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ORIGINAL ARTICLE

Growth of *Salmonella enterica* in foliar pesticide solutions and its survival during field production and postharvest handling of fresh market tomato

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foliar pesticide solutions, food safety, preharvest water, *Salmonella* serovars, survival, tomato.

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Abstract

Aims: To evaluate *in vitro* the growth kinetics of *Salmonella enterica* in pesticide solutions labelled for fresh market tomato and the effect of ag-chemical application with contaminated water to tomatoes during field production.

Methods and Results: The capacity of pesticide formulations in the survival of *S. enterica* was evaluated *in vitro* and on tomato surfaces during field production. Most pesticides had ability to maintain the growth of *Salmonella,* however, specific pesticides can also support its growth, which was also dependent on the water composition and temperature to which pesticide solutions were held. *Salmonella* applied to field grown tomatoes through pesticide application was able to survive up to 15 days in up to 80% of the collected samples, even more postharvest washing with sodium hypochlorite was insufficient to completely mitigate the presence of *Salmonella* on tomato surfaces.

Conclusions: This study provides further evidence that pesticides may support the growth of *Salmonella,* if introduced with source water and may elevate risk during foliar contact application beyond that of the water source alone.

Significance and Impact of Study: The study points out the importance of the microbiological quality of foliar contact water as a critical point to prevent contamination of fruits and vegetables from early stages of field production.

Introduction

Consumption of contaminated produce with foodborne pathogenic micro-organisms has become a major concern in public health. The increasing number of foodborne illness caused by diverse enteric pathogens, in particular, has brought increased concern and scrutiny among regulators and public health agencies as well as research attention and focus among the scientific community to identify risks and to control causes and sources of produce contamination (FDA 2011). Nonconsumer-based contamination of produce with spoilage and pathogenic micro-organisms can occur at any point from the cultivation stages to processing and commercial food preparation (Tournas 2005; Hanning *et al.* 2009). If contamination occurs at any point of primary production in the supply chain, prevention and mitigation strategies in other downstream processing or distribution steps for produce consumed in a fresh form will not be successful. Thus, it is necessary to further elucidate preharvest risks to implement standards and strategies that allow prevention of contamination at the critical stages of fresh fruit and vegetable production.

Preharvest contamination, during crop production, has been associated with wildlife, soil amendments and irrigation water (Suslow *et al.* 2003; Franz and van Bruggen 2008; Suslow 2010). Production of horticultural crops necessarily requires some form of irrigation and other uses of foliar contact water during the growing season (Pachepsky *et al.* 2011). Broadly taken, preharvest water use applies to irrigation and other crop management practices, including the application of pesticides,

fertilizers, nutrients, growth regulators, frost control aids, farm dust abatement and microenvironment management (Suslow 2010). Irrigation water, along with any other form of foliar applied water that will be in contact with edible portions of the crop, is considered a potential source for produce contamination, particularly if inadequate and microbiologically uncharacterized water sources are utilized.

Salmonella enterica is a leading cause of foodborne illness in the United States with over 70 produce-related outbreaks since 1950 (CDC 2011), and in recent years, its increasing attribution to illness and outbreaks linked to fresh produce consumption has become alarmingly evident (Hanning *et al.* 2009). In addition to several environmental factors that contribute to its persistence, irrigation water has been commonly found as a source of this micro-organism in several outbreaks (Hanning *et al.* 2009). Although the information is limited, microbiological assessment of irrigation water has demonstrated the prevalence of *Salmonella* in irrigation water (Duffy *et al.* 2005; Espinoza-Medina *et al.* 2006; Rajabi *et al.* 2011). As previously mentioned, irrigation source water may also be used for other crop production management practices, including the application of fertilizers and pesticides which are often reconstituted and diluted with water in the farm (Ng *et al.* 2005). As many of these agricultural products are applied in direct contact with plant and edible portions, often very close to harvest and thus limited time is available for pathogen death. The source and quality of water for reconstitution of such products is pivotal to prevent contamination with micro-organisms from water sources at preharvest stages.

Pesticides by themselves, in general, are not considered a source of microbial contamination (Ng *et al.* 2005) as they go through a strict quality control during their manufacture; however, once in suspension with water, microbial growth can occur. Previous studies have demonstrated that indigenous micro-organisms as well as other enteric pathogens such as *Salmonella* can survive and in some cases grow in several commercial pesticides (Guan *et al.* 2001, 2005; Ng *et al.* 2005; Stine *et al.* 2011), which could impact the microbial quality of the treated crops. Investigations from several outbreaks of foodborne pathogens, particularly *Salmonella enterica*, have traced the implicated strain to an irrigation water source, and in the investigations, it has been pointed out that those water sources were also utilized for reconstitution of pesticides (Herwaldt and Beach 1999; Horby *et al.* 2003; Greene *et al.* 2008; Mody *et al.* 2011). Although produce safety standards for water utilized in application of agricultural products to foliar surfaces typically specify potable sources as a best practice, specific data are often lacking as a foundation to behavioural change among producers. In this study, tomato was chosen as a crop due to its economic importance and its involvement in several *Salmonella* outbreaks (Cummings *et al.* 2001; Gupta and Crowe 2001; Gupta *et al.* 2007; Greene *et al.* 2008; Hanning *et al.* 2009). Specifically, the growth of *Salmonella enterica* was evaluated in different pesticide solutions, labelled for application to fresh market tomato production, to determine the influence on its growth and survival under different conditions of water quality and temperature as well as the impact on its survival during field tomato production and postharvest processing.

