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Ecotoxicity of binary mixtures of cholinium-based ionic liquids and salts to microalgae†

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Due to the widespread use of ionic liquids (ILs) in various applications and their frequent combination with salts in processes or products, mixtures of ILs and salts are likely to be present in aquatic environments. The present study aimed to assess the combined ecotoxicity of mixtures of cholinium-based ILs (cholinium bicarbonate, benzyldimethyl(2-hydroxyethyl)ammonium chloride, cholinium bitartrate and cholinium dihydrogencitrate) and salts (potassium phosphate tribasic and sodium citrate dihydrate) to the microalga *Raphidocelis subcapitata*, a highly sensitive microalgal species. The 96 h-EC_{50} values for the endpoint yield increased as follows: [Chol][DHCit] (EC_{50} = 85.2 mg L^{-1}) < [Chol][Bit] (EC_{50} = 110.8 mg L^{-1}) < [Chol][Bic] (EC_{50} = 310.5 mg L^{-1}) < [BzChol]Cl (EC_{50} = 766.3 mg L^{-1}), generally following the expected increase of ecotoxicity with increased hydrophobicity. Both CA and IA models could describe the observed ecotoxicity, but a better fit was achieved with the CA model, with antagonistic interactions observed in 5 of the 8 tested mixtures. The salt K_3PO_4 was found to be less ecotoxic than $NaCit{\cdot}2H_2O$ and, simultaneously, to promote stronger antagonism, and is thus recommended to be used in future processes or product design with cholinium-based ILs, hence supporting the advancement of green chemistry. Synergism was not significant in any mixture, despite being observed under specific conditions, particularly when the IL was dominant in the mixture and above 1 TU. The antagonism observed for most of the mixtures, associated with the hormesis observed for all mixtures, suggests that mixtures of ILs and salts will likely be less environmentally hazardous than predicted based on their individual toxicities. Since ILs commonly present high water solubility and good stability, further studies addressing the effects of mixtures with ILs should be performed, contributing to improving the risk assessment of ILs.

Green foundation

1. Due to the extensive use of ILs and their frequent combination with salts in process development, their mixtures are likely to enter aquatic environments. This study aimed to evaluate the combined ecotoxicity of mixtures containing cholinium-based ILs and salts on a highly sensitive microalgal species, under the scope of IMPACT coverage.
2. The (eco)toxicity of cholinium-based IL and salt mixtures, commonly studied in downstream processes, was evaluated. Although considered sustainable, these ILs combined with salts may be harmful. This work highlights the need for careful salt selection using toxicity models to support greener choices.
3. This methodology should be applied to other aquatic species and more complex, ecologically relevant mixtures. The same approach is needed for eutectic solvents, as assuming their safety based solely on individual components and forgetting that they are mixtures is misleading.

Introduction

Ionic liquids (ILs) are salts with low melting points that have been the focus of much scientific work and industrial interest due to their particularly unique characteristics.[1] They have been proposed for a wide range of applications in areas such as (electro)chemistry, biotechnology, chemical and material engineering and the pharmaceutical industry.[1,2] Despite being

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labeled as "green" solvents, several studies have shown that some ILs can be highly toxic to aquatic biota.[3,4]

Salts have been used with ILs in liquid–liquid separation processes, namely in aqueous biphasic systems (ABS), allowing the separation of a wide variety of substances, including drugs, biomolecules, antibiotics, food colorants and textile dyes (ref. 5, 6 and references cited therein). Among the most commonly used salts are sodium citrate and potassium phosphate tribasic (ref. 5 and references cited therein). Sodium citrate has been successfully used as a salting-out agent in the extraction of proteins[7] and textile dyes,[6] whereas potassium phosphate tribasic (K_3PO_4) has been used for the extraction of astaxanthin from shrimp waste released by the food industry,[8] biomolecules,[9,10] textile dyes[11] and for cellulose precipitation in biorefinery applications,[12] among others. For this reason, it is expected that ILs and salts occur simultaneously in aqueous effluents. Early studies[13–15] have also suggested the use of salts as a plausible process for the treatment of aqueous effluents containing ILs, contributing to their recovery and further reuse, as recently reviewed by Khoo *et al.* (2024).[2] Indeed, some salts exhibit a strong salting-out ability, thus reducing the concentration of ILs in aqueous solutions.[2] The possibility of using inorganic salts in the treatment of effluents contaminated with ILs was further addressed by Ventura *et al.* (2010),[16] who proposed the use of aluminium sulfate to remove $[C_3mim][NTf_2]$ from contaminated effluents. Consequently, there is a likely possibility of generating aqueous effluents containing both ILs and salts.

The interest in IL toxicity has increased pronouncedly during the last decade, but most works only address exposures to single ILs, aiming to identify those that are less environmentally toxic. Studies addressing the combined toxicity of ILs and other compounds are scarce. As an example, the combined ecotoxicity of cholinium laurate and a biosurfactant was assessed in zebrafish, with the mixture classified as non-toxic.[17] Mixtures of a methylimidazolium tetrafluoroborate-based IL and four carbamate pesticides were assessed in the freshwater photobacterium *Vibrio qinghaiensis* sp.-Q67, showing a clear antagonistic interaction for most of the mixtures.[18] In contrast, binary mixtures of ILs and metals were shown to interact synergistically in the same freshwater bacterium.[19] However, as far as we know, the potential toxicity of mixtures of ILs and salts to the aquatic biota has been overlooked so far.

