Assessment of sanitation efficacy against *Escherichia coli* O157:H7 by rapid measurement of intracellular oxidative stress, membrane damage or glucose active uptake

Andrea Cossu^{1,#}, Phuong Le^{2,#}, Glenn M. Young¹ and Nitin Nitin^{1,2,*}

1 Food Science and Technology Department, University of California - Davis, Davis, CA 95616 USA

2 Department of Biological and Agricultural Engineering, University of California-Davis, Davis, CA 95616 USA.

Equal contribution

* Corresponding author

Corresponding author:

Nitin Nitin, Ph.D.

Professor and Barrett Faculty Fellow

Departments of Food Science and Technology And Biological and Agricultural Engineering

University of California Davis, Robert Mondavi Institute South, 392 Old Davis Road

Phone: +1-530-752-6208

Fax: +1-530-752-4759

Email: nnitin@ucdavis.edu

ABSTRACT

To reduce cross contamination with foodborne pathogens during washing procedures, sodium hypochlorite (NaOCl) and hydrogen peroxide (H₂O₂) are widely used in the fresh produce industry. To ensure food safety and minimize excessive use of sanitizers and water during sanitation, it is critical to develop a rapid method to assess sanitation efficacy. This study examines the potential for employing oxidative stress, membrane damage and glucose uptake measurements to assess the antimicrobial efficacy of NaOCl (0 - 70.4 ppm) and H₂O₂ (0 - 1.6% v/v) toward *Escherichia coli* O157:H7. The relative amount of intracellular reactive oxygen species (ROS) was measured using Aminophenyl fluorescein (APF) and 2',7'- dichlorodihydrofluorescein diacetate (H₂DCFDA) for cells treated with NaOCl and H₂O₂ respectively. Results from these ROS sensitive probes revealed a limited correlation between these oxidative stress measurements and inactivation of bacteria measured using the plate counting method. Sanitation experiments, conducted with contaminated pre-cut lettuce leaves in water, were also carried out. Measurement of the bacterial membrane integrity was assessed using the membrane permeable probe propidium iodide (PI) and by evaluating effects on active transport by the the glucose transport system using 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (2-NBDG). The results demonstrate that the relative increase in membrane permeability using PI correlated with NaOCl, but did not correlate with H₂O₂ induced reduction of bacterial survival quantified by the plate counting method. In contrast to the other approaches, monitoring the loss of glucose transport system function by measuring the uptake of 2-NBDG displayed strong correlation with the reduction of bacterial survival for both NaOCl and H₂O₂ treatment. Overall, the study demonstrated potential of glucose uptake measurements with 2-NBDG to serve as surrogate method for the traditional plate counting, which is the current gold standard used by the Food and Drugs Administration, to provide a faster analysis of sanitation processes.

KEYWORDS

Rapid measurement; oxidative stress; membrane damage; glucose uptake; *Escherichia coli* O157:H7

HIGHLIGHTS

- Oxidative stress by NaOCl or H₂O₂ in *E. coli* O157:H7 was measured with APF or H₂DCFDA
- Membrane damage and glucose uptake were measured using PI and 2-NBDG
- Oxidative stress probes did not show significant correlation with microbial reduction
- PI permeation had significant correlation with microbial reduction when NaOCl was used
- Measurement of 2-NBDG uptake was shown as the best surrogate for plate counting

1. INTRODUCTION

Sanitation of process water and equipment is a critical step to ensure the safety and quality of food (Gil et al, 2009). The significance of sanitation is highlighted by the fact that numerous foodborne outbreaks have been traced back to pathogens surviving in food process facilities, resulting in cross-contamination of the food (Luo et al, 2011). Therefore, rapid assessment of sanitation efficacy is highly desirable for many operations in food processing. These rapid assays can improve food safety, operational efficiency, and reduce the excessive use of sanitizers and water during sanitation.

Food process operations are designed to be time and cost efficient with strict attention to maintaining safety. For fresh ready-to-eat products, such as produce, the timeline from harvest through processing, packing and shipment is often 1 – 2 days. Within these operations sodium hypochlorite (NaOCl) and hydrogen peroxide (H₂O₂) are very effective oxidizers commonly used as sanitizers (Gil et al, 2009). To measure the efficacy of these sanitizers, the current monitoring approaches, such as the standard plate count, provide only retrospective assessments for bacterial contamination that often take several days to complete. Chemical based methods are available to measure sanitizer concentration, total carbon content, oxidation reduction potential (ORP), turbidity and pH of the aqueous phase (Suslow, 2004; Le et al, 2015). However, these measurements are not effective in measuring bacterial reduction in complex environments because of potential fouling of electrodes as well as interference due to organic matter (Suslow, 2004). Improvement would be gained by rapid assays that directly measure the effectiveness of sanitizers on target bacteria based on robust technologies that function in complex environments such as wash water flumes and associated produce sanitation equipment. A rapid assay that would measure the real biological effect on the bacterial target rather than simply a chemical parameter of the wash water, would be

definitely well accepted by the market. With this motivation, prior studies have measured changes in various physiological markers in bacterial cells using fluorescence spectroscopy, flow cytometry, and imaging (Wang et al, 2010; Kramer and Muranyi, 2014; Baatout et al, 2006). The physiological markers evaluated in prior studies included membrane damage (membrane permeabilization, membrane potential, and intracellular pH) and reduction in enzymatic activity such as esterase activity. These results were correlated with standard plate counting method. Based on these outcomes, it was suggested that physiological measurements have a potential to predict the efficacy of sanitation, but the key challenge was in the identification of an optimal biomarker for a selected sanitizer compound (Wang et al, 2010). Prior studies measured the effect of sanitizers on membrane damage and enzymatic activity, but the knowledge from studies that directly measured bacterial oxidative stress induced by sanitizers in bacteria is limited (Wang et al, 2010; Kramer and Muranyi, 2014; Baatout et al, 2006). Since antimicrobial activity of most of the current sanitizers including NaOCl and H₂O₂ is based on oxidative stress induced in bacteria, direct assessment of oxidative stress induced by sanitizers may be an indicator of sanitizer efficacy.