Materials and methods

Bacterial strains

Bacterial strains and inoculum preparation

Salmonella enterica sv Newport (PTVS73), Poona (PTVS026) and Michigan (PTVS42) selected for rifampicin resistance via spontaneous mutation and Typhimurium (aPTVS177) strains were used in this study. Strain aPTVS177 is an avirulent strain that lacks adenylate cyclase and cyclic AMP receptor protein rendering it avirulent yet still immunogenic (Hassan and Curtiss 1990). aPTVS177 is an antibiotic-resistant derivative strain from aPTVS150 for tolerance to rifampicin (80 mg l^{-1}), and it was isolated via spontaneous mutation, which facilitated detection and recovery and minimized interference with other background bacteria during field studies (Lopez-Velasco *et al.* 2012a). The use of aPTVS177 in field studies was approved by the Office of Environmental Health and Safety (EH&S) of the University of California, Davis.

All *Salmonella* serovars were cultured at 37°C for 18 h on tryptic soy agar (TSA; BD Diagnostics, Sparks, MD, USA), supplemented with 80 mg l^{-1} of rifampicin (rif, Fisher Scientific, Hampton, NH, USA) and 1 g l^{-1} of sodium pyruvate {$C_3H_3NaO_3$;(TSARP)}. Approximately, five colonies were resuspended in 5 ml of Butterfield's phosphate-buffered saline (BPBS) (Whatman Inc., Piscataway, NJ, USA). A total of 100 μl was spread onto TSARP and incubated for 18 h to allow the formation of a uniform confluent lawn. Cells were harvested by gently scraping the agar surface with a sterile rubber spatula, which was suspended in BPBS. Bacterial suspension was centrifuged at 1500 ***g*** for 10 min. The pellet was washed twice in BPBS and resuspended in BPBS to adjust the optical density at 600 nm of approximately 0·750, which corresponds to log 9 CFU ml^{-1}. The inoculum containing a cocktail of serovars Newport, Poona and Michigan was then diluted to the desired concentration for the inoculation of pesticide solutions and serovar Typhimurium (aPTVS 177) for the inoculation of irrigation water utilized to reconstitute pesticides to labelled application rates during field studies.

In vitro growth of *Salmonella enterica* in pesticide solutions influenced by temperature, water composition and *Salmonella* serovar

Pesticides were reconstituted from commercial formulation concentrates to approved labelled dose rates in non-sterile tap water, water supplemented with 10 ml l^{-1} of a sterile-filtered bovine serum solution (BSA 60 mg l^{-1} protein; Sigma, St. Louis, MO, USA) as a controlled organic bio-burden matrix, previously described by Park *et al.* (2009), or in surface irrigation water, collected from a typical irrigation district distribution canal within San Joaquin Valley region of California. . The concentration of the pesticide was established in accordance with U.S. Environmental Protection Agency (USEPA) and California Environmental Protection Agency (CALEPA) registration labelling (Table 1). Fifty millilitres, in triplicate, of the pesticide solutions was inoculated with log 2 CFU ml^{-1} of a cocktail of *S. enterica* sv. Newport, Poona and Michigan. In addition to association with tomato and cantaloupe outbreaks, these isolates could be readily differentiated by pulse field gel electrophoresis (PFGE). The individual strains of the cocktail were previously quantified to adjust the number of cells before inoculation. Inoculated pesticide solutions were incubated in the darkness at 10, 25 and 37°C. Quantification and colony confirmation of the *Salmonella* cocktail population was determined after 0, 24, 48, 72 and 96 h of inoculation by direct plating of 100 μl of the suspension on TSARP followed by incubation for 24 h at 37°C. Plates were further examined after returning to 37°C for an additional 20–24 h to observe for delayed new colony development. If necessary, 10-fold serial dilutions were prepared and held overnight at 2·5°C to quantify the viable population of *Salmonella*. Each treatment was repeated in triplicate. Additionally, a total of 115 colonies recovered after 96 h from pesticide solutions, which were resuspended in irrigation water and stored at 25°C, were randomly collected and analysed through pulse field gel electrophoresis (PFGE) to identify their unique band patterns and to determine the distribution of predominant surviving strains within the cocktail. The protocol for PFGE analysis was followed according to the PulseNet USA protocol (Ribot *et al.* 2006) using *Xba*I as restriction enzyme.

Survival of *Salmonella enterica* on tomato fruits during field production

After evaluation of the behaviour of *Salmonella* when suspended in different pesticides solutions, pesticides Radiant (CAS), Success (CAS), Cabrio (CAS), Admire (CAS) and a control which consisted of surface irrigation water without amendment, were chosen for either causing a neutral, inhibitory or stimulatory effect on Salmonella growth during *in vitro* studies. A total of 4 l of reconstituted pesticide solutions was prepared using irrigation source water that was first inoculated to achieve a concentration of log 3 CFU ml^{-1} of an attenuated strain of *S. enterica* sv Typhimurium (aPTVS177). A field trial was performed during summer-fall 2011 at the University of California Davis Plant Sciences Research Farm facility. The field consisted of five beads of 60 m in length each and 1·5 m of width from furrow-centre to furrow-centre. Tomato-seedling plants typical for CA for the production of fresh market tomatoes (var. Bobcat) were produced in the greenhouse, hardened for 10 days in an outdoor lath-house and transplanted to the field with a spacing of approximately 60 cm between each plant. When tomato fruits reached a Mature Green stage of development, inoculated pesticides solutions were applied using a tractor-mounted agricultural sprayer for crop management. Applications were made with twin fan-jet nozzles across one bed at a time

