CENTER for PRODUCE SAFETY

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FINAL PROJECT REPORT

Project Title

Science-based monitoring for produce safety: Comparing indicators and pathogens in water, soil and crops

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Abstract

Using treated wastewater (TWW) for crop irrigation represents an important opportunity for ensuring adequate food production in light of growing freshwater scarcity worldwide. However, the environmentally sustainable approach of using TWW for irrigation can lead to contamination of produce with fecal pathogens that may remain in treated water. The overall goal of this research was to evaluate the correlation between the presence of fecal indicator bacteria (FIB) and that of a suite of human pathogens in TWW, the irrigated soil, and crops. Field experiments were conducted to compare secondary and tertiary TWW with dechlorinated tap water for irrigation of tomatoes, a typical commercial crop, in Israel, a semi-arid country. Human pathogens including bacteria (*Salmonella*), protozoa (*Cryptosporidium* and *Giardia*), and viruses (Adenovirus [AV Types A, B, C & 40/41] and Enterovirus [EV71 subtypes]) were monitored in two field trials using a combination of microscopic, cultivation-based, and molecular (qPCR) techniques.

Results from the field trials indicate that microbial contamination on the surface of tomatoes did not appear to be associated with the source of irrigated waters; FIB contamination was not statistically different on tomatoes irrigated with TWW as compared to tomatoes irrigated with potable water. In fact, Indicator bacteria testing did not predict the presence of pathogens in any of the matrices tested. High concentrations of FIB were detected in water and on tomato surfaces from all irrigation treatment schemes, while pathogen contamination on tomato surfaces (*Cryptosporidium* and *Salmonella*) was only detected on crops irrigated with TWW. These results suggest that regular monitoring for pathogens should take place to accurately detect presence of harmful microorganisms that could threaten consumer safety.

A notable result from our study is that the large numbers of FIB in the water did not appear to lead to FIB accumulation in the soil. With the exception of two samples, *E. coli that* was present at 10^3 to 10^4 cells/100 mL in the water, was not detected in the soil. Other bacterial targets associated with the enteric environment (e. g., *Proteus* spp.*)* as well as protozoal pathogens were detected in the TWW, but not in the soil. These findings suggest that significant microbial transfer to the soil from TWW did not occur in this study. The pattern of FIB contamination on the surfaces of tomatoes was the same for all treatment types, and showed a temporal effect with more contamination detected as the duration of the field trial increased. An important observation revealed that water quality dramatically deteriorated between the time of its release from the wastewater treatment plant and the time it was utilized for irrigation, highlighting the importance of performing water quality testing throughout the growing season at the cultivation site.

Background

Demand for freshwater (FW) often exceeds availability in many geographic regions. Globally, populations are forecast to increase, which will most likely compound the issues around beneficial water uses. Even developed nations are in peril as water scarcity could potentially affect related technologies, such as hydroelectric power in addition to general water supplies (Shannon et al., 2007). Contemporary research offers potential for reduced consumption through various conservation and treatment technologies via water desalinization, disinfection and decontamination (Shannon et al., 2007). In particular, the use of treated wastewater (TWW) has the potential for additional conservation, specifically in the form of crop irrigation (Toze, 2006). The reasons are evident when considering that the amount of water used globally for agricultural purposes has increased significantly in the last 50 years (IWMI, 2006).

Using TWW for crop irrigation represents an important opportunity in ensuring adequate sustenance for industrialized countries and food security in developing regions. In fact, diminishing sources of FW supplies needed for irrigation pose a serious challenge to agriculture practices. In arid and semi-arid regions (such as Israel and portions of the United States), freshwater supplies are extremely limited, and as populations and the demand for FW have grown in the municipal sector, agricultural policy has had to adapt. For example, in Israel, over the past three decades this adaptation has come in the form of reducing overall FW available to the agricultural sector and increasing the amount of TWW supplied to farmers to use only for the irrigation of fruit trees. At present, 91% of all municipal sewage in Israel is treated, 73% of which is reclaimed [versus 2.5% in the United States (National Academy of Science, 1996)], contributing roughly one-fifth of Israel's total water supply. Reclaimed water irrigation is a cornerstone of Israel's long-term water management strategy, and is expected only to increase in volume and application (Fig. 1)

The environmentally sustainable approach of using treated wastewater for irrigation can lead to contamination of produce with fecal pathogens that may remain in treated water. It has been well established that irrigation with contaminated water may increase the risk of bacterial, parasitic and viral infections (Shuval et al. 1985, 1986, 1989; Fattal et al. 1986; Campbell et al. 2001; Doyle & Erickson 2008; Nygard et al. 2008). Fresh produce has been implicated as the major vehicle for food borne pathogenic outbreaks in the past decade (Doyle & Erickson, 2008), mostly leafy greens, sprouts and low growing fruits, such as tomatoes (Warriner & Namvar, 2010). Yet no conclusive evidence exists that would make TWW a greater risk factor for crop irrigation than water sourced otherwise. ***This project was, therefore, designed to compare secondary and tertiary TWW with dechlorinated tap water for irrigation of tomatoes, a typical commercial crop, in a semi-arid country.***

Historically, water quality monitoring guidelines have targeted fecal indicator bacteria instead of fecal pathogens due to the simpler and cheaper methods involved in detecting them. However, numerous studies have shown that the presence of fecal indicators in TWW does not always correlate with the presence of disease-causing microorganisms (Payment et al., 2001; Harwood et al., 2005; Ottoson et al., 2006). Bacterial pathogens including *Salmonella*, *Campylobacter*, *Vibrio cholerae*, *Escherichia coli* strain O157:H7, protozoal pathogens such as *Cryptosporidium* and *Giardia*, and viruses such as Adenovirus and Enterovirus may be present in TWW effluents, and can be associated with severe disease in humans (Mead et al, 1999; Nachamkin, 1999; Tison, 1999). The lack of correlation between pathogens and indicators of fecal pollution that are currently used in microbiological monitoring standards of irrigation waters may lead to unpredictable and serious risks to public health.

In addition to direct contamination of produce with TWW, soil can serve as a vehicle for transferring pathogens to produce because some pathogens may persist in soil following irrigation of the soil matrix with TWW, as well as via fertilization practices. Pathogens within soil may contaminate crops directly, when heavy rain or sprinkler irrigation cause leaf splash, or indirectly, by penetrating the plant tissues. Fecal pathogens survive for long periods (Blumenthal, 2000) and in some instances propagate in the soil until crops are planted (Bernstein et al., 2007; Heaton and Jones, 2008), increasing the likelihood of crop contamination during the plant's growth cycle or through the harvest process.

Guidelines to wastewater reuse in agriculture

With higher crop yield efficiencies and greater demand for reclaimed water, guidelines have been established for its use in agriculture by both the United States Environmental Protection Agency (USEPA, 2004) and the World Health Organization (WHO; Blumenthal et al., 2000) as summarized below. Israel has stricter guidelines and the basic standard for irrigation of freshly eaten vegetables cannot exceed 10^1 *E. coli* cells/100 ml of TWW as compared with 10^3 *E. coli*/100 ml that is recommended by the WHO.

Despite the implementation of stricter standards, this criterion may not adequately protect consumers because it relies on a single fecal indicator organism, *E. coli*. In addition, the actual correlation between levels of indicator fecal organisms in irrigation water and presence of pathogens on plant tissues is largely unknown. In 2006, major disease outbreaks involving spinach, lettuce, and tomatoes contaminated with fecal bacteria were reported in the United States, and the epidemiologic investigations pointed to proximity of the crops to irrigation wells and surface waterways exposed to feces from cattle and wildlife (Doyle and Erickson, 2008). However, the exact mode of pathogen contamination of the crops remains unknown. Various human pathogens have been recovered from tomatoes (Table 1), yet the contributing sources (e.g. irrigation water or soil) of these pathogens remain unknown. In this study we compared

pathogens and indicators present in TWW, the irrigated soil, and crops, providing data that can be used towards developing science-based monitoring guidelines to recommend sampling of TWW, soil or both, to accurately predict the presence of enteric pathogens on edible plant tissues.

As more and more TWW is used for agriculture needs due to regional water scarcity and competing demands, standards need to be broadened to encompass a wider range of target organisms in monitoring schemes. This is critical not only due to the fact that the demand for TWW will rise in the agricultural sector in the U.S.A. and abroad, but also because the sources of WW are becoming more varied. WW is now deemed just as valuable as freshwater and so sources of WW other than from municipal use, such as from industry, stormwater, and agricultural runoff are being considered. An effluent standard based on a single fecal indicator is thus insufficient.

Research Objectives

The overall goal of this research was to evaluate the correlation between fecal indicator organism and the presence of pathogens in TWW, the irrigated soil, and crops. To this end, we identified three objectives to better understand the relationship between levels of fecal indicator bacteria and presence of pathogenic fecal bacteria, protozoa and viruses in reclaimed wastewater, and in the soil and crops that are irrigated with TWW. Specifically, the project evaluated if current monitoring techniques that target fecal indicator organisms accurately predict the presence of fecal pathogens on produce using wastewater irrigated tomatoes as our model plant, cultivated in experimental field test plots.

Objective 1. Simultaneously sample irrigation water, soil, and crops for two growing cycles at an experimental plot hosting tomato plants irrigated with secondary TWW, tertiary-TWW, and potable water.

Objective 2. Perform physico-chemical water and soil analyses and standard microbiological testing of water, soil, and plant tissues for fecal indicator bacteria.

Objective 3. Process water, soil, and plant samples for pathogen detection. Analyze samples for fecal enteropathogenic and opportunistic bacteria, enteric viruses, and fecal protozoa.

Research Methods and Results

Methods

Pathogen detection assay validation and spiking experiments

In order to validate the methods used for this project, spiking experiments were conducted using one replicate for secondary and tertiary TWW and potable water matrices (100 L), and three to five replicates for soil and tomato matrices. Each experiment was repeated at least three times (n=3). We used a subset of the

bacterial and protozoan pathogens that were targeted in cultivation experiments (Table 2). Non-pathogenic bacterial and viral "surrogate" organisms that were utilized as internal recovery controls were also included.

