CENTER for PRODUCE SAFETY

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FINAL PROJECT REPORT

Project Title
Occurrence and accumulation of potentially infectious viruses in process water and impact of water disinfection practices to minimize viral cross-contamination

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Objectives

1. *Detection and quantification of potentially infectious enteric viruses and coliphages in process water used from industrial partners*
2. *Inactivation studies to evaluate the efficacy of chlorine and non-chlorine based sanitizers on human enteric viruses and coliphages*
3. *Validation of the established water disinfection practices for enteric viruses and coliphages in commercial facilities – to be carried out on-line in three disinfectant-produce combinations (i.e., leafy greens and sodium hypochlorite, peppers and PAA and tomatoes and ClO2)*
4. *Establishment of coliphages as a suitable indicator of enteric viruses in commercial facilities.*

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FINAL REPORT

Abstract

The presence of human enteric viruses in produce has been reported extensively. However, the role of the virological quality of process wash water (PWW) used by the produce industry in the produce contamination has received limited attention. As a first step to overcome technical limitations in monitoring viruses in PWW, the analytical performance of a dead-end hollow fiber ultrafiltration method was assessed to concentrate viral particles from 20 L of spiked PWW. The selected method used for virus concentration of PWW was carefully validated, thus enabling the accurate quantification and estimation of viral titres of human enteric viruses and phages by molecular techniques and plate assays, respectively. Furthermore, PWW samples from the whole and fresh-cut fresh produce industry were collected periodically from the washing tanks of commercial facilities. The occurrence of crAssphage (cross-assembly phage) and coliphages and their relationship with human enteric viruses were determined. The analysis of somatic coliphages and F-specific RNA phages was performed by plaque assay, while occurrence of enteric viruses and crAssphage was determined by molecular techniques. Significant differences were observed in the physicochemical composition of PWW, mostly because of the different nature of the type of fresh produce and the differences in the sanitizer used in commercial operations.

Furthermore, this project aimed to explore the antiviral disinfection efficiency of chlorine (free chlorine, FC), chlorine dioxide (ClO_2) and peracetic acid (PAA) at regulated operational limits in PWW using cultivable norovirus surrogates, including murine norovirus (MNV), Tulane virus, and hepatitis A virus (HAV), and MS2 phages. Defined commodity representative crops (leafy greens, bell peppers, and veggie mix) associated with specific water-based processes were tested using batch experiments and a dynamic system (DS) that allows performance of experiments incorporating an inoculum of a mix of human enteric viruses and coliphages as well as the constant entrance of disinfectant solution and organic matter to simulate the conditions of a fresh produce washing tank.

Batch-scale experiments showed that 20 ppm FC and 3 ppm ClO_2 completely inactivated norovirus surrogates and MS2 within one min regardless of the type of PWW assayed. However, infectivity of HAV was reduced by less than 2 log after 1 min, with complete inactivation not observed within 10 min in both cases, FC and ClO_2. In contrast, residual viral infectivity was observed following 80 ppm PAA treatments despite ca. 2 log reductions that were measured in veggie mix for norovirus surrogates.

In the DS, treatments with 5 ppm FC and 2-3 ppm ClO_2 avoided the accumulation of MS2 below the detection limit for the entire duration of the tests (60 min). As observed in the batch experiments, the addition of 80 ppm PAA was unable to prevent the accumulation of MS2 and MNV although 2 log reductions were observed in baby leaves and bell pepper PWW. Selected data from batch experiments (Tulane infectivity cell culture assays) were fitted using different types of nonlinear regression models. In the case of PWW treated with FC and ClO_2, data were adjusted using an asymptotic regression model. On the other hand, for PWW treated with PAA, data were adjusted using an exponential decay model. To characterize the inactivation kinetics in the DS, modeling studies were performed to mechanistically describe the inactivation performance of FC, ClO_2 and PAA operated on simulated washing tanks with three types of PWW from commercial industrial plants. In the case of PWW treated with FC and ClO_2 as well as the control conditions without sanitizers, data were adjusted using Chick (first order reaction) model, while for PWW treated with PAA, the Chick model was modified to include the loss of effectiveness when COD concentration increased.

Minimum operational limits identified in the lab-scale trials were validated in the industry processing lines. Trials were performed for baby leaves as well as bell pepper PWWs. The validation studies confirmed the efficacy of the selected treatments. Finally, data was analyzed with the aim of establishing the suitability of total and F-specific RNA phages as well as crAssphage, as potential indicators of human enteric viruses in commercial facilities. Accumulation of crAssphage and coliphages was observed in PWW obtained from industrial processing lines, but correlation with human enteric viruses was not possible due to the low prevalence of these pathogens in the PWW analyzed. More research is needed to establish any potential correlation and threshold value for these indicators.

Background

Despite accounting for one of the major causes of foodborne illness outbreaks in high-income countries, human enteric viruses have received comparatively less attention than other foodborne pathogenic bacteria. Human enteric viruses are the most common etiologic agents identified in produce-associated outbreaks (54%), frequently linked with food-handling issues (Bennett et al., 2018). The presence of human enteric viruses in irrigation waters has been extensively reported (Ashbolt, 2015; López-Gálvez et al., 2016; Randazzo et al., 2016; Tian et al., 2017; Li et al., 2018; López-Gálvez et al., 2018; Truchado et al., 2021). Among others, the viruses most commonly detected in irrigation waters include human norovirus, astrovirus (HAstV), rotavirus A (RV), and hepatitis A virus (HAV). Moreover, the USA included norovirus in the list of water contaminants that need to be regulated in drinking water (US EPA). Physical and chemical parameters, together with classical microbial indicators such as fecal indicator bacteria (FIB), including fecal coliforms, *Escherichia coli*, and enterococci, have been widely used to assess the quality of process wash water (PWW) used in different postharvest unit operations. However, the presence of human enteric viruses has not been fully implemented for this purpose.

Process wash water has been defined as water resulting from washing raw materials, rinsing water, or water used for cooling or transport, which usually accumulates organic matter including microorganisms (Suslow, 1997). Process water in the fruit and vegetable sector is highly variable in terms of quality parameters, such as dissolved solids, chemical oxygen demand, and microbiological quality. This fact makes it a challenge to implement a standard treatment fit for all purposes. The occurrence of potentially infectious enteric viruses in PWW used by the fresh produce industry is likely possible and thus, it needs to be closely examined. Several considerations must be taken into account to address this issue, **i)** relatively low levels of human enteric viruses introduced will be randomly distributed into large volumes of water and may not be detectable using protocols that include small volume collection, **ii)** sampling points in commercial facilities are critical for pathogen detection (Kearns et al., 2019), **iii)** molecular-based methods, currently used for human enteric virus detection in food (ISO 15216-1:2017) cannot discriminate between inactivated and potentially infectious enteric viruses, and **iv)** organic fresh produce market has tremendously increased in the last years, and the food safety perception of consumers must be assured.

Due to the difficulties associated with direct detection of pathogens in water, bacteriophages infecting enteric bacteria, such as coliphages, have been suggested as a viral indicator in irrigation water because they mimic viruses better than any other group of indicators showing moderate resistance to treatments and persistence in the environment (Agulló-Barceló et al., 2016). Coliphages are viruses that have been used as viral indicators and can infect *E. coli*. They are split into two categories based on the route of bacterial host infection: somatic coliphages and male-specific (F+) coliphages (F-RNA & F-DNA) (McMinn et al., 2017). Recently, crAssphage (cross-assembly phage) has been suggested as a novel viral indicator of fecal contamination, as

it presents in a high abundance compared to human enteric viruses. Recent data indicate that crAssphage could be used to detect human fecal contamination on environmental surfaces and hands (Park et al., 2020). However, the usefulness of this indicator in PWW is still unknown.

Monitoring and maintaining the quality of PWW during postharvest operations is considered important for the safety of end-products. In this project, we monitored the occurrence of most relevant human enteric viruses, coliphages and crAssphage in PWW collected from three different handling and processing plants of whole and fresh-cut fruits and vegetables over six sampling times from July to December 2020 and December 2021 to January 2022. To monitor PWW, a rapid, user-friendly, and reliable protocol to concentrate human enteric viruses, as well as bacteriophages, in large PWW volumes was initially developed. Importantly, limits of detection were established using model human enteric viruses and MS2 phage.

Moreover, this project aimed to evaluate the efficacy of current water disinfection practices to minimize viral cross-contamination. Thus, we investigated the inactivation of infectious viruses and MS2 phage in response to chlorine (free chlorine, FC), chlorine dioxide (ClO_2) and peracetic (PAA) at established operational limits for the disinfection of different types of PWW (i.e., baby leaves, bell peppers and vegetable mix) to avoid cross-contamination of the fresh produce. To evaluate the inactivation rates of viruses exposed to sanitizers in PWW, our approach included batch-scale and dynamic system (DS) experiments. Finally, batch infectivity data sets and DS data were used to develop predictive viral inactivation models for the implementation of disinfection strategies in produce industry settings. Validation of the minimum operational limits were done in selected processing lines.

Research Methods & Outcomes and Accomplishments

Objective 1: Detection and quantification of potentially infectious enteric viruses and coliphages in process water used from industrial partners

Methods

Viruses, phages, cells and bacteria

Feces positive for norovirus GI, norovirus GII, and HAstV (courtesy of Dr. Buesa from Hospital Clínico Universitario, University of Valencia, Spain) were resuspended (10%, wt/vol) in phosphate-buffered saline (PBS) containing 2 M $NaNO_3$ (Panreac), 1% beef extract (Conda), and 0.1% Triton X-100 (Fisher Scientific) (pH 7.2), vortexed and centrifuged at 1000 ×g for 5 min. The supernatant was stored at −80°C in aliquots. Mengovirus was used as the process control for sample concentration validation (Randazzo et al., 2019). The cytopathogenic HM-175 strain of HAV (ATCC VR-1402) and the human RV strain Wa (ATCC VR-2018), and mengovirus vMC0 (CECT 100000) were propagated in FRhK, MA-104, and HeLa cell monolayers, respectively. Semi-purified stocks were then produced in the same cells by low-speed centrifugations of infected cell lysates (3000 ×g for 20 min). The *E. coli* strains CECT 9198 and CECT 5695 were obtained from the Spanish Type Culture Collection and used for the quantification of total and F-specific RNA phages, respectively. Wild-type MS2 bacteriophage DSM 13767 was obtained from the German Collection of Microorganisms and Cell Cultures.

Physicochemical properties of process wash water

The pH, oxidation reduction potential (ORP, mV) and electrical conductivity (EC, µS/cm) were measured using a pH and redox multimeter (Crison). Organic matter was measured as chemical oxygen demand (COD, mg/L) determined by the standard photometric method (APHA, 1998) using a Spectroquant NOVA 60 photometer. The turbidity was measured with the Turbiquant®

from Merck and expressed in nephelometric turbidity units (NTU). Sanitizer concentrations were determined by using a Kemio™ instrument (Palintest), that uses an electrochemical technique (known as chronoamperometry) and Kemio™ sensors to measure FC, ClO_2 or PAA in the water samples. For quenching the disinfectant residuals, sodium thiosulfate (Panreac) was used for FC and ClO_2, and a mix of sodium thiosulphate and catalase was prepared in phosphate buffer (Merck) for PAA.

Concentration procedure

Process wash water from washing shredded lettuce was generated in the laboratory, mimicking the industrial conditions described previously (Tudela et al., 2019). Briefly, to generate the PWW, lettuce heads were obtained from a local supermarket, cut into 6 mm pieces after outer leaves were manually removed, and washed in tap water. A total of 6 kg of cut lettuce was washed in 8 L of tap water. The PWW obtained had similar physicochemical characteristics to those of commercial processing lines. Then, PWW was artificially inoculated with the MS2 bacteriophage (10^6 plaque-forming units PFU/mL), a single-stranded RNA virus, and mengovirus to evaluate the performance of viral concentration methods. For the primary concentration of viruses, a volume of 20 L of process water was processed by dead-end hollow-fiber ultrafiltration (DEUF) using single-use Asahi Kasi REXEED 25A filters (Cuevas-Ferrando et al., 2020, 2021). To recover the viruses, the filter was backflushed using 500 mL of backflush solution (0.01% Tween 80, 0.01% sodium polyphosphate and 0.001% antifoam). The backflush volume was concentrated using polyethylene glycol (PEG) precipitation and the final concentrate was used for the extraction of viral RNA. Then, mengovirus was quantified by RT-qPCR according to ISO 15216-1:2017, and MS2 was enumerated using the host strain *E. coli* CECT 9198 and the double layer agar method. In parallel, 1 L of artificially inoculated PWW was concentrated using an aluminum hydroxide adsorption-precipitation method (Randazzo et al., 2019).

Detection limit of enteric viruses and MS2 in process wash water

Process wash water (20 L) from a leafy green line was collected 4-5 h after the process started and transported to the lab. Once in the lab, PWW was characterized as previously mentioned. Free chlorine (FC) and total chlorine (TC) levels were determined by the DPD method using the Spectroquant NOVA 60 photometer (Merck) and the corresponding test kits (APHA, 1998). Combined chlorine (CC) values were calculated by the differences in the measurements between TC and FC. In the case of residual concentrations of the disinfectants present in the PWW, as mentioned before, a solution of sodium thiosulphate pentahydrate (Scharlau) was used for quenching the disinfectant residuals. Process water was then artificially inoculated with different ten-fold serially diluted concentrations (starting from 6 $\log_{10}$ IU/ 20 L) of norovirus genogroup (GI), norovirus GII, and rotavirus, establishing the detection limit and recoveries of the procedures. In addition, mengovirus was used as process control. Primary virus concentration was performed using DEUF with Rexeed-25A filters.

