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Water reuse of treated domestic wastewater in agriculture: Effects on tomato plants, soil nutrient availability and microbial community structure

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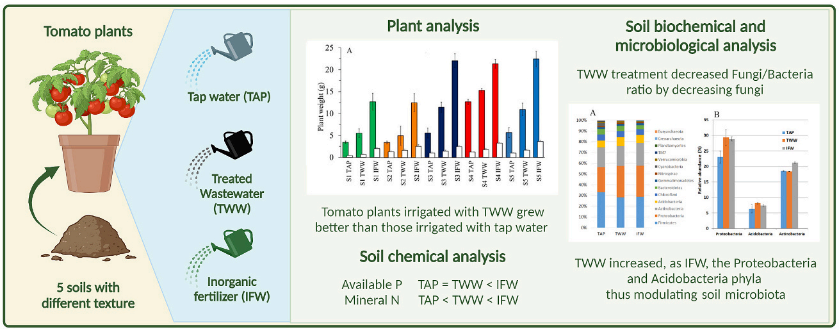
HIGHLIGHTS

- Soils irrigation with tap water, treated wastewater or inorganic nutrient solution.
- Tomato plants irrigated with treated wastewater grew better compared to tap water.
- Treated wastewater did not affect soil available P but increased mineral N.
- Treated wastewater shaped soil microbial community structure, mainly fungi.
- Treated wastewater increased, along with IFW, Proteobacteria and Acidobacteria phyla.

GRAPHICAL ABSTRACT



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ABSTRACT

The reuse of treated wastewater (TWW) in agriculture for crop irrigation is desirable. Crop responses to irrigation with TWW depend on the characteristics of TWW and on intrinsic and extrinsic soil properties. The aim of this study was to assess the response of tomato (*Solanum lycopersicum* L.) cultivated in five different soils to irrigation with TWW, compared to tap water (TAP) and an inorganic NPK solution (IFW). In addition, since soil microbiota play many important roles in plant growth, a metataxonomic analysis was performed to reveal the prokaryotic community structures of TAP, TWW and IFW treated soil, respectively. A 56-days pot experiment was carried out. Plant biometric parameters, and chemical, biochemical and microbiological properties of different soils were investigated. Shoot and root dry and fresh weights, as well as plant height, were the highest in plants irrigated with IFW followed by those irrigated with TWW, and finally with TAP water. Plant biometric parameters were positively affected by soil total organic carbon (TOC) and nitrogen (TN). Electrical conductivity was increased by TWW and IFW, being such an increase proportional to clay and TOC. Soil available P was not affected by TWW, whereas mineral N increased following their application. Total microbial biomass, as well as, main microbial groups were positively affected by TOC and TN, and increased according to the following order: IFW > TWW >

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TAP. However, the fungi-to-bacteria ratio was lowered in soil irrigated with TWW because of its adverse effect on fungi. The germicidal effect of sodium hypochlorite on soil microorganisms was affected by soil pH. Nutrients supplied by TWW are not sufficient to meet the whole nutrients requirement of tomato, thus integration by fertilization is required. Bacteria were more stimulated than fungi by TWW, thus leading to a lower fungi-to-bacteria ratio. Interestingly, IFW and TWW treatment led to an increased abundance of Proteobacteria and Acidobacteria phyla and *Balneimonas, Rubrobacter,* and *Steroidobacter* genera. This soil microbiota structure modulation paralleled a general decrement of fungi versus bacteria abundance ratio, the increment of electrical conductivity and nitrogen content of soil and an improvement of tomato growth. Finally, the potential adverse effect of TWW added with sodium chloride on soil microorganisms depends on soil pH.

1. Introduction

The problems with water supply and decrease in water quality are critical global issues (Lahlou et al., 2021). Water scarcity is causing significant challenges worldwide, particularly in arid and semiarid regions; it is predicted that by 2025, approximately 1.8 billion people will reside in areas with limited access to water (Hashem and Qi, 2021). These risks are expected to worsen soon due to factors such as population growth, climate change, and increased demand for water from various sectors (Andrews et al., 2016; Hashem and Qi, 2021). This question is a substantial threat to agricultural productivity, food security, and mankind stability. The agricultural sector's over-dependence on water resources causes worries about its long-term sustainability and resilience. Currently, irrigation alone consumes 80 % of freshwater resources, and this demand is expected to increase in the near future. This will exacerbate water shortages in regions already affected by water scarcity, enforcing the exploration of innovative techniques to optimize water use (Lahlou et al., 2021). In this context, treated wastewater (TWW) is becoming increasingly required in agriculture.

Wastewater helps to meet the growing water demand and contributes to mitigating environmental pollution (Aziz and Farissi, 2014; Saliba et al., 2018). Indeed, if appropriately treated, wastewater can become microbiologically safe, making it suitable for crop irrigation within a circular economy perspective (Elbana et al., 2019; Ofori et al., 2021; Lucia et al., 2022). Moreover, wastewater contains nutrients, which constitute an opportunity for agriculture to reduce dependence on chemical fertilizers and minimize effluent discharge into water bodies (Urbano et al., 2017; Lahlou et al., 2021). Exploiting these nutrients, wastewater can serve as a valuable resource, promoting sustainable agricultural practices. However, several advanced wastewater treatment technologies that ensure microbiological safety, the current global use of treated wastewater remains very limited, with <1 % reusing of the total water sanitized (Salgot and Folch, 2018; Saliba et al., 2018). These limitations are related to social, economic, and legislative factors (Mannina et al., 2021a).

An issue regarding TWW for agricultural irrigation may be the high concentration of dissolved ions, particularly sodium, magnesium, potassium, calcium, and chloride. Gao et al. (2021), conducted a meta-analysis to evaluate the effects of irrigation with TWW on crops and soil. The authors pointed out that using treated water for irrigation can significantly benefit crops, improving their growth and yield by providing nutrients such as nitrogen, phosphorus, and potassium. However, they also pointed out that the high salt content in treated water, particularly sodium and chloride, has been observed by several authors to inhibit plant growth (Raveh and Ben-Gal, 2016). Urbano et al. (2017) evaluated the effect of irrigation with treated domestic wastewater on lettuce, focusing on the physical, chemical, and microbiological characteristics of the soil, as well as the yield and quality of the lettuce. In their study, they tested drinking water plus conventional fertilization and TWW plus partial conventional fertilization. The results showed that irrigation with TWW led approximately to a doubling in the soil concentration of exchangeable basic cations, particularly Mg^{2+} and Ca^{2+}. No traces of *Escherichia coli* bacteria were found on the lettuce leaves or in the soil. Additionally, irrigation with TWW did not cause damage to the physical properties of the soil but contributed to improving macronutrient availability, such as Mg^{2+}, Ca^{2+} and K^+. Moreover, there were not macronutrients (N, P, K, Ca, Mg, S) and micronutrients (B, Cu, Fe, Mn, Zn) significant differences in lettuce leaves between drinking water plus conventional fertilization and TWW plus partial conventional fertilization.

