. The decrease in bioluminescence with increasing sample concentration serves as an integrated measure of physiological impairment of the bacteria, thereby demonstrating the toxic effect of the studied compound [12].

Two different classifications were employed in this study to discern the toxicity of the compounds. The first classification, widely used and proposed by Passino and Smith [26], is based on the values of EC_{50} at 30 min. Thus, ecotoxicity is classified into five levels according to EC_{50}:

EC_{50} > 1000 mg/L means that the compound is relatively harmless;
1000 mg/L > EC_{50} > 100 mg/L, practically harmless;
100 mg/L > EC_{50} > 1 mg/L toxic;
1mg/L > EC_{50} > 0.1 mg/L highly toxic;
0.1 mg/L > EC_{50} > 0.01 mg/L extremely toxic;
EC_{50} < 0.01 mg/L supertoxic.

The other classification is based on the studies of Chang et al. [27], who introduced the concept of toxicity units, calculated by Equation (1):

$$TU = \frac{100}{EC_{50}} \quad (1)$$

where EC_{50} (in mg/L) is measured after 15 min of exposition. Thus, the toxicity steps are defined as follows: TU < 1 Non-toxic; 1 < TU< 10 Toxic; 10 < TU < 100 Very Toxic; TU > 100 Extremely Toxic.

3. Results and Discussion

Figure 3 shows, as an example, the inhibition of bioluminescence response for 30 min of exposure against the logarithm of the concentration of the ILs with common C_4C_1Pyrr cation and ammonium nitrate family. All the inhibition responses against the concentration for all the analyzed ILs were fitted to a logistic equation, as indicated in the Section 2. From these fittings, the values of EC_{50}, EC_{20}, and EC_{10} after 5 min, 15 min, and 30 min of exposition were calculated and are presented in Tables 2–4. As previously mentioned, EC_{10} and EC_{20} serve as initial benchmarks for estimating the lowest observed effect concentration. In particular, EC_{10} stands out as a reliable parameter for the effects independently of concentration or for the identification of compounds with minimal environmental risks [28]. The bacterial bioluminescence reactions are widely recognized as indicators of cellular metabolism in bacteria, where a decrease in bioluminescence corresponds to a reduction in cellular respiration [29]. Therefore, the toxicity trend can be determined based on the ionic nature. It should be noted that the trend of toxic effects does not depend on the exposure time, which suggests a common mechanism in the effect on bacteria throughout the exposure.

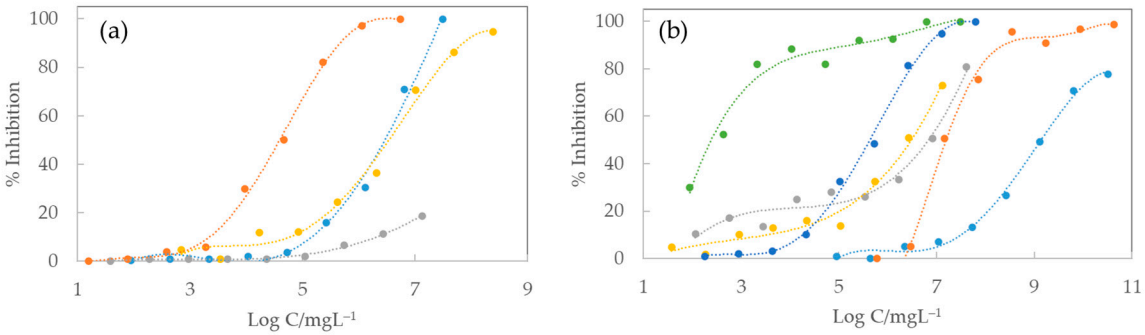

Figure 3. Inhibition of bioluminescence for 30 min of exposure against logarithm of concentration of the butylmethylpyrrolidinium cation-based ILs (**a**): (●) C_4C_1Pyrr TFSI, (●) C_4C_1Pyrr FAP, (●) C_4C_1Pyrr C_4FAP and (●) C_4C_1Pyrr C_8FAP, and nitrate anion-based ILs (**b**): (●) EAN, (●) PAN, (●) BAN, (●) PEAN, (●) HEAN and (●) OAN.

