Sustainable Agricultural and Forestry Systems

and Food Security

XXXV Cycle

Recovery of hydrothermal liquefaction wastewater: potential valorisation for agronomic purpose

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Thesis abstract

The competition for fresh water among industry, agriculture, and public utilities is increasing due to population growth and climate change. On average, 40% of total water abstraction in Europe is used for industry and energy production, 15% for public water supply and 44% for agriculture. Agriculture is therefore the major user of freshwater. At the same time, agriculture is the sector most affected by water scarcity, especially in Mediterranean countries due to an aggravation of arid climatic conditions. In this scenario, the key to ensure water security, sustainability, and resilience is the "water reuse", also commonly known as water recycling or water reclamation. Water reuse reclaims water from a variety of sources that include municipal and industrial wastewater, stormwater, agriculture runoff and return flows

Industrial wastewater obtained from hydrothermal liquefaction (HTL-WW) of food wastes for biofuels production could represent a source of crop nutrients since it is characterized by a high amount of organic and inorganic substances. Therefore, in the present PhD thesis the potential valorisation of HTL-WW for agronomic purpose was assessed. In particular, an overview of the main biomass conversion strategies with a particular emphasis on hydrothermal liquefaction process was discussed in the first chapter. HTL is an innovative eco-friendly technology for bioenergy production that utilises water at high temperature and high pressure to break down the bonds of macromolecules contained in the biomass. The main products are bio-oil (15%), biochar (15%) and wastewater (70%). Considering the great concentration of this wastewater released during each production cycle, it is necessary to evaluate various valorisation strategies. Based on its composition, HTL-WW could be recycled in different biological processes such as anaerobic digestion or microalgae cultivation. However, the HTL-WW obtained from the organic fraction of urban wastes, is characterised by a neutral pH, a high content of nutrients and minerals and organic matter as well as it is free of human pathogens and hazardous chemicals. Because of these features, the HTL-WW from organic wastes could be a good candidate as water irrigation. In fact, The HTL-WW from organic wastes, is rich in nitrogen, phosphorus, potassium, organic carbon and minerals. However, the concentration of some

chemical elements, such as electrical conductivity, chemical oxygen demand or ammonia were beyond the official threshold values (DM183/2003).

Therefore, in the second chapter, the feasibility of the use of HTL-WW, deriving from the "Waste to fuel" technology employed by Eni S.p.A., was assessed in agriculture as irrigation water using *Nicotiana tabacum* L. as a model plant. Its impact on root-associated microbiota was determined and described evaluating the diversity and richness of prokaryotic and eukaryotic communities occurring following HTL-WW treatment. In detail, tobacco plants were grown in a greenhouse under controlled conditions and daily irrigated with diluted HTL-WW. Rhizosphere and plants were weekly sampled to evaluate, over time, the effect of wastewater irrigation both on soil microbiota, through culture-independent methods, as well as on the tobacco plants development, through the measurement of different biometric indices. The total genomic DNA was extracted from rhizosphere and bulk soil samples and preliminarily analysed by Denaturing Gradient Gel Electrophoresis (PCR-DGGE) to determine the prokaryotic and eukaryotic communities' structure. Amplicon based metagenomic sequencing was also employed to describe differences in microbial composition among treated and non-treated tobacco rhizosphere. The sequences were analysed with QIIME2 software. Taxonomic assignment was obtained by the RDP classifier and the Greengenes or UNITE database for bacterial 16S rRNA and fungal ITS sequences.

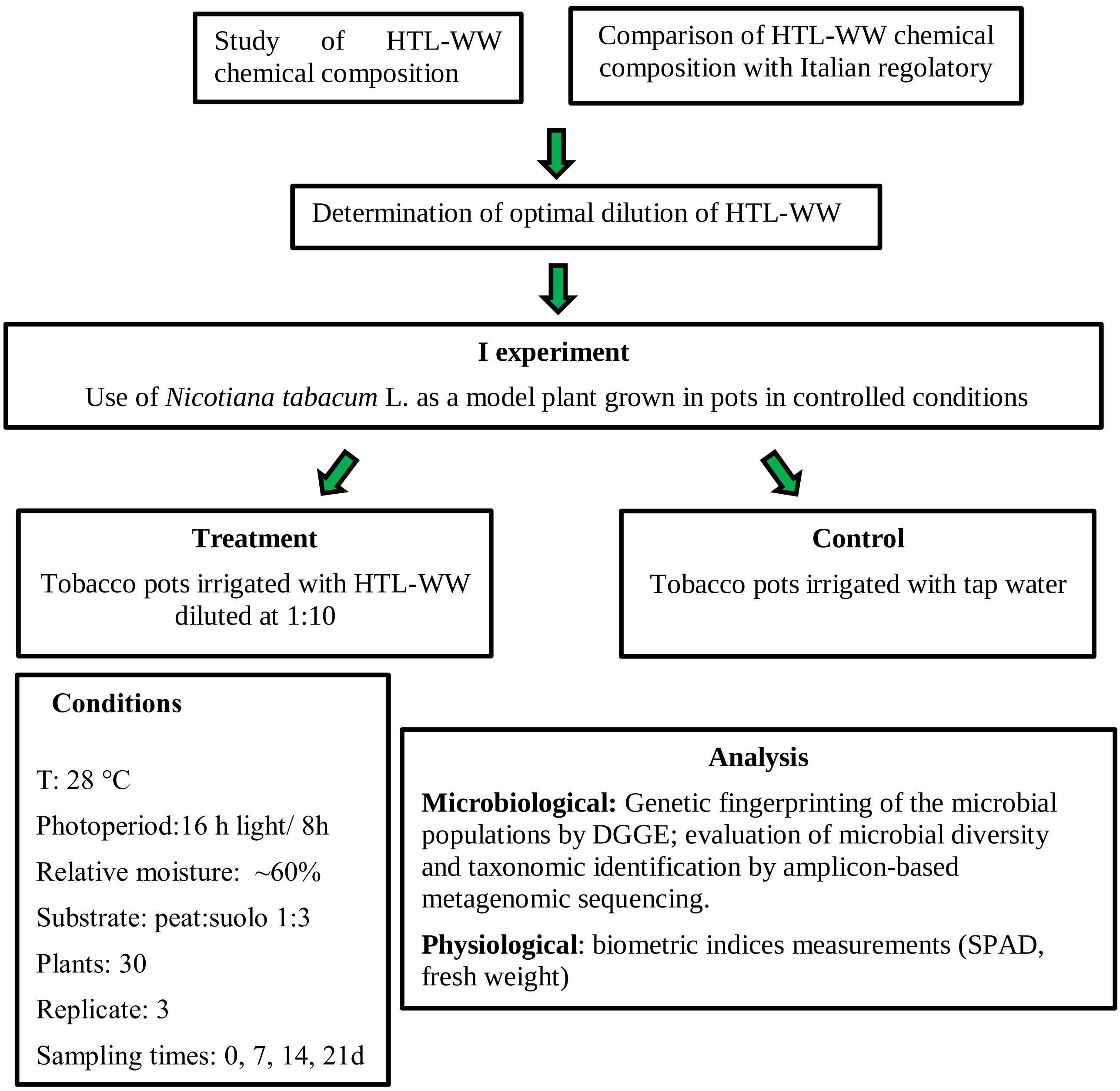
Based on the obtained results, a second experiment was carried out in open field conditions using *Ricinus communis* L. plants, which is currently a key species for bioenergy production, as described in the third chapter. Moreover, to improve crops development, a selected plant growth-promoting bacteria (PGPB) consortium was also inoculated to the plants, individually or in combination with HTL-WW. Therefore, the experimental design consisted of four different conditions as follows: 1) plants irrigated with tap water; 2) plants inoculated with PGPB consortium and irrigated with tap water; 3) plants irrigated with HTL-WW; 4) plants inoculated with PGPB consortium and irrigated with HTL-WW. Biometric indices and gas exchanges measurements as well as soil chemical analysis were performed. The composition of the rhizosphere-associated microbiota was also assessed by high-throughput sequencing. Data analysis of quality filtered reads was performed using R v4.0.1. Taxonomy assignment was performed using the RDP naive Bayesian classifier through the dada2 method with the SILVA database for prokaryotes.

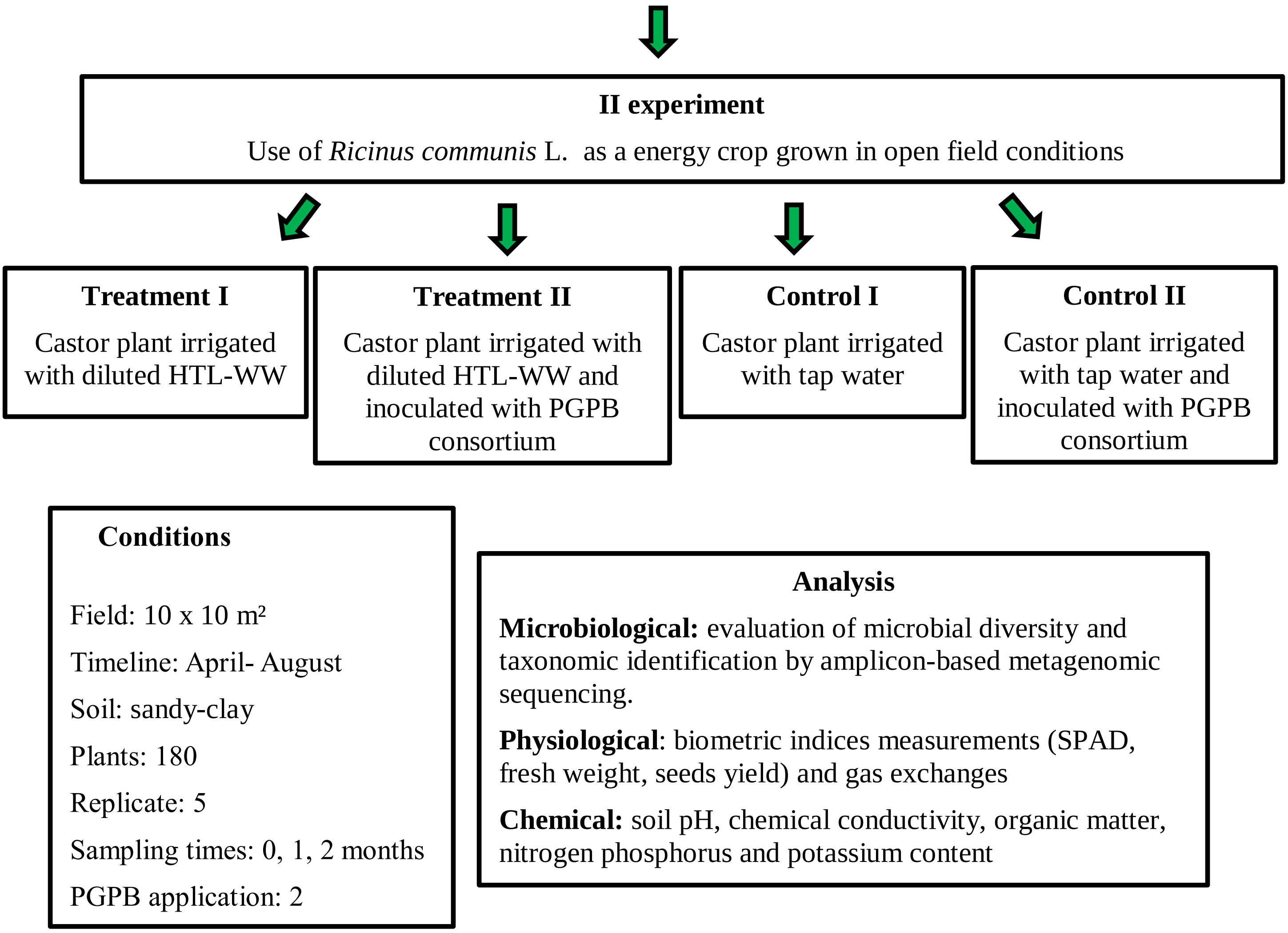
At last, in the **final chapter,** a conclusion of the entire PhD project was reported.

Objectives

- Assess the feasibility of the use of HTL-WW as irrigation water in agriculture using *Nicotiana tabacum* L. and *Ricinus communis* L. plants.
- Evaluate the response of the root-associated microbiota and plant to HTL-WW treatment as well as changes in soil properties.
- Investigate the effect of the simultaneous application of a selected plant growth-promoting bacterial consortium and hydrothermal liquefaction wastewater to *Ricinus communis* L. on autochthonous soil microbiota and plants.

Experimental design




II experiment
Use of *Ricinus communis* L. as a energy crop grown in open field conditions
Treatment I
Castor plant irrigated with diluted HTL-WW
Treatment II
Castor plant irrigated with diluted HTL-WW and inoculated with PGPB consortium
Control I
Castor plant irrigated with tap water
Control II
Castor plant irrigated with tap water and inoculated with PGPB consortium
Conditions
Field: 10 x 10 m²
Timeline: April- August
Soil: sandy-clay
Plants: 180
Replicate: 5
Sampling times: 0, 1, 2 months
PGPB application: 2
Analysis
Microbiological: evaluation of microbial diversity and taxonomic identification by amplicon-based metagenomic sequencing.
Physiological: biometric indices measurements (SPAD, fresh weight, seeds yield) and gas exchanges
Chemical: soil pH, chemical conductivity, organic matter, nitrogen phosphorus and potassium content

1. Introduction

In 2021 Italy held the Presidency of the G20, the international forum that brings together the world's major economies to face up the great challenges of today such as climate change, land degradation, biodiversity loss and freshwater shortage. The Earth's freshwater shortage due to consumption and pollution of resources drawing serious concerns, also because only a small percentage of water is available for human use. Indeed, about 96-97.5% of the Earth's water is found in the ocean as salt water, while the remaining 2.5-4% is freshwater. This latter is additionally subdivided into ice and snow (2-3% of globally available water) and surface water as well as groundwater (0.5-1%) (Filimonau and Barth, 2016). The most important uses, in terms of total abstraction, have been identified as urban (households and industry connected to the public water supply system), industry, agriculture and energy (cooling in power plants). On average, 40% of total water abstraction in Europe is used for industry and energy production (cooling in power plants), 15% for public water supply and 44% for agriculture. Nowadays, agriculture is the major user of freshwater; farming affects both the quantity (accounting for 70% of global freshwater withdrawals for irrigation) and quality (e.g. through fertiliser/pesticide pollution) of freshwater resources (Safe water, 2022). At the same time, agriculture is one of the first sectors to be affected of water scarcity, especially in Mediterranean basin countries due to an aggravation of arid climatic conditions (Figure 1) (Sofroniou and Bishop, 2014). The Mediterranean Basin is a region particularly prone to the effects of climate change and it was characterized as one of the hot-spots areas of the 21st century. Future warming rates in the Mediterranean area are expected to be 20% higher than globally in summer even up to 50% and increasing inter-annual variability in the warm season is projected (Vogel et al., 2021). The rise of temperatures is combined with precipitation and snow decreasing, that is crucial to sustaining the river flow, accounting for nearly a third of Italy's agricultural production. In fact, the drought in

Italy has caused serious problems and an economic loss of 1.4 billion € in the last few years (Villani et al., 2022).