Regarding the impact on the soil, an overall increase in salinity and exchangeable sodium activity was predominantly observed, which may negatively affect soil quality (Pedrero et al., 2010) and crop grown. In general, irrigation with treated wastewater increases soil electrical conductivity, but the effects on soil pH and crop yield may be incoherent (Leonel et al., 2022). Therefore, Gao et al. (2021) suggested that it is crucial to carefully consider the level of wastewater treatment (primary, secondary, or tertiary) and the physical and chemical characteristics of the soil when using treated wastewater for irrigation. Furthermore, Zhang et al. (2018) observed that exposure to chloride and mixed chloride-sulphate salts when applied to soil can negatively affect on the soil microbial community. They observed the impact of these salts on *Bacillus* spp. and bacterial and fungal community structures in the soil. Their results determined specific threshold salt concentrations for maintaining soil physiological function, with distinct values of 1.26 and 0.45 dS m^{-1} for the mixed Cl^-/SO_4^{2-} and Cl^- salts, respectively, while those for soil microbial community structural diversity were 0.70 dS m^{-1} for Cl^- and 1.75 dS m^{-1} for Cl^-/SO_4^{2-}. In other words, they suggested that soil physiological function is more sensitive to salt levels than the structural diversity of the soil microbial community.

We conducted a comprehensive investigation to evaluate the effect of TWW, to which sodium hypochlorite had been added for disinfection, on the growth of tomato plants, the soil chemical properties and the microbial community structure. Unlike the previous studies that mainly focused on comparing wastewater with tap water or that used wastewater only as a solvent for dissolving fertilizers, this study also investigated the influence of physical and chemical characteristics of the soil as a new variable. For this purpose, five soils with different chemical (e.g. total organic carbon) and physical (e.g. clay content) properties were chosen to evaluate the fertilizing effect of TWW compared to simple tap water irrigation and the traditional inorganic N:P:K fertilization (20,10,20) for plant growth. Also, the soil microbial groups and community structure were characterized by using metataxonomic and fatty acids analyses, respectively.

2. Materials and methods

2.1. Experimental setup

The experiment was conducted in a greenhouse located at the University of Palermo (Palermo, Sicily, Italy; 38°10′67.16” N; 13°35′03.24″ E), from May to July 2022. Tomato (*Solanum lycopersicum* L.) plants were chosen as test due to their ability to withstand high temperatures. The experimental design followed a complete randomized scheme, with three irrigation treatments (tap water, TAP; treated wastewater, TWW; inorganic fertilized water, IFW) through five different soils and three replicates, resulting in a total of forty-five pots. Soils were collected from the superficial layer of five agricultural sites in Sicily (S1: 38°04′54.5”N

13°04′23.0″E, S2: 38°10′09.7"N 13°21′22.1″E, S3: 38°02′07.8"N 13°27′52.0″E, S4: 37°54′32.2"N 13°45′19.4″E, S5: 38°06′27.3"N 13°21′09.5″E), Italy, at a depth of 0–20 cm. Chemical and physical properties of soils are reported in Table 1.

Pots (8 × 8 × 15 cm) were filled firstly with expanded clay (about 10 g) and then with 600 g of soil sieved at 4 mm. The IFW treatment consisted of water solution of N:P:K inorganic fertilizers (20:10:20) at a concentration of 1 g L^{-1} applied once a week. The total amount of nutrients supplied to each IFW pot during the experiment was 200 mg of N (50 % as NH_4^+ and 50 % as NO_3^-), 44 mg of P as P_2O_5 and 166 mg of K as K_2O. To ensure that the irrigation requirements of the tomato plants were adequately met, the soil moisture was maintained at field capacity. Manual irrigation was performed three times per week with two daily applications, at 11:00 am and 6:00 pm, to prevent water percolation. The total volume of water supplied during the whole experiment was 2.8 L in S1, 4.4 L in S2, 5.6 L in S3, 7.8 L in S4 and 3.3 L in S5.

2.2. Properties of water used in this study

Tap water was obtained directly from the storage tank serving the greenhouse where the experiment was carried out (Mannina et al., 2021b). Treated wastewater was obtained from the pilot wastewater treatment plant located at the Department of Engineering of the University of Palermo (Italy), as part of the Wider Uptake H2020 project (Mannina et al., 2021b). Wastewater was treated with sodium hypochlorite at a concentration of 5 mg L^{-1} for sanitization purposes.

The main physical and chemical characteristics of both TAP and TWW are reported in Table 2 and Table S1, whereas the amount of nutrients supplied by irrigation treatments are reported in Table 3.

2.3. Post pot-experiment analyses

After 56 days, plant height, root length, and dry and fresh weights were recorded to gather information about plant health. In addition, the soil of each pot was fully mixed, air dried, sieved at <2 mm, and then analysed. Soil reaction (pH) and electrical conductivity (EC) of soil extracts (1:2.5, *w/v*) were determined by a pHmeter (FiveEasy, Mettler Toledo Spa, Milan, Italy) and a conductometer (HI5321, Hanna Instruments Italia srl, Padua, Italy), respectively. Ammonium and NO_3^- were quantified on 0.5 M K_2SO_4 soil extracts (1:4, w/v) through colorimetric analysis employing the Berthelot method (Mulvaney, 1996) and the Spectroquant® Nitrate test, using a spectrophotometer (UVmini-1240, Shimadzu Italia srl, Milan, Italy) after the formation of a yellow-green and red complex, respectively. Additionally, P Olsen was measured using the colorimetric Olsen method with sodium bicarbonate extraction at pH 8.5, using the same spectrophotometer after the formation of a blue complex (Murphy and Riley, 1962).

The main soil microbial groups were analysed by the direct extraction of the ester-linked fatty acids (ELFAs), following the method proposed by Schutter and Dick (2000). A Thermo Scientific FOCUS™ gas chromatograph equipped with a flame ionization detector and a fused-silica capillary column Mega-10 (50 m × 0.32 mm I.D.; film thickness 0.25 μm) was used for ELFA detection and quantification. The gas chromatograph temperature was set as follows: initial isotherm at 140 °C for 5 min, increase at a rate of 1.5 °C per minute from 140 to 230 °C and final isotherm at 230 °C for 2 min. The identification of FAME peaks was based on comparing retention times with known standards (Supelco Bacterial Acid Methyl Esters mix cat no. 47080-U and Supelco 37 Component FAME mix cat no. 47885-U). FAMEs were expressed as nmol g^{-1} dry soil. FAs with <14 carbon atoms or >19 carbon atoms were excluded as originating from non-microbial sources (Frostegård and Bååth, 1996; Laudicina et al., 2012). The FAs i15:0, a15:0, 15:0, i16:0, i17:0, 17:0, cy17:0, 18:1ω7 and cy19:0 were considered to represent bacterial biomass. Saprotrophic and ectomycorrhizal fungi (hereafter fungal biomass) were represented by 18:2ω6,9 and 18:1ω9 (Olsson, 1999). Gram-positive bacteria (BacG+) were quantified by summing the FAs i15:0, a15:0, i16:0 and i17:0, while Gram-negative bacteria (BacG-) were obtained by summing FAs 18:1ω7, cy17:0 and cy19:0.

