ABSTRACT

Title of Dissertation: *SALMONELLA ENTERICA STRATEGIES FOR PERSISTENCE ON TOMATO (SOLANUM LYCOPERSICUM) AND SEROVAR DYNAMICS IN SURFACE AND RECLAIMED WATER*

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While select aspects of *Salmonella enterica* subspecies *enterica* persistence in agricultural matrices have been illustrated, serovar specific survival strategies in surface water, transmission, and persistence on plants are multifaceted and remain only partially examined. In the present work, we utilized an interdisciplinary approach to illustrate novel mechanisms by which *S. enterica* may adapt to plants as an alternative host. Furthermore, we leveraged the wealth of diversity in *S. enterica* serovars to investigate specific dynamics and drivers of persistence in water and transfer onto produce crops. Through biochemical, gene expression, and plant challenge assays of both tomato (*Solanum lycopersicum*) vegetative and fruit organs, we found that plant-derived NO was generated in response to *S.* Newport recognition. Furthermore, bacterial gene expression on both leaves and fruit was indicative of adaptation to a novel environment including upregulation in NO detoxification machinery, indicating plant-derived NO as a novel bacterial stress. NO tolerance of various *S. enterica* was then evaluated to investigate drivers of “produce associated’

S. enterica adaptation to the plant niche. We identified that plant derived NO can negatively affect titers of all *S. enterica* serovars tested and that serovar specific tolerance to NO *in vitro* was apparent in a concentration and exposure time dependent manner. Finally, the survival of various *S. enterica* in surface and reclaimed water was investigated while evaluating the potential for transition to viable but non-culturable (VBNC) organisms. Furthermore, surface water used for irrigation, a common water environment for *S. enterica*, was investigated as a priming reservoir for various *S. enterica* serovars for enhanced transmission onto tomato crops. Persistence in water included VBNC subpopulations and was driven by water type. Transfer success onto tomato was driven by serovar, and prolonged incubation in water increased the transfer ability of serovars that initially transferred poorly onto tomato. Finally, attachment to polystyrene and water oxidation-reduction potential were identified as possible indicators of foodborne pathogen transfer success onto tomato. Moving forward, a greater understanding of the environmental queues used by *S. enterica* subspecies *enterica* responding to the agricultural environment will aid researchers in developing *S. enterica* targeted on-farm management techniques to ensure safe yet sustainable fresh produce cultivation practices.

SALMONELLA ENTERICA STRATEGIES FOR PERSISTENCE ON TOMATO (*SOLANUM LYCOPERSICUM*) AND SEROVAR DYNAMICS IN SURFACE AND RECLAIMED WATER

by

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Dedication

This work is dedicated to the memory of Rebecca Selleck.

You have taught me so much, I am so grateful to have known you.

Acknowledgements

I would firstly like to express the sincerest gratitude to Dr. Micallef for taking me on as a graduate student. Your guidance and support to help me find and then finely tune my scientific voice has been invaluable. Thank you for listening, for nurturing my intellectual curiosity, and for providing the structure and strategy to navigate these last 5 years.

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List of Abbreviations

NO	Nitric oxide
RNS	Reactive nitrogen species
H_2O_2	Hydrogen peroxide
ROS	Reactive oxygen Species
PAMP	Pathogen associated molecular patterns
PTI	PAMP triggered immunity
SPER/NONO	Spermine nonoate
$CaCl_2$	Calcium chloride
cPTIO	2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide
DAF2-DA	Diaminofluorescein-diacetate
DAB	3,3’-diaminobenzidine
CM-H_2DCFDA	6-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester
Rif	Rifampicin
VBNC	Viable but non-culturable
PMA	Propidium monoazide
FSMA PSR	Food Safety Modernization Act Produce Safety Rule
GAPs	Good agricultural practices

Chapter 1: Introduction

The demand for eating fresh, local, sustainable fruits and vegetables has increased in recent years (Sparling, 2018; Tropp, 2014), and with it, foodborne illness outbreaks attributed to fresh produce consumption. From 1998 to 2016, multistate foodborne illness outbreaks in fresh produce caused by *S. enterica* subspecies *enterica* have increased relative to meat, dairy, and nut products (Foodborne Outbreak Online Database (FOOD Tool), 2016), with recurrent serotypes frequently implicated. In 2018 this trend is sustaining, with *S. enterica* illnesses from seeded vegetables alone accounting for the second highest number from any one pathogen-commodity pair, only surpassed by *S. enterica* in eggs (Jackson et al., 2013; Marder et al., 2018; Tack et al., 2019). Several factors may contribute to this observation, including the increased consumption of fresh produce with 94% growth in million tons of global production from 1980-2004, more centralized processing and distribution of fresh produce products, better outbreak detection methods, and pathogen adaptation to plant environments (Olaimat and Holley, 2012). While all factors may play a role, *S. enterica* has been firmly established as a prevalent microbe in agricultural environments from both environmental research surveys as well as historic salmonellosis outbreak traceback investigations successfully identifying farm production environments as the origin of contamination (Greene et al., 2008, McEgan et al., 2014 Bell et al., 2015; Callahan et al., 2019; Micallef et al., 2012). While there is a diversity of salmonellae in these agricultural matrices surveyed, outbreaks with produce are more likely to occur with select serotypes (Jackson et al., 2013).

Research within the farm to fork continuum has revealed key components necessary for persistence of select *S. enterica* serovars including; plant genotype and surface phytochemical profiles (Barak et al., 2011; Han and Micallef, 2014), bacterial attachment appariti (Barak et al., 2005, 2007; Salazar et al., 2013; Tan et al., 2016), and nutrient acquisition strategies (de Moraes et al., 2017, 2018; Goudeau et al., 2013). However, serovar specific survival strategies of this persistent yet elusive pathogen in surface water, transmission, and persistence on plants are multifaceted and remain only partially examined. A thorough literature review in **Chapter 2** provides the current state of knowledge and emphasizes where research is needed to improve our understanding of enteric bacterial ecology in crop production environments.

Leveraging the unique ability of interdisciplinary research, the present work aimed to better define survival strategies of *Salmonella enterica* as this foodborne pathogen negotiates diverse environments from farm to fork under the following hypotheses:

1. Reciprocal responses between plants and *S. enterica* allows this bacterial pathogen to use plants as an alternative non-animal host.
2. *S. enterica s*erovar specific tolerance to nitric oxide, a common plant derived immune signaling molecule, contributes to preferential serovar – produce commodity associations.
3. The ability to persist in surface irrigation water is a key function for successful transmission of *S. enterica* to produce.

To test these hypotheses, the model system of tomato (*Solanum lycopersicum)* and *S. enterica* was utilized due to the regular association of this pathogen-commodity pair (Bennett et al., 2015; Jackson et al., 2013; Painter et al., 2013). Following this effort, behavior of *S. enterica* serovars in the context of two agricultural matrices, plant and surface irrigation water, were then investigated. Together, this work evaluated the influence of key aspects of the agricultural environment on *S. enterica* persistence from farm to fork, contributing critical information for the development of regionally specific, efficient, effective, and sustainable food safety risk mitigation strategies for seamless incorporation into a holistic farm management plan.

Previous research in our lab has corroborated the ability of *Salmonella enterica* to colonize plants, and transcriptomic study of *Salmonella* colonizing tomato revealed upregulation of genes needed for abiotic stress mitigation, detoxification of reactive oxygen species (ROS) and nitric oxide (NO) (Han and Micallef, 2014, 2016), Han et al., unpublished). Reports of upregulation of plant defense genes as well as induction of ROS including hydrogen peroxide (H_2O_2) have been documented in tobacco and *Arabidopsis* upon *S. enterica* challenge (Meng et al., 2013; Shirron and Yaron, 2011). However, no studies to date have implicated nitric oxide, another component in plant immune signaling, in the *Salmonella*-plant interaction. Therefore, its effect on *S. enterica* populations or colonization strategy in the phyllosphere is unknown. As such**,** in **Chapter 3** Objective 1 was addressed by evaluation of tomato – *Salmonella* Newport interactions mediated by plant derived NO and ROS, as well as the ensuing bacterial response**.** Fluorescent probes, targeted gene expression assays, and the use of NO and H_2O_2 modulators commonly employed

for fungal pathogen work provided the framework to investigate the extent of *S*. Newport – tomato interactions with the following specific aims:

Specific aim 1-1*:* Release of NO, ROS from tomato fruits and seedlings upon *Salmonella Newport* challenge was detected.

Specific aim 1-2: The effect of modulating tomato fruit and seedling levels of ROS, NO on *Salmonella Newport* survival was evaluated.

Specific aim 1-3: Targeted *Salmonella* Newport gene expression profiles while colonizing tomato fruit and seedlings including queries for NO, ROS stress perception and mitigation were constructed.

Although *Salmonella* enterica comprises of over 2500 different serotypes, 10 are responsible for the majority of illnesses (**Chapter 4**). Furthermore, these 10 serovars are not equally distributed among different food commodity associated illnesses. Indeed, research as well as epidemiologic data suggest select *Salmonella* serovars may be source specific (Strawn et al., 2014; Tack et al., 2019), warranting investigation into drivers of adaptation of these organisms to different environmental niches. To add interactions with plants as a driver of serovar commodity associations to the current body of research, in **Chapter 4** Objective 2 was assessed via determination of *S. enterica* serovar specific differences in response to nitric oxide, a common phyllosphere stressor**.** This was accomplished under the following:

Specific aim 2-1: *S. enterica* serovar-specific colonization ability on tomato seedlings were investigated via seedling colonization assays.

Specific aim 2-2*:* The relative NO tolerance of produce-associated and non-produce-associated *Salmonella enterica* serovars was determined *in vitro* and *in planta*.

Finally, surface water used for irrigation is a well-established harbor of *Salmonella,* profiled extensively in the mid-Atlantic (Bell et al., 2015; Callahan et al., 2019; Micallef et al., 2012). However, it is less understood how the hazard of *S. enterica* in water translates to food safety risk of produce irrigated with this water. In **Chapter 5**, Objective 3 was investigated through evaluation of various *Salmonella enterica* serovars population dynamics in MD surface water and reclaimed water sources as well as transfer potential onto tomatoes. Time course studies, culture independent methods, and transfer assays to tomato fruit were employed to evaluate the importance of surface water in the *Salmonella* farm to fork continuum via the following aims:

Specific aim 3-1*:* Bacterial persistence in non-tidal fresh, tidal brackish, pond, and reclaimed water samples was assessed, and culture-based and culture-independent approaches were compared to determine temporal effects on bacterial viability.

Specific aim 3-2*:* The ability of environmental, clinical, and poultry house associated strain(s) of *Salmonella enterica* to transfer from water to tomato fruit were compared both as a function of water source and incubation time in water.

Specific Aim 3-3- Physicochemical data from water sources were analyzed and utilized to determine if any relationships were present between these profiles and *S. enterica* persistence, transfer, or attachment to abiotic surfaces.

In **Chapter 6**, highlights of the present findings are discussed, as well as broader impacts to on-farm food safety management and educational approaches. Limitations of the methodologies employed are discussed for each study, and a path forward for future applied research to address novel or unanswered questions is proposed.

Chapter 2: Literature Review

1. Current trends in the fresh commodity agricultural industry

The demand for sustainable, local, fresh produce is increasing, evidenced in part by fresh fruits and vegetables comprising the top selling category of organic food products (Organic Market Overview, 2017) (USDA, 2017) and the movement to direct-to-consumer fresh produce purchasing (Low et al., 2015; Sparling, 2018; Tropp, 2014). However, the increase in interest of eating more fruits and vegetables is coupled with the increased risk of foodborne illness from fresh produce, due to these products not receiving a commercial "kill" step. Between 2009 – 2015 the Food Disease Outbreak Surveillance System (FDOSS) reported 100,939 illnesses associated with foodborne outbreaks, with 10 percent in seeded vegetables alone (2,572 illnesses) (Dewey-Mattia et al., 2018). In terms of the produce category as a whole, one study across all etiologic agents from 1998 – 2008 found 46 % of all foodborne illnesses were attributed to produce commodities, corroborated by another study from 1973 -2010 finding leaf vegetables and fruits together responsible for 26% of multistate outbreaks, a higher percentage than even beef (22%) (Nguyen et al., 2015).

Foodborne illness is a heavy economic burden for the producer and consumer alike. Using foodborne illness estimates from a 2011 report (Scallan et al., 2011) the USDA economic research service (ERS) estimates that the total economic burden to the US public is 15.5 billion dollars annually (Hoffman et al., 2015). Even more interesting, *Salmonella enterica* subspecies *enterica, Listeria monocytogenes*,

Toxoplasma gondii, and Norovirus attribute to 90 % of this burden (Hoffman et al., 2015). *S. enterica* subspecies *enterica*, the second largest cause of foodborne outbreaks preceded only by Norovirus (Callejón et al., 2015; Dewey-Mattia et al., 2018), is reported to have an average yearly burden of 3.67 million dollars, encompassing loss of productivity, hospitalization costs, and willingness to pay to prevent premature death (Hoffman et al., 2015).

Across all foodborne pathogens, it is estimated that the economic burden to the restaurant sector alone is $3,968 to $2.6 million. This estimate is dependent on the number of lawsuits, outbreak size, lost revenue, and pathogen type (Bartsch et al., 2018). This cost could amount to as much as 5970% of an establishment's annual marketing costs and up to 101% of annual profits (Bartsch et al., 2018). A similar meta-analysis of the cost to primary fruit and vegetable producers has not been undertaken, but examining case studies of fresh produce outbreaks – the 2006 spinach O157:H7 outbreak as well as the 2008 *S. enterica* outbreaks of muskmelon and tomato -- estimate the short-term farm level costs varied from 5.8 million to 25 million dollars (Ribera et al., 2012). Other indirect economic costs, such as the increased import rate of product and the depressed price of commodities after an outbreak also burden the primary producer in the long run (Ribera et al., 2012). Interestingly, this study estimates that the cost of the outbreak greatly outweighs the cost of prevention (Ribera et al., 2012). Taken together, research into strategies to minimize foodborne illness outbreaks from *S. enterica* especially are needed not only to promote public health, but also to sustain a healthy agricultural industry.

2. *S. enterica* and fresh produce: a growing problem

2.1 *S.* enterica *subspecies* enterica *background and clinical significance*

Phylogenetic studies estimate that *S. enterica* and *Escherichia coli* diverged from a common ancestor around 120 – 150 million years ago (Groisman and Ochman, 1996). This event was marked by the former acquiring *S. enterica* pathogenicity island 1 (SPI-1), among other factors (Groisman and Ochman, 1996). *S. enterica* encompasses two species, *Salmonella enterica* (n~2,557) and *S. bongori* (n=22). *S. bongori* is adapted to cold blooded animals, and divergence from *S. enterica* is marked by the acquisition of SPI-2 in the latter. (Groisman and Ochman, 1996). *S. enterica* is further separated into 6 subspecies of around 2500 serovars: *arizonae* (n=99 serovars), *diarizonae* (n=336), *houtenae* (n=73), *indica* (n=15), *salmae* (n=505), and *enterica* (n= 1531) (Grimont and Weill, 2007), with only *diarizonae* and some *indica* having the ability to ferment lactose. The *S. enterica* subspecies *enterica*, (hereinafter referred to as *S. enterica*) is responsible for the majority of human diseases. *Salmonella enterica* serovars can be divided into typhoidal (not zoonotic), causing an estimated 7.5 million illnesses per year globally (Havelaar et al., 2015) and non-typhoidal (zoonotic), accounting for 93.8 million cases a year globally. Of these, an estimated 80.3 million cases are foodborne gastroenteritis (Majowicz et al., 2010). Further differentiation of non-typhoidal illnesses find that 10 *S. enterica* serovars cause the majority of illnesses, comprising 59% of annual infections in the US and around 70% in both Canada and the European Union (Arya et al., 2017) (**Figure 1**). Interestingly, *S. enterica* serovars *S.* Enteriditis, *S.* Typhimurium, *S.* Heidelberg, *S.* 4,[5], 12:i:- , *S.* Javiana, and *S.* Newport are

among the most common *S. enterica* among each country with the exception of *S.* Heidelberg prevalence in the European Union (Arya et al., 2017).

Gastroenteritis, the principle manifestation of disease from non-typhoidal *S. enterica*, requires an infectious dose of at least 10,000 cells on average (van der Heijden and Finlay, 2012). Gastroenteritis is characterized by a relatively short incubation period (8-72 h), followed by non-bloody inflammatory diarrhea, vomiting, nausea, headache, abdominal cramps, and myalgia (Coburn et al., 2007). Symptoms have been historically recorded as self-limiting (Eng et al., 2015), however, because *S. enterica* is an intracellular pathogen there is an increased risk of bacteremia if not resolved (Coburn et al., 2007). This can lead to extraintestinal complications of the lung, urinary tract, endocardium, and meninges (Eng et al., 2015). Furthermore, sequelae, or the advent of chronic complications from an acute illness, has been documented in the aftermath of *S. enterica* outbreaks in the form of reactive arthritis (Arnedo-Pena et al., 2010; Porter et al., 2013). This drives home two important points; firstly, that the cost of foodborne pathogen outbreaks are not limited to product compromised from the marketplace, and secondly that foodborne illness is serious and can drastically alter human quality of life.

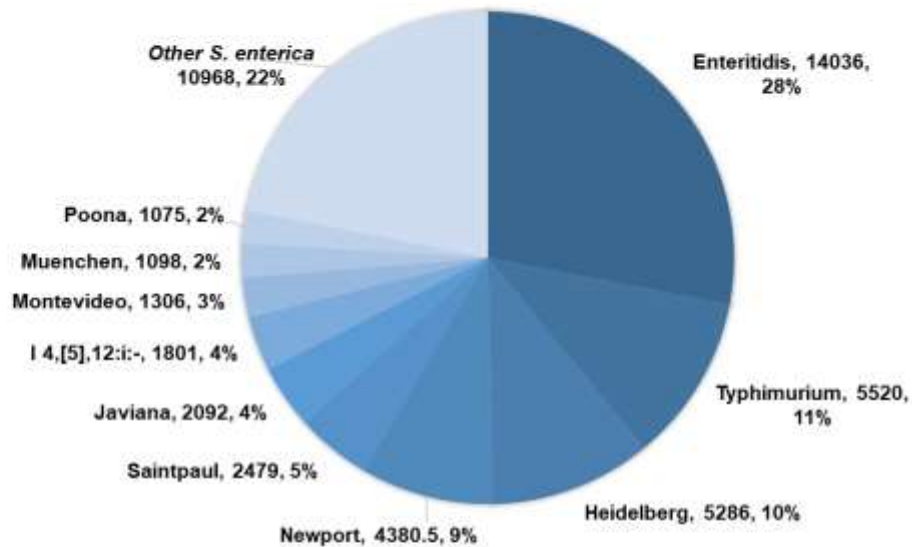


Figure 1: Top 10 *S. enterica* causing foodborne illness in the United States across all food commodities from 1998 – 2017. Number of illnesses are provided as well as percent of total illnesses. Data adapted from CDC National Outbreak Reporting System https://wwwn.cdc.gov/norsdashboard/.

2.2 S. enterica *foodborne outbreak trends*

While *S. enterica* has been found to associate with a diverse range of food commodities from dried chia seed powder (Harvey et al., 2017) to poultry (Antunes et al., 2016) and papayas (Gibbs et al., 2009), *S. enterica* and seeded vegetables are a formidable pathogen commodity pair. *S. enterica* was second to only Norovirus in illnesses from fresh fruits and vegetables from 2004-2012 (Callejón et al., 2015). FDOSS and the National Outbreak Reporting System (NORS) reporting 2018 data from just 10 US laboratory sites ranked illnesses with this pathogen commodity pair second and third respectively behind *S. enterica* in chicken and *S. enterica* in pork

(Dewey-Mattia et al., 2018; Tack et al., 2019). Further, one study identified vegetable row crops, seeded vegetables, and fruits as the three most common sources of *S. enterica* associated produce outbreaks from 1998 -2013 (Bennett et al., 2018). Tomatoes were by far the most common produce item within the seeded vegetable category, with 42 out of a total 66 outbreaks (Bennett et al., 2018). This was corroborated by another study of *Salmonella* outbreaks between 1998- 2008, where in 19 vine stalk vegetable outbreaks, 90% were from tomatoes (Jackson et al., 2013).

Serovar specific associations in outbreaks can also be gleaned from epidemiological data. In a cross section of illnesses from NORS data of *S. enterica* illnesses in 2018, *S.* Typhimurium, *S.* Newport, and *S.* Enteriditis were the three most common isolates, following similar trends observed in 2008 and 2015. (Jackson et al., 2013; Marder et al., 2018; Tack et al., 2019). Further, epidemiological evidence supports serovar specific associations with some food commodities over others. One report found more than 80% of outbreaks caused by the serovars *S.* Enteriditis, *S.* Heidelberg, and *S.* Hadar were attributed to eggs and poultry during the given study period (Jackson et al., 2013). In the case of *S.* Javiana, *S.* Mbandaka, *S.* Muenchen, *S.* Poona, and *S.* Senftenberg more than 50% of outbreaks were from plant commodities (Jackson et al., 2013). Interestingly, this research reported *S.* Typhimurium and *S.* Newport were associated with diverse food types and were therefore generalists, although 29% of vine stalk vegetable outbreaks were due to *S.* Newport (Jackson et al., 2013). Similarly, study of the 15 US multistate tomato outbreaks between 1990 and 2010 revealed *S.* Newport as the most implicated serovar (n= 6 outbreaks) followed by *S.* Braenderup (n=2), *S.* Enteriditis, *S.* Javiana, *S.* Montevideo, *S.*

Thompson, and *S.* Typhimurium (n=1 each) (Bennett et al., 2015). Taken together, epidemiological data suggests that *S. enterica* outbreak trends reflect key serovar – commodity associations, therefore identifying the sources of these serovars and drivers behind their establishment or preference for different food commodities are important for reducing *S. enterica* outbreaks.

2.3 *Outbreak traceback establishes* S. enterica *as a hazard of pre-harvest crop production areas*

Finally, while changes in produce production, distribution, and consumption trends coupled with the increased ability of finely tuned molecular methods to detect outbreak could account for the observed increase in produce illnesses (Olaimat and Holley, 2012), there is no denying that the agricultural environment may be an amenable niche for this organism. This In key historic outbreaks *S. enterica* were not traced back to the point of consumption, retail, or packinghouse, but rather the crop production environment. Outbreaks with tomato, peppers and papaya have been successfully traced back to irrigation or wash water (CDC, 2008; Gibbs et al., 2009; Greene et al., 2008). Furthermore, sampling of irrigation surface water sources has also identified *S. enterica* with identical Pulse Field Gel Electrophoresis (PFGE) patterns to outbreak isolates, found in the case of irrigation ponds in Georgia harboring *S.* Thompson, *S.* Enteriditis, and *S.* Javiana (Li et al., 2014a). Moreover, research also reports that surveys of livestock rarely find plant associated outbreak strains of *S. enterica* (Foley et al., 2008), suggesting that *S. enterica* may be specifically adapting to the produce agricultural environment as an amenable niche.

Additionally, geographic factors may play a role in *S. enterica* outbreaks. For example, two mid-Atlantic states-- Pennsylvania and Virginia-- were regularly implicated (9 and 10 outbreaks, respectively) out of the 15 tomato multistate tomato outbreaks from 1990 – 2010 (Bennett et al., 2015). The Delmarva region specifically has hosted tomato outbreaks in 2002, 2005, 2006, and 2007 all with *S. enterica* Newport PFGE XbaI pattern JPX01.0061 (Bennett et al., 2015). The mid-Atlantic coastal region has furthermore been pinpointed as high risk for negative impacts on food safety due to climate change, where increased extreme temperature and rain events are associated with increased risk of salmonellosis (Jiang et al., 2015). For these reasons, evaluating *S. enterica* dynamics or adaptation to the agricultural environment in the mid-Atlantic is imperative to continually improve management practices to minimize enteric bacteria contamination and persistence on fresh produce.

3. Current efforts to control *S. enterica* on the farm

3.1 *Prior to 2010: voluntary standards and best practices for minimizing foodborne pathogen contamination during crop production*

Good agricultural practices (GAPs) are a series of farm production techniques and standard operating procedures that are devised to minimize the risk of microbial contamination and proliferation on the edible portions of fresh fruit and vegetables, as detailed in a 1998 guide published by the Food and Drug administration (FDA) (Services, 1998). When adopted, GAPs have been shown to improve farm efficiency, increase product quality, create better market access, and be protective against

commodity associated outbreaks (Bihn and Gravani, 2006). The practices were written in general terms with no reference to any specific agricultural system, such that they could apply to a diverse range of commodities, farm scale, and cropping systems. These practices are divided into four main areas that impart high probability of pathogen hazard; **W**ater, animal **W**aste, **W**ildlife, livestock and domesticated animals and **W**orkers – often dubbed the 4 W's.

According to GAPs, water used in production should be of appropriate microbial quality for the intended use (Services, 1998). To accomplish this, farmers should consider the historic and current use of their land, document weather events, maintain their water delivery and holding systems in good working conditions, and query their microbial water quality through periodic testing (Services, 1998). Use of the fecal indicator *E. coli* is recommended to farmers to assess their water quality, selected for its low ability to multiply in the environment and because it shares a common source with pathogens of interest (US EPA, 1986). Generally, municipal water has high microbial quality and therefore would not usually require any intervention prior to use, although it is costly to obtain. Surface water sources such as lakes, ponds, rivers, creeks, and shallow wells on the other hand are exposed to the environment and subject to many factors that can cause fluctuations in their microbial quality, therefore careful monitoring of these water sources is warranted. If microbial testing deems the water to be of low quality, GAPs suggest farmers implement mitigation procedures to minimize potential contamination of edible portions of the crop with this "risky" water, which could potentially harbor pathogenic microbes. This can include installation of a sand filter, employment of drip irrigation lines, or implementing

microbial die off periods between last irrigation and crop harvest (Services, 1998). Mitigation procedures can also include water treatment, although costly and usually adopted as a last resort. Acceptable irrigation sanitizers include ethanol, isopropanol, calcium hypochlorite, chlorine dioxide, and sodium hypochlorite (National Organic Program, 2000). Peroxyacetic acid (PAA) can be used for controlling blight and pathogenic bacteria, seed disinfection, and food contact surface cleaners (National Organic Program, 2000). SaniDate 12.0, a 12% PAA solution, is a regularly used commercial form of peroxyacetic acid used in irrigation line treatment (Arancibia et al., 2017). While there is no doubt these efforts have minimized some on-farm food safety hazards, continuing outbreaks in fresh produce have revealed that a better understanding is needed surrounding pre-harvest interactions between pathogens and crops to identify gaps in this approach where contamination and persistence of human pathogens can still occur.

3.2 *From reactionary to preventative: the goal of FSMA PSR*

With the implementation of the Food Safety Modernization Act Produce Safety Rule (FSMA PSR) signed into law December 2010, several of the previously recommended GAPs became mandatory for farmers based on their operation size and income (Standards for the Growing, Harvesting, Packing, and Holding of Produce for Human Consumption). Included in this rule are strict standards for agricultural water- - any water that contacts the edible portion of a "covered[1]" crop. Farmers are now required to assess the microbial water quality of their agricultural water through

[1] Covered here meaning a commodity which is legally covered in the FSMA PSR, or a Raw Agricultural Commodity (RAC).

routine *E. coli* testing throughout the growing season for each irrigation source, developing a microbial water quality profile (MWQP) (Standards for the Growing, Harvesting, Packing, and Holding of Produce for Human Consumption). Because surface water is deemed more variable, these sources must be sampled at a higher frequency (N=20 samples with 5 new each year) compared to groundwater sources. Furthermore, the regulation has numerical criteria of *E. coli* which these water samples must fall under to deem appropriate for use without mitigation steps applied. For agricultural water, *E. coli* geometric mean of the samples must be below 126 CFU/ 100 mL water, and no one sample can peak above the statistical threshold value of 410 CFU/ 100 mL water (Standards for the Growing, Harvesting, Packing, and Holding of Produce for Human Consumption). Mitigation steps if water is of insufficient quality are similar to what is recommended by GAPs, and have similar drawbacks (Standards for the Growing, Harvesting, Packing, and Holding of Produce for Human Consumption).

It is important to consider the burden growers must shoulder in complying with the PSR. The FDA environmental impact statement calculated that the cost of compliance could be estimated at $4,477 per year for very small farms, $12,384 per year for small farms, and $29,545 per year for large farms (Lichtenberg and Page, 2016). As such, small and mid-sized farms may be disproportionately burdened with implementation tasks (especially copious water sampling) as well as the cost of PSR compliance compared to larger farm operations (Lichtenberg and Page, 2016). It is anticipated that these farms would be the most likely to make detrimental management decisions to keep production under a certain threshold so that they

remain excluded or can apply for a qualified exemption from the provisions of the PSR. These management decisions could adversely affect marketing channels and potential for future growth of small farms. Furthermore, by being excluded from the Rule these farms may not be engaging in practices to minimize food safety risk on the farm – a public health concern certainly against the tenants of the PSR to promote an "on-farm food safety culture." Agricultural Extension in many parts of the US has realized this issue, and is adopting a strategy to target these small farms for GAP trainings as a foundation for and relieving some burden to FSMA PSR compliance (Marine et al., 2016). However, opportunities also exist for on-farm research to support farmers by providing data which can be directly applied to sensible food safety policy making decisions.

4. *S. enterica* in the specialty crop production environment: interactions in surface water used for irrigation

4.1 S. enterica *presence from farm to fork*

It is well known that enteric bacteria can persist on plants, in soil, and in water systems (Ongeng et al., 2015). This success can be affected by farm location, agricultural practices, temperature, season, humidity, resident microbiota, soil chemistry, and nutrient availability (Gu et al., 2018; Martínez-Vaz et al.). *Salmonella enterica* reservoirs include environmental matrices as well as asymptomatic and symptomatically shedding wildlife and livestock (Cummings et al., 2009). For example, *S. enterica* can survive in manure for 21 days at 33°C and up to 227 days at 7°C (Semenov et al., 2007). Animals and their feces, hair and feathers can harbor and

transmit foodborne infectious disease agents (Beuchat, 2006). Furthermore, prevalence in animals may vary by region and by animal species (Olaimat and Holley, 2012). Apart from manure, pathogens may persist in soil and sediment. *S. enterica* and pathogenic *E.coli* have been shown to survive in soil from 7 to 25 weeks depending on soil type, climatic conditions, moisture level, temperature, and farm management practices of biological soil amendments of animal origin (Erickson et al., 2010; Guo et al., 2002; Lang and Smith, 2007; Marine et al., 2015). Furthermore, survival in soil for some pathogens can be extended by low substrate pH, low water availability, high nutrient availability and soil composition with preference towards loamy soils (Bech et al., 2010; Franz et al., 2005).

All said, surface waters, such as ponds, lakes and creeks, are the most common water source for harboring and persistence of foodborne pathogens (Maffei et al., 2016). This may be due to residency ability in water itself, or the continued shedding of S*almonella* from harborages into these systems. Elucidating the interactions among plants, human bacterial pathogens, and the agricultural environment is important to fully understanding *S. enterica* food safety risk to fresh crops grown in such environments.

4.2 Irrigation water sources available for fresh produce crops

Water is used in many activities from farm to fork- from irrigation, frost protection, evaporative cooling, to cooling and washing produce, sanitizing tools and handwashing. Many water uses contact the edible portion of crops and therefore opportunities exist for pathogen transfer if the water is contaminated. Irrigation water has been regularly implicated as a vehicle for foodborne pathogens, with differential

microbial risk associated for various water sources. Ground water sources that are properly designed and maintained are generally consistent in microbial quality (Gerba, 2009). However from a sustainability standpoint, groundwater aquifers are a precious natural resource under increased withdrawal pressure (Perlman, 2016). Reports from Maryland monitoring efforts have identified increased use of these aquifers for irrigation has led to lower levels in recent years (Groundwater Protection Program: Annual Report to the Maryland General Assembly, 2012). Further, one aquifer in the Maryland coastal plain (upper Patapsco) will take so long to recharge it is effectively rendered a non-renewable resource (Groundwater Protection Program: Annual Report to the Maryland General Assembly, 2012). Therefore, it is extremely important that in addition to safety, irrigation management decisions are made with sustainability in mind.

Around many areas in Maryland groundwater use for irrigation of crops is prevalent and increasing (**Figure 3**). Notwithstanding, surface water is used for irrigation in key Maryland counties, with the majority on the eastern shore (**Figure 3**). This variability is reflected on other areas on the east coast; a survey in Georgia, South Carolina, and Virginia reported 12.4% of farmers use surface water to irrigate specialty crops (Harrison et al., 2013). In New York, although the study did not state which crops, 57% of farmers in 2008- 2009 reported using surface water for irrigation (Bihn et al., 2013).

Another option for irrigation currently being explored is the use of reclaimed water for fresh produce irrigation. Reclaimed water is waste water that has gone through secondary or tertiary treatment processing to regain high microbiological

quality (Samer, 2015; US Environemental Protection Agency, 1998). Reclaimed water is not currently available for use in Maryland on fresh produce crops, but is emerging as an alternative source of irrigation water other places in the United States such as Arizona with strict guidelines for appropriate and inappropriate use (Rock et al., 2012). It is important to note that *S. enterica* may still be present in this water, as one study from Spain found three *S. enterica* positive samples post tertiary treatment (Santiago et al., 2018). However, proper management can minimize this risk (Wu et al., 2009). As an emerging sustainable option for water, more research into *S. enterica* specific harborage in reclaimed water and transfer risk to produce is warranted.

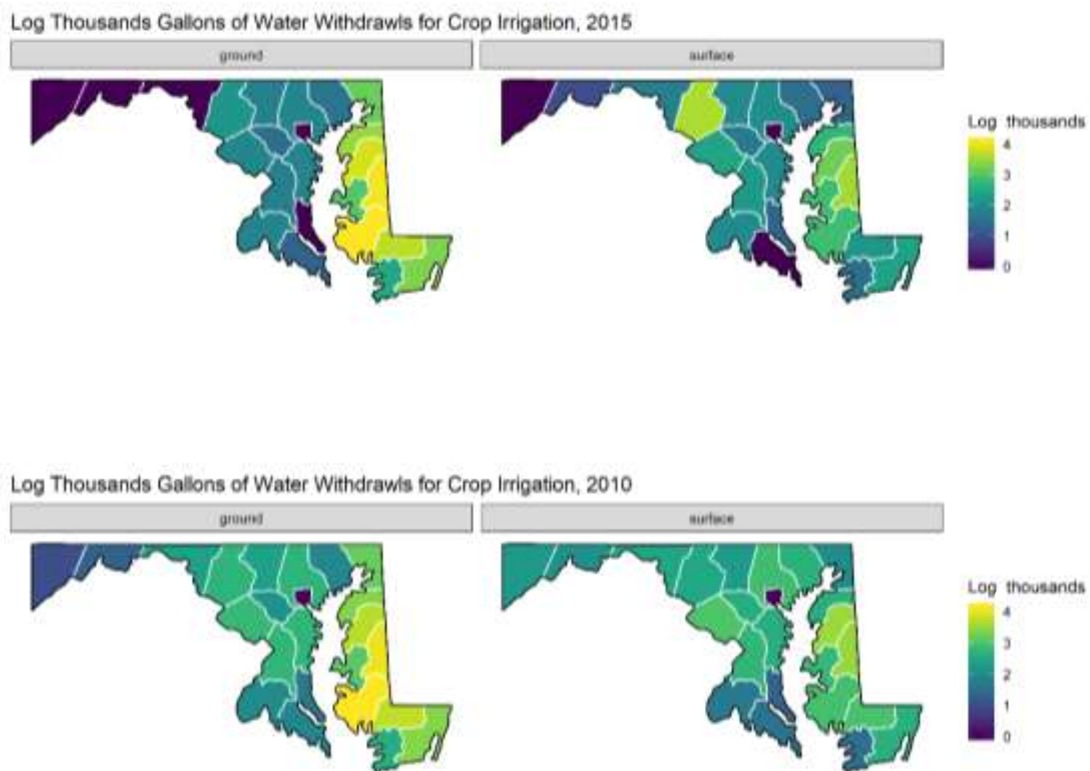


Figure 2: Surface water and ground water withdrawals in Maryland in 2015 and 2010 for crop irrigation, in log thousand gallons per day. Adapted from Dieter, C.A., et. al. U.S. Geological Survey data release, https://doi.org/10.5066/F7TB15V5.

