Abstract

The reduced susceptibility of biofilm to disinfectants presents a challenge to the successful reprocessing of medical equipment. This study examined the effect of residual biomass remaining after previous PAA disinfection on tolerance of subsequent mature *P. aeruginosa* biofilms to PAA. The effect of enzymatic degradation of specific components of *P. aeruginosa* biofilm EPS on the effectiveness of PAA disinfection was also evaluated.

The presence of residual biomass from previously disinfected biofilms significantly enhanced the tolerance of a subsequent biofilms. A 96hr old "secondary biofilm" formed on disinfected biomass survived PAA concentrations of 4000 ppm, which exceeds the concentrations used in practice for high-level disinfection.

These observations indicate that, under certain circumstances recolonisation of residual EPS can cause failure of disinfection of medical equipment such as endoscopes and emphasises the importance of cleaning of endoscopes prior to disinfection.

Keywords: word; biofilm; high-level disinfection; endoscope; extracellular polymeric substance; residual biomass; peracetic acid

Introduction

Various studies have demonstrated biofilm formation in endoscope lumens (Pajkos et al. 2004; Ren-Pei et al. 2014; Johani et al. 2018), an aggregation of bacterial cells embedded in a self-produced extracellular polymeric substance (EPS) (Blackledge et al. 2013). This phenotypic characteristic has been suggested as a cause of high-level disinfection failure for reprocessed endoscopes (Bajolet et al. 2013; Kovaleva et al. 2013; McCafferty et al. 2018). Our work has shown that biofilms are tolerant to disinfectants, including peracetic acid (PAA), an agent used in high-level disinfection of heat sensitive semi-critical medical equipment (Akinbobola et al. 2017). Endoscopes have a small margin of safety owing to their complex narrow channels as well as contamination with pathogenic microorganism and soils from patients after use (Cowen, 2001; Edmiston and Spencer, 2014). Also endoscope reprocessing entails multiple steps, thus, any slight deviation from their standard reprocessing procedures may result in reprocessing failure and subsequently infection transmission between patients

An important mechanism of biofilm tolerance to disinfectants is thought to involve the ability of biofilm EPS to limit the penetration of compounds into biofilm (Mah and O'Toole 2001; Bridier, Briandet, et al. 2011). This phenomenon results either in non-exposure or exposure to a sub-lethal concentration of cells embedded in biofilm EPS (Bridier, Dubois-Brissonnet, et al. 2011). Several studies have demonstrated this phenomenon in disinfectants (Stewart et al. 2001; Grobe et al. 2002). As disinfectants tend to react more with the organic materials present in biofilm EPS, there may be a more significant reduction in activity of disinfectants when compared to less reactive agents such as antibiotics (Lambert and Johnston 2001; Stewart PS 2015). In addition, unlike antibiotics, disinfectants are intended to act within a relatively short time period so any delay in penetration into biofilm may compound the effect by reducing contact time. Biofilm resistance may also be due to enhanced efflux pump

activity and reduced metabolic activity (Donlan 2000; Mah and O'Toole 2001; Stewart P et al. 2001), but these mechanisms are probably less significant in biofilm tolerance to high-level disinfectants. Thus, biofilm EPS, which constitutes more than 90% of the total biomass of biofilms, is the most important factor responsible for reduced biofilm susceptibility to disinfectants (Flemming and Wingender 2010; Marshall et al. 2014).

Tote et al. (2010) demonstrated the inability of a wide range of disinfectants to significantly reduce the biomass of *Staphylococcus aureus* and *Pseudomonas aeruginosa* biofilms at concentrations which significantly reduce the viability of the biofilm. Such residual disinfected biofilm biomass may have significant impact on the tolerance of subsequent, secondary biofilms which recolonise the residual biomass left by the disinfected biofilm. The impact of this residual biofilm biomass on the tolerance of biofilm to high-level disinfection has not been previously studied. Understanding the implication of such residual biomass for high-level disinfection is particularly important when considering endoscopes, which owing to their complex design and narrow channels present difficulties for the physical removal of biomass. Oxidizing agents such as PAA are now being increasingly used as an alternative to aldehyde-based disinfectants in the high-level disinfection of endoscopes (Vizcaino-Alcaide et al. 2003; Spinzi et al. 2008; Soares et al. 2011; Chenjiao et al. 2016). Oxidizing agents, despite their high antimicrobial activity, are also very reactive. Therefore, their activity can be significantly reduced by reaction with organic substances, such as the components of biofilm EPS (Jaglic et al. 2012; Kampf et al. 2014). The influence of PAA disinfected residual biomass on the PAA tolerance of biofilm recolonising residual biomass was examined in this study.

We have previously demonstrated that increase in biofilm biomass with increase in biofilm age cannot alone explain the increase in tolerance of *P. aeruginosa* biofilm to PAA

(Akinbobola et al. 2017). This suggests that the composition of biofilm EPS may also be a significant factor in biofilm resistance to disinfection. Extracellular DNA (eDNA), protein and polysaccharides have all been identified as common components of biofilm EPS, although their relative abundance varies depending on the organism and their growth conditions (Flemming and Wingender 2010; De Melo et al. 2013; Reichhardt and Cegelski 2014). Xue et al. (2014) demonstrated that monochloramine is less effective in penetrating and killing protein based biofilm of *P. putida* compared to a polysaccharide based biofilm of *P. aeruginosa*, due to its higher affinity to react with protein compared to polysaccharides. This indicates that resistance of a biofilm to disinfectants will depend not only on the biomass present, but also its composition and the chemical nature of the disinfectant. Therefore, understanding the effect of selective degradation of components of biofilm EPS could aid in the enhancement of pre-disinfection processing of endoscope, to further enhance the disinfection processes and to mitigate the adverse effects of any residual biomass. Results presented here showthat selective enzymatic degradation of the eDNA, protein and alginate components of the EPS of *P. aeruginosa* biofilm has different effect on its biomass and susceptibility to PAA.

Materials and Method

Inoculum preparation: for each experiment, fresh overnight cultures of *P. aeruginosa* PA14 were prepared by inoculating a single colony of the isolates in 10 mL of sterile Luria-Bertani(LB) broth (Fluka, St. Louis USA), which was incubated overnight at 37ºC. Cells were recovered from the overnight culture through the centrifugation of 1 mL of the overnight culture at 10,000 x g for 5 min. The resulting pellets were re-suspended in 1 mL phosphate buffered saline (PBS) and washed twice by centrifugation at 10,000 x g for 5 min.

The colony forming unit (CFU) of the inoculum for each experiment was standardised based on their absorbance 570nm.

