

# Antibacterial activity testing of cotton medical textiles sonochemically impregnated with metal oxide nanoparticles

Singh, Gagandeep

*Award date:*
2014

*Awarding institution:*
Coventry University

# ANTIBACTERIAL ACTIVITY TESTING OF COTTON MEDICAL TEXTILES SONOCHEMICALLY IMPREGNATED WITH METAL OXIDE NANOPARTICLES

**By**

**GAGANDEEP SINGH**

**PhD**

**December 2014**

# ANTIBACTERIAL ACTIVITY TESTING OF COTTON MEDICAL TEXTILES SONOCHEMICALLY IMPREGNATED WITH METAL OXIDE NANOPARTICLES

**By**

**GAGANDEEP SINGH**

**December 2014**

**A thesis submitted in partial fulfilment of the University's requirements for the Doctorate of Philosophy**

**Table of Contents**

Acknowledgements ..... i

Abstract..... ii

Published work..... iv

1 Introduction and Literature Review..... 1

1.1 Bacteria..... 1

1.2 Description of bacterial ultrastructure..... 2

1.2.1 Cell wall..... 3

1.2.2 Cytoplasmic membrane..... 5

1.2.3 Capsule..... 5

1.3 Control of microbial growth..... 6

1.3.1 Antiseptics and Antibiotics..... 7

1.3.2 Antibiotic resistance..... 8

1.4 Hospital acquired infections (HAIs)..... 10

1.4.1 *Staphylococcus aureus*..... 13

1.4.2 *Pseudomonas aeruginosa*..... 14

1.4.3 *Escherichia coli*..... 15

1.4.4 *Acinetobacter baumannii*..... 15

1.4.5 *Klebsiella pneumoniae*..... 16

1.4.6 Biofilms and their role in nosocomial infections..... 17

1.5 Infection prevention..... 19

1.5.1 Medical textiles and infections..... 20

1.5.2 Survival of pathogens on textile surfaces..... 24

1.5.3 The role of antimicrobial textiles..... 25

1.5.4 Antimicrobial agents for textiles..... 26

1.5.5 Organic antimicrobial agents ..... 26
1.5.6 Triclosan ..... 27
1.5.7 Quaternary ammonium compounds (QACs) ..... 28
1.5.8 Chitosan ..... 29
1.5.9 Polyhexamethylene biguanide hydrochloride (PHMB) ..... 30
1.5.10 Inorganic antimicrobial agents ..... 30
1.5.11 Silver ..... 31
1.5.11.1 Research into silver antimicrobial textiles ..... 32
1.5.12 Zinc oxide (ZnO) ..... 36
1.5.12.1 Research into ZnO antimicrobial textiles ..... 37
1.5.13 Copper oxide (CuO) ..... 40
1.5.13.1 Research into CuO antimicrobial textiles ..... 41
1.6 Textile application methods ..... 43
1.6.1 Confinement of antibacterial agents in resins ..... 44
1.6.2 Pad-dry-cure method ..... 44
1.6.3 Sonochemical method of impregnating textile fabrics ..... 45
1.6.4 Ultrasound ..... 46
1.6.5 Principles of ultrasonics sonochemistry ..... 47
1.6.5.1 Cavitation in a liquid medium ..... 47
1.6.5.2 Cavitation at or near to a solid surface ..... 48
1.6.6 Sonochemical synthesis and impregnation of textile fabrics with metal oxide nanoparticles ..... 49
1.6.7 The SONO project ..... 50
2 Research aims and objectives ..... 54

2.1 Objectives ..... 55
3 Methodology ..... 57
3.1 Quality control and safety ..... 57
3.2 Equipment and apparatus ..... 58
3.3 Bacterial strains ..... 59
3.4 Materials used ..... 60
3.4.1 Reagents and culture media ..... 60
3.4.1.1 Nutrient broth (NB) ..... 60
3.4.1.2 Soyabean-Casein Digest broth with Lecithin and Polysorbate 80 medium ..... 60
3.4.1.3 Plate count agar (PCA) ..... 61
3.4.1.4 Nutrient agar (NA) ..... 62
3.4.1.5 Sterile saline ..... 63
3.4.1.6 Phosphate buffered saline ..... 63
3.4.1.7 Staining buffer (Flow cytometery) ..... 63
3.5 Preparation of inoculum (ISO 20743:2007) ..... 63
3.5.1 Inoculum for growth on solid media ..... 63
3.5.2 Preparation and incubation of test inoculums ..... 64
3.5.3 Calibration of bacterial suspensions ..... 65
3.6 Preparation of fabric samples ..... 65
3.7 Antibacterial assessment of test fabrics ..... 67
3.7.1 Absorption method (ISO 20743) – plate count enumeration ..... 68
3.7.2 Serial dilutions ..... 69
3.7.3 Agar plating ..... 70

3.7.3.1 Pour plating ..... 70
3.7.3.2 Spread plating ..... 71
3.7.4 Test results interpretation ..... 72
3.7.4.1 Judgment of test effectiveness ..... 72
3.7.5 Calculation of antibacterial activity value ..... 72
3.7.6 Calculation of percentage reduction ..... 74
3.7.7 Calculation of growth reduction ..... 74
3.8 Disc diffusion method (Semi-quantitative test) ..... 75
3.9 Shake flask tests ..... 76
3.9.1 Shake flask in nutrient broth (Semi-quantitative test) ..... 76
3.9.2 Shake flask test in saline (Quantitative method) ..... 77
3.10 Saline leaching tests ..... 78
3.11 Inductively coupled plasma - optical emission spectrometer (ICP – OES) .. 78
3.11.1 Measurement of the total concentration of metals on the fabrics ..... 80
3.11.2 Measurement of the concentration of metals in the leachates ..... 80
3.12 Shake flask tests with different sized fabric pieces ..... 80
3.13 Effect of sample preparation on viable cell numbers ..... 82
3.14 Preparation and incubation of test inoculums ..... 82
3.15 Flow cytometry (FC) ..... 83
3.15.1 Storage and handling of staining solutions ..... 83
3.15.2 Preparation of live and dead bacterial suspensions ..... 83
3.16 Bacterial suspension and staining ..... 84
3.17 Data acquisition and analysis ..... 86
3.18 Determination of sensitivity of detection by flow cytometry ..... 87
3.19 Fluorescence microscopy ..... 87

