IJISET - International Journal of Innovative Science, Engineering & Technology, Vol. 6 Issue 6, June 2019
ISSN (Online) 2348 – 7968
www.ijiset.com

Effects of *Citrus sinensis* Irrigation with Treated Wastewater on Microbiological Quality of Soil and Fruits

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Abstract

The study aimed to evaluate bacterial contamination of soil and orange fruits when secondary TWW are used for irrigation. The study was carried out in north eastern Tunisia. The orchard was equipped for drip irrigation. The experimental plot was compared to a control irrigated by groundwater. Hygienic impact assessments on soil and fruits lasted three years (from 2015 to 2017). The results showed that irrigation with TWW caused significant contamination of the soil surface by faecal indicators (*E. coli*: 3.7 x 10^3 MPN g^{-1}ds). Most faecal bacteria introduced into the soil were retained at the 0-60 cm layer. These microorganisms did not survive for a long period. Five weeks after the last irrigation of the season, they have been almost totally eliminated. Soil and fruit samples were free of *Salmonella*. Fruit contamination by faecal indicators was negligible, which suggested that TWW can be useful as an alternative for drip irrigation of citrus orchards in water-scarce areas.

Keywords: Wastewater reuse, bacterial contamination, faecal indicators, citrus fruits, Health hazards.

1. Introduction

Land application of wastewater has become attractive from both an environmental and economic perspective. This practice is particularly prevalent in countries suffering from water scarcity. According to several authors [1, 2, 3, 4, 5], the use of urban wastewater for crop irrigation has been in recent decades a realistic way to reduce water shortages in many Mediterranean countries. With climate change, periods of drought are becoming longer and more frequent, affecting larger and larger areas. As a result, the reuse of wastewater for agricultural purposes is accelerating and is reaching more countries around the world [3, 6, 7, 8].

In addition to its contribution to mitigate the effects of water scarcity, wastewater irrigation is a means of combating pollution caused by the discharge of wastewater into the natural environment [9, 10]. It is also a free source of nutrients that can improve plant growth and yield [11, 12, 13] and reduce the use of chemical fertilizers [4, 14]. This decreases the costs of agricultural production and allows farmers to make a better financial return [12, 15].

In Tunisia, treated wastewater reuse in agriculture has become an integral part of the national strategy of mobilization and development of water resources for several years. This practice started in 1965 on a limited area where ground water irrigation could not continue because of the increasing salinity. At the beginning of the 80s, a real policy of wastewater reuse was launched and several irrigated schemes have been created across the country. Currently, the area equipped for reclaimed wastewater reuse is 8,500 ha [16]. Future programs planned to be realized in the short term will help create new areas of 7,000 ha as well as extending of old schemes of 5,000 ha. However, despite the efforts made and long experience in this field, the level of wastewater reuse is relatively low compared the potentialities. In 2017, the volume of treated wastewater reached 260 million m3, but only 24% of this volume was reused [16], the rest was discarded at sea or in rivers. The reuse of wastewater should experience rapid and significant development in the coming years because Tunisia is part of the WANA region countries where fresh water scarcity is reaching alarming levels [17]. The annual per capita renewable water supply is roughly 450 m^3, well below the United Nations threshold for classifying regions as water scarce (1,000 m^3 per year). The country is already consuming 99% of its renewable water resources. Moreover, climate change is already being felt in Tunisia through higher temperatures, more frequent heat waves, reduced rainfall and increased frequency of prolonged droughts [18]. In the coming years, the satisfaction of water needs of all sectors especially agriculture should be based largely on the massive development of non-conventional water. Since the desalination of sea water requires the use of expensive

technologies, TWW will be the main relay of fresh water in the agricultural sector.

Despite all the advantages mentioned above, agricultural wastewater reuse has some drawbacks: The nutrient richness of wastewater can pose a risk to crops. If not taken into consideration, the nutrients brought by the wastewater can cause a nutritional imbalance that could lead to problems of fruit maturity or a decrease in yields and product quality [9]. Some chemical constituents of wastewater are highly persistent and toxic even at very low dose. The most studied are heavy metals, Polycyclic Aromatic Hydrocarbons (PAHs), Polychlorinated Biphenyls (PCBs), and Persistent organic pesticides (POPs) that pose a real challenge to developing countries [4, 19]. Wastewater also carries various types of pathogenic microorganisms [20, 21, 22]. Some of these pathogens are able to escape treatment and be found in reclaimed wastewater [22, 23, 24, 25, 26]. So there are crucial health and environmental risks associated with using wastewater in agriculture. The health risk is particularly high when untreated wastewater is used for irrigating vegetables consumed raw [27, 28]. In order to protect public health and the natural environment, various measures can be taken including the construction of treatment plants as well as the development of standards and regulations. Some countries in the Mediterranean region such as Italy, Greece, Cyprus, Turkey and Jordan have developed their own standards for TWW irrigation [4, 10] while some others continue to draw on the flexible WHO guidelines [29, 30] to mitigate the risks.