Biofilm formation: biofilms were prepared in 24-well plates (CellBIND Clear polystyrene Corning). Briefly, *P. aeruginosa* PA14 cells were recovered from overnight culture as highlighted above. Adjusted cultures were serially diluted in LB broth to obtain a final bacterial cell concentration of $\approx 1 \times 10^6$ CFU/mL. Wells of the 24-well plate were inoculated with 1 mL of diluted inoculum for biofilm formation. Plates were incubated at 37ºC for 96 hours with spent media aseptically replaced every 24 hours.

Characterisation of PAA effect on biofilm: to elucidate the impact of residual biofilm biomass on the tolerance of biofilm grown on disinfected biofilm to PAA, the total biomass, protein and polysaccharide component of PAA treated biofilm compared to untreated control biofilm was investigated. For the effect of PAA treatment on biofilm biomass, the residual biomass of 96 hr old *P. aeruginosa* biofilm killed with 3500 ppm of PAA was compared to an untreated control using the crystal violet assay. Crystal violet quantification of biofilm biomass according to O'Toole (2011) with slight modification was used to quantify the biomass of treated biomass and control. Briefly, treated biofilms were washed with sterile distilled water and stained with 0.1% w/v crystal violet for 30 min. The crystal violet stain was removed, and stained biofilm biomass washed with sterile distilled water twice to remove excess stain. Stain binding to the biofilm biomass was dissolved in 95% v/v ethanol for 30 min. The absorbance of dissolved stain was quantified at 570nm in 96-well plate reader (Tecan Infinite F200 PRO Switzerland). The effect of PAA on polysaccharide and protein component of the EPS of PAA treated biofilm and a control was quantified using the phenol-sulphuric acid or Bradford assay respectively (Masuko et al. 2005; Chiba et al. 2015). For the phenol-sulphuric assay, 50 µL of EPS extracted from biofilm using 1M NaCl as

described by Chiba et al. (2015) was added to 150 µL of concentrated sulphuric acid in a 96 well microplate. Subsequently, 30 µL of 5% phenol was added to the mixture and incubated for 5 min at 90^0C in a water bath. The absorbance of the mixture at 490nm was estimated in a 96-well plate reader (Tecan Infinite F200 PRO, Switzerland) after they were allowed to cool to room temperature. The µg/mL of the polysaccharide component of the extracted EPS was estimated based on the standard curve generated using known concentrations of glucose (Sigma St. Louise USA). The protein component of the extracted EPS was estimated by adding equal volume (50 µL) of the extracted EPS and Bradford reagent (sigma St. Louise, USA). The absorbance of 100 µL of the mixture at 595 nm was estimated in flat bottom 96-well plate using a 96 well plate reader (Tecan Infinite F200 PRO, Switzerland) after the mixture was allowed to react for 10 minutes at room temperature. The µg/L of the protein component of the extracted EPS was likewise estimated based on a standard curve initially generated using known concentrations of Bovine serum albumin (BSA) (sigma St. Louise USA).

Evaluation of the impact of disinfected biofilm biomass on biofilm susceptibility to PAA: to evaluate the impact of biomass of PAA killed biofilm on PAA susceptibility of re-colonising biofilm, 96 hr old biofilm grown in 24-well plates as explained above was killed by treatment with 3500 ppm of PAA for 5 min before neutralisation with the addition of PAA neutralisation mixture (1.0% w/v sodium thiosulphate, 0.5% w/v sodium dodecyl sulphate, 1.0% w/v granular lecithin, 15% w/v of Polysorbate 80 and 0.1% w/v L-histidine), which has been previously shown to be effective in quenching the activity of PAA and not toxic to bacteria cells (Akinbobola et al. 2017). The residual biomass of the disinfected biofilm was re-inoculated with 1 mL 10^6 CFU/mL of *P. aeruginosa* and biofilm allowed to regrow for 24, 48 or 96 hours. The control biofilm for this experiment was grown in the same way in fresh wells of a 24-well plate. The biofilms were subsequently treated with different

concentrations of PAA for 5 min and neutralised as described above. The neutraliser was removed, and biofilms were scraped into PBS (Bridier et al. 2012) and sonicated at 37Hz for 5 min prior to serial dilution and inoculation onto LB agar in triplicate using the Miles and Misra drop plate method(Miles et al. 1938). Inoculated plates were incubated at 37ºC for 24 hours and the CFU of the surviving bacterial population was subsequently calculated.

Visualisation of the distribution of viable and dead cells in re-inoculated PAA killed biofilm biomass: *P. aeruginosa* biofilms (96 hr old) grown in 6 well plates were killed with 3500 ppm of PAA, as earlier explained, and re-colonised with *P. aeruginosa* cells for 24 hours before being stained and viewed by confocal microscope. Sterile LB broth rather than LB culture of *P. aeruginosa* planktonic cells was added to the control biofilm used for this study. The prepared biofilms were stained with Syto9/propidium iodide Live/Dead viability kit (Invitrogen, Paisley UK) for 15 min at room temperature in the dark. Stained biofilms were subsequently fixed with 4% w/v paraformaldehyde and 5% w/v LB broth for 20 min before they were viewed using the x40 objective of a Lecia SP5 confocal microscope (Wetzlar, Germany) with x5 magnification.

Evaluation of enzyme effect on biofilm biomass:DNase I (Sigma St. Louis USA) was dissolved in 0.15M sodium chloride supplemented with 50 Mm magnesium chloride to form a stock solution of 2 mg/mL. Alginate lyase (Sigma St. Louis USA) and proteinase K (Sigma St. Louis USA) were dissolved in sterile distilled water to also form a stock solution of 2 mg/mL. The stock solutions were sterilised through filtration with a 0.2 µm pore size filter and subsequently diluted in LB broth to required concentrations. The effect of a range of concentrations of the different enzymes used in this study on biofilm biomass was evaluated using crystal violet quantification of biofilm biomass. For this, 96 hr old biofilms formed in 24-well plates were washed with sterile distilled water after the removal of the growth media,

various concentrations of each enzyme or solvent (diluted in LB) were subsequently added to biofilm and incubated at 37ºC for 2 hours. Biofilm biomass was quantified from each treatment, as described above.

***Impact of biofilm EPS components enzymatic degradation on biofilm susceptibility to peracetic acid*:** the effect of enzyme pre-treatment on biofilm susceptibility to PAA was evaluated using the Miles and Misra drop plate technique (Miles et al. 1938). Biofilms were treated with pre-determined concentrations of enzymes as described above. Enzyme treated biofilms and an untreated control were exposed to either 2000 ppm of PAA or sterile distilled water for 5 min before neutralisation as earlier described. The neutraliser was removed and biofilms were scraped into PBS and sonicated at 37Hz for 5 min prior to serial dilution and inoculation onto LB agar in triplicate (Bridier et al. 2012). Inoculated plates were incubated at 37ºC for 24 hours and the CFU of the surviving bacterial population was subsequently calculated.

