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# An integrated electrolysis – electrospray – ionization antimicrobial platform using Engineered Water Nanostructures (EWNS) for food safety applications

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ABSTRACT

Engineered water nanostructures (EWNS) synthesized utilizing electrospray and ionization of water, have been, recently, shown to be an effective, green, antimicrobial platform for surface and air disinfection, where reactive oxygen species (ROS), generated and encapsulated within the particles during synthesis, were found to be the main inactivation mechanism. Herein, the antimicrobial potency of the EWNS was further enhanced by integrating electrolysis, electrospray and ionization of de-ionized water in the EWNS synthesis process. Detailed physicochemical characterization of these enhanced EWNS (eEWNS) was performed using state-of-the-art analytical methods and has shown that, while both size and charge remain similar to the EWNS (mean diameter of 13 nm and charge of 13 electrons), they possess a three times higher ROS content. The increase of the ROS content as a result of the addition of the electrolysis step before electrospray and ionization led to an increased antimicrobial ability as verified by *E. coli* inactivation studies using stainless steel coupons. It was shown that a 45-min exposure to eEWNS resulted in a 4-log reduction as opposed to a 1.9-log reduction when exposed to EWNS. In addition, the eEWNS were assessed for their potency to inactivate natural microbiota (total viable and yeast and mold counts), as well as, inoculated *E. coli* on the surface of fresh organic blackberries. The results showed a 97% (1.5-log) inactivation of the total viable count, a 99% (2-log) reduction in the yeast and mold count and a 2.5-log reduction of the inoculated *E. coli* after 45 min of exposure, without any visual changes to the fruit. This enhanced antimicrobial activity further underpins the EWNS platform as an effective, dry and chemical free approach suitable for a variety of food safety applications and could be ideal for delicate fresh produce that cannot withstand the classical, wet disinfection treatments.

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## 1. Introduction

Food borne disease as a result of consuming microbiologically contaminated food is a major public health problem, with the annual worldwide toll reaching an alarming 600 million cases and 420,000 deaths (WHO, 2015). Between 2004 and 2012, the United States experienced 377 major food related outbreaks, with Norovirus, *Salmonella* spp. and E. *coli* being the biggest culprits (Callejón et al., 2015). By a 2014 USDA estimation, foodborne illnesses cost the U.S. economy $15.6 billion annually (USDA, 2014). In addition to safety concerns, food waste, for reasons including microbial spoilage, is also reaching epidemic levels, with estimates in the USA being in the order of 30–50% of the food produced (Tscharntke et al., 2012). The food industry is challenged to find solutions to a fast-changing food production environment, dictated by new consumer preferences for 'green' and organic foods, including consumption of more fresh fruits and vegetables (Motarjemi & Käferstein, 1999; Van Boxstael et al., 2013).

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Currently, there are a number of antimicrobial strategies used by the food industry across the "farm to the fork" chain. These mainly include: 1) chlorine-elemental or hypochlorite (Parish et al., 2003); 2) chlorine dioxide (Costilow, Uebersax, & Ward, 1984; Pao, Kelsey, Khalid, & Ettinger, 2007); 3) peracetic acid (Ernst et al., 2006; Fraisse et al., 2011); 4) hydrogen peroxide liquid or vapor (Gulati, Allwood, Hedberg, & Goyal, 2001; Rudnick, McDevitt, First, & Spengler, 2009); 5) ozone - gaseous and aqueous (Horvitz & Cantalejo, 2014); 6) irradiation (UV and gamma) (WHO, 2008); and 7) electrolyzed water (Izumi, 1999; Koseki, Yoshida, Isobe, & Itoh, 2004; Park, Hung, Doyle, Ezeike, & Kim, 2001). Some of these methods (chlorine-elemental, hypochlorite, chlorine dioxide) leave behind chemical residues, are ineffective with a heavy organic load and are not approved for use with organic products (Karaca & Velioglu, 2007). Moreover, some of the aforementioned methods can induce visible damage and negative sensory effects to products such as fresh produce (e.g., ozone) (Rico, Martín-Diana, Barat, & Barry-Ryan, 2007). Some interventions are also associated with high energy costs and significant environmental footprints (Ruder, 2006).

Furthermore, there is a class of products, popular among consumers due to health benefits, namely the berries (strawberries, blackberries, raspberries, blueberries), that cannot tolerate the traditional, wet type treatment methods due to their delicate nature and subsequent mold proliferation potential (Keutgen & Pawelzik, 2008). Also, the increased demand for organic produce and organic certification further restraints the use of the classical, chemical disinfection interventions. It should be mentioned that the U.S. FDA and USDA have imposed a long list of restrictions on acceptable chemicals and their concentrations for organic produce (USDA, 2016). In addition, microorganisms are constantly adapting to current antimicrobial technologies, leading to ineffectiveness of conventional treatments (Andersson & Hughes, 2010; Austin, Bonten, Weinstein, Slaughter, & Anderson, 1999).

The food industry is, therefore, in need of novel, effective, green and low cost intervention methods, in line with the new sustainable environmental approaches and emerging consumer preferences. Such methods should have the capability to be applied with ease at various stages from "farm to fork", **and replace or supplement** existing technologies and enhance food safety and quality (Newell et al., 2010).

In the last two decades, nanotechnology has shown that it can enhance our arsenal of methods in the battle against pathogenic and spoilage microorganisms. Indeed, nanotechnology-based approaches, such as antimicrobial food surfaces, nano-enabled sensors, active/intelligent packaging and novel disinfection platforms, are finding their way within the agri/food/feed sector, bringing great new opportunities to the food industry (Eleftheriadou, Pyrgiotakis, & Demokritou, 2017).

Recently, the authors have developed a novel, dry, organic chemical free, nanotechnology-based antimicrobial platform utilizing Engineered Water Nanostructures (EWNS) synthesized as an aerosol using a combined electrospray and ionization process. These EWNS have been shown to effectively inactivate a wide range of food related microorganisms on food surfaces, on food contact surfaces and in air (Pyrgiotakis et al., 2015, 2016; Pyrgiotakis, McDevitt, Yamauchi, & Demokritou, 2012; Pyrgiotakis, McDevitt, Bordini, et al., 2014; Pyrgiotakis, McDevitt, Gao, et al., 2014). These EWNS particles possess a unique set of physico-chemical properties that make them an effective antimicrobial agent. They have an average charge of 10–40 electrons per structure and an average nanoscale size of 25 nm (Pyrgiotakis et al., 2012, 2015, 2016; Pyrgiotakis, McDevitt, Bordini, et al., 2014; Pyrgiotakis, McDevitt, Gao, et al., 2014). Earlier studies have shown that they contain a large number of reactive oxygen species (ROS), primarily hydroxyl ($OH\cdot$) and superoxide ($O_2^-$) radicals which are highly microbicidal (Pyrgiotakis et al., 2016).

