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Ecotoxicological Evaluation of Ethylammonium Nitrate and Aluminium Salt Mixture †

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Abstract: The ecotoxicity of a mixture of the ionic liquid ethylammonium nitrate (EAN) and aluminum nitrate salt ($Al(NO_3)_3$), as well as the corresponding pure components, was studied in this work. This mixture is of singular interest as electrolytes in electrochemical applications and data on the effects of mixture and components on the environment can hardly be found in the literature. Changes in the bioluminescence of the *Aliivibrio fischeri* (Beijerinck) Urbanczyk bacteria, determined through a Microtox® test, and microbial activity, measured by microcalorimetry, of two soils with different organic matter contents when exposed to solutions of different concentrations of these compounds were analyzed.

Keywords: ecotoxicity; inorganic salt; microcalorimetry; electrolyte; Microtox; microbial activity

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1. Introduction

Ionic liquids (ILs) are salts with an organic cation and an organic or inorganic anion with melting points lower than 100 °C. ILs are a group of synthetic compounds with unique properties (low inflammability, low vapor pressure, variable solubility, low viscosity, high thermal and chemical stability, high ionic conductivity and a wide electrochemical window) that make then attractive for use in numerous industrial applications such as advanced materials for smart electrochemical devices—e.g., supercapacitors, dye-sensitized solar cells or lithium and aluminum batteries [1–4]. ILs are considered as a good alternative to the conventional volatile organic solvents due to their abovementioned negligible vapor pressure reducing the possible risk of atmospheric contamination. Romero et al. [5] have stated that some imidazolium ILs can show higher toxicity than common organic solvents. This statement can be explained by taking into account their water solubility and biodegradability resistance, which could make them strong pollutants and persistent in the environment, and therefore they could pose a danger for the aquatic organisms, plants and soil microorganisms considering probable spills into the environment [6–8]. Despite this fact, and although the applicability of ILs has spread rapidly, toxicity studies of these compounds are very unusual. Thus, ILs could enter into commercial mass production without full understanding of their ecotoxicity [9].

The IL used in this work, ethylammonium nitrate (EAN), is considered as an room-temperature IL (RTIL); this compound presents water-like properties [10] and, mainly due to its ability to form hydrogen bounds, some studies use it as an electrolyte, additive, detergent or precipitating agent among other applications, which can be found in the recent literature [11–13]. However, in spite of their applicability and the high probability of spills and filtration into terrestrial and aquatic environments, studies on their toxicity in these environments are very scarce.

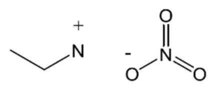
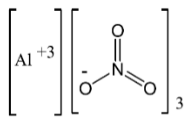
In this work, the ecotoxicity of ethylamonium nitrate, the aluminum nitrate salt ($Al(NO_3)_3{\cdot}9H_2O$) and the saturated mixture of both components was studied using two different tests. The first one is a standardized method based on the variation of the bioluminescence generated by the bacteria *Aliivibrio fischeri* (Beijerinck) Urbanczyk through a Microtox® test. Moreover, a good way to analyze the impact of a substance on soil microorganisms or even isolated organisms [14] is through the variation of their metabolic activity, and microcalorimetric techniques with high power sensibility can be used for this propose; in particular, isothermal microcalorimetry is an generally used technique as it measures heat production rate, which accompanies nearly all physical, chemical and biological processes. The use of this technique has increased during recent years in the microbiological field due to its simplicity, versatility and fast analysis [15]. In this study, changes in the heat released by microorganisms of two soils with different organic matter contents as a consequence of the application of different amounts of the pure compounds (EAN, Al) and of aluminum-saturated EAN were analyzed.

2. Material and Methods

2.1. Chemicals

The main characteristics—namely, CAS (Chemical Abstracts Service) registration number, structure, molecular mass, purity and provenance of the selected ionic liquid and salt—are indicated in Table 1. Further purification of the pure IL was carried out by performing a high vacuum procedure until a water content below 100 ppm was obtained.

Table 1. Identification number, chemical structure, molecular mass and purity of ethylammonium nitrate (EAN) and aluminum salt.

