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RESEARCH ARTICLE

Biochemical and mineral changes in leaves and roots of treated wastewater irrigated olive trees

Wiem Sdiri[a,b], Dhekra Toumi[a], Houda Akremi[b], Rym Hassani [d] and Hedi Ben Mansour [a,c]

[a]Research Unit of Analysis and Process Applied on the Environment – APAE UR17ES32, Higher Institute of Applied Sciences and Technology, University of Monastir, Mahdia, Tunisia; [b]Institut National des Technologies et des Sciences du Kef, Université de Jendouba, Le Kef, Tunisia; [c]Instituto Universitario de Estudios Ambientales y Recursos Naturales (i-UNAT), Universidad de Las Palmas de Gran Canaria, Las Palmas de Gran Canaria, Spain; [d]Environment and Nature Research Centre, Jazan University, Jazan, Saudi Arabia

ABSTRACT
The treated wastewater (TWW) is seen as a potential alternative for crop irrigation in areas with limited freshwater resources. This alternative water source may present a fertilising effect for plants and soils. The present study aims to assess young olive trees' physiological and biochemical behaviour as a result of dairy TWW irrigation. Therefore, olive tree parts are analysed following irrigation. Moreover, the two irrigation systems' efficiency is studied in the present work. *Olea europaea* L. cv. Chemlali leaves and roots were sampled from young olive trees irrigated for 12 months with TWW using surface drip irrigation (SDI) or manual irrigation (MI) systems. Treated wastewater seemed to improve significantly mineral amounts, especially Mg, K and Ca in olive leaves and roots. Moreover, this practice leads to ameliorating plant physiological performance by improving chlorophyll synthesis. These positive effects were observed following the SDI system application. On the other hand, the MI system promoted heavy metal translocation to plant roots and leaves. This phenomenon was blocked by the SDI system. The present results highlight that TWW seems to be an interesting bio-fertiliser of young *Olea europaea* L. cv. Chemlali trees.

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Introduction

Water shortage is a chronic problem worldwide. Several countries suffer from high evapotranspiration demands. These phenomena were observed in the Mediterranean basin (Soda et al. 2017). Moreover, a reduction in freshwater availability was observed. It was attributed to agricultural and industrial uses such as irrigation and food production. In this situation, to sustain production and secure food supplies, growers seek out an alternative water source. Thus, the monitoring of water quality used in agricultural activities was required for growers by applying regulations such as the Food Safety Modernisation Act (FSMA) (FDA 2018).

CONTACT Hedi Ben Mansour hdbenmansour@gmail.com; Rym Hassani rhassani@jazanu.edu.sa

Food industries, especially the dairy industry, consume and generate large water volumes. Imperatively, seawater desalination and wastewater treatment would be considered as alternative solutions to complement conventional freshwater sources (Ofori et al. 2021).

In arid and semi-arid regions, wastewater reuse has become an increasingly popular practice because of limited good-quality water supplies. In these regions, a negative effect of wastewater use for irrigation was observed. According to (Ganjegunte et al. 2017), low-quality water used as an alternative irrigation source may affect negatively agricultural products. The possibility and practicality of wastewater reuse were demonstrated in many parts of the world, such as in Tunisia and China (Ofori et al. 2021). Globally, treated municipal wastewater was used in agricultural fields to irrigate forests and horticultural crops (Al-Absi 2010). Worldwide, several research works have shed more light on wastewater reuse in irrigation. These studies have been conducted at both field and laboratory scales. Indeed, the economic feasibility of the impacts of wastewater reuse on public health, soil as well as water and crops have been investigated (Ganjegunte et al. 2017; Mkhinini et al. 2018).

Many studies showed that irrigation with treated wastewater causes the major plants' metabolic process disruption. In this situation, salts or heavy metals present in irrigation water produce damage to plants. These last chemicals develop several mechanisms like osmolyte accumulation (Gómez-Bellot et al. 2013; Talaat and Shawky 2014).

