



Evaluation of the possible persistence of potential human pathogenic bacteria in olive orchards irrigated with treated urban wastewater

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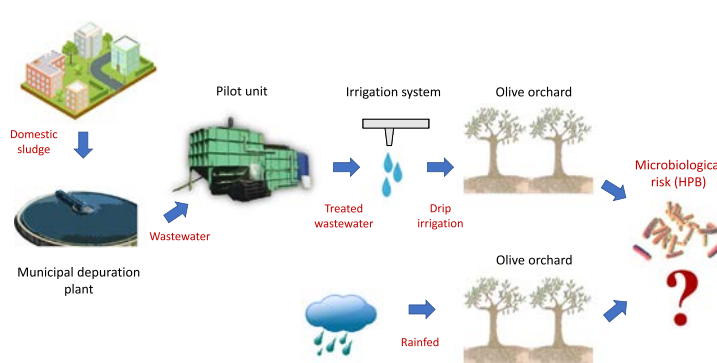
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HIGHLIGHTS

- Treated urban wastewater (TWW) can contain human pathogenic bacteria (HPB).
- A TWW-irrigated (WW_{tr}) and a rainfed (RF_{tr}) olive orchard were compared.
- 16S-rRNA-microbiological analyses on soil, xylem sap, and phyllosphere were done.
- WW_{tr} soil contained more potential HPB than xylem sap and phyllosphere.
- Urban TWW, if adequately-treated and applied, does not constitute health risks.

GRAPHICAL ABSTRACT



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ABSTRACT

Under suitable conditions, low-quality, treated urban wastewater (TWW) is an additional water resource for irrigation in water-scarce environments but its use in agriculture requires a careful monitoring of a range of hygiene parameters, including human pathogenic bacteria (HPB). DNA-based microbiological analyses on soil, xylem sap, and leaves surface (phyllosphere) were carried out in an olive (*Olea europaea* L.) grove located in Southern Italy (Basilicata region). The experimental grove has been managed in two plots for 18 years. The experimental plot (WW_{tr}) was drip irrigated daily with TWW ($2800 \text{ m}^3 \text{ ha}^{-1} \text{ year}^{-1}$), while the control plot (RF_{tr}) was rainfed. The results of the 16S-rRNA-based metagenomic analysis demonstrated that the phyllosphere had the lowest number of potential HPB (6), compared to soil (22) and xylem (26) compartments. Gammaproteobacteria, including potential HPB, like *Pseudomonas* and *Acinetobacter* spp., were significantly higher in WW_{tr} soil and xylem sap, compared to RF_{tr} . A similar trend was observed for *Burkholderia* spp. (Betaproteobacteria) and *Mycobacterium* spp. (Actinobacteria). The Firmicutes *Enterococcus*, *Staphylococcus* and *Streptococcus* spp. were more abundant in WW_{tr} xylem sap. The pathogenic *Clostridium perfringens* was found higher on WW_{tr} leaves (relative abundance 7.17 in WW_{tr} and 1.33 in RF_{tr}) and *Enterococcus faecalis* in WW_{tr} xylem sap (93.22 in WW_{tr} and 7.08 in RF_{tr}). On the basis of the results obtained, the irrigation with TWW can be considered a realistic and safe agronomic practice in Mediterranean orchards, and an opportunity for farmers and consumers.

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1. Introduction

Olive trees (*Olea europaea* L.) represent one of the most important oil crops world-wide, which have characterized the Mediterranean landscape since ancient times. In 2018, on an area of 10.65 Mha, an amount of 19.27 Mt of olives was harvested world-wide (FAOSTAT, 2018). Considering the olive orchards are mainly located in areas characterized by water scarcity, the need for a sustainable approach in irrigation management has become evident in order to improve or maintain soil quality, health and fertility (Sofo et al., 2014; Pascazio et al., 2015). As a good example Bedbabis et al. (2015) successfully adopted treated urban wastewater (TWW) for irrigating olive trees grown under a semi-arid climate, so obtaining several benefits in terms of increased yield and soil organic matter, and faster olive ripening, with consequent high levels of phenols, tocopherols and pigments in fruits. Thus, under suitable conditions, TWW can be an additional water resource in agriculture.

On the other side, the use of TWW can cause an increase in the genetic and functional and metabolic diversity of soil bacterial communities, as demonstrated by Becerra-Castro et al. (2015) and Lüneberg et al. (2018), and more specifically in olive groves by Sofo et al. (2010), with evident benefits to soil quality and crop production. On the other hand, the adoption of WW_{tr} in agriculture requires a careful monitoring of a range of hygiene parameters, including the presence of potential human pathogenic bacteria (HPB) (Palese et al., 2009; Becerra-Castro et al., 2015). The advantages of using TWW for irrigation has been extensively studied in olive groves but not always the potential consequences for public health and environmental quality have been taken into account (Qadir et al., 2010; Courault et al., 2017). In leafy green production, the use of irrigation water deriving from drainage ditches causes significantly increases in *E. coli* (Allende et al., 2018). In soils irrigated with untreated urban WW, HPB can be a serious threat, as recently pointed out by Lüneberg et al. (2018), who found soil contaminations of potentially active and harmful *Acinetobacter*, *Bacillus* and *Nocardia* spp.

Palese et al. (2009) already studied the effects of TWW on the microbiological quality of soil and olive fruits adopting a traditional culture-based method. Here, in the same system, we adopted a metagenomic analysis based on 16S-rRNA gene sequencing with the aim of identifying potential HPB in soil, xylem sap and leaf surface (phyllosphere), that might be a source of fruit contamination and a health risk for the population. On the basis of other authors' work and point of view, previous research from our research group, and original data, we here present our opinions on this argument, based on experimental data.

2. Materials and methods

2.1. Experimental site and olive grove management

The trial was carried out in a 2-ha mature olive grove (*Olea europaea* L., cv. 'Maiatica'; plants with an age of approximately 70 years, trained to vase at a distance of 8×8 m; NE orientation) located in Ferrandina (Southern Italy, Basilicata region; N 40°29', E 16°28'). The area has a semi-arid climate, annual precipitation of 565 mm (mean 1995–2015), falling mostly in the winter, and mean annual temperature ranging from 15 to 17 °C.

In the experimental plot (WW_{tr}), olive plants were drip-irrigated from March to October ($2800 \text{ m}^3 \text{ ha}^{-1} \text{ year}^{-1}$) with TWW. Six drip emitters discharging 8 L h^{-1} over a 1-m radius were placed for each plant. The distribution pipes and emitters were placed 1.5 m above the ground, in order to facilitate soil tillage. A pilot unit (Fig. S1) was used to treat urban wastewater (about $50 \text{ m}^3 \text{ day}^{-1}$) according to the simplified scheme described in Palese et al. (2009). In brief, sewer water was collected in the municipal depuration plant, that included grilling, desalting, primary sedimentation, biological oxidation with activated sludge, denitrification, and secondary sedimentation.

