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Development and first evaluation of new Natural Deep Eutectic Solvents (NaDES) formulations for applications in the agronomic field

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Table of contents

Abstract

Following the concept of sustainable development, Green Chemistry is actively seeking for new solvents to replace conventional ones that present inherent toxicity, flammability and hazardous environmental fate. In this contest, Natural Deep Eutectic Solvents (NaDES) have recently emerged as new generation solvents; NaDES are eutectic mixtures of naturally occurring hydrogen bond donors (HBD, e.g choline chloride) and hydrogen bond acceptors (HBA, e.g. sugars, organic acids, amino acids) that display peculiar solubilization behaviour. In the present study, the possibility of use NaDES as eco-compatible bioactive formulations to deliver high value compounds in agricultural field was evaluated. The investigated NaDES were composed by betaine and choline chloride as HBA and HBD with different reactivity: acid (citric acid, lactic acid, malic acid), basic (urea) and neutral (glycerol and ethylene glycol). NaDES formulations were produced by extraction of highly-valuable macromolecules from algae biomasses (*Arthrospira platensis* and *Phaeodactylum tricornutum*) and grape by-products (grape pomace and grape seeds). Since NaDES ecotoxicological profile is poorly known, the toxicity of NaDES was investigated on soil animals (*Eisenia andrei*) and plants (*Lepidium sativum*, *Avena sativa, Ocimum basilicum and Lactuca sativa*) and their biodegradability were assessed. Results revealed NaDES high extraction efficiency and promising ability as antioxidants and inhibitors of the enzyme urease. NaDES formulations did not seem to produce promoting effects on plants and exhibited different toxicological profiles based on the tested organisms and the solvent composition. Overall, NaDES containing acids and urea as HBD showed a significant toxicity on the tested organisms.

Keywords: Natural Deep eutectic solvents, polyphenols, algae, toxicity, biodegradability.

Acronyms

- Active pharmaceutical ingredients (API)
- American Food and Drug Administration (AFDA)
- Best Available Technology (BAT)
- Betaine (Bet)
- Biochemical Oxygen Demand (BOD)
- Channel Catfish Ovary (CCO)
- Choline chloride (ChCl)
- Citric acid (CA)
- COD (Chemical oxygen demand)
- Deep Eutectic Solvents (DES)
- Dichloromethane and methanol (DMC)
- Environmental Protection Agency (EPA)
- Ethylene glycol (EG)
- Fatty acids (FAME)
- Food and Agriculture Organization of the United Nations (FAO)
- Fourier transform infrared spectroscopy (FTIR)
- Gas chromatography coupled with mass spectrometry (GC-MS)
- Glycerol (G)
- Global Harmonized System (GHS)
- Half Maximal Inhibitory (IC50)
- Hydrogen bond acceptor (HBA)
- Hydrogen bond donor (HBD)
- Instant controlled pressure drop (DIC)
- Ionic liquids (IL)
- Lactic acid (LA)
- Lactic acid (LC)
- Lipids (LP)
- Liquid polymers (LP)
- Malic acid (MA)
- Median Effective Concentration (EC50)
- Microwave-assisted extraction (MAE)
- Molarity (M)
- Natural Deep Eutectic Solvents (NaDES)
- Nuclear magnetic resonance spectroscopy (NOESY)
- Phenolic compounds (PC)
- Plants growth regulators (PGR)
- Polyethylene glycol (PEG)
- Polyphenols (PP)
- Polysaccharides (PS)
- Proteins (PR)
- Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH)
- Safety, Health and Environment (SHE)
- Supercritical fluids (SCF)
- Tetrahydrofuran (THF)
- Thermogravimetric analysis (TGA)
- Total phenolic content (TPC%)
- Ultrasound-assisted extraction (UAE)
- United States Environmental Protection Agency (US EPA)
- Urea (U)
- Volatile organic compounds (VOC)

1 Introduction

1.1 Green Chemistry: the sustainable approach to chemistry

The concept of green chemistry was introduced in the 1990s as a natural evolution of various pollution prevention initiatives. Indeed, the worldwide effort to improve crop protection, produce commodities and pharmaceuticals for the humans needs and meet the global energy requirement caused and, is still causing, severe side effects to our planet [1]. With the aim of reversing the tendency of a global environmental deterioration, the *World Commission on Environment and Development's* (Brundtland Commission) introduced for the first time the concept of Sustainable Development within the report ''Our Common Future'' in 1987: ''*sustainable is the development that meets the needs of the present without compromising the ability of future generations to meet their own needs*'' [2]. Chemistry and chemical engineering play a significant role in sustainable development, acting as two of the major contributors to the worldwide economic development, but representing at the same time harmful human activities [3,4,5]. Therefore, developing a sustainable chemistry has been considered an important objective to improve the quality of the environment for present and future generations (Rio Declaration and Agenda 21) [6,7].

The establishment of the green ghemistry as a recognized scientific field took place two decades ago, through the enactment of the *Pollution Prevention Act* (1990) in the United States [1]. The term "*Green Chemistry*" was coined by Paul Anastas and John Warner, staff of the Environmental Protection Agency (EPA), Office of Pollution Prevention and Toxins, sowing in that way the seeds of productive collaboration between government, industry, and academia [5,8]. The concept of green chemistry was at first defined as the ''*design of chemical products and processes to reduce or eliminate the use and generation of hazardous substances*'' [9,10]. Green chemistry and chemical engineering seek to reinvent the production and use of chemicals to maximize efficiency and minimize health and environmental hazards throughout every stage of a chemical's life cycle [11,12]. It's important to note that the aim of this approach goes beyond concerns over hazards from chemical toxicity and includes consideration such as energy conservation, waste reduction, the use of more sustainable or renewable feedstocks and designing for end of life or the final disposition of the product [1]. Consequently this "green revolution" has been developed into

a globe-spanning endeavour aimed at meeting the ‘‘triple bottom line’’: sustainability in environmental but also economic and social performance [11].

This environment-friendly chemistry involves an interdisciplinary approach, guided by the principle ‘‘benign by design’’ [10,11]. Green chemistry attempts to redesign the materials that make up the basis of our society and our economy in ways that are benign for humans and the environment and possess intrinsic sustainability [10]. Design and implementation of safe and environmental-friendly products and processes is an enormous challenge for chemistry [13] because of the number of chemicals synthesis pathway and the difficulty of describing in a unique way what is sustainably and what is not. A fundamental guideline arrived at the end of the twentieth century, when Anastas and Warner formulated The Twelve Principles of green chemistry (*Fig. 1*): design criteria that provide chemists achieve the intentional goal of sustainability [7,9]. They are an overreaching construct for the design of safe chemical products and chemical transformations, applying to all aspects of the process life-cycle from the raw materials used to the efficiency and safety of the process, the toxicity and biodegradability of products and reagents used12. Green chemistry strives to achieve the goal of sustainability by careful planning of chemical synthesis and molecular design with restricted adverse consequences [12,14].

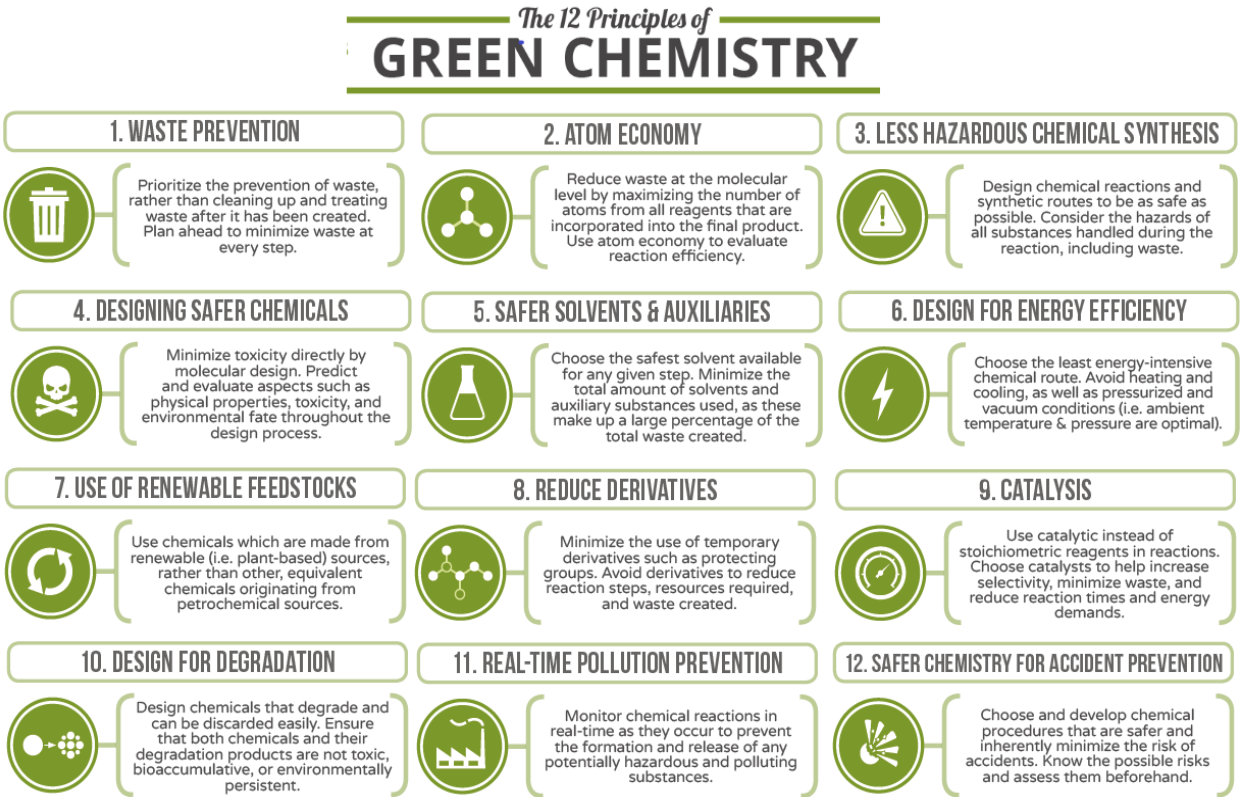


Fig. 1. The Twelve Principles of Green Chemistry.[14]

The advent of green chemistry launched a globalized challenge for the creation of an environmental protection strategy which has been set out with the adoption of revolutionary legislative acts. The European scenario radically changed in 2006 when the REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) Regulation was adopted. REACH aims to ensure a high level of protection of human health and the environment through a better and early identification and registration of the intrinsic properties of chemical substances produced or imported by the EU chemicals industry [15]. A further step towards non-sustainable industry decline was made two years later within the IPPC Directive, concerning integrated pollution prevention and control [16]. The 2008/CE directive underlines the fundamental necessity of safe process for reducing industry impact by defining the notion of BAT (Best Available Technology) for each professional sector [17].

1.2 Green extraction of natural compounds

In the context of sustainable chemical revolution, the development of green technological innovations that break away from the past represents one of the major items of scientific research [18,19]. Among the chemical procedures, the extractions, in particular extractions of natural compounds, are one of the process that have been widely modified in the attempt to follow the principles of green chemistry. Extraction is the first step to separate the desired natural compound from the raw materials. Etraction methods include solvent extraction, distillation method, pressing and sublimation according to the extraction principle. Solvent extraction is the most widely used method. The extraction of natural products progresses through the following stages:

1. the solvent penetrates into the solid matrix;
2. the solute dissolves in the solvents;
3. the solute is diffused out of the solid matrix;
4. the extracted solutes are collected [20].

The extraction of high added value compounds from natural biomass, for example antioxidants, stimulants, perfumes, was considered “clean” when compared with heavy chemical industries, but researchers and professional specialists found that its environmental impact is far greater than first appeared [18]. Although the overall environmental footprint produced by an industrial extraction cycle is difficult to evaluate precisely, it is known that it is a highly energy consuming step that requires at least 50% of

the energy of the whole industrial process [21]. Contrary to what we might expect from an energy-intensive, time-consuming process, with high demand for raw materials and a large amounts of solvents, the final yields of conventional extractions are often low [20,21]. Furthermore, once the substance of interest has been extracted, if have to be applied in the cosmetic, pharmaceutical or food industry, the final product must be free from the solvents used for extraction, and this process requires further energy, consumption and therefore produces greater environmental impact. However, recent trends in extraction techniques have largely focused on finding solutions that minimize these drawbacks. In fact while in 1990's it was not easy to find literature concerning efforts to make traditional extraction protocols green [21], over the last decade the design of green, efficient and sustainable extraction methods has become a hot topic [21,19] for the scientific community [18]. Nowadays, the design of extraction process must be modelled strongly considering all the potential side effects, to ensure its safety to humans and the environment [19,18].

Consequently, the main challenges for the green extraction approach are:

- To increase extraction processes efficiency (yield and selectivity towards the compounds of interest)
- To have the lowest possible impact on the environment (by reducing or eliminating petrochemical solvents, together with moderate energy consumption)
- To plan the recycling or reuse of substances and products, in a life cycle assessment view(coproducts, biodegradability, etc.) [19,18,21].

The previous considerations show that green extraction has been developed based on the principles of green chemistry but also in closed association with the twenty principles of Green Engineering on which modern "sustainable processes" are founded [22]. Referring exclusively to green extraction of natural product Chemat et al. have proposed a new definition: "*Green Extraction is based on the discovery and design of extraction processes which will reduce energy consumption, allows use of alternative solvents and renewable natural products, and ensure a safe and high-quality extract/product*" [12]. In association with the definition a list of "six principle of Green Extractions for Natural Products" has been developed:

- *Principle 1*: Innovation by selection of varieties and use of renewable plant resources.
- *Principle 2*: Use of alternative solvents and principally water or agro-solvents.

- *Principle 3*: Reduce energy consumption by energy recovery and using innovative technologies.
- *Principle 4*: Production of co-products instead of waste to include the bio- and agro-refining industry.
- *Principle 5*: Reduce unit operations and favour safe, robust and controlled processes.
- *Principle 6*: Aim for a non-denatured and biodegradable extract without contaminants.

Those principles must be considered not as rules but a as useful guidelines than could be applied in scientific research and industry as an innovative approach in all aspects of the extraction process [23,21].

Even more extraction technique research has focused on finding unconventional solutions that minimize the use of solvent and energy. Great improvements can be achieved with the use of innovative green extraction techniques [19] such as supercritical fluid extraction, ultrasound-assisted extraction (UAE) [23] and microwave-assisted extraction (MAE) [24] and instant controlled pressure drop (DIC) [25]. Each method has advantages and limits, so many factors must be considered to identify the appropriate extractions technique for the matrix in object [21].

1.3 Sustainable solvents, an application of green chemistry

Solvents represent one of the most important group of chemicals in the industry, due to the great quantities that are used annually in various areas including analytical and synthetic chemistry, processing food industry and in the material and coating sector. The annual industrial-scale production of organic solvents has been estimated at almost 20 million metric tons. For example, solvents can be up the 85% of the mass material used in a pharmaceutical process to prepare active pharmaceutical ingredients (API) [26,27]. Chemical processes use solvents to facilitate separation and purification, to facilitate mass and heat transfer, as reactive medium or diluent. Additionally they represent a primary component in adessives, cleaning agents and coatings [18]. Despite their usefulness is undeniable, solvents rise innumerable environmental concerns because they are often flammable, persistent and accumulative substances, hazardous for human health, animals and plants. Moreover, most of them are volatile compounds (VOC) that are able to form

low-level ozone and smog through free radical air oxidation process (e.g., pentane or diethyl ether) [28,18].

Fig. 2. Main features of ideal green solvents.

1.3.1 Red, yellow and green solvents: to avoid and benign alternatives

The fifth principle of green chemistry asks to "use safer solvents and auxiliaries", i.e. avoid using of auxiliary substances whenever possible and, if needed, choose the safest solvents available for any given step [8]. Moreover, solvents use conflicts with other principles of green chemistry, as consequence the chemistry research looking forward greener and alternative solvents has greatly increased in the last thirty years [29]. For this purpose, the scientific community has made efforts to create a tool capable to quantify and qualify the most sustainable of a wide range of solvents [30,31]. Literature proposed several solvent selection guides with the purpose of promoting the use of more sustainable solvent [32,33,34]. Among these, the CHEM12 metrics toolkit proposes a quick method of identifying problematic solvents and then recommending ecofriendly alternatives [35] (e.g. replacing harmful organic solvents, like chloroform, with less harmful organic solvents, as ethanol). The rank is based on set criteria that are in line with the Global Harmonized System (GHS) and European regulations:

- Safety (e.g. flash point, tendency to form explosive and auto-ignition temperature)
- Health, that reflects the occupational hazard
- Environment (e.g. toxicity towards aquatic life, bioaccumulation, ability to generate VOC, CO_2 footprint).

The metrics has been applied to 51 solvents, both conventional solvents commonly used at the industrial level and fully registered according to REACH and less common

solvents, such as bio-derived solvents. For each of the three criteria a score of 1 to 10 (where 10 represents the highest hazard in each category) has been assigned to all the analysed solvents. These three SHE (Safety, Health and Environment) scores have been combined to create a colour-code rank for any solvent (*Table 1*) [36]:

- **Recommended (green solvents)**: The least harmful solvents in each category, thus the solvents to be preferred for any process.
- **Problematic (yellow solvents)**: these solvents can be used in process but their implementation in the production scale needed further consideration due to the significant energy consumption.
- **Hazardous (light res solvents):** solvents that show significant limitation on scale-up, so during the process development the replacement of these solvents is recommended
- **Highly hazardous (red solvents)**: solvents to be avoided, even in the laboratory.

Table 1. Ranking of main solvents categorized from safe (green) to hazardous (red).

Combined ranking	Solvent
Recommended	Water, EtOH, *i* -PrOH, *n* -BuOH, EtOAc, *i* -PrOAc, *n* -BuOAc, anisole, sulfolane.
Recommended or problematic?	MeOH, *t* -BuOH, benzyl alcohol, ethylene glycol, acetone, MEK, MIBK, cyclohexanone, MeOAc, AcOH, Ac_2O.
Problematic	Me-THF, heptane, Me-cyclohexane, toluene, xylenes, chlorobenzene, acetonitrile, DMPU, DMSO.
Problematic or hazardous?	MTBE, THF, cyclohexane, DCM, formic acid, pyridine.
Hazardous	Diisopropyl ether, 1,4-dioxane, DME, pentane, hexane, DMF, DMAc, NMP, methoxy-ethanol, TEA.
Highly hazardous	Diethyl ether, benzene, chloroform, CCl_4, DCE, nitromethane.

1.3.2 Environmental-friendly solvents as tools for green extractions

One of the main goals of green chemistry applied in the extraction field is to reduce the solvent volumes used and to replace standard extraction solvents with environmental-friendly substances. The second principle of green extractions demands the replacement of conventional solvents, often hazardous and toxic, with water or bio-based solvents, as

suitable, safer and more environmental-friendlier [23]. Different types of solvents used in extraction procedure are grouped in a hierarchical approach (*Fig. 3*): from bottom, petroleum-based (e.g. benzene, toluene, xylene) and VOC, the most hazardous solvents that must be avoided, to the top, considering the solvent-free extractions as the greenest solution [30,29].

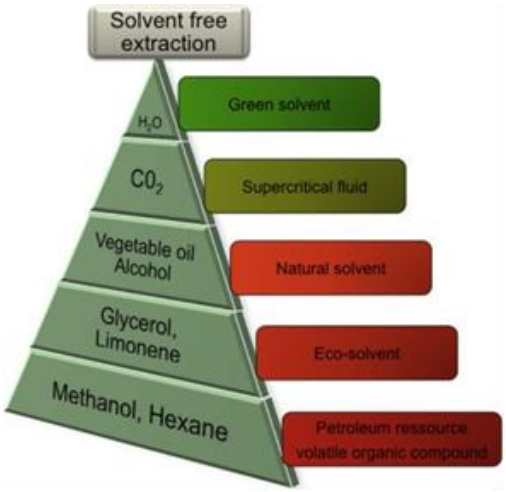


Fig. 3. Hierarchical approach to the type of solvents used in extraction procedures from a green analytical point of view [12].

*Table 2. G*reen extractions: hazardous (bottom), safe (top), and main features [17].

Solvent	Extraction Technique (Application)	Solvent Power			Health & Safety	Cost	Environmental Impact
		Polar	Weakly Polar	Non-Polar			
Solvent-free	Microwave Hydrodiffusion and Gravity (antioxidants, essential oils)	+++	+		+++	+	+++
	Pulse Electric Field (antioxidants, pigments)	+++	+		+++	+	+++
Water	Steam distillation (essential oils)	++	+		+	++	+
	Microwave-assisted distillation (essential oils)	+++	+++	+	+	+	++
	Extraction by sub-critical water (Aromas)	+	++		+	+	+
CO_2	Supercritical fluid extraction (decaffeination of tea and coffee)	−	+	+++	+	+	+
Ionic liquids	Ammonium salts (Artemisinin)	−	+	+++	−	−	++
Agrosolvents	Ethanol (pigments and antioxidants)	+	+	−	−	++	+
	Glycerol (polyphenols)	+	+	−	−	+	+
	Terpenes such as *d*-limonene (fats and oils)	−	−	++	−	+	+
Petrochemical solvents	*n*-Hexane (fats and oils)	−	+	+++	−−−	++	−−−

1.3.3 The most prominent green solvents in use today

The introduction of cleaner solvents has become a major topic of interest in both academia and chemical industry [37]. As consequence over the past three decades research on benign alternatives has growth exponentially, highlighting green solvents already known or developing a width range of new eco-friendly solutions for different process and applications *(Fig. 4).*

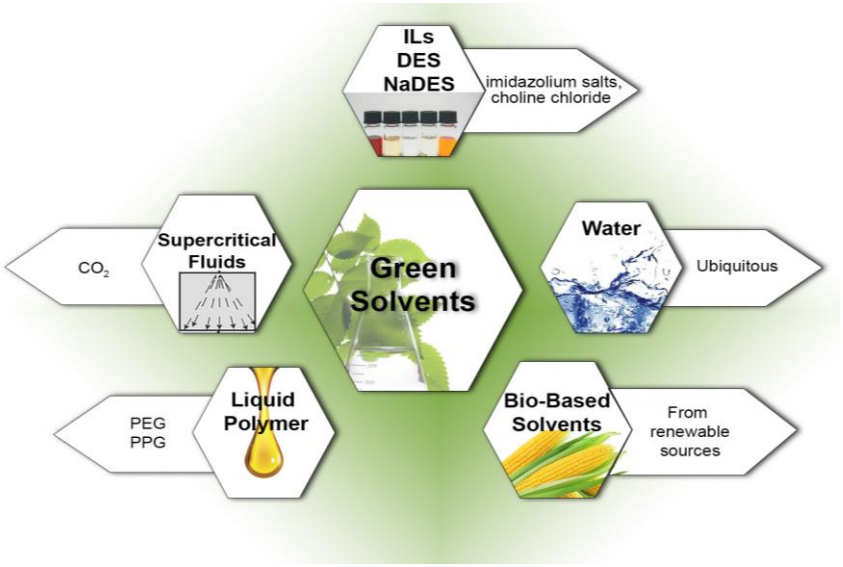


Fig. 4. Main green solvents used nowadays.

1.3.3.1 Solvent-free process

The best solvent would be no solvent at all [50,50]. In the last decade most of the green chemistry effort focused on the “perfect green solvents”: benign for the environment, safe for human, with high reaction yield and cheap. For these reasons the challenge of most chemists is to design reactions that proceed under solvent-free conditions such as solid-state, gas-phase and ultrasound and microwaves assisted reactions [50]. The last techniques have been widely applicated for extraction (e.g essential oil, colours and antioxidants) and synthesis [38]. This approach is based on the ability of a specific material (e.g. solvent and/or reagents) to absorb microwave energy and to convert it into heat after the application of microwave irradiation [39]. It often used as a pre-treatment in combination with other solvents, such as Deep Eutectic Solvents, improving the yield of the extraction of bioactive compound [40]. However, there are some disadvantages in solvent-free extraction, for example microwave reactions required expensive equipment and, up to now, they are unsuitable for the scale-up production because of problems with handling samples of large volumes [41]. Similar drawbacks are shown in gas-phase extraction, in

addition its extraction efficiency is often scarce due to the poor interaction between the sample and the gas [42].

1.3.3.2 Water

Beyond using no solvent, water should be considered the best alternative because it is greener solvent for excellence. Due to its high solvent proprieties water is already used quite widely in chemical industry, particularly in hydrodistillations and in emulsion polymerization processes [46]. Water is an extremely polar solvent due to its high dielectric constant (ε_r ~ 80, at ambient temperature and pressure), as a result of this feature water is an excellent solvent for inorganic substances and natural water-soluble products (e.g. sugars, proteins and low molecular weight acids) [23]. Moreover, water contains extensive hydrogen bonding, this means that ionic compounds dissolve easily and that boiling and melting point of water are much higher than those of acetone, ethanol or other organic compound, increasing the safety of this ubiquitarian solvent [18].

1.3.3.3 Bio or renewable solvents

Looking for a valid green alternative, bio-solvents play a central role due to their high solvent power, biodegradability, non-toxicity and renewable sources (e.g corn, wheat, starch) [23,40]. Plant biomass, derived from crop such as corn, agricultural wastes or agroforestry products, is currently considered the primary source of renewable fuels and solvents for the chemical industry [46]. Biomass could be used as raw material to produce a wide variety of renewable solvents [43]:

- *Ethanol*: is a bio-alcohol obtained from fermentation of sugar-rich material such as corn or sugar cane. Due to availability in high purity, low price, biodegradability and low toxicity compared with other alcohols, ethanol is the most common bio-solvent (production of bioethanol exceeded 22 billion gallons/year in 2010) [44].
- *Glycerol*: a polyol derivable from the trans-esterification of vegetable oils. Widely use because able to dissolve many organic and inorganic compounds [44] .
- Terpenes: *β-pinene, α-pinene, p-cymene,* and *d-limonene* are terpenes derived from pine and citrus fruits, largely used because their high solvent power and low polarity [45].
- 2-MeTHF: is the sustainable alternative of *Tetrahydrofuran* (THF) due to its lower toxicity, higher stability and lower volatility. It can be synthesized from xylose and

glucose, derived from biomass or agricultural waste, and intermediates such as levulinic acid and furfural [46].

Methyl esters of fatty acids (FAME): these bio-based solvents from vegetable oil are widely used to replace mineral spirits because of similar solvent ability but are non-toxic, non-volatile and biodegradable [47].

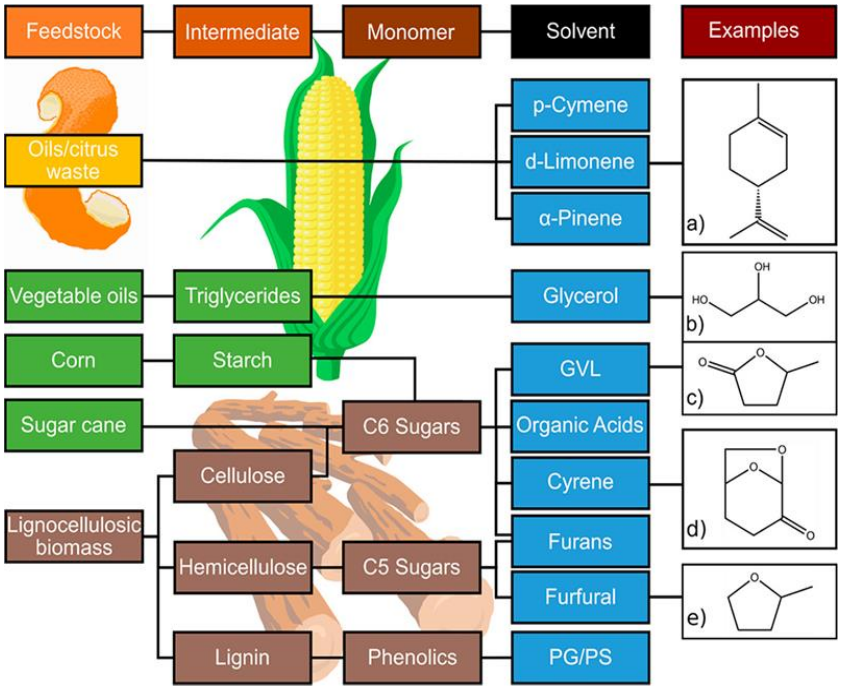
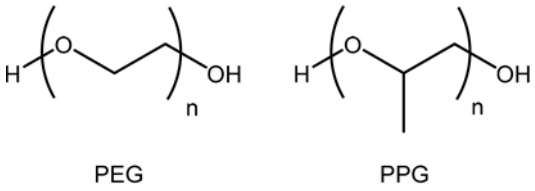


Fig. 5. A wide variety of renewable solvents cold be derived from biomass. Structural examples show (a) d-limonene, (b) glycerol, (c) γ-valerolactone, (d) cyrene, and (e) 2-MeTHF [50].

1.3.3.4 Liquid polymers

Polyethylene glycol (PEG) and *polypropylene glycol* (PPG) *(Fig. 6*) are liquid polymers (LP) that might be a promising replacement for conventional solvents due to their inherent negligible volatility, low toxicity profile and low flammability. While PEG is readily biodegradable by several soil bacteria, PPG has a lower biodegradability [48]. PEG is mainly synthesised from ethylene glycol, that could be produced in a sustainable way directly from lignocellulosic, sugar or bacterial biomass, in addition PEG from agricultural waste is already commercially available. PEG is used in various commercial applications ranging from care products to food additives, indeed it has been approved by the U.S. Food and Drug Agency for international compounds [49].



PEG PPG

Fig. 6. Chemical structures of some liquid polymers used as solvents: (left to right) polyethylene glycol (PEG), polypropylene glycol (PPG)[50].

1.3.3.5 Neoteric solvents or non-traditional green solvents

Within the composite word of sustainable solvents, despite conventional solvents (e.g. *water, methanol, ethanol, glycerol*) are still the most employed in chemical industries, neoteric (neoteric= recent, modern) solvents are slowly being integrated into industrial processes due to their desirable, less hazardous, new proprieties, multiple advantages over organic, or aqueous, solvents, e.g. improving product separation [38]. Examples of neoteric solvents include supercritical fluids (SCF), ionic liquids (IL) and Deep Eutectic Solvents (DES) [50].

Supercritical fluids (SCFs)

Supercritical fluids are a well-established alternative technique in industry due to their unusual proprieties. SCF are substances that get into the supercritical phase, exceeding their critical temperature and pressure point, named *critical point* [51]. Thus, SCF exhibit liquid-like and gas-like features simultaneously: high densities, low viscosity and high diffusivities respectively. Those property make them suitable for reactions involving gaseous substances and increase the reaction rates [38]. Supercritical carbon dioxide ($scCO_2$: Tc = 31 °C, pc = 72,8 atm) is the most used SCT in green chemistry, because is renewable, non-flammable and safe [44]. CO_2, an odourless non-flammable gas, is used to extract weakly polar compounds of low molecular weight (e.g. carotenoids, triglycerides, fatty acids) with no trace of solvents [18]. Indeed, $scCO_2$ has been broadly used in the cosmetic, food, pharmaceutical sectors (e.g decaffeination coffee process) [52].

Ionic Liquids (IL): **proprieties, uses and environmental impact**

Ionic liquids (IL) are organic salts composed at least of two components, an organic cation (e.g. *imidazolium, pyrrolidinium, pyridinium, ammonium, phosphonium*) and an organic or inorganic anion (e.g. Cl^-, B^-, PF_6^-, BF_4^-), with melting point below 100°C [53].

Typically, IL are characterized by an asymmetric ionic structure and a dispersion of their charge, which leads to a low intermolecular attraction, and hence they are often liquid at room temperature [54,55].

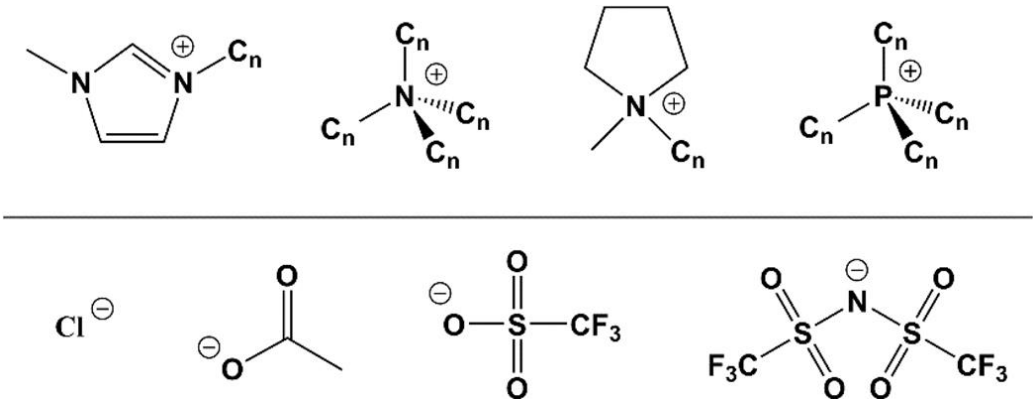


Fig. 7. Common cations (top) and anions (bottom), which may be paired in any combination to form ILs 50.

IL have multiple fascinating proprieties which allowed these media to perform a wide range of chemical processes, such as separation and purification, organic synthesis, electrochemical, biochemical and catalytic reaction [57]. Therefore, they are attractive media and an intensive research subject for scientific community and industry. Green chemistry proposed them as promising benign solvents due to their multiple positive characteristic, in fact IL:

- Show negligible vapor pressure at room temperature, as consequence they do not evaporate and hence, compared with traditional organic solvents, they cannot produce volatile organic compounds (VOC).
- Are safe solvent because they have low or no flammability and no flash point, thus the potential of explosion is minimized [56].
- Are thermally and chemically stable and possess a notable solvating capacity with many types of molecules (they dissolve polar end not polar species) [56], making them a widely appreciated green solvents [57,58]. Thus, those 'green' solvents are widely used to extract a variety of substances such as organic and bio- molecules, metal ions, organosulfur from fuels and gases [59,60].
- Can be recovered and recycled, in many cases [60].
- Are commonly defined as "designer solvents" or "task specific IL" [61] because changing anion-cation combination can be defined a particular solvent with a

specific end use or to own a particular set of physicochemical proprieties [38,56]. In fact, the broad term “ionic liquid” embody multifunctional chemical tools: great solvents [62], stable energetic materials, lubricants, catalyst [63], good working electrolytes, drug delivery system [64], agro-chemicals [65]. Recently researches are investigating IL as an eco-friendly alternative to currently used environmental harmfully agrochemicals, a bioactive component acting as bactericides, fungicides, herbicides, wood preservation agents, plants stimulants, growth regulators [65].

Despite the innumerable environmentally friendly properties that characterize IL, many researches show that they are not intrinsically green as often assumed [66]. For example LCA of common imidazolium-based ionic liquids shows that the production of IL requires volatile solvents, thus replacing organic solvents with IL does not reduce the environmental impact of conventional solvents but simply shifts the problem from the use to the production [67]. Moreover, the high chemical stability of IL makes them poorly biodegradable [68] and potentially persistent organic pollutants, especially concerning aquatic environments [69]. Many studies tried to understand the toxicology profile of IL testing biological indicator, that include *Vibrio fischeri* [70], *Daphnia magna* [71,72], Gram-positive bacteria, fungal strains [73], algae, and plants [65,74]. Those studies have demonstrated that IL are a broad family of compounds that consist of both “toxic” and “non-toxic” salts. Most of the literature for example reports that conventional IL as imidazolium-based ones, are poorly biodegradable [75], toxic and costly [76]. The toxicity changes enormously with different IL, and organisms. Thus, the prediction of a correlation between structure and toxicity is a crucial challenge for a safe application of IL [65]. Moreover, understanding the metabolic pathways of ionic liquids is a crucial challenge, because metabolites possess environmental behaviours and toxicity profiles different from those of the parent ionic liquids [51,50].

Table 3. Advantages a disadvantage of ionic liquid

Pros	Cons
Low vapour pressure	Toxicity
Low flammability	High viscosity
Low flash point	Poor degradability
Melting point below 100°C	High Cost (5-20 times more than conventional solvents)
High Solvating proprieties	Polarity
Thermal stability	
Do not release VOCs	
Task-specific (ILs “designer solvents”)	

1.4 Deep Eutectic Solvents: green alternative solvents

As consequence of the debate on the real greenness of IL, scientist have mobilized to investigate other solvents able to retain the desirable properties of IL while being economically competitive and with minimal environmental side-effects [77]. In this framework, precisely in 2004, Deep Eutectic Solvents (DES) have emerged [78]. DES are eutectic mixtures of hydrogen bond donors (HBD, e.g. quaternary ammonium salts) and hydrogen bond acceptors (HBA, e.g organic acid, polyalcohols, amides) with a melting point lower than that of either of the individual component (highlighting noncovalent affinities at the molecular level) [79,80,81]. The charge delocalization occurring through hydrogen-bond interaction seems to be the main cause of the freezing-point depression of the mixture [82,83,84].

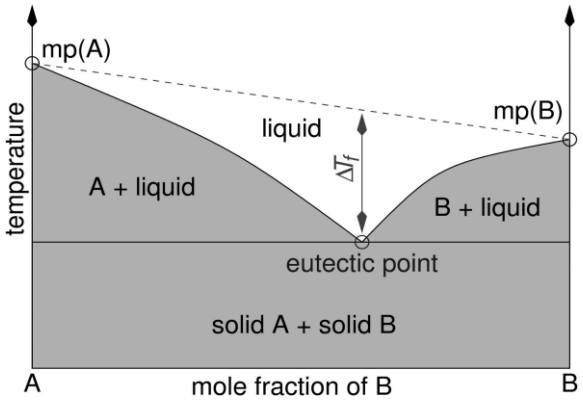


Fig. 8. Schematic representation of a eutectic point on a two-components phase diagram, mp (melting point). The difference in the freezing point at the eutectic composition of a binary mixture of A+B compared to that of a theoretical ideal mixture, ΔTf, is related to the magnitude of the interaction between A and B. The larger the interaction; the larger will be ΔTf [82].

Most DES exhibit fascinating physiochemical proprieties, similar to those of IL: very low melting point (liquid at room temperature or temperature lower the 100°C), low vapour pressure, high conductivity, high flash point, high dissolving power, high water solubility [85]. Moreover, most DES show remarkable advantages compared with IL, since they are [86,80,87,88,89]:

- easy to synthesize (DES can be easily prepared by mixing two or more, often solid, reagents and heating them to around 80° to form a liquid product);

- economically competitive (DES are usually prepared from cheap and accessible raw materials that are available as bulk chemicals, among them the most commons are urea, citric acid, succinic acid and glycerol.);
- non-toxic or only weakly toxic;
- biodegradable;
- biocompatible, thanks to the safety of the individual components;
- usable in large-scale processes;
- non-reactive with water;
- less prone to waste generation.

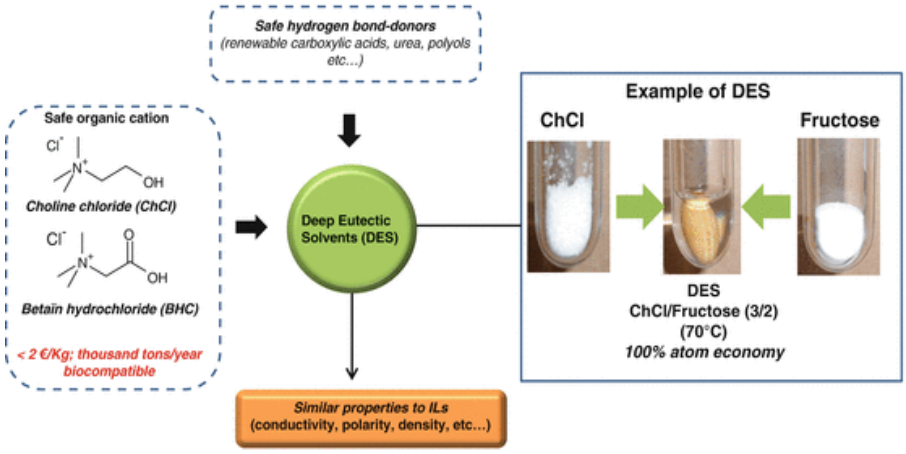


Fig. 9. Advantages and green chemistry principles in DES preparation [93].

1.4.1 DES: a tuneable solvent for several applications

Due to their physiochemical features DES are efficient solvents, already successfully applied in various fields including extraction [90], biomass processing [91], electrochemistry [95], CO_2 adsorption [92], organic synthesis, catalysis, biomass pre-treatment and processing, enzyme-catalysed reactions, new material preparation, biotechnology [93,94,95]. Essentially, DES have similar applications to IL, but their accessible and biocompatible ingredients have allowed them to be used in food and nutraceuticals/pharmaceuticals [85].

1.5 NaDES: entirely nature-based Deep Eutectic Solvents

When DES are formed by natural compounds abundantly present in organisms, they are named Natural Deep Eutectic Solvents (NaDES) [96,97]. Organic acids and bases, amino

acids, sugars and polyalcohols represent some of the primary metabolites, commonly synthesized by plants, that are successfully mixed to create NaDES. NaDES are eutectic mixtures of two or more natural components, usually solid, in peculiar molar ratio that became liquid and generally keep liquid state at room temperature [100] (*Fig. 10*) [98]. Compared with DES, NaDES offer all the same suitable physicochemical properties that qualify DES (low vapor pressure, high stability, high solubilizing power and miscibility with water, among others) [110], but additionally its total natural origin allows to propose NaDES as "greener" solvents. NaDES constituents shows different environmental-friendly features [101,97]:

- Cheap
- From removable sources
- Readily biodegradable
- Low or Non-toxic

However, is important to bear in mind that the properties of a NaDES can be different from the properties of the single ingredients [47].

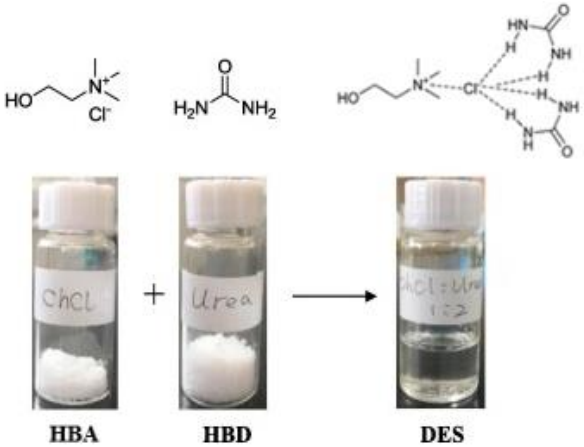


Fig. 10. Transformation of the solid state NADES components into a liquid state eutectic mixture [98], modified.

The term NaDES was coined in 2011 by Choi et al. who speculated the possibility of NaDES to be the third liquid medium in organisms apart from water and lipid. The existence of this liquid phase would explain a great number of biological mechanisms that occur in all organisms, otherwise difficult to explain, such as biosynthesis, transport and storage of poorly water-soluble macromolecules and metabolites in the aqueous environment of cells or the survival of organisms in extreme conditions such as salt stress,

drought and low temperatures. Potentially NaDES could be used as a truly green solvent for extraction and as medium for chemical and enzymatic reactions [99].

Over the past two decades NaDES have gained increasing interest from the scientific community as the potential new generation of green solvents, improving the pathway outlined by DES [100]. The number of published articles regarding NaDES has grown exponentially, revealing the effort of scientist directed towards the understanding of the characteristics and the capability profile of this new family of designer safer solvents [101]. Nowadays a literature search in Scopus using the keywords “natural deep eutectic solvents” provides more than 2800 references (*Fig. 11*).

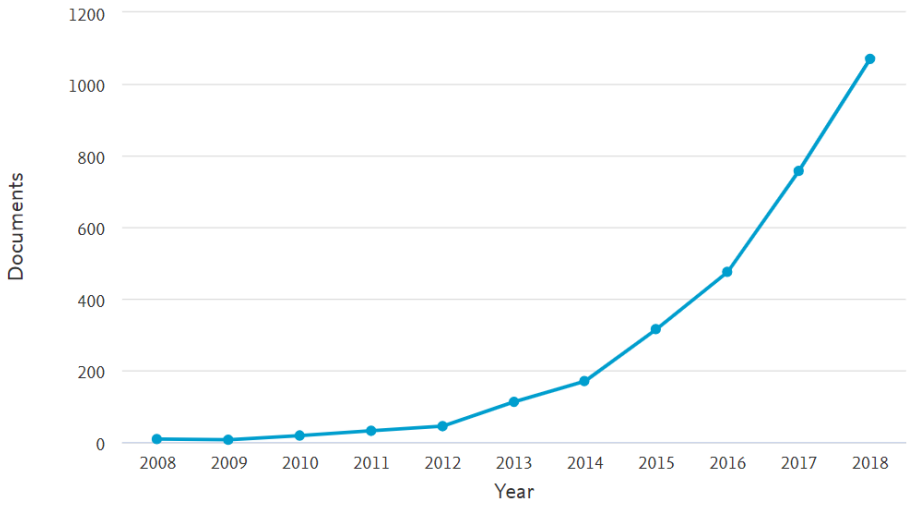


Fig. 11. Number of publications using the keywords “natural deep eutectic solvents” in the past eight years (source www.scopus.com).

1.5.1 The supermolecular structure of NaDES

The structure of different NaDES was investigated with two-dimensional nuclear magnetic resonance spectroscopy (NOESY) and Fourier transform infrared spectroscopy (FTIR) [98,114], revelling the presence of extensive intermolecular hydrogen bonds between the components within NaDES, that form a supermolecular structure. Hydroxyl, carboxylic and amine groups, abundant in amino acids, organic acids, sugars or choline derivatives, are the main functional groups involved in hydrogen bond interaction [102]. Those strong hydrogen bond networks are the key to NaDES formation and the cause of the high melting point depression of the individual precursors in the eutectic mixture [98].