Scarce studies of acute toxicity tests on *Alivibrio fischeri* for the studied ILs can be found in the previous literature. Similar values of EC_{50} to those reported here for C_2C_1Im TFSI, C_4C_1Im TFSI, C_6C_1Im TFSI, C_8C_1Im TFSI, $C_4C_1C_1$Im TFSI, C_3C_1Pip TFSI, AC_1Im Cl, C_4Py TFSI, and C_8C_1Pyrr TFSI ILs were found in previously published papers [30–35].

Although many studies indicate that the cation has more influence than the anion on toxicity, our results show an important influence of both anion and cation on the toxicity of ILs, observing that the highest values of EC_x (x = 10, 15, 30), i.e., the less toxic ILs, correspond to EAN and PAN, independently of the time of exposure. On the contrary, the most harmful ILs, which have the lowest EC_x values, have been observed in OAN and C_8C_1Im TFSI.

Table 2. EC_{50} effective concentration values in mg/L and the respective 95% confidence intervals, obtained after 5, 15, and 30 min of exposure of the marine bacteria *A. fischeri*.

IL	EC_{50} 5 min/mg/L (Lower; Upper) Limits	EC_{50} 15 min/mg/L (Lower; Upper) Limits	EC_{50} 30 min/mg/L (Lower; Upper) Limits
C_2C_1Im TFSI	367.43 (247.06; 487.80)	189.97 (104.68; 275.27)	113.08 (41.74; 184.42)
C_4C_1Im TFSI	78.48 (36.48; 120.48)	54.85 (24.91; 84.79)	46.58 (14.68; 78.48)
$C_4C_1C_1$Im TFSI [12]	150.44 (72.43; 228.49)	113.32 (82.29; 144.35)	98.70 (82.39; 115.01)
C_6C_1Im TFSI	26.29 (24.00; 28.57)	23.64 (21.83; 25.45)	29.60 (27.66; 31.53)
C_8C_1Im TFSI	3.57 (3.43; 3.71)	4.23 (3.86; 4.59)	5.97 (4.85; 7.10)
AC_1Im TFSI	655.63 (417.77; 893.49)	337.68 (235.35; 440.01)	249.30 (175.09; 323.52)
AC_1Im Cl	1399.39 (463.05; 2335.73)	842.87 (531.77; 1153.97)	715.21 (479.39; 951.03)
AC_1Im DCA	1181.58 (866.64; 1496.51)	639.62 (538.85; 740.39)	534.19 (454.01; 614.38)
C_4C_1Im FAP	97.64 (70.21; 125.1)	77.43 (59.59; 95.27)	74.37 (58.05; 90.69)
C_1C_1Im DMP	1186.31 (1071.08; 1300.92)	1198.33 (1111.72; 1285.07)	1254.24 (1172.95; 1337.75)
C_2Py TFSI	314.24 (175.08; 453.40)	133.72 (54.34; 213.11)	74.31 (0.00; 150.51)
C_4Py TFSI	150.21 (133.75; 166.66)	106.84 (93.51; 120.17)	92.90 (78.26; 107.54)
C_6Py TFSI	44.16 (40.40; 47.92)	40.30 (36.29; 44.30)	45.84 (41.92; 49.76)
C_3C_1Pip TFSI	215.24 (161.47; 269.01)	138.13 (104.77; 171.49)	117.07 (82.16; 151.98)
C_4C_1Pip TFSI	150.63 (140.30; 160.95)	119.23 (110.40; 128.07)	107.37 (101.19; 113.56)
C_4C_1Pyrr TFSI [12]	1463.91 (1162.13; 1765.69)	964.58(791.32; 1137.88)	714.43 (577.92; 851.21)
C_6C_1Pyrr TFSI	88.95 (79.22; 98.69)	70.29 (62.57; 78.00)	75.26 (66.25; 84.27)
C_8C_1Pyrr TFSI	15.71 (13.10; 18.31)	15.80 (13.03; 18.57)	23.30 (16.95; 30.25)
C_4C_1Pyrr FAP	805.87 (554.86; 1056.83)	707.70 (562.28; 853.11)	604.90 (516.64; 693.16)
$C_1OC_2C_1$Pyrr FAP	62.37 (31.43; 93.31)	37.94 (23.69; 52.19)	31.59 (22.00; 41.19)
C_4C_1Pyrr C_4FAP	96.75 (71.83; 121.66)	62.28 (36.83; 87.74)	50.54 (22.51; 78.57)
C_4C_1Pyrr C_8FAP	--	--	5430.07 (1845.01; 8224.73)
Ph_3t FAP	3555.25 (2429.44; 4605.18)	1096.36 (569.37; 1623.35)	805.63 (417.84; 1193.42)
EAN [12]	12,582.07 (8186.64; 16977.50)	10,665.47 (6650.14; 14680.80)	9711.63 (6561.46; 12860.79)
PAN [23]	8314.99 (7268.61; 9361.37)	5932.88 (5043.45; 6822.30)	5827.78 (4998.72; 6656.84)
BAN [23]	1491.99 (636.69; 2347.04)	1066.71 (551.52; 1581.90)	1017.14 (478.49; 1555.78)
PEAN [23]	1116.9 (945.1; 1288.8)	1073.6 (836.3; 1311.0)	1029.8 (792.5; 1267.1)
HEAN [23]	85.69 (77.71; 93.68)	57.54 (52.98; 62.10)	50.12 (44.85; 55.39)
OAN [23]	9.70 (6.37; 13.03)	7.33 (5.23; 9.43)	7.38 (5.51; 9.25)
C_2Im NO_3 [12]	612.55 (395.90; 828.01)	573.77 (372.29; 774.55)	597.89 (408.00; 785.08)

