FINAL PROJECT REPORT

Project Title: Assessment of overhead cooling practices for apple food safety

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*Dr.'s Zhu and Hanrahan served as CO-PI's of this study after Dr. Killinger took a new position with the FDA in July 2015

Cooperators: This study involved partnerships with the WSU Wenatchee Tree Fruit Research and Extension Center and WSU Prosser Research and Extension Center for field studies, as well as industry partners and input from regulatory personnel. We acknowledge the generous donation from Wilson Irrigation.

Acknowledgements: Manoella Mendoza utilized data from year 1 of the study in her MSc. thesis and her contribution to the success of the project is acknowledged. WSU staff with major project contribution: Tonia Green, Lauren Walter, Kyu Ho Jeong, Andy Liao. WTFRC seasonal staff has contributed greatly to the success of this project and the effort is highly appreciated.

Total Project Funding: **Year 1:** $92,363 **Year 2:** 97,887 **Year 3:** $104,183

Other funding sources

Agency Name: Western Center for Food Safety
Amt. requested/awarded: $80, 768 (requested) / $80,768 (awarded)
Notes: The Western Center for Food Safety, an FDA Center of Excellence, provided funding for validation of field experimental methods and selection of appropriate surrogate organisms. Dr. Killinger attended meeting with scientists funded by the Western Center for Food Safety to discuss methods used in field experiments in order to better align methods between investigators nationally and discuss future strategies for research.

Agency Name: Washington Specialty Crop Block Grant
Amt. requested/awarded: $45,304 (awarded)
Notes: Funding from a Washington Specialty Crop Block Grant related to irrigation water treatment provided funds for additional testing of irrigation water.

Budget History:

WTFRC Collaborative expenses (projected):

Item	2014	2015	2016
Wages	6,400	15,000	16,000
Benefits	1,600	2,000	2,500
Total	8,000	17,000	18,500

Footnotes: Wages and benefits for assistance from WTFRC staff as originally proposed. Actual numbers vary, depending on year. Permanent staff time was not included into original budget. Total WTFRC collaborative expenses for 2016, incl. all staff costs, benefits, travel and equipment amounted to $ 48,867.

Organization Name: WSU **Contract Administrator: Carrie Johnston**
Telephone: (509) 335-4564 **Email address: carriej@wsu.edu**

Item	2014	2015	2016
Salaries		41,615	48,961
Benefits		16,682	20,156
Wages	5,800	3,000	1,500
Benefits	563	66	33
Equipment	65,000	8,000	
Supplies	10,000	10,000	12,000
Travel	3,000	1,524	3,033
Plot Fees			
Miscellaneous			
Total	84,363	80,887	85,683

Footnotes:
[1] Technical support and undergraduate students in Pullman.
[2] Equipment for field and laboratory experiments.
[3] Fruit, chemicals, measurement devices, microbial supplies and analysis/management fees.
[4] Travel to central Washington for inoculation studies and fruit collection.

OBJECTIVES

1) Investigate foodborne pathogen and surrogate survival in laboratory studies and develop inoculation methods for field experiments

2) Examine non-pathogenic surrogate survival in field studies to understand potential risks associated with standard overhead cooling water application practices

SIGNIFICANT FINDINGS

Objective 1 (inoculation method development):

- For the field study, highly sensitive methods and generic *E. coli* strains were selected, optimized and utilized to align with studies having similar objectives in other regions and with other commodities. Rifampicin resistance to regionally acquired *Salmonella* spp. and *E. coli* O157:H7 was developed for growth curve analysis. Growth curve methods were optimized.

- This project developed field inoculation methods coupled with specific staff training programs to generate a consistent method for application of generic *E. coli* surrogates in an orchard setting.

Objective 2 (surrogate survival under field conditions):

- Additional treatment with overhead, evaporative cooling did not appear to impact survival of generic *E. coli* on apples within the first four days after inoculation, compared to the response on control apples that did not receive overhead cooling application.
- Experimental data on mature Gala and Golden Delicious suggests *E. coli* were reduced at a rate greater than or equivalent to the 0.5 log per day reduction proposed by FDA for overhead evaporative cooling (EC) treated varieties for at least four days after inoculation when applied within one week of commercial harvest.
- Generally, the greatest reduction rate of generic *E. coli* on apples occurred within the first 8-10 hours of inoculation, with additional reduction at a slower rate between 24 – 106 hours. In some cases, increases in generic *E. coli* levels were observed between 24 hours and 176 hours.
- Based on initial analysis (without statistical analysis for all three years), the following factors did not appear to consistently impact reduction of generic *E. coli* levels at all time points: type of EC system (traditional vs. misting), and inoculum level (approximately 10 million/31,000,000 CFU, 7.5 log) vs. a lower inoculum level (approximately 3,100 CFU, 3.5 log).
- Some factors appeared to have an effect at certain time points within an experiment and warrant more detailed statistical analysis and continued investigation in future studies, including canopy location of fruit, training system of the orchard, weather conditions, fruit developmental stage (mature vs. immature), and yearly variability.
- The reduction of generic *E. coli* varied dramatically among individual apples within the same variety at any given time point. Typical standard deviations for most time points after 2 hours were greater than 1 log. Therefore, at any given time point, some individual apples had higher generic *E. coli* levels observed than the overall averages reflect (in some cases between 3.5 – 6.0 log CFU/apple, or approximately 3,000 – 1,000,000 generic *E. coli* remaining at the end of the sampling periods in the experiments.

