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Accumulation of pathogenic bacteria in agricultural soil irrigated with treated wastewater and their relationship with selected soil fertility variables

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Abstract
The use of treated wastewater (TWW) has been widely accepted globally offering significant benefits including alleviating water scarcity. However, TWW is also a potential source of pathogens whose fate and link to soil fertility variables are not well established. Thus, this study focused on establishing the level of bacterial pathogens accumulated and their relationship with soil fertility in agricultural soil irrigated with TWW and tap water (TP). Selected groups of pathogenic bacteria (*E.coli*, enteric bacteria, *Staphylococcus spp* and *Pseudomonas aeruginosa*) were isolated from the irrigation treatments and soil samples. In addition, soil fertility variables (electrical conductivity (EC), soil organic carbon (SOC), nitrate (NO_3), phosphorus (P), potentially mineralisable nitrogen (PMN), and pH) were analysed from the soil samples. The results revealed that using TWW for irrigation leads to a highly significant accumulation of the selected pathogens. But diluting TWW with TP reduced the accumulation of all the selected bacterial pathogens. In addition to the accumulation of pathogens, the use of TWW leads to an increase in P, EC, SOC, NO_3 and pH. However, the accumulation of the selected pathogens corresponded with the decrease in N, PMN and SOC.

Keywords: *water scarcity; wastewater treatment; soil quality; pathogenic bacteria.*

Introduction

Water scarcity is one of the greatest environmental challenges the agricultural sector is currently facing (Farhadkhani *et al.*, 2018). Since water acts as a solvent and a medium for nutrients, this has led to a decline in the yield of irrigated agriculture (Hejazi *et al.*, 2018). Consequently, leading to the increased use of TWW for irrigation, resulting in enhanced water security and improved yields (Cui and Liang, 2019). It has been proven that TWW contains a significant load of particulate and dissolved organic matter, as well as mineral macronutrients like phosphorus (P) and nitrogen (N) (Durán-lvarez and Jiménez-Cisneros, 2014; Khaskhoussy *et al.*, 2022). As a result of nutrient delivery, TWW acts as a fertilising agent, reducing the need for chemical fertiliser use (Farhadkhani *et al.*, 2018). Also, using TWW has been shown to improve soil bacterial activity (Becerra-Castro *et al.*, 2015; Muscarella *et al.,* 2024). Despite the aforementioned benefits, the water is considered a reservoir and vehicle for bacterial pathogens (Cui and Liang, 2019), therefore, using TWW poses risks to both human health and the environment. Irrespective of the reclamation process used to remove pathogens from wastewater, some of the resulting TWW still contain bacterial pathogens beyond the acceptable limits stated by the Food Agricultural Organization (FAO) (Cui *et al.*, 2020). The accumulation of bacterial pathogens in the soil cannot be decided based on the bacterial loads in the water used for irrigation because of several factors that act as filters (Farhadkhani *et al.*,

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2018). The soil is considered a natural filter (also known as a self-purifying resource), which can reduce the population of bacterial pathogens by inactivating them upon reaching the soil (Bernstein, 2011). It could either be through predation by the indigenous microbiota such as *Lactobacillus spp* and *Streptomyces spp* within the soil, or unfavourable environmental conditions (Durán-Álvarez and Jiménez-Cisneros). These environmental conditions include soil moisture, pH, temperature, organic matter content and antagonism with indigenous soil microorganisms (Farhadkhani *et al.*, 2018). However, some bacterial pathogens can survive under harsh environmental conditions and may compete for nutrients and sites of interaction with the beneficial bacteria (Ibekwe *et al.* 2018; Sarma *et al.*, 2023), which can lead to a decline in the beneficial microbial count and nutrients (Becerra-Castro *et al.*, 2015). Soil fertility is a crucial factor in ensuring sustainable crop production and ecosystem stability in agriculture (Li *et al.,* 2023). The delicate balance between beneficial and pathogenic bacteria living in the soil ecosystem is one of the major factors affecting soil fertility variables (Hayat *et al.*, 2010). So, the use of TWW for irrigation can lead to the accumulation of pathogenic bacteria in the soil (Farhadkhani *et al.*, 2018). Which have the potential to disturb the microbial equilibrium through competition with the beneficial bacteria for the nutrients in the soil. Since beneficial bacteria perform numerous ecosystem functions, which include promoting nutrient. mineralisation and availability this could lead to a decline in soil nutrients (Bernstein, 2011; Becerra-Castro *et al.*, 2015). However, TWW as a fertilising agent is rich in P, carbon and N which are essential for soil fertility (Ibekwe *et al.* 2018). As a result, high soil nutrients have been reported due to the use of TWW for irrigation (Ibekwe *et al.* 2018). But the cumulative impact of pathogenic bacteria from TWW on the fertility status of agricultural soil is not well established. A recent study conducted by (Kgopa *et al.*, 2021) reported high loads of bacterial pathogens in TWW used for irrigation from a night dam at the Uni-versity of Limpopo experimental farm (ULEF). Ho-wever, the accumulation of these pathogens in the soil and their relationship with soil fertility variables has not been explored at the farm. Therefore, there is a need to study the fate of these bacterial pathogens in the soil (Cui and Liang, 2019). Thus, this study was aimed at establishing the level of accumulation of pathogenic bacteria and their relationship with the fertility variables of agricultural soil irrigated with TWW.

Materials and Methods

Study area description

The study was conducted at the University of Limpopo (UL) in the Soil Science Laboratory and the shade house adjacent to the laboratory. The UL (23°88'71" S; 29°73'84" E) is based in South Africa (30°33'34.16" S; 22°56'15.06" E) under Limpopo pro-

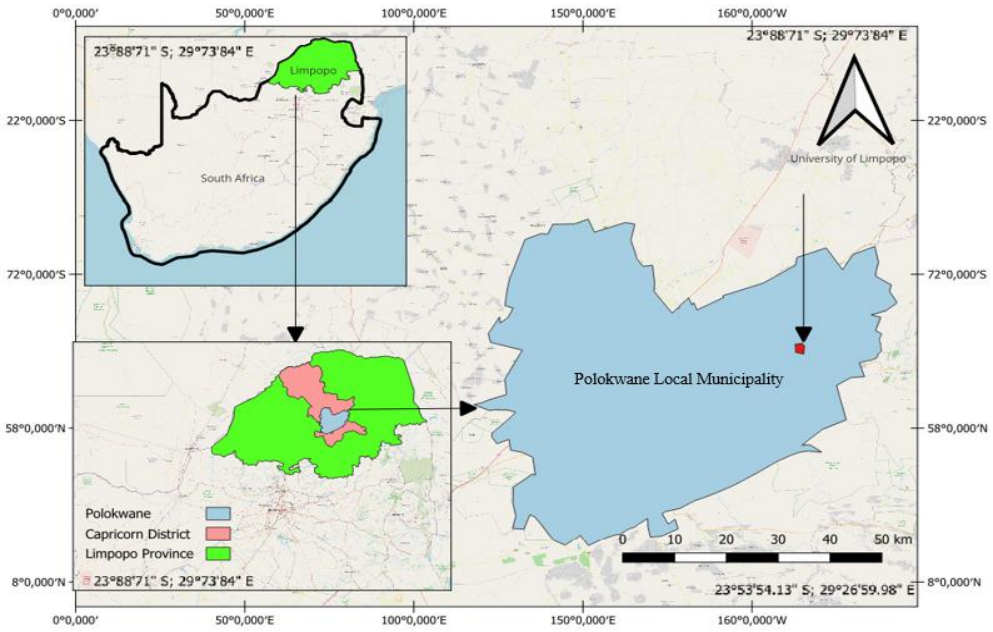

Figure 1
Study area map of the University of Limpopo.