The foreseen increased number of people to feed (more than 2 billion) implies to produce 60% more food, because of which total global water withdrawals for irrigation are projected to increase at 20–30% by 2050 (Zucchinelli et al., 2021). The key for water security, sustainability, and resilience is the "water reuse", also commonly known as water recycling or water reclamation. Water reuse reclaims water from a variety of sources that include municipal and industrial wastewater, stormwater, agriculture runoff and return flows (EPA,2021). About 380 billion m^3 of water can be recovered from the annual volumes of wastewater produced. This type of water recovery is expected to reach 470 billion m^3 by 2030 and 574 billion m^3 by 2050 (Unesdoc.unesco.org., 2022). The use of wastewater in agriculture offers a lot of benefits that include: a solution to irrigation water scarcity; the availability of large amounts throughout the year; the possibility of reserving better-quality water for human consumption; a potential reduction of fertilizers needed due to the nutrients contained in some wastewaters; protection of the environment; the reduction of effluent waters in the surrounding area; avoid the overexploitation of marine water in coastal areas. The wastewater recycling not only offers an alternative source for crop irrigation, but also the opportunity to recover fertilizing elements, such as nitrogen (N), phosphorous (P), potassium (K), organic matter, minerals, and micronutrients into agricultural soils. Nevertheless, in Europe only 2.4% of wastewater (700 Mm^3/year) is used, mostly in Spain, and this is clearly not enough (Petousi et al., 2019). Until 2020, the major barriers preventing a wider spreading of this practice in EU were the limited awareness of potential benefits among stakeholders, and the lack of a supportive and coherent framework for water reuse. According to Directive 91/271/EEC - Article 12, "*treated wastewater must be reused whenever appropriate and disposal routes must minimize any adverse effects on the environment*". However, the document does not specify the minimum standards for wastewater reuse (Petousi et al., 2019). Recently, the EU commission approved a new regulation on minimum requirements for water reuse in agricultural irrigation (EU 2020/741) that will be applied from 26 June 2023. This will eventually encourage and facilitate water reuse across Europe.

In Italy, the agricultural use of wastewater is currently regulated by Ministerial Decree no.185/2003, which regards only the municipal and agro-industrial effluents. However, nowadays, there so many innovative technologies for biomass conversion and energy production, which allow the recovery of wastewater with better and safer features than the municipal effluents.

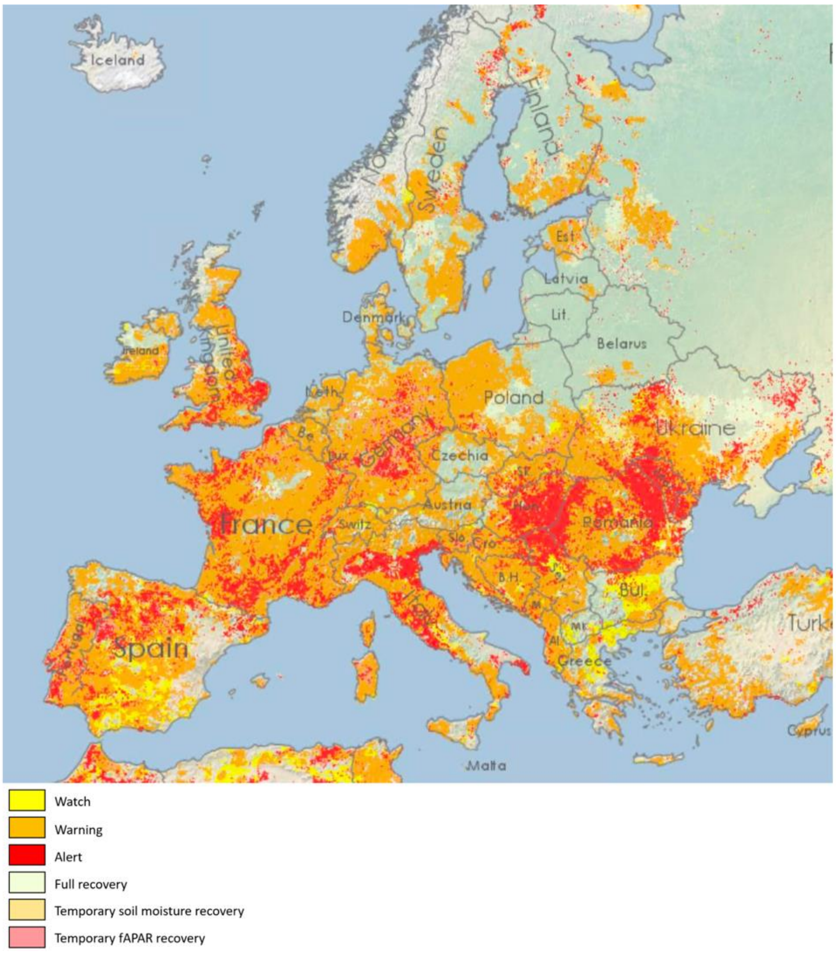


Figure 1. Combined Drought Indicator (CDI) in Europe in the first ten days of August 2022. Provided by the Global Drought Observatory (GDO) of the Copernicus Emergency Management Service (CEMS) - https://edo.jrc.ec.europa.eu/gdo.

2. Biomass conversion strategies and water recovery

2.1. Introduction

The twenty-first century is affected by one of the worst environmental crises in history, which have the potential to alter the natural course of life on this planet. Pollution, climate change, global warming, waste disposal and natural resource reduction are frightening challenges that may threaten next generations' future if governments, industrialists, and scientist do not face up to them promptly. These global problems are predominately related to the energy, since up today serious impacts on environment resulting from production, transport, and consumption of energy. According to International Energy Agency (IEA, 2017), over 60% of power production derived from fossil-based resources, such as oil, coal, and natural gas, which consists of hydrocarbon compounds situated under the earth's crust. The extensive exploitation of these elements and all the handling operations, negatively impact the quality of soil, air, and water. For instance, the coal mining led to dramatic degradation of soil by excavating, blasting, drilling rocks, and further resealing high quantity of toxic substances and heavy metals (Kumar and Singh, 2016). More dangerous is the water pollution, called acid mine drainage, which occurs when sulphide-rich rocks that contain target ores like gold and copper are exposed to water. The sulphides form sulfuric acid, which dissolves surrounding rock, releasing harmful metalloids into the groundwater near the mine. This pollution can spread through streams and rivers contaminating drinking water sources (Perera, 2017). Moreover, during the petroleum extraction process could occur oil spills, thus exposing wildlife and marine life to toxic hydrocarbons. This phenomenon not only causes the death of thousands of species but introducing these harmful elements into the food chain exposing human population to serious health risks (Horn, 2021). Furthermore, the electricity and heat consumption from fossil source, leads to the emission of carbon dioxide nitrogen oxides and sulphur dioxide which are responsible for acid rains, damaging vegetation and aquatic ecosystems (Gralla et al., 2017). Nevertheless, the transport sector (including road, aviation, and shipping) accounting for almost 96% of oil supply, remains the major source of environmental pressures in Europe (EEA, 2019). In fact, the fossil fuel combustion releases high concentration of gases that trap heat in the atmosphere, called greenhouse gases (GHGs), which are composed by 79% of carbon dioxide (CO_2), 11% of methane (CH_4), 7% of nitrous oxide (N_2O) and 3% of fluorinated gases. GHGs occur naturally and are part of our atmosphere keeping the planet at a habitable temperature of about 15°C (59 °F) on overage. Despite that, the increased concentration of these heating-trap gases leads to temperature of Earth's air and oceans rise, in the range of 1 to 1.2°C since 1850 (US EPA, 2022). Besides these catastrophic environmental effects, the fossil energy sources are also non-renewable and unevenly distributed around the world, further, the globally reserves are rapidly depleting (Martins et al., 2019).

The constant increasing population together with the increasing energy demand, lead to an unreasonable rise price. Hence, to mitigate market instability and environmental threats, in December 2019 the European Union introduced the European Green Deal, a set of policy initiatives to foster the transition towards the climate-neutral economy by reducing GHGs emissions towards 55% by 2030 and achieving carbon neutrality by 2050 (Sikora, 2021). Part of this package is the Renewable Energy Directive (EU 2018/2001), aimed to increase the shares of renewable energy sources in an integrated energy system.

Furthermore, the recent surge in demand due to COVID-19 pandemic and the war in Ukraine, caused a 60% and 400% price rises of oil and natural gas, respectively, prompted the European Commission to revise the Renewable Energy Directive increasing the use of energy from renewable sources up to 40% by 2030 (Butler, 2022). Thus, the European Commission is driving the UE State Members for constructing a new renewable-based energy system financing an unprecedented level of investment to promote the Energy Transition. As mentioned before, the aim of Energy Transition is to increase the diffusion of renewable energy into the energy supply mix, gradually replacing the oil, natural gas and coal with clean energy resources like biomasses, wind, solar, as well as lithium-ion batteries (Europe Bioenergy, 2022).

The circular bioeconomy is a promising approach to achieving the change required by the European Green Deal and decoupling economic growth from resource use. Indeed, a recent study estimates that applying circular bioeconomy principles has the potential to increase the EU total value of all goods and services produced (gross domestic product or GDP) by an additional 0.5% by 2030 and creating around 700 000 new jobs (Mhatre et al., 2021). The concept of "circular bioeconomy" combined the circular economy principles (reusing, repairing, and recycling) with the bioeconomy, which utilizes renewable biological resources to produce energy (Tan et al., 2021). Further, the circular bioeconomy goes beyond simply switching fossil resources with renewable, biological resources. It requires low-carbon energy inputs, sustainable supply chains, and promising disruptive conversion technologies for the sustainable transformation of renewable bioresources to high-value bio-based products (Giampietro, 2019).

The use of biomass as a feedstock for valuable chemicals and biofuels is crucial for the conversion from the fossil-based economy into a bio-based economy. However, the biomass conversion technologies are not intrinsically sustainable just because it is based on renewable resources (Pfau et al., 2014; Gawel et al., 2019). In fact, a non-sustainable process can cause various environmentally conflicts. For example, an increase in biofuel demand will lead to an increase in biomass demand, which in turn will lead to competition for arable land use (i.e., land-grabbing for biomass feedstock production), freshwater consumption, and even food production (i.e., food *vs*. fuel), resulting in social unrest or social sustainability concerns (Tan et al., 2021). On the environmental sustainability aspect, there will also be negative impact due to the increase in land demand for biomass production, including more GHGs emission due to indirect land-use changes, such as deforestation for growing energy crops (Plevin et al., 2010). The sustainable bioeconomy is not just about substituting fossil resources with renewable resources; it will require sustainable biomass feedstock production, biomass conversion processes, and products. Therefore, the main goal is to improve bioprocesses utilising organic waste materials as primary, sustainable and low-cost feedstock.

Waste biorefining is one of the eco-friendly and economically strategies of the bio-circular economy, that closes the loop of organic wastes valorisation, water and nutrients recovery, production of various marketable products, carbon management and GHGs mitigation. Various kind of waste materials such as food waste, side stream from industries (e.g., paper and pulp industry, beer and wine industry, starch, and juice industry), agro-industrial by-product, forest and agricultural waste, lignocellulosic material as well as wastewater or sludge, have been efficiently valorised into biofuel and bioproducts (Rehan et al., 2019; Leong et al., 2021). The techniques for biomass conversion and valorisation are eco-friendly and at "zero wastes", because are the most efficient to regain the residues such as water fraction and organic residues which could be further utilized.

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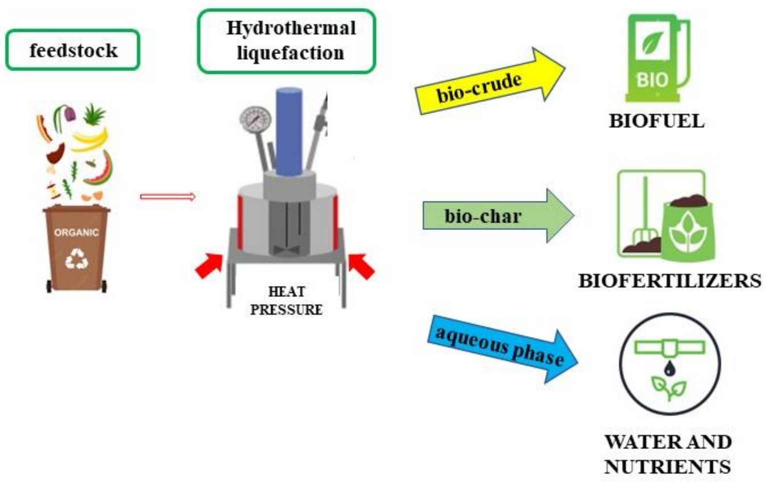

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[redacted] is produced daily in greater quantities, with high disposal costs. Although, this effluent [redacted] which regulates the agricultural use of wastewater, [redacted] Ministerial Decree no. [redacted] transferred to HTL-WW in terms of their high solubilities Gu et al., 2019). Thus, [redacted]

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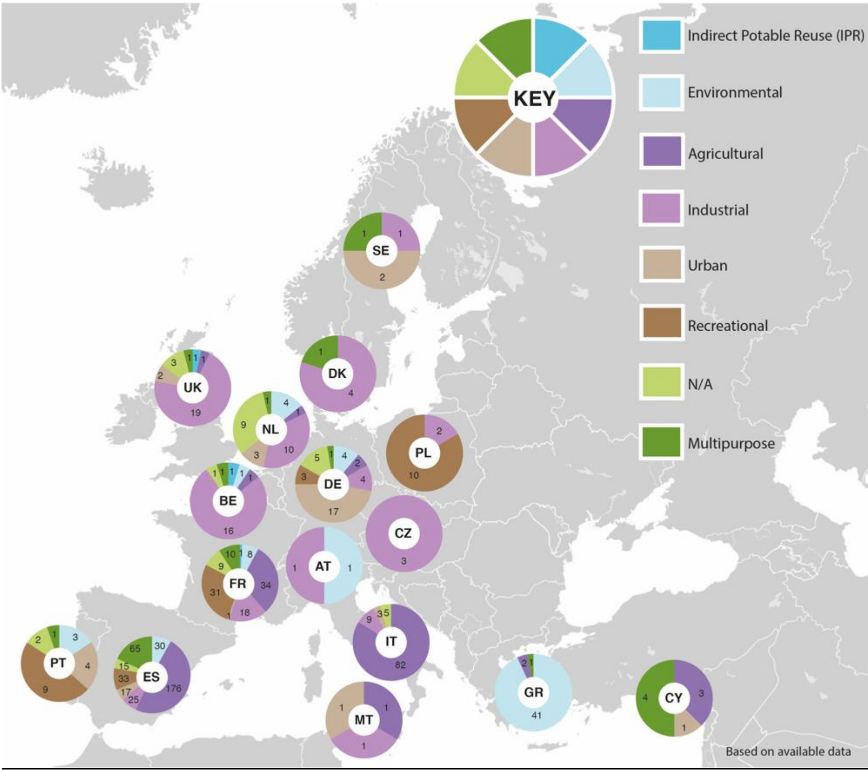

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3. Use of hydrothermal liquefaction wastewater in agriculture: effects on tobacco plants and rhizosphere microbiota

3.1. Introduction

The competition for fresh water among industry, agriculture, and public utilities is increasing due to population growth and climate change (Zucchinelli, et al., 2021). Agriculture is therefore the major user of freshwater and the most affected sector by water scarcity. Thus, to ensure water source for fields irrigation is necessary resorting to the use of wastewater.