Statistical analysis:all data are presented as mean of data from three independent replicates ±standard deviation from the mean. Statistical analysis of data was performed using GraphPad Prism 6.0, with $p < 0.05$ considered as statistically significant after the data were tested for normal distribution using excel statistics function. Significant difference in the effect of PAA on biofilm biomass was evaluated using a t-test while the PAA effect on the polysaccharide and protein components of biofilm EPS was compared using a two-way ANOVA and Tukey's multiple comparison test. One-way ANOVA and Tukey post hoc test were used for the statistical analysis of enzyme effect on biofilm biomass and the effect of enzyme pre-treatment on biofilm susceptibility to PAA.

Results

The effect of PAA on the biomass and EPS components of 96 hr old *P. aeruginosa* biofilm is shown in figure 1. The concentration of PAA known to kill 96 hr old biofilm of *P. aeruginosa* (3500 ppm) has poor effect on the biomass of the biofilm. As shown in figure 1a, although 3500 ppm of PAA caused a significant reduction in biomass of the PAA treated biofilm ($p<0.001$), approximately 60% of the biomass persisted post PAA treatment. Evaluation of the specific impact of PAA on the protein and polysaccharide component of the PAA treated biofilm compared to the control showed that PAA only significantly affected the protein components of the PAA treated biofilm biomass ($p\leq0.001$), but not the polysaccharide components of the biomass (figure 1b).

The PAA tolerance of biofilm resulting from the recolonisation of the residual biomass of PAA disinfected biofilm is as shown in figure 2. Biofilms grown on disinfected biofilm biomass were more tolerant to PAA compared to the control biofilm. For instance, while the 48 hr old control biofilm did not survive treatment with 1000 ppm, 48 hr secondary biofilm survived treatment with 2000 ppm of PAA and only killed at 4000 ppm. The 96 hr old secondary biofilm was only killed at 8000 ppm of PAA, while the control biofilm of the same age was killed at 4000 ppm.

Confocal microscopy visualisation of the distribution of live and dead cells in the biomass of biofilm killed with 3500 ppm of PAA and recolonised with *P. aeruginosa* PA14 cells is shown in figure 3. Live cells are evenly distributed throughout the recolonised biomass rather than forming a new layer of secondary biofilm on the biomass surface.

The effect of adding enzymes able to break down components of the EPS is shown in figure 4. DNase I and alginate lyase degraded the biomass of the 96 hr *P. aeruginosa* biofilm, in a dose dependant way. Treatment of the biofilm with DNase I and alginate lyase resulted in

approximately 40% reduction in biofilm biomass, Proteinase K had no significant impact on the biomass of the enzyme treated biofilm, with less than 10% reduction in biomass. A concentration of 400 Kunitz/mL of DNase I, 5U/mL of alginate lyase and 30 U/mL of proteinase K were subsequently used to evaluate the impact of enzyme pre-treatment on biofilm susceptibility to 2000 PPM PAA. The results are shown in figure 5. DNase I and alginate lyase sensitised *P. aeruginosa* biofilm to PAA killing compared to the control (p value ≤ 0.05 and ≤ 0.001, respectively). Pre-treatment with 5U/mL of alginate lyase showed the most significant effect resulting in more than 8 $\log_{10}$ reduction in biofilm viability as measure by its CFU ($p < 0.001$). Pre-treatment with 400 Kunitz/mL of DNase I also had significant effect on biofilm susceptibility to PAA (p value ≤ 0.05), but this was not significantly different to the effect of alginate lyase pre-treatment. Pre-treatment with proteinase K did not sensitise *P. aeruginosa* biofilm to PAA killing as there was no significant difference in the susceptibility of proteinase K pre-treated biofilm to PAA compared to non-enzyme pre-treated biofilm.

Discussion and Conclusion

Previous studies have shown the ability of mature biofilm to survive treatment with high-level disinfectants (Alfa and Howie 2009; da Costa Luciano et al. 2016; Neves et al. 2016; Akinbobola et al. 2017). This suggests that if a mature biofilm is allowed to form on complex semi-critical medical equipment like endoscopes it may have significant impact on the effectiveness of high-level disinfection used in reprocessing. In this study, the role of biofilm EPS related tolerance to a high-level disinfection and the benefit of using biofilm EPS degrading enzymes prior to high-level disinfection were examined. A situation where biofilms were killed by high-level disinfectant but not physically removed was modelled in

order to study how residual biomass affects the susceptibility of recolonising biofilm to high-level disinfection.

PAA concentrations which killed the biofilm used only caused around 40% reduction in its biomass. Although the inability of various disinfectants used at a concentration which reduces viability or kills the biofilm to achieve corresponding reduction in biomass have been previously reported (Tote et al. 2010; Yamaguchi et al. 2013), this is the first report of the effect of high-level disinfectant at a concentration used clinically for high-level disinfection on the biomass and EPS component of biofilm. Further investigation of the effect of PAA impact on the polysaccharide and protein subcomponents of the *EPS* showed that PAA only significantly reduced the protein component causing more than 50% reduction in protein component of biofilm EPS. This observation demonstrates that the protein component of *P. aeruginosa* biofilm may play a less significant role, compared to the polysaccharide component, in maintaining the structure of the *P. aeruginosa* biofilm biomass.

Comparison of the PAA susceptibility of secondary biofilm of different ages growing on residual PAA killed biofilm biomass showed that the presence of residual biofilm biomass remaining after high-level disinfection enhances biofilm tolerance to PAA. In fact, more than twice the concentration of PAA was needed to kill biofilm growing on residual biofilm biomass. When we modelled a 96 hr old biofilm grown on residual biomass of 96 hr biofilm killed with 3500 PPM of PAA to depict a worst-case scenario the biofilm formed was not killed at concentrations of PAA used clinically for high-level disinfection. The result from this study also showed that while young bacterial biofilm may be susceptible to high-level disinfection their tolerance can be enhance by residual biomass accumulating over time in complex semi-critical medical equipment like endoscopes. These observations showed that

disinfection is only one part of endoscope reprocessing and should only follow thorough physical cleaning

It was unclear whether the recolonising cells would be embedded in the biomass or form a new layer of biofilm on the it. For residual biomass on surfaces to enhance the tolerance of recolonising biofilm it would have been expected that the cells would need to be embedded within the pre-existing EPS. Visualisation of the recolonised biofilm using confocal microscopy after a live/dead staining showed that this is in fact the case for the recolonised *P. aeruginosa* biofilm used in this study. The 2D and 3D images of the recolonised biomass showed that green fluorescing Syto9 stained viable cells were evenly distributed throughout the recolonised biomass. Similar interactions of *Streptococcus mutans* cells recolonising biofilm killed with 70% isopropyl alcohol was demonstrated by Ohsumi et al. (2015) using confocal fluorescence microscopy.

