CENTER for PRODUCE SAFETY

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FINAL PROJECT REPORT

Project Title
Investigation of risk criteria and foodborne pathogen reduction practices for irrigation water

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Objectives
1) Investigate practical criteria for the prediction of foodborne pathogens in irrigation pond and well water.
2) Evaluate the efficacy of commercial sanitizers on the decontamination of three common foodborne pathogens, Salmonella spp., E. coli O157:H7, and Listeria monocytogenes, in irrigation well and pond water.
3) Provide education and training to stakeholders on improved agricultural practices to reduce the food safety risks in irrigation water.

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FINAL REPORT

Abstract

Current outbreaks of foodborne illnesses associated with vegetables and fruits attract concerns about produce safety; irrigation water is considered to be one of the main contamination sources of foodborne pathogens on fresh produce. *Salmonella enterica*, *Escherichia coli* O157:H7, and *Listeria monocytogenes* are three common enteric pathogens that may exist in irrigation water and are reported to be associated with produce contamination. Science-based data are needed to generate practical and accurate prediction methods as well as effective decontamination strategies for irrigation water to mitigate contamination of produce by human pathogens at the production level. In this study, we detected the dynamics of *Salmonella* population and distribution in irrigation ponds and wells on the Eastern Shore of Virginia. The results provide growers with information that is necessary for them to determine if a contamination problem exists. Correlations between foodborne pathogens (*Salmonella* spp. and *Listeria monocytogenes*) and contextual factors, including water parameters, weather information, and fecal indicators, were analyzed. Bacterial community analysis through metagenomics study provides meaningful clues to identify suitable indicators and potential suppressors of foodborne pathogens in irrigation water. In addition, the efficacy of commercial sanitizers with different active ingredients used for irrigation water treatment was evaluated. Relevant results derived from this study have been shared with vegetable growers and other stakeholders through extension talks, field days, and scientific meetings. The outcomes of this research will benefit stakeholders especially vegetable and fruit industries to reduce the contamination risks of foodborne pathogens during the production and achieve the new requirements of the Food Safety Modernization Act on produce safety.

Background

Fresh produce is increasingly recognized as a common vehicle for transmission of foodborne pathogens (Brandl, 2006; Franz and van Bruggen, 2008). Ranked in the top five pathogens contributing to illnesses, hospitalizations, or deaths in the United States during the outbreaks of foodborne diseases, *Salmonella* spp., *E. coli* O157:H7, and *L. monocytogenes* are considered to be common foodborne pathogens associated with fresh produce (CDC, 2011). Consumption of *Salmonella* contaminated produce has led to several multistate and international outbreaks in recent years (CDC, 2005; CDC, 2007; Greene et al., 2008). *E. coli* O157 is typically responsible for most infections (80%) among the Shiga-toxin-producing bacteria in the U.S. and in some European countries (Muniesa et al., 2006; Paton and Paton, 1998). *L. monocytogenes* was responsible for a 2011 produce-borne outbreak in the U.S., with 147 illnesses, 33 deaths, and 1 miscarriage, due to consumption of cantaloupe (FDA, 2012). In addition, a considerable number of produce recalls have occurred in the past years as a result of contamination by the three pathogens (FDA, 2004–2014).

Irrigation water may play an important role in contaminating vegetables and fruits with foodborne pathogens (Hintz et al., 2010; Islam et al., 2004; Solomon et al., 2002). Waste with the bacteria can enter the water through different ways, including sewage overflows, polluted storm water runoff, and agricultural runoff. The 2005 multistate *Salmonella* Newport outbreak associated with tomato, and the 2006 multistate *E. coli* O157 outbreak associated with spinach, were reported to be related to contaminated irrigation water (Bidol et al., 2007; Gelting et al., 2011). After irrigation with contaminated water, the bacteria can adhere to plants, enter into plants, and translocate to other organs of the infested plants, which would be difficult to completely sterilize during typical washing and disinfection procedures, and may persist and multiply at any point along the farm-to-fork continuum from production to consumption (Barak et al., 2011; Danyluk, 2013; Franz and van Bruggen, 2008; Gu et al., 2011; Gu et al., 2013c; Miles et al., 2009; Solomon et al., 2002; Zheng et al., 2013). Therefore, minimizing the risk of contamination by human bacterial pathogens during the pre-harvest period would also be essential to reducing foodborne illness risks.

Fecal pollution, including *Salmonella* spp., *E. coli* O157:H7 and *L. monocytogenes*, is traditionally evaluated by detecting fecal indicator bacteria like fecal coliforms and generic *E. coli* (EPA, 2002). However, contradictory results have been reported as to the correlation between index or indicator organisms and the occurrence of human pathogens in surface water (Ahmed et al. 2009; Benjamin et al., 2013; Burton et al., 1987; Chandran and Hatha, 2005; Chandran et al., 2011; Gu et al., 2013a; McEgan et al., 2013; Rhodes and Kator, 1988). Chigor et al. (2010) reported that some environmental factors, including water turbidity and concentrations of nitrate, phosphate, and chloride, were positively correlated with the population of fecal coliforms in a river used for fresh produce irrigation in Nigeria. A previous study evaluating irrigation ponds in Georgia showed positive correlation between temperature, rainfall, populations of fecal coliform, and culturable bacteria and the occurrence of *E. coli* O157, and negative relationship between the total nitrogen concentration, oxidation reduction potential (ORP), and dissolved oxygen concentration and the occurrence of this pathogen ($P < 0.05$) (Gu et al., 2013a). Another survey conducted at Central Florida presented different results on the relationship of environmental factors to the prevalence of *Salmonella* in surface water (McEgan et al., 2013). These studies showed regional difference for both occurrence/population of detected foodborne pathogens and the efficacy of indicators to predict the prevalence of the pathogens (Ahmed et al., 2009; Benjamin et al., 2013; Gu et al., 2013a; McEgan et al., 2013). Additional studies would be necessary to evaluate and validate the efficacy of biological index organisms and physicochemical indicators on the prediction of foodborne pathogen contamination in various major agricultural regions in U.S. Furthermore, previous studies about the impacts of bacterial communities on the occurrence/population of foodborne pathogens in irrigation ponds showed the probability of identifying alternative indicator microorganisms to economically and reliably predict the presence or absence of foodborne pathogens in irrigation water and the on-farm agricultural environment (Gu et al. 2013a; Gu et al. 2013b).

Currently, as part of the Food Safety Modernization Act (FSMA), the U.S. Food and Drug Administration (FDA) issued a Proposed Rule for Produce Safety, which includes mandatory standards for growing, harvesting, packing and holding produce on domestic and foreign farms (FDA, 2013). Standards based on the presence/population of generic *E. coli* have been proposed for agricultural water, including irrigation water. Even though farmers are allowed to use alternatives to requirements for testing water and taking action based on the results, solutions to the issues, such as prevention strategies and suitable decontamination methods to treat the irrigation water if contamination occurs, are not provided in the proposed rule for produce farmers. Former studies have reported multiple methods to disinfect foodborne pathogens in the postharvest period (Abadias et al., 2011; Gil et al., 2009; Selma, 2008; Suslow, 2001; Tomás-Callejas et al., 2012). In another similar area, information about practical decontamination methods to control foodborne pathogens for large irrigation systems is limited. The efficacy of commercial sanitizers, especially the products approved by the U.S. Environmental Protection Agency (EPA) and/or the Organic Materials Review Institute for use in irrigation water, has not been evaluated. In addition, the impacts of water quality from various irrigation water sources (pond or well water at different locations) on the reduction of different foodborne pathogens in irrigation water have not been sufficiently analyzed. These factors, such as water pH, conductivity, ORP, and population/diversity of microbial communities, could potentially affect the decontamination efficacy of sanitizers, and increase the minimum effective dose.

To better understand the prevalence of foodborne pathogens in irrigation water and assess the food safety risks, science-based data about the occurrence/population of enteric pathogens in major agricultural regions associated produce-borne illnesses as well as the correlation to potential biological or physicochemical indicators are needed. The proposed study comparing EPA-approved commercial sanitizers with investigation of the optimal treatment concentration under various water qualities will lead to practical and economical solutions for reducing contamination and achieving the new standards of FSMA on produce safety.

Research Methods and Results

OBJECTIVE 1. Investigate practical indicators for the prediction of foodborne pathogens in irrigation pond and well water.

Methods:

Water sample collection. The study area is the Eastern Shore of Virginia (ESV), an important agricultural region in the U.S., including the top tomato production county in Virginia. From January 2015 to December 2015, 4-liter pond and well irrigation water samples were collected weekly from four vegetable farms (Farms A-D) on ESV for *Salmonella* spp. isolation. Additional 4-liter water samples were collected monthly from the same irrigation ponds and wells in 2015 for *Listeria monocytogenes* detection. Collected water samples were stored on ice in the field and transported to lab for further experiments. Totally 392 weekly pond and well irrigation water samples (2 water types × 4 farms × 49 weeks) were collected for *Salmonella* detection, and 96 monthly pond and well water samples (2 water types × 4 farms × 12 months) were collected for *Listeria monocytogenes* detection in this study.

Water parameter measurement, weather information collection, and fecal indicator detection. During sampling, water samples were tested for temperature, pH, specific conductance, dissolved oxygen percentage, dissolved oxygen concentration, dissolved oxygen charge, and ORP with an YSI® 6600 Multiparameter Sonde (Yellow Springs, OH). Water turbidity was tested by HI98713 turbidity meter (Hanna instruments, Woonsocket, RI). MPN values of generic *E. coli* and fecal coliform were assayed using Colilert® (IDEXX Laboratories, Inc., Westbrook, ME) by following product instruction. HOBO Micro Station (Onset Computer Corporation, Bourne, MA) was set up at each tested farm. Temperature (^{o}C) and rainfall data (mm) 1 week prior to sampling at four farms were collected for further analysis.

***Salmonella* detection and most probable number (MPN) analysis.** A most probable number (MPN) method was used to assess *Salmonella* concentration in collected water samples (Luo et al., 2014). In brief, water samples (500, 100, and 10 mL) were added to equal volumes of lactose broth (BD Biosciences, San Jose, CA) at 2× concentration in quadruplicate and incubated at 37°C overnight. One mL of each culture was transferred into 9 mL tetrathionate broth (TT broth; Dot Scientific, Burton, MI) for selective enrichment at 37°C overnight. TT broth cultures were then streaked onto *Salmonella* selective XLT4 agar (BD Biosciences, San Jose, CA) plates and incubated at 40°C overnight. Positive colonies (black or black centered with a yellow periphery) were confirmed by a cross-streaking method on CHROMagar *Salmonella* plates (DRG International, Springfield, NJ) and by PCR amplification of *InvA* gene. Up to four positive colonies per plate were stored in 20% glycerol in a −80°C freezer.

***L. monocytogenes* detection and MPN analysis.** Similarly, *L. monocytogenes* was detected by the similar MPN method as mentioned above. In brief, water samples (500, 100 and 10 mL) were added to equal volumes of sterile buffered *Listeria* enrichment broth (BLEB, Becton, Dickinson and Co., Sparks, MD) at 2× concentration in quadruplicate and incubated at 30°C for 24 h. 50 µL of each enrichment was then streaked onto *Listeria monocytogenes* plating medium (LMPM, R&F Laboratories 0550M) for 48 h at 35°C. Positive colonies (turquoise convex) were confirmed by a cross-streaking method using modified Oxford agar (MOX) (Becton, Dickinson) and by PCR amplification of *sigB* gene. Up to four positive colonies per plate were stored in 20% glycerol in a −80°C freezer.

Molecular serotyping analysis. *Salmonella* serotypes of isolated strains were determined using the CDC standard protocol for the molecular determination of *Salmonella* serotype (CDC, 2009). Briefly, DNA from a pure culture was isolated using Instagene (BioRad). Multiplex PCR was set up using Qiagen HotStar Master Mix (Qiagen), 1 µL of DNA and thermocycled under the following conditions: 95°C, 15 min; 30 cycles of 94°C for 30 sec, 48°C for 90 sec, 72°C for 90 sec; then 72°C for 10 min. DNA from the PCR reactions were then hybridized to the beads with specific O- and H-Ag probes before addition of strepavidin-R-phycoerythrin (Invitrogen div. Life Technologies, Grand Island, NY). After incubation the samples were read using the Bio-Plex instrument (BioRad). Positives were determined based on the ratio of signal to noise using a no template DNA negative control. Serotypes were determined based on which

antigens are positive for each sample. *S. enterica* serovar Typhimurium strain ATCC 14028 was used as positive control. Sterile water was used as negative control.

Antimicrobial susceptibility test. 96-well Sensititer Gram Negative Narms plates (Thermo Fisher Scientific, Waltham, MA) were used for antimicrobial susceptibility tests according to the manufacturer's instructions. Results were interpreted and antibiotic-resistant strains were defined according to the National Committee for Clinical and Laboratory Standards criteria.

Metagenomic analysis. 200 mL of each collected water sample was vacuum filtered through a 0.22-µm sterile nitrocellulose membrane (Millipore Corporation, Billerica, MA). Genomic DNA of bacteria captured by the membranes was extracted using a PowerWater DNA Isolation Kit (MOBIO Laboratories, Inc., Carlsbad, CA) and stored at −80°C until use. Selected water DNAs (one set per month for both *Salmonella* and *L. monocytogenes* detection) were processed for shortgun and amplicon sequencings using Nextseq and Miseq. Diversity of bacterial community as well as presence/population of bacterial species were analyzed by 16s PCR amplicon sequencing using Miseq (Illumina). Raw 16S rRNA amplicon sequences were trimmed for quality using Trimmomatic, and high-quality read pairs were merged using the FLASH tool. High-quality R1 read pairs that did not merge (due to a low quality R2 pair) were also included in downstream analysis. Final trimmed sequences were required to be at least 200bp in length. During preprocessing sequences were screened for PhiX contaminant as well as Chloroplast sequences using the RDP classifier trained on the GreenGenes 16S database.

Passing high-quality 16S sequences were analyzed using the Resphera Discovery protocol (Baltimore, MD). Briefly, sequences were clustered into operational taxonomic units using UCLUST (de novo) with a 97% identity threshold. Representative members of each OTU were assigned a consensus taxonomic lineage using the RDP classifier trained on the Resphera Discovery 16S database (minimum confidence 80%). Prior to downstream comparative analysis, samples were rarefied to 10,000 sequences per sample. Differential abundance analysis included the Mann-Whitney test.

Results:

There were spatial (farm location) and temporal (weekly) differences for *Salmonella* occurrence in surface pond water (Fig. 1). The prevalence of *Salmonella* spp. in the tested four ponds of farms A, B, C and D were 18.4, 12.3, 24.5, and 22.5 %, respectively. The average MPN values of *Salmonella* in the four ponds during the study were 0.43, 0.28, 0.75, and 1.82 MPN/L, respectively. There were no significant differences for *Salmonella* prevalence among seasons ($p > 0.05$). The major *Salmonella* serovars in Ponds A–D were Thompson, Typhimurium, Thompson, and Newport, respectively (Fig. 6). The top three serovars isolated from all pond water samples were Newport, Thompson, and Typhimurium in 2015 (Fig. 7). All wells of the tested farms, except farm B, were *Salmonella* positive at certain times in 2015 (Table 1). Thompson was the major serovar of isolated *Salmonella* strains in well water samples, followed by Typhimurium and Javiana (Fig. 8). *Salmonella* Newport was only isolated from well water in Farm C.

Compared with the data of a pre-study performed in 2014, the observed prevalence of *Salmonella* in pond irrigation water was approximately the same in 2014 (19.9%) and 2015 (19.4%), while the average concentration in the water increased from 0.49 ± 1.66 MPN/L in 2014 to 0.82 ± 5.14 MPN/L in 2015. Same as in 2015, all wells were *Salmonella* positive except from farm B at certain time points in 2014, especially in farm D. The prevalence (<4%) of *Salmonella* in well water was significantly lower than that for pond water ($p < 0.01$), and had an average level value of 0.03 ± 0.15 MPN/L in 2014 and 0.05 ± 0.36 MPN/L in 2015.