- Reduction of generic *E. coli* was influenced by apple varieties. In general the reduction in generic *E. coli* on Fuji apples during the first 10 hours after inoculation was lower (slower rate) than for other varieties examined in the study (Gala and Golden Delicious). Fuji apples showed a 1.1 – 1.8 log reduction compared to 2.1-2.9 log for Gala/Golden Delicious, possibly due to the different harvest season of this variety, mid-October versus late August to early September.

RESULTS & DISCUSSION

Objective 1 (inoculation method development): Following is a description of the field inoculation protocol developed by the team as part of the project in Year 1:

Rifampicin-resistant generic *E. coli* strains (TVS 353, TVS 354, TVS 355, LJH 1238) were obtained from UC Davis and used for the inoculum cocktail. For the cocktail, lawns of each strain were grown on MacConkey agar with 50µg/ml rifampicin and removed by adding 0.1% peptone water followed by carefully dislodging the lawn. The liquid inoculum was collected from each plate, combined into a cocktail and transported to the orchard on ice for final inoculum preparation. Immediately prior to inoculation, the cocktail was combined with 9.6L 0.1% peptone water in a backpack sprayer and mixed.

Preliminary field experiments were performed in all years to optimize the field inoculation method and ensure consistent inoculum levels on apples. Individuals with the most consistent technique for inoculation were identified through preliminary experiments to perform inoculation during the field experiment.

Inoculation was performed after sunset to reflect the last application of potentially contaminated water prior to harvest as well as the highest risk for bacterial attachment and survival. Apple harvest time points reflected industry harvesting practices (e.g. start of picking at dawn). For inoculation, an individual navigating a ladder in the dark with a backpack sprayer of inoculum sprayed individual apples on each tree. Teams of "spotters" with flashlights communicated with the "sprayer" to ensure thorough inoculation. Emphasis was given to crew training and quality control throughout the study, by strictly adhering to a detailed experimental plan to optimize sampling consistency at each time point, performance assessment and corrections of all personnel, de-briefing after each experiment, and in general maintenance of a constant feedback loop between lab and field team members.

Enumeration of rifampicin-resistant generic *E. coli* (flow diagram provided in Figure 1) was performed at the WSU Pullman campus. For each apple, 10ml of 0.1% peptone water was added then rubbed for 1 minute, shaken for 30 seconds, and rubbed 1 minute. Media for enumeration involved CHROMagar™ ECC with 50µg/ml rifampicin with and without filtering as well as pre-enrichment in tryptic soy broth (TSB). Time 0 samples were plated onto CHROMagar™ ECC with rifampicin (Figure 1) for quantification. Samples at later time points were also plated onto CHROMagar™ ECC with rifampicin for enumeration as well as pre-enriched in TSB and the remainder of the sample was filtered. If samples had counts below the countable range, the TSB pre-enrichment was plated onto CHROMagar™ ECC with rifampicin. The detectable limit was 1 CFU/apple.

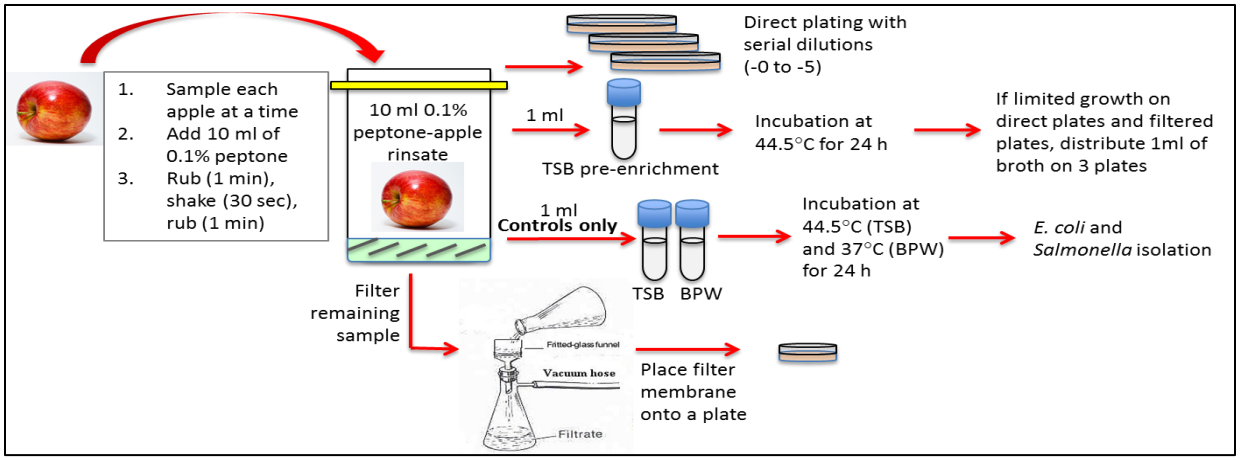


Figure 1: Flow diagram summary of enumeration method

Objective 2 (surrogate survival under field conditions):

WSU research orchards located in two regions representing a significant segment of the tree fruit growing region in Washington were utilized. Several apple varieties (Fuji, Gala, Golden Delicious) were examined with inoculation near harvest and earlier in the season in the Wenatchee and Prosser region, following the general inoculation protocol described in Objective 1. Prior to inoculation, trees were carefully selected for moderate crop load, all damaged fruit was removed prior to inoculation.