Tunisia is a good example for constructing higher number of sewage treatment plants when compared to other countries in the region. By the end of 2017, the rate of connection to the public sanitation network in urban areas reached 85.9% and the number of treatment plants was 119 [16]. Moreover a regulatory framework has been developed since decades. Water law of 31 March 1975 prohibits the use of raw wastewater in agriculture. Treated wastewater intended for agricultural reuse must be in accordance with the Tunisian standard that sets an upper limit for many parameters such as COD, BOD_5, TSS, heavy metals and intestinal nematodes [31]. Treated wastewater can be used for restricted irrigation: All types of crops can be grown except vegetables, whether eaten raw or cooked. Regulation also stipulates that in case of fruit trees irrigation with TWW, fruits should be not collected from the ground [32]. Despite all these regulatory measures, the health risks associated with wastewater irrigation remains a major concern for farmers, policy makers and consumers. The potential for survival and accumulation of pathogens in soil, their movement to ground water and the transmission of infectious diseases through the consumption of contaminated agricultural products constitute a particular concern. Although many studies have been carried out in different countries on the fate of pathogenic microorganisms in agricultural soils and on various crops [5, 13, 33, 34, 35, 36, 37, 38], additional work must be done under the Tunisian climatic and environmental conditions.

The objective of this study was to investigate the microbiological contamination of soil during irrigation season and to assess the persistence of bacterial indicators after stopping irrigations. The hygienic quality of orange fruits produced on plots drip irrigated with TWW was also evaluated.

2. Materials and methods

2.1. The experimental site

The study was conducted in an experimental field station located in north eastern Tunisia near the coastal city of Nabeul. The altitude is 25 m above sea level. The climate is semi-arid, characterized by a rainy and frost-free winter. The average minimum temperature varies between 7.2 and 21.3°C depending on the month. The average maximum temperature is between 15.8 and 31.1°C. The prevailing winds are from north / northwest during the period from November to March. The station is located in a zone considered as relatively windy and the average annual rainfall is 427 mm.

The experimental plot is an orange orchard with an area of 1.8 ha and a sandy soil. The orchard was divided in two blocs: The first served as control and was irrigated by groundwater (GW) while the second was irrigated by treated wastewater (TWW). The spacing between the shafts is 6 x 4 m. Each orange tree row was irrigated by one double drip lateral. The emitters had a flow rate of 4l/h and were spaced 0.5 m on the drip laterals. TWW were secondary effluents obtained from an activated sludge treatment plant (SE4). The origin of wastewater was mainly urban. The orchard was irrigated from mid-April to mid- September. Around 250 mm of water were applied during each irrigation season.

2.2. Waters, soil and orange fruit sampling

The sampling and analysis were conducted during three successive years (from 2015 to 2017). Wastewater and groundwater samples were taken weekly during the irrigation season. They were collected randomly in the field from 10 emitters using 1000 ml sterile glass bottles and transported to the laboratory in a cool box at 4°C. When obtaining water samples from drippers, they were alcohol treated and flame sterilized.

Soil samples were collected from the GW and TWW irrigated plots before the start of the irrigation campaign, after 48 hours of the first irrigation and during the days following the last irrigation of the season. They were taken near the emitters at the surface level and at depths of 30, 60 and 90 cm. Sample collection was done using a manual auger. At the time of sampling, soil temperature was measured at the studied depths using a digital thermometer connected to a temperature probe. Before each soil sampling, the auger was cleaned with alcohol and sterilized with the flame of a blowtorch in order to prevent cross-contamination. Each sample taken from an experimental plot consisted of 5 subsamples randomly sampled in zigzag.