In Italy, the agricultural use of wastewater is currently regulated by Ministerial Decree no. 2003/185, which regards only the municipal and agro-industrial effluents. Most research on testing the use of wastewater in agriculture refers to treated municipal wastewater, olive mill wastewater, sewage sludges and digestates (FAO, 2022). However, there are additional industrial processes which deliver, as side products, high levels of liquid wastes that could be recovered and valorised for agricultural uses. In particular, the hydrothermal liquefaction is a high-performance and eco-sustainable thermochemical technology to produce bioenergy from organic biomass and wastes. This is a green and cost-effective process since it does not require high energy input to dry out the feedstock as in other thermochemical techniques (Gu et al., 2019). The hydrothermal liquefaction is also an environmentally friendly method because it does not require additional chemicals, since it relies on water as a reaction medium and it minimizes problems associated with waste disposal (Usman et al., 2019). The Eni's Renewable Energy and Environmental R&D Centre has recently developed a continuous pilot plant within the project *Waste to Fuel* (Figure 4) whose aim is to produce biofuel from organic wastes (https://www.eni.com/en-IT/operations/waste-to-fuel.html). This pilot plant can process about 700 kg of Organic Fraction of Municipal Solid Waste (OFMSW) per day and produce from 3% to 16% of bio-oil, which can be used directly as low sulphur fuel to be shipped or further refined to create high-performance biofuels. The process generates also up to 95% of hydrothermal liquid wastewater (HTL-WW) with high concentrations of organic and inorganic compounds/elements. Therefore, the valorisation of this liquid co-product is a crucial step in hydrothermal liquefaction development since its discharge into civil wastewater treatment plants requires high extra-costs, making this process no longer economic viable (Posmanik et al., 2017). Moreover, HTL-WW may have some interesting properties as irrigation water, since it is rich in plant macro and micronutrients as well as organic carbon. The HTL-WW does not contain pathogens, pesticides and emerging contaminants including analgesics, antihypertensive drugs and antibiotics which may be found in municipal wastewater or sludge (Jaramillo and Restrepo, 2017). Nevertheless, the presence of organic and inorganic matter in wastewaters could affect the soil physic-chemical properties including the electrical conductivity (EC), hydrophobicity, heavy-metal concentrations, pH as well as organic carbon content, humus, nitrogen, phosphate and potassium levels and should be adequately monitored (Muamar et al., 2014). Moreover, wastewater applications are expected to alter the soil microbiota, because it is particularly sensitive to human-induced perturbations or environmental stress compared to higher organisms due to their close relations with the surroundings and because of higher surface area to volume ratio (Karimi et al., 2017). Investigating the soil microbiota composition and the interactions with plant systems could provide useful information on both crops and soils productivity and health status (Ventorino et al., 2018a). In this context, the aim of this study was to assess the feasibility of the use of HTL-WW as irrigation water in agriculture using *Nicotiana tabacum* L. as a model plant as well as to determine and describe the impact of HTL-WW on root-associated microbiota by evaluating diversity and richness variations of prokaryotic and eukaryotic communities. To the best of our knowledge, this is the first work reporting the use of wastewater deriving from hydrothermal liquefaction for crop irrigation purpose.

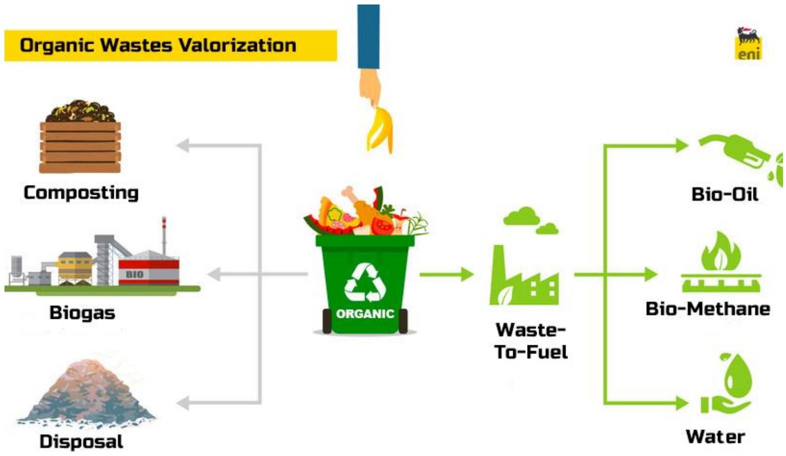

Figure 4. Schematic representation of Waste to fuel project developed by Eni S.p.A. (www.eni/wastetofuel.com).

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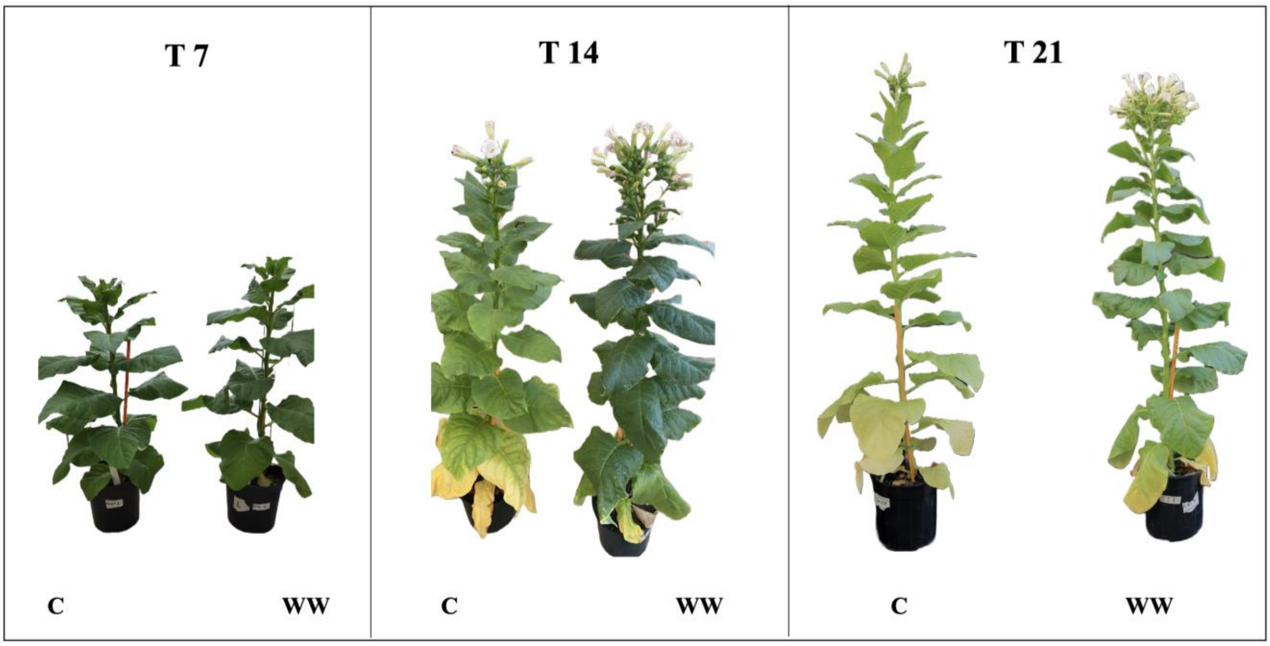

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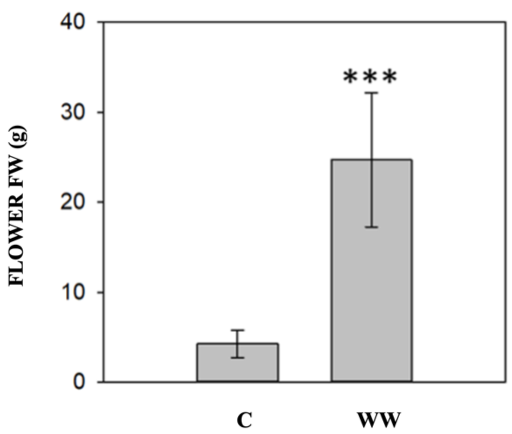

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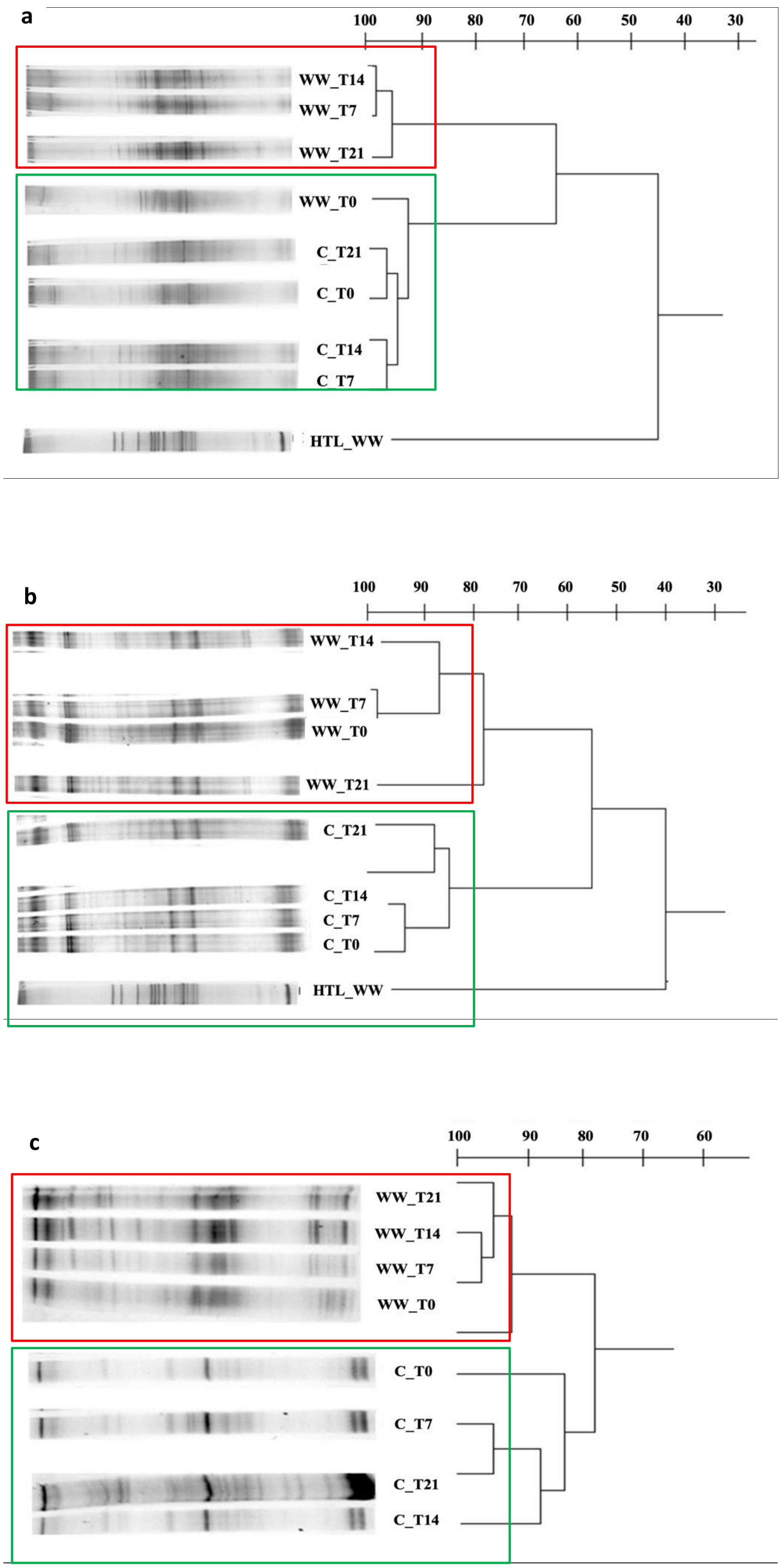
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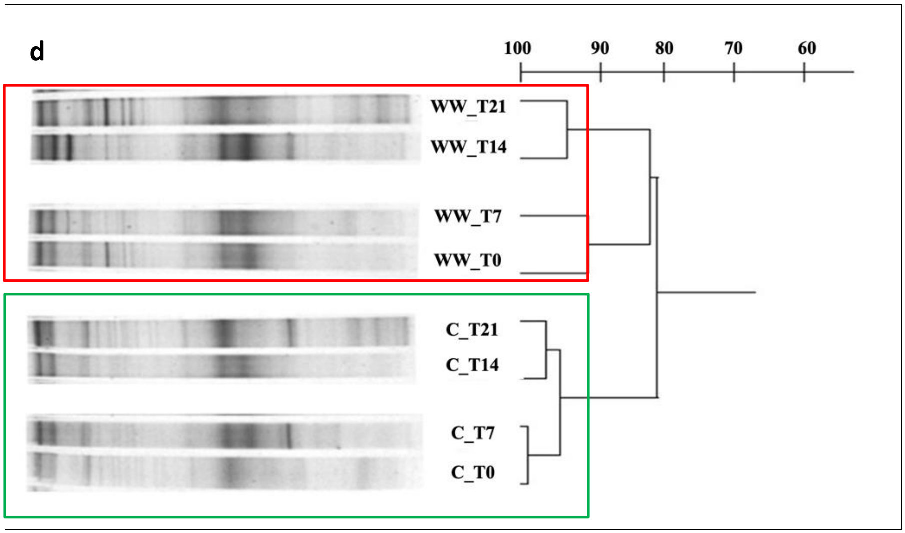

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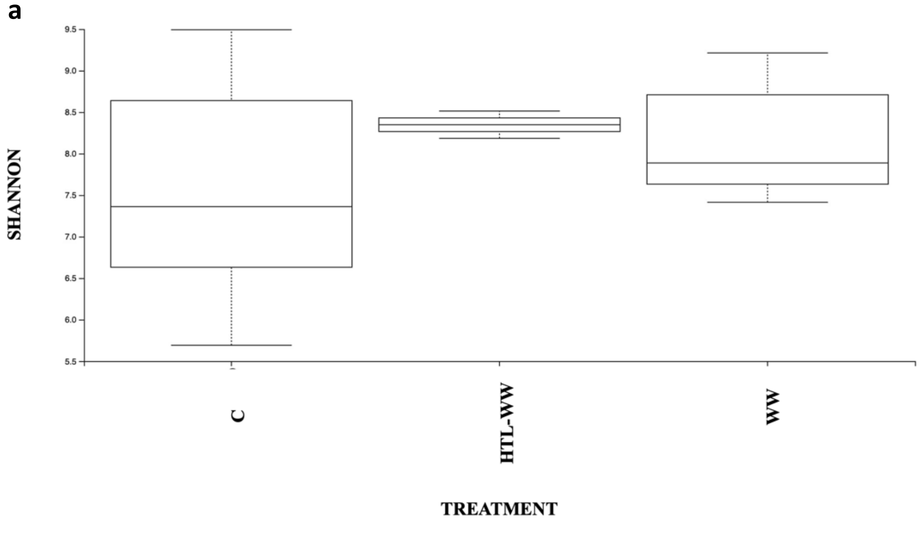