The number and the type of hydrogen bond acceptors and donors, the spatial structure of their groups and the consequent interaction influence the formation, the stability and the

physical, thermal, chemical and biological properties of the solvent. Thus, NaDES arise as a tailor-made new solvent that could be specifically designed for a different enormous type of application [98]. The actual challenge is to predict and investigate empirically which mixture, molar ratio and temperature will originate the most adequate NaDES for a given purpose.

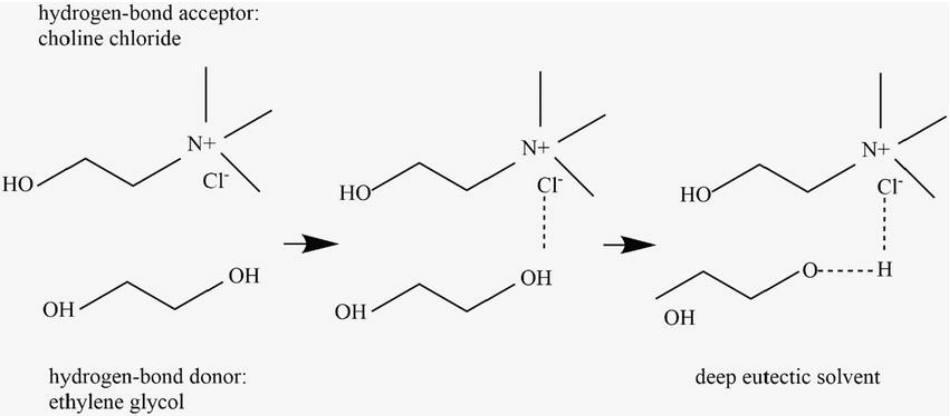


Fig. 12. Hydrogen bond formation: in this example choline chloride acts as hydrogen-bond donor and ethylene glycol is the hydrogen-bond accepros,[84]

1.5.2 Type of NaDES: a mixture of different natural compounds

Usually, NaDES are easily prepared by mixing and heating (~ 70°C) two or more natural compounds under a certain molar ratio until a homogeneous and transparent liquid is achieved at room temperature. Moreover, this mixture does not require any additional purification. NaDES composition is given as molar ratio (mol/mol), which directly reflects the contribution of components [103]. According to their components, NaDES can be categorized in five clusters [98]:

- acid or base;
- neutral;
- sugar-based mixtures with an acid;
- sugar-based mixtures with a base;
- sugar-based mixtures with an amino acid.

1.5.2.1 Hydrogen Bond Donors (HBD)

The HBD in NaDES are mostly primary metabolites naturally present in all types of cells: sugar (e.g. glucose, sucrose, fructose, etc), organic acids (e.g. lactic, malic, citric acids), amino acids, alcohols (e.g. glycerol, ethylene glycol) and urea. Some of them act both as hydrogen-bond donors and acceptors, for example fructose/citric acid, malic acid/proline and D(+) glucose, L(+) tartaric acid among others [98].

1.5.2.2 Hydrogen Bond Acceptors (HBA)

Most of the mixtures reported in literature have choline chloride (ChCl) as HBA [101]. Choline chloride is a quaternary ammonium salt with choline cation and chloride anion. It has a role as animal growth promoter, essential nutrient (known as vitamin B4) and precursor for phospholipids and acetylcholine. ChCl is readily biodegradable according to OECD-criteria (reaching 93 % degradation within 14 days), weakly toxic (tests on animals and human cell show LD50s of 3150-5000 mg/kg), cheap (~2 €/kg) [93,104,105].

Recently few researches have selected betaine-based NaDES as suitable solvents [97 106]. Betaine (Bet) is a low-cost chemical (~3€/Kg)[107], readily biodegradable in water (88% of mineralisation in 28 days, OECD guideline), not toxic to sludge micro-organisms, not bioaccumulative and not persistent [108]. Moreover, Glycine betaine (N,N,N-trimethyl glycine) is widely known in agriculture field acting as a protector of plants against the damaging effects of environmental stress, such as drought, excessive salt, cold, heat and freezing. Some plants, including spinach and barley, accumulate high quantity of GB in their chloroplasts, to maintain the integrity of membranes against external hazard [109].

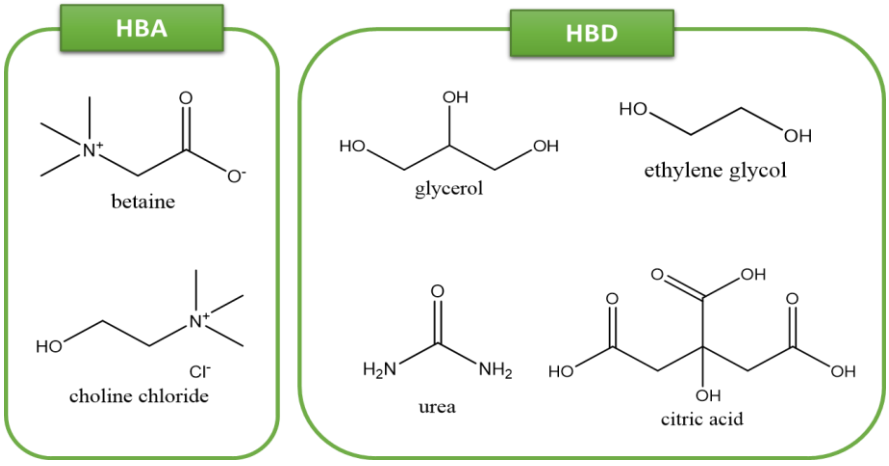


Fig. 13. Examples of some hydrogen-bond acceptor and donors widely used for NaDES synthesis

1.5.2.3 Water

Most of NaDES are made up involving only two components creating the most investigated binary system (e.g. CC/glucose), but a third chemicals can be added generating a ternary system (e.g Bet/urea/water). In fact water is often added to the eutectic mixtures because of its ability to tailor NaDES physicochemical properties such as viscosity, conductivity, density, polarity and extracting efficiency [102]. Thus, by modulating the water content, the solvation power can be adjusted to specific needs. The supermolecular structure of NaDES changes after addition of water as consequence of progressive rupture of the hydrogen bonding between HBA and HBD [112]. Experiments verified that when of water is add at molar proportion similar to those of other constituents, it is able to form hydrogen-bond with other compounds and becomes an integral constituent of the supermolecular structure of NaDES [104]. However, an excessive addition of water has a negative impact on the supermolecular structure, as a consequence of the progressive rupture of the hydrogen bonding between HBA and HBD. When the water content exceeds 50 wt%, a complete dissociation of the eutectic networks occurs, leading to the individual ingredients solubilized in water. However, this threshold may diverge from NaDES to NaDES [112].

Table 4. Examples of different combinations and molar ratios in natural deep eutectic

Components			Mole ratio
Component 1	Component 2	Component 3	
Choline chloride	Lactic acid		1:1
Choline chloride	Malonic acid		1:1
Choline chloride	Maleic acid		1:1, 2:1,
Choline chloride	DL-Malic acid		1:1, 1.5:1,
Choline chloride	Citric acid		1:1, 2:1,
Choline chloride	Aconitic acid		1:1
Choline chloride	L-(+)-Tartaric acid		2:1
Choline chloride	Glycol		1:1,1:2
Choline chloride	1,2-Propanediol		1:1, 1:1.5, 1:2, 1:3
Choline chloride	1,2-Propanediol		2:1[a]
Choline chloride	Glycerol		1:1, 3:2
Choline chloride	*meso*-Erythritol		2:1[a]
Choline chloride	Xylitol		5:2
Choline chloride	Adonitol		5:2
Choline chloride	Ribitol		5:2
Betaine	D-(+)-Glucose		5:2 [a]
Betaine	Sucrose		4:1, 1:1[a]
Betaine	Sucrose		2:1
Betaine	D-(+)-Trehalose		4:1
Betaine	D-Sorbitol		3:1[a]
Betaine	DL-Malic acid		1:1
Betaine	L-(+)-Tartaric acid		2:1
Betaine	D-Mannose		5:2
Betaine	Inositol	Raffinose	9:1:1[a]
Betaine	Sucrose	Proline	1:1:1
Betaine	Sucrose	Proline	5:2:2
Betaine	D-(+)-Glucose	Proline	1:1:1
Betaine	DL-Malic acid	D-(+)-Glucose	1:1:1
Betaine	DL-Malic acid	Proline	1:1:1
Betaine	DL-Malic acid	Inositol	1:1:1[a]
Betaine	Oxalic acid	D-(+)-Glucose	1:1:1
Betaine	Citric acid		1:1

1.5.3 NaDES features affecting their efficiency

NaDES have an enormous potential as neoteric green solvents, nevertheless, the increasing research on these promising solvents has raised some important disadvantages of NaDES, compared with conventional solvents. Shortcomings like high viscosity and polarity could be a restriction in various applications, especially in extraction procedures, and could prevent industrial adaptations [110]. However, literature describes this issues as “adjustable”, through the modulation of specific parameters, such as [98,100]:

- Temperature
- Water content
- HBD e HBA composition

1.5.3.1 Viscosity, a problem with different solutions

Viscosity is the major obstacle for the application of NaDES [113] because it slows mass transfer in extraction or dissolution processes, involves time-consuming solvent transfer operations and has detrimental effects on reaction rates and handling especially in extractive processes [111,30]. An obviously desirable propriety of NaDES is the low viscosity. NaDES viscosity typically ranges from 200-500 mm^2 s^{-1} at 40°C, much higher than the most common molecular solvents, [112] and can be 20−1000 times higher than that of water at room temperature [102]. The high viscosity is caused by the strong hydrogen-bonding interaction between NaDES compounds limiting the mobility of the free species inside the NaDES. Several studies concerning extraction of natural compounds by NaDES show that extraction yield is deeply affected by viscosity of the solvent [113]. For example, NaDES have been used to extract flavonoids from food sample, and the experiment highlighted that the decrease in solvent viscosity allows to an increase in diffusivity, accelerating release of secondary metabolites from the sample matrix to the solvent [113]. The same behaviour occurs using ChCl-based NaDES for the extraction of bioactive compounds, (α-mangostin) from *Garcinia mangostana*. The high viscosity hampers the breaking of intermolecular bonding between HBA and HBA, and the formation of new bonds with mangostin; as consequence longer extraction time is required than conventional organic solvents. *Fig. 14* clearly highlights difference in extraction times between ethanol, that has a low viscosity, and ChCl-based NaDES, in which the high viscosity slows the dissolution of the analyte. As described by the Stokes-Einstein equation, conductivity and viscosity (η) are inversely proportional, so high viscosity means low diffusion coefficients (D) [114]:

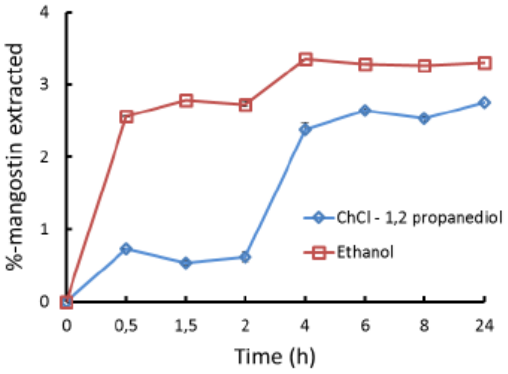


Fig. 14. Effect of time on mangostin yield using ClCl-1,2-propandiol as NaDES and ethanol as reference solvent: viscosity decrease the conductivity and so increase extraction time [114].

Viscosity is widely called “adjusted propriety”, because there are different factors that can be changed to reduce it, thus increasing extraction yields:

- Composition: the viscosity of NaDES differs enormously according to their composition: glycerol, urea, lactic acid, malonic acid, sucrose e fructose are only few examples. so is necessary to investigate various combination of HBA and HBD to find the better mixture [113].
- Temperature: viscosity linearly changes with temperature. As show in *Fig. 15* an increase of temperature generates a low viscosity NaDES, so a more desirable solvent [98].
- Water content: a third widely investigated parameter is the content of water [112]. It shows the same effect of temperature: NaDES viscosity decreases as the amount of water increases [27,76]. An addition of water leads to a large decrease in the viscosity of NaDES as a result of the gradually weakened hydrogen-bonding interactions between the components, and consequently improve NaDES solubilization ability. This theory is confirmed by a research for the extraction of K-carrageenan from the seaweed *Kappaphycus alvarezii*. As displaced in *Table5* the yields of K-carrageenan obtained by using 10% hydrated ChCl/Gly (1:2) was twice as high compared to the anhydrous NaDES. Thus, NaDES great solubilizing ability could be enhanced increasing the temperature or adding water. Nevertheless, viscosity is only one of the parameters that influences extraction efficiency [76].

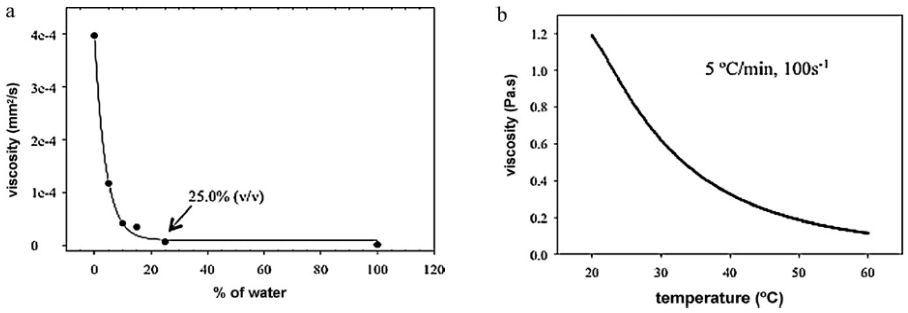


Fig. 15. Relationship between (a) viscosity and added water percentage (v/v). (b) viscosity and temperature of glucose-choline chloride-water [96].

Table5. Yield of K-carrageenan obtained from *Kappaphycus alvarezii* using different solvent systems.

Solvent system/method	Yield % (±S.D)	Viscosity (cP) of solvent at 25 °C
Choline-Chloride-Urea 1:2	37.60 ± 1.20	289.10
10% Hydrated choline-Chloride-Urea 1:2	53.64 ± 1.25	34.49
Choline-Chloride-Glycerol 1:2	30.93 ± 0.90	266.60
10% Hydrated choline-Chloride-Glycerol 1:2	60.25 ± 1.10	52.64
Water	46.87 ± 2.00	0.90

1.5.3.1.1 Difficulties in recovery from NaDES matrix

The low vapour pressure of the solvents and the strong H-bond occurring between NaDES molecules and extracted compounds, are both a drawback and an advantage in extraction procedure [110]. A very low vapour pressure is a desirable propriety of NaDES, because the extraction process could be performed within a wide range of temperatures without considering solvent evaporation and the creation of hazardous VOC [104]. On the other hand, since the conventional extraction use evaporation as recovery method, the non-volatility of NaDES become a drawback. Furthermore, the ability of NaDES to create strong intermolecular interactions with the target compound, which is the reason of their outstanding extraction power, represents a hindrance to the separation phase. The higher is the ability of the solute to interact with the NaDES network, the higher is the extraction yield, but also the difficulty to isolate the extract from the solvent [110].

As consequence, after NaDES extraction additional processing strategies are required to recover the pure target analyte. In the literature it is possible to find many examples of the recovery of bio-compounds from NaDES extracts with chromatographic approaches and elution with ethanol, methanol or acetone [117]. The results obtained with conventional solvents (ethanol and methanol) show an efficient isolation of phenolic and polyphenol metabolites, terpenoids, and saponins [118] form the NaDES matrix. However, the use of conventional solvents after NaDES mediated extractions is clearly a contradiction and a misalignment from green chemistry principles. NaDES have been developed with the aim to find an efficient environmentally friendly substitute to the hazardous and toxic solvents used so far and not to postpone their use.

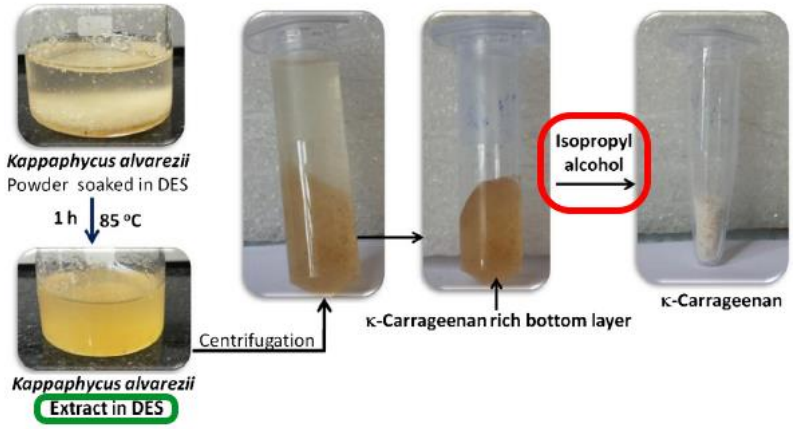


Fig. 16. An extraction procedure from algae biomass that include both NaDES (based on urea, ethylene glycol, glycerol and choline chloride) and conventional solvents (acetone) to obtain the pure compounds, modified.

An option to bypass this problem is the use of NaDES first as extraction media and after as formulation systems, producing a new useful material composed of NaDES with enhanced concentrations of bioactive molecules. In this way NaDES turn into carriers of a precise functional metabolite. The starting components of NaDES are metabolites that naturally occur in organisms, safe, non-toxic and even edible thus theoretically NaDES are fully compatible with cosmetics applications, pharmaceutical formulations, or food manufacturing [110]. For example, NaDES can act as a vehicle in drug-delivery systems increasing the concentration and thus the bioavailability of poor water-soluble drugs. Some studies demonstrated that solubility of five model drugs increases 5 to 22,000-fold in urea or malic acid ChCl-based NaDES [115]. Recent studies have investigated the application of

NaDES in agrochemical industry, focusing on their potential as solvent carrier systems for plant-derived crop protection agents. NaDES such as, lactic acid/1,2- propanediol (2:1), 1,2-propanediol/ChCl/water (1:1:1 and 1:1:3) have demonstrated considerable improvement in solubility of poorly soluble insecticides, proving to be promising vehicles to deliver these plant protection products. Moreover, some researchers proposed the possibility to combine the benefit provided by the extracted bioactive compound, with the suitable proprieties of NaDES itself, achieving a double beneficial effect in one solution. For example, grape skin extracts, abundant in phenolic compounds, increases significantly their antioxidant activity when combined with ChCl/malic acid NaDES. The phenomena could be explained by the beneficial effect of the NaDES itself: malic acid and other organic acids have various pharmacological effects, including, anti-inflammatory, antiplatelet and antioxidant, making these extracts suitable for pharmacological application [117]. In line with these observations, recent studies show that the antioxidant activity of the phenolic molecules improves if combined with NaDES, allowing a better phytoactive profiles [110]. Another study demonstrated the safety of NaDES when used as drugs delivery. Thirty different types of NaDES, based on sugars, organic acids and amino acids, were used to extracts rutin, a flavonoid used as nutraceutical for its health-promoting activity, an create formulations that have been administered orally to rats in moderate doses, demonstrating to be non-toxic for the model organism. Moreover, NaDES solution of rutin resulted in higher and more persistent levels of this molecule in plasma as measured by LCMS/MS, demonstrating that NaDES formulation increase the adsorption of rutin by gastrointestinal tract compared to water [118].

1.5.3.2 Polarity

The polarity of NaDES has also to be considered an important property, based on the ability of the solvent to interact with solutes, that could affect its solubilizing capacity, and thereby limiting its extraction abilities. Most NaDES are organic acid based (polarity ~45 kcal mol^{-1}), followed by pure sugar and amino acid based NaDES with a polarity similar to water (~50 kcal mol^{-1}), and few of them are polyalcohol based NaDES, the most polar, displaying a polarity similar to that of methanol (~55 kcal mol^{-1}). Thus, NaDES show a broad polarity range and have been reported to dissolve and extract a wide range of metabolites from polar to medium or low polar, that show low solubility in water, such as many bioactive natural compounds, gluten, starch and DNA [98]. Despite that, NaDES are

primarily made up by polar ingredients thus, they are not able to efficiently solubilize and extract non-polar compounds. This principle aligns with results achieved in the extraction of total anthocyanin from grape skin. Anthocyanins are polar molecules, and the best extraction yield was achieved through ChCl/oxalic acid NaDES (the most polar organic acid-based NaDES), whereas both sugar and polyalcohol-based NaDES, like ChCl/Glycerol, are less polar and consequently less effective in extraction of anthocyanins, in accordance to the like dissolves like principle.

Nevertheless, NaDES are tailorable solvents, and polarity could be partially modulated by changing the HBA/HBD combination [110] and the water content [102] to be target-specific and to optimize selectivity [118]. Water dilution increase NaDES polarity, resulting in a polarity similar to water itself. The extraction efficiency of phenolic compounds of a broad range of polarity, from *Carthamus tinctorius* L., have been tested with seven diluted NaDES, showing that NaDES with a high water content performed better for polar compounds while NaDES with low water content are suitable for the extraction of less polar compounds [110].

1.5.3.3 Thermal behaviour

Solvents are often use as media for chemical and biological reactions that might require high temperature to be achieved. Considering that some NaDES components are usually thermally unstable, as is the case of sugars or amino acids, the thermal behaviour of thirteen NaDES have been investigated using thermogravimetric analysis (TGA) and differential scanning calorimetry. All the thirteen NaDES investigated remained stable until 100°C but showed evident decomposition at temperature higher than 135°C and a glass transition under 50°C [98]. Other studies reported the glass transition temperatures of twenty-six NaDES, which varied from 78 to °C to 13°C. As results, NaDES should be used as solvents in the temperature range from 13° C to 100°C [121].

1.5.4 Applications of NaDES as green technology media

Having been established as convenient green alternatives to aqueous and conventional solvents, NaDES potential applications cover a wide spectrum of sectors. The major research efforts have been focused in several fields: extraction, synthesis, analytics, polymer, metal processing, biomass pretratment and nanomaterials sciences among the

others [124]. For example, NaDES have been extensively applied as green solvent media in polymer synthesis for industry or medicine applications [116]. As solvents NaDES have been successfully used for the pre-treatment of rice straw, one of the most abundant lignocellulosic biomass residues worldwide. Different NaDES have been tested, showing high solubility for lignin and poor or negligible solubility for cellulose: for example, the ChCl-lactic acid mixture dissolves only lignin and the solubility increase with a higher lactic acid proportion, presumably because acid in the NaDES allows stronger hydrogen bonding to lignin [117]. Apart from solvents, NaDES have been used as functional additives and monomers as well. In fact, ChCl–urea and ChCl–glycerol eutectic mixtures were incorporated as additives in the preparation of corn starch- and cellulose-based polymer electrolytes, as well as in the fabrication of agar films. advantages such as sustainability, low cost, lower toxicity of the plasticizing processes, along with superior morphological, thermal and chemical integrity, and conducting characteristics were highlighted in these formulations.

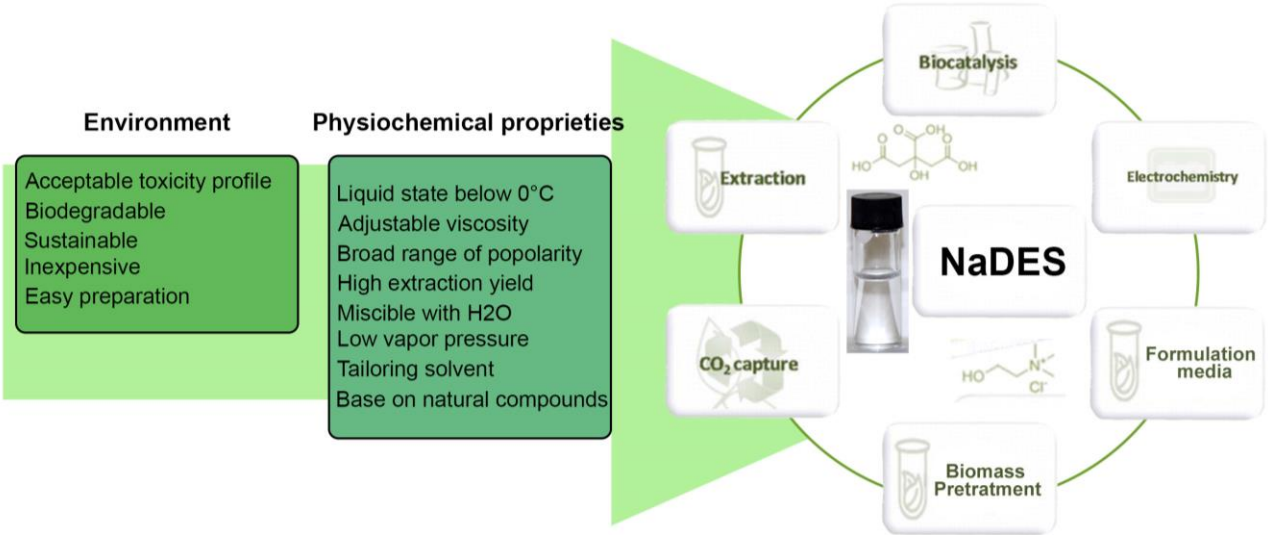


Fig. 17. Suitable proprieties of NaDES and their multiples applications.

1.5.4.1 NaDES as extraction media

Considering stabilization and solubilizing talents of NaDES, is not surprising that they are firstly applied as extraction media [86]. NMR study suggest that intense strong hydrogen bonding interactions between NaDES molecules and solutes should the primary reasonable of this great extractive capacity. Several studies used NaDES to carry on extraction processes from plants, food, biowaste and other natural matrices to obtain high

added value chemical compounds request in several fields, such as pharmaceutics, cosmetics, nutraceutical, and agricultural [84].

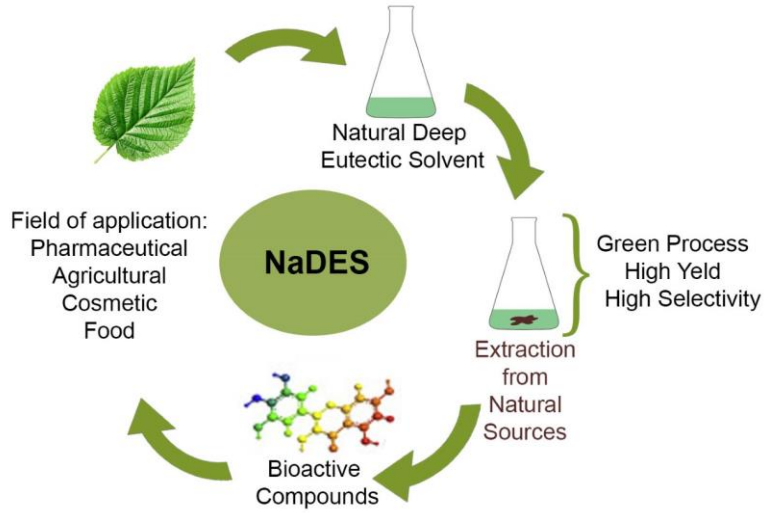


Fig. 18. Simplification the extraction process of bioactive compound from natural sources with NaDES and their applications [87], modified.

Phenolic compounds extraction

Phenolic compounds (PC) are the most investigated group of phytochemicals extracted through NaDES [98]. In particular, polyphenol are organic molecules characterized by the presence of large multiples of phenol structural units (aromatic ring bonded to a hydroxyl groups). These compounds are secondary metabolites mainly concentrate in the plant kingdom, where play important role in seed germination, growth, reproduction, defence and take part in resistance responses during environmental stress factors [118]. Polyphenols are widely used by plants as useful phytochemicals against:

- Oxidative stress: polyphenols including flavonoids (e.g. anthocyanins, tannins, flavonols, flavanols, proanthocyanidins) and nonflavonoids (e.g. stilbenes, phenolic acids, gallotannins, ellagitannins, lignins) are primary natural sources of antioxidant, an efficient system developed by plants in order to protect their own tissues [118]. The excellent antioxidant activities provide by polyphenols allows plants to increase their oxidative stress tolerance by the capture of reactive oxygen species (ROS), components harmful to plant cells because lead to damage of proteins, lipids and nucleic acids, and thus pathological condition [119].

- Excessive sun exposure: flavonoids are often colour compounds (e.g quercetin give the characteristic yellow tint) able to absorb radiation of high energy. Studies have observed that plants radiated with UV light excess increased flavonoids synthesis and accumulation in epithelial cells, with the aim of protect deeper-lying tissues against destructive radiation.
- Heavy metal stress: some plants such as corn (*Zea mays L.*) produce in the root exudates high level of some flavonoids (e.g *catechin* and *quercetin*) able to chelate transition metals (e.g. Fe, Cu, Ni, Zn) and provide protection against the toxic effects of high concentrations of these metals [118].
- Defence against infection and pathogen: useful polyphenols synthesized in plants tissues in response to infection by microorganisms (bacteria, fungi, viruses) are phytoalexins. These compounds limit spore germination and phytopathogenic fungi hyphal growth and possess bactericidal proprieties, reducing in this way the spread of the pathogenic microorganism infection [121]. Rutin and quercetin, represent one of the most studied plant defence flavonoids thanks to their abundant occurrence and well documented toxicity to numerous insects [118].

A very large number of publications reported the ability of NaDES in the isolation and fractionation of phenolic compounds appeared over the past two decades, confirming that NaDES could be a good replacement for conventional solvents (water and ethanol) [102]. NaDES were used to extract efficiently polyphenols from a wide range of natural biomass:

- Wine waste: NaDES have been developed to recycle the wine lees of the wine industry, as a cheap source of phenolic compounds, especially anthocyanins (*Fig. 19*). ChCl-based NaDES with malic acid provide a more efficiently extraction of anthocyanins compared with conventional solvents [120].
- Vanilla: Another experiment was conducted on the extraction ability of phenolics from vanilla pods using different NaDES, including ChCl and betaine. All the combination provides more efficient extraction than ethanol, moreover a NaDES solution suitable for flavouring food products was also produced [121].
- Food: Seventeen types of NaDES, including choline chloride and betaine-based solvents, were used to develop a method for the extraction of flavonoids from food samples. The procedures showed high recovery performances, small amount of

solvent needed for extraction, short analysis times and simple extraction procedures [122].

All the studies highlight that different parameters of the extraction, such as water content, temperature and compounds combination, need to be tailored to optimize the recovery and obtain better stability of the extracts [85]. Moreover, NaDES generally act as preservatives, which allows to conserve polyphenols for long time avoiding their degradation or damage.

Fig. 19. Grapes are one of the main natural sources of polyphenols

Concerning agriculture field, recently NaDES has been proposed as new green crop protection agents: a solvent carrier system for the delivery of flavonoid, a secondary metabolite with notable pesticidal behaviour. The principal issue in the uses of those insecticidal metabolites, as a safe substitute to conventional and harmful insecticides, was their poor water solubility. Otherwise, these metabolites display solubility in NaDES 29 to 195-fold more, compared to aqueous solubility. Thus, NaDES is proposed as a functional media for the application of insecticidal plant secondary metabolites to protect agriculture crops mainly in the earlier and vulnerable stages [123].

Micro and macroalgae extraction

The most popular extraction method for recovering high value products from microalgae is solvent extraction because its high yield [124], however so far, literature concerning algae extraction using NaDES is limited.

The ChCl/1,2-propanediol/water (1:1:1) NaDES coupled with ball mill or ultrasound assisted extraction has been tested to extract carbohydrates, proteins, chlorophylls, carotenoids and polar lipids from wet samples of the microalga *Scenedesmus dimorphus*.

In general, NaDES show satisfactory extractive ability for photosynthetic pigments, carbohydrates and proteins but a low recovery for nonpolar compounds as lipids [124].

ChCl-based NaDES with urea, ethylene glycol and glycerol and their hydrated counterparts were used for the selective extraction of the sulphated polysaccharides κ-*carrageenan* from the carrageenophyte *Kappaphycus alvarezii.* The extraction efficiency of NaDES was equivalent to the conventional process and higher than using water as solvent. The reason of the high extraction capacity of NaDES is probably the strong interaction between carrageenan (a polysaccharide that can play as HBD) and choline chloride.

Macromolecules extraction

Extraction of RNA and DNA is not easy because they are macromolecules, generally difficult to solubilize and susceptible to degradation with heat and in the presence of certain chemical substances. ChCl-based NaDES combined with, *glycerol, ethylene glycol, sorbitol, resorcinol* or *levulinic acid* have been used to extract successfully salmon DNA and demonstrated to be recyclable many times without lost in efficiency extraction. Among the tested NaDES, *ChCl/glycerol* or *ethylene glycol* showed higher extraction yields and stability of DNA [125].

Extraction of gluten with NaDES, in combination with an ultrasound-assisted procedure, has been proposed as feasible substitutes to conventional ethanol-water solution extractions used in food processing. The results demonstrated that the solubilized proteins maintained their structures without significant changes. The greatest yield has been obtained from hydrated fructose/citric NaDES, additionally, the presence of citric acid help to prevent proteins from oxidation [126,127].

1.6 Toxicity of NaDES

Even if NaDES consist of components with a presumed safety profile, the interaction between the individual ingredients could result in a synergic effect and thus a different toxicological profile [127]. Since NaDES have proven their great potential as extraction solvent or functional carrier of bioactive compounds for the direct and indirect human consumption, an assessment of their toxicological profile for different trophic levels is stringently required [77].

1.6.1 Cytotoxicity

The toxicity of NaDES has been mainly evaluated at cellular level, thanks to the repeatability, low cost and short time required of these tests.

The first cytotoxicity test evaluated the vitality of the fibroblast-like model cell line L929 placed in contact with eleven different NaDES and two different IL. Despite the results did not display a clear toxicity trend, both tartaric and citric acid showed a detrimental effect on the cells activity. This phenomenon is probably caused by the drop in pH inducted by the presence of organic acids, as confirmed by the literature [77,128].

Three ChCl-based NaDES were studied by testing their cytotoxicity towards fish (Channel Catfish Ovary (CCO) cells) and human tumour (MCF-7) cell lines. For both cell lines toxicity data indicate a low toxicity for ChCl/glucose and ChCl/glycerol, while ChCl/oxalic acid resulted to possess moderate cytotoxicity [77].

Three human (HelaS3, CaOV3, and MCF-7) and one mouse (B16F10) cancer cell line have been used to test cytotoxicity of NaDES with ChCl as HBA and glucose, sucrose, glycerol, fructose and malic acid as HBD. All NaDES showed some degree of toxicity to cells, and the study identified the source of the cytotoxicity in four main factors:

1. Physical proprieties of NaDES: in the study a trend between high viscosity of NaDES and increased lethality was observed. In fact, ChCl/glycerol/H_2O (1:2:1) was both the least viscous and the least toxic to the three human and one mouse cell lines.
2. pH: ChCl/malonic acid is the most toxic among the five NaDES, probably the cause lies in the excessive decrease in pH level and consequent deleterious effect, when organic acids are used as HBD.
3. Cellular metabolic pathways*:* choline, glycerol and sugar are compounds required for the cellular metabolism (e.g. synthesis of phospholipids membranes, source of energy), therefor the cells high tolerance of those raw material may explain why the respective NaDES were less toxic. In contrast, malonic acid is an inhibitor of some metabolic pathways (e.g. citric acid cycle), and this may lead to the high toxicity exerted by NaDES that contain this acid.
4. Interaction between NaDES and cellular membrane: a simulator of the interactions between cellular membranes phospholipids and NaDES suggested that NaDES strongly interacted with some functional groups on cell surfaces, allowing their

penetration into the cytoplasm and aggregation or accumulation, thus expressing their cytotoxicity [128].

In summary, NaDES may be both deleterious and benign at the cellular level and may, thus accordingly to the above-mentioned results it is clear that the relationship between cytotoxic mechanism and NaDES must be further investigated to understand their toxicity behaviour.

Table 6. Toxicity comments for mixtures of NaDES on different cell lines.

NaDES	Cell lines	Toxicity comments	Reference
ChCl/glucose ChCl/oxalic acid ChCl/glycerol (2:1, 1:1, 1:2)	Fish cells (CCO) and human cell lines (MCF-7 cells)	NaDES showed low or moderate toxicity	Radošević et al. (2015) [77]
ChCl/lactic acid/water (5:2:5) ChCl/sucrose/water (4:1:4) ChCl/glycerol/water (1:2:1) ChCl/fructose/water (5:2:5) ChCl/malonic acid/water (1:1)	Three human (HelaS3, CaOV3, and MCF-7) and one mouse (B16F10) cancer cell lines	ChCl/glycerol/water was the least toxic, but also acid NaDES showed high toxicity toward cell lines.	Hayyan et al. (2016) [128]

1.6.2 Toxicity to bacteria

The toxicity of NaDES based on ChCl combined with sugars, amines, alcohols and organic acids have been evaluated using the growth inhibition of two Gram-positive (*Staphylococcus aureus* and *Listeria monocytogenes*) and two Gram-negative (*Escherichia coli* and *Salmonella enteritidis*) bacteria. All the tested amine-, alcohol-, and sugar-based NaDES did not show side-effects on bacteria growth, so could be labelled as benign to the model organism. On the contrary all seven organic acid-based NaDES had a significant inhibitory effect (*Fig. 20*), suggesting their use as antibacterial agents [76]. Authors suggested that the main cause on growth inhibitory was the low pH due to acid components: when pH of NaDES were readjusted to the optimal bacteria growth range, no inhibitory effects on bacteria were observed [130].

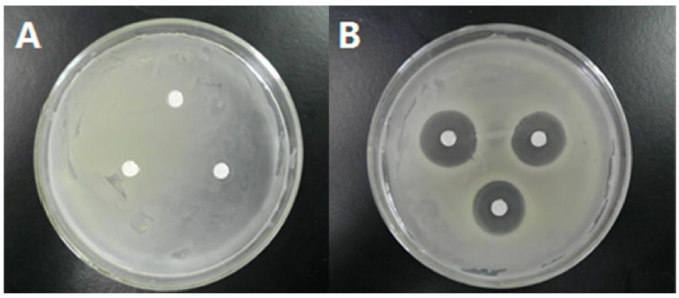


Fig. 20. Effect of different NaDES on Escherichia coli in a Petri dish: with inhibition (A) ChCl/urea, with inhibition (B) ChCl/citric acid, without inhibition [78].

Aligned with the previously observation, a recent study assessing the ecotoxicity of NaDES based on ChCl combined with different organic acids, namely acetic, citric, lactic, and glycolic acids, against the bacterium *Vibro fischeri*, observed that toxicity increased with the acid content. The NaDES investigated showed essentially identical results to those of the corresponding acid, tested individually. Thus, the effect of acids HBD is preponderant in toxicity, probability due to the negative effect on cell activity through the denaturation of proteins. In this way NaDES was able to pass cell membrane and exert its toxic effect [66].

The results carried out testing NaDES based on ChCl combined with urea, glycerol, ethylene glycol and acetamine in different molar ratio (1:2, 1:1, 2:1) are consistent with these observations. NaDES at low level may be considered non-toxic to bacteria, while when administered at high concentration (0,75 M) all of them exhibited significant inhibitory effects on the model organism (*Escherichia coli*). Moreover, the results suggested that the anti-bacterial property of the eutectic mixtures was much more intense than that of their individual components [129].

Another toxicological test on Gram positive and Gram-negative bacteria was carried out using NaDES based on ChCl combined with four hydrogen bond donors, namely glycerine, ethylene glycol, triethylene glycol and urea. Results revealed no toxic effect on bacteria, confirming the benign effects of NaDES. Despite that, all the investigated NaDES had high toxicity compared with their individual components, indicating different toxicological behaviour [130].

Table 7. Toxicity comments for mixtures of NaDES on different bacteria.

NaDES	Bacteria	Toxicity comments	Reference
20 kinds of NADES	*Staphylococcus aureus, Listeria monocytogenes, Escherichia coli, Salmonella enteritidis*	All the NaDES except for acid-containing NaDES showed no toxic effect on the bacteria	Zhao et al. (2015) [76]
ChCl/lactic acid ChCl/glycolic acids ChCl/acetic acid ChCl/citric acid (1:1, 2:1, 1:2)	*Vibro fischeri*	Toxicity against bacteria increased with the acid content (HBD).	De Morais et al. (2015) [66]
ChCl or ChOAc/urea (1:1) ChCl or ChOAc/glycerol (1:1) ChCl or ChOAc/acetic acid (1:1) ChCl or ChOAc/ethylene glycol (1:1)	*Escherichia coli*	0,75 mol/L NaDES resulted in annhibition index of 72,8–93,8% for the bacterium, and single components.	Wen et al. (2015) [129]
ChCl/glycerol (1:3) ChCl/ethylene glycol (1:3) ChCl/urea (1:3) ChCl/triethylene glycol (1:3)	*Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus subtilis, Artemia salina*	All the NaDES showed no toxic effect on the bacteria. The cytotoxicity of NaDES was much higher than their was much higher than their individual components	Hayyan et al. (2013) [130]

1.6.3 Phytotoxicity and toxicity to fungi

While NaDES toxicity at cellular level have been largely investigates, so far literature reports only few tests assisting the toxicity profile of these eutectic solvents on plants and fungus.

The toxicity of different NaDES based on ChCl combined with urea, ethylene glycol and glycerol were evaluated towards the fungus *Aspergillus niger*. The results found that NaDES exhibited higher anti-fungal property (higher inhibition index) at different concentrations compared to their individual components [131].

Phytotoxicity towards wheat (*Triticum aestivum*) was evaluated using three ChCl-based NaDES with glucose, glycerol, and oxalic acid as the HBD. Wheat seeds were treated with different concentrations for one week; afterward the effect of NaDES on germination and early growth of wheat was determined. The results revealed that all three NaDES exhibited low toxicity on wheat seed germination (EC_{50} > 5000 mg L^{-1}) and a higher toxicity on root and shoot growth (EC_{50} values were at least 5,5 times lower than those for germination inhibition). In particular, NaDES with citric acid exhibited the highest growth inhibition, probably caused by the very low pH (2,8) value of the solution. Phytotoxicity has been related to oxidative stress caused by the perturbation of antioxidant enzymes. Moreover, a positive correlation between chlorophyll content and growth was found [77].

Garlic (*Allium satiuvum*) has been also used to evaluate the phytotoxicity of some ChCl-based NaDES and their components (urea, ethylene glycol and glycerol), by the evaluation of root lengths (*Fig. 21* and *Fig. 22*) and abnormality of root tip cells. The results revealed that the presence all the tested NaDES mat be toxic to some extent towards for garlic following some common features:

- All the tested NaDES or they components can lead inhibitory effects to the model organism by induction of shorter root growth and formation of damaged cells.
- All the individual components exerted high toxicity compared with the corresponding NaDES.

The ChCl-based NaDES display a higher inhibitory effect than the NaDES with choline acetate as HBA [129].

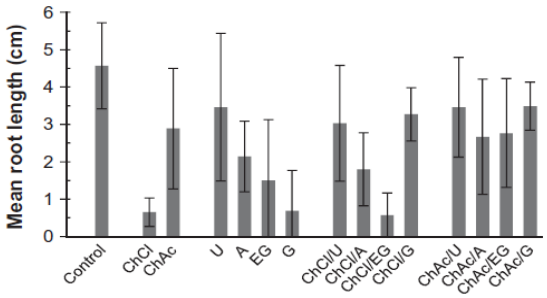


Fig. 21. Root growth of A. sativum in the presence of different NaDES (1:1) or their components [129].

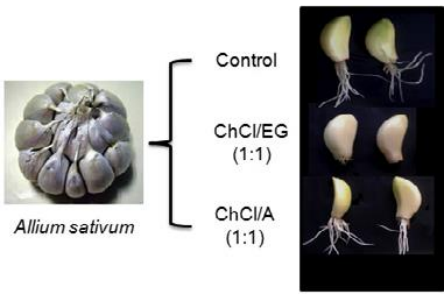


Fig. 22. Effect of different ChCl-base NaDES on garlic roots length [129].

1.6.4 Toxicity to animals

The same NaDES tested on garlic were used to estimate the toxicity profile on *Hydra sinensis*, a freshwater invertebrate used as test animal due to its extremely high sensitivity to chemical contamination. The results suggest that NaDES may exhibit a toxic effect on hydra with features like those identified for garlic. When placed in contact with a solution

contained the individual HBA, HBD or NaDES, hydras survival times were significantly shortened compared to normal growth medium. The four HBD (urea, acetamide, glycerol and ethylene glycol) did not drastically reduce the hydra's survival time, while the cholinium salts (chloride- and acetate- choline), generally considered eco-friendly, were highly deleterious to hydra causing tentacles contraction and eventually the complete disintegration of the animals (*Fig. 23*). However, the authors revealed that the detrimental effect of the cholinium salts can be reduced by incorporation into a NaDES [129].

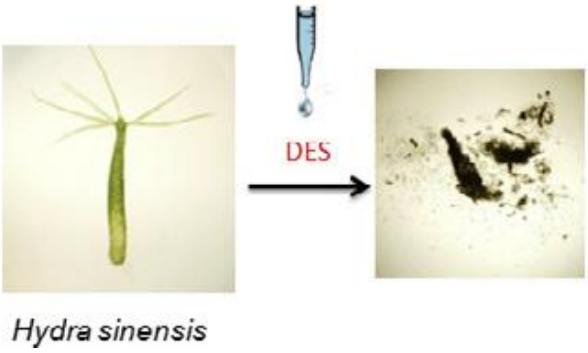


Fig. 23. Effect of NaDES toxicity on *Hydra sinensis* [129].