The present study aims to assess the toxicity of binary mixtures of four cholinium-based ILs and two salts to the green microalga *Raphidocelis subcapitata*. The selected ILs were cholinium dihydrogencitrate, cholinium bicarbonate, cholinium bitartrate and benzyldimethyl(2-hydroxyethyl)ammonium chloride. These cholinium-based ILs were selected based on a previous work to represent ILs with a wide range of toxicity values to the microalga, with yield-EC_{50} values varying between 27 mg L^{-1} (cholinium bitartrate) and 232 mg L^{-1} (cholinium bicarbonate).[20] The first three share the same cation, varying solely in the anion moiety, thus allowing us to study the effect of the anion on the ecotoxicity of the single chemical and, subsequently, on the ecotoxicity of mixtures. The selected salts were potassium phosphate tribasic and sodium citrate dihydrate. The first one is a high charge density salt with high potential for use together with cholinium-based ionic liquids for phase separation.[21] The second one is also a salt with high salting-out ability when compared to other organic salts,[22] and is considered to be more eco-friendly and biocompatible compared to potassium phosphate.[6] The microalga *R. subcapitata* was chosen as a model species due to its ubiquity in freshwater ecosystems and its key role in aquatic trophic webs. Being the basis of aquatic trophic webs as producers, any effects on these organisms will be propagated throughout the food webs due to decreased availability and/or quality of food for higher trophic levels, thus extending individual responses to the functional regulation of the aquatic systems. Moreover, this species is a model organism in regulatory ecotoxicology and risk assessment of chemicals, and it is among the most sensitive ones to ILs.[23]

Materials and methods

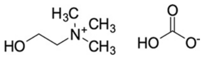
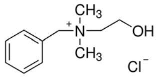
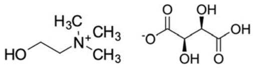
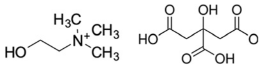
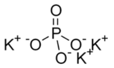
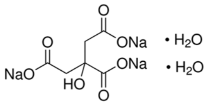
Chemicals

The ILs cholinium bicarbonate ([Chol][Bic], CAS number 78-73-9, 80% purity, Sigma-Aldrich), cholinium bitartrate ([Chol][Bit], CAS number 87-67-2, 99% purity, Sigma-Aldrich), cholinium dihydrogencitrate ([Chol][DHCit], CAS number 77-91-8, 98% purity, Sigma-Aldrich) and benzyldimethyl(2-hydroxyethyl)ammonium chloride ([BzChol]Cl, CAS number 7221-40-1, 97% purity, Fluka) were used in the present study. The salts potassium phosphate tribasic (K_3PO_4, CAS number 7778-53-2, 97 wt%, ACROS ORGANICS) and sodium citrate dihydrate ($NaCit\cdot 2H_2O$, CAS number 6132-04-3, no purity described, distributed by José M. Vaz Pereira, S.A., Portugal) were combined with the abovementioned ILs in the mixture toxicity tests. The chemical structures and main properties of the tested ILs and salts are presented in Table 1. The chemicals used to prepare the microalga culture medium were of analytical grade. The ILs and salts were dried in a vacuum (0.1 Pa and 353.15 K) for 48 h prior to use.

Chemical analysis

Chemical analyses were performed to determine the actual concentrations of the major ions: sodium, citrate, potassium, phosphate, cholinium, bicarbonate, and bitartrate. Samples for chemical analysis were prepared at the beginning of the experiments, following the same procedure and using the same solutions as those used in the experimental tests, with the exception that ultrapure water was used instead of Woods Hole MBL medium.[25] This procedure was adopted to allow more straightforward quantification of the chemicals of interest. Using the Woods Hole MBL medium would lead to numerous peaks in each chromatogram, which would bias the identification of the peaks corresponding to the chemicals of interest. Samples for each ion were collected in all the experimental tests, *i.e.*, different mixtures and different treatments. Overall, the number of tested samples per ion varied between 17 (cholinium ion) and 74 (phosphate ion). The ions were quantified by ion chromatography (Thermo Scientific Dionex™ ICS-5000

Table 1 Nomenclature, chemical structure and logarithm of the octanol–water partition coefficient (log K_{ow}) of the tested chemical compounds

Abbreviation	Name	Chemical structure	Log K_{ow}
[Chol][Bic]	Cholinium bicarbonate		−3.70(ref. 24)
[BzChol]Cl	Benzyldimethyl(2-hydroxyethyl)ammonium chloride		−2.54 (ref. 21)
[Chol][Bit]	Cholinium bitartrate		−1.43 (ref. 24)
[Chol][DHCit]	Cholinium dihydrogencitrate		−1.32 (ref. 24)
K_3PO_4	Potassium phosphate tribasic		—
NaCit·2H_2O	Sodium citrate dihydrate		—

coupled with a DC detector). For quantification of the cations (cholinium, sodium and potassium), a Dionex IonPaC® CS16-HC (4 × 250 mm) analytical column was used, using 30 mM methanesulfonic acid (MSA) as the eluent at 0.36 mL min^{-1} for the cholinium ion and 30 mM potassium hydroxide (KOH) as the eluent at 0.2 mL min^{-1} for the remaining cations. The anions (bitartrate, bicarbonate, dihydrogencitrate, phosphate, chloride and citrate) were quantified using a Dionex IonPac® AS11 (4 × 250 mm) analytical column, with 30 mM potassium hydroxide (KOH) as the eluent at 0.2 mL min^{-1}. The injection volume was 1.00 µL for all samples.

