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Treated wastewater as irrigation source: A microbiological and chemical evaluation in apple and nectarine trees

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ABSTRACT

The use of secondary treated wastewater (STW) for irrigation is considered as a strategy to mitigate water shortage in summer periods. Unfortunately, the utilization of STW in agriculture is not exempt from environmental and health risks, being a potential source of toxic chemicals (e.g. heavy metals) and human pathogens (e.g. *Salmonella* spp. and *Escherichia coli*). The aim of this work is to verify whether irrigation of apple and nectarine trees with STW may lead to heavy metal and microbial contamination in shoot, leaf and fruit tissues. Three-year old trees were grown in pots and drip irrigated separately, for one season, using either tap water (TW) or STW. STW irrigation did not affect heavy metal and trace element concentration (e.g. B, Na, Zn) both in leaves and fruits. Heavy metal concentrations in fruit tissues were lower than international limits for human consumption in both species. Independently from the species, the total bacterial count (TBC) on shoot was almost the double in STW irrigated plants compared to the TW-irrigated ones. No *E. coli* was found in shoot and fruit; few coliforms (TC) were detected in shoot tissues and in nectarine fruits, far below European microbiological limits for foodstuff. Finally, a laboratory trial, aimed at evaluating possible mechanisms of *E. coli* translocation inside plants, was carried out on 3-month old peach rootstock plants (i.e. GF 677), artificially inoculated with two *E. coli* strains, characterized by a different level of pathogenicity *(*disarmed DH5α and *E. coli 1576)*. Populations of both *E. coli* strains were isolated as epiphytic and as endophytic only at root level. Nonetheless, the endophytic population of the *E. coli* 1576 was generally higher than the DH5α strain. Colonization of the epigeal part was never observed. These results are promising for the use of STW, especially for drip irrigated orchards, since water does not wet the canopy and consequently the final product.

1. Introduction

Due to ongoing climate changes and the increasing frequency of drought conditions in temperate environments, wastewater could represent an advantageous alternative to face the increasing water scarcity and irrigation demand, especially in areas with summer water restriction (Pereira et al., 2002).

However, wastewater re-use in agriculture may also lead to environmental and health risks, being a potential source of dangerous chemicals (Muchuweti et al., 2006; Fatta-Kassinos et al., 2011) and human pathogens (Bernstein, 2011). In particular, high levels of heavy metals as well as pathogenic bacteria, such as *Escherichia coli* and *Salmonella* spp., are recognized as major threats to human health related to irrigation with wastewater (Petterson et al., 2001; Palese et al., 2009; Cirelli et al., 2012; Forslund et al., 2012; Szkup-Jablonska et al., 2012). *E. coli* strains, as example, are responsible for outbreaks of infectious diarrhoea and a number of deaths in developing countries (EFSA and ECDC, 2018).

Highly contaminated wastewaters may be a reservoir of human enteropathogenic microorganisms, which can be transferred to the soil ecosystem first and then to plant tissues (Berg et al., 2005). Some bacterial species may be up-taken by roots, transported through the xylem vessels, become endophytes and retrieved into the fruits (Deering et al., 2012). Endophytes are usually considered plant symbionts that may contribute to plant adaptability and promote plant growth and resistance against biotic and abiotic stresses (Brader et al., 2017; Perpetuini et al., 2019). The big concern is related to potential virulent *E. coli* and *Salmonella* spp. whose behaviour into the soil, in particular their ability to become endophytes, is not predictable. Several studies, especially on horticultural crops, have shown that human

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pathogens can penetrate internal plant tissues through roots (Franz et al., 2007; Mootian et al., 2009; Hintz et al., 2010), translocating and surviving within plants (Lapidot and Yaron, 2009; Martinez et al., 2015). However, the mechanisms allowing bacteria to enter plant roots (i.e. passive or active mechanism), the colonization pathways and their persistence inside the plant have not been elucidated yet (Tyler and Triplett, 2008; Hirneisen et al., 2012; Gu et al., 2013) and are still anecdotal, especially as regard to human pathogens towards fruit tree crops.

The internalization of microorganisms depends on several factors such as the specificity of bacterial strains or serotype, plant age, composition of root exudates, soil type and climate conditions (Hirneisen et al., 2012; Hofmann et al., 2014). Therefore, the transmission risk of virulent bacterial species cannot be underestimated. Many environmental microorganisms can be detected in wastewater (*i.e.* species belonging to the genera *Pantoea, Pseudomonas, Enterobacter, Herbaspirillum, Ochrobactrum, Ralstonia* and *Stenotrophomonas*). The same genera also include species, which are reported as plant endophytes, thus suggesting that the ability of colonizing plant tissues could be widespread also in environmental microorganisms.

As regard to heavy metals, they can be important co-factors or essential micronutrients, if in trace. In this case, they can be directly released into the root xylem and freely, or as chelated ions, translocated to aerial organs, including leaves, fruits or seeds through the xylem transpiration stream (Kirkham, 1977; Krijger et al., 1999; Bhatia et al., 2005; Page and Feller, 2005a, b; Page et al., 2006). Instead, the excess of heavy metals is a serious threat, but plants have evolved several mechanisms to detoxify their excess such as insolubilization (Kosegarten and Koyro, 2001; Bravin et al., 2008) or cell compartmentation at root level (Yang et al., 2006; Richau et al., 2009).

Translocation of heavy metals (Chien et al., 2006; Salah and Barrington, 2006; Schück and Greger, 2019) or bacteria (Solomon et al., 2002a; Cooley et al., 2003) may be subjected to xylem transport and therefore it can be related to the plant transpiration activity. The transpiration stream is mainly related to environmental conditions (e.g. vapour pressure deficit) and to plant anatomical features such as the xylem hydraulic conductance or the surface conductance of different organs (e.g. leaf and fruit) (Higgins et al., 1992). Indeed, Gorbatsevich et al. (2013) reported that *Salmonella enterica*, in sweet basil, was identified with a higher incidence of internalization in the vegetative organs than in the reproductive ones (i.e. inflorescence), supporting the concept of bacterial transport directly related to the tissue transpiration rate. As regard to apple and peach fruits, it is known that they behave differently in terms of evaporative demand (Morandi et al., 2012). Apples, because of their lower epidermis surface conductance, are characterized by lower transpiration rates that imply lower xylem flows compared to stone fruit species (e.g. peach). Moreover, apple fruits (Drazeta et al., 2004), like kiwi berries (Dichio et al., 2003) and unlike peach fruits, are characterized by the loss of the fruit xylem functionality during the second part of the season, at about 120 days after full bloom (DAFB). This different fruit growth mechanism and the loss in xylem functionality in apple might then exert a barrier-like effect against pollutant translocation towards the fruit.

Although the World Health Organization (WHO) recommends a flexible guideline for a direct use of treated wastewater in agriculture at global level, most Countries decided to use more restricted internal regulations (BIO, 2015). In Italy, for instance, the only treated wastewater that can be directly used in agriculture is the tertiary treated treatment (TTW) (i.e. Italian Legislative Decree No. 185/03) with very strict law limits similar to those for drinking water (Angelakis et al., 2007). Secondary treated wastewater (STW) (Italian Legislative Decree No. 152/06) cannot be used as an alternative irrigation source, even if it represents the most available wastewater source in Italy (https://www.istat.it/), having good intrinsic parameters and benefit/cost ratio for irrigation purposes (Molinos-Senante et al., 2010; Pistocchi et al., 2017).

