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Biodegradability and Ecotoxicity Profiles of Choline Acetate, Betaine, and L-Proline NADESs: A Hidden Threat for Eutrophication?

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Abstract

Deep Eutectic Solvents (DESs) and in essence naturally available DESs (NADESs) are considered to be green solvents due to their low vapor pressure, non-flammability, thermal stability, good solvent power and low oxicity. These properties make them attractive as safer and more environmentally acceptable solvent options. Green Chemistry promotes the use of renewable and biocompatible compounds such as amino acids, lipids and acids of natural origin to yield more sustainable DESs, which yields their application in several industrial processes. Driven by the current requisite for sustainable progress, along with overcoming dependence on fossil-based resources, the current work details important findings pertaining to the design of sustainable NADESs from the perspective of green chemistry to exhibit suitable physico-chemical properties and a low toxicological profile. Biodegradation studies using OECD 301D closed bottle test (CBT) were performed to observe the biodegradability of 15 selected NADESs. Toxicity controls were run along with the CBT run to observe the behavior of these NADESs in the environment. In this framework, the present paper investigates the development of safer NADESs. The results obtained suggest that our synthesized NADESs, have high biodegradability and low toxicity towards microalgae. Although a conventional threat to the environment would seem out of reach, it must be hypothesized that such compounds might act as enhancers of eutrophication phenomena.

Keywords: naturally available deep eutectic solvents; biodegradation; ecotoxicity; *Raphidocelis subcapitata*; closed bottle test; hydrogen bond donor; hydrogen bond acceptor

1. Introduction

In the advent of sustainability and green chemistry era there is an ongoing quest to discover safer and more environmentally friendly solvents to replace traditional ones derived from fossil-derived chemicals [1]. These conventional solvents pose significant environmental threats during

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production, use, and disposal [2]. The end-of-life cycle for these solvents is a problem because they are not biodegradable. This means they must be burned, which creates CO_2 emissions as they are often non-biodegradable. In addition, the impact of these solvents on human health has recently become a cause for concern in various industrial sectors. For example, *N*,*N*-dimethyl formamide (DMF) has recently been highlighted for its reproductive toxicity[3]. Consequently, its use, particularly in the pharmaceutical industry, has been prohibited within the European Union. In this context, the investigation of sustainable alternatives to fossil-based solvents has intensified in recent years. Among the neoteric solvents,the deep eutectic solvents (DESs) represent a versatile alternative to volatile organic solvents, including those of natural origin [4].The recent popularity of DES is mainly due to their simple preparation and their relatively low-cost [5,6]. DESs are typically obtained by the mixture of two or more compounds [7], which determines a decrease of the melting point of the mixture below that expected for the ideal eutectic [8]. At the basis of this deviation there is the strong interaction between a hydrogen bond donor (HBD) and a hydrogen bond acceptor (HBA) that creates a strong microheterogeneity at the molecular level [9]. However, the real key to the success of DES is their tuneability, which allows for the customization of the system to suit specific needs. Simply modifying the chemical composition of the mixture allows its chemical and physical properties to be adapted for a specific application. An additional degree of modularity can be achieved by adjusting the molar ratio between the components [10]. A subset of DESs, inspired by nature and derived from plant-based products and other bio resources, are called Natural Deep Eutectic Solvents (NADESs) [11]. In fact, they are composed of natural substances, which is the reason behind their biocompatibility [12–14].

Although the definition of DESs does not have a direct implication on most features, initially, the green characteristics of DESs were assumed based on the pharmaceutically acceptable profile that was reported in the material safety data sheets of the selected individual components [15]. Later, a few studies have been devoted to investigating the DESs toxicity profile [16]. Indeed, bio-based chemicals may not be completely biodegradable hence not 100% environmentally friendly. However, despite the great interest of academia and industry on DESs and NADESs applications for gas capture [17], biomass valorisation [18,19], wastewater entrapment [20] and wastewater treatment [21], material synthesis [22], the toxicity of these compounds is still an underrated issue. Limited recent research studies have investigated NADESs toxicity on different targets, such as human cells [23–25], bacteria [25–28], yeasts [25], invertebrates and microalgae [12,26,27], and fish [29], sometimes reporting conflicting results. Indeed, what emerged from different papers was a pool of different behaviors of HBA/HBD if assessed singularly or, instead, in a NADESs composition [12]. Moreover, several studies support the idea of DESs as “eco-friendly”, assuming they are not toxic given the fact they are formed by naturally-occurring ingredients and, for this reason, bio-renewable, biodegradable and bio-assimilable [30,31], while other publications report a clear toxicity linkable to some DESs components [23].

