HYDROUSA

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Brief Description	This report includes the description of the activities regarding the soil and crops in the HYDROs to determine the HYDRO effectiveness and replicability in other Mediterranean regions. First, the soil characterization (physicochemical characteristics, trace elements, nutrients, ions) as well as the evaluation of biodiversity are reported. Moreover, soils and crops were searched also in terms of selected organic micropollutants (pharmaceutical active and endocrine disrupting compounds) and heavy metals. Finally, it reports the results of the organic pest control methods. Part of the information produced in this deliverable is then applied in the Deliverable D6.4 (Environmental and human health assessment) with the aim to provide tangible results that the produced crops are safe to consume.
Keywords	Biodiversity; crops; organic micropollutants; pest control; soil.

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ABBREVIATIONS

B	**Boron**
BPA	**Bisphenol A**
BPs	**Bisphenols**
Ca	**Calcium**
CEC	**Cation exchange capacity**
CT	**Control treatment**
Cu	**Copper**
CW	**Constructed wetlands**
d.w.	**Dry weight**
dSPE	**Dispersive Solid Phase Extraction**
EC	**Electrical conductivity**
EDCs	**Endocrine-Disrupting Chemicals**
ELT	**Ecolodge Tinos**
Fe	**Iron**
FT	**Full Treatment**
ICRA	**Catalan Institute for Water Research**
K	**Potassium**
Mg	**Magnesium**
Mn	**Manganese**
MS	**Mangroove Still System**
N	**Nitrogen**
Na	**Sodium**
NI	**Negative Ionization mode**
NSAIDs	**Non-Steroidal Anti-Inflammatory Drugs**
NTUA	**National Technical University of Athens**
OMPs	**Organic Micropollutants**
P	**Phorhorus**
PI	**Positive Ionization mode**
PSA	**Primary Secondary Amine**
PT	**Partial Treatment**
SOM	**Soil organic matter**
SRM	**Selected Reaction Monitoring**
TBEP	**Tris-(2-butoxyethyl) phosphate**
TCEP	**Tris-(2-chloroethyl) phosphate**
TCPP	**Tris-(2-butoxyethyl) phosphate**
TST	**Target Scan Time**
UASB	**Up-flow Anaerobic sludge blanket**
UHPLC-MS/MS	**Ultra High Performance Liquid Chromatography–tandem mass spectrometry**
WP	**Work Package**
Zn	**Zinc**

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EXECUTIVE SUMMARY

The HYDROUSA project aims at maximizing the benefits of different water treatment systems to reuse water from various non-conventional sources of water. This is shown in six different demonstration sites located in three Greek islands, which are referred to as HYDRO1-6: demo sites 1 and 2 are located in the island of Lesvos, demo sites 3 and 4 are located in the island of Mykonos, and demo sites 5 and 6 are located in the island of Tinos.

This deliverable is directly related to all the HYDROs, with more focus on HYDRO1 and HYDRO2. HYDRO1 describes the development of an innovative system for domestic wastewater treatment, whereas HYDRO2 is an “agroforestry” system irrigated with nutrient-rich treated wastewater which is the resultant/output from HYDRO1 system. The implementation of upflow anaerobic sludge blanket (UASB) coupled to a constructed wetland (CW) and fertigation of crops can result in a self-sustaining wastewater management system with significant economic benefits from the agroforestry system, closing the loop and creating a resilient ecosystem. The deliverable is divided into 4 chapters, with the main scope of each one described as follows.

The Soil analysis chapter dealt with the general soil characteristics, physicochemical characteristics / trace elements / nutrients, major ions and heavy metals of the HYDROs 2-6. According to the findings it is anticipated that fertigation (HYDRO2) can fully promote the soil nutrient state and was beneficial for plant growth, while in the cases of water originated from other sources (rainwater, stormwater, seawater, etc) the soil characteristics were not affected and therefore appreciable plant growth was achievable.

In the Biodiversity analysis chapter, in the context of treated wastewater irrigation, biodiversity can serve as a key indicator of soil health and the effectiveness of the treatment processes. Soil health was assessed in terms of soil microorganisms, nematodes, and macrofauna. After two years of macrofauna biomonitoring, it was observed that the use of treated wastewater did not decrease the abundance of the main taxa. The climate significantly impacted the soil macrofauna, the application of treated wastewater led to a higher abundance of soil nematodes, which enhanced soil fertility while the presence of different plant species did not have an impact on soil macrofauna.

The Micropollutants analysis for food safety assessment chapter involved soil and crops analysis to investigate selected micropollutants uptake and food safety. Fertigation of soil where three crops (lettuce, oregano, and lavender) were planted took place with either tap water, reclaimed water (i.e., treated wastewater) or partially treated reclaimed water, in two sampling campaigns, fall and summer at the Lesvos agroforestry site (HYDRO2). Results showed significant differences in soil organic matter levels between soils irrigated with PT of water and the other treatments across all samples and seasons. The presence of micropollutants varied, with antibiotics exhibiting the lowest concentrations, whereas classes such as diuretics and antihypertensives did exhibit high concentrations in the summer campaign. Around 6% of the analysed compounds were detected in the crops, where summer conditions raised the total levels as well. Overall, psychiatric drugs, antihypertensives, antibiotics, and β-blockers showed a preference of retention in the roots rather than the leaves, whereas NSAIDs, EDCs and diuretics were retained in both plant parts. Heavy metal analysis was also included in all crops and no significant difference was observed between crops irrigated with tap water or fully treated water.

Finally, in the Pest control strategy chapter, various infestations as well as issues resulting from soil nutrient deficiencies, as well as climate fluctuations, were dealt with in all HYDROs 2-6. A variety of forestry trees, orchards/bushes, herbs, and annual crops developed issues such as foliage and root rot in oregano, mealy-bugs and aphids in tropical fruit, and all cases were successfully treated with the appropriate interventions,

such as application of natural oil formulations, pyrethroids and other chemicals, as well as a chicken coop in HYDRO6 for fertilization and composting of the system, minimizing the use of fungicides/pesticides.

HYDROUSA has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 776643.

1. INTRODUCTION

The aim of HYDROUSA European Project is to find new possible sources of freshwater, considering multiple water sources: wastewater, rainwater, and seawater. This deliverable on food safety issues and pest control reports the activities related to four main topics, related mainly to HYDROUSA soils and crops: soil analyses (in terms of general characteristics and parameters), biodiversity analyses, organic micropollutants analyses for food safety and heavy metals (in soil and crops), and pest control strategy.

Different typologies of trees, bushes, and crops were planted in the HYDROs 2-6 irrigated with: reclaimed water (i.e., treated wastewater) (HYDRO2), rainwater and/or stormwater (HYDRO3, HYDRO4, HYDRO6), or with the mangrove still desalination system and saltwater evaporation greenhouse system (HYDRO5). A general characterization in terms of soil analysis and pest control strategy was applied to all the corresponding HYDROs with responsibility of NTUA and the corresponding HYDRO leaders.

A deeper focus on HYDRO2 involved two main topics to evaluate food safety: biodiversity (led by NTUA) and micropollutants in soil and crops, as well as antibiotic resistance in the soil (led by ICRA-CERCA). HYDRO2 is the HYDRO with the largest agroforestry surface, and it is applied for wastewater reclamation. Less concern is expected in this context for the HYDROs and the corresponding crops, dealing with rainwater/stormwater, as it can be also extrapolated from the low content of organic micropollutants presented in D5.9 (Report on monitored micropollutants and pathogens) for HYDROs 3-6. Three crops were chosen for a more in-depth analysis (fulfilling the corresponding KPI): lettuce (largely present in the market and with wide scientific literature, also in terms of emerging contaminants), and oregano and lavender (present in most HYDROs). Selected emerging contaminants like pharmaceutical active compounds (including antibiotics and selected EU watch list 2015/495 and 2022/1307 compounds) and endocrine disrupting chemicals (including bisphenol A) were investigated in the soil and in the crops. The final list consisted of 88 compounds, fulfilling the corresponding KPI. To be noted that the preliminary campaign presented in Deliverable D5.9 also included pesticides. Nonetheless, they were not found, or at very small concentrations in Antissa, where HYDRO1 is placed and, hence, pesticides were excluded from the list of screened emerging contaminants in soil and crops.

2. SOIL ANALYSIS

2.1. Soil sample collection and analysis

Soil samples were collected at the end of the growing season to measure all the parameters needed. The sampling was conducted with a soil sampler at different parts of the fields. Soil samples were transferred in plastic bags to the laboratory, then air-dried, crushed, and sieved with a 2-mm sieve. The 2 mm sieve is necessary as the 2 mm and smaller soil particles are the ones that define the characteristics of an agricultural soil.

Several parameters were measured in the soil samples such as particle size distribution, soil pH, the electrical conductivity, soil organic matter, macronutrients percentage, in particular phosphorus (P) and nitrogen (N), exchangeable cations, cation exchange capacity (CEC), micronutrient and metal content and C/N ratio. More specifically, the particle size distribution was determined by the hydrometer method (Bouyoucos, 1951), while the pH was measured in a 1:2.5 (w/v) soil/water ratio with the use of a pH meter. The electrical conductivity was determined through the measurement of the total salinity of the soil and the method of electrical resistance of the saturated soil paste (Rhoades, 1989). Soil organic matter (SOM) content was obtained by the Walkley-Black's procedure (Nelson & Sommers, 1982). The percentage of the nitrogen (N) included in the samples was measured by the Kjeldahl method (Bremner, 1960), while the available phosphorous (P) was obtained by the Olsen method (Olsen, 1954). Cation exchange capacity (CEC) was determined by the sodium acetate method and the exchangeable cations Ca, K, Na, Mg by the ammonium acetate method (Rhoades, 1982). The micronutrients and metals were measured with the DTPA method (FAO, 2022) and lastly, the C/N ratio was calculated with the division of the percentage of C with the percentage of N (Cornell University, 1996)

2.2. HYDRO2

2.2.1. Demo-site overview and sampling points

HYDRO2 is an agroforestry system on Lesvos Island which includes a variety of trees, shrubs, medicinal plants, and annual crops. The demonstration site is irrigated with reclaimed water which is produced in HYDRO1 demo site that processes Antissas' village municipal wastewater. The irrigation water produced in HYDRO1 is of Class A water quality which complies with the EU legislation on unrestricted agricultural use and contains a significant nutrient content in terms of nitrogen and phosphorus. The fact that the irrigation water contains vital nutrients for the plants is important for HYDRO2 site as the need for commercial fertilizers is minimized.

The site occupies an area of about 1 ha and is divided into 2 fields. The main field of HYDRO2 is located at the north-east of the HYDRO1 site and the second field is located on the other side of the road close to HYDRO1 (Figure 2.1).

Figure 2.1. Overview of existing WWTP, location of HYDRO1&2, as well as fields of HYDRO2

The main demonstration site occupies about 0.8 ha including more than 70 different plant species (Figure 2.2). The second, smaller, field (0.25 ha) includes a crop rotation system of annual plantations of maize and barley co-cultivated with medicinal plants and trees. The second field is separated in 4 plots while each plot includes 3 randomly placed on the site replications, so in total it contains 12 replications (Figure 2.3). From the previously mentioned plots the two are irrigated with conventional fresh (tap) water and the other two with the reclaimed water produced in HYDRO1 for research and comparison purposes, in order to evaluate the effect of the reclaimed water on plants' growth, health, yield etc.

Figure 2.2. HYDRO2 main field

Figure 2.3. HYDRO2 second field

Agroforestry systems have many competitive advantages and benefits in comparison to the conventional farming systems as they combine the advantages of agriculture and forestry through the creation of systems where trees and shrubs are integrated with agricultural crops (in alleys or at the perimeter of the field) and/or livestock in a dynamic system. The advantages include the diversification of income, increased production, biodiversity, higher soil health and water quality, etc.

The agroforestry system is irrigated mainly through a drip irrigation system while at the same time traditional stone channels coupled with furrow irrigation were constructed and tested during the 2 years of operation. The irrigation of the main field is regulated by an irrigation panel and the second field is irrigated by an autonomous irrigation system which turns the irrigation on and off depending on the measurements of the soil moisture sensors established.

The soil samples were collected from the points that are indicated as red dots in Figure 2.4. For the purpose of the analysis of the laboratory results, these samples are considered as replications within the field. Moreover, samples were collected from the same spots as in 2019 (pre-cultivation year), or from spots as adjacent as possible to the same ones, in 2021 and in 2023.

Figure 2.4. HYDRO2 main field soil sampling spots

The statistical analysis (ANOVA, SPSS version 24) of these samples compared the means for each parameter tested, for the same location and for the duration of the experiment (sampling years 2019, 2021 and 2023). Means and standard deviation (STDEV) are presented in Table 2.1. For all variables with the same letter, the difference between the means is not statistically significant. If two variables have different letters, they are significantly different.

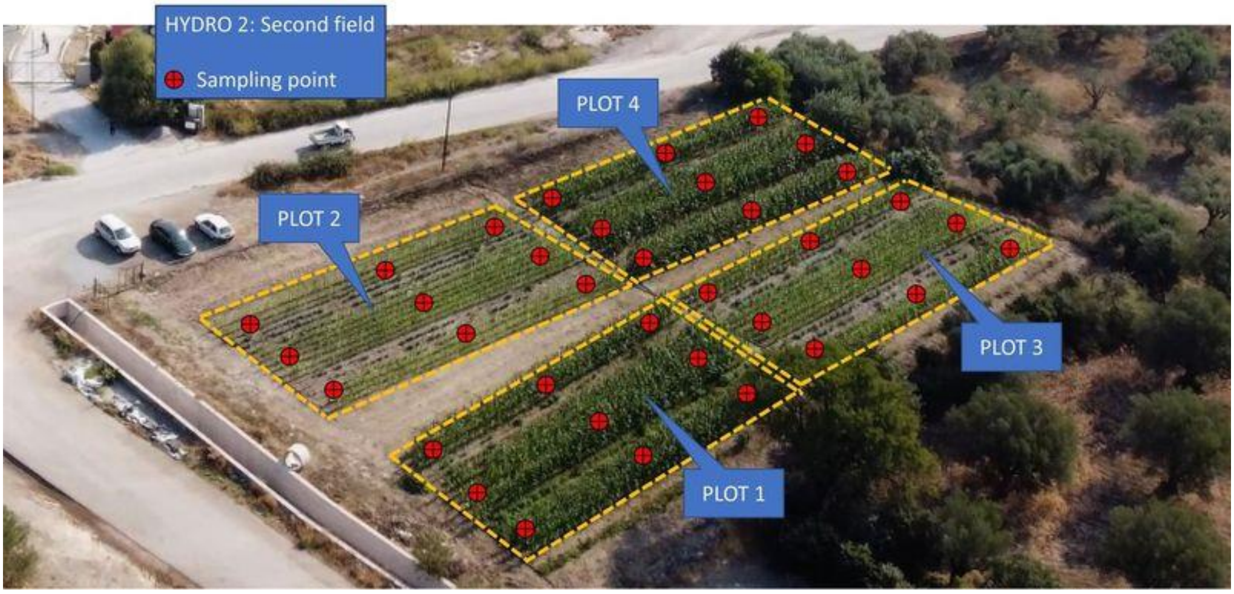

Figure 2.5. HYDRO2 second field soil sampling spots

2.2.2 Results and discussion

The soil samples collected were used to evaluate two different questions. The first included the comparison for the nutrients and other physicochemical parameters of the soil in three cultivating seasons, 2019 (benchmarking year with no crops in place), 2021 and 2023 in the large field which was irrigated with reclaimed water (i.e., treated wastewater) and is presented in section 2.2.2.1. The other one included the comparison between the two different water qualities used for irrigation in the small field, the fresh water and the treated water, which is presented in section 2.2.2.2.

2.2.2.1 Results from the large field

Table 2.1 summarizes the results of the soil analysis within the three different sampling years. The year 2019 is before the establishment of the cultivations (benchmarking) and the years 2021 and 2023 are during the cultivations.

Table 2.1. Soil analysis results comparing the different years of sampling[1]

Parameter	Sampling season		
	2019	2021	2023
Soil moisture (%)	4.38 ± 0.77 a	2.69 ± 0.35 b	3.77 ± 0.3 c
pH	6.87 ± 0.21 a	7.24 ± 0.44 a	6.68 ± 0.33 a
E.C. (μS/cm)	152.42 ± 59.8 a	582.5 ± 160.9 b	406.6 ± 65.7 b
Organic matter (%)	0.53 ± 0.35 a	2.26 ± 0.27 b	3.57 ± 0.88 c
N (%)	-	0.1 ± 0.04 a	0.16 ± 0.05 b
P (mg/kg)	17.42 ± 4.8 a	43.32 ± 20.5 b	51.84 ± 39 b
K (Ex.cat.) (ppm)	178.36 ± 90 a	236.22 ± 46.23 a	289.84 ± 88.9 a
Na (Ex.cat.) (ppm)	22.54 ± 2.6 a	238.6 ± 36.5 b	104.8 ± 26.5 c
Ca (Ex.cat.) (ppm)	2076.55 ± 321.8 a	1183.6 ± 627 b	938.5 ± 664.9 b
Mg (Ex.cat.) (ppm)	333.8 ± 110.3 a	283.4 ± 142.4 a	392 ± 167.2 a
CEC ($cmol_c$/kg)	13.7 ± 2.13 a	11 ± 4.3 a	13.1 ± 4.9 a
Fe (ppm)	15.8 ± 3.1 a	18.2 ± 3.9 a	18.07 ± 2.3 a
Zn (ppm)	0.77 ± 0.5 a	2.51 ± 1.4 b	2.48 ± 0.97 b
Mn (ppm)	11.9 ± 5.5 a	35.5 ± 8.5 b	13.16 ± 5.4 a
Cu (ppm)	1.5 ± 0.6 a	1.47 ± 0.3 a	1.33 ± 0.4 a
B (ppm)	0.45 ± 0.2 a	0.29 ± 0.08 a	0.63 ± 0.5 a
C/N ratio	-	13.9 ± 8.4 a	11.3 ± 2 a

[1] Mean values in the same line followed by a different letter differ significantly at $p \leq 0.05$.
For all variables with the same letter, the difference between the means is not statistically significant.

According to Table 2.1, soil moisture exhibited some variability between the different years of the cultivation. Considering that soil moisture is highly influenced by local weather conditions, these differences could be attributed to weather variability (less or more rain) as well as the differences in soil organic matter content.

Soil pH was not significantly different between the different years. However, soil pH is related to soil organic matter content (SOM); even if soil pH is not statistically different, in 2023 it is lower where SOM is greater compared to the other years. All pH values vary between 6 and 7.5 which are considered acceptable values for plant growth.

The electrical conductivity (E.C.) was lower the first year of cultivation when compared to the other two years that had no significant difference between them. The electrical conductivity is a result of many factors like the organic matter content of the soil, the salt content of the soil, the type of the soil and the soil moisture content (FAO, 1985). As shown in Table 2.1, a correlation between the organic matter and Na content is evidenced, while neither the soil structure nor the soil moisture content can be regarded as crucial factors affecting the E.C. The elevated E.C. within the treated wastewater is probably also contributing to this increase.

Soil organic matter was statistically different between the three years of sampling. More specifically, in 2021 Soil Organic Matter (SOM) was 4.5 times greater than 2019 while in 2023 was 7 times greater than 2019. This increase is an indicator of the positive contribution of the specific agricultural management to SOM and the use of treated wastewater to irrigate the plots.

The percentage of soil nitrogen (N) measured showed significant differences between the years 2021 and 2023 while soil phosphorus (P) showed a significant difference between 2019 and both 2021 and 2023. Therefore, it can be commented that both N and P content increases by time, possibly as a result of the fertigation practice.

The exchangeable cations measured were potassium (K), sodium (Na), calcium (Ca) and magnesium (Mg). Soil potassium (K) exchangeable cations levels, even though not significantly different, gradually increased in time (from 2019 to 2021). Sodium (Na) exchangeable cations showed a significant statistical difference between the years, with its concentrations increased between 2019 and 2021. Calcium (Ca) (exchangeable) was different between 2019 and both 2021 and 2023. However, Ca content showed a different course concerning its concentrations, as they were found to decrease from 2019 to 2021 and 2023. The magnesium (Mg) exchangeable cations were not significantly different between the three years. These results show that K concentrations are adequate, while Na and Mg concentrations were higher than the expected concentrations of a typical soil. On the other hand, Ca concentration seems to be low. These differences can be attributed to the non-usage of fertilizers and the characteristics of the treated wastewater used for irrigations.

Similarly, Cation Exchange Capacity (CEC) exhibited no significant statistical difference between 2019 and 2023. CEC is affected by the type, pH and organic matter (SOM) content of the soils. Based on results the strongest relation in this case seems to be the one within CEC and SOM since, in general, when SOM is higher CEC is also high. CEC indicates soil's ability to hold positively charged ions so, analogously, its low values reflect its low cation exchange capacity (Cornell University, 2007). This result was to be expected since the parameters affecting CEC, like SOM, are similarly low. Similar results are also reported for soils on Greek islands (Massas et al, 2008).

From the analysis of the micronutrients contained in the soil, iron (Fe) content showed no significant difference between the three years while zinc (Zn) showed a significant statistical difference between 2019 and both 2021 and 2023. Manganese (Mn) showed a significant statistical difference between 2021 and both 2019 and 2023, while copper (Cu) soil content was almost the same in all years. Boron (B) content did not exhibit any remarkable variability over the years. Soil Fe levels are adequate for plants' growth, while Zn concentration is adequate for 2021 and 2023 but low for 2019. Mn concentration is very high for 2021 and is sufficient in both 2019 and 2023, whereas Cu concentration is sufficient for all years. Lastly, concentrations of B are almost sufficient for 2019 and 2023, while being rather low for 2021. It should be mentioned that the concentrations of micronutrients are mostly dependent on the physicochemical characteristics of the reclaimed water used for the irrigation.

Finally, the C/N ratio was not significantly altered between 2021 and 2023, which is indicative of the stability provided by the cultivation practices applied, including fertigation.

2.2.2.2 Results from the small field

Table 2.2 summarizes the results of the soil analysis for the cultivation year of 2021. Plots 1 and 3 are on the right side of the field and plots 2 and 4 on the left as shown previously in Figure 2.3.

Table 2.2. Soil analysis results comparing treated and fresh water effect[1]

Parameter	Plots irrigated with treated water		Plots irrigated with fresh water	
	Plot 1	Plot 4	Plot 2	Plot 3
Moisture (%)	18.6 ± 3.62 a	12.28 ± 5.98 b	10.22 ± 5.23 b, c	5.33 ± 4.66 c
pH	7.12 ± 0.2 a	7.27 ± 0.17 a	7.43 ± 0.08 b	6.95 ± 0.33 c
N (%)	0.03 ± 0.08 a	0.05 ± 0.03 a	0.02± 0.01 a	0.04± 0.02 a
P (%)	0.31 ± 0.08 a	0.76 ± 1.09 a	0.33 ± 0.17 a	0.22 ± 0.12 a
Organic C (%)	2.86 ± 0.47 a	1.79 ± 0.54 b	2.85 ± 0.29 a, b	2.38 ± 3.53 a, b
Ca (Ex.cat.) (meq/100g)	18 ± 6.88 a	7.63 ± 1.07 b	18.07 ± 5.44 a	9.77 ± 1.73 c
K (Ex.cat) (meq/100g)	0.48 ± 0.05 a	0.30 ± 0.03 b	0.47 ± 0.06 a	0.42 ± 0.04 a
Na (Ex.cat) (meq/100g)	1.59 ± 0.43 a	1.82 ± 0.4 a	0.99 ± 0.28 b	0.79 ± 0.21 b
Mg (Ex.cat) (meq/100g)	8.29 ± 0.28 a	7.85 ± 0.18 b	8.23 ± 0.32 a	7.78 ± 0.24 b
CEC (meq/100g)	28.36 ± 7 a	17.60 ± 1.37 b	27.75 ± 5.72 a	18.76 ± 1.77 b
Mn (ppm)	4.81 ± 0.33 a	7.26 ± 0.71 b	5.35 ± 0.34 a	8.32 ± 0.61 b
Zn (ppm)	2.71 ± 0.63 a	2.96 ± 0.43 a	1.96 ± 0.35 a	2.31 ± 0.63 a
Fe (ppm)	6.71 ± 0.97 a	7.83 ± 1.55 a	5.97 ± 0.4 a, b	8.15 ± 1.29 a, c
Cu (ppm)	1.03 ± 0.05 a	1.22 ± 0.1 b	1.09 ± 0.12 a, b	1.30 ± 0.14 a, b
C/N ratio	94.02 ± 17.87 a	52.67 ± 38.78 b	139.96 ± 48.04 c	106.1 ± 148.08 c

[1] Mean values in the same line followed by a different letter differ significantly at $p \leq 0.05$.
For all variables with the same letter, the difference between the means is not statistically significant.

According to the results, soil moisture significantly differed between the plots irrigated with reclaimed water and the ones irrigated with fresh water. This can be attributed to the fact that the plants irrigated with reclaimed water showed a greater overall growth during the cultivation period and, therefore, the dense biomass decreased soil moisture evaporation.

Soil pH was significantly different between the plots irrigated with treated and fresh water but also between the plots irrigated with fresh water. In particular, soil pH of the plots receiving treated water, was on average 7 whereas the ones receiving fresh water had soil pH of 7.3. These differences could be attributed to a combination of factors including cations' content, soil microorganisms (as affected by the different nutrient's input via irrigation/fertigation) and organic matter content.

As for the other results, the percentage of nitrogen (N) and phosphorus (P) measured in the field did not show significant differences between all plots. It can be assumed that this was due to the higher quantities demanded by the corn plants for their growth.

The organic carbon content showed a significant difference between the replications with the reclaimed water when both of the replications with fresh water showed similarities with both of the replications with the treated water. These low percentages were something to be expected as the soils in Greece seem to have low organic content in general as a result of the Mediterranean environment (Yassoglou et al., 2017) and also no extra material containing organic carbon was added.

The exchangeable cations did not exhibit any statistically significant difference with the exception of sodium.

The Cation Exchange Capacity (CEC) showed the same results as in the previous case with the two plots on the left statistically differing to the plots on the right. CEC is affected by the type of the soil, soil pH and the soil organic matter (SOM) content. In this specific case there seems to be a correlation between the CEC and the SOM since, in all plots, when the former is high then the latter is also high. For example, CEC is highest in plot 1 and so is SOM in the same plot.

From the analysis of the micronutrients contained in the soil, zinc (Zn) showed no significant difference among all treatments, while manganese (Mn) showed a significant difference between the left and the right part of the field (plots 1 and 2 with 3 and 4). Iron (Fe) content of the soil did not exhibit any significant difference among the two different irrigation water types, but showed a difference among the freshwater replications. Copper (Cu) soil content showed a significant difference only between the two replications irrigated with the reclaimed water. Therefore, it is anticipated that despite the differences occurring during the statistical analysis, the concentrations of Cu, Zn and Mn are adequate even though no extra fertilization was added, while only Fe concentration is lower than expected.

The C/N ratio was high in all plots, especially plot 2 which is indicative of a high C concentration and especially a low N concentration. As an overall conclusion the C/N ratio in the plots irrigated by fresh water is high indicating unfavourable conditions for soil microbes and N mineralization.

In view of the above it is concluded that the use of reclaimed water instead of tap water resulted to comparable soil conditions without creating any problems that might hinder crops development. Furthermore, this promising irrigation water resource contributes to the better management of the fresh water distribution and usage, relieving the fresh water stress that is already an issue of concern all over the world and especially the Mediterranean region.

2.3. HYDRO3

2.3.1. Demo site overview and sampling points

HYDRO3 is implemented in Mykonos Island in Ano Mera, and it is about 0.4 ha in area. The field starts at the end of a big slope where rainwater was harvested by vertical collection (Figure 2.6). There is also an approximately 10% slope on the ground without the presence of intense and sharp fluctuations. Also, at the southwest side, there are two old stone buildings that are used as storage facilities for agricultural tools and

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small machinery, the protection of HYDRO3 automation and electronic automation systems - control panel, pumping system, and data logging.

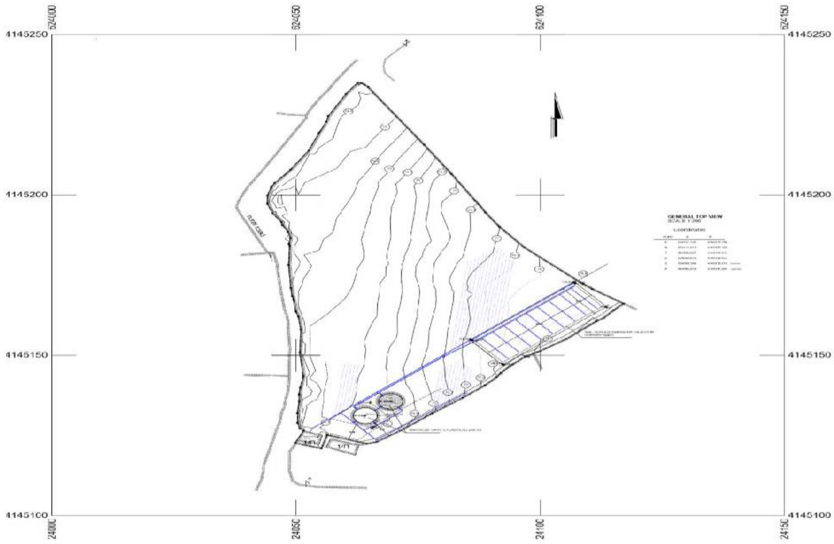

Figure 2.6. HYDRO3 master plan showing the positioning and elevation of the field and location of water collection tanks and irrigation pipes.

The soil samples in 2023 were collected from the spots that can be seen as red dots in Figure 2.7. Based on the analysis performed, these samples were considered as replications within the field and their average values are used. The samples collected in 2019 (benchmarking year with no cultivation) from the same spots were mixed and unified (one sample regarded at the end).

