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Corso di Laurea Magistrale in
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Development of urease enzyme inhibitors and assessment of their efficacy and ecotoxicity

Tesi di Laure Magistrale in
Bioraffinerie e Sostenibilità

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1. Introduction

1.1 Sustainable development and the green approach

The first time that we have heard of sustainable development has been with World Commission on Environment and Development's (Bruntland Commission),which introduced this concept in the report "Our Common Future" in 1987 defining it like " that development able to ensure the satisfaction of the needs of the present generation without compromising the possibilities of future generations"[1].

The term “environmentally sustainable” refers to biophysical conditions of the Earth and the use that is made of its resources. The main concept that based on sustainability is that planet resources cannot be exploited or used infinitely.

Biophysical conditions of Earth and its resources are basic growth factors and irreplaceable and unlike what it might seem, sustainability is not an anti-growth model but rather a model that pursues development in such a way that resources are respected and used wisely.

A step forward has been done with the introducing of the concept of “green chemistry” after the approval of the Pollution Prevention Act (1990) in the USA[2]. It was born in the 1990s with the purpose to reverse the global environmental deterioration and it was recognized as a scientific field in the 2000s. The term “green chemistry” was mint by Paul Anastas and John Warner, staff of the Environmental Protection Agency (EPA), who saw the right way for a collaboration between government, industry and university.

The first definition of “green chemistry” was " design of chemical products and processes to reduce or eliminate the use and generation of hazardous substances" and based on this definition, chemists began to re-evaluate all life cycle of chemicals to maximize efficiency and reduce health and environmental hazards[3].

It is important to highlight that this green approach goes beyond the concern of chemical toxicity risks but also includes energy conservation, waste reduction, use of renewable sources and designing life cycles[4] including the end of life of products. Therefore, this approach has been developed not just to have sustainability in the environment but also economic and social performances.

Green chemistry tries to redesign the materials that make up the basis of our society and our economy in a way that is benign for humans and the environment, but this is a big challenge because there is a variety of chemicals synthetic pathways and to describe what is sustainably and what is not is very difficult. Help is given by Anastas and Warner, who formulated the Twelve Principles (*Fig.1*) of green chemistry[5,6], criteria that provide chemists achieve the goal of sustainability. They are a guideline for the design of safe chemicals and chemical transformations to be applied to all aspects of the life cycle of the products. Green chemistry tries to achieve the goal of sustainability by a careful planning of chemical synthesis and molecular design with limited damage[5,7].

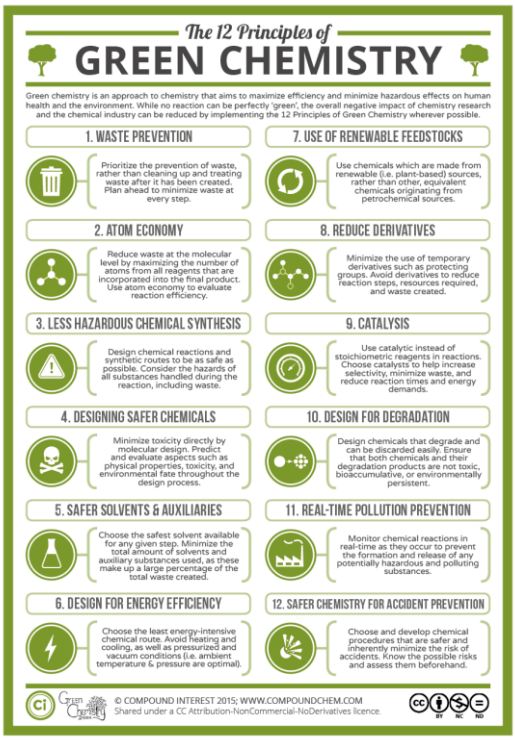


Fig. 1 The Twelve Principles of Green Chemistry

Therefore, the introduction of green chemistry did yes to create a global challenge for environmental protection adopting a strategy which has been set out with the adoption of legislative acts. Indeed, the two main changes were the adoption of REACH (Registration, Evaluation, Authorisation and Restrict of Chemicals) regulation in 2006, which assures a high protection of human health and the environment across the identification and registration of properties of chemicals produced or imported in EU[8], and the introduction of IPPC Directive in 2008[9] which underlines the

fundamental necessity of safer processes for reducing industry impact and defines the concept of BAT (Best Available Technologies) for each professional sector.

1.2 Application of green chemistry

In the framework of green chemistry, a problem that scientific research tries to resolve is the development of innovative technologies that break away from the old methods[10]. In particular, the processes of extraction of natural compounds have been modified following the principles of green chemistry. Among the various methods there are solvent extraction, distillation, pressing and sublimation according to the extraction principle. Extraction is the first step to separate the desired compounds to raw material and the solvent extraction is the most used method. The extraction process follows these four stages[11]:

1) solvent penetrates solid matrix
2) solute dissolves in the solvent
3) solute is diffused out of the solid matrix
4) the extracted solutes are collected

The traditional methods to extract compounds of high value (e.g antioxidants, perfumes) require a lot of solvents and high energy, but the yields are often lo[11]. If we must use these extracted compounds in cosmetic, pharmaceutical or food industry we must separate the solvent because the final product must be free from solvents and this process requires further energy, consumption and produces another environmental impact.

Over the last decade, scientific community is developing new green and efficient methods considering all the potential effects and to ensure safety to humans and the environment. The green extraction approach has three main challenges[10]:

1) to increase extraction process efficiency for compounds of interest
2) to have the lowest possible impact on the environment
3) to plan recycling or reuse of substances and products, in a life cycle thinking.

The focus is to find solutions that minimize the use of solvents and in literature we find many example of green extraction techniques like supercritical fluid extraction (e.g. supercritical CO_2, supercritical H_2O), ultrasound-assisted extraction (UAE)[12], microwave assisted extraction (MAE)[13,14] and instant controlled pressure drop[15].

Each of these methods has advantages and limits, which must be considered to identify the appropriate techniques for the compounds and matrix of interest[16].

1.3 Sustainable solvents

Solvents are chemicals that dissolve solutes and form solutions, facilitate many reactions. They are used for everything from extractions to dry cleaning to paints and much more they can be as benign as water or as hazardous as dichloromethane. Because they are so ubiquitous, using toxic solvents affects millions of workers every year and has implications for consumer and the environment.

Solvents are a key priority in green chemistry because they are used in high volumes and many are volatile organic compounds (VOC). Their use creates large amounts of waste, air pollution, and other health impacts[17]. Finding safer, more efficient alternatives or removing solvents altogether is one of the best ways to improve a process or product.

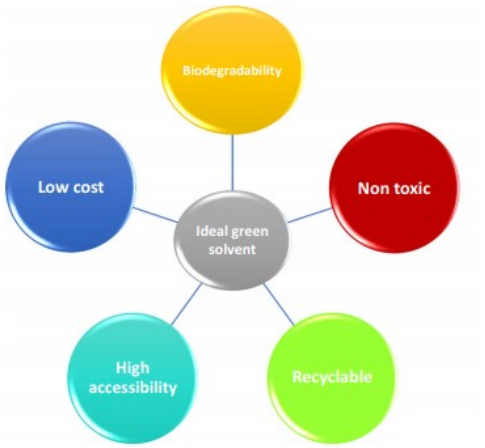


Fig. 2 Ideal solvent characteristics

The green chemistry principles that regard sustainable solvents are three[2]:

1) to use safer solvents and auxiliaries, the greenest solvents are the ones that are not used, but choosing innocuous ones when needed is a key component of green chemistry,
2) inherently safer chemistry for accident prevention, using safer solvents and preventing hazardous waste minimizes the potential for chemical accidents, including releases, explosions, and fires
3) eliminate and minimize hazards and pollution, choosing inherently safer solvents (or no solvents at all) prevents the generation of unnecessary

hazardous waste while improving the safety of the chemical processes involved.

In the literature there are several solvent selection guides with the purpose of promoting the use of more sustainable solvents[18,19]. For example, there is a guide developed by CHEM21[19], a European consortium which promotes sustainable biological and chemical methodologies. This guide defines four classes based on a set of criteria that put together Safety (e.g. flash point, tendency to form explosive and auto-ignition temperature), Health (reflected the occupational hazard) and Environment (e.g. toxicity towards aquatic life, ability to generate VOC). For each criterion, a score of 1 to 10 has been assigned to all the solvents analysed. The scores of each criterion have been combined to create a colour-code rank as follows and reported in *Fig.3*:

- Recommended: the least harmful solvents in each category, thus the solvents to be preferred for any process
- Problematic: these solvents can be used in process but their implementation in the production scale needed further consideration due to the significant energy consumption.
- Hazardous: solvents that show a significant limitation, so during process development it is recommended to replace these solvents.
- Highly hazardous: solvents to be avoided, even in the laboratory.

There are then two other intermediate classes (recommended or problematic and problematic or hazardous), with solvents still being assessed for their hazardousness or safety.

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.

Fig. 3 Ranking of solvents from green (safe) to red (hazardous)

1.4 Green solvents used today

The research of green alternative in the last three decades has growth exponentially, showing green solvents already known or developing a wide range of new eco-friendly solutions for different process and applications (*Fig. 4*).

Fig. 4 Principal green solvents

We will focus the discussion on neoteric solvents, used in this study. The name neoteric means recent, modern and this class of green solvents include supercritical fluids (SCF), ionic liquids (IL) and deep eutectic solvents (DES)[20]. Despite conventional solvents (e.g. water, methanol, ethanol, glycerol) are still the most employed in chemical industries, neoteric solvents are slowly being integrated into industrial processes due to their desirable, less hazardous, new proprieties and for the multiple advantages over organic, or aqueous, solvents, e.g. improving product separation.

1.4.1 Deep eutectic solvents

In 2004, the term DES began to appear in literature. Because of its similar properties to IL, they are often confused with them, but it is necessary to specify that they are two different types of solvents[21].

DESs are a system formed by a eutectic mixture of Lewis and Bronsted acids and bases which can contain a variety of atomic and/or cationic species, while ILs are formed by systems composed primarily of one type of discrete anions and cations. Although the physical properties of DESs are like other ILs, their chemical properties suggest application areas which are significantly different.

DES are formed by 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 (Fig. 5)[22,23]. The charge delocalization occurring through hydrogen-bond interaction seems to be the main cause of the freezing-point depression of the mixture[23,24].

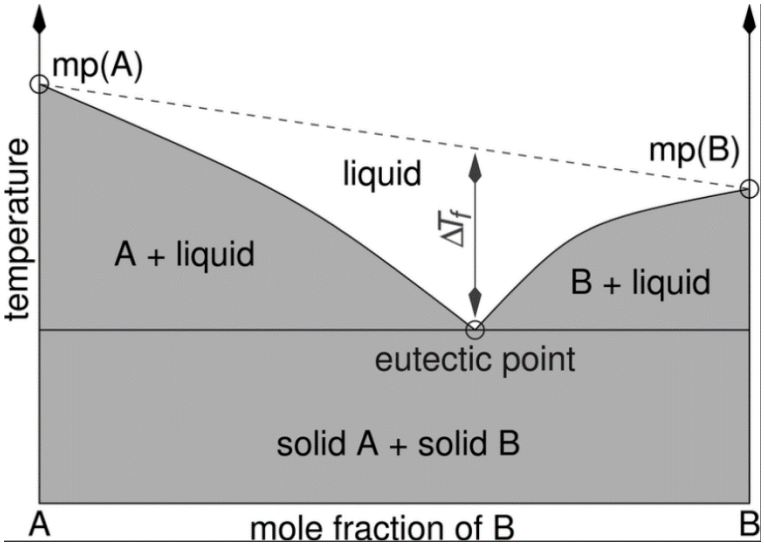


Fig. 5 Schematic representation of eutectic point

Most DES exhibit fascinating physiochemical properties, like those of IL: very low melting point (liquid at room temperature or temperature lower at 100°C), low vapour pressure, high conductivity, high flash point, high dissolving power, high water solubility. Anyway, a lot of DES show remarkable advantages compared with IL, they are[25–27]:

- easy to synthesize, since they are a combination of two or more compounds, often solid, that react and heated to around 80°C to form a liquid product
- economically competitive, since they are usually prepared from cheap and accessible raw materials that are available as bulk chemicals, like as urea, citric acid, glycerol
- nontoxic or only weakly toxic
- biodegradable
- biocompatible
- usable in large-scale process
- non-reactive with water
- less prone to waste generation

Thanks to their physiochemical features DES are efficient solvents, successfully applied in various fields including extraction[28], biomass processing[29], CO_2 adsorption[30], organic synthesis, catalysis, biomass pre-treatment and processing, new material preparation, biotechnology[31–33]. Essentially, DES have similar applications to IL, but their accessible and biocompatible ingredients have allowed them to be used in food and nutraceuticals/pharmaceuticals[34].

1.5 NaDES: natural-based solvents

When DES are formed by natural chemical compounds present in the meadows of living organisms, they are named Natural Deep Eutectic Solvents (NNDES)[35,36].

NaDES are eutectic mixtures of two or more natural components (e.g. organic acids, amino acids, sugars, polyalcohols), which we usually find in the solid phase, that mixed in peculiar molar ratio become liquid and generally keep the liquid phase at room temperature[37].

Beyond the properties that identify like DES, they have in additional a total natural origin that allows to propose NaDES as "greener" solvents.

It is important to keep in mind that the properties of DES can be different from properties of compounds that form them.

1.5.1 Molecular structure of DES

Intermolecular interactions in DES were determined by Fourier-transform infrared spectroscopy (FT-IR) and NMR spectroscopy[38,39]: the presence of extensive intermolecular hydrogen bonds between the component of DES has been clearly highlighted (*Fig. 6*). The main groups involved in hydrogen bond interaction are hydroxyl, carboxyl and amino groups present in many amino acids, organic acids or choline derivatives.

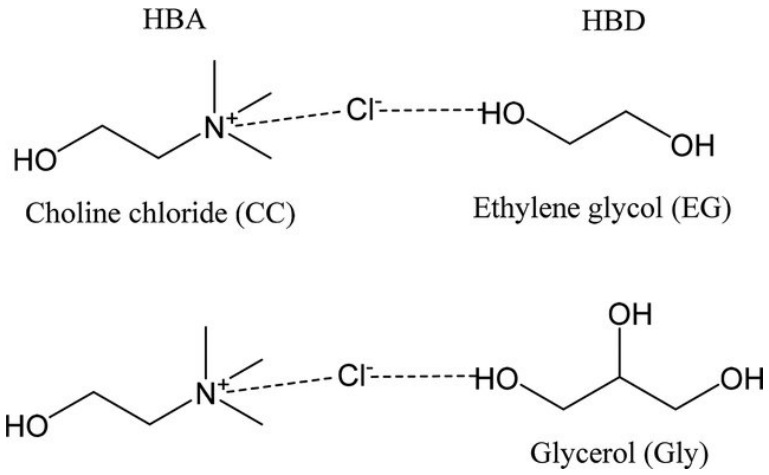


Fig. 6 Hydrogen bond formation: interaction between choline chloride as hydrogen bond acceptor, and ethylene glycol and glycerol as hydrogen bond donor[40]

Those strong hydrogen bonds are the key to DES formation and the cause of the high depression melting point of the individual precursors in the eutectic mixture[39]. Depending on the number and type of hydrogen bond acceptors and donors, the spatial arrangement of the molecules and the resulting interaction, the physical, thermal, chemical and biological properties of the solvent are affected.

1.5.2 Type of HBD and HBA

DES are easily prepared by mixing and heating to moderate temperatures (~ 70/80°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. DES composition is given as molar ratio (mol/mol), which directly reflects the contribution of the components[41].

The HBDs in DES are mostly primary metabolites naturally present in all types of cells: sugars (e.g. glucose, sucrose, fructose, etc), organic acids (e.g. lactic, malic, citric acids), amino acids, alcohols (e.g. glycerol) and urea. Some of them act both as hydrogen-bond donors and acceptors[23].

In the class of HBAs, the most used compound is choline chloride (ChCl) (*Fig. 7*)[23]. This 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 (75% degradation within 5 days)[42], weakly toxic (LD50s between 3150 - 5000 mg/kg observed on animals and human cells)[42], and cheap (~ 2 €/kg)[43].

Recently, also betaine-based NaDES have been proposed as suitable solvents[36,44]. Betaine (Bet) is a low-cost chemical (~3 €/Kg)[45], readily biodegradable in water, 88% mineralized in 28 days, like OECD guideline says, not toxic to sludge micro-organisms, not bioaccumulative and not persistent[46]. In addition, Glycine betaine (N,N,N-trimethylglycine, GB) is widely known in agriculture as a plant protection agent against environmental stresses such as drought, excess salt, cold, heat and frost[47,48].