The objectives of this study were to evaluate a range of potential physiological measurements that may strongly correlate with reduction in bacterial survival by: (a) measuring bacterial oxidative stress induced by NaOCl and H₂O₂ using *Escherichia coli* O157:H7; and (b) measure changes in membrane damage and active uptake of glucose metabolite upon treatment of *E. coli* O157:H7 with the selected sanitizers with and without the presence of pre-cut lettuce. These measurements were compared with the standard bacterial plate counting method to evaluate the potential of developing one of these approaches into a technology for predicting reduction in bacterial survival, defined here as the ability to form a colony on standard growth medium. Currently, these oxidative stress measurements using free radical sensitive fluorophores such as 2',7'-dichlorofluorescein and MitoSOX red have been mostly used in mammalian cells with limited application in bacteria (Hosoki

et al, 2012; Bergamini et al, 2012; Nakazato et al, 2007). To investigate intracellular generation of reactive oxygen species (ROS) in bacteria, the Aminophenyl fluorescein (APF) dye or the hydroxyl radical sensitive dye 2',7'- dichlorodihydrofluorescein diacetate (H₂DCFDA) were chosen. APF is a fluorescein derivative, which is specific for measuring levels of $\cdot\text{OCl}$ and $\cdot\text{OH}$ in cells upon exposure to HOCl or NaOCl (Choi and Hu, 2008). This ROS reporter dye has been previously used in mammalian cells to measure ROS generated by endogenous hypochlorous acid (Nakazato et al, 2007). Upon interacting with $\cdot\text{OCl}$ ions and $\cdot\text{OH}$ radicals, the non-fluorescent APF dye is activated resulting in an increase in fluorescence signal intensity. Similar to the APF dye, H₂DCFDA is also a fluorescein derivative and is a cell permeable dye with specificity for hydroxyl radicals (Lyon et al, 2008). After permeating into cells, this dye molecule is cleaved by intracellular esterases which prevents the release of the entrapped dye from bacterial cells. Upon reaction with hydroxyl radicals, the non-fluorescent dye molecules in the bacteria are converted to a fluorescent DCF (2',7'- dichlorodihydrofluorescein) form.

In addition to measuring oxidative stress in bacteria, two physiological markers were also selected in this study in order to compare changes in their signals and reduction in bacterial survival. The structural integrity of bacteria was assessed based on changes in membrane permeability by propidium iodide (PI). The intracellular uptake of glucose metabolite was selected as an indicator of metabolic activity, using a fluorescent analog of deoxy-glucose. Specificity of intracellular uptake of this analog through glucose transporters has been validated in both bacterial and mammalian systems (Yoshioka et al, 1996; Nitin et al, 2009). Metabolic activity was selected in this study as previous studies in both bacterial and mammalian systems have indicated the significance of changes in metabolic activity to predict reduction in cell viability (Yoshioka et al, 1996; Luo et al, 2014). The results of this study will aid in development of rapid assays for the measurement of the

efficacy of standard sanitizers in environments such as wash water of fresh produce avoiding time consuming procedures and costs associated with expensive food recalls.

2. MATERIAL AND METHODS

2.1 Bacterial culture

A Rifampicin-resistant Shiga toxin negative derivative *Escherichia coli* O157:H7 ATCC#700728 (*E. coli* O157:H7) was kindly provided by Dr. Linda Harris (University of California, Davis, USA) and stored in liquid nitrogen with 50% v/v glycerol. For selective growth of *E. coli* O157:H7, Rifampicin (TCI America, Portland, OR, USA) was added to the medium at 50 µg/mL. The liquid nitrogen *E. coli* stock was streaked onto a Luria Bertani Agar (LBA) (Fisher Scientific, Pittsburg, CA, USA) plate and grown overnight at 37°C. A colony was picked from the agar plate, cultured in Luria Bertani (LB) broth (Fisher Scientific, Pittsburg, CA, USA) for 16 h and then sub-cultured in LB broth for 16 h at 37°C, 250 rpm agitation, before use in each experiment. In the subculture step, *E. coli* was grown to the stationary growth phase (OD = 1.4 at 600 nm). The bacteria were washed twice with phosphate buffered saline (PBS) (Fisher Scientific, Pittsburg, CA, USA) by centrifuging the sample at $3,100 \times g$ for 10 minutes at room temperature and resuspending the sample in PBS for further analysis.

2.2 Exposure of *Escherichia coli* O157:H7 to sodium hypochlorite or hydrogen peroxide

The stationary growth phase bacteria were diluted in PBS to OD = 1 at 600 nm (1×10^8 Colony forming units (CFU)/mL) before each experiment. Sodium hypochlorite (NaOCl) (Sigma-Alrich, St. Louis, MO, USA) or hydrogen peroxide (H₂O₂) (Fisher Scientific, Pittsburg, CA, USA) were used as sanitizers for the inactivation. The final concentrations of NaOCl used were 0, 2.2, 4.4, 8.8, 17.6, 35.2, and 70.4 ppm, while the final concentrations of H₂O₂ used were 0, 0.1, 0.2, 0.4, 0.8, 1.6 % v/v in 1 mL of reaction volume. These concentrations were chosen based on previous works that showed similar ranges being effective to achieve non-lethal, sub-lethal and lethal activity in water

against *E. coli* (Luo et al, 2011; Le et al, 2015). After treatment with NaOCl for 10 minutes, sodium thiosulfate (Sigma-Aldrich, St. Louis, MO, USA) with a final concentration of 0.1% w/v was added to the *E. coli* O157:H7 suspension to stop further activity of NaOCl. Similarly, catalase (Sigma-Aldrich, St. Louis, MO, USA) with a final concentration of 5 µg/mL was added to the *E. coli* O157:H7 suspension after 10 minutes treatment to stop further activity of H₂O₂. A previous study has shown that sodium thiosulfate in the concentration range of 0.01% to 5% does not affect *E. coli* survival (Kemp and Schneider, 2000).

