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Acute toxicity and biodegradability of *N*-alkyl-*N*-methylmorpholinium and *N*-alkyl-DABCO based ionic liquids

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ABSTRACT

N-Alkyl-*N*-methylmorpholinium and *N*-alkyl substituted 1,4-diazabicyclo[2.2.2]octane (DABCO) based ionic liquids (ILs), *N*-alkyl-DABCO, bearing short alkyl chains are characterised by a low toxicity to *Vibrio fischeri*, although toxicity significantly increases on increasing the alkyl chain length. Alkyl chain length affects also biodegradability in the 28 days tests; the higher level of biodegradation was found in both the series in the case of the ethyl (C₂) derivatives. In the case of *N*-ethyl DABCO based IL, although biodegradability is still around 40%, and consequently this IL cannot be classified as “readily biodegradable”, this value is similar to the more biodegradable functionalized imidazolium based ILs.

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1. Introduction

The bioassays-based approach represents a fundamental point in the definition of ecological risk in the aquatic environment for promising chemicals as ionic liquids (ILs). In the registration process for all chemicals produced in or imported into the European Union above one metric tonne per year, the new regulatory framework for the Registration, Evaluation and Authorisation of Chemicals (REACH) adopted by the EU (2006) indicates the requirement of (eco)toxicological assessment. The predicted no-effect concentration (PNEC) represents one of the major indicators required for the environmental hazard assessment of chemicals. PNEC consists of the identification of the environmental effects of chemicals on organisms and the determination of chemical concentration below which adverse effects in the environment (e.g., aquatic) are not expected to occur. The calculation starts from the available chemical and ecotoxicity data, derived

from test organisms, such as bacteria, algae, crustaceans and fish in both acute and chronic toxicity experiments.

Over the past years, ILs have received considerable attention as potential green solvents or as materials for a wide range of applications in the area of green chemistry and engineering. This is prevalently due to their low vapour pressure (if any) and non-flammability that reduce air emission and improve operational safety with respect to the molecular solvents. However, the environmental risk assessment in terms of (eco)toxicity appears to be needful in order to evaluate the sustainability production of chemicals before any possible large-scale application. Recently, important data (both experimental and calculated) on the environmental impact of ILs have been published, showing that the ionic nature not necessarily confers an eco-friendly behaviour. Surely, the water solubility of many ILs may allow their widespread in the aquatic compartment and several ILs have been found to be toxic for different aquatic organisms, such as bacteria (Ranke et al., 2004; Docherty and Culpa, 2005; Garcia et al., 2005; Couling et al., 2006; Wells and Coombe, 2006; Matzke et al., 2007; Stolte et al., 2007; Samori et al., 2007; Luis et al., 2010), unicellular algae (Latala et al., 2005; Matzke et al., 2007; Stolte et al., 2007; Cho et al., 2008; Kulaki and Lamberti, 2008), crustaceans (Bernot et al., 2005a; Couling et al., 2006; Wells and Coombe, 2006; Samori et al., 2007), molluscs (Bernot et al., 2005b) and fish (Pretti et al., 2006).

In principle this might have important consequences, such as, accidental spills and effluent discharges, in particular if their

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application in large-scale processes increases (e.g. accidental spills, effluent discharges). Fortunately, ILs not only have different degrees/levels of toxicity to aquatic organisms depending on species, but toxicity is also strongly affected by IL structure. More in particular, it depends on the head group (Pretti et al., 2009), side-chain length (Bernot et al., 2005a, 2005b) and anion nature (Ranke et al., 2004; Matzke et al., 2007). Three extensive reviews on sustainability of ILs reporting an overview on ecotoxicological data have been recently published (Ranke et al., 2007; Pieraccini et al., 2007; Pham et al., 2010). The heterogeneity within the ILs gives an almost unlimited number of possible compounds, which complicates the hazard assessment, but increases the possibility to design more environmental sustainable ionic compounds. However, the biological activity of ILs on the organisms of aquatic ecosystems has been tested only in acute toxicity tests: the lack of chronic toxicity data induces a high uncertainty level on the real impact of ILs. The release of non-biodegradable chemicals, even with a low acute toxicity, could result in chronic toxic effects through a bioaccumulation mechanism in aquatic environment.

