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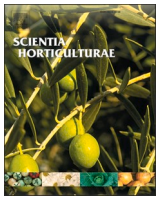

The fate of bacteria in urban wastewater-irrigated peach tree: a seasonal evaluation from soil to canopy

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ABSTRACT

Irrigation with wastewater can be a solution to preserve and mitigate freshwater demand, in particular during drought periods. Unfortunately, wastewater, although being treated at different levels, could be a carrier of human pathogens (*e.g., E. coli*) and potentially contaminate crops for human consumptions.

This study investigated the seasonal microbiological concentrations, on soil, shoot and fruit tissues of potted peach trees, following two irrigation treatments: freshwater (FW) and secondary urban wastewater without the final disinfection treatment (SW).

E. coli was only detected in SW irrigated soil, whereas total coliforms (TC) and total bacteria counts (TBC) were similar in both treatments throughout the season. Endophytic *E. coli, Salmonella* spp. and TC were not detected in shoot and fruit, but a higher presence of total bacteria (TBC) was observed in SW-irrigated tree compared to FW-irrigated tree. In particular, SW shoots had a higher load compared to fruits, thus showing a potential effect of leaf transpiration, that promoted the transfer of water-borne bacteria from soil to the epigeal part (shoot). The adoption of low-quality SW (even above the microbiological limits of the European Regulation 2020/741 for wastewater re-use in agriculture), when a drip irrigation method is applied, could be a valid alternative to save fresh water without compromising fruit safety.

1. Introduction

Water scarcity is becoming one of the major challenges of this century, especially for crop production. Agriculture represents the sector with the highest use of fresh water with an average of 70% of all water uses, reaching peaks of 90% in many developing countries (FAO, 2017). Due to the shortage of traditional water sources, alternative strategies are urgently needed (Deng et al., 2019). Wastewater reuse could be a solution, in particular in arid and semi-arid regions but it also represents a support to traditional irrigation in areas affected by frequent water shortage (*e.g.*, Italy) (Mancuso et al., 2020; Singh, 2021). Wastewater irrigation is independent from seasonal drought and weather variability, being able to cover peaks of water demand and reducing risks of crop shortage and income losses. Furthermore, several studies confirmed its fertigation role, being rich in macro and micronutrients for plant nutrition (Ofori et al., 2021). On the other hand, it could be heavily contaminated by chemical compounds (*e.g.,* heavy metals, toxic elements, emerging contaminants) and harmful pathogens (*e.g.*, *E. coli, Salmonella* spp.) deriving from organic wastes with related risks for soil, plant and human health (Singh, 2021). *E. coli* is the main indicator of fecal contamination in most of international legislation for water quality (Baudišová, 1997); moreover it provides a simple standardized method to assess the efficacy of wastewater treatment processes (Motlagh and Yang, 2019; Nwaneri et al., 2018). Bacterial content in wastewater can have a broad concentration range (1–10 log CFU L^{-1}), depending on its origin, environmental parameters (*e.g.,* temperature, C/N ratio) treatment level and disinfection system (*e.g.,* peracetic acid (PAA), UV) (Singh, 2021; Chen et al., 2021).

Water-borne bacteria can reach the soil trough wastewater, where they can survive and find a niche in the rhizosphere (Orlofsky et al., 2016; Zolti et al., 2019). There is evidence that plant root bacteria endophytes are mainly recruited from soil, which then ascend to stems and leaves via the apoplast in xylem vessels (Chi et al., 2005). However, their presence in the roots does not directly correlate with their presence in

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crops edible or foliar tissues (Perulli et al., 2021). Moreover, this process is highly selective, involving actively the rhizosphere, the rhizoplane and the endosphere that perform a screening role in the acquisition process (Chi et al., 2005).

According to some studies, the risk of bacterial pathogens uptake through the roots from contaminated soil is relatively low (Hirneisen et al., 2012).

However, the water transpiratory flux seems to be the main bacterial driver from the roots to the canopy (Gorbastevich et al., 2013). Indeed, the presence of *E. coli* and/or *Salmonella* spp. is higher in vegetative tissues (*e.g.,* leaves) compared to reproductive ones (*e.g.,* fruits) in horticultural crops such as tomato (Hintz et al., 2010; Gorbastevich et al., 2013; Ocaña de Jesús et al. 2018).

As concern fruit tree crops, literature is still poor of investigations and the few available studies looked at the external fruit contamination, excluding the root internalization pathway (Vivaldi et al., 2013; Pedrero et al., 2020).

In our first preliminary investigation, we evaluated the presence of endophytic bacteria in nectarine trees irrigated with an urban wastewater (subjected to a disinfection treatment) at harvest time (Perulli et al., 2021). In the present study, which represents a follow-up of Perulli et al. (2021), we drip-irrigated potted peach trees with heavily microbiologically polluted urban wastewater where we assessed the fate of bacteria, in soil and inside plant tissues, throughout a whole irrigation season. Furthermore, the relationship between plant internalized bacteria and leaf gas exchanges (*i.e.,* transpiration, stomatal conductance) was investigated. This research was performed also in the light of the less strict microbiological limits established by the new European Regulation (EU) 2020/741 (active starting from June 2023) on minimum requirements for water reuse in agriculture (European Comission, 2020). This would allow a better understanding of wastewater potential risks on fruit tree irrigated with a drip irrigation system when the wastewater quality is even lower than the class C of the EU, 2020/741 (European Comission, 2020).