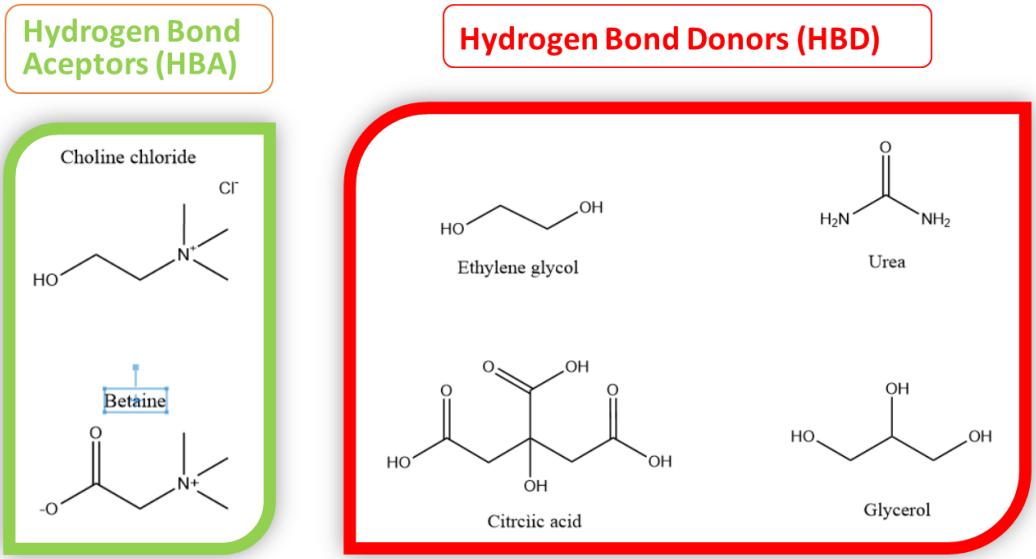


Fig. 7 Some types of hydrogen bond acceptors (HBA) and hydrogen bond donors (HBD)

Most of DES are made up only two components creating the most investigated binary system (e.g. ChCl/ethylene glycol), but a third chemicals can be added generating a ternary system. The third component very often used is water, which thanks to its ability can modify the physical-chemical properties of DES such as viscosity, conductivity, density, polarity and extraction efficiency[49]. Thus, by changing the water content, the solvation power can be adapted to specific requirements. Experiments have verified that when water is added in a molar proportion like that of the main constituents, it is able to form hydrogen bonds with other compounds and becomes an integral component of the supramolecular structure of DES. However, an excess of water can have a negative impact on the structure and cause the progressive breaking of the hydrogen bond between HBA and HBD[35].

1.6 Properties of NaDES that influence its effectiveness

DES have a big potential as green solvents, but the increasing research on these solvents, has highlighted some disadvantages which are within their own properties. Properties like as viscosity and polarity could be a restriction in various applications, especially in the extraction procedures, and could prevent industrial adaptations[50]. This problem, in literature, is described as "adjustable" through the adjustment of specific parameters, such as[51]:

- temperature
- water content
- HBD e HBA composition

1.6.1 Viscosity

Viscosity is the main obstacle for the application of DES, because it slows down the extraction or dissolution processes, leading to additional operations that take a long time and has negative effects on the reaction speed in the extraction processes[37].

The viscosity of DES typically varies from 200-500 $mm^2\ s^{-1}$ at 40°C, much higher than most common molecular solvents. The high viscosity is caused by the strong interaction of the hydrogen bond between DES compounds which therefore limits the mobility of free species within the DES structure. In fact, in one study, three different choline chloride DES were prepared with different molar ratios to assess viscosity. It was seen that mixtures with high viscosity were linked to low water content, while with 25% water content the mixtures became highly hydrated and consequently with low viscosity[52]. The viscosity also leads to extraction problems as in the case of α-mangostin from *Garcinia mangostana*. The high viscosity and the consequent low diffusivity of DES-2P3 (ChCl-1,2-propanediol) make the infiltration of the eutectic mixture into the matrix difficult; *Fig.8* clearly shows the differences in extraction times between ethanol, which has a low viscosity, and ChCl-based DES, in which the high viscosity slows down the dissolution of the analyte. The two properties, conductivity and viscosity, are in fact inversely proportional as demonstrated in Stokes-Einstein's equation, so high viscosity means low diffusion coefficients and consequently longer extraction times are required compared to conventional organic solvents[53].

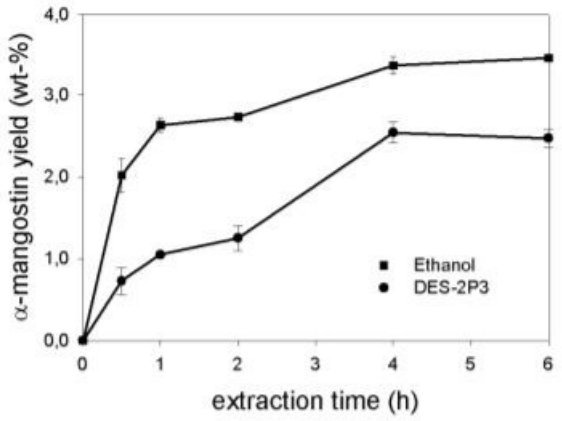


Fig. 8 Effect of time on mangostin yield using ChCl-1,2-propandiol (DES-2P3) as DES and ethanol as reference solvent [53]

Despite viscosity is a disadvantage, this can be overcome with the change of some factors that increase the extraction yields, indeed viscosity is called "adjusted propriety". Factors that can be modified are:

- composition of mixture: viscosity of DES differs enormously according to their composition. Is necessary to investigate various combination of HBA and HBD to find the best mixture[54].
- Temperature: viscosity linearly changes with temperature. The figure (*Fig.9*) shows that an increase in temperature generates a low viscosity DES, therefore a more appropriate solvent[35].
- Water content: the third parameter that can be changed is the water content[51]. It has a similar behaviour with temperature, the viscosity of NaDES decreases as the amount of water increases[52]. The addition of water leads to a decrease in the viscosity of NaDES, as the hydrogen-bonded interactions between the components of the mixture weaken, and consequently improves the solubilization capacity of NaDES[55]. Thus, the viscosity of NaDES could be improved by increasing the temperature or adding water. However, it is not the only property that can influence the extraction efficiency[26].

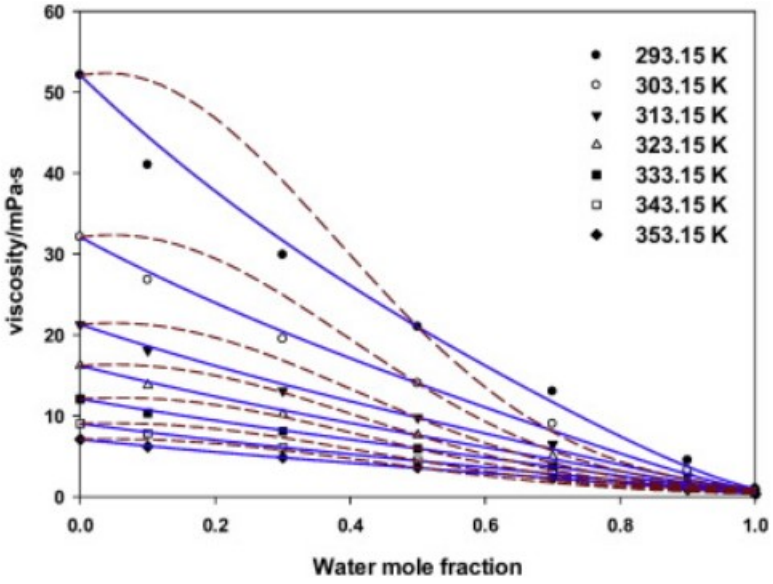


Fig. 9 Relationship between viscosity, water content and temperature on Reline, DES formed by chloride and urea choline[52].

1.6.2 Polarity

The other constant property of DES is the polarity which affects the solubilization capacity and consequently the extraction capacity. The most polar DES are those based on organic acids (polarity ~45 kcal mol^{-1}), followed by DES based on pure sugar and amino acids with a polarity similar to that of water (~50 kcal mol^{-1}), while the less polar ones are DES based on polyalcohol, which show a polarity similar to that of methanol (~55 kcal mol^{-1}). Thus, DES show a wide range of polarities and are able to dissolve and extract a wide range of polar to medium to low polar metabolites, which show low solubility in water, like many natural bioactive compounds, gluten, starch and DNA[35].

In fact, DES are mainly composed of polar compounds that are not able to solubilize and extract non-polar compounds efficiently. This is in line with the results obtained in anthocyanin extraction[56]. Anthocyanins are polar molecules, and the best extraction yield was obtained from DES composed of ChCl/malic acid, while both sugar-based and polyalcohol-based DES, such as ChCl/Glycerol, being less polar, have consequently obtained less effective extractions.

Nevertheless, DES are customisable solvents, and the polarity can be adjusted by changing the HBA/HBD combination and water content[57]. The dilution of water increases the polarity of DES. This was seen in a study on flavonoid extraction,

where the increase in water content led to increased polarity affecting the interactions between DES and flavonoids, making them less efficient in terms of extraction yield[58].

1.6.3 Thermal Behaviour

Solvents are often use as media for chemical and biological reactions that might require high temperature to be achieved. Considering that some DES components are usually thermally unstable, as is the case of sugars or amino acids, the thermal behaviour DES have been investigated using thermogravimetric analysis (TGA) and Differential Scanning Calorimetry (DSC). Analysis shows that, all the DES can be heated to 100 °C, without any evident decomposition. DES made by sugars have a low decomposition temperature of approximately 135 °C but others have a decomposition temperature that is even above 200 °C. All DES evaluated by Differential Scanning Calorimetry (DSC) revealed that they have glass transition points below −50 °C, which confirms that those DES are supramolecular complexes, with a stable liquid status over a wide temperature range. It implies that DES can be used as solvents in a range between at least 0 and 100 °C[35]

1.7 Applications of NaDES

DES potential applications cover a wide range of sectors (*Fig.10*). The main research areas are extraction, synthesis, analysis, polymers, metalworking, biomass pre-treatment and nanomaterials sciences. As solvents, DES have been successfully used for the pre-treatment of rice straw, one of the most abundant residues of lignocellulosic biomass. Several DES have been tested, showing high solubility for lignin and poor solubility for cellulose[59]. DES have been used as functional additives and monomers as well. DES have been used as functional additives and monomers. In fact, some eutectic mixtures, including ChCl/urea, have been implemented in the preparation of polymers used both in the medicinal[60] and industrial[61] fields.

Despite some problems related to the properties described above that can be fixed, DES have significant advantages such as sustainability, low cost and lower toxicity compared to conventional solvents.

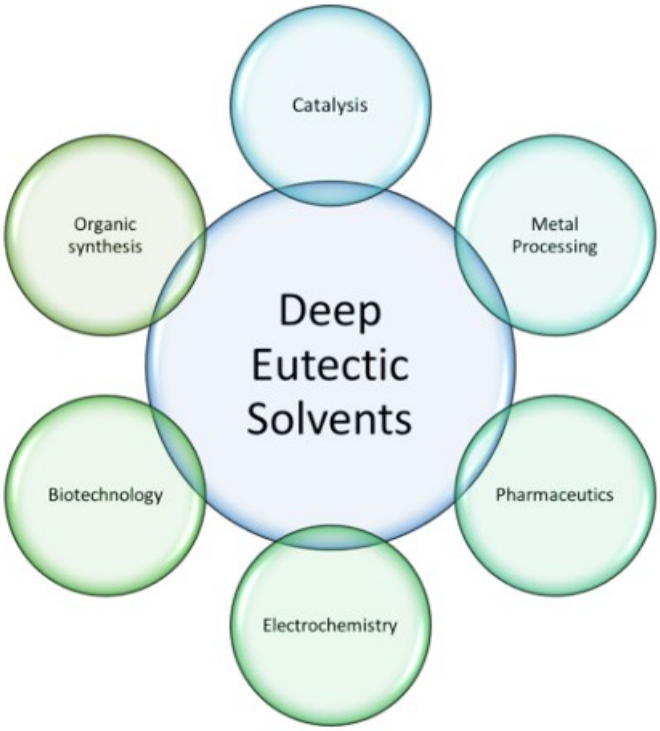


Fig. 10 Principal applications of DES.

1.8 NaDES in extraction field

Thanks to their properties, DES have been applied in many studies like as extraction media. They were used to carry on extraction process from plants, food, biowastes and other natural matrices to obtain high value compounds request in several fields such as pharmaceutics, cosmetic, nutraceutical and agricultural[62].

1.8.1 Polyphenols extraction

Phenolic compounds are the most investigated group through the extraction with NaDES[63,64]. Polyphenols are organic molecules characterized by the presence of phenol structural units, aromatic rings bonded to a hydroxyl groups. These compounds are present as secondary metabolites in the plant kingdom, where they play an important role for different functions from seed germination to defence and resistance against environmental stress factors. Polyphenols, for example, are used towards:

- Oxidative stress: some flavonoids (e.g. quercitin and rutin) containing the hydroxyl group were able to chelate heavy metals and consequently detoxify ROS[65], components that are harmful to plant cells because they lead to damage to proteins, lipids and nucleic acids.
- Heavy metal stress: for example, one study saw the formation of a complex between aluminium and tannin (a type of polyphenol) in the *Lotus pedunculatus* forage legume, where aluminium was detoxified at the root tip and this removal of toxicity then allowed the roots to grow [66].
- Defence against infection and pathogen: some polyphenols, such as rutin, quercitin[67] and phytoalesins[68], present in plants manifest their function to defend the plant from infections and pathogenic organisms.

In the literature, there are many publications that reported the ability of DES in the isolation and fractionation of phenolic compounds, confirming that DES could be good surrogate for conventional solvents[69]. DES have been used to extract polyphenols from:

- Wine waste: DES have been developed to recycle the wine lees of the wine industry, as a cheap source of phenolic compounds, especially anthocyanins. ChCl-based DES with oxalic acid provide a more efficiently extraction of anthocyanins compared with conventional solvents[70].
- Vanilla: various ChCl and Bet-based DES proved to be more efficient extraction media than ethanol for recovering phenolics from vanilla pods, in addition, a DES solution suitable for flavouring food products has also been produced[71].
- Food: several types of DES were used to develop a method for the extraction of flavonoids from food samples. With small amounts of DES there were high yields of extractions[54].

In the field of agriculture, DES has been proposed as a transport solvent for the supply of flavonoids, a secondary metabolite with remarkable pesticide behaviour, thus protecting crops. The main problem in the uses of these metabolites as insecticides, replacing them with conventional and harmful insecticides, was their poor solubility in water, but these metabolites show a higher solubility in DES than water[72]. Thus, DES are proposed as functional transport pathways for the application

of secondary metabolites to protect agricultural crops primarily in the most vulnerable stages.

1.9 Toxic profile of NaDES

We know that DES are constituted by components that are supposed to be safe, but the interaction between the individual ingredients could result in a synergic effect and thus a different toxicological profile[73]. Since DES were used as extraction media or functional carrier of bioactive compounds for direct or indirect human consumption, we must delineate a toxicological profile at different trophic levels[74].

1.9.1 Toxicity to bacteria

The toxicity (inhibition of growth) of ChCl-based DES on bacteria, was evaluated by using two Gram-positive (*Staphylococcus aureus* and *Listeria monocytogenes*) and two Gram-negative (*Escherichia coli* and *Salmonella enteritidis*) (*Fig. 11*). Tested DES based on amine, alcohol and sugar showed no side effects on the growth of the bacteria, so they could be declared as benign for the target organism. On the other hand, DES based on organic acids have had a significant inhibitory effect, however, suggesting their use as antibacterial agents[75]. These results suggest that the main cause of growth inhibition is due to the low pH of the acidic components, in fact when the pH of DES has been adjusted to the optimal growth range of the bacteria no inhibitory effects have been observed[76].