There was no significant correlation between most water parameters and *Salmonella* occurrence in the tested pond and well water ($p > 0.1$, data not shown due to large size). *Salmonella* population in pond irrigation water was positively corrected water turbidity (Table 3). However, the significant correlation was mainly contributed by tested water samples of pond water in farm D ($p < 0.01$). Water turbidity was not significantly correlated with *Salmonella* MPN values in ponds A–C ($p > 0.05$). No significant correlations were identified between tested weather parameters (temperature and rainfall) and *Salmonella* occurrence prevalence and levels in 2015.

Populations of fecal indicators, including coliform and generic *E. coli*, in irrigation pond and well water samples were detected during the study (Figs. 3, 4). There were no significant correlations between fecal indicators and the prevalence/population of detected *S. enterica* spp. Population density of coliform in most pond water samples exceeded the maximum detection limit (2,419.6 MPN/100 ml), which indicates it is not a suitable indicator to evaluate the risks of foodborne pathogen contamination in surface pond water. However, coliform was positively correlated with *Salmonella* in well water (Table 4), even though the significance was mainly contributed by the high correlation in well water of farm D ($p < 0.01$). Similarly, the concentration of generic *E. coli* in well D was also significantly positively corrected with *Salmonella* population ($p < 0.01$). It is notable that pond D has the highest *Salmonella* MPN values, and well D has the largest prevalence ratio compares with ponds and wells in other farms. The relative high levels of *Salmonella* in this farm might be caused by distinct contamination sources compared with other farms on ESV, which results in significant correlation to water turbidity in pond and fecal indicator populations in well water. Nevertheless, even though farm D was observed to have relatively higher risk of *Salmonella* contamination in pond and well water, the levels of fecal indicators, coliform and generic *E. coli* are not the highest among all tested farms. The geometric mean and statistical threshold values of generic *E. coli* in tested pond and well in farm D also achieved (lower than) the microbial standard of water quality for produce safety as per FSMA. The geometric means of ponds A–D and wells A–D in the sampling period (with a total of 49 samples each) were 22.5, 6.3, 8.8, 5.7, 1.1, 1.0, 1.2, and 1.3, respectively. The statistical threshold values of ponds A–D and wells A–D in the sampling period (with a total of 49 samples each) were 770, 62, 681, 28.7, 2.3, 0, 71.8, and 18.4, respectively. The high variance of the correlation of foodborne pathogens to contextual factors among different farms makes it challenging to identify a universal indicator to predict contamination risks.

Similarly, occurrence and population of *L. monocytogenes* in irrigation pond and well water was tested monthly from January 2015. There was an observable difference for *L. monocytogenes* occurrence among ponds (Fig. 2). The prevalence of *L. monocytogenes* in the tested four ponds of farms A, B, C and D were 18.4, 12.3, 24.5, and 22.5 %, respectively. The average MPN values of *L. monocytogenes* in the four ponds during the study were 0.43, 0.28, 0.75, and 1.82 MPN/L, respectively. For well water samples, *L. monocytogenes* was only isolated from farm D in April and October (Table 2). Probably due to limited samples size (monthly instead of weekly detection), no contextual factors measured in this study was significantly correlated to L. *monocytogenes* population and prevalence (data not shown).

To analyze the correlation between *Salmonella* spp. and *L. monocytogenes*, *Salmonella* MPN data of the specific week in which *Listeria* detection was conducted were selected to compare the two foodborne pathogens. In addition, the average of *Salmonella* MPN/L of each month was also calculated for comparison. However, the population/prevalence of neither the selected *Salmonella* MPN values per month nor the average *Salmonella* values were correlated with *L. monocytogenes* population/prevalence in both pond and well water samples. Based on the monthly detection data, the average prevalence and population of *L. monocytogenes* was similar to that of *Salmonella*. However, this pathogen has not been associated with any outbreaks traced back to EVS, which may be contributed by the lower survival and transfer rate of *L. monocytogenes* in the food commodities produced and transported in this region.

A total of 220 *Salmonella* isolates, including 171 from irrigation ponds and 49 from irrigation wells or creek water, were selected for antimicrobial susceptibility testing. Fourteen of the 220 *Salmonella* isolates (6.4%) were resistant isolates, including 10 isolates from pond water and 4 from well/creek water samples. All resistant isolates collected from irrigation water were only resistant to one of the tested antibiotics. Tested isolates were resistant to ceftriaxone (n=6; 2.7%), streptomycin (n=2; 0.9%), tetracycline (n=1; 0.5%), cefoxitin (n=1; 0.5%), ampicillin (n=1; 0.5%), amoxicillin/clavulanic acid 2:1 (n=1; 0.5%), ciprofloxancin (n=1; 0.5%), and ceftiofur (n=1; 0.5%). Even though most of the environmental isolates (93.6%) from irrigation water were not resistant to the tested antibiotics, the varying antimicrobial resistance of *Salmonella* isolates recovered from localized areas in ESV is cause for concern.

Based on 16s amplicon sequencing results, hierarchical clustering of samples based on the 50 most dominant class level taxa revealed a strong clustering associated with water source. The heatmap scale denotes log transformed proportion values (e.g. -1 ~ 10%, -2 ~ 1%, -3 ~ 0.1%) (Fig. 9). Similarly, hierarchical clustering of samples based on the 50 most dominant genus level taxa revealed a strong clustering associated with water source. According to the heatmap scale values, some taxa appear uniquely enriched in the pond samples (e.g., Linmohabitans) while others are most abundant in the well samples (e.g., Dechloromonas) (Fig. 10). Beta-diversity (i.e., principal coordinates analysis) was conducted based on Bray-Curtis distances, and points are colored to present dominant factors associated with community composition: (i) water source status and (ii) location and source combination. We observed an effect of both water source and location in the plot on the right. For example, among well samples, WD has a distinct community, while WA and WB are much closer in community composition. There was substantial overlap in composition among pond samples from all four locations (Fig. 11). Relative abundance of dominant taxa of sampled irrigation water varied among different water types and locations (Fig. 12).

Based on the taxonomic database, correlation analysis and Mann-Whitney test were conducted to identify potential indicators for *Salmonella* and *L. monocytogenes* contamination in irrigation water. Due to the different distribution and occurrence of the tested foodborne pathogens, it was not beyond our expectation that different bacterial taxa were correlated with these two foodborne pathogens in sampled irrigation water. Abundance of the Bacteroides class is associated with *Salmonella* positive status ($p = 0.003$), while the Spirochetes class ($p = 0.007$) and the Rhodocyclaceae family ($p < 0.001$) are negatively correlated with *L. monocytogenes* prevalence (Fig. 13). Abundance of the Sphingobacteria class is associated with *L. monocytogenes* positive status ($p = 0.027$), while the Gammaproteobacteria class ($p = 0.014$) and Acinetobacter genus ($p = 0.002$) are negatively correlated with *L. monocytogenes* prevalence (Fig. 14). Based on the data, bacterial species of the Bacteroides and Sphingobacteria classes could be further tested as alternative/suitable indicator microorganisms for *Salmonella* and *L. monocytogenes*, respectively. The bacteria with a negative correlation with the tested foodborne pathogens could be potential biocontrol agents to mitigate contamination risks. Deeper sequencing and further computational analysis are underway to identify certain microbial communities/patterns that are associated with both foodborne pathogens for risk evaluation.

OBJECTIVE 2. Evaluate the efficacy of four commercial sanitizers on the decontamination of three common foodborne pathogens, *Salmonella* spp., *E. coli* O157:H7, and *Listeria monocytogenes*, in irrigation well and pond water.

Methods:

Water sample collection. During the growth season of 2015 (May to October), 10-L well and pond water samples were collected from the same four vegetable farms on ESV. In total, 24 water samples were collected for the experiments under objective 2.

Preparation of *Salmonella* Newport, *E. coli* O157:H7 and *L. monocytogenes*. *S. enterica* serovar Newport strain J1892, *E. coli* O157:H7 strain Ec06 and *L. monocytogenes* strain 0104, isolated from previous outbreaks, were originally obtained from the Centers for Disease Control and Prevention (CDC) in Atlanta, GA. Bacterial cultures were stored in Luria-Bertani (LB) broth containing 20% glycerol at −80°C. Before each experiment, a loopful of the stored bacterial culture was added to shake cultures of LB broth, and grown for about 20 h at 37°C. The culture was harvested by centrifugation at 4,000 rpm for 15 min at 22°C. Pre-inoculum solution of target pathogens with concentration of 8 log CFU/mL was prepared by suspending bacterial pellets with irrigation well or pond water to an optical density (450 nm) of 0.3. Ten ml of 8 log pre-inoculum was then added in 250-mL glass flasks with 100 mL irrigation well or pond water to reach initial bacterial concentration of 7 log CFU/ml. A 100-fold serial dilution was conducted to prepare bacterial solutions at 5 log and 3 log CFU/mL levels by transferring 1 mL bacterial solution into 99 mL irrigation water.

Sanitizer treatments of irrigation water. Disinfectants XY-12 (Ecolab Inc., St. Paul, MN**),** CDG Solution 3000 (CDG Environmental, LLC, Bethlehem, PA), Sanidate 5.0 (BioSafe Systems, LLC, East Hartford, CT), and Sanidate 12.0 (BioSafe Systems) were tested in this study (Table 5). XY-12 is labeled for water chlorination with the active ingredient of sodium hypochlorite. It was reported that electrolyzed oxidizing water prepared by adding sodium hypochlorite could significantly increase water ORP value and inactivate *E. coli* O157:H7 strains (Kim et al., 2000). However, this product is not labeled by EPA for use in irrigation water. CDG Solution, Sanidate 5.0, and Sanidate 12.0 are approved by EPA for agricultural water and water system treatments (EPA, 2011; EPA, 2013a; EPA, 2013b).

For each treatment investigated, a 250-mL glass flask containing a magnetic stir bar and 100 mL target bacterial solution with different initial concentrations as mentioned above were placed on an active stir-plate at room temperature (~ 25°C). Each sanitizer was applied at the maximum label concentration rate for irrigation water treatment (Table 1). Two application doses of Sanidate 5.0 and 12.0 were tested. High concentration was suggested for contaminated water treatment, and low concentration for clean water maintenance. Sterilized tap water was used as a control. Each treatment was conducted in triplicate.

After 1-min or 30-min contact times, a 1-mL suspension of each sample was added to 9 mL of sodium thiosulphate 5-hydrate (0.5 %, w/v; Fisher Scientific International) for neutralization (Abadias et al., 2011). The aliquots were diluted serially by 10-fold in 0.1 M phosphate-buffered saline solution. Three 100-µL aliquots of each sample at each dilution level were plated onto XLT4 agar (for *Salmonella* Newport detection), Chromagar *E.coli* O157 (for *E. coli* O157:H7 detection) or LMPM medium (for *L. monocytogenes* detection) using the Eddy Jet 2 spiral plater (IUL Instruments, Barcelona, Spain) at mode c100, and plates were incubated at 40°C for 24 h. Positive colonies on each plate were counted by Flash & Grow colony counter (IUL Instruments). Also, an enrichment step was carried out by following the pathogen detection methods described above for objective 1. In brief, 1 mL of each neutralized solution was added to 9 mL corresponding enrichment broth (tryptic soy broth for *E. coli* O157:H7 detection) and then streaked onto selective plates after incubation for the detection of each targeted pathogen. Fecal indicator, water parameter, and bacterial community analyses of water samples were performed as above.

Available active ingredient measurement. Active ingredients of the sanitizers in irrigation water were tested for each treatment, including the control. Free and total chlorine, chlorine dioxide, and hydrogen peroxide were tested by Hach Pocket, Colorimeter II AccuVac Ampuls, and HYP-1 test kits, respectively (Hach, Loveland, CO). Peroxyacetic acid was measured by MP-9700 test kit (Masters Company, Inc., Wood Dale, IL).

Results:

Efficacy of sanitizers on foodborne pathogen deduction. *Salmonella* concentration was significantly decreased after 1-min application of all sanitizers compared with the control at all three inoculation levels of 7, 5, and 3 log CFU/ml (Fig. 15). However, the efficacy of low dose Sanidate 5.0 and 12.0 applications was significantly lower compared with most of other treatments after the short contact time. There were no significant differences between pond and well water samples for *Salmonella* levels after each treatment. But it is notable that the pathogen was not detectable after CDG treatments in well water samples at all three inoculation levels, whereas corresponding pond water samples were still *Salmonella*-positive after enrichment. Similar results were observed for XY application at 5 and 3 log inoculation levels and high dose Sanidate 5.0 application at 7 and 5 log inoculation levels. *Salmonella* was not detectable after 30-min contact time for most treatments, except CDG application at an initial *Salmonella* concentration of 7 log CFU/ml in pond water (Fig. 16).

L. monocytogenes and *E. coli* O157:H7 were relatively more tolerant to the tested sanitizers (Figs. 17–20). After 1-min contact time, *L. monocytogenes* was detectable under most treatments (Fig. 17). *L. monocytogenes* concentration in both pond and well water samples under low dose Sanidate 5.0 and 12.0 applications was significantly reduced compared with the control. After 30-min contact time, all treatments efficiently reduced the pathogen populations in both pond and well water samples (Fig. 18). *L. monocytogenes* was not detectable in all treated water samples with initial concentration of 3 log CFU/ml.

After 1-min treatment, *E. coli* O157:H7 was still detectable under all treatments (Fig. 19). *E. coli* O157:H7 level was not significantly reduced under low dose Sanidate 5.0 and 12.0 applications at 5 and 3 log inoculation levels compared with the control, except well water with Sanidate 12.0 treatment at the 3 log inoculation level. Similar to *Salmonella* and *L. monocytogenes* deduction tests, after 30-min contact time, all treatments efficiently reduced *E. coli* O157:H7 populations in both pond and well water samples (Fig. 20). The pathogen was still detectable after enrichment in pond water samples applied with sodium hypochlorite (XY) at all 3 inoculation levels, while well water samples with the same treatment were *Salmonella* negative. Similar results were observed for S 5.0-HD, S 12.0, and S 12.0-HD at 7 log inoculation level, S 5.0, S 5.0-HD, S 12.0, and S 12.0-HD at 5 log inoculation level, and CDG at 3 log inoculation level. Considering the low population densities of foodborne pathogens in the irrigation water of this region (less than 2 log MPN/L), all tested sanitizers are qualified for irrigation water decontamination.

pH changes after disinfectant treatments. Sodium hypochlorite (NaClO) and chlorine dioxide (ClO2) are the main active ingredient of XY and CDG, respectively, and can produce hydroxide (OH^-) after dissolving in water. In contrast, peroxyacetic acid could reduce water pH. As expected, water pH values were generally increased after treatment with NaClO (XY) and ClO2 (CDG), and decreased after treatment with hydrogen peroxide and PAA (Sanidate 5.0 and 12.0) treatment (Tables 6, 10, 14). Most Sanidate 5.0 and 12.0 treatments, especially at the high dose, resulted in significant pH deduction compared with the control. Irrigation water pH value in the fields was associated with plant growth and pesticide applications. Most nutrients for plant growth are plant-available in the soil solution when the pH range is 6.0 to 7.5. In this study, pH values of collected water samples were above 7. Since pH deduction values after application of tested sanitizers were all less than 1, the pH of treated irrigation water was still in the proper range for plant growth.

Active ingredient residual percentage. The residual percentage of active ingredients of tested sanitizers varied after 30-min treatment (Tables 7, 11, 15). The depletion of free chlorine and chlorine dioxide in XY and CDG were significantly higher than PAA in the Sanidate products. Most of the active ingredient residual percentage values were not significantly different between pond and well water samples. The significantly higher PAA residual level in well water with S 5.0-HD treatment at 5 log inoculation level (Table 15) was consistent with the detection difference of *E. coli* O157:H7 in respective water samples (Fig. 20).

Impact of water parameters. Water turbidity was the only tested parameter significantly lower in well water compared with pond water samples in all foodborne pathogen sanitization experiments (Tables 8, 12, 16). The higher turbidity in pond water may be associated with the detection difference of tested foodborne pathogens between certain pond and water samples under the same sanitizer treatment as mentioned above.

