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Comparable levels of microbial contamination in soil and on tomato crops after drip irrigation with treated wastewater or potable water

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ABSTRACT

To evaluate the impact of treated wastewater (TWW) irrigation for produce safety, field experiments were conducted to compare secondary and tertiary TWW with potable water using tomatoes as a model crop. Human pathogens including a suite of obligate and opportunistic bacterial pathogens (*Campylobacter*, *Pseudomonas*, *Salmonella*, *Shigella*, *Staphylococcus*), protozoa (*Cryptosporidium* and *Giardia*), and viruses (*Adenovirus* and *Enterovirus*) were monitored in two field trials using a combination of microscopic, cultivation-based, and molecular (qPCR) techniques. Results indicate that microbial contamination on the surface of tomatoes was not associated with the source of irrigation waters; fecal indicator bacteria (FIB) contamination was not statistically different on tomatoes irrigated with TWW or potable water. In fact, indicator bacteria testing did not predict the presence of pathogens in any of the matrices tested. Indicator bacteria and the opportunistic pathogens were detected in water, soil and on tomato surfaces from all irrigation treatment schemes, and were positively correlated with duration of time in the field ($p < 0.0001$). Pathogen contamination (*Cryptosporidium*) was detected in secondary TWW (3/14 samples) and on the surface of a TWW irrigated tomato (1/41 samples). Furthermore, the *Enterobacteriaceae* species in the TWW were markedly different from those found in soil and tomato. The results indicate that (surface drip) irrigation with TWW did not result in the transfer of fecal indicator bacteria or microbial pathogens to the irrigated soil or crop. Moreover, parallel testing for pathogens with traditional culture-based and quantitative PCR indicates that specific and rapid molecular testing of pathogens appears to be a more appropriate strategy than fecal indicator testing for the determination of reclaimed water safety.

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1. Introduction

In many geographic regions, demand for freshwater (FW) often exceeds availability. Globally, human populations are forecasted to increase, which will most likely compound beneficial water use issues and exacerbate regional conflicts over water resources (Sofer, 1999). Contemporary research offers potential for reduced consumption through various conservation and treatment technologies such as water desalination, disinfection and decontamination. The use of treated wastewater (TWW) has the potential for additional conservation, specifically in the form of

crop irrigation (Toze, 2006), considering that the amount of water used globally for agricultural purposes is increasing while the resources are limited or even diminishing (Boelee, 2013; Sofer, 1999).

Using TWW for crop irrigation represents an important opportunity for ensuring adequate sustenance in industrialized countries and food security in developing regions. An example for the implantation of this practice is found in Israel, where over the past three decades FW available to the agricultural sector was reduced while the amount of TWW supplied to farmers to irrigate fruit trees increased. At present, 96% of all municipal sewage in Israel is treated, 80% of which is reclaimed [versus 10.6% in Spain (Iglesias et al., 2010) or 2.5% in the United States (Page et al., 1996)], contributing about one-fifth of Israel's total water supply (Kfir et al., 2012). A crucial impediment to this environmentally sustainable approach is the possible contamination of produce with fecal pathogens that may be present in TWW, which carries an associated risk for foodborne illness to produce consumers.

Regardless of irrigation regimen, fresh produce eaten raw has been implicated as the major vehicle for foodborne pathogenic outbreaks in the past decade (Doyle and Erickson, 2008), mostly due to contaminated leafy greens, sprouts and low growing fruits, such as tomatoes (Warriner and Namvar, 2010). Soil might serve as a vehicle for transferring pathogens to produce due to pathogen persistence for long periods in soil following irrigation with TWW, fertilization practices (Bech et al., 2010; Gorbatsevich et al., 2013), or contaminated runoff (Ramirez et al., 2009). Some fecal pathogens may also propagate in the soil until crops are planted (Bernstein et al., 2007; Heaton and Jones, 2008), increasing the likelihood of contamination during the plant's growth cycle. Pathogens within the soil may contaminate crops directly, for instance when sprinkler irrigation cause leaf splash (Monaghan and Hutchison, 2012), or indirectly, by penetrating the plant tissues (Bernstein et al., 2007).

It has been well established that irrigation with raw WW increases the risk for bacterial, parasitic and viral infections in consumers (Campbell et al., 2001; Doyle and Erickson, 2008; Fattal et al., 1986; Nygård et al., 2008; Shuval et al., 1989). Yet, there is no conclusive evidence implicating TWW as a risk factor for crop irrigation. In fact, reports from around the world indicate that irrigation with TWW presents no greater risk than irrigation with potable water (Bichai et al., 2012; Christou et al., 2014; Cirelli et al., 2012; Forslund et al., 2012, 2010; Jang et al., 2013; Martínez et al., 2013; Shuval, 2010). However, these reports rely either on epidemiological data (reviewed in Shuval, 2010) or mainly on fecal indicator bacteria (FIB) such as coliforms or *Escherichia coli* to assess possible health risks (Bichai et al., 2012; Christou et al., 2014; Forslund et al., 2012, 2010; Jang et al., 2013), neglecting major pathogen groups such as viruses and protozoa. The lack of correlation between pathogens and FIB, currently used in microbiological monitoring standards (Bitton, 2011; Edberg et al., 2000; WHO, 2006), is well established (Harwood et al., 2005; Ottoson et al., 2006; Payment et al., 2001) and may lead to under- or over-estimation of the risks to public health.

In this study we used biochemical, molecular and microscopic methods to follow pathogens and indicators from TWW to the irrigated soil and crops. We hypothesized that the presence of FIB would not accurately predict the presence of pathogens in the crops; this lack of correlation may apply to bacterial pathogens as well as protozoa and viruses. We further postulated that soil and crops irrigated with TWW rather than potable water are more likely to be contaminated by fecal microorganisms, i.e., fecal contamination of the soil and crops would be directly correlated to the quality of the water used for irrigation.

2. Methods

2.1. Field cultivation experimental design

Tomato (*Solanum lycopersicum* L. cv Smadar) was used as a model vegetable crop to evaluate microbial contamination on crops irrigated with TWW and potable water. Lachish, the experimental station (operated by the Israeli Ministry of Agriculture), is located near the municipality of Kiryat Gat in the south of Israel. The tomato seedlings (obtained from Hishtil, Nehalim, Israel) were planted under a screen house in 11×2 m plots. Two independent field experiments were conducted with five replicates to each treatment (Fig. S1). In the first cultivation experiment (April–August, 2011), a total of 10 plots were planted and irrigated with potable water or secondary TWW effluents. In the second cultivation experiment (April–August, 2012), a total of 15 plots were planted and irrigated with potable water, secondary TWW, or tertiary TWW. Each plot experiment lasted for approximately 20 weeks from the planting of the seedling to the termination of the plats in the field.

Using a random experimental design, up to three plots were planted along a bed (10 m long and 1.9 m wide) with 1.5 m of non-irrigated soil separating plots within a bed (to ensure that roots of plants from one plot will not invade a neighboring plot). One dry bed separated adjacent beds. Each bed included one row of tomato plants, two plants per running meter, and one surface drip irrigation lateral.