Protozoal spiking experiments

The recovery efficiency and limits of detection (LOD) of *Cryptosporidium parvum* oocysts and *Giardia lamblia* cysts from the experimental matrices were evaluated using immunomagnetic separation (IMS) followed by direct fluorescence antibody (DFA) staining. Serial dilutions ranging from approximately 5 to 1000 (oo)cysts were applied to 5 g soil, 100 L water, and the surfaces of 5 tomatoes, and were subsequently processed in the manner applicable to each matrix, as detailed below.

Protozoal recoveries for water and tomato surfaces were approximately 40-50%, and the LOD were determined to be approximately 1 (oo)cyst L^{-1} and 5 (oo)cysts $tomato^{-1}$ (Table 3). The soil matrix proved to be a more challenging matrix, mostly because of the large amount of background debris present that interfered with magnetic bead and fluorescent antibody binding. A modified sedimentation method that yielded improved recoveries was developed, and was employed as the method of choice for detection of *Cryptosporidium* and *Giardia* from soil for the remainder of the project. This method removes magnetic particles from the soil sample prior to addition of the magnetic antibody-coated beads, as well as increases the number of elution washes of the beads. The net suspension contains little sediment and has a high yield (up to 70%, Table 3).

Bacterial spiking experiments

The recoveries of bacteria (indicators, pathogens, and surrogate species) were accomplished by cultivation dependent methods and/or qPCR on nucleic acid (NA) extracts from the experimental matrix (Table 4). For the soil and tomato experiments, an extract of the spike solution (a concentrated 10 mL cocktail of organisms used to spike the soil or tomato) was used to determine the amount of bacteria applied to the matrix. In the water experiment, a direct feed extract (0.5 mL) was used to measure the amount of bacteria actually inoculated into the system. In water, recoveries of spiked bacteria tended to be higher than 100%, while in soil and tomato matrices lower recoveries were detected (20-80%, Table 4).

Salmonella: Further spiking experiments were conducted with *Salmonella*, to evaluate several methods for detection of this bacterial pathogen in cultivation experiments. The standard cultivation based-method involving enrichment and plating on selective media was successful when used in un-inoculated media, i.e. sterile matrices, but was subject to a large amount of interference by colonies with similar gross morphology in the non-sterile environmental matrix. We found that a basic enrichment (0.1% Buffered Peptone Water, BPW) followed by whole cell extraction and qPCR, was successful in detecting low levels of *Salmonella* in the tomato, soil and water matrices (<5 CFU per tomato, g soil and 500 mL, respectively).

Finally, experimentation with qPCR on non-enriched samples showed recoveries of approximately 85% for water and tomato wash, but lower recoveries in soil (15%, Table 4).

Field cultivation experimental design

Using the methods validated in the preliminary spiking studies, cultivation experiments using tomatoes as a model vegetable crop were conducted to evaluate microbial contamination on crops irrigated with recycled effluents. Experimental plots were planted in an agricultural experimental station in Israel that specializes in the study of effluent irrigation of agronomic produce. The tomato seedlings (Smadar F1) were planted under a screen house in 11 × 2 m plots, according to the routine agricultural practice in Israel. In the first field experiment, a total of ten plots were planted: five control plots irrigated with potable water, and five treatment plots irrigated with secondary TWW effluents (Fig 2). In the second field experiment, a total of 15 plots were planted, with five replicates, irrigated with potable water, secondary TWW, or tertiary TWW (Fig 3). Using a random experimental design, up to three plots were planted in-line along a bed, where 1.5 m of non-irrigated soil separated plots in a bed, and one dry bed separated adjacent beds (Fig 4). Each bed included one row of tomato plants, 2 plants per running meter, and one drip irrigation line.

Wastewater source and treatment

The experiment applied treated wastewater effluents that are similar to effluents used for tree irrigation in many agricultural fields in Israel. The treated municipal WW originated from the Gat WW treatment plant (Gat WWTP) of the town of Kiryat-Gat. The WW was treated with aerobic activated sludge and batch reactors that cycle between anoxic (10-20% of O_2) and aerated (80-90% of O_2) conditions with a retention time of about 28 hrs. The new Israeli standards (since 2010) for wastewater effluents call for strict regulations such as 10-10-25 (Biological Oxygen Demand (BOD), Total Suspended Solids (TSS) and Nitrogen [N], respectively), among a total of 36 parameters for unrestricted irrigation (Inbar, 2007; Ministry of Environmental Protection, 2010). Upon release from the WWTP, the secondary TWW can exceed the parameters in certain cases (e.g. Fecal Coliform of <10 CFU 100 mL^{-1}), provided that it is treated appropriately at the irrigation site. Accordingly, the secondary TWW meets the criteria of BOD, TSS, and N before it is released, but other parameters such as fecal coliforms are not quantified on a routine basis at the WWTP.

From the treatment plant, the water passes into a shallow one-day reservoir and from there into a larger 480,000 m^3 reservoir. Both of these reservoirs are under a management separate than that of the treatment facility. It is from the 480,000 m^3 reservoir that the farm receives the TWW for irrigation. At the farm, the secondary TWW is stored in a 110 m^3 tank, which is approximately large enough to provide for one day's irrigation requirements. In general, the secondary TWW are not used as is, but are chlorinated to 0.1-0.5 ppm chlorine. If tertiary treatment is required, the TWW is passed through a sand filtration column. In the

first cultivation experiment, secondary TWW were chlorinated upon transport from the treatment plant, and were not re-chlorinated at the research site (representing a worst case-scenario). In the second cultivation experiment, two separate treatments were applied, 1) chlorination, and 2) filtration through a sand column. Thus, there were three effluent water qualities tested: 1) secondary TWW, non-chlorinated, 2) secondary TWW, chlorinated, 3) tertiary TWW, sand-filtered and chlorinated. The TWW was applied using drip irrigation, a common barrier step applied when using TWW for irrigation of fruit trees in Israel.

Fecal indicator bacteria and pathogen testing

Fecal indicator bacteria (FIB) and pathogens in the irrigation water, soil, and on the tomato surfaces were monitored during both growing cycles using methods validated in the preliminary experiments. Sampling was conducted from the center of the bed to ensure root exposure only to the target water quality.

Irrigation water, soil, and tomato samples were tested for:

1. FIB including fecal coliforms, total coliforms, *E. coli* and *Enterococci.*
2. Fecal pathogens including bacteria (*Salmonella*), protozoa (*Cryptosporidium* and *Giardia*), and viruses (Adenovirus [AV Types A, B, C & 40/41] and Enterovirus [EV71 subtypes]).

Water Collection and Concentration

One hundred L of the treated effluents and potable water were collected in four 25 L jerricans from the experimental farm, and transported to the laboratory at the Zuckerberg Institute of Water Research in Sde Boker. At the time of collection, sodium thiosulfate at a final concentration of 0.1% (v/v) was added to neutralize any chlorine present in the water (Kemp and Schneider, 2000). Testing for FIB took place immediately upon return to the lab, and before concentration of the water. For pathogen detection, water was concentrated using ultrafiltration (FX-100 dialyzer, Fresenius Medical Care, Germany), to retain particles larger than 35 kD present in the 100 L "feed" into a final concentrated volume of 200 mL "retentate" (Fig. 5). Fifty mL of retentate was stored at 4°C for up to 72 hours for the determination of protozoan pathogens as described below. An aliquot of the retentate was tested for the presence of *Salmonella* by enrichment followed by plate culture and (in the second growing season) qPCR (see detailed protocol below). The remaining retentate volume was mixed with glycerol (20% v/v) and stored at -80°C until further processing (nucleic acid extraction). Ultrafiltration recoveries were estimated with appropriate surrogate particles for each of the three pathogen classes (bacteria, protozoa, and viruses) as described in Table 2.

Soil and crops collection

Soil and crop samples were collected from each replicate plot by randomly selecting five sampling points within the 5 m long designed sampling zone in each replicated plot. Soil was sampled within the row, 20 cm from the dripper. Each soil sample was collected aseptically by removing approximately 100 g of soil from the

upper 10 cm of the profile, using a sterile spatula. The samples were placed in individual sterile plastic bags (Whirl-Pak, USA), transported to the laboratory and processed within 24 hours (overnight storage at 4°C). In the laboratory, the samples from each replicate plot were homogenized and combined for chemical testing and microbial analyses. To test for presence of FIB and pathogens on the irrigated tomato crop surface, the fruit was collected within the designated sampling zone as described above. Tomatoes were harvested, and five fruits from each plot were sampled for pathogen testing by washing the surfaces with sterile water + 0.01% Tween80, and subjecting the washing solution to analyses for FIB and pathogens as described above.

Laboratory detection methods

The targeted FIBs, pathogens, respective surrogates and detection methods used for estimating recoveries are summarized in Table 5.

FIB quantification

The indicator bacteria E. coli, total and fecal coliforms, and Enterococcus were detected and quantified from water, soil and tomato samples, utilizing either US EPA accepted cultivation methods (membrane filtration) EPA 9223 B and ASTM D6503-99) or by using the Colisure and Enterolert kits respectively (IDEXX). In-house validation verified that the tests yielded almost identical results. In the second growing season the indicator bacteria were exclusively tested with the Colisure and Enterolert kits.

