This article was published in Food and Bioproducts Processing, 98, 333-340, 2016
http://dx.doi.org/10.1016/j.fbp.2016.02.008

Disinfection with neutral electrolyzed oxidizing water to reduce microbial load and to prevent biofilm regrowth in the processing of fresh-cut vegetables

I. Machado, A. Meireles, R. Fulgêncio, F. Mergulhão, M. Simões, L.F. Melo *

LEPABE, Department of Chemical Engineering, Faculty of Engineering, University of Porto, Rua Dr Roberto Frias, s/n, 4200-465 Porto, Portugal

Abstract

Product decontamination is one of the most important processes of the hygienic practice in food industries such as Minimally Processed Vegetables (MPV) plants and sodium hypochlorite (NaOCl) solutions are commonly used as a biocide for disinfection. Although it may be corrosive and irritating when compared to alternative biocides, this biocide is frequently applied at high concentrations. This work aims at studying the use of lower concentrations of chlorine by testing neutral electrolyzed oxidizing water (NEOW) as a chlorine-source disinfectant in fresh-cut salad processing. Assays were performed at industrial and laboratory scale. Results showed that lower doses of chlorine from NEOW (30 ppm) are as effective as higher concentrations of the traditional chlorine from NaOCl (80 ppm) in the reduction of total microbial population at industrial scale. Moreover, in laboratory studies, the NEOW chlorine was also more effective in biofilm eradication, as well as a biofilm preventive agent. NEOW can thus be a successful alternative water disinfection technique, reducing the free chlorine concentration needed to sanitize salads, also decreasing water consumption whilst taking into account environmental and food quality impacts.

1. Introduction

The consumption of ready-to-eat salads has increased drastically in recent years. In order to ensure the hygienic safety of a product that is consumed raw, salad washing has become a critical step in the production process (De Giusti et al., 2014). High volumes of water are frequently used to achieve microbial load reductions and, in order to maintain good hygienic practices, chlorine compounds (such as sodium hypochlorite) are frequently used as disinfectant agents. However, the free chlorine concentrations in this food industry are very high and the doses applied may fluctuate, leading to lack of knowledge regarding the water disinfection efficiency and the microbial load that remains in the process water and in the washing tanks surface. Regarding the use of hypochlorite, several concerns have been raised, namely the release of toxic chlorine by-products, accumulation of chloramines, and generation of chlorine off-gas in the processing environment (Shen et al., 2012; Vandekinderen et al., 2009) that may pose significant environmental and health risks (Ölmez and Kretzschmar, 2009).

In order to reduce water and chlorine consumption while maintaining salad-washing efficiency and avoiding the production of disinfection by-products like organochlorinated compounds, neutral electrolyzed oxidizing water (NEOW) has been proposed as a sanitizing agent. Electrolyzed oxidizing (EO) water has been regarded as a new sanitizer in recent years in the food sector, for industrial equipment and in the processing of vegetables, fruit, poultry, meat and seafood (Huang et al., 2008).

EO water is generated by electrolysis (Fig. 1) only with water and salt (sodium chloride) as raw materials, having the following advantages over other traditional cleaning agents: effective disinfection, simple operation, relatively inexpensive, environmentally friendly and safe (Huang et al., 2008). Also, the chlorine-based biocide can be produce *in* situ and, therefore, it does not require special handling, storage or transportation of relatively dangerous concentrated chemicals. It is therefore a good alternative to the traditional sodium hypochlorite solutions.

The extent of bacterial transfer and cross-contamination from the water containing disinfectants to the washing tank surfaces (and reciprocally) that can occur during the salad washing remains poorly understood. Many organisms present in the water tend to easily adhere to the tank stainless steel surfaces and to develop communities of cells protected by a self-produced matrix of extracellular polymeric sub- stances called biofilms (Stoodley et al., 2002). These microbial communities have the ability to live in extreme conditions and to resist cleaning procedures, being a persistent source of contamination that can lead to food spoilage (Speranza et al., 2011). Moreover, little is known regarding the biofilm behaviour after water disinfection and biofilm persistence in such food surfaces after the use of an antimicrobial. Therefore, the purpose of this study was to investigate the antimicrobial potential of NEOW when compared with the traditional sodium hypochlorite method as a water disinfecting agent at industrial scale. Additionally, this work also addresses the effect of NEOW on biofilms formed on stainless steel surfaces and their ability to regrow after disinfection.

2. Materials and methods

2.1. *Industrial scale*

2.1.1. *Sampling points and industrial scale scheme*

For NEOW production, Aqualution UK Ltd assembled the generator on site as shown in Fig. 2.

A water softener (Fig. 2, #1) and a holding tank (Fig. 2, #2) containing high-grade quality salt were mounted. The NEOW generating cell (Fig. 2, #3) had the capacity to produce 1 L of NEOW solution every 90 s.

In the NEOW generator, two types of processing water were produced (Fig. 1), electrolyzed oxidizing water (EO), which is acidic (pH 2.3–2.7), and electrolyzed reduced water (ER), which is alkaline (pH 10.0–11.5). Both solutions were mixed in the hypochlorous tank (Fig. 2, #4) in a proportion to generate a hypochlorous solution with chlorine concentration that ranged from 150 to 180 ppm whilst washing trials were performed by adjusting this concentration to 30–40 ppm of free chlorine (NEOW, pH 6.0) by means of a dosing pump (Fig. 2,#5). The NEOW solution was directly injected (Fig. 2, #9) into a closed ring of circulating water to the sanitizing tank.

The Compact Chlorometer Duo (Palintest, USA) was used for measuring free chlorine concentrations in the hypochlor- ous acid storage tank, and in the sanitizing tank.

Concentrated hypochlorous solution was monitored twice daily to ensure stability whilst free chlorine levels in the sanitizing tank were measured at 10-min intervals during trials.