Table 1 List of commercial pesticides utilized in this study

Pesticide brand	Function	Active compound	% of active ingredient	Supplier	Concentration of reconstituted pesticide
Rally®40WSP	Fungicide	Myclobutanil	40	Dow AgroSciences LLC, Indianapolis, IN, USA	0·18 g l^{-1}
Admire®	Insecticide	Imidacloprid	42·8	Bayer Crop Sciences, Research Triangle Park, NC, USA	0·15 g l^{-1}
Asana	Insecticide	Esfenvalerate	8·4	Dupont™, Wilmington, DE, USA	0·06 g l^{-1}
Avaunt	Insecticide	Indoxacarb	30	Dupont™	0·08 g l^{-1}
Bravo WeatherStick	Fungicide	Chlorothalonil	54	Syngenta, Greensboro, NC, USA	1·8 g l^{-1}
Cabrio®EG	Fungicide	Pyraclostrobin	20	BASF, Florham Park, NJ, USA	1·20 g l^{-1}
Coragen®	Insecticide	Chlorantraniliprole	18·4	Dupont™	0·08 g l^{-1}
Intrepid2F	Insecticide	Methoxyfenozide	22·6	Dow AgroSciences	0·30 g l^{-1}
Radiant®SC	Insecticide	Spinetoram J&L	11	Dow AgroSciences	0·10 g l^{-1}
Ridomil	Fungicide	Mefexonam	45·3	Syngenta	0·6 ml l^{-1}
Success®Naturalyte®	Insecticide	Spinosyn A&D	22·8	Dow AgroSciences	0·31 ml l^{-1}
Sulfur	Fungicide	Sulfur	98	Loveland Products, Loveland, CO, USA	3·5 g l^{-1}

according to rates calibrated to pesticide supplier instructions. After application of each pesticide, the spray tank and lines were thoroughly rinsed and flushed to prevent carry-over contamination. Each bed was subdivided into four subplots for time interval sampling collection. Due to EPA- and CALEPA-labelled postapplication re-entry restrictions, sampling could be performed only after 3 days of pesticide application, and thus, sprayed mature green tomato fruits were collected after 3, 7 and 15 days postpesticide application. A total of five samples was collected from each subplot, and each sample consisted in three tomatoes that were aseptically placed on a sterile bag (n = 20 samples of three tomatoes per treatment).

Samples were taken to the laboratory and processed within 1 h after collection. A total of 50 ml of sterile potassium phosphate buffer (3·9 mmol l^{-1} KH_2PO_4 and 6·1 mmol l^{-1} K_2HPO_4) supplemented with 0·05% Tween 20 (Fisher, Fair Lawn, NJ) was added to the bag containing the three fruits. The skin of the tomatoes was vigorously rubbed manually to detach bacteria, and a total of 1 ml of the bacterial suspension was centrifuged at 3000 ***g*** for 2 min to concentrate the recovered cells, and 800 μl of the supernatant was discarded, and the remaining volume was utilized to resuspend the formed pellet. The pellet was plated on TSARP supplemented with 5 mg l^{-1} of pentachloronitrobenzene (TSARPP) (Amvac Chemical Co., Newport Beach, CA, USA) and incubated at 37 C for 24 h for quantification of the attenuated *Salmonella*. Additionally, the remaining cell suspension was combined with and equal double strength of buffered peptone water (2 × BPW) (BD Diagnostics) supplemented with 80 mg l^{-1} (2 × BPW/rif) and incubated at 37°C for 18–24 h for the enrichment of viable populations. A total of 40 μl of the resultant enrichments was spotted onto *Salmonella* differential and selective agar Xylose Lactose Tergitol 4 (XLT-4) (BD Diagnostics) supplemented with 80 mg l^{-1} of rifampicin (XLT-4/rif) and incubated up to 24 h at 37°C for culture recovery, confirmation and subsequent colony purification.

Survival of *Salmonella enterica* after pesticide application, harvesting and postharvest washing

After 3, 7 and 15 days of pesticide application to field grown on-vine tomatoes, a total of 10 tomatoes from each bed were collected along with 30 tomatoes that were neither inoculated nor treated with pesticides but had developed to the same maturity stage. Treated tomatoes were previously marked at harvest with indelible ink to identify them from nontreated fruits. A water bath containing 5 l of tap water with 50 mg l^{-1} of sodium hypochlorite was prepared to mimic commercial tomato washing in dump tank (Tomas-Callejas *et al.* 2012). The bath was adjusted at a temperature of approximately 42°C and the pH adjusted to 6·5 with citric acid and an oxidation-reduction potential (ORP) larger than 700 mV. Treated and nontreated tomatoes were dumped into the bath and manually agitated for 2 min. After that time, 4 ml of 1 N sodium thiosulfate (Sigma) was added to the bath to ensure total neutralization of free chlorine, previously determined to be sufficient to eliminate residual chlorine in this volume and un-reacted free chlorine with no tomatoes added (data not shown). During the washing time, ORP and pH were monitored using portable calibrated metres (Thermo Fisher Scientific, Waltham, MA, USA). After thorough washing, new bath solution was prepared for each pesticide-treated group. A total of five samples of two fruit each were collected from each group (treated or nontreated tomatoes were collected from each bath) and analysed for quantification and detection of *Salmonella* as described previously. Additionally, a total of 200 ml of water from each bath was collected after neutralization and enriched with 2 × BPW/rif. Confirmation of *Salmonella* was done as described earlier.

Statistical analysis

Data generated during *in vitro* studies was transformed from CFU ml^{-1} to log CFU ml^{-1} for normalization. All statistical analysis was performed using Statistical Analysis System (SAS. V 9.2; SAS Institute, Cary, NC, USA). Analysis of variance was performed following a factorial design using the GLM procedure followed by mean separation using Tukey's test comparison. Significant difference between treatments was accepted as established when the P-value was <0·05. A correlation matrix was calculated to establish significant correlation among the represented factors using the CORR procedure. For field trials, the presence of the attenuated *Salmonella* aPTVS177 was done through sample enrichment, and thus, the percentage of positive detection within each subplot and pesticide treatment was calculated. Percentages were compared using a generalized linear mixed model for categorical data using the GLIMIX procedure and Tukey for treatment separation to determine whether significant difference in *Salmonella* survival associated with different pesticide application had occurred (Lopez-Velasco *et al.* 2012a).