Protozoal pathogen detection

Detection of *Cryptosporidium* and *Giardia* spp. was performed using IMS followed by DFA staining according to EPA Method 1623 (US EPA, 2001), as performed during the preliminary spiking studies described above. A novel approach for preparation of soil for subsequent IMS DFA testing was developed in this study, and is briefly summarized here: 5 g of soil was incubated in PBS Tween-80 (0.1%) for 24 hrs, washed, and aspirated, leaving at least 10 mL of liquid above the soil phase. Next, natural magnetic particles present in the soil were removed prior to the addition of the magnetic beads from the kit. This was done by attaching the sample tubes to a magnetic particle concentrator (Invitrogen, Norway) and allowing the native magnetic particles to affix to the back of the tube. Then the supernatant was poured to a new tube. The protocol then followed manufacturer's guidelines for IMS with the addition of the magnetic anti-*Cryptosporidium* and anti-*Giardia* beads with slight modifications, including additional washes of the beads prior to acid-elution, and adding an extra acid-elution step. Parasite staining using DFA was accomplished with EasyStain C+G combo (BTF-bio, Australia) according to the manufacturer's instructions, except that the mounting medium provided by the EasyStain kit was replaced by a mounting medium provided by Waterborne Inc. (USA). Detection of protozoa from tomato washing was achieved by combining wash material from a batch of 5 tomatoes. The tomatoes were washed in sequence in the same volume of 50 mL PBS Tween80 0.01%. The pellet from the 50 mL was

collected, washed in DDW and aspirated, leaving 10 mL of volume above the pellet. Up to 0.5 mL of pellet was transferred for IMS following manufacturer's guidelines. Detection of protozoa from irrigation waters was performed by IMS DFA as previously described (Hogan et al., 2012).

Samples that had suspected protozoal parasites based on the IMS DFA analysis were shipped to UC Davis for further molecular characterization. At UCD, samples were 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. *Cryptosporidium* genotyping was performed by PCR amplification of a conserved 18S rRNA encoding gene (Morgan et al., 1997) and the nested PCR protocol described by Xiao et al. (1999). Similarly, microscopy-positive *Giardia* samples were characterized using a hemi-nested PCR and DNA sequence analysis of the glutamate dehydrogenase (GDH) encoding gene (Read et al., 2004).

Bacterial pathogen detection

To assess best detection methods for *Salmonella* spp., two methods were evaluated and compared: culture isolation and qPCR. Culture techniques involve standard methodologies that utilize pre-enrichment and selective enrichment in 0.1% buffered peptone water (BPW) and Rappaport Vasilliadis-R10, respectively (Andrews et al., 2011). Bacteria were also identified using qPCR (Table 5) because this method offers several advantages compared to traditional culture techniques including rapid results, sensitivity, and ability to provide quantitative results, thus allowing the detection of bacteria that are viable but not culturable (Call et al., 2001). The nucleic acid (NA) extraction technique utilized in this study is based on chloroform/phenol suspensions coupled with bead-beating (Biospec, USA) and is expected to maximize the yield of both DNA and RNA as adapted from Angel and Conrad (2009). In general, the protocol of the qPCR assay followed the methods described by Schriewer et al. (2010). All qPCR assays were performed on a Bio-Rad CFX-1000 (Biorad, USA) and utilized TaqMan® fluorescent probes (Metabion, Germany). Probes were labeled with different fluorescent dyes (FAM, Cy5, Texas Red, or Yakima yellow; Table 6), which allowed for multiplex qPCR, i.e. detection of different targets within the sample reaction tube. A single qPCR reaction utilized the following reagents: Absolute Blue PCR Master mix (100 µM; ThermoScientific), forward and reverse primers and probe (100 nM; Metabion), BSA (0.01%), and the template DNA (~1 ng). The number of gene copies was determined with a standard curve generated by serial dilutions of a plasmid extract containing the target fragment.

Viral pathogen detection

For detection of viral pathogens, NAs extracted from the water, soil, and tomato wash samples were shipped in RNAstable tubes (Genvault, USA) to UCD where all viral assays were performed (Wuertz laboratory). Molecular detection using TaqMan qPCR targeted Adenovirus (AV Types A, B, C and 40/41) and Enterovirus

spp. (EV71 subtypes) using previously established methods (Monpoeho et al., 2000; Leruez-Ville et al., 2004; Rajal et al., 2007).

Quality Control and Recovery Estimates

Multiple quality control measures were incorporated into the procedures for sample handling and analysis to minimize and monitor for cross-contamination. To assess detection capabilities, spiking with surrogate particles were performed to account for recovery efficiency of target organisms in water, soil, and tomato samples (Table 5). Known numbers 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. Reagent and field blanks were processed through the entire analytical procedure of the first growing season in a manner identical to the samples to assess for potential contamination at the field and/or during the sample processing in the laboratory.

Additional Water and Soil Quality Parameters

At each sampling event, chemical and physical quality parameters were collected (Table 7). Irrigation water was collected in a 2L Nalgene bottle for analyses in the laboratory including total suspended solids (TSS), dissolved organic carbon (DOC), total organic carbon (TOC), TSS-nitrogen and TCC-carbon. Soil chemical and physical properties were determined following standard soil analytic methods: Soil pH, electrical conductivity, Cl, Na and K were analyzed in SSE (Na and K by atomic A/E and cl by a Chloridometer); N as nitrate and ammonium and P in Olsen extract were analyzed by an Autoanalyzer; soil texture, cation exchange capacity, and organic matter content by dichromate method.

Data analysis

Statistical analyses were performed using Statistica software to perform non-parametric tests to compare FIB levels between plot samples irrigated with different water types. In the first experiment, a Mann Whitney statistical test was used to compare FIB levels between samples collected from potable and secondary treated effluents. In the second cultivation study, a Kruskal Wallis test was initially applied to test for differences in FIB levels between samples irrigated with potable, secondary effluents, and tertiary effluents. Comparisons that yielded significantly different results among groups were followed by post-hoc pair-wise testing. P values <0.05 were considered significant.

Results

Cultivation experiment 1: Potable water vs. secondary TWW

Water analyses

Protozoal pathogens were detected once during the duration of the experiment, in secondary TWW collected at 3 weeks from cultivation (0.5 *Cryptosporidium* oocyst and 0.5 *Giardia* cyst L^{-1}, Table 8). *Salmonella* was detected with qualitative cultivation-based methods in secondary TWW at 18 weeks from planting, but not prior to that (Table 8). QPCR on un-enriched extracts of the water retentates detected *Pseudomonas, Salmonella, Shigella* and *Staphylococcus* at levels that were too low to be quantifiable. It should be noted that the positive control organism *Acinetobacter,* was successfully detected in the samples, and indicated an approximately 80% recovery for the qPCR assay (Table 4 for QPCR data). It should be noted that *Proteus* spp. (an opportunistic pathogen) was detected in the secondary TWW using cultivation methods at all sampling events. This organism was incidentally identified due to the similar morphological appearance to *Salmonella* on SS agar, confirmed using biochemical testing as *Proteus* spp.

Soil analyses

FIB levels were high but not statistically different between soil irrigated with potable water or secondary TWW (Table 9). *E. coli* was not detected, except once in a secondary irrigated plot at 15 weeks from planting. *Salmonella* was detected using cultivation-based methods, but not quantifiable, due to high numbers of biochemically similar organisms (Table 9). With the exception of *Pseudomonas*, other bacteria (indicators and pathogens) were not detected using qPCR on soil samples and were therefore omitted from the second cultivation experiment.

Tomato analyses

FIB levels were high but not statistically different between tomato surfaces irrigated with potable water or secondary TWW (Table 10). As in soil, *E. coli* was not detected. *Salmonella* was detected on the surfaces of tomatoes irrigated with both potable and secondary irrigated water. The levels of *Salmonella* were too low to be quantifiable using cultivation-based methods, again partly due to the presence of biochemically similar interfering organisms (Table 9). Efforts for *Salmonella* quantification with qPCR on non-enriched extracts were also unsuccessful, but did detect this pathogen (Table 10). With the exception of *Pseudomonas*, other bacterial targets were not detected at quantifiable levels using qPCR and were therefore omitted from the second cultivation experiment. Viral pathogens were not detected in any samples tested during the first field experiment.

Cultivation experiment 2: Potable water, secondary TWW, and tertiary TWW

Water analyses

FIB were not detected in potable irrigation water, with the exception of 10 total coliforms 100 mL^{-1} measured in a sample collected at week 20 (Table 11). The secondary and tertiary treated wastewater effluents in this study were characterized by high fecal indicator bacterial contamination (4-5 log per 100 mL, Fig 6). The median FIB concentrations appeared higher in secondary TWW compared with tertiary TWW, but the difference was not statistically significant ($P>0.1$). Using IMS DFA, protozoal pathogens were detected in effluent irrigation waters, with *Giardia* detected once in secondary TWW, and *Cryptosporidium* detected once in secondary and once in tertiary TWW (Table 11). Of these suspect protozoal positive samples sent to UCD for molecular characterization, only a single sample yielded amplified DNA that provided a clear sequence: a secondary TWW sample with *Cryptosporidium* oocysts visualized by DFA were confirmed to have sequences (Morgan and Xiao DNA products) identical to *C. hominis*.

Salmonella and viral pathogens were not detected in any irrigation water samples. The water quality parameters measured on irrigation waters collected at the farm were higher than allowed for application on crops according to the water quality regulations set by the Israeli ministry of health (30/20 ppm for secondary and 10/10 for tertiary treated wastewater). By comparison with the water quality measurements recorded on the same effluents at the time they were released from the wastewater treatment plant, the wastewater applied for irrigation in this study was of markedly reduced in quality (Table 7).

Soil analyses

The physic-chemical characteristics of the soil used in the cultivation experiments were: pH, 8.2, Conductivity (ds/m) 0.95, chloride (ppm) 4.1, sodium (ppm) 5.2, chalk 24.2%, sand 25%, silt 24.3%, clay 48.7%. Concentrations of fecal and total coliforms were up to 4.5 log g^{-1}. While high concentrations of *E. coli* were detected in the TWW, *E. coli* was only detected in one sample of non-irrigated soil at 4950 MPN g^{-1} (Fig 7; Table 12). *Salmonella* was tentatively detected by qPCR in enriched samples in 1, 4, 6 and 8 of non-irrigated, potable, secondary and tertiary TWW irrigated samples, respectively. By comparing estimated C_T values obtained by qPCR with those obtained during spiking studies, it appears that the *Salmonella* concentrations in the environmental soil samples were always <5 CFU g^{-1}. Protozoal parasites were not detected in soil samples. Enteric viruses were presumably detected in 2, 3, and 4 soil samples collected from potable, tertiary TWW, and secondary TWW irrigated plots; however these results could not be confirmed by sequencing efforts.