Successively, wastewater was pumped to the pilot unit taking it from three different points of the depuration plant (sewer water, water before the denitrification unit, and water from the final sedimentation plant, at a proportion of 1:2:2, v/v). Here, it was subjected to clariflocculation, further sedimentation and bland disinfection with peracetic acid 2.5 mg L^{-1} for 60 min. The scheme partially excluded the biological processes for the removal of organic carbon (C) and nitrogen (N) from the wastewater, in order to recover them and use them as fertilizing substances. The flow squeeze from the pre-denitrification unit allowed to use water subjected to a reduction of only the rapidly biodegradable organic matter fraction, that is often cause of soil anoxic conditions. In WW_{tr} , the average annual amounts of organic C, N, P and K distributed by the treated wastewater were $124, 54, 3$ and $50 \text{ kg ha}^{-1} \text{ year}^{-1}$, respectively (mean 2000–2016). An integrative amount of 40 kg ha^{-1} of N-NO_3^- per year was distributed by fertigation during fruit set and pit hardening phase, in order to entirely satisfy olive nutrient needs. The composition of TWW is shown in Table 1. According to earlier published data (Palese et al., 2009) obtained using culture-based techniques, *Escherichia coli* concentration in the TWW varied considerably from 0 to $78,000 \text{ CFU } 100 \text{ mL}^{-1}$, *Salmonella* was never detected in the TWW, and variable levels of enterococci (from 0 to $28,000 \text{ CFU } 100 \text{ mL}^{-1}$) and sulphite-reducing *Clostridium* spores (from 10 to $31,000 \text{ CFU } 100 \text{ mL}^{-1}$) were observed.

An adjacent plot, characterized by soil and trees having similar features, was taken as control. This plot was naturally rainfed (RF_{tr}) (about $35 \text{ m}^3 \text{ water plant}^{-1} \text{ year}^{-1}$) and a mineral fertilization was carried out once per year, during fruit set and pit hardening phase, using ternary compounds (NPK 20-10-10 fertilizer at doses ranging from 300 to $500 \text{ kg ha}^{-1} \text{ year}^{-1}$; mean 2000–2016).

2.2. Soil and plant tissue sampling

In May 2017, soil sub-samples for both the treatments (WW_{tr} and RF_{tr}) were picked at 0–30 cm depth in ten different points around (over a 1-m radius) each plant ($n = 3$, three plants per plot) and pooled on site to constitute a composite soil sample of about 2 kg. In order to avoid border interferences, plants located in the central part of each plot and far 24 m each other were randomly chosen. Three composite soil samples ($n = 3$) for each treatment were collected and stored at $-20 \text{ }^\circ\text{C}$ for DNA extraction.

Leaves were collected for both WW_{tr} and RF_{tr} . For each treatment, three composite leaf samples were randomly collected in the upper part of the canopy using sterile gloves and equipment from the same plants used for collecting the soil. The same plants used for collecting the soil were selected. Leaf samples were stored immediately at $4 \text{ }^\circ\text{C}$ in sterile plastic bags and brought to the laboratory, where they were stored at $-20 \text{ }^\circ\text{C}$ without any further processing. Bacteria from the phyllosphere were desorbed from a mix of 50 leaves per plant by the method of Redford and Fierer (2009). Leaves were washed with 50 mL of detergent solution (Tris-HCl 20 mM, EDTA 10 mM and Triton

Table 1

Chemical parameters (\pm standard error) of the treated urban wastewater applied in the olive orchard ($n = 10$, one per month). Period of sampling: March–October 2016 and March–May 2017. COD: chemical oxygen demand.

Parameter	Unit of measure	Value
pH	–	7.6 ± 0.3
Conductivity	($\mu\text{S cm}^{-1}$)	884.0 ± 43.4
Organic C	(mg L^{-1})	20.4 ± 5.5
N ($\text{NO}_3^- + \text{NH}_4^+$)	(mg L^{-1})	18.3 ± 4.7
Na	(mg L^{-1})	121.3 ± 32.1
Mg	(mg L^{-1})	13.8 ± 2.5
Ca	(mg L^{-1})	67.8 ± 8.1
B	(mg L^{-1})	0.7 ± 0.1
K	(mg L^{-1})	17.2 ± 3.6
P	(mg L^{-1})	1.4 ± 0.4
COD	(mg L^{-1})	180.4 ± 29.0

0.024) by mechanical agitation at 100 rpm for 15 min on a rotary shaker (Thomas Scientific, Inc., Swedesboro NJ, USA). The washing solution was centrifuged at $5000 \times g$ for 10 min at 4°C , the supernatant was discarded, and the resulting pellet was air dried for 2 h under laminar flow fan and then stored at -20°C .

The xylem sap was extracted from shoots of olive trees of both WW_{tr} and RF_{tr} using Scholander-type hand-made chambers pressurized with N_2 (Fig. S2). The same plants used for collecting the soil and the leaves were selected. Two shoots, approximately 15–20 cm in length, were taken from each of the four cardinal points per plant using sterile cutting shears and a pool of plant material was collected, put in plastic bags, transported to the laboratory, and stored under at -20°C before the use. For each shoot, a 1-cm wide bark strip was removed in the proximal part with a sharp knife sterilized with 75% ethanol in order to exclude the phloem sap and to prevent external contamination. The cut end of the stem was placed, through the hole with a gasket, in the Scholander pressure chamber lid with approximately 3 cm of the cut end facing out; this part of the stem was connected to a thin plastic tube through which the liquid was let flow into a 1.5-mL microcentrifuge tube. The foliage of the shoot was placed in the pressure chamber and the lid was locked down. The foliage of the cutting was placed in the pressure chamber. High pressure was applied (approximately from 50 to 70 bar) to force xylem sap from the tissue at the proximal end of the cutting. After, discarding the first drops, xylem sap was collected into 1.5-mL microcentrifuge tubes (the procedure took about 15–20 min per shoot) and stored at -20°C before analysis.

2.3. DNA extraction

Total bacterial DNA was extracted from 0.5 g of each soil and phyllosphere pellet sample according to the protocol of the FastDNA® SPIN Kit for soil in combination with the Thermo Savant FastPrep® System homogenizer (MP Biomedicals LLC, Cleveland, OH, USA). Sap samples were centrifuged at $12,000 \times g$ for 10 min at 4°C . The pellet and the respective supernatant were stored at -20°C separately. The pellet was used for total DNA extraction using the “DNeasy Blood Tissue” Qiagen kit.

The quality and concentration of DNA extracts were determined at 260, 280 and 230 nm using a NanoDrop®ND-1000 UV–Vis spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The fragmentation of the DNA was checked by agarose gel electrophoresis

(0.7% w/v agarose–0.5× Tris–Borate–EDTA) and UV visualization of the gels stained with Gel Red™ (Biotium, Inc., Hayward, CA, USA).

