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NEUTRAL ELECTROLYSED OXIDIZING WATER AS ANTI-BIOFOULING ALTERNATIVE TO TRADITIONAL CHLORINE

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ABSTRACT

Biofouling is the undesirable accumulation of microorganisms in solid wet surfaces, constituting a problem for industry. This undesirable accumulation of microorganisms on equipment surfaces and pipes reduces heat transfer efficiency and increases maintenance and operational costs. In this work sodium hypochlorite (SH) and neutral electrolyzed oxidizing water (NEOW) were tested on the control of planktonic and sessile *Escherichia coli* cells. The free chlorine stability of disinfectants was also assessed. The most efficient disinfectant in the control of planktonic and sessile cells was NEOW. Furthermore, to have the same antimicrobial activity of NEOW, SH has to be used in a 13 times higher concentration. Moreover, concerning the free chlorine content, NEOW was more stable than SH. In conclusion, NEOW is an effective chlorine-based disinfectant for the removal of both planktonic and sessile cells and is also more stable than the traditional SH.

INTRODUCTION

Microbiological fouling in cooling systems (biofouling) is the result of an abundant growth of bacteria, algae and fungi on solid wet surfaces. This microbial accumulation leads to the formation of biofilms. If uncontrolled, biofouling adversely affects equipment energy performance and promotes metal corrosion (Bott 2011a). These problems can be controlled through proper biomonitoring and application of appropriate antimicrobials (Sriyutha Murthy et al. 2005). SH is the most common antimicrobial agent used in cooling systems to disinfect water and sanitize surfaces, though it produces unhealthy and toxic by-products (Bott 2011b, Harms and O'Brien 2010). It is usually produced by the mixture of chloramine gas and water (Hahn and Weber 2014) or the absorption of gaseous chlorine in sodium hydroxide solutions (Fukuzaki 2006), but it can also be produced electrochemically from seawater (Saleem 2011). SH is very effective, but its bactericidal activity is reduced in the presence of organic matter. Furthermore, it can lead to the formation of organochlorinated compounds, some of them being potentially carcinogenic (Hahn and Weber 2014, Meireles et al. 2015). In aqueous solution, chlorine exists in three forms: hypochlorous acid (HOCl), the species responsible for the germicidal action, that dissociates into H^+ and hypochlorite ion (^-OCl), also responsible for the cleaning efficacy; and aqueous Cl_2 that can be released to the atmosphere (Fukuzaki 2006).

NEOW has been lately used as antimicrobial agent in different applications, and is generally recognized as safe and very effective. This solution is formed by electrodialysis of a sodium chloride solution in an electrolysis chamber with an anode and a cathode separated by a membrane (Cheng et al. 2012, Demirci and Bialka 2010). The reaction forms an acid and an alkaline solution (Hricova et al. 2008, Ongeng et al. 2006). The acid solution is formed at the anode and it comprises HCl, HOCl, Cl_2, ^-OCl, and O_2. The alkaline solution is formed at the cathode by hydroxyl ions that can form sodium hydroxide (Cheng et al. 2012, Hricova et al. 2008). NEOW is formed by the mixture of these two solutions (Cheng et al. 2012). The main advantages of using NEOW are related with its on-site (on demand) production and low economic cost (the raw material is an aqueous solution of NaCl), which make it a feasible antimicrobial treatment. Moreover, the formation of unwanted by-products is minimized when compared to SH, being also less corrosive when in contact with metal surfaces (Demirci and Bialka 2010). In this work, SH and NEOW were tested in the control of planktonic and sessile *E. coli*. The free chlorine stability of disinfectants was also assessed.

MATERIAL AND METHODS

Microorganism and Culture Conditions

E. coli CECT 434 was the selected microorganism. The bacterium was obtained from overnight cultures grown in 100 mL flasks with 25 mL of Mueller–Hinton broth (MHB) (Merck, Germany), incubated at 30 ºC and 120 rpm (CERTOMAT® BS-1, Sartorius AG, Germany).