2.3 Measurement of intracellular reactive oxidative species during *Escherichia coli* O157:H7 inactivation

Direct measurements of intracellular reactive oxidative species (ROS) during NaOCl sanitation was performed with Aminophenyl fluorescein (APF) (Life Technologies, Carlsbad, CA, USA). One milliliter containing 1×10^8 CFU/mL of *E. coli* O157:H7 was pre-incubated with 10 µM final concentration of APF at room temperature for 30 minutes. After incubation with APF, bacteria were washed with PBS and resuspended in 1mL PBS. The bacterial suspension was then treated with 0 to 70.4 ppm of NaOCl for 10 minutes. Sodium thiosulfate (Sigma-Aldrich, St. Louis, MO) at the final concentration of 0.1% w/v was added to neutralize the excess NaOCl after the 10 minute treatment.

Similarly, to measure the ROS species generated during treatment of bacteria with H₂O₂, *E. coli* O157:H7 was pre-incubated with 20 µM final concentration of 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) in a 1 mL volume (Life Technologies, Carlsbad, CA, USA) at 37°C for 1 hour. Following the incubation with H₂DCFDA, bacteria were washed with PBS and then resuspended in PBS. The *E. coli* O157:H7 suspension was then treated with 0 to 1.6% v/v H₂O₂ for 10 minutes. Catalase enzyme from bovine liver (Sigma Aldrich, St. Louis, MO, USA) at the final concentration of 5

µg/mL was added to quench the excess of H₂O₂. Suspension was pelleted by centrifugation and resuspended in 1 mL of PBS.

For both sanitation treatments, 100 µL of the *E. coli* O157:H7 pre-incubated with the ROS probe were transferred into each well of a 96-well plate (Corning, Tewksbury, MA, USA). The fluorescence intensity was measured at excitation/emission wavelengths of 488/520 nm using a fluorescence plate reader (Spectrafluor Plus, Tecan, Durham, NC, USA). The fluorescence intensity ratio was calculated as fluorescence with sanitizer/fluorescence without sanitizer.

2.4 Sanitation of cut lettuce leaves artificially contaminated with *Escherichia coli* O157:H7

E. coli O157:H7 was resuspended in water and added at a final concentration of 1×10^8 CFU/mL to a suspension of water containing 1×1 cm² cut lettuce (*Lactuca sativa*) leaves from a local retail market to a ratio of 1 g per 10 mL of the volume. A concentration of 1×10^8 CFU/mL of bacteria was selected to ensure significant signal to noise ratio and to be able to confirm an ideal goal of sanitation of 5-6 log reduction in bacterial count by plate counting methods. NaOCl and H₂O₂ were added at the same concentrations used for the experiments described above. After treatment for 10 minutes, NaOCl was inactivated with sodium thiosulfate and H₂O₂ with catalase as mentioned above. Ten milliliter of the liquid suspension were then passed through a 5 µm filter (Tisch Scientific, Cleves, OH, USA) to remove plant cells and the filtrate was collected and centrifuged at $11,000 \times g$. Supernatant was discarded and pellet was then used for the following analysis of plate counting and measurement of membrane damage and glucose uptake.

2.5 Measurement of bacterial membrane damage and active glucose uptake

Membrane damage in *E. coli* O157:H7 following treatment with sanitizers with or without the presence of cut lettuce was measured using propidium iodide (PI) dye (Sigma Aldrich, St. Louis, MO,

USA). Changes in glucose uptake were measured using a fluorescent analog of glucose, 2-NBDG (2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose) (Life Technologies, Eugene, OR, USA). After *E. coli* O157:H7 was treated with NaOCl or H₂O₂, bacteria were incubated with either 5 µg/mL of PI at room temperature or 2-NBDG at 37°C for 20 minutes in a volume of 1mL. The microbes were then washed once with PBS and resuspended in PBS. One hundred microliters of the *E. coli* O157:H7 suspension was transferred into each well of a 96-well plate and fluorescence intensity was measured at excitation/emission wavelengths of 530/580 nm or 488/520 nm for PI and 2-NBDG, respectively. The signal/background ratio was calculated as fluorescence with sanitizer/fluorescence without sanitizer.

2.6 Statistical Analysis

Statistical analyses of the data were carried out using Microsoft Excel (Microsoft, Redmond, WA, USA). The statistical significance was determined using the two-tailed unpaired t-test, assuming unequal variances with an alpha (statistical significant level) of 0.05. The Pearson Correlation Coefficient (PCC) and relative p-value between markers and logarithmic reductions were calculated using the Pearson correlation tool from Wessa.net (Wessa, 2016).