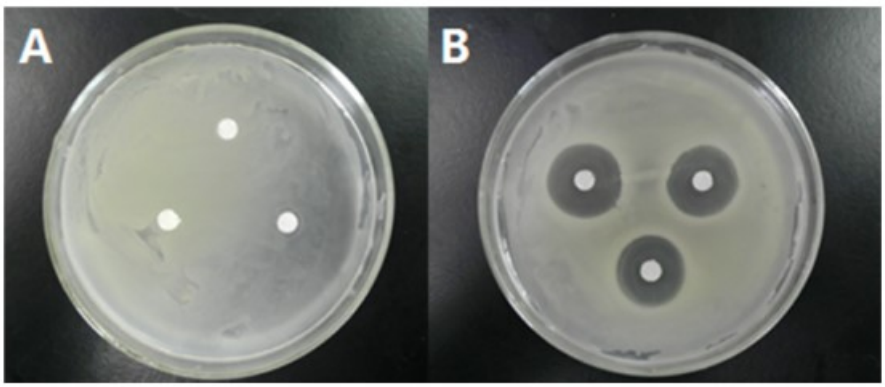


Fig. 11 Effect of different DES on Escherichia coli. A) Non-inhibitory effect of ChCl/urea; B) Inhibitory effect of ChCl/ citric acid[75]

A study conducted on *Vibro fischeri* revealed that the toxicity of acidic DES increases by increasing the acid content, due to the negative effects on cellular activity through protein denaturation. Therefore, DES can pass through the cell membrane and exert a toxic effect[77].

Several DES based on ChCl and urea, glycerol, ethylene glycol and acetamines combined in different molar ratios have been tested on *Escherichia coli*. The results show that DES at low concentration levels can be considered non-toxic on bacteria, but if we increase the concentrations, all DES show an inhibitory effect. Despite this, the results suggest that the antibacterial properties of the eutectic mixture are more intense than the properties of the individual components[78].

1.9.2 Toxicity to fungi and plants

The toxicity of DES is little tested on fungi and plants. The toxicity of several DES based on ChCl combined with several HBDs have been assessed against the fungus *Aspergillus niger*. The results reveal that NaDES showed higher antifungal properties (higher inhibition index) at different concentrations than their individual components[74].

Garlic (*Allium satiuvum*) has also been used to assess the phytotoxicity of some ChCl-based DES and their components (urea, ethylene glycol and glycerol), through the evaluation of root length (*Fig. 12*). The results showed that the DES tested are toxic to some extent to garlic in particular:

- the DES tested or their components can bring inhibitory effects to the body, shorter root growth or the formation of damaged cells
- the individual components exerted a high toxicity compared to corresponding eutectic mixtures.

ChCl-based eutectic mixtures show a higher inhibitory effect than DES with choline acetate as HBA[78].

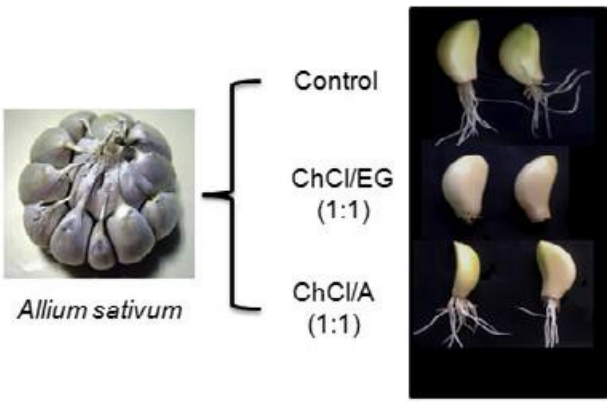


Fig. 12 Effect of NaDES on garlic[78]

In a study on watercress[79] (*Lepidium sativum)*, where its germination was evaluated, it was found that none of the DES investigated had any influence on seed germination,

while some specific combinations of DES and and the formulations of DES and polyphenols (DES-PF) showed an influence on root growth. On oats (*Avena sativa)*, however, in the same study[79], the endpoints evaluated were shoot length, dry shoot weight and visible effects on plants. DES and formulations of DES and polyphenols based on citric acid and ethylene glycol as HBD showed no effect on oat growth.

1.9.3 Toxicity to animals

Together with the evaluation of the toxicity on garlic, the same DES were evaluated on *Hydra sinensis*, a freshwater invertebrate used as a test organism for its high sensitivity to chemical contamination.

The results show that DES shows a toxic effect on hydra with characteristics such as those identified for garlic (*Fig. 13*). When placed in contact with a solution containing the individual HBAs, HBDs or mixed, the survival time of the hydra was significantly reduced compared to the normal growth medium. The four HBDs (urea, acetamide, glycerol and ethylene glycol) do not have hydra survival times, while both choline chloride and choline acetate, known as ecological, were severely detrimental to hydra, causing the tentacles to contract and in the worst case, complete disintegration of the animals. However, the authors have revealed that the harmful effect of choline salts can be limited by incorporation into a NaDES[78].

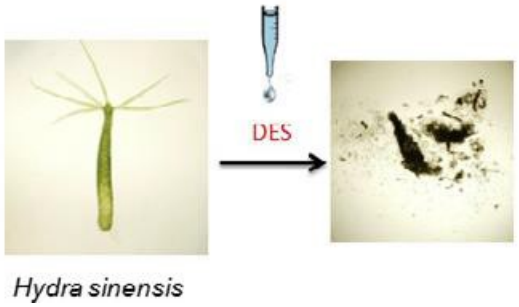


Fig. 13 Effect of NaDES on Hydra sinensis[78]

The ecotoxicological test performed on *Daphnia magna* with ChCl/urea NaDES shows that the individual components have a low toxicity on model organism, even though ChCl has a higher toxicity than urea. In addition, the resulting combination of the two components appears to show similar toxicity to choline chloride[80].

These results do not allow DES to be labelled as non-toxic or low-toxic, but many DES show different toxic behaviours that could result as a different combination of HBA and HBD, molar ratio, water content, concentrations or organism model

tested[73,81]. The toxic effects on cells, microorganisms, plants and animals must therefore be further investigated to determine the safety of DES.

The effects of DES were also analysed on the earthworm *Eisenia andreai* by assessing growth, reproduction and survival[79]. The DES tested were based on both chloride choline and betaine. DES consisting of choline chloride and ethylene glycol were found to be moderately toxic and compromised egg development, while DES consisting of betaine and ethylene glycol was found to be non-toxic to any of the endpoints evaluated.

1.10 The urea issue

The present Thesis falls within the field of green chemistry and focuses on the study of urea and urea fertilization issues.

Urea is the main source of N in worldwide crop production thanks to several commercial and safety advantages if compared to other N-fertilizers such as ammonium nitrate. In 2018 around 241 thousand tons of urea-based fertilizers were sold.

In the soil there is an enzyme, named urease, which is of critical importance in the global nitrogen cycle by hydrolysing urea to eventually yield NH_3 and CO_2[82,83] a reaction that causes an overall increase of soil pH[84,85]. Soils contain large quantities of urease, both inside living cells of plants and microbes, and as extra-cellular enzyme adsorbed onto organic and inorganic soils components.

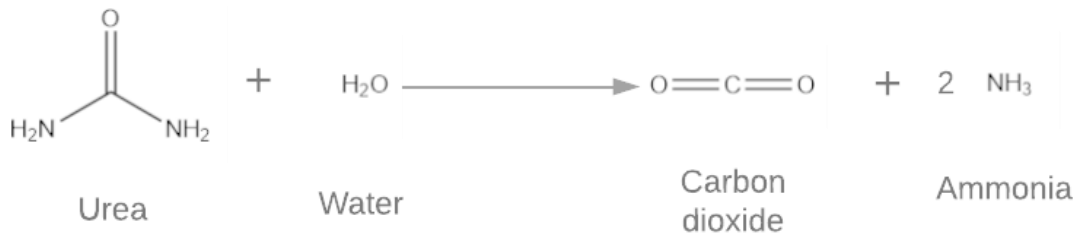


Fig. 14 Hydrolysis of urea in the soil

Urea in soil is hydrolysed by the enzyme urease, but its rapid hydrolytic decomposition catalysed by soil urease results in a substantial decrease of the efficiency of urea-based soil fertilization due to fast ammonia volatilization process

that leads to loss of N to the atmosphere[86].Moreover, the rapid pH increase associated to urea decomposition negatively impacts on plants germination or growth, further decreasing the amount of ammonium absorption by plants roots. Ammonia volatilization has also impacts on both local and international scales, contributing to atmospheric pollution with the production of NH_4^+ in the atmosphere by reacting with acid pollutants like SO_2 and NOx, that is a secondary pollutant and is the major fraction of PM2.5 aerosols[87]. The enzyme urease is important not only for the soil, but also for the human body as its action is effective in catalysing the hydrolysis of urea in serious infections caused by certain bacteria in the gastric and urinary tract.

The practice of using urease inhibitors as N-stabilizers has been implemented to counterbalance these negative aspects in various ecosystems. The most widely used urease inhibitor for agronomic purposes is N-(n-butyl)-thiophosphoric triamide (NBPT), that belongs to the class of those inhibitors that directly bind to the Ni^{2+} ions in the active site of the enzyme[88,89]. The discovery of NBPT as a urease inhibitor began in the 1970s, where a study showed that some organic compounds containing phosphorus had an inhibitory effect on soil urease. In the same decade was discovered the direct involvement of phosphoramidate in the binding with nickel ion in the active site of *Jack bean* urease (JBU). Nowadays the most effective inhibitors are amines and esters derived from phosphoric and thiophosphoric acid. Structural and kinetic investigations on urease inhibition in the presence of N- (n-butyl) thiophosphoric triamide (NBPT), show that the monodeaminated form is the one that interacts with the enzyme through a slow inhibition mechanism which is related to the time[90]. The efficacy of the urease inhibitor has been demonstrated in several studies, with a lowering in percentage terms of ammonia loss due to volatilization. Despite its effectiveness, characterized both in vitro and in vivo[90,91], and the determination of the mechanism of inhibition at an molecular level[90], NBPT seems not to meet the essential characteristics necessary for agronomic applications, especially in terms of low phytotoxicity, since it causes leaf necrosis in several crop plants[92,93]. A second group of inhibitors, to which catechol belongs, is able to covalently bind to a conserved cysteine-residue located on a protein flap (involved in the catalytic mechanism by modulating substrate/product exchange across the active site cavity), blocking the enzyme activity[94]. The mechanism of urease-inactivation by catechol

has been suggested to be radical-promoted, with a mode of action hypothesized to be a common denominator for aromatic poly-hydroxylated urease inhibitors like polyphenols.

There is no doubt that nature is a vast source of natural products of that exhibit a variety of biological activities. The diversity of chemical structure makes natural products very valuable to pharmaceutical industries and agricultural segments as well. Natural products from plants have been a great source of inspiration for improving human and animal life quality as disease therapeutics and also for increasing food resources. In this context, the investigation of the potential of plant derived natural products as urease inhibitors can be valuable for the development of therapeutics for diseases associated with intense urease activity and improved nitrogen fertilizer formulations to increase food production.

Polyphenols are bioactive plant metabolites, potentially exploitable in a broad range of human-related applications. Polyphenols constitute one of the most abundant and structurally diverse classes of compounds, which are highly bioavailable in medicinal and edible plants. Among the variety of useful biological properties (e.g. anticancer, anti-inflammatory, antibacterial, cardio-protective, anti-osteoporotic properties), polyphenols exhibit also enzyme-inhibitory activities, like against the enzyme urease itself. This became the background of many patents claiming the use of different herbs and seaweed extracts with high polyphenolic content for example, the application of nutraceuticals in the treatment of gastric and urinary tract infections (and their consequences) induced by urease-producing pathogens like *Helicobacter pylori*. For example, ethanolic extracts enriched in polyphenols obtained from perennial and deciduous trees (from the bark of *Acacia decurrens*, *Acacia caven* and *Pinus radiate*, or from the seed coat of *Terminalia chebula*), as well as whole plant (*Camelia sinensis* and *Azadirachia indica*) were effective against soil-urease[95,96].

2 Aim of the study

The aim of this study is to evaluate the potentiality of polyphenols extracted from waste biomass as urease inhibitors in the agricultural field and the possibility of using DES as ingredients for new bioactive formulations and eco-compatible to deliver these high value compounds. Polyphenols are known to be effective against bacterial urease associated to human diseases, but the study of their effect on soil urease is less deepened; moreover, although NaDES are considered green, non-toxic and environmentally benign solvents, the knowledge about their ecotoxicological profile is extremely limited and there is scarce information about their effect on the soil compartment.

In the first part of the study, we have focused our attention on the potential inhibition of urease in soil by polyphenols compared to the activity of a commercial inhibitor (NBPT), aiming to reduce the fast ammonia volatilization after soil fertilization. The polyphenols that we used are extracted from white grape pomace with DES formed by choline chloride (HBA) and ethylene glycol (HBD) in a molar ratio 1:2.

The second part of the study focuses on the ecotoxicological evaluation of DES. The aim was to find a relationship among the toxicity and DES composition to provide a preliminary assessment of their safety in terrestrial environment and environmental persistency. We have evaluated the toxicity on flora and fauna model organisms:

- *Lepidium sativum* and *Avena sativa*: seeds germination and growth tests were used to evaluate the inhibitory or promoting behaviour of DES.

- Microarthropods of soil: survival of organisms was used to evaluate the potential toxic effects of DES.

3. Materials and methods

All solvents used in this study were obtained from Sigma-Aldrich, with >98% purity and used without any further purification.

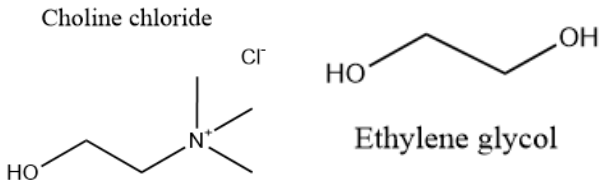
Grape pomace was supplied by Caviro s.r.l., black tea and green tea obtained from residues of different teas after their use, coffee obtained from residual coffee grounds after use. Watercress seeds (*Lepidium sativa*) distributed by Fioral and seeds for oat were supplied by a local organic farmer (Forlì).

The Limus used is that of Compo Expert s.r.l. commercially sold under the name Nexur® 46.

3.1 NaDES preparations

NaDES have been prepared by mixing choline chloride (ChCl) as HBA and ethylene glycol (EG) as HBD in the correct molar ratio (1:2) (*Fig. 15*).

The two components were transferred into a glass vial and heated to about 90° until a liquid and transparent compound was obtained (about 15/20 minutes). Afterwards they were cooled down to room temperature and then 30% H_20 was added to the total weight of the mixture.



DES	HBA	HBD	molar ratio	mol HBA	mol HBD	g HBA	g HBD	g H2O
ChCl + EG	Choline cloride	ethylene glycol	1:2	0.06	0.12	8.13	7.23	7.68

Fig. 15 Graphic representation of the two components and their molar and weight ratios

3.2 Extraction polyphenols from dried biomass

Polyphenols were extracted using conventional methods (EtOH:H_2O mixture 1:1 v/v or H_2O alone) and NaDES from various freeze-dried biomasses: white grape pomace, red grape pomace, black tea, green tea, coffee.

900 mg of biomass were placed in a tube and put in contact with 18 g of solvent. The mixture was left stirring at room temperature for 24 hours. Afterwards, the sample was centrifuged for a few minutes to separate the polyphenolic extract (supernatant) from the rest of the biomass. The supernatant was taken and subsequently used for

the ecotoxicity test, the urease enzyme inhibition test, the total polyphenol content (TPC) and the antioxidant activity of polyphenols.

3.2.1 Determination of total polyphenols content

The Folin-Ciocâlteu method was used to determine the total polyphenol content. Folin-Ciocalteu reagent is a mixture in aqueous solution of phosphomolybdate and phosphotungstate used in analytical chemistry for the determination of phenols and polyphenols. 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 the dark at room temperature for 30 minutes. The spectrophotometric analyses were performed using a DR/2010 portable spectrophotometer (*Fig. 16*) calibrated at 720 nm and using different calibration standards (catechol with concentrations from 2.5 ppm to 40 ppm with R^2= 0.99, floroglucinol from 10 ppm to 100 ppm with R^2= 0.98, epigallocatechin gallate from 2.5 to 100 ppm with R^2= 0.99 and gallic acid from 2.5 ppm to 80 ppm with R^2=0.99).

Fig. 16 Spectrophotometer used for the analyses described in the following paragraphs 3.2.1

3.2.2 Determination of antioxidant activity

The DPPH method has been applied to determine the antioxidant activity of polyphenols extracted from biomass. DPPH (2,2-diphenyl-1-1picrylhydrazyl) is a dark coloured powder composed of stable free radical molecules that is commonly applied as an antioxidant assay based on electron transfer that produces a purple solution in ethanol.