Disinfectants

The equipment (ECAse) used to produce NEOW was provided by Loehrke (Germany). A solution of sterilized saline (2.5 g/L) was used to produce NEOW. SH 13% (w/w) was obtained from Acros Organics (Belgium).

Planktonic Tests

An overnight-grown culture of *E. coli* was centrifuged (4000 g, 15 min) and washed one time with saline solution (8.5 g/L NaCl). Afterwards, bacteria were ressuspended in

saline solution to obtain an OD_{600} (optical density at 600 nm) of 0.1±0.02 corresponding to 3.9×10^7 CFU (colony forming units)/mL. These cells were centrifuged again (4000 g, 15 min) and the saline solution was replaced by NEOW and SH in the concentrations tested (5 and 20 ppm of free chlorine). When required NEOW and SH were diluted in saline solution. After 20 minutes of exposure time the necessary dilutions were performed to determine the number of CFU using the motion drop method in plate count agar (PCA, Merck, Germany) plates (Reed and Reed 1948). The plates were incubated overnight, at 30 ^{0}C. Before plating, a antimicrobial neutralization step was performed by dilution to sub-inhibitory concentrations (Johnston et al. 2002).

Biofilm Tests

Biofilms were formed on coupons of stainless steel (SS, AISI 316) and polystyrene (PS) (dimensions of 1.0×0.9×0.1 cm). The coupons were placed in 48-wells flat-bottomed PS tissue culture plates (Thermo Fisher Scientific, Korea) using a total volume of 1000 µL with an initial number of cells of 3.9×10^7 CFU/mL. The plates were incubated for 24 hours for biofilm formation, at 30 ºC and 120 rpm (CERTOMAT® BS-1, Sartorius AG, Germany). After the incubation period, the medium was removed and replaced by the disinfectant solution (NEOW and SH at 5 and 20 ppm) for 20 min, at 30 ºC and 120 rpm (CERTOMAT® BS-1, Sartorius AG, Germany). After the disinfectant exposure, the coupons were placed in 5 mL of saline solution, the cells were removed by vigorously vortex and the neutralisation step was performed by dilution to sub-inhibitory concentrations (Johnston et al. 2002). The necessary dilutions were performed to determine the number of CFU using the motion drop method in PCA (Merck, Germany) plates (Reed and Reed 1948) after overnight incubation at 30 ^{0}C.

Antimicrobial Susceptibility

These tests were performed with *E. coli* obtained from overnight cultures in 96-wells flat-bottomed PS tissue culture plates (Orange Scientific, USA) using a total volume of 200 µL. The initial OD_{600} was 0.04, corresponding to 2.8×10^7 CFU/mL. After 24 h the broth was removed and the wells were washed with saline solution. A new saline solution (180 µL) was mixed with the disinfectants (SH 260 ppm and NEOW 20 ppm) in the 96-wells plate. After 20 min of contact time, the disinfectant was removed, and the wells were washed. Finally, the wells were scraped with a metal scalpel and the necessary dilutions were performed to determine the number of CFU using the motion drop method in PCA (Merck, Germany) plates (Reed and Reed 1948), after overnight incubation at 30 ^{0}C.

Free Chlorine Measurement

The stability of the solutions was evaluated one month after being opened with a free chlorine portable meter (Hanna Instruments) at 25 ^{0}C.

Statistical Analysis

The results were analysed using paired samples *t*-test from the statistical software SPSS 22.0 (SPSS Inc., Chicago, IL, USA). Statistical calculations were based on a confidence level of ≥ 95% ($P<0.05$ was considered statistically significant).

RESULTS AND DISCUSSION

In this work, *E. coli* biofilms and planktonic cultures were developed and the antimicrobial action of SH and NEOW at 5 and 20 ppm was compared. The free chlorine stability of both disinfectants was also assessed.