Mixture design

Prior to the mixture experiments, the ecotoxicity of each chemical was assessed individually. This allowed us to estimate the corresponding median effective concentration values (EC_{50} values, *cf.* section 2.5), and thus to express their concentration in relative Toxic Units (TU) when addressing mixture toxicity (see section 2.5 for details on the TU approach). For the mixtures, a full factorial design was used, with each of the four ILs being tested in combination with each of the two salts, thus corresponding to eight combinations, *i.e.*, eight binary mixtures. For each mixture, a ray design was used, in which the IL : salt ratio across each ray was maintained constant but the overall toxic strength (expressed in TU) of the mixture was increased; the following ratios were tested: 1 : 1, 1 : 2, 1 : 4, 1 : 8, 2 : 1, 4 : 1 and 8 : 1. Besides the control, a total of 5 treatments per ratio were used, as illustrated in Fig. S1 (ESI†), representing mixture strengths between 0.22 TU and 4.6 TU. An exception was made for the ratio 1 : 1, for which a total of 7 concentrations plus control were used, representing mixture strengths between 0.10 TU and 4.6 TU (Fig. S1†).

Growth inhibition experiments

Growth inhibition of the microalgae was assessed following the OECD Guideline 201,[26] adapted for the use of 24-well microplates.[20] Tests were started with 1.0 × 10^4 cells per mL in the log exponential growth phase and were carried out in triplicate. Each replicate consisted of the chemical solution (either individual or in mixture), the test medium (Woods Hole MBL[25]), and the microalgae, sourced from a long-term semi-static, non-axenic laboratory culture. The chemical solution was prepared by dissolving the IL and/or salt in Woods Hole MBL medium, followed by dilution to the desired concentrations. An experimental control (microalgae in blank MBL) and a test medium control (no microalgae; used for mixture stability verification and as a spectrophotometric blank at the end of the test) were also included for each treatment. Exposure to individual chemicals was also performed in the mixture tests to complete the ray design (see Fig. S1†). Exposure was performed at 23 ± 1 °C under continuous light (cool-white fluorescent) for 96 h. After this period, the absorbance at 440 nm was measured by spectrophotometry (UV-1800 spectrophotometer, Shimadzu Corporation, Japan) and converted to cell density (algae cells per mL) using a previously established calibration curve, except for tests with [Chol][Bit] and [Chol][DHCit]. As these ILs interfered with absorbance measurements at 440 nm, microalgal densities were determined by counting under a microscope using a Neubauer hemocytometer.[23] Cell densities were used to calculate both the biomass yield and the average specific growth rate, based on the increase in *R. subcapitata* cell density during the exposure period, following OECD Guideline 201.[26]

The concentrations eliciting 50%, 20% and 10% inhibition (EC_{50}, EC_{20} and EC_{10}, respectively) for the endpoint yield and growth rate were estimated following nonlinear regression, using the least-squares method to fit the data to a 3-parameter logistic equation (STATISTICA 8.0, StatSoft Inc.).

$$\frac{c_A}{EC_{x_A}}+\frac{c_B}{EC_{x_B}}=1 \qquad (1)$$

where c_A and c_B represent the concentration (or dose) of chemicals A and B, respectively, and EC_{x_A} and EC_{x_B} represent the concentration of that chemical causing x% of effect if applied individually.[28] Fractions c_A/EC_{x_A} and c_B/EC_{x_B} are designated as "toxic units" (TU) and represent the concentration of that chemical in the mixture scaled for its relative potency.[27]

On the other hand, the IA model accounts for the dissimilarity in the mode of action among chemicals in a mixture, thus assuming that the effect of one chemical is independent of the other.[27] For a binary mixture of chemicals A and B, the IA model can be mathematically described (eqn (2)) according to the joint probability of statistically independent events as:

$$E(c_{\text{mix}}) = 1 - [(1 - E(c_{\text{A}})) \times (1 - E(c_{\text{B}}))] \qquad (2)$$

where c_{mix} represents the total concentration of the mixture, and $E(c_{\text{mix}})$ is the IA-expected overall effect (scaled to the range of 0–1) of that mixture; $E(c_{\text{A}})$ and $E(c_{\text{B}})$ represent the effect of chemicals A and B if applied individually at concentrations c_{A} and c_{B}, respectively.[27]

After assessing the fit of the reference models to the experimental data, the potential deviations from the baseline models were assessed by comparing the observed ecotoxicity (experimental data) with the ecotoxicity predicted by baseline models incorporating deviation terms that define synergism/antagonism (S/A), the dose ratio (DR) and the dose level (DL), as established in ref. 28. S/A refers to the observed effects being more severe or less severe, respectively, than expected based on the predictions of the baseline models. DR refers to the interactive effect (synergism or antagonism) dependent on the mixture composition, *i.e.*, the ratio of each chemical in the mixture. DL refers to the interactive effect dependent on the dose level, *i.e.*, on the strength of the mixture. Further details on the mixture modelling theory and on their biological significance can be obtained in the literature.[28]

The fits of the experimental data to each model (baseline and those with added deviation functions) were compared through likelihood testing. The best fit was selected based on the results of the Chi-square test (which statistically compares the residual sum of squares, $p < 0.05$) and the coefficient of determination (which represents the adjustment between the predicted and the experimental data).