When pH values were too low (2.5–3.0), the sodium hydroxide solution produced at the cathode side of the NEOW device was automatically added to prevent the volatilization of chlorine gas from the sanitizing tank.

At industrial scale, 400 kg of the different types of salad were washed in each trial. After a period of 20 min of chlorine concentration stabilization in sanitizing tank (4000 L water capacity), salad was fed onto the washing line.

Water samples were taken from the washing tank (only water, without antimicrobial agent) and from the sanitizing tank (Fig. 2) in three consecutive days. In the first day, NaOCl concentration ranging from 60 to 90 ppm of free chlorine was used in the sanitizing tank. The concentration of free chlorine from NEOW was 40 ppm. In the second day of sampling, NaOCl was tested as in the first day, and the chlorine concentration from NEOW was adjusted to 30 ppm. In the last day of sampling, only NaOCl in a concentration of 80 ppm was tested. The total microbial load was determined for each sampling port. Selective media were used to quantify the diversity of microbial population in the washing and sanitizing tanks.

2.2. *Total microbial load*

Three sample volumes of 10 mL were collected aseptically in each point being serially diluted and plated in plate count agar (PCA, Merck, VWR Portugal)) according with the tracking plate technique in order to achieve 10–100 CFU per track. Plates were incubated at 30 °C for 24, 48 and 72 h.

2.3. *Population characterization*

Heterotrophic counts were carried out to characterize the microbial population present in the water sampled in the different points. One milliliter of sample was plated onto selective media prepared according the manufacturers' instructions. Plates were incubated aerobically at 37 ± 1 °C for 18–24 h. The following media were used:

2.4. *Laboratory studies*

2.4.1. *NEOW production*

In the lab experiments, NEOW was produced using an electrolyzed water generator ECase (LOEHRKE, Germany). Deionized water and lab quality NaCl were used to feed the generator, by preparing a saline concentration with 2.5 g L^{-1} (brine). The feed flow was adjusted to obtain 26 L h^{-1} of brine, being divided into approx. 13 L h^{-1} for the anolyte and 13 L h^{-1} for the catholyte. The current intensity of the anode was adjusted to 8 A (max). The NEOW solution (pH 7.0, and free chlorine concentration

90 mg L^{-1}) was collected and stored until needed, during a maximum period of 48 h, keeping its chlorine concentration. The free chlorine concentration was confirmed before and during each experiment using the free chlorine ion specific meter HI-93701 (Hanna Instruments, USA).

2.5. *Strain and culture conditions*

Escherichia coli strain JM109 (DE3) was used as a model bacterium at laboratory scale, as it has been routinely used for biofilm research in previous works (Moreira et al., 2015; Teodósio et al., 2013). Bacteria were stored at −80 °C in 20% (v/v) glycerol stocks and propagated by streaking a loopfull of cells onto PCA, and incubated at 30 °C for 24 h. These stocks were stored at 4 °C for no longer than two weeks. For all experiments, batches (30 mL in 125 mL flasks) of sterile concentrated nutrient medium (CNM) consisting of 5 g L^{-1} of glucose, 2.5 g L^{-1} of peptone and 1.25 g L^{-1} of yeast extract, in 0.2 M phosphate buffer (PB) (KH_2PO_4; Na_2HPO_4) at pH 7 (Merck, VWR Portugal) were inoculated with freshly grown cells in an orbital shaker at 30 °C overnight. Cells were harvested by centrifugation and washed in sterile saline. Standardized cell suspensions (1×10^6 cells mL^{-1}) in the appropriate growth medium were prepared.

2.6. *Biofilm formation and regrowth in a Flow Cell system*

The flow cell system used in the lab consisted of a 3.5 L bioreactor, two vertical Perspex Flow Cells (2 cm × 1 cm) operating in parallel, one 0.5 L bioreactor (Bioreactor II), peristaltic and centrifuge pumps (Fig. 3). A pure culture of *E. coli* was used to inoculate the chemostat (Bioreactor I), containing glucose rich medium) that operated continuously, dripping into Bioreactor II at a flow rate of 10 mL h^{-1}. Bioreactor II was fed with a diluted medium that consisted of a 1:100 dilution of the CNM in 0.2 M PB at a flow rate of 0.833 L h^{-1}. The applied dilution rate ensured that biofilm formation predominated over plank- tonic growth. Coupons of stainless steel AISI 316 (SS) were glued onto the removable parts of the flow-cell and bacterial suspension from Bioreactor II recirculated in the flow-cell at a Reynolds number (Re) of 5400 in order to form biofilms. Biofilms were developed and analyzed during 5 d, ensuring the formation of steady state biofilms in the two parallel flow cell reactors.

After biofilm formation, the flow was stopped and the feeding solution was replaced by NaOCl with 150 ppm of free chlorine from NaOCl or by NEOW solution with 90 ppm of free chlorine during 20 min (biofilm treatment) maintaining the flow conditions used for biofilm formation. Biofilms developed on the SS coupons were analyzed regarding mass, weight and number of biofilm cells (CFU cm−2), before (each day, during 5 days) and after 20 min of treatment. The biocide was removed, the system was restarted with the same operating conditions described previously, and biofilms were left to regrow for 24 h. Sampling was performed at 5, 18 and 24 h after finishing the biocidal treatment in order to assess the potential of biofilm regrowth after antimicrobial treatment.

2.7. *Planktonic load*

The microbial load that remained in Bioreactor II (Fig. 1) was quantified in terms of CFU mL^{-1} in all the sampling times, by plating serial dilutions on PCA. Colony forming units (CFU) were counted after 24 h incubation at 30 °C.