NaDES with ChCl as HBA and urea, glycerol and ethylene glycol as HBA were tested on *Cyprinus carpio* fish, a widely used model vertebrate for acute toxicity test. The investigated aqueous NaDES ranged from relatively harmless (ChCl/ethylene glycol and ChCl/glycerol) to moderate toxic (ChCl/urea). In line with outcome of the hydra experiment all the NaDES were clearly less toxic than their individual components [131].

Preliminary ecotoxicological assay on *Daphnia magna* using ChCl/urea NaDES display that the individual components have a low toxicity towards *D. magna*, though ChCl has a higher toxicity than urea. Furthermore, the DES resulting from the combination of the two seems to exhibit a similar toxicity to that of choline chloride [132].

These results highlight that NaDES may be considered non or weakly toxic in some cases, but a wide range of different toxicity behaviours could resulted as different combination of HBA and HBD, molar ratio, water content, concentrations or organism model tested [131,128]. There is no doubt that more investigation regarding toxic effects on cells, microorganisms, plants and animals are required to determine NaDES safety for organisms and to discover the relationship between toxicity of NaDES and their structures.

2 Aim and scope of the study

A sustainable agriculture "*conserves land, water, plant and animal genetic resources, is environmentally non-degrading, technically appropriate, economically viable and socially acceptable*" (Food and Agriculture Organization of the United Nations (FAO) Council, 1989).

The aim of this projects is to evaluate the possibility of using NaDES as ingredients for new eco-compatible bioactive formulations to deliver high value compounds, extracted from natural biomasses, in agricultural field. In spite of presenting a promising potential for crop enhancement and protection, NaDES and NaDES-formulations environmental impact and fate (e.g., biodegradation and (eco)toxicity) have to be extensively and critically evaluated before their application. In fact, although NaDES are considered green, non-toxic and environmentally benign solvents, the knowledge about their ecotoxicological profile is extremely limited and there is no information about their effect on the soil compartment.

To provide information about the real applicability of NaDES and NaDES-formulations in agricultural field, this study made a systematic assessment of extraction abilities and environmental impact of different type of NaDES.

NaDES can be produced from a wide variety of compounds, but the choice of HBA (hydrogen bond acceptors) and HBD (hydrogen bond donors) used as well as their molar ratio greatly influence their chemical-physical properties and therefore their extraction capacity. Therefore, the first part of the present study focused on the synthesis of hydrated NaDES with various composition with the aim to evaluate the extractive potentials in relation to the chemical structure, thus finding possible structure-activity relationship. Betaine and chlorine chloride were selected as HBA and combined with HBD exhibiting different reactivity:

- Acid: citric acid (HBA:HBD molar ratio = 1:1) lactic acid (1:1) and malic acid (1:1)
- Basic: urea (1:2)
- Neutral: glycerol (1:2) and ethylene glycol (1:2)

Then these NaDES were used to extract different natural biomass to prepare NaDES-formulations:

- Grape pomace and grape seeds: two of the richest natural sources in polyphenols, organic compounds that play important role in seed germination, growth, reproduction, defence against infection, pathogens, and take part in resistance responses during environmental stress factors.
- *Arthrospira platensis* and *Phaeodactylum tricornutum*: microalgae that enable to improve plant resistance to environmental stress, enhance plant defences against pests and pathogens and facilitate the recovery of damages caused by insects and bacterial or fungal.

The last part of this work focuses on the ecotoxicological evaluation of various NaDES, tested alone and as formulations on organisms of different trophic levels, and in terms of biodegradability in water. The aim was to find a relationship among toxicity and NaDES composition to provide a preliminary assessment of their safety in terrestrial environment and environmental persistency. The toxicity tests were performed on soil flora and fauna model organisms:

- *Eisenia andrei*: this earthworm species was used to run a 56 days reproductive toxicity test with six different NaDES.
- *Avena sativa:* effects on the emergence and early growth in soil of this higher plant were tested to evaluate phytotoxicity of pure NaDES and NaDES formulations and their potential beneficial influence on crops growth.
- *Lepidium sativum*: seeds germination tests of this herbaceous plant were used to evaluate the inhibitory or promoting behaviour of NaDES.
- *Ocimum basilicum and Lactuca sativa:* phytotoxicity and water stress resistance were tested using Bet-based NaDES and NaDES-formulations carrying polyphenols.

3 Materials and methods

This thesis work consisted of two main phases:

1. A first chemical step concerning the synthesis of NaDES, NaDES formulations and the choice of the most appropriate candidate for the final purpose.
2. A second ecotoxicological step regarding the evaluation of bio-stimulating or inhibitory effects of the selected NaDES formulations on various organisms occurring in the agricultural field.

Accordingly, the tests carried out and the materials used were divided into two interconnected sections.

3.1 Synthesis and selection of NaDES formulations

3.1.1 Materials

All solvents and chemicals used in this study were obtained from Sigma–Aldrich (purities > 98%) and used without further purification.

The grape pomace and seeds biomass used comes from *Vitis vinifera* cultivations and were supplied by Caviro Distillerie s. r. l.

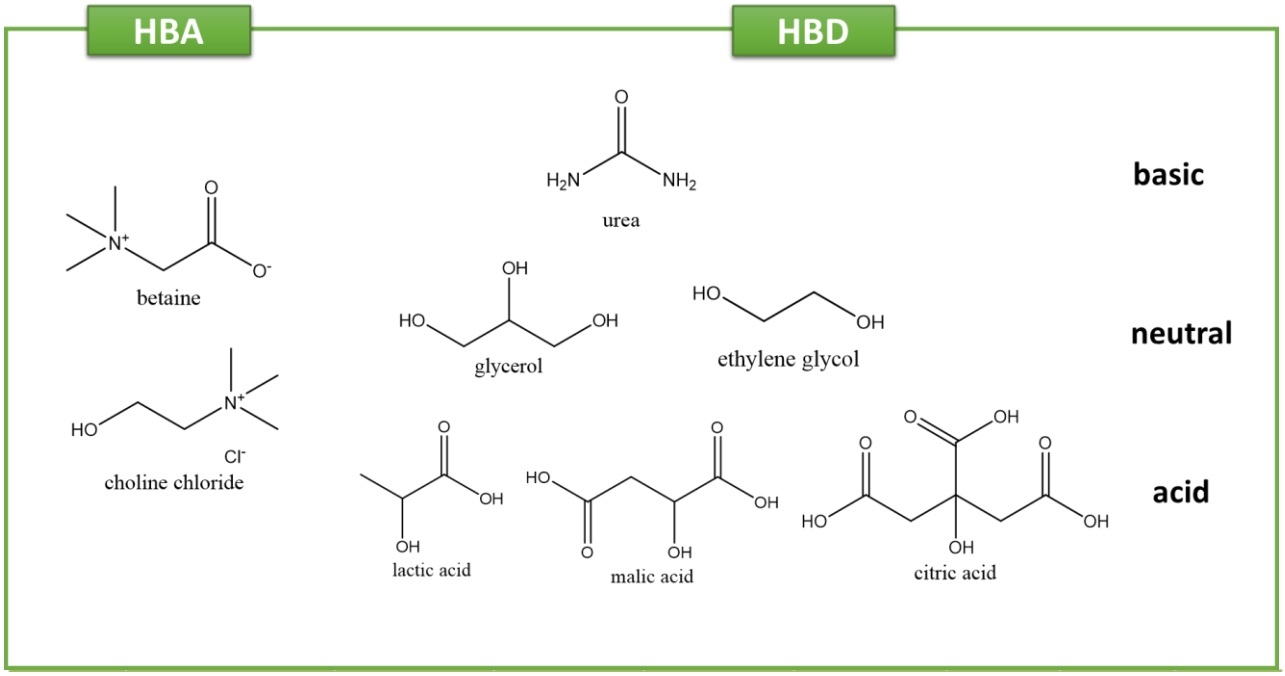
The freeze-dried biomass of the microalgae *Arthrospira platensis* and *Phaeodactylum tricornutum* were supplied by the Algology laboratory of the Department of Geological, Biological and Environmental Sciences (University of Bologna, Ravenna campus).

3.1.2 NaDES preparation

NaDES were prepared by mixing choline chloride (ChCl) or betaine (Bet), as HBA, with different HBD such as citric acid (CA), malic acid (MA), lactic acid (LA), urea (U), glycerol (G) and ethylene glycol (EG), in the required amounts, to obtain an adequate molar ratio (*Table 8*).

For each mixture, the two-components were transferred to a glass vial and heated to ~ 90 °C, with constant stirring, for the time required until the formation of a clear liquid (about 15 min). The mixture was allowed to cool down to rt. Subsequently, a quantity of water equal to the 40% weight of the final mixture was added.

Table 8. Molar ratio and weight of the components that form the different NaDES



NaDES	HBA	HBD	molar ratio	mmol HBA	mmol HBD	HBA (g)	HBD (g)	H_2O (g)
Bet+CA	Betaine	Citric acid	1:1	10,24	10,24	1,20	1,97	2,11
Bet+MA	Betaine	Malic acid	1:1	10,24	10,24	1,20	1,37	1,72
Bet+LA	Betaine	Lactic acid	1:1	10,24	10,24	1,20	0,92	1,42
Bet+U	Betaine	Urea	1:2	10,24	20,49	1,20	1,23	1,62
Bet+G	Betaine	Glycerol	1:2	10,24	20,49	1,20	1,89	2,06
Bet+EG	Betaine	Ethylen glycol	1:2	10,24	20,49	1,20	1,27	1,65
ChCl+CA	Choline chloride	Citric acid	1:1	8,59	8,59	1,20	1,65	1,90
ChCl+U	Choline chloride	Urea	1:2	8,59	17,19	1,20	1,03	1,49
ChCl+G	Choline chloride	Glycerol	1:2	8,59	17,19	1,20	1,58	1,86
ChCl+EG	Choline chloride	Ethylen glycol	1:2	8,59	17,19	1,20	1,07	1,51

3.1.3 Extraction and determination of the polysaccharide fraction

The method by Myklestad (1972) was used to extract the cellular polysaccharides from the starting dried biomasses. For each analysed biomass, samples of 10 mg were weighed and subsequently incubated with 1 mL of H_2SO_4 (12 M) at 37 °C for 1 hour (*Fig. 24*a, *Fig. 24*b). Afterwards, each sample was diluted with distilled water to reach a total volume of 11 mL and then mixed on vortex for 30 seconds. The samples were again incubated at 100 °C for 2 hours and subsequently cooled in ice for 30 minutes (*Fig. 24c*). After being centrifuged at 3000 xg for 10 minutes the supernatant was isolated and analysed to determine the concentrations of the polysaccharides.

The determination of polysaccharides was carried out on pure NaDES, NaDES extracts and biomass extracts following the colorimetric method by Dubois [133] . In the presence of

concentrated H_2SO_4 the polysaccharides form furfatals that condenses with phenol giving coloured products (*Fig. 24d*). Consequently, the intensity of the coloration changes linearly with polysaccharides concentrations. 100 µL of the supernatant were placed in glass tubes and 1 mL of phenol solution 2,5% and 2,5 mL of H_2SO_4 96% were added. After 30 minutes, a spectrophotometric measurement at a wavelength of 490 nm was carried out. The concentration of polysaccharides in the sample was obtained based on a glucose standard curve calibration.

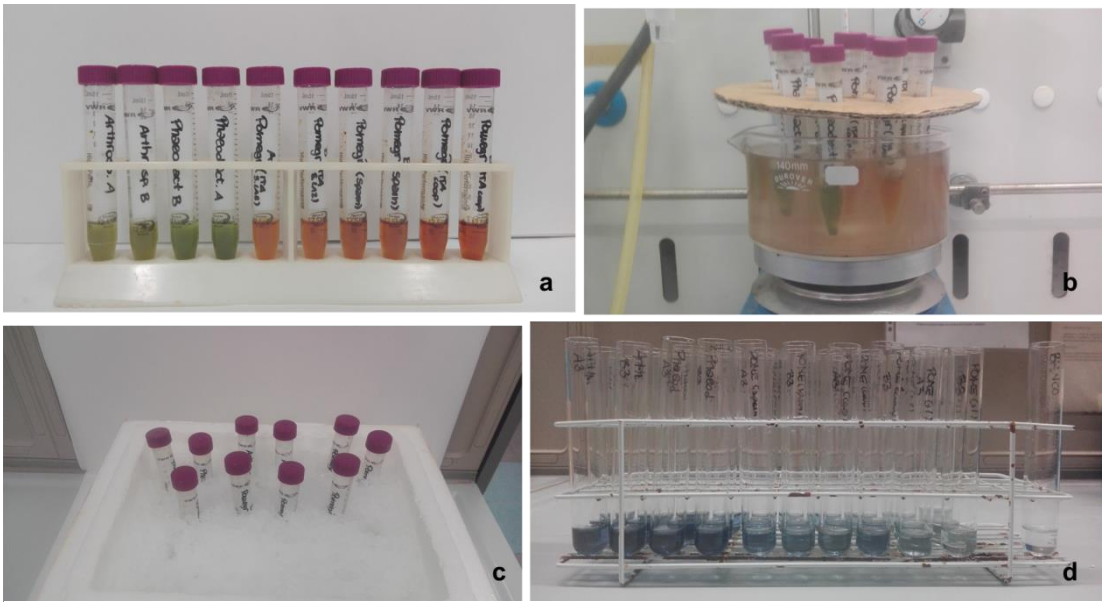


Fig. 24. Main steps required to extract and determin the polysaccharide fraction from dried biomasses.

3.1.4 Extraction and determination of the protein from dried biomass

To assess the protein content in natural biomass 10 mg of the samples were placed in 15 mL tubes where 3 mL of NaOH (0,5 M) were added. In order to facilitate cell breakage, the solution was agitated on vortex for few seconds. Subsequently, the samples were incubated at 90°C for 8 minutes and then cooled in ice for 2 minutes. The samples were then centrifuged at 3000 xg for 10 minutes at 4°C. The supernatant was moved to a new 15 mL tube. These extraction steps were repeated 2-3 times to optimize the result.

Lowry method [134] was used to determine the protein content in pure NaDES, NaDES extracts and biomasses. To each sample a NaOH solution enough to reach a volume of 10 mL was added. Three solutions were subsequently prepared:

- solution n°1: 5 g of Na_2CO_3 in 50 mL of NaOH 0,5M
- solution n°2: 0,5 g of tartrate NaK in 50 mL of H_2O

- solution n°3: 50 mg of $CuSO_4$ in 10 mL of "solution n°2"

Based on these solutions were prepared:

- solution A: 20 mL of solution 1 with 1 mL of solution 3
- solution B: 6 mL of Folin reagent (mainly consisting of phosphomolybdate, phosphotungstate and copper salts, with amino acids containing aromatic rings) with 54 mL of distilled water.

1 mL of each sample (opportunely diluted with distilled water if required) was inserted into glass tubes and after was added 1 mL of "solution A". After 10 minutes 3mL of the "solution B" were added. After 30 minutes, spectrophotometers measurements were made with a wavelength of 750 nm. The calibration curve used was obtained with a standard bovine serum albumin (BSA).

3.1.5 Extraction and determination of the lipid fraction

The extraction of the lipid fraction was performed only on the dried algal biomasses using the Bligh et Dyer method (1959). 0,05 g of biomass and 20 mL of a mixture of dichloromethane and methanol in ratio 2:1 were placed in a round bottom flask and kept stirred at 80°C for 1.50 hours. The organic phase was then concentrated using rotavapor by evaporation of the solvent. The procedure was repeated twice to increase the extraction yield. The total quantity of extracted lipids was determined in a percentage ratio with respect to the weight of the analysed dry biomass.

3.1.6 Analysis of methyl ester of fatty acids (FAME)

2 mg of lipid extract were added with 0,4 mL of dimethyl carbonate, 0,1 mL of dimethoxy propane and 0,1 mL of potassium methoxide (1,2 M), the mixture was then placed under magnetic stirring for thirty minutes at 70°C. Subsequently 0,7 mL of MeOH-BF_3 were added and the sample was heated among 70°C for 30 minutes under magnetic stirring. The samples were cooled to room temperature for five minutes and then added 1 mL of hexane, 2 mL of an NaCl aqueous solution and 50 µL of internal standard (methyl nonadecanoate, 1000 ppm) were added.

Thus, a two-phase liquid separation was obtained; the supernatant, containing the lipid fraction as fatty acid methyl esters, was analysed by gas chromatography coupled with mass spectrometry (GC-MS).

The interpretation of the resulting chromatograms was performed in relation to the peak area of the internal standard, the resulting value has been converted and expressed as a percentage on dry weight of the lipid extract.

Fig. 25. vial containing the fatty acid extract, the two-phase separation is evident.

3.1.7 Extraction of polyphenols by conventional methods and NaDES

50 mg of grape by-products (red grape pomace, white grape pomace and grape seeds) were placed in glass tubes and extracted with 2 g of solvent (NaDES, water ethanol, water: ethanol 1:1 v/v). The mixture was stirred at room temperature for 24 hours. The samples were then centrifuged for a few minutes allowing the separation of the polyphenolic extract (supernatant) from the residual biomass. The supernatant was taken and subsequently analysed to determine the total polyphenolic content (TPC) and used in phytotoxicity tests with both oats and cress.

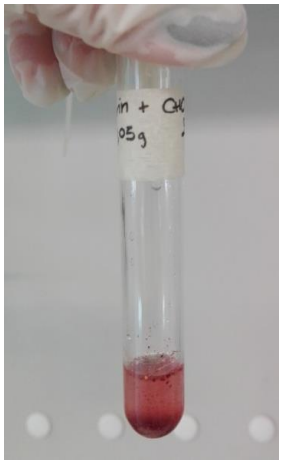


Fig. 26. Red marc extract with NaDES.

3.1.8 Algae extraction using NaDES

Both *A. platensis* and *P. tricornutum* were extracted by placing 0,1 g of biomass and 2 g of NaDES in centrifuge tubes. The samples were kept in constant stirring at room temperature for 24 h providing the rupture of algal cell walls. Unlike the grape by-product biomass, the centrifugation was not carried out because this procedure generated a scarce separation of the extract from the residual biomass. The whole mixture produced was used as a bioactive formulation in phytotoxicity tests both on oats and cress.

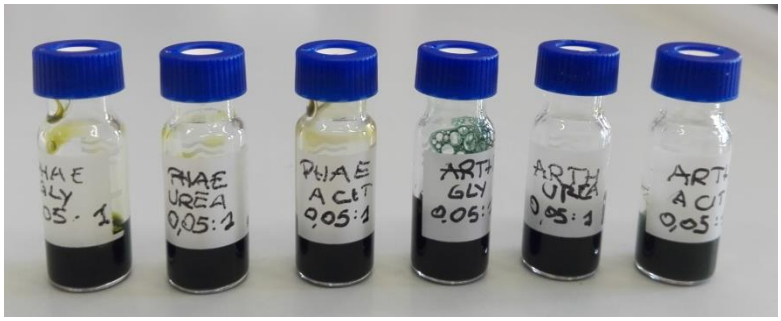


Fig. 27. Formulation based on algae biomasses

3.1.9 Determination of the total polyphenols component

The total polyphenol concentration was evaluated on 10 µL of extract from both conventional extracts and NaDES extracts. To this sample, the following solutions were added in sequence:

1. 190 µL of a 70% aqueous solution of acetone
2. 200 µL of Folin-Ciocâlteu reagent diluted with water in a 1:1 ratio
3. 4 mL of a 6% Na_2CO_3 aqueous solution.

The mixture was left in dark at room temperature for 30 minutes.

The spectrophotometric analyses were performed using a Jasco V-650 UV-vis spectrophotometer calibrated at 720 nm. The concentrations were referred to a calibration curve made with floroglucinol solutions (1,3,5-trihydroxybenzene).

3.2 Assessment of ecotoxicological properties

3.2.1 Artificial soil

Toxicity tests on the monocotyledon grass *Avena sativa* and on the earthworm *Eisenia andrei* were performed using the same artificial soil, which was prepared, in accordance with the OECD guideline 222 [135], with the following composition (based on components, dried to a constant weight at 40°C):

- 10% of sphagnum peat: Irish sphagnum peat purchased from Vigorplant Italia srl, Via A. Volta 2 - 26861 Fombio (LO). After being dried it was ground and sieved with a DARGENT SA cylinder mill with 2 mm mesh.
- 20% kaolin clay: washed and micronized kaolin purchased from Mineral Techniqa, F-8, Phase III, Riico Industrial Area, Beawar, Rajasthan 305901, India
- 70% quartz sand: fine Po river sand purchased from Bacchi, via Argine Cisa 19/A, 42022 Boretto (RE), dried and manually sieved with a 500 µm mesh sieve to reach the particles diameters required by the guideline (predominantly fine sand with more than 50% of the particles between 50 and 200 µm).

The dry constituents of the soil were mixed using a spatula inside plastic containers. 2 kg were the maximum amount of dry ingredients that could easily be mixed into the container to obtain a homogeneous final product.

Immediately before the start of each experiment, the dry soils were moistened by adding water to reach the final water content of 30 mL/100 mg dry soil, for the phytotoxicity test, and 50 mL/100 mg dry soil for the toxicity test with earthworms. The amount of water had to be such as to produce a substrate that was moist and suitable for the life of earthworms and the growth of plants but had no standing or free water when compressed in the hand.

The artificial soil was chosen as the best substrate to be used in all subsequent tests, for the following reasons:

1) The OECD Guidelines 222 [135] recommend the use of artificial soil for the earthworm reproduction tests, also the norm ISO 11268-2 [136] allowed the use of artificial soil to perform toxicity test on plants.
2) The use of artificial soil allowed comparison with other literature studies and between earthworms and phytotoxicity test with *A. sativa.*

3) An artificial soil is less prone the growth of plants not used in the test or organisms that could interfere with the investigated substances.

3.2.2 Earthworm reproduction test

The earthworm *Eisenia andrei* (Bouchè, 1972) was used to run a 56 days reproduction toxicity test according to the OECD guideline 222 [135] and the ISO standard 11268-2 [136]. The aim was to investigate the effect on soil fauna of six NaDES composed by betaine and choline chloride as HBA combined with citric acid, urea and ethylene glycol as HBD. Earthworms are widely used as model organism to assess soil ecotoxicity, because they are relevant and sensitive; in particular *Eisenia andrei* is often selected as the test species.

This test assessing lethal and sub-lethal effect over a period of eight weeks and was divided into two main steps:

1) Mortality and growth effects on the adult worms were determined after 28 days of exposure by counting the number of survivals and measuring their weight.
2) The adults were then removed from the soil and effects on reproduction were assessed after further 28 days of exposure based on the number and the weight of juveniles (offspring) and the number of laid cocoons present in soil.

3.2.2.1 Test organisms

E. andrei (Oligochaeta, Lumbricidae), the common "red" worm, are segmented worms, bilaterally symmetrical, with an external gland (clitellum), a pale-or dark-coloured swollen band located behind the genital pores, for producing the egg case (cocoon). In optimum conditions the length of their life cycles (from newly-laid cocoon through clitellate adult earthworm) ranges from 45 to 51 days. They are hermaphrodite animals, and reproduction normally occurs through copulation and cross-fertilization, following which each of the mated individuals produces cocoons containing 1-20 fertilized eggs (rate of cocoon production is 0.35–0.5 day^{-1}). The number of young earthworms hatching from viable cocoons varies from 2,5 to 3,8 depending on the temperature. Hatchling earthworms, unpigmented and only a few millimetres long when emerging from the cocoons, gain their adult pigmentation within a few days. Additionally, earthworms are considered excellent bioindicator of the quality of the soil as they are sensitive to a large number of contaminants, ubiquitous and in direct contact with the substrate in which they live. The ways of exposure of earthworms to the tested substances present in the soil are

predominantly by absorbing interstitial water directly through the cuticle-free body surface and to a lesser extent from the ingestion of soil and organic matter in it and from the interstitial air breathing [137].

Eisenia andrei were purchased from a verimiculturist (Lombricoltura Compagnoni, Mandello del Lario, (LC), Italy) and maintained in the laboratory for several weeks prior to running the experiments. The earthworms were allowed to acclimatize in the controlled temperature chamber settled with the same condition required for the test: temperature 20±1 °C, light-dark cycle 16:8 h, illumination 700 lx. The original breeding earthworms were selected, divided and settled in four 10 L tanks in order to guarantee the correct growth of the worms up to the size required to perform the test; 260 individuals were placed in each tank. The substrate was a mixture of 1 kg sphagnum peat, 1 kg natural soil, 1 kg distilled water. Earthworms were weekly fed with oat flakes and bran and the soil was aerated and moistened with distilled water until reached the optimal humidity. Earthworms selected for the experiments were adults, at least 8 weeks old, with a well-developed clitellum (a specific region of the worm body easily visually detectable) and of similar size.

3.2.2.2 Soil and tested substances

Accordingly to the OECD guideline 222 and norm ISO 11268-2, for the test was performed using the artificial soil described in paragraph 3.2.1.

Considering that NaDES were never tested on earthworm before and taking into account the long exposure times required by the assay, it was decided to test only one concentration of the highest possible number of substances instead of several concentrations of one or few substances. For the same reason only pure NaDES and no NaDES extracts were considered. The tested concentrations of NaDES were the equivalent of 4 g of HBA in 1 L of water. It is worth mentioning that the concentrations of NaDES used in these experiments could be greater than the concentrations that could affect the target organisms in the environment. In fact, in the field it is reasonable to assume a mode of exposition through foliar application in water solution, that could correspond to a lower NaDES dose/lower availability due to the presence of pore water and the interactions with the soil components. On the other hand, in this Thesis artificial soil was directly soaked with water solutions of NaDES.

For this test the amount of water needed to moisten sufficiently the volume of soil used for a single replicate was 200 mL. Therefore, considering this volume of water, the corresponding quantity of HBA was calculated and then, based on *Table 8,* the quantities of HBD and water needed to prepare NaDES were identified. In *Table 9* were showed the molarity (mM) of each NaDES to which the earthworms of a single replicate were exposed.

Table 9. NaDES used in the *Eisenia andrei* reproduction toxicity test. The table shows: the molar ratios between HBA and HBD and their respective molarity (mM), the quantities (g) of the individual components, the quantity (g) of NaDES in 200 ml of H_2O. NaDES are divided according to their reactivity.

NaDES	molar ratio HBA:HBD	HBA (mM)	HBD (mM)	HBA (g)	HBD (g)	H_2O (g)	NaDES in 200 mL H_20 (g)
Bet+CA	1:1	34	34	0,80	1,31	1,41	3,52
ChCl+CA	1:1	34	34	0,95	1,31	1,51	3,78
Bet+U	1:2	34	68	0,80	0,82	1,08	2,70
ChCl+U	1:2	34	68	0,95	0,82	1,18	2,96
Bet+EG	1:2	34	68	0,80	0,85	1,10	2,75
ChCl+EG	1:2	34	68	0,95	0,85	1,20	3,00

3.2.2.3 Preparation of the test

At the beginning of the experiment (day 1), 500 g of the artificial dry soil was placed in glass containers (20×2 cm, height 8 cm), noting the weight of the container both empty and full. Each container represented a single replica. The quantity of appropriate NaDES (*Table 9*) was weighed inside glass flasks, then it was made up to a volume of 200 mL with distilled water. Subsequently the solution was poured over the dry soil in the glass containers. The same volume of distilled water (200 mL) without NaDES was added to the controls. About 5 hours later, time required for the soil to completely moisten, the substrate was mixed in order to homogeneously distribute the NaDES solution in the soil. In each unit the food, prepared mixing 2 g of oat flour with 5 g of distilled water, was added.

All the treatments, control included, were performed with 4 replicates. Due to the large number of units, the entire test procedure was carried out on the first two replicates one day apart from the other two. An extra replicate for each treatment was prepared to assess the pH of the soil over all the period of the experiment. A control with clean soil (containing everything except test NaDES) was also included in the test.

3.2.2.4 Experimental exposure and life cycle endpoints measurement

Worms in good health condition and sexually mature (showing a well-developed clitellum) were taken from the rearing tanks, briefly immersed in water to remove soil, and then separated into groups of 10 individuals. Each group was weighed (live weight) and randomly assigned to an experimental container. The containers were then closed with transparent perforated polyethylene lids and placed in a thermostatic climate chamber under the controlled conditions previously described. The positions of the containers were systematically rotated twice a week.

The described procedure was performed for the containers used for the reproductive test and form those used for pH measurement. The day after the start of the exposure and then every two weeks, two earthworms were taken and frozen for future analysis and 100 g of wet soil were removed from each container established for the pH evaluation and set in a fridge of 4°C to prevent NaDES degradation.

Fig. 28. Containers used for the test placed in the thermostatic climate chamber.

Every week, throughout 28 days from the start of the experiment, the worms were fed with 2 g of oat flour suspended in 5 g of water. With the same frequency day water loss was checked by weighing the containers and replenished with distilled water, if necessary. Adults who died during this period were counted and removed from the containers to prevent interferences caused by the release of decomposition substances. In some cases, the decomposition state was advanced, and the number of dead worms was not accurate.

On the day 28 the first part of the test was concluded all adult worms were removed from the container and the following effects on the adult worms were measured:

- mortality (number of survivors out of the 10 exposed adult worms for each replicate);
- growth (total body weight of the survivors).

The same day the second phase of the test started. Food was provided to each containers and soil, containing any cocoons and juveniles that had been produced, was incubated for further 28 days at the same environmental conditions. For the next four weeks the food was not supplied, only water loss was checked weekly and restored when necessary by adding distilled water. On day 56, at the end of the experiment period, the effects on reproduction were assessed counting by hand sorting:

- the number of hatched juvenile earthworms;
- total dry weight of the juveniles, after 24 h at 60 °C;
- the number of hatched (empty) and unhatched (full) cocoons.

The number of cocoons escaped to the visual inspection of the wet soil was evaluated air drying the soil of each container for several days and then sifting the dry soil using a sieve with a 2000 µm mesh size.

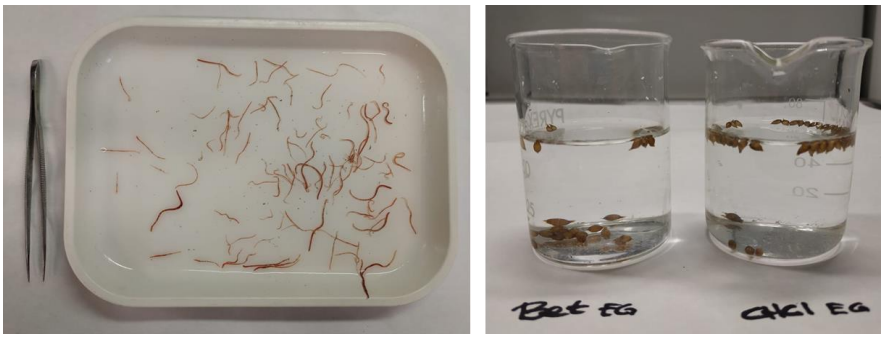


Fig. 29. On the left juveniles. On the right Unhatched cocoons (on the bottom of the becher) and hatched cocoons (on the surface of water)

Fig. 30. schematization of the main steps for the reproductive test on *Eisenia andrei*: day 0) preparation of the test; day 18) assessment of surviving adults and their removal from the containers; day 56) evaluation of the number of unhatched and hatched eggs, number and dry weight of juveniles.

3.2.3 *Lepidium sativum*: phytotoxicity tests in Petri dishes

3.2.3.1 Test organisms

Cress (*Lepidium sativum*, family Brassicaceae) is a dicotyledon annual herb about 15-70 cm tall, that produces red seeds of about 2 mm of size. *L. sativum* was chosen as model organism because is a sensitive test species recommended in in UNI 11357:2010 and widely used in the toxicity tests due to it is rapidly growing and because it is cheap and easy to analyse.

3.2.3.2 Tested substances

Growth and germination tests, assessing NaDES solutions phytotoxicity, were performed on cress seeds accordingly to the procedure described in UNI 11357:2010.

The principle for calculating the amount of NaDES used is the same as described in the paragraph 3.2.2.2. The only exception was NaDES formulations with algae: given the high amount of biomass contained in the extracts (equal to 10% of the final formulation) it was decided to use a concentration of algae formulation 10% higher than that of pure NaDES used in the same test. For the red grape-based formulations, only the supernatant of the extract was used, thus the quantity of biomass contained in the formulation was minimal, consequently the amount of formulation used in the test was equal to the quantity of pure NaDES. The same principle was used to identify the quantities of formulation to be used in the test with oat.

For each treatment to be tested, 50 mL of stock solution were prepared by diluting the pure NaDES and the NaDES formulations in distilled water (*Table 10*). Aliquots of 5 mL were then used for each replicate.

Table 10. NaDES and NaDES formulations used in the germination toxicity test with *Lepidium sativum*. The table shows: the molar ratios between HBA and HBD and their respective molarity (mM), the quantities (g) of the individual components, the quantity (g) of NaDES in 50 ml of H_2O. NaDES are divided according to their reactivity.

NaDES	molar ratio HBA:HBD	HBA (mM)	HBD (mM)	HBA (g)	HBD (g)	H2O (g)	NaDES in 50 mL H20 (g)
Bet+CA (high conc.)	1:1	34	34	0,20	0,33	0,35	0,88
Bet+CA (low conc.)	1:1	2	2	0,01	0,02	0,02	0,04
Bet+CA+pol	1:1	34	34	0,20	0,33	0,35	0,88
Bet+CA+Arth	1:1	34	34	0,22	0,36	0,39	0,97
Bet+CA+Phaeo	1:1	34	34	0,22	0,36	0,39	0,97
Bet+LA (high conc.)	1:1	34	34	0,20	0,15	0,24	0,59
Bet+LA (low conc.)	1:1	2	2	0,01	0,01	0,01	0,03
Bet+MA (high conc.)	1:1	34	34	0,20	0,23	0,29	0,71
Bet+MA (low conc.)	1:1	2	2	0,01	0,01	0,01	0,03
ChCl+CA	1:1	34	34	0,24	0,33	0,38	0,94
ChCl+CA+pol	1:1	34	34	0,24	0,33	0,38	0,94
Bet+U	1:2	34	68	0,20	0,20	0,27	0,67
Bet+U+pol	1:2	34	68	0,20	0,20	0,27	0,67
Bet+U+Arth	1:2	34	68	0,22	0,22	0,30	0,74
Bet+U+Phaeo	1:2	34	68	0,22	0,22	0,30	0,74
ChCl+U	1:2	34	68	0,24	0,20	0,29	0,74
ChCl+pol	1:2	34	68	0,24	0,20	0,29	0,74
Bet+EG	1:2	34	68	0,20	0,21	0,27	0,69
Bet+EG+pol	1:2	34	68	0,20	0,21	0,27	0,69
Bet+EG+Arth	1:2	34	68	0,22	0,23	0,30	0,76
Bet+EG+Phaeo	1:2	34	68	0,22	0,23	0,30	0,76
Bet+G	1:2	34	68	0,20	0,31	0,27	0,79
Bet+G+pol	1:2	34	68	0,20	0,31	0,27	0,79
Bet+G+Arth	1:2	34	68	0,22	0,35	0,30	0,87
ChCl+EG	1:2	34	68	0,24	0,21	0,30	0,75
ChCl+EG+pol	1:2	34	68	0,24	0,21	0,30	0,75
ChCl+G	1:2	34	68	0,24	0,31	0,37	0,92
ChCl+G+pol	1:2;	34	68	0,24	0,31	0,37	0,92
Bet		34		0,20			0,20
ChCl		34		0,24			0,24
EG			68		0,21		0,21

3.2.3.3 Experimental exposure

The tests were conducted by incubating 50 seeds of cress with 5 mL of test solution. The seeds and the tested mixture were positioned onto a Whatman cellulose filter paper disc and placed in a Petri dishes and sealed with paraffin film. All Petri dishes were incubated in a thermostatic climate chamber (21±1 °C) in absence of light for 72 h. Tests were performed in parallel with a control prepared with deionized water. Four replicates were used for each test substance and for the control.

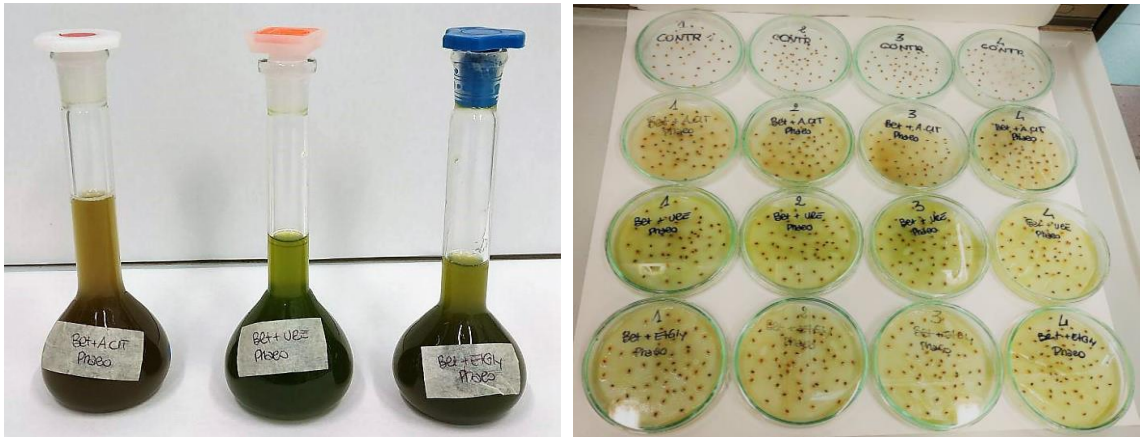


Fig. 31. NaDES solution based on NaDES algae formulations used in *Lepidium sativum* phytotoxicity tests (photo on the left). Petri dishes containing cress seeds and NaDES solutions

3.2.3.4 Phytotoxicity endpoints and statistical analysis

After three day of exposure the number of seeds germinated, root length and shoot length of seedlings were assessed.

For the germination assessment all the 50 seeds in each Petri dish were considered. Visible root development, regardless of length, was used as the operational definition of seed germination.

For measurements of growth endpoint in plants only 25 seeds for each Petri dish were evaluated. In case of all the seeds of a single Petri showed a roots or shoots length equal or inferior to 0,5 cm, for all the seeds, even if not germinated a length value equal to 0,5 cm, was assigned. In the case of to all the seeds of a Petri were assigned the same value of 0,5 cm, it was not possible to include the treatment in the statistical analysis (ANOVA) because of the absence of variance within the values. In this case the treatment was considered significantly different from the control and from all the other treatments. Apart from this exception, the same considerations described for germination parameter were used for the statistical analysis. For the statistical analysis the values measured on single seedlings within each Petri dish were averaged, thus the analysis was always based on four independent replicates per treatment.

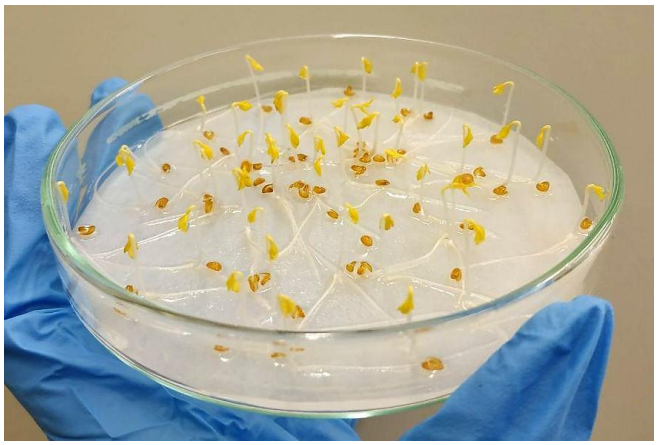


Fig. 32. Germinated seeds after three days of exposure, the picture reported refers to the control.

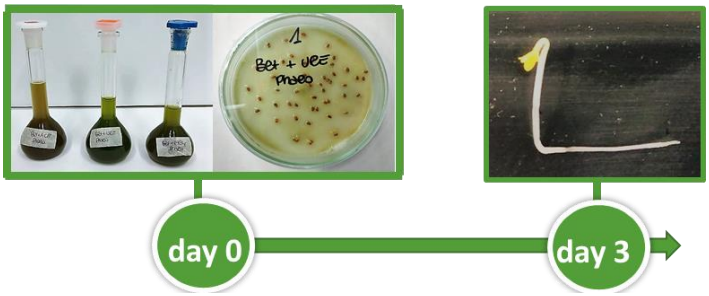


Fig. 33. Schematization of the main steps for the germination test on Lepidium sativum: day 0 preparation of the test day 18 evaluation of root and stem length and number of germinated seeds.

3.2.4 *Avena sativa*: early growth toxicity test in soil

Phytotoxicity of NaDES was assessed by the measuring the emergence and early growth of oat (*Avena sativa*) in soil, accordingly to the ISO standard 11269-2:2012 [138].

3.2.4.1 Test organisms

Seeds for oat were supplied by a local organic farmer (Forlì). *Avena sativa* was selected as model organism because was an important crop for food production and one of the test species recommended by the U.S. EPA, the OECD guidelines 208 (2006), and the U.S. Food and Drug Administration (U.S. FDA) [139,140]. This higher plant species is widely used as model organism in toxicity tests because its use in herbicide bioassays, heavy metal screening, salinity or mineral stress tests indicates its sensitivity to a wide variety of stressors.

Avena sativa L., commonly known as oat, is a monocotyledons annual grass, member of the Poaceae (Gramineae) family, that typically is from 0,70 to 1,25 m tall. The species is hermaphrodite (has both male and female organs) and is pollinated by wind. Oat is cultivated by spreading their edible seeds (botanically a type of simple dry fruit called a caryopsis), directly into the soil. Oats' plants prefer a poor dry soil and tolerate cool moist conditions and pH of 4.5 to 8.6 [141].

3.2.4.2 Soil and tested substances

All tests were performed using the artificial soil described in paragraph 3.2.1. Water solutions with pure NaDES, NaDES formulations and single NaDES components were assessed, each one in a single concentration. Considering that so far only two studies have tested the phytotoxicity of NaDES [77,129], it was preferred to evaluate only one concentration of the highest possible number of substances instead of several concentrations of one or few substances. Furthermore, considering that the aim of the in the present study was the evaluation of NaDES as system to deliver bioactive components, also NaDES formulations from algae and red grape pomace were tested, in order to evaluate their possible phytotoxicity as well as their potential stimulating effect of plant growth. The individual components of NaDES containing urea were also tested to evaluate if the cause of their evident phytotoxicity on plants should be referred to the single components or to the eutectic mixture. All the tested NaDES and the related concentrations or molarity used in each single vessel were shown in *Table 11*.

Table 11. NaDES, NaDES formulations and single components of NaDES used in the early growth toxicity test with Avena sativa. The table shows: the molar ratios between HBA and HBD and their respective molarity (mM), the quantities (g) of the individual components, the quantity (g) of NaDES in 60 ml of H2O. NaDES are divided according to their reactivity.

NaDES	molar ratio HBA:HBD	HBA (mM)	HBD (mM)	HBA (g)	HBD (g)	H_2O (g)	NaDES in 60 mL H_20 (g)
Bet+CA	1:1	34	34	0,24	0,39	0,42	1,06
Bet+CA+pol	1:1	34	34	0,24	0,39	0,42	1,06
Bet+CA+Arth	1:1	34	34	0,26	0,43	0,46	1,16
Bet+CA+Phaeo	1:1	34	34	0,26	0,43	0,46	1,16
ChCl+CA	1:1	34	34	0,29	0,39	0,45	1,13
ChCl+CA+pol	1:1	34	34	0,29	0,39	0,45	1,13
Bet+U	1:2	34	68	0,24	0,25	0,32	0,81
Bet+U+pol	1:2	34	68	0,24	0,25	0,32	0,81
Bet+U+Arth	1:2	34	68	0,26	0,27	0,36	0,89
Bet+U+Phaeo	1:2	34	68	0,26	0,27	0,36	0,89
ChCl+U	1:2	34	68	0,29	0,25	0,35	0,89
ChCl+pol	1:2	34	68	0,29	0,25	0,35	0,89
Bet+EG	1:2	34	68	0,24	0,25	0,33	0,82
Bet+EG+pol	1:2	34	68	0,24	0,25	0,33	0,82
Bet+EG+Arth	1:2	34	68	0,26	0,28	0,36	0,91
Bet+EG+Phaeo	1:2	34	68	0,26	0,28	0,36	0,91
ChCl+EG	1:2	34	68	0,29	0,25	0,36	0,90
ChCl+EG+pol	1:2	34	68	0,29	0,25	0,36	0,90
Bet		34		0,24			0,24
ChCl		34		0,29			0,29
U			68		0,25		0,25
EG			68		0,25		0,25

3.2.4.3 Experimental exposure

Plastics pots with a top internal diameter of 10 cm were used as tests containers. A filter disk was settled at the bottom of each pot to prevent loss of water, soil or growth of roots through the holes. Subsequently, all the pots were filled with 200 g of artificial soil. The solutions were prepared weighing an adequate quantity of NaDES (*Table 11)* and after making up to 60 mL with distilled water. 60 mL represented the quantity of solution that was evaluated by preliminary tests as adequate to moisten the artificial soil as required by OECD guideline 222 and in accordance with ISO 11274 (3.2.1). The solution was poured over the dry soil contained in each vessel. The same volume of distilled water (60 mL) without NaDES was added to the controls. About 5 hours later, time required for the soil to completely moisten, the substrate was mixed in order to homogeneously distribute the solution with the NaDES in the soil. Immediately after, ten uniform seeds, were planted into 10 mm deep holes previously drilled in the soil. Under each pot a plastic petri dish was placed as a saucer, to prevent any excess solution or water from leaking through the holes

on the bottom of the pot, potentially removing part of the NaDES from the ground. Each treatment was prepared in four replicates. Every single replicate was weighed together with the saucer, and throughout the whole test period every two days, each vessel was manually watered until reaching the weight noted on day 0. In this way soil moisture was adjusted to maintain the predetermined value throughout the exposure period. The pots were placed in a controlled temperature chamber settled at 20 ± 1 °C with a light-dark cycle 16:8 h and with illumination of 7000 lx (ISO 11268-2:2012). The position of the vessels was systematically rotated twice a week.