Viral extraction, detection and quantification

Nucleic acids from each concentrated PWW were extracted following the NucleoSpin® RNA virus kit (Macherey-Nagel GmbH & Co.) manufacturer's instructions with some modifications. In short, 150 µL of each concentrated sample was added with 25 µL Plant RNA Isolation Aid (Ambion) and 600 µL of lysis buffer from the NucleoSpin® RNA virus kit and subjected to pulse-vortexing. Then, the homogenate was centrifuged for 5 min at 10,000 ×g for debris removal. The supernatant was subsequently processed according to the manufacturer's instructions. The presence of norovirus GI and GII, HAV, HAstV, RV and mengovirus was detected in 96-well plates using the RNA UltraSense One-Step kit (Invitrogen SA), whilst crAssphage occurrence was resolved through qPCR Premix Ex Taq™ kit (Takara Bio Inc). For both RT-qPCR and qPCR assays, LightCycler® 480 instrument (Roche Diagnostics) was used for amplification and detection analysis. Moreover,

undiluted and ten-fold diluted nucleic acid were tested to check for inhibitors. Different controls were used in all assays, including negative process control consisting of PBS, whole process control to monitor the process efficiency of each sample (spiked mengovirus), and positive and negative RT-qPCR controls. Primers, probes, and RT-qPCR conditions used in this study are listed in **Table 1**. Standard curves were determined according to the Public Health England (PHE) Reference Materials for Microbiology for norovirus GI (batch number 0122-17), norovirus GII (batch number 0247-17) and HAV (batch number 0261-2017) and reported as genomic copies (GC), while standard curves for RV, mengovirus, and HAstV were generated by amplifying ten-fold serial dilutions of viral suspensions in quintuplicates and calculating the number of PCR units (PCRU). Standard DNA material for crAssphage standard curve generation relied on a customized gBlock gene fragment containing target sequences for crAssphage (Integrated DNA Technologies) (Stachler et al., 2017). Standard DNA material for crAssphage standard curve generation relied on a customized gBlock gene fragment (Integrated DNA Technologies) containing target sequence for CPQ_064 crAssphage primers set (Stachler et al., 2017).

Types of process water analyzed

Each of the three industrial collaborators, including two vegetable processors of baby leaves and veggie mix and a handler and packer of bell peppers, were visited six times from July to December 2020. Free chlorine was used as a sanitizer for baby leaves, while PAA was used for washing peppers, whereas no sanitizer was used either for the pre-washing of peppers or the washing of veggie mix. A volume of 20 L of PWW from the pre-washing and washing tanks was collected 4-5 h after the production started and transported to the lab in less than 45 min. Once in the lab, PWW was characterized as previously described. Samples of PWW were concentrated and analyzed for coliphages, human enteric virus, and crAssphage detection and quantification as previously described.

Objective 1 Outcomes and Accomplishments

Optimization of the concentration procedure of process water for enteric virus detection

The surveillance of PWWs for the presence of human enteric viruses requires procedures sensitive enough to detect the low levels of viruses expected. Initially, two different approaches previously used for human enteric virus detection in wastewaters were compared using PWW. Mengovirus mean recoveries in PWW ranged from 23.73±0.21% in the DEUF procedure using 20 L of PWW to 23.56±0.53% in aluminum precipitation using 1 L of PWW, which are slightly higher than the mengovirus recovery rates using Rexeed 25AX ultrafiltration reported previously in tap water, seawater and surface water (Cuevas-Ferrando et al., 2020, 2021a). In the case of MS2, mean recoveries were 31.7±11.5 and 47.9±19.5 for DEUF and aluminum precipitation, respectively. In light of those results, the DEUF protocol was selected to determine the detection limit due to the higher volume able to be processed (20 L versus 1 L). This is a tremendous advantage in samples expected to contain low concentrations of pathogens, as well as to recover viable phages.

Determination of the detection limit in PWW for enteric viruses and MS2

One major limitation in determining the virological quality of PWW is the lack of standardized and validated methods. Thus, the detection limits of human enteric viruses and MS2 in PWW were examined through the analysis of serial diluted spiked samples. Primary virus concentration was performed using DEUF with Rexeed-25A filters, resulting in an average eluate volume of 609.25 ± 60.40 mL. DEUF ultrafiltration combined with secondary PEG precipitation resulted in the mean recovery of 27.1%, 27.3%, 36.6% and 39.0% for norovirus GI, GII, rotavirus and MS2. An average recovery rate of 19% for mengovirus was achieved, which meets the quality control requirements of standardized methods. The ISO 15216-1 requires 1% recovery of the process control and

Method 1615 from the US Environmental Protection Agency (EPA) allows 5–200% recoveries for the process control for the concentration of environmental and drinking water samples for the quantification of human enteric viruses. The LoD95% of the procedure in PWW calculated according to Wilrich and Wilrich, (2009) was 1.1 $\times 10^3$ gc/L, 1.7 $\times 10^3$ gc/L, 4.3 $\times 10^2$ gc/L and 2.6$\times 10^2$ pfu/L for norovirus GI, GII, rotavirus and MS2, respectively, reporting a similar performance of 6.2 $\times$ 10^3 gc/L when the method was applied for HEV in drinking water (Cuevas-Ferrando et al., 2020). Farkas et al. (2018) reported LoD of 50 gc/L for norovirus using a similar procedure; however, validation was performed using deionized water only (Farkas et al., 2018).

Occurrence of coliphages and human enteric viruses in process water

Three commercial processing plants, including two vegetable processors of baby leaves and veggie mix and a handler and packer of bell peppers, were visited six times from July to December 2020. The physicochemical characteristics of the PWWs are shown in **Table 2**.

For baby leaves, the mean ratio of produce/wash water was 1.6 kg per liter of water. The concentration of FC was maintained between 0-59 mg/L and 3-125 mg/L in the pre-washing and washing tanks, respectively. The pH of the chlorinated water was 8.5, which was higher than the recommended one (pH=6.5) to reach the maximum concentration of hypochlorous acid when sodium hypochlorite is used as a water disinfectant. This means that the residual FC concentration with the maximum antimicrobial capacity would be lower than that expected at the optimum pH. The ORP was higher than 650 mV, indicating that there were reactive oxidizing species, except in one sampling where no FC was present and the ORP was lower than 650 mV (463 mV). The content of organic matter was low (188 and 75 mg/L maximum in the pre-washing and washing tanks, respectively), as were UV254 absorbance and turbidity. When the levels of total and F-specific RNA coliphages were determined in the pre-wash and washing PWW of baby leaves, it was observed that no recovery occurred of either total and F-specific RNA coliphages in any of the pre-wash or the washing tanks. However, detection of crAssphage was observed in 50% of the samples (**Table 3**). The absence of viable phages in these samples indicates that detection of crAssphage by qPCR was most likely targeting crAssphage DNA traces rather than viable phages. Furthermore, the absence of viable phages was probably due to the residual FC level even though the pH was higher than the recommended.

For bell peppers, the mean ratio of produce/wash water was 71 kg per liter of water, which is an extraordinarily low volume of water for a considerably high amount of product. This fact influenced the physicochemical quality of the pre-washing water with no PAA as well as the washing water with PAA (**Table 2**). Peracetic acid concentration was very high, with mean values of 334 mg/L vs. 80 mg/L, the recommended one. We observed a very high COD, with a mean value of 455 and 1490 mg/L in the pre-washing and washing tanks, respectively. In the pre-washing water, the EC was 743 µS cm^{-1} and the turbidity 392 NTU. In the PWW obtained from the washing tank, the EC was 765 µS cm^{-1} and the turbidity 159 NTU. These physicochemical characteristics influenced the UV254 absorbance, showing high levels (1.4 Abs) and low redox potential. When using PAA, the ORP is generally lower than 650 mV, opposite to what is found in chlorinated water (> 650 mV). It is remarkable to mention the high turbidity of the pre-washing water, due to the high amount of peppers washed in the same volume of water without any water replenishment and the consequent accumulation of organic and inorganic residues from field contamination of the product surface. High levels of coliphages, total, and F-specific RNA, of about 4 log pfu/L were found in the pre-washing tank (**Table 3**). The high levels of coliphages in the pre-washing water were probably due to the absence of sanitizer and the high ratio of produce/water. However, when a high residual concentration of PAA was maintained in the washing tank, the counts of total and F-specific RNA coliphages in the pre-washing tank decreased by 1 log, with no differences between total and F-specific RNA coliphages (**Table 3**).

The veggie mix included tomatoes, peppers, cucumbers, and onions that all entered the washing line before the blending process. The main difference of this PWW was that no sanitizer was added. The physicochemical characteristics of the PWW showed that the quality was very satisfactory because of the low COD value (166 mg/L), the pH was close to neutral (mean of 7.3), the ORP was very low (mean of 218 mV), as were the UV254 (0.08), and turbidity (19 NTU). Counts of total and F-specific RNA coliphages varied considerably over the samplings from non-detected coliphages to high counts (4.4 log pfu/L), with no significant differences between them (total and F-specific RNA coliphages). These high levels of coliphages were probably due to the absence of sanitizer in the water tank (**Table 3**).

During the surveillance of human enteric viruses in PWW, all samples tested negative for the presence of human norovirus GI, GII, astrovirus, rotavirus, and HAV, except for one PWW sample from bell peppers, in which rotavirus was detected. Overall, detection of crAssphage was observed in 70 and 60% of samples from the pre-washing and washing tanks, respectively. Concentrations of crAssphage changed from non detected to up to 3 logs pfu/L.

The results obtained showed that depending on the product, water ratio, type of product washed in the water, and residual concentration of the sanitizer, the prevalence and concentration of bacteriophages varied significantly. Based on the limit of detection for enteric viruses, it may be possible that the viruses were present, but the method's sensitivity was not adequate for their detection and quantification. More research should be done to lower the detection limit to confirm the low potential risk linked to the accumulation of enteric viruses in PWW when a residual sanitizer is present.

Objective 2: Inactivation studies to evaluate the efficacy of chlorine and non-chlorine based sanitizers on human enteric viruses and coliphages

Methods

Viruses, phages, cells and bacteria

The cytopathogenic murine norovirus MNV-1 strain (provided by Prof. H.W. Virgin, Washington University School of Medicine), the Tulane virus (TV) (provided by Prof. Farkas, Louisiana State University) and HAV, HM-175/18f strain (ATCC VR-1402) were propagated and assayed in RAW 264.7 (ATCC TIB-71), LLC-MK2 (ATCC CCL-7) and FRhK-4 cells (ATCC CRL-1688), respectively. Viruses were harvested and enumerated by endpoint dilution and cytopathic effect determination using the tissue culture infective dose fifty ($TCID_{50}$) method as described by Falcó et al. (2018). Wild-type MS2 bacteriophage DSM 13767 was quantified as previously described.

Batch-scale inactivation experiments

Inactivation experiments were performed in PWW from processing lines of baby leaves, bell peppers and veggie mix from the industrial collaborators of the project. Physicochemical characteristics are reported in **Table 4** and correspond to PWW sampled on 10/27/2020. The sanitizer solutions tested consisted of sodium hypochlorite (free chlorine, FC), PAA and chlorine dioxide (ClO_2). Sanitizer concentrations were measured using Kemio® (Palintest) based on chronoamperometry and the corresponding sensors. All viral inactivation experiments were conducted at 4°C in sterile chlorine demand-free 250-mL beakers containing an initial volume of 200 mL of PWW matrix and 2 mL of MNV, TV, HAV and MS2 phage. For the sanitizers, the volume added was that required to reach an initial concentration of around 10-20 mg/L for FC, 80 mg/L for PAA and 2-3 mg/L for ClO_2. Flasks were continuously mixed throughout the experiment.

Changes in levels of viruses were measured at different time intervals. For the inactivation experiments, samples of 1 mL were taken from the treated PWW and transferred into tubes

containing 9 mL of a neutralizing solution (DMEM supplemented with 10% fetal calf serum) for viruses and sodium thiosulphate for MS2 phage for quenching the residual concentration of the sanitizers. Then, samples were ten-fold diluted and titrated by cell culture using RAW 264.7, LLC-MK2 and FRhk-4 cells for MNV, TV and HAV, respectively. MS2 was enumerated using the host strain *E. coli* CECT9198 and the double layer agar method. To avoid contamination with PWW microbiota, PWW samples were decontaminated by sequential filtering through 0.45 µm and then 0.22 µm (Spin Centrifuge Tube Filters, Corning). Each sample was tested in triplicate, and viral inactivation calculated as $\log_{10}(N_x/N_0)$, where N_0 is the initial infectious virus titer in untreated samples and N_x is the infectious virus titer for disinfectant-treated samples at each time point.

Models development for the batch inactivation experiments

Experimental infectivity data obtained by cell culture assays for TV from specific combinations of type of PWW and type of sanitizer were used for the development of the models during treatment time. Selected data were fitted using different types of nonlinear regression models. In the case of PWW treated with FC and ClO_2, data were adjusted using an asymptotic regression model. On the other hand, in the case of PAA, data were adjusted using an exponential decay model.

Dynamic system inactivation experiments

The three industrial collaborators were visited twice at the end of the working process (4-6 h from the beginning of the washing) to sample PWWs with poor microbiological and physicochemical quality from washing a large amount of products in the same volume of water. Two trials were performed for each type of PWW. The physicochemical parameters of the PWWs obtained in the washing tanks from washing baby leaves, bell peppers and veggie mix are shown in **Table 5**. Each PWW was inoculated with MS2 at 10^4-10^5 infectious units per liter and MNV was inoculated in PWW from baby leaves and bell peppers. The inactivation experiments were carried out in a cold room (5-10°C) using a dynamic system that simulates the conditions of a commercial washing tank in which the organic matter and the microorganisms are accumulated in the PWW over time (Gómez-López et al., 2014). This system allowed the constant entrance of PWW with a high organic matter content and the inoculum. There was also the entrance of the sanitizer solution (FC, PAA, ClO_2) to maintain the operational limits set for FC (5 mg/L), PAA (80 mg/L), and ClO_2 (2-3 mg/L) constant over time while COD and the inoculum progressively accumulated in the washing tank. Samples were taken every 15 min and physicochemical parameters were measured as described above. The inactivation rate was measured after samples were concentrated using PEG precipitation, and the final concentrate was analyzed by enumerating PFUs for MS2 phage and $TCID_{50}$ for MNV as previously mentioned.

Model development for the dynamic system inactivation experiments

Modeling studies were performed to mechanistically describe the inactivation performance of FC, ClO_2 and PAA operated on simulated washing tanks with three types of PWW from commercial industrial plants. In the case of PWW treated with FC and ClO_2 as well as the control conditions without sanitizers, data were adjusted using Chick (first order reaction) model, while for PWW treated with PAA, the Chick model was modified to include the loss of effectiveness when COD concentration increased.

Statistical analysis

The statistical analysis was carried out by the post-hoc Tukey's method ($p < 0.05$) to compare and determine the differences among sanitizers and a Student's t-test was used to compare average values of sanitizers in the dynamic system alone and combined. Statistical software version 10 (StatSoft Inc., Tulsa, OK, USA) was used for statistical analyses.

