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Irrigating Lettuce with Wastewater Effluent: Does Disinfection with Chlorine Dioxide Inactivate Viruses?

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Abstract

Reclaimed water obtained from urban wastewater is currently being used as irrigation water in water-scarce regions in Spain. However, wastewater can contain enteric viruses that water reclamation treatment cannot remove or inactivate completely. In the present study, greenhouse-grown baby lettuce (*Lactuca sativa* L.) was irrigated with secondary treatment effluent from a wastewater treatment plant untreated and treated using chlorine dioxide (ClO_2). The effect of ClO_2 treatment on the physicochemical characteristics and the presence of enteric viruses in irrigation water and lettuce was assessed. The presence of human noroviruses genogroups I and II (NoV GI and NoV GII), and human astroviruses (HAstV), was analyzed by real-time polymerase chain reaction (RT-qPCR). Additionally, to check for the loss of infectivity induced by the disinfection treatment, positive samples were re-analyzed after pretreatment with the intercalating dye PMAxx before RNA extraction and RT-qPCR. There were no significant differences in the proportion of positive samples and the concentration of enteric viruses between treated and untreated reclaimed water without PMAxx pretreatment ($p > 0.05$). A significantly lower concentration of NoV GI was detected in ClO_2–treated water when samples were pretreated with PMAxx ($p < 0.05$), indicating that inactivation was due to the disinfection treatment. Laboratory-scale validation tests indicated the suitability of PMAxx-RT-qPCR for discrimination between potentially infectious and ClO_2–damaged viruses. Although the applied ClO_2 treatment was not able to significantly reduce the enteric virus load of the secondary effluent from the wastewater treatment plant, none of the lettuce samples analyzed ($n = 36$) was positive for the presence of NoV or HAstV.

Core Ideas

- Reclaimed water used for irrigation can contain enteric viruses.
- Current detection methods do not distinguish infectious from noninfectious viruses.
- PMAxx treatment can help to get information on the infectivity of viral particles.
- Treatment of reclaimed water with ClO_2 did not improve irrigation water safety.

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Access to adequate water quality is challenging in several regions of the world, and the situation is expected to worsen as climate change exacerbates water scarcity. Water recycling and reuse is a strategy to save water resources. In some parts of the world, water reclaimed from wastewater will need to be used for irrigation to sustain agriculture (Becerra-Castro et al., 2015). Among other human pathogens, enteric viruses can be found in the aquatic environment, mainly by the discharge of wastewater treatment plant (WWTP) effluents or the failure of depuration processes (Morsy et al., 2007; Hewitt et al., 2011; Rusiñol et al., 2015).

In the growing trend in foodborne outbreaks associated with the consumption of leafy greens, irrigation water is usually suspected as the main contamination source (Li et al., 2015). The presence of human norovirus (NoV) and human astroviruses (HAstV) in irrigation water is one cause of fresh produce contamination with these pathogenic microorganisms (Bosch et al., 2014; Van Haute et al., 2015). Viruses present in contaminated irrigation water can attach to leaf surfaces and survive at the preharvest step (Wei et al., 2010; Hirneisen and Kniel, 2013). In addition, other studies conducted on soil-grown lettuce (*Lactuca sativa* L.) and strawberry (*Fragaria* L.) reported the internalization of NoV surrogates via plant roots from contaminated soils and the subsequent dissemination to the leaves and fruits (DiCaprio et al., 2015a, 2015b).

Disinfection is a mitigation strategy that can be used to reduce the health risk associated with the presence of microbial pathogens in irrigation water, although little information is available on its effectivity on human enteric viruses (Hamilton et al., 2006). One of the disinfection technologies that can be applied for irrigation water is chlorine dioxide (ClO_2) (Reitz et al., 2015), which has the advantage of not forming organohalogen by-products and is a more powerful oxidant than sodium hypochlorite (Gómez-López et al., 2009; López-Gálvez et al.,

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Abbreviations: HAstV, human astrovirus; NoV, norovirus; ORP, oxidation–reduction potential; PBS, phosphate-buffered saline; PMA, propidium monoazide; RT-qPCR, real-time polymerase chain reaction; WWTP, wastewater treatment plant.