2.2. TWW source and treatment

The source of the irrigation water was either potable water or secondary or tertiary treated effluent originating from the municipal WW treatment plant (WWTP) of the town of Kiryat-Gat (operated by Kal-Binyan, Caesarea, Israel). The WW was treated in an activated sludge system cycling between anoxic and aerobic conditions with a hydraulic retention time of about 28 h. The TWW was chlorinated upon leaving the WWTP. At the farm, the secondary TWW was stored in a 110 m^3 tank and for tertiary treatment was passed through a sand filtration column. In the first cultivation experiment the secondary TWW were used as is (without chlorination) mimicking a worst case-scenario. Results for this case helped to focus the detection efforts in following experiments. During the second cultivation experiment secondary and tertiary effluents were chlorinated (1 mg L^{-1} residual) at the entry point to the field. Thus, four treatments were applied in the field: in the (1) non-chlorinated secondary TWW (first cultivation study); (2) chlorinated secondary TWW; (3) chlorinated tertiary TWW by sand-filtration of the secondary TWW (second cultivation study); and (4) potable water irrigation was used as a control in both cultivation studies. We note that in accordance with the Inbar guidelines (Inbar, 2007), barriers were applied during the use of TWW for irrigation including surface drip irrigation (all treatments), chlorination (three treatments), and sand filtration (one treatment).

2.3. Method validation and limits of detection (LOD)

Limits of detection for bacteria, protozoa and viruses were estimated with preliminary spiking studies of the targets applied to the different matrices used in this study (i.e., water types, soil and tomato crops). Spiking studies were performed at high (1×10^3) and low (1–10) concentrations of biological agents of interest per test unit to estimate the recovery efficiency and LOD of each method and target combination (Table S1). Internal control surrogate microorganisms were added to the feed (*Acinetobacter baylyi* for the bacteria (Schriewer et al., 2010) and *Pseudomonas aeruginosa* phage PP7 (Rajal et al., 2007) for viruses) and their LOD monitored

in preliminary experiments. Subsequently, these surrogates were added to all samples during the course of the experiments.

2.4. Quality control

Multiple quality control measures were incorporated into the procedures for sample handling and analysis to minimize and monitor for cross-contamination. In both seasons, five field blanks (i.e., controlled, uncontaminated samples of deionized water, autoclaved soil and surface sterilized tomatoes) were exposed to the same sampling conditions as the experimental samples and were processed in a manner identical to the experimental samples to assess potential contamination in the field and/or during sample processing in the laboratory (Keith et al., 1983). Method blanks and reagent controls were examined throughout the duration of the study to ensure reagent and method performance.

2.5. Recovery estimates

Known concentrations of surrogate particles or microorganisms were added to the collected samples and processed in an identical manner as the field samples. By calculating the percent recovery of the spiked organisms, an estimate was made regarding the true concentration of microorganisms in the field samples (Eq. (1)).

$$\frac{\text{Detected organism mL}^{-1}}{\text{Spiked organism mL}^{-1}} = \text{Percent Recovery} \tag{1}$$

Each recovery procedure was associated with a particular dilution or concentration factor, as described in the relevant sections (2.6, 2.8, 2.10), and used to normalize the detected organism concentration per mL.

2.6. Water sampling

For indicator bacteria and chemical parameter testing, one liter of each water type was collected in sterilized Nalgene bottles (Rochester, NY) containing sodium thiosulfate (0.1% v/v, Sigma, New Orleans, MO) to neutralize chlorine (Kemp and Schneider, 2000). The water was transported to the laboratory on ice, stored in 4 °C and analyzed within 24 h. For pathogen detection, one hundred liters of the treated effluents and potable water were collected in four 25-L jerricans and transported to the laboratory. There, the water was filtered through an FX-100 dialyzer (Fresenius Medical Care, Bad Homburg, Germany) to retain particles larger than 35 kDa and concentrated into a final volume of 100 mL retentate, following a previously described filtration protocol (Rajal et al., 2007). Aliquots of the retentate were tested for pathogens as follows (described in more detail in Sections 2.11–2.17): 1 mL was added to 9 mL buffered peptone water (BPW; BD, Franklin Lakes, NJ) for Salmonella enterica pre-enrichment. Up to 10 mL (depending on solids content) was used for protozoal detection by immunomagnetic separation (IMS) and direct fluorescence antibody (IFA) staining as described previously (Hogan et al., 2012; US EPA, 2005) and 0.5 mL was added to a screw-capped 2-mL tube containing glass beads, and frozen at −80 °C for molecular analyses.

2.7. Water chemical analysis

Chemical parameters including biological oxygen demand (BOD), chemical oxygen demand (COD), total nitrogen (TN), total phosphorous (TP), electrical conductivity (EC), and pH were measured at the WWTP and also tested by standard methods (APHA, 2012) for the water collected at the farm.

2.8. Soil sampling

Soil sampling always took place within a day of irrigation. Soil from a non-irrigated, nearby plot was sampled in parallel to the first sampling event in each season to serve as a reference for background FIB contamination. Soil (1 kg) was collected from the interior 10 m of each replicate plot by randomly selecting five sampling points. The soil was gathered from the upper 10 cm of the profile with a disinfected spatula, 20 cm from the dripper along the irrigation line. The samples were placed in individual Whirl-Pak sterile plastic bags (Nasco, Fort Atkinson, WI), transported to the laboratory in ice, stored in 4 °C and processed within 24 h. Samples were homogenized, sieved (0.1 cm) and detritus was removed. Soil aliquots were drawn as follows: 5 g of soil was suspended in PBS (1:5 w:v) (Sigma), shaken (200 RPM X 30 min) and after 10 min of settling the suspension was used for culture of indicators and pathogens including FIB, total coliform, P. aeruginosa and Staphylococcus aureus as previously described (Benami et al., 2013). Five gram was also used for protozoal detection according to Orlofsky et al. (2013). One gram was transferred to 9 mL BPW for S. enterica pre-enrichment. Lastly, 0.5 g was transferred to a screw-cap microcentrifuge tube containing glass beads, spiked with the surrogate organisms (A. baylyi and PP7), and frozen for molecular analyses (see below Sections 2.11–2.17).

2.9. Soil chemical and physical analyses

Chemical and physical properties were measured as previously described (Sacks and Bernstein, 2011). In short, soil pH and EC (in saturated paste extract) were determined with pH and conductivity meters, respectively. Na and K in saturated paste extracts were analyzed by atomic A/E and Cl by a Chloridometer; N as nitrate and ammonium and P were analyzed by an Autoanalyzer; soil texture was determined by densitrometer; cation exchange capacity was measured by the ammonium acetate method; and organic matter content by the dichromate method.

2.10. Crop sampling

Five tomatoes were collected at random from each plot and placed in paper bags. The samples were transported to the laboratory on ice, stored in 4 °C and processed within 24 h. Prior to processing, the surfaces of each tomato replicate (consisting of 5 tomatoes) were spiked with the bacterial and viral surrogates. After one hour, the five tomatoes from each plot were washed in sequence in 50 mL 0.01%, Tween-80 (Sigma) in PBS by vigorous shaking in a biohazard bag (Romical, Haifa, Israel). Aliquots of the wash were used for pathogen detection and FIB quantification. One milliliter was mixed with 9 mL BPW for S. enterica pre-enrichment, 25 mL was used for protozoal detection using IMS/DFA, and 0.5 mL was frozen for molecular analyses.