To compensate for non-germinating seeds, a higher number of seeds were planted in each pot than plants required for the test. After 50% of the seedlings in the control have emerged, for *Avena sativa* about three to five days, emergence rates were determined, and seedlings were pulled out to give a total of five evenly spaced representative specimens of the plants in the pots. Indeed, it was important that the density of plants in a test vessel did not limit normal growth. The test terminated 14 days after having thinned out the seedlings. Each plant was then gently separated from the ground by manual manipulation and then washed with water to eliminate eventual residual soil in the roots. For each treatment and on each vessel the following endpoints were detected:

- Percentage of germination, data were expressed as relative percentage of germination (germination (%)) with respect to the control (deionized water). A seed was considered to have germinated when shoot sprouts were observed.
- Shoot length of each of the five seedlings in the single pot.
- The biomass of the shoots expressed as dry weight per pot. The shoots of the five seedlings were placed in aluminium containers and dried in an oven at 60°C until constant weight was reached (about 48 h).
- Visual observation of phytotoxicity effect or anomality of shoot (e.g. depigmentation, wilting, leaf and stem deformations).
- Visual observation of phytotoxicity effect or anomality of roots. The length and dry weight of the roots were not considered in the statistical analyses as they were considered excessively affected by measurements errors. In fact, the oats roots were very thin and tangled, therefore, despite the soil were removed manually, some roots could break and traces of soil remained in the sample, affecting the final result.

For the statistical analysis, the values measured on single seedlings within each pot were averaged, thus the analysis was always based on four independent replicates per treatment.

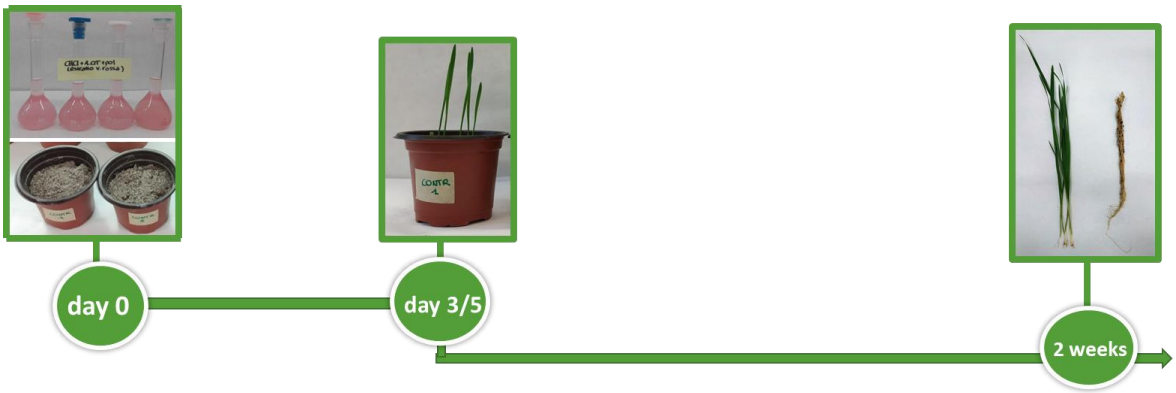


Fig. 34. Schematic representation of the main steps of the toxicity test with oats in soil: day 0) preparation of the experiment; day3/5) Thinning of the seedlings; 2 weeks) End of the test and evaluation of the endpoints.

The same procedure was also performed using cress seeds, this test was carried out to evaluate whether the toxicity shown by NaDES Bet+CA performed in Petri dishes was maintained even in soil. For this species the plant physiology allowed to evaluate the following endpoints:

- Shoot length of each of the five seedlings in the single pot.
- Shoot weight as average per pot.
- Root length of each of the five seedlings in the single pot.
- Roots weight as average per pot.
- For the statistical analysis, the values measured on single seedlings within each pot were averaged, thus the analysis was always based on four independent replicates per treatment.

3.2.5 Statistical analysis

The determination of statistically significant differences between treatment means was performed using one-way analysis of variance (ANOVA) followed by the Newman-Keuls post hoc test. The one-way ANOVA is used to determine whether there are any statistically significant differences between the means of two or more groups. Homogeneity of variance, a critical assumption of ANOVA, was checked through the Cochran test. One-way ANOVA detects whether at least one group is significantly different

from all the other being compared. Since in the present study it was necessary to compare more than two groups and understand which specific groups differed from each other, the Newman-Keuls post hoc test was performed too.

Differences between the means were considered statistically significant for p-values < 0,05. The error bars in the bar charts indicated standard error. In all figures, significant p-values of differences between any treatments and the control are marked with asterisks (*p < 0,05; **p < 0,01; ***p < 0,001). All the experiments were conducted in quadruplicate.

All the statistical analyses were performed using the software StatSoft Statistica version 10.

3.2.6 *Lactuca sativa* and *Ocimum basilicum*: phytotoxicity and water stress resistance assay

3.2.6.1 Phytotoxicity test

Lattuce (*Lactuca sativa L.)* and basil (*Ocimum basilicum L.)* were used as model organisms to test the safety and effectiveness of NaDES solutions. The plants already grown, about 10 cm height, were bought from a local plant nursery and placed in a semi-protected environment, in sunlight but sheltered from the rain. The plants were left in the same peaty soil in which they were purchased and irrigated during all the test period to maintain optimal moisture for plants growth. The NaDES solutions were produced by mixing the appropriate amount of pure NaDES or a NaDES formulation carrying polyphenols (*Table 12*) with 1 L of water. The quantity of NaDES was defined with the same principle described in the paragraphs 3.2.3 and 3.2.3.2. All tested NaDES were betaine-based NaDES combined with HBD showing different reactivity: three pure NaDES and three red grape pomace NaDES formulations. Each solution was then sprayed manually on the leaves of a group of six plants until dripping, two applications were carried out, one week apart. Tests were performed in parallel with plants growing in normal conditions (sprayed only with water). At the end of the experiment (day 14) the safety of products (absence of phytotoxicity symptoms) and the stimulus to the growth and development of plants in general were assessed through the evaluation of the following endpoints:

- Presence of residues of the NaDES solution on the leaf surface (an agronomic parameter for the qualitative evaluation of the product)

- Variations in the colour of the leaves compared to the control
- Differences in development compared to control
- Wet weight of the biomass obtained from the six vessels used for each treatment

On these endpoints no statistical analyses were performed but only visual observations.

3.2.6.2 Water stress resistance assay

Ocimum basilicum plants were used to test the capacity of NaDES as water stress resistance inducers. The plants used in this test were purchased from the same nursery previously mentioned. Basil plants were placed in a controlled environment with artificial lighting, a temperature of 25 °C and a relative humidity of 45%. The plants were left in the same peaty soil in which they were purchased. In order to test the resistance to water stress, each vessel was irrigated until saturation of the water capacity of soil (day 0) but after that the plants were no longer irrigated. For each treatment four vessels were used, for this test the solutions of pure NaDES and NaDES formulations previously described (*Table 12*) were applied in the same way as the phytotoxicity experiment. Starting from day 7, every two days, visual evaluations of the degree of wilting were carried out.
Both tests were performed by CBC (Europe) S.r.l.

Table 12. NaDES and NaDES formulations used in the toxicity tests and water stress resistance assay with *Ocimum basilicum and Lactuca sativa*. The table shows: the molar ratios between HBA and HBD and their respective molarity (mM), the quantities (g) of the individual components, the quantity (g) of NaDES in 1 L of H_2O. NaDES are divided according to their reactivity.

NaDES	molar ratio HBA:HBD	HBA (mM)	HBD (mM)	HBA (g)	HBD (g)	H_2O (g)	NaDES in 1 L H_20 (g)
Bet+CA	1:1	34	34	4	6,56	7,04	17,60
Bet+CA+pol	1:1	34	34	4	6,56	7,04	17,60
Bet+U	1:2	34	68	4	4,10	5,40	13,50
Bet+U+pol	1:2	34	68	4	4,10	5,40	13,50
Bet+G	1:2	34	68	4	6,29	6,86	17,15
Bet+G+pol	1:2	34	68	4	6,29	6,86	17,15

3.2.7 pH

Electrical conductivity (EC) and pH were both determined with a XS Instruments PC 70 combined meter. The pH and EC of the NaDES solutions applied in cress germination tests was directly measured by placing the glass electrode or conductivity probe into the suspension. These NaDES solutions were analysed before being applied on cress seeds but also at the end of the test by taking the solution with Pasteur pipettes directly from the

petri dishes. The pH of NaDES solutions used in early growth to toxicity tests with *Avena sativa,* before their application in soil, were tested in the same way. Instead the pH of soils recovered at the end of the same phytotoxicity tests and the pH of soils used for the reproductivity test with *Eisenia andrei* were performed accordingly to the EPA method 9045D [142]. To 20 g of soil were add 20 mL of distilled water in a beaker, cover, and continuously stir the suspension for 5 min. The soil suspension was let to stand for about 1 h to allow most of the suspended clay to settle out from the suspension. Then the suspension was centrifuge in centrifuge glass tubes and the pH and EC were measured by placing the glass electrode or conductometer into the liquid suspension phase.

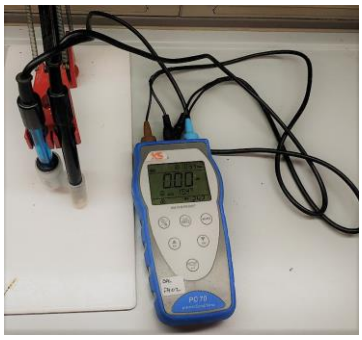


Fig. 35. pHmeter used to pH and EC evaluations.

3.2.8 Assessment of biodegradability

Three betaine-based NaDES were tested to assess their biodegradability:

- Bet+CA
- Bet+U
- Bet+EG

NaDES biodegradability was tested adding the test substances in mineral medium, that was inoculated with a wastewater microorganism from a mixed population. For this assay aerobic bacteria (activated sludge) were taken from the local purifier and set in the laboratory for 4 days at 20°C with continuous aeration. Fours stock solutions were prepared dissolving in water the following reagents:

a) 8,5 mg L^{-1} KH_2PO_4, 21,75 mg L^{-1} K_2HPO_4, 33,4 mg L^{-1} $Na_2HPO_4 \cdot 2H_2O$ and 0,5 mg L^{-1} NH_4Cl
b) 27,5 mg L^{-1} $CaCl_2 \cdot 2H_2O$
c) 22,5 mg L^{-1} $MgSO_4 \cdot 7H_2O$
d) 0,25 mg $L^{-1}$$FeCl_3$.

The mineral medium was prepared mixing 1mL of solutions (a) with 1mL of (b), (c) and (d) and then make up to 1 litre of water.

Each NaDES individually (see *Table 8* for the concentration) and 2,5 mL of activated sludge (diluted 1:10 with mineral medium) were make up to 250 mL with the mineral medium. A control with 2,5 mL of inoculum, without NaDES addition, was conducted as the oxygen blank, while as a reference substance glucose was used. The mixture was then packed in different capped bottles, all being stored in the dark at constant temperature 20 ± 1 °C. All samples were prepared in duplicate.

Table 13. Concentration of the tested substances

Tested substance	Concentration (mg/L)
Bet+CA	100
Bet+U	100
Bet+EG	60
Glucose	100

Fig. 36. Activated sludge from the local purifier

3.2.9 COD analysis

The biodegradation of the tested substances was assessed by measuring the COD (Chemical oxygen demand) of the mixtures every 2 or 5 days. The test took 18 days (time required to degrade substances over 90%). Biodegradability, corrected for values of the blank inoculum run in parallel, was expressed as a percentage of COD. COD of the samples was measured by thermal oxidation at 1200°C with detection of the oxygen

consumption using a COD analyser QuickCODLab (LAR Process Analyzer AG). 400 µL of the homogenized sample were injected directly into the reactor where it was completely oxidized at 1200°C under air/nitrogen flow, and continuously analysed with O_2 detector. The COD was calculated as g O_2 L^{-1} by comparison of signal areas, corresponding to the O_2 consumption in ppm, with those of known standard solution of glucose.

4 Results and discussions

4.1 NaDES preparation

The aim of this study is to develop no-hazardous NaDES formulations carrying bioactive molecules that can be applied in the agricultural field to promote advantages on crops as protectors against pathogens or growth biostimulators. Thus, NaDES were at first used as eco-sustainable solvents to efficiently extract bioactive molecules from different natural sources. By changing the HBA and HBD combination, it is possible to tune the polarity and reactivity of NaDES, and thus tuning the solubilization capacity of specific bioactive natural compounds.

The components and the molar ratios used in this study to synthetises NaDES are shown in *Table 8.* NaDES were selected according to literature researches and previous studies carried out in this laboratory which demonstrated their extractive efficiency [132].

Two naturally occurring HBA were used (*Fig. 37*):

- Choline chloride: the most used substance acting as hydrogen bond acceptor in NaDES synthesis. It was chosen because its proved ability to extract compounds from natural biomass. This quaternary ammonium compound occurs naturally in large quantities in plants and animal tissues, it is a constituent of membrane phospholipids and therefore is an essential metabolite in plants for growth and development [143].
- Betaine: a limited number of studies has tested this molecule as NaDES component, however it was demonstrated to form solvents with high stability. Betaine is a metabolite used by plants in defence processes against environmental stress, thus it was tested in this study to prove its potential added value as well as its extractive abilities [106,109].

Various natural/potentially bio-based HBD with different reactivity were selected to synthesise NaDES with the following behaviour (*Fig. 37*):

- Acid: citric acid, lactic acid, malic acid
- Basic: urea
- Neutral: glycerol, ethylene glycol

The mixtures were prepared by mixing the HBA with the required amount of HBD to obtain the eutectic mixture with the selected molar ration (1:1 or 1:2), which directly reflects the contribution of hydrogen bond donors and acceptors. Moreover, 40% by weight

of water was added to all NaDES. Literature demonstrates that hydrated NaDES have better extractive abilities than anhydrous, moreover water allows to reduce some NaDES drawbacks such as viscosity [112].

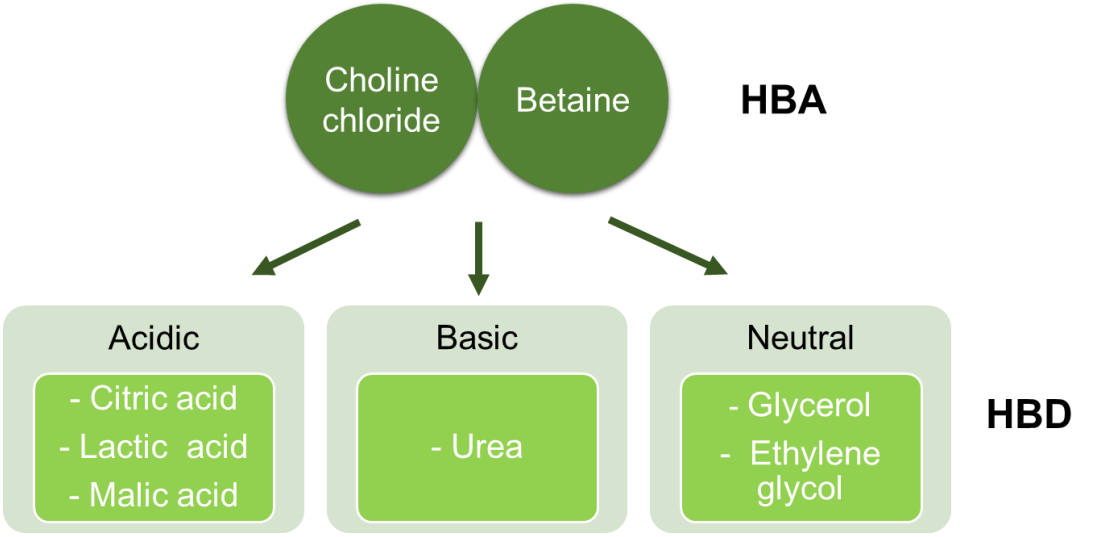


Fig. 37. List of hydrogen bond donors and hydrogen bond acceptors used for NaDES synthesis. HBD are grouped based on their reactivity.

Three main methods can be used for transforming solid state NaDES components into a liquid state eutectic mixture:

- Freeze-drying [144]: the components are dissolved in water and then subjected to freeze-drying
- Vacuum evaporation: the components are dissolved in water before being evaporated at 50°C with a rotary evaporator [96].
- Thermal mixing: the components are mixed at about 80-90°C on a hot plate with magnetic stirring until a homogeneous colourless clear liquid is formed. After cooling to room temperature, a transparent uniform mixture indicates that the designed NaDES has been obtained [76]. This method was selected for the present study

All NaDES synthesized in this work were stable and liquid at room temperature. The only exception was NaDES combined with lactic acid as a hydrogen bond donor, which was solid at room temperature. This was probably due to the molar ratio used (1:1), but no further investigations have been performed to verify this hypothesis.

Fig. 38. Schematization of the main steps required for the preparation of NaDES

4.2 Composition of the biomasses of interest

Two different biomasses were used for the extraction of bioactive compounds and the production of NaDES-based formulations: grape by-products of wine industry and microalgae (*Fig. 39*).

- White grape pomace, red grape pomace and grape seeds from *Vitis vinifera* were selected thanks to their large amount of polyphenols, compounds that have been proven to enhance plants stress resistance and growth. More generally, polyphenols represent the third most abundant constituent in grapes, after carbohydrates and fruit acids [118].
- *Phaeodactylum tricornutum* and *Arthrospira platensis* are two types of microalgae and could play an innovative role in agriculture as biofertilizer and resistance inductors, like already-in-use macroalgae (e.g. *Ascophyllum nodosum*) [145].

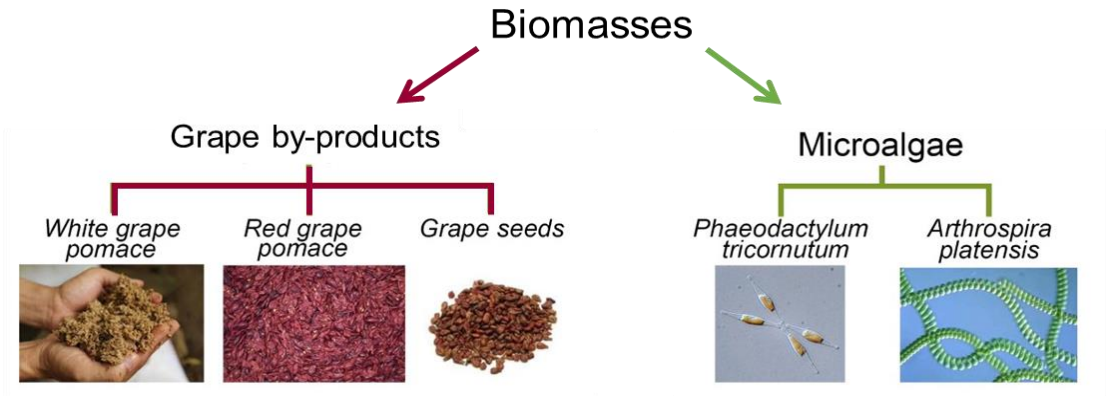


Fig. 39. Biomasses tested in this work.

Since these biomass can vary in composition, according to the conditions of algal cultivation (temperature, nutrients, light:dark rate, cultivation system) and to grape's variety, location of production and harvesting season, an initial characterization of their

biochemical composition was performed in terms of proteins, polysaccharides, polyphenols and lipids.

4.2.1 Grape pomace and grape seeds

Pomace represents the remaining material containing seeds, skins and stems after wine grapes pressing. The grape pomace cell wall is a complex system composed of 30% of neutral polysaccharides, 20% of acidic pectin substances, 15% of insoluble proanthocyanidins, lignin, and less than 5% structural proteins and phenols [146]. Polyphenols have both hydrophobic aromatic rings and hydrophilic hydroxyl groups with the ability to strictly link to cell-wall polysaccharides and proteins by hydrogen bonds and hydrophobic interactions [147] [148]. In this study, both red and white pomace were evaluated, the colour reflects the original type of grape used.

Grape seeds represent about 5% of the berries weight, thus are produced in large quantities by the winemaking industry. This by-product has become a valuable raw material for extraction of polyphenols, due to their high concentrations in this matrix (5-8 wt% depending on the variety [149]). Apart from polyphenols, grape seed are rich in lipids.

4.2.1.1 Determination of the polysaccharide fraction

The intracellular polysaccharide content present in red and white grape pomace and grape seeds was directly quantified by the conventional colorimetric method of Dubois [133], a procedure based on the ability of polysaccharides to form furfural in the presence of concentrated sulfuric acid, which bonds to phenolic reagent giving coloured products.

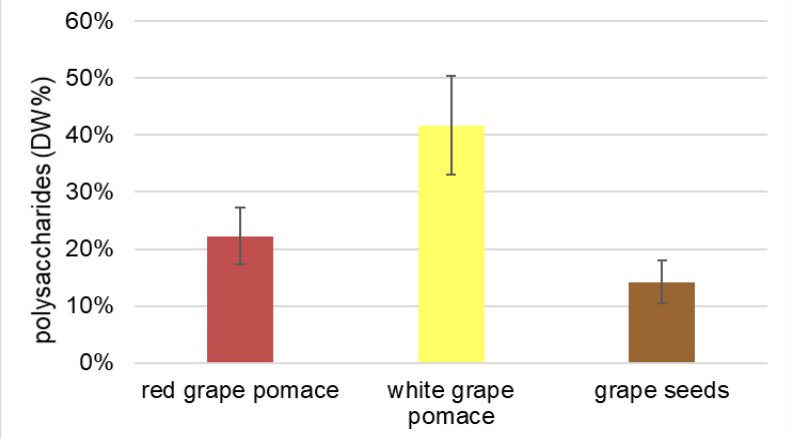


Fig. 40. Polysaccharides (dry weight percentage, DW%) present in grape by-products dried biomasses analysed. Values are means of three replicates ± standard deviation (SD).

As display in *Fig. 40* the three different matrixes analysed showed a content of polysaccharides by dry weight (PS DW%) ranging between 14,2±3,7% and 41,7±8,6%. While results obtained from red grape pomace (22,3±5,0%) and grape seeds (14,2±3,7%) were aligned with the ones obtained from literature, the white grape pomace showed a greater polysaccharide content (41,7±8,6%) [148].

4.2.1.2 Determination of the protein fraction

The proteins content within the dried biomasses was determined by applying the conventional Lowry method [134], based on a colorimetric reaction given by the Folin reagent with the amino acids contained in the aromatic rings.

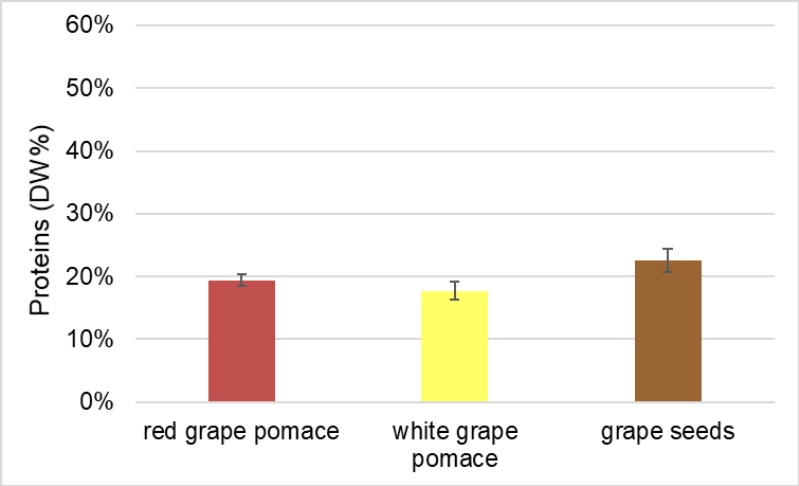


Fig. 41. Proteins (dry weight percentage, DW%) present in grape by-products dried biomasses analysed. Values are means of three replicates ± standard deviation (SD).

The grape pomace samples investigated had a protein content by dry weight (PR DW%) of 17,7±1,4% and 19,4±0,9% for red and white grape pomace respectively, and values of 22,6±1,9% for grape seeds *(Fig. 41).* The quantity revealed in this thesis were about twice as large as compared to the amount found in other studies: 8,49% 11% and 14% for red and white grape pomace and seeds respectively [150].

Compared to literature data, those differences in protein and polysaccharide composition can be attributed to two main factors:

- the analytical methods used to evaluate the compounds from biomass within this and other studies were not fully comparable.

- the biomass composition may differ considerably depending on the type of grape waste, the grape variety and climatic and cultivation conditions [146].

In this study the lipid content of grape by-product biomass wasn't evaluated because the polar nature of the NaDES wouldn't allow its extraction. Consequently, NaDES formulation from grape by-products doesn't contain the lipid as bioactive fraction.

4.2.2 Microalgae

In this study the blue-green cyanobacterium *A. platensis* (common name Spirulina) and the marine diatom *P. tricornutum* were used as biomass because of their documented bio-stimulating and bio-inducer abilities. The positive effect of biofertilization techniques using microalgae are mainly explained by the high content of growth regulatory compounds (Plant Growth Regulator) and the large portion of bioactive substances contained in their cells [151]. The abilities of those microalgae to induct resistance against some phytopathogen are based on substances with varied chemical nature (linoleic acid, oligosaccharin and chitosans among the other), but in general they are polysaccharides, lipids and proteins [152]. For example, it has already been demonstrated that polysaccharide containing sulphate or carboxylate groups (e.g. ulvans, fucoidans, alginates and carrageenan) extracted from macroalgae were able to stimulate the plant defence responses against pathogens and, thanks to their chelating properties, act as carrier of micro-nutrients in fertilizers [153].

4.2.2.1 Determination of the polysaccharide and protein fraction

The polysaccharides and proteins content present in *A. patensis* and *P. tricornutum* were quantified with the same colorimetric methods (Dubois [133] and Lowry [134]) used for the previous biomasses.

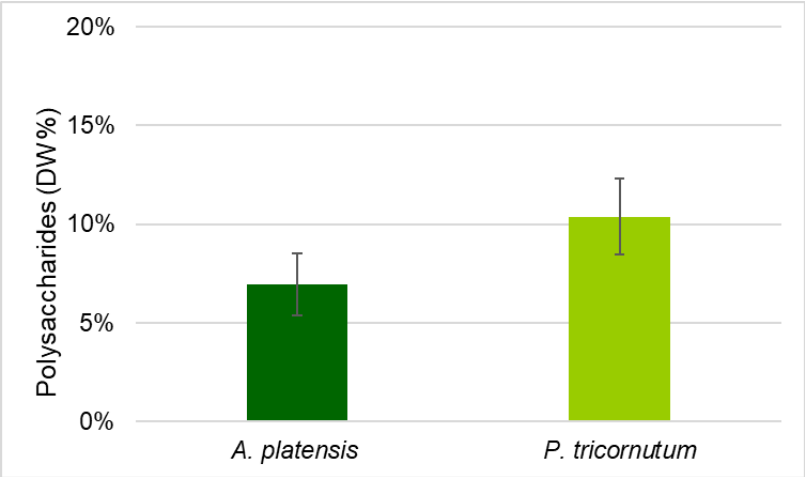


Fig. 42. Polysaccharides (dry weight percentage, DW%) present in *A. platensis* and *P. tricornutum* dried biomasses analysed. Values are means of three replicates ± standard deviation (SD).

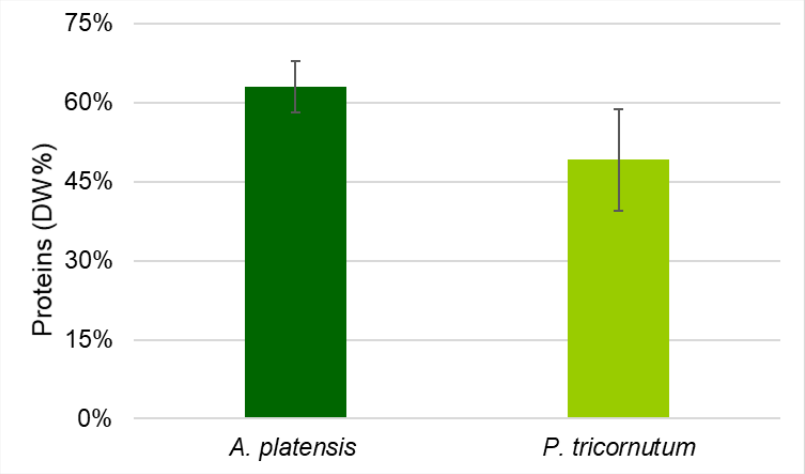


Fig. 43. Proteins (dry weight percentage, DW%) present in *A. platensis* and *P. tricornutum* dried biomasses analysed. Values are means of three replicates ± standard deviation (SD).

Fig. 42 and *Fig. 43,* show respectively the percentage of polysaccharides and proteins from the dried biomass (PS and PR DW%) of *A. platensis* and *P. tricornutum*:

- The analysis displayed that *A. platensis* and *P. tricornutum* possessed similar polysaccharide content (7±1,6% and 10±1,9%, respectively). These values are about half of that found in other studies, that reported about 20% of the total biomass in *A. platensis* and 36% in *P. tricornutum* [154,155], presumably due to different growth phases
- In both the algae protein represented a high percentage of the total biomass: in particular *A. platensis* showed a high protein content (63±4,9%) compare to *P. tricornutum* (49±9,6%), a trend confirmed by literature data. *A. platensis* is known

to be a high-protein algae, about 60%, while in *P. tricornutum* protein usually makes up only 26% of the dried biomass [154,155], a mean value lower than that found in this study.

This deviation from the literature may be due to the strong variability in the composition of microalgae under different cultivation conditions (temperature, light: dark ratio, nutrients and cultivation system).

4.2.2.2 Determination of the lipid fraction

The extraction of lipids was performed following the conventional methodology described by Bligh & Dyer (1959) based on the use of a mixture of chloroform and methanol. The extract obtained with this method consists of a mixture of components with different polarity such as triglycerides, free fatty acids, phospholipids, glycolipids, chlorophylls, sterols and phytols, and represents the percentage of lipids portion in comparison to the initial dried biomass determined by gravimetry (lipid content DW%)

To identify the fatty acid composition present in the extract, a GC-MS analysis was performed, and the percentage of total fatty acids present in the lipid extract (TFA DW%) and in the initial dried biomass (TFA biomass DW%), and their qualitative composition determined by GC-MS analysis

Table 14. Evaluation of the lipid portion of *A. platensis and P. tricornutum* on dry basis.

Algae	lipid content (DW%)	TFA (DW%)	TFA biomass (DW%)
A. platensis	26%	12%	3%
P. tricornutum	36%	37%	13%

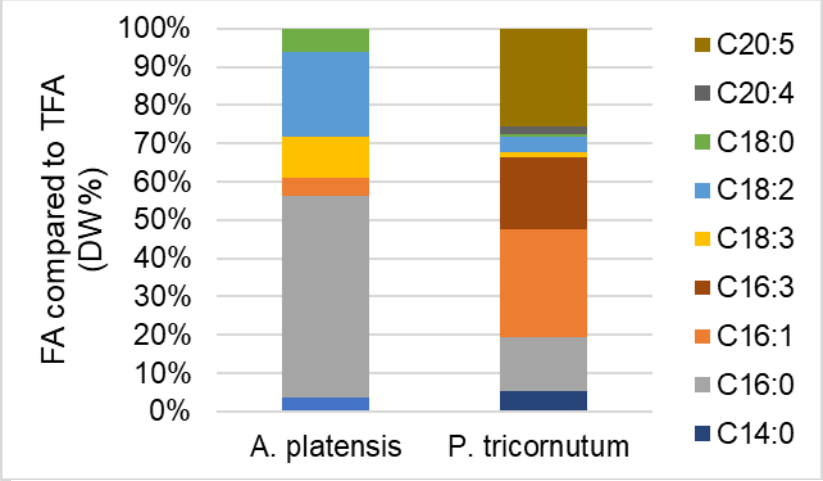


Fig. 44. Percentage composition of fatty acids (FA) compared to total fatty acids (TFA) present in the lipid portion of *A. platensis* and *P. tricornutum* dry biomass.

From analyses carried out on the lipid fraction of *A. platensis* were highlighted the following observations:

- The lipid component represents 26% of the dried biomass (*Table 14),* well above the 6% detected in literature. This high lipid content could be a consequence of microalgae stressed growth conditions, which led to a change in the metabolism and an accumulation of lipidic material [156].
- Fatty acids constitute 12% of the lipid fraction and 3% of the dried biomass. Their relative composition is not significantly different from the ones reported in literature [154,155]. The main fatty acids were palmitic (C16:0) and linoleic (C18:2), respectively 53% and 22% of total fatty acids (*Fig. 44)*.

Investigation of the lipid component of *P. tricornutum* biomass display that:

- The lipid component represents 36% of the total dried biomass (*Table 14*), almost twice the values of 18% reported in literature [155]. The reason could be related to the above-mentioned microalgae behaviour under stressed growth conditions [156].
- Fatty acids made up 37% of the lipidic extract and 13% of the dried biomass, of which the most abundant are palmitoleic (C16:1) and eicosapentaenoic fatty acids which are respectively 28% and 26% of total fatty acids (C20:5) (*Fig. 44).*

These results supported the used of *A. platensis* and *P. tricornutum* as started biomass for NaDES formulations synthesis, thanks to the great content of bioactive compounds in the form of polyphenols, proteins and fatty acids.

4.3 NaDES formulations

4.3.1 NaDES formulations from grape by-products

NaDES formulations carrying polyphenols were prepared by extracting the grape by-products biomasses using NaDES. The biomass and the solvent, in the ratio of 0,05 g : 2 g respectively, were constantly stirred for 24 hours, then the mixture was centrifuged to allow the separation of the residual biomass from the supernatant containing the polyphenols, which represented the final formulation used in this study. On contrary, as described in

paragraph *4.3.2*, in algae formulations the whole biomass was processed and maintained within the final formulation.

4.3.1.1 Selection of the biomass with the highest polyphenol content

The three grape by-product biomasses considered (red and white grape pomace and grape seeds) were evaluated with the aim to find the richest in extractable polyphenols, and therefore to find the best candidate for the synthesis of bioactive formulations.

The total polyphenols content expressed as percentage of the initial dried biomasses (TPP DW%) were evaluated on extracts performed by:

- Three conventional solvents: water, ethanol and a mixture of water and ethanol (1:1). In particular, the mixture of water and ethanol (1:1) should countian all the extractable polyphenolic components present in the various matrices. Thus, this value was considered as references of the total content of polyphenols in the analysed biomasses, namely, 100% of the polyphenols contained in the grape by-waste.
- Three NaDES: betaine-based NaDES combined with citric acid (Bet+CA, 1:1), urea (Bet+U, 1:2) and glycerol (Bet+EG, 1:2) as HBD were used as green extractants. While ChCl-based NaDES were already assessed to extract polyphenols efficiently, so far only one paper reports the use of betaine-based NaDES [157].

Usually, the polyphenols are extracted with conventional solvents such as ethanol, methanol, dichloromethane, acetone, hexane and ethyl acetate [158]. Although their extraction yields are high, some of these solvents are harmful both to human health and to environment. Otherwise, NaDES could be used at first as green solvents to extract the polyphenols efficiently from the biomasses and then directly applied the formulation carrying the bioactive compounds, thus bypassing the solvent removal and purification steps.

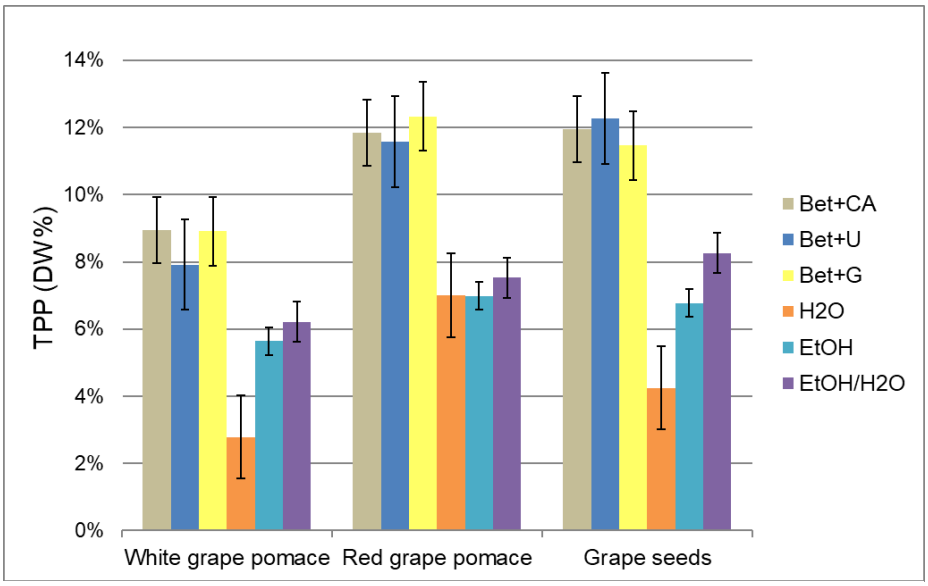


Fig. 45. Total polyphenols content (TPP, dry weight percentage DW% compared to the initial biomass) extracted from the dried biomass of three grape by-products using conventional methods (water, ethanol and ethanol/water mixture in a 1:1 ratio) and NaDES (Bet+CA, Bet+U, Bet+G). Values are means of three replicates ± standard deviation (SD).

The extractions were carried out at 2,5 wt% of biomass concentration. After stirring for 24 hours, the mixture was centrifuged and the supernatant, containing the polyphenols extracted, was analysed using Folin-Ciocâlteu colorimetric method and finally analysed with the spectrophotometer.

Fig. 45 shows the percentages of polyphenols extracted on biomass dried-weight basis (TPP DW%). From the analysis of the histograms it is possible to highlight some observations:

- As expected, among traditional solvents the following extraction efficiency trend was observed: H_2O < EtOH < EtOH/H_2O. The highest percentage of polyphenols (8,3÷0,4%) was extracted with EtOH/H_2O from grape seeds. (*Fig. 45)*.
- The polyphenols extracted from traditional solvents range between 2,8±0,4% to 6,2±0,8% in white grape pomace, from 7±0,4% to 7,5±0,8% in red grape pomace and from 4,2±0,4% to 8,3±0,8% in grape seeds. While the polyphenols evaluated in NaDES extracts range among 7,9±0,8 to 8,9±0,7%, from 11,6±1,3% to 12,30±1,5% and from 11,5±0,1% to 12,3±0,3% respectively for the above-mentioned biomasses. Generally, NaDES give extraction performance comparable with those of conventional protocols. The slightly higher values determined in NaDES extracts

could be due to the presence of biological interferents, such as proteins and polysaccharides, which interact with the Folin reagent used in the colorimetric method, producing a higher output when analysed by the spectrophotometer. Otherwise conventional solvents were probably not able to extract these biological interferents, and therefore showed lower values. These hypotheses are supported by the results displayed in *Table 15* and *Table 16*: both Bet- and ChCl-based NaDES combined with CA, U and EG showed high recovery efficiency of polysaccharides and proteins, ranging between 56-100% for polysaccharides and between 75-100% for proteins.

- White grape pomace possessed the lowest amount of polyphenols extracted within all the solvents used, both traditional and alternative. Those results are confirmed by literature: an experiment based on extraction assisted by ultrasonic evaluated the polyphenolic content in different wine by-products including grape pomace and seeds, was observed that generally the red grape varieties present higher values of total polyphenol content than the white one [159].

Bearing in mind these observations NaDES were considered promising materials to produce formulations carrying bioactive compounds from grape by-products, thanks to their extraction capacity comparable to those of traditional solvents. Moreover, according to literature [160] red grape pomace was evaluated as the richest biomass in extractable polyphenols and for this reason it was chosen as the best candidate to test the efficiency of NaDES as formulation media.

4.3.1.2 Polyphenols extraction from red grape pomace with different NaDES

Once the red grape pomace was identified as the biomass with the highest polyphenol content, it was used as candidate to further investigate the extractive capacities of different NaDES. For this purpose, eight hydrated NaDES were tested. HBD with different reactivity were selected:

- Acid: citric acid
- Basic: urea
- Neutral: ethylene glycol and in glycerol

Each of these hydrogen bond acceptors were mixed with both betaine and choline chloride as HBA. In this way it was possible to evaluate the effect of both HBD and HBA

on polyphenols extraction. All the extractions were performed as described in the previous paragraph.

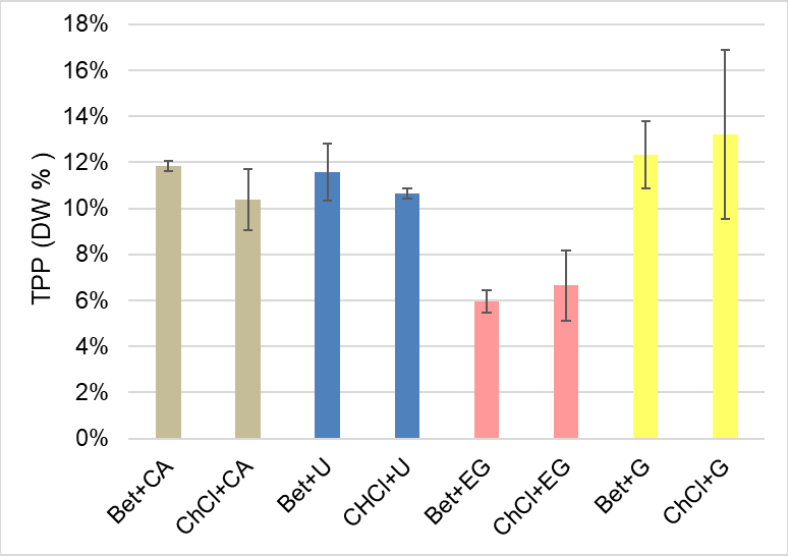


Fig. 46. Total polyphenol content (TPP, dry weight percentage, DW%) extracted by different NaDES from red grape pomace. Bars with the same colour refer to NaDES with the same HBD: acidic (gray_CA), basic (blue_U), neutral (pink_EG, yellow_G). Values

The extraction of the total polyphenols content (TPP) from the red grape by-product was performed in the same way described in paragraph 4.3.1.1. The values are expressed as a percentage of the treated dry biomass (DW%).

The analysis of *Fig. 46* highlighted some trends of NaDES behaviours:

- NaDES with the same HBD, regardless of whether they are betaine- or choline chloride-based, showed extremely similar extraction capacity:
  - Acidic NaDES showed a polyphenol content of 11,8±0,2% and 10,4±1,3% by dry biomass respectively form Bet- and ChCl-based NaDES, corresponding to 0,3% of polyphenols contained in NaDES formulations.
  - Basic NaDES combined with Bet showed a polyphenol content of 11,6±1,3% and a 10,6±0,2% when combined with ChCl, therefore both NaDES formulations with urea were composed for 0,3% of these bioactive components.
  - Neutral NaDES containing EG exhibited a polyphenol content of 6,0±0,5% (Bet-based) and 6,6±1,5% (ChCl-Based) by dry biomass, and in parallel a 0,2% and 0,2% in NaDES formulation. On the other hand, NaDES combined with glycerol

showed a 12,3±1,5% (Bet-based NaDES) and 13,2±3,7% (ChCl-based NaDES) of TPP referring to dried grape pomace, and respectively a 0,3% and 0,4% content in NaDES formulation (*Fig. 46*).

This trend (comparable extraction capacity performed by NaDES with same HBD) was exhibited by all NaDES with the same hydrogen bond donors, regardless of their reactivity. Therefore, could be assumed that the HBD was the solvent component that most influenced the extraction capacities. So far literature doesn't report studies testing the extraction activity of NaDES with different HBA but equal HBD, thus this hypothesis remains exclusive of this study.

- Both glycerol-based NaDES showed the highest extraction rates: 12,3±1,5% (Bet+G) and 13,2±3,7% (ChCl+G) by dry biomass (*Fig. 46*), therefore the NaDES formulations tested contained 0,38% of polyphenols (ChCl+G). Literature report an example where ChCl/G (1:2) NaDES gave the highest extraction yields of total phenolic compounds from olive oil. Another study testing the extraction of quercetin-3-Oglucoside (a phenolic compound) from grape skin reported that the best result was achieved with ChCl/G (1:2). In contrast, in the same article, the worst extraction yield of total phenols was obtained with ChCl/G, whereas the best extraction yield belonged to ChCl-based NaDES containing acid compounds as HBD (ChCl/malic acid, 1:2) [141]. Other study confirmed that the best extraction efficiency of anthocyanins (phenolic compounds) was obtained with acid-based NaDES: ChCl/malic acid (1:2), ChCl/oxalic acid (1:1) and ChCl/citric acid (1:2) [120]. These data indicated that NaDES with acids as hydrogen bond donors were the most suitable solvents to extract these phenolic compounds. The acidity of the solvents could be the key in the efficient extraction of anthocyanins because low pH is extremely important in the anthocyanin stability [110]. Results highlighted in *Fig. 46* and previously studies carried out on the same substances in this laboratory [132] were consistent with the literature results, showing high extraction yield of phenolic compounds with acidic NaDES.
- NaDES containing urea showed an effective extraction rates of 11,6±1,3% (Bet-based) and 10,6±0,2% (ChCl-based) by dry biomass, similar to those produced by acidic NaDES (11,8±0,2% and 10,4±1,3%) (*Fig. 46*). These results would be explained by the occurrence in red grape pomace biomass of polyphenolic compounds able to bind successfully with urea, thus allowing their accumulation in

the solvent. In order to confirm this hypothesis, further investigations would be necessary.