The primary objective, to examine the impact of EC versus untreated fruit on generic *E. coli* levels was examined over 2-3 years in replicated field blocks for 3 different varieties that represented different harvest seasons; mature Gala and nearly mature Golden Delicious apples were examined near harvest over three years, and mature Fuji were examined over two years. Each treatment (untreated control (UC), standard evaporative cooling (EC) and mist (Prosser, Fuji only)) included two field replications. The impact of canopy location was included as a variable in each year of these studies (approach differed slightly in 2015, see below for more detail). Notes on weather related variables during and following inoculation were documented by field staff. Additionally, WSU Ag WeatherNet data will be included in final data analysis.

During the course of the project, smaller scale studies were also performed to examine the following variables: canopy location of fruit, training system of the orchard, type of EC system used (traditional vs. misting), a high inoculum level (approximately 10 million/31,000,000 CFU, 7.5 log) vs. a lower inoculum level (approximately 3,100 CFU, 3.5 log), and fruit developmental stage (mature vs. immature) (more details in Table 1). As a result, an extensive data set including 25 experiments over three years was developed.

At the onset of each experiment, untreated control apples (20 of each variety and treatment) were collected from buffer rows between treatment blocks and examined for total coliforms, generic *E. coli* as well as pathogenic *E. coli* and *Salmonella*. For inoculated apples, immediately after an entire tree was inoculated, apples were randomly picked at each canopy position and placed into individual bags for enumeration of the initial inoculation level (time 0). The remaining apples were picked, bagged, and transported at specific time points. Time points differed slightly between years. Generally, fruit were sampled for up to one week after inoculation at the following time points: 0, 2, 10, 18, 34, 42, 58, 82, 106, 154 hours.

Table 1: Experimental variables included into the assessment of overhead cooling practices for apple food safety

	2014			2015			2016		
	Gala	**Golden**	**Fuji**	**Gala**	**Golden**	**Fuji**	**Gala**	**Golden**	**Fuji**
Mature fruit	**X**	**X**		**X**	**X**	**X**	**X**	**X**	**X**
Immature fruit				**X**	**X**				
Region(s)/tree architecture/training system						**X**			**X**
Misting system						**X**			**X**
Fruit position									
Fruiting wall (top/bottom)	**X**	**X**					**X**	**X**	
Fruiting wall (top/bottom inside/bottom outside)				**X**	**X**	**X**			
Traditional training system (full sun vs. shade)									**X**
Lower inoculum level (3-4 log)*									**X**

*typical inoculum levels for all other experiments: ~7.5 log

General observations: The reduction of generic *E. coli* varied dramatically among individual apples within the same variety at any given time point (detailed descriptions shown in year 1 & 2 reports). It is important to note that the standard deviations associated with each mean is typically around 1 log (detailed discussion provided in year 2 continuing report). Statistical analysis will be performed to fully evaluate project results. All results should be considered preliminary; for example, it is likely that seasonal influences impacted differences between varieties, and the statistical analysis will be useful in assessing this observation.

A total of 465 apples of untreated control fruit were examined between 2014 and 2016. For detection of foodborne pathogens, *E. coli* O157 was never detected, but 2 apples were positive for *Salmonella spp*. (Fuji, Oct. 3, 2016; Prosser). In addition, 37 (8%) apples had detectable levels of generic *E. coli* (32 Fuji, 3 Gala and 2 Golden Delicious, majority from Prosser) and 174 (37%) apples had detectable levels of total coliforms.

Mature fruit, Wenatchee region, Sunrise orchard: Averaged over three years of data for untreated **Gala** apples, at 10 hours, the average reduction in generic *E. coli* was 2.5 log and for treated Gala with evaporative cooling at 10 hours, the average reduction was 2.9 log (Figure 2). For untreated Gala apples, average values increased between 18 and 24 hours; a slight increase was also observed in treated Gala apples. The greatest reduction in generic *E. coli* levels on mature Gala apples inoculated near harvest were observed within the first 8-10 hours of inoculation, with additional reduction at a slower rate between 34 – 106 hours.

Averaged over three years of data for untreated **Golden Delicious** apples, at 10 hours, average reduction in generic *E. coli* was 2.1 log and for treated Golden Delicious (EC) at 10 hours, average reduction was 2.8 log (Figure 3). The greatest reduction in generic *E. coli* levels was observed within the first 8-10 hours of inoculation, with additional reduction at a slower rate between 34 – 106 hours for generic *E. coli* inoculated near harvest. For almost all time points, average generic *E. coli* levels for treated Golden Delicious apples tended to be slightly lower than corresponding values for untreated

Figure 2. Generic *E. coli* levels on inoculated Gala apples with (EC: treated) and without (UTC: untreated) overhead evaporative cooling water from an open surface water source near Wenatchee, WA from 2014 to 2016. Values reported in $\log_{10}$ colony forming units/apple.