The aim of this study was to evaluate the potential risks and benefits of STW drip irrigation in apple and nectarine. For this purpose, the potential transfer of pollutants (i.e. heavy metals) and microbial contaminants (e.g. *E. coli*) to the vegetative and reproductive plant tissues were monitored both in field and controlled conditions. Nectarine and apple trees were chosen as models for two different fruit growth behaviours: a) nectarine, less selective and with a continuous functionality of fruit xylem up to harvest (Morandi et al., 2007); b) apple, more selective and characterized by a xylem functionality that ends in the mid-season (Drazeta et al., 2004).

2. Materials and methods

2.1. Experimental potted trial

A 1-year experiment was performed in semi-field conditions at the experimental station of the University of Bologna, located in Cadriano (BO), on 3-year old nectarine trees (*Prunus persica* L. Batsch.) "Big Top" grafted on GF 677 and apple trees (*Malus domestica* L.) "Gala Schniga" grafted on M9. Trees were individually grown in 40-L pots manually filled, for avoiding any possible modification of physical and hydraulic soil properties such as soil compacting, with an alkaline, poorly fertile sandy-loamy soil (Tab. S1). Trees were trained as slender spindle, protected by a shading hail net and subjected to standard pruning, pest, disease and thinning according to the ICM, 2010.

For each species, five trees were arranged in a randomized design with two irrigation treatments: a) secondary treated wastewater (STW) and b) tap water (TW). STW, subjected to the Italian Legislative Decree No. 152/06, was locally obtained by the urban civil wastewater treatment plant (HERA S.p.a - Italian multi-utility).

Trees were micro-irrigated (four drippers 2 L h^{-1}per tree) twice a day to compensate evapotranspiration (ET_C). Irrigation was managed according to the "Irrinet" irrigation scheduling system (www.irriframe.it). Treatments received the same irrigation volume (360 L $tree^{-1}$ $season^{-1}$) along the season, from May to harvest (half of July for nectarine and beginning of August for apple).

2.1.1. Leaf and fruit heavy metals and trace elements concentration

During mid-summer (July 17th), at 60 day from the beginning of the experiment, ten mature leaves per tree were collected from randomly selected annual shoots, immediately enclosed into polyethylene bags and transported to the laboratory in a portable refrigerator. Petioles were removed. Leaves laminas were washed in a HCl (0.1 N) and surfactant (Tween 20, Sigma–Aldrich, Milan, Italy) (0.1 %) solution, rinsed 3 times in tap water and finally in deionized water then oven dried (65 °C) (Sorrenti et al., 2012). Dried leaves were milled (0.2 mm mesh) and analysed for heavy metals (HMs), such as Al, Cu, Fe, Ni, Pb, Sn, Zn and trace elements (TEs) B, Ba, Mn, Na concentrations. The metals and trace elements were extracted by wet mineralization according to US EPA Method 3052 (Kingston, 1988). 0.5 g dw of tissue were treated with 8 mL of nitric acid (65 %) and 2 mL of hydrogen peroxide (30 %) at 180 °C in an Ethos TC microwave lab station (Milestone, Bergamo, Italy) and measurements performed by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) (Ametek Spectro Arcos EOP, Kleve, Germany). The same analysis was done on four representative fruits per tree (20 per treatment), collected at commercial harvest, to determine fruit peel and fruit pulp mineral concentration.

2.1.2. Water microbiological and chemical analyses

Microbiological analyses of STW were carried out weekly by sampling water directly from the wastewater treatment plant. STW was an-

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alyzed for the presence of *E. coli* and *Salmonella* spp. as indicated by the Italian Legislative Decree No. 152/06; additionally, total coliforms (TC) and total bacterial counts (TBC) were assessed. The same analyses were performed on tap water (TW). Water samples were collected in 1 L sterile glass bottles and duplicate aliquots of 100, 10, 1.0 and 0.1 mL of each sample were filtered through nitrocellulose membranes (0.45 μm pore size, 47 mm diameter, Sartorious). Membranes were placed onto Chromocult agar (VWR, Milan, Italy) and incubated at 37 °C for 24 h. *E. coli* identity (colonies appear as blue/purple) was then confirmed by checking indole production and cytochrome oxidase activity. *Salmonella* spp. was detected according to the UNI EN ISO 19250:2013 procedure. Results were recorded as colony forming units (CFU) 100 m L^{-1} for *E. coli* and TC, and absence/presence for *Salmonella* spp. TBC was enumerated by plate counting in Plate Count Agar (PCA, Biolife, Milano, Italy) in serially diluted water samples (incubation at 22 °C and 37 °C, 3–5 days) and results expressed as CFU mL^{-1}.

For each irrigation treatment, water samples were analysed for water quality characterization (7 samples year $^{-1}$). Samples were collected in glass bottles, transported in an ice chest to the lab and stored at 5 °C. The concentration of HMs and TEs were determined by Inductively Coupled Plasma (ICP-OES, England) and pH was measured with a pH-meter XS PH510 (Eutech Instruments, Singapore). EC was determined using the METERLAB, CDM 210 (Radiometer Analytical, France), the sodium absorption ration (SAR) was calculated using the following equation (with concentrations in meq L^{-1}) (Richards 1954): SAR = $[(Na^{+})/ [(Ca^{2++} Mg^{2+})/ 2] 1/2$.

2.1.3. Microbiological analysis on shoots, fruits and soil samples

At harvest, shoot and fruit samples were collected from individual trees. Three samples of shoots and fruits per tree were randomly chosen, transported to the laboratory in sterile plastic bags and immediately processed. Samples were surface-sterilized with consecutive washes in 70 % ethanol, water, 2% NaCl, followed by 3 times washing with sterile distilled water. An aliquot of the final rinse was plated on PCA to ensure the surface sterilization efficiency. Using a sterile scalpel, 10 g of shoots and 25 g of fruits were aseptically weighted into a sterile plastic bag with 90 and 225 mL of BPW (Buffered Peptone Water), respectively. Homogenization was carried out in a stomacher (Stomacher 400 circulator, Seward Ltd, Technology Centre, Worthing, West Sussex, UK) for 1 min, then bags were stored at room temperature for 30 min to allow bacterial cell recovery.

Soil samples (between 0 and 30 cm of depth) were collected, for both species, from STW and TW irrigated pots at the end of the irrigating season. Four soil cores per pot were taken and pooled. 10 g soil were homogenized in a stomacher, as described above, with 90 mL buffered peptone water (BPW; Merck, Darm-stadt, Germany).

Serial dilutions of shoot, fruit and soil suspensions were prepared and plated both on PCA amended with 200 mg L^{-1} cycloheximide (Sigma–Aldrich, Milan, Italy) and Chromocult agar. Plates were incubated 3–5 days at 30 ± 1 °C and 24 h at 37 ± 1 °C, respectively. Each target sample was replicated twice. After incubation, the number of CFU g^{-1} was recorded, transformed into log^{-1} of soil and means and standard deviations calculated. From Chromocult plates, some colonies of uncertain identity (13 for apple and 17 for nectarine samples) were picked up, re-streaked and stored at - 80 °C for molecular characterization.