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vince (23°24'04.66" S; 29°25'04.55" E), Polokwane Local Municipality (23°53'54.13" S; 29°26'59.98" E) as shown on Figure 1. The soil used in this study was classified as Chromic Luvisol using the World Reference Base (WRB) soil classification system (IUSS, 2014). Whereas the texture was loamy sand textured respectively soil. The soil was collected from a fallowed field while TWW was collected from a night dam (Fig. 2). These are located at the University of Limpopo Experimental Farm (ULEF), Syferkuil, in the Capricorn District Municipality, South Africa. The night dam receives TWW from Mankweng Wastewater Treatment Plant (MWTP) adjacent to ULEF (23°50'42.86" S; 29°42'44.35" E). The effluent at the MWTP was received from various settlements in the Mankweng vicinity, including Mankweng Hospital, the University of Limpopo and two shopping centres (Mankweng complex and Paledi mall) as well as filling stations (Kgopa *et al.*, 2021). The climate of the study area is classified as semi-arid, with the mean tempera-ture of 10°C and 25°C for winter and summer respecti-vely. The long-term annual rainfall received is between 350 mm and 500 mm and occurs mostly in the summer months of October to March (Mogale *et al.*, 2022).

Figure 2
The night dam and the fallowed field at the University of Limpopo Experimental Farm.

Experimental design

The experiment was a 2 x 5 factorial where the first factor was temperature which consisted of controlled temperature that was kept at 37°C throughout the trial (incubator) and uncontrolled temperature in the shade house. The second factor was irrigation treatments which were as follows: [100% Treated wastewater (100%TWW), 100% tap water (100%TP), 50% treated wastewater diluted with 50% tap water (50% TWW + 50% TP), 75% treated wastewater diluted with 25% tap water (75% TWW + 25% TP), and 25% treated wastewater diluted with 75% tap water (25%T WW + 75% TP)]. The irrigation treatments were arranged in a randomized complete block design (RCBD) in the shade house and a completely randomised design (CRD) in the incubator experiment.

Soil and water sampling

A composite soil sample was obtained by collecting core samples from six sampling positions within a uniform 1 hectare field following the random sampling method at a 30 cm depth. This was followed by the sampling of TWW and TP from the night dam exit at the farm and a tap next to the shade house at UL, respectively. The tap water was mixed with TWW to create the abovementioned irrigation treatments. The pre-soil samples for analysis were stored in sterile plastic zip lock bags which were placed in a fridge kept at 4°C, and then TP and TWW were analysed immediately after collection for the selected counts of pathogenic bacteria.

Experimental procedures

This study was conducted in summer and then repea-

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ted in winter to observe the response in two seasons. The experiment was established the same way for both seasons in the shade house and the Soil Science Laboratory (incubator). For the incubator experiment, twenty 500 ml glass beakers were labelled with a marker based on the five irrigation water treatments. For the shade house experiment, twenty truncated planting pots with 17 cm height, 13.5 cm base diameter and 19.8 cm top diameter were labelled using a marker based on the irrigation water treatments. An amount of 3746 g of soil was weighed into each of the planting pots and 468.25 g into each beaker. The soils were irrigated up to field capacity throughout the experiment. Then, the planting pots were placed in the shade house, and the beakers were placed in the incubator at a controlled temperature of 37°C. The experiment was conducted for a period of twelve (12) weeks.

Pathogenic bacteria isolation from the irrigation treatments and the soil

Selected groups of pathogenic bacteria were isolated from the irrigation treatments and pre and post-irrigation soil samples using their selective growing media. The selective growing media used were as follows centrimide agar base for the isolation of *Pseudomonas aeruginosa* (*P. Aeruginosa*), MacConkey agar (MAC) for Enteric bacteria, mannitol salt agar for *Staphylococcus spp* and m-TEC agar for *Escherichia coli* (*E.coli*). Spread plate using the glass spreader method was followed to grow the pathogenic bacteria in their respective agars (Sanders, 2012). The number of colonies on the growing media were counted, recorded and used to calculate the colony forming units (CFU) per gram of soil using the following equation:

$$\frac{\text{(no. of colonies) x (dilution factor)}}{\text{(volume of culture plated) x (weight of soil diluted through serial dilution)}} \quad [1]$$

Whereas CFU/ml of the irrigation treatments were calculated using this formula:

$$\frac{\text{(no. of colonies) x (dilution factor)}}{\text{(volume of culture plated) x (amount of water diluted through serial dilution)}} \quad [2]$$

The CFU/g of the bacterial pathogens were transformed using the $Log_{10}(x+1)$ homogenise the variance (Kuske *et al.*, 1998).

Soil physicochemical properties

Soil texture for the composite soil sample prior to contamination was determined using the hydrometer method (Bouyoucos, 1962). Pre- and post-trial soil samples were analysed for soil pH in water using the electrometric method (van Reeuwijk, 1992). Electrical conductivity (EC) was determined using an EC meter in a 1:2.5 soil to water ratio solution (Page, 1982). Soil organic carbon (SOC) was determined using the Walkley-Black method (Walkley and Black, 1934). Soil nitrate nitrogen (NO_3-N) was extracted using the colorimetric method by (Olsen, 1954) while phosphorus was extracted following the Olsen P method (Bremmer and Mulvaney, 1996) then analysed using ICP-OES (Hesse, 1971). Potentially mineralisable nitrogen (PMN) was determined following the phenate method (Benedetti and Sebastiani, 1996).

Statistical analysis

All data was subjected to two-way analysis of variance (ANOVA) using General Statistics 18th edition software (GenStat). Mean separation for significantly affected parameters was determined using Waller Duncan's Multiple Range Test at a probability level of 5% significance level. In addition, Pearson's correlation analysis was performed to assess the relationship between the accumulated pathogens and selected soil fertility variables.

