International Journal of Food Microbiology 269 (2018) 128–136

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Efficacy of chlorine dioxide on *Escherichia coli* inactivation during pilot-scale fresh-cut lettuce processing

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ARTICLE INFO

Keywords:
Water disinfection
Microbial cross-contamination
Disinfection by-products
Fresh-cut produce wash
Pilot

ABSTRACT

Controlling water quality is critical in preventing cross-contamination during fresh produce washing. Process wash water (PWW) quality can be controlled by implementing chemical disinfection strategies. The aim of this study was to evaluate the pilot-scale efficacy of chlorine dioxide (ClO_2) during processing on the reduction of *Escherichia coli* in the PWW and on processed fresh-cut 'Lollo Rossa' lettuce. The objective was to have a residual target concentration of either 5 or 3 mg/L ClO_2 in the washing tank (3.5 m^3) before and during 800 kg of lettuce processing (90 min). After 90 min., a nonpathogenic, non-Extended Spectrum Beta-Lactamase (ESBL) *E. coli* inoculum from an overnight culture broth (37 °C) was added to the tank resulting in an approximate final level of 10^6 CFU/mL. PWW and lettuce samples for microbiological and chemical analyses were taken before and after the input and supply halted. ClO_2 concentrations quickly decreased after ClO_2 input halted, yet a residual concentration of ≥ 2.5 mg/L and ≥ 2.1 mg/L ClO_2, respectively for 5 and 3 mg/L pilots, was present 12 min after the supply halted. No detectable levels of *E. coli* (limit of detection 5 log) were determined in the water within 1 min after *E. coli* was added to the ClO_2 containing wash water. Results demonstrated that ClO_2 use at the semi-commercial pilot scale was able to reduce the *E. coli* peak contamination in the PWW. After storage (5 days, 4 °C), background microbial communities (*i.e.,* fluorescent Pseudomonads and total heterotrophic bacteria) grew out on lettuce. Overall, ClO_2 decreased the potential for cross-contamination between batches compared to when no sanitizer was used. Chlorate levels of the lettuce sampled before entering the wash water ranged from 7.3–11.6 μg/kg. The chlorate levels of the lettuce sampled after being washed in the ClO_2 containing wash water, as well as after rinsing and centrifugation, ranged from 22.8–60.4 μg/kg; chlorite levels ranged from 1.3–1.6 mg/kg, while perchlorate levels were below the limit of quantification (LOQ, < 5 ng/g). In this study, we report the semi-commercial pilot-scale evaluation of ClO_2, for its ability to maintain the PWW quality and to prevent cross-contamination in the washing tank during fresh-cut lettuce processing. Furthermore, we provide quantitative values of ClO_2 disinfection by-products chlorate and chlorite as well as of perchlorate from PWW and/or lettuce samples.

1. Introduction

The ability to guarantee fresh(−cut) produce safety has become arduous despite control measures at all stages of the chain. Callejón et al. (2015) have provided evidence that pathogenic contamination of fresh produce significantly contributes to the overall burden of foodborne disease. One of the main obstacles in supplying safe and high quality fresh(−cut) produce, which thereby prevents foodborne disease, originates from the overwhelming responsibility required by all actors along the fresh produce chain (*i.e.,* from farm to fork) to ensure safety despite the limited control measures available for such minimally processed products. Despite their ongoing efforts, there are several sources or pathways by which pathogens can be introduced. Although current practices for actors, such as Good Agricultural Practices (GAP) for primary producers and Food Safety Management Systems (FSMS) for processors, aim to prevent the potential for (cross−) contamination, the burden is still present (Kirezieva et al., 2013). Particularly, the post-harvest processor is substantially responsible for the safety and quality albeit fresh(−cut) produce may only be minimally processed.

During post-harvest processing, produce washing can help to

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https://doi.org/10.1016/j.ijfoodmicro.2018.01.013
Received 27 July 2017; Received in revised form 23 December 2017; Accepted 10 January 2018
Available online 31 January 2018

remove soil and debris. However, washing can also serve as a potential pathway for pathogenic (cross−) contamination in the wash tank. This can occur when bacterial cells release from contaminated produce into the PWW and then attach to uncontaminated produce; thus, leading to batch contaminations (Banach et al., 2017; Danyluk and Schaffner, 2011; Gil et al., 2009; Jensen et al., 2015; López-Gálvez et al., 2010a; Luo et al., 2014; Van Haute et al., 2013). Despite this washing conundrum, the processor can influence the potential for pathogenic (cross−) contamination by implementing technical and managerial controls on the water used during processing. These controls may include wash water disinfection strategies such as the use of chemical sanitizers in the PWW. Therefore, fresh(−cut) produce washing, that is with the use of chemical sanitizers, may be a potential solution to combat cross-contamination *via* the PWW.

Several research groups have argued that the aim of PWW disinfection - with sanitizers - during produce processing is to reduce the likelihood of cross-contamination (Allende et al., 2008; Baert et al., 2009; Banach et al., 2015; Chardon et al., 2016; Danyluk and Schaffner, 2011; Davidson et al., 2013; Gil et al., 2009; Gil et al., 2015; Holvoet et al., 2012; López-Gálvez et al., 2009; López-Gálvez et al., 2010b; Luo et al., 2014; U.S. Food and Drug Administration (USFDA), 2009; Van Haute et al., 2015b; Zhao et al., 2009). Although the application of chlorine during fresh (−cut) produce washing has been a prominent choice for industry, potential health, and environmental concerns as raised by *e.g.*, European countries, like the formation of carcinogenic compounds (*e.g.*, trihalomethanes (THMs)), have prompted research for alternatives (Artés et al., 2009; Gil et al., 2009; Gómez-López, 2012; Joshi et al., 2013).

This study aims to evaluate the pilot-scale efficacy of chlorine dioxide (ClO_2) during processing on the reduction of *Escherichia coli* in the PWW and on processed fresh-cut 'Lollo Rossa' lettuce. Foremost, we assess the impact that a residual concentration of ClO_2 had during washing (*in-situ*) on the microbiological and chemical safety of the PWW. Secondarily, we examine the efficacy of ClO_2 on processed fresh-cut lettuce to demonstrate if ClO_2 can prevent cross-contamination in the washing tank. Our study presents an innovative method of investigating the pilot-scale application of ClO_2 as a PWW sanitizer during fresh-cut lettuce processing while considering the potential presence of disinfection by-products (DBPs) like chlorate and chlorite as well as of perchlorate.