2010). Chlorine-washing procedures are reported to be almost ineffective in removing human NoV surrogates from fresh produce (Cromeans et al., 2014; DiCaprio et al., 2015c), while several studies have reported the sensitivity of a range of viruses to ClO_2 (Butot et al., 2008; Jin et al., 2012; Lim et al., 2010; Li et al., 2015; Xue et al., 2013). However, no information on HAstV has been reported so far.

Currently, real-time polymerase chain reaction (RT-qPCR) is the gold-standard method for the detection and quantification of enteric viruses in water and food samples (Bosch et al., 2011). In 2017, ISO 15216 was released as standard procedure for hepatitis A virus and NoV detection in food and bottled water (International Organization for Standardization, 2017). However, this technique cannot distinguish between infectious and noninfectious viral particles (Knight et al., 2013; Hamza et al., 2011). Lim et al. (2010), for instance, reported that the virus inactivation by ClO_2 measured by cell culture using NoV surrogates did not correlate with short and long template RT-PCR assays, resulting in a significant underestimation of the inactivation. Thus, to assess viral infectivity by RT-qPCR, some authors recommend the use of intercalating dyes such as ethidium monoazide, propidium monoazide (PMA), or PMAxx (Biotium) (Coudray-Meunier et al., 2013; Prevost et al., 2016; Randazzo et al., 2016). The application of intercalating dyes has been proposed as a strategy for a better interpretation of quantitative results in terms of viral infectivity in foods (Moreno et al., 2015; Randazzo et al., 2016, 2018a,b) and different types of water samples (Fuster et al., 2016; Parshionikar et al., 2010; Quijada et al., 2016; Randazzo et al., 2016).

The aim of the present study was to assess ClO_2 disinfection of a secondary effluent of wastewater used for lettuce irrigation. We examined (i) occurrence of NoV and HAstV in irrigation water, (ii) NoV and HAstV inactivation in irrigation water, and (iii) occurrence of NoV and HAstV in lettuce.

Material and Methods

Experimental Setup

Red oak leaf lettuce was grown in trays of 294 alveoli with peat as substrate. Greenhouse characteristics and irrigation system were described previously in López-Gálvez et al. (2016). Irrigation water of two qualities was compared: secondary treatment effluent from a WWTP (secondary wastewater treatment was performed as described in López-Gálvez et al., 2016), and ClO_2–treated water from the same source. A total of eight lettuce trays with 294 plants each were used per water type in each trial, and two trials were performed between June and August 2016. Minimum and maximum temperatures inside the greenhouse were 26.7 and 32.2°C, with an average of 28.1 ± 1.5°C. The relative humidity in the greenhouse ranged from 55.1 to 99.9%, with an average of 80.8 ± 10.2%. Irrigation water was applied at ≈300 L d^{-1} in two irrigation periods of 20 min each. The growth cycle of baby lettuce lasted 38 d in the first trial (June–July), and 26 d in the second trial (July–August).

Water samples were taken weekly during the entire growth cycle. Due to the small plant size, samples of baby lettuce were taken weekly starting 2 wk before harvest. Each sampling day, two water samples of each type of water and three lettuce samples per water type were taken. Water samples (2 L) were taken in 2.7-L aseptic plastic bottles. Sodium thiosulfate was added to the ClO_2–treated water to quench residual disinfectant. Lettuce samples (100 g) were taken in aseptic conditions and placed in plastic bags. Water and lettuce samples were transported to the laboratory in refrigerated conditions. A total of 24 water samples (12 per water type) and 36 lettuce samples (18 per water type) from the two trials combined were analyzed for the presence of NoV (GI and GII) and HAstV.