3.20 Cytotoxicity studies ..... 88
3.20.1 Cell culture ..... 88
3.20.2 Cytotoxicity by indirect contact ..... 88
3.20.3 MTT assay ..... 89
3.21 Statistics ..... 90
4 Results and Discussion ..... 91
4.1 Testing of lab scale ZnO and CuO impregnated cotton fabrics ..... 91
4.1.1 Antibacterial efficacy testing of lab scale ZnO and CuO cotton fabrics using the absorption method from ISO 20743:2007 ..... 92
4.1.2 Antibacterial activity of PEC fabrics coated with silver and triclosan .. 101
4.1.3 Assessment of the wash durability of ZnO, CuO, silver and triclosan fabrics in terms of antibacterial activity ..... 105
4.1.4 Enzymatic pre-treatment of cotton prior to ZnO impregnation ..... 107
4.1.5 Antibacterial activity testing of lab scale ZnO and CuO fabrics by a disc diffusion method ..... 109
4.1.6 Shake flask testing to assess the antibacterial activity of lab scale ZnO and CuO fabrics ..... 113
4.1.6.1 Shake flask test in nutrient broth ..... 114
4.1.6.2 Shake flask test in saline ..... 117
4.1.7 Determination of minimum inhibitory concentration of lab scale fabrics in terms of sample size ..... 120
4.1.8 ICP analysis of lab scale fabrics to measure the amount of zinc and copper on fabrics and in leachates ..... 122
4.1.8.1 Antibacterial activity of Zn and Cu ions by shake flask method ..... 125
4.1.8.2 Antibacterial activity of metal salts against *E. coli* ..... 128

4.2 Testing of CuO fabrics from the pilot scale machines ..... 130
4.2.1 Antibacterial efficacy testing of CuO impregnated PEC fabrics from the prototype machines using the absorption method ..... 132
4.2.2 Antibacterial activity testing of pilot scale CuO PEC fabrics by disc diffusion method ..... 137
4.2.3 Antibacterial activity of pilot scale CuO PEC fabrics by shake flask method in saline ..... 139
4.2.4 Determination of minimum inhibitory concentration of pilot scale PEC fabrics in terms of sample size ..... 140
4.2.5 Measurement of the amount of copper on fabrics and in leachates by ICP ..... 144
4.2.5.1 Antibacterial activity of saline leachates from pilot scale CuO samples.. ..... 146
4.2.5.2 Leaching of copper in to nutrient broth ..... 150
4.2.6 Differences in the homogeneity of the impregnation (Klopman vs Davo) ..... 152
4.2.6.1 Antibacterial activity of fabric E from pale and dark area of coating 153
4.3 Flow cytometry (FC) ..... 153
4.3.1 Effect of sample preparation on bacterial viable count (FC experiments).. ..... 154
4.3.2 Measurement of the sensitivity of cell detection by FC on the BD FACS system ..... 160
4.3.3 Comparing the enumeration of viable cells from lab scale ZnO and CuO fabrics by FC and agar plate counting ..... 164

4.3.4 Comparing the enumeration of viable cells from 4 pilot scale CuO PEC fabrics by FC and agar plate counting ..... 173
4.4 Cytotoxicity studies on pilot scale ZnO and CuO fabrics ..... 182
5 Conclusion ..... 187
6 Future work ..... 200
7 References ..... 203
Appendices See attached disc

**List of Figures**

Figure 1 Typical structure of a prokaryotic cell ..... 2

Figure 2 Gram stain images of Gram positive (A) *Staphylococcus aureus* and (B) Gram negative *Escherichia coli* ..... 3

Figure 3 The cell wall structure of Gram positive and Gram negative bacteria ..... 4

Figure 4 Examples of biological mechanisms of antibiotic resistance ..... 9

Figure 5 SEM image of morphology of *Staphylococcus aureus* ..... 13

Figure 6 SEM image of morphology of *Pseudomonas aeruginosa* ..... 14

Figure 7 SEM image of morphology of *Escherichia coli* ..... 15

Figure 8 SEM image of morphology of *Acinetobacter baumannii* ..... 16

Figure 9 SEM image of morphology of *Klebsiella pneumoniae* ..... 17

Figure 10 Differences between planktonic cells and biofilms ..... 18

Figure 11 Potential contaminated surfaces in a hospital room ..... 20

Figure 12 The sites and surfaces at home based on the level of risk involved in the transmission of infection ..... 23

Figure 13 Structure of triclosan ..... 27

Figure 14 Structure of quaternary ammonium cations ..... 28

Figure 15 Structure of chitosan ..... 29

Figure 16 Structure of polyhexamethylene biguanide hydrochloride (PHMB) ..... 30

Figure 17 Fabric coating by pad-dry-cure method ..... 45

Figure 18 Different ranges of ultrasonic sound frequencies ..... 47

Figure 19 Acoustic cavitation: generation of the bubble ..... 48

Figure 20 Collapse of a cavitation bubble at or near a solid surface ..... 49

Figure 21 Chemical processes for the sonochemical conversion of copper acetate into CuO nanoparticles ..... 50

Figure 22 A schematic diagram of the pilot sonochemical reactor used for impregnating fabric ..... 52
Figure 23 One of the prototype machines used to produce medical textiles based on results generated by the SONO project ..... 53
Figure 24 A quadrant streak of *Staphylococcus aureus* on nutrient agar plate ..... 64
Figure 25 Standardization of culture by serial dilutions ..... 69
Figure 26 Colonies formed on agar by pour plating method ..... 70
Figure 27 An example dot plot to show FL1 vs FL3 discrimination of bacterial cells 86
Figure 28 Antibacterial assessment of lab scale ZnO impregnated fabrics against (A) *S. aureus*, (B) *E. coli*, (C) *A. baumannii*, (D) *P. aeruginosa* and (E) *K. pneumoniae* by the absorption method from ISO 20743 ..... 96
Figure 29 Antibacterial assessment of lab scale CuO impregnated fabrics against (A) *S. aureus*, (B) *E. coli*, (C) *A. baumannii*, (D) *P.aeruginosa* and (E) *K. pneumoniae* by the absorption method from ISO 20743 ..... 100
Figure 30 Antibacterial activity of PEC fabrics coated with silver and triclosan against (A) & (C) *E. coli* and (B) & (D) *S. aureus* ..... 102
Figure 31 Antibacterial activity of 60/40% polyester cotton fabrics coated with silver and triclosan tested against *K. pneumoniae* ..... 103
Figure 32 Antibacterial activity values of different fabrics against *E. coli* after 10 wash cycles according to ISO 6330 ..... 106
Figure 33 Antibacterial activity of modified cotton fabrics impregnated with ZnO NPs against *A. baumannii* ..... 109
Figure 34 Disc diffusion plates showing the antibacterial activity of ZnO & CuO impregnated cotton against (A) *E. coli*, (B) *S. aureus* & (C) *A. baumannii* ..... 113

