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SECONDARY TREATED WASTEWATER AS A VALUABLE AND SAFE SOURCE FOR DRIP IRRIGATING TREE CROPS

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Ci sono soltanto due possibili conclusioni: se il risultato conferma l'ipotesi, allora hai appena fatto una misura. Se il risultato è contrario alle ipotesi, allora hai fatto una scoperta.

(Enrico Fermi)

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ABSTRACT

Wastewater represents a support source for irrigation and mineral nutrients supply in agricultural systems, offering agronomical and environmental advantages. This work investigates the effect of STW (secondary treated wastewater) irrigation on apple and nectarine crops. Physiological, nutritional status and contaminants accumulation in vegetative and reproductive organs were assessed based on the different physiological traits of the two species. Trees were grown, for two consecutive seasons, in pots and drip irrigated with: Tap water (TW), Tap water plus an addition of mineral fertilizer (TW+MF) and Secondary treated wastewater (STW). Furthermore a laboratory trial was carried out to asses two *E.coli* strains internalization on young GF 677 micropropagated plants. Regarding the pots trial, apple and nectarine tree physiological, vegetative and fruit growth/quality parameters were in most of the cases promoted by STW, compared to TW trees, although TW+MF trees showed the highest values. This response is related to the different amount of nutrients supplied to the trees. Although STW provided a “fertigation-like” effect, results suggest that it did not completely fulfil tree nutrient demand. Treatments affected mainly leaf rather than fruit mineral concentration, with concentrations mostly in the optimal range for all treatments, except the TW, which showed nutritional deficiencies. STW irrigation improved nectarine fruit growth rate and influenced positively apple fruit quality parameters. Heavy metal concentration was unaffected by STW-irrigation with concentrations in fruit tissues within international limits imposed for human consumption in both species. No *E.coli* and few total coliforms were detected in the vegetative and reproductive tissue of both species. As for the laboratory trial, *E.coli* strains was able to enter roots but without any translocation in the areal part of the plant, not representing a hazard for human health. The overall results indicate STW as a convenient and safe source for drip irrigating tree crops.

CHAPTER I

INTRODUCTION

1.1. Climate change

Scientific evidences are showing changes in global climate, principally caused by the concentration increase in greenhouse gases, especially CO_2, in the atmosphere, which has been induced by industrial development and human activities over time. Climate change could modify the level of temperature on the ground surface, rainfalls and regional water supplies (Fig.1).

Many areas of the Earth will experience a rapid warming as well as the fact that weather events are more and more often taken to extremes (Auci and Vignani, 2014).

Impacts of climate change are mainly detected through changes in precipitation, temperature and higher degrees of variability of climatic conditions. Although climate change is affecting many economic sectors, agriculture is the most susceptible one as weather heavily affects crop production trends, yield variability and reduction of suitable areas for cultivation. Climate change represents a "*challenge*" that the European agriculture has to face in the immediate future, being subjected to relevant risks generated by new local meteorological conditions (Auci and Vignani, 2014). In fact, in many countries, temperatures have become more extreme and economic losses due to extreme weather events and decreased water availability have risen considerably in the last decade. The intensity of rainfalls and snowfalls has increased with more frequent floods in Northern Europe, while in Southern areas, rain has decreased substantially and drought periods are more frequent than in the past (Auci and Vignani, 2014).

While on one hand, an increasing length of spring and summer, and the related increase of temperatures, could favour crop productions in northern temperate latitude sites, on the other, higher temperatures and water scarcity could heavily reduce yields and threaten crop productivity in southern latitude areas. In this context, farmers have to deal with these risks in presence of more competitive global market conditions and modest policy support programs finalized to adaptation to climate change in European countries (Auci and Vignani, 2014).

1.2. Climate change and agriculture in Europe

Weather experts affirm that climate variability and extreme weather events are the major causes of production level alteration, higher yield variability and reduction of cultivation areas in Europe, especially in regions with a lower latitude.

Many studies have evaluated the effects of climate change on agriculture in Europe taking into account important regional differences (Olesen and Bindi, 2002; Reidsma and Lansink, 2007; Iglesias et al., 2009, Gornall et al., 2010). As a whole, in Europe an increase in the length of the growing season – defined as frost-free period – was observed in the last thirty year (Auci and Vhgnani, 2014). While some of the envisaged consequences could be beneficial for agriculture in the Northern areas of Europe (lengthening of the growing season and improvements in agricultural production due to milder weather conditions) it is expected that in the countries of the Mediterranean basin most of the consequences will be negative and will bring economic losses (Ventrella, 2012; EEA 2013b). In particular in Italy, Portugal, Greece, southern France and Ireland a significant reduction of cumulated rain amounts during winter was recorded (Auci and Vignani, 2014). Moreover, Italy and southern France showed a reduction of rain in summer. The combined effect of significant increase in temperature and reduction in rainfall has determined an increasing irrigation demand and has contributed to increased water deficit (Rosenzweig and Tubiello, 1997). Water shortage represents the most important consequence of these meteorological phenomena on the agricultural production of southern European countries. For these reasons, increased plant heat stress was recorded in Spain, Italy and in the Black Sea area like Turkey. In these countries, the agricultural sector must absolutely improve its water use efficiency in order to counter the costs associated with the increased use of this input (Auci and Vignani, 2014).

1.3. Climate change and agriculture in Italy

Italy is strongly affected by the negative consequences of climate change which could lead to resource limitations and constraints for the agricultural sector. These problems are mainly due to the geographical location of Italy and to the features that characterize the Italian agricultural sector, such as small farms with low resilience capability, facing increased evapotranspiration and increasing water scarcity (Auci and Vignani., 2014). Moreover, in this framework, national institutions have not put in place suitable environmental management policies and an agricultural sector governance to deal efficiently with the negative effects of climate change. In the last twenty years, a growing number of extreme weather events occurred and a rising shortage of water in several areas, traditionally suited to agriculture activities, threatened crops and areas suitable for cultivation with substantial losses (Auci and Vignani., 2014). In particular, in some areas in southern Italy, desertification has continued to increase since 1970 forcing the abandonment of local crops and the choice of new cultivations more heat resistant in summer time (Auci and Vignani., 2014; Ventrella et al., 2012).

In Italy, irrigated agriculture is the major water user accounting for more than 60% of total extractions (OECD, 2006). In the South of Italy, the high water demand from agriculture and from the population is exacerbated by the limited natural availability of water resources and by high climatic variability (MGWWG, 2005; Ventrella et al., 2015). Unfortunately, climate change is expected to intensify problems of water scarcity and irrigation requirements in all the Mediterranean region and in Italy in particular, as explained above (IPCC, 2007, Goubanova and Li, 2006; Rodriguez Diaz et al., 2007; Ventrella et al., 2017).

1.4. Agriculture water demand

Agriculture is the main water user in many European countries, accounting for around 33 % of total water use (EEA, 2012a). However, this proportion can be much higher in certain regions – for example, in some parts of Southern Europe (e.g. Spain), it accounts for up to 80% of all freshwater abstractions, with food crop irrigation being the dominant use (EEA, 2012a). In the arid and semi-arid areas of Europe (southern France, Italy, Greece, Portugal, Cyprus and Spain) irrigation is an essential component of production, helping to increase yield. In other European countries (central and northern regions), the proportion of water abstracted for crop irrigation is much lower but still significant, given the increasing and competing demands vs the limited water resources (Mudgal et al., 2015). Here, irrigation is primarily used to guarantee high quality features of the production, as well as consistent supplies of produce to retailers and processors (Knox et al., 2012). Most of the water used for irrigation is abstracted from surface or groundwater resources and used directly with relatively little on-farm storage (reservoirs) (Mudgal et al., 2015).

1.5. Water scarcity and wastewater reuse

Freshwater resources in Europe are under increasing stress, with a worrying mismatch between demand for, and availability of, water resources across both temporal and geographical (spatial) scales (EEA, 2012a). Water stress affects one third of the European territory all year round (EC, 2012). In summer, water scarcity is more pronounced in Southern European river basins but it is also becoming increasingly important in Northern river basins, including UK and Germany. Even in areas where water stress indicators are well below the thresholds, water saving is an important concern, in particular for domestic consumption, due to the energy consumption linked with water distribution, use and treatment. The frequency and intensity of drought events and their environmental and economic damages appear to have increased over the past thirty years (EC, 2012). South-eastern Europe is increasingly facing extended periods of drought, and both Northern and Western Europe have been affected in more recent years (EEA, 2012a).

Resource availability is further compromised by poor or unsuitable water quality which can significantly increase the financial supply costs (Mudgal et al., 2015). This is not only an issue for arid regions with low rainfall and high population density that are prone to increasing water stress; temperate areas with intense agricultural, tourism and industrial activities also suffer from frequent water shortages and/or expensive supply solutions (Rodriguez et al., 2007a). Global climate change is already exacerbating these problems with projections indicating significant and widespread impacts over the medium to long term (EEA, 2005). Growing competition for water resources between different water use sectors is already emerging, with high quality resources being protected and reserved for drinking water supply. Protecting water resources also has benefits for other resources such as biodiversity, soil or energy. The capacity of Europe to respond to the increasing risks of water scarcity and drought could be enhanced by foreseeing a wider reuse of treated wastewater for agricultural, industrial and urban uses. Water reuse is an accepted practice in several countries subjected to water scarcity (e.g. Italy, Cyprus, Spain), where it has become an integral and effective component of long-term water resources management (Mudgal et al., 2015). Water reuse may have a lower environmental impact than alternative water supplies such as water transfers or desalination, under certain conditions, and may offer a range of environmental, economic and social benefits. However, at present, the uptake of water reuse solutions remains limited in comparison with their potential (Mudgal et al., 2015). This appears to be due to a number of factors, including low economic attractiveness, low public acceptance of reuse solutions and limited awareness of their benefits, lack of common European environmental/health standards for reused water, and poor coordination of the professionals and organisations who design, implement and manage such schemes (Mudgal et al., 2015).

1.6. Wastewater characteristics

1.6.1. Sources of wastewater

In general, municipal wastewater comes from domestic wastewater, industrial wastewater, rain water, and by groundwater seepage entering the municipal sewage network. Domestic wastewater consists of effluent discharges from households and commercial buildings. Industrial wastewater is the effluent discharged by manufacturing units and food processing plants.

1.6.2. Urban wastewater

Urban wastewater is defined as 'domestic water or the mixture of domestic wastewater with industrial wastewater and/or run-off rain water'.

Reclaimed water is usually defined as former wastewater that has been treated to remove solids, organic matter and other types of impurities. Such water is treated to a certain quality that matches the intended use, in most cases, at a lower standard than drinking water quality.

The main objective of urban wastewater treatment plants (WWTPs) is to remove suspended solids, organic matter, and, in sensitive areas, also nutrients (e.g. N, P) prior to discharge of the water to a receiving water body (typically a river). These are the key families of pollutants, which are regulated by the EU directive for discharging treated wastewater into the environment. In order to comply with the directive requirements, a two-step treatment process is necessary ('primary treatment' followed by 'secondary treatment'). When treated wastewater is intended to be reused, in most cases there is a need for an additional treatment in order to minimise health and environmental risks and adjust the water quality to the planned use. This additional treatment step, called 'tertiary treatment', mainly consists of the removal of pathogens (e.g. bacteria, faecal coliforms, viruses and helminth eggs) and chemical contaminants (e.g. heavy metals and emerging contaminants) (Hussain et al., 2002). Given the advanced level of technology in wastewater treatment and the diversity of techniques available, it is worth noting that secondary treated wastewater can have a bacteriological quality that is similar to surface freshwater and even sometimes better than some surface water bodies (AFSSA, 2008).

1.6.3. Characteristics of wastewater treatments

The basic function of wastewater treatment is to speed up the natural processes by which water is purified. There are two main stages in the treatment of wastes, primary and secondary, possibly followed by a tertiary:

- Primary Treatment

The sewage enters the treatment plant and flows through a screen, which removes large floating objects. After the sewage has been screened, it passes into a grit chamber, where cinders, sand, and small stones settle to the bottom (Fig.2).

After the first screening is completed and grit has been removed, sewage still contains organic and inorganic matter along with other suspended solids. These solids are minute particles that can be removed from sewage in a sedimentation tank. When the speed of the flow through one of these tanks is reduced, the suspended solids will gradually sink to the bottom, where they form a mass of solids called '*raw primary biosolids*' formerly sludge. Biosolids are usually removed from tanks by pumping, afterwards they can be further treated for use as fertilizer or disposed of in a land fill or incinerated (EPA, 1998).

- Secondary Treatment

The secondary stage of treatment removes about 85% of the organic matter in sewage thanks to bacteria. The main secondary treatment technique is the activated sludge process. After the effluent leaves the sedimentation tank in the primary stage it flows or is pumped to a facility to undergo the next process (Fig.2).

The activated sludge process speeds up the microbiological degradation of organic matter by bringing air and sludge heavily laden with bacteria into close contact with sewage.

After the sewage leaves the settling tank in the primary stage, it is pumped into an aeration tank, where it is mixed with air and sludge, loaded with bacteria and allowed to remain for several hours. During this time, the bacteria break down the organic matter into harmless by-products.

The sludge, now activated with additional billions of bacteria and other micro-organisms, can be used again by returning it to the aeration tank for mixing with air and new sewage. From the aeration tank, the partially treated sewage flows to another sedimentation tank for removal of excess bacteria (Fig.2).

To complete the secondary treatment, effluent from the sedimentation tank is usually disinfected before being discharged into receiving waters. The disinfectant is added to the water to kill pathogenic bacteria, and to reduce odour (EPA, 1998).

- Tertiary Treatment (Advanced Treatment)

Advanced waste treatment techniques (Fig.2) are capable of removing nitrogen and phosphorus using physical-chemical separation techniques such as filtration, carbon adsorption, distillation, and reverse osmosis (EPA, 1998).

1.7. Wastewater applications

A wide range of reuse applications for treated urban or industrial water exist:

- Agricultural irrigation: crops irrigation (e.g. food and non-food);
- Industrial uses: process water, aggregate washing, cooling water, concrete making, dust control;
- Non-potable urban uses: landscape irrigation (e.g. public parks, golf courses, sporting facilities, private gardens), fire protection systems, street cleaning, vehicle washing, toilet flushing, air conditioners;
- Environmental and recreational uses: recreational impoundments (e.g. boating, fishing), aquatic ecosystem restoration or creation of new aquatic environments, stream augmentation, aquifer recharge (for saline intrusion control and delayed abstraction to increase water resources in quantity and quality), aquaculture and artificial-snow production;
- Increasing water availability for potable water production through the deliberate incorporation of reclaimed water into a raw water supply such as a river, catchment reservoir or aquifer resulting in mixing and assimilation thus providing an environmental buffer (before potable treatment).

Depending on the intended application and the initial quality of the reclaimed water, additional treatments of the reclaimed water may be required to adjust its quality to the application-specific requirements. A wide variety of additional treatment processes are available to respond to different applications and different economic and environmental contexts (Hussain et al., 2002).

1.8. Benefits of wastewater reuse in agriculture

1.8.1 Environmental and agricultural benefits

The use of treated wastewater in agriculture benefits the environment, the human health and the economy. This use represents an alternative practice that has been adopted in different regions confronted with water shortages and growing urban populations with increasing water needs (Winpenny et al., 2013; Bacerra et al., 2015; UNESCO, 2017) related to the decline in surface and groundwater resources caused by climate variability and climate change. The availability of water resources is also affected by wastewater-source pollution, as such water is not always treated before reaching surface channels, and by associated aquifer pollution (Banco Mundial, 2002; Winpenny et al., 2013; UNESCO, 2017).

One of the most recognized benefits of wastewater use in agriculture is the associated pressure decrease on freshwater sources. Thus, wastewater serves as an alternative irrigation source (Winpenny et al., 2013), especially for agriculture, the greatest global water user, which consumes 70% of available water (Pimentel et al., 2008).

Furthermore, wastewater reuse increases agricultural production in regions experiencing water shortages, thus contributing to food safety (Corcoran et al., 2010). Approximately 805 million people, one-ninth of the global population, suffer from hunger. However, according to FAO's latest estimations, a decreasing trend in hunger supports the possibility of halving the number of undernourished people. However, to be successful, it is first necessary to adopt a comprehensive approach that includes public and private investment aimed at increasing agricultural productivity, in addition to increasing and improving the availability of water resources and protecting vulnerable groups (FAO, 2015).

Depending on the local situation, another benefit associated with agricultural wastewater reuse could be the avoided cost of the extraction costs of groundwater resources. In this regard, it is worth noting that the energy required to pump groundwater can represent up to 65% of the costs of irrigation activities (Cruz et al., 2009).

Moreover, the nutrients naturally present in wastewater allow savings on fertilizer expenses (Drechsel et al., 2010; Winpenny et al., 2013; Corcoran et al., 2010; Moscoso et al., 2017), thus ensuring a closed and environmental friendly nutrient cycle that avoids the indirect return of macro- (especially nitrogen and phosphorous) and micro-elements to water bodies. Depending on the nutrients contained, wastewater may be a potential source of macro (i.e. N, P and K), micro-nutrients (i.e. Mg, B, Mg, Fe, Mn, Zn) and organic carbon (Barreto et al., 2013; Henze and Comeau, 2008; Liu and Haynes, 2011). Indeed, wastewater reuse has been proven to improve crop yield thanks to its nutrient effects (Moscoso, 2017; Jimenez, 1995; Lal et al., 2013; Matheyarasu et al., 2016; Oliveira and Von Sperling, 2008) and results in the reduced use of fertilizers in agriculture (Adrover et al., 2012; Fatta-Kassinos et al., 2011; Toze, 2017; Umana, 2011). Therefore, eutrophication conditions in water bodies would be reduced, as well as the expenses for agrochemicals to be used by farmers (Jaramillo, 2014; Candela et al., 2007).

Furthermore, a decrease in wastewater discharge helps improving the quality of receiving water bodies (Bixio and Wintgens, 2006; Toze, 2017). Moreover, groundwater reservoirs are preserved, as agricultural wastewater reuse recharges these sources with higher-quality water (Moscoso et al., 2002).

Wastewater reuse could also be beneficial for protecting the groundwater resources from saline intrusion, particularly in island and costal areas (through groundwater recharge). Additionally, an increased use of wastewater could contribute to the installation and optimization of treatment facilities to produce effluents of the desired quality for irrigation purposes, representing an economic benefit to sanitation projects (Zambrano et al., 2012).

In those areas where climatic and geographic characteristics allow it, low-cost wastewater treatment systems might also be a viable option, achieved using certain technological options that fulfil the objective of agricultural reuse (Winpenny et al., 2013). Moreover, decreasing the level of purification/treatment necessary for discharging wastewater would reduce the energy consumption associated with the water treatment. In addition, wastewater use in agriculture helps liberate capital resources through the payment of economic tools by the actors of different countries (Jaramillo et al., 2014). An implicit economic benefit of agricultural wastewater reuse is the promotion of the treated water discharged for human consumption, as this use is considered to be of highest priority. In some countries, wastewater reuse contributes to reducing the municipal cost of searching for water sources using more expensive means (Silva et al., 2008). Agricultural wastewater reuse can contribute to the justification of suitable investment policies and financing mechanisms for pollution control and prevention (Hernàndez et al., 2010).

1.9. Risks of wastewater reuse in agriculture

1.9.1. Environmental, crop-related and food safety risks

The use of treated or untreated wastewater in agriculture is not exempt from adverse effects on the environment, especially on soil, that represents the first water receiver.

The scientific literature includes evidence of alterations in the physicochemical parameters of soil (Bacerra et al., 2015). Additionally, in recent research, variations have been observed in the structure and magnitude of microbial biomass in soil, as well as an increase in microbial activity caused by agricultural wastewater reuse (Bacerra et al., 2015). Altering physicochemical parameters (i.e. pH, organic matter, nutrients, salinity and contaminants) and soil microbiota can affect fertility and productivity, thus disturbing soil sustainability due to inadequate irrigation with wastewater (Bacerra et al., 2015).

Irrigation with poor quality wastewater may also create undesirable effects on plants, negatively affecting their growth or productivity. This phenomena could be mainly attributed to the salinity levels of the wastewater that together with other contaminants could also cause plant phytotoxicity.

Crop plants irrigated with treated wastewater have also been found to absorb and accumulate excess heavy metals in the edible parts beyond maximum permissible limits (MPLs) (EC, 2001; WHO/FAO, 2007), set for guidance of their safety (Muchuweti et al., 2006; Khan et al., 2008; Singh et al., 2010a). Moreover, the presence of bacteria in wastewater irrigated crops edible parts such as *Escherichia coli*, and other human health related pathogens, is also a potential concern (Petterson et al., 2001; Palese et al., 2009; Cirelli et al., 2012; Forslund et al., 2012). In this regard, comprehensive guidelines and criteria have been established in order to safeguard environmental sustainability and public health as a result of wastewater irrigation (WHO, 2006; Brissaud, 2008; U.S. Environmental Protection Agency, 2012).

The Food and Agriculture Organization of the United Nations (FAO) has also developed several guidelines relevant to the use of wastewater in agriculture. These guidelines relate the degree of restriction of water use to salinity, infiltration and toxicity parameters of specific ions (Ayers and Wescott, 1985). In 1999, the FAO published the suggested guidelines for the “agricultural reuse of treated waters and treatment requirements”. In these guidelines, the type of agricultural reuse was classified on the basis of the type of irrigated crop (FAO, 2017) (Table 1).

1.10. Social aspects

1.10.1 Health risks of wastewater reuse in agriculture

Risks to public health are one of the key concerns associated with the reuse of reclaimed water. These risks may occur through direct or indirect exposure of the public with microbiological agents (pathogens) or chemical substances that are usually present or may be present in reclaimed water (Mudgal et al., 2015). Health impacts of water reuse depend upon the wastewater origin, the conditions imposed on the treatment and the subsequent use of the reclaimed water.

The composition of reclaimed water may vary depending on the origin of the collected wastewater, season, health status of the population and treatment applied (ANSES, 2012). Many pathogens can survive for long periods of time in soil or on crop surfaces to be transmitted to humans or animals. The most resistant pathogens in the environment are helminth eggs, which in some cases can survive for several years in the soil.

There are different possible exposure pathways, including in particular:

- Ingestion of reclaimed water or inhalation of droplets of reclaimed water, especially when the water is used for urban or recreational purposes;
- Ingestion of food products harvested from crops irrigated with reclaimed water;
- Ingestion of meat from animals grazing on pastures irrigated with reclaimed water or fed with forage crops irrigated with reclaimed water.

Human health and environmental risks associated with reclaimed water reuse are described in publications such as Deliverable D15 of the AQUAREC project (Salgot et al., 2006) or the WHO guidelines (WHO, 2006), with additional examples of exposure pathways for potential chemical and biological contaminants. According to the WHO, for the reuse of water in agriculture, the greatest health risks are associated with crops that are eaten raw (e.g. salad crops), especially root crops (e.g. radish and onion) or crops that grow close to the soil (e.g. lettuce and zucchini) (WHO, 2006).

The concentration levels and types of pathogens and chemical substances present in wastewater vary by region, according to the sanitary and socioeconomic conditions of a particular community (Gerba and Rose, 2003).

The concentration of viruses, protozoan parasites and helminths in wastewater can be 10–1000 times higher in developing countries than in developed countries (Jiménez et al., 2010). Table 2 presents the primary types of enteric pathogens and substances of sanitary interest that can be found in wastewater used for agricultural irrigation (Jaramillo and Restrepo., 2017)

Wastewater-borne diseases can also be chronic or acute (Craun et al., 1996). Acute risk corresponds to the possibility of becoming ill in the short-term when exposed to low infectious doses of a pollutant, whereas chronic risk refers to the presence of pollutants of chemical nature that affect human health after long periods of exposure (Guerra de Macedo, 1993). Additionally, microbial diseases can be directly or indirectly transmitted by water. Globally, such diseases have significantly contributed to premature mortality, especially in developing countries (Craun et al., 1996).

Other compounds present in irrigated wastewater that may pose risks to human health are emerging contaminants (ECs). ECs are molecules with biological activity on different organisms, and their physicochemical properties determine their persistence in the environment and facilitate their bioaccumulation (Jaramillo and Restrepo, 2017). ECs include analgesics, antihypertensive drugs and antibiotics, among others.

Such substances, of complex nature, even if contaminants of great concern, are still not considered in the legislation policies related to public health and wastewater treatment and reuse systems (Jaramillo and Restrepo, 2017).

However, there are very few health risk quantification studies and epidemiological studies on the reuse of reclaimed water; most epidemiological studies have addressed the reuse of raw sewage (where the contamination risks are much higher). The literature, however, does not report cases of human diseases caused by reclaimed water in Europe (Mudgal et al., 2015)

1.11. Public acceptance

The reuse of wastewater raises issues in terms of public acceptance, especially for drinking water production applications. The type of application for which water is reused is an important factor for public acceptance. Public acceptance decreases when public health is at stake or when there is a risk of contact or ingestion of reclaimed water (Mudgal et al., 2015). For instance, public acceptance for reusing water to irrigate crops that are intended to be eaten or to wash clothes can be low while reusing water for bioenergy cropping will not cause serious public concerns (IEEP et al., 2012).

The survey conducted as part of the AQUAREC project, revealed that in the view of some public administrations and of the population, treated wastewater basically remains wastewater. Furthermore, according to water industry experts, it is not widely known that in many urban and semi-urban areas in Europe surface or ground waters have bacterial quality worse than that of a secondary-treated wastewater, and that some agricultural areas are irrigated with self-abstracted water whose quality is lower than secondary-treated water (Mudgal et al., 2015). It is not widely known either that, in many urbanized catchments, the water cycles actually include indirect, unplanned and uncontrolled reuse of, sometimes even untreated, wastewater (Bixio et al., 2006).

Public acceptance is difficult to achieve as long as citizens are not fully aware of the need to reuse treated wastewater and consider it an efficient solution to address water scarcity and to reserve high quality water supplies for drinking water purposes (Mudgal et al., 2015). The first stage of acceptance of the use of reclaimed water is community awareness of the need. In this case, the use of reclaimed water becomes a solution to a problem and this, in turn, is an important driver of public perception (UK Water Research Industry, 2003).

Public acceptance also strongly relies on the understanding of the local water cycle. An important consideration is the question of when does wastewater cease to be wastewater and becomes just another water resource (UK Water Research Industry, 2003). Users perception of reclaimed water can improve significantly once they receive information about the holistic water cycle and the existence of unplanned potable reuse. The survey also revealed that the terminology continues to have a strong influence on the level of acceptability (e.g. ‘purified water’ much better perceived than ‘treated wastewater’) and that more information on monitoring and testing is needed to increase trust (Mudgal et al., 2015).

1.12. Economic aspects

1.12.1. Pricing and cost recovery

Pricing for water services in Europe is defined within Article 9 of the Water Framework Directive according to the principle of cost recovery (including environmental and resource costs) as well as the polluter-pays principle (proportionality to the pressures imposed on aquatic ecosystems by the main water users) (Mudgal et al., 2015). Available evidence suggests that, in the best case scenarios, only financial costs of water treatment and distribution are included in the fees: few countries apply direct charges to polluters for the purification of their wastewater as well as other activities that impact on water quality, and charging for the resource costs of water abstraction is rare (EEA, 2013). Furthermore, whilst financial cost recovery is high for domestic users, in agriculture low levels of cost recovery (20-80%) point to heavy subsidisation of freshwater use, even in water-scarce Mediterranean countries (EEA, 2013).

Prices would discourage both water efficiency and water reuse by failing to account for the full external costs of freshwater abstraction and wastewater discharge. Because these external costs are typically borne by taxpayers, price support measures for water reuse may be justified, to enhance its competitiveness (Mudgal et al., 2015).

1.12.2. Consumer demand

Evidence from countries with strong reuse of treated effluent points to a high differential against freshwater prices, owing to perceptions of weak demand. It follows that increasing demand and correspondingly higher willingness to pay might reduce the price differential over time (Mudgal et al., 2015). Another important demand factor is a consistent regulatory regime. The existence (or absence) of different quality standards for reclaimed water across Europe represents a barrier to both agricultural trade and consumer confidence. Risk perceptions may be highly influential in depressing willingness to pay for reused water. Evidence from several studies (e.g. Menegaki et al., 2007; Tsagarakis and Georgantizis, 2003) suggests that whilst irrigation and agricultural demand is primarily sensitive to price signals and the relative costs of reused water, for other uses (consumption of agricultural products and indirect domestic consumption) demand is sensitive to levels of knowledge regarding the risks and benefits of reclaimed water. This indicates that there is a socially optimal level of reused water uptake, and that better standards and awareness could reduce the need for price differentials against freshwater (Mudgal et al., 2015).

1.12.3 Delivery models

Fragmentation of the water supply and wastewater disposal cycle is a major obstacle for supply and demand coordination. Responsibilities for both regulating and supplying water services and wastewater treatment and disposal are typically separated, obscuring costs such as water pollution control, which accounts, for example in Europe, for 50% of the environmental spending (EUWI, 2007). Water reuse can be seen as a cost-effective alternative to some point-source pollution abatement measures required under the Urban Waste Water Directive (Bixio, et al., 2006). Separation of water supply and wastewater disposal may act as a constraint on the supply of treated effluent for reuse, both in terms of infrastructure – major investments may be needed to link treatment plants to consumers – and the relative distribution of costs and benefits. For water suppliers, reuse benefits are largely limited to financial returns (if any), and reducing demand for freshwater may impact on overall investment in water infrastructure (Fatta et al., 2005). For water suppliers, a degree of 'benefit leakage' may occur, with few obvious methods for compensation. The main externalities from water reuse are presented in

Table 3. Evidence suggests how the economic returns on water reuse can significantly outweigh costs, when such externalities and public goods are accounted for (Molinos-Senante et al., 2011; Wilson et al., 2011). Quantifying these benefits can strengthen the case for reuse schemes and public support, but does not address the practical issue of how to allocate benefits, and in particular up-front costs and risks, within a reuse project (Mudgal et al., 2015).

1.13. Main problems related to an insufficient wastewater reuse

- Inadequate water pricing

To date, price differentials between reused water and freshwater are insufficient; they are increased by a lack of full cost recovery within most water markets and the presence of public subsidies to conventional water resources in many countries. This is both a regulatory failure and a market failure as prices of conventional resources and reused water do not reflect their actual cost. This situation leads to a limited economic attractiveness of water reuse projects and improper decisions by water users and decision makers (Mudgal et al., 2015).