Results

Effect of water composition, time and storage temperature in the survival of *Salmonella* in pesticide solutions

Overall, the effect of water composition, temperature and time of storage as well as their respective two- and

three-way interactions were significant as related to the growth kinetics of *Salmonella* in the presence of approved concentrations of ag-chemicals commonly applied to fresh market tomatoes. In accordance with the pattern of behaviour outcomes, death-survival-growth, of *Salmonella* in the various pesticide solutions, the pesticides evaluated were classified in two major groups (Table 2). Group 1, into which most pesticides were assigned, corresponded to those formulations in which a slight positive growth of *Salmonella* was determined. For chemistries fitting this group, it was considered that although most pesticides supported the sustained viability of the introduced *Salmonella enterica* serovars they do not contribute to a significant growth potential (Fig. 1a,b). For this particular group, the effect of solution temperature fit a binomial model as the best correlative fit to predict the quantitative increase in *Salmonella*; larger populations were determined at 25°C compared with 10 or 37°C (Fig. 1b). In contrast, those pesticides classified within group 2 were able to maintain and support the growth of *Salmonella* during the duration of the study (Fig. 2). In this case, a direct correlation between growth and temperature was determined (Fig. 2b) with a 3–4 log-increase in the population, particularly when stored at 37°C. Additionally, the effect of the water constituent composition was also significant but the interaction with the temperature was the dominant factor. The growth of *Salmonella*, at 25°C, was greatest in irrigation water from a surface source as compared to BSA, and an overall sustained population, characterized as no net growth, was observed when suspended in tap water. At a holding temperature of 37°C, the effect of water composition was not significant.

In both groups, the storage at low temperature (10°C) caused a net decline in the population when pesticides were suspended in tap water and the population remained unchanged in both irrigation water and BSA solution. Additionally, the presence of organic matter in the solution provided by the diluent suspension media (BSA or irrigation water) favoured the maintenance and/or growth of *Salmonella* (Figs 1a and 2a) as populations determined in chloramine-neutralized tap water were always lower ($P < 0{\cdot}05$) regardless of the holding temperature.

The growth of *Salmonella* in the controls, which consisted of the different sources of water without the

Table 2 Description and classification of pesticides labelled for fresh market tomatoes in relation to the behaviour of a *Salmonella* cocktail when resuspended in water of different composition and stored at various temperatures

		Growth of *Salmonella* in pesticide solutions*		
Group classification	Pesticides	Effect of water composition	Effect of temperature	Correlation of growth with time of storage
0	*Water control*	Population did not change in BSA or irrigation water ($P > 0{\cdot}05$) Significant decline in population in tap water ($P < 0{\cdot}05$)	A quadratic polynomial fit the data ($P < 0{\cdot}05$) Larger populations were quantified at 25°C than at 10 or 37°C ($P < 0{\cdot}05$)	Population tended to decline but the correlation was no significant ($P > 0{\cdot}05$)
1	*Asana*[i] *Avaunt*[i] *Bravo*[f] *Coragen*[i] *Intrepid*[i] *Radiant*[i] *Ridomil*[f]	No significant difference between BSA or irrigation water ($P < 0{\cdot}05$) Significant decline in population in tap water ($P < 0{\cdot}05$)	A quadratic polynomial fit the data ($P < 0{\cdot}05$) Larger populations were quantified at 25°C than at 10 or 37°C ($P < 0{\cdot}05$)	At 10°C, negative correlation was determined ($P < 0{\cdot}05$) At 25°C, significant increase in population (0·7 to 1·5 log) At 37°C, no change in population or significant decline
2	*Rally® 40 WSP*[f] *Admire*[i] *Cabrio*[f] *Success*[i] *Sulfur*[f]	Larger populations in BSA or irrigation water compared with tap water ($P < 0{\cdot}05$) At 37°C, the effect of water composition was no significant ($P > 0{\cdot}05$) except for Rally® 40WSP	Positive correlation between temperature and population of *Salmonella* ($P < 0{\cdot}05$)	At 10°C, population is maintained or has a slight decrease ($P > 0{\cdot}05$) At 25 and 37°C, significant increase in population (up to 4 log)

*The growth of a cocktail of *Salmonella* was determined by direct plating. Data were analysed by the analysis of variance to determine the main effect of the three factors considered in this study; water composition (BSA, irrigation water and tap water), time (0, 24, 48, 72 and 96 h) and temperature (10, 25 and 37°C) of incubation. The two- and three-way interactions of the factors were also significant ($P < 0{\cdot}05$). Insecticide (i) and Fungicide (f).

