



# **Changes in the Microbial Properties of Olive Cultivated Soils under Short, Medium and Long-term Irrigation with Treated Wastewater**

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## **Authors' contributions**

*This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.*

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## **ABSTRACT**

**Aims:** In Tunisia, Climatic changes and water shortage has led to the reuse of treated municipal wastewater (TMWW) in the agricultural sector since the sixties. This work was intended to study the short, medium, and long-term impacts of this practice on soil microbial properties.

**Study Design:** Five different experimental fields were chosen which had been irrigated with TMWW for 10, 20, 25, and 28 years, respectively. A pluvial irrigated field was selected as a control.

**Place and Duration of Study:** The study was conducted in Zaouit Sousse (Tunisian Sahel region) located in the south of Sousse City (longitude: 35°47', latitude: 10°38' and of altitudes: 20 m N.G.T.). The soil sampling campaign was carried out at the end of the dry season (September 2014). This study was undertaken in a semi-arid area that is facing a water crisis (water shortage and irreversible seawater intrusion).

**Methodology:** Soil fecal pollution indicators were determined with the most probable number MPN method. Bacterial and fungal enumeration was done by the plate count agar method. Pathogenic bacteria was determined using the conventional bacteria identification methods.

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**Results:** Irrigation with TMWW (for more than ten years) induced a significant increase in soil microbial biomass (heterotrophic bacteria and filamentous fungi). Soil microbial contamination was assessed by measuring Total and Fecal Coliforms, *E. coli*, and *Faecal Streptococci* at three studied soil layers (0-20; 20-40 and 40-60 cm) show's a significant increase in TMWW irrigated plots compared to the control. *Salmonella* spp. and *Shigella* spp. screening revealed the absence of those pathogens in all studied soils. This result is true for the three soil horizons (0-20; 20-40 and 40-60 cm). This result seems to be due to the short survival period of these pathogens in the soil.

**Conclusion:** TMWW irrigation had positive effects on soil fertility. However, this practice has led to a deterioration of the soil sanitary quality. The quality of the wastewater treated in Sousse Sud plant must be improved to ensure the reduction of emerging bacterial pathogens to non-detectable levels or to levels that have not been associated with human health risk.

*Keywords: Impact; irrigation; treated wastewater; microbial biomass; faecal pollution indicators bacteria; pathogens bacteria.*

## 1. INTRODUCTION

The Mediterranean region is one of the most affected areas by the water shortage in the world [1,2], yet the water request was expanding due to population growth, rising living standards, urbanization as well as the increase of economic activities and expanding areas of irrigated agriculture [3].

Tunisia is among the most affected Mediterranean countries by water shortage with available water resources of about 480 m<sup>3</sup>/capita/year [4]. Main water demand is represented by the agricultural sector (about 80% of the total demand), while the drinking sector consumes 14%, industry accounts for 4% and 2% for the tourism sector [4]. The agriculture sector plays a major role in the economy of the country, employing 17% of its workforce. Olive growing is Tunisia's main agricultural activity and plays a very important socio-economic role. The crop is closely linked to the traditions of Tunisia, about 82 million olive trees are spread over approximately one third of the country's crop area, constituting 1.835 million hectares [5,6]. Ninety-five percent of olive growing is rainfed in varying climatic conditions. Tunisian olive production fluctuates considerably from one year to the next, due to the phenomenon of the alternate bearing of olive trees and extremely unpredictable climatic conditions and water scarcity particularly in arid and semi-arid regions [7].

Tunisia is also facing water quality problems (anthropic salinization) and seawater intrusion in coastal areas due to excessive groundwater pumping. Therefore, better water demand management and the development of new water resources are urgently required. At this point, treated municipal wastewater reuse for irrigation

can be a valuable alternative alleviating the pressure on freshwater resources [8].

During the last part of the 20<sup>th</sup> century, wastewater reuse was a common practice in many countries of the world and the scientific literatures has recognized its benefits [9-16]. Treated municipal wastewater (TMWW) may be appropriate for a large variety of applications. Among the most common reuse applications are irrigation, residential uses, urban and recreational use, groundwater recharge, bathing water, aquaculture, industrial cooling water, and drinking water production [17]. It estimated that the last 10% of the global population consumed food produced by irrigation with untreated, partly treated/diluted or treated wastewater [18] and more than 20 million hectares are irrigated with untreated, partly treated/diluted or treated wastewater around the world [19].

TMWW reuse for irrigation has been largely applied to agriculture due to the advantages related to nutrient recovery possibilities, socio-economic implication, reduction of fertilizer application and effluent disposal [11,20-24].

However, this practice was related not only to the number of benefits, in regards to water balances, management and preservation, but, also, to a number of question marks. Approximately 70% of treated wastewater is used for agriculture [25,26], which may have harmful effects on the environment and human health [27-29,22,30].

Relatively little is known about the influence of long-term wastewater application on the nutrient stock and microbiological quality in soil. Such studies are significant to fill knowledge gaps related to the potential effects that the wastewater practices might induce on human health and the environment [31-33].

The present scientific work was aimed to investigate the short, medium and long-term effects of TMWW reuse for irrigation on soil microbial properties: (i) Heterotrophic bacteria, filamentous fungi and faecal pollution indicators counting, (ii) Screening for the presence of pathogenic bacteria, and (iii) Carbon and nitrogen microbial biomass.

## 2. MATERIALS AND METHODS

### 2.1 Experimental Site and Soil Sampling

The study was conducted in Zaouit Sousse (Tunisian Sahel region) located in the south of Sousse City (longitude: 35°47', latitude: 10°38' and of altitudes: 20 m N.G.T.). The regional climate was semi-arid with a mean annual temperature of 20°C and precipitation around 400 mm in the north and 300 mm in the southern part of the studied valley [34]. The study site was irrigated since 1989. Irrigation was realized by flooding through furrowing. Experiments were carried out in five cultivated olive plots (*Var. Chemlali*) with intercropped fodder crops (sorghum, berseem, etc.) (Fig. 1). Four fields had been irrigated with TMWW, respectively, for 10, 20, 25 and 28 years. One field, pluvial irrigated, was selected as a control.

Irrigation was realised by flooding through furrowing. Mean annual irrigation rates vary between 200 and 400 mm depending on the used crop and water availability [32].

Soil sampling campaign was carried out using a drill at the end of the dry season (September 2014). Each site was divided into three blocks. In each block, soil subsamples were set down in a zigzag pattern and pooled. Composite soil samples were taken from three depths 0-20, 20-40 and 40-60 cm, respectively. The soil sample was sieved (2 mm) in order to eliminate rocks and roots fragments, placed inside plastic ventilated bags and stored at 4°C until the microbiological analyses.

This study was undertaken in a semi-arid area that is facing a water crisis (water shortage and irreversible seawater intrusion).

### 2.2 Treated Wastewater Quality

Municipal wastewater used throughout this study was treated in the Southern Sousse wastewater treatment station. It was just an activated sludge-extended aeration plant with a mechanical screen, grit removal tanks, primary

sedimentation, extended aeration, and finally sedimentation tanks. The characteristics of Treated Municipal WasteWater (TMWW) used for irrigation varied within and among the application years. All experiments (pH, COD, BOD, EMC) were performed according to the standard method book [35].