2.11. Microbial targets and detection methods

In the first season, we targeted fecal coliforms (FC), E. coli, Enterococci, and total coliforms as well as pathogenic bacteria (S. enterica, Shigella spp. and Campylobacter jejuni), opportunistic pathogens (P. aeruginosa, S. aureus), protozoal pathogens (Cryptosporidium spp. and Giardia spp.), and viral pathogens (Adenovirus [AV Types A, B, C & 40/41] and Enterovirus [EV71 subtypes]). In the second season (after analysis of the "worst case scenario" results from irrigation with non-chlorinated secondary TWW), the opportunistic pathogens P. aeruginosa and S. aureus and the obligate pathogens Shigella spp. and C. jejuni were omitted from the detection effort.

2.12. FIB quantification and characterization

In the first season, the indicator bacteria *E. coli*, total and fecal coliforms, and *Enterococcus* were quantified from serial dilutions of soil suspension and tomato wash using membrane filtration (APHA 9222; EPA 1600) and selective media (HiMedia, Mumbai, India). The water was tested using the Colisure and Enterolert kits (IDEXX Laboratories, Westbrook, ME) in a 15-tube MPN format (APHA, 2012). In-house validation verified that the plating and Colisure/Enterolert methods yielded identical results for the experimental matrices (Table S1). We found the use of Colisure/Enterolert to be more time-efficient and less labor intensive for rapid detection of FIB in a large number of samples. Thus, in the second growing season the indicator bacteria in all matrices were exclusively tested with the kits utilizing the Quantitray setup (IDEXX). Sub-samples of bacteria from all matrices that grew on media specific for *Enterobacteriaceae* (mFC, SS agar) were further classified using biochemical testing with Hy-Enterotest (Hylabs, Rehovot, Israel), according to the manufacturer's instructions. A sub-sample of enterococci was confirmed by growing colonies in brain-heart infusion (HiMedia) at 60 °C, and in 6.5% NaCl at 45 °C (APHA, 2012).

2.13. Opportunistic pathogen detection

Serially diluted aliquots of soil suspension, tomato wash and water samples were tested for the absence/presence of the opportunistic pathogen *S. aureus* with membrane filtration on Baird Parker selective agar (HiMedia) followed by confirmation with coagulase test (HiMedia). The opportunistic pathogen *P. aeruginosa* was detected on *Pseudomonas* isolation agar and confirmed with green fluorescence under UV light. *S. aureus* and *P. aeruginosa* were also detected using molecular methods (Table S2).

2.14. Protozoal pathogens detection

Detection of *Cryptosporidium* and *Giardia* was performed using immunomagnetic separation (IMS) followed by direct fluorescence antibody (DFA) staining according to EPA Method 1623 (US EPA, 2001) and utilized the C&G Combo kit (Life Technologies, Carlsbad, CA). Detection of protozoa from irrigation water types and tomato wash was performed as previously described (Hogan et al., 2012). Detection in soil utilized an IMS and DFA method that was recently validated as a sensitive tool for protozoal detection in this matrix (Orlofsky et al., 2013). Parasite staining using DFA was accomplished with EasyStain C+G combo (BTF-Bio, Sidney, Australia) according to the manufacturer's instructions, except that the mounting medium provided by the kit was replaced by No-Fade mounting medium (Waterborne Inc., New Orleans, LA).

Samples that had suspected protozoal parasites based on the IMS/DFA analysis were subjected to further molecular characterization. In order to prepare a concentrated sample containing parasites, IMS was performed for a second time on a fresh aliquot of the putatively contaminated environmental sample, without acid-elution of the parasites from the magnetic beads. The samples were then subjected to one freeze-thaw cycle to rupture (oo)cyst walls and DNA was then extracted following manufacturer's instructions using the Qiagen tissue extraction kit (Venio, Limburg, the Netherlands). *Cryptosporidium* genotyping was performed by PCR amplification of a conserved 18S rRNA encoding gene (Morgan et al., 1998) in a nested PCR protocol. Similarly, microscopy-positive *Giardia* samples were characterized using a semi-nested PCR and DNA sequence analysis of the glutamate dehydrogenase (GDH) encoding gene (Read et al., 2004).

2.15. S. enterica detection

In the first season, the presence of *S. enterica* was tested from all matrices by a modified version of the method described in the Bacteriological Analytical Manual (USFDA, 2011), and validated with spiking studies in each experimental matrix (Table S1). Samples were incubated in BPW (1:10 v/v, 37 °C, 24 h), followed by transfer of 1 mL of the non-specific enrichment to 9 mL Rappaport-Vassiliadis R-10 medium (BD) and incubation for 24 h at 41 °C. The culture was then inoculated on SS agar (HiMedia). Suspected colonies were tested biochemically with Hy-Enterotest (Hy-Labs) according to the manufacturer's instructions. Colonies that did not produce indole, produced acid and gas (from the fermentation of glucose), produced H_2S, and were motile and urease-negative were tentatively considered *S. enterica* strains, and were further tested for the presence of the *invA* gene by qPCR amplification (Benami et al., 2013). In an effort to obtain quantitative data, nucleic acid extracts from non-enriched samples were also tested for the presence of *invA* by qPCR (Benami et al., 2013). In the second experiment, a fast screening procedure was added to the culture method, utilizing nucleic acid extracted from enriched samples as template for qPCR analysis (Krämer et al., 2011).

2.16. Total nucleic acid (TNA) extraction

A phenol/chloroform (Sigma–Aldrich, Buchs, Switzerland) and bead-beating (BioSpec, Dover, FL) TNA extraction procedure (Angel, 2012) was used to maximize recovery of recalcitrant organisms from the environmental matrices. TNA extracts were passed through S-200 HR MicroSpin Columns (GE Healthcare, Little Chalfont, UK) then purified further using DNA extraction kit (Bioneer, Seoul, South Korea). The TNA were quantified with a NanoDrop 1000 spectrophotometer (Thermo, Waltham, MA) and frozen at −80 °C until used.

2.17. qPCR amplification

Detection of bacterial pathogens utilized the NAs extracted from the water, soil, and tomato samples using TaqMan® qPCR targeting *A. baylyi* (Schriewer et al., 2010), *P. aeruginosa*, *S. enterica* (Shannon et al., 2007), *S. aereus* (Elizaquível and Aznar, 2008), *Shigella* spp. (Thiem et al., 2004), and *C. jejuni* (Nogva et al., 2000), applying previously optimized qPCR methods (Benami et al., 2013).

For detection of viral pathogens, total nucleic acids extracted from the water, soil, and tomato samples were analyzed using TaqMan® qPCR targeting Pseudomonas phage PP7, Adenovirus (AV Types A, B, C and 40/41) and Enterovirus spp. (EV71 subtypes) applying previously established methods (Monpoeho et al., 2000; Leruez-Ville et al., 2004; Rajal et al., 2007). The sequences of the primer and probe combinations targeting bacteria and viruses are listed in Table 1.