2.1.4. Microbiological genotyping of isolates and molecular identification

Two-mL aliquots of selected overnight cultures, derived from isolation on Chromocult agar, were processed for DNA extraction, using the Wizard Genomic DNA Purification Kit (Promega, Madison, USA). Molecular biology-based grouping of isolates was performed by ERIC-PCR with primers ERIC-1 (5′ ATGTAAGCTCCTGGGGATTCAC-3′) and ERIC-2 (5′AAGTAAGTGACTGGGGTGAGCG-3') according to Gaggìa et al. (2013). After electrophoresis (2% w/v agarose gel at 75 V), gels were stained with ethidium bromide and visualized with the gel documentation system Gel DocTM XR (Bio-Rad, Hercules, CA, USA). Images were elaborated with Gel Compare software v. 6.6 (Applied Maths, Sint-Martens-Latem, Belgium) and a cluster analysis was carried out by the unweighted pair group method with arithmetic mean (UPGMA) algorithm based on the DICE coefficient with an optimization coefficient of 1 %.

Based on genotypic grouping, representative isolates were selected for 16S rRNA amplification and delivered to Eurofins MWG Operon (Ebersberg, Germany) for sequencing (Gaggìa et al., 2013). Sequence assignment to species or genera was investigated by matching them against all catalogued bacterial 16S rRNA sequences, by using the nucleotide BLAST (Basic Local Alignment Search Tool; http://www.ncbi.nlm.nih.gov/BLAST/).

2.2. Experimental trial in controlled conditions (root internalization trial)

Experiments were carried out indoor at the University of Bologna, on 3-month old micropropagated GF 677 rootstock hybrid (*Prunus persica x Prunus amigdalus*) grown in pots filled with a mixture of peat and sand (1:1, v/v) and regularly irrigated. Plants were initially kept in a phytotron 3 days under artificial light (8 h of light followed by 16 h of dark) at a constant air temperature of 23 °C and relative humidity of 90 % for establishing favorable conditions for plant bacteria colonization. Hereafter, plants were moved inside the greenhouse for 30 days under natural light conditions where average values of maximum and minimum temperature and relative humidity were 24 °C, 28.8 °C, 17.6 °C and 85.5 %, 99 %, 56 %, respectively.

E. coli DH5α-GFPuv was used as artificial inoculum of plants. The strain was transformed with the plasmid pDSK-GFPuv to allow *in vivo* microscopical observation of the plant colonisation (Cellini et al., 2014).

To monitor the colonization of plant tissues by *E. coli* 1576 and *Pseudomonas syringae* pv. *syringae* 4364, double mutants for streptomycin and rifampicin resistance were produced by natural selection on LA plates with increasing concentration of the single antibiotic. Pure culture of both strains were obtained by the German Collection of Microorganism and Cell Cultures (DSMZ).

E. coli 1576, a Biosafety level 2 organisms because of the absence of any verotoxin genes (stx1 and stx2), was chosen since it is considered as a surrogate organism of *E. coli* O157:H7 which have been reported to contaminate horticultural crops (Evrendilek et al., 1999; Li and Zhang, 2004).

Inocula were prepared by cultures grown in liquid Luria-Bertani (LB) medium under moderate shaking at 27 °C. After a 24 h incubation, cells were centrifugated at 500 rpm for 10 min then resuspend in 10 mM $MgSO_4$. The titre of bacterial suspensions was determined by plating 10 μL drops of serial 1:10 dilutions on LB medium containing 15 g L^{-1} of agar amended with cycloheximide (200 mg L^{-1}) and specific antibiotics for each strain.

Two different methods for plant inoculation were adopted. In a preliminary trial aimed at verifying whether *E. coli* and *Pseudomonas syringae* pv. *syringae* could be translocated passively by the xylem flow, each plant was cut at the collar level and the cut was immersed in 50 mL of bacterial suspension and kept in the phytotron for 1 and 3 days. In this way, xylem vessels were directly exposed to the microbial suspension.

A second experiment was performed by immersing the bare root plants into the bacterial suspension. The inoculum concentration is reported in Tab. S3. Bacterial concentration values were kept higher with respect to the Italian legislation limit for treated wastewater reuse in agriculture (Italian Legislative Decree No. 185/03).

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Endophytic bacterial population was enumerated starting from first not immersed portion of the stem (point 0). After 1 and 3 days of inoculation, the portion above the 0 point was divided into segments of 0–10 cm. Endophytic bacterial population was assessed according to Michelotti et al., 2018.

Finally, in bare root plants to assess the evolution of possible symptoms linked to the internalization of *E. coli*, after 3 days of exposure to the bacterial suspension, plants were potted in sterile soil and kept in greenhouses for 30 days. *Pseudomonas syringae* pv. *syringae* 4364, which is a known plant pathogen, was used as positive control in all the experiments.

2.3. Statistical analysis

Data of mineral concentration and TBC-TC concentrations were analysed according to a randomized block design (ANOVA). When the analysis of variance showed a statistical effect, means were separated by the SNK Test using SAS 9.0 (SAS Institute Inc., Cary, NC, USA).

Data of *E. coli* DH5alfa, *E. coli* 1576 and *P. syringae* 4364 epiphytic and endophytic bacteria concentration were compared using a one-way ANOVA analysis followed by the Tukey HSD test. Analyses were carried out using R software (www.r-project.org).

3. Results and discussion

3.1. Effect of the irrigation treatment on leaf, fruit peel and pulp HMs and TEs concentration

Although a higher concentration of HMs and TEs was found in STW rather than in TW (Table 1) and despite generally favourable soil characteristics (i.e. high sand percentage, low cation exchange capacity and organic matter; Tab. S1) for HMs solubility and bioavailability (Gupta et al., 2019; Khawla et al., 2019), leaf concentration was not affected by irrigation treatments. Only Fe leaf concentration, was slightly increased in STW (54.7 μg/g) compared to the TW (48.5 μg/g) treatment. In any case, leaf Fe concentration was slightly below the standard range requirements for adult nectarine tree (Ricci et al., 2006) and above the deficient level for adult apple tree (Shunfeng et al., 2018). Hence, no Fe-related phytotoxic symptoms, as bronzing and leaf stippling were observed (Reeves and Baker, 2000).

Table 1
Chemical (heavy metal and trace elements) and microbiological quality of tap water (TW) and secondary treated wastewater (STW) (n = 7 ± SE).

Chemical Parameters	Irrigation water	
	[1]TW	[2]STW
pH	7.43 ± 0.04	8.31 ± 0.92
EC (dS m $^{-1}$)	0.47 ± 0.01	1.21 ± 0.04
SAR	0.63 ± 0.03	1.85 ± 0.04
Al (μg L $^{-1}$)	[3]nd	24.0 ± 1.63
B (μg L $^{-1}$)	83.7 ± 4.71	180 ± 6.47
Ba (μg L $^{-1}$)	55.5 ± 0.50	63.3 ± 2.98
Cu (μg L $^{-1}$)	6.08 ± 1.10	15.9 ± 1.39
Fe (μg L $^{-1}$)	6.00 ± 0.50	22.9 ± 2.31
Mn (μg L $^{-1}$)	[3]nd	1.00 ± 0.25
Na (mg L $^{-1}$)	20.7 ± 0.73	82.9 ± 1.04
Ni (μg L $^{-1}$)	[3]nd	3.68 ± 0.14
Pb (μg L $^{-1}$)	[3]nd	13.0 ± 0.71
Sn (μg L $^{-1}$)	[3]nd	3.54 ± 0.18
Zn (μg L $^{-1}$)	10.4 ± 1.70	42.9 ± 7.20
Microbiological Parameters		
E. coli (CFU 100 m L $^{-1}$)	4 ± 5	absent
Total Coliforms (CFU 100 m L $^{-1}$)	4245 ± 416	absent
Salmonella spp. (presence/absence)	absent	absent
Total Bacteria Count 22 °C (CFU mL $^{-1}$)	13725 ± 2258	absent
Total Bacteria Count 37 °C (CFU mL $^{-1}$)	7950 ± 1527	absent

[1] Tap Water.
[2] Secondary Treated Wastewater.
[3] not detected: concentration were below the instrumental detection limit as for Ag, As, Be, Cd, Co, Cr, Hg, Li, Mo, Sb, Se, Sr, Ti, and V.