- Aligned with the results of previous studies carried out in this laboratory [132]. NaDES containing EG showed the lowest extraction yields: 6,0±0,5% and 6,6±1,5%, whit betaine and choline chloride respectively, that means that NaDES formulation used in this study contained a minimum of 0,2% of polyphenols (Bet+EG) (*Fig. 46*) . The comparison with *Fig. 45* highlighted that the percentages of total polyphenols extracted with NaDES containing EG were similar to those extracted with water (7±0,4%), ethanol (7±0,8%) and ethanol/water mixture (7,5±0,4%). As mentioned before, probably these lower values are due not to a lower extraction capacity of the bioactive compounds but to their poor ability to extract molecules able to infer with the colorimetric measurement and thus able to produce a higher final yield. This could mean a higher selectivity towards polyphenolic compounds than for other molecules

These results confirmed NaDES as suitable solvents for the extraction of phenolic compounds. After the comparison with literature data was evident that the heterogeneity of the phenolic compounds and the great variation of their extractive capacities with different NaDES, does not allow the definition of a specific solvent as the most appropriate for polyphenols extractions. The most suitable solvent can be defined empirically considering the specific phenolic composition of the biomass of interest. In this study, all NaDES considered as acidic, basic and neutral showed good and similar extraction capacity.

4.3.1.3 Recovery of proteins and polysaccharides by NaDES

The same methods used to calculate the protein and polysaccharide content in red grape biomass were used to characterize NaDES formulations. The results were expressed as a percentage of recovery (*Fig. 15 and Fig. 16*), namely the percentage of proteins and polysaccharides extracted from the red grape pomace dry biomass using NaDES, compared to the quantities extracted from the same biomass treated with traditional methods (*Fig. 40 and Fig. 41*), that conventionally represent 100% of the content in biomass. The values shown in *Table 15* and *Table 16* refer exclusively to the content of proteins and polysaccharides recovered from the red grape pomace biomass, actually the contributions made by pure NaDES (blank) were subtracted from the value of

the corresponding formulation. Recovery rates of polysaccharide performed by red grape pomace NaDES formulations.

Table 15 .Recovery rates of polysaccharide performed by red grape pomace NaDES formulations

NaDES formulation	Recovery %
Bet+CA+pol	64%
Bet+U+pol	100%
Bet+EG+pol	83%
ChCl+CA+pol	88%
ChCl+U+pol	71%
ChCl+EG+pol	56%

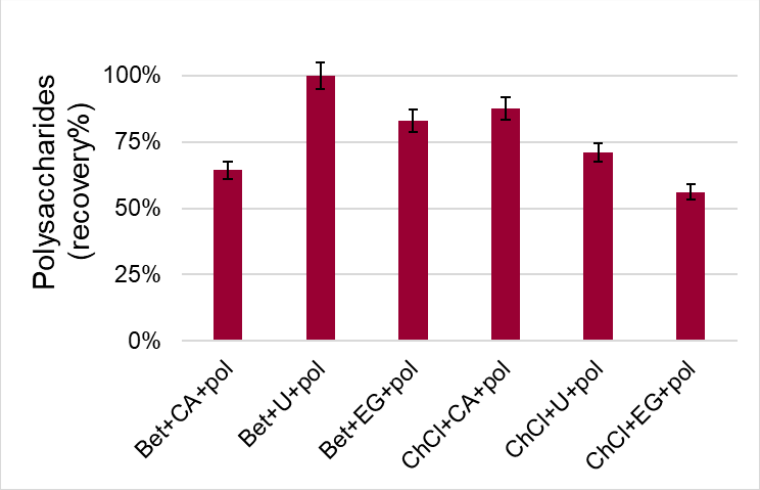


Fig. 47. Bars chart showing the recovery rates of polysaccharides performed by acidic (CA), basic (U) and neutral (EG) NaDES combined with Bet and ChCl.

Observing *Table 15* it is possible to draw some observations:

- NaDES formulation showed high recovery rate of polysaccharide that ranged from 56% (ChCl+EG+pol, showing the lower recovery%) to 100% (Bet+U+pol, exhibiting the higher recovery%). As provided by these results, most of the polysaccharides contained in the initial biomass were recovered and then accumulated in NaDES formulations.
- NaDES formulation containing the same HBD did not exhibit a well-defined recovery trend. Bet- and ChCl-based NaDES showed respectively a recovery percentage of:
  - 65% and 88% when combined with CA
  - 100% and 71% when mixed with U

- 83% and 56% when combined with EG

Based on these data, HBD did not affect the recovery rate of polysaccharides, but, apart from acid formulations, the percentages of recovery of Bet-based NaDES were slightly higher than those of ChCl-based ones.

Table 16. Recovery rates of proteins performed by red grape pomace NaDES formulations. ND: not detected

NaDES formulation	Recovery %
Bet+CA+pol	ND
Bet+U+pol	ND
Bet+EG+pol	ND
ChCl+CA+pol	100%
ChCl+U+pol	75%
ChCl+EG+pol	76%

Table 16, showing the proteins recovery percentage performed by the same NaDES formulation previously considered, highlight some main features:

- During the analytical protocol all the Bet-based formulations formed a precipitate that prevented the correct determination of proteins and therefore the percentage of recovery *(Fig. 48*).
- All the ChCl-based NaDES formulations showed high extraction efficiency: NaDES made up with urea and ethylene glycol showed a recovery rate of 75% and 76% respectively, while ChCl+CA NaDES extracted the totality of protein presented in the red grape pomace biomass that represented 19,4% by dry weight.

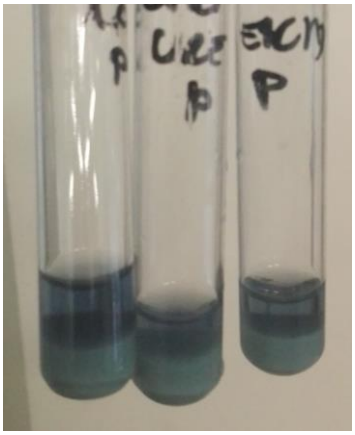


Fig. 48. Precipitate formed in all the betaine-based NaDES formulations during the protein determination.

4.3.1.4 Antioxidant activity of rad grape pomace NaDES extracts

Several plant diseases are related to oxidative stress, caused by an imbalance between antioxidant systems and production of oxidizing agents, including reactive oxygen species (ROS). Some studies have shown that biomass extracts with high levels of phenolic compounds and flavonoids have strong antioxidant activities. The by-products of the wine-producing activity have a considerable potential to be used as sources of natural antioxidants since they contain most of the polyphenolic compounds [159,161].

According to the literature, NaDES that contained individual components known for their antioxidative activity, such as malic acid, citric acid, proline and betaine, were also antioxidative [162]. For example, NaDES comprising betaine combine with malic acid and proline (1:1:1) have antioxidant activity. In support of this fact, literature studies have shown how the phytoextracts with NaDES have a greater antioxidant activity due to the presence of NaDES [163].

Considering what reported in the literature, the antioxidant activity of NaDES extracts from red grape pomace, namely the biomass from wine by-products that showed the highest polyphenols content, was determined in collaboration with the University of Torino, Italy, by using the DPPH 2,2-Diphenyl-1-picrylhydrazyl free radical protocol.

The evaluation of the antioxidant activity of these extracts was carried out with the aim of evaluate the potential of NaDES as a functional product enriched in polyphenols, useful for enhance plants defence from oxidative stress. The same DPPH assay was also performed on EtOH red grape pomace extracts as a reference conventional solvent (*Table 17*).

The antioxidant activity of NaDES extracts were performed by the DPPH assay. The DPPH (*2,2-Diphenyl-1-picrylhydrazyl*) free radical is one of the few stable organic nitrogen radicals, which bears a deep purple colour. This assay was based on the measurement of the reducing ability of antioxidants toward DPPH. The addition of an antioxidant results on a loss of DPPH, thus the percentage of remaining DPPH is proportional to the antioxidant concentration. The extract concentration needed to inhibit 50% of initial DPPH is defined as EC_{50}, *Table 17*). For the precise calculation of the EC_{50} a specific regression algorithm (Probit) was used and EtOH extracts were taken as EC_{50} reference. The parameter used to express DPPH assay results was TEAC index (Trolox Equivalent Antioxidant Capacity), where the antioxidant capacity of NaDES extracts was compared to that of the standard antioxidant Trolox (*6-hydroxy-2,5,7,8 tetramethylchroman-2-carboxylic acid*) an analogous

hydro-soluble of Vitamin E [164]. These measurements of antioxidant strength based on Trolox, were reported in units called Trolox Equivalents (TE). In this study the values expressed in equivalent Trolox were calculated using a calibration line constructed from the Trolox/DPPH interaction, moreover the TE values were normalized on 1mg/mL of NaDES extract (*Table 17*).

Table 17. Antioxidant activity results by DPPH assay on red grape pomace NADES extracts. For each extract were showed EC_{50} values (mg/mL) and the measure of antioxidant strength expressed as µg/mL Trolox Equivalents (TE) per 1mg/mL of extract.

NaDES extract	EC50 (mg/mL)	TE (µg/mL) (normalized on 1mg/mL extract)
Bet+CA+pol	3,7395	0,0503
Bet+U+pol	0,8793	0,1491
Bet+EG+pol	1,5012	0,1488
ChCl+CA+pol	3,4580	0,0691
ChCl+U+pol	13,7632	0,0230
ChCl+EG+pol	0,5635	0,1751
EtOH	1,5537	0,1480

- Considering all the samples, the two extracts with the best antioxidant activity (lower EC_{50}) were the ChCl+EG+pol (0,5635 mg/mL) and Bet+U+pol (0,8793 mg/mL) extracts, while the extracts with lower activity antioxidant (higher EC_{50}) were ChCl+U+pol (13,7632 mg/mL) and Bet+CA+pol (3,7395 mg/mL).
- Overall, considering the HBA of the extracts, the EC_{50} values show the following trend:
    - ChCl+EG+pol > EtOH > ChCl+CA+pol > ChCl+U+pol
    - Bet+U+pol > Bet+EG+pol > EtOH > Bet+CA+pol

  The comparison between these trends did not highlight a dominant tendency generated by HBD. Thus, HBD did not seem to influence the antioxidant power of NaDES pomace extracts. Moreover, these results weren't aligned with the extraction capacity of the total polyphenols previously evaluated (*Fig. 46*): for example, EG-based NaDES showed the lower extraction capacities of total polyphenols from the red grape pomace, while the antioxidant activity (EC_{50}) of the same extracts showed always medium-high values, in particular the extract based on ChCl+EG NaDES showed the highest values among all. Furthermore, the results do not agree with the literature data reporting the high anti-oxidant capacities of

acidic NaDES [162], which in this work showed the lowest antioxidant activities (3,7395 mg/mL and 3,4589 mg/mL) after ChCl+U+pol.

- Similarly, by the comparison between extracts with the same HBD was not evident a homogeneous trend confirming a greater antioxidant activity for betaine-based or choline chloride-based extracts.
- Extracts with EtOH showed an antioxidant activity only superior to NaDES with citric acid and to ChCl+U extract.
- All the observations outlined from EC_{50} data are consistent with the antioxidant activity results expressed as Trolox Equivalent (TE). In this case, however, the higher values of the extracts correspond to a greater antioxidant activity, while the lower values showed an inferior antioxidant activity.

In general, extracts with NaDES showed an interesting antioxidant performance, in some cases even higher than those of conventional solvents extracts. However, there wasn't a clear trend linking antioxidant capacities with a specific NaDES compounds, neither with the total polyphenol content extracted. Although the NaDES extracts richer in polyphenols confirmed their potential as antioxidant formulations, further studies are needed to better understand the source of this activity.

4.3.1.5 Grape by-products NaDES extracts as potential inhibitors of urease

Recent studies reported for the first time the ability of grape by-products NaDES extracts to act as inhibitors of the enzyme urease. These experiments were performed in collaboration with the Agricultural Department (University of Bologna) which carried out the analyses of anti-urease activity on extracts produced by the candidate in the laboratories of the Ravenna Campus (University of Bologna).

Urease is an enzyme produced by plant roots and soil microorganisms that is able to hydrolyse urea forming ammonia and carbon dioxide. An excess of this enzyme in soil can generate serious problems in the agricultural sector (by reducing the concentration of urea in the soil, one of the main fertilizers used in agriculture due to its ability to release nitrogen) and the environment (by releasing ammonia, a greenhouse gas, into the atmosphere). The NaDES formulation rich in polyphenols could potentially be used to inhibit the activity of the enzyme urease present in soils and reduce these issues.

For this purpose, betaine and choline chloride-based NaDES extracts carrying polyphenols and combined with citric acid, urea and ethylene glycol as HBA, were evaluated.

- Pure NaDES did not induce inhibition of urease enzyme activity, which showed 100% of the activity (*Fig. 49*, black bars).
- The results showed that NaDES formulations containing urea, combined with betaine and choline chloride, were not able inhibit enzyme urease (*Fig. 49a, Fig. 49b*), while betaine and ChCl-based NaDES formulation containing citric acid (*Fig. 49c, Fig. 49d*),and NaDES formulation made up with betaine and ethylene glycol (*Fig. 49e*),were able to reduce by about 50% of the urease activity. However, the most promising extract was ChCl+EG+pol, capable of inhibiting enzymatic activity by about 90% (*Fig. 49f).*

Further studies are required to evaluate the anti-urease activity of the extracts applied directly on the microorganism from which the enzyme has been isolated and in the agricultural field. It is necessary to evaluate whether this promising enzyme-inhibiting response produced by the NaDES extracts is preserved even when applied to other systems.

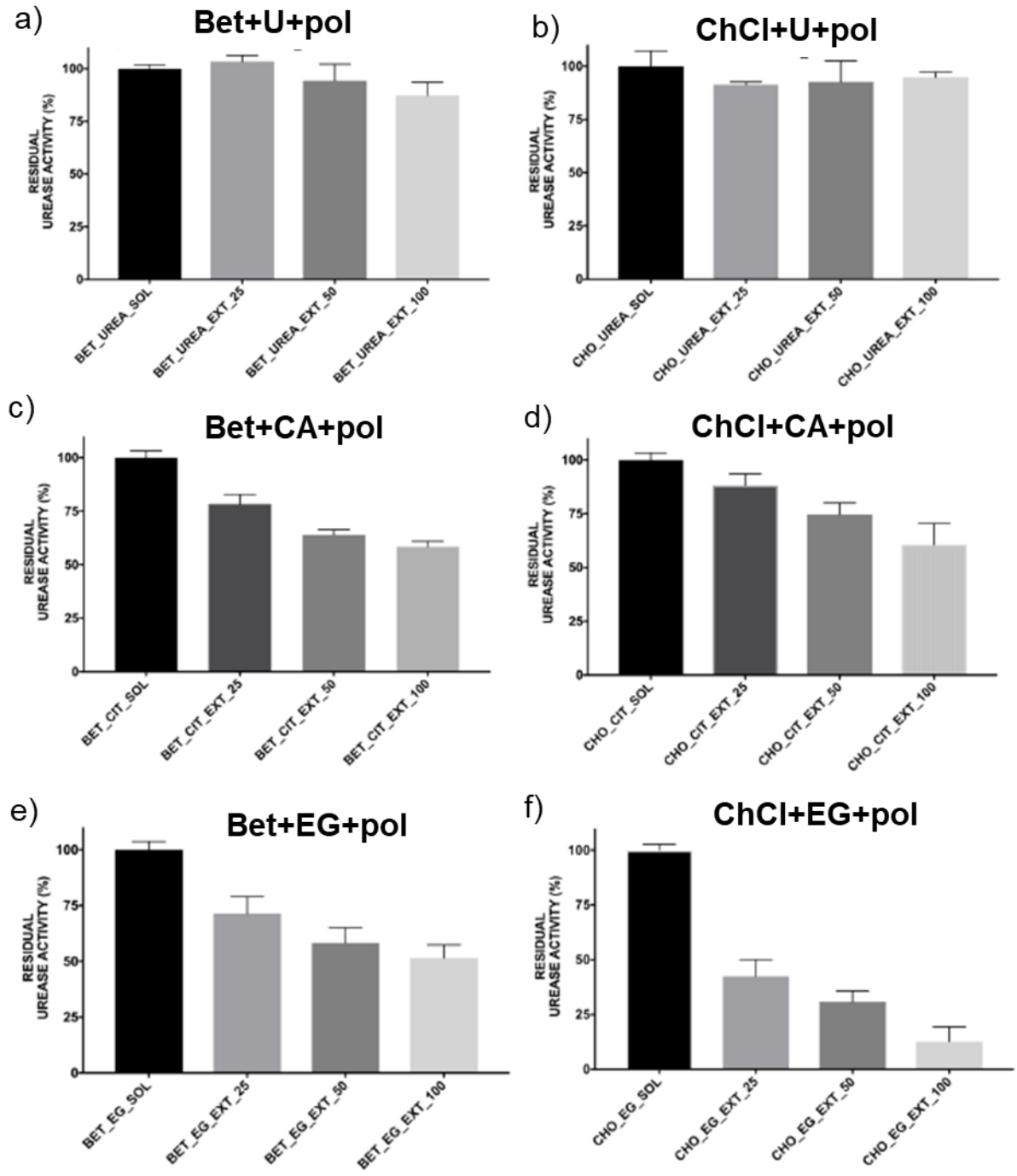


Fig. 49 Urease activity at increasing concentrations (25, 50 and 100 µL) formulations extracted with Bet- and ChCl-based NaDES combined with (a)(b) urea (c)(d) citric acid (e)(f) ethylene glycol (labelled as XXX_EXT in the bar charts). Data are shown as normalized values on the respective value of pure NaDES (XXX_SOL) and expressed as percentage. Results provided by the Agricultural Department (University of Bologna).

4.3.2 Algae: extraction and synthesis of formulation media with NaDES

Algae were selected as second natural matrix used in this study as beneficial component to produce bioactive formulations. Indeed, algae derivates are currently used word wide as useful agricultural products [165] because they contain a variety of bioactive compounds including polysaccharides (e.g *laminarin*), pigments (e.g carotenoids), lipids (e.g PUFA), phytohormones (e.g *ethylene*), vitamins and minerals that have been confirmed to have beneficial influence on crops: increased biomass production, improved resistance to environmental stress, increased photosynthetic efficiency and carbon assimilation, enhancement of plant defences against pests and pathogens and facilitated recovery from damages caused by insects, bacterial or fungi [166]. Microalgae (e.g *Arthrospira platensis*) [152] and macroalga [145] e (e.g *Ascophyllun nodosum*) [145] extracts have been tested to enhance crop resistance against pathogen and increase the yields of food production. Stimulation of roots growth, and the consequent increase in biomass, have been observed in *Arabidopsis sp.* after the application of very low concentrations of extract obtained from *Ascophyllun nodosum*. In particular, the highest effect was obtained when the extracts were applied in an early stage of growth. Foliar application of sewed extracts on soya resulted in higher yields, more vigorous growth and better nutrients uptake [167]. Recently a formulation for agricultural use derived from microalgae (e.g *Phaeodactylum tricornutum* and *Arthrospira platensis*) has been shown to be capable of controlling plants diseases by the induction of systemic resistance mechanisms against pathogens. The formulation contained the entire original content of polysaccharides of the microalgae which has been shown to have an important effect in plant defences responses. The test was performed on vine plants affected by *Plasmopara viticola* (peronospora) and showed an increase in the content of pathogenesis-related (PR) proteins, indicating an increased tolerance of vine plants against the pathogen and thus the potential for resistance induction provided by the product [152].

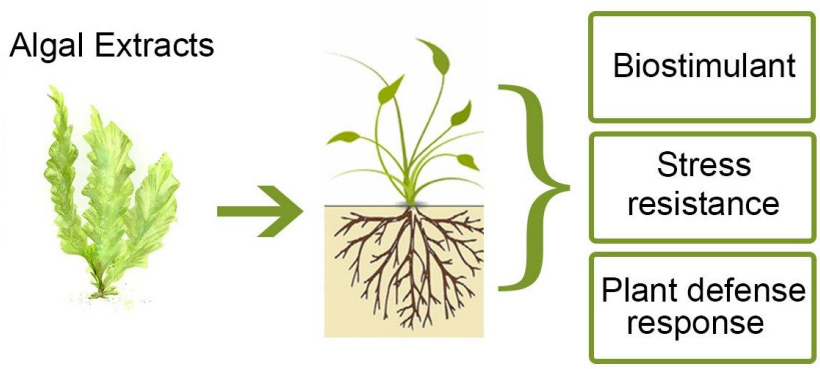


Fig. 50. Benefits provides by Seaweed extracts in plants and crops.

The microalgae *A. platensis* and *P. tricornutum* were selected as suitable species for NaDES formulations synthesis because of the promising benign effects tested on crops [152] and the well-known biology and system production [168,169].

NaDES with both betaine and choline chloride as hydrogen accept donor were selected as solvents, coupled with the following HBD:

- Acid: citric acid
- Basic: urea
- Neutral: ethylene glycol and in glycerol

Since the production of algal extracts with NaDES is an innovative process, a preliminary step to establish the most suitable concentrations for the synthesis the algae-based NaDES formulation was required. In this first phase the same biomass concentration (2,5 wt%) applied to extract grape by-products was used: The solution was kept under constant stirring for 24 hours to facilitate the breaking of algal cells. Subsequently the samples were centrifuged, but unlike what happened using grape matrix, the solution did not form two separate phases but showed only a slight separation (*Fig. 51*). The sample was then further filtered with cotton wool to separate the extract from the residual biomass, but this operation was not effective. The homogeneous products that were formed after NaDES extraction was probably caused by the strong interaction that occurred between the alga biological compounds and NaDES constituents. Once the cell was mechanically fragmented, both the intracellular and cell wall components were able to interact with the HBA and HBD creating a homogeneous and viscous solution.

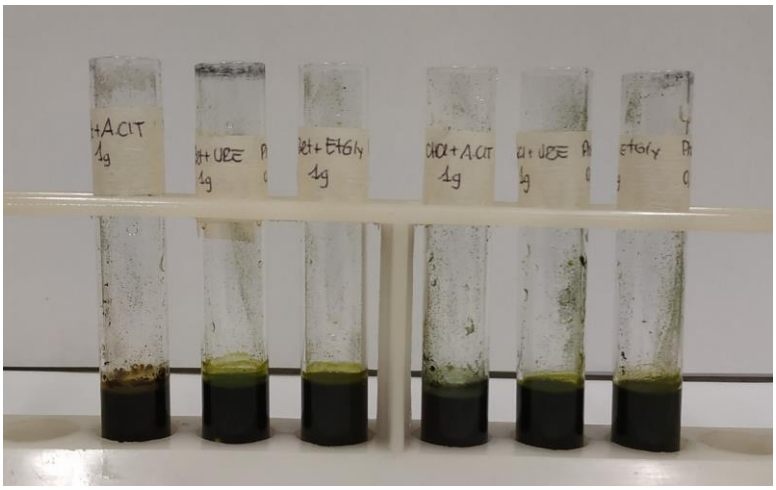


Fig. 51. Algal extract with betaine and choline chloride-based NaDES. All the tested NADES did not produce a clear separation into two distinct phases.

Based on these preliminary results it was decided to synthesise the algal-based formulations without separation, thus the whole algal biomass was kept maintained in the final material.

In the second steps, the most suitable biomass to solvent ratio was defined. Formulations containing 30, 20 and 10wt % of algal biomass with respect to the final product were produced. A concentration of 10 wt% was considered the most suitable to gain the purpose of this work.

Considering that the entire algal biomass became part of the final formulation, no further investigations were made to determine the extraction capacity of the different NaDES. For the same reason the content of polysaccharides, proteins and lipids extracted with NaDES was not carried out: the components and the concentrations that characterized the algal biomass were the same of the algal formulations.

4.3.3 Composition of NaDES formulations media

In the present study NaDES were used as delivery system to convey bioactive compounds useful for plants. Therefore, the composition of NaDES formulations media was reported as percentage of pure NaDES (NaDES), proteins (PR), polysaccharides (PS), polyphenols (PP) and lipids (LP). In this way was possible to correlate the benign or toxic effect tested on plants and animals, exhibited within the ecotoxicological assay, with a specific composition of the formulation applied.

Table 18. Composition of the red grape pomace NaDES formulations as percentage of polysaccharides (PS), proteins (PR), polyphenols (PP) and pure NaDES.

Components	**Red grape pomace NaDES formulations**					
	Bet+CA+pol	Bet+U+pol	Bet+EG+pol	ChCl+CA+pol	ChCl+U+pol	ChCl+EG+pol
PS	0,4%	0,6%	0,5%	0,5%	0,4%	0,3%
PR	ND	ND	ND	0,5%	0,4%	0,4%
PP	0,3%	0,3%	0,2%	0,3%	0,3%	0,2%
NaDES	99,3%	99,1%	99,3%	98,7%	99,0%	99,2%

Table 18 shows the composition of bioactive formulations produced by treating red grape biomass with betaine and ChCl-based NaDES combined with citric acid, urea and ethylene glycol. From these analyses some main features were highlighted:

- Undoubtedly most of the formulation was made of pure NaDES: the lowest value showed by ChCl+CA+pol represented 98,7% of the final formulation, while the highest percentage of pure NaDES were presented in Bet-based formulation media combined with U and EG, where it reached 99,3% of the global composition.
- The polyphenols, that were the bioactive compounds of greatest interest in these extracts, always represent 0,3% of the final formulation, apart from those containing EG in which they constitute 0,2%.
- The proteins content was homogeneous within ChCl-based extracts, where it represented 0,5% in the acid formulations and 0,4% in the basic and neutral systems. The protein composition of betaine formulations was not determined because of the precipitate formed during the samples' analyses.
- The polysaccharides were present into the different formulations in similar percentages: the lowest values were showed in ChCl+EG+pol (0,3%) while Bet+U+pol displayed the highest content (0,6%).

Table 19. Composition of the algae NaDES formulations as percentage of polysaccharides (PS), proteins (PR), lipids (LP) and pure NaDES.

Components	**Algae NaDES formulations**	
	A. platensis	*P. tricornutum*
PS	0,6%	0,9%
PR	6,0%	4,4%
LP	2,4%	3,3%
NaDES	91,0%	91,4%

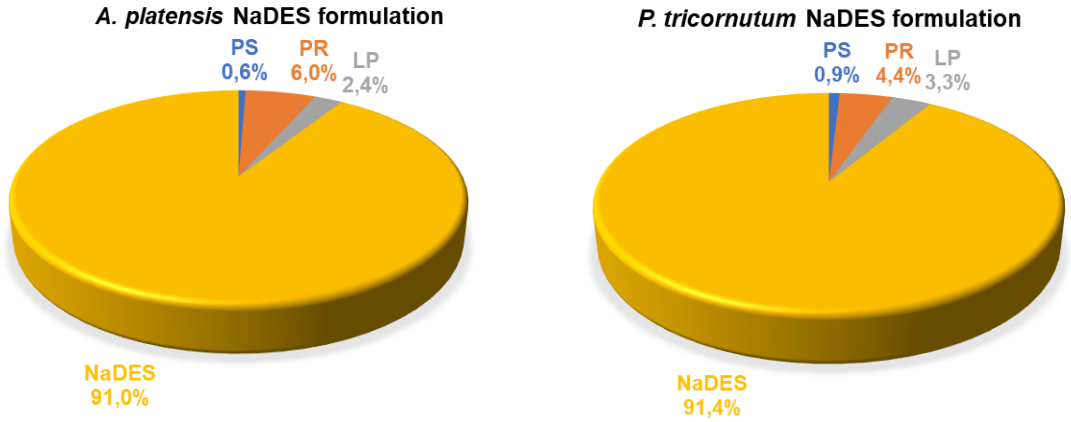


Fig. 52. Pie charts representing the composition of the algae formulations as percentage of polysaccharides (PS_blue), proteins (PR_orange), lipids (LP_grey) and pure NaDES (NaDES_ yellow).

Differently from grape extracts, the whole algal matrix becomes part of the final system in the formulations based on algae (*Table 19* and *Fig. 52*). Therefore, the percentage composition of PS, PR and LP did not vary among different NaDES, but reflects the composition of the type of algae used to prepare the formulations. From the observation of *Table 19* and *Fig. 52* some main behaviours can be summarized:

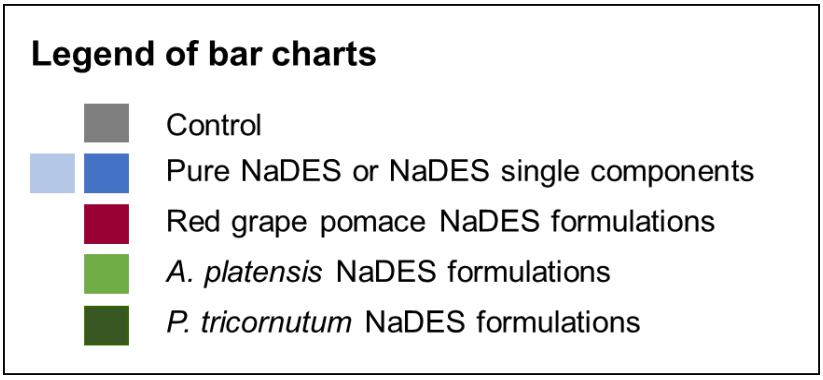
- In both algae-based formulations the content of pure NaDES is relevant: 91,0% and 91,4% respectively in the *A. platensis* and *P. tricornutum* extract.
- The lipid content was high in both formulations and represents 2,4% in the *A. platensis* extract and 3,3% of the *P. tricornutum* extract.
- The proteins constitute the most abundant part of the formulation apart NaDES: 6% and 4,4% respectively in the *A. platensis* and *P. tricornutum* formulations.
- The polysaccharides represent instead the smallest portion of the formulation: 0,6% 0,9% respectively in the *A. platensis* and *P. tricornutum* formulations.

Generally, NaDES systems carrying bioactive compounds extracted from red grape pomace and algae did not showed high compositional differences within the same biomasses. Most of the phytotoxic or bio-stimulant effects provided by these formulations could probably be ascribed to the different HBA and HBD involved, considering that more than 91% of the formulations were composed of pure NaDES.

4.4 Ecotoxicity assessment

Even though literature describes NaDES as safe and non-toxic substances the knowledge about their ecotoxicological profile are extremely limited, moreover the application of NaDES in the soil compartment has never been investigated. In view of the potential use of NaDES in agriculture it was considered essential to evaluate their toxicity towards soil organisms. NaDES and NaDES formulations phytotoxicity or bio-stimulant effects were estimated at the germination level in Petri dishes (*Lepidium sativum*) and early growth stages in soil (*Avena sativa*). The effects of NaDES on the soil fauna was estimated by chronic reproductive test on earthworm (*Eisenia andrei*). All toxicity tests performed were regulated by standardized methods, ISO or OECD guidelines, and therefore applied on organisms known for their high sensitivity. All NaDES were applied in toxicity tests as aqueous solutions, reproducing the way in which NaDES formulations would be applied in the agricultural field. The amount of NaDES appropriate to the test was calculated based on the amount of water needed to humidify the volume of soil or filter paper used in the test as described in paragraph (*Table 20*). The goal of this step of the study was to take up a systematic assessment on NaDES toxicity to obtain a structure-based understanding of their effects through different living organism and life cycle stages.

Table 20. Colour legend for bar charts of the ecotoxicological tests.



4.4.1 Earthworm toxicity test (*Eisenia andrei*)

All the results reported below refers to treatments performed with six pure NaDES (*Table 9*) prepared using betaine and choline chloride as HBA and combined with substances showing the following reactivity:

- Acidic: citric acid

- Basic: urea
- Neutral: ethylene glycol

4.4.1.1 Mortality

All treatments, except for urea-based NaDES, showed no lethal effect on earthworm: all ten worms placed in the soil at the beginning of the test were found alive after 28 days of exposure to the substances. In contrast, treatments containing urea, both combined with betaine and choline chloride, have produced profound lethal effects since the first days of exposure. On day 7, about half of the individuals treated with urea-based NaDES were found dead on the soil surface in a high state of decomposition. After 28 days of exposure, no individual survived, apart from three worms treated with Bet+U found in the same container.

4.4.1.2 Growth

The growth response was assessed by recording for each treatment the total wet weight of the earthworms before and after 28 days of exposure to the NaDES solution. Figures *Fig. 53* show respectively the total weight per treatment at day 0 (*Fig. 53*a) and at day 28 (*Fig. 53*b).

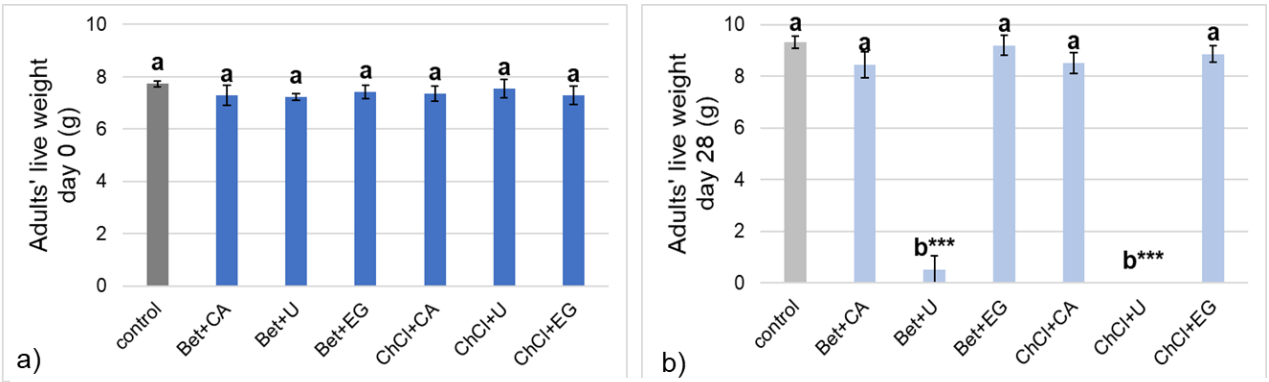


Fig. 53 a) Total weight of adult earthworms at day 0 b) and at day 28. Each point is the mean of four replicates, bars are ± standard error. Treatments marked with the same letter are not significantly different from each other ($p \geq 0,05$, Newman–Keuls post hoc test). Asterisks indicate a significant difference between a treatment and the control (* $p < 0,05$, ** $p < 0,01$, *** $p < 0,001$).

As displayed in *Fig. 53a,* the total weight of earthworms on day 0 showed no significant differences. In fact, in order to reduce the variability between treatments for the tests, homogeneous individuals were chosen for reproductive status and size, a parameter confirmed by this analysis of the variance.

The analysis of *Fig. 53b* highlights that there are no significant differences between treatments, except for the NaDES with urea which differ significantly from all other treatments. This result is a direct consequence of the high mortality rate produced: at day 28 the ChCl+U treatment showed no survivors, therefore the weight of the worms was equal to 0 g, while in the treatment Bet+U only three individuals survived, whose overall weight is shown in the graph *Fig. 53b*.

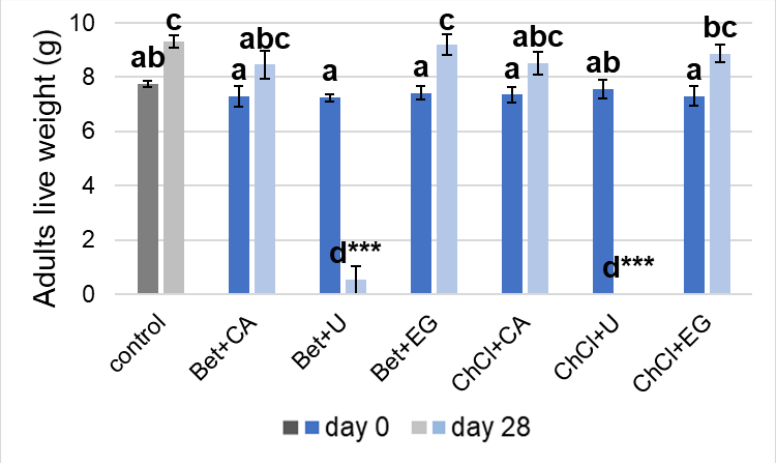


Fig. 54 Comparison among live weight of adult's earthworms at day 0 and day 28. Each point is the mean of four replicates, bars are ± standard error. Treatments marked with the same letter are not significantly different from each other ($p \geq 0,05$, Newman–Keuls post hoc test). Asterisks indicate a significant difference between a treatment and the control (* $p < 0,05$, ** $p < 0,01$, *** $p < 0,001$).

*Table 21.*Growth expressed as a percentage of the mean weight at day 28 compared to weight at day 0.

Treatment	growth %
control	20%
Bet+CA	16%
Bet+EG	24%
ChCl+CA	16%
ChCl+EG	21%

Fig. 54 shows the comparison between the weight of the earthworms at the beginning of the test and after 28 days of exposure to NaDES. From its analysis we can highlight some main trends:

- The control and treatments with EG, both combined with betaine and choline chloride, show a statistically significant increase (*Fig. 54*) compared to day 0.

The control organisms showed an average weight increase of 20% over 28 days, while treatments with EG augmented by 24% and 21% respectively when combined with betaine and choline chloride (*Table 21*).

- NaDES containing CA showed a 16% increase in weight both when combined with ChCl and when combined with Bet (*Table 21*). Despite this from the analysis of variance this increase is not statistically significant (*Fig. 54*).

Reproduction

Effects of NaDES on earthworm's reproduction were analysed by counting the number of deposited cocoons (eggs) and the number of hatched juveniles (new-born earthworms) at the end of the exposure phase.

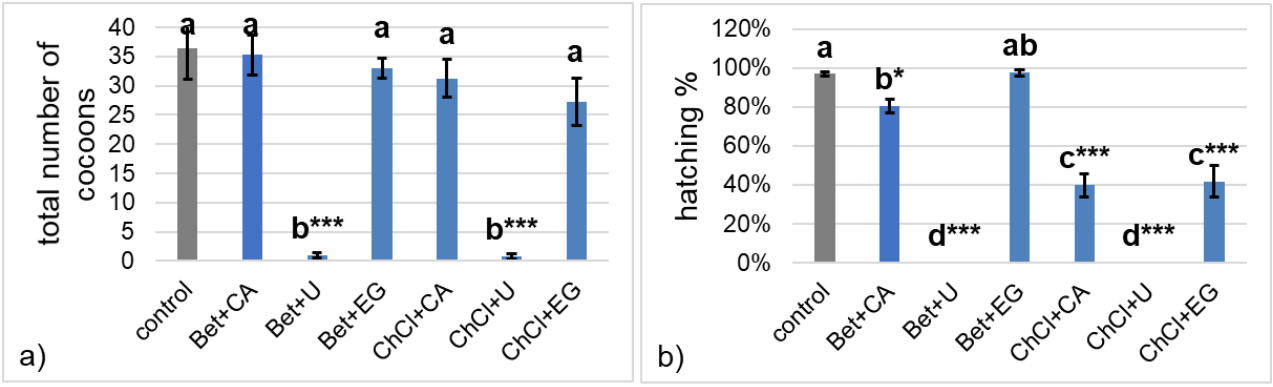


Fig. 55 a) Total cocoons counted at the end of the 56 days of exposure. b) Percentage of hatched cocoons compared to the total deposited. Each point is the mean of four replicates, bars are ± standard error. Treatments marked with the same letter are not significantly different from each other ($p \geq 0,05$, Newman–Keuls post hoc test). Asterisks indicate a significant difference between a treatment and the control (* $p < 0,05$, ** $p < 0,01$, *** $p < 0,001$).

Fig. 55a and *Fig. 55b* respectively report the values measured on day 56 of: the average of total cocoons (both empty and full) laid for each treatment and the percentages of hatching of the cocoons for each treatment, calculated as the ratio between the empty cocoons (hatched) compared to the total cocoons. From the results obtained it is possible to make the following observations:

- Apart from treatments containing urea, there were no significant differences in the number of laid cocoons between the control and the treatments, thus NaDES did not produce toxic effects in the deposition phase *Fig. 55a*. The control showed the maximum number of cocoons produced on average (36,5), followed by Bet+CA (35,3), Bet+EG (33), ChCl+CA (31,3) and ChCl+EG (27,3).

- In contrast, NaDES containing urea showed significant differences with respect to all treatments, with a mean of eggs deposited of 1 and 0,8 respectively for the Bet and ChCl-based NaDES, which represented the lowest number of eggs produced among all treatments (*Fig. 55a*).
- Almost all treatments showed a percentage of hatching significantly lower in comparison to the 96% exhibited by the control. The only exception was Bet+EG in which 98% of cocoons hatched producing juveniles. Moreover, the graph shows that all treatments based on ChCl, regardless of the related HBD, have a significantly lower hatching percentage both to control and to all treatments made up with Bet (not considering the case of urea). This experiment indicated that NaDES may affected cocoons hatching mainly due to the presence of ChCl, in fact while ChCl+CA showed a hatching of 40%, the corresponding acid with betaine exhibited an 80%, in the same way in the treatments with ChCl+EG only 42% of eggs were empty, less than half compared to 98% of the respective neutral NaDES with betaine (*Fig. 55b*).

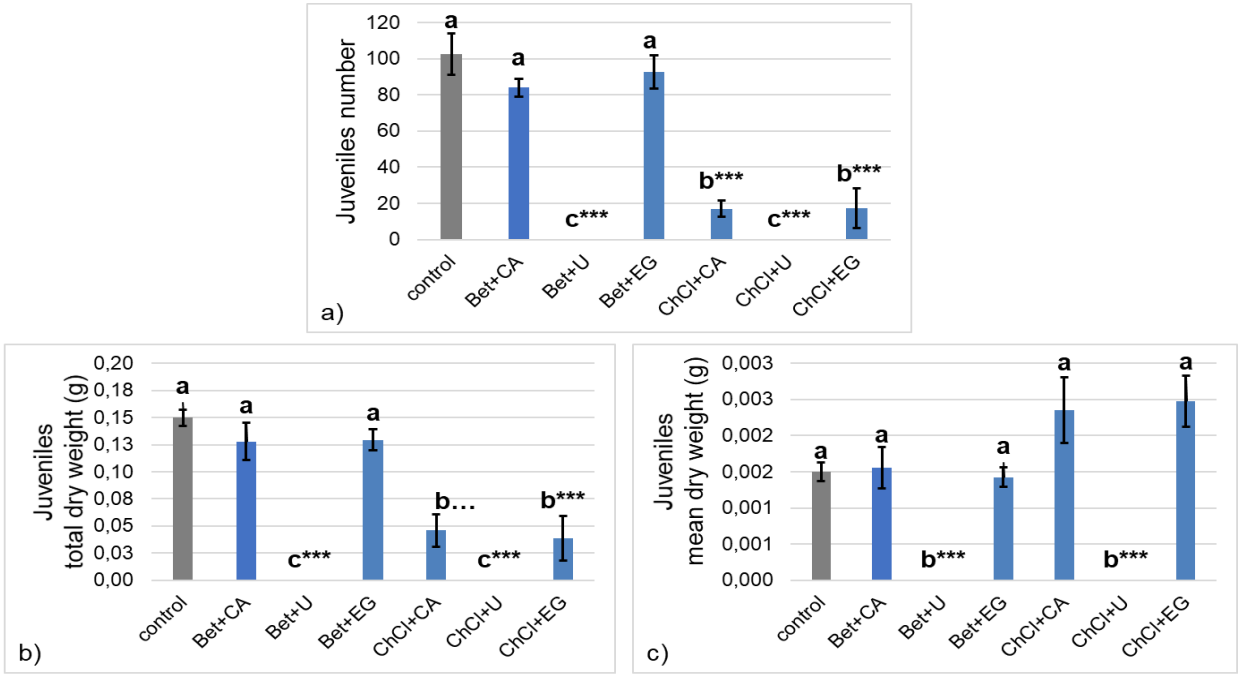


Fig. 56 a) Number of juveniles hatched from the cocoons b) Tototal dry weight of the juveniles on average c) Dry weight of a single juveniles on mean. Each point is the mean of four replicates, bars are ± standard error. Treatments marked with the same letter are not significantly different from each other ($p \geq 0,05$, Newman–Keuls post hoc test). Asterisks indicate a significant difference between a treatment and the control (* $p < 0,05$, ** $p < 0,01$, *** $p < 0,001$).

At the end of the experiment period for each treatment the following reproduction endpoints were also measured (*Fig. 56a)* the number of juveniles hatched from the cocoons *Fig. 56b* the total dry weight of the juveniles on average *Fig. 56c* the dry weight of a single juveniles on mean). From the analysis of the graphs (*Fig. 56*) it is possible to highlight the following observations:

- Treatments containing urea, both when combined with Bet or ChCl, were statistically different to all other treatments. No cocoons were able to hatch (*Fig. 55b)* as a result no juveniles were found in soils treated with these NaDES (*Fig. 56a*).
- In all the three reproductive parameters analysed Bet-based NaDES, both when combined with CA and with EG, showed no significant differences with respect to the control. It is therefore possible to state that these NaDES have no toxic effect on the reproduction of model organisms (*Fig. 56a, Fig. 56b, Fig. 56c*).
- In contrast, soils treated with the corresponding ChCl-based NaDES the number of juveniles (*Fig. 56a)* and their total dry weight (*Fig. 56b)* were statistically different and much lower than both control and Bet-based treatments, highlighting the toxic effect of this HBA on the reproduction of earthworms. The mean weight of a single juvenile was higher in the ChCl+CA and ChCl+EG treatments than in other treatments, control included, even if the differences were not statistically significant, due to the high variability within the treatments. Based on these data, it was possible to deduce that ChCl-based treatments showed a lower number of juveniles but with a greater growth of the individual compared to control and Bet-based NaDES.