Chlorine Dioxide Treatment and Physicochemical Analysis of Water

A concentrated solution of ClO_2 (≈6000 mg L^{-1}) AGRI DIS (Servicios Técnicos de Canarias) was diluted to 100 to 300 mg L^{-1} ClO_2 using tap water in a 20-L opaque plastic tank according to manufacturer instructions. The diluted ClO_2 solution was prepared at the irrigation head and dosed using a peristaltic pump for treating the secondary effluent of the WWTP. The leftover diluted solution was disposed daily before performing the first irrigation, and a new one was prepared. Time lapse from the ClO_2 application point to the sprinklers (contact time) was about 6 min. To avoid interference from the water remaining in the irrigation system from previous irrigations, a protocol was established before each irrigation event. First, trays were removed from the tables, and then the system was filled with freshly treated water and the irrigation started for a few minutes. Then the irrigation was stopped, the trays were returned to the tables, and the irrigation started again. Chlorine dioxide measurements were performed daily by chronoamperometry analysis (Palintest) in untreated and ClO_2–treated water samples taken during irrigation at the sprinklers. Temperature, pH, and oxidation–reduction potential (ORP) were measured with a multimeter (pH and redox 26, Crison Instruments). Water filtered through 0.45-μm syringe nylon filters (Fisherbrand-Fisher Scientific) was used for measuring the absorbance at 254 nm using an ultraviolet-visible spectroscopy spectrophotometer (Jasco V-630) and quartz cuvettes with a path length of 1 cm (Hellma). Total organic carbon was assessed using a multi N/C 3100 analyzer (Analytik Jena) after filtration through ashless filter paper (Albet Labscience).

NoV and HAstV Analysis in Water

Recovery of viruses from water was performed as described in López-Gálvez et al. (2016). Briefly, magnesium chloride ($MgCl_2$) was added to each water sample and pH was adjusted to 3.5 with hydrogen chloride (HCl). Samples of 200 mL were filtered through 0.45-μm membrane filters (Sartorius). Filters were then transferred to sterile tubes containing 5 mL of elution buffer, and pH was adjusted to 9.5 using sodium hydroxide (NaOH). Tubes were shaken for 1 min in a vortex, kept for 4 min in an ultrasonic bath, and shaken again in a horizontal orbital shaker at 250 rpm for 10 min, and then pH was adjusted to 7 using HCl (10%). After that, samples were kept at −70°C until analysis. To monitor the virus concentration procedure and RNA extraction, 200 mL of each secondary effluent water sample was spiked with a known concentration of mengovirus M0 strain before processing as process control virus following ISO 15216 guidelines (International Organization for Standardization, 2017; Costafreda et al., 2006). RNA extraction was performed using the NucleoSpin

RNA virus kit (Macherey-Nagel GmbH & Co.) according to the manufacturer's instructions and with the plant RNA isolation aid treatment as detailed in Randazzo et al. (2016). Human NoV and HAstV genome copies were quantified in duplicate by one-step RT-qPCR assay using the RNA UltraSense One-Step quantitative system (Invitrogen SA) with a half-scale modification of the manufacturer's protocol and the LightCycler 480 instrument (Roche Diagnostics) following the standardized ISO procedure (ISO 15216-1:2017, International Organization for Standardization, 2017) for NoVs, and the primers and probe for HAstV as suggested by Sano et al. (2010).

Additionally, positive samples for NoV GI, NoV GII, or HAstV were extracted again, and a PMAxx-Triton pretreatment was applied in parallel before RNA extraction (Randazzo et al., 2016). Briefly, 100 μL of concentrated samples was mixed with 50 μM PMAxx and 0.5% (v/v) Triton (PMAxx-Triton), incubated in the dark at room temperature for 10 min in a shaker at 150 rpm, and immediately exposed to continuous LED light (Led-Active Blue, GenIUL) for 15 min. After intercalating-dye pretreatment, RNA was extracted using the NucleoSpin RNA virus kit according to the manufacturer's instructions. Real-time PCR was performed as described above. As a control, 100 μL of concentrated water samples was processed without performing the PMAxx-Triton pretreatment.