2.18. Data analysis

Statistical analyses were performed using R (R Development Core Team, 2008). Samples were pooled by irrigation water type and Fisher's exact test was used to compare the occurrence of pathogens and FIB, according to Forslund et al. (2012), since non-detects occurred in many samples. The heteroscedasticity of log-transformed FIB concentrations (where quantitative data was available) were tested with Breusch–Pagan test (Breusch and Pagan, 1979) and the data found to meet the conditions for comparison with a simple regression on $\log(x)$. Means were then compared with a t-test (geometric mean testing). For soil chemical data (one season of "worst case"), a repeated measures ANOVA (with time as factor and plot number as ID) was used in order to minimize the

Table 1
Chemical characteristics of water types used to irrigate tomato plots. The values are the average and standard deviation of nine independent measurements.

Irrigation water type	BOD$_5$ [mg O$_2$ L^{-1}]	COD [mg O$_2$ L^{-1}]	pH	EC [mS cm^{-3}]	TSS [mg L^{-1}]	TN [mg L^{-1}]	TP [mg L^{-1}]
Potable	2.7 ± 2.4	7.0 ± 4.0	7.3 ± 0.7	0.7 ± 0.3	0.3 ± 0.5	3.4 ± 4.2	<1
Secondary TWW at WWTP	13.7 ± 4.5	30.8 ± 14.0	7.1 ± 0.3	1.1 ± 0.2	11.8 ± 6.3	10.5 ± 5.4	<1
Secondary TWW at farm	25.3 ± 5.8	80.0 ± 13.0	7.9 ± 0.5	2.0 ± 0.4	19.4 ± 13.7	9.4 ± 2.9	<1
Tertiary TWW at farm	22.5 ± 5.0	45.6 ± 14.0	8.1 ± 0.3	1.9 ± 0.4	13.4 ± 5.8	8.5 ± 4.6	<1

WWTP, wastewater treatment plant; BOD, biological oxygen demand; COD, chemical oxygen demand; EC, electrical conductivity; TSS, total suspended solids; TN, total nitrogen; TP, total phosphorus.

within subject error component, thereby increasing the power of the ANOVA. For water chemical data, one-way ANOVA on pooled data (excluding the time element) was used, since the sampling scheme did not include enough replicates to allow for within subject variance testing. *p* values < 0.05 were considered significant. Plots were generated with SigmaPlot v 6.1 (Systat Software, San Jose, CA).

3. Results

3.1. Water chemical properties

Potable water at the field conformed to permissible value ranges for all measured parameters by Israeli drinking water standards (Table 1; Israeli Ministry of Health, 2013). TWW was measured at four stages: immediately after secondary treatment at the WWTP, at the field without further treatment, at the field after chlorination and at the field after tertiary treatment (sand-filtration and chlorination). The secondary TWW at the WWTP was of superior quality to the secondary TWW at the field, and even the tertiary TWW (Table 1). Tertiary treatment was only partially effective at restoring the water quality. Thus, while the TWW discharged from the WWTP generally met average BOD/TSS/TN levels mandated by standard guidelines for unrestricted irrigation of 10/10/10 mg L^{-1} (Inbar, 2007), the TWW sampled at the field failed to meet these standards, with secondary and tertiary water reaching >30 and >25 mg L^{-1} BOD, respectively. The color of the secondary and tertiary TWW suggested that algal growth was present (data not shown).

3.2. Soil chemical and physical properties

The chemical and physical properties of the soils irrigated by TWW and potable water are summarized in Fig. S2. Three factors were examined: (1) the effect of the irrigation water treatment (irrigation water), (2) the temporal (weeks) effect on the chemical parameters, and (3) an interaction term between the two factors (irrigation water × weeks). The ANOVA results, including *F*-statistic, degrees of freedom and associated *p*-values, are presented in Table S3. In the case of a significant time related trend, a linear regression was used to further elucidate the relationship. It should be noted that the fertilization regimen used took into account the higher levels of nutrients in the TWW compared to the potable water.

The soil pH (Fig. S2A) was unaffected by the irrigation water alone, but the effect of time and the interaction between time and irrigation water was significant ($p < 0.001$ and $p = 0.03$, respectively). The pH varied only slightly throughout the season (7.8–7.5) reflecting the local calcareous soil buffer capacity, and therefore could be regarded as a relatively insignificant change overall. The pH of potable irrigated soil declined more noticeably than that in TWW irrigated soil ($R^2 = 0.58$ and 0.1, respectively), perhaps due to the lower pH in potable water as compared to TWW (Table 1).

The effect of time on EC was significant (Fig. S2B, $p = 0.001$) and increased during the growing season (from 1.2–5 ms cm^{-1}, $R^2 = 0.13$). This finding is similar to those reported by previous studies that applied TWW irrigation in Israel (Bernstein et al., 2009; Sacks and Bernstein, 2011). There was no effect of irrigation water or the interaction of time and irrigation water.

As expected, Cl$^-$ was higher under TWW irrigation (Fig. S2C, $p = 0.021$), with a significant difference first occurring at the 12th week of irrigation. Average values throughout the season were 158 mg kg^{-1} Cl in potable irrigated soil versus 278.8 mg kg^{-1} Cl in TWW irrigated soil. The effect of time alone was barely significant ($p = 0.052$), though the interaction between time and irrigation water was slightly more significant ($p = 0.02$). The levels of EC and Cl were within the acceptable range for tomatoes in Israel (Yermiyahu et al., 2010).

Ammonia (NH$_4$-N) and nitrate-N (NO$_3$-N) levels were unaffected by treatment type or by the interaction of irrigation water and time (Fig. S2D and S2E), but the levels of both were significantly affected by time ($p < 0.001$), with nitrate-N increasing slightly (slope = 1.65) and ammonia slightly decreasing (slope = −0.5) over the growing season. Phosphorous concentrations were similar in the TWW and the potable water irrigated soil (Fig. S2F, 33–40 mg L^{-1}), and remained stable throughout the growing season.

Potassium concentrations were affected by the irrigation water type, time and the interaction between them (Fig. S2G, $p < 0.001$). Potassium concentrations rose quickly after the start of cultivation and were higher on average in the TWW irrigated than potable irrigated soils (657.8 versus 464.9 mg kg^{-1}). This is a high value for potassium in soil and may be more than necessary for optimal tomato plant growth (Zalom, 2003).

Organic matter (OM) content was affected by time, and to a lesser extent, the interaction of time and irrigation water (Fig. S2H, $p < 0.001$ and $p = 0.011$, respectively). OM increased slightly more in the TWW than the potable irrigated soils (slopes = 0.11 and 0.095, respectively), probably due to higher organic matter content in the water source. However, the similar rise in OM in both soil types indicates that growth of microbes spurred by irrigation may be an important source of organic carbon.