3. RESULTS

3.1 Assessment of *E. coli* O157:H7 survival after NaOCl or H₂O₂ sanitation

Sanitation experiments were performed using bacteria treated alone or in co-suspension with cut lettuce. For samples containing the lettuce, the analysis was limited to assessing viability of bacteria that were recovered from the suspension. The results in Fig. 1 (A) show no significant reduction in survival of *E. coli* O157:H7 until the concentration of NaOCl in water reaches 8.8 ppm ($p < 0.05$). Survival of *E. coli* did display a linear reduction (log scale) as the concentration of NaOCl increased in the range between 8.8 ppm and 35.2 ppm. In the presence of cut lettuce, the minimum concentration of NaOCl required for a significant reduction ($p < 0.05$) of survival was 35.2 ppm. At the highest concentration of NaOCl (70.4 ppm), there was a 5.7 log CFU/mL and 2.3 log CFU/mL reduction in *E. coli* survival after 10 min of incubation in water and in the presence of lettuce, respectively.

The results in Fig. 1 (B) show no significant reduction in survival of bacteria upon treatment with H₂O₂ in the concentration range of 0 - 0.4% (v/v) both in water and in suspension with cut lettuce. Whether the *E. coli* was suspended in water or water with lettuce, a concentration of at least 0.8% H₂O₂ was required to observe a significant decrease ($p < 0.05$) in survival. At 1.6% H₂O₂ there was a 4.7 log CFU/mL and 3.1 log CFU/mL reduction in survival after 10 min of incubation in water and in the presence of cut lettuce, respectively.

3.2 Measurement of oxidative stress in *E. coli* O157:H7 with ROS probes during NaOCl or H₂O₂ treatment of bacteria suspension in water

Fig. 2 (A) shows that exposure of APF labeled bacteria in water to NaOCl resulted in a significant increase in fluorescence signal, indicating generation of intracellular ROS in bacteria

immediately upon exposure to NaOCl in the concentration range of 0 - 4.4 ppm. Above 4.4 ppm, there was no significant increase ($p > 0.05$) in the fluorescence intensity in bacterial cells with an increase in concentration of NaOCl. The correlation between the APF signal and the logarithmic reduction of bacteria count did not show a significant correlation ($p = 0.3406$) with a PCC of 0.4260 and a R^2 of 0.1814 (Fig. 2 A and C).

The results in Fig. 2 (B) show that the fluorescence intensity ratio of the H₂DCFDA labeled cells increased with respect to the control cells (not incubated with H₂O₂) with an increase in H₂O₂ concentration. This indicates activation of the fluorescent dye molecules by hydroxyl radicals and peroxide molecules. The fluorescence intensity ratio as a function of H₂O₂ showed a trend with multiple saturation levels from 0.2 to 0.4% and from 0.8 to 1.6% H₂O₂. The correlation analysis between the H₂DCFDA fluorescence in treated cells and the microbial logarithmic reduction in H₂O₂ showed a weak correlation between the two variables ($p = 0.048$) with a PCC of 0.8153 and a R^2 of 0.6647 (Fig. 2 B and D). Since both the selected oxidative stress indicators did not show significant correlation with reduction in bacterial survival or indicated the critical concentration above which significant reduction in bacterial count was achieved, these markers were not further investigated for the bacterial cells suspended in the fresh cut lettuce samples.

3.3 Assessment of membrane damage and reduction of *E. coli* 0157:H7 survival after sanitation of water and pre-cut lettuce

As a complementary to the direct measurement of oxidative stress, physiologically-relevant changes in membrane integrity and activity of the bacterial glucose transport system were monitored in the bacterium following exposure to sanitizers. The analysis was conducted in water and in the presence of pre-cut lettuce to gain insight on the possible effect that plant-based organic constituents might have on the outcome.

Fig. 3 (A) shows that exposure of bacteria to NaOCl resulted in an increase in normalized fluorescence intensity ratio of the PI dye at a NaOCl concentration level equal or higher than 8.8 ppm ($p < 0.05$). In the concentration range of 0 - 4.4 ppm no significant increase ($p > 0.05$) in fluorescence intensity ratio of the PI dye was observed. A similar trend was also found in bacteria subjected to NaOCl sanitation in the presence of cut lettuce (Fig. 3 (A)) at a NaOCl concentration level equal or higher than 17.6 ppm. Increase in the PI labeling of bacteria in the presence of fresh cut lettuce was lower than as compared to the increase in water, indicating a relative lower antimicrobial effect of sanitizers in the presence of fresh cut lettuce. Correlation analysis between the bacterial log reduction and increase in fluorescence labeling of bacteria cells with PI dye both in water and in the presence of fresh cut lettuce showed a good correlation ($p < 0.05$) with PCCs of 0.9621 and 0.9735 and R^2 of 0.9256 and 0.9477, respectively (Fig 3 A, C and D).

The results in Fig. 3 (B) demonstrated only a small (1.4 fold) but statistically significant increase in membrane permeability when the *E. coli* O157:H7 was treated with 1.6% H_2O_2 as compared to the control. However, there was no statistically significant difference in membrane permeability between 0 to 0.2% H_2O_2 , after 0.4% the PI signal was saturated. The correlation between microbial logarithmic reduction and the increase in PI signal was statistically significant ($p = 0.0074$) with a PCC of 0.9289 and R^2 of 0.8628 (Fig 3 B and E). The signal of PI fluorescence upon treatment of bacteria with H_2O_2 in the presence of cut lettuce did not show any statistical significant difference among the H_2O_2 concentrations (Fig. 3 (B)). Correlation analyses confirmed the absence of correlation between the logarithmic reduction of the bacterial count and the PI signal in the presence of cut lettuce.