Various studies have reported the increased susceptibility of detached biofilm cells to antimicrobials compared to intact biofilm (Behnke and Camper 2012). Therefore, degrading biofilm EPS should enhance the antimicrobial activity of disinfectants against biofilms by allowing increased disinfectant penetration into the biofilm. In this study, the ability of DNase I, alginate lyase and proteinase K to degrade the biomass of *P. aeruginosa* biofilm and therefore sensitise it to PAA high-level disinfection were evaluated. Both DNase I and alginate lyase were able to achieve an approximate 40 % reduction in the biomass of 96 hr biofilm after two hours treatment while treatment with the proteinase K resulted in less than 10% reduction in biofilm biomass. The effect of EPS degrading enzymes on the biofilm of other bacteria species has been examined. (Nguyen and Burrows 2014) demonstrated that both DNase I and proteinase K can degrade established biofilms of *Listeria monocytogene*s. This differs from the result with *P. aeruginosa* biofilm in this study and may be due to the

differences in both composition and architecture of *L. monocytogenes* and the *P. aeruginosa* PA14 biofilms. Other clear differences between the susceptibility of different bacterial species to biofilm EPS degrading enzymes have been reported. (Izano et al. 2008) showed that DNase I was able to degrade *S. aureus* biofilmbut had no effect on *S. epidermidis* biofilm. In *P. aeruginosa,* DNase I has been shown to reduce the biomass of *P. aeruginosa* biofilm after 24 hr treatment (Tetz et al. 2009). Alginate lyase has also been shown to disperse established biofilm (Germoni et al. 2016), but no previous study has examined how this biofilm degradation may act synergistically with PAA disinfection.

Results from this study have shown that pre-treatment of 96 hr old *P. aeruginosa* biofilm with alginate lyase and DNase I enhanced the activity of PAA against the biofilm achieving greater than 8 log_{10} reduction in biofilm CFU. Proteinase K pre-treatment however did not enhance the susceptibility of pre-treated biofilm to PAA. Several studies have examined the effect of biofilm EPS component degrading enzymes on biofilm susceptibility to antimicrobials but most of the studies examined the anti-biofilm efficacy of antibiotics rather than disinfectants. Hatch and Schiller (1998) demonstrated that alginate lyase enhanced the penetration of the alginate barrier by aminoglycoside thereby enhancing its activity against alginate embedded bacterial cells. Abdi-Ali et al. (2006) using a sandwich cup method demonstrated that antibiotics showed different abilities to penetrate alginate suggesting that the ability of an antimicrobial agent to penetrate biofilm depends on the chemical nature of the biofilm and antimicrobial agent. The slight difference in the susceptibility of alginate lyase and DNase I pre-treated biofilm to PAA may be due to the different structural roles of alginate and eDNA in aged *P. aeruginosa* biofilm. Alginate has been shown to play a crucial role in maintaining cell viability in the biofilm of *P. aeruginosa* PA01, although this may be strain specific (Ghafoor et al. 2011). Alginate interaction with ions such as Ca^{2+} or protein can result in the formation of a gel-like barrier in biofilm which can more effectively retard

antimicrobial penetration. Degradation of such a protection barrier by alginate lyase may explain the enhance susceptibility of alginate lyase pre-treated biofilm to PAA as observed in this study.

Findings from this study indicate that polysaccharide like alginate may be the most important component of biofilm EPS retarding PAA penetration into *P. aeruginosa* biofilm. Biofilm EPS consists of different types of polysaccharide which vary between microorganism and condition of growth. *P. aeruginosa* biofilm produces three main types of polysaccharides *Psl*, *Pel*, and alginate (Billings et al. 2013). The polysaccharide component of biofilm has been demonstrated to play an important role in biofilm development, maturation and in maintaining biofilm structure (Ma et al. 2006; Colvin et al. 2011; Ghafoor et al. 2011). Biofilm polysaccharide have also been shown to play an important role in biofilm ability to withstand environmental and antimicrobial stress (Limoli et al. 2015). A common example is the enhanced resistance of mucoid *P. aeruginosa* to antibiotics due to overproduction of alginate, a factor responsible for the persistence of *P. aeruginosa* infection in the lungs of the cystic fibrosis patient (Hatch and Schiller 1998; Hentzer et al. 2001).

Only the contribution of alginate polysaccharide to the enhanced biofilm tolerance to PAA was investigated in this study. Previous studies have demonstrated that the role of different biofilm polysaccharide in biofilm tolerance to antimicrobial stress varies between organism and even strains. Colvin et al. (2011) demonstrated that while *pel* is important in the development of the biofilm of *P. aeruginosa* PA14 and its resistance to aminoglycoside antibiotics, it plays no significant role in the biofilm phenotype of *P. aeruginosa* PA01. Therefore, investigating the interaction between PAA and different types of polysaccharide commonly found in biofilm can clarify the role of biofilm polysaccharide in reduced susceptibility of biofilm to PAA.

In conclusion, the results from this study show that residual disinfected biomass may, under certain conditions, lead to the development of a secondary biofilm which will not be killed by commonly used concentrations of PAA. This thus demonstrated that while young bacterial biofilm may be susceptible to high-level disinfection their tolerance can be enhance by residual biomass accumulating over time in complex semi-critical medical equipment like endoscopes. These observations showed that disinfection is only one part of endoscope reprocessing and should only follow thorough physical cleaning. Also, observation from the study that the degradation of alginate and eDNA sensitised 96 hr biofilm to PAA may have important impact on current guidance for endoscope reprocessing. While agents which have proteolytic ability are currently recommended in many endoscope reprocessing guidelines. The use of agents also capable of degrading polysaccharides and other components of biofilm EPS should also be considered in the pre-treatment of endoscopes prior to high-level disinfection especially where storage time may have allowed the development of mature biofilms within these instruments. The multi-well plate single specie biofilm used in this study may not be as complex as biofilms in channels of medical equipment. Medical equipment associated biofilm may involve multiple species and develop over a long period, however, results from this study highlight the risk residual biofilm poses to effective reprocessing of semi critical medical equipment.

References

Abdi-Ali A, Mohammadi-Mehr M, Alaei YA. 2006. Bactericidal activity of various antibiotics against biofilm-producing Pseudomonas aeruginosa. International journal of antimicrobial agents. 27(3):196-200.