Fruit sampling was done at harvesting period which takes place between early December and late January according to the year and climatic conditions. From each of the two plots (control and TWW irrigated plot), 20 samples were taken each year. Fruits were picked directly from the trees, transported to the laboratory in sterile plastic bags and stored at 4 °C before analysis. Each sample was composed of five fruits.

2.3. Analytical procedures

All samples were analyzed within 24 h of sample collection. The bacteriological analysis consisted to enumerate the faecal indicators bacteria (FIB) and detect the presence of Salmonella. Soil moisture was determined on the same samples by drying at 105°C for 48 h.

Total coliforms (TC), faecal coliforms (FC), *Escherichia coli* (*EC*) and faecal streptococci (FS) were tested by multiple tube fermentation procedure and following a 3 replication x 5 dilutions scheme [39]. Results were expressed as most probable number (MPN) per 100 ml. The determination for Salmonella in water samples was performed as described by ISO 19250 [40]. Biochemical confirmation of presumptive Salmonella colonies was performed using the identification system for Enterobacteriaceae and other gram-negative rods: API 20E (Biomérieux). Serotype identification was performed by slide agglutination using monovalent and polyvalent anti-Salmonella serums (Sanofi).

Soil samples were subjected to an extraction-dilution step as follows: 10 g of each sample was weighed and added to 90 ml buffered peptone water, then homogenized in a stomacher for 3 minutes. The decimal dilutions in buffered peptone water were prepared and analysed with the same method than water samples. Salmonella determinations were carried on 10 g soil samples inoculated in 90 ml of Buffered Peptone Water (Merck) and incubated overnight at 37°C. Afterwards a 100 µl aliquot of culture was transferred to 10 ml Rappaport Vassiliadis Novobiocin broth and incubated for 24 h at 42°C. Isolation and identification of strains were conducted in the same way as for water samples.

For fruit analysis, 25 grams from each sample (peel and pulp) was weighed out and put into a sterile bag with 225 ml of buffered peptone water and homogenized in a stomacher for 3 minutes. Decimal dilutions of fruit samples were carried out using Tryptone Broth. Bacterial indicators were tested using the multiple tube fermentation procedure. Detection of *Salmonella* in fruit samples was performed according to the international standard ISO 6579-1 [41].

Soil and fruit results were expressed as MPN g^{-1}, respectively on dry and fresh weight basis.

2.4. Statistical analysis

Analysis of the results was performed using SPSS 20.0 software for windows (SPSS Inc., Chicago, IL, United States).

To characterize the bacteriological quality of irrigation water, geometric means ($GM = 10^x$, where x = the mean of $\log_{10}$-transformed values) were calculated from the results of the indicator counts.

To characterize the state of soil bacterial contamination before irrigation starts, and after the first and last irrigation of the season, geometric means were calculated from the results obtained during the three seasons 2015, 2016 and 2017. Before being processed, all the microbiological data from soil were logarithmically transformed ($\log_{10}$ X+1). When bacterial indicators were not detected in the soil, the corresponding levels were set equal to zero. The addition of the unit value to X was necessary to avoid impossible solution in case of X=0. The differences between means were tested with the Student-Newman-Keuls test at $p<0.05$

The bacteriological quality of the fruits was described by the arithmetic means of the results obtained from the samples taken each year.

3. Results and discussion

3.1. Bacteriological quality of irrigation water

TWW used for the irrigation of the experimental plots was heavily contaminated by faecal indicators. The geometric mean of *E. coli* varied from 7.7 x 10^3 to 2.5 x 10^5 MPN/100 ml, whereas that of faecal streptococci varied from 1.1 x 10^4 to 1.1 x 10^5 MPN/100 ml (table1). These concentrations are above the limits for wastewater irrigation in some countries such as Italy, Turkey, Jordan and island of Crete [4, 5, 10]. Tunisian standard [31] does not set a limit for faecal indicators in TWW reused for

restricted irrigation. Therefore, TWW from the SE4 plant can be used for citrus irrigation.