Table 22. Summary table of the transformations performed on endpoints data and degree of significance of Cochran's C and p value of ANOVA analysis. (* p < 0,05; ** p < 0,01; *** p < 0,001). The information refers to the data shown in *Fig. 53-56.*

Endpoint	Transformation	Cochran's C	ANOVA
Adults live weight, day 0 (g)	none	*	ns
Adults live weight, day 28 (g)	none	ns	***
Adults live weight (g)	none	ns	***
Total number of cocoons	none	**	***
Hatching %	none	*	***
Juveniles number	none	*	***
Juveniles total dry weight (g)	none	ns	***
Juveniles means dry weight (g)	none	ns	***

Considering that the pH of soil plays an important role in the survival and health of organisms, seven replicas containing the same substances used in the toxicity tests and the same number of earthworms were prepared in parallel to the toxicity test. For the whole period of the experiment and every two weeks, 100 g of soil were taken from each of these replicas to evaluate the pH value. As shown in *Table 23*, the pH values were relatively homogeneous between all the treatments and during the entire time period considered, exhibiting values around 7,5, therefore the pH of soil ranged between neutral and weakly alkaline. The lowest pH values, 7,0, were measured at the end of the experiment in the treatment with ChCl+U, while the highest value, 8,5, was evaluated at day 14 always in the same treatment. These results were expected in the case of treatments with U and EG, respectively substances with basic and neutral reactivity, while the acid NaDES showed unexpected values. Probably the pH of the soil was predominantly influenced by its composition (clay, quartz sand and peat) and only minimally by the NaDES used in the treatments. Indeed the pH of the controls, that contain everything except the NaDES, showed extremely similar values to soils treated with solvents. Therefore, the toxic effects evaluated in this test are not attributable to the pH of the soil.

Table 23. pH of the soils treated with different NaDES solutions and control. Values measured on soil samples taken every 14 days for the entire period of the experiment.

Treatment	pH				
	day 1	day 14	day 28	day 42	day 56
control	7,1	7,8	7,4	7,4	7,3
Bet+CA	nd	7,8	7,6	7,1	7,2
Bet+U	8,0	8,3	7,3	7,6	7,1
Bet+EG	nd	7,9	7,5	7,5	7,2
ChCl+CA	7,6	7,6	7,6	7,4	7,1
ChCl+U	8,0	8,5	7,4	7,7	7,0
ChCl+EG	7,5	7,7	7,5	7,5	7,4

Up to date there is no specific investigation on the toxicity of Bet and ChCl-based NaDES on earthworms. Therefore, the comparison of these results with the literature data is limited. However, from the analysis of this data is possible to highlight can identify some main trends:

- All NaDES with U, at the tested concentration of 34 mM for the HBD (*Table 9*), were toxic to earthworms, with lethal effects on adults and consequent negative effects

on all other reproductive parameters tested. These results were consistent with literature data reporting lethal effects of high concentrations of urea on earthworms. Since urea is widely used in agriculture as a plant nutrient (N-source), the effects of long-term use (20 years) of urea fertiliser at 60, 120 and 180 kg N/ha/year was assessed on lumbricid earthworms in uncultivated turfgrass on loamy sand soil. The test concluded that application of nitrogenous fertilisers for long periods could reduce earthworm numbers and biomass and lowered the pH. Earthworms (*Eisenia fetida*) was studied using the soil-culture method to evaluate the relationship between the mortality of earthworms and excessive use of urea as nitrogen fertilizers. When the concentration of urea applied to the soil was 500–1000 mg kg^{-1}, the mortality of earthworms was still low. Once the concentration of urea was higher than 1500 mg kg^{-1}, the mortality approached 100%. The LD50 of the tested earthworms after a 14 days exposure to urea was 1065,9 mg kg^{-1} or 0,017 mol kg^{-1} if expressed as molar concentration of urea in soil [170]. These results are consistent with the present study: almost every organism died after the same period of exposure to urea-based NaDES containing 0,027 mol kg^{-1}. The toxic effect of urea water solutions on earthworm (*E. fetida*) was also evaluated adopting the simple paper contact acute toxicity method (OECD, 1984) where filter papers on the Petri plate bed are used as substrate medium. The lethal concentration for 48 h was 10 mg/5 ml, equivalent to 10 mM, thus, the toxicity grade of urea was categorized as “very toxic” to *E.fetida* The authors of the study attribute the high toxicity shown in the experiment, in part, to the direct contact of the organism with the solution investigated [171].

However, it should be highlighted that the concentrations of NaDES used in these experiments are presumably higher than those to which the organism would come into contact in case of real applications, thus the lethal effect produced by urea in these experiments overestimated the effects that would occur in the reality.

The test concentration used in the present study were based on the concentration of betaine currently used in commercial formulations for agriculture (4 g L^{-1}). As a result of 1:2 HBA:HBD molar ratio in the NaDES, the concertation of urea (HBD) in the test solution was 4.1 g L^{-1}. Exposing the worms to this concentration assumed that the use of the NaDES-based product could bring the soil pore water to the same concentration of the solution used in the treatments.

Additionally, toxicity tests on animals showed that the toxicity of NaDES was strictly dependent on the tested concentrations [131]. It would be therefore necessary to test different concentrations of these urea-based solvents to establish their EC50 and to compare them with field concentrations expected as a result of realistic applications, in order to fully understand the impact of urea-based NaDES on the earthworm populations.

- The HBA choline chloride seems to provoke harmful effects only against cocoons, by inhibiting their hatching capacity. NaDES composed of ChCl, excluding those containing U, did not show toxic effects on adult, moreover, once the juveniles had hatched, they presented a growth rate even greater than the other treatments. It is therefore hypothesized that ChCl has a toxic effect only on certain stages of the life cycle of these model organisms. The toxic effects of cholinium salts as HBA component of NaDES were also observed in and other study assessing ChCl toxicity on the growth of hydra. Hydra exposed to a medium containing 0.01 M of ChCl showed highly deleterious effect on the organisms, causing their tentacles to be contracted and eventually their bodies disintegrated within a few hours [129]. The same behaviour was observed on *Cyprinus carpio* fish: the authors ascribed the higher toxicity of the aqueous solutions in which fish were exposed to the existence of cholinium salts which was more toxic than both individual HBD evaluated (EG and U) and the respective eutectic mixtures [131]. However, NaDES and their individual components exhibit different toxicological profile based on the method and test organisms, thus the toxicity of ChCl should therefore be individually tested on *E. andrei* to confirm that the toxicity detected is due to the individual component and not to the eutectic mixture. Moreover, no toxicological tests have been carried out using pure Bet-based NaDES, therefore the observations here reported are only assumptions that require detailed investigations to be confirmed.
- Betaine-based NaDES combined with CA and EG seem to be the most promising medium to be used as carrier to deliver bioactive compounds in agriculture, almost none of the life cycle parameters of earthworms were affected by the presence of these substances.

In general, the results of this test highlight well-defined toxicological trends. Further studies could provide better understanding of toxic mechanisms of these substances and

thus guide to the design and develop truly green solvent with low toxicity on soil organisms.

4.4.2 Seeds germination test in Petri dishes (*Lepidium sativum*)

The toxicity and biostimulator effect of pure NaDES, NaDES formulations from red grape pomace and algae biomasses (*Arthrospira platensis* and *Phaeodactylum tricornutum*) and some individual components were tested on *Lepidium sativum*. The test NaDES were prepared (*Table 10*) using both betaine and choline chloride as HBA, mixed with substances showing different reactivity:

- Acidic: citric acid, lactic acid, malic acid
- Basic: urea
- Neutral: ethylene glycol, glycerol

4.4.2.1 Individual tests

Germination

In all the tests the percentage of germination was calculated as the number of seeds germinated in each treatment compared to the total treated seeds. From the analysis of the variance none of the treatments showed significantly differences from the control, apart for two exception:

- Test n°4: The germination of *L. sativum* was inhibited by ChCl+CA+pol, that showed significant differences compared to the control *(Fig. 62d*).
- Test n°5: all Bet-based formulations containing *P. tricornutum* biomass showed a significantly lower percentage of germination in comparison to the control. From the analysis of the data the following trend was highlighted: control > Bet+CA+Phaeo > Bet+EG+Phaeo > Bet+U+Phaeo. *(Fig. 63*).
- Test n°6: the germination rate showed by Bet+CA formulations containing *A. platensis* biomass was significantly lower than the untreated seeds *(Fig. 64d*).

Overall, the pure NaDES, NaDES formulations and the single components tested did not exhibit adverse effect son *L. sativum's* germination. The bar graphs representing the germination percentages are shown below from *Fig. 57* to *Fig. 67* and always indicated with the letter (*d*), however these parameters will no longer be commented.

Root and shoot length

Test n°1: pure Bet-based NADES

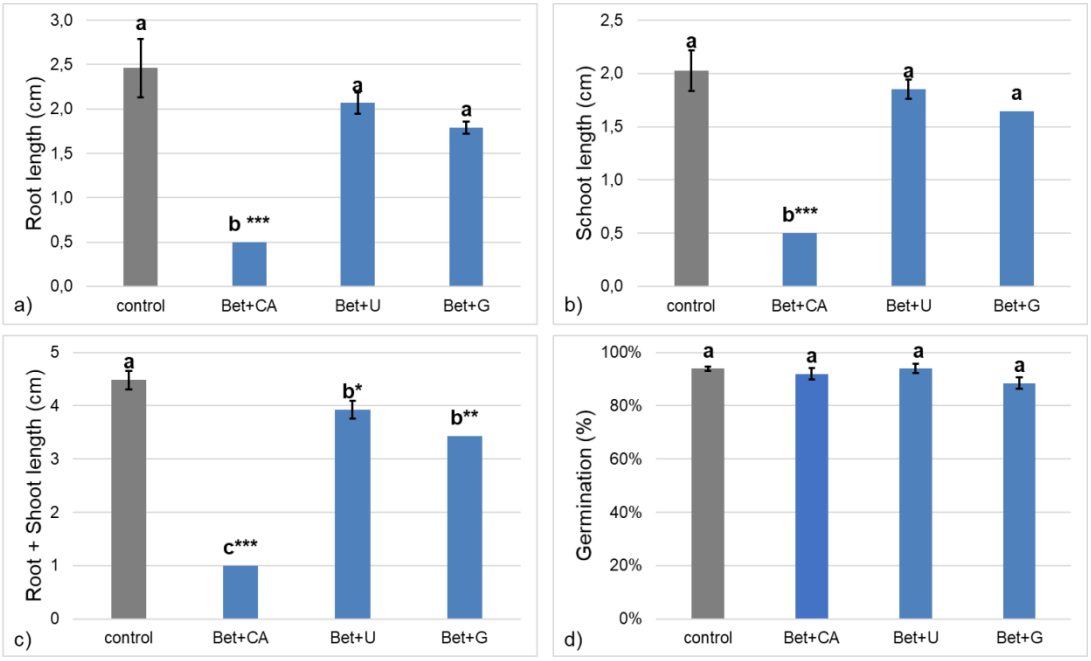


Fig. 57 The bar charts show a) the root length b) the shoot length c) total length of seeds germinated and b) percentage of germination (calculated as the number of seeds germinated in each treatment in comparison to the total) of the untreated seeds and the seedings treated with Bet-based NaDES. Each point is the mean of four replicates, bars are ± standard error. Treatments marked with the same letter are not significantly different from each other ($p \geq 0,05$, Newman–Keuls post hoc test). Asterisks indicate a significant difference between a treatment and the control (* $p < 0,05$, ** $p < 0,01$, *** $p < 0,001$).

Table 24. Summary table of the transformations performed on endpoints data and degree of significance of Cochran's C and p value of ANOVA analysis. (* $p < 0,05$; ** $p < 0,01$; *** $p < 0,001$). The information refers to the data shown in Fig.57

Endpoint	Transformation	Cochran's C	ANOVA
Root length (cm)	log_{10}	ns	ns
Shoot length (cm)	none	ns	ns
Root + shoot length (cm)	none	ns	**
Germination (%)	none	ns	ns

Fig. 57 shows the results obtained by exposing the seeds of *L. sativum* to aqueous solutions of Bet-based NaDES combined with basic (U) neutral (G) and acidic (CA) HBD, from its analysis it was possible to make some observations:

- Bet+CA showed always significantly differences from the control and the other treatments, therefore this NaDES exhibited high toxicity to *L. sativum*. The 50 ml of

the prepared solution used in this test contained 34 mM of the HBD, which represents the highest concentration between those evaluated in the present study. Moreover, on the cellulose filter paper discs of the Petri dishes treated with the solution containing CA, different type of moulds has grown. Similar moulds, in variable amounts, have been observed in almost all the treatments performed using NaDES or formulations containing citric acid (*Fig. 57*). It was therefore assumed that this compound could be involved in the metabolism of these organisms, creating optimal conditions for their growth. Microscopic analyses were performed on moulds' samples, however the species could not be identified (*Fig. 58 and Fig. 59)*

- Bet+U and Bet+G did not show an inhibitory effect on shoots and roots lengths, if considrerd separately, while observing the overall length of the sprout the analysis of variance showed a significant reduction with respect to the control.

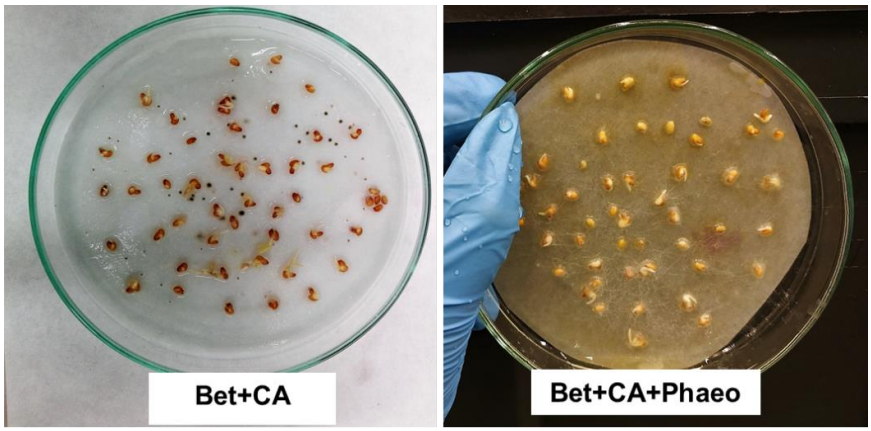


Fig. 58 Two types of moulds grown on cellulose filter paper discs of the Petri dishes containing NaDES solutions with citric acid (on the left, seeds exposed to Bet+CA, on to the right seed treated with to the P. tricornutum formulation with citric acid)

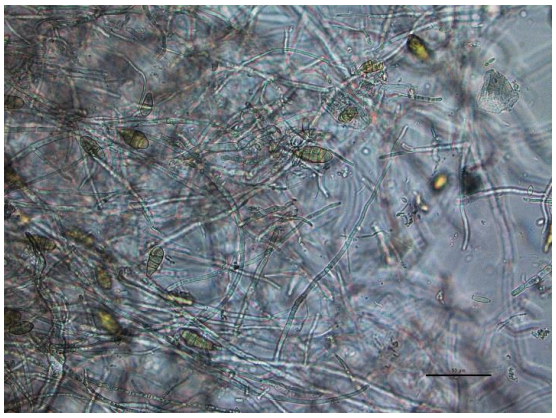


Fig. 59 Microscopic image of a mould taken from treated petri dishes containing citric acid.

Test n°2: Bet-based NaDES formulations carrying polyphenols

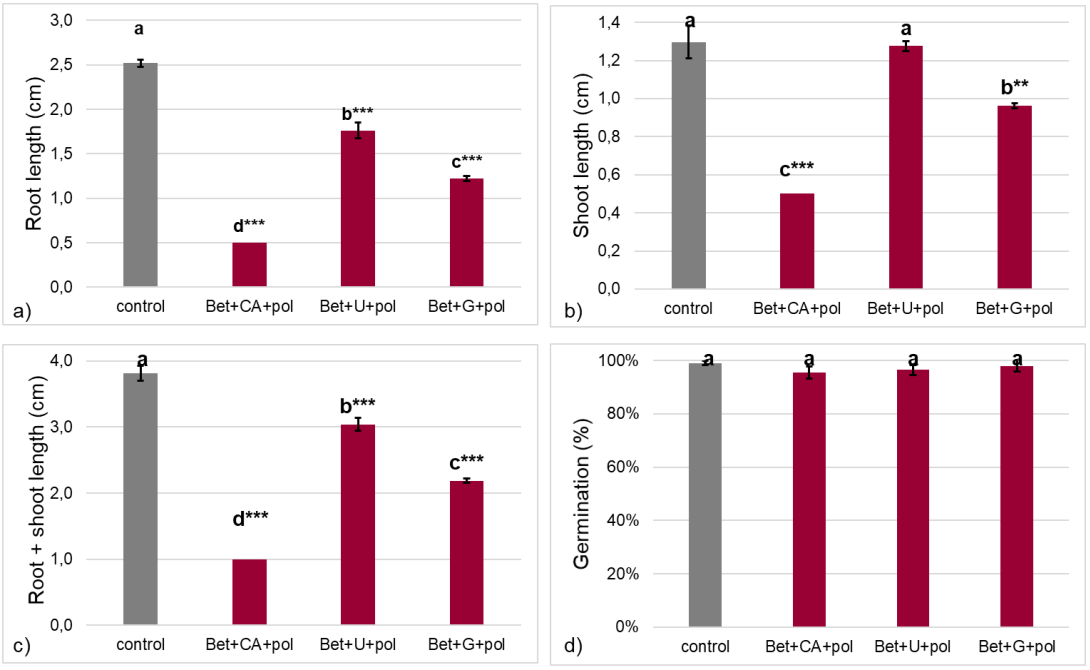


Fig. 60 The bar charts show a) the root length b) the shoot length c) total length of seeds germinated and b) percentage of germination (calculated as the number of seeds germinated in each treatment in comparison to the total) of the untreated seeds and the seeds treated with Bet-based NaDES carrying polyphenols. Each point is the mean of four replicates, bars are ± standard error. Treatments marked with the same letter are not significantly different from each other (p ≥ 0,05, Newman–Keuls post hoc test). Asterisks indicate a significant difference between a treatment and the control (* p < 0,05, ** p < 0,01, *** p < 0,001).

Table 25. Summary table of the transformations performed on endpoints values, the degree of significance of Cochran's C and p value of ANOVA analysis. (* p < 0,05, ** p < 0,01, *** p < 0,001). The information refers to the data shown in Fig. 60.

Endpoint	Transformation	Cochran's C	ANOVA
Root length (cm)	none	ns	***
Shoot length (cm)	none	*	***
Root + shoot length (cm)	none	ns	***
Germination (%)	none	ns	ns

The bar charts in *Fig. 60* displayed the results obtained from the treatments performed with the red grape pomace formulations obtained by extractions with Bet-based NaDES combined with CA, U and G. Apart from Bet+U+pol, all the formulations tested produced inhibiting effects on the sprout growth, in fact the analysis of variance highlighted significant differences with respect to control (*Fig. 60*a, *Fig. 60b, Fig. 60c*).

Test n°3: pure ChCl-based NADES

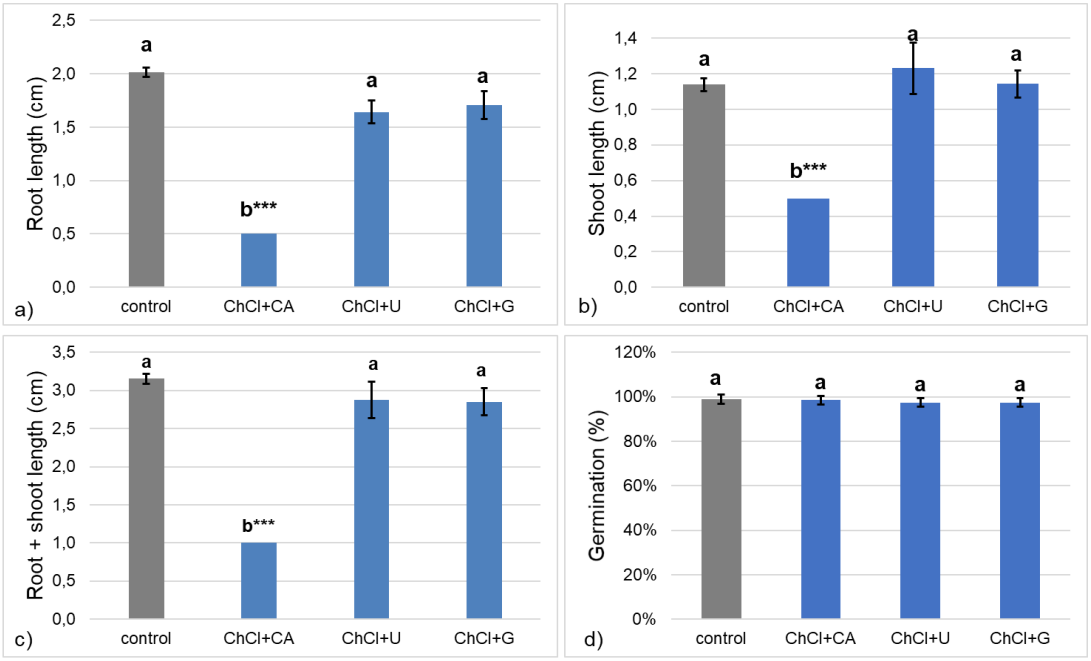


Fig. 61 The bar charts show a) the root length b) the shoot length c) total length of seeds germinated and b) percentage of germination (calculated as the number of seeds germinated in each treatment in comparison to the total) of the untreated seeds and the seeds treated with ChCl-based NaDES. Each point is the mean of four replicates, bars are ± standard error. Treatments marked with the same letter are not significantly different from each other (p ≥ 0,05, Newman–Keuls post hoc test). Asterisks indicate a significant difference between a treatment and the control (* p < 0,05, ** p < 0,01, *** p < 0,001).

Table 26. Summary table of the transformations performed on endpoints values, the degree of significance of Cochran's C and p value of ANOVA analysis. (* p < 0,05, ** p < 0,01, *** p < 0,001). The information refers to the data shown in Fig. 61.

Endpoint	Transformation	Cochran's C	ANOVA
Root length (cm)	none	ns	ns
Shoot length (cm)	none	ns	ns
Root + shoot length (cm)	none	ns	ns
Germination (%)	none	ns	ns

The effect on pure NaDES combined with citric acid, urea and glycerol as HBD, was also tested. From the results showed in *Fig. 61* it was possible to highlight that:

- In line with the previous results, the NaDES containing CA produced a strongly inhibiting effect on all the parameters evaluated, as evidenced by the analysis of the variance, that showed significant differences compared to the control *(Fig. 61a, Fig. 61b, Fig. 61c).*
- Otherwise, ChCl+U and ChCl+G were non-hazardous for *L. satimum*. The bar charts were labelled with the same letter "a" of the control, indicating non-significant

differences compared to the values obtained from the untreated seeds *(Fig. 61a, Fig. 61b, Fig. 61c).*

Test n°4: ChCl-based NaDES formulations carrying polyphenols

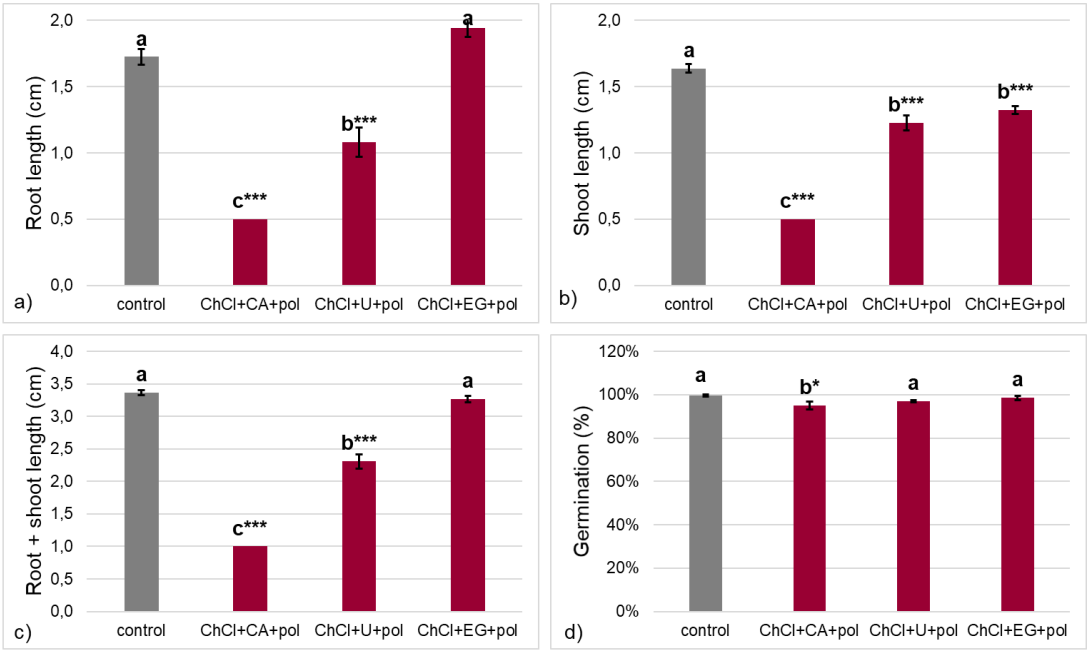


Fig. 62 The bar charts show a) the root length b) the shoot length c) total length of seeds germinated and b) percentage of germination (calculated as the number of seeds germinated in each treatment in comparison to the total) of the untreated seeds and the seeds treated with ChCl-based NaDES carrying polyphenols. Each point is the mean of four replicates, bars are ± standard error. Treatments marked with the same letter are not significantly different from each other (p ≥ 0,05, Newman–Keuls post hoc test). Asterisks indicate a significant difference between a treatment and the control (* p < 0,05, ** p < 0,01, *** p < 0,001).

Table 27. Summary table of the transformations performed on endpoints values, the degree of significance of Cochran's C and p value of ANOVA analysis. (* p < 0,05, ** p < 0,01, *** p < 0,001). The information refers to the data shown in Fig. 62.

Endpoint	Transformation	Cochran's C	ANOVA
Root length (cm)	none	ns	***
Shoot length (cm)	none	ns	***
Root + shoot length (cm)	none	ns	***
Germination (%)	none	ns	ns

Fig. 62 shows the results measured on seeds of *L. sativum* exposed to formulations obtained from the extraction of red grape pomace using ChCl-based NaDES combined with CA, U, EG. The statistical analyses performed on these values allowed to make some observations:

- ChCl+CA+pol was always significantly different in comparison to the control considering all the parameters evaluated. This means that the formulations containing polyphenols showed a high deleterious effect on the seed's growth. Similar results were obtained on the seeds exposed to the ChCl+U+pol: although bar charts showed values higher than those measured on seeds exposed to acid formulations, the differences compared to the untreated seeds were significant, thus these formulations produced deleterious effect on seed's growth *(Fig. 62a, Fig. 62b, Fig. 62c).*
- Formulations containing EG did not showed inhibitory effect on root length (*Fig. 62a*) and overall length of sprout (*Fig. 62*) while they had a significant inhibitory effect on stem growth (*Fig. 62b*).

Test n°5: Bet-based NaDES formulations from *A. platensis* biomass

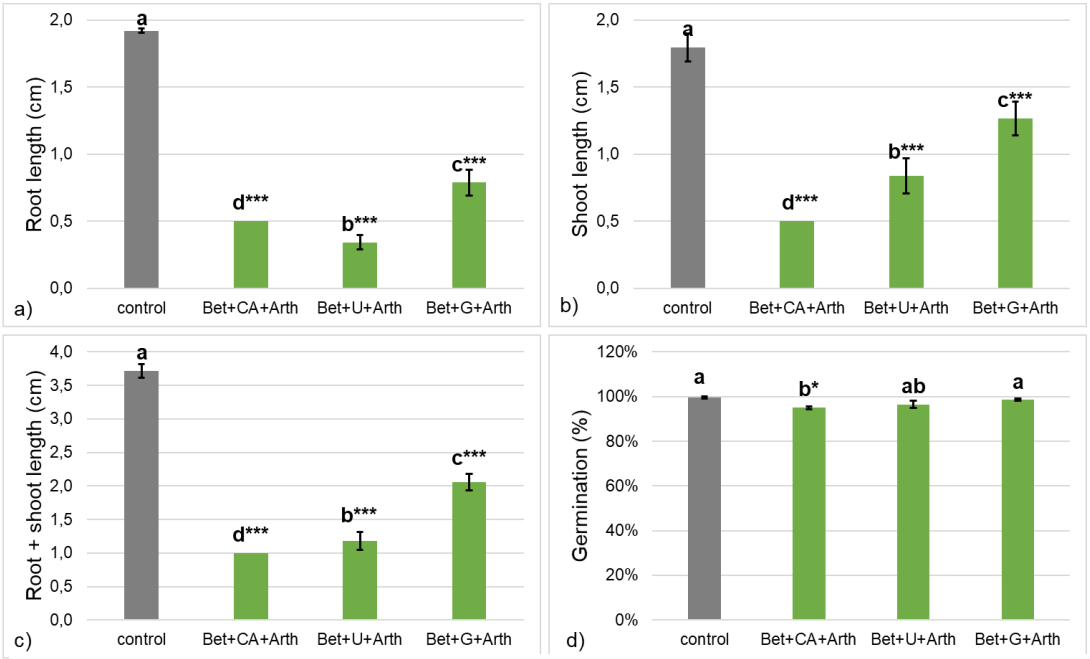


Fig. 63 The bar charts show a) the root length b) the shoot length c) total length of seeds germinated and b) percentage of germination (calculated as the number of seeds germinated in each treatment in comparison to the total) of the untreated seeds and the seeds treated with Bet-based NaDES formulations containing *A.platensis* biomass. Each point is the mean of four replicates, bars are ± standard error. Treatments marked with the same letter are not significantly different from each other (p ≥ 0,05, Newman–Keuls post hoc test). Asterisks indicate a significant difference between a treatment and the control (* p < 0,05, ** p < 0,01, *** p < 0,001).

Table 28 Summary table of the transformations performed on endpoints values, the degree of significance of Cochran's C and p value of ANOVA analysis. (* $p < 0,05$, ** $p < 0,01$, *** $p < 0,001$). The information refers to the data shown in Fig. 63.

Endpoint	Transformation	Cochran's C	ANOVA
Root length (cm)	log_{10}	ns	**
Shoot length (cm)	none	ns	**
Root + shoot length (cm)	none	ns	***
Germination (%)	none	ns	*

Fig. 63 shows the results obtained using formulations from the extraction of *A. platensis* using Bet-based NaDES combined with CA, U, G. From the analysis of the graphs it was evident that all the algal formulations tested produced a highly toxic effect on *L. sativum*. As showed by the analysis of the variance, all the formulations considered, in all the evaluated parameters showed significant differences compared to the control, due to the strong inhibiting effect on the roots and shoot growth of the model organism *(Fig. 63a, Fig. 63b, Fig. 63c).* Overall, the three formulations presented a toxicity pattern in the following order: Bet+CA+Arth > Bet+U+Arth > Bet+EG+Arth (*Fig. 63b, Fig. 63c*) or Bet+U+Arth > Bet+CA+Arth > Bet+EG+Arth (*Fig. 63a*).

Test n°6: Bet-based NaDES formulation from *P. tricornutum* biomass

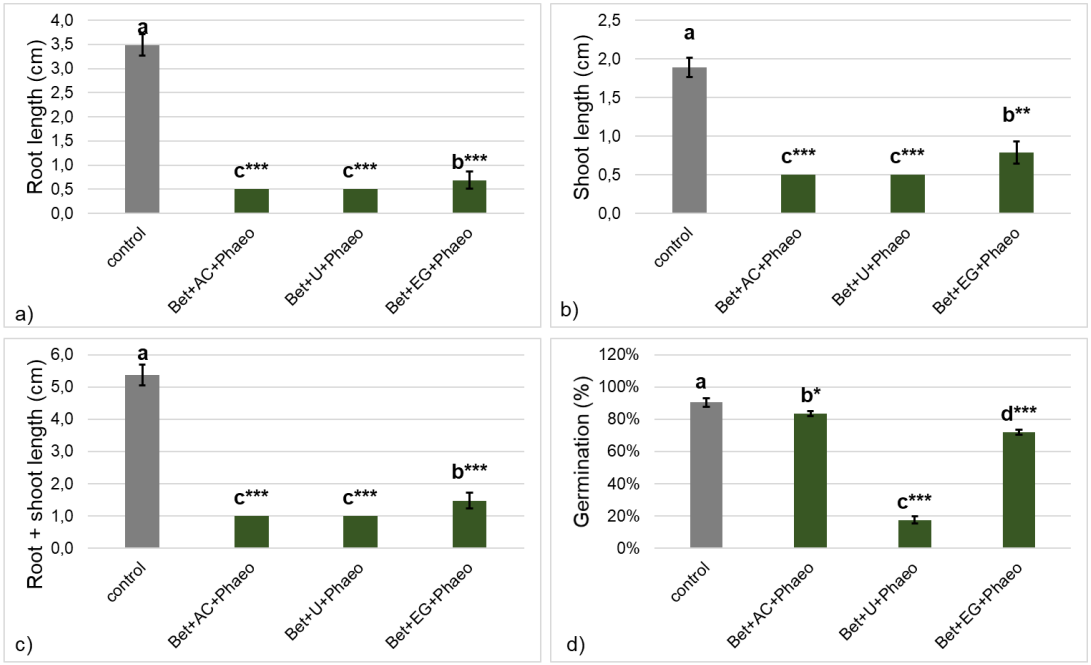


Fig. 64 The bar charts show a) the root length b) the shoot length c) total length of seeds germinated and b) percentage of germination (calculated as the number of seeds germinated in each treatment in comparison to the total) of the untreated seeds and the seeds treated with Bet-based NaDES formulations containing *P. tricornutum* biomass. Each point is the mean of four replicates, bars are ± standard error. Treatments marked with the same letter are not significantly different from each other (p ≥ 0,05, Newman–Keuls post hoc test). Asterisks indicate a significant difference between a treatment and the control (* p < 0,05, ** p < 0,01, *** p < 0,001).

Table 29. Summary table of the transformations performed on endpoints values, the degree of significance of Cochran's C and p value of ANOVA analysis. (* p < 0,05, ** p < 0,01, *** p < 0,001). The information refers to the data shown in Fig. 64.

Endpoint	Transformation	Cochran's C	ANOVA
Root length (cm)	none	ns	***
Shoot length (cm)	none	ns	**
Root + shoot length (cm)	none	ns	***
Germination (%)	none	ns	***

The toxicity of algal formulations containing *P. tricornutum* were also tested (*Fig. 64*). Similarly, the extraction was performed with Bet-based NaDES, however in this case CA, U and EG were used as HBD. The results obtained (*Fig. 64)* were consistent to those observed with the formulations containing *A. platensis (Fig. 63*). All the tested formulations showed significant differences compared to the control, due to the strong toxic effect produced on the entirely growth parameters evaluated. The graphs show a homogeneous toxicity pattern of the formulations: Bet+CA+Phaeo = Bet+U+Phaeo > Bet+Eg+Phaeo

Test n°7: Acidic Bet-based NaDES (low concentration)

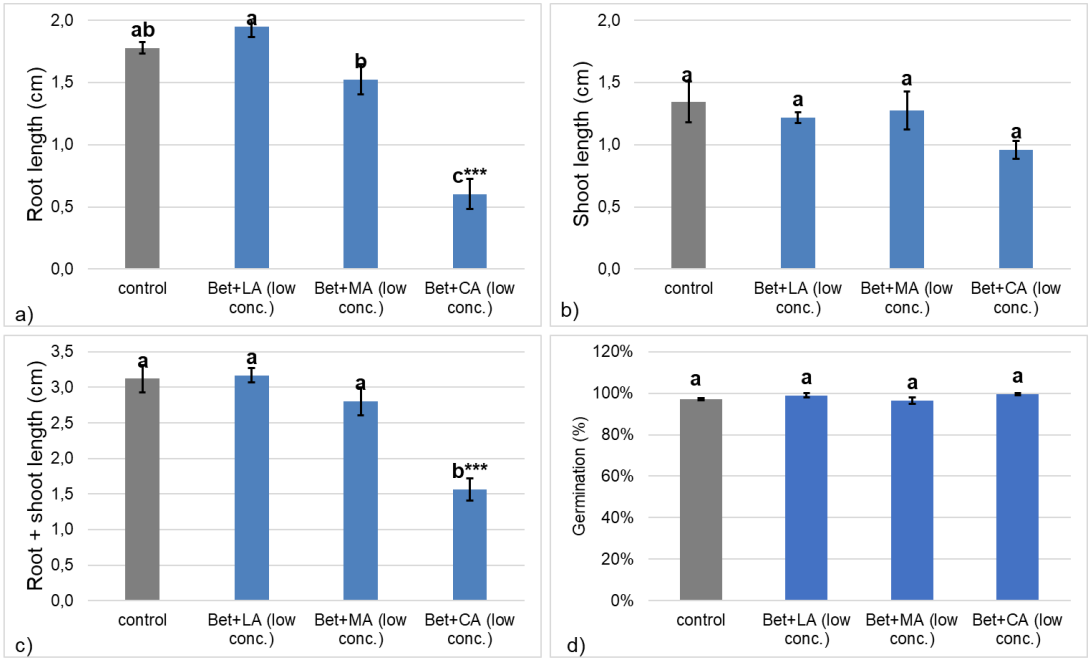


Fig. 65. The bar charts show a) the root length b) the shoot length c) total length of seeds germinated and b) percentage of germination (calculated as the number of seeds germinated in each treatment in comparison to the total) of the untreated seeds and the seeds treated with Bet-based NaDES formulations combined with acid HBD evaluated in low concentration. Each point is the mean of four replicates, bars are ± standard error. Treatments marked with the same letter are not significantly different from each other ($p \geq 0,05$, Newman–Keuls post hoc test). Asterisks indicate a significant difference between a treatment and the control (* $p < 0,05$, ** $p < 0,01$, *** $p < 0,001$).

Table 30. Summary table of the transformations performed on endpoints values, the degree of significance of Cochran's C and p value of ANOVA analysis. (* $p < 0,05$, ** $p < 0,01$, *** $p < 0,001$). The information refers to the data shown in Fig. 65.

Endpoint	Transformation	Cochran's C	ANOVA
Root length (cm)	none	ns	***
Shoot length (cm)	none	ns	ns
Root + shoot length (cm)	none	ns	***
Germination (%)	none	ns	na

Fig. 65 shows the results of the toxicity test performed by exposing the seeds of L. sativum to three different Bet-based NaDES containing organic acid as HBD: lactic acid (LA), malic acid (MA) and citric acid (CA). In this test, the seeds were exposed to aqueous solutions containing low concentrations of HBD, 2 mM, compared to the subsequent test (n°8) in which the same substances were evaluated at high concentrations, using 34 mM of each HBD. Observing the bar charts, it was possible to highlight that:

- The NaDES containing CA showed values always lower than all the other treatments: these differences are statistically different in all the parameters evaluated, except for the shoot length (*Fig. 65b*).
- Bet LA never showed inhibiting effect on any of the parameters considered. Also Bet+U did not produce deleterious effects on the root's growth and on the overall length of the sprout, while it exhibited inhibiting effect at root level, where it showed significant differences with respect to the control.
- In general, the results showed the following ascending toxicity pattern: Bet+LA < Bet+MA < Bet+CA. These differences, however, were not always highlighted by the analysis of variance.

Test n°8: Acidic Bet-based NaDES (high concentration)

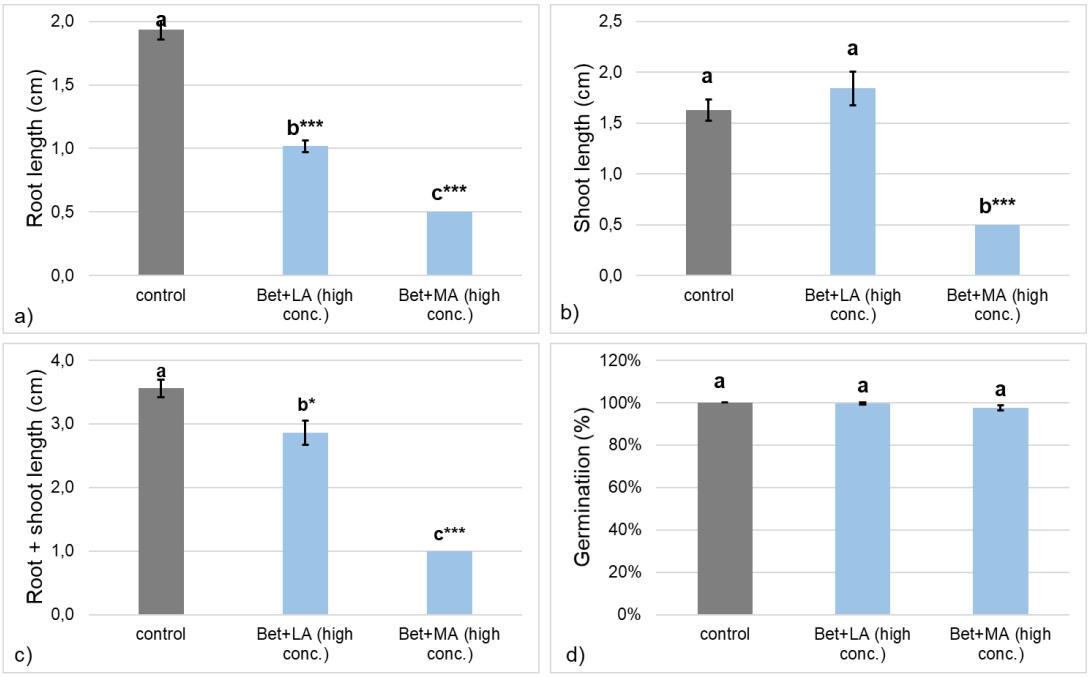


Fig. 66 The bar charts show a) the root length b) the shoot length c) total length of seeds germinated and b) percentage of germination (calculated as the number of seeds germinated in each treatment in comparison to the total) of the untreated seeds and the seeds treated with Bet-based NaDES formulations combined with acid HBD evaluated in high concentration. Each point is the mean of four replicates, bars are ± standard error. Treatments marked with the same letter are not significantly different from each other ($p \geq 0,05$, Newman–Keuls post hoc test). Asterisks indicate a significant difference between a treatment and the control (* $p < 0,05$, ** $p < 0,01$, *** $p < 0,001$).

Table 31. Summary table of the transformations performed on endpoints values, the degree of significance of Cochran's C and p value of ANOVA analysis. (* p < 0,05, ** p < 0,01, *** p < 0,001). The information refers to the data shown in Fig. 66.

Endpoint	Transformation	Cochran's C	ANOVA
Root length (cm)	none	ns	***
Shoot length (cm)	none	ns	***
Root + shoot length (cm)	none	ns	***
Germination (%)	none	ns	ns

This experiment is parallel to the previously (n°7; *Fig. 66*), but in this case were tested only NaDES containing lactic acid and malic acid, both at high concentrations (34 mM). NaDES containing CA have not been evaluated because the values estimated for Bet+CA (HBA 34 mM) had already been assessed in the test n°1 (*Fig. 57*). *Fig. 66* displays the following results:

- At high concentrations, all the acidic NaDES evaluated showed deleterious effects on the *L. sativum* growth: in all the parameters the values measured of Bet+LA and Bet+MA were significantly lower with respect to the control *(Fig. 66a, Fig. 66b, Fig. 66c),* except for Bet+LA in the "shoot length" parameter (*Fig. 66c*).
- The toxicity exhibited by Bet+MA was always statistically greater than that produced by Bet+ LA.