Wastewater can contain significant amounts of heavy metal whose accumulation in the rooting medium retards plant growth. In addition, physiological processes disturbance can be observed like perturbations in cellular metabolism and element uptake. Then, fruit number and crop yield decrease were revealed. An excess of metallic ions can damage plant structure and toxicity symptoms like wilting and necrosis can be observed (Kasprzak et al. 2003; Rather et al. 2020). The excess of these chemicals also produces direct oxidative damage to the pigments and their biosynthesis inhibition. This last destructive effect influences photosynthesis pigmentation functionality and content (Chow et al. 2021). The use of effluents for crop irrigation depends on their quality and needs efficient management. Effluent heavy metals resistance and their toxic effects cause a serious environmental problem (Khalil et al. 2007). Low-quality wastewater leads to heavy metal accumulation in agricultural soils. Therefore, treated wastewater can be used as an irrigation source only if all trace element concentrations are within guidelines for crop irrigation (Shatanawi and Fayyad 1996). Indeed, many plants are considered heavy metals, such as Zn, Cu and Pb bioaccumulators. Furthermore, following heavy metal accumulation in both plant and animal organs, cellular damage and oxidative stress increase were reported in the literature (Wilson and Pyatt 2007; Paithankar et al. 2021).

On the other hand, wastewater is rich in potassium (K), nitrogen (N) and micro-nutrients. Thus, wastewater irrigation reduces the need for chemical fertilisers (Martínez et al. 2013). In Tunisia, TWW is considered an inexpensive and renewable irrigation water source. In addition, this practice leads to recharging groundwater and fertilsing soil (Hechmi et al. 2023). According to Kiziloglu et al. (2008), wastewater irrigation leads to enhanced mineral availability to plants which increases cauliflower and red cabbage yields. Moreover, treated wastewater contains considerable amounts of dissolved organic carbon. Then, several research works showed that treated wastewater irrigation improves olive tree growth and photosynthetic capacity (Chaganti et al. 2020; Tekaya et al. 2016). In the Mediterranean region, olive (*Olea europaea* L.) is a major tree crop because of increasing olive oil consumption. Olive oil presents high nutritional value. In Tunisia, wastewater irrigation has been practised for several decades to support economic yields such as olive oil production (Mollahoseini 2013).

It was mentioned that before wastewater irrigation, some criteria should be taken into account such as water quality, economic consideration and farming conditions. It seems that olive tree irrigation with treated wastewater saves costs and provides benefits to farmers (Feigin et al. 2012; Mansir et al. 2024). Other criteria, such as soil capacity to release water source into its solution and culture's physiological adaptations, should be taken into account before treated wastewater application (Hoogendijk et al. 2023).

In freshwater scarcity situations, water use for irrigation should be managed. In this situation, surface drip irrigation method is recommended and may, therefore, help to reduce evaporation and to conserve water. When this method is applied, water is distributed directly near the roots (Hashem and Qi 2021). According to Malash et al. (2005), the use of wastewater is a safe practice when the drip irrigation method is applied. Besides, Orlofsky et al. (2016) reported that a surface drip irrigation system is suitable for tomato-treated wastewater irrigation. Several studies have focused on olive tree physiological changes in response to treated wastewater irrigation. The present study expands the scope of this work to include the metals, salts and micronutrient accumulation. In this way, this study aims to, firstly, monitor biochemical parameters such as total phenols, soluble sugar and chlorophyll contents on olive leaves and root extracts in response to TWW irrigation. Secondly, this work aims to understand elements translocation throughout olive tree parts.

Methods

Wastewater collection

Treated wastewater was collected from the wastewater treatment plant of the dairy industry. This industry generates around 2000 m^3 of wastewater daily (Sdiri et al. 2018). This water was used throughout all processing steps of the dairy industry, including cleaning, sanitisation and cleaning of external areas. This source is treated at a secondary level using biological processes that consist of eliminating the biodegradable matter by their transformation into microbial residues. Aerobic biological treatment is performed by aerobic microorganisms that metabolise the organic matter in the wastewater, thereby producing more microorganisms and inorganic end products (CO_2, NH_3 and H_2O) (Gharsallaoui et al. 2011). This water is pumped out after treatment to a river. Grab sampling was carried out. Representative samples were collected from the treatment plant decanter. Samples were brought immediately for analysis.