Table 1: Microbiological quality of treated wastewater and ground water used for irrigation

Year	Water type	*Escherichia coli* *			Faecal Streptococci *			Salmonella
		M	Min	Max	M	Min	Max	
2015	TWW	7.7×10^3	7.4×10^1	9.3×10^5	1.1×10^4	9.3×10^2	2.3×10^5	-
	GW	2.2×10^1	1.5×10^1	4.3×10^1	1.5×10^2	2.3×10^1	2.4×10^2	-
2016	TWW	2.5×10^5	9.2×10^3	2.4×10^6	1.1×10^5	3×10^2	2.4×10^6	+
	GW	$7{,}7 \times 10^1$	$4{,}3 \times 10^1$	$9{,}3 \times 10^1$	4.7×10^2	2.4×10^2	9.3×10^2	-
2017	TWW	8.6×10^4	3×10^3	9.3×10^5	5.8×10^4	9.2×10^3	9.3×10^5	+
	GW	7×10^1	2×10^1	2.3×10^2	1.6×10^2	4.3×10^1	7.5×10^2	-

n=20; *****: MPN/100 ml; TWW: Treated wastewater; GW: Ground water; M: Geometric mean; Min: Minimum; Max: Maximum.

Salmonella were found in 3 of 60 TWW samples analysed during the study period. These pathogens were detected twice in 2016 and once in 2017. The isolated strains belonged to two serotypes which were *S. enteritidis* and *S. Kentucky*. The presence of Salmonella in TWW samples was predictable because these pathogens are often found in urban wastewater [23, 42, 43, 44] and no disinfection treatment was applied to the effluents of SE4 plant prior to their reuse in irrigation. The frequency of Salmonella in TWW was rather low, with only 5% of positive samples. This result could be explained by the storage of wastewater before reuse: At the exit of the treatment plant, the TWW are directed towards a large basin that feeds the irrigation scheme where the experimental station is located. In the experimental field station, the TWW are stored in a second basin before being used for the irrigation of the experimental plots. The residence time of TWW within the two basins was not fixed because it varied according to the demand for irrigation water, but this storage probably helped eliminate some of the pathogens. Indeed, several studies have demonstrated a beneficial effect of reservoir storage on the microbiological quality of wastewater [45, 46, 47].

Ground waters used as control contained a relatively small number of faecal indicators. The average values of *E. coli* were less than 100 MPN/100 ml (Table 1). The faecal streptococci were a little more numerous, but their geometric mean did not reach 500 MPN/100 ml. All the analysed samples were free of salmonella. Groundwater contamination was relatively low compared to the numbers of faecal indicators frequently found in conventional water which may be used for irrigation of crops that are normally eaten raw [29, 48].

3.2. Bacteriological quality of soil

3.2.1. Initial contamination of soil with bacterial indicators

The initial bacteriological quality of the soil was evaluated two weeks before the start of the irrigation season. The obtained results (Table 2) showed that bacterial contamination level was low: The number of all studied indicators did not exceed 5 bacteria g^{-1}dry soil. No Salmonella was detected.

The low rates of soil moisture recorded particularly at shallow depths could partially explain the level of faecal contamination. Indeed, moisture is an essential component of bacterial life; its level in the soil has a considerable influence on the survival of microorganisms [49]. As sampling and analysis were performed before the first irrigation of the year, the soil of the two plots (control and TWW irrigated plot) had received during the last month only rainwater that does not carry faecal bacteria hence the low contamination level.

Table 2: Initial number of bacteria in the studied soils (MPN g^{-1} dry soil) and humidity (%)

Soil irrigated with groundwater						
Depth (cm)	TC	FC	E. coli	FS	Salmonella	H (%)
0	0.63 ± 0.1	Abs	Abs	0.82 ± 0.2	-	1.9 ± 0.3

30	0.74 ± 0.2	0.53 ± 0.1	0.53 ± 0.1	0.95 ± 0.1	-	6.8 ± 1.1
60	1.86 ± 1.1	1.06 ± 0.7	0.92 ± 0.3	2.1 ± 1.1	-	9.2 ± 0.1
90	0.52 ± 0.3	0.41 ± 0.1	Abs	1.6 ± 0.1	-	10.3 ± 0.2
Soil irrigated with treated wastewater						
0	1.61 ± 0.2	0.43 ± 0.2	0.43 ± 0.4	0.56 ± 0.2	-	2.1 ± 0.5
30	0.58 ± 0.1	0.92 ± 0.1	0.92 ± 0.1	0.97 ± 0.5	-	5.4 ± 0.6
60	1.75 ± 0.7	1.23 ± 0.6	1.23 ± 0.8	0.99 ± 0.6	-	8.7 ± 0.4
90	0.64 ± 0.4	0.15 ± 0.1	Abs	0.22 ± 0.1	-	9.8 ± 0.3

MPN: Most Probable Number; TC: Total coliform; FC: Faecal coliform; FS: Faecal streptococci; H: Humidity

3.2.2. Vertical distribution of faecal indicator bacteria in the soil after irrigation

After irrigation, the number of the studied microorganisms increased at all depths both in control soil and in the soil irrigated by TWW. Figure 1 illustrates the distribution of bacterial indicators in the profiles 48 hours after the first irrigation of the season. Compared to the initial state (Table 2), the increase of bacterial concentrations was generally low in control soil.