Objective 2 Outcomes and Accomplishments

Batch-scale inactivation experiments

Changes in the concentrations of FC, ClO_2 and PAA in PWWs from baby leaves, bell peppers and veggi mix were recorded over the 20 min period that the batch-scale inactivation experiments were conducted. Under these conditions, the complete inactivation of MNV and MS2 at 10-20 mg/L FC in all PWWs was observed after 1 min contact time (**Figure 1**). However, infectivity of HAV was reduced only by 2 log after 1 min in the PWW of veggie mix, and complete inactivation was not observed even after 10 min (**Figure 1**). Infectivity/viability of MNV, HAV and MS2 was merely reduced by 80 mg/L PAA treatment in PWWs of peppers and baby leaves (**Figure 2**). On the other hand, ClO_2 at 2-3 mg/L was effective in reducing MNV infectivity below the limit of detection in PWWs of peppers and veggie mix after 1 min, but required 5 min in PWW of baby leaves (**Figure 3**). Results indicated that 4 log reduction in infectivity was achieved for MS2 in PWWs of peppers and veggie mix treated with 2 mg/L ClO_2 for 1 min while 2.7 log reduction was reported in PWW of baby leaves at the same ClO_2 concentration (**Figure 3**).

Results on TV inactivation showed that 20 mg/L FC and 2-3 mg/L ClO_2 completely inactivated TV regardless of the type of PWW assayed (**Figure 4**). Moreover, their antiviral effect was likely instantaneous as no infectious viral particle was detected after 1 min. However, PAA reduced TV titer by only 2 log in baby leaves and bell pepper PWWs after 15 and 20 min, respectively, while a similar reduction was observed in veggie mix PWW after 5 min (**Figure 4**). Thus, sanitation with PAA did not complete the viral inactivation, as infectious TV was recovered in all types of PWWs even though at concentrations bordering the limit of detection (LoD=15.8 $TCID_{50}$/mL)

Model development for the batch inactivation experiments

The type of sanitizer influenced the model that was most suitable to explain the behavior of TV in the different types of PWW. In the case of PWWs treated with FC and ClO_2, data were adjusted using an asymptotic regression model, defined by the equation:

$$y = a - (a - b) \cdot e^{-cx}$$

where *a* is the smallest value that *y* can take, which matches with the value associated with the horizontal asymptote of the curve; *b* is the value of *y* at *x*=0; and *c* is a value proportional to the relative rate of *y* that changes with the increase of *x*.

On the other hand, in the case of PAA and mostly due to its low inactivation efficacy, data were adjusted using an exponential decay model, defined by the equation:

$$y = a + (b - a) \cdot e^{\frac{-x}{c}}$$

where *a* is the value associated with the horizontal asymptote of the curve; *b* is the value of *y* at *x*=0; and *1*/*c* is the relative change of *y* for a unit increase of x, i.e. decay constant.

Kinetic modeling of enteric viral and coliphage reduction was developed. The models emphasized the significant differences between the viral inactivation capacity of the tested disinfectants, including FC, ClO_2 and PAA (**Figures 5 and 6**). While FC and ClO_2 reached the maximum infectivity reduction around 1 min of contact time, PAA required more than 10 min to achieve a minimal viral inactivation in bell pepper PWW. A more significant infectivity reduction was observed when FC was applied (1.7-3.7 log) compared to PAA (0.25-1.0). Regarding the susceptibility of the different viruses to the tested sanitizers, it was observed that the highest infectivity reduction was shown for MNV. These results indicate that FC is the sanitizer with better inactivation kinetics for viral inactivation. Additionally, the results indicated that the model can

predict the behavior of the disinfectant more accurately when compared to PAA. There is significant uncertainty associated with the efficacy of PAA in process water, which makes prediction of the viral inactivation very difficult.

Previous studies proposed the use of mechanistic models to describe the antiviral capacity of sanitizers in PWW. For instance, Dunkin et al. (2017) reported that the incomplete gamma Hom function effectively represents MS2 infectivity reduction in whole leaf and shredded iceberg PWWs sanitized with FC, while Hom-Power law model was the best fit for chopped romaine PWW. The inactivation processes studied in this study are nonlinear phenomena that are appropriately modelled by nonlinear regression equations. Moreover, the selection of nonlinear regression models versus mechanistic models is mostly due to the relevant attributes as parsimony, easy interpretation, prediction, and flexibility of nonlinear regression models, which can adopt a variety of shapes using data-driven methods of successive approximations.

Taking these data together, it has been demonstrated that current operational limits for FC and ClO_2 are satisfactory to inactivate TV in PWWs and prevent cross-contamination, while higher concentrations should be needed for PAA. The predictive models based on TV described in this study expand the current knowledge of interest to implement post-harvest produce safety procedures in industry settings, and therefore protect public health.

Dynamic system inactivation experiments

The inactivation experiments using the dynamic system were performed twice with PWWs obtained in the two visits in each processing plant. The dynamic system had the advantage versus the batch experiments that the progressive accumulation of organic matter, inoculum and sanitizer were maintained simulating the conditions that happened in a commercial washing tank. Changes in the physicochemical characteristics of PWWs without sanitizer (control) and with each sanitizer (FC, ClO_2 and PAA) were recorded every 5 min for 1 h. Results for PWWs are shown in **Table 6** for baby leaves, in **Table 7** for bell peppers and in **Table 8** for the veggi mix. The analysis of the PWW at time 0 confirmed the absence of MS2 before the inoculum was added.

To study the inactivation kinetics, MS2 was inoculated at the initial cell density of around 10^4-10^5 infectious units per liter into the simulated washing tank. Treatments with 5 mg/L FC and 2-3 ppm ClO_2 avoided the accumulation of MS2 below the detection limit for the entire duration of the tests (1 h) independently of the PWW (**Figures 7 and 8**). However, as observed in the batch experiments, the addition of 80 mg/L PAA was unable to prevent the accumulation of MS2 and only a moderate effect of a 2 log reduction was observed in bell pepper PWW (**Figure 9**). Similarly, 80 mg/L PAA was unable to prevent the accumulation of MNV in PWW, although 2 log reductions were observed in baby leaves and bell pepper PWWs (**Figure 10**).

Model development for the DS inactivation experiments

Models were developed for the prediction of the inactivation of coliphages in the dynamic system. Results are shown in **Figure 11** for baby leaves, in **Figure 12** for bell peppers and in **Figure 13** for the veggi mix PWWs. In the case of PWW treated with FC and ClO_2, as well as the control conditions without sanitizers, data were adjusted using Chick (first order reaction) model, defined by the equation:

$$\frac{dN}{dt} = \frac{F}{V}(N^I - N) - kNC$$

where N is the virus concentration in the tank, F is the flux incoming in the tank (and equal to the flux outcoming due to overflow), V is the tank volume, C is the sanitizer concentration, N^I is the concentration of virus in the incoming PWW into the tank, and k is the disinfection rate constant.

For PWW treated with PAA, the previous equation was modified to include the loss of effectiveness when COD concentration increased. This is included in the last term of the equation, which refers to the COD.

$$\frac{dN}{dt} = \frac{F}{V}(N^I - N) - ke^{(-\mu COD)NC}$$

The lack of effectiveness of PAA to prevent viral accumulation is the result of the model development and reflects the relevant differences in the effectiveness depending on the sanitizer and the PWW matrix. The effectiveness of PAA in reducing foodborne bacteria has been reported at laboratory and industrial scales (Banach et al., 2020). In comparison to other chemical sanitizers, PAA is an oxidizing agent but, depending on PWW characteristics, it is not a suitable sanitizer that can be recommended for preventing viral cross-contamination. The potential cross-contamination of viral particles in the washing tank was highly dependent on the PWW quality characteristics and sanitizer (**Figures 11-13**). While the impact of suspended solids on chemical disinfection kinetics has been widely recognized for PAA, a detailed modeling framework for assessing their contribution to disinfection efficiency in PWW is not yet available. In this objective, we conducted experimental and modeling studies to mechanistically describe the inactivation performance of FC, ClO_2 and PAA operated on simulated washing tanks with three types of PWW from commercial industrial plants. Several studies described that suspended solids impact the disinfection efficacy and strongly reduce the inactivation by PAA (Domínguez Henao et al., 2018; Elhalwagy et al., 2021). Suspended solids affecting PAA effectiveness has been related to two mechanisms: (i) consumption of PAA entailing a reduction of the available concentration for disinfection and, thus, a lower PAA exposure dose for viral inactivation, which is not our case as we maintained the residual concentration by pumping; and (ii) shielding of virus against the action of the sanitizer as microbial aggregates and microorganisms attached to or embedded into particles demonstrated increased resistance to inactivation by PAA compared to non-attached (Domínguez Henao et al., 2018).

Objective 3: Validation of the established water disinfection practices for enteric viruses and coliphages in commercial facilities – to be carried out on-line in three disinfectant-produce combinations (i.e., leafy greens and sodium hypochlorite, peppers and PAA, and tomatoes and ClO_2)

Methods

Challenges faced during the performance of this objective

The validation activity was initially planned in three processing lines, to check the performance of the three selected sanitizers (i.e., FC, ClO_2 and PAA). However, problems arose when searching for companies using ClO_2 as a water disinfection treatment. The validation of ClO_2 in the tomato packing line planned when the proposal was submitted could not be carried out due to the change in the disinfection practice implemented by the company that moved from ClO_2 to PAA. Thus, for the validation studies, two trials were conducted, one in the commercial processing plant of baby leaves that used FC and another in the bell pepper packing line that used PAA.

Experimental design

Sampling of PWWs was performed 4-6 h after the start of washing. Two replicates of 20 L each were taken at the same time. Some physicochemical parameters such as residual concentrations of sanitizer (FC and PAA), pH, ORP, and EC were measured *in situ* while organic matter as COD was measured in the lab after transporting the samples (30 miles). The concentration procedure for PWWs was conducted as explained in detail in Cuevas-Ferrando et al. (2021b).

Objective 3 Outcomes and Accomplishments

The optimal conditions identified during the lab-scale experiments were transmitted to the fresh-cut processing plant to be applied during the validation assays (**Table 10**). However, the recommendations made to the industrial partners were not maintained during the whole duration of the validation assay. The physicochemical characterization of PWWs from washing baby leaves and bell peppers is included in **Table 11**. Total and F-specific coliphages were absent in the PWW from washing baby leaves with FC in which the residual concentration was maintained at 30 mg/L. The efficacy of FC was remarkable even though the pH was a little high for its maximum effectiveness (**Table 11**). The levels of total and F-specific coliphages in the PWW of bell peppers with PAA were around 3-4 log. Human enteric viruses (i.e., human norovirus GI, GII, astrovirus, rotavirus, and HAV) were absent in both PWWs from washing baby leaves and bell peppers (**Table 12**). Moreover, detection of crAssphage was reported in both washing tanks, with crAssphage levels 1 log higher in washing tanks from bell peppers. The effect of total suspended solids (TSS) on the inactivation/reduction of bacteria by PAA has been described (Domínguez Henao et al., 2018). However, the effect of suspended solids affecting PAA disinfection of phages and human enteric viruses has not been previously investigated. The validation results confirmed the contribution of the high COD (995 mg/L), particularly the high suspended matter present in the PWW of peppers supporting microbial aggregates attached to or embedded into the suspended solids, demonstrating the high resistance to inactivation and the relatively low impact of PAA on water disinfection. The mechanism of protection afforded by suspended solids to bacteria has been hypothesized to be due to PAA decay, leading bacteria to be exposed to lower doses of disinfectant. In our validation study, as the residual concentration of PAA was very high (376 mg/L), the occurrence of interaction phenomena (aggregation) between phages and human enteric viruses and suspended solids could explain the defense mechanism against disinfection.

Objective 4: Establishment of coliphages as a suitable indicator of enteric viruses in commercial facilities.

Methods

The experimental set used to determine potential correlation and threshold values for viral indicators in PWW are the same as those described for Objective 1. PWW from the pre-washing and washing steps of baby leaves, bell pepper and veggie mix of commercial lines subjected to different conditions were tested, including the use of FC and PAA as well as the absence of sanitizer. Samples of PWW were concentrated and analyzed for levels (log pfu/L) of coliphages, human enteric viruses, and crAssphage detection and quantification, as previously described for Objective 1. Samples were taken at different sampling times and points to determine the most suitable sampling procedures. The Tukey honest significant difference (HSD) test was applied for multiple comparisons. Each group was indicated by different letters (a, b, c). Except when stated otherwise, P values ≤ 0.05 were considered statistically significant. Correlation between the count of indicators and the human enteric viruses was evaluated using Pearson's correlation, with a confidence interval set established at 95%.

Objective 4 Outcomes and Accomplishments

Establishment of the threshold values for somatic coliphages and F-specific RNA phages that can be correlated with a higher probability of enteric virus in process water

Correlation between the prevalence and concentration of coliphages (total and F-specific RNA phages) and enteric viruses was explored. Due to the low prevalence of human enteric viruses, even very high concentrations of bacteriophages (up to 4.4 log pfu/L) could not be correlated with the presence of pathogenic viruses. Based on the data observed, correlations of pathogenic

viruses with total or F-specific RNA coliphages, either detected by culture or PCR, could not be established. Among all the positive samples for crAssphage, only one sample was positive for rotavirus (pepper PWW). In this case high and low levels of crAssphage have been associated with the presence of human enteric viruses (**Table 3 and Table 12**). Therefore, crAssphage cannot be suggested as a good indicator for the presence of enteric viruses in PWW.

Based on the results obtained it was not possible to establish a threshold value for any of the tested indicators that could be correlated with a higher probability of the prevalence of human enteric viruses. Therefore, the absence of crAssphage cannot be unmistakably associated with the absence of human enteric viruses. A similar study was performed in irrigation water, where levels of human enteric viruses was higher. In this case, those samples with a coliphage concentration above the quantification limit (1 log pfu/100 mL), showed a good correlation between coliphage concentrations and norovirus (ρ=0.63 for norovirus GI, ρ=0.61 for norovirus GII) (Truchado et al., 2021). This study indicates that there is a higher likelihood of norovirus in those samples with high concentrations of coliphages. Meta-analyses conducted in wastewater matrices pointed out that bacteriophages, particularly somatic coliphages, are good surrogates of human enteric viruses (Chacón et al., 2020). However, the low prevalence of human enteric viruses found in the current study did not allow the establishment of a similar correlation.

In general, correlations between the levels of coliphages and human enteric viruses in water have been studied, but contradictory results have been found. One reason could be that human enteric viruses are quantified using molecular methods, which do not allow differentiation between infectious and non-infectious norovirus but genetic material. However, in the case of coliphages, the methodology allows the quantification of infectious phages. On the other hand, based on the limit of detection for enteric viruses, it may be possible that enteric viruses were present, but the sensitivity of the method was not low enough for their detection and quantification.