The method described by Band-Williams[97] was used to measure the antioxidant activity. The sample is reacted with DPPH and the reaction mixture is composed as follows:

1) 0.5 mL of sample
2) 3 mL absolute ethanol
3) 0.3 mL of DPPH 0.05M

For the blank, 0.5 mL of sample are added to 3.3 mL ethanol, while the control mixture consists of 3.5 mL ethanol and 0.3 mL DPPH radical solution. The mixtures were reacted in the dark for 100 minutes, and the colour change from purple to yellow was then read through absorbance (Abs) with Jasco V-650 UV-vis spectrophotometric calibrated at 517 nm. The antioxidant activity in percent was then determined using the formula:

$$AA\% = 100 - [(Abs\ sample - Abs\ blank) * 100] / Abs\ control$$

3.3 Setting up urease enzyme inhibition test

3.3.1 System tuning

The air-termination system was set up to test the tightness of the system. Two holes were drilled on the plastic box cover, one for the passage of the air that was flushed inside by an engine and one for the outflow of ammonia that is collected inside a 40 g/L boric acid sequestering solution thanks to the air. A solution of NH_4Cl and NaOH put in a petri dish was initially used to set up the entire system and find the appropriate conditions for volatilizing and capturing NH_3 (*Fig. 17*). Within 24 hours, almost all the ammonia has been evaporated and collected.

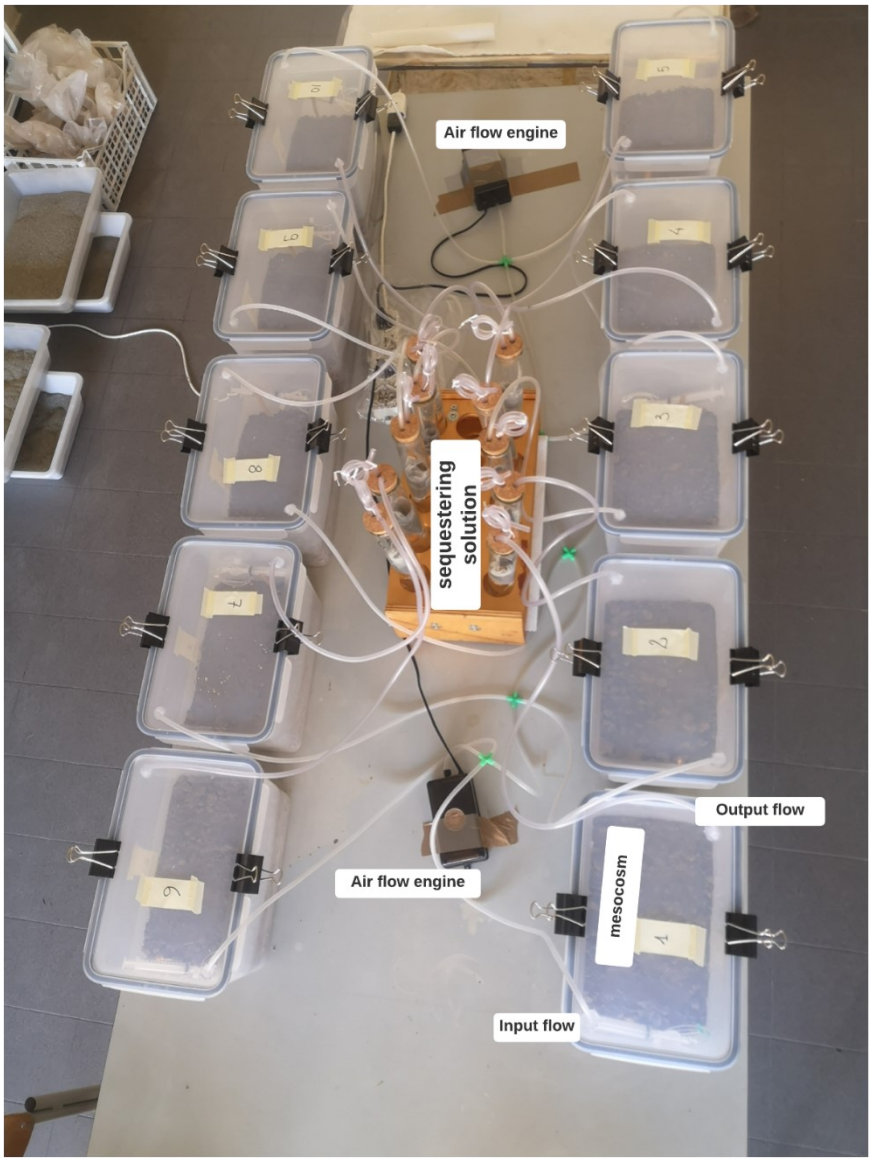


Fig. 17 System used for the capture of volatile ammonia

3.3.2 Urease enzyme inhibition test

For the evaluation of urease inhibition, 10 mesocosms were set up in plastic containers, each containing 4.682 Kg of soil taken from the apple orchard of the Istituto Tecnico Agrario Statale (ITAS) in via dell'Agricoltura, 5, Ravenna (IT). In each mesocosm 45 seeds of *Lepidium sativum* were planted and fertilized with 3.39 g of urea. Two replicates were carried out for each of the following five tests: control (without any inhibitor), DES alone, H_2O plus polyphenols, DES plus polyphenols and Limus (NBPT). The soil was irrigated several times during the experiment. Two air holes were drilled in the lid of each container to circulate an airflow through each mesocosm and carry the evaporating ammonia in a glass test tubes with a solution of

boric acid (H_3BO_3) 40 g/L. To quantify the evaporated ammonia, 10 mL of sequestering solution were taken, 0.1 mL of colorimetric indicator composed of bromocresol green and methyl red was added and a direct titration with 0.05M sulphuric acid (H_2SO_4) was carried out. This equipment, with appropriate modifications, is like what already experienced by Soares[98].

3.4 Nitrogen determination

The different nitrogen pools present in the soil before and after the test in the mesocosms were investigated. All soil chemical parameters concentrations were expressed per kg of dry matter (dm), the latter determined by placing the samples in the oven at 105°C for 24 hours. The soil nitrogen pools were analysed in three replicates for the soil at time zero (without any treatment) and in single replicates for the soils of each mesocosm at the end of the test.

Unlike these, the nitrogen forms in the leached water collect during the test, were analysed only in the mesocosms.

Figure *18* shows a block diagram with the procedures followed to determine the different nitrogen pools in soil and in leached water.

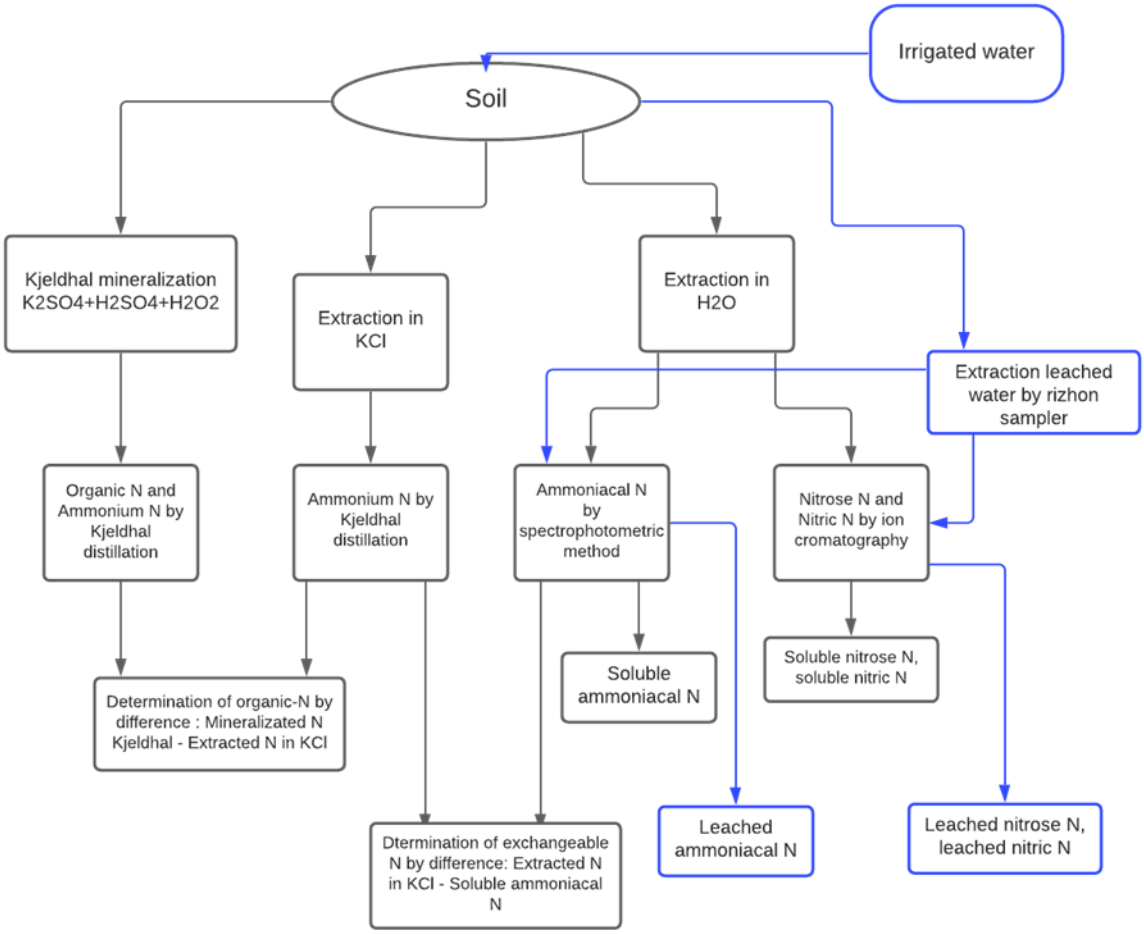


Fig. 18 Schematic representation of the different analyses carried out on soil and leaching water to determine nitrogen forms

The amounts of nitrogen pools, in input at the beginning of the test (soil N and fertilized N) were compared with the amounts of pools in output at the end of the test (soil N and lost N through volatilization and leaching). This allowed to quantify the effects of nitrogen fertilization and to characterize the nitrogen mass balance in the mesocosms. The methods used to determine nitrogen pools are shown as follows.

3.4.1 Leached forms of nitrogen

For the capture of leaching water, Rizhon soil moisture sampler by Eijkelkamp ® were used. This tool consists of a porous septum inserted into the soil and connected through a capillary to a syringe. The creation of a pressure vacuum makes the water rise inside the syringe. The collected water was then stored in plastic containers and the main anions (fluorides, chlorides, nitrites, nitrates, phosphates and sulphates) were determined using an ion chromatograph. Through the colorimetric method of

salicylate ammonium by spectrophotometric reading, soluble ammoniacal nitrogen ($N\text{-}NH_3$) was also determined in the percolation water.

The anion and cation concentrations found in the water leached after irrigation were expressed in milliequivalents per litre (meq/L), while, measuring the volume of the extracted water by rhizon, the absolute amount of each leached nitrogen pools were calculated.

3.4.2 Determination of Kjeldahl nitrogen (organic plus ammoniacal nitrogen)

The determination of Kjeldahl nitrogen in soils was determined using method XIV.4 described in the Gazzetta Ufficiale (G.U)[99] and adapted according to the Unit Distillation Kjeldahl (UDK) 126A from Velp Scientific ® laboratory instrument handbook. The method allows determining organic nitrogen plus ammoniacal nitrogen. It involves a first digestion step of 5g of soil from each test with the addition of 7g of potassium sulphate (K_2SO_4), 10 mL of concentrated sulphuric acid (H_2SO_4) at 96% and 5 mL of hydrogen peroxide (H_2O_2). The whole is digested for 30 minutes at 420°C on a boiling plate. Afterwards, the digestate is cooled and 50 mL of H_2O is added to dilute. The second step of the method involves distillation through a steam current distiller with UDK equipment (*Fig. 19*). 70 mL of sodium hydroxide (NaOH) at 32% is added to the digested sample and the distillate is collected in a Herlenmeyer flask containing 25 mL of boric acid (H_3BO_3) solution at 40g/L. The distillate is then titrated with 0.05M sulphuric acid with the addition of 7 drops of indicator.

Fig. 19 Kjeldahl distillation unit (UDK) 126A used for Kjeldahl nitrogen determination

3.4.3 Determination of KCl extractable nitrogen (exchangeable plus soluble ammoniacal nitrogen)

The determination of extractable nitrogen was carried out by extraction with a 2M potassium chloride (KCl) solution and Kjeldahl distillation following the method XIV.4 in the GU[99]. This method allows determining exchangeable nitrogen plus soluble ammoniacal nitrogen. The KCl extraction involved mixing 15 g of soil with 150 mL of KCl solution (soil/solution ratio 1:3) and stirring for about 1 hour. At the end of stirring the solution is filtered, sodium hydroxide is added and distilled with a steam distiller where the distillate is collected in a flask with 25 mL of boric acid. The distilled solution is then titrated with H_2SO_4 0.05M.

3.4.4 Determination of soluble nitrogen

Soluble nitrogen is determined by the method IV.2 described in the G.U[99]. For each test 7 g of soil is taken and mixed with 70 mL of deionised H_2O (soil/ H_2O ratio 1:10). The solution is then stirred for 1 hour and then filtered. The main anions (fluorides, chlorides, nitrites, nitrates, phosphates and sulphates) are determined using the ion chromatograph reading Metrohm IC 883 Basic plus (*Fig. 20*). Using the salicylate ammonium colorimetric method, soluble ammoniacal nitrogen ($N\text{-}NH_3$) was also determined in soil extracts with H_2O.

Fig. 20 Ion chromatograph (Metrohm IC 883 Basic plus) used for the determination of soluble forms of nitrogen

3.5 Determination of pH and Electrical Conductivity (EC) of soil

In order to verify if there was a pH and EC change in the soil before and after the test in each mesocosm, method IV.2 of the G.U[99] was used. To 20 g of soil sample 50 mL of deionised H_2O is added and stirred for two hours. The solution is then centrifuged, and a pH meter and conductivity meter are used to read the pH and electrical conductivity (EC) of the aqueous soil extracts. At this soil water ratio (1:2.5 w/v) the soil electrical conductivity is commonly indicated as EC 1:2.5.

3.6 Ecotoxicity tests

The effects of the NaDES and of the other treatments used in the microcosm experiment on soil microarthropods and on the emergence and early growth of plants were tested.

3.6.1 Effects on soil Microarthropod fauna

The mesofauna of the soil is made up of all those animal organisms between 0.2 and 2 mm, in length, and this description includes a high number of species belonging to the phylum Arthropoda, which are collectively indicated as microarthropods.

At the end of the microcosm experiment one integrated soil sample was obtained from each container. Each sample was obtained by dividing the plastic container of the mesocosms into 4 parts and collecting a soil core from the centre of each portion. Two paired plastic scoops were used as the corer. The four cores were then combined into an integrated sample of the approximate volume of 1,5 L.

Each sample was placed on a Berlese-Tullgren funnel to extract the soil microarthropods, as shown in *Fig. 21*. The device works by creating a temperature gradient on the sample such that mobile organisms move away from the high temperature zone until they fall into a collection container with 80% alcohol, that kills and preserves the specimens. The light bulbs placed over each soil sample was left turned off for the first two days, to avoid drying the soil too quickly. The lights bulbs were then turned on and the extraction continued further eight days.

Fig. 21 Representation of the Berlese funnels used for the experiment. Photos (a) replicates from 1 to 5, photos (b) replicates from 6 to 10

The extracted specimens were observed and counted under a stereoscopic microscope. The main taxa were identified, and their abundances determined. In addition, within Acarina and Collembola, several groups were defined based on morphology and the number of specimens in each group was counted. These groups were not taxonomically identified. Each group, presumably corresponded to a single species, was used as an operational taxonomic unit in the data analysis, and was labelled using the name of the major taxon it belonged, followed by a number (e.g.: Acarina 1, Acarina 2, Collembola 1, Collembola 2).

Differences in the microarthropod abundances among treatments were tested using PERMANOVA[100,101]. PERMANOVA is a multivariate non-parametric method analogue to the analysis of variance (ANOVA) that allows data to be analysed and tested for statistical significance based on any resemblance measure, by using permutation techniques. As a complement to PERMANOVA, non-metric multidimensional scaling (MDS) was used to graphically represent the relationships among samples. The Bray-Curtis distance was used as the resemblance measure, after square root transformation of the taxa abundances.

In addition, various diversity indices were evaluated including, Margalef species richness index (d), Pielou uniformity index (J'), Shannon diversity index (H') and Simpson diversity index (1-λ).