TMWW samples were collected in sterilized glass bottles from the wastewater treatment plant. Samples were collected and transported directly to the laboratory at +4°C and kept in the refrigerator for later analysis.

The samples were examined within 24 h for screening pathogenic bacteria (*Salmonella*, *Staphylococcus* and *Pseudomonas*) [36]. Faecal Coliforms and *Streptococcus* enumeration were carried out by using the Most Probable Number (MPN) method and following the 3 replications 5 dilution scheme [35]. Enterococci and *E. coli* are widely recognised as a useful indicator for contamination, because of their resistance to disinfection and environmental factors and their ability to survive for long periods in the environment [37,38].

### 2.3 Soil Analysis

#### 2.3.1 Soil microbial enumeration

The soil microbial density in the different studied plots was evaluated by counting bacteria and fungi on culture media (plate count agar method). Briefly, soil aliquots (5 g) were plated onto 10 fold-diluted tryptic soy agar (Bio-Rad, France). Plates were incubated at 25°C for 3 days after spreading of 100 µl of appropriate dilution [39,40]. Bacterial colonies were counted after 48 h of incubation at 28°C. Only plates with between 30 and 300 colonies per plate were examined. For fungi enumeration, the appropriate soil dilution was spread on Malt Extract Agar. The number of developed colonies was recorded after 7 days of incubation at room temperature. Soil microbe's enumeration was an expression of the number of colony forming units (CFU) per gram of dry watered soil.

#### 2.3.2 Soil pathogenic bacteria screening

The isolated organisms, from soil-studied samples, were purified through repeated subculture method. Streak plate methods were used for this purpose. Nutrient agar was used as media. When a plate yielded only one type of colony, the organisms were considered to be pure. The purification of the isolates was also confirmed by microscopic observation.

Biochemical characteristics accompanied with colony characteristics on different selective medium were observed for the identification of bacterial isolates [41].

## 2.4 Statistical Analysis

Analysis of variance was carried out using SPSS software (SPSS for Windows, version 20; SPSS Inc., Chicago, IL, USA) and means were separated by the least significant difference according to the Student-Newman-Keuls test. All results represented the mean of three determinations.

## 3. RESULTS AND DISCUSSION

### 3.1 Treated Wastewater Quality

#### 3.1.1 Chemical characteristics

TMWW was, on average, alkaline with a basic pH value of 7.71 ( $\pm 0.12$ ). The obtained pH values fall within the Food and Agriculture Organisation guidelines standard limits 6.5-8.4 [42]. Chemical and Biochemical Oxygen Demand (COD and BOD respectively) values were 392 ( $\pm 31.2$ ) and 139.3 ( $\pm 17.9$ )  $\text{mg}\cdot\text{L}^{-1}$ , respectively. Note that organic charge was positively correlated with total bacterial counts [42,30]. The BOD<sub>5</sub>/COD ratio was about 0.35 ( $\pm 0.017$ ). TMWW used in our study was easily biodegradable. Mean Electrical Conductivity (EC) was 3.37 ( $\pm 0.15$ )  $\text{mS}\cdot\text{cm}^{-1}$ , lower than the limit recommended by the Tunisian Standards (EC = 7  $\text{mS}/\text{cm}$ ). EC value indicating a severe degree of restriction on the use of this wastewater in irrigation (Table 1). Moreover, the average Na<sup>+</sup> and Cl<sup>-</sup> concentrations was around 338.2 ( $\pm 43.13$ ) and 597.5 ( $\pm 17.02$ )  $\text{mg}\cdot\text{L}^{-1}$  respectively.

TMWW Metallic Elements Contents (MEC) were found to be in the limit recommended by the Tunisian Standards and FAO Standards (Table 1).

Southern Sousse municipal wastewater represented a source for major nutrients. They contains an organic load much higher than the limit recommended by the Tunisian Standards. COD and BOD<sub>5</sub> limit values (NT 106.03) were equal respectively to 90 and 30  $\text{mg}\cdot\text{L}^{-1}$  (Table 1), which leads to the risk of clogging of irrigated soil. It is to highlight that irrigation using saline water can add salt concentration to the soils. Salinity is the result of all dissolved anions and cations in water [43]. It cause increase of the osmotic pressure of soil solution, harming the ability of plants to absorb water and nutrients

[44]. Therefore, it is necessary to control the soil salinity when using treated wastewater for irrigation [43].

### 3.1.2 Microbial characteristics

#### 3.1.2.1 Fecal pollution indicators

Bacterial characteristics of the TMWW used in this study were given in Fig. 2. The most probable number per 100 ml (MPN/100 ml) was about 27 ( $\pm 3.2$ )  $10^7$  and 22 ( $\pm 1.15$ )  $10^7$  of fecal coliforms and *E. coli*, respectively for the water sampled from the outlet of the treatment plant (TMWW1). In the stabilization pond (TMWW2), the bacteriological load decreases to reach 13 ( $\pm 2$ )  $10^7$  and 11 ( $\pm 0.6$ )  $10^7$  of fecal coliforms and *E. coli*, respectively. This result seems to be due to the combined effect of the settling phenomenon and solar ultra violet rays having a disinfecting effect [45]. Arriving at the level of the plot (irrigation valve, TMWW3), the bacteriological quality of the water revealed a slight deterioration (Fig. 2). This result seems to be due to the residual and stagnant organic load in the water distribution network [45].

Based on these results, we can conclude that the wastewater from the Zaouia wastewater treatment plant carries a faecal bacteriological load that is much greater than the limit set by WHO standards. These results seems to be due to the significant organic load in this poorly treated water. It should be noted that Tunisian standards (NT 106.03) did not report the bacteriological quality of wastewater that can be used in agriculture.

#### 3.1.2.2 Bacterial pathogens

Bacteria isolated from the wastewater sampled from the outlet of the treatment plant (TMWW1), stabilization pond (TMWW2) and irrigation valve (TMWW3). Totally 182 colonies were isolated on the nutrient agar plate.

Thirty-eight predominant individual colonies were selected and identified based on Morphological characteristics, Gram staining and biochemical characteristics according to the key of Bergey's Manual of Determinative Bacteriology. All the selected bacterial colonies were streaked on the different specific agar plates. In Cetrimide agar plates for *Pseudomonas* sp screening, in MSA plates agar for *Staphylococcus* sp and S-S plates agar for *Salmonella* screening. Isolated colonies were examined under microscope after the Gram staining, Mobility test (Table 2), and Biochemical analysis (Table 3). Based on the microscopic

examination and biochemical analysis, selected 17 bacteria isolates from the TMWW1 were identified: S1-1, S1-4, S1-8, S1-9, S1-12, S1-16 and S1-17 as *Pseudomonas* sp., S1-2, S1-5, S1-7, S1-10, S1-13 and S1-15 as *Staphylococcus* sp, S1-3, S1-6, S1-11 and S1-14 as *Salmonella* sp. From the TMWW2, 10 selected bacteria were identified: S2-3, S2-6, S2-8 and S2-9 as *Pseudomonas* sp., S2-1, S2-4 and S2-7 as *Staphylococcus* sp., S2-2, S2-5 and S2-10 as *Salmonella* sp.. From the TMWW3, 09 selected bacteria were identified: S3-1, S3-3, S3-4, S3-7 as *Pseudomonas* sp., S3-2, S3-6, S3-8 as *Staphylococcus* sp., S3-5, S3-9 as *Salmonella* sp.