Figure 35 Change in absorbance over time (3 hours) for nutrient broth shake flask tests against (A) *S. aureus*, (B) *E. coli* and (C) *A. baumannii* ..... 116
Figure 36 Antibacterial efficiency of ZnO and CuO impregnated fabrics against different bacteria using a saline shake flask test method ..... 119
Figure 37 Absorbance (OD) values after 24 hours incubation at 37 ºC by different sized pieces of cotton fabrics impregnated with ZnO and CuO nanoparticles against (A) *E. coli*, (B) *S. aureus* and (C) *A. baumannii* ..... 122
Figure 38 (A) Total concentration of Zn and Cu on the cotton fabrics and (B) concentration of metals leached in the saline leachate solution ..... 124
Figure 39 Shake flask tests with saline leachates from ZnO and CuO impregnated cotton fabrics ..... 127
Figure 40 Antibacterial activity of zinc chloride and copper sulphate solution against *E. coli* ..... 129
Figure 41 Fabrics impregnated with CuO nanoparticles from both the pilot plants; Klopman and Davo ..... 131
Figure 42 Scanning electron microscopic images of the fabrics from the prototype machine at Klopman (A) *in-situ* fabric (B) TTS fabric ..... 132
Figure 43 Antibacterial activity of pilot scale CuO PEC fabrics against MRSA and *P. aeruginosa* by absorption method (ISO 20743) ..... 135
Figure 44 Antibacterial activity of pilot scale CuO PEC fabrics against (i) MRSA and (ii) *P. aeruginosa* assessed by disc diffusion method ..... 138
Figure 45 Antibacterial efficiency of pilot scale CuO PEC fabrics A, B, D and E against MRSA and *P. aeruginosa* using shake flask method in saline ..... 140
Figure 46 Antibacterial activity shown by different sized pilot scale CuO PEC fabrics from Klopman and Davo against *P. aeruginosa* and MRSA ..... 143

Figure 47 (A) Total concentration of Cu on the 4 pilot scale PEC fabrics and (B) Cu concentrations in saline leachate solutions ..... 146
Figure 48 Number of viable cells obtained by agar plating from the leached saline solutions incubated with *P. aeruginosa* ..... 147
Figure 49 Number of viable cells obtained by agar plating from the leached saline solutions incubated with MRSA ..... 148
Figure 50 Antibacterial activity of saline leachates against *E. coli*. Numbers of viable cells were obtained by plating. Fabrics A and B – Klopman TTS and *in-situ* ..... 149
Figure 51 Concentration of leached Cu content in nutrient broth ..... 150
Figure 52 Photos of CuO fabrics in SCDLP medium. The coloured fabrics have leached completely into SCDLP medium turning into white fabrics ..... 151
Figure 53 (a) CuO fabrics produced by Klopman and Davo using the pilot scale machines – squares 5 x 5 cm (b) concentrations of Cu on random, dark and pale regions of fabric E ..... 152
Figure 54 Comparison of the antibacterial activity of samples from the pale and dark regions of the impregnations against *P. aeruginosa* ..... 153
Figure 55 Effect of different sample preparations on bacterial viable counts for (A) *E. coli*, (B) *A. baumannii*, (C) *S. aureus*, (D) MRSA and (E) *P. aeruginosa* ..... 157
Figure 56 Changes in the morphology of *E. coli* after different treatments; (A) Untreated sample; (B) Centrifuged sample; (C) Sonicated sample ..... 158
Figure 57 Mean viable cell counts by FC from 3, 4, 5 and 6 fold dilutions of 3 hour cultures (~$10^8$ cells per ml) to assess the sensitivity of cell detection by flow cytometry for (A) *S. aureus*, (B) *E. coli*, (C) *A. baumannii*, (D) MRSA and (E) *P. aeruginosa* ..... 162

Figure 58 Comparison of viable counts from flow cytometry and agar plating for dilutions of 3, 4, 5 & 6 fold of a 3 hour culture of (A) *S. aureus*, (B) *E. coli*, (C) *A. baumannii*, (D) MRSA and (E) *P. aeruginosa*..... 164

Figure 59 An example FC dot plot with *E. coli* showing event populations for 2 different fluorescence emissions (FL1 and FL3) ..... 165

Figure 60 Antibacterial activity of lab scale ZnO fabrics assessed by agar plate counting and flow cytometry against (A) *E. coli*, (B) *A. baumannii* and (C) *S. aureus* ..... 168

Figure 61 Flow cytometry dot plots with FL1 versus FL3 for *E. coli* comparing control cotton fabric against ZnO impregnated fabric..... 170

Figure 62 Flow cytometry dot plots with FL1 versus FL3 for *A. baumannii* comparing control cotton fabric against ZnO impregnated fabric..... 170

Figure 63 Flow cytometry dot plots with FL1 versus FL3 for *S. aureus* comparing control cotton fabric against ZnO impregnated fabric..... 170

Figure 64 Flow cytometry dot plots with FL1 versus FL3 for *E. coli* comparing control cotton fabric against CuO impregnated fabric ..... 172

Figure 65 Flow cytometry dot plots with FL1 versus FL3 for *A. baumannii* comparing control cotton fabric against CuO impregnated fabric..... 172

Figure 66 Flow cytometry dot plots with FL1 versus FL3 for *S. aureus* comparing control cotton fabric against CuO impregnated fabric..... 172

Figure 67 Antibacterial activity of lab scale CuO fabrics assessed by viable agar plate counting and flow cytometry against (A) *E. coli*, (B) *A. baumannii* and (C) *S. aureus* ..... 173

Figure 68 Flow cytometry dot plots with FL1 versus FL3 for MRSA comparing control cotton fabric against CuO impregnated fabric ..... 175

Figure 69 Antibacterial activity of pilot scale CuO PEC fabrics (A, B, D & E) assessed with viable cell enumeration by agar plate counting and flow cytometry against MRSA ..... 176
Figure 70 Fluorescence photomicrographs showing green live and red dead MRSA cells in post incubation samples from the absorption testing (x200 magnification) 177
Figure 71 FC dot plots with FL1 versus FL3 for *P. aeruginosa* comparing control cotton fabric against ZnO impregnated fabric..... 178
Figure 72 Antibacterial activity of pilot scale CuO PEC fabrics assessed by agar plate counting and flow cytometry ..... 179
Figure 73 Fluorescence photomicrographs showing bacterial viability against CuO impregnated fabrics against *P. aeruginosa* at x200 magnification ..... 180
Figure 74 Human dermal fibroblast cells viability after (A) 1 day and (B) 7 day exposure with the suspension from ZnO and CuO impregnated fabrics ..... 183
Figure 75 Human hepatocellular carcinoma cells (HepG2) viability after (A) 1 day and (B) 7 day exposure with the suspension from ZnO and CuO impregnated fabric ..... 185

**List of Tables**

Table 1 Differences between the cell walls of Gram positive and Gram negative bacteria ..... 5

Table 2 Microorganisms commonly associated with hospital acquired infections .... 11

Table 3 Reports on the presence of microorganisms on hospital textiles..... 22

Table 4 Commercially available silver based antimicrobial agents and fibres ..... 32

Table 5 Metal ion standards for calibration of the ICP (PPM = parts per million) ..... 79

Table 6 Mean viable bacterial counts (CFU/ml) after 24 ± 3 hours contact time showing growth reduction with ZnO impregnated cotton samples compared to control cotton samples ..... 95

Table 7 Mean viable bacterial counts (CFU/ml) after 24 ± 3 hours contact time showing log growth reduction with CuO impregnated cotton samples compared to control cotton samples ..... 99