In the TWW irrigated soil, the increase of bacterial concentrations compared to their initial state was relatively large; it exceeded 2 log units for all the studied microorganisms between surface layer and 60 cm depth. This result confirmed earlier work that has shown a marked increase in microbial contamination of the soil surface after irrigation with treated wastewater [35]. At the depth 90 cm, the concentrations of the studied bacteria were slightly higher than those observed in the initial state, but the increase was less than 1 log unit. Wastewaters were probably stripped of most bacteria they carried before reaching this depth; their transition to this level did not result in significant soil contamination.

In TWW irrigated soil, TC, FC, EC and FS were slightly more numerous at 30 cm depth than at the surface, but the difference was not statistically significant. This result can be explained by the action of environmental factors such as heat, wind and sunlight that affect bacteria on the soil surface leading to their elimination. Côté and Quessy [50] observed an exponential decrease of *E. coli* number on the soil surface after manure application to a sandy loam soil.

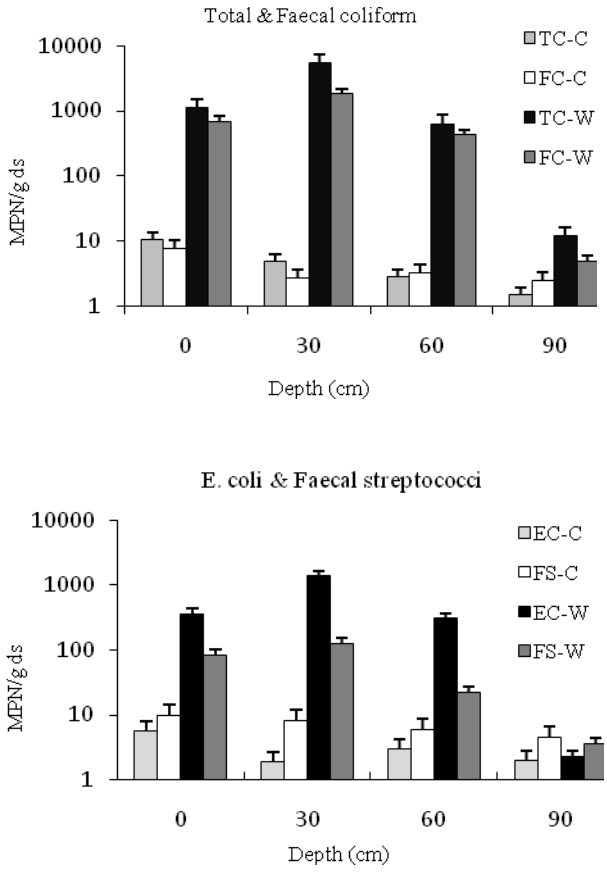

Fig. 1 Vertical distribution of bacterial indicators in experimental soils. TC: Total Coliform, FC: Faecal Coliform, EC: E. coli, FS: Faecal Streptococci, C: Control soil, W: Wastewater irrigated soil.

Other authors [51] found an almost complete elimination of bacteria and viruses from soil surface two days after irrigation with TWW. In this study, the adverse effect of environmental factors was not clearly visible after 48 hours because ground surface at sampling locations was shaded by tree foliage. The soil surface received only a very small proportion of solar radiation to which microorganisms are vulnerable. Moreover, no climatic phenomenon likely to

change quickly the state of the soil surface such as wind or excessive heat was recorded at the beginning of the studied irrigation seasons.