2. Materials and methods

2.1. Experimental trial and weather conditions

The study was performed outdoors, inside the urban wastewater treatment plant (HERA S.p.a- Italian multi-utility), located in Cesena (Emilia-Romagna, Italy), on 3-year-old bearing peach trees (*Prunus persica* L. Batsch) "Aliblanca" grafted on "GF 677″.

Trees were individually grown in 60L pots filled with an alkaline (pH 7.9), clay loam (23% sand, 48% silt, 29% clay) soil. Trees were trained as a spindle and protected by an exclusion hail net (20% shading).

Trees were arranged in a randomized block design (6 trees block^{-1}) with two irrigation treatments (9 trees treatment^{-1}): a) freshwater (FW) and b) secondary treated urban wastewater (SW).

SW was subjected to the Italian Decree of Ministry for Environment (2006), but bypassing, for the scope of this research, the final disinfection treatment, normally achieved with PAA. Chemical characteristics of the water sources were analysed in Perulli et al. (2022). SW was directly pumped to the potted trees through a dedicated irrigation pipeline system furnished by Irritec (https://www.irritec.it/). Trees were micro-irrigated (two drippers 2L h^{-1}per tree) and received the same volume (440L tree^{-1}) and the same nutritional input (nitrogen, N; phosphorus, P; potassium, K) along the season, from 37 days after full bloom (DAFB) to 174 DAFB, as reported in Perulli et al. (2022). Full bloom was registered on the 25th of March. Meteorological parameters (*e.g.,* rainfall, VPD) and soil temperature were daily measured, throughout the season, by a standard weather station (Winet s.r.l) and by Sentek TriSCAN probes (https://sentektechnologies.com/), respectively (Fig. 1).

2.2. Water microbiological analyses

SW microbiological analyses were carried out along the season by sampling water directly from the outlet tank of the secondary wastewater. In particular, *E. coli* monitoring was performed at: 67, 76, 82, 90, 96, 111, 125, 129, 139, 151 DAFB. SW was also analysed for the presence of *Salmonella* spp., total coliforms (TC) and total bacterial counts (TBC).

Water samples were collected in 1L sterile glass bottles and duplicate aliquots of 100mL and 1:10, 1:100 and 1:1000 dilutions of each sample were filtered through nitrocellulose membranes (0.45μm pore size, 47mm diameter, Sartorius). Membranes were placed onto Chromogenic Coliform Agar (Oxoid, Thermofisher, Milan, Italy) and incubated at 37°C for 24h. *E. coli* identity (5–10 blue/purple colonies from the countable dilution) was then confirmed by checking indole production and cytochrome oxidase activity. *Salmonella* spp. was detected according to the UNI EN ISO 19,250:2013 procedure. Results were recorded as colony forming units (CFU) 100 mL^{-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 30°C, 3–5 days) and results expressed as $\log_{10}$ CFU 100 mL^{-1}.

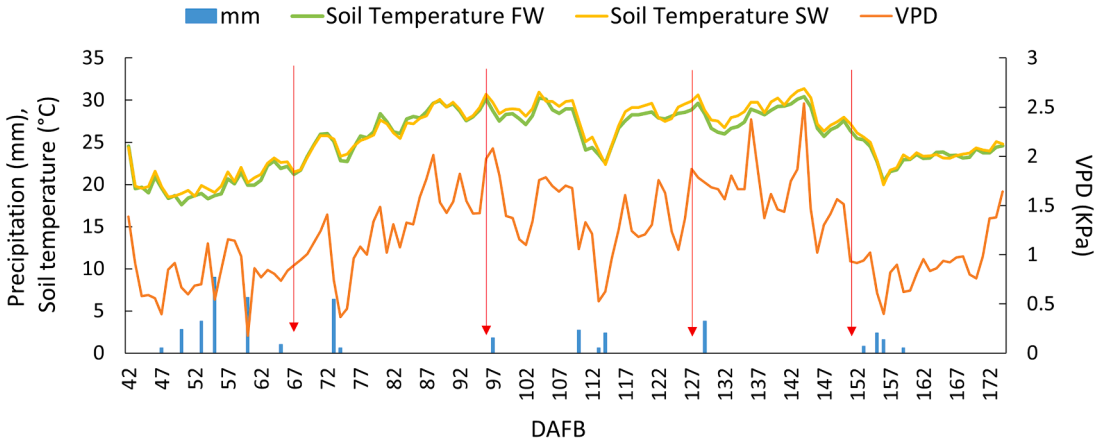

Fig. 1. VPD (orange line), rain (light-blue bars) and soil temperatures of the two treatments (FW, green line; SW, yellow line) recorded from 42 to 172 DAFB. Red arrows indicate the times when microbiological and physiological measurements were performed. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.3. Soil, shoot and fruit microbiological analyses

During the season, at 67, 96, 129 and 151 DAFB (Fig. 1), soil, shoot and fruit samples were collected from each tree, corresponding to 9 replicates for each matrix in both treatments. Two soil cores (0.0–0.15m of depth) per pot were taken and pooled together (9 samples per treatment) and processed as in Perulli et al. (2021).