Contour plots illustrating the interactive effects between the IL and the salt on *R. subcapitata*, following the best fit, were created to improve data interpretation.

Results and discussion

Chemical analysis

In general, there was good agreement between the measured concentration and the nominal concentration, as the measured concentrations were within 12% of the nominal values, except for bicarbonate (Table S1†). The fact that the measured concentration of bicarbonate was prominently lower than the nominal one may be due to its degradation or conversion to carbonate during the experiments and/or chemical analyses.[29]

Ecotoxicity of the individual chemicals

The median effective concentration (96 h-EC_{50}) for each of the tested chemicals is depicted in Fig. 1. Data regarding 96 h-EC_{20} and 96 h-EC_{10} are presented in Table S2.† The ecotoxicity of the tested chemicals increased in the following order: [BzChol]Cl < K_3PO_4 < NaCit·$2H_2O$ < [Chol][Bic] < [Chol][Bit] < [Chol][DHCit], with the EC_{50} values varying between 766.3 mg L^{-1} and 85.2 mg L^{-1}, respectively, for the endpoint yield. The 96 h EC_{50} yield inhibition values for [Chol][Bic] and [Chol][DHCit] are similar to those found in previous studies in our laboratory, 87.2 mg L^{-1} and 232.4 mg L^{-1}, respectively.[20] However, the EC_{50} values obtained for [BzChol]Cl and [Chol][Bit] in the present study were 4-fold higher than those reported in ref. 20. A possible explanation for this difference is the exposure period: 72 h in the study reported in ref. 20 and 96 h in the present study. Although the extension of the microalga growth inhibition test is accommodated in the followed standard guideline,[26] it is reasonable to

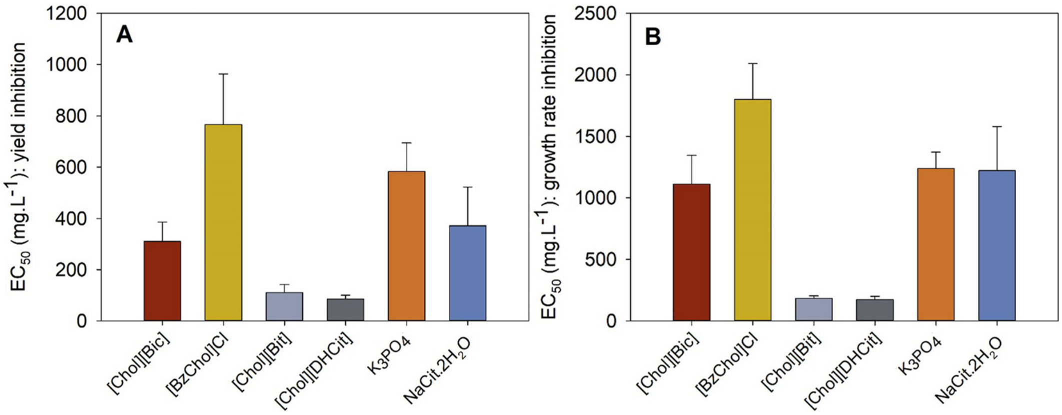


Fig. 1 Median effective concentration (EC_{50}) values of each tested chemical (*i.e.*, [Chol][Bic], [BzChol]Cl, [Chol][Bit], [Chol][DHCit], K_3PO_4 and NaCit·$2H_2O$) to the microalga *R. subcapitata* after 96 h of exposure, considering the endpoint yield (A) and growth rate (B). Error bars represent standard deviation. Data were obtained from single exposures in the mixture toxicity tests.

consider that longer exposure periods translate into decreased EC_{50} values. In general, the EC_{50} values match the expected increase in toxicity with increasing hydrophobicity of ILs,[23,30] commonly attributed to the higher capacity of more hydrophobic ILs to interact with cell membranes.[30] Indeed, hydrophobicity increases as [Chol][Bic] < [BzChol]Cl < [Chol][Bit] < [Chol][DHCit] (Table 1), whereas ecotoxicity increases as [BzChol]Cl < [Chol][Bic] < [Chol][Bit] < [Chol][DHCit] (Fig. 1; Table S2†). The lack of a perfect match between the increased hydrophobicity and the increased ecotoxicity is not surprising, which might be related to the species and the experimental conditions. In this regard, for instance, the inverted trend was observed for the endpoint yield and growth rate based on the frond number of *Lemna minor* (Table 2), where the least hydrophobic chemicals exhibited the highest ecotoxicity.