2. Materials and methods

2.1. Experimental design

Preliminary pilot experiments evaluated the chemical parameters of the PWW (pH, ammonium-N (NH_4 –N), nitrate-N ((NO_3 + NO_2) –N), phosphate-P (PO_4 –P), and total organic carbon (TOC)) during 180 min of processing (data not shown). Since the TOC after 90 min (43 mg/L) was similar to that after 180 min (45 mg/L), 800 kg of 'Lollo Rossa' lettuce processed in 90 min was investigated during the ClO_2 pilots to allow an accumulation of organic compounds in the PWW.

Based on the potential practical application of a minimum ClO_2 concentration and the results of previous lab experiments, ClO_2 concentrations of 5 and 3 mg/L were assessed (data not shown); each concentration was tested in duplicate during the pilots. The application of ClO_2 (5 mg/L), also given minimum effective concentrations, warranted further research as a potential process wash water disinfectant during pilot-scale processing (Banach et al., 2017). ClO_2 concentrations were evaluated against *E. coli* by processing an 800 kg batch of 'Lollo Rossa' lettuce in 90 min through a 3.5 m^3 commercial washer (Flotation washer, Remie, build year mid-1997), after which PWW was inoculated with *E. coli* to achieve a final level of 10^6 CFU/mL *E. coli*. Pilot trials with sanitizer-free water and non-supplemented *E. coli* served as controls. During lettuce processing, water and lettuce samples were collected and quantitatively examined for the presence of *E. coli* as well as chlorate, chlorite, and/or perchlorate. Water samples were also analyzed for pH, NH_4 –N, NO_3 + NO_2 –N, PO_4 –P, TOC, and chemical oxygen demand (COD). Pre-processing and post-processing swabs of the processing line after routine cleaning were quantified for *E. coli*.

2.2. 'Lollo Rossa' lettuce

Crated shipments of a loose leaf-type lettuce (*Lactuca sativa* var. crispa 'Lollo Rossa') grown in Spain were obtained from a supplier of the processor; one supply per experimental run was used. Lettuce was stored in a 4 °C walk-in cooler and used within 2–3 days of delivery. Directly before the lettuce entered the processing line, it was cored with a knife, and any damaged outermost leaves were removed (pre-trimmed). In short, lettuce was processed by pre-trimming (by hand), shredding, conveying, vibrating, washing, rinsing, centrifuging, and when applicable, were packaged.

2.3. Bacterial strain preparation

A nonpathogenic, non-Extended Spectrum Beta-Lactamase (ESBL) *E. coli* strain (12–123.2) was obtained from the National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands. The strain had been isolated from surface water. The strain was maintained at −80 °C in Luria Broth (LB; L1704 LB Broth High Salt, Duchefa Biochemie B.V., Haarlem, The Netherlands) supplemented with 25% (v/v) glycerol. Bacteria were streaked on Brilliance *E. coli* coliform selective agar (BECSA; CM1046, Oxoid Ltd., Basingstoke, United Kingdom).

Cultures were prepared by inoculation of a single colony from BESCA by growing them overnight (16-18 h) in 25 mL LB at 37 °C in a 200 rpm shaking air incubator. Afterwards, 0.5 mL of the culture was added to 2 L Erlenmeyer flasks filled with 500 mL of LB. After growth for 18 (± 1) h, to obtain stationary phase cells, the liquid cultures (c.a. 10^9 CFU/mL) were collected in a 5 L plastic container. All liquid (c.a. 4.5 L) was added directly to the wash tank.

2.4. Processing line

A semi-commercial lettuce processing line, housed at 10 °C, and capable of processing 400 kg/h, or approximately 800 kg/90 min, was used. It consisted of a lettuce shredder, step conveyer, infeed vibrator, washer, and hand centrifuge. Tap water (7 °C, optimally 4 °C, pH ~7.2–7.6) was pumped into a 3.5 m^3 stainless steel washer, which had a refreshment rate of 1000–1500 L/h. The washer was modified with a stainless steel cover with a sliding inlet, for *E. coli* supply. ClO_2 was produced *in situ* under controlled conditions (in a specially developed generator – P3-Oxy-Gen^{Plus} 170, Ecolab B.V., Nieuwegein, The Netherlands) by mixing and dilution of two precursors: P3-Oxonet, a sodium chlorite ($NaClO_2$) solution, and P3-Oxodes a hydrochloric acid (HCl) solution; hence, it is referred to as the acid-chlorite reaction. This reaction is described as follows:

$$5NaClO_2 + 4HCl \rightarrow 4ClO_2 + 5NaCl + 2H_2O$$

ClO_2 and fresh water were supplied *via* air inlets to the washer furthest from the product inflow and *E. coli* supply. Water flow was in a circular motion (front to back to front) in the washing tank, and product inflow was facilitated with the use of product transport paddles. Product outflow was collected and centrifuged before packaging on-site.

2.5. Semi-commercial pilots

Pilot control and treatment trials (n = 6) were performed in duplicate. For treatment trials, we aimed to have a ClO_2 residual concentration of either 5 or 3 mg/L in the washing tank before and during 800 kg of 'Lolla Rossa' lettuce processing. Half of the trials were

Table 1
Pilot control and treatment trials (n = 6), in duplicate, including the day of the trial, measured chlorine dioxide (ClO_2) concentrations (mg/L) at 80 min in the process wash water (PWW) and final level of *E. coli* (log CFU/mL) at 90 min in the washer.

Pilot trial[a]	Day of trial	ClO_2 (mg/L) at 80 min	*E. coli* (log CFU/mL) added at 90 min
Control A.1	2	–	–
Control A.2	4	–	–
Control B.1	1	–	6.5
Control B.2	3	–	5.8
Treatment A.1	1	5.2	–
Treatment A.2	3	3.4	–
Treatment B.1	2	6.7	6.2
Treatment B.2	4	4.5	6.1
Treatment C.1	5	3.1	–
Treatment C.2	6	2.8	–
Treatment D.1	5	3.6	6.3
Treatment D.2	6	2.8	6.2

[a] Each control or treatment trial was performed in duplicate as indicated by 1 and 2. Control trials were performed without addition of ClO_2 and either without (Control A) or with (Control B) *E. coli*. Treatment trials were performed with ClO_2, of which either 5 mg/L (Treatments A and B) or 3 mg/L (Treatments C and D) were tested. Of the treatment trials, *E. coli* was added during Treatments B and D.

evaluated with inoculated *E. coli* (Table 1). The concentration of ClO_2 was monitored and manually adjusted to obtain the desired concentration; measurements were performed every 15–20 min and repeated for verification as required. After 800 kg of lettuce had been fed into the shredder, the produce inflow and the ClO_2 supply halted to be able to distinguish the effect of ClO_2 had *versus* that from a dilution effect. During the pilots with *E. coli* addition, the strain (~4.5 L) was simultaneously added to the tank at the time the ClO_2 input and lettuce input halted; this resulted in a final level of approximately 10^6 CFU/mL. During this time, the processing line continued operating, resulting in the remaining outflow of lettuce.