Results

Counts of selected bacterial pathogens isolated from the irrigation treatments

Selected pathogenic counts in the irrigation treatments used for irrigation are outlined in Table 1. The highest counts of *Staphylococcus spp*, enteric bacteria, *E.coli* and *P.aeruginosa* were observed in 100% TWW. On the other hand, 100% TP had the lowest counts of *P.aeruginosa* and *Staphylococcus spp* but had no counts of enteric bacteria and *E.coli*. An increase in the proportion of TWW in the irrigation treatments led to an increase in the counts showing that the two have a directly proportional relationship.

Counts of selected bacterial pathogens in the soil before irrigation

Selected pathogenic counts of the soil before irrigation using TWW are outlined in Table 2. The highest counts of pathogenic bacteria observed were those of

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Table 1. *Counts of pathogenic bacteria in the irrigation treatments used to irrigate the soil*

Pathogenic bacteria (CFU/ml)	100% TP	100% TWW	75%TWW+25%TP	50%WW+50%TP	25%TWW+75%TP
Staphylococcus spp	0	3700	2700	1900	900
Enteric Bacteria	2200	8300	6300	4200	2000
E.coli	0	46000	37100	23200	15500
P.aeruginosa	1100	28600	21400	14300	7100

100% TWW = 100% treated wastewater, 50% TWW + 50% TP = 50% treated wastewater + 50% tap water, 75% TWW + 25% TP = 75% treated wastewater + 25% tap water, 25% TWW + 75% TP = 25% treated wastewater + 75% tap water treated and 100% tap water = 100% TP.

Staphylococcus spp (45800 CFU/g) and the lowest counts observed were those of enteric bacteria (14300 CFU/g) in the pre sample.

Table 2. *Selected pathogenic counts in the soil before irrigation*

Pathogenic bacteria	Bacterial pathogenic counts
Staphylococcus spp (CFU/g)	45800
Enteric bacteria (CFU/g)	14300
E. coli (CFU/g)	30000
P. aeruginosa (CFU/g)	35000

The selected fertility variables of the soil before irrigation

The selected soil fertility variables analysed before irrigating with treatments are outlined in Table 3. The SOC was 0.62% whereas the NO_3-N level was 0.32 mg/kg with PMN being 0.06 mg/kg. While the soil EC was 107.60 µS/cm which shows that the soil is not saline and the P content of the soil was 26.90 mg/kg. The pH of the composite sample before irrigation was alkaline with a level of 7.56.

Table 3. *Selected soil fertility variables before irrigation*

Physicochemical properties	Status	Standard deviation
Sand (%)	70.00	2.00
Silt (%)	16.67	1,00
Clay (%)	13.33	0,58
pH (H_2O)	7.56	0,40
EC (µS/cm)	107.60	2,60
SOC (%)	0.62	0,06
NO_3-N (mg/kg)	0.32	0,03
PMN (mg/kg)	0.06	0,01
P (mg/kg)	26.90	1,85

EC = electrical conductivity, SOC = soil organic carbon, NO_3-N = nitrate nitrogen, PMN = potentially mineralisable nitrogen and P = phosphorus.

Accumulation of pathogenic bacteria on agricultural soil irrigated with treated wastewater under varying temperature conditions

The interaction of temperature and irrigation treatments had a highly significant effect ($p<0.01$) on *E.coli* and enteric bacteria during summer and winter trials. Likewise the independent factors, irrigation treatments and temperature had a highly significant effect ($p<0.01$) on enteric bacteria and *E.coli* during the summer and winter trials. Generally, using 100% TWW for irrigation resulted in the highest accumulation of *E.coli* and enteric bacteria during both trials. The highest count of *E.coli* observed was 31500000 CFU/g (7.50 transformed) in soils irrigated with 100% TWW under uncontrolled temperature in summer. While the lowest count, 300000 CFU/g (5.48 transformed) was observed in soils irrigated with 100% TP under controlled and uncontrolled temperature in both trials (Figure 3a and b). During the winter trial, the highest count of enteric bacteria, 1775000 CFU/g (6.25 transformed), was observed under controlled temperature in soils irrigated with 100% TWW. The lowest count observed was 100000 CFU/g (5 transformed) under controlled temperature during the summer trial in soils irrigated with 100% TP (Figure 3c and d). Notably, the accumulation of *Staphylococcus spp* was not significantly affected ($p>0.05$) by the interaction of temperature and irrigation treatments during both trials. But the irrigation treatments had a significant effect ($p<0.05$) on the accumulation of *Staphylococcus* spp during the summer trial and a highly significant effect ($p<0.01$) during the winter trial. Whereas temperature had a highly significant effect ($p<0.01$) on *Staphylococcus spp* during the winter trial and a significant effect ($p<0.05$) during the summer trial. The highest count 1872250 CFU/g (6.27 transformed) of *Staphylococcus spp* was observed in soil irrigated with 100% TWW under

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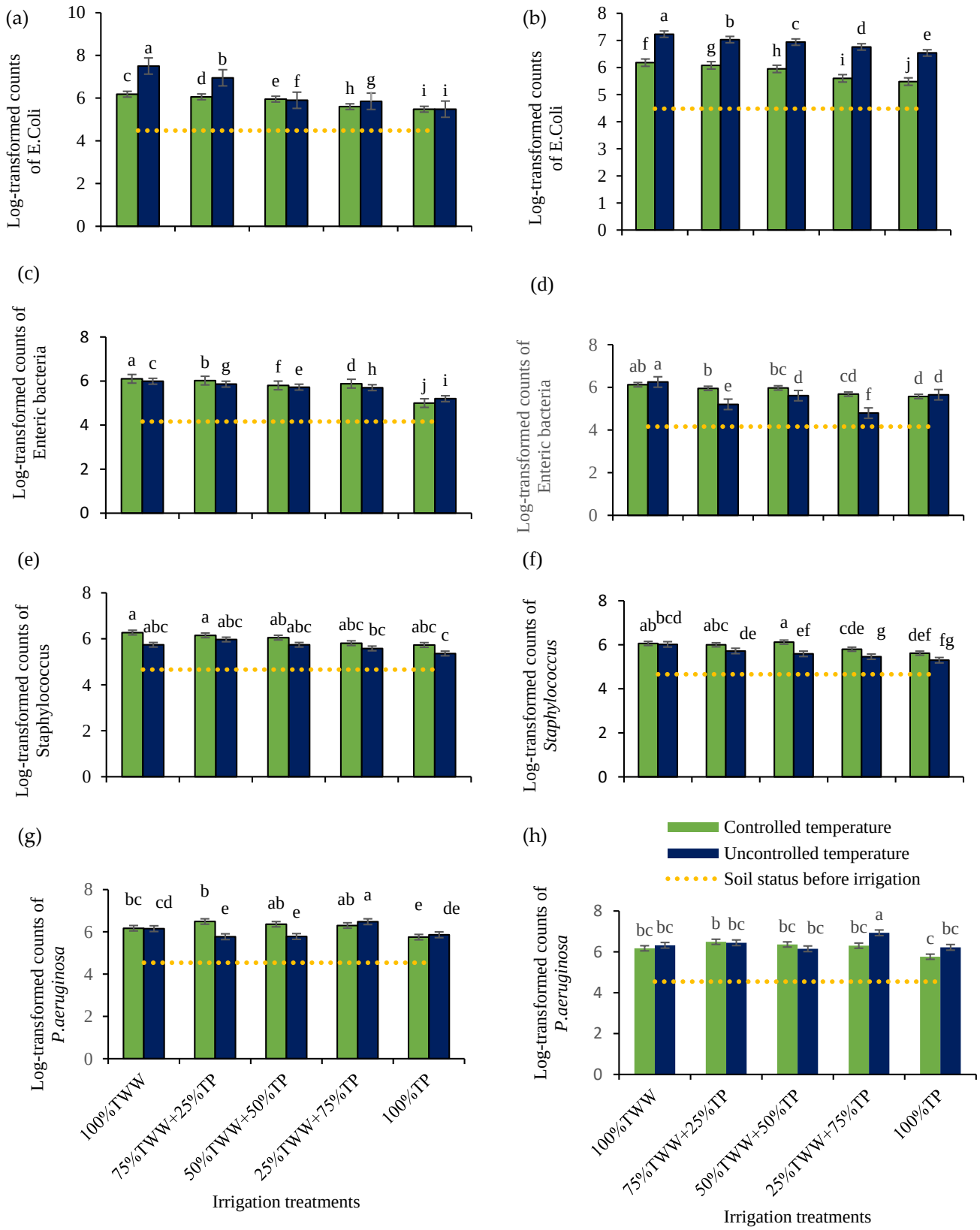