No significant differences were also observed by Segal et al. (2011) and Petousi et al. (2015) in olive leaves HMs content, between reclaimed wastewater and fresh water irrigated-tree. Erel et al. (2019) found no increases in olive leaf B, Fe, Cu, Mn and Zn content in wastewater irrigated olive trees. Even Vivaldi et al. (2019) and Pedrero et al. (2014), found no differences in HMs and TEs (e.g. Zn, Cu, Fe, Mn) leaf concentration between wastewater and fresh water irrigated-trees, in almond and mandarin tree, respectively. Similar results, on Fe, Mn and Zn leaf concentration, were also achieved by Pedrero et al. (2015) in saline grapefruit irrigated-trees.

The overall lack of HMs and TEs increase in STW leaf concentration is encouraging considered the STW water quality, with higher concentrations both in HMs and TEs compared to TW (Table 1). Indeed, most of the elements (e.g. B, Cu, Fe, Na, Zn) retrieved in STW were between two and four times higher than in TW (Table 1). In any case, the analysed STW water quality had a generally lower concentration of HMs and TEs compared to other similar studies on fruit tree crops (Pedrero and Alarcón, 2009; Christou et al., 2014; Vivaldi et al., 2015a, b; Petousi et al., 2019a, b; Vivaldi et al., 2019). For Fe in reclaimed water, Pedrero and Alarcón (2009), Vivaldi et al. (2015a, b) and Vivaldi et al. (2019), found values between 40 and 230 μg/L, between two and ten times higher if compared to the present study (22.9 μg/L).

In any case, even if more chemically polluted wastewater were used for irrigation of fruit tree crops, previous studies showed HMs and TEs (e.g. B, Na) leaf concentration increases (Pedrero et al., 2015; Nicolás et al., 2016; Pedrero et al., 2018; Petousi et al., 2019a, b) but usually without never exceeding the toxic thresholds established for the considered species (e.g. nectarine, citrus, mandarine, grape, olive, almond) and with any observed leaf detrimental effects.

The accumulation of HMs and TEs in plant tissues depends on multiple factors, starting from soil characteristics such as soil pH, texture, cation exchange capacity (CEC) and organic matter (OM) (Khawla et al., 2019). In the present study, even if the soil texture (60.9 % of sand), low CEC and OM were considered favourable conditions for metal solubility and bioavailability (Gupta et al., 2019), the slightly alkaline soil pH (7.6; Tab. S1) could have limited this effect. Alkaline pH is considered to be the main factor to decrease metals soil solubility (Sheoran et al., 2016).

Furthermore, the HMs and TEs uptake from the soil solution to plant tissues depends also on plant-related traits such as species, cultivars, rootstocks, plant age, plant morphology/anatomy and plant physiological stage (Avci and Deveci, 2013; Petousi et al., 2019a, b; Pedrero et al., 2018; Vivaldi et al., 2019) and to physical, chemical and biological involved processes (e.g. root uptake, sequestration/compartmentalization, transpiration flux) (Cataldo and Wildung, 1978).

Significative differences on leaf concentration were indeed mainly found between the two species considered. Ba, Cu, Ni and Pb concentrations were higher in apple leaves compared to nectarine while Na and Zn increased in the leaf tissues of nectarine trees (Table 2).

As regard to both fruit peel and pulp HMs and TEs concentrations, no significant differences were observed between STW and TW treatments in both species, with factor interaction (species and water source) for B, Fe, Mn and Zn (Table 2). Irrigation with STW slightly increased Zn concentration in nectarine peel (17.8 μg/g STW; 12.1 μg/g TW) (Table 2). Zn is known to have a high exchangeable soil capacity and a high xylem mobility for possible accumulation in plant edible parts (e.g. fruit) (Feller, 2015; Khawla et al., 2019). In any case, HMs concentrations retrieved in the analysed tissues were within the

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Table 2
Effect of the water source and fruit tree species on the leaf, fruit peel and pulp heavy metal and trace element concentrations.

Treatment	Al	B	Ba	Cu	Fe	Mn	Na	Ni	Pb	Sn	Zn
Leaf											
TW	24.3	29.3	30.4	7.55	48.5 b	27.3	70.5	0.74	1.17	1.19	17.0
STW	22.4	26.3	34.5	8.00	54.7 a	30.5	68.3	0.99	1.14	1.16	17.7
Significance	ns	ns	ns	ns	*	ns	ns	ns	ns	ns	ns
Species											
Nectarine	23.8	29.9	18.8 b	6.39 b	53.1	30.8	80.6 a	0.63 b	1.02 b	1.14	19.6 a
Apple	22.9	25.7	46.1 a	9.16 a	50.2	27.0	62.8 b	1.10 a	1.29 a	1.21	15.1 b
Significance	ns	ns	***	***	ns	ns	**	**	*	ns	**
Interaction	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Fruit peel		Apple Nectarine			Apple Nectarine	Apple Nectarine					Apple Nectarine
TW	12.9	24.6 b 43.3 a	1.53	3.20	37.6 b 49.4 a	5.80 c 9.03 a	37.6	0.73	1.55	1.07	2.43 c 12.1 b
STW	9.46	14.8 c 43.6 a	1.37	3.10	50.3 a 39.3 b	6.55 c 7.90 b	37.8	0.66	1.59	1.04	2.75 c 17.8 a
Significance	ns		ns	ns			ns	ns	ns	ns	
Species											
Nectarine	11.6		0.98 b	4.95 a			52.7 a	0.90 a	1.40 b	1.13 a	
Apple	10.6		1.84 a	1.60 b			24.8 b	0.53 b	1.71 a	0.99 b	
Significance	ns		***	***			***	***	*	*	
Interaction		**			*	*					*
Fruit pulp		Apple Nectarine			Apple Nectarine						
TW	3.33	31.8 a 25.6 b	0.45	1.92	6.17 b 9.10 a	2.25	37.1	0.57	1.48	0.87	3.20
STW	3.41	14.5 c 27.7 b	0.39	2.01	8.4 a 6.43 b	2.00	29.1	0.57	1.66	0.86	4.11
Significance	ns		ns	ns		ns	ns	ns	ns	ns	ns
Species											
Nectarine	3.13		0.28 b	2.23 a		3.05 a	34.5	0.82 a	1.52	0.93	5.68 a
Apple	3.59		0.53 a	1.74 b		1.31 b	31.3	0.36 b	1.63	0.80	1.99 b
Significance	ns		***	**		***	ns	***	ns	ns	***
Interaction		***			**						

ns, *, ** and ***: effect not significant or significant at $p \leq 0.05$, $p \leq 0.01$ or $p \leq 0.001$, respectively. Within the same element, means followed by the same letter are not statistically different ($p \leq 0.05$, SNK Test). Ag, As, Be, Cd, Co, Hg, Mo, Sb, Ti, Tl, and V concentration were was below the instrumental detection limit (dl).

acceptable limits imposed by the FAO heavy metal regulations for contaminants and toxins in food and feed (FAO/WHO Codex Alimentarius, 2007).