2.8. *Biofilm sampling and analysis*

E. coli biofilms grown on SS surfaces were characterized during formation, after treatment and for their regrowth potential. Coupons were removed from the flow cell reactor and thickness and dry weight were first determined according the procedure described by Teodosio et al. (2011). Thickness was assessed, using a needle connected to a digital micrometer (VS-30H, Mitsubishi Kasei Corporation). Ten measurements were made (with a magnifying lens to detect the upper and lower limits of the biofilm) at random points on each coupon and the average value was determined. The dry weight of the biofilm was determined by the total volatiles solids (TVS) of the homogenised biofilm suspensions according the procedure described by Simoes et al. (2003). For cell enumeration, coupons were immersed in saline solution and biofilms were scraped using a sterile scalpel and homogenized in a vortex for 1 min. The biofilm number of cultivable cells was assessed, after proper dilution, in terms of colony forming units (CFU cm^{-2}) in PCA (Merck, Portugal).

2.9. *Statistics*

Statistical analysis was performed using GraphPad Prism, version 6.0 software for Macintosh. Normality of data distribution was tested by the D'Agostino's *K*-squared test. Statistical significance values of the groups' means of planktonic microbial load, biofilm biomass, thickness and weight were evaluated using a one-way analysis of variance. The statistical analyses performed were considered significant when $p < 0.05$.

3. Results

3.1. *Industrial scale*

3.1.1. *Microbial load and population characterization at* different sampling points

In order to assess the efficacy of NEOW as a water disinfection agent at industrial scale, the total microbial load and microflora were characterized. The efficiency of chlorine from NEOW and traditional chlorine from NaOCl was estimated from the values of the overall load in the washing and sanitizing tanks (Table 2). The log reduction was calculated considering the average of CFU mL^{-1} in the washing tank, at each sampling point, and the CFU mL^{-1} in the sanitizing tank. The population distribution by microorganism type in both sampling points was characterized and is shown in Fig. 4.

As seen in Table 1, at industrial scale, the values obtained for microbial load reduction ranged between 1 and 5 log CFU mL^{-1}. The highest reduction of the microbial load was attained with traditional chlorine from NaOCl (4.22 log), the lowest reduction being observed after water sanitation also with NaOCl (1.27 log). This difference is probably due to the different products washed. It should be noted that the microbial reduction

obtained with 40 ppm free chlorine content from NEOW (3.17 log) was close to the highest reduction achieved when using a much higher concentration (80 ppm) of traditional chlorine from NaOCl solution (4.22 log).

The results obtained with the population characterization show that Streptococci and Lactobacilli, as well as *Enterobacter* spp., are the genera that predominate in the overall microbial population (Fig. 4, triangles and squares). The most efficient reduction of the population was achieved in the second and third days of analyses, the microorganisms prevailing after treatment being *Streptococcus*[a])pp. and *Lactobacillus* spp., how- ever in values very close to the method detection limit (Fig. 4).

3.2. *Laboratory studies*

In order to better characterize biofilm formation, their behavior after contacting the chlorinated water and their regrowth ability on SS surfaces, several parameters were evaluated. One of these parameters was the planktonic microbial load in the Bioreactor II before and after NaOCl (150 ppm) and NEOW (90 ppm free chlorine) recirculation, as well as during the biofilm regrowth period (Fig. 5).

The results show that the number of bacteria that remain in suspension in the bioreactor increased slightly over time before biofilm treatment (around 8 log CFU mL^{-1}). Immediately after biofilm treatment, with the two agents, the bioreactor microbial load was reduced to values close to and under the method detection limit. However, during the first 24 h of regrowth after treatment, the microbial load in the bioreactor increased again to 8 log CFU mL^{-1}.

3.3. *Biofilm formation and regrowth in a flow cell* system

Biofilm formation ability and resilience to chemical treatment were tested. For biofilm characterization, the thickness, dry mass and number of cells were assessed. Results are shown in Fig. 6.

Biofilm thickness was stable during 5 days of biofilm formation (approx. 0.2 mm). After 20 min of treatment, chlorine from NEOW and NaOCl (90 ppm and 150 ppm respectively) did not show a significant effectiveness in reducing biofilm thick- ness ($p > 0.05$). During the regrowth period of time, again no significant changes were observed for this parameter ($p > 0.05$). Regarding dry biomass, biofilms formed in the flow cell system kept a similar average weight during the 5 days of biofilm formation (around 1.4 mg/cm^{-2}). After biofilm treatment with chlorine from NEOW, the values obtained for biofilm dry weight where smaller and statistically different from the ones obtained after traditional chlorine treatment ($p < 0.05$) (Fig. 6 b). From this result, it can be stated that NEOW has some antimicrobial effect on biofilm mass reduction, causing a reduction of about 0.5 mg cm^{-2}. Still, biofilm regrowth during the subsequent 24 h (after finishing the treatment) reached dry mass values similar to the ones observed in the first 5 days of biofilm formation particularly for NaOCl ($p > 0.05$).

The number of biofilm entrapped cells increased more than 1 log CFU cm^{-2} over time along the 5 days of biofilm formation ($p < 0.05$). The reduction of viable cells was significant when chlorine from both antimicrobial agents was applied ($p < 0.05$) (Fig.

6c), causing a reduction of 2 log CFU cm^{-2} in the number of cells. The effects of chlorine from traditional NaOCl solution and NEOW were similar ($p > 0.05$). After treatment and following 24 h of biofilm regrowth, a total recovery of the number of bacteria ($p > 0.05$) was observed for the two antimicrobials tested. Also, the number of biofilm cells after regrowth was higher when biofilms were previously exposed to chlorine from NEOW ($p < 0.05$). This can be probably due to the lower chlorine dose applied.

Overall, results show that after antimicrobial application, biofilms are able to recover in the following 24 h the number of cells and thickness, reaching values similar to the ones observed in the first 5 days of formation. The only exception was observed for the dry mass of biofilms treated with chlorine from NEOW (90 ppm, Fig. 6b). In fact, after 24 h of regrowth, biofilm weight was circa half of the value obtained for biofilms developed during the first five days of the experiment.