The Margalef index (d), quantifies species richness in relation to the total number of individuals in the sample. The values of index are obtained from the formula:

$$d = \frac{(S-1)}{log_2(N)}$$

where S is the number of species and N is the number of individuals.

The Pielou index of evenness (J'), is a measure of how abundance is evenly distributed among the species that exist in a community. it is possible to find the value of the index through the formula:

$$J' = \frac{H'}{\log(S)}$$

where H' is the Shannon index as described below and S is the number of species.

The Shannon-Wiener index (H'), is an index that assumes that individuals are randomly sampled from an indefinitely large community and that all species are represented in the sample. The index can be estimated using the formula:

$$H' = \Sigma\, p_i * log_2 p_1$$

where p_i, is the proportion of individuals of species i in the sample and can be estimated by doing the ratio between Ni/N where Ni is the number of individuals of species i and N is the total number of individuals.

The original Simpson index is a dominance index (λ). A diversity index, which considers both evenness and richness, can be derived as follows:

$$1 - \lambda = 1 - \Sigma\, p_i^2$$

where $p_i$is the proportion of the i[th] species in a sample.

The multivariate analysis and the computation of the diversity indices were performed using the software PRIMER 6 with the PERMANOVA+ add-on.

The QBS-ar index of biological quality, according to Parisi[102], was also calculated. The method is based on classification of the soil microarthropods into biological forms. Each identified biological form is given a score ranging from 1 to 20 depending on whether the biological form considered is very little or completely suitable for the soil; this score is called the ecomorphological index (EMI. The scores of all the biological forms in the soil sample added together, determine the value of the QBS-ar index

3.6.2 Phytotoxicity: effects on the emergence and early growth of plants

The effects of the NaDES and of the other treatments on the emergence and early growth of plants were performed in accordance with ISO 11269-2:2012[103]. Oat (*Avena sativa L.*) was selected as the test species. The test was performed as a separate experiment, independent from the microcosm experiment.

Plastic pots with a diameter of about 8 cm were used as containers in the test. A paper filter was placed at the base of each pot to prevent soil, water or roots growing through the holes in the bottom of the container.

The soil used in this test was collected from the same site and at the same time as that used in the mesocosms and then treated separately. The treatments tested were the same as those used in the enzyme inhibition test and 3 replicates were made for each treatment. About 200g of soil, were placed in each pot and 10 oat seeds were sown in each pot.

The growth of the plants took place in a temperature controlled and stabilised chamber at 20°C ± 1°C, 7000 lx and a light/dark cycle of 16:8 h. After 50% of the control seedlings have emerged, about 5 days, they have been taken out so that a total of only five representative specimens, equally spaced, remain inside the pot.

The test ended 14 days after thinning the plants. Each plant was then gently separated from the soil by manual handling and then washed with water to remove any soil residues in the roots. The following endpoints were detected for each treatment and on each pot:

- shoot length
- dry shoot weight, after drying at 60°C for 48h
- visual observation of the effects on plants

4.Results and discussion

4.1 DES preparations

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

- Freeze-drying[104]: 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[105].
- Thermal mixing: the components are mixed at about 100°C on a hot plate with magnetic stirring until a homogeneous colourless clear liquid is formed (*Fig. 22*). After cooling to room temperature, a transparent uniform mixture indicates that the designed DES has been obtained[106]. This method has been selected for the preparation of DES.

Fig. 22 Representation of the DES preparation phases

4.2 Quantification of the total polyphenol content and antioxidant activity

We know that the total content of polyphenols analysed with the Folin-Ciocalteau procedure does not give us a clear picture the chemical nature of polyphenols, but we have tried to give a polyphenolic composition to our extracts using four calibration standards (catechol, epigallocatechin gallate, fluoroglucinol, gallic acid) that could be representative of a variety of polyphenols commonly present in natural matrices.

First, several biomasses were analysed to determine their polyphenol content and find the most promising source of urease inhibitors: white and red grape marc, spent coffee grounds, spent black and green tea have been analysed. The total polyphenol content is expressed as a percentage of the initial dried biomass (TPC DW%).

Usually, polyphenols are extracted with conventional solvents such as ethanol, methanol, dichloromethane, acetone, hexane[107]. Although their extraction yields are high, some of these solvents are harmful to both the environment and humans.

Thus, we decided to use the “green” mixture ethanol/water (1:1 v/v) as benchmark for comparing the extracts with ethanol/water (1:1 v/v) of the various biomasses with different calibration curves.

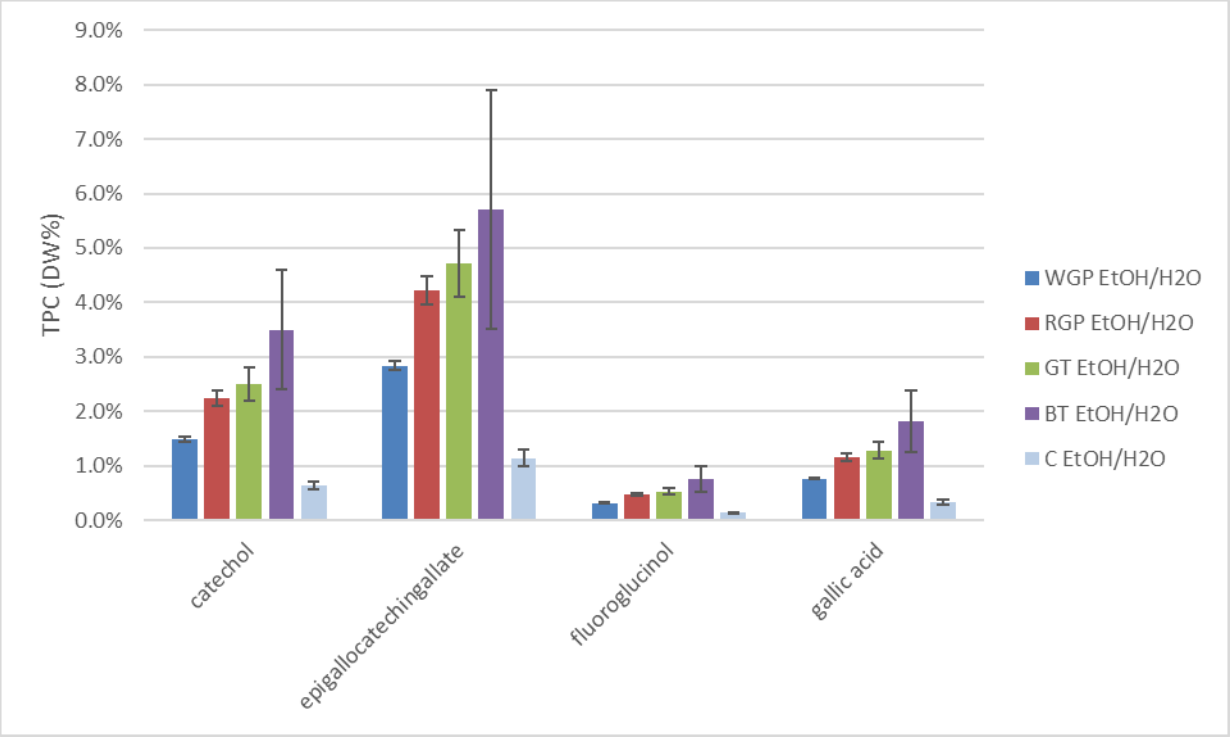


Fig. 23 Total polyphenols (dry weight percentage %) in different biomasses.(WGP= white pomace; RGP= red pomace; GT= green tea; BT= black tea; C= coffee).

Figure 23 shows the percentages of polyphenols extracted on dried weight basis (TPC DW%). All the biomasses analysed have a quite relevant polyphenol content except for coffee; black tea has the highest total polyphenol content according to all the different polyphenol standards, especially epigallocatechin gallate, which is representative of the polyphenol family (catechins) capable to inhibit urease in the human gastrointestinal tract[108]. These extracts were preliminary tested in an “in vitro” urease inhibition test performed on *Jack bean* urease (JBU) by prof. Stefano Ciurli and Dr. Luca Mazzei of Dipartimento di Farmacia e Biotecnologie (FABIT), University of Bologna (IT), with the aim of correlating the polyphenol content of each biomass with their potential inhibitory effect. These preliminary results (*Fig. 24*) are expressed

as residual urease activity after incubation with the same concentration of each extract.

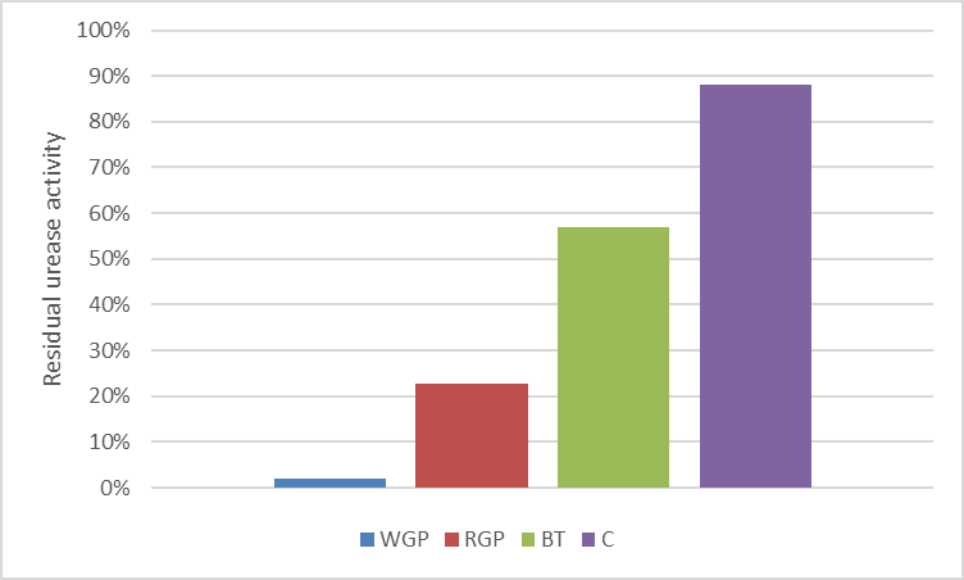


Fig. 24 Residual ureasic activity of the various biomasses tested. (WGP= white pomace; RGP= red pomace; BT= black tea; C= coffee).

As it is possible to see from the *Fig. 24*, coffee does not inhibit enzyme activity, it has 90% residual ureasic activity and this is related to its low polyphenol content. On the other hand, the white grape pomace is a surprise because from the *Fig.23* it does not seem to have many polyphenols but in the *Fig.24* it is the one that has the lowest percentage of residual ureasic activity, less than 1% of the activity is maintained.

The antioxidant activity of all the hydroalcoholic extracts considered was also evaluated and compared to confirm the trend found with the “in vitro” enzymatic assay (*Fig. 25*). The results of the analysis using the DPPH method were expressed as a percentage of antioxidant activity (AA%).

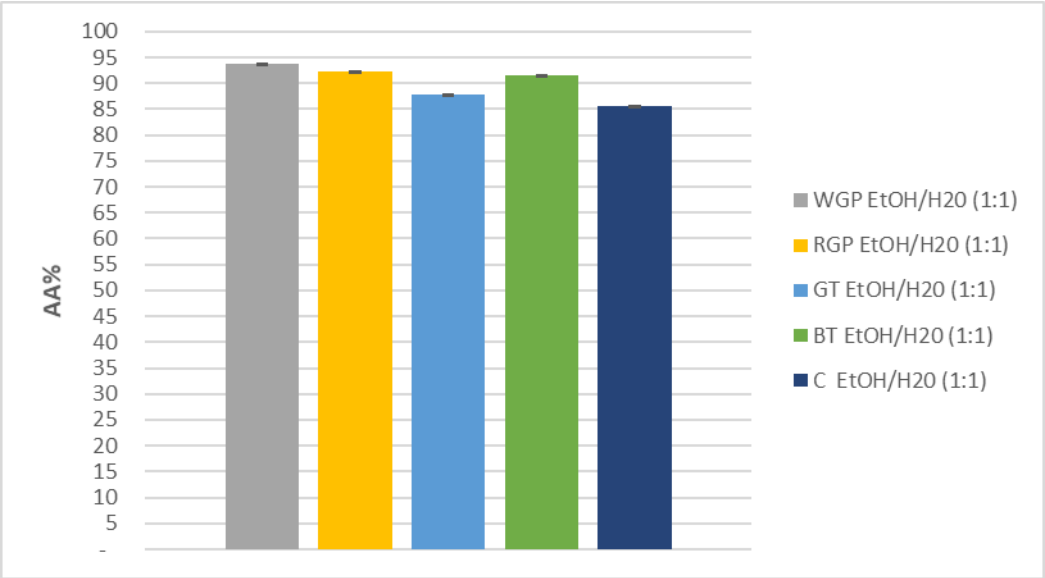


Fig. 25 Antioxidant activity expressed in %. (WGP= white pomace; RGP= red pomace; GT= green tea; BT= black tea; C= coffee).

All the extracts analysed have a high antioxidant power with a range that varies from 86% (spent coffee grounds) to 94% (white grape pomace), in line with the preliminary data on urease inhibition capability: the higher is the percentage of antioxidant activity, the greater is the inhibition of enzyme activity.

Therefore, white grape pomace was chosen as our reference biomass to be used in the test and made a comparison between different extraction protocols with different solvents (*Fig.26*). The extraction yield with NaDES (CHCl:EG) 1.6 ± 0.2% is two times higher than the yield achieved with the conventional EtOH/H2O mixture (0.8± 0.02%) and about eight times higher than the results with water alone (0.2± 0.05%). This result agrees with other studies carried out on the extraction of polyphenols with deep eutectic solvents (DES)[64,70].

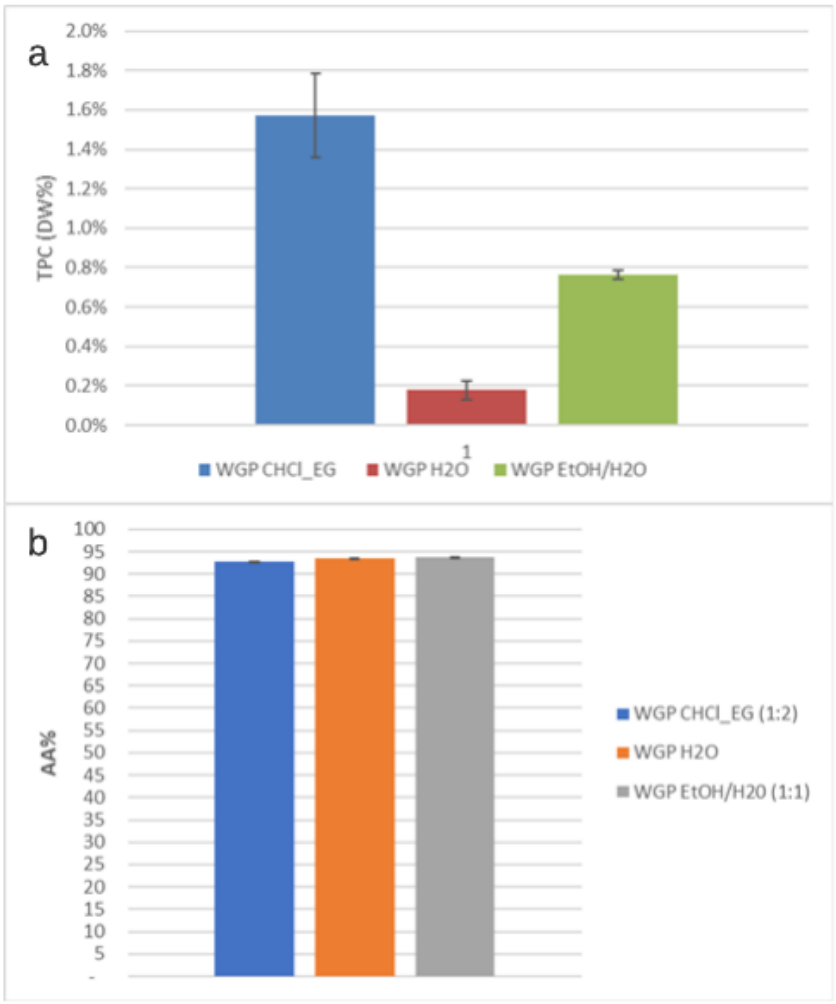


Fig. 26 (a) Total polyphenols (dry weight %) (b) antioxidant activity (%) of white grape pomace (WGP) by three different extraction methods.