Three samples of shoot (of about 0.20m length each) and fruits per tree were randomly chosen, transported in sterile plastic bags inside a fridge box and immediately processed. Samples were surface-sterilized, pooled together and processed according to Perulli et al. (2021); to optimize homogenization, 10g of shoots were added to 90mL of Buffered Peptone Water (BPW, VWR, Milan, Italy) and treated with a blender.

Serial dilutions of soil, shoot and fruit suspensions were set up and plated on both PCA amended with 100mg L^{-1} cycloheximide (Sigma–Aldrich, Milan, Italy) and Chromogenic Coliform Agar. Plates were incubated 3–5 days at 30±1°C and 18–24h at 37±1°C, respectively. Each analysis was replicated twice; after incubation, the number of CFU g^{-1} was recorded and transformed into $\log_{10}$ CFU g^{-1}. Means, standard deviations and standard errors were then calculated.

The bacterial transfer factor (TF) was then calculated as the ratio, respectively between shoot/fruit TBC, with soil TBC concentration.

2.4. Leaf gas exchanges

Leaf transpiration (E), stomatal conductance (g_s) and photosynthesis (A) were determined at 12.00h on the same days and times of microbiological samples collection. Measurements were performed using a portable gas analyser (Li-COR 6400, LICOR, Lincoln, Nebraska, USA) equipped with a light emitting diode (LED) source and an external photosynthetic photon flux density (PPFD) sensor. Measurements were carried out on one leaf per plant (9 per treatment). During each measurement, CO_2 concentration and light intensity inside the cuvette were maintained constant by setting CO_2 concentration at 400ppm and the light intensity at level of the incident one as recorded by the PPFD sensor immediately before the measurements (1500μmol m^{-2} s^{-1}).

2.5. Statistical analysis

Soil, shoot, fruit TBC, shoot TF, fruit TF and soil TC concentrations were analysed according to a randomized block design using a one-way ANOVA analysis. A Principal Components Analysis (PCA) was performed for both treatments using the seasonal data of soil, shoot and fruit TBC, leaf photosynthesis (A), leaf transpiration (E) and stomatal conductance (g_s). Analyses were carried out using R software (www.r-project.org).

3. Results and discussion

3.1. Wastewater microbiological quality

The seasonal evaluation of *E. coli* showed a homogenous trend in SW with an average of about 4 $\log_{10}$ CFU 100 mL^{-1} (10.000 CFU 100 mL^{-1}) (Fig. 2). Obtained values were, as expected, much higher compared to those of the actual Italian law (Decree of Ministry for Environment, 2003) for wastewater reuse (<10 CFU 100 mL^{-1}), and to the new EU legislation for wastewater reuse (European Comission, 2020) for drip irrigating tree crops (< 1000 CFU 100 mL^{-1}; class C). The applied wastewater originated from a secondary treatment effluent without final disinfection (Decree of Ministry for Environment, 2006).

Salmonella spp. were never detected in SW, in agreement with the Italian legislation (Decree of Ministry for Environment, 2003).

TC registered the highest values at 67 and 82 DAFB (5.6–5.5 $\log_{10}$ CFU 100mL^{-1}), while a 1 log decrease was found at 96 and 129 DAFB (Fig. 2). *E. coli* and TC concentrations are supposed to be strictly dependent on several factors such as the intrinsic characteristic of the wastewater treatment plant (*e.g.,* activated sludge, membrane bioreactor) and, when applied, to the disinfection system adopted (*e.g.,* PPA, hypochlorite, UV) (Bonetta et al., 2022). Indeed, Perulli et al. (2021), found *E. coli* and TC, 1000 and 100 times reduced in a wastewater deriving from a different treatment plant.

TBC showed a similar pattern than TC, with values ranging from 4.8 $\log_{10}$ CFU 100 mL^{-1} at 96 DAFB to 6.2 $\log_{10}$ CFU 100 mL^{-1} at 67 DAFB.

Literature on TC and TBC amount in wastewater is scarce, as these parameters are not included among the microbiological indicators for wastewater reuse in agriculture (FAO/WHO Codex Alimentarius Commission, 2007). However, these data could be pivotal to understand the microbial impact when heavily contaminated water are used for plant irrigation.

3.2. Soil E. coli, TC and TBC seasonal pattern

E. coli was detected only in SW-irrigated soils (Fig. 3), thus showing a direct contribution of SW on *E. coli* soil contamination. The seasonal average concentration was 1.15 $\log_{10}$ CFU g^{-1}; these results are in agreement with Petousi et al. (2019) and Vivaldi et al. (2013) who