According to the United Nations Globally Harmonized System of Classification and Labelling Chemicals (GHS),[31] considering the growth rate inhibition values of microalgae, all the tested chemicals can be classified as non-toxic, as their EC_{50} values are above 100 mg L^{-1}, which agrees with the "green" character commonly attributed to ILs. However, note that 96 h-EC_{50} values for the endpoint yield were below 100 mg L^{-1} for [Chol][DHCit], which suggests that this IL is not completely devoid of ecotoxicity. [BzChol]Cl was the least toxic IL to the microalgae, with a 96 h-EC_{50} yield inhibition above 700 mg L^{-1} (Fig. 1; Table S2†). The lowest ecotoxicity of this IL might be related to the molecular structure of its cation, supporting the role of the cation as the major driver of IL toxicity.[23,32] Indeed, compared to the cholinium cation, [BzChol] has an additional benzyl group (Table 1). Despite that adding an aromatic ring commonly makes ILs more ecotoxic,[33,34] the opposite trend was observed in the present study, which agrees with a previous study reporting lower toxicity of [BzChol]Cl compared to [Chol]Cl toward *R. subcapitata.*[20] Such an inconsistent trend could be explained by the larger/longer cation [BzChol] and the concomitant difficulty to cross the cell wall of microalgae.[20]

Interestingly, the salt $NaCit{\cdot}2H_2O$, which was previously reported to be more eco-friendly and biocompatible than K_3PO_4,[6] was indeed more toxic than K_3PO_4 for the tested microalgal species.

Considering the cholinium-based ILs [Chol][Bic], [Chol][Bit] and [Chol][DHCit], a 3.6-fold variation in the 96 h-EC_{50} yield inhibition values was observed, corroborating the important role of the anion in IL toxicity.[20,23] This effect is more pronounced in ILs with short cation alkyl chains, as in the case of the cholinium cation.[35] In the present study, the IL with the anion [Bic] was pronouncedly less ecotoxic than the ILs with [DHCit] and [Bit], which agrees with previous studies with cholinium-based ILs using the same anions (Table 2). Indeed, Santos *et al.*[20] also observed that [Chol][Bic] was less toxic than [Chol][DHCit] to *R. subcapitata*. Moreover, Ventura *et al.*[36] observed that [Chol][DHCit] showed similar toxicity to [Chol][Bit] regarding the bioluminescence inhibition to the marine bacterium *Aliivibrio fischeri*, but far above that of [BzChol]Cl. The lower ecotoxicity conferred by the anion [Bic] compared to [Bit] and [DHCit] agrees with the "side-chain effect", which states that toxicity increases with the size of the alkyl side chain.[20,23] Indeed, the anion [Bic] has a shorter chain and less ramification than the others (Table 1). [Chol][Bic] exhibits higher hydrophilicity (a lower $\log K_{ow}$ is associated with lower hydrophobicity; see Table 1) and, thus lower lipophilicity, with consequent less interaction with the biological membranes and their embedded proteins.[30] Thus, the present results corroborate previous studies, reinforcing the important role of the anion in the IL toxicity to aquatic species.

Ecotoxicity of ILs and salt mixtures

The effects of the binary mixtures of ILs and salts on the yield of *R. subcapitata* are depicted in Fig. 2 through isobolograms corresponding to the mixture toxicity model that better fits each binary (see Table S3† for the estimated parameters and the statistical summary). This is complemented by Table 3, concerning relevant parameters for the interpretation of mixture toxicity.

Table 2 Summary of the ecotoxicological data (EC_{50} values, expressed in mg L^{-1}) available for the ILs tested in the present work towards primary producers

IL	*R. subcapitata*	*Chlorella vulgaris*	*Lemna minor*	*Lemna gibba*
[Chol][Bic]	72 h EC_{50} (yield): 232.4[20] 72 h EC_{50} (GR): 1375[20]		7 d EC_{50} (yield-FN): 483.6[20] 7 d EC_{50} (GR-FN): 658.1[20] 7 d EC_{50} (yield-DW): 2589[20]	
[BzChol]Cl	72 h EC_{50} (yield): 196.2[20] 72 h EC_{50} (GR): 456.2[20]		7 d EC_{50} (yield-FN): 11.86[20] 7 d EC_{50} (GR-FN): 13.88[20] 7 d EC_{50} (yield-DW): 26.30[20] 7 d EC_{50} (GR-DW): 49.20[20]	
[Chol][Bit]	72 h EC_{50} (yield): 27.26[20] 72 h EC_{50} (GR): 125.3[20]		7 d EC_{50} (yield-FN): 1063[20] 7 d EC_{50} (GR-FN): 1632[20] 7 d EC_{50} (yield-DW): 1197[20] 7 d EC_{50} (GR-DW): 5694[20]	
[Chol][DHCit]	72 h EC_{50} (yield): 87.16[20] 72 h EC_{50} (GR): 155.2[20]	96 h EC_{50} (yield): 524.0[23]	7 d EC_{50} (yield-FN): 1863[20] 7 d EC_{50} (GR-FN): 5649[20]	7 d EC_{50} (yield-FN): 880.9[23] 7 d EC_{50} (yield-DW): 1631[23]

GR: growth rate; FN: the endpoint frond number; DW: the endpoint dry weight.