NoV and HAstV Analysis in Baby Lettuce

Recovery of viruses from fresh produce samples was performed as described in López-Gálvez et al. (2016), with some modifications. Briefly, lettuce samples (25 g) were placed in sterile stomacher bags with filter and soaked in 40 mL of elution buffer for 20 min at room temperature with constant shaking. The fluid was recovered from the bag and centrifuged at 8500 *g* for 30 min at 4°C. Then, pH of the decanted supernatant was adjusted to 7.2 ± 0.2 using HCl. Polyethylene glycol (PEG) 6000 (Acros Organics) and sodium chloride (NaCl) (Panreac Química) were added to the neutralized supernatant. Subsequently, fluid was incubated for 2 h at 4°C. Viruses were concentrated by centrifuging the solution at 8500 *g* for 30 min at 4°C. The supernatant was discarded, and additional centrifugation was performed at 8500 *g* for 5 min at 4°C to compact the pellet. The pellet was finally resuspended in 1 mL of phosphate-buffered saline and stored at −75°C before RNA extraction. Mengovirus was added as process control. RNA extraction and RT-qPCR reactions from lettuce samples were performed as described above for water samples.

Performance of PMAxx Pretreatment to Predict Viral Inactivation in Phosphate-Buffered Saline and Secondary Effluent Waters Treated with Chlorine Dioxide

A test was performed to validate the suitability of the PMAxx treatment to predict viral inactivation under the experimental conditions used. Fecal samples containing NoV GI Genotype 4 (GI·P4) and GII Genotype 4 (GII.4 variant Den Haag 2006b), and HAstV were suspended (10%, w/v) 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 used to inoculate 500-mL aliquots of PBS (pH 7.2) and a WWTP secondary treatment effluent. Chlorine dioxide was added to achieve an initial concentration of 5.5 or 11 mg L^{-1}, and samples were incubated for 6 min at room temperature (≈25°C) under agitation on a magnetic stirrer (300 rpm). After the contact time of 6 min, sodium thiosulfate (2.75 g L^{-1}) was added to neutralize ClO_2. The ClO_2 concentration was measured by chronoamperometry using a portable device (Chlordioxense; Palintest). Aliquots of inoculated untreated PBS and secondary wastewater were kept at room temperature as a control. Viral inactivation was determined by examining the loss of genome copy titers by comparing RT-qPCR and PMAxx-RT-qPCR as described above. Physicochemical characteristics of treated PBS and secondary water were also assessed. pH, temperature and ORP were monitored during the treatment using a portable multimeter (sensION+ MM150, Hach). Absorbance at 254 nm of secondary wastewater was measured as explained above. Chemical oxygen demand of secondary water was measured by the standard photometric method (APHA, 2012).

Statistical Analysis

Norovirus and HAstV quantification was adjusted with the mengovirus recovery for each sample (Costafreda et al., 2006). In particular, final NoV and HAstV concentrations were corrected by taking into account the recovery of mengovirus calculated before and after the concentration and extraction steps for each type of water. Thus, NoV and HAstV titers were log-transformed and introduced in a Microsoft Excel 2010 spreadsheet (Microsoft). IBM SPSS statistics 20 (IBM) was used for statistical analysis. The Kolmogorov–Smirnov test and Levene's test were used to assess normality and equality of variance, respectively. When normality could be assumed, a *t* test was performed to evaluate differences. When data did not follow a normal distribution, nonparametric tests were applied. To compare the prevalence of positive samples for viruses between ClO_2 treated and untreated irrigation water, the chi-square (χ^2) test was used.

Results and Discussion

Chlorine Dioxide Treatment and Physicochemical Characteristics of Irrigation Water

The doses of ClO_2 that were applied were selected based on preliminary tests with the aim of reaching levels of *Escherichia coli* in the water below 2 log colony-forming units 100 mL^{-1}, which corresponds to the *E. coli* threshold recommended in several good agricultural practices (GAP) guidelines for irrigation water (EC, 2017). To achieve this goal, the initial ClO_2 concentration in treated water ranged between 3 and 11 mg L^{-1}. Mean residual ClO_2 concentration in ClO_2–treated irrigation water at the sprinklers was 0.53 ± 0.40 and 0.42 ± 0.43 mg L^{-1} in the first and the second growing cycles, respectively. The average residual concentration adjusted was close to the recommended dose for the continuous application in greenhouse production (WEAH, 2016). The residual concentration of ClO_2 in the treated water varied between 0.04 and 1.28 mg L^{-1} in the first trial and between 0.03 and 1.51 mg L^{-1} in the second trial. The observed variation in the residual concentration can be attributed to the differences in the sanitizer demand by the secondary effluent provided by the WWTP. The ClO_2 treatment did not change the physicochemical characteristics of the irrigation water significantly (Table 1).