- Insufficient control over freshwater abstraction

Conventional water resources, in many situations, are insufficiently controlled by public authorities, resulting in both over-allocation (abstraction permits going beyond available resources, including situations where no maximum amount is set in permits) and illegal abstraction (when permits are not enforced, in particular because of no monitoring of actual abstractions). This issue can be considered as a regulatory failure (Mudgal et al., 2015).

- Economic and technical uncertainties for decision-makers

There are a number of information, regulatory and technical failures, as well as societal issues, which limit consumer willingness to pay for reused water and hence wastewater ability to compete with freshwater resources:

- A lack of stakeholders’ awareness concerning the benefits of water reuse;
- A lack of public acceptance towards water reuse;
- A fear of potential trade barriers on agricultural goods that have been grown using reclaimed water.

These issues create uncertainties for potential project developers or investors interested in water reuse (Mudgal et al., 2015).

- Too strict water reuse standards

In some countries (i.e. Italy, France), the strictness of the existing water reuse standards has been reported to be an obstacle to the further uptake of water reuse solutions, due to high administrative burden and associated costs for local authorities, reclaimed water suppliers and users. The situation is likely to remain unchanged in future years (i.e. very few new water reuse projects) in the absence of action related to standards' harmonisation and simplification (Mudgal et al., 2015).

- Reuse not seen as a component of integrated water management approaches

Integrated water management is not sufficiently implemented and it is still in its infancy in some countries. This is characterised by a fragmentation of responsibilities and authorities over different parts of the water cycle and a lack of communication and cooperation among the stakeholders involved in the whole water cycle in certain regions, in particular between water supply and sanitation stakeholders. This can be considered as a regulatory failure. Reuse is rarely considered in the design and choice of location of wastewater treatment plants (Mudgal et al., 2015).

- Technical challenges and scientific uncertainties

The water reuse sector seems to be mature, technical solutions are well known and available to cover a wide range of applications and environments. However, these solutions are not always cheap and a few technical challenges remain, in particular: the removal of emerging contaminants (e.g. pharmaceuticals, drug metabolites, household chemicals, etc.) by conventional treatment techniques; the need for rapid monitoring techniques that are reliable and cost-effective (Mudgal et al., 2015).

1.14. Wastewater reuse in Italy, an overview

The Italian total treated effluent flow is estimated at 233 M m^3 yr^{-1} of usable water. Because of the regulatory obligation to achieve a high level of treatment, the medium to large-sized plants (>100.000 inhabitants), accounting for approximately 60% of urban wastewater flow can provide re-usable effluents for a favourable cost/benefit ratio (Angelakis et al., 2007).

Treated wastewater is mainly used for agricultural irrigation covering over 4.000 ha. However, the controlled reuse of urban wastewater in agriculture is not yet developed in most Italian regions and has decreased due to the low water quality.

The total cost (plant construction, operation and maintenance) for the wastewater treatment, in addition to the costs for the distribution and the monitoring of the whole reuse system, is difficult to recover and probably only for large WWTPs, thus reducing the benefit of reclaiming water and hampering the development of wastewater reuse practices for the smaller ones (Mudgal et al., 2015).

In Italy, legal quality standards have been set at the national level for agricultural, urban and industrial applications (Ministerial Decree no.185/2003), but regional authorities may impose stricter quality standards. This has led to a situation where, in many regions, the quality standards for reclaimed water were similar to those for drinking water even for non-potable uses (Angelakis et al., 2007), limiting the economic attractiveness of water reuse schemes for potential investors. Moreover, the fertilizing potential of wastewater is almost lost in a tertiary treatment, where the levels of the main crop nutrients (i.e. nitrogen and phosphorous) are significantly reduced by the cleaning treatment (Pescod, 1992). The requirement for full disinfection for all applications is also considered as too stringent by many stakeholders. Reportedly, complying with the standards involves significant costs, especially as existing WWTPs need to be refurbished. Another issue is the high number of quality parameters to be monitored (which exceeds 50), their significantly low threshold (if compared to international guidelines) and the high sampling frequency required in certain regions, entailing high monitoring costs (Mudgal et al., 2015). This approach is considered as highly precautionary, driven by a strong demand from consumers, farmers and food retailers to have a high level of safety (reuse of water is not well perceived in general).

For this reason, it is quite limiting to take into account only the utilization of wastewater subjected to the quality standard of the Italian Ministerial Decree no.185/2003, which is considered a highly cautious tertiary treatment. Because of the high availability of other different wastewater sources, meeting good intrinsic quality parameters, such as secondary treated wastewater (Ministerial Decree No. 152/06), it would be possible to increase and enhance the available amount of water reuse in this country. Indeed, secondary treated wastewater could be a beneficial water source for agriculture (e.g. fertilizer savings), for the environment (e.g. reducing eutrophication) and for reducing the cost of wastewater plants

(Molinos-Senante et al., 2010). In any case, wastewater use in agriculture should be restricted only to certain types of crops (i.e. crops whose edible part is not directly wet by irrigation to avoid any microbiological or chemical contamination), without creating any risks for the final consumer. Further research is needed to optimize the levels of water filtration on secondary treated wastewater in order to maintain its potential nutritional value, while guaranteeing the chemical and microbiological safety of the harvested products.

1.15. Aim of the study

Secondary treated wastewater (STW) represents an opportunity to mitigate water shortage in summer time and a rational approach for reducing fertilizer utilization.

The aim of this research was to assess the still unexploited effects of drip irrigation with STW on two of the main worldwide cultivated horticultural crops: apple and peach, which are characterized by a different physiological and nutritional traits.

The effect of STW was investigated considering its potential to promote plant nutritional (i.e. leaf and fruit macro-micro element content) and physiological status (i.e. vegetative growth, water relation, leaf gas exchanges and fruit quality parameters). Furthermore, possible risks due to heavy metals accumulation and microbial internal translocation (i.e. *E.coli* and total coliforms) were determined in plant vegetative and reproductive organs (i.e. fruit), based on the different plant physiology and anatomy (e.g. apple fruit xylem dysfunctionality). A further study was performed, on GF677 micropropagated plants, to better clarify the internalization and translocation process by a common waterborne pathogen bacterium as *E. coli*.

This study represents a first step to encourage low cost treatment systems, such as STW, and for promoting wastewater use with a crop-based approach, suppling wastewater of different qualities depending on the morpho-physiological features of each horticultural tree crop.

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1.16. Figures and Tables

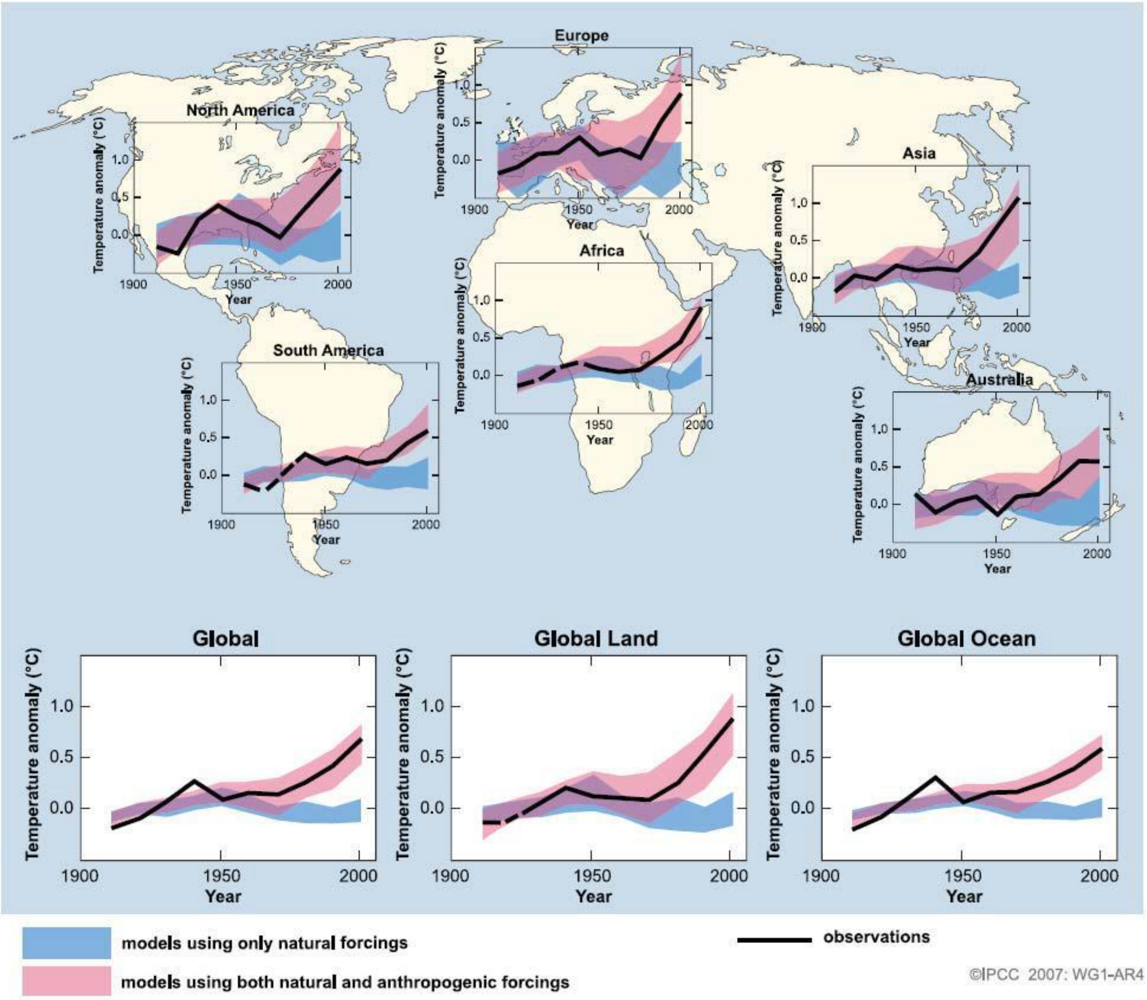


Figure 1. Temperature change on global and continental scale. Source: IPCC data.

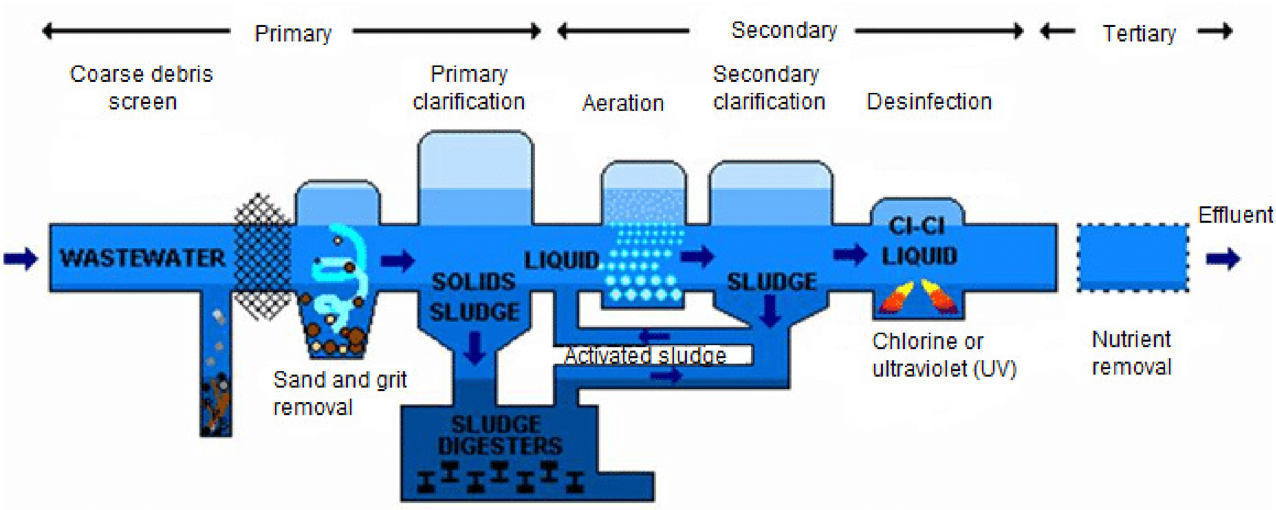


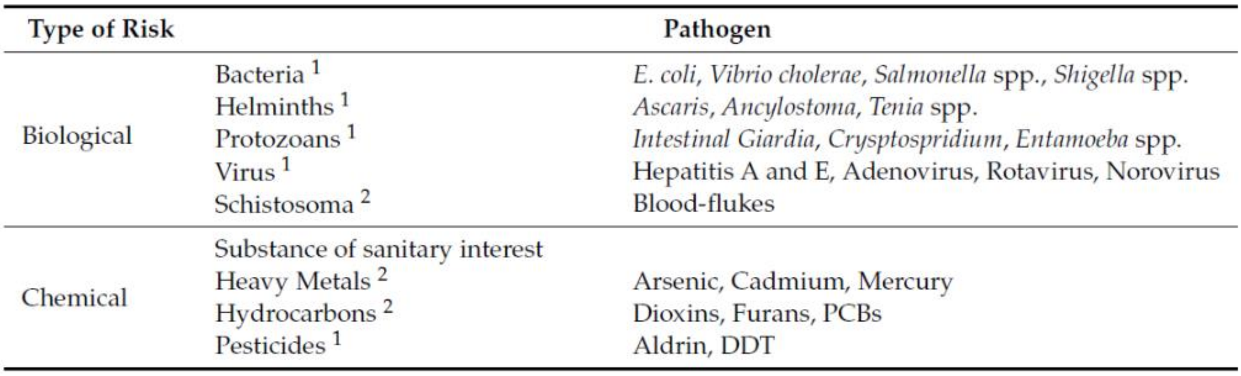
Figure 2. Diagram of a mechanical-biological wastewater treatment plant. Source: Zăbavă B.ȘT et al 2016.

Table 1. FAO guidelines for the agricultural reuse of treated water.

Type of Agricultural Reuse	Type of Treatment	Quality Criterion
Agricultural reuse in crops that are consumed and not processed commercially.	Secondary Filtration—Disinfection	pH = 6.5–8.4 BOD < 10 mg/L <2 UNT <14 NMP *E. coli*/100 mL <1 Egg/L
Agricultural reuse in crops that are consumed and not processed commercially.	Secondary—Disinfection	pH = 6.5–8.4 BOD < 30 mg/L SS < 30 mg/L <200 NMP *E. coli*/100 mL
Agricultural reuse in crops that are not consumed.	Secondary—Disinfection	pH = 6.5–8.4 BOD < 30 mg/L SS < 30 mg/L <200 NMP *E. coli*/100 mL

Source: FAO. Wastewater Treatment and Use in Agriculture. Avaliable online: http://www.fao.org/docrep/T0551E/ T0551E00.htm (accessed on 30 April 2017).

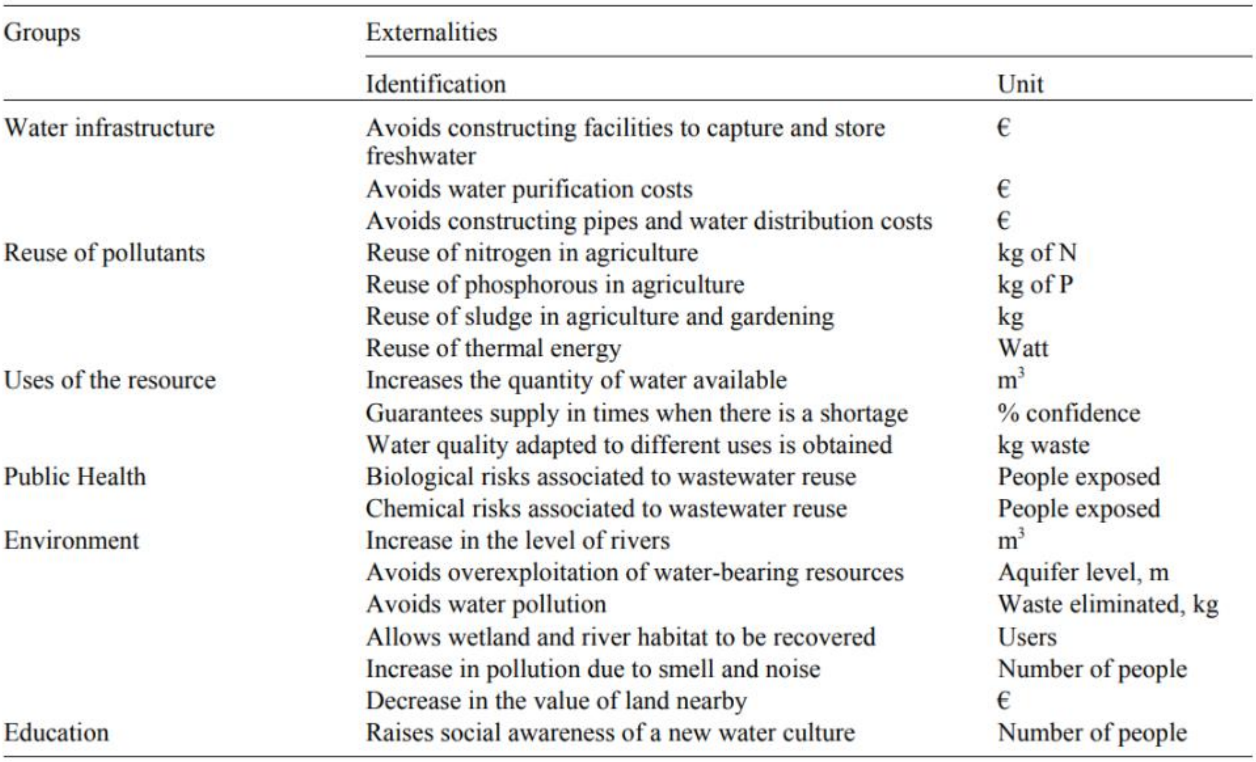
Table 2. Biological and chemical risks associated with the use of raw wastewater in agriculture



Type of Risk		Pathogen
Biological	Bacteria [1]	*E. coli, Vibrio cholerae, Salmonella* spp., *Shigella* spp.
	Helminths [1]	*Ascaris, Ancylostoma, Tenia* spp.
	Protozoans [1]	*Intestinal Giardia, Crysptospridium, Entamoeba* spp.
	Virus [1]	Hepatitis A and E, Adenovirus, Rotavirus, Norovirus
	Schistosoma [2]	Blood-flukes
Chemical	Substance of sanitary interest	
	Heavy Metals [2]	Arsenic, Cadmium, Mercury
	Hydrocarbons [2]	Dioxins, Furans, PCBs
	Pesticides [1]	Aldrin, DDT

Contact and/or consumption; 2 Consumption; Source: WHO. WHO. Guidelines for the Safe Use of Wastewater. Excreta and Greywater in Agriculture. Volume 2. Wastewater Use in Agriculture; WHO Press: Geneve, Switzerland, 2006.

Table 3. Identification and valuation of externalities



Groups	Externalities	
	Identification	Unit
Water infrastructure	Avoids constructing facilities to capture and store freshwater	€
	Avoids water purification costs	€
	Avoids constructing pipes and water distribution costs	€
Reuse of pollutants	Reuse of nitrogen in agriculture	kg of N
	Reuse of phosphorous in agriculture	kg of P
	Reuse of sludge in agriculture and gardening	kg
	Reuse of thermal energy	Watt
Uses of the resource	Increases the quantity of water available	m^3
	Guarantees supply in times when there is a shortage	% confidence
	Water quality adapted to different uses is obtained	kg waste
Public Health	Biological risks associated to wastewater reuse	People exposed
	Chemical risks associated to wastewater reuse	People exposed
Environment	Increase in the level of rivers	m^3
	Avoids overexploitation of water-bearing resources	Aquifer level, m
	Avoids water pollution	Waste eliminated, kg
	Allows wetland and river habitat to be recovered	Users
	Increase in pollution due to smell and noise	Number of people
	Decrease in the value of land nearby	€
Education	Raises social awareness of a new water culture	Number of people

Source: Hernandez et al., 2006.

CHAPTER II

BENEFICIAL EFFECT OF SECONDARY TREATED WASTEWATER IRRIGATION ON NECTARINE TREE PHYSIOLOGY

2.1. Introduction

Agriculture is the mostly threatened sector by the effects of climate change, due to the rise of temperature and the decrease in precipitation frequency, occurring especially during the summer period, when evapotranspiration requirements are higher and water is needed for irrigation. For this reason, the use of treated wastewater as an alternative source for irrigation represents a viable strategy to avoid water scarcity during the warmest periods of the season, as a support to traditional irrigation management, not only in arid and semi-arid environments but also in areas affected by occasional water shortages.

Beside its irrigation value, treated wastewater could have a positive effects on soil and plant nutrition, improving soil fertility (soil structure; water holding capacity; carbon storage) and reducing the need for mineral fertilization, due to its intrinsic richness in organic matter and mineral elements such as N, P and K (Khurana and Singh 2012). On the other hand, treated wastewater may imply environmental risks, both in terms of soil pollution (heavy metals contamination), safety of harvested crops (presence of potential bacterial human pathogens) and social acceptance (Muchuweti et al. 2006; Bernstein 2011; Fatta-Kassinos et al. 2011). In addition the reiterate use of salt-rich treated wastewater may increase the risk of salinization, in particular due to Na and Cl accumulation, which can compromise the growth and yield of cultivated plants (Dridi et al., 2017; Hapeshi et al., 2014; Lü et al., 2012; Ben Mahmoud et al., 2006).

Normally, wastewater is subjected to several cleaning processes before its reuse but it still maintains a certain level of toxic elements and microorganisms, with potential negative effects on soil quality, crop and human safety (Christou et al. 2014). Despite that, the use of treated wastewater in fruit crops has already been successfully applied on species cultivated in drought environments like *Olea europaea* (Petousi et al. 2015), mandarin trees (*Citrus clementina*) (Pedrero et al. 2013), lemon trees (*Citrus limon*) (Pedrero and Alarcon. 2009), nectarines (*Prunus persica*) (Vivaldi et al. 2013), grapevines (*Vitis vinifera*) (Mendoza-Espinoza et al. 2008). Most of the studies regarding water reuse for irrigation investigate the use of tertiary treated wastewater (TTW), currently, the maximum level of cleaning treatment used for wastewater recovery, and are mainly focused on pollutants contamination aspects, mainly investigating soil chemical and microbiological proprieties, leaf mineral status, productivity (Christou et al. 2014; Pedrero et al., 2015; Nicolàas et al., 2016; Nicolàs et al., 2017), fruit quality and fruit safety (Pedrero et al. 2012; Pedrero et al., 2018). The main conclusions from these studies are that tertiary treated wastewater (TTW) can be used as an additional water resource for tree irrigation in water-scarce Mediterranean environments.

From the legislative point of view, the European Union still misses a general agreement about the maximal pollution thresholds allowed for the use of treated wastewater in agriculture and different regulations are applied in each country. In Italy, for example, very low pollution thresholds are allowed for the use of treated wastewater in agriculture (Italian Legislative Decree No. 185/03) and no discrimination in the pollutant thresholds is foreseen for the different cultivated crops (BIO by Deloitte 2015). For this reason, it is necessary to investigate on the consequences deriving also from the use of STW, which is still not allowed in Italy for irrigation purposes. STW is currently the treated wastewater resource which is mostly available in Italy (https://www.istat.it/); and is already characterized by good intrinsic parameters for irrigation purposes, thus potentially representing a water resource with a high benefit/cost ratio.

The STW water resource could then be applicable for the irrigation of crops whose edible part is not wet by irrigation to avoid any microbiological contamination of the final product.

In addition, the potential nutritional effect of STW is still underestimated, as it could lead to significant fertilizer savings due to its high level of macronutrients (Chen et al. 2008) and micronutrients. STW application might ensure the transfer of fertilizing elements, such as nitrogen (N), phosphorous (P), potassium (K), organic matter as well as micro-nutrients into agricultural soils (WCED 1987). This fertilizing potential is partially lowered for TTW, where the levels of nutrients, especially nitrogen and phosphorous, are significantly reduced by the cleaning treatment (Pescod, 1992).

Further studies are needed to optimize the levels of water filtration on STW in order to maintain the potential nutritional value of treated wastewater, while guaranteeing the chemical and microbiological safety of the harvested products.

The majority of the studies on treated wastewater irrigation have focused mainly on reclaimed urban effluents, especially TTW. However, research on the physiological performance of tree crops irrigated with STW is still lacking as studies have been conducted just on TTW and only on some species like mandarin (Pedrero et al. 2014; Nicolás et al. 2016; Nicolàas et al., 2017) and grapefruit (Romero-Trigueros at al., 2014; Pedrero et al., 2013; Paudel et al., 2018).

The aim of this work is to investigate the potential physiological and nutritional effects of using STW (treated according to the Italian Legislative Decree No. 152/06) as an alternative irrigation source on young nectarine trees, which is one of the main cultivated fruit species worldwide. The use of STW could thus represent an opportunity to mitigate water shortage in summer time and a rational approach for reducing fertilizer use, which would otherwise be wasted in the environment, preventing potential contamination of both surface and groundwater (Asano, 1998).

2.2. Material and Methods

2.2.1. Experimental set up

The trial was carried out during two subsequent years: 2016 and 2017, on 15 potted nectarine trees (*Prunus persica*; cv. Big Top, grafted on GF 677) from May to the beginning of September. The experiment was set up outdoors, under a shading 20% hail net during summer, at the experimental farm of the University of Bologna located in Cadriano (Bologna) (44°33′00.09″N, 11°23′37.81″E) in the Emilia-Romagna region. Two years old tree seedlings were grown in 15 identical 40L pots filled in with sandy loam soil (United States Department of Agriculture classification) with the following characteristics: sand 60.9%; loam 26.6%; clay 12.5%; carbonates 199.8 g kg^{-1} and arranged in 3 rows, spaced 0.6 m, with the plants at a distance of 1.0 m apart along each single row. During the growing seasons, standard agronomic practices for fruit crops in the area were performed.

Starting from 65 days after full bloom (DAFB; full bloom: March, 20th), three irrigation treatments were applied, each to 5 plants: 1) irrigation with secondary treated wastewater (STW) 2) irrigation with tap water plus an addition of 14.2, 2.35, 8.96, and 0.72 g $tree^{-1}$ of N, P, K and Mg, respectively as commercial mineral fertilizers, split in 3 interventions from 65 DAFB (TW+MF) (in the first season fertilizer amount was equal to: 8.5, 2.1, 8.21 and 0.57 g $tree^{-1}$ of N, P, K and Mg respectively) and 3) irrigation with tap water without any addition of fertilizer (TW). The secondary treated wastewater (regulated by the Italian Legislative Decree No. 152/06) came from the close urban wastewater treatment plant of Granarolo (Bo), managed by HERA S.p.a (Italian multi-utility). The water is then ready to overflow from the treatment plant. In this study, the STW was transported and stored for a maximum of 15 days to the experimental site through a 1 m^3 tank and subsequently used for irrigation. Treatments were assigned according to a randomized block design. A drip irrigation system with four drippers per pot, at 2 L h^{-1}flow rate each, was used for the irrigation. The amount of water applied to each treatment during the whole crop cycle was 240 L tree $^{-1}$ on the first year and 360 L tree $^{-1}$ on the second year, with the water volume at each irrigation varying from 1.5 L tree $^{-1}$ to 4 L tree $^{-1}$, two times per day, depending on ETc.

Etc for the plants used in the experiment was calculated according to the Irrinet irrigation scheduling system, developed and made available over the Internet by the "Consorzio per il Canale Emiliano Romagnolo (CER)" of the Emilia-Romagna Region (www.irriframe.it). The environmental parameters needed as inputs by this web-based platform were obtained from the experimental farm weather station.

2.2.2. Water analysis

For each irrigation treatment, water samples were collected every two weeks, both in 2016 and 2017, in order to characterize the water quality (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 macronutrients, micronutrients and heavy metals were determined by Inductively Coupled Plasma (ICP-OES, England); pH was measured with a pH-meter XS PH510 (Eutech Instruments, Singapore); EC was determined using the METERLAB, CDM 210 (Radiometer Analytical, France); TOC was measured with the following instrumentation: Shimadzu TOC-V CPN. The sodium absorption ration (SAR) was calculated using the following equation (with concentrations in meq L^{-1}) (Richards 1954): SAR = $[(Na^+)/ [(Ca^{2++} Mg^{2+})/ 2] 1/2$.

Microbiological analysis were performed on STW samples on *E. coli* and *Salmonella* spp., by the membrane filtration method. Triplicate aliquots of 100, 10, 1.0 and 0.1 mL of each water sample were filtered through 0.45 μm pore sized (47 mm diameter) nitrocellulose membranes (Whatman, Maidstone UK). For *E. coli* enumeration, the membranes were placed onto Chromocult ES (VWR) agar and incubated at 37 °C for 24 h. The same water samples were also analyzed for *Salmonella* spp., with their detection performed according to procedure UNI EN ISO 19250:2013.

2.2.3. Tree nutritional status

During mid-summer 2017, ten mature leaves per tree were collected from randomly selected annual shoots. Petioles were removed, leaves were treated as described in Sorrenti et al. (2012)

and then analyzed for macro-micronutrients and heavy metals concentration. Briefly, P, K, Ca, Mg, S, Fe, Cu, Zn, B, Cu, Fe, Na, Zn were determined by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES, Ametek Spectro Arcos EOP, Kleve, Germany), after digestion with nitric acid (HNO_3) by a microwave lab station (Ethos TC-Milestone, Bergamo, Italy), while N was determined by the Kjeldahl method. The same analyses were performed at commercial harvest (i.e. 124 DAFB), on 4 representative fruit per replicate, to determine peel and pulp mineral concentrations.

2.2.4. Allometry measurements

Basal diameter and length of 3 lateral shoots per tree, randomly chosen, were recorded at regular time intervals during the two seasons. Shoot diameter measurements were taken using a digital caliper provided with an external memory (http://www.hkconsulting.it/) while shoot length was assessed using a classical measuring tape. Based on shoot diameter data, it was possible to calculate the Stem Cross Sectional Area (SCSA) for each shoot.

Trunk diameter (measured 5 cm above the grafting point) was collected for each tree at the beginning (70 DAFB 2016), in the middle (50 DAFB 2017) and at the end (170 DAFB 2017) of the trial. Based on the trunk diameter it was possible to calculate the Trunk Cross Sectional Area (TCSA).