The biodegradability of different ILs has been recently investigated from several authors. In particular, an extensive review has been published by Coleman and Gathergood (2010). It has been shown that imidazolium-based ILs with linear alkyl chains or containing an amide functionality show a low biodegradability, whereas ester side chains result in a significant enhancement of biodegradation (Gathergood and Scammells, 2002; Gathergood et al., 2004). An increase in biodegradation was also observed by the incorporation of the octylsulfate anion (Garcia et al., 2005). Certain lipophilicity seems to be necessary for enhancing the biodegradation rate of imidazolium based ILs, even if a high lipophilicity affects negatively the toxicity (Stolte et al., 2008)). On the other hand, comparative studies showed that pyridinium are more biodegradable than imidazolium-based ILs (Docherty et al., 2007) and lipophilic anions do not reduce the biodegradability of pyridinium ILs (Harjani et al., 2008, 2009a). It is noteworthy that a pyridinium based IL with an ester side chain and a saccharinate anion was also identified as readily biodegradable (Stasiewicz et al., 2008). A potential biodegradation pathway has been therefore recently proposed for pyridinium ILs (Pham et al., 2009). In continuation of earlier investigations, Harjani et al. (2009b) have revisited the biodegradability of imidazolium based ILs introducing a variety of functional groups on the cation and testing different anions. However, none of the introduced functional groups increased biodegradability; whereas, an increase in the biodegradation rate was observed on increasing the chain length on the alkyl sulphate anion. Biodegradable naphthenic acid ILs have also been reported, even if they present a low thermal stability (Yu et al., 2008). Finally, the biodegradability of a range of phosphonium ILs has been recently tested. It is noteworthy that in contrast to dialkylimidazolium and alkylpyridinium ILs with incorporated ester moieties and octylsulfate anions, the analogously functionalized phosphonium ILs showed relatively low levels of biodegradability (Atefi et al., 2009).

In this paper, we report experimental data on the aquatic toxicity and biodegradability of *N*-alkyl-*N*-methylmorpholinium (Morph) and *N*-alkyl substituted 1,4-diazabicyclo[2.2.2]octane (DABCO) based ILs as function of the alkyl chain length. Only few sporadic data of toxicity have been reported for dialkylmorpholinium based ILs (Docherty and Culpa, 2005; Pretti et al., 2009; Stolte et al., 2007), whereas alkyl-DABCO based ILs have never been investigated.

The hazard assessment on aquatic environment of the two series of ILs was estimated using the standard Microtox[®] Acute Toxicity test system, determining the effective concentration at 50% to the marine bacterium *Vibrio fischeri*. Ultimate biodegradability was evaluated by the CO₂ headspace test, using the test substances as the only source of carbon for the inoculated environmental microorganisms.

2. Materials and methods

2.1. Ionic liquids

2.1.1. Synthesis of *N*-alkyl-*N*-methylmorpholinium bromides

The appropriate bromoalkane was added dropwise over 1 h to an equimolar solution of *N*-methyl morpholine in acetonitrile (ca. 0.5 mmol in 200 ml of solvent) while stirring vigorously, and N₂ was bubbled through the solution. In the case of bromoethane, as the boiling point of this compound is very low, 2% excess was taken in the reflux. The mixture was refluxed for 8 h at 70 °C. The salt was decanted, washed three times with 100 ml of CH₂Cl₂ and dried for 1 h at 45 °C under low pressure. If the product was found to have traces of brown or yellowish colour, then it was washed with acetone and recrystallised in this solvent (Fig. 1).

2.1.2. Synthesis of *N*-alkyl-DABCO bromides

To a colourless solution of DABCO (86 mmol) in ethyl acetate (50 ml) a solution of the appropriate bromoalkane (80–82 mmol) in the same solvent (50 ml) was added dropwise. Then, the reaction was stirred at room temperature for 12 h. The formed solid was separated by filtration, initially washed with ethyl acetate, dried in vacuum and then re-washed with anhydrous tetrahydrofuran and dried in vacuum (Chiappe et al., 2009).