Pilot trials occurred over six different days. There were two pilot trials per day, of which the first one was without the supplemented *E. coli* and the second one with the supplemented *E. coli*. There were four days with ClO_2 pilot trials, two with 5 mg/L and two with 3 mg/L as the targeted disinfectant concentration (Table 1). The equipment was cleaned between each run and was thoroughly sanitized at the end of the day. Equipment was swabbed with swab rinse kits (SRK; 922C,CR, SRK 10 mL TRIPLE PACKED, Copan Italia SpA, Brescia, Italy) pre- and post- processing for inoculated *E. coli* at the (i) infeed vibrator, (ii) front wall of the washer, (iii) rear wall of the washer, and (iv) output band of the washer to verify hygiene. Subsequently, 100 μL of the undiluted swab fluid was plated on BECSA and incubated at 37 °C for 24 h. Furthermore, during the 3 mg/L pilots, swab samples were quantified on King's B Agar (KB; K5165 KB Medium, Duchefa Biochemie B.V., Haarlem, The Netherlands) and R2A Agar (218262 Difco™ R2A Agar, BD Diagnostics, Breda, The Netherlands) to determine background microbial communities, respectively, fluorescent Pseudomonads and total heterotrophic plate counts. Plates were incubated at 35 ± 2 °C for 42–48 h.

2.6. Sample collection

PWW samples for microbiological analyses were collected (2 L) from the wash tank at 80 min (*i.e.,* before the lettuce input and ClO_2 supply halted at 90 min) as well as at 91, 93, 96, and 102 or 110 min (Table 2). Samples to determine the ClO_2 concentration of the PWW were collected (50 mL) in duplicate periodically throughout processing. These samples were processed on-site. PWW samples for chemical analyses (pH, NH_4 –N, NO_3 + NO_2 –N, PO_4 –P, TOC, COD, chlorate, chlorite, and perchlorate) were collected at the same time points as for the microbiological analyses. Additionally, water from the centrifuged lettuce samples that were processed on-site was collected during the pilots with 3 mg/L (*i.e.,* Treatments C and D in Table 1) and analyzed for the potential presence of chlorate and perchlorate. All water samples were collected in sealed containers and transported under refrigerated conditions to the laboratory.

Lettuce samples for microbiological analyses were sampled before lettuce entered the wash tank (*i.e.,* after the lettuce was pre-trimmed and shredded), after lettuce exited the wash tank at 80 min (*i.e.,* before the lettuce input and ClO_2 supply halted at 90 min) as well as at 91, 93, and 95 min (Table 2). These samples were processed on-site as described in Section 2.7. Additionally, lettuce samples were collected for storage and further analyses before the lettuce entered the wash tank and between 2 and 3 min after the lettuce input and ClO_2 supply halted (*i.e.,* between 92 and 93 min). These samples were subsequently rinsed and centrifuged before packaging, to dilute out ClO_2 and to remove unattached *E. coli* cells. All samples were packaged on-site and transported under refrigerated conditions to the laboratory for microbiological and chemical analyses. Packaged lettuce samples were stored for 5 days at 4 °C before microbiological analyses. During the pilots with 3 mg/L, chlorate and perchlorate levels of the lettuce were analyzed to assess the potential transfer. These lettuce samples were stored at 4 °C and extracts from 3 to 5 samples of the packaged lettuce of about 68 g (SD ± 15 g) each, were prepared within 5 days. Extracts were stored at −20 °C until analyses.

2.7. Microbiological analyses

Preliminary pilot experiments were performed before pilot trials; they evaluated the microbiological parameters of the PWW on Luria Broth agar (LBA) plates containing 1.2% agar Luria Broth (LBA; No. 241420 Difco™ Luria Broth Base, BD Diagnostics, Breda, The Netherlands) and BECSA. Plates were incubated at 37 °C for 24 h with daily inspection of colonies for up to 5 days to check if potentially damaged cells could eventually grow out. Total viable counts demonstrated a negligible increase over time and averaged 4 log CFU/mL during processing (data not shown). There was no observed difference between CFUs quantified on LB and BECSA (data not shown). Hence during the pilot trials, PWW was determined on BECSA. Samples were collected as described in Section 2.6.

During the pilot trials, directly after collection of the PWW samples, 100 μL was directly plated, and 1 mL was serially diluted into a peptone physiological salt solution (PPS; Tritium Microbiologie B.V., Eindhoven, The Netherlands). Then, 100 μL of the appropriate dilutions were plated. Plates were incubated at 37 °C for 24 h with daily inspection of colonies for up to 5 days to check if potentially damaged cells could eventually grow out.

Furthermore, during the pilot trials, lettuce samples for on-site analyses were collected from the outflow of the processing line and were directly hand-centrifuged (Zyliss Smart Touch Salad Spinner, Farnborough, United Kingdom). Approximately 10 g of lettuce was washed twice with potable water to simulate commercial processing conditions. Packaged lettuce samples were collected and processed as described in Section 2.6. Lettuce samples were transferred to BioReba (10 mL volume) bags (Bioreba AG, Reinach, Switzerland) in which 10 mL of sterile Ringer's solution (BR0052; 177 Oxoid, part of Thermo Fisher Scientific, Breda, The Netherlands) was added and gently homogenized. Tenfold serial dilutions of Ringer's solution were made from the lettuce homogenates. Then, 100 μL of undiluted and diluted lettuce homogenates were pipetted onto BECSA. Afterward, liquid drops were spread over the agar surfaces to allow enumeration of individual CFUs of *E. coli* following incubation at 37 °C for 24 h. Additionally, during the 3 mg/L pilot trials, lettuce samples were quantified on KB and R2A, to determine fluorescent Pseudomonads and total heterotrophic plate counts, respectively, and incubated at 35 ± 2 °C for 42–48 h. For lettuce samples, the effect of ClO_2 before and after addition of *E. coli* to the PWW on fluorescent Pseudomonads (KB) and total heterotrophic bacteria (R2A) was calculated and used for

Table 2
Overview of sampling plan during pilot trials.