Southern Sousse treated wastewater carries a load of fecal coliforms that exceeds the limits of use restriction recommended by World Health Organization (>1000 MPN/100 ml) [46]. Pathogenic bacteria (*Pseudomonas*, *Staphylococcus* and *Salmonella*) also, contaminate these waters. These expected results ( $BOD_5=139.3 \pm 17.9$  mg/L) seem to be due to the state of overload known at the Southern Sousse treatment plant. This plant, with a capacity of 10,000 cubic meters, receives around 30,000 cubic meters of wastewater per day. This situation will be resolved by commissioning, in the zone of another wastewater treatment plant (Sousse Hamdoune) with a capacity of 40,000 cubic meters per day.

### 3.2 Impact of Wastewater Irrigation on Soil Properties

#### 3.2.1 Physicochemical parameters

No significant variations in soil texture or total calcareous level were observed among soils sampled from the different sites (Table 4). This result validated the choice of the studied sites that present no plot-to-plot soil heterogeneity that might have masked the impact of irrigation management.

##### 3.2.1.1 Soil pH

The soil pH values measured in water ( $pH_w$ ) was illustrated in Fig. 3. The soil pH increased systematically in plots irrigated with TMWW for more than ten years. As no basic amendment input was added to the studied soils, it can be concluded that irrigation with TMWW raised the soil pH by approximately 1 unit. The average pH values at the control plot were ranged from 7.32 to 7.39. The slight alkali values were probably a consequence of the buffering capacity of such Tunisian soils rich in limestone and with an

intense degree of ammonification [22]. There was a significant increase in pH values after 28 years of TMWW irrigation (pH ranged from 8.14 to 8.79). This finding concord with these published by Tarchouna et al., Bedbabis et al., Chen et al. [15,22,47]. Macino and Pepper [48] attributed such a pH rise to (i) the high content of basic cations such as  $Na^+$ ,  $Ca^{2+}$  and  $Mg^{2+}$  of the TWW, which raised the alkaline reserve of the soil, and (ii) an enhanced rate of denitrification that produced hydroxyl ions. Other researchers reported that irrigation with TWW decreased the pH when compared with irrigation with freshwater in sandy soil [49,50]. Differences can be attributed to the oxidation of organic compounds and nitrification of ammonium [51,52].

##### 3.2.1.2 Soil salinity

Soil salinity, measured as electrical conductivity (EC) of 1:5 soil extract in  $mS\ cm^{-1}$  was low in the control plot for the two surface horizons (0-20 and 20-40 cm). The EC values increased significantly after an irrigation period with TWW of 10 years and it reaches its maximum value after 30 years. Overall, our results show two salinity trends: (i) Significant increase of salinity from the top (0–20 cm) to bottom (40–60 cm) with an important soil salt concentrations at the deep horizons (Fig. 4), explained by sea water intrusion, and (ii) Significant increase of salinity according to the length of the TWW irrigation period (a spatiotemporal distribution). Clearly visible for the two surface horizons (0-20 and 20-40 cm). Mohamed and Mazahreh [51] stated that the increase in EC for soil irrigated with TMWW was a result of the original high-level total dissolved salts (TDS) of the TMWW. These results were conforming to some previous finding [22,53,54].

##### 3.2.1.3 Soil Organic Matter (SOM)

The Soil Total Organic Carbon (STOC) content increased markedly with the increase according to the length of the TWW irrigation period (Fig. 5). The concentration goes from simple to double after 30 years of irrigation (from 0.828% to 1.56%). These findings can be directly attributed to the composition of the water, which presented high values of BOD (139.3 mg/L) and COD (392 mg/L). These results are different from other studies which reported a decrease of the SOC in soil irrigated with domestic TWW [15,55]. According to these authors, SOC increase was related to an intensification of microbial activity due to labile C and N supplied by TWW. Although SOC contents accumulated more in the

upper soil horizon. The significantly higher SOC contents at the deeper soil horizon (40–60 cm) showed that the effect of irrigation was not limited to the surface layer, and could be explained by the sandy texture, leading to a weak discrimination between the soil horizons in depth [32,56,57].

#### 3.2.1.4 Soil Total Nitrogen (STN), available Phosphorus content ( $P_2O_5$ ) and Potassium content ( $K_2O$ )

High concentrations of STN were detected for the surface horizon (0-20 cm) in the plots irrigated by wastewater for a period equal or greater than 10 years (Fig. 6). The highest value was recorded for the plot irrigated by TMWW for 25 years. This value is of the order of 2.68%. This suggests that wastewater contained nitrogen in excess of plants requirements [58].

Available P content follows the same evolution as STN. An increase has been recorded in treated wastewater irrigated plots. This result is true, essentially, for the two surface horizons (0-20 and 20-40 cm). This result suggested that a certain fertilizing effect of TMWW was possible as a consequence of (i) high soluble P content and (ii) organic matter adsorption, as already found by previous works [22,58,59]. Indeed, TMWW may be a source of N, P and K can have ecological and economical advantages avoiding or reducing the use of P and K fertilisers [22,60].

### 3.2.2 Soil microbiological parameters

#### 3.2.2.1 Soil Mesophilic aerobic bacteria (MAB) and filamentous fungi counting

The average numbers of MAB in the topsoil (0-20 cm) ranged from  $112 (\pm 2) 10^4$  to  $221 (\pm 1) 10^6$  Colony Forming Units (CFU)/g dewatered soil. The highest values were recorded in TMWW irrigated plots. For the two horizons in depth (20-40 and 40-60 cm), the MBA numbers follow the same variation recorded for the surface horizon but with a lower intensity (Table 5). This result seems to be due to the low availability of organic matter and oxygen gas at depth.

Similar significant variations were recorded for fungi, both between the control and TMWW irrigated plots (Table 5). This result, visible mainly for the surface horizon, is also valid for the depth horizons.

Altogether, these data revealed that long-term irrigation with wastewater induced a significant

increase in soil microbial abundance. This growth of microorganisms might be explained by the ready source of easily degradable compounds in the oligotrophic soil environment brought about by wastewater irrigation [61]. Indeed, microorganisms are mainly heterotrophic and carbon-limited in soil and the observed differences could be due to a higher availability and quality of the carbon source supplied by wastewater irrigation. In fact, high correlation coefficients, of the order of 0.70; 0.74 and 0.89 respectively for the three-studied soil layers (0-20; 20-40 and 40-60 cm).