The current study describes our efforts to further enhance the inactivation efficacy of these EWNS particles by integrating the electrolysis of de-ionized water in their synthesis process. The combined electrolysis-electrospray-ionization synthesis process and the resulting Enhanced Engineered Water Nanostructures, referred as eEWNS, are presented here. These eEWNS particles were characterized physico-chemically using state-of-the art methods. Furthermore, their increased antimicrobial efficacy was assessed against *E. coli* inoculated onto stainless steel coupons and on the surface of delicate fruits (blackberries), as well as, in the reduction of the natural bioburden (total viable and yeast and mold counts) of blackberries.

## 2. Materials and methods

### 2.1. Synthesis of Enhanced Engineered Water Nanostructures (eEWNS)

The synthesis of the eEWNS was a two-step process. In more detail:

#### 2.1.1. Synthesis of eEWNS

The combined electrolysis (STEP 1), electrospray/ionization (STEP 2) of the eEWNS synthesis process is presented in Fig. 1a. In more detail:

For the electrolysis of de-ionized water (STEP 1), 500 mL highly purified de-ionized water (18.2 MΩ $cm^{-1}$, Thermo Scientific Nanopure) underwent electrolysis in a stirred beaker, with a tungsten oxide plate as the anode and a stainless-steel plate as the cathode (12.2 × 3.6 cm) (Fig. 1a). The electrolysis was powered in a galvanostat mode by an electrical power supply (Sorensen DCS 600–1.7, Ametek Programmable Power, San Diego CA), with a fixed current of 0.2 A. The electrolysis lasted for 1 h, with the beaker immersed in a dry ice bath to prevent heating of the water. During electrolysis, the voltage and current values, as well as, the water temperature were recorded in real time. After electrolysis of de-ionized water, the water was used in step 2 for the synthesis of eEWNS.

The basic EWNS generation setup (STEP 2) is the same as reported in our previous work (Pyrgiotakis et al., 2016). In summary, the generation of the EWNS is a combination of two phenomena, electrospray and ionization. In a typical experiment, a high voltage (in the kV range) is applied between a metal capillary that contains the liquid (Electrolysis water in this case or DI water in the case of simple EWNS) and a grounded counter electrode. The strong electric field between the two electrodes causes the formation of a conical meniscus at the outlet of the capillary, the so-called Taylor cone (Meesters, Vercoulen, Marijnissen, & Scarlett, 1992; Taylor, 1964). From the tip of the Taylor cone, highly charged water droplets continue to break into smaller particles as they are drawn by the electrical field towards the counter electrode. These as-produced aerosols often show a remarkably narrow size distribution, which is considered to be monodispersed. At the same time, the high electric field causes some water molecules to split and can strip off electrons (ionization), resulting in a high number of reactive oxygen species (ROS).

Fig. 1b presents the eEWNS generation system developed and used for both the physico-chemical characterization and microbial inactivation experiments (described below). The fluid (~150 mL), stored in sealed bottle, was fed through a Teflon tubing (Internal Diameter 2 mm) to a 30 Gauge stainless steel needle which serves as the metal capillary (Hamilton Robotics, Reno NV). The flow of the fluid through the needle is controlled by adjusting the pressure of

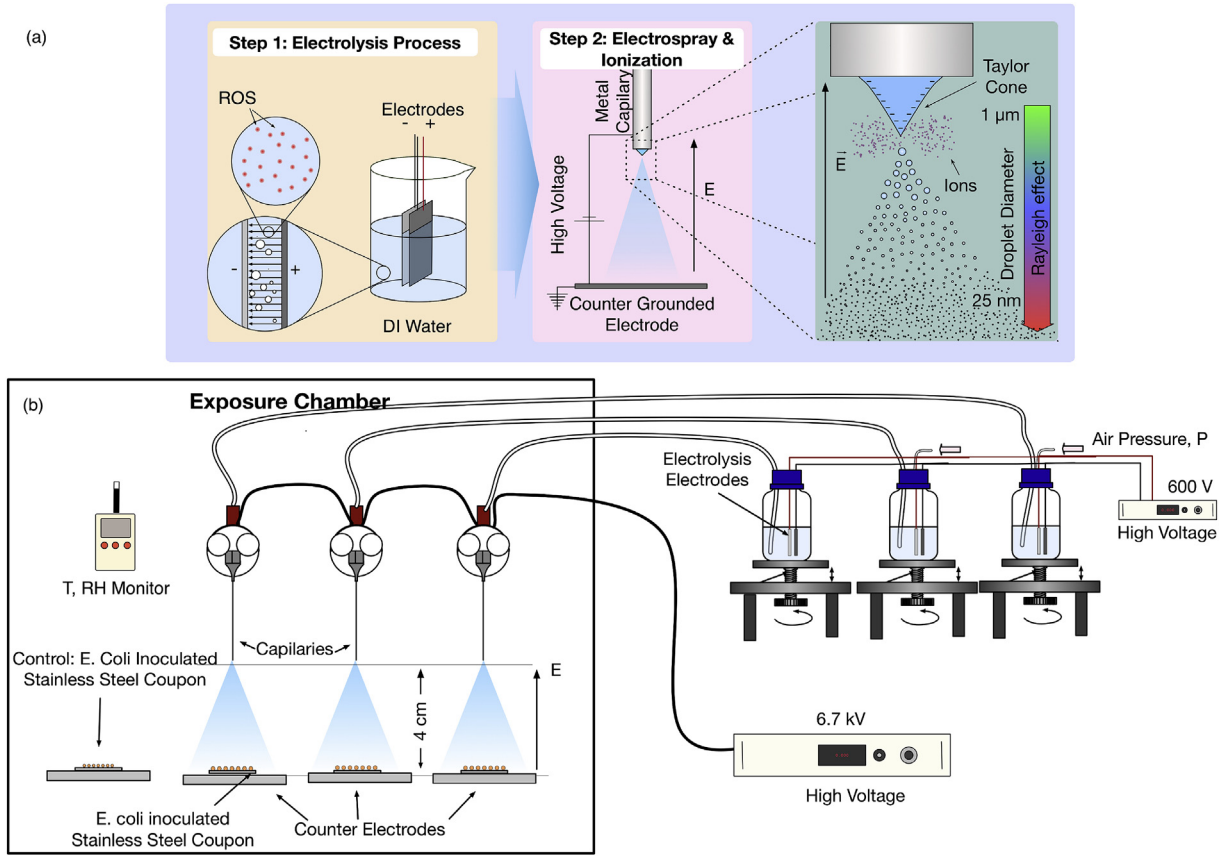


**Fig. 1. The eEWNS generation and antimicrobial treatment setup.** (**a**) Electrolysis of de-ionized water followed by electrospray and ionization. The electrolysis produces reactive oxygen species in the deionized water. Electrospray takes place when a high voltage is applied to a capillary containing a liquid and grounded counter electrode. The application of the high voltage results into two distinct phenomena: (i) the electrospray of the water and (ii) generation of reactive oxygen species (radicals) that are trapped in the EWNS. (**b**) The treatment of *E. coli* inoculated coupons with the eEWNS aerosol, showing the vertical electric field which results in a target specific delivery of electrically charged eEWNS particles onto the surface of interest (stainless steel or blackberries).

air inside the bottle (Fig. 1b). The applied voltage (V), the distance between the needle and counter electrode (L), and the flow of the fluid through the capillary ($\phi$), were adjusted to obtain a stable Taylor cone. The needle was held at a distance of about 4 cm above the counter electrode, with an applied voltage of ~6.7 kV, based on our previous optimization study (Pyrgiotakis et al., 2016). The counter electrode is a polished aluminum disk. If the EWNS aerosol needs to be sampled (e.g., for characterization), an aluminum disk with an opening in the center (1.29 cm) is used instead. Beneath the counter electrode there is an aluminum sampling funnel that is connected through grounded brass tubing to the sampling apparatus. The same experimental setup is used for the production of EWNS (DI water used as the fluid) and eEWNS (water produced by electrolysis used as the fluid).