Name	Abbreviation CAS Number	Structure	M_W (g mol^{-1})	Purity Provenance
Ethylammonium nitrate	EAN 22113-86-6		108.096	>0.97 Iolitec
Aluminum nitrate	$Al(NO_3)_3{\cdot}9H_2O$ 7784-27-2		374.996	>0.999 Merck

EAN and $Al(NO_3)_3{\cdot}9H_2O$ saturated solution were obtained by mixing both components, with the help of an ultrasound bath if needed, for 24 to 48 h and by increasing molality in 0.5 mol kg^{-1} intervals until 2 mol kg^{-1} (saturation point) at room temperature [16].

2.2. Microtox Test

The toxicity was assessed by a Microtox® Toxicity Test kit (Modern Water, London, UK) using the luminescence production of the marine bacteria *Aliivibrio fischeri* Beijerinck Urbanczyk, a metabolic effect used to test treatments which comprise a range of aqueous solutions (0–81.9% of a 2 molal stock solution) of each compound. The light output of the luminescent bacteria following 5, 15 and 30 min of exposure was read and compared to that of a blank control. Concentrations promoting 50, 20 and 10% luminescence inhibition (EC_{50}, EC_{20} and EC_{10}), and the corresponding 95% confidence intervals were estimated through a nonlinear regression, using the least-squares method to fit the data to the logistic equation [16].

2.3. Microcalorimetric Test

These experiments were performed using an isothermal microcalorimeter Thermal Activity Monitor (TAM III) Thermometric AB. The effects of different solutions (0, 1, 10, 25, 50 and 75%) of pure compounds and the IL-salt mixtures in distilled water and the heat released by microorganisms of two soils with different organic matter (OM) contents and similar pHs were studied in order to determine the influence of the OM on the effects of the contaminants. The measurements were carried out at 25 °C, with a quantity of field-moist soil equivalent to 2 g of air-dried soil spiked with 0.2 mL of the 0 to 75% solutions indicated above. Immediately before the measurements, 0.2 mL of a glucose solution in water with a concentration of 6.25 g L^{-1} was added to activate the metabolism of soil microorganisms. The heat released by soil microorganisms until the total consumption of glucose was measured [14].

Table 2 shows the main characteristics of the two soils used for the study. The first soil corresponds to a corn field collected in A Pedra (A Coruña) on 17/07/2018; the second one is a forest soil collected in Negreira on 25/10/2018. When they were collected, their humidity values were 13.54 and 27.72%, respectively.

Table 2. Main characteristics of the soils used in the study.

Soil	pH $_{H_2O}$	pH $_{KCl}$	%Ct	%N_t	C/N	%sand	%silt	%clay	Texture
A Pedra	4.55 ± 0.06	3.67 ± 0.06	2.17 ± 0.05	0.21 ± 0.00	10	66	23	11	S/L
Negreira	4.17 ± 0.07	3.28 ± 0.01	12.43 ± 0.06	0.65 ± 0.02	19	71	16	13	S/L

S/L: Sandy loam.

3. Results

3.1. Microtox Test

The results of mean effective concentration values to obtain a reduction of 10, 20 and 50% of bioluminescence of the bacteria after 5, 15 and 30 min of exposition, determined by Microtox® test, are shown in Table 3. As can be expected, these values decrease with the time of exposition, indicating that toxicity increases. The highest EC values were found for EAN, and the lowest for $Al(NO_3)_3$, whereas the mixture showed slightly higher values than the pure salt.

Table 3. Mean effective concentration values (EC_{10}, EC_{20}, EC_{50}) in mg L^{-1} and the respective 95% confidence intervals (CIs), obtained after exposure of the marine bacteria *Allivibrio fischeri* to the studied compounds for 5, 10 and 30 min.