4.3 S. enterica *prevalence and persistence in surface irrigation water*

Salmonella enterica specifically has been recognized as a potentially endemic taxon in surface waters in important agricultural regions of the US. Creeks, ponds, and rivers on the east coast have reported an *S. enterica* prevalence rate between 8.4% (Virginia eastern shore) to 79% (Georgia river basin) (Bell et al., 2015; Haley et al., 2009). Reported prevalence is affected both by season, with more positive samples occurring in August (Haley et al., 2009; Li et al., 2014a; Luo et al., 2015), and positive associations with rainfall events (Jones et al., 2014; Luo et al., 2015). Commonly identified serotypes include *S.* Newport, *S.* Javiana and *S.* Typhimurium (Bell et al., 2015; Callahan et al., 2019; Haley et al., 2009; Jones et al., 2014; Li et al., 2014a; Luo et al., 2015; McEgan et al., 2014; Micallef et al., 2012). These observations have been repeatedly found in other areas of the US and globally (Benjamin et al., 2013; Huang et al., 2014). This high environmental prevalence may have interesting implications in public health, as Georgia also endures an increased prevalence of salmonellosis compared to the national average (24 cases / 100,000 vs 15 cases per 100,000) (Maurer et al., 2015). These data further necessitate research into understanding the connections between environmental prevalence and impact on the public health of the surrounding community.

To address these data gaps, research in persistence and transfer ability to crops has been undertaken to understand foodborne pathogen risk to human health from

irrigation of *S. enterica* harboring water. This body of work has revealed that river and marine water can support *S. enterica* for up to 45 days, the level of which is strain dependent and may have included non-culturable metabolic states (Roszak et al., 1984; Santo Domingo et al., 2000). In pond water, *S. enterica* was found to be viable and culturable after 168 days (Topalcengiz et al., 2019). Furthermore, *S. enterica* can survive well in water sediments (Moore et al., 2003). Formation of biofilms in water, water sediment, and water distribution systems can increase persistence of multiple Enterobacteriaceae including *S. enterica* (Pachepsky et al., 2012; Sha et al., 2011). Taken together, this continued source of inoculum could be a risk to irrigated crops, although many questions around the mechanisms and probabilities remain.

4.4 S. enterica *transferability from surface irrigation water to crops*

The current state of research concerning transfer ability of foodborne pathogens to crops illustrate the influence of irrigation method, contamination load, plant and bacterial genotype can influence successful transfer. For example, *E. coli* O157:H7 was successfully transferred from overhead applied water to lettuce, but rapid bacterial declines occurred in days (Xu et al., 2016). Virulent *S*. Typhimurium persisted on parsley for 28 days and only 2 days with lower inoculum levels (Kisluk and Yaron, 2012). By contrast, irrigation with attenuated *S*. Typhimurium contaminating parsley and lettuce persisted for 63 and 121 days, respectively (Islam et al., 2004). Further still, a group investigating *S*. Enteriditis found it did not transfer to tomatoes at all (Jablasone et al., 2004). Finally, repeated contamination through irrigation spray has been shown to increase pathogen titers in the phyllosphere, suggesting continued irrigation with contaminated water can increase transfer success

(Solomon et al., 2003; Van der Linden et al., 2013). It is interesting to note that pathogen transfer is not limited to irrigation practices, as one study showed foliar pesticide constructed from a contaminated water source could support *S. enterica* persistence and allow for transfer to crops (Lopez-Velasco et al., 2013).

The necessary mechanisms needed for bacterial transfer are an active area of study, with most headway made in illustrating the importance of attachment apparati. For example, *S.* Typhimurium attachment to parsley following irrigation is dependent on curli and to a lesser extent cellulose production (Lapidot and Yaron, 2009). Biofilm production has been also identified as potentially important, as seen in lettuce models (Kroupitski et al., 2009). Interestingly, other groups have identified biofilm formation and attachment to produce may be serovar specific features. For example, Patel et al. identified poultry associated serovars were poor biofilm producers and attachers to spinach compared to produce isolates (2013). Taken together, there are numerous experimental factors which require controlling for robust and realistic study into transfer dynamics of foodborne pathogens. Once undertaken, these data can be more directly applied in risk modeling to inform safe irrigation practices without the explicit recommendation to switch to a less sustainable water source.

4.5 Efforts in using E. coli *to monitor water quality*

For foodborne pathogen risk mitigation, much research has been devoted to assessing the accuracy of biological indicators to provide fast, comparatively inexpensive data on the relative quality of a water source. *E. coli* has been an accepted indicator for fecal contamination and water quality for many years (US EPA, 1986), however recent research is uncovering that the efficacy of using this

organism as an indicator is variable. Over a 12-month period sampling Florida ponds, creeks, and rivers, McEgan et al. corroborated the adequacy of *E. coli* as an indicator for *S. enterica* using logistic regression (2013), as did a study from southern Georgia and Florida (Luo et al., 2015). Conversely, a 3 year sampling effort of Florida ponds revealed that water quality parameters, including *E. coli,* did not guarantee the absence of *S. enterica* (Topalcengiz et al., 2017). Furthermore, in California pond and stream sediment *E. coli* prevalence was not correlated with *S. enterica* presence (Benjamin et al., 2013). Aside from *S. enterica,* one Canadian pond study found that *E. coli* was inversely correlated with *Listeria monocytogenes* presence (Wilkes et al., 2009). Metadata assimilation of indicator studies in 2015 found that overall, the correlation coefficient between the presence of *S. enterica* and indicators was generally weak and region specific (Pachepsky et al., 2016). The season, water body size, water type, flow rate, past rain events, and sample size were all shown to influence indicator *E. coli* concentrations (He et al., 2007; Wu et al., 2011). Indeed, one of the aforementioned studies which asserted positive correlation between *S. enterica* and *E. coli* recommended that the sample size for such determination be greater than 20 samples due to high variability (Havelaar et al., 2017). From a produce grower's perspective, the current FSMA PSR requirement of 20 samples (with an additional 5 each year) is already too burdensome, rendering recommendations for additional sampling ineffective.

For selection of an appropriate indicator, and the scientific community must carefully consider robustness of the method, ease of sampling as well as ease of interpretation of results for maximum public health benefit. Some studies

investigating the effect of rain events on water bacterial community dynamics have indicated that there may be other candidates for indicators (Chen et al., 2018), as one study in Philadelphia found that following such events *Bacteroides* and not *E. coli* correlated with human pathogen prevalence in urban creeks (McGinnis et al., 2018). Through another metadata study, Wu et al. found that while no single indicator from a suite of viral and bacterial candidates correlated best with the presence of human pathogens, a combination of a few indicators including F specific coliphage[2] and *C. perfringes* may more appropriately correlate with human pathogen presence (2011). Indeed, further study into both foodborne pathogen and potential indicator population dynamics in water and response to environmental, anthropogenic, geographic, and climatic variables is warranted.

5. *S. enterica* in the specialty crop production environment: interactions in the phyllosphere

For *S. enterica* to successfully make the connection between persistence in agricultural water and contamination on fresh produce, this organism must attach, colonize, and mitigate stressors on the plant surface. Elucidating the interaction between plants and enteropathogens is a burgeoning area of research, where the scientific community has capitalized on interdisciplinary collaboration to answer increasingly fine-tuned questions. From this research, several important components have been found to drive plant-bacterial associations; plant genotype, bacterial

[2] "male" specific bacteriophage, infector of *E. coli*

genotype, ambient environment, and bacterial interaction with plant surface features both chemical and biotic [for review, see (Martínez-Vaz et al., 2014)].

5.1 Plant factors

One major determinant of colonization success lies in plant genotype and tissue site. Concerning the tomato plant structure and genotype, *S. enterica* colonization has been shown to be cultivar dependent with type 1 trichomes as preferred colonization sites (Barak et al., 2011; Han and Micallef, 2014). Furthermore, sugars, sugar alcohols, and organic acids differentially present on tomato cultivar surfaces were shown to be positively correlated with *S. enterica* growth, while fatty acids, including palmitic and oleic acids, were inversely correlated (Han and Micallef, 2016).

Interaction with both epiphytes and plant pathogens have been found to be beneficial for *S. enterica* persistence on produce (Brandl and Mandrell, 2002; Potnis et al., 2015). Lettuce studies with *S.* Montevideo reported increased recovery from plants with healthy resident epiphyte populations, perhaps due in part to resident release of extracellular polysaccharides which *S. enterica* can utilize (Poza-Carrion et al., 2013). More interesting is the thought that interactions with plant pathogens on produce could "prime" *S. enterica* by eliciting upregulation of certain virulence factors. One study investigating *S.* Typhimurium colonization of lettuce soft rot reported an upregulation of multiple genes necessary for colonization on chicken host models including *hmpA*, a nitric oxide dioxygenase, and *ycfR* (Goudeau et al., 2013).

5.2 Bacterial factors

Recently, interest has centered around the genetic profile required for *S. enterica* successful colonization and persistence on crops. Principally, bacterial serotype itself may play a role in determining successful colonization, as some serovars display successful persistence compared to others in sprout, lettuce, herb, and tomato models (Cui et al., 2018; Guo et al., 2002; Klerks et al., 2007; Patel and Sharma, 2010; Reed et al., 2018; Shi et al., 2007; Zheng et al., 2013). A host of bacterial genes involved in successful interactions with produce have also been elucidated which fall under the following categories: cell surface structure, virulence, motility, evasion and mitigation of plant defense, biofilm formation, and an overall downshift in bacterial metabolism [for comprehensive reviews, refer to (Brandl et al., 2013; Martínez-Vaz et al.)]. While interrelated, these studies can be loosely grouped into two primary aims 1) investigating adaptation to the plant environment as an alternate host with a distinct suite of traits needed and 2) endeavoring to draw parallels between plant colonization and colonization strategies of *S. enterica* in the human gut. Endophytic studies in plant wounds and soft rots have identified iron acquisition, amino acid biosynthesis, ascorbate metabolism and cell structure maintenance as important in *S. enterica* persistence (de Moraes et al., 2017, 2018; Goudeau et al., 2013). On phyllosphere surfaces and plant cell wall models, *S. enterica* preferentially exhibits chemotaxis towards photosynthetically active areas like stomata (Kroupitski et al., 2009) and attachment apparati including flagella, O-antigen capsule assembly, curli nucleator, cellulose, and production of extracellular matrix (Barak et al., 2005, 2007; Salazar et al., 2013; Tan et al., 2016) also drive

colonization success. *ycfR, sirA, yigG,* genes for stress regulation, biofilm formation, and virulence (in the case of *sirA*) are important for chlorine stress response and attachment to spinach leaf and grape tomato in *S*. Saintpaul and *S*. Typhimurium (Salazar et al., 2013). Other virulence genes such as flagellin *fliC* are important for success in the basil model on a serovar specific basis -- with a mutation in *S*. Senftenberg significantly negatively impacting attachment whereas *S.* Typhimurium remained unaffected (Berger et al., 2009). Taken together, this suggests *S. enterica* persistence may include actively strategizing amenable niches for colonization of plants as an alternative host.

5.3 Bacterial and plant interaction under plant immunity: PTI, ETI, NO, ROS

Plants can engage in a range of interactions with resident epiphytes and endophytes, from assistance with plant growth promotion, nutrient availability, and gaining protection from abiotic and biotic stress to name a few (Chagas et al., 2018). Another driving interaction of plant-microbe associations is interaction with plant immunity. Plants have elegant mechanisms to not only distinguish self from non-self, but also to differentiate between beneficial bacteria from non-beneficial or pathogenic bacteria. Two scenarios of immunity have been classically described in plants; PAMP triggered immunity (PTI) and effector triggered immunity (ETI) (Jones and Dangl, 2006). In PAMP triggered immunity, pathogen associated molecular patterns (PAMPs) or microbe associated molecular patterns (MAMPs) are recognized by pathogen recognition receptor proteins (PRR) on the plant cell wall (Jones and Dangl, 2006) such as flg22, elf18, or chitin (Felix et al., 1999; Hayafune et al., 2014; Kunze et al., 2004). PRR interaction with PAMPs causes an influx of calcium and hydrogen

ions to initiate a signal cascade within the cell (Frederickson Matika and Loake, 2014), triggering a release of nitric oxide (NO) and reactive oxygen species (ROS). The release of these two signaling molecules serve multiple purposes for the plant. ROS burst can directly control potential pathogen threat and may interact with some Mitogen activated protein kinase (MAPK) signaling, while through promotion of phosphatidic acid NO production engages in signaling downstream MAPK (Frederickson Matika and Loake, 2014; Raho et al., 2011), Calmodulin protein kinase (CDPK), and transcription factors which initiate transient defense responses (pathogenesis related protein 1 (PR-1), salicylic acid production) that comprise PTI (Bigeard et al., 2015).

Some plant pathogens have evolved to use a second wave of proteins, called effector proteins, to disrupt the signaling cascade and PTI (Jones and Dangl, 2006). Furthermore, these effectors can be used to promote plant cell nutrient leakage and pathogen dispersal (Jones and Dangl, 2006). Some effector proteins are plant hormone mimics, and can therefore disrupt developmental cellular processes (Lindeberg et al., 2012; Thomma et al., 2011). This disrupts plant immune signaling, allowing the pathogen to spread and cause disease. In another evolutionary push, some plants developed resistance or “R” proteins to interact with specifically released effectors from the pathogen in order to block effector protein modes of action (Jones and Dangl, 2006). This interaction maintains the integrity of the plant immune signal cascade, therefore eliciting an immune response dubbed effector triggered immunity, ETI. While interactions with PTI and ETI are described in the context of plant pathogens, plant-enteric pathogen recognition and response are less well known.

It is important to note that NO and ROS are involved in multiple plant physiological processes. ROS is produced in multiple cell types in plants by Respiratory burst oxidase homologs (RBOH) -NADPH oxidase genes (Zhang et al., 2018). NO production in plants is currently still under investigation, however it is postulated NO can be generated both enzymatically and non-enzymatically. Nitrate reductase-dependent NO is important for stomatal movements, hormone responses, salt, osmotic, cold stress, root and floral development (Astier et al., 2018a). Inducible nitric oxide synthases (NOS) were once thought to be responsible for immune related production of NO as there are homologous enzymes in mammalian host immunity, however studies which claimed to isolate the enzyme actually isolated a GTPase (Moreau et al., 2008), and in a study of 1000 land plant transcriptomes no homologs of NOS were found (Jeandroz et al., 2016).

NO partakes in three main post-translational modifications of proteins that all impact the conformation, activity, or localization of the protein via 1) reversibly binding of a thiol group in cysteine residues (N-nitrosylation), 2) nitrosylation of tyrosine, and 3) interacting with the heme center of metalloproteins (Astier et al., 2018b) This activity allows NO to interfere with phytohormone signaling pathways such as cytokinin, giberillin, abcisic acid, and auxin (Feng et al., 2013; Prakash et al., 2018; Sanz et al., 2014; Signorelli and Considine, 2018), providing a pathway by which NO is involved in regulation of many plant developmental activities.

Signaling crosstalk between NO and ROS has been identified. Research has revealed interaction between the two molecules are essential for leaf cell death (Lin et al., 2012), to delay senescence (Iakimova and Woltering, 2015), root growth and

development (Liao et al., 2009), stomatal closure (Huang et al., 2015), and pollen tube growth (Serrano et al., 2012). It is not yet clear production of which chemical precedes the other. In the case of stomatal closure, ROS stimulates NO production, as seen in tomato (Shi et al., 2015). However in other processes NO may stimulate H_2O_2 production, as shown during rooting behavior of *Tagetes erecta*, the Texas marigold (Liao et al., 2011). Furthermore, NO is required for H_2O_2 production in *Arabidopsis thaliana* elicited by *Botrytis* oligosaccharides (Rasul et al., 2012). Finally, as mentioned earlier, NO production is partly required for phosphatidic acid (PA) which interacts with CDPK's and PDK's to activate downstream transcription factors, as well as RBOHD. Addition of PA has been shown to create an ROS burst, further suggesting the pathways are connected (Bigeard et al., 2015). Taken together, this illustrates NO ROS crosstalk is a complex signaling network which research to date has not completely elucidated all necessary players to fully understand these phenomena.

5.4 Documented S. enterica *response to plant immunity*

To determine the existence of a significant interaction between plants and enteric pathogens, demonstration of plant recognition to the select agent is key. To this effect, flagella from *S. enterica* Typhimurium has been shown to elicit MAPK signaling in *A. thaliana* leaves (Garcia et al., 2014). In this study, pretreatment of plants with *S*. Typhimurium flg22 triggered resistance against *Pseudomonas syringae* DC 3000 colonization. This was demonstrated by interaction with MAPK 3,4,6 and correlated with the upregulation of transcription factors, both hallmarks of PTI (Garcia et al., 2014). Low induction of MAPK and defense associated gene PR-1

expression from vacuum infiltrated *S*. Typhimurium challenged *A. thaliana* (Schikora et al., 2008) and PR-1 expression from drip inoculated *S.* Dublin on lettuce has also been documented (Klerks et al., 2007). A study conducted by Shirron and Yaron found that *S. enterica* Typhimurium does indeed elicit ROS production (corroborated by Meng et al. in tomatoes) when syringe infiltrated into tobacco leaves, however significantly more ROS was produced when the leaves were challenged with LPS, flagella or chloramphenicol treated cells alone (2011, 2013). Finally, in comparing plant stomatal responses to *E. coli* and *S. enterica* it was found that stomatal immunity was only transiently displayed for *S.* Typhimurium in a plant cultivar dependent manner (Roy and Melotto, 2019) but robust for *E. coli* O157:H7 (Roy et al., 2013). These studies suggest *S. enterica* may either weakly initiate plant defense systems or employ mechanisms to mitigate plant defense related activity.

Interestingly, investigation of *S. enterica* - plant recognition studies have revealed some cellular components elicit variable, at times serovar specific responses. In one study, *S*. Typhimurium flagella elicited such a response while *S*. Senftenberg flg22 failed to produce similar results (Garcia et al., 2014). To delve deeper into this, *in silico* it was found that *S*. Typhimurium flg22 had four amino acid residue differences from the FliC of *Pseudomonas syringae* pv tomato (Meng et al., 2013). *S. enterica* can express two distinct flagellar filament proteins, FliC and FljB, leading to phase variation (Bonifield and Hughes, 2003). These two proteins are conserved in the amino and carboxy termini, yet have a variable middle region leading to different antigenic capacities in the mouse infection model (Bonifield and Hughes, 2003). Using *fliC* and *fljB S. enterica* mutants infiltrated into *Nicotiana benthamania*, it was

determined that the *fliC* gene was a major contributor to PTI response and not the *fljB* gene phase (Bonifield and Hughes, 2003; Meng et al., 2013). As *S. enterica* is a Gram-negative bacterium, it would also be reasonable to hypothesize that the lipopolysaccharide layer (LPS) in the outer wall of the cell could elicit PTI, however currently little research indicates significant solicitation of PTI from the LPS of *S. enterica* serovars Typhimurium, Montevideo, Dublin, Muenster, or Anatum (Meng et al., 2013). Thus, while the flagella may be a significant immune response initiation factor, research into recognition of other cellular components across serotypes is warranted.

Other plant defense molecules downstream of PAMP recognition have also been investigated in *S. enterica* plant interactions. Knowledge of *S. enterica* as an intracellular pathogen utilizing type 3 secretion systems (T3SS) (van der Heijden and Finlay, 2012) prompted investigation into analogous interactions in the plant phyllosphere. It has been shown that *S. enterica* with a defective T3SS colonizes plants significantly less efficiently, (Garcia et al., 2014; Iniguez et al., 2005). Furthermore, the *S. enterica* effector SpvC which is needed for host infection (Haneda et al., 2011) was shown to be active in protoplasts and upregulated plant defenses (Neumann et al., 2014). However, to date no realistic mechanism for *S. enterica* effector delivery during plant surface colonization has been described.

Other downstream PTI activity, such as salicylic (SA), jasmonic acid (JA) and ethylene (ET) have also been described. One study reported increased transcription of hormone regulated defense proteins PR-1, PR-2, and PR-4 when challenged with *S.* Typhimurium, suggesting pathogen induction of salicylic acid and jasmonic acid

signaling activity (Schikora et al., 2008). Furthermore, when *S.* Typhimurium was inoculated via dip onto *NahG*, *ein2*, and *coi1* plants (salicylic acid, ethylene, and jasmonic acid signaling deficient mutants) it was reported that JA and ET mutants harbored significantly more bacteria than the wild type *A. thaliana*, (Schikora et al., 2008).

5.5 *Opportunity for identifying cross kingdom associations in* S. enterica *plant interactions in the context of NO and ROS stress mitigation*

S. enterica mitigation of murine model host NO and ROS production for successful invasion has been well documented (Spector and Kenyon, 2012; van der Heijden et al., 2015; Vazquez-Torres and Fang, 2001). In early stages of infection, *S. enterica* must contend with ROS produced as a part of the gut inflammatory response (Diaz-Ochoa et al., 2016) and later in macrophages (van der Heijden et al., 2015). While *S. enterica* is known to enter dendritic cells and M cells (phagocytic cells) one hallmark of *S. enterica* infection is that it gains entry into the epithelial cell lumen via activity of the T3SS, which secretes effectors to promote host cell cytoskeleton rearrangement. This in turn induces phagocytosis of *S. enterica* into the cell and the development of an *S. enterica* containing vacuole (SVC) which can protect the replicating *S. enterica* during infection (van der Heijden and Finlay, 2012). Also interesting to note, *S. enterica* suppresses apoptosis of its host to establish a stable intracellular niche via one of its many effectors, SpvC which interferes with host immune signaling, specifically MAP kinases (van der Heijden and Finlay, 2012). Once phagocytized, *S. enterica* within the SCV must employ NO and ROS detoxification machinery to overcome NO, O_2-, and H_2O_2 mediated cytotoxic effects

minimizing damage to proteins with metal centers, thiols, and DNA bases, by detoxifying NO to N_2O, NH_3 or NO_3^- and H_2O_2 to water (Imlay, 2013; Karlinsey et al., 2012; Reniere, 2018). Outside of mammalian models, few studies have investigated the interaction of other exogenous sources of NO and ROS with *S. enterica* in an environmental setting, with an exception of utilizing NO as a biofilm clearing agent on food contact surfaces (Marvasi et al., 2014) and post-harvest food grade ROS producing sanitizers against *S. enterica* (Singh et al., 2018; Ukuku and Sapers, 2001). Due to the ability of *S. enterica* to detoxify NO in the mammalian host model, opportunity for research into cross-kingdom connections is tantalizing.

6. Alternative persistence strategies of foodborne pathogens for long-term success in the agricultural environment

Other than persisting in the culturable fraction of the agricultural environment, foodborne pathogens may enter a viable but non-culturable state, known as VBNC, to achieve successful persistence. When a population of cells are subjected to an exogenous stress, a subsection may not exhibit the same response (Oliver, 2010; Ramamurthy et al., 2014). A heterogeneity or diversification of the population in response to stress ensures the longevity of some individuals, therefore preserving genetic viability and the potential to produce progeny (Ayrapetyan et al., 2015; Helaine and Holden, 2013). Viable but non-culturable organisms are microbes which respire and metabolize but cannot be cultured using classical microbiological techniques (Li et al., 2014b). These microbes are not considered dead because they have intact cellular membranes with undamaged genetic information and have a basal rate of metabolic activity, albeit a dramatic decrease in transcription rates (Fu et al.,

2015; Lesne et al., 2000). This can be evidenced by incorporation of amino acids 3H-Leucine and production of proteins (del Mar Lleo et al., 1998). Transition into VBNC states involves the perception of environmental stress signals, such as UV, chlorine, reactive oxygen species, high salinity, and desiccation (Asakura et al., 2002; Lesne et al., 2000; Morishige et al., 2017; Oliver et al., 2005), all agents commonly found within an agricultural environment. Advantages of a VBNC strategy include resistance to sonication, pH, chlorine, and antibiotics. Furthermore, bacteria can spend a considerable amount of time in this state with no damage to their genetic information (Oliver, 2010). This strategy has been described for various species including *Campylobacter jejuni, Vibrio parahaemolyticus*, *Vibrio vulnificus, Mycobacterium smegmatis*, *Salmonella enterica*, and *Enterobacter faecalis* (Li et al., 2014b). In terms of *S. enterica* VBNC virulence, while in the dormant state cells have been reported as poor epithelial cell invaders (Passerat et al., 2009), passage through a host gut, growth in eukaryotic cell culture, co-incubation with cell free supernatants, or perception of quorum sensing autoinducers may resuscitate these agents to culturable organisms and restore invasive ability (Asakura et al., 2002; Ramamurthy et al., 2014; Senoh et al., 2012).

Another alternative strategy for survival available to bacteria is entering into the persister state. Persisters are said to be similar to VBNC cells yet are revived more readily (Ayrapetyan et al., 2015). Debate exists in the scientific community as to if these two states are actually the same dormant phenotype (Kim et al., 2017), while others assert the existence of a “dormancy continuum” where VBNC are in a deeper dormant state compared to persisters (Ayrapetyan et al., 2016). Indeed, study has

shown both states can be induced via the same stress mechanisms, acidity, oxidative stress, and antimicrobial pressure (Kim et al., 2017). In the mammalian host, persisters have been identified as a stress mitigation mechanism of some *S. enterica* in the SCV from acidification- leading to long term chronic infections (Helaine et al., 2014). Outside of the host, persister states have been described as a subpopulation type in *S. enterica* biofilms (Miyaue et al., 2018). Interestingly, cells in this state could be removed from the biofilm and maintained up for up to 4 weeks at 37 °C (Miyaue et al., 2018), a finding which has important implications in an agricultural context. For example, persisters in water sediment if dislodged during irrigation can remain viable, more resistant to environmental stress, and could contaminate crops. Indeed, the transition of foodborne pathogens to a non-culturable state would allow for long term chronic persistence in water in ponds, creeks, and the surface of crops if contamination was to occur.

Select studies have suggested *S. enterica* could exist in a VBNC state in river and marine water (Roszak et al., 1984; Santo Domingo et al., 2000), findings which have important public health implications. If non-culturable states are a regular persistence strategy for *S. enterica* in the environment, this may result in under-estimation or false negatives of environmental samples for enteric pathogens. For food safety professionals to adequately serve the grower community, understanding the degree to which enteric pathogens can exist in a VBNC state and the relative risk of VBNC organisms on fresh produce is paramount for devising effective on-farm food safety monitoring and risk management practices.

Chapter 3: *Salmonella enterica* Elicits, Responds to, and Is Restricted by Tomato Immune Response Signals Nitric Oxide and Reactive Oxygen Species

1. Introduction

Between 2004 and 2012, non-typhoidal *Salmonella enterica* was the second most implicated causal agent of illness from fresh fruits and vegetables behind Norovirus (Callejón et al., 2015). *Salmonella enterica* is frequently isolated from water and soil in agricultural settings (Bell et al., 2015; Callahan et al., 2019; Micallef et al., 2012) and foodborne illness outbreaks have been traced back to crop production areas (Bennett et al., 2015; Greene et al., 2008), suggesting the possibility of human pathogen-crop associations initiating during the crop cultivation stages. Human pathogen-plant interactions continue to be understudied, despite the far-reaching public health and economic consequences.

Previous work has reported *S. enterica* can survive and multiply on plants (Brandl and Mandrell, 2002), the success of which is influenced by multiple factors (Brandl et al., 2013), including plant genotype and organ (Barak et al., 2011; Han and Micallef, 2014), age (Brandl and Amundson, 2008; Zheng et al., 2013), surface chemical profiles (Han and Micallef, 2016), as well as resident epiphytes (Poza-Carrion et al., 2013). The genetic profile of *S. enterica* itself may also influence successful colonization and persistence on crops. Attachment apparati (Barak et al., 2005, 2007; Salazar et al., 2013; Tan et al., 2016), nutrient acquisition (Kroupitski et al., 2009), and other specific metabolic pathways have been identified as important in

Salmonella persistence in the plant environment. For example, in endophytic studies including wounds and soft rots, iron acquisition, amino acid biosynthesis, ascorbate metabolism and cell structure maintenance have been identified as important in *S. enterica* persistence (de Moraes et al., 2017, 2018; Goudeau et al., 2013). As far as stress mitigation is concerned, *ycfR, sirA, yigG,* genes for stress regulation, biofilm formation, and virulence (in the case of *sirA*) are important for both chlorine stress response and *S.* Saintpaul and *S.* Typhimurium attachment to spinach leaf and grape tomato (Salazar et al., 2013). Research within our group has identified that *S.* Typhimurium upregulates multiple stress response genes when colonizing tomato plants including nitrosative and oxidative stress detoxification genes (Han et al, unpublished). These findings point to a possible bacterial counter-response to a plant immune response.

One well-documented mechanism of plant-microbe interactions is in the context of plant immunity. Plants recognize potential microbial pathogens through detection of pathogen-associated molecular patterns (PAMPs) or microbe associated molecular patterns (MAMPs) (Jones and Dangl, 2006). If the microbe does not secrete effectors, the main mode of plant recognition is through pathogen recognition receptor protein (PRR) interaction with these PAMPs. This recognition causes several strong yet transient signaling events to occur initiating with an influx of calcium ions into the cell (Ranf et al., 2011) which induces a burst of reactive oxygen species (ROS) and nitric oxide (NO) (Bigeard et al., 2015). Mitogen associated protein kinases (MAPKs) are activated, leading to defense related gene transcription and ethylene biosynthesis (Meng and Zhang, 2013). The release of reactive oxygen

species (ROS) and the more recently identified nitric oxide (NO) serve multiple purposes for the plant. The ROS burst can directly control the potential pathogen threat and together with NO may signal the upregulation of transcription factors that initiate transient defense responses that comprise PAMP triggered immunity (PTI) (Bigeard et al., 2015).

A growing body of evidence suggests *Salmonella enterica,* which is not a plant pathogen, may interact with parts of the plant immune system. *S.* Typhimurium and its flagella have been shown to induce an ROS burst in tobacco and tomato leaf disks (Meng et al., 2013; Shirron and Yaron, 2011). Flagellin from *S*. Typhimurium is recognized by tobacco and *Arabidopsis thaliana* through the FLS2 receptor, inducing plant defenses similar to the well-studied flg22 epitope (Garcia et al., 2014). Furthermore, flagellar mutants of *S*. Typhimurium were shown to better colonize wheat, alfalfa and *A. thaliana*, suggesting that attenuation of PAMPs favours bacterial colonization (Iniguez et al., 2005).

While the *S. enterica* - plant association profile is developing, gaps remain in our understanding of the reciprocal responses in this interaction. While even the extent of this interaction is not well described, the effects of this plant response to *S. enterica* PAMP recognition remains limited to the finding that several genes needed to detoxify ROS and NO are transcribed to higher levels in *S. enterica* cells associating with tomato leaf and fruit surfaces (Han et al., submitted). Moreover, data relevant to agricultural situations, which relate directly to salmonellosis outbreak-causing *S. enterica* strains are not available. *S. enterica* mitigation of host-derived NO and ROS is crucial for successful invasion in animal host models. These processes

have been well documented (Spector and Kenyon, 2012; van der Heijden et al., 2015; Vazquez-Torres and Fang, 2001; Zheng et al., 2011a), compounding the importance of investigating the presence of analogous interactions in *S. enterica* -plant associations.

To address these data gaps, and further investigate the possibility that in addition to ROS, NO may be an important signal in the *S. enterica* -tomato interaction, a *Salmonella* Newport – tomato model was used to represent the commonly associated enteropathogen-plant commodity pair in salmonellosis outbreaks (Anderson et al., 2011; Bennett et al., 2015). We investigated PTI induction in tomato seedling leaves and fruit in response to *Salmonella* Newport association by measuring NO and ROS production, the reciprocal bacterial response, and the effect of surface modulation of these molecules on *S. enterica* colonization of tomato leaves and fruit.

2. Materials and Methods

2.1 Cultivation of plant material

Tomato seeds cv. 'Heinz-1706' were obtained from the Tomato Genetics Resource Center (TGRC) from the University of California, Davis. TGRC seeds were germinated at 25℃ after pre-treatment in 30% w/v polyethylene glycol solution at room temperature with shaking for 72 h. Germinated seeds were transferred to potting media (Sunshine LC1; Sungro Horticulture, Canada) supplemented with fertilizer and subjected to a 16 h-light/8 h-dark photoperiod and 26°C day temperature/18°C night temperature with 70% humidity (RH) at the University of Maryland Research

Greenhouse. Tomato seedlings were grown to 5 true leaves before experimentation unless otherwise noted. For fruit, plants were either grown in the field at the Wye Research and Education Centre, Queenstown, MD, or transplanted into 1.7 gallon/ 6L pots to be grown in the greenhouse once they reached the 5-leaf stage. In the greenhouse, plants were fertilized once a week and treated with non-organophosphate containing pesticide once every two weeks for aphid and white fly management. Fruit was collected immediately prior to experimentation and rinsed with sterile water unless otherwise stated.

2.2 Bacterial strains

The *Salmonella enterica* Newport (*Se*N) strain used was an environmental isolate collected from an irrigation pond that matched a recurring tomato outbreak strain (Greene et al., 2008). *Se*N had been previously adapted to rifampicin (rif) and was therefore maintained at -80°C in Brucella Broth (BD, Sparks MD) containing 15% glycerol and 50 µg/mL rifampicin (Tokyo Chemical Industry, Portland OR). For each experiment, cultures of *Se*N were grown overnight on Trypticase Soy Agar (TSA; BD)+rif at 35°C. A single colony was selected, suspended in sterile water, and diluted to OD_{600}=0.34 - approximately 8.5 log CFU/mL. Serial dilutions for inoculation were performed in sterile water for the inoculum and 0.1% peptone water for enumeration (BD Difco, Sparks MD). Cells were enumerated on TSArif. *Pseudomonas syringae* pv. maculicola ES4326 was generously provided by Dr. Shunyuan Xiao and prepared identically to *S. enterica* without Rifampicin.