Akinbobola A, Sherry L, Mckay WG, Ramage G, Williams C. 2017. Tolerance of Pseudomonas aeruginosa in in-vitro biofilms to high-level peracetic acid disinfection. Journal of Hospital Infection.

Alfa MJ, Howie R. 2009. Modeling microbial survival in buildup biofilm for complex medical devices. BMC infectious diseases. 9:56.

Bajolet O, Ciocan D, Vallet C, de Champs C, Vernet-Garnier V, Guillard T, Brasme L, Thiefin G, Cadiot G, Bureau-Chalot F. 2013. Gastroscopy-associated transmission of extended-spectrum beta-lactamase-producing Pseudomonas aeruginosa. Journal of Hospital Infection. 83(4):341-343.

Behnke S, Camper AK. 2012. Chlorine dioxide disinfection of single and dual species biofilms, detached biofilm and planktonic cells. Biofouling. 28(6):635-647.

Billings N, Millan MR, Caldara M, Rusconi R, Tarasova Y, Stocker R, Ribbeck K. 2013. The extracellular matrix component Psl provides fast-acting antibiotic defense in Pseudomonas aeruginosa biofilms. PLoS pathogens. 9(8):e1003526.

Blackledge MS, Worthington RJ, Melander C. 2013. Biologically inspired strategies for combating bacterial biofilms. Current opinion in pharmacology. 13(5):699-706.

Bridier A, Briandet R, Thomas V, Dubois-Brissonnet F. 2011. Resistance of bacterial biofilms to disinfectants: a review. Biofouling. 27(9):1017-1032.

Bridier A, Dubois-Brissonnet F, Greub G, Thomas V, Briandet R. 2011. Dynamics of the action of biocides in Pseudomonas aeruginosa biofilms. Antimicrobial agents and chemotherapy. 55(6):2648-2654.

Bridier A, Sanchez-Vizuete MdP, Le Coq D, Aymerich S, Meylheuc T, Maillard J-Y, Thomas V, Dubois-Brissonnet F, Briandet R. 2012. Biofilms of a Bacillus subtilis Hospital Isolate Protect Staphylococcus aureus from Biocide Action. Plos One. 7(9).

Chenjiao W, Hongyan Z, Qing G, Xiaoqi Z, Liying G, Ying F. 2016. In-Use Evaluation of Peracetic Acid for High-Level Disinfection of Endoscopes. Gastroenterology Nursing. 39(2):116-120.

Chiba A, Sugimoto S, Sato F, Hori S, Mizunoe Y. 2015. A refined technique for extraction of extracellular matrices from bacterial biofilms and its applicability. Microbial biotechnology. 8(3):392-403.

Colvin KM, Gordon VD, Murakami K, Borlee BR, Wozniak DJ, Wong GC, Parsek MR. 2011. The pel polysaccharide can serve a structural and protective role in the biofilm matrix of Pseudomonas aeruginosa. PLoS Pathog. 7(1):e1001264.

Cowen AE. 2001. The clinical risks of infection associated with endoscopy. Canadian Journal of Gastroenterology and Hepatology. 15(5):321-331.

da Costa Luciano C, Olson N, Tipple AFV, Alfa M. 2016. Evaluation of the ability of different detergents and disinfectants to remove and kill organisms in traditional biofilm. American journal of infection control. 44(11):e243-e249.

De Melo WC, Avci P, De Oliveira MN, Gupta A, Vecchio D, Sadasivam M, Chandran R, Huang Y-Y, Yin R, Perussi LR. 2013. Photodynamic inactivation of biofilm: taking a lightly colored approach to stubborn infection. Expert review of anti-infective therapy. 11(7):669-693.

Donlan RM. 2000. Role of biofilms in antimicrobial resistance. ASAIO journal. 46(6):S47-S52.

Edmiston CE, Spencer M. 2014. Endoscope reprocessing in 2014: why is the margin of safety so small? AORN journal. 100(6):609-615.

Flemming H-C, Wingender J. 2010. The biofilm matrix. Nature Reviews Microbiology. 8(9):623-633.

Germoni L, Bremer P, Lamont I. 2016. The effect of alginate lyase on the gentamicin resistance of Pseudomonas aeruginosa in mucoid biofilms. Journal of applied microbiology.

Ghafoor A, Hay ID, Rehm BH. 2011. Role of exopolysaccharides in Pseudomonas aeruginosa biofilm formation and architecture. Applied and environmental microbiology. 77(15):5238-5246.

Grobe K, Zahller J, Stewart P. 2002. Role of dose concentration in biocide efficacy against Pseudomonas aeruginosa biofilms. Journal of Industrial Microbiology and Biotechnology. 29(1):10-15.

Hatch RA, Schiller NL. 1998. Alginate Lyase Promotes Diffusion of Aminoglycosides through the Extracellular Polysaccharide of MucoidPseudomonas aeruginosa. Antimicrobial agents and chemotherapy. 42(4):974-977.

Hentzer M, Teitzel GM, Balzer GJ, Heydorn A, Molin S, Givskov M, Parsek MR. 2001. Alginate overproduction affects Pseudomonas aeruginosa biofilm structure and function. Journal of bacteriology. 183(18):5395-5401.

Izano EA, Amarante MA, Kher WB, Kaplan JB. 2008. Differential roles of poly-N-acetylglucosamine surface polysaccharide and extracellular DNA in Staphylococcus aureus and Staphylococcus epidermidis biofilms. Applied and environmental microbiology. 74(2):470-476.

Jaglic Z, Červinková D, Vlková H, Michu E, Kunová G, Babák V. 2012. Bacterial Biofilms Resist Oxidising Agents Due to the Presence of Organic Matter. Czech Journal of Food Science. 30(2).

Johani K, Hu H, Santos L, Schiller S, Deva AK, Whiteley G, Almatroudi A, Vickery K. 2018. Determination of bacterial species present in biofilm contaminating the channels of clinical endoscopes. Infection, Disease & Health.

Kampf G, Fliss PM, Martiny H. 2014. Is peracetic acid suitable for the cleaning step of reprocessing flexible endoscopes? World journal of gastrointestinal endoscopy. 6(9):390.

Kovaleva J, Peters FT, van der Mei HC, Degener JE. 2013. Transmission of infection by flexible gastrointestinal endoscopy and bronchoscopy. Clinical microbiology reviews. 26(2):231-254.

Lambert R, Johnston M. 2001. The effect of interfering substances on the disinfection process: a mathematical model. Journal of applied microbiology. 91(3):548-555.

Limoli DH, Jones CJ, Wozniak DJ. 2015. Bacterial extracellular polysaccharides in biofilm formation and function. Microbiology spectrum. 3(3).