Time	Event and/or sample[a]
$t_{pre-trials}$	Swab pre-trials Locations: input band, inside of the wash tank – front and rear, output trill
$t_{start} = t_0 - 90$ min (0 min)	**Start** process line and monitor input
	Start ClO_2 supply
	Monitor ClO_2 levels Check and record the ClO_2 concentration (water) and short-term exposure limits (air) at 10-15 minute intervals. Adjust the pump when necessary (record).
	Control sample: take lettuce samples after shredding
$t_0 - 10$ min (80 min)	**1st samples**: take water and lettuce samples
	MO: process 1st lettuce sample
	MO: analyze 1st wash water sample
	CHEM: analyze ClO_2 content 1st wash water sample
t_0 (90 min)	**Stop** input of lettuce
	Stop ClO_2 supply
	MO: addition of *E. coli* (when applicable), supplied furthest from ClO_2 inlet
$t_0 + 1$ min (91 min)	**2nd samples**: take water and lettuce samples
	MO: process 2nd lettuce sample
	MO: analyze 2nd wash water sample
	CHEM: analyze ClO_2 content 2nd wash water sample
$t_0 + 2/3$ min (92–93 min)	**Take packaged samples for packaging (microbiological) and chemical analyses**
$t_0 + 3$ min (93 min)	**3rd samples:** take water and lettuce sample
	MO: process 3rd lettuce sample
	CHEM: take and analyze ClO_2 content 3rd wash water sample
$t_0 + 5$ min (95 min)	**4th samples:** take lettuce samples
	MO: process 4th lettuce sample
$t_0 + 6$ min (96 min)	**4th samples:** take water samples
	MO: analyze 4th wash water sample
	CHEM: analyze ClO_2 content 4th wash water sample
$t_0 + 12$ min or $t_0 + 20$ min (102 or 110 min)	**5th samples:** take water sample
	MO: analyze 5th wash water sample
	CHEM: analyze ClO_2 content 5th wash water sample
$t_{end} = t_0 + 13$ min or $t_0 + 21$ min (103 or 111 min)	Store plates and wash water samples for chemical analyses
	Empty and clean process line
$t_{post-trials}$	Swab post trials Locations: input band, inside of the wash tank – front and rear, output trill

[a]MO = microbiological; CHEM = chemical.

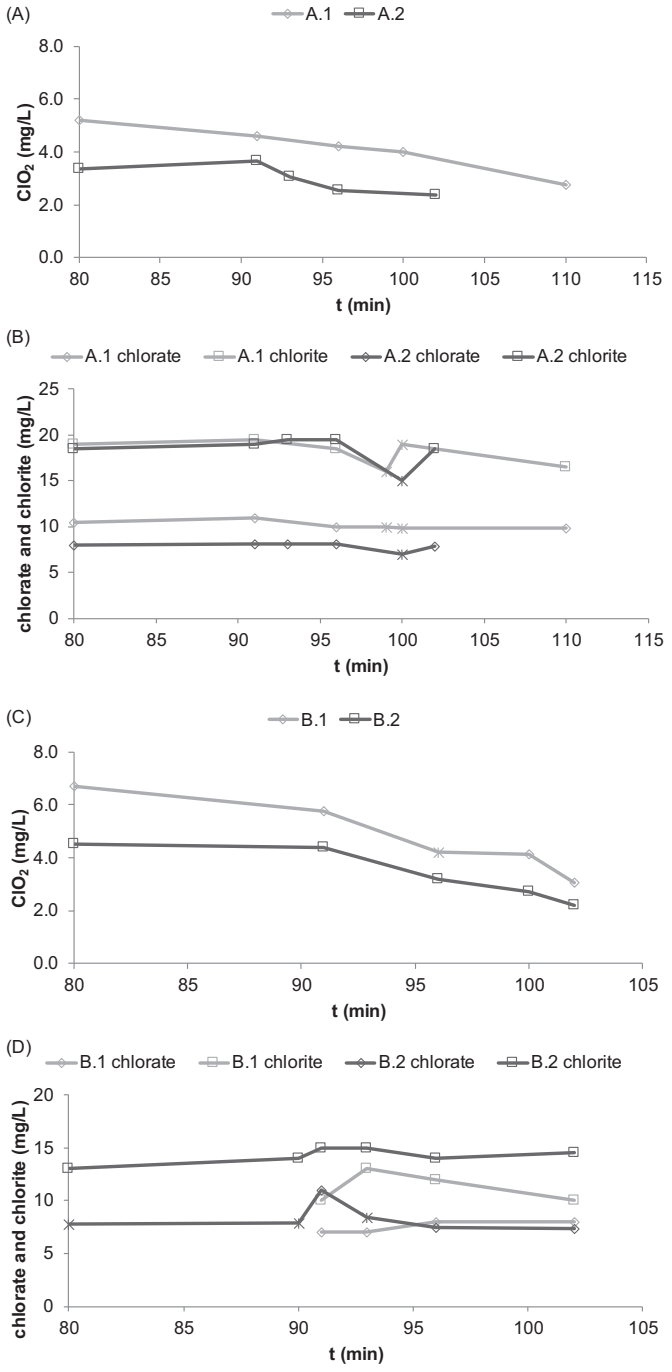

Fig. 1. Pilot trials at 5 mg/L chlorine dioxide (ClO_2): (A–B) Treatment A without *E. coli* and (C–D) Treatment B with *E. coli*. Each pilot treatment was performed twice. (A, C) represent the concentration of ClO_2 in the PWW during the trials. Data represent duplicate measurements; single measurements are starred. (B, D) represent the concentration of chlorate (◊) and chlorite (□) in PWW during the pilots. A.1, A.2, and B.2 are in duplicate; single measurements are starred. B.1 are single measurements.