2.3 Detection of H_2O_2 in leaves and fruit

To detect the amount of H_2O_2 produced by tomato seedling leaves following *Se*N challenge, a method of staining with 3,3'-diaminobenzidine (DAB) was adapted from Bindschedler et al. (2006). Briefly, a third emerged leaflet from freshly watered 5-leaved 'Heinz-1706' seedlings was syringe infiltrated on the abaxial surface with 500 uL of either *Se*N in sterile water at 8 Log CFU/mL, heat-killed *Se*N, or sterile water (no *Se*N control). Positive controls were conducted with *Pst* (data not shown). Inoculated plants were incubated in a growth chamber subjected to a 16 h-light/8 h-dark photoperiod, 26°C and 70% RH. At 0 and 24 hours post-inoculation (hpi), inoculated leaflets were excised and submerged in 5 mL DAB solution (1 mg/mL aqueous DAB (Alfa Aesar, Ward Hill MA), 200 mM Na_2HPO_4 (VWR, Westchester PA), 0.05% Tween 20 (Amresco, Solon OH) and 100 uL 3 N HCl). Samples were vacuum-infiltrated for 4 min, then incubated in the dark with shaking at 50 rpm for 4 h. At the end of staining, decolorizer solution was added (3:1:1 95% ethanol, glycerol, glacial acetic acid) and samples were incubated in a boiling water bath for 15 min. Decolorized leaflets were fixed to paper and imaged with an Epson V330 photo scanner. The stain, corresponding to the H_2O_2 produced, was analyzed for intensity via ImageJ2 FIJI package (Schindelin et al., 2012). Optical density in leaves was calculated using the formula $OD = \log_{10}(\max intensity \div mean\ intensity\ of\ leaf\ area)$.

To detect a range of ROS produced from *Se*N challenge on fruit, staining with 6-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA; Invitrogen, Eugene OR) was adapted from Shin and Schachtman (2004).

Briefly, 3 mm sections of tomato exocarp were excised with a sterile razor and placed in black 96-well plates (Corning, Nazareth PA). Aliquots of 150 µL deionized water were delivered to the sample wells and incubated overnight in the dark at 4°C to allow for dissipation of any ROS production due to injury. Immediately before experimentation, wells were washed with 100 µL sterile water. One hundred µL of 8.0 log CFU/mL *Se*N, heat killed *Se*N (14 h only) or sterile water were delivered to sample wells, vacuum-infiltrated for 5 min, then shaken at 100 rpm at 27°C for 3 and 14 hpi. At the time of sampling, 25 µM CM-H_2DCFDA in water was added to each well. The fluorophore was able to react for 30 min in the dark with shaking at 50 rpm before being imaged with a Synergy HTX Microplate reader (BioTek, Winooski VT) at 485 nm excitation, 520 nm emission with 50 gain.

2.4 Detection of NO in leaves and fruit

To measure amounts of nitric oxide release from tomato when challenged with *Se*N, 4,5-diaminofluorescein diacetate (DAF-2 DA) (Fisher Scientific, Hampton NH) was used for its ability to complex intercellular NO as well as NO in solution (Rasul et al., 2012). For measurements on leaves, leaflets of 'Heinz-1706' plants grown in the research greenhouse were punched with a 3 mm hole punch and cut tissue was placed in wells in a black 96-well plate (Corning, Nazareth PA). For measurements on fruit, 3 mm sections of tomato exocarp were excised with a sterile razor. For both experiments, 150 µL deionized water were delivered to the sample wells and samples were incubated overnight in the dark at 4°C to allow for dissipation of injury related NO signal. Prior to inoculation, tissues were washed twice with sterile water, then challenged with 100 µL of 8 log CFU/mL *Se*N, heat

killed *Se*N, distilled water or 8 log CFU/mL *Pst.* Plates were vacuum-infiltrated for 5 min, then shaken at 100 rpm at 27°C. At 0.1, 1 and 3 h a final concentration of 15 µM DAF-2 DA in 50 mM Tris HCl pH 7.5 was delivered to the wells. Plates were incubated in the dark for 30 min at 27°C with shaking at 50 rpm and immediately read on the Synergy HTX (BioTek, Winooski VT) at 485 nm excitation, 520 nm emission with 50 gain.

2.5 Targeted q-RT-PCR of SeN genes colonizing seedling and fruit surfaces

To evaluate genes involved in nitrosative and oxidative stress responses in *Se*N colonizing the tomato phyllosphere, 3 leaf 'Heinz-1706' seedlings were pre-treated with water (referred to as "native" environment), 2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO, "limiting" NO environment) or $CaCl_2$ ("excess" NO environment), then challenged with *Se*N. To achieve this, 48 plants grown in autoclaved LC-1 potting media (Sunshine LC1; Sungro Horticulture, Canada) were separated into 3 groups and aerosol-sprayed with either 1 mL 0.5% $CaCl_2$, ddH_2O or 0.2 mM cPTIO. The plants were allowed to air-dry for 30 min. The second emerged leaf was challenged with 7 log CFU/mL *Se*N, delivered as ten 2-µL spots onto the leaf surface. Plants were incubated in a growth chamber subjected to a 16 h-light/8 h-dark photoperiod, 26°C and 70% RH. At 6 hpi, inoculated leaves of 4 plants were pooled to comprise one composite sample (N=4/treatment) and immediately fixed in 2:1 RNAProtect Bacteria (Qiagen, Germantown MD):ddH_2O. Samples were sonicated on a 8510 Branson Sonicator at full strength for 2 min to dislodge surface attached bacteria.

To evaluate the role of genes involved in colonization of tomato fruit, 'Heinz-1706' mature red fruit were washed with 200 ppm sodium hypochlorite and triple rinsed with ddH_2O. Fruit were then syringe-injected at the calyx with either 500 µL ddH_2O or 0.25 mM ascorbic acid. Seven log CFU/mL *Se*N was delivered as five 20-µL spots on the fruit surface. Fruit were incubated in 80% RH at 23°C. At 6 hpi, 5 fruit from each treatment were pooled to comprise one composite sample (N=4/treatment) and placed in RNALater stabilization solution (Invitrogen, Carlsbad CA). Fruit were vigorously vortexed for 3 min to dislodge attached cells. Washate containing *Se*N was transferred to a fresh tube and processed as described below.
In both experiments, 0.5 mL *Se*N inoculum in water was immediately fixed with RNAProtect to serve as the baseline for gene expression. All samples were centrifuged 5,000 *g* for 30 min and total RNA was extracted using the Qiagen RNeasy Mini kit (seedlings) (Qiagen, Germantown MD) or the Purelink RNA Isolation kit (fruit) (Invitrogen, Carlsbad, CA) with 45 min on-column DNA digestion (Invitrogen, Carlsbad CA). Resulting RNA was evaluated on the Nanodrop 1000 (ThermoFisher,) for quality. PCR of target genes was performed using 1uL RNA samples to ensure depletion of gDNA (data not shown). cDNA was synthesized with Verso cDNA kit (Thermo Scientific, Waltham MA) and 1 ng samples were subjected to q-PCR of genes using primers listed in **Table 1**. Primers were used at 100 nM concentration and verified to be 90-105% efficient. Plant material was verified to produce no off-target amplification (data not shown). In a series of experiments using TSB amended with treatment reagents, SeN gene expression gene expression was confirmed to be reflective of epiphytic habit on tomato surface and not an artifact of

interaction with elicitor or scavenger (**Supplementary Figure 1**). Amplification was conducted on a ABI Step-One Plus (Applied Biosystems, Foster City CA) with SYBR as a reporter using the following parameters: 50°C for 2 min, 95°C for 2 min, followed by 40 cycles of 95°C for 15 s and 59°C for 30 s. Melt curve analysis was included to ensure product specificity. Data were analyzed on the ABI Step One Plus instrument using the ΔΔCt method with sigma factor *rpoD* as the endogenous control (Pfaffl, 2001). The cutoff Ct was set to 35.5 cycles.

Relative gene expression was compared to expression in *Se*N inoculum after internal normalization to *rpoD* expression. Genes were determined to be differentially regulated if they experienced significantly lower expression in one plant environment (excess, limiting, or native levels of elicitor or scavenger) compared to inoculum ($p < 0.05$).

Table 1: List of genes, their qPCR primers, and efficiencies used to examine *S.* Newport gene expression on tomato leaves and fruits.

Functional Category	Gene	Function	5′- 3′ Sequence	Reference	Efficiency
ROS response	*ahpC*	Peroxiredoxin	TCGCTTCGCCTTCTTTCCAT GACCTTTGTTGTTGACCCGC	This Study	101%
	katG	Catalase	GACTCACCGACACCCTGAAG CACGGTCTCTTCGTCGTTCA	This Study	102%
NO Detoxification and response	*hmpA*	Flavohemoglobin, nitric oxide dioxygenase activity	GAACATTTCGTCCAGCGTCG ATCAGCGTGAAGCCCTGTTT	This Study	95%
	yoaG	Cytoplasmic protein in NsrR regulon	ATAGCAACGGCGTCTCTGTG GGTATCGTAGGAACGCACGG	This Study	101%
Virulence	*phoP*	Virulence regulator	CGACTTTATCCTGCCAGCCT GCCTTTCCTTAATACGCCGC	This Study	91%
	phoQ	Virulence regulator, membrane-bound sensor kinase	TATGGTGTGGAGCTGGTTCG CGGCGATCCACAGTAAAGGA	This Study	91%
	sdiA	Virulence regulator, quorum-sensing regulator	GATGAGGTCTTCCCTTCCGC TACGCTGCTCCTCGTTTACC	This Study	90%
Environmental fitness	*marA*	DNA-binding transcriptional activator for antibiotics resistance operon MarRAB	TACGGCTGCGGATGTATTGG CGAGGATAACCTGGAGTCGC	This Study	105%
	nmpC	Outer membrane porin protein, cell wall biogenesis	GTCCGTCCATCGCTTACCTG GCTTTGGTGAAGTCGCTGTC	This Study	94%
	trpE	Tryptophan biosynthesis protein	CGCTTTTTCACCAGGTCTGC AACGCCTGAATGGTGACAGT	This Study	102%
Housekeeping	*rpoD*	RNA polymerase sigma factor	GTGAAATGGGCACTGTTGAACTG TTCCAGCAGATAGGTAATGGCTTC	Karlinsey et. al 2012	101%

2.6 Modulation of endogenous hydrogen peroxide, nitric oxide levels and plant colonization assays

To investigate the effect of plant derived H_2O_2 and NO on *Se*N survival on tomato surfaces, the third emerged leaf on 5-leaved 'Heinz-1706' seedlings or mature fruit were treated with reagents to either scavenge surface ROS (Bradley et al., 1992; Lee et al., 1999) or NO (Keshavarz-Tohid et al., 2016; Małolepsza and Różalska, 2005), or elicit production of NO (Chakraborty et al., 2016), then subsequently inoculated with *Se*N. The reagents employed, concentrations and application methods for leaves and fruit are detailed in **Table 2**. After application of cPTIO and $CaCl_2$, fruit and leaves were left to air-dry for 4 h at 25°C. Ascorbic acid-treated leaves were

left to dry for 2 h and fruit were left to dry for 30 min prior to *Se*N inoculation. Following pretreatment, a suspension of 5.5 log CFU/ml *Se*N in water was applied to the surface of the leaflets in 10 2-µL spots. Samples were incubated in 80% RH for 12 h. To retrieve viable *Se*N, inoculated leaflets or carposphere were excised, diluted in 0.1% peptone water, and either hand-massaged (leaflets) or vortexed (fruit) for 2 min before serially plating dilutions onto TSArif and incubating at 35°C for 20 h.

Table 2: Chemicals and application methodology for modulation of Tomato leaf and fruit NO, ROS levels.

Pretreatment Purpose	**Tissue**	**Chemical**	**Source**	**Application Method**
H_2O_2 Scavenger	Fruit	0.25 mM ascorbic acid	Sigma, St. Louis MO	Calyx syringe injection
	Leaves	2.5 mM ascorbic acid	Sigma, St. Louis MO	Abaxial syringe infiltration
NO Scavenger	Leaves and Fruit	0.22 mM 2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide	Enzo Life Sciences, Farmingdale, NY	Adaxial aerosol spray
NO Elicitor	Leaves and Fruit	0.5% $CaCl_2$	Sigma, St. Louis MO	Adaxial aerosol spray

2.7 Statistical analysis

All experiments had at least 3 biological replicates and were repeated twice. All experiments were performed in a completely randomized design (CRD). Statistical analysis was performed in JMP version 14.1, assessing treatment effects for significance using ANOVA and post-hoc Dunnett's test (α=0.05) for *Se*N compared to water unless otherwise noted. Targeted transcriptomic data was analyzed via Dunnett's test (inoculum vs. on-plant environments), Tukey's Honestly

Significant Difference (cPTIO vs. $CaCl_2$ vs. H_2O leaf environments), and Student's T-test (Ascorbic Acid vs. H_2O tomato fruit environments) all at α=0.05. For on-plant challenge assays, Student's T-test was employed to compare the chemical modulator treatment to water treatments (α=0.05).

3. Results

3.1 Salmonella *Newport elicits H_2O_2 production in tomato seedlings and fruit*

Immediately following inoculation of leaves with *Se*N (0.1 hpi), a dark brown precipitate - indicative of H_2O_2 production - was detected in all samples, with calculated OD measurements ranging from 0.21-0.61 (**Figure 1A**). The brown precipitate deposited in *Se*N-challenged (0.47±0.10) or heat-killed *Se*N-challenged leaves (0.45±0.08) was darker (p=0.067, dead S*e*N) and significantly darker (p=0.037, live S*e*N) than in water challenged leaves. Twenty-four hpi, H_2O_2 production was still measurable from all samples ranging from 0.14-0.64, with greater discrepancy among treatments. Heat-killed *Se*N (0.20±0.05) and water control (0.23±0.08) had comparable measurements, while leaves treated with live *Se*N exhibited darker staining (0.35±0.18) compared to water (p=0.072) treated leaves.

At 3 h post-challenge, more ROS was detected in *Se*N-treated fruit exocarp than in water-treated exocarp (p=0.062, Student's T-test) (**Figure 1B**). At 14 hpi, significantly more ROS was detected in exocarp samples treated with heat-killed (98.18±51.0 Au) and live *Se*N (82.82±17.0 Au) compared to the water-treated controls (51.41±15.5 Au) (p=0.05). Heat-killed *Se*N measurements, while not significantly different from measurements taken from live *Se*N-treated samples, were

both the highest at 14 hpi and exhibited the largest variation among samples. These measurements suggest that live *Se*N induced an ROS burst in both tomato leaf and fruit exocarp tissue.

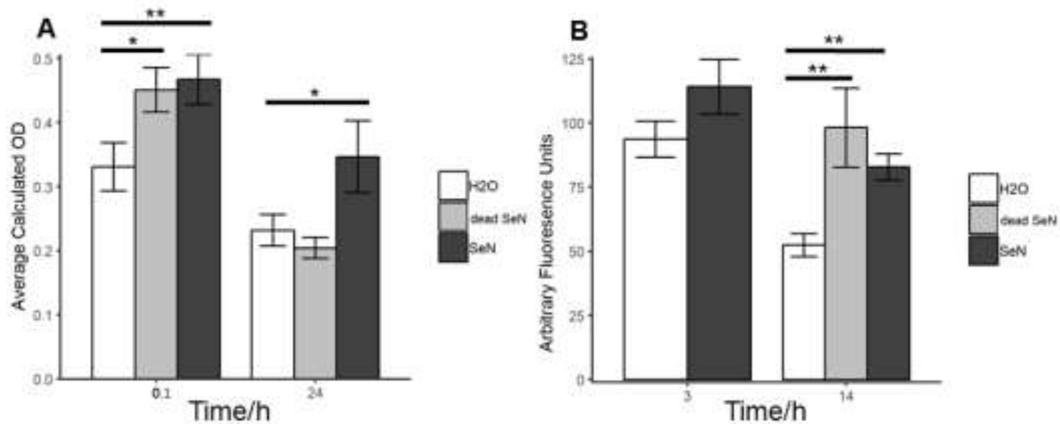


Figure 1: Plant derived ROS produced following challenge with sterile water (H_2O), heat killed *S*. Newport (dead *Se*N), or *S*. Newport. (A) Calculated optical density showing degree of 1mg/mL DAB stain in tomato leaves following treatment via syringe infiltration at 0, 24 hpi. N=3, 2 technical replicates. OD was calculated using FIJI ImageJ package. (B) Average fluorescence from 25 µM CM-H_2DCFDA of tomato fruit peel challenged with sterile water or 8.0 log CFU/mL *S.* Newport (SeN) at 3, 14 hpi followed by imaging on Synergy HTX 485 nm excitation, 520 nm emission. N=5, 3 technical replicates. Asterisks denote significance (*=$p<0.1$ or **= $p\leq0.05$) between comparisons via post-hoc Dunnett's test unless otherwise noted with water serving as control ($\alpha\leq0.05$). Error bars represent standard error of the means (SEM).

3.2 Salmonella *Newport elicits NO production in tomato seedlings, but not in fruit*

Following leaf challenge, signal for NO detection increased over time for all treatments, with the smallest average increase detected in the water control (Δ 20.8 Au) and the largest in *Pst*-treated leaf sections (Δ 29.4 Au). As expected, the tomato pathogen *Pst* induced significantly more NO than the water control at all sampling times ($p<0.001$) via orthogonal contrasts. At 0.1 hpi, *Se*N signal was not statistically significantly different to water, but at 1 and 3 hpi *Se*N induced a stronger signal in leaves compared to water at (100.6±13.4 Au; $p=0.003$), (112.9±18.8 Au; $p=0.001$) respectively **(Figure 2A)**. A low level of NO was detected in leaf tissue challenged with heat-killed *Se*N, however the signal was only statistically significant when compared to water-treated leaves at 3 hpi (108.0±13.2 Au; $p<0.01$).

Conversely to leaves, fruit tissue signal for all treatments decreased over time by an average of 100.1 Au, with the smallest average decrease observed in live *Se*N-treated fruit tissue (Δ -54.67Au) and the largest change in *Pst* treated leaf sections (Δ -186.39 Au) **(Figure 2B)**. *Pst* produced significantly higher NO than the water control at 5 min post-inoculation but not at 3 hpi ($p<0.001$, $p>0.05$). No significant exocarp production of NO was detected in *Se*N-treated fruit exocarp compared to the water control, either at 0.1 (323.6±180.7) and 3 (268.9±102.1) hpi. Heat-killed *Se*N did prompt some NO production, however the signal was not significantly different than water or live *Salmonella.*

Fruit exocarp tissue produced a stronger NO signal than leaf sections regardless of treatment (Student's *t*-test, $p<0.001$). However, exocarp measurements

exhibited a larger variation with a coefficient of variation (CV) of 0.52 compared to 0.36 for leaf sections. Taken together, these data suggest that live *Se*N can induce release of H_2O_2 and NO consistent with a PTI response in tomato seedling leaves.

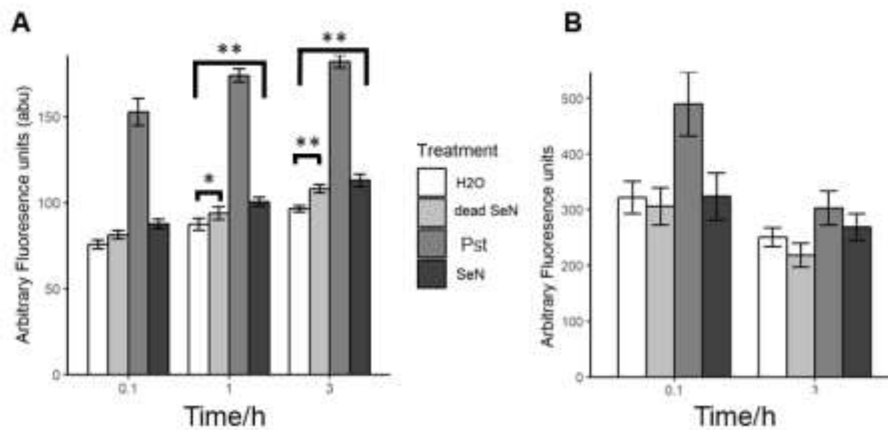


Figure 2: Plant derived NO produced following 100µL water (H_2O), heat killed *S*. Newport (dead S*e*N), assay control *Pseudomonas syringae* (*Pst*), or *S.* Newport on (A) tomato leaves or (B) tomato fruit. NO measured by addition of 15 µM fluorophore diaminofluorescein diacetate (DAF-2DA) at 0.1, 1, and 3 hpi followed by imaging on Synergy HTX 485 excitation, 520 emission. N=5 plants, 3 technical replicates each. Seedling leaf data is pooled between 2 experimental replicates, whereas tomato fruit data is a representative subset of 2 experimental replicates. Error bars represent SEM. Asterisks denote significance (*=p<0.1 or **= p≤0.05) between comparisons via post-hoc Dunnett's test with water serving as control (α≤0.05).

3.3 Differential regulation of S. enterica *genes responsible for ROS and NO detoxification*

To investigate specific bacterial responses to the observed elicited H_2O_2 on tomato leaves and fruit and NO on leaves only, a targeted transcriptomic analysis approach was employed, assaying for expression of genes responsible for NO detoxification, ROS mitigation and other environmental fitness factors (Table 2). On leaves, 78% of samples displayed ≥ |2|-fold change in expression compared to the inoculum culture. Genes involved in the nitrosative stress response *hmpA, katG, yoaG* a well as virulence *phoP* and *sdiA* were differentially transcribed among seedling treatments; native, excess, and limiting NO (**Figure 3**). Gene expression of *hmpA* and *yoaG* in *Se*N was significantly higher on native leaves and leaves producing excess NO compared to baseline, but not on NO-scavenged leaves. Expression of *hmpA* increased 3-fold (p<0.05) in *Se*N associating with native leaves and NO-excess leaves. The *yoaG* gene followed a similar trend with no increase in levels of transcription in *Se*N on NO-limiting leaves but transcribed at higher levels, to an almost 3-fold increase on native leaves and a 4-fold increase on NO-excess leaves (p<0.05). In the case of *hmpA* and *yoaG*, expression in NO excess and native environments were comparable, suggesting *Se*N can serve as a strong NO elicitor in the tomato seedling.

Multiple antibiotic resistance transcriptional regulator MarA was also significantly upregulated in NO excess and native plant environment. The gene *ahpC*, which encodes the enzyme alkyl hydroperoxide reductase C that protects cells from oxidative stress by catalyzing the reduction of hydrogen peroxide and other organic

peroxides, was up-regulated in all treatments compared to inoculum (p<0.05), but was not found to be differentially expressed among treatments. Taken together, these findings suggest that *Se*N is inducing, recognizing and responding to plant derived NO and ROS.

The virulence factors *phoQ* and *sdiA* both exhibited an increase in expression in native and NO excess environments, with *sdiA* increasing 4-fold in NO excess from NO-limiting environments and *phoQ* increasing 3-fold on native leaves from NO limiting leaves. Other fitness factors tested, *marA, nmpC* and *trpE* - displayed uniform significant upregulation in all treatments compared to inoculum (p< 0.05), suggesting that these genes may be important for colonization of the phyllosphere, but are not impacted by NO stress.

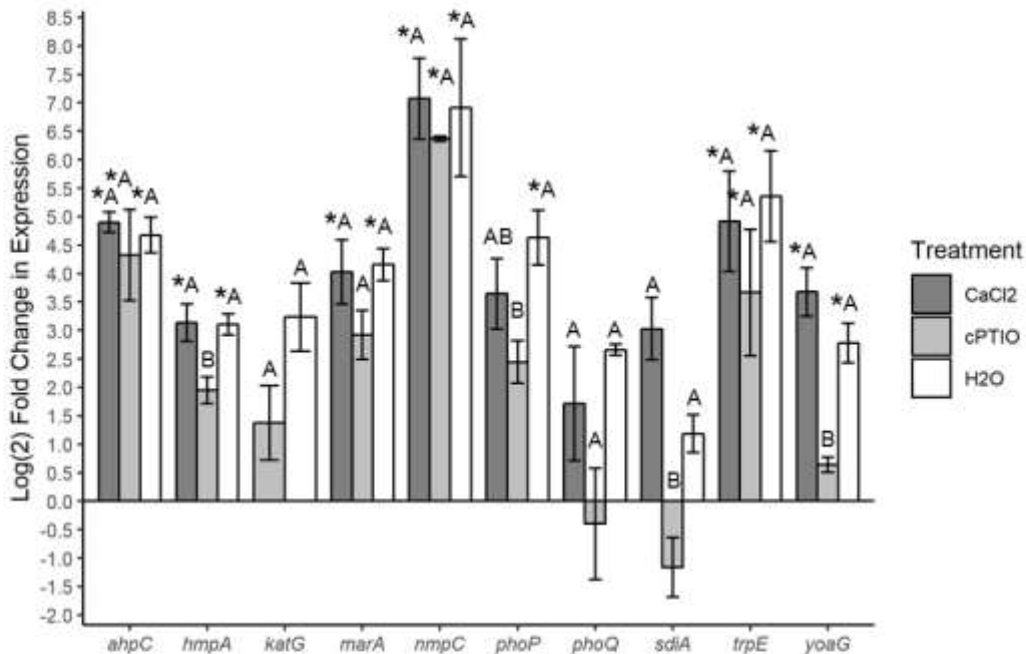


Figure 3: Log base 2-fold change in expression of select *S.* Newport genes compared to expression in water inoculum. ΔΔCt results of *S.* Newport colonizing 3 leaf Heinz seedlings pretreated to reflect the native environment (H_2O), NO limiting (cPTIO), or NO excess ($CaCl_2$) environments normalized with the sigma factor *rpoD*. N=4 groups of 4 pooled plants per treatment. Error bars=SEM. Asterisks denote significance in genetic expression compared to inoculum according to Dunnett's test (α=0.05). Letters denote significant differences in expression among treatments of one target gene query via Tukey's Honestly Significant Difference (α=0.05).

3.4 NO, ROS detoxification also observed in S. *Newport colonizing tomato fruits*

This approach was repeated on mature Heinz fruit to investigate NO and ROS detoxification responses in *Se*N colonization of fruit. Here, ascorbic acid was employed as a scavenger of fruit ROS ("limiting" environment) before challenging with *S*. Newport and comparing expression profiles to *Se*N on water treated tomato fruit ("native" environment). As observed on seedling leaves, S*e*N in 72% of fruit samples displayed a ≥ |2|-fold change in expression compared to water inoculum (**Figure 4**). *Se*N on native fruit exhibited significant up-regulation of *hmpA, yoaG* and *ahpC* compared to the inoculum (p<0.05). These genes were also up-regulated in the native fruit environment compared to the ROS limiting environment (p<0.05). Taken together, this again suggests a bacterial response to plant derived NO and ROS. Also similar to seedling samples, transcription levels of *marA* were higher in both fruit environments (p<0.05) compared to *Se*N inoculum and appeared unaffected by modulation of ROS. However, in contrast to *Se*N on seedlings both fruit

environments displayed significant down-regulation of *nmpC* ($p < 0.05$), compared to inoculum.

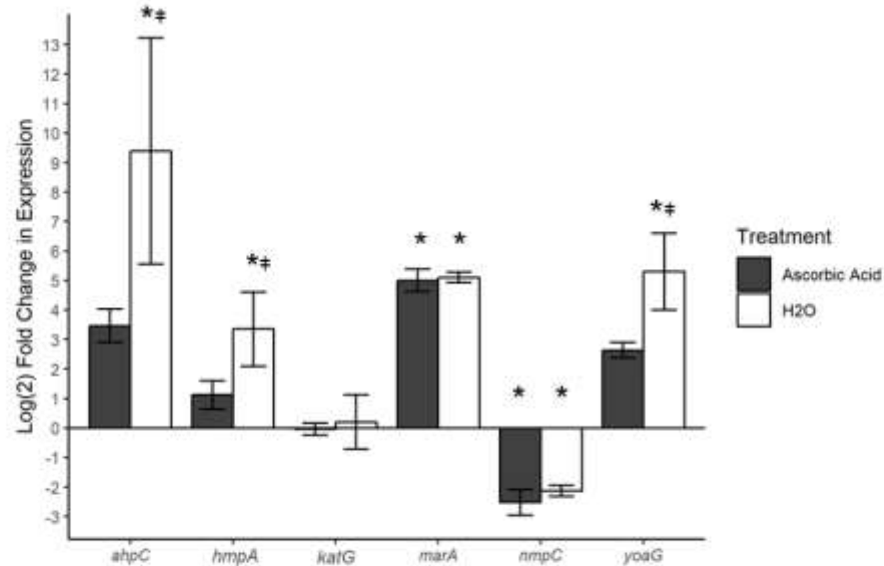


Figure 4: Log base 2-fold change expression of selected *S.* Newport genes compared to expression in water inoculum. ΔΔCt results of *S.* Newport colonizing Heinz fruit pretreated to reflect the native fruit environment (H_2O) or ROS limiting (AscA) environments normalized with the sigma factor *rpoD.* N=4 groups of 4 pooled plants per treatment. Error bars=SEM. Asterisks denote significance in *Se*N regulation compared to inoculum via Dunnett's test (α=0.05). Double crosses denote significant differences in transcription between fruit environments of one target gene query (Student's T-test, α=0.05).

3.5 Scavenging hydrogen peroxide results in higher survival of Salmonella *on tomato fruit and leaves*

To evaluate whether *Se*N colonization of tomato surfaces was significantly affected by modulated levels of plant-derived H_2O_2 and NO, a series of 12 h on-plant *Se*N challenge assays were conducted on plants pre-treated with endogenous elicitors or scavengers of these molecules. When the hydroxyl radical and H_2O_2 were scavenged from plant fruit and leaf samples with ascorbic acid, significantly more *Se*N was retrieved from scavenged leaflets (4.56 ± 0.10 log CFU/tomato leaf) and fruit surfaces (3.27±0.47 log CFU/tomato) compared to sterile water pre-treated fruit (p<0.05; **Figure 5C**). On fruit, regardless of treatment, *Se*N retrieval was 0.8 log CFU/unit lower than on leaves, with larger variation in retrieval from fruit compared to leaves (CV_{fruit} =0.20 and CV_{leaves} =0.07).

3.6 Modulating tomato surface NO levels significantly affects S. *Newport colonization success on leaves, but not fruit*

After 12 hpi *Se*N counts recovered from NO-scavenged leaves were higher (4.85±0.5 log CFU/leaflet) compared to sterile water treated leaf tissue (p<0.05; **Figure 5B**). This effect was not observed on fruit. *Se*N counts recovered from leaves pre-elicited to produce endogenous NO were almost 2 log lower at 12 hpi (p<0.001), measured at 3.32 ±0.2 log CFU/ leaflet compared to 5.15±0.3 log CFU/leaflet recovered from mock treated leaves (**Figure 5A**).

Regardless of pre-treatment *Se*N was retrieved at higher titres with smaller variation on leaves compared to fruit samples, in both NO scavenged tissue

(CV_{fruit}=0.39 and CV_{leaves}=0.10) and NO elicited tissue (CV_{fruit}=0.42 and CV_{leaves}=0.24).

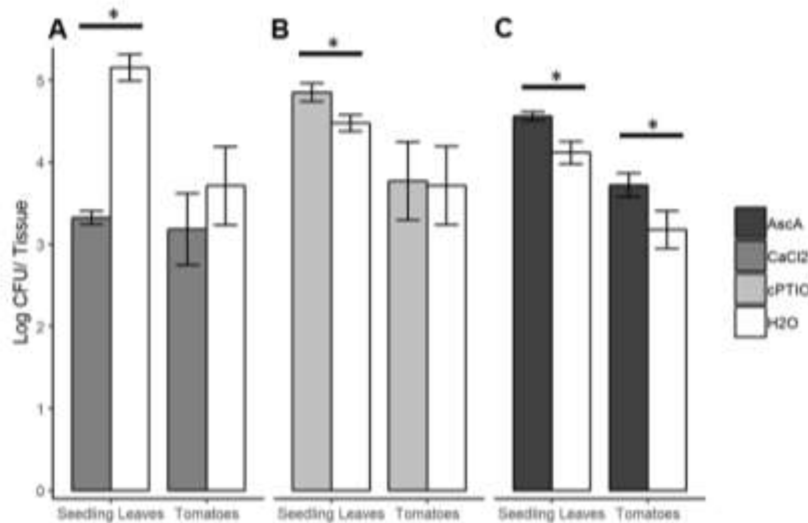


Figure 5: Surface populations of *S.* Newport after pre-treatment of plant tissue with (A) NO elicitor, (B) NO scavenger, or (C) H_2O_2 scavenger. Samples were incubated at 25°C maintaining 80% humidity for 12 hpi before retrieval. Error bars represent SEM, significance was determined using Student's T-test between treatments and water control (α= 0.05). (A) Plant tissue was aerosol sprayed with 1 mL 0.5% $CaCl_2$ and dried 4 h before surface inoculation with *S.* Newport. N=4 plants, N=10 tomato fruits. (B) Plant tissue was aerosol sprayed with 1 mL 0.2mM cPTIO and dried 4 h before surface inoculation with *S.* Newport. N=18 plants, N=10 tomato fruits. (C) Plant tissue was syringe infiltrated with 0.5mL 2.5mM (leaves) or 0.25mM (fruit) ascorbic acid, and dried 30 min before surface inoculation with *S.* Newport. N=3 plants, N=11 tomato fruits.

4. Discussion

In the present study, biochemical, targeted bacterial gene expression, and on-plant colonization assays of both vegetative tissue and ripe mature fruit provided evidence to suggest that the tomato plant can not only recognize *Salmonella* Newport, but mount a response that the bacteria in turn interprets as a stress which requires mitigation to minimize inflicted damage. ROS was elicited in both leaf and fruit samples, and the reciprocal bacterial response effect was consistent with recognition and response to these stimuli as colony restricting stressors. On the other hand, NO induction and an adverse effect of NO modulators on *Se*N colonization was detected on leaves, but not fruit.

The present work found evidence to suggest that plant-derived NO is generated in response to *S. enterica* recognition. Up-regulation of *S. enterica* NO detoxification genes during tomato surface colonization indicates that this environmental *Salmonella* strain perceives plant-derived NO as a stressor, corroborated by lower colonization outcomes of *S. enterica* on NO elicited seedling leaves. The NsrR regulon, controlled by the nitric oxide sensing transcriptional repressor NsrR, plays an important role in nitrosative stress resistance during infection. Within this regulon, the flavohaemoglobin HmpA is identified as the main protein responsible for NO detoxification activities in the presence of an oxygenated environment (Karlinsey et al., 2012). Supporting this, in the present study two genes in the NsrR regulon were measured that were differentially transcribed compared to all native fruit and leaf environments, *hmpA* and *yoaG.* The NO detoxification-associated genes *hmpA, nfrA* and *ygbA* were also found to be up-regulated in soft rot

macerated cilantro and lettuce leaf tissue caused by the plant pathogen *Dickeya dadantii* (Goudeau et al., 2013). In the present study, evidence of NO detoxification was reported in the absence of a plant pathogen or tissue injury, and tomato plants produced NO· upon perception of *S. enterica* which, in turn, led the bacteria to switch on reactive nitrogen species (RNS) detoxification machinery. It is therefore hypothesized that this action may be necessary in order for *Se*N to successfully persist on some plant tissue.