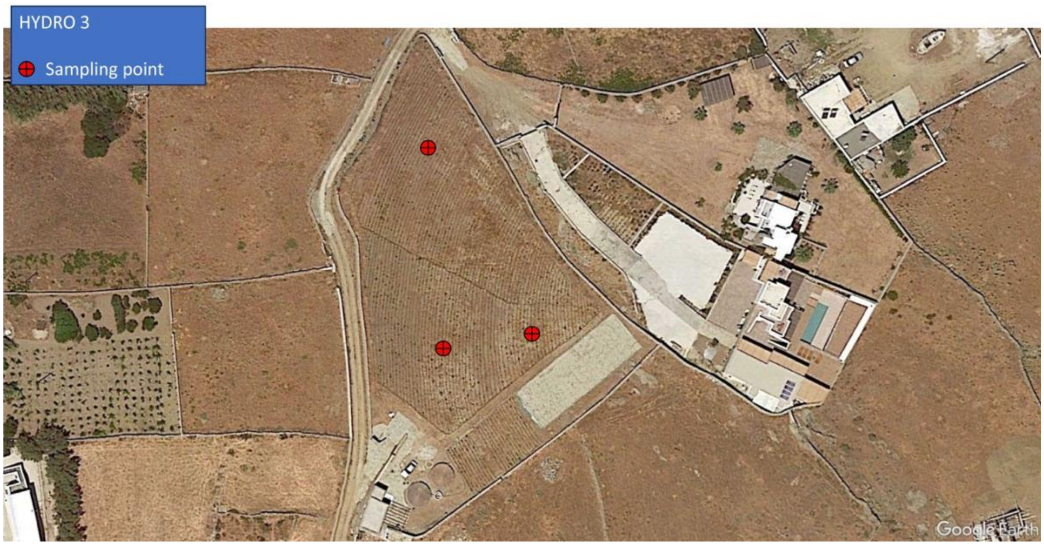

Figure 2.7. HYDRO3 soil sampling spots

2.3.2. Soil analysis Results

The soil samples collected were used to evaluate the differences between the nutrients and other parameters of the soil in 2019 and 2023. The soil samples were collected from an oregano plantation which was irrigated with rainwater harvested within HYDRO3. Table 2.3 summarizes the results of the laboratory analyses.

Table 2.3. Soil analysis results comparing the sampling years 2019 and 2023

Parameter	Cultivating season	
	2019	2023
Soil moisture (%)	1.01	0.7 ± 0.58
pH	7.42	7.11 ± 0.47
E.C. (μS/cm)	291	284 ± 34.39
Organic matter (%)	0.9	2.82 ± 0.89
N (%)	-	0.15 ± 0.05
P (mg/kg)	8.38	11.55 ± 4.72
K (Ex.cat.) (ppm)	111.11	45.04 ± 16.25
Na (Ex.cat.) (ppm)	28.28	224.08 ± 14.46
Ca (Ex.cat.) (ppm)	727.29	297.7 ± 256.85
Mg (Ex.cat.) (ppm)	129.3	63.33 ± 76.43
CEC ($cmol_c$/kg)	5.11	4.62 ± 1.79
Fe (ppm)	29.39	16.12 ± 5.14
Zn (ppm)	1.48	0.78 ± 0.16
Mn (ppm)	3.9	5.21 ± 1.96
Cu (ppm)	0.33	5.21 ± 0.11
B (ppm)	0.05	0.20 ± 0.06
C/N ratio	-	9.58 ± 0.43

Based on the results, soil moisture was higher in 2019. It is well known that soil moisture is highly dependent on the local weather conditions and therefore the slight difference recorded between 2019 and 2023 can be equally attributed to weather conditions and the different organic matter content of the soil. Accordingly, soil pH values are quite similar and at ranges acceptable to support plant growth and the same is valid for electrical conductivity.

On the opposite, an appreciable increase in soil organic matter (SOM) was evidenced for 2023. This increase is an indicator of the positive contribution of the specific agricultural management to SOM and is associated both to the addition of a soil ameliorant, containing 78% organic matter and 45% organic carbon, in 2023 and the fact that part of the organic matter deriving from the oregano plants was integrated in the field during the cultivational season.

The nitrogen (N) content of the soil measured in 2023 is rather low. This can be explained as the plants need the nitrogen for their vegetational growth even if a fertilizer containing N was used. With the utilization of the fertilizer, it was assured that there would not be any nutrient deficiency.

The phosphorus (P) content of the soils samples presents an increase between 2019 and 2023. This increase is associated with the addition of a fertilizer in the mid period. However, even after the addition of the fertilizer the phosphorus content of soils is rather moderate compared to the concentrations that a healthy soil should have (25-50 ppm) (University of Nebraska, 2015), so the fertilization with the organic fertilizer that was used should continue.

The exchangeable cations measured, as in the previous HYDROs, were potassium (K), sodium (Na), calcium (Ca) and magnesium (Mg). Concerning the potassium (K) exchangeable cations there was a decrease in 2023 compared to 2019, when sodium (Na) exchangeable cations showed an increase 2023. Calcium (Ca) (exchangeable) was higher in 2019 compared to 2023 and the same applies for magnesium (Mg) exchangeable cations.

The Cation Exchange Capacity (CEC) presents comparable values for both periods while this was not the case for the micronutrients. Iron (Fe) and zinc (Zn) content was greater in 2019 compared to 2023. On the other hand, manganese (Mn), copper (Cu) and boron (B) content was greater in 2023. This can be attributed to the usage of an organic liquid fertilizer that was applied in the field which provided the soil with these micronutrients but in different concentrations. For example, Fe and Zn content in the fertilizer was comparable low compared to the other micronutrients.

Conclusively the application of the HYDRO3 collected rainwater to the field did not affect or alter the physicochemical conditions of the soil thus providing for the development of healthy soil conditions that can support plant growth.

2.4 HYDRO4

2.4.1. Demo site overview and sampling points

HYDRO4 is in the small village of Ano Mera, on Mykonos Island and the agricultural area cultivated is about 0.2 ha. This site is situated in the premises of a house with little to almost no slope. The HYDRO4 system is based on collection of rainwater and surface runoff which is stored in tanks and into the aquifer.

Figure 2.8. HYDRO4 field

The soil samples were collected from the spots that can be seen as red dots in Figure 2.9 in 2019 and in Figure 2.10 in 2023. These samples in the statistical analysis that follows are considered as replications within the field.

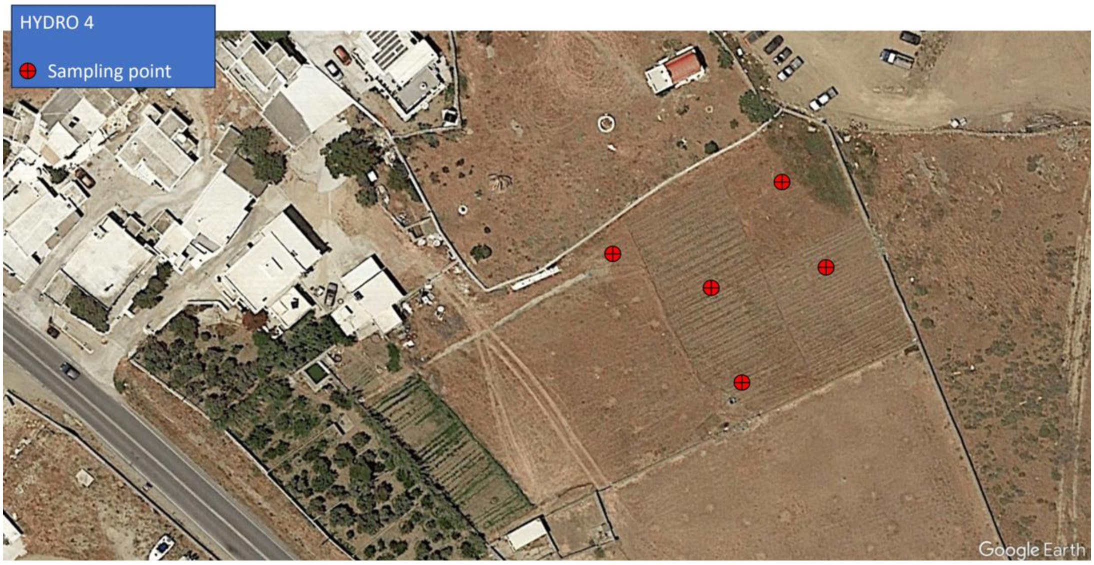

Figure 2.9. HYDRO4 soil sampling spots in 2019

Figure 2.10. HYDRO4 soil sampling spots in 2023

The statistical analysis of these samples was realized through the comparison of the samples taken from the same spots for both sampling years (2019 and 2023) with the help of the ANOVA procedure. Average values of the replications are shown in Table 2.3, as well as the standard deviation (STDEV).

2.4.2. Soil analysis Results

The soil samples collected were used to evaluate the differences between the nutrients and other parameters of the soil in 2019 (benchmarking year with no cultivations) and 2023. The soil samples were taken from a lavender plantation and the results of the soil analysis are shown in Table 2.4.

Table 2.4. Soil analysis results comparing the sampling years 2019 and 2023[1]

Parameter	Cultivating season	
	2019	2023
Soil moisture (%)	1.15 ± 0.23 a	1.24 ± 0.03 a
pH	7.06 ± 0.65 a	6.99 ± 0.25 a
E.C. (μS/cm)	335.28 ± 56.5 a	383 ± 8.5 a
Organic matter (%)	0.83 ± 0.05 a	3.63 ± 0.39 b
N (%)	-	0.2 ± 0.02
P (mg/kg)	3.09 ± 1.18 a	20.2 ± 2.8 b
K (Ex.cat.) (ppm)	118.9 ± 25.07 a	79.6 ± 21.5 a
Na (Ex.cat.) (ppm)	29.9 ± 4.8 a	259.8 ± 25.7 b
Ca (Ex.cat.) (ppm)	1264.1 ± 1395.7 a	435.25 ± 512.4 a
Mg (Ex.cat.) (ppm)	423.2 ± 46 a	141.7 ± 154.6 b
CEC ($cmol_c$/kg)	10.24 ± 7.3 a	5.75 ± 5.4 a
Fe (ppm)	63.84 ± 40.2 a	39.21 ± 12 a
Zn (ppm)	1.25 ± 0.45 a	1.69 ± 0.37 a
Mn (ppm)	7.43 ± 2.37 a	4.05 ± 0.28 a
Cu (ppm)	0.8 ± 0.52 a	0.5 ± 0.04 a
B (ppm)	0.22 ± 0.22 a	0.28 ± 0.06 a
C/N ratio	-	8.94 ± 0.18

[1] Mean values in the same line followed by a different letter differ significantly at $p \leq 0.05$.
For all variables with the same letter, the difference between the means is not statistically significant.

Based on the results it is concluded that both soil moisture, pH and electrical conductivity values between 2019 and 2023 are practically similar.

On the other hand, soil organic matter (SOM) values present a 4-fold increase in 2023 which is associated with the addition of a soil ameliorant, containing 78% organic matter and 45% organic carbon, in 2023. Another source of SOM can be the biomass deriving from the lavender plants during the cultivation season.

The nitrogen content of soil in 2023 was comparably low, while the significant increase of phosphorus content in 2023 is also associated to the addition of the fertilizer.

The exchangeable cations measured were potassium (K), sodium (Na), calcium (Ca) and magnesium (Mg). Potassium (K) exchangeable cations concentration decreased in 2023 compared to 2019, while sodium (Na) exchangeable cations increased in 2023. Calcium (Ca) (exchangeable) was higher in 2019 compared to 2023 and the same applies for magnesium (Mg) exchangeable cations. The fact that all three exchangeable cations, K, Ca and Mg, were higher in 2019 while Na was lower and when Na increased in 2023 all the other concentrations dropped, is indicative of the selective absorption of the cations by the plants (Gedroiz, 1930). The high concentrations in 2019 are probably due to the very young stage of the plants, where they did not need to absorb great quantities of these cations. In 2023 the lavender plants are fully developed and thus they have greater needs in nutrients also explaining the lower concentrations despite the addition of the previously mentioned fertilizer.

Furthermore, the Cation Exchange Capacity (CEC) decreased in 2023 compared to 2019. In general CEC is affected by the type of the soil, soil pH and the SOM content. In this case the relation of CEC and the pH is obvious as when pH is higher, CEC is also higher. The same does not seem to apply for SOM and CEC as it should follow the same course: when one increases the other increases too, and for CEC and soil type, as the soil type did not change. From the analysis of the micronutrients contained in the soil, iron (Fe) manganese (Mn) and copper (Cu) content was greater in 2019 compared to 2023. On the other hand, zinc (Zn) and boron (B) content was greater in 2023. In general, the concentrations of Fe were quite high in 2019 and in 2023, even though a high concentration of a micronutrient is not necessarily connected with a nutrient toxicity (Espinosa, 2021). On the other hand, Zn concentrations were sufficient in both cases, while Mn concentrations were low in both sampling years. Cu concentrations in 2019 are considered adequate while in 2023 are quite low and finally B concentration is low both in 2019 and 2023. In view of the above it is anticipated that the practices followed in HYDRO4 can guarantee appreciable soils conditions that can support plants' growth.

2.5. HYDRO5

2.5.1. Demo site overview and sampling points

The demo site of HYDRO5 is located within the premises of the municipal desalination plant in Agios Fokas, on Tinos Island in Greece. Concerning the technologies used in HYDRO5, a Mangrove Still System (MS) has been established. This technology uses evaporation of water deriving from seawater with the help of solar power and direct solar radiation and almost immediately condenses distilled water on a cooler surface and guides the almost pure water into tanks for storage and further use. Except for the desalination system, a production greenhouse (PGH) was developed for the development of common tropical fruit crops all year round. The irrigation is achieved through the water generated by the Mangrove Still system.

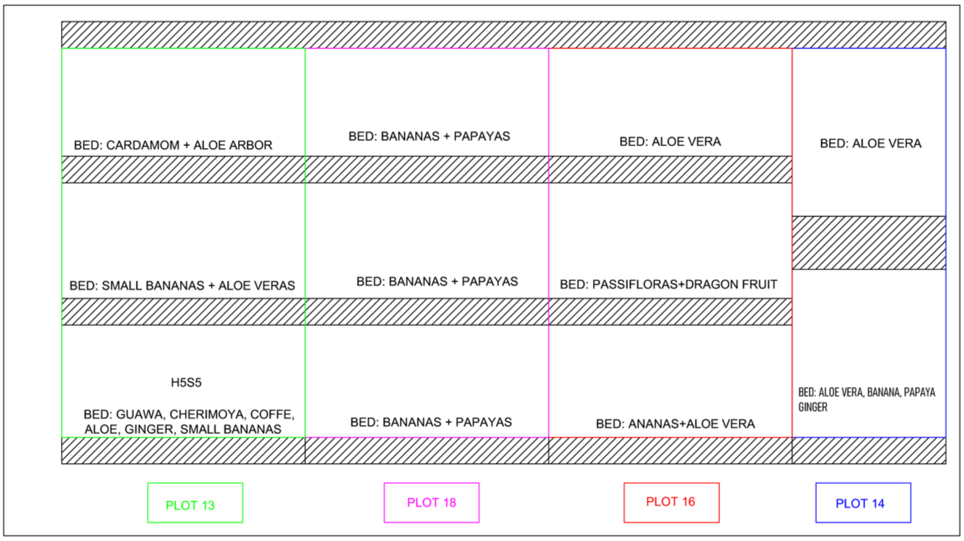

Figure 2.11. HYDRO5 schematic representation of the Production Greenhouse

The schematic layout of the site is shown in Figure 2.11. The MS-System takes up an area of 189 m^2 and the PGH about 201 m^2. The expected output of the MS system would be, on average, enough to supply the PGH with irrigation water. In case of need, the irrigation water was complemented by tap water from the municipal desalination plant. The irrigation was controlled by an intelligent system which uses soil humidity sensors and electronic valves powered by solar power cells and delivers irrigation water only when it is really needed. The soil samples were collected in 2023 from the spots that can be seen as red dots on Figure 2.12.

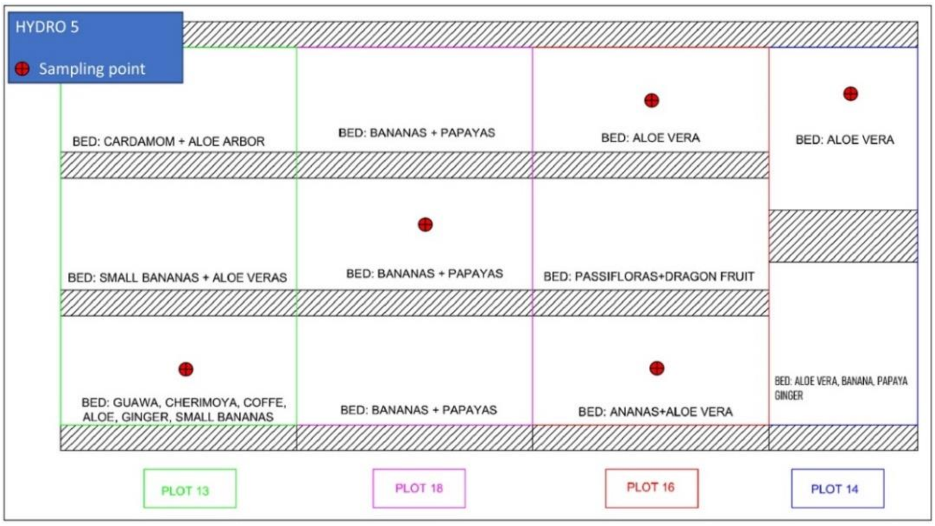

Figure 2.12. HYDRO5 soil sampling spots in 2023

2.5.2. Soil analysis Results

The soil samples collected were used to evaluate the differences between the different sampling spots in 2023. Table 2.5 summarises the results of the laboratory analyses.

Table 2.5. Soil analysis results of HYDRO5 in the different spots in 2023

Parameter	Sample				
	1	2	3	4	5
	Aloe vera	Aloe vera	Aloe + Ananas	Bananas + Papaya	Various
Soil moisture (%)	3.53	3.3	1.67	1.54	1.31
pH	6.31	6.57	7.44	7.5	7.42
E.C. (μS/cm)	793	795	257	231	222
Organic matter (%)	0.73	0.59	3.21	2.57	2.77
N (%)	0.08	0.02	0.12	0.07	0.12
P (mg/kg)	18.95	7.33	23.89	11.17	28.26
K (Ex.cat.) (ppm)	35.82	70.66	149.9	68.64	212.8
Na (Ex.cat.) (ppm)	491.8	773.9	547.8	413.3	387.4
Ca (Ex.cat.) (ppm)	666.8	2132.1	3.664.2	3911.2	3023.0
Mg (Ex.cat.) (ppm)	314.7	814.0	195.2	97.48	89.15
CEC (cmol$_c$/kg)	16.43	26.04	22.65	22.28	18.04
Fe (ppm)	30	6.92	8.89	9.85	7.6
Zn (ppm)	5.39	2.28	3.54	3.34	3.66
Mn (ppm)	36.05	13.02	4.74	6.23	4.5
Cu (ppm)	1.39	0.61	1.15	1.41	1.26
B (ppm)	2.21	1.07	4.21	2.26	4.52
C/N ratio	4.52	15.02	13.5	17.24	11.53

Based on the results, the soil moisture was higher in the plot with the Aloe vera crop. As in this case we are commenting over a greenhouse, the influence of the rainfall cannot be included as a factor affecting the moisture. Also, the organic matter does not seem to be a factor affecting the soil moisture because in this plot the organic matter was low. On the other hand, an explanation of the differences noted among the plots can be the cultivation itself. Judging by the results, in both plots with only Aloe vera plantations soil moisture is higher than in the plots with two, or more, different plant species. This is normal since Aloe vera does not have high demands in irrigation water and, thus, the soil has more water stored.

Soil pH was higher in the Bananas & Papaya plot, followed by the Aloe & Ananas plot and then the plot with the various plant species. It is interesting that, in the plots with only the Aloe cultivation, pH values are lower than in the other plots. Nevertheless, Aloe can grow in a wide range of pH values and this way pH was not affecting the plants. Concerning the plots with the Bananas & Papaya and the one with the mixture of plants species, the pH is also mostly appropriate for the cultivation providing this way good conditions for the growth of the plants. On the other hand, the plot containing the Aloe & Ananas had higher pH compared to the pH needed for the Ananas cultivation. Soil pH is adversely related to soil organic matter content, that in this case is not obvious. All these results can be explained when having in mind the fact that compost, farmland topsoil and correction agents for pH were added, thus, in general, the plants in the greenhouse showed typical growth and health.

The electrical conductivity (E.C.) was higher in both the plots with the Aloe vera. The electrical conductivity is influenced by soil organic matter content, soil salt content, the type of the soil and soil moisture content. In this case, and as shown in Table 2.5, the strongest correlation exists between the E.C. and soil moisture content, while the SOM, soil salt content and soil structure, did not affect the E.C. More specifically, it is obvious that in the plots where E.C. is higher, soil moisture is also higher and vice versa.

Soil organic matter (SOM) was lower in both plots with Aloe vera while it was higher in all the other plots presented in Table 2.5. These high concentrations can be attributed to the addition of compost, farmland topsoil and chicken manure. Another source of SOM can be biomass deriving from the fruits that were produced and not collected during the growing season. The lack of much organic matter in the plots where Aloe is cultivated is positive as organic matter tends to hold more water which would have a negative effect on the Aloe plants.

The percentage of soil nitrogen (N) measured was in general high, except for the second plot with Aloe. Even though N is necessary for plant growth, when its concentration is very high it can cause problems in the absorption of other nutrients such as K, Zn, Fe, Ca and B. These high concentrations can be attributed to the addition of chicken manure and compost. It can be assumed that the quantity added was not the same in all the plot from which the soil samples were collected.

Soil phosphorus (P) was higher in the sample from the plot containing the variety of tropical plants, while the lowest concentration was found to be in the second sample (plot) with the Aloe vera plant.

The exchangeable cations measured were potassium (K), sodium (Na), calcium (Ca) and magnesium (Mg). Potassium (K) exchangeable cations were higher in the plot with the variety of tropical plants. As mentioned before, N concentration affects K absorbance from the plants and, in this case, the correlation between the two is apparent. As shown in Table 2.5 the two plots with highest percentages of N (0.12 %) contained in the soil, show the greatest concentrations of K as it was not absorbed by the plants. Sodium (Na) exchangeable cations were higher in the second Aloe plot: high concentrations are expected since the water from the desalination system may contain some concentrations of this element. Calcium (Ca) exchangeable cations were lower in the plots with the Aloe plants and higher in the rest of the plots from which samples were collected. Lastly, magnesium (Mg) exchangeable cations were found in higher concentrations in two plots with Aloe with the highest being in the second plot. Mg affects the availability of K and Ca for plants in general but in this case, there does not seem to be any strong correlation among these parameters, thus, the differences can be attributed to the organic materials added.

Concerning the analysis of the micronutrients, contained in the soil, iron (Fe), zinc (Zn) and manganese (Mn) levels were higher in the soil samples collected from the first Aloe vera plot. All the other plots contained similar concentrations of these micronutrients. The soil in the plot with Banana & Papaya had the highest concentration in copper (Cu) and the soil in the plot containing the variety of the plants, had the highest boron (B) concentration. Fe concentration in the first plot is considered high, while the concentration in the other plots can be characterized as low. Zn concentration was found to be within normal range only in the second Aloe plot and all other plots from where the samples were collected contained high concentrations that could cover future needs for plants' growth. Mn concentration was high in the first two plots and low in the other 3 sampled plots. Cu content was found in normal concentration (for plants' growth) in the soil of the plot with the Aloe & Ananas, low in the second Aloe plot and high in all the other plots. Finally, B content was found in normal concentrations in the soil of the second Aloe vera plot, while all other plots were found to contain soils with high B concentrations. Boron's absorbance can be affected by the quantity of N contained in the soil. This is obvious in Table 2.5 where the highest concentrations of B were found in the soils of the plots that also contained high concentrations of N. In general, the results shown in Table 2.5 can also be attributed to the addition of the extra organic materials and probably in slightly different quantities which affected the concentrations of these micronutrients.

Lastly, the C/N ratio shows that in the first field (Aloe vera plantation) the decomposition of the organic matter is fast which allows the soil to have more N available for the plants while in all the other four plots the decomposition of the organic matter is slower leading to a faster N removal from the soil.

Conclusively the characteristics of the soil on the different plots reveal the effectiveness of the applied practices in HYDRO5 that guarantee appropriate soil conditions for tropical plants development.

2.6. HYDRO6

2.6.1. Demo site overview and sampling points

The demo site is located at Potamia, Akeratos, in Tinos Island on the premises of the Tinos Ecolodge, which is a touristic destination. Visitors can participate in plant growing activities and sustainable resource management while the agricultural areas of the Ecolodge serve also as a demonstration site for this project (Figure 2.13).

Figure 2.13. HYDRO6 demo site image

The area is on hilly terrain and the local soil is, generally, stoney, sandy and of limited fertility. Terraced terrain with enriched soil with organic matter is used for cultivation. A 120 m^2 greenhouse is also included in the site that mainly serves to produce fresh vegetables all year round (Figure 2.14).

The irrigation water concept follows various strategies. Collected and stored rainwater is used for plots with edible vegetables. Reclaimed water from showers and handwash basins is used for watering plots with herbs and medicinal plants. On the other hand, terracing and stone wall placements help to retain surface runoff water from rainfall, infiltrate it and store it in the enriched soil.

Figure 2.14. Illustration of HYDRO6

The soil samples were collected from the spots that can be seen as red dots in Figure 2.15 and were grouped (based on the crop species and use) as shown within the yellow frames. These samples in the statistical analysis that follows are considered as replications within the field.

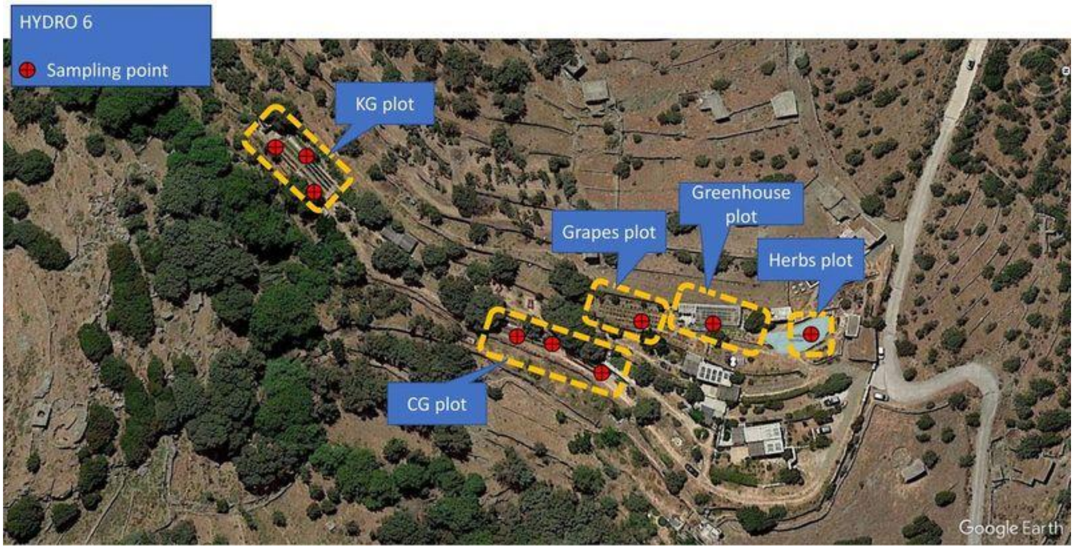

Figure 2.15. HYDRO4 soil sampling spots in 2019

The statistical analysis compared the samples taken from the indicated locations for both sampling years (2019 and 2023) by the ANOVA procedure. The results (mean and standard deviation) are presented in Table 2.6.

2.6.2. Soil analysis Results

Table 2.6 summarizes the results of the soil analysis.

Table 2.6. Soil analysis results of HYDRO6 in 2019 and 2023 [1]

Parameter	Cultivating season	
	2019	2023
Soil moisture (%)	1.64 ± 0.45 a	2.89 ± 1.5 a
pH	7.19 ± 0.71 a	7.36 ± 0.32 a
E.C. (μS/cm)	379.08 ± 110.7 a	1391.78 ± 1896.9 a
Organic matter (%)	3 ± 1.05 a	4.97 ± 2.07 b
N (%)	-	0.32 ± 0.13
P (mg/kg)	36.07 ± 34.63 a	86.23 ± 46.94 b
K (Ex.cat.) (ppm)	109.09 ± 34.72 a	215.08 ± 139.9 a
Na (Ex.cat.) (ppm)	32.04 ± 24.28 a	202.59 ± 48.46 b
Ca (Ex.cat.) (ppm)	1929.9 ± 1457.01 a	2505.7 ± 886.25 a
Mg (Ex.cat.) (ppm)	230.04 ± 256.7 a	133.52 ± 101.12 a
CEC ($cmol_c$/kg)	11.96 ± 8.96 a	15.32 ± 5.62 a
Fe (ppm)	25.8 ± 23.57 a	24.15 ± 10.9 a
Zn (ppm)	1.77 ± 0.68 a	4.98 ± 1.94 b
Mn (ppm)	6.05 ± 2.99 a	5.93 ± 1.99 a
Cu (ppm)	0.91 ± 0.23 a	2.12 ± 0.94 b
B (ppm)	1.01 ± 0.47 a	5.16 ± 4.39 b
C/N ratio	-	8.34 ± 3.81

[1] Mean values in the same line followed by a different letter differ significantly at $p \leq 0.05$.
For all variables with the same letter, the difference between the means is not statistically significant.

Based on the results, soil moisture was higher in 2023 probably due to the different weather conditions prevailed in 2023. The higher values of electrical conductivity (E.C.) in 2023 are well correlated with the different soil moisture content, SOM and soil salt content.

The increased soil organic matter (SOM) in 2023 is mostly due to the addition of compost, an organic fertilizer and chicken manure in the plots. Another source of the increased SOM can be biomass deriving from the cultivated crops during the growing (cultivation) season. In general, the concentration of organic matter in both sampling years is high and can further support any upcoming cultivations.