Field site and plant material

This study was carried out in the province of Mahdia (Dkhila), Tunisia (35° 31N, 10° 58E, 7 m above sea level). This region is characterised by a Mediterranean climate whose conditions have been described in our previous work (Sdiri et al. 2020). Seven-month-old and uniform olive transplants of *Olea europaea* L. cv. Chemlali were planted for this experimental study which was carried out during 12 months.

Experimental design and irrigation schedule

The impact of industrial treated wastewater was examined on olive trees which were planted beside the dairy industry. Soil physico-chemical characteristics have been described in our previous work (Sdiri et al. 2023).

This industry was the source of the treated wastewater used for olive tree irrigation. Young olive trees were arranged in rows; each row (irrigated either with tap water or treated wastewater) was composed of fifteen trees (n = 15) with a distance between individual trees of 2.5 m. The distance between rows was 5 m. Two irrigation systems and two rows for each system were applied. The irrigation period lasted 12 months (from March 2017 to February 2018). The thirty-one trees were irrigated with a surface drip irrigation system with two drip emitters per plant (one per side) delivering both 14 L/plant/irrigation. Irrigation was applied two times per week from June to August and weekly during other months of the year and lasted 24 min. The other thirty trees were sustained a manual irrigation and plants received the same doses which were previously described.

Doses were chosen based on crop evapotranspiration rate (ET_c). Annual requirements were covered by irrigation applied and rainfall effective (FAO 1976). Water requirements were calculated considering the recommended crop coefficient (K_c) and reduction coefficient (K_r) depending on the percentage of the ground surface covered by the crop (FAO 1998; Masmoudi-Charfi et al. 2004).

Treated wastewater quality

Treated wastewater used in the present study contains highly essential nutrients such as potassium (K), nitrogen (N) and phosphorus (P). In addition, this water is devoid of pesticides and heavy metals and presents physico-chemical parameters (electrical conductivity (EC), chemical oxygen demand (COD), pH, biological oxygen demand (BOD) …) within limits established for treated wastewater use for irrigation (NT 106.03). Dairy-treated wastewater presents considerable nitrogen content. Irrigation water quality was described in our previous work (Sdiri et al. 2018).

Total phenols concentration

Leaves and roots total phenol concentration were determined using the method described by Montedoro et al. (1992). Total phenols were extracted from 0.5 g of olive leaves or roots dissolved in 10 mL of methanolic solution. Then, the Folin–Ciocalteu reagent was used and absorption was measured with a spectrophotometer at 765 nm (Perkin Elmer Lambda 25).

Quantification of soluble sugars

Following treatment application, olive leaves and roots were sampled and soluble sugar contents were determined. Dry crushed samples (0.1 g) were dissolved in 10 mL of ethanol. Soluble sugars were extracted and quantified at 485 nm following the phenol-sulphuric acid method application which was described by Dubois et al. (1956).

Chlorophyll pigments determination

According to Arnon (1949), 0.25 g of fresh olive leaves were homogenised in 80% acetone. The optical density was measured spectrophotometrically at 647, 663 and 470 nm for chlorophyll a, chlorophyll b, total chlorophylls and carotenoids contents, respectively. Pigment content was measured using a spectrophotometer (Perkin Elmer Lambda 25).