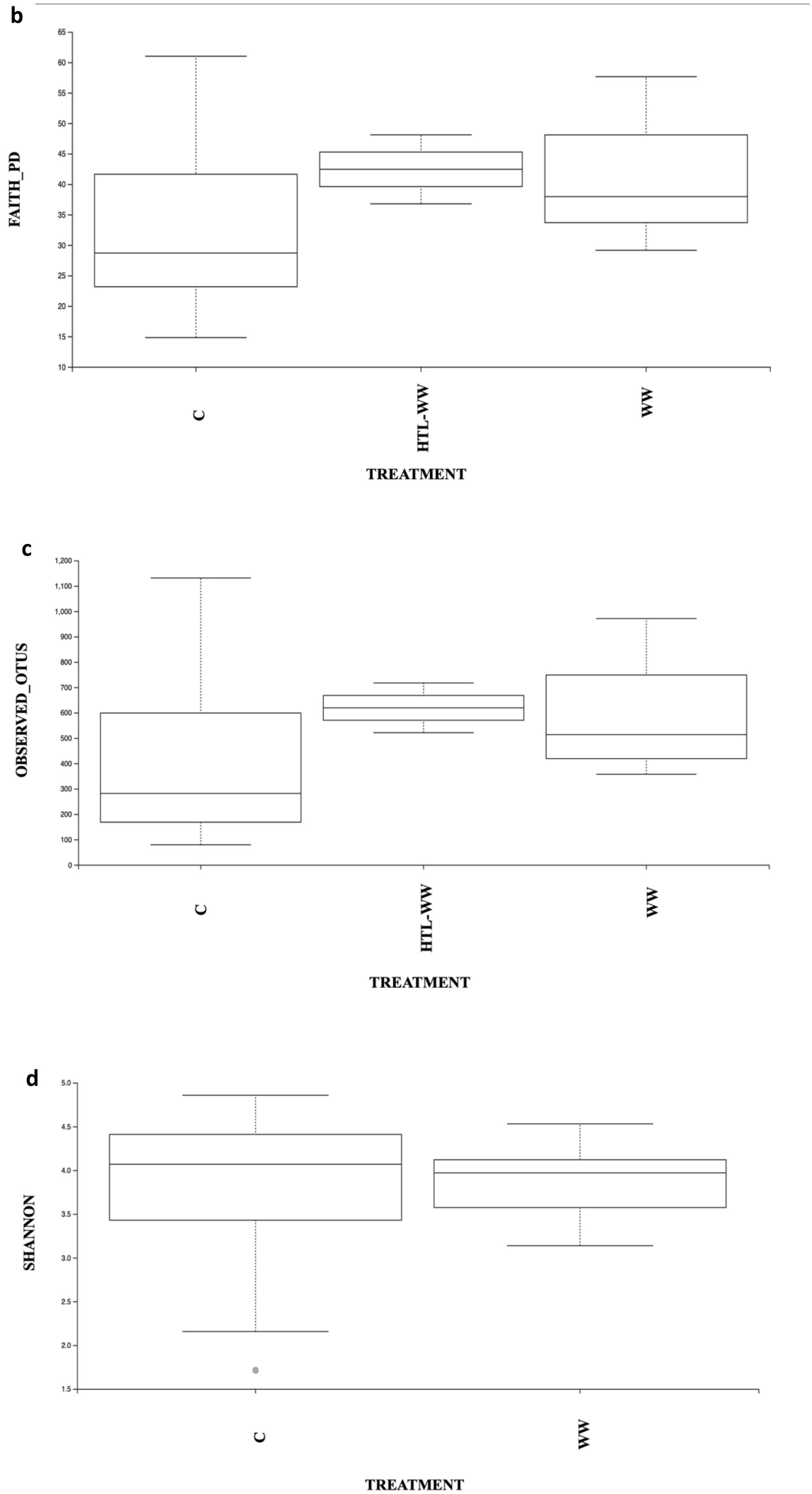
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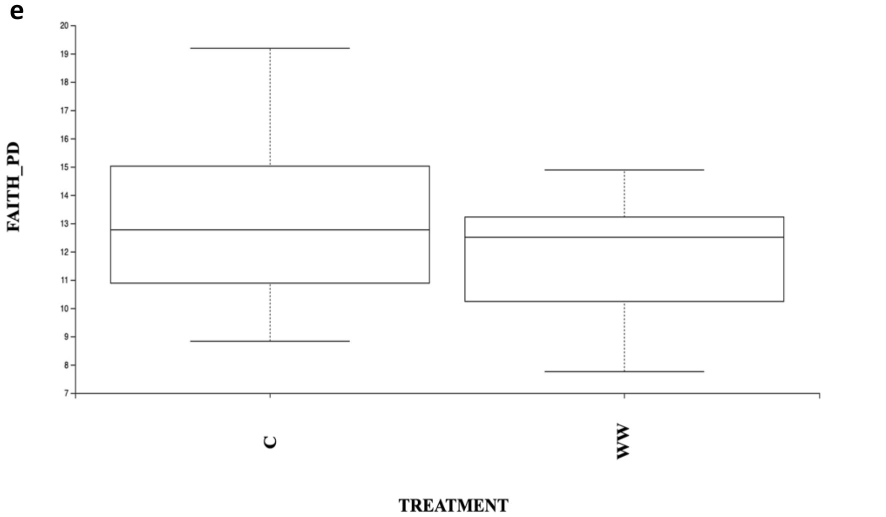
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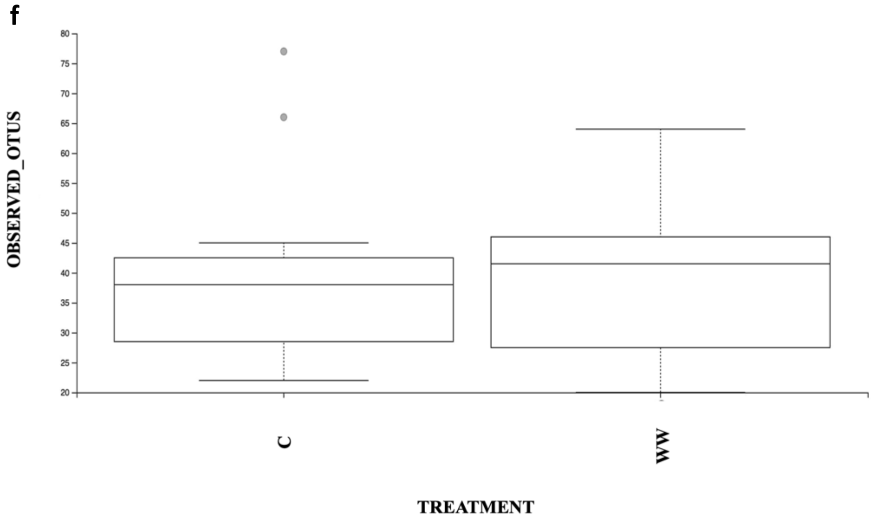
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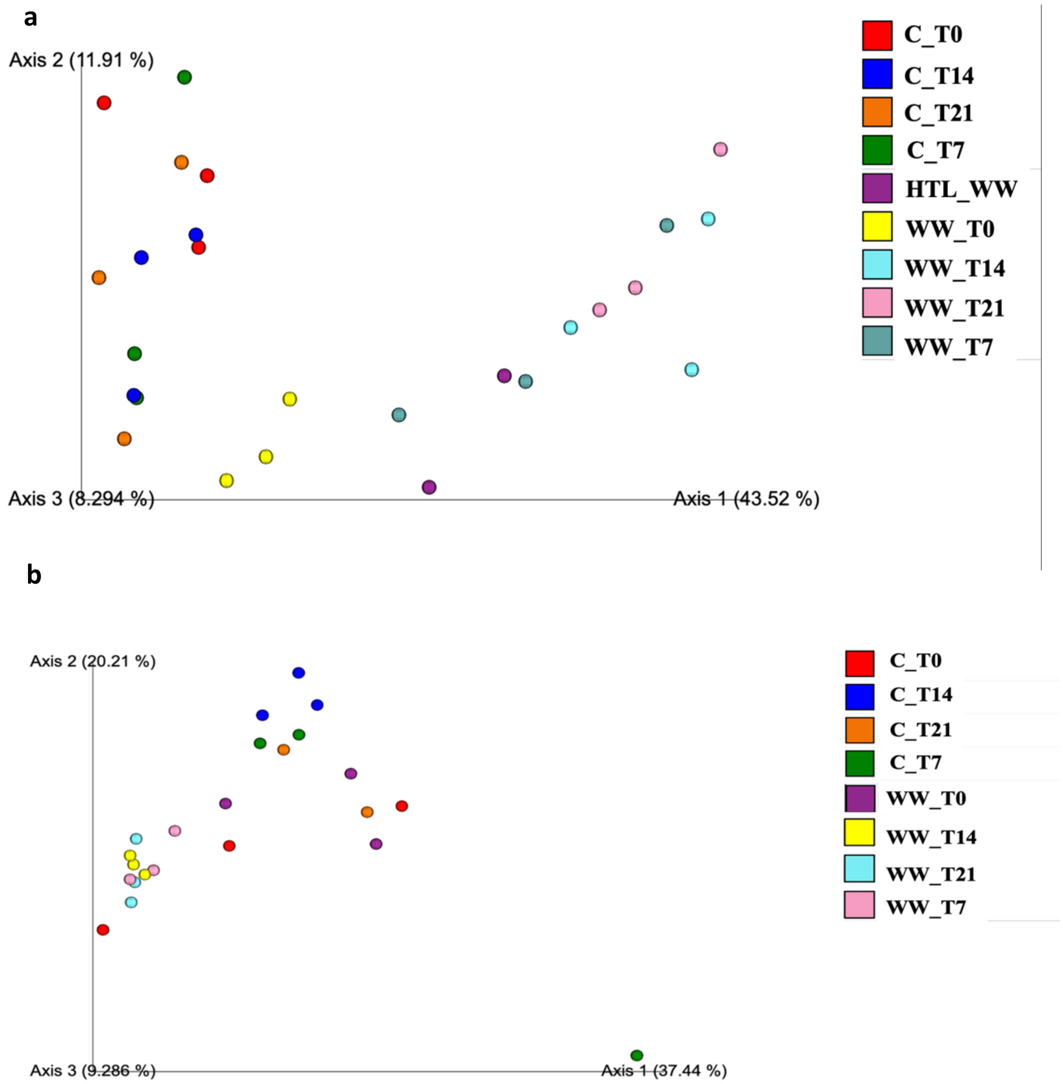