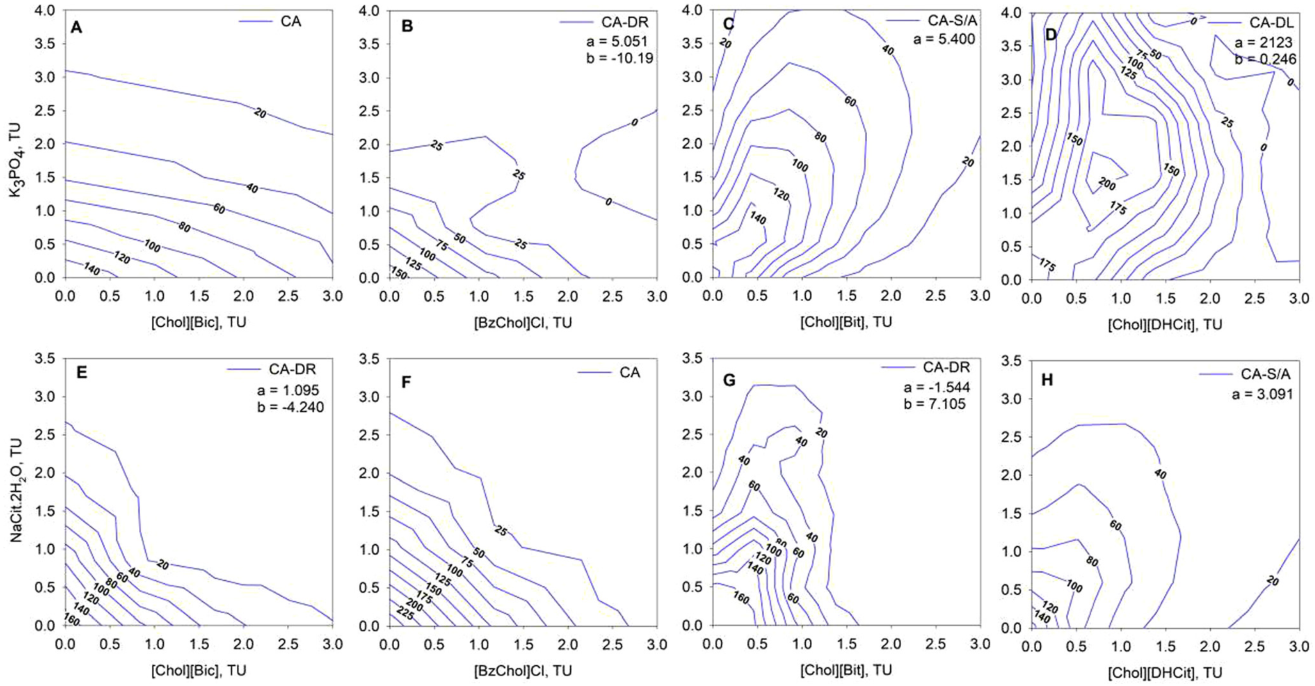


Fig. 2 Isobolograms representing the variation of the yield (× 10^4) of *R. subcapitata* under exposure to mixtures of the salt potassium phosphate tribasic (K_3PO_4) and each IL (A: [Chol][Bic]; B: [BzChol]Cl; C: [Chol][Bit]; D: [Chol][DHCit]) or the salt sodium citrate dihydrate (NaCit·$2H_2O$) and each IL (E: [Chol][Bic]; F: [BzChol]Cl; G: [Chol][Bit]; H: [Chol][DHCit]), according to the model/deviation that best fits the experimental data (see top of each figure). The isoboles represent equi-effective levels. Points below the straight line connecting equi-effective concentrations of both mixture components represent a synergistic interaction between the mixture components; points above that line represent an antagonistic effect; points in the line represent an additive effect. The chemical concentrations are expressed in TU (see Table 3 for the correspondence TU – mg L^{-1}).

The baseline model that best fit the experimental data was the CA model in all mixtures (Table S3†). No significant deviation from the baseline model was found for 2 mixtures, whereas DR, S/A and DL deviations were observed for 3, 2 and 1 mixtures, respectively, from the 8 tested mixtures (Table 3). Despite the statistically significant effect of the deviations on the baseline model, it was found that the baseline model already explained, to a good extent, the observed variability of the experimental data (Table S3†). A previous study with the microalga *Scenedesmus quadricauda* also reported that the predictions of the CA and IA models were close to the observed ecotoxicity for binary mixtures of ILs and graphene.[37]