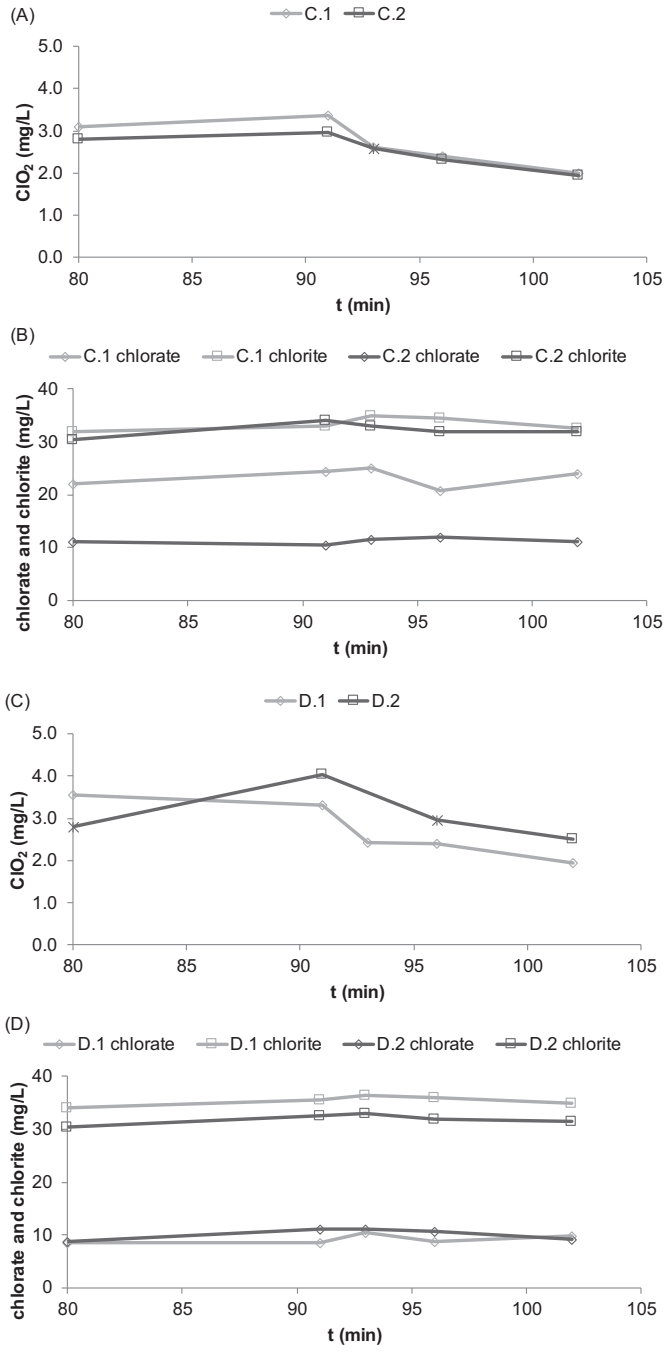

Fig. 2. Pilot trials at 3 mg/L chlorine dioxide (ClO_2): (A–B) Treatment C without *E. coli* and (C–D) Treatment D with *E. coli*. Each pilot treatment was performed twice. (A, C) represent the concentration of ClO_2 in the PWW during the trials. Data represent duplicate measurements; single measurements are starred. (B, D) represent the concentration of chlorate (◊) and chlorite (□) in PWW during the pilots. Data represent duplicate measurements.

statistical comparison using Student's *t*-test (GenStat release 12.1, Hemel Hempstead, United Kingdom).

2.8. Chemical analyses

ClO_2 was analyzed with the DULCOTEST® DT4B and DT1B photometer (ProMinent Verder B.V., Vleuten, The Netherlands) and the Hach Lange DR 2800™ spectrophotometer (Hach, Tiel, The Netherlands). The pH was determined with a Beckman Φ34 pH meter; NH_4 –N, NO_3 + NO_2 –N, and PO_4 –P were analyzed, all in 0.01 M $CaCl_2$, with a Skalar segmented flow analyzer (SFA; model SAN + +); and TOC was analyzed with a Skalar SFA, model SAN + + in accordance with NEN-EN 1484 (Chemical Biological Soil Laboratory, Wageningen, The Netherlands). COD was determined according to the sealed tube method in accordance with NEN-ISO 15705 (Waterlab Noord, Glimmen, The Netherlands). Chlorite concentrations were determined by ion chromatography in accordance with NEN-EN-ISO 10304-4 (Vitens N.V., Leeuwarden, The Netherlands). Chlorate and perchlorate concentrations were determined by liquid chromatography–tandem

mass spectrometry (LC–MS/MS) according to the Quick Polar Pesticides (QuPPE) Method, as described by Anastassiades et al. (2013), at RIKILT Wageningen University & Research, Wageningen, The Netherlands.

3. Results

3.1. Pilot process washing water assessment

An overview of the pilot control and treatment trials including the day of the trial, the concentration of ClO_2 measured at 80 min in the PWW, and the final level of *E. coli* in the wash tank, which was added at 90 min, is shown in Table 1. For Control A, Treatment A, and Treatment C pilot trials, *E. coli* had not been supplemented to the washing water, nor was it detected in the water samples measured thereafter. For Control B pilot trials, *E. coli* was added at 90 min to the non-ClO_2 containing PWW; results showed no reduction of *E. coli* in the water samples measured thereafter (Table 1). Results of pilot trials with 5 mg ClO_2/L (Treatment B) and 3 mg ClO_2/L (Treatment D), during which *E. coli* had been added to the PWW at 90 min, showed that *E. coli* were not detected in the PWW when analyzed on BESCA 1 min after *E. coli* was added to the PWW (*i.e.,* at 91 min) (data not shown). Furthermore, PWW samples measured thereafter (*i.e.,* at 93, 96, 102, or 110 min) showed that *E. coli* were not detected, and thus, at least a 5 log reduction occurred (data not shown).