By taking epigallocatechingallate as a benchmark, an evaluation of the relationship between the different classes of polyphenols in white grape pomace was also performed: the polyphenols of the epigallocatechingallate family are about double the polyphenols of the catechol family, 9 times higher than the polyphenols of the fluoroglucinol family and about 4 times higher than the polyphenols of the gallic acid family (*Fig. 27*).

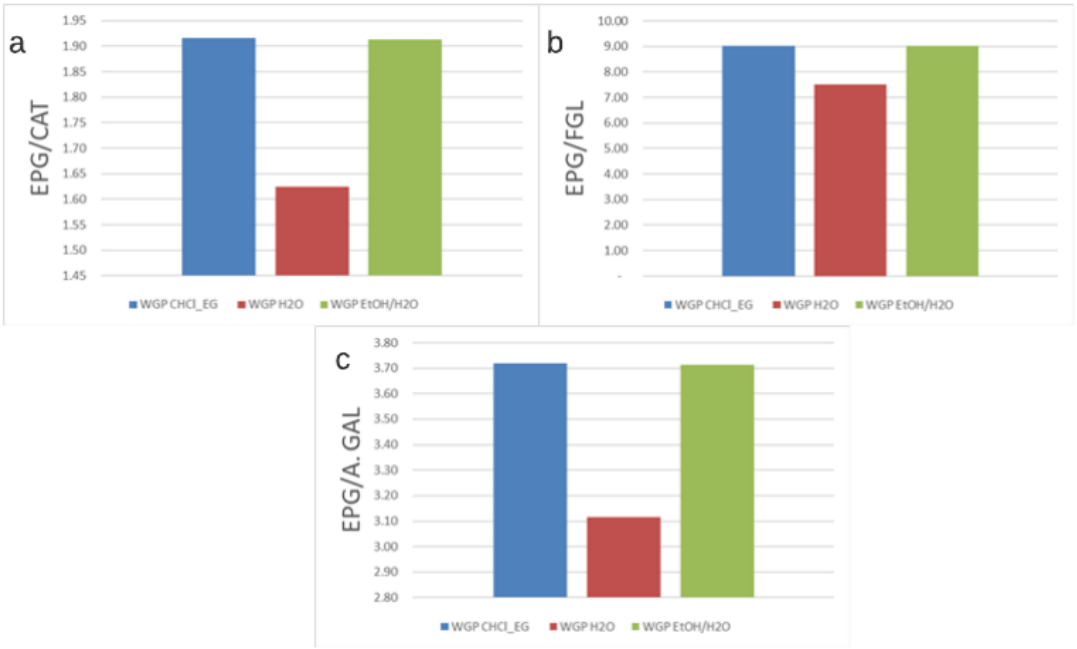


Fig. 27 Representation of the relationships between the families of polyphenols considered. a) epigallocatechingallate/catechol; b) epigallocatechingallate/fluoroglucinol; c) epigallocatechingallate/ gallic acid.

This result is in accordance with a study[109], that determined the catechin family as the most abundant one in various grape pomace by HPLC analysis, confirming the considerable potential of the by-products of the wine-producing as sources of natural antioxidants.

4.3 Soil analysis before and after the inhibition test

The results of the chemical-physical parameters analyzed in the soil before and after the mesocosm test are shown below. The analysis allowed to evaluate the effects of fertilization and inhibitors on nitrogen pools and other chemical parameters related to them within mesocosms.

4.3.1 pH, EC, dry matter and Kjeldahl nitrogen

Table *2* shown a slightly decrease of soil pH in the mesocosms, compared with soil at the beginning of the test (Soil 0), decreasing from moderately alkaline to weakly alkaline. The acidification process could be due to the high increase of nitrite and nitrate concentration in the mesocosms (*Table 2*), following the oxidation of part of nitrogen supplied by urea. However, decrease of pH is limited, as these soils have a

high concentration of calcium carbonate[110]. In fact, according to the literature, the dissolution of the latter as calcium bicarbonate induces a strong buffering power, limiting pH changes[111].

On the other hand, the increase in electrical conductivity (EC) is due to the abundant presence of ions in the soil extracts as shown in *Tab.3*. In fact, EC is a measure of all the ions that conduct electricity in aqueous solutions. The greater the quantity of ions present in the solution, the greater the electricity conducted by that solution. The soil at time zero (Soil 0) presents non-saline conditions (EC 1:2.5 < 0.5), while in the mesocosms weakly saline (0.5 < EC 1:2.5 < 1) to saline (1 < EC 1:2.5 < 2) conditions are reached.

The amount of dry soil within each mesocosm (4,252 kg) is 90.81% of the weight of non-dry soil (4,682 kg), while the dry matter in percentage decreases significantly due to the irrigations: 1240 mL, corresponding to a rain of 120 mm.

The Kjeldahl nitrogen (composed by organic and ammoniacal nitrogen) observed in the soil at time zero (Soil 0) presents the typical normal values of an agricultural orchard soil.

On the post-treatment soil samples, we observe an increase in the concentration of Kjeldahl nitrogen, in a range from 1400 mg/kg dm to 1800 mg/Kg dm, compared to Soil 0, which has an amount of 1370 mg/Kg dm. This increase may be due to soil fertilization with urea in all mesocosms and, in some of them, to the presence of DES used (ChCl:EG), as choline chloride is a quaternary ammonium salt.

To support this increase in organic and ammoniacal nitrogen there are the mineralization reactions that start from an organic compound containing nitrogen (in this case, urea), which is hydrolysed in NH_3 which can be protonated in the form NH_4^+ or volatilized, as shown in the equation[112]:

Replica/ box	Samples	Reaction in H2O	CE 1:2.5 25°C	Dry matter (dm) *	Amount of soil per mesocosm	Kjeldahl N	Extracted N in KCl *
		pH	mS/cm	%	kg dm	mg/kg dm	mg/kg dm
0.1	Soil 0	8.14	0.1984	90.81		1383	16.9
0.2	Soil 0	8.12	0.1913	±		1368	±
0.3	Soil 0	8.12	0.1958	0.34		1368	0.006
1	DES - 1	7.58	1.1504	75.14		1783	199.9
2	H2O+Pol - 1	7.58	0.8933	75.42		1626	35.7
3	Control 1	7.65	0.7835	76.00		1420	14.0
4	Limus - 1	7.63	0.8439	74.97	4.252	1466	33.8
5	DES+Pol - 1	7.70	0.9447	75.95		1672	94.6
6	Control 2	7.77	0.7080	75.34		1479	33.4
7	Limus - 2	7.79	0.5889	74.18		1449	26.9
8	DES+Pol - 2	7.72	0.9156	74.21		1575	60.6
9	DES - 2	7.77	0.8125	71.41		1678	112.9
10	H2O+Pol - 2	7.82	0.6018	75.79		1311	39.6

* : Dry matter and N in KCl values are referred to different replicas

Table 1 pH, EC, Kjeldahl N and N extracted in KCl for each mesocosm compared to Soil 0

4.3.2 Extracted N in KCl

From KCl extracts, by distillation, the adsorbed ammoniacal N in the colloidal exchange sites, plus the soluble N, were quantified. Basically, these represent the reduced forms of soil mineral nitrogen. It is observable that in all mesocosms there has been a strong increase of KCl extractable N compared to the initial condition. In mesocosms where urea plus NaDES were administered an increase of about 10 times the concentration of KCl extractable nitrogen present in the soil at time zero is visible.

4.3.3 Soluble ammoniacal nitrogen and soluble anions

The soluble ions inside the soil have been expressed in milliequivalents per Kg of dry matter (meq/kg dm). With these analyses, we wanted to evaluate the change in the composition of the main soluble anions and the amount of ammonium ion (NH_4^+) compared to the initial soil conditions (Soil 0). From *Table 2* we can see that in all mesocosms there is a strong increase of nitrite (NO_2^-) concentrations and over all of nitrate (NO_3^-) concentration, compared to the starting soil. That is most likely due to the oxidation performed by bacteria inside the soil[112]. Attention should be paid to tests in which both DES alone and DES with the inhibitor were administered. In fact, as we see a sharp increase in chloride concentration due to the release of this anion

from the choline chloride into the soil. For the same reason we see a sharp increase in ammonium ion concentration because the used DES is a quaternary ammonium salt.

Replica/ box	Samples	F-	Cl-	NO2-	NO3-	PO43-	SO42-	NH4+
		meq/kg dm	meq/kg dm	meq/kg dm	meq/kg dm	meq/kg dm	meq/kg dm	meq/kg dm
0.1	Soil 0	0.252	0.368	0.066	0.148	0.056	0.283	0.09
0.2	Soil 0	0.311	0.372	0.066	0.107	-	0.264	0.07
0.3	Soil 0	0.249	0.364	0.066	0.110	-	0.258	0.12
1	DES - 1	0.155	5.622	2.407	15.829	-	0.262	6.65
2	H2O+Pol - 1	0.182	0.327	0.836	10.739	0.075	0.329	0.74
3	Control 1	0.162	0.430	1.493	21.276	-	0.406	0.77
4	Limus - 1	0.199	0.393	2.063	20.523	-	0.407	1.44
5	DES+Pol - 1	0.160	4.272	1.501	14.297	-	0.275	3.18
6	Control 2	0.158	0.412	0.789	15.051	-	0.357	0.44
7	Limus - 2	0.177	0.461	1.787	11.950	-	0.378	0.45
8	DES+Pol - 2	0.174	4.935	1.646	13.700	-	0.297	2.58
9	DES - 2	0.160	5.143	1.833	15.245	-	0.302	3.97
10	H2O+Pol - 2	0.204	0.558	1.012	12.992	-	0.452	0.66

"-" : not detectable value

Table 2 Soil soluble anions and cations, comparison between mesocosms and Soil 0

4.3.4 Water soils extracted by rizhon

The main anions in the leached water composition are NO_3^-, Cl^- and NO_2^-, while ammonium cations (NH_4^+) found are in very low concentrations The high Cl^- and NO_3^- concentrations in leached water, are correlated to the concentrations found in the soil soluble fraction (*Table 3*). Therefore, it is possible that a fraction part of these ionic forms has been moved by water[112].

Box	Sample	CE 1:10 25°C	F-	Cl-	NO2-	NO3-	SO42-	Anions sum	NH4+	Collected volumes
		mS/cm	meq/L	meq/L	meq/L	meq/L	meq/L	meq/L	meq/L	mL
1	DES - 1	2.030	0.014	10.422	0.460	4.736	0.337	15.969	0.047	56
2	H2O+Pol - 1	1.677	0.014	1.076	0.817	8.219	0.458	10.584	0.013	32
3	Control 1	1.470	0.000	1.218	0.705	4.355	0.334	6.612	0.008	50
4	Limus - 1	1.322	0.015	1.114	0.678	4.890	0.394	7.090	0.009	31
5	DES+Pol - 1	1.503	0.015	5.003	0.388	2.921	0.364	8.690	0.044	52
6	Control 2	1.308	0.027	1.865	0.933	5.833	0.718	9.377	0.009	33
7	Limus - 2	1.307	0.016	1.804	0.793	5.564	0.572	8.751	0.008	32
8	DES+Pol - 2	2.578	0.040	10.408	0.284	6.726	0.527	17.984	0.009	45
9	DES - 2	1.734	0.063	6.013	0.182	2.843	0.522	9.623	0.014	49
10	H2O+Pol - 2	1.220	0.021	1.190	0.754	3.996	0.431	6.392	0.017	32

Table 3 Anions and cations soluble in smoothed water, comparison between mesocosms and Soil 0

4.3.5 Concentration of soil nitrogen pools

From the various soil analyses carried out, a summary has been made of the concentrations of the various nitrogen pools found in the soil both at time zero and in each mesocosm after the test (*Table 4*). All the results are expressed in milligrams of nitrogen per kilogram of dry matter (mg/Kg $_{dm}$).

In *Table 5* we see that the most abundant form of nitrogen is organic, while the most abundant mineral forms are NO_3^- and NH_4^+, the latte in both exchangeable and soluble forms, in all mesocosms. In all cases, there is an increase of both the total nitrogen concentration and the mineral nitrogen pools, with respect to the soil at time zero (Soil 0). This is more evident in the treatments where we have administered NaDES which, being composed by choline chloride, a quaternary ammonium salt, had some influence on the final nitrogen concentration, increasing both organic and ammonium nitrogen forms.

Box	Samples*	Organic N	Exchangeable N-NH4+	Soluble N-NH4+	Soluble N-NO2-	Soluble N-NO3-	Mineral N	Total N
		mg/kg dm	mg/kg dm	mg/kg dm	mg/kg dm	mg/kg dm	mg/kg dm	mg/kg dm
	Soil 0	1356	2.8	1.3	0.9	1.7	6.7	1363
1	DES - 1	1583	106.7	93.2	33.7	222	455	2038
2	H2O+Pol - 1	1591	25.3	10.3	11.7	150	198	1788
3	Control 1	1406	3.3	10.7	20.9	298	333	1738
4	Limus - 1	1432	13.6	20.2	28.9	287	350	1782
5	DES+Pol - 1	1578	50.1	44.5	21.0	200	316	1893
6	Control 2	1445	27.2	6.2	11.0	211	255	1700
7	Limus - 2	1422	20.6	6.3	25.0	167	219	1641
8	DES+Pol - 2	1514	24.4	36.2	23.0	192	275	1790
9	DES - 2	1565	57.3	55.5	25.7	213	352	1917
10	H2O+Pol - 2	1272	30.3	9.2	14.2	182	236	1507

* : For "Soil 0" was considered a medium value because replicas was not came out from the same aliquots.

Table 4 Concentration of nitrogen pools at the end of the test compared to Soil 0.

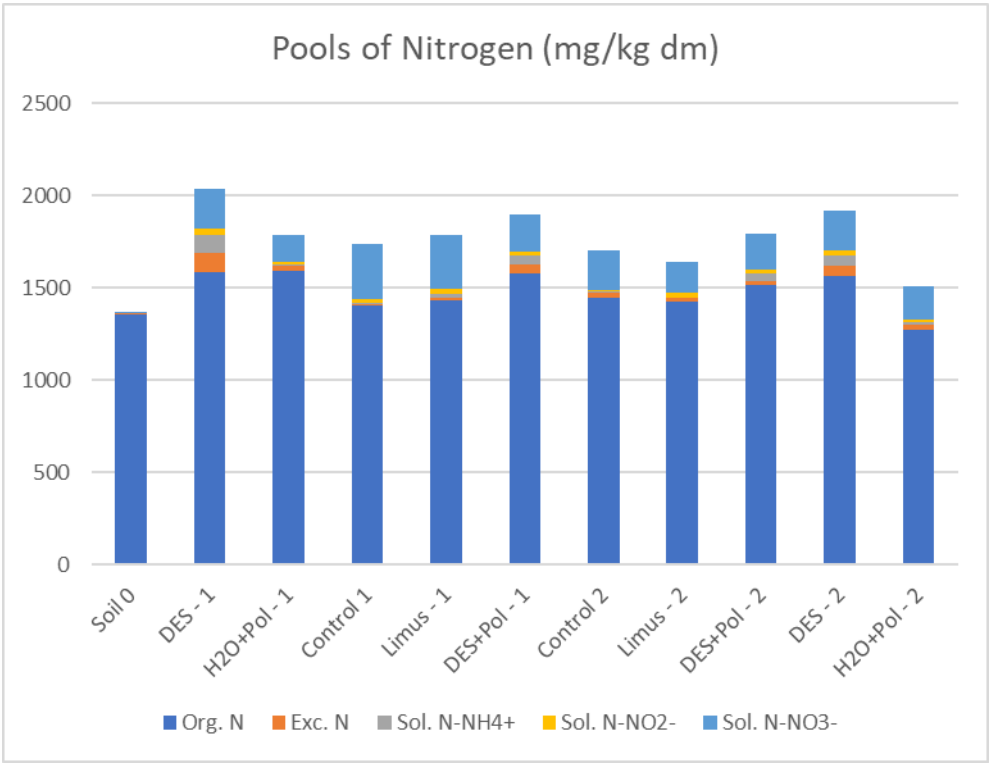


Fig. 28 Histogram with the distribution of the various nitrogen pools.

4.4 Ammonia volatilization

The focal point of our study was to test the inhibition effect of the polyphenolic extracts (with DES and H_2O) on urease enzyme activity. The released amounts of NH_3 were compare with the values obtained with those of the NBPT (Limus) inhibitor already on the market. The results reported in *Fig. 29* derive from a 18-day analysis of the amount of NH_3 collected with the capture system compared to the nitrogen administered with fertilisation as input (%N-NH_3 seq/ N-$CO(NH_2)_2$.