Table 8 Comparison of the antibacterial A value of ZnO and CuO impregnated fabrics to silver and triclosan fabrics..... 104

Table 9 Antibacterial activity values (A) of fabrics after 10 wash cycles at 92°C (cotton) or 75°C (PEC) without detergent against *E. coli*. ..... 106

Table 10 Antibacterial assessment in terms of zone of inhibition for ZnO and CuO cotton fabrics by disc diffusion method..... 112

Table 11 Shake flask results on lab scale fabrics in terms of percentage growth reduction in absorbance ..... 115

Table 12 ICP analysis of total Zn and Cu on fabrics and in leachates ..... 124

Table 13 Mean viable bacterial counts (CFU/ml) after 24 ± 3 hours and log reductions for CuO impregnated PEC samples compared to untreated PEC samples against MRSA and *P. aeruginosa* ..... 134

Table 14 Antibacterial assessment in terms of zone of inhibition of pilot CuO fabrics by disc diffusion method ..... 138
Table 15 Total amount of copper on pilot scale samples and the amount released in to saline leachates ..... 146
Table 16 Mean viable plate counts recovered after FC sample preparation steps against different bacterial species ..... 157
Table 17 Mean viable bacterial counts (CFU/ml) by both FC and plate counting after 24 ± 3 hours contact time for ZnO samples and control samples against *E. coli*, *A. baumannii* and *S. aureus* and their antibacterial A values ..... 168
Table 18 Mean viable bacterial counts (CFU/ml) by both FC and plate counting after 24 ± 3 hours contact time for CuO samples and control samples against *E. coli*, *A. baumannii* and *S. aureus* and their antibacterial A values ..... 173
Table 19 Mean viable bacterial counts (CFU/ml) by both FC and plate counting after 24 ± 3 hours contact time for pilot scale CuO PEC samples and control samples against against MRSA ..... 176
Table 20 Mean viable bacterial counts (CFU/ml) by both FC and plate counting after 24 ± 3 hours contact time for pilot scale CuO PEC samples and control samples against *P. aeruginosa* ..... 179

**Acknowledgements**

I am deeply indebted to my director of studies, Dr. Eadaoin Joyce and my supervisor Dr. Jamie Beddow for their excellent and very patient supervision and encouragement throughout the course of this research. They have made my experience as a postgraduate student an enjoyable one. I would also like to thank the director of Sonochemistry Centre, Professor Timothy Mason for his additional support.

Appreciations are extended to the EU FP7 programme for providing the funding for this project (SONO, FP7 project number: 228730) and the Faculty of Health and Life Sciences at Coventry University for the additional support.

I wish to thank all my colleagues in JS217 especially Patrick King and Rhianna Briars for helpful discussions and support throughout the years.

Thanks to the technical staff of the faculty in particular Daniel Comeskey, for all the help and advice over the years.

Finally, I would like to acknowledge the appreciation and love that I have received from all my family during this research. I am very grateful to my parents (Mr. Jagtar Singh and Mrs. Parminder Kaur), brother (Gurpreet Singh), my wife (Gagandeep Kaur) and my son (Karanveer Singh) for their support and patience during stressful times and for going the extra mile to help me in completing my thesis.

I would also like to acknowledge all my friends who have not been mentioned here. Thank you very much.

**Abstract**

The Sonochemistry Centre at Coventry University is one of a group of organisations working on a project to develop a new technology for producing antimicrobial textiles. This technology involves the use of an ultrasonic process (sonochemical) to generate and impregnate fabrics with antibacterial metal oxide nanoparticles. The expectation is that these textiles can be produced at an affordable price for routine use in hospitals as uniforms, curtains, hospital bed sheets and linen.

The aim of this PhD project was to assess the antibacterial activity of fabrics impregnated with ZnO and CuO NPs against a variety of Gram positive and Gram negative bacteria. The testing was principally carried out according to the absorption method from ISO 20743:2007. Research was also extended to compare different methods of assessing antibacterial activity of textile fabrics. These included disc diffusion tests and shake flask tests in saline or nutrient broth.

Overall the results from absorption tests demonstrated that both the ZnO and CuO impregnated fabrics showed very good levels of antibacterial activity (A>2) against the test bacteria (*Staphylococcus aureus*, Methicillin-resistant *Staphylococcus aureus*, *Acinetobacter baumannii*, *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*).

During the optimisation of lab scale process to the pilot scale, two different types of CuO fabrics were produced to test and compare the antibacterial activity. One type of fabrics were impregnated with pre-made CuO nanoparticles by a 'throwing the stones' technology termed TTS and the other with sonochemically formed nanoparticles (*in-situ*), same as the lab process. The results indicated that

the fabrics impregnated with sonochemically formed NPs displayed better antibacterial activity than the pre-made NPs.

Leaching of the antibacterial agents in to saline was investigated using a shake flask method. CuO and ZnO coated fabrics prepared at laboratory scale were tested against *Staphylococcus aureus*, *Acinetobacter baumannii* and *Escherichia coli*. It was found that leachates prepared by shaking the fabrics in saline for 3 hours showed no antibacterial activity for CuO fabrics. However, leachates from ZnO fabrics showed an excellent activity after 24 ± 3 hours against all three bacterial species.

Flow cytometry (FC) was investigated as an alternative to standard agar plate count (PC) methods for the determination of viable cell numbers. There was a general agreement between the results from agar plate counts and flow cytometry except that post incubation counts were greater with FC. The higher numbers of viable cells detected with FC may have been due to the presence of viable but not culturable cells (VBNC). Viable cells were observed by fluorescence microscopy in post incubation samples in which no viable cells were detected on nutrient agar plates.

Cytotoxicity studies were conducted on ZnO and CuO fabrics from the pilot scale (both *in-situ* and TTS) against human dermal fibroblast cells (HDF) and human hepatocellular carcinoma cells (HepG2) using a MTT assay to determine cell viability. The results showed that ZnO and CuO are not toxic to HDF cells. However, cytotoxicity was seen in HepG2 cells with cell viability decreasing by > 14% for all the fabrics after 24 hours.