The nitrogen (N) content in soil is high which indicates that there is no need for any extra N addition. This high concentration of N can be attributed to the previously mentioned addition of compost, organic fertilizer, and chicken manure which is also well related to the increase of the phosphorus content in 2023 measurements.

The exchangeable cations measured were potassium (K), sodium (Na), calcium (Ca) and magnesium (Mg). Potassium (K), sodium (Na) and calcium (Ca) exchangeable cations were higher in 2023 compared to 2019, while magnesium (Mg) exchangeable cations were lower in 2023 and higher in 2019. As mentioned in the previous paragraph, N (which is essential for plant growth) can cause disorders in the absorption of other nutrients such as K, Zn, Fe, Ca, and B when its concentration is very high. This could be assumed in this case since both K and Ca may not have been absorbed and, thus, higher concentrations were measured. Finally, Mg exchangeable cations were found in lower concentration in 2023 probably because due to higher absorbance of this element by the crops and the vegetation in general. These parameters can also be affected by the addition of the compost, the organic fertilizer, and the chicken manure.

From the analysis of the micronutrients contained in the soil, iron (Fe) and manganese (Mn) content was greater in 2019 compared to 2023, however without any statistical difference. On the other hand, zinc (Zn) copper (Cu) and boron (B) content was greater in 2023 and in all three cases this difference was statistically important. It is possible that Zn and B higher concentrations are caused by the high N content which does not allow their fast absorption, but it could also be due to the addition of the compost, the organic fertilizer, and the chicken manure. This addition could also explain the higher concentration of the Cu contained in the soil samples. On the other hand, it could be concluded that Fe and Mn concentrations were higher in 2019 because there were not great quantities added with the additional organic matter that was applied and, thus, the concentrations that were measured in 2019 were absorbed by 2023 where the other sampling was conducted.

Conclusively it is anticipated that the practices followed in all plots of HYDRO6 resulted in the development of satisfactory soil conditions that can fully support plant development.

3. BIODIVERSITY ANALYSIS

The use of treated wastewater for agricultural irrigation is becoming increasingly common in many parts of the world due to water scarcity and the need for sustainable water management practices. However, the use of treated wastewater for irrigation may pose a potential risk to the environment due to the presence of contaminants such as heavy metals, and organic compounds. To ensure the safety of using treated wastewater for irrigation, it is essential to monitor the quality of water and the effectiveness of the treatment processes.

One critical aspect of this monitoring is the consideration of soil biodiversity. Biodiversity, which refers to the variety of living organisms in a particular ecosystem, plays a crucial role in maintaining ecosystem functions and services. In the context of treated wastewater irrigation, biodiversity can serve as a key indicator of soil health and the effectiveness of the treatment processes. Soil biodiversity can reflect the presence or absence of contaminants, and the impact of those contaminants on the ecosystem.

Therefore, it is crucial to incorporate biodiversity into monitoring efforts to ensure the safety and sustainability of using treated wastewater for irrigation. This approach can help identify potential risks and ensure that the treated wastewater meets the required standards for safe irrigation. Additionally, it can contribute to maintaining the health and functioning of the ecosystem, ensuring sustainable water management practices for future generations.

Soil health is essential for maintaining sustainable agricultural production, and assessing soil health is becoming increasingly important for agriculture management. Soil health is a complex concept that encompasses various physical, chemical, and biological properties of soil. In recent years, soil microorganisms, nematodes, and macrofauna have emerged as crucial indicators of soil health due to their roles in maintaining soil fertility and nutrient cycling.

3.1 Scope of the analysis

Biological indicators have emerged as a valuable tool for monitoring the quality of treated wastewater for irrigation. These indicators are organisms or their biological processes that can reflect the health and functioning of the ecosystem. They can provide information on the presence of contaminants and their impact on the ecosystem. Biological indicators can be used to evaluate the effectiveness of the treatment processes and the suitability of the treated wastewater for irrigation.

Microbial indicators can provide insights into soil properties such as organic matter content, nutrient availability, and soil structure. Changes in microbial abundance and diversity can indicate changes in soil health and productivity. Additionally, microbial indicators can be used to assess the impact of agricultural management practices on soil health, such as using fertilizers, pesticides, and irrigation water. Therefore, incorporating microbial abundance and diversity into soil health monitoring programs can provide a cost-effective and efficient way to assess soil health and inform management decisions. This approach can contribute to sustainable agriculture by promoting soil health and productivity, reducing environmental impact, and ensuring the long-term sustainability of agricultural systems.

Nematodes and macrofauna also play crucial roles in the soil ecosystem. Nematodes are microscopic worms abundant in soil and are essential in nutrient cycling and decomposition. Their abundance and diversity can reflect the overall health of the soil ecosystem. Nematode's primary role is breaking down organic matter and releasing nutrients such as nitrogen, phosphorus, and potassium. They also feed on other microorganisms, such as bacteria and fungi, releasing nutrients plants can take up. Their movements also contribute to soil structure by creating pores and channels. This enhances soil aeration, water infiltration, and drainage, essential for plant growth. Macrofauna, such as arthropods, are larger soil organisms that also play important roles in soil health, contributing to nutrient cycling, soil structure, and water retention.

3.2 Experimental design

Over a two-year period, between May 2021 and March 2023, 8 seasonal sampling events were carried out, one every three months (May, September, December, and March). Within the area of the HYDRO1, square experimental plots were created (each with an area of 64 m^2) in which the plant species (Olive, Pomegranate, Anise, and Lavender) had been sown according to the layout described in Figure 3.1. Half were irrigated with tap water, while the other half were irrigated with treated municipal wastewater, as obtained from the treatment foreseen by the HYDROUSA research project. Eight experimental plots were created (A, B, C, D), and 4 for each treatment (irrigation management).

Soil nematodes are small-bodied (0.5-3.0 mm long and 15-100 μm wide) worm-like metazoans that are very abundant and diverse and widely distributed in all soil ecosystems. The sampling method followed for the analysis of soil nematodes included four composite soil samples from each plant and irrigation treatment per sampling period. In particular, three soil cores (soil sampling depth 0-20 cm) were collected randomly but in proximity to the plants and mixed in one plastic bag to form one composite sample. Soil samples were stored at 4°C until further processing. Soil nematodes were extracted from each soil sample using Cobb's sieving and decanting method, modified by S'Jacob and van Bezooijen (1984). The soil sample (approximately 150 ml of soil) was hand mixed in a water-filled beaker, breaking the soil aggregates and detaching the nematodes from them. The nematode suspension was then poured off and sieved through a series of sieves of decreasing mesh size. The residues retrieved from the sieves were placed on nematode filters, and the latter were placed on extraction plates containing 100 ml of clean water. Extraction plates were then stored at 15–17°C for 48 h, sufficient time for nematodes to move through the filters to the clean water. Clean water containing the nematodes was collected, and total abundance was counted under a stereoscope. Nematodes were fixed on 4% formaldehyde, and at least 100 individuals per sample were identified to the genus level using a microscope and the taxonomic key of Bongers and Bongers (1998). Nematode genera were assigned to trophic groups and classified along the colonization–persistence gradient (c–p values) following Bongers (1990).

With regards to the macrofauna, sixteen pit-fall traps were placed on each experimental plot at random spots close to the plants during each sampling period. At each sampling, all trapped macrofauna individuals from the traps were stored in alcohol bottles for preservation and then transferred to the laboratory to process and identify the organisms under stereoscopy.

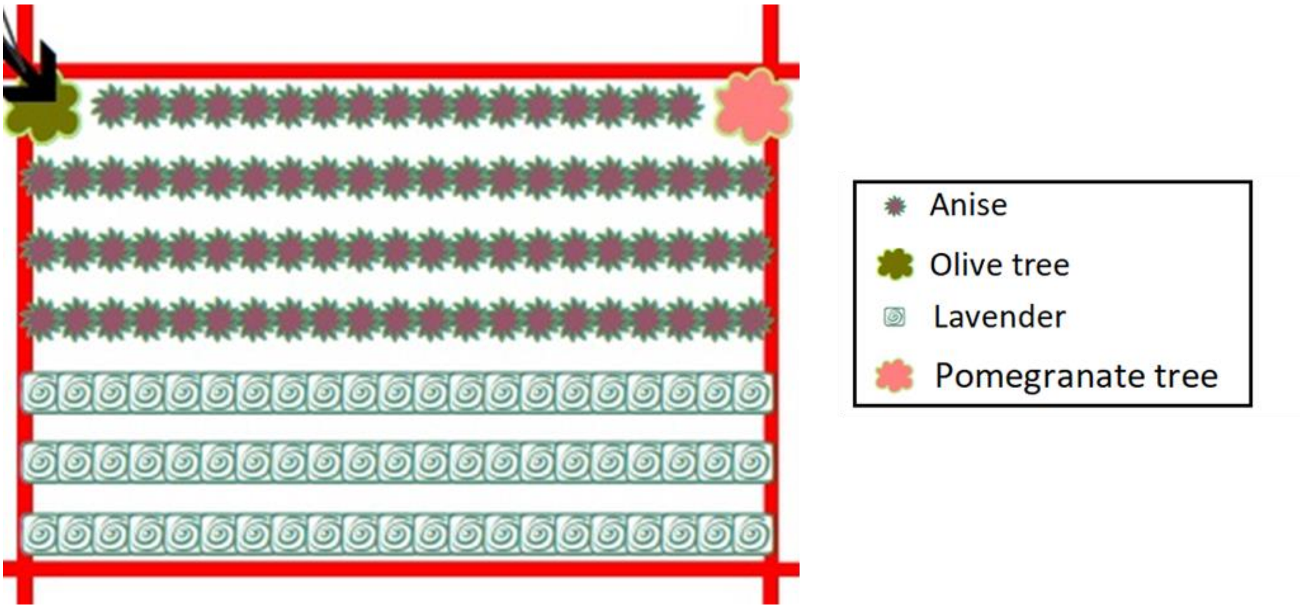

Figure 3.1. Example of the arrangement of plant species in a square experimental plot.

3.3 Analysis

3.3.1. Macrofaunal groups studied

The groups that were the most abundant were the following: Araneae, Opiliones, Chilopoda, Diplopoda, Isopoda, Collembola, Coleoptera, Dermaptera, Heteroptera, Orthoptera, Mantodea, Blattodea, Diptera, Hymenoptera.

<u>Abundance and diversity of macrofauna</u>

Each taxa abundance was estimated per treatment in each sampling occasion. Two-way ANOVA was applied to our dataset to examine the effect of the irrigation treatment regime (treated wastewater vs. clean water), the effect of the sampling period, and that of the plant species on the populations of macrofaunal taxa.

Estimation of site diversity was carried out using the Renyi diversity index. This diversity index shows a variable sensitivity to rare and abundant species as the parameter α (alpha) changes (RICOTTA, 2000). Graphing the index value against the parameter α gives the diversity curve of the biocommunity. Thus, for value $\alpha = 0$, the index equals the number of species. For $\alpha = 1$, the index is proportional to the Shannon diversity index. For value $\alpha = 2$, the index is proportional to Simpson's diversity index. As the value of α increases and tends to infinity, the index begins to rely more heavily on the equal distribution of species. Therefore, according to the above, when the curves of two biological communities differ at low values of the parameter α, this is because they have different numbers of species, while when they differ at high values, it means that they differ in the pattern of species dominance. The calculation of the Renyi parametric index was carried out using Past software.

<u>Seasonal variation in average invertebrate abundance for each type of irrigation</u>

Our results had a similar pattern for the duration of the experiment in both sampling years (Figure 3.2). The total macrofaunal abundance did not show a statistically significant difference in irrigation type. On the contrary, the effect of seasonality on the variation of total abundance was significant. The highest mean values were found in the March sampling conducted in early March, while the values of the December sampling were consistently the lowest.

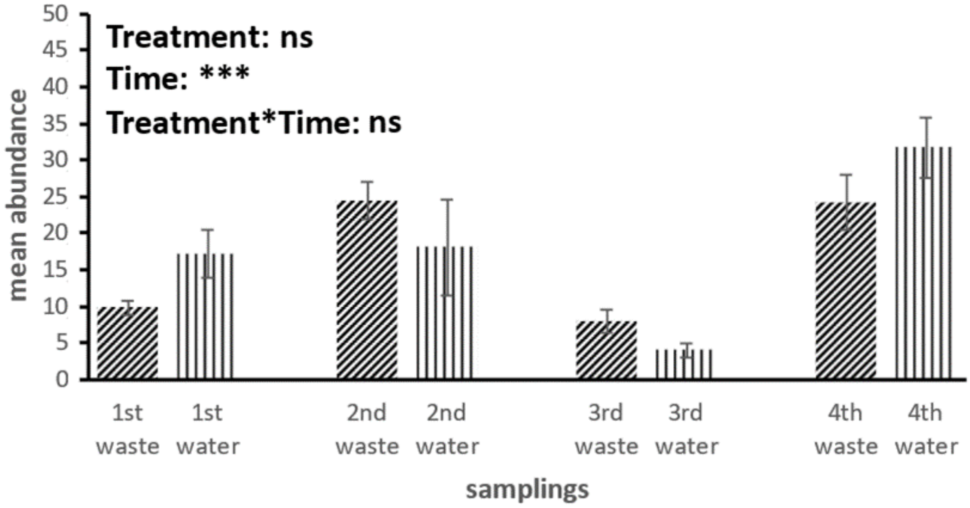

Figure 3.2. Mean value (±standard error) of the mean invertebrate abundance between the different treatments (Waste: Treated Wastewater and Water: Water) for each sampling (1st=May, 2nd=September, 3rd=December, 4th=March 2022). Two-way ANOVA showed statistically significant differences (*p<0.001, ns: non-significant).**

<u>Percentage participation of groups for the two types of irrigation in each sampling</u>

The main groups found in our samples were Araneae, Collembola, and Coleoptera, showing a high percentage of participation (Figure 3.3). Seasonality altered the composition of bio-communities. Spiders and Coleoptera dominated the May sampling. Coleoptera dominated the second sampling (September), while the winter sampling in December showed higher participation of Coleoptera and spiders. Finally, in March faunal community seemed to be more evenly distributed among the groups.

This project has received funding from the European Union's Horizon 2020 Research and Innovation Programme under Grant Agreement No 776643

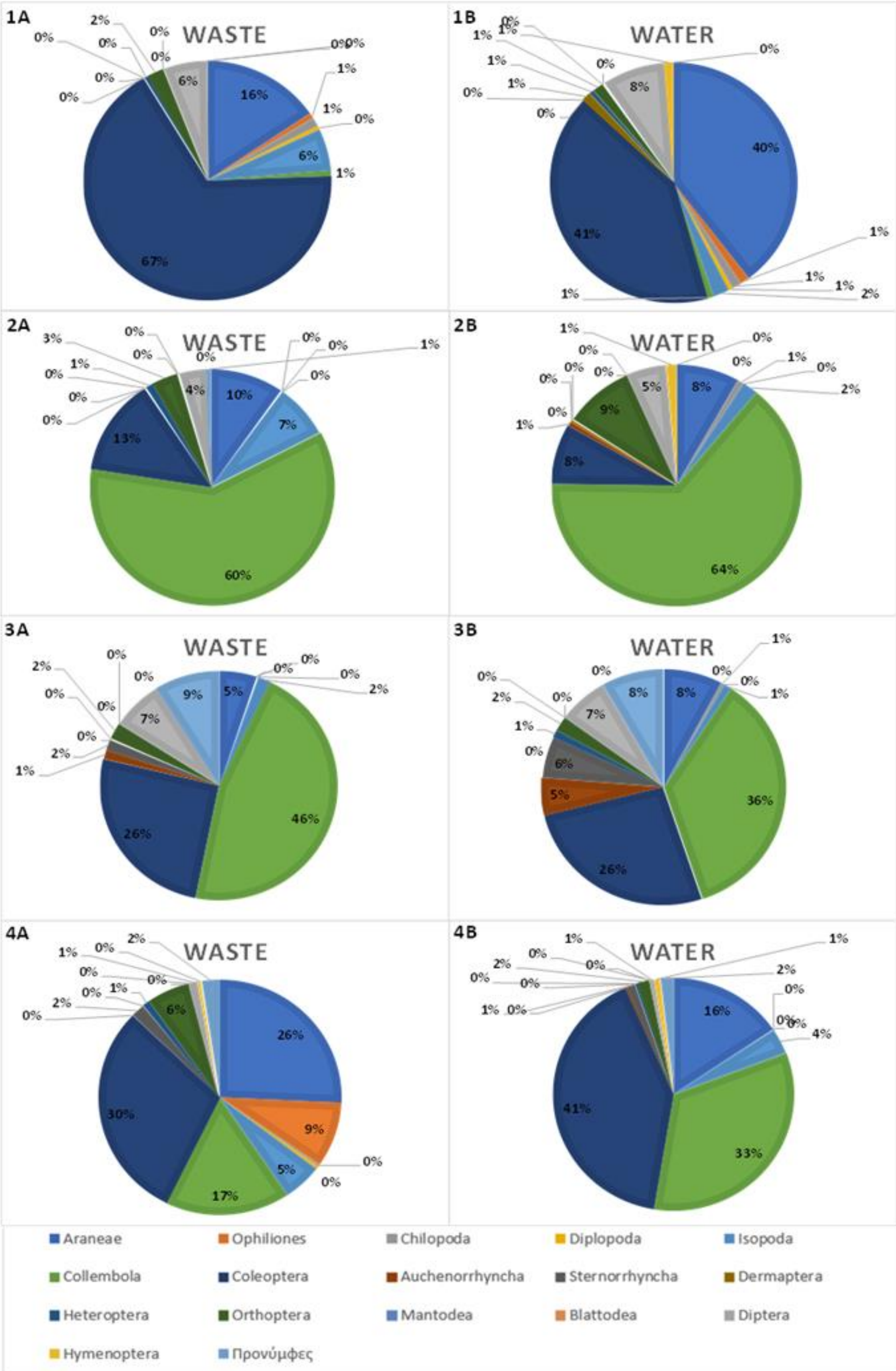

Figure 3.3. Percentage of each taxon in samples irrigated either with treated wastewater (A) or water (B) for each sampling event. The numbers before the letters correspond to the four sampling events (1 = May, 2 = September, 3 = December, 4 = March).

In addition to the effect of seasonality, differences in the communities between the two types of irrigation were also detected, especially in the May and March sampling. The plots treated with wastewater in May (Figure 3.3-1A) were dominated by Coleoptera (percentage contribution of 67%). In contrast, in the clean water irrigated plots (Figure 3.3-1B), we found a different pattern where spiders and Coleoptera had equal participation (40-41%). In the March sampling, the community of the plots irrigated with clean water community showed a more even distribution, with significant participation of Coleoptera (30%), spiders (26%), and collembola (17%), in the wastewater-treated plots, a similar pattern was recorded as these three groups presented similar percentages (41% for Coleoptera), (33% for collembola) and (16% for spiders).

No statistically significant difference was recorded regarding irrigation regime management in any of the three major taxa (Figure 3.4-A-C). However, in all three taxa, the effect of seasonality is strong. For example, spiders (Figure 3.4-A) and coleoptera (Figure 3.4B) had their maximum abundance mainly during the May (1st sampling) and March (4th sampling) periods, in contrast to the collembola, whose maximum abundance was found in the second sampling (September).

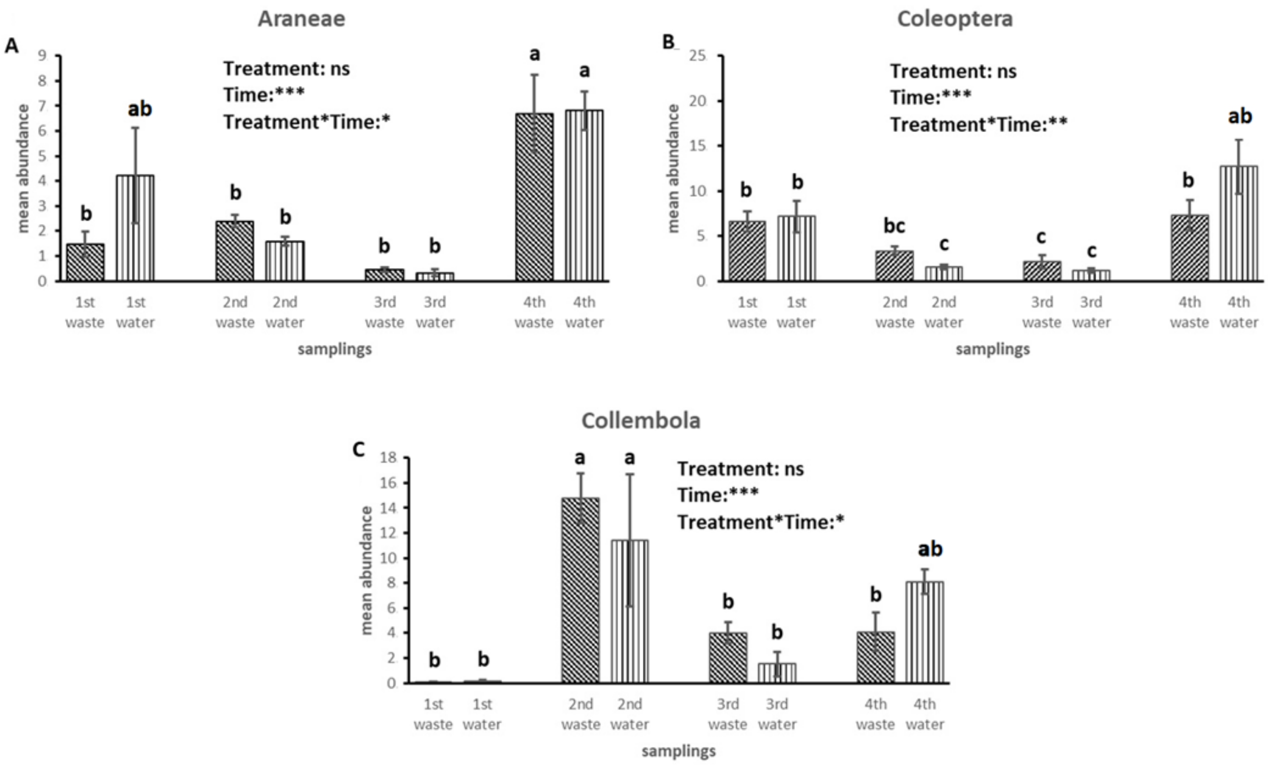

Figure 3.4. Mean abundance values (±standard error) of spiders/Araneae (A), Coleoptera (B), and Collembola (C) among the different treatments (Waste: Treated Wastewater and Water: Water) for each sampling (1st= May, 2nd= September, 3rd= December, 4th= March), for the two sampling years. Different letters above each column indicate statistically significant differences, as revealed by Two-way ANOVA (*p<0.05,p<0.01,***p<0.001).**

Estimation of biodiversity using the parametric diversity index Renyi

An estimate of the diversity of the sampling areas using the Renyi diversity index is presented in Figure 3.5. By observing the index values for $\alpha = 0$, we can see how diversity (number of morphospecies was affected by seasonality. The highest number of morphospecies was collected in the May and March samples (Figure 3.5A and 3.5D, respectively), while fewer morphospecies were detected in September and especially in December (Figure 3.5B and 3.5C, respectively). Regarding the two types of irrigation regimes, the plots irrigated with treated wastewater in the May, December, and March sampling occasions showed greater diversity in morphospecies distribution. On the contrary, in the second sampling in December, although the treated wastewater irrigated plots had more morphospecies, a better iso-distribution was present in the areas irrigated with water. Overall, for the most part, more morphospecies were detected in the treated wastewater plots, and at the same time, the evenness was better with a reduced dominance pattern.

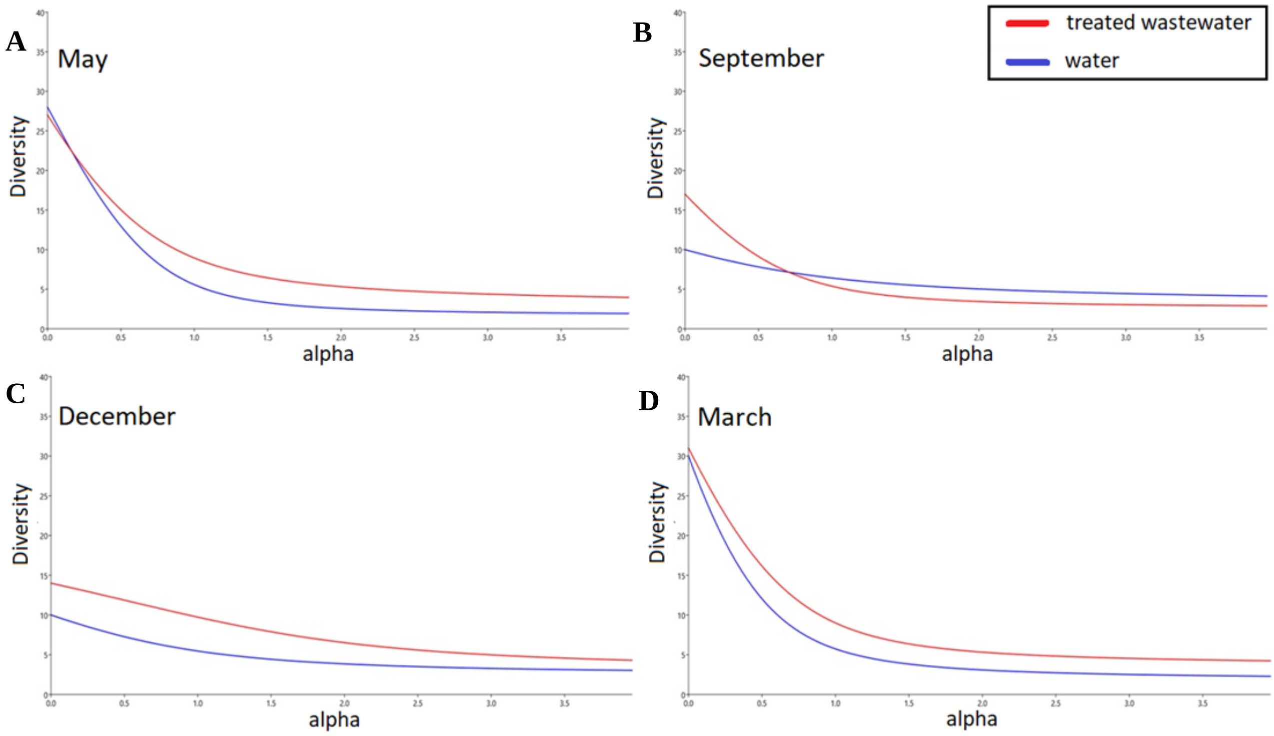

Figure 3.5. Estimation of plot macrofaunal diversity based using the Renyi diversity index. The diversity index shows a changing sensitivity to rare and abundant morphospecies as the parameter α (alpha) changes. Plotting the index value against the parameter α gives the diversity curve of the biocommunity. The index equals the number of species for a value of α = 0. For α = 1, the index is proportional to the Shannon diversity index. For a value of α = 2, the index is proportional to the Simpson diversity index.

Nematodes

The results of the first year of the monitoring have shown nematode abundances to increase over time with significant differences recorded during May and December sampling periods (Figure 3.6). Higher abundances of nematodes were observed in soils irrigated with treated wastewater compared to those irrigated with freshwater. With regards to the sampling periods, May did show the lowest nematode abundances, September and March an intermediate abundance while, December exhibited the highest nematode abundance.

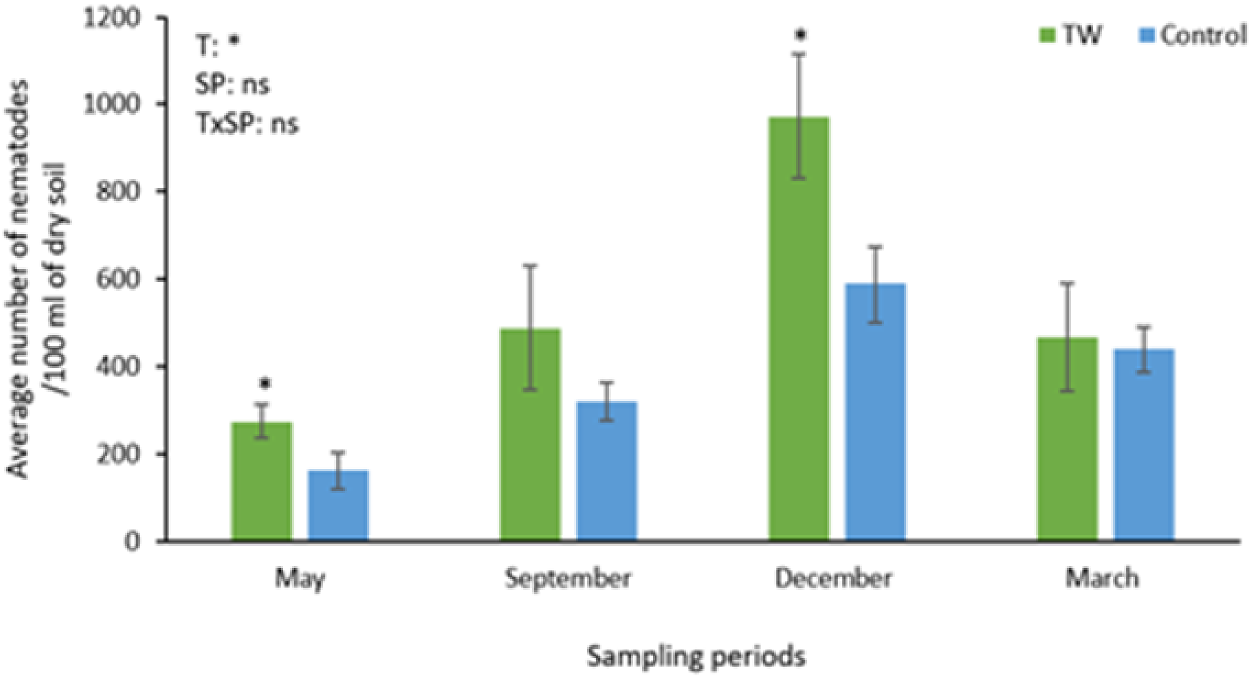

Figure 3.6. Average number of nematodes per 100 ml of dry soil across different sampling periods and results of repeated-measures ANOVA regarding "Treated wastewater irrigation" (TW), "Freshwater-control" (Control) and their interactive effect (TxSP). (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ns: $p > 0.05$).