Figure 3. *Log transformed mean counts of pathogenic bacteria, (a) E.coli (c) Enteric bacteria (e) Staphylococcus spp and (g) P.aeroguinosa during the summer trial and (b) E.coli (d) Enteric bacteria (f) Staphylococcus spp and (h) P.aeroguinosa during the winter trial. According to Duncan's Multiple Range Test, the means followed by the same letter were not different (p>0.05). The yellow dotted lines indicate the bacterial counts that were in soil before the use of all the irrigation treatments to irrigate the soil. The error bars represent the standard error of the means.*

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controlled temperature during the summer trial. Likewise, the highest count 1160000 CFU/g (6.06 transformed) was observed in soils irrigated 100% TWW under controlled temperature during the winter trial. As expected, the lowest counts of *Staphylococcus spp* were observed in soils irrigated with 100%TP (Figure 3e and f). The interaction of temperature and irrigation treatments had a highly significant ($p<0.01$) effect during the summer trial on the accumulation of *P.aeruginosa* and a significant effect ($p<0.05$) during the winter trial. Similarly, the irrigation treatments had a highly significant effect ($p<0.01$) on the accumulation of *P.aeruginosa* in summer and a significant effect ($p<0.05$) in winter. Temperature had no significant effect ($p>0.05$) on *P.aeruginosa* during both trials. Unexpectedly the highest counts of *P.aeruginosa* were observed in soils irrigated using 75 TWW%+ 25%TP during both trials while the lowest counts were observed in soils irrigated with 100%TP (Figure 3g and h).

Interactive effects of temperature and the irrigation treatments on soil pH, EC and SOC

The combined effects of temperature and irrigation treatments had a highly significant impact ($p<0.01$) on EC levels during the summer season. Conversely, soil EC during the winter trial, pH (H_2O) and SOC during the summer and winter trials were not significantly affected ($p>0.05$) by the interaction of temperature and irrigation treatments. The irrigation treatments had a highly significant effect ($p<0.01$) on EC levels during both trials and on SOC during the summer trial. In contrast, SOC was not significantly affected ($p>0.05$) by the irrigation treatments during the winter trial. Temperature regulation did not show any significant effect ($p>0.05$) on EC during the summer and winter season. Soil EC was notably high in soils irrigated with 100% TWW under both controlled and uncontrolled temperature conditions, while it was low in soils irrigated with 100% tap water (TP) for both trials. Soil EC ranged from 136.8 µS/cm to 193.8 µS/cm under controlled temperature during the summer season and under uncontrolled, it ranged between 121.8 µS/cm and 275.5 µS/cm. Whereas, during the winter season the soil EC under controlled temperature ranged between 136.9 µS/cm and 193.8 µS/cm. While it ranged from 120.0 µS /cm to 184.5 µS/cm under uncontrolled (Figure 4c and d). Soil pH was significantly affected ($p<0.05$) by the irrigation treatments during the winter trial while no significant effect ($p>0.05$) was observed during the summer trial.

Unlike the irrigation treatments, temperature had a highly significant impact ($p<0.01$) on soil pH (H_2O) during both trials. The highest pH (H_2O) was observed in soils irrigated under uncontrolled temperature (8.47) and low in soils under controlled temperature (7.70) during the summer trial. Similarly, during the winter trial pH (H_2O) was high in soils irrigated under uncontrolled temperature (8.15) and low in soils irrigated under controlled temperature with a pH of 7.74 (Figure 4a and b). Interestingly, temperature had a highly significant effect ($p<0.01$) on SOC during the summer trial. However, no significant effect ($p>0.05$) was observed on SOC during the winter season. The highest SOC was observed in soils irrigated under controlled temperature with 50% TWW+ 50% TP (0.90%) and lowest in soils irrigated with 100% TP (0.11%) under uncontrolled temperature during the summer trial. During the winter trial SOC was high in soils irrigated under uncontrolled temperature with 50%TWW+ 50%TWW (0.93%) and lowest in soils irrigated with 25%TWW+ 75%TP (0.42%) under controlled temperature (Figure 4e and f).

Interactive effects of temperature and irrigation treatments on soil NO_3-N, PMN and P

A highly significant effect ($p<0.01$) caused by the interaction of temperature regulation and irrigation treatments was observed on soil nitrogen, PMN and P during summer and winter trials. Like the interaction, irrigation treatments significantly affected NO_3-N, PMN and P during trials. Similarly, a highly significant effect ($p<0.01$) of temperature regulation on soil nitrogen, PMN and P was observed for both summer and winter trials. The highest phosphorus content was observed in soil irrigated with 100% TWW (38.10 mg/kg) under controlled temperature during the winter season (figure 5). The lowest P content was observed in soils irrigated with 25% TWW + 75% TP (16.20 mg/kg) under uncontrolled temperature (Figure 5). The highest NO_3-N content in the soil was observed in soils irrigated with 100% TP (0.19 mg/kg) under controlled temperature. While the lowest nitrogen content was observed in soils irrigated in with 25% TWW+ 75% TWW (0.54 mg/kg) under uncontrolled temperature during the summer season. Similarly, during the winter trial soils irrigated with 25% TWW+ 75% TWW resulted in low NO_3-N content (0.54 mg/kg) under uncontrolled temperature. While low NO_3-N