Table 1. Physicochemical characteristics of the secondary effluent of a wastewater treatment plant (untreated) and ClO_2–treated secondary effluent (ClO_2 treated) used for lettuce irrigation. Data are mean ± standard deviation of two trials performed between June and August 2016.

Parameters†	Untreated	ClO_2 treated
pH	7.7 ± 0.1	7.4 ± 0.1ns‡
Temperature (°C)	27.2 ± 1.4	27.2 ± 1.5ns
ORP	343 ± 96	411 ± 178ns
UV254 (cm^{-1})	0.26 ± 0.06	0.24 ± 0.06ns
TOC (mg L^{-1})	13.1 ± 3.3	14.4 ± 2.8ns
Initial ClO_2 (mg L^{-1})§	<0.02	5.6 ± 2.4
Residual ClO_2 (mg L^{-1})	<0.02	0.51 ± 0.41**

** Significant difference ($p < 0.05$) between untreated and treated samples.

† ORP, oxidation–reduction potential; TOC, total organic carbon; UV254, absorbance at 254 nm.

‡ ns, not significant.

§ The initial concentration of ClO_2 was calculated taking into account the irrigation water flow and the volume and concentration of the ClO_2 solution dosed.

The average temperature of the water was similar to the average temperature measured inside the greenhouse (≈28°C). Although the presence of oxidants has been reported to increase both ORP and UV254 (APHA, 2012), no significant differences in these parameters were detected in the present study between ClO_2–treated and untreated water (Table 1). In contrast, higher ORP and UV254 values have been reported for ClO_2–treated irrigation water even though the ClO_2 residuals were lower than those detected in the present study (López-Gálvez et al., 2018). These differences could be due to differences in water-quality characteristics between the surface water from the mentioned study and the reclaimed water from the present study.

Occurrence of Viruses in Irrigation Water

All the water samples were positive for the sample process control (mengovirus) with a percentage of recovery of 48.2 ± 22.9%. da Silva et al. (2007) reported extraction efficiencies generally above 10% of mengovirus in wastewater samples. The occurrence of NoV GI, NoV GII, and HAstV in untreated and ClO_2–treated water samples analyzed without PMAxx pretreatment is shown in Table 2. Comparing both NoV genogroups in untreated water samples, the concentration of NoV GII was significantly higher than that of NoV GI ($p < 0.05$). In contrast, levels of NoV GI have been reported to be higher than those of NoV GII (López-Gálvez et al., 2016). This difference highlights the temporal fluctuations in virus concentration in WWTP effluents. Kitajima et al. (2014) observed in a 1-yr study that in some months there was a higher concentration of NoV GI compared with NoV GII in WWTP effluent, while in other months the reverse was true. On the other hand, in untreated water, there was a higher percentage of samples positive for NoV GI than for NoV GII (Table 2). The same relationship was observed in a secondary effluent in a previous study (López-Gálvez et al., 2016). In the present study, treatment of secondary effluent with ClO_2 reduced the proportion of samples positive for NoV GI and GII (Table 2). However, there was no significant difference in the concentration of NoV between untreated and ClO_2–treated samples ($p > 0.05$). As in our study, Katayama et al. (2008) did not detect differences in NoV levels between secondary treated wastewater and WWTP effluent (chlorinated) samples. In the case of HAstV, there was no difference in the percentage of positive samples between untreated and ClO_2–treated water samples (Table 2). Similar results were recently reported by Kobayashi et al. (2017), where a down-flow hanging sponge reactor for the treatment of effluent was evaluated as a source of water for agricultural irrigation. They reported the presence of HAstV in 36 and 32% of influent and effluent water samples analyzed, respectively. Real-time PCR is not able to distinguish between infectious and noninfectious viruses (Knight et al., 2013), which could explain the lack of difference in the virus concentration between untreated and ClO_2–treated water samples.