Among NADESs components, choline is the most used compound to prepare DESs [32], due to its high biodegradability [33] and low toxicity [31] as well as low cost and widespread availability [34]. Although found in living organisms, choline cation currently on the market is of fossil origin and is obtained from trimethylamine and ethylene oxide [33]. In particular, choline acetate (ChA) and bitartrate (TA) are much less commonly used than choline chloride (ChCl) in the preparation of DES, but they are a valuable halogen-free alternative. For example, DES based on ChTA have recently been used to increase the solubilisation of drugs [35], plasticize starch [36] and extract flavonoids [37]. ChA-based DESs have also recently been studied from a physico-chemical and application perspective [38–41]. For example, ChA:glycolic acid (GA), ChA:levulinic acid (LA) and ChA:imidazole (Im) showed excellent performance in hemicellulose solubilisation and were used in Kraft cellulose purification. [42] On the other hand, betaine (trimethylglycine, Bet), a zwitterionic compound bearing both formal positive and negative charges has also been used to prepare DESs [43] and has been even suggested as the universal HBA [44]; like choline and betaine it is also biocompatible [45], has low toxicity profile [46,47], and is readily biodegradable. However, in contrast

 doi:10.20944/preprints202512.1548.v1

to ChCl, Bet can be derived from renewable sources and in particular by transformation of by-product of sugar production [42]. The use of Bet-based DES has grown significantly in recent years and are being used in numerous applications [48–51].

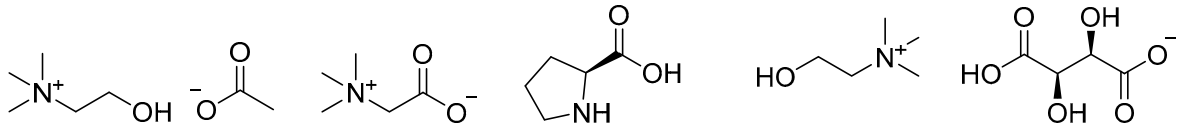
The proteinogenic amino acid L-proline (L-Pro) has received attention recently for designing ILs [52–54] and NADESs [55,56] for different applications. The ability of L-Pro to form enamines or imines, when reacting with carbonyl groups, as well as its ability to induce chirality promoted by the cyclic structure of the molecule, have been exploited in a wide range of organic synthetic methodologies. L-Pro can promote a very broad diversity of transformations, which can be explained by the multiple catalytic roles allowed by its structural features.

Similarly, L-Pro can behave both as an acid or Brønsted base, or even show both behaviors during a mechanism, being therefore a bifunctional catalyst. It is the only natural amino acid with a secondary amine functionality, a feature that raises the nitrogen pK_a and increases its nucleophilicity in comparison to other amino acids. L-Pro has several additional advantages such as being inexpensive, commercially available, non-toxic and easily recoverable, which are important properties from the point of view of green chemistry [57]. L-Pro has recently become widely used as an ingredient in supplements, health foods and cosmetics. The use of L and D-Pro as an HBA in the preparation of DES solvents allows to develop new reaction media with a dual solvent/catalyst role [58].

In this study, a series of 15 different choline, betaine, and L-proline-based NADESs (Table S1) and their single components (Figure 1), was assessed for their biodegradability using the aerobic biodegradation method [59], in order to evaluate their persistence in aquatic systems and the potential release of nutrients, such as N and P by molecule dissolution.

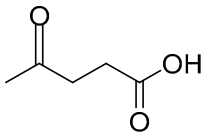
Moreover, we evaluated the potential ecotoxic effect of these compounds and their respective single components in terms of freshwater microalgae *Raphidocelis subcapitata* growth stimulation and/or inhibition, hypothesizing an underrated effect of NADESs as potential eutrophication substances.

A. Hydrogen bond acceptors:

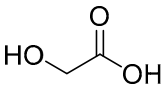


Choline acetate (**ChA**) Betaine (**Bet**) L-proline (**L-Pro**) Choline bitartrate (**ChTA**)

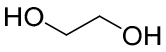
B. Hydrogen bond donors:



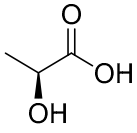
Levulinic acid (**LA**)



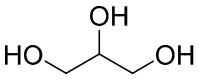
Glycolic acid (**GA**)



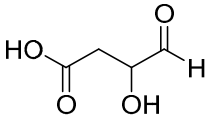
Ethylene glycol (**EG**)



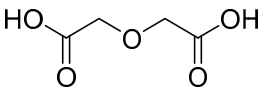
L-lactic acid (**L-LacA**)



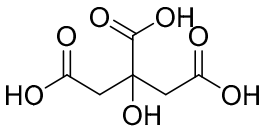
Glycerol (**Gly**)



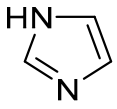
D,L-malic acid (**MA**)



Diglycolic acid (**DGA**)



Citric acid (**CA**)



Imidazole (**Im**)

Figure 1. Chemical structures of hydrogen bond acceptors (A) and hydrogen bond donors (B) and their abbreviations used used in this work.