2.4. Library preparation, sequencing and bioinformatics analysis

DNA samples isolated from soil, phyllosphere and xylem sap were characterized up to the species level of bacterial communities. Two amplification steps in the library workflow were performed: an initial PCR amplification using locus specific 16S PCR primers and a subsequent amplification that integrated relevant Illumina flow-cell binding domains and unique indices (NexteraXT Index Kit, FC-131-1001/FC-131-1002). In particular, the variable V3 and V4 regions of the 16S rRNA gene were amplified to determine bacterial community compositions. For the 16S locus, the target sequences were those reported by Klindworth et al. (2013). Libraries were sequenced in a MiSeq run in paired end with 300-bp reads producing about 100,000 sequences per sample by IGA Technology Services S.r.l. (Udine, Italy).

The bioinformatic pipeline QIIME2 (<https://view.qiime2.org/>) was used to process and analyze the obtained 16S amplicon sequences. The preliminary processing of the data included de-multiplexing and quality filtering with a minimum length of 200 bp. Following the QIIME2, the USEARCH algorithm (version 8.1.1756) allowed to perform the following steps: chimera filtering, grouping of replicate sequences, sorting sequences per decreasing abundance and operational taxonomical units (OTUs) identification. For OTUs' picking, the open reference protocol was used. Reads were aligned against reference database, a modified version of GreenGene (version 2013_8). Only matches with a minimum identity of 94% were retained and clustered representing the centroid. Query sequences not sharing similarity with the centroid constituted a novel OTU and the most abundant and long reads in each OTU were selected as representative sequences. In this step, the clustering threshold was set at 97% and OTUs were generated with a minimum of two sequenced fragments. The RDP classifier and GreenGene database were used to assign taxonomy with a minimum confidence threshold of 0.50. Rarefaction curves end-points and normalization of counts for diversity analysis were set to 50% of the target sequencing coverage. Due to different size of the samples, a cutoff of 20,000 high-quality reads was selected. Samples not satisfying the count threshold were not included in standard alpha- and beta-diversity estimators. The total count was retained for taxonomic abundances estimation and used accordingly for ad-hoc statistical testing of

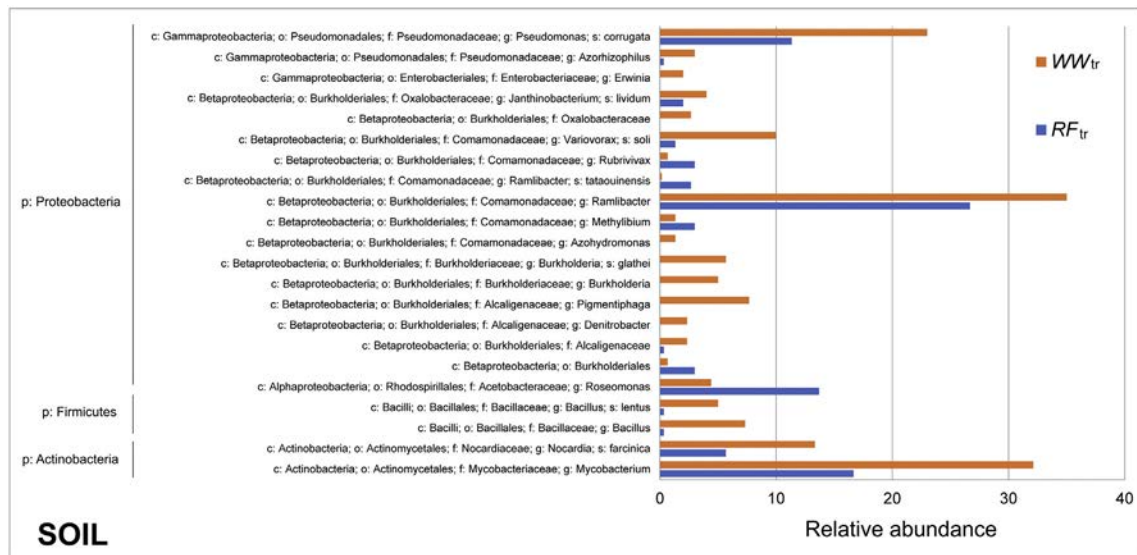


Fig. 1. Relative abundances of the bacterial operational taxonomical units (OTUs) of potential human pathogenic bacteria (HPB) that were statistically different in soils of the olive orchard irrigated with treated urban wastewater (WW_{tr}) and the rainfed olive orchard (RF_{tr}). Only the OTUs with a statistical difference ($p < 0.05$) between WW_{tr} and RF_{tr} were considered. p, phylum; c, class; o, order; f, family; g, genus; s, species.

taxonomic abundance. Overall raw results of the analysis are presented in Tables S1–S3.

The potential HPB were chosen on the basis of the ‘Inventory list of human pathogenic microorganisms in plant production systems’ (<https://docs.google.com/spreadsheets/d/13qDivF5Hi8GrhTWalubfd18NVIFINAwENbVTMSbxwls/edit?usp=sharing>) (COST HUPLANT control, 2018) and of the bibliography consulted (see References section). Only the HPBs with an abundance significantly different at $p < 0.05$ between WW_{tr} and RF_{tr} were reported.

3. Results and discussion

The metagenomic analysis (showed in Fig. 1 for soil, and in Fig. 2A, B for xylem sap and phyllosphere, respectively) revealed statistically higher abundance ($p < 0.05$) of some potential HPB belonging to the phyla Actinobacteria, Firmicutes and Proteobacteria in the soil and

xylem sap, and to Firmicutes and Proteobacteria in the phyllosphere. Leaf surface had the lowest number of potential HPB (6), compared to soil (22) and xylem (26) compartments. This suggests that olive phyllosphere, more subjected to environmental variables and also for this reason far for having constant conditions (Whipps et al., 2008), is not a habitat suitable for HPB, whereas xylem, being more stable, could be a reservoir of HPB. The richness in HPB in soil is surely due to the fact that most of the wastewater ends up directly in the soil and only a minimal part to the canopy by aerosol (drip emitters are 1.5-m high and canopy tops are on average 4.5 m high; Fig. S3). Thus, roots are the main entrance to the xylem for microorganisms and so the transmission of bacteria to the phyllosphere is internal. On the other side, this could also likely be due to the fact that the colonization of internal tissues normally occurs from the soil to aerial plant parts through xylem vessels but also vice versa through the stomata (Compant et al., 2005), making xylem a compartment with a higher microbial diversity.