Element determination by ICP-MS

Leaves and root element contents were determined according to the method described by Di Bella et al. (2016). Before analysis, lyophilised samples were digested by H_2O_2 (30%) in addition to 0.5 g of each sample. Then, HNO_3 (65%) was added for mineralisation using a closed-vessel microwave digestion system (CEM Microwave™ Digestion System, Discovery SP-D, CEM Corporation, Mathews, NC). Mineral and metal contents were determined using Inductively Coupled Plasma-Mass Spectrometry (ICP-MS), using an iCAP Q (Thermo Scientific, Waltham, MA) spectrometer equipped with an autosampler ASX520 (Cetac Technologies Inc., Omaha, NE).

Statistical analysis and data representation

To examine wastewater and irrigation system effects on olive leaves and roots composition, the software SPSS, release 23.0, for Windows (SPSS, Chicago, IL, USA) was used. Values of measured parameters are shown as means ± standard deviations (n = 15). The statistical significance level was fixed at $p < 0.05$. Differences between means were tested by Duncan's test application.

The analyses and visualisations, including the representation of heatmap matrices, were performed using Colab, an interactive Python environment hosted in the cloud, with the matplotlib, seaborn and pandas libraries (McKinney 2022).

Results

Total phenols and soluble sugar contents

It can be seen in Figure 1 that the TWW application, using the MI system, didn't affect significantly leaves total phenol content compared to the TW application. The total phenol amounts were about 27971.5 mg kg^{-1} and 27629 mg kg^{-1}, respectively. However, the use of the SDI system leads to an increase in leaves' total phenols content following TWW irrigation to rich a value of 27383.33 mg kg^{-1}. In terms of root phenol content, an enhancement was obtained only in response to the MI system. This elevation was independent of water resources. On the other hand, Figure 1 oesn't show the soluble sugar amount enhancement in the leaves and roots of plants irrigated with TWW for those when TW was used.

Chlorophylls and carotenoids

Chlorophylls a, chlorophylls b, total chlorophylls and carotenoid contents were compared under different treatment applications in the leaves of olive plants (Figure 2). According to

Figure 1. Heatmap of the effect of irrigation treatments on olive leaves and roots' total phenols and soluble sugar contents (mg/kg). Data represents mean values (n = 15) ± standard deviation. Horizontally, values with the same letter are not significantly different at a 5% probably level according to Duncan's test. TWMI = Tap Water irrigation using a Manual Irrigation system; TWWMI = Treated Wastewater irrigation using a Manual Irrigation system; TWSDI = Tap Water irrigation using a Surface Drip Irrigation system; TWWSDI = Treated Wastewater irrigation using a Surface Drip Irrigation system. Values are represented by colours. Each cell in the heatmap corresponds to a specific value and the colour of the cell indicates the intensity or magnitude of that value. The colours typically follow a gradient, ranging from darker tones for lower values to brighter tones for higher values, making it easier to identify patterns, relationships or trends within the data.

the obtained results, TWWSDI treatment application increases significantly ($p < 0.05$) these parameters values compared to the other treatments. The obtained amounts of chlorophylls a, chlorophylls b, total chlorophylls and carotenoids, following TWWSDI treatment application, were 23.44 mg kg^{-1}, 8.67 mg kg^{-1}, 35.82 mg kg^{-1} and 7.18 mg kg^{-1}, respectively.

Minerals and heavy metals

As shown in Figure 3 and 4, the TWWSDI treatment application increases significantly Mg leaves and root contents compared with the TWWMI treatment. This increase was about 38.65% and 68.17% in olive tree leaves and roots, respectively.

Moreover, the TWWSDI treatment influences positively K and Ca leaves' contents compared with the other treatments enhancing K and Ca amounts to reach 7481.98 mg kg^{-1} and 3506.64 mg kg^{-1}, respectively. For Na, the same treatment (TWWSDI) enhances its content but in olive roots up to a concentration of 5730.09 mg kg^{-1}. Leaves and roots P contents are also shown in Figure 3 and 4. It was