2.2.5. Water relations

Leaf and stem water potentials were monitored at 72, 106, 127, 161 DAFB in 2016 and at 93, 132 and 167 DAFB in 2017. On each date, measurements were performed at 5.00, 9.00, 12.00 and 16.00 hour in 2016 and at 12.00 hour in 2017, using a Scholander pressure chamber (Soilmoisture Equipment Corp., Santa Barbara, CA, USA). Leaf water potential was measured on one well exposed leaf per tree (5 leaves per treatment) following the recommendations of Turner and Long (1980). Similarly, stem water potential was measured on one leaf per tree which was previously covered with aluminium foil and placed in plastic bags for at least 90 minutes, to allow equilibration with the stem, according to the methodology described by McCutchan and Shackel (1992) and Naor et al. (1995).

2.2.6. Leaf gas exchanges

Leaf net photosynthesis (A) and transpiration (E) were determined at about 9.00, 12.00 and 16.00 hour on the same day when water potentials were recorded, using a portable gas analyser (Li-COR 6400, LI-COR, 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 (5 leaves per treatment). During each measurement, light intensity inside the cuvette was maintained constant during the measurement by setting the level of light at the incident light level as recorded by the PPFD sensor immediately before the measurements.

Cumulative daily photosynthesis (∑A) and transpiration (∑E) (from 9:00 to 17:00) were also calculated, as described by Losciale et al. (2010) and Cano et al. (2014) using the following equation:

$$\sum_{y} = \sum_{i=t0}^{t1} (\frac{(yto + yt1)}{2})i + \sum_{i=t1}^{t2} (\frac{(yt1 + yt2)}{2})i \qquad (1)$$

where y is the variable A or E, t_0 corresponds to 9:30, t_1 to 13:30 and t_2 to 16:30.

For each plant, cumulative daily photosynthesis (∑A) and transpiration (∑E) were then multiplied by total leaf area, averages were then calculated for each treatment.

To estimate total leaf area, at the end of the season, each plant was defoliated while all leaves were weighted. A leaf sample of 20 g per tree, replicated three times, was collected and weighted, while its leaf area was determined using a leaf area meter (LI-3000 A, LI-COR, Lincoln, Nebraska, USA). A correlation between leaf weight and leaf area was then built among the leaf samples (R^2 =0.994) and was used to calculate the total leaf area per tree.

2.2.7. Fruit growth, yield and quality

The diameter of 8 fruit per treatment, randomly chosen, was recorded at regular time intervals during the season 2017, at: 77, 85, 93, 100, 111 and 121 DAFB, using a digital caliper provided with an external memory (http://www.hkconsulting.it/).

At 124 DAFB, final yield (i.e. fruit $tree^{-1}$) was assessed for each treatment and the vegetative/reproductive ratio was calculated by dividing the tree leaf area (m^2 $tree^{-1}$) by the number of fruits for each tree.

The main fruit quality parameters (dry matter content, firmness, soluble solid content, color lightness, chroma (a*), chroma (b*)) were assessed on 12 fruit per treatment at the physiological ripening stage. Colorimetric analysis were performed using a Minolta CR-400 (Konica Minolta Sensing Americas, Inc, USA), assessing the classical parameters for skin fruit colour: 'L', 'a*' e 'b*'. 'L' showing the lightness, 'a' showing the shades from the green (negative value) to the red (positive value) and ‘b’ showing the shades from the blue (negative value) to the yellow (positive value). Fruit firmness was assessed thanks to the 53220 FTA Fruit Texture Analyser (T.R. Turoni srl, Italy). Soluble solids content was determined by a digital refractometer (ATAGO CO., LTD, Japan). Fruit dry matter content was determined on fruit slices which were dried at 65°C for several days and weighted with a precision Mettler scale, Model PE3600 (METTLER TOLEDO LLC, USA).

2.2.8. Statistical analysis

SCSA, shoot length and fruit seasonal growth were analysed using a linear mixed model function.

Fruit quality, fruit yield, vegetative/reproductive ratio, TCSA, daily leaf and stem water potential and daily leaf gas exchanges were compared among treatments using a one-way ANOVA analysis followed by a Tukey HSD test. Analyses were carried out using R software (www.r-project.org). The same software was used for creating graphs.

Data of tissues mineral concentration were instead analyzed as in a randomized block design and when analysis of variance showed a statistical effect, means were separated by the SNK Test using SAS 9.0 (SAS Institute Inc., Cary, NC, USA). Statistical significance was established for $P < 0.05$. For each data means and standard error (SE) were calculated.

2.3. Results

2.3.1. Water quality

The quality of the two water sources was significantly different in NH_4, NO_3, P, K, Ca, Mg, S, Na, Cu, Fe, B, Zn and TOC concentrations as well as pH, EC and SAR (Tab. 1). The values of these parameters were significantly higher in the STW than in the TW (Table 1). The average NO_3-N, P, and K concentrations detected in STW were almost 4, 100 and 5 times higher than the average concentrations measured in TW, respectively. The electrical conductivity over the experimental period was 1.21 in STW and 0.47 dS m^{-1} in TW. The SAR index was 1.85 in STW and 0.63 in TW. *E. coli* average concentration in STW was equal to 4 CFU 100 mL^{-1}. No Salmonella was detected in any of the STW samples.

2.3.2. Tree nutritional status

Treatments significantly affected tree nutritional status, with stronger effects on leaves than on fruit tissues (Tab. 2). N concentration in TW leaves was below the optimal threshold (Toselli et al., 2006) (Tab. 2), while the overall mineral concentration in TW+MF and STW leaves were in the optimal range for this nectarine variety (Sorrenti et al., 2016). Moreover, despite the larger canopy size and the higher amount of fruit tissue (peel and pulp), N concentration was statistically increased by the supply of mineral N from TW+MF, with intermediate values recorded in the leaves and fruit peel of trees irrigated with STW (Tab. 2). Among micronutrients, mineral fertilization slightly reduced leaf B concentration and increased that of Cu, while, regardless of the treatment, no effects emerged on leaf Fe, Mn, Na and Zn concentrations (Tab. 2). The same trend was observed in the fruit peel and fruit pulp for B concentration (Tab. 2). Notably, heavy metal concentration in leaves and fruit tissues was statistically comparable among treatments, except for Zn, which was higher only in the fruit peel of STW trees

2.3.3. Vegetative growth

In 2016, shoot length (Fig.1a) showed an initial fast increase, especially for TW+MF and STW treatments, reaching a steady state at about 100 DAFB. TW+MF trees reached their maximum shoot length very early in the season, at 97 DAFB, with an average value of 54.8 cm. STW shoots were characterized by a similar growth pattern, with a more gradual increase and reached a plateau 25 days later, at 122 DAFB, with an average shoot length of 42.0 cm. Instead, shoots on TW trees were always shorter than on TW+MF and STW trees, showing a limited and slow growth during the whole season and reaching a maximum length of 18.8 cm. A similar pattern was recorded in 2017 (Fig.1b), but, in this case, almost no differences were detected between TW and STW trees. In 2017, a delay in reaching the maximum shoot length values compared to 2016 was also recorded for all treatments (19.8 cm at 110 DAFB for TW+MF, 14.4 cm and 8.5 cm at 145 DAFB for STW and TW treatments, respectively).

In 2016, the seasonal SCSA growth pattern (Fig. 1c) showed an almost constant increase for TW+MF and STW trees, reaching values of 44.47 and 33.48 mm^2, respectively, at 191 DAFB. TW trees showed a completely different pattern as their SCSA increased very slowly during the season, with a final value of 13.38 mm^2. TW+MF and STW trees SCSAs were statistically higher than TW during almost all the season. In 2017, SCSA seasonal pattern (Fig. 1d) showed a linear increase followed by an exponential trend in the final part of the season, for all treatments. Higher SCSAs were recorded in TW+MF trees compared to STW trees, from 85 to 145 DFAB, but no differences were recorded at 174 DAFB, with values of 16.31, 11.95 and 12.55 mm^2 for

TW+MF, STW and TW trees, respectively.

TCSA measurements performed at 70 DAFB-2016, at 50 DFAB-2017 and at 170 DAFB-2017 showed how TW+MF trees maintained larger trunks while STW trees maintained intermediate values during the whole trial, although at the beginning no statistical difference was detected among treatments. TCSA average values at the end of the experiment were indeed: 255, 490 and 360 mm^2 for TW, TW+MF and STW trees, respectively (Fig. 2).

2.3.4. Water relations

In 2016, leaf and stem water potentials showed the same decreasing pattern from early morning to midday on all dates of measurements. Instead, from midday to afternoon they followed an opposite trend with values becoming more positive for the leaves and more negative for the stem (Fig. 3). This trend was similar on all dates of measurement except for the stem at 127 and 161 DAFB, where STW trees started to recover in the afternoon, and for the leaves at 72 and 161 DAFB when a higher water potential with a faster recover in the afternoon was shown for the control treatment.

Few differences were detected among treatments, especially in the morning, at 106 and 127 DAFB, when TW+MF trees showed lower stem and leaf water potential values, especially during the afternoon.

In 2017, TW and STW were not different in terms of stem water potentials during all the season (Fig.4a), while, at 93 and 132 DAFB, the TW+MF treatment showed more negative values, with appreciable statistical differences compared to TW and STW trees. Instead, midday leaf water potential (Fig. 4b) showed an increasing trend at the beginning (from 93 to 132 DAFB), followed by a decreasing pattern at the end of the season (from 132 to 167 DAFB). The only significant differences for leaf water potential were detected at 93 DAFB, between TW and TW+MF treatments.

2.3.5. Leaf gas exchanges

In all 2016 dates of measurements, except at 127 DAFB, leaf photosynthesis showed a morning increase, reaching a peak around midday, followed by an afternoon decrease. A similar pattern was recorded for transpiration, in almost all dates of measurement, except at 72 DFAB (Fig. 5). At 127 DAFB, leaf photosynthesis showed a decreasing pattern from 9:00 hour on, with statistical differences between treatments at 09.00 and 12.00 hour, with higher values recorded for STW trees, followed by TW and finally by TW+MF. On the same date, transpiration followed the typical daily pattern, with a peak at midday, followed by stomata closure in the afternoon, for TW and STW treatments. TW+MF trees showed a different behaviour, keeping very low transpiration values (lower than 2 mmol H_20 $m^{-2}s^{-1}$) during the whole day. At 127 DAFB, TW trees always maintained higher transpiration values than TW+MF, at all hours of measurements.

In 2017, leaf photosynthesis and transpiration showed the same trend for TW and STW treatments (Fig. 6a,b). This pattern was characterized by 2 positive peaks at 93 DAFB and 167 DAFB and a negative peak at 132 DAFB. The TW+MF treatment did not show any positive peak at 93 DAFB, maintaining low values of photosynthesis and transpiration during the whole season. Statistical differences appeared at 93, 132 and 167 DAFB mostly for TW+MF trees which maintained lower photosynthesis than TW and STW trees.

Cumulative daily photosynthesis for the whole canopy estimated at the end of the 2017 growing season highlighted differences among treatments (Fig.7a). Indeed, TW+MF trees reached the highest daily CO_2 assimilation with 20.68 g d^{-1}, then followed by STW that reached 10.84 g d^{-1} and eventually by TW trees with 5.37 g d^{-1}. Each treatment was significantly different from each other. The same pattern was shown by daily transpiration, although in this case no statistical differences between TW and STW trees appeared (Fig.7b).

2.3.6. Fruit growth, yield and fruit quality

The seasonal pattern of fruit diameter showed the same trend for all treatments, with reduced growth for TW and TW+MF trees, at 93 and 100 DAFB (Fig. 8). The STW treatment kept highest values in fruit dimeter for almost all the season, if compared to the other two. Statistical differences were recorded at 85, 93, 100, 121 DAFB mainly between the TW and STW, but no differences were detected at 111 DAFB. The total fruit yield was more than doubled for the TW+MF and STW if compared to the TW. The average tree fruit number was indeed 17.2 fruit $tree^{-1}$ ± 2.9, 16.7 fruit $tree^{-1}$ ± 3.0 and 7.6 fruit $tree^{-1}$± 1.1 for the TW+MF, STW and TW treatments, respectively.

At the end of the 2017 season, TW+MF trees exhibited the highest vegetative/reproductive ratio (0.11 m^2 $fruit^{-1}$ ± 0.019) compared to the much lower values of STW (0.05 m^2 $fruit^{-1}$ ± 0.007) and TW (0.02 m^2 $fruit^{-1}$ ± 0.002).

The fruit quality analysis showed how the irrigation treatments affected in a consistent way almost all traits (dry matter, firmness and soluble solids content) (Fig. 9). TW+MF fruit always showed the highest values for these parameters, while TW and STW fruit showed similar values. For all the others parameters, fresh weight, chroma (a*), chroma (b*) and lightness (L) no statistical difference among treatments was recorded.

2.4. Discussion

The chemical features of the STW and TW waters, measured during the experiment, revealed several differences in their composition. STW was characterized by a higher concentration for almost all fertility elements like N, P, K, Ca, Mg, and organic matter. This natural richness in mineral elements represents an important and free source of nutrients for improving soil fertility, plant growth, crop yield and preventing lack of macro and micro-nutrients. The availability of these elements could allow a significant reduction in fertilizer application while still meeting tree nutrient requirements, as it has been reported from other studies on fruit trees (Vivaldi et al. 2017; Pedrero et al. 2012). In our experimental conditions, the supply of STW as irrigation source, allowed to save 28.3, 49.0 and 77.7% of N, P and K, respectively, compared to TW+MF. In any case the potential nutrient supply is highly heterogeneous among treated wastewater sources, depending mostly on the quality of the water row arriving to the treatment plant and to the reclaiming process (Levy et al., 2011). For these reasons, fertilizer savings can highly differ among treated wastewater sources.

Even if STW showed higher pH, SAR, EC, heavy metal (e.g. Zn, Fe and Cu) contents and *E. coli* load than TW (Table. 1), it did not exceed the Italian thresholds for reclaimed water for agricultural purpose (Ministerial Decree no. 185/2003). These data highlight how this STW could be safely used also on crops that are considered salt stress sensible, such as nectarine, without showing any phytotoxic effect or salt stress sensibility (Ayers and Westcot, 1985; Maas and Grattan, 1999). The low salinity effects recorded in our trial could be due both to the low EC values of STW, compared to other treated wastewater sources (Qian and Mecham, 2005)), and to the soil properties (e.g. soil texture, pH, $CaCO_3$ content) that probably reduced the possible soil salinization and heavy metal soil accumulation. However, as our trial was carried out for only two years we cannot exclude the possibility for long term accumulation of salt in the soil, when treated wastewater is applied for several years, in open field conditions.

Concerning tree nutritional status, STW could not reach the TW+MF level, even though it provided a higher amount of macro and micro-nutrients, either in inorganic or organic forms, compared to TW. This is confirming that nectarine is a high-demanding species for N and that N delivered exclusively by STW was not enough to satisfy tree requirements. On the contrary, leaf P, Ca, Mg and B concentrations significantly decreased in TW+MF trees. This was likely due to the dilution and partitioning effect induced by a larger vegetative biomass. This is in line with the highest leaf P, K, Ca, Mg and B concentration recorded in TW trees (Tab. 2), which seems related to their reduced canopy growth and lower crop load. These data indicate how STW irrigation may contribute to a partial fulfilment of plant nutrient requirements. However, beside providing water, the amount of nutrients supplied by STW could be substantial and must be taken into consideration in the fertilization schedule. Moreover, the fruit heavy metal concentrations were within the thresholds set for heavy metal by the FAO regulation for contaminants and toxins in food and feed (FAO/WHO Codex Alimentarius, 2003), even for Zn which was the most present heavy metal. This result is particularly important for peach fruit which is known to maintain a functional xylem until harvest (Morandi et al., 2007) and it represents a further confirmation towards the potential use of STW for fruit-crops irrigation.

The application of treated wastewater to nectarine trees positively affected vegetative growth during the season in terms of both shoot length and SCSA. In particular, shoot growth data recorded in 2016 (Fig.1a,c) were not reduced in trees irrigated with STW compared to TW+MF trees, thus highlighting the positive nutritional effect of this alternative source for irrigation STW-irrigated plants showed a more balanced shoot growth, with a lower source/sink ratio compared to TW+MF trees, likely due to the fertilization contribution provided by the STW. A similar response for both shoot length and SCSA was recorded in 2017 (Fig. 1b, d), although this season was characterized by a lower vegetative growth, compared to the previous year, for all treatments, being potted plants already at their third leaf. The good performances of TW+MF and STW trees in terms of vegetative growth were confirmed by TCSA data (Fig. 2), representing a typical and reliable index for estimating tree growth, during both years.

In 2016, predawn stem water potential (Fig. 3) did not show any differences among treatments and no negative effect of STW irrigation was detected in STW trees, at any time during the season (161 DAFB). This result suggests how the possible higher values of salt added to the soil by STW did not cause any osmotic stress, even after 4 months of continuous irrigation (Fig.3). Similar results were achieved on mandarin trees irrigated with a tertiary treated wastewater TTW by Nicolás et al. (2016). However, during the day, both stem and leaf water potentials occasionally showed more negative values for TW+MF and STW treatments, starting from 106 DFAB. This behaviour could be mainly attributed to the higher availability of nutrients, in both treatments, which might have decreased the osmotic potential of the leaf tissues. In 2017, stem water potential (Fig. 4) confirmed the trend recorded during the last dates of 2016, with TW+MF trees showing reduced midday values, below -1.5 MPa, thus indicating a drought stress condition. This effect can be attributed to the higher vegetative growth recorded during this season in TW+MF trees, which caused a higher water demand, despite water availability was guaranteed to all trees during the trial. The lower water potentials recorded in TW+MF trees might also be caused by the addition of fertilizers few days prior measurements, which temporarily increased soil salinity, limiting plant water uptake This probably increased the soil osmotic potential, with a further negative impact on stem WP, that became more negative compared to TW and STW trees. The same trend appears also for leaf WP, although in this case a higher variability is recorded within each treatment, especially at 167 DAFB. In open field conditions and commercial environment, this possible salinity effect might be buffered by the wider soil space explored by the roots and by the bigger tree size, however further studies are necessary to asses possible salinity effects deriving from long term irrigation with treated wastewater.

As for leaf gas exchanges, no limitation in CO_2 assimilation and evapotranspiration were recorded during the first half of the season in 2016 (Fig. 5), indicating no phytotoxic effect induced by irrigation with STW. Indeed, above threshold concentrations of B (Gimeno et al. 2012) and Na (Navarro et al. 2011) in the soil solution can lead to stomatal closure, which in turn may reduce transpiration rate and photosynthesis (García-Sánchez and Syvertsen 2006). Similar and positive results were achieved by Nicolás et al. (2016) on mandarin trees. However, as a consequence of their reduced water potentials, TW+MF trees showed a reduction in both A and E at 127 DAFB indicating the presence of some water stress.

However, in 2017, TW+MF trees are the ones showing the best performance in terms of total amount of CO_2 assimilated (Fig. 7), although this result is mainly related to their highest leaf canopy area. It is interesting to notice how STW trees still maintain a higher assimilation capacity compared to TW, due to the beneficial nutritional effect of irrigation with STW, with no negative effect in vegetative growth and water potentials. As a result of this optimal balance between vegetative and reproductive sinks, STW trees showed the highest fruit growth rates during the season (Fig. 8) and final yield comparable to TW+MF trees. This is particularly interesting from the commercial point of view as size is currently the only quality trait that is paid to the grower. Besides, STW fruit maintained similar quality levels in terms of dry matter percentage, firmness and colour than what recorded for TW fruit, but increased their soluble solids content, indicating an overall positive effect of STW irrigation on nectarine fruit quality (Ahmed et al. 2009). On the contrary, TW+MF trees showed reduced fruit growth rates during the season. This must be attributed to the higher competition with the vegetative growth, as a consequence of the higher vegetative/reproductive ratio compared to STW and TW trees. A further reason for limiting fruit growth in TW+MF trees is represented by their midday stem water potential, that were below the thresholds of water stress (Naor et al.1999). Despite the lower fruit size, the presence of stress in TW+MF trees positively affected other fruit quality traits such as dry matter percentage and soluble solids content. This effect must be attributed to the lower stem water potential, which might have reduced xylem flows to the fruit (Morandi et al. 2007) thus increasing the relative contribution of the phloem to nectarine fruit growth.

2.5. Conclusions

This study considers for the first time the physiological, nutritional and productive effects of using STW as an alternative source of irrigation in fruit crops. Results show how STW irrigation for two subsequent seasons did not induce any negative effect on plant leaf water status and gas exchanges, thus suggesting the lack of phytotoxic effects and heavy metal accumulation neither in the vegetative (leaves) nor in the reproductive (fruit) tissues. On the contrary, it induced a positive effect on total canopy assimilation and improved tree source/sink ratio, with positive effects on fruit growth, fruit yield and quality, due to the beneficial nutritional effect of STW at total canopy level.

Indeed, STW could partially contribute to fulfil plant nutrient requirements, even though fertilization must necessarily be integrated by alternative sources (e.g. mineral or organic fertilizers). These positive results suggest how STW could be used as a potential alternative source for irrigation of fruit trees in presence of water scarcity and how it may represent a free source of nutrients, reducing the need of mineral fertilizers and being, at the same time, beneficial also for the environment, limiting eutrophication problems. The use of STW as an alternative source for irrigation is particularly suitable for orchards irrigated with typical drip irrigation systems, as fruit are not wet by irrigation, thus decreasing the risk for external contaminations. However, further studies are necessary to investigate on the potential translocation of pollutants (e.g. human pathogenic bacteria) to the edible tissues in order to guarantee the safety of the fruit harvested. Moreover, interdisciplinary feasibility studies should be conducted in open field orchards for a longer period in order to avoid possible limitations typical of potted trees and evaluate possible long term effects deriving from the continuous irrigation with treated wastewater.

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2.7. Figures and Tables

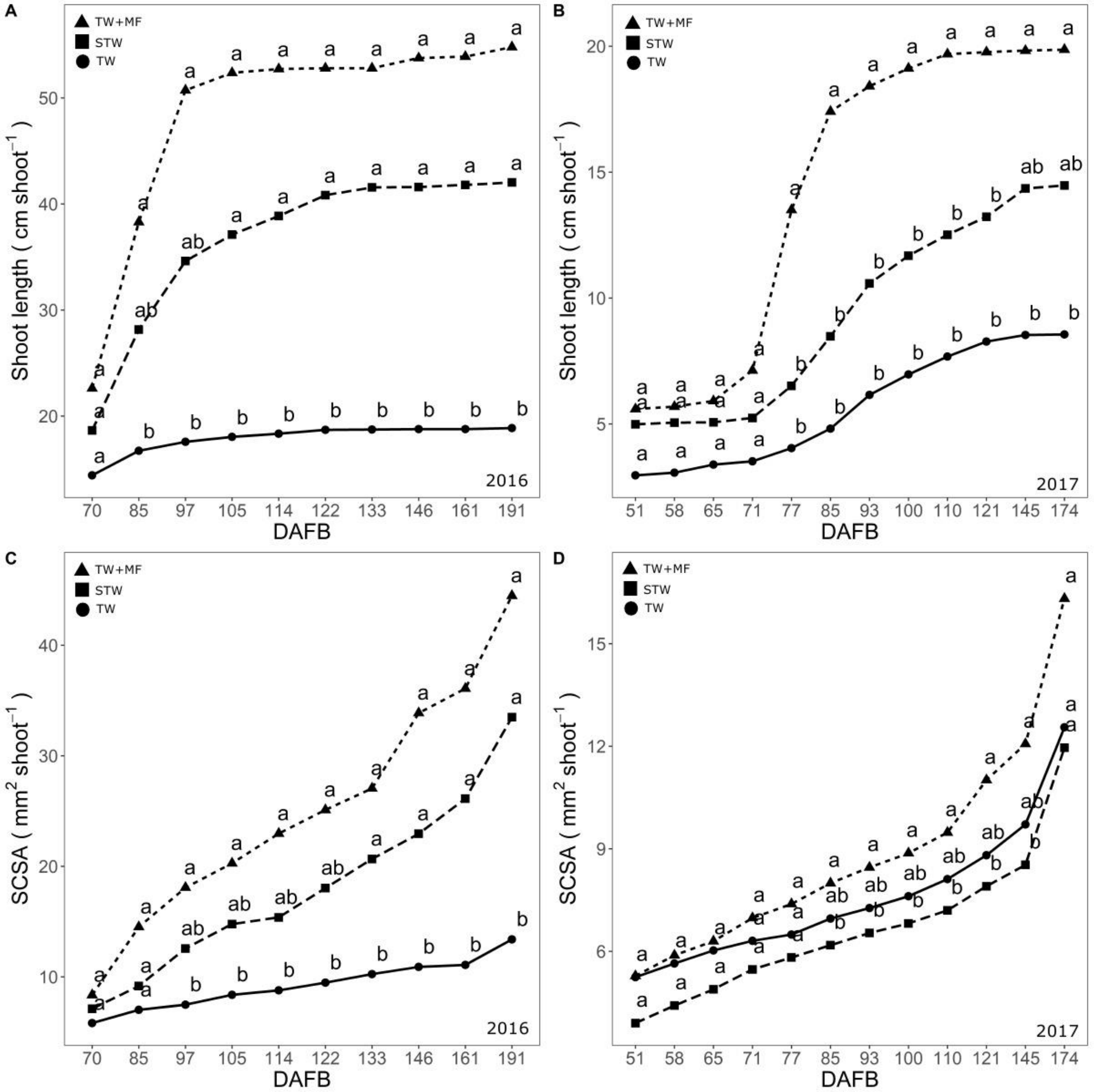


Fig.1. Seasonal mean shoot growth (± SE) (cm shoot^{-1}) (A, 2016; B, 2017) and Stem Cross Sectional Area (± SE) (mm^2 shoot^{-1}) (C, 2016; D, 2017), for TW (circle and continuous line), TW+MF (triangles and short- dashed line) and STW (squares and long-dashed line) treatments. Each value corresponds to the mean of 15 measurements. Different letters indicate significant differences among treatments with a p value <0.05.

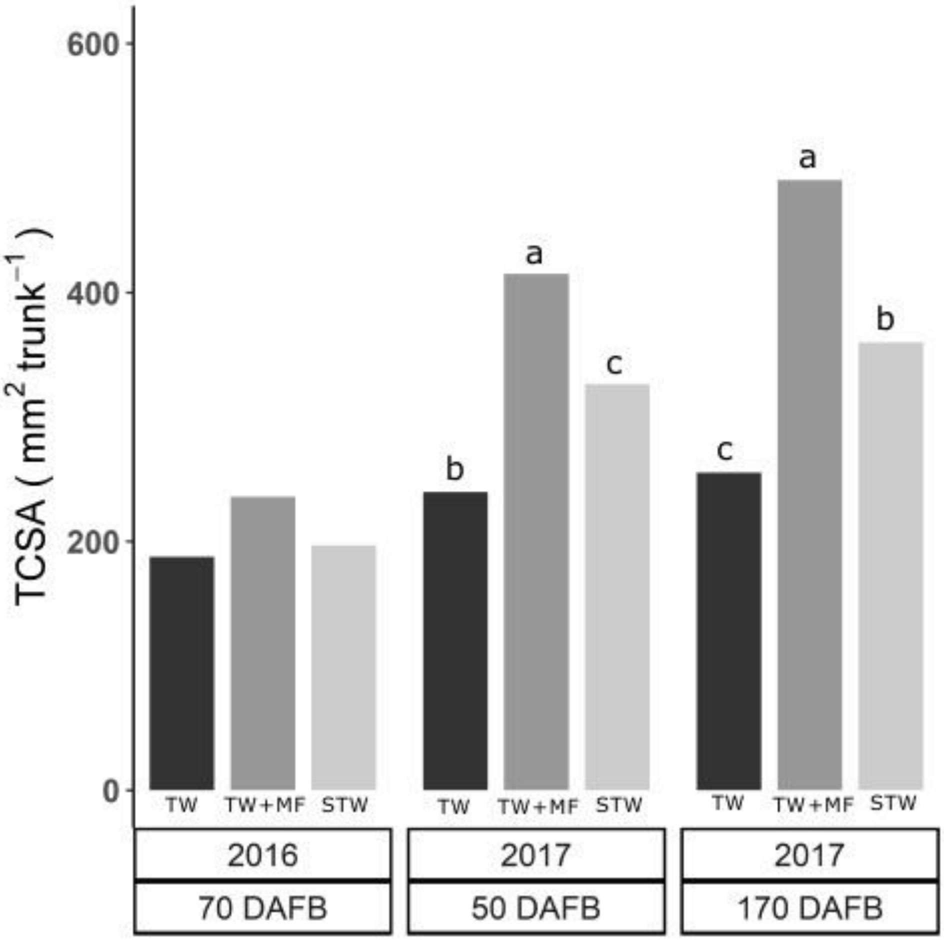


Fig.2. Average Trunk Cross Sectional Area (± SE) (mm^2 trunk^{-1}) at the beginning of the first (70 DAFB) and of the second (50 DAFB) season and at the end of the trial (170 DAFB). Each bar represents the mean of 5 measurements. Different letters indicate significant differences among treatments with a p value <0.05.