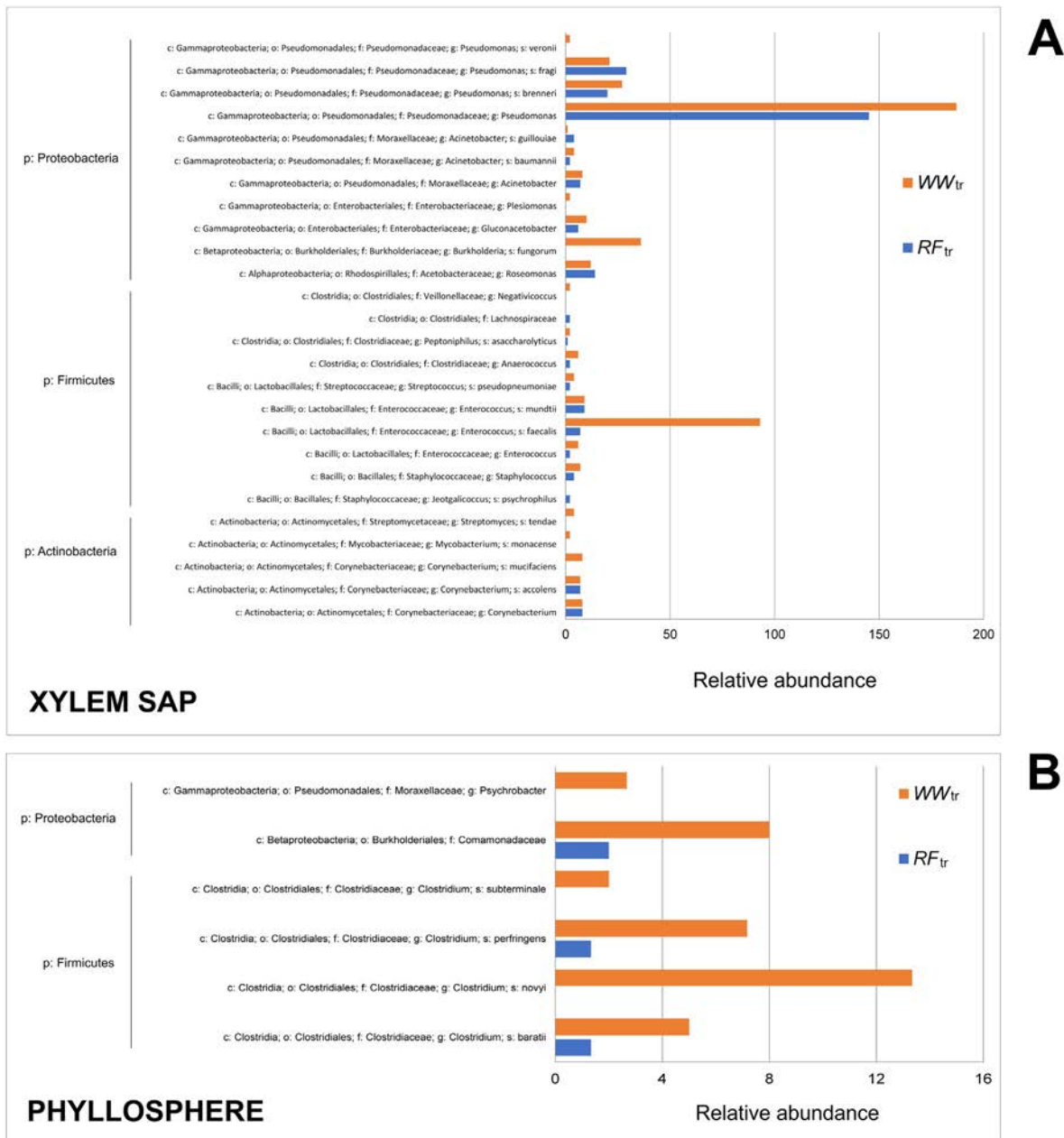


Fig. 2. Relative abundances of the bacterial operational taxonomical units (OTUs) that were statistically different in (A) the xylem sap and (B) phyllosphere (leaf surface) of olive plants irrigated with treated urban wastewater (WW_{tr}) or rainfed (RF_{tr}). Only the OTUs with a statistical difference ($p < 0.05$) between WW_{tr} and RF_{tr} were considered. p, phylum; c, class; o, order; f, family; g, genus; s, species.

This is particularly true for Firmicutes, where at the class level xylem sap contained both Bacilli and Clostridia, the former likely coming from the soil and the latter from the phyllosphere.

Gammaproteobacteria, including potential pathogenic like *Pseudomonas* and *Acinetobacter* spp., were found significantly higher in WW_{tr} soil and xylem sap. Among Betaproteobacteria, Burkholderiales order was over-represented in WW_{tr} , with the presence of OTUs (2 in soil and 1 in xylem sap) belonging to *Burkholderia* spp., a genus potentially pathogenic for humans (Becerra-Castro et al., 2015). Among Firmicutes, *Enterococcus*, *Staphylococcus* and *Streptococcus* spp. (3, 1 and 1 OTUs, respectively) were found to be significantly higher in WW_{tr} xylem sap. These latter groups probably came from the soil, where they found harshest conditions compared to xylem and have a high competition with other microorganisms (Lakshmanan et al., 2014), so being not detected here. *Mycobacterium* spp., belonging to Actinobacteria, were more abundant in both WW_{tr} soil and xylem sap. Regarding the certain and harmful HPB, *Clostridium perfringens* was found higher on WW_{tr} leaves (relative abundance 7.17 in WW_{tr} and 1.33 in RF_{tr}) and *Enterococcus faecalis* more abundant in WW_{tr} xylem sap (93.22 in WW_{tr} and 7.08 in RF_{tr}). Considering the strict relationship between leaf surface (phyllosphere) and fruit surface (carposphere) (examined in olive by Pascazio et al., 2015) and the close contact between xylem sap and fruit pulp, the presence of these two HPB could be taken into account for TWW irrigation practices.

In conclusion, the results confirmed that, excepting for two cases (*Clostridium* and *Enterococcus*) that should be ascertained with more sampling throughout the year and in different years, fertigation with urban TWW did not cause a substantial and significant increase in HPB. In the same system, Palese et al. (2009) found *Escherichia coli*, *Enterococcus* spp. and *Clostridium* spp. in the TWW used but not in topsoil (10-cm depth). The same authors did not detect any microbial contamination on olive fruits harvested directly from the canopy. Considering a) the discontinuity of the urban TWW composition and distribution, often concentrated in the hot and dry period (Becerra-Castro et al., 2015), b) the culture-based data previously obtained by Palese et al. (2009) in olive, c) the fact that in a DNA-based analysis we have data on bacterial presence more than on vitality and persistence, as recently reviewed by Morgan et al. (2017) in TWW-irrigated grapevine, and d) taking into account the HPB mortality and inactivation due to soil resilience and competition with endogenous bacteria (Whipps et al., 2008; Lakshmanan et al., 2014), we can assert that the irrigation with adequately-treated and applied urban wastewater can be considered a realistic and safe agronomic practice in Mediterranean orchards, and an opportunity for farmers and consumers.

Author contributions

AS conceived the conceptual idea for the study. CX, BD and CC designed the study, and CF and MS carried out the field work. ANM, CF and AS carried out the data analysis with help from MS and CC. The first draft of the manuscript was written by AS, with all authors contributing substantially to revisions.

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Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scitotenv.2018.12.264>.