4. Discussion

Sodium hypochlorite solutions have been used routinely at industrial scale to inactivate pathogens on fresh produce (Baert et al., 2009) as well as on food processing surfaces (Rossoni and Gaylarde, 2000). The bactericidal activity of NEOW is due to the relatively high available chlorine concentration and a high positive oxidation–reduction potential (ORP) (Park et al., 2004). Some studies have shown the effectiveness of electrolyzed water against different foodborne pathogens grown in suspension (Abadias et al., 2008; Cheng, 2012; Tomas- Callejas et al., 2011), but only few studies have been conducted to determine its potential application as surface sanitizer (Park et al., 2002; Ayebah and Hung, 2005) or as anti-biofilm agent (Ayebah et al., 2005).

The main purpose of this work was to evaluate the antimicrobial potential of NEOW to replace traditional chlorine from NaOCl as an industrial water disinfecting agent. Moreover, the influence of traditional NaOCl and of NEOW on the control of biofilms developed on SS surfaces was assessed, as well as the ability of biofilms to regrow after antimicrobial treatment. NEOW has been reported to show strong bactericidal effects on many pathogenic bacteria like *E. coli* (Liao et al., 2007), *Listeria monocytogens* and *Salmonella enteritidis* (Venkitanarayanan et al., 1999) and seems to be a useful alternative to classical bactericidal agents and other control measures (Vazquez- Sanchez et al., 2014).

In this work, it was shown that the water disinfection potential of NEOW was similar to the one of NaOCl in total bacteria inactivation (Table 2) at industrial scale. NEOW seems to be more efficient than the traditional NaOCl since the chlorine concentration tested (NEOW, 30–40 ppm) was much lower than the free chlorine content of NaOCl (80 ppm), achieving a similar bacterial load reduction. Other authors reported that 30 ppm of NEOW can be applied during lettuce washing (5 min) to reduce the use of sodium hypochlorite without affecting salad organoleptic properties (Vandekinderen et al., 2009; Abadias et al., 2008). The same authors also reported that a small concentration of NEOW (50 mg L^{-1} free chlorine) was as effective as 120 mg L^{-1} of chlorine from NaOCl in the reduction of the total aerobic mesophilic count in the minimal processed vegetables washed. Industrially, traditional chlorine-based decontamination procedures include 50–200 ppm free chlorine in a vast majority of minimally processed product

manufacturers (Luo et al., 2011) with a contact time of 1–2 min (Beuchat et al., 1998). At the industrial plant studied in this work, sodium hypochlorite is regularly used up to a maximum concentration of 90 ppm of free chlorine. As assays described in this work were carried out at industrial scale, external parameters like organic load and microbial population variability were considered. Results showed that the water disinfection efficiency (regarding population diversity) was more dependent on the initial microbial load, which was straight- forwardly related with the type of product washed (Fig. 4). The population variability may be due to the differences in the roughness of the leaf surface, weather conditions or the soil in which each type of salad had been planted. Overall, the most significant fact is that the total log reduction achieved was similar to 30–40 ppm of NEOW or 80 ppm of NaOCl. In this work, the stability of NEOW should also be mentioned, since this antimicrobial agent was produced locally at the factory site and stored in a container, maintaining its antimicrobial potential and free chlorine concentration. The production of NEOW on site reduces costs associated with transport and storage and avoids the hazards from storing high volumes of NaOCl concentrated solutions for weeks, since NEOW production can be scheduled on demand. In fact, according to the work of Hsu and Kao (2004), NEOW solutions maintain chlorine concentration, pH and ORP values approximately constant during 4 days of storage. The free chlorine concentration produced by NEOW is thus more easily controlled and stable, showing no fluctuations and allowing the exact knowledge of chlorine that is used, conversely to what happens with traditional NaOCl biocidal solutions. This highlights an additional advantage of NEOW in fresh-cut salad sanitizing procedures, because this type of product processing releases copious amounts of vegetable "juice" into the washing water, drastically increasing the water organic load. Such high organic load in the water may also lead to an increased formation of toxic chlorine byproducts and generation of chlorine gas (Luo et al., 2011). Since the NEOW technique enables a tighter control of free chlorine concentrations, it contributes for the minimization of toxic organochlorinated by-product formation.

In addition to the capacity of chlorine to reduce the viable microbial bioburden in the salad washing water, it is also frequently used as a sanitizer during equipment cleaning and disinfection, thus preventing biofilm formation (Shen et al., 2012). NEOW solution is not so aggressive to skin, or metallic surfaces when compared with sodium hypochlorite and its use on SS (the most commonly used material for food contact surfaces) has been tested with promising results. Ayebah and Hung, (2005) reported that electrolyzed water (pH of 6.12, ORP of 774 mV and 50 ppm of free chlorine) did not have any corrosiveness effect on SS surfaces even when applied during 8 days.

The results obtained in the laboratorial experiments showed that when using NEOW as a water disinfection agent, both the planktonic microbial load and the biofilms formed in food processing surfaces were mitigated in the same extension as when chlorine from NaOCl was used. Moreover, the ability to regrow in the 18 subsequent hours of the remaining mass of biofilm was reduced when NEOW was applied (Fig. 6). The efficacy of chlorine from NEOW was also reported by other authors. Guentzel et al. (2008) found that the application of a low concentration of free chlorine (20 ppm) obtained from NEOW during 10 min was able to reduce 6 log CFU mL^{-1} of mixed species biofilms (*E. coli*, *Salmonella typhimurium*, *Staphylococcus aureus*, *L. monocytogenes*, and *Enterococcus faecalis*) on several types of surfaces. In a work of

Deza et al. (2005), more than 5 log CFU cm^{-2} reduction of *E. coli* biofilms on SS surfaces was achieved with only 5 min treatment with approx. 60 ppm of NEOW free chlorine. This study was developed in accordance with the European standard UNE-EN 1276 (Vazquez-Sanchez et al., 2014), which indicates that a surface disinfection agent should be able to reduce the microbial load in 4 log per cm^2 in a period of 5 min. All these studies assessed antimicrobial potential of NEOW over adhered cells or biofilms developed in microtiter plates, and thus the log reductions achieved in short periods of exposure cannot be directly com- pared with the results obtained in the present work where mature biofilms were formed in flow-cell devices under high hydrodynamic shear stress conditions. Such biofilms are characterised for their high cohesiveness, matrix density and high resistance to antimicrobial treatments (Teodosio et al., 2011). In the work here reported, the application of NEOW as a water disinfectant reduced the biofilms formed on SS surfaces in about 2 log cm^{-2}, similarly to the effect of the traditional chlorine from NaOCl at much higher concentrations. It was also shown that, after chemical stress relief, biofilm mass was not able to regrow (within 18 h) to the values observed during formation.