3.3. Method validation and limit of detection

Methods were validated with preliminary spiking studies using serial dilutions (10^4–10^0) of organisms in the experimental matrices (Table S1). For bacteria and protozoa, method limits of detection (LOD) based on microscopy and culture were as low as 1–10^1 cell per unit sample (1 g soil, 1 L water, 1 tomato surface). For soil and tomato wash, the qPCR method LOD for the bacteria was up to two orders of magnitude higher (i.e., less sensitive) than using culture-based methods (10^2 gene copies per unit sample), due to the dilution series needed to observe linear amplification responses and the smaller sample size used for TNA extraction as compared to culturing. In water samples, the higher LOD of the qPCR was mitigated by ultrafiltration concentration. Viral method LODs were in the range of 10^2–10^3 gene copies (GC) per unit sample. Our results were sufficiently sensitive to detect the targeted protozoal and bacterial pathogens, which have low infectious doses (Table S1). Method agreement was observed between membrane
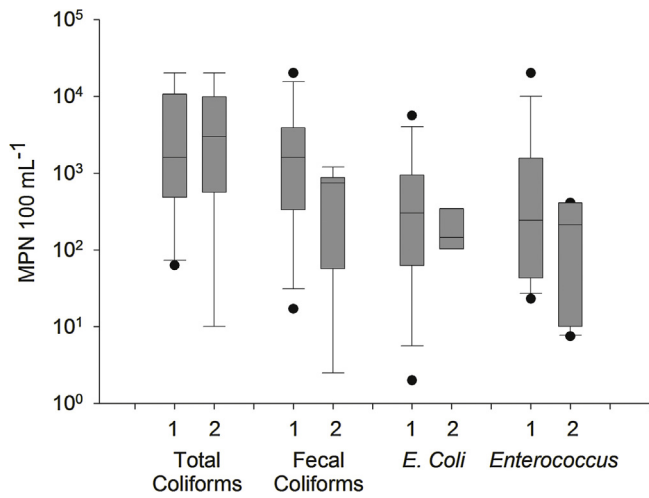
Fig. 1. Fecal indicator bacteria in the TWW used in the study during the second cultivation study ($n = 7$) in (1) secondary TWW (activated sludge followed by chlorination) and (2) tertiary TWW (activated sludge, sand-filtration and chlorination). Boxes represent 25–75% of data, solid line the median, whiskers the minimum and maximum (excluding outliers), outliers (1.5 time lesser or greater than lower or upper quartiles) represented by dots.

filtration and the Colisure/Enterolert systems for the measured FIB, and thus these data validated the interchangeability of these tests.

3.4. Fecal indicator bacteria detection

Fecal indicator bacteria (FIB) were rarely detected in potable water, with only a single detect of total coliform recorded during the second cultivation study (10 CFU 100 mL^{-1}, Table 2). By contrast, both the secondary and tertiary TWW were characterized by a high prevalence of FIB. The maximum values for FIB were higher in the secondary TWW than the tertiary TWW by approximately 1.5 logs (2×10^4 and 1.2×10^3, respectively, Table 2, Fig. 1), yet comparison of the geometric means indicated that similar overall quantities of FIB were present in both TWW types ($p > 0.05$). Similarly, the prevalence and quantity of FIB were not significantly different in soil or on the surface of tomatoes irrigated with the different water types (Figs. 2 and 3, Table 2, $p > 0.05$). Interestingly, over the course of both seasons, E. coli was rarely detected in
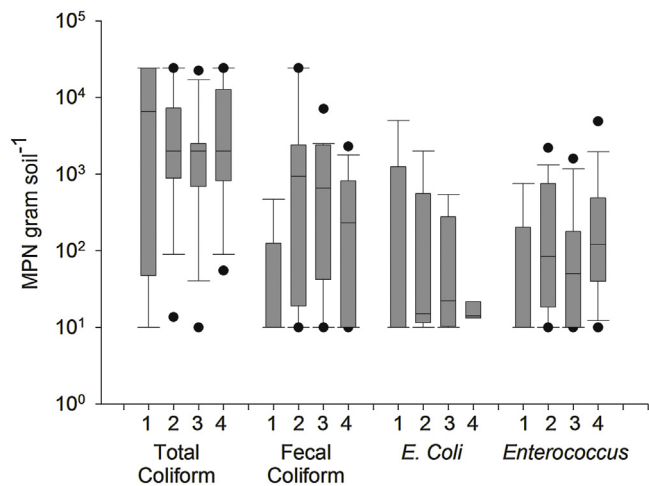

Fig. 2. Fecal indicator bacteria in (1) non-irrigated ($n = 5$), (2) potable, (3) secondary and (4) tertiary TWW irrigated soil ($n = 25$ for each irrigated treatment) from the second cultivation study. For explanation of boxplot symbols, see Fig. 1.

Table 2
Fecal indicator bacteria and pathogens in irrigation water, the irrigated soil and on tomato surfaces from two growing seasons (differences between seasons were insignificant by Fisher's Exact Test and Geometric Mean t-test).

| Sample Type	Irrigation water	Total coliforms		Fecal coliforms		Escherichia coli		Enterococcus		Pathogens
		Detects/total no.	Geometric mean (max value)	Detects/total no.	Geometric mean [max value]	Detects/total no.	Geometric mean [max value]	Detects/total no.	Geometric mean	[Detects/total no.]
Water	Potable	1/13	10 (10)	0/11	ND	0/14	ND	0/14	ND	ND
	Secondary TWW	13/13	1544 [20,050]	8/11	911.3 [20,050]	10/14	199 [5600]	12/14	320.3 [20,050]	C. parvum* [2/14], G. lamblia [1/14]
	Tertiary TWW	5/6	1239.6 [20,050]	5/7	166.3 [1203.3]	3/7	173.7 [410]	6/7	68.4 [410.6]	C. parvum [1/14]
Soil	Non-irrigated	4/5	1179 [24,190]	1/5	467 [467]	1/14	4950 [4950]	3/5	24.7 [749]	ND
	Potable	35/36	2610 [24,190]	28/41	2218.7 [24,190]	5/56	10.8 [1986]	42/56	193.2 [11667]	ND
	Secondary TWW	36/36	1774 [26,500]	29/41	1258 [10,112]	4/56	11 [667]	37/56	190.3 [5550]	ND
	Tertiary TWW	20/20	2375.9 [24,190]	11/26	571 [2500]	3/26	3.6 [4.1]	24/26	85.7 [10,000]	ND
Tomato	Potable	28/30	752.1 [2500]	17/30	674.6 [2500]	1/40	100 [100]	28/40	423 [10,000]	ND
	Secondary TWW	26/31	1275.8 [3555.6]	18/31	920.1 [2400]	2/41	6.9 [16.1]	27/41	333.6 [3888.9]	C. parvum [1/41]
	Tertiary TWW	15/15	2001.2 [2906]	7/20	577.7 [2400]	2/20	2.9 [4.1]	20/20	217.6 [5000]	ND

ND, not detected. *Confirmed by molecular testing.
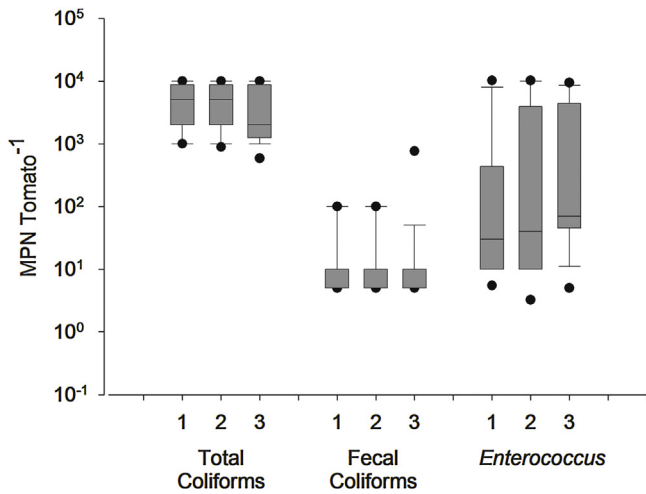
Fig. 3. Fecal indicator bacteria on the surface of potable (1), secondary (2) and tertiary (3) treated TWW irrigated tomatoes from the second cultivation field experiment ($n = 20$ for each treatment). For explanation of boxplot symbols, see Fig. 1.