Adrover et al. [62] obtained similar results. According to these authors, soil microbial biomass was significantly higher in soils 20 years irrigated with TWW, when compared to that found in well water irrigated soils. Friedel et al, [63] observed a similar increase in microbial biomass and dehydrogenase activity in Vertisols which had been irrigated on a long-term basis with untreated wastewater. [63,64] reported a significant increase of alkaline phosphatase in soils irrigated with treated wastewater over shorter periods of time (4 and 3 years, respectively) and Truu et al. [65] observed an enhancement of various enzymatic activities in soils irrigated with treated wastewater over 10 years. The positive effect of treated wastewater irrigation on soil microbial biomass and its associated activities can be attributed to the addition of easily decomposable organic matter and nutrients [62,65]. These modifications are not necessarily beneficial and the stimulation of the soil microbial abundance and activity may have negative impacts on soil properties [66]. For instance, Becerra-Castro et al. [67] observed that the bacterial growth stimulated by irrigation with wastewater led to the formation of biofilms, with the concomitant clogging of the pore spaces between particles, with implications in the soil hydraulic conductivity.

#### 3.2.2.2 Fecal indicators bacteria count

Soil microbial contamination was assessed by measuring Total and Fecal Coliforms, *E. coli*, and Fecal Streptococci at three-studied soil layers (0-20; 20-40 and 40-60 cm). The average of the main bacteriological parameters measured during the experimental trial are shown in Table 6. Data observed show a significant increase in the indicators of fecal pollution indicators in TMWW irrigated plots compared to the control. This result, recorded for the upper soil layer (0-20 cm), is true for all depth horizons (20-40 and 40-60 cm). Our results seem to be

due to the mediocre bacteriological quality of the wastewater treated in Southern Sousse plant. These waters carry a bacteriological load that greatly exceeds the limit value recommended by the World Health Organization (FC=1000 cfu/100 ml). Our results agree those

recorded in several subsequent studies [30,68-70]. According to these authors, the accumulation and persistence of faecally sourced microbes from wastewater in soil is one of the major concerns associated with this practice.

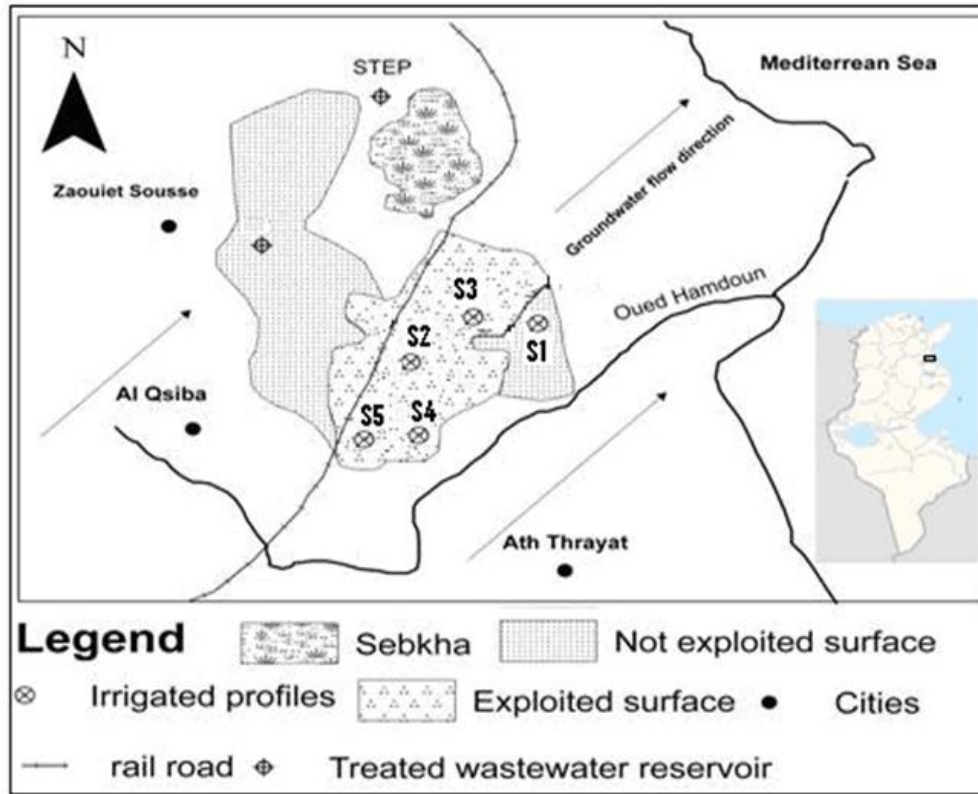


Fig. 1. Localisation of the irrigated perimeter Zaouit Sousse (Tunisian Sahel area) and different studied profiles [57]

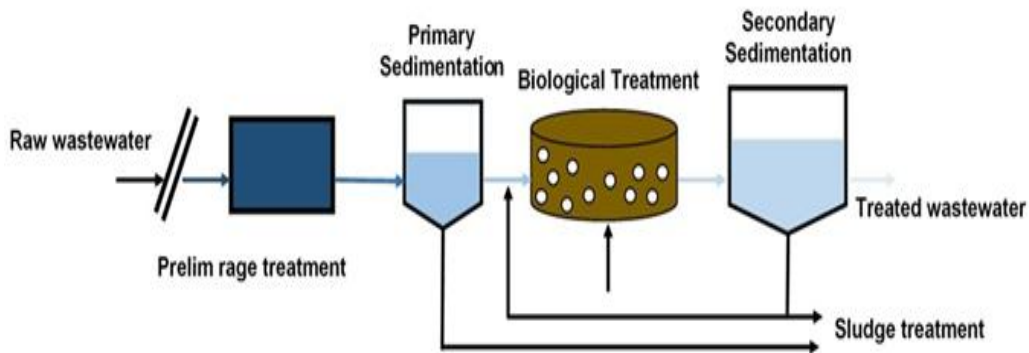
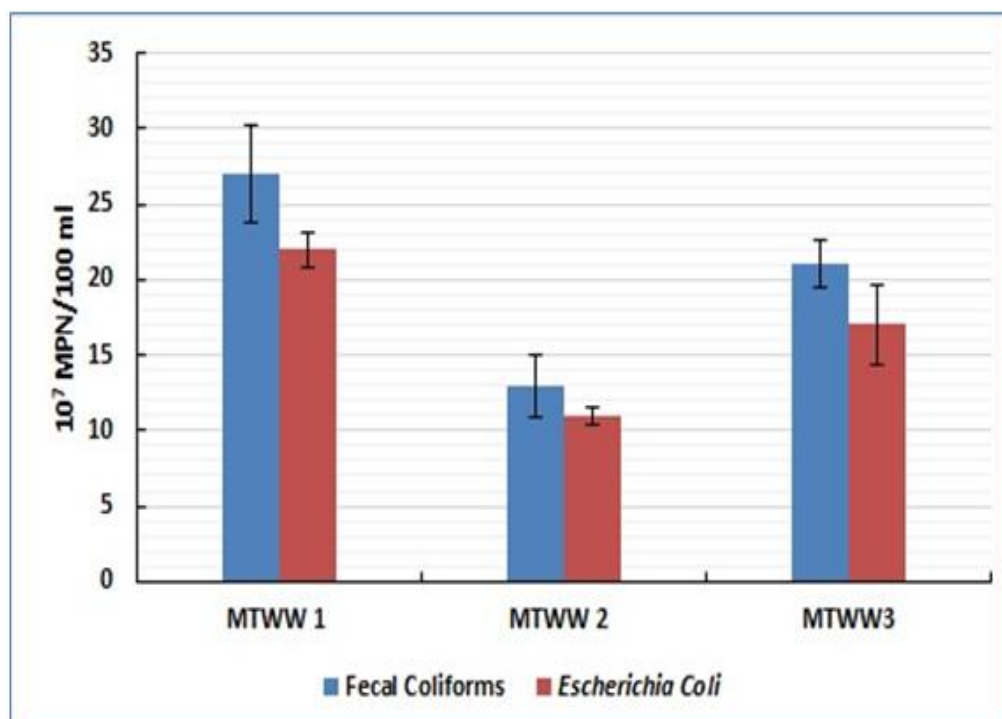