For all synthesised ILs, purity (> 99%) was checked by NMR (Bruker Advance II 250) and electrospray ionisation mass spectrometry ESI-MS (LCQ Advantage spectrometer equipped with an ion-trap analyser, ThermoElectron Company) (Bini et al., 2007). To ensure that the amount of water and volatile compounds in these ILs was reduced as much as possible, each IL was kept under vacuum (pressure around 10⁻³ mbar) at 40–50 °C for 24 h before each test. ¹H NMR and headspace GC-MS analyses were used to check for residues of unreacted reagents or solvents (Fig. 1).

2.2. CO₂ headspace test

To evaluate the ultimate biodegradability of the test ILs, the “CO₂” Headspace test (ISO 14593, 1999) was applied. This method allows the evaluation of the ultimate aerobic biodegradability of an organic compound in an aqueous medium at a given concentration of microorganisms by analysis of inorganic carbon. The tested ILs, as the sole source of carbon and energy, was added at a concentration of about 20 mg/L of total organic carbon (TOC) to a mineral salt medium. These solutions were inoculated with activated sludge from a municipal wastewater treatment plant, washed and aerated prior to use and incubated in sealed vessels with a headspace of air. Biodegradation (mineralisation to CO₂) was determined by measuring the net increase in the TOC levels over time compared with not amended blanks. Sodium benzoate was used as reference substance, and the degradation curve is reported in Fig. 2 showing curves both in filtered and not filtered samples to evaluate the natural activity of the microbial population and the adsorption of this molecule to the microbial sludge. Analyses were performed both in filtered (0.45 μm) and not filtered sample to determine the % of biodegradability and the % of biosorption of the IL to the microbial sludge as difference between total levels measured at each time and total dissolved ones. The test was run for 28 days, sampling every 7 days, analyses were performed in three replicates. The extent of biodegradation was expressed as a percentage of the theoretical amount of inorganic carbon (ThC) based on the amount of test compound added initially.

Aqueous degradation rates, could be significantly affected by abiotic factors such as temperature, pH and oxygenation levels. During the experiments, these factors were maintained fixed, closed to natural ones in freshwaters and constantly monitored. Temperature was fixed to 22.0 ± 0.5 °C, and experiments were performed inside a temperature controller incubator. Oxygenation levels were maintained oversaturated during the whole experiments. Oversaturation was guaranteed by the selection of opportune headspace-solution ratios. This favoured the aerobic activity of bacteria. pH levels were measured in sludge and to vessels (7.5 ± 0.3).

2.3. Microtox[®] bioassay (*V. fischeri*)

The Microtox[®] Test was performed according to the standard operating procedure using the Basic protocol (Azur Environmental, 1995), based on the ISO

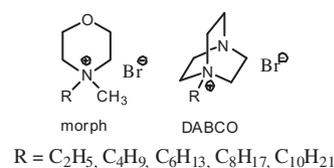


Fig. 1. Structure and alkyl chain length of methylalkylmorpholinium and alkyl-DABCO (*N*-alkyl substituted diazabicyclo[2.2.2]octane) based ILs.

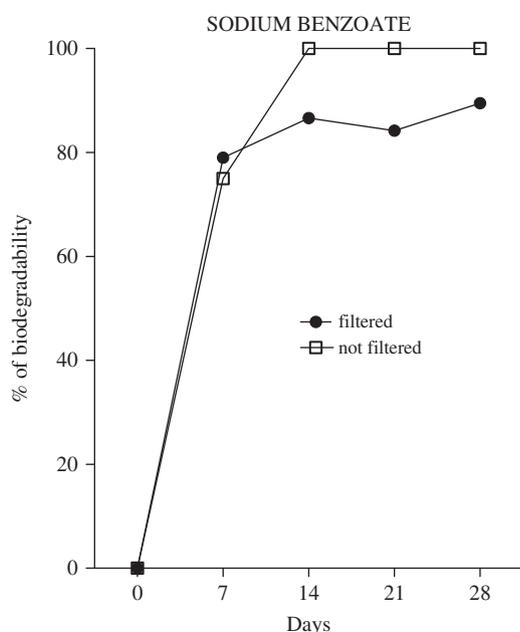


Fig. 2. Percentage of biodegradability of sodium benzoate calculated in filtered and not filtered samples. Observations were performed at 0, 7, 14, 21 and 28 days from the inocula. Experiments were carried out in triplicate; levels were reported as average data ($n=3$).

procedures (ISO 11348-3, 2007). The bacteria (*V. fischeri*) were obtained from Ecotox LDS (Pregnana Milanese, MI, Italy) as freeze-lyophilised cells.