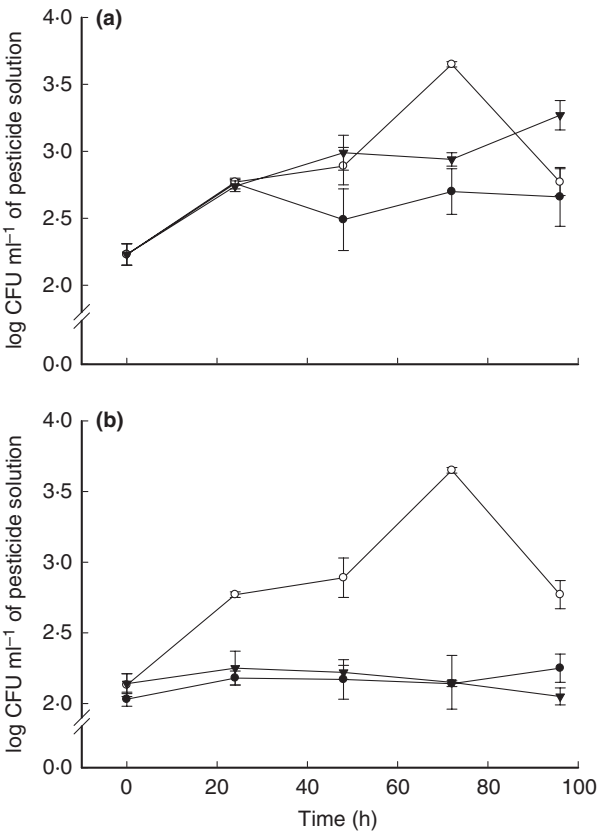

Figure 1 Effect of (a) water composition and (b) temperature on the growth of *Salmonella enterica* in pesticide solutions in group 1. Each point represents the mean and standard deviation of $n = 3$ independent replicates of the population of a cocktail of *S. enterica* in pesticide Avaunt. Figure (a) is a representation of the growth of *Salmonella* in different water sources incubated at a constant temperature of 25°C. Figure (b) is a representation of the growth of *Salmonella* at different incubation temperatures when pesticide was suspended in irrigation water. (a) (●) Tap water; (○) irrigation water and (▼) BSA. (b) (●) 10°C; (○) 25°C and (▼) 37°C.

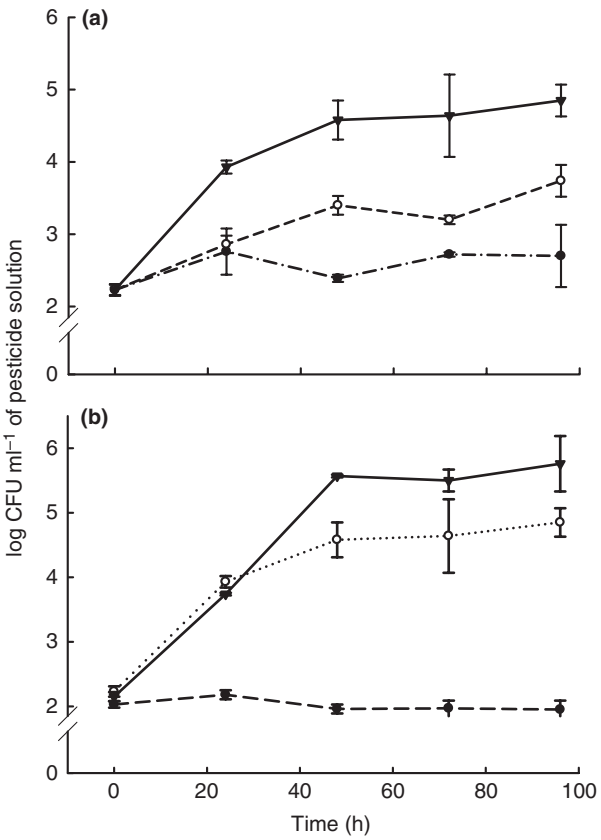

Figure 2 Effect of (a) water composition and (b) temperature on the growth of *Salmonella enterica* in pesticide solutions in group 2. Each point represents the mean and standard deviation of $n = 3$ independent replicates of the population of a cocktail of *S. enterica* in pesticide Cabrio. Figure (a) is a representation of the growth of *Salmonella* in different water sources incubated at a constant temperature of 25°C. Figure (b) is a representation of the growth of *Salmonella* at different incubation temperatures when pesticide was suspended in irrigation water. (a) (—●—) Tap neutralized water; (—○—) BSA and (—▼—) Irrigation water. (b) (--●--) 10°C; (--○--) 25°C and (—▼—) 37°C.

addition of the pesticides, showed a decline of population in tap water, in comparison with groups 1 and 2 in which the net population is maintained, while in BSA or irrigation water the population did not change. This indicates that the presence of organic matter, and potentially small amounts of dissolved inorganic nutrients, can favour the maintenance of microbial cells, although they do not seem to provide any significant nutrient source for growth. It seems likely that small incremental increases in population could be attributed to nonsynchronous division and completion of cell binary fission events or cryptic growth from minor residual nutrients in culture preparations. Additionally, it was observed that the presence of certain pesticides can favour bacterial multiplication (Group 2) or simply can contribute to cell maintenance (Group 1), but they do not seem to have a detrimental effect. Thus, important considerations about the water source, temperature conditions and duration in which suspended pesticides are held for field or glasshouse application are variables well worth controlling in production operations.

Genotyping of colonies obtained from the pesticides solutions stored at 25°C was determined after 96 h of incubation to determine whether differences in survival can be associated with a specific *Salmonella* serovar form among the three utilized for this study. Overall, serovar's Newport and Michigan were more prevalent than Poona which comprised approximately 15% of all the randomly collected isolates tested ($n = 115$ colonies) (Fig. 3). Although the prevalence distribution of each representative serovar differs depending on the pesticide (Fig. 3a) or the water composition (Fig. 3b), serovar Newport