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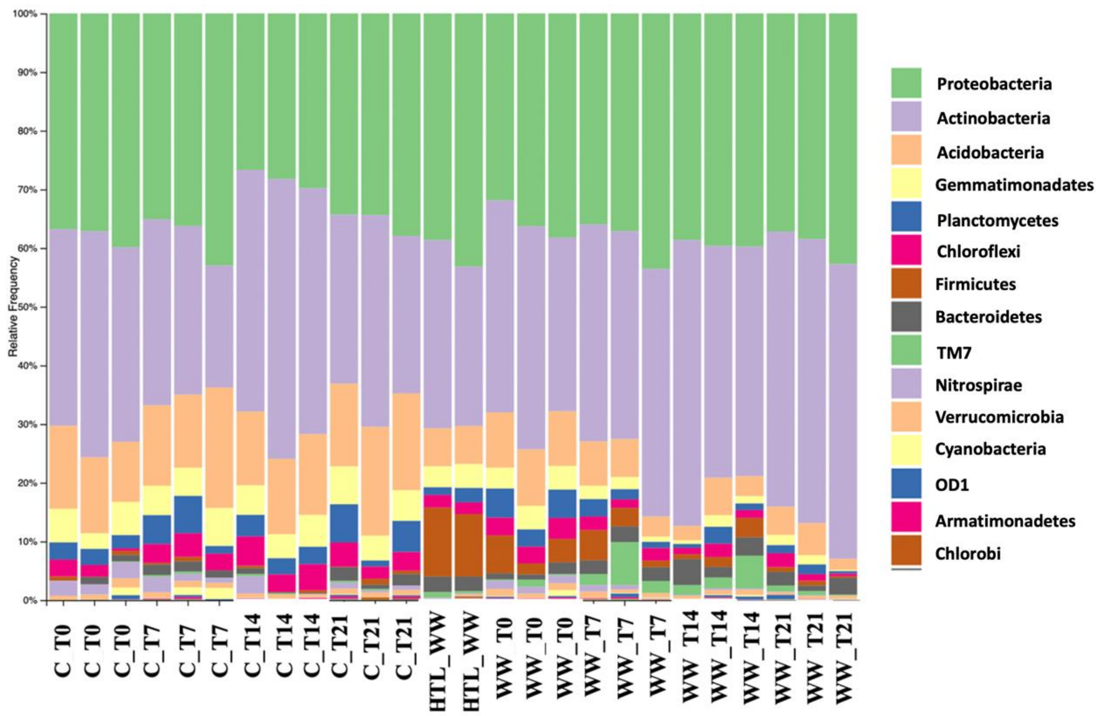
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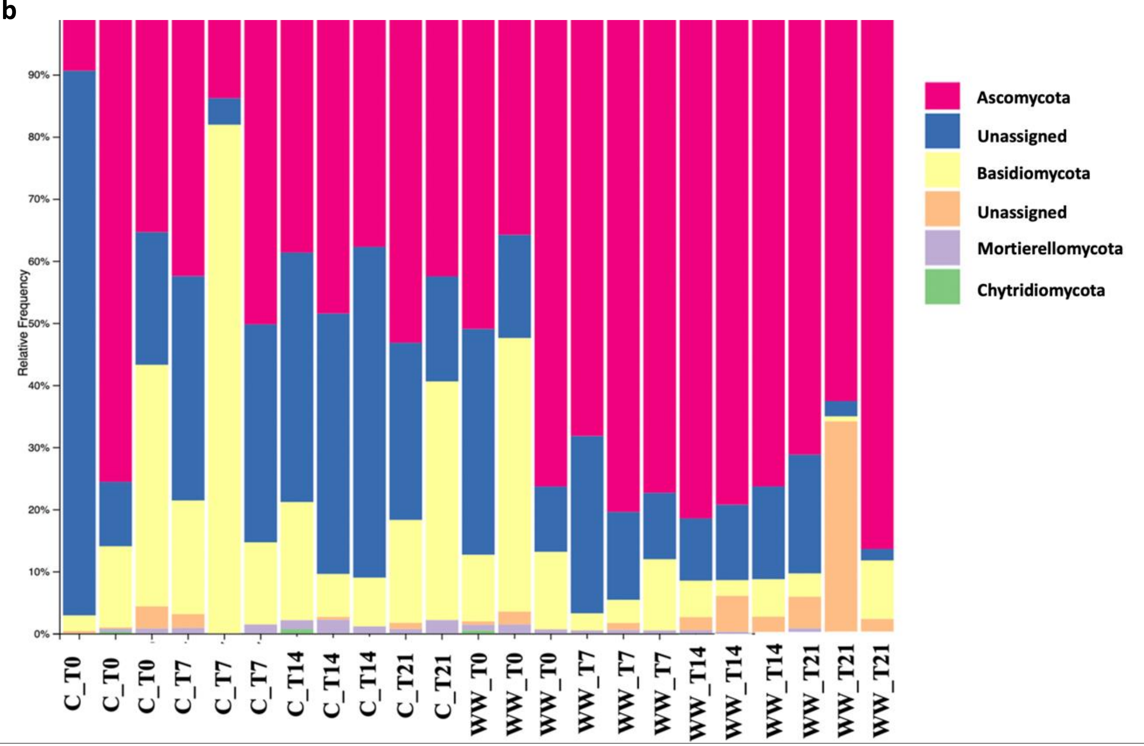
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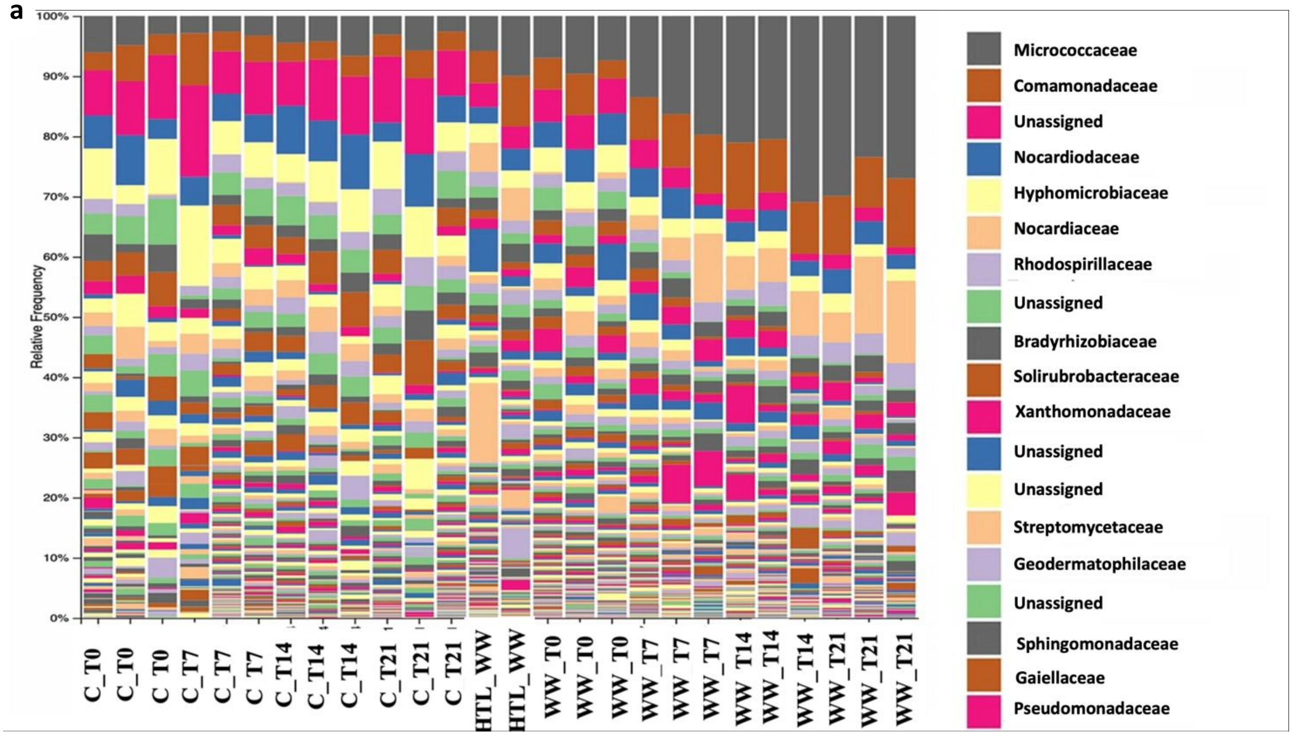
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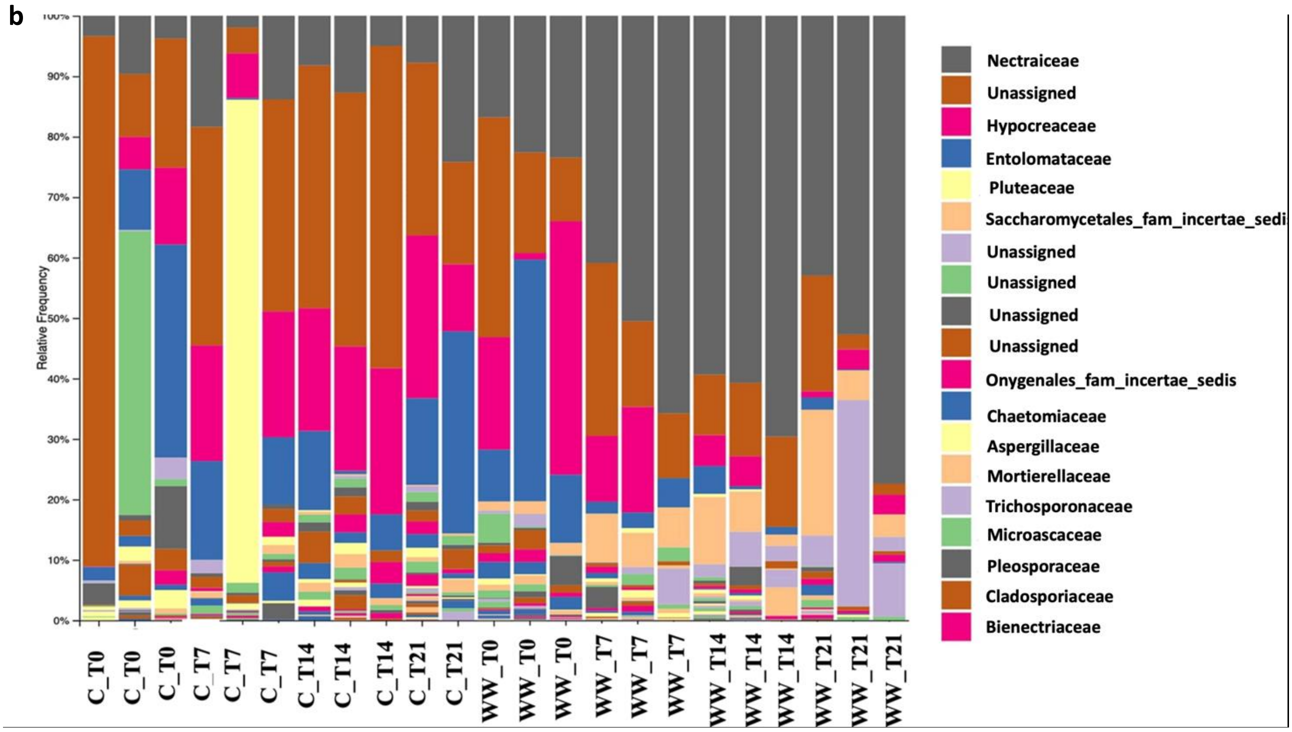
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4. Simultaneous application of hydrothermal liquefaction wastewater and a plant growth-promoting bacterial consortium on castor bean plants in an open field experiment

4.1. Introduction

Based on the results obtained on plants of *Nicotiana Tabacum* L. discussed in the previous chapter, a new experiment was set up to test the use of the wastewater coming from hydrothermal liquefaction of organic waste (HTL-WW) to irrigate plants of *Ricinus communis* L. In fact, today there is an increasing interest in the cultivation of castor bean plants for biofuel production to replace the palm oil which the EU has decided to phase out by 2030. In addition, the intensive cultivation of palm, rapeseed, and sunflower have raised many socio-economic and ecological concerns such as land competition with food crops and high water consuming (Demirbas et al., 2016). To mitigate these problems, the use of castor bean plant seems to be a valid alternative since it is one of the most promising non-edible oil and hardy plants which requires low fertilizer input, and it could be cultivated in marginal and degraded soils and it is also resistant to drought (Chatzakis et al., 2011). This plant belonged to the family *Euphorbiaceae*, reaching a seed and oil yield of about 1.100/1.800 kg and 500/600 L per *hectare*, respectively (Demirbas et al., 2016). The oil obtained is rich in ricinoleic fatty acid ($C_{18}H_{34}O_3$), which makes it suitable for industrially biodiesel production assuring low production costs (McKeon et al., 2016). The castor biodiesel is biodegradable, non-toxic, and renewable, and it can be also used alone and further, its production released the 80% less carbon dioxide emissions and less sulphur and hydrocarbons content compared with the convectional diesel production (Osorio-González et al., 2020). Thus, the castor plant cultivation is an attractive alternative feedstock for this industrial process, and its global demand is rising constantly at 3–5% per annum. In fact, the ENI S.p.A. forged a partnership with Tunisian and Congolese governments for the large-scale production of castor bean plants on a pre-desert area, to provide feedstock for its biorefinery system.

Despite studies on the use of wastewater for castor plant cultivation are still limited, the results obtained are very encouraging. As reported by previous works, the municipal wastewater irrigation did not have any negative impact on castor growth, soil parameters and biodiesel quality (Tsoutsos et al., 2013; Barreto et al., 2013, Abbas et al., 2015; Pereira et al., 2016; Nasr et al., 2018).

Nevertheless, using the HTL-WW irrigation as the only source of nutrients may results in plant nutrient deficiency and ionic imbalance, reducing crop development and yields. Research has strongly focused on the use of eco-friendly principles to minimize potentially harmful chemical inputs and manage ecological relationships and biodiversity, as the use of plant growth-promoting microbes (PGPM). They are defined by the EU Regulation 2019/1009 as "*products stimulating plant nutrition processes independently of the product's nutrient content with the sole aim of improving one or more of the following characteristics of the plant or the plant rhizosphere: nutrient use efficiency, tolerance to abiotic stress, quality traits, availability of confined nutrients in soil or rhizosphere*" (Fusco et al., 2022). The inoculation of PGPM in agricultural crops is considered an environmental-friendly alternative to chemical fertilization and a win-win cost-effective strategy, since the global fertilizer prices are at near record levels and may remain elevated throughout the entire 2023 (Chojnacka et al., 2023). The price rises have been driven largely by global pressures including increased demand, the war in Ukraine and higher energy costs. According to the Agricultural Marketing Service (AMS), anhydrous ammonia prices have increased up to $ 743 per ton, diammonium phosphate has increased of $ 295 and potash fertilizer (potassium) has risen up to $ 381 per ton (Schnitkey et al., 2022). Thus, the use of microbial inoculants to ensure crop yield and nutritional quality, by enhancing the availability of nutrients, the regulation of phytohormones, and by increasing plant tolerance against biotic and abiotic stresses (Lopes et al., 2021), seems to be a valid alternative. Moreover, the application of a microbial consortium could have a synergic effect on plant development.

In this context, the aim of this study was to determine and describe the impact of the use of HTL-WW as irrigation water on castor plant in a field experiment. A bacterial consortium consisting of four strains (*Azotobacter chroococcum* 76A, *Kosakonia pseudosacchari* TL13, *Bacillus megaterium* EL5, and *Methylobacterium populi* VP2) belonging to the microbial collection of the Division of Microbiology (Department of Agricultural Sciences, University of Naples Federico II) was also used. These strains were selected based on their plant growth-promotion activities. In detail, *K. pseudosacchari* TL13 had multiple plant growth promotion activities as production of indole-3-acetic acid (IAA), siderophores, ammonia, and 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase as well was able to solubilize phosphate and to exert antimicrobial activity against plant pathogens (Romano et al., 2020). *A. chroococcum* 76A is a free-living nitrogen fixer able to produce siderophores and phytohormones such as auxins (Viscardi et al., 2016). Moreover, this strain could colonize the rhizosphere successfully and enhance plant adaptation to drought and salt stress (Viscardi et al., 2016; Van Oosten ate al., 2018). *M. populi* VP2 was able to produce IAA and siderophores, solubilise phosphate, and produce a biofilm in the presence of polycyclic aromatic hydrocarbon (PHA) and alleviate PHA stress in seeds (Ventorino et al., 2014). At last, *B. megaterium* EL5 was capable to produce siderophores and to solubilise phosphate exhibiting its promotion activities also in contaminated environments (Ventorino et al., 2018).

Following HTL-WW irrigation and microbial inoculum application, the effect of the different treatments on root-associated microbiota were assessed evaluating the diversity and richness of prokaryotic community. Moreover, crop physiological parameters such as biometric indices, gas exchanges and water relative content, as well as physic-chemical properties of soil were also measured. To the best of our knowledge, this is the first work reporting the use of wastewater deriving from hydrothermal liquefaction in soil for irrigation of plants for energy purpose.

4.2. Material and methods

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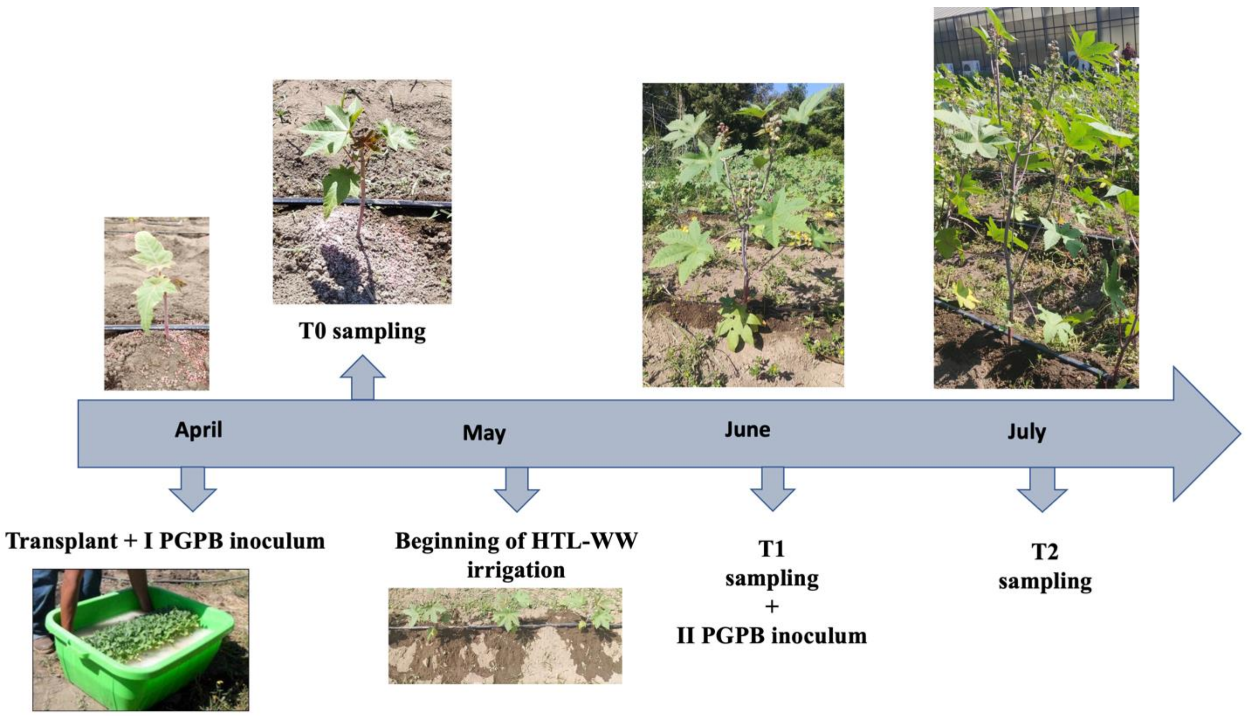