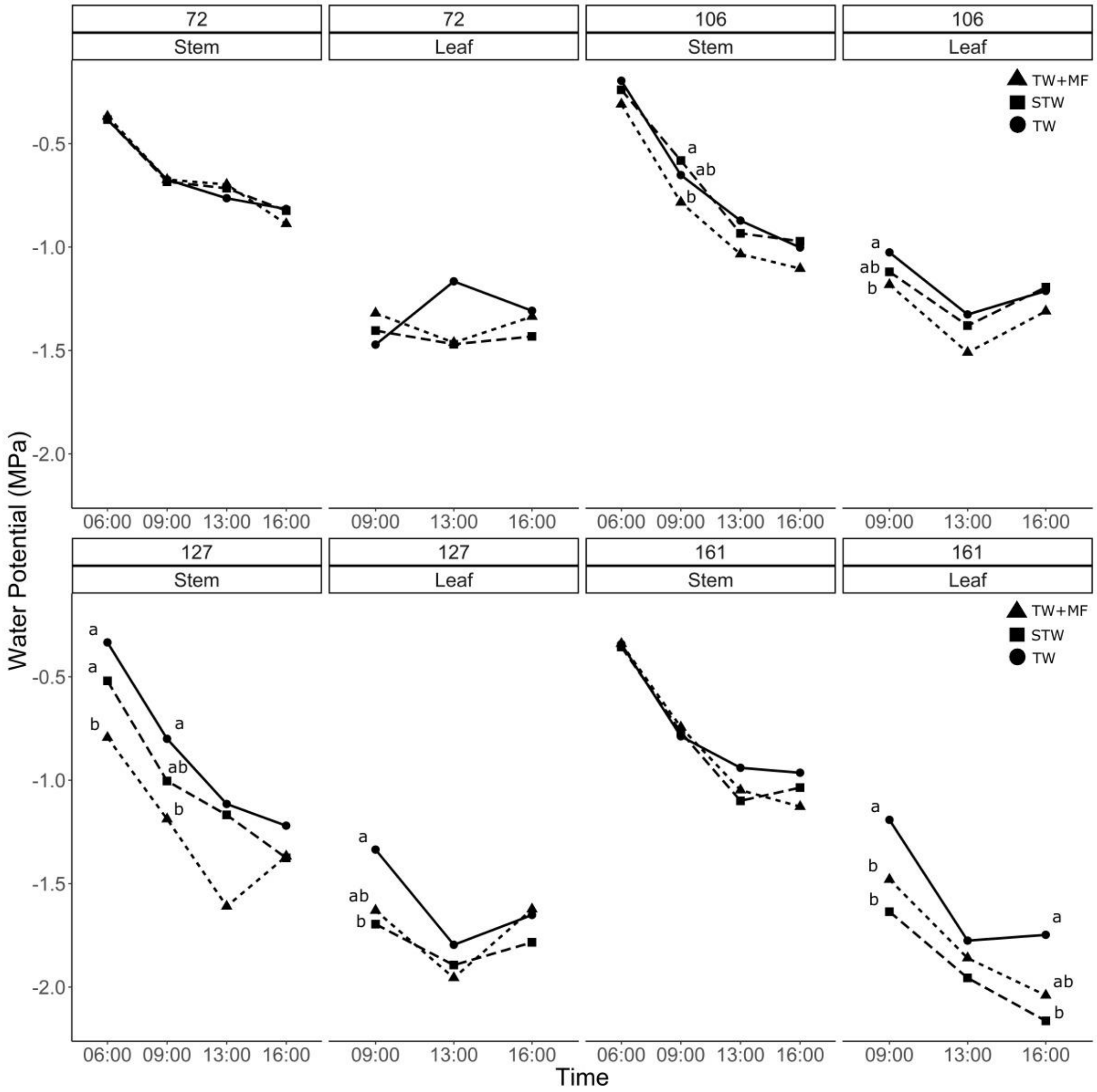


Fig.3. Daily 2016 patterns of Stem Water Potential (± SE) (MPa) (A) and Leaf Water Potential (± SE) (MPa) (B) for TW (circle and continuous line), TW+MF (triangles and short-dashed line) and STW (squares and long-dashed line). Each point represents the mean of 5 measurements. Different letters indicate significant differences among treatments with a p value <0.05.

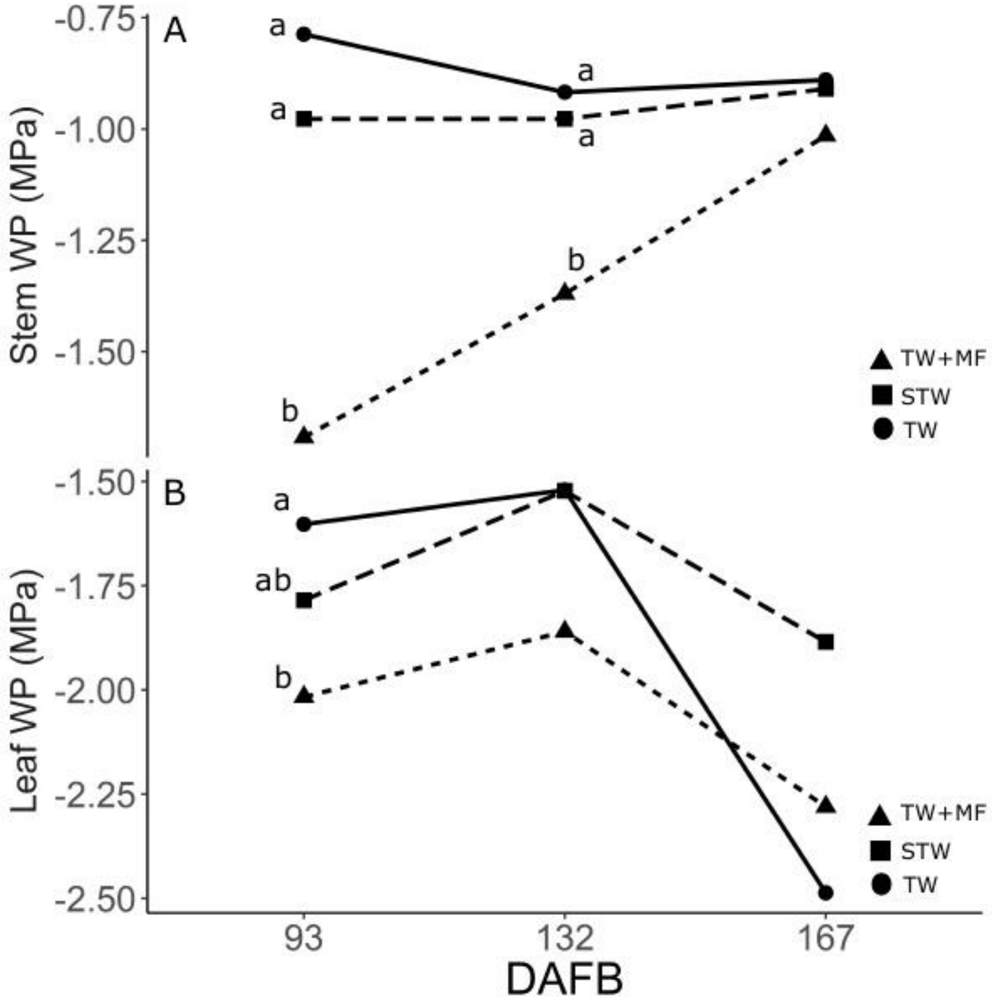


Fig.4. Midday 2017 seasonal pattern of Stem Water Potential (± SE) (MPa) (A) and Leaf Water Potential (± SE) (MPa) (B) for TW (circle and continuous line), TW+MF (triangles and short-dashed line) and WW (squares and long-dashed line). Each point represents the mean of 5 measurements. Different letters indicate significant differences among treatments with a p value <0.05.

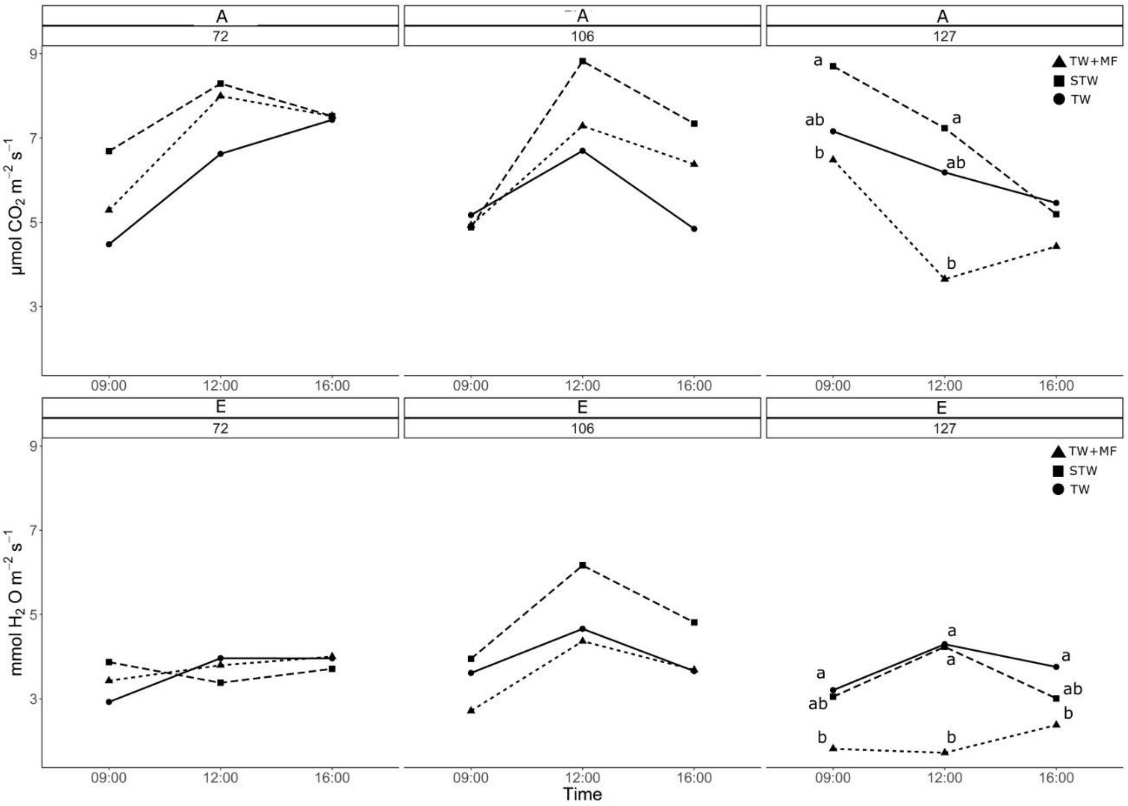


Fig.5. Daily 2016 seasonal pattern of Assimilation (± SE) (µmol CO$_2$ m^{-2} s^{-1}) (A) and Evapotranspiration (mmol H$_2$O m^{-2} s^{-1}) (± SE) (E) for TW (circle and continuous line), TW+MF (triangles and short-dashed line) and STW (squares and long-dashed line). Each point represents the mean of 5 measurements. Different letters indicate significant differences among treatments with a p value <0.05.

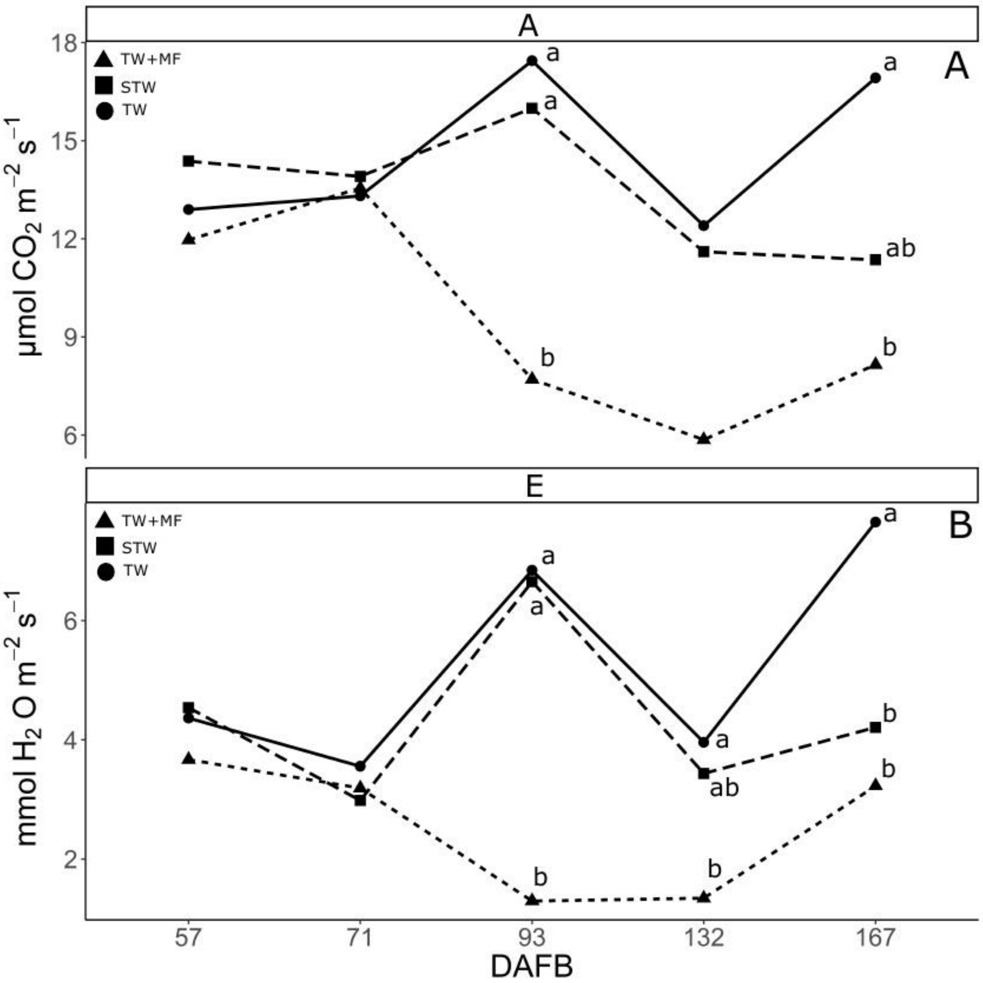


Fig.6. Midday 2017 seasonal patterns of Assimilation (± SE) (μmol CO_2 m^{-2} s^{-1}) (A) and Evapotranspiration (mmol H_2O m^{-2} s^{-1}) (± SE) (E) for TW (circle and continuous line), TW+MF (triangles and short-dashed line) and STW (squares and long-dashed line). Each point represents the mean of 5 measurements. Different letters indicate significant differences among treatments with a p value <0.05.

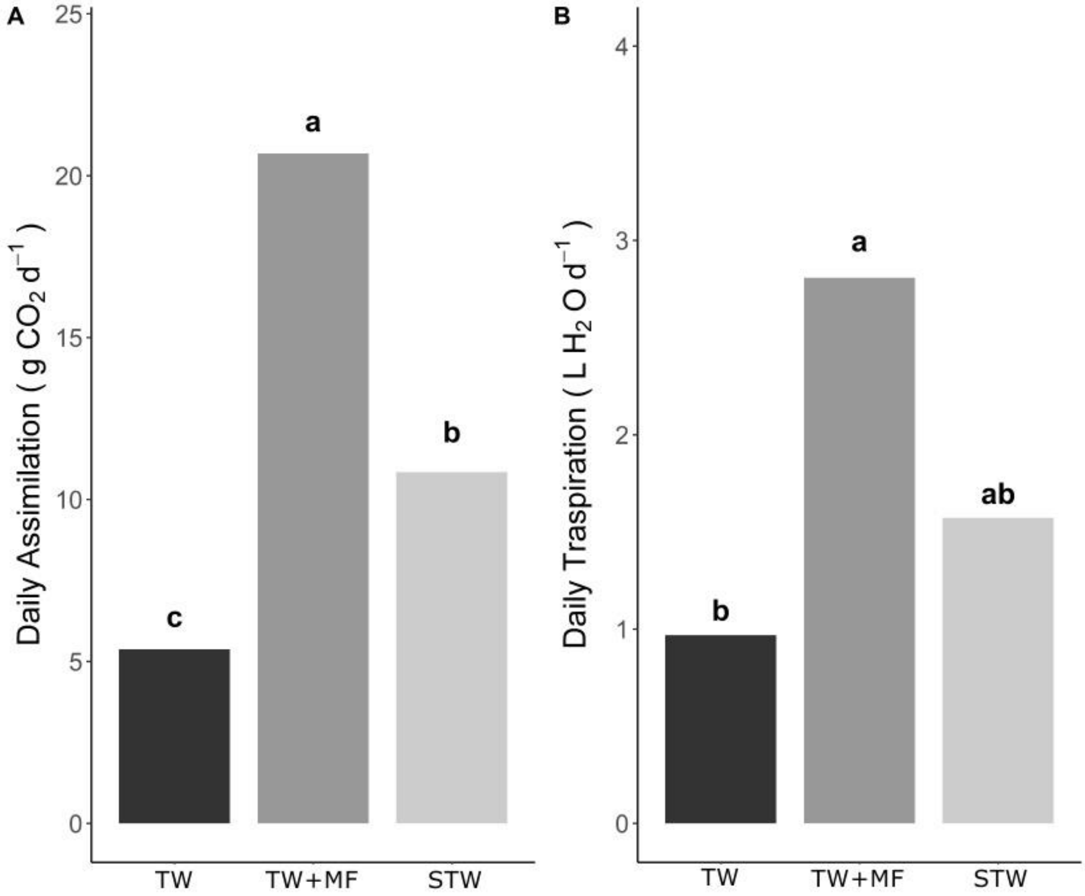


Fig.7. Cumulative Daily Assimilation (± SE) (gCO_2 d^{-1}) (A) and Evapotranspiration (± SE) (LH_2O d^{-1}) (E) for the whole canopy at the end of the 2017 season. Each bar represents the mean of 5 measurements. Different letters indicate significant differences among treatments with a p value <0.05.

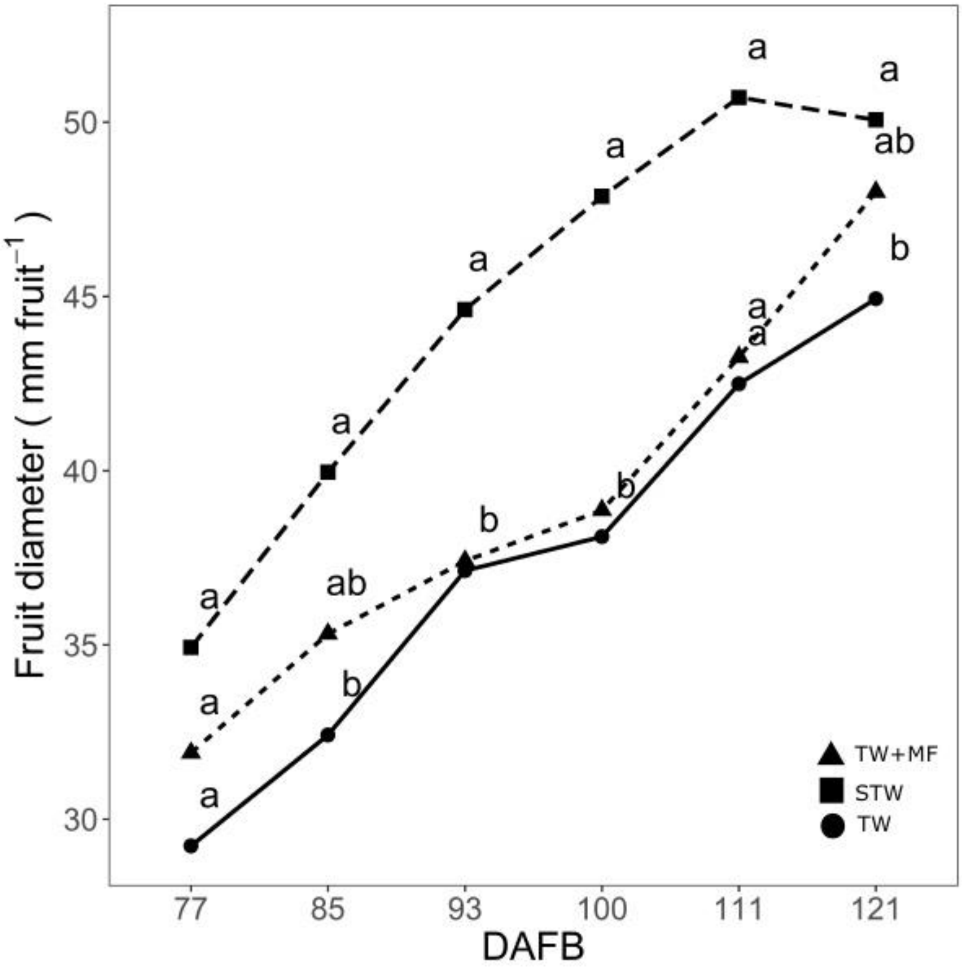


Fig.8. Seasonal pattern of fruit growth (± SE) (mm fruit^{-1}), for TW (circle and continuous line), TW+MF (triangles and short- dashed line) and STW (squares and long-dashed line) treatments. Each value corresponds to the mean of 8 measurements. Different letters indicate significant differences among treatments with a p value <0.05.

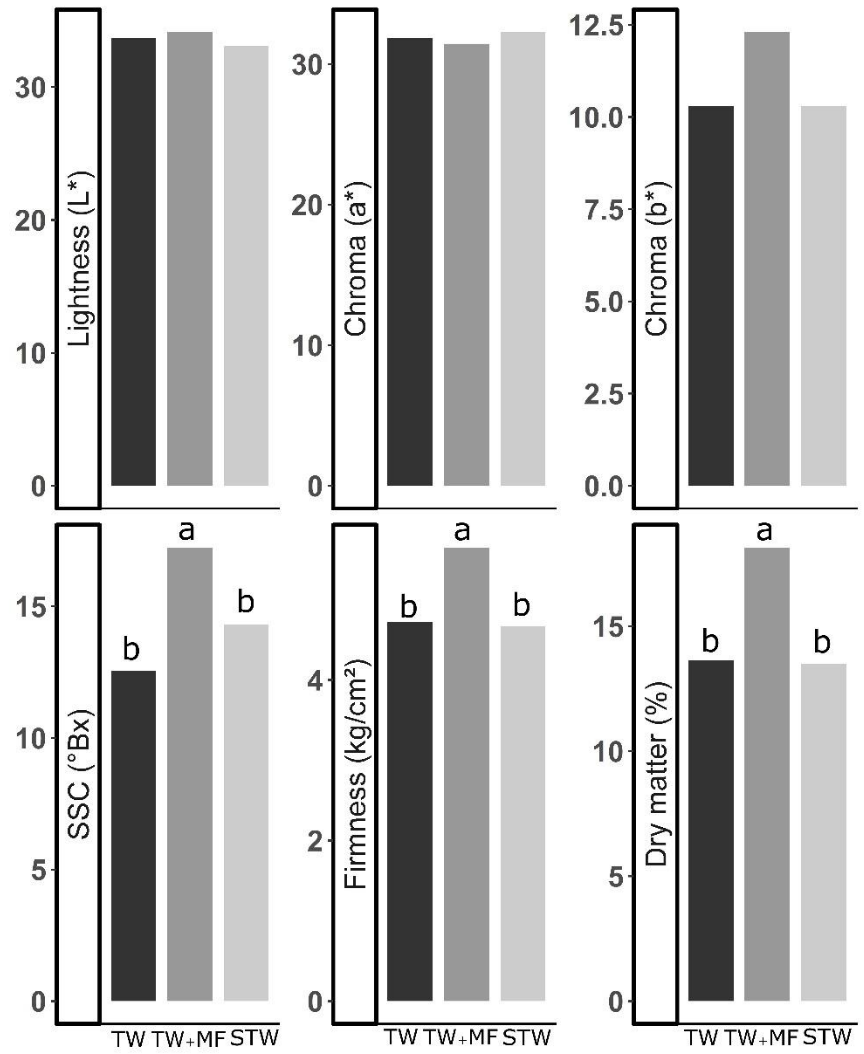
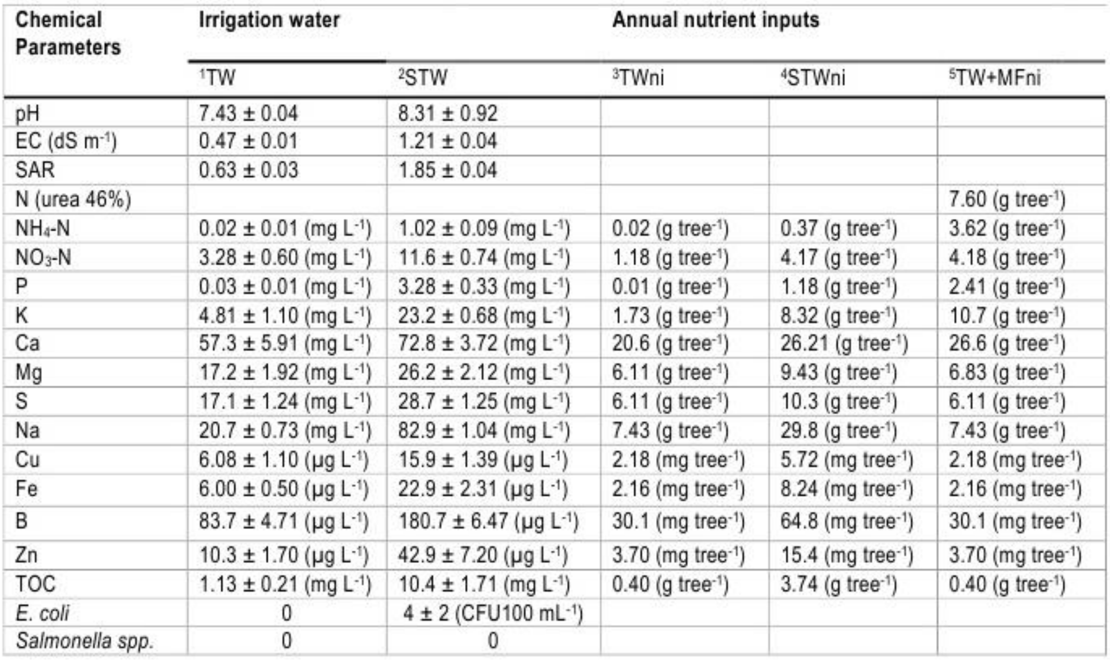


Fig.9. Fruit quality parameters: dry matter (± SE) (%), firmness (± SE) (kg cm^{-2}), soluble solid content (± SE) (°Bx), lightness (± SE) (L*), chroma (± SE) (a*) and chroma (± SE) (b*). Each value corresponds to the mean of 12 values. Different letters indicate significant differences between treatments with a p value <0.05.

Table 1. Chemical and microbiological characteristics of the water sources used as irrigation water (n=14 ± SE) and annual nutrient inputs from water sources and from the mineral fertilization.

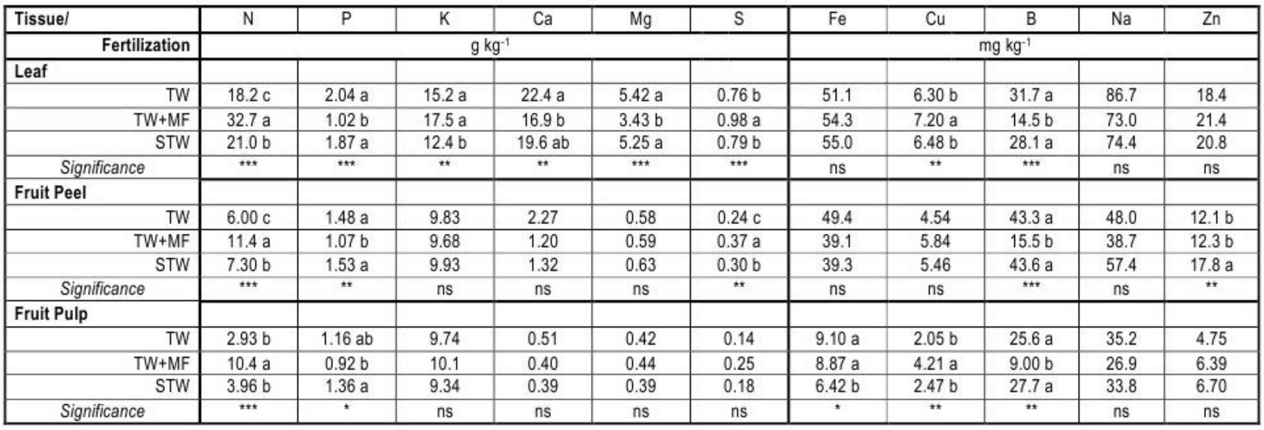


Chemical Parameters	**Irrigation water**		**Annual nutrient inputs**		
	[1]TW	[2]STW	[3]TWni	[4]STWni	[5]TW+MFni
pH	7.43 ± 0.04	8.31 ± 0.92			
EC (dS m^{-1})	0.47 ± 0.01	1.21 ± 0.04			
SAR	0.63 ± 0.03	1.85 ± 0.04			
N (urea 46%)					7.60 (g $tree^{-1}$)
NH_4-N	0.02 ± 0.01 (mg L^{-1})	1.02 ± 0.09 (mg L^{-1})	0.02 (g $tree^{-1}$)	0.37 (g $tree^{-1}$)	3.62 (g $tree^{-1}$)
NO_3-N	3.28 ± 0.60 (mg L^{-1})	11.6 ± 0.74 (mg L^{-1})	1.18 (g $tree^{-1}$)	4.17 (g $tree^{-1}$)	4.18 (g $tree^{-1}$)
P	0.03 ± 0.01 (mg L^{-1})	3.28 ± 0.33 (mg L^{-1})	0.01 (g $tree^{-1}$)	1.18 (g $tree^{-1}$)	2.41 (g $tree^{-1}$)
K	4.81 ± 1.10 (mg L^{-1})	23.2 ± 0.68 (mg L^{-1})	1.73 (g $tree^{-1}$)	8.32 (g $tree^{-1}$)	10.7 (g $tree^{-1}$)
Ca	57.3 ± 5.91 (mg L^{-1})	72.8 ± 3.72 (mg L^{-1})	20.6 (g $tree^{-1}$)	26.21 (g $tree^{-1}$)	26.6 (g $tree^{-1}$)
Mg	17.2 ± 1.92 (mg L^{-1})	26.2 ± 2.12 (mg L^{-1})	6.11 (g $tree^{-1}$)	9.43 (g $tree^{-1}$)	6.83 (g $tree^{-1}$)
S	17.1 ± 1.24 (mg L^{-1})	28.7 ± 1.25 (mg L^{-1})	6.11 (g $tree^{-1}$)	10.3 (g $tree^{-1}$)	6.11 (g $tree^{-1}$)
Na	20.7 ± 0.73 (mg L^{-1})	82.9 ± 1.04 (mg L^{-1})	7.43 (g $tree^{-1}$)	29.8 (g $tree^{-1}$)	7.43 (g $tree^{-1}$)
Cu	6.08 ± 1.10 (µg L^{-1})	15.9 ± 1.39 (µg L^{-1})	2.18 (mg $tree^{-1}$)	5.72 (mg $tree^{-1}$)	2.18 (mg $tree^{-1}$)
Fe	6.00 ± 0.50 (µg L^{-1})	22.9 ± 2.31 (µg L^{-1})	2.16 (mg $tree^{-1}$)	8.24 (mg $tree^{-1}$)	2.16 (mg $tree^{-1}$)
B	83.7 ± 4.71 (µg L^{-1})	180.7 ± 6.47 (µg L^{-1})	30.1 (mg $tree^{-1}$)	64.8 (mg $tree^{-1}$)	30.1 (mg $tree^{-1}$)
Zn	10.3 ± 1.70 (µg L^{-1})	42.9 ± 7.20 (µg L^{-1})	3.70 (mg $tree^{-1}$)	15.4 (mg $tree^{-1}$)	3.70 (mg $tree^{-1}$)
TOC	1.13 ± 0.21 (mg L^{-1})	10.4 ± 1.71 (mg L^{-1})	0.40 (g $tree^{-1}$)	3.74 (g $tree^{-1}$)	0.40 (g $tree^{-1}$)
E. coli	0	4 ± 2 (CFU100 mL^{-1})			
Salmonella spp.	0	0			

[1]Tap Water. [2]Secondary Treated Wastewater. [3]TWni Tap water annual nutrient input. [4]STWni Secondary treated wastewater annual nutrient input. [5]TW+MFni Tap water plus mineral fertilized annual nutrient input.