3.4 Glucose uptake and reduction of *E. coli* O157:H7 survival after sanitation of water and pre-cut lettuce

The results in Fig. 4 (A) show a significant drop in glucose uptake in the bacterial cells at the NaOCl concentration level of 8.8 ppm. At concentrations of NaOCl higher than 8.8 ppm, no significant further decrease in the uptake of 2-NBDG was observed. In the presence of lettuce, the significant decrease in 2-NBDG signal was detected only at 17.6 ppm or higher following a linear reduction. Correlation analyses in both cases indicated a statistically significant correlation between the logarithmic reduction in CFU counts and 2-NBDG signal in bacterial cells without ($p = 0.0174$; $PCC = -0.8424$; $R^2 = 0.7097$) or with cut lettuce during water sanitation ($p = 0.0003$; $PCC = -0.9703$; $R^2 = 0.9414$).

The results of glucose analog uptake following treatment with H_2O_2 (Fig. 4 (B)) demonstrated that upon exposure to sub-lethal concentration of H_2O_2 (up to 0.4% as measured based on reduction in survival of bacteria) in water, the intracellular uptake of 2-NBDG increased by almost two fold. Upon exposure to lethal dosage (higher than 0.4% hydrogen peroxide), there is a rapid decrease in the intracellular uptake of 2-NBDG. This decrease in the intracellular uptake of 2-NBDG was linear and statistically significant between the individual concentration levels tested in this study ($p < 0.05$). In the presence of cut lettuce in water, no significant change in the intracellular uptake of the 2-NBDG was detected at sub-lethal levels of concentration below 0.8% H_2O_2 , but a statistically significant decrease in the intracellular uptake of 2-NBDG was measured at or above 0.8% H_2O_2 . Correlations between logarithmic reduction in CFU count in water and in the presence of fresh cut lettuce and changes in 2-NBDG signal were statistically significant ($p < 0.05$) with PCCs equal to -0.8649 and -0.8352, respectively.

4. DISCUSSION

The results of this study demonstrated that the reduction in bacterial survival in water was suppressed by the presence of fresh cut lettuce. This difference in sanitation efficacy was more pronounced in the case of NaOCl as compared to H₂O₂, since the minimum concentration of NaOCl required for inducing significant reduction in microbial count was 4 times higher in the presence of fresh cut lettuce (35.2 ppm) as compared to sanitation in water (8.8 ppm). This result can be explained based on interaction of active chlorine with the organic content in the pre-cut lettuce suspension (Rodgers et al, 2004). For H₂O₂, a smaller decrease in sanitation efficacy was observed with the addition of pre-cut lettuce to water. This result supports the conclusion that the sanitation efficacy of H₂O₂ is not strongly influenced by the organic load. Moreover, it reinforces previous observation that loss of NaOCl antimicrobial activity is due to organic carbon removal (Mikutta et al, 2005).

Results in Fig. 1 (A) indicate that the concentration of NaOCl required for inducing statistically significant reduction of the survival of *E. coli* O157:H7 in water was higher than 8.8 ppm of NaOCl, which is consistent to a similar result achieved with free chlorine between 5 and 25 ppm (Luo et al, 2011; Van Haute et al, 2013; Gómez-López et al, 2014). The results from APF dye labeled bacteria could only assess the increase in oxidative stress at sub-lethal concentration levels of NaOCl (0 - 4.4 ppm). The current analysis revealed that the ROS measurement using the APF dye was not effective in predicting the critical concentration of NaOCl above which significant reduction in *E. coli* O157:H7 survival was achieved. This conclusion is supported by the correlation analyses, which demonstrated no significant relationship between the reduction in bacterial survival quantified by standard plate count (changes in logarithmic CFU) and changes in fluorescence signal from the APF labeled cells. We speculate that the limited dynamic range of APF is due to the limited permeation of APF dye into

Gram-negative bacteria. The limited permeation of the dye across the outer membrane of Gram-negative bacteria such as *E. coli* O157:H7 can reduce the intracellular concentration of the dye and in turn limit the potential full dynamic range response of the APF (Breeuwer and Abee, 2000). Interestingly, however, the results did reveal APF can be a probe to detect ROS generated by sub-lethal concentrations of NaOCl. Measurement of oxidative stress induced by H₂O₂ in bacterial cells using H₂DCFDA dye indicated a non-linear response with multiple saturation levels at both sub-lethal and lethal concentration levels. Pearson correlation analyses of the data suggested only a weak relationship between the H₂DCFDA fluorescence and the logarithmic reduction in bacterial survival. Based on these results, we deduce that both APF and H₂DCFDA could be used to measure the oxidative stress from NaOCl and H₂O₂ within bacteria at sub-lethal concentrations, but that it may be difficult for these probes to estimate the critical concentration of NaOCl or H₂O₂ to induce reduction of survival of bacteria.

Membrane damage induced by NaOCl was measured using PI, a membrane impermeable DNA interchelating dye. PI enters into bacterial cells only when the cell membrane is compromised. Following entry, PI migrates to and interacts with DNA which results in enhanced fluorescence intensity (Kramer and Muranyi, 2014). There was a significant relationship, based on correlation analyses, between logarithmic reduction of bacterial survival and the fluorescence-based detection of PI penetration of bacterial cells. These results indicate that lethal concentrations of NaOCl induce significant membrane damage, which may be a primary mechanisms for reduction in bacterial survival. These results are in strict agreement with previous studies that also indicated a key role of membrane damage for the inactivation of bacteria upon treatment with lethal concentrations of NaOCl (Gray et al, 2013; Virto et al, 2005). Significantly, the outcome of this set of experiments provides a proof-of-concept for entering into additional developmental studies to translate PI into an element of a technology that can be used for evaluating the sanitation efficacy of NaOCl-based wash

water flumes used for fresh cut produce. Consistent with the idea that PI is specifically useful in situation where membrane damage is a primary mechanism for bacterial killing, Fig. 3 (B) indicates that PI is not useful for the monitoring of loss of bacterial survival by treatment achieved by H₂O₂. Treatment with H₂O₂ is not considered to result in significant membrane damage since only a marginal increase in cell permeability occurred for bacteria suspended in water, with no significant changes in membrane permeability occurring for bacteria in the presence of pre-cut lettuce. Together these results indicate that the increased membrane permeability might not be the leading mechanism for inducing reduction of survival of bacteria treated with H₂O₂. Whether H₂O₂ causes membrane damage is still unclear since only a few studies have investigated this subject (Imlay, 2003; Linley et al, 2012). Furthermore, these few studies were only conducted at concentration levels of H₂O₂ much lower than those used in sanitation.