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New.CleanUp.ReferenceOTU6533	0.0495	2.00	7.67	k: Bacteria; p: Actinobacteria; c: Thermoleophila; o: Solirubrobacterales; f: Conexibacteraceae; g: Conexibacter
New.CleanUp.ReferenceOTU7276	0.0495	1.33	5.00	k: Bacteria; p: Actinobacteria; c: Thermoleophila; o: Solirubrobacterales; f: Solirubrobacteraceae; g: Solirubrobacter; s: soli
New.CleanUp.ReferenceOTU8084	0.0495	14.33	44.33	k: Bacteria; p: Proteobacteria; c: Gammaproteobacteria; o: Xanthomonadales; f: Sinobacteraceae; g: Steroidobacter
New.CleanUp.ReferenceOTU8125	0.0495	15.00	6.67	k: Bacteria; p: Actinobacteria; c: Actinobacteria; o: Actinomycetales
New.CleanUp.ReferenceOTU8809	0.0495	19.33	53.33	k: Bacteria; p: Actinobacteria; c: Thermoleophila; o: Solirubrobacterales; f: Solirubrobacteraceae; g: Solirubrobacter; s: soli
New.CleanUp.ReferenceOTU9301	0.0495	1.67	9.67	k: Bacteria; p: Proteobacteria; c: Alphaproteobacteria; o: Rhodospirillales; f: Acetobacteraceae; g: Roseomonas
New.CleanUp.ReferenceOTU10319	0.0495	4.33	9.67	k: Bacteria; p: Proteobacteria; c: Alphaproteobacteria; o: Rhizobiales
New.CleanUp.ReferenceOTU10323	0.0495	4.00	13.33	k: Bacteria; p: Actinobacteria; c: Thermoleophila; o: Solirubrobacterales; f: Solirubrobacteraceae; g: Solirubrobactersoli
New.CleanUp.ReferenceOTU10652	0.0495	26.33	41.00	k: Bacteria; p: Verrucomicrobia; c: Spartobacteria; o: Chthoniobacteriales; f: Chthoniobacteraceae; g: Chthoniobacter; s: flavus
New.CleanUp.ReferenceOTU10720	0.0495	6.33	17.33	k: Bacteria; p: Actinobacteria; c: Actinobacteria; o: Actinomycetales; f: Geodermatophilaceae; g: Modestobacter
New.CleanUp.ReferenceOTU10949	0.0495	10.00	4.00	k: Bacteria; p: Proteobacteria; c: Alphaproteobacteria; o: Rhizobiales; f: Hyphomicrobiaceae; g: Rhodoplanes
New.CleanUp.ReferenceOTU12002	0.0495	2.67	22.00	k: Bacteria; p: Chloroflexi; c: Anaerolineae; o: Caldilineales; f: Caldilineaceae; g: Caldilinea
New.CleanUp.ReferenceOTU13252	0.0495	17.33	4.67	k: Bacteria; p: Actinobacteria; c: Actinobacteria; o: Actinomycetales; f: Nocardioidaceae
New.CleanUp.ReferenceOTU14007	0.0495	7.33	2.00	k: Bacteria; p: Actinobacteria; c: Actinobacteria; o: Actinomycetales
New.CleanUp.ReferenceOTU14515	0.0495	1.00	9.67	k: Bacteria; p: Bacteroidetes; c: Sphingobacteriia; o: Sphingobacteriales; f: Chitinophagaceae; g: Flavisolibacter
New.CleanUp.ReferenceOTU14655	0.0495	2.33	7.33	k: Bacteria; p: Proteobacteria; c: Alphaproteobacteria; o: Rhizobiales; f: Beijerinckiaceae; g: Chelatococcus; s: daeguensis
New.CleanUp.ReferenceOTU15439	0.0495	2.00	24.67	k: Bacteria; p: Actinobacteria; c: Actinobacteria; o: Actinomycetales; f: Cellulomonadaceae; g: Cellulomonas
New.CleanUp.ReferenceOTU15717	0.0495	3.00	10.33	k: Bacteria; p: Actinobacteria; c: Actinobacteria; o: Actinomycetales; f: Geodermatophilaceae; g: Blastococcus; s: aggregatus
New.CleanUp.ReferenceOTU15725	0.0495	4.67	11.67	k: Bacteria; p: Actinobacteria; c: Actinobacteria; o: Actinomycetales
New.CleanUp.ReferenceOTU17130	0.0495	10.67	1.00	k: Bacteria; p: Proteobacteria; c: Alphaproteobacteria; o: Rhodospirillales; f: Acetobacteraceae
New.CleanUp.ReferenceOTU17264	0.0495	1.00	7.00	k: Bacteria; p: Actinobacteria; c: Actinobacteria; o: Actinomycetales; f: Geodermatophilaceae; g: Modestobacter
New.CleanUp.ReferenceOTU17577	0.0495	2.33	8.00	k: Bacteria; p: Proteobacteria; c: Alphaproteobacteria; o: Rhizobiales; f: Methylobacteriaceae; g: Methylobacterium
New.CleanUp.ReferenceOTU17820	0.0495	5.33	31.33	k: Bacteria; p: Chloroflexi; c: Anaerolineae; o: Caldilineales; f: Caldilineaceae; g: Caldilinea
New.CleanUp.ReferenceOTU19164	0.0495	19.67	10.00	k: Bacteria; p: Bacteroidetes; c: Sphingobacteriia; o: Sphingobacteriales; f: Chitinophagaceae
New.CleanUp.ReferenceOTU19964	0.0495	37.00	68.67	k: Bacteria; p: Proteobacteria; c: Alphaproteobacteria; o: Rhizobiales; f: Methylobacteriaceae; g: Methylobacterium
New.CleanUp.ReferenceOTU20126	0.0495	14.67	29.33	k: Bacteria; p: Actinobacteria; c: Actinobacteria; o: Actinomycetales; f: Nocardioidaceae; g: Nocardioides
New.CleanUp.ReferenceOTU20510	0.0495	31.67	2.67	k: Bacteria; p: Actinobacteria; c: Thermoleophila; o: Solirubrobacterales; f: Conexibacteraceae; g: Conexibacter