Tomato analyses:

Total coliform bacteria were detected at 2-4 log MPN per tomato surface. However, unlike the first cultivation experiment, fecal coliform bacteria were rarely detected (Table 13). *E.* coli was not detected on tomato samples. *Enterococcus* was detected in the third tomato sampling event (20 weeks from planting) at 2-4 log MPN per tomato surface, while in the first cultivation experiment, *Enterococci* levels were lower at 0-100 MPN per tomato surface. The source of this contamination is unknown. It should be noted that the bacterial indicator levels on tomato surfaces were not significantly different between the water irrigation treatment plots (Kruskal-Wallis, P>.05, Fig 8), although a trend was observed with higher *Enterococci* concentrations detected on tomatoes from all plots in later sampling points (Fig 9). One sample of secondary TWW irrigated tomatoes was found to contain a single *Cryptosporidium* oocyst as visualized by DFA, however molecular testing at UCD was not successful at amplifying DNA for sequence analysis. *Salmonella* was cultivated from the surface of a tomato sample irrigated with secondary TWW from the third tomato-sampling event (20 weeks from planting). The strain was both biochemically and molecularly confirmed to be *Salmonella enterica*, with Hy-enterotest (Hy-labs, Israel) and qPCR targeting the *invA* gene, respectively. Additional tomato samples appeared to be contaminated with *Salmonella* based on qPCR assays, but they were not cultivable (Table 12). By comparing qPCR C_T results with those obtained during the spiking studies, the *Salmonella* detected on the tomatoes appeared to be <5 CFU per tomato. Viruses were not detected on tomato surfaces.

Outcomes and Accomplishments

This project's aims were to determine 1) If irrigating food crops with TWW poses an increased hazard to human health compared with crop irrigation using potable water; and 2) if FIB levels in irrigation water or soil could accurately predict the type and degree of produce pathogen contamination. Our objectives were achieved through completion of two field cultivation experiments in which we monitored FIB and pathogen contamination in irrigation water and soil and on the surface of tomatoes. This study represents the first experiment of its kind that compares microbial contamination in a vegetable produce eaten raw that has been irrigated with potable water, secondary TWW, and tertiary TWW. As originally proposed in our objectives, concurrent sampling of diverse matrices for a wide range of FIB and pathogen classes was successfully completed, and has yielded the most comprehensive data set to date on presence of protozoal, bacterial, and viral pathogens on tomatoes irrigated with potable water compared with TWW.

The collaborative nature of this project was unique, in that all field work and the majority of laboratory efforts were executed by our Israeli collaborators at Ben Gurion University (BGU), with the UC

Davis (UCD) team providing intellectual expertise on assay development and validation, training on protozoal detection methods, and analyses of samples shipped from Israel for viruses and protozoal molecular confirmation. An unexpected challenge that arose due to the collaborative setup was maintaining adequate progress of the project according to the original timeline proposed; because the scientists equipped with pathogen detection expertise were based at UCD, while the core researchers carrying out the experimental objectives were at BGU, there was a disconnect between the skills and knowledge needed to implement the study objectives and the physical nature of the study being located in Israel. Two factors that helped address this challenge were 1) the time spent by Dr. Shapiro who traveled from UCD to BGU to set up Dr. Gillor's laboratory for protozoal pathogen detection and provide training to the graduate student charged with performing these assays; and 2) monthly Skype meetings that were held between collaborators in the two countries, which generated meeting minutes that were circulated following each group discussion.

While certain challenges arose due to the international nature of this project, the overall outcome of this research represents a significant advancement to the emerging field of produce safety in face of water scarcity: While prior studies evaluated the effect of irrigation with raw wastewater to irrigate crops (Minhas et al., 2006; shuval 1991; Rosas et al., 1984), fewer studies have examined the implications of applying TWW for irrigation of vegetable crops. WW treatment is intended to reduce organic matter, nitrogen, and the microbial contamination present in wastewater (WHO, 2006). Indeed, we found that the water quality of the TWW before the start of the growing season in early spring met the quality regulations set by the Israeli ministry of health. Fecal coliforms were undetectable (<1/100 mL), BOD was <10 mg/L and TN was approximately 5 mg/L. However, when cultivation began, a major increase in FIB concentration and a general decline in water quality overall was recorded. It should be noted that the wastewater treatment plant (WWTP) reported fairly consistent water quality parameters for this period. Thus, our assumption is that TWW contamination occurred subsequent to release from the treatment plant, potentially at the regional storage reservoir.

A notable result from our study is that the large numbers of FIB in the water did not appear to lead to FIB accumulation in the soil. In fact, *E. coli*, present at 10^3 to 10^4 cells/100 mL in the water, was not detected in the soil with the exception of two samples. Other bacterial targets associated with the enteric environment (e. g., *Proteus* spp.*)* as well as protozoal pathogens were detected in the TWW, but not in the soil. These findings suggest that significant microbial transfer to the soil from TWW did not occur in this study. While pathogens were only detected rarely on crops, the only instances of detection of protozoal pathogens and *Salmonella* occurred on tomatoes irrigated with TWW. However, presence of pathogens on tomatoes was not predicted by FIB concentrations on any matrix. The pattern of FIB contamination on the surfaces of

tomatoes was the same for all treatment types, and showed a temporal effect, i.e., more contamination with increasing time in the field (FIG 9).

Summary of Findings and Recommendations

1. Microbial contamination on the surface of tomatoes did not appear to be associated with the source of irrigated waters; FIB contamination was not statistically different on tomatoes irrigated with TWW as compared to tomatoes irrigated with potable water.
2. Indicator bacteria testing did not predict the presence of pathogens in any of the matrices tested. High concentrations of FIB were detected in water and on tomato surfaces from all irrigation treatment schemes, while pathogen contamination on tomato surfaces (*Cryptosporidium* and *Salmonella*) was only detected on crops irrigated with TWW. These results suggest that ***regular monitoring for pathogens should take place to accurately detect presence of harmful microorganisms that could threaten consumer safety.***
3. Water quality can change dramatically between the time of its release from the wastewater treatment plant and the time it is utilized for irrigation. Therefore it is important to perform water quality testing throughout the growing season at the cultivation site.
4. Methods-based accomplishments:

a. An improved method for detection of protozoal pathogens (*Giardia* and *Cryptosporidium* spp.) in soil was developed.
b. Short enrichment scheme for rapid and sensitive detection of a variety of enteropathogenic bacteria using real-time PCR was evaluated. Bacterial detection was sensitive even with high numbers of heterotrophic bacteria present, as validated for *Salmonella* and *Shigella* in TWW.
c. Multiplex qPCR assays were validated for a variety of bacterial targets in water, soil and tomato surface washes.

5. An unexpected result from the study is that bacteria from the water did not appear to transfer to the soil or the crop surface. This feature has been noted in other studies such as with antibiotic-resistant bacteria (Gatica and Cytryn, 2013). The effect of soil conditions (such as soil type and other microorganism populations) on persistence of pathogenic bacteria in the soil should be better elucidated.

APPENDICES

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Publications and Presentations (required)

Ezra Orlofsky, Karen Shapiro, Woutrina Miller, Nirit Bernstein, Stefan Wuertz and Osnat Gillor. Microbial pathogens and fecal indicator bacteria in an effluent Irrigated tomato field. Poster presentation, 2nd Water Research Conference, Singapore, 20-23 January, 2013.

Ezra Orlofsky, Karen Shapiro, Woutrina Miller, Nirit Bernstein, Stefan Wuertz and Osnat Gillor. Microbial pathogens and fecal indicator bacteria in an effluent Irrigated tomato field. Poster Presentation, 4th Conference on Drylands, Deserts and Desertification, Ben Gurion University, Sede Boqer, Israel, 12-15 November, 2012.

Ezra Orlofsky, Karen Shapiro, Woutrina Miller, Nirit Bernstein, Stefan Wuertz and Osnat Gillor. Microbial pathogens and fecal indicator bacteria in an effluent Irrigated tomato field. Oral presentation, Microbial Ecology Meeting (sandwich club), Agricultural Research Organization, Bet Dagan, Israel, 8 July 2012

Stefan Wuertz. Science-based monitoring for produce safety: Comparing indicators and pathogens in water, soil, and crops. Poster at 3rd Annual Produce Research Symposium, Davis, CA, 27 June, 2012, presented by Arti Kundu.

Ezra Orlofsky, Karen Shapiro, Woutrina Miller, Nirit Bernstein, Stefan Wuertz and Osnat Gillor. Microbial pathogens and fecal indicator bacteria in an effluent Irrigated tomato field. Oral presentation, Conference on Active Research by Environmental Science Students (CARESS), Weizmann Institute, Rehovot, Israel, 15 June 2011

Ezra Orlofsky, Karen Shapiro, Woutrina Miller, Nirit Bernstein, Stefan Wuertz and Osnat Gillor. Microbial pathogens and fecal indicator bacteria in an effluent Irrigated tomato field. Oral presentation, 19th International Conference on Environmental Indicators, Technion, Haifa, Israel, September 11-14th 2011

Stefan Wuertz. Science-based monitoring for produce safety: Comparing indicators and pathogens in water, soil, and crops. Oral presentation at 2nd Annual Produce Research Symposium, Miami, Florida, 28 June, 2011.

Ezra Orlofsky, Karen Shapiro, Woutrina Miller, Nirit Bernstein, Stefan Wuertz and Osnat Gillor. Microbial pathogens and fecal indicator bacteria in an effluent Irrigated tomato field. Oral presentation, World Wide Workshop for Young Environmental Scientists, (WWW-YES, 11th Edition), Arcueil, France, 5-10 June 2011.

Several publications are currently in preparation

Budget Summary (required)

Wuertz CEE:
Funds from CPS were used to support salaries ($31,477.45), benefits ($2,818.71), supplies ($18,363.75) and tuition ($9,016.80) to support graduate student participation. Indirect costs totaled $6,167.81.