Considering the isobolograms, it is observed that the yield achieved values above 100% at the lowest concentrations for all mixtures. Such increased yield compared to controls reflects the increased microalgal growth observed at low concentrations for all mixtures, *i.e.*, a hormetic effect (stimulation occurring in response to low levels of exposure to compounds that are harmful at high levels of exposure). The most remarkable example was the mixture of [BzChol]Cl and NaCit·$2H_2O$, for which the yield values reached up to 225% compared to controls. The hormetic effect has been previously observed for several species, namely bacteria,[38] nematodes,[39] freshwater microalgae, including *R. subcapitata* exposed to a methylimidazolium-based IL and a methylpyrrolidinium-based IL,[40] *Scenedesmus obliquus* exposed to a pyridinium-based IL[41] and to three imidazolium-based ILs[42] and *Scenedesmus quadricauda* exposed to a methylimidazolium-based IL,[43] as well as the marine microalgal species *Dunaliella tertiolecta* exposed to methylimidazolium-derived ILs.[44] Indeed, at low concentrations, ILs may act as plant growth hormones, as observed in several terrestrial plants,[45] explaining the hormetic effect. Cholinium-based ILs have been reported to promote hormesis in the yeast species *Saccharomyces cerevisiae*[46] but, as far as we are aware, the present study is the first to report hormesis of cholinium-based ILs in microalgae. Hormesis is a relevant issue since aquatic organisms will likely be exposed to mixtures of chemicals at low concentrations, which might have significant implications for environmental risk assessment. For this reason, the possible occurrence of hormesis in natural environments should not be disregarded when appraising the hazardous potential of contaminants prospectively. Still, some studies reported that the hormetic (stimulatory) effect might be time-dependent, changing after longer exposure periods.[40,43]

Antagonism was the main interaction effect observed in the mixtures' toxicity, as given by the positive *a* values (Table 3) and supported by the convex shape of the curves (Fig. 2). Based on the *a* values, antagonism was more pronounced for the mixture of [Chol][DHCit] and K_3PO_4 (*a* = 2123), followed by [Chol][Bit] and K_3PO_4 (*a* = 5.400) and by [BzChol]Cl and K_3PO_4 (*a* = 5.051). As antagonism is a deviation characterized by the effects of the chemicals in the mixture being less hazardous

Table 3 Estimated parameters for the best fitting model-deviation to the experimental data, considering the effect of binary mixtures of IL salts on the yield of *R. subcapitata* after 96 h of exposure. Refer to Table S3† for details on the fitting and selection of the best fit model

	[Chol][Bic] × K_3PO_4 (Fig. 2A)	[Chol][Bic] × NaCit·$2H_2O$ (Fig. 2E)	[BzChol]Cl × K_3PO_4 (Fig. 2B)	[BzChol]Cl × NaCit·$2H_2O$ (Fig. 2F)	[Chol][Bit] × K_3PO_4 (Fig. 2C)	[Chol][Bit] × NaCit·$2H_2O$ (Fig. 2G)	[Chol][DHCit] × K_3PO_4 (Fig. 2D)	[Chol][DHCit] × NaCit·$2H_2O$ (Fig. 2H)
Best fit model	CA	CA–DR	CA–DR	CA	CA–S/A	CA–DR	CA–DL	CA–S/A
Y_{max}	158.0	174.5	167.8	270.5	168.9	179.4	191.5	167.7
EC_{50} IL	661.2	475.6	794.4	811.3	47.95	50.83	71.38	33.74
EC_{50} salt	493.9	346.7	389.6	314.2	320.4	286.2	428.5	283.87
β IL	1.105	1.906	2.372	2.636	2.163	3.765	2.494	1.418
β salt	1.911	2.443	2.432	2.428	2.279	3.578	3.476	1.358
a	—	1.095	5.051	—	5.400	−1.544	2123	3.091
b	—	−4.240	−10.19	—	—	7.105	0.246	—
Mixture interaction[a]	—	Antagonism, except when the mixture ratio is approximately equi-effective, where there is synergism	Antagonism, except when the mixture ratio is dominated by [BzChol]Cl, where there is synergism	—	Antagonism	Synergism, except when the mixture ratio is dominated by NaCit·$2H_2O$, where there is antagonism	Antagonism at a low dose level and synergism at a high dose level, with the change at a higher dose level than the EC_{50}	Antagonism

r^2 is the coefficient of determination; *p* (χ^2 or *F*-test) indicates the outcome of the likelihood ratio test (for comparing the adjustment of different models) or the outcome of the *F*-test (for the baseline model; tests the null hypothesis that the experimental data do not follow the respective baseline model); Y_{max} is the control response (yield, cells per mL); β is the slope of the individual dose–response curve; EC_{50} (in mg L^{-1}) is the median effect concentration; *a* and *b* are the parameters in the deviation functions; CA is the concentration addition; S/A is synergism/antagonism; DL is the dose level-dependent deviation from the reference; and DR is the dose ratio-dependent deviation from the reference. [a] Based on ref. 28.

than predicted based on their individual ecotoxicity, such a mixture behavior represents a more favorable environmental condition when compared to additivity or synergism. Having in mind that K_3PO_4 was less ecotoxic than NaCit·$2H_2O$, and considering the antagonism observed for the mixtures with this salt, we recommend the use of the former instead of the latter in several applications, namely for separation processes (ABS) and for wastewater treatment. However, this recommendation is limited by the need to further study its environmental impact on other aquatic species and its degradation kinetics. The lack of ecotoxicological data of the tested salts to other species prevents a deeper discussion.