Fig. 2. Flow diagram (Zaouia wastewater treatment plant)

**Table 1. Characteristics of treated wastewater used for irrigation**

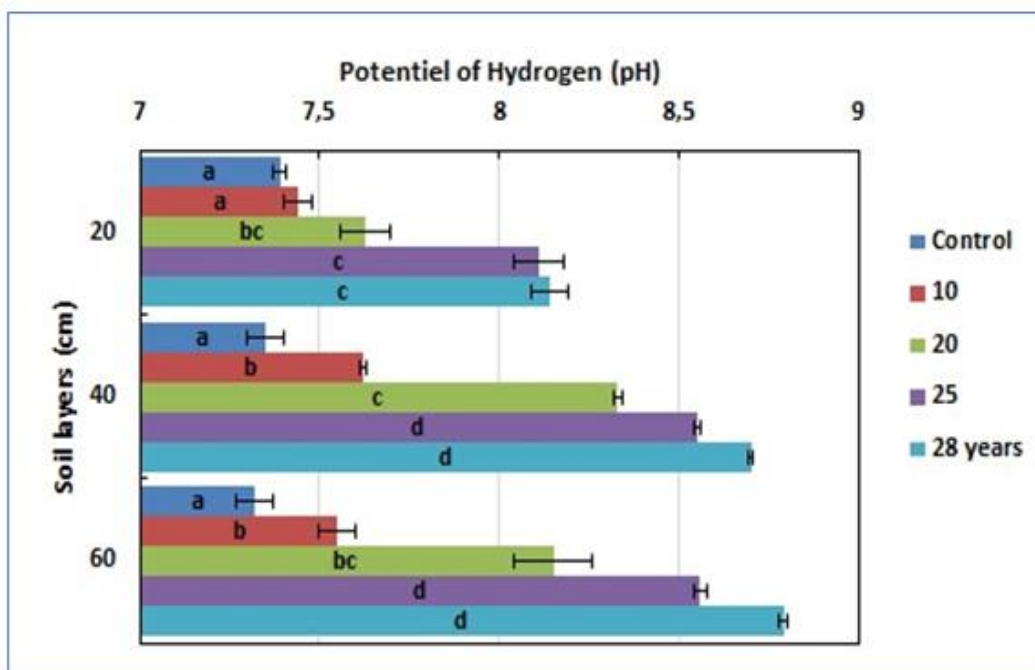
Parameters	Unit	Mean	Tunisian Standards	FAO
			NT 106-03	Standards
pH	-	7.57	6.5-8.5	6.5-8.4
Electrical conductivity (EC)	mS.cm <sup>-1</sup>	3.37	7	0.7-3
Chemical Oxygen Demand (COD)	(mg.L <sup>-1</sup> )	374	90	-
Biochemical Oxygen Demand (BOD <sub>5</sub> )	(mg.L <sup>-1</sup> )	129	30	30
Total Suspended Solid (TSS)	(mg.L <sup>-1</sup> )	152	30	30
Ammonia	(mg.L <sup>-1</sup> )	109.8	-	-
Chlorides	(mg.L <sup>-1</sup> )	614	2000	2000
Sodium oxide	(mg.L <sup>-1</sup> )	313.3	-	30
Potassium oxide	(mg.L <sup>-1</sup> )	48.7	-	-
Calcium	(mg.L <sup>-1</sup> )	131.33	-	-
Magnesium oxide	(mg.L <sup>-1</sup> )	69.43	-	-
Phosphate pent-oxide	(mg.L <sup>-1</sup> )	17.8	-	-
Sodium adsorption ratio (SAR)	-	5.48	-	-
Cadmium (Cd)	(mg.L <sup>-1</sup> )	0.00002	0.01	0.01
Lead (Pb)	(mg.L <sup>-1</sup> )	0.019	1.00	5.00
Cobalt (Co)	(mg.L <sup>-1</sup> )	0.058	0.1	0.05
Chromium (Cr)	(mg.L <sup>-1</sup> )	0.06	0.1	0.1
Copper (Cu)	(mg.L <sup>-1</sup> )	0.36	0.5	0.1
Nickel (Ni)	(mg.L <sup>-1</sup> )	0.095	0.2	0.02
Iron (Fe)	(mg.L <sup>-1</sup> )	0.371	5.00	5.00
Zinc (Zn)	(mg.L <sup>-1</sup> )	0.87	5.00	2.00



**Fig. 3. Municipal Treated WasteWater (MTWW) microbiological quality (fecal coliforms and *Escherichia coli* enumeration)**

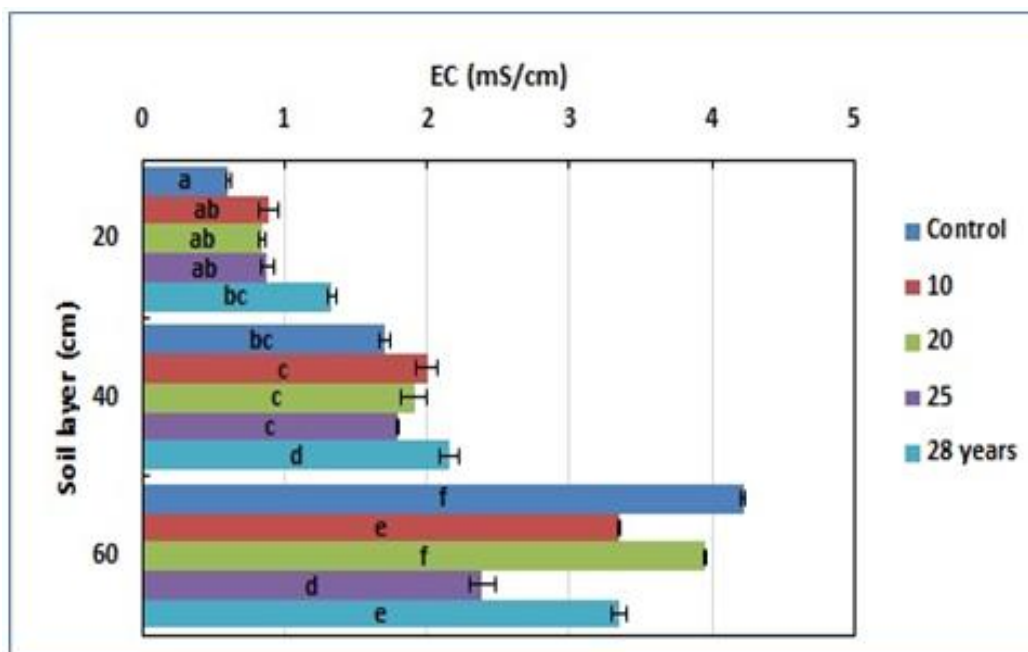
MTWW1: Water sampled from the outlet of the treatment plant; MTWW2: Water sampled from the stabilization pond; MTWW3: Water sampled from the irrigation valve; Each value is the mean of 3 replicates (n = 3) samples