Evidence of ROS elicitation by *S. enterica* was also observed, consistent with other reports of PAMP -response recognition interactions with this foodborne pathogen (Garcia et al., 2014; Klerks et al., 2007; Meng et al., 2013; Schikora et al., 2008; Shirron and Yaron, 2011). Furthermore, evidence of bacterial mitigation of ROS stress while colonizing plants was observed via up-regulation of *S. enterica* catalase (*katG)* on leaves and *ahpC* in *S. enterica* on leaves and fruit. As the primary scavenging enzyme in non-stressed environments, AhpC is a two-component thiol-based peroxidase which transfers electrons from NADH to H_2O_2, producing water (Imlay, 2013). Conversely, catalase G, *katG* is heme dependent and only weakly expressed during active growth, however is strongly induced (via oxyR) when cells are stressed by exogenous H_2O_2 (Imlay, 2013). In the present study, these data can be taken to suggest while baseline ROS may be perceived by *S. enterica* on both leaves and fruit in native environments evidenced by *ahpC* activity, leaves may be producing more ROS evidenced by up-regulation of *katG.*

Other than NO and ROS stress, gene expression of *Se*N on both leaves and fruit were indicative of adaptation to a novel environment. In leaves, *trpE*, which has

been associated with biofilm development (Hamilton et al., 2009), was upregulated in all environments. Biofilm formation is known to enhance the capacity of pathogenic bacteria to survive stresses in the environment and during host infection. Thus, the present work provides more evidence to the growing body of work which define attachment as paramount to survival in the phyllosphere (Barak et al., 2005, 2007). *MarA* was also upregulated in all leaf and fruit environments. In addition to their importance as regulators of xenobiotic efflux, *marRAB* may have indirect effects on expression in the enzymatic systems of, iron metabolism, membrane composition, and the stress related sigma factor *rpoS.* (Lee et al., 2015). In *S. enterica* transcriptomic surveys (Brankatschk et al., 2014; Wang et al., 2010), the use of such machinery is pertinent to survival in stress-inducing environments. Interestingly, *nmpC (*also referred to in the literature as *ompD*) displayed differential expression among plant tissue types, upregulated compared to inoculum in all leaf samples and down-regulated on the tomato fruit surface. NmpC *(*ompD) is one of the most abundant outer membrane porins of *S. enterica*, used to passively transport nutrients into the cell and toxins out of the cell (van der Heijden et al., 2016). Additionally, OmpD may be needed for adherence and recognition of *S*. Typhimurium to human macrophages and epithelial cells during the initial stages of infection (Hara-Kaonga and Pistole, 2004). Currently there are conflicting reports as to whether or not nmpC (ompD) is a main contributor to H_2O_2 diffusion in and out of the cell, and it may be that environmental conditions dictate the contribution of nmpC to H_2O_2 transport across the outer membrane (Ipinza et al., 2014; van der Heijden et al., 2016). In our study, down-regulation of *nmpC* in fruit coupled with up-regulation of *ahpC* may

suggest the hypothesis of *nmpC (ompD*) involvement in H_2O_2 diffusion. However, up-regulation of both *nmpC (ompD*) and ROS detoxification machinery on leaves may point to an additional unknown function of this porin, perhaps engaging in efflux activity of other xenobiotics. Taken together, targeted gene expression on both tissues provide evidence for *S. enterica* needing to attach and mitigate various stressors, potentially for niche development.

In this study, some outcomes were unexpected. For example on fruit, while up-regulation of bacterial NO detoxification genes was observed, fruit NO generated from challenge with various biotic treatments including *Salmonella* produced a fluorescent response greater than that of leaves but was not treatment specific. In other fruit experiments, *S. enterica* colonization outcomes were affected with ascorbic acid mediated modulation, but not NO modulation. These unexpected results may be due in part by the interplay of ROS and NO as signaling molecules in other pathways important to plant development and metabolism. For example, large amounts of NO detected from fruits (Figure 2B) across all treatments may be due to exocarp senescence (Mur et al., 2013), causing a significant amount of noise in the assay and rendering no detection of treatment-based signal. Further on fruit, the observation of ascorbic acid significantly affecting *S. enterica* colonization outcomes whereas NO modulation did not could be attributed to NO and ROS endogenous levels in mature red fruit at the time of study. Ripe red fruit are known to have lower concentrations of nitric oxide compared to mature green fruit, as NO is involved in regulating ethylene production and thus facilitating the ripening process (Ya'acov et al., 1998). Furthermore, the transition from green to red fruit is marked by accruement of high

ROS concentrations (Kumar et al., 2016). Thus, the ascorbic acid injected in our plant colonization assays could have been targeting ripening-related ROS, nonetheless providing a more hospitable environment for colonizing *S. enterica*. Taken together, these data suggest plant tissue-dependent mechanisms of *S. enterica* restriction may exist in addition to plant non-host immune response, and that they may be connected to or confounded by the ripening process. While some research groups have measured ROS detoxification efforts from fungal pathogens colonizing red ripe fruit (Petrasch et al., 2019), most NO modulation studies in fruit responding to plant pathogens are routinely conducted with mature green fruit (Lai et al., 2011; Zheng et al., 2011b, 2011a; Zhu and Tian, 2012). One reason for this could be that mature ripe fruit tissue have lower levels of pathogen recognition response capacity compared to vegetative tissue, perhaps due in part to the breakdown of cellular wall components during ripening (Cantu et al., 2009). This could result in the failure or high variability of exogenous chemicals to modulate these levels but nevertheless allowing for micro-niche responses to exist. *S. enterica* studies on ripe and unripe tomato fruit have found the organism proliferates more readily in ripe red compared to mature green tomato fruit (Barak et al., 2011), an observation that could be explained by the robustness of mature green non-host immune response in addition to availability of surface nutrients. Regardless, more research is needed to evaluate the interconnectivity between ripening and pathogen defense, both in the contexts of plant and human pathogens.

Overall, higher titres of *S*. Newport were more consistently retrieved from leaves compared to fruits, an observation which has been reported elsewhere (Barak

et al., 2011). This variability could be due to the plant immune response, relative abundance of nutrients on the surface of the different plant organs, or a combination of both physiological processes which renders the tomato fruit a less hospitable long-term environment compared to the leaves. Supporting evidence can be found in *Salmonella* field sampling studies. For example in one multi-year field study researchers sampling tomato leaves and fruit for wild *S. enterica* colonization found only leaves returned positive *S. enterica* result, never fruits (Gu et al., 2018). In a previous study from our group, we identified that Heinz fruit aqueous fraction of surface chemicals had higher proportions of fatty acids compared to seedling shoots or mature leaves, a feature which was negatively correlated with *S. enterica* growth (Han and Micallef, 2016). It was furthermore identified that *Se*N retrieval from leaves was less variable than fruit, suggesting a potential heterogeneity of response to unique stressors present on this plant organ. Diversification of stress response or "bet hedging" has been documented in *S. enterica* intercellular interaction with ROS and other stress agents (Burton et al., 2014; Helaine et al., 2014; Helaine and Holden, 2013). Taken together, this suggests that tomato fruit may be a unique yet harsh environment for colonizing human pathogens for which "bet hedging" may be a significant strategy to ensure long term survival.

While the leaves of the tomato are not eaten, in the field leaves and tomato fruits are in constant contact with one another, serving as a contamination source for fruit. As is current common practice, this vegetative matter may be harvested along with tomato fruit and could lead to widespread contamination if appropriate Good Agricultural Practices are not followed; for example using recirculated water to wash

the tomatoes without the appropriate concentration of sanitizer. Therefore, one effective way to minimize foodborne pathogen risk in tomato cultivation is to implement standard operating procedures which minimizes the presence of vegetative matter co-mingling with fruit during harvest and post-harvest.

Perception and mitigation of NO stress may be one important process making up, with other important stress responses, a distinct strategy for successful plant colonization. Mitigation of these stresses, including NO and ROS which can be short-term restricting agents, may lead to more established persistence long term in the field. This has been documented in the mammalian host model, where presence of RNS in the gut increased overall colonization fitness of *S. enterica*, possibly because it can outcompete some resident microflora (Stecher et al., 2007). As ROS may be present in many aspects of the agricultural environment (Diaz and Plummer, 2018), stress mitigation strategies may be key for enteric organisms to survive in multiple areas of the agricultural environment on their way to the mammalian gut. Future work to investigate if this ability is shared by all *S. enterica*, or if it is specific to *S. enterica* serovars that are regularly implicated in produce outbreaks is imperative to continue to elucidate adaptation to alternative host environments. Investigation of minute plant- *S. enterica* interactions to further characterize the environmental lifestyle of this enteric pathogen is imperative to provide holistic, science-based farm management decisions for risk reduction during specialty crop cultivation.

Chapter 4: *Salmonella enterica* Serovar Specific Tolerance to Nitric Oxide Stress *in vitro* and in the Tomato Phyllosphere

1. Introduction

S. enterica subspecies *enterica* is comprised of over 2,500 different serovars, with around 10 responsible for 77% of foodborne illnesses (2010- 2016) (Foodborne Outbreak Online Database (FOOD Tool), 2016). Within common serovars implicated in foodborne illness outbreaks, recent epidemiological research suggests associations between certain serovars and food commodity categories exist (Jackson et al., 2013). For example, *S.* Enteriditis, while present in the environment (Callahan et al., 2019), is disproportionately associated with outbreaks from meat and eggs compared to produce (Jackson et al., 2013). *S.* Newport on the other hand has been identified as a generalist, yet is the most common serovar in vine vegetable outbreaks (Jackson et al., 2013). These data raise questions on the existence and drivers of specific serotype – food commodity relationships. Previous work investigating bacterial ability to persist on produce crops has revealed colonization success not only varies by produce type, but also by serotype (Cui et al., 2018; Guo et al., 2002; Klerks et al., 2007b; Patel and Sharma, 2010; Reed et al., 2018; Shi et al., 2007; Zheng et al., 2013). Indeed, studies with tomatoes – a frequently implicated commodity in salmonellosis outbreaks (Bennett et al., 2015; Jackson et al., 2013) – report *S.* Newport and *S.* Javiana as more prevalent than other serovars like *S.* Typhimurium, *S.* Enteriditis and *S.* Dublin, which are considered less likely to be ‘produce-associated’ (Shi et al., 2007; Zheng et al., 2013). Furthermore, plant

metabolites may be involved in these interactions, as was shown in the positive correlation of *S.* Senftenberg persistence in the basil phyllosphere with persistence in basil oil compared to *S.* Typhimurium (Kisluk et al., 2013). Furthermore, correlations have been identified between *S.* Newport growth on tomato surfaces and tomato exudates (Han and Micallef, 2016).

Research into the genetic basis for 'produce adaptation' has identified differential traits among serovars in attachment to surfaces and ability to form biofilms. For example, Patel et al. showed that *S. enterica* isolated from produce formed significantly more biofilm in microbroth, polycarbonate, and stainless steel surfaces compared to poultry isolates, and is consistent with spinach colonization ability (2013). Mechanistically, it has been identified that *fliC* may be important for attachment onto leafy greens on a serovar specific basis (Berger et al., 2009). Deletion of *ycfR* and *yigG,* two genes necessary for attachment to spinach and grape tomatoes, resulted in serovar-specific deficiencies in aggregation and tolerance to chlorine (Salazar et al., 2013). Furthermore, serovar-specific interactions with sugar residues in plant cell walls may affect attachment to plants (Tan et al., 2016).

Study into other factors that may contribute to serovar-specific persistence on plants is sparse, but several *S. enterica*-plant interactions have been reported that are important for bacterial colonization. In our previous work, we identified genetic mechanisms that are induced in *S.* Newport when associating with tomato surfaces which help mitigate plant-derived nitric oxide (NO) and reactive oxygen species released by plants upon recognition of *S. enterica* (see **Chapter 3**). NO in the traditional *S. enterica* pathogenesis model is released by animal host cells during *S.*

enterica invasion in an attempt to damage bacterial DNA and proteins (van der Heijden et al., 2015). *S. enterica* must detoxify this threat for successful pathogenesis, however save one study in chicken cells which found *S.* Typhimurium and *S.* Enteriditis adept at inhibiting host NO production, it is currently unclear if different *Salmonella* serovars mitigate NO stress with similar efficacy (He et al., 2012). In the phyllosphere, NO burst is an important component of the early stages of plant immunity (Frederickson Matika and Loake, 2014). In combination with other abiotic stresses such as desiccation and UV exposure, serovar-specific differences in NO mitigation may significantly drive serotype-specific persistence outcomes on plants.

The objective of this study was to evaluate if different *S. enterica* serovars possessed the ability to detoxify nitric oxide *in vitro* and to investigate whether the ability to mitigate this stress is associated with colonization outcomes on plants. With this effort, we hope to link potential factors driving serovar specific adaptations to non-animal host environments. Such work can aid the development of serovar-targeted intervention strategies to minimize food safety risk in cultivation of fresh produce crops, especially in areas where many diverse serovars of salmonellae may be present in the agricultural environment.

2. Materials and Methods

2.1 Cultivation of plant material

Tomato seeds cv. ‘Heinz-1706’were obtained from the Tomato Genetics Resource Center (TGRC) from the University of California, Davis. TGRC seeds were germinated at 25°C after pre-treatment in 30% w/v polyethylene glycol solution at

room temperature shaking for 72 h. Germinated seeds were transferred to potting media (Sunshine LC1; Sungro Horticulture, Canada) supplemented with fertilizer and subjected to a 16 h-light–8 h-dark photoperiod, 26°C day temperature /18°C night temperature with 70% relative humidity in a plant growth chamber. Tomato seedlings were grown to the 5-true leaf stage before experimentation.

2.2 Selection of serotypes for study

An epidemiological assessment of *S. enterica* outbreaks from 1998-2017 was conducted to determine representative serovars in different food commodity classifications for the present study. Food tool data for the period 1998-2015 was acquired on 1-20-2016 and 2016-2017 data was acquired on 12-10-18 from the National Outbreak Reporting System Dashboard (NORS) https://wwwn.cdc.gov/norsdashboard/. All non-typhoidal *S. enterica* outbreaks from 1998-2017 were included. Outbreaks which did not have an etiologic agent, were not confirmed, or did not have a food vehicle were filtered out. Outbreaks with multiple etiologic agents had illnesses divided equally among the agents. Food vehicles were classified into one of the following commodity classifications: produce, dairy, eggs, meat, nuts, and other. Any complex food vehicles (i.e. 'deli sandwich') or foods which could be grouped into multiple commodity classifications were labeled as 'other'. Seeds were included in the 'nuts' category, frozen produce and sprouts were treated as produce. Ice cream was treated as dairy unless otherwise specified. To compare influence of serovars on illnesses, a 5% cutoff was employed within each food commodity to return serovars responsible for 95% of illnesses.

2.3 Bacterial strains

S. Newport *XbaI* pattern JJPX01.0061 (Greene et al., 2008), *S.* Typhimurium LT2 (ATCC 700720), *S.* Heidelberg, *S.* Enteriditis (both isolated from a poultry house environment), *S.* Javiana ATCC BAA-1593 (clinical isolate from a tomato outbreak) and *S.* Typhimurium (isolated from bird faeces; Micallef et al., 2012) were used for this study. Strains were maintained at -80°C in Brucella Broth (BD, Sparks MD) containing 15% glycerol and 50 µg/mL rifampicin (Rif; Tokyo Chemical Industry, Portland OR). For each experiment, cultures of *S. enterica* were grown overnight on Trypticase Soy Agar *(*TSA; BD) with Rif at 35°C. A single colony was selected from each culture, suspended in sterile water and diluted to OD_{600}= 0.34, approximately 8.5 log CFU/mL. Serial dilutions were prepared in 1X PBS for *in vitro* inocula and sterile water for on-plant inocula. Remaining dilutions were performed in 0.1% peptone water for enumeration on TSA Rif by standard plate counting.

2.4 Growth curve of S. enterica *in presence of NO*

Fifty µL of *S.* Newport or *S.* Typhimurium LT2 in 1X Phosphate Buffered Saline (PBS) were inoculated into 5 mL tubes of Trypticase Soy Broth (TSB; BD) to obtain a final concentration of 4.3 log CFU/mL. To these suspensions, spermine NONOate (SPER/NONO; Calbiochem, Millipore Sigma), an NO donor, was suspended in 1X PBS and added to half of the tubes to a final concentration of 1 mM. Control tubes received equal volumes of 1X PBS. Tubes were incubated at 28°C with shaking at160 rpm for 24 h. Following incubation, 100 µL were retrieved at 0.5, 2 and every subsequent 2 h thereafter up to 24 h for dilution plating and enumeration on TSA Rif.

2.5 In vitro determination of S. enterica *NO tolerance*

One hundred and fifty µL of 7.8 –8.0 log CFU/mL of *S. enterica* suspensions in 1X PBS were inoculated into flat-bottom 96-well polystyrene plates (Sigma Aldrich St. Louis, MO). SPER/NONO was suspended in 1X PBS and added to the following final concentrations [0, 125, 250, 500, 1000, and 2000 µM]. Plates were sealed with parafilm and incubated at 28°C with shaking at 50 rpm. At 1, 4, and 24 h post-inoculation (hpi), wells were destructively sampled, serially diluted in 0.1% peptone water and plated on TSA+ Rif or TSA without antibiotic for bacterial quantification. The following calculation for "log decline" was applied for normalization across different inoculums and to correct for baseline persistence in PBS: (log CFU /$mL_{inoculum}$ - log CFU/$mL_{X\ SPER/NONO\ concentration}$) – log CFU/$mL_{0\ \mu M\ SPER/NONO}$.

2.6 Serotype response to modulation of tomato nitric oxide levels

To investigate serovar specific survival on tomato plant surfaces, the third emerged leaf on 5-leaved 'Heinz-1706' seedlings was treated with either 1 mL 0.5% v/v of $CaCl_2$ (Sigma, St. Louis MO) to elicit endogenous production of NO (Chakraborty et al., 2016) or sterile water. Treatments were aerosol sprayed onto the leaf, then left to dry for 4 h at 25°C. Once pretreated, a suspension of 5.5 log CFU/ml *S. enterica* was applied to the surface of one leaflet in 10 x 2 µL spots. Samples were incubated in 80% RH for 12 h in a biosafety cabinet. To retrieve viable *S. enterica* cells, inoculated leaflets were excised, diluted in 0.1% peptone water and hand massaged for 30 s followed by 1 min sonication using an 8510 Branson Sonicator. Serial dilutions were then plated onto TSA Rif for bacterial quantification.

2.7 Statistical analysis

All statistical analysis was performed using JMP 14.1. For epidemiological data, chi square tests were performed to determine the likelihood of independence in outbreak distribution at the commodity level across all *S. enterica* and across six serovars of interest (α=0.05).

Growth curve experiments were performed as a repeated measures design with three biological replicates per treatment. Data were fitted to the Huang full growth model through IPMP 2013 (Huang, 2014) software, as described in the following equation:

$$Y(t) = Y_0 + Y_{max} - \ln\{e^{Y_0} + [e^{Y_{max}} - e^{Y_0}]e^{-\mu \max B(t)}\}$$

$$B(t) = t + \frac{1}{\alpha}\ln\frac{1 + e^{-\alpha(t-\lambda)}}{1 + e^{\alpha\lambda}}$$

where $Y(t)$, Y_0 , $Y_{\max}$ are bacterial populations in natural logarithm, at initial, maximum , and time (t). μmax refers to the growth rate, and λ refers to lag time with the transition coefficient of $\alpha=4$. Growth parameters among different treatments were analyzed for significance with ANOVA and pairwise comparisons were conducted using Student's *t*-test (α=0.05).

In vitro experiments were performed as a completely randomized design with three biological replicates per treatment combination, and each serovar was tested in at least two experimental replicates. The homogeneity of variances was evaluated using Brown-Forsythe's test, and pairwise comparisons between treatments were performed via Welch's test, followed by post-hoc Kruskal-Wallis / Wilcoxon rank test (α=0.05).

On-plant challenge assays were performed as a Randomized Complete Block Design, with placement in the biosafety cabinet serving as the Block. Each plant treatment comprised of 4 biological replicates. The significance of treatment effects were assessed using Student's T-test to compare leaf treatments and orthogonal contrasts to compare serovars within each leaf treatment (all at $\alpha=0.05$).

3. Results

3.1 Incidence of illness by serotype and food category

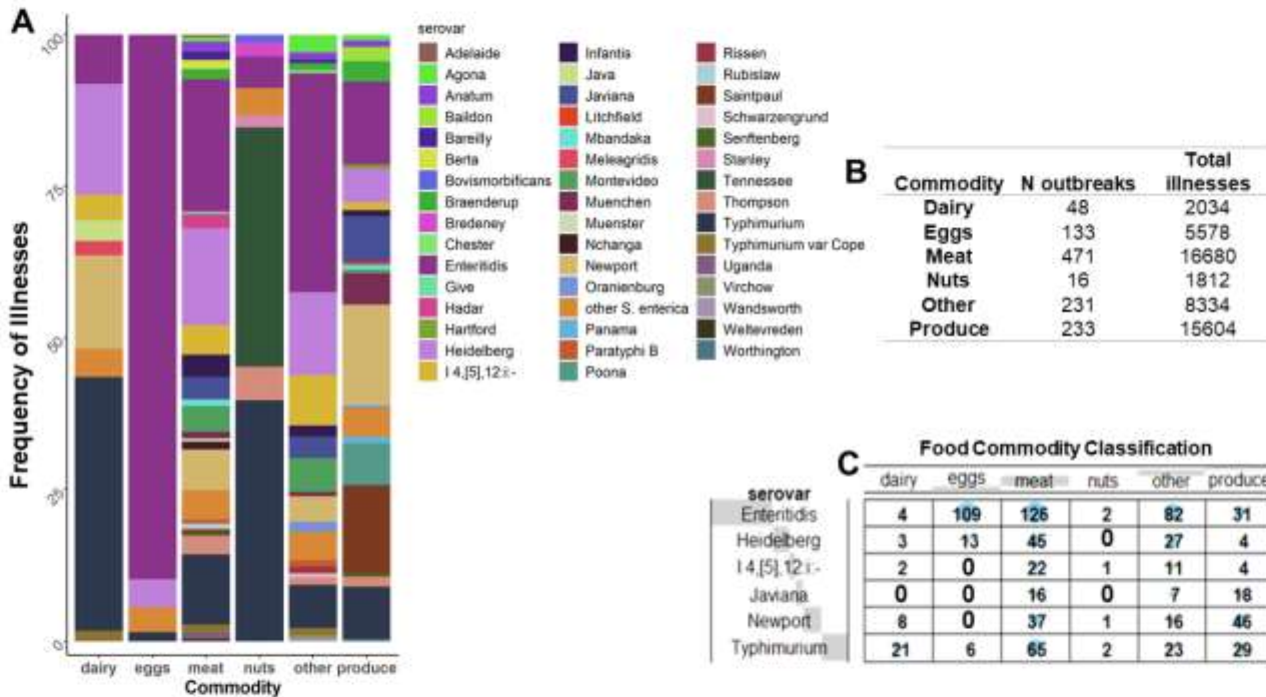
In total, 1,132 unique foodborne illness outbreaks were returned from the NORS query, amounting to 50,042 illnesses from *S. enterica* outbreaks between 1998-2017 caused by 90 serotypes (**Figure 1**). The commodity "meat" had the highest number of outbreaks and illnesses (471 outbreaks, 16,680 illnesses) followed by the "produce" commodity (233 outbreaks, 15,604 illnesses) (**Figure 1B**). Across all commodities, 10 *Salmonella* serovars were responsible for 78% of illnesses. *S.* Enteriditis was responsible for 14,036 illnesses, followed by *S.* Typhimurium (5,520), *S.* Heidelberg (5,286), *S.* Newport (4,380), *S.* Saintpaul (2,479), *S.* Javiana (2,092), *S.* I 4,[5],12:i:- (1,801), *S.* Montevideo (1,306), *S.* Muenchen (1,098) and *S.* Poona (1,097). Interestingly, the number of outbreaks caused by a serovar was significantly correlated with the number of illnesses ($R^2=0.97$, $p<0.001$).

95% of all *S. enterica* illnesses during 1998- 2017 returned 1,069 outbreaks and 47,706 illnesses. *S.* Enteriditis in "eggs" had the highest frequency of associated illnesses (89%) compared to any other serovar-commodity classification (**Figure 1A**). Consequently, eggs had the fewest number of serovars responsible for 95% of

illnesses. *S.* Enteriditis was also the serovar exhibiting the highest frequency of illnesses in the "meat" (21%) and "other" (36%) categories at the 95% level. *S.* Typhimurium was the serovar in "nuts" (39%) and "dairy" (42%) displaying the highest frequency of illnesses, and *S.* Newport was the serovar in the "produce" commodity that had the highest frequency of illnesses (16.6%). Serovar-specific commodity associations at the 95% level were apparent, with *S.* Newport and *S.* Javiana both responsible for a higher frequency in illnesses associated with "produce" (16.6% and 7.5%, respectively) compared to meat (6.7% and 3.7%, respectively). Conversely, *S.* Heidelberg was more abundant in meat-associated illnesses (16.0%) compared to produce (5.5%).

Comparing the number of outbreaks caused by *S. enterica* etiologic agent, it was found that *S. enterica* serovars were not equally likely to cause an outbreak across each food commodity type (X^2 (5, N=1132) = 792.1, $p<0.001$), providing further evidence that serovar – commodity associations may exist. Investigation of outbreaks caused by six serovars of interest (**Figure 1C**); *S.* Enteriditis, *S.* Heidelberg, *S.* Javiana, *S.* Newport, *S.* Typhimurium and *S.* 4,[5],12:i:- revealed within this subsection of serovars, it was not likely that outbreaks were distributed evenly across food commodities (X^2 (25, N=781) = 248.0 $p<0.001$), with *S.* Newport, *S* Javiana, *S.* Typhimurium and *S.* Enteriditis more likely to be implicated in a produce outbreak compared to *S.* Heidelberg or *S.* 4,[5],12:i:-. Moreover, 50% of *S.* Newport outbreaks were attributed to produce compared to 4% of *S.* Heidelberg outbreaks. These serovar-commodity associations informed serovar selection for

evaluating differential NO detoxification among serovars, choosing to evaluate *S.* Enteriditis, *S.* Heidelberg, *S.* Typhimurium, *S.* Javiana and *S.* Newport.



Commodity	N outbreaks	Total illnesses
Dairy	48	2034
Eggs	133	5578
Meat	471	16680
Nuts	16	1812
Other	231	8334
Produce	233	15604

serovar	dairy	eggs	meat	nuts	other	produce
Enteritidis	4	109	126	2	82	31
Heidelberg	3	13	45	0	27	4
I 4,[5],12:i:-	2	0	22	1	11	4
Javiana	0	0	16	0	7	18
Newport	8	0	37	1	16	46
Typhimurium	21	6	65	2	23	29

Figure 1: Distribution and abundance of illnesses caused by *S. enterica* in each commodity classification, from 1998 – 2017. Data adapted from the CDC National Outbreak Reporting System https://wwwn.cdc.gov/norsdashboard/. (A) Summary of 95% of illnesses caused by *S. enterica* in a given commodity classification. (B) Total illnesses and outbreaks across all serovars from 1998 – 2017. (C) Contingency table of the number of outbreaks from six serovars of interest between 1998-2017 by food commodity classification.

3.2 Effect of 1 mM Spermine NONOate on S. enterica *growth*

Comparing the growth model parameters of *S*. Newport and *S.* Typhimurium LT2 in TSB with or without 1 mM NO, all cultures had the same starting inoculum (Y_0) (**Table 1**). Both serovars challenged with SPER/NONO exhibited significant increases in lag time compared to unamended TSB, from 1.9 and 1.3 h to 10.0 and 10.8 h in the presence of SPER/NONO for *S*. Newport and S. Typhimurium, respectively (p<0.001). No significant difference in lag time was detected between serotypes grown in TSB, but the discrepancy approached significance between serotypes in the presence of the NO donor (p=0.055). *S.* Newport exhibited a faster growth rate compared to *S*. Typhimurium regardless of SPER/NONO challenge (p<0.001). When normalized to TSB, the growth rate declined slightly more for *S.* Typhimurium in the presence of the NO donor compared to *S.* Newport, but this was not statistically significant. Serovar specific differences were apparent with respect to final population attained (Y_{max}) in SPER/NONO-amended TSB, with *S.* Newport growing to 1.1 log CFU/mL higher concentrations compared to *S.* Typhimurium (p<0.001).

Table 1: IPMP Huang Growth Curve Model Parameters of *S. enterica* in 1mM SPERNONO stress

Treatment	RMSE	± Std Dev	y_0	± Std Dev	Lag (h)	± Std Dev	y_{max}	± Std Dev	μ_{max}	± Std Dev
SPER/NONO-LT2	0.169	0.04	4.191	0.02	10.801	0.05	8.008	0.12	0.821	0.08
SPER/NONO-SeN	0.640	0.86	4.131	0.15	10.040	0.49	9.124*	0.09	1.152*	0.04
TSB-LT2	0.205	0.01	4.200	0.15	1.316	0.41	9.125	0.03	0.932	0.01
TSB-SeN	0.194	0.04	4.355	0.11	1.889	0.30	9.335	0.05	1.239*	0.04

* Denotes significant differences between serotypes within treatments via Student's T test (α=0.05).

3.3 Effect of increasing NO concentrations on S. enterica *serovar survival over 24 hours*

Across all serovars, 250 µM SPER/NONO was the minimum concentration at which retrieval was significantly lower compared to 0 µM, hereinafter referred to as the minimum concentration to reduce growth (MCRG). This activity was first detected at 4 hpi ($p<0.05$ Tukey HSD). (**Figure 2A**). At 1 hpi, 500 µM was detected as the MCRG ($p<0.05$ Tukey HSD). Interestingly, a significant ($p<0.05$) positive effect of 15 µM NO on *S. enterica* growth was found at 24 hpi. We set the range of concentrations for subsequent experiments from 250 to 2000 µM to assess NO tolerance among five serotypes. Population decline progressed in a SPER/NONO concentration-dependent manner (**Figure 2B**). In general, log decline from 1-24 hpi in 250 µM NO donor was constant, whereas in 500 µM the maximum log decline was reached at 4 hpi , except in the case of *S.* Typhimurium which continued to experience log declines to the 24 hpi timepoint. Populations of cells exposed to the higher concentrations of 1000 and 2000 µM SPER/NONO displayed continuous decline without reaching a maximum from 1 to 24 h for all serotypes except *S.* Heidelberg.

Differences in the ability to tolerate NO were affected by serovar type and exposure time to SPER/NONO. Differences between serovar tolerance were first evident at 1 hpi at 500 ($p=0.121$), 1000 ($p=0.157$) and 2000 µM ($p<0.05$) of SPER/NONO (**Figure 2B**), with *S.* Typhimurium initially displaying more tolerance to NO compared to other serovars ($p<0.05$). However, this ability was not sustained and *S.* Typhimurium lost this advantage at 24 hpi, exhibiting significantly more log

decline in solutions of 250, 500 and 1000 μM compared to *S*. Heidelberg and *S*. Javiana (p<0.05). This suggests that *S*. Typhimurium may be adept at mitigating initial NO stress, while *S*. Javiana and *S*. Heidelberg may be more competitive under long term stress. Other serovar specific differences were evident with *S*. Javiana. At 4 hpi, this serovar displayed higher tolerance to NO compared to *S*. Heidelberg and *S*. Enteriditis at 250 and 500 μM ($p < 0.05$ for *S*. Heidelberg at 250 and *S*. Enteriditis at 500 μM).

Serovar specific differences in decline following long term (24 hpi) exposure were highly dependent on NO concentration. At 250 μM, *S*. Heidelberg and *S*. Javiana experienced significantly less decline than *S*. Newport (p<0.05). In 500 μM solution, *S*. Javiana and *S*. Heidelberg experienced less decline than *S*. Typhimurium, *S*. Enteriditis, and *S*. Newport (p<0.05). In 1000 μM solution however, *S*. Javiana and *S*. Heidelberg seemed to lose tolerance with *S*. Enteriditis instead displaying less log decline compared to *S*. Javiana (p<0.05), *S*. Newport (p>0.05), and *S*. Typhimurium (p<0.05). At 24 hpi, variances among samples increased with increasing NO concentrations, evidenced by significant Brown-Forsythe's test at 2000 μM (**Figure 2C**). Consequently, 2000 μM samples did not display specific differences in the log decline. *S*. Heidelberg notably showed the highest increase in variance, attributed to significant variation introduced by one experimental replicate (**Figure 2D**). In this replicate (**Figure. 2D**), *S*. Heidelberg unexpectedly exhibited the least log decline compared to other serovars at all timepoints, an observation that was not reproducible but that skewed the overall data at the higher concentrations for this serotype.

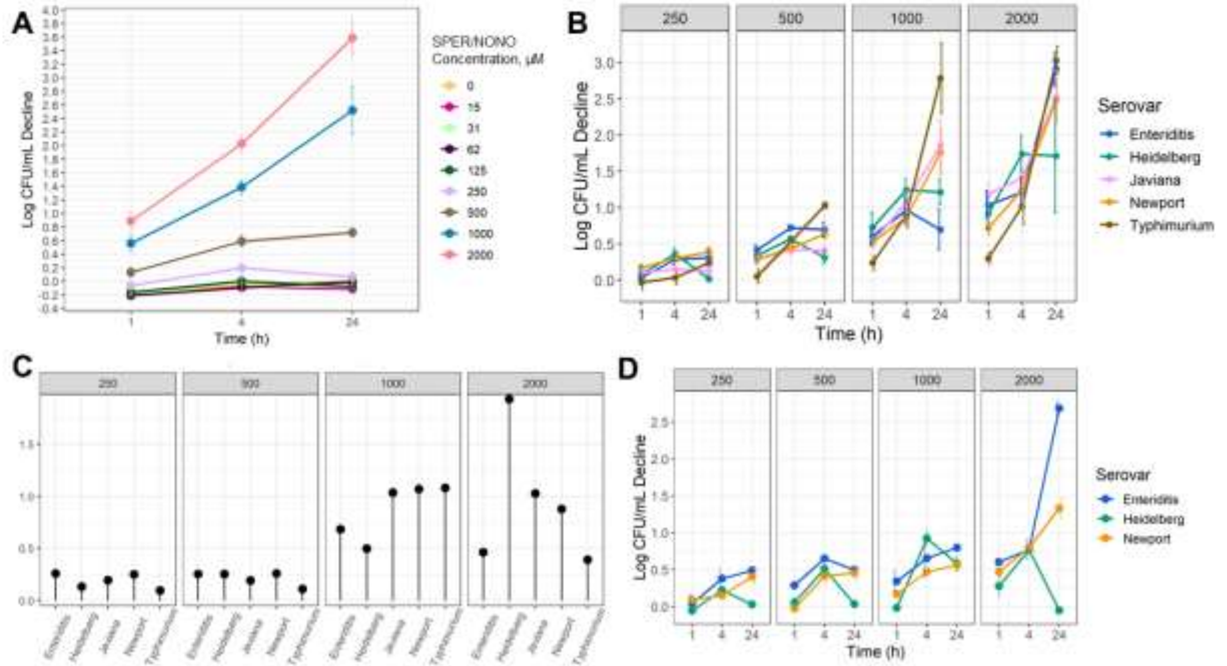


Figure 2: Log decline from inoculum of five *Salmonella enterica* serovars at various SPER/NONO concentrations after 1, 4, and 24 h post inoculation. (A) Concentrations of NO across serovars tested to find functional range of NO for subsequent in vitro assays. (B) Log decline from inoculum, corrected for log decline in 0 µM for each sample at each sampling time, of five serovars tested at 1, 4, and 24 in hpi 96 well plates of PBS amended with various concentrations of NO. N=3 biological replicates, with at least two technical replicates per serovar. (C) Standard deviations at 24 h of each serovar at each SPER/NONO concentration, averaged over 4 experimental replicates. (D) Third experimental replicate pulled out to illustrate inter-experimental variability of *S*. Heidelberg.