With regard to the toxicity order for anions, the following trend is observed: TFSI > FAP > NO_3 > DCA > Cl> DMP. The cation families can be also ordered according to the results obtained as follows: imidazolium > pyperidium > piperidinium >pyrrolidinium > phosphonium > ammonium; however, as expected, the alkyl chain length is the key factor influencing toxicity, and the toxicity of the IL increases with longer alkyl chain lengths, as will be detailed below. These results agree with the previous idea that the protic and non-aromatic ILs are less toxic than the aprotic and aromatic ones [12,13]. For instance, the EC_{50} values at 15 min for C = 2 compounds are 289.97 and 133 mg/L for APILs C_2C_1Im TFSI and C_2Py TFSI, respectively, while for EAN and C_2Im NO_3, as PILs, the values are 10,665.47 and 573 mg/L, respectively, which clearly shows the difference between PILs and APILs and between ammonium cation, which is less toxic, and imidazolium cation, which is more toxic.

Table 3. EC_{20} effective concentration values in mg/L and the respective 95% confidence intervals, obtained after 5, 15, and 30 min of exposure of the marine bacteria *A. fischeri*.

IL	EC_{20} 5 min/mg/L (Lower; Upper) Limits	EC_{20} 15 min/mg/L (Lower; Upper) Limits	EC_{20} 30 min/mg/L (Lower; Upper) Limits
C_2C_1Im TFSI	97.11 (37.29; 156.94)	43.33 (7.85; 78.81)	22.82 (0.00; 48.22)
C_4C_1Im TFSI	19.51 (1.67; 37.35)	13.45 (1.05; 25.86)	10.50 (0.00; 22.63)
$C_4C_1C_1$Im TFSI [12]	46.34 (5.74; 86.95)	39.09 (20.78; 57.40)	36.45 (26.05; 46.85)
C_6C_1Im TFSI	12.60 (10.59; 14.60)	12.43 (10.69; 14.17)	17.15 (15.22; 19.08)
C_8C_1Im TFSI	1.49 (1.37; 1.60)	1.77 (1.48; 2.06)	2.50 (1.68; 3.33)
AC_1Im TFSI	110.00 (46.34; 173.66)	68.95 (27.41; 110.49)	61.21 (25.35; 97.07)
AC_1Im Cl	376.21 (0.00; 797.78)	310.13 (106.25; 514.02)	268.03 (108.68; 427.38)
AC_1Im DCA	436.58 (262.10; 611.06)	309.55 (225.00; 394.10)	291.63 (217.70; 365.56)
C_4C_1Im FAP	24.57 (12.19; 36.94)	21.24 (13.01; 29.47)	19.05 (11.28; 28.62)
C_1C_1Im DMP	917.35 (839.85; 994.63)	970.14 (914.78; 1026.19)	997.02 (945.86; 1050.47)
C_2Py TFSI	54.17 (9.12; 99.22)	26.67 (0.00; 54.39)	12.37 (0.00; 34.03)
C_4Py TFSI	64.42 (52.13; 76.71)	45.45 (35.55; 55.35)	39.90 (28.93; 50.87)
C_6Py TFSI	21.69 (18.49; 24.88)	20.00 (16.57; 23.44)	24.66 (21.03; 28.29)
C_3C_1Pip TFSI	66.74 (36.70; 96.77)	46.42 (26.57; 66.26)	40.91 (19.42; 62.41)
C_4C_1Pip TFSI	67.57 (59.56; 75.58)	57.01 (49.72; 64.29)	55.22 (49.76; 60.69)
C_4C_1Pyrr TFSI [12]	684.04 (441.90; 926.09)	416.73 (286.18; 545.93)	289.18 (192.91; 386.85)
C_6C_1Pyrr TFSI	39.71 (32.17; 47.25)	33.50 (27.14; 39.87)	41.44 (32.94;49.95)
C_8C_1Pyrr TFSI	6.84 (4.87; 8.81)	7.51 (5.22; 9.80)	13.36 (7.02; 19.70)
C_4C_1Pyrr FAP	428.33 (201.78; 654.88)	385.65 (252.92; 518.38)	337.60 (256.32; 418.89)
$C_1OC_2C_1$Pyrr FAP	20.47 (2.84; 38.10)	13.00 (4.67; 21.33)	11.36 (5.71; 17.01)
C_4C_1Pyrr C_4FAP	49.88 (28.06; 71.70)	28.72 (8.44; 49.09)	21.95 (1.27; 42.63)
C_4C_1Pyrr C_8FAP	--	--	1267.78 (446.89; 2088.66)
Ph_3t FAP	959.49 (513.08; 1045.91)	402.74(151.78; 653.70)	324.91 (37.84; 611.99)
EAN [12]	4314.31 (1548.95; 7081.66)	3236.68 (951.77; 5522.60)	3012.33 (1264.99; 4761.67)
PAN [23]	4309.57 (3391.12; 5228.02)	3116.85 (2332.68; 3901.02)	3301.43 (2521.37; 4081.48)
BAN [23]	326.04 (0.00; 669.80)	318.35 (30.59; 606.11)	287.02 (0.00; 575.25)
PEAN [23]	381.72 (274.82; 488.63)	351.09 (208.36; 493.83)	346.65 (200.17; 493.12)
HEAN [23]	49.72 (42.04; 57.40)	34.67 (29.90; 39.43)	32.30 (26.60; 38.01)
OAN [23]	4.24 (1.70; 6.78)	3.85 (1.93; 5.78)	5.02 (2.61; 7.43)
C_2Im NO_3 [12]	195.44 (79.12; 312.90)	194.19 (79.98; 310.53)	223.45 (105.10; 342.82)

