1 Research Group on Quality, Safety and Bioactivity of Plant Foods
2 Dept. Food Science and Technology
3 CEBAS-CSIC
4 Building 25, Campus de Espinardo, 30100, Murcia, SPAIN
5
6

7 P.O. Box 47,
8 6700 AA Wageningen,
9 the Netherlands

10 25th June 2018

11 Dear **Nynke Hofstra**,

12 Please find enclosed the second revision of the manuscript “Irrigating lettuce with
13 wastewater effluent: Does disinfection with chlorine dioxide inactivates viruses?”
14 submitted for publication in the Journal of Environmental Quality, to the special issue
15 “Microbial water quality: monitoring and modeling”. The paper has been amended
16 following the editor and reviewer comments. We would like to thank the editor for the
17 valuable comments which have helped us to improve the quality of the manuscript.

18

19 Thank you for your consideration.

20 Yours sincerely,

21

22 Ana Allende

23

24

25 **Irrigating lettuce with wastewater effluent: Does disinfection with chlorine dioxide**
26 **inactivates viruses?**

27

28 F. López-Gálvez^{1*}, W. Randazzo^{2,3}, A. Vásquez⁴, G. Sánchez², L. Tombini Decol⁵, R. Aznar^{2,3},
29 M.I. Gil¹, Ana Allende¹

30

31 ¹Research Group on Quality, Safety and Bioactivity of Plant Foods, Department of
32 Food Science and Technology, CEBAS-CSIC, Campus Universitario de Espinardo, 25,
33 30100, Murcia, Spain.

34 ²Department of Biotechnology, IATA-CSIC, Av. Agustín Escardino, 7, 46980,
35 Valencia, Spain.

36 ³Departament of Microbiology and Ecology, University of Valencia. Av. Dr. Moliner,
37 50. 46100 Burjassot. Valencia. Spain.

38 ⁴Faculty of Animal Science and Food Engineering, University of São Paulo, Av. Duque
39 de Caxias Norte, 225. 13635-900 Pirassununga, São Paulo, Brazil.

40 ⁵Laboratório de Microbiologia e Controle de Alimentos, Instituto de Ciência e
41 Tecnologia de Alimentos, Universidade Federal do Rio Grande do Sul (ICTA/UFRGS).
42 Av. Bento Gonçalves 9.500, prédio 43212, Campos do Vale, Agronomia, CEP: 91501-
43 970, Porto Alegre/RS, Brazil.

44

45

46

47 *Corresponding author: Dr. Francisco López-Gálvez. E-mail: flopez@cebas.csic.es

48 **Core Ideas**

- 49 - Reclaimed water used for irrigation can contain enteric viruses.
- 50 - Current detection methods do not distinguish infectious from non-infectious
- 51 viruses.
- 52 - PMAxx treatment can help to get information on the infectivity of viral particles.
- 53 - Treatment of reclaimed water with chlorine dioxide did not improve irrigation
- 54 water safety.

55 **Abstract**

56 Reclaimed water obtained from urban wastewater is currently being used as
57 irrigation water in water-scarce regions in Spain. However, wastewater can contain
58 enteric viruses that water-reclamation treatment cannot remove or inactivate completely.
59 In the present study, greenhouse-grown baby lettuce was irrigated with secondary
60 treatment effluent from a wastewater treatment plant untreated and treated using
61 chlorine dioxide (ClO₂). The effect of ClO₂ treatment on the physicochemical
62 characteristics and the presence of enteric viruses in irrigation water and lettuce was
63 assessed. Presence of human noroviruses genogroups I and II (NoV GI and NoV GII),
64 and human astroviruses (HAstV), was analyzed by real-time PCR (RT-qPCR).
65 Additionally, to check for the loss of infectivity induced by the disinfection treatment,
66 positive samples were re-analyzed after pre-treatment with the intercalating dye PMAxx
67 before RNA extraction and RT-qPCR. There were no significant differences in the
68 proportion of positive samples and the concentration of enteric viruses between treated
69 and untreated reclaimed water without PMAxx pre-treatment ($p>0.05$). A significantly
70 lower concentration of NoV GI was detected in ClO₂-treated water when samples were
71 pretreated with PMAxx ($p<0.05$), indicating that inactivation was due to the disinfection
72 treatment. Laboratory-scale validation tests indicated the suitability of PMAxx-RT-
73 qPCR for discrimination between potentially infectious and ClO₂-damaged viruses.
74 Although the applied ClO₂ treatment was not able to significantly reduce the enteric
75 virus load of the secondary effluent from the wastewater treatment plant, none of the
76 lettuce samples analyzed ($n=36$) was positive for the presence of NoV or HAstV.

77

78 *Keywords:* wastewater; norovirus; astrovirus; vegetables; irrigation water; infectivity.

79

80 **Introduction**

81 Access to adequate water quality is challenging in several regions of the world,
82 and the situation is expected to worsen as climate change exacerbates water scarcity.
83 Water recycling and reuse is a strategy to save water resources. In some parts of the
84 world, water reclaimed from wastewater will need to be used for irrigation to sustain
85 agriculture ([Becerra-Castro et al., 2015](#)). Among other human pathogens, enteric viruses
86 can be found in the aquatic environment, mainly by the discharge of wastewater
87 treatment plant (WWTP) effluents or the failure of depuration processes ([Morsy et al.,](#)
88 [2007](#); [Hewitt et al., 2011](#); [Rusiñol et al., 2015](#)).