soil irrigated with either water type, although the various TWWs contained significant amounts of this organism. It is further noteworthy that *E. coli* was infrequently detected on the tomato surface (up to 10% of samples) and its concentration did not differ among treatments (Table 2). No correlation between the irrigation regime and the FIB concentrations in the soil or tomatoes was detected. FIB counts on the surface of tomato (though not in the soil) increased over time in all treatments, and this trend is especially clear in the case of *Enterococcus*, where increases of 3–4 orders of magnitude were detected in the final compared to the initial sampling events ($F_{2,42} = 34.99$, $p < 0.0001$, Fig. 4). Interestingly, biochemical characterization of the *Enterobacteraciae* revealed separate communities in the TWW compared to the soil and tomato surface (Fig. 5). The TWW was dominated by *Proteus vulgaris* while FC from the soil and tomato surfaces mainly consisted of *Enterobacter* spp. and *Citrobacter freundii*. Thus, there was no detectable effect of extended TWW irrigation of soil and crops on either the amount or type of *Enterobacteraciae* in general, or FIB in particular, detected in these matrices.
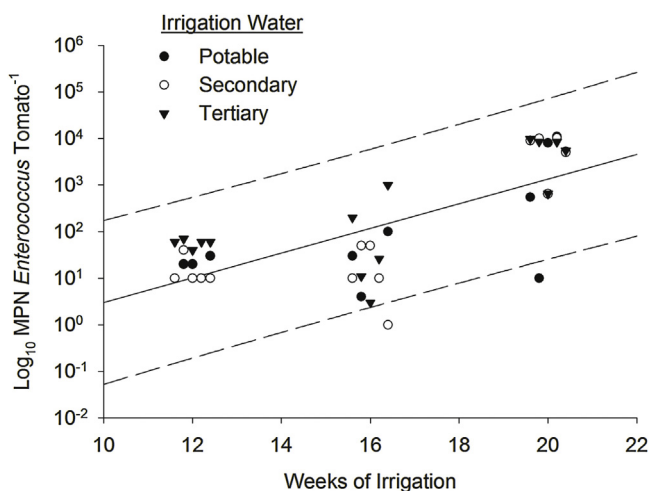

Fig. 4. Temporal changes of *Enterococcus* on the surface of tomatoes sampled during the second cultivation study. The regression ($\pm 95\%$ CI) is an average of all treatments as the individual slopes did not differ significantly ($p > 0.05$) $n = 5$.
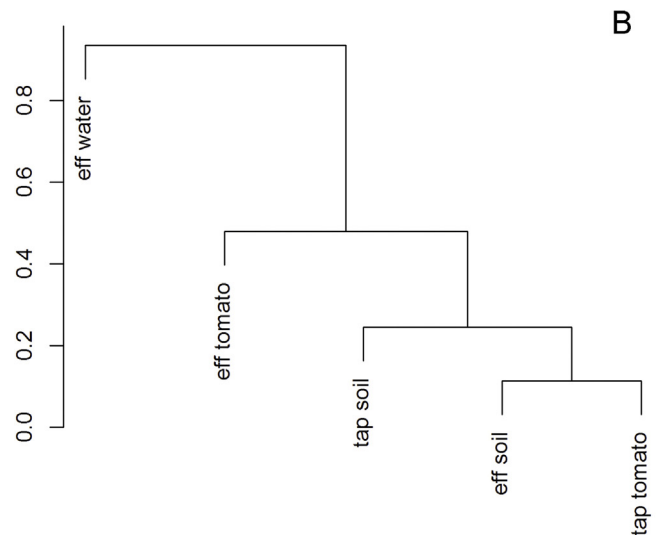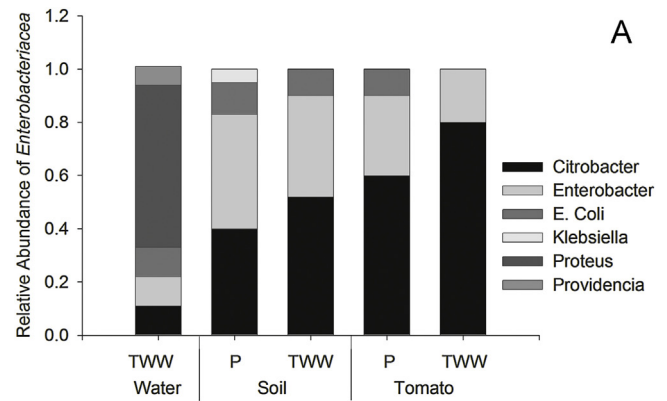

Fig. 5. (A) Relative abundance of Enterobacteriaceae in potable (P) or secondary TWW irrigated soil and tomatoes ($n = 20/60$ isolates per sample) from the first growing season. (B) Cluster dendrogram of the samples in (A) by Bray–Curtis distance matrix.

3.5. Opportunistic pathogens

The opportunistic pathogens *P. aeruginosa* and *S. aureus* were detected in TWW but not in potable water. Nevertheless, the prevalence of both opportunistic pathogens (measured by plate culture) did not differ significantly in the soil or on the crop exposed to the different irrigation waters ($p > 0.05$, Table 3). The concentration of *P. aeruginosa* increased from an initial concentration of up to 10^3 GC g soil^{-1} in non-irrigated soil to 4×10^5 GC g soil^{-1} and 7×10^4 in TWW- and potable water-irrigated soil, respectively, changing significantly during the cultivation period ($p = 0.011$). However, after 8 weeks of irrigation, the concentration of *P. aeruginosa* stabilized at $10^5–10^6$ GC g soil^{-1} in both irrigation regimes (Fig. 6). From that point on, concentrations did not change until the 20th week, when the bacterial concentrations (in both irrigation regimes) reached 10^7 GC g soil^{-1}. Analysis of opportunistic pathogens by qPCR in tomato wash showed a higher prevalence of *P. aeruginosa* as compared to culture based analysis (21/25 and 23/25 for potable and TWW irrigated samples, respectively, Table 3). The concentrations were identical in both treatments throughout the growing season ($p > 0.05$) and increased in concentration from 10^2 GC tomato surface^{-1} at week 12–5×10^4 GC tomato surface^{-1} at week 24 (Fig. 7). In water, *P. aeruginosa* was detected by qPCR in both water types (6/7 and
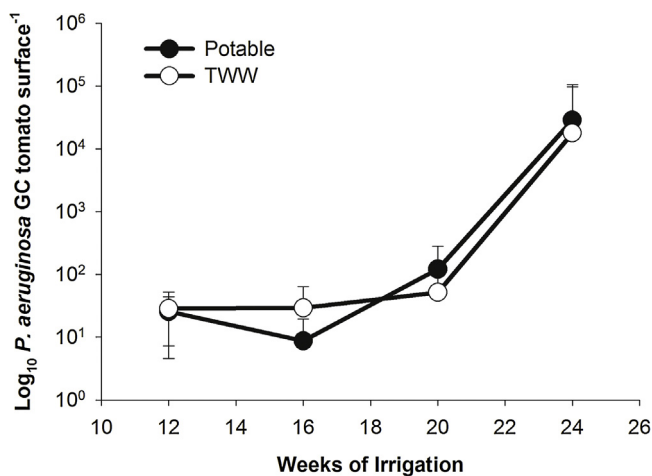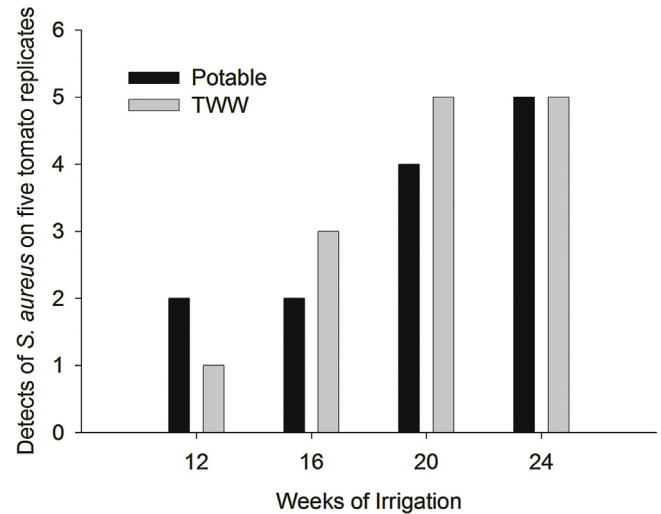
Table 3
Prevalence of *Pseudomonas aeruginosa* and *Staphylococcus aureus* in irrigation water, soil and tomato wash (culture based data) from one growing season.