Figure 4 shows the values of EC_{50} after 15 min of exposure for IL with common ions: anion FAP (a), NO_3 (c), and TFSI (e), and cation ammonium (b), pyrrolidinium (d), and imidazolium (f). From this figure, it is easy to conclude that the increase in the alkyl chain leads to an increase in toxicity. An important finding of this work corresponds to the fact that the reduction in EC_{50} with the increase in the length of the alkyl chain is especially drastic and significant when C = 6 is achieved. See, for example, Figure 4c, where similar values of EC_{50}, higher than 1000 mg/L, can be found for BAN and PEAN (C = 4 and C = 5, respectively), but EC_{50} for HEAN (C = 6), the value falls to 57.54 mg/L and continues decreasing dramatically, reaching the value 7.33 mg/L for the OAN (C = 8) which represents a very significant increase in toxicity. Similar behavior can be observed for APILs C_xC_1Im TFSI, C_xPy TFSI, and C_xC_1Pyrr TFSI: the change from C = 4 to C = 6 can result in a reduction of up to 90% in the EC_{50}. To our knowledge, this behavior has not been reported previously in IL toxicity studies. Nevertheless, several authors have stated the existence of a critical alkyl size (CAS) on different thermodynamic properties, beyond which further increases in the alkyl chain length do not significantly alter the polar network (anion–cation) interaction and the structural organization of the ILs in the crystal [21,35–38].

Table 4. EC_{10} effective concentration values in mg/L and the respective 95% confidence intervals, obtained after 5, 15, and 30 min of exposure of the marine bacteria *A. fischeri*.

IL	EC_{10} 5 min/mg/L (Lower; Upper) Limits	EC_{10} 15 min/mg/L (Lower; Upper) Limits	EC_{10} 30 min/mg/L (Lower; Upper) Limits
C_2C_1Im TFSI	44.54 (7.06; 82.03)	18.24 (0.07; 38.18)	8.94 (0.02; 22.05)
C_4C_1Im TFSI	8.63 (0.00; 18.97)	5.91 (0.00; 13.03)	4.39 (0.00; 11.01)
$C_4C_1C_1$Im TFSI [12]	23.25 0.00; 50.07)	20.96 (7.96; 33.95)	20.34(12.63; 28.05)
C_6C_1Im TFSI	8.19 (6.42; 9.96)	8.53 (6.91;10.16)	12.46 (10.54; 14.37)