The last part of the study focused on monitoring the activity of the *E. coli* glucose transport system using 2-NBDG. The result in Fig. 4 (A) shows that reduction of the intracellular uptake of the glucose analog 2-NBDG following treatment of bacteria with NaOCl is a step change process as compared to a linear change observed in the case of membrane permeability measurements. These results indicate that reduction in the intracellular uptake of glucose could be an indicator for the critical concentration of NaOCl at and above which significant reduction in survival can be achieved. The reduction in the intracellular uptake of glucose could be attributed to changes in activity of intracellular enzymes and glucose transporters upon treatment of bacteria with NaOCl (Loi et al, 2015) since the activity of intracellular enzymes including glycolytic enzymes could strongly influence the metabolic state of cells (Leichert et al, 2008). Furthermore, the function of glucose transporters could also be influenced by membrane damage induced by NaOCl treatment (Drazic et al, 2015).

An increase in uptake of glucose in *E. coli* exposed to low concentration (sub-lethal) of H₂O₂ and tellurite has been reported earlier (Valdivia-González et al, 2012). The previous study has suggested that an increase in the intracellular uptake of glucose may provide protection against oxidative stress by increasing the intracellular levels of NADPH (Gray et al, 2013). At lethal dosage, the rapid decrease in the intracellular uptake of 2-NBDG can be attributed to potential oxidative damage to glucose transporters, intracellular enzymes and metabolites. In the case of glucose transporters, treatment of bacteria with H₂O₂ can potentially induce oxidative damage to glucose transport and phosphorylation machinery such as oxidation of phospho-cysteine residues of the phosphotransferase unit. Similarly, oxidative damage to metabolic enzymes and critical co-factors can potentially decrease the rate of intracellular uptake of glucose. The linear regression analyses of the two cases of H₂O₂ sanitation tested in this work showed that similarly to the case with NaOCl, the 2-NBDG can be a good marker for the detection of the lethal concentration of the sanitizer for the microbial reduction also in contact with pre-cut lettuce. Based on the results of this study, changes in the intracellular uptake of glucose measured using the 2-NBDG probe was the best marker for evaluating the sanitation efficacy of water in the presence of fresh cut lettuce.

5. CONCLUSIONS

The results of this study show that glucose uptake, measured by 2-NBDG, can assess the critical concentration of the selected sanitizers required for achieving a significant reduction of survival of the bacteria. The results also demonstrate that direct oxidative stress measurements using ROS sensitive dyes are useful at sub-lethal concentrations of selected sanitizers; however, their predictive ability was limited due to saturation in the signal intensity (APF dye) and non-linear response (H₂DCFDA). Membrane damage, measured by PI, correlates well with survival reduction of *E. coli*

O157:H7 when treated with NaOCl but not in the case of H₂O₂. Further studies are required to evaluate extension of this approach to different strains of bacteria under different physiological states and diverse environments.

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REFERENCES

- Baatout, S., De Boever, P., & Mergeay, M. (2006). Physiological changes induced in four bacterial strains following oxidative stress. *Prikladnaia Biokhimiia I Mikrobiologiia*, 42(4), 418–427.
- Bergamini, C., Moruzzi, N., Sblendido, A., Lenaz, G., & Fato, R. (2012). A water soluble CoQ10 formulation improves intracellular distribution and promotes mitochondrial respiration in cultured cells. *PloS One*, 7(3), e33712. <http://doi.org/10.1371/journal.pone.0033712>
- Breeuwer, P., & Abee, T. (2000). Assessment of viability of microorganisms employing fluorescence techniques. *International Journal of Food Microbiology*, 55(1-3), 193–200.
- Choi, O., & Hu, Z. (2008). Size dependent and reactive oxygen species related nanosilver toxicity to nitrifying bacteria. *Environmental Science & Technology*, 42(12), 4583–4588.
- Drazic, A., Kutzner, E., Winter, J., & Eisenreich, W. (2015). Metabolic Response of *Escherichia coli* upon Treatment with Hypochlorite at Sub-Lethal Concentrations. *PLoS ONE*, 10(5). <http://doi.org/10.1371/journal.pone.0125823>
- Gil, M. I., Selma, M. V., López-Gálvez, F., & Allende, A. (2009). Fresh-cut product sanitation and wash water disinfection: problems and solutions. *International Journal of Food Microbiology*, 134(1-2), 37–45. <http://doi.org/10.1016/j.ijfoodmicro.2009.05.021>

Gómez-López, V. M., Lannoo, A.-S., Gil, M. I., & Allende, A. (2014). Minimum free chlorine residual level required for the inactivation of *Escherichia coli* O157:H7 and trihalomethane generation during dynamic washing of fresh-cut spinach. *Food Control*, 42, 132–138. <http://doi.org/10.1016/j.foodcont.2014.01.034>

Gray, M. J., Wholey, W.-Y., & Jakob, U. (2013). Bacterial responses to reactive chlorine species. *Annual Review of Microbiology*, 67, 141–160. <http://doi.org/10.1146/annurev-micro-102912-142520>

Hosoki, A., Yonekura, S.-I., Zhao, Q.-L., Wei, Z.-L., Takasaki, I., Tabuchi, Y., ... Zhang-Akiyama, Q.-M. (2012). Mitochondria-targeted superoxide dismutase (SOD2) regulates radiation resistance and radiation stress response in HeLa cells. *Journal of Radiation Research*, 53(1), 58–71.