### *2.2. Physicochemical characterization of electrolysis water and eEWNS*

#### *2.2.1. Physical characterization of the water produced by electrolysis (STEP 1)*

During the electrolysis of water, 1 mL samples were collected every 10 min and the conductivity was measured using the Zetasizer (Malvern Instruments, Westborough MA). The pH of the water during the electrolysis step was also measured using a pH meter (SI Analytics, College Station TX).

#### *2.2.2. Physical characterization of the eEWNS produced (STEP 2)*

**Particle number and size distribution and charge measurement**: Fig. S1a summarizes the aerosol particle number concentration and charge characterization of eEWNS. A Scanning Mobility Particle Sizer (SMPS) (Model 3936, TSI, Shoreview, MN) and a Faraday Aerosol electrometer (Model 3068B, TSI, Shoreview, MN) (Fig. S1a) were used to measure the aerosol particle number concentration and charge, respectively. The SMPS was used to measure the particle number and size distribution concurrently. It is worth noting that it was shown in our previous studies that while the SMPS provides an estimate of the total particle number concentration of the EWNS aerosol, it does not measure accurately the EWNS size distribution due to the fact that the SMPS sampled aerosol charge will be reduced to the Boltzmann equilibrium (Ji, Bae, & Hwang, 2004; Kim, Woo, Liu, & Zachariah, 2005) using a radioactive source. It was shown in our previous and other studies that a reduction in the electric charge of the water particle will result to lower surface tension and increased evaporation which can result to smaller particle sizes (Nielsen, Maus, Rzesanke, & Leisner, 2011). As a result, we have previously employed Atomic Force Microscopy (AFM) to estimate the size distribution (Pyrgiotakis et al., 2016). Here by comparing the SMPS derived size distributions of both the EWNS and eEWNS, one can get an idea whether the size distribution of the latter differs from the previously reported one in our studies.

The Faraday aerosol electrometer was used to measure the aerosol current, and thus, the electrical charge of the nanoparticles. Both instruments had a sampling flow of 0.5 L per minute (lpm). The particle number concentration and the aerosol current were measured for a duration of 120 s. This measurement was repeated

30 times. From the current measurement, the total electric charge of the aerosol was calculated and the average EWNS electric charge was estimated for the given total number of EWNS particles sampled. The average EWNS charge $q$ can be calculated with equation (1):

$$q = \frac{I_{El}}{N_{SMPS} \times \varphi_{El}} \quad (1)$$

where $I_{El}$ (Amperes) is the measured current, $N_{SMPS}$ (#/m$^3$) is the number concentration measured with the SMPS, and $\varphi_{El}$ (m$^3$/s) is the flow of the aerosol into the electrometer. The total mass of the particles was also measured through SMPS. The temperature and relative humidity (RH) were maintained at 21 °C and 25%, respectively.

### 2.2.3. ROS characterization of water produced by electrolysis (STEP 1)

Two methods were employed to measure ROS levels of electrolysis step. In more detail:

*2.2.3.1. ROS measurement using the Trolox method.* The Trolox Method (Miller, Rice-Evans, Davies, Gopinathan, & Milner, 1993) was employed to quantitatively detect the presence of ROS, including short lived ROS such as hydroxyl radical (OH·) and superoxide ($O_2^-$) and also hydrogen peroxide ($H_2O_2$). As a water-soluble Vitamin E analog, Trolox reacts with short lived radicals such $O_2^-$ and OH· to form Trolox Quinone which can be subsequently analyzed by LC-MS/MS (Miller et al., 1993). However, the reaction rate of Trolox with the longer lived $H_2O_2$ is very slow (practically negligible within 30 min). Hence, in order to detect $H_2O_2$, horse radish peroxidase (HRP) was added to catalyze the decomposition of the $H_2O_2$, which as a result produced hydroxyl radicals (van den Berg, Haenen, van den Berg, & Bast, 1999). The hydroxyl radicals can then be measured. With this method, the total ROS in the solution, including both short lived ROS and $H_2O_2$ can be measured.

Here, Trolox was added to a beaker containing 500 mL of deionized water to reach a concentration of 0.1 mM. The water underwent electrolysis for 1 h. Aliquots of 1 mL samples were taken during the electrolysis phase at 1, 2, 5, 10, 20, 40, and 60 min. After the electrolysis process was completed, the remaining water was removed from the electrolysis setup and placed at room temperature. More samples were taken at 15, 30, 45, and 60 min following electrolysis, to determine the post-treatment concentrations of ROS species. The detailed methodology is described in detail in the Supplemental Information.

*2.2.3.2. ROS measurement using the electron spin resonance method (ESR).* In addition to the Trolox method described above, ROS characterization was also performed using ESR method. ESR has been used in many studies to detect specific ROS species (Lecour et al., 1998). Spin-trapping reagents react with short-lived radicals to produce long-lived radicals called spin-adducts (Kohno, 2010). By observing the ESR spectra of these spin-adducts, specific species of ROS can be identified (Frejaville et al., 1995). The ROS speciation and semi-quantitative analysis of concentration can be analyzed from the peak position and average peak height of the spectra. Furthermore, since $H_2O_2$ is a longer-lived species, the Fenton reaction is used to detect the presence of $H_2O_2$ in the solution. The Fenton reaction is shown below:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH + OH^-$$

$FeSO_4$ was added to the spin trap solution that the samples were bubbled through. ESR measurements were conducted using a Bruker EMX spectrometer (Bruker Instruments Inc. Billerica, MA) and a quartz flat cell assembly. The Iron sulfate ($FeSO_4$) used for Fenton Chemistry and the spin trap 5,5-Dimethyl-1-Pyrroline-N-Oxide (DMPO) were purchased from Sigma-Aldrich (St. Louis, MO).

Samples of the water produced during electrolysis were taken at 1, 10, 20, 40 and 60 min during the electrolysis process. 1 mL of each sample was added to a solution containing 500 mM DMPO to which 5 mM $FeSO_4$ was then added. For the detection of superoxide radical, samples were bubbled for 5 min though DMPO. After 3-min incubation (for OH·) or 1 min incubation (for $O_2^-$) both sample types were transferred to a quartz flat cell (Wilmad Glass, Vineland, NJ) and placed in the ESR cavity for measurement. ESR settings for both sample types were: center field 3510 ± 100, frequency 9.75 GigaHertz, power 126.6 mWatts, gain 1 × 10^4, modulation frequency 100 kHz, modulation amplitude 1.0 G, time constant 40 ms.