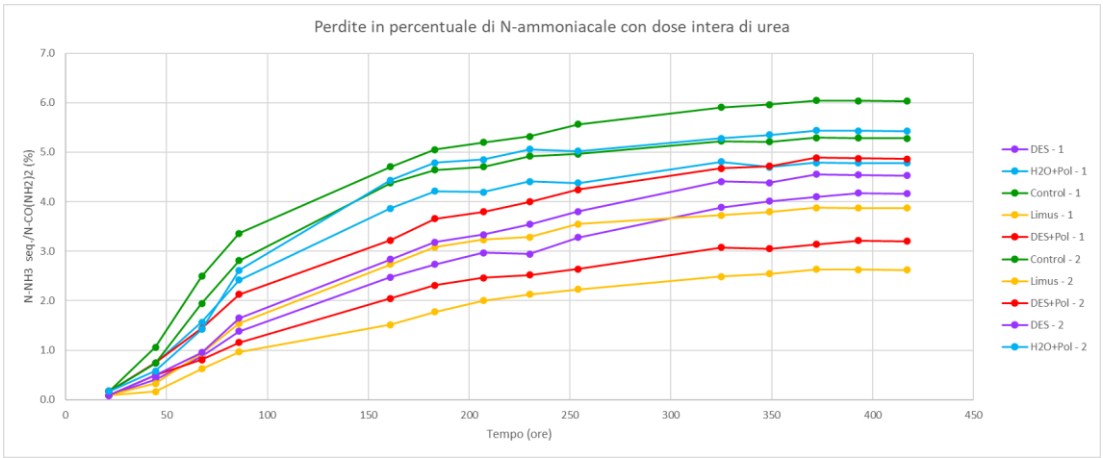


Fig. 29 Graphic representation of the ammonia seized during the 18 days of analysis.

Despite the number of replicates that has to be increased in further experiments, some preliminary considerations can be done:

- NBPT present in the Limus formulation, already on the market, has been confirmed as a good urease inhibitor, resulting to be the compound with the lowest release of volatile ammonia, as already claimed in other studies[113];
- the performance of DES plus polyphenols (DES+Pol) is the most like the one of Limus, but the variability of the data is the largest among the tested compounds (*Fig. 30*). Moreover, since the used DES is an organic compound formed by a HBA (choline chloride) bound with a HBD (ethylene glycol), there could be the possibility that the presence of another HBD (urea) present in our system gives rise to other hydrogen bonds with ChCl: the direct effect of this hypothesis could be that ChCl incorporates urea creating a new DES ChCl/U[27,81], making urea less available for the enzyme (consequently a lower ammonia release would be observed);
- the same holds true for the treatment with DES alone (DES);
- the treatment with water and polyphenols (H_2O + Pol) does not seem to have any effect on the inhibition of the urease enzyme. This happens because the water alone extracts too little or extracts polyphenols that are not able to inhibit the enzyme.

Sample	% N-NH3 seq/ N-CO(NH2) 2 %
DES - 1	4.2
DES - 2	4.5
H2O+Pol - 1	4.8
H2O+Pol - 2	5.4
Control - 1	6.0
Control - 2	5.3
Limus - 1	2.6
Limus - 2	3.9
DES+Pol - 1	4.9
DES+Pol - 2	3.2

Sample	Mean %	St.dv %
DES	4.3	0.3
H2O+Pol	5.1	0.5
Control	5.7	0.5
Limus	3.2	0.9
DES+Pol	4.0	1.2

Fig. 30 Percentage of ammonia sequestered in each mesocosm, mean and standard deviation

4.5 Mass balance of nitrogen pools

The analyses carried out have the aim, among the others, to understand the distribution in the various nitrogen forms of the nitrogen that we have administered through urea.

Knowing all the concentrations of the nitrogen forms and the amount of soil put in each mesocosm, all the amounts of nitrogen pools involved during the test were calculated. As shown in Fig.31, the nitrogen pools in input and output, related to the chemical forms indicated in brackets, have been considered.

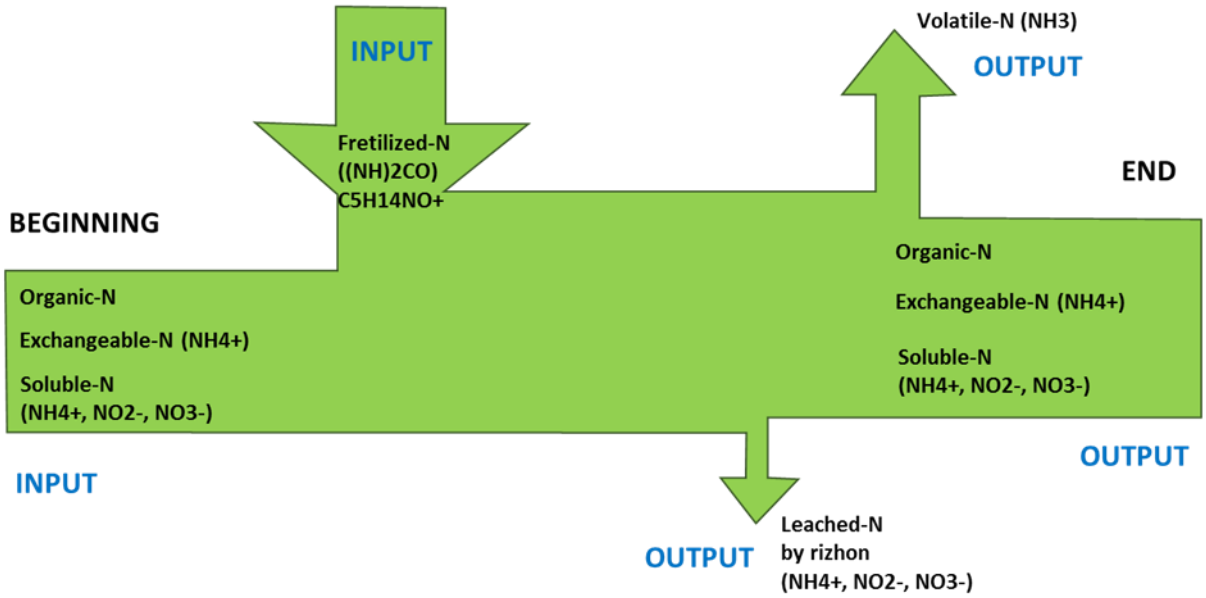


Fig. 31 Schematic representation of the inputs and outputs of the various nitrogen pools in our mesocosms.

The tables (*Table 5 a/b*) show all the inputs and outputs of the various nitrogen forms expressed in milligrams (mg), both in the mesocosms with the various treatments performed and in the soil at time zero (Soil 0). As can be seen in almost all mesocosms, the total nitrogen content obtained analytically (Total-N analysed) is however similar to the total nitrogen content calculated from the algebraic sum of nitrogen at time zero plus nitrogen administered as urea and choline chloride minus leaching and volatilization losses: (Total-N theoretical). The amount of organic nitrogen increases in almost all mesocosms and represents most of the nitrogen in the soil. It is possible that the lower organic nitrogen content in mesocosm No. 10 is due to unrepresentative sampling and not to an abnormal and unlikely mineralization.

The characterization of nitrogen pools in mesocosms has also been done in order to understand into which forms of nitrogen the administered urea is transformed.

The form of nitric nitrogen (NO_3^-) represents the most abundant form after organic nitrogen and in a real context could be a problem for groundwater contamination. Nitrogen lost through volatilization compared to the total is a modest amount. Finally, looking at the quantities of the different pools, it could be hypothesized that part of the urea nitrogen supplied is hydrolysed by the urease enzyme. A fraction of formed NH_3 is volatilized and a more conspicuous part is oxidized in the nitric and nitrous forms (*Fig. 32*).

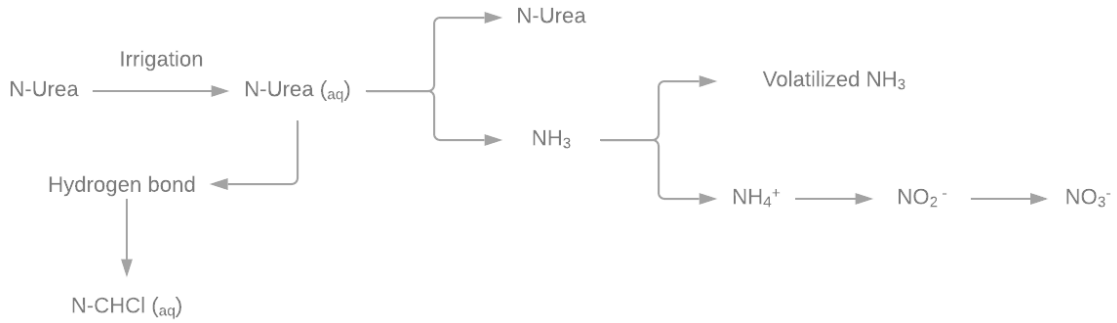


Fig. 32 Schematic representation of possible distributions of urea after hydrolysation

a

		Soil 0		1 - DES 1			2 - H2O+Pol 1		3 - Control 1		4 - Limus 1		5 - DES + Pol 1		
Pools	Chem. Form	Initial amounts	Input	Input	Losses	Final amounts	Losses	Final amounts	Losses	Final amounts	Losses	Final amounts	Input	Losses	Final amounts
		mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg
Organic-N		5766				6731		6763		5976		6090			6707
Excangeable-N	NH4+	66.2				454		108		14		58			213
Soluble-N	NH4+	5.6				396		43.8		45.7		86.0			189
	NO2-	3.9				143		49.7		88.9		123			89.4
	NO3-	7.3				942		639		1266		1222			851
Fertilized-N	(NH2)2CO		1581.6												
	C5H14NO+			420									420		
Volatile-N	NH3				108.5		120.3		142.0		76.8			130.1	
Leached-N (extracted by Rhizon)	NH4+				0.037		0.006		0.005		0.004			0.03	
	NO2-				0.36		0.37		0.49		0.29			0.28	
	NO3-				3.71		3.68		3.05		2.12			2.13	
Total-N (analyzed)		5849	1582		113	8666	124	7604	146	7391	79	7578		133	8050
Total-N (theoric)						7738		7307		7286		7352			7719

b

		Soil 0		6 - Control 2		7 - Limus 2		8 - DES + Pol 2			9 - DES 2			10 - H2O + Pol 2	
Pools	Chem. Form	Initial amounts	Input	Losses	Final amounts	Losses	Final amounts	Input	Losses	Final amounts	Input	Losses	Final amounts	Losses	Final amounts
		mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg	mg
Organic-N		5766			6145		6044			6438			6652		5407
Excangeable-N	NH4+	66.2			116		88			104			244		129
Soluble-N	NH4+	5.6			26.3		26.7			154			236		39.3
	NO2-	3.9			47.0		106			98			109		60.3
	NO3-	7.3			896		711			815			907		773
Fertilized-N	(NH2)2CO		1581.6												
	C5H14NO+							420			420				
Volatile-N	NH3			123.3		98.8			85.4			122.7		136.3	
Leached-N (extracted by Rhizon)	NH4+			0.004		0.004			0.006			0.01		0.007	
	NO2-			0.43		0.36			0.18			0.12		0.34	
	NO3-			2.70		2.49			4.24			1.95		1.79	
Total-N (analyzed)		5849	1582	126	7230	102	6977		90	7609		125	8149	138	6408
Total-N (theoric)					7305		7329			7761			7726		7293

Table 2 Calculation of theoretical total nitrogen considering inputs and outputs of nitrogen pools in each mesocosm. a) mesocosms from 1 to 5; b) mesocosms from 6 to 10.

4.6 Effects on soil Microarthropod fauna

Acarina (mites) was the most abundant among the major taxa in all treatments, ranging from 12 to 90 individuals per sample. The second most abundant taxon was Collembola (from 1 to 9 individuals per sample).

The PERMANOVA test did not detect significant differences in the abundances of the operational taxonomic units among the five soil treatments (P = 0.446, *Table 6*).

Source of variation	df	SS	MS	Pseudo-F	P
Treatment	4	191.59	47.88	10705	0.446
Residual	5	223.75	1074.00		
Total	9	415.34			

Table 3 PERMANOVA test results for the abundances of operative taxonomic units of microarthropods

From the table elaborated through the Primer v6 software we can see that the p value we obtained is very far from the required level of significance ($p<0.05$) and therefore we can say that the differences between treatments are not significant. The MDS plot (*Fig. 33*) visually confirms that the treatments did not affect the microarthropod fauna, since the replicates of the different treatments are interspersed in the ordination plane (i.e. there is no separation between the treatments)

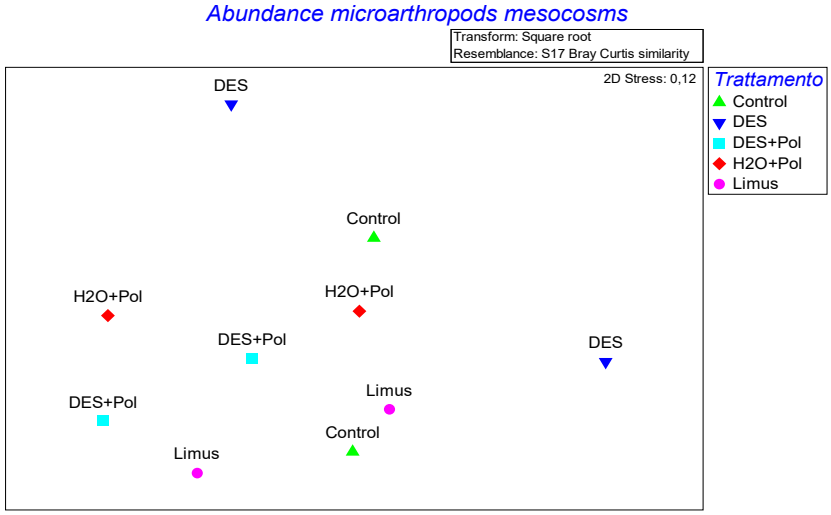


Fig. 33 Non-metric multidimensional scaling (MDS) plot based on square root transformed abundances of operational taxonomic units of microarthropods and Bray-Curtis distances.

As Acarina (mites) was the most abundant major taxon in all treatments, we wanted to see if there were significant differences between the various treatments as to the abundances of the operational taxonomic units within this group of microarthropods. Also here the PERMANOVA analysis was applied and as we can see from *Table 7,* the p value obtained is very far from the required level of significance ($p<0.05$), so we can say that also here the differences between the treatments are not significant.

Source of variation	df	SS	MS	Pseudo-F	P
Treatment	4	714.56	178.64	1.0803	0.483
Residual	5	826.78	165.36		
Total	9	1541.3			

Table 4 PERMANOVA test results for the abundances of operative taxonomic units of Acarina..

Acarina 1 was the most abundant operational taxonomic unit in all treatment. In the graph of *Fig. 34* we see its distribution within the treatments with the circles indicating the abundance found in each treatment.

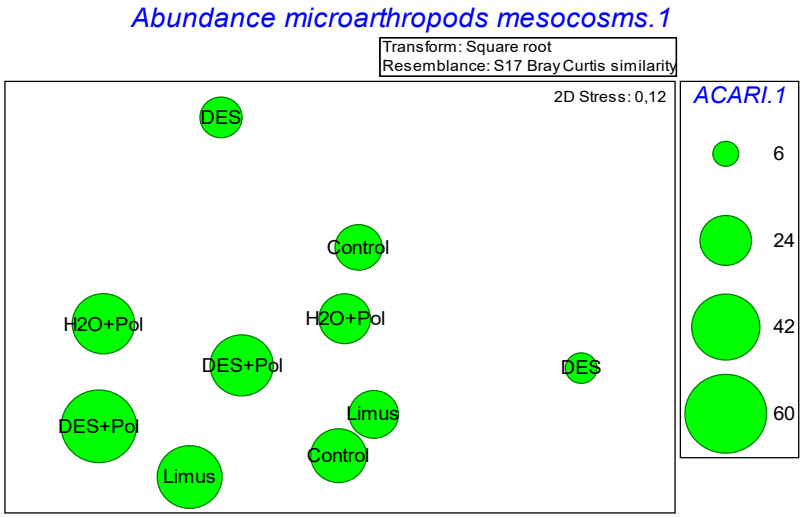


Fig. 34 MDS plot of Fig. 33, with superimposed circles representing abundance of the operative taxonomic unit Acarina 1.

4.6.1 Evaluation of the different diversity indices

Based on our data, we have also calculated the different diversity indices (*Table 8*).