Ag, Al, As, Be, Cd, Co, Cr, Hg, Mo, Sb, Sn, Ti, Tl, and V concentration either in STW or TW was below the instrumental detection limit (dl).

Table 2. Effect of the fertilization strategy on the leaf, fruit peel and fruit pulp macro and micronutrient concentration. Leaves and fruit were collected in full summer, at commercial harvest (i.e. 124 DAFB) in 2017.



Tissue/ Fertilization	N	P	K	Ca	Mg	S	Fe	Cu	B	Na	Zn
	$g\ kg^{-1}$						$mg\ kg^{-1}$				
Leaf											
TW	18.2 c	2.04 a	15.2 a	22.4 a	5.42 a	0.76 b	51.1	6.30 b	31.7 a	86.7	18.4
TW+MF	32.7 a	1.02 b	17.5 a	16.9 b	3.43 b	0.98 a	54.3	7.20 a	14.5 b	73.0	21.4
STW	21.0 b	1.87 a	12.4 b	19.6 ab	5.25 a	0.79 b	55.0	6.48 b	28.1 a	74.4	20.8
Significance	***	***	**	**	***	***	ns	**	***	ns	ns
Fruit Peel											
TW	6.00 c	1.48 a	9.83	2.27	0.58	0.24 c	49.4	4.54	43.3 a	48.0	12.1 b
TW+MF	11.4 a	1.07 b	9.68	1.20	0.59	0.37 a	39.1	5.84	15.5 b	38.7	12.3 b
STW	7.30 b	1.53 a	9.93	1.32	0.63	0.30 b	39.3	5.46	43.6 a	57.4	17.8 a
Significance	***	**	ns	ns	ns	**	ns	ns	***	ns	**
Fruit Pulp											
TW	2.93 b	1.16 ab	9.74	0.51	0.42	0.14	9.10 a	2.05 b	25.6 a	35.2	4.75
TW+MF	10.4 a	0.92 b	10.1	0.40	0.44	0.25	8.87 a	4.21 a	9.00 b	26.9	6.39
STW	3.96 b	1.36 a	9.34	0.39	0.39	0.18	6.42 b	2.47 b	27.7 a	33.8	6.70
Significance	***	*	ns	ns	ns	ns	*	**	**	ns	ns

ns, *, ** and ***: not significant or significant at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively. Per each element and within the same tissue, means followed by the same letter are not statistically different ($p \leq 0.05$, SNK Test).

CHAPTER III

SECONDARY TREATED WASTEWATER AS A SUPPORT STRATEGY FOR TREE CROPS IRRIGATION: NUTRITIONAL AND PHYSIOLOGICAL RESPONSES ON APPLE TREE

3.1. Introduction

Recycling treated municipal wastewater for agricultural irrigation purposes may reduce the water volumes extracted from natural water sources especially in areas facing water shortages. This practice contributes to recycle nutrients, improve soil health and cut the amount of pollutants discharged into the waterways (Hanjra et al., 2012). For instance, through the irrigation with wastewater, most of the eutrophication-related elements (i.e. N and P) can be conveniently reused as fertilizers rather than lost in fresh-water bodies. Nowadays, the reuse of treated wastewater in agriculture is highly encouraged as the amount of collected and treated wastewater is likely to increase considerably with population growth and urbanization. However, treated wastewater must be carefully managed to protect the environment and public health. Scientific knowledge of such practice on both annual and perennial crops intended for human consumption are highly required (Pedrero et al., 2010), especially when its use in agriculture is increasing in the Mediterranean Countries.

Compared to freshwater, treated wastewater has a higher mineral and organic matter concentration, representing a precious source of nutrients to "fertigate" crops which in turn can provide benefits on plant physiological and nutritional status (Khurana and Singh, 2012). Literature mostly confirms that tertiary treated wastewater (TTW) can be suitably reused as water resource to irrigate tree crops in water-scarce Mediterranean areas (Mendoza-Espinoza et al., 2008; Pedrero and Alarcon, 2009; Pedrero et al., 2013; Vivaldi et al., 2013; Petousi et al., 2015). In Europe a univocal legislation regulating the reuse of treated wastewater in agriculture is currently missing and each Country adopts its own regulation. In Italy, for instance, the reuse of secondary treated wastewater (STW) for irrigation purpose is still not admitted.

The fertilization effect of STW on cultivated crops remains underestimated. Indeed, STW supplies significant amount of organic matter as well as plant-available nutrients (Chen et al., 2008). Thus, a large-scale utilization of STW to irrigate crops would reduce the need of chemical inputs in agriculture. The use of wastewater in agriculture has been demonstrated to positively affect soil fertility and productivity (WCED, 1987). However, most of these studies were addressed using TTW, in which the amount of nutrients, especially nitrogen and phosphorous, were significantly depleted as a consequence of the cleaning treatments (Pescod, 1992), while the effect of the STW is only beginning to be explored. On the other hand, although the agronomic validity would need to be demonstrated, the use of the STW in agriculture implies environmental (i.e. soil pollution, phytotoxicity), food safety risks and social acceptance obstacles as well (Muchuweti et al., 2006; Bernstein, 2011; Fatta-Kassinos et al., 2011).

Although irrigation of fruit crops does not wet the plant canopy (preventing external contamination), investigations on the potential consequences of STW irrigation on the tree-root absorption pathway are required before its diffusion on a large scale. Finally, these studies are of extreme importance to support the legislator in promulgating new regulations about treated wastewater reuse in agriculture.

The aim of this work was to investigate the effect of STW (treated according to the Italian Decree of Ministry for Environment, No. 152/2006) as irrigation water on the nutritional and physiological responses of bearing apple trees, one of the mostly cultivated fruit tree crops in Europe. We hypothesized that STW, as exclusive source of irrigation water and fertilizer source, may positively affect nutritional and physiological status of apple trees grown in controlled environment.

3.2. Materials and Methods

3.2.1. Experimental set up

We carried out a 1-year experiment at the experimental farm of the University of Bologna located in Cadriano (BO), on 15 bearing 3-year old apple trees (*Malus × domestica Borkh*) cv. Gala grafted on M9. Trees were grown in 40-L pots each, filled with an alkaline, poorly fertile sandy-loamy soil (USDA classification) and maintained under a shading hail net. They were trained as slender spindle, irrigated by four drippers per tree of 2 L h^{-1} and managed according to the local Integrated Standard Crop Management practices (ICM, 2010) for pruning, thinning, pest and disease management.

Starting from 48 days after full bloom (DAFB), three irrigation treatments were set up, with 5 replicates (single tree) each: 1) irrigation with secondary treated wastewater (STW) 2) irrigation with tap water (TW) and 3) irrigation with tap water and fertilization with mineral inputs (TW+MF). Trees irrigated with STW did not receive additional fertilizer sources. STW (Decree of Ministry for Environment, No. 152/2006) was provided by the local urban wastewater treatment plant, managed by HERA S.p.a (Italian multi-utility). Along the season, TW+MF trees received 7.83, 1.56, 5.97, and 0.49 g tree^{-1} of N, P, K and Mg as commercial mineral fertilizers, respectively, split in 3 interventions starting from 48 DAFB. Trees were irrigated twice a day to balance crop evapotranspiration (ETc) rate. ETc for the plants used in the experiment was calculated according to the Irrinet irrigation scheduling system, developed and made available over the Internet by the "Consorzio per il Canale Emiliano Romagnolo (CER)" of the Emilia-Romagna Region (www.irriframe.it). The environmental parameters needed as inputs by this web-based platform were obtained from the experimental farm weather station.

3.2.2. Irrigation water chemical and microbiological characterization

Samples of STW and TW were collected at two weeks intervals throughout the irrigation season for chemical analyses, then stored at 5 °C. Mineral concentration was determined by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) (Ametek Spectro Arcos EOP, Kleve, Germany) on liquid samples as such. pH was measured with a pH-meter XS PH510 (Eutech Instruments, Singapore) whereas electrical conductivity (EC) was determined by a conductimeter (METERLAB, CDM 210, Radiometer Analytical, France). Finally, total organic carbon (TOC) and total dissolved N were measured in the water samples by an elemental analyzer TOCVcpn- TNM1 (Shimadzu Corp., Kyoto, Japan).

The abundance of *E. coli* and *Salmonella* spp., was determined on STW samples by the membrane filtration method. Briefly, for *E. coli* enumeration, membranes were placed onto a Chromocult ES (VWR) agar and incubated at 37 °C for 24 h. *Salmonella* spp. Relative abundance was performed according to procedure UNI EN ISO 19250:2013.

The annual nutrient input was calculated multiplying the concentration of the dissolved elements in TW and STW water by the amount of water provided throughout the season. The TW+MF annual nutrient input is the contribution of the TW annual nutrient input plus the mineral fertilizer supply.

3.2.3. Tree nutritional status

Leaf mineral concentration was assessed on ten fully expanded leaves per replicate, randomly selected from annual shoots on the second half of July. Petioles were removed, then leaf limbs were washed, oven-dried, weighed, milled and analysed. N was determined by the Kjeldahl method (Schuman et al., 1973) while P, K, Ca, Mg, S, Fe, Cu, B, Na, Zn, Mn were determined by ICP-OES after digestion with nitric acid (HNO_3) by a microwave lab station (Ethos TC-Milestone, Bergamo, Italy). The same procedure was adopted to asses mineral concentration of fruit peel and fruit pulp on fruit sampled at commercial harvest.

3.2.4. Vegetative growth and daily photosynthetic assimilation rates assessment

Three shoots per tree were selected and their length was recorded at 34, 41, 48, 54, 60, 68, 76, 83, 93, 104, 128 and 157 DAFB. Furthermore, for each tree leaf net assimilation rate (A) was measured at about 9:00, 13:00 and 16:00 hours at 174 DAFB using a portable gas analyser (Li-COR 6400, LI-COR, Lincoln, Nebraska, USA). Measurements were carried out on one fully-expanded leaf per plant. Light intensity inside the cuvette was maintained constant as recorded by the photosynthetic photon flux density (PPFD) sensor immediately before the measurements. Cumulative daily photosynthesis (∑A) (from 9:00 to 17:00) was then calculated as described by Tozzi et al. (2018) using the following equation (Eq.1):

$$\sum_{y} = \sum_{i=t0}^{t1} \left(\frac{(yto+yt1)}{2}\right) i + \sum_{i=t1}^{t2} \left(\frac{(yt1+yt2)}{2}\right) i \qquad (1)$$

where y is the variable A whereas t_0, t_1 and t_2 correspond to the A values recorded at 9:30, 13:30 and 16:30, respectively. Cumulative daily photosynthesis (∑A) was then multiplied by the total leaf area per tree. Leaf area was estimated by multiplying the total leaf number by the average leaf area, measured by a leaf area meter (LI-3000 A, LI-COR, Lincoln, Nebraska, USA) on three replicates per tree, each of 30 grams of leaves.

3.2.5. Tree leaf and stem water potential

The daily patterns of leaf and stem water potentials (WP) were assessed at 115 and 150 DAFB (post-harvest). Measurements were performed at 6:00, 9:00, 13:00 and 16:00 hour using a Scholander pressure chamber (Soil Moisture Equipment Corp., Santa Barbara, CA, USA). Leaf water potential was measured on a well exposed leaf per tree following the recommendations of Turner and Long (1980). Similarly, stem water potential was measured on leaves previously covered with aluminium foils and placed in plastic bags for at least 90 minutes prior measurements, to allow equilibration with the stem (Naor et al., 1995).

3.2.6. Fruit growth rate, yield and quality

The diameter of 8 fruit per treatment, randomly chosen, was recorded at 60, 68, 76, 83, 94, 104, 117 and 128 DAFB, using a digital caliper provided with an external memory (http://www.hkconsulting.it/). At commercial harvest, yield was assessed for each tree. Fresh weight, dry matter content, flesh firmness, soluble solids content (SSC), colour lightness (L*) and chroma (a*; b*) was assessed on the harvested fruit. Flesh firmness was assessed by a 53220 FTA Fruit Texture Analyser (T.R. Turoni srl, Italy) equipped with a 11 mm plunger. Soluble solids content was determined on the fruit juice by a digital refractometer (ATAGO CO., LTD, Japan) and peel colour components was performed using a Minolta CR-400 (Konica Minolta Sensing Americas, Inc, USA).

3.2.7. Statistical analysis

Shoot length and fruit growth were analysed using a linear mixed model function. A one-way ANOVA followed by a Tukey HSD test using R software (www.r-project.org) was used to establish differences among treatments for daily leaf and stem water potential, daily cumulative photosynthesis, yield and fruit quality parameters. Data of the tissue mineral concentration were analysed according to a complete randomized block design. When the analysis of variance showed a statistical effect, means were separated by the SNK Test (SAS 9.0, SAS Institute Inc., Cary, NC, USA).

3.3. Results and Discussion

3.3.1. Water quality

As expected, mineral concentration was lower in TW than STW (Tab. 1). This latter showed a moderately alkaline pH and a relatively low EC and SAR indexes, indicating low risks of soil salinization. Values were even within the Italian legal thresholds for a direct utilization of treated wastewater sources, as TTW, in the agricultural sector (Decree of Ministry for Environment, No. 185/2003). TOC was almost 10-fold higher in STW compared to TW, with potential benefits on soil microbial activity, CEC and nutrient availability (Beutler et al., 2014). Dissolved mineral nutrients supplied through STW irrigation allowed to save 50.3 % and 75.1 % of N and P, respectively, compared to the reference mineral fertilized treatment (TW+MF). *E. coli* mean concentration in STW was 4 CFU 100 mL^{-1}, below the Italian *E. coli* threshold for irrigation water (Decree of Ministry for Environment, No. 185/2003). No *Salmonella* spp. were detected in STW water samples.

3.3.2. Tree nutritional status

Trees irrigated with TW (without fertilization) exhibited a leaf N concentration far below the optimal threshold (Tab. 2), while the overall values of leaf mineral concentration found in TW+MF and STW irrigated trees were close to the optimal range for the same variety (Cheng and Raba, 2009). Leaf and fruit N concentrations were statistically enhanced by the TW+MF, despite a larger canopy development. Intermediate values were recorded in trees irrigated with STW (Tab. 2). This indicates that N exclusively provided by STW (< 6.0 g tree^{-1}) was not enough to satisfy tree nutrient requirements. Indeed, leaf N concentration of mineral-fertilized trees were significantly higher as a consequence of higher N inputs (9.0 g tree^{-1}). On the contrary, leaf P and Ca concentration in TW+MF trees were significantly decreased, likely due to the dilution and partitioning effect induced by a larger vegetative biomass. An opposite trend was exhibited in the TW trees, with higher concentration for P and Ca (Tab. 2). Concerning micronutrients, TW+MF increased leaf Fe and Mn concentrations while no effects was detected on leaf Cu, B and Na concentrations, regardless of the irrigation treatment (Tab. 2). An increased concentration of Fe, Cu and Mn was induced by TW+MF in fruit peel and pulp (Tab. 2). Instead, a decreasing trend was detected in P, Ca, B and Na concentrations from TW+MF to TW (Tab. 2).

The reiterate supplied of STW as irrigation water likely promoted an increased in the soil microbial biomass, due to the naturally high microbial abundance and biodiversity of this water source. Thereby, other than the direct nutritional contribution provided by the STW (nutrients under mineral forms dissolved in the water), the effect of the STW-derived microorganisms on the native soil organic matter on tree uptake, cannot be disjointed.

It is worth to mention that heavy metals accumulation in STW vegetal tissues (i.e. leaves and fruits) was not observed, excluding potential contamination risk for human health.

3.3.3. Vegetative growth and daily photosynthetic assimilation rate

TW+MF shoot length was characterized by a fast increase until 60 DAFB, while afterwards shoot growth rate was much slower and proceeded until 157 DAFB. Shoots on TW+MF trees were statistically longer from 60 DAFB on, compared to the other treatments, reaching an average length of 32.0 cm shoot^{-1} at the end of season. Shoot growth on STW and TW irrigated trees showed comparable growth patterns, with limited and slow growth rates, reaching a maximum length of 15.2 cm shoot^{-1} and 9.9 cm shoot^{-1}, respectively.

The different shoot growth rate is a consequence of the total N that trees received in the different treatments. Indeed, TW+MF trees received a higher amount of N, which likely sustained tree growth. This indicates that irrigation with STW may partially contribute to fulfil plant nutrient requirements. Therefore, nutrients supplied by STW should be taken into account in the fertilization schedule. In our conditions, results suggest that the use of STW cannot replace traditional fertilization for young apple trees and mineral nutrients must be integrated by alternative sources. On the other hand, irrigation with STW was not detrimental to plant growth and nutritional status, indicating that STW is a potential water source to irrigate apple trees.

Treatments significantly affected tree photosynthetic daily assimilation rate (Fig. 2). Compared to the TW-irrigated trees, irrigation with STW more than doubled the cumulative amount of assimilated C estimated at the end of the season (Fig. 2) with values of 13.4 and 5.04 g CO_2 d^{-1} in STW and TW irrigated trees, respectively. However, the C assimilated in TW+MF trees was the highest, with a value of 19.8 g CO_2 d^{-1} (Fig. 2). These differences are likely the consequence of the different nutrient supplies and canopy areas among the irrigation treatments. Tree canopy area was on average 0.68 ± 0.07 m^2 $tree^{-1}$, 0.29 ± 0.04 m^2 $tree^{-1}$ and 1.42 ± 0.09 m^2 $tree^{-1}$ for the STW, TW, and TW+MF irrigated trees, respectively.

3.3.4. Water relations

Leaf and stem water potentials (WP) showed a decreasing pattern from early morning to afternoon on both the day of measurements (Fig. 3). In pre-harvest (115 DAFB) leaf WP on STW and TW+MF trees were statistically more negative in comparison to TW trees (Fig. 3, b). This difference seems related to the higher water demand of STW and TW+MF trees, which can be attributed to their larger leaf area and fruit yield compared to TW. No difference was found among treatments on the post-harvest leaf WP (150 DAFB), except at 9:00 A.M. In this case, STW trees showed slightly more negative water potentials.

Stem WP at 115 DAFB revealed more negative values on STW trees compared to the other treatments (Fig.3 a) during the whole day, except at midday. This result may indicate a slight salinity stress (Acosta-Motos et al., 2017) induced by the irrigation with STW. Indeed, apple tree is considered among the most sensitive tree crops to soil salinity (FAO, 1985). Such effect was confirmed on the post-harvest measurement, at 150 DAFB, when trees are characterized by a physiological recovering process (Fig.3). Indeed, STW trees showed lower stem WPs if compared to TW+MF and TW treatments, except at 9.00 A.M. This behaviour agrees with the hypothesis of the salinity stress observed during pre-harvest.

3.3.5. Fruit growth, yield and quality

The seasonal pattern of fruit growth was not statistically different among treatments (Fig. 4). In any case, STW trees showed slightly higher values in fruit dimeter for almost all the season compared to TW and TW+MF. Moreover, STW recorded a more than double yield if compared with the TW+MF trees while few fruit were collected from trees irrigated with TW. The average fruit number was 10.4 ± 2.3 fruit tree^{-1}, 5.1 ± 2.8 fruit tree^{-1} and 2.4 ± 0.9 fruit tree^{-1} for the STW, TW+MF, and TW treatments, respectively. Data indicate that STW did not negatively affect seasonal fruit development, even considering the higher crop load.

Treatments affected most of the fruit quality parameters (Fig. 5). Fruit from TW+MF treated trees showed statistically higher chroma (b*) and lightness (L*) values compared the other two treatments. Concerning dry matter and soluble solids content, TW+MF and STW trees showed statistically higher values if compared to TW trees. Chroma (a*) was statistically higher in the STW trees, followed by TW and TW+MF, respectively. Instead, fruit firmness was higher in TW trees if compared to the other two treatments. No differences were detected in the fruit weight (data not shown).

The higher dry matter and soluble solid contents in STW-irrigated fruit, which were characterized also by a higher crop load, could be related to the chemical element concentrations and EC of the STW water (Tab. 1). In fact, many plants adapt to salt stress by enhancing the concentration of sugars, organic acids, proteins and amino acids which act as osmolytes to maintain plant turgor under salt stress. The presence of these metabolites often increases the nutritive quality and marketability of fruit and vegetables (Ahmed et al., 2009; Ahlem et al., 2011). It has been demonstrated on different crops (tomatoes, muskmelon, and cucumber), that fruit quality parameters such as soluble solid content, improved in fruits irrigated with reclaimed water (Basiouny, 1984; Crisosto et al., 1994; Lurie et al., 1996; Biernbaum and Argo, 1995; Pedrero et al., 2012). Our data indicate that even if the STW used was not highly saline, fruit quality parameters were positively affected by irrigation with this water.

3.4. Conclusions

Results suggest that STW represents a valuable water source for tree crops management, as it contributes to fulfil tree nutrient requirements. The amount of nutrients dissolved in STW can promote the plant nutritional and physiological status of apple trees, without any adverse effect.

Other than organic matter and nutrients, STW may contain heavy metals which are toxic to plants beyond a certain limit. In our conditions, irrigation with STW did not increase heavy metal concentration both in leaf and fruit tissues, indicating limited risks also for human health. However, data indicate a moderate salinity stress induced by the STW which indirectly improved fruit quality parameters (i.e. dry matter percentage, soluble solids content). We can conclude that recycling STW in agriculture would allow to recover the amount of N and P typically contained in treated wastewater sources and to reduce the use of mineral fertilizer inputs, providing positive ecological (e.g. limiting eutrophication problems) and agronomical implications.

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3.6. Figures and Tables

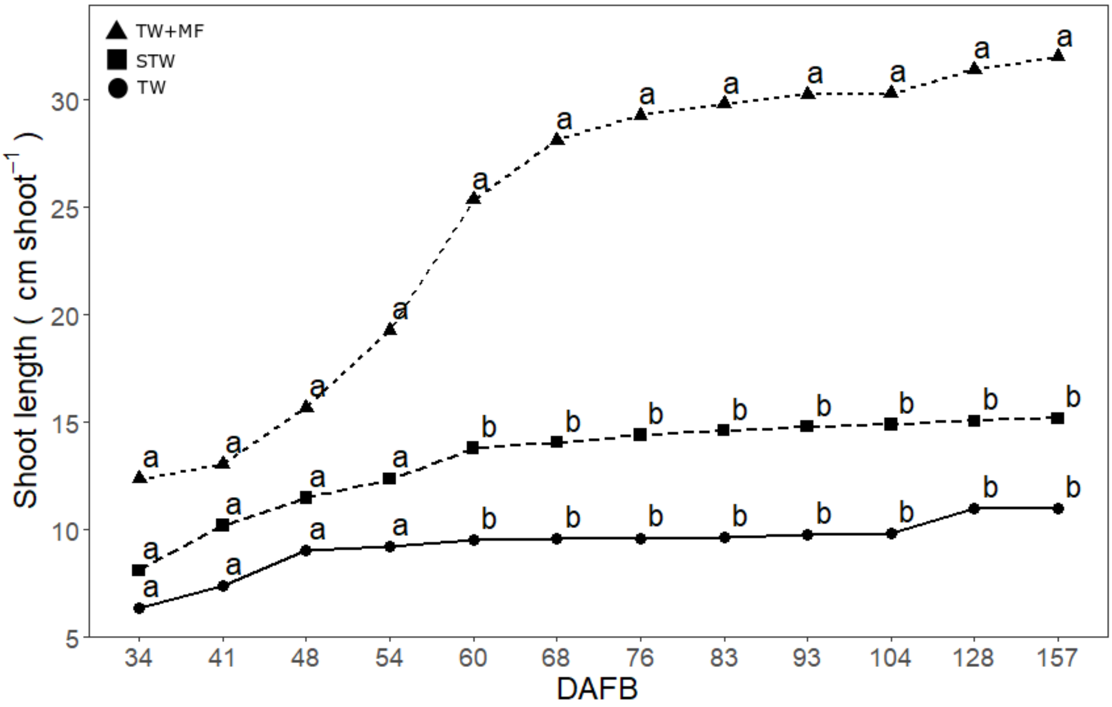

Fig. 1. Seasonal pattern of shoot growth (n=15; Avg.) for TW+MF, STW and TW. Different letters indicate significant differences with p value <0.05.

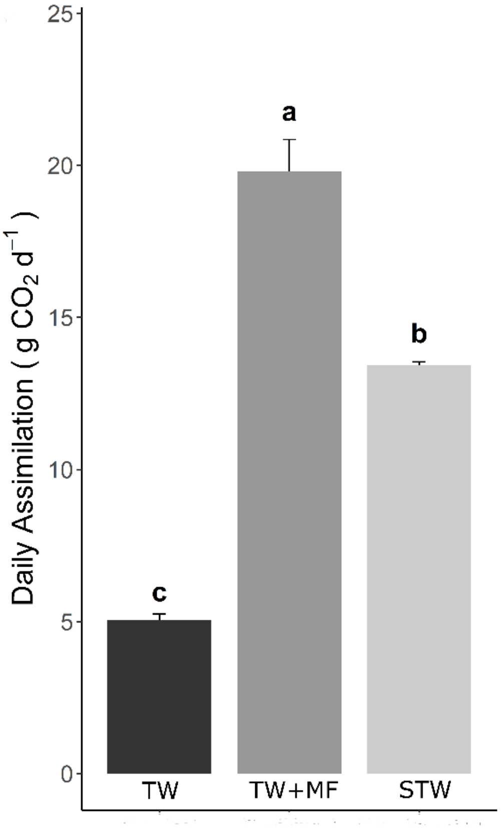

Fig. 2. Effect of the irrigation and fertilization treatment on the cumulative daily canopy CO_2 assimilation (n=5; Avg. ± SE) measured at the end of the season. Columns with different letters indicate significant differences at $p < 0.05$.

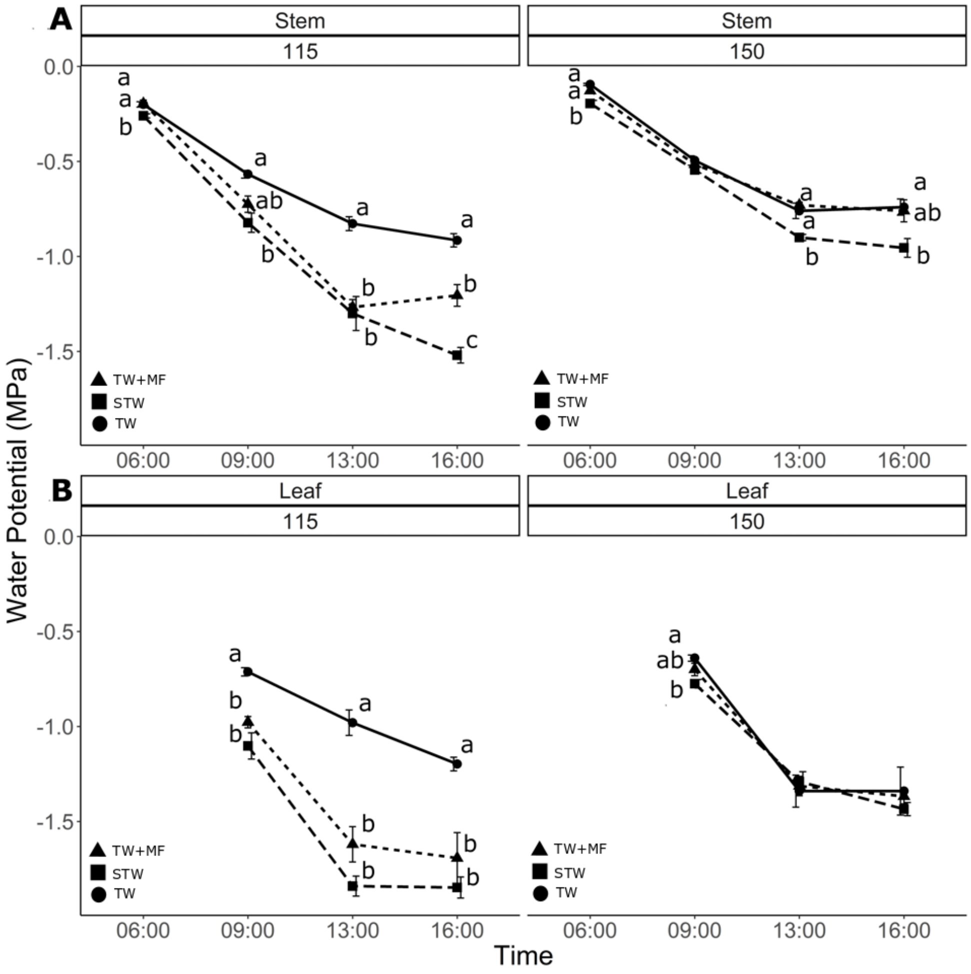

Fig.3. Daily patterns of stem (A) and leaf water potentials (B) in TW, STW and TW+MF irrigated trees, measured at 115 and 150 DAFB. Each point represents the mean of 5 measurements. Within the same time, values with different letters indicate significant differences at $p < 0.05$.

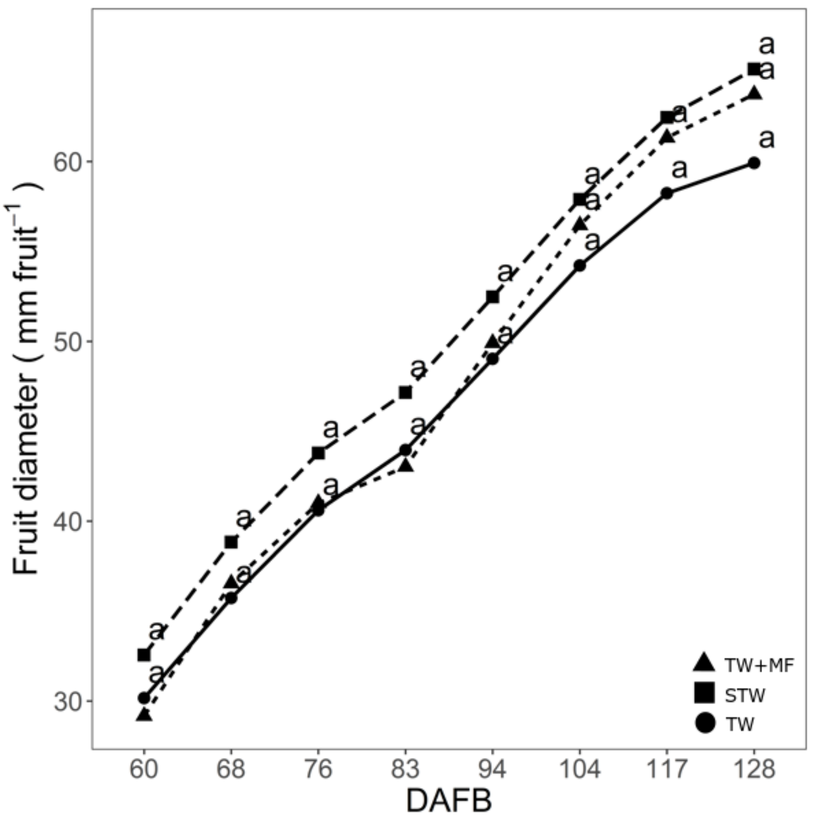

Fig. 4. Seasonal pattern of fruit growth (mm) on TW+MF, STW and TW irrigated trees (n=8; Avg.). Different letters indicate significant differences with p value <0.05.