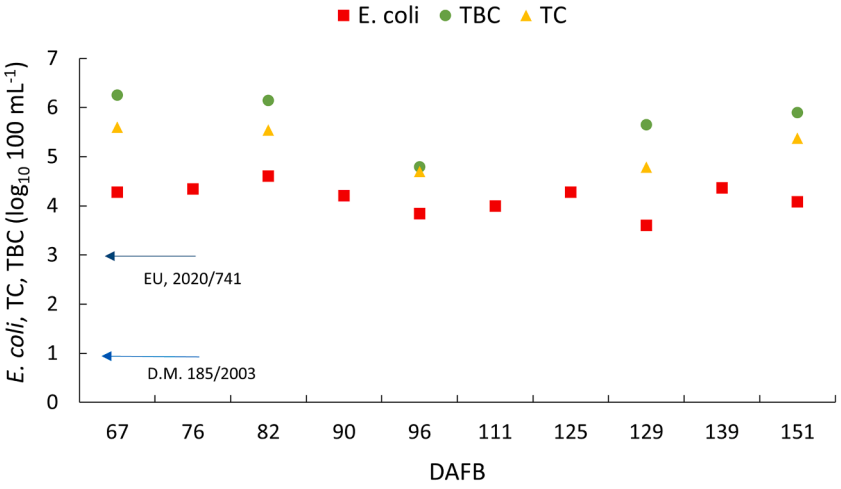

Fig. 2. *E. coli* (square red dots), TC (triangle orange dots) and TBC (green circle dots) concentrations retrieved in the secondary wastewater (SW) along the season (61–151 DAFB). Dark blue and light blue arrows indicate the European class C (European Comission, 2020) and Italian (Decree of Ministry for Environment, 2003) *E. coli* limits for treated wastewater reuse in agriculture, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

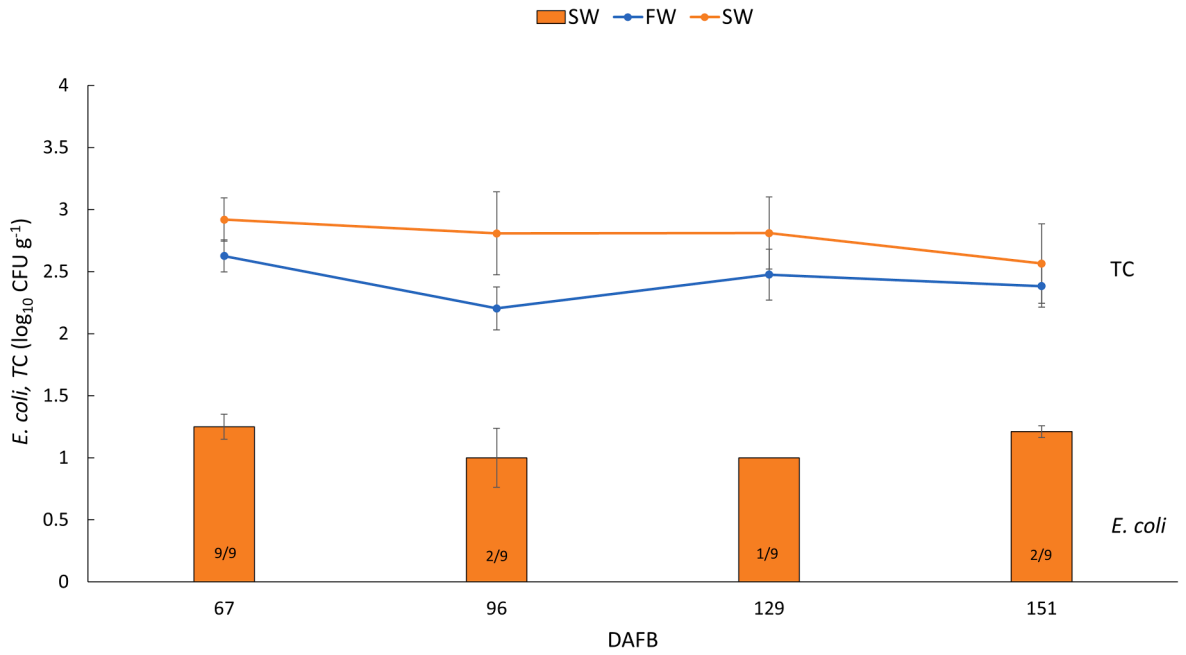

Fig. 3. Total Coliforms (TC) for freshwater FW (blue line) and secondary wastewater (SW) (orange line) treatments (n=9; Avg. ±SE) in soil samples. Orange histograms represent *E. coli* soil concentrations and the ratio between positive samples and total samples in SW treatment. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

found, in soil irrigated with SW, *E. coli* fluctuating between 1 and 2.1 log CFU g^{-1} and from 2.0 to 3.1 $\log_{10}$ CFU g^{-1}, respectively.

One of the main factors affecting the contamination of *E. coli* in the soil is its concentration in the irrigation water (Vergine et al., 2015). Vergine et al. (2015) detected *E. coli* in topsoil when irrigating with water containing 3000 CFU 100 mL^{-1} of *E. coli* but not when using a water with a lower *E. coli* amount (70 CFU 100 mL^{-1}). This is also in agreement with Perulli et al. (2021). The detected 1 log *E. coli* reduction between water and soil, suggested a scarce ability of *E. coli* to survive and persist in the soil. Furthermore, the number of positive soil samples decreased along the season, from 9/9 (67 DAFB) to 2/9, 1/9 and 2/9, respectively at 96, 129 and 151 DAFB. Different factors can influence cell viability and colonization, such as the competition with other bacteria, soil pH and temperature, enhancing a natural *E. coli* die-off (Forslund et al., 2012; Vergine et al., 2015). Soil temperature is the main driver of such decrease, since it can directly affect human pathogens survival; high temperatures generally negatively influence *E. coli* growth and its survival in soil (Blaustein et al., 2013). Indeed, in the present study, *E. coli* reduction was observed in the sampling time characterized by higher soil temperatures (Fig. 1). Furthermore, the above discussed results were obtained in pots where water leaching was prevented (Perulli et al., 2022). When adopting *E. coli* polluted water its of extreme importance to manage correctly irrigation (limit over irrigation practices) for avoiding *E. coli* contamination of water table with potential environmental risks associated with pathogen dispersal by drainage (Bernstein, 2011).