Sample	S	N	d	J'	H'	1-λ
Control 1	10	32	2.60	0.62	1.42	0.60
Control 2	8	51	1.78	0.66	1.38	0.65
DES 1	12	43	2.92	0.79	1.97	0.81
DES 2	3	12	0.80	0.66	0.72	0.44
DES+ Pol 1	9	53	2.01	0.54	1.18	0.52
DES+ Pol 2	13	103	2.59	0.64	1.63	0.70
H2O+Pol1	6	53	1.26	0.63	1.14	0.54
H2O+Pol2	8	35	1.97	0.57	1.18	0.52
Limus 1	9	68	1.90	0.65	1.43	0.65
Limus 2	3	31	0.58	0.72	0.79	0.46

Table 5 Indices of diversity in the various treatments. (S = number of species; N= number of individuals; d= Margalef index; J'= Pielou index; H'= Shannon index; 1- λ= Simpson index).

The Margalef index (d) ranged from 0.58 in Limus2 to 2.60 in Control1. But there are no proposed or established reference values, and it is almost often difficult to understand.

The Pielou index (J') ranges between 0 and 1, where 1 represents a community with perfect uniformity of taxa abundances and decreases to 0 as the abundances deviate from uniformity[114]. In our case the values of J' varied between 0.54 and 0.79 and we can say that we have a medium-high uniformity.

The Shannon-Wiener index (H'),does not have a theoretical maximum but in general varied in a range from 0 to 5[114], where 0 indicates that there is no diversity while the maximum value is obtained when all species have the same frequency within the sample. In our case we have an index of diversity in the various treatments between 0.72 and 1.97.

The Simpson index (1- λ) varied between 0.44 and 0.81 which tell us that we have little abundance of species.

We carried out the analysis of the variance (Anova) to see if there were differences in the treatments according to the index calculation.

Source of variation	df	SS	MS	F	p
Treatment	4	1.5	0.37	0.481	0.751
Residual	5	3.9	0.77		
Total	9	5.4			

Source of variation	df	SS	MS	F	p
Treatment	4	0.026	0.007	1.761	0.273
Residual	5	0.019	0.004		
Total	9	0.045			

Source of variation	df	SS	MS	F	p
Treatment	4	0.155	0.039	0.178	0.94
Residual	5	1.089	0.218		
Total	9	1.244			

Source of variation	df	SS	MS	F	p
Treatment	4	0.015	0.004	0.184	0.937
Residual	5	0.104	0.021		
Total	9	0.119			

Table 6 ANOVA test results for the diversity indices. From top to bottom: Margalef index (d), Pielou index (J'), Shannon index (H'), Simpson index (1-λ)

As seen in *Table 9*, all four indices considered have obtained a value of p very far from the required level of significance ($p<0.05$). Therefore, we can say that the differences between the treatments are not significant.

4.6.2 Biological soil quality with microarthropods: QBS-ar

The results obtained with this method show that we have a minimum QBS-ar value of 20 obtained in the Limus2 replicate and a maximum value of 66 obtained in the Control1 replicate. For all other replicates the values remain homogeneous, varying between 31 and 42. By analysing our data through ANOVA we see that the p value we obtain is very far from the required level of significance ($p<0.05$) and therefore we can confirm that there are no significant differences between the different treatments.

The single QBS-ar value obtained in Control1 might seem anomalous comparing it with the average obtained in the other samples, but if we apply the average also between the two control replicas we see that we obtain a QBS-ar value of about 40 which is in line with the results of the other treatments. This anomalous value could therefore be due to randomness in the number of organisms in that mesocosm and not representative of the effects of the treatment administered.

Assuming that the soil type used in our study can be defined as "orchard", the reference QBS-ar value is, from literature, 103[115]. The values we obtained, as we can see, are largely below the reference value, possibly because of the manipulations to which the soil was subjected in the mesocosm experiment. The soil was firstly homogenized, and then a treatment was applied for several days with an aeration not equivalent to the natural one bringing therefore to a greater stress condition our organisms.

For a correct evaluation of the impact of our treatments through QBS-ar, the substances should be tested by eliminating those variables that could cause the stress that led to such low QBS-ar values.

4.7 Phytotoxicity

A first evaluation of the phytotoxicity of the DES and of the other treatments was carried out on watercress (*Lepidium sativum*) by assessing its growth within the mesocosms. After a first growth of the plants occurred about 4 days after sowing, the plants started not to grow anymore and many of them died while others did not really germinate. The possible cause for this outcome could be the poor light plants had during the experiment, as they were closed in a laboratory and thus not exposed to direct sunlight. A second cause could have been the massive amount of urea administered. We cannot therefore say anything about the phytotoxicity of the DES

formulations used in this test because the behaviour of the cress was the same in the other treatments where DES was not present. In other studies, however, there have been several DES formulations that have been shown to have no influence on seed germination[79].

Consequently, to assess the toxicity of DES on plants, an experiment on oats (*Avena sativa*) was conducted with the same treatments used in mesocosms. Shoot weight and length at the end of the exposure in the five treatments are compared in *Fig. 35*. The lengths of the shoots have similar values, with a range from 30 cm (Limus) to 33 cm (Control), while for the weight the values vary from 190 mg (Limus) to 256 mg (Control).

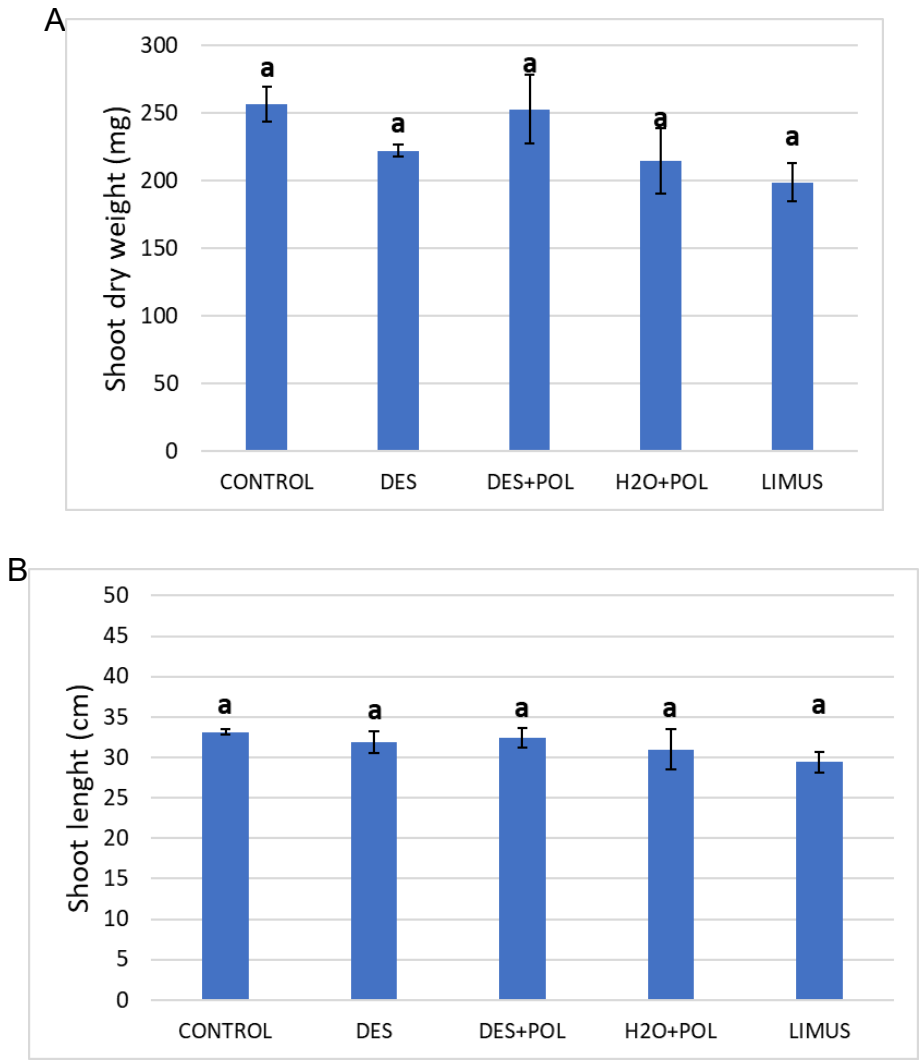


Fig. 35 Effect of different soil treatments on Avena sativa shoot weight (A) and shoot length (B); values are reported as mean ± standard error (n = 3). Treatments marked with the same letter are not significantly different from each other

Source of variation	df	SS	MS	F	p
Treatment	4	6381.9	1595.5	1.472	0.282
Residual	10	10838.4	1083.8		
Total	14	17220.3			

Source of variation	df	SS	MS	F	p
Treatment	4	24.6	6.1	0.96	0.471
Residual	10	64.2	6.4		
Total	14	88.8			

Table 10 ANOVA test results for shoot dry weight (a) and shoot length (b).

Applying ANOVA, as seen that the differences in shoot length and dry weight between the treatments were not statistically significant, in fact, as shown in *Table 10* where a p value was obtained that was far from the required level of significance ($p<0.05$). In addition, there were no visible differences in root growth (*Fig. 36*). This confirms the low toxicity of choline chloride-based DES[74].

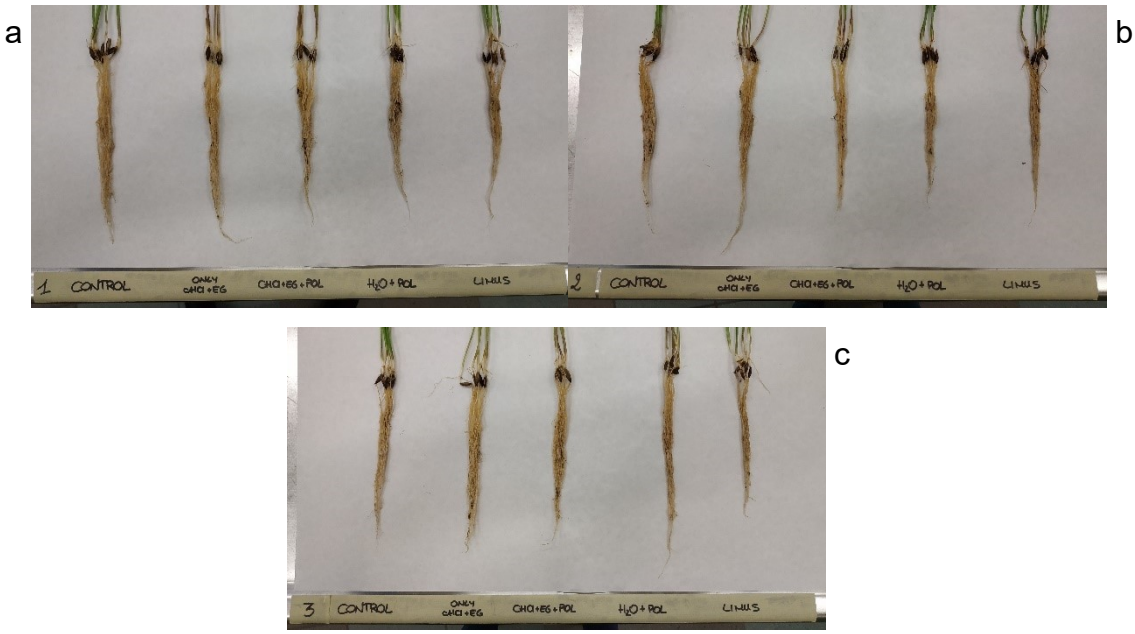


Fig. 35 Root growth in the different treatments. a) first replicate; B) second replicate; c) third replicate

No differences among treatments were evident in the yellowing of the tip of the shoot, which took place in all plants (*Fig. 37*).

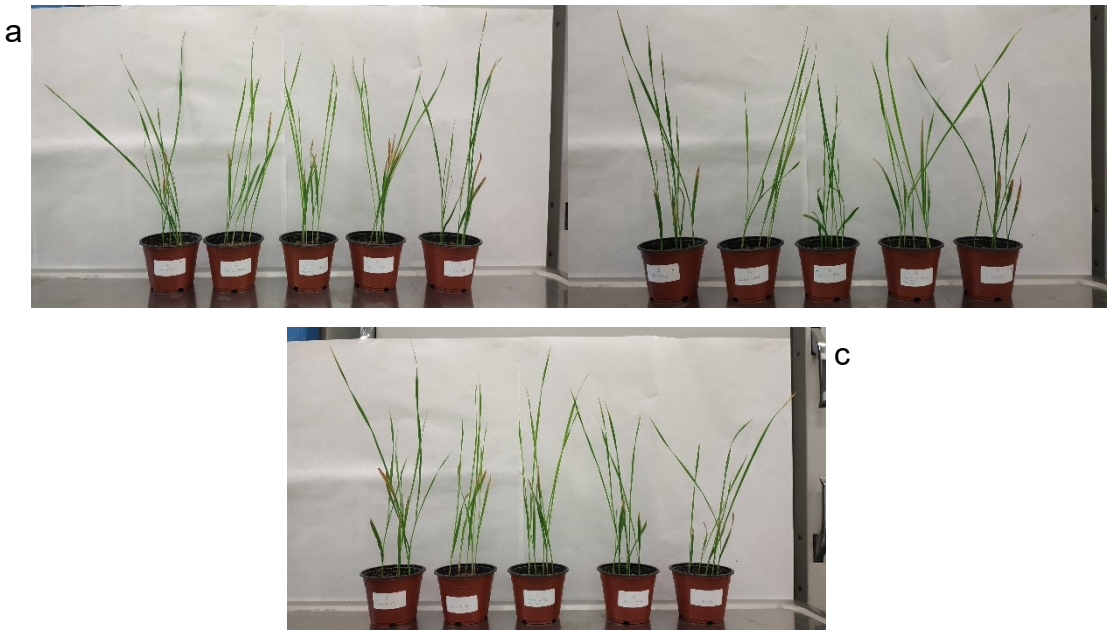


Fig. 36 Yellowing of the shoot seen on oat plants. a) first replicate; B) second replicate; c) third replicate

5.Conclusions

The aim of the study was to evaluate the efficacy of polyphenolic compounds, extracted with DES, as inhibitors of the activity of the enzyme urease in the soil and to compare them with the inhibitor already used in commerce (NBTP). The toxicity of DES on terrestrial organism and the destiny of nitrogen supplied by the urea-based fertilizer were also evaluated.

For this study, an initial characterization of the biomasses (red and white pomace, green and black tea, coffee grounds) was carried out, evaluating their polyphenol content and antioxidant activity. These results were then correlated to the preliminary in vitro assessment of ureasic activity. From these comparisons, white grape pomace was found to have the highest inhibiting power (>98% reduction of enzymatic activity) and was chosen as the biomass source of polyphenols in the test. Once the biomass of reference was chosen, the extraction capacity of DES based on choline chloride and ethylene glycol (ChCl:EG) was evaluated compared to conventional methods (H_2O and EtOH/H_2O mixture). Extraction with DES was found to be the best performing method compared to the other two methods. In addition, an evaluation of the antioxidant activity was carried out on white grape pomace extracts obtained with the three methods, demonstrating that they have approximately the same antioxidant power (~93%) regardless of the method and solvent used.

Polyphenol extracts were then tested in the evaluation of the effectiveness of urease inhibition through the capture system of volatile ammonia produced by hydrolysis of urea in the soil. Encouraging data were obtained regarding our DES formulation plus polyphenol extract, very close to the performance obtained with Limus (4% ammonia captured by DES + polyphenols against 3.2% captured by Limus (NBPT)).

A complete picture of the various nitrogen pools was then obtained by determining the concentrations of the nitrogen components in soil and leaching water, both before and after the test. A substantial N part was found in the soil in the form of organic nitrogen (range from 1300 to 1600 mg/Kg dm), while among the inorganic forms the one with the greatest influence was nitrogen in the form of nitrates (NO_3^-) (range from 170 to 300 mg/Kg dm). Ammonium ion (NH_4^+) concentrations, both exchangeable on soil colloids and water-soluble, were higher especially in mesocosms where DES was used as a means of transporting polyphenols

The ecotoxicity results, both on microarthropods and plants, show that none of the treatments had adverse effects in comparison with the control, thus supporting the idea that the use of DES in agriculture is eco-compatible.

In conclusion, DES have been shown to be good extractors of phenolic compounds from biomass and have shown good application in agriculture for inhibition of the urease enzyme. However, further investigation and improvement can be made by finding the best combination of HBA and HBD that will give even better performance on urease inhibition.

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