The evaluation of different sampling points for PWW samples in the processing plants studied showed that those steps where sanitizers are not added have a higher probability of being contaminated, and might help in the detection of contaminated produce. Pre-washing and washing operations in which no sanitizer was used would be the selected washing steps suitable as sampling points in commercial facilities. Previous studies suggested the analysis of the centrifuge effluent water as an alternative strategy to the sampling of fresh produce for the detection of microorganisms (López-Gálvez et al., 2019; Castro-Ibañez et al., 2016). However, these claims are based on lab-scale studies using artificial inoculation of fresh produce. In this study, none of the processing plants included in the study had a dewatering step using a centrifuge. The baby lettuce processing line was equipped with an air drying tunnel, while the bell peppers and mixed vegetables were not subjected to drying. Therefore, the suitability of centrifuge effluent water as a good sampling point to detect human enteric viruses could not be assayed as an alternative to determine the contamination of fresh produce with human enteric viruses. However, this is a strategy should be explored in the future.

Summary of Findings and Recommendations

1. Overall, the project provided critical data on (i) levels of coliphages and human enteric viruses in process wash water (PWW) and, (ii) effects on norovirus surrogates, hepatitis A virus (HAV), and MS2 phage of free chlorine (FC), chlorine dioxide (ClO_2) and peracetic acid (PAA) treatments at established operational limits for the disinfection of different types of PWW.
2. Depending on the product, the water ratio, type of product washed in the water, and residual concentration of the sanitizer, the prevalence and concentration of bacteriophages varied significantly.
3. The study provides industry with important data on (i) current industrial washing practices using FC that are effective for infectious virus and phage control, and (ii) depending on the type of product washed, product/water ratio, and residual concentrations of the sanitizers, the prevalence and concentration of bacteriophages changed significantly.
4. A key finding of this study is that MS2 inactivation follows similar profiles as norovirus surrogates, so it will facilitate validation in industrial settings.
5. It has been demonstrated that current operational limits for FC and ClO_2 are satisfactory to inactivate human enteric viruses in PWWs and prevent cross-contamination, while higher concentrations would be needed for PAA.
6. The validation results confirmed the contribution of the high COD (995 mg/L), particularly the high suspended matter present in the PWW of bell peppers supporting microbial aggregates attached to or embedded into the suspended solids, demonstrating the relatively low impact of PAA on water disinfection.
7. It was not possible to establish a threshold value for any of the tested indicators that could be correlated with a higher probability of the prevalence of human enteric viruses. The absence of crAssphage or bacteriophages cannot be unmistakably associated with the absence of human enteric viruses.

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APPENDICES

Publications and Presentations

E. Cuevas-Ferrando, A. Allende, A. Pérez-Cataluña, P. Truchado, N. Hernández, M.I. Gil, G. Sánchez. Occurrence and accumulation of human enteric viruses and phages in process water from the fresh produce industry. Foods 10(8): 1853 (2021) https://doi.org/10.3390/foods10081853

J. A. Férez-Rubio, J. Santamaria, J. A. Tudela, D. Rodríguez-Lázaro, R. Aznar, M.A. Gil, G. Sánchez, A. Allende, W. Randazzo. Inactivation of Tulane virus in response to chlorine, chlorine dioxide and peracetic acid treatments in process wash water. *Submitted to Food Control*.

Publication in preparation
I. Falcó, A. Pérez-Cataluña, P. Truchado, A. Garrido, I. Girón-Guzmán, M.I. Gil, A. Allende, G. Sánchez. Effect of chemical disinfectants on human enteric viruses and phages in process wash water from the fresh produce industry. *In preparation*.

Presentations
This work has been presented at the annual Center for Produce Safety's Research Symposium in 2020 (lightning round talk and poster) and 2021 (full presentation and poster).
This work was also presented on the CPS video webpage: (https://www.centerforproducesafety.org/article/203/Virus_study_seeks_improved_detection_method_and_sanitizer_efficacy_data.html).
Final results will be presented at the 2022 CPS Research Symposium in La Jolla, CA.

This work will be presented at the 2022 ISFEV meeting in Santiago de Compostela (May 2022). Chloride and non-chloride sanitizers in vegetable post-harvest wash water to control viral contamination. I. Falcó, Al. Pérez-Cataluña, W. Randazzo, P. Truchado, I. Girón-Guzmán, M.I. Gil, A. Allende, G. Sánchez.

Budget Summary
The project was awarded a total of $310,304 in research funds: $158,391 to IATA-CSIC and $151,913 to CEBAS-CSIC (subaward). Expenditures were primarily for salaries and fringe benefits, supplies, and some travel. Funding was sufficient to complete the project.

Tables 1–12 and Figures 1–13 (see below)

Table 1. Primers, probes, and RT-qPCR conditions used in this study.

Virus	Primers and probe	Sequence	RT-qPCR conditions	Reference
Norovirus GI	QNIF4	CGC TGG ATG CGN TTC CAT	RT: 55 °C for 60 min, Preheating: 95 °C for 5 min PCR (45 cycles) 95 °C for 15 s, 60 °C for 60 s, 65 °C for 60 s.	ISO - ISO 15216-1:2017 - Microbiology of the food chain — Horizontal method for determination of hepatitis A virus and norovirus using real-time RT-PCR — Part 1: Method for quantification
	NV1LCR	CCT TAG ACG CCA TCA TCA TTT AC		
	NVGG1p	[FAM]-TGG ACA GGA GAY CGC RAT CT-[BHQ]		
Norovirus GII	QNIF2	ATG TTC AGR TGG ATG AGR TTC TCW GA	RT: 55 °C for 60 min, Preheating: 95 °C for 5 min PCR (45 cycles) 95 °C for 15 s, 60 °C for 60 s, 65 °C for 60 s.	ISO - ISO 15216-1:2017 - Microbiology of the food chain — Horizontal method for determination of hepatitis A virus and norovirus using real-time RT-PCR — Part 1: Method for quantification
	COG2R	TCG ACG CCA TCT TCA TTC ACA		
	QNIFs	[FAM]-AGC ACG TGG GAG GGC GAT CG-[BHQ]		
HAV	HAV68	TCA CCG CCG TTT GCC TAG	RT: 55 °C for 60 min, Preheating: 95 °C for 5 min PCR (45 cycles) 95 °C for 15 s, 60 °C for 60 s, 65 °C for 60 s.	ISO - ISO 15216-1:2017 - Microbiology of the food chain — Horizontal method for determination of hepatitis A virus and norovirus using real-time RT-PCR — Part 1: Method for quantification
	HAV240	GGA GAG CCC TGG AAG AAA G		
	HAV150	[FAM]-CCT GAA CCT GCA GGA ATT AA-[MGBNFQ]		
RV	JVKF	CAG TGG TTG ATG CTC AAG ATG GA	RT: 50 °C for 30 min, Preheating: 95 °C for 15 min PCR (45 cycles) 94 °C for 10 s, 55 °C for 30 s, 72 °C for 20 s.	Jothikumar et al., 2009
	JVKR	TCA TTG TAA TCA TAT TGA ATA CCC A		
	JVKP	[FAM]-ACA ACT GCA GCT TCA AAA GAA GWG T-[BHQ]		

Virus	Primers and probe	Sequence	RT-qPCR conditions	Reference
HAstV	AstVorf1b+	AAG CAG CTT CGT GAC TCT GG	RT: 55 °C for 60 min, Preheating: 95 °C for 5 min PCR (45 cycles) 95 °C for 15 s, 58 °C for 60 s, 65 °C for 60 s.	Bennett et al., 2018
	AstVorf1b-	AGC CAT CAC ACT TCT TTG GTC		
	AstVorf1bp	[FAM]-AGA GCA ACT CCA TCG CAT TT-[BHQ]		
MgV	Mengo 110	GCG GGT CCT GCC GAA AGT	RT: 55 °C for 60 min, Preheating: 95 °C for 5 min PCR (45 cycles) 95 °C for 15 s, 60 °C for 60 s, 65 °C for 60 s.	ISO - ISO 15216-1:2017 - Microbiology of the food chain — Horizontal method for determination of hepatitis A virus and norovirus using real-time RT-PCR — Part 1: Method for quantification
	Mengo 209	GAA GTA ACA TAT AGA CAG ACG CAC AC		
	Mengo 147	[FAM]-ATC ACA TTA CTG GCC GAA GC-[MGBNFQ]		
crAssphage	064F1	TGT ATA GAT GCT GCT GCA ACT GTA CTC	Preheating: 95 °C for 5 min PCR (45 cycles) 95 °C for 5 s, 60 °C for 30 s.	Stachler et al., 201
	064R	CGT TGT TTT CAT CTT TAT CTT GTC CAT		
	064P1	[FAM]-CTG AAA TTG TTC ATA AGC AA-[MGBNFQ]		

Table 2. Physicochemical characteristics including pH, oxidation-reduction potential (ORP), absorbance at 254 nm (UV254), turbidity, chemical oxygen demand (COD), electrical conductivity (EC), and temperature of wash water from the pre-washing and washing steps of baby leaves, bell peppers and veggie mix in commercial lines using chlorine, peracetic acid (PAA) and without sanitizer, respectively. NA: Not Available. Values are the mean ± standard deviation (n = 3).

Process Wash Water		Date	Ratio Produce/ water (kg/L)	COD	pH	ORP (mV)	UV254 (Abs.)	Turbidity (NTU)	EC (µS cm^{-1})	Temp (ºC)
Baby leaves	Pre-Washing	7/21/2020	1.6	188 ± 39	7.9 ± 0.1	705 ± 13	0.16 ± 0.01	172 ± 3	918 ± 1	12 ± 0
		9/30/2020	1.2	67 ± 8	7.8 ± 0.1	463 ± 34	0.06 ± 0.00	57 ± 1	781 ± 2	9 ± 0
		10/13/2020	1.7	54 ± 25	8.1 ± 0.0	710 ± 5	0.07 ± 0.00	23 ± 3	866 ± 2	6 ± 0
		10/27/2020	1.8	42 ± 7	8.1 ± 0.1	717 ± 17	0.06 ± 0.00	24 ± 3	1156 ± 2	14 ± 1
		11/10/2020	1.6	32 ± 17	8.5 ± 0.0	755 ± 1	0.09 ± 0.00	45 ± 1	997 ± 2	9 ± 0
		11/24/2020	1.8	71 ± 6	8.3 ± 0.1	738 ±13	0.11 ± 0.01	51 ± 5	1106 ± 2	7 ± 1
	Washing	7/21/2020	1.6	75 ± 35	7.8 ± 0.1	724 ± 6	0.05 ± 0.00	31 ± 1	803 ± 2	11 ± 0
		9/30/2020	1.2	23 ± 3	7.7 ± 0.1	643 ± 12	0.02 ± 0.00	14 ± 1	725 ± 4	10 ± 0
		10/13/2020	1.7	35 ± 3	8.3 ± 0.1	732 ± 3	0.13 ± 0.00	15 ± 1	1108 ± 1	7 ± 0
		10/27/2020	1.8	19 ± 3	8.2 ± 0.0	753 ± 3	0.09 ± 0.00	12 ± 0	1397 ± 2	13 ± 0
		11/10/2020	1.6	14 ± 12	8.5 ± 0.1	748 ± 3	0.15 ± 0.00	21 ± 1	1288 ± 9	10 ± 0
		11/24/2020	1.8	26 ± 16	8.4 ± 0.0	759 ± 3	0.15 ± 0.00	19 ± 1	1341 ± 6	7 ± 0
Bell peppers	Pre-Washing	7/21/2020	31.0	845 ± 106	NA	NA	0.40 ± 0.01	305 ± 15	NA	NA
		9/29/2020	32.0	301 ± 14	7.4 ± 0.1	205 ± 11	0.29 ± 0.00	241 ± 3	783 ± 2	19 ± 0
		10/13/2020	4.0	414 ± 10	7.5 ± 0.3	216 ± 22	0.29 ± 0.01	376 ± 33	718 ± 1	15 ± 0
		10/27/2020	137.7	281 ± 8	8.0 ± 0.1	438 ± 58	0.40 ± 0.01	425 ± 9	713 ± 4	19 ± 0
		11/10/2020	88.0	548 ± 0	6.7 ± 0.4	226 ± 3	0.77 ± 0.02	538 ± 24	761 ± 2	20 ± 0
		11/24/2020	136.0	341 ± 20	6.8 ± 0.5	189 ± 1	0.47 ± 0.00	467 ± 3	742 ± 4	17 ± 0
	Washing	7/21/2020	31.0	1020 ± 35	3.5 ± 0.0	438 ± 3	1.09 ± 0.00	60 ± 1	815 ± 3	21 ± 0
		9/29/2020	32.0	1541 ± 98	4.0 ± 0.0	465 ± 8	0.94 ± 0.07	49 ± 5	711 ± 6	22 ± 0
		10/13/2020	4.0	2118 ± 43	4.0 ± 0.0	513 ± 9	1.25 ± 0.01	92 ± 2	715 ± 2	16 ± 0
		10/27/2020	137.7	1207 ± 46	4.0 ± 0.0	385 ± 0	1.39 ± 0.00	152 ± 4	728 ± 6	20 ± 0
		11/10/2020	88.0	1900 ± 0	3.8 ± 0.0	382 ± 2	1.93 ± 0.01	390 ± 99	836 ± 8	21 ± 0
		11/24/2020	136.0	1192 ± 25	3.7 ± 0.0	380 ± 1	1.58 ± 0.00	209 ± 2	786 ± 4	17 ± 0
Veggie mix	Washing	9/29/2020	5.6	171 ± 8	7.5 ± 0.3	147 ± 9	0.07 ± 0.00	18 ± 0	666 ± 0	20 ± 0
		10/14/2020	18.4	144 ± 2	7.2 ± 0.4	244 ± 19	0.07 ± 0.00	16 ± 1	882 ± 1	19 ± 0
		10/27/2020	2.9	147 ± 8	7.4 ± 0.2	273 ± 5	0.07 ± 0.00	18 ± 2	732 ± 4	19 ± 0
		11/24/2020	9.1	169 ± 16	7.7 ± 0.2	164 ± 14	0.07 ± 0.00	23 ± 2	917 ± 2	14 ± 0
		12/9/2020	7.8	179 ± 6	7.0 ± 0.3	254 ± 12	0.09 ± 0.00	18 ± 0	1059 ± 2	15 ± 1
		12/22/2020	13.1	185 ± 1	7.2 ± 0.4	226 ± 20	0.09 ± 0.00	19 ± 1	1017 ± 1	14 ± 0

Table 3. Levels (log pfu/L) of total and F-specific RNA coliphages, crAssphage and human enteric viruses (norovirus GI and GII, HAV, HAstV, RV) of PWW from the pre-washing and washing steps of baby leaves, bell pepper and veggie mix of commercial lines using free chlorine (FC) peracetic acid (PAA) and without sanitizer, respectively. Nd: non-detected.