Golden Delicious apples.

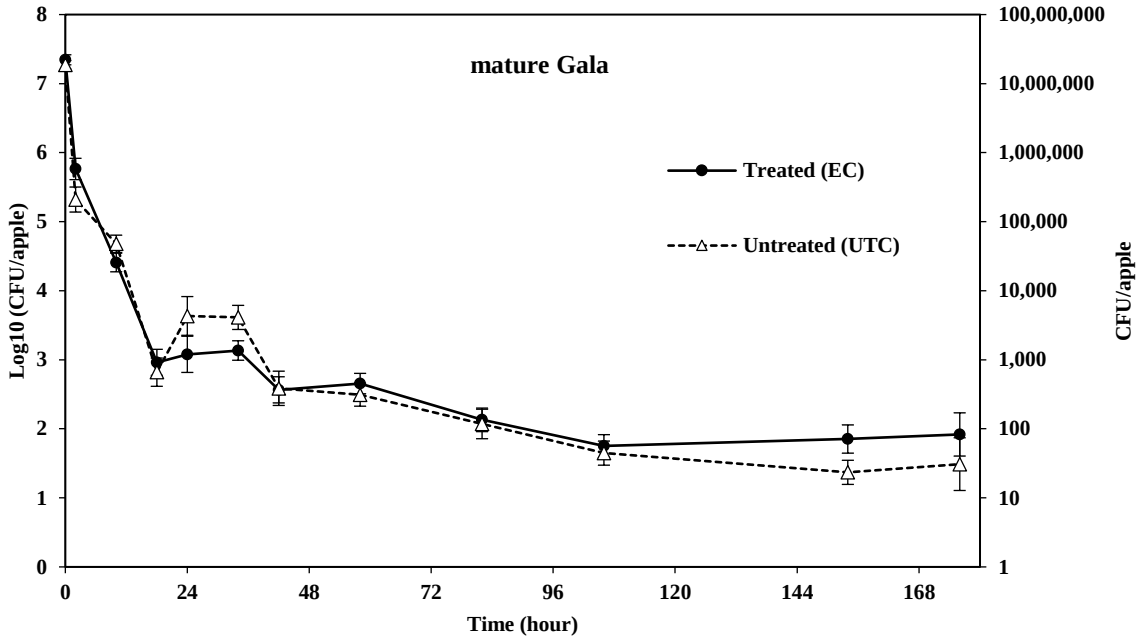
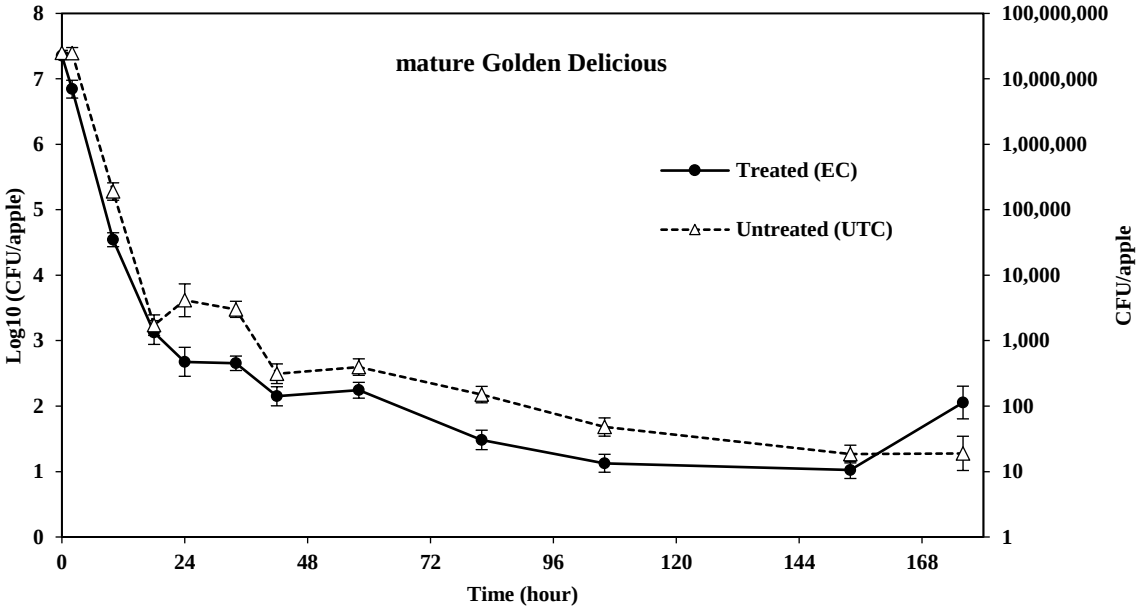
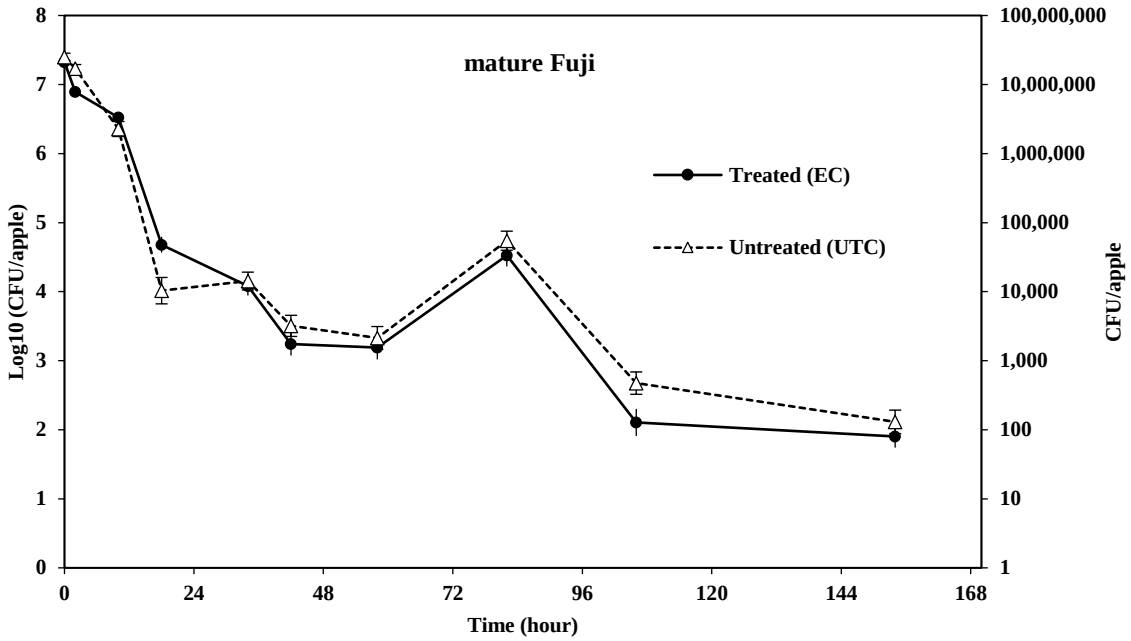