3.4 S. enterica *strain retrieval from tomato seedlings elicited to produce excess NO*

Different *S. enterica* strains were inoculated onto tomato surfaces to identify serovar specific differences in the capacity to associate with the tomato phyllosphere while negotiating either augmented or native plant concentrations of NO. Exogenous elicitation of NO in seedlings significantly decreased *S. enterica* survival by an average of 2.0 log CFU/seedling across all serovars (p<0.001, Student's T-test). The most decrease between control and elicited seedlings was found in *S.* Heidelberg (2.46 log CFU/seedling) and the least observed in *S.* Enteriditis (1.70 log CFU/seedling).

Serovar specific differences in retrieval from seedlings in both treatment environments was also identified. *S*. Javiana was retrieved from seedlings in higher concentrations compared to *S*. Newport (p=0.170) in the native environment, and significantly higher compared to Heidelberg in both the native and excess NO plant environments (p=0.022), (p<0.001), respectively (**Figure 3**). In Fact, *S.* Heidelberg retrieval from NO-excess seedlings was significantly reduced compared to all other serovars tested (p<0.05 via orthogonal contrasts).

No significant differences in variances for each treatment by strain were found using Brown-Forsythe's test, however, in NO excess environments differences in variances approached significance (p=0.063), with *S.* Enteriditis (std dev =0.500) and *S.* Heidelberg (std dev =0.250) variances higher than *S.* Javiana (std dev =0.139) and *S.* Newport (std dev =0.163). It was interesting to note serovar specific trends were apparent in the magnitude of variance depending on seedling treatment; where the variances in *S.* Javiana and *S.* Newport increased from $CaCl_2$ samples to water

samples, and the variances of *S.* Heidelberg and *S.* Enteriditis displayed the opposite trend.

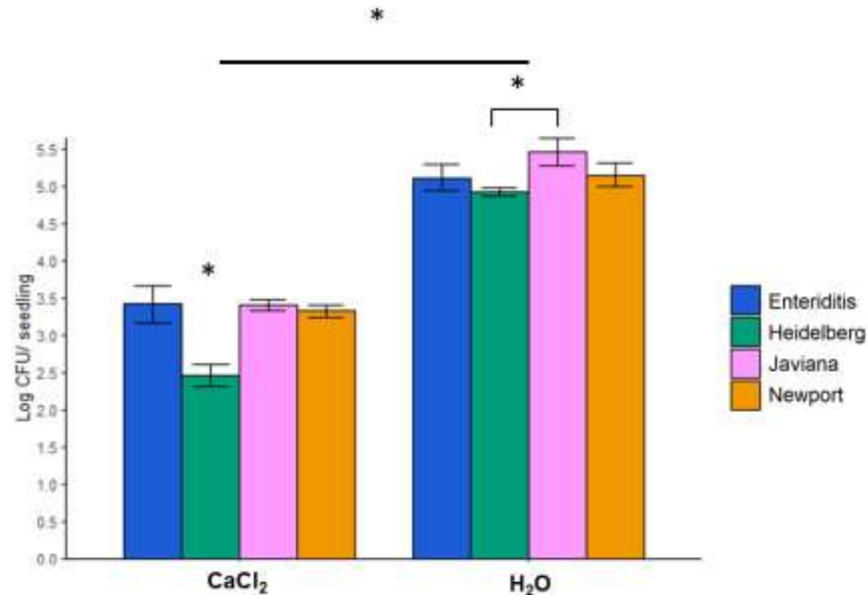


Figure 3: Surface populations of *S. enterica* after pre-treatment of plant tissue with H_2O_2 or NO elicitor. Five leaf seedlings were aerosol sprayed with 1 mL sterile water or 0.5% CaCl2 and dried 4 h before surface inoculation with *S. enterica*. Error bars represent SEM, significance was determined using Student's T-test and orthogonal contrasts (α= 0.05). N=4 plants per treatment. One *S.* Heidelberg outlier was removed from analysis.

4. Discussion

Investigating enteric pathogen-plant interactions is a burgeoning area of scientific research with recent interest in evaluating bacterial genotype as a factor for persistence. For example. Patel and Sharma reported that a cocktail of *S. enterica*

produce isolate strains persisted longer and at higher concentrations when irrigated on spinach compared to poultry isolate cocktails (2013). However, comparatively few studies have investigated phenotypic and genetic factors behind these observed associations. Epidemiological results from the present work indicate that serovar specific associations exist for certain food commodity classifications, such that *S.* Newport and *S.* Javiana are more likely to be attributed to produce commodity illnesses and outbreaks compared to meat commodities. Initial growth curves comparing *S. enterica* serovar specific tolerance to NO found that the total population maximum in the presence of NO stress was serovar dependent. Furthermore, serovar specific tolerance to NO *in vitro* was apparent in a concentration and exposure time dependent manner such that one produce associated serovar and a generalist serovar, *S.* Javiana and *S.* Typhimurium, displayed increased tolerance to NO at lower concentrations (≤ 500 µM) and at shorter exposure times (≤ 4hpi). However, at long exposure times and high NO concentrations, *S.* Enteriditis displayed increased tolerance. Finally, it was identified that plant derived NO can negatively affect colonization outcomes of all *S. enterica* serovars tested, a finding which necessitates future investigation into microbial responses to plant-released signals.

It was expected that serovars *S.* Newport and *S.* Javiana to perform well both in the *in vitro* and the on-plant studies. Previous work investigating strain specific persistence on red ripe tomatoes reported that during storage *S.* Newport could persist at levels similar to *S.* Javiana, both higher than *S.* Enteriditis and *S.* Typhimurium (Shi et al., 2007). Further, from a five strain inoculation of *S.* Montevideo, *S.* Javiana, and *S.* Typhimurium and *S.* Newport, the latter was regularly recovered from inoculated

leaves at concentrations similar to *S.* Javiana, and both were recovered significantly more compared to *S.* Typhimurium (Zheng et al., 2013). Similar results were retrieved from fruit that was inoculated at the blossom stage. In the present study, *S.* Javiana did in part display an advantage over other serovars, evidenced at low SPER/NONO concentrations *in vitro* and also retrieval from plants consistent with previous work (Zheng et al., 2013). This could suggest that S. Javiana may be a hearty persister in phyllosphere environments, although longer term study (greater than 12 h) is needed to further explore this hypothesis. *In vitro S.* Newport did not follow this trend as expected, and *S.* Heidelberg instead displayed similar NO tolerance to *S.* Javiana. Furthermore, at high SPER/NONO concentrations in the *in vitro* study *S.* Enteriditis and *S.* Heidelberg and not *S.* Javiana displayed advantages compared to other serovars tested. One study in chicken macrophages may provide some insight to these findings. In this work, the authors found *S.* Enteriditis was able to completely block production of macrophage NO, providing evidence to support this observation (Balan and Babu, 2017; He et al., 2012). Interestingly, on tomato seedlings, *S.* Heidelberg was retrieved in lower concentrations compared to *S.* Javiana. However, in the *in vitro* study *S.* Heidelberg was rarely different in its tolerance to NO except for 1 replicate of 2,000 μM at 24 hours. This discrepancy in tolerance across experimental conditions has been replicated in one other study: in chicken macrophage cell lines, while *S.* Typhimurium was able to abrogate NO production, it did not have significantly higher invasion rates in the host macrophage cells (He et al., 2012). Taken together, this may indicate that the interaction of

multiple factors governs colonization outcomes; either tied to NO (e.g. the degree of plant NO induction to certain serovars), or perhaps other unknown factors.

Long term exposure (24 hpi) of extremely high concentrations of NO (2000 μM) led to no serovar specific differences in tolerance and high variability among response, especially prominent in *S.* Javiana, Newport and Heidelberg. Heterogeneity of stress response within populations may be a survival strategy for long term persistence, and has been observed in murine infection models (Helaine et al., 2014). Furthermore, it may be reasonable to expect an erasure of all serovar specific responses as especially when levels extremely high levels of NO concentrations are reached which likely overwhelm cellular detoxification machinery. Nanomolar to two micromolar levels of NO are expected to be released from plants as part of their immune response when challenged with pathogens (Mur et al., 2011). In the present study using plate counts there was no observed effect on colony levels at the lowest level of SPER/NONO used, however cellular level transcriptomic effects may be present and are an opportunity for further investigation.

Salmonella can mitigate NO stress in both aerobic and anaerobic conditions through direct detoxification or through efflux systems (Henard and Vazquez-Torres, 2011). Most studies investigating NO detoxification machinery are with *Salmonella* Typhimurium (Crawford and Goldberg, 1998; Fitzsimmons et al., 2018; Karlinsey et al., 2012), however variations in nitrosative stress response may exist among *S. enterica* serovars. For example, *Salmonella* Saintpaul does not have the *ssrB* region (needed to detect NO stress), and within *S.* Typhimurium clinical isolates there is a clade characterized by mutations in *hmpA* and *katE,* leading to a loss of fitness in

nitrosative and oxidative stress (Hayden et al., 2016). Serovar specific differences by time and NO concentration in the present study may be indicative of *S. enterica* serovars employing variable strategies and efficiencies to mitigate nitric oxide. It would be valuable in future work to identify if one machinery is favored over the other in various NO-producing environments.

Unlike other papers which investigate *Salmonella* Newport in the phyllosphere and associated tomato fruits (Shi et al., 2007; Zheng et al., 2013), this study did not find that *Salmonella* Newport was in the strongest persister group. There may be two possible explanations for this observation. One possible reason for this could lie in differential plant response to *Salmonella* serovars. For example, plants may produce more NO after contact with *S.* Newport compared to other serovars, creating a comparatively more stressful environment for colonization of this serovar and leading to fewer recovered cells. In support of this hypothesis previous work has identified *S.* Typhimurium flagellin flg22 which induces PAMP triggered immunity as evidenced by up-regulation of immunity related transcription factors, oxidative burst, and salicylic acid accumulation over time while *S.* Senftenberg flagellin failed to elicit a similar response (Garcia et al., 2014). The notion that only some *Salmonella* may elicit plant responses is highly interesting and could provide evidence of plant-enteropathogen reciprocal responses leading to strain specific adaptation to specific phyllosphere environments. To further investigate this, it would be prudent to investigate the ability of Salmonellae to elicit NO production.

Another explanation for this result could be due to the tomato cultivar used. In a previous study by Han et al., it was identified that epiphytic *S. enterica* persistence

was significantly influenced by tomato cultivar, with cv. 'Heinz 1706' resulting in significantly less retrieval of *Salmonella* Newport on leaves and fruit (2014). It was hypothesized these differences may be due in part to the plant immune response system in this cultivar, and therefore chose to use cv. 'Heinz-1706' for the present study. *S.* Newport persistence on this cultivar may be due to plant factors other than plant immunity, as it is well known that a suite of other factors drive bacterial persistence on plants (Brandl et al., 2013). In another study by our group, we investigated *S. enterica* persistence in surface exudates of various tomato cultivars. The study found that Heinz fruit surface washes were enriched in fatty acids in higher proportion to other compounds including sugar and sugar alcohols, and that these compounds were correlated with reduced *S.* Newport growth (Han and Micallef, 2016). Therefore, it would be reasonable to suspect that aside from the immune response system, other components and chemicals making up physical barriers and physiological processes could also significantly affect *S. enterica* persistence on a serovar specific basis. Taken together, these results suggest further investigation is needed to explore colonization dynamics in the context of plant surface features.

In this study it was found that seedling-derived NO significantly decreased colonization outcomes for all serovars tested. This highlights an important discovery that plant activity can significantly influence enteric pathogen activity, such as colonization. Furthermore, *S.* Heidelberg, a serotype that has not been implicated in fresh produce outbreaks, was more affected by plant-derived NO than other serovars, as evidenced by lower retrieval compared to other serovars. A similar phenomenon has been documented where *S.* Typhimurium had similar to low viability in chickens

but *in vitro* almost completely inhibited NO production in HD11 cells (He et al., 2012). From this, investigation into other factors which interact with NO to negatively affect *S. enterica* colonization is warranted.

It is also important to consider how plant cultivar, age, and tissue type would affect serovar specific dynamics. For example, it has been reported that recovery of various *S. enterica* serovars from tomato seeds was significantly lower than fenugreek, alfalfa, and lettuce seeds (Cui et al., 2018). In terms of plant genotype, while studies in tomato and basil found Typhimurium uncompetitive in the phyllosphere (Kisluk et al., 2013; Zheng et al., 2013) this serovar was robust in endophytic colonization of lettuce leaves (Klerks et al., 2007a). These studies signify that there may be more factors at play that may determine success in non-animal hosts which remain to be elucidated for *S. enterica*.

In conclusion, this work identified that serovar specific differences in NO tolerance may exist, dictated by both exposure time and concentration of nitric oxide. All serovars persisted on Heinz seedling leaves and were negatively affected by plant derived NO, and the meat associated *S.* Heidelberg strain was retrieved in lower titers from the phyllosphere following NO elicitation compared to other serovars tested. We also found that *S*. Newport, an isolate commonly associated with tomato outbreaks, was retrieved at lower bacterial concentrations compared to other strains. More work is needed to understand this phenomenon, including carrying out experimentation for longer periods of time to investigate the presence of serovar specific long term survival outcomes on plants. Furthermore, additional study into other plant factors and their interaction with the diverse serovars of *S. enterica* must be pursued to

further investigate drivers behind "produce-associated" and "meat-associated" serovars.

Bridging environmental stressors *S. enterica* may encounter with persistence outcomes on plants is imperative to provide a sufficient profile of the hurdles each foodborne pathogen must interact with to establish itself on fresh produce crops. It is likely plant derived NO may be one stimulus included in a wider symphony of plant-enteric pathogen interactions, all working in concert to produce an outcome of colonization. Understanding these environmental queues for adaptation to the agricultural environment will aid researchers and farmers to develop targeted management techniques to ensure safe cultivation of food.

Chapter 5: *S. enterica* Serovar Specific Dynamics in Surface and Reclaimed Water and Transferability onto Tomato Fruit

1. Introduction

When assessing routes of vegetable contamination with foodborne pathogens during cultivation, irrigation water has been regularly implicated as a reservoir for *Salmonella enterica*. Evidence comes from successful trace-back investigations of foodborne illness outbreaks and the isolation of *S. enterica* from water sources that match clinical strains (Bell et al., 2015; Bennett et al., 2015; Callahan et al., 2019; Greene et al., 2008).

In an effort to minimize food safety risk imposed by irrigation water sources, the US Food Safety Modernization Act Produce Safety Rule (FSMA PSR) has compiled stringent guidelines for water quality to be used for irrigating the edible portion of fresh fruit and vegetables (Standards for the Growing, Harvesting, Packing, and Holding of Produce for Human Consumption). This includes developing a water quality profile based on generic *Escherichia coli*, a bacterial indicator of fecal contamination, for all irrigation sources used on produce covered by the regulation, as well as employing mitigation steps for water sources that fail to meet the *E. coli* standard. Mitigation steps can include initiating a wait period between irrigation and harvest which may impose unfeasible restrictions on growers selling to farmers' and other direct markets. Farmers can also opt to treat the water to reduce microbial loads- requiring added production cost, labor, and time, as well as compromising sustainable farming. Farmers can also choose to switch to other water sources such as

groundwater, but this places a burden on a precious natural resource (Perlman, 2016). For these reasons, protecting the quality of water used for irrigation is the most cost-effective and environmentally friendly approach to reducing the risk of water-related foodborne pathogen contaminating crops.

Even more troubling, the food safety risk to crops that foodborne pathogens like *S. enterica* pose if present in these waters is not well understood (Benjamin et al., 2013; Winfield and Groisman, 2003). Current research suggests *S. enterica* may or may not co-exist or correlate with *E. coli* levels in water (Benjamin et al., 2013; McEgan et al., 2013; Payment and Locas, 2011; Wilkes et al., 2009; Wu et al., 2011). Further, studies in autoclaved river water samples have illustrated *S. enterica* can persist in river and marine waters, the level of which is strain dependent and may include non-culturable metabolic states (Roszak et al., 1984; Santo Domingo et al., 2000). However, no work has been completed on mid-Atlantic surface water comparing “produce associated” with other commodity associated strains. Furthermore, there is a lack of information on foodborne pathogen persistence in reclaimed (recycled) water – an increasingly attractive alternative water source to alleviate pressure on groundwater resources.

Investigation into how *S. enterica* prevalence in water translates to probability of crop contamination is a new area of research. Some study of transfer potential from environmental media to crops has been undertaken, reporting highly variable persistence from 2 – 121 days (Xu et al., 2016, Islam et al., 2004, Kisluk and Yaron, 2012) which seems to be affected by contamination load, pathogen, and crop type. It has also been shown that culturing media for bacteria can positively influence

persistence on plants, such as pre-incubation of *E. coli* O157:H7 with manure extracts (Seo and Matthews, 2014), but impact of environmental media on *S. enterica* has not been established. Finally, preferential plant colonization of some *S. enterica* serovars relative to others has been documented (Zheng et al., 2013), but whether previous adaptation to surface water environments impacts this proclivity still remains to be investigated. Data are needed to better understand the agricultural risk of *S. enterica* presence in surface irrigation waters to develop strategies enable farmers to continue to use surface waters for irrigation of fresh produce, rather than resorting to expensive and energy-consuming remedial actions or expanding use of groundwater, a finite natural resource. Furthermore, expanding farmer access to highly treated reclaimed water would reduce pressure on groundwater resources, however additional research is needed to understand how to enable safe reclaimed water use for fresh produce irrigation in the mid-Atlantic.

The dearth of knowledge provides a strong rationale for further investigation into 1) *S. enterica* serovar-specific interactions in water and 2) connections between survival in water to persistence on plants. The objectives of this study were to investigate the presence of serovar specific differences in persistence in various types of surface water present in the mid-Atlantic, and to determine if persistence in water included the formation of viable but non-culturable states. This study also aimed to investigate whether attachment ability and incubation time in various types of water influenced serovar ability to transfer to tomato fruit. Finally, physicochemical parameter data of surface water when sampled was collected and evaluated via bivariate analysis to assess associations among persistence in water, transfer onto

tomatoes, or attachment to polystyrene. Investigating the influence of colonization in water environments on transference onto crops is essential to understanding the true risk pathogen presence in irrigation water environments poses to fresh produce crops and is critical to the development of practical and sustainable management strategies.

2. Materials and Methods

2.1 Bacterial strains

S. enterica 4,[5], 12:i:-, *S.* Newport, *S.* Typhimurium, and multidrug resistant *S.* Newport and *S.* Typhimurium ("MDR+", resistant against Ampicillin, Clavulanic acid, Cefoxitin, Ceftriaxone, Sulfisoxazole, and Tetracycline) were collected from rivers in Maryland (Callahan et al., 2019). *S.* Heidelberg was isolated from a poultry house environment and was kindly provided by FDA (Laurel MD). *S.* Javiana ATCC BAA-1593 strain is a clinical isolate from a tomato foodborne illness outbreak. The *S. enterica* strains were maintained at -80°C in Brucella Broth (BD, Sparks MD) containing 15% glycerol. For each experiment, cultures of *S. enterica* were grown overnight on Trypticase Soy Agar (TSA; BD) at 35°C. A single colony was selected from each culture, suspended in sterile water and diluted to OD_{600}= 0.34, approximately 8.5 log CFU/mL. Serial dilutions were performed in sterile water and cells were enumerated on TSA by standard plate counting.

2.2 Water preparation

Surface and reclaimed water (**Table 1**) was aseptically collected from sites as previously described (Allard et al., 2019a, 2019b) in sterile 1 L Nalgene containers. Immediately after collection, reclaimed water samples were quenched of free chlorine

via addition of 1 mL of 10% sodium thiosulfate (Alfa Aesar, Heysham, England). Water was kept at 4°C prior to 0.22 µm polyethersulfone membrane filtration (Corning, VWR). Filtered water was then stored at 4°C until experimentation. At the time of collection, a ProDSS digital sampling system (YSI, Yellow Springs, OH, USA) was submerged into the surface water body at a depth of 30 cm or into a 20 L carboy containing reclaimed water to collect triplicate measurements for the following physicochemical parameters: water temperature (°C), % dissolved oxygen, conductivity (SPC uS/cm), pH, oxidation/reduction potential (mV), turbidity (FNU), nitrate (mg/L), and chloride (mg/L). Salinity was indirectly calculated from conductivity using the following resource (https://jsta.shinyapps.io/cond2sal_shiny/).

Table 1: Surface water types and water codes used in this study

Water Code	Water Type
MA03	Non-tidal Fresh River Water
MA04	Tidal Brackish River Water
MA05	Non-tidal Fresh River Water
MA06	Reclaimed Water
MA10	Pond Water

2.3 Cultivation of plant material

Tomato seeds cv. 'Heinz-1706' were obtained from the Tomato Genetics Resource Center (TGRC) from the University of California, Davis. Seeds were germinated at 25°C after pre-treatment in 30% w/v polyethylene glycol solution at room temperature with shaking for 72 h. Germinated seeds were transferred to LC-1

soil (Sunshine LC1; Sungro Horticulture, Canada) supplemented with fertilizer and subjected to a 16 h-light/8 h-dark photoperiod and 26°C day temperature/18°C night temperature with 70% humidity (RH) at the University of Maryland Research Greenhouse. Tomato seedlings were transplanted at the into 1.7 gallon / 6 liter pots once they reached 5 leaf stage. Plants were irrigated via drip line. The plants were fertilized once a week and treated with non-organophosphate containing pesticide once every two weeks for aphid and white fly management. Fruit was collected immediately before experimentation and rinsed with ddH_2O. In summer months, 'Heinz-1706' where grown in the field at the Wye Research and Education Centre, Queenstown, MD.

2.4 Attachment capacity on polystyrene

Crystal violet staining was used to evaluate the ability of *S. enterica* strains to attach to a surface. All strains and water types were used for this experiment. Aliquots of 150 µL of filtered surface water or distilled water (control) were delivered to the central inner wells of a flat bottom 96-well polystyrene plates (Corning, Nazareth PA) followed by inoculation with 50 µL *S. enterica* in sterile water to a final concentration of 7.9 log CFU/mL. The wells comprising the perimeter of the plate were filled with 200 µL distilled water to prevent evaporation. Plates were sealed with parafilm and statically incubated at 24°C for 48 h before washing and staining. Wells were carefully washed once with distilled water before staining with 0.2% w/v crystal violet for 30 min. Stained wells were carefully washed thrice with distilled water, then solubilized in 200 µL of 30% v/v glacial acetic acid for 15 min prior to reading OD at 600 nm on a Synergy HTX microplate reader (BioTek, Winooski VT). For

each sample, an "attachment index" was calculated for each serovar in each surface water by normalizing for attachment in TSB using the formula $OD_{sample} / OD_{Average\ TSB}$. These data were used for statistical analysis. This experiment was repeated thrice for all water types except MA05 and MA06 for which this was repeated twice. JMP 14.0 (SAS Institute Inc., Cary, NC) was used to perform ANOVA and Tukey's Honestly Significant Difference (α=0.05) to determine the effect of water type and serovar on attachment index. Serovar specific differences in attachment for each water type were determined by ANOVA or Welch's test following a significant Brown-Forsythe test indicating homogeneity of variances was violated. Pairwise comparisons performed by Tukey's HSD or Wilcoxon test, respectively (α=0.05).

2.5 Persistence in surface water samples over 90 days via agar plating

S. enterica strains were singly inoculated into 9.9 mL of 0.22 µm filtered surface water to a final concentration of 3.70 (replicate 1) or 4.65 (replicate 2) log CFU/mL (see **Table 2** for more information on strains and water used for each experiment). Samples were incubated at 24°C with shaking at 100 rpm for 90 days with caps on but not tightened to allow for oxygen flow. Samples were taken at day 1, 3, 5, 10, 20, 30, 60, 90 for replicate 1; day 3 and 5 were deleted from the sampling scheme of replicate 2 due to lack of change in population. At each sampling time, 100 µL aliquots were collected for preparation of 10-fold dilutions that were plated on Trypticase Soy Agar. This experiment was repeated twice with three experimental replicates per treatment combination. Bacterial decline was modeled using Buchanan's two-phase log-linear model (Buchanan and Golden, 1995) via IPMP 2014 (Huang, 2014) software, as described in the following equation:

$$y = y_0, \qquad t \leq t_{shoulder}$$

$$y = y_0 - \frac{t - t_{shoulder}}{D}, \quad t > t_{shoulder}$$

where Y is the population of bacteria at day x; y_0 is the log normalized population of cells at each day during the lag time before a decline in agar plate count commences; $t_{shoulder}$ is the time in days at which linear decline begins; and D is the negative reciprocal of the rate of decay.

Parameters obtained as well as the Root mean squared error (RMSE) and R^2 were compared with linear decay models generated in DMfit 3.5 (https://www.combase.cc/index.php/en/8-category-en-gb/21-tools) based on the Baranyi linear reduction model (Baranyi and Roberts, 1994). Due to DMfit 3.5 over-estimation in lag and larger variations in RMSE, Buchanan's two-phase log-linear model output was selected for biological analysis (data not shown). Total log inactivation over 90 days (calculated via $LogCFU/mL_{Day\ 1}$ - $LogCFU/mL_{Day\ 90}$), lag time, and the rate of decay were used to compare bacterial persistence in water by water type, serovar, and experimental replicate using JMP 14.0 (SAS Institute Inc., Cary, NC) via ANOVA and Tukey's Honestly Significant Difference test (α =0.05).

Table 2: Details of water, water sampling date, and serovars used for each experiment in the present study

Experiment	Serovars used	Water used	Water Sampling Dates
Biofilm- attachment to 96 well plate	All	All	10/30/2017, 12/12/17, 4/9/2017
Culturable decline 90 days	All	All	10/30/2017, 6/18/2018
Culturable decline 90 days + Culture independent study	Removed Newport MDR+	MA03, MA06, MA10	9/10/2018, 10/1/2018
Tomato Transfer 1- 30 days	Removed Newport MDR+	MA05	8/6/2018, 6/18/2018
Tomato Transfer 30 days	Removed Newport MDR+	MA03, MA06, MA10	8/20/2018, 10/1/2018

2.6 Comparing agar plate counts with total viable cells using culture-independent methods for evaluating persistence in water

S. enterica strains were singly inoculated into 30 mL aliquots of 0.22 µm filtered surface water to a concentration of either 4.69 log CFU/mL or 5.5 log CFU/mL **(Table 2)**. The samples were incubated as detailed above. The experimental design was a completely randomized design (CRD) with three experimental replicates per treatment combination. Samples were destructively sampled at day 1, 30, 60 and 97. At each sampling time, conical tubes were centrifuged at 7,000 rpm for 8 min at room temperature. The supernatant was decanted, and 1 mL of sterile water was added. Conical tubes were vigorously vortexed for 30 s to dislodge the cell pellet. Six hundred µL were taken for dilution plating on TSA, hereinafter referred to as "plate count" data, and 400 µL was reserved in clear 1.5 mL eppendorf tubes for propidium monoazide (PMA; Biotium, Fremont CA) treatment, hereinafter referred to "culture-independent count" (Banihashemi et al., 2012; Li and Chen, 2013). One hundred µL PMA enhancer was delivered to culture independent samples, followed by 2µL PMA for a final concentration of 25 µM. PMA treated samples were incubated in darkness with shaking at 90 rpm for 10 min. Samples were then placed on aluminum foil lined with ice and exposed for cross-linking to a 500 W halogen lamp at a distance of 21 cm for 12 min, rotated once at 6 min. Cells were pelleted by 5000 *g* for 10 min, supernatant was decanted and tubes inverted onto an aseptic laboratory wipe to remove any excess supernatant from the pellet. DNA was extracted using the Purelink Genomic DNA mini kit (Invitrogen, Carlsbad CA) and eluted in 40 µL with 10 mM Tris HCl pH 8.0. One µL was used for amplification of the *rpoD* gene (RNA

polymerase σ factor D) via qPCR (forward primer 5′-GTGAAATGGGCACTGTTGAACTG-3′, reverse primer 5′-TTCCAGCAGATAGGTAATGGCTTC-3′ yielding a 131 bp product (Karlinsey et al., 2012)). A standard curve was used to determine total viable CFU/mL in each sample. Q-PCR was performed on an ABI Step-One Plus (Applied Biosystems, Foster City CA) system with SYBR as a reporter using the following cycling parameters: 50°C for 2 min, 95°C for 2 min, followed by 40 cycles of 95°C for 15 s, and 59°C for 30 s, followed by Melt Curve analysis to ensure specificity. PMA and primers were validated for efficiency, specificity, and sensitivity prior to experimentation (data not shown).

Agar plate total log inactivation over 90 days and PMA-qPCR total log inactivation over 90 days were calculated via $LogCFU/mL_{Day\ 1}$ - $LogCFU/mL_{Day\ 90}$. Agar plate decay was modeled as described above using Buchanan's two-phase log-linear model (Buchanan and Golden, 1995) through IPMP 2014 (Huang, 2014). Total log inactivation, lag time, and the rate of decay were used to compare persistence by water type, serovar, retrieval method (PMA-qPCR or plate count), and experimental replicate through JMP 14.0 (SAS Institute Inc., Cary, NC) via Analysis of Variance and Tukey's Honestly Significant Difference (α =0.05). Orthogonal contrasts were used to compare retrieval methods for each serovar x water type combination (α =0.05).

2.7 Evaluating transfer of S. enterica *strains from surface water to tomato fruit*

To assess transfer potential onto tomatoes, *S.* Heidelberg, *S.* Javiana, *S.* 4,[5], 12:i:-, *S.* Newport, *S.* Typhimurium MDR +, or *S.* Typhimurium were singly

inoculated into 30 mL aliquots of 0.22 µm filtered MA05 water (fresh water creek) to a final concentration of 6.5 log CFU/mL and allowed to persist for 30 days. Samples were incubated as detailed above. At day 1, 5, 10, and 30 an aliquot of inoculated water ("test") was centrifuged 7000 rpm for 8 min, washed 1x with sterile water. Twenty µL was inoculated onto cv. 'Heinz-1706' tomatoes which had been washed with 100 ppm sodium hypochlorite, triple rinsed and dried. To assess the effect of water habitation on serovar transferability, the following control was constructed for each serovar at each time point: one day prior to transfer on day 5, 10, and 30, 4 mL of each water x serovar x replicate combination was 0.22 µm filter sterilized using a cellulose acetate filter (VWR, Radnor PA). Into this medium, suspensions of fresh culture of the same *S. enterica* strain were singly inoculated (at an equivalent concentration as that of the "test" samples) and allowed to incubate for 24 h. These samples will hereinafter be referred to as "control" samples. Cell centrifugation, washing, resuspension and inoculation onto tomatoes proceeded concurrently with "control" and "test" water sample samples. Tomato was treated as a random effect due to inter-tomato variability; all "test" and "control" inoculations for one water type were performed on one tomato, with replicates. Tomatoes were incubated at 24˚C and 75% RH for 14 h before inoculated exocarp was aseptically removed with sterile razors. Tissue was vortexed in 0.1% peptone for 3 min (Difco, Sparks MD), and dilutions plated on TSA for enumeration. Thirty day incubations and transfers were repeated for MA03, MA06 and MA10. When plate counts were below the limit of detection, 46 ˚C Xylene-Lysine-Tergitol-4 (XLT-4, Difco, Franklin Lakes NJ) were pour plated onto fruit peels to ensure bacteria did not remain attached.

This experiment was repeated thrice with at least 5 tomatoes per water type for each experimental replicate. Bacterial populations retrieved from tomatoes were normalized with each respective serovar x tomato inoculum level via (log $CFU/mL_{Tomato\ inoculum}$ - $logCFU/mL_{Tomato\ peel}$) to produce ‘Log decline from inoculum’ heretofore described as ‘transferability’ or “transfer success” where lower log declines from inoculum were regarded as more successful. “Test” and “control water” transferability were compared by water type, serovar, incubation time in water and experimental replicate using JMP 14.0 (SAS Institute Inc., Cary, NC). Orthogonal contrasts between the same serovar x water combinations were used to compare transferability of serovars in “test” versus “control water” samples (α=0.05). Main effect differences of water type and serovar were determined by ANOVA and serovar specific differences in transferability in each water type were determined using Tukey’s Honestly Significant Difference (α =0.05).

2.8 Evaluating relationships among bacterial inactivation, attachment, transfer and water physicochemical parameter data

To explore relationships among data collected, physicochemical parameter data of the water bodies at the time of sampling was paired to each experiment based on water sampling date **(Table 2)**. Datasets were constructed for total log inactivation (all 4 replicates of 90-day decay assays, Agar plate count only), attachment index and transfer to tomato. Statistical analyses were performed using R version 3.5.2. First, a correlogram was made to visually assess any significant correlations between the bacterial parameter of interest and physicochemical data with the R package “Performance Analytics” using Pearson’s correlation coefficient calculated (α=0.05).

Then, linear regression models were evaluated for each bacterial output as a function of significant physicochemical parameters ($\alpha=0.05$). The fit of the model was examined with residuals, q-q plots and statistical power of the model (data not shown).

3. Results

3.1 Surface water physicochemical profiles

Each biological experiment was the result of at least two independent water sampling events, resulting in 28 unique water samples. During water sampling, dissolved oxygen (DO%), conductivity (SPC uS/cm), pH, oxidation-reduction potential (ORP mV), turbidity (FNU), nitrate (mg/L) and chloride (mg/L) measurements were obtained (**Table 3**). Across all sampling times, MA03 and MA05 – the two non-tidal freshwater samples, had statistically similar physicochemical profiles. Comparing all water types, it was found that conductivity, nitrate, and chloride attributes varied widely among MA04, MA05 and MA06. When variances were equal, salinity varied significantly by water type ($p<0.001$) with MA06 displaying statistically higher salinity compared to other water types. Significant differences in water type conductivity, nitrate and chloride were also observed ($p<0.05$ via Welch's test). Conductivity of reclaimed water was significantly higher than either non-tidal fresh or pond water. Further, reclaimed and tidal brackish water were found to have significantly higher chloride levels compared to MA03. Finally, reclaimed water had significantly higher nitrate levels compared to MA04. Taken together, this indicates that each type of surface water; non-tidal fresh, tidal brackish, pond and reclaimed, all

had unique physicochemical profiles, with reclaimed water being the most distinct. It is important to note that reclaimed water physicochemical parameters were measured before sodium thiosulfate quenching, which likely changed some aspects of the physicochemical parameter profile. For this reason, MA06 physicochemical parameter data was not used in bivariate analyses with experimental data.