The planktonic tests (Fig. 1) showed that NEOW and SH at 5 ppm had a very similar activity ($P>0.05$), reducing 2.15 and 2.10 log CFU/mL, for NEOW and SH, respectively. When applying higher concentrations of the disinfectants (20 ppm), the results obtained demonstrated that NEOW was more efficient than SH ($P<0.05$). NEOW 20 ppm completely eliminated planktonic *E. coli* (7.88 log CFU/mL reduction), while SH 20 ppm only reduced 2.68 log CFU/mL. The antimicrobial action of NEOW could be explained by the additional secondary species formed in its production, such as HCl and NaOH (Cheng et al. 2012).

When testing the disinfectants in biofilms formed on SS and PS surfaces the results showed a similar tendency (Fig. 2). At 5 ppm, both disinfectants caused similar log CFU/cm^2 reduction (less than 1.00 log CFU/cm^2) ($P>0.05$). NEOW was more efficient for higher concentrations (20 ppm) on both surfaces (2.20 and 1.73 log CFU/cm^2 reduction for PS and SS, respectively) ($P<0.05$).

On the other hand, SH showed a weaker antimicrobial activity. In fact, with the increase of SH concentration (20 ppm) the CFU reduction achieved was not proportional to the NEOW action at the same concentration ($P<0.05$). The higher concentration (20 ppm) only caused additional 0.77 and 0.45 log CFU/cm^2 reductions on PS and SS surfaces, respectively ($P<0.05$). Furthermore, biofilm formation was higher for PS surfaces ($P<0.05$), as already demonstrated by Meireles et al. (2015).

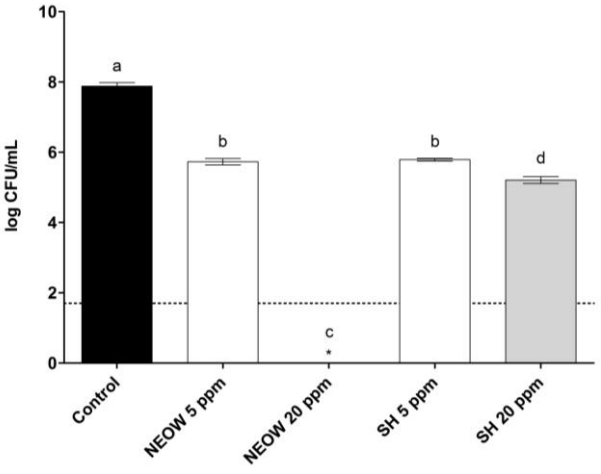

Fig. 1 Log CFU/mL reduction achieved after the application of NEOW and SH at 5 and 20 ppm against planktonic *E. coli*. The line indicates the detection limit of the method (1.70 log CFU/mL). * indicates that no CFU was detected. Different letters represent statistically different values ($P<0.05$).