Produce	Sampled water	Sampling date	Sanitizer	Sanitizer concentration (mg/L)	Total coliphages[1]	F-specific RNA coliphages[1]	crAssphage[1]	Human enteric viruses[1]
Baby leaves	Pre-Washing	7/21/2020	Chlorine	18 ± 1	Nd	Nd	Nd	Nd
		9/30/2020	Chlorine	0 ± 0	Nd	Nd	2.18 ± 0.00	Nd
		10/13/2020	Chlorine	36 ± 1	Nd	Nd	Nd	Nd
		10/27/2020	Chlorine	37 ± 2	Nd	Nd	Nd	Nd
		11/10/2020	Chlorine	57 ± 6	Nd	Nd	2.97 ± 0.06	Nd
		11/24/2020	Chlorine	59 ± 0	Nd	Nd	2.46 ± 0.04	Nd
	Washing	7/21/2020	Chlorine	12 ± 1	Nd	Nd	Nd	Nd
		9/30/2020	Chlorine	3 ± 0	Nd	Nd	Nd	Nd
		10/13/2020	Chlorine	98 ± 8	Nd	Nd	Nd	Nd
		10/27/2020	Chlorine	72 ± 1	Nd	Nd	1.66 ± 0.00	Nd
		11/10/2020	Chlorine	120 ± 3	Nd	Nd	2.93 ± 0.04	Nd
		11/24/2020	Chlorine	125 ± 5	Nd	Nd	2.62 ± 0.14	Nd
Bell peppers	Pre-Washing	7/21/2020	None	-	4.4 ± 0.0	4.4 ± 0.0	Nd	Nd
		9/29/2020	None	-	3.4 ± 0.0	3.1 ± 0.0	3.08 ± 0.02	Nd
		10/13/2020	None	-	3.8 ± 0.1	3.9 ± 0.0	1.18 ± 0.0	Nd
		10/27/2020	None	-	3.6 ± 0.1	3.9 ± 0.0	Nd	Nd
		11/10/2020	None	-	4.3 ± 0.0	4.0 ± 0.0	3.02 ± 0.04	Nd
		11/24/2020	None	-	3.5 ± 0.0	3.3 ± 0.0	3.14 ± 0.20	Nd
	Washing	7/21/2020	PAA	416 ± 96	4.4 ± 0.0	4.4 ± 0.0	Nd	Nd
		9/29/2020	PAA	233 ± 2	Nd	Nd	Nd	Nd
		10/13/2020	PAA	326 ± 9	2.9 ± 0.0	3.0 ± 0.0	1.18 ± 0.0	Nd
		10/27/2020	PAA	330 ± 7	3.3 ± 0.1	3.8 ± 0.0	Nd	Nd
		11/10/2020	PAA	370 ± 9	3.7 ± 0.2	3.6 ± 0.0	Nd	Nd
		11/24/2020	PAA	332 ± 9	2.7 ± 0.1	3.1 ± 0.1	2.94 ± 0.09	5.55*
Veggie mix	Washing	9/29/2020	None	-	3.9 ± 0.1	4.1 ± 0.0	Nd	Nd
		10/14/2020	None	-	2.0 ± 0.0	2.0 ± 0.0	Nd	Nd
		10/27/2020	None	-	Nd	Nd	2.11 ± 0.08	Nd
		11/24/2020	None	-	3.8 ± 0.1	3.7 ± 0.0	Nd	Nd
		12/9/2020	None	-	3.7 ± 0.1	3.6 ± 0.1	2.82 ± 0.04	Nd
		12/22/2020	None	-	2.7 ± 0.1	2.0 ± 0.0	2.33 ± 0.21	Nd

[1] Bacteriophage load expressed as log pfu/L and viral load as log genome copies/L.
*Only rotavirus detection. LoD95% 4.28 ×10^2
"-", No sanitizer added.

Table 4. Physiochemical characterization of produce wash water (PWW) used for batch experiment.

PWW	pH	ORP	EC	COD	Turbidity
Baby leaves	8.1±0.3	743±15	1110±286	23.4±7.9	19±7
Bell peppers	3.8±0.2	410±39	765±54	1496±437	112±67
Veggie mix	7.3±0.3	218±51	879±155	166±16.7	19±2

Abbreviations: PWW, produce wash water; ORP, oxidation-reduction potential (mV); EC, electric conductivity (µS/cm); COD, chemical oxygen demand (mg/L); Turbidity (NTU, nephelometric turbidity unit).

Table 5. Physicochemical characteristics of the process wash water from washing baby leaves, bell peppers and a veggie mix sampled at two sampling dates from commercial lines. Data include pH, oxidation-reduction potential (ORP, mV), chemical oxygen demand (COD, mg/L), absorbance at 254 nm (UV254), turbidity (NTU, nephelometric turbidity unit) and electrical conductivity (EC, μS cm^{-1}).

Process Wash Water	Date	pH	ORP	COD	UV254	Turbidity	EC
Baby leaves	04/21/2021	8.3 ± 0.0	321.5 ± 12.0	105.0 ± 9.9	-	-	1024.0 ± 4.2
Baby leaves	05/24/2021	8.1 ± 0.1	276.3 ± 3.8	91.7 ± 7.1	0.11 ± 0.01	49.7 ± 1.5	1112.3 ± 1.5
Bell peppers	04/26/2021	7.2 ± 0.0	283.2 ± 5.6	381.6 ± 12.3	0.80 ± 0.01	315.2 ± 6.1	567.7 ± 2.5
Bell peppers	05/10/2021	7.4 ± 0.1	180.7 ± 9.1	383.3 ± 12.3	1.08 ± 0.02	323.9 ± 5.4	602.0 ± 1.6
Veggie mix	04/19/2021	7.4 ± 0.1	188.0 ± 11.3	150.3 ± 12.3	0.47 ± 0.01	30.3 ± 0.9	979.0 ± 3.7
Veggie mix	05/03/2021	7.1 ± 0.0	159.0 ± 4.0	252.6 ± 2.5	0.41 ± 0.01	30.3 ± 0.9	987.0 ± 2.9

Table 6. Physicochemical characteristics including sanitizer concentration, pH, oxidation-reduction potential (ORP), chemical oxygen demand (COD) and electrical conductivity (EC) of process wash water from washing **baby leaves** without sanitizer (control), and with sodium hypochlorite (free chlorine, FC), chlorine dioxide (ClO_2) and peracetic acid (PAA) when increasing organic matter (COD) for 1 h. Results are means of at least two repetitions ± standard deviation.

Time (min)	Sanitizer	Sanitizer concentration (mg/L)	pH	ORP (mV)	COD (mg/L)	EC (µS cm^{-1})
0	Control	-	8.2 ± 0.0	623.0 ± 49.5	5.0 ± 0.0	840.5 ± 6.4
5	Control	-	8.3 ± 0.0	598.0 ± 82.0	-	864.5 ± 9.2
10	Control	-	8.3 ± 0.0	547.5 ± 112.4	-	887.0 ± 7.1
15	Control	-	8.3 ± 0.0	479.5 ± 137.9	-	911.0 ± 5.7
20	Control	-	8.3 ± 0.1	447.5 ± 129.4	-	927.0 ± 4.2
25	Control	-	8.3 ± 0.1	423.0 ± 111.7	-	945.0 ± 7.1
30	Control	-	8.3 ± 0.1	394.0 ± 82.0	53.5 ± 3.5	963.5 ± 4.9
35	Control	-	8.3 ± 0.0	377.0 ± 62.2	-	975.5 ± 2.1
40	Control	-	8.3 ± 0.0	360.0 ± 45.3	-	983.0 ± 9.9
45	Control	-	8.3 ± 0.0	343.5 ± 29.0	-	1002.0 ± 2.8
50	Control	-	8.3 ± 0.0	333.5± 21.9	-	1012.0 ± 4.2
55	Control	-	8.3 ± 0.0	327.5 ± 17.7	-	1019.5 ± 4.9
60	Control	-	8.3 ± 0.0	321.5 ± 12.0	105.0 ± 9.9	1024.0 ± 4.2
0	FC	5.0 ± 0.2	5.3 ± 0.0	833.0 ± 2.8	5.0 ± 0.0	849.0 ± 1.4
5	FC	4.5 ± 0.3	5.4 ± 0.1	840.5 ± 0.7	-	871.5 ± 10.6
10	FC	5.6 ± 0.1	5.4 ± 0.1	841.0 ± 0.0	-	897.5 ± 0.7
15	FC	5.5 ± 0.1	5.6 ± 0.1	836.0 ± 4.2	-	921.0 ± 9.9
20	FC	5.3 ± 0.1	5.6 ± 0.0	826.0 ± 9.9	-	937.5 ± 7.8
25	FC	4.9 ± 0.2	5.7 ± 0.1	813.5 ± 13.4	-	951.5 ± 4.9
30	FC	5.2 ± 0.3	5.8 ± 0.0	803.5 ± 6.4	50.0 ± 1.4	966.0 ± 24.0
35	FC	5.3 ± 0.1	5.6 ± 0.1	807.0 ± 15.6	-	973.0 ± 21.2
40	FC	5.2 ± 0.6	5.6 ± 0.0	804.0 ± 15.6	-	984.5 ± 23.3
45	FC	5.2 ± 0.1	5.7 ± 0.2	780.5 ± 37.5	-	1001.5 ± 29.0
50	FC	5.0 ± 0.2	5.7 ± 0.0	785.5 ± 21.9	-	1003.5 ± 41.7
55	FC	4.8 ± 0.2	5.6 ± 0.1	790.5 ± 31.8	-	1016.5 ± 34.6
60	FC	4.9 ± 0.3	5.7 ± 0.0	780.0 ± 24.0	99.0 ± 2.8	1021.0 ± 41.0
0	ClO_2	2.4 ± 0.2	7.9 ± 0.1	775.0 ± 1.4	5.0 ± 0.0	873.5 ± 2.1
5	ClO_2	2.0 ± 0.2	7.8 ± 0.8	771.5 ± 3.5	-	891.5 ± 0.7
10	ClO_2	1.8 ± 0.2	7.8 ± 0.0	768.5 ± 3.5	-	914.5 ± 2.1
15	ClO_2	1.9 ± 0.1	7.7 ± 0.0	765.5 ± 2.1	-	942.5 ± 10.6
20	ClO_2	1.9 ± 0.9	7.7 ± 0.0	762.5 ± 2.1	-	956.0 ± 1.4
25	ClO_2	2.0 ± 0.1	7.7 ± 0.0	759.5 ± 2.1	-	977.0 ± 2.8
30	ClO_2	1.9 ± 0.2	7.7 ± 0.0	757.0 ± 0.0	68.0 ± 8.5	944.5 ± 4.9
35	ClO_2	1.9 ± 0.2	7.7 ± 0.0	754.5 ± 0.7	-	1012.5 ± 7.8
40	ClO_2	2.0 ± 0.1	7.7 ± 0.0	753.0 ± 2.8	-	1025.5 ± 9.2
45	ClO_2	2.1 ± 0.1	7.7 ± 0.0	752.5 ± 0.7	-	1039.0 ± 9.9
50	ClO_2	1.8 ± 0.0	7.7 ± 0.0	750.5 ± 0.7	-	1050.0 ± 8.5
55	ClO_2	1.9 ± 0.0	7.7 ± 0.0	749.0 ± 0.0	-	1063.0 ± 11.3
60	ClO_2	2.0 ± 0.1	7.7 ± 0.0	749.5 ± 0.7	103.0 ± 11.3	1069.5 ± 12.0
0	PAA	78.0 ± 2.8	6.1 ± 0.6	327.5 ± 4.9	233.5 ± 17.7	796.5 ± 75.7
5	PAA	72.0 ± 2.8	5.9 ± 0.3	326.5 ± 10.6	-	843.5 ± 33.2
10	PAA	76.5 ± 6.4	5.9 ± 0.2	325.0 ± 8.5	-	861.0 ± 24.0
15	PAA	79.5 ± 9.2	5.9 ± 0.2	329.0 ± 7.1	-	871.0 ± 9.9
20	PAA	81.5 ± 6.4	5.8 ± 0.2	329.0 ± 5.7	-	893.0 ± 24.0
25	PAA	81.0 ± 1.4	5.8 ± 0.0	330.5 ± 3.5	-	910.0 ± 18.4
30	PAA	85.5 ± 4.9	5.8 ± 0.1	332.0 ± 1.4	493.5 ± 41.7	930.0 ± 25.5
35	PAA	80.0 ± 1.4	5.9 ± 0.1	330.5 ± 4.9	-	930.0 ± 8.5
40	PAA	80.0 ± 2.8	5.9 ± 0.0	330.0 ± 4.2	-	941.5 ± 2.1
45	PAA	83.0 ± 7.1	6.1 ± 0.1	326.5 ± 0.7	-	1002.0 ± 1.4
50	PAA	74.0 ± 1.4	6.2 ± 0.0	324.5 ± 3.5	-	1008.0 ± 5.7
55	PAA	73.5 ± 2.1	6.2 ± 0.1	324.5 ± 2.1	-	1015.5 ± 6.4
60	PAA	84.0 ± 5.7	6.0 ± 0.2	328.0 ± 7.1	718.0 ± 79.2	1021.5 ± 6.4

Table 7. Physicochemical characteristics including sanitizer concentration, pH, oxidation-reduction potential (ORP), chemical oxygen demand (COD) and electrical conductivity (EC) of process wash water from washing **bell peppers** without sanitizer (control), and with sodium hypochlorite (free chlorine, FC), chlorine dioxide (ClO_2) and peracetic acid (PAA) when increasing organic matter (COD) for 1 h. Results are means of at least two repetitions ± standard deviation.