Figure 2. Heatmap of the effect of irrigation treatments on olive leaves chlorophyll a, chlorophyll b, total chlorophyll and carotenoid contents (mg/kg). Data represent mean values (n = 15) ± standard deviation. Horizontally, values with the same letter are not significantly different at a 5% probably level according to Duncan's test. TWMI = Tap Water irrigation using a Manual Irrigation system; TWWMI = Treated Wastewater irrigation using a Manual Irrigation system; TWSDI = Tap Water irrigation using a Surface Drip Irrigation system; TWWSDI = Treated Wastewater irrigation using a Surface Drip Irrigation system. Values are represented by colours. Each cell in the heatmap corresponds to a specific value, and the colour of the cell indicates the intensity or magnitude of that value. The colours typically follow a gradient, ranging from darker tones for lower values to brighter tones for higher values, making it easier to identify patterns, relationships or trends within the data.

revealed that olive trees' TWW irrigation using the MI system (TWWMI) didn't induce significant (p>0.05) P accumulation compared with amounts obtained following TW irrigation using the same system.

Statistically, significant differences were observed in terms of leaves and roots for Fe, Cu and Se amounts in response to TWWSDI and TWWMI treatment application. It seems that the MI system leads to higher leaves and roots Fe, Cu and Se accumulation than the SDI system. For leaves Fe, Cu and Se amounts, this enhancement was about 62.7, 10.6 and 30%, respectively. In terms of roots Fe, Cu and Se contents, an increase of 237.6, 9152.45 and 1850%, was recorded respectively.

Discussion

Secondary metabolites provide plants' protection against environmental stresses, especially, phenols whose contents increase in response to biotic or abiotic stresses.