Similar results were confirmed by Vivaldi et al. (2015a, b), on nectarine fruit, who did not observed any increment on HMs (Cu, Mn, Zn) and TEs (B, Fe, Na), although a higher concentration was evidenced in the secondary-treated municipal wastewater.

As concern the overall fruit trace element concentration (e.g. Cu, Na, Ni and Sn), significant differences were found between species, with generally higher values in nectarine fruit tissues (Table 2). This evidence could be due to the volume of water transpired by nectarine fruits, having a higher amount of xylem sap entering the fruit compared to apple (Huguet et al., 1998). Therefore, the different behaviour in accumulating heavy metals in leaves and fruit may be a consequence of their different transpiration rate (Higgins et al., 1992; Grifferty and Barrington, 2000; Morandi et al., 2012).

HMs and TEs are mainly known to be transported passively from root to shoot through xylem vessels (Oropeza-Garcia et al., 2014). Zheljazkov and Nielsen (1996) found that the concentrations of heavy metals in vegetables per unit of dry matter generally follow the order: leaves > fresh fruits > seeds.

The higher fruit transpiration in nectarine compared to apple fruit seems to represent the key driver for HMs accumulation, even if the different genetic characteristics of plants in absorption, transport and accumulation of HMs and TEs in the upper parts of plants should not be excluded (Halim and Deveci, 2013).

This is highlighted by the fact that extremely xylem mobile heavy metals, such as Cu, Mn, Zn (Feller, 2015) and trace elements as B (Brown and Shelp, 1997), had higher concentrations in nectarine fruit peel compared to apple fruit peel. Khawla et al. (2019) found similar results in corn edible tissue, with high accumulation for Cu and Zn in wastewater-irrigated plants.

In addition, nectarine fruit HMs concentration (peel and pulp) followed the sequent decreasing order: Zn > Mn > Cu, as found by Nworie et al., 2019 in shoot of wild herbaceous plants grown in contaminated sites. The high root-shoot translocation rate, for Zn, Mn and Cu was also observed by Page et al. (2005); Chandra et al. (2009); Han et al. (2013); Kamari et al. (2014) and Christou et al. (2014).

As concerns B fruit concentration, a higher amount was found in nectarine fruit than in apple as for Zn, Mn and Cu. Indeed, B is known to be a highly xylem-mobile element (Brown and Shelp, 1997). Furthermore, despite all the other elements, B showed more elevated concentrations in nectarine fruit than in leaves. This could be explained by the fact that nectarine is known to be a B-phloem-mobile tree and tends to remobilize from sources to sinks (e.g. fruit) (Pedrero et al., 2018).

As for apple fruit, the lower HMs concentration retrieved is likely determined by its apoplasmic phloem-unloading during the whole season (Zhang et al., 2004). Heavy metals are generally poorly mobile in the phloem (Mapanda et al., 2007), with direct penalization on the re-translocation process (leaf to fruit) and thus resulting in lower accumulation (Gupta et al., 2019). Moreover, the xylem dysfunctionality that affects apple fruits at about 120 days after full bloom (DAFB) (Drazeta et al., 2004) represents a further limit to the potential xylem-driven transport of HMs to fruit.

Eid and Shaltout (2016) found similar results in tomato plants, with a higher metal content in leaves and shoots compared to fruits, indicating the presence of a physiological barrier in the transfer of these metals from roots/shoot tissues to fruits, probably ascribable by the fact that tomato growth, like apple, is characterized mainly by an apoplasmic phloem unloading during the last growth stages (Van Ieperen et al., 2003).

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Researches by Lonigro et al. (2007) and Christou et al. (2014) did not find significant differences in tomato fruit HMs concentration in wastewater irrigated-plants compared to conventional water irrigation.

Our results, as most of the studies related to wastewater-irrigated fruit tree crops (Pedrero et al., 2015; Nicolás et al., 2016; Pedrero et al., 2018; Petousi et al., 2019a, b), suggest that reclaimed water can be safely adopted for irrigation, without any HMs and TEs-related phytotoxic symptoms. It should always be taken into consideration that we are dealing with perennial crops where the accumulation rate is generally lower than in leafy vegetables (Gupta et al., 2019). Furthermore, concerning the fruit, no contamination was found even on nectarine fruit, where growth is mainly transpiration-driven throughout the xylem, the preferential pathways for HMs and TEs translocation.

Even if this research brought to promising results on potted plants, long-term studies should be performed in field conditions to better assess plant toxicity effects and potential fruit contamination. Simultaneously, case by case evaluations of soil characteristics and of wastewater quality should be carefully assessed. This would guarantee a safe wastewater reuse for the environment and for public health.

3.2. Wastewater microbiological quality

Table 1 reports the main microbiological safety indicators of the used STW. Seasonal average enumeration of *E. coli* was 4 ± 5 CFU 100 m L^{-1}, whereas *Salmonella* spp. was never detected. *E. coli* recovery respected the Italian threshold (< 10 CFU 100 m L^{-1}) for treated wastewater irrigation purposes (Italian Legislative Decree No. 185/2003). The additional data of TC, TBC 22 °C and TBC 37 °C were 4245 CFU 100 m L^{-1}, 13,725 CFU mL^{-1} and 7950 CFU mL^{-1}, respectively (Table 1). The Italian legislation does not establish any threshold concerning these parameters; however, analyses have been performed to have wider information about STW bacterial load.

Table 3
Enumeration of bacterial indicators in STW and TW-irrigated soil samples (n = 5 ± SE).

Soil	Nectarine			
Treatment	TW	STW	TW	STW
Target microorganisms				
E. coli (CFU g^{-1})	absent	absent	absent	absent
TC (log CFU g^{-1})	3.06 ± 0.30	2.13 ± 0.62	1.22 ± 0.51	2.29 ± 0.06
TBC 30 °C (log CFU g^{-1})	6.55 ± 0.09	6.39 ± 0.12	5.27 ± 0.06	5.14 ± 0.02

3.3. Soil microbial characterization

Soil microbial analyses did not show statistical effects either by water source or plant species (Table 3), with TBC values ranging from 5.1–6. 6 log CFU g^{-1} and TC values between 2.3 log CFU g^{-1} and 3.0 log CFU g^{-1}. Values for TC are in agreement with Vivaldi et al. (2013) who performed a similar trial on nectarine trees in open field conditions. However, these results are strictly influenced by the intrinsic water and soil properties. The microbial load (i.e. TBC and TC) retrieved in the treated wastewater (Table 1) seemed not to influence the soil microbiological parameters, indicating that the introduced microorganisms could not adapt to the harsh environment of the soil and to perturb its complex and stable microbial equilibrium of the soil (Van Elsas et al., 2011). *E. coli* was never isolated in the soil samples, although it was present in the treated wastewater. This result confirms the data obtained by Palese et al. (2009) in a topsoil (0–0.10 m depth) irrigated with *E. coli* contaminated wastewater source. Similar results were also reported by Lonigro et al. (2015) who did not find any contamination effect on soil samples irrigated with three treated wastewaters.