	Water		Irrigated Soil		Tomato	
	Potable	TWW	Potable	TWW	Potable	TWW
P. aeruginosa	0/7	2/7	17/30	20/30	5/20	4/20
S. aureus	0/7	4/7	22/30	16/30	13/20	11/20

TWW, treated wastewater.
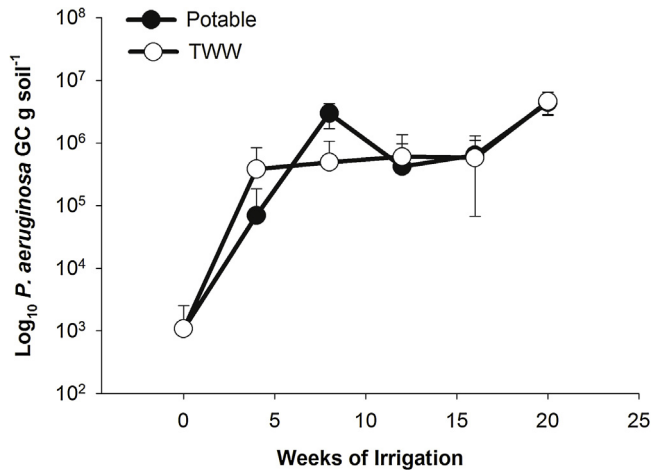
Fig. 6. *Pseudomonas aeruginosa* in soil irrigated with potable water and secondary TWW from 0 to 20 weeks of irrigation in the first growing season, determined by qPCR (*n* = 5). Error bars indicate standard deviation from average.

Fig. 8. Detection of *Staphylococcus aureus* on tomato surfaces by qPCR during the first cultivation season (*n* = 5 for each sampling event).

4/7 detects for potable and TWW water, respectively, Table 3), but quantitation appeared to be unreliable due to non-linear replication between log-dilutions (data not shown). *S. aureus* was not detected by qPCR in soil during the first 0–4 weeks of irrigation, but after 8–24 weeks it was found in all soil samples, save 3 from each treatment (17/25 samples, Table 3). On tomatoes, *S. aureus* was detected by qPCR in 13 and 14 of 20 samples for potable and TWW irrigated samples, respectively (Fig. 8). A clear trend of increasing detects of *S. aureus* was noticed from the 12th to 24th week of irrigation in both treatments (Fig. 8). In summary, the prevalence and magnitude of the opportunistic pathogen concentrations in soil and on tomato was not affected by the type of the irrigation water but was positively affected by the time in the field.

Fig. 7. *Pseudomonas aeruginosa* on tomato surfaces irrigated with potable water and secondary TWW from 12 to 24 weeks of irrigation (first growing season), determined by qPCR (*n* = 5). Error bars indicate standard deviation from average.

3.6. Microbial pathogens

Bacterial and viral pathogens including *C. jejuni*, *S. enterica*, *Shigella* spp, *Adenovirus* and *Enterovirus* were not detected in potable water or TWW (see LODs in Table S1). *Cryptosporidium parvum* was detected twice in secondary TWW (0.5 and 2.3 oocysts/L) and once in tertiary TWW (0.3 oocysts/L). Molecular genotyping of the oocysts from the secondary TWW revealed 100% homology of the 18S rRNA encoding gene with *Cryptosporidium hominis*, an obligate human parasite. Suspect *Giardia lamblia* cysts were detected twice in secondary TWW by IMS/DFA (0.5 and 0.1 cysts/L) but molecular typing was not conclusive. No pathogenic protozoa were detected in the soil. A single suspected oocyst of *C. parvum* was found on the surface of a secondary TWW irrigated tomato by microscopic methods during the second season, but molecular confirmation was unsuccessful. Thus, aside from the isolated *C. parvum* findings, none of the targeted microbial pathogens were conclusively detected in any of the matrices (Table 2).

4. Discussion

Fecal indicator bacteria and a range of microbial pathogens (bacteria, viruses, and protozoa) were monitored in soil and tomato crops irrigated with TWW over the course of two growing seasons. While FIB are used as the primary means of establishing water microbiological quality (USEPA/USAID, 2012; WHO, 2006), we found that their number and type (*E. coli*, *Enterococcus*, fecal coliforms and total coliforms) did not predict pathogen presence in any of the matrices tested (Table 2). Furthermore, it was found that irrigation with TWW had no effect on quantities of FIB and a variety of opportunistic or obligate human pathogens in the soil or on the crop (Tables 2 and 3, Figs. 2 and 8).

While several previous studies have evaluated the effect of application of raw WW to irrigate crops (Fattal et al., 1986; Minhas et al., 2006; Rosas et al., 1984) and determined it unfit, few studies

have examined the implications of applying TWW for irrigation of vegetable crops (Christou et al., 2014; Cirelli et al., 2012; Forslund et al., 2010, 2012; Martinez et al., 2013). Treatment of WW is intended to reduce organic matter, nitrogen, and microbial contaminants (Bitton, 2011). Indeed, we found that the water quality of the TWW at the point of exit from the WWTP (before the regional reservoir was filled) met the stringent regulations set by the Israeli ministry of health for unrestricted irrigation that mandate the "10/10/10/1 rule" referring BOD/TSS/FC/chlorine levels (Inbar, 2007; Israeli Ministry of Health, 2002). Fecal coliforms were undetectable (<1/100 mL), BOD and TSS were <10 mg L^{-1} and TN was approximately 5 mg L^{-1} (Table 1). However, in this study crops were cultivated during the long and dry Mediterranean summers and while monitoring the TWW we detected a general decline in water quality overall, and in particular a major increase in FIB concentrations (Tables 1 and 2, Fig. 1). It should be noted that the WWTP reported fairly consistent water quality parameters during the period of cultivation when experiments took place. Thus, any TWW contamination occurred subsequent to release from the treatment plant, potentially at the regional storage reservoir or from biofilms in the conveyance or irrigation pipes, as has been suggested by previous reports (Juanico and Shelef, 1994; Kfir et al., 2012; Rebhun et al., 1987).