Table 3: Physicochemical parameter data for water used in the present study from all collection dates associated with experimentation

	MA03			MA04			MA05			MA06			MA10		
Parameter	**N**	**mean**	**±Std dev**	**N**	**mean**	**±Std dev**	**N**	**mean**	**±Std dev**	**N**	**mean**	**±Std dev**	**N**	**mean**	**±Std dev**
DO%	7	89.20	19.98	4	89.45	6.24	4	93.51	6.38	4	70.43	46.92	7	101.10	9.28
Conductivity (SPC uS/cm) ǂ	7	176.56^{b}	15.89	4	401.74^{ab}	348.01	4	197.35^{b}	45.67	6	810.09^{a}	97.03	7	129.68^{c}	27.49
pH	7	7.02	0.58	4	7.08	0.25	4	7.49	0.48	4	7.16	0.48	7	7.89	0.77
ORP mV	7	218.79	48.48	4	173.60	23.60	4	125.31	43.37	4	148.29	213.39	7	158.64	58.56
Turbidity (FNU)	5	2.22	4.49	4	8.20	3.55	3	9.33	11.30	6	9.68	6.32	7	2.10	4.87
Nitrate (mg/L) £	6	4.77^{ab}	2.08	3	1.92^{b}	0.23	2	0.63^{ab}	0.36	5	8.22^{a}	4.92	2	0.22^{ab}	0.01
Chloride (mg/L) £	6	11.55^{b}	4.57	3	107.13^{a}	92.10	1	42.92^{ab}	--	4	98.02^{a}	66.06	2	32.25^{ab}	5.25
Salinity ǂ	7	0.08^{b}	0.01	4	0.20^{b}	0.17	4	0.10^{b}	0.02	3	0.42^{a}	0.04	4	0.07^{b}	0.01

ǂ Indicates a significant ANOVA
£ Indicates a significant Welch's test, performed when data violated homogeneity of variances through a significant Brown-Forsythe test ($p<0.05$).
Letters indicate significant differences within each physicochemical parameter (rows), performed with Tukey HSD or Wilcoxon's test ($\alpha=0.05$) where appropriate.

3.2 Agar plate decline in surface water samples over 90 days

3.2.1 Evaluating rate of decay, lag time, and agar plate total log inactivation of *S. enterica* in 90 day surface water samples

After modeling the dataset using the Buchanan two-phase model, >0.50 RMSE values were identified in MA03 and MA10 (**Supplementary Figure 1**). In these instances, the data fit better to the Buchanan 3 phase model which incorporates a "tail," indicating maximum inactivation was reached and a new level of persistence was initiated. Parameters from the Buchanan two-phase model were utilized to compare serovar specific differences in persistence among water types.

Based on plate count data in replicated experiments, water type was a significant driver of bacterial decline rate, lag time and agar plate total log inactivation (t_{90}-t_0) ($p<0.05$, $p<0.001$, $p<0.001$, respectively). Total log decline was more pronounced in replicate 1 compared to replicate 2 ($p<0.005$) (**Figure 1**). Excluding MA06, replicate 1 and 2 rates of decay were significantly correlated with agar plate total log inactivation (R^2=0.55, $p<0.005$, R^2 = 0.12, $p<0.001$) (**Supplementary Figure 2**). Across all serovars, reclaimed water (MA06) displayed the lowest rate of decay and total log inactivation and was the most consistent between experimental replicates. Brackish water (MA04) displayed the largest difference between rates of decay and total log inactivation from replicated experiments (1.65 ± 0.46 and 0.60 ± 0.35). Pond water (MA10) was the least favourable for *S. enterica* persistence in replicate one, with the largest log declines reported. Replicates using non-tidal fresh water (MA03 and MA05) were consistent.

Across all serovars, a significant interaction between water type and replicate was detected for rate of decay ($p<0.001$), with MA10 displaying significantly higher decay rates in replicate one ($p<0.05$). This may have contributed to this water type also displaying largest average total log inactivation across all water types in replicate one as well. Furthermore, across all serovars lag time (shoulder) was significantly impacted by experimental replicate ($p<0.001$) (**Figure 2B**). With the exception of MA10, every water type in replicate two had a significantly longer shoulder compared to the same water type in replicate one (p<0.05). This may contribute to lower R^2 values in replicate two (**Supplementary Figure 2**), as longer lag times may

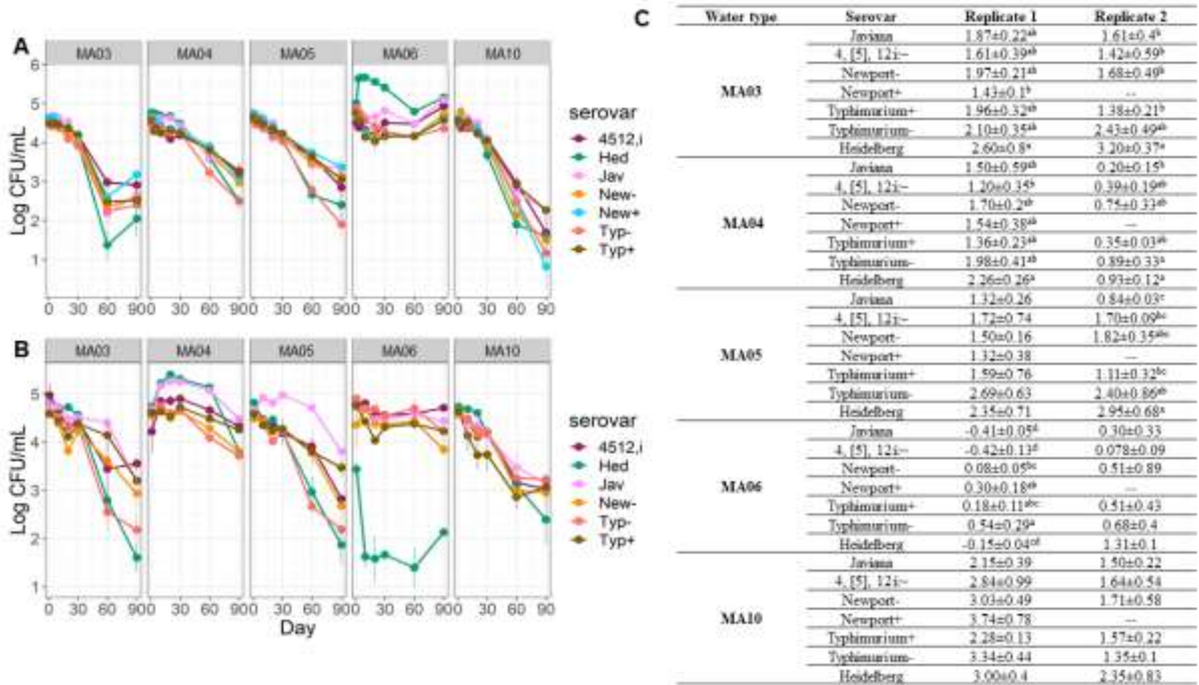
have contributed to less agar plate total log inactivation but may not have affected rate of decay.

3.2.2 Evaluating serovar specific dynamics in rate of decay, lag time, and total log inactivation of *S. enterica* in 90-day surface water samples

Serovar specific differences in agar plate total log inactivation were detected in both replicate one and two in MA03 and MA04, and in replicate two only in MA05 water (**Figure 1C**). In replicate 1, *S.* Heidelberg was seen to have significantly more total log inactivation compared to *S.* 4,[5], 12:i:– in MA04 and *S.* Newport MDR+ in MA03 ($p<0.05$). The latter was the only serovar specific difference in decline that was consistent between experimental replicates. In replicate two, although there was less total inactivation overall compared to replicate one, there were more serovar specific differences in total log inactivation. *S.* Heidelberg again exhibited the most agar plate total log inactivation compared to *S.* Javiana in MA03, MA04, and MA05 and additionally compared to *S.* Typhimurium MDR+ and *S.* 4, [5], 12:i:– in MA03 and MA05.

Concerning rate of decay, *S.* Heidelberg displayed significantly more severe rates of decay compared to *S.* 4, [5], 12:i:– and *S.* Newport in MA03 and MA04 in replicate one, and *S.* Typhimurium MDR +/- and *S.* Newport in MA04 during replicate two (**Figure 2A, Supplementary Tables 1 &2**). Concerning lag time, only MA05 in replicate two saw serovar specific differences in lag with *S.* Javiana displaying a significantly longer lag time than both *S.* Typhimurium MDR+ and MDR- (**Figure 2B**). Taken together, this suggests lag time and rate of decline are experimentally specific, but total log inactivation is more consistent with *S.*

Heidelberg displaying more total log inactivation compared to at least one other serovar in non-tidal fresh or tidal brackish water in both experimental replicates (**Figure 1C**).



Water type	Serovar	Replicate 1	Replicate 2
MA03	Javiana	1.87±0.22^{ab}	1.61±0.4^{b}
	4, [5], 12i–	1.61±0.39^{ab}	1.42±0.59^{b}
	Newport-	1.97±0.21^{ab}	1.68±0.49^{b}
	Newport+	1.43±0.1^{b}	--
	Typhimurium+	1.96±0.32^{ab}	1.38±0.21^{b}
	Typhimurium-	2.10±0.35^{ab}	2.43±0.49^{ab}
	Heidelberg	2.60±0.8^{a}	3.20±0.37^{a}
MA04	Javiana	1.50±0.59^{ab}	0.20±0.15^{b}
	4, [5], 12i–	1.20±0.35^{b}	0.39±0.19^{ab}
	Newport-	1.70±0.2^{ab}	0.75±0.33^{ab}
	Newport+	1.54±0.38^{ab}	–
	Typhimurium+	1.36±0.23^{ab}	0.35±0.03^{ab}
	Typhimurium-	1.98±0.41^{ab}	0.89±0.33^{a}
	Heidelberg	2.26±0.26^{a}	0.93±0.12^{a}
MA05	Javiana	1.32±0.26	0.84±0.03^{c}
	4, [5], 12i–	1.72±0.74	1.70±0.09^{bc}
	Newport-	1.50±0.16	1.82±0.35^{abc}
	Newport+	1.32±0.38	–
	Typhimurium+	1.59±0.76	1.11±0.32^{bc}
	Typhimurium-	2.69±0.63	2.40±0.86^{ab}
	Heidelberg	2.35±0.71	2.95±0.68^{a}
MA06	Javiana	-0.41±0.05^{d}	0.30±0.33
	4, [5], 12i–	-0.42±0.13^{d}	0.078±0.09
	Newport-	0.08±0.05^{bc}	0.51±0.89
	Newport+	0.30±0.18^{ab}	--
	Typhimurium+	0.18±0.11^{abc}	0.51±0.43
	Typhimurium-	0.54±0.29^{a}	0.68±0.4
	Heidelberg	-0.15±0.04^{cd}	1.31±0.1
MA10	Javiana	2.15±0.39	1.50±0.22
	4, [5], 12i–	2.84±0.99	1.64±0.54
	Newport-	3.03±0.49	1.71±0.58
	Newport+	3.74±0.78	--
	Typhimurium+	2.28±0.13	1.57±0.22
	Typhimurium-	3.34±0.44	1.35±0.1
	Heidelberg	3.00±0.4	2.35±0.83

Figure 1: Agar plate counts from 1-90 days of various *Salmonella enterica* in 10mL samples of 0.22 µM filtered mid-Atlantic surface water. N=3 for each serovar and water type combination. Panels represent experimental replicate one (A) and two (B). Error bars represent the standard error of the mean (SEM). (C) Agar plate total log inactivation from 1 -90 days of *S. enterica* survival in surface water multiplied by negative one. Std dev represent the standard deviations. Letters denote serovar specific differences in agar plate total log inactivation within water types through Analysis of Variance and Tukey's Honestly Significant Difference (α =0.05). – denotes the removal of *S.* Newport MDR + from experimental replicate 2.

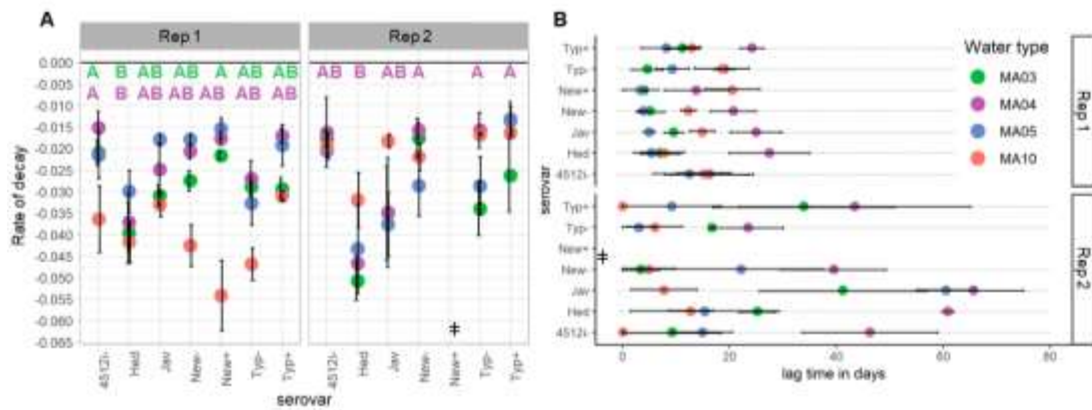


Figure 2: Rate of decay(A) and Lag time (λ) (B) in days of various *Salmonella enterica* in 9.9 mL samples of 0.22 µM filtered surface water. Decay parameters were obtained from Buchanan's two-phase log-linear inactivation model (IPMP 2014). N= 3 experimental replicates, error bars represent SEM. Letters, colored by water type, denote serovar specific differences in rate of decay within water types through Analysis of Variance and Tukey's Honestly Significant Difference (α=0.05). ǂ denotes the removal of *S.* Newport MDR + from experimental replicate 2.

3.3 Comparing culture-based data to culture independent bacterial persistence data in surface water

To assess if the decline observed in the 90-day sample study resulted from bacterial death of transition to a VBNC state, the study was repeated using water from MA03, MA06 and MA10. Here, bacterial plate counts were compared with a PMA-q-PCR method that would detect not only culturable cells but also viable but non-culturable cells. Agar plate count data were again modeled using Buchanan's two-phase log-linear model, resulting in similar trends with regard to water type and

serovar specific dynamics in rates of decay as the 9.9mL sample study (for more detail, see **Supplementary Figure 3A-D**). As seen in the previous two replicates, again agar plate count total log inactivation was significantly affected by water type, serovar and experimental replicate ($p<0.001$ for all, **Supplementary Figure 3E**). It is important to note that in both replicates *S.* 4, [5], 12:i:– in MA03 exhibited significantly lower agar plate total log inactivation compared to *S.* Heidelberg, consistent with the 9.9 mL 90-day persistence study.

Comparing both types of recovery methods for viable cells across all water types and serovars revealed PMA-q-PCR reported significantly less total log inactivation compared to plate counting ($p<0.001$). Although population levels measured by the two methods were equivalent at t_0, the curves started to diverge after t_{60}. One exception was MA06 in replicate two (**Figure 3**). In this water type, across all serovars, plate counts and PMA-q-PCR results were statistically similar. This was expected because MA06 in culture studies did not report appreciable decline throughout the course of the previous two studies (**Figure 1**). In fact, pairwise comparisons of plate count versus PMA-q-PCR for each serovar within each water type and replicate found that MA03 had the most discrepancies between plate count and PMA treated qPCR recorded total log inactivation, suggesting water type may significantly influence transition into VBNC state ($p<0.05$ **Figure 4**). Interestingly, while all serovars in MA03 indicated subpopulations entering VBNC states, only select serovars in MA06 and MA10 indicated transition to VBNC. For example, in MA06 and MA10 of experimental replicate one, *S*. Heidelberg displayed significant differences between plate count and qPCR, whereas *S.* 4, [5], 12:i:– and *S*. Javiana

did not. This suggests that serotype x water type interactions may play a significant role in the decision of subpopulations to transition to VBNC persistence.

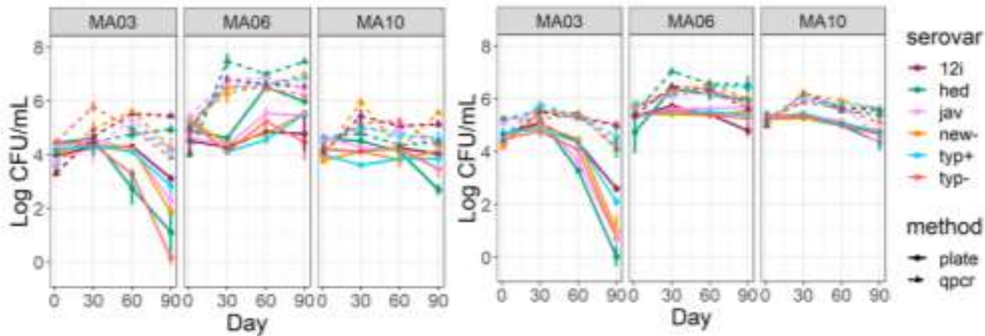


Figure 3: **Agar plate count (solid line) and PMA treated qPCR (dashed line) persistence over 90 days of various *Salmonella enterica* in 30 mL samples of 0.22 µM filtered mid-Atlantic surface water.** Left panel represents experimental replicate 1, and right replicate 2. For each experimental replicate, N=3 for each serovar and water type combination. Each sample was split for qPCR and culture-based plate count, as described in the materials and methods. Error bars represent the SEM.

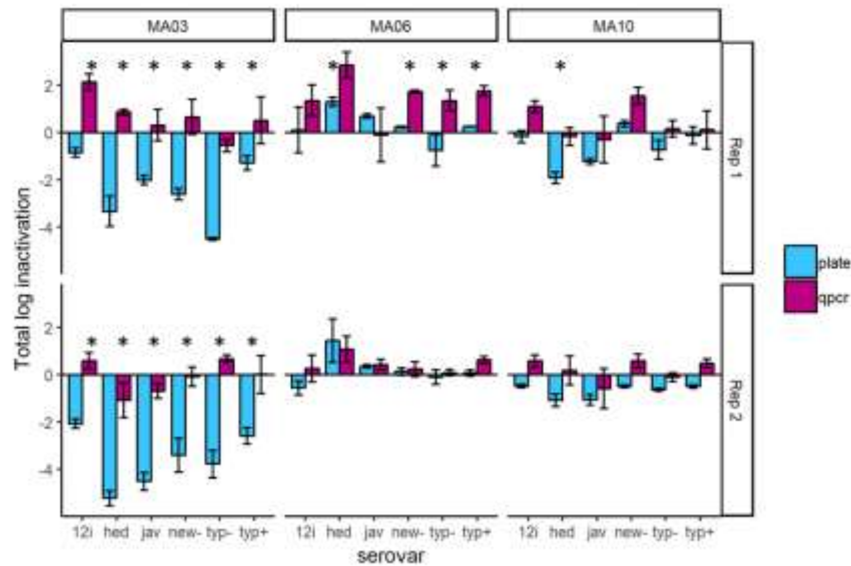
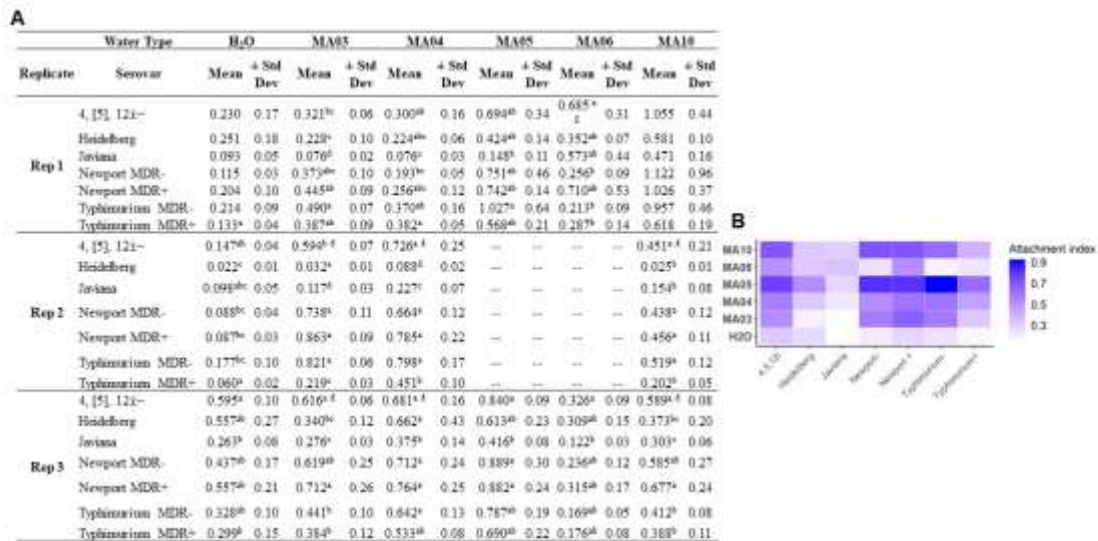


Figure 4: Total log inactivation from 1-90 days of *S. enterica* obtained from PMA treated qPCR (blue) and plate counts (purple). N=3 for each serovar and water type combination. Each sample was split for qPCR and plate count, as described in materials and methods. Error bars represent the SEM. Asterisks denote significant discrepancies in total log inactivation recorded by agar plate compared to PMA treated qCR (orthogonal contrasts α=0.05).

3.4 Surface water mediated attachment to polystyrene

Water type and serovar were significant driving factors of attachment capacity (*p*=0.001). Across all serotypes tested, reclaimed water (MA06) harbored a significantly lower attachment index than pond and non-tidal freshwater (MA10 and MA05) in replicate one and three (**Figure 5**). Across all water types, *S.* Newport MDR - and *S.* 4, [5], 12:i:– displayed a significantly higher attachment index than *S.* Javiana and *S.* Heidelberg.

Reproducible serovar specific differences in attachment indices were observed for every surface water type except for sterile water. *S.* 4, [5], 12:i:– displayed reproducibly significantly higher attachment indices compared to *S.* Javiana in MA03, MA04, and MA10, and significantly higher than *S.* Heidelberg in MA03 and MA10 (p<0.05). Furthermore, *S.* Typhimurium MDR- also displayed significantly higher attachment indices compared to *S.* Javiana in MA03 and MA04, and *S.* Heidelberg in MA03 (p<0.05). *S.* Newport MDR + displayed variable attachment indices dependent on surface water type; this organism performed similarly to *S.* Javiana in MA03, yet in MA10 was statistically similar to *S.* 4, [5], 12:i:–. No advantage of multidrug resistance on attachment was registered for *S.* Newport. Differences between *S.* Typhimurium MDR + and MDR – were only registered in replicate two of the study. In this replicate, *S.* Typhimurium MDR – displayed a significantly higher attachment index to polystyrene compared to *S.* Typhimurium MDR + in MA03, MA04, and MA10 (p<0.05). Taken together, these results suggest that the interaction of bacterial genotype incubating in different water types could significantly affect attachment success to polystyrene.



A

Replicate	Water Type / Serovar	H₂O Mean	H₂O + Std Dev	MA03 Mean	MA03 + Std Dev	MA04 Mean	MA04 + Std Dev	MA05 Mean	MA05 + Std Dev	MA06 Mean	MA06 + Std Dev	MA10 Mean	MA10 + Std Dev
Rep 1	4, [5], 12:i−	0.230	0.17	0.321^{bc}	0.06	0.300^{ab}	0.16	0.694^{ab}	0.34	0.685^{a} £	0.31	1.055	0.44
	Heidelberg	0.251	0.18	0.228^{c}	0.10	0.224^{abc}	0.06	0.424^{ab}	0.14	0.352^{ab}	0.07	0.581	0.10
	Javiana	0.093	0.05	0.076^{d}	0.02	0.076^{c}	0.03	0.148^{b}	0.11	0.573^{ab}	0.44	0.471	0.16
	Newport MDR-	0.115	0.03	0.373^{abc}	0.10	0.193^{bc}	0.05	0.751^{ab}	0.46	0.256^{b}	0.09	1.122	0.96
	Newport MDR+	0.204	0.10	0.445^{ab}	0.09	0.256^{abc}	0.12	0.742^{ab}	0.14	0.710^{ab}	0.53	1.026	0.37
	Typhimurium MDR-	0.214	0.09	0.490^{a}	0.07	0.370^{ab}	0.16	1.027^{a}	0.64	0.213^{b}	0.09	0.957	0.46
	Typhimurium MDR+	0.133^{a}	0.04	0.387^{ab}	0.09	0.382^{a}	0.05	0.568^{ab}	0.21	0.287^{b}	0.14	0.618	0.19
Rep 2	4, [5], 12:i−	0.147^{ab}	0.04	0.594^{b} £	0.07	0.726^{a} £	0.25	--	--	--	--	0.451^{a} £	0.21
	Heidelberg	0.022^{c}	0.01	0.032^{a}	0.01	0.088^{d}	0.02	--	--	--	--	0.025^{b}	0.01
	Javiana	0.098^{abc}	0.05	0.117^{d}	0.03	0.227^{c}	0.07	--	--	--	--	0.154^{b}	0.08
	Newport MDR-	0.088^{bc}	0.04	0.738^{a}	0.11	0.664^{a}	0.12	--	--	--	--	0.438^{a}	0.12
	Newport MDR+	0.087^{bc}	0.03	0.863^{a}	0.09	0.785^{a}	0.22	--	--	--	--	0.456^{a}	0.11
	Typhimurium MDR-	0.177^{bc}	0.10	0.821^{a}	0.06	0.798^{a}	0.17	--	--	--	--	0.519^{a}	0.12
	Typhimurium MDR+	0.060^{a}	0.02	0.219^{c}	0.03	0.451^{b}	0.10	--	--	--	--	0.202^{b}	0.05
Rep 3	4, [5], 12:i−	0.595^{a}	0.10	0.616^{a} £	0.06	0.681^{a} £	0.16	0.840^{a}	0.09	0.326^{a}	0.09	0.589^{a} £	0.08
	Heidelberg	0.557^{ab}	0.27	0.340^{bc}	0.12	0.662^{a}	0.43	0.613^{ab}	0.23	0.309^{ab}	0.15	0.373^{bc}	0.20
	Javiana	0.263^{b}	0.08	0.276^{c}	0.03	0.375^{b}	0.14	0.416^{b}	0.08	0.122^{b}	0.03	0.303^{c}	0.06
	Newport MDR-	0.437^{ab}	0.17	0.619^{ab}	0.25	0.712^{a}	0.24	0.889^{a}	0.30	0.236^{ab}	0.12	0.585^{ab}	0.27
	Newport MDR+	0.557^{ab}	0.21	0.712^{a}	0.26	0.764^{a}	0.25	0.882^{a}	0.24	0.315^{ab}	0.17	0.677^{a}	0.24
	Typhimurium MDR-	0.328^{ab}	0.10	0.441^{b}	0.10	0.642^{a}	0.13	0.787^{ab}	0.19	0.169^{ab}	0.05	0.412^{b}	0.08
	Typhimurium MDR+	0.299^{b}	0.15	0.384^{b}	0.12	0.533^{ab}	0.08	0.690^{ab}	0.22	0.176^{ab}	0.08	0.388^{b}	0.11

Figure 5: (A) Serovar specific attachment to 96 well plate during static incubation in various water types and (B) averaged across three experimental replicates. N=6. £ Indicates a significant Welch's test, performed when data violated homogeneity of variances through a significant Brown-Forsythe test *(p*<0.05), otherwise comparisons were compiled with ANOVA. Letters indicate significant differences in serovar attachment index within a given water type (rows), performed with Tukey HSD or Wilcoxon's test (α=0.05) where appropriate. -- denotes data was not analyzed for these samples.

3.5 Evaluating the effect of prolonged incubation in surface water on transferability onto tomato fruit

3.5.1 Effect of increased incubation time in MA05 water on tomato transfer success

Evaluating the effect of increased incubation in water on transfer success found as little as 10 days incubation in water ("test" samples) was shown to significantly increase transferability onto tomatoes compared to incubation in water for 24 hours ("control" samples), as evidenced by Typhimurium MDR – (p=0.034) in replicate one (**Figure 6**). When replicated, these tendencies were consistent, with 10-day incubation additionally positively influencing transfer in *S.* Heidelberg, *S* Javiana, and *S.* Typhimurium MDR + ($p \leq 0.0002$ for all). This advantage was expanded 30 days after incubation for *S.* 4, [5], 12:i:– and *S.* Heidelberg in replicate one, and every serovar except for *S.* Typhimurium MDR+ compared to 24 h of incubation in replicate two ($p<0.05$ via orthogonal contrasts).

Serovar type was also a significant factor for transfer success. Serovar specific differences in transferability onto tomatoes were evident from day 1 to day 30 in both experimental replicates, but were reproducible starting at day 10 (**Figure 6**). In "control" samples, *S.* Javiana, *S.* Newport, and *S.* 4, [5], 12:i:– transferred to tomatoes significantly better than *S.* Heidelberg on day 10 and 30 in both experimental replicates. Interestingly, even though *S.* Heidelberg transfer improved with prolonged incubation in water, it was still retrieved in the lowest concentrations from tomato fruits compared to other serovars after 30 days of incubation in water. When evaluating multidrug resistant differences in tomato transfer, a significant reproducible MDR + advantage on tomato transfer was registered on day 30 "control" samples ($p<0.05$), but prolonged incubation improved *S.* Typhimurium MDR- transfer to *S.* Typhimurium MDR+ levels (replicate one $p<0.05$, replicate two $p>0.05$).

3.5.2 Comparing the Effect of Incubation in Multiple Water Types on Tomato Transfer Success

This study was repeated with the 30-day transfer time point to investigate if, in addition to prolonged incubation, water type also influenced serovar specific transfer success to tomatoes. For this experiment MA03, MA06 and MA10 water was used **(Table 2).** Water type was found to be a significant factor in *S. enterica* transfer success to tomato, variable by experimental replicate. In the first experimental replicate, regardless of incubation time and serovar tested, MA06 conferred higher transfer success compared to other water types ($p>0.05$) (**Figure 7A**). Conversely, in replicate one and two, MA10 exhibited the least transfer success compared to MA03 and MA06 ($p<0.05$) (**Figure 7A-B**).

Across all serovars in the second ($p=0.056$) and third ($p<0.001$) replicates, bacteria which were incubated for 30 days in water had more successful transfer to tomatoes compared to bacteria incubated for 24 hours (**Figure 7B-C**). Evaluating this by water type revealed across all serovars MA06 supported significantly higher retrieval of "test" samples from tomatoes compared to "control" samples ($p<0.05$) (**Figure 7C).** Increased incubation time in water significantly and reproducibly improved transfer of *S.* Typhimurium MDR- in MA06 water ($p<0.05$, orthogonal contrasts) (**Figure 7B-C**). Positive influence of prolonged incubation was observed for other serovars as well, exhibited by *S.* Heidelberg (MA03, MA06), *S.* Typhimurium MDR- (MA10) and *S.* Javiana (MA06), but varying with experimental replicate.

Serovar specific differences in transfer success were evident for every experimental replicate in at least one water type. Regardless of incubation time in water or experimental replicate, *S.* Heidelberg transferred to tomato with the least success compared to at least one other serovar in every water type which registered serovar specific differences in transfer. In MA03 and MA10, *S.* 4, [5], 12:i:– displayed significantly more transfer compared to *S.* Heidelberg. In MA06, *S.* Javiana displayed more transfer than *S.* Heidelberg ($p<0.05$, Tukey HSD). Similarly to the MA05 time course transfer study, a significant reproducible MDR+ advantage on tomato transfer was identified on day 30 "control" samples in at least one experimental replicate of MA03 and MA10 water. Prolonged incubation improved *S.* Typhimurium MDR- transfer to the same level as *S.* Typhimurium MDR+. Interestingly, prolonged incubation did not result in the rescue of *S.* Heidelberg transfer success.

Other serovar specific differences in transfer were observed among water type tested, but they varied by experimental replicate. Interestingly across all replicates, a larger range of serovar specific differences is present in "control" samples and was distilled to fewer differences after 30 days of incubation. For example, in addition to *S.* 4, [5], 12:i:– outperforming *S.* Heidelberg, in MA03 the serovars *S.* Typhimurium MDR+ and *S.* Javiana also transferred to tomato with significantly more efficiency in "control" samples (**Figure 7A, C**). Also in control samples of MA10 and MA05, *S.* Newport outperformed *S.* Heidelberg (**Figure 7A, C**). After 30 days of incubation in water, serovar specific differences in ability to transfer to tomato were evident in MA06 and MA10. In both cases, *S.* Heidelberg was retrieved at significantly lower

titers after transfer compared to *S.* Javiana, and in MA10 also compared to *S.* Typhimurium MDR+ and *S.* 4, [5], 12:i:–

Taken together, it is evident that serovar drives transfer success onto tomato more consistently than water type, with *S.* Heidelberg and *S.* Typhimurium MDR – displayed significantly less transferability compared to all other serovars tested, when corrected for inoculum level. Furthermore, while increased incubation time in water increases transfer to tomato-providing evidence for water as a “priming” reservoir for successful persistence on crops- this was serovar specific. This suggests that at least in this study *S.* Heidelberg may not utilize water as a priming reservoir.

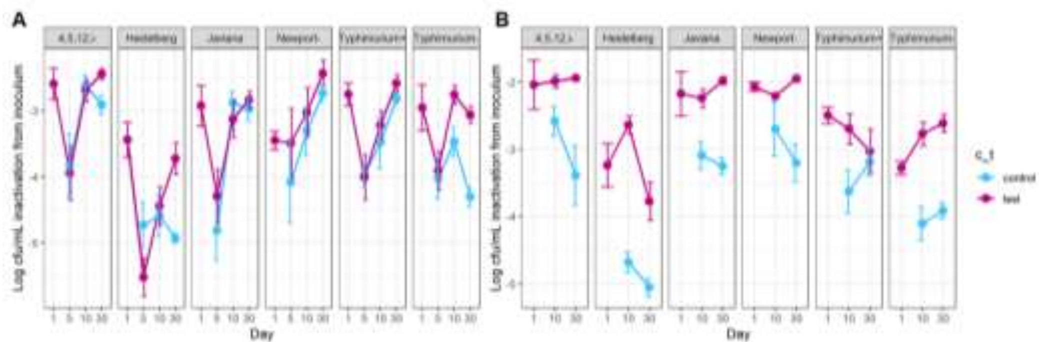


Figure 6: Tomato transferability of multiple *S. enterica* serovars to cv. ‘Heinz’ fruit after 1, 5, 10 and 30 day incubation in 0.22 µM filtered non-tidal fresh water MA05. Multi-day incubated “test” (purple) samples are compared to 24 h incubation in 0.22 µM “control,” (blue) water samples in (A) replicate one or (B) replicate 2. N=5 tomatoes per treatment combination, error bars represent SEM.