Association with fecal indicators. The concentration of coliforms and generic *E. coli* were significantly higher in pond water than in sampled well water (Tables 9, 13, 17). However, the difference did not affect the general effects of tested sanitizers on the decontamination of foodborne pathogens. There were no statistically significant differences for the deduction of foodborne pathogens between pond and well waters for most sanitizer treatments. However, as mentioned above, the tested foodborne pathogens were still detectable after enrichment in certain pond water samples, while the corresponding well water samples under the same treatments were negative for target pathogens. Coliforms and generic *E. coli* in tested water samples were completely eliminated after all sanitizer treatments.

OBJECTIVE 3. Provide education and training to stakeholders on improved agricultural practices to reduce the food safety risks in irrigation water.

At the beginning of the project, a survey was conducted by Drs. Strawn and Rideout at regional extension meetings. Survey results (n=256) showed an average response of “fair” for FSMA’s Produce Safety and Preventative Control Rule awareness. While many stakeholders are mildly familiar with FSMA, the

average response of "I don't know" was selected in response to whether "a grower's operation would be covered under the FSMA Produce Safety Rule." Additionally, 91% of growers who responded to the survey felt they were most concerned or worried about the standards for agricultural water. Many growers requested a need for specialized training for only agricultural water standards, specifically for untreated surface water applications. As shown in the following publication and presentation section, results derived from this project about foodborne pathogen contamination in water samples and practical methods on irrigation water decontamination have been reported to stakeholders during field day and multiple extension meetings. Final results will also be reported at the CPS 2016 Symposium and IAFP annual meetings.

Outcomes and Accomplishments

According to CDC reports, at least three outbreaks of salmonellosis associated with contaminated tomatoes have been traced back to ESV from 2002 to 2010. *Salmonella* contamination on tomatoes also raised questions about the safety of the water used for irrigating these products in this region. Producers are frequently asked by retailers to certify product quality. In this study, we detected the dynamics of *Salmonella* population and distribution in ESV irrigation ponds and wells. The results provide growers with information that is necessary for them to determine if a contamination problem exists. Correlations between foodborne pathogens (*Salmonella* spp. and *Listeria monocytogenes*) and contextual factors, including water parameters, weather information, and fecal indicators, were analyzed. Bacterial community analysis through metagenomics study provides meaningful clues to identify suitable indicators and potential suppressors of foodborne pathogens in irrigation water. This information is vital as the FDA is going to mandate uniform regulations for food safety, inclusive of irrigation water standards. In addition, the efficacy of commercial sanitizers with different active ingredients used for irrigation water treatment was evaluated. Relevant results derived from this study have been shared with vegetable growers and other stakeholders through extension talks, field days, and scientific meetings. The outcomes of this research will benefit stakeholders, especially vegetable and fruit industries, to reduce the contamination risks of foodborne pathogens during irrigation and achieve the requirements of FSMA on produce safety. With education about food safety and good agricultural practices on produce production, no outbreaks of salmonellosis have been linked to the tomatoes produced in Virginia in recent years.

Summary of Findings and Recommendations

This study found that *Salmonella* populations of selected samples per month and the average MPN values for each month were not correlated ($p > 0.1$). Weekly differences of *Salmonella* occurrence and populations in water samples among the same detection month indicated that intensive sampling would be necessary to identify the accurate spatial and temporal pattern of the foodborne pathogen in irrigation water. In addition, the low probability and concentration of *Salmonella* in collected water samples indicate that large sample amount or additional replicates (>3) would be important to find out the real prevalence in environmental samples.

Distribution and diversity of *Salmonella* serovars varies among different types of water samples and in two sampling years, i.e., the dominant *Salmonella* serovar in water samples collected in 2014 was Newport (data for 2015 will be provided at the CPS 2016 Symposium). Further genotypic analyses using whole genomic sequencing of selected *Salmonella* strains isolated from this study will be conducted and the data will be compared with identified or sequenced clinical and environmental strains from previous studies. In addition, survival comparison study and genetic analysis will be performed to compare different strains isolated from this agricultural area, like strains of serovars Newport and Typhimurium, to identify specific bacterial properties and/or mechanisms that contribute to colonization and survival variances of *Salmonella* in different environments.

The low correlation coefficient between fecal indicators, including generic *E. coli*, and foodborne pathogens in irrigation water suggests further efforts to identify more suitable indicator microorganisms

or other biological markers. In addition, the prevalence and population difference of different foodborne pathogens (*Salmonella* spp. and *L. monocytogenes* in this study) in the same water samples as well as the high variance among different farms (location) bring challenges to just using one universal indicator or standard to evaluate contamination risks in agricultural water.

Microbial community analysis through metagenomics provides clues to identify suitable alternative indicators and/or potential suppressors of foodborne pathogens in agricultural water for produce production. Additional searches for *Listeria* and *Salmonella enterica* were performed using the high-resolution Resphera Insight protocol; however, additional sample data are required to provide sufficient frequency in the dataset to provide a complete confident positive call. Due to budget and time limitations, only monthly water samples tested for both foodborne pathogens were selected for metagenomic analysis. Further 16s amplicon sequencing of all weekly samples (392) and subsequent computational analysis are needed to achieve a final definite conclusion. In addition, metagenomic analyses to identify eukaryotes and metabolites in irrigation water samples are underway to investigate their relationship with foodborne pathogens and to evaluate their potentials as suitable predictors. Further data analysis will be performed by principal component analysis, partial least square, stepwise and canonical discriminant analyses to identify variables and OTUs or a certain group (pattern) of variables that contributed most to the classification (pathogen presence/absence or different levels of *Salmonella* and *Listeria monocytogenes* population). Additional results will be reported at the CPS 2016 Symposium.

Results derived from this study provide information for local farmers and other stakeholders to recognize the contamination risks of agricultural production with irrigation in the major horticultural area of Virginia. The prevalence, population and diversity of *Salmonella* strains in major produce production regions, like VA, CA, FL, GA, NY etc., need to be compared to analyze the variance of foodborne pathogens for future outbreak prediction and mitigation.

Irrigation water was hypothesized to be one of the main contamination sources of *Salmonella* within production fields. Suitable decontamination methods for large irrigation water systems to control foodborne pathogens became an urgent request from vegetable and fruit farmers. The instant efficacy of hydrogen peroxide and PAA (Sanidate 5.0 and 12.0) at low application levels on the disinfection of foodborne pathogens in irrigation water is relatively lower compared with sodium hypochlorite (XY-12) and chlorine dioxide (CDG300). For 1-min contact time, the population of tested foodborne pathogens treated with hydrogen peroxide and PAA was significantly higher than water samples treated with sodium hypochlorite and chlorine dioxide. However, the residual concentration of active ingredient of PAA was significantly higher than that of sodium hypochlorite and chlorine dioxide after 30-min contact time, which could prevent subsequent contaminations. Further studies can be performed by inoculating additional foodborne pathogens into treated water samples after 30-min contact time to evaluate the prevention efficacy. So for instantaneous treatment, use of sodium hypochlorite and chlorine dioxide products would be recommended. However, constant detection of active ingredients and retreatment with these products would be necessary to maintain pathogen-free agricultural waters.

In conclusion, considering the low population densities of foodborne pathogens in the irrigation water of this ESV region as detected in objective 1 (less than 2 log MPN/L), all test sanitizers would be qualified for irrigation water decontamination. Further studies would be necessary to investigate the effects of various disinfectants and the application in large irrigation systems in vegetable and fruit farms for food safety management.

FDA collaboration: We appreciate the support from Dr. Andrea Ottesen and Padmini Ramachandran for metagenomics experiments and data analysis. We would also like to thank Dr. Jie Zheng and Elizabeth Reed for their assistance in molecular serotyping and antimicrobial susceptibility tests of *Salmonella* isolates collected from this study.

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APPENDICES

Publications and Presentations

Meeting abstracts

di Stefano, J., E. Brown, M. Duong, L. Yang, R. Boyer, G. Gu, S. Rideout. 2016. Antimicrobial Resistance of *Salmonella enterica* Environmental Isolates from the Eastern Shore of Virginia. International Association of Food Protection (IAFP) annual meeting abstracts (submitted).

Gu, G., A. Ottesen, J. Zheng, D. Oryang, R. Boyer, L. Strawn, and S. L. Rideout. 2016. Diversity and dynamics of *Salmonella enterica* spp. in irrigation water and poultry litter amended fields on the eastern shore of Virginia. IAFP annual meeting abstracts (submitted).

Gu, G., J. Zheng, M. Reiter, L. Strawn, and S. L. Rideout. 2015. Prevalence and survival of *Salmonella enterica* spp. in irrigation water, poultry litter and amended soils on the Eastern Shore of Virginia. IAFP annual meeting abstracts 104: T7-12.

Allard, S., G. Gu, E. Reed, S. Rideout, J. R. White, S. Micallef, A. Ottesen and S. Gorham. 2015. Bacteria associated with tomato microbiomes: Ripe vs. Unripe. American Society of Microbiology general meeting abstracts 104: P1-187.

Presentations

Strawn, LK, G. Gu, and S. L. Rideout. Research Efforts on the Eastern Shore of Virginia involving *Salmonella*. Delmarva Food Safety Task Force. Jan 2016.

Strawn, LK, G Johnson, D Martin, K Kniel, S Rideout, C Walsh. “Best Management Practices for Del-mar-va Growers”. 1st Delmarva Food Safety Task Force Annual Meeting. Melfa, VA. 09 Dec 2015.

Strawn, LK, R Pfuntner, L Truitt, G Gu, S Rideout. “*Salmonella* Research Targeted for Del-mar-va Growers”. Delaware State Agriculture Week. Harrington, Delaware, USA. 12 Jan 2016.

Strawn, LK, R Williams, and S. L. Rideout. “Sanitizer Use Part 1 & 2”. Produce Packinghouse Workshop. Dayton, Virginia, USA. 12 Nov 2015.

Strawn, LK, and S. L. Rideout. “Good Agricultural Practices: Pre- and Postharvest Water”. Wegmans/VCE sponsored Good Agricultural Practices Training. Spotsylvania, VA, USA. 27 Feb 2015.

Gu, G., and S. Rideout. Parameterizing QPRAM with data from *Salmonella enterica* contamination of Tomatoes. Food safety project webinar. 26 October 2015.

Gu, G., and S. Rideout. Tomato, irrigation and fertilization studies for food safety research update. Eastern Shore AREC's Annual Field Day, Painter, VA. 11 August 2015.

Gu, G., and S. Rideout. Prevalence and survival of *Salmonella enterica* spp. in irrigation water, poultry litter and amended soils on the Eastern Shore of Virginia. The 26th Eastern Shore Ag Conference and Trade Show. 11 February 2015.

Publications

We anticipate submission of two papers on indicator investigation for *Salmonella* spp. and *Listeria monocytogenes* in irrigation water, and one paper on sanitizer evaluation for irrigation water treatment.

Budget Summary

Funds utilized:

Virginia Tech:	
Personnel (Salaries and Fringe benefits):	$26,875
Supplies:	$120,270
Travel:	$1,592
Indirect Costs:	$1,043
Total Expenses:	$149,780

All funds awarded except travel and publication funding in year 2 have been used for this project.

Tables and Figures

Table 1. Occurrence and MPN values of *Salmonella* spp. in irrigation well water.

	Sampling date	MPN/L	Upper limit*	Lower limit
Well A	3/23/2015	3.4	9.3	1.3
Well B				
Well C	6/1/2015	0.42	3.2	0.0055
Well D	9/21/2015	1.5	4.9	0.43
	9/28/2015	0.46	3.2	0.065
	12/21/2015	3.4	9.3	1.3

Table 2. Occurrence and MPN values of *Listeria monocytogenes* in irrigation well water.

	Sampling date	MPN/L	Upper limit*	Lower limit
Well D	4/20/2015	24	8	72
	10/5/2015	0.46	3.2	0.065

Table 3. Correlation coefficients between environmental factors and the population of *Salmonella spp.* isolated from irrigation ponds.

Water parameters	**Se MPN correlation coefficient**	**P value**
Temperature	0.028619863	0.690586
Conductivity	-0.022679506	0.753205
Dissolved Oxygen percentage	-0.015714816	0.827112
Dissolved Oxygen charge	0.05324747	0.458622
pH	-0.030228895	0.674346
pHmV	0.001772284	0.980357
Oxidation Reduction Potential	-0.024194071	0.737406
Turbidity	0.178114753	**0.012504**
Temperature	0.057310855	0.424949
Rainfall	-0.037480388	0.602762
Temperature-1	0.058021483	0.419226
Rainfall-1	-0.07548581	0.29356
Coliform	0.035183962	0.624473
g*E. coli*	-0.023775576	0.741608

Table 4. Correlation coefficients between environmental factors and the prevalence and population of *Salmonella spp.* isolated from irrigation wells.

Water parameters	**Se MPN correlation coefficient**	**P value**
Temperature	-0.016725192	0.816289
Conductivity	0.027387653	0.703248
Dissolved Oxygen percentage	-0.001896953	0.980024
Dissolved Oxygen charge	-0.02925719	0.684543
pH	-0.058643371	0.414584
pHmV	0.044567272	0.535154
Oxidation Reduction Potential	0.075559036	0.292598
Turbidity	-0.005999806	0.934589
Temperature	-0.06090302	0.396475
Rainfall	0.031963391	0.656543
Temperature-1	-0.036002162	0.616414
Rainfall-1	0.126490946	0.077289
Coliform	0.207758907	**0.00348**
g*E. coli*	0.081067518	0.258712

Table 5. List of sanitizers and active ingredients.

Trade Name	**Active Ingredients**	**Concentration (mg/L)**	**Application Dose (µL/100 mL)**
Ecolab XY-12 (XY)	8.4 % sodium hypochlorite	4*	4.8
CDG Solution 3000™ (CDG)	0.3% chlorine dioxide	5	167
Sanidate 5.0 (S 5.0)	23.0 % hydrogen peroxide/ 5.3 % peroxyacetic acid	10/2.4	4.5
Sanidate 5.0 High Dose (S 5.0-HD)	23.0 % hydrogen peroxide/ 5.3 % peroxyacetic acid	84/20	36.4
Sanidate 12.0 (S 12.0)	18.5 % hydrogen peroxide/ 12.0 % peroxyacetic acid	4.6/3	2.5
Sanidate 12.0 High Dose (S 12.0-HD)	18.5 % hydrogen peroxide/ 12.0 % peroxyacetic acid	14/9	7.7
Control (CK)	Sterilized tap water	/	/

*, the maximum residual disinfectant level under the Safe Drinking Water Act (http://www.epa.gov/laws-regulations/summary-safe-drinking-water-act).

Table 6. Water pH values and changes after sanitizer treatment in *Salmonella* deduction experiment.

Initial concentration	**Sanitizer**	**Pond (mean ± sd)**	**Well (mean ± sd)**
7 log	CK	**7.25 ± 0.036**	**7.323 ± 0.026**
	XY	0.01* ± 0.009	0.025 ± .013
	CDG	0.018 ± 0.024	0.018 ± 0.29
	S 5.0	-0.19 ± 0.028 Δ	-0.288 ± .041 Δ
	S 5.0-HD	-0.548 ± 0.030 Δ	-0.665 ± .041 Δ
	S 12.0	-0.233 ± 0.030 Δ	-0.335 ± .031 Δ
	S12.0-HD	-0.313 ± 0.020 Δ	-0.428 ± .027 Δ
5log	CK	**7.088 ± 0.030**	**7.155 ± 0.034**
	XY	0.118 ± 0.027	0.095 ± 0.018 Δ
	CDG	0.168 ± 0.036	0.103 ± 0.015 Δ
	S 5.0	-0.04 ± 0.009	-0.055 ± 0.010 Δ
	S 5.0-HD	-0.405 ± 0.039 Δ	-0.358 ± 0.044 Δ
	S 12.0	-0.228 ± 0.040 Δ	-0.198 ± 0.022 Δ
	S12.0-HD	-0.328 ± 0.048 Δ	-0.3 ± 0.046 Δ
3 log	CK	**7.1 ± 0.054**	**7.105 ± 0.044**
	XY	0.085 ± 0.021	0.03 ± 0.012
	CDG	0.06 ± 0.020	0.09 ± 0.017
	S 5.0	-0.048 ± 0.026	-0.078 ± 0.035
	S 5.0-HD	-0.443 ± 0.053 Δ	-0.415 ± 0.037 Δ
	S 12.0	-0.313 ± 0.050 Δ	-0.28 ± 0.032 Δ
	S12.0-HD	-0.358 ± 0.059 Δ	-0.333 ± 0.040 Δ

*, water pH changes compared to control.