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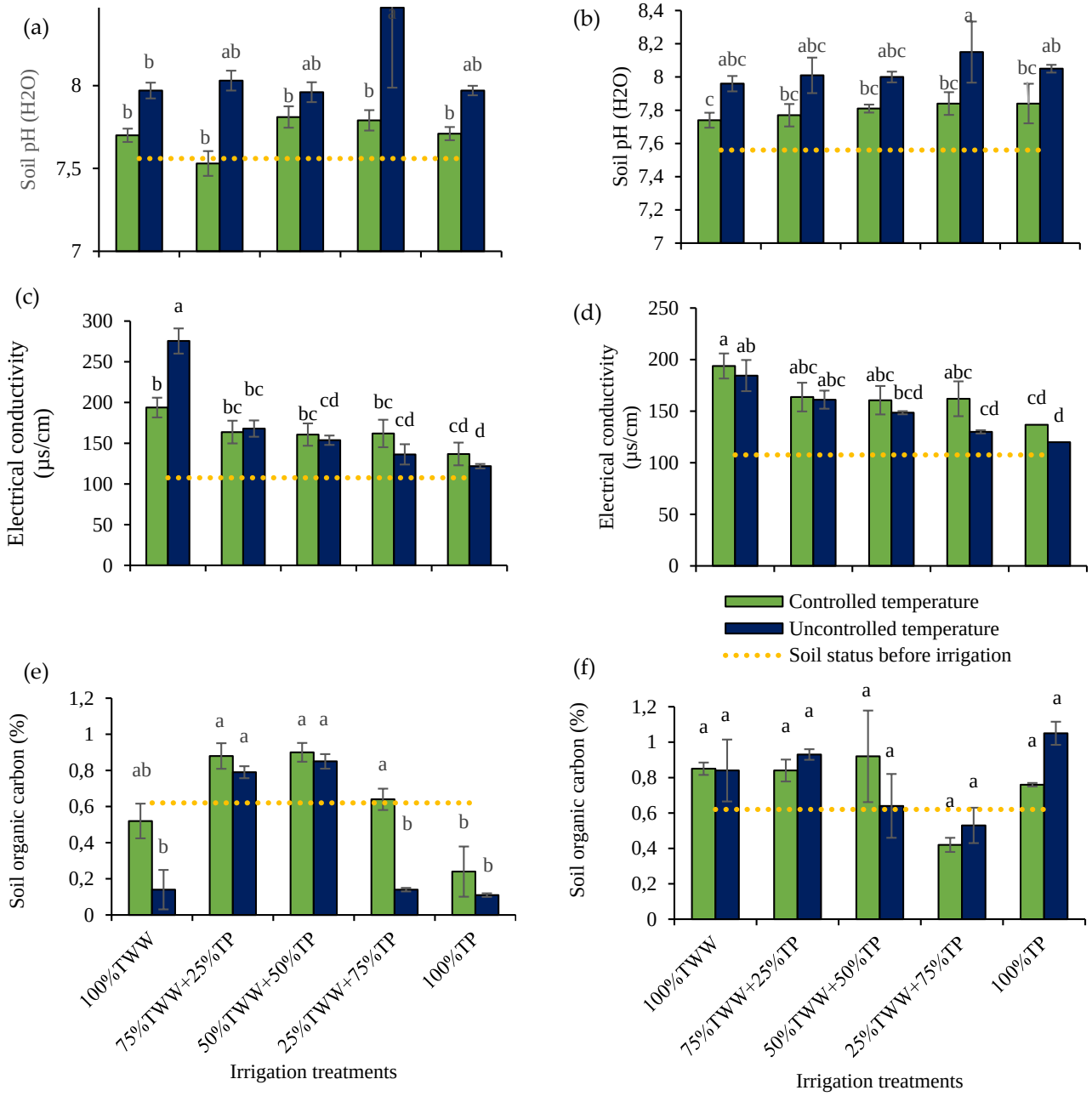

Figure 4. *Mean values of selected soil fertility variables: (a) soil pH, (c) EC, (e) SOC during the summer trial and (b) soil pH (d) EC, and (f) SOC during the winter trial. Means followed by the same letter were not different (p>0.05) according to Duncan Multiple Range Test. The yellow dotted lines indicate the status of the soil pH, EC and SOC before the use of all the irrigation treatments to irrigate the soil. The error bars represent the standard error of the means.*

content was observed in soils irrigated with 100% TP (0.23 mg/kg) under controlled temperature (Figure 5). The highest PMN was observed in soils irrigated with 50% TWW+ 50% TP (0.18 mg/kg) and 25% TWW+ 75% TP (3.13 mg/kg) under controlled and uncontrolled temperature, respectively, during the winter trial. Similarly, during the summer trial, the highest PMN content was observed in soils irrigated with 50% TWW+ 50% TP (0.02 mg/kg) and 25% TWW+ 75% TP (0.29 mg/kg) under controlled and uncontrolled temperature respectively (Figure 5).

The relationship between accumulated pathogenic bacteria and selected fertility variables