Potential Infectivity of Viruses in Irrigation Water

The protocol used in the present study was demonstrated to be appropriate to reduce the RT-qPCR signal of hepatitis A virus, NoV GI, and NoV GII in irrigation and wastewater samples (Randazzo et al., 2016; Randazzo et al., 2018a,b). Some authors reported pretreatment with intercalating dyes (e.g., PMA, PMAxx) coupled with RT-qPCR as a useful tool to rapidly assess the infectivity of enteric viruses in water inactivated by heat treatments (Randazzo et al., 2016) or chemical disinfectants (Parshionikar et al., 2010; Leifels et al., 2015; Fuster et al., 2016). However, when the present study was performed, the suitability of PMA-RT-PCR to assess the inactivation of ClO_2–treated viruses had not been confirmed by previous studies.

The two ClO_2 concentrations tested in our laboratory-scale study represented the average (≈5.5 mg L^{-1}) and the highest initial levels (≈11 mg L^{-1}) of ClO_2 applied during the greenhouse tests. Figure 1 shows the concentration of viral genomes detected by RT-qPCR and by PMAxx-RT-qPCR after ClO_2 treatment was performed in PBS and secondary-treatment effluent waters. Results show the ability of PMAxx pretreatment in discriminating potentially infectious and ClO_2–damaged viruses. Comparing the results obtained by RT-qPCR

Table 2. Prevalence of human norovirus genogroup I (NoV GI) and II (NoV GII) and human astrovirus (HAstV) analyzed by real-time polymerase chain reaction in the secondary effluent of wastewater treatment plant (untreated) and ClO_2–treated secondary effluent (ClO_2 treated) used for lettuce irrigation. Samples were not pretreated with PMAxx.

	Untreated				ClO_2 treated			
	Prevalence†	Median	Minimum	Maximum	Prevalence	Median	Minimum	Maximum
	%	log genome copies L^{-1}			%	log genome copies L^{-1}		
NoV GI	75.0	5.00	4.08	5.15	58.3ns‡	4.86	4.12	5.25
NoV GII	66.7	5.53	4.43	5.94	50.0ns	5.30	5.15	5.65
HAstV	33.3	5.52	4.04	7.29	33.3ns	6.64	6.39	6.80

† Proportion of positive samples from each type of water ($n = 12$)

‡ ns, not significant.