Time (min)	Sanitizer	Sanitizer concentration (mg/L)	pH	ORP (mV)	COD (mg/L)	EC (µS cm^{-1})
0	Control	-	7.8 ± 0.2	509.0 ± 9.0	26.0 ± 0.0	882.5 ± 11.5
5	Control	-	7.8 ± 0.1	401.5 ± 15.5	-	858.5 ± 13.5
10	Control	-	7.7 ± 0.1	341.0 ± 29.0	-	842.5 ± 12.5
15	Control	-	7.6 ± 0.2	294.0 ± 43.0	238.0 ± 52.0	824.5 ± 13.5
20	Control	-	7.5 ± 0.1	268.5 ± 41.5	-	813.0 ± 16.0
25	Control	-	7.5 ± 0.1	242.0 ± 27.0	-	797.0 ± 18.0
30	Control	-	7.5 ± 0.1	224.5 ± 19.5	244.0 ± 52.0	797.0 ± 30.0
35	Control	-	7.5 ± 0.1	211.5 ± 11.5	-	772.5 ± 17.5
40	Control	-	7.5 ± 0.1	202.5 ± 6.5	-	762.5 ± 19.5
45	Control	-	7.5 ± 0.1	199.0 ± 8.0	268.0 ± 48.0	749.0 ± 21.0
50	Control	-	7.5 ± 0.1	197.5 ± 9.5	-	742.0 ± 21.0
55	Control	-	7.4 ± 0.1	196.0 ± 11.0	-	735.5 ± 20.5
60	Control	-	7.5 ± 0.0	195.0 ± 10.0	316.0 ± 16.0	732.0 ± 19.0
0	FC	5.3 ± 0.0	5.4 ± 0.1	834.5 ± 6.5	28.0 ± 2.0	911.0 ± 43.0
5	FC	4.6 ± 0.2	5.0 ± 0.5	782.5 ± 28.5	-	887.0 ± 31.0
10	FC	4.4 ± 0.3	5.6 ± 0.0	735.0 ± 26.0	-	886.0 ± 44.0
15	FC	5.4 ± 0.3	5.7 ± 0.0	762.5 ± 9.5	68.0 ± 0.0	885.5 ± 29.5
20	FC	4.6 ± 0.2	5.7 ± 0.0	760.5 ± 2.5	-	882.5 ± 33.5
25	FC	5.0 ± 0.3	5.7 ± 0.0	743.5 ± 19.5	-	874.0 ± 35.0
30	FC	4.8 ± 0.5	5.8 ± 0.0	740.0 ± 18.0	182.0 ± 2.0	866.5 ± 32.5
35	FC	5.8 ± 0.3	5.7 ± 0.0	740.5 ± 18.5	-	865.0 ± 23.0
40	FC	5.3 ± 0.2	5.7 ± 0.0	733.0 ± 7.0	-	847.5 ± 16.5
45	FC	4.7 ± 0.2	5.7 ± 0.0	729.0 ± 14.0	255.0 ± 55.0	849.5 ± 17.5
50	FC	4.5 ± 0.1	5.7 ± 0.0	736.0 ± 4.0	-	840.5 ± 4.5
55	FC	4.8 ± 0.5	5.7 ± 0.1	721.0 ± 23.0	-	832.5 ± 12.5
60	FC	4.3 ± 0.0	5.7 ± 0.0	733.5 ± 12.5	310.0 ± 5.0	835.0 ± 7.0
0	ClO_2	2.2 ± 0.0	7.6 ± 0.1	771.0 ± 5.0	26.0 ± 0.0	894.5 ± 5.5
5	ClO_2	1.3± 0.0	7.6 ± 0.1	722.5 ± 10.5	-	874.0 ± 11.0
10	ClO_2	2.3 ± 1.2	7.4 ± 0.1	697.5 ± 6.5	-	874.5 ± 13.5
15	ClO_2	2.4 ± 0.6	7.3 ± 0.1	695.5 ± 13.5	86.0 ± 0.0	871.0 ± 11.0
20	ClO_2	1.9 ± 0.1	7.2 ± 0.1	683.0 ± 22.0	-	867.5 ± 9.5
25	ClO_2	2.2 ± 0.1	7.1 ± 0.1	692.0 ± 11.0	-	872.5 ± 15.5
30	ClO_2	2.3 ± 0.3	7.1 ± 0.1	680.5 ± 22.5	138.0 ± 12.0	870.0 ± 13.0
35	ClO_2	2.2 ± 0.2	7.0 ± 0.1	682.0 ± 13.0	-	867.0 ± 18.0
40	ClO_2	2.4 ± 0.0	6.9 ± 0.1	690.0 ± 3.0	-	883.5 ± 37.5
45	ClO_2	2.0 ± 0.4	6.9 ± 0.2	688.5 ± 0.5	224.0 ± 34.0	892.0 ± 50.0
50	ClO_2	2.3 ± 0.3	6.9 ± 0.2	677.0 ± 7.0	-	882.5 ± 45.5
55	ClO_2	1.8 ± 0.1	6.9 ± 0.2	675.5 ± 4.5	-	887.0 ± 54.0
60	ClO_2	2.2 ± 0.0	6.8 ± 0.2	672.5 ± 4.5	267.0 ± 13.0	883.0 ± 53.0
0	PAA	77.5 ± 0.5	5.9 ± 0.2	311.5 ± 7.5	217.5 ± 3.5	883.0 ± 5.0
5	PAA	69.0 ± 8.0	5.9 ± 0 .1	311.0 ± 2.0	-	858.0 ± 19.0
10	PAA	84.0 ± 9.0	5.5 ± 0.0	315.5 ± 5.5	-	841.0 ± 16.0
15	PAA	66.0 ± 0.0	5.4 ± 0.1	326.5 ± 3.5	328.0 ± 0.0	820.0 ± 13.0
20	PAA	71.0 ± 1.0	5.2 ± 0.4	337.5 ± 7.5	-	789.0 ± 15.0
25	PAA	85.0 ± 6.0	4.9 ± 0.3	343.0 ± 11.0	-	772.5 ± 27.5
30	PAA	80.5 ± 1.5	4.8 ± 0.4	348.0 ± 12.0	424.0 ± 2.0	759.0 ± 34.0
35	PAA	79.0 ± 12.0	4.7 ± 0.3	353.0 ± 12.0	-	753.5 ± 33.5
40	PAA	80.5 ± 6.5	4.6 ± 0.3	358.0 ± 8.0	-	750.0 ± 33.0
45	PAA	84.5 ± 0.5	4.6 ± 0.2	357.5 ± 4.5	533.0 ± 37.0	738.0 ± 37.0
50	PAA	85.5 ± 4.5	4.5 ± 0.2	363.5 ± 6.5	-	735.5 ± 35.5
55	PAA	83.0 ± 2.0	4.5 ± 0.2	364.0 ± 7.0	-	723.5 ± 43.5
60	PAA	83.0 ± 0.0	4.5 ± 0.2	365.0 ± 5.0	555.0 ± 43.0	721.5 ± 43.5

Table 8. Physicochemical characteristics including sanitizer concentration, pH, oxidation-reduction potential (ORP), chemical oxygen demand (COD) and electrical conductivity (EC) of process wash water from washing a **veggie mix** without sanitizer (control), and with sodium hypochlorite (free chlorine, FC), chlorine dioxide (ClO_2) and peracetic acid (PAA) when increasing organic matter (COD) for 1 h. Results are means of at least two repetitions ± standard deviation.

Time (min)	Sanitizer	Sanitizer concentration (mg/L)	pH	ORP (mV)	COD (mg/L)	EC (µS cm^{-1})
0	Control	-	7.7 ± 0.0	286.0 ± 0.0	31.0 ± 5.5	892.0 ± 0.0
5	Control	-	7.7 ± 0.0	258.0 ± 0.0	-	900.0 ± 0.0
10	Control	-	7.6 ± 0.0	239.0 ± 0.0	-	908.0 ± 0.0
15	Control	-	7.5 ± 0.0	224.0 ± 0.0	144.0 ± 88.0	914.0 ± 0.0
20	Control	-	7.4 ± 0.0	216.0 ± 0.0	-	922.0 ± 0.0
25	Control	-	7.4 ± 0.0	211.0 ± 0.0	-	926.0 ± 0.0
30	Control	-	7.4 ± 0.0	207.0 ± 0.0	166.0 ± 70.0	930.0 ± 0.0
35	Control	-	7.4 ± 0.0	203.0 ± 0.0	-	934.0 ± 0.0
40	Control	-	7.3 ± 0.0	202.0 ± 0.0	-	938.0 ± 0.0
45	Control	-	7.4 ± 0.0	204.0 ± 0.0	169.0 ± 61.0	940.0 ± 0.0
50	Control	-	7.4 ± 0.0	201.0 ± 0.0	-	943.0 ± 0.0
55	Control	-	7.4 ± 0.0	201.0 ± 0.0	-	947.0 ± 0.0
60	Control	-	7.4 ± 0.0	202.0 ± 0.0	191.0 ± 53.0	950.0 ± 0.0
0	FC	4.8 ± 0.5	5.7 ± 0.2	812.0 ± 4.0	26.5 ± 1.5	876.5 ± 4.5
5	FC	3.5 ± 0.3	5.5 ± 0.0	815.0 ± 13.0	-	881.0 ± 10.0
10	FC	4.7 ± 1.0	5.5 ± 0.2	817.0 ± 7.0	-	888.0 ± 6.0
15	FC	5.6 ± 0.7	5.5 ± 0.1	827.5 ± 14.5	67.5 ± 0.5	896.5 ± 8.5
20	FC	4.3 ± 0.7	5.6 ± 0.2	830.5 ± 16.5	-	907.5 ± 4.5
25	FC	4.6 ± 0.4	5.6 ± 0.2	833.5 ± 17.5	-	912.5 ± 0.5
30	FC	4.3 ± 0.7	5.5 ± 0.1	838.5 ± 17.5	112.0 ± 24.0	925.0 ± 2.0
35	FC	4.4 ± 0.5	5.5 ± 0.1	843.0 ± 13.0	-	936.0 ± 2.0
40	FC	4.6 ± 0.4	5.5 ± 0.2	844.0 ± 16.0	-	945.5 ± 4.5
45	FC	5.0 ± 0.1	5.6 ± 0.2	845.0 ± 7.0	136.0 ± 20.0	958.0 ± 12.0
50	FC	5.2 ± 0.5	5.5 ± 0.2	846.5 ± 5.5	-	957.5 ± 7.5
55	FC	5.1 ± 0.2	5.6 ± 0.2	840.5 ± 5.5	-	965.0 ± 11.0
60	FC	5.6 ± 0.0	5.6 ± 0.2	842.5 ± 5.5	173.0 ± 31	964.0 ± 7.0
0	ClO_2	2.2 ± 0.0	7.7 ± 0.1	769.0 ± 2.0	21.5 ± 3.5	870.0 ± 2.0
5	ClO_2	1.7 ± 0.1	7.6 ± 0.1	742.5 ± 5.5	-	899.0 ± 8.0
10	ClO_2	1.8 ± 0.4	7.5 ± 0.1	741.0 ± 2.0	-	911.0 ± 17.0
15	ClO_2	2.4 ± 0.2	7.3 ± 0.0	733.5 ± 0.5	74.0 ± 18.0	922.5 ± 17.5
20	ClO_2	2.3 ± 0.1	7.3 ± 0.0	725.0 ± 0.0	-	927.0 ± 15.0
25	ClO_2	2.1 ± 0.0	7.2 ± 0.0	715.0 ± 5.0	-	929.5 ± 17.5
30	ClO_2	2.1 ± 0.1	7.1 ± 0.0	716.5 ± 2.5	101.5 ± 24.5	942.0 ± 15.0
35	ClO_2	2.2 ± 0.1	7.1 ± 0.0	716.5 ± 1.5	-	952.0 ± 18.0
40	ClO_2	2.1 ± 0.0	7.1 ± 0.0	714.0 ± 0.0	-	960.5 ± 19.5
45	ClO_2	2.2 ± 0.2	7.1 ± 0.0	711.0 ± 0.0	147.0 ± 33.0	967.0 ± 21.0
50	ClO_2	1.9 ± 0.2	7.1 ± 0.0	707.0 ± 2.0	-	968.5 ± 18.5
55	ClO_2	2.1 ± 0.0	7.1 ± 0.0	705.5 ± 3.5	-	977.5 ± 23.5
60	ClO_2	2.2 ± 0.1	7.1 ± 0.0	704.5 ± 2.5	176.0 ± 44.0	978.5 ± 21.5
0	PAA	74.0 ± 0.0	6.0 ± 0.1	318.5 ± 0.5	211.0 ± 9.0	872.0 ± 16.0
5	PAA	80.5 ± 7.5	6.1 ± 0.1	317.0 ± 0.0	-	876.0 ± 17.0
10	PAA	90.5 ± 2.5	6.0 ± 0.3	319.5 ± 5.5	-	880.0 ± 19.0
15	PAA	93.5 ± 5.5	6.0 ± 0.3	319.5 ± 6.5	282.5 ± 41.5	878.5 ± 20.5
20	PAA	75.0 ± 1.0	6.0 ± 0.4	319.0 ± 8.0	-	889.0 ± 14.0
25	PAA	78.0 ± 7.0	5.7 ± 0.3	326.0 ± 4.0	-	883.0 ± 25.0
30	PAA	89.5 ± 6.5	5.4 ± 0.4	336.0 ± 4.0	345.0 ± 69.0	877.0 ± 30.0
35	PAA	73.5 ± 1.5	5.4 ± 0.4	336.0 ± 6.0	-	868.5 ± 25.5
40	PAA	84.5 ± 2.5	5.3 ± 0.5	339.5 ± 9.5	-	865.5 ± 19.5
45	PAA	84.0 ± 5.0	5.1 ± 0.5	344.0 ± 7.0	422.0 ± 92.0	854.0 ± 15.0
50	PAA	77.5 ± 8.5	5.2 ± 0.5	343.5 ± 9.5	-	845.5 ± 6.5
55	PAA	77.5 ± 1.5	5.2 ± 0.6	344.0 ± 14.0	-	832.0 ± 5.0
60	PAA	76.5 ± 2.5	5.0 ± 0.4	350.0 ± 10.0	461.0 ± 90.0	824.5 ± 5.5

Table 9. Values of the equation constants, including the disinfection rate constant (k) and the loss of effectiveness (μ) of the models in dynamic system inactivation experiments with PWW from washing baby leaves, bell peppers and veggie mix.

Constant	Process Wash Water (PWW)	Chlorine	ClO_2	PAA	Control
k	Baby leaves	$1 \cdot 10^4$	$1 \cdot 10^4$	$2.6 \cdot 10^2$	0
	Bell peppers	$1 \cdot 10^4$	$1 \cdot 10^4$	$2.2 \cdot 10^2$	0
	Vegetables mix	$1 \cdot 10^4$	$1 \cdot 10^4$	$2.0 \cdot 10^{12}$	0
μ	Baby leaves	NA	NA	0.012	NA
	Bell peppers	NA	NA	0.013	NA
	Vegetables mix	NA	NA	0.077	NA

NA: Not applicable.

Table 10. Recommended conditions for the fresh produce processing lines established based on the lab-scale studies.

Produce	Sampling place	Sanitizer	Residual concentration (mg/L)	pH	ORP
Baby leaves	Washing tank	FC	15-20	5.5 – 6.0	>650
Bell peppers	Washing tank	PAA	80	3.5 - 4.0	-

[1] ORP: oxidation reduction potential.