The influence of the accumulated selected pathogenic

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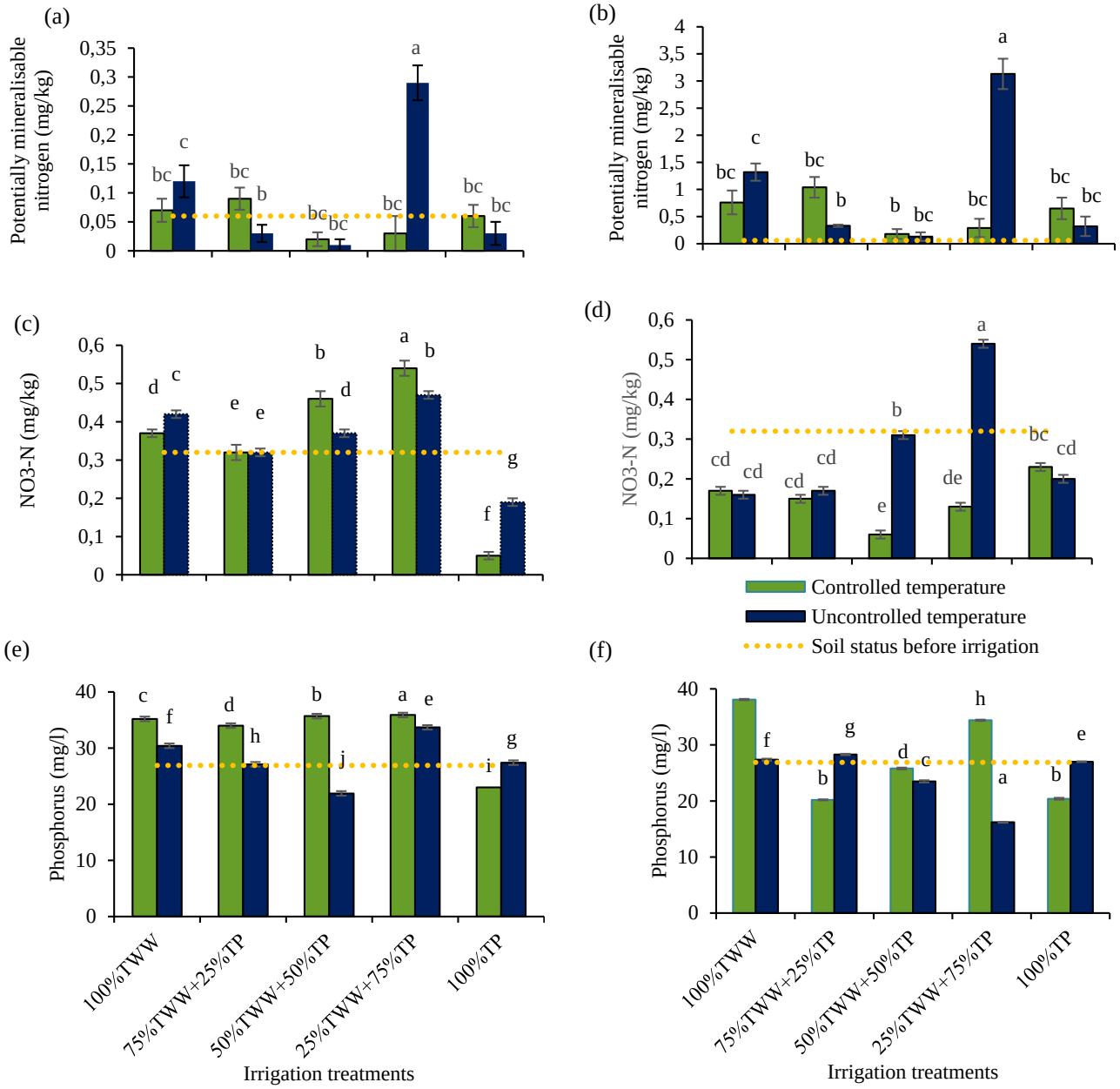

Figure 5. *Mean values of selected soil fertility variables: (a) potentially mineralisable nitrogen (PMN), (c) nitrate nitrogen (NO_3-N), (e) phosphorus (P) during the summer trial and (b) potentially mineralisable nitrogen (PMN) (d) nitrate nitrogen (NO_3-N), and (f) phosphorus (P) during the winter trial. Means followed by the same letter were not different (p>0.05) according to Duncan Multiple Range Test. The yellow dotted lines indicate the status of the PMN, NO_3-N and P and SOC before the use of all the irrigation treatments to irrigate the soil. The error bars represent the standard error of the means*

counts on soil fertility during summer trial is outlined in Table 4. The accumulated counts of *E. coli* had a significant positive correlation with EC only. While counts of *P. aeruginosa* had a significant positive correlation with NO_3-N, P and PMN. The accumulated counts of *Staphylococcus spp* had a significant negative correlation with SOC. The counts of enteric bacteria had a significant positive correlation with SOC, NO_3-N and P. The influence of the accumulated selected pathogenic counts on soil fertility during winter trial is outlined in Table 5. The accumulated counts of *E.coli* had a significant positive correlation with pH. Whereas counts of *Staphylococcus spp* and enteric bacteria had a significant negative correlation with pH and NO_3-N, a positive correlation with EC and P was observed. These pa-

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thogens also have a significant positive correlation between them indicating that they can coexist in the soil without negatively affecting one another. The counts of *P.aeruginosa* had a significant positive correlation with NO_3-N and PMN. But, had a signifi-cant negative correlation with counts of *E.coli* indica-ting their ability to suppress each other.

Table 4. *Pearson correlation analysis of the accumulated pathogenic bacteria and fertility variables from soil irrigated with irrigation treatments during the summer trial.*

	Staphylococcus spp	Enteric bacteria	*Escherichia. coli*	*Pseudomonas aeruginosa*
EC	0.22	-0.19	0.75	0.08
pH	-0.14	-0.01	0.08	0.23
SOC	-0.32	0.58	-0.03	-0.09
NO_3-N	0.07	0.52	0.26	0.64
P	0.18	0.53	0.04	0.62
PMN	-0.13	0.18	0.12	0.45
Staphylococcus spp	1.00	0.22	0.22	-0.07
Enteric bacteria	0.22	1.00	-0.07	0.22
Escherichia coli	0.22	-0.07	1.00	-0.11
Pseudomonas aeruginosa	-0.07	0.22	-0.11	1.00

EC=Electrical conductivity. SOC= Soil organic carbon. NO_3-N =Nitrate nitrogen. PMN=potentially mineralisable nitrogen. P=phosphorus. Bolded correlation values are significant at P ≤0.05.

Table 5. P*earson correlation analysis of the accumulated pathogenic bacteria and fertility variables from soil irrigated with irrigation treatments during the winter trial*

	Staphylococcus spp	Enteric bacteria	*Escherichia coli*	*Pseudomonas aeruginosa*
EC	0.42	0.51	0.03	-0.09
pH	-0.46	-0.45	0.45	0.07
SOC	0.14	0.02	0.13	-0.11
NO_3-N	-0.60	-0.62	0.13	0.51
P	0.45	0.49	-0.12	-0.27
PMN	-0.24	-0.24	0.20	0.38
Staphylococcus spp	1.00	0.58	-0.30	-0.16
Enteric bacteria	0.58	1.00	-0.17	-0.35
Escherichia coli	-0.30	-0.17	1.00	0.20
Pseudomonas aeruginosa	-0.16	-0.35	0.20	1.00

EC=Electrical conductivity. SOC=organic carbon. NO_3-N=nitrate nitrogen. PMN=potentially mineralisable nitrogen. P=phosphorus. Bolded correlation values are significant at P ≤0.05.