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2. Results

2.1. Biodegradation Results of NADESs

The biodegradability tests conducted for all of the test substances for 28 days were proven to be valid as the control, readily biodegradable sodium acetate was eliminated by at least 60% by the end of 14 days

All the tested NADESs, as well their single components, were observed to be classified as "readily biodegradable", showing biodegradation values > 60 % after a 28-days Closed Bottle Test (Table S2). The only exception was Im, with a biodegradation value of 23 % as an indisvidual HBD component, although when in combination with ChAc (NADES 11), it seemed to not affect whole NADES's biodegradability score (67 %). The biodegradability profiles observed for studied NADES are presented on Figure 2a-o.

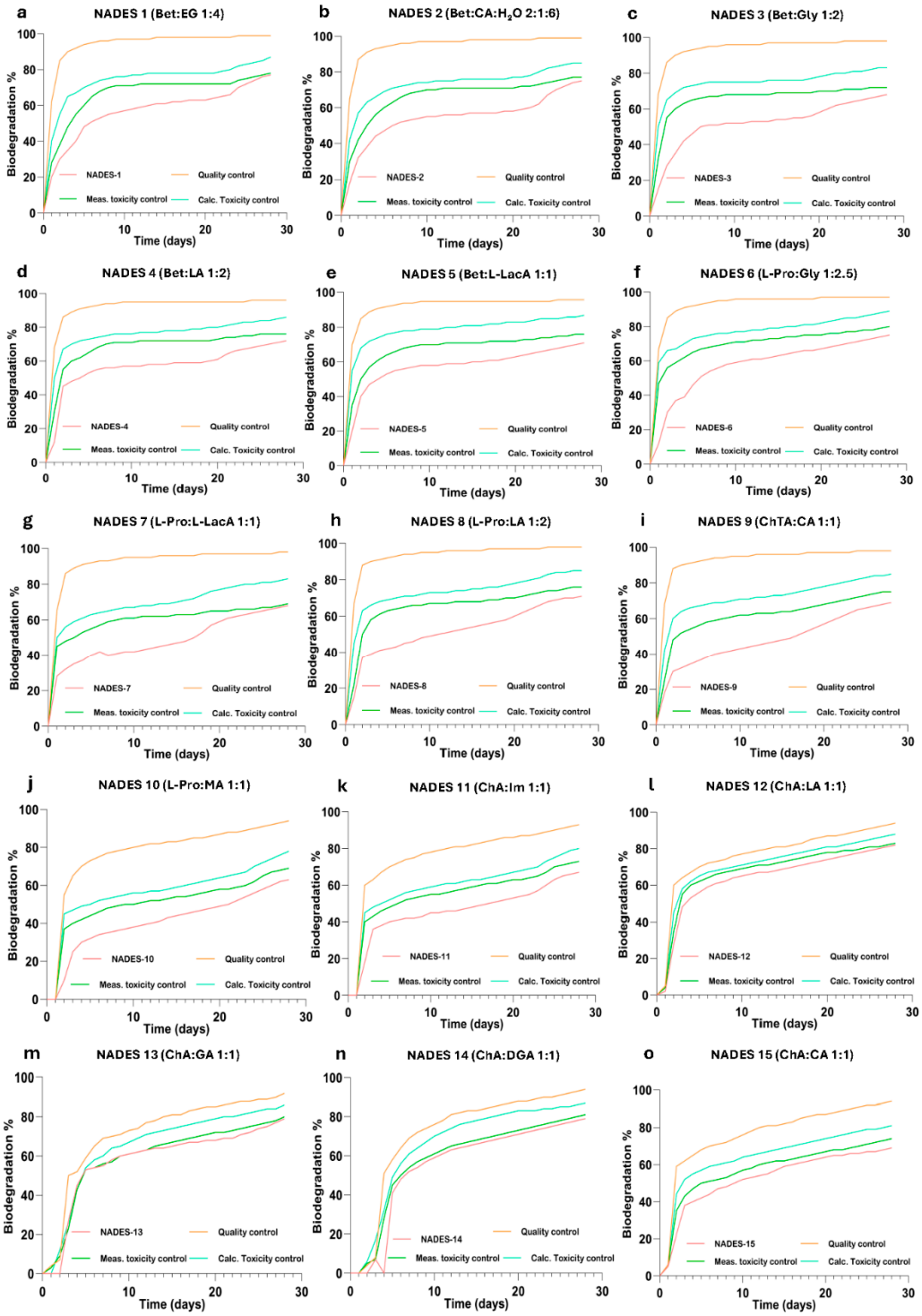

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Figure 2. Biodegradability profiles of Closed Bottle Test (CBT OECD 301D, 28 days) for studied NADES: . *a*) Bet:EG; *b*) Bet:CA; *c*) Bet:Gly; *d*) Bet:LA; *e*) Bet:L-LacA; *f*) L-Pro:Gly; *g*) L-Pro:L-LacA; *h)* L-Pro:LA; *i*) ChTA:CA; *j*) L-Pro:MA; *k*) CA:Im; *l*) ChA:LA; *m*) ChA:GA; *n*) ChA:DGA; *o*) ChA:CA.

2.2. Raphidocelis subcapitata Growth Bioassay

The Figure 3 represents the growth behaviour of *R. subcapitata* algae exposed to different concentrations of 15 NADESs. Each graphic showed the differences, in terms of concentration response trend, between NADESs and their components. The concentration-response curve showed an algal growth stimulation, with increasing concentrations, in thirteen NADESs compared to control. The only 2 tested NADESs where the algal growth was not different from control were NADES 4 (Bet : LA) (Figure 3d) and NADES 11(ChA : Im) (Figure 3k).