Shapiro VM:PMI:
Funds from CPS were used to support salaries ($73,324.61), benefits ($17,640.93), supplies ($10,464.13) and travel ($2,861.87) to fund Dr. Shapiro's trip to Israel in 2010 to set up protozoal testing capacity in Dr. Gillor's laboratory. Indirect costs totaled $10,409.77.

Tables and Figures (optional)

Tables

Table 1. Reported tomato related outbreaks of bacterial and viral gastroenteritis (adapted from Heaton & Jones 2008)

Year	Pathogen	Reference
1998–99	*Salmonella baildon*	Anon 2001
2002	*S. Javiana*	CDC 2002
2004	*S. Braenderup*	CDC 2005
2004	*S. Javiana*	Anon 2005
2006	*S. Newport*	Anon 2007
2006	*S. Typhimurium*	CDC 2006
1992	Norovirus	Anon 2005
1994	Hepatitis A virus	Anon 2001

Table 2. Organisms used in the spiking studies.

Group	Organism	Detection Method
Protozoa	*Cryptosporidium*	IMS/DFA
	Giardia	IMS/DFA
Bacteria	*Shigella*	qPCR
	Salmonella	qPCR & Culture
	Campylobacter	qPCR
	*Acinetobacter**	qPCR
Virus	Bacteriophage PP7*	qPCR

*Surrogate organisms used for recovery estimates in the field experiment

Table 3. Recoveries of 1000 applied (oo)cysts and limit of detection (LOD) of Protozoa by IMS/DFA obtained from spiking experiments

Organism	Water (ultrafiltration) (N=3)		Soil (novel protocol) (N=4)		Tomato (Washing) (N=3)	
	Recovery % ± SD	LOD	Recovery % ± SD	LOD	Recovery % ± SD	LOD
C. parvum	38 ± 5	1 L^{-1}	71 ± 10	2 g^{-1}	46 ± 4	5 $tomato^{-1}$
G. lamblia	54 ± 18	1 L^{-1}	32 ± 3	3 g^{-1}	59 ± 14	5 $tomato^{-1}$

Table 4. Bacterial recoveries of 1000 CFU and LOD from water (100 L), soil (5 g), and tomato surfaces obtained from spiking experiments

		Matrix (processing method)					
Organism		**Water (UF) (N=3)**		**Soil (Suspension) (N=5)**		**Tomato (Washing) (N=3)**	
		Recovery (% ± SD)	**LOD**	**Recovery (% ± SD)**	**LOD**	**Recovery (% ± SD)**	**LOD**
Acinetobacter[a]		170 ± 17	10-50 gc L^{-1}	51±16	10-20 gc g^{-1}	67 ± 28	10-20 gc $tomato^{-1}$
Shigella[a]		155 ± 24	10-50 gc L^{-1}	51.8 ± 5	10-20 gc g^{-1}	84 ± 26	10-20 gc $tomato^{-1}$
Salmonella[a,b]	qPCR direct extraction	85 ± 10	10-50 gc L^{-1}	15 ± 13	10-20 gc g^{-1}	81 ± 33	10-20 gc $tomato^{-1}$
	Direct plating Culture-without enrichment	90 ± 5	1-3 CFU L^{-1}	91.5 ± 15’	1.3 g^{-1}	85 ± 10	1-5 CFU $tomato^{-1}$
	qPCR enrichment extraction	100 ± 0	1-5 CFU L^{-1}	100 ± 0	1-5 CFU g^{-1}	100 ± 0	1-5 CFU $tomato^{-1}$
Campylobacter[a]		104 ± 67[a]	10-50 gc L^{-1}	NA	NA	23.5 ± 8	10-20 gc $tomato^{-1}$
E. Coli/Fecal coliforms[b]	Plating	95 ± 10	100 L^{-1}	92 ± 10	1 g^{-1}	NA	NA
	Colilert-18/Colisure	96 ± 8	10 L^{-1}	90 ± 8	1 g^{-1}	NA	NA
Enterococcus[b]	Enterolert	92 ± 11	10 L^{-1}	NA	NA	NA	NA

[a] qPCR recovery
[b] Culture dependent recovery
[c] NA=Not assessed

Table 5. Targeted indicators and pathogens analyzed in cultivation experiments. Listed are the targeted organisms, the detection method and the surrogates used to estimate experimental losses during the sample processing.

Organism	**Detection method**	**Recovery surrogate**
Fecal Indicator Bacteria		
E. Coli	Colisure/Enterolert (IDEXX, USA)	N/A
Enterococci		N/A
Fecal coliforms		N/A
Total coliforms		N/A
Pathogenic Bacteria		
Salmonella spp.	Culture via SS Agar (Difco, USA) & qPCR (invA gene)	*Acinetobacter*
Protozoa		
Cryptosporidium parvum	IMS/DFA	Microspheres (Bangs, USA)
Giardia lamblia	IMS/DFA	Microspheres (Bangs, USA)
Viruses		
Adenovirus (AV Types A, B, C & 40/41)	qPCR	Bacteriophage PP7
Enterovirus (EV71 subtypes)	qPCR	Bacteriophage PP7

Table 6. Primers and probe sequences used for bacteria detection using qPCR.

Organism	Forward and Reverse (F, R) Primers and Probe (Pr) Sequences (5'-3')	QPCR gene symbol/ GenBank sequence accession number (or reference)	Color of probe/channel used for detection *
Acinetobacter spp.	F: GAT GCA ACG CGA AGA ACC TTA R: TTC CCG AAG GCA CCA ATC PR: CTG GCC TTG ACA TAG TAG AAA CTT TCC	Schriewer et al., 2010	Cy5/Red
Campylobacter spp.	F: CTG AAT TTG ATA CCT TAA GTG CAG C R: AGG CAC GCC TAA ACC TAT AGC T PR: TCT CCT TGC TCA TCT TTA GGA TAA ATT CTT TCA	*cadF*/ NC_002163.1	Texas Red/Orange
Salmonella spp.	F: CGT TTC CTG CGG TAC TGT TAA TT R: AGA CGG CTG GTA CTG ATC GAT AA Pr: CCA CGC TCT TTC GTC T	*invA*/ NC_003197.1	Yakima Yellow/Yellow
Shigella spp.	F: ACC ATG CTC GCA GAG AAA CT R: TAC GCT TCA GTA CAG CAT GC Pr: TGG CGT GTC GGG AGT GAC AGC	*ipaH*/ NC_007607.1	FAM/Green
Staphylococcus aureus	F: CGT ATT AGC AGA GAG CCA ACC A R: GTG AAT TTA CTC GCT TTG TGC AA Pr: ACC CTA CGC CAG ATG A	*mecA*/ NC_002952.2	FAM/Green
Pseudomonas aeuroginosa	F: TGC TGG TGG CAC AGG ACA T R: TTG TTG GTG CAG TTC CTC ATT G Pr: CAG ATG CTT TGC CTC AA	*regA (AKA toxR)/ NC_008463.1*	FAM/Green

* The probe color is subject to change in order to maximize the number of assays that can be run simultaneously. All probe sequences begin with a fluorescent dye (listed in the last column) and end with a quencher, BHQ-1/2-

Table 7. A selected representation of water quality parameters for secondary and tertiary TWW (second season) measured at the time of release from the wastewater treatment plant (WWTP), and at the farm where produce was irrigated.

Cultivation Week	BOD (mg/L) Average monthly value ± SD			TSS (mg/L) Average monthly value ± SD			Total N_2 Average Monthly value ± SD		
	Secondary (at WWTP)	Secondary (at farm)	Tertiary (at farm)	Secondary (at WWTP)	Secondary (at farm)	Tertiary (at farm)	Secondary (at WWTP)	Secondary (at farm)	Tertiary (at farm)
0-4	8 ± 2.9	>30 ± N/A	>30 ± N/A	10 ± 3	29 ± 4.3	15 ± 4	15 ± 6	9.5 ±2	5.2 ± 3
16-20	15.8 ± 2.7	22.5 ± 6.1	15.4 ± 5.3	12.9 ± 2.6	22.1 ± 6	7.8 ± 4.3	8 ± 4	10 ± 5.1	9.5 ± 4

Cultivation Results

First Cultivation Experiment

Table 8. FIB and pathogen detection in irrigation water collected during the first tomato cultivation study in which treatment plots are irrigated with secondary treated wastewater effluents, and control plots are irrigated with potable water. FIB were enumerated on samples prior to concentration using ultrafiltration, while pathogens were tested on concentrated retentate samples.

Sampling time	Irrigation source	Fecal indicator bacteria MPN / 100ml (95% confidence intervals)				Pathogens / L	
		Total coliforms	Fecal coliforms	*E. coli*	*Enterococci*	*Salmonella*	*Cryptosp / Giardia*
Pre-planting	Effluent	900 (300-2900)	NA[a]	<1	240 (100-940)	<2	<1
	Potable	<1	NA	<1	<1	<2	<1
3 wks	Effluent	>1600	NA	<1	17 (7-46)	<2	0.5/0.5
	Potable	<1	NA	<1	<1	<2	<1
6 wks	Effluent	1600 (600-5300)	NA	300 (100-1300)	<1	<2	<1
	Potable	<1	NA	<1	<1	<2	<1
9 wks	Effluent	>1600	<1	2 (1-11)	1600 (600-5300)	<2	<1
	Potable	<1	<1	<1	<1	<2	<1
12 wks	Effluent	110 (40-300)	17 (7.0-46)	11 (4-29)	30 (10-110)	<2	<1
	Potable	<1	<1	<1	<1	<2	<1
15 wks	Effluent	>1600	>1600	80 (10-390)	23 (9-86)	<2	<1
	Potable	<1	<1	<1	<1	<2	<1
18 wks	Effluent	>1600	>1600	>1600	240 (100-940)	P[b]	<1
	Potable	<1	<1	<1	<1	<2	<1

[a] NA=Not assessed

[b] Present, determined using non-quantitative enrichment culture assays

Limits of detection determined with spiking studies in water (*E coli*= 1 CFU/100 mL; *Salmonella*=2 CFU/L protozoa= 1 (oo)cyst/L); Targeted viruses (Adenovirus and Enterovirus) were not detected (LOD=0.25-4 gc/mL)

Table 9. FIB and pathogen detection in soil samples collected during the first tomato cultivation study in which treatment plots are irrigated with secondary treated wastewater effluents, and control plots are irrigated with potable water. Results presented as median of 5 replicate plots.