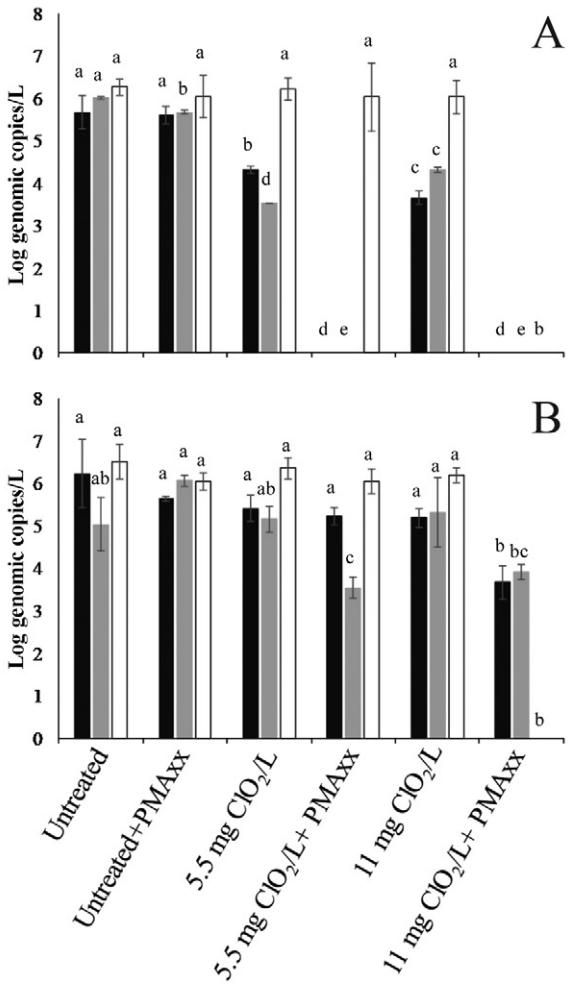

Fig. 1. Performance of real-time polymerase chain reaction (RT-qPCR) and PMAxx-RT-qPCR to discriminate between potentially infectious and ClO_2–treated norovirus GI (black bars), GII (gray bars), and astrovirus (white bars) in (A) phosphate-buffered saline and (B) secondary effluent water. Different letters denote significant differences among treatments for each virus ($p < 0.05$).

and PMAxx-RT-qPCR in ClO_2–treated PBS suspensions (Fig. 1A), it could be concluded that PMAxx pretreatment allowed detection of the effect of ClO_2 treatment on the integrity of viral structure. This effect was significant in NoV GI and GII for the two ClO_2 concentrations tested, whereas it was significant in HAstV only for the highest disinfectant concentration (11 mg L^{-1}). Wigginton et al. (2012) and Sigstam et al. (2013) reported extensive degradation of viral proteins by ClO_2, while Yeap et al. (2016) showed that ClO_2 inactivation of murine norovirus included disruption of viral structure.

When ClO_2 treatments were applied to viral suspensions prepared using secondary-treatment effluent water at the laboratory scale, the results obtained were different from those obtained in PBS suspensions. The comparison of the results obtained by RT-qPCR and PMAxx-RT-qPCR in ClO_2–treated secondary effluent suspensions (Fig. 1B) suggests that the integrity of the viral structure was damaged in the case of NoV GII for both ClO_2 concentrations, while it was affected only by the highest concentration for HAstV and NoV GI.

The difference in the results obtained in PBS and secondary effluent water could have been caused by the different disinfectant demand of these two media. The higher ClO_2 demand of secondary effluent water caused a larger decrease in ClO_2 concentration during treatment (Supplemental Material). With less disinfectant available, the damage on the viral capsid would have been milder in the secondary effluent water compared with PBS. Kingsley et al. (2014) suggested that human noroviruses are quite resistant to different disinfectants, including ClO_2. They observed only a 2.8 log reduction in human norovirus after a 60-min treatment with 350 mg L^{-1} ClO_2. In contrast, in our study, reductions of 5 to 6 log units in NoV GI and GII were obtained by treating PBS with an initial dose of 5.5 mg L^{-1} ClO_2. However, as explained above, smaller reductions were detected when secondary effluent water was treated.

Regarding the greenhouse tests, Table 3 shows the concentrations (log genome copies L^{-1}) of NoV GI and GII in water samples positive for the presence of NoV and HAstV that were reanalyzed with and without PMAxx-Triton pretreatment. In the case of ClO_2–treated secondary effluent water, the concentration of NoV GI in PMAxx-Triton pretreated samples was significantly lower ($p < 0.01$) than non-pretreated samples (Table 3), indicating that the capsid of viruses was damaged due to disinfection with ClO_2. However, no significant differences were observed for NoV GII and HAstV ($p > 0.05$), indicating that the viral particles remained uninjured after the disinfection treatment with ClO_2. As would be expected, no significant differences were observed for NoV and HAstV between PMAxx-pretreated and non-pretreated samples in untreated irrigation water (Table 3). In a previous study, the RT-qPCR signal of NoV GII was more easily removed than the signal of NoV GI using the PMAxx pretreatment, which was attributed to a better environmental persistence of NoV GI (Randazzo et al., 2016).

Sales-Ortells et al. (2015) assessed the health risks derived from consumption of lettuce irrigated with tertiary effluent containing NoV in Catalonia, Spain, stating that reductions of 4.3 log should be ensured to reduce the disease burden to 10^{-6} disability adjusted life years (DALYs) per person per year, as recommended (WHO, 2006). In the present study, a maximum reduction of 0.65 log for NoV GI in secondary-treatment effluent water was detected by PMAxx-RT-qPCR during the greenhouse tests. In the laboratory-scale tests, the reductions obtained in secondary effluent water were higher but were still far from the target of 4.3 log.