The concentration of ClO_2, as well as byproducts chlorate and chlorite in the PWW, are shown in Fig. 1 and Fig. 2 for pilot treatments with a targeted ClO_2 concentration of 5 and 3 mg/L, respectively, including trials without *E. coli* (A-B) and with *E. coli* (C-D). For these pilot trials, before 'Lollo Rossa' lettuce processing began, the washing tank had a starting concentration of $ClO_2 \geq 5$ or 3 mg/L, respectively. The concentration of ClO_2 in the PWW ranged from 3.4–6.7 mg/L and 2.8–3.6 mg/L after 80 min of processing, respectively for 5 and 3 mg/L pilots (Fig. 1 and Fig. 2). A downward trend in ClO_2 in the PWW after 90 min was visualized for pilots tested without and with *E. coli*, yet a residual concentration of ClO_2 being ≥ 2.5 mg/L and ≥ 2.1 mg/L, respectively for 5 and 3 mg/L pilots, was observed at 102 min (*i.e.,* 12 min after the input of lettuce and ClO_2 supply halted) (Fig. 1 and Fig. 2). Chlorate and chlorite in the PWW ranged from 8–11 mg/L and 13–19 mg/L, respectively, after 80 min of processing for the 5 mg/L pilots (Fig. 1). Similarly, for the 3 mg/L pilots, chlorate and chlorite ranged from 9–22 mg/L and 31–34 mg/L, respectively (Fig. 2). Additionally, during 3 mg/L pilots, the concentration of perchlorate was < 20 mg/L (limit of detection, LOD) for all sampled time points in the PWW.

In addition, several quality parameters of the PWW (NH_4 –N, NO_3 + NO_2 –N, PO_4 –P, pH, COD, and TOC) are shown for pilot control trials without ClO_2, *i.e.,* Control A without *E. coli* (A, B) and Control B with *E. coli* (C, D) (Fig. 1S), as well as for 5 and 3 mg/L ClO_2 pilot treatment trials, respectively (Fig. 2S and Fig. 3S). In general, PWW from the pilot trials had a pH range of 7.1–8.0 after 80 min of processing. The pH during processing without ClO_2 (Fig. 1S) were similar to the PWW previously measured during preliminary pilot experiments (data not shown), while the pH during processing with ClO_2 (Fig. 2S and Fig. 3S) was lower, yet demonstrated an increase after 90 min (*i.e.,* after the ClO_2 supply halted). Pilot results for NH_4 –N, NO_3 + NO_2 –N, PO_4 –P after 80 min of processing ranged from 0.1–0.6 mg/L, 3.8–6.1 mg/L, and 0.6–1.2 mg/L, respectively. COD and TOC ranged from 84–800 mg/L and 79–150 mg/L, respectively after 80 min of processing. The TOC after 80 min of processing without ClO_2 (Fig. 1S) was higher (86–132 mg/L) than that of the PWW previously measured during preliminary pilot experiments (data not shown). An upward trend in COD and TOC between 91 and 93 min was observed for pilot trials tested with *E. coli* because of the addition of *E. coli* to the PWW at 90 min.

3.2. Pilot 'Lollo Rossa' lettuce assessment

During pilot trials, the lettuce leaf surface was not inoculated beforehand; rather the *E.coli* was added as a suspension to the wash tank. After addition, *E. coli* cells adhered to leaf surfaces and the difference in adherence was observed between treatment trials with ClO_2 and control trials.

During the 5 mg/L ClO_2 pilot trials (Treatment B), a 2.3 log reduction of *E. coli* was observed on lettuce leaf samples at 93 min compared to Control B pilot trials (without supplemented ClO_2, yet with added *E. coli* at 90 min); *i.e.,* 2.8 log CFU/g leaf – 0.5 log CFU/g leaf = 2.3 log CFU/g leaf (Table 3). Also, the centrifuged rinse water of the lettuce samples analyzed on-site that were measured after the second centrifugation had a 1.2 log reduction of *E. coli* at 93 min compared to Control B pilot trials at 93 min. Stored lettuce samples, collected during 92–93 min of processing, had on average a 2.5 log reduction of *E. coli* compared to Control B pilot trials (Table 3).

Table 3
Effect of control and 5 mg/L chlorine dioxide (ClO_2) treatment pilot trials on *E. coli* (BECSA) for lettuce, lettuce centrifuge rinse water, and after lettuce storage (5 days, 4 °C).

Pilot trial[a]	Treatment		Time	*E. coli* on lettuce ± SD	*E. coli* in 2nd centrifuge rinse water	*E. coli* after storage 5 days, 4 °C
	ClO_2	*E. coli*	(min)	(log CFU/g leaf)[b]	(log CFU/mL water)[b]	(log CFU/g leaf)[b]
Control B.1	No	Yes	80	< 1	< 1	
			91	3.00 ± 0.10	3.84	
			93	2.75 ± 0.051	3.06	4.7
			95	3.32 ± 0.0059	3.49	
Control B.2	No	Yes	80	< 1	< 1	
			91	3.28 ± 0.021	3.43	
			93	2.76 ± 0.023	2.15	4.4
			95	2.66 ± 0.75	3.98	
Treatment B.1	Yes	Yes	80	< 1	< 1	
			91	2.62 ± 0.019	2.00	
			93	0.750 ± 1.06	1.04	0.95
			95	1.14 ± 0.64	< 1	
Treatment B.2	Yes	Yes	80	< 1	< 1	
			91	2.21 ± 0.089	2.66	
			93	0.15 ± 0.21	1.71	3.0
			95	0.15 ± 0.22	< 1	

[a] During Control A (no ClO_2) and Treatment A pilots (5 mg/L ClO_2), *E. coli* was not supplemented to the washing water.
[b] *E. coli* was below the limit of detection (*i.e.,* < 1 log CFU/g on lettuce or < 1 log CFU/mL water).

Table 4
Effect of 3 mg/L chlorine dioxide (ClO_2) treatment pilot trials on *E. coli* (BECSA), on fluorescent Pseudomonad (KB), and on heterotrophic plate counts (R2A) for lettuce analyzed on-site.