C_8C_1Im TFSI	0.88 (0.80; 0.98)	1.07 (0.83; 1.30)	1.50 (0.84; 2.17)
AC_1Im TFSI	38.67 (2.85; 74.48)	27.19 (3.07; 51.31)	26.89 (4.41; 49.37)
AC_1Im Cl	174.31 (0.00; 435.31)	172.68 (17.45; 327.90)	150.85 (28.69; 273.00)
AC_1Im DCA	243.68 (91.39; 395.97)	202.36 (124.95; 279.78)	204.59 (133.20; 275.98)
C_4C_1Im FAP	10.95 (3.62; 18.28)	10.19 (4.96; 15.43)	8.38 (3.86; 12.89)
C_1C_1Im DMP	763.66 (679.73; 846.46)	847.12 (794.31; 921.86)	901.85 (845.03; 958.37)
C_2Py TFSI	39.23 (29.18; 49.29)	27.55 (19.52; 35.59)	24.32 (15.38; 33.26)
C_4Py TFSI	19.35 (0.00; 40.98)	10.38 (0.00; 24.55)	4.33 (0.00; 14.24)
C_6Py TFSI	14.30 (11.49; 17.11)	13.27 (10.23; 16.31)	17.15 (13.77; 20.53)
C_3C_1Pip TFSI	42.26 (35.50; 49.01)	37.00 (30.62; 43.38)	37.41 (32.40; 42.41)
C_4C_1Pip TFSI	33.61 (13.56; 53.97)	24.51 (10.51;38.50)	22.10 (6.61; 37.60)
C_4C_1Pyrr TFSI [12]	438.08 (225.18; 650.98)	254.32 (146.51; 362.18)	170.23 (93.44; 247.12))
C_6C_1Pyrr TFSI	24.76 (18.44; 31.09)	21.71 (16.15; 27.26)	29.22 (21.09; 37.36)
C_8C_1Pyrr TFSI	4.20 (2.59; 5.82)	4.86 (2.88; 6.84)	9.57 (3.44; 15.71)
C_4C_1Pyrr FAP	295.81 (79.77; 511.84)	270.28 (142.70; 397.86)	23.992 (161.01; 318.83)
$C_1OC_2C_1$Pyrr FAP	10.66 (0.81; 19.79)	6.94 (1.09; 12.79)	6.24 (2.26; 10.22)
C_4C_1Pyrr C_4FAP	33.83 (13.75; 53.92)	18.26 (2.88; 33.62)	13.47 (1.04; 24.25)
C_4C_1Pyrr C_8FAP	--	--	540.79 (114.86; 1000.97)
Ph_3t FAP	445.54 (162.01; 729.08)	224.03 (15.24; 457.16)	190.91 (3.87; 305.99)
EAN [12]	2304.89 (248.43; 4361.05)	1609.79 (560.06; 3163.56)	1517.65 (332.07; 2703.22)
PAN [23]	2932.68 (2072.90; 3792.46)	2138.10 (1402.95; 2873.26)	2366.64 (1600.94; 3132.34)
BAN [23]	136.59 (0.00; 354.65)	160.30 (0.00; 369.51)	139.74 (0.00; 341.90)
PEAN [23]	203.54 (121.38; 285.71)	182.43 (75.64; 289.23)	183.21 (72.35; 294.07)
HEAN [23]	36.14 (28.64; 43.65)	25.76 (20.96; 30.57)	24.98 (19.07; 30.89)
OAN [23]	2.62 (0.53; 4.71)	2.64 (0.87; 4.42)	4.00 (1.36; 0.66)
C_2Im NO_3 [12]	100.10 (21.33; 179.99)	103.80 (22.16; 184.19)	127.39 (37.59; 214.25)