**Published work**

1. **Singh, G**., Beddow, J., Joyce, E. M., and Mason, T. J. (2012) 'Evaluation of Antibacterial Activity of ZnO Nanoparticles Coated Sonochemically onto Textile Fabrics'. ***Journal of Microbiology, Biotechnology and Food Sciences 2 (1), 106-120***

2. Beddow, J., **Singh, G**., Blanes, M., Molla, K., Perelshtein, I., Gedanken, A., Joyce, E., and Mason, T. (2012) 'Sonochemical Coating of Textile Fabrics with Antibacterial Nanoparticles'. ***AIP Conference Proceedings 1433, 400-403***

3. **Singh, G**., Beddow, J., Joyce, E. M., and Mason, T. J. (2013) 'Production and efficacy testing of antimicrobial fabrics for use in hospitals'. ***Antimicrobial Resistance and Infection Control 2 (1), 184***

4. Perelshtein, I., Ruderman, Y., Perkas, N., Beddow, J., **Singh, G**., Vinatoru, M., Joyce, E., Mason, T. J., Blanes, M., and Mollá, K. (2013) 'The Sonochemical Coating of Cotton Withstands 65 Washing Cycles at Hospital Washing Standards and Retains its Antibacterial Properties'. ***Cellulose 20 (3), 1215-1221***

# 1 Introduction and Literature Review

## 1.1 Bacteria

Bacteria are single celled organisms that perform a variety of functions vital to life. Their influence on the environment is vast and profound and they constitute a major portion of biomass on earth (LaMorte 2012). These unicellular prokaryotic cells are a complex group of living organisms and play a vital role in the functioning of our biosphere. They establish relationships with higher organisms that can be either beneficial or harmful (Paniker 2005). Bacteria are ubiquitous i.e. they can survive at temperatures above the boiling point of water (>100$^{\circ}$C) and in cold below freezing point (< 0$^{\circ}$C). One important feature that has enabled them to spread so widely is their ability to become dormant under adverse conditions for extended periods of time (Waggoner and Speer 2006). Bacteria can also live or feed on many materials including plastic, wood, fabrics and a variety of other organic materials (Paniker 2005).

Bacteria are detrimental when they lead to infection (Poxton 1993). Most bacteria that form the normal flora are also opportunistic pathogens and can cause infections if suitable conditions arise. For example, *Staphylococcus aureus* is an opportunistic pathogen and is a leading cause of bacterial infections in humans. However, it is carried by about 20 – 30% of the population and can be easily transmitted from the nasal membrane of a carrier to a new susceptible host. It can cause infections of the skin, heart, lungs and brain. *Streptococcus pneumoniae* occupies the upper respiratory tract as part of the normal flora. In immunocompromised people, it can colonise the lower respiratory tract and cause

pneumonia. *Streptococcus pneumoniae* is responsible for 95% of all bacterial pneumonia infections (Todar 2009).

## 1.2 Description of bacterial ultrastructure

The typical structure of a prokaryotic cell is depicted in Figure 1. The outer layer of a bacterial cell consists of a cell wall and plasma membrane. The main function of the cell wall is to prevent cells from rupturing due to changes in osmotic pressure and to maintain cell shape (LaMorte 2012). The plasma membrane surrounds the essential components of a bacterial cell including cytoplasm, ribosomes, mesosomes, vacuoles, granules and the nuclear body. Unlike eukaryotes, the genetic material (DNA) in bacteria is not contained in a nucleus but is free in the cytoplasm (Paniker 2005).



**Figure 1 Typical structure of a prokaryotic cell. Image taken from http://biology.about.com**

### 1.2.1 Cell wall

Gram staining is the most commonly used staining technique for the identification of bacteria using light microscopy. The staining characteristics of bacterial cell walls are used to differentiate bacteria into two broad categories: Gram positive and Gram negative. Gram positive cells stain a deep blue/purple colour with a solution of crystal violet. Gram negative cells do not retain the dye and are instead counter stained with a red dye such as safranin (Figure 2).

In Gram positive bacteria the cell wall contains a thick multilayer protein and carbohydrate called peptidoglycan which makes up approximately 90% of the cell wall structure (Figure 4). Due to the high amount of peptidoglycan in the cell wall of Gram positive bacteria, they retain crystal violet. In contrast, Gram negative bacteria have a much thinner cell wall making up less than 20% of the structure and do not retain the crystal violet stain (Fix 2013).



**Figure 2 Gram stain images of Gram positive (A) *Staphylococcus aureus* and (B) Gram negative *Escherichia coli*. *S. aureus* retains crystal violet and appears as purple cells and *E. coli* retains safranin showing as red/pink colonies under a microscope (Cotter 2006)**

The differences between Gram positive and Gram negative cell walls are summarised in Table 1. Gram negative bacteria possess an outer lipid bilayer made up of phospholipids and lipopolysaccharide moieties called the outer membrane. This outer lipid layer is linked to a thin peptidoglycan layer in the periplasmic space (Figure 3). Gram positive bacteria have teichoic acids in their cell walls which consist of polyhydric alcohol (ribitol or glycerol) and phosphate groups. There are two classes of teichoic acids; lipoteichoic acid and wall teichoic acid. Lipoteichoic acids transverse the peptidoglycan layer and are physically connected to lipids in the cytoplasmic membrane. The wall teichoic acids do not transverse the peptidoglycan layer but are physically connected to it. Teichoic acids are negatively charged because of the phosphate group in their molecular structure. This adds rigidity to the cell wall by binding to cations such as magnesium and sodium. They are also involved in regulating the movement of cations into and out of the cell (Todar 2009).



**Figure 3 The cell wall structure of Gram positive and Gram negative bacteria (Cotter 2006)**

**Table 1 Differences between the cell walls of Gram positive and Gram negative bacteria (Cotter 2006)**



### 1.2.2 Cytoplasmic membrane

The cytoplasmic membrane or plasma membrane is a lipid bilayer 5 – 10 nm thick lining the inner surface of the cell wall. It acts as a permeability barrier and contains a range of different transport systems that mediate the inflow and outflow of metabolites to and from the protoplasm (LaMorte 2012). Prokaryotic cells lack intracellular organelles for respiration or photosynthesis, but many species possess the ability to conduct these processes as a function of their plasma membrane (Todar 2009).

### 1.2.3 Capsule

Some bacterial species possess a third protective layer of complex carbohydrates (polysaccharides) covering the outside of the cell wall that help to protect the bacteria from phagocytosis by larger micro-organisms. This layer is called

the capsule or glycocalyx. The capsule is considered to be a major virulence factor, as it enhances the ability of bacteria to cause infection (Paniker 2005). The capsule also protects bacteria from engulfment by white blood cells (phagocytes) and from attack by antimicrobial agents of plant or animal origin (Todar 2009).

### 1.3 Control of microbial growth

"Control of microbial growth" here refers to the preventive measures used to inhibit or restrict microbial growth. This can be done either by killing or by inhibiting the growth of microorganisms. The agents which kill bacteria are called bactericidal and agents which inhibit the growth of bacteria are referred to as bacteriostatic. A bactericide kills bacteria; a fungicide kills fungi and so on.

It is very important to control microbial growth in many practical situations such as in medicine, food science, water and in agriculture. In terms of medical microbiology, uncontrolled growth of microbes can lead to infections and outbreaks that can cause death. In a laboratory environment working under aseptic conditions (sterile conditions) is very important to obtain accurate results.

Sterilization in microbiology is a process of complete destruction of microbes. There is no degree of sterilization: either an object is sterile or it is not. Microbial growth can be eliminated using physical or chemical methods which either kill or inhibit growth. Physical methods include the use of heat, radiation, filtration, boiling and autoclaving. Chemicals used to decontaminate objects are termed disinfectants. Examples of chemicals used for sterilization include; formaldehyde, ethylene oxide, hydrogen peroxide, phenol, ozone and peroxyacetic acid. These compounds are biocides that destroy microorganisms but due to their toxicity, their application is restricted to cleaning products (Denyer and Stewart 1998). Microbial control in living

tissues requires compounds with higher selectivity and lower toxicity than that of disinfectants (Denyer and Stewart 1998).