Pilot trial	Treatment		Time	*E. coli*[a] ± SD	Fluorescent Pseudomonad ± SD	Heterotrophic plate counts ± SD
	ClO_2	*E. coli*	(min)	(log CFU/g lettuce)	(log CFU/g lettuce)	(log CFU/g lettuce)
Treatment C.1	Yes	No	80	< 1	3.64 ± 0.11	3.90 ± 0.36
			91	< 1	2.82 ± 0.86	3.05 ± 1.05
			93	< 1	2.59 ± 0.26	3.21 ± 0.23
			95	< 1	285 ± 2.01	2.88 ± 0.23
Treatment C.2	Yes	No	80	< 1	3.17 ± 0.092	4.38 ± 0.11
			91	< 1	3.52 ± 0.38	4.31 ± 0.011
			93	< 1	2.80 ± 0.18	3.34 ± 0.21
			95	< 1	3.19 ± 0.16	3.48 ± 0.10
Treatment D.1	Yes	Yes	80	< 1	2.73 ± 0.61	3.22 ± 0.20
			91	1.81 ± 0.012	2.29 ± 0.034	3.10 ± 0.41
			93	1.50 ± 0.43	3.48 ± 0.83	3.24 ± 0.80
			95	1.49 ± 1.24	3.28 ± 0.15	3.36 ± 0.064
Treatment D.2	Yes	Yes	80	< 1	3.08 ± 0.073	3.08 ± 0.12
			91	2.61 ± 0.075	3.44 ± 0.11	3.58 ± 0.18
			93	< 1	3.28 ± 0.23	3.56 ± 0.23
			95	1.08 ± 1.09	3.56 ± 0.067	3.56 ± 0.08

[a] *E. coli* was below the limit of detection (*i.e.,* < 1 log CFU/g on lettuce).

During the 3 mg/L ClO_2 pilot trials (Treatment D), a similar reduction, about 2.0 log reduction of *E. coli*, was observed on lettuce leaf samples at 93 min compared to Control B pilot trials (without supplemented ClO_2, yet with added *E. coli* at 90 min); *i.e.,* 2.8 log CFU/g leaf – 0.8 log CFU/g leaf = 2.0 log CFU/g leaf (Table 3, Table 4). During these pilots, a clear effect of ClO_2 on background microbial communities, non-*E. coli* cells on KB and R2A, after *E. coli* administration was not observed (Table 4). It is clear that the background microbial communities, *i.e.,* fluorescent Pseudomonads and total heterotrophic bacteria, remain on the lettuce after ClO_2 treatments as observed during the 3 mg/L pilots (Table 4) and after storage (Table 5). According to Student's *t*-test, the effect of ClO_2 before and after addition of *E. coli* to the PWW is not significant at $p < .05$, meaning there is no measurable effect of ClO_2 on these microbial communities. Moreover, ClO_2 influenced *E. coli* counts on lettuce (Table 3, Table 4) and in the second centrifuged wash water (Table 3). Wash water disinfection during pilot trials with supplemented *E. coli* did not prevent eventual outgrowth of *E. coli* in packaged lettuce (Table 3, Table 5); however, background microbial communities were able to grow out at these temperatures due to their psychrophilic nature (Table 5). The impact of outgrowth these microbial communities on *E. coli* was not further investigated as it was out of the scope of this research.

Furthermore, during the 3 mg/L pilot trials, chlorate levels of the lettuce sampled before entry into the wash tank were on average 7.3 μg/kg (SD ± 2.1 μg/kg) for Treatment C.2 and 11.6 μg/kg (SD ± 2.9 μg/kg) for Treatment D.2. Levels on the lettuce after washing, rinsing, and centrifugation were on average 23.9 μg/kg (SD ± 6.7 μg/kg) and 60.4 μg/kg (SD ± 17.7 μg/kg) for Treatment C.1 and C.2, respectively, and on average 22.8 μg/kg (SD ± 0.9 μg/kg) and 58.8 μg/kg (SD ± 31.7 μg/kg) for Treatment D.1 and D.2, respectively (data not shown). Chlorite was analyzed for lettuce samples taken post-disinfection during Treatment C.2 and D.2; values were 1.6 and 1.3 mg/kg, respectively. Perchlorate analyses for Treatments C and D demonstrated levels < 5 ng/g (limit of quantification, LOQ). For first centrifuged lettuce rinse water samples at 80, 91, 93, and 95 min, chlorate values ranged from 3.0–14.1 mg/L (Treatment C.1 and D.1). For second centrifuged lettuce rinse water samples at 80, 91, 93, and 95 min, chlorate values ranged from 0.0340–0.073 mg/L (Treatment C.2 and D.2). Perchlorate samples for centrifuged samples from Treatments C and D, regardless of the time point, were < 20 mg/L.

Table 5
Effect of 3 mg/L chlorine dioxide (ClO_2) treatment pilot trials on *E. coli* (BECSA), on fluorescent Pseudomonad (KB), and on heterotrophic plate counts (R2A) for lettuce analyzed after storage (5 days, 4 °C).

Pilot trial	Treatment		Time	*E. coli*[a] ± SD	Fluorescent Pseudomonad ± SD	Heterotrophic plate counts ± SD
	ClO_2	*E. coli*	(min)	(log CFU/g lettuce)	(log CFU/g lettuce)	(log CFU/g lettuce)
Treatment C.1	Yes	No	5	< 1	5.96 ± 0.12	6.23 ± 0.24
			93	< 1	5.83 ± 0.33	5.72 ± 0.23
			93	< 1	5.37 ± 0.12	5.65 ± 0.064
			93	< 1	5.61 ± 0.26	5.57 ± 0.081
Treatment C.2	Yes	No	5	< 1	6.42 ± 0.059	6.37 ± 0.036
			93	< 1	6.34 ± 0.094	6.40 ± 0.19
			93	< 1	6.52 ± 0.019	6.50 ± 0.14
			93	< 1	5.86 ± 0.059	6.05 ± 0.040
Treatment D.1	Yes	Yes	5	< 1	6.04 ± 0.20	6.08 ± 0.17
			93	2.60 ± 1.84	5.74 ± 0.13	5.39 ± 3.81
			93	1.92 ± 0.31	5.35 ± 0.055	5.25 ± 3.71
			93	2.10 ± 0.31	5.69 ± 0.014	5.71 ± 0.081
Treatment D.3	Yes	Yes	5	< 1	6.20 ± 0.18	6.45 ± 0.31
			93	1.00 ± 0.97	5.81 ± 0.66	5.89 ± 0.64
			93	0.87 ± 0.41	6.23 ± 0.39	6.28 ± 0.30
			93	0.94 ± 0.66	6.12 ± 0.048	6.12 ± 0.025

[a] *E. coli* was below the limit of detection (*i.e.,* < 1 log CFU/g on lettuce).