The laboratorial data showed an interesting feature regarding the dry weigh of the biofilms treated with NEOW, which should be further explored in future work. In fact, when applying NEOW, the dry weight decreased significantly more than when the traditional NaOCl was used. The lower dry weight of the NEOW-treated biofilm was kept during regrowth. Since the thickness remained the same in the two cases (around 200 micron), this means that the biofilm treated with NEOW became more porous (physically more fragile) than the one treated with NaOCl, which tends to favor the removal of the attached biomass from the surface in the first case (by applying higher shear stresses and/or detergents).

5. Conclusions

The present work aimed to reduce the free chlorine content used in the MPV industry and to study the possible replacement of NaOCl. The free chlorine concentration used in the NEOW method was quite lower (30 ppm) than the one of the traditional NaOCl solution (80 ppm), but the efficacy was similar (2 log CFU/cm^2 reduction). This has a significant impact, since lower doses of chlorine need to be produced and used as water disinfection agents, reducing the presence of this product in the discharge water and the risk of by-product formation. The regrowth tests proved that *E. coli* was able to recover the number of cells and thickness after antimicrobial treatment. However, in the biofilms treated with NEOW (90 ppm) it was possible to observe that the biofilm weight was half of the value obtained before treatment with NEOW.

Overall, the results from industrial and laboratorial tests presented in this work suggest that the NEOW technique can be used in water disinfection to replace the traditional NaOCl solution.

Acknowledgements

This work was undertaken as part of the European Research Project SUSCLEAN (Contract n FP7-KBBE-2011-5, project number: 287514). This work was financially supported by: Project UID/EQU/00511/2013-LEPABE, by the FCT/MEC with national funds and co-funded by FEDER in the scope of the P2020 Partnership Agreement; Project NORTE-07-0124-FEDER-000025 - RL2 Environment&Health, by FEDER funds through Programa Operacional Factores de Competitividade–COMPETE, by the Programa Operacional do Norte (ON2) program and by national funds through FCT - Fundação para a Ciência e a Tecnologia (FCT) for Ana Meireles PhD Grant (SFRH/BD/52624/2014).

References

Abadias, M., et al., 2008. Efficacy of neutral electrolyzed water (NEW) for reducing microbial contamination on minimally-processed vegetables. Int. J. Food Microbiol. 123 (1-2), 151–158.

Ayebah, B., Hung, Y.C., 2005. Electrolyzed water and its corrosiveness on various surface materials commonly found in food processing facilities. J. Food Process Eng. 28 (3), 247–264.

Ayebah, B., Hung, Y.C., Frank, J.F., 2005. Enhancing the bactericidal effect of electrolyzed water on Listeria monocytogenes biofilms formed on stainless steel. J. Food Prot. 68 (7), 1375–1380.

Baert, L., et al., 2009. Efficacy of sodium hypochlorite and peroxyacetic acid to reduce murine norovirus 1: B40-8, *Listeria monocytogenes*, and *Escherichia coli* O157:H7 on shredded iceberg lettuce and in residual wash water. J. Food Prot. 72 (5), 1047–1054.

Beuchat, L.R., et al., 1998. Efficacy of spray application of chlorinated water in killing pathogenic bacteria on raw apples: tomatoes, and lettuce. J. Food Prot. 61 (10), 1305–1311.

Cheng, K.C., 2012. Electrolyzed oxidizing water for microbial decontamination of food. In: Demirci, A., Ngadi, M.O. (Eds.), Microbial Decontamination in the Food Industry. Woodhead Publishing, pp. 563–591.

De Giusti, M., et al., 2014. Temporal pattern of microbial indicators of ready-to-eat rocket salads during shelf life. Ann. Ist. Super. Sanita 50 (1), 90–95.

Deza, M.A., Araujo, M., Garrido, M.J., 2005. Inactivation of *Escherichia coli*: *Listeria monocytogenes*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* on stainless steel and glass surfaces by neutral electrolysed water. Lett. Appl. Microbiol. 40 (5), 341–346.

Guentzel, J.L., et al., 2008. Reduction of bacteria on spinach: lettuce, and surfaces in food service areas using neutral electrolyzed oxidizing water. Food Microbiol. 25 (1), 36–41.

Hsu, S.Y., Kao, H.Y., 2004. Effects of storage conditions on chemical and physical properties of electrolyzed oxidizing water. J. Food Eng. 65 (3), 465–471.

Huang, Y.R., et al., 2008. Application of electrolyzed water in the food industry. Food Control 19 (4), 329–345.

Liao, L.B., Chen, W.M., Xiao, X.M., 2007. The generation and inactivation mechanism of oxidation–reduction potential of electrolyzed oxidizing water. J. Food Eng. 78 (4), 1326–1332.

Luo, Y., et al., 2011. Determination of free chlorine concentrations needed to prevent *Escherichia coli* O157:H7

cross-contamination during fresh-cut produce wash. J. Food Prot. 74 (3), 352–358.

Moreira, J.M.R., et al., 2015. The impact of material properties: nutrient load and shear stress on biofouling in food industries. Food Bioprod. Process. 95 (0), 228–236.