Figure 3. Generic *E. coli* levels on inoculated Golden Delicious apples with (EC: treated) and without (UTC: untreated) overhead evaporative cooling water from an open surface water source near Wenatchee, WA from 2014 to 2016. Values reported in $\log_{10}$ colony forming units/apple.



Averaged over two years of data for **Fuji** apples, at 10 hours, average reduction in generic *E. coli* was 1.1 log for untreated Fuji and for fruit treated with evaporative cooling at 10 hours, average reduction was 0.8 log (Figure 4). For both treated and untreated Fuji apples, average values increased between 58 and 82 hours in 2015. Fluctuations in generic *E. coli* survival were observed in both years of the study in both locations between 42 – 82 hours; further analysis is ongoing. The key reasons to include Fuji in the study was to capture the response of fruit in different locations and at different times of the year (aka cooler weather close to harvest). Although the use of overhead cooling would be less frequent near harvest for Fuji, some orchards utilize overhead irrigation practices which may be utilized near harvest.

Figure 4. Generic *E. coli* levels on inoculated Fuji apples with (EC: treated) and without (UTC: untreated) overhead evaporative cooling water from an open surface water source near Wenatchee, WA from 2015 and 2016. Values reported in $\log_{10}$ colony forming units/apple.



It is important to note that the standard deviations associated with each mean is typically around 1 log (detailed discussion provided in year 2 continuing report). Statistical analysis will be performed to fully evaluate project results. All results should be considered preliminary; for example, it is likely that seasonal influences impacted differences between varieties, and the statistical analysis will be useful in assessing this observation.

Mature fruit, Prosser region, Roza: Averaged over two years of data for untreated **Fuji** apples, at 10 hours, average reduction in generic *E. coli* was 1.1 log and for treated fruit with evaporative cooling at 10 hours, average reduction was 1.7 and 1.8 log respectively for EC and mist (Figure 5).

In general, the reduction in generic *E. coli* was lower (slower rate) for Fuji in both Sunrise and Roza orchards than for the other varieties included in the study, possibly due to the different harvest season of this variety (Figure 6). For example, during the first 10 hours after inoculation a 1.1 log reduction versus 2.1-2.6 log was observed.

Figure 5. Generic *E. coli* levels on inoculated Fuji apples with (EC: treated), without (UTC: untreated) and mist overhead evaporative cooling water application from an open surface water source near Prosser, WA in 2015 and 2016. Values reported in $\log_{10}$ colony forming units/apple.