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Among NADESs components, the one with the most stimulating effect in terms of algal growth was choline acetate, which represents the HBA of NADESs 11-12-13-14-15 (Figure 3 k,l,m,n,o). Following the same trend, L-Pro in NADES 7 and NADES 10 (Figure 3g and 3j, respectively) showed a slight growth stimulation at the maximum tested concentration, followed by a reduction in algal growth at 75 %, 50 % and 25 %. The same compound, tested at concentrations as in NADESs 6 and 8 (Figure 3f and 3h, respectively), showed an inhibiting effect until 50 %. This is linked to the fact that the concentration of L-Pro in NADES 6 and 8 at 50 % was similar to L-Pro in NADES 7 and 10 at 25 %. A similar trend, as for L-Pro in NADESs 7 and 10, was observed for MA and L-LacA (Figure 3 e,g,j). However, in these cases, the inhibition effect was limited to the concentration of 50 % and 75 % for MA and only at 75 % for L-LacA.

Bet and ChTA displayed a similar effect on algal growth, having both shown biostimulation at the 100 % concentration of NADESs 1,2,3,5 and 9 (Figure 3 a,b,c,e,i). In addition, a biomass increase was also found at 50 and 75 % of Bet in NADES 5, and at 75 % of ChTA in NADES 9. Contrastingly, Bet exhibited a different algal growth pattern in NADES 4 (Figure 3d), where three peaks of biostimulation were found at 10, 75 and 100 %, and an inhibition peak was displayed at 2.5 %.

Concerning other tested components, they showed no effect as growth patterns are very similar to those of the respective controls.

ANOVA results, with Tukey's post-test for multiple comparisons, for all concentration-response effect of each NADES are collected in Supporting Information (File S2).