Table 1
Physical and chemical properties of soils (S1, S2, S3, S4 and S5) used in this study.

Soil property	S1	S2	S3	S4	S5
Clay (%)	2	25	32	43	15
Silt (%)	6	25	18	29	22
Sand (%)	92	50	50	28	63
Water holding capacity (%)	12	20	25	35	15
pH (in H_2O)	7.0	7.2	7.8	8.0	7.9
Electrical conductivity (μS cm^{-1})	258	261	269	331	200
Total carbonates (%)	8	3	23	33	17
Cation exchange capacity ($cmol_+$ kg^{-1})	5	15	24	33	35
Total organic carbon (g kg^{-1})	8	13	13	31	34
Total nitrogen (g kg^{-1})	0.4	1.3	1.7	2.6	3.5
Available P (mg kg^{-1})	39	42	43	42	45

Table 2
Chemical characteristics of the tap (TAP) and treated wastewater (TWW) used for tomato irrigation.

Water property	TAP	TWW
pH	7.5 ± 0.1	8.1 ± 1
EC (dS m^{-1})	0.10 ± 0.0	1.1 ± 0.1
Total Hardness (°f)	8 ± 1	32 ± 3
N (as NH_4^+; mg L^{-1})	0.1 ± 0.0	0.5 ± 0.0
N (as NO_3^-; mg L^{-1})	0.1 ± 0.0	0.2 ± 0.0
Total P (mg L^{-1})	0.02 ± 0.00	0.21 ± 0.0
Fe (mg L^{-1})	0.1 ± 0.0	3.0 ± 0.2
Mn (mg L^{-1})	0.001 ± 0.000	0.041 ± 0.000
Ca (mg L^{-1})	14 ± 2	69 ± 6
K (mg L^{-1})	1.0 ± 0.2	22 ± 3
Mg (mg L^{-1})	2.0 ± 0.2	19 ± 3
Na (mg L^{-1})	3.0 ± 0.2	155 ± 27

Table 3
Amounts (mg) of nutrients (N, P, K) added by different irrigation treatments to each of the three pots filled with 600 g of soil. The amount of nutrients supplied by tap (TAP) and treated wastewater (TWW) was calculated by multiplying the concentration of each nutrient in water by the total volume of water added to irrigate tomato plants during the experiment.

Soil	Water	N	P	K
S1	TAP	0.6	0.06	2.8
	TWW	2.0	0.6	62
	IFW	200	44	166
S2	TAP	0.9	0.09	4.4
	TWW	3.1	0.9	93
	IFW	200	44	166
S3	TAP	1.1	0.11	5.6
	TWW	3.9	1.2	123
	IFW	200	44	166
S4	TAP	1.6	0.16	7.8
	TWW	5.5	1.6	172
	IFW	200	44	166
S5	TAP	0.7	0.07	3.3
	TWW	2.3	0.7	73
	IFW	200	44	166

2.4. Prokaryotic metataxonomics

Analysis of the prokaryotic microbiota structure of soil was performed by metataxonomics based on next-generation sequencing (NGS) analysis of 16S rRNA gene amplicons obtained from metagenomic DNA. The analysis was carried out on S5 soil, i.e. soil with the highest concentration of total organic C (Table 1). Two samples per water treatment were analysed (i.e. S5 TAP, TWW, and IFW in duplicate). The extraction of DNA from the soil microbial community was carried out using the DNeasy PowerSoil® Pro Kit (Qiagen) following the manufacturer's instructions. The DNA extractions were evaluated by 1 % (*w/v*) agarose gel electrophoresis analysis, with the addition of 0.5 μg mL^{-1} ethidium bromide for visualization using a UV lamp. The concentrations of the DNA, extracted from soil samples and the corresponding tenfold serial dilutions were measured by reading absorbance at 260 nm with a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The purity of the extracted DNA was evaluated by measuring the absorbance ratios (260/280 and 260/230 nm) to indicate contamination due to proteins and organic compounds or chaotropic agents, respectively (Sambrook et al., 1989). The following primers were used for PCR amplification of the V3-V4 regions of the 16S gene: PRO 341F CCTACGGGNBGCASCAG and PRO 805R GACTACNVGGGTATCTAATCC. For PCR, DreamTaq DNA polymerases (ThermoFisher Scientific) was used following the manufacturer's instructions. The thermal profile used was: 95 °C for 2 min; 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, elongation at 72 °C for 30 s; final elongation at 72 °C for 5 min. Amplification products were sequenced in one 300 bp paired end run on an Illumina MiSeq platform at BMR Genomics (Padova, Italy). The raw 16S rDNA data were processed by using the QIIME2 software (https://qiime2.org/ accessed on 22.12.2023) as paired-end sequences. In the denoising approach, overlapping paired-end reads were processed with the plugin DADA2. Unique amplicon sequence variants (ASVs) were assigned and aligned to the Greengenes reference database at 99 % sequence similarity (https://greengenes.secondgenome.com/). The number of ASVs and the percentages of relative abundances of domain, phyla, orders, classes, families, genus and specie were determined. In this work domains, phyla and genera are reported and discussed.

Principal coordinate analysis (PCoA) was chosen as a multivariate statistical approach and was performed using the Bray–Curtis distance matrix and the Emperor software. METAGENassist (http://www.metagenassist.ca) was used to analyze the predicted metabolic capacities of the microbiota based on the abundances of the residing prokaryotic genera.

2.5. Statistical analyses

Reported results are arithmetic means of three soil sample replicates and are expressed on an oven-dry weight basis (105 °C). Two-way analysis of variance (ANOVA) of the measured variables was performed with soil type (S1, S2, S3, S4 and S5) and irrigation treatment (TAP, TWW and IFW) as main factors. Significant statistical differences within the same soil type, among three irrigation treatments, and at the same irrigation treatments, among five soil types were established by Tukey test ($P < 0.05$). Before performing parametric statistical analyses, normal distribution and variance homogeneity of the data were checked by Kolmogorov–Smirnoff goodness-of-fit and Levene's tests, respectively. Residual maximum likelihood variance components were also performed to determine which of the two main factors, or their interaction, accounted for the majority of the variation in each of the measured variable. All statistical analyses were carried out using SPSS 13.0.

3. Results and discussion

3.1. Plant biometric data

Water type used for the irrigation of tomato plants was the factor that explained the greatest amount of variance (at least >39 %) of the biometric plant properties (Table 4).