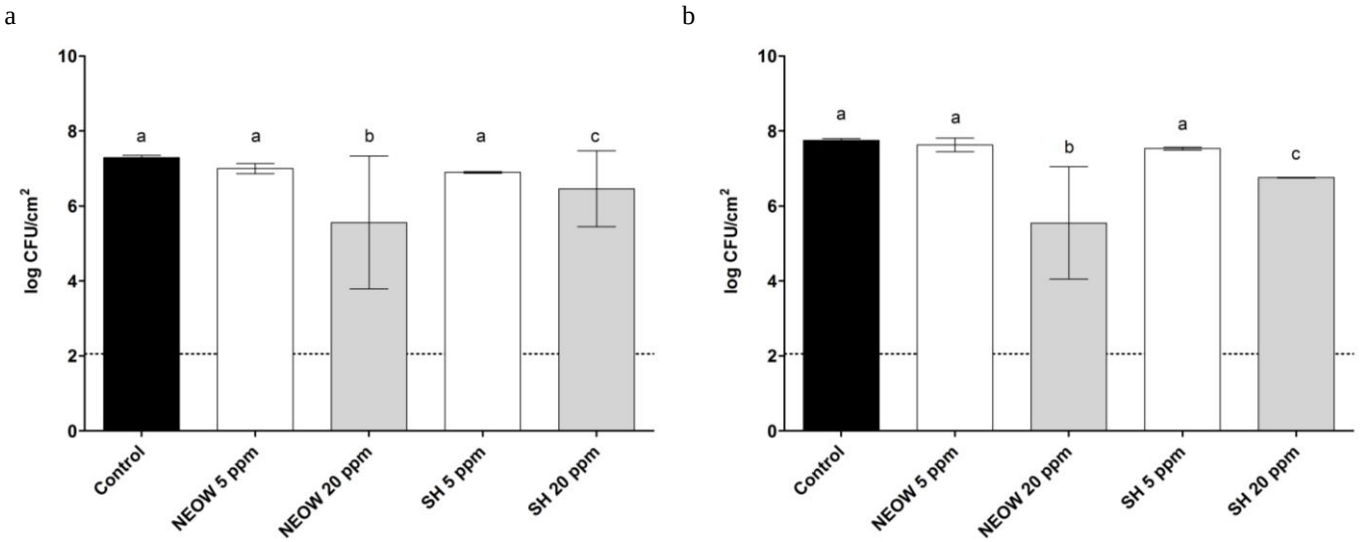

Fig. 2 Log CFU/cm^2 reduction achieved after the application of NEOW and SH at 5 and 20 ppm against *E.coli* biofilms on SS (a) and PS (b) surfaces. The line indicates the detection limit of the method (2.06 log CFU/cm^2). Different letters represent statistically different values within each surface ($P<0.05$).

In what concerns the antimicrobial susceptibility (Table 1), the results demonstrate that it is necessary a 13 times higher concentration of SH to obtain the same (approximately) log CFU/cm^2 reduction (≈ 3 log CFU/cm^2). This reinforces the previous results, i.e. higher SH concentrations are required to promote similar CFU reduction to NEOW. Previous works also demonstrate this antimicrobial potential of NEOW. Abadias et al. (2008) concluded that using NEOW with 50 ppm of free chlorine was equally effective as using 120 ppm of chlorine solution. Rico et al. (2008) showed that 60 ppm of NEOW were equivalent to 120 ppm of SH. The present study also shows that, even if chlorine-based products are recognized as good disinfectants, effective biofilm control requires the use of significantly higher disinfectant concentrations than those effective against planktonic cells.

Table 1. Log CFU/cm^2 reduction of *E. coli* using NEOW at 20 ppm and SH at 260 ppm.

Disinfectant	Log CFU/cm^2 reduction
NEOW 20 ppm	3.1
SH 260 ppm	3.6

In terms of stability, the values of free chlorine in the SH solution were never constant/stable, ranging from upper to lower values. The free chlorine values in NEOW were maintained in the first days and decreased over time. The initial free chlorine content was equal for the two solutions (100 ppm) and after 30 days of opening, both solutions had 60 ppm of free chlorine content. In conclusion, NEOW demonstrated to be a promising disinfectant for the control of both planktonic and sessile cells and it is also more stable than SH.

NEOW has potential to be used in heat exchangers as surface cleaning and disinfecting agent. However, further studies are needed to validate this *in vitro* data with NEOW *in-situ* application, particularly in terms of surface corrosion.

CONCLUSIONS

1. Industrial biofilm formation on equipment and pipes surfaces is a major challenge on the development of effective cleaning and disinfection procedures.
2. This study demonstrates that NEOW (20 ppm) was more efficient in controlling planktonic and sessile *E. coli* cells than SH.
3. It was found that SH has to be used in a concentration 13 times higher than NEOW to have the same antimicrobial activity.
4. The overall results propose that NEOW is a more stable and efficient disinfectant than SH to be used as surface disinfectant.

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NOMENCLATURE

CFU Colony forming units
NEOW Neutral electrolyzed oxidizing water
OD Optical density
PCA Plate count agar

PS Polystyrene
SH Sodium hypochlorite
SS Stainless steel

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