Ma L, Jackson KD, Landry RM, Parsek MR, Wozniak DJ. 2006. Analysis of Pseudomonas aeruginosa conditional psl variants reveals roles for the psl polysaccharide in adhesion and maintaining biofilm structure postattachment. Journal of bacteriology. 188(23):8213-8221.

Mah T-FC, O'Toole GA. 2001. Mechanisms of biofilm resistance to antimicrobial agents. Trends in microbiology. 9(1):34-39.

Marshall JM, Flechtner AD, La Perle KM, Gunn JS. 2014. Visualization of extracellular matrix components within sectioned Salmonella biofilms on the surface of human gallstones. PloS one. 9(2):e89243.

Masuko T, Minami A, Iwasaki N, Majima T, Nishimura S-I, Lee YC. 2005. Carbohydrate analysis by a phenol–sulfuric acid method in microplate format. Analytical biochemistry. 339(1):69-72.

McCafferty CE, Aghajani MJ, Abi-Hanna D, Gosbell IB, Jensen SO. 2018. An update on gastrointestinal endoscopy-associated infections and their contributing factors. Annals of clinical microbiology and antimicrobials. 17(1)1-6

Miles AA, Misra SS, Irwin JO. 1938. The estimation of the bactericidal power of the blood. Journal of Hygiene. 38(06):732-749.

Neves MS, da Silva MG, Ventura GM, Côrtes PB, Duarte RS, de Souza HS. 2016. Effectiveness of current disinfection procedures against biofilm on contaminated GI endoscopes. Gastrointestinal Endoscopy. 83(5):944-953.

Nguyen UT, Burrows LL. 2014. DNase I and proteinase K impair Listeria monocytogenes biofilm formation and induce dispersal of pre-existing biofilms. International journal of food microbiology. 187:26-32.

O'Toole GA. 2011. Microtiter dish biofilm formation assay. JoVE (Journal of Visualized Experiments).(47):e2437-e2437.

Ohsumi T, Takenaka S, Wakamatsu R, Sakaue Y, Narisawa N, Senpuku H, Ohshima H, Terao Y, Okiji T. 2015. Residual structure of Streptococcus mutans biofilm following complete disinfection favors secondary bacterial adhesion and biofilm re-development. PloS one. 10(1):e0116647.

Pajkos A, Vickery K, Cossart Y. 2004. Is biofilm accumulation on endoscope tubing a contributor to the failure of cleaning and decontamination? Journal of Hospital Infection. 58(3):224-229.

Reichhardt C, Cegelski L. 2014. Solid-state NMR for bacterial biofilms. Molecular physics. 112(7):887-894.

Ren-Pei W, Hui-Jun X, Ke Q, Dong W, Xing N, Zhao-Shen L. 2014. Correlation between the growth of bacterial biofilm in flexible endoscopes and endoscope reprocessing methods. American journal of infection control. 42(11):1203-1206.

Soares J-B, Gonçalves R, Banhudo A, Pedrosa J. 2011. Reprocessing practice in digestive endoscopy units of district hospitals: results of a Portuguese National Survey. European journal of gastroenterology & hepatology. 23(11):1064-1068.

Spinzi G, Fasoli R, Centenaro R, Minoli G, Group SLW. 2008. Reprocessing in digestive endoscopy units in Lombardy: results of a regional survey. Digestive and Liver Disease. 40(11):890-896.

Stewart P, Rayner J, Roe F, Rees W. 2001. Biofilm penetration and disinfection efficacy of alkaline hypochlorite and chlorosulfamates. Journal of Applied Microbiology. 91(3):525-532.

Stewart PS. 2015. Antimicrobial tolerance in biofilms. Microbiology spectrum. 3(3).

Tetz GV, Artemenko NK, Tetz VV. 2009. Effect of DNase and antibiotics on biofilm characteristics. Antimicrobial agents and chemotherapy. 53(3):1204-1209.

Tote K, Horemans T, Berghe DV, Maes L, Cos P. 2010. Inhibitory effect of biocides on the viable masses and matrices of Staphylococcus aureus and Pseudomonas aeruginosa biofilms. Applied and environmental microbiology. 76(10):3135-3142.

Vizcaino-Alcaide MJ, Herruzo-Cabrera R, Fernandez-Acenero MJ. 2003. Comparison of the disinfectant efficacy of Perasafe((R)) and 2% glutaraldehyde in in vitro tests. Journal of Hospital Infection. 53(2):124-128.

Xue Z, Lee WH, Coburn KM, Seo Y. 2014. Selective reactivity of monochloramine with extracellular matrix components affects the disinfection of biofilm and detached clusters. Environmental science & technology. 48(7):3832-3839.

Yamaguchi M, Noiri Y, Kuboniwa M, Yamamoto R, Asahi Y, Maezono H, Hayashi M, Ebisu S. 2013. Porphyromonas gingivalis biofilms persist after chlorhexidine treatment. European journal of oral sciences. 121(3pt1):162-168.

Figure 1: Evaluation of the effect of PAA on the total biomass (A) as well as the polysaccharide and protein components of the ECM (B) of 96 hr old *P. aeruginosa* biofilm. The PAA concentrations used failed to remove biofilm biomass despite being able to achieve a 100 % reduction in biofilm viability. PAA at the concentration used only significantly reduced the protein componnet of the ECM of PAA treated biofilm (*** pvalue $\leq$ 0.001).

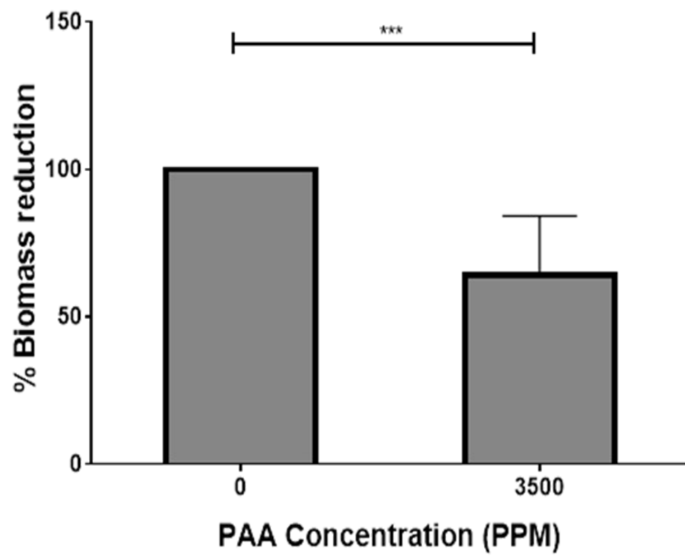
Figure 2: Evaluation of the impact of residual biomass of killed *P. aeruginosa* biofilm on the PAA tolerance of biofilm of different ages formed by recolonising the disinfected biofilm biomass. The biofilm growing on the disinfected biomass showed higher tolerance to PAA compare to biofilm of the same age growing on wells of 24-well plate. The dash lines depict the range of PAA concentrations used clinically for endoscope disinfection.