### 2.2.4. ROS measurement of EWNS and eEWNS produced (STEP 2)

Two methods were employed to measure ROS levels of both EWNS and eEWNS. In more detail:

*2.2.4.1. ROS measurement using the Trolox method.* Briefly, a 5 mL of Trolox solution (0.1 mM, in 0.05 M pH 7 phosphate buffer) was placed in an impinger (Midget Impinger, SKC Inc., Eighty Four PA), which was connected to the outlet of the EWNS/eEWNS sampling system (Fig. S1b). A sampling flow rate of 0.5 lpm was maintained. The particles were produced and three distinct sampling times, 5, 10 and 15 min were used. The loss of particles within the impinger was determined by the difference between the measured particle number concentrations with and without the impinger. Two aliquots of 1 mL were taken from the impinger after the reaction time. The first aliquot was processed without further modification to detect the short-lived ROS, and the second aliquot was spiked with HRP (100 unit/mL final concentration), for the detection of $H_2O_2$, as described above. Samples were incubated for 30 min at 37 °C prior to analysis as described previously (Section 2.2.3.1).

*2.2.4.2. ROS detection in EWNS and eEWNS using ESR method.* 1 mL solution of 500 mM DMPO was placed in an impinger, which was connected to the outlet of the EWNS/eEWNS sampling system (Fig. S1b). A sampling flow rate of 0.5 lpm was maintained. The EWNS/eEWNS particles were bubbled through the DMPO solution for 5 min. The samples were then analyzed with ESR to detect the OH· and $O_2^-$ peaks.

## 2.3. Microbial inactivation experiments

Fig. 1b illustrates the experimental design of the microbial inactivation experiments. In more detail:

### 2.3.1. E. coli inoculum preparation

Strains of *Escherichia coli* (ATCC #27325) and *Escherichia coli* (ATCC #25922) were used for the inoculation experiments. Before each experiment, a few colonies of each strain were added to 20 mL Tryptic Soy Broth (TSB) (Becton Dickinson, Franklin Lakes NJ) and the culture was grown overnight at 37 °C inside a shaker incubator. The cells were centrifuged at 3000 rpm for 20 min and the resulting pellet was re-suspended in deionized water. The concentration of the suspension was adjusted to 1 × 10$^8$ cfu/mL by measurement of Optical Density at 600 nm. This was used as the inoculum for further experimentation, ATCC #27325 for the inoculation of the coupons and ATCC #25922 for inoculation of the blackberries.

### 2.3.2. Blackberries samples

Fresh, packaged, organic blackberries were purchased from

stores locally, transported to the lab and stored at 4 °C until experimental work was performed on the same day.

*2.3.3. Inoculation of coupons with E.coli*

Circular Stainless-Steel coupons with mirror finish (stainless steel 304, diameter 1.82 cm, Stainless Supply, Monroe NC) were used. The coupons were added to a mild soap solution and sonicated for 1 h. They were subjected to a three-step sonication process each with ethanol, isopropyl alcohol and methanol for 10 min. The coupons were then autoclaved at 121 °C in self-sealing sterilization pouches (VWR International, Radnor PA).

10 μL of the original $10^8$ cfu inoculum *E. coli* (ATCC #27325) (equivalent to $10^6$ cfu) was inoculated onto the surface of stainless steel coupons by adding 10 droplets of 1 μl each in a concentric fashion near the center of the coupon. The coupons were then kept inside small petri dishes and transferred in a biosafety cabinet where the inoculum was allowed to dry (for approximately 30 min). The coupons were then placed in closed sterile petri dishes until further experimentation as described in 2.3.5.

*2.3.4. Inoculation of blackberries with E.coli*

Before inoculation, the blackberries were dipped in a solution of 70% Ethanol (VWR International, Radnor PA) for 2 min. They were then placed inside the biosafety cabinet and treated with UV light for 20 min, being turned over once for uniform treatment. Afterwards, 10 μL of the original $10^8$ cfu *E. coli* (ATCC#25922) inoculum (equivalent to $10^6$ cfu) was inoculated onto the surface of each blackberry by adding 10 droplets of 1 μl each near the apex of the blackberry on the smooth surfaces. The inoculum was allowed to dry (for approximately 30 min) in a biosafety cabinet before transfer to the exposure chamber.

*2.3.5. Exposure of E.coli inoculated coupons to eEWNS*

Three inoculated coupons were placed directly below each of the three electrospray needles in the exposure chamber (with the bacterial inoculum side facing the needle), on the counter ground electrode (Fig. 1b). The charged eEWNS particles were generated and travelled towards the coupon in the electrical field. The EWNS aerosol particle number concentration was measured immediately before and after treatment. An additional three inoculated coupons were left untreated and kept away from the electrospray needles, under the same temperature and RH conditions (22 °C, 25% RH) within the exposure chamber. Two separate exposure treatments were performed, one for 15and the other for 45 min. After each time period exposure, both the control and exposure coupons were removed from the chamber in order to determine the extent of *E. coli* inactivation as described in section 2.3.8 below. For comparison purposes and to be able to estimate enhancement, a similar treatment was performed with inoculated coupons exposed to the original EWNS particles, produced without the electrolysis step in the synthesis process.

*2.3.6. Exposure of E.coli inoculated blackberries to eEWNS*

Inoculated blackberries were exposed to eEWNS as in 2.3.5. Three berries were used as treatment berries in each experiment. One berry each was placed directly underneath each electrospray needle (Fig. 1b) and treated for 15 min and 45 min in separate experiments. For each experiment three additional inoculated berries were held as controls. These were placed inside the chamber but away from the electrospray needle, under the same temperature and RH conditions (22 °C, 25% RH). eEWNS aerosol particle number concentration was monitored and measured during treatment. Immediately after the exposure, berries were aseptically removed for further analysis to determine *E. coli* inactivation as described in 2.3.8 below.

*2.3.7. Exposure of blackberries to eEWNS for natural microbiota inactivation*

In parallel to inoculation experiments, non-inoculated blackberries were exposed to eEWNS to evaluate natural microbiota inactivation (total viable count and yeast and mold count). Blackberries of the same brand, lot, size (weight) and color, and with no visible damage or mold growth were selected. Three berries were used as treatment berries for each experiment. The treatment berries were placed underneath each electrospray needle and were exposed to eEWNS for 15 and 45 min, in separate experiments (turned once on other side). Three additional blackberries, not exposed to the treatment, served as the comparison to evaluate reductions.

*2.3.8. Recovery of inoculated E. coli*

**From coupons:** For the recovery and estimation of the surviving *E. coli*, the coupons were placed in individual 50 mL centrifuge tubes. 5 mL of PBS was added to each tube containing a coupon. The tubes were then vortexed at medium speed for 30 s on the vortex mixer (VWR Scientific, Radnor PA). The rinsate was serially diluted and plated on Tryptic Soy Agar (TSA) (Becton Dickinson, Franklin Lakes NJ). The plates were placed in a 37 °C stationary incubator and grown overnight before colony counting. The results were expressed as cfu/ml.