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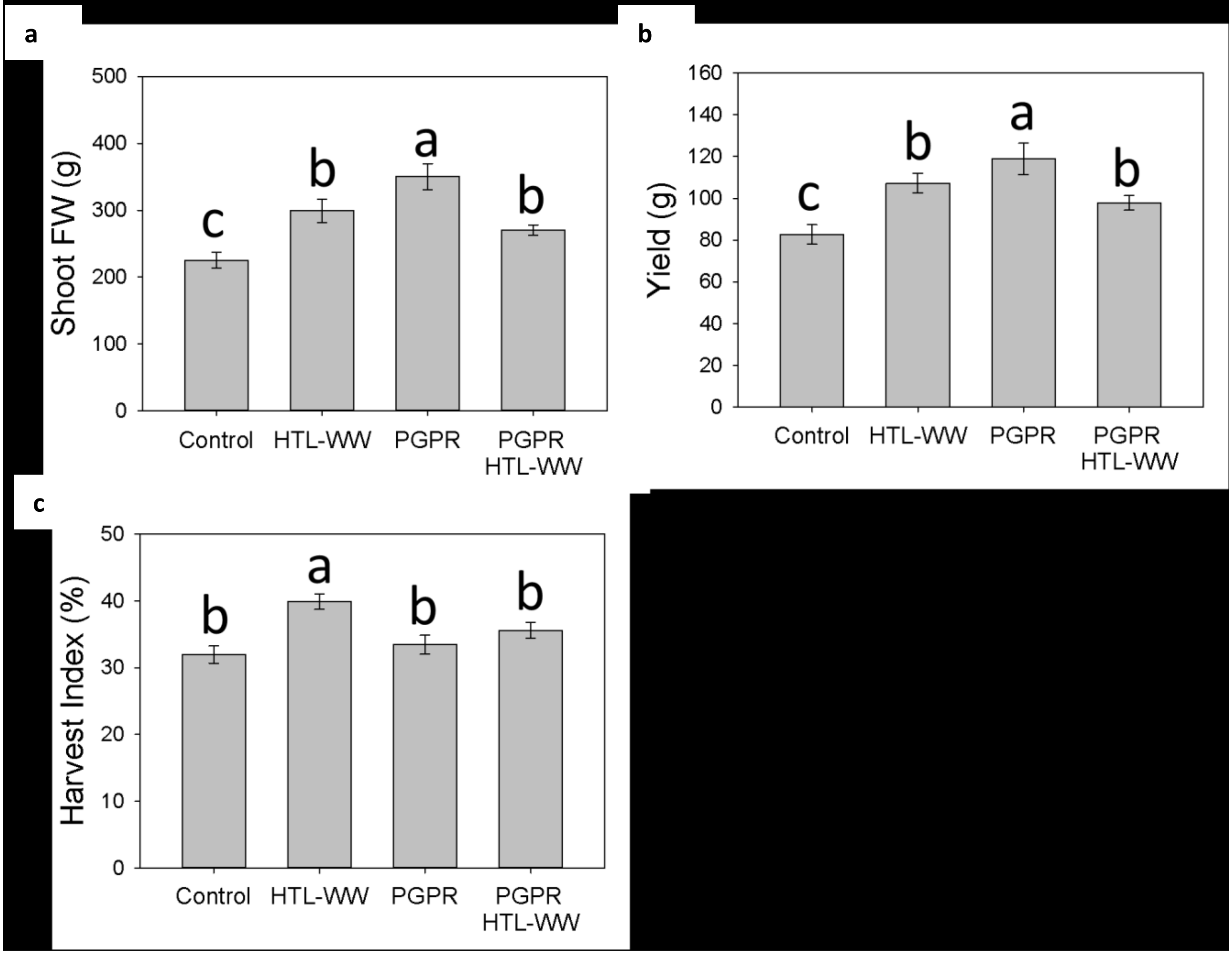

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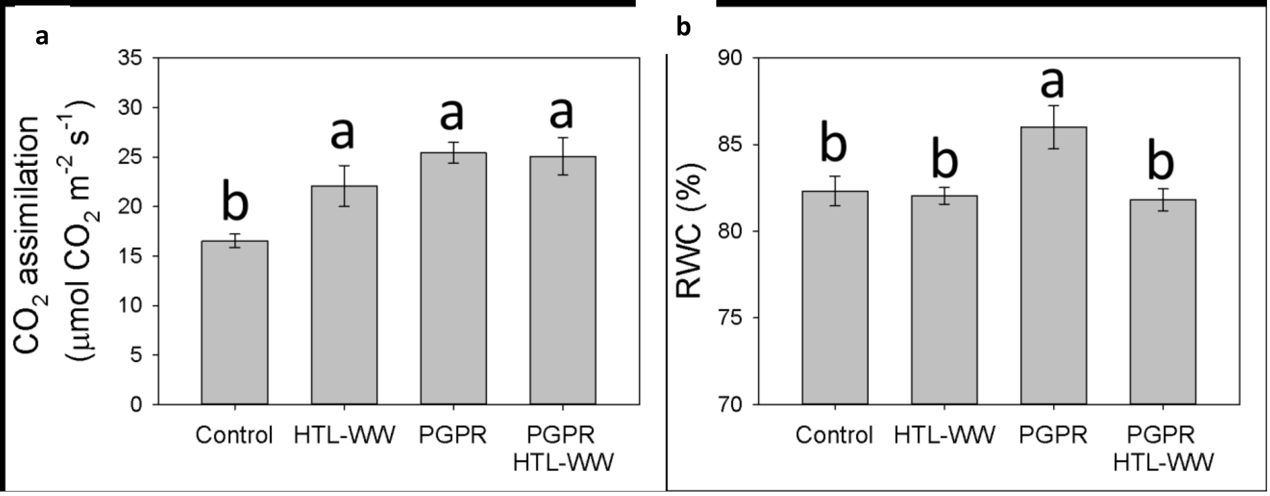

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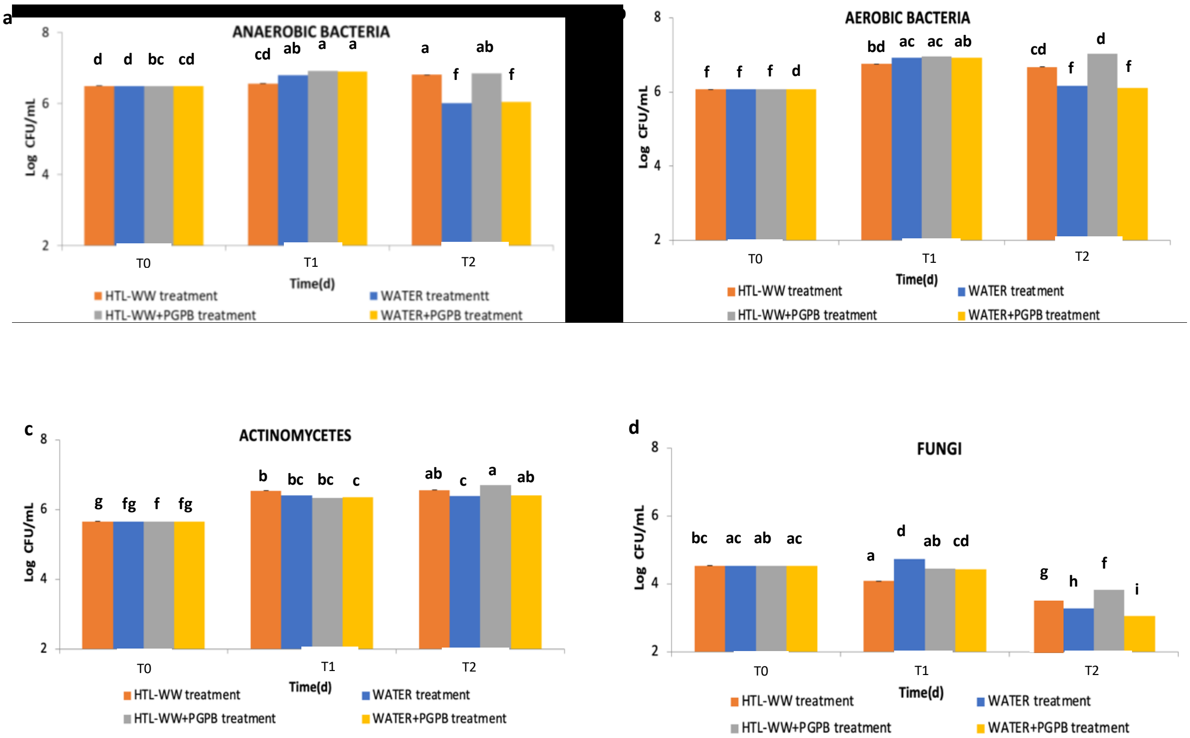

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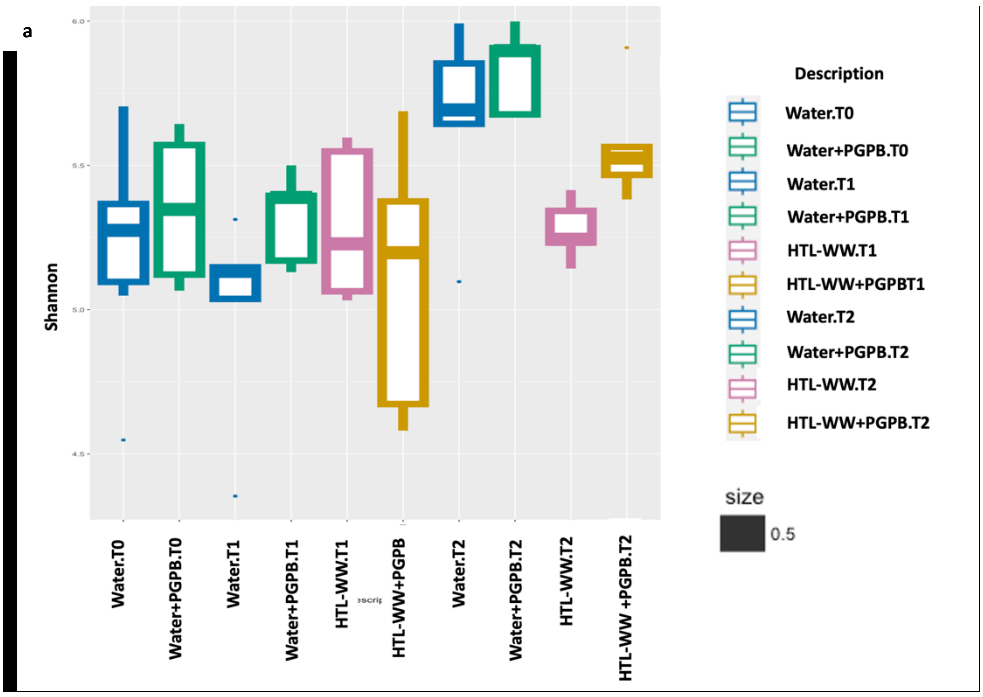
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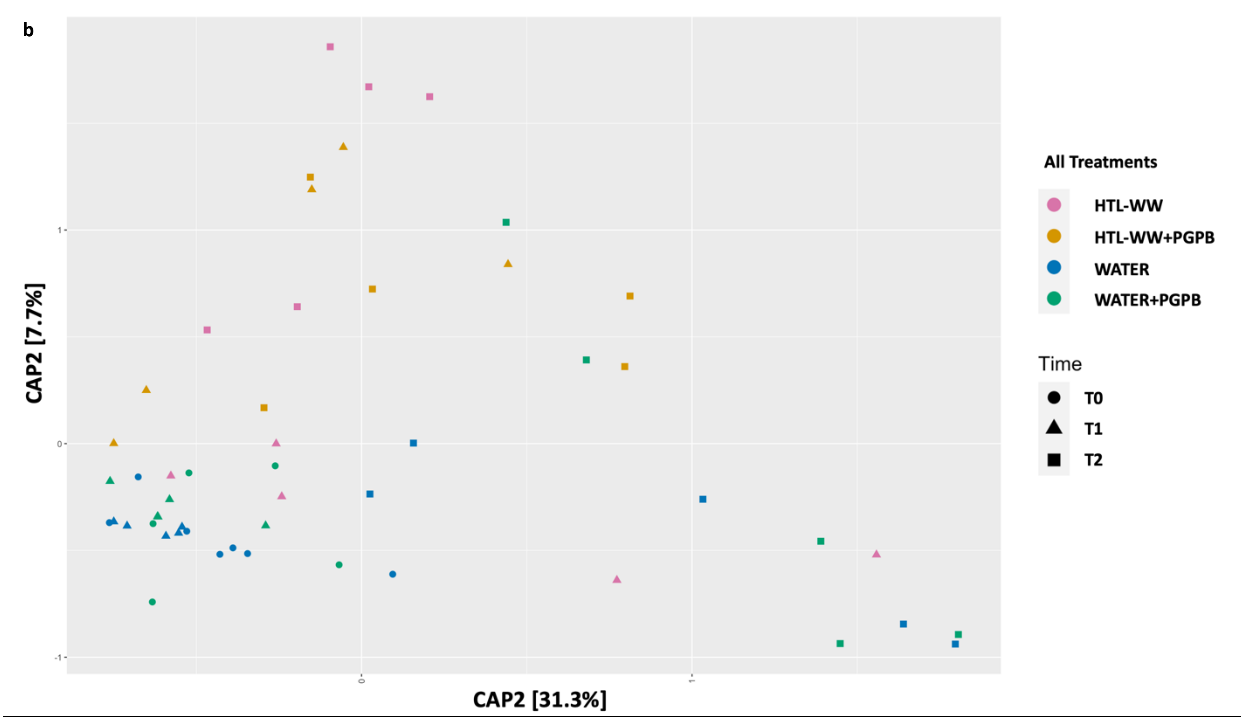
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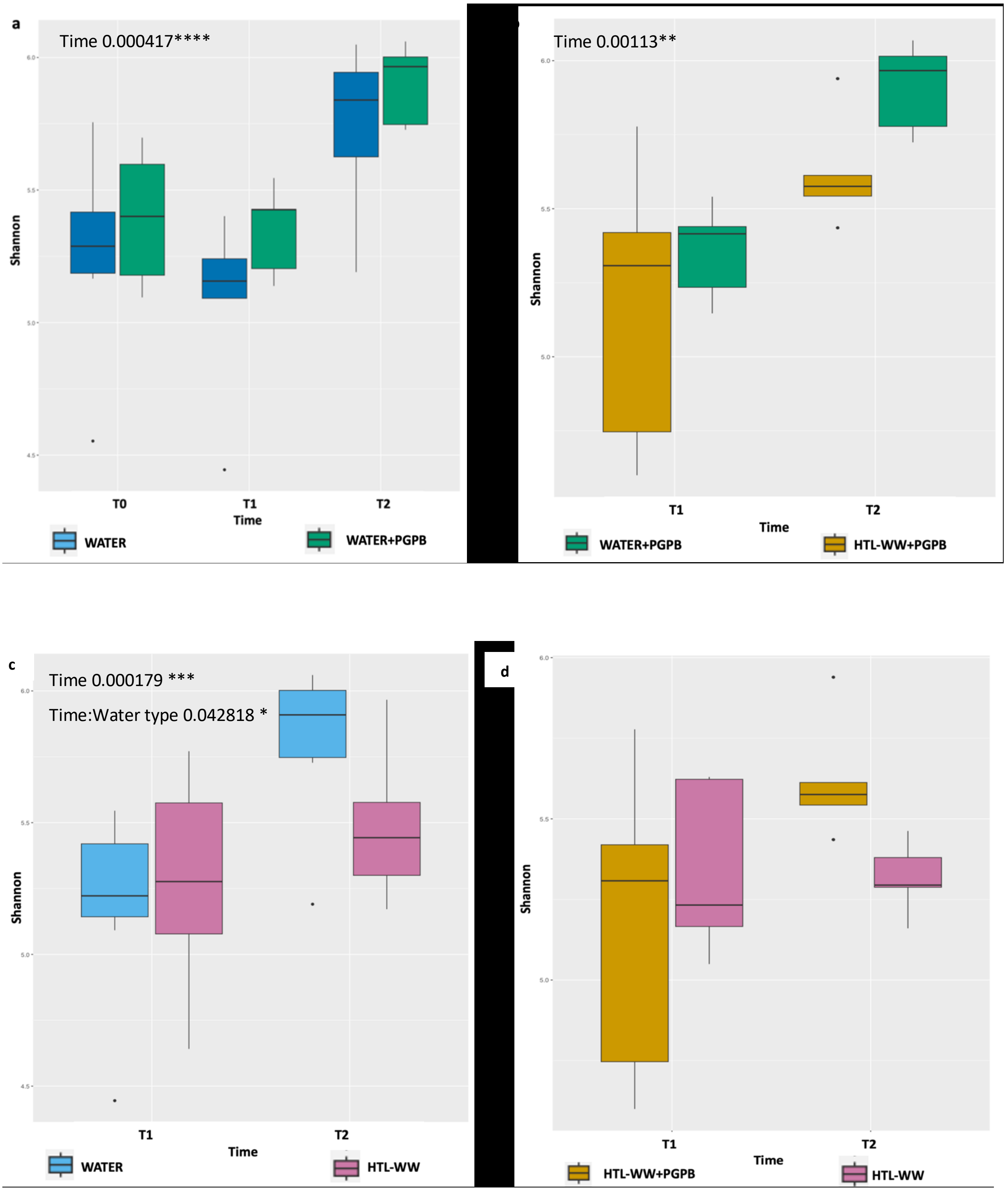
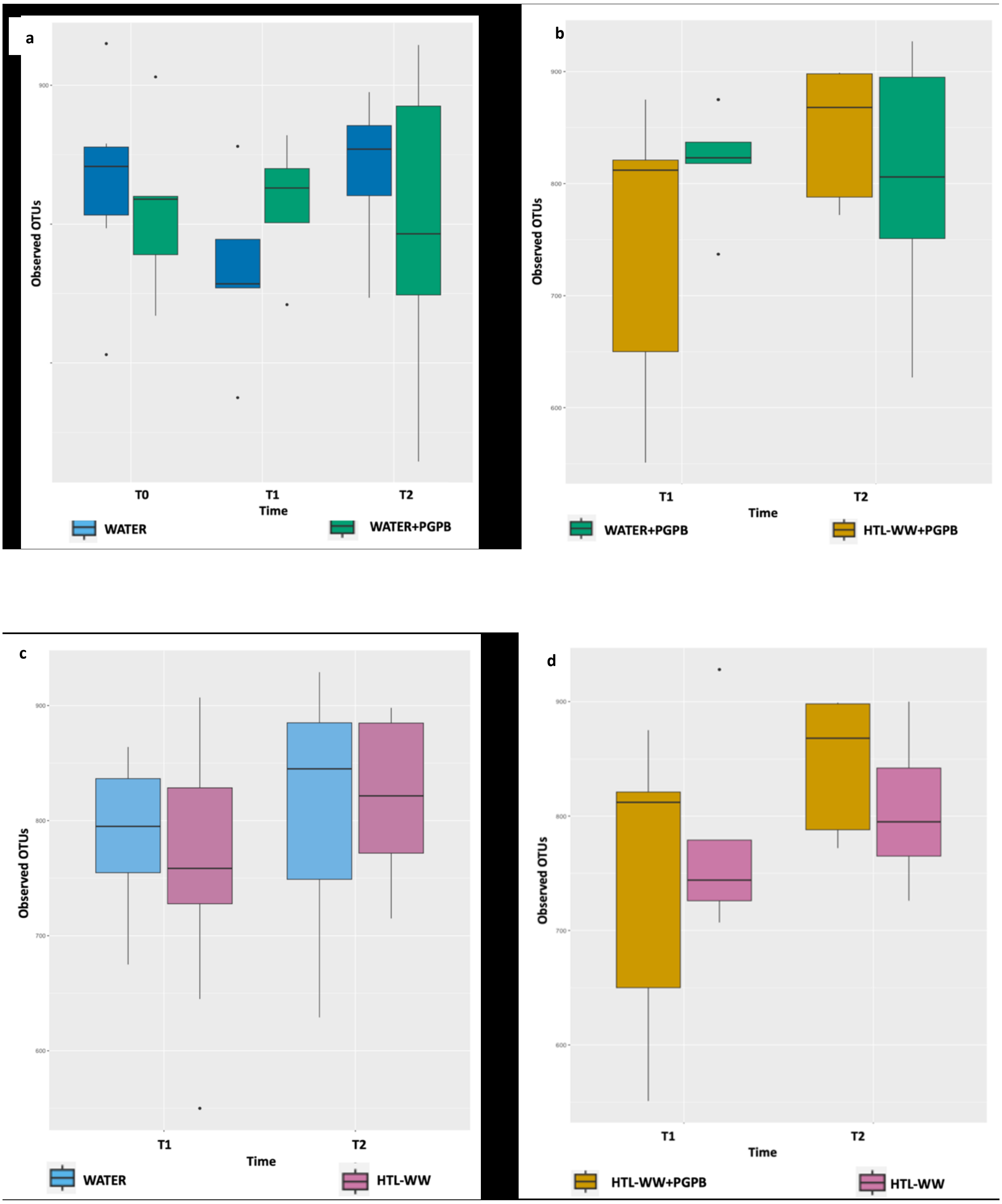
T0
T1
T2