Table 3: Temperature and humidity of soils irrigated with GW and TWW at studied depths

Depths (cm)	Temperature (°C)		Humidity (%)	
	GW	TWW	GW	TWW
0	25.5 ± 0.2	26.7 ± 0.24	11.8 ± 0.2	11.5 ± 0.1
30	25.9 ± 0.2	27 ± 0.25	11.8 ± 0.15	11.4 ± 0.13
60	25.9 ± 02	27.1 ± 0.2	11.3 ± 0.1	11.1 ± 0.11
90	25.8 ± 0.1	26.9 ± 0.15	10.9 ± 0.1	10.3 ± 0.1

GW: Ground water; TWW: Treated wastewater

The soil temperature and humidity varied little with depth (Table 3) indicating roughly similar conditions across the studied soil layer. No significant correlation was found between the number of bacterial indicators and soil temperature. The variation of soil temperature according depth did not exceed 0.4°C which is too weak to cause a measurable effect on bacteria. Consequently, the bacterial concentrations recorded at the surface were not significantly lower than those determined in the soil where microorganisms were protected from the aggressive action of environmental factors in particular solar radiation which plays an essential role in the removal of faecal bacteria [52].

Fig. 1 shows that most of the bacteria introduced into the soil by irrigation water were retained in the 0-60 cm layer and that from the depth of 30 cm, the concentration of indicators decreased with increasing depths. These results were in agreement with those of other authors [49] who observed high reduction of the concentration of microorganisms when wastewater moves through the soil. According to these results, the soil performs as an efficient barrier, decreasing microorganism's concentration and therefore sanitary risk.

3.2.3. Persistence of bacterial indicators in the soil

3.2.3.1. Persistence in soil surface

Fig. 2 shows the bacterial contamination evolution of the experimental soils during the days following the last irrigation of the season. Examination of the overall results (not shown here) revealed that the first three indicators (TC, FC and EC) evolved in similar ways whereas faecal streptococci were generally more persistent than the other groups. To maintain the clarity of the graph, only two parameters were represented: *E. coli* and faecal streptococci.

In the control soil, the number of *E. coli* recorded after irrigation was relatively low. It decreased with time and reached values close to zero on the 6th day. The number of faecal streptococci was of a few tens of bacteria per gram of dry soil. It was maintained at about the same level during the days following irrigation. The minimum was reached after 36 days.

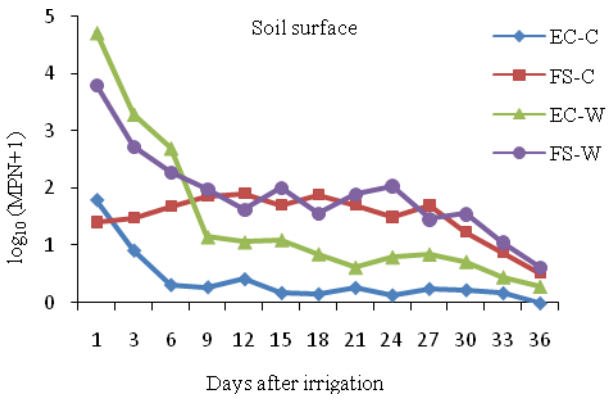

Fig. 2 Persistence of *E. coli* (EC) and faecal streptococci (FS) in soil surface. (C: Control soil; W: TWW irrigated soil)

After irrigation with TWW, the soil surface was heavily contaminated by faecal indicators the number of which decreased considerably during the first ten days to reach values similar to those recorded in the control soil. From the 15th day, the two faecal indicators followed the same evolution as that recorded in the control soil. The maximum reduction was reached 36 days after irrigation. It was of 4.42 and 3.18 log units respectively for *E. coli* and faecal streptococci. These results are in agreement with those of other authors [53] who found a significant decrease in the abundance of faecal bacteria in surface horizon after five weeks of irrigation with TWW from stabilization ponds. In this study, the decrease in the abundance of indicators was significant after 6 days of irrigation with TWW. The relatively rapid removal of faecal bacteria may be due to the use of a drip irrigation system. Some works [51] have shown that the application

of TWW by a drip system affected the survival of microorganisms.

3.2.3.2. Persistence in the soil profile

In the control soil profile, contamination by the faecal indicators varied slightly depending on sampling depth (Fig. 3). At the start of monitoring, the number of bacteria was of a few tens per gram dry soil whatever the indicator and the soil depth considered. Since the first days, EC decreased rapidly and reached very low values after 6 to 9 days while FS remained substantially at the same level for about two weeks. Their number then decreased to a minimum after 36 days.