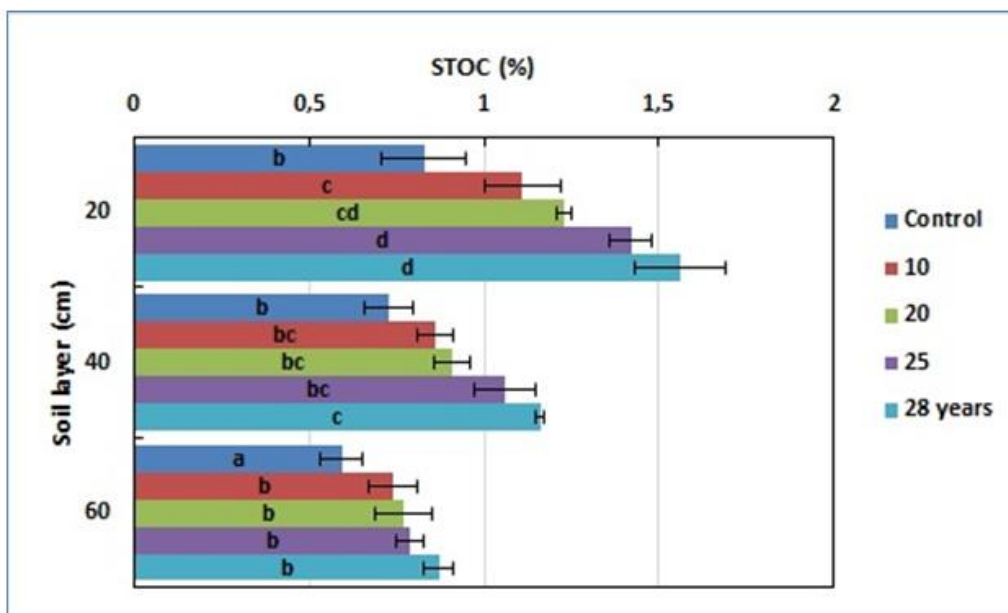
**Fig. 4. Soil Hydrogen potential (pH) as affected by the duration of wastewater application (years) and soil depth**

Each value is the mean of 3 replicates ( $n = 3$ ) samples. Means marked with the same letter (a, b, c ...) are not significantly different according to the Student-Newman-Keuls test ( $P = 0.05$ )



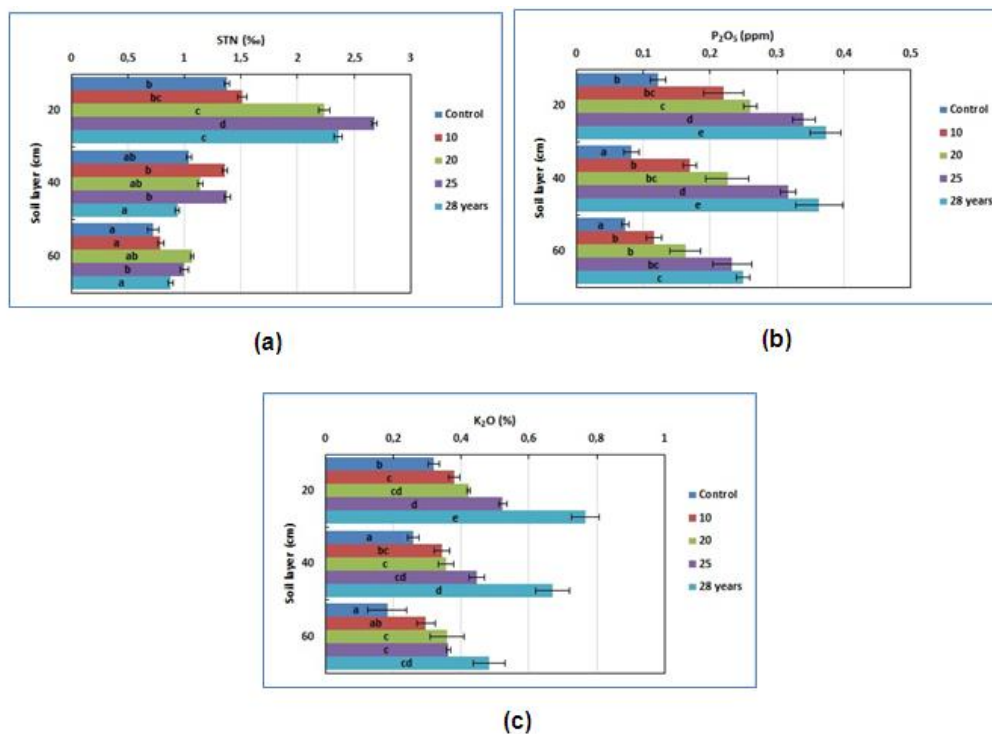
**Fig. 5. Soil Electrical Conductivity (EC) as affected by the duration of wastewater application (years) and soil depth**

Each value is the mean of 3 replicates ( $n = 3$ ) samples. Means marked with the same letter (a, b, c ...) are not significantly different according to the Student-Newman-Keuls test ( $P = 0.05$ )



**Fig. 6. Soil Total Organic Carbon (STOC) as affected by the duration of wastewater application (years) and soil depth**

Each value is the mean of 3 replicates ( $n = 3$ ) samples. Means marked with the same letter (a, b, c ...) are not significantly different according to the Student-Newman-Keuls test ( $P = 0.05$ ).



**Fig. 7 Soil Total Nitrogen (STN), available phosphorus content ( $P_2O_5$ ) and Potassium content ( $K_2O$ ) as affected by the duration of wastewater application (years) and soil depth**

Each value is the mean of 3 replicates ( $n = 3$ ) samples. Means marked with the same letter (a, b, c ...) are not significantly different according to the Student-Newman-Keuls test ( $P = 0.05$ ).

**Table 2. Microscopic Examination of Bacterial Isolates**

Bacterial isolates	Gram straining	Spore staining	Mobility test
S1-1 ; S1-4 ; S1-8 ; S1-9 ; S1-12 ; S1-16 ; S1-17.	Gram Negative, Rod	Non-spore forming	Motile
S1-2 ; S1-5 ; S1-7 ; S1-10 ; S1-13 ; S1-15.	Gram Positive, Cocci	Non-spore forming	Non-Motile
S1-3 ; S1-6 ; S1-11 ; S1-14.	Gram Negative, Rod	Non-spore forming	Motile
S2-3 ; S2-6 ; S2-8 ; S2-9.	Gram Negative, Rod	Non-spore forming	Motile
S2-1 ; S2-4 ; S2-7.	Gram Positive, Cocci	Non-spore forming	Non-Motile
S2-2 ; S2-5 ; S2-10.	Gram Negative, Rod	Non-spore forming	Motile
S3-1 ; S3-3 ; S3-4 ; S3-7.	Gram Negative, Rod	Non-spore forming	Motile
S3-2 ; S3-6 ; S3-8.	Gram Positive, Cocci	Non-spore forming	Non-Motile
S3-5 ; S3-9.	Gram Negative, Rod	Non-spore forming	Motile

S1 : bacteria isolates from the TMWW1, S2 : bacteria isolates from the TMWW2, bacteria isolates from the TMWW3.