Δ, significant differences (p = 0.05) between treatment and control.

Table 7. Active ingredient residual percentages of sanitizers after treatment in *Salmonella* deduction experiment.

Initial concentration	**Sanitizer**	**Pond (%)**	**Well (%)**
7 log	XY	3.875 ± 3.217 a	7.063 ± 4.839 a
	CDG	8.15 ± 5.889 a	18.05 ± 6.779 a
	S 5.0	47.396 ± 18.559 c	69.896 ± 16.755 bc
	S 5.0-HD	59.101 ± 8.232 c	55.830 ± 10.141 c
	S 12.0	46.268 ± 16.044 bc	79.685 ± 19.399 c
	S12.0-HD	64.607 ± 14.749 c	65.933 ± 17.393 c
5 log	XY	8.563 ± 6.691 a	17.5 ± 9.017 a
	CDG	12.95 ± 10.167 a	36.05 ± 10.011 ab
	S 5.0	68.75 ± 22.750 bc	82.917 ± 15.435 c
	S 5.0-HD	70.733 ± 15.139 bc	71.638 ± 7.430 c
	S 12.0	87.471 ± 7.446 c	78.870 ± 13.054 c
	S12.0-HD	86.651 ± 1.633 c	81.603 ± 13.939 c
3 log	XY	15.25 ± 7.887 a	32.333 ± 10.804 a
	CDG	15.8 ± 7.940 a	30.533 ± 10.110 a
	S 5.0	77.917 ± 18.430 b	92.083 ± 1.917 b
	S 5.0-HD	69.5 ± 15.313 b	67.433 ± 12.597 b
	S 12.0	77.889 ± 16.547 b	94 ± 0.490 b
	S12.0-HD	81.074 ± 5.897 b	85.704 ± 4.636 b

Different lowercase letters indicate significant differences (p = 0.05) between treatments within each of the experiments.

Table 8. Water parameters of sampled irrigation ponds and wells in *Salmonella* deduction test.

	Pond	**Well**
Temperature	25.069 ± 1.832 a	22.503 ± 2.031 b
Conductivity	0.212 ± 0.026 a	0.237 ± 0.066 a
Dissolved Oxygen percentage	107.225 ± 25.449 a	77.331 ± 20.049 b
Dissolved Oxygen charge	47.469 ± 15.627 a	44.2 ± 13.553 a
pH	7.309 ± 0.215 a	7.235 ± 0.111 a
pHmV	-70.796 ± 12.019 a	-66.076 ± 6.223 a
Oxidation Reduction Potential	81.638 ± 16.087 a	45.263 ± 29.337 b
Turbidity	8.248 ± 5.156 a	2.329 ± 1.581 b

Different lowercase letters indicate significant differences (p = 0.05) between Pond and Well water samples.

Table 9. Population of fecal indicators in sampled irrigation pond and well water in *Salmonella* deduction test.

MPN/100 ml	**Pond**	**Well**
Coliform	2259.65 ± 130.431 a	22.503 ± 2.031 b
Generic *E. coli*	218.356 ± 293.211 a	0.237 ± 0.066 b

Different lowercase letters indicate significant differences (p = 0.05) between Pond and Well water samples.

Table 10. Water pH values and changes after sanitizer treatment in *Listeria monocytogenes* deduction experiment.

Initial concentration	Sanitizer	Pond (mean ± sd)	Well (mean ± sd)
7 log	CK	**7.76** ± 0.053	**7.53** ± 0.036
	XY	0.005* ± 0.043	0.06 ± 0.031
	CDG	0.028 ± 0.028	0.04 ± 0.026
	S 5.0	-0.23 ± 0.073	-0.133 ± 0.069
	S 5.0-HD	-0.858 ± 0.036 Δ	-0.443 ± 0.104
	S 12.0	-0.653 ± 0.025 Δ	-0.368 ± 0.073 Δ
	S12.0-HD	-0.64 ± 0.041 Δ	-0.408 ± 0.076 Δ
5log	CK	**7.223** ± 0.073	**7.165** ± 0.050
	XY	0.175 ± 0.042	0.225 ± 0.040 Δ
	CDG	0.183 ± 0.037 Δ	0.135 ± 0.012 Δ
	S 5.0	-0.028 ± 0.006 Δ	-0.013 ± 0.022
	S 5.0-HD	-0.433 ± 0.019 Δ	-0.313 ± 0.053 Δ
	S 12.0	-0.258 ± 0.038 Δ	-0.1 ± 0.021 Δ
	S12.0-HD	-0.233 ± 0.032 Δ	-0.13 ± 0.022 Δ
3 log	CK	**7.213** ± 0.075	**7.175** ± 0.074
	XY	0.178 ± 0.044	0.173 ± 0.040
	CDG	0.13 ± 0.026 Δ	0.205 ± 0.024 Δ
	S 5.0	-0.01 ± 0.007	-0.018 ± 0.004 Δ
	S 5.0-HD	-0.39 ± 0.046 Δ	-0.34 ± 0.055 Δ
	S 12.0	-0.238 ± 0.039 Δ	-0.178 ± 0.034 Δ
	S12.0-HD	-0.195 ± 0.034 Δ	-0.175 ± 0.028 Δ

*, water pH changes compared to control.

Δ, significant differences (p = 0.05) between treatment and control.

Table 11. Active ingredient residual percentages of sanitizers after treatment in *Listeria Monocytogenes* deduction experiment.

Initial concentration	**Sanitizer**	**Pond (%)**	**Well (%)**
7 log	XY	3.125 ± 1.277 a	14.438 ± 7.483 a
	CDG	1.6 ± 0.548 a	4.45 ± 1.576 a
	S 5.0	47.708 ± 14.212 b	61.979 ± 7.363 bc
	S 5.0-HD	59.413 ± 6.559 bc	48.713 ± 5.977 b
	S 12.0	65.667 ± 13.374 bc	74.083 ± 14.290 bc
	S12.0-HD	78.944 ± 4.872 c	65.944 ± 19.369 bc
5 log	XY	5.375 ± 2.982 b	24.625 ± 9.986 b
	CDG	2.2 ± 1.219 b	**20.7 ± 6.402 b**
	S 5.0	86.667 ± 29.001 c	88.125 ± 11.162 c
	S 5.0-HD	69.65 ± 2.164 c	66.325 ± 17.607 c
	S 12.0	75.583 ± 16.674 c	81.667 ± 9.205 c
	S12.0-HD	74.333 ± 5.062 c	75.389 ± 4.715 c
3 log	XY	6.938 ± 3.930 a	25.188 ± 5.968 a
	CDG	2.7 ± 0.614 a	8.05 ± 2.391 a
	S 5.0	64.688 ± 13.551 b	78.229 ± 7.530 bc
	S 5.0-HD	64.775 ± 3.156 b	70.6 ± 3.978 bc
	S 12.0	77.083 ± 17.018 bc	92.583 ± 14.428 c
	S12.0-HD	82.694 ± 7.329 bc	85.194 ± 9.391 bc

Different lowercase letters indicate significant differences (p = 0.05) between treatments within each of the experiments. Bold number indicates significant difference (p = 0.05) between Pond and Well water samples.

Table 12. Water parameters of sampled irrigation ponds and wells in *Listeria monocytogenes* deduction test.

	Pond	**Well**
Temperature	28.877 ± 1.029 a	24.983 ± 2.180 b
Conductivity	0.251 ± 0.233 a	0.182 ± 0.080 a
Dissolved Oxygen percentage	28.094 ± 99.529 a	13.244 ± 76.302 a
Dissolved Oxygen charge	55.813 ± 32.259 a	58.494 ± 30.348 a
pH	7.525 ± 0.191 a	7.354 ± 0.164 a
pHmV	-71.044 ± 34.160 a	-73.288 ± 9.189 a
Oxidation Reduction Potential	50.056 ± 19.636 a	39.994 ± 46.179 a
Turbidity	29.161 ± 22.139 a	4.519 ± 4.360 b

Different lowercase letters indicate significant differences (p = 0.05) between Pond and Well water samples.

Table 13. Population of fecal indicators in sampled irrigation pond and well water in *Listeria monocytogenes* deduction test.

MPN/100 ml	**Pond**	**Well**
Coliform	2241.294 ± 207.510 a	24.983 ± 2.180 b
Generic *E. coli*	190.581 ± 201.319 a	0.182 ± 0.080 b

Different lowercase letters indicate significant differences (p = 0.05) between Pond and Well water samples.

Table 14. Water pH values and changes after sanitizer treatment in *Escherichia coli* O157:H7 deduction experiment.

Initial concentration	Sanitizer	Pond (mean ± sd)	Well (mean ± sd)
7 log	CK	**7.643** ± 0.035	**7.64** ± 0.053
	XY	0.07 ± 0.018	0.098 ± 0.020 Δ
	CDG	0.085 ± 0.022	0.09 ± 0.030
	S 5.0	-0.04 ± 0.010	-0.09 ± 0.018 Δ
	S 5.0-HD	-0.645 ± 0.079 Δ	-0.513 ± 0.049 Δ
	S 12.0	-0.258 ± 0.045 Δ	-0.193 ± 0.040 Δ
	S12.0-HD	-0.34 ± 0.029 Δ	-0.32 ± 0.056 Δ
5log	CK	**7.568** ± 0.111	**7.455** ± 0.056
	XY	0.068 ± 0.014 Δ	0.08 ± 0.017 Δ
	CDG	0.03 ± 0.005 Δ	0.063 ± 0.016
	S 5.0	-0.185 ± 0.021 Δ	-0.21 ± 0.036 Δ
	S 5.0-HD	-0.96 ± 0.113 Δ	-0.643 ± 0.039 Δ
	S 12.0	-0.478 ± 0.035 Δ	-0.293 ± 0.039 Δ
	S12.0-HD	-0.538 ± 0.016 Δ	-0.395 ± 0.045 Δ
3 log	CK	**7.613** ± 0.023	**7.488** ± 0.053
	XY	0.028 ± 0.009	0.073 ± 0.013
	CDG	0.04 ± 0.016	0.06 ± 0.029
	S 5.0	-0.323 ± 0.056 Δ	-0.158 ± 0.032 Δ
	S 5.0-HD	-0.98 ± 0.117 Δ	-0.695 ± 0.116 Δ
	S 12.0	-0.31 ± 0.046 Δ	-0.198 ± 0.029 Δ
	S12.0-HD	-0.465 ± 0.026 Δ	-0.318 ± 0.046 Δ

*, water pH changes compared to control.

Δ, significant differences (p = 0.05) between treatment and control.

Table 15. Active ingredient residual percentages of sanitizers after treatment in *Escherichia coli* O157:H7 deduction experiment.

Initial concentration	**Sanitizer**	**Pond (%)**	**Well (%)**
7 log	XY	11.625 ± 3.618 a	9.375 ± 4.140 a
	CDG	3.65 ± 1.063 a	5.8 ± 1.747 a
	S 5.0	55.625 ± 9.589 bcd	44.479 ± 11.331 bc
	S 5.0-HD	39 ± 4.417 b	47.738 ± 6.734 bc
	S 12.0	70.167 ± 9.567 d	58.833 ± 9.723 cd
	S12.0-HD	68.583 ± 3.234 d	69.222 ± 2.762 d
5log	XY	8.5 ± 3.501 a	16.813 ± 8.286 ab
	CDG	6.15 ± 2.707 a	7.65 ± 3.902 a
	S 5.0	75.104 ± 24.106 cd	97.396 ± 31.448 d
	S 5.0-HD	53.563 ± 2.743 bc	**66.025 ± 1.087 cd**
	S 12.0	72.583 ± 19.746 cd	67.917 ± 9.263 cd
	S12.0-HD	74.639 ± 5.412 cd	72.417 ± 4.321 cd
3 log	XY	8 ± 3.866 a	17.688 ± 5.179 a
	CDG	5.7 ± 1.310 a	8.05 ± 3.540 a
	S 5.0	55.833 ± 15.778 b	57.083 ± 10.789 b
	S 5.0-HD	68.15 ± 3.114 b	62.45 ± 4.175 b
	S 12.0	55.417 ± 10.058 b	62.083 ± 7.132 b
	S12.0-HD	71.556 ± 7.057 b	64.278 ± 7.576 b

Different lowercase letters indicate significant differences (p = 0.05) between treatments within each of the experiments. Bold number indicates significant difference (p = 0.05) between Pond and Well water samples.

Table 16. Water parameters of sampled irrigation ponds and wells in *Escherichia coli* O157:H7 deduction test.

	Pond	**Well**
Temperature	20.043 ± 2.883 a	19.489 ± 1.982 a
Conductivity	8.088 ± 15.366 a	0.239 ± 0.069 a
Dissolved Oxygen percentage	101.988 ± 23.261 a	87.194 ± 23.019 a
Dissolved Oxygen charge	56.338 ± 6.233 a	49.819 ± 7.676 a
pH	7.853 ± 0.863 a	7.634 ± 0.902 a
pHmV	-38.65 ± 48.280 a	-39.838 ± 31.817 a
Oxidation Reduction Potential	108.838 ± 29.243 a	47.269 ± 68.274 b
Turbidity	22.544 ± 20.741 a	6.792 ± 6.989 b

Different lowercase letters indicate significant differences (p = 0.05) between Pond and Well water samples.

Table 17. Population of fecal indicators in sampled irrigation pond and well water in *Escherichia coli* O157:H7 deduction test.

MPN/100 ml	**Pond**	**Well**
Coliform	2288.719 ± 261.763 a	19.489 ± 1.982 b
Generic *E. coli*	75.867 ± 104.522 a	0.239 ± 0.069 b

Different lowercase letters indicate significant differences (p = 0.05) between Pond and Well water samples.

Figure 1. *Salmonella* MPN values in pond irrigation water.

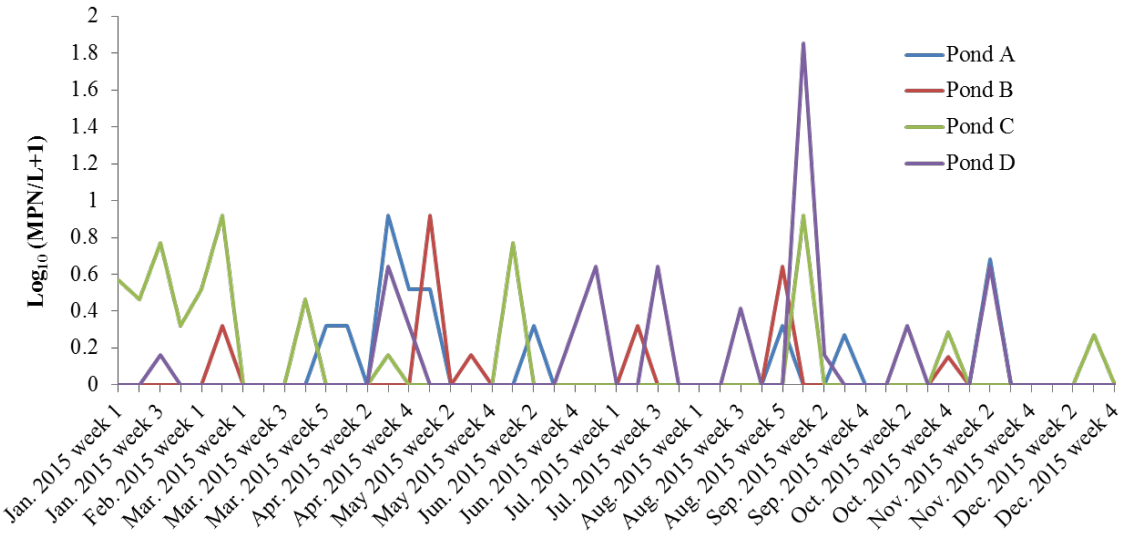


Figure 2. *Listeria monocytogenes* MPN values in pond irrigation water.