**From blackberries:** For the recovery and estimation of the surviving *E. coli,* each blackberry was added to a sterile zipper bag containing 20 mL Maximum Recovery Diluent (MRD) (Oxoid Ltd, Basingstoke, UK) where it was manually rubbed for 2 min. It is worth noting that stomaching could also be used especially in large scale experiments but we believe the developed protocol used in this study is equally scientifically appropriate and the efficacy of similar wash method has been assessed with various fruits and vegetables before (Burnett & Beuchat, 2001). The rinsate was then serially diluted and 1 ml inoculum was pour-plated with Tryptone Bile X-Glucuronide (TBX) Agar (Sigma Aldrich, St. Louis MO). The plates were placed inside a 37 °C stationary incubator and grown overnight. After incubation, typical blue-green colonies were counted and results were expressed as CFU/fruit.

*2.3.9. Enumeration of natural microbiota (total viable count and yeast and mold count)*

Each blackberry, treated or not treated, was put in a sterile zipper bag containing 20 mL of sterile Maximum recovery diluent (MRD) (Oxoid Ltd, Basingstoke, UK) where it was manually rubbed for 2 min. The resulting wash solutions were serially diluted in sterile MRD and pour plated to enumerate the Total viable count (TVC) and the yeast and mold count. Plate count agar (PCA) (Teknova Inc., Hollister CA) was used to assess TVC, incubated at 30 °C for 72 h and Potato Dextrose Agar (PDA) (Hardy Diagnostics, Albany NY) acidified to pH 3.5 with 10% w/v L (+) Tartaric acid (Spectrum Chemical, New Brunswick NJ), incubated at room temperature for 5 days for the yeast and mold count. At the end of incubation, colonies were counted and results were expressed as CFU/fruit.

*2.3.10. Statistical analysis of log reduction for microbial inactivation experiments*

All experiments were performed in triplicates. Log-reductions for coupon inoculation experiments were calculated for each treatment condition (control, eEWNS, EWNS) according to the equation:

$$Log\ Reduction = Log\left(\frac{C(t)}{C(0)}\right) \quad (2)$$

where C (0) is the concentration of the bacteria at time = 0 (i.e. the bacteria recovered from the coupons immediately after drying) and C(t) is the concentration of bacteria recovered after time t of exposure. To account for the natural decay of the bacteria at timepoint *t*, the Log-Reduction was calculated as compared to the control at time point *t* as follows:

$$Log\ Reduction = Log_{10}\left(\frac{C_{Exposed}\ (t)}{C_{Exposed}\ (t) - C_{Control}\ (t)}\right) \quad (3)$$

where, $C_{control}$ (t) is the bacteria concentration of the control coupon at time t while $C_{Exposed}$ (t) is the concentration of the exposed bacteria at time t.

For the blackberry experiments, the average value of the log count and the standard deviation were calculated for each treatment condition. Results were plotted for each treatment condition and the reduction in the concentration of viable microorganisms was calculated in absolute percentages and in log values.

## 3. Results

### 3.1. Characterization of water produced by electrolysis (step 1 in synthesis process)

During the electrolysis process, the voltage across the electrodes gradually decreased. At the beginning of the electrolysis, the voltage was 360 V with the current set to be 0.2 A. The conductivity of the water increased almost five times, from 0.004 mS/cm at the beginning to 0.019 mS/cm after 60 min. The final values of current and voltage were recorded to be 0.2 A and 160 V, respectively. The voltage vs. current curve supports the increase of conductivity during the electrolysis (Fig. S3). The pH remained nearly unchanged (~5.8) as measured by a pH meter.

### 3.2. Physical characterization of EWNS and eEWNS

Fig. 2 summarizes the results for the particle number concentration and charge measurements of the EWNS and eEWNS. The data represent the average of 30 concurrent measurements of number concentration ($N_{SMPS}$, #/cm$^3$) and current ($I_{el}$, fA). As observed from Fig. 2a and b, the "single needle" system generates EWNS and eEWNS aerosols with similar number concentrations, 40755 ± 4042 and 38675 ± 4689 #/cc respectively. Both size distributions are similar to that measured and reported in our earlier work (Pyrgiotakis et al., 2016), with a mean diameter of ~13 nm and a mode diameter of ~14 nm which is indicative that the electrolysis step does not change the particle number concentration and size distribution. The size distribution is log-normal with geometric standard deviation of 1.34 and 1.32 respectively for EWNS and eEWNS. Fig. 2c and d shows the corresponding current profiles for EWNS and eEWNS measured by the aerosol electrometer that are in very similar levels of −741 ± 32 and −681 ± 36 fA ($10^{-15}$ A), respectively. Converting these values to charge shows that the EWNS and eEWNS have similar average charges per particle (14 ± 2 $e^-$ for EWNS vs. 13 ± 2 $e^-$ for eEWNS). These values were not statistically significantly different (p-Value = 0.0577). The total dose rate per minute was also calculated by multiplying the total mass of the particle, as obtained from the SMPS, by the sampling flow rate. The dose rates were 0.328 ng/min, for EWNS and 0.114 ng/min, for eEWNS.

### 3.3. Detection and quantification of ROS in water produced by electrolysis

Using the Trolox method, we quantified the amount of short-lived (fast reacting) ROS species (OH· and $O_2^-$) and $H_2O_2$ in the electrolysis water as a function of time (Fig. 3a). The sum of these two components yields the total ROS concentration. Both the concentrations of short lived ROS species and $H_2O_2$ in the electrolysis water increased over time during the electrolysis ($p < 0.001$). The concentration of short lived ROS reached 51.36 μM at the end of electrolysis. The concentration of $H_2O_2$ followed a similar trend, increasing throughout the electrolysis process, reaching 76.44 μM. The data also indicates that the concentration of both the short lived ROS and $H_2O_2$ increased for the first sample post electrolysis (15 min), reaching the maximum value detected, 155.49 μM $H_2O_2$ equivalent for total ROS, of which 94 μM were $H_2O_2$. The values of the total ROS and $H_2O_2$ maintained steady levels 30 min post treatment. The values stabilized at 128 μM $H_2O_2$ equivalent for total ROS and 57 μM $H_2O_2$ equivalent for $H_2O_2$ concentration.

Fig. 3b indicates the results of the ESR detection of OH· from the Fenton Reaction, which demonstrates the presence of $H_2O_2$. In contrast, there was no OH· detected in the deionized water used in the electrolysis. The concentration of $H_2O_2$ is indicated by the strength of the peak height of the ESR signal. The results indicate that the concentration of OH· increases as the time of electrolysis increased ($p < 0.01$).