Imlay, J. A. (2003). Pathways of oxidative damage. *Annual Review of Microbiology*, 57, 395–418. <http://doi.org/10.1146/annurev.micro.57.030502.090938>

Kemp, G. K., & Schneider, K. R. (2000). Validation of thiosulfate for neutralization of acidified sodium chlorite in microbiological testing. *Poultry Science*, 79(12), 1857–1860.

Kramer, B., & Muranyi, P. (2014). Effect of pulsed light on structural and physiological properties of *Listeria innocua* and *Escherichia coli*. *Journal of Applied Microbiology*, 116(3), 596–611. <http://doi.org/10.1111/jam.12394>

Le, P., Zhang, L., Lim, V., McCarthy, M. J., & Nitin, N. (2015). A novel approach for measuring resistance of *Escherichia coli* and *Listeria monocytogenes* to hydrogen peroxide using label-free magnetic

resonance imaging and relaxometry. *Food Control*, 50, 560–567.
<http://doi.org/10.1016/j.foodcont.2014.09.038>

Leichert, L. I., Gehrke, F., Gudiseva, H. V., Blackwell, T., Ilbert, M., Walker, A. K., Strahler, J. R., Andrews, P. C., Jakob, U. (2008). Quantifying changes in the thiol redox proteome upon oxidative stress in vivo. *Proceedings of the National Academy of Sciences of the United States of America*, 105(24), 8197–8202.
<http://doi.org/10.1073/pnas.0707723105>

Linley, E., Denyer, S. P., McDonnell, G., Simons, C., & Maillard, J.-Y. (2012). Use of hydrogen peroxide as a biocide: new consideration of its mechanisms of biocidal action. *The Journal of Antimicrobial Chemotherapy*, 67(7), 1589–1596. <http://doi.org/10.1093/jac/dks129>

Loi, V. V., Rossius, M., & Antelmann, H. (2015). Redox regulation by reversible protein S-thiolation in bacteria. *Frontiers in Microbiology*, 6. <http://doi.org/10.3389/fmicb.2015.00187>

Luo, Y., Nou, X., Yang, Y., Alegre, I., Turner, E., Feng, H., Abadias, M., & Conway, W. (2011). Determination of free chlorine concentrations needed to prevent *Escherichia coli* O157:H7 cross-contamination during fresh-cut produce wash. *Journal of Food Protection*, 74(3), 352–358.
<http://doi.org/10.4315/0362-028X.JFP-10-429>

Luo, Z., Loja, M. N., Farwell, D. G., Luu, Q. C., Donald, P. J., Amott, D., Truong, A. Q., Gandour-Edwards, R., & Nitin, N. (2014). Widefield optical imaging of changes in uptake of glucose and tissue extracellular pH in head and neck cancer. *Cancer Prevention Research (Philadelphia, Pa.)*, 7(10), 1035–1044.
<http://doi.org/10.1158/1940-6207.CAPR-14-0097>

Lyon, D. Y., Brunet, L., Hinkal, G. W., Wiesner, M. R., & Alvarez, P. J. J. (2008). Antibacterial activity of fullerene water suspensions (nC60) is not due to ROS-mediated damage. *Nano Letters*, 8(5), 1539–1543. <http://doi.org/10.1021/nl0726398>

Mikutta, R., Kleber, M., Kaiser, K., & Jahn, R. (2005). Review: Organic matter removal from soils using hydrogen peroxide, sodium hypochlorite, and disodium peroxodisulfate. *Soil Science Society of America Journal*, 69(1), 120–135. <http://doi.org/10.2136/sssaj2005.0120>

Nakazato, T., Sagawa, M., Yamato, K., Xian, M., Yamamoto, T., Suematsu, M., Ikeda, Y., Kizaki, M. (2007). Myeloperoxidase is a key regulator of oxidative stress mediated apoptosis in myeloid leukemic cells. *Clinical Cancer Research*, 13(18 Pt 1), 5436–5445. <http://doi.org/10.1158/1078-0432.CCR-07-0481>

Nitin, N., Carlson, A. L., Muldoon, T., El-Naggar, A. K., Gillenwater, A., & Richards-Kortum, R. (2009). Molecular imaging of glucose uptake in oral neoplasia following topical application of fluorescently labeled deoxy-glucose. *International Journal of Cancer*. 124(11), 2634–2642. <http://doi.org/10.1002/ijc.24222>

Rodgers, S. L., Cash, J. N., Siddiq, M., & Ryser, E. T. (2004). A Comparison of Different Chemical Sanitizers for Inactivating *Escherichia coli* O157:H7 and *Listeria monocytogenes* in Solution and on Apples, Lettuce, Strawberries, and Cantaloupe. *Journal of Food Protection*, 67(4), 721–731.