Ölmez, H., Kretzschmar, U., 2009. Potential alternative disinfection methods for organic fresh-cut industry for minimizing water consumption and environmental impact. LWT - Food Sci. Technol. 42 (3), 686–693.

Park, H., Hung, Y.C., Chung, D., 2004. Effects of chlorine and pH on efficacy of electrolyzed water for inactivating *Escherichia coli* O157: H7 and *Listeria monocytogenes*. Int. J. Food Microbiol. 91 (1), 13–18.

Park, H., Hung, Y.C., Kim, C., 2002. Effectiveness of electrolyzed water as a sanitizer for treating different surfaces. J. Food Prot. 65 (8), 1276–1280.

Rossoni, E.M., Gaylarde, C.C., 2000. Comparison of sodium hypochlorite and peracetic acid as sanitising agents for stainless steel food processing surfaces using epifluorescence microscopy. Int. J. Food Microbiol. 61 (1), 81–85.

Shen, C., et al., 2012. Enhanced inactivation of *Salmonella* and *Pseudomonas* biofilms on stainless steel by use of T-128: a fresh-produce washing aid, in chlorinated wash solutions. Appl. Environ. Microbiol. 78 (19), 6789–6798.

Simoes, M., Pereira, M.O., Vieira, M.J., 2003. Effect of different concentrations of ortho-phthalaldehyde on biofilms formed by *Pseudomonas fluorescens* under different flow conditions. Biofouling 19 (5), 287–295.

Speranza, B., Corbo, M.R., Sinigaglia, M., 2011. Effects of nutritional and environmental conditions on *Salmonella* sp. biofilm formation. J. Food Sci. 76 (1), M12–M16.

Stoodley, P., et al., 2002. Biofilms as complex differentiated communities. Annu. Rev. Microbiol. 56, 187–209.

Teodósio, J.S., et al., 2013. Flow cells as quasi-ideal systems for biofouling simulation of industrial piping systems. Biofouling 29 (8), 953–966.

Teodosio, J.S., et al., 2011. Flow cell hydrodynamics and their effects on *E. coli* biofilm formation under different nutrient conditions and turbulent flow. Biofouling 27 (1), 1–11.

Tomas-Callejas, A., et al., 2011. Neutral and acidic electrolyzed water as emergent sanitizers for fresh-cut mizuna baby leaves. Postharv. Biol. Technol. 59 (3), 298–306.

Vandekinderen, I., et al., 2009. Moderate and high doses of sodium hypochlorite, neutral electrolyzed oxidizing water, peroxyacetic acid, and gaseous chlorine dioxide did not affect the nutritional and sensory qualities of fresh-cut Iceberg lettuce (*Lactuca sativa* var. *capitata* L.) after washing. J. Agric. Food Chem. 57 (10), 4195–4203.

Vazquez-Sanchez, D., Cabo, M.L., Rodriguez-Herrera, J.J., 2014.

Single and sequential application of electrolyzed water with benzalkonium chloride or peracetic acid for removal of *Staphylococcus aureus* biofilms. J. Food Saf. 34 (3), 199–210.

Venkitanarayanan, K.S., et al., 1999. Efficacy of electrolyzed oxidizing water for inactivating Escherichia coli O157:H7: *Salmonella enteritidis*, and *Listeria monocytogenes*. Appl. Environ. Microbiol. 65 (9), 4276–4279.

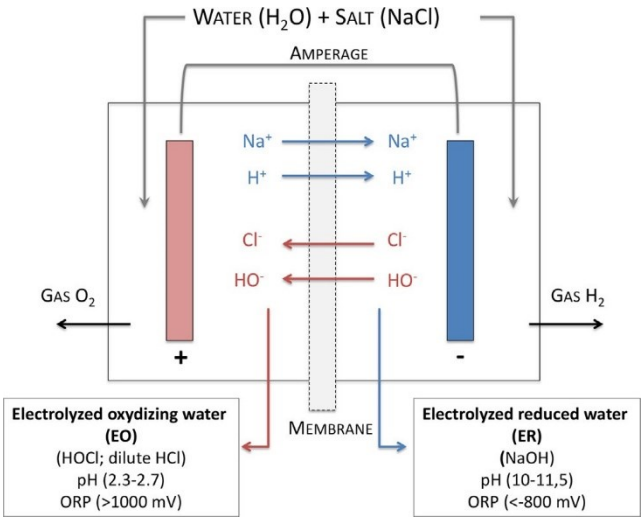

Fig. 1 – Schematic representation of electrolyzed oxidizing water production [adapted from (Huang et al., 2008)]

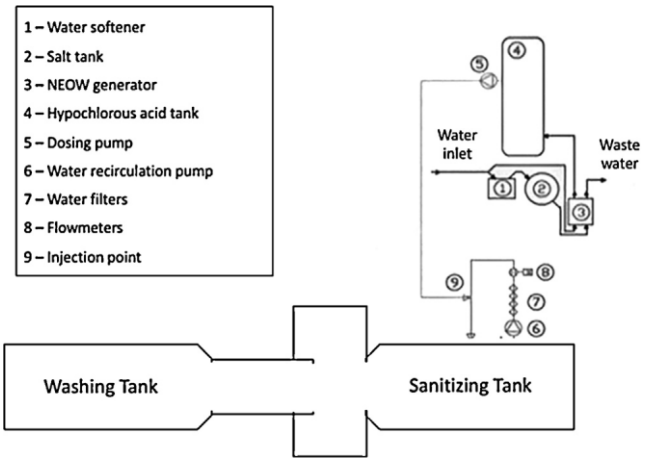

Fig. 2 – Schematic representation of the industrial washing and sanitizing tanks.