3.4. Internal shoot and fruit bacteria concentration

Overall, results on TBC in shoots (Fig. 1) suggest a significant increase of bacteria in both apple and nectarine trees irrigated with STW compared to TW. The same trend, even if not statistically different, was also found on TBC fruit concentration (Fig. 1). This effect may be related to the microbial load provided by treated wastewater (Table 1) that may have enriched the endophytic community of STW plants (Zolti et al., 2019). Moreover, the increasing nutrients provided by STW (Perulli et al., 2019) could have promoted the survival and growth of already existing endophytes, as well as the concentration in-

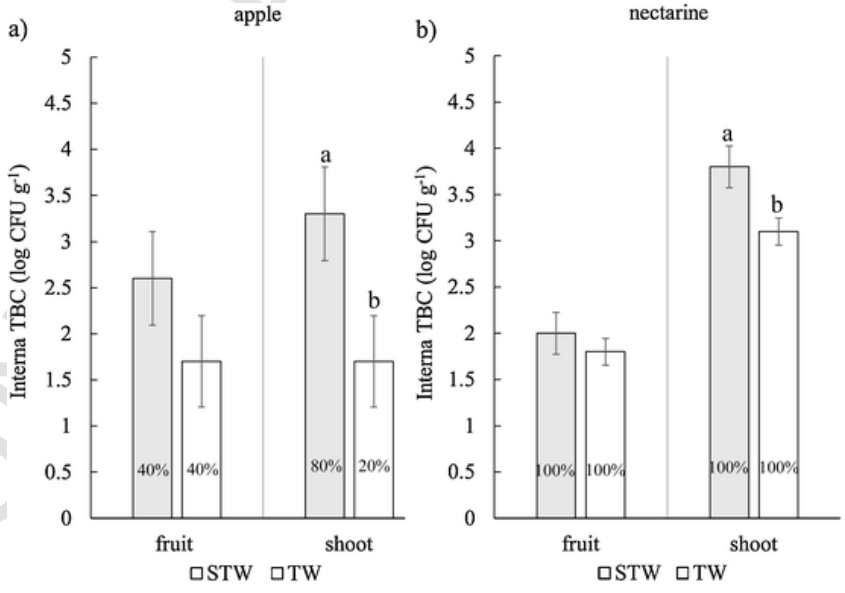

Fig. 1. a) Total bacteria population (TBC; log CFU g^{-1}) and frequency in apple fruit and shoot irrigated with STW and TW respectively. b) Total bacteria population (TBC; log CFU g^{-1}) and frequency in nectarine fruit and shoot irrigated with STW and TW respectively. For each species a *t*-Student test was applied between tissues irrigated with the two different water sources.

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crease of some heavy metals. In addition, STW-derived signalling molecules could enhance the colonization of soil-associated bacteria (Sorrenti et al., 2017). Several studies claim that the rhizosphere soil is the primary source of endophytic colonization (Hallmann et al., 1997) with endophytic bacteria translocated to the areal part of the plant (James et al., 2002; Compant et al., 2005) by the water transpiration flux, which is the main driving force of the xylem stream (Zimmermann et al., 2002). This is supported by results in Table 4 where TBC load was higher in nectarine than in apple, probably because of its higher leaf transpiration (Higgins et al., 1992). This assumption is further strengthened by the presence of TC (ubiquitous aerobic microorganisms) only in trees (shoot and fruit) irrigated with STW, while no TC detection was observed in TW-irrigated trees (Fig. 2). These data strongly suggest that most of the influence on plant endophytic microbial load could derived from the STW supply.

Another important aspect is the higher TBC on shoots compared to fruits (Table 4), which seems probably related to the lower transpiration rate of fruits (Higgins et al., 1992; Morandi et al., 2012). It is mostly accepted that roots are the main bacteria entrances to the xylem vessels (Hardoim et al., 2008; Turner at el., 2013) and, once inside, their main driver to the areal part of the plant is the water transpiration flux. Gorbatsevich et al. (2013) showed, on sweet basil, a higher incidence of bacteria internalization (i.e. *S. enterica*) in vegetative organs compared with reproductive ones. As reported by Ocaña de Jesús et al. (2018), *E. coli* O157:H7, *E. coli* O157:H16 and *E. coli O105ab* strains were found in higher concentrations in shoot compared to fruit in inoculated roots of tomato plant. *E. coli* presence in tomato fruit, as reported by Windt et al. (2009), could be related to the xylem water inflow. These results confirm a likely direct effect of the transpiration stream to promote bacteria translocation to the areal part of trees (Solomon et al., 2002b; Cooley et al., 2003).

In any case, it cannot be excluded the effect of the harsher microenvironment for bacterial colonization in fruits due to presence of organic acids and antimicrobial compounds (Fattouch et al., 2008; Cevallos-Casals et al., 2006), even if some studies reported that some endophytic bacteria colonize flowers, fruits and seeds (Hallmann, 2001, Spinelli et al., 2005). Under natural conditions the majority of fruits does not contain endophytic bacteria at all or only at very low population (Hallmann, 2001), reaching up to 10^2 10^3 CFU g^{-1} of fresh weight (S. Compant, unpub. results), in line with our findings. It is likely that only specialized endophytic strains can colonize and survive into the reproductive organs (Mundt and Hinkle, 1976).

In both species, it is evident how STW irrigation had a significant impact on TBC load compared to TW irrigation (Table 4). This difference was statistically verified in shoots (Fig. 1), whereas no statistical evidence was found for fruits, even if the trend was maintained. Based on our knowledge, there are no available data regarding the TBC and TC concentration in shoot and fruit tissues, on fruit perennial crops. Vivaldi et al. (2015a, b) performed a similar experiment on nectarines, but results are not comparable since authors investigated the exterior microbial load on fruit peel. Furthermore, Petousi et al. (2019a, b) evaluated the total coliform and *E. coli* concentrations in grapefruit irrigated with STW, but without differentiating the endophytic and epiphytic bacterial population. However, McInroy and Kloepper (1994) reported that the common population size of indigenous endophytic population ranged in different annual crops from 10^4 to 10^6 CFU g^{-1} of fresh weight, in line with our results.

This conclusion is validated by the results of the TC load. In this case, statistical differences were again confirmed for the treatment effect, with increased concentrations in STW compared to TW (0.7 log CFU g^{-1} *vs* 0.0 log CFU g^{-1}) (Table 4). As concern species and tissue

Table 4
Effect of the water source, species and type of tissue on TBC and TC concentration. Expand

	TBC (log CFU mL $^{-1}$)	TC (log CFU mL $^{-1}$)
Treatment		
TW	1.51 a	0.00 a
STW	2.39 b	0.69 b
Significance	**	*
Species		
Apple	1.20 a	0.31
Nectarine	2.70 b	0.38
Significance	***	ns
Tissue		
Shoot	2.50 b	0.57
Fruit	1.40 a	0.12
Significance	**	ns
Interaction	ns	ns

ns, *, ** and ***: effect not significant or significant at $p \leq 0.05$, $p \leq 0.01$ or $p \leq 0.001$, respectively. Within the same parameter, means followed by the same letter are not statistically different ($p \leq 0.05$, SNK Test).