Another important observation is related to the fact that EC values of $C_1OC_2C_1$Pyrr FAP are more than ten times lower than that the corresponding to C_4C_1Pyrr FAP, which means that the presence of an oxygen atom in the cation seems to induce higher toxicity to the IL. This statement was previously reported by Grzonkowska et al. [36], who attributed this effect to an increase in the number of polar functional groups.

It is also important to note that phosphorous-based moieties show the highest values of EC_{50} in Figure 4a (ILs with the common anion FAP) and 4f (ILs with the common cation family imidazolium), which makes them especially interesting for developing greener and safer industrial applications, for example, fuel desulfurization, novel electrolytes, and lubrication [37,38].

Among the scarce literature on the toxicity of FAP-based ILs is the work of Weyhing-Zerrer et al. [13], who proposed the interesting evidence named "reverse side-chain effect", wherein an increase in the cation hydrophobicity chain results in decreased toxicity of FAP-based ILs. Similar conclusions to those of these authors can be obtained by the comparison between the EC_{50} (15 min) for C_4C_1Im FAP (77.43 mg/L), here analyzed, and the corresponding C_2C_1Im FAP, reported by Viboud et al. [34] (12.7 mg/L). A possible explanation of this observation, reported by Weyhing-Zerrer et al. [13] is that the associated ion pair is less permeable into or through the cell membrane, and therefore, the active [FAP] anion cannot reach the cell, resulting in being less toxic or harmless as the alkyl chain size increases. Furthermore, following with the analysis of FAP-based ILs, the same interesting toxicity pattern also emerged with the increase in alkyl chain length on the FAP anion, with the values of EC_{50} after 30 min of exposure of 50.54 mg/L and 5430 mg/L for C_4C_1Pyrr

C_4FAP and C_4C_1Pyrr C_8FAP, respectively. This unexpectable behavior is also consistent with the observations of Weyhing-Zerrer et al. [11].