The structure of the nematode community in terms of trophic groups (Figure 3.7) was dominated by bacterial-feeders in all sampling periods and irrigation treatments, with the exception of May treated wastewater irrigation treatment where fungal-feeders were the major trophic group. With regards to the winter, autumn, and spring sampling periods, treated wastewater irrigation treatments showed significant proportions of omnivores, predators, parasitic and non-parasitic plant-feeders, while the mentioned trophic groups are least represented in freshwater irrigation treatments. However, fungal-feeders in autumn and spring sampling periods were more abundant in FW irrigation treatments compared to treated wastewater treatments.

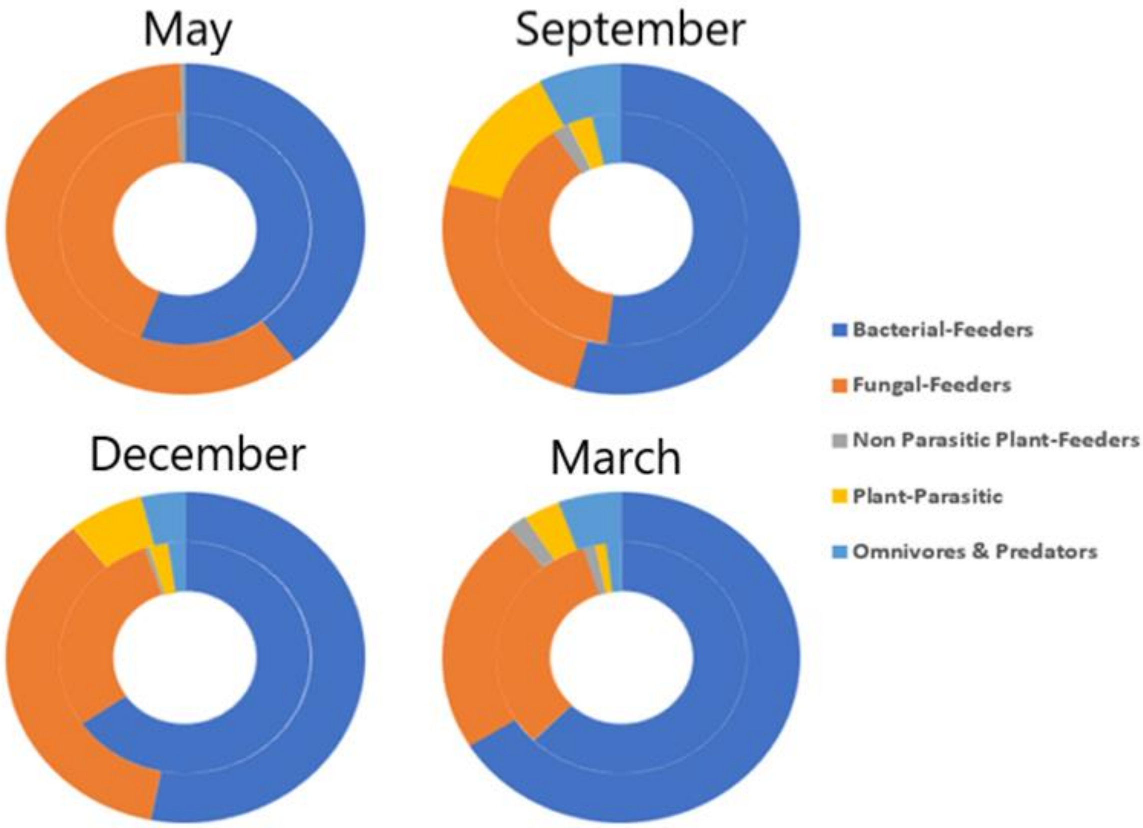

Figure 3.7. Percentage contribution of trophic groups for all sampling periods and irrigation treatments. The outer circle stands for treated wastewater irrigation, while the inner circle stands for freshwater irrigation.

The composition of the nematode community at all sampling periods and the two irrigation treatments is given in the rank abundance graphs (Figure 3.8). The most abundant genera recorded in all cases belonged to bacterivores and fungivores nematodes. More specifically, Acrobeloides (cp-2 bacterivore) was the dominant genus in all sampling periods and irrigation treatments, with the exception of May and December, which were irrigated with freshwater and treated wastewater, respectively. The latter treatments were dominated by Aphelenchus (cp-2 fungivore), with minor difference with Acrobeloides, which were the second most abundant genus. In terms of diversity, the sampling period of May recorded the lowest genera diversity, in both irrigation treatments, while September recorded the highest in treated wastewater irrigation treatment.

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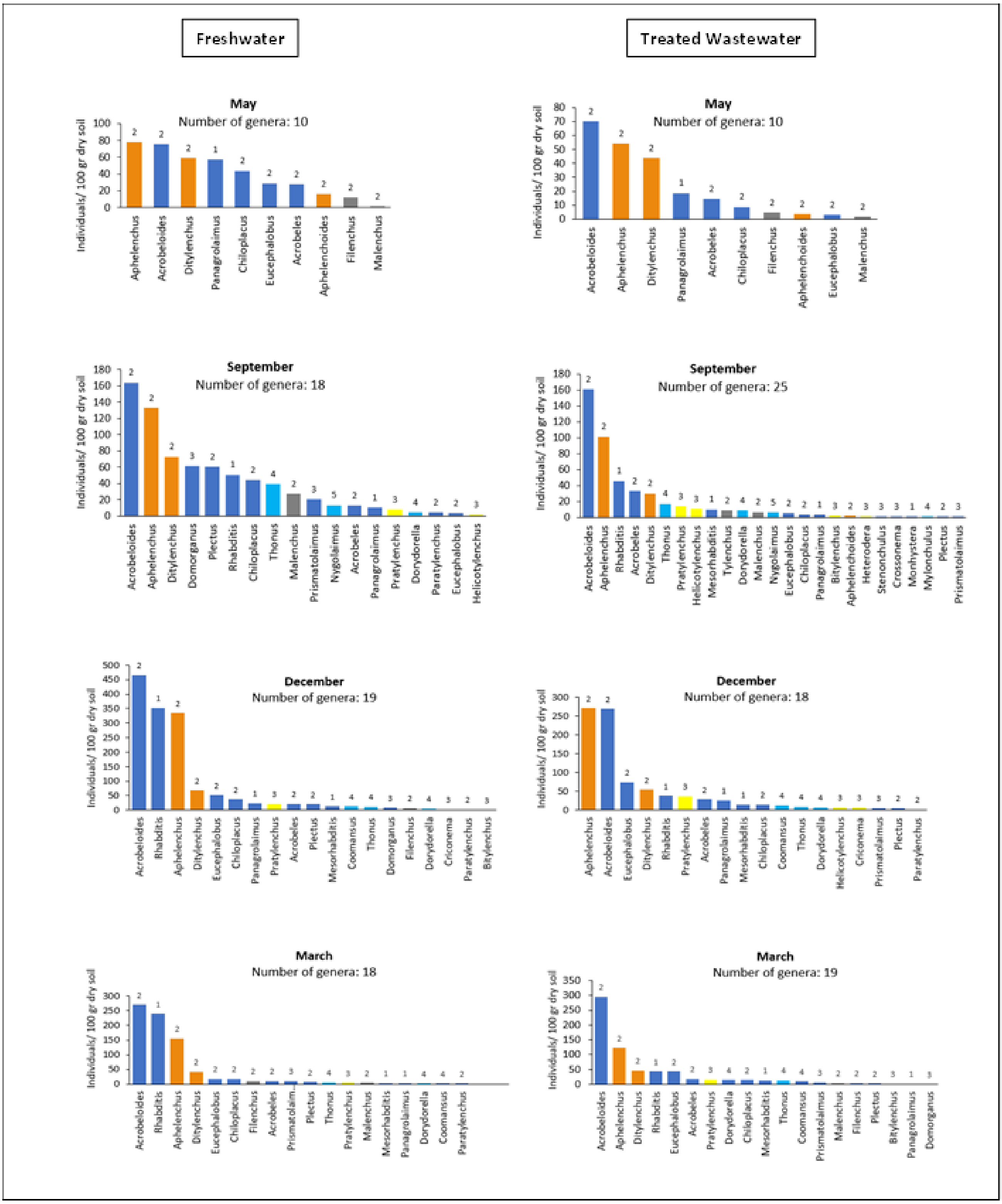

Figure 3.8. Rank abundance graphs for nematode genera at different sampling periods and irrigation treatment. Genera are ranked from the most to the least abundant. The numbers above bars indicate the c–p value of each genus. Each bar colour indicates the trophic group each nematode genera belongs to (Blue: Bacterial-Feeders; Orange: Fungal-Feeders; Grey; Non-parasitic Plant-Feeders; Yellow: Plant-Parasitic; Light blue: Omnivores & Predators).

The food web analysis according to the EI/SI ratio (Figure 3.9) has shown that all sampling periods of freshwater-control treatment and the first two sampling periods (May and September) of treated wastewater treatment were ordinated in the lower left quadrant, indicating that all soil samples were in a stage of depletion. On the contrary, the last two sampling periods of treated wastewater treatment were ordinated in the upper left quadrant, indicating that these soil samples passed into the Nitrogen enrichment phase.

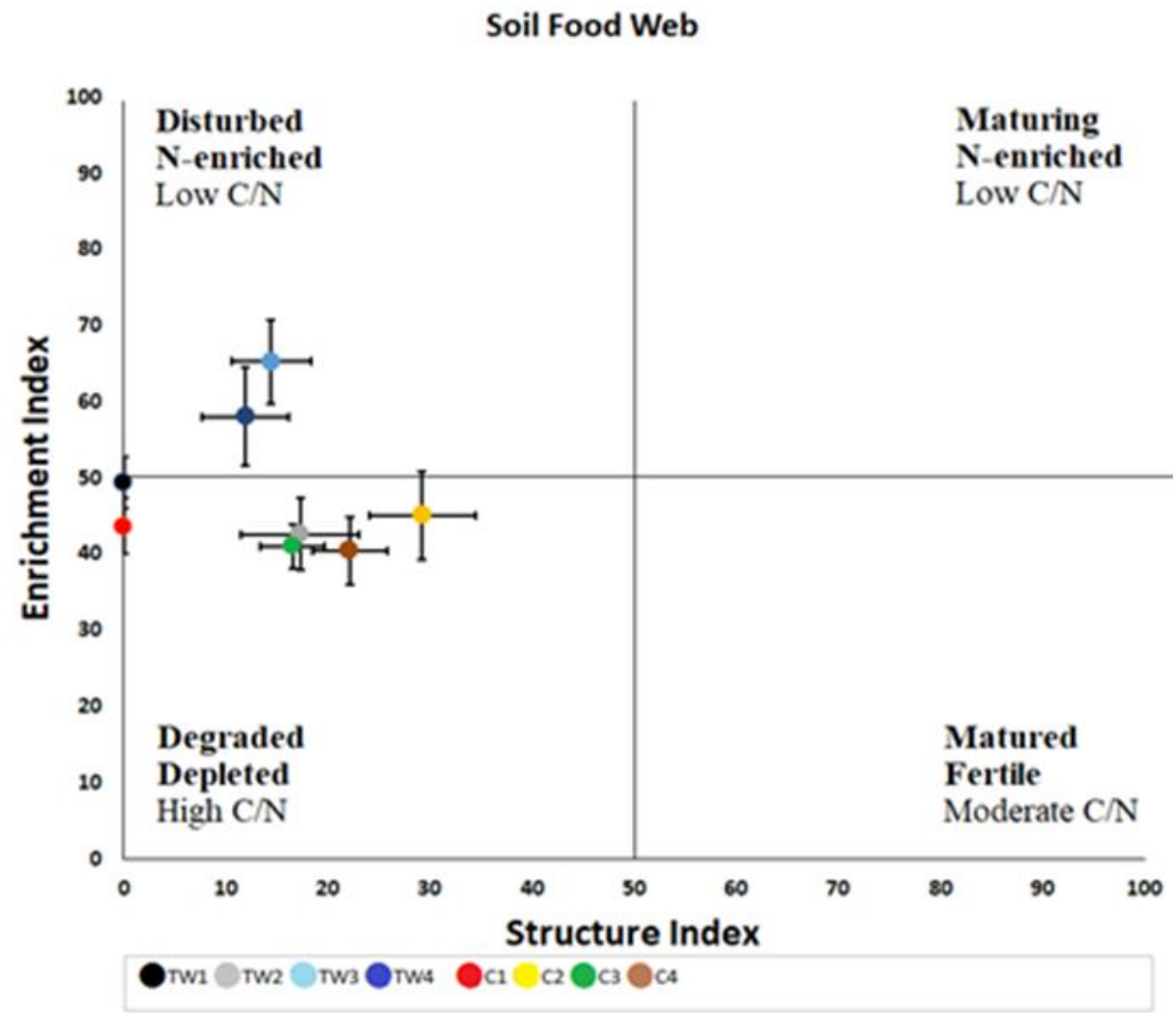

Figure 3.9. Food web analysis according to the ordination of the samples based on the EI and SI values of the different irrigation treatments. The crosses point to the mean value of the ratio and the respective standard deviation. Different colours and numbers show different treatment and sampling periods. “Treated wastewater irrigation” (TW), “Freshwater-control” (C), 1=May, 2=September, 3=December, 4=March.

3.4 Conclusions

Macrofauna

After two years of macrofauna biomonitoring, our results showed that using treated wastewater did not decrease the abundance of the main taxa of soil macrofauna, as no significant differences were recorded compared to the clean water irrigation management. The most critical taxa (e.g., spiders, Coleoptera, and collembola) are groups often used as biomarkers in scientific work and had the highest abundances and contribution to the overall abundance. Their significance as crucial role players in soil health has been highlighted in the past, indicating the importance of their presence in a soil system (Liu et al., 2007).

Our Renyi results showed that using treated wastewater favoured the abundance of specific taxa and the biodiversity of these plots, except for the September sampling, treated wastewater led to biocommunities with better evenness and a limited pattern of dominance, indicating a more stable community. This indicates that the use of treated wastewater at these concentrations resulted in improved soil conditions. However, much attention must be paid to the amount of treated wastewater used for irrigation. Tessaro et al. (2013) also found a relative increase in arthropod diversity following the use of treated wastewater—however, a further increase in effluent density led to the opposite effect. In addition, high doses are related to greater substrate availability, which can become a limiting factor (toxicity) at higher doses.

In both years, the most abundant samplings occasions were May and March, while the December sampling presented the lowest overall abundance and about half of the morphospecies compared to the other two. The differentiation in macrofauna total abundance and community richness between seasons is usually interpreted as related to variations in climatic factors such as temperature, precipitation, or day length, especially in Mediterranean regions with strong seasonality (Lionello et al., 2006). The increased temperatures recorded in September and the low ones in December seem to significantly impact the soil macrofaunal communities on the island of Lesvos. On the contrary, the mild climatic months (March and May) favour them.

The presence of different plant species did not have an impact on soil macrofauna. Our results showed that two years is considerably short for the selected plants to form their microclimatic conditions, altering the composition of the taxa residing under their canopy and close to their roots.

Nematodes

The application of treated wastewater for irrigation has been found to result in a higher abundance of soil nematodes compared to soils irrigated with conventional freshwater. This can be attributed to the elevated levels of nutrients and organic matter found in treated wastewater, which provide a conducive environment for nematode proliferation and survival. Nematodes are known to be highly responsive to changes in soil nutrient levels, and the increased availability of nitrogen, phosphorus, and other essential nutrients in treated wastewater can lead to population growth. Additionally, the high organic matter content in treated wastewater can increase soil moisture retention, which can further support nematode activity. Moreover, precipitation levels can affect nematode abundance in soil. Nematodes are sensitive to changes in soil moisture levels, and precipitation can directly influence soil moisture content. In general, nematodes are more abundant in soils that are consistently moist, as these conditions provide a favourable environment for nematode growth and reproduction. However, prolonged drought can reduce nematode populations, as it can lead to a decrease in soil moisture levels and limit their access to nutrients. Therefore, the higher nematode abundance observed in December, for both irrigation treatments, is justified by the increased precipitation rates at that period in the specific area.

Soil moisture can also play an important role in determining the relative abundance and activity of nematodes. The dominance of bacterivorous nematodes in all sampling periods and treatments is attributed to the tolerance they demonstrate in dry soil conditions, typical of the Aegean islands. Bacterivorous nematodes can maintain their activity and reproduction at lower levels of soil moisture, as long as there is enough available water to maintain bacterial populations. In contrast, fungivores nematodes are more sensitive to dry soil conditions and require higher levels of soil moisture to maintain fungal populations and support their activity and reproduction.

The presence of omnivore and predator nematodes in soils irrigated with treated wastewater can indicate a healthy and diverse soil food web. Omnivore nematodes, as their name suggests, are able to feed on a wide variety of food sources, including bacteria, fungi, and other nematodes. They play an important role in regulating the populations of other soil organisms and can contribute to nutrient cycling by breaking down organic matter. Predator nematodes, on the other hand, feed primarily on other nematodes and small soil animals, such as mites and springtails. They are important regulators of nematode populations in soil and can contribute to maintaining a balanced and diverse soil food web.

The presence of omnivore and predator nematodes in soil can indicate that there is a diverse array of food sources available to support a complex soil food web. This can help to maintain a healthy soil ecosystem with robust nutrient cycling and a balance of different soil organisms. Additionally, the presence of these nematodes can indicate that the soil is able to support a range of different trophic levels, which can be an important indicator of soil health and fertility.

In summary, both irrigation treatments reflect a disturbed soil system however, soils irrigated with wastewater tend to improve over time as nematode abundances increase along with the number of genera mainly because of the Nitrogen enrichment in those soils. Therefore, the use of treated wastewater for irrigation can be an effective means of enhancing soil fertility and promoting soil health through the stimulation of beneficial nematode populations.

4. MICROPOLLUTANTS ANALYSIS FOR FOOD SAFETY ASSESSMENT

The integrated system of HYDRO1 + HYDRO2 of the HYDROUSA project on the island of Lesvos (Greece) can be seen in Figure 4.1. The agroforestry system of HYDRO2 in Antissa (Lesvos, Greece) takes advantage of the reclaimed water from HYDRO1, recycling both water and nutrients (by means of fertigation).

Three types of crops, lettuce (*Lactuca sativa romana*), oregano (*Origanum vulgare*), and lavender (*Lavandula angustifolia*) were planted as part of HYDRO2. They were fertigated with either:

1) tap water as control treatment (CT),

2) water discharged from the HYDRO1 wastewater treatment plant (FT), which consists of an upflow anaerobic sludge blanket coupled to a constructed wetland and tertiary treatment (UV),

3) wastewater from a partial treatment train, including UASB and tertiary treatment UV but that bypasses the constructed wetlands (PT).

Two sampling campaigns were established, one starting in the fall of 2021 (1st campaign) and one in the summer of 2022 (2nd campaign). Harvest took place after approximately 7 weeks for lettuce for both campaigns, whereas for both oregano and lavender it took 6 and 8 months for the harvest of the 1st and 2nd campaign, respectively.

Analysis of 88 selected organic micropollutants (OMPs) - with a likelihood of contaminant uptake in the edible part of the plants - took place in the planted soil (section 4.1) as well as in the leaves and roots of the fertigated crops (paragraph 4.2). All the analyses were performed at ICRA-CERCA (Institut Català de Recerca de l'Aigua, Spain). The list of micropollutants analysed can be found in the Excel file ''ANNEX CROPS'' (in the tab "compound list"). Selected heavy metals (Zn, Cu, Fe, Mn, Cr, Pb, Cd, Ni) were also analysed in lettuce, oregano, and lavender at NTUA (National Technical University of Athens, Greece).

It should be mentioned that the information of this Deliverable 4.6 provides the data for the food safety assessment that is presented in Deliverable 6.4 (Environmental risk assessment model).

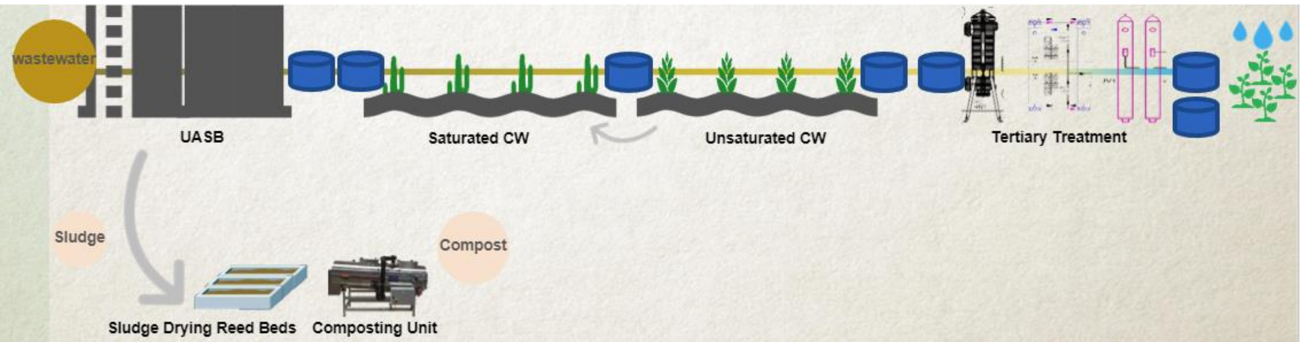

Figure 4.1. The integrated agroforestry system of HYDRO1 + HYDRO2 in Antissa (Lesvos, Greece).

4.1 Organic micropollutants (OMPs) in soils

4.1.1 Methodology

a) Soil sampling

Samples from the HYDRO2 wastewater reclamation demo-site of the HYDROUSA project on the island of Lesvos (Greece) involved soils and crops irrigated with either: 1) tap water as a control experiment (CT), 2) water discharged from the full HYDRO1 wastewater treatment train (FT), which consists of an upflow anaerobic sludge blanket coupled to a constructed wetland and a UV-treatment, or 3) wastewater from the partial treatment train that bypasses the constructed wetlands (PT). To be noted that water from point 3 had a much longer retention time in the tank (before its use for irrigation) than water from point 1; with likely degradation and/or sorption and/or other factors (e.g., evaporation) of organic micropollutants (OMPs), thus their occurrence was not comparable between the two treatments (a more in-depth analysis of the OMP results in water is addressed in the Deliverable 5.9). Consequently, the characterization of soil and crops here is not meant to compare the FT and PT wastewater treatment trains but to relate the occurrence of emerging pollutants in soils and crops with the corresponding irrigation waters.

The soil study focused solely on the plots for lettuce cultivation, and the sampling was adapted to the growth times of this crop. Soils were sampled at three different stages:

- **Initial time** (t0): samples collected before planting the lettuces:
  1st campaign – Fall 2021: 20/10/21
  2nd campaign – Summer 2022: 19/06/22
- **Intermediate time** (t1): samples collected two weeks after planting the lettuces:
  1st campaign – Fall 2021: 06/11/21
  2nd campaign – Summer 2022: 08/07/22
- **Final time** (tf): samples collected at the harvesting time of the lettuces (c.a. 7 weeks after planting):
  1st campaign – Fall 2021: 10/12/21
  2nd campaign – Summer 2022: 02/09/22

On each plot, 3 random replicates of **bulk soil (BS)** samples were collected near the drippers and stored in aluminum containers. For each replicate, a square-shaped section (25 cm/side) was determined, and 5 subsamples were collected from the corners and the centre (Figure 4.2). Sub-samples were mixed and homogenized *in-situ* to generate a composite sample (100 grams). Soil was preserved at -20ºC until shipping. Upon arrival at the laboratory, the samples were divided into two portions. One fraction (for micropollutants analysis) underwent freeze-drying, while the other fraction (for moisture, soil organic matter, and DNA analysis) was kept as it was. Both fractions were then stored at -20ºC until the corresponding analysis was conducted.

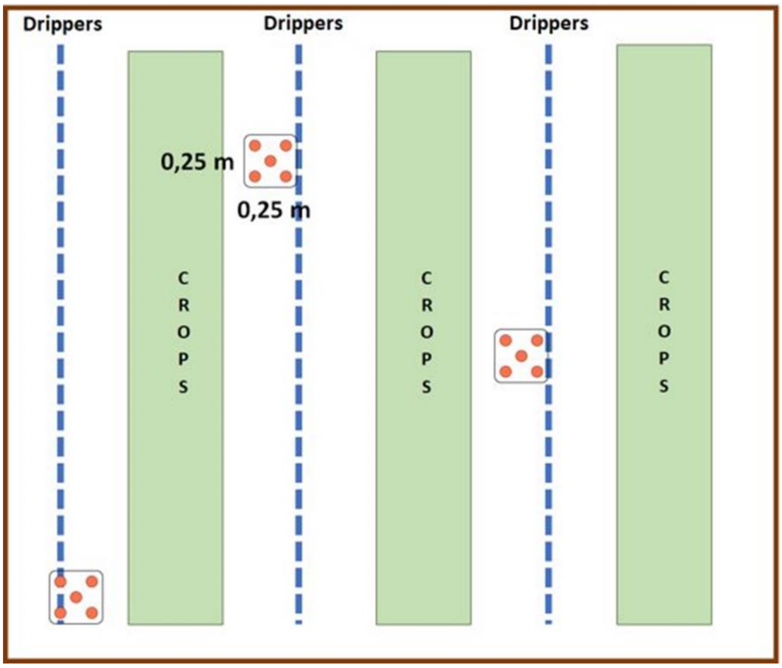

Figure 4.2. Scheme of soil sampling in a studied plot. The blue striped line represents the irrigation line and the green squares the position of the crops. The location for the collection of 3 replicates of a study plot is represented by the white squares, and the orange dots indicate the subsamples.

b) Moisture and soil organic matter

The soil organic matter (SOM) content was determined by loss of ignition (Albers et al., 2009). Briefly, 2 g of each sample were weighted and dried overnight (105ºC) to determine the moisture. Afterwards, the samples were heated to 550ºC for 2 h. The SOM% was obtained according to the equation below:

$$SOM\ (\%) = \frac{Soil\ (550^{\circ}C) mass\ (g)\ x\ 100}{Soil\ initial\ mass\ (g)}$$

c) Organic micropollutants extraction and analysis in soils

Freeze-dried samples were sieved with a 2 mm sieve. Samples were stored at -20ºC until further processing. The analytical procedures were performed according to Gros et al. (2019). Briefly, 1 g of freeze-dried solid was sequentially mixed with buffer McIlvaine and MeOH, followed by an ultrasound cycle and centrifugation. The procedure was repeated three times in total, and the supernatant fractions were combined, mixed with EDTA 0.1 N solution and diluted to 200 mL. The solutions were filtered and finally purified by SPE, with Oasis Accell™ Plus QMA (500 mg, 6 mL) cartridges in tandem with Oasis HLB (200 mg, 6 mL) cartridges. The cartridges were eluted with MeOH, and the extracts were spiked with a 50µL of a 1000 ng/µL mix solution of isotopically labelled standards. The solvent was evaporated, and the sample was reconstituted in 1 mL of a mixture of MeOH/HPLC water (1:1, v/v).

Pharmaceuticals

The analysis of pharmaceuticals in the soil extracts was performed with a Waters Acquity Ultra-Performance™ liquid chromatography system coupled to a 5500 QTRAP hybrid triple quadrupole-linear ion trap mass spectrometer (Applied Biosystems, Foster City, CA, USA) with a turbo Ion Spray source. The pharmaceuticals were analysed in positive and negative ionization modes (Castaño-Trias et al., 2023):

- Positive ionization mode: chromatographic separation was achieved using an Acquity HSS T3 column (50 × 2.1 mm i.d., 1.8 µm particle size, Waters Corporation). The mobile phase consisted of ACN as A and 0.1% formic acid in MS grade water as B.
- Negative ionization mode: an Acquity BEH C18 column (50 × 2.1 mm i.d., 1.7 µm particle size) was used for the chromatographic separation. ACN was selected as eluent A and 5mM ammonium acetate/ammonia (pH 8) as eluent B.

The quantification of analytes was performed by SRM by monitoring two mass transitions between the precursor ion and the most abundant fragment ions for each compound. The one at higher intensity was used for quantification purposes, while the second one was used for confirmation of the compound identification. Data acquisition and processing was performed with Analyst 1.5.1 software.

A total of 54 pharmaceuticals were analysed in soils and the analytical quality parameters can be found in the "ANNEX SOILS". The limits of detections for the whole set of analytes ranged from 0.01 to 1.4 ng/g (dry weight, d.w.). Recovery tests were performed for each treatment plot (CT, FT, PT), with values ranging from 20 to 148%. Analytes with recoveries lower than 20% were not considered for the analysis.

Endocrine disrupting chemicals (EDCs)

A second UHPLC-MS/MS methodology for the analysis of EDCs in the soil extracts was performed according to Becker et al. (2017). For the chromatographic separation, an EQuan MAX Plus chromatographic system (Thermo Fisher Scientific), which comprises an Accela Open AS auto sampler and two mixing quaternary pumps (eluting pump and loading pump) was used. For the detection of the analytes, the system was coupled to a TSQ Vantage triple quadrupole mass spectrometer, equipped with an electrospray ionization source (Thermo Fisher Scientific). Chromatographic separation was achieved using an Acquity UHPLC BEH C18 column (50 x 2.1 mm i.d.; 1.7 µm particle size). The mobile phase for both ionization modes (positive and negative) consisted of MeOH (A) and water MS grade (B). For both ionization modes, the acquisition was performed in SRM. Data acquisition and processing was performed with Xcalibur 2.2 software.