Bacteria were exposed to a dilution series of the sample and their light emission was determined after incubation. The light emission of the bacteria in the samples was measured after 5, 15 and 30 min and compared to an aqueous control. The tests were performed at 15 °C (pH 8) and with three replicates and four controls. All measurements were performed using the M500 luminometer equipped with the appropriate cells. The instrument is interfaced with PC operating with the Microtox[®] Omni 1.16 software, for acquisition and data handling. Phenol was used as the positive toxicity control for the Microtox assay. EC_{50} values, expressed as $\log_{10}(EC_{50}/\mu M)$ were reported as the means of three replicate determinations \pm standard deviation.

3. Results

3.1. *V. fischeri*: acute toxicity

The ecotoxicological test data are shown in Fig. 3 (expressed as $\log_{10} EC_{50}/\mu M$). DABCO- and morpholinium-based bromides, bearing the same alkyl side-chains as substituents on nitrogen, were characterised by similar EC_{50} values. Nevertheless, in both the series a strong correlation between toxicity and the alkyl side-chain length was found; a monotonic decrease in EC_{50} values for the bioluminescence activity of *V. fischeri* with increasing chain length of ILs has been observed. The lowest EC_{50} values were 1.3 ± 0.1 for both DABCO C10 and *N*-methylmorpholinium C10, corresponding to an EC_{50} of $\sim 20 \pm 4.5$ expressed as μM . The highest EC_{50} values were 5.6 ± 0.1 and 5.2 ± 0.1 for DABCO C2 and *N*-methylmorpholinium C2, respectively. The corresponding values expressed as μM were 378.582 ± 71 and 161.90 ± 31 for DABCO C2 and *N*-methyl morpholinium C2, respectively. Parent compounds were also tested: the EC_{50} were 2.4 ± 0.4 and 3.8 ± 0.2 for DABCO and *N*-methylmorpholine, respectively.

3.2. Biodegradability

The biodegradability of morpholinium and DABCO-based bromides was assessed by the CO₂ headspace test. Concerning the sodium benzoate (reference substance) curve, a difference in the efficiency of biodegradation of about 10% was observed between filtered and not filtered samples, probably related to the occurrence of