89 In the growing trend in foodborne outbreaks associated with the consumption of
90 leafy greens, irrigation water is usually suspected as the main contamination source ([Li](#)
91 [et al., 2015](#)). Presence of human norovirus (NoV) and human astroviruses (HAstV) in
92 irrigation water is one cause of fresh produce contamination with these pathogenic
93 microorganisms ([Bosch et al., 2014](#); [Van Haute et al., 2015](#)). Viruses present in
94 contaminated irrigation water can attach to leaf surfaces and survive at the pre-harvest
95 step ([Wei et al., 2010](#); [Hirneisen and Kniel, 2013](#)). Moreover, other studies conducted
96 on soil-grown lettuce and strawberry reported the internalization of NoV surrogates via
97 plant roots from contaminated soils and the subsequent dissemination to the leaves and
98 fruits ([DiCaprio et al., 2015a, 2015b](#)).

99 Disinfection is a mitigation strategy that can be used to reduce the health risk
100 associated with the presence of microbial pathogens in irrigation water, although little
101 information is available on its effectivity on human enteric viruses ([Hamilton et al.,](#)
102 [2006](#)). One of the disinfection technologies that can be applied for irrigation water is
103 chlorine dioxide (ClO₂) ([Reitz et al. 2015](#)), which has the advantage of not forming
104 organohalogen by-products and is a more powerful oxidant than sodium hypochlorite
105 ([Gómez-López et al. 2009](#); [López-Gálvez et al., 2010](#)). Chlorine-washing procedures
106 are reported to be almost ineffective in removing human NoV surrogates from fresh
107 produce ([Cromeans, 2014](#); [DiCaprio, 2015c](#)), while several studies have reported the
108 sensitivity of a range of viruses to ClO₂ ([Butot et al., 2008](#); [Jin et al., 2012](#); [Lim et al.,](#)
109 [2010](#); [Li et al., 2015](#); [Xue et al., 2013](#)). However, no information on HAstV has been
110 reported so far.

111 Currently, real-time RT-PCR (RT-qPCR) is the gold-standard method for the
112 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 (HAV) and NoV detection in food and bottled water ([International Organization for Standardization, 2017](#)). However, this technique cannot distinguish between infectious and non-infectious viral particles ([Knight et al., 2013](#); [Hamza et al., 2011](#)). For instance, [Lim et al. \(2010\)](#), reported that the virus inactivation by ClO₂ measured by cell culture using norovirus 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 (EMA), propidium monoazide (PMA) or PMAxx ([Coudray-Meunier et al., 2013](#); [Prevost et al., 2016](#); [Randazzo et al., 2016, 2017](#)). 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, 2017](#)).

The aim of the present study was to assess ClO₂ disinfection of a secondary effluent of wastewater used for lettuce irrigation. We examined: (i) occurrence of NoV and HAsV in irrigation water, (ii) NoV and HAsV inactivation in irrigation water, and (iii) occurrence of NoV and HAsV in lettuce.

Material and Methods

Experimental setup

Red oak leaf lettuce (*Lactuca sativa*, L.) 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 wastewater of two qualities was compared: untreated secondary effluent from a wastewater treatment plant (WWTP; secondary wastewater treatment was performed as described in [López-Gálvez et al., 2016](#)) and ClO₂-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 °C and 32.2 °C, with an average of 28.1±1.5 °C. The relative humidity (RH) in the greenhouse ranged from 55.1% to 99.9%, with an average of 80.8±10.2%. Irrigation water applied at ≈300 L/d in two irrigation periods of 20 min

each. The growth cycle of baby lettuce lasted 38 days in the first trial (June-July), and 26 days in the second trial (July-August).

Water samples were taken weekly during the whole growth cycle. Due to the small plant size, samples of baby lettuce were taken weekly starting two weeks 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 lab 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.

ClO_2 treatment and physicochemical analysis of water

A concentrated solution of ClO_2 (≈ 6000 mg/L) AGRI DIS[®] was diluted to 100-300 mg/L ClO_2 using tap water in a 20-L opaque plastic tank according to manufacturer instructions (Servicios Técnicos de Canarias, Las Palmas de Gran Canaria, Spain). ClO_2 -treated water 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 carried out daily by chronoamperometry analysis (Palintest, Gateshead, UK) in untreated and ClO_2 -treated water samples taken during irrigation at the sprinklers. Temperature, pH, and ORP were measured with a multimeter (pH and redox 26, Crison, Barcelona, Spain). Water filtered through 0.45- μm syringe nylon filters (Fisherbrand-Fisher Scientific, Waltham, USA) was used for measuring the absorbance at 254 nm using a UV-VIS spectrophotometer (Jasco V-630, Tokyo, Japan) and quartz cuvettes with a path length of 1 cm (Hellma, Müllheim, Germany). Total organic carbon (TOC) was assessed using a multi N/C 3100 analyzer (Analytik Jena,