A total of 10 EDCs, including bisphenols (BPs), were considered for the soil analysis (analytical quality parameters provided in the "ANNEX SOILS"). The limits of detections for the whole set of analytes ranged from 0.01 to 0.11 ng/g (dry weight, d.w.). Recovery tests were performed for each treatment plot (CT, FT, PT), with values ranging from 20 to 80%. Analytes with recoveries lower than 20% were not considered for the analysis.

d) DNA extraction and analysis of antibiotic resistance genes (ARG)

Soils samples were thawed, and DNA was extracted using a commercial kit (DNeasy Power Soil Kit, Qiagen) and quantified using NanoDrop 2000 (Thermo Scientific, Wilmington, DE). Real-time polymerase chain reaction (qPCR) assays were performed to quantify the abundance of *intI1 (*1 integron-integrase), as a tracer of anthropogenic pollution and horizontal gene transfer, and several genes encoding resistance to the main antibiotic groups used in human (*sul1, ermB).* Copy numbers of 16s rRNA gene were applied to normalize the copy numbers of the ARG. Based on their DNA concentration, serial dilutions of the extracts were made to prepare the qPCR standard curves, ranging from 10^9 to 10^2 gene copies per ll. Quantification was performed following the conditions described in Subirats et al. (2017). For the qPCR assays, SYBR green detection chemistry was used, on a MX3005 system (Agilent Technologies; Santa Clara, CA, USA), as described in Marti et al. (2013). Standard curves and negative controls were included in each run, and the samples were analyzed by duplicate. Specificity of amplification was determined by analysis of the melting curves and gel electrophoresis of amplified products.

4.1.2 Results

a) Soil organic matter

The results of the soil organic matter (SOM) analysis are presented in Figure 4.3. A detailed overview of the results of SOM and moisture is presented at the “Soil parameters” tab in the "ANNEX SOILS”. An analysis of variance was conducted to examine the variations in SOM content among different treatments. No significant variations were found between the fall and summer campaigns. The findings revealed significant differences ($p<0.05$) in SOM levels between PT soils and the other treatments across all samples and seasons. This discrepancy could be attributed to the fact that it was the first time that PT soils were utilized for agricultural purposes, whereas CT and FT soils had previous cultivation history, which includes fertigation periods.

Among CT and FT treatments, CT soils exhibited a higher initial SOM content ($p<0.05$) in both seasons. However, no significant differences were observed at the final time of sampling (harvesting). The input of reclaimed water in the FT soils may have contributed to an increase in organic matter content after 7 weeks of irrigation.

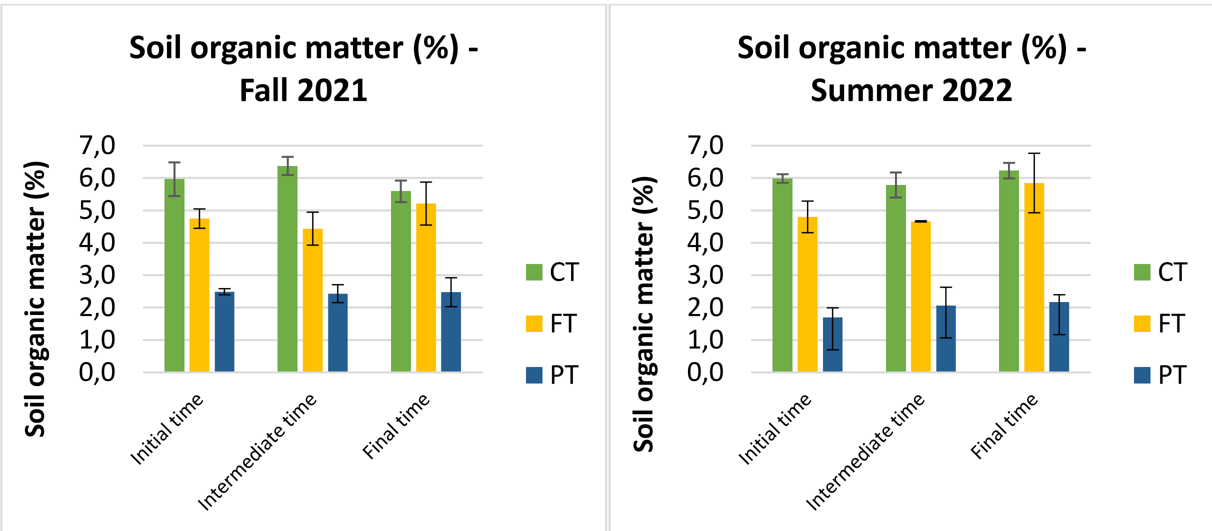

Figure 4.3. Soil organic matter (SOM) in the fall 2021 and summer 2022 campaigns. The 3 soil treatments are grouped by sampling time.

b) Organic micropollutants and EDCs

Soils from the HYDRO2 system, irrigated with reclaimed water provided by HYDRO1, were analysed in two sampling campaigns (Fall 2021 and Summer 2022). The summarized results of pharmaceutical classes and the EDCs are shown in Figure 4.4. For a detailed overview of the concentration data for each sample and the analytical quality parameters for each compound, please refer to the excel file “ANNEX SOILS” in the corresponding tab for each campaign (“Fall 2021 ng-g” and “Summer 2022 ng-g”).

1st campaign: fall 2021

Soils irrigated with all three water treatments exhibited the presence of the studied compounds. Caffeine was detected in all samples. At the initial time, before irrigation started, the analgesics and anti-inflammatories (NSAIDs) therapeutic class comprised most of the pharmaceuticals mass load detected in the season. The ibuprofen metabolite, 2OH-ibuprofen (max: 5.75 ng/g d.w.), was detected in the 3 irrigated plots at the initial time. At the initial time, acetaminophen was not found in the CT soils, but it was detected in the FT (13.6 ng/g d.w.) and PT plots (3.52 ng/g d.w.). Other compounds detected in the initial time were the antihypertensive irbesartan (1.6 ng/g d.w) and the diuretic hydrochlorothiazide (0.67 ng/g d.w.), which are usually prescribed together as a formulation to treat high blood pressure. The pair was also found at the intermediate and final time for the FT plots at the same order of magnitude. Similarly, it was observed for the psychiatric drug carbamazepine (0.45 ng/g d.w.). For the PT-plot, another antihypertensive, losartan (0.19 ng/g d.w.) was found. Regarding the bisphenols, both BPA and BPS were detected at the initial time in all 3 plots. BPS was found at higher levels than BPA in the CT and FT plots, although it has been reported to exhibit less retention in soils than BPA (Shi et al.2019). The levels detected in the CT soils were higher than those detected in the soils irrigated with the reclaimed waters (FT and PT). The retention of both BPA and BPS in soils was positively correlated to the SOM content (Shi et al., 2019), and for the studied plots, higher levels of SOM (Figure 4.3) were found for the CT soils. As for the rest of studied EDCs, only the parabens were detected at the initial time (when lettuce was planted), with methylparaben occurring at the 3 studied plots, but propylparaben only in the PT soils.

In the intermediate time (2 weeks after planting), a similar profile of compounds was found for the 3 plots. A decrease of the total mass load of pharmaceuticals was observed for CT, mainly due to the lower concentration of BPs. Acetaminophen was detected in the CT soils, at a lower concentration than those found in the FT and PT ones. Erythromycin, a macrolide antibiotic, was found in the FT soils (1.30 ng/g d.w.) at the intermediate time, being the only antibiotic detected at this campaign.

At the final time (coinciding with lettuce harvesting), acetaminophen and the metabolite 2-OH-ibuprofen were the most abundant pharmaceuticals in all 3 plots. The 3 pharmaceuticals previously detected at the FT soils were found again at the final time: carbamazepine, one of the most reported pharmaceuticals in soils irrigated with reclaimed water (Mordechay et al., 2021), and the pair irbesartan/ hydrochlorothiazide. Two compounds occurred for the first time: the β-blocker metoprolol and the psychiatric drug paroxetine were detected in the FT and PT soils, respectively, at the final time. Methylparaben and the BPs were detected at similar levels than in the previous sampling times, while propylparaben was no longer detected.

2nd campaign: summer 2022

In between both campaigns, the agroforestry system was irrigated for the growth of other crops (lavender and oregano). However, it was a discontinuous regime due to the occurrence of heavy rains and snow falls during the period. The second lettuce campaign started on 19/06/2022. The new batch of lettuces were planted on the same plots as in the 2021 campaign.

A greater spectrum of analytes and higher concentrations were found in all 3 treated plots during this campaign, with each soil displaying a different profile of compounds. NSAIDs comprised most of the mass load detected at the CT plots, while a more diversified profile was found for the reclaimed water plots (FT and PT). As observed in fall 2021 (the previous season), 2-OH-ibuprofen and acetaminophen exhibited in summer 2022 the highest concentrations, followed by diclofenac and ibuprofen, which were not detected previously in these soils. The antibiotic ofloxacin was found at the initial sampling of the summer campaign in all 3 plots, while erythromycin was only detected in the soils receiving reclaimed water (FT and PT). Overall, the concentrations found in the FT plot were higher than those detected in the other plots. Among the possible causes, the residence time of the reclaimed water from the PT treatment in the tank (over 15 days) may have influenced the *in-situ* dissipation of the micropollutants in the tank. Also, the higher SOM in the FT soils (compared to the PT) might have enhanced the sorption and retention of most of the pollutants. The pair irbesartan/hydrochlorothiazide comprised a great fraction of the mass load from the FT soil. Low concentrations were found for β-blockers (0.34 – 3.0 ng/g d.w.), with several detections below the LOQ. For the non-pharmaceuticals, BPs and parabens were found, with the highest levels for BPS and methylparaben, respectively. Other compounds such as 1H-benzotriazole (a versatile substance, mainly used as an anticorrosive) and the hormone estrone were detected in all 3 soils.

In contrast to observations in fall 2021, the pharmaceutical profiles in the intermediate and final sampling times of this second campaign were also different among the three soil types (Figure 4.4). A similar pattern was found for the CT soils (dominated by analgesics and anti-inflammatories) during the summer 2022 campaign, just as observed in fall 2021. However, the total mass load at the intermediate and final times of summer 2022 was approximately 5.2-5.5 times higher than in the first sampling campaign. Conversely, both FT and PT plots showed different behaviours, mainly due to the greater numbers of compounds detected at all the sampling times, compared to CT. Although the detected analytes at the final time for FT and PT were 28, their concentrations led to a significant difference between both soils, with the total mass load of pharmaceuticals in the FT being 2-3 times higher than those in the PT plots (Figure 4.4). The main contribution to this difference is due to the presence of hydrochlorothiazide and the large increase in antihypertensives drugs. Hydrochlorothiazide showed the highest concentration of the study at the three studied times, in the FT, with a maximum of 68.6 ng/g (d.w.) at the intermediate time, followed by irbesartan, whose concentration increased from 14.0 to 59.4 ng/g (d.w.) from the intermediate to the final sampling. Hydrochlorothiazide has a low expected sorption behaviour in terms of its organic carbon-water partition coefficient K_{oc} (K_{oc} = 12), but it has been reported to sorb strongly in soils with a clay content > 20% (in the range of the studied soils from this work). Also, the persistence of this diuretic in soils has been reported (Biel-Maeso et al., 2019). Irbesartan has a high K_{oc} (K_{oc} = 7700), which indicates an expected sorption into soil (enhanced by the higher organic matter in the FT soil), favouring its retention but with a low expected bioavailability. Also, a significant increase was found for other pharmaceutical classes in the soils irrigated with reclaimed waters (FT, PT): the psychiatric drugs, mainly due to the presence of anticonvulsive carbamazepine (ubiquitous along the summer 2022 campaign), venlafaxine and its metabolite O-desmethyl-venlafaxine; and at a lower concentration, the β-blockers.

As for the BPs and EDCs, no significant variations were observed in their concentrations, with BPS, 1H-benzotriazole, estrone and methylparaben at the same range of concentrations throughout the lettuce growth period.

This project has received funding from the European Union's Horizon 2020 Research and Innovation Programme under Grant Agreement No 776643

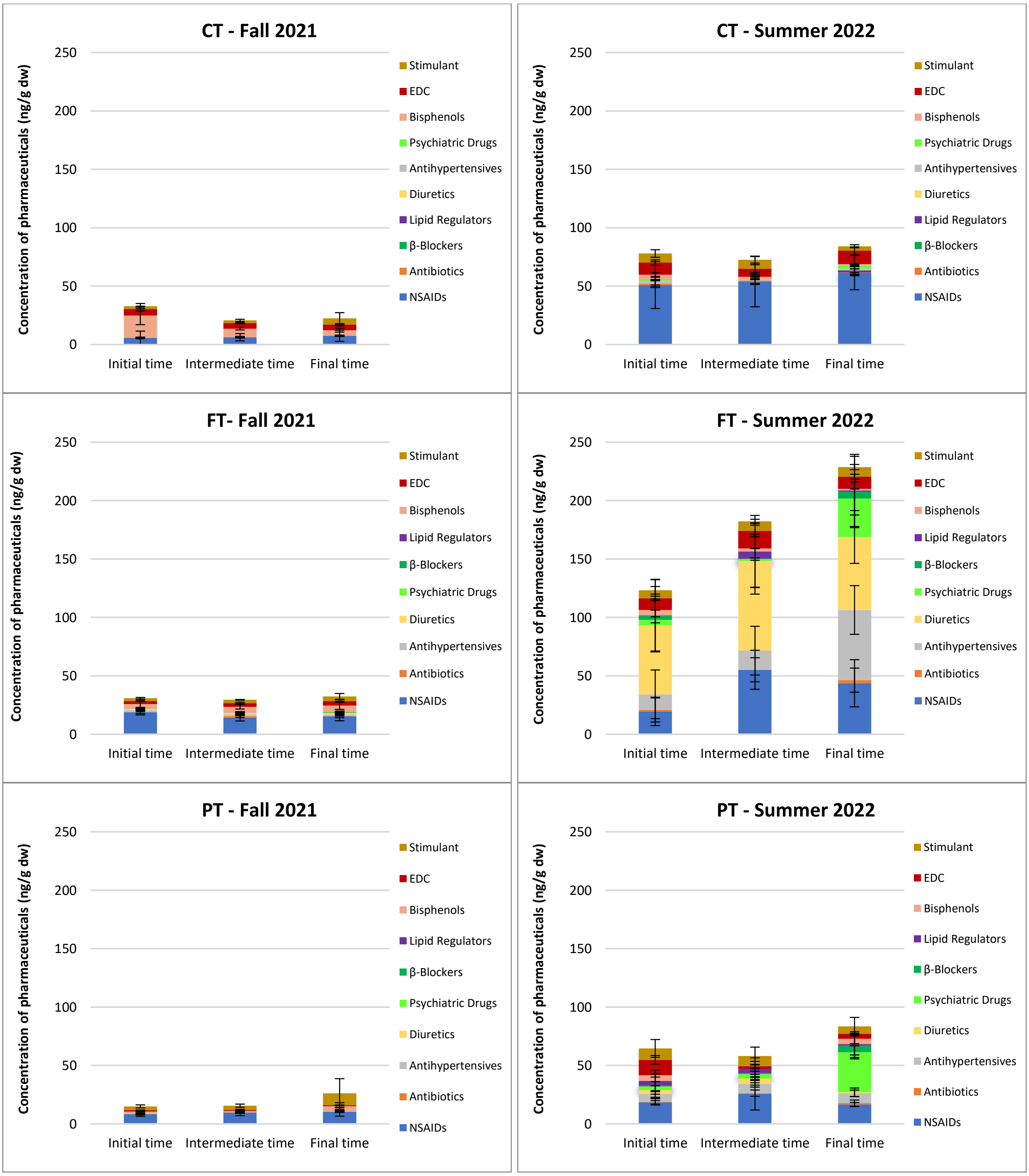

Figure 4.4. Sum concentrations (ng/g, dry weight - d.w.) of the studied pharmaceutical classes found in the soils irrigated with the CT - control treatment FT - full treatment and PT - partial treatment. On the left, results from the 1st sampling campaign (fall 2021). On the right, results from the 2nd sampling campaign (summer 2022).

4.1.3 Results: antibiotic resistance genes

The number of copies of 16s rRNA is presented in Figure 4.5. The abundance of 16s rRNA ranged from 2.86 x 10^6±1.1 x 10^6 to 5.98 x 10^7±1.27 x 10^7 (fall 2021) and 2.88 x 10^7±6.34 x 10^6 to 8.36 x 10^7±6.54 x 10^6 (summer 2022). A higher detection of the 16s rRNA gene was found in the summer 2022 campaign for most of the treatments, except the final time of CT soils. In the fall season, the abundance of the 16s rRNA increased in the final time for all 3 plots. Instead, no pattern was observed during the summer season (Figure 4.5).

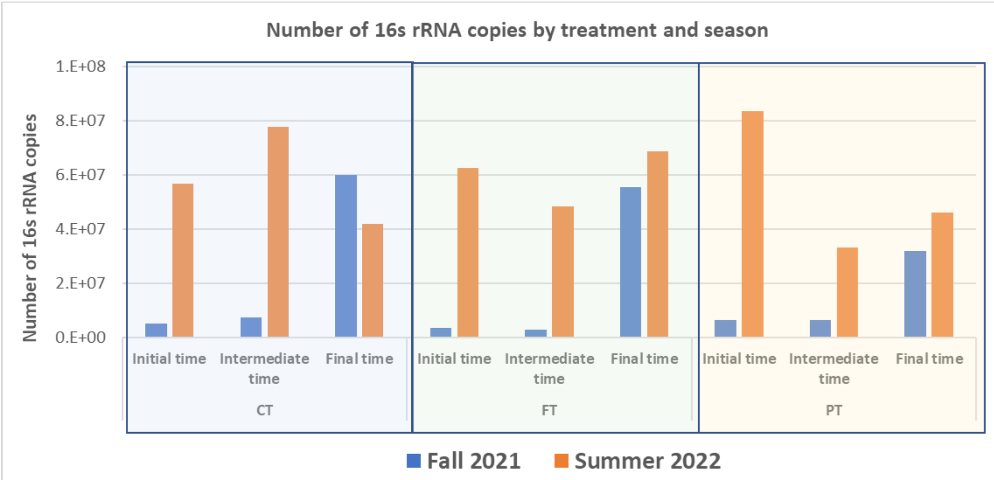

Figure 4.5. Abundance of 16s rRNA gene in the soil samples from the different plots.

For the analysis of 16s rRNA, serial dilutions of the samples were tested in an inhibition test, to assess the correct concentration for the qPCR. Due to the intense effect of inhibition found in the sample, 1:50 and 1:70 dilutions had to be applied. The same pattern of dilution was applied for the rest of the genes, but unfortunately, the combination of a low sensitivity of the analysis and a low quantity of ARG in the sample, led to a detection of less than 1000 copies for common ARGs as sul(I) and erm(B). As a control, the mobility integron (int1) was also analysed, and the same effect was found.

For qualitative purposes only, the results of ARG found after the analysis are shown in Figure 4.6. It can be observed that the detection of int1 was not consistent in the different samples, as well as the detection of sul(I). As for erm(B), it was found at t1 and tf of the PT plots for both campaigns, with a higher occurrence in the fall 2021 season.

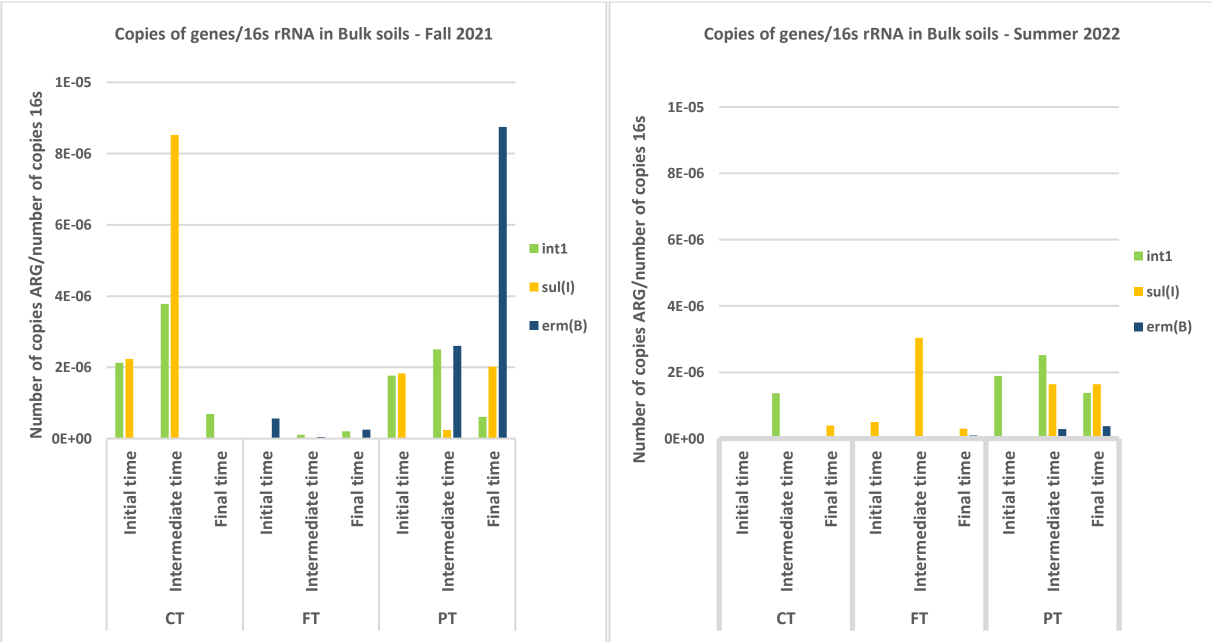

Figure 4.6. Abundance of the studied genes (int1 and the ARGs sul(I) and erm(B)), normalized by the abundance of the 16s RNA gene, in the fall 2021 campaign (up) and summer 2022 campaign (down).

4.2 Emerging pollutants and heavy metals in crops

4.2.1 Methodology

a) Crops sampling

Immediately after harvest, the crops were separated into leaves and roots, both rinsed with NaCl solution to remove soil particles and surface microbes, air-dried, and stored at -20°C until shipping. Upon receipt, samples were freeze-dried and stored at -20°C until analysis.

b) Micropollutant extraction and analysis in crops

Sample preparation involved extraction by QuEChERS and dSPE PSA-C18 clean-up (adapted protocol, Montemurro et al., 2020). In short, 1 g of freeze-dried and milled crop leaves was placed in a 50 mL falcon tube and hydrated with 9 mL HPLC water. The tubes were vortexed for 2 min at 2500 rpm and left to hydrate for 1 h. 10 mL of acetonitrile and 50 µL of formic acid were added in the tubes, vortexing was repeated and the extraction salts (1 g NaCl and 4 g $MgSO_4$) were added in the tubes. The mixture was instantly shaken to prevent crystalline agglomerates formation. Tubes were vortexed as before and centrifuged at 4°C for 10 min at 4000 rpm. The supernatant, containing the organic phase, was transferred into glass tubes, and left overnight at -20°C for the precipitation of fatty acids and waxes. The following day, the clean-up step involved the transfer of 6 mL of the supernatant into the PSA (primary secondary amine) tubes (150 mg PSA, 150 mg C18, 900 mg $MgSO_4$) and the mixture was vortexed and centrifuged at 4°C for 5 min. The same process was followed for the extraction of roots, replacing the hydration step with EDTA solution instead of water, omitting the formic acid addition and the clean-up step. For all samples, 1 mL of the supernatant was spiked with the internal standard mix at a concentration of 20 µg/L, the sample was evaporated until dryness under nitrogen at room temperature and then reconstituted with 1 mL of water/20% methanol solution. To remove any possible particles formed from precipitation, a final centrifugation step at 7000 rpm for 10 min was added and the samples were injected for UHPLC-MS/MS analysis.

<u>Pharmaceuticals analysis</u>

The analytical parameters for the pharmaceuticals and antibiotics analysis are described in section 4.1. A total of 88 micropollutants were analysed in lettuce, oregano, and lavender; 49 pharmaceuticals, 15 antibiotics, 6 BisPhenols (BPs) and 18 Endocrine-Disrupting Chemicals (EDCs). The detailed analytical quality parameters for each compound and matrix can be found in the document ''ANNEX CROPS''.

<u>Endocrine disrupting chemicals (EDCs) analysis</u>

A UHPLC-MS/MS methodology for the analysis of EDCs was performed according to Turull et al. (2023). A Waters Acquity Ultra-PerformanceTM liquid chromatography system, equipped with two binary pumps systems (Milford, MA, USA) and coupled to a 5500 QTRAP hybrid triple quadrupole-linear ion trap mass spectrometer (Applied Biosystems, Foster City, CA, USA) with a turbo Ion Spray source was used. Chromatographic separation was achieved using a Kinetex Biphenyl column (50 x 2.1 mm i.d.; 2.6 µm particle size) equipped with a pre-column. For positive ionization mode (PI), the mobile phase consisted in methanol (A) and water MS grade (B), while for negative ionization mode (NI) was used methanol (A) and water MS grade pH 9 (adjusted with ammonia) (B). For both ionization modes, the flow rate was 0.4 mL/min and a total run of 8 min; the elution gradient was as follows: 0-5 min, 20-100% A; 5-6 min, 100% A; 6-6.5 min, return to initial conditions; 6.5-8 min, equilibration of the column. The column temperature was set at 40 ºC and an injection volume of 5 µL was used. The mass spectrometry parameters consisted in the following conditions: in both ionization modes, target scan time (TST) was set at 1 s, with a SRM detection window of 60 s; the resolution at the first quadrupole (Q1) was set at unit, and the third quadrupole (Q3) was set at low, and the pause between mass ranges was 5 ms. The settings for source-dependent parameters were set-up as follows: for PI mode, curtain gas, 30 V; Nitrogen collision gas medium; source temperature of 600 ºC; ion spray voltage at 5000 V; ion source gases GS1 and GS2 set at 60 and 40 V, respectively. For NI mode, such parameters were: curtain gas, 30 V; Nitrogen collision gas medium; source temperature of 600 ºC; ion spray voltage at -3000 V; ion source gases GS1 and GS2 set at 60 and 40 V, respectively. The entrance potential was set at 10.

All data were acquired and processed using Analyst 1.5.1 software. In most cases, the quantification of analytes was performed by SRM by monitoring two mass transitions between the precursor ion and the most abundant fragment ions for each compound. The one at higher intensity was used for quantification purposes, while the second one was used for confirmation of the compound identification. For seven compounds – norfloxacin, acetaminophen, levonorgestrel, salicylic acid, ibuprofen, 2-OH-ibuprofen and ketoprofen – the molecule gives only one dominant fragment, and in those cases only that fragment is used for quantification purposes.

c) Heavy metal analyses in crops

During the 2nd sampling campaign, plants from the FT and CT plots were analysed for selected heavy metals (Zn, Cu, Fe, Mn, Cr, Pb, Cd, Ni). The heavy metals were analysed in roots and leaves of lettuce and oregano, as well as in flowers and roots of lavender, following Method D (Asher et al., 2020). All the samples were washed with high-quality reagent water to eliminate impurities, dried for 48 h at 60°C and finally grounded in a blender. Approximately 2 g of dried powdered samples of lettuce, oregano, and lavender were weighed into 250 mL Erlenmeyer flasks and the exact weight was recorded. In the same flasks 7 mL of newly produced acid mixture (HNO3: H2SO4: HCl (5:1:1)) is added, and the volume is increased to 50 mL with distilled water. The mixture was allowed to gradually boil on a heated plate (at 100 °C) for three hours. After cooling, solutions were filtered using Whatman membranes 0.45 µm made up again to 50 mL. The metal content of Cu, Pb, Ni, Cd, Cr, As, Zn, Fe was determined using an absorption spectrophotometer coupled to a graphite furnace (Model Z900 Pinnacle, Perkin Elmer).

4.2.2 Organic micropollutants results in crops

The results from the OMPs in all three crops and for both campaigns are presented in terms of micropollutant classes and are discussed as such below (Figure 4.7). A total of 88 OMPs were analysed in all samples and at least 71, 66 and 68 of them presented sufficient recoveries in lettuce, oregano and lavender, respectively, in both campaigns and at least in one of the two parts (leaves/roots) of the crop. The number of compounds actually detected in each crop for the fall campaign were 14, 13 and 4, for lettuce, oregano and lavender, respectively, and for the summer campaign 15, 11 and 14, respectively. Averaging the two campaigns, for all three crops, 52-70% of the OMPs detected were retained exclusively in the roots, with 24-32% exclusively in the leaves and 4-16% retained in both. Thus, it becomes clear that the majority of OMPs found in all cases is preferentially retained in the roots, the non-edible part of the crops.

The recoveries and limits of detection (LODs) levels for each crop and plant part (leaves/roots) can be found in Table 4.1. For a detailed overview of individual concentration data, please refer to the excel file ''ANNEX CROPS'' in the corresponding tabs for each crop and campaign.

Table 4.1. Recoveries (RCs) and limits of detection (LODs) for each crop, plant part and campaign.

	Roots		Leaves	
	Recovery (%)	LOD (ng/g d.w.)	Recovery (%)	LOD (ng/g d.w.)
FALL 2021				
LETTUCE	24-100	0.03-3.5	22-100	0.04-9.1
OREGANO	23-100	0. 11-13.5	20-100	0.16-13.7
LAVENDER	23-100	0. 11-13.5	21-100	0.06-13.7
SUMMER 2022				
LETTUCE	21-100	0.11-13.5	20-100	0.15-7.5
OREGANO	21-100	0.03-8.2	22-100	0.2-13.7
LAVENDER	21-100	0.03-8.2	22-100	0.02-13.7

Lettuce

1st campaign: Fall 2021

When lettuce was irrigated with tap water (CT), the compounds detected in the **leaves** were analgesics/anti-inflammatories (ibuprofen, naproxen) and the calcium channel blocker furosemide (3.6 – 37 ng/g d.w.). Naproxen was the only compound detected when reclaimed water (FT) was used for irrigation and no compound was above detection limit when reclaimed water from the partial treatment (PT) was used.