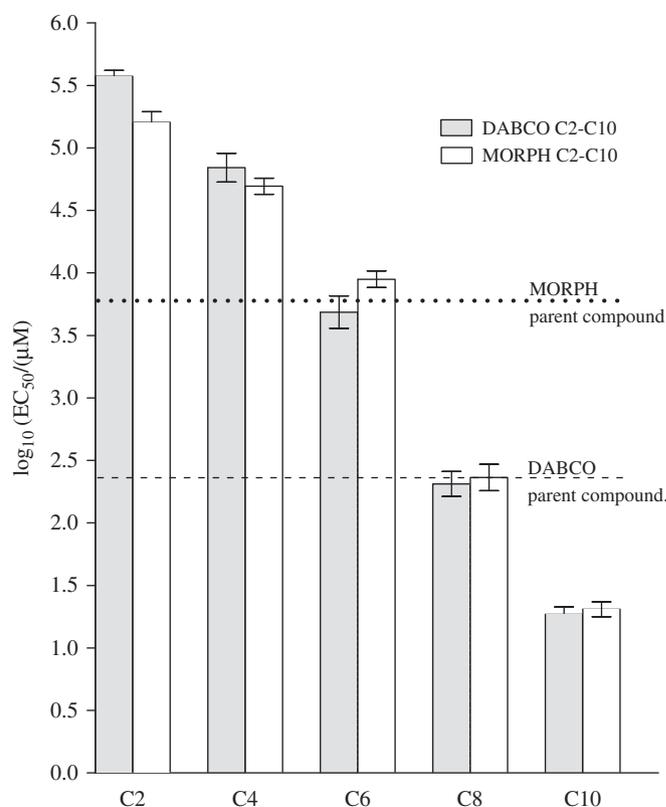


Fig. 3. Acute toxicity (*V. fischeri*) values (EC_{50}) for DABCO-based and morpholinium ILs with different lateral chain lengths (C2–C10) after 30 min of incubation. Results are expressed as average $\log_{10}(EC_{50}/M) \pm$ standard deviation ($n=3$). Dot line represents the EC_{50} values of parent compounds (DABCO and *N*-methylmorpholine). Results are expressed as average $\log_{10}(EC_{50}/M) \pm$ standard deviation ($n=3$). Significance, tested by one way ANOVA, Tukey's multiple comparison test ($p < 0.05$), evidenced that each DABCO-bar significantly differs from others DABCO-bars and that each morpholinium-bar significantly differs from others morpholinium-bars.

adsorption phenomena. Under the experimental conditions, the reference substance reached the maximum level of biodegradation (90% in not filtered samples) between day 7 and day 14 and all biodegradation rates of tested ILs were referred as percentage of reference, as shown in Figs. 4 and 5. Two different experiments for evaluating the biodegradability rate of DABCO and morpholinium ILs were performed. All investigated ILs showed a low percentage of biodegradation ($< 60\%$), with low differences between DABCO and morpholinium ILs and the alkyl chain length. Moreover, in both series the biodegradation trend showed that the higher percentage of biodegradation is reached between day 15 and day 21 after sampling. For DABCO ILs, the percentage of biodegradation varied from 40% (C2, day 28) to 22% (C6, day 28). Morpholinium ILs showed a biodegradation efficiency lower than those of DABCO ILs: the percentage of biodegradation varied from 30% (C2, day 28) to 3–5% (C6, C8 and C10, day 28). In this case, no significant differences were found as a function of the alkyl side chain on going from C6 to C10. Observed time trends related to the total dissolved organic carbon measured in the experimental vials have been reported in Fig. 6 (DABCO) and Fig. 7 (morpholinium). Residual values of about 10% (day 28) were observed for DABCO molecules, whereas morpholinium evidenced residual values higher than 30% (day 28).

4. Discussion

About the acute toxicity towards *V. fischeri*, these results represent the only source of data for DABCO-based ILs whereas

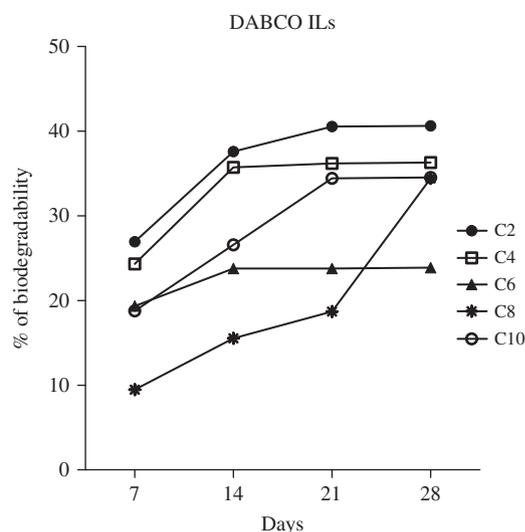


Fig. 4. Percentage of biodegradability calculated for DABCO ILs with different lateral chain length (C2–C10). Observations were performed after 7, 14, 21, and 28 days from the inoculums. Experiments were carried out in triplicate, levels were reported as average data ($n=3$).