4. Discussion

This study demonstrates that ClO_2 use at the semi-commercial pilot scale reduced the *E. coli* peak contamination that entered the PWW, and thus, supports the ability for ClO_2 to prevent cross-contamination in the washing tank during fresh-cut 'Lollo Rossa' lettuce processing. Our results concur with a recent publication that investigated the efficiency of ClO_2 in standardized process water (*i.e.,* laboratory prepared water). Van Haute et al. (2017) reports that with 5 mg/L of ClO_2, *E. coli* was reduced > 5 orders of magnitude after 3 min (COD 1130 mg O_2/L) and with 4 mg/L of ClO_2 *E. coli* was reduced > 5 log units after 1 min (COD 625 and 734 mg O_2/L). The COD of the PWW determined during our pilots was lower than that reported by Van Haute et al. (2017), most presumably due to the residual concentration of ClO_2 available throughout processing and water refreshment of the system. Besides this, the general differences in experimental design and equipment used in our study compared to Van Haute et al. (2017) can exhibit other factors that may influence the difference in COD reported. During the pilot treatment trials, interactions between the organic components and ClO_2 presumably occurred before addition of *E. coli*. The residual concentration of ClO_2 available during the pilot treatment trials was therefore available to interact with incoming microorganisms and other organic matter in contrast to what was observed during the Control B pilot trials without ClO_2.

Our results demonstrate that ClO_2 treatments did not prevent *E. coli* attachment to the lettuce; however, the use of ClO_2 decreased the probability for cross-contamination between lettuce batches during a point contamination (6 log CFU/mL) compared to when no sanitizer was applied. Moreover, the background microbial communities investigated remained on the lettuce, as observed during the 3 mg/L pilots and after storage (5 days, 4 °C). Our results concur with Allende et al. (2008) who observed that the extent of *E. coli* cross-contamination was influenced by wash water quality, particularly when fresh-cut escarole was highly contaminated (5.1 log CFU/g). Our results further demonstrated that the application and residual concentration of ClO_2 in the wash water could be maintained during fresh(−cut) processing. In brief, the quality of the PWW during processing can be questionable regarding microbiological safety when controls regarding the water quality, such as water disinfection strategies, are not implemented.

Furthermore, our study shows the concentrations of chlorate and chlorite *in situ* given real-time processing conditions and procedures. According to the World Health Organization (WHO), provisional guidelines for chlorate and chlorite in drinking water are 0.7 mg/L for each DBP, yet authorities indicate that the use of ClO_2 as a disinfectant may result in the value being exceeded, and thus, stress that difficulties in meeting such a guideline value should not constitute compromising adequate disinfection (World Health Organization (WHO), 2005). More recently, the European Food Safety Authority (EFSA) has evaluated the presence of chlorate in food; given a hypothetical maximum residue limit (MRL) of 0.7 mg/kg for foodstuffs and 0.7 mg/L for drinking water, both chronic and acute exposures, based on the available occurrence data, would only minimally reduce exposure and associated risks (European Food Safety Authority (EFSA), 2015). Until the European Commission has re-evaluated the maximum residue limit (MRL) for chlorate in foods, member states have the authority to dictate national levels. The results from our study can serve to minimize the data gaps concerning the impact of food processing on chlorate and chlorite residues in food. For example, our study demonstrates that the 0.7 mg/L guideline for both chlorate and chlorite is exceeded in the PWW, but not in the lettuce, when ClO_2 was used at 5 and 3 mg/L. Nevertheless, regarding the potential burden on public health, given both a microbiological and toxicological standpoint, ClO_2 application in the PWW may be favorable given additional processing parameters like a final rinse step with potable water. For example, the USFDA designates that ClO_2 treatment of fruits and vegetables shall be followed by either a potable rinse or another preservative method (U.S. Food and Drug Administration (USFDA), 2016). Future research that quantifies the effect that a final rinse step has on public health, *e.g.*, as observed during pilots, is warranted. In our pilot treatment trials with 3 mg/L ClO_2, we quantified the levels of chlorate on the lettuce pre- and post-washing, rinsing, and centrifugation demonstrating that results were below the previous default MRL of 0.01 mg/kg set by Regulation (EC) 396/2005 (European Commission (EC), 2005). These results coincide with a previous study that evaluated the commercial-scale application of sodium hypochlorite as a PWW sanitizer, demonstrating that chlorate residues on the washed fresh (−cut) lettuce after a 1 min tap water rinse were below LOQ (0.0024 mg/L) even when chlorate levels in the PWW were as high as 13 mg/L (Gil et al., 2016). Future research can investigate the use of multiple wash tanks including analysis of the DBPs in the PWW of the subsequent washers.

As with any chemical, worker safety and health alongside processing precautions should be considered (Parish et al., 2003) as well as the environmental impact, associated costs (Van Haute et al., 2015a), and sustainability of the method. Some restrictions for the use of ClO_2 as a PWW sanitizer include the operational costs and maintenance requirements, the operating skills and training needed to apply the technology, and the safety management of the technology at the processing site (on-site formation of hazardous compounds, monitoring ambient concentration levels, *etc.*) (Van Haute et al., 2015a). Despite these restrictions, other suitable technologies to treat the PWW (*e.g.*, ozone, peracetic acid) exhibit comparable limitations on either technical, managerial, and/or sustainability aspects (Uyttendaele et al., 2015; Van Haute et al., 2015a). Further research and modeling on the efficacy of sanitizers at both the laboratory and pilot scales are warranted to optimize the residual concentrations, among other parameters, in practice. Additionally, research into the use of other sustainable chemical and/or physical methods that meet food safety and quality objectives are warranted. Overall, cross-contamination prevention *via* the washing water remains a critical step during produce processing and application of ClO_2 at the industrial scale is attainable with continuous application and monitoring.

Acknowledgements

This research has been financed by the Dutch Ministry of Economic Affairs (EZ) under the project Microbiology in Horticulture (PPS 296, BO-33.05-001-001) from the TopSector Horticulture and Starting Materials. We gratefully acknowledge C. van Kekem, L.B.J.M. Berendsen, C.H. Lombaers-van der Plas, C. van Tongeren, M.T.J. Helmond (Wageningen University & Research), and R. Dolmans, G. van Wezel, M. Scheffer, A. van Empel (Ecolab B.V.) for their technical assistance. H.M. Vollebregt (Wageningen University & Research) is kindly thanked for her contribution to this project including setting up the pilot trials. M. H. Zwietering (Wageningen University & Research) is kindly thanked for his suggestions on an earlier version of the paper. The authors acknowledge the contributions from the Dutch fruit and vegetable industry, the Fresh Produce Centre (GFH), and in particular, Hessing B.V. for the pilot plant facilities.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2018.01.013.

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