Shoot and root dry and fresh weights, as well as plants height, were the highest in plants irrigated with IFW followed by those irrigated with TWW, and finally with TAP water (Fig. 1). Such results were proportional to the amounts of nutrients supplied by the three irrigation treatments, which at decreasing order were IFW > TWW > TAP. Similar positive results using TWW have been found by other authors. Leonel et al. (2022) reported that irrigation with TWW improved almost five times the wheat grains yield probably as a consequence of the higher nutrient supply, such as N and P, compared to conventional water. Zema

Table 4
Percentage of explained variance of the assessed biometric parameters of tomato cultivated on five different soils and irrigated with tap water (TAP), inorganic nutrient solution (IFW), and treated wastewater (TWW).

Biometric parameters	Soil (S)	Water (W)	S x W
Plant height	29**	39***	NS
Root weight (dry)	15*	74***	NS
Root weight (fresh)	30***	52***	NS
Shoot weight (fresh)	18*	73***	6*
Shoot weight (dry)	18*	64***	7*

* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

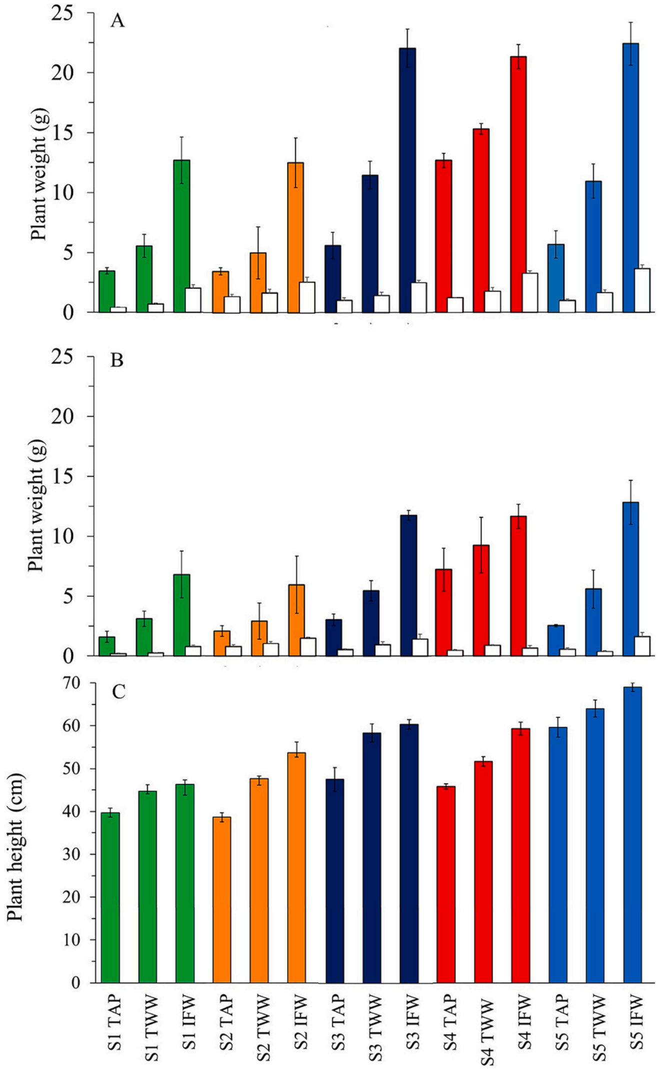

Fig. 1. Fresh (A) and dry (B) weights of shoot (full histograms) and roots (empty histograms), and plant height (C) of tomato cultivated in pots after 56 days. Tested experimental factors were: soil type (S1, S2, S3, S4, S5), water type (TAP, tap water; IFW, inorganic nutrient solution; TWW, treated wastewater) for irrigation, and their interaction. Standard deviations are reported as vertical bars.

et al. (2012) reported an increase of 26 %, 87 % and 63 % in plant (lettuce) height, leaf area index, and biomass yield, respectively, in TWW compared to their conventional water counterpart.

Aziz and Farissi (2014) suggested as well that improved yield and growth of plants can be ascribed to higher nutrients availability for plant uptake. Indeed, TWW had more N and P than by TAP water. In particular, N was supplied as nitrate (NO_3^-) and ammonium (NH_4^+), i.e. in the most readily available plant assimilation forms. Similarly, phosphorus and potassium, which are supplied such as orthophosphate and potassium ion, are very easily assimilable by plants (Ofori et al., 2021).

Finally, it is to note that despite tomato experienced different water volumes during the experiment, i.e. different amounts of sodium hypochlorite supplied, this did not prejudice crop growth. Such results depended on the low concentration of sodium hypochlorite (NaOCl) used for wastewater sanitization (<5 mg L^{-1}). Indeed, Lykogianni et al. (2023) as well, using a nutrient enriched solution treated with sodium hypochlorite at 2.5, 5 and 7.5 mg L^{-1}, did not found any adverse effect on growth, leaf gas exchange, fruit yield and tissue mineral composition in tomato. A previous study suggested that the greater the residual sodium hypochlorite, the worse the plant damage (Lonigro et al., 2017).

Also soil type played a key role in affecting plant biometric parameters. Shoot and root dry and fresh weights, as well as plants height, increased by increasing the fertility of soils used for the experiment, i.e. with the highest levels of total N and organic C (soils S3, S4 and S5; Table 1; Fig. 1).

Overall, based on biometric plant response to water irrigation treatments, such results suggested that TWW could not meet the whole nutrients requirement of tomato, thus nutrients integration by inorganic fertilization is required. However, the use of TWW for irrigation purpose can contribute to reduce the external input of nutrients during the growth period.

3.2. Effects of water irrigation on chemical soil properties

Irrigation treatment and soil type differently affected the response of soil chemical properties assessed at the end of the tomato plants growth. Soil reaction was mainly affected by soil type (Table 5).

Table 5

Chemical parameters of soils after 56 days. Tested experimental factors were: soil type (S1, S2, S3, S4, S5), water type (TAP, tap water; IFW, inorganic nutrient solution; TWW, treated wastewater) for irrigation, and their interaction. Along a column, different capital letters indicate significant differences ($P < 0.05$) among irrigation treatment within the same soil; lowercase letters indicate significant differences ($P < 0.05$) among soils within the same irrigation treatment. Level of significance for explained variance: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Soil (S)	Water (W)	pH	Electrical conductivity µS cm^{-1}	Available P mg kg^{-1}	Mineral N mg kg^{-1}
S1	TAP	7.4 Ab	264 Ba	41 Bb	0.9 Bb
	TWW	7.6 Ac	524 Ac	46 Bb	8.8 Ac
	IFW	7.1 Bd	549 Acd	76 Aa	8.2 Ac
S2	TAP	7.5 Ab	262 Aa	33 Bb	0.9 Cb
	TWW	7.5 Ac	542 Ac	38 Bb	8.6 Bc
	IFW	7.4 Ad	498 Ad	100 Aa	11.2 Ab
S3	TAP	7.9 Aa	278 Aa	47 Bb	1.1 Cb
	TWW	8.0 Ab	675 Ab	50 Bb	9.9 Bb
	IFW	7.8 Ac	693 Ab	109 Aa	12.5 Aa
S4	TAP	8.3 Aa	334 Aa	27 Bb	1.0 Bb
	TWW	8.5 Aa	841 Aa	32 Bb	11.6 Aa
	IFW	8.3 Aa	747 Aba	96 Aa	12.8 Aa
S5	TAP	8.1 Aa	210 Ab	45 Bb	1.8 Ca
	TWW	8.1 Aab	698 Ab	48 Bb	9.7 Bb
	IFW	8.1 Abc	604 Ac	118 Aa	11.2 Ab
Soil (S) %		82***	12*	NS	NS
Water (W) %		NS	79***	88***	92***
S × W %		5*	6*	NS	5*