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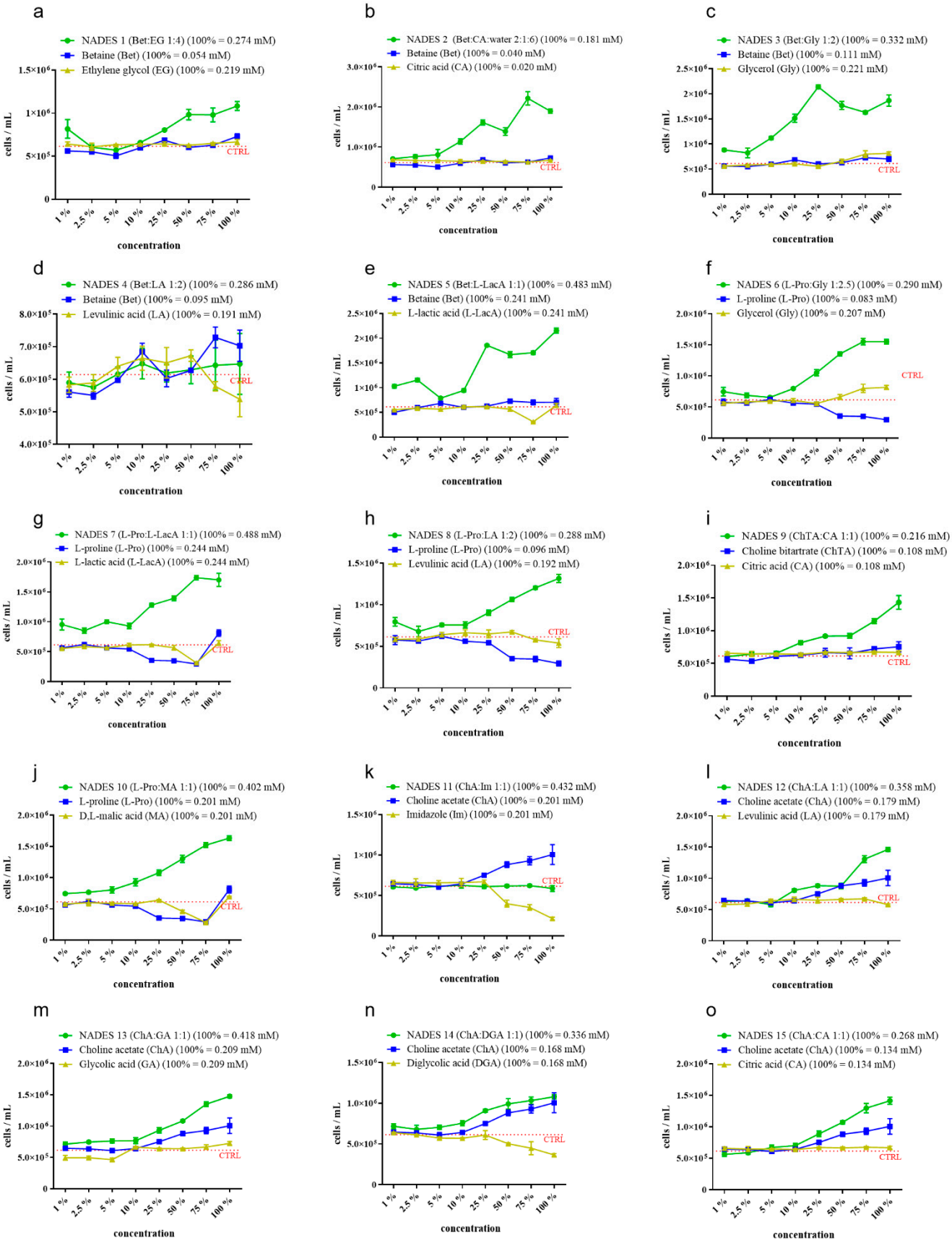

Figure 3. Concentration-response graphs of *R. subcapitata* exposed to NADESs and to their single components at the relative concentration as in the whole compound at the same concentration. Red dotted line is the mean algal

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concentration measured in controls. *a*) Bet:EG; *b*) Bet:CA; *c*) Bet:Gly; *d*) Bet:LA; *e*) Bet:L-LacA; *f*) L-Pro:Gly; *g*) L-Pro:L-LacA; *h)* L-Pro:LA; *i*) ChTA:CA; *j*) L-Pro:MA; *k*) CA:Im; *l*) ChA:LA; *m*) ChA:GA; *n*) ChA:DGA; *o*) ChA:CA. Results are expressed as mean algal concentration (cells mL^{-1}) ± standard deviation (n=3) for each tested concentration of each tested substance. Statistical analyses of differences among whole compound, components and control are reported in Supporting Information (File S2).

3. Discussion

Considering the continuously growing necessity of finding new green solutions aiming at decreasing the impact of human activities on the environment, the present study took in considering a pool of 15 NADES, claimed as non-toxic systems, to evaluate their biodegradability potential and their effects on freshwater algal growth. The same analyses were accrued out on the single components to highlight possible differences when mixed in the NADES.

The current study represents the natural development of our previous research work on NADES [12], where the pool of 15 NADES was evaluated in terms of ecotoxicological effects on two batteries of tests, a marine test and a freshwater test. Due to interesting results obtained in terms of microalgal growth biostimulation, particularly emphasized in the freshwater environment, a deeper investigation on biodegradability and single component effect, in respect to the formed DES system, was considered necessary. Observing as example biodegradability percentages of NADES single components after 28 days, we may notice that Im was the one with the lowest biodegradation value (23%). We may suggests that the inoculum used was likely deficient in key breakdown pathways for this particular chemical, since in the NADES with choline acetate the biodegradability risen to 67%, underlining complex interactions between components in whole NADES formulation

Despite the very limited toxic effect of NADES observed with tests involving invertebrates, their potential impact on the environment, if improperly disposed, may lie in their feature as nutrient source, thus increasing the already large pool of substances with the potential to disrupt biogeochemical cycles at a global scale [60]. This disruption might be intensified by extreme natural events linked to a Global Change Scenario, such as thermal heatwaves [61] and ocean acidification [62]. Regarding the latter, it was observed how NADES in aqueous solutions are able to reduce pH value and, as a consequence, enhance algal growth [12,63].