179 Jena, Germany) after filtration through ashless filter paper (Albet Labscience, Dassel,
180 Germany).

181

182 *NoV and HAsV analysis in water*

183 Recovery of viruses from water was performed as described in [López-Gálvez et](#)
184 [al. \(2016\)](#). Briefly, magnesium chloride (MgCl_2) was added to each water sample and
185 pH was adjusted to 3.5 with hydrogen chloride (HCl). Samples of 200 mL were filtered
186 through 0.45- μm membrane filters (Sartorius, Göttingen, Germany). Filters were then
187 transferred to sterile tubes containing 5 mL of elution buffer, and pH was adjusted to 9.5
188 using sodium hydroxide (NaOH). Tubes were shaken for 1 min in a vortex, kept for 4
189 min in an ultrasonic bath, and shaken again in a horizontal orbital shaker at 250 rpm for
190 10 min, and then pH was adjusted to 7 using HCl (10%). After that, samples were kept
191 at -70 °C until analysis. To monitor the virus concentration procedure and RNA
192 extraction, 200 mL of each secondary effluent water sample was spiked with a known
193 concentration of mengovirus M0 strain prior to processing as process control virus
194 following ISO 15216 guidelines ([International Organization for Standardization, 2017](#);
195 [Costafreda et al., 2006](#)). RNA extraction was performed using the NucleoSpin® RNA
196 virus kit (Macherey-Nagel GmbH & Co.) according to the manufacturer's instructions
197 and with the plant RNA isolation aid treatment as detailed in [Randazzo et al. \(2016\)](#).
198 Human NoV and HAsV genome copies were quantified in duplicate by one-step RT-
199 qPCR assay using the RNA UltraSense One-Step quantitative system (Invitrogen SA)
200 with a half-scale modification of the manufacturer's protocol and the LightCycler 480
201 instrument (Roche Diagnostics) following the standardized ISO procedure ([ISO 15216-](#)
202 [1:2017, International Organization for Standardization, 2017](#)) for NoVs, and the primers
203 and probe for HAsV as suggested by [Sano et al. \(2010\)](#).

204 Additionally, positive samples for NoV GI, NoV GII, or HAsV were extracted
205 again, and a PMAXx-Triton pretreatment was applied in parallel before RNA extraction
206 ([Randazzo et al., 2016](#)). Briefly, 100 μL of concentrated samples was mixed with 50 μM
207 PMAXx and 0.5% Triton (PMAXx-Triton), incubated in the dark at room temperature
208 for 10 min in a shaker at 150 rpm, and immediately exposed to continuous LED light
209 (Led-Active Blue, GenIUL) for 15 min. After intercalating-dye pretreatment, RNA was
210 extracted using the NucleoSpin® RNA virus kit according to the manufacturer's
211 instructions. RT-qPCR was performed as described above. As a control, 100 μL of

concentrated water samples was processed without performing the PMAxx-Triton pretreatment.

NoV and HAsV 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, Geel, Belgium) and sodium chloride (NaCl) (Panreac Química, Barcelona, Spain) 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 carried out at 8500 g for 5 min at 4 °C to compact the pellet. The pellet was finally re-suspended 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 PBS and secondary effluent waters treated with ClO₂

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 HAsV were suspended (10%, wt/vol) in phosphate-buffered saline (PBS) containing 2 M NaNO₃ (Panreac), 1% beef extract (Conda), and 0.1% Triton X-100 (Fisher Scientific) (pH 7.2), vortexed and centrifuged at 1000 xg for 5 min. The supernatant was used to inoculate 500-mL aliquots of PBS (pH 7.2) and a WWTP secondary treatment effluent. ClO₂ was added to achieve an initial concentration of 5.5 or 11 mg/L, 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) was added to neutralize ClO₂. ClO₂ concentration was measured by chronoamperometry using a portable device (Chlordioxense; Palintest, Gateshead, UK). 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-RTqPCR 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, Loveland, Colorado, USA). 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

NoV 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, Redmond, WA, USA). IBM SPSS statistics 20 (IBM, Armonk, NY, USA) 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, non-parametric tests were applied. To compare the prevalence of positive samples for viruses between ClO₂ treated and untreated irrigation water, the chi-square (χ^2) test was used.

Results and Discussion

ClO₂ treatment and physicochemical characteristics of irrigation water

The doses of ClO₂ that were applied were selected based on preliminary tests with the aim of reaching levels of *Escherichia coli* in the water below 2 log cfu/100 mL, which corresponds to the *E. coli* threshold recommended in several GAP guidelines for irrigation water (EC, 2017). To achieve this goal, the initial ClO₂ concentration in treated water ranged between 3 and 11 mg/L. Mean residual ClO₂ concentration in ClO₂ treated irrigation water at the sprinklers was 0.53±0.40 and 0.42±0.43 mg/L 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 ClO₂ in the treated water varied between 0.04 and 1.28 mg/L, and between 0.03 and 1.51 mg/L in the first and the second trials, respectively. 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 ClO₂ treatment did not change the physicochemical characteristics of the irrigation water significantly (Table 1). 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 ClO₂-treated and untreated water (Table 1). In contrast, higher ORP and UV254 values have been reported for ClO₂-treated irrigation water even though the ClO₂ 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.

294

295 *Occurrence of viruses in irrigation water*

All the water samples were positive for the sample process control (Mengovirus) with a percent 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 ClO₂-treated water samples analyzed without PMAxx pre-treatment 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-year study that in some months there was a higher concentration of NoV GI compared to 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 ClO₂ 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 ClO₂-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 ClO₂-treated water samples (**Table 2**). Similar results were recently reported by [Kobayashi et al. \(2017\)](#), where a down-flow hanging sponge (DHS) 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. RT-qPCR is not able to distinguish between infectious and non-infectious viruses ([Knight et al., 2013](#)), which could explain the lack of differences in the virus concentration between untreated and ClO₂-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 HAV, NoV GI and NoV GII in irrigation and wastewater samples ([Randazzo et al., 2016](#); [Randazzo et al., 2018ab](#)). 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, 2017](#)) 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 ClO₂-treated viruses had not been confirmed by previous studies.