Sampling time	**Irrigation source**	**Fecal indicator bacteria Median CFU / g (range) [detects]**				**Pathogens / g**
		Total coliforms	**Fecal coliforms**	***E. coli***	***Enterococci***	***Salmonella*** [c]
Pre-planting	Effluent	NA[a]	NA	<1	NA	NA
	Potable	NA	NA	<1	NA	NA
3 wks	Effluent	NA	NA	<1	3333 (556-10000) [5/5]	<1.3
	Potable	NA	NA	<1	1111 (0-3330) [4/5]	<1.3
6 wks	Effluent	>10000 (200->10000) [5/5]	NA	<1	0 (0-556) [1/5]	<1.3
	Potable	6500 (1550-10000) [5/5]	NA	<1	556 (0-10000) [3/5]	P [b] [1/5]
9 wks	Effluent	N/A	NA	<1	0 (0-400) [1/5]	<1.3
	Potable	N/A	NA	<1	225 (0-900) [4/5]	P [1/5]
12 wks	Effluent	2220 (1110-5556) [5/5]	556 (277-4722) [5/5]	<1	30 (15-117.5) [5/5]	P [3/5]
	Potable	1389 (830-4440) [5/5]	1111 (0-1667) [4/5]	<1	163 (25-400) [5/5]	P [2/5]
15 wks	Effluent	4450 3330-5556 [5/5]	10000 (3300-10000) [5/5]	0 0-66 [1/5]	400 (250-550) [5/5]	P [3/5]
	Potable	6667 5000-10000 [5/5]	3300 (1944-5000) [5/5]	<1	200 20-500 [5/5]	P [2/5]
18 wks	Effluent	>10,000 [5/5]	>10,000 [5/5]	<1	500 [5/5]	P [5/5]
	Potable	>10,000 [5/5]	>10000 (5550->10000) [5/5]	<1	500 (375-500) [5/5]	P [1/5]

[a] NA=Not assessed

[b] Present, determined using non-quantitative enrichment culture assays

Limits of detection determined with spiking studies in soil (*Salmonella*= 1.3 CFU/g); Protozoal (LOD=2 *Cryptosp*/3 *Giardia* (oo)cyst/g) and viral pathogens (Adenovirus and Enterovirus, LOD=260-4,000 gc/g) were not detected.

Table 10. FIB and pathogen detection in washings from tomato samples collected during the first tomato cultivation study in which treatment plots are irrigated with secondary treated wastewater effluents, and control plots are irrigated with potable water. Results presented as median, range and number of detects of 5 replicate plots.

Sampling time	**Irrigation source**	**Fecal indicator bacteria** Median CFU / tomato (range) [detects]				**Pathogens/ tomato**
		Total coliforms	**Fecal coliforms**	***E. coli***	***Enterococci***	***Salmonella*** **[detects]**
9 wks	Effluent	NA[a]	NA	<1	<2	<5
	Potable	NA	NA	<1	<2	<5
12 wks	Effluent	0 (0-56) [1/5]	NA	<1	0 (0-3889) [1/5]	P[c] [1/5]
	Potable	1778 (0-2500) [3/5]	NA	<1	<2	P [1/5]
15 wks	Effluent	>10,000 [5/5]	>10,000 [5/5]	<1	3611 (388-10000) [5/5]	<5
	Potable	>10,000 [5/5]	>10,000 [5/5]	<1	>10000 (8333-10000) [5/5]	P [1/5]
18 wks	Effluent	8611 (3055-10000) [5/5]	>10000 (2500-10000) [5/5]	<1	>500 [5/5]	P [1/5]
	Potable	>10,000 [5/5]	>10,000 [5/5]	<1	>500 [5/5]	P [2/5]

[a] NA=Not assessed

[c] Present, determined using non-quantitative enrichment culture assays

Limits of detection determined with spiking studies on tomato surfaces (*E coli* = 1 CFU/tomato; *Salmonella*=5 CFU/tomato); Protozoal (LOD = 5 (oo)cysts/tomato) and viral pathogens (Adenovirus and Enterovirus, LOD=2,600-40,000 gc/tomato) were not detected.

Second cultivation experiment

Table 11. FIB and pathogen detection in irrigation water samples collected during the second tomato cultivation study in which treatment plots were irrigated with secondary or tertiary treated wastewater effluents, and control plots were irrigated with potable water.

Sampling time	Irrigation source	Fecal indicator bacteria MPN/100ml (95% CI)				Pathogens / L
		Total coliforms	**Fecal coliforms**	***E. coli***	***Enterococci***	***Cryptosp/ Giardia***
Pre-planting	Secondary	80 (45-171)	<1	<1	<1	<1
	Tertiary	10 (3-56)	<1	<1	<1	<1
	Potable	<1	<1	<1	<1	<1
4 wks	Secondary	6760 (5350-8330)	2980 (1950-4340)	727 (519-1010)	3330 (2702-4017)	0.1 G/L
	Tertiary	3000 (1960-4270)	1203.3 (810.8-1750.7)	88.2 (62.9-120.2)	410 (287-607)	<1
	Potable	<1	<1	<1	<1	<1
8 wks	Secondary	12033 (8108-17507)	602 (441-800)	216 (141-312)	40 (11-89)	NA [a]
	Tertiary	6488 (4245-9415)	763 (588-962)	145 (86-234)	20 (3-56)	NA
	Potable	<1	<1	<1	<1	NA
12 wks	Secondary	>20,050 (14610-∞)	>20050 (14610-∞)	5600 (4100-7500)	>20050 (14610-∞)	<1
	Tertiary	750 (578-946)	738 (540-962)	410 (277-588)	410.6 (260.6-618.9)	<1
	Potable	<1	<1	<1	<1	<1
16 wks	Secondary	20050 (14610-∞)	64 (30-139)	<1	1445 (1023-2241)	NA
	Tertiary	20050 (14610-∞)	75 (37-155)	<1	406 (280-595)	NA
	Potable	<1	<1	<1	<1	NA
20 wks	Secondary	63 (25-127)	<1	<1	52 (18-108)	*2.6 C /L
	Tertiary	<1	<1	<1	10 (1-55)	0.13 C/ L
	Potable	10 (1-55)	<1	<1	<1	<1

[a] NA=Not assessed

* Confirmed as *Cryptosporidium hominis* through molecular testing and sequence analyses

Limits of detection determined with spiking studies in water (*E coli*= 1 CFU/100 mL; protozoa= 1 (oo)cyst/L); *Salmonella* (LOD=2 CFU/L) and viral pathogens (Adenovirus and Enterovirus, LOD=0.25-4 gc/mL) were not detected.

Table 12. FIB and pathogen detection in soil samples collected during the second tomato cultivation study in which treatment plots are irrigated with secondary or tertiary treated wastewater effluents, and control plots are irrigated with potable water. Results presented as medians of 5 replicate plots

Sampling time	**Irrigation source**	**Fecal indicator bacteria Median MPN/g (Range) [detected/tested]**				**Pathogens / g**
		Total coliforms	**Fecal coliforms**	***E. Coli***	***Enterococci***	***Salmonella*** [a]
4 wks	Non-irrigated	6488 (<1-24190) [4/5]	<1 (<1-467) [1/5]	<1 (<1-4950) [1/5]	1 (<1-256.7) [3/5]	<1.3 [1/5]
	Secondary	6867 (10-24190) [5/5]	1810 (550-10112) [5/5]	<1 [0/5]	31 (<1-109) [3/5]	<1.3 [0/5]
	Tertiary	24190 (12633-24190) [5/5]	319 (130-523) [5/5]	<1 [0/5]	121 (40-183) [5/5]	<1.3 [1/5]
	Potable	24190 (3282-24190) [5/5]	24190 (566-24190) [5/5]	<1 [0/5]	31 (0-173) [4/5]	<1.3 [1/5]
8 wks	Secondary	1184 (111-2005) [5/5]	42 (<1-164) [3/5]	<1 [0/5]	200 (100-1370) [5/5]	<1.3 [2/5]
	Tertiary	124 (42-2005) [5/5]	<1 [0/5]	<1 [0/5]	1500 (310-2070) [5/5]	<1.3 [1/5]
	Potable	364 (<1-2005) [4/5]	42 (<1-164) [2/5]	<1 [0/5]	200 (100-1370) [5/5]	<1.3 [0/5]
12 wks	Secondary	2005 [1/1]	2005 [1/1]	<1 [0/1]	<1 [0/1]	<1.3 [0/1]
	Tertiary	2005 [1/1]	945 [1/1]	<1 [0/1]	10 [1/1]	<1.3 [1/1]
	Potable	>2005 [1/1]	>2000 [1/1]	<1 [0/1]	<1 [0/1]	<1.3 [0/1]
16 wks	Secondary	NA	<1[0/5]	<1[0/5]	<1[0/5]	<1.3 [3/5]
	Tertiary	NA	<1[0/5]	<1[0/5]	1 (<1-4) [4/5]	<1.3 [5/5]
	Potable	NA	<1[0/5]	<1[0/5]	1 (1-4) [3/5]	<1.3 [3/5]
20 wks	Secondary	>10000 [5/5]	<1[0/5]	<1[0/5]	<1 (<1-2000) [2/5]	<1.3 [1/5]
	Tertiary	>10000 [5/5]	<1 [0/5]	<1 [0/5]	200 (<1-10000) [4/5]	<1.3 [3/5]
	Potable	>10000 [5/5]	<1 (<1-200) [1/5]	<1 [0/5]	0 (<1-750) [2/5]	<1.3 [0/5]

[a] Positive *Salmonella* detects from qPCR; *Salmonella* was not detected in soil using culture techniques. LOD is given for culture-based techniques.