Synergism was not significant in any mixture. However, it was observed in some mixtures under specific conditions, commonly when the IL was dominant and above 1 TU, as observed for the mixture between [Chol][Bit] and NaCit·$2H_2O$ (Fig. 2). Given the individual ecotoxicity of each component of this mixture and the observed trend for synergism, we do not recommend the industrial use of this mixture for environmental reasons, unless other mixtures, more environmentally friendly, are not available to achieve the same performance in the intended application. Moreover, the mixture of [BzChol]Cl and K_3PO_4 also showed a trend for synergism when the mixture was dominated by the IL (Fig. 2). These results raise concern about the effects of these mixtures when the IL is dominant in the mixture.

In natural environments, it is not expected that concentrations of ILs and salts will exceed 0.5–1.0 TU since, as previously mentioned, commonly, chemicals are found at low individual concentrations forming complex mixtures. The isobolograms show that mixtures of ILs and salts at concentrations below 0.5–1.0 TU commonly follow the additive model, as given by the linearity of the lines, with the exception of the mixtures including [Chol][Bit] and [Chol][DHCit] with either of the tested salts, but most pronouncedly for the salt K_3PO_4, for which antagonism is observed even at these low concentrations (Fig. 2). However, these ILs were the most toxic to the tested microalgal species. It is thus evident that the selection of a mixture as potentially interesting from an environmental perspective must consider not only the interaction of chemicals (preferentially antagonism) but also their individual toxicity and the conditions of use and disposal (namely the expected concentration in aqueous effluents and dilution rates in the recipient environmental compartment). Thus, the recommendation of a specific mixture as the most environmentally friendly for industrial applications cannot be made generically but for specific purposes under specific conditions, and this is where reliable mixture toxicity models can assist decision making according to each context.

Previous studies have also reported antagonistic interactions in mixtures of ILs and other chemicals. For instance, Wang *et al.*[37] reported that, for the freshwater microalga *Scenedesmus obliquus*, mixtures of graphene and methylimidazolium-based ILs were additive at low mixture strengths but antagonistic at high strengths, which agrees with the results of the present study. Also, three binary mixtures of an imidazolium-based IL and carbamate pesticides showed antagonism at relatively low effect regions, while one mixture exhibited additive action against the freshwater photobacterium *Vibrio qinghaiensis* sp.-Q67.[18] Another study also reported antagonistic interactions between several ILs (dimethylimidazolium-based and pyridinium-based ILs) and the pesticide dichlorvos in the same bacterial species.[47] Moreover, binary mixtures of ILs (imidazolium-based IL and a pyridinium-based IL) and pesticides (desmetryn and dichlorvos) exhibited a similar toxicity interaction pattern, showing synergism in a high concentration region, additivity in a medium concentration region, and antagonism in a low concentration region,[48] which matches the behavior observed for the mixture of [Chol][DHCit] and K_3PO_4. On the other hand, the literature suggests that mixtures of ILs and metals will likely show synergistic interactions. For instance, a mixture of an imidazolium-based IL and cadmium showed a synergistic interaction for the freshwater macroalga *Scenedesmus vacuolatus* and for the plant *Triticum aestivum.*[49] Also, mixtures of metals (Cd, Ni, Cu and Zn) and imidazolium-based ILs showed synergistic interactions for the photobacterium *Vibrio qinghaiensis.*[19] Overall, these studies provide evidence that the co-existence of ILs can differentially affect or be affected by the toxicity of other contaminants. There is also evidence that environmental stressors, such as salinity, can affect the physiological response of microalgae against ILs.[50] Given the high solubility and stability of ILs, there is the risk of accumulation and persistence in soils[45] and in aquatic systems, which highlights the ecological relevance of including these chemicals in mixture toxicity studies of regulatory interest, not only concerning microalgae but also other aquatic species. Future studies should also address more complex mixtures, which are ecologically more relevant.

Conclusions

The present study assessed the combined ecotoxicity of mixtures of ILs and salts, often used together in separation processes and product formulations, to the microalga *R. subcapitata*. The individual ecotoxicity of the ILs was low as expected (96 h-EC_{50} yield inhibition values between 85.2 mg L^{-1} and 766.3 mg L^{-1}), verifying the general heuristic rule of increased toxicity with increased hydrophobicity. When mixing ILs and salts, the baseline CA model invariably explained more of the experimental data variation than the IA model, with an antagonistic interaction observed for most of the tested mixtures. The salt K_3PO_4 showed to be less ecotoxic than $NaCit{\cdot}2H_2O$ (96 h-EC_{50} yield inhibition values were 583.0 and 372.2 mg L^{-1}, respectively) and, simultaneously, to promote stronger antagonism, and is thus recommended to be used in future applications, when possible. Synergism was not dominant in any mixture, despite being observed for some mixtures under specific conditions. Although the antagonistic effect observed for most mixtures indicates that combinations of ILs and salts are less toxic than individual chemicals, and given that hormesis was observed for all mixtures, these results should be analyzed with caution, as they concern a single species, representing a single functional level of aquatic ecosystems. Since ILs commonly present high water solubility and stability, further studies addressing the effects of mixtures with ILs should indeed be performed, contributing to improving the prospective/regulatory risk assessment of ILs.

Conflicts of interest

There are no conflicts to declare.

Data availability

The data for this article, including the results of the growth inhibition tests with *Raphidocelis subcapitata*, are available through the institutional data repository of the University of Aveiro, DunAs, at **https://doi.org/10.48527/3QAY9H**.

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