The two ClO₂ concentrations tested in our lab-scale study represented the average (≈ 5.5 mg/L) and the highest initial levels (≈ 11 mg/L) of ClO₂ applied during the greenhouse tests. Figure 1 shows the concentration of viral genomes detected by RT-qPCR and by PMAXx-RT-qPCR after ClO₂ treatment was performed in PBS and secondary treatment effluent waters. Results show the ability of PMAXx pretreatment in discriminating potentially infectious and ClO₂-damaged viruses. Comparing the results obtained by RT-qPCR and PMAXx-RT-qPCR in ClO₂-treated PBS suspensions (Fig. 1A), it could be concluded that PMAXx pretreatment allowed detection of the effect of ClO₂ treatment on the integrity of viral structure. This effect was significant in NoV GI and GII for the two ClO₂ concentrations tested, whereas it was significant in HAstV only for the highest disinfectant concentration (11 mg/L). [Wiggington et al. \(2012\)](#) and [Sigstam et al. \(2013\)](#) reported extensive degradation of viral proteins by chlorine

dioxide, 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 lab 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 HAsV 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 (Supplementary information 1). 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 ppm chlorine dioxide. 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 ClO_2 . However, as explained before, smaller reductions were detected when secondary effluent water was treated.

Regarding the greenhouse tests, **Table 3** shows the concentrations (log genome copies/L) of NoV GI and GII in water samples positive for the presence of NoV and HAsV that were re-analyzed with and without PMAxx-Triton pre-treatment. 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 HAsV ($p > 0.05$), indicating that the viral particles remained uninjured after the disinfection treatment with ClO_2 . As could be expected, no significant differences were observed for NoV and HAsV between PMAxx-pretreated and non-pretreated samples in untreated irrigation water (**Table 3**). In a previous study, RT-qPCR signal of NoV GII was more

378 easily removed than the signal of NoV GI using the PMAxx pretreatment, which was
379 attributed to a better environmental persistence of NoV GI ([Randazzo et al. 2016](#)).

380 [Sales-Ortells et al. \(2014\)](#) assessed the health risks derived from consumption of
381 lettuces irrigated with tertiary effluent containing norovirus in Catalonia (Spain), stating
382 that reductions of 4.3 log should be ensured to reduce the disease burden to
383 10^{-6} Disability Adjusted Life Years (DALYs) per person per year, as recommended
384 ([WHO, 2006](#)). In the present study, a maximum reduction of 0.65 log for NoV GI in
385 secondary treatment effluent water was detected by PMAxx-RT-qPCR during the
386 greenhouse tests. In the lab-scale tests, the reductions obtained in secondary effluent
387 water were higher, but were still far from the target of 4.3 log.

388

389 *Occurrence of viruses in lettuce*

390 All the analyzed lettuce samples were positive for the mengovirus process
391 control, with average recoveries of $10.2 \pm 8.5\%$, in accordance with the ISO 15216
392 recommendations ([International Organization for Standardization, 2017](#)), which
393 establishes acceptable recoveries greater than 1%. In line, [Hennechart-Collette et al.](#)
394 [\(2015\)](#) reported similar recoveries of mengovirus from lettuce (10.8%). In the present
395 study, no lettuce samples positive for NoV GI, NoV GII, or HAstV (n=36) were
396 observed. As viruses were detected in irrigation water and they can survive in pre-
397 harvest conditions, such results were unexpected, especially taking into account the
398 sprinkler irrigation system used ([Hirneisen and Kniel, 2013](#)). The lack of positive
399 results in lettuce could be explained by the low recovery rates compared with those
400 obtained in water samples. Furthermore, the protocol used for virus recovery could not
401 detect internalized viral particles if present. [Pérez-Rodríguez et al. \(2014\)](#) detected two
402 positive samples for NoV GI and four positive samples for NoV GII when analyzing 60
403 lettuce samples (both unprocessed and ready-to-eat lettuce), which corroborates the low
404 prevalence in fresh produce. In accordance with these results, [Kokkinos et al. \(2012\)](#)
405 found only two positives out of 149 samples of lettuce analyzed for NoV GI, and one
406 positive sample out of 126 of lettuce analyzed for NoV GII. [Baert et al. \(2011\)](#) found
407 186 samples positive for NoV out of 653 samples (28.5%). Furthermore, at
408 temperatures as high as those observed during the trials, high reduction of virus titers in
409 fresh produce surfaces was expected (3-4 log at 30 °C in 24 h; [Carratalà et al., 2013](#)).

410

411 **CONCLUSIONS**

412 RT-qPCR analysis of water samples without PMAxx pretreatment did not show
413 significant differences in virus load between non-disinfected and ClO₂-disinfected
414 secondary effluent. A lab-scale validation experiment confirmed the ability of PMAxx
415 pretreatment in discriminating enteric viruses inactivated by ClO₂. Pretreatment using
416 PMAxx and Triton significantly reduced the RT-qPCR signals of NoV GI in ClO₂-
417 treated water when compared to the non-pretreated samples. This could indicate virus
418 inactivation by ClO₂. For NoV GII and HAstV these differences were not detected,
419 suggesting lack of viral inactivation in water samples. In our study, the ClO₂ treatment
420 was only minimally effective in reducing the load of enteric viruses in reclaimed water
421 assessed by RT-qPCR coupled with PMAxx pretreatment. However, NoV and HAstV
422 could not be detected in the lettuce irrigated with contaminated water, probably due to
423 the limited recovery achieved. In any case, ClO₂ treatment should be optimized to
424 achieve the reductions needed to make reclaimed water microbiologically safe for its
425 use in irrigation (e.g., using longer contact times or higher ClO₂ doses). The efficacy of
426 the water-disinfection treatments should be assessed using novel techniques that allow
427 differentiation between infectious and non-infectious viral particles.