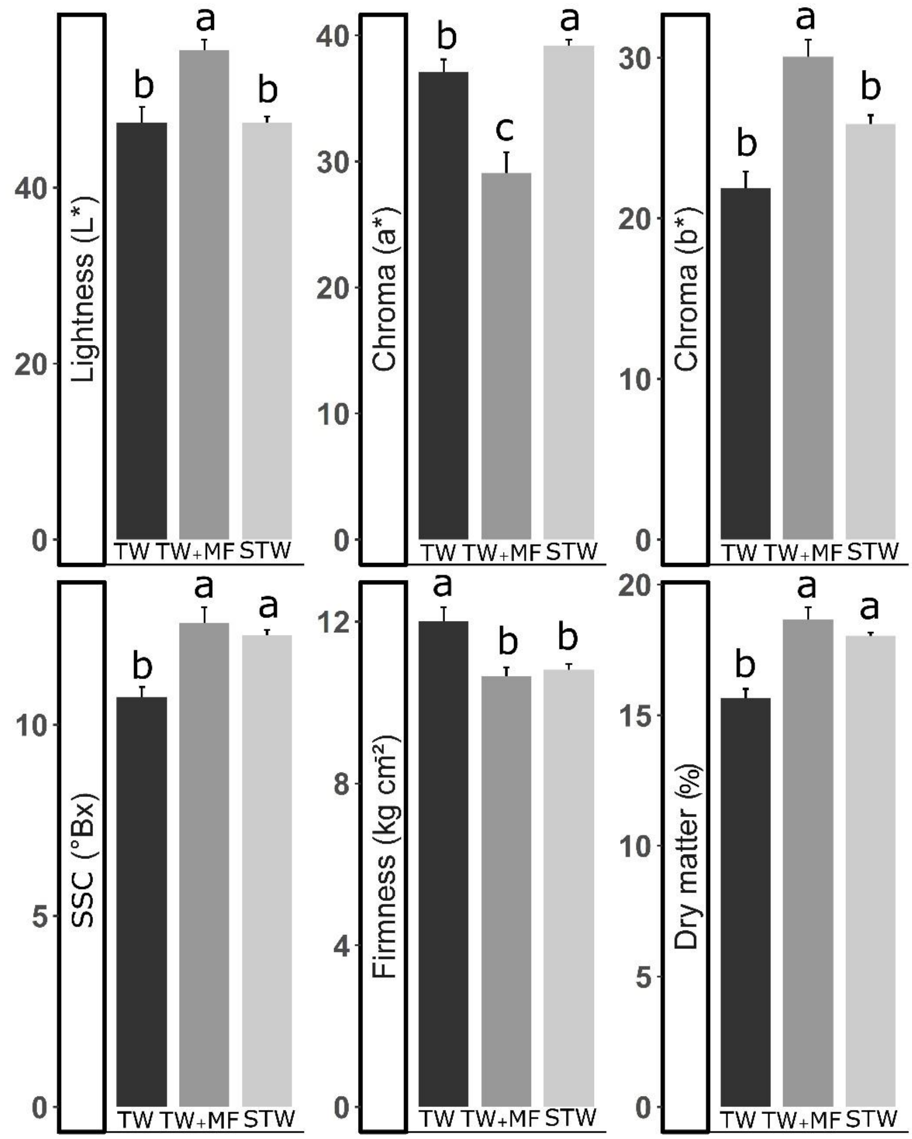

Fig. 5. Effect of the irrigation and fertilization treatment on the fruit lightness (L*), chroma (a*; b*), soluble solid content, flesh firmness, and dry matter at commercial harvest (n=12; Avg. ± SE). Black, dark-grey and grey bars indicate TW, TW+MF and STW trees, respectively. Within the same parameter, columns with different letters indicate significant differences at $p<0.05$.

Table 1. Chemical and microbiological parameters of tap water (TW) and secondary treated wastewater (STW) (n=7 ± SE) and estimated annual nutrient inputs supplied trough the water source (TWni, STWni) and from the mineral fertilizers (TW+MFni).

Chemical Parameters	**Irrigation water**		**Annual nutrient inputs**		
	[1]TW	[2]STW	[3]TW	[4]STW	[5]TW+MF
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			
N (urea 46%)					5.52 (g $tree^{-1}$)
NH_4-N	0.02 ± 0.01 (mg L^{-1})	1.02 ± 0.09 (mg L^{-1})	0.02 (g $tree^{-1}$)	0.37 (g $tree^{-1}$)	1.88 (g $tree^{-1}$)
NO_3-N	3.28 ± 0.60 (mg L^{-1})	11.6 ± 0.74 (mg L^{-1})	1.18 (g $tree^{-1}$)	4.17 (g $tree^{-1}$)	1.63 (g $tree^{-1}$)
P	0.03 ± 0.01 (mg L^{-1})	3.28 ± 0.33 (mg L^{-1})	0.01 (g $tree^{-1}$)	1.18 (g $tree^{-1}$)	1.57 (g $tree^{-1}$)
K	4.81 ± 1.10 (mg L^{-1})	23.2 ± 0.68 (mg L^{-1})	1.73 (g $tree^{-1}$)	8.32 (g $tree^{-1}$)	7.70 (g $tree^{-1}$)
Ca	57.3 ± 5.91 (mg L^{-1})	72.8 ± 3.72 (mg L^{-1})	20.6 (g $tree^{-1}$)	26.21 (g $tree^{-1}$)	20.6 (g $tree^{-1}$)
Mg	17.2 ± 1.92 (mg L^{-1})	26.2 ± 2.12 (mg L^{-1})	6.11 (g $tree^{-1}$)	9.43 (g $tree^{-1}$)	6.60 (g $tree^{-1}$)
S	17.1 ± 1.24 (mg L^{-1})	28.7 ± 1.25 (mg L^{-1})	6.11 (g $tree^{-1}$)	10.3 (g $tree^{-1}$)	6.11 (g $tree^{-1}$)
Na	20.7 ± 0.73 (mg L^{-1})	82.9 ± 1.04 (mg L^{-1})	7.43 (g $tree^{-1}$)	29.8 (g $tree^{-1}$)	7.43 (g $tree^{-1}$)
Cu	6.08 ± 1.10 (µg L^{-1})	15.9 ± 1.39 (µg L^{-1})	2.18 (mg $tree^{-1}$)	5.72 (mg $tree^{-1}$)	2.18 (mg $tree^{-1}$)
Fe	6.00 ± 0.50 (µg L^{-1})	22.9 ± 2.31 (µg L^{-1})	2.16 (mg $tree^{-1}$)	8.24 (mg $tree^{-1}$)	2.16 (mg $tree^{-1}$)
B	83.7 ± 4.71 (µg L^{-1})	180.7 ± 6.47 (µg L^{-1})	30.1 (mg $tree^{-1}$)	64.8 (mg $tree^{-1}$)	30.1 (mg $tree^{-1}$)
Zn	10.3 ± 1.70 (µg L^{-1})	42.9 ± 7.20 (µg L^{-1})	3.70 (mg $tree^{-1}$)	15.4 (mg $tree^{-1}$)	3.70 (mg $tree^{-1}$)
TOC	1.13 ± 0.21 (mg L^{-1})	10.4 ± 1.71 (mg L^{-1})	0.40 (g $tree^{-1}$)	3.74 (g $tree^{-1}$)	0.40 (g $tree^{-1}$)
E. coli	0	4 ± 2 (CFU100 mL^{-1})			
Salmonella spp.	0	0			

[1]Tap Water. [2]Secondary Treated Wastewater. [3]TWni Tap water annual nutrient input. [4]STWni Secondary treated wastewater annual nutrient input. [5]TW+MFni Tap water plus mineral fertilized annual nutrient input.

Ag, Al, As, Be, Cd, Co, Cr, Hg, Mo, Sb, Sn, Ti, Tl, and V concentration either in STW or TW was below the instrumental detection limit (dl).

Table 2. Leaf, fruit peel and pulp macro and micronutrient concentration in TW+MF, STW and TW irrigated trees.

Tissue	N	P	K	Ca	Mg	S	Fe	Cu	B	Na	Zn	Mn
Treatment	g kg^{-1}						mg kg^{-1}					
Leaf												
TW	11.9 c	2.41 a	14.5	13.5 a	2.56	0.70 b	45.8 b	8.80	27.0	63.4	15.4 a	23.0 b
TW+MF	19.9 a	1.15 b	14.0	10.9 b	2.27	0.98 a	90.0 a	9.80	25.2	52.8	10.8 b	34.4 a
STW	16.6 b	2.65 a	13.5	13.3 a	2.32	0.93 a	54.4 b	9.40	24.6	62.2	14.6 a	31.2 a
Significance	***	***	ns	*	ns	***	***	ns	ns	ns	*	**
Fruit Peel												
TW	2.00 b	0.30 b	3.50 c	1.34 a	0.89 a	0.14 c	37.5	1.90	24.6 a	27.1	2.44	5.80 c
TW+MF	2.72 a	0.31ab	3.85 a	0.82 c	0.81 b	0.28 a	44.1	2.10	11.9 c	19.6	2.44	7.46 a
STW	2.31 b	0.33 a	3.70 b	0.99 b	0.84 ab	0.22 b	50.3	1.40	14.8 b	23.1	2.73	6.55 b
Significance	*	*	**	***	*	***	ns	ns	***	ns	ns	***
Fruit Pulp												
TW	1.01 b	0.61 a	6.05 a	0.36 a	0.22	0.08 c	6.20 c	1.76 b	31.8 a	39.0 a	1.75	1.13 c
TW+MF	2.72 a	0.43 c	5.12 b	0.23 c	0.21	0.14 a	11.0 a	2.34 a	10.0 c	22.6 b	0.70	1.70 a
STW	1.34 b	0.56 b	5.40 b	0.29 b	0.22	0.11 b	8.40 b	1.75 b	14.5 b	25.5 b	2.15	1.44 b
Significance	**	***	**	**	ns	***	***	**	***	**	ns	***

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

CHAPTER IV

SECONDARY TRETED WASTEWATER FOR IRRIGATION: CHEMICAL AND MICROBIOLOGICAL RISK ASSESSMENT ON APPLE AND NECTARINE TREES

4.1. Introduction

Wastewater re-use in agriculture is a topical subject as it represents a strategy to deal with water scarcity (Pereira et al., 2002). Due to climate changes and the increasing frequency of drought conditions in temperate countries, wastewater could represent an advantageous alternative to face the increasing water scarcity and irrigation demand, a concern which is common in areas with water restriction.

Treated wastewater is also a potential source of mineral nutrients and organic compounds (Khurana and Singh, 2012), thereby its use as irrigation water can have major effects on the soil physical, chemical and biological properties (Jueschke et al., 2008). As a consequence, simulating a “fertigation-like” effect, the use of wastewater in agriculture could contribute in reducing the mineral inputs for plant fertilization. Moreover, wastewater provides significant amount of organic carbon (C). These benefits are mostly associated with the secondary treated wastewater (STW), (Chen et al. 2008) although STW is not accessible in many countries as irrigation water source. On the other hand, the fertilization effect of tertiary treated wastewater (TTW) is much lower due to poorer mineral concentrations (Pescod, 1992).

Literature mostly report studies with TTW reuse in horticulture (Christou et al. 2014; Pedrero et al. 2012), often concluding how TTW can be safely adopted as an additional water resource for orchards irrigation.

Nevertheless, little is known about the potential use of STW as irrigation purposes (Pedrero et al. 2014; Nicolás et al. 2016). This lack of knowledge is also the consequence of the strict rules applied by many countries on wastewater legislation for irrigation purposes. This is the case of Italy, where only TTW reuse is permitted in agriculture.

However, wastewater re-use in agriculture may also be associated with environmental and health risks, given the potential source of dangerous chemicals (e.g. heavy metals) and pathogens (e.g. *E. coli* and *Salmonella spp.*) (Muchuweti et al., 2006; Bernstein, 2011; Fatta-Kassinoset al., 2011). In particular, high levels of heavy metals as well as bacteria such *E. coli* and *Salmonella spp.*, in the food are recognized as mayor factors negatively affecting human health safety (Szkup-Jablonska et al., 2012; Wang et al.,2012(Petterson et al., 2001; Palese et al., 2009; Cirelli et al., 2012; Forslund et al., 2012). Health risks are associated with the potential transfer of such organic and inorganic contaminants to the edible parts, including fruit epicarp and mesocarp, depending on the irrigation system (e.g. overcanopy, sprinkler or microirrigation). Drip irrigation, which is widely used in horticultural crops, is likely to avoid the external contamination of edible parts, given that drops are localized under the canopy, although contaminant uptake by roots cannot be excluded.

From a microbiological point of view, highly contaminated wastewaters are potentially a "reservoir" of human faecal pathogenic microorganisms like *E. coli* and *Salmonella spp*., which may be transferred first to the soil ecosystem, then into plant tissues (Berg et al., 2005). Fortunately, not all the strains possess virulent characteristics, being most of them commensal bacteria of the human gastrointestinal tract. However, the transmission risk of virulent strains cannot be underestimated. Organic contaminants may be uptaken by roots, transported through the xylem, become endophytic microorganisms and retrieved into the fruits (Deering et al., 2012). Recently, many authors reported the widespread presence of human pathogens in the environment and their ability to overcome the natural plant barriers and colonize plant tissues (Berg et al., 2005). Endophytic microorganisms are usually not harmful to plants and they may play different roles regarding plant health and growth (Brader et al., 2017). Many environmental microorganisms can be detected in STW (i.e. strains of *Pseudomanas, Enterobacter, Herbaspirillum, Ochrobactrum, Ralstonia, and Stenotrophomonas, Pantoea*). These may colonize plant tissues and, to some extent, be beneficial to the plant host.

Heavy metals are usually subjected to different screening mechanisms in plants, such as insolubilization (Kosegarten et al., 2001; Bravin et al., 2008) and cell compartmentation at root level (Yang et al., 2006; Richau et al., 2009). Sometimes, they can be directly released into the root xylem and freely, or as chelated ions, translocated to the aerial part, including leaves, fruits or seeds through the xylem transpiration stream (Page et al., 2006; Page and Feller, 2005; Bhatia et al., 2005). The transpiration stream is mainly related to the crop anatomical features (e.g. hydraulic conductance, leaf/fruit surface conductance) and to environmental conditions (e.g. vapour pressure deficit). These represent the main factors affecting heavy metal accumulation rates in plant tissues. For instance, it is known that apple and peach trees 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, which that imply lower xylem flows compared to stone fruit species. Moreover, apple fruits (Drazeta at 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 behaviour between species can be associated to the difference in the mineral uptake and accumulation (e.g. stone fruit species are more nutrient demanding). On the other hand, the loss in xylem functionality might exert a barrier-like effect against pollutant accumulation on fruit tissues.

Given that, even if the WHO and FAO guidelines are quite flexible regarding the re-use of wastewaters in agriculture, most countries impose their own legislation, with different chemical and microbiological thresholds for contaminants, which are kept precautionary and indiscriminately very low (Mugdal et al., 2015). In Italy, the use of reclaimed water in agriculture is subjected to very strictly rules regarding polluting compounds (Italian Legislative Decree No. 185/03), despite evidences of potential polluting effects induced by STW as irrigation water in perennial species are still scarce.

In this context, the aims of this study was to assess the effect of irrigating fruit crops with STW (Decree of Ministry for Environment, No. 152/2006). The trial was conducted on two perennial crops, characterized by two different mechanisms of fruit growth. STW effects were evaluated on the potential partitioning of minerals and heavy metals to vegetative and reproductive tissues and on the bacterial translocation/accumulation of waterborne pathogens (i.e. *E.coli*) in different plant tissues (i.e. shoot and fruits).

4.2. Materials and methods

4.2.1 Experimental design

A1-year pot experiment outdoors was performed at the experimental station of the University of Bologna, located in Cadriano (BO), on 3-years old nectarine trees (*Prunus persica* L. Batsch.) cv. Big Top grafted on GF 677 and apple trees (*Malus domestica* L.) cv. Gala Schniga, grafted on M9. Trees were individually grown in 40-L pots filled with an alkaline, poorly fertile sandy-loamy soil, trained as slender spindle, protected by a shading hail net. They were irrigated by micro-irrigation (four drippers per tree of 2 L h^{-1}) and managed in terms of pruning, pest and disease management and thinning according the regional Integrated Crop Management practices (ICM, 2010).

For each species, five replicates (single trees) were arranged in a randomized block design with three irrigation/fertilization strategies: a) secondary treated wastewater without external fertilization inputs (STW); b) tap water and mineral fertilization (TW+MF); c) an unfertilized control irrigated with tap water (TW). Irrigation started 65 and 48 days after full bloom (DAFB) for nectarine and apple trees, respectively. STW, in line with the Italian Legislative Decree No. 152/06, was locally obtained by the urban civil wastewater treatment plant (HERA S.p.a - Italian multi-utility). Briefly, incoming wastewater underwent a preliminary treatment to remove the coarse organic waste, then effluent water was directed into an equalization tank for the secondary biological treatments. Finally, peracetic acid was added to reduce the residual bacteria population prior to overflow outside.

Trees were irrigated twice a day to return the evapotranspiration (ETc) rate. ETc for the plants used in the experiment was calculated according to the Irrinet irrigation scheduling system, developed and made available over the Internet by the "Consorzio per il Canale Emiliano Romagnolo (CER)" of the Emilia-Romagna Region (www.irriframe.it). The environmental parameters needed as inputs by this web-based platform were obtained from the experimental farm weather station.

Treatments received the same irrigation volume (360 L $tree^{-1}$ $season^{-1}$) along the season of tap water (TW+MF and TW treatments) or secondary treated wastewater (STW). Nectarine trees of the TW+MF treatment received 14.2, 2.35, 8.96, and 0.72 g $tree^{-1}$ of N, P, K and Mg, respectively while apple trees received 7.83, 1.53, 5.97, and 0.49 g $tree^{-1}$ of N, P, K and Mg respectively, as commercial mineral fertilizers split in 3 interventions along the season.

4.2.2. Water chemical analysis

The main chemical parameters of STW and tap water (TW) were determined on samples collected biweekly and stored at 5 °C. The water pH was measured with a pH-meter XS PH510 (Eutech Instruments, Singapore) under stirring conditions and EC (electrical conductivity) was determined by a conductimeter (METERLAB, CDM 210; Radiometer Analytical, France); Concentration of macro micronutrients and heavy metals of liquid samples was determined by Inductively Coupled Plasma (ICP-OES, England); TOC (Total Organic Carbon) (Shimadzu TOC-

V CPN) and TN (Total Nitrogen) (Shimadzu TNM-1)SAR was calculated according to (Richards 1954) using the formula (with concentrations in meq L^{-1}): SAR = [(Na+)/ [(Ca^{2++} Mg^{2+})/ 2] 1/2.

4.2.3. Tree organ mineral partitioning

During mid-summer, ten mature leaves per tree were collected from randomly selected annual shoots. Petioles were removed, leaves were treated as described in Sorrenti et al. (2012) and then analyzed for macro and micronutrients concentration. Briefly, P, K, Ca, Mg, S, Fe, Cu, Zn, Mn, Al, B, Ba, Cr, Cu, Fe, Mn, Na, Ni, Pb, Sn, Sr, Zn were determined by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES), (Ametek Spectro Arcos EOP, Kleve, Germany), after digestion with nitric acid (HNO_3) by a microwave lab station (Ethos TC-Milestone, Bergamo, Italy).

At commercial harvest, 4 representative fruits per replicate were collected and used to determine fruit peel and fruit pulp mineral concentration, as previously described.

4.2.4. Wastewater microbiological parameters

Microbiological analyses of STW were carried out periodically by sampling water directly from the wastewater treatment plant.

The microbiological quality of TW respected the Italian Legislative Decree n. 31/2001.

Water was investigated 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. Samples were collected in 1 L sterile glass bottles and immediately brought to the laboratory for the microbiological analysis. 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* typically appears as blue/purple colonies, whereas coliforms appear as red/rose colonies. Testing for indole production and citocrome oxidase activity gave further confirmation of *E. coli* identity. *Salmonella* spp. detection was performed according to the procedure UNI EN ISO 19250:2013. Results for *E. coli* and TC were recorded as colony forming units CFU 100 mL^{-1} and absence/presence for *Salmonella* spp. TBC was enumerated in Plate Count Agar (PCA, Biolife, Milano, Italy) in water samples previously diluted (incubation at 22 °C and 37 °C, 3-5 days) and results expressed as CFU mL^{-1}.

4.2.5. Microbiological analysis on shoots, fruits and soil

At ripening, shoot and fruit samples were collected from each tree from TW+MF and STW treatments. A number of 3 samples (both 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 knife and scissors, 10 g of shoots and 25 g of fruits were aseptically weighted into a sterile bag and 90 and 225 mL of BPW (Buffered Peptone Water), respectively, were added. Homogenization was carried out in a stomacher for 1-2 min and bags were stored at room temperature for 30 min to allow bacterial cell recovery.

Soil samples (10-30 cm depth) were collected, from TW+MF and STW treatments, four days after the end of the irrigation season. From each pot 4 different samples were collected and 10 g were added to 90 mL of buffered peptone water (BPW; Merck, Darm-stadt, Germany), the dilution was shaken in a stomacher for 1-2 min and bags were then stored at room temperature for 30 min to allow bacterial cell recovery.

Serial dilutions of shoot, fruit and soil suspensions were prepared and plated on PCA with 2 g/L cycloheximide (Sigma–Aldrich, Milan, Italy) and Chromocult agar. Plates were then incubated 3-5 days at 30 ± 1°C, then 24 h at 37 ± 1°C. Each target sample was replicated twice. After incubation, the number of colony forming units CFU g^{-1} was recorded, transformed into $\log_{10}$ values g^{-1} of soil and means and standard deviations calculated. From Chromocult plates, suspected *E. coli* (13 for apple and 17 for nectarine) were picked up, re-streaked and stored at - 80 °C for molecular characterization.

4.2.6. Microbiological genotyping of isolates and molecular identification

A 2 mL quantity of overnight cultures was sampled and 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 and the 16S rDNA amplification performed with universal primers 8f and 1520r. The amplified products were then purified (PCR clean-up; Macherey-Nagel GmbH & Co. KG, Germany) and delivered to Eurofins MWG Operon (Ebersberg, Germany) for the sequencing. Sequence chromatograms were edited and analyzed using the software programs Finch TV version 1.4.0 (Geospiza Inc., Seattle, WA, USA). DNAMAN software (Version 6.0, Lynnon BioSoft. Inc., USA) was applied to obtain consensus sequences, whose assignment to species or genera was investigated by matching them against all catalogued bacterial 16S rDNA sequences, by using the nucleotide BLAST (Basic Local Alignment Search Tool; http://www.ncbi.nlm.nih.gov/BLAST/).

4.2.7. Statistical analysis

Mineral concentration data were analyzed according to a randomized block design and averages were separated by 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).

4.3. Results and Discussion

The chemical elements concentration in the irrigation water was always higher in STW than in TW (Tab. 1) for all analysed parameters. STW showed a moderately alkaline pH, while the relatively limited EC and SAR index indicate low salinization risks. These values remained within the Italian legal thresholds for agricultural purpose (Decree of Ministry for Environment, No. 185/2003). TOC was almost 10-fold higher in STW, while concentration of N (particularly the nitric form), P, K and Zn was quite higher respect to the TW.

4.3.1. Effect of irrigation strategy and genotype on the leaf, fruit peel and pulp mineral concentration

Leaf nutritional status (macronutrient concentration) was overall positively affected by the mineral fertilization and by STW-irrigation, compared to the unfertilized control (Perulli et al., *in press*), with values within the optimal range for the same nectarine variety (Sorrenti et al., 2016) and apple species (Aichner and Stimpfl, 2002). The positive effect induced by STW on tree nutritional status, compared to the unfertilized control is likely a consequence of the plant-available nutrient content dissolved in the wastewater, which contributed to fertilize trees.

Without interaction between factors (genotype and irrigation strategy), leaf Cu, Na, Sn and Sr concentration resulted statistically higher in nectarine trees. On the contrary, the concentration of Ni and Pb was higher in apple leaves (Tab. 3). Regardless of the genotype, leaf Al concentration showed significantly lower values in mineral-fertilized trees, whereas no effects on leaf Cr, Cu, Ni, Pb, Sn and Sr concentration were attributed to the irrigation strategy (Tab. 3). Factors interacted for the leaf B, Ba, Fe, Mn and Zn concentrations (Tab. 3), without a clear trend attributable to the species or to the irrigation treatments. However, it is worth mentioning that leaf mineral concentration values were far below toxic levels, indicating how wastewater did not promote undesirable heavy metal accumulation in leaf tissues.

Without interaction, genotype did not alter fruit peel Al and Sn concentration (Tab. 4). Similarly, concentrations of Al, Cu, Ni, Pb, Sn and Sr in the fruit peel resulted unaffected by irrigation strategies (Tab. 4). However, regardless of the water source, fruit peel Cu, Ni and Sr concentrations showed higher values in nectarine than in apple fruits, whereas this latter was characterized by higher values of Ba and Pb (Tab. 4). Interactions between factors occurred for fruit peel B, Fe, Mn, Na, Sb and Zn concentration (Tab. 4). For these elements, irrigation with wastewater induced values generally comparable with those of trees irrigated with tap water, either mineral fertilized or unfertilized, in both species. Slightly higher values were retrieved for B, Mn, Na and, in particular, for Zn in nectarine compared to apple fruit peel. Finally, the supply of wastewater increased only fruit peel Fe and Zn concentration in apple and nectarine fruits, respectively (Tab. 4).

Generally, the mineral concentration values were higher in fruit peel than in the pulp. However, trends observed in the fruit peel did not mirror those of fruit pulp (Tab. 5).

Except for Ba, Mn, Ni, Sr and Zn concentrations were significantly higher in nectarine than in apple fruit pulp, while comparable values emerged for Na, Pb, Sb and Sn (Tab. 5).

Regardless of the species, a significantly higher concentration of Ba was measured in the pulp of unfertilized compared to mineral fertilized trees, while intermediate values were observed in the wastewater-irrigated trees. Instead, no effects induced by the wastewater were appreciable on the concentration of Cu, Ni, Pb, Sn and Sr in the fruit pulp.

Irrigation strategies interacted for Al, Cu and Fe, although differences were of limited extent, unlikely referable to a specific species or irrigation strategy.

It is well proved that wastewater is considered a rich source of organic matter and other mineral nutrients for plant uptake. On the other hand, wastewater can be contaminated with trace elements, such as lead (Pb), copper (Cu), zinc (Zn), boron (B), cobalt (Co), chromium (Cr), arsenic (As), molybdenum (Mo) and manganese (Mn), many of which are toxic to plants, animals and human beings over time (Kanwar and Sandha,2000).

For this reason, the re-use of wastewater (e.g. sewage water) as irrigation water often rise the levels of heavy metals, such as Fe, Mn, Cu, Zn, Pb, Cr, Ni, Cd and Co, in the receiving soils (Rattan et al., 2005; Khan et al., 2008; Ullah et al., 2012), in vegetables and cereals and in their subsequent transfer to the food chain, causing potential health risks for consumers (McGrath et al., 1994; Kumar Sharma et al., 2007). As a consequence, there is an increasing risk of public exposure to heavy metals because of the consumption of food grown in field irrigated with wastewater (Sharma et al.,2007; Khan et al., 2008; Srinivasan and Reddy, 2009; Tijani, 2009; Hani et al., 2010; Chary et al., 2008). For instance, heavy metal concentration in plants grown in wastewater-irrigated soils was higher than grown in control soils (Khan et al., 2008; Singh et al., 2010). The same authors concluded that even the use of treated wastewater for irrigation could cause potential health risks in the long term, as these increased the contamination with Cd, Pb and Ni in the edible portions of vegetables. Similarly, Sachan et al., (2007) and Khan et al. (2012) retrieved that the bioaccumulation of Pb and Cr in vegetables was above the critical concentrations for plant growth.

However, this did not occur in our study. Indeed, independently of the water and fertilizer sources used to grown trees, mineral concentration, including heavy metals, assessed in the different tissues of both species remained within the acceptable limits imposed by the FAO heavy metal regulations for contaminants and toxins in food and feed (FAO/WHO Codex Alimentarius, 2003). This response is likely a consequence of the scarce mineral concentration (including that of the heavy metals) measured in the waters used as irrigation source, with values below limits imposed by legislation (Italian Legislative Decree No. 152/06).

For some elements (e.g. Ba, Mn) the high concentration measured in the tissues of the unfertilized trees compared to those of the other treatments can be due to the dilution and partitioning effect induced by a larger vegetative biomass of these trees.

As expected, concentration values were overall higher in nectarine than apple trees, confirming that stone fruit species are generally more nutrient-demanding that pome fruits. The last assumption was verified also for fruits, despite nectarine fruits were harvested 1 month earlier than apple. This behaviour could be also related to the to the xylem dysfunctionality that affects only apple fruits at about 120 DAFB.

4.3.2. Wastewater microbiological quality

The analyses of the main microbiological safety indicators related to STW are shown in table 8. Seasonal average enumeration of *E. coli* was 4 CFU 100 mL^{-1} in the STW. Although being a secondary treated wastewater, the detected number is even under the Italian limit values for a tertiary treated wastewater (Ministerial Decree no. 185/2003; value < 10 CFU 100 mL^{-1}). These data were also in accordance with the analyses performed by the local wastewater management company (data not shown). The second main indicator, *Salmonella* spp., was never detected. The additional data of TC, TBC 22 °C and TBC 37 °C were 4245 (CFU 100 mL^{-1}), 13725 (CFU mL^{-1}) and 7950 (CFU mL^{-1}), respectively (Tab. 8). Italian legislation does not establish any threshold concerning these parameters; however, analyses have been performed to have information about STW bacterial load.