[redacted]

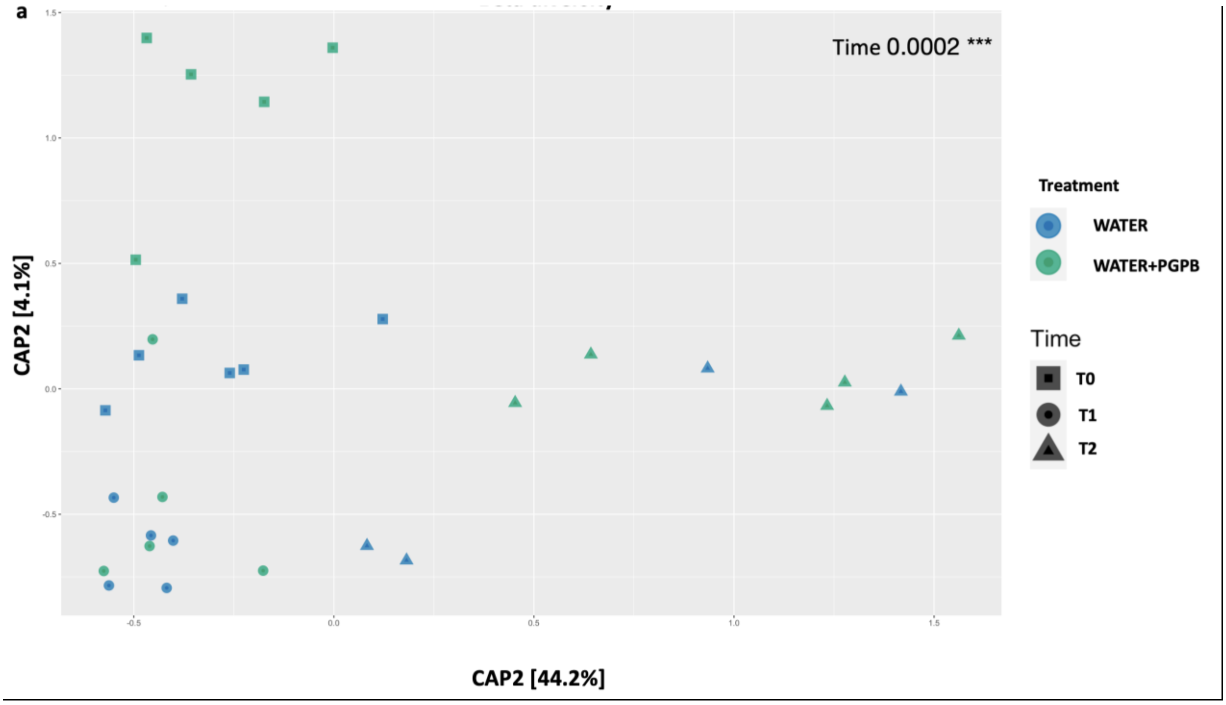

a
Time 0.000417****
Shannon
T0
T1
T2
Time
WATER
WATER+PGPB
Time 0.00113**
Shannon
T1
T2
Time
WATER+PGPB
HTL-WW+PGPB
c
Time 0.000179 ***
Time:Water type 0.042818 *
Shannon
T1
T2
Time
WATER
HTL-WW
d
Shannon
T1
T2
Time
HTL-WW+PGPB
HTL-WW

[redacted]


a
Observed OTUs
T0
T1
T2
Time
WATER
WATER+PGPB
b
Observed OTUs
T1
T2
Time
WATER+PGPB
HTL-WW+PGPB
c
Observed OTUs
T1
T2
Time
WATER
HTL-WW
d
Observed OTUs
T1
T2
Time
HTL-WW+PGPB
HTL-WW

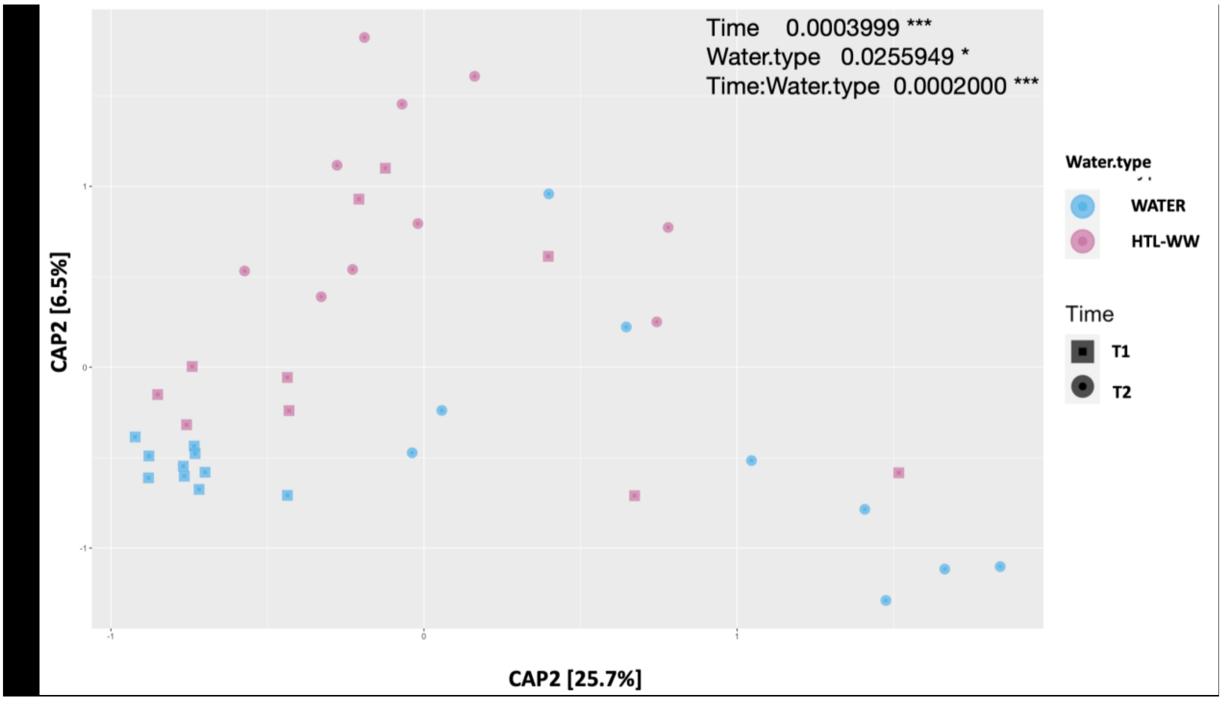
[redacted]