428

429 **Acknowledgments**

430 Authors are thankful for the financial support from the Center for Produce Safety
431 Grant Agreement (Projects 2015-374 and 2017-01), the MINECO (Projects AGL2013-
432 48529-R and AGL2016-75878-R) and the Spanish National Institute for Agriculture
433 and Food Research and Technology (INIA) co-financed by the European Social Fund
434 (Project RTA2014-00024-C03). Financial support has been co-sponsored by the
435 European Regional Development Fund (FEDER). Support provided by the Fundación
436 Séneca (19900/GERM/15) and Proyecto Intramural 201670E056 is also appreciated.
437 This work was carried out during a scholarship supported by the Doctoral Program
438 Sandwich Exterior (PDSE), process number 88881.132189 / 2016-01, financed by
439 CAPES - Brazilian Federal Agency for Postgraduate Support and Evaluation at the
440 Brazilian Ministry of Education. We also thank Dr. Albert Bosch (University of Barcelona,

Spain) for providing the Mengovirus M0 strain, and Dr. Buesa (University of Valencia, Spain) for providing the fecal samples.

443

444 **References**

445 APHA (American Public Health Association). 2012. Standard Methods for the
446 Examination of Water and Wastewater, 22th ed. American Public Health
447 Association, Washington, D.C.

448 Baert, L., K. Mattison, F. Loisy-Hamon, J. Harlow, A. Martyres, B. Lebeau, et al. 2011.
449 Review: Norovirus prevalence in Belgian, Canadian and French fresh produce: A
450 threat to human health? *Int. J. Food Microbiol.* 151:261-269.

451 Becerra-Castro, C., Lopes, A.R., Vaz-Moreira, I., Silva, E.F., Manaia, C. M., Nunes,
452 O.C., 2015. Wastewater reuse in irrigation: A microbiological perspective on
453 implications in soil fertility and human and environmental health. *Environ.*
454 *International*, 75, 117–135.

455 Bosch, A., R.M. Pintó, and S. Guix. 2014. Human Astroviruses. *Clin. Microbiol. Rev.*
456 27:1048-1074.

457 Bosch, A., G. Sánchez, M. Abbaszadegan, A. Carducci, S. Guix, F. Le Guyader, et al.
458 2011. Analytical methods for virus detection in water and food. *Food Anal.*
459 *Method.* 4:4-12.

460 Butot, S., T. Putallaz, and G. Sánchez. 2008. Effects of sanitation, freezing and frozen
461 storage on enteric viruses in berries and herbs. *Int. J. Food Microbiol.* 126:30-5.

462 Carratalà, A., J. Rodriguez-Manzano, A. Hundesa, M. Rusiñol, S. Fresno, N. Cook, and
463 R. Girones. 2013. Effect of temperature and sunlight on the stability of human
464 adenoviruses and MS2 as fecal contaminants on fresh produce surfaces. *Int. J.*
465 *Food Microbiol.* 164:128-134.

466 Costafreda, M.I., A. Bosch, and R.M. Pintó. 2006. Development, evaluation, and
467 standardization of a real-time TaqMan reverse transcription-PCR assay for
468 quantification of hepatitis A virus in clinical and shellfish samples. *Appl. Environ.*
469 *Microb.* 72:3846-3855.

- 470 Coudray-Meunier, C., A. Fraisse, S. Martin-Latil, L. Guillier, and S. Perelle. 2013.
471 Discrimination of infectious hepatitis A virus and rotavirus by combining dyes
472 and surfactants with RT-qPCR. *BMC Microbiol.* 13:216.
- 473 Cromeans, T., G.W. Park, V. Costantini, D. Lee, Q. Wang, T. Farkas, et al. 2014.
474 Comprehensive comparison of cultivable norovirus surrogates in response to
475 different inactivation and disinfection treatments. *Appl. Environ. Microb.*
476 80:5743-5751.
- 477 Da Silva, A.K., J.-C. Le Saux, S. Parnaudeau, M. Pommepuy, M. Elimelech, and F.S.
478 Le Guyader. 2007. Evaluation of removal of noroviruses during wastewater
479 treatment, using real-time reverse transcription-PCR: Different behaviors of
480 genogroups I and II. *Appl. Environ. Microb.* 73:7891-7897.
- 481 DiCaprio, E., D. Culbertson, and J. Li. 2015a. Evidence of the internalization of animal
482 caliciviruses via the roots of growing strawberry plants and dissemination to the
483 fruit. *Appl. Environ. Microb.* 81:2727-2734.
- 484 DiCaprio, E., A. Purgianto, and J. Li. 2015b. Effects of abiotic and biotic stresses on the
485 internalization and dissemination of human norovirus surrogates in growing
486 romaine lettuce. *Appl. Environ. Microb.* 81:4791-4800.
- 487 DiCaprio, E., A. Purgianto, Y. Ma, J. Hughes, X. Dai, and J. Li. 2015c. Attachment and
488 localization of human norovirus and animal caliciviruses in fresh produce. *Int. J.*
489 *Food Microbiol.* 211:101-108.
- 490 EC (European Commission). 2017. Commission notice on guidance document on
491 addressing microbiological risks in fresh fruits and vegetables at primary
492 production through good hygiene (2017/C 163/01). *Official Journal of the*
493 *European Union* 163: 1-40.
- 494 Fuster, N., R.M. Pintó, C. Fuentes, N. Beguiristain, A. Bosch, and S. Guix. 2016.
495 Propidium monoazide RTqPCR assays for the assessment of hepatitis A
496 inactivation and for a better estimation of the health risk of contaminated waters.
497 *Water Res.* 101:226-232.