4.3.3. Soil microbiology

Soil microbiological analyses showed similar values for both treatments and plant species (Tab. 9), with TBC ranging from 5.0 to 6.5 $\log_{10}$ UFC g^{-1} and TC around 2.5 $\log_{10}$ CFU g^{-1}. Values for TC are in agreement with Vivaldi et al. (2013) who performed a similar trial on nectarines trees in open field condition. However, these results are strictly influenced by the intrinsic water and soil properties. The high microbial load found in wastewater (Tab. 8) seemed not to influence the soil microbiological parameters, indicating that the incoming microorganisms were quickly subjected to death or injuries that probably prevented their lab cultivability (Langet al., 2007; Van Elsas et al., 2011). *E. coli* was not isolated in any of the analyzed soil samples, although some CFU were present in the wastewater.

4.3.4. Shoot and fruit microbiology

Figures 1 and 2 show the internal microbial content related to shoots and fruits after sterilization of the external surface. All the rinses analyzed to test the efficacy of the sterilization were negative in all samples (data not shown), thus showing the complete efficacy of the surface sterilization step. As shown in Fig. 1, nectarine fruits data on TBC reported similar values for both treatments with a count for all samples relatively low between 0.5 and 2.5 $\log_{10}$ CFU g^{-1}. On the contrary, TBC in shoot samples reached an average value ranging from 3.0 to 3.5 $\log_{10}$ CFU g^{-1} in TW+MF and STW, respectively. TC were absent in both shoots and fruits of the TW+MF treatment, whereas in the STW-irrigated tree TC were found only in two plants and, more precisely, in the fruit of W5 and shoots of W5 and W1 (2.5, 2.7 and 2.6 $\log_{10}$ CFU g^{-1}, respectively) (Fig.2).

Concerning apple fruits (Fig. 1), TBC was positive in only two plants (out of five) for both treatments (values ranging from 1.2 to 2.2 $\log_{10}$ CFU g^{-1} in TW+MF and STW, respectively). TBC in apple shoots was 1.8 $\log_{10}$ CFU g^{-1} only in one plant of the TW+MF and an average of 3.0 $\log_{10}$ CFU g^{-1} in four of the treated plants. As for nectarines, TC in TW+MF plants were completely absent in both shoots and fruits and in the fruits of STW plants (Fig.2). Shoots (2 out of 5) had an average of TC of about 3 $\log_{10}$ CFU g^{-1}.

Analysis on nectarine trees showed that all plants had an endophytic community, as revealed by the TBC in the TW+MF (Fig. 1). Overall results on TBC and TC suggest a general increase, even if not significant, of bacteria in plants irrigated with STW, except for peach fruits, where TBC is almost unvaried. This effect is surely due to the higher microbial load provided by wastewater in the soil solution that may enrich the endophytic community of STW plants. This behavior is also highlighted in TC count, where coliforms, that are ubiquitous aerobic microorganisms and abundant in secondary treated wastewater, have been found in two STW plants. Another important aspect is the higher TBC of shoots compared to fruits, thus indicating the lower water potential gradients ($\Delta\Psi$) and hydraulic conductance (K) of the xylem-to-fruit pathway and probably a more adverse microenvironment for bacterial colonization in fruits due to presence of organic acids and antimicrobial compounds in the growing fruits (Moing et al., 1998; Ryu, and Beuchat, 1998; Uljas and Ingham 1998).

Analysis on apple trees showed a similar situation concerning the increase of TBC in plants irrigated with STW. However, the endophytic microbial community was found to be rather low in TW+MF shoots, the TBC being detectable only in one plant, whereas in STW trees TBC it was positive in four plants (Fig.1). These data confirm that on shoots most of the influence on endophytic microbial load is due to the STW supply. Moreover, TC are completely absent in the TW+MF trees (shoots and fruits) and in the fruits of STW trees, whereas they have been found in apple shoots (W1 and W2) irrigated with STW (Fig.2). The most relevant result for apple trees is the absence of TC in the fruits probably due to the fruit growth strategy, where the xylem becomes inactive at about 120 DAFB.

In both species it is evident how STW-irrigated trees had an impact on the total microorganism content in shoots, whereas no evidence was found for fruits. Based on our knowledge, there is no available data on perennial crops. Vivaldi et al. (2015) performed a similar experiment on nectarines but results were not comparable since authors only investigated the exterior microbial load on fruit peel.

However, comparing the two crops, nectarines showed a higher TBC than apples with all the samples showing bacteria presence (Fig.1). Moreover, no apple fruit was affected by the presence of TC (Fig.2). Even if, these results were not statistically significant, the different physiological processes related to the water potential gradients (ΔΨ), the plant hydraulic conductance (K) and the different fruit growth strategy (Morandi et al., 2016) of the two species, could help to explain the final bacteria plant internal colonization. In particular, leaf nectarine transpiration is higher compared to apple leaves with fruits that, unlike apple, have a higher water loss and are not subjected to xylem dysfunctionality during the season (Drazeta at al., 2004). Indeed in apple the water transport is then limited, determining, as a consequence, a reduced bacterial internalization.

4.3.5. Microbiological molecular identification

Another important aspect derived from the analysis was the isolation of thirty suspected *E. coli* colonies in Chromocult plates, that were detected only in STW-irrigated trees. However, the sequencing results did not confirm the assignment to the species *E. coli*. First, the cluster analysis allowed the identification of three sub-clusters for nectarine isolates and five for apple isolates (Tab.10). The further sequencing of the selected strain (one for each sub-clusters) and the consequent sequence alignment in the NCBI tools (Fig.3, 4) led to the identification of these bacterial strains as *Pantoea* spp. with a similarity of 99% (Tab.10).

Chromocult agar is a bacteriological growth medium developed for the simultaneous detection of total coliforms and *E. coli* due to the inclusion of two chromogenic substrates. Dark blue colonies resulting from salmon-galactoside and X-glucuronide cleavage by β -D-galactosidase and β -D-glucuronidase are usually classified as presumptive *E. coli* colonies. Our results outlined the presence of false positive as already reported by Finney et al. (2003) who found, among enterobacteriaceae, species showing β –glucuronidase activity.

From these results irrigation with wastewater would not cause potential health problems, since no *E. coli* have been found in the vegetative and reproductive tissues of the plants. TC have been isolated in four out of ten plants irrigated with STW but in limited number and most of them only in shoot tissues.

TC and *Pantoea spp*. detection probably derived from the STW irrigation continuously applied to STW trees for all the season, since no TC and *Pantoea spp*. have been isolated in the TW+MF plants.

Although among these bacterial species some strains could be virulent for human and plants, they are not considered as potential foodborne hazardous organisms. Actually, coliform bacteria and *Pantoea spp.* are ubiquitous in the environment and they can persist as endophytic microorganism, therefore not harmful, in plant tissue. In some cases, most of them can also possess Plant Growth Promoting activity and therefore be beneficial for plants (El-Gleel Mosa, 2016).

4.4. Conclusions

Treated wastewaters as a source of irrigation water in agriculture is mentioned among the most important alternatives for renewable water in many countries, especially those that suffer from a shortage of traditional water resources. In our conditions, the use of STW did not promote accumulation of potentially toxic elements (i.e. Cd, Cu, Fe, Mn, Pb) in both shoots and fruits of nectarine and apple trees, with concentration values within limits internationally imposed for the human consumption.

Regarding soil and plant microbiological parameters, results showed that the use of STW in irrigating nectarine and apple crops was not a hazard for the environment and for the food safety. The absence of *E. coli* and the stable load of TC into the soil were particularly important since the soil system could be a reservoir of harmful bacteria. The absence of *E. coli* and the few TC detected in the vegetative and reproductive plant tissues were not representing a food hazard for human health. Our results indicate that STW, if properly controlled, can be safely adopted as water source to irrigate perennial crops, also based on their different anatomical and physiological features (i.e. xylem dysfunctionality). Although this study may represent a first step to promote STW re-use in agriculture, the long term effect of the STW irrigation in the potential heavy metal and bacterial soil and plant accumulation remains unexplored.

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4.6. Figures and Tables

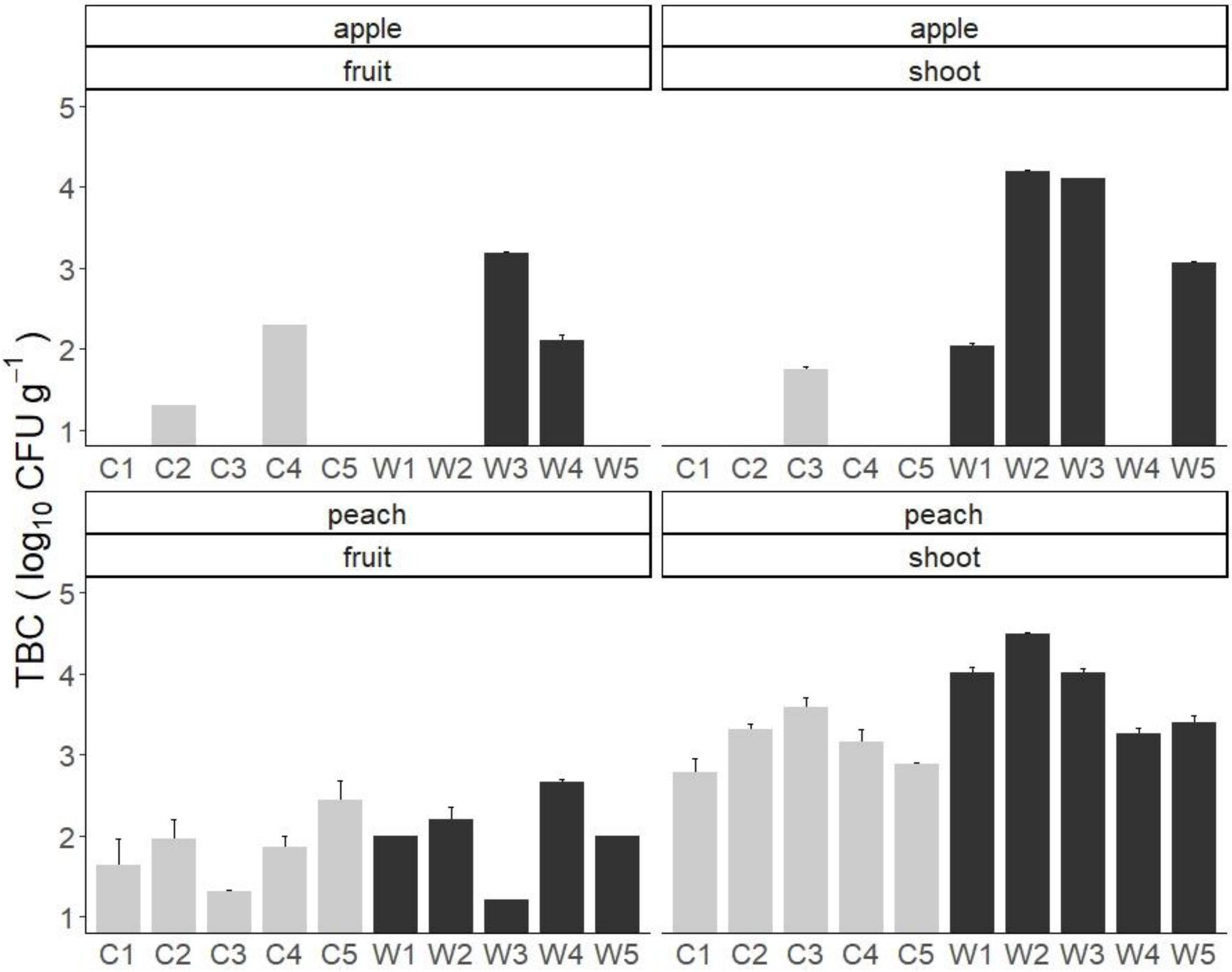

Fig.1. Total bacteria count in apple (fruits and shoots) and nectarine (fruit and shoots). C and W stand for mineral-fertilized and wastewater strategy. Each bar represents a mean of the two replicates.

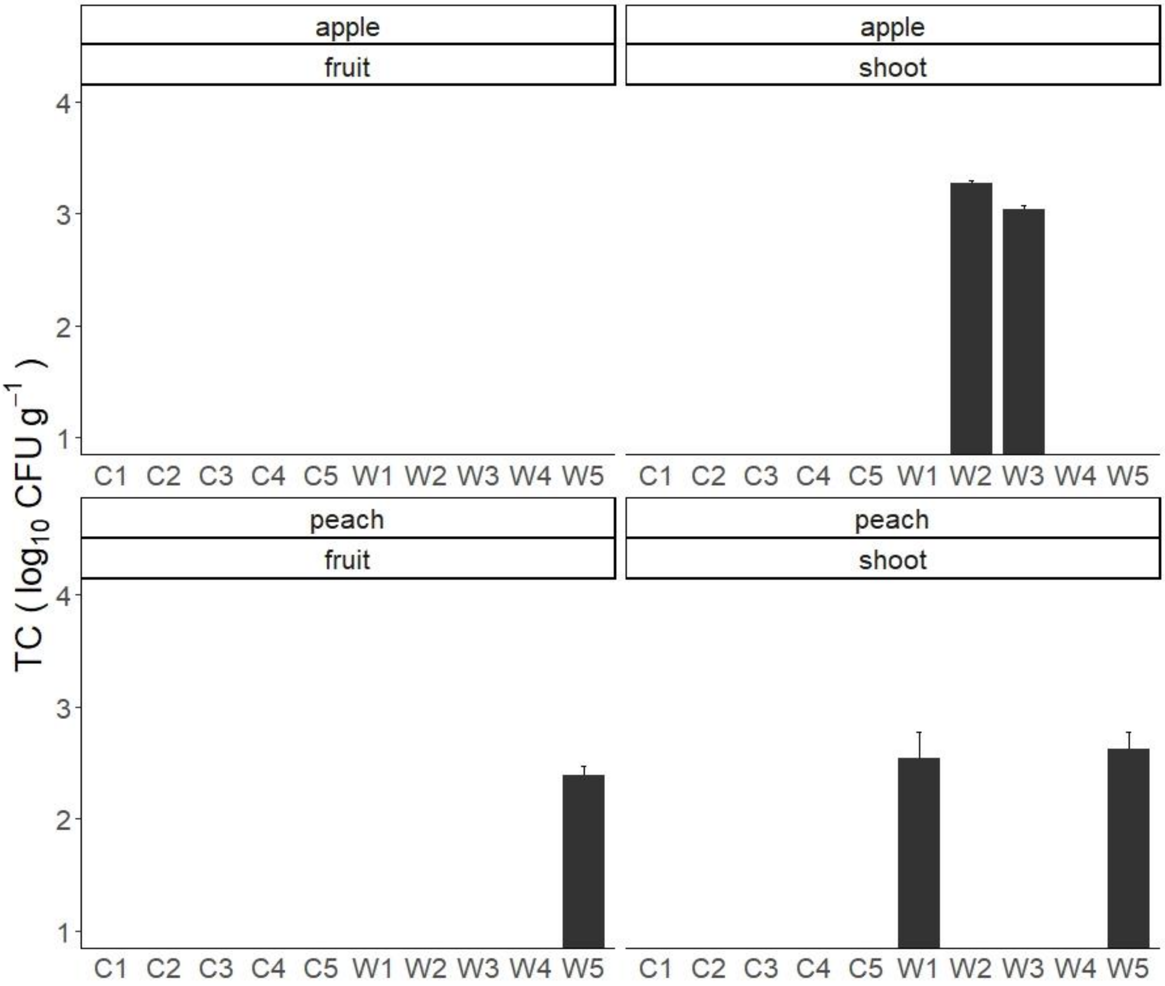

Fig. 2. Total coliforms in apple (fruit and shoot) and nectarine (fruit and shoot). C and W stand for mineral-fertilized and wastewater strategy. Each bar represents a mean of the two replicates.

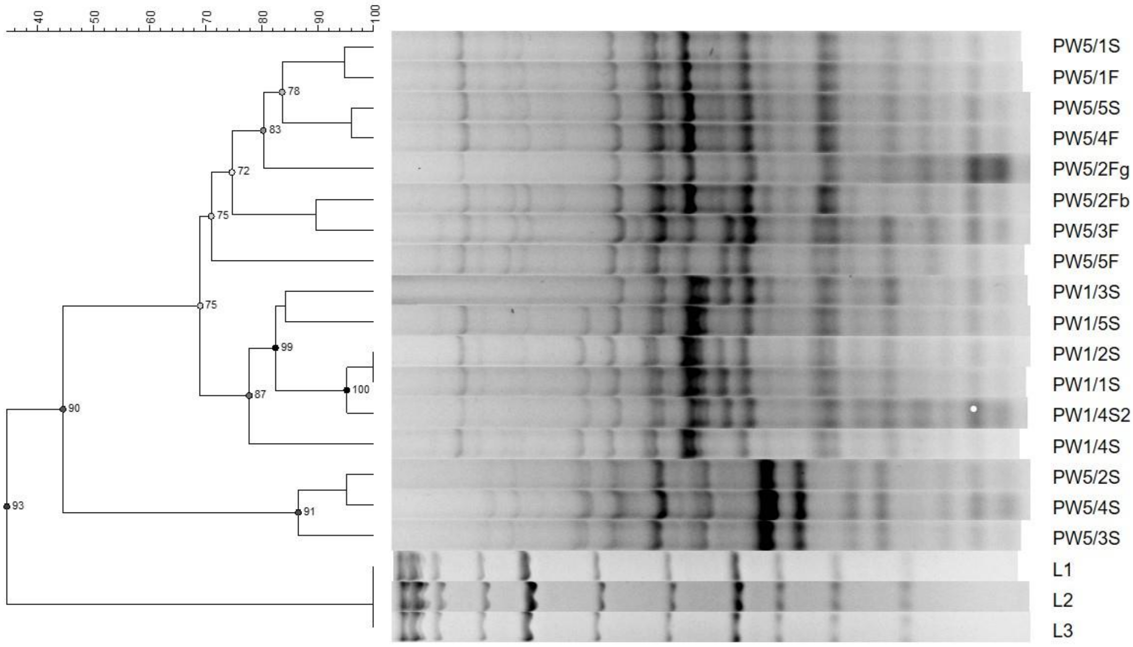

Fig. 3. Cluster analysis of ERIC-PCR fingerprints of isolated strains of nectarine shoots and fruits irrigated with STW.

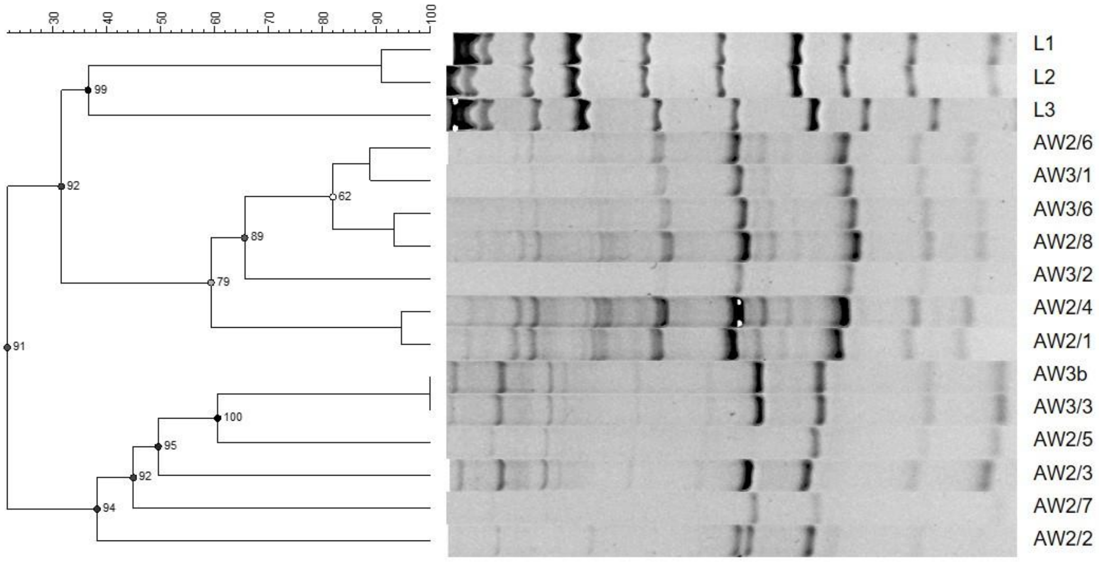

Fig. 4. Cluster analysis of ERIC-PCR fingerprints of isolated strains in shoots of apple trees irrigated with STW.

Table 1. Chemical characteristics of the irrigation water (n=7 ± SE).

Chemical Parameters	**Irrigation water**	
	[1]STW	[2]TW
pH	8.32 ± 0.92	7.43 ± 0.04
EC (dS m^{-1})	1.25 ± 0.04	0.47 ± 0.01
SAR	1.85 ± 0.04	0.63 ± 0.03
NH_4-N (mg L^{-1})	1.30 ± 0.09	0.02 ± 0.01
NO_3-N (mg L^{-1})	12.3 ± 0.74	3.30 ± 0.60
P (mg L^{-1})	4.50 ± 0.33	0.03 ± 0.01
K (mg L^{-1})	22.1 ± 0.68	4.81 ± 1.10
Ca (mg L^{-1})	98.6 ± 3.72	57.3 ± 5.91
Mg (mg L^{-1})	32.1 ± 2.12	17.2 ± 1.92
S (mg L^{-1})	34.0 ± 1.25	17.1 ± 1.24
Na (mg L^{-1})	82.9 ± 1.04	20.7 ± 0.73
Cu (μg L^{-1})	11.7 ± 1.39	6.10 ± 1.10
Fe (μg L^{-1})	22.9 ± 2.31	6.10 ± 0.50
B (μg L^{-1})	192.2 ± 6.47	83.7 ± 4.71
Zn (μg L^{-1})	50.7 ± 7.20	10.3 ± 1.70
TOC (mg L^{-1})	10.4 ± 1.71	1.13 ± 0.21

[1]Secondary Treated Wastewater, [2]Tap Water

Ag, Al, As, Be, Cd, Co, Cr, Hg, Mo, Sb, Sn, Ti, Tl, and V concentration either on STW or TW was below the instrumental detection limit (dl).

Table 3. Effect of the irrigation strategy and genotype on the leaf mineral concentration.

Leaf	Al	B		Ba		Cr	Cu	Fe		Mn		Na	Ni	Pb	Sn	Sr	Zn	
Irrigation		Peach	Apple	Peach	Apple			Peach	Apple	Peach	Apple						Peach	Apple
	mg kg^{-1}																	
TW	24.4 a	31.8 a	27.0 a	17.8 c	43.2 a	0.30	7.60	51.2 b	45.8 b	31.8 ab	23.0 b	75.0 a	0.73	1.18	94.5	15.6	18.5 bc	12.2 d
STW	22.5 a	28.0 a	24.0 a	20.0 c	49.0 a	0.33	7.90	54.8 b	32.6 b	30.0 ab	31.2 ab	68.3 ab	0.98	1.14	99.5	17.2	20.2 b	16.2 cd
TW+MF	16.0 b	14.0 b	25.2 a	13.2 c	30.4 b	0.29	8.50	54.2 b	90.0 a	27.2 ab	34.4 a	62.9 b	0.97	1.12	86.5	17.1	31.3 a	19.7 b
Significance	***					ns	ns					*	ns	ns	ns	ns		
Genotype																		
Nectarine	21.3					0.32	9.33 a					78.0 a	0.66 b	1.00 b	115 a	20.1 a		
Apple	20.6					0.29	6.66 b					59.4 b	1.14 a	1.29 a	72.1 b	13.2 b		
Significance	ns					ns	***					***	***	***	***	**		
Interaction	ns	***		*		ns	ns	***		**			ns	ns	ns	ns	**	

ns, *, ** and ***: effect not significant or significant at p ≤0.05, p ≤0.01 or p ≤0.001, respectively. Within the same element, means followed by the same letter are not statistically different (p ≤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).

Table 4. Effect of the irrigation strategy and genotype on the fruit peel mineral concentration.

Fruit Peel	Al	B		Ba	Cu	Fe		Mn		Na		Ni	Pb	Sb		Sn	Sr	Zn	
Irrigation		Peach	Apple			Peach	Apple	Peach	Apple	Peach	Apple			Peach	Apple			Peach	Apple
	mg kg^{-1}																		
TW	12.9	43.3 a	24.6 b	1.53 a	3.21	49.4 ab	37.5 b	9.04 a	5.79 c	48.0 ab	27.1 cd	0.72	1.56	0.80 b	1.02 a	1.08	4.47	12.1 b	2.45 c
STW	9.57	43.6 a	14.8 c	1.31 ab	3.38	39.3 ab	50.3 a	7.87 ab	6.55 bc	57.4 a	23.1 d	0.68	1.57	0.80 ab	0.96 ab	1.04	3.22	17.8 a	2.73 c
TW+MF	9.93	15.5 c	11.9 c	1.08 b	3.96	39.1 ab	44.1 ab	6.73 bc	7.46 abc	38.7 bc	19.6 d	0.66	1.64	0.88 ab	0.83 ab	0.98	2.89	12.3 b	2.44 c
Significance	ns			*	ns							ns	ns			ns	ns		
Genotype																			
Nectarine	11.3			0.93 b	5.25 a							0.87 a	1.46 b			1.09	4.29 a		
Apple	10.2			1.68 a	1.77 b							0.51 b	1.71 a			0.97	2.76 b		
Significance	ns			***	***							***	*			ns	*		
Interaction	ns	***		ns	ns	*		***		*		ns	ns	*		ns	ns	***	

ns, * and ***: effect not significant or significant at p ≤0.05 or p ≤0.001, respectively. Within the same element, means followed by the same letter are not statistically different (p ≤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).

Table 5. Effect of the irrigation strategy and genotype on the fruit pulp mineral concentration.

Fruit Pulp	Al		B		Ba	Cu		Fe		Mn	Na	Ni	Pb	Sb	Sn	Sr	Zn
Irrigation	Peach	Apple	Peach	Apple		Peach	Apple	Peach	Apple								
	mg kg^{-1}																
TW	3.44 ab	3.23 ab	25.6 b	31.8 a	0.45 a	2.06 b	1.76 b	9.10 ab	6.19 c	2.26	37.1 a	0.57	1.49	0.68	0.86	1.48 a	3.21
STW	2.81 b	3.84 a	27.7 ab	14.6 c	0.38 ab	2.40 b	1.75 b	6.43 bc	8.40 abc	2.10	29.6 b	0.60	1.64	0.87	0.87	1.09 ab	4.43
TW+MF	3.27 ab	2.95 b	9.00 d	10.1 cd	0.32 b	4.21 a	2.34 b	8.77 abc	11 a	2.33	24.8 b	0.62	1.55	0.76	0.75	1.05 b	3.50
Significance					***					ns	***	ns	ns	ns	ns	**	ns
Genotype																	
Nectarine					0.26 b					3.03 a	31.9	0.83 a	1.53	0.71	0.87	1.37 a	5.89 a
Apple					0.51 a					1.42 b	29.1	0.37 b	1.59	0.83	0.79	1.04 b	1.54 b
Significance					*					***	ns	***	ns	ns	ns	**	***
Interaction	*		***		ns	**		***		ns	ns	ns	ns	ns	ns	ns	ns

ns, *, ** and ***: effect not significant or significant at p ≤0.05, p ≤0.01 and p ≤0.001, respectively. Within the same element, means followed by the same letter are not statistically different (p ≤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).

Tab. 6. Enumeration of bacterial indicators in the secondary treated wastewater (STW) (n=7 ± SE).

Water source	STW
Target microorganisms	
E. coli (CFU 100 mL^{-1})	4 ± 2
Total Coliforms (CFU 100 mL^{-1})	4245 ± 416
Salmonella spp. (presence/absence)	0
Total Bacteria Count 22 °C (CFU mL^{-1})	13725 ± 2258
Total Bacteria Count 37 °C (CFU mL^{-1})	7950 ± 1527

Tab. 7. Enumeration of the bacterial indicators in soil samples in TW+MF and STW treatments. TW+MF and STW stand for mineral-fertilized and wastewater strategy. Each value represent the mean of two samples, each of them replicated twice.

Soil	Nectarine		Apple	
Treatment	TW+MF	STW	TW+MF	STW
Target microorganisms				
TC (CFU 100 mL^{-1})	3.06 ± 0.30	2.13 ± 0.62	1.22 ± 0.51	2.29 ± 0.06
TBC 30 °C (CFU mL^{-1})	6.55 ± 0.09	6.39 ± 0.12	5.27 ± 0.06	5.14 ± 0.02

Tab. 8. Sequencing results from isolates of apple and nectarine fruits (F) and shoots (S) tissues irrigated with STW.

Tree species	Tissue	Cluster	Sequenced isolate	Identification
Nectarine	S	W1-4	W5-1F	*Pantoea* spp. (99%)
	S	W5-1		
	F	W5-1		
	S	W5-5		
	F	W5-6		
	F	W5-2		
	F	W5-4		
	S	W1-5		
	F	W5-5		
	F	W5-3		
Nectarine	S	W1-1	W1-2	*Pantoea* spp. (99%)
	S	W1-3		
	S	W1-4		
	S	W1-2		
Nectarine	S	W5-3	W5-2	*Pantoea* spp. (99%)
	S	W5-2		
	S	W5-4		
Apple	S	W2-3	W2-3	*Pantoea* spp. (99%)
	S	W2-8		
Apple	S	W2-2	W2-5	*Pantoea* spp. (99%)
	S	W2-7		
	S	W2-5		
	S	W2-4		
	S	W2-1		
Apple	S	W2-6	W2-6	*Pantoea* spp. (99%)
Apple	S	W3-1	W3-5	*Pantoea* spp. (99%)
	S	W3-2		
	S	W3-4		
	S	W3-6		
	S	W3-5		
	S	W3-3		
	S	W3-7		
	S	W3-8		
Apple	S	W3-9	W3-9	*Pantoea* spp. (99%)

CHAPTER V

INVESTIGATING *ESCHERICHIA COLI* ROOT INTERNALIZATION ON GF 677 *(PRUNUS PERSICA X PRUNUS AMIGDALUS)*

5.1. Introduction

Research has recently paid attention to understanding the interactions between human enteropathogens and cultivated plants. The internalization of microorganisms depends on numerous factors such as the biology and serotype of bacteria, type, morphology, age of the plant, composition of root exudates, type of soil and climatic conditions (Hirneisen et al., 2012; Hoffman et al., 2014). *E. coli* is capable of evolving and colonizing new sites to respond to environmental variations and thus adapt to new habitats. Post-genomic studies demonstrate the ability of these agents to continuously evolve and form unrecorded serotypes (Newell et al., 2010). The survival capacity of these human enteric bacteria also allows a long-term persistence in the rural environment (Côté et al., 2005). The developmental stage of the root system highly affects the ability to internalize enteric bacteria, because pathogens enter very easily, especially through young roots and root hairs (Mootian et al., 2009). Radical exudates can influence the *E. coli* mechanism of internalization, both positively and negatively, given their variable composition that change depending on the plant species (Weir et al., 2010).