New.CleanUp.ReferenceOTU21506	0.0495	5.33	17.33	k: Bacteria; p: Verrucomicrobia; c: Spartobacteria; o: Chthoniobacteriales; f: Chthoniobacteraceae; g: Chthoniobacter; s: flavus
New.CleanUp.ReferenceOTU22832	0.0495	1.00	10.67	k: Bacteria; p: Bacteroidetes; c: Sphingobacteriia; o: Sphingobacteriales; f: Chitinophagaceae; g: Flavisolibacter
New.CleanUp.ReferenceOTU23556	0.0495	4.00	12.67	k: Bacteria; p: Planctomycetes; c: Planctomycetia; o: Gemmatales; f: Isosphaeraceae; g: Singulisphaera
New.CleanUp.ReferenceOTU23640	0.0495	3.00	10.33	k: Bacteria; p: Actinobacteria; c: Thermoleophila; o: Solirubrobacterales; f: Solirubrobacteraceae; g: Solirubrobacter
New.CleanUp.ReferenceOTU23751	0.0495	4.67	24.67	k: Bacteria; p: Proteobacteria; c: Alphaproteobacteria; o: Rhodospirillales; f: Acetobacteraceae; g: Roseomonas
New.CleanUp.ReferenceOTU23862	0.0495	11.00	32.33	k: Bacteria; p: Actinobacteria; c: Acidimicrobia; o: Acidimicrobiales; f: Acidimicrobiaceae
New.CleanUp.ReferenceOTU24528	0.0495	19.33	34.67	k: Bacteria; p: Chloroflexi; c: Anaerolineae; o: Caldilineales; f: Caldilineaceae; g: Caldilinea
New.CleanUp.ReferenceOTU24794	0.0495	1.00	7.33	k: Bacteria; p: Verrucomicrobia; c: Spartobacteria; o: Chthoniobacteriales; f: Chthoniobacteraceae; g: Chthoniobacter; s: flavus
New.CleanUp.ReferenceOTU24864	0.0495	2.33	13.67	k: Bacteria; p: Proteobacteria; c: Alphaproteobacteria; o: Rhodospirillales; f: Acetobacteraceae; g: Roseomonas
New.CleanUp.ReferenceOTU24894	0.0495	8.33	46.33	k: Bacteria; p: Proteobacteria; c: Alphaproteobacteria; o: Rhodospirillales; f: Acetobacteraceae; g: Roseomonas
New.CleanUp.ReferenceOTU25542	0.0495	2.67	11.67	k: Bacteria; p: Planctomycetes; c: Planctomycetia; o: Planctomycetales; f: Planctomycetaceae; g: Planctomyces
New.CleanUp.ReferenceOTU26246	0.0495	31.67	3.00	k: Bacteria; p: Actinobacteria; c: Actinobacteria; o: Actinomycetales; f: Micromonosporaceae
New.CleanUp.ReferenceOTU26274	0.0495	1.67	6.00	k: Bacteria; p: Proteobacteria; c: Gammaproteobacteria; o: Chromatiales; f: Ectothiorhodospiraceae; g: Methylostratum; s: kenyaense
New.CleanUp.ReferenceOTU26425	0.0495	19.33	58.00	k: Bacteria; p: Actinobacteria; c: Actinobacteria; o: Actinomycetales; f: Geodermatophilaceae; g: Geodermatophilus
New.CleanUp.ReferenceOTU26611	0.0495	2.33	14.67	k: Bacteria; p: Proteobacteria; c: Alphaproteobacteria; o: Rhodospirillales; f: Acetobacteraceae; g: Roseomonas
New.CleanUp.ReferenceOTU26710	0.0495	36.33	18.33	k: Bacteria; p: Proteobacteria; c: Alphaproteobacteria; o: Rhizobiales; f: Phyllobacteriaceae; g: Mesorhizobium
New.CleanUp.ReferenceOTU27209	0.0495	17.67	48.33	k: Bacteria; p: Actinobacteria; c: Actinobacteria; o: Actinomycetales; f: Nocardioidaceae; g: Nocardioides
New.CleanUp.ReferenceOTU27313	0.0495	3.00	7.00	k: Bacteria; p: Proteobacteria; c: Alphaproteobacteria; o: Rhizobiales; f: Methylocystaceae
New.CleanUp.ReferenceOTU28138	0.0495	45.00	19.67	k: Bacteria; p: Actinobacteria; c: Acidimicrobia; o: Acidimicrobiales; f: Acidimicrobiaceae; g: Acidimicrobium
New.CleanUp.ReferenceOTU28529	0.0495	24.33	6.67	k: Bacteria; p: Actinobacteria; c: Actinobacteria; o: Actinomycetales; f: Nocardioidaceae; g: Aeromicrobium
New.CleanUp.ReferenceOTU28751	0.0495	15.67	5.33	k: Bacteria; p: Planctomycetes; c: Planctomycetia; o: Gemmatales; f: Isosphaeraceae; g: Singulisphaera
New.CleanUp.ReferenceOTU28788	0.0495	6.67	30.00	k: Bacteria; p: Actinobacteria; c: Rubrobacteria; o: Rubrobacterales; f: Rubrobacteraceae; g: Rubrobacter
New.CleanUp.ReferenceOTU28803	0.0495	2.33	8.00	k: Bacteria; p: Actinobacteria; c: Actinobacteria; o: Actinomycetales; f: Geodermatophilaceae; g: Blastococcus; s: aggregatus
New.CleanUp.ReferenceOTU28855	0.0495	2.00	7.33	k: Bacteria; p: Armatimonadetes; c: Armatimonadia; o: Armatimonadales; f: Armatimonadaceae; g: Armatimonas
New.CleanUp.ReferenceOTU29140	0.0495	2.00	11.67	k: Bacteria; p: Actinobacteria; c: Thermoleophila; o: Solirubrobacterales; f: Conexibacteraceae; g: Conexibacter
New.CleanUp.ReferenceOTU30047	0.0495	70.00	27.33	k: Bacteria; p: Proteobacteria; c: Alphaproteobacteria; o: Rhizobiales; f: Hyphomicrobiaceae; g: Hyphomicrobium
New.CleanUp.ReferenceOTU30562	0.0495	6.00	12.00	k: Bacteria; p: Actinobacteria; c: Acidimicrobia; o: Acidimicrobiales; f: Acidimicrobiaceae