### 1.3.1 Antiseptics and Antibiotics

Antiseptics are antimicrobial substances that are chemically non-toxic to living tissue and are applied to reduce the chance of infection. Antiseptics include iodine, silver nitrate, alcohol and hydrogen peroxide. Antiseptics generally act in a non-specific manner on bacterial cells, disrupting cell membranes. They injure the plasma membrane and denature proteins resulting in the inactivation of enzymes (Demezas 1999).

Antibiotics are drugs that either kill or inhibit the growth of bacteria. They can be natural, synthetic or semi-synthetic compounds. Antibiotics are classified using various schemes such as their bacterial spectrum of activity (broad or narrow spectrum) or the type of activity they display (bacteriostatic or bactericidal). The main classes of antibiotics are: β-lactams, macrolides, tetracycline and aminoglycosides (Todar 2009). Antibiotics principally operate by interfering with bacterial chemosynthesis: i.e. the synthesis of DNA, RNA, proteins and the cell wall. To minimize toxicity during use, the target of the antibiotic should be very selective.

Many antibiotics act against the cell walls and cell membranes of bacteria. The cell wall of bacteria continuously expands by the synthesis of peptidoglycan (a polymer of sugar), N-acetyl glucosamine (NAG) and amino acid, N-acetylmuramic acid (NAM) strands. These strands are linked together by an enzyme called trans-peptidase to form a rigid cell wall. For cells to grow in size and reproduce, the cell wall must be able to expand with the growing interior. Some antibiotics interfere with the synthesis of peptidoglycan by preventing cross linkage of the strands. This

reduces the strength of the cell wall and can result in osmotic lysis of the cells (Fox 2010).

β-lactams such as penicillin target cell walls in this way. The β-lactam ring gives the antibiotics its function. It is structurally similar to the substrate of the enzyme involved in the last stage of cell wall synthesis. The antibiotic binds irreversibly to the enzyme preventing the formation of cross links in the peptidoglycan. Other side chains or modifications to the β-lactam rings can confer traits such as resistance to enzymes that degrade penicillin, a change to the mechanism of cell wall penetration or a reduction in toxic side effects (Fox 2010).

Besides the β-lactam antibiotics that target the cell wall, the largest group of antibiotics are those that inhibit protein synthesis. Protein synthesis is a process that involves RNA transcription and translation. Many classes of antibiotics target ribosomal subunits or critical enzymes to prevent translation and inhibit growth. Most antibiotics target RNA translation. In this process, bacterial ribosomes read the messenger (mRNA) sequence and bind to transfer RNA molecules (tRNA) carrying the amino acids to be added to the growing protein chain. Antibiotics are able to specifically target this step because the ribosomes of prokaryotes are sufficiently different from those of eukaryotes (Ware 2012).

### 1.3.2 Antibiotic resistance

Bacterial resistance to antibiotics has seriously hampered bacterial growth control. Resistance mechanisms include: changes in the permeability of the cell envelopes, enzymatic degradation of antibiotics, chemical modification of the antimicrobial agent and the presence of membrane efflux systems which pump out the antimicrobial agents from the cytosol (Köhler, Pechère and Plésiat 1999, Russell

1995, Wright 2003). Microorganisms can acquire resistance either by acquisition of genetic material (plasmids) from another cell or by mutation (Figure 4).



**Figure 4 Examples of biological mechanisms of antibiotic resistance. Image taken from http://www.nature.com/nm/journal/v10/n12s/box/nm1145_BX4.html**

Extensive and often unwarranted use of antibiotics has led to the emergence of new strains of antibiotic-resistant bacteria. Methicillin-resistant *Staphylococcus aureus* (MRSA) is perhaps the best known example, it shows resistance to multiple antibiotics and causes infections that are very hard to treat (Cohen 1992, Levy and Marshall 2004). Furthermore, increased resistance to some antimicrobial agents (QACs, chlorhexidine and diamidines) has been found in MRSA strains carrying genes encoding resistance to gentamicin, an aminoglycoside antibiotic (Russell 1995).

There are a number of reasons to be concerned about antimicrobial resistance. Firstly the fact that the resistant bacteria especially Staphylococci sp.,

Enterococci sp., *Klebsiella pneumoniae* and Pseudomonas sp. are becoming a major part of a hospital environment (Edmond *et al.* 1999, Jones *et al.* 2002, Karlowsky *et al.* 2003). The emergence of these resistant strains poses a major threat to humans and the environment. Since the introduction of penicillin by Fleming in 1929, various new classes of antibiotics have been introduced to treat bacterial infections (Ter Meulen 2009). However, due to excessive misuse of antibiotics, resistance has been reported against nearly all currently available antibiotics (Gopal Rao 1998). Thus there is an important need to discover antimicrobials which can be used solely or in combination with other natural antimicrobial agents.

Secondly, in regards to the financial pressures felt in today's healthcare systems, antibacterial drug resistance has a major economic impact on hospitalization costs (McGowan Jr. 2001). Despite the increase in antimicrobial resistance, the discovery of new antibiotics has slowed significantly during the last 50 years. As a result of the low potential for profitability, the development of new antibiotics has been considered to be too expensive for pharmaceutical companies. Moreover, governments have also reduced investment in research on infectious diseases, due to them being largely viewed as easily treatable (Conly and Johnston 2005).

## 1.4 Hospital acquired infections (HAIs)

Hospital acquired infections are infections associated with treatment in a hospital or any other health care setting. They are also referred to as nosocomial infections. The word nosocomial is derived from the Greek words 'nosos' meaning "disease" and 'komein' meaning "to take care of" (Vincent 2003). An infection is considered to be nosocomial if it appears after 48 hours of hospital admission or

within 30 days of discharge. A wide range of bacteria, fungi and viral pathogens are responsible for such infections. Many of the species involved are part of the normal flora (Table 2) (Inweregbu, Dave and Pittard 2005). These pathogenic microorganisms can cause infections of the skin, heart, joints, wound sites, operation sites, lungs (pneumonia), urinary tract and other parts of body.

**Table 2 Microorganisms commonly associated with hospital acquired infections (HAIs) (NNISS Jan 1989 – Jun 1998) (Inweregbu, Dave and Pittard 2005)**



In recent years, hospital acquired infections have become a major issue of concern in European health care systems. The health care sector is at particularly high risk for several reasons. Already ill patients are often more vulnerable to further infections and can be more likely to develop other serious complications. Hospital environments are also home to many antibiotic resistant bacteria. Although many

procedures are in place to ensure patient safety in hospitals, nosocomial infections are a significant burden to the health care sector and to society as a whole (Ter Meulen 2009).