In addition to these statements, a ready biodegradability was observed for the majority of assessed NADES, with biodegradation values at 28 days between 60 % and 81 % for NADES compounds, and between 23 % and 79 % for their single components.

Our data coincides with the report on NADESs based ChCl as HBA and a series of HBD (glycerol, ethylene glycol, urea, glucose, malonic acid, and lactic acid) studied by CBT [33], demonstrated biodegradability beyond 60% in all cases. Irrespectively of number of hydroxyl groups per mole or the presence of carboxylic derivatives, such as acids, esters, or amides the biodegradability profiles were considered as “readily biodegradable”. This feature may lead the general opinion to consider NADES as completely “green” and “eco-friendly” substitutes to conventional organic solvents. However, the fact that they are readily biodegradable may enhance the load of dissolved ions/molecules with nutritional value, such as choline, known also as vitamin B4, and its degradation products, emphasizing the occurrence of eutrophication phenomena [64].

Due to lack of literature treating NADES effects on microalgae, to better understand current observations, it is important to deeply analyse all obtained results and compare NADES effects with their single component behaviour. Among NADES, those showing the highest induction of algal growth were NADES 2 (Bet : CA), 3 (Bet : Gly) and 5 (Bet : L-LacA). All of these three systems have betaine in their formulation, which, notwithstanding a lack of effect while tested as single component, strongly enhanced algal proliferation when combined with Gly, CA, or L-LacA. Moreover, also NADES containing L-Pro as HBA, which tend to slightly reduce algal load while assessed as single component at high concentrations (> 0.29 mM), displayed a growth stimulating effect when in combination with other HBDs (NADES 6-7-8-10). Differently from those cases, effects of NADES with

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ChA (NADES 11-12-13-14-15), appeared to be more influenced by this latter component respect to all other HBAs, given the similar shape of concentration-effect curves.

Moving the focus specifically to NADES components, it is possible to divide them into three main groups, depending on their effect. The first group, which comprehends the majority of assessed compounds (betaine, ethylene glycol, citric acid, glycerol, glycolic acid, choline bitartrate, L-lactic acid, malic acid and levulinic acid), is composed by substances without significant effects neither in enhancing nor inhibiting algal growth. The second group is composed by Im, L-Pro and DGA. These three compounds showed a limited inhibiting effect on algal growth when tested at concentrations > 0.08 mM for L-Pro, > 0.107 mM for Im, and > 0.1 mM for DGA (concentrations in the relative NADES solution, assessed at dilutions between 25 % and 100 %). The last group is composed by the ChA only, which is present in NADES 11-15 formulations (Figure 3 k,l,m,n,o). As stated before, this particular compound showed a clear algal growth enhancing effect, which followed the same concentration-response curve of the relative NADES, appearing to be the main responsible for algal growth, if compared with concentration-response curves of each assessed whole NADES. However, algal biomass measured at each ChA assessed concentration resulted in lower values, if compared with the relative whole system. This, again, led us to hypothesize a synergistic effect between ChA and the other DES component, like in other assessed NADES. The only exception was represented by NADES 11, where the two opposite effects of Im and ChA seemed to interact by simple addition (Figure 3 k).

This hypothetical synergistic effect of the two components in the NADES composition, observed as an emphasized "overgrowth" effect of the entire systems if compared with both single compounds until 25 % as threshold concentration, has already been observed for ChCl-based DESs [65,66], but also for other toxic DESs, such as glyceline, ethaline and reline [27]. This last result was observed also in the present work with NADES 11 (ChA : Im 1:1) which, as reported before, has Im in its formulation. Im is known to be linked to aquatic toxicity also in other kind of NADES-related compounds, such as ionic liquids [12]. However, while Im showed a certain degree of algal growth inhibition, no relevant effects were observed when it was assessed in combination with ChA as whole DES.

Regarding biodegradability, with the exception of the previously discussed imidazole, no other relevant differences in terms of biodegradability percentages were observed between single components and NADES, with all values still over 60%. However, the readily biodegradability of these compounds, considering that several of them, such as L-proline, betaine and choline acetate, contain nitrogen in their formulation, and the synergistic effect between some components in enhancing microalgal growth, may act as a perfect fertilizer with potential eutrophication effect [67].