### 3.4. Detection and quantification of ROS in EWNS and eEWNS

The total ROS content per particle was calculated by dividing the total ROS (as $H_2O_2$ equivalent from the Trolox method) over the total particle numbers for the measurement time of 5, 10, and 15 min. The result is shown in Fig. 4a. The ROS content remained nearly unchanged during the sampling time of 15 min. Each eEWNS particle has 4.12 ± 0.41 × $10^{-16}$ mol $H_2O_2$ equivalent of ROS, which is more than 3 times that of each EWNS particle (1.21 ± 0.14 × $10^{-16}$ mol $H_2O_2$ equivalent). More interestingly, the $H_2O_2$ concentration, while at extremely low levels in eEWNS, is significantly higher than in EWNS. There is 0.81 ± 0.06 × $10^{-16}$ mol of $H_2O_2$ contained in each eEWNS particle, whereas there is almost no $H_2O_2$ in EWNS detected (0.06 ± 0.09 × $10^{-16}$ mol $H_2O_2$ per particle averaged for the three sampling points).

Fig. 4b indicates that the eEWNS produced a stronger $O_2^-$ signal than the EWNS. The comparison of peak heights for EWNS and eEWNS shows that the concentration of superoxides in eEWNS is doubled that in EWNS ($p = 0.19$). The peak height of $O_2^-$ signal in EWNS was detected to be 36.75 a. u. (±6.71751), whereas the peak height in eEWNS was detected to be 71.75 a. u. (±7.42462). Fig. 4c displays representative ESR spectra from acquisition. Here, the entire spectrum is shown, and it can be seen that the eEWNS signal is stronger indicating more $O_2^-$ generation. The spectra for the Fenton reaction, which indicates OH· generation and for Xanthine/Xanthine Oxidase, which generates $O_2^-$ is also shown.

### 3.5. E.coli inactivation on coupons

Fig. 5 shows the results of the *E. coli* inactivation experiments on coupons. The output concentration of EWNS/eEWNS from each needle was ~40,000 #/cc. The control coupons showed 0.0171 logs/min ($R^2 = 0.92$) decay over the 45 min of treatment time and a total reduction of 0.74 logs. The EWNS treatment produced 0.0519 logs/min ($R^2 = 0.91$) inactivation rate. For the 45 min timepoint, the observed inactivation compared to control was 1.8 logs ($p < 0.001$). For the eEWNS, the results show a significant increase in their

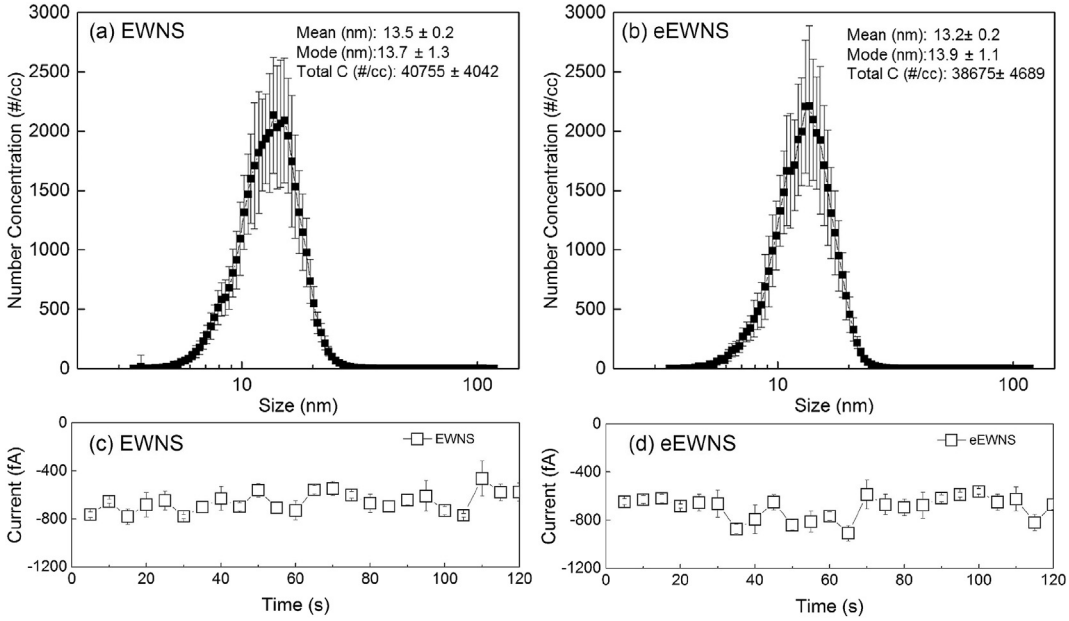


**Fig. 2.** Particle number concentration as a function of size measured by SMPS: **(a)** EWNS; **(b)** eEWNS; a typical current profile for EWNS **(c)** and eEWNS **(d)** measured by the aerosol electrometer to determine the particle charge.

inactivation potency. The overall rate of inactivation was 0.097 logs/min ($R^2 = 0.91$). For 45 min, the lowest detection limit of the method was reached. The inactivation at 45 min was 4 logs compared to controls ($p < 0.001$).

### 3.6. Treatment of blackberries with eEWNS

The results of the *E. coli* inoculation on blackberries are shown in Fig. 6a. For the control, blackberries not treated with eEWNS, the recovery was 654 cfu/fruit (Standard Deviation ±368.90) for 15 min and 348 cfu/fruit (Standard Deviation ± 364.30) for 45 min. For the eEWNS treated Blackberries, the 15 min timepoint showed 93% (1.1 logs) inactivation. While there was complete inactivation (2.5 logs) for the 45 min timepoint.

Fig. 6b and c shows the results of the natural microbiota treatment experiments. Fresh blackberries not exposed to eEWNS had a mean Total viable count of $3.0 \times 10^4$ cfu/fruit and a mean yeast and mold count of $6.1 \times 10^4$ cfu/fruit. The inactivation efficiency following a 15-min treatment of fruit to eEWNS was 62% (0.42 log) for the TVC and 93.6% (1.19 log) for yeast and molds. At 45 min of exposure inactivation increased reaching 97% (1.5 log) for TVC and 99% (1.99 log) for yeast and molds. It is worth noting that in this limited antimicrobial assessment performed in this study, no visual effects (i.e. color, texture) on exposed blackberries were observed (data not shown). More comprehensive organoleptic studies should be performed in future studies.

## 4. Discussion

In this study, a modification to the EWNS generation and treatment paradigm was implemented. The EWNS were modified by integrating an additional electrolysis step in their synthesis. Indeed, the resulting eEWNS from the integrated electrolysis-electrospray-ionization process, as proven by inactivation studies of coupons inoculated with *E. coli*, more than doubled the antimicrobial potency, reaching almost 4 log reduction in 45 min of exposure as compared to 1.9 log reduction for the same length of treatment with EWNS. These results clearly show that the electrolysis step doubled the inactivation as evidenced also by the increase in the total ROS content of eEWNS.