Irrigation of lettuce with reclaimed water (FT) resulted in a wider variety of compounds and in higher levels in **roots** (2-150 ng/g d.w.): the psychiatric drugs lorazepam, the antihypertensive irbesartan, the calcium channel blocker hydrochlorothiazide, and four β-blockers (atenolol, metoprolol, metoprolol acid, sotalol). Small amounts of EDCs (TBEP, progesterone, estrone) were detected only in the roots when either tap water (CT) or reclaimed water from the partial treatment (PT) were used (3.3-18.2 ng/g d.w.).

Overall, NSAIDs and calcium channel blockers were detected in both leaves and roots, whereas antihypertensives, β-blockers, psychiatric drugs and EDCs were retained exclusively in the roots. The only time that the calcium channel blockers furosemide and hydrochlorothiazide were detected in this study was in the lettuce plants of the fall campaign.

2nd campaign: Summer 2022

When the sampling was repeated the following summer, irrigation with tap water (CT) resulted in a few compounds in the lettuce **leaves**, with acetaminophen found in all three treatments, and its metabolite 2-OH-ibuprofen found in the FT treatment. Small amounts of sotalol, progesterone and TBEP were also detected across the three treatments (4.0-27.9 ng/g d.w.).

Ibuprofen and ketoprofen were detected in the lettuce **roots** across treatments (with FT levels higher than the rest). Similar to the first campaign, psychiatric drugs (venlafaxine) and β-blockers (atenolol, metoprolol, metoprolol acid, sotalol) appeared exclusively in the roots, in higher levels in FT than PT treatment by 2.2 times. Venlafaxine exhibited an exceptionally high concentration in the FT case (1072.9 ng/g d.w.) with an almost 5x lower concentration for PT. BPs and EDCs were found in the roots as well, in the range of 5.4-36.8 ng/g (d.w.) across the treatments.

Oregano

1st campaign: Fall 2021

When oregano was irrigated with tap water (CT), only the NSAID ketoprofen and the EDC tolyltriazole were detected in the **leaves** (35.4 and 12.2 ng/g d.w., respectively). The amount of ketoprofen doubled in the FT treatment, where a small amount of azithromycin was also detected. Irrigation with PT treated water resulted in 47.8 ng/g (d.w.) of ketoprofen and 1.2 ng/g (d.w.) TBEP. In summary, ketoprofen was the only dominant micropollutant in the leaves of oregano in the fall campaign, with small amounts of EDCs present as well.

Apart from ketoprofen that was found in all three treatments for **roots**, two more NSAIDs, ibuprofen and its metabolite 2-OH-ibuprofen, were detected in the roots irrigated with tap water (CT). Irrigation with FT and PT water resulted in doubled levels of 2-OH-ibuprofen compared to CT, whereas ibuprofen was absent, indicating its conversion to its metabolite. As with the lettuce, psychiatric drugs (carbamazepine, venlafaxine) and β-blockers (atenolol, metoprolol acid, sotalol) appeared exclusively in the roots. The psychiatric drugs were detected also in the PT case, whereas β-blockers were the most dominant compounds and were present only for the FT, in the range of 215.4-670.5 ng/g (d.w.). The total concentration of all compounds quantified in FT was 6.6x the one for PT and 4.5x the one for CT. Small amounts of TCEP were also found in the CT and FT cases in the roots (41.0-59.4 ng/g d.w.).

2nd campaign: Summer 2022

Irrigation of oregano with tap water (CT) in the summer campaign resulted in only 2 compounds across all three treatments in the oregano **leaves**: ketoprofen and the EDC levonorgestrel (29.0-161.0 and 82.2-139.3 ng/g d.w., respectively, across treatments).

Fewer compounds but a wider variety of compounds was found in the **roots**, compared to the fall campaign, where 2-OH-ibuprofen (NSAID), ofloxacin (antibiotic), carbamazepine (psychiatric drug), and tolyltriazole (EDC) were found across the three treatments, whereas hydrochlorothiazide (diuretic), metoprolol acid (β-blocker), and BPA (bisphenol) were detected only in the FT case. The total concentration of all compounds quantified in FT was 2x the one for PT and 4.5x the one for CT. The highest concentrations observed were for 2-OH-ibuprofen in all treatments (175.9-512.0 ng/g d.w.).

Lavender

1st campaign: Fall 2021

Fewer samples were processed for lavender, due to issues with the blooming of the plants in some cases, insufficient matrix in others, as well as the inability to process some root samples. Therefore, no roots from the FT treatment and no samples at all from the PT treatment were processed for this campaign. When lavender was irrigated with tap water (CT), only the antibiotic ciprofloxacin and BPA were detected in the **flowers** (68.3 and 39.2 ng/g d.w., respectively). FT treatment resulted in ciprofloxacin and carbamazepine detection (64.8 and 110.7 ng/g d.w., respectively). Tap water irrigation led to the uptake of ibuprofen and carbamazepine in the **roots**, with ibuprofen exhibiting the highest concentration for lavender overall in the fall campaign (646.8 ng/g d.w.).

2nd campaign: Summer 2022

Irrigation of lavender plants in the summer campaign with tap water (CT) resulted in ketoprofen, progesterone, and benzylparaben in the lavender **flowers**, with only progesterone detected in the FT. The highest concentration was attributed to benzylparaben (207.5 ng/g d.w.), and this was the only time where benzylparaben was detected in this study.

A wider range as well as levels of micropollutants were quantified in the **roots**, especially for the FT treatment, as observed for the other two crops for both campaigns. Ketoprofen and carbamazepine were found across the three treatments, whereas hydrochlorothiazide, venlafaxine, and the β-blockers (metoprolol, metoprolol acid, propranolol, sotalol) were found exclusively in the FT of the roots and dominated the root composition (318.0-852.2 ng/g d.w.). BPs and EDCs again had the lowest levels (7.0-74.8 ng/g d.w.). Total concentration of micropollutants for the FT was 6.4x and 5x higher than the PT and CT treatments, respectively.

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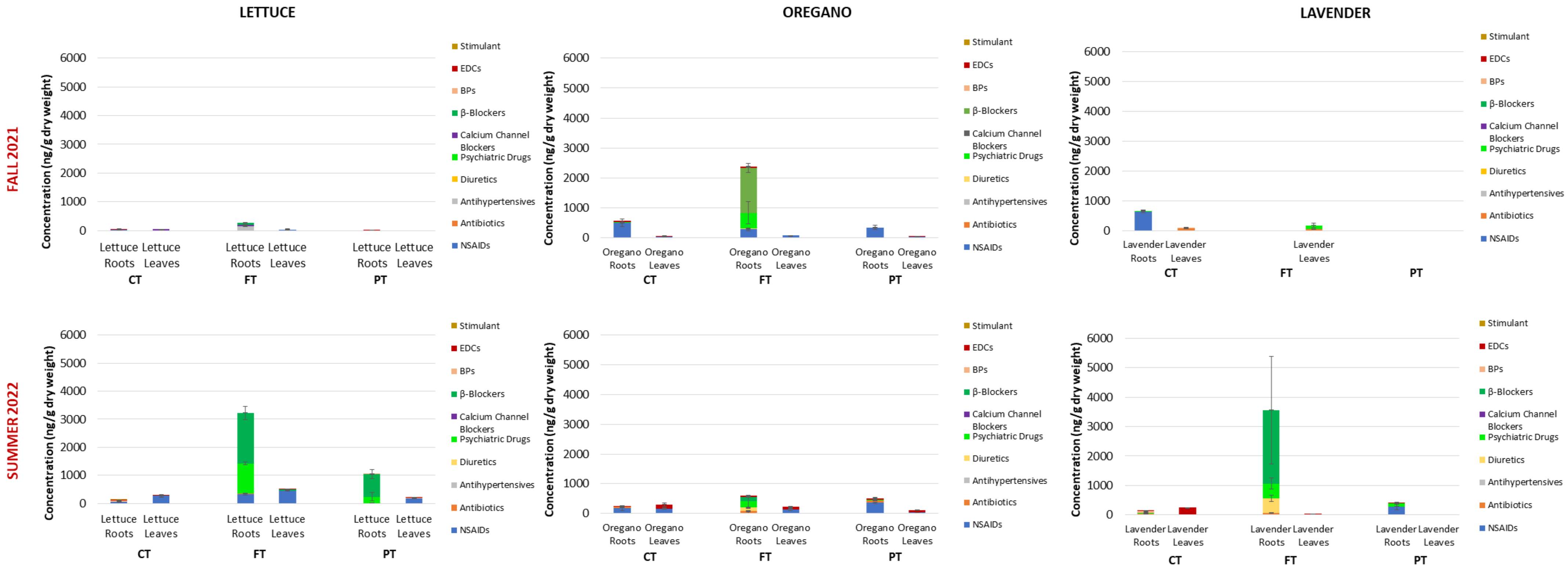

Figure 4.7. Concentrations (ng/g dry weight) of micropollutants recovered in at least one part of the crop and detected in lettuce, oregano, and lavender, for the fall (2021) and summer (2022) campaigns, irrigated with tap water (CT), fully-treated wastewater (FT) or partially-treated wastewater (PT), presented per class. A total of 71, 66, and 68 OMPs presented sufficient recoveries in lettuce, oregano, and lavender, respectively, in both campaigns and at least in one of the two parts (leaves/roots) of the crop. No lavender samples for the PT treatment and no lavender roots for FT treatment were collected.

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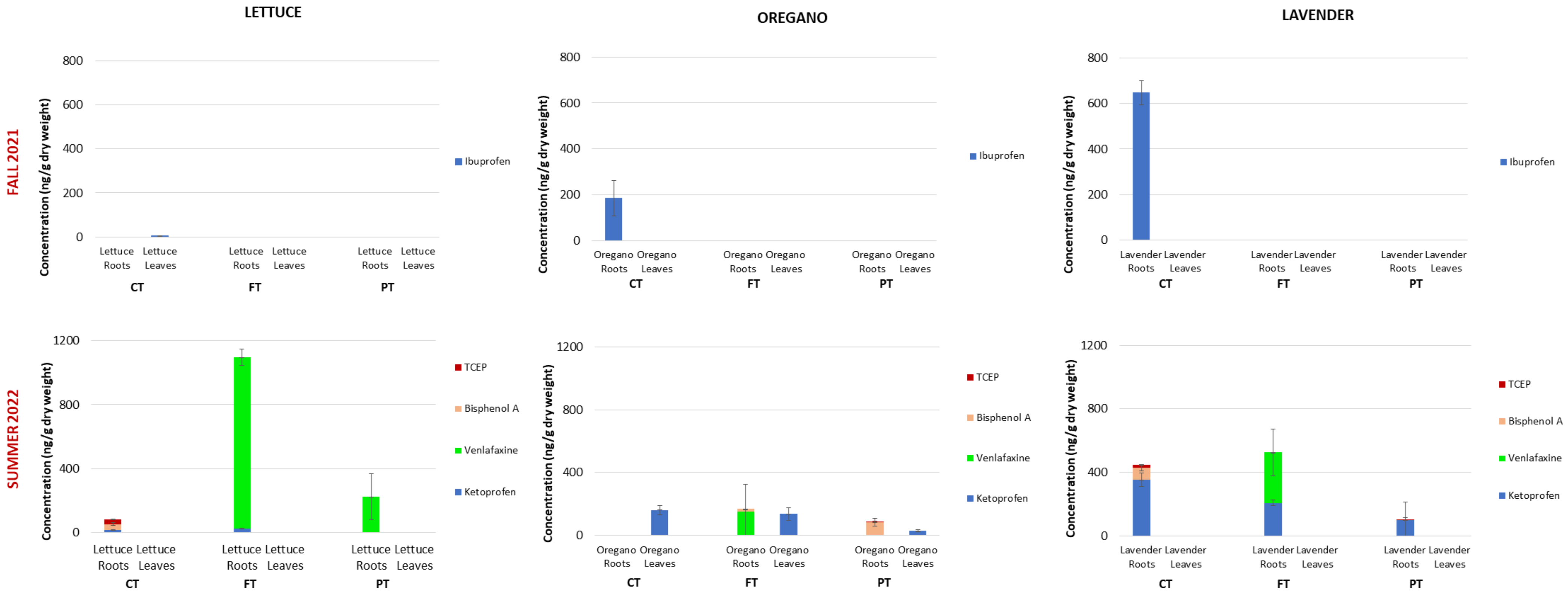

Figure 4.8. Concentrations (ng/g dry weight) of micropollutants recovered in at least one part of the crop and detected in all three crops (lettuce, oregano, and lavender), for the fall (2021) and summer (2022) campaigns, irrigated with tap water (CT), fully-treated wastewater (FT) or partially-treated wastewater (PT). A total of 71, 66, and 68 OMPs presented sufficient recoveries in lettuce, oregano, and lavender, respectively, in both campaigns and at least in one of the two parts (leaves/roots) of the crop. No lavender samples for the PT treatment and no lavender roots for FT treatment were collected.

4.2.3 Heavy metal results in crops

During the 2nd sampling campaign, plants from the FT and CT plots were analysed for selected heavy metals (Zn, Cu, Fe, Mn, Cr, Pb, Cd, Ni). The heavy metals were analysed in roots and leaves of lettuce and oregano, as well as in flowers and roots of lavender. The results for lettuce, lavender, and oregano are presented in Table 4.2 where all the concentrations provided for heavy metals are per dry weight and in Table 4.3 where the results are per wet weight.

Table 4.2. Heavy metals (as dry weight) in lettuce, lavender and oregano roots and leaves irrigated with tap water and fully treated water.

	Roots		Leaves	
	Control (tap water)	Fully treated water	Control (tap water)	Fully treated water
Lettuce				
Zn (mg/kg)	36	38	21	19
Cu (mg/kg)	20	15	6.8	5.3
Fe (g/kg)	4.6	8.5	77	137
Mn (mg/kg)	173	413	136	83
Cr (mg/kg)	20	35	0.24	0.20
Pb (mg/kg)	4.2	7.5	0.094	0.082
Cd (mg/kg)	0.17	0.25	0.34	0.27
Ni (mg/kg)	8.1	17	0.31	0.28
Lavender				
Zn (mg/kg)	35	32	25	28
Cu (mg/kg)	17	12	22	27
Fe (g/kg)	1.4	2.5	115	138
Mn (mg/kg)	77	92	25	31
Cr (mg/kg)	2.3	3.4	0.38	0.48
Pb (mg/kg)	1.7	2.1	0.15	0.15
Cd (mg/kg)	0.084	0.032	0.0064	0.0088
Ni (mg/kg)	4.3	5.2	0.9	1.2
Oregano				
Zn (mg/kg)	47	57	29	35
Cu (mg/kg)	19	18	12	13
Fe (g/kg)	6.7	4.2	174	397
Mn (mg/kg)	286	196	38	43
Cr (mg/kg)	8.8	7.0	0.56	0.64
Pb (mg/kg)	6.6	3.8	0.11	0.14
Cd (mg/kg)	0.088	0.13	0.0050	0.013
Ni (mg/kg)	8.2	4.7	1.3	1.4

Table 4.3. Heavy metals (as wet weight) in lettuce, lavender and oregano roots and leaves irrigated with tap water and fully treated water.

	Roots		Leaves	
	Control (tap water)	Fully treated water	Control (tap water)	Fully treated water
Lettuce				
Zn (mg/kg)	3.2	3.4	3.6	0.8
Cu (mg/kg)	1.8	1.4	0.3	0.2
Fe (g/kg)	0.4	0.8	3.1	5.5
Mn (mg/kg)	15.6	37.2	5.4	3.3
Cr (mg/kg)	1.8	3.2	0.0	0.0
Pb (mg/kg)	0.4	0.7	0.0	0.0
Cd (mg/kg)	0.0	0.0	0.01	0.01
Ni (mg/kg)	0.7	1.5	0.0	0.0
Lavender				
Zn (mg/kg)	12.3	11.2	9.5	8.5
Cu (mg/kg)	6.0	4.2	9.2	7.5
Fe (g/kg)	0.5	0.9	46.9	39.1
Mn (mg/kg)	27.0	32.2	10.5	8.5
Cr (mg/kg)	0.8	1.2	0.2	0.1
Pb (mg/kg)	0.6	0.7	0.05	0.05
Cd (mg/kg)	0.0	0.0	0.0	0.0
Ni (mg/kg)	1.5	1.8	0.4	0.3
Oregano				
Zn (mg/kg)	18.8	22.8	13.3	11.0
Cu (mg/kg)	7.6	7.2	4.9	4.6
Fe (g/kg)	2.7	1.7	150.9	66.1
Mn (mg/kg)	114.4	78.4	16.3	14.4
Cr (mg/kg)	3.5	2.8	0.2	0.2
Pb (mg/kg)	2.6	1.5	0.05	0.04
Cd (mg/kg)	0.0	0.1	0.0	<0.0050
Ni (mg/kg)	3.3	1.9	0.5	0.5

Lettuce (Lactuca sativa)

Figure 4.9 presents the results of the laboratory analyses regarding the heavy metals content (in dry weight) in the roots and leaves of lettuce of the plots being irrigated with full treated water - FT and tap water TW.

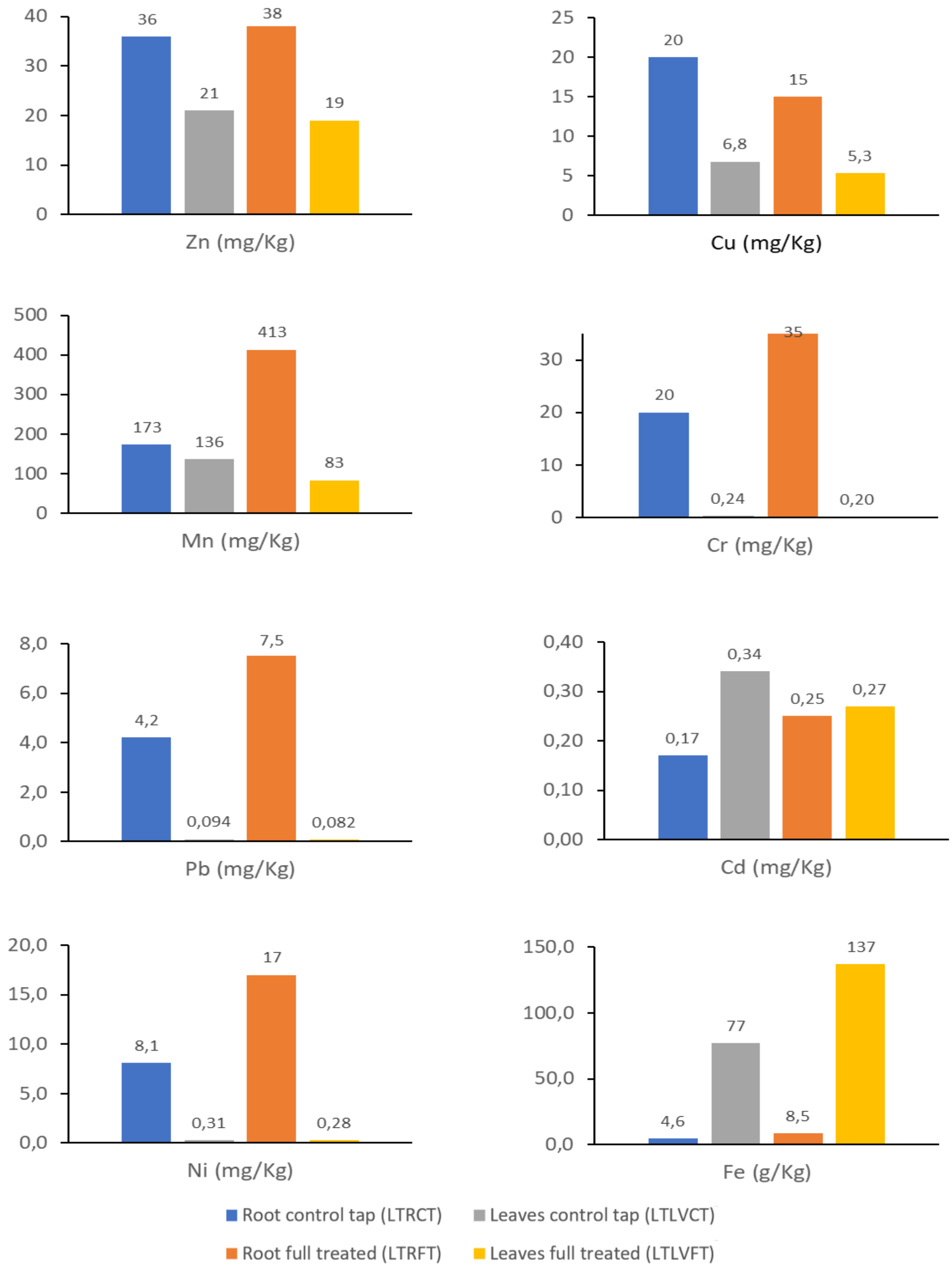

Figure 4.9. Heavy metals concentration (mg/kg dry solid) found in lettuce irrigated with tap water (CT) and full treatment water (FT)

Based on the results it is concluded that the roots of the lettuce irrigated with full treated water - FT contained higher concentrations of Zn, Mn, Cr, Pb, Cd, Ni and Fe compared to the roots irrigated with tap water - TW (increase to the order of 75-138%); on the contrary Cu concentrations were higher in the roots of plants irrigated with tap water.

This was not the case for the leaves of lettuce where heavy metals concentrations were comparable for both plots being irrigated with treated wastewater and tap water, with exceptions being Mn which was higher in the lettuce leaves irrigated with tap water (60%), and Fe which was higher in lettuce leaves irrigated with full treated water (77%).

In general, the concentrations observed in lettuce leaves concerning Ni, Cr and Pd are close to those mentioned by other studies (e.g. Pérez-Figueroa et al., 2023). Cu and Zn content was lower than the concentrations mentioned by Ferri et al. (2016), while Mn concentrations were higher. The values measured in the present study are comparable with the ones reported by Mensah et.al. (2009) for Pb, Ni, Fe, Cu and Zn.

Based on the results it is anticipated that Cr, Pb, and Ni are heavy metals that are mainly concentrated in the roots, while Cu, Mn, and Cd appear at similar concentrations in both the roots and leaves. Accordingly, Fe seems to be mostly concentrated in the leaves of lettuce.

Lavender (Lavandula spp.)

Figure 4.10 displays the results of heavy metal concentrations (in dry weight) in lavender crops and roots that were irrigated with full treated water and tap water.
The lavender roots irrigated with full treated water exhibit a higher concentration of Mn, Pb, Cr, and Ni (to the order of 14%) compared to the roots irrigated with tap water. However, the concentrations of Zn, Cu, and Fe in the roots are similar regardless of the irrigation water source.

In the case of lavender leaves, there is no significant difference in the concentrations of most heavy metals (Cu, Cr, Pb, Ni, Cd, Zn, Mn) between those irrigated with tap water and those irrigated with full treated water. The exception is Fe, which is approximately 12% higher in the lavender leaves irrigated with full treated water. The Pb values of the present study are lower than the concentrations mentioned by Serban et al. (2022), while Cd and Zn content is also lower compared to the concentrations mentioned by Angelova at al. (2015). Concerning the concentrations of Fe even though they were higher in the lavender leaves irrigated with full treated water the concentrations are still lower than the ones found by Sabina et al. (2019).

When comparing the roots and leaves, Mn, Cr, Pb, Cd, and Ni are heavy metals that are mainly concentrated in the roots, while Zn and Cu appear at similar concentrations in both the roots and leaves. Finally, Fe seems to be mainly concentrated in the leaves of lavender.

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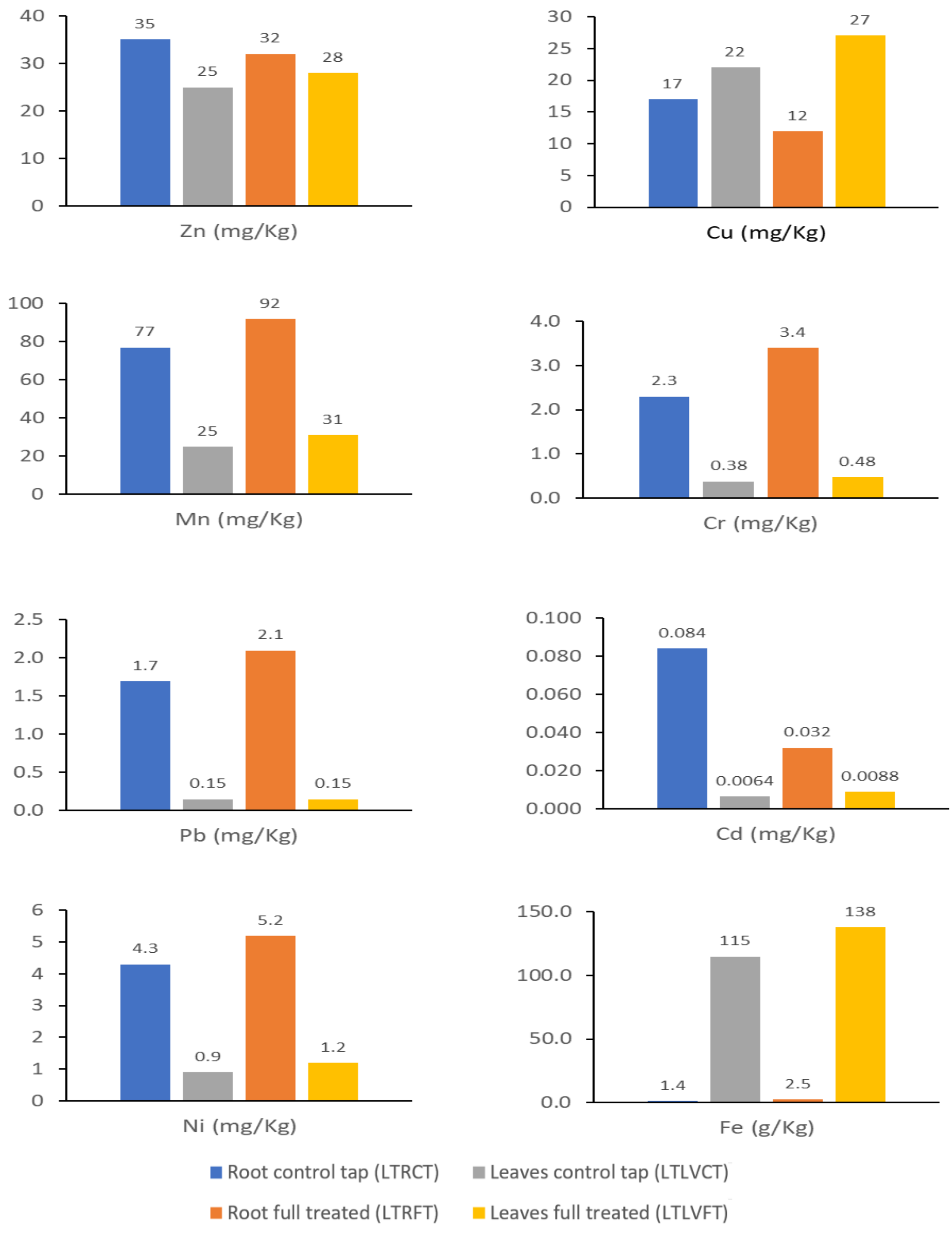

Figure 4.10. Metals concentration (mg/kg dry solid) found in lavender irrigated with either tap water (CT) and full treatment water (FT)

Oregano (Origanum Vulgare L.)

Figure 4.11 displays the results of the main heavy metals (in dry weight) in the roots and leaves of oregano that were irrigated with full treated water and tap water.

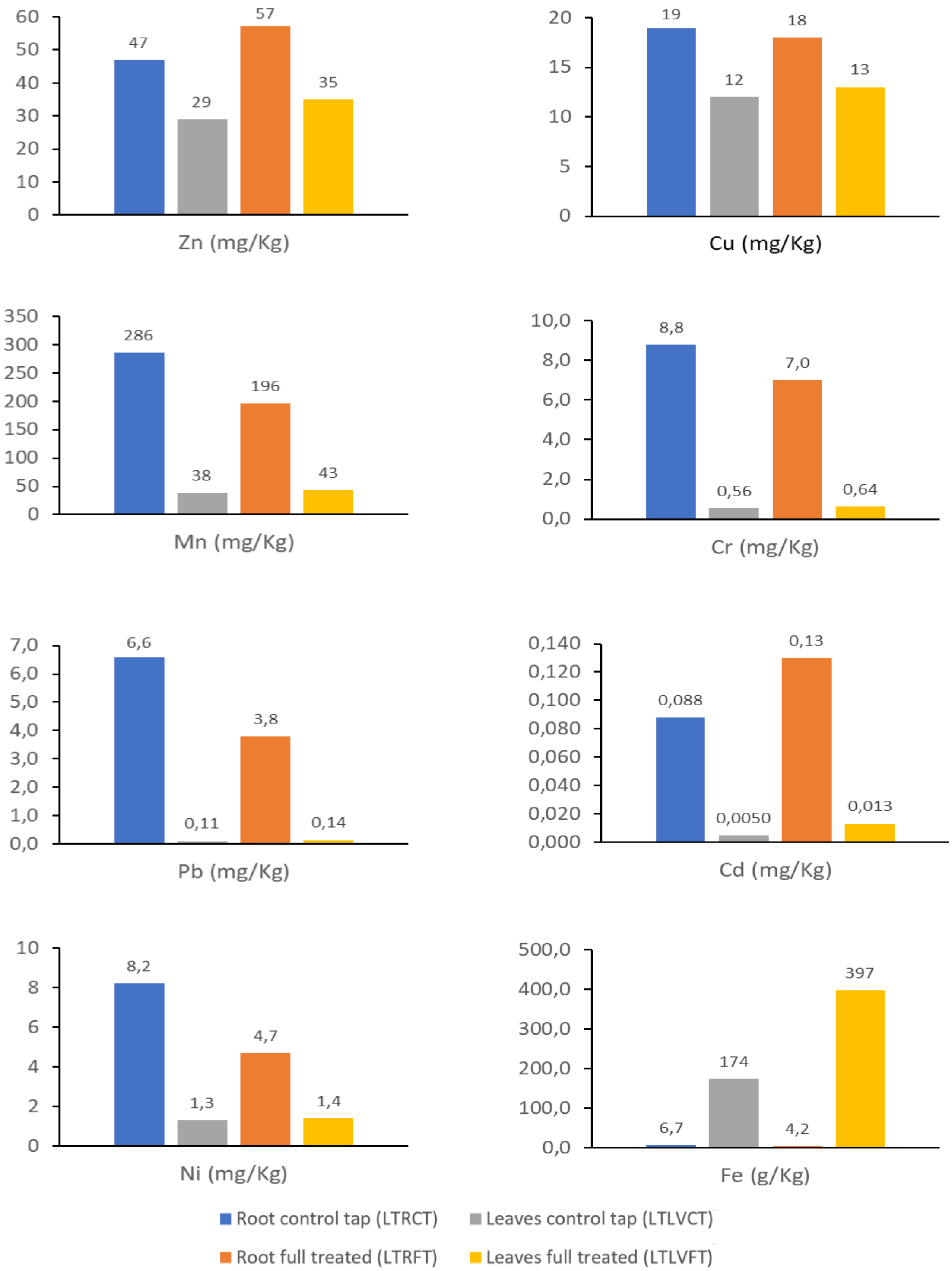

Figure 4.11. Metals concentration (mg/kg dry solid) found in Oregano irrigated with either tap water (CT) and full treatment water (FT)

The oregano roots irrigated with full treated water exhibited a lower concentration (decrease to the order of 45-75%) of Mn, Pb, and Ni compared to the roots irrigated with tap water. On the other hand, the concentrations of Zn, Cu, Cr, and Fe in the roots were relatively similar regardless of the irrigation water source.