The European Commission estimates that in the EU (Yaneva-Deliverska 2011):

- 1 in 10 patients are affected by nosocomial infections
- 50,000 people die each year of healthcare-related infections
- 3 million deaths are partly caused by healthcare-related infections

The financial impacts of these infections counteract benefits made from medical advances and add expensive medical treatments. Healthcare costs are also raised due to increases in the length of hospital stay by at least 8 days on average per affected patient (Yaneva-Deliverska 2011). Across Europe, this adds up to more than 10 million additional days of patient stay in hospitals each year, which results in an increased mortality rate and additional healthcare costs (Yalcin 2003).

Some of the most common bacterial species responsible for causing nosocomial infections are:

- *Staphylococcus aureus*
- Methicillin-resistant *Staphylococcus aureus* (MRSA)
- *Pseudomonas aeruginosa*
- *Escherichia coli*
- *Acinetobacter baumannii*
- *Klebsiella pneumonia*

### 1.4.1 *Staphylococcus aureus*

*Staphylococcus aureus* is a Gram-positive spherical cocci measuring approximately 1 μm in diameter and is arranged in characteristically grape like clusters. It is a common cause of hospital and community acquired infections. Methicillin-resistant strains (MRSA) display resistance to β-lactam antibiotics and other antibiotics and present very challenging problems for treatment and eradication control (Johnston and Bryce 2009). It has been recovered from various surfaces within a hospital environment including floors, linens, air vents, tourniquets, bed sheets, dry mops, bandages, etc. In burns units, staphylococci have been found as a major contaminant on surfaces acting as a reservoir for infection (Shiomori *et al.* 2002). The evolutionary change in microorganisms due to increased use of antimicrobials has led to a rapid spread of MRSA globally. The worldwide prevalence of MRSA in blood cultures, varying from 0.6% in Netherlands to 66.8% in Japan, shows that this is an emerging strain in the community rather than just a hospital pathogen (Deurenberg *et al.* 2007).



**Figure 5 SEM image of morphology of *Staphylococcus aureus*. Image taken from http://www.bacteriainphotos.com/StaphylococcusaureuselectronmicroscopyHTML**

### 1.4.2 *Pseudomonas aeruginosa*

*Pseudomonas aerugionsa* is an aerobic, Gram-negative, rod shaped, non-spore forming bacilli measuring 1.5 - 3 x 0.5 µm, actively motile by a polar flagellum. It is a highly opportunistic nosocomial pathogen. According to data collected by the Centers for Disease Control (CDC) and Prevention of National Nosocomial Infections Surveillance system between 1990 - 1996, *Pseudomonas aeruginosa* was the second most common cause of nosocomial pneumonia (17%), third most common cause of urinary tract infection (11%), fourth most common cause of surgical site infection (8%), fifth most common isolate (9%) obtained overall and the seventh most commonly isolated pathogen from the blood stream (El Solh and Alhajhusain 2009). It commonly colonizes moist areas of the human body such as the axilla, ear and perineum. Moisture is a crucial aspect of its occurrence within hospital reservoirs and it has been isolated from disinfectants, sinks, mops, bandages, respiratory equipment and vegetables (Diekema, Pfaller and Jones 2002).



**Figure 6 SEM image of morphology of *Pseudomonas aeruginosa*. Image taken from https://globalmedicaldiscovery.com/wp-content/uploads/2014/10/Pseudomonas-aeruginosa.jpg**

### 1.4.3 *Escherichia coli*

*Escherichia coli* is a facultative anaerobic Gram-negative bacilli measuring approximately 1.1 - 1.5 x 2 - 6 µm, commonly present in the intestine of humans and animals. Most strains of *E. coli* are harmless but some serotypes such as O15:H7 are pathogenic. Multidrug resistance *E. coli* strains are a serious problem causing nosocomial infections via direct person to person transmission, via environmental contamination or through water or food contamination (Paniker 2005).



**Figure 7 SEM image of morphology of *Escherichia coli*. Image taken from http://www.bbc.co.uk/news/health-13639241**

### 1.4.4 *Acinetobacter baumannii*

*Acinetobacter baumannii* is an aerobic Gram-negative pleomorphic bacillus commonly isolated from hospitalized patients or hospital environments. *A. baumanni* has recently emerged as a significant threat due to its developed resistance to many common antibiotics, making it multi-drug resistant bacteria (Yang *et al.* 2010). The ability of *A. baumannii* to colonize a variety of hospital surfaces, such as catheters and surgical drains, has caused major concerns in the healthcare market. It is a

prevalent human pathogen which mostly affects patients with compromised immune systems. It can grow under any conditions that are abiotic, wet or dry. Strain 19606 of *A. baumannii* can even form biofilms on glass surfaces via pili formation. This strain can survive on a range of different surfaces in a hospital including furniture, catheters, respiratory tubes and bed sheets (Manchanda, Sanchaita and Singh 2010).



**Figure 8 SEM image of morphology of *Acinetobacter baumannii*. Image taken from http://www.acinetobacter.org/**

### 1.4.5 *Klebsiella pneumoniae*

*Klebsiella pneumoniae* is non-motile, encapsulated, lactose fermenting, facultative anaerobic Gram-negative bacteria found in the normal flora of human skin, mouth and intestine. It is one of the most common hospital acquired pathogens and an important member of Klebsiella genus of Enterobacteriaceae family. It has become an important pathogen in nosocomial infections, causing urinary tract infections, pneumonia and intra-abdominal infections. It is also seen as a prospective

community-acquired pathogen. *Klebsiella pneumoniae* tends to affect people with low immune response and can colonize invasive instruments such as intravenous catheters, urinary catheters and breathing tubes (Jarvis, Munn and Highsmith 1985).



**Figure 9 SEM image of morphology of *Klebsiella pneumoniae*. Image taken from http://klebsiella-pneumoniae.org/klebsiella_pneumoniae_urinary_tract_infection.html**

### 1.4.6 Biofilms and their role in nosocomial infections

A biofilm is a group of microorganisms in which cells adhere to each other on a surface. Biofilms are commonly embedded in an extracellular matrix consisting of polysaccharides, proteins and DNA. This slimy, glue like substance helps the cells to adhere to any kind of surface such as metals, plastics, soil particles, medical implant materials or biological tissues. A biofilm can be formed on almost any surface which is exposed to bacteria and some moisture (Bjarnsholt 2013).

**Figure 10 Differences between planktonic cells and biofilms. A biofilm is composed of microbial cells attached within a matrix of extracellular polymeric secretions (EPS), which surround and protect cells. The EPS matrix is typically composed of polysaccharides, proteins, lipids, and extracellular DNA. Image taken from http://www.nature.com/scitable/content/a-biofilm-is-composed-of-attached-microbial-100450918**

Historically, bacteria have primarily been characterized as planktonic or freely suspended cells. It was Van Leeuwenhoek, who first described the phenomenon that bacteria have the ability to attach to and grow on exposed surfaces as a sessile aggregate. He referred to this as the biofilm mode of growth (Donlan 2002). Given the appropriate conditions, all bacteria have the ability to grow as a biofilm.