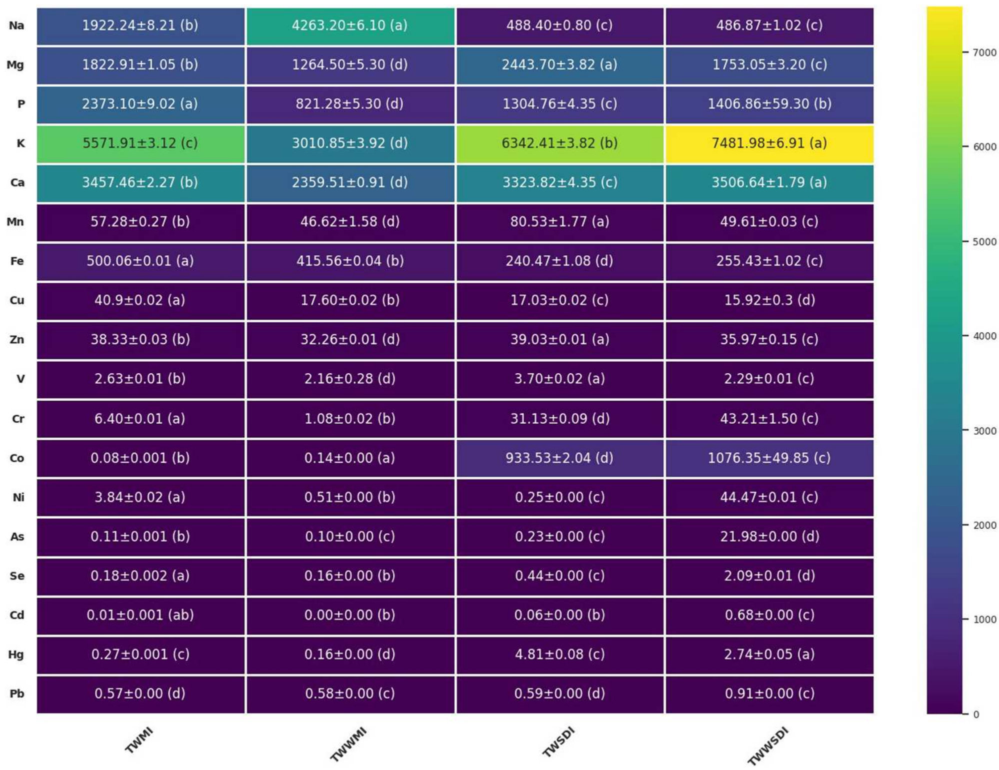

Figure 3. Heatmap of the effect of irrigation treatments on olive leaves' mineral contents (mg/kg). Data represent mean values (n = 15) ± standard deviation. Horizontally, values with the same letter are not significantly different at a 5% probably level according to Duncan's test. TWMI = Tap Water irrigation using a Manual Irrigation system; TWWMI = Treated Wastewater irrigation using a Manual Irrigation system; TWSDI = Tap Water irrigation using a Surface Drip Irrigation system; TWWSDI = Treated Wastewater irrigation using a Surface Drip Irrigation system. Values are represented by colours. Each cell in the heatmap corresponds to a specific value and the colour of the cell indicates the intensity or magnitude of that value. The colours typically follow a gradient, ranging from darker tones for lower values to brighter tones for higher values, making it easier to identify patterns, relationships or trends within the data.

Moreover, Petridis et al. (2012) reported an enhancement in plants' phenol content following abiotic stresses. In response to treated wastewater irrigation, Tekaya et al. (2016) revealed a phenol content decrease in 'Chemlali' cultivar olive leaves. In the present study, olive trees' TWW irrigation (TWWMI) didn't cause leaves and roots' polyphenol content changes. According to Zandi and Schnug (2022), phenols are known as metal chelators and reactive oxygen species scavengers. Phenols present antioxidant properties in stress conditions in response to reactive oxygen species elevation in plant tissues. Therefore, it can be asserted from our data that treated wastewater doesn´t cause olive trees stress. In this regard, Pratyusha (2022) and Kısa et al. (2016) showed that the total phenol content tends to increase when the plants, such as corn plants, are under heavy metal stress. Thus, it can be asserted that the unchanged phenol content, revealed in the present work, reflects the wastewater's good quality which is devoid of heavy metals. Moreover, soluble sugar amounts didn't increase in the leaves and roots of