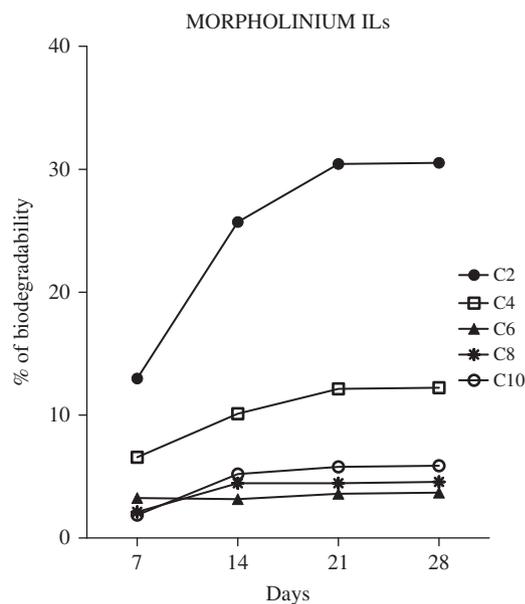


Fig. 5. Percentage of biodegradability calculated for morpholinium ILs with different lateral chain length (C2–C10). Observations were performed after 7, 14, 21, and 28 days from the inoculums. Experiments were carried out in triplicate, levels were reported as average data ($n=3$).

for morpholinium-based ILs an EC_{50} value of 4.3 (expressed as $\log_{10} EC_{50}/\mu M$) was reported by Stolte et al. (2007) for *N*-methyl-*N*-butylmorpholinium bromide (Morph C4). This value is in agreement with our results giving an EC_{50} (expressed as $\log_{10} EC_{50}/\mu M$) of 4.7 for the same IL, although Stolte et al. (2007) used a different detection method (LUMISTox[®]).

On the basis of the data represented in Fig. 3, it is evident that the transformation of both DABCO and *N*-methyl morpholine into the corresponding bromide salts bearing short alkyl chains reduces toxicity towards *V. fischeri* except to C8 and C10. The comparison of the EC_{50} values characterizing starting amines and the corresponding salts also shows that small amounts of the unreacted starting substrate (DABCO or *N*-Methylmorpholine) cannot significantly affect toxicity of the corresponding salts.

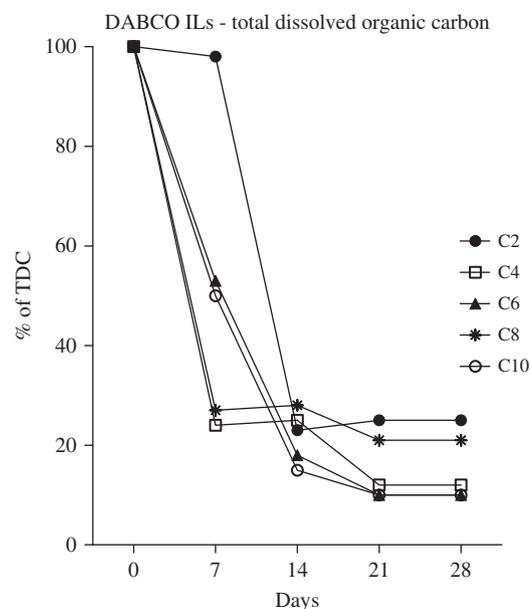


Fig. 6. Percentage of total dissolved organic carbon (TDC) calculated for DABCO ILs with different lateral chain length (C2–C10). Results are expressed as % of each DABCO point of the curve set at 100% (Fig. 4). Observations were performed at 0, 7, 14, 21, and 28 days from the inoculums. Experiments were carried out in triplicate, levels were reported as average data ($n=3$).

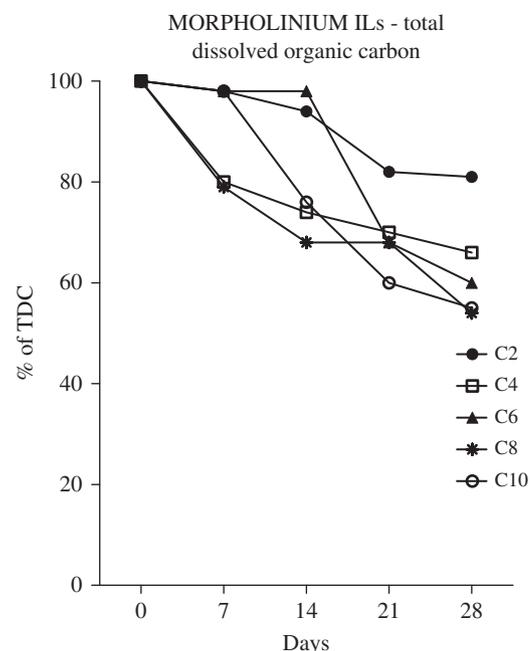


Fig. 7. Percentage of total dissolved organic carbon (TDC) calculated for morpholinium ILs with different lateral chain length (C2–C10). Observations were performed at 0, 7, 14, 21, and 28 days from the inoculums. Experiments were carried out in triplicate, levels were reported as average data ($n=3$).