A notable result from our study is that the high numbers of FIB in the water did not lead to FIB accumulation in the soil. In fact, *E. coli*, though present at 10^3–10^4 cells/100 mL in the TWW, was detected in the soil only in 10% of the samples and at concentrations that were similar across all irrigation regimes (Figs. 1 and 2, Table 2). The accumulation and persistence of fecally sourced microbes from WW in soil is one of the major concerns associated with WW irrigation (Bernstein et al., 2009), and has been shown to occur under simulated conditions (Bernstein et al., 2007; Monaghan and Hutchison, 2012; Ramirez et al., 2009). Yet in this study utilizing TWW under field conditions, no evidence for this phenomenon was found.

Microbial survival in soil and on fomites is often cited as a reason to approach WW irrigation with caution (Bernstein et al., 2007; Shuval, 2010). In this study, bacterial and protozoal targets associated with the enteric environments were detected in the TWW, but were not conclusively transferred to the soil or crop. In fact, only *Cryptosporidium* was detected once on tomato surface irrigated with secondary TWW. In this case, the usage of the conservative FIB proxy for pathogen contamination appears to be less reliable than targeted detection of pathogens. Interestingly, the major members of the FC group in TWW were composed of different species than in soil and on the tomato surface (Fig. 5). These findings suggest that if significant microbial transfer from TWW to the soil occurred, then the bacteria did not persist. Other studies have noted similar patterns when FIB and pathogens (Benami et al., 2013; Christou et al., 2014; Forslund et al., 2012) as well as antibiotic-resistant genes (Gatica and Cytryn, 2013) were monitored in TWW and the irrigated soil. It has previously been noted that bacterial communities in soil change under TWW irrigation, including a decrease in the relative abundance of *Actinobacteria* and an increase in gammaproteobacteria abundance (Frenk et al., 2014). Families included within the gammaproteobacteria class are the *Enterobacteriaceae*, *Vibrionaceae* and *Pseudomonadaceae*. In this study, the prevalence and concentration of FC (primarily *Citrobacter* spp. and *Enterobacter* spp) and *P. aeruginosa* increased in soil under all irrigation regimens (Figs. 4 and 5). The detection of *P. aeruginosa* in potable water may be due to biofilms in irrigation plumbing equipment (Mena and Gerba, 2009); nevertheless, the negative culture data indicates that either extracellular DNA or viable but non-culturable cells are responsible for the qPCR detects.

Irrigation water quality as well as soil physico-chemical properties, notably nitrogen, salt and organic matter content, have been correlated with microbial persistence (Bernstein et al., 2007; Bech et al., 2010); thus we endeavored to catalog these relevant parameters throughout the first growing season. Moreover, the physico-chemical effects of TWW irrigation on soil include salinization of the lithosphere, lowering the hydrophilicity of soil and excess nutrient loading (Sacks and Bernstein, 2011). In this study we noticed some of these effects, such as higher chloride content and potassium content in TWW irrigated soil (Fig. S2). However, as the fertigation regimens of the irrigation waters were closely matched, there were no noticeable differences in the nitrogen (ammonia and nitrate-N) levels in the irrigated soils. Yet, ending values of nitrate-N (as high as 180 mg kg^{-1}) were higher than necessary for tomato plant growth (Heckman, 2002) and should be carefully considered since nitrate accumulation can be hazardous to the environment as well as poisonous. Except for organic matter that increased in the soil by 4-fold within several weeks of beginning irrigation, the other parameters changed slowly, if at all. This reflects the high buffering capacity of the high-clay soil and it appears that soil physico-chemical properties cannot be used to approximate microbial contamination.

Pathogens were not detected on crops (except for one incidence of *Cryptosporidium*) and FIB contamination was not statistically different on tomatoes irrigated with TWW as compared to tomatoes irrigated with potable water (Table 2, Fig. 3). High concentrations of FIB were detected on tomato surfaces regardless of the irrigation treatment. We conclude that the presence of pathogens on tomatoes was not directly predicted by FIB concentrations associated with any matrix. In particular, the presence of *C. hominis* in the secondary TWW was found at three to four orders of magnitude lower than the FIB (Table 2). Therefore, in comparison to direct pathogen testing, basing the assessment of irrigation water quality on FIB levels alone, as is the case in most policy documents regarding TWW worldwide, may be overprotective, especially in light of the lack of microbial transfer to plant surfaces, and soil. This conclusion is supported by the growing body of evidence, including in this study, that indicators do not correlate necessarily with the pathogens in the TWW, irrigated soil or crop. It might be advisable to monitor indicators and/or pathogens in water at the cultivation site and not only at the WWTP, a requirement not explicitly legislated in any TWW use guidelines (Inbar, 2007; USEPA/USAID, 2012; WHO, 2006). Moreover, the ambiguity in documentation regarding FIB monitoring location should be resolved as regrowth can occur when the water is stored prior to use. Thus, effluents that meet TWW unrestricted quality guidelines upon leaving the WWTP may fail to meet the same guidelines when used in the field. As noted elsewhere (Blumenthal et al., 2000; Shuval, 2010), unnecessarily strict guidelines may cause farmers to intentionally disregard guidelines that seem unfair or unattainable. Therefore, it may be important to consider FIB density at any given moment as only one of a variety of microbial quality indicators, such as the type of treatment the WW was subjected to prior to use (Inbar, 2007).

5. Conclusions

The present work enhances previous studies on potatoes (Forslund et al., 2010), rice (Jang et al., 2013), melons (Martinez et al., 2013), tomato (Christou et al., 2014; Cirelli et al., 2012; Forslund et al., 2012) and eggplants (Cirelli et al., 2012) crops irrigated with TWW by expanding the tracked microorganisms from primarily FIB to include viral, bacterial and protozoan pathogens. This is the first field report to show that the fecal indicator bacteria far exceeded the pathogens from the major microbial groups, concurrently in TWW, soil and crop surfaces. Furthermore, this study synthesizes several techniques that are simple and practical for pathogen detection in the environment. This work adds to

increasing evidence, such as molecular fingerprinting of *E. coli* in water and crops (Forslund et al., 2012), showing that irrigation with TWW may hold promise from a microbial pathogen standpoint. Controlled studies have shown that pathogens could invade the roots from the soil and colonize plants (Barak and Liang, 2008; Barak et al., 2011; Yaron and Römling, 2014), yet the high bacterial counts introduced in these studies (10^7–10^9) were never encountered in the TWW or soil in the present study (Table 2). Thus, further analyses should be conducted by agronomists and public health officials aiming of test the irrigation of TWW on a brad array of soil types and vegetables.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.agee.2015.08.008.

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