As concerns TC, no statistical difference was found between the two treatments throughout the season (Fig. 3), thus indicating no influence of the irrigation source on TC soil concentration. These similar TC concentrations evidenced that TC is mostly related to the soil endogenous bacterial population. However, soil TC in SW soils was slightly higher than in FW soils (Fig. 3).

TC detection progressively decreased in both treatments, with values of 2.4 $\log_{10}$ CFU g^{-1} and 2.6 $\log_{10}$ CFU g^{-1} at the end of the season (151 DAB), for FW and SW treatments, respectively. Again, as for *E. coli*, the increased soil temperature during the season (Fig. 1), likely influenced the TC survival rate in both treatments.

These values are in agreement with Perulli et al. (2021) who did not find significant differences in soil TC between FW (3.6 $\log_{10}$ CFU g^{-1}) and SW (2.13 $\log_{10}$ CFU g^{-1}) irrigated soils. Such results are in agreement with Petousi et al. (2019), who counted 3–4 $\log_{10}$ CFU g^{-1} of TC in SW soils. On the other hand, Vivaldi et al. (2013) reported higher soil TC concentration in SW treatment (2.7- 3.5 $\log_{10}$ CFU 100 g^{-1}) compared to FW (1.3 −2.4 $\log_{10}$ CFU 100 g^{-1}), for two consecutive years.

Regarding TBC, FW and SW treatments showed a similar trend during all the season. However, values were slightly higher in SW soil (5.96 to 6.57 $\log_{10}$ CFU g^{-1}) than in FW (5.72 to 6.43 $\log_{10}$ CFU g^{-1}) (Fig. 4). Probably, the daily impact of the TBC transported through SW did not influence the load of the existing soil heterotrophic bacteria, being the soil TBC average (5.9 $\log_{10}$ CFU g^{-1}) itself about 2 log higher compared to that retrieved in SW water (3.9 $\log_{10}$ CFU^{-} mL^{1}). This finding is in accordance with what reported by Perulli et al. (2021) who found similar values in soil TBC between SW and FW irrigated potted soils at the end of the irrigating season (6. 39 CFU g^{-1}vs 6.55 CFU g^{-1}).

3.3. Endophytic TBC assessment and its relationship with leaf gas exchanges

Neither *E. coli* nor TC were found in the epigeal part (shoot and fruit) of SW trees, despite their high concentration (17.000 CFU 100 mL^{-1}) in the wastewater source. These results are promising considering the *E. coli* wastewater microbiological limits in the new EU regulation for the drip irrigation of tree crops (class C: < 1000 CFU 100 mL^{-1}). Samples of shoot and fruit tissues were also free of *Salmonella* spp. It is known that plant roots act as habitat filters, selecting and recruiting beneficial microorganisms and expelling those that do not provide benefits (Chi et al., 2005).

Investigations on the internalization of human pathogens on fruit tree crops are still scarce; the few available works analysed the presence of potential human pathogens on the external surface of fruits or without discriminating between inside and outside (Vivaldi et al., 2013; Christou et al., 2014). Only recently, some authors started evaluating the presence (by root internalization) of bacteria from wastewater to internal plant tissues; neither Perulli et al. (2021), nor Sofo et al. (2019) found *E. coli* inside apple and nectarine shoot/fruit tissues and in the xylem sap of olive trees. The assessment of *E. coli* in wastewater irrigated