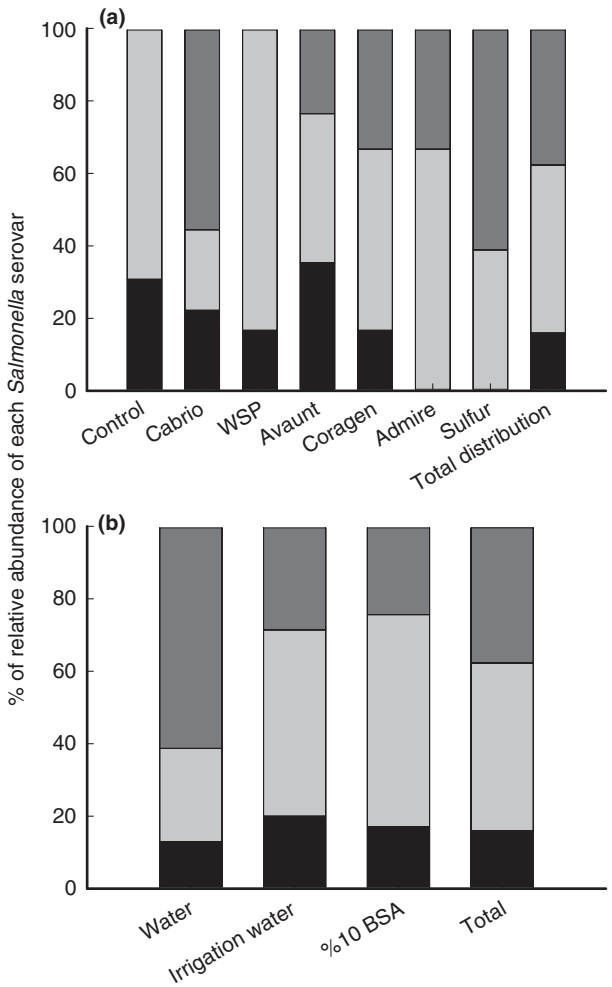

Figure 3 Distribution of different *Salmonella* serovars elucidated by PFGE, after incubation in pesticide solutions (a) by pesticide solution (b) by water source (n = 115 isolates). Isolates were randomly collected from TASRP plates after 96 h of incubation from pesticides suspended in irrigation water and incubated at 25°C. (▬) Poona; (▭) Newport and (▬) Michigan.

showed the greatest overall prevalence, including in the control solution, suggesting that differential genetic determinants allows a better fitness, or incrementally faster growth rate, under the conditions used in this study.

Survival of *Salmonella* on tomato surface after application through pesticide solutions

The survival of an attenuated strain of *Salmonella* on tomatoes was monitored after its application in the field when suspended in different pesticide solutions. Due to the low inoculation dose (approximately log 2 CFU per fruit), it was not practical to quantitatively determine the population of viable cells, and thus, all samples were analysed through qualitative enrichment culture. The percentage of positive samples recovered after 3 days of sampling was not significantly different between the four applied pesticides and the control (Fig. 4). After seven and 15 days of the application of contaminated pesticides, the recovery of the attenuated strain was significantly lower in the control, which consisted of irrigation water without pesticides, as well in the presence of *Admire* as compared to suspending cells in Cabrio, Radiant or Success. This indicates that the presence of certain pesticides could favour the survival and persistence of a contaminating Salmonella introduced with a source water of inappropriate microbiological quality. The recovery of the attenuated *Salmonella* during the time course of this field study, though displaying an overall trend of decline in the percentage of positive samples, was not significantly different indicating the ability to persist on the surface onc applied, even under conditions of high temperature, high UV flux and low RH present during the production season (Fig. 4). In the case of *Radiant* and *Admire* that were classified in groups 1 and 2, during the *in vitro studies* (Table 2), a significantly lower survival rate on fruit surfaces was determined for Admire than for all the other pesticides. This could indicate that the effect of individual pesticide interactions with *Salmonella* in application-dose solution concentration may not necessarily mirror of predict behaviour on plant or fruit

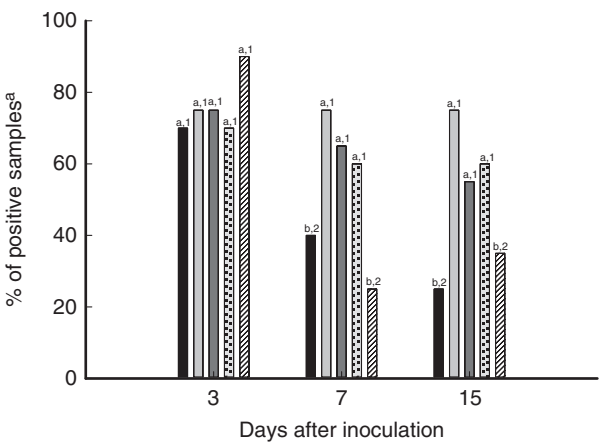

Figure 4 Detection of attenuated *Salmonella* on field-grown tomatoes after pesticide application in reconstituted contaminated irrigation water. [(a)]Results represent the average percentage of samples with positive detection of *Salmonella* after sample enrichment, the sampling consisted of a total of four subplots of five samples of three tomatoes each, for every pesticide treatment. Different letters denote significant difference ($P < 0{\cdot}05$) in the number of positive samples among pesticides. Different numbers denote significant difference among sampling days after inoculation ($P < 0{\cdot}05$). Statistical analysis was carried out using generalized linear mixed model for categorical data. (▬) Control; (▭) Radiant; (▬) Success; (▭) Cabrio and (▨) Admire.

surfaces. This outcome points out the importance of verifying *in vivo* studies under practical and relevant agroecoregion field conditions.

Persistence of *Salmonella* after postharvest washing

During the field studies, a subpopulation of tomatoes graded as Mature Green were collected during each assessment time interval (3, 7 and 15 days). Detection of *Salmonella* was carried out after washing inoculated and noninoculated tomatoes with 50 μg l^{-1} of sodium hypochlorite (from 12·5% stock solution) at pH 6·5. With exception of tomatoes that were treated with a contaminated solution of *Cabrio*, *Salmonella* was detected in 20–60% of the collected samples (Table 3), and in one occasion, it was present in all sampled tomatoes. Although it was not possible to establish a trend between the prevalence of *Salmonella* and the time of exposure to the inoculum in the field, it was possible to establish that once the fruit has been exposed to *Salmonella*, the postharvest washing might not be sufficient to completely eliminate this risk of viability at the point of consumption. In addition, when the likelihood of crosscontamination was evaluated, it was possible to detect the attenuated strain in two of 15 different water samples as well as in noninoculated tomatoes (Table 3). Thus, even if *Salmonella* is present in low prevalence among harvested fruit and small populations per contaminated fruit risk of crosscontamination through dump and flume tanks during postharvest management is an important focal point of control.