Compound	Time (min)	EC_{50} (mg L^{-1})	EC_{20} (mg L^{-1})	EC_{10} (mg L^{-1})
EAN	5	11,590 (7294; 15,887)	3727 (1189; 6264)	1918 (122; 3713)
	15	10,653 (7365; 13,941)	3243 (1352; 5135)	1616 (330; 2903)
	30	10,333 (7484; 13,182)	3283 (1586; 4980)	1677 (497; 2858)
$Al(NO_3)_3$	5	17.67 (11.66; 23.68)	4.46 (1.51; 7.41)	1.99 (0.21; 3.76)
	15	14.39 (10.52; 18.26)	7.51 (4.04; 10.98)	5.13 (1.92; 8.33)
	30	12.91 (10.10; 15.72)	8.48 (5.41; 11.55)	6.63 (3.41; 9.85)
EAN+Al	5	61.77 (44.33; 79.20)	20.78 (10.89; 30.67)	10.98 (4.12; 17.84)
	15	51.00 (45.21; 56.80)	31.01 (24.80; 37.22)	23.17 (16.93; 29.41)
	30	44.17 (40.83; 47.52)	26.56 (22.57; 30.55)	19.72 (15.82; 23.61)

Taking into account that higher values of EC indicate lower toxicity, pure $Al(NO_3)_3$ and the mixture (EAN+Al) can be considered slightly toxic (10 mg L^{-1} < EC_{50} < 100 mg L^{-1}) and EAN can be classified as relatively harmless (greater than 1000 mg L^{-1}) using the Passino and Smith classification [17]. Other works also highlighted the harmless or moderately toxic effects of different ILs towards *A. fischeri* and detected the influence on the toxicity of the chemical elements attached to the IL, such as the increase in the toxicity when an alkyl side chain increases, which is not the case for EAN. In Table 1, it can be seen that this IL is not as bulky as pyrrolidinium or imidazolium can be, and this could be the reason for its low toxicity [18].

3.2. Microcalorimetric Test

Figure 1 shows the power time curves of both studied soils treated with different concentrations of pure IL, pure salt and a mixture of both.