- 498 Gómez-López, V.M., A. Rajkovic, P. Ragaert, N. Smigic, and F. Devlieghere. 2009.
499 Chlorine dioxide for minimally processed produce preservation: a review. *Trends*
500 *Food Sci. Technol.* 20:17-26.
- 501 Hamilton, A.J., F. Stagnitti, R. Premier, A.-M. Boland, and G. Hale. 2006. Quantitative
502 microbial risk assessment models for consumption of raw vegetables irrigated
503 with reclaimed water. *Appl. Environ. Microb.* 72:3284-3290.
- 504 Hamza, I.A., L. Jurzik, K. Überla, and M. Wilhelm. 2011. Methods to detect infectious
505 human enteric viruses in environmental water samples. *Int. J. Hyg. Envir. Heal.*
506 214:424-436.
- 507 Hennechart-Collette, C., S. Martin-Latil, L. Guillier, and S. Perelle. 2015.
508 Determination of which virus to use as a process control when testing for the
509 presence of hepatitis A virus and norovirus in food and water. *Int. J. Food*
510 *Microbiol.* 202:57-65.
- 511 Hewitt, J., M. Leonard, G.E. Greening, and G.D. Lewis. 2011. Influence of wastewater
512 treatment process and the population size on human virus profiles in wastewater.
513 *Water Res.* 45:6267-6276.
- 514 Hirneisen, K.A., and K.E. Kniel. 2013. Norovirus Surrogate Survival on Spinach
515 During Preharvest Growth. *Phytopathology* 103:389-394.
- 516 International Organization for Standardization. 2017. ISO 15216-1:2017. Microbiology
517 of food and animal feed. Horizontal Method for Determination of Hepatitis A
518 Virus and Norovirus in Food Using Real-Time RT-PCR. Part 1: Method for
519 quantification.
- 520 Jin, M., Z.-G. Zhao, X.-W. Wang, Z.-Q. Shen, L. Xu, Y.-M. Yu, et al. 2012. The 40-
521 80nt region in the 5'-NCR of genome is a critical target for inactivating poliovirus
522 by chlorine dioxide. *J. Med. Virol.* 84:526-535.
- 523 Katayama, H., E. Haramoto, K. Oguma, H. Yamashita, A. Tajima, H. Nakajima, and S.
524 Ohgaki. 2008. One-year monthly quantitative survey of noroviruses,
525 enteroviruses, and adenoviruses in wastewater collected from six plants in Japan.
526 *Water Res.* 42:1441-1448.

- 527 Kingsley, D.H., Vincent, E.M., Meade, G.K., Watson, C.L., Fan, X. 2014. Inactivation
528 of human norovirus using chemical sanitizers. *Int. J. Food Microbiol.* 171:94-99.
- 529 Kitajima, M., B.C. Iker, I.L. Pepper, and C.P. Gerba. 2014. Relative abundance and
530 treatment reduction of viruses during wastewater treatment processes -
531 Identification of potential viral indicators. *Sci. Total Environ.* 488-489:290-296.
- 532 Knight, A., D. Li, M. Uyttendaele, and L.A. Jaykus. 2013. A critical review of methods
533 for detecting human noroviruses and predicting their infectivity. *Crit. Rev.*
534 *Microbiol.* 39:295-309.
- 535 Kobayashi, N., M. Oshiki, T. Ito, T. Segawa, M. Hatamoto, T. Kato, et al. 2017.
536 Removal of human pathogenic viruses in a down-flow hanging sponge (DHS)
537 reactor treating municipal wastewater and health risks associated with utilization
538 of the effluent for agricultural irrigation. *Water Res.* 110:389-398.
- 539 Kokkinos, P., Kozyra, I., Lazic, S., Bouwknecht, M., Rutjes, S., Willems, K., et al. 2012.
540 Harmonised Investigation of the Occurrence of Human Enteric Viruses in the
541 Leafy Green Vegetable Supply Chain in Three European Countries. *Food*
542 *Environ. Virol.* 4:179-191.
- 543 Leifels, M., L. Jurzik, M. Wilhelm, and I.A. Hamza. 2015. Use of ethidium monoazide
544 and propidium monoazide to determine viral infectivity upon inactivation by heat,
545 UV- exposure and chlorine. *Int. J. Hyg. Environ. Health* 218:686-93.
- 546 Li, D., A., De Keuckelaere, and M. Uyttendaele. 2015. Fate of foodborne viruses in the
547 "farm to fork" chain of fresh produce. *Compr. Rev. Food Sci. Food Saf.* 14:755-
548 770.
- 549 Lim, M.Y., J.-M. Kim, and G.P. Ko. 2010. Disinfection kinetics of murine norovirus
550 using chlorine and chlorine dioxide. *Water Res.* 44:3243-3251.
- 551 López-Gálvez, F., A. Allende, A. Martinez-Sanchez, J.A. Tudela, M.V. Selma, and M.I.
552 Gil. 2010. Suitability of aqueous chlorine dioxide versus sodium hypochlorite as
553 an effective sanitizer for preserving quality of fresh-cut lettuce while avoiding by-
554 product formation. *Postharvest Biol. Tec.* 55:53-60.