In TWW irrigated soil, the evolution of bacterial contamination rate at 30 cm depth was different from that observed in the surface layer. Bacterial concentrations determined at the start of monitoring were lower than those recorded the same day in the surface; the difference was of 1 to 1.2 log units according to the indicator. A portion of the bacterial load carried by irrigation water was stopped at the surface layer that acted as a first filter. During the days following irrigation, the decrease in the frequency of the two indicators was slower than at the soil surface. After 6 days of irrigation the decrease was of 0.3 log for EC and 0.22 log for FS whereas it was respectively of 2 and 1.5 log at the soil surface. In the soil the indicators are protected from drying and solar rays that entail their quick removal in the surface. Furthermore, the decrease in the number of indicators compared to the level observed after one day of irrigation became significant after 12 days for EC and after 27 days for FS. This result confirmed the ability of FS to survive longer than EC because they are more resistant to harsh environmental conditions [54].

At 60 cm deep the numbers of faecal indicators after the irrigation were lower than those determined in the surface layer and at 30 cm. The evolution of the number of EC and FS showed some similarities with that observed at 30 cm depth: A decrease was observed during the first 15 days after irrigation. The concentrations changed more slowly thereafter and reached a minimum between 33 and 36 days both in control and in TWW irrigated soils.

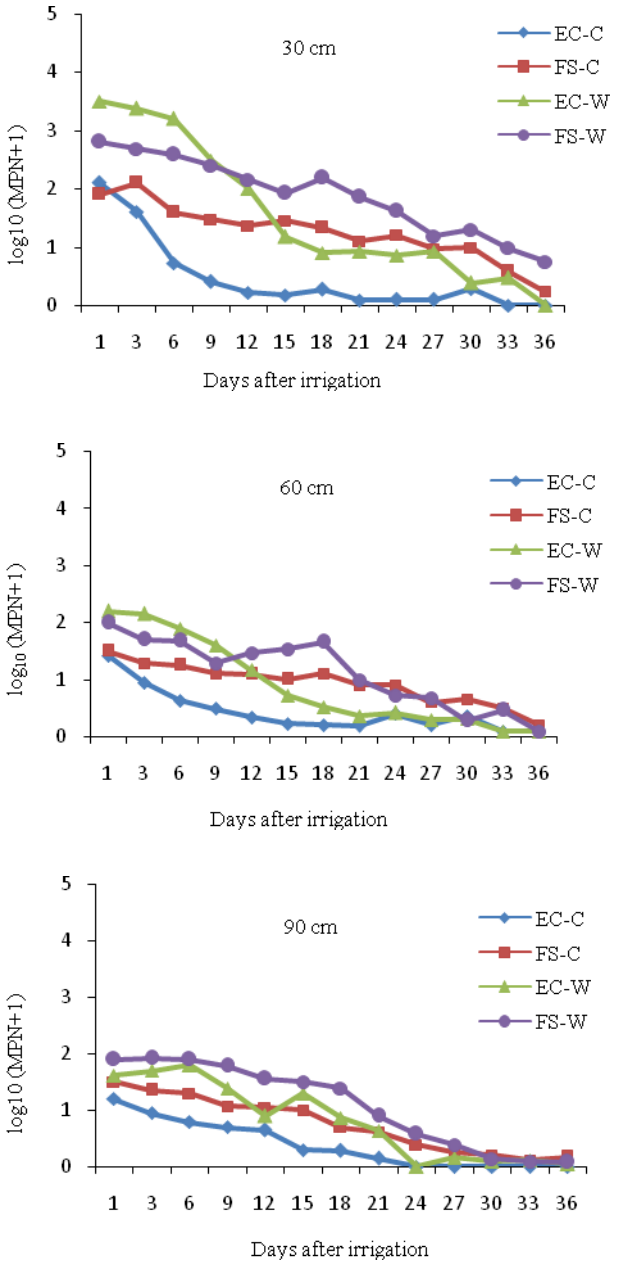

Fig. 3 Persistence of *E. coli* (EC) and faecal streptococci (FS) in the soil profile. (C: Control; W: TWW irrigated soil)

The reduction of bacterial concentrations measured after 36 days of irrigation with TWW was of 2.11 and 1.9 log units respectively for EC and FS.

At 90 cm depth, bacterial counts recorded at the start of monitoring were lower than those corresponding to all the other studied depths. The concentration of indicators in control and TWW irrigated soils were fairly close. They stayed at the same level for about two weeks and then a slight downward trend was observed. The minimum level

was reached after 24 days of irrigation for EC and after 30 days for FS.