Internalization mechanisms can be divided in two categories: passive and active (Hirneisen et al., 2012). In the passive process, bacteria can enter the plant through the absorption of water and move inside the root system thanks to the mass flow of water (Matthews et al., 2014). This type of mechanism does not require direct action by bacteria. Several studies, especially on horticultural crops, have shown how human pathogens can penetrate internal plant tissues through the roots (Franz et al., 2007; Mootian and Matthews, 2009; Hintz et al, 2010), translocating and surviving then within plants (Lapidot and Yaron 2009; Martinez et al., 2015).

On the other hand, the active mechanism occurs when the process of internalization requires an active biological process from bacteria (Cooley et al., 2003). For example, when flagella are used to position themselves near the growing lateral roots to increase their possible internalization. This suggests that the active type process is the result of the interaction between plant and bacteria. In this case, bacteria can enter through the natural openings on the plant surface, which are usually present on the stem, in the roots and in the leaves such as lenticels, stomata (Kroupitski et al., 2009; Gomes et al., 2009), wounds (Brandl et al., 2002) or caused by biological or physical agents (Burnett et al., 2000). This ability of pathogens to attack plants is determined by the activity of hydrolytic enzymes (Kudryavtseva et al., 2013; Yarullina et al., 2016).

Concerning fruit trees, research has focused mainly on post-harvest contamination (Penteado et al, 2004; Mditshwa 2017). The processing methods such as cutting and packaging for fourth and fifth range fruit and vegetable products can represent another source of contamination and consequently a risk to public health (Augustin and Guillier 2018). Pathogenic enterobacteria such as *E. coli* can use plants as hosts or secondary carriers to subsequently infect animals or humans (Martınez et al., 2015; Garge et al., 2018). The internalized bacteria are protected from being removed by washing and disinfecting, so they could be a primary source of infection (Ximenes et al., 2017).

While we are sure that phytopathogenic genomes encode effector proteins to suppress the plant's innate immune response (Jones and Dangl, 2006), it is still necessary to ascertain that even human pathogenic bacteria possess capable effectors to suppress these immune responses of the plant. The human pathogenic bacteria must compete with the microflora present on the surface of the host plant but also with the microflora present inside (Schuenzel and Harrison, 2002). The native bacterial community has the potential to inhibit the growth of internalized human pathogenic bacteria (Liao and Fett, 2001). However, in some circumstances it has been shown that co-inoculation of human pathogens with some epiphytic microorganisms leads to an increase in the level of growth by human pathogens (Brandl, 2006). This positive synergism could be given by nutritional factors, due to the aggregation of several species in biofilms that improve survival (Brandl, 2006; Ximenes et al., 2017).

Pathogenic enterobacteria such as *E.coli* are normally found as a waterborne pathogen in wastewater sources. Their concentration limits change in respect to each country legislation for wastewater reuse in agriculture. In Italy, for example, the legislation threshold of *E.coli* concentration in wastewater to be reutilized in agriculture is 10 CFU/100 mL (Italian Legislative Decree No. 185/03). As regard to wastewater sources that are still not available to be reutilized in agriculture, as secondary treated wastewater, the *E.coli* Italian legislation threshold is 5000 CFU/100 mL (Italian Legislative Decree No. 152/06). We decided to test the effect of two strains of *E. coli* with concentration values, even higher than the Italian Legislative

Decree No. 152/06 and four fold higher in respect to the Italian legislation limit for wastewater reuse in agriculture. This study was performed to asses, in stressed conditions compared to the real wastewater *E.coli* concentration, on a horticultural rootstock such as GF677 (*Prunus persica x Prunus amigdalus*), the possible internalization and translocation process of one of the most hazardous enteropathogenic bacteria for human health as *E. coli*.

5.2. Materials and Methods

5.2.1 Experimental set-up

Experiments were carried out in the laboratory of DiSTAL, University of Bologna, in controlled conditions, on young, 3-month old, micropropagated plants of GF 677 (*Prunus persica x Prunus amigdalus*) potted in a mixture of peat and sand (1:1, v/v). Standard irrigation was applied.

During the experiments (Test 1-2), plants were kept in the phytotron for 4 days under artificial light and stable temperature (23 °C) and relative humidity (90 RH%) conditions. Test 3 was performed at the end of May in the greenhouse for 35 day under natural light conditions and with the following average, maximum and minimum values of temperature and relative humidity, respectively: 24 °C, 28.8 °C, 17.6 °C and 85.5 %, 99%, 56 %.

5.2.2. Bacteria strains

The *E. coli* DH5α-GFPuv strain used for artificial inoculation were transformed with the plasmid pDSK-GFPuv (Wang et al., 2007). The plasmid stability and the growth rate of the transformed strain were tested *in vitro* on Luria-Bertani medium.

Mutants of *E. coli strain 1576* and *Pseudomonas syringae* pv*. syringae 4364* used in this experiment were obtained by natural selection for antibiotics resistance on Luria agar plates.

Inocula were prepared by cultures grown in liquid Luria-Bertani medium under moderate shaking at 27 °C. After a 24 h incubation cells were centrifugated at 500 rpm for 10 minutes 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 Luria-Bertani medium containing 15 g L^{-1} of agar amended with cyclohexamide (100 mg L^{-1}) and specific antibiotics for each strain:

- *E. coli* DH5α-GFPuv kanamycin 100 mg L^{-1}
- *E. coli 1576* streptomycin 100 mg L^{-1} and rifampicin 50 mg L^{-1}
- *Pss 4364* streptomycin 100 mg L^{-1}

5.2.3. Plant inoculation

Test 1 and 2 were performed on 2 plants for each immersion time. Test 3 was performed on 3 plants. Plants were immersed in 50 mL of bacterial suspension and kept in the phytotron for 24 and 72 hours. The initial inoculum had the characteristics reported in Tab.1.

The first shoot portion not immersed in the suspension was considered the point 0. After 24 and 72 hours from inoculum, the entire non-submerged aerial part of the plant was divided into segments of 0-5 cm (for the root test) and 0-10 cm (for the shoot test) and the bacterial population was assessed by washing in $MgSO_4$ and plating the sequential dilutions. The submerged part /roots were instead evaluated separately.

The bacteria population on the different portions was assessed by washing, homogenizing in MgSO4 and plating the sequential dilutions. To assess endophytic populations, roots samples after washing stage, were surface-sterilized by washing in ethanol (70%, 2 minutes) and NaOCl (0.1%, 2 minutes), followed by two rinses in sterilized water. Then they were homogenized in 10 mM $MgSO_4$ and the bacterial population was assessed as previously described. The complete efficacy of the sterilization method was confirmed by comparing the epiphytic population of unsterilized and sterilized roots.

5.2.4. Shoot test (Test 1)

GF677 plants were cut at the collar and immersed in the bacterial suspension and evaluated at 24 and 72 hours from the inoculation, in order to promote a systemic plant invasion.

5.2.5. Root test (Test 2)

The second experiment followed the same protocol of the first test, but utilizing rooted plants instead of shoot cuts.

Finally, to assess the evolution of possible symptoms linked to the internalization of *E. coli* a sub-set of uncut plants coming from Test 2 were potted and kept in greenhouses for 35 days (Test 3). Plants were inoculated at the root level, via bacterial suspension, under controlled conditions. The point of the plant collar that was no longer wet by the solution is indicated as point 0.

For all tests, in order to have a reference model, a *Pseudomonas syringae* pv. *syringae* 4364 strain was used to observe its absorption and translocation in comparison to that of *E. coli*.

5.3. Results

5.3.1. Test 1 - Shoot internalization

At 24 hours in the external part of the submerged shoots, bacteria were assessed in the following concentrations: 2.35, 5.1, 2.45 Log_{10} CFU g^{-1} for DH5α, *E. coli 1576* and *Pss 4364*, respectively.

E. coli 1576 was the most present epiphytic microorganism.

At 72 hours, *DH5α* increased its concentration (3.3 Log_{10} CFU g^{-1}) while *E. coli 1576* and *Pss 4364* kept their values stable as epiphytes (5 and 2.7 Log_{10} CFU g^{-1}). Instead, the same bacteria decreased as endophyte, from 4.97 to 3.56 Log_{10} CFU g^{-1} for *E.coli 1576* and from 2.80 to 0.87 Log_{10} CFU g^{-1} for *Pss 4364*, respectively.

E. coli 1576 was the most present microorganism behaving as endophyte after 24 h of immersion (4.9 Log_{10} CFU g^{-1}) but decreasing its concentration already after 72 h (3.5 log10 CFU g^{-1}).

DH5α was not found as an endophyte bacterium after 24 h, unlike *E. coli*. *DH5α* was detected inside the plant (2.4 Log_{10} CFU g^{-1}) only after 72 h form the inoculation, showing higher values compared to *Pss 4364* (0.87 Log_{10} CFU g^{-1}).

No *DH5α* or *E.* coli *1576* were found in the aerial part in both biological replicates. Only Pss *4364* was detected above the zero point.

5.3.2 Test 2 - Root internalization

In the root system *DH5α, E. coli 1576* and *Pss 4364* proved to behave both as epiphytes and endophytes. All bacteria showed a higher concentration in the root surface at both 24 (2.4, 5.6 and 7.4 log_{10} CFU g^{-1} for DH5α*, E. coli 1576* and *Pss 4364,* respectively*)* and at 72 (4, 6.4 and 4.6

$\log_{10}$ CFU g^{-1} for DH5α, *E. coli 1576* and *Pss 4364,* respectively*)* hours. At 72 hours, *DH5α* and *E. coli 1576* increased as epiphytes (4 and 6.4 Log_{10} CFU g^{-1} respectively) compared to *Pss 4364* which decreased to 4.6 Log_{10} CFU g^{-1}.

The endophytic colonization for all the bacteria was always lower than the epiphytic, both at 24 hours (1.3, 4.4, 5.3 Log_{10} CFU g^{-1} for DH5α, *E. coli 1576* and *Pss 4364,* respectively) and at 72 hours (3.3, 5.2, 3.2 Log_{10} CFU g^{-1} for DH5α, *E. coli 1576* and *Pss 4364,* respectively).

DH5α and *E.* coli 1576 showed higher concentration as endophytes after 72 hours (3.4 and 5.2 Log_{10} CFU g^{-1}) compared to 24 hours (1.3 and 4.4 Log_{10} CFU g^{-1}), with higher values if compared to *Pss 4364* which instead decreased its initial concentration, from 5.2 to 3.2 Log_{10} CFU g^{-1}.

Above the zero point, in the aerial part of the plant, no presence of *DH5α* and *E. coli 1576* was found, as shown in table 2. The presence of *Pss 4364* was recorded in a single plant.

5.3.3. Test 3 - Root internalization after 35 days

After 35 days from the initial inoculation, *DH5α* was completely absent in both the external and internal part of the roots.

Only *E. coli 1576* and *Pss 4364* were found, as epiphytic, in the root system with the following concentrations: 1.3 $\log_{10}$ CFU g^{-1} and 6.2 Log_{10} CFU g^{-1}, respectively.

No bacteria were recorded in the areal part, above the zero point.

5.4. Discussion

These results highlight that *E. coli 1576* was able to internalize plant roots, but not to translocate to the aerial part of the plant (Tab 1 and 2).

Bacteria indeed can penetrate through young secondary roots, until the Casparian strip, where their movement is blocked. At the casparian strip level, water is indeed forced to move only through the cell membranes present in the symplast and just been then able to reach the xylem (Warriner *et al.,* 2003). Indeed, *E. coli* seems to colonize more easily the root environment (Fig. 3,4) than the shoot environment (Fig. 1,2).

Concentration and type of root exudates may influence the pathogenic chemotaxis to the roots (Klerks et al., 2007) and support bacterial growth within the rhizosphere.

Human pathogens can grow using root exudates as nutrients. Roots can show higher levels of colonization and internalization than the cut plant (Jablasone et al. 2005), as they might provide radical exudates and openings through which bacteria can be internalized in the plant.

Pseudomonas syringae pv*. syringae 4364* was chosen as a reference as this bacterium is a well known pathogen, known to attack all the commercially grown stone fruit species (Ogawa and English, 1991). *P. syringae* can survive in aquatic habitats in association with the roots of plants and therefore it can be absorbed by the roots.

E.coli epiphytic concentration, especially in the roots trial (Fig. 3,4) seems initially to increase (from 24 to 72h) but after 35 days it is more than halved. Generally, *E.coli* finds an hostile environment for its survival within the plant tissues. This is highlighted by the complete absence of *DH5α* both epiphytically and endophytically in the root system after 35 days from the initial inoculation. *E.coli 1576* instead was found only epiphytically in a low concentration (Fig. 5).

In the root surface, the survival of enteric pathogens can be hampered by competition for niches and for root exudates caused by indigenous epiphytes of the soil (Cooley *et al*., 2006).

Inside the plant tissue, the implementation of the plant defence systems and the competition between *E. coli* and the endophytic microflora (Liao and Fett, 2001; Deering *et al.* 2012) could be the main factors determining the lowest survival in this environment.

To reduce the microbiological plant-related contamination it is important to study the interaction mechanisms between the human enteric pathogens and the plants, as well as their internalization and persistence in the plant organism (Hirneisen et al., 2012; Hoffman et al., 2014).

5.5. Conclusions

This study highlighted how *E. coli 1576* was able to colonize and internalize plant roots. However, this bacterium was able to enter only roots, failing then to move inside the plant. It is interesting to notice how *E. coli 1576* was initially able to colonize the internal and external part of the roots but after 35 days, it was found only externally and in a low concentration. Its seems that *E.coli 1576* could be hampered by competition factors with the indigenous microflora present in the soil and in the plant (Schuenzel and Harrison, 2002).

These data are encouraging the application of secondary treated wastewater (Italian Legislative Decree No. 152/06) as an alternative source for irrigation. This water is still not available as a water source in agriculture, in open field conditions, especially for horticultural trees. Indeed horticultural tree irrigation is normally provided through a drip irrigation system, drastically reducing possible risks of external contamination of the tree canopy.

In any case future internalization studies should be conducted with enteric pathogens including realistic plant growth conditions, along with pathogen contamination levels encountered in a production system subjected to a reclaimed water irrigation.

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5.7. Figures and Tables

Tab.1. Inoculum concentrations for each bacteria (*DH5α-gpf, Escherichia coli 1576, Pseudomonas syringae* pv. *syringae 4364*) and for the control.

Inoculum	**Concentration (CFU mL $^{-1}$)**
DH5α-gpf	5×10^5
Escherichia coli 1576	1.23×10^5
Pseudomonas syringae pv. *syringae* 4364	1.93×10^5
Control	$MgSO_4$

Tab.2. Detection of *DH5α-gpf*, *E. coli* 1576 and *Pseudomonas syringae* pv. *Syringae 4364* in the aerial part of the plant. Data in the table refer to a single sample. Asterisk indicates the mean of 2 replicates.

	24h				**72h**		
cm	*DH5α-gpf*	*E. coli* 1576	*Pss* 4364	***cm***	*DH5α-gpf*	*E. coli* 1576	*Pss* 4364
0-10	0	0	2.3 Log_{10} CFU g^{-1}	0-10	0	0	2.2 Log_{10} CFU g^{-1}
10-20	0	0	0	10-20	0	0	0
20-35	0	0	0	20-35	0	0	3.6 Log_{10} CFU g^{-1} *

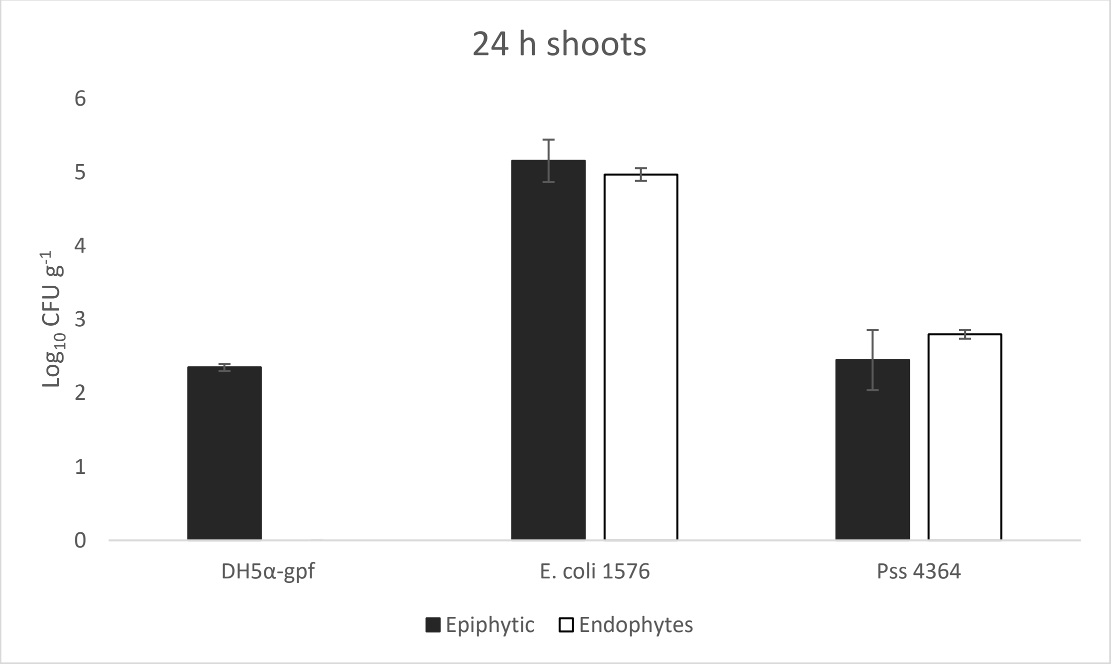

Fig 1. Mean (± SE) of *DH5α-gpf*, *E. coli* 1576 and *Pseudomonas syringae* pv. *Syringae 4364* concentration inside and on GF 677 external shoot surface of GF 677, after 24 hours of inoculation. Each value corresponds to the mean of 2 replicates.

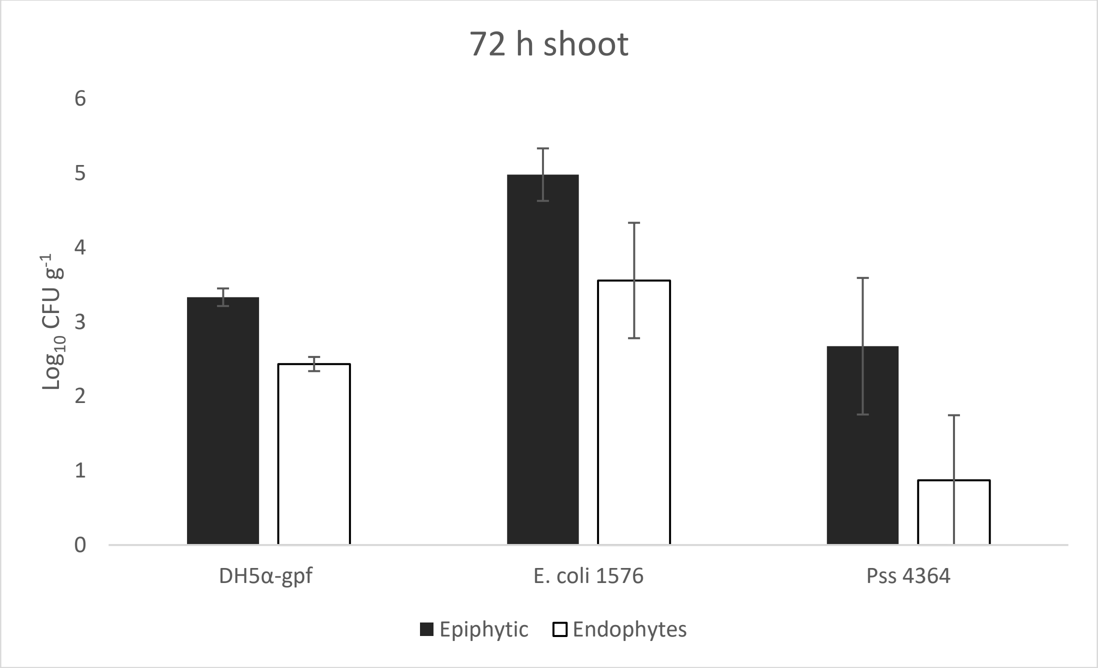

Fig 2. Mean (± SE) of *DH5α-gpf*, *E. coli* 1576 and *Pseudomonas syringae* pv. *Syringae 4364* concentration inside and on GF 677 external shoot surface of GF 677, after 72 hours of inoculation. Each value corresponds to the mean of 2 replicates.

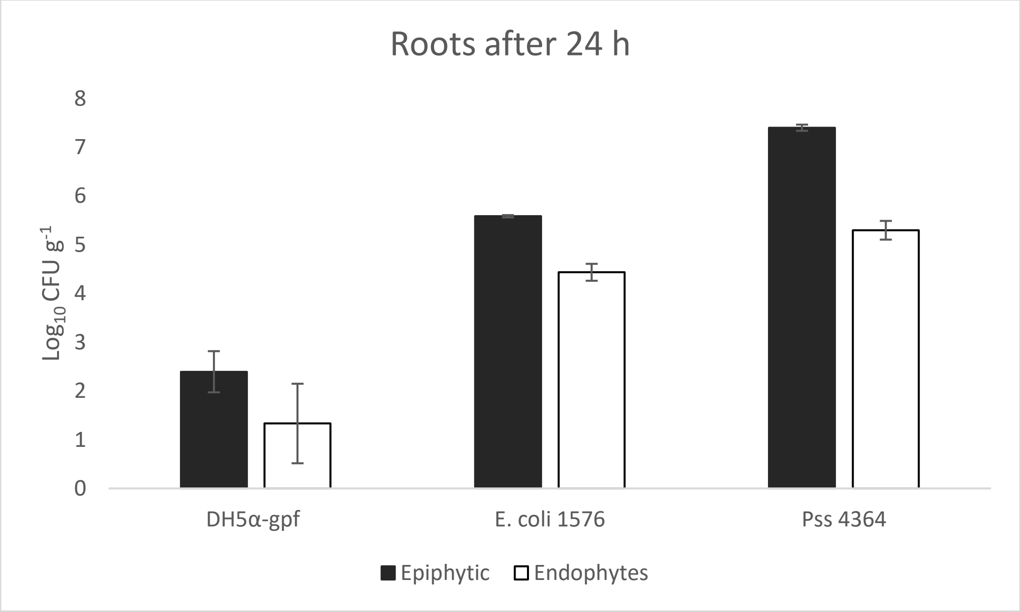

Fig 3. Mean (± SE) of *DH5α-gpf*, *E. coli* 1576 and *Pseudomonas syringae* pv. *syringae 4364* concentration inside and on GF 677 external root surface of GF 677, after 24 hours of inoculation. Each value corresponds to the mean of 2 replicates.

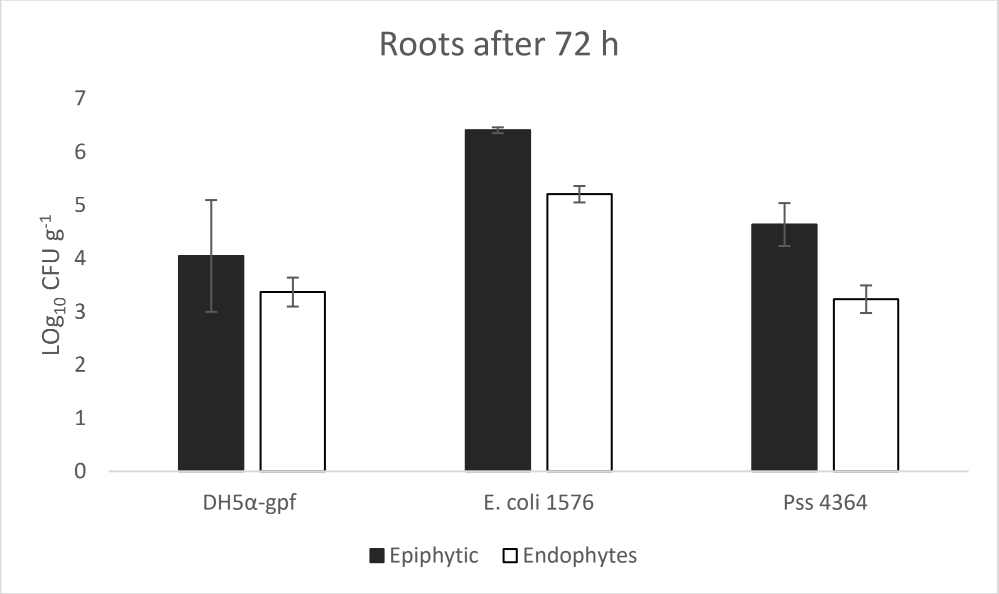

Fig 4. Mean (± SE) of *DH5α-gpf*, *E. coli* 1576 and *Pseudomonas syringae* pv. *Syringae 4364* concentration inside and on GF 677 external shoot surface of GF 677, after 72 hours of inoculation. Each value corresponds to the mean of 2 replicates**.**

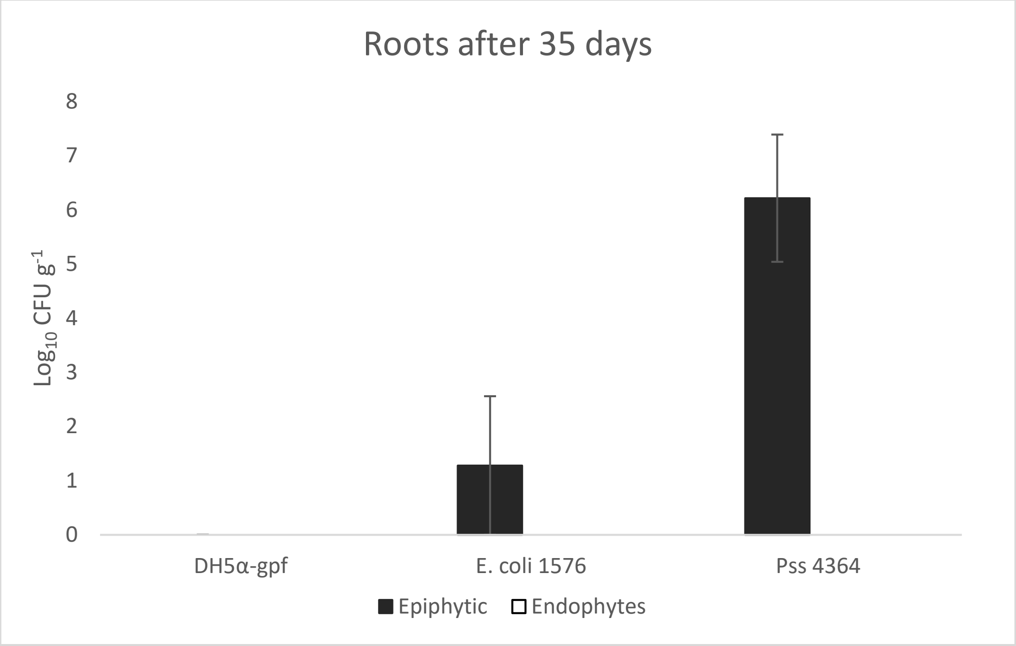

Fig 5. Mean (± SE) of *DH5α-gpf*, *E. coli* 1576 and *Pseudomonas syringae* pv. *Syringae 4364* concentration inside and on GF 677 external root surface of GF 677, after 35 days from the initial inoculation. Each value corresponds to the mean of 3 replicates.

CHAPTER VI

CONCLUSIONS

Results indicate how STW may be conveniently used as an alternative water source in drip irrigation and fertigation strategies of perennial crops, grown in temperate environments. Nectarine and apple trees irrigation with STW, for two subsequent seasons, did not induce any adverse responses on plant water status, leaf gas exchanges and tree vegetative growth performances, suggesting the lack of phytotoxic effects at tree level. A positive effect was also assessed on total canopy CO_2 assimilation with, no detrimental effects on fruit growth and quality parameters, for both species. STW positively influenced the nutritional status of both nectarine and apple trees (e.g. leaf N). Furthermore the physiological and anatomical differences between the two species on leaf and fruit growth mechanisms (i.e. xylem hydraulic conductance) could be seen as a discrimination factor to decide the quality level allowed for wastewater irrigation of one specific crop.

In general, the exclusive use of STW as irrigation source did not increase heavy metal accumulation, neither in the vegetative (i.e. leaves) nor in the reproductive tissues (i.e. fruit pulp) for both species, with concentration values within limits internationally imposed for the human consumption. STW may contribute but not fully meet plant nutrient requirements, which must necessarily be integrated by alternative sources (e.g. mineral or organic fertilizers). This has direct positive ecological and agronomical implications. In fact, recycling STW in agriculture allows at the same time to recover most of the N and the P typically contained in the wastewater and to reduce the need of mineral fertilizer in agriculture. Simultaneously, the use of STW would be beneficial also for the environment, limiting eutrophication problems.

Concerning soil and plant microbiological parameters, results showed that the use of STW for the irrigation of nectarine and apple trees was not a hazard for the environment and for food safety. The absence of *E. coli* and the stable load of TC into the soil were results particularly important since the soil system could be a reservoir of harmful bacteria. No *E. coli* and a few TC counts were detected in the vegetative and reproductive tissues of both species with very low concentration.

Moreover, the ability of *E. coli 1576*, in the GF 677 micropropagated plants, to colonize and internalize only roots and failing to move inside the plant was an additional confirmation of the previous results. These data are encouraging a widespread use of STW as a safe strategy in tree crop drip irrigation, according both to its chemical and microbial load and to the anatomical and physiological features of the plant.

Further studies are foreseen to deeply investigate how STW may influence tree physiology and pollutants translocation in trees grown in open field condition and for a prolonged time.