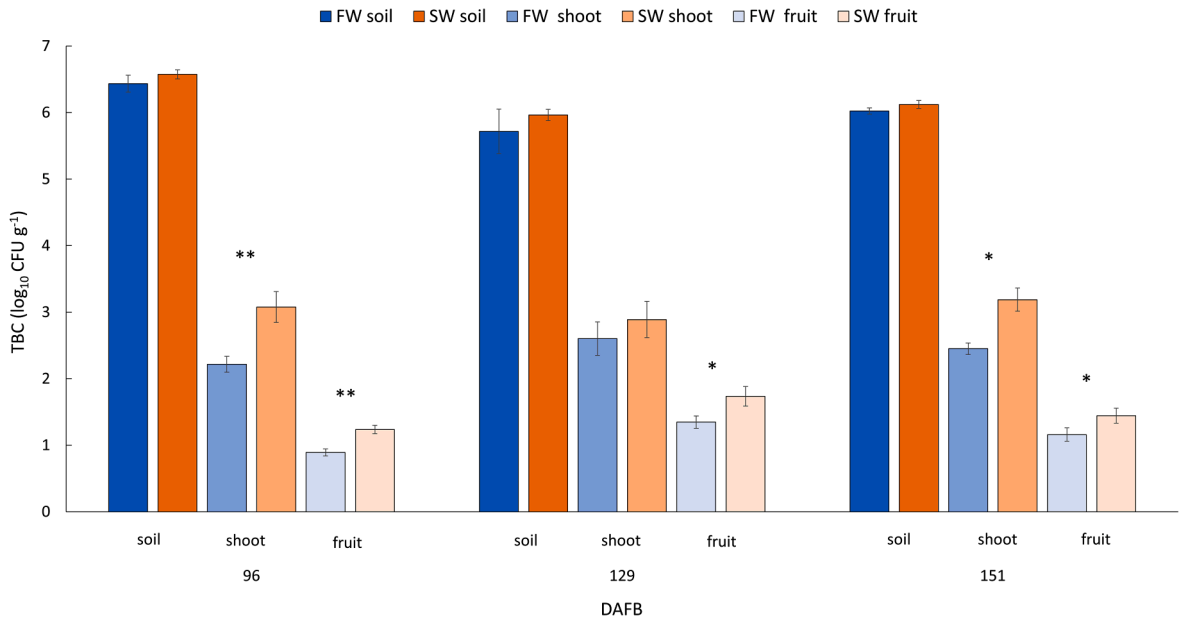

Fig. 4. Total bacteria count (TBC) for FW (blue palette) and SW (orange palette) treatments (n=9; Avg. ±SE) in soil, shoot and fruit, respectively. * and **: effect significant at $p \leq 0.05$ and $p \leq 0.01$, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

crops is a crucial issue that needs to be deeply investigated, in light of several outbreak occurred in Europe due to the spread of harmful bacteria in the edible part of plants (Soon et al., 2013).

Although shoot tissues were negative for *E. coli, Salmonella* spp. and TC, shoot TBC was significantly increased in SW treated pots with stable values along the season. In particular, TBC was statistically different at 96 (2.2 $\log_{10}$ FW-TBC g^{-1} and 3.1 $\log_{10}$ SW-TBC g^{-1}) and at 151 DAFB (2.4 and 3.2 $\log_{10}$ TBC g^{-1}, respectively for FW and SW) with higher value in SW shoots (Fig. 4). This last result is in accordance with Perulli et al. (2021) who found, at harvest time, a significant increase of shoot TBC in both nectarine and apple trees irrigated with secondary treated wastewater.

The assessed shoot TBC in the SW treatment (1 log higher than shoot FW) is a consequence of the higher microbial load provided by SW water source absorbed by the root apparatus. Roots represent one of the main ways of entry for bacteria in the plant, and the water transpiration flux would allow its transport through the xylem vessels to the areal part of the plant (Hardoim et al., 2008; Turner et al., 2013). Water is indeed the vehicle by which bacteria can move inside plant tissues (Zolti et al., 2019).

Similar findings were achieved also for fruit TBC, where SW-irrigated pots displayed statistically higher values compared to FW-irrigated pots (1.2, 1.7, 1.4 $\log_{10}$ TBC g^{-1}*vs* 0.9, 1.3, 1.2 $\log_{10}$ TBC g^{-1}), respectively at 96, 129 and 151 DAFB (Fig. 4).

In both fruit and shoots, the overall TBC count did not increase along the season, even after 109 days of continuous SW supply, suggesting a likely absence of bacteria proliferation in these tissues. This result is in line with what reported in literature (Perulli et al., 2021).

Fruits normally do not contain endophytic bacteria or, if so, only at very low concentration (Hallmann et al., 1997). Furthermore, the fruit is