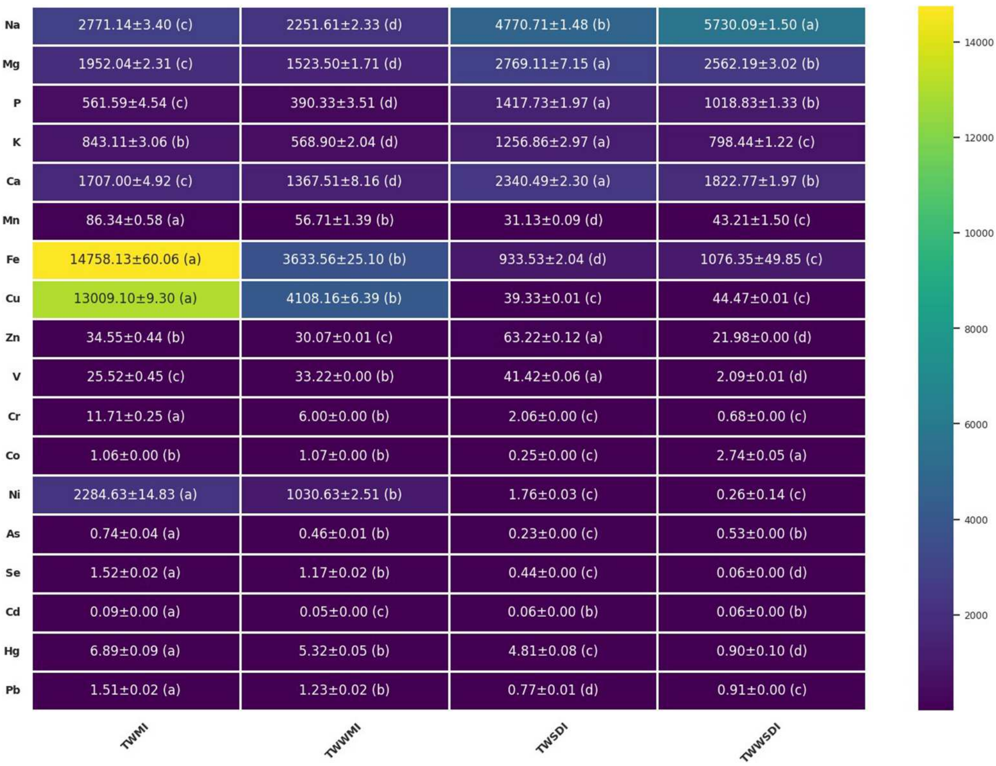

Figure 4. Heatmap of the effect of irrigation treatments on olive roots mineral contents (mg/kg). Data represent mean values (n = 15) ± standard deviation. Horizontally, values with the same letter are not significantly different at a 5% probably level according to Duncan's test. TWMI = Tap Water irrigation using a Manual Irrigation system; TWWMI = Treated Wastewater irrigation using a Manual Irrigation system; TWSDI = Tap Water irrigation using a Surface Drip Irrigation system; TWWSDI = Treated Wastewater irrigation using a Surface Drip Irrigation system. Values are represented by colours. Each cell in the heatmap corresponds to a specific value, and the colour of the cell indicates the intensity or magnitude of that value. The colours typically follow a gradient, ranging from darker tones for lower values to brighter tones for higher values, making it easier to identify patterns, relationships or trends within the data.

wastewater-irrigated trees compared with those registered in samples obtained from tap water-irrigated trees. A positive correlation between plants' stress tolerance and soluble sugar accumulation was reported in the literature (Rosa et al. 2009; Sami et al. 2016). Mouri et al. (2012)'s finding revealed an osmoprotective role of sugars in mitigating abiotic stress in plant cells. Ben Hassena et al. (2018) demonstrated soluble sugar content enhancement in olive leaves following TWW tree irrigation. These authors referred to sugar content enhancement to starch degradation to satisfy the energy requirements for plants' physiological activity.

The present data showed high leaves and roots Mg accumulation following the TWWSDI application compared with results registered in response to the TWWMI application. This input induced probably total chlorophyll accumulation in the same leaf type cited previously. The TWWSDI treatment may increase Mg bioavailability in olive trees and its vertical transfer to leaves. According to Tekaya et al. (2016), Mg^{2+} is essential for chlorophyll biosynthesis. Our results are in line with those obtained by Helaly et al. (2018) which indicated a total chlorophyll content enhancement in mango trees (*Mangifera indica* L.) irrigated with treated wastewater. Tekaya et al. (2016) reported that wastewater irrigation improves olive leaves' photosynthetic parameters and therefore ameliorates plant physiological performance. This improvement results from essential nutrient input following wastewater application. In addition, Ben Hassena et al. (2018) suggests that wastewater is a rich source of essential nutrients contributing to ameliorating the photosynthetic capacity of young olive trees.

However, the present results are not in line with a previous work about chlorophyll content for the olive 'Koroneiki' cultivar. Petousi et al. (2015) didn't report any modification in terms of this agronomic trait content following wastewater irrigation. Then, our results are not in accordance with the results obtained by other investigators showing photosynthetic apparatus damage in lantana (*Lantana camara* L.) plant leaves irrigated with treated wastewater (Banón et al. 2011).

Mansir et al. (2024) considered olive tree TWW irrigation as a safe practice and didn't affect leaves' physiological parameters. Moreover, the same authors revealed photosynthesis rate enhancement following TWW irrigation. Therefore, they recommend TWW use by farmers for many benefits such as cost reduction.

Our results reported a significant increase in leaves K and Ca contents, suggesting a certain fertilising effect of TWW irrigation using the SDI system (TWWSDI). This increase was registered compared with the other treatments and results from K and Ca cation adsorption and its translocation until olive trees aerial parts. According to Anwar et al. (2016), mint (*Mentha arvensis* L.) TWW irrigation improved the uptake of K which plays an important role in plant growth and development.