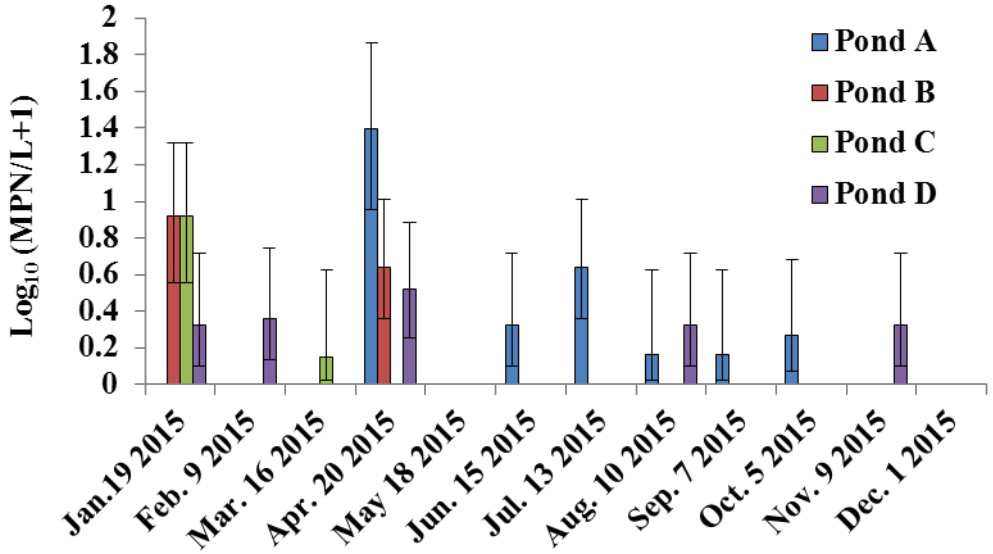


Figure 3. MPN values of coliform and generic *E. coli* in pond water.

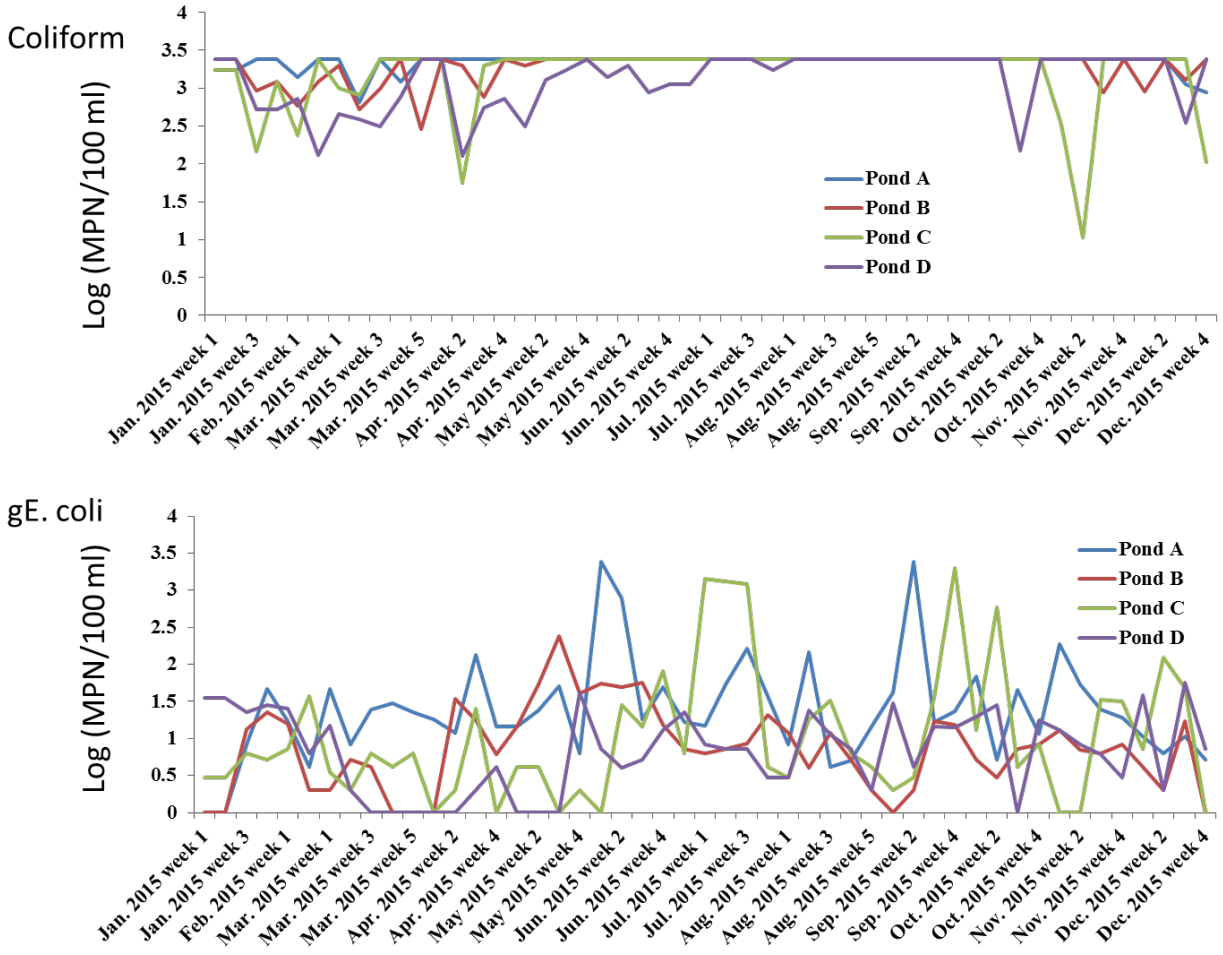


Figure 4. MPN values of coliform and generic *E. coli* in well water.

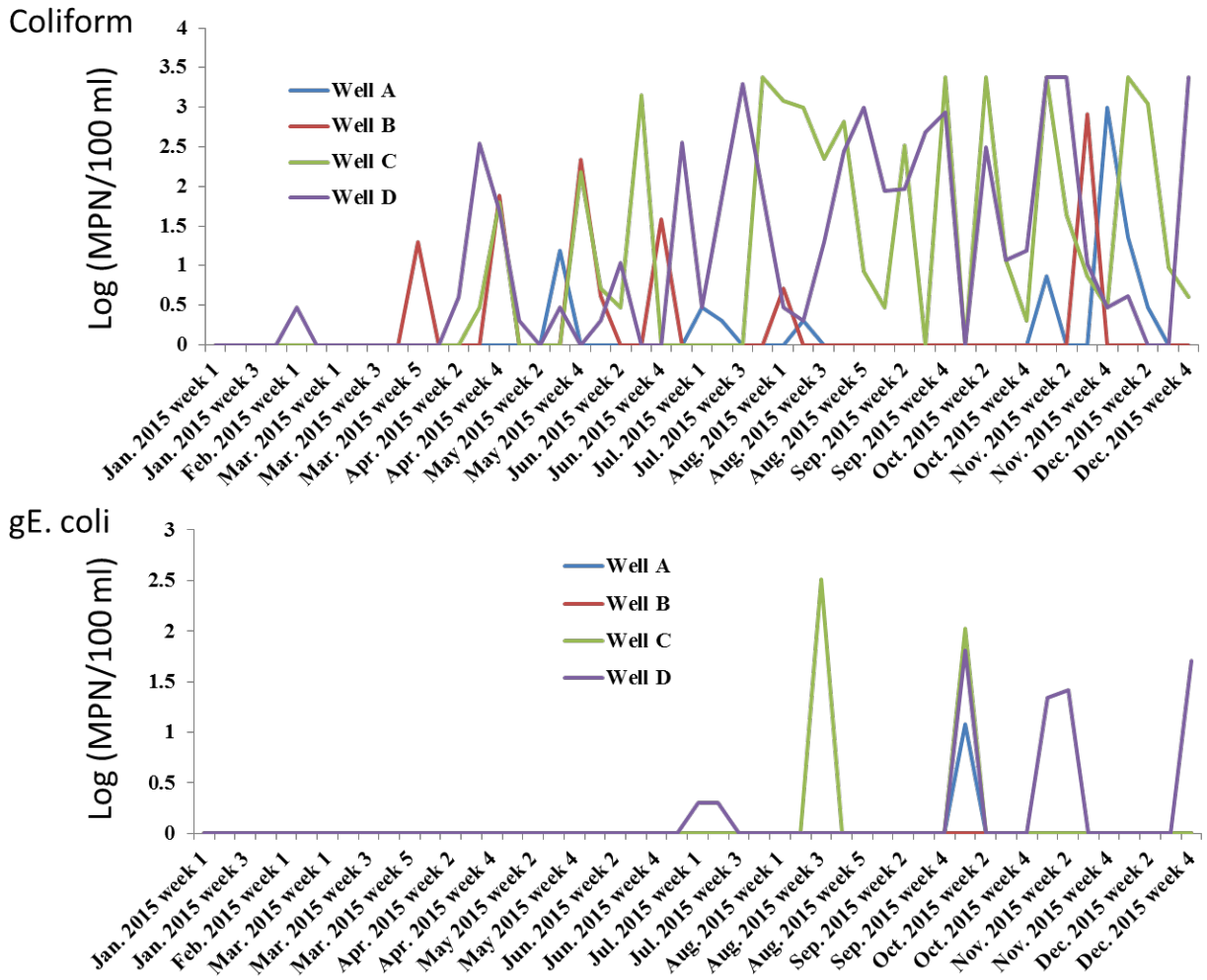


Figure 5. Dynamics of temperature and total rainfall of tested farms.

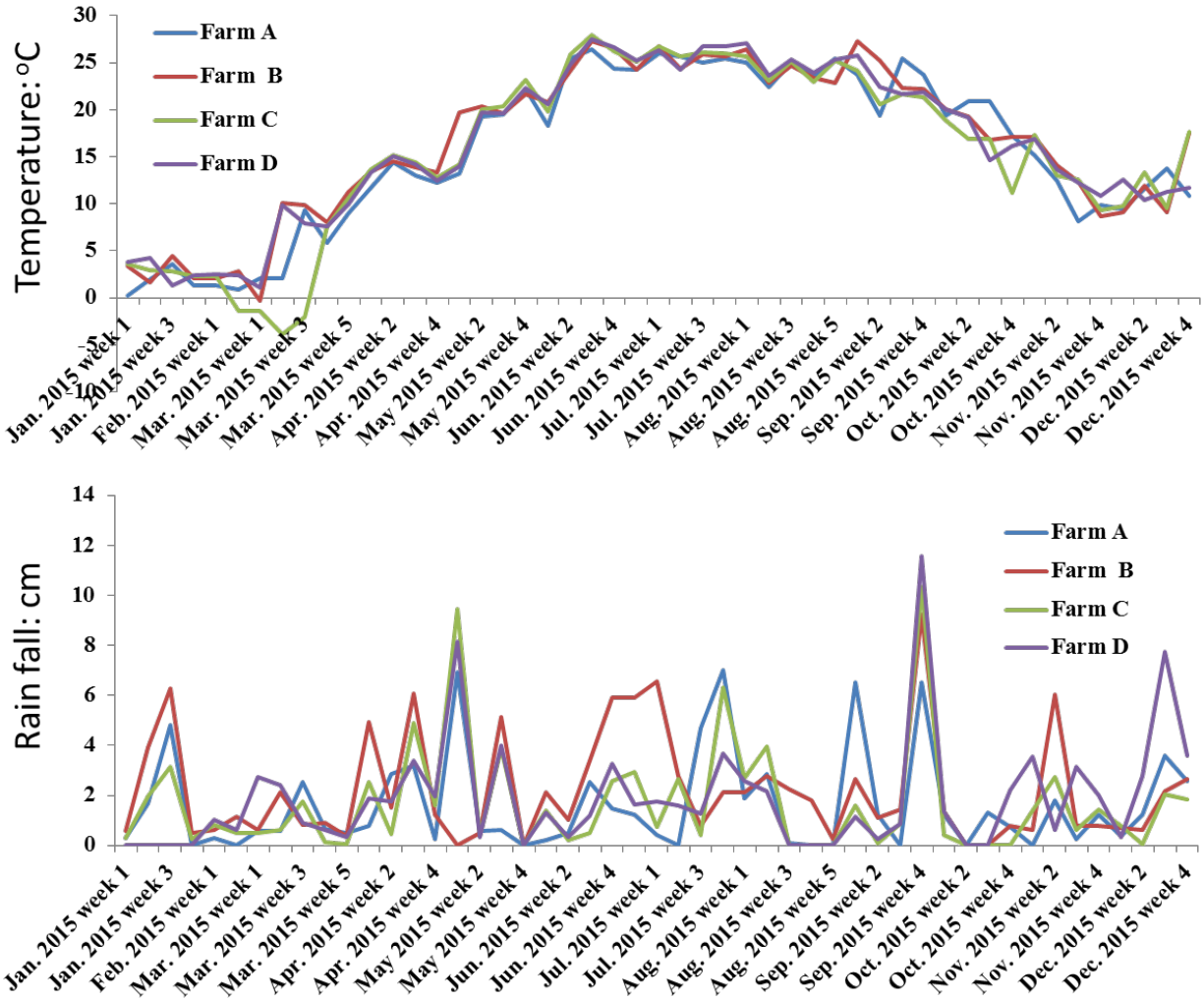


Figure 6. Serovar diversity of *Salmonella* spp. in the four tested ponds.

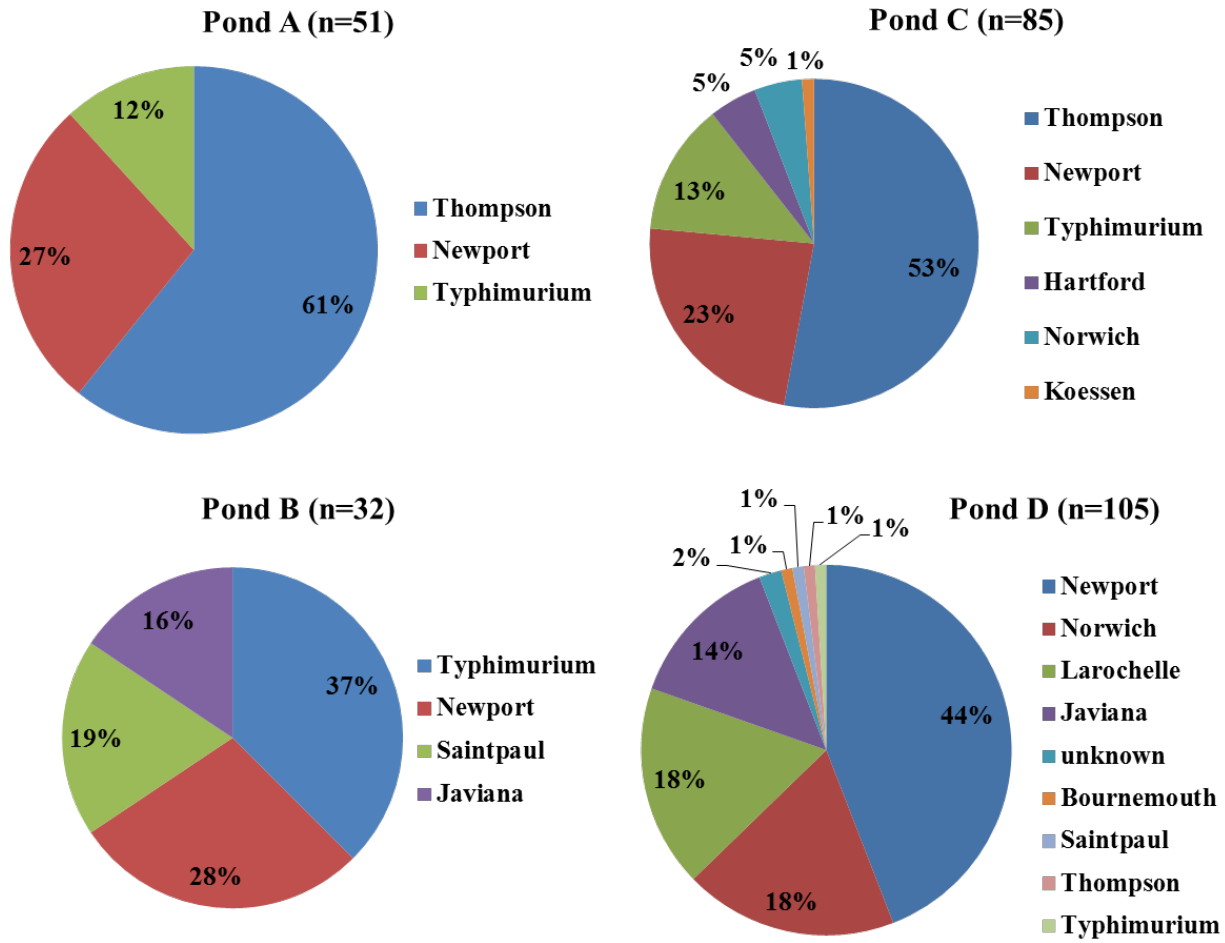


Figure 7. Serovar diversity of *Salmonella* spp. in irrigation pond water on ESV.