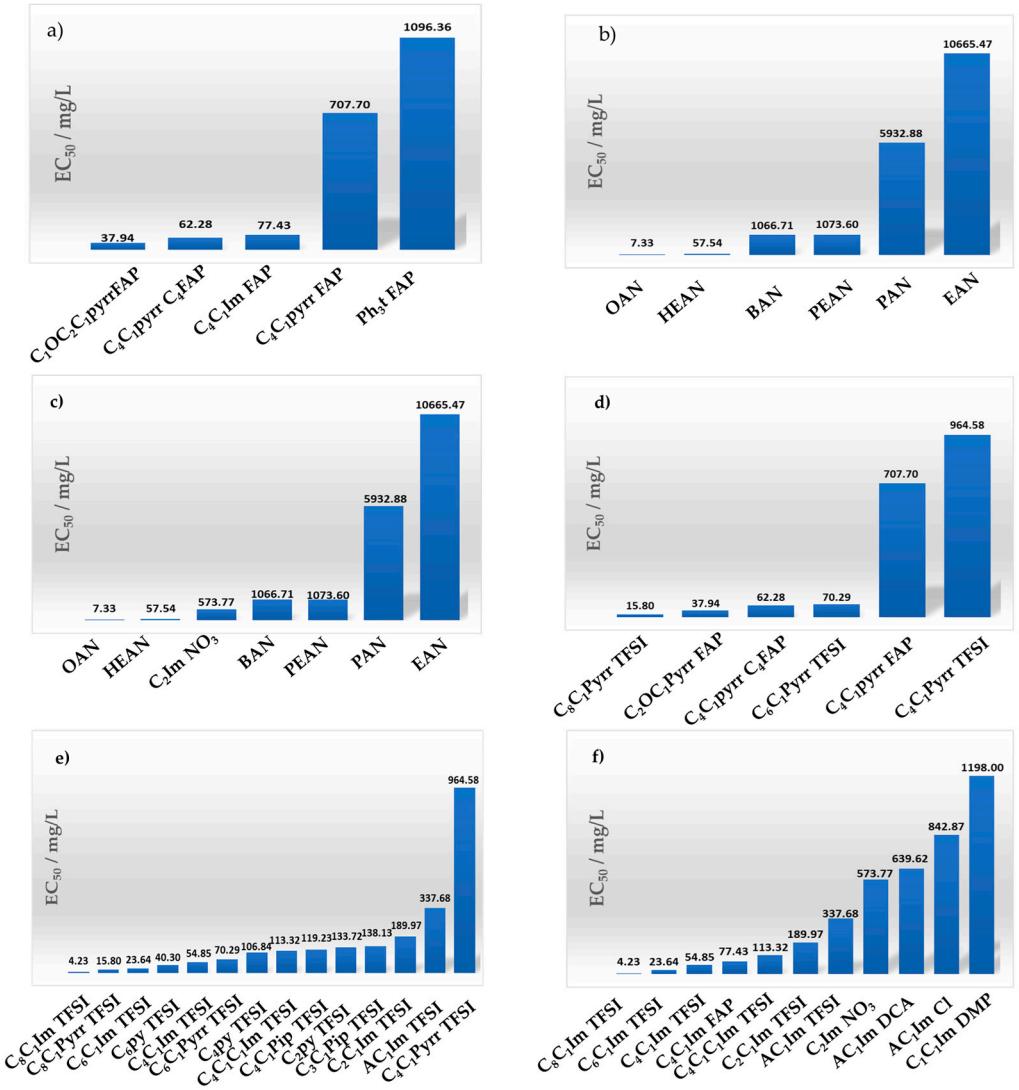

Figure 4. EC_{50} values at 15 min of exposure for the different studied ions: (**a**) FAP anion-based, (**b**) ammonium cation-based, (**c**) Nitrate anion-based, (**d**) pyrrolidinium cation-based, (**e**) TFSI anion-based, and (**f**) imidazolium cation-based.

As mentioned earlier, two toxicity classifications were employed to categorize the selected ILs. Table 5 shows the classification of the 30 ILs, revealing that none of them falls under the categories of highly toxic or extremely toxic, based on both criteria.