Figure 3: Confocal microscope visualisation of live and dead cells distribution in residual biofilm of *P. aeruginosa* biofilm killed with 3,500 PPM of PAA and re-inoculated with *P. aeruginosa* cells (A) and a non-reinoculated control (B). The images of the re-inoculated biofilm showed that the re-colonising cells were evenly distributed throughout the biofilm rather forming a layer of biofilm on the disinfected biomass

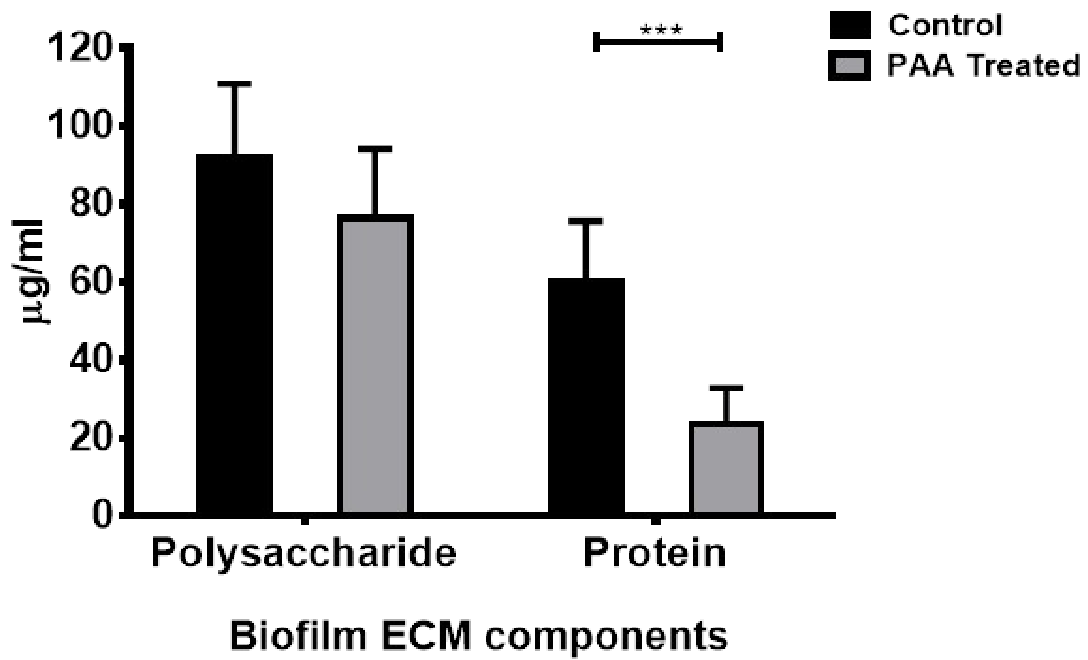
Figure 4: Evaluation of biofilm ECM components degrading enzymes on the biomass of 96 hr old *P. aeruginosa* biofilm. To determine the optimal concentration of enzyme required to degrade biofilm biomass, the effect of different concentrations of DNase I (A), alginate lyase (B) and proteinase K (C) on the biomass of 96 hr old *P. aeruginosa* biofilm was evaluated using the crystal violet assay.

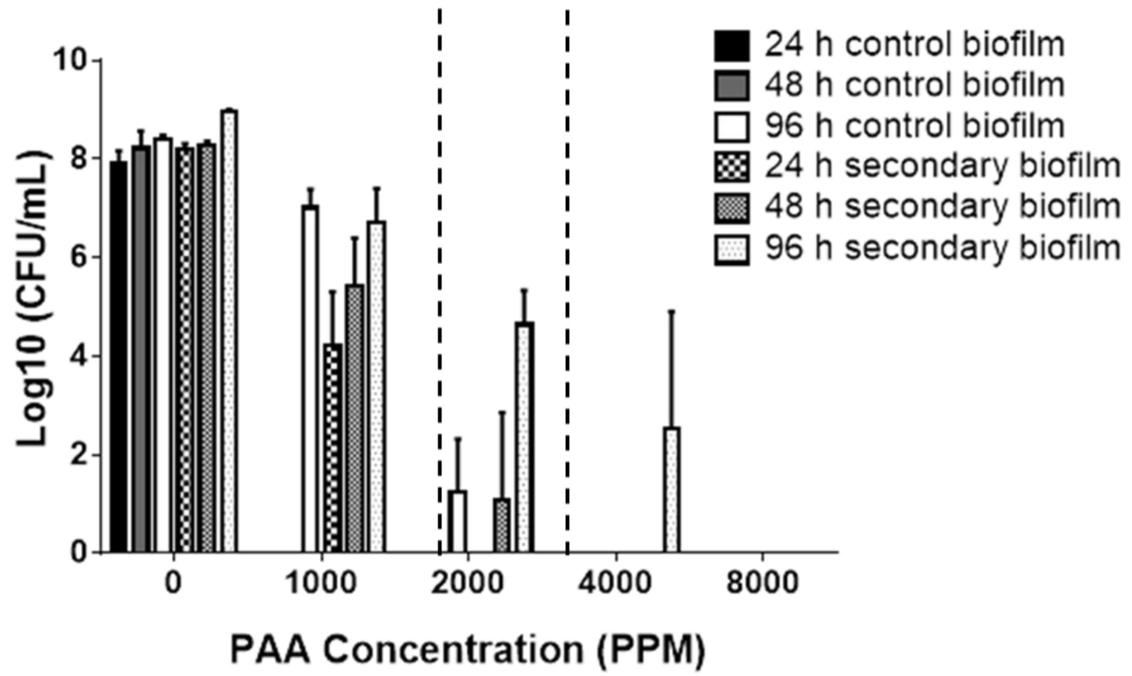
Figure 5: Effect of DNase I (400 Kunitz/mL), alginate lyase (5U/ mL) and proteinase K (30 U/mL) pre-treatment on *P. aeruginosa* biofilm susceptibility to 2000 PPM of PAA. Alginate lyase and DNase 1 pre-treatments enhanced biofilm susceptibility to PAA. The same letter indicates no significant difference while different number indicate significant difference between treatment.