Such finding was reasonable being reaction an intrinsic soil property, i.e. depending mainly on the soil colloids (clay and organic matter contents), that is their buffer capacity, thus, soil pH is slightly or not affected by irrigation water. Indeed, the highest pH shift was observed for soil S1 that had the lowest content of clay and of organic C. Similar results were reported also by Farhadkhani et al. (2018) and Guo et al. (2017).

Electrical conductivity was mainly affected by irrigation treatment (Table 5). Indeed, soil irrigated with TWW and IFW showed the highest EC values, reasonably because soluble salts were added to soil by both irrigation waters (Romaneckas et al., 2023). The increase of soil salinity following the irrigation with TWW is one of the main drawbacks reported by many authors (e.g. Chaganti et al., 2020; De las Heras and Mañas, 2020) because salt accumulation negatively affects soil particles aggregation and, hence, permeability, hydraulic conductivity and microbial community (Leonel and Tonetti, 2021). In this study, however, at the end of the experiment, soil EC values were always below the threshold value of 2 dS m^{-1}, thus suggesting that soils can be considered cultivable (Weil and Brady, 2008). The nature and properties of soils (Vol. 13, pp. 662–710). Upper Saddle River, NJ: Prentice Hall.). Moreover, considering that the study was carried out in pots, such EC increase could be considered transitory in open field, where rainfall may contribute to soluble salt leaching. Soil cultivated with tomato in open field and irrigated with treated wastewater experienced temporal high EC values during the summer period but return to normality at the end of the winter period (Vergine et al., 2017). The authors attributed the temporal increase to high irrigation regime and lack of rainfall which caused the retention of salts from the TWW.

Soil type played a key role also affecting EC values (Table 5). The main soil driver affecting EC values was clay content, at least for two reasons. On the one hand, the higher the clay content (S3 and S4), the greater the amount of soluble salts retained. On the other hand, having clayey soils higher water holding capacity (WHC) than other soils (S1, S2 and S5), they experienced higher volume of water for crop irrigation that entailed higher amounts of soluble salts added. Similar results have been reported by many authors (Kallel et al., 2012; Klay et al., 2010; Shakir et al., 2017). Thus, when TWWs are used for irrigation purpose, an appropriate evaluation of soil type has to be done and electrical conductivity has to be monitored to assess the attainment of critical values for crops.

Available P was greatly affected only by irrigation treatment, in particular by IFW treatment which, on average, doubled the amount of soil available P by TAP and TWW treatments. Such results are reasonable since tap and treated wastewater held a very low amount of total P that, when added to soil, likely underwent to precipitation process practically in all soils (pH $\geq$ 7.0) or was used by plants for their development, thus reducing P precipitation (Leonel et al., 2022).

Also, mineral N ($NH_4^+ + NO_3^-$) was mainly affected by irrigation treatment as it was always extremely the highest in IFW treatment; moreover, contrarily to what observed for available P, mineral N was slightly higher in TWW treatment compared to TAP one as treated wastewater held more than two-fold nitrogen, as ammonium, compared to tap water. Also, Xu et al. (2010) observed an increase of N levels but after eight and twenty years of TWW addition. In our case, the observed increase after 56 days could be ascribed to the specific conditions of our pot experiment, which did not include significant percolation.

3.3. Effects of water irrigation on main microbial groups

Total microbial biomass, as well as main microbial groups assessed by ELFAs, were affected mainly by soil type and to some extent by irrigation water (Table 6). Soil with higher concentrations of total organic C and total N (S4 and S5) showed higher amounts of microbial biomass. The increase of soil microbial biomass following irrigation with

Table 6

Total ester linked fatty acids (ELFAs) and main microbial groups (nmol FA kg^{-1}) of soils (S1, S2, S3, S4, S5) assessed at the end of the experiment lasted 56 days. Tested experimental factors were: soil type (S1, S2, S3, S4, S5) and water type (TAP, tap water; IFW, inorganic nutrient solution; TWW, treated wastewater) for irrigation, and their interaction. Along a column, different capital letters indicate significant differences ($P < 0.05$) among irrigation treatment within the same soil; lowercase letters indicate significant differences ($P < 0.05$) among soils within the same irrigation treatment. Level of significance for explained variance: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Soil (S)	Water (W)	Total ELFAs	Bacteria	Fungi	Fungi/bacteria
S1	TAP	178 Ce	111 Ce	28 Cc	0.26 Ac
S1	TWW	299 Be	176 Bc	34 Bc	0.20 Bc
S1	IFW	344 Ad	223 Ad	63 Ad	0.28 Ac
S2	TAP	256 Bd	167 Bd	51 Bb	0.31 Ab
S2	TWW	308 Bd	191 Bc	49 Bbc	0.26 Bb
S2	IFW	385 Ad	262 Ac	85 Acd	0.32 Ab
S3	TAP	323 Cc	203 Cc	58 Bb	0.29 Abc
S3	TWW	449 Bc	270 Bb	56 Bb	0.21 Bc
S3	IFW	575 Ac	380 Ab	115 Abc	0.30 Abc
S4	TAP	380 Cb	220 Cbc	95 Ca	0.43 Aa
S4	TWW	548 Bb	301 Bb	114 Ba	0.38 Ba
S4	IFW	745 Aa	455 Aa	187 Aa	0.41 ABa
S5	TAP	464 Ca	283 Ca	120 Ba	0.43 Aa
S5	TWW	611 Ba	354 Ba	129 Ba	0.36 Ba
S5	IFW	688 Ab	440 Aa	186 Aa	0.42 Aa
Soil (S) %		55***	46**	64**	76***
Water (W) %		36***	45***	30**	18**
S × W %		6*	6*	5*	ns

TWW and IFW may be ascribed to the supply of available nutrients (Laudicina et al., 2012).

Also, irrigation treatment affected microbial biomass and main microbial groups. Generally, microbial biomass due to bacteria and fungi was the highest according to the following order IFW > TWW > TAP, except in soil S2. As previously suggested, such a pattern may be linked to the available nutrients added by IFW, which is higher than TWW, in turn more than TAP. The increase of soil microbial biomass is a positive feedback of the use of TWW; indeed, since microorganisms drive a high number of processes, including nutrient cycling and soil organic matter decomposition, such an increase may lead to an improvement of soil fertility.