New.CleanUp.ReferenceOTU1122	0.0495	11	8	k: Bacteria; p: Proteobacteria; c: Betaproteobacteria; o: Burkholderiales; f: Oxalobacteraceae; g: Cupriavidus; s: pauculus
New.CleanUp.ReferenceOTU1187	0.0495	3	2	k: Bacteria; p: Proteobacteria; c: Betaproteobacteria; o: Burkholderiales; f: Oxalobacteraceae; g: Janthinobacterium
New.CleanUp.ReferenceOTU1238	0.0495	2	2	k: Bacteria; p: Proteobacteria; c: Betaproteobacteria; o: Burkholderiales; f: Oxalobacteraceae; g: Janthinobacterium
New.CleanUp.ReferenceOTU1348	0.0495	10	16	k: Bacteria; p: Proteobacteria; c: Betaproteobacteria; o: Burkholderiales; f: Oxalobacteraceae; g: Janthinobacterium; s: agaricidamnosum
New.CleanUp.ReferenceOTU1433	0.0495	203	176	k: Bacteria; p: Proteobacteria; c: Betaproteobacteria; o: Burkholderiales; f: Oxalobacteraceae; g: Janthinobacterium; s: lividum
New.CleanUp.ReferenceOTU1471	0.0495	1	1	k: Bacteria; p: Proteobacteria; c: Gammaproteobacteria; o: Alteromonadales; f: Chromatiaceae; g: Alishewanella
New.CleanUp.ReferenceOTU1502	0.0495	3	9	k: Bacteria; p: Proteobacteria; c: Gammaproteobacteria; o: Alteromonadales; f: Shewanellaceae; g: Shewanella; s: baltica
New.CleanUp.ReferenceOTU1584	0.0495	10	6	k: Bacteria; p: Proteobacteria; c: Gammaproteobacteria; o: Enterobacteriales; f: Enterobacteriaceae; g: Gluconacetobacter
New.CleanUp.ReferenceOTU1585	0.0495	2	0	k: Bacteria; p: Proteobacteria; c: Gammaproteobacteria; o: Enterobacteriales; f: Enterobacteriaceae; g: Plesiomonas
New.CleanUp.ReferenceOTU1591	0.0495	8	7	k: Bacteria; p: Proteobacteria; c: Gammaproteobacteria; o: Pseudomonadales; f: Moraxellaceae; g: Acinetobacter
New.CleanUp.ReferenceOTU1610	0.0495	4	2	k: Bacteria; p: Proteobacteria; c: Gammaproteobacteria; o: Pseudomonadales; f: Moraxellaceae; g: Acinetobacter; s: baumannii
New.CleanUp.ReferenceOTU1762	0.0495	1	4	k: Bacteria; p: Proteobacteria; c: Gammaproteobacteria; o: Pseudomonadales; f: Moraxellaceae; g: Acinetobacter; s: guillouiae
New.CleanUp.ReferenceOTU2091	0.0495	2	0	k: Bacteria; p: Proteobacteria; c: Gammaproteobacteria; o: Pseudomonadales; f: Moraxellaceae; g: Enhydrobacter
New.CleanUp.ReferenceOTU2135	0.0495	187	145	k: Bacteria; p: Proteobacteria; c: Gammaproteobacteria; o: Pseudomonadales; f: Pseudomonadaceae; g: Pseudomonas
New.CleanUp.ReferenceOTU2340	0.0495	27	20	k: Bacteria; p: Proteobacteria; c: Gammaproteobacteria; o: Pseudomonadales; f: Pseudomonadaceae; g: Pseudomonas; s: brenneri
New.CleanUp.ReferenceOTU2400	0.0495	21	29	k: Bacteria; p: Proteobacteria; c: Gammaproteobacteria; o: Pseudomonadales; f: Pseudomonadaceae; g: Pseudomonas; s: fragi
New.CleanUp.ReferenceOTU2435	0.0495	2	0	k: Bacteria; p: Proteobacteria; c: Gammaproteobacteria; o: Pseudomonadales; f: Pseudomonadaceae; g: Pseudomonas; s: veronii
New.CleanUp.ReferenceOTU2454	0.0495	12	9	k: Bacteria; p: Proteobacteria; c: Gammaproteobacteria; o: Xanthomonadales; f: Xanthomonadaceae; g: Stenotrophomonas; s: geniculata
New.CleanUp.ReferenceOTU2482	0.0495	0	4	k: Bacteria; p: Proteobacteria; c: Gammaproteobacteria; o: Xanthomonadales; f: Xanthomonadaceae; g: Xanthomonas
New.CleanUp.ReferenceOTU2535	0.0495	8	5	k: Bacteria; p: Thermi; c: Deinococci; o: Deinococcales; f: Deinococcaceae; g: Deinococcus
New.CleanUp.ReferenceOTU2582	0.0495	5	1	k: Bacteria; p: Thermi; c: Deinococci; o: Thermales; f: Thermaceae; g: Meiothermus; s: silvanus