- 555 López-Gálvez, F., P. Truchado, G. Sánchez, R. Aznar, M.I. Gil, and A. Allende. 2016.
 556 Occurrence of enteric viruses in reclaimed and surface irrigation water:
 557 relationship with microbiological and physicochemical indicators. *J. Appl.*
 558 *Microbiol.* 121:1180-1188.
- 559 López-Gálvez, F., Gil, M.I., Meireles, A., Truchado, P., and Allende, A. Demonstration
 560 tests of irrigation water disinfection with chlorine dioxide in open field cultivation
 561 of baby spinach. *J. Sci. Food Agric.* 98: 2973-2980.
- 562 Moreno, L., R. Aznar, and G. Sánchez. 2015. Application of viability PCR to
 563 discriminate the infectivity of hepatitis A virus in food samples. *Int. J. Food*
 564 *Microbiol.* 201:1-6.
- 565 Morsy El-Senousy, W., S. Guix, I. Abid, R.M. Pinto, and A. Bosch. 2007. Removal of
 566 astrovirus from water and sewage treatment plants, evaluated by a competitive
 567 reverse transcription-PCR. *Appl. Environ. Microbiol.* 73:164 –167
- 568 Parshionikar, S., I. Laseke, and G.S. Fout. 2010. Use of propidium monoazide in
 569 reverse transcriptase PCR to distinguish between infectious and noninfectious
 570 enteric viruses in water samples. *Appl. Environ. Microbiol.* 76:4318–4326.
- 571 Pérez-Rodríguez, F., P. González-García, A. Valero, M. Hernández, and D. Rodríguez-
 572 Lázaro. 2014. Impact of the prevalence of different pathogens on the performance
 573 of sampling plans in lettuce products. *Int. J. Food Microbiol.* 184:69-73.
- 574 Prevost, B., M. Goulet, F.S. Lucas, M. Joyeux, L. Moulin, and S. Wurtzer. 2016. Viral
 575 persistence in surface and drinking water: Suitability of PCR pre-treatment with
 576 intercalating dyes. *Water Res.* 91:68-76.
- 577 Quijada, N.M., G. Fongaro, C.R.M. Barardi, M. Hernández, and D. Rodríguez-Lázaro.
 578 2016. Propidium monoazide integrated with qPCR enables the detection and
 579 enumeration of infectious enteric RNA and DNA viruses in clam and fermented
 580 sausages. *Front. Microbiol.* 7(DEC), 2008.
- 581 Randazzo, W., F. López-Gálvez, A. Allende, R. Aznar, G. Sánchez. 2016. Evaluation of
 582 viability PCR performance for assessing norovirus infectivity in fresh-cut
 583 vegetables and irrigation water. *Int. J. Food Microbiol.* 229:1-6.

- 584 Randazzo, W., Piqueras, J., Rodríguez-Díaz, J. Aznar, R., Sánchez, G. 2018a.
 585 Improving efficiency of viability-qPCR for selective detection of infectious HAV
 586 in food and water samples. *J. Appl. Microbiol.* 124, 958-964.
- 587 Randazzo, W., Khezri, M., Ollivier, J., Le Guyader, F.S., Rodríguez-Díaz, J., Aznar, R.,
 588 Sánchez, G. 2018b. Optimization of PMAxx pretreatment to distinguish between
 589 human norovirus with intact and altered capsids in shellfish and sewage samples.
 590 *Int. J. Food Microbiol.*, 266, 1-7.
- 591 Reitz, S.R., R.A. Roncarati, C.C. Shock, H. Kreeft, and J. Klauzer. 2015. Chlorine
 592 dioxide injection through drip irrigation reduces *Escherichia coli*. 2015
 593 ASABE/IA Irrigation Symposium: Emerging Technologies for Sustainable
 594 Irrigation. Proceedings of the 10-12 November 2015 Symposium, Long Beach,
 595 California USA. Published by ASABE St. Joseph, Michigan, USA. Publication
 596 date November 10, 2015. ASABE Publication No. 701P0415.
- 597 Rusiñol, M., X. Fernandez-Cassi, N. Timoneda, A. Carratalà, J.F. Abril, C. Silvera, et
 598 al. 2015. Evidence of viral dissemination and seasonality in a Mediterranean river
 599 catchment: Implications for water pollution management. *J. Environ. Manage.*
 600 159: 58-67.
- 601 Sales-Ortells, H., X. Fernandez-Cassi, N. Timoneda, W. Durig, R. Girones, and G.
 602 Medema. 2014. Health risks derived from consumption of lettuces irrigated with
 603 tertiary effluent containing norovirus. *Food. Res. Int.* 68:70-77.
- 604 Sano, D., R.M. Pinto, T. Omura, and A. Bosch. 2010. Detection of oxidative damages
 605 on viral capsid protein for evaluating structural integrity and infectivity of human
 606 norovirus. *Environ. Sci. Technol.* 44:808-812.
- 607 Sigstam, T., Gannon, G., Cascella, M., Pecson, B. M., Wigginton, K. R., and Kohn, T.
 608 2013. Subtle differences in virus composition affect disinfection kinetics and
 609 mechanisms. *Appl. Environ. Microbiol.* 79:3455-3467.
- 610 Van Haute, S., I. Samper, L. Jacxsens, and M. Uyttendaele. 2015. Selection Criteria for
 611 Water Disinfection Techniques in Agricultural Practices. *Crit. Rev. Food Sci.*
 612 11:1529-1551.