Table 11. Physicochemical characterization[1] of wash water from the washing tanks of baby leaves and bell peppers in commercial lines using free chlorine (FC) and peracetic acid (PAA), respectively.

Produce	Sampling place	Sanitizer	Residual concentration (mg/L)	pH	ORP	EC	COD
Baby leaves	Washing tank	FC	31 ± 10	8.3 ± 0.1	749 ± 5	987 ± 99	238 ± 11
Bell peppers	Washing tank	PAA	376 ± 9	4.0 ± 0.0	-	750 ± 10	995 ± 45

[1] ORP: oxidation reduction potential, EC: electrical conductivity (µS/cm) and COD: chemical oxygen demand (mg/L).

Table 12. Levels of total and F-specific RNA coliphages, crAssphage and human enteric viruses (norovirus GI and GII, HAV, HAstV, RV) of wash water from the washing tanks of baby leaves and bell peppers in commercial lines using chlorine (FC) and peracetic acid (PAA), respectively.

Process Wash Water	Sampling place	Sampling date	Sanitizer	Total coliphages[1]	F-specific RNA coliphages[1]	crAssphage[1]	Human enteric virues[1]
Baby leaves	Washing tank	25/01/2022	FC	Nd[2]	Nd	2.11 ± 0.08	Nd
Bell peppers	Washing tank	09/11/2021	PAA	3.84 ± 0.01	3.15 ± 0.06	3.34 ± 0.48	Nd

[1] Bacteriophage load expressed as log pfu/L and viral load as log genome copies/L.
[2] Nd: no detected.

Figure 1. Effect of free chlorine (FC) on murine norovirus (MNV), hepatitis A virus (HAV) (panel A) and MS2 (panel B) infectivity/viability in PWW from peppers, veggie mix and baby leaves.

** Orange lines: MNV titers; Green lines: HAV titers; Yellow lines: MS2 titers; Blue dashed lines: level of sanitizer; Grey lines represent viral concentrations below limit of detection of the assay*

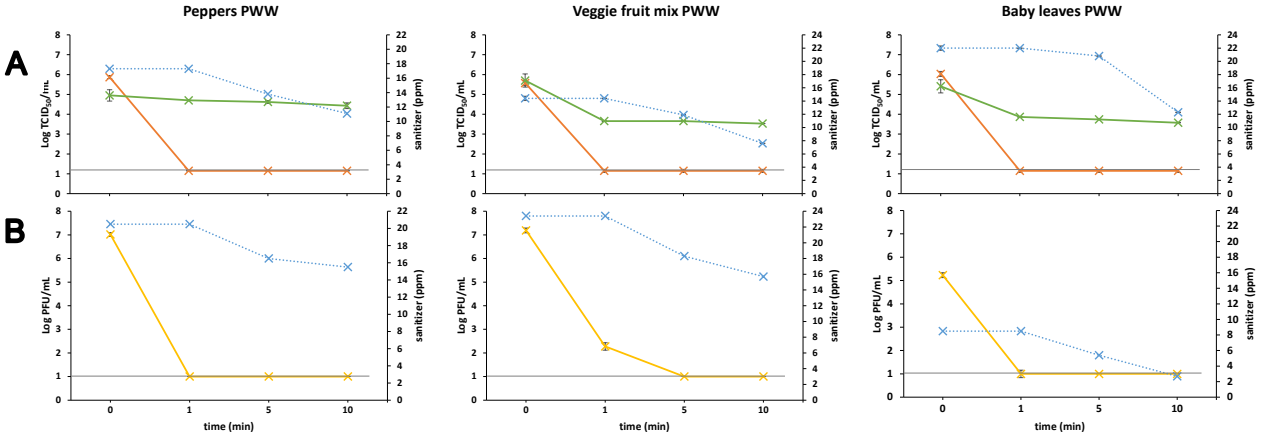

Figure 1. Effect of peracetic acid (PAA) on murine norovirus (MNV), hepatitis A virus (HAV) (panel A) and MS2 (panel B) infectivity/viability in PWW from peppers, veggie mix and baby leaves.

**Orange lines: MNV titers; Green lines: HAV titers; Yellow lines: MS2 titers; Blue dashed lines: level of sanitizer; Grey lines represent viral concentrations below limit of detection of the assay*

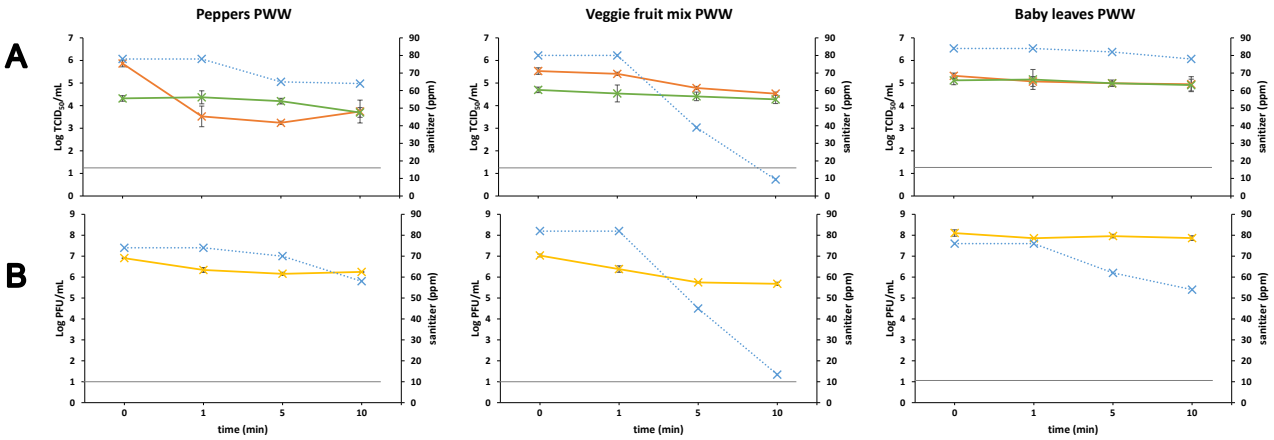

Figure 2. Effect of chlorine dioxide (ClO_2) on murine norovirus (MNV), hepatitis A virus (HAV) (Pane A) and MS2 (Panel B) infectivity/viability in PWW from peppers, veggie mix and baby leaves.

**Orange lines: MNV titers; Green lines: HAV titers; Yellow lines: MS2 titers; Blue dashed lines: level of sanitizer; Grey lines represent viral concentrations below limit of detection of the assay*

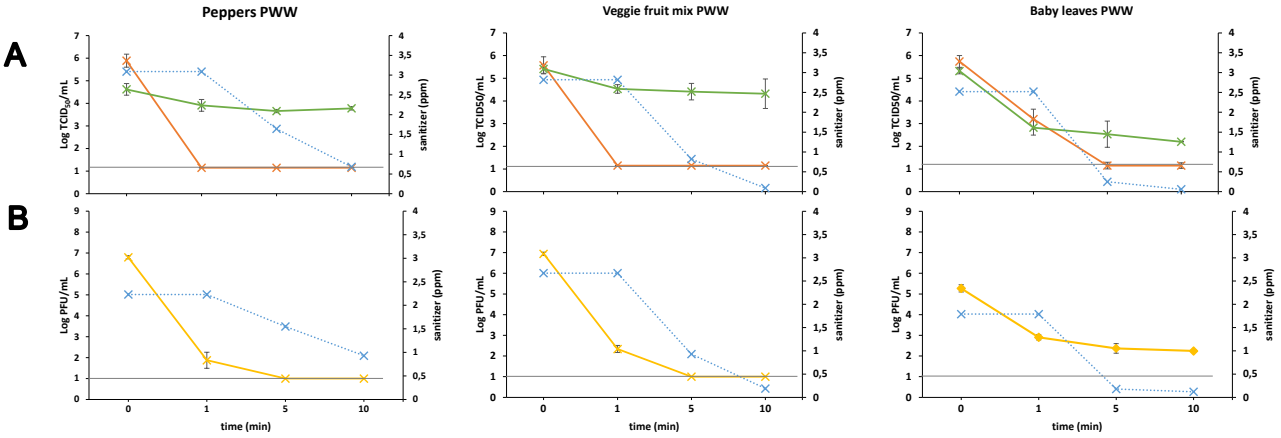

Figure 4. Decay of free chlorine (FC), chlorine dioxide (ClO_2) and peracetic acid (PAA) and Tulane virus (TV) inactivation in PWW from peppers, veggie mix and baby leaves.

*Red lines: TV titers; Blue dashed lines: level of sanitizer; Grey lines represent viral concentrations below the limit of detection of the assay

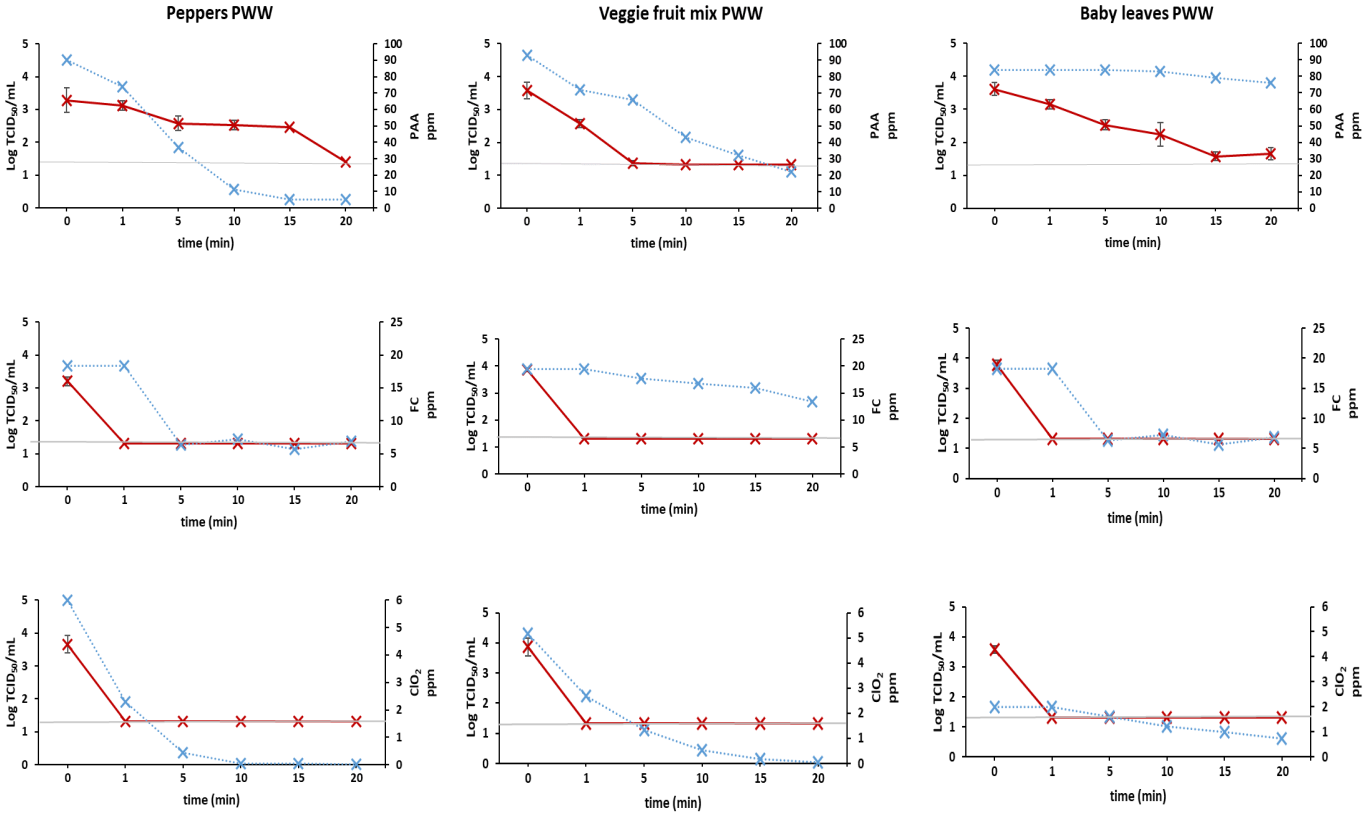

Figure 5. Asymptotic regression models predicting Tulane virus inactivation and free chlorine (FC) sanitizer decays in PWWs. Panel A: Baby leaves PWW sanitized using free chlorine (FC); Panel B: Bell pepper PWW sanitized using chlorine dioxide (ClO_2); Panel C: Veggie fruit mix PWW sanitized with chlorine dioxide (ClO_2).

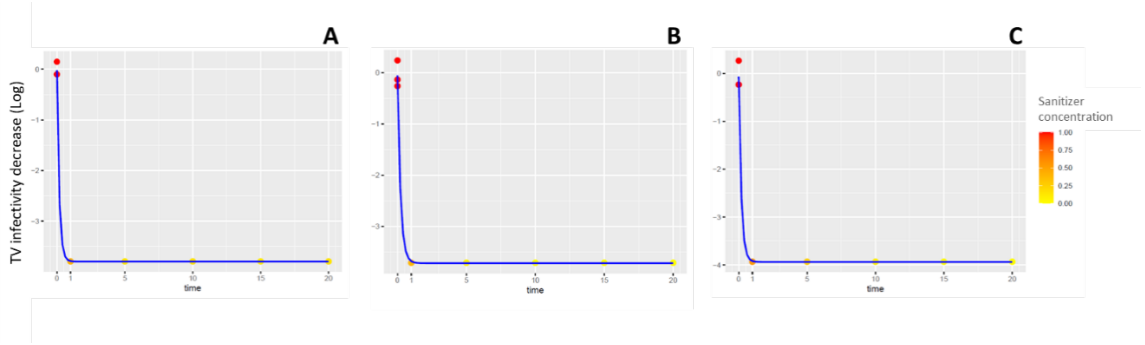

Figure 6. Exponential decay model predicting Tulane virus inactivation and peracetic acid (PAA) decay in bell pepper (Panel A) and veggie fruit mix PWWs (Panel B).