Our findings agreed with those of Adrover et al. (2012) who investigated the effect of secondary-treated municipal wastewater on the chemical properties and biological activity of 21 arable soils, irrigated for >20 years. They found that soil water-soluble organic carbon, soil microbial biomass and β-glucosidase and alkaline phosphatase activities increased under TWW irrigation. Also, Elifantz et al. (2011) assessed the impact of TWW when used as an alternative for irrigation of agricultural crops. They found that the microbial hydrolysis activity in soils irrigated with TWW was significantly higher compared to that of soil irrigated with freshwater. Moreover, Elifantz et al. (2011) reported an improvement of the nitrification potential in TWW irrigated soils. However, the potential activity of the microbial community returned to the initial level during the rainy season, thus suggesting that periodic use of TWW did not permanently change the soil microbial activity.

The increase of bacteria and fungi in response to the irrigation treatment did not occur similarly. Indeed, the fungi-to-bacteria ratio was lowered in soil irrigated with TWW compared to those irrigated with TAP and IFW, regardless of soil type. Such decrease suggested a slight adverse effect of TWW over fungi. Recently, Song et al. (2019), investigating the environmental risk of chlorine-controlled clogging in drip irrigation system using reclaimed water, found that fungi were less abundant in the soil and gradually decreased with increased chlorination concentration. Here, although a different amount of sodium hypochlorite was supplied to soils following the addition of TWW on the basis of their WHC, no correlation was evident with fungal biomass. Instead, fungi decreased in all soils irrigated with TWW. Fungi and bacteria play an important role in soil organic matter decomposition and nutrient cycling, generally with different efficiency, i.e. fungi are more efficient than bacteria (Laudicina et al., 2012; Fanin et al., 2019). The lower the fungi-to-bacteria ratio, the lesser the stability of soil ecosystem (Wardle et al., 2004; Orwin et al., 2018). Hence, an increase of fungal biomass lower than that of bacterial biomass, leading to a lower fungi-to-bacteria ratio, may be considered, at least from an ecological point of view, a negative feedback of TWW treatment.

On the other hand, however, bacteria give to biomass proportionately greater contribution than fungi to the energy flow in soil food webs. The lower fungi-to-bacteria ratio does not indicate that fungi are less important than bacteria because they play different roles and are usually non-substitutable in ecosystems (Wang et al., 2019).

Overall, such results showed that irrigation treatment, included TWW, change the relative percentage of the main microbial groups and more long-term studies are required to elucidate the effect of TWW on bacteria and fungi.

Also soil type played a key role in affecting soil microorganisms. Indeed, microbial biomass and main microbial groups were more abundant in soils (S3, S4, S5) with high fertility. Interestingly, such soils were those irrigated with the highest volume of TWW during the experiment and, hence, supplied with the highest amount of sodium hypochlorite. The latter when added to TWW acts as chlorine producing hypochlorous acid. Then, such acid undergoes oxidation reactions that lead to deactivation of pathogens in the water, like bacteria, viruses and protozoa, preventing them from being able to reproduce or pose a risk to human health (Fukuzaki, 2006). If on the one hand sodium hypochlorite was effective in the disinfection of TWW, on the other hand, when added to soil, its effect on soil microbial biomass was negligible. This likely occurred for many concurrent reasons among which a) the quantity of sodium hypochlorite added to the TWW (5 mg L^{-1}) was not sufficient to inactivate soil microorganisms, and b) the high fertility of soil S3, S4 and S5 counteracted the adverse effect of sodium hypochlorite.

However, also soil reaction could have weakened the ability of sodium hypochlorite in affecting soil microorganisms. Indeed, in water, NaClO dissolves as follows:

$$NaClO + H_2O \rightarrow Na^+ + ClO^- + H_2O \tag{1}$$

Reacting with water, ClO^- acts as a base to form:

$$ClO^- + H_2O \leftrightarrows HClO + OH^- \tag{2}$$

In soil, the equilibrium (2) depends on soil pH. The higher the alkalinity, the greater the concentration of ClO^-, and vice versa. Ionized ClO^- has a poor germicidal activity because of its inability to diffuse through microbial plasma membrane, and it exerts an oxidizing action only from outside of the cell. On the other side, the hypochlorous acid (HClO) can penetrate the lipid bilayer in the plasma membrane by passive diffusion due to its electrical neutrality. Then, HClO can attack the microbial cell both from the outside and inside the cell, which is responsible for the potent germicidal activity of HClO (Fukuzaki, 2006). The effect of soil pH on germicidal activity of sodium hypochlorite is of great importance and noteworthy of further investigations. In fact, in presence of TWW heavily loaded of pathogens, it is desirable to use high quantity of sodium hypochlorite. The resulting TWWs could then be applied to alkaline or subalkaline soils without affecting the soil microbial community.

3.4. *Structure of prokaryotic communities of soil*

Most (85.7 %) of the ASVs have been assigned to the Bacteria domain while the remaining (14.3 %) to the Archaea domain. The prokaryotic composition of the soil was comparatively analysed considering the relative abundances at the phylum and genus levels (Figs. 2 and 3, Tables S2 and S3). Metataxonomic analysis revealed that bacteria

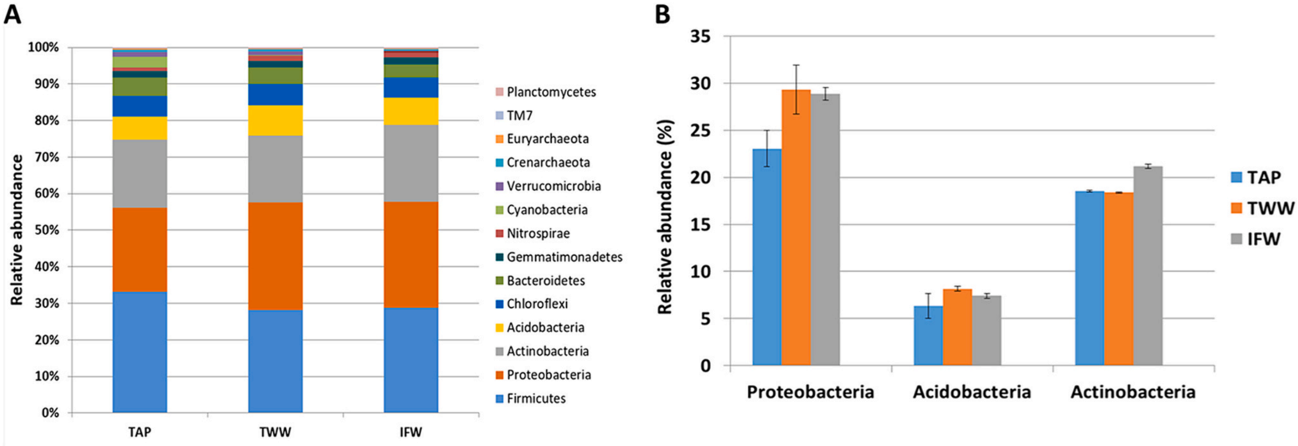

Fig. 2. Histograms reporting the taxonomic composition of S5 soil samples irrigated with TAP (tap water), TWW (treated wastewater) and IFW (inorganic NPK solution) at the taxonomic level of phylum. (A) The relative abundance is the percentage of each phylum with respect to all the identified phyla. (B) A magnification of the relative values of the three phyla Proteobacteria, Acidobacteria and Actinobacteria is reported.