Table 3 Detection of attenuated *Salmonella* on field-grown tomatoes after pesticide application and postharvest washing with sodium hypochlorite

	Control	Radiant	Success	Cabrio	Admire
DPI	% of samples with positive detection of *Salmonella* (% of nontreated tomatoes that showed crosscontamination after washing)*·†				
3	20 (0)	40 (20)	0 (0)	0 (0)	0 (0)
7	20 (0)	60 (0)‡	100 (30)	0 (0)	40 (0)‡
15	0 (0)	0 (0)	60 (0)	0 (0)	40 (0)

*Results represent the total number of samples that were positives for *Salmonella* by enrichment after washing in a bath with 50 mg l^{-1} of sodium hypochlorite at pH 5·6. Average physicochemical parameters in baths were (pH 6·59 ± 0·17, ORP 721 ± 26·8 mV before dumping fruit and 639 ± 45·2 mV after 2 min washing and temperature of 41·8 ± 1·30°C).

†A total of 10 tomatoes collected from the field that were treated with contaminated pesticide solutions were mixed in the tank with 30 nontreated tomatoes.

‡Positive detection of *Salmonella* in water samples from each bath (n = 2).

Discussion

Pesticides and other foliar applied materials are often aqueous formulations chemical preparations that are widely used in the cultivation of fruits and vegetables and the microbiological quality of the source water is an important food safety consideration (Suslow *et al.* 2003; Suslow 2010). Their application in the horticultural food industry is of great importance in crop management, although several risk associated with their exposure to human health are often discussed. In addition, pesticides have been questioned for their contribution to an increased microbiological risk during the produce production supply chain (Guan *et al.* 2001; Ng *et al.* 2005; Olaimat and Holley 2012), this is mostly attributed to the use of nonpotable or agricultural water that is of inappropriate microbiological quality (Guan *et al.* 2001; Stine *et al.* 2011) and the fact that bacterial contaminants in water can exhibit significant growth in some pesticide preparations (Ng *et al.* 2005). In some regions, agricultural source water, sometimes inappropriately used for foliar treatments very temporally near harvest, has a very high prevalence of contamination with *Salmonella enterica* (Rajabi *et al.* 2011). In this study, pesticides used for application in fresh tomato production and resuspended in water contaminated with a cocktail of different *Salmonella* serovars were evaluated to determine their capacity to permit survival and growth of *Salmonella* using a multifactorial approach and extending this assessment to the further postapplication survival during field tomato production.

Based on the behaviour of the introduced *Salmonella*, evaluated pesticides were classified into two groups; group 1 that includes pesticides in which only seem to support the initial population of *Salmonella* (log 2 CFU ml^{-1}) and group 2 in which significant growth was determined (Table 2). In the two groups, the storage temperature or the pesticides formulations as well as the water composition used for resuspension had a major effect on the net outcome of effects on populations of *Salmonella*. The results support the findings reported by Guan *et al.* 2001 in which the growth of *E. coli* O157:H7, *Salmonella, Shigella, and Listeria* were also capable of growth in selected pesticides. Other studies have showed that the growth of native bacteria present on irrigation water can be stimulated and support by the presence of some pesticides (Ng *et al.* 2005). Therefore, the presence of ag-chemicals, the water composition and the conditions in which these solutions are held during use can significantly contribute the potential growth of human

pathogens. In this particular case, we were able to determine that the presence of dissolved or suspended constituents, including organic matter, present in the irrigation water or by addition of BSA favoured the growth *Salmonella* when compared to its growth in neutralized tap water (Table 2 and Table S1). *Salmonella* is known for having high survival rates in aquatic environment (Winfield and Groisman 2003) and several studies have shown survival in water of different origin including municipal water (Wang *et al.* 1996; Santo Domingo *et al.* 2000; Ng *et al.* 2005). This could be attributed to the presence of minimal amounts of nutrients in water, cryptic growth or attachment to suspended particles, which allow the population to be maintained. In addition, the presence of pesticides, even at labelled doses, could also play a role in its survival. Whether the source of growth was attributed to the specific pesticide active ingredient or the nondisclosed "inert ingredients" of the formulation was not determined. Several studies have found that pesticides can be degraded by microbial activity and the break down products metabolized as a unique sources of nitrogen, carbon or sulfur (Aislabie and Lloyd-Jones 1995). Although specific information about *Salmonella* in relation to pesticide utilization is limited, the possibility of acquisition of genes through transposable elements or plasmids in the environment to utilize novel compounds should not be excluded. In contrast, there is also evidence that pesticides have the ability to inactivate micro-organisms such as *Escherichia* coli (Pham *et al.* 2004), however, that should only be considered as a secondary effect based on the design of this study and previously cited research.

The presence of inerts, which are part of pesticides formulations, could also participate in the growth of bacteria when they are resuspended (Hochbert 1996; Guan *et al.* 2001; Ng *et al.* 2005). Inerts could be emulsifiers, surfactants, clays etc. that could offer attachment surfaces as well as nutrients (Hochbert 1996), and therefore, they could also favour the growth in pesticides. In this study, pesticides from group 1 : 40 WSP, Cabrio, Success and Sulfur, in which *Salmonella* showed a significant growth, corresponded to pesticides that are provided in a solid or semisolid state, and thus, it is likely that suspended particles could provide sites for physical or chemical attachment or nutrient sources that favoured their growth (Fig. 2a,b).