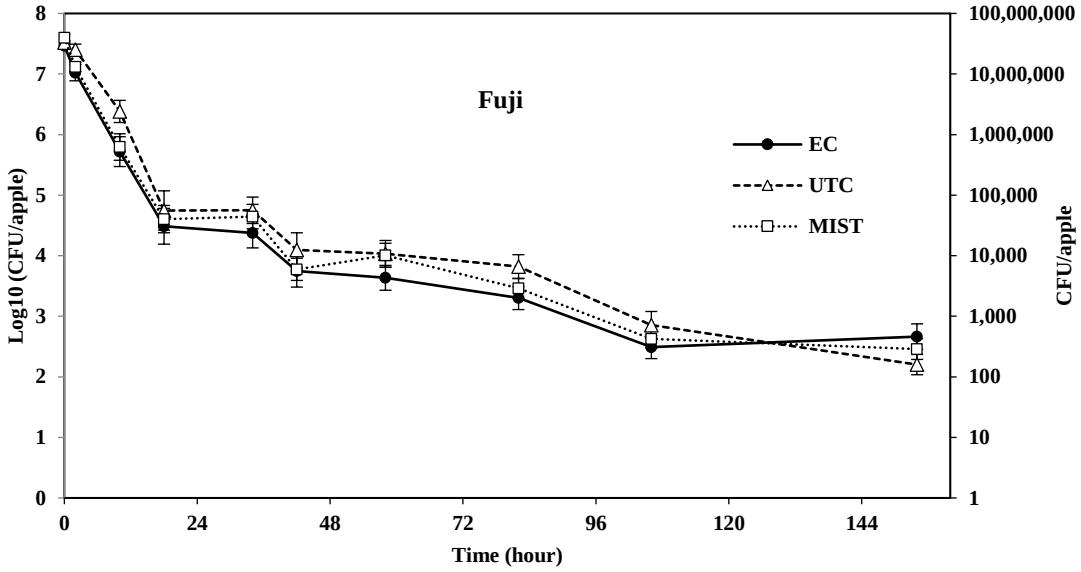
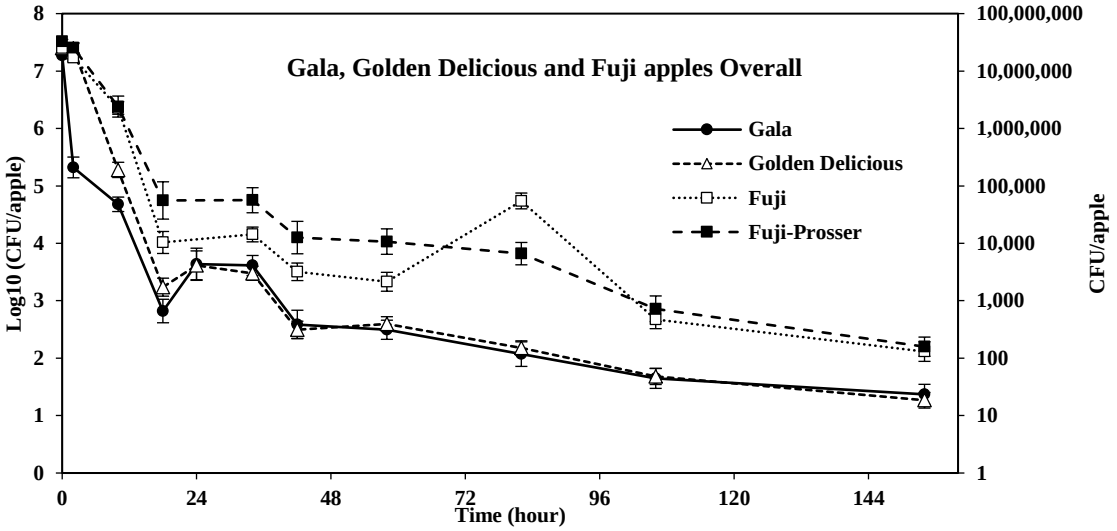


Figure 6. Generic *E. coli* levels on inoculated Gala, Golden Delicious and Fuji apples without (UTC: untreated) overhead evaporative cooling water from an open surface water source near Wenatchee and Prosser, WA in 2014 to 2016 (Fuji 2015-26 only). Values reported in $\log_{10}$ colony forming units/apple.



Immature fruit, Wenatchee region, Sunrise orchard (Gala, Golden Delicious): In 2015, a 2.7 and 3.8 log reduction of generic *E.coli* was observed during the first eight hours after inoculation in Golden Delicious and Gala treated immature apples, respectively (Table 2). Initial reduction rates were greatest in the first

eight hours after inoculation, and microbial reduction between treatments were similar. At most time points, generic *E. coli* levels were not influenced by EC (Table 2).

Table 2. Overall average of generic *E. coli* levels (log CFU/apple) on inoculated, immature Gala and Golden Delicious apples with EC practice (EC) or without (UC) in 2015. Values reported in $\log_{10}$ CFU/apple. Letters sharing the same subscript within a time point do not differ significantly at $P < 0.1$.

Variety	Treatment	Hours after inoculation								
		0	2	8	32	56	80	104	152	320
Gala	UC	7.25 a	5.35 a	3.53 a	2.73 a	NA	1.17 a	0.78 a	0.69 b	0.34 a
	EC	7.18 a	5.76 a	3.34 a	2.33 a	NA	1.14 a	1.03 a	1.23 a	0.28 a
Golden Delicious	UC	7.50 a	6.19 a	4.69 a	3.31 a	2.35 a	NA	1.83 a	0.70 a	0.43 a
	EC	7.41 a	5.90 a	4.66 a	2.50 b	2.21 a	NA	1.62 a	0.77 a	0.47 a

Canopy position: Type of canopy (traditional and modern) and fruit position within the canopy (high versus low) have the potential to impact microbial survival. In certain years of the study, both factors were examined. At Sunrise (a modern fruiting wall), two approaches were used to examine this factor: a) dividing the canopy into two (high vs. low) in 2014 and 2016 or b) three (high, low outside, low inside) distinct fruit locations in 2015. In all three years, canopy location appeared to have some influence on generic *E. coli* survival at specific time points, which warranted further investigation throughout the study. At the Roza orchard (traditional, free standing trees with dense canopy), in 2016, immature fruit located either in full sun or full shade were inoculated in two replications both in the field and over time. Generic *E. coli* levels were similar from inoculation up to 10 hours after inoculation (Figure 7); however, after 58 hours, generic *E. coli* levels were approximately one log higher for apples in full shade compared to apples in areas of full sun (4.2 log versus 3.5 log CFU/apple) (Figure 7). When performing studies on microbial survival on tree fruit, canopy location should continue to be evaluated to determine if and how canopy position influences survival of generic *E. coli*.