A



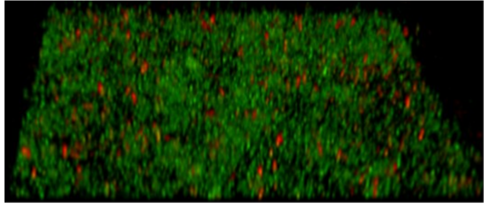
B

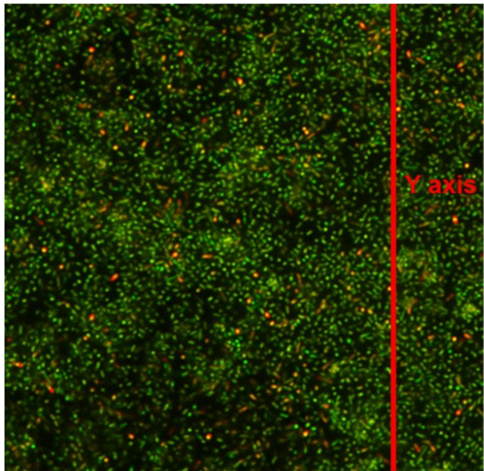


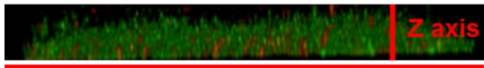


24 h control biofilm
48 h control biofilm
96 h control biofilm
24 h secondary biofilm
48 h secondary biofilm
96 h secondary biofilm
Log10 (CFU/mL)
10
8
6
4
2
0
0
1000
2000
4000
8000
PAA Concentration (PPM)

A

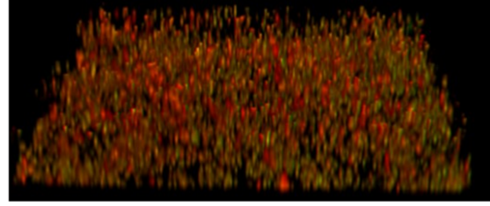


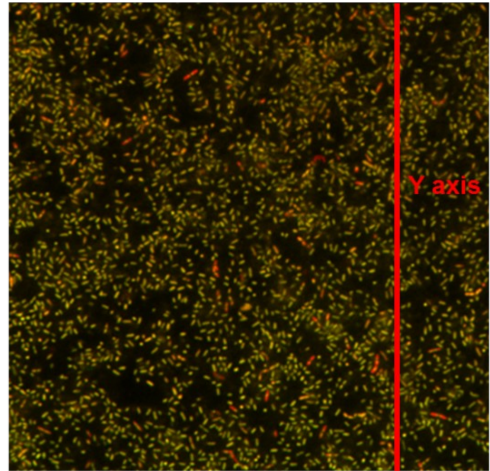


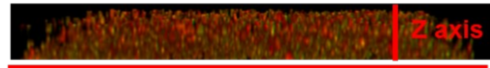


X axis

B

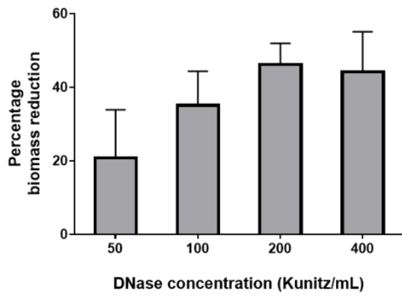


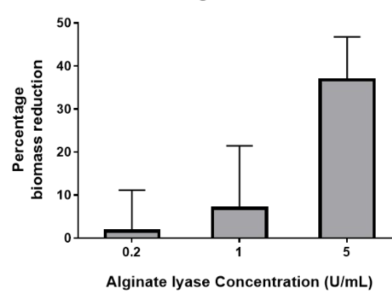


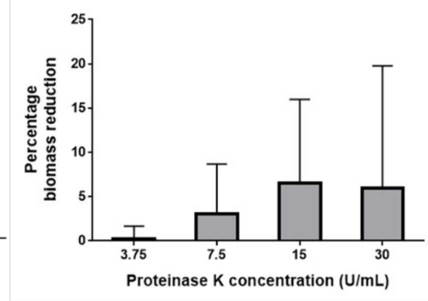


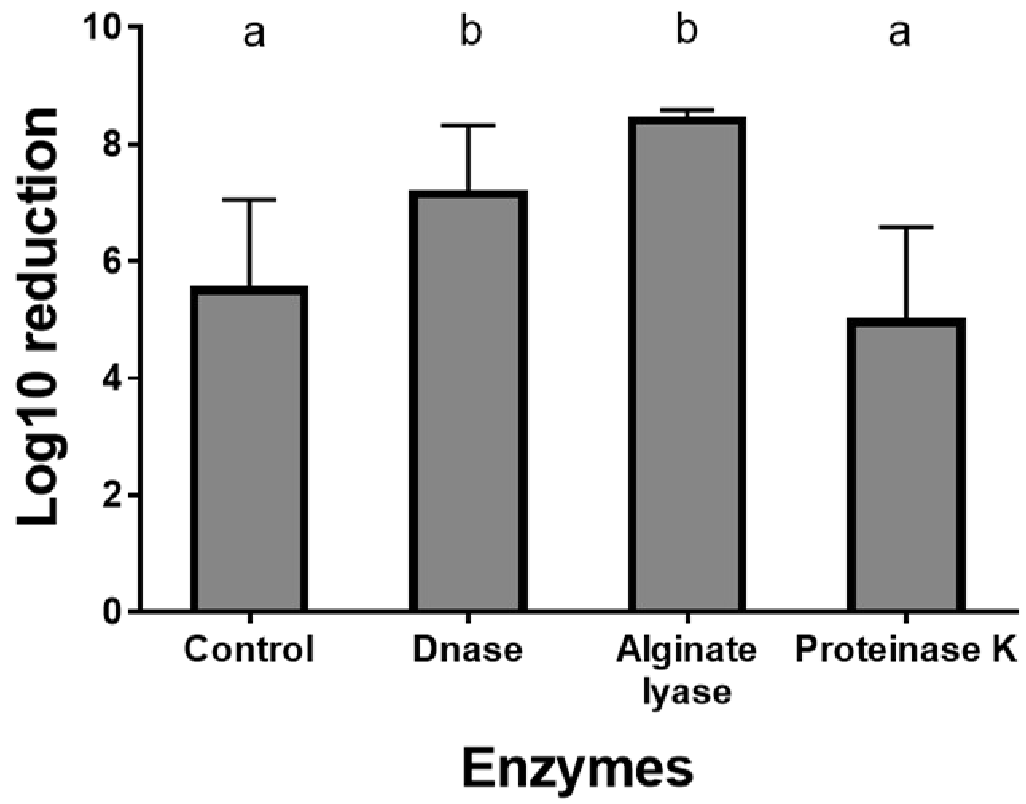
X axis

A **B** **C**








10
8
6
4
2
0
Log10 reduction
a
b
b
a
Control
Dnase
Alginate lyase
Proteinase K
Enzymes