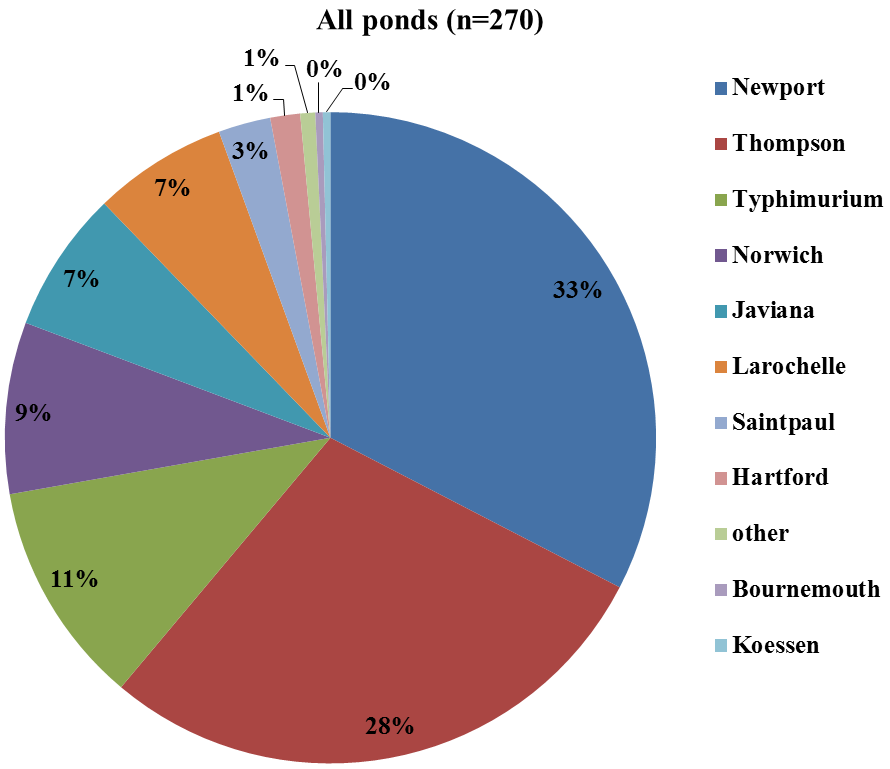


Figure 8. Serovar diversity of *Salmonella* spp. in irrigation well water on ESV.

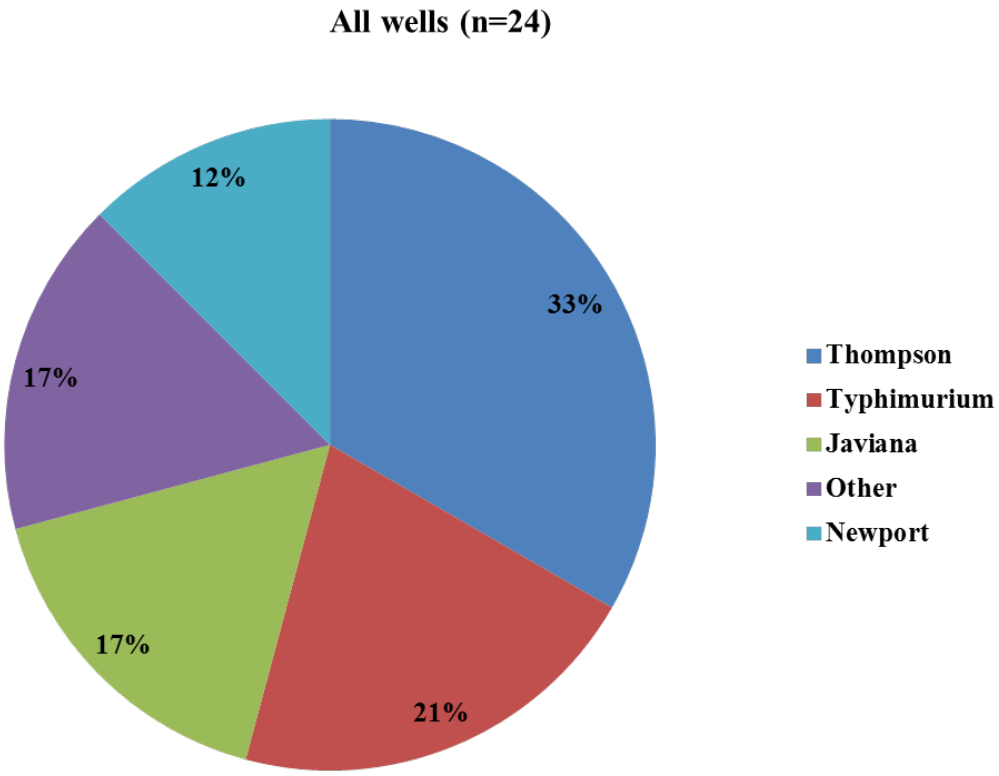


Figure 9. Hierarchical clustering of samples based on the 50 most dominant class level taxa revealing a strong clustering associated with water source.

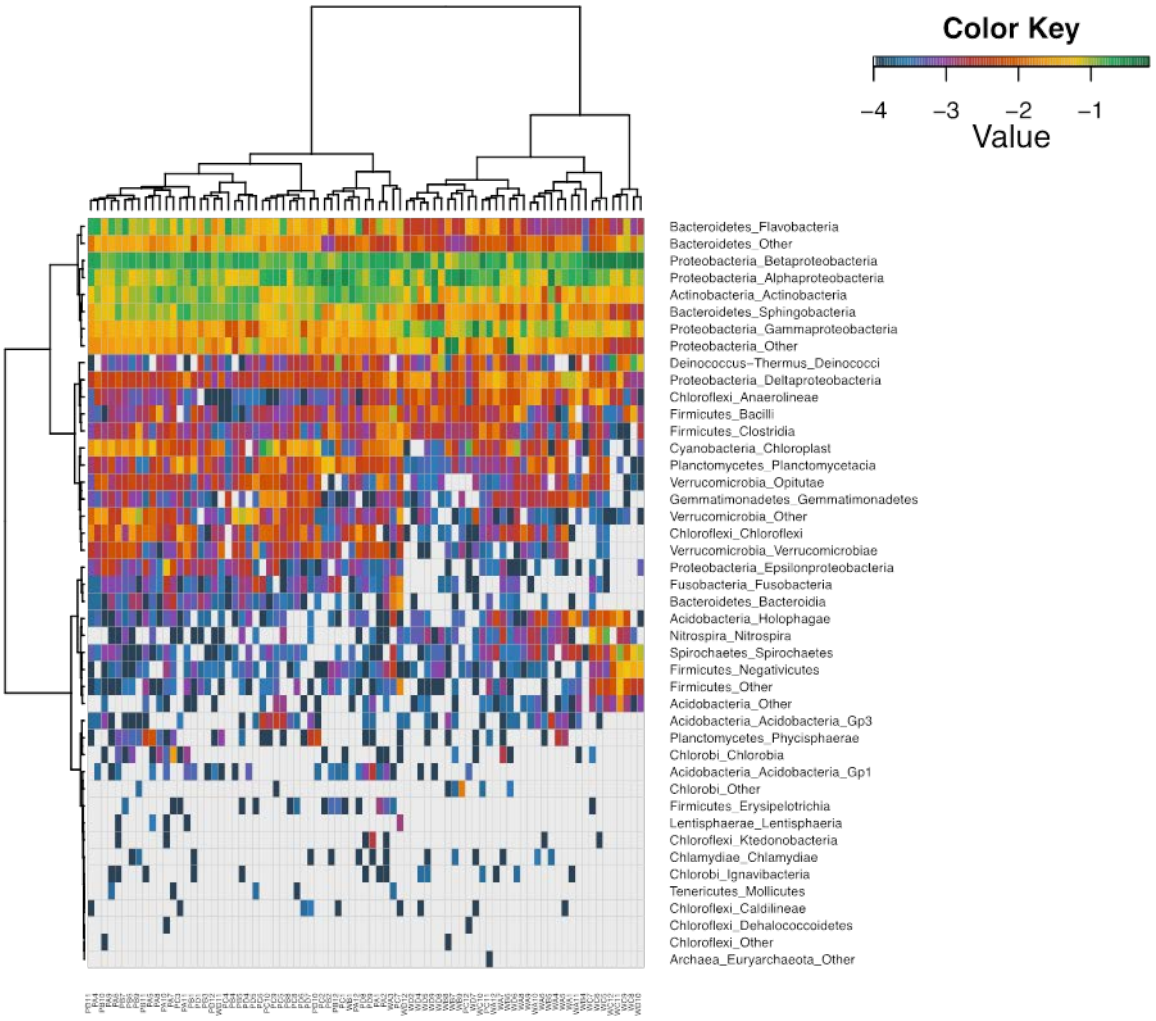


Figure 10. Hierarchical clustering of samples based on the 50 most dominant genus level taxa revealing a strong clustering associated with water source.

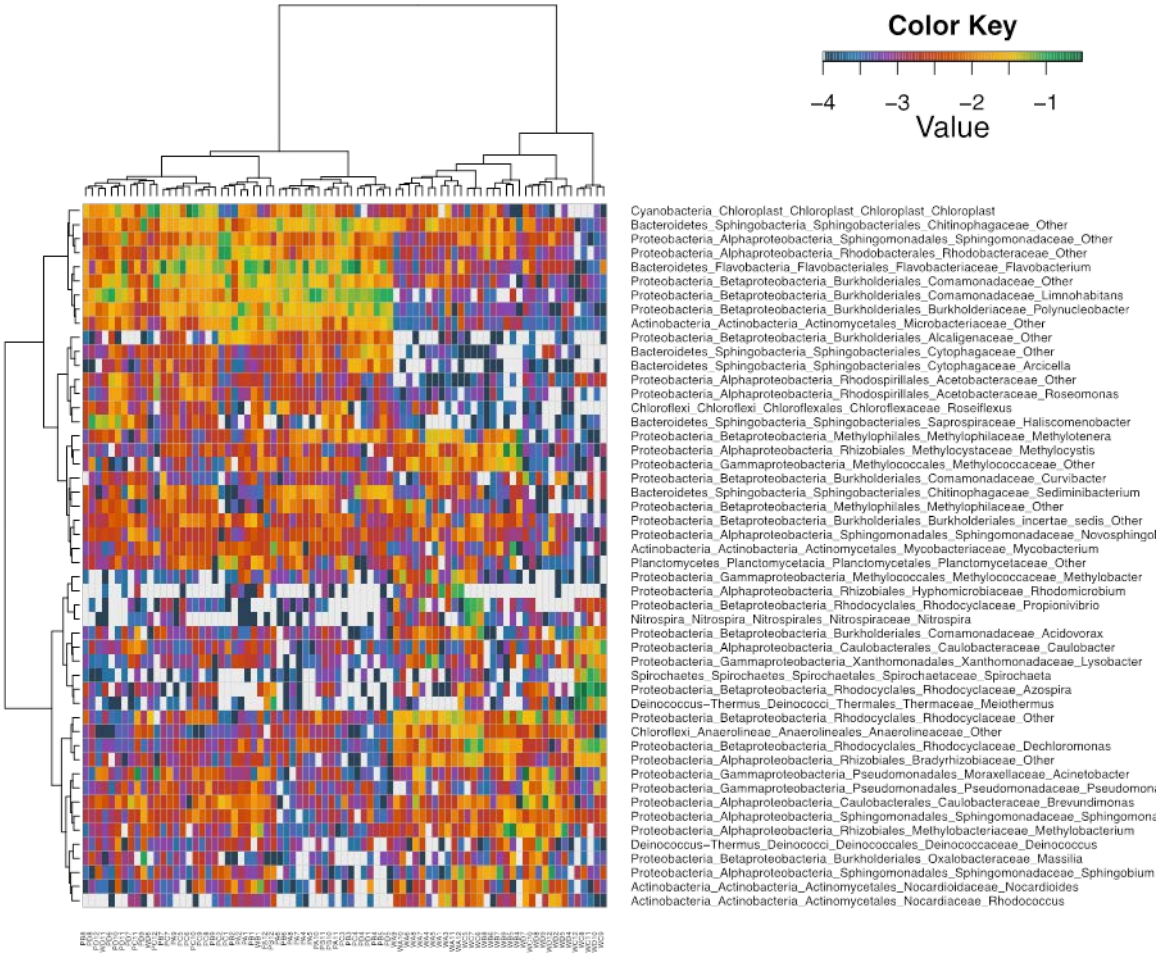


Figure 11. Beta-diversity (Principal coordinates analysis) based on Bray-Curtis distances and points are colored by (i) water source status and (ii) location and source combination.