Supplementary Table 3. Relative abundances of the bacterial operational taxonomical units (OTUs) that were statistically different in the phyllosphere (leaf surface) of olive plants irrigated with treated urban wastewater (WW_{tr}) or rainfed (RF_{tr}). Only the OTUs with a statistical difference ($p < 0.05$) between WW_{tr} and RF_{tr} were considered. k, kingdom; p, phylum; c, class; o, order; f, family; g, genus; s, species.

PHYLLOSHERE

OTU ID	p level	Relative abundance		Taxonomic group
		WW_{tr}	RF_{tr}	
JN559538.1	0.0253	2.00	0.00	k: Bacteria; p: Firmicutes; c: Clostridia; o: Clostridiales; f: Clostridiaceae; g: Clostridium; s: subterminale
HM315314.1	0.0253	1.00	0.00	k: Bacteria; p: Proteobacteria; c: Alphaproteobacteria; o: Rhizobiales; f: Methylobacteriaceae; g: Methylobacterium; s: adhaesivum
FM873458.1	0.0253	0.00	1.00	k: Bacteria; p: Actinobacteria; c: Actinobacteria; o: Actinomycetales; f: Micrococcaceae; g: Kocuria;
HM272697.1	0.0339	0.00	1.33	k: Bacteria; p: Actinobacteria; c: Actinobacteria; o: Actinomycetales; f: Geodermatophilaceae; g: Modestobacter
HM278382.1	0.0339	2.33	0.00	k: Bacteria; p: Proteobacteria; c: Betaproteobacteria; o: Burkholderiales; f: Comamonadaceae; g: Variovorax; s: paradoxus
JF020944.1	0.0339	0.00	1.33	k: Bacteria; p: Proteobacteria; c: Alphaproteobacteria; o: Rhizobiales; f: Methylobacteriaceae; g: Methylobacterium; s: adhaesivum
JF049593.1	0.0339	0.33	2.00	k: Bacteria; p: Proteobacteria; c: Alphaproteobacteria; o: Rhizobiales; f: Methylobacteriaceae; g: Methylobacterium; s: adhaesivum
New.CleanUp.ReferenceOTU542	0.0339	2.00	0.33	k: Bacteria; p: Bacteroidetes; c: Sphingobacteriia; o: Sphingobacteriales; f: Flexibacteraceae; g: Hymenobacter
New.CleanUp.ReferenceOTU1744	0.0339	0.00	1.67	k: Bacteria; p: Cyanobacteria; c: Oscillatorophycideae; o: Chroococcales; f: Phormidiaceae; g: Microcoleus; s: vaginatus
New.CleanUp.ReferenceOTU2054	0.0339	0.00	1.67	k: Bacteria; p: Proteobacteria; c: Alphaproteobacteria; o: Rhizobiales
New.CleanUp.ReferenceOTU4091	0.0339	1.67	0.00	k: Bacteria; p: Actinobacteria; c: Actinobacteria; o: Actinomycetales; f: Kineosporiaceae; g: Kineococcus
New.CleanUp.ReferenceOTU9499	0.0339	2.67	0.00	k: Bacteria; p: Proteobacteria; c: Gammaproteobacteria; o: Pseudomonadales; f: Moraxellaceae; g: Psychrobacter
New.CleanUp.ReferenceOTU12968	0.0339	1.33	0.00	k: Bacteria; p: Proteobacteria; c: Gammaproteobacteria; o: Xanthomonadales; f: Xanthomonadaceae
New.CleanUp.ReferenceOTU14372	0.0339	1.33	0.00	k: Bacteria; p: Firmicutes; c: Clostridia; o: Clostridiales; f: Clostridiaceae; g: Clostridium; s: perfringens
New.CleanUp.ReferenceOTU15461	0.0339	2.67	0.00	k: Bacteria; p: Actinobacteria; c: Actinobacteria; o: Actinomycetales; f: Micromonosporaceae; g: Couchioplanes
AY558587.1	0.0369	13.33	0.00	k: Bacteria; p: Firmicutes; c: Clostridia; o: Clostridiales; f: Clostridiaceae; g: Clostridium; s: novyi
AJ576423.1	0.0369	0.00	2.00	k: Bacteria; p: Actinobacteria; c: Actinobacteria; o: Actinomycetales; f: Micrococcaceae; g: Arthrobacter
AB78676.1	0.0369	8.00	2.00	k: Bacteria; p: Proteobacteria; c: Betaproteobacteria; o: Burkholderiales; f: Comamonadaceae
New.CleanUp.ReferenceOTU3788	0.0369	2.33	0.00	k: Bacteria; p: Actinobacteria; c: Actinobacteria; o: Actinomycetales
New.CleanUp.ReferenceOTU5257	0.0369	2.33	0.00	k: Bacteria; p: Proteobacteria; c: Alphaproteobacteria; o: Rhodospirillales; f: Acetobacteraceae; g: Acidisphaera
AF371840.1	0.0431	5.00	1.33	k: Bacteria; p: Firmicutes; c: Clostridia; o: Clostridiales; f: Clostridiaceae; g: Clostridium; s: baratii
New.CleanUp.ReferenceOTU13625	0.0431	1.67	3.67	k: Bacteria; p: Proteobacteria; c: Alphaproteobacteria; o: Sphingomonadales; f: Sphingomonadaceae; g: Sphingomonas
AF385532.1	0.0463	3.67	0.33	k: Bacteria; p: Actinobacteria; c: Actinobacteria; o: Actinomycetales; f: Micrococcaceae; g: Kocuria; s: rhizophila
AF371845.1	0.0463	13.00	2.67	k: Bacteria; p: Firmicutes; c: Clostridia; o: Clostridiales; f: Clostridiaceae; g: Clostridium; s: perfringens
EU071484.1	0.0463	8.00	1.33	k: Bacteria; p: Proteobacteria; c: Alphaproteobacteria; o: Sphingomonadales; f: Sphingomonadaceae; g: Sphingomonas
AJ555244.1	0.0463	2.67	6.33	k: Bacteria; p: Proteobacteria; c: Alphaproteobacteria; o: Rhizobiales; f: Beijerinckiaceae; g: Methylocella
DQ071104.1	0.0463	3.00	0.33	k: Bacteria; p: Proteobacteria; c: Alphaproteobacteria; o: Sphingomonadales; f: Sphingomonadaceae; g: Sphingomonas; s: asaccharolytica
New.ReferenceOTU9	0.0463	7.67	16.00	k: Bacteria; p: Bacteroidetes; c: Sphingobacteriia; o: Sphingobacteriales; f: Flexibacteraceae; g: Hymenobacter
New.CleanUp.ReferenceOTU8413	0.0463	2.33	9.67	k: Bacteria; p: Actinobacteria; c: Actinobacteria; o: Actinomycetales; f: Kineosporiaceae; g: Kineococcus
New.CleanUp.ReferenceOTU15290	0.0463	2.33	9.00	k: Bacteria; p: Proteobacteria; c: Alphaproteobacteria; o: Rhizobiales
HM335388.1	0.0495	98.67	25.33	k: Bacteria; p: Bacteroidetes; c: Sphingobacteriia; o: Sphingobacteriales; f: Flexibacteraceae; g: Hymenobacter
DQ129612.1	0.0495	124.33	29.33	k: Bacteria; p: Proteobacteria; c: Alphaproteobacteria; o: Sphingomonadales; f: Sphingomonadaceae; g: Sphingomonas
HM322346.1	0.0495	92.67	68.67	k: Bacteria; p: Bacteroidetes; c: Sphingobacteriia; o: Sphingobacteriales; f: Flexibacteraceae; g: Hymenobacter
HQ115548.1	0.0495	38.33	18.33	k: Bacteria; p: Proteobacteria; c: Betaproteobacteria; o: Burkholderiales; f: Comamonadaceae
HM312958.1	0.0495	26.67	42.00	k: Bacteria; p: Bacteroidetes; c: Sphingobacteriia; o: Sphingobacteriales; f: Flexibacteraceae; g: Hymenobacter
AB015566.1	0.0495	573.00	837.67	k: Bacteria; p: Proteobacteria; c: Alphaproteobacteria; o: Rhizobiales; f: Methylobacteriaceae; g: Methylobacterium; s: adhaesivum
JF077740.1	0.0495	874.67	509.67	k: Bacteria; p: Bacteroidetes; c: Sphingobacteriia; o: Sphingobacteriales; f: Flexibacteraceae; g: Hymenobacter
HM270012.1	0.0495	20.67	1.33	k: Bacteria; p: Actinobacteria; c: Actinobacteria; o: Actinomycetales; f: Micromonosporaceae; g: Couchioplanes
New.ReferenceOTU0	0.0495	1714.00	3023.00	k: Bacteria; p: Proteobacteria; c: Alphaproteobacteria; o: Rhizobiales
New.ReferenceOTU4	0.0495	201.00	449.00	k: Bacteria; p: Proteobacteria; c: Alphaproteobacteria; o: Rhodospirillales; f: Acetobacteraceae
New.ReferenceOTU16	0.0495	15.33	33.67	k: Bacteria; p: Proteobacteria; c: Betaproteobacteria; o: Burkholderiales; f: Burkholderiaceae; g: Burkholderia
New.ReferenceOTU44	0.0495	389.67	760.00	k: Bacteria; p: Bacteroidetes; c: Sphingobacteriia; o: Sphingobacteriales; f: Flexibacteraceae; g: Hymenobacter
New.ReferenceOTU22	0.0495	55.33	9.33	k: Bacteria; p: Bacteroidetes; c: Sphingobacteriia; o: Sphingobacteriales; f: Flexibacteraceae; g: Hymenobacter
New.CleanUp.ReferenceOTU5149	0.0495	36.67	5.33	k: Bacteria; p: Bacteroidetes; c: Sphingobacteriia; o: Sphingobacteriales; f: Flexibacteraceae; g: Hymenobacter
New.CleanUp.ReferenceOTU11231	0.0495	1.00	6.33	k: Bacteria; p: Proteobacteria; c: Alphaproteobacteria; o: Rhodospirillales; f: Acetobacteraceae

Supplementary Figure 1

Pilot unit for urban wastewater treatment



Irrigation with urban wastewater treated by a pilot unit.

Secondary treatment (excepting sand filtration as a tertiary treatment).

Treated urban wastewater distributed from May to October by drip irrigation.

Amount of treated urban wastewater applied = $2800 \text{ m}^3 \text{ ha}^{-1} \text{ year}^{-1}$ (mean 2000-2016).

Supplementary Figure 2

Xylem sap extraction method

- **Equipment:** Scholander chambers with N₂.
- **Pressure applied:** 50-70 bar for 15-20 minutes per shoot.



Catia Fausto, one of the authors, while is extracting xylem sap.

Analysis of sap samples

- DNA extraction
- 16S rRNA gene amplified by PCR
- Metagenomic analysis of bacterial communities: DNA sequencing (Illumina MiSeq platform), and OTU (Operational Taxonomic Unit) identification.

Supplementary Figure 3

Drip irrigation system

- Six **drip emitters** discharging 8 L h^{-1} over a 1-m radius for each plant.
- **Canopies** approximately 4.5 m high.
- The **distribution pipes and emitters** were placed approximately 1.5 m above the ground.