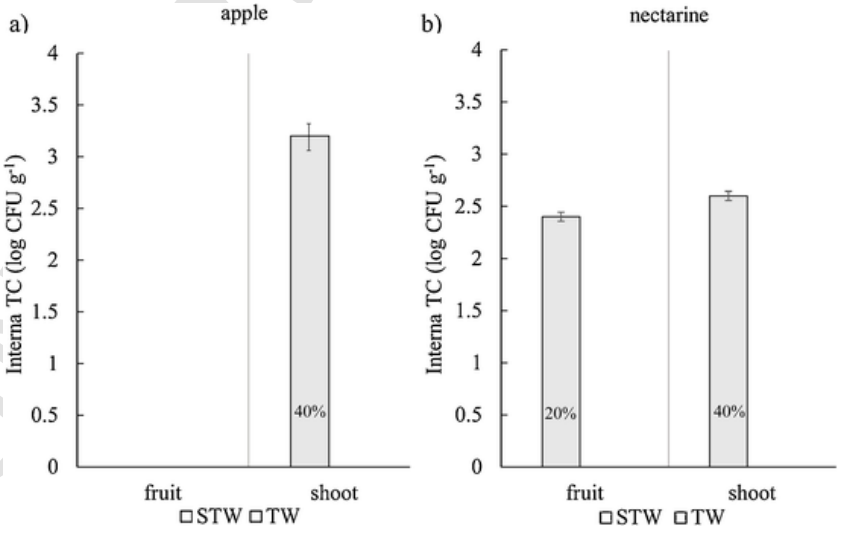

Fig. 2. a) Total coliforms population (TC; log CFU g^{-1}) and frequency in apple fruit and shoot irrigated with STW and TW respectively. b) Total coliforms population (TC; log CFU g^{-1}) and frequency in nectarine fruit and shoot irrigated with STW and TW respectively. For each species a *t*-Student test was applied between tissues irrigated with the two different water sources.

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TC load, the same TBC trend (higher concentration in nectarine and shoot, respectively in species and tissue) was maintained, even if not statistically significative (Table 4)

TC were completely absent in all apple fruits compared to nectarine fruits where 20 % of trees was found positive (Fig. 2b). Differences in the transpiration rate and in the xylem vessels conductivity (e.g. xylem dysfunctionality) during fruit development between the two species (Morandi et al., 2016) could explain the different bacteria fruit colonization.

Indeed, nectarine fruits, unlike apple, are not subjected to xylem dysfunctionality during development and have a higher water loss during the season (Drazeta et al., 2004). Although nectarine fruits seem more susceptible to bacterial contamination, our results suggested that even after four months of continuous irrigation with STW, only few TC colonies (2.4 log CFU g^{-1}) were found in the 20 % of trees, with values far below the limits fixed by Commission Regulation (EC) (EC) No 2073/2005, 2005 for microbiological criteria for foodstuffs. This result confirms the safety of nectarine and apple fruit produced by irrigation with STW. Similar results were achieved by Palese et al. (2009) who showed a very weak olive-drupes *E. coli* contamination (10 CFU 100 g^{-1} fresh weight) on the whole fruit. Although, at least in nectarine, potential human pathogenic bacteria could reach the areal part of the plant, their persistence in the plant tissue is unlikely due to the competition with the endo- and epiphytic microbiota (Schuenzel and Harrison, 2002), that could prevent their multiplication and spread (Liao and Fett, 2001).

3.5. Molecular identification of bacterial isolates

The thirty isolated colonies (suspected coliforms or *E. coli*) from Chromocult plates and detected only in STW-irrigated trees did not belong to the species *E. coli*. The cluster analysis allowed the identification of three sub-clusters for nectarine isolates and two for apple isolates (Tab. S2). The sequencing of the selected strains (one for each sub-clusters) led to the identification of these bacterial strains as *Pantoea agglomerans* in nectarine and *Pseudomonas punonensis* and *Pseudomonas orizyhabitans* in apple (Tab.S2)

Chromocult agar is a bacteriological growth medium developed for the simultaneous detection in water samples of total coliforms and *E. coli* due to the inclusion of two differential chromogenic substrates. Dark blue colonies resulting from salmon-galactoside and X-glucuronide cleavage by β -D-GALACTOSIDASE AND B -D-glucuronidase are generally referred to as putative *E. coli* colonies. Our results outlined the presence of false positive as already reported by Finneya et al. (2003) who found, among enterobacteriaceae, species showing β –glucuronidase activity. In fact, several *Pantoea* ssp. isolates present β –glucuronidase activity.

These results suggest that irrigation with secondary treated wastewater does not cause any risk of fruit contamination by potential human pathogen bacteria, since no *E. coli* have been found in the vegetative and reproductive tissues of the plants. Similar results were achieved by Sofo et al. (2019), which did not find *E. coli* in the xylem sap of olive trees irrigated with treated urban wastewaters. TC were isolated in 40 % of trees irrigated with STW at very low concentration and mostly in shoots.

Undoubtedly, the continuous irrigation with STW slightly affected the overall tree endophytic community, being the TW-irrigated plants TC free. Sofo et al. (2019) showed that *Pseudomonas* and *Acinetobacter spp*. were significantly higher in wastewater irrigated xylem sap of olive trees, compared to the rainfed trees.

Several bacterial genera belonging to the Enterobacteriaceae family are plant associated bacteria (Hallmann et al., 1997) and they include both pathogenic species, such as *Erwinia amylovora (*Kim et al., 2001*)* and plant symbionts, such as *Pantoea agglomerans* (Walterson and Stavrinides, 2015). Bell et al. (1995) described *Pantoea* spp. as one of the endophytic bacteria isolated in the stem of grapevine, while Chen et al., 2017 found that *Pantoea alhagi* could promote growth and drought tolerance in wheat. Moreover, coliform bacteria and *Pantoea* spp. are ubiquitous in the environment and able to persist as endophytic microorganism (Gao et al., 2019). In some cases, enterobacteriaceae can also possess Plant Growth Promoting activity and be beneficial to plants (El-Gleel Mosa et al., 2016; Perpetuini et al., 2019). As regard to *Pseudomonas* spp., it is known that it represents a key component of ecological processes able to suppress plant diseases in agricultural and natural environments (Weller et al., 2002), and several strains are used commercially to manage plant diseases in agriculture (Stockwell and Stack, 2007). *P. punonensis* strain D1−6 is known to produce 1-aminocyclopropane-1-carboxylate deaminase (ACC deaminase) (Lafi et al., 2017), while *P. orizyhabitans* was reported as promising biological agents towards various plant root pathogens and plant-parasitic nematodes of the genus *Meloidogyne* (Vagelas et al., 2007).

3.6. Root internalization test

In the root system, *E. coli* DH5α, *E. coli* 1576 and *P. syringae* 4364 were isolated both as epiphytes and endophytes. At day 1 post-inoculation, the epiphytic populations were statistically different among the three bacteria, *P. syringae* 4364 exhibiting the highest value, followed by *E. coli* 1576 and DH5α. On the root surface, both *E. coli* strains showed an increasing concentration from 1 to 3 days post inoculum. Instead, *Pss* 4364 epiphytic population decreased during time (Fig. 3).

The results clearly discriminated between the plant pathogenic behaviour of *P. syringae* 4364 and the casual plant association of *E. coli* strains. Indeed, plant pathogenic bacteria, being adapted to the plant environment, are able to attain high population in the epiphytic niche (Ogawa and English, 1991; Cellini et al., 2019; Donati et al., 2018). Similarly, the reduction of *P. syringae* 4364 epiphytic population over time suggest internalisation of the pathogen after reaching the infection threshold (Donati et al., 2020 in press). The higher population of *P. syringae* 4364 in comparison to *E. coli* may also be due to the chemiotaxis exerted by root exudates on plant pathogens (Van Overbeek and Van Elsas, 1995). Nonetheless, root exudates could also be exploited by human associated bacteria, such as *E. coli*, as a nutrient source (Jablasone et al., 2005). As far as *E. coli* is concerned, the armed strain *E. coli 1576* showed a population more than two times higher than the disarmed strain *E. coli* DH5α.