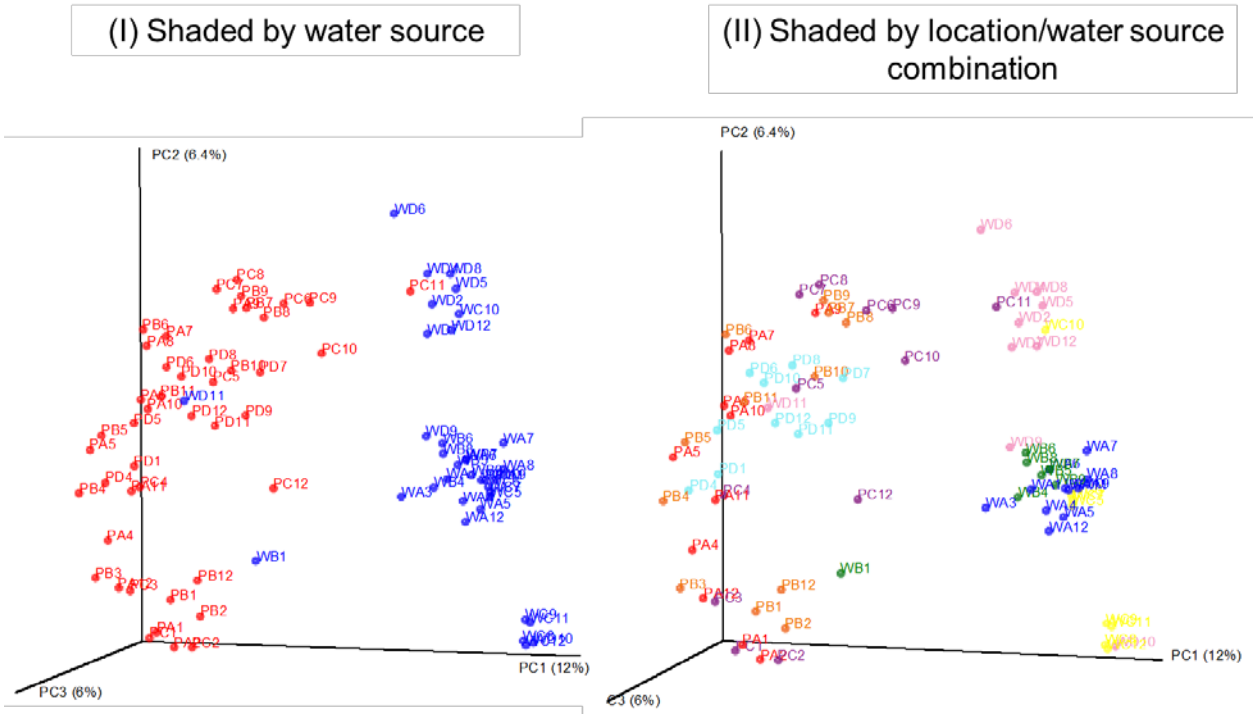
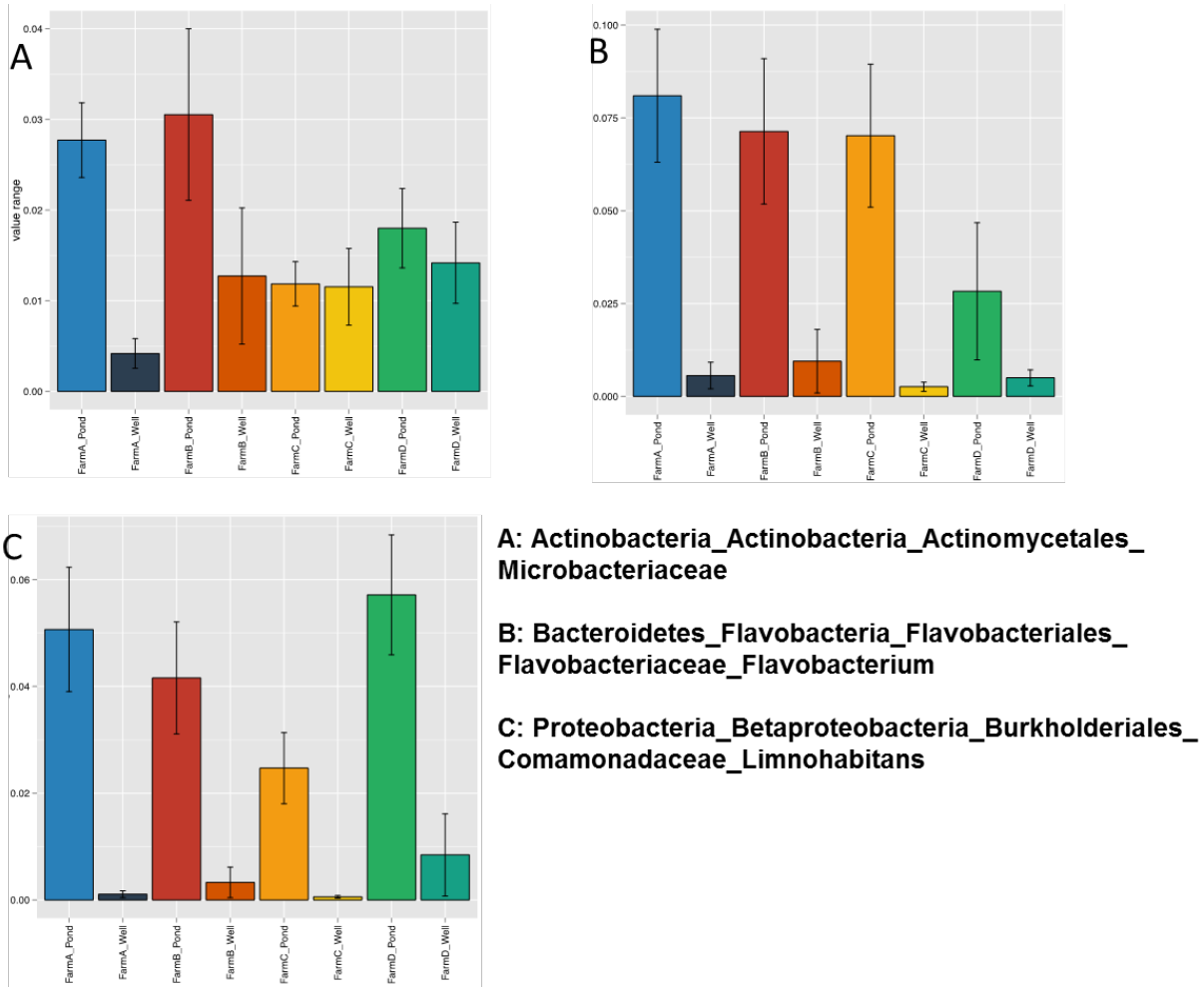


Figure 12. Relative abundance of dominant taxa of sampled irrigation ponds and wells.



A: Actinobacteria_Actinobacteria_Actinomycetales_ Microbacteriaceae

B: Bacteroidetes_Flavobacteria_Flavobacteriales_ Flavobacteriaceae_Flavobacterium

C: Proteobacteria_Betaproteobacteria_Burkholderiales_ Comamonadaceae_Limnohabitans

Figure 13. Abundance of taxa correlated with *Listeria monocytogenes* negative and positive samples.

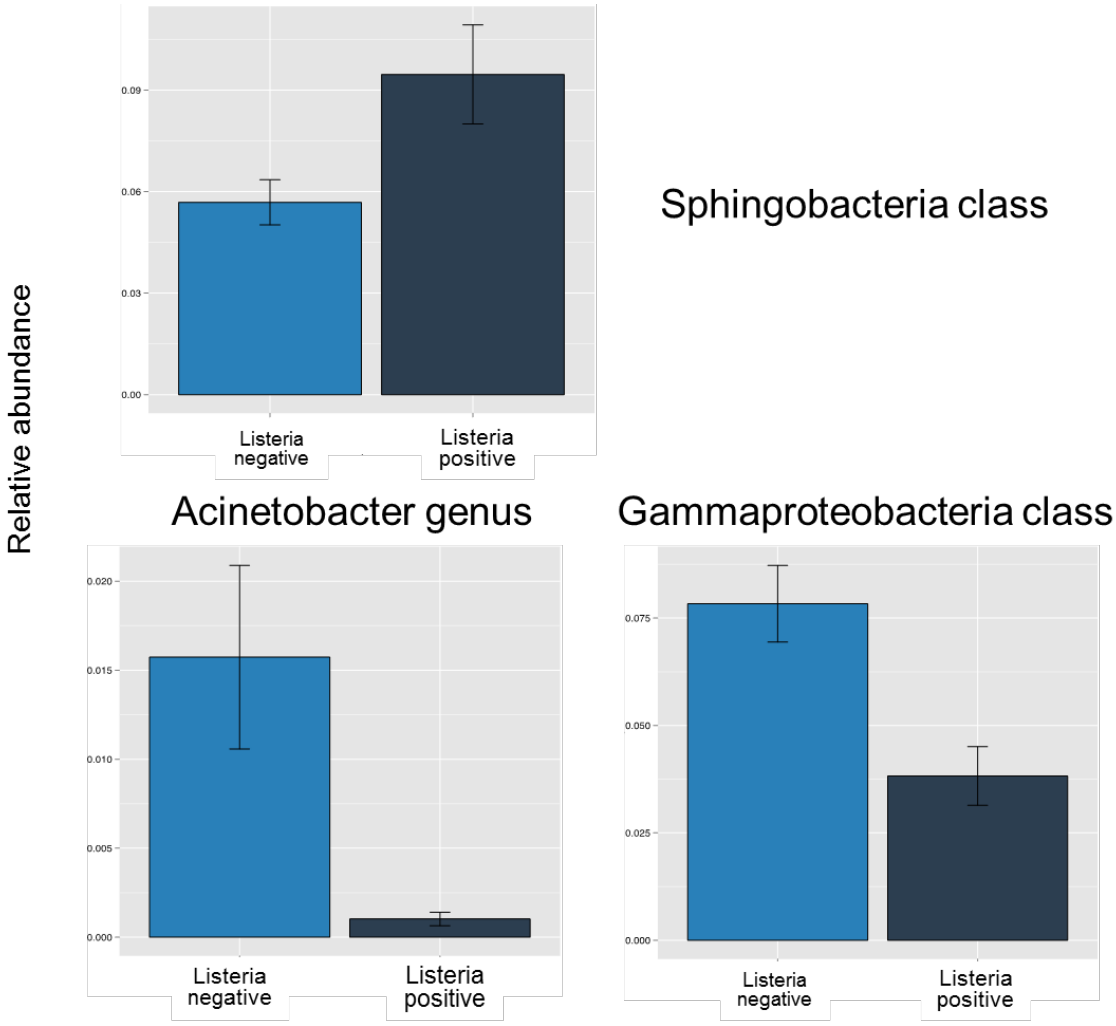


Figure 14. Abundance of taxa correlated with *Salmonella* negative and positive samples.

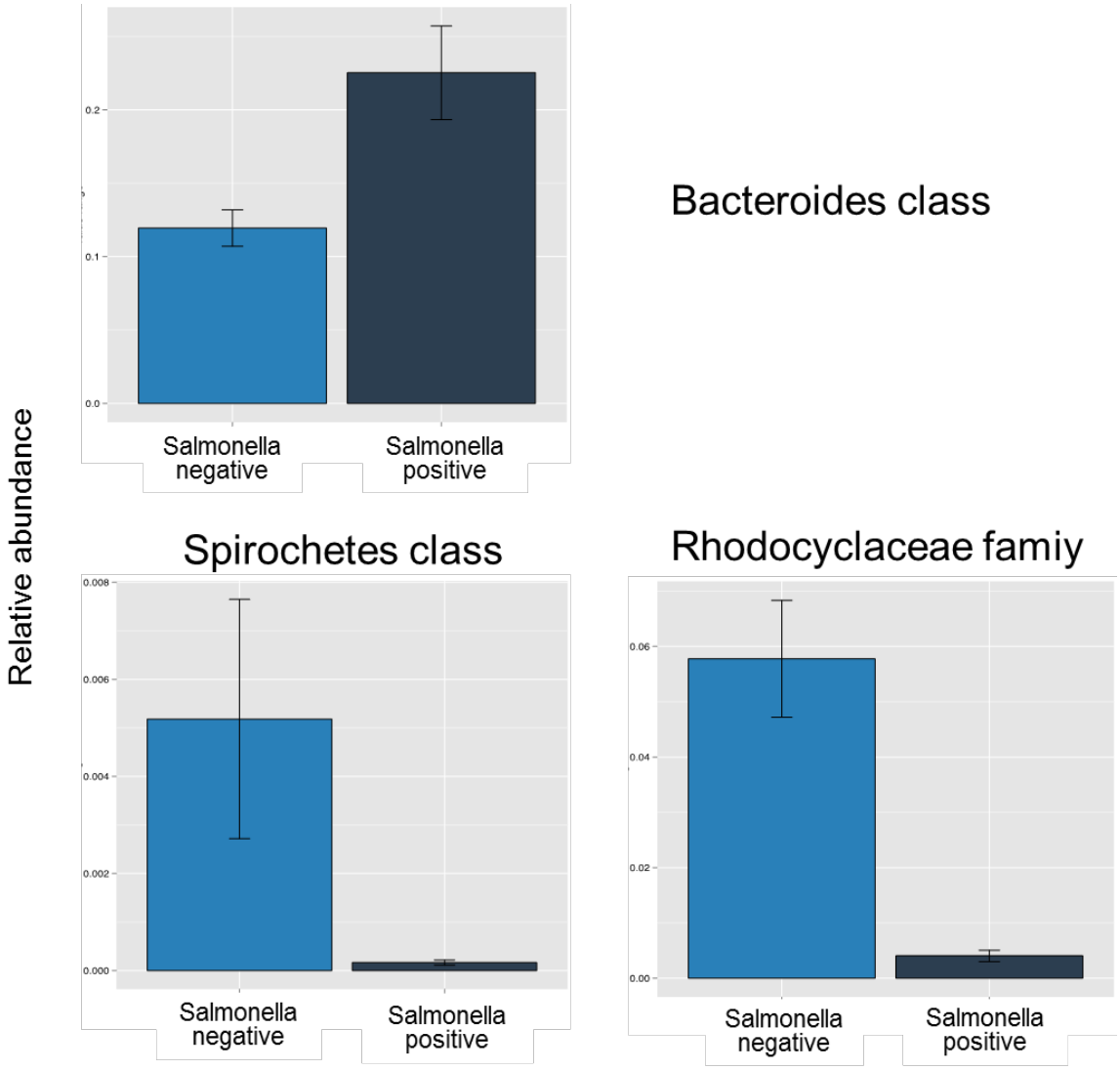


Figure 15. *Salmonella* levels after sanitization with 1 min contact time.

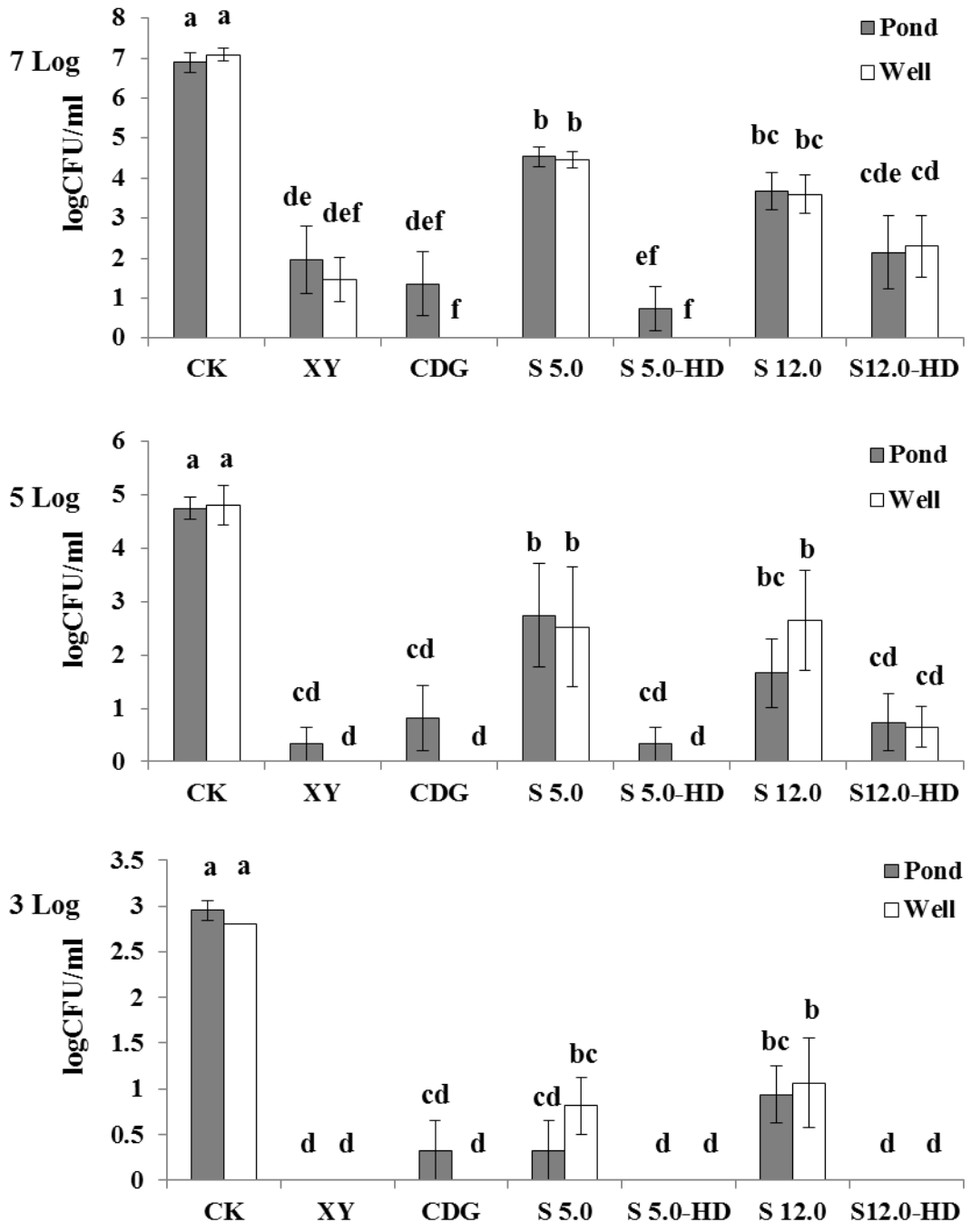


Figure 16. *Salmonella* levels after sanitization with 30 min contact time.