All the results obtained at different depths showed that after irrigation with TWW, bacterial concentrations were increasingly low for greater depths. This means that during the passage through the soil, the number of microorganisms carried by wastewater decreased considerably. This result was consistent with that of other work [35, 49]. We also found that the higher the number of indicators was at baseline, their elimination was more rapid. This phenomenon could be explained by the competition and microbial antagonism that are all the stronger than the number of microorganisms is high. It was also noted that FIB showed better persistence in soil than at its surface. This result was in agreement with that of another study [53] and was due to the protection against drying and sunlight afforded by the soil to bacteria. *Salmonella* have never been detected in the soil samples analyzed during the three years of the trial. This result was probably due to its low frequency in irrigation water. Wastewater heavily contaminated with Salmonella should not be used for irrigation before undergoing disinfection treatment because *salmonella* can persist long in the soil [50, 55].

3.3 Hygienic quality of orange fruits

Bacteriological analyses carried out on orange fruit samples revealed a very low contamination by faecal indicators (Table 4). All samples analysed contained no *E. coli* and Salmonella. Their quality was therefore satisfactory according to the rules of the European Commission on microbiological criteria for pre-cut fruits and vegetables ready-to-eat [56]. The total coliforms ranged from zero to 2.3 g^{-1}.

Table 4: Microbial contamination of orange fruits during three seasons (MPN g^{-1})

Year	Water type	Total coliform			*Escherichia coli*			Faecal streptococci			Salmonella
		M	Mn	Mx	M	Mn	Mx	M	Mn	Mx	
2015	GW	0.3	0	2.3	0	-	-	0.1	0	0.9	Absence
	TWW	0.3	0	2.3	0	-	-	3.3	0	24	Absence
2016	GW	0.2	0	0.9	0	-	-	9.8	0	93	Absence
	TWW	0.4	0	2.3	0	-	-	0.6	0	4.3	Absence
2017	GW	0	-	-	0	-	-	0.2	0	0.9	Absence
	TWW	0.02	0	0.4	0	-	-	0.3	0	23	Absence

n= 20; GW: Ground water; TWW: Treated wastewater; M: Mean; Mn: Minimum; Mx: Maximum

The number of faecal streptococci showed greater variation since the maximum reached 93 MPN g^{-1}. However, 97.5% of the samples analyzed during the three years trial contained less than 5 MPN g^{-1}. This low bacterial contamination was due to the impossibility of contact between fruits and the contaminated water used for irrigation. The water was distributed by a drip system and irrigation was completed several weeks before harvest. The recorded occasional fruit contamination by total coliforms and faecal streptococci could be due to the effect of natural vectors of microorganisms such as birds and insects. Some authors [36] have stressed the important role of some uncontrollable bacterial sources (such as roaming wild animals and birds, runoff from agricultural areas) responsible of the contamination of soil and fruits in rain fed plots. The obtained results suggested that under suitable conditions, irrigation of citrus orchards by secondary treated wastewater does not lead to negative effects on the microbiological quality of fruits. Similar results were obtained in different Tunisian regions where other fruit trees such as pear, plum, peach, pomegranate and almond trees were drip irrigated with treated wastewater [57].

4. Conclusions

In north eastern Tunisia, TWW has been successfully used for citrus orchards irrigation for many years. The effects of this practice on the microbiological quality of soil and fruits were evaluated in an experimental field station located in this region. The results showed that wastewater treated to the secondary level was contaminated with coliforms, *E. coli*, faecal streptococci and in rare cases, with Salmonella.

The use of TWW for drip irrigation resulted in a significant bacterial contamination of the soil compared to the control irrigated with GW. The vertical distribution of microorganisms determined two days after the irrigation showed that most of the bacteria applied to the land were retained at the 0-60 cm layer. The risk of bacterial contamination of the groundwater was low in the conditions of this test since the water table was 15 meters deep. The study of the microorganism's persistence at different depths showed that five weeks after the last

irrigation of the season, removal of microorganisms was almost complete.

The bacteriological quality of fruits sampled from TWW irrigated plot was always similar to that of fruits sampled from control plot. All the fruits produced on the experimental orchard had a satisfactory quality according to the rules of the European Commission on microbiological criteria for foodstuffs.

This work has confirmed that under suitable conditions, secondary TWW may be useful as an alternative water resource for drip irrigation of citrus in Mediterranean water-scarce areas. However, periodic monitoring of soil and fruit quality is required to ensure successful and safe long-term TWW irrigation.

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