Discussion

Accumulation of selected bacterial pathogens on soil irrigated with treated wastewater

The irrigation treatments resulted in the accumulation of different counts of *E.coli* in the soil, with TWW contributing to the high counts in the irrigated soil. An increase in the TWW proportion in the TWW and TP (TWW+TP) dilution irrigation treatments led to an increase in the accumulation of *E.coli* in the soil. This increase was observed as a result of the high counts of *E.coli* in TWW (showing an addition of the pathogen to the soil ecosystem), while TP had no counts of *E.coli*. These findings were in agreement with (Vergine *et al*., 2015*)*, who reported that irrigation with TWW leads to the accumulation of *E.coli*. Uncontrolled temperature favoured the accumulation of *E.coli* for both winter and summer trial. The counts were high under uncontrolled temperature, but lower under controlled for all the irrigation treatments. This could be as a result of other antagonistic bacteria (such as *Pseudomonas spp)* that may exist in the soil which might have suppressed the counts of *E.coli*, given that controlled temperature (37°C) is favourable to most bacteria species (Seidu *et al*., 2008). These findings are similar to those observed by (Van Elsas *et al*., 2011), where the counts of *E.coli* increased in agricultural soils irrigated under uncontrolled temperatures. Enteric bacteria counts in the soil increased significantly

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as a result of the use of TWW for irrigation with temperature contributing to their survival in the soil. These findings correlate with those of (Malkawi and Mohammad, 2003), which showed that irrigating with TWW of poor biological quality (having high pathogenic bacteria counts) leads to the accumulation of enteric bacteria. In contrast, a study by Li *et al.* (2023), revealed that no significant effects were observed that could be attributed to TWW. The difference in the findings can be attributed to the quality of TWW used for irrigation because, according to (Obayomi *et al.*, 2019), the water acts as a carrier and transporter of enteric bacteria. The results of this study showed that TWW had high counts of enteric bacteria while TP had the lowest counts. Thus, TWW introduced more counts of enteric bacteria than the other irrigation treatments. In this study, soils irrigated under controlled temperature during the summer trial had the highest microbial counts of enteric bacteria compared to uncontrolled, indicating that the controlled temperature favoured the accumulation and survival of enteric bacteria. But during the winter trial, some treatments, such as 100% TWW and 100% TP, had high counts under uncontrolled temperature which was mostly below 37°C. This observation can be attributed to the ability of this pathogen to thrive well even under unsuitable temperature conditions during the winter season. In addition, unsuitable temperature conditions reduce counts of the antagonistic bacteria in the soil according to (LaBauve and Wargo, 2012) thereby reducing their level of suppression on counts of enteric bacteria. Using TWW for irrigation increased the counts of *Staphylococcus spp* during summer and winter trial. Since TP had no counts of *Staphylococcus spp* (Table 1) diluting TWW with TP before irrigation greatly reduced the counts accumulated in the soil. These findings correlate with those of (Obayomi *et al.*, 2019), which showed that irrigating with TWW that contains counts of *Staphylococcus spp* introduces and consequently increases the counts of *Staphylococcus spp* in the soil. Irrigation using 100% TP did not introduce any counts of *Staphylococcus spp* because this water does not have this bacterial pathogen. The *Staphylococcus spp* counts observed are the ones that were already in the soil before irrigation took place. In this study, controlled temperature favoured the increase in bacterial counts of *Staphylococcus spp*, hence the irrigation treatments led to higher counts compared to uncontrolled. According to (Malkawi and Mohammad, 2003), controlled temperature favours the survival and accumulation of Staphylococcus spp in the soil, whereas uncontrolled temperature filters *Staphylococcus spp* to a certain extent. Under uncontrolled conditions, the tem-perature fluctuates and it can go as low as 10°C and 25°C during winter and summer respectively. According to (Onyango *et al.*, 2012), the suitable temperature for the survival and proliferation of *Staphylococcus spp* is between 30°C and 37°C which is way above what the shade house at UL experiences. Thus, during low temperatures, the proliferation gets inhibited while the accumulation of *Staphylococcus spp* gets reduced hence the low counts observed under uncontrolled compared to controlled temperature.

The highest counts of *P.aeruginosa* were observed in soil irrigated with 25% TWW while the lowest counts were observed in soils irrigated with 100% TP under uncontrolled temperature during the both trials. (Culotti and Packman, 2014) reported that *E.coli* can survive in soils with limited nutrients and can outcompete *P.aeruginosa*. So the high counts of *P.aeruginosa* observed in soils irrigated with 25% TWW+ 75%TP can be attributed to the fact that the accumulation of *E.coli* was much lesser so this led to a minimal competition for nutrients hence favouring the use of the nutrients by *P.aeruginosa* and its survival in the soil. In contrast, under controlled temperature high counts were observed for soils irrigated with 75% TWW+ 25%TP. This outcome for both seasons does not correlate with the analysis of the water (Table 1). This shows that other factors might have led to this variation such as the microbial diversity of the soil which might have had a higher population of antagonist bacteria which played a crucial role in filtering *P.aeruginosa* and mostly the pathogen's response to variation in temperature. According to (Farhadkhani *et al.*, 2018), the accumulation of bacterial pathogens in the soil cannot be decided based on the bacterial loads in the water, due to the varying capabilities of the soil to filter pathogens. Controlled temperature led to high bacterial counts in soils irrigated with 100% TWW and 50% TWW + 50% TP when compared to uncontrolled during the winter season. Conversely, irrigating with 25% TWW + 75% TP and 100% TP led to high counts under uncontrolled compared to controlled temperature, this is a conflicting result.

The influence of treated wastewater on soil selected fertility variables

Irrigating the soil using different water treatments increased soil pH during both summer and winter trial with temperature contributing to the variations