The endophytic populations were generally lower than the epiphytic ones, both at 1 and 3 days after inoculation (Fig. 4). Endophytic population increase during time for both *E. coli DH5α* and *E. coli 1576,* while it remained constant for *P. syringae 4364*. The lack of increase of endophytic *P. syringae 4364* population does not contrast the hypothesis of the pathogen internalisation into the host tissues and it may be due to the migration form roots to other plant organs (Table 5) (Ogawa and English, 1991; Cellini et al., 2019; Donati et al., 2020)

Concerning *E. coli*, at 1 day after inoculation, the endophytic population reflected the epiphytic one showing the same statistical discrimination between the two strains. This result suggests that both the *E.coli* strains are able to enter the root apoplast through root emergence sites, wounds, cracks (Reinhold-Hurek and Hurek, 1998), but with a higher capacity for the armed *E. coli 1576* strain in colonising the tissues (Oca et al., 2018).

At 3 days post-inoculum, both the *E. coli* strains increased their concentrations, suggesting that they could exploit the nutrients dissolved in the apoplast.

Even at 30 days post inoculation, both *E. coli DH5α* and *1576* strains were never isolated from the above ground parts of the plant, suggest-

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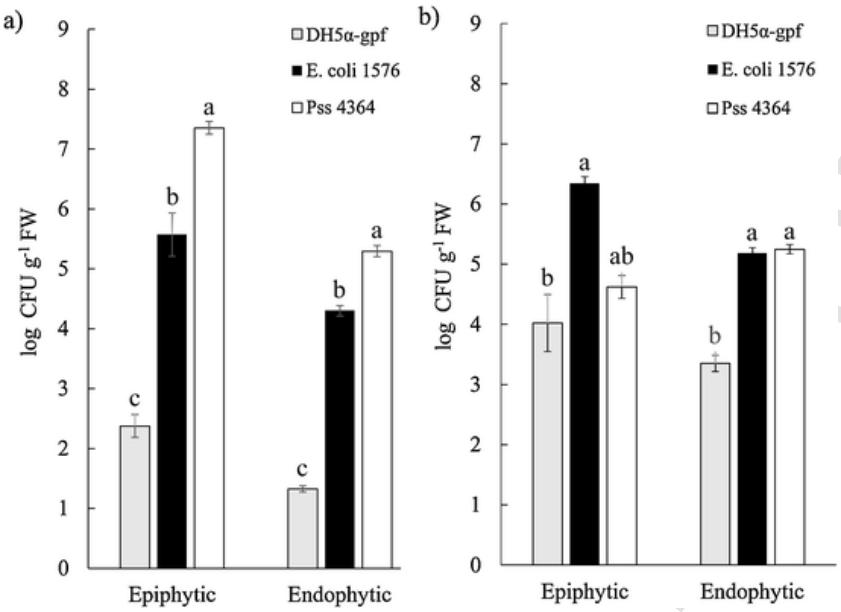

Fig. 3. Epiphytic and endophytic population at inoculation site (roots) assessed at 1 (a) and 3 (b) days post inoculum in peach rootstock (i.e. GF 677). Each value corresponds to the mean (ME ± SE) of log CFU g^{-1} fresh weight. Different letters indicate significant differences between strains, inside the same category (i.e. epiphytic or endophytic), according to Tukey's HSD test ($P < 0.05$).

Table 5
Detection of endophytic *E. coli* DH5α-gpf and, 1576 and *Pseudomonas syringae pv. syringae 4364* above inoculation point in GF677 peach rooted plants at 1, 3 and 30 days post inoculation. Expand

	1 d			3 d			30 d		
	log CFUg $^{-1}$ FW			log CFUg $^{-1}$ FW			log CFUg $^{-1}$ FW		
cm	DH5α-gpf	E. coli 1576	Pss 4364	DH5α-gpf	E. coli 1576	Pss 4364	DH5α-gpf	E. coli 1576	Pss 4364
0–10	0	0	0	0	0	8.2 ± 4.1	0	0	0
10–20	0	0	26 ± 6.1	0	0	0	0	0	0
20–35	0	0	0	0	0	0	0	0	8.1 ± 4.1
35–50	0	0	0	0	0	0	0	0	18 ± 9.2

Inoculation was performed on peach roots immerged in the appropriate bacteria suspension (1.8 × 104 CFU mL $^{-1}$). Each value corresponds to the mean (ME ± SE) of log CFU g $^{-1}$ fresh weight.

ing the inability of this species to translocate and migrate in the plant (Table 5). *E. coli* was absent also in cutting directly exposed to a bacterial suspension, thus suggesting that even passive translocation inside the xylem, driven by transpiration, did not occur (Tab. S4).

On the other hand, the plant pathogenic strain *P. syringae 4364* was able to translocate to the areal part starting from the first day after inoculation. Furthermore, *P. syringae 4364* migrated up to plant apical buds (30–50 cm, above ground) (Table 5).

After 30 days, *E. coli DH5α* was completely absent also at the epiphytic root level, while *E. coli 1576* was only epiphytically present in the root system even though its population decreased more than twice (Fig. S2). The survival of enteric pathogens on the root surface can indeed be hampered by competition for niches and for root exudates caused by indigenous epiphytes (Cooley et al., 2006).

As regard the root endophytic colonization (Fig. S2), the implementation of the plant defence systems and the competition between *DH5α, E. coli 1576* and the endophytic microbiota (Liao and Fett, 2001; Matos and Garland, 2005; Schuenzel and Harrison, 2002; Deering et al., 2012) could be the main factors determining the inability of these strains to permanently colonize and migrate to the upper part of the plant, avoiding any potential risk of bacterial contamination of the areal part of the plant.

4. Conclusions

The use of treated wastewater for irrigation could be one of the most promising and sustainable strategies for alleviating water shortage issues in many countries. In our conditions, irrigation with STW did not promote the accumulation of potential toxic elements (e.g. B, Na, Zn) both in leaves and fruits of nectarine and apple trees. Most of the differences in fruit heavy metal and trace element concentrations were related to the species, slightly higher in nectarine.

Additionally, STW did not affect soil microbiological population. The absence of *E. coli* and the low TBC and TC in soil is of interest since wastewater usually represents a potential reservoir of harmful bacteria of fecal origin that could enrich in the soil and get in touch with the plant system. The plant tissues did not contain any *E. coli*. Coliforms, which can be indicators of fecal contamination, were detected in the vegetative and reproductive tissues only of nectarine. However, their counts were well below the limit for microbiological criteria for foodstuff (EU Commission Regulation (EC) (EC) No 2073/2005), indicat-

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ing that food safety is not compromised by using this irrigation strategy.

Moreover, even with artificial inoculation with very favorable conditions for plant colonization, *E. coli DH5α* and *E. coli 1576*, even if initially able to colonize epiphytically and endophytically GF 677 roots, failed to move inside the areal part of the tree, even at 30 days post-inoculation. These results are particularly important since they exclude the *E. coli* contamination of fruit from plant grafted on GF 677, which is one of the rootstocks most commonly used in peach and nectarine cultivation.

Although this study endorses STW re-use in agriculture, longer-term and open field conditions investigations are required to evaluate the endophytic population dynamics and their potential effects on plant growing performances. Moreover, a wider screening of contaminants of emerging concern (CECs) (i.e. pesticides, hormones, antibiotics and antibiotic resistant microorganisms) should be carried out at both soil and plant level.

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Uncited references

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.agwat.2020.106403.

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