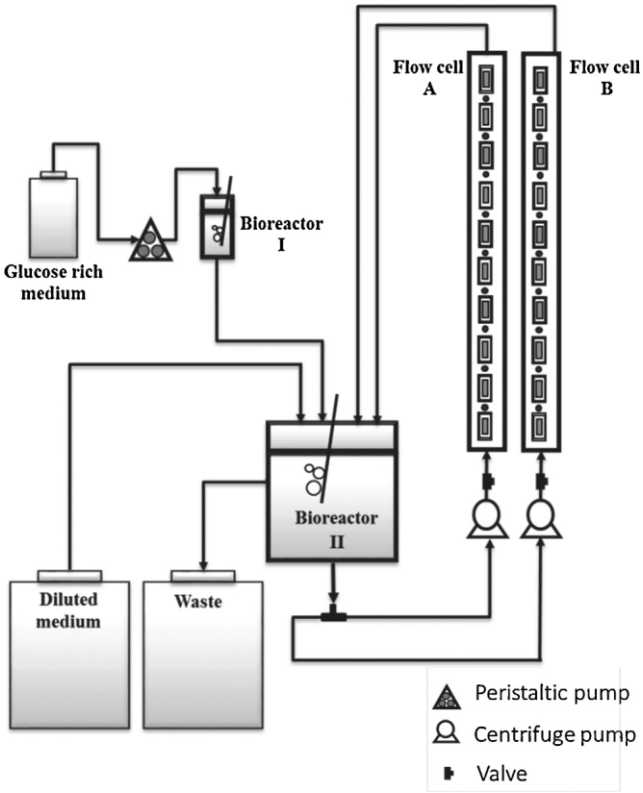

Fig. 3 – Schematic representation of the flow cell system.

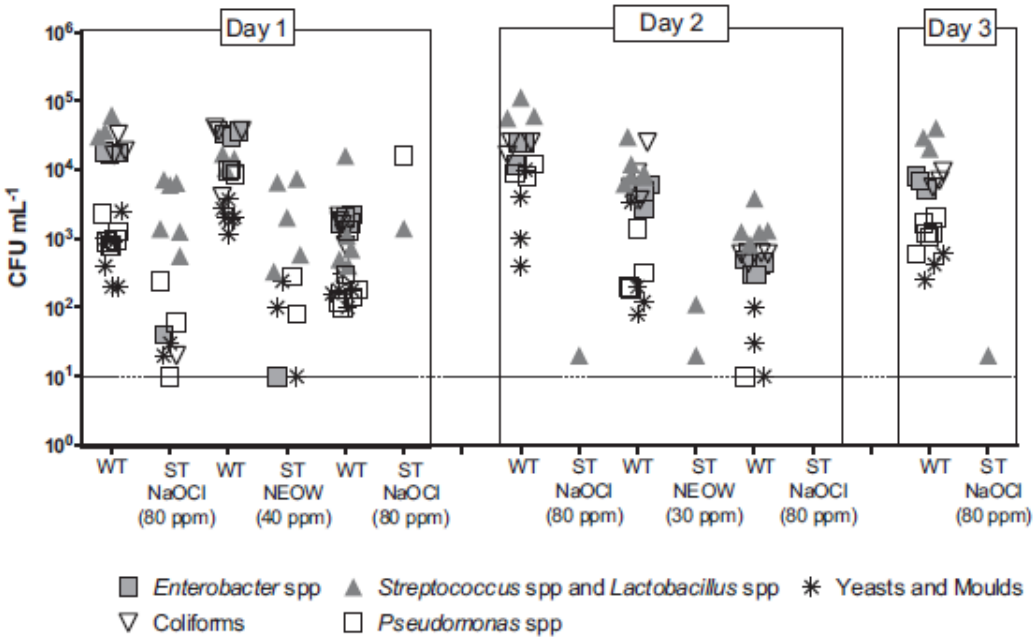

Fig. 4 – Microbial population characterization. *Enterobacter* spp. (•), *Streptococcus* spp. and *Lactobacillus* spp. (.._), Yeasts and moulds (*), Coliforms (v) and *Pseudomonas* spp. (D). Detection limit is 10 CFU mL^{-1}. WT–Washing tank; ST- Sanitizing tank; NaOCl-traditional chlorine from sodium hypochlorite; NEOW–chlorine from neutral electrolyzed oxidizing water.

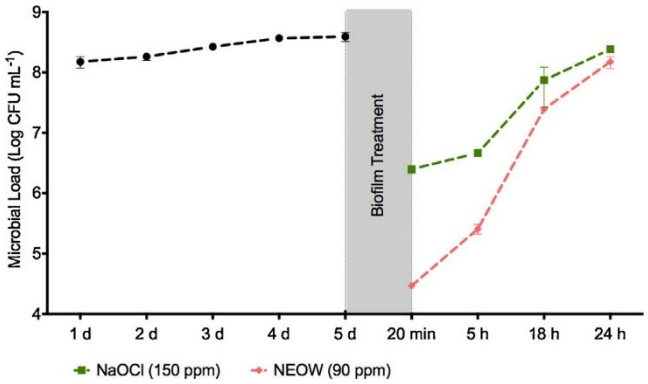

Fig. 5 – Planktonic microbial load on Bioreactor II. Biofilms formed during 5 days (•) were treated during 20 min with NaOCl (•, 150 ppm) and NEOW (+, 90 ppm). Biofilm regrowth was analyzed after 5 h, 18 h and 24 h following antimicrobial exposure. Method detection limit is 6 log mL^{-1}