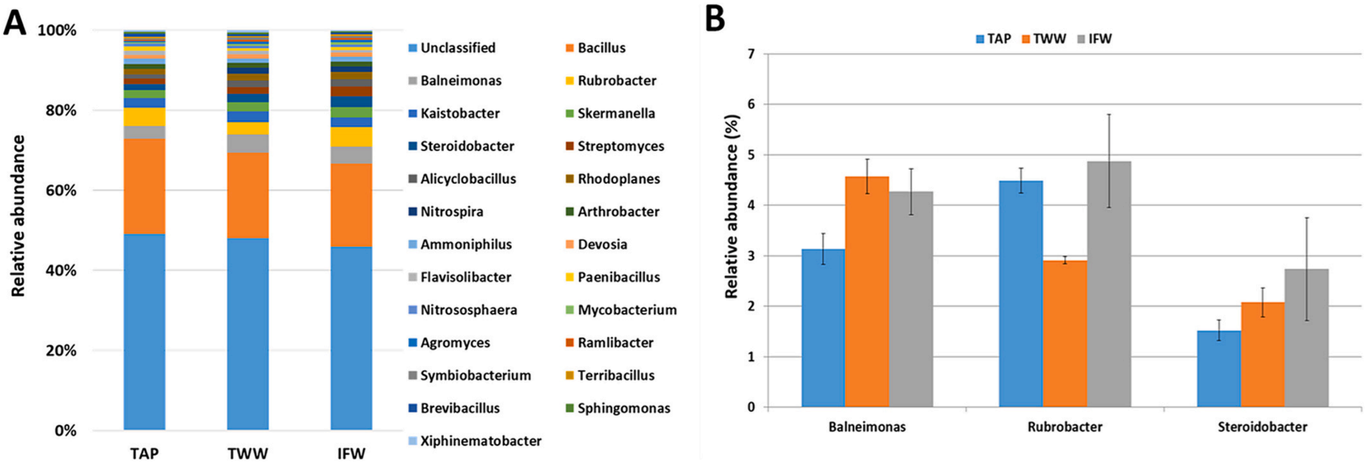

Fig. 3. Histograms reporting the taxonomic composition of S5 soil samples irrigated with TAP (tap water), TWW (treated wastewater) and IFW (inorganic NPK solution) at the taxonomic level of genus. (A) The relative abundance is the percentage of each genus with respect to all the identified genera. (B) A magnification of the relative values of the three genera *Balneimonas, Rubrobacter* and *Steroidobacter* is reported.

belonging to the Firmicutes, Proteobacteria, and Actinobacteria phyla are dominant in all irrigation treatments (Fig. 2). This result agrees with other studies, demonstrating the concurrent presence of these three bacterial phyla in both soil and TWW microbial communities (Becerra-Castro et al., 2015). Firmicutes, Proteobacteria, and Actinobacteria play crucial roles in plant growth and maintain constant soil parameters under stress conditions, especially in the case of tomatoes, as demonstrated by recent studies (Lee et al., 2021; Pokluda et al., 2021; Faddetta et al., 2023). The treatments with TWW and IFW caused, as previously described, an increase in microbial biomass to the detriment of the fungal community, especially in the case of the plants irrigated with TWW.

The metataxonomic analysis revealed distinct prokaryotic community structures in the samples as highlighted by principal coordinate analysis (PCoA) (Supplementary Fig. S1), confirming that the use of different irrigation treatments in tomato plant soil can shape soil microbial biodiversity in a short term period. Studies aimed at clarifying the role of the microbial community structure on the growth of tomato plants demonstrate that the soil microbiota exploits different metabolic capabilities to ensure the growth and health of tomato plants. In this regard, an increase in the abundance of Acidobacteria was observed in conjunction with a growth-promoting effect on tomato plants (Kalam et al., 2017). In fact, Acidobacteria can promote plant growth by solubilizing zinc, producing siderophores and enzymes useful for plant growth and finally producing growth-regulating phytohormones that promote various phases of growth starting from germination and differentiation of tomato tissues (Kalam et al., 2017). In addition, it is known that Proteobacteria are capable of colonizing the root system of plants, improving the growth and health of the host organisms (Bruto et al., 2014). Resendiz-Nava et al. (2023) observed a close biological interaction between tomato plants and Proteobacteria that possess high genomic plasticity, easily adapting to stress and successfully colonizing plant niches. Furthermore, it has been shown that Proteobacteria promote processes beneficial to plants such as nitrogen fixation and phosphate solubilization. Indeed, the metataxonomic analysis (Fig. 2) showed that the relative abundance of Gram-negative bacteria, in particular those belonging to Proteobacteria and Acidobacteria phyla, increased in soils irrigated with TWW (29.32 % and 8.16 %, respectively) and IFW (28.85 % and 7.42 %) in comparison to those irrigated with TAP water (23.04 % and 6.32 %). This finding suggested that TWW can modulate soil microbiota. Such a result agreed with Wafula et al. (2015)'s report that found an increase in the relative abundance of Proteobacteria and Acidobacteria and a decrease of Actinobacteria in soil irrigated with TWW compared to not irrigated soil. Indeed, in this study, a decrease in Actinobacteria was observed in TWW with respect to IFW (Fig. 2B). A strict correlation between Acidobacteria and

Proteobacteria from the irrigated WW samples and total N concentration was already reported (Wafula et al., 2015). Some Proteobacteria, such as purple phototrophic bacteria, are considered microorganisms that can purify wastewater because their ability in degrading harmful substances in wastewater, such as hydrocarbons (Nhi-Cong et al., 2021). Unfortunately, the increase of Proteobacteria could represent a problem for human health, since it was previously reported that wastewater irrigation leads to an increased abundance of potentially harmful Proteobacteria, such as *Pseudomonas, Stenotrophomonas*, and *Acinetobacter* spp. (Broszat et al., 2014). In the soils analysed in this work, these bacteria were not found, but their presence in WW cannot be ruled out since it can be dependent on bacterial loading and quality of the irrigation water. Thus, the bacterial composition and, in particular, Proteobacteria and harmful bacteria should be carefully monitored in TWW before its use. The analyses conducted in this study revealed that there are not any bacterial genera present potentially dangerous to human health. However, to effectively mitigate the risk of their presence in TWWs and guarantee the safety of human health, disinfection methods, such as UV radiation, paracetic acid, ozonation, or the use of sodium hypochlorite could be exploited (Bonetta et al., 2021). Chlorination is the most used method for disinfecting wastewater, as it is effective and inexpensive. This treatment inactivates several pathogens but can cause an increase in substances such as total organic halogens which are cytotoxic. Therefore, physical disinfection methods, such as UV radiation, that do not produce toxic by-products are increasingly used. However, it is known that some bacteria can have protective effects, reducing the microbial load of pathogenic bacteria (Thao et al., 2021).