On the other hand, in agreement with the behaviour already evidenced with other ILs (imidazolium, pyridinium, ammonium, pyrrolidinium, piperidinium and so on), a correlation between toxicity to *V. fischeri* and lipophilicity can be envisaged: toxicity increases on increasing the alkyl chain length. However, when ILs bearing alkyl chains of comparable length are considered, morpholinium and DABCO-based ILs show toxicity towards *V. fischeri* significantly lower than imidazolium and pyridinium salts (Stolte et al., 2007).

The investigated DABCO and morpholinium based ILs did not show high levels of ultimate biodegradability: mineralisation in 28 days was for all examined ILs lower than 60%; consequently, none could be defined as “readily biodegradable”. However, the values characterizing the salts of both the series bearing the shortest alkyl chain (ethyl) were significantly higher than those found for other widely used imidazolium-, phosphonium-, pyridinium-based ILs (Pham et al., 2010). Percentages of biodegradation around 35% have been reported only for imidazolium based ILs containing an ester side group (Harjani et al., 2008), which is known to increase significantly the biodegradability. Generally, DABCO based ILs are more biodegradable than the corresponding morpholinium salts bearing comparable alkyl chains.

The observed biodegradation trend showed an apparently strange behaviour of C8 DABCO. In fact, compared to the other ones there is a strong increase in the ultimate biodegradability from day 21 onwards. However, all the degradation patterns are included in a narrow range of total biodegradation and the observed effect could be considered as a natural fluctuation of the density of the microbial population.

Concerning DABCO, after 14 days of incubation, the residual fraction of total dissolved organic carbon (TDC) was minor than 30% for all the tested molecules with different chains. On the contrary, concerning morpholinium, observed TDC values remained close to 60% till the end of the incubation. This behaviour could be both due to differences in the equilibrium processes and due to the different efficiency of microbial population in the degradation of the dissolved fraction of the tested molecules.

Nevertheless, it is noteworthy that in DABCO and morpholinium based ILs, at variance with previously investigated imidazolium salts, biodegradability does not increase on increasing alkyl chain length on going from butyl to octyl (C4–C8); consequently, the more biodegradable ILs are also less toxic to *V. fischeri*. This suggests the presence of peculiar sites of biodegradation attack with respect to alkyl substituted imidazolium, pyridinium and ammonium salts. Moreover, considering that in the case of C2 and C4 substituted salts the percentage of biodegradation corresponds to the organic carbon content present in sites of the head group near to the oxygen (morpholinium) or to the tertiary nitrogen (DABCO-based), the hypothesis that these heteroatoms may direct biodegradation favouring the attack by mono-oxygenases can be advanced. On the other hand, in the case of morpholinium and DABCO ILs bearing longer alkyl chains, the reduced contribution of the head group carbons to the total amount of carbon and the possible biocidal effects could explain the lower biodegradation.

5. Conclusions

The aim of this study was the investigation of the environmental impact of two classes of ILs based on aliphatic cyclic quaternized amines, both bearing a further heteroatom (nitrogen or oxygen) on the cycle, on increasing alkyl chain length determining the effect on the marine bacteria *V. fischeri* and their biodegradability using the CO₂ headspace test. All the tested salts had bromide as counter-anion to reduce to the number of parameters that could affect the environmental impact evaluation. In conclusion, morpholinium and DABCO based ILs bearing short alkyl chains are characterised by a low toxicity to *V. fischeri*, although toxicity significantly increases on increasing the alkyl chain length. Alkyl chain length affects also biodegradability in the 28-day tests; the higher level of biodegradation was found in both the series in the case of the ethyl (C2) derivatives, i.e. the salts having the lower toxicity. In the case of *N*-ethyl DABCO based IL, although biodegradability is still around 40% and consequently this IL cannot be classified as “readily

biodegradable”, this value is similar to the more biodegradable functionalized imidazolium based ILs.

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