These findings confirm that both criteria show similar conclusions, and thus, protic ILs exhibit broadly lower toxicity in comparison with aprotic ones, and non-aromatic ILs are less toxic than aromatic ones. The alkyl chain length plays a fundamental role in the toxicity of the ILs, with six carbons being the critical size to mark the transition from non-toxic to toxic in many cases, although further studies in this line should be performed to verify this conclusion.

Table 5. Toxicity level for the selected ILs obtained by Passino and Smith [26] and Chan et al. [27] criteria.

IL	Passino and Smith [26]	Chang et al. [27]
C_2C_1Im TFSI	Practically harmless	Non-toxic
C_4C_1Im TFSI	Toxic	Toxic
$C_4C_1C_1$Im TFSI [12]	Practically harmless	Non-toxic
C_6C_1Im TFSI	Toxic	Toxic
C_8C_1Im TFSI	Toxic	Very toxic
AC_1Im TFSI	Practically harmless	Non-toxic
AC_1Im Cl	Relatively harmless	Non-toxic
AC_1Im DCA	Relatively harmless	Non-toxic
C_4C_1Im FAP	Toxic	Toxic
C_1C_1Im DMP	Relatively harmless	Non-toxic
C_2Py TFSI	Practically harmless	Non-toxic
C_4Py TFSI	Practically harmless	Non-toxic
C_6Py TFSI	Toxic	Toxic
C_3C_1Pip TFSI	Practically harmless	Non-toxic
C_4C_1Pip TFSI	Practically harmless	Non-toxic
C_4C_1Pyrr TFSI [12]	Relatively harmless	Non-toxic
C_6C_1Pyrr TFSI	Toxic	Toxic
C_8C_1Pyrr TFSI	Toxic	Toxic
C_4C_1Pyrr FAP	Practically harmless	Non-toxic
$C_1OC_2C_1$Pyrr FAP	Toxic	Toxic
C_4C_1Pyrr C_4FAP	Toxic	Toxic
C_4C_1Pyrr C_8FAP	--	--
Ph_3t FAP	Relatively harmless	Non-toxic
EAN [12]	Relatively harmless	Non-toxic
PAN [23]	Practically harmless	Toxic
BAN [23]	Practically harmless	Toxic
PEAN [23]	Relatively harmless	Non-toxic
HEAN [23]	Toxic	Toxic
OAN [23]	Toxic	Very toxic
C_2Im NO_3 [12]	Practically harmless	Non-toxic

4. Conclusions

In this study, the ecotoxicity of a set of ILs with different ionic natures was evaluated. The assessment was based on the inhibition of the bioluminescence of the bacteria *Aliivibrio fischeri* with different concentrations of the ILs using the Microtox® standard toxicity test.

The most remarkable findings of this study are the following:

- This study found that 16 of the 30 compounds were either non-toxic or practically harmless, and none were at the highest levels of the two classifications considered. No preferential effect of cation or anion on toxicity have been found, although the effect on bacteria is determined by the combination of both.
- Protic ILs exhibit lower toxicity compared to aprotic ones at the shorter alkyl chain length, and non-aromatic ILs generally demonstrate lower toxicity than aromatic ones. Additionally, water solubility plays a significant role, with lower toxicity associated with higher hydrophilicity within each group.
- Ionic liquids with ammonium cations presented the lower toxicity, while the imidazolium-based ILs are more harmful for the shortest alkyl chain length ILs, although the toxicity increases with this alkyl chain, with OAN and C_8C_1Im TFSI being the most toxic ILs, both with C = 8, the longest chain considered in this work.
- The toxicity of similar cations ranged from the TFSI anion, the most toxic, to nitrate-based ILs, the least toxic.
- FAP-based ILs, which present the opposite behavior regarding the chain length to the other anion ILs; i.e., the more toxic ILs correspond to the ILs with the shorter alkyl chain.

- The identification of a critical alkyl size (CAS) at C = 6 was documented in this study, defining a tipping point in toxicity behavior related to the length of the chain. The presence of six or more carbons in the alkyl chain results in a significant increase in toxicity levels.

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