It has been shown (Frenk et al., 2014) that the soil microbiota is susceptible to irrigation treatments, especially in a short-term upon the use of TWW for irrigation; however a return to a untreated state demonstrates that the soil community is resilient in the long term to the anthropic impact imposed by the quality of water for irrigation (Frenk et al., 2014). Furthermore, no change was detected in the predicted metabolic capability of the prokaryotic community due to the three treatments considered (Supplementary Material-Fig. S2). Indeed, these predicted metabolic activities were similar in all the treatments with a high percentage of unknown metabolic pathways (about 66 %), sulfate reducing bacteria (about 35 %), sulfide oxidizer (about 30 %) and nitrite reducer (about 30 %). Anyhow, it is not possible to exclude that the category Unknowns could probably include plant growth promoting capacities that can have a role in the stimulation of tomato plant growth in TWW in the respect of TAP. Some soil characteristics, such as salinity EC and pH may have modulated the soil microbial composition. EC is known to be able to prominently modulate soil bacterial community structure. In particular, in the short term, soils with high EC are characterized by lower bacterial diversity (Zhao et al., 2020). For example, it is reported that at high ECs (for example around 6 dS m^{-1}) Bacteroidetes, Gemmatimonadetes and Firmicutes can increase their abundance. In S5 soil, the pH is 8.1 for all the TAP, TWW and IFW treatments while the highest EC was obtained with the TWW treatment but this difference is not consistent with a high EC values.

Apart from the unclassified bacteria, at the genus level, 13 taxa showed an average percentage abundance higher than 1 % with *Bacillus* being the most abundant genus in all three irrigation treatments (Fig. 3). It has been seen, in previous studies evaluating the role of the *Bacillus* genus in the wastewater that this bacterial genus is able to tolerate not only basic pH, but also high salinity level. Members of *Bacillus* can confer tolerance to abiotic stresses to plants, also generating symbiosis with microalgae, and have been proposed as agricultural biofertilizers, promoting plant growth (Bui-Xuan et al., 2022; Yong et al., 2021). Anyhow, also at the genus level, some variations of the relative abundances can be ascribed to the irrigation treatment. Among them, the most interesting abundance variations concern *Balneimonas, Rubrobacter,* and *Steroidobacter.* In particular, the genera *Balneimonas* and *Steroidobacter* increased in both TWW and IFW treatments compared to the TAP treatment, while *Rubrobacter* increased in IFW and decreased in TWW compared to TAP. In other studies, it is reported that bacteria belonging to *Balneimonas* and *Rubrobacter* genera are important for promoting plant growth and nutrient cycling. Their relative abundances decreased in soil under drought conditions while increasing in irrigated soils (Trivedi et al., 2021). Similarly, bacteria belonging to *Steroidobacter* halve drought and positively affect crop growth. Previous studies have reported that the growth of *Steroidobacter* in the soil is stimulated by the presence of nitrogen as it favours the synthesis of glycosides beneficial for plant growth. Thus, the increase of *Steroidobacter* relative abundance may be related to the high level of N and improved growth in TWW in comparison with TAP treatment. Therefore, the improved plant growth in soil irrigated with TWW compared to those irrigated with TAP, may be associated with a modulation of soil microbiota structure that for some bacterial taxa resembled that of IFW treatment which, however, showed a stronger plant-growth stimulating effect in comparison to TAP and TWW irrigations. These findings suggest a strong relationship between TWW irrigation, soil nutrient availability and soil microbiota structures and deserve further investigations to fully elucidate the key parameters controlling tomato plant-growth and soil microbiota structure.

4. Conclusions

The reuse of treated wastewater for crop irrigation is desirable to overcome the issue of water shortages as a consequence of the acceleration of urbanization, population growth and industrial development. However, the reuse of treated wastewater in agriculture is not free of consequences, being some of them adverse with regard to plant growth and soil ecosystem. This comprehensive study examined many of the implications involved with the reuse of treated wastewater on the soil-plant system. Based on the obtained results, the following conclusions can be drawn:

1. Regardless soil type, irrigation of tomato with treated wastewater compared to tap water improved biometric plant properties likely due to the higher supply of available nutrients, mainly available P and mineral N. However, nutrient supply by treated wastewater was not sufficient to meet the whole nutrients requirement of tomato, thus nutrients integration by fertilization is required. In addition, the different amount of sodium hypochlorite supplied by TWW did not show any adverse effect on plant growth.
2. Soil reaction was not affected by irrigation treatment and, hence, by TWW. On the other hand, electrical conductivity was mainly affected by irrigation treatment and was higher in soil irrigated with treated wastewater and nutrient inorganic solution due to the soluble salts added. However, at the end of the experiment, soils were non-saline. The main soil drivers affecting EC were clay and organic matter content that were able to retain soluble salts. Hence, soil EC has to be monitored to avoid salinization.
3. Treated wastewater supplied only mineral N to soil-plant system, whereas P added by TWW underwent to precipitation process or was used by plants for their development, thus not accumulating in soil.
4. Nutrients added by treated wastewater modulated soil microbial biomass, as well as bacteria and fungi. However, the increase of bacteria and fungi in response to the irrigation treatment did not occur proportionally, being bacteria more stimulated than fungi. Such behaviour led to lower fungi-to-bacteria ratio in soil irrigated with treated wastewater. Such an aspect needs further investigation due to the ecological role played by fungi in soil organic matter mineralization and nutrient cycling, thus suggesting that treated wastewater shape the composition of the main microbial groups.
5. Soil pH played a key role in affecting the response of soil microbial biomass to sodium hypochlorite added via TWW. Indeed, the higher the alkalinity of soil, the lower the germicidal activity of sodium hypochlorite.

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CRediT authorship contribution statement

Sofia Maria Muscarella: Writing – original draft, Methodology, Investigation, Formal analysis. **Rosa Alduina:** Writing – review & editing, Formal analysis. **Luigi Badalucco:** Writing – review & editing. **Fanny Claire Capri:** Formal analysis. **Ylenia Di Leto:** Writing – original draft, Formal analysis. **Giuseppe Gallo:** Writing – review & editing, Writing – original draft, Formal analysis. **Vito Armando Laudicina:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Conceptualization. **Sara Paliaga:** Writing – original draft, Formal analysis. **Giorgio Mannina:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Giorgio Mannina reports financial support was provided by European Union Horizon 2020. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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