Concerning the oregano leaves, there was not a great difference in the concentration of most heavy metals (Cu, Cr, Pb, Ni, Cd, Zn, Mn) whether they were irrigated with tap water or full treated water, except for Fe, which was higher (128% increase) in the leaves irrigated with full treated water. Compared to other studies (e.g. Dghaim et al., 2015), the concentrations of Zn, Cu, Pb, Cd and Fe measured in oregano in HYDRO2 are comparable. On the other hand, the Cd and Pb content in the oregano leaves deriving from plants both irrigated with tap and full treated water were lower than the concentrations reported by Winiarska-Mieczan et al. (2022) and the permissible limits in various countries as mentioned by Vuong (2020). Furthermore, the concentrations of Ni and Zn were within the concentrations measured by Behmen et al. (2022) in oregano samples collected from various locations in Bosnia-Herzegovina. The Mn concentration in the oregano leaves was also comparable with the reported values of Elsokkary and Aboukila (2020). Lastly, as the oregano plants have shown the capacity of bioaccumulating Cr (Levizou et al., 2018) the concentrations measured in this specific case are not considered great.

When comparing the roots and leaves, Mn, Cr, Pb, Cd, and Ni are heavy metals that are primarily concentrated in the roots, while Zn and Cu appear at similar concentrations in both the roots and leaves. Finally, Fe seems to be mainly concentrated in the leaves of oregano.

Taking into account the **metals** that was measured in the three different plants (leaves and roots**),** no large difference was observed between crops irrigated with tap water or fully treated water in terms of heavy metals. In some cases, the concentration in tap water irrigated crops was slightly higher than with full treated water.

As to Zn, the concentration in crops (as wet weight) leaves was usually below 15 mg/kg (with a maximum of 13.3 mg/kg for oregano irrigated with tap water) and in the range 3.2-22.8 mg/kg for roots, without large difference between the two applied irrigation water. Cu was in the range 1.4-7-6 mg/kg in the roots of the three crops, within a similar range (4.6-4.9 mg/kg) in oregano leaves, lower in lettuce leaves (0.2-0.3 mg/kg), and slightly higher in lavender leaves (7.5-9.2 mg/kg).
Mn was at higher concentrations (as wet weight) in roots, in particular of oregano (78.4-114.4 mg/kg), than in lavender and lettuce roots (15.6-37.2 mg/kg), and crops leaves (3.3-16.3 mg/kg). Fe, conversely, was at remarkably lower concentrations in roots (0.2-2.7 g/kg) than in leaves (3.1-150.9 g/kg) with maximum concentrations in oregano leaves. Ni was at concentration in the range 0.7-3.3 mg/kg in the roots, and below 0.5 mg/kg in the leaves.

Cr was always below 0.2 mg/kg in crops leaves, without differences in terms of irrigation. Larger differences were observed in the roots with concentrations up to 3.2, 1.2, and 3.5 mg/kg in the roots of lettuce, lavender, and oregano, respectively. Pb concentration was always below 0.05 mg/kg in leaves and below 2.6 mg/kg in roots. Cd was always below 0.01 mg/kg in all the roots and leaves samples. It can be mentioned that the concentration of Pb and Cd in the leaves was aways below 0.3 and 0.1 mg/kg, respectively, complying the Commission Regulation (EU) 2023/915 of 25 April 2023 on maximum levels for certain contaminants in food and repealing Regulation (EC) No 1881/2006.

4.3 Discussion and conclusions on micropollutants analysis for food safety assessment

4.3.1 Soils

The analysis of micropollutants in soils revealed seasonal variations, demonstrating a wider range of substances and higher concentrations during the summer campaign. The number of substances detected at the final time in the FT soils went from 11 in fall to 26 in summer, and the total OMP concentration increased from 31.7 ng/g (fall) to 222 ng/g (summer). The study site, located in Antissa, Lesvos, experiences a high tourism influx during this season. Consequently, the consumption patterns of pharmaceuticals are altered, resulting in an impact on the profile of pollutants detected in HYDRO1, as discussed in Deliverable 5.9 (Report on monitored micropollutants and pathogens).

The relevance of NSAIDs in the soil is noteworthy, as they were detected on every plot and in both seasons. These compounds have been detected in a range of 3.0-60 ng/mL in irrigation waters, reaching levels of 11-82 ng/g d.w. in soils. Generally, these compounds exhibit moderate to high adsorption levels and low half-lives, suggesting that their detection over time may be associated with repeated levels of entry through irrigation water rather than accumulation. On the other hand, clindamycin, among other antibiotics, exhibited high detection levels in the influent wastewater (reaching up to 17.8 ng/mL in the summer for the FT treatment). However, its maximum concentration in soils was found to be 2.7 ng/g dry weight (d.w.). This suggests a rapid dissipation of clindamycin, as reported by Koba et al. (2017) in a study involving 12 soils. The findings propose degradation as the primary process undergone by this substance, rather than its transport to lower underground layers. Other noteworthy pharmaceuticals include the diuretic hydrochlorothiazide and the antihypertensive irbesartan, which exhibited the highest values during the summer campaign. These compounds were found in higher concentrations in the FT soils compared to the PT soils; however, the same variation in levels found in the irrigation water between both treatments was not observed. Other factors may have influenced the greater retention (and hence, accumulation) of these compounds, such as the difference in organic matter content of the plots (CT and FT soils having 2 to 3 times more %SOM than PT soils; Figure 4.3) (Chefetz et al., 2008).

The presence of these pollutants in soils can lead to several harmful consequences, since they can undergo a variety of processes leading to environmental or human risks. For this specific case, the environmental risk assessment (related to terrestrial biota exposure to micropollutants) is addressed on the Deliverable 6.4 (Environmental risk assessment model), whereas their potential uptake by edible crops (lettuce) is discussed in the following sections of the current report.

4.3.2 Crops

A total of 88 OMPs were analysed for all crop samples, with an average of 68 compounds among crops and campaigns with satisfactory recoveries in at least one part of the crop (leaves/roots). Out of those, only 14, 13 and 4 OMPs in total were detected in the fall campaign in lettuce, oregano and lavender, respectively, and 15, 11 and 14, respectively, in the summer. The total concentration of all OMPs found in both parts of each crop was as follows: for lettuce 1,380 ng/g (d.w.) and 6,863 ng/g (d.w.) in fall and summer campaign, respectively; for oregano 3,608 ng/g (d.w.) and 2,493 ng/g (d.w.) in fall and summer campaign, respectively; and for lavender 948 ng/g (d.w.) and 4,316 ng/g (d.w.) in fall and summer campaign, respectively. In all cases, more than half of the OMPs detected were retained exclusively in the roots, with the rest retained in either leaves or both leaves and roots.

From a **crop** perspective, lettuce retained NSAIDs, BPs, and other EDCs in both leaves and roots. Additionally, antibiotics, psychiatric drugs, calcium channel blockers, β-blockers and antihypertensives were retained only in the roots. Oregano retained NSAIDs, antibiotics, and EDCs in both leaves and roots, and psychiatric drugs, β-blockers and diuretics in the roots. Lavender retained NSAIDs, antibiotics, BPs and EDCs in both leaves and roots, and psychiatric drugs, β-blockers and diuretics in the roots (Figure 4.7). In most cases, summer conditions exhibited raised levels of micropollutants compared to fall. This observation is expected, due to the increased touristic activity on the island which increases but also diversifies the population that is contributing to the composition of the wastewater used for fertigation. Summer findings are marked by the presence of antibiotics, diuretics, as well as higher concentrations of β-blockers, possibly tied to the increase in the population number and diversity during the summer months.

From a **therapeutic class** perspective, NSAIDs were found across all crops, and almost all treatments and periods, which can be explained by the easy accessibility of these compounds by the public and their multitude of use. β-blockers were found in all three crops in both seasons, at higher concentrations for lettuce and lavender in the summer than in winter and always exclusively in the roots. The only antihypertensive detected was irbesartan and was retained only in the roots of lettuce in the fall campaign. Diuretics were retained in oregano and lavender, and psychiatric drugs in all three crops, but both classes exclusively in the roots. EDCs were found mostly in oregano and in the summer period.

Comparing the results for the micropollutants detected in common in all three crops studied (Figure 4.8), for the **fall** sampling campaign, only ibuprofen was commonly found. Ibuprofen was detected in leaves of lettuce, but in the roots of oregano and lavender. Its levels widely varied among the crops, with 3.6, 184.9 and 646.8 ng/g (d.w.) in lettuce, oregano and lavender, respectively.

In the **summer**, four compounds were commonly found in the three crops, ketoprofen, venlafaxine, BPA and TCEP. Ketoprofen was found in the roots of lettuce and lavender, and in the leaves of oregano. Venlafaxine, BPA and TCEP were found exclusively in the roots of all three, with lettuce exhibiting the highest levels, which were also the highest levels detected of any compound in this study.

For seven compounds, norfloxacin, acetaminophen, levonorgestrel, salicylic acid, ibuprofen, 2-OH-ibuprofen and ketoprofen, some of which exhibited high concentrations in some cases in this study (consult ''ANNEX CROPS'' for specific values) the molecule gives only one dominant fragment with high intensity, therefore only that fragment is used for quantification purposes. Furthermore, regarding concentrations of compounds in oregano and lavender roots, introduction of error cannot be excluded due to the fact that lettuce root matrix calculations (i.e., calibration curves and recovery values) were applied to both oregano and lavender roots data. This can potentially explain the consistently high concentrations of venlafaxine and the β-blockers metoprolol, metoprolol acid, propranolol and sotalol.

Finally, it should be noted that the ranges of micropollutants found in the literature in the different parts of various plants irrigated with reclaimed water depend largely on the composition of the irrigation water used in each case, and can be as low as 0.01-4 ng/g (d.w.) (Wu et al., 2014, 19 micropollutants in 8 vegetables) to 1-200 ng/g (d.w.) (Li et al., 2021, phenols in vegetables; Hurtado et al., 2016, micropollutants in lettuce), and even up to levels above 2000 ng/g (d.w.) (Mordechay et al., 2021, carbamazepine in parsley).

The highest concentrations observed across crops and campaigns were found in the roots when reclaimed water from the full-treatment train was used, which is in line with literature. This is a favourable result for the health risk assessment of the edible part of lettuce and oregano that was conducted based on the data presented here. The highest concentrations observed specifically in the leaves were in the occasion of lettuce, for salicylic acid (181.5-224 ng/g d.w.) in the winter and for acetaminophen (101.3-264.3), 2-OH-ibuprofen (358.8 ng/g d.w.) and salicylic acid (265.6-403.9 ng/g d.w.) in the summer period.

4.3.3 Combined overview

In order to obtain a more spherical understanding of the micropollutants journey stemming from the reclaimed water to the soils and eventually all the way to the roots and leaves of the lettuce, the water-soil-lettuce continuum was investigated as a combined overview of the results obtained. Even though the extraction methodologies for every matrix differed, the overall findings are consistent and permit the deduction of some important conclusions. For this reason, two comparative figures for the two campaigns are presented (Figure 4.12 and Figure 4.13; different y-scales to bear in mind), including the results on micropollutants detection and quantification in soil and lettuce, respectively (sections 4.1 and 4.2 of the current deliverable) and the data of the irrigation water presented in Deliverable 5.9 (Report on monitored micropollutants and pathogens). To solely focus on the behaviour of the water-soil-lettuce continuum, these graphs include only the compounds that were analysed in all three matrices on each season (the number and type of compounds varied with each method) and detected at least once. Those compounds that were analysed in only one or two of the matrices were excluded from the graphs. It should be noted that the CT values in water (tap water) given here stem from a one-time sampling collected at the start of the irrigation period, thus not representative of the total irrigation period (detailed information can be found in Deliverable D5.9, Annex "HYDRO1", tabs "b", "c", "d", "e"). It should also be kept in mind that the levels of micropollutants in both soils and crops might be accumulative over the period of the irrigation time, which in the case of the lettuce was 7 weeks.

In the fall campaign (Figure 4.12), the only compound found in the control samples (i.e., tap water) was the β-blocker metoprolol acid and in the PT the NSAIDs value corresponds only to ibuprofen. The profiles of the water and soil regarding the FT treatment are very similar, whereas for the PT only the antihypertensive irbesartan is found in the soils. The class of antihypertensives corresponds only to irbesartan, whose concentration was higher for the FT than for the PT for all matrices. Irbesartan, a drug with a high tendency to be retained in soils, made its way to the roots of the lettuce when reclaimed water was used for irrigation. Diuretics also appeared to be resilient and transitioned from the water to the soil and eventually to the lettuce. Two diuretics, furosemide and hydrochlorothiazide, were detected in lettuce, with the former retained in the leaves and the latter only in the roots. A similar case was found for the β-blockers, which reached the soils and were found in the roots as well. EDCs were not detected in soil, but they were detected in the lettuce, suggesting complete migration to the crop, and partitioning to both of its parts. NSAIDs were retained primarily in the leaves.

In the summer campaign (Figure 4.13), six classes of compounds were detected in the irrigation waters in the control samples, all at very low levels. When reclaimed water from both the full and partial treatment was analysed, an array of compounds was found, belonging mainly to NSAIDs, psychiatric drugs and β-blockers. All of them seem to make it to the soil where the lettuce was grown and irrigated, and to the parts of the lettuce as well. Psychiatric drugs, antibiotics and β-blockers were absent in the control samples but present in soils irrigated with reclaimed water. Though the concentrations in water seem to be low, the levels detected in soils reveal its capacity for the accumulation of certain drugs, as can be especially noted for the antibiotic clarithromycin and the psychiatric drug venlafaxine, both in the FT and PT soils. Except for the β-blockers, higher levels of the other classes were found in the FT irrigation water compared to the PT. The same pattern is observed for diuretics. Psychiatric drugs, antihypertensives, antibiotics and β-blockers showed a preference of retention in the roots rather than the leaves, whereas NSAIDs, EDCs and diuretics were retained in both plant parts.

One final observation is the consistently higher levels for the FT compared to PT in both campaigns for all three matrices, water, soil, and crops. The expected behaviour would be the opposite, with a more enhanced removal performance of the FT treatment, where the constructed wetlands are included, compared to PT where this step is bypassed. The question now becomes, are the FT levels higher than PT when it comes to our data? We suggest three factors possibly involved:

- Residence time of the effluent water in tanks: after HYDRO1 systems, reclaimed water was stored in separated tanks before soil irrigation. While for the FT the storage period ranged from hours to a few days, for the PT irrigation the water was stored for weeks before its use. Under the latter conditions, it is possible that a fraction of the micropollutants decreased in terms of degradation (hydrolysis, photolysis, among other processes).

- As previously discussed for soils, the lower amount of soil organic matter content in the PT plots (first time used as agricultural soils, 2-3%) might be related to lesser accumulation of micropollutants, when compared to control (CT) or FT soils (6-8%). Also, a higher content of organic matter can reduce the bioavailability of these compounds to the soil bacteria, decreasing the extent of the biodegradation.
- Several unexpected weather conditions affected the agricultural site in the fall period, including snow and heavy rain. These conditions can enhance certain processes, such as surface runoff, leading to the transport of micropollutants all over the fields.

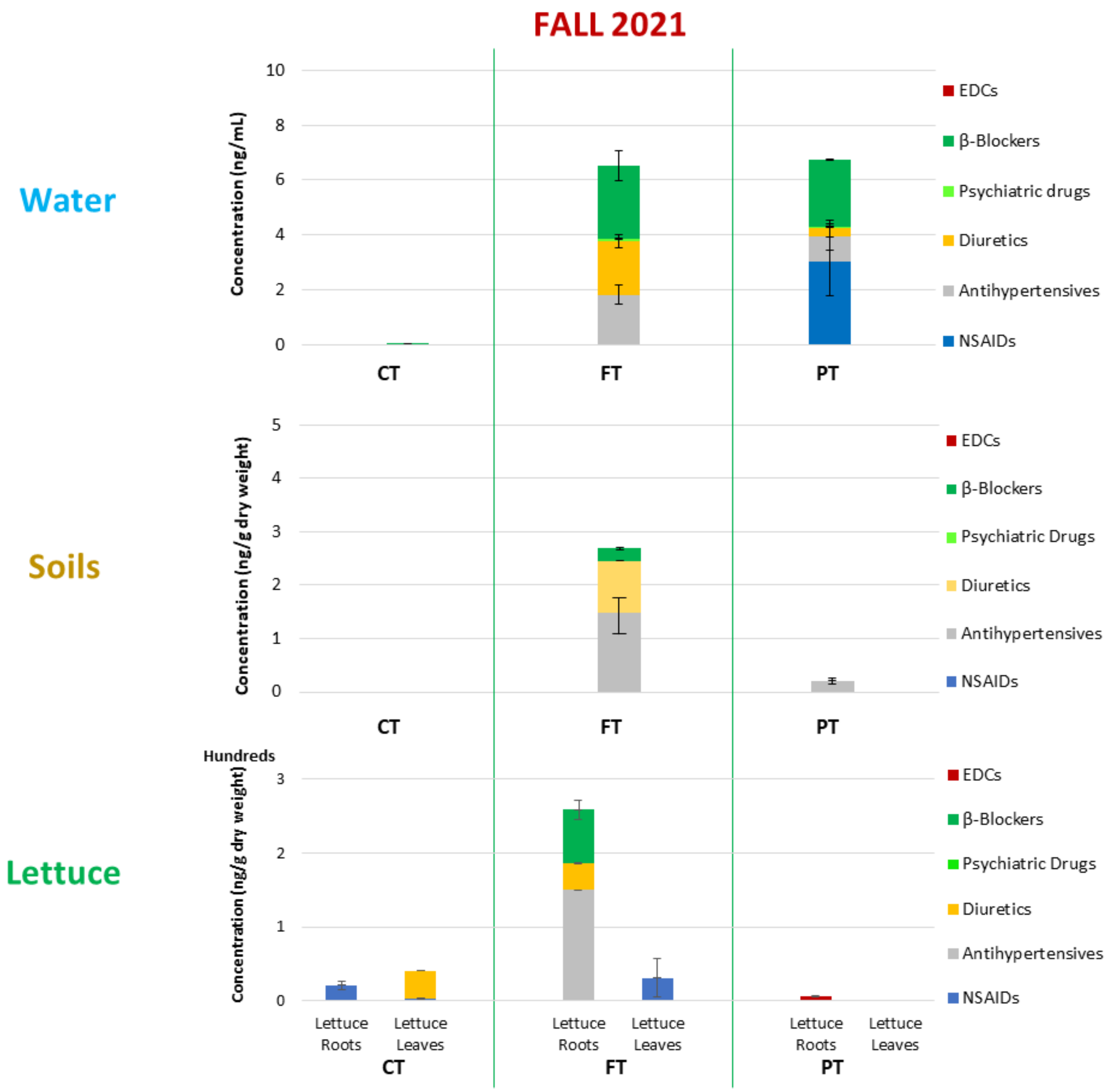

Figure 4.12. Comparative levels of micropollutants found in common in irrigation water, soil, and lettuce in the fall 2021 campaign.

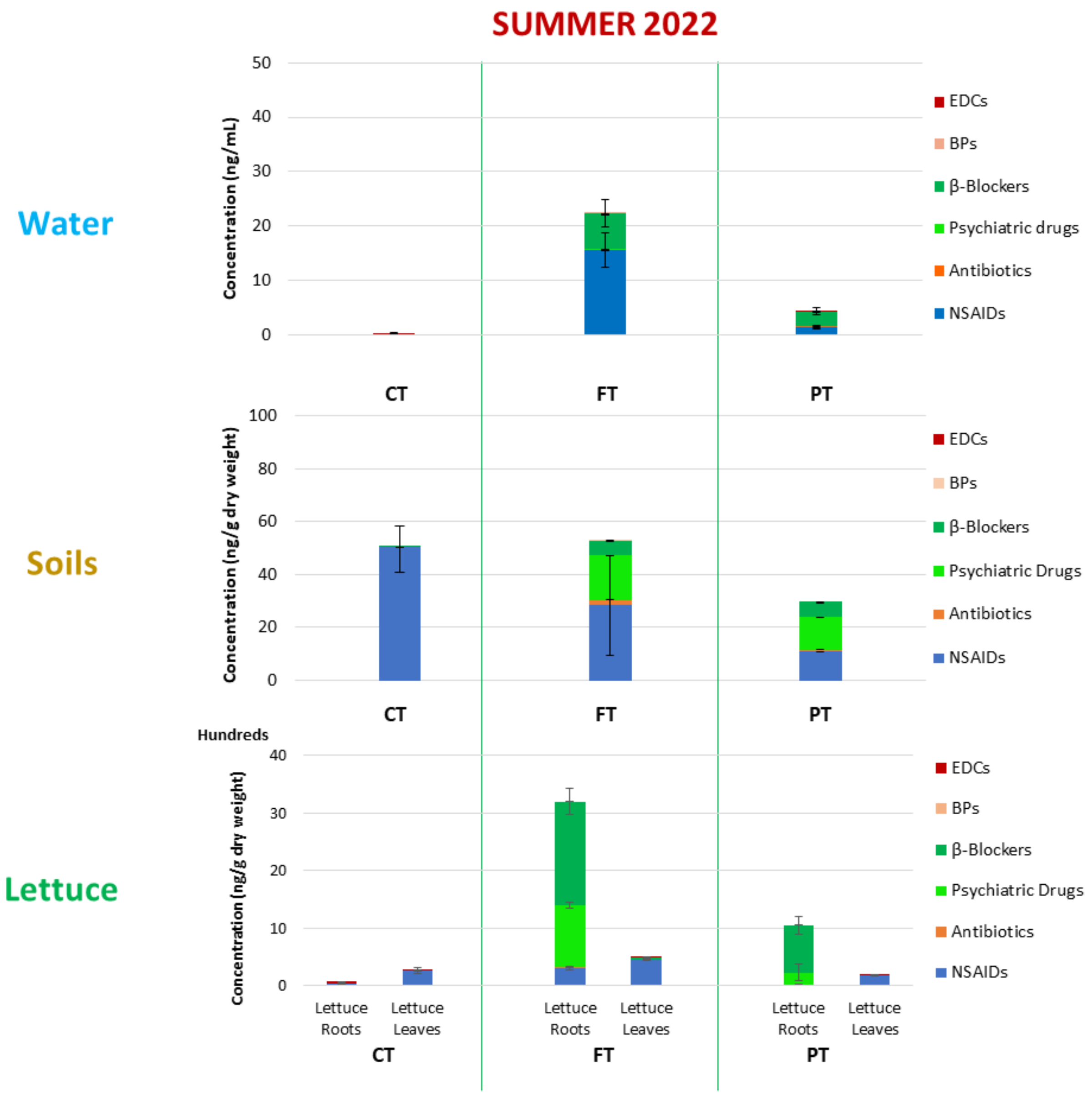

Figure 4.13. Comparative levels of micropollutants found in common in irrigation water, soil, and lettuce in the summer 2022 campaign.

5. PEST CONTROL STRATEGY

5.1 HYDRO2

<u>Phytopathogens and pest control in HYDRO2</u>

During the 2 years of HYDRO2 operation, various infestations were encountered in almost all the plant species from fungi, bacteria, viruses, insects, and soil nutrient deficiencies, as well as consequences from extreme temperatures like frost and intense heat.

<u>The measures used to tackle these problems were:</u>

- Mechanical removal of the infested parts of the plant or removal of the whole plant
- Control of the factors causing and/or favouring the infestation with measures like restrictions in the quantity of the irrigation water, pruning, removal and destruction of weeds
- Local interventions with biological or natural products/formulations
- Complete coverage of all the parts of the plant by category with the help of natural/biological pesticides
- Enhancement of the natural defence of the plants through natural bio-stimulants
- Utilization of beneficial fungi and parasitic nematodes

<u>Clarifications:</u>

- The interventions based on the intensity of the infestation were repeated two to three times
- Special attention was given in the deficiencies tending to weaken the plants
- No beneficial insects, such as the ladybugs (*Coccineleade*) or beneficial bees and wasps, were harmed with interventions like full coverage sprayings
- The weeds were not destroyed continuously or completely. There was always a small quantity of them in the in-between or perimetrically to host the beneficial insects
- The infestations may have been repeated in various time intervals.
- Before and during the interventions, formulations for the enhancement of plant defence and nutrition were applied
- No chemical formulations were used

In Table 5.1 the infestations are presented by plant species and the measures taken to tackle the problem.

Table 5.1. Infestation met, plant categories and intervention applied.

	Infestation	Plant	Intervention
1	*Tetranychus sp. Acari*	Oregano, Basil, Echinacea, Melissa, Apple, Tangerine, Oak, Hazel, Fig, Almond and Olive trees, Blackberries, Tomatoes, Eggplants, Watermelon, Melon, Zucchini, Maize, Physalis	-Copper (systemic formulation) -Plant extracts to tackle Acari -Natural oils -Potassium salts
2	*Aphididae*	Basil, Tomatoes, Maize, Melons, Watermelons, Zucchini	-Plant extracts -Potassium salts
3	*Pentatomoidea*	Oregano	Natural pyrethrin
4	*Margaronia (Lepidoptera)*	Olive trees	*Bacillus thuringiensis*
5	*Stephanitis pyri*	Apple trees, Aronia	Natural pyrethrin
6	*Thrips*	Tomatoes, Peppers, Physalis	-Plant extracts -Potassium salts
7	*Epilachna chrysomelina*	Fig trees	Natural pyrethrin
8	*Helicoverpa sp*	Maize (corn)	-*Bacillus thuringiensis* -Beneficial nematodes
9	*Sesamia nonagrioides (Lepidoptera)*	Maize (corn)	*Bacillus thuringiensis*
10	*Rapalosiphum maidis (Aphididae)*	Maize (corn)	-Plant extracts -Potassium salts
11	*Fusarium sp.* *Pythium sp.*	Lavender, Aronia, Pomegranate, Rosemary, Maize, Tomatoes, Melons, Watermelons, Raspberries, Blackberries,	-*Mycorrhizae* -Beneficial soil fungi -Plant extracts -Copper (systemic formulation)
12	*Colletotrichum Anthracnose*	Basil, Calendula, Melons, Watermelons	Copper (systemic formulation)
13	*Peronosporales*	Basil, Anise, Oregano	Copper (systemic formulation)
14	*Ustilago maydis*	Maize (corn)	Copper (systemic formulation)
15	*Cycloconium oleagineum*	Olive trees	Copper (systemic formulation)
16	*Erysiphales*	Goji Berry, Calendula, Roses, Tomatoes	Sulphur formulation Romeo

17	*Puccinia*	Blackberries	-Copper (systemic formulation) -Chlorine oxides Romeo
18	*Bacterium*	Watermelon, Melon, Basil, lavender	Copper (systemic formulation)
19	Viruses	Figs, Tomatoes, Pomegranates	Plant removal and destruction
20	Frost	Lavender, Melissa, Spearmint, Pomegranates	Anti-frosting formulations

The previously mentioned infestations and diseases were recurrent and depending on the biological cycle of each one, interventions were made as early as possible before many cycles had the time to be completed and/or repeated.

The success of the interventions was based on the time precision, the appropriate spraying apparatus (pressure sprayer), the repetition and the cultivation practices for the plant protection.

Some minor infestations were noted (*Aphids* and *Sciaridae* mainly) and were tackled with the help of beneficial insects.

The infestations were numerous and in a very short period of time, something that can be attributed to the particular weather conditions, the existence of plants diseases already from the previous year, the existence of pathogens in the surrounding area etc. Continuous observation is needed and immediate interventions to face these problems, with biological formulations.

5.2 HYDRO3

In June 2021, following a recent planting of 1000 oregano plants in April 2021, a significant loss of 50% of the plants was observed. This was accompanied by the emergence of black spots on the foliage and the decay of their root system. After conducting sample analyses, the agronomist determined that the application of Bordeaux mixture was necessary. Bordeaux mixture, officially known as a combination of bluestone and lime, falls under the category of fungicidal treatments. Specifically, it functions as a fungicidal agent due to its copper content.

Regarding its application, Bordeaux mixture is recommended for the prevention of fungal infestations in plants and the mitigation of various diseases that are either caused by these fungi or by bacteria. Consequently, its effectiveness diminishes when employed subsequent to the establishment of fungal or bacterial issues.

For instance, one of the numerous applications of Bordeaux mixture is its immediate use after pruning plants. This aids in their growth and development.

Figure 5.1. From left to right: new oregano plant to be planted showing leaf colour change due to environmental conditions; drying of the root system of the plant; sprinkling with organic algae juice to avoid shock in the new planting.