MTWW1: Water sampled from the outlet of the treatment plant; MTWW2: Water sampled from the stabilization pond; MTWW3: Water sampled from the irrigation valve

**Table 3. Biochemical tests of Bacterial Isolates from Treated Municipal WasteWater (TMWW)**

Strains	Indole	MR	VP	Citrate	TSI	Oxidase	Catalase	Urease	CFT
S1-1	-	-	-	+	G <sup>+</sup> H <sub>2</sub> S <sup>-</sup>	-	+	-	-
S1-2	-	-	-	-	G <sup>+</sup> H <sub>2</sub> S <sup>-</sup>	+	+	+	+
S1-3	-	+	-	-	G <sup>+</sup> H <sub>2</sub> S <sup>+</sup>	+	+	-	+
S1-4	-	-	-	+	G <sup>+</sup> H <sub>2</sub> S <sup>-</sup>	-	+	-	-
S1-5	-	-	-	-	G <sup>+</sup> H <sub>2</sub> S <sup>-</sup>	+	+	+	+
S1-6	-	+	-	-	G <sup>+</sup> H <sub>2</sub> S <sup>+</sup>	+	+	-	+
S1-7	-	-	-	-	G <sup>+</sup> H <sub>2</sub> S <sup>-</sup>	+	+	+	+
S1-8	-	-	-	+	G <sup>+</sup> H <sub>2</sub> S <sup>-</sup>	-	+	-	-
S1-9	-	-	-	+	G <sup>+</sup> H <sub>2</sub> S <sup>-</sup>	-	+	-	-
S1-10	-	-	-	-	G <sup>+</sup> H <sub>2</sub> S <sup>-</sup>	+	+	+	+
S1-11	-	+	-	-	G <sup>+</sup> H <sub>2</sub> S <sup>+</sup>	+	+	-	+
S1-12	-	-	-	+	G <sup>+</sup> H <sub>2</sub> S <sup>-</sup>	-	+	-	-
S1-13	-	-	-	-	G <sup>+</sup> H <sub>2</sub> S <sup>-</sup>	+	+	+	+
S1-14	-	+	-	-	G <sup>+</sup> H <sub>2</sub> S <sup>+</sup>	+	+	-	+
S1-15	-	-	-	-	G <sup>+</sup> H <sub>2</sub> S <sup>-</sup>	+	+	+	+
S1-16	-	-	-	+	G <sup>+</sup> H <sub>2</sub> S <sup>-</sup>	-	+	-	-
S1-17	-	-	-	+	G <sup>+</sup> H <sub>2</sub> S <sup>-</sup>	-	+	-	-

Strains	Indole	MR	VP	Citrate	TSI	Oxidase	Catalase	Urease	CFT
S2-1	-	-	-	-	G <sup>+</sup> H <sub>2</sub> S <sup>-</sup>	+	+	+	+
S2-2	-	+	-	-	G <sup>+</sup> H <sub>2</sub> S <sup>+</sup>	+	+	-	+
S2-3	-	-	-	+	G <sup>+</sup> H <sub>2</sub> S <sup>-</sup>	-	+	-	-
S2-4	-	-	-	-	G <sup>+</sup> H <sub>2</sub> S <sup>-</sup>	+	+	+	+
S2-5	-	+	-	-	G <sup>+</sup> H <sub>2</sub> S <sup>+</sup>	+	+	-	+
S2-6	-	-	-	+	G <sup>+</sup> H <sub>2</sub> S <sup>-</sup>	-	+	-	-
S2-7	-	-	-	-	G <sup>+</sup> H <sub>2</sub> S <sup>-</sup>	+	+	+	+
S2-8	-	-	-	+	G <sup>+</sup> H <sub>2</sub> S <sup>-</sup>	-	+	-	-
S2-9	-	-	-	+	G <sup>+</sup> H <sub>2</sub> S <sup>-</sup>	-	+	-	-
S2-10	-	+	-	-	G <sup>+</sup> H <sub>2</sub> S <sup>+</sup>	+	+	-	+
S3-1	-	-	-	+	G <sup>+</sup> H <sub>2</sub> S <sup>-</sup>	-	+	-	-
S3-2	-	-	-	-	G <sup>+</sup> H <sub>2</sub> S <sup>-</sup>	+	+	+	+
S3-3	-	-	-	+	G <sup>+</sup> H <sub>2</sub> S <sup>-</sup>	-	+	-	-
S3-4	-	-	-	+	G <sup>+</sup> H <sub>2</sub> S <sup>-</sup>	-	+	-	-
S3-5	-	+	-	-	G <sup>+</sup> H <sub>2</sub> S <sup>+</sup>	+	+	-	+
S3-6	-	-	-	-	G <sup>+</sup> H <sub>2</sub> S <sup>-</sup>	+	+	+	+
S3-7	-	-	-	+	G <sup>+</sup> H <sub>2</sub> S <sup>-</sup>	-	+	-	-
S3-8	-	-	-	-	G <sup>+</sup> H <sub>2</sub> S <sup>-</sup>	+	+	+	+
S3-9	-	+	-	-	G <sup>+</sup> H <sub>2</sub> S <sup>+</sup>	+	+	-	+

CFT : carbohydrate fermentation test, S1 : Strain identified from the TMWW1 (Outlet of the treatment plant), S2 : Strain identified from the TMWW2 (Stabilization pond), S3 : Strain identified from the TMWW3 (Irrigation valve)

**Table 4. Soil physicochemical parameters of the different studied sites**

<b>Sites</b>	<b>Depth (cm)</b>	<b>Clay (%)</b>	<b>Silt (%)</b>	<b>Sand (%)</b>	<b>CaCO<sub>3</sub> tot (%)</b>
Site 1 (control)	0-20	15.2	17.94	66.86	10.8
	20-40	27.01	19.29	53.7	14.4
	40-60	24.51	21.38	54.11	23.4
Site 2(10 years)	0-20	16.8	16.34	66.86	13.05
	20-40	23.38	18.16	58.46	16.2
	40-60	24.06	20.2	55.74	24.3
Site 3(20 years)	0-20	16.57	19.06	64.37	12.15
	20-40	21.08	19.75	59.17	19.8
	40-60	20.65	18.84	60.51	25.2
Site 4(25 years)	0-20	16.57	18.37	65.06	11.7
	20-40	24.74	22.01	53.24	17.1
	40-60	21.33	20.2	58.47	20.7
Site 5(28 years)	0-20	14.4	17.25	68.01	12.6
	20-40	22.51	20.39	57.1	21.6
	40-60	22.78	20.11	57.11	23.4