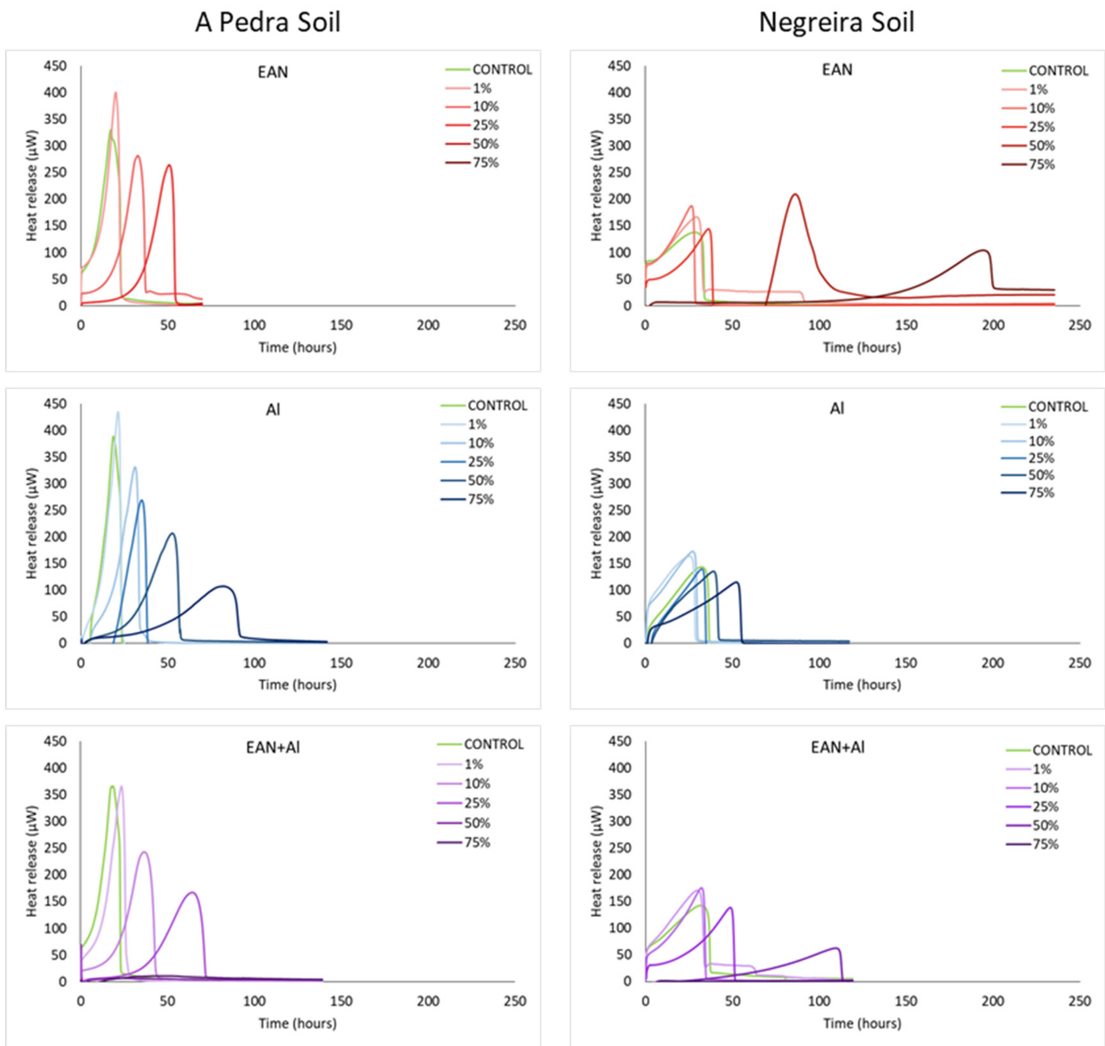


Figure 1. Power–time curves of microbial activity of the two selected soils after the addition of the different concentrations of ethylammonium nitrate (EAN), $Al(NO_3)_3$ (Al) and the mixture of both (EAN+Al).

Chen et al. [19] suggested the division of the power–time curves in four phases: lag, log, stationary and decline phases. Similar shapes can be observed in our experiments where the increase in the lag phase happened in the two soils for all the compounds together with a slower and progressive log phase for the highest doses. The stationary phase stays similar for all the doses and compounds, and the same happened with the decline phase, with just a few exceptions in both cases.

The main conclusion is that pure EAN and the mixture present the highest toxicity in both soils, where no signal was obtained beyond 25% for both compounds in the soil with low organic matter, and a delay of the heat released peak was observed for 50 and 75% doses for the soil with high organic matter content. On the contrary, the aluminum salt is the least toxic compound, showing heat release signals for all the studied concentrations. This does not agree with the toxicity of these compounds shown by Microtox analysis. Ucha et al. [20] detected a very high EAN toxicity, either alone or mixed with Al, on the germination of three tree species. The toxicity was already significant for doses of 2.5% of EAN or EAN+Al in water. However, it must be considered that the seeds were sown in a Petri dish without adding any amount of soil. This would explain why the toxicity was even higher than that obtained in the present work by microcalorimetry for the two studied soils, reinforcing the idea that organic matter could partially buffer the toxicity of this ionic liquid. This also suggests that Microtox analysis is less sensitive, although faster, than microcalorimetry and seed germination analysis shows the toxicity of the compounds investigated in this study.

This study also demonstrates an inverse relationship between organic matter content and IL toxicity exposed by Salgado et al. [14] and Sixto et al. [21], in agreement with a similar inverse relationship observed for other organic compounds toxic to soil microorganisms [22].

4. Conclusions

In this work, ecotoxicity of the ionic liquid ethylammonium nitrate (EAN) and aluminum nitrate salt ($Al(NO_3)_3$), as well as the mixture of both compounds, was analyzed in terms of the bioluminescence of the marine bacteria *A. fischeri* and the microbial growth of two soils after the addition of several concentrations of these complexes. Ecotoxicity studies showed that EAN is very toxic to soil microorganisms, unlike for the marine bacteria *A. fischeri*. On the contrary, $Al(NO_3)_3$ showed higher toxicity for the marine bacteria than for the soil microorganisms. In addition to this, the toxicity for the mixture of both is always similar to that of the more toxic compound in each case.

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