Also, in this publication we experimented and presented for the first time a completely new concept which has to do with the use of a new EWNS platform to deliver, in a targeted and precise manner, “active ingredients” in aqueous suspension. In this case, the active ingredient was ROS generated by electrolysis of de-ionized water. The EWNS particles and the enhanced EWNS (called eEWNS particles) resulting from this new approach were compared to each other in terms of ROS content and in inactivation experiments by an exposure setup where they were directly “sprayed onto the fruit/surface”. It is important to mention that, our earlier publication regarding Optimization of EWNS referred to optimization of the synthesis operational parameters (electric field, voltage, air flows etc) to increase EWNS ROS potential while the exposure of the inoculated surfaces took place using an entirely different exposure system, a draw thorough approach where the EWNS aerosol and particles were deposited to surface/fruits using an Electrostatic Precipitation System (EPES). This EPES methodology, although effective in bringing about 3.8 log reductions in *E. coli*, has certain drawbacks, such as the need for a separate exposure chamber with energy intensive second electric field for deposition of these particles. In this study, we did not compare those earlier results as they were primarily a function of the treatment methodology and not the antimicrobial content of the EWNS.

It is worth noting that in our earlier work with the EWNS, ROS have been identified as the major antimicrobial species, that cause the destruction of the outer microbial cell envelope (Pyrgiotakis et al., 2015). Herein, two methods of ROS identification and quantification were employed to further understand the different chemistry of both the water produced by electrolysis and the resulting eEWNS, as compared to EWNS. As shown, the electrolysis step results in the generation of traces of $H_2O_2$ (94 μM Equivalent), as confirmed by both Trolox and ESR methods (Fig. 3a and b). This is likely part of the reason why in eEWNS, traces of $H_2O_2$ were also detected (accounting for 20 ± 3% of the total ROS). It is worth noting that no $H_2O_2$ levels were detected on EWNS in the absence of electrolysis step. Additionally, the ESR characterization

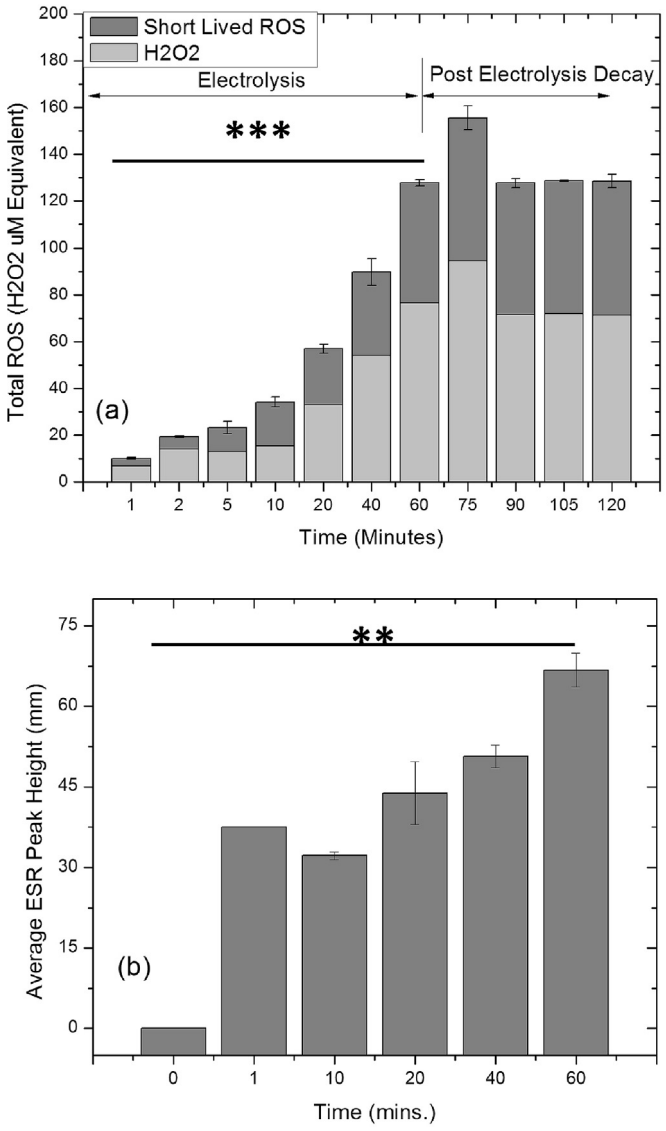


**Fig. 3.** Detection of ROS in Water undergoing electrolysis through **(a)** Trolox Method and **(b)** ESR.

experiments also confirmed our previously reported data (Pyrgiotakis et al., 2016) that OH· and $O_2^-$, other than $H_2O_2$, are the major components of ROS in the original EWNS, as no $H_2O_2$ was detected using the Trolox method (Fig. 4a). In summary, the eEWNS have 3 times more total ROS as compared to EWNS (4.12 vs. $1.21 \times 10^{-16}$ mol $H_2O_2$ equivalent per particle). The dose rate of the eEWNS aerosol was 114 pg per minute, further emphasizing the "Dry" nature of this treatment.

In addition to the comparative experiments using *E. coli* inoculated on coupons mentioned above, the inactivation of *E. coli* inoculated onto blackberries showed 2.5 log reduction in 45 min. Furthermore, fresh non-inoculated blackberries showed a promising reduction of TVC by a 1.5 log and of the yeast and Mold count by 2 logs, after 45 min of treatment. Other researchers have found 0.9 log reduction of Total Viable Counts after gamma irradiation of raspberries at 1 Kilogray (Verde et al., 2013). These are promising preliminary pilot data and once our technology is scaled up we will pursue similar studies in greater depth. It is worth noting that the observed variability as shown in the standard deviations in the assessment of antimicrobial activity, is due to the limited number of berries used (6 berries) per the developed protocol. In the near