Inoculation level: In 2016, the impact of initial inoculation levels on survival of generic *E. coli* was performed using Fuji apples at the Roza location. The high inoculum level used for most experiments (approximately 10 million/31,000,000 CFU, 7.5 log) vs. a lower inoculum level (approximately 3,100 CFU, 3.5 log) were used in this study. Results are shown in Figure 8. Initial response differed between the high and low inoculum treatments between 2 and 10 hours post-inoculation. For the lower inoculum treatment, generic *E. coli* levels increased between 2-10 hours after inoculation (3.4 to 3.7 log CFU/apple), while for the higher inoculum treatment, generic *E. coli* levels decreased slightly (7.6 to 7.3 log CFU/apple). This trend generally differs from data from other experiments. For the remainder of the experiment, both fruit treated with high or with low inoculum levels showed a decrease in generic *E. coli* levels that was fairly consistent for both inoculum levels. For example, at 58 hours fruit from both treatments had achieved an average reduction in generic *E. coli* of 2.7-3.0 logs. For the low inoculum treatment, the majority of fruit reached the level of detection of the method by 42 hours. The use of a high inoculum level allows for quantitative analysis of microbial survival for longer periods of time after inoculation.

Figure 7. Generic *E. coli* levels on inoculated immature Fuji apples located in full sun (sun) or full shade (shade) within the canopy near Prosser, WA in 2016. Values reported in $\log_{10}$ colony forming units/apple.

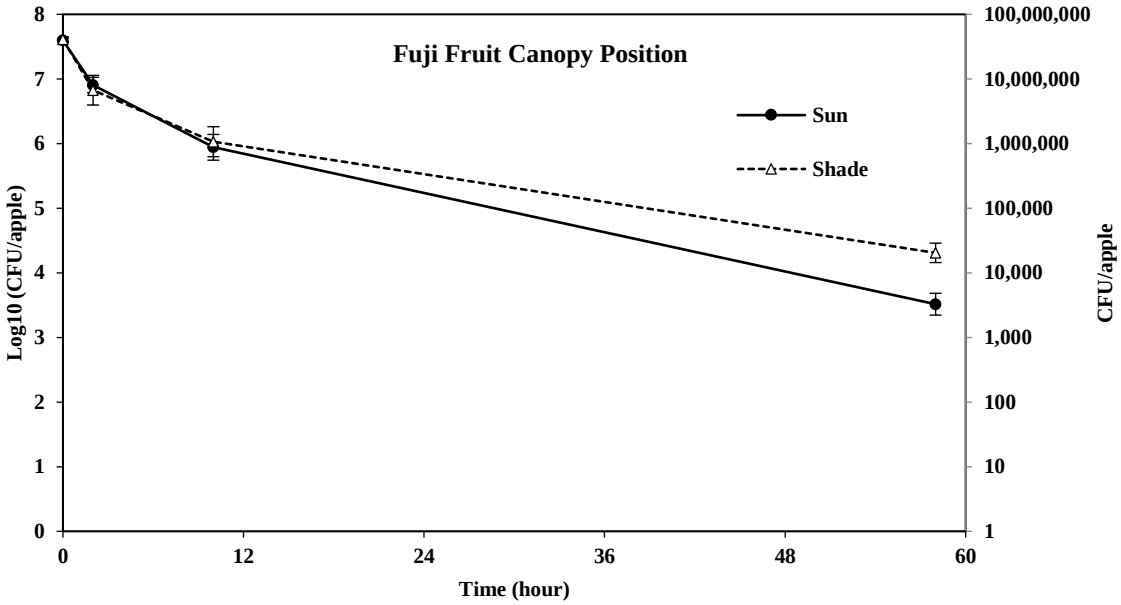
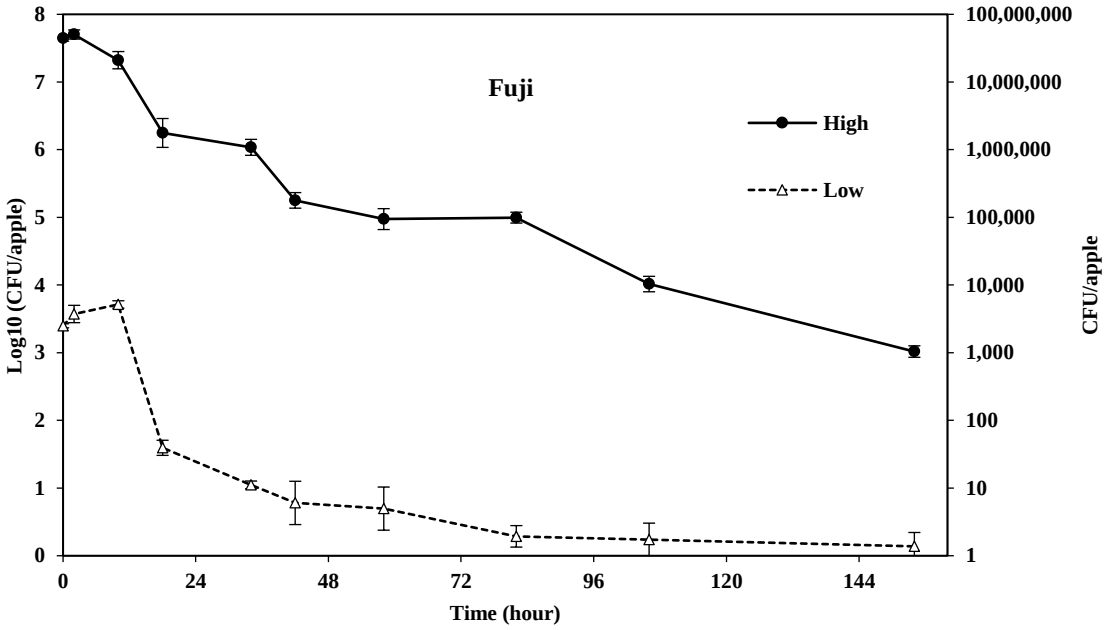