4. Materials and Methods

The NADESs used in this study and their composition, along with the ratio of the mixture and their chemical structure, as well as the NADESs components are listed in Table S1.

4.1. Chemicals

Ultrapure deionized water (Milli-Q Direct Water Purification System) was used for all solutions and media preparation. Potassium dichromate, $K_2Cr_2O_7$ (1 g/L) was purchased as dehydrated salt (ACS reagent grade, purity ≥ 99.0 %) from Sigma-Aldrich and used as reference toxicant for algal growth inhibition assay. For NADESs preparation, both hydrogen bond acceptors (HBAs) (Bet, L-Pro, ChTA, ChA) and hydrogen bond donors (HBDs) (EG, CA, Gly, LA, L-LacA, MA, Im, GA, and DGA) were purchased from Alfa Aesar GmbH. Composition and molar ratios of NADESs tested in this study are reported in Table S1. NADESs were prepared following the methodologies described in the literature [68,69].

4.2. Biodegradability Assessment

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In the assessment of the biodegradability of organic compounds, the initial and simple test used is the closed bottle test (modified OECD 301D) usually referred to as the aerobic biodegradation test [70,71].

Aerobic biodegradation testing was done using the modified closed bottle test (CBT), based on OECD 301D guidelines [71,72]. CBT setup with modifications, where biological oxygen consumption is measured with an optode oxygen sensor system using PTFE-lined PSt3 oxygen sensor spots (Fibox 3 PreSens, Regensburg, Germany), allows measuring BOD without dispensing it from the stock solution each time for each test and thereby reducing the number of parallels as once we open a bottle to measure its BOD we cannot use it anymore because its exposed to the atmosphere, so in that case we would keep a stock solution and pour it into new measuring flask each time. The modified setup has also shown to improve reproducibility compared to the original OECD 301D guideline[71,73]. Compared to other standard aerobic biodegradation tests, CBT is better suited for testing compounds with different physico-chemical properties. It is also one of most stringent OECD tests on biodegradability, as the amount of inoculum added is very low and, thus, compounds passing CBT 301D should show good biodegradation not only under artificial wastewater treatment conditions but also in soil and groundwater systems.

Experimental Setup. Each CBT run consisted of four different series each of which was run in duplicates. First was „reference series „in which readily biodegradable sodium acetate in a known concentration (6.41 mg/L) was added to a flask of mineral medium inoculated with effluent from a wastewater treatment plant. As sodium acetate is known to be rapidly biodegradable it acted as a reference and control for monitoring activity of microbes in the inoculum. In the test series a studied compound as a sole source of carbon was added to the inoculated mineral medium. The test compound was added in a concentration corresponding to theoretical oxygen demand (ThOD) of approximately 5 mg/L. ThOD was calculated assuming nitrification would take place as each of the 25 studied compounds included nitrogen atom(s) in their structure. Toxicity control series, containing both sodium acetate and test compound in their respective concentrations, were used to evaluate test compounds' toxicity against inoculum – if biodegradation values in these bottles were significantly lower compared to reference series, it was concluded that the test compound could be inhibiting or even being toxic to microbes in WWTP effluent. To negate the effect of inoculum itself, blank bottles containing only inoculum and mineral medium were added to each CBT run and the value of these bottles was subtracted from all the other bottles. To make sure seasonal variations in inoculum composition did not have an effect on biodegradation results, a total of 6 CBT runs from June to November were performed.

Inoculum. Effluent from wastewater treatment plant was collected from a municipal wastewater treatment plant in Tallinn, Estonia (Paljassaare wastewater treatment plant, 59°27'55.5"N 24°42'08.8"E). WWTP effluent was filtered through a cellulose filter (membrane ø 240 mm) before being used as inoculum for aerobic biodegradation testing.

Results from each run were accepted if the following criteria were met: (i) difference of extremes of replicate values at the plateau is less than 20 %, (ii) oxygen concentration in test series bottles must not fall below 0.5 mg/L at any time, iii) sodium acetate in reference series must be degraded ≥ 60 % by day 14. Blank bottles oxygen consumption was also monitored to avoid the possibility of the system turning from aerobic to anaerobic. In none of the CBT runs oxygen consumption in blank bottles exceeded 34 % compared to the initial oxygen concentration.

4.3. Ecotoxicity Assessment

Stock and working solutions

3N-BBM + V, Bold Basal Medium

3N-BBM+V (Bold Basal Medium with 3-fold Nitrogen and Vitamins), as algal culture medium, was prepared according to CCAP (Culture Collection of Algae and Protozoa) guidelines. Medium pH value was corrected to 8.00 ± 0.1.