Limits of detection determined with spiking studies in soil (*Salmonella*= 1.3 CFU/g); Protozoal (LOD= 2 *Cryptosporidium*/3 *Giardia* (oo)cyst/g) and viral pathogens (Adenovirus and Enterovirus, LOD=260-4,000 gc/g) were not detected.

Table 13. FIB and pathogen detection in tomato samples collected during the second tomato cultivation study in which treatment plots are irrigated with secondary or tertiary treated wastewater effluents, and control plots are irrigated with potable water. Results presented as medians of 5 replicate plots.

Sampling time	**Irrigation source**	**Fecal indicator bacteria MPN/Tomato[a] (Range) [detects]**				**Pathogens / tomato**	
		Total coliforms	**Fecal coliforms**	***E. coli***	***Enterococci***	***Salmonella*** [a]	**Cryptosp/ Giardia**
12 wks	Secondary	2005 (1461-∞) [5/5]	<1 [0/5]	<1 [0/5]	<1 (<1-40) [2/5]	<5 [0/5]	<5 [0/5]
	Tertiary	2005 (1461-∞)	<1 [0/5]	<1 [0/5]	58 [5/5]	<5 [0/5]	<5 [0/5]
	Potable	>2005 (1461-∞)	<1 [0/5]	<1 [0/5]	20 [4/5]	<5 [0/5	<5 [0/5]
16 wks	Secondary	>2005 (1461-∞)	<1 [0/5]	<1 [0/5]	27.75 [4/5]	<5 [1/5]	NA [b]
	Tertiary	>2005 (1461-∞)	<1 [0/5]	<1 [0/5]	60 [4/5]	<5 [1/5]	NA
	Potable	>2005 (1461-∞)	<1 [0/5]	<1 [0/5]	38.8 [5/5]	<5 [0/5]	NA
20 wks	Secondary	>10000	<1 [0/5]	<1 [0/5]	7130 [5/5]	[1/5]*	1 C [1/5]
	Tertiary	>10000	<1 [0/5]	<1 [0/5]	2160 [5/5]	[2/5]	<5 [0/5]
	Potable	>10000	<1 [0/5]	<1 [0/5]	6387.5 [5/5]	<5	<5 [0/5]

[a] Positive *Salmonella* detects from qPCR; **Salmonella* was detected on tomato surfaces once using culture techniques
[b] NA=Not assessed
Limits of detection determined with spiking studies on tomato surfaces (*E coli* = 1 CFU/tomato; *Salmonella*=5 CFU/tomato; protozoa= 5 (oo)cyst/tomato); viral pathogens (Adenovirus and Enterovirus, LOD=2,600-40,000 gc/tomato) were not detected.

Figures

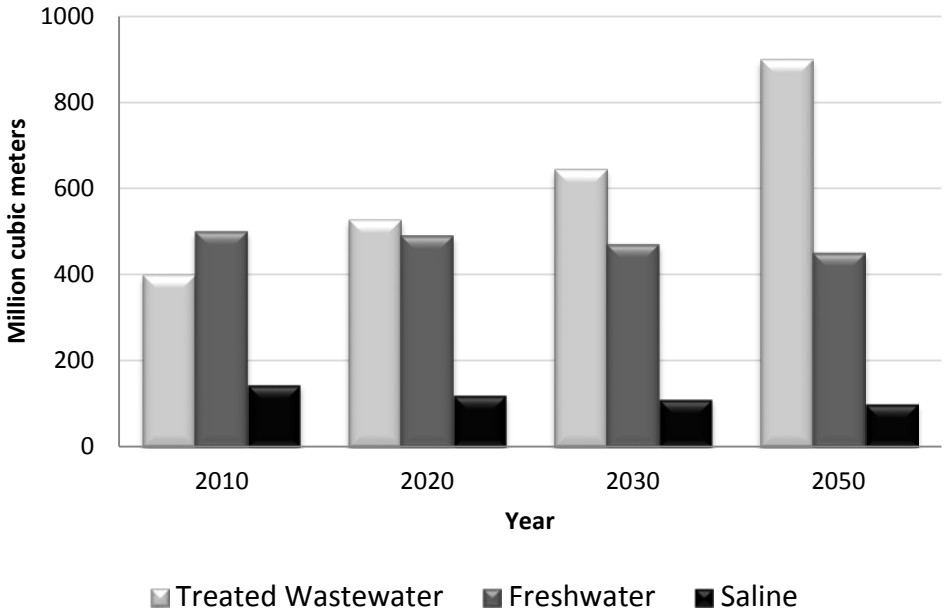
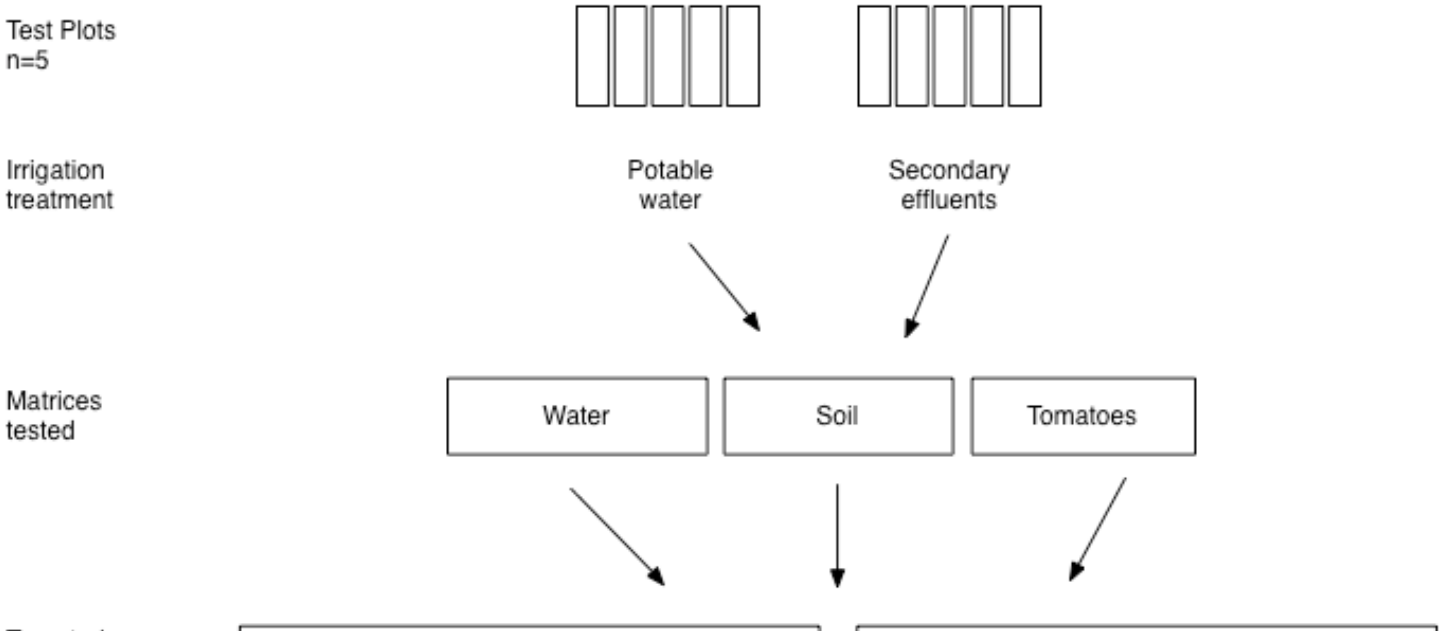


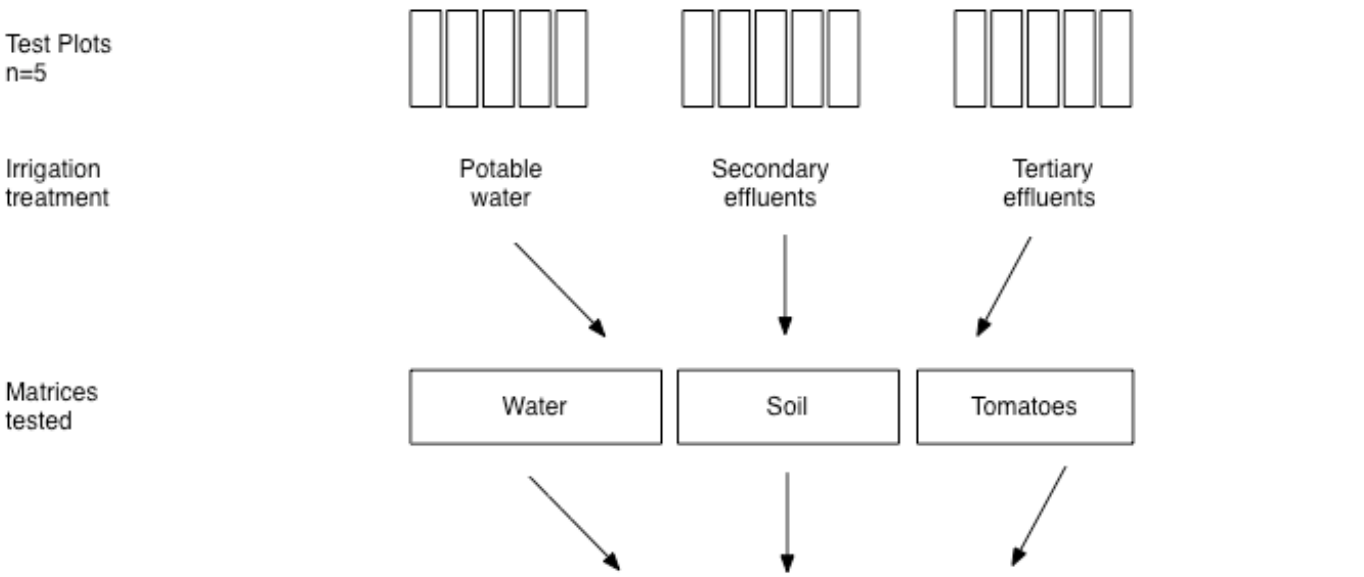
Figure 1: Israel's long-term strategy for reclaimed wastewater irrigation (Source: Israel Water Authority, 2011)



Indicator	Method
E coli	Colisure (water) Plating (soil/tomatoes)
Total coliforms	Colisure (water) Plating (soil/tomatoes)
Fecal coliforms	Colisure (water) Plating (soil/tomatoes)
Enterococcus	Enterolert (water) Plating (soil/tomatoes)

Pathogen	Pathogen
Cryptosporidium	IMS DFA
Giardia	IMS DFA
Salmonella	qPCR and Culture
Shigella	qPCR
Campylobacter	qPCR
Enteroviruses	qPCR
Adenoviruses	qPCR

Figure 2. Flow diagram depicting study design and sampling scheme for the first cultivation experiment, in which plots (n=5) were irrigated with potable water and secondary TWW.