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amongst the treatments. The soil pH range for both summer and winter trials under controlled and uncontrolled temperature falls within the alkaline range (7.0-8.5) outlined by (Maier and Pepper, 2009). Which according to (Maier and Pepper, 2009) falls within the ideal range for microbial growth and activity. In addition, it is favourable to the availability of soil nutrients such as nitrogen, phosphorus and potassium, which act as substrates for bacteria in the soil. The highest increase was observed in soils irrigated with TWW. These findings correlate with those of (Becerra-Castro et al., 2015), where the highest increase was observed in soils irrigated with TWW. As a result, of TWW having high salts which increased the soil pH following irrigation with the water, which leads to, salts building up in the soil (Durán-Álvarez and Jiménez-Cisneros, 2014). The soil EC varied amongst the soils irrigated with different irrigation treatments. Irrigation using 100% TWW resulted in high soil EC during both winter and summer trial whereas 100% TP resulted in the lowest EC. The findings in this study are similar to those of (Cui and Liang, 2019), which state that the use of TWW for irrigation increases soil salinity. In addition, a study by (Durán-Álvarez and Jiménez-Cisneros, 2014), shows that mixing TP and TWW decreases EC in the water thereby improving its quality for irrigation. The results in Figures 4c and d indicate that all the soils irrigated with the different irrigation treatments are not saline based on the critical values outlined by (Zaku *et al.*, 2011), which shows that soils that have EC greater than 2000 µS/cm are saline. According to a study by (Tsigoida and Argyrokastritis. 2020), irrigation using TWW leads to an increase in the soil EC as a result of the water having high levels of salts. Soil salinity is directly linked to the decrease in microbial activities and population which also causes a decline in nutrient mineralisation (Becerra-Castro *et al.*, 2015). A decrease in the proportion of TWW in the TWW/TP dilutions led to a decrease in EC. This showed that the TWW and soil EC have a directly proportional relationship. Soil organic carbon content increased in soils irrigated with 50%TWW+ 50%TP under both controlled and uncontrolled temperature during the summer trial. This may be due to the fact TWW is rich in organic carbon and when used for irrigation acts as a fertilizing agent (Ibekwe *et al.* 2018). Also diluting the raw TWW reduced the accumulation of the pathogens (Figure 3) thereby reducing their competition for carbon which they use as a source of energy hence the accumulation of carbon was higher than the amount used by the pathogens. Thus, irrigation using 50%TWW+50%TP increased the SOC. According to (Becerra-Castro *et al.*, 2015), pathogenic bacteria compete for SOC in the soil thus a decrease was observed in soil irrigated with 100%TWW during the summer trial. Soils irrigated under controlled temperature had high organic carbon content compared to uncontrolled. As a result, of uncontrolled temperature favouring microbial activity during the summer trial and the increase in pathogenic bacteria, which in turn may have resulted in the decrease of SOC. An increase in the counts of pathogenic bacteria could have led to a decrease in SOC. This is supported by the negative significant correlation observed between *staphylococcus* and SOC showing that these two variables have an inversely proportional relationship. Unlike the summer trial contrasting results were observed during the winter trial. The highest content of organic carbon was observed in soils irrigated with 100%TP under uncontrolled, but under controlled temperature organic carbon was high in soils irrigated with 50%TWW. Under both controlled and uncontrolled temperature SOC was lowest in soils irrigated with 25%TWW. The observation was as a result of temperature variation and also may be due to the SOC content of the TWW+TP dilutions. The accumulation and activity of pathogenic bacteria is dependent on temperature (Becerra-Castro *et al.*, 2015). Hence though 25%TWW had a low count of pathogens, irrigating with this treatment resulted in low organic carbon content. The use of TWW for irrigation increased the NO_3-N content of the soil. These results are in agreement to those of (Farhadkhani *et al.*, 2018), where irrigation with TWW increased NO_3-N. But unexpectedly in this study the highest NO_3-N was observed in soil irrigated with 25%TWW + 75% TP. This may be attributed to the lower accumulation of pathogens (*E.coli* and enteric bacteria) which resulted in the reduction of the level of NO_3-N consumed by the pathogens for their metabolism. In addition, the negative correlation observed between the accumulated pathogens (*Staphylococcus spp* and enteric bacteria) and the NO_3-N content of the soil showed that an increase in the pathogenic bacteria results in the depletion of nitrogen. Denitrifying bacteria (*P.aeruginosa*) was observed to be in high counts on soils irrigated with TP. As a result, soils irrigated with 100% TP had the lowest nitrogen content. This bacterium can convert the available form of nitrogen (nitrate) into nitrogen gas resulting

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in a decline in soil nitrogen (Diggle and Whiteley, 2020) and according to (Durán-Álvarez and Jiménez-Cisneros, 2014), unlike TWW, TP is not rich in nitrogen. Thus, in this study soils irrigated with 25%TWW + 75%TP had the highest nitrogen content, since the water introduced fewer counts of pathogens resulting in a less disturbed ecological balance between pathogenic and beneficial bacteria. In this study irrigating the soil using TWW resulted in an increase of PMN except for these dilutions 75% TWW + 25%TP during the summer trial under uncontrolled temperature, 50% TWW+50% TP during the summer trial and 25% TWW+75% TP under controlled temperature during the summer trial. This variation observed may be attributed to the high accumulation of pathogens and high temperatures experienced during the summer trial. The high temperatures are very suitable for the competition of nutrients by the accumulated bacterial pathogens. Irrigating using 50%TWW +50% TP resulted in the lowest nitrogen content of the soil possibly due to the high number of pathogens accumulated in the soil. They compete with nitrogen mineralising bacteria for nitrogen. Whereas the use of 25% TWW + 75% TP led to the highest PMN. A possible explanation for this might be that these irrigation treatments introduced fewer bacterial pathogens thereby minimising their competition. The decrease observed in soils irrigated with treatments that have a higher proportion of TWW can be attributed to the high counts of pathogens in this water. This study revealed TWW results in the highest accumulation of pathogenic counts which then outcompeted mineralising bacteria thereby decreasing PMN. This, according to (Durán-Álvarez and Jiménez-Cisneros, 2014), is due to the pathogenic bacteria outcompeting the nitrogen mineralising bacteria in the soil for nutrients. Irrigating the soil with 25% TWW+ 75% TP resulted in the lowest accumulation of pathogens. Therefore there was minimal competition for phosphorus in the soil as shown by the non-significant negative correlation between *E.coli* and phosphorus. As a result, soil irrigated with 25% TWW + 75% TP had high phosphorus content under controlled and uncontrolled temperature in summer. In contrast to the observations in summer, high phosphorus content was observed in soils irrigated with 100% TWW and 75% TWW + 25% TP under controlled and uncontrolled temperature respecttively during the winter trial. This could be due to the fertilising abilities of TWW since it has been proven that the water is rich in phosphorus when used for irrigation. Which improves soil fertility without the use of synthetic fertilizers. Similar findings were reported by (Malkawi and Mohammad, 2003) were the study revealed that irrigating with 100% TWW results in an increase in soil phosphorus. The contradictory results observed between the summer and winter trial is due to the temperature variation.

Conclusions

The TWW from the ULEF night dam contains a significant load of pathogens beyond the permissible limits and irrigating with this water resulted in their transfer into the soil. However, the results of this study demonstrated that diluting TWW with TP reduces the counts of the studied pathogenic bacteria in the water, so this improved the biological quality of the water. In addition, the findings proved that the accumulation of the bacterial pathogens in soils irrigated with the dilutions is lesser compared to those irrigated with undiluted TWW. Generally controlled temperature favoured the accumulation of pathogens, while uncontrolled temperature decreased their accumulation. The use of TWW led to a significant increase in P, EC, SOC, NO_3 and pH. However, the accumulation of the pathogens correlated negatively with the SOC, N, PMN and NO_3 signifying that there could be an existent competition between the pathogens `for these nutrients that is causing their depletion. The irrigation treatment 50%TP+ 50%TWW had minimal negative effects making it suitable for maintaining the soil fertility variables. Therefore, it is advisable to dilute TWW with TP to improve the quality of the water before using it for irrigation. Despite the significant findings mentioned above, this study did not investigate the long-term effects of accumulated pathogens on soil fertility status. Moreover, it does not cover the impact that different crop types may have on the accumulation of pathogens in the soil. Therefore, future research should focus on possible interactions between various crops and TWW that may influence the accumulation of bacterial pathogens. Also, future work should try to establish the long term effects of using TWW on the accumulation of pathogens and how in turn this can affect the health of the soil. Such studies could provide valuable insights into managing soil health and crop safety in agricultural systems that use TWW.

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