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4.4. Naturally Available Deep Eutectic Solvents (NADES)

For all NADESs, a set of working concentrations was prepared, starting form 100 mg/L, which represents our 100%. Selected dilutions for each compound were: 100 - 75 - 50 - 25 - 10 - 5 - 2.5 - 1 %. Dilutions were carried out with algal culture medium 3N-BBM + V.

4.5. HBAs and HBDs

For each HBA and HBD, 1 g/L stock solution was prepared in ultrapure water. These stock solutions were further diluted to test the concentration of each individual component, matching the concentration they had in the corresponding NADES mixture. Dilutions were carried out with algal culture medium 3N-BBM + V. Results were plotted as percentage indicating the relative amount of compound at that NADES tested concentration.

4.6. Raphidocelis subcapitata Growth Bioassay

R. subcapitata, was purchased from the reference center CCAP (Culture Collection of Algae and Protozoa - Scottish Association for Marine Science/SAMS Research Services Ltd). Axenic cultures were kept in 100 mL glass flask stored at 20 ± 2 °C, under natural white illumination (6000–8000 lx) with photoperiod 16:8 dark:light. 3N-BBM + V was used for culturing *R. subcapitata*. Cultures were renewed every two weeks. The growth assessment of the freshwater alga *R. subcapitata* (batch: CCAP 1052/1A) was performed following ASTM procedures [74].

Before the test started, an algal working batch was prepared by inoculating 2 mL of maintenance cultures in 20 mL of fresh medium, maintaining it at 20 ± 2 °C under continuous illumination (6000–8000 lx), in order to obtain a logarithmic-phase algal culture. After 72 h, algal concentration in the working batch was measured and diluted to reach a concentration of 10^6 cell/mL. For the growth inhibition bioassay, all samples at all concentrations were prepared in triplicate, in sterile 24-well plastic plates. 20 µL of diluted algal working batch were inoculated in each 2 mL replica of all samples and negative controls (medium), previously distributed in. Plates were incubated at 20 ± 2 ◦C, under continuous illumination (6000–8000 lx) for 72 h.

After 72 h, absorbance (λ = 670 nm) was measured in each well with a spectrophotometer (Jenway Genova Plus), using 1 cm optic-path plastic cuvettes. Algal concentration (Cells/mL) was calculated using the following equation, previously obtained by the CIBM (Livorno, Italy) research group.

$$\boldsymbol{Cells/mL = \frac{Abs_{670}}{8 * 10^{-8}}}$$

The reference toxicant was potassium dichromate. Stock solution was prepared by dissolving the dehydrated salt in ultrapure water at the concentration of 1 g/L. From stock solution, five different concentrations of potassium dichromate were prepared, respectively directly in algal growth medium (1.8 – 1 – 0.56 – 0.32 – 0.18) mg/L to check the reliability of the test. Results obtained for this assay fell into the laboratory control chart (EC_{50} 0.742 mg /L (0.648 – 0.808).

4.7. Statistical Analysis

For statistical analysis, two-way ANOVA was performed, followed by Tukey multicomparison test to valuated algae growth difference between 1) DES and control 2) HBA /HBD and control 3) DES and HBA/HBD 4) HBAs and HBDs. Statistical analysis was performed with Graph-Pad Prism 7 software (GraphPad Software, La Jolla California, USA, www.graphpad.com). Statistically significant differences were reported with asterisks: $p< 0.05$ (*), $p<0.01$ (**), $p<0.001$ (***).

5. Conclusions

All the studied NADESs and the absolute majority of their components showed good biodegradation values (i.e. >60% = readily biodegradable). However, although they could be labelled

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as "green" and "safe" from the chemical point of view, they showed a clear stimulating effect of *Raphidocelis subcapitata* growth. This effect was not always observed when single components were assessed at the same concentration as in the relative whole NADES, suggesting a putative synergistic effect for most of the substances while in mixture. In particular, NADESs with the most accentuate synergistic behavior were those containing betaine and proline, followed by those containing choline acetate and, as the less effective, choline bitartrate. Observing different HBA/HBD combinations, results suggest that the hypothetical synergistic effect may be mainly linked to HBA contribution. Considering what reported above, it appears quite clear that to label NADES as "green" or "eco-friendly" a deeper investigation on both molecular behaviour in solutions and interactions at several organization level, from molecules to ecosystem, is necessary, in order to prevent unpredicted negative effects on the environment.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. File S1: Table S1. NADESs composition and molar ratios; Table S2. NADESs and their single components, with the relative raw formula, molar mass, concentration for biodegradability assessment, and biodegradation achieved by CBT 301D. File S2: Algal growth test statistic data.

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Declaration: of competing interest. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. .

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