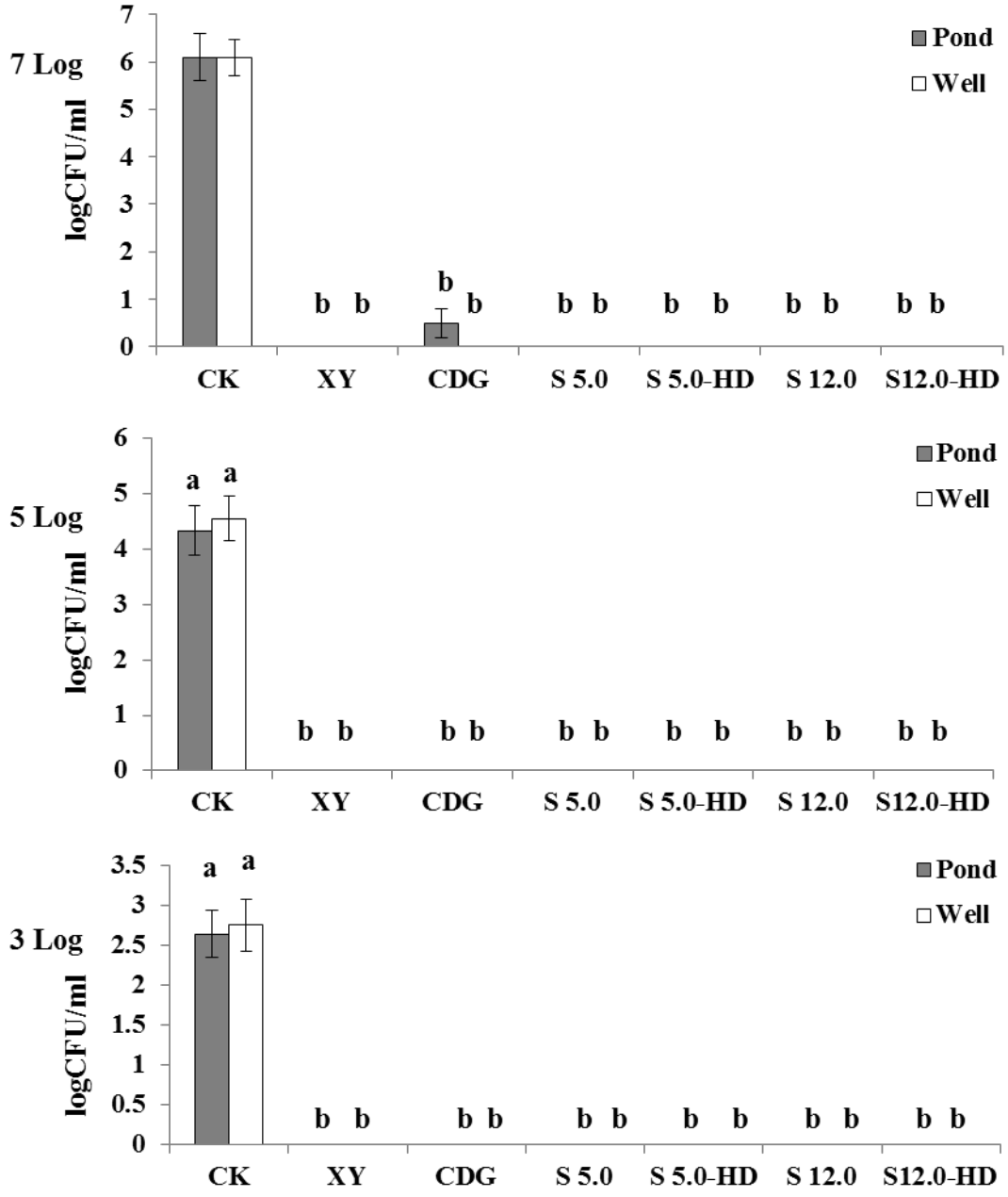


Figure 17. *Listeria monocytogenes* levels after sanitization with 1 min contact time.

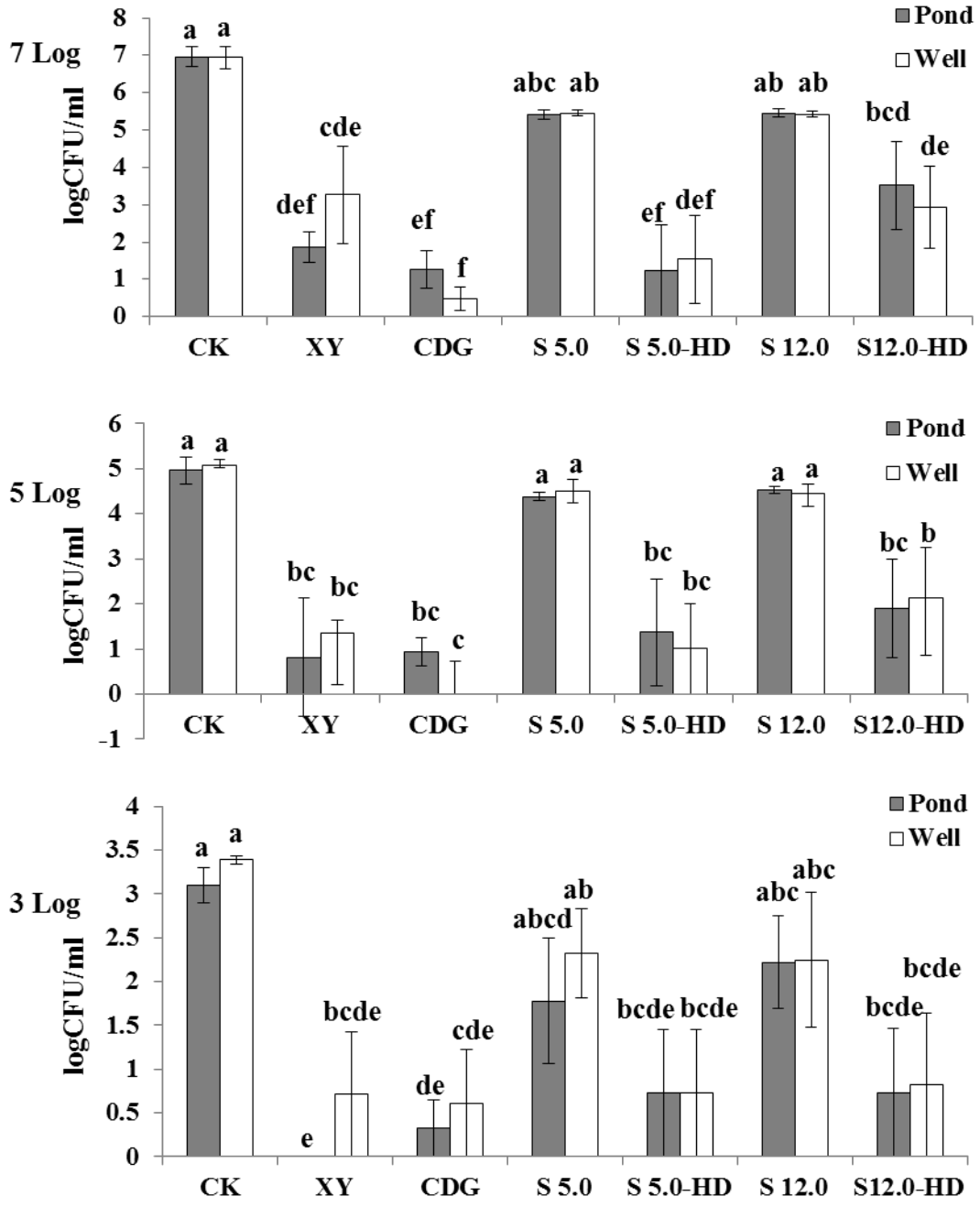


Figure 18. *Listeria monocytogenes* levels after sanitization with 30 min contact time.

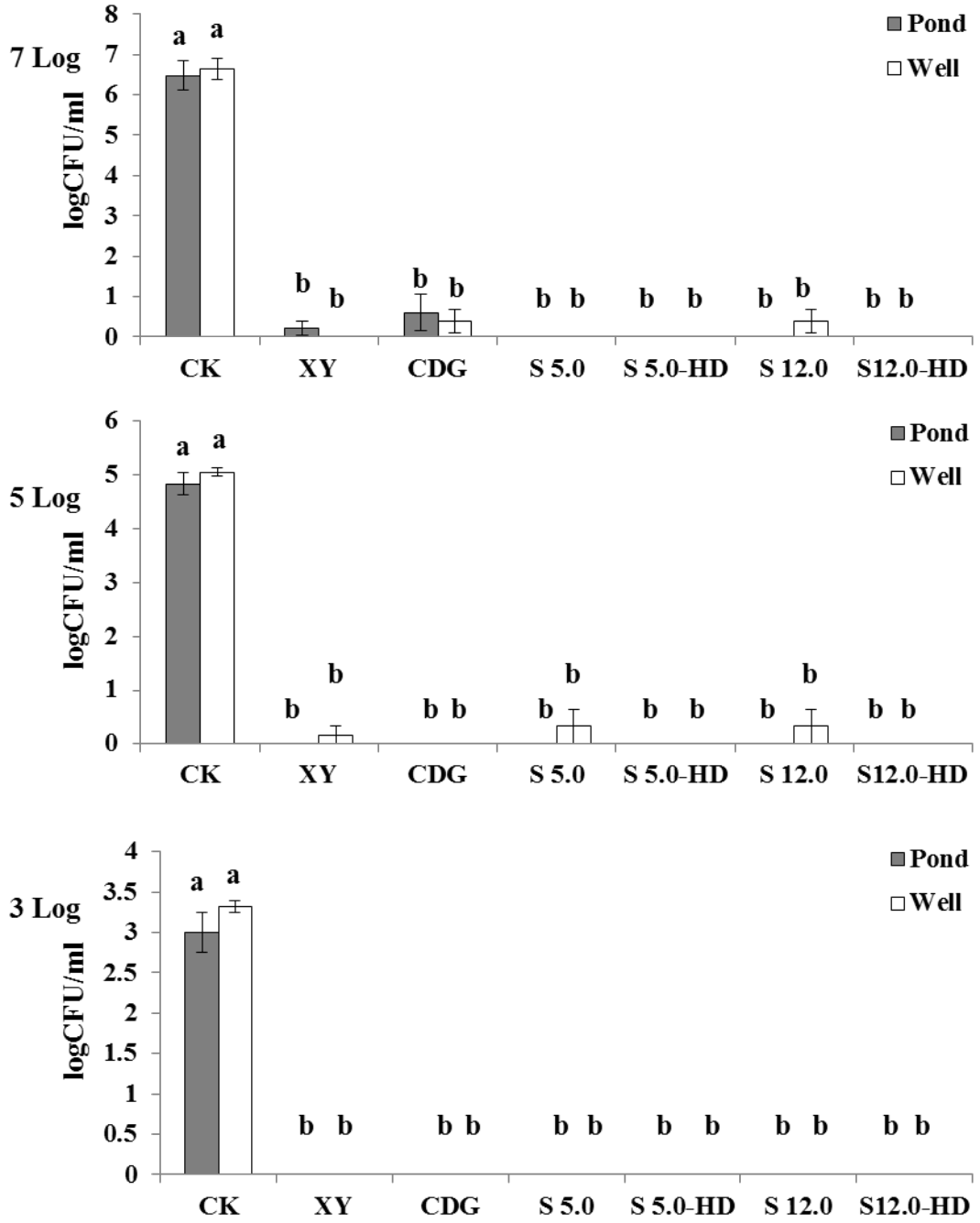


Figure 19. *Escherichia coli* O157:H7 levels after sanitization with 1 min contact time.

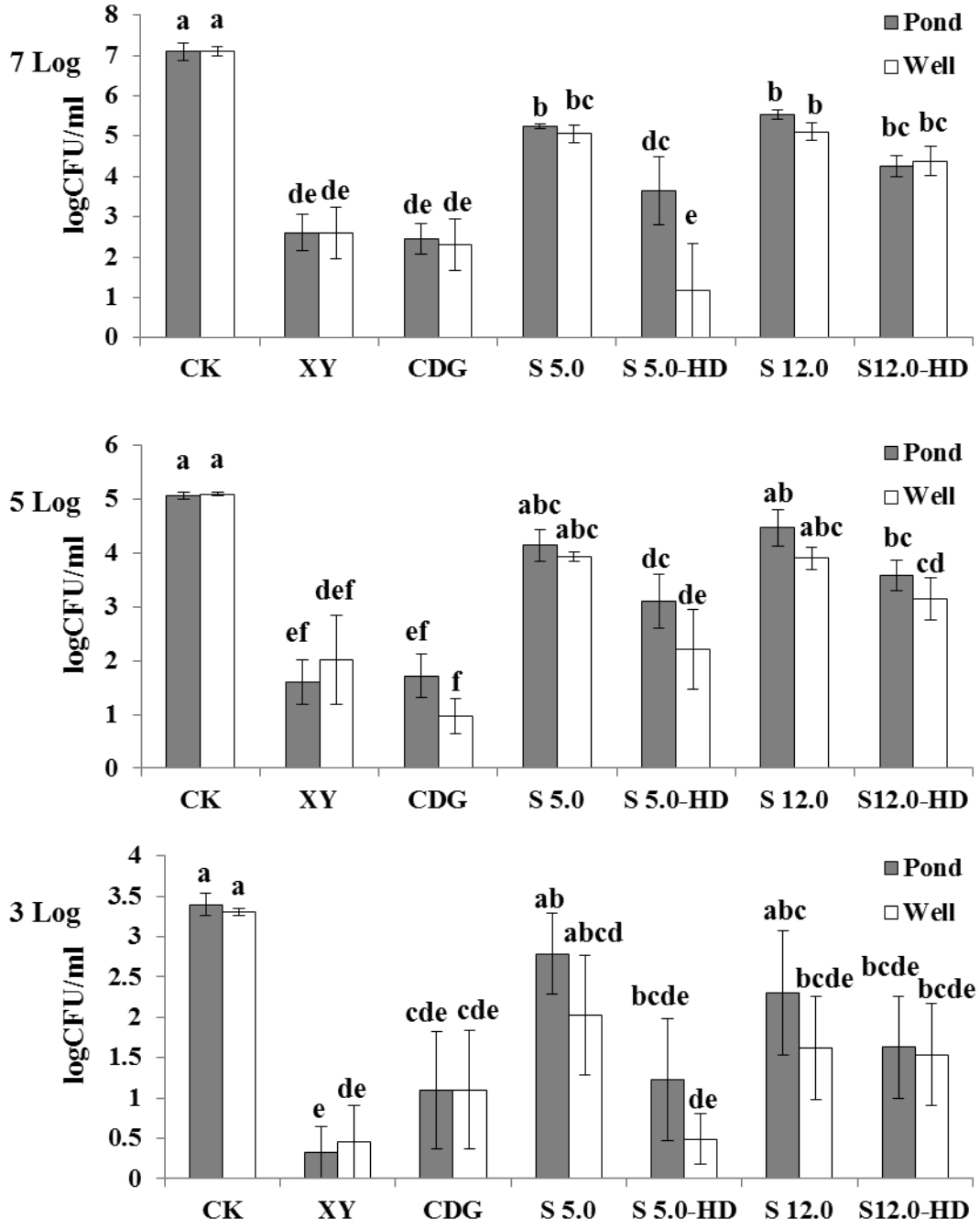


Figure 20. *Escherichia coli* O157:H7 levels after sanitization with 30 min contact time.