Occurrence of Viruses in Lettuce

All the analyzed lettuce samples were positive for the mengovirus process control, with average recoveries of 10.2 ±

Table 3. Log genome copies per liter (mean ± SD) in the secondary effluent of wastewater treatment plant (untreated) and ClO_2–treated secondary effluent (ClO_2 treated) used for lettuce irrigation samples analyzed by real-time polymerase chain reaction with and without the use of PMAxx-Triton.

	Untreated		ClO_2 treated	
	PMAxx	No PMAxx	PMAxx	No PMAxx
	log genome copies L^{-1} (mean ± SD)			
NoV GI	4.65 ± 0.54	4.75 ± 0.39ns†	4.19 ± 0.37	4.84 ± 0.34*
NoV GII	5.47 ± 0.42	5.28 ± 0.23ns	5.43 ± 0.09	5.21 ± 0.28ns
HAstV	7.04 ± 0.15	7.14 ± 0.20ns	6.50 ± 0.31	6.62 ± 0.19ns

* Significant difference ($p < 0.01$) between samples treated and not treated with PMAxx.

† ns, not significant.

8.5%, in accordance with the ISO 15216 recommendations (International Organization for Standardization, 2017), which establishes acceptable recoveries greater than 1%. In line, Hennechart-Collette et al. (2015) reported similar recoveries of mengovirus from lettuce (10.8%). In the present study, no lettuce samples positive for NoV GI, NoV GII, or HAstV (n = 36) were observed. As viruses were detected in irrigation water and they can survive in preharvest conditions, such results were unexpected, especially taking into account the sprinkler irrigation system used (Hirneisen and Kniel, 2013). The lack of positive results in lettuce could be explained by the low recovery rates compared with those obtained in water samples. Furthermore, the protocol used for virus recovery could not detect internalized viral particles if present. Pérez-Rodríguez et al. (2014) detected two positive samples for NoV GI and four positive samples for NoV GII when analyzing 60 lettuce samples (both unprocessed and ready-to-eat lettuce), which corroborates the low prevalence in fresh produce. In keeping with these results, Kokkinos et al. (2012) found only 2 positives out of 149 samples of lettuce analyzed for NoV GI, and 1 positive sample out of 126 of lettuce analyzed for NoV GII. Baert et al. (2011) found 186 samples positive for NoV out of 653 samples (28.5%). Furthermore, at temperatures as high as those observed during the trials, a high reduction of virus titers in fresh produce surfaces was expected (3–4 log at 30°C in 24 h; Carratalà et al., 2013).

Conclusions

Real-time PCR analysis of water samples without PMAxx pretreatment did not show significant differences in virus load between nondisinfected and ClO_2–disinfected secondary effluent. A laboratory-scale validation experiment confirmed the ability of PMAxx pretreatment in discriminating enteric viruses inactivated by ClO_2. Pretreatment using PMAxx and Triton significantly reduced the RT-qPCR signals of NoV GI in ClO_2–treated water when compared to the non-pretreated samples. This could indicate virus inactivation by ClO_2. For NoV GII and HAstV, these differences were not detected, suggesting lack of viral inactivation in water samples. In our study, the ClO_2 treatment was only minimally effective in reducing the load of enteric viruses in reclaimed water assessed by RT-qPCR coupled with PMAxx pretreatment. However, NoV and HAstV could not be detected in the lettuce irrigated with contaminated water, probably due to the limited recovery achieved. In any case, ClO_2 treatment should be optimized to achieve the reductions needed to make reclaimed water microbiologically safe for its use in irrigation (e.g., using longer contact times or higher ClO_2 doses). The efficacy of the water-disinfection treatments should be assessed using novel techniques that allow differentiation between infectious and noninfectious viral particles.

Supplemental Material

Supplemental table: Physicochemical characteristics of PBS and secondary effluent water before and during treatment with ClO2 at the laboratory scale.

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