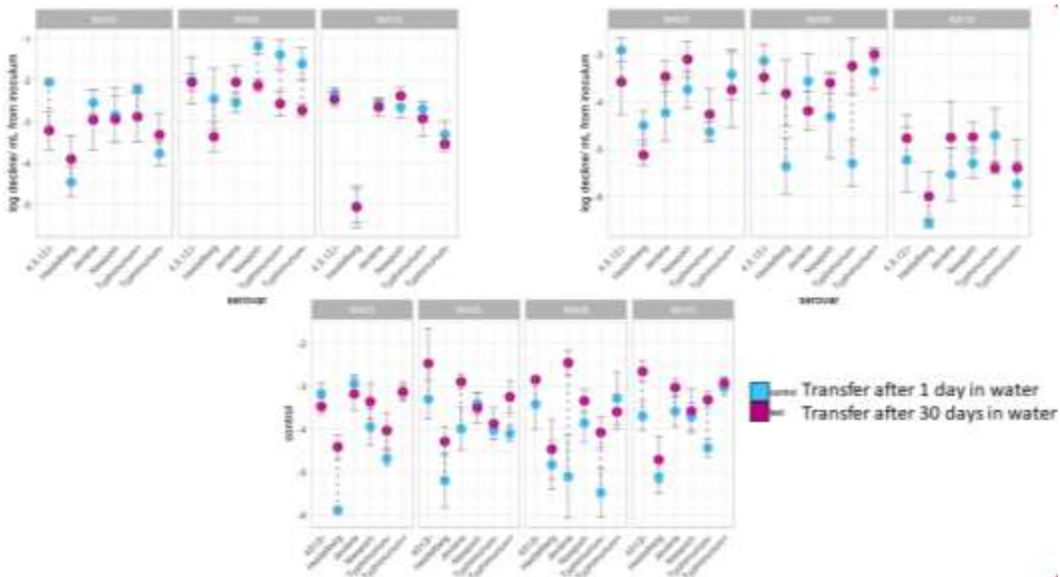


Figure 7: Tomato transferability of multiple *S. enterica* serovars to cv. 'Heinz' tomatoes after 1, 5, 10, and 30 day incubation in 0.22 µM filtered MA03, MA06, MA05, MA10. Multi-day incubation "test" (purple) samples are compared to 24 h incubation in 0.22 µM "control" (blue) water samples in replicate one (A) two (B), or three (C). N=5 tomatoes per treatment combination, error bars represent SEM.

3.6 Evaluating relationships among surface water physicochemical profile, total log inactivation in water over 90 days, attachment to polystyrene, and transfer onto tomato

3.6.1 Surface water physicochemical relationships with experimental data

First, it was investigated if there were any associations with physicochemical parameters of the surface waters at time of sampling and experimental data acquired. Through correlograms (**Supplementary Figures 4, 5, and 6**) it was identified that water oxidation reduction potential was significantly associated with both attachment to polystyrene and tomato transfer success, but not total log inactivation data

($p<0.05$). Total log inactivation data correlated strongly with dissolved oxygen and nitrate concentration ($p<0.05$).

Simple linear regression models were then used to assess the associations between significant physicochemical parameters and experimental data. Evaluating the association of attachment index to polystyrene, it was found that with one standard deviation decrease in ORP, attachment index increased by 0.48 standard deviations ($p<0.001$). It was also identified that water ORP significantly affected the transferability onto tomato, where one standard deviation decrease in water ORP decreased total log inactivation on tomato from inoculum by 0.35 standard deviation ($p=0.009$). Interestingly, ORP was not significantly associated with transfer capacity from organisms incubated in “control” water treatments. Finally, it was revealed that water DO significantly affected agar plate total log inactivation in water, in which one standard deviation increase in water DO was associated with 0.44 standard deviation decrease in total log decline over 90 days ($p<0.001$) This indicated that higher DO may associated with high concentrations of colonies retrieved from agar plates. Close inspection of each water shows that this association was mainly driven by MA10 ($R^2=0.51$) and MA04 ($R^2=0.72$). Interestingly, MA10 and MA04 each exhibited greater agar plate count log declines during the fall replicate of the 90-day persistence experiment compared to the spring, consistent with the change in the dissolved oxygen levels recorded at the time of sampling. These results have two implications, 1) that high levels of water DO are associated with high *S. enterica* persistence, and 2) that lower levels of DO may be an important factor in the decision for sub-populations to transition to VBNC survival strategies.

3.6.2 Relationships among Inactivation, Attachment to Polystyrene, and Tomato Fruit Transfer Success

Through correlograms (**Supplementary Figure 7**), it was identified that transfer success onto tomato after prolonged incubation in water was significantly correlated with attachment indices ($p<0.05$). One standard deviation increase in attachment index was associated with 0.4 standard deviation less log decline from inoculum onto tomato ($p<0.05$). This was driven more by serovar than by water type, as the average R^2 fit by water type was 0.12±0.11 compared to 0.61±0.31 for serovar (**Supplementary Figure 8**). *S.* Javiana, interestingly, was the only serovar which did not follow this trend, displaying low attachment indices on polystyrene but had strong transfer to tomato. This suggests that there may be other *S*. Javiana-tomato specific interactions which determine tomato fruit attachment. Interestingly, bacteria which were only incubated for 24 h were not correlated with attachment indices. Finally, no significant associations were found between attachment or "test" transfer samples and total log inactivation over 90-day water incubation.

4. Discussion

Much study has been devoted to understanding *S. enterica* prevalence in surface water body systems (Bell et al., 2015; Callahan et al., 2019; Haley et al., 2009; Li et al., 2014; Luo et al., 2015; McEgan et al., 2014; Micallef et al., 2012), however less is known about *S. enterica* serovar specific ecology in water, including persistence strategies, associations with water physicochemical parameters, and transfer potential onto crops from water which the present study aimed to address. Results from this study indicate that persistence in water was principally driven by

water type and may include viable but non-culturable states over prolonged periods. Furthermore, transfer success onto tomato fruits was driven by serovar more than water, and prolonged incubation in water increased the transferability of all serovars, including those that initially transferred poorly onto tomato fruits. Finally, linear regression analysis of experimental data with water physicochemical variables revealed that attachment to polystyrene and water oxidation reduction potential should be further evaluated as potential indicators of foodborne pathogen transfer probability onto tomato.

Assessing physicochemical parameter profiles, it was apparent that both non-tidal freshwater sites were the most alike, and reclaimed water was the most dissimilar of all water types profiled. This is expected, as reclaimed water goes through an aerobic digestion process, filtration, and in the case of this site, chlorination (Wu et al., 2009). Interestingly, brackish water along with reclaimed water were highly variable in the parameters tested over sampling time. This is expected for brackish water site MA04, as this water source is subjected to tidal currents. Variation in reclaimed water is much less understood, although it could be very plausible that over the course of sampling varied inputs into the wastewater treatment plant during different months could cause physicochemical parameters to vary widely, as it is known nutrients like nitrogen and phosphorous concentrations can vary in reclaimed water over seasons (Fan et al., 2014).

In the present study, *S. enterica* persisted in all water types tested for at least 90 days. Furthermore, persistence in water included viable but non-culturable states for all *S. enterica* serovars tested in non-tidal fresh and pond water, a finding which

agrees with previous work in *S. enterica* persistence in marine and river water (Roszak et al., 1984; Santo Domingo et al., 2000). This finding has pertinent public health implications in that there is an increased risk of under-estimating *S. enterica* prevalence in these water systems, especially in non-tidal fresh water (MA03) where the discrepancies between culturable populations and VBNC were consistently significant. Moving forward, it is recommended that more exploration into inexpensive, rapid, and reproducible culture independent methods for assessing *S. enterica* presence in these waters is further explored to accurately profile water hazards.

Over the course of this study, reclaimed water retained the highest populations of viable and culturable cells while non-tidal fresh and pond water exhibited the most decline from agar plate counts. One reason for this observation could be lower nutrient levels in pond and non-tidal fresh water types could result in higher VBNC turnover to mitigate starvation (Riedel et al., 2013). Furthermore, tertiary treatment of the reclaimed water, which is employed to reduce potential pathogens in the water, could also have resulted in a significantly different “microbial fingerprint” of exudates or secondary metabolites compared to un-treated pond and non-tidal fresh water, which could influence *S. enterica* persistence. Finally, at the time of sampling, sodium thiosulfate was added to reclaimed water to quench any remaining chlorine. *S. enterica* is known to utilize the oxidized form thiosulfate, tetrathionate, as a terminal electron acceptor when persisting in anerobic and microaerophilic environments (Winter et al., 2010). Since all water samples were not tightly closed allowing for air

flow and aeration from shaking, it is unlikely this contributed to increased persistence in reclaimed water, but is presented for consideration nonetheless.

Biofilm attachment to polystyrene study showed the clinical isolate *S.* Javiana and poultry house isolate *S.* Heidelberg attached to polystyrene poorly when incubated in surface water compared to other isolates tested. This could possibly be explained by the source nature of the other isolates used in the study – all of these strains were isolated from mid-Atlantic water (Callahan et al., 2019). As the ability to form biofilms in an environment has been shown to aid in environmental persistence, (Moore et al., 2003; Sha et al., 2011), it is reasonable therefore to expect that waterborne *S. enterica* isolates have increased ability to form biofilms in the surface waters tested. It was also interesting that reclaimed water did not support attachment onto polystyrene, but also did not significantly hinder transfer onto tomatoes. These observations point to interesting and possibly unique unknown water – serovar – tomato dynamics in play, which more research should undertake to increase understanding of *S. enterica* transition to different environmental niches.

Serovar specific propensity for crop may exist (Patel and Sharma, 2010; Zheng et al., 2013), so it was hypothesized that transfer success from irrigation water to produce would vary between poultry house isolate *S.* Heidelberg and produce outbreak isolate *S.* Javiana. Indeed, **a**cross the transfer study, it was found that *S.* Heidelberg and *S.* Typhimurium MDR- consistently transferred poorly onto tomatoes compared to other serovars. Interestingly, prolonged incubation in water significantly increased the transferability of these serovars, but not enough in the case of *S.* Heidelberg to render it equivalent to other serovars tested. It is interesting to postulate

the genetic changes taking place over 30 days' time leading to increased transfer success from an aqueous to a desiccated, perhaps more harsh environment of a tomato fruit. Some studies have investigated the effect of previous niches on foodborne pathogen survival in diverse environments; pathogenic *E. coli* after passing through a cow intestine has been shown to persist longer in water (Scott et al., 2006), and *S. enterica* passing through the protist tetrahymena gut has emerged acid tolerant and more resistant to hypochlorite (Brandl et al., 2005; Gourabathini et al., 2008; Rehfuss et al., 2011). Indeed, nutrient starvation and stress mitigation may all be important factors in "priming" these serovars for increased transfer to tomato fruits providing opportunities for future research.

In the case of *S.* Heidelberg, despite longer incubation increasing in transfer success, this serovar was still recovered at the lowest concentrations compared to other strains after 30 days incubation in water. This result was validated with another poultry house isolated *S.* Heidelberg in our collection when singly inoculated into water (**Supplementary Figure 9**). This observation points to a lack of adaptation to the tomato surface, possibly arising from insufficient attachment machinery or poor nutrient acquisition machinery once on the surface of tomato. Interestingly, when both *S.* Heidelberg strains in the collection were 50: 50 mixed into water samples and left for incubation, prolonged (30 day) incubation resulted in transfer success similar to that of a two strain *S.* Newport cocktail. The reason for this is unclear but could indicate that in addition to incubation in a harsh environment perhaps a mutual hardening off, competition, horizontal gene transfer, or synergistic cooperation of the

two strains together in water over time could contribute into increased transfer success.

In the present work, *S.* Javiana and *S.* 4, [5], 12:i:– were the dominant serovars in tomato transfer, and not *S.* Newport. it was expected that *S.* Newport would have a competitive advantage on fruit due to the observed pathogen-commodity associations this serovar has with tomatoes, as evidenced by the disproportionate number of historic outbreaks (Bennett et al., 2015). However, in the present studies while *S.* Newport performed better than *S.* Heidelberg, it was by no means the superior isolate. This could mean that in the environment, other factors may be at play other than initial transfer success that ultimately result in more *S.* Newport outbreaks. Such factors could include: long term persistence ability on plants including competition ability with other microbes or environmental stress (UV and desiccation), the relative abundance of *S.* Newport in the watershed compared to other serovars (due to local anthropogenic activity or other reasons), or even post-harvest factors including susceptibility during washing, handling, and storage. Indeed, more study is needed to clearly tease apart *S.* Newport – tomato outbreak clustering.

In early experiments, no difference in result was registered between MDR + and MDR – *S.* Newport, so the remainder of the study was executed with MDR – *S.* Newport only. This is also because most *S.* Newport associated salmonellosis illnesses are from pan-susceptible isolates (Crim et al., 2018), so using this organism could reflect the outbreak strain more realistically. Considering the two *S.* Typhimurium strains manipulated in this study, when differences in experimental outcome between MDR + and – variants existed, it was more often found that MDR +

was more competitive compared to MDR- variants. This was unexpected at first, as it was hypothesized that acquiring antimicrobial resistance may lead to trade-offs in survival and persistence, as has been documented [for a review, please see (Melnyk et al., 2015)]. However, this was not the case. Supporting these findings, one study assessing *rpoB* fitness costs in *E. coli* populations found that in nutrient limiting conditions, mutations had a positive fitness effect (Maharjan and Ferenci, 2017). Finding a fitness advantage of multidrug resistance isolates has pertinent public health implications, as this makes the MDR+ strain more likely to be established in a niche and therefore may be more likely to contaminate crops (Bengtsson-Palme et al., 2017). Moving forward, it may be important to regularly assess antimicrobial resistance profiles of waterways to better understand persistence of antimicrobial resistance ecology and investigate if this is a good indicator for pathogen transfer or general water quality.

In the present study multiple associations among experimental results were identified. Positive correlations between transfer of microbes to tomatoes after 24 hours of incubation in water and prolonged incubation in water was encouraging to observe, which suggests transfer after incubation in water for one day can potentially be predictive of transfer after a prolonged period of time. Furthermore, total agar plate log inactivation was associated with low transfer success after 1 day water incubation. This can suggest low culturability in water may indicate a lower likelihood of transfer success onto tomato fruits. Finally, through three independent replications of data, the average transfer success onto tomato after prolonged incubation, and not transfer data from 24 h incubation in water, was significantly

positively associated with attachment to polystyrene. This finding provides evidence that biofilm formation may not only be predictive of transfer to tomato fruits but is also a key plant persistence trait which previous history in water affords microbes. Previous studies have identified that biofilm formation, mediated by CsgD and including curli, cellulose capsule, and O antigen formation, are important for *S. enterica* successful establishment on plants (Barak et al., 2005, 2007; Kroupitski et al., 2009; Lapidot and Yaron, 2009; Patel et al., 2013). However, this is the first study which related biofilm formation in water to transfer success on plants. This is extremely encouraging and provides evidence to suggest water is not a static reservoir but acts as driving stimulus for *S. enterica* to exhibit increased fitness on plants. It is exciting to postulate the reasoning for this; nutrient starvation, interaction with xenobiotics, stress, or activation of other processes like motility that enhances attachment could all contribute to this phenomenon. More work is certainly needed in this realm to further understand driving mechanisms behind pathogen transfer from sources to food crops.

Leveraging water source physicochemical data at the time of sampling revealed that transfer to tomato fruits after 30-day incubation was significantly correlated with attachment onto polystyrene and water ORP. Oxidation reduction potential has been previously investigated as an indicator for *S. enterica* prevalence in water with limited success (Haley et al., 2009; McEgan et al., 2014), however this is the first report to our knowledge where ORP is associated with *S. enterica* attachment to polystyrene in surface water and transfer onto tomato fruits. Oxidation reduction potential is currently used to monitor efficacy of water disinfection parameters in

post-harvest agricultural water (Suslow, 2004). This is because at significantly high ORP levels (>450mV), strong oxidants can remove electrons from the cellular membrane, destabilizing the membrane and having significant negative effects on bacteria (Suslow, 2004). However, lower levels of ORP (<450mV) have also been identified as important for biofilm formation, as studies have shown decreased biofilm formation when in an environment overwhelmed by reductants as opposed to having access to oxidants for microbial electron transfer (Gomez-Carretero et al., 2017). Therefore, there may be a Goldilocks mechanism in play, as evidenced in a previous study evaluating *P. fluoresens* biofilm formation, where an optimal level of ORP is required for biofilm formation, between -200mV < x < 450mV (Busalmen and de Sánchez, 2005). Moving forward, oxidation reduction potential in water bodies should be further investigated as an indicator of successful pathogen transfer. The knowledge of this can translate to useful on-farm technologies to better profile water quality.

While microbial safety of fresh produce is a priority, the current obligation under the Produce Safety Rule for growers to comply with *E. coli* standards for water quality is imposed without a comprehensive knowledge of the true food safety risk that irrigating fresh produce in a *S. enterica* endemic region poses. This study begins to bridge the gaps between *S. enterica* persistence dynamics in surface water and on-plant interactions, which could inform long term persistence strategies. More research is needed to investigate the genetic responses of *S. enterica* negotiating transition from water to crops, both in crop specific and region-specific realms. These data can then be integrated into comprehensive risk assessments to accurately identify

regionally specific hazards and practices needed to efficiently, effectively, and sustainably mitigate food safety risk in fresh produce production.

Chapter 6: Conclusions, Limitations, and Future Directions

As research in each individual silo of science advances, the scientific community now has an unparalleled opportunity to engage in transdisciplinary collaboration and tackle increasingly complex and interconnected research questions in the pursuit of a safe and sustainable fresh food supply. Trans-disciplinary work lends itself well to a "One Health" approach to food safety whereby research can now integrate multiple variables into a single study such as water, plant, and bacterial genotype, to provide better understanding of enteropathogen environmental interactions. In the present work, we utilized this approach to illustrate novel mechanisms by which *S. enterica* may adapt to plants as an alternative host. Furthermore, we leveraged the wealth of diversity in *S. enterica* serovars to investigate specific dynamics and drivers of persistence in water and transfer onto produce crops.

Study Highlights

Novel insight into strategies *S. enterica* may utilize for survival along the farm to fork continuum are provided in the present work. In **Chapter 3, *it*** was illustrated that *Salmonella* Newport elicits and actively responds to not only plant-derived hydrogen peroxide, but also nitric oxide. This finding has major implications in how the interaction between human pathogens and plants is viewed. Opportunity now exists to expand this realm of research to investigate other mechanisms of enteropathogen-plant mutual responses. In this study it was observed that the majority of NO reciprocal responses were present on tomato leaves compared to fruit, and that

overall higher concentrations of *S*. Newport were more consistently retrieved from leaves compared to fruits. While this may indicate that survival strategies on fruit may include additional factors not explored in the current work, it also suggests that leaves could be the preferred niche of culturable pre-harvest associated *S. enterica*. Therefore, it is advantageous to augment current tomato harvesting GAPs to include procedures which minimize contact between fruit and leaves, especially during key contamination multiplier events; at harvest, in post-harvest dunk tanks, and storage.

Investigating *S. enterica* serovar dynamics in surface and reclaimed water revealed that persistence in water is principally driven by water type and may include viable but non-culturable states. Furthermore, data was provided to suggest transfer success onto tomato is driven by serovar more than water, and residency in water may increase the transfer ability of *S. enterica* (**Chapter 5)**. This indicates that water may act as a priming reservoir for select *S. enterica* for downstream increased success on plants. Further, linear regression of experimental data with water physicochemical variables revealed that attachment to polystyrene and water oxidation reduction potential should be further evaluated as potential indicators of foodborne pathogen transfer probability onto tomato (**Chapter 5**). In an age where we are still evaluating the effectiveness of sampling for fecal indicators to assess water quality, investigation into additional methods for water quality estimations is very important.

In our studies, one thing is clear – serovars of *Salmonella enterica* are unique. In **Chapter 4** a differential nitric oxide stress effect was observed across serovars which was concentration-dependent. Importantly, it was identified that increasing plant-derived nitric oxide in the phyllosphere had a negative effect on *Salmonella*

retrieval. In terms of *Salmonella* Newport, while we identified active mutual recognition and response present between this organism and tomatoes (**Chapter 3**), this serovar did not outperform others on tomato seedling or fruit assays (**Chapters 4 and 5,** respectively). Furthermore, it was expected that *S.* Heidelberg- the poultry associate isolate- would perform poorly in every experiment, yet it persisted well in water (**Chapter 5).** Furthermore, along with *S.* Enteriditis, *S.* Heidelberg had increased tolerance to nitric oxide at higher concentrations (**Chapter 4**). Finally, surprisingly *S.* 4,[5], 12:i:-, a serovar not historically isolated from tomatoes, transferred extremely well from each water source to tomato fruit. Taken together, these data suggest that *Salmonella enterica* is a diverse species and the probability of one specific serovar causing an outbreak is likely due to a symphony of interconnected variables, some synergistic, some antagonistic, ultimately leading to contamination and persistence through the food chain. In the present work we were able to provide insights into driving factors behind currently observed epidemiologic trends of serovar- source specificity, however more research must be conducted before we can understand a clearer picture of why one serovar over another ultimately causes an outbreak. For instance – serovar specific interaction with the resident epiphytic community, manipulation of plant surface exudates, and mitigation of abiotic stress may all factor into success or failure to persist in the agricultural environment. Research, which has traditionally focused on *S.* Typhimurium and a few other select serovars, should work to incorporate a wider breadth of *S. enterica* serovars to more accurately obtain this profile.

Limitations

This work is not without its limitations and challenges, however, each challenge identified also presents renewed opportunity for future work with increasingly finer tuned questions in the farm to fork continuum under a "One Health" approach to solutions for on-farm produce safety.

In our tomato experiments, the inter-variability of individual plants and responses to chemical modulation was a challenge for experimental reproducibility, and a considerable amount of time was taken to devise the best standardization technique. In working with NO, variability in results has been previously documented (effectiveness of the treatment ranging from 0 to 100%, sometimes in the same model) (Astier et al., 2018) and underscores the complexity of this system in plants. To address these challenges, in **Chapter 3** multiple replications of experiments were conducted, whereas in **Chapter 5** robust experimental design and statistical treatment addressed this issue.

Another challenge in **Chapter 3** arose from efforts to create a *Salmonella* knockout *hmpA* mutant. There was effort during this process to construct a Newport knockout mutant via the lambda red system in the principle NO detoxification enzymes flavoahaemoglobin *hmpA* and *yoaG*, however due to the nature of this serovar this study was unsuccessful. Investigation into other methods of constructing competent cells and mutagenesis procedure, such as conjugation via a suicide vector (Morrison et al., 2012) are main avenues to consider in future efforts.

Our plant experimental system could also be viewed as a limiting factor, as all experiments were completed with 'Heinz-1706' tomato, a canning variety. We chose

this variety based on previous work showing that *S*. Newport colonized seedling leaves significantly poorly compared to other cultivars and embarked on the dissertation journey to investigate if this discrepancy was in part due to immune response signaling molecules (**Chapter 3**). Moving forward, it seems imperative to evaluate if this interplay between *Salmonella* and plant NO is restricted to this cultivar of tomato, *Solanum lycopersicum* only, or if this is a relevant interaction in other *Salmonella* -plant crop interactions. One could suppose another limitation in the experimental system chosen is that select experiments in the phyllosphere study were completed with seedling leaves. Seedling leaves were chosen to work with due to ease of experimentation, but it is important to consider the age-based biases of this work with young plant material. Several studies have profiled plant age as a factor in effector triggered immunity studies, where older plants observe more resistance against plant pathogenic agents (Shah et al., 2015). While the mechanism of age related immunity in plants is not fully profiled, work by Carella et al. reports that baseline immune function is not altered by age related resistance, however engagement with effector "R" proteins from a pathogen is affected. Therefore plant age may be more important in ETI studies (2015). However, this question certainly sparks interest in another aspect of foodborne pathogens in the context of the farm to fork continuum; how do foodborne pathogens adapt to a changing plant as it grows and develops? Time course studies may elucidate more interesting information about *Salmonella* niche development in the context of plant development.

Another limitation can be observed in our water type comparison studies. In **Chapter 5**, water samples were 0.22 µm filtered in order to eliminate competition

and the possibility of protozoal grazers which would skew results (Fischer et al., 2006). Furthermore, we thought that adding additional antimicrobial resistance marker through a plasmid may be unstable and skew results. It has been documented that persistence in sterilized or treated water may not be identical to untreated water (Santo Domingo et al., 2000, Topalcengiz et al., 2019). Moving forward, construction of antibiotic resistance in the chromosome of strains of interest and evaluating persistence in water with either native communities or via spiking in a standardized microbial "community" after cycloheximide treatment to eliminate protozoa may provide opportunity to be able to draw more real-world laboratory water study.

Finally, while evidence of active stress negotiation and niche adaptation was observed in *S.* Newport -tomato interaction (**Chapter 3**), parallel targeted transcriptomic study to evaluate serovar specific adaptation strategies from water to tomato using a similar approach were unsuccessful due to insufficient concentrations of RNA in ~30% of the samples. In bacterial transcriptomic studies, it is recommended that 10^8 cells are retrieved for optimal RNA concentrations in downstream experimentation (Sirsat et al., 2011). However, it is a challenge to achieve this, yet perform an experiment with a real world concentration of *Salmonella* contamination (5 or 6 log CFU/mL at most). Moving forward, increasing the number of pooled samples and decreasing the number of treatments can allow for successful investigation of genetic machinery necessary to negotiate transition from water to a tomato surface.

Broader Impacts and Future Directions

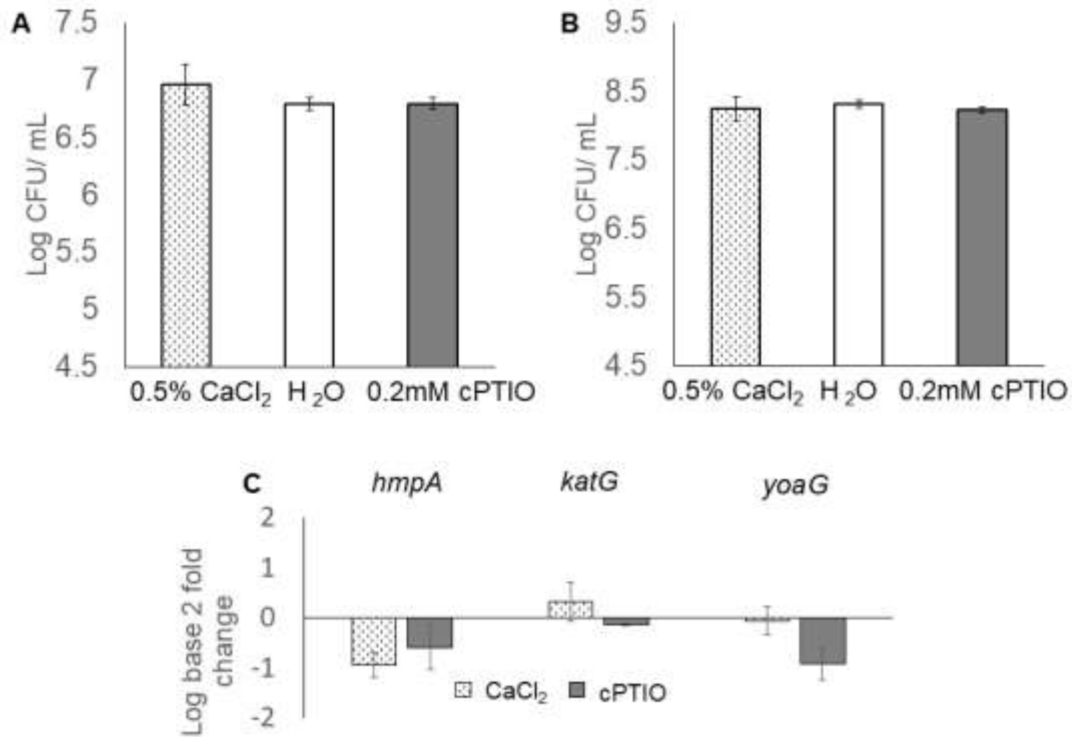
Our studies revealed key water, plant organ, and bacterial factors that influence contamination and persistence on food crops, however further exploration remains a necessity. For example, to further explore serovar specific reactions to plant-derived nitric oxide, additional experiments employing DAF fluorescence detection could be undertaken to relate NO tolerance to the amount of NO each serovar elicits from the seedling material. This work and parallel efforts are important steps in the growing body of work illustrating serovar specific propensity for specific niches, which can lead to serovar specific mitigation strategies based on the *Salmonella* ecology of a given agricultural environment in the near future.

Another major outcome of this work is that there is a wealth of study opportunity in *Salmonella* – water interactions that may have direct profound outputs for mid-Atlantic farmers. In our studies we found that VBNC states may be a strategy for persistence. This physiological state has been under-evaluated in an agricultural context. Moving forward, during routine microbial testing of water sources, it will be important to consider that water samples which test negative for *S. enterica* may be false negatives and instead may harbor cells in a VBNC state that are not detectable by culture methods. Moreover, investigation of other drivers or environments in the farm to fork continuum used by *Salmonella* to engage in this persistence strategy remains to be explored. In terms of translatable outputs, knowledge gained from the present work on *S. enterica* associated risks in surface irrigation water, as well as updates on current efforts to augment water quality measurements could be disseminated to the grower constituency on a regular basis.

One year into compliance of the FSMA PSR, especially for smaller more diversified farms (like we have here in Maryland), “one size” adaptation of the Rule will not fit all. This has been recognized on a limited commodity basis; as Mushroom Good Agricultural Practices, Tomato Good Agricultural Practices, and the Leafy Green Marketing Agreement exist to translate food safety principles into sensible on-farm practices. While these resources are important in helping growers tailor food safety needs to their farm, there is a sense that the next step must be taken in the development of regionally and operational-scale specific guidance whose goals are to enhance food safety minded practices based on individualized hazards and varying technology available for risk mitigation. The present work endeavored to more holistically track one organism, *S. enterica,* as it moves through the agro-environment, focusing on key negotiation strategies used to combat hurdles from farm to fork. While we provide some insight on *S. enterica* dynamics, more research is needed to investigate the genetic responses of this and other foodborne pathogens negotiating the transition from water to crops, both in crop-specific and region-specific realms. Expanding the scope of research to understand 1) new and emerging crop production strategies such as hydroponics, greenhouse cultivation, permaculture, and urban farming and 2) current and future pressures on the farm environment such as climate change and the impact of surrounding anthropogenic activity is crucial for the future of food safety research. With these perspectives, a better understanding can be gained of current hazards and can be used to anticipate future issues from *S. enterica,* other foodborne pathogens, and chemical contaminants which threaten the safety and sustainability of our food and water supply. This variation of a “One

health” approach to interdisciplinary public health research can elevate on-farm food safety practices, driving past the previous goal of compliance to regulation towards a farming and research culture which incorporates equitability, innovation, sustainability, and safety.

Appendix I



Supplementary Figure 1: (A) Plate counts of 6 hr *S.* Newport incubation in PBS amended with plant scavenger or elicitor of NO. (B) Plate counts of 6 hr *Salmonella* incubation in TSB amended with plant scavenger or elicitor of NO. Inoculum was 7 log CFU/mL for both A and B, with no significant differences in growth after 6h. (C) 1/2 strength TSB *S.* Newport control- *hmpA, katG,* and *yoaG* expression relative to *rpoD*. All average log based 2-fold change in expression are $-1 < x < 1$.

Appendix II

Supplementary Table 1: Log decline from inoculum of various *S. enterica* when exposed to different concentrations of Spermine NONOate, corrected for 0 µM at each time point. Letters denote significant differences among serovars at each time point and concentration via Kruskal-Wallis / Wilcoxon rank test (α=0.05). This is the average of 4 experimental replicates.

		mean	± Stdev	mean	± Stdev	mean	± Stdev	mean	± Stdev
hpi	serovar	250 µM		500 µM		1000 µM		2000 µM	
1	Javiana	0.101	0.121	0.277	0.193	0.519	0.186	1.187^{b}	0.462
	Newport	0.170	0.164	0.297	0.246	0.526	0.268	0.717^{b}	0.306
	Heidelberg	0.085	0.340	0.338	0.238	0.727	0.621	0.920^{b}	0.656
	Enteriditis	0.037	0.133	0.418	0.164	0.608	0.327	1.037^{b}	0.478
	Typhimurium	-0.030	0.241	0.053	0.238	0.242	0.246	0.301^{a}	0.190
4	Javiana	0.151^{a}	0.096	0.420^{ab}	0.242	1.084	0.463	1.410	0.843
	Newport	0.306^{ab}	0.197	0.435^{a}	0.206	0.867	0.521	1.244	0.838
	Heidelberg	0.359^{b}	0.255	0.571^{b}	0.132	1.243	0.448	1.740	0.750
	Enteriditis	0.288^{ab}	0.181	0.721^{c}	0.096	0.967	0.464	1.215	0.506
	Typhimurium	0.033^{a}	0.203	0.533^{ab}	0.138	0.901	0.440	1.018	0.610
24	Javiana	0.126^{ab}	0.194	0.410^{a}	0.189	1.857^{b}	1.036	2.522	1.028
	Newport	0.390^{c}	0.249	0.639^{b}	0.257	1.763^{abc}	1.068	2.483	0.878
	Heidelberg	0.018^{a}	0.133	0.312^{a}	0.252	1.215^{ab}	0.499	1.715	1.932
	Enteriditis	0.310^{bc}	0.258	0.696^{b}	0.253	0.697^{a}	0.684	2.909	0.460
	Typhimurium	0.239^{bc}	0.096	1.031^{c}	0.109	2.783^{c}	1.082	3.028	0.390

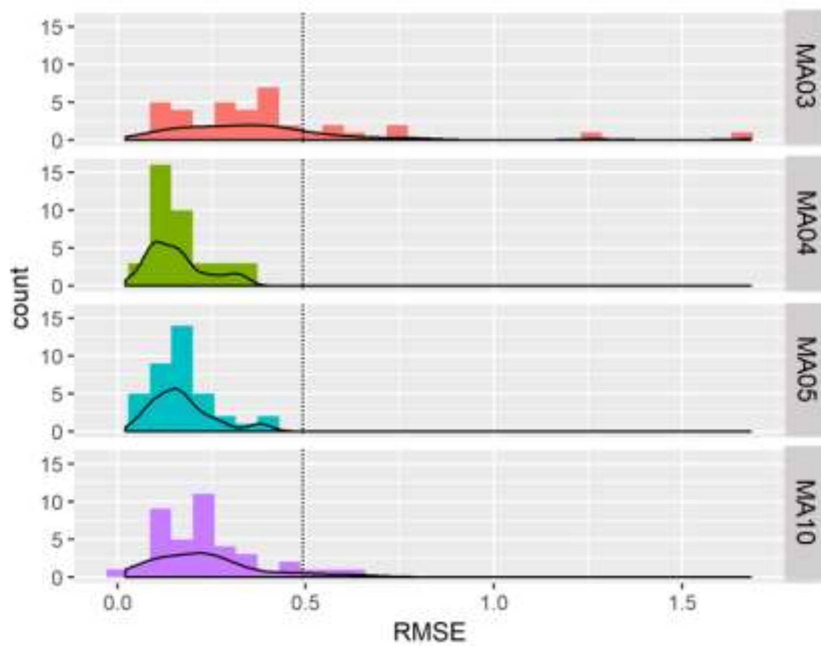
Appendix III

Supplementary Table 1: IPMP Buchanan's two-phase log-linear model parameters for 90 day persistence in surface water measured by agar plate count, replicate one. The average lag time in days, rate of decay * 100, Sum of squares due to error (SSE) and root mean square error (RMSE) are reported.