a

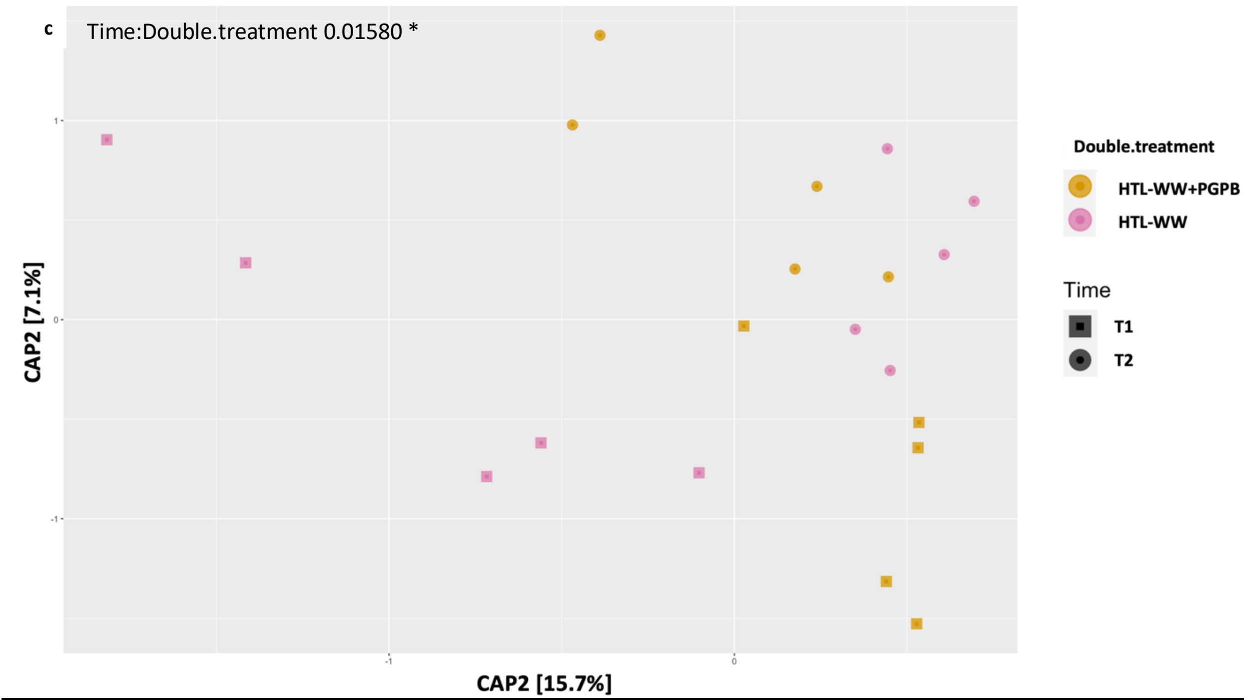


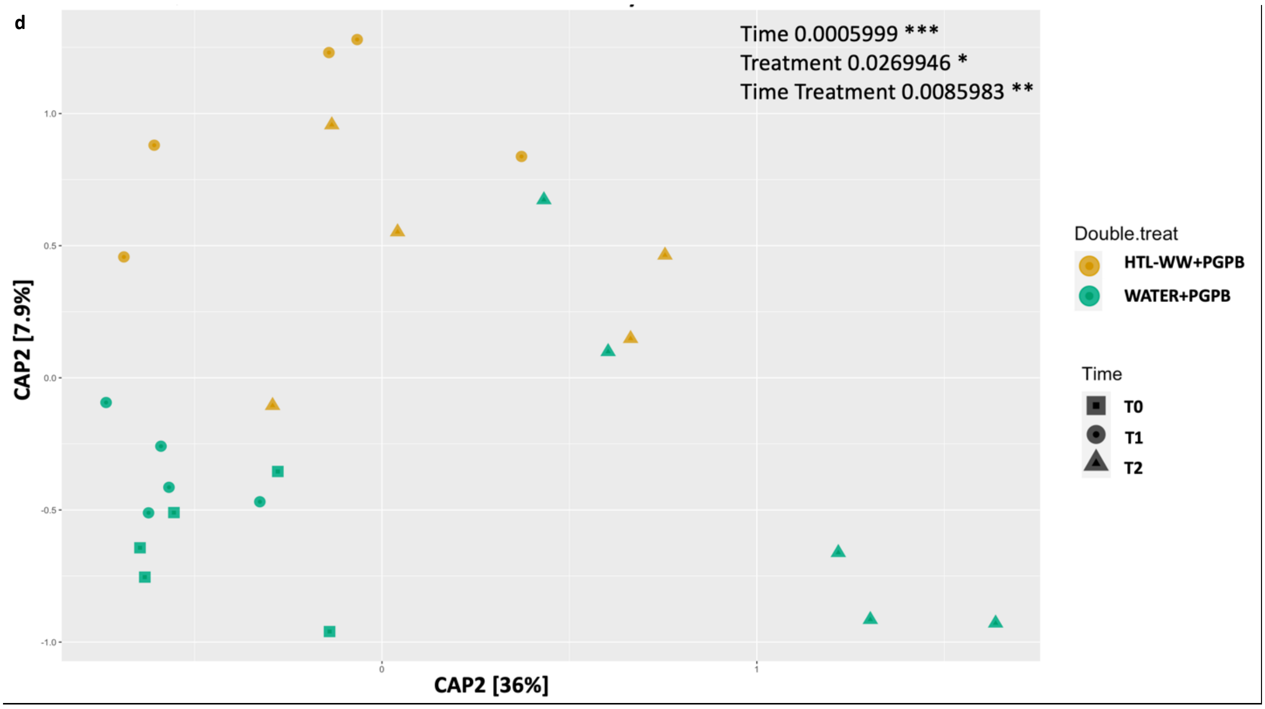
Time 0.0002 ***
Treatment
WATER
WATER+PGPB
Time
T0
T1
T2
CAP2 [4.1%]
CAP2 [44.2%]

b


Time 0.0003999 ***
Water.type 0.0255949 *
Time:Water.type 0.0002000 ***
CAP2 [6.5%]
CAP2 [25.7%]
Water.type
WATER
HTL-WW
Time
T1
T2

c


Time:Double.treatment 0.01580 *
CAP2 [7.1%]
CAP2 [15.7%]
Double.treatment
HTL-WW+PGPB
HTL-WW
Time
T1
T2



[redacted]

[redacted]

[redacted]

[redacted]

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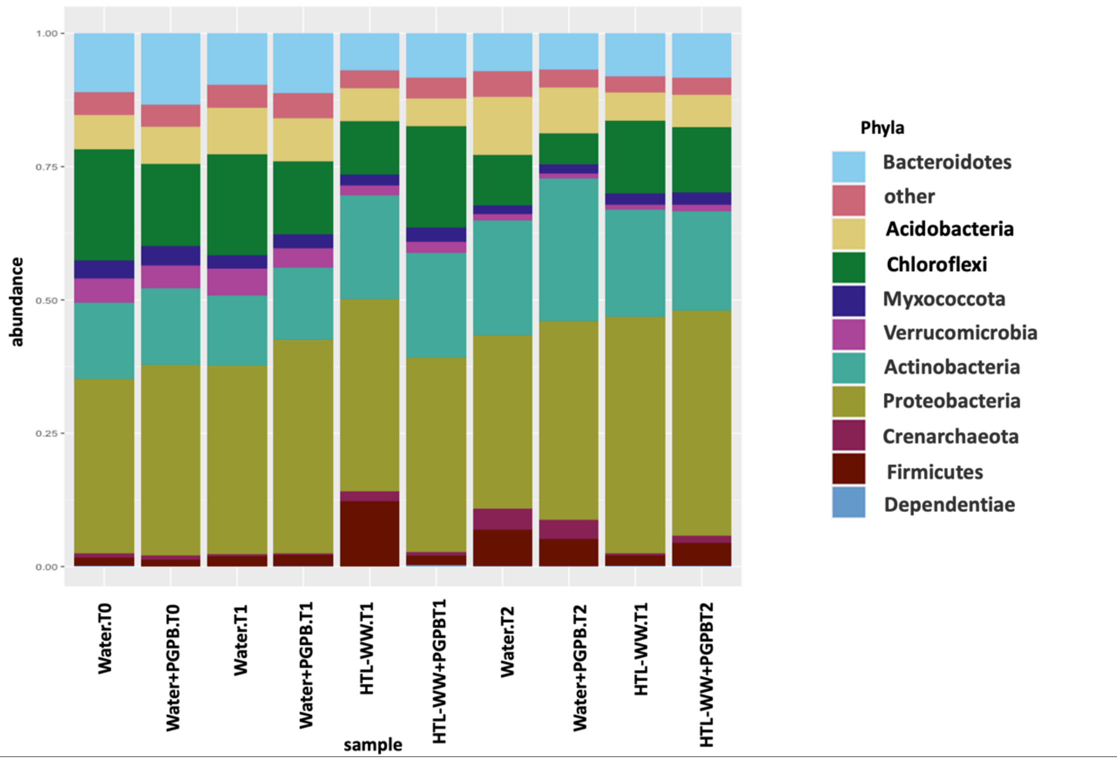
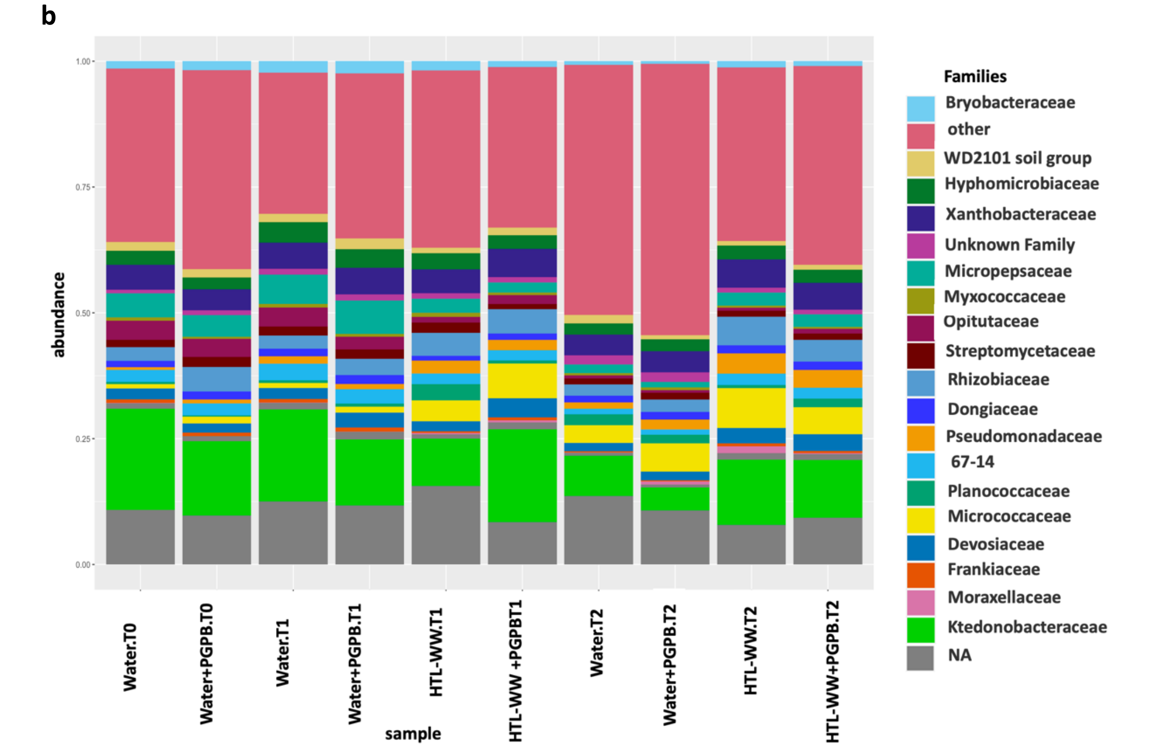
[redacted]

[redacted]

a


abundance
1.00
0.75
0.50
0.25
0.00
Water.T0
Water+PGPB.T0
Water.T1
Water+PGPB.T1
HTL-WW.T1
HTL-WW+PGPBT1
Water.T2
Water+PGPB.T2
HTL-WW.T1
HTL-WW+PGPBT2
sample
Phyla
Bacteroidotes
other
Acidobacteria
Chloroflexi
Myxococcota
Verrucomicrobia
Actinobacteria
Proteobacteria
Crenarchaeota
Firmicutes
Dependentiae



[redacted]

[redacted]

[redacted]

[redacted]

[redacted]

[redacted]

[redacted]

[redacted]

[redacted]

[redacted]

[redacted]

[redacted]

[redacted] the positive effect exhibited by HTL-WW irrigation on plant development could be due to an increase in bacterial families able to promote plant growth such as *Rhizobiaceae*, *Pseudomonadaceae* and *Micrococcaceae*. [redacted] *Chloroflexi* phylum includes heterotrophs, lithotrophs and phototrophs adapted to different environments and extreme conditions ([redacted]

[redacted]

[redacted]

[redacted]

[redacted]

[redacted]

[redacted]

[redacted]

Moreover, the application of HTL-WW on soil did not influence the soil parameters as confirmed also by previous works on short-term wastewater irrigation. Indeed, Chatzakis et al. (2011) as well as Farhadkhani et al. (2018) monitored the soil parameters under municipal wastewater effluent irrigation and no change in soil pH, soil organic matter (SOM), total nitrogen (TN) and soil salinity was detected at the end of the experiment compared to the initial values.

[redacted]

The use of hydrothermal liquefaction wastewater [redacted] to satisfy water requirements of [redacted] could be a valuable tool for recycling and valorise this liquid by-product following the closed-loop economy model. However, its use may critically affect the overall soil biological fertility and therefore its impact on soil [redacted] using *Ricinus communis* L. bioenergy crop. Moreover, [redacted] to enhance the crops' growth and productivity following sustainable agriculture principles.

[redacted]

5. Conclusion

This PhD thesis, taking into the account what was previously described in Chapter 1 about the freshwater depletion and the pressure on the agriculture, offers a new alternative resource for industrial crops irrigation. The wastewater reuse is one of the best strategies for water security, sustainability, and resilience. To date, the municipal wastewater was the most widely used in agriculture, however, nowadays there so many innovative technologies for biomass conversion and energy production, which allow the recovery of wastewater with better and safer features than the municipal effluents. As described in Chapter 2, among the biomass conversion strategies, the most cost effective and eco-friendly process is the hydrothermal liquefaction, which operates at high temperature and pressure to convert the biomasses into biofuel. This technology was exploited by ENI S.p.A. that are developing a new project, called *Waste to Fuel*, producing biofuel from organic fraction of municipal solid waste. During this process is also produced tons and tons of wastewater that carried all the organic compounds included in the feedstock. In fact, this hydrothermal liquefaction wastewater (HTL-WW) is rich in nitrogen, phosphorus and sulphur as well as micronutrients and minerals. This wastewater is already used as feedstock for anaerobic digestion or as substrate for microalgae cultivation. However, based on its chemical composition and on data obtained from literature, the HTL-WW showed a great potential as water irrigation for agricultural purpose. Moreover, the HTL-WW does not contain human pathogen and hazardous contaminants, although comparing the composition with the Italian Ministerial Decree about the wastewater application on field (185/2003) some elements such as chemical oxygen demand and electrical conductivity are higher than legislative limits. Thus, in Chapter 3, an optimal dilution at 10% was applied to the HTL-WW and used to daily irrigate the model plant of *Nicotiana tabacum* grown on greenhouse. Therefore, to evaluate the impact of diluted HTL-WW irrigation the effect on autochthonous microbiota as well as on plant development was analyzed. The diluted HTL-WW irrigation improved tobacco health state increasing the SPAD values and the flower biomass at the end of the experiment. Moreover, the wastewater irrigation improved the growth of several bacterial families as *Micrococcaceae*, *Nocardiaceae* and *Bacillaceae,* which are well-known halotolerant bacteria with a great potential for plant growth-promotion and also play a crucial ecological role in nature in the recycling of organic matter. Within the fungal families after HTL-WW an enrichment of and *Nectriaceae*, *Saccharomycetales-incertae-sedis* and *Trichosporonaceae* was observed. These families participate to the decomposition and mineralization of recalcitrant and labile compounds as well as to the bioremediation of nitrogen heavy metals.

Based on these encouraging results, in Chapter 4 was described a second experiment in which the use of WW-HTL was tested for the cultivation of the energy crop *Ricinus communis* L., in an open field experiment. Moreover, to enhance the crops' growth and productivity the PGPB inoculation strategy individually and in combination with HTL-WW was also tested. The plants treated with PGPB and irrigated with top water, showed the best effects on plant physiology also enriched the bacterial richness and evenness in the rhizosphere of castor plants. However, also the treatment with HTL-WW improved the shoot biomass and the CO_2 assimilation rate and transpiration compared to untreated plants. The yield of castor beans was higher in plants under HTL-WW and PGPB treatments. The HTL-WW application, individually or in combination with the bacterial inoculum, significantly affected the bacterial community, as demonstrated by the proliferation of *Rhizobiaceae*, *Pseumonodaceae* and *Micrococcaceae* bacterial families involved in nitrogen fixation, hormones production, phosphate solubilisation and bioremediation. Although, the biodiversity decreased in the rhizosphere of plants treated with HTL-WW, this effect was alleviated by PGPB inoculum.

The studies presented in this work, focused on wastewater valorisation in agronomic field following the principles of sustainable agriculture and closed business loop model. The agriculture sector in Mediterranean countries is facing the water shortage, and the wastewater reuse is the most promising solution to this problem. In this work was investigated the effectiveness of HTL-WW irrigation analysing the main component of agro-ecosystem such as rhizosphere-associated microbiota through metagenomic analysis, plants' growth and production through biometric indices and gas exchanges measurements, and soil physic-chemical parameters. Although the two crops used in this research, such as tobacco and castor bean, grown under different conditions they were positively influenced by the wastewater irrigation, individually or in combination with PGPB consortium, showing also higher production potential than control samples. Moreover, the HTL-WW irrigation exerted a selection pressure on indigenous microbiota leading to the establishment of a new microbial community promoting the growth of specific microorganism that rapidly adapted to the new environmental condition, taking over to the other microbial species. These microorganisms belong to bacterial and fungal families that include many microbial taxa involved in plant growth promotion, bioremediation and stress tolerance.

This work proposed a new strategy for industrial crop management suggesting the simultaneous application of wastewater derived from hydrothermal liquefaction and microbial bio-stimulants to decrease the input of chemical fertilizers and improving production, following the sustainable agriculture principles.

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