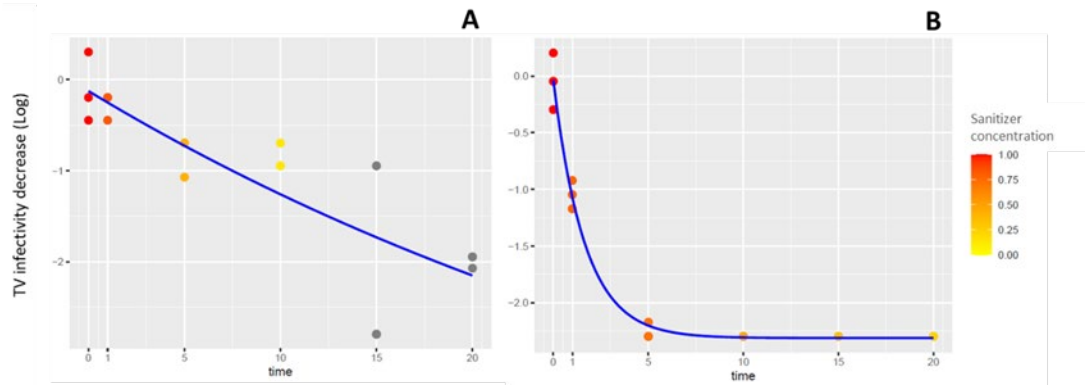

Figure 7. Changes in MS2 during free chlorine disinfection (5 ppm) of PWW from baby leaves (A), bell peppers (B) and veggie fruit mix (C) when increasing chemical oxygen demand (0 – 500 mg/L) along the time (60 min). Results are means of at least two repetitions ± standard deviation.

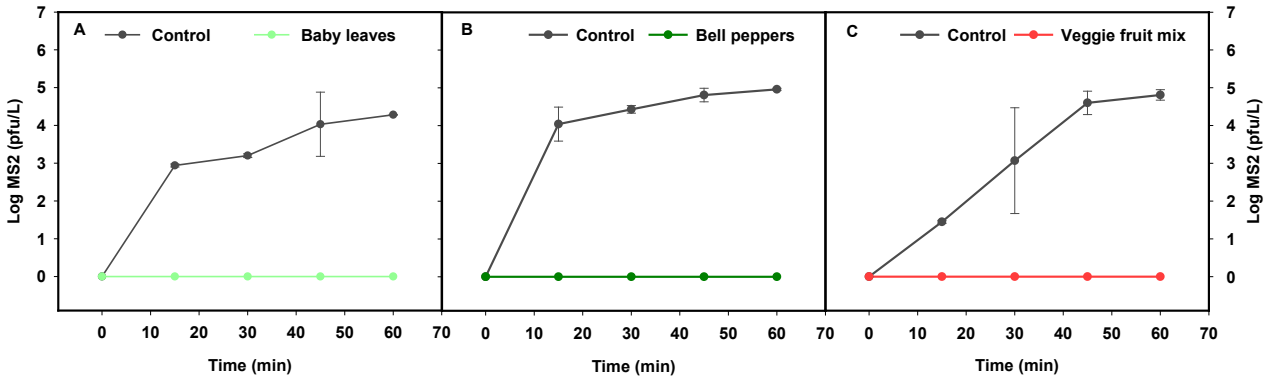

Figure 8. Changes in MS2 levels during ClO_2 (2-3 mg/L) of PWW from baby leaves (A), bell peppers (B) and veggie fruit mix (C) when increasing chemical oxygen demand (0 – 500 mg/L) along the time (60 min). Results are means of at least two repetitions ± standard deviation.

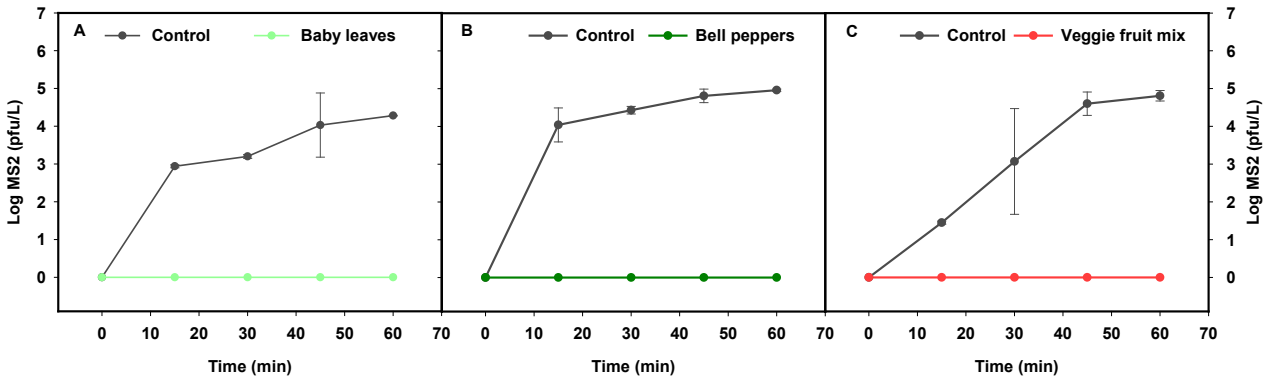

Figure 9. Changes in MS2 levels during PAA (80 mg/L) disinfection of PWW from baby leaves (A), bell peppers (B) and veggie fruit mix (C) when increasing chemical oxygen demand (0 – 500 mg/L) along the time (60 min). Results are means of at least two repetitions ± standard deviation.

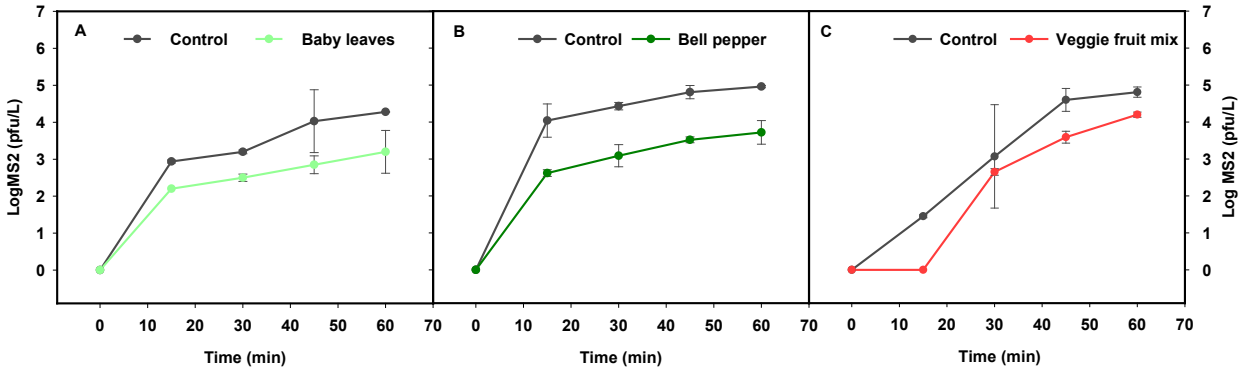

Figure 10. Changes in MNV during PAA (80 mg/L) disinfection of process wash water from washing baby leaves and bell peppers when increasing chemical oxygen demand along the time. Results are means of at least two repetitions ± standard deviation.

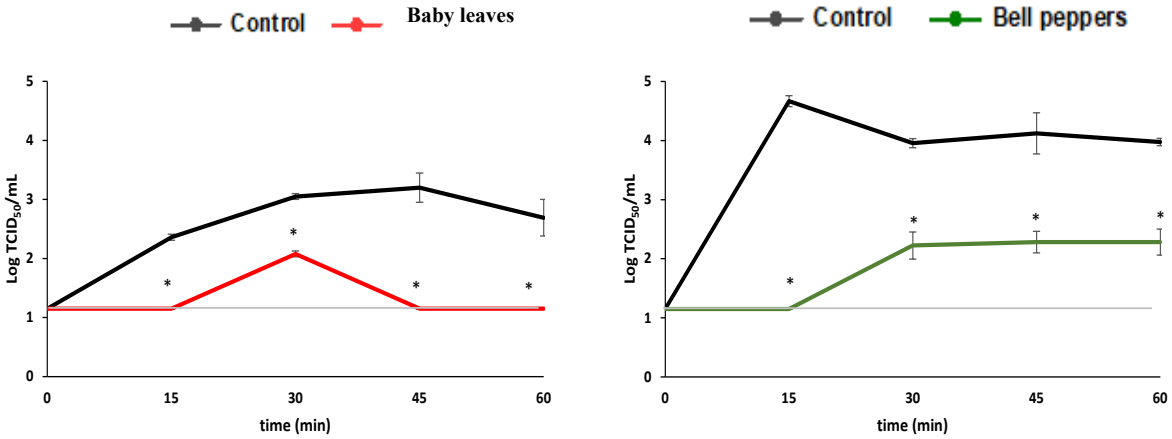

Figure 11. Kinetics of coliphage MS2 in a dymanic system (DS) feeded with a continuous flux of water from washing baby leaves inoculated with MS2. Lines represent the best fit of the model to the observed accumulation data (dots) when using sanitizers such as 5 $mg \cdot L^{-1}$ free chlorine ( ), 2 $mg \cdot L^{-1}$ ClO_2 ( ), 80 $mg \cdot L^{-1}$ PAA ( ), and control ( ) without sanitizer.

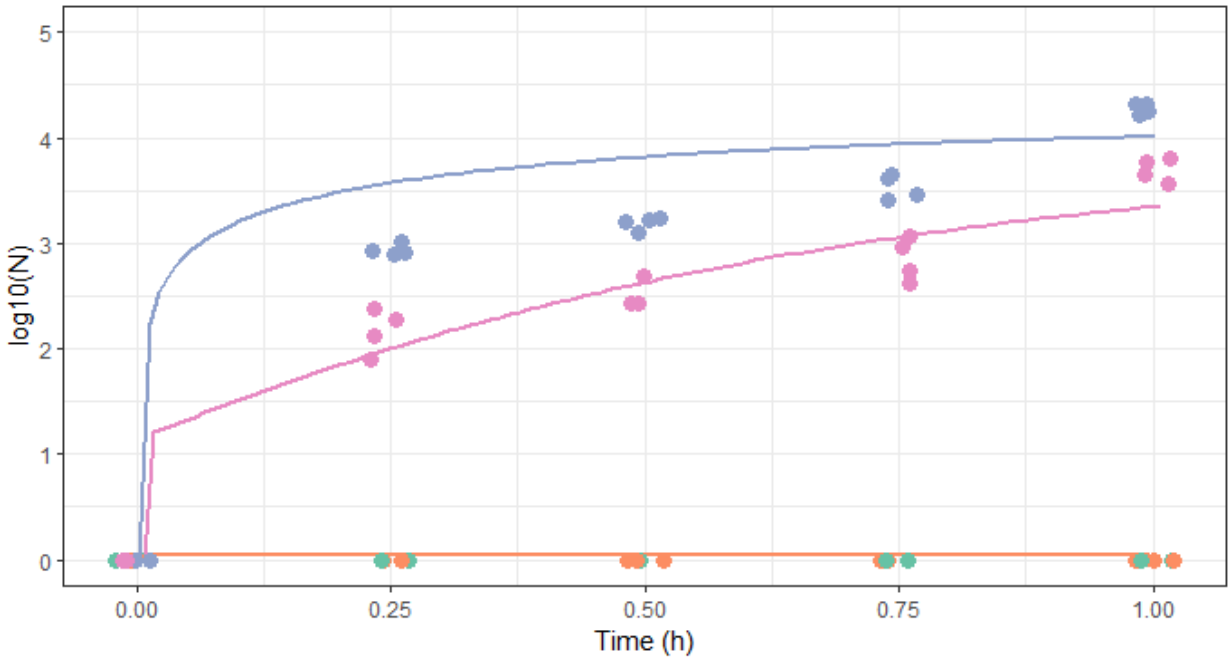

Figure 12. Dynamics of coliphage MS2 in a dynamic system feeded with a continuous flux of water from washing bell pepper with MS2. Lines represent the best fit of the model to the observed accumulation data (dots) when using 5 mg·L^{-1} free chlorine ( ), 2 mg·L^{-1} ClO_2 ( ), 80 mg·L^{-1} PAA ( ), and control ( ) without sanitizer.

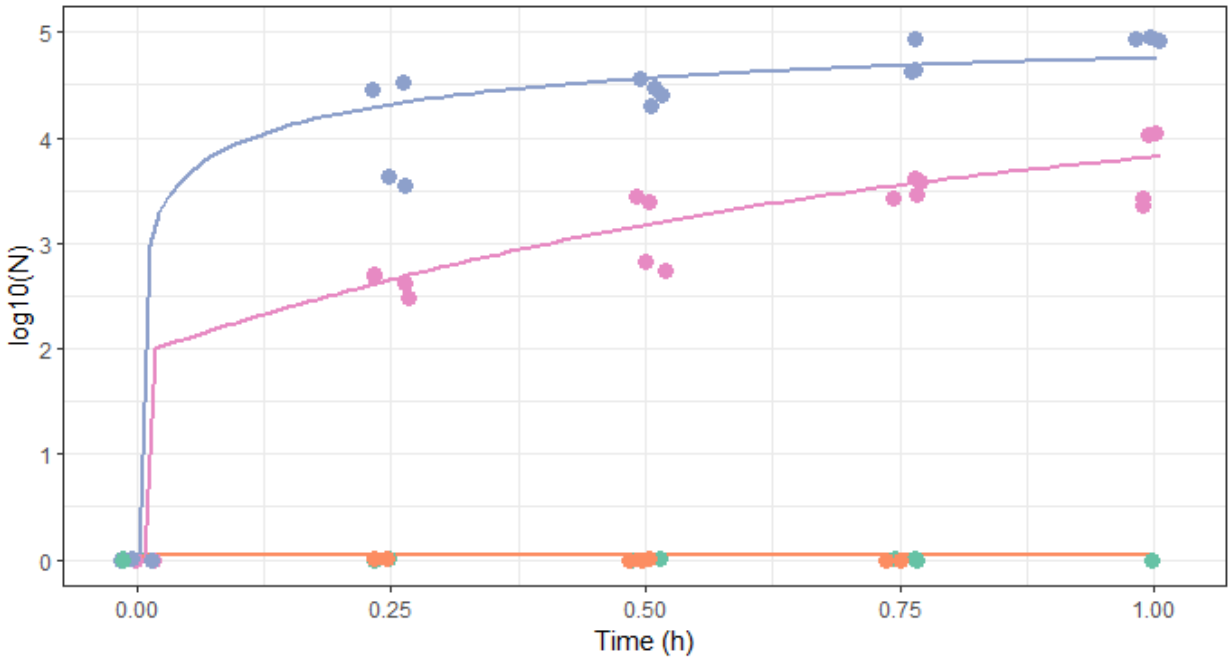

Fig. 13. Dynamics of coliphage MS2 in a dynamic system feeded with a continuous flux of water from washing a vegetable mix inoculated with MS2. Lines represent the best fit of the model to the observed accumulation data (dots) when using 5 mg·L^{-1} free chlorine ( ), 2 mg·L^{-1} ClO_2 ( ), 80 mg·L^{-1} PAA ( ), and control ( ) without sanitizer.