Figure 8. Generic *E. coli* levels on immature Fuji apples in an orchard near Prosser after high and low log inoculation, WA in 2016. Values reported in $\log_{10}$ colony forming units/apple.



EXECUTIVE SUMMARY

Overhead evaporative cooling (EC) using untreated surface water is frequently used in Washington to decrease sunburn in apples. While this technique prevents economic losses for farmers, its influence on food safety risk is uncertain as water is often applied near harvest. This study examined non-pathogenic surrogate survival in field studies, in order to understand potential risks associated with standard overhead cooling water application practices.

An important outcome of this study was the development of field inoculation methods for tree fruit, coupled with a specific staff training program to generate a highly consistent method for application of generic *E. coli* surrogates in an orchard setting. The development of this method and training program benefits long-term food safety research efforts involving field inoculation studies and strengthened the ability to draw conclusions from the study results.

This was the first long-term field study to examine survival of generic *E. coli* and impact of EC on multiple apple varieties. Collection of data over three years increased the strength of the data to draw conclusions by replicating over time and will allow for evaluation of weather conditions over several seasons. Mature Gala and Golden Delicious varieties (untreated and EC treated) were examined over three years in replicated field blocks; data to examine the impact of fruit location in the canopy and weather impacts were also collected. Time points differed slightly between years, but generally, fruit were sampled for up to one week after inoculation at the following time points: 0, 2, 10, 18, 34, 42, 58, 82, 106, 154 hours, and harvest times were selected to align with commercial practices, with picking starting at dawn. Generally, the greatest reduction rate of generic *E. coli* on apples occurred within the first 8-10 hours of inoculation, with additional reduction at a slower rate between 24 – 106 hours. Averaged over three years of data for untreated Gala and Golden Delicious, at 10 hours, average reduction in generic *E. coli* averaged 2.1-2.6 log CFU/apple for untreated and 2.8-2.9 log CFU/apple for EC treated apples. Additional EC water applications with overhead, evaporative cooling did not appear to impact average generic *E. coli* levels on apples within the first 96 hours (four days) after inoculation compared to the response on control apples that did not receive overhead cooling application; this observation represents an economic benefit to the apple industry, as the use of EC prevents economic losses due to sunburn and did not appear to negatively influence food safety risk within the first four days after inoculation. Additional statistical analysis will be performed to investigate the influence of EC, weather, fruit maturity, apple variety, orchard and canopy location to enhance study findings. All results should be considered preliminary; for example, it is likely that seasonal influences impacted differences between varieties, and the statistical analysis will be useful in assessing this observation.

By examining different varieties, generic *E. coli* response at different harvest time periods was determined. In general, the reduction in generic *E. coli* on Fuji apples during the first 10 hours after inoculation was slower (slower rate) than other varieties (Gala and Golden Delicious) examined in the study. These findings are important because there may be differences in generic *E. coli* survival on apples harvested later in the year, which is relevant for growers harvesting late season varieties, particularly if they utilize overhead irrigation practices.

A 2.7 and 3.8 log reduction of generic *E.coli* was observed during the first eight hours after inoculation in Golden Delicious and Gala treated immature apples, respectively. Microbial reduction between treatments were similar. Initial generic *E. coli* reduction was greatest in the first eight hours after inoculation, and at most time points, generic *E-coli* levels were not influenced by EC. Generally, average reduction of generic *E. coli* on immature fruit were nearly equivalent to or greater than those on mature fruit for both treated and untreated Gala and Golden Delicious.

Lastly, in all experiments, the reduction of generic *E. coli* varied dramatically among individual apples within the same variety at the same time point. Therefore, while average levels of generic *E. coli* generally appear to decline over time, certain harvested apples have the potential to carry high levels of generic *E. coli*.