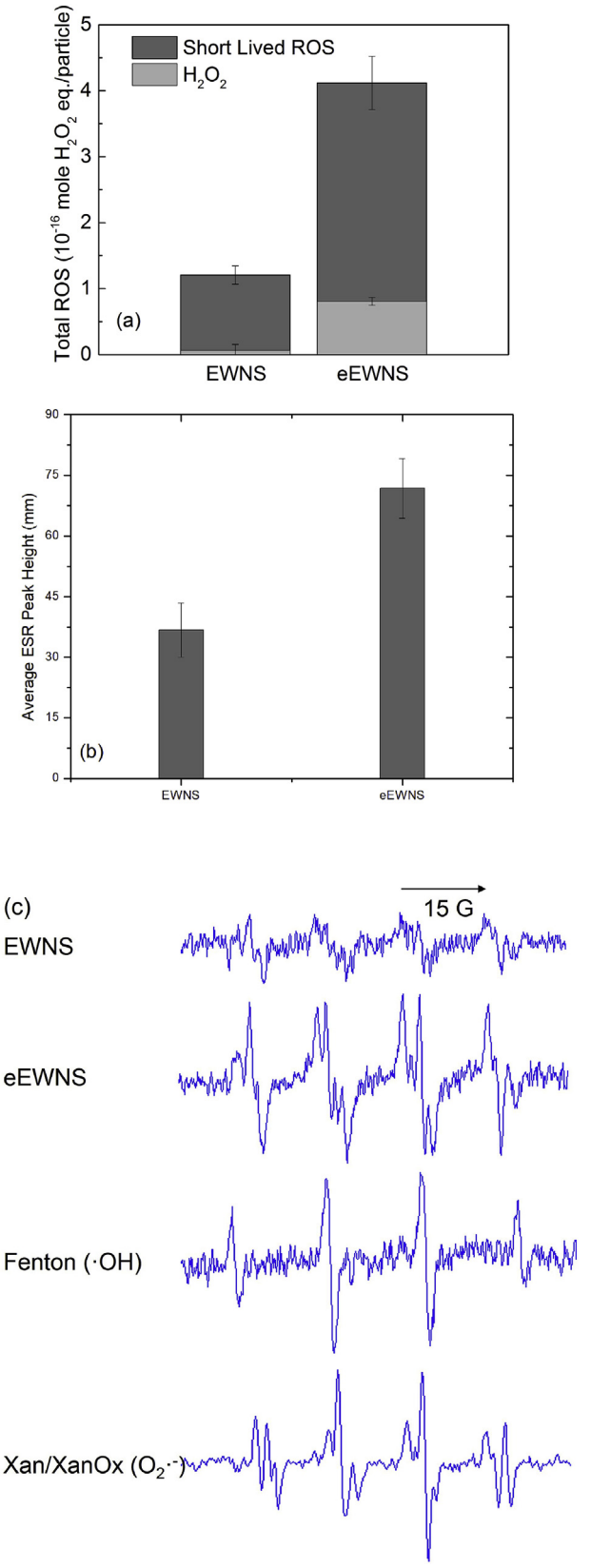


**Fig. 4.** Detection and quantification of ROS in eEWNS and their comparison with EWNS **(a)** Trolox Method, **(b)** ESR peak height values (as an index of abundance) for Superoxide detection and **(c)** ESR spectra showing the comparison of eEWNS and EWNS.

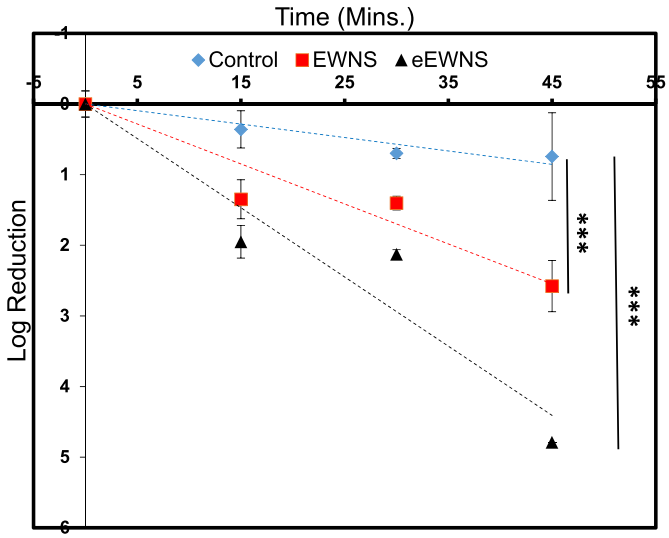


**Fig. 5.** Inactivation of *E. coli* on coupons after exposure to eEWNS and comparison with EWNS. The error bars represent the standard deviation of means. Trendlines represent the best linear fit.

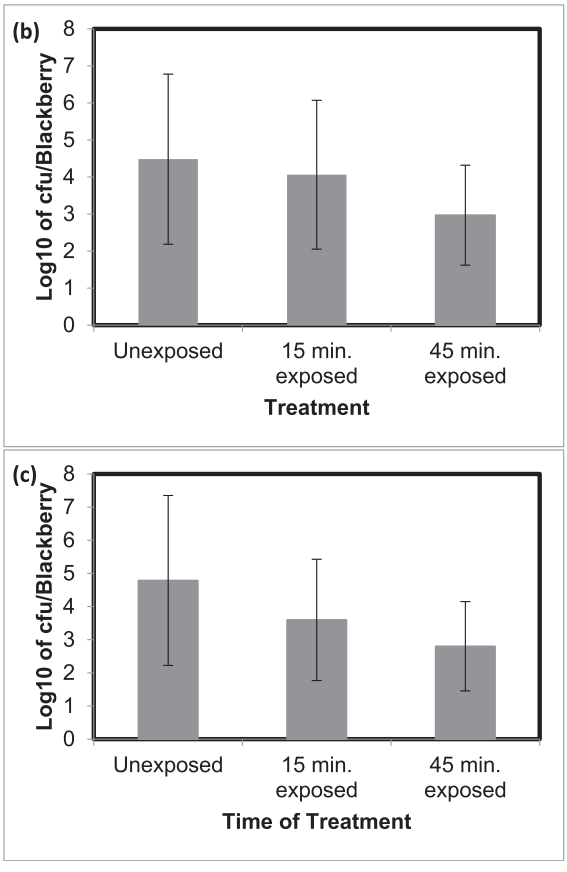


**Fig. 6.** Treatment of Blackberries with eEWNS: **(a)** Inactivation of *E. coli* inoculated on blackberries. The results represent control: inoculated untreated (light grey bars) and eEWNS treated (dark grey bars); **(b)** Inactivation of Total Viable Microorganisms; **(c)** Inactivation of Yeasts and Molds. Error bars represent standard deviation of means.

future, scale up of the EWNS generation system will be performed and a larger scale antimicrobial study using greater number of berries and other pathogens will be performed. Such study will be reported in a companion manuscript.

To avoid confusion, it is worth mentioning that the eEWNS method presented here has nothing to do with Electrolyzed Water (EW), which has been known to have bactericidal effects against many pathogenic bacteria (Issa-Zacharia, Kamitani, Morita, & Iwasaki, 2010; Kim, Hung, & Brackett, 2000; Park et al., 2001). In the production of electrolyzed water, sodium chloride (NaCl) is usually added to produce chlorine-based radicals (Kiura et al., 2002) which however, are not suitable for organic produce as they can leave behind chemical traces. EW is also a wet disinfection approach which consists of dipping and spraying EW onto food surfaces of interest. This has two major disadvantages: it requires large volumes of EW that increases the cost of the technology and also deems it unsuitable for sensitive produce that cannot undergo a wet treatment (e.g. berries). In the approach presented here, no additive or electrolytes were added in the de-ionized water during the electrolysis step of the EWNS particle synthesis, maintaining the green profile of the technology. The EWNS method on the other hand, due to the a few hundred picogram amounts of water being targeted to the surface of interest is a very gentle, "dry" disinfection method that leaves no chemical traces in the exposed product.

## 5. Conclusion

The presented integrated electrolysis - electrospray and ionization method for the synthesis of eEWNS is suitable for a variety of food safety applications. Such an organic, chemical free, dry method can be ideal for disinfection treatment of delicate fresh and organic produce such as berries.

The microbial inactivation results found in these studies are promising, given that the platform is a lab-based one and not yet scaled up. Future planned upscaling of the technology to a multi needle; high EWNS concentration platform will enable implementation at various critical control points (CCPs) across the farm to the fork chain.

## Disclaimer

The findings and conclusions in this report are those of the author(s) and do not necessarily represent the views of the National Institute for Occupational Safety and Health.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.foodcont.2017.09.034.

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