Test n°9: NaDES with EG as HBD and corresponding individual components

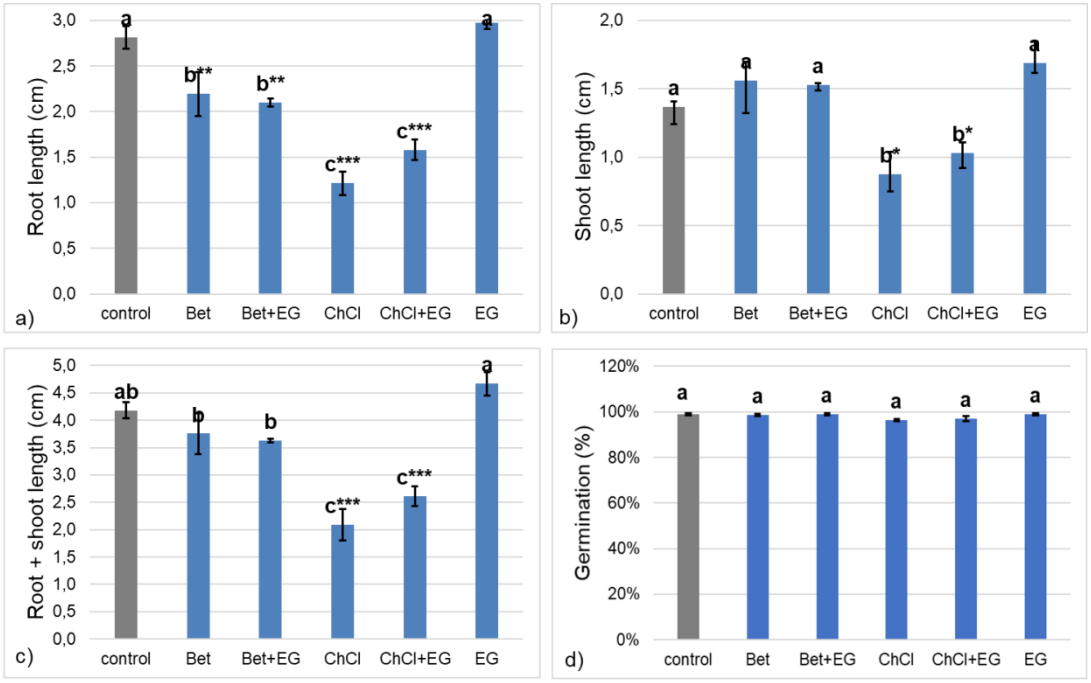


Fig. 67 The bar charts show a) the root length b) the shoot length c) total length of seeds germinated and b) percentage of germination (calculated as the number of seeds germinated in each treatment in comparison to the total) of the untreated seeds and the seeds treated with EG-based NaDES and the corresponding individual components. Each point is the mean of four replicates, bars are ± standard error. Treatments marked with the same letter are not significantly different from each other (p ≥ 0,05, Newman–Keuls post hoc test). Asterisks indicate a significant difference between a treatment and the control (* p < 0,05, ** p < 0,01, *** p < 0,001).

Table 32. Summary table of the transformations performed on endpoints values, the degree of significance of Cochran's C and p value of ANOVA analysis. (* p < 0,05, ** p < 0,01, *** p < 0,001). The information refers to the data shown in Fig 67.

Endpoint	Transformation	Cochran's C	ANOVA
Root length (cm)	none	ns	***
Shoot length (cm)	none	**	***
Root + shoot length (cm)	none	ns	***
Germination (%)	none	ns	ns

The aim of these test was to evaluate the source of the growth promoting effect evaluated in some NaDES containing ethylene glycol (*Fig. 67*). For this purpose, the single substances composing the EG-based NaDES (ChCl, Bet and EG) and the corresponding eutectic mixtures (Bet+EG and ChCl+EG) were tested. Clearly, the concentration of the single components evaluated was the same as that present in the NaDES mixtures (*Fig. 67*). From the results shown in fig you can highlight a specific trend:

- The seeds exposed to an aqueous solution containing only EG *(Fig. 67a, Fig. 67b, Fig. 67c)* showed a higher growth compared to the control in all the parameters evaluated.
- Overall, the following trend was repeated within all the parameter evaluated: control = EG ≥ Bet = Bet+EG > ChCl = ChCl+EG.
- Furthermore, in all the evaluated parameters the ChCl-based NaDES showed an inhibitory effect on sprouts' growth statistically significant with respect to the control and to the Bet-based NaDES. Otherwise, the NaDES containing betaine did not exhibit inhibitory effect on the growth of the stem and on the general length of the shoot, while they present values that are statistically lower than the control in the "root length", highlighting an inhibitory effect on roots.

In conclusion, from the results obtained by the single experiments is possible to summarize some main trends:

- From an overall analysis of the results it was observed that germination was the least sensitive parameter among those tested, consistent with results reported by the study assessing ChCl-based NaDES phytotoxicity using wheat 77.
- NaDES containing CA as HBD showed the lower values in all the parameters evaluated, confirming their harmful effect on germination.
- NaDES formulations did not show a biostimulating effect on the sprouts, on the contrary, all the formulations containing algae produced inhibitory effects on the growth of the seeds of L. sativum.
- Aqueous solutions containing pure EG seems to improve seeds growth *(Fig. 67a, Fig. 67b, Fig. 67c).* Even if these differences are not considered statistically significant by the analysis of variance, these results could be considered in line with the literature information: *Ethylene is a natural plant growth regulator often produced in sufficient quantities to alter cellular and developmental processes in a characteristic hormonal manner. Almost all phases of plant development are affected, including germination, growth, flowering, dormancy, abscission, senescence and sex expression*[172]*.* However, observing the toxicity trend showed *in the test n°9,* control = EG ≥ Bet = Bet+EG > ChCl = ChCl+EG., it was possible to hypnotised that the effect shown in the eutectic mixture containing EG is mainly caused by the effect of HBA while a minor contribution was produced by the HBD.

4.4.2.2 Comparisons among results from different experiments

Based on the results obtained from the individual tests, comparative tests were also carried out. The values of the endpoints obtained from each replicate of the treatments tested in the single experiments were expressed as percentage of the mean of their respective control.

root length (%), shoot length (%), root+shoot length (%) = (mean of the root, shoot or root+shoot length in the treatment / mean of root, shoot or root+shoot length in the respective control) x 100

This normalization allowed statistical comparisons among results obtained from different experiments. The purpose of the comparative tests was to highlight any stimulating or inhibiting effects produced by the formulations with respect to pure NaDES and to better understand the toxicity of acid NaDES on *L. sativum* by comparing HBD with different acid reactivity and concentration.

Seeds germination

In a similar way to the results obtained in the individual tests, even in the comparative tests, the percentage of seeds germination in the treatments were always not significantly different compared to the control, highlighting their non-toxicity to *L.sativum* germination (results showed as following from *Fig. 68* to *Fig. 72* with the letter (d)). However, some pure NaDES and NaDES formulations diverged from this general trend, showing significant differences with respect to the control:

- ChCl+CA+pol *Fig. 69*.
- Bet+CA (in the comparative test with formulations containing *A. platensis* (*Fig. 70*) and in the test assessing carboxylic acid toxicity (*Fig. 72*).
- Bet+U+Phaeo and Bet+EG+Phaeo (*Fig. 71*).

Root and shoot elongation

Pure NaDES and NaDES formulation carrying polyphenols

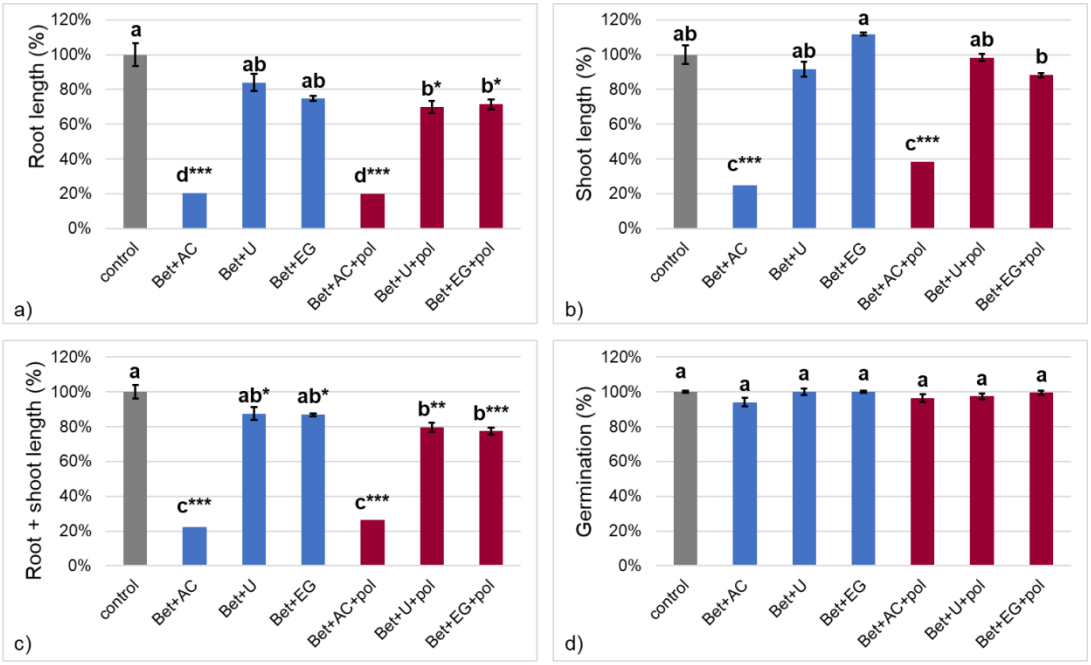


Fig. 68 Comparison among pure Bet-based NaDES and NaDES formulation from red grape pomace considering a) the root length (%) b) the shoot length (%) c) total length of seeds germinated (%) and b) germination (%) calculated as percentage of the mean control value. Each point is the mean of four replicates, bars are ± standard error. Treatments marked with the same letter are not significantly different from each other ($p \geq 0,05$, Newman–Keuls post hoc test). Asterisks indicate a significant difference between a treatment and the control (* $p < 0,05$, ** $p < 0,01$, *** $p < 0,001$).

Table 33. Summary table of the transformations performed on endpoints values, the degree of significance of Cochran's C and p value of ANOVA analysis. (* $p < 0,05$, ** $p < 0,01$, *** $p < 0,001$). The information refers to the data shown in Fig 68.

Endpoint	Transformation	Cochran's C	ANOVA
Root length (%)	none	**	***
Shoot length (%)	$\log_{10}$	**	*
Root + shoot length (%)	none	ns	***
Germination (%)	none	ns	ns

Fig. 68 shows the comparison between pure Bet-based NaDES and the corresponding formulations from red grape pomace. Observing these results, it was possible to highlight that:

- All the acidic NaDES were significantly lower to all treatments considering all the parameters evaluated, indicating their high toxicity on *L. sativum* (*Fig. 68a, Fig. 68b, Fig. 68c).*
- In all the parameters evaluated, the formulations carrying polyphenols did not show significant differences compared to the pure NaDES, the only exception was Bet+EG+pol that showed a shoot length significantly lower than Bet+EG.

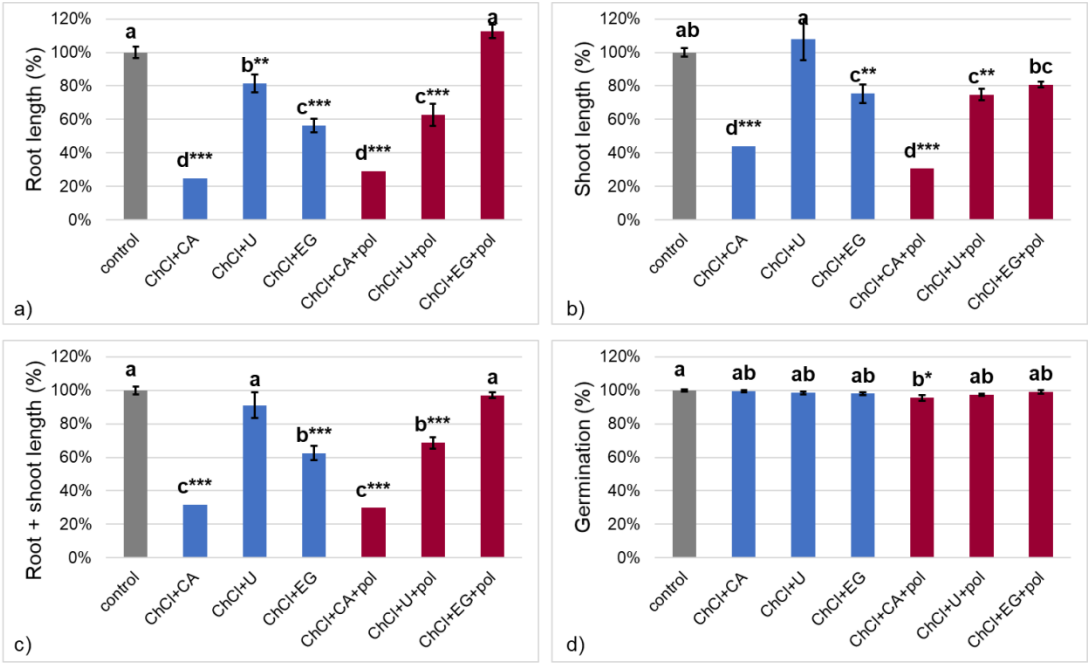


Fig. 69 Comparison among pure ChCl-based NaDES and NaDES formulation from red grape pomace considering a) the root length (%) b) the shoot length (%) c) total length of seeds germinated (%) and b) germination (%) calculated as percentage of the mean control value. Each point is the mean of four replicates, bars are ± standard error. Treatments marked with the same letter are not significantly different from each other ($p \geq 0,05$, Newman–Keuls post hoc test). Asterisks indicate a significant difference between a treatment and the control (* $p < 0,05$, ** $p < 0,01$, *** $p < 0,001$).

Table 34. Summary table of the transformations performed on endpoints values, the degree of significance of Cochran's C and p value of ANOVA analysis. (* $p < 0,05$, ** $p < 0,01$, *** $p < 0,001$). The information refers to the data shown in Fig 69.

Endpoint	Transformation	Cochran's C	ANOVA
Root length (%)	none	ns	***
Shoot length (%)	$\log_{10}$	*	***
Root + shoot length (%)	none	ns	***
Germination (%)	none	ns	*

Fig. 69 display the comparison between pure ChCl-based NaDES and the corresponding formulations from red grape pomace. Detecting these bar charts is possible to outline some main observations:

- All the ChCl-based NaDES combined with CA as HBD exhibited high toxicity on through the model organism tested: the values of root, shoot, root + shoot in percentage were always significantly lower to compare both to the control and pure NaDES (*Fig. 69a, Fig. 69b, Fig. 69c*).
- Considering the all the parameter the ChCl-based formulations containing polyphenols showed not homogeneous differences in comparison the corresponding to pure NaDES. On the other hand, by comparing the tested substances within the same HBD was possible to observe a general trend:
    - The formulations containing CA were never significantly different from the equivalent pure NaDES.
    - The basic formulations, with U as HBD, were always significantly lower than the corresponding pure NADES.
    - The neutral formulations, containing EG, exhibited values always significantly higher than the equivalent pure NaDES, the only exception for the shoot length parameter (%) (*Fig. 69b*) in which the same letter indicates non-significant differences.

  However, these values did not allow to establish whether the formulations had a promoter or inhibiting effect on *L. sativum* germination with respect to neutral NaDES.

Pure NaDES and NaDES formulation from algae biomasses

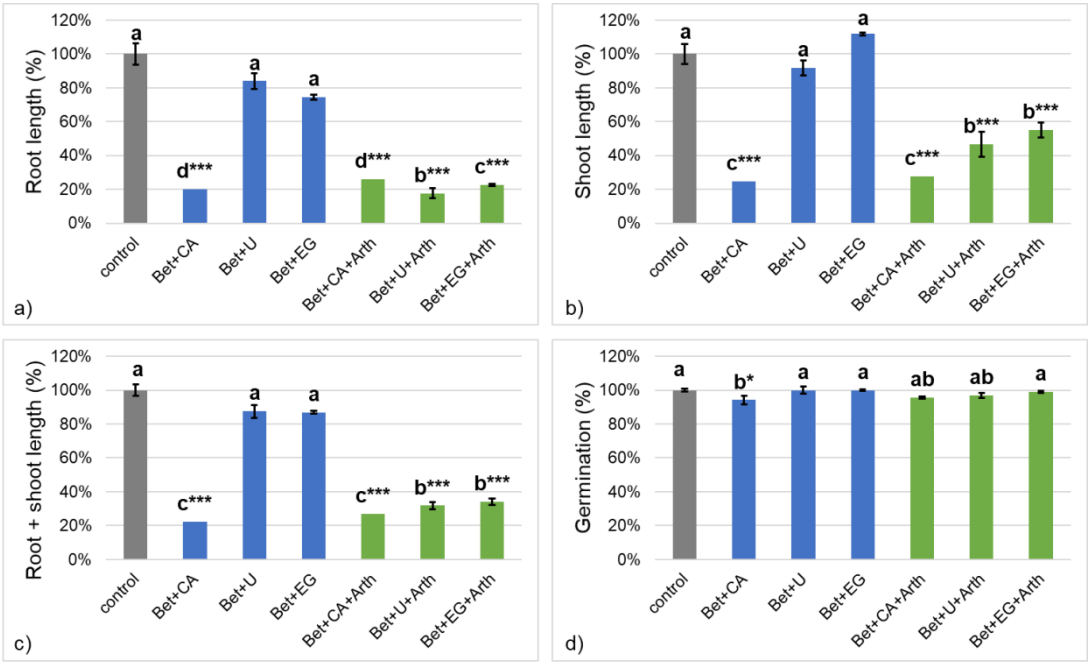


Fig. 70 Comparison among pure Bet-based NaDES and NaDES formulation *containing A. platensis* biomass considering a) the root length (%) b) the shoot length (%) c) total length of seeds germinated (%) and b) germination (%) calculated as percentage of the mean control value. Each point is the mean of four replicates, bars are ± standard error. Treatments marked with the same letter are not significantly different from each other ($p \geq 0,05$, Newman–Keuls post hoc test). Asterisks indicate a significant difference between a treatment and the control (* $p < 0,05$, ** $p < 0,01$, *** $p < 0,001$).

Table 35. Summary table of the transformations performed on endpoints values, the degree of significance of Cochran's C and p value of ANOVA analysis. (* $p < 0,05$, ** $p < 0,01$, *** $p < 0,001$). The information refers to the data shown in Fig 70.

Endpoint	Transformation	Cochran's C	ANOVA
Root length (%)	$\log_{10}$	**	***
Shoot length (%)	none	*	***
Root + shoot length (%)	none	ns	***
Germination (%)	none	*	*

Fig. 70 shows the comparison between pure Bet-based NaDES and the corresponding formulations from *A. platensis* biomass. Observing these results, it was possible to highlight some main trends:

- All the substances tested containing citric acid exhibited a high toxic effect on the *L. sativum* sprouts, in fact in all the parameters considered they showed significantly lower differences compared to the control and to all other treatments (*Fig. 70a, Fig. 70b, Fig. 70c*).

- All formulations based on algae biomass showed statistically significant differences compared to control and all other treatments. These formulations can be defined as highly toxic for *L. sativum* (*Fig. 70a, Fig. 70b, Fig. 70c*).

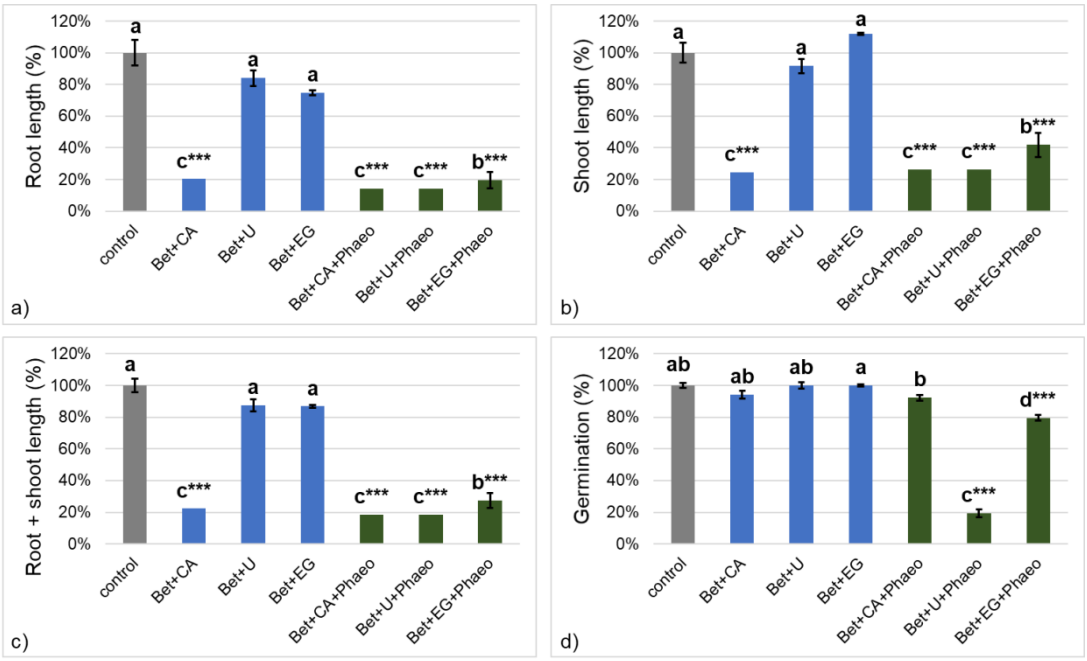


Fig. 71 Comparison among pure Bet-based NaDES and NaDES formulation *containing P. tricornutum* biomass considering a) the root length (%) b) the shoot length (%) c) total length of seeds germinated (%) and b) germination (%) calculated as percentage of the mean control value. Each point is the mean of four replicates, bars are ± standard error. Treatments marked with the same letter are not significantly different from each other ($p \geq 0,05$, Newman–Keuls post hoc test). Asterisks indicate a significant difference between a treatment and the control (* $p < 0,05$, ** $p < 0,01$, *** $p < 0,001$).

Table 36. Summary table of the transformations performed on endpoints values, the degree of significance of Cochran's C and p value of ANOVA analysis. (* $p < 0,05$, ** $p < 0,01$, *** $p < 0,001$). The information refers to the data shown in Fig 71.

Endpoint	Transformation	Cochran's C	ANOVA
Root length (%)	none	*	***
Shoot length (%)	none	*	***
Root + shoot length (%)	none	ns	***
Germination (%)	none	ns	***

Fig. 71 shows the comparison between pure Bet-based NaDES and the corresponding formulations from *A. platensis* biomass.

In *Fig. 71* the same NaDES evaluated in the previously comparison (*Fig. 70*), namely Bet+CA, Bet+U, Bet+EG) were compared, but in this case the formulations were prepared using the biomass of P. tricornutum:

- Acidic NaDES were always highly toxic for *L. sativum*, in all the tested parameters (*Fig. 71a, Fig. 71b, Fig. 71c*).
- Algae formulations showed always significantly lower values than the pure NaDES and the control, highlighting their hazardous effect on the model organisms (*Fig. 71a, Fig. 71b, Fig. 71c*).

Acid Bet-based NaDES

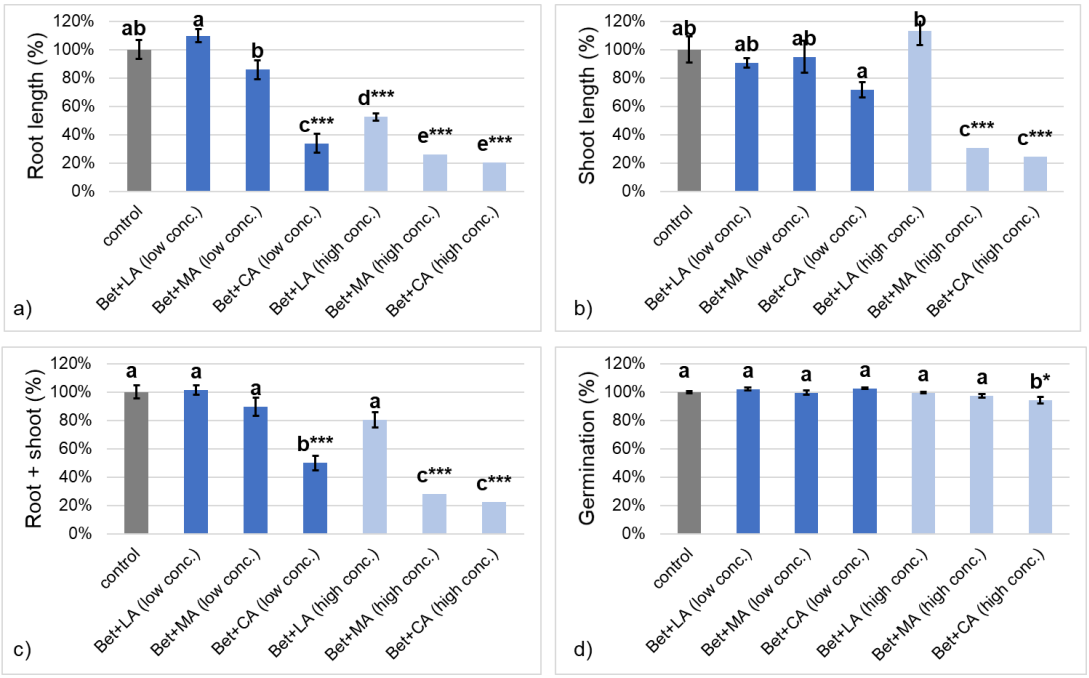


Fig. 72 Comparison among Bet-based NaDES combined with different carboxylic acid (LA, MA, CA) evaluated at high concentration (blue) and low contrition (light blue). Bar charts show a) the root length (%) b) the shoot length (%) c) total length of seeds germinated (%) and b) germination (%) calculated as percentage of the mean control value. Each point is the mean of four replicates, bars are ± standard error. Treatments marked with the same letter are not significantly different from each other ($p \geq 0,05$, Newman–Keuls post hoc test). Asterisks indicate a significant difference between a treatment and the control (* $p < 0,05$, ** $p < 0,01$, *** $p < 0,001$).

Table 37. Summary table of the transformations performed on endpoints values, the degree of significance of Cochran's C and p value of ANOVA analysis. (* $p < 0,05$, ** $p < 0,01$, *** $p < 0,001$). The information refers to the data shown in Fig 72.

Endpoint	Transformation	Cochran's C	ANOVA
Root length (%)	none	ns	***
Shoot length (%)	none	ns	***
Root + shoot length (%)	none	ns	***
Germination (%)	none	ns	***

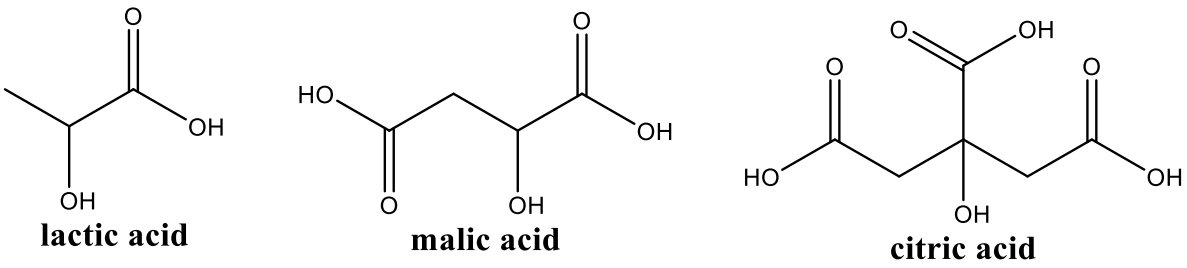


Fig. 73 Chemical structure of the carboxylic acids used as HBD. carboxylic acid functional group

Fig. 72 shows the comparison among three different acid NaDES evaluated at low concentrations (blue bars) and at high concentrations (light blue bars). This comparative study was carried out in order to evaluate:

1. The relationship between the structure of carboxylic acid and phytoxicity.
   Literature data show that the toxicity of NaDES increased with the increase in the number of acid functions [66]. In order to test this theory, acids with an increasing number of acid functions have been used: lactic acid, malic acid and citric acid, that showed respectively 1, 2 and 3 carboxyl groups. All the tested NaDES were prepared with the same molar ratio among HBA and HBD (1:1), therefore considering that NaDES contained the same quantities of betaine, the differences among phytotoxic effects could be related to the type of carboxylic acid used.
2. The effect of acid concentration on phytotoxicity.
   The toxicity of NaDES appears to increase as the acid concentration increases in the eutectic mixture [66]. In this test the same three acidic NaDES were then tested at low and high concentrations: in low concentration tests, the 50 ml of aqueous solutions the acidic NaDES was prepared using 2 mM of the acidic HBD, while for the high concentration test the 34 mM of the acids evaluated were used the eutectic mixture.

From the results shown in the *Fig. 72* it was possible to highlight some observations:

- In all the evaluated parameters the three acid NaDES tested, both with high and low concentrations, showed the following toxicity trend: Bet+LA < Bet+MA < Bet+CA. Despite these differences were evident by comparing the height of the bar charts, the analysis of the variance did not classify these differences as statistically significant (*Fig. 72*a, *Fig. 72*b, *Fig. 72*c).

- Similar to what observed in all previous tests performed, NaDES containing citric acid always showed significant differences with respect to the control. These acidic mixtures exhibited a highly toxic effect on *L. sativum* both at high concentrations and at low concentrations (*Fig. 72*a, *Fig. 72*b, *Fig. 72*c).
- NaDES containing MA in high concentrations' tests also showed significant differences compared to control in all the parameters evaluated. Also the NaDES produced using this acidic HBD exhibit strong phytotoxic effects (*Fig. 72*a, *Fig. 72*b, *Fig. 72*c).
- Overall, all acid NaDES, with the exception of the values of Bet+LA in the shoot length and root+shoot length parameter show high phytotoxic effects when tested at high concentration, with significant differences compared to the control (*Fig. 72*b, *Fig. 72*c).

In conclusion, from the analysis of these results it is possible point out some observations:

- None of the NaDES formulations exhibit promoting effects on *L.sativum*'s sprouts (from *Fig. 68* to *Fig. 71*). Considering the beneficial effect of polyphenols on plants these results are unexpected: polyphenols are indeed primary sources of antioxidant and provide protection against pathogens, infection and environmental stress [118,119]. Probably this test and the evaluated endpoints are not suitable to highlight the beneficial effect produced by these compounds. It should also be kept in mind that the red grape pomace formulations contain, in addition to the polyphenols, other biological compounds, namely proteins and polysaccharides (*Table 18*) which were successfully extracted by all the tested NaDES (*Table 15* and *Table 16*). Thus, the effects produced on *L. sativum* could also be attributed to the occurrence of these bioactive compounds. Further investigations must be carried out to clarify this phenomenon.

  All treatments exposed to algae formulations exhibited toxic effects. These results are unexpected considering that algae are a bioactive constituent of fertilizers worldwide used in agriculture [165]. Moreover, the same microalgae were already tested as effective resistance inducers against pathogens (*Plasmopara viticola* (peronospora) in plants without producing side effects [152]. The divergent results obtained in this thesis work may be due to several factors:

- The formulations tested as inductors of resistance against phytopathogens was produced using a phosphate buffer (KH_2PO_4) [152] while in this thesis the formulations were based on NaDES. Perhaps the substances extracted with the buffer were different from those present in the formulations based on NaDES, which contained the whole biomass of the algae.
- Different plants and plant's life stage evaluated: germination phase on *L. sativum* in this thesis work and adult *V. vinifera* plants in the other experiment.

- All acid NaDES produced significant inhibitory effects on *L. sativum* independently of the HBA used in the eutectic mixture (from *Fig. 68* to *Fig. 72*). The overall consistency of the results suggested the HBD as the main driving force behind the toxicity of acidic NaDES. Other study, carried out on model organisms at different trophic level (wheat [77], bacteria [66,78], cell lines [80]) confirm that the effect of acid NaDES is preponderant in toxicity. Some of the studies hypnotised that the toxic effect is probably due to the acidic nature of the HBD, consequently the corresponding NaDES were expected to have particularly low pH, detrimental to the survival of the organisms tested [77,128,66].

  Overall, acid NaDES showed high phytotoxic effects when tested at high concentrations (*Fig. 72*). These results were consistent with literature data, toxicity tests carried out on *Vibrio fisheri* demonstrated that the toxicity of ChCl-based NaDES increased with content of the acidic HBD tested (acetic acid, citric acid, lactic acid, glycolic acid), following the same ascending pattern of toxicity in all the experiments: ChCl << ChCl+acid (2:1) < ChCl+acid (1:1) < acid. In other words, any studied NaDES had an intermediate value of toxicity when compared to the starting materials (acids and ChCl), and the toxicity increased with the acid content [66].

  Moreover, as showed in *Fig. 72* the toxicity of NaDES seems to increase alongside the number of the functional groups constituting the carboxylic acid increased, showing the following toxicity trend: LA < MA < CA. These data confirm the results obtained in the ecotoxicological test on *V. fischeri*, which highlight that within the NaDES tested the ascending toxicity pattern is related with the structure of the acid: acetic acid < lactic acid < glycolic acid < citric acid. In line with the results of our test, also [66].

Table 38. pH values of some of the pure NaDES, NaDES formulations and individual component used in the germination tests with *L. sativum*.

NaDES	pH	NaDES	pH	NaDES	pH	Individual componets	pH
Bet+CA	2,5	Bet+U	6,2	Bet+EG	6,0	Bet	6,2
Bet+CA+pol	2,7	Bet+U+pol	7,0	Bet+EG+pol	6,4	ChCl	6,3
Bet+CA+Arth	2,6	Bet+U+Arth	8,2	Bet+EG+Arth	5,6	EtGly	6,1
Bet+CA+Phaeo	2,8	Bet+U+Phaeo	9,3	Bet+EG+Phaeo	7,4		
ChCl+CA	2,6	ChCl+U	6,4	ChCl+EG	6,1		
ChCl+CA+pol	2,6	ChCl+pol	5,9	ChCl+EG+pol	5,5		

Based on literature information, also in this study the pH values of the NaDES solutions used in the germination toxicity test on *L. sativum* were assed. As expected, the solutions containing citric acid showed particularly low pH values, from 2,5 (Bet+CA) to 2,8 (Bet+CA+Phaeo) (*Table 38*) consistent with the known importance of the HBD on the acidity of the corresponding eutectic mixture [80]. These data confirm that the dominant role of CA on the observed NaDES toxicity was probably due to its pH, far below the optimal pH required for *L.sativum* growth. This species prefers a relatively narrow pH range between 6,0, values comparable to all the other neutral or basic NaDES solutions (*Table 38*) that generally showed moderate or no toxic effect on *L. sativum* germination [77].

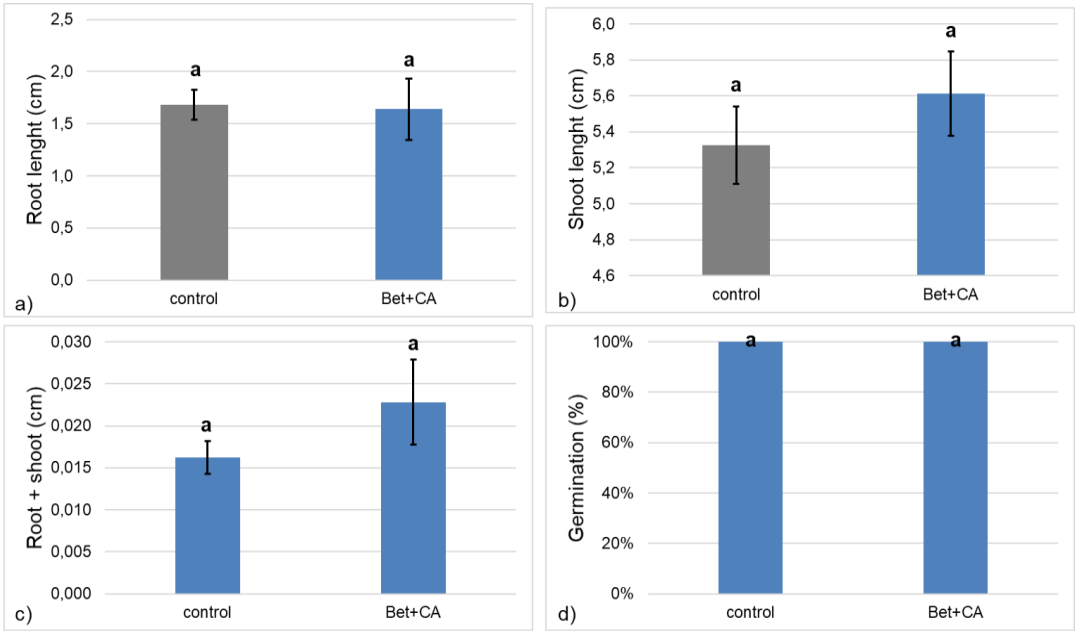


Fig. 74 The bar charts show a) the root length b) the shoot length c) total length of seeds germinated and b) percentage of germination (calculated as the number of seeds germinated in each treatment in comparison to the total) of the untreated seeds and the seeds treated with Bet+CA in soil. Each point is the mean of four replicates, bars are ± standard error. Treatments marked with the same letter are not significantly different from each other ($p \geq 0,05$, Newman–Keuls post hoc test). Asterisks indicate a significant difference between a treatment and the control (* $p < 0,05$, ** $p < 0,01$, *** $p < 0,001$).

Table 39. Summary table of the transformations performed on endpoints values, the degree of significance of Cochran's C and p value of ANOVA analysis. (* p < 0,05, ** p < 0,01, *** p < 0,001).

Endpoint	Transformation	Cochran's C	ANOVA
Root length	none	ns	ns
Shoot length	none	ns	ns
Root+shoot length	none	ns	ns
Germination %	none	ns	ns

Considering that the formulations used in the present study were designed with the purpose to be applied in agricultural field, the same experiment carried out in Petri dishes was reproduced in soil to assess whether the toxicity of citric acid was maintained. The same quantity of Bet+CA applied on *L. sativum* in Petri's tests at high concentration (*Table 9*) was mixed to 200 g of artificial soil in which ten cress' seeds were placed. After about 14 days the endpoints evaluated in the Petri tests and the dry weight of the total biomass were evaluated. The results (*Fig. 74*) showed that the NaDES containing CA when applied in soil lose the evident toxicity exhibited in the Petri's test, none of the parameters in fact was significantly different from the control. The inhibition on germination, and on root and stem's growth showed in Petri's tests could be explained by the direct contact between the NaDES solution and the seed, while, in the pot's tests, the soil would act as an intermediary between the seeds and the NaDES. In addition, as shown in the table, the pH of the soil at the end of the experiment was about 6, therefore optimal for the growth of *L. sativum*. Probably the components that made up the soil (sand, clay and peat) have levelled the pH increasing its value.

4.4.3 Seedling emergence and early growth test in soil (*Avena sativa*) – Terrestrial toxicity

The impact of NaDES on crops was assessed evaluating the emergence and early growth of oat's seeds (*Avena sativa)* exposed to the tested solvents after to be mixed with artificial soil. For this phototoxicity assay the following substances were tested (*Table 11*):

- Bet and ChCl-based NaDES combined with CA, U, EG, and the corresponding NaDES formulations carrying polyphenols (extracts from red grape pomace)
- Algae formulations prepared by Bet-based NaDES combined with CA, U and EG as HBD.
- NaDES individual components such as Bet and ChCl (34 mM), U and EG (68 mM)

Seedling emergence

Table 40. Seeds germination in each treatment, expressed as mean of the number of seeds emerged. From the right: Bet-based NaDES (with and without polyphenols) ChCl-based NaDES (with and without polyphenols), Bet-based algae formulations, Components used in final experiment to asses urea toxic effect on *Avena sativa*.

Treatment	n° seeds germ.	Treatment	n° seeds germ.	Treatment	n° seeds germ.	Treatment	n° seeds germ.
control	9,0	control	9,0	control	9,0	control	9,0
Bet+CA	9,3	ChCl+CA	9,3	Bet+CA+Arth	9,5	Bet+U	9,3
Bet+U	9,3	ChCl+U	9,3	Bet+U+Arth	8,5	ChCl+U	9,3
Bet+EG	9,8	ChCl EG	9,8	Bet+EG+Arth	9,3	Bet	9,8
Bet+CA+pol	9,5	ChCl+CA+pol	9,5	Bet+CA+Phaeo	9,8	ChCl	9,5
Bet+U+pol	8,3	ChCl+U+ pol	8,3	Bet+U+Phaeo	8,8	U	8,3
Bet+EG+pol	9,3	ChCl+EG+pol	9,3	Bet+U+Phaeo	9,0	EG	9,3

Table 41. Summary table of the transformations performed on endpoints values, the degree of significance of Cochran's C and p value of ANOVA analysis. (* $p < 0,05$, ** $p < 0,01$, *** $p < 0,001$). The information refers to the data shown in Fig.

Endpoint	Transformation	Cochran's C	ANOVA
n° seeds germ.	none	ns	ns

The number of seeds germinated after the exposure to NaDES, NaDES formulations and individual components are showed in *Table 40*. Bear in mind that in every pot a total of ten seeds were planted just above the soil. From the results presented in *Table 40* it was possible to highlight that:

- Emergence rates ranged from 8,3, representing the lower value assessed in ChCl+U+pol, to 9,8, the highest mean number of seeds emerged, showed in Bet, Bet+EG, CHCl+EG and Bet+CA+Phaeo.
- From the analysis of the variance performed on these values no significant differences were highlighted, thus none of the treatments tested produced adverse effects on *Avena Sativa* at germination level (*Table 41*).

Seedling growth

Bet-based pure NaDES and NaDES formulation carrying polyphenols

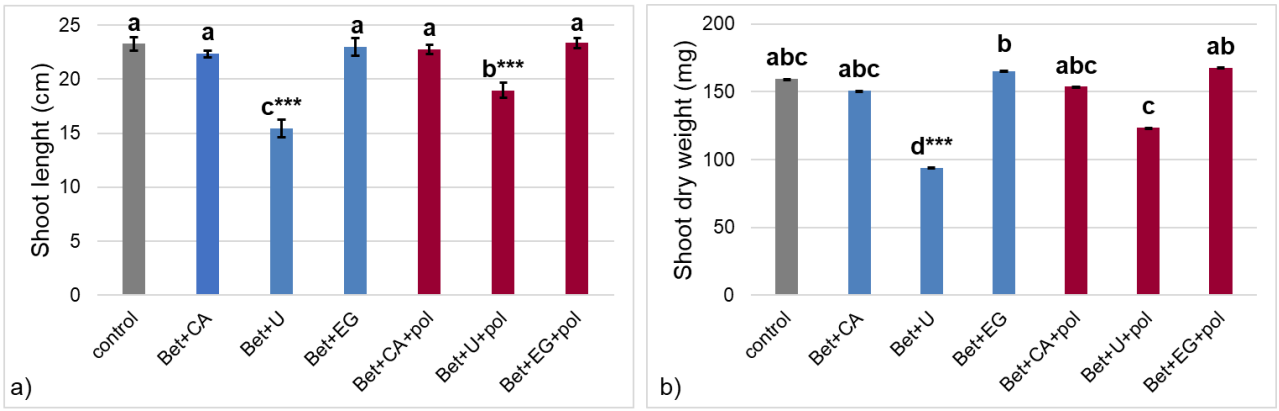


Fig. 75. The bar charts show a) the length of the shoot (cm) and b) dry weight of the shoot (mg) of the untreated seedlings, the seedings treated with Bet-based NaDES and the corresponding formulations with polyphenols. Each point is the mean of four replicates, bars are ± standard error. Treatments marked with the same letter are not significantly different from each other ($p \geq 0,05$, Newman–Keuls post hoc test). Asterisks indicate a significant difference between a treatment and the control (* $p < 0,05$, ** $p < 0,01$, *** $p < 0,001$).

Table 42. Summary table of the transformations performed on endpoints data and degree of significance of Cochran's C and p value of ANOVA analysis. (* $p < 0,05$, ** $p < 0,01$, *** $p < 0,001$).

Endpoint	Transformation	Cochran's C	ANOVA
Shoot length (cm)	none	ns	***
Shoot dry weight (cm)	none	ns	***

Fig. 75 and *Fig. 76* show the effects produced on seedling exposed to Bet-based NaDES and the corresponding formulations carrying polyphenols. In particular, *Fig. 75a* and *Fig. 75b* display respectively the plant's shoots lengths and dry weight for each treatment, from their analysis it is possible to highlight some main observations:

- In both parameters all treatments, apart from NaDES containing urea, did not show significant differences compared to controls. Therefore, was possible to state that these NaDES did not exhibit phytotoxic effects on these parameters (*Fig. 75*).
- Pure NaDES and NaDES formulations with urea as HBD, showed strong phytotoxic effects both in terms of elongation and of biomass of the shoots (*Fig. 75*). The value of Bet+U+pol shoot dry weight deviate from this trend, in fact, despite showing a lower mean weight than the control, this difference has not been calculated as statistically significant by ANOVA analysis, therefore it did not show a phytotoxic effect on shoot's biomass (*Fig. 75b*).

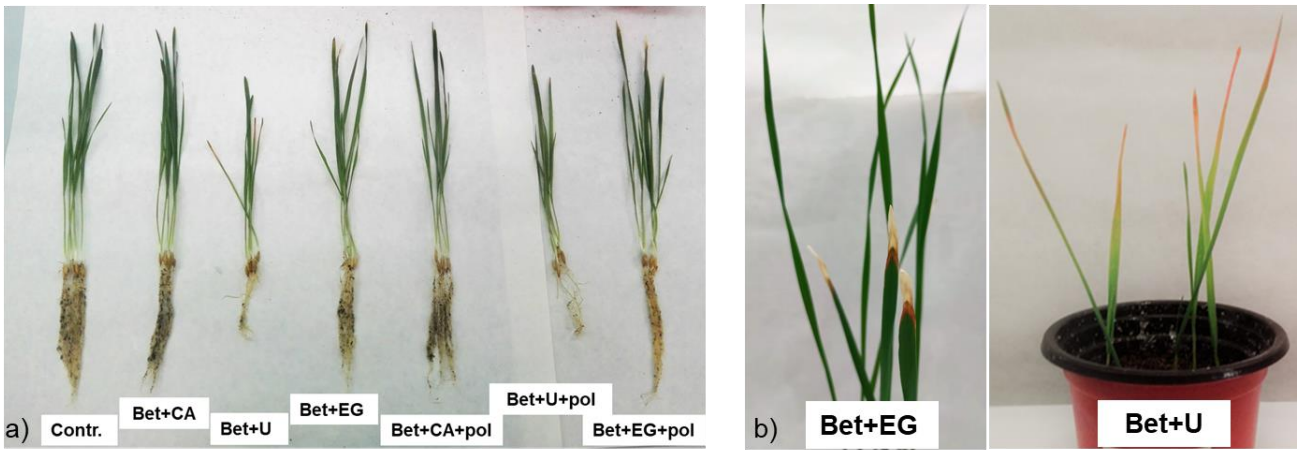


Fig. 76 a) From left to right, photographs of untreated seedlings, seedlings treated with Bet-based NaDES and corresponding formulations with polyphenols highlighting the phytotoxic effect of NaDES with urea on the roots. b) Detail of the yellowed and dried shoot of the seedling treated with Bet+EG and Bet+U.