Indicator	Method
E coli	Colisure
Total coliforms	Colisure
Fecal coliforms	Colisure
Enterococcus	Enterolert

Pathogen	Method
Cryptosporidium	IMS DFA
Giardia	IMS DFA
Salmonella	qPCR and Culture
Enteroviruses	qPCR
Adenoviruses	qPCR

Figure 3. Flow diagram depicting study design and sampling scheme for the second cultivation experiment, in which plots (n=5) were irrigated with potable water, secondary TWW, and tertiary TWW effluents.

1 Potable		**4 Effluent**		**7 Potable**		**10 Effluent**
Dry (1.5 m)	Dry bed	Dry (1.5 m)	Dry bed	Dry (1.5 m)	Dry bed	Dry (1.5 m)
2 Effluent		**5 Potable**		**8 Effluent**		
Dry (1.5 m)		Dry (1.5 m)		Dry (1.5 m)		
3 Potable		**6 Effluent**		**9 Potable**		

Figure 4. Diagram depicting experimental plot arrangement in the first cultivation experiment

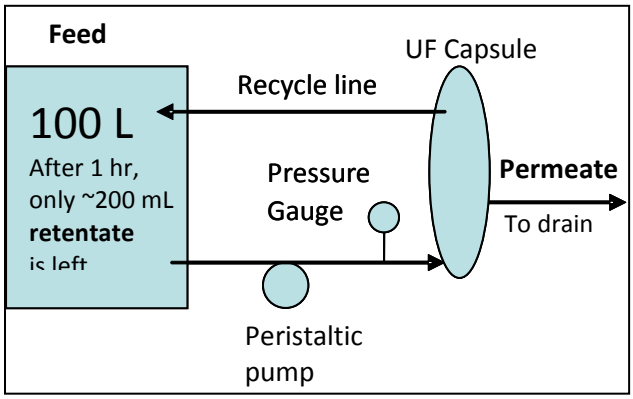


Figure 5. Schematic of water ultrafiltration method. The arrows denote the direction of water pumping.

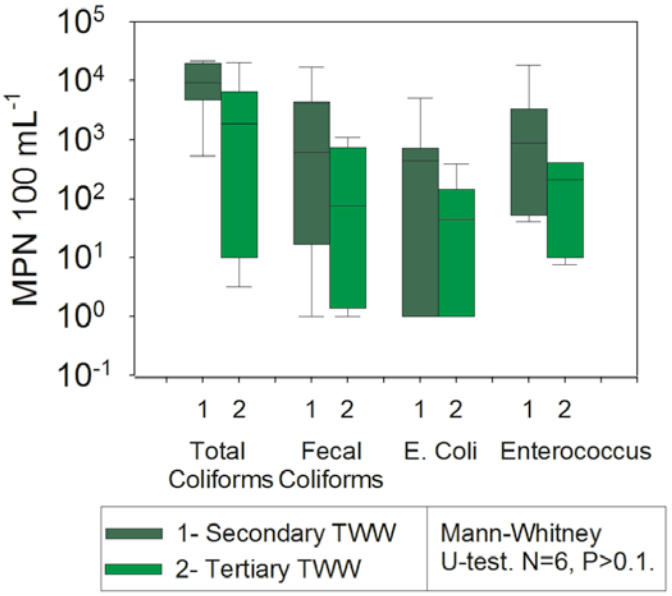


Figure 6. Fecal Indicator Bacteria in secondary and tertiary TWW (second cultivation experiment)

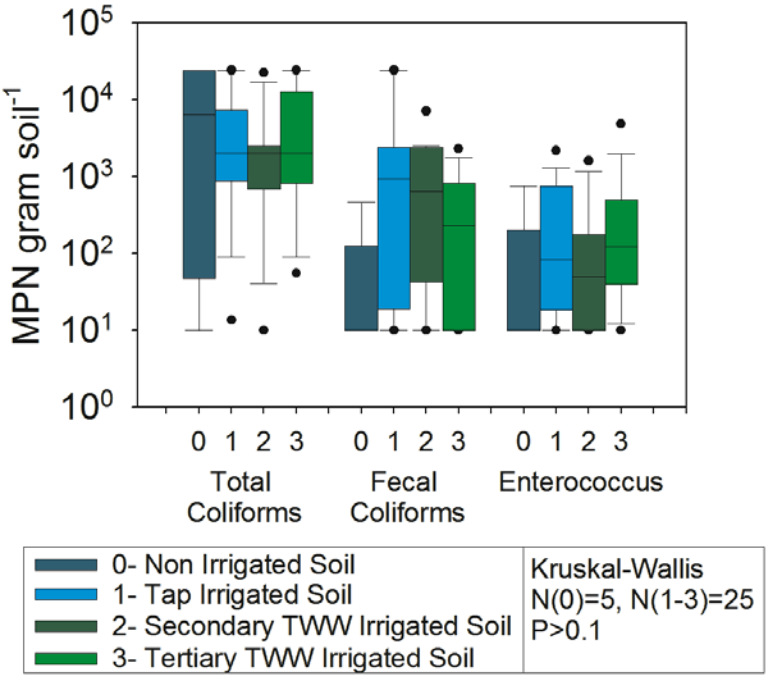


Figure 7. Fecal indicator bacteria measured in soil irrigated with tap water, secondary effluents, or tertiary effluents (second cultivation experiment)

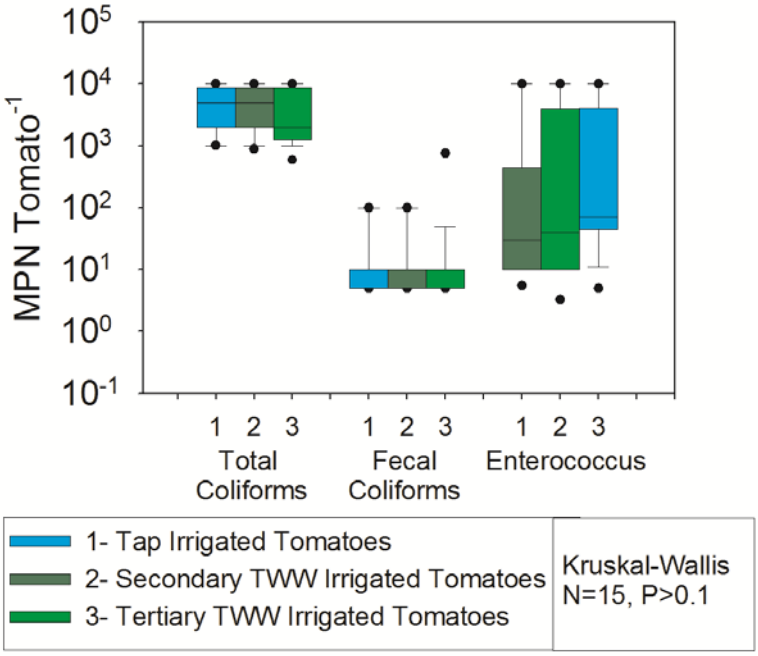


Figure 8. Fecal indicator bacteria measured on tomato surfaces irrigated with tap water, secondary effluents, or tertiary effluents (second cultivation experiment)

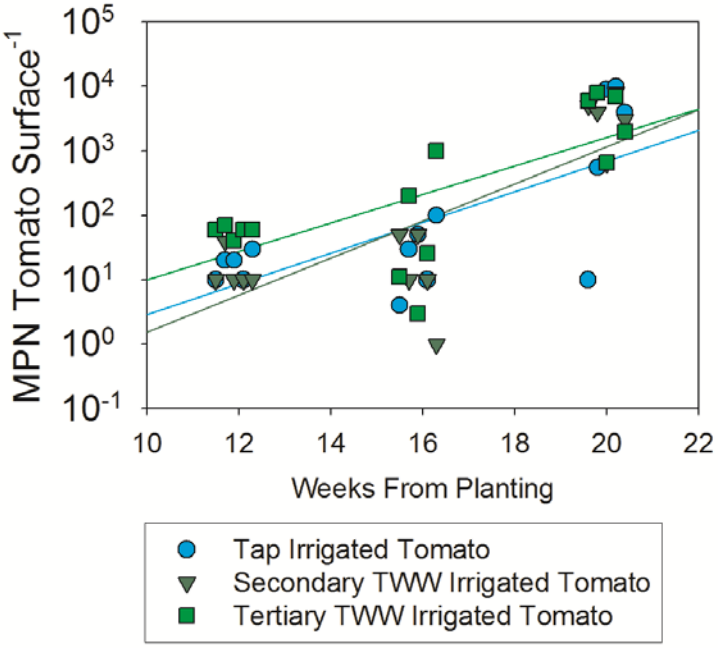


Figure 9. A time series representation of *Enterococcus* concentrations measured on tomato surfaces irrigated with tap water, secondary TWW, or tertiary TWW (second cultivation experiment)

Suggestions to CPS (optional)

For future CPS-BARD collaborations we recommend some flexibility regarding transfer of funds between the partner institutions, in the event that one of the partners may encounter unexpected expenses. This happened in our project when the fixed costs for utilization of experimental plots in the second field trial were increased by new management thus affecting our Israeli partners' ability to proceed with the field experiment. As a result fewer pathogen measurements were conducted than had been originally planned.