Suslow, T. V. (2004). Oxidation-reduction potential (ORP) for water disinfection monitoring, control, and documentation. *University of California. Division of Agriculture and Natural Resources. Publication 8149.*

Valdivia-González, M., Pérez-Donoso, J. M., & Vásquez, C. C. (2012). Effect of tellurite-mediated oxidative stress on the *Escherichia coli* glycolytic pathway. *Biometals*, 25(2), 451–458. <http://doi.org/10.1007/s10534-012-9518-x>

Van Haute, S., Sampers, I., Holvoet, K., & Uyttendaele, M. (2013). Physicochemical quality and chemical safety of chlorine as a reconditioning agent and wash water disinfectant for fresh-cut lettuce washing. *Applied and Environmental Microbiology*, 79(9), 2850–2861. <http://doi.org/10.1128/AEM.03283-12>

Virto, R., Mañas, P., Alvarez, I., Condon, S., & Raso, J. (2005). Membrane damage and microbial inactivation by chlorine in the absence and presence of a chlorine-demanding substrate. *Applied and Environmental Microbiology*, 71(9), 5022–5028. <http://doi.org/10.1128/AEM.71.9.5022-5028.2005>

Wang, Y., Claeys, L., van der Ha, D., Verstraete, W., & Boon, N. (2010). Effects of chemically and electrochemically dosed chlorine on *Escherichia coli* and *Legionella beliardensis* assessed by flow cytometry. *Applied Microbiology and Biotechnology*, 87(1), 331–341. <http://doi.org/10.1007/s00253-010-2526-2>

Wessa, P. (2016). Free Statistics Software, Office for Research Development and Education, version 1.1.23-r7, URL <http://www.wessa.net/>

Yoshioka, K., Takahashi, H., Homma, T., Saito, M., Oh, K. B., Nemoto, Y., & Matsuoka, H. (1996). A novel fluorescent derivative of glucose applicable to the assessment of glucose uptake activity of *Escherichia coli*. *Biochimica Et Biophysica Acta*, 1289(1), 5-9.

FIGURE CAPTIONS

Figure 1. Microbial reduction of *E. coli* O157:H7 upon sanitation treatment with NaOCl or H₂O₂. CFU logarithmic count is shown for 10 min sanitation experiments with NaOCl (A) or H₂O₂ (B) at different concentrations in water (white bars) or in contact with pre-cut lettuce (gray bars). Data are shown as mean $\pm$ standard deviation of triplicate experiments. Asterisks indicate statistical significance ($p < 0.05$).

Figure 2. Measurement of oxidative stress with ROS probes in *E. coli* O157:H7 during sanitation treatment with NaOCl or H₂O₂. Oxidative stress in bacteria upon treatment with NaOCl was measured by APF probe fluorescence (A) while ROS production upon H₂O₂ sanitation was measured by H₂DCFDA signal fluorescence (B). Normalized fluorescence intensity ratios were plotted against the concentration of sanitizer used. The Pearson correlation coefficient (PCC) between the logarithmic reduction achieved by NaOCl and the APF signal or between the logarithmic reduction achieved by H₂O₂ and the H₂DCFDA fluorescence signal are also reported together with the p-value below each graph. Asterisks indicate statistical significance ($p < 0.05$). Scatter plots of the logarithmic reduction achieved by NaOCl and the APF signal (C) or between the logarithmic reduction achieved by H₂O₂ and the H₂DCFDA fluorescence signal (D) are also shown.

Figure 3. Measurement of permeated propidium iodide (PI) in *E. coli* O157:H7 after treatment with NaOCl or H₂O₂ in water or in contact with lettuce leaves. Permeation of PI was measured in bacterial cells subjected to 10 min NaOCl (A) or H₂O₂ (B) sanitation in water (white bars) or in contact with lettuce (gray bars). Data are shown as normalized fluorescence intensity ratios for all concentrations tested. The Pearson correlation coefficient (PCC) between the

logarithmic reductions and PI fluorescence in bacteria treated with NaOCl in water or in contact with lettuce leaves are shown below the (A) graph as well as the Pearson correlation coefficient (PCC) between the logarithmic reductions and PI fluorescence in bacteria treated with H₂O₂ in water or in contact with lettuce leaves are shown below the (B) graph. All PCCs are shown together with the measured p-value for each correlation test. The inset caption in B represents data from B graph with a smaller y scale to graphically highlight differences among the series. Asterisks indicate statistical significance ($p < 0.05$). Scatter plots of the logarithmic reductions and PI fluorescence in bacteria treated with NaOCl in water (C) or in contact with lettuce leaves (D) are shown as well as between the logarithmic reductions and PI fluorescence in bacteria treated with H₂O₂ in water (E) or in contact with lettuce leaves (F).

Figure 4. Measurement of 2-NBDG uptake in *E. coli* O157:H7 after treatment with NaOCl or H₂O₂ in water or in contact with lettuce leaves. Uptake of 2-NBDG was measured in bacterial cells subjected to 10 min NaOCl (A) or H₂O₂ (B) sanitation in water (white bars) or in contact with lettuce (gray bars). Data are shown as normalized fluorescence intensity ratios of intracellular 2-NBDG in bacteria subjected to all the concentrations tested. The Pearson correlation coefficient (PCC) between the logarithmic reductions and 2-NBDG fluorescence in bacteria treated with NaOCl in water or in contact with lettuce leaves are shown below the (A) graph as well as the PCCs between the logarithmic reductions and 2-NBDG fluorescence in bacteria treated with H₂O₂ in water or during contact with lettuce leaves are shown below the (B) graph. All PCCs are shown together with the measured p-value for each correlation test. Asterisks indicate statistical significance ($p < 0.05$). Scatter plots between the logarithmic reductions and 2-NBDG fluorescence in bacteria treated with NaOCl in water (C) or in contact with lettuce leaves (D) are shown as well as between the logarithmic reductions and 2-NBDG fluorescence in bacteria treated with H₂O₂ in water (E) or during contact

Figure 1

Figure 2

	PCC value	p-value
APF / Log reduction by NaOCl in water	0.4260	0.3406

	PCC value	p-value
H2DCFDA / Log reduction by H2O2 in water	0.8153	0.0480

Figure 3

Figure 4