*CaCO<sub>3</sub> tot: total calcareous level*

**Table 5. Soil microbial biomass (bacterial and fungal) counting on the studied sites**

<b>Sites</b>	<b>Depth cm</b>	<b>Mesophilic Aerobic Bacteria (MAB) CFU/g d. soil</b>	<b>Filamentous Fungi Counting (FFC)</b>
Site 1 (control)	0-20	112 ( $\pm 2$ ) $10^4$ b	34 ( $\pm 3.5$ ) $10^2$ a
	20-40	82 ( $\pm 3.5$ ) $10^4$ ab	42 ( $\pm 2$ ) $10^2$ a
	40-60	34 ( $\pm 2$ ) $10^4$ a	25 ( $\pm 1$ ) $10^2$ a
Site 2 (10 years)	0-20	164 ( $\pm 5.3$ ) $10^6$ bc	46 ( $\pm 2$ ) $10^5$ c
	20-40	142 ( $\pm 4$ ) $10^6$ b	58 ( $\pm 4$ ) $10^4$ b
	40-60	68 ( $\pm 2$ ) $10^6$ a	78 ( $\pm 2$ ) $10^2$ a
Site 3 (20 years)	0-20	208 ( $\pm 4$ ) $10^6$ c	87 ( $\pm 5$ ) $10^5$ cd
	20-40	177 ( $\pm 5$ ) $10^6$ bc	66 ( $\pm 3.5$ ) $10^5$ c
	40-60	84 ( $\pm 2$ ) $10^6$ a	39 ( $\pm 2.3$ ) $10^2$ a
Site 4 (25 years)	0-20	219 ( $\pm 2.3$ ) $10^6$ c	102 ( $\pm 3$ ) $10^5$ d
	20-40	190 ( $\pm 3.5$ ) $10^6$ bc	96 ( $\pm 1$ ) $10^5$ d
	40-60	89 ( $\pm 3$ ) $10^6$ ab	159 ( $\pm 2$ ) $10^2$ a
Site 5 (28 years)	0-20	221 ( $\pm 1$ ) $10^6$ c	83 ( $\pm 2$ ) $10^5$ cd
	20-40	193 ( $\pm 3$ ) $10^6$ bc	65 ( $\pm 4$ ) $10^5$ c
	40-60	92 ( $\pm 4$ ) $10^6$ b	138 ( $\pm 3$ ) $10^2$ a

*n* = 3; (In brackets): standard deviation; Means followed by the same letter (a, b c ...) within a line are not significantly different according to the Student-Newman-Keuls test at  $P < 0.05$ ; CFU/g. d. soil: Colony forming unit per gram dry soil

**Table 6. Soil faecal pollution indicators on the different studied sites**

Sites	Depth cm	CT MPN/g d. soil	CF	E. coli	SF
Site 1 (control)	0-20	133 ( $\pm$ 21) a	66 ( $\pm$ 5) a	43 ( $\pm$ 15) a	1633 ( $\pm$ 321) ab
	20-40	67 ( $\pm$ 6) a	46 ( $\pm$ 12) a	37 ( $\pm$ 6) a	1200 ( $\pm$ 173) a
	40-60	57 ( $\pm$ 15) a	36 ( $\pm$ 6) a	33 ( $\pm$ 6) a	900 ( $\pm$ 321) a
Site 2 (10 years)	0-20	2933 ( $\pm$ 58) d	2067 ( $\pm$ 58) cd	1900 ( $\pm$ 265) bc	26667 ( $\pm$ 3214) c
	20-40	1833 ( $\pm$ 289) c	1433 ( $\pm$ 58) c	1200 ( $\pm$ 173) b	11667 ( $\pm$ 2516) c
	40-60	120 ( $\pm$ 17) a	67 ( $\pm$ 6) a	57 ( $\pm$ 15) a	1733 ( $\pm$ 312) ab
Site 3 (20 years)	0-20	3300 ( $\pm$ 608) d	2633 ( $\pm$ 462) d	2167 ( $\pm$ 115) c	46667 ( $\pm$ 5773) d
	20-40	3267 ( $\pm$ 635) d	2467 ( $\pm$ 289) d	1700 ( $\pm$ 264) a	37667 ( $\pm$ 4041) d
	40-60	147 ( $\pm$ 6) a	133 ( $\pm$ 21) b	113 ( $\pm$ 25) ab	1667 ( $\pm$ 378) ab
Site 4 (25 years)	0-20	3667 ( $\pm$ 577) d	3267 ( $\pm$ 635) d	2667 ( $\pm$ 321) c	53333 ( $\pm$ 15275) e
	20-40	3267 ( $\pm$ 635) d	2633 ( $\pm$ 289) d	2067 ( $\pm$ 58) bc	41000 ( $\pm$ 8544) d
	40-60	203 ( $\pm$ 6) a	103 ( $\pm$ 12) b	90 ( $\pm$ 20) ab	2667 ( $\pm$ 321) b
Site 5 (28 years)	0-20	3333 ( $\pm$ 577) d	3267 ( $\pm$ 635) d	2700 ( $\pm$ 321) c	60000 ( $\pm$ 15275) e
	20-40	3200 ( $\pm$ 173) d	2667 ( $\pm$ 321) d	2133 ( $\pm$ 58) c	50000 ( $\pm$ 10000) e
	40-60	240 ( $\pm$ 36) b	163 ( $\pm$ 32) b	133 ( $\pm$ 21) ab	15233 ( $\pm$ 21448) ab

*n* = 3; (In brackets): standard deviation; Means followed by the same letter (a, b c ...) within a line are not significantly different according to the Student-Newman-Keuls test at *P* < 0.05; g. d. soil: gram dry soil; TC: Total Coliforms; FC: Faecal Coliforms; E. coli: Escherichia coli; FS: Faecal Streptococci.

### 3.2.2.3 Opportunistic pathogen detection

*Salmonella spp* and *Shigella spp* screening revealed the absence of those pathogens in all studied soils. This result is true for the three soil horizons (0-20; 20-40 and 40-60 cm). This result seems to be due to the short survival period of these pathogens in the soil. According to [69] the survival period of pathogenic bacteria in soil or crops does not exceed a few days. However, it should be noted that large inputs of organic matter and nutrients could enhance the growth of microbial organisms [71]. Hence the health risk for the agricultural population which is in direct contact with poorly treated wastewater.

## 4. CONCLUSION

TMWW irrigation contributed to modify the physicochemical and microbiological soil properties (the amount of organic nutrients, the lowest risk of soil salinization, faecal indicators bacteria count...), the magnitude and specificity of these changes being significantly correlated with the duration of such practice. Microorganisms growth might be explained by the ready source of easily degradable compounds in the oligotrophic soil environment brought by TMWW irrigation.

The TMWW irrigation can have positive effects, not only in aspects of soil quality (the amount of organic nutrients), but also in social terms (improves the incomes of small farmers), as it allows the maintenance of irrigated agriculture in areas where groundwater has been polluted by seawater intrusion. However, proper management of TMWW irrigation and periodic monitoring of soil fertility and quality parameters are required to ensure successful, safe and long-term reuse of TMWW irrigation.

In our case, it should be noted that the treatment strategy used in the studied wastewater treatment plant (Southern Sousse) must be revised to ensure the reduction of emerging bacterial pathogens to non-detectable levels or to levels that have not been associated with human health risk. Southern Sousse plant, with a capacity of 10,000 cubic meters, receives around 30,000 cubic meters of wastewater per day. This situation will be resolved by commissioning, in the zone of another wastewater treatment plant (Sousse Hamdoune) with a capacity of 40,000 cubic meters per day.

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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