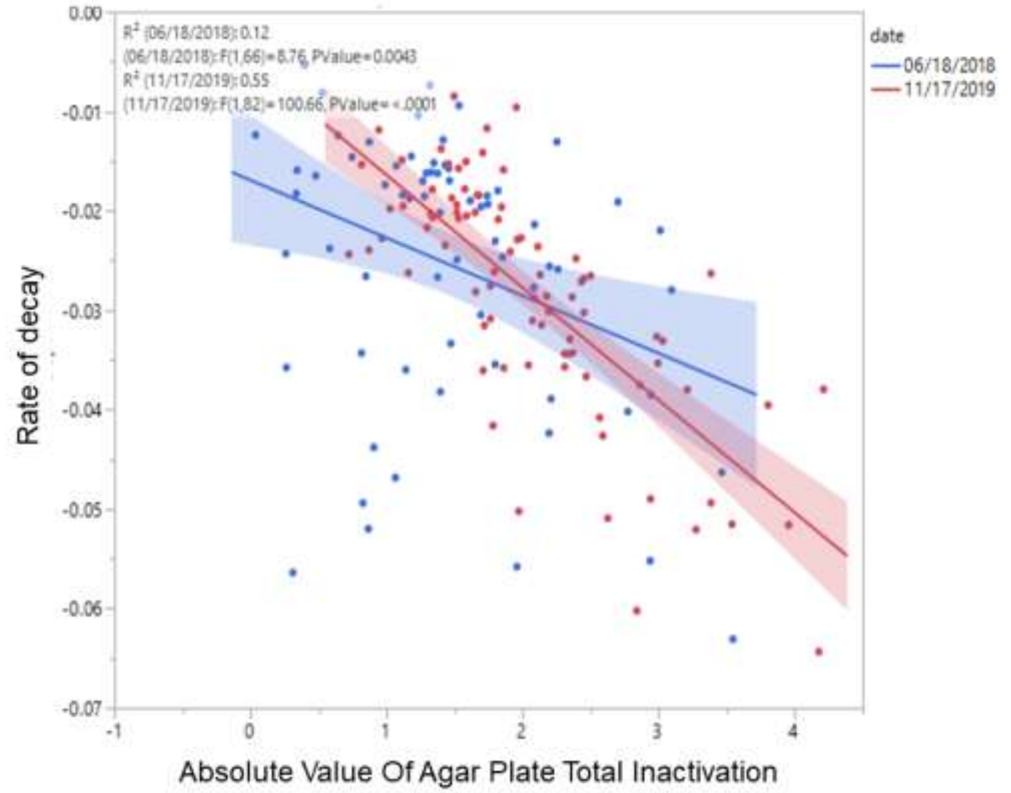
			Lag		μ max *10²		SSE		RMSE	
Date	**Water**	**Serovar**	mean	std dev	mean	std dev	mean	std dev	mean	std dev
Rep 1	**MA03**	**4, [5], 12:i:-**	16.19	12.70	-2.10	0.52	5.84	9.62	0.72	0.83
		Heidelberg	7.00	6.50	-3.96	1.24	2.08	0.81	0.64	0.12
		Javiana	9.52	3.10	-3.09	0.43	1.24	0.53	0.49	0.12
		Newport MDR-	5.16	4.52	-2.74	0.41	1.06	1.60	0.37	0.34
		Newport MDR+	4.04	1.00	-2.16	0.16	0.86	0.13	0.41	0.03
		Typhimurium MDR-	4.59	5.05	-2.88	0.36	0.65	0.28	0.36	0.08
		Typhimurium MDR+	11.19	5.50	-2.93	0.44	0.58	0.32	0.33	0.11
	MA04	**4, [5], 12:i:-**	16.12	14.39	-1.52	0.66	0.10	0.05	0.14	0.04
		Heidelberg	27.46	13.11	-3.71	1.20	0.05	0.03	0.09	0.04
		Javiana	24.98	8.30	-2.48	1.03	0.12	0.13	0.14	0.08
		Newport MDR-	20.70	7.43	-2.06	0.30	0.13	0.03	0.16	0.02
		Newport MDR+	13.77	10.44	-1.76	0.25	0.08	0.05	0.13	0.04
		Typhimurium MDR-	19.03	3.32	-2.70	0.71	0.15	0.11	0.16	0.07
		Typhimurium MDR+	24.21	3.83	-1.70	0.29	0.10	0.06	0.14	0.04
	MA05	**4, [5], 12:i:-**	12.49	11.71	-2.16	0.93	0.12	0.02	0.16	0.01
		Heidelberg	5.36	5.82	-2.99	0.84	0.29	0.02	0.24	0.01
		Javiana	4.95	1.89	-1.79	0.24	0.15	0.08	0.17	0.05
		Newport MDR-	3.86	1.50	-1.79	0.22	0.05	0.05	0.10	0.05
		Newport MDR+	3.33	5.77	-1.54	0.43	0.06	0.06	0.10	0.06
		Typhimurium MDR-	9.29	5.43	-3.27	0.87	0.08	0.05	0.12	0.04
		Typhimurium MDR+	8.18	8.23	-1.92	0.83	0.07	0.04	0.12	0.04
	MA10	**4, [5], 12:i:-**	15.25	8.60	-3.64	1.34	0.13	0.11	0.15	0.07
		Heidelberg	7.74	6.70	-4.17	0.82	0.67	0.51	0.35	0.14
		Javiana	14.87	4.14	-3.30	0.51	0.26	0.20	0.22	0.10
		Newport MDR-	12.30	2.45	-4.25	0.82	0.44	0.53	0.26	0.19
		Newport MDR+	20.56	8.87	-5.42	1.42	0.37	0.28	0.25	0.12
		Typhimurium MDR-	18.52	8.86	-4.69	0.65	0.21	0.08	0.20	0.04
		Typhimurium MDR+	12.96	2.89	-3.10	0.22	0.10	0.09	0.12	0.09

Supplementary Table 2: IPMP Buchanan's two-phase log-linear model parameters for 90-day persistence in surface water measured by agar plate count, replicate two. The average lag time in days, rate of decay * 100, Sum of squares due to error (SSE) and root mean square error (RMSE) are reported.

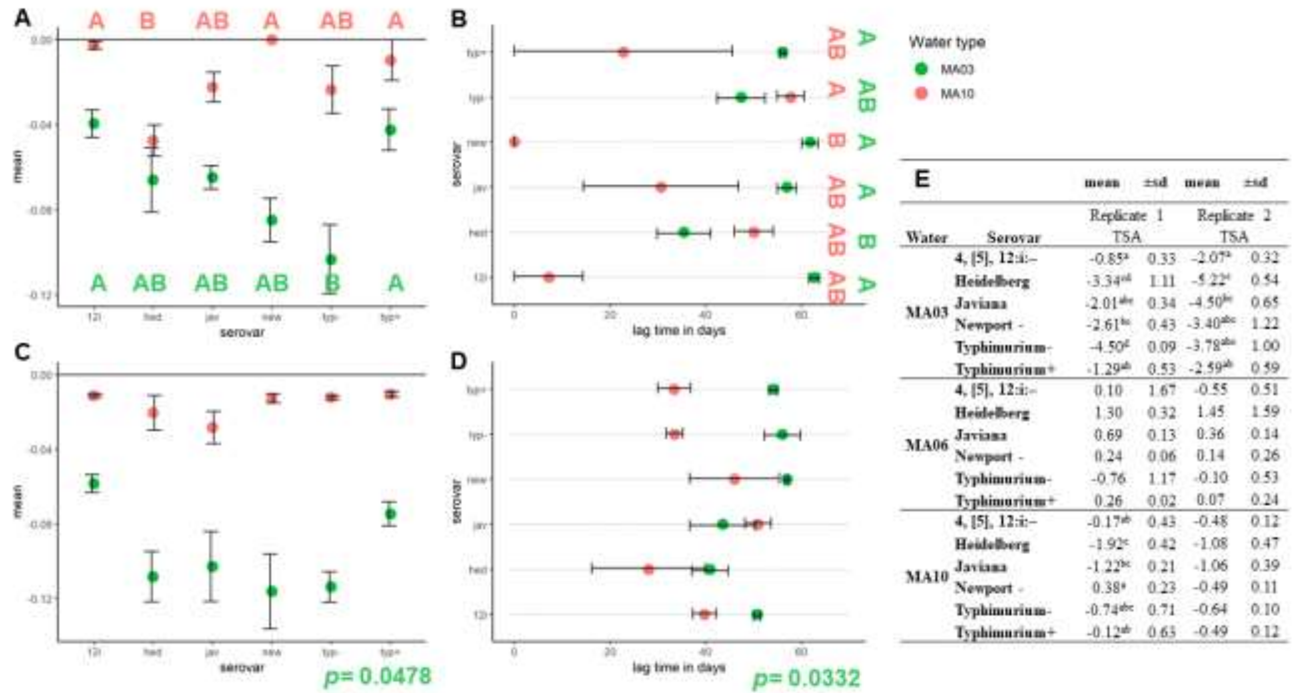
			Lag		μ max $*10^2$		SSE		RMSE	
Date	**Water**	**Serovar**	mean	std dev	mean	std dev	mean	std dev	mean	std dev
Rep 2	**MA03**	**4, [5], 12:i:–**	9.22	15.98	-1.78	0.34	1.85	2.58	0.65	0.54
		Heidelberg	25.20	4.88	-5.07	0.63	0.23	0.30	0.28	0.27
		Javiana	41.19	27.02	-3.50	1.89	0.10	0.09	0.17	0.08
		Newport MDR-	3.39	5.87	-1.75	0.79	0.26	0.13	0.29	0.08
		Typhimurium MDR-	16.72	0.62	-3.39	0.88	0.26	0.31	0.26	0.20
		Typhimurium MDR+	33.87	29.34	-2.62	1.46	0.15	0.14	0.20	0.10
	MA04	**4, [5], 12:i:–**	46.28	18.00	-1.62	1.15	0.06	0.01	0.15	0.01
		Heidelberg	60.87	2.00	-4.67	0.28	0.27	0.05	0.30	0.03
		Javiana	65.66	16.37	-3.48	2.20	0.23	0.09	0.28	0.05
		Newport MDR-	39.52	17.02	-1.58	0.31	0.03	0.02	0.09	0.05
		Typhimurium MDR-	23.51	11.26	-1.57	0.71	0.04	0.04	0.12	0.05
		Typhimurium MDR+	43.42	37.61	-1.32	0.69	0.12	0.17	0.17	0.14
	MA05	**4, [5], 12:i:–**	14.92	9.64	-2.05	0.22	0.19	0.18	0.23	0.11
		Heidelberg	15.36	24.17	-4.33	1.80	0.18	0.23	0.21	0.15
		Javiana	60.49	9.45	-3.76	1.30	0.08	0.04	0.16	0.04
		Newport MDR-	22.14	26.49	-2.86	1.23	0.12	0.10	0.19	0.09
		Typhimurium MDR-	3.07	4.34	-2.87	0.95	0.33	0.14	0.33	0.07
		Typhimurium MDR+	9.20	15.93	-1.35	0.57	0.08	0.05	0.16	0.05
	MA10	**4, [5], 12:i:–**	0.04	0.07	-1.94	0.56	0.19	0.10	0.25	0.06
		Heidelberg	12.73	7.24	-3.19	1.11	0.08	0.11	0.15	0.10
		Javiana	7.74	10.74	-1.82	0.27	0.21	0.30	0.22	0.18
		Newport MDR-	4.92	8.52	-2.19	0.56	0.40	0.52	0.31	0.24
		Typhimurium MDR-	6.02	8.93	-1.67	0.05	0.08	0.07	0.17	0.10
		Typhimurium MDR+	0.00	0.00	-1.64	0.14	0.54	0.63	0.39	0.22



Supplementary Figure 1: Histogram of Root Mean Squared Error (RMSE) values calculated from Buchanan's two-phase log-linear model, by water type. Cutoff for a "high RMSE" was determined to equal = 0.5. High RMSE values for 2 phase fit were identified in MA03 and MA10. In these instances, the data fit better to Buchanan's three phase log linear model of decay, indicating the presence of a tail.

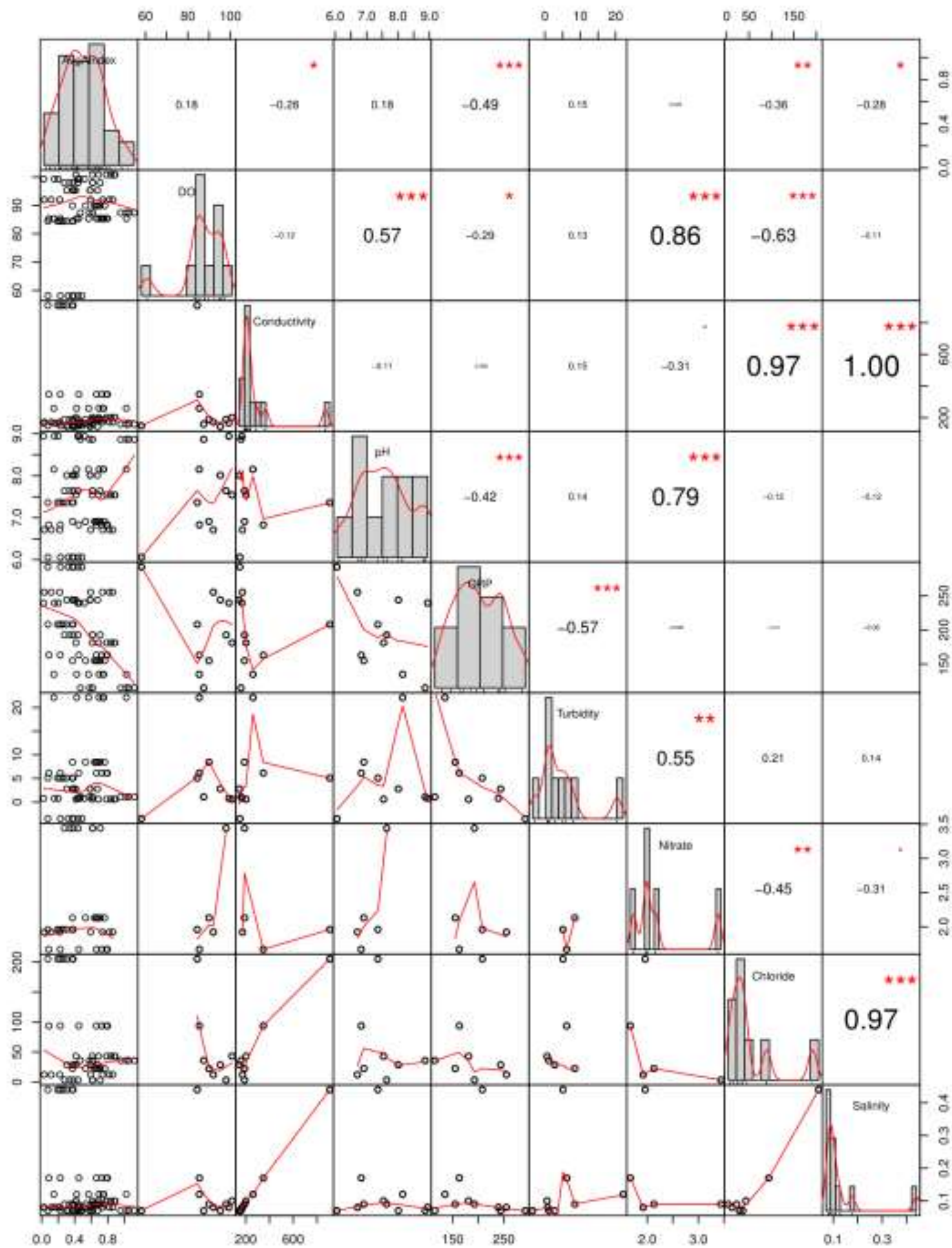


Supplementary Figure 2: Correlation of each experimental replicate agar plate total log inactivation and rate of decay. Red data represent replicate one and blue represents replicate two. R^2 values reported reflect Pearson's correlation coefficient (α=0.05).



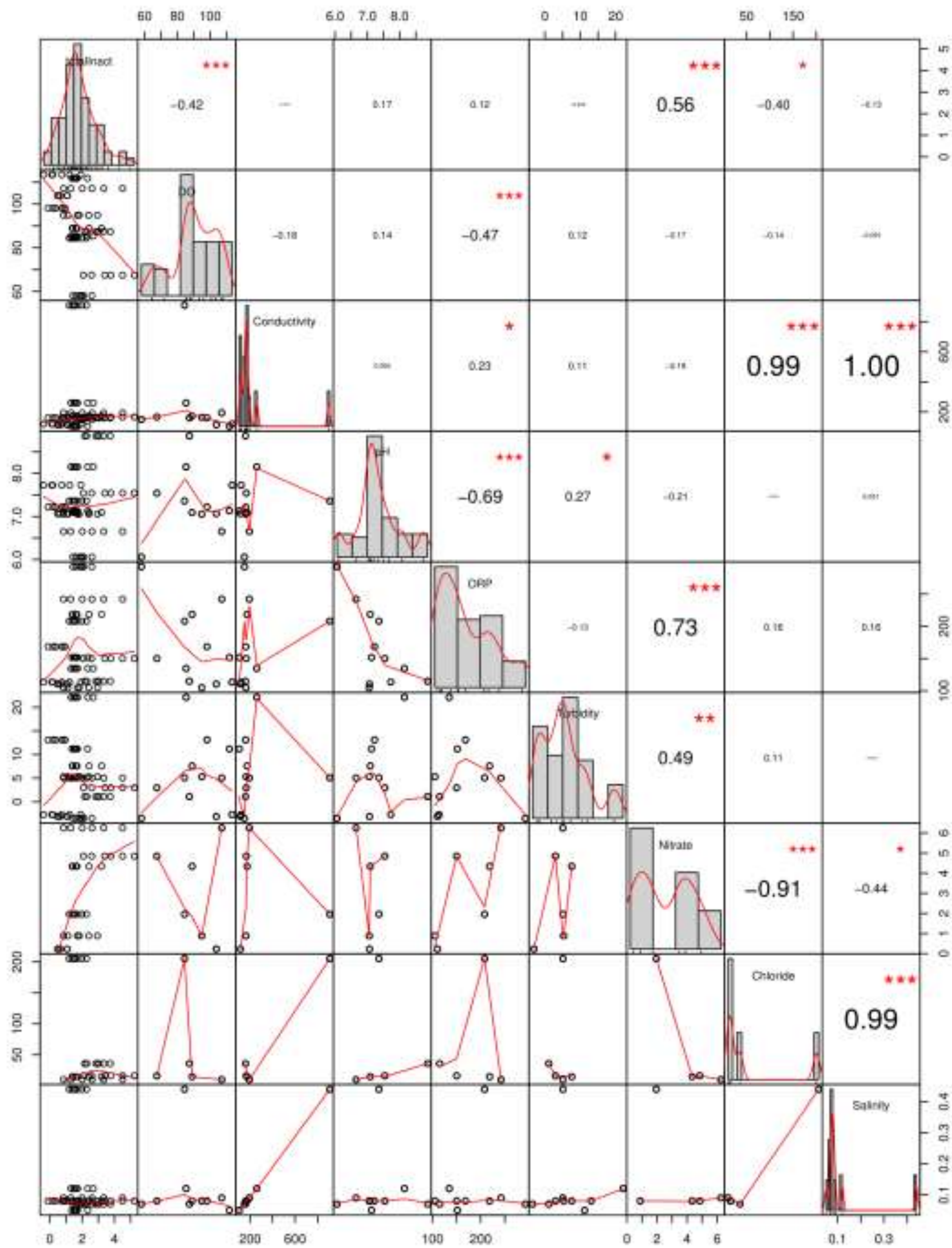
E		mean	±sd	mean	±sd
		Replicate 1		Replicate 2	
Water	Serovar	TSA		TSA	
MA03	4, [5], 12:i:–	-0.85[a]	0.33	-2.07[a]	0.32
	Heidelberg	-3.34[cd]	1.11	-5.22[c]	0.54
	Javiana	-2.01[abc]	0.34	-4.50[bc]	0.65
	Newport -	-2.61[bc]	0.43	-3.40[abc]	1.22
	Typhimurium-	-4.50[d]	0.09	-3.78[abc]	1.00
	Typhimurium+	-1.29[ab]	0.53	-2.59[ab]	0.59
MA06	4, [5], 12:i:–	0.10	1.67	-0.55	0.51
	Heidelberg	1.30	0.32	1.45	1.59
	Javiana	0.69	0.13	0.36	0.14
	Newport -	0.24	0.06	0.14	0.26
	Typhimurium-	-0.76	1.17	-0.10	0.53
	Typhimurium+	0.26	0.02	0.07	0.24
MA10	4, [5], 12:i:–	-0.17[ab]	0.43	-0.48	0.12
	Heidelberg	-1.92[c]	0.42	-1.08	0.47
	Javiana	-1.22[bc]	0.21	-1.06	0.39
	Newport -	0.38[a]	0.23	-0.49	0.11
	Typhimurium-	-0.74[abc]	0.71	-0.64	0.10
	Typhimurium+	-0.12[ab]	0.63	-0.49	0.12

Supplementary Figure 3: Rate of decay (A, C) and lag time in days (B, D) of various *Salmonella enterica* in 30 mL samples of 0.22 µM filtered MA03, MA10 in replicate 1 (A, B) and 2 (C, D) of Agar plate – qPCR PMA study. Agar plate counts over 90 days were modeled via Buchanan's two-phase log-linear inactivation equation (IPMP 2014). N=3 for each serovar and water type combination. Error bars represent the SEM. Letters denote serovar specific within water types through Analysis of Variance and Tukey's Honestly Significant Difference (α =0.05). (E): Agar plate total log inactivation from 1-90 days of *S. enterica* in 30 mL water samples. Letters denote serovar specific differences in total bacterial death within water types for culture-based counts through Analysis of Variance and Tukey's Honestly Significant Difference (α=0.05).

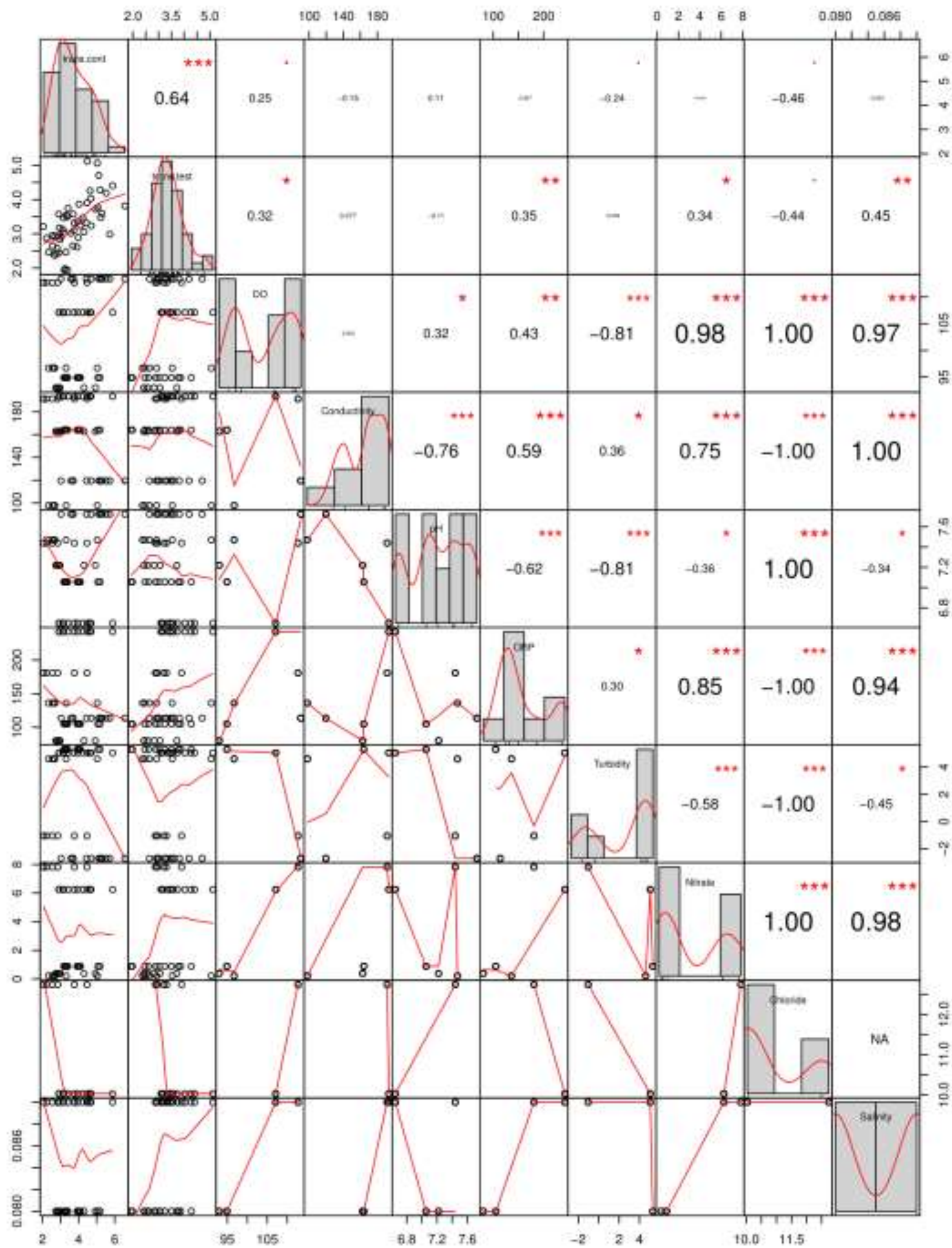


Supplementary Figure 4: Correlogram of physicochemical parameter data at the time of sampling from water sources used for experimentation and attachment index to polystyrene, "AvgAindex." Attachment indices for each serovar x water type

combination were averaged over two experimental replicates for MA05, and three experimental replicates for all other water types. Triple asterisks denote significant Pearson's correlation coefficient (p<0.001).

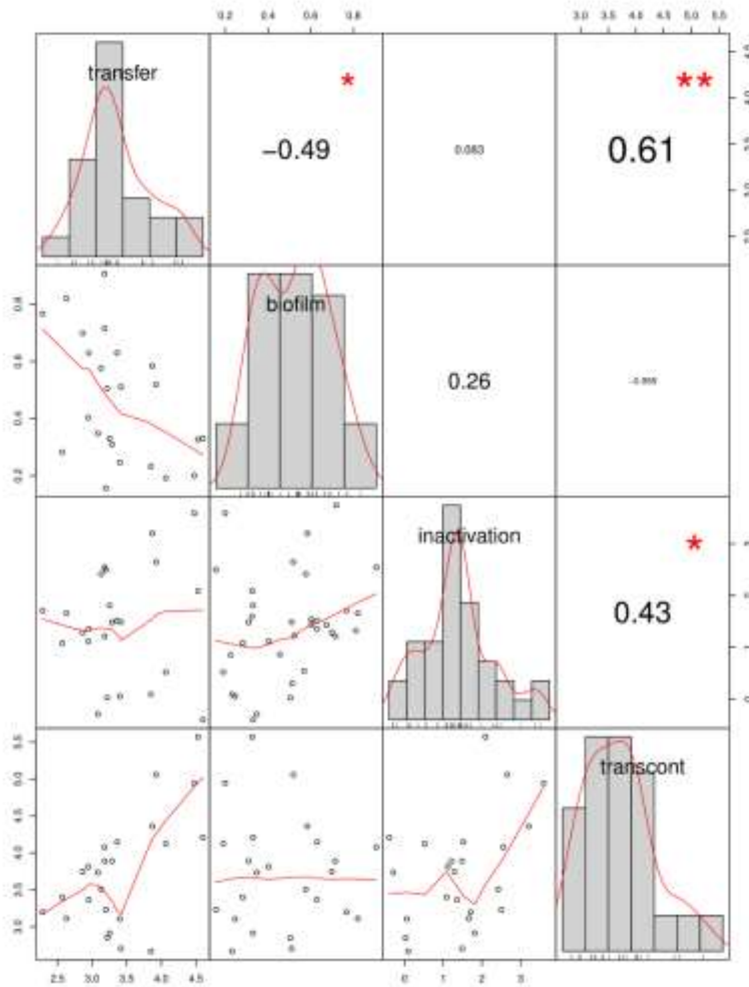


Supplementary Figure 5: Correlogram of physicochemical parameter data at the time of sampling from water sources used for experimentation and plate count total inactivation from N=4 water sample studies, "Totalinact." Experimental data were averaged over two experimental replicates for MA04 and MA10, and four experimental replicates for all other water types. Triple asterisks denote significant Pearson's correlation coefficient ($p<0.001$).

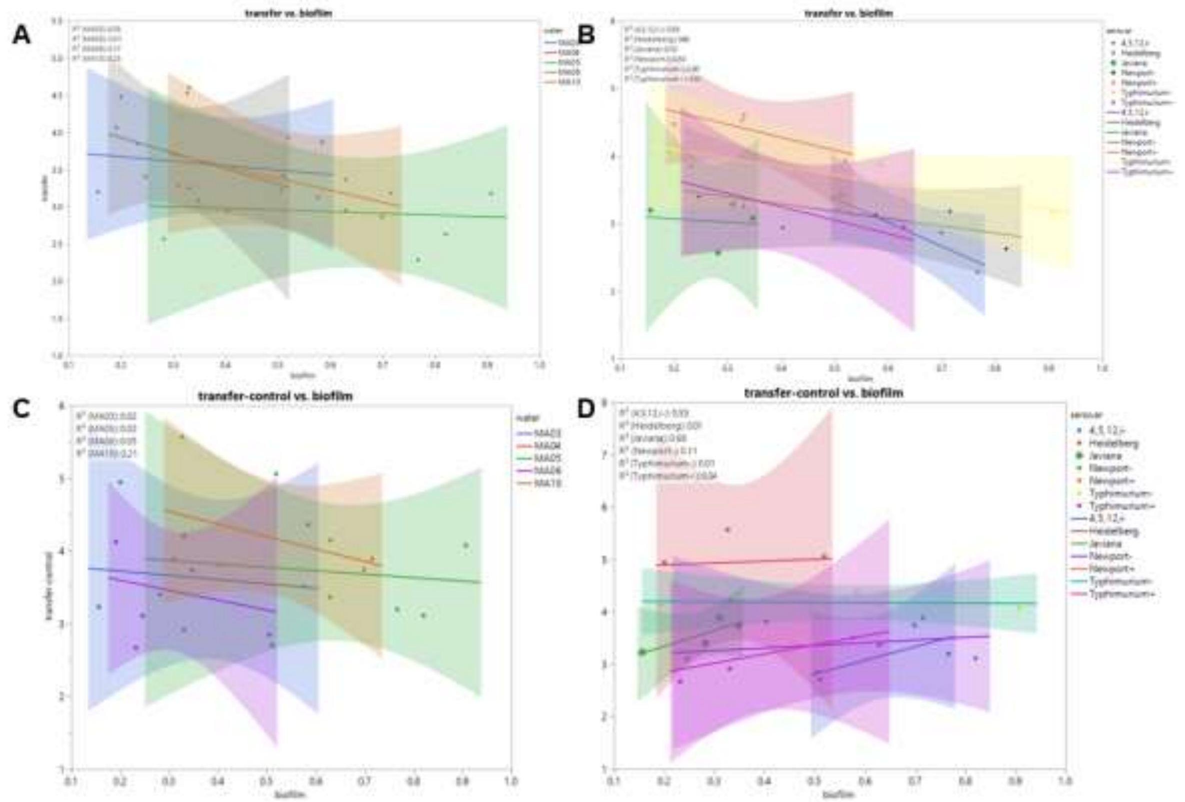


Supplementary Figure 6: Correlogram of physicochemical parameter data at the time of sampling from water sources used for experimentation and transfer success onto tomato after 24 h in water, “trans.cont” or 30 days in water “trans.test.”

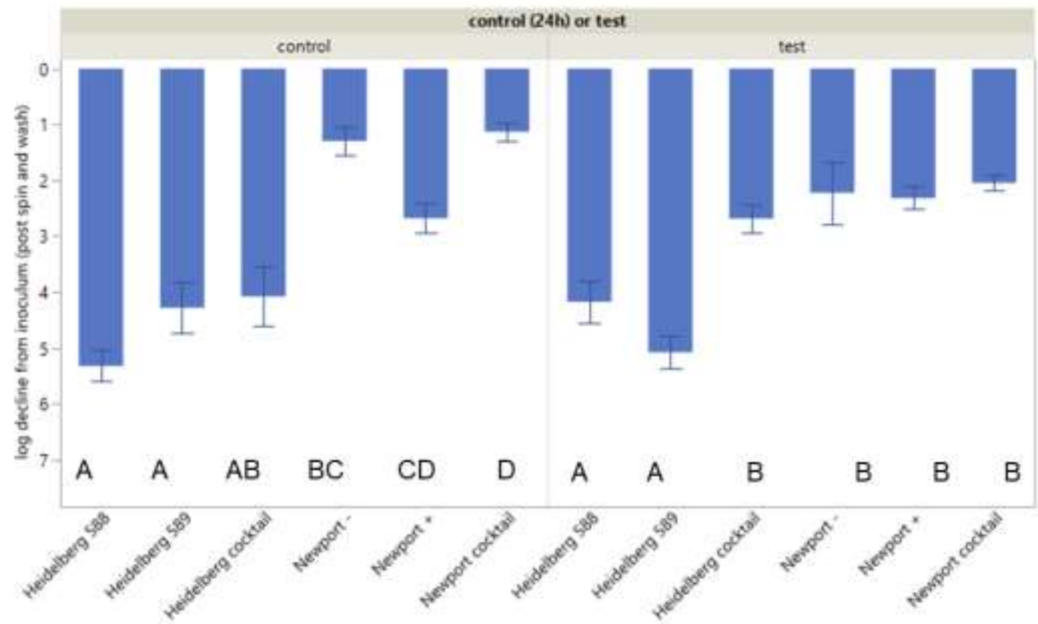
Experimental data are averaged over three experimental replicates. Triple asterisks denote significant Pearson's correlation coefficient ($p<0.001$).



Supplementary Figure 7: Correlogram of attachment index to polystyrene "AvgAindex", plate count total inactivation over 90 days "Totalinact," transfer success onto tomato after 24 h in water, "trans.cont" and after 30 days in water "trans.test." Data were paired by water type. Triple asterisks denote significant Pearson's correlation coefficient ($p<0.001$).



Supplementary Figure 8: Correlations and R^2 fits between attachment index and transfer success onto tomato. Panel A and B depict transfer success from serovars incubating in water for 30 d; Panel C and D depict transfer success from serovars incubating in water for 24 h (“spent”). Fits are clustered by water type (Panel A, C) and serovar (B, D).



Supplementary Figure 9: Transfer success of two *S.* Heidelberg strains and two *S.* Newport strains 50:50 mixed in MA05 samples (denoted by "cocktail"), or singly inoculated *S. enterica*. Samples were incubated for 24 h "control" or 30 d "test" in MA05. N=3, error bars represent the SEM. Letters denote significant differences in transfer within water incubation treatment (Tukey's HSD α=0.05).

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