The effect of the storage temperature was also studied (Figs 1b and 2b). Pesticides manufacturers recommend that pesticide solutions to be applied within 24 h once, and they are reconstituted; however, a 7-day maximum may also be stated (Guan *et al.* 2005), and thus, storage conditions once in resuspension are important risk factors. Although it is not a common practice to hold resuspended pesticides in application tanks, it is a frequent anecdotal comment among growers and empirical observation (Suslow, pers. obs.) that tank-mixes are left out in the field or orchard where high summer temperatures can be reached, or residual materials left in tanks and spray lines and may promote conditions for pathogen multiplication. Under conditions of inadequate daily cleaning and sanitation practices residual contaminated solutions can effectively inoculate and contaminate a new lot of incoming foliar material, perhaps this time from a clean well or municipal source rather than surface water.

For all pesticides tested, storage at 10°C resulted in a net decline in population, which could be attributed to death or loss of viability under these conditions, thus holding resuspended ag-chemicals at low temperatures if application extends over more than several hours carries a lower anticipated risk. Of all pesticides tested, an increase in temperature to 25°C allowed the growth of *Salmonella*, however, for pesticides classified in group 1 (Fig. 1a,b), a further increase in temperature to 37°C cause the decline of the population of recoverable *Salmonella*. This effect has been previously observed for other pathogenic bacteria when pesticides were resuspended in saline solution and stored at 30°C (Guan *et al.* 2005). Likely an increase in temperature could promote an initial trigger for microbial growth, however, due to low nutrient availability and presumptive stressful conditions associated to the presence of pesticides, *Salmonella* cannot adapt to these conditions and rapidly exhaust available resources for multiplication and either die or transition to viable but not culturable (VBNC) state (Dinu *et al.* 2009).

Salmonella cells that survived storage in pesticide solutions at 25°C were collected after 96 h and subjected to genotyping to distinguish among the three serovars present in the initial inoculum (Fig. 3). Differences between the three serovars indicate that serovar Newport and Michigan, originally isolated during tomato outbreak and from irrigation water, respectively, seem to have better fitness than serovar Poona when inoculated in water as well as in pesticide solutions. Differential serovar survival and fitness in several studies suggest that some response mechanisms rather than a passive establishment favour the survival of particular micro-organisms (Guan *et al.* 2005; Tomas-Callejas *et al.* 2011; Lopez-Velasco *et al.* 2012b).

Once the likelihood of cell maintenance or growth of *Salmonella* in pesticide solutions was determined, the survival of low levels attenuated *Salmonella* (log 2 CFU ml^{-1}), applied through pesticide spraying, was evaluated on tomato surfaces during field production (Fig. 4) and after postharvest processing (Table 3). Currently, they are few options for intentional inoculation of demonstrably avirulent strains of Salmonella in an open

environment field experiment. The isolate selected, aPTVS177, and approval by the UCD IBC relied on the detailed stability and reversion frequency studies reported by Curtiss and Kelly (1987). While restoration of CRP-cAMP-mediated metabolic phenotypes was noted at very low frequency, these revertants retained their avirulence in mice inoculations with high cell doses. Though selections of other stable and environmentally fit virulence-attenuated isolates of Salmonella are being developed, aPTVS177 was considered acceptable in both biosafety and fidelity to wild-type behaviour in survival and persistence for field trials.

During field production, the number of tomatoes that tested positive for the presence of *Salmonella,* though tending to decline, it was not significant different after 15 days of inoculation. Other studies also observed that not significant differences in net population of *Salmonella* were present when inoculated in tomato plants indicating that once established the levels of the microorganism are mostly constant (Barak *et al.* 2011). A significant difference was determined between those tomatoes treated with pesticides and the control group that consisted of irrigation source water alone. This may suggest that the presence of pesticides could favour the survival of *Salmonella,* which is not necessarily related to the presence of the pesticide but to other inert carries in the commercial formulation as mentioned previously (Hochbert 1996). These observations are different from those reported by Guan *et al.* (2005), in which *E. coli* and *Salmonella* survived longer on tomato plants when sprayed in saline solution than when sprayed with the specific pesticide Bravo, which could be attributed to a different behaviour dependent on the applied pesticides or the *Salmonella* isolate. Compared with *in vitro* experiments performed in this study, tomatoes treated with Admire had fewer tomatoes that tested positive for *Salmonella* than Radiant, a material that did not support the growth during *in vitro* studies. This suggests that the behaviour of pathogens could be influenced by the differences in the matrix utilized (liquid suspension vs solid surface). However, the attenuated Typhimurium strain was also utilized to determine its growth and survival as described for the *Salmonella* cocktail and it showed a similar behaviour during *in vitro* assays (data not showed). Thus variations are not necessarily associated with the applied *Salmonella* serovar.

After application of contaminated pesticides and further exposure to field conditions, tomatoes were subjected to postharvest washing and disinfection with sodium hypochlorite (Table 3). *Salmonella* persisted on tomatoes after postharvest washing, and surviving cells were able to cause crosscontamination to noninoculated tomatoes in two of the 15 different baths prepared for this experiment. Although the application of a disinfectant agent is necessary to minimize the risk of crosscontamination (United Fresh 2008), washing with 50 mg l^{-1} of chlorine was not sufficient to eliminate the presence of *Salmonella* on the surface, and actually, current water disinfection procedures does not ensure a reduction in the microbial load (Tomas-Callejas *et al.* 2012). Collectively, the outcomes of this study further support the pivotal importance of preventing contamination from the early stages of crop production, and especially, close to harvest.

Conclusions

Application of certain pesticides after reconstitution in contaminated water can allow persistence or will support the growth of *Salmonella*, which may elevate the risk, beyond that of the water source alone, of subsequent foodborne illness as a result of foliar contact applications. Ensuring and verifying the use of appropriate water sources for suspension of ag-chemicals, including pesticides or any other crop production materials that might be directly applied or come in contact with the edible parts of horticultural foods, typically or characteristically consumed without cooking, should be carefully considered within a comprehensive food safety plan.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 Effect of incubation temperature and water composition in the growth kinetics of *Salmonella enterica*.