Starting from February 2021, a bi-weekly routine was established involving the removal of weeds and the application of algae and amino acids. This practice serves to naturally stimulate plant growth by providing essential vitamins and amino acids. The utilization of fertilizers derived from algae, amino acids, or a combination of fish-derived components greatly aids in reinforcing plant resilience, enabling them to better withstand stress-inducing situations such as high temperatures or the aftermath of a hailstorm. Additionally, this type of fertilizer enhances flowering and fruiting processes by promoting elevated sugar levels and improving overall coloration.

5.3 HYDRO4

Concerning the cultivation of lavender in the HYDRO4 system, several issues have been encountered. Notably, adverse climatic conditions have hindered the recovery of plants from winter dormancy, leading to their inability to regain vitality. Additionally, losses have been incurred due to the root system succumbing to rot caused by stagnant water. However, there have been no reported problems with pest infestations.

Starting from February 2022, a bi-weekly routine was initiated, involving the regular removal of weeds and the application of algae and amino acids. This practice serves to naturally stimulate plant growth by providing essential vitamins and amino acids. The utilization of fertilizers derived from algae, amino acids, or a combination of fish-derived components greatly aids in reinforcing plant resilience, enabling them to better withstand stressful situations such as high temperatures or the drought following a hailstorm. Moreover, the use of this fertilizer contributes to the enhancement of flowering and fruiting processes, as it leads to an increase in sugar levels and an improvement in the vibrancy of plant colours.

Figure 5.2. From left to right: new lavender plants that are dormant (they remained 2 months in the environment dormant before blossoming); new lavender cutting; planting lavender in the field.

5.4 HYDRO5

At HYDRO5, the cultivation of tropical and subtropical crops on Tinos Island offered several advantages. Since the crops were grown in an area far away from their native regions, the occurrence of "indigenous" diseases or pests that could exert substantial pressure on the crops was minimal. Within the greenhouse environment, protection against strong wind and rainfalls was maintained, and water supply relied exclusively on drip irrigation. This strategy ensured that the above-ground foliage of the plants remained consistently dry, thereby making it challenging for fungal diseases to take hold.

However, challenges were encountered due to occasional high relative humidity within the greenhouse, which could lead to condensation forming on the leaves. Additionally, the low winter temperatures posed another challenge. Despite these hurdles, most of the crops experienced few issues with diseases or infestations caused by biological agents such as bacteria, insects, or viruses.

Spider mite infestations occasionally occurred, particularly affecting the Pepino crop, especially during autumn and winter. These infestations were exacerbated by closed greenhouse windows and significant temperature fluctuations between daytime and nighttime, creating ideal conditions for spider mite proliferation. Attempts were made to control these pests using synthetic pyrethroids, certified for organic farming, with only partial success. The Pepino crop suffered substantial losses, but other crops like papaya, Passiflora, and banana managed to recover once the Pepino was removed, infestation pressure reduced, and the effects of pyrethroid sprays took effect.

Instances of mealybugs and aphids occasionally appeared on certain crops, often linked to delayed weed control. These pests were managed through the application of natural oil formulations, such as rape-seed oil, and by allowing beneficial insects to become active within the greenhouse during warmer periods by keeping the doors open. The same synthetic pyrethroid sprays used to control spider mites also proved effective against mealybugs and aphids.

The cold winter temperatures did result in plant and leaf tissue weakening, making them susceptible to opportunistic fungal diseases like botrytis or rot fungi. To counteract these diseases, copper-based formulations similar to those used in organic wine farming were employed through spraying.

The low winter temperatures could sometimes cause a brown necrosis on the leave borders of banana and papaya and yellow area discoloration in papaya. Since there was no heating in the greenhouse, the only option was to allow the plants to recover by themselves in spring with higher temperatures. Sometimes affected leaves had to be removed and foliar nutrient sprays helped the plants recover quicker. This can be considered a temperature induced physiological disorder. Given the only minor damage to the plants it is unclear if the benefits of active heating and therefore energy consumption during cold winter nights would outweigh the economic and environmental costs.

Figure 5.3. Leaf discoloration on banana during winter (on the left); severe spider mite infestation on pepino (on the right).

Conversely, during periods of notably high summer temperatures within the greenhouse, certain banana plants were susceptible to heat-related damage. Some young leaves would exhibit browning or blackening, resembling the aftermath of a lightning strike. This phenomenon occurred infrequently and affected only a limited number of plants. The occurrence of this damage was primarily linked to instances when exceptionally strong winds, characteristic of Tinos' summer months, hindered the wider opening of greenhouse windows due to the risk of the wind displacing the roof. To ensure proper ventilation, all doors needed to be kept open.

In conclusion, HYDRO5 faced minimal risks and concerns regarding food safety. On one hand, the irrigation source, which was distilled seawater, constituted nearly pure water with minimal microbiological content. On the other hand, the chosen tropical crops all bore fruits with substantial peels, providing an additional protective barrier against the transmission of pathological microorganisms through means such as dust or other vectors.

5.5 HYDRO6

In the poly cropping agricultural approach of HYDRO6 the Pest control strategy is based on very short scouting cycles trying to observe a problem in its earliest possible stage. After that follows the identification, if possible, of the active cause. In the case of insects this is most of the time successful, while fungal, bacterial or viral infections are much harder or even impossible to identify with certainty. After that a record is kept with the key parameters like where, what and when, for reoccurring infestations. The decision of intervening or not is made according to previous operating experiences of potential success of the possible action. This is an important assessment because interventions are time intensive, costly and always tend to have side

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effects. If there is little chance of a cure our experience has shown that, if possible, it is more beneficial to wait for feedback of the ecosystem solving the problem which is often very efficient. The main question here is how high the potential damage and economic loss is by losing a certain crop. Through the high variety of different crops produced in a small area at ELT (Ecolodge Tinos) many times the decision can be made to further observe the evolution of the infestation. An example of this is Aphid infestations that given a certain time, are solved very effectively by ladybug populations increasing through higher food supply by the Aphid population. Another action path, if the outcome is unknown or the previous experience has shown that it is only possible to suppress and not cure the infestation, ending in a cycle of application-reapplication of certain substances, is to abandon a certain crop and replant a new crop. This is of course less problematic with annual cultivation's that have fast turnovers compared to perennials where the loss can be significant.

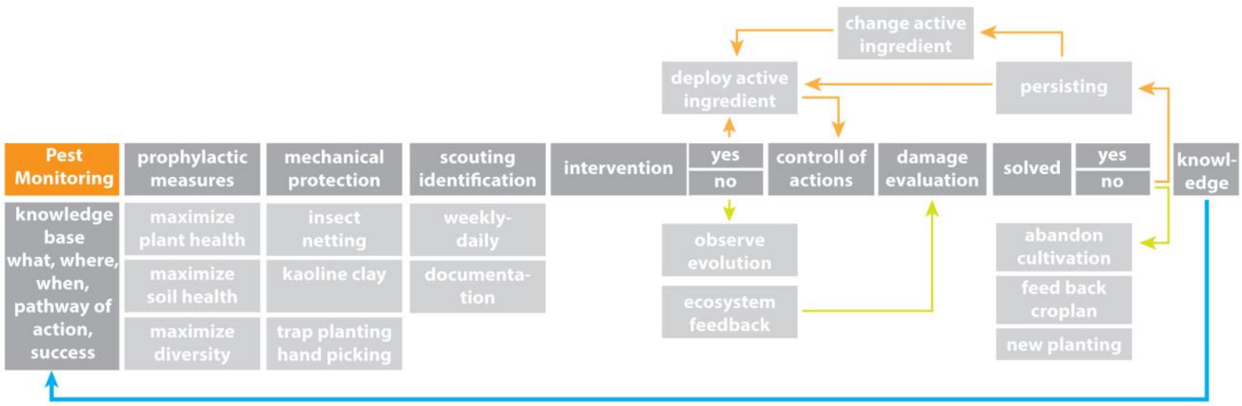

Figure 5.4. Flow diagram of pest control developed in HYDRO6 by ELT.

In the first growing season of 2019, ELT was consulted by an organic certified agronomist who had composed a basic collection of organic pesticides and fungicides. Mainly consisting of sulphur, Copper, Soap and Pyrethrin based products. A fairly strict regime of prophylactic sulphur and systemic ionic copper applications was sprayed on sensitive cultivations like the family of Solanaceae, Cucurbitaceae and others. When a pest manifestation was observed nonetheless Copper and Pyrethrin were applied. The observations showed that rarely a disease could be cured but, in many cases, it could be slowed down while keeping UP a certain application rhythm. This was generally unsatisfactory from the point of view taken by ELT on organic agriculture. Because the tested approach followed the common practice to treat the symptoms rather than researching the underlying cause leading to the problem in the first place. Also, it is an unpleasant experience to apply even organic certified agrochemicals with a backpack sprayer every 14 days. These experiences throughout the 4 growing seasons accomplished, led to a customized pest control strategy adopted to the micro climatic conditions, the needs of the customers of the produced crops and finally the ethical standpoint of ELT.

1. **<u>Prevention-Avoidance</u>**
   Is the most effective pest control measure whenever possible to apply. It consists of the understanding of the micro climatic conditions of the location and the ones within the location itself. For example, avoiding places with higher humidity levels for plants pruned to powdery mildew. Crop rotation is highly effective on a long-term base where the host plants are rotated in order to prevent buildup of different pests to harmful levels. This is implemented by the crop planning process considering the past and future placement of plant families within the growing area over the course of several years. Adjusting the cropping pattern to known pests throughout the season effectively avoids co-existence of a pest and its host plant or prevents the second or third generation of a pest building up to increased pressure.

Managing the overall habitat by increasing biodiversity and fostering habitats where predators can thrive ensures an intact feedback loop of the ecosystem. At HYDRO6 the installation of 10 birdhouses and bushes providing food for small birds, which mainly feed on different insects, started to increase the bird population on site. Also, areas of wildflowers and shrubs are cultivated around the growing areas providing habitat and food source for a wide variety of different species. A healthy soil has proven to be the foundation of a healthy plant development. Here compost applications, mulching, No-till, cover cropping and other measures described in further detail in Deliverable 4.5 (Report on yields, health of crops and derived products) have reduced the overall pest pressure. The crop variety selection can further help avoid certain infestations due to the higher resistance of certain cultivars against observed pests. In the Hybrid F1 varieties the gamma of tolerant or even resistant plants is fairly high while in open pollinated not copyright protected varieties it is fairly poor. Inter-cropping is another successfully applied strategy where two or more crops are grown near each other fostering a beneficial relation. Also, the avoidance of bought in plants from nurseries importing already infected plants has shown effective.

2. **Intervention**

   Deciding when to intervene requires careful consideration. Among the successful interventions tested, mechanical protection using insect netting has proven effective, particularly for low-growing leafy crops. The implementation of low tunnels to support the netting, secured to the ground, has demonstrated nearly 100% effectiveness against insect infestations. However, this approach demands heightened management efforts for installing, opening, and closing the nets throughout the cultivation cycle.

   Manual control is efficacious for larger, slower-moving insects situated predominantly on the surface, facilitating hand-picking. The use of trap plants has notably streamlined the handpicking process by concentrating a specific insect population on these designated plants. For instance, flowering parsley attracts a significant number of shield bugs, which can be effectively managed through manual control. These trap plants also serve as easily monitored indicators of pest presence.

   To combat many soil-borne diseases, early inoculation with indigenous and purchased beneficial microorganisms has proven effective, particularly when applied during the plant's nursery stage. Pesticide application is restricted to the utilization of biopesticides, and only when deemed absolutely necessary for situations where replacement crops aren't feasible with reasonable effort. Bacillus thuringiensis ssp. is frequently employed against caterpillar and moth populations. Pyrethrin is used to combat white fly infestations in oregano plants and aphids within the greenhouse. Diatomaceous earth is effectively used to manage certain arthropod and gastropod populations. Additionally, prophylactic application of kaolin clay serves as a preventive measure, primarily addressing insect-related issues.

Table 5.2 summarises all the agents used to control different pests at HYDRO6 throughout the four growing seasons accomplished within the project.

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Table 5.2. Pest control agents used and tested.

Pest control at Hydro6											
Insects	Order, Genus, Family	Anti insect netting	Baccilus Thurigensis	Hand picking	Soap & Alcohol	Kaolin clay	Diatomaceous earth	Pyrethrum	Nematrap	Sulfur	Copper
Aphid	*Aphididae*				P	O	O	O			
Cabbage maggot	*Delia*	P									
Potato beetle	*Leptinotarsa*	P		O							
Cutworm	*Agrotis*		O	P							
Flea beetle	*Chrysomelidae*	P					O	O			
Leek moth	*Acrolepiopsis*		O	P							
Mustard white			P								
Catterpillars	*Lepidoptera*		P	O							
Swede midge	*Contarinia*	P						O			
Thrip	*Thrips*				O		O	P			
Stink bug	*Halyomorpha*	P		O			O				
Shield bugs	*Hemiptera*			O			O				
White fly	*Aleyrodidae*	O						O			
Root knot nematode	*Meloidogyne*								P		
Spider mite	*Tetranychus*						O				
Fungi											
Downy mildews	*Peronosporales*									P	O
Powdery mildew	*Peronosporales*									P	O
Early blight	*Peronosporales*									P	O
Viruses	*Plant removal and burning of debris*										
Prefered solution	P	Also effective	O								

Equipment

For the application of foliage sprays different pressure sprayers were used throughout the project. They mainly differ in size, carrying type and build quality. In the beginning a cheaper and smaller product was bought commonly used in advanced amateur to semi-professional settings. This rendered with 8 litres too small for the size of the garden being too time consuming due to the need of refilling. After this a 15-liter backpack sprayer with crank hand pump was purchased. It was of lower quality but at a cheaper price point in order to test this kind of sprayer. In general, the type and size were sufficient but the build quality and the ergonomics were bad. The conclusion is that for a small but professionally operated farm a backpack sprayer of 15-20 litres is optimal considering refill rhythm to weight on the shoulders. It should be designed for professional use from a well-established company supplying spare parts and detailed technical drawings for service ability. The design and documentation play an important role in how efficient the device can be operated and maintained. All sprayers have the tendency to clog at various points within the construction. The cleaning in a badly designed sprayer can become very time-consuming and depending on the frequency renders the product finally useless. Another important factor observed is that with all hand pumped sprayers it is difficult and fairly tedious to maintain even pressure in order to maintain a well atomized spray which is important to achieve good coverage. A battery-operated sprayer with pressure control is beneficial to the effort and quality of the application. Another point is the availability of spray nozzles that create well defined droplet size, radius and distance. For applying kaolin clay or diatomaceous earth in the form of powder a hand duster is used.

The identification of very small insects and different fungal diseases found on the plant surface is supported by a digital microscope. Many of these pests are so small that the human eye cannot observe them with sufficient precision. It also helps to identify or observe the effectiveness of foliage spray application by observing how many insects have survived on sampled leaves. Also, different life cycles and number of generations can be identified.

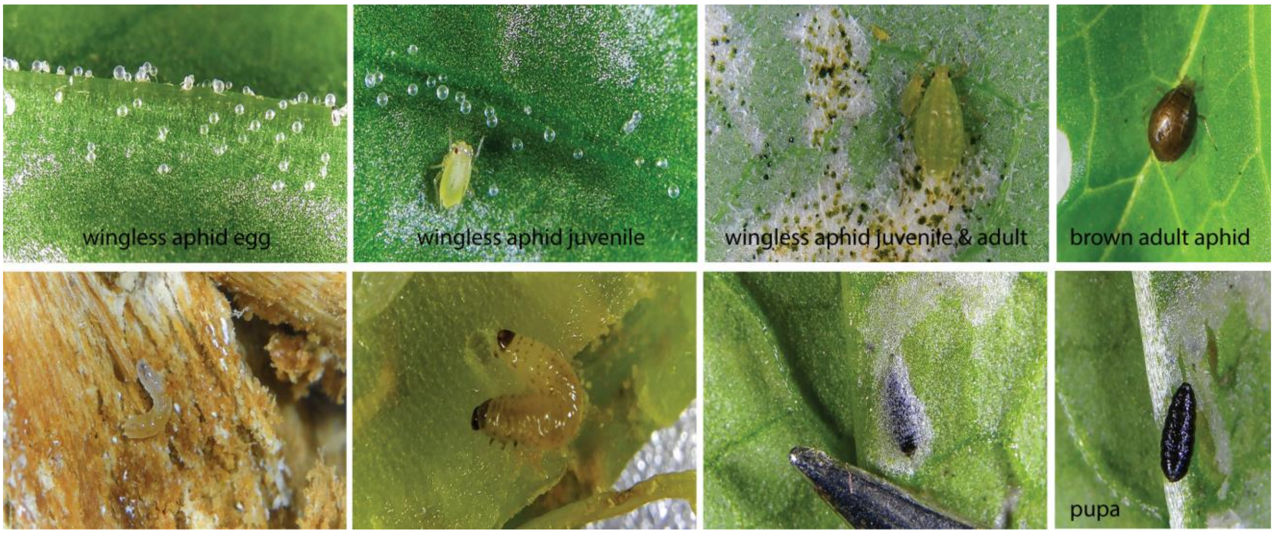

Figure 5.5. Pest pictures taken with microscope.

Chicken Coop

Chickens serve a dual purpose within the agricultural framework of HYDRO6. Firstly, they are harnessed to create nutrient-rich compost. Secondly, during the winter-spring season, they are allowed to roam freely to hunt insects and insect larvae-pupae, primarily located in the litter layer or the topmost centimetres of the soil. Chickens display a broad appetite for various insects, provided they lack specialized defence mechanisms.

Introduced to the agroecosystem in 2019, the chicken flock played a pivotal role. Hatchlings were nurtured using an egg incubator and comprised an assortment of unidentified breeds obtained from a local chicken owner. These chicks were reared within the greenhouse environment, and a dedicated chicken coop was constructed on-site. The coop's entrance is automated, operated either by preset timings or a light sensor from dusk till dawn. This automation minimizes the effort required to manage the chickens' ingress and egress. The inaugural chicken run was established in the planned expansion area of the market garden.

Figure 5.6. Building the first chicken coop, raising chicks and first chicken run.

This was done in order to prepare the ground, enrich the soil with organic matter and nutrients and reduce the weed pressure and seed bank in the soil. An electric fence was bought in order to be able to move the chicken to certain areas and confine them while being protected from local predators. The fence is electrified by a mobile power unit charged via a photovoltaic panel. Chickens can fall to pray on the island to predators such as raptors and badgers. The raptors are faced by providing the chicken with coverage by trees which gives them hiding spots and makes it impossible for the raptor to attack from the air. The badger is a more difficultly managed carnivore and throughout every season some chickens are lost. Only metal fencing has worked so far as protection from the badgers while the electric fence helps but on its own is insufficient.

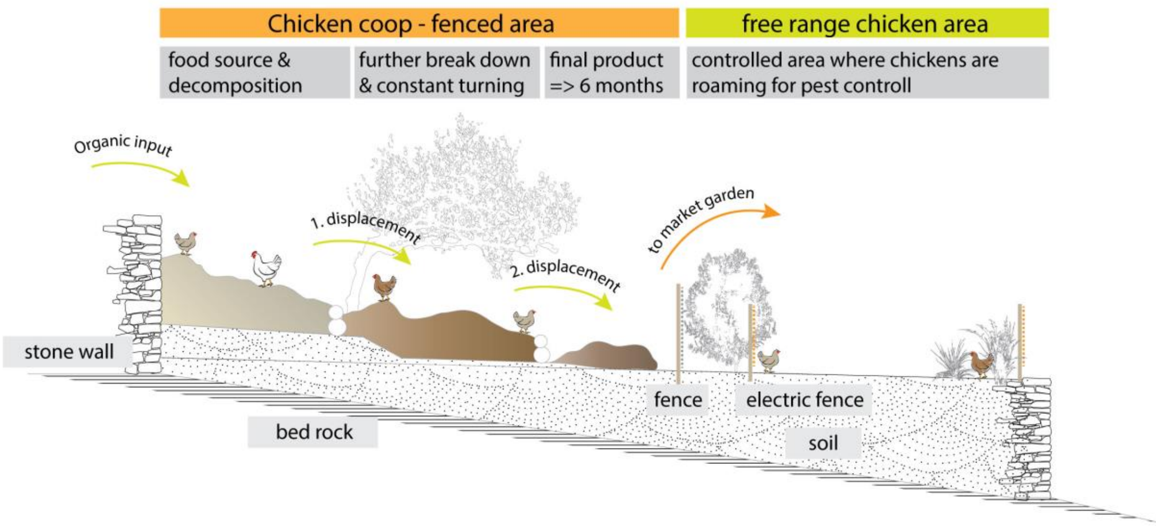

Figure 5.7. Schematic section cut illustrating chicken compost system.

The high predation rate in the first year made it necessary to restock the flock. Given the opportunity it was decided to purchase two breeds with good overall characteristics concerning laying, friendly character, robust old variety and their ability to survive outside. The Plymouth and Australorp breeds were finally chosen. From this time on the flock is maintained to consist out of 5-15 chicken which is an optimal flock count for the available space, cost to benefit ratio and the amount of eggs that can be used. This means that once or twice a year fertilized eggs are hatched in the incubator and around 10 new chicks are raised. Due to the 50% male-female distribution the males are fed around 6 months to be slaughtered as meat birds.

Figure 5.8. Chicken flog and free ranging chicken.

In 2020 the chicken run and coop were moved to a new location in order to establish the second market garden area on the prepared ground. The new area was fenced, and two terraces were created forming three small terraces within the run which is located on the stone wall of the above terrace. This provides the opportunity to easily throw organic matter from the above stone terrace into the chicken area landing on the highest terrace within the area. This terrace slowly fills up with organic matter from the agricultural areas and brought in wood shavings from local carpenters. The chickens' natural behaviour of scratching and tilling the ground is utilized to turn the organic matter constantly and displacing it to the next lower terrace and so on. When the material has reached the lowest terrace, it is controlled for the stage of decomposition. If it is ready, it is transported out into the market garden beds as fertilizer. If not, it is shuffled up again to the highest terrace to pass the cycle once again, in order to ensure proper decomposition.

Figure 5.9. Soil building and composting with chickens.

A major problem faced in the free ranging pest controlling chickens was the persistence and smartness of the badgers. Leading to shorter time spans of roaming due to the need of attending or at least controlling the return of all birds to the safety of the chicken coop at night. Another issue is controlling the chicken movement, which is difficult in the landscape of Tinos, avoiding plant damage caused by animals escaping the confinement of the electric fence. Especially in summer the soil becomes so dry that the electric grounding of the fence stops working leading to malfunction. Also, the fences used for chickens are low which makes it possible for the birds to fly over them if motivated enough. This can be avoided by clipping the lower wing feathers confining the animals to the ground but increases the management effort.

Figure 5.10. Moving the chicken coop and run to a new area in 2020.

Pest monitoring and control is one of the very difficult tasks within organic agriculture. The background information and experience needed to perform these tasks adequately is enormous. The many interacting drivers of pests make the system complex and hard to manage in a holistic way. This leads many times to actions and applications targeting only the manifestation of a problem while the underlying cause is not further investigated. Time pressure, economic loss and limited understanding combined with false hope are some of the experienced reasons. At HYDRO6 we have decided that plant health deriving from well managed cultivation practices combined with a high priority on soil health as well as a diverse biotope forms the foundation of pest control management. Mechanical protection is very efficient and has very little side effects on the ecosystem and is therefore the preferred prophylactic solution whenever possible. Fast evaluation of possible alternative cultivation combined with an estimate of financial impact has shown many times the better alternative to prolonging harvest by repeated active compound application. While some use of fungicide and pesticide seems unavoidable, the four growing seasons have shown that it can be greatly reduced while maintaining a productive system with minor negative impacts. The chickens are an integral part of ELT's agricultural system by providing fertilizer, organic matter composting, an additional food source in the form of eggs and meat and their tireless appetite and curiosity help keep insect populations in balance.

CONCLUSIONS

The use of treated wastewater for agricultural irrigation is becoming increasingly common in many parts of the world due to water scarcity and the need for sustainable water management practices. However, the use of treated wastewater for irrigation may pose a potential risk to the environment in many various aspects that were studied in this deliverable and their conclusions are discussed below.

Soil analysis

The Soil analysis revealed that fertigation (HYDRO2) can fully promote the soil nutrient state and was beneficial for plant growth, while in the cases of water originated from other sources (rainwater, stormwater, seawater, etc) the soil characteristics were not affected and therefore appreciable plant growth was achievable.

Biodiversity analysis

Soil biodiversity can serve as a key indicator of soil health and the effectiveness of the water management treatment processes. Soil microorganisms, nematodes, and macrofauna have emerged as crucial indicators of soil health due to their roles in maintaining soil fertility and nutrient cycling. After two years of macrofauna biomonitoring, results showed that using treated wastewater did not decrease the abundance of the main taxa of soil macrofauna, as no significant differences were recorded compared to the clean water irrigation management. Using treated wastewater favoured the abundance of specific taxa and biodiversity, led to more stable bio-communities and improved soil conditions. Attention must be paid to the amount of treated wastewater used for irrigation. The climate significantly impacted the soil macrofaunal. The presence of different plant species did not have an impact on soil macrofauna. The application of treated wastewater for irrigation resulted in a higher abundance of soil nematodes compared to soils irrigated with conventional freshwater, and soil fertility and soil health were enhanced through the stimulation of beneficial nematode populations.

Micropollutants analysis for food safety assessment

Three types of crops, lettuce (*Lactuca sativa romana*), oregano (*Origanum vulgare*), and lavender (*Lavandula angustifolia*) were planted as part of the HYDRO2 wastewater reclamation demo-site of the HYDROUSA project on the island of Lesvos (Greece). Fertigation took place with either tap water, reclaimed water or partially treated reclaimed water, in two sampling campaigns, fall 2021 and summer 2022. Potential uptake of 88 selected organic micropollutants in both planted soil and the planted soil and crops was investigated.

Soils were sampled at three different times. Significant differences ($p<0.05$) in SOM levels between PT soils and the other treatments across all samples and seasons were observed, possibly because that was the first time that PT soils were utilised for agricultural purposes. The presence of OMPs in the reclaimed water resulted in a broad range of soil concentrations for most compounds. These occurrences and levels were particularly elevated during the summer campaign. NSAIDs were the most commonly found class. The retention of OMPs in soils is heavily influenced by soil organic matter, with higher organic matter content in soils leading to higher concentrations of specific pharmaceutical classes, such as diuretics and antihypertensives. On the other hand, compounds such as antibiotics, which were highly detected in water, underwent rapid dissipation processes, and as a result, their concentrations in soils were low.

Regarding OMPs in crops, only around 6% of the compounds well recovered in all crops and campaigns and in at least one part of the crop were actually detected in all cases. More than half of the detected compounds were detected in the roots of all crops, with the rest partitioning either in the leaves or in both parts of the plant. This result is also favourable regarding the health risk assessment (Deliverable 6.4) which considers only the edible part of lettuce and oregano. Lettuce retained NSAIDs, BPs and EDCs in both leaves and roots, and antibiotics, psychiatric drugs, calcium channel blockers, β-blockers and antihypertensives only in the roots. Oregano retained NSAIDs, antibiotics and EDCs in both leaves and roots, and psychiatric drugs, β-blockers and diuretics in the roots. Lavender retained NSAIDs, antibiotics, BPs and EDCs in both leaves and roots, and psychiatric drugs, β-blockers and diuretics in the roots. Overall, psychiatric drugs, antihypertensives, antibiotics and β-blockers showed a preference of retention in the roots rather than the leaves, whereas NSAIDs, EDCs and diuretics were retained in both plant parts. In most cases, summer conditions exhibited raised levels of micropollutants compared to fall. This observation was expected, due to the increased touristic activity on the island which increases and diversifies the population that is contributing to the composition of the wastewater used for fertigation.

One final observation for both soil and crops was the consistently higher levels for the FT compared to PT in both campaigns for all three matrices, and three possible factors were suggested: the residence time of the effluent water in tanks, amount of soil organic matter content in the different plots, and the several unexpected weather conditions.

Selected heavy metals (Zn, Cu, Fe, Mn, Cr, Pb, Cd, Ni) were also analysed in lettuce, oregano and lavender. In general, no large difference was observed between crops irrigated with tap water or fully treated water with HYDRO1 in terms of heavy metals.

Pest control strategy

HYDRO2: During the 2 years of HYDRO2 operation, various infestations were encountered in almost all the plant species from fungi, bacteria, viruses, insects, and soil nutrient deficiencies, as well as consequences from extreme temperatures like frost and intense heat. The success of the interventions was based on the time precision, the apparatus used, the repetition and the cultivation practices.

HYDRO3: For oregano, foliage and rotting of their root system was observed and the use of Bordeaux mixture was deemed necessary. Sprinkling with algae and amino acids was also carried out, to enhance flowering and fruiting, and to improve colour and raise sugar levels.

HYDRO4: Regarding the cultivation of lavender, no problems with pests were observed. It was helpful to use a fertilizer made from algae, amino acids or a mixture of fish, which contributes to strengthening the plants against stressful situations.

HYDRO5: The tropical and subtropical crops had little problems with diseases or infestations. Occasionally there were mealy-bugs occurrences, as well as some aphids, which was controlled with spraying of natural oil formulations (rape-seed oil) and keeping the greenhouse doors open to allow beneficial insects to become active in the greenhouse. Synthetic pyrethroids were used to control spider mites. The low winter temperatures weakened the plants, which led to fungal diseases. Those were controlled with copper-based formulations. Overall, no risks and concerns were observed for food safety, since irrigation source was almost pure water with hardly any microbiological load, and also the tropical crops' peels offered an additional physical barrier against pathological microorganisms.

HYDRO6: A variety of different crops were produced in a small area at Ecolodge Tinos. A chicken coop was established in 2019, providing fertilizer, organic matter composting, additional food source in the form of eggs and meat and a way to keep insect populations in balance. While low usage of fungicide and pesticide was unavoidable, it can be greatly reduced while maintaining a productive system with minor negative impacts.

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