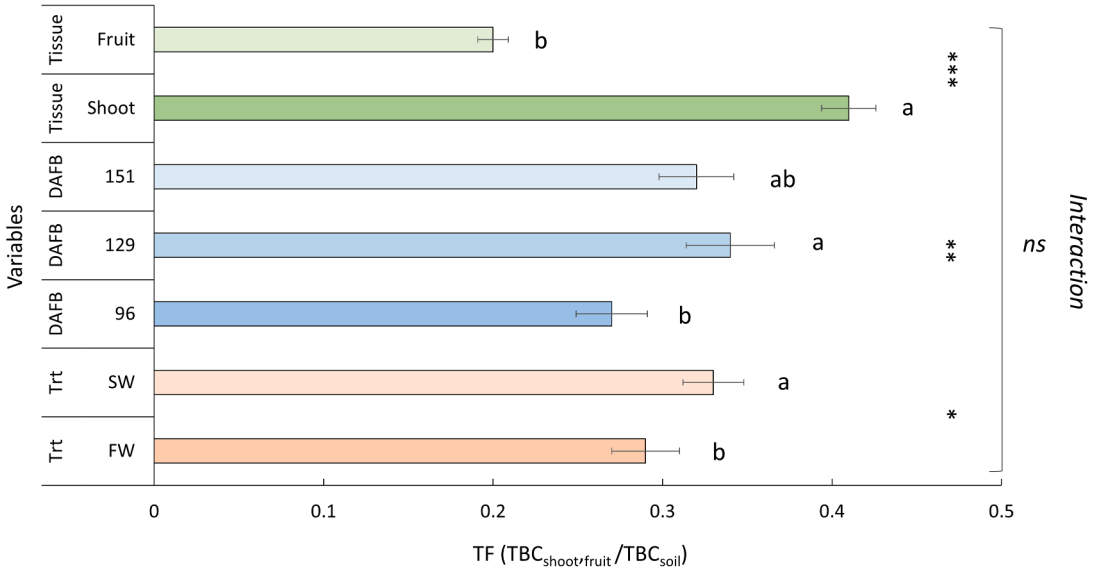

Fig. 5. Effect of the water source (FW, SW), tissues (shoot, fruit) and sampling time (96, 129, 151 DAFB) on the TBC transfer factor (TF) capacity from soil to the tree epigeal tissues (shoot and fruit) (n=9; Avg. ±SE). ns, *, ** and ***: effect not significant or significant at $p \leq 0.05$, $p \leq 0.01$ or $p \leq 0.001$, respectively.

a more complex tissue compared to shoots and it cannot be excluded the effect of a harsher environment, injures, stress factors which may affect their viability (Schuenzel and Harrison, 2002; Liao and Fett, 2001; Cevallos-Casals et al., 2006; Fattouch et al., 2008).

The analysis through the TF (Transfer Factor) further evidenced the different endophytic presence between shoots and fruits; shoot TF (0.41) was more than double higher compared to fruit TF (0.20), despite the water treatment (Fig. 5).

Studies on horticultural crops (*e.g.,* sweet basil, tomato) confirmed the higher presence of internalized bacteria (*e.g., E. coli, Salmonella* spp.) in vegetative organs (shoot) compared to reproductive ones (fruit) (Gorbatsevich et al., 2013; Ocaña de Jesús et al., 2018; Windt et al., 2009); investigations on fruit tree crops, to the best of our knowledge, are instead limited to Perulli et al. (2021).

Nevertheless, the TF analysis also showed statistically higher values in SW (0.33) than in FW (0.29) tissues (Fig. 5). Since the soil TBC did not differ between the treatments, the most probable factor influencing the higher endophytic TBC in SW irrigated-plants, could be the continuous intake of a microorganism-rich polluted water with a daily microbial content of about 7 $\log_{10}$ TBC L^{-1}. Meanwhile, also the sampling time influenced the TF, since different TF values have been registered at 96, 129 and 151 DAFB (Fig. 5), thus showing that further parameters as the daily water quality, environmental conditions and plant physiological performances could affect the TF.

The PCA evidenced that the Principal Component 1 (PC1) explained 52.2% of the variance of the results and Principal Component 2 (PC2) explained 19.3% (Fig. 6). In SW treatment (orange ellipse) the main contributors positively associated to shoot TBC were soil TBC, leaf transpiration (E), stomatal conductance (g_s) and leaf photosynthesis (A) as represented in the right side of PCA, while fruit TBC was negatively associated to shoot TBC (Fig. 6). On the contrary, when FW was adopted for irrigation (blue ellipse), the PCA did not evidence strong interactions among all the involved factors (soil, shoot, fruit TBC, E, g_s, A) (Fig. 6). The PCA analysis led to hypothesize that SW-borne microorganisms in the soil were likely to be promptly absorbed by the plant root apparatus and directly translocated in the transpiring tissues (*e.g.*, shoots), as consequence of the leaves transpiration rate. This result could partially confirm the higher presence of internalized bacteria in vegetative organs in SW vegetative tissues (Windt et al., 2009; Gorbatsevich et al., 2013; Ocaña de Jesús et al., 2018). However, these data should be furtherly confirmed with dedicated experiments focusing on a real-time monitoring of microbiological water quality and leaf and fruit transpiration rate at different environmental conditions (*e.g.*, VPD).

Indeed, fruits, contrarily to shoots, were independent to leaf gas exchanges and to soil TBC. This could be inferred, as already mentioned, both to the harsher environment of fruits compared to shoot but likely also to peach fruit growth mechanism and to its lower fruit transpiration rate compared to shoot (Higgins et al., 1992; Morandi et al., 2010). During the morning, the xylem inflow to the fruit is reduced to its minimum value while it resumes its growth in late afternoon, reaching the highest fruit growth rates when the xylem inflow increases and balances the transpiration water losses (late afternoon and night) (Morandi et al., 2007). The sampling time (*i.e.,* during the day) could then likely be a further factor influencing the TBC concentrations recovered in the different plant tissues.

These results confirmed what deduced from the TF analysis; the wastewater bacterial load transient through the soil, together with the plant transpiration rate, seem to be the main drivers of endophytic bacteria recovery within plant tissues.

Therefore, it has to be outlined that the continuous intake of a high-contaminated wastewater was able to influence the overall tree endophytic community, without threatening fruit microbiological quality (*E. coli, Salmonella* spp.). However, a better knowledge on wastewater-mediated bacteria able to colonize plant tissues is extremely important. Several bacterial species carried out from polluted waters and recruited from plant roots can also bring positive features since those waters may also be a reservoir of potential environmental bacteria with plant growth promoting activities (*e.g., Bacillus* spp., *Pseudomonas* spp.).