Contrarily, TWWSDI application induced a higher accumulation of Na in roots than in leaves analysed in the present study. Our results are in line with those obtained by Hassena et al. These authors reported olive plant roots' role as an effective barrier against transport and excessive accumulation of Na in leaves following TWW irrigation.

With respect to tap water (TW), our previous work (Sdiri et al. 2018) showed that treated wastewater (TWW) presents a higher P amount. Following the TWWMI treatment application, irrigated soils didn't show P accumulation (Sdiri et al. 2023). On the other hand, the present data didn't show P accumulation in olive tree leaves and roots. These results may be explained by P migration due to the high demand of P from the fruits for oil biosynthesis and because of its role in the reproductive processes (Erel et al. 2008; Erel et al. 2011; Bedbabis et al. 2014). According to Hoogendijk et al. (2023), the possibility of irrigating crops with wastewater depends on the composition of the waste load. For example, contaminated wastewater with heavy metals can present a potential risk for soil physico-chemical properties. In our study, plants that received the TWWSDI treatment were characterised by leaves and roots Fe, Cu and Se contents lower than those registered in plants leaves and roots that received the TWWMI treatment. The latter remained cultivated in less contaminated soils following irrigation compared with soils that received the TWWSDI treatment. These results reflect that olive trees under the TWWMI treatment, present capacity to absorb heavy metals which are then concentrated in olive leaves and roots. In this case, Fe, Cu and Se translocation to leaves can result from their influx increase into the xylem vessels so their transfer to the leaves (Hajihashemi et al. 2020). It can be revealed that Fe, Cu and Se elements' mobility to olive tree parts is maintained by a manual irrigation system (MI) and blocked by a surface drip irrigation system (SDI). Furthermore, in our study, a decrease in Zn and Cr contents was recorded in olive leaves and roots irrigated with TWW. Mkhinini et al. (2018) reported that an exceeded dose of Zn and Cr in TWW could cause phytotoxicity.

The present results are not in accordance with an earlier study by Petousi et al. (2015), showing that wastewater irrigation doesn´t modify olive leaves nutrients and heavy metal contents compared with freshwater irrigation. On the other hand, long-term irrigation with TWW can be applied with caution and oil quality should be assessed. Al-Habahbeh et al. (2021) considered olive trees as heavy metal accumulators. Ofori et al. (2025) reported that pollutant translocation and uptake depend on plant physiology. It can be asserted that TWW, used in the present work, doesn´t induce young olive trees' abiotic stress manifested by phenols and sugar content modification in the plant parts. Furthermore, this agronomic practice can improve plant physiological performance by enhancing its total chlorophyll content. These results may be considered courageous to use treated wastewater safely. Contrarily, it was reported in the literature that industrial effluent use affects plant physiology (Gufran Khan et al. 2011).

Conclusion

In light of these results, it is suggested that the use of wastewater can be an option to save water resources for domestic use. Treated wastewater irrigation doesn´t affect olive roots and leaves physiological parameters like phenols and sugar contents. This work explains that TWW irrigation using the SDI system suggests a certain fertilising effect by enhancing minerals amounts like Mg, K and Ca in olive leaves and roots. Therefore, this treatment (TWWSDI) application leads to ameliorating plant physiological performance by improving chlorophyll synthesis. However, TWWSDI treatment enhances Na accumulation in olive roots. In terms of heavy metals translocation to olive tree parts, it seems that this phenomenon is maintained by the MI system and blocked by the SDI system. The current results are promising and encouraging to recommend the use of treated wastewater as an alternative option for orchard irrigation.

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Author contributions

Wiem Sdiri: Methodology and Writing – original draft; Dhekra Toumi: Writing – review & editing; Houda Akremi: heatmap preparation; Rym Hassani: Investigation, Formal analysis; Hedi Ben Mansour: Conceptualisation, Supervision.

Disclosure statement

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ORCID

Rym Hassani http://orcid.org/0000-0002-9340-8835
Hedi Ben Mansour http://orcid.org/0000-0001-7936-9828

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