Fig. 77. Detail of the roots, to the left root of an untreated seedling and right root of a seedling treated with Bet + U

In a similar way, the same effects described above were reflected on the root system (*Fig. 76a*). Two main types of effects on roots' growth were detected: the reduction of roots´ length was observed as the principal symptom of phytotoxicity, moreover an overall anomaly development of root system occurred in plants exposed to urea-based NaDES. These roots were characterized by a main root with very few secondary roots, on the contrary, the roots of the control or plants treated with acid and basic NaDES showed a much more complex mass of the root apparatus (*Fig. 76*).

In most of the seedlings treated with NaDES the terminal part of the stem was drier and yellowed. These detrimental effects were particularly evident in the plants exposed to urea-based NaDES were the shoot were marked with reddish-yellow colour that start at the tip and moves to the bottom of the leaf, while plants treated with EG-based NaDES showed drying and burn tips. The same effects were shown, in a major or minor extent, on all the seedlings treated with NaDES and NaDES with polyphenols (*Fig. 75*).

ChCl-based pure NaDES and NaDES formulation carrying polyphenols

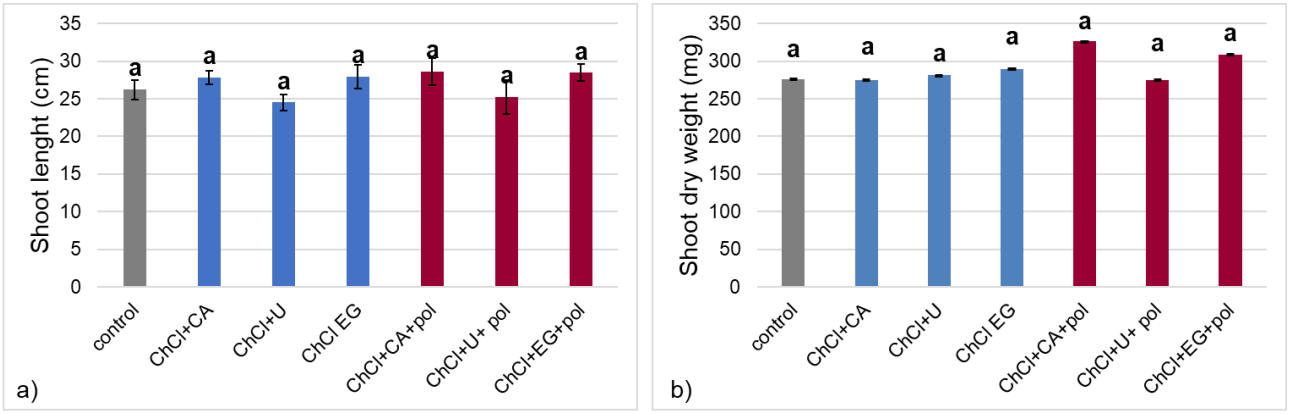


Fig. 78. The bar charts show a) the length of the shoot (cm) and b) dry weight of the shoot (mg) of the untreated seedlings, the seedings treated with ChCl-based NaDES and the corresponding formulations with polyphenols. Each point is the mean of four replicates, bars are ± standard error. Treatments marked with the same letter are not significantly different from each other ($p \geq 0,05$, Newman–Keuls post hoc test). Asterisks indicate a significant difference between a treatment and the control (* $p < 0,05$, ** $p < 0,01$, *** $p < 0,001$).

Table 43. Summary table of the transformations performed on endpoints data and degree of significance of Cochran's C and p value of ANOVA analysis. (* $p < 0,05$, ** $p < 0,01$, *** $p < 0,001$).

Endpoint	Transformation	Cochran's C	ANOVA
Shoot length (cm)	none	ns	ns
Shoot dry weight (g)	none	ns	ns

The same parameters previously described were statistically analysed on the shoots of the seedlings treated with ChCl-based NaDES and corresponding formulated with polyphenols. As showed in *Fig. 78* each bar is labelled with the same letter, this means that the analysis of the variance did not highlight any significant difference both between treatments and compared to control, therefore is possible to state that all tested ChCl-based NaDES had no toxic effect on *Avena sativa*'s shoots.

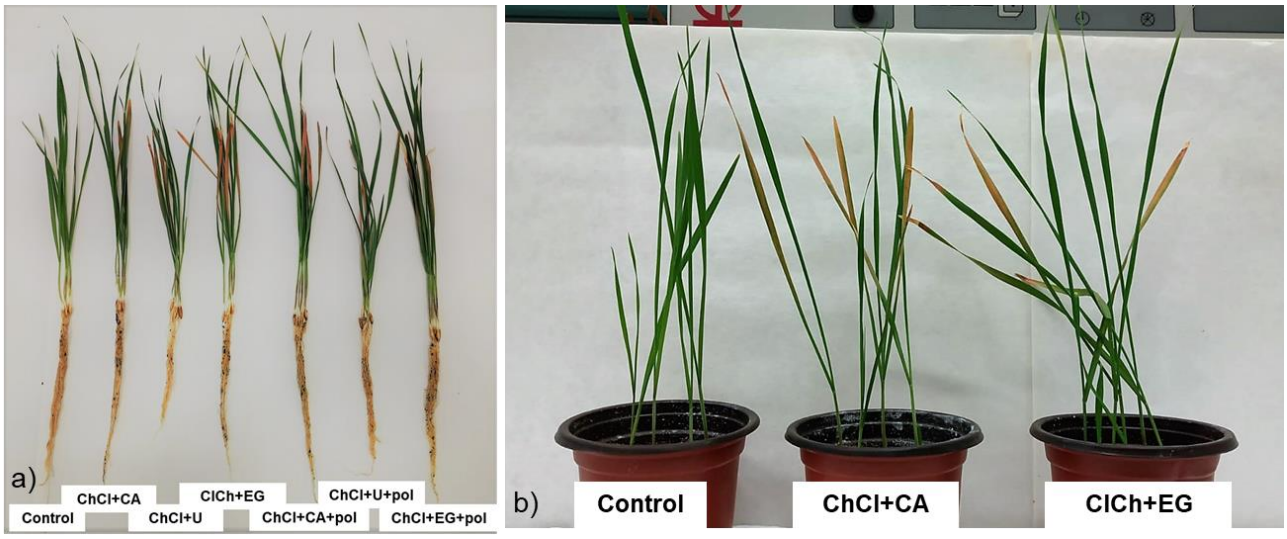


Fig. 79. a) From left to right, photographs of untreated seedlings, seedlings treated with ChCl-based NaDES and corresponding formulations with polyphenols highlighting the phytotoxic effect of NaDES with urea on the roots. b) Detail of the yellowed and dried shoot of the seedling treated with ChCl+CA and ChCl+EG in comparison to shoot's control that do not exhibit detrimental effect.

Consistent with the effects produced by Bet-based NaDES *Fig. 79* showed an adverse results produced by ChCl-based NaDES containing urea: plants exposed to ChCl+U and ChCl+U+pol resulted in a moderate inhibition of roots system compared to other treatments (*Fig. 79a*). Moreover, seedlings treated with ChCl-based NaDES show the yellowed shoot's tip compared to the control in most cases regardless of the NaDES used (*Fig. 79b*).

NaDES formulations from algae biomasses

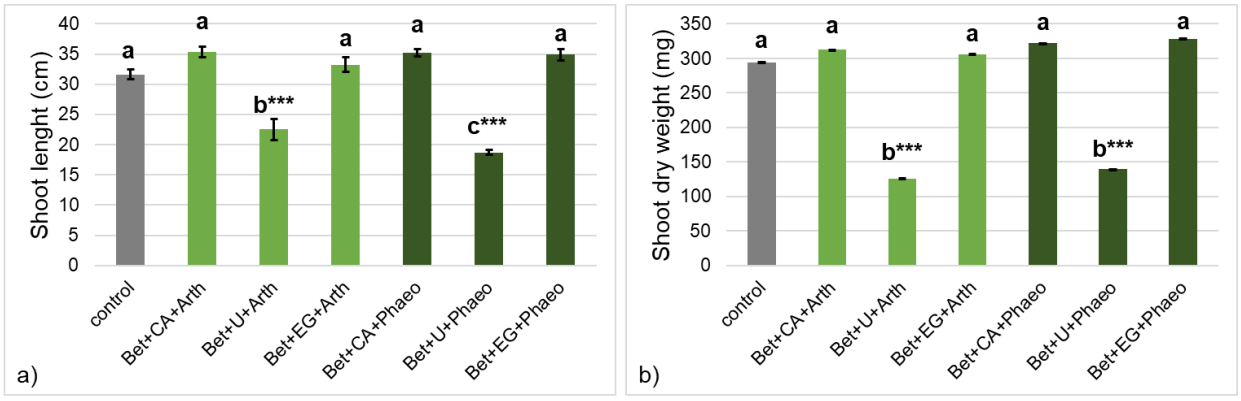


Fig. 80. The bar charts show a) the length of the shoot (cm) and b) dry weight of the shoot (mg) of the untreated seedlings, the seedings treated with Bet-based NaDES formulations with *A. platensis* (light green) *P. tricornutum* (dark green). Each point is the mean of four replicates, bars are ± standard error. Treatments marked with the same letter are not significantly different from each other ($p \geq 0,05$, Newman–Keuls post hoc test). Asterisks indicate a significant difference between a treatment and the control (* $p < 0,05$, ** $p < 0,01$, *** $p < 0,001$).

Table 44. Summary table of the transformations performed on endpoints data and degree of significance of Cochran's C and p value of ANOVA analysis. (* p < 0,05, ** p < 0,01, *** p < 0,001).

Endpoint	Transformation	Cochran's C	ANOVA
Shoot length (cm)	none	ns	***
Shoot dry weight (g)	none	ns	***

In addition to the NaDES formulations carrying polyphenols, the phytotoxicity of algal based formulations (both with *A. platensis* and *P.tricornutum*) has also been tested in soil medium. *Fig. 80a* and *Fig. 80b* show the effects of Bet-based algae formulations compared to control. In this case the phytotoxic effects of pure NaDES were not evaluated as they had already been tested with the two previous tests (*Fig. 75* and *Fig. 78*).

From the statistical analyses results performed on the shoot's length and dry weight (*Fig. 80*) it is possible to highlight some trends:

- Likewise to the outcomes obtained in the previous tests (*Fig. 75* and *Fig. 78*) the algal formulations produced with NaDES combined with CA and EG as HBD, did not show significant differences from the control, both considering the lengthening of the stem and the dry biomass (*Fig. 80*a and *Fig. 80*b).
- Consistent with results in *Fig. 75*, all formulations containing urea produced statistically significant effects compared to control in both the shoots endpoints evaluated (*Fig. 80*). These data highlight the toxic effect of this HBD in the early stages of seedling growth.

Fig. 81. a) From left to right, photographs of untreated seedlings, seedlings treated with Bat-based NaDES formulations from *A. platensis* and form *P. tricornutum* biomasses, highlighting the phytotoxic effect of NaDES with urea on the roots. b) Detail of the yellowed and dried shoot of the seedling treated with algae formulations.

*Fig. 81*a confirm the negative influence of urea-based NaDES on early growth of plants: roots system exposed to Bet+U+Arth and Bet+U+Phaeo were significantly underdeveloped and growth was severely inhibited compared to control and formulations containing CA or EG, that did not exhibit toxic effect on development of root system.

As in the previous cases (*Fig. 76* and *Fig. 79*) the shoots exhibited burned tips when exposed to EG-based NaDES, while with NaDES combined with CA and U shoot showed dried tips and variations in the colouring. Although these effects were clearer in the plants treated with NaDES.

NaDES containing urea and individual component

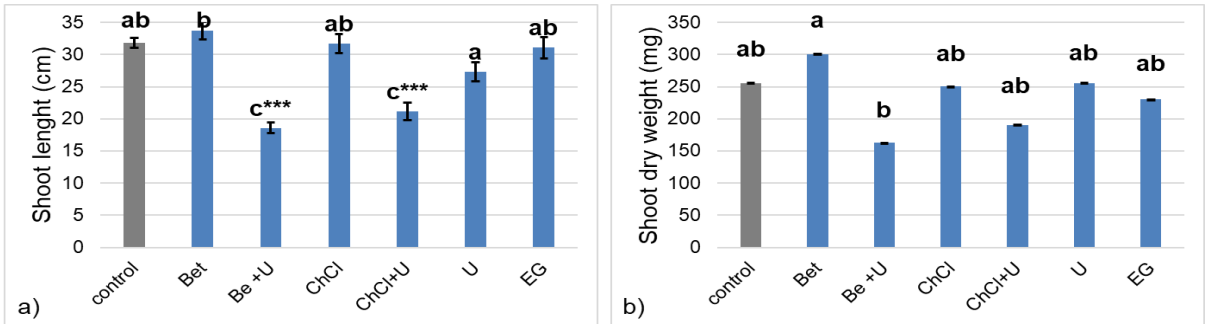


Fig. 82. The bar charts show a) the length of the shoot (cm) and b) dry weight of the shoot (mg) of the untreated seedlings, the seedings treated with individual components of NaDES (Bet, ChCl, U, EG) and urea-based NaDES. Each point is the mean of four replicates, bars are ± standard error. Treatments marked with the same letter are not significantly different from each other ($p \geq 0,05$, Newman–Keuls post hoc test). Asterisks indicate a significant difference between a treatment and the control (* $p < 0,05$, ** $p < 0,01$, *** $p < 0,001$).

Table 45. Summary table of the transformations performed on endpoints data and degree of significance of Cochran's C and p value of ANOVA analysis. (* p < 0,05, ** p < 0,01, *** p < 0,001).

Endpoint	Transformation	Cochran's C	ANOVA
Shoot length (cm)	none	ns	***
Shoot dry weight (g)	none	ns	*

Considering the evident deleterious effects of NaDES containing urea observed in phytotoxicity tests in soil and the lethal effects provided by the same substances on adult earthworms (*E. andrei*), it was decided to design a specific test to investigate the toxicity of urea-based NaDES. The phytotoxicity of the individual components (Bet, ChCl and URE) and the corresponding eutectic mixtures (Bet+U and ChCl+U) were tested to understand if the origin of the toxicity had to be attributed to the individual substances themselves or to a synergistic effect resulting from their combination. The EG was also tested because of its promoting effects on root and shoot growth observed in cress plants.

From the statistical analysis of shoot data *Fig. 82*a and *Fig. 82*b and observing *Fig. 83*, it is possible to make some observations:

- EG did not show significant differences compared to the control. (*Fig. 82).*
- Treatment exposed to urea-based NaDES, both combined with Bet and with ChCl, exhibited a statistically significant effect on shoot elongation: the shoot length of these plants was substantially lower than both the control and the individual components that make up the NaDES (Bet, ChCl and U). While, the corresponding HBA e HBD tested individually did not show significant differences compared to the control and therefore do not have a phytotoxic effect on the shoot growth (*Fig. 82a*). Similarly, the shoot's dry weight of the eutectic mixtures was lower than the corresponding individual components. However, even if these differences were manifest from the bar charts, the analysis of the variance identified only the NaDES based on Bet as statistically significant compared to Betaine, therefore with a lower dry weight of the biomass, while ChCl+U was not significantly different from the corresponding HBA (ChCl). Furthermore, none of the evaluated eutectic mixtures shows significant differences either with respect to the HBD (urea) nor to the control. (*Fig. 82*b).

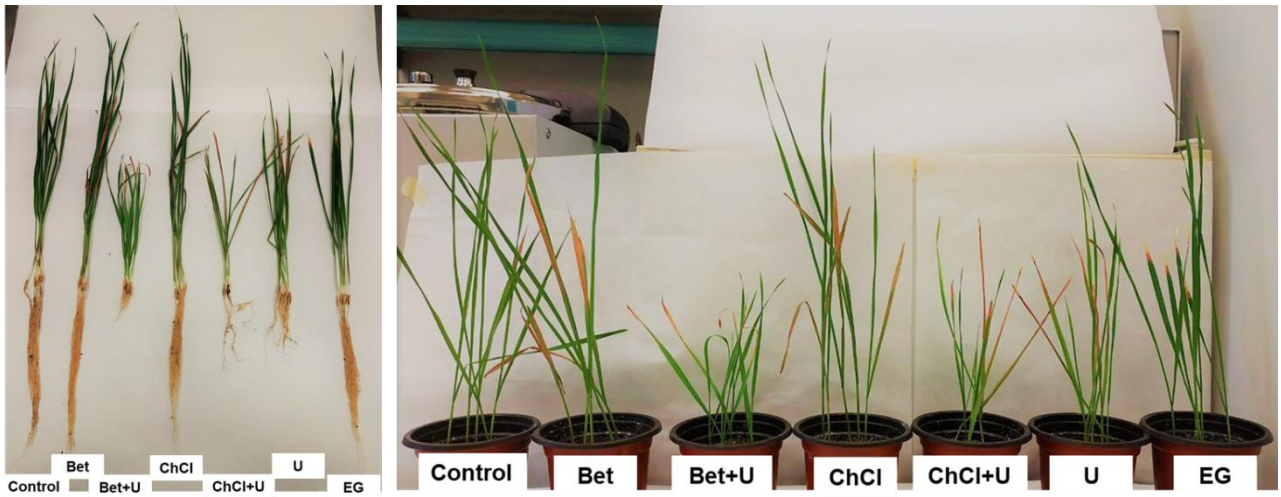


Fig. 83. a) Photographs highlighting the phytotoxic effect of urea and NaDES with urea on the roots in comparison to effect produced by the individual HBA and control. b) Detail of the yellowed and dried shoot of the seedling.

Likewise from the visual observations from the root system (*Fig. 83*a) it was evident how the exposure of the seedlings to the NaDES Bet+U and ChCl+U have provoked a drastic inhibition of the root development and therefore a phytotoxic effect, while the seedlings treated with the HBA solutions individually did not show differences on root's growth compared to control and therefore did not seem to induce any deleterious effect on the plants. Unlike the results obtained by considering the stem of the plants (*Fig. 83*a and *Fig. 83*b), the roots of the seedlings exposed to a solution of pure urea showed high symptom of phytotoxicity.

This could be the first time that NaDES phytotoxicity is assessed in soil system. So far, the impact of synthesized NaDES tested on wheat, garlic and fungi was tested using only ChCl-based NaDES on Petri dishes and filter paper or gauze [80] [146]. Even though Petri dishes tests are an established method used to assess the toxicity of substances on the flora, however these experiments ignore the contribution of soil media. The use of NaDES in the present study is projected in a direct application in the agricultural field, it is therefore important to evaluate the inhibitory and promotor effects of NaDES on plants within the soil system to recreate as far as possible a realistic condition of use.

Considering all the tests described so far, it is possible to outline some general trends of NaDES' effects in the early growth of plants:

- None of the treatments tested produced adverse effects on Avena Sativa at germination level. However, it is important to note that early growth emergence's rate is, in almost all cases, the less sensitive endpoint [151,80]
- None of the tested formulas, neither containing polyphenols nor algal biomass, have shown evident biostimulating effects on the elongation of plant's shoots or roots or on the increase in biomass produced. These results are unexpected considering the beneficial effect of polyphenols on plants described by other studies [119]. At the same time the results observed in study by algal-based NaDES diverge from the effects obtained by other products containing algal biomass widely used in agriculture as fertilizers for their growth-promoting properties of crops [173,174]. However, in these formulations commercially available, the algae's bioactive components were not delivered by NaDES but with other mediums. Thus, the lack of beneficial effect of these formulations is probably due to the solvent used as deliverer or to its components, even though both betaine and choline chloride are used in fertilizers for their biostimulating properties[175,176], furthermore the HBD used in this work are generally considered non-toxic.
- On the other hand, apart from products containing urea, none of the tested NaDES has produced toxic effects on the assessed endpoints. These results are aligned with phytotoxicity studied performed on wheat that revealed the non-toxic effects of tested ChCl-based NaDES on seed germination. However, it is important to note that the NaDES used in this test (ChC+OA, ChCl+Gly and ChCl+Glc), and the method used diverge from this study [77].
- Most of the stems of the seedling treated with NaDES showed died tips and an evident loss of the green colour, the leaf tended to yellow from the extremity towards the base of the stem. These phenomena could be correlated with the study assessing NaDES effects on wheat: they observed that the higher the NaDES toxicity, the increase in MDA content (an indicator of oxidative stress) and the decrease of chlorophyll content (other biochemical stress indicators) was grater 77. Perhaps also in this study an enhanced level of oxidative stress produced by NaDES exposition may contribute to photosynthetic system damage and thus chlorophyll degradation. In this study, however, no further tests have been carried out to verify, thus what has been said remains only a supposition. Moreover, in some of the experiments the same leaf discoloration was observed in the controls,

therefore this phytotoxic effect could be expression of nutrient deficiency [138]. In fact, none of the tested plants were treated with fertilizers to avoid interactions with the solvents tested.

- All the tested NaDES containing urea and urea tested individually had a harmful influence on root system of tested plants, resulting in a severe inhibition of their development. Overall, also plant's shoots exhibited toxic effect after the exposition to NaDES with urea, showing lower lengths and dry weight biomasses capered to control. These results aligned with literature data that reported the adverse effects of urea fertilizers on seed germination and seedling growth in soil. Aligned with the result found in this work another study observed that after the application in pot of equivalent of 70 or 700 lb. of urea per acre, at high nitrogen-rate urea applied on maize seed planted in soil produced severe root damage: "*the roots were very short* [..] *and ended in a scorched brown tip*". The author attributed the phytotoxicity to seedlings after the application of urea to soil due to the local accumulation of toxic amounts of free ammonia produced by the hydrolysis of urea [177].

  The results in *Fig. 82* showed that generally NaDES containing urea possess higher toxicity than their individual components toward the target model, this behaviour could be due to a synergic effect of forming NaDES. Some authors claim that the physicochemical propriety, namely the toxicity, of the HBA:HBD complex are different from those of the starting materials due to the hydrogen bonds created between both the HBA and HBD: "*the whole is other than the sum of the parts*" [130]. However, this effect was unexpected considering that high rate of water dilution of NaDES, as occurred within the aqueous solution used in this work, may lead to the complete disruption of the supermolecular structure of the eutectic mixture producing as result a simple solution of the free forms of the individual components in water [112]. Another study testing the toxicological activity of different aqueous solutions of ChCl-based NaDES on fungi, observed that while U and ChCl showed no inhibition as individual material, their final product as ChCl+U showed inhibition toward some fungi [131]. Other study explains that the difference in toxicity between NaDES and their individual components, tested in aqueous solutions, could be attributed to the extensive hydrogen bonding network throughout the NaDES, it could be sufficiently strong to prevent NaDES dissociation in aqueous solution,[178]. However literature data about synergic effect of NaDES are non-homogeneous,

therefore, is possible to say that the eutectic mixture and their individual components exhibit different toxicological profile based on the method followed and tested organisms in which each system exhibits different response, and the results, cannot be generalized over other systems or organism which each system exhibits different response [77].

In order to find an explanation for the toxicity found in plants also the pH of the soils after the end of the experiment was evaluated. Despite the low pH value of acidic NaDES solutions (*Table 38*) the soils treated with these products did not showed toxic effect on the early growth of plants in soil, as already demonstrated using *A. sativa* and *L. sativum* as model organism (*Fig. 74*) . On the contrary, NaDES containing urea in aqueous solution had a pH coherent with *A. sativa* optimal growth conditions (pH 6,3 for Bet+U and pH 6,2 for ChCl+U) (*Table 38*), showed phytotoxicity on the early growth of the plants. In order to better understand the influence of pH on the toxicity of plants in soil, at the end of each experiments soil's pH values were measured (*Table 46*): the pH of the soil ranges from a minimum of 6,0 to a maximum of 7,40, all these pH values are consistent with the optimal growth conditions for *A. sativa* and coherent within the limits set by the ISO standards for the test [138]. From these results pH was excluded as a possible source of toxicity for early growth plants in soil.

Table 46. pH values of some of the pure NaDES, NaDES formulations and individual component used in the germination tests with A. sativa.

NaDES	pH	NaDES	pH	NaDES	pH	Individual componets	pH
Bet+CA	7,2	Bet+U	7,0	Bet+EG	6,0	Bet	7,4
Bet+CA+pol	6,9	Bet+U+pol	7,3	Bet+EG+pol	6,4	ChCl	7,2
Bet+CA+Arth	7,4	Bet+U+Arth	7,4	Bet+EG+Arth	7,4	U	7,3
Bet+CA+Phaeo	7,4	Bet+U+Phaeo	7,3	Bet+EG+Phaeo	7,4	EG	7,2
ChCl+CA	6,5	ChCl+U	7,4	ChCl+EG	6,3		
ChCl+CA+pol	6,5	ChCl+pol	6,8	ChCl+EG+pol	6,5		

4.4.4 Phytotoxicity and water stress resistance assay with NaDES (*Ocimum basilicum* and *Lactuca sativa*)

Phytotoxicity assay

In order to evaluate the safety and efficacy of NaDES in the most complete way, were performed other phytotoxicity essays using two sensitive species, basil (*Ocimum Basilicum*) and lettuce (*Lactuca sativa*) [179] as model organisms.

For this test, six NaDES and corresponding formulations carrying polyphenols were tested. All the tested products had betaine as HBA combined with HBD showing different reactivity:

- Acid (citric acid)
- Basic (urea)
- Neutral (glycerol).

Table 12 shows the quantities of NaDES used to produce 1 L of solution which was then applied until dripping. In this case, only visual evaluations of plant's shoot were recorded.

At the end of the experiment (day 14) the safety of the products (absence of phytotoxicity symptoms) and the bio-stimulating effects in terms of expansion of the leaf surface and development of the seedlings were evaluated in both the model organisms, consequently the following observations were outlined:

- No residues of the product were observed on the leaf surface in any of the treated plants (*Fig. 84*).
- No symptoms of phytotoxicity emerged in any of the treated plants (*Fig. 84Fig. 84*).
- There were no variations in the colour of the leaves between the treated seedlings and the control (*Fig. 84*).
- No differences in the development or vigour were observed in basil plants exposed to NaDES and formulations compared to the control. Differently, lettuce plants exposed to Bet+CA and Bet+CA+pol showed a reduced development compared to the control and to the other treatment.
- Basil's plants exposed to Bet+CA and Bet+CA+pol showed some symptoms of Peronospora, an important disease in word-wide *O. basilicum* production, caused

by *Peronospora belbahrii* a types of oomycete microbes (organisms that belong to kingdom fungi) that is obligate parasites of plants [180].

- Observing the values of the wet weight biomass obtained at the end of the experiments (*Table 47*) was possible to observe a general trend in both basil and lettuce plants: Bet+G+pol > control > Bet+U+pol > Bet+CA+pol. A similar effect was obtained in basil's plants treated with pure NaDES: Bet+G = control > Bet+U > Bet+CA. All the plants treated with pure NaDES or formulations containing citric acid always showed the lowest biomass values, while all the plants treated with Bet+G showed values that were always higher than those of the control. It was not possible to highlight a clear trend from the comparison of pure NaDES and corresponding formulas.

In conclusion we can highlight some main trends:

- The plants treated with NaDES based on citric acid seem to be generally inhibited exhibiting reduced foliar growth, lower biomass and the presence, in some cases, of pathogenic organisms. These observations were consistent with the results obtained on the previously experiments on cress: NaDES containing CA constantly caused an inhibitory effect on plants germination reducing the length of the stems and the roots. The results obtained in this work aligned the literature data that show a greater toxicity of the acidic NaDES compared to those with different reactivity [77,128].
- NaDES containing U or G did not seem to show phytotoxic effects on plants.
- Consistent with the previously test on *L. sativum* and *A. sativa*, plants treated with NaDES formulations carrying polyphenol did not show clear advantages compared to the pure NaDES counterparts.

Fig. 84. a) *O. basilicum* and b) L. sativa plants at the end of the assay (day 14).

Table 47. The fresh weight of the biomass obtained from the six plants of *O. basilicum* and 60 plants of *L. sativa* treated with NaDES, with polyphenol formulations and untreated.

Treatment	***Shoot wet weight (g)***	
	O. basilicum	***L. sativa***
Bet+CA	148	148
Bet+CA+pol	157	184
Bet+U	161	254
Bet+U+pol	158	191
Bet+G	169	240
Bet+G+pol	176	225
Control	169	219

Water stress resistance assay

On the basil seedlings the capacity of the same Bet-based NaDES and formulations with polyphenols used in the phytotoxicity test were tested to induce resistance to water stress.

From day 7 and then every two days, photographic records were made to allow the observation of water stress resistance effects produced by NaDES and NaDES formulations. From the results obtained it is possible to make the following observations:

- The seedlings treated with Bet+G and Bet+G+pol presented a withering earlier than all the other treatments (*Fig. 85*a).
- Bet-based NaDES formulations carrying polyphenols did not produce an evident resistance to water stress in comparison to pure NaDES. These tests do not confirm literature data that indicate a positive relationship between accumulation of betaine and improvement of the plant stress tolerance to drought and other environmental stress[181].
- Over two weeks all the plants showed an irreversible wilt (*Fig. 85*c).

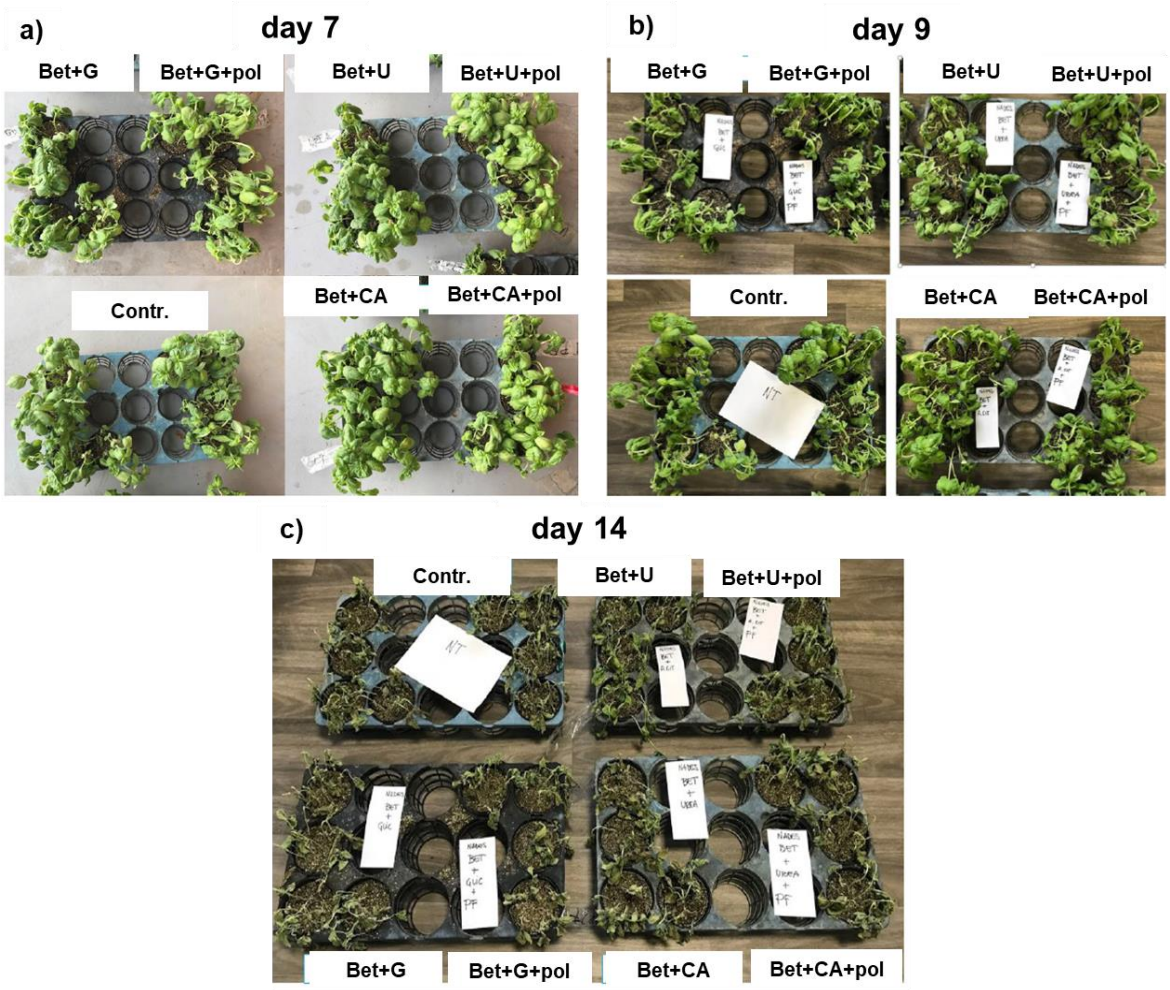


Fig. 85. Photographs of the water stress resistance performed on *O. basilicum.*

In conclusion both the pure NaDES and the NaDES with polyphenols do not seem to induce resistance to water stress in the seedlings. Further tests using different NaDES application methods and different substance concentrations are needed to better understand the use of betaine-based NaDES as resistance to hydric stress.

4.5 Biodegradability of NaDES

Since the aim of this work was to evaluate of the potential application of NaDES formulations in the agricultural field it was necessary to estimate the biodegradability of these substances, confirming their short persistence in the environment and thus their eco-compatible nature. NaDES biodegradability was tested using wastewater microorganisms inoculated in an aqueous mineral medium in which the NaDES to be tested were added. The degree of biodegradation was determined periodically over time by the detection of the COD (Chemical Oxygen Demand) decrease. The biodegradability of NaDES has been assessed using aquatic organisms due to the following reasons:

- To allow a comparison with the literature data. In fact, all the information concerning NaDES biodegradability were assessed using aerobic wastewater microorganisms [77, 129].
- It was reasonable to think that once applied NaDES formulations to the soil compartment these substances could migrate in the water sector, as result of leaching, and move from groundwater to aquatic ecosystems.
- NaDES have a polar and hydrophilic character, therefore they tend to accumulate in aqueous phases.

The NaDES selected for this test were:

- Bet+CA (acidic)
- Bet+U (basic)
- Bet+EG (neutral)

The glucose biodegradability was as well determined as a reference substance.

For the biodegradability assay aerobic bacteria (activated sludge) were taken from the local purifier and set in the laboratory for 4 days at 20°C. The concentrations of the substances tested are listed in *Table 13*.

As reported by ECHA, all the individual components of the tested NaDES were classified as readily biodegradable substances: betaine reached 88% mineralisation after 28 days [182], citric acid showed 97% degradation within the same time period [183], urea is completely biodegraded after 21 days [184] and after 10 days more than 90 % degradation of ethylene glycol was determined [185].

So far, the biodegradability of betaine-based NaDES has never been determined.

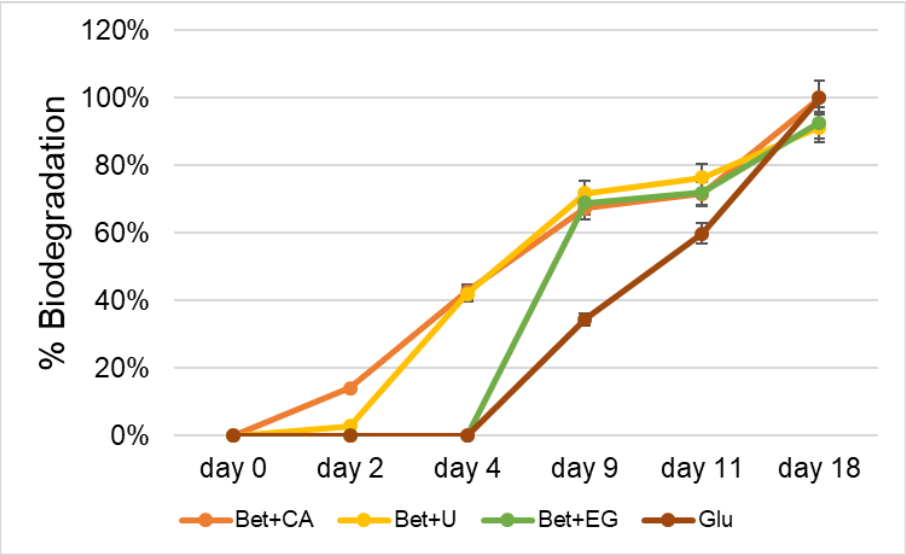


Fig. 86. Biodegradability of four betaine-based NaDES and glucose determined as COD decreasing in the mixture from day 0 (start of test) to day 18 (end of test). The percentage of biodegradability was calculated in comparison with to the initial COD values (day 0, showing 0% biodegradation).

Analyzing the test's results showed *Fig. 86* it was possible to highlight some main observations:

- The results of this test could be considered valid since the glucose, the reference substance known to be readily biodegradable in short time period, was completely degraded (100%) over 18 days (*Fig. 86*).
- All NaDES achieved almost complete biodegradability within 18 days from the start of the test: the acid NaDES showed the highest degradation (100%), followed by the neutral NaDES, Bet+EG, (93%) and the basic NaDES, Bet+U, (91%) (*Fig. 86*). Studies assessing the biodegradability of ChCl-based NaDES by using the closed bottle test (OECD 301D) confirm that DES containing citric acid (1:1) and urea (1:1) were classified as completely biodegradable. In support of this, previous work performed in this laboratory using the same biodegradability test (OECD 301D) showed that NaDES composed of choline chloride and urea (1:1) were completely degraded within only 3 days.
- The glucose and NaDES containing ethylene glycol started to degrade only between day 4 and day 9 (*Fig. 86*). This phenomenon was probably caused by the low winter temperature that affected the metabolic activity of the inoculum used in the test. It took a few extra days to get the wastewater bacteria fully acclimatized and able to start the degradation.

- Observing the biodegradation rates over time, glucose was the substance that showed the most linear trend: from day 4, in which the degradation started, the percentages of degradation increased in parallel with time. In contrast, NaDES containing urea e citric acid showed a high rate of biodegradation from day 0 to day 9, in which all NaDES were degraded more than 65%, while from day 9 to day 18 the degradation rates were lower, as showed by the less leaning curve (*Fig. 86*).

These data confirm that NaDES are highly biodegradable substances and therefore they could potentially be used in agriculture field without negative effect on groundwater compartment. Although the amount of available data concerning the biodegradability of NaDES was limited, it seems that the biodegradability of the individual components was maintained even within the NaDES, as confirmed by this study.

Future studies are needed to test the effective permanence of the NaDES and NaDES-formulations in the agricultural field. A too short period of biodegradation could be a disadvantage from an application point of view, because the culture would not be exposed enough time to the formulation to guarantee its effectiveness.

5 Conclusions

In the work the use NaDES as eco-compatible carriers for the development of bioactive-formulations and the delivery of natural high value compounds in agricultural field was evaluated.

Various terrestrial and aquatic biomass/by products were investigated as source of bioactive molecules. To this purpose, three grape by-products (red grape pomace, white grape pomace and grape seeds) and two microalgae (*Arthrospira platensis* and *Phaeodactylum tricornutum*) were initially characterized in terms of polysaccharides, proteins, lipids and polyphenols.

Among the three grape by-products evaluated, the red grape pomace biomass was the richest in polyphenols (7,2%, on dry weight basis, DW) and thus was chosen as most suitable for the creation of the NaDES formulations. Subsequently, the extraction capacity of different betaine and choline chloride-based NaDES combined with HBD showing different reactive reactivity was evaluated. The use of NaDES for the extraction of polyphenols from grape by-products biomass was promising, since the achieved extractions yields were similar to those obtained with conventional solvents. In particular NaDES based on glycerol as HBD exhibited the highest extraction ability (12,3%, on dry weight basis, DW). Apart from polyphenols, NaDES were also capable to extract grape pomace proteins and polysaccharides: the recovery percentages ranged from 56% to 100%, confirming the high extraction capacities of all evaluated NaDES. NaDES composed by Bet+U and ChCl+CA have the highest recovery capacities (100%) for polysaccharides and proteins respectively.

The formulations rich in polyphenol displayed an interesting antioxidant activity, in some cases higher than those of conventional solvents tested (EtOH). The two extracts with the best antioxidant activity were ChCl+EG+pol, showing an EC_{50} of 0,6 mg/mL and Bet+U+pol, with a value of 0,89 mg/mL. Moreover, the same formulations showed an interesting inhibition activity of the enzyme urease: the most promising extract was ChCl+EG+pol, capable of inhibiting enzymatic activity by about 90%. These results highlight promising potential applications of the red grape pomace formulations in the agricultural field.

The analysis displayed that both algae' biomasses possessed similar percentage of polysaccharide, 7% in *A. platensis* and 10% *P. tricornutum* (on dry weight basis, DW), and a high protein content, 63% and 49% (on dry weight basis, DW) respectively. The lipid fraction extracted from *P. tricornutum* (36% DW) consisted of 37% fatty acid rich in palmitoleic acid (16:0) which represented 28% of TFA. The biomass of *A. platensis* contained a lower lipid percentage (26%) with 12% of TFA, which the most abundant was palmitoleic (C16:1). Since the extraction of algal biomass with NaDES formed a homogeneous product, and not two separate phases as for grape pomace extracts, thus the whole algal matrix becomes part of the final formulations. Therefore, the percentage composition of PS, PR and LP in the algae-based formulations reflects the composition of the type of algae used for the extraction.

The obtained data on toxicity against earthworms, cress, oat, basil and lettuce indicate that pure Bet- and ChCl-based NaDES, NaDES formulations and their individual components have different toxicological profiles according to the tested organisms, the method of administration (e.g. mixed with ground, foliar-applications) and the matrix in which the organisms were tested (in soil or directly on filter paper). Each system exhibited different response, consequently it was not possible to outline and general and homogeneous toxicological trend.

For what concerns the toxicity test on *Eisenia andrei* it is possible to highlight the following considerations:

- Almost none of the life-cycle parameters was influenced by Bet-based NaDES combined with CA and EG, which can be defined as non-toxic to earthworms.
- All NaDES containing urea were highly toxic to the model organism, causing lethal effects from the first days of exposure.
- The HBA choline chloride seems to provoke harmful effects only against cocoons, by inhibiting their hatching capacity, while it does not cause negative effects at other stages of the life cycle.

The results obtained on *Lepidium sativum* in Petri did not show biostimulant effects produced by the NaDES formulations utilized, neither based on algal nor containing polyphenols. On the other hand, the tests show little or no toxicity of Bet- and ChCl-based

NaDES containing urea and ethylene glycol, confirming to be "safe" for plants. On the contrary, all the tests carried out confirm that the effect of acid HBD is preponderant in *L. sativum* toxicity. In line with literature data, the following toxicity pattern of acid HBD was shown: lactic acid < malic acid < citric acid. However, the toxic effect of NaDES Bet+CA was completely eliminated by reproducing the test in soil, presumably because the soil matrix is able to mitigate the toxicity produced by these solvents.

The results obtained from phytotoxicity tests in soil with *Avena sativa*, have shown parallelisms with both previous tests. As observed in *L. sativum*, the formulations containing bioactive compounds did not seem to induce growth-promoting effects on the plants. On the other hand, both Bet- and ChCl-based NaDES containing ethylene glycol and citric acid, and the corresponding compounds did not show an inhibitory effect, supporting the thesis of the NaDES as eco-compatible solvents. Consistent with the results obtained on *E. andrei* all urea-based NaDES produced a strong toxic effect on oat, but it is worth mentioning that the concentrations of NaDES used in these experiments could be greater than the concentrations that could affect the target organisms in the environment. In fact, in the field it is reasonable to assume a mode of exposition through foliar application in water solution, that could correspond to a lower NaDES dose/lower availability due to the presence of pore water and the interactions with the soil components.

Betaine-based NaDES formulations carrying polyphenol did not show clear advantages on *Ocimum basilicum* and *Lactuca sativa* growth. On the other hand, the experiments confirmed the safety of NaDES containing urea and glycerol that did not produce phytotoxic effects on the plants, while plants treated with products comprising citric acid seem to be generally inhibited. Furthermore, pure NaDES and the NaDES with polyphenols do not seem to induce resistance to water stress on *O. basilicum*.

To conclude, many of the NaDES-based formulations seem to possess excellent characteristics from an eco-compatibility point of view of, showing low or no toxicity and high biodegradability. From these preliminary results, however, they do not seem to be able to produce beneficial effects on agricultural crops, but they have excellent potential as inhibitors of the enzyme urease, which represents a major problem at the agricultural level.

Moreover, this study has shown the enormous importance in the choice of HBA and HBD used for the synthesis of NaDES and the toxic effect produced: since the same NaDES can show different effects according to the evaluated organism, it is necessary to keep the area in which it must be applied, thus choose the least impacting constituents.

However, the ecotoxicological evaluation of NaDES must be investigated deeper because the data available are not enough to predict their behaviour in soil compartment and, in general, in the environment. Further studies could provide better understanding of the relationship between the toxicity/biodegradability of NaDES and their components, thus guiding to the design of truly green solvents with a better ecotoxicological profile, while still keeping desired physiochemical proprieties of the solvents.

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