Unfortunately, to date, studies aimed at identifying the overall bacterial community in plant vegetative tissues, subjected to wastewater irrigation, are limited. Sofo et al. (2019) illustrated that *Pseudomonas* and *Acinetobacter* spp. were significantly higher in wastewater irrigated

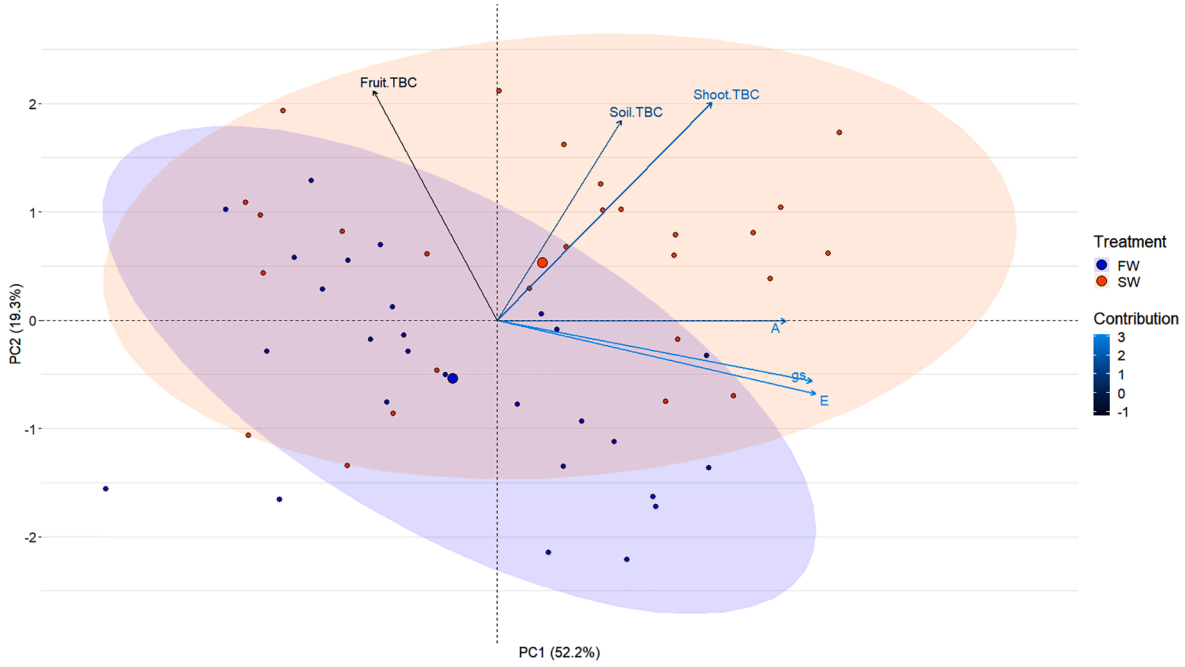

Fig. 6. Principal component analysis (PCA) describing soil TBC, shoot and fruit endophytic TBC and midday physiological performances (leaf transpiration (E), stomatal conductance (g_s) and leaf photosynthesis (A)) for tree irrigated with SW (orange ellipse) and FW (blue ellipse), respectively. The contribution of a variable to the principal components is the length of the vector. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

xylem sap of olive trees, compared to the rainfed trees. Perulli et al. (2021) isolated *Pantoea agglomerans* in both nectarine shoots and fruits, and *Pseudomonas punonensis* and *Pseudomonas orizyhabitans* in apple shoots, when wastewater was adopted as irrigation source.

4. Conclusions

Results of the present study allowed at the conclusion that pathogenic microorganisms (*e.g., E. coli*), retrieved in wastewater, are not a risk for food security. Although the very high *E. coli* concentrations (ten times above the EU regulation 2020/741) in secondary urban wastewater, the tree areal parts (*e.g.,* shoot and fruits) were free of *E. coli* and *Salmonella* spp. when wastewater was applied with a drip irrigation system. Furthermore, *E. coli* concentration and persistence decreased into the soil along the season. The soil TC and TBC was not influenced by the bacterial load carried out by wastewater; but the endophytic community into shoots and fruits increased in SW-irrigated peach plants.

The use of urban wastewater was able to influence the plant endophytic microbiota, with a higher concentration in shoots than fruits but with steady pattern along the whole season. The leaves transpiration rate seemed to be main driver of bacteria transport from the soil to the canopy.

These results are extremely encouraging in the perspective of using the secondary treated wastewater source, following the microbiological guidelines of the new European Regulation EU 2020/741, for drip irrigating tree crops. In any case, further studies are necessary to better investigate and characterize the overall retrieved endophytic microbial community, in order to avoid any risk for food consumption and to know potential positive effects of water-borne bacteria on plant physiological performances.

CRediT authorship contribution statement

Giulio Demetrio Perulli: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **Francesca Gaggia:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Luigi Manfrini:** Investigation, Supervision, Writing – review & editing. **Diana Di Gioia:** Investigation, Supervision, Supervision, Validation, Writing – review & editing. **Attilio Toscano:** Investigation, Supervision, Writing – review & editing. **Brunella Morandi:** Investigation, Supervision, Validation, Writing – review & editing.

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.

Data availability

Data will be made available on request.

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