- 613 WEAH (Water Education Alliance for Horticulture). 2016. Treatment Technologies:
 614 Chlorine dioxide. Available at: <http://watereducationalliance.org/keyinfo.asp>.
 615 Accessed 26 October 2016.
- 616 Wei, J., Y. Jin, T. Sims, and K.E. Kniel. 2010. Manure- and biosolids-resident murine
 617 norovirus 1 attachment to and internalization by romaine lettuce. *Appl. Environ.*
 618 *Microbiol.* 76:578-583.
- 619 WHO (World Health Organization). 2006. Wastewater Use in Agriculture. In: World
 620 Health Organization, editor. Guidelines for the safe use of wastewater, excreta and
 621 greywater, Vol. II. WHO, Geneva.
- 622 Wigginton, K.R., Pecson, B.M., Sigstam, T., Bosshard, F., and Kohn, T. 2012. Virus
 623 inactivation mechanisms: impact of disinfectants on virus function and structural
 624 integrity. *Envir. Sci. Tech.* 46:12069-12078.
- 625 Xue, B., M. Jin, D. Yang, X. Guo, Z. Chen, Z. Shen, et al. 2013. Effects of chlorine and
 626 chlorine dioxide on human rotavirus infectivity and genome stability. *Water Res.*
 627 47:3329-3338.
- 628 Yeap, J.W., Kaur, S., Lou, F., DiCaprio, E., Morgan, M., Linton, R., Li, J. 2016.
 629 Inactivation kinetics and mechanism of a human norovirus surrogate on stainless
 630 steel coupons via chlorine dioxide gas. *Appl. Environ. Microbiol.* 82:116-123.

631 **Figure captions**

632 **Figure 1.** Performance of RT-qPCR and PMAxx-RT-qPCR to discriminate between
633 potentially infectious and ClO₂-treated norovirus GI (black bars), GII (grey bars) and
634 astrovirus (white bars) in PBS (panel A) and secondary effluent water (panel B).
635 Different letters denote significant differences among treatments for each virus (p <
636 0.05).

Table 1. Physicochemical characteristics of the secondary effluent of a wastewater treatment plant (untreated) and ClO₂ treated secondary effluent (ClO₂ treated) used for lettuce irrigation. Data are mean±standard deviation of two trials performed between June and August 2016. ORP: Oxidation-reduction potential; TOC: Total organic carbon; UV254: Absorbance at 254 nm.

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)	13.1±3.3	14.4±2.8ns
Initial ClO ₂ (mg/L)*	<0.02	5.6±2.4
Residual ClO ₂ (mg/L)	<0.02	0.51±0.41**

*The initial concentration of ClO₂ was calculated taking into account the irrigation water flow, and the volume and the concentration of the ClO₂ solution dosed; **significant difference (p<0.05) between untreated and treated samples. ns: not significant.

Table 2. Prevalence of human norovirus genogroup I (NoV GI) and II (NoV GII) and human astrovirus (HAstV) analyzed by RT-qPCR in the secondary effluent of wastewater treatment plant (untreated) and ClO₂ treated secondary effluent (ClO₂ treated) used for lettuce irrigation. Samples were not pre-treated with PMAx. Data are expressed as log genome copies/L.

	Untreated				ClO ₂ treated			
	Prevalence (%) ^a	Median	Minimum	Maximum	Prevalence (%)	Median	Minimum	Maximum
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

^aProportion of positive samples from each type of water (n=12). *significant difference (chi-square test, p<0.01) in the prevalence between untreated and treated samples. ns: not significant.

Table 3. Log genome copies/L (Mean $\pm$ SD) in the secondary effluent of wastewater treatment plant (untreated) and ClO₂ treated secondary effluent (ClO₂ treated) used for lettuce irrigation samples analyzed by RT-qPCR with and without the use of PMAxx/Triton. Data are expressed as log genome copies/L.

	Untreated		ClO ₂ treated	
	PMAxx	No PMAxx	PMAxx	No PMAxx
NoV GI	4.65 $\pm$ 0.54	4.75 $\pm$ 0.39ns	4.19 $\pm$ 0.37	4.84 $\pm$ 0.34*
NoV GII	5.47 $\pm$ 0.42	5.28 $\pm$ 0.23ns	5.43 $\pm$ 0.09	5.21 $\pm$ 0.28ns
HAstV	7.04 $\pm$ 0.15	7.14 $\pm$ 0.20ns	6.50 $\pm$ 0.31	6.62 $\pm$ 0.19ns

*significant difference (p<0.01) between samples treated and untreated with PMAxx. ns: not significant.

Figure 1.

Supplementary information 1. Physicochemical characteristics of PBS and secondary effluent water before and during treatment with ClO₂ at lab scale.

Suspension media	Initial ClO ₂ dose (mg/L)	Time (min)	pH	ORP (mV)	Temperature (°C)	ClO ₂ (mg/L)
PBS	5.5	0*	7.32	234	25.4	3.8
		1	7.35	742	25.5	
		3	7.39	747	25.6	
		6	7.42	741	25.7	
Secondary treatment effluent water	5.5	0*	7.43	399	24.4	0.03
		1	7.41	688	24.5	
		3	7.40	659	24.6	
		6	7.39	607	24.7	
PBS	11	0*	7.44	278	25.6	9.29
		1	7.44	764	25.6	
		3	7.44	765	25.7	
		6	7.52	764	25.8	
Secondary treatment effluent water	11	0*	7.81	402	25.6	3.22
		1	7.56	723	25.7	
		3	7.53	712	25.8	
		6	7.54	703	25.9	

*Before adding ClO₂.