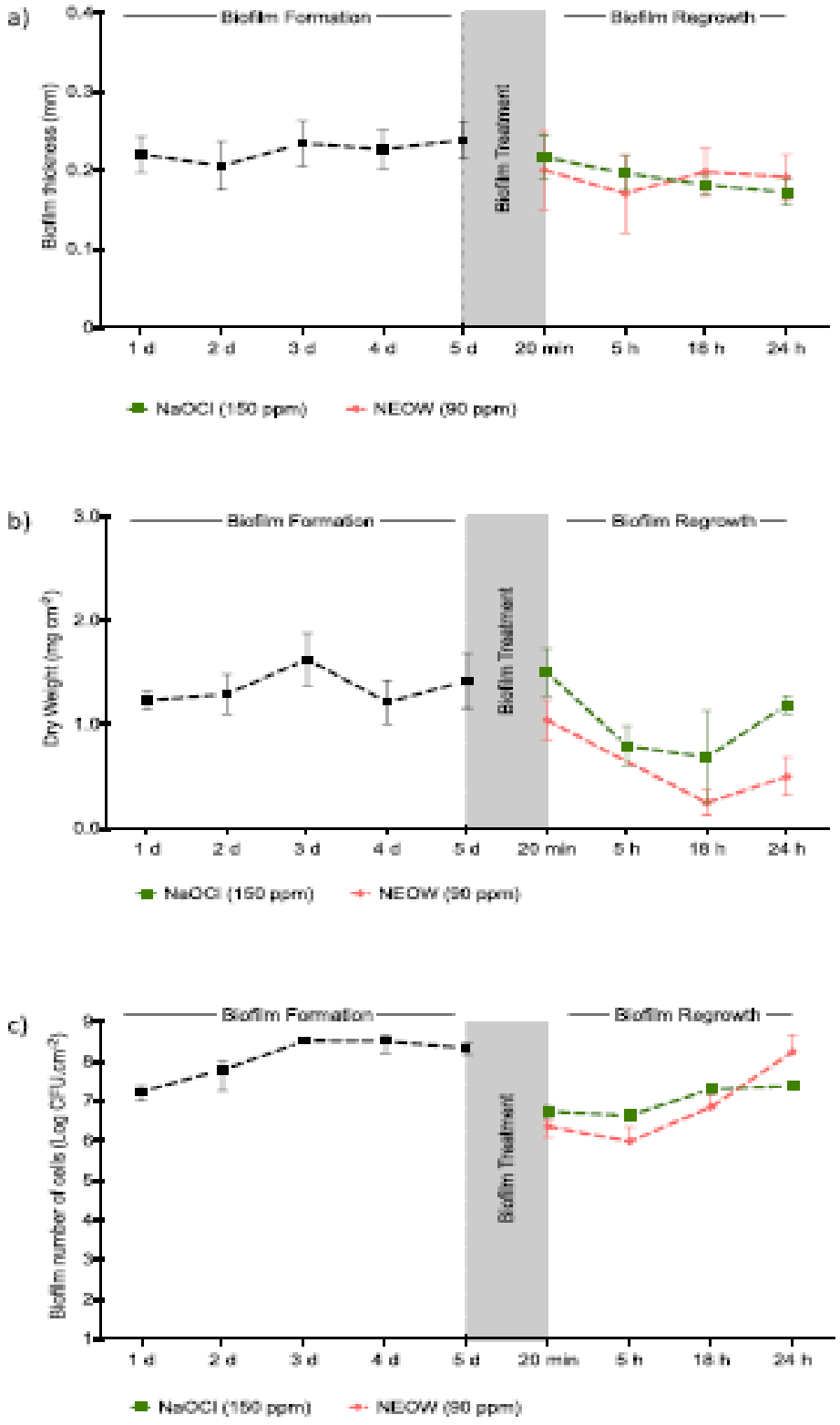

Fig. 6 – Biofilm formation during 5 days (•) and regrowth ability after treatment during 20 min with chlorine from sodium hypochlorite (•, NaOCl, 150 ppm), and chlorine from neutral electrolyzed oxidizing water (+, NEOW, 90 ppm); a) biofilm thickness, b) biofilm dry weight, c) biofilm number of cells.

Table 1 – Selective media used in the population characterization at industrial scale.

Media		Enumeration of
Elliker agar	DIFCO (Ref: 212183)	*Lactobacillus* spp.; *Streptococcus* spp.
Pseudomonas agar supplemented with Cetrimide, Fucidin, Cephalordine (CFC)	Liofilchem (Ref: 610071) + Liofilchem (Ref: 81049)	*Pseudomonas* spp.
Yeast glucose chloramphenicol agar	Liofilchem (Ref: 610070)	Yeasts and molds
Violet red bile lactose agar	Liofilchem (Ref: 610058)	Coliforms
Violet red bile glucose agar	Liofilchem (Ref: 610059)	*Enterobacter* spp.

Table 2 – Microbial load (CFU mL^{-1}) and log reduction of microorganisms during the sampling days. Salad Mixture: baby leaf batavia, baby green tango lettuce and baby green romaine.

	Antimicrobial tested	Sampling point		Microbial load (CFU mL^{-1})		log reduction
				Average	Deviation	
Day 1	NaOCl (80 ppm)	Washing Tank	Baby leaf batavia	6.07E+05	3.27E+05	4.22
		Sanitizing tank		3.67E+01	1.53E+01	
	NEOW (40 ppm)	Washing Tank	Baby green tango lettuce	2.65E+05	1.56E+05	3.17
		Sanitizing tank		1.80E+02	4.24E+01	
	NaOCl (80 ppm)	Washing Tank	Salad mixture	4.97E+04	3.88E+04	1.27
		Sanitizing tank		2.65E+03	2.12E+02	
Day 2	NaOCl (80 ppm)	Washing Tank	Salad mixture	9.93E+05	3.11E+05	3.67
		Sanitizing tank		2.10E+02	2.69E+02	
	NEOW (30 ppm)	Washing Tank	Baby leaf batavia	5.54E+05	4.71E+05	2.20
		Sanitizing tank		3.46E+03	5.66E+03	
	NaOCl (80 ppm)	Washing Tank	Salad mixture	7.95E+03	2.91E+03	3.08
		Sanitizing tank		6.67E+00	5.77E+00	
Day 3	NaOCl (80 ppm)	Washing Tank	Watercress	1.64E+07	6.12E+04	2.43
		Sanitizing tank		6.07E+04	9.48E+04	