Most of the research on bacterial pathogenesis has been focussed on acute infections. These are assumed to be caused by planktonic bacteria and are generally treatable with appropriate antibiotics. However, research has shown that the biofilm mode of growth is very important in infections, particularly chronic ones (Bjarnsholt 2013). The presence of biofilms has profound implications for the patient because of their ability to resist host immunity, antibiotics and other antimicrobial agents. Unlike

planktonic cells, the cells embedded in the biofilm structure are physically protected biologically and chemically by the extracellular matrix (Bryers 2008, Percival and Bowler 2004).

It has been estimated that biofilms are associated with 65 percent of nosocomial infections. Most of the bacterial species that cause these infections are members of the normal micro flora of humans and form biofilms at sites where they exist as harmless commensals. Therefore, it is now appreciated that in order for antibacterial agents to effectively counter infections they should be active against both biofilms and planktonic cells (Percival and Bowler 2004).

## 1.5 Infection prevention

The most common routes for the spread of infections within hospital settings are via airborne aerosols or via indirect contact. For example spread by indirect contact can occur if an infected patient touches and contaminates an object, an instrument or a surface and that same surface is touched by a second person. Indirect contact can be mediated through patient bedding, clothes, wound dressing, staff lab coats, surgical instruments and other surfaces (Ayliffe *et al.* 1992, Burke 2003). Some of the surfaces that can potentially become contaminated and spread infection within hospital settings are shown in Figure 11.

According to Ayliffe *et al.* (1992), there are three basic principles that should be taken into account to control and prevent infection:

i. Removal of the source infection by treating infections and proper decontamination procedures
ii. Using aseptic procedures (good hand hygiene) and appropriate isolation (wherever necessary) to prevent transfer of infection

iii. Appropriate antibiotic prophylaxis or vaccination

One of the most effective ways to combat the spread of infections is by following hand hygiene protocols such as thorough hand washing and/or the use of alcohol rubs before and after each patient contact. Infection control programs including such practices have proved to be very successful in reducing rates of nosocomial infections (Collins 2008).



**Figure 11 Potential contaminated surfaces in a hospital room. Image taken from http://news.cnet.com/8301-27083_3-20068434-247/**

### 1.5.1 Medical textiles and infections

Direct contact is the most likely route for the transmission of infection; however, the role of the environment should not be ignored within hospital settings. Fabric surfaces in hospitals may be a route for the spread of nosocomial infections

(Bureau-Chalot *et al.* 2004). Hence, textiles are an important factor to consider in an integrated infection control program of every healthcare facility (Tinker 2010). Healthcare textiles include blankets, bed sheets, lab coats, gowns, towels, personal clothing and drapes for surgical procedures. Medical textile surfaces can be heavily contaminated with microorganisms from substances like urine, blood, stool, sweat and other body fluids (Fijan and Turk 2012).

In terms of pathogen transmission, textiles play a crucial role in the chain of infection caused by pathogenic microorganisms like bacteria, fungi and viruses (Borkow and Gabbay 2008). Textiles of all varieties are considered to be very proficient at carrying bacteria and serving as a reservoir for the transmission of infection (Tinker 2010). However, the survival of pathogens on textile substrates varies considerably. For example, some spore forming bacteria are highly durable and can survive for long periods on fabrics while other bacteria die within a few minutes of contact due to poor moisture conditions (Neely and Maley 2000, Neely and Orloff 2001).

A list of reports of the presence of microorganisms on hospital textiles is shown in Table 3. It is clear that a demand exists for antimicrobial finished textiles that are able to limit or avoid fabric degradation, odour generation and most importantly bacterial incidence and spread (Ibrahim, Refaie and Ahmed 2010, Mahltig, Haufe and Böttcher 2005, Parthasarathi and Borkar 2007, Rajendra *et al.* 2010, Ramachandran, Rajendrakumar and Rajendran 2004).

**Table 3 Reports on the presence of microorganisms on hospital textiles**

| Surviving microorganisms | Hospital textile | Time | Reference |
|---|---|---|---|
| Moulds | Sheets, pyjamas | After use by patients | Bureau-Chalot *et al.* 2004 |
| Coagulase - negative staphylococci, *Bacillus* spp. | Sheets, pyjamas, uniforms | After laundering in hospital laundry | Fijan *et al.* 2007 |
| *Staphylococcus aureus*, *Clostridium difficile* | Nurse's u iforms | After 24h shift | Perry, Marshall and Jones 2001 |
| *Acinetobacter baumannii*, MRSA | Bed linen, uniforms and curtains | After laundering in hospital launderies | Weinstein and Hota 2004 |
| Vancomycin resistant enterococci (VRE) | Bed linen | 11 weeks after inoculation | Hochmuth, Magnuson and Owens 2005 |

A number of studies have shown that hospital textiles are a potential route for transmission of pathogens (Duffy *et al.* 2010). A key reservoir for the spread of pathogens in hospitals has been found to be the patient's bed. The Centre for Disease Control and Prevention (CDC) reported outbreaks of nosocomial zygomycosis, where deaths occurred. Zygomycotic infections are fungal infections of the head, lungs or digestive tract, which are very hard to treat. The sources of pathogens reported in these outbreaks were pillow case, linen and healthcare workers coats (Duffy *et al.* 2010).

In 2006, Kramer *et al.* reported on the persistence of hospital pathogens associated with nosocomial infections (Kramer, Schwebke and Kampf 2006). *Staphylococcus aureus* was found to be present in a significant percentage of the sites tested: 12.6% of cotton coats, 9.2% of plastic aprons, 15% of the care workers'

uniforms and 15% of areas in an isolation ward. Thiry (2005) showed that *S. aureus* can survive on hospital curtains for up to 90 days.

Reif *et al.* (1989) reported on a case where inadequate cleaning of textiles contaminated with faeces resulted in an outbreak of Cryptosporidiosis (Reif *et al.* 1989). Cryptosporidiosis is a serious diarrheal disease caused by an intestinal parasite *Cryptosporidium*.

Lidwell *et al.* (1974) undertook a study in a UK hospital where it was found that the strains of *S. aureus* on nurse's dresses were transferred on to patient's bed clothes and their hands. This was also demonstrated by Hambreus (1973).

Bloomfield (2007) analysed risk assessment data to assess the frequency and probability of transfer of contamination to which family members are most commonly exposed to within a home. From this data, certain sites and surfaces were ranked according to the level of risk involved. Bloomfield suggested hands as the most critical point of spread of infection (Figure 12). From the hands the infection can spread via cleaning cloths, clothing and household linens, which form a bridge in the spread of a pathogen around the home. Bloomfield also considered proper handling and cleaning of textile products, as a critical factor in maintaining hygienicity (Bloomfield 2007).



**Figure 12 The sites and surfaces at home based on the level of risk involved in the transmission of infection. Image taken from http://www.eurosurveillance.org**

### 1.5.2 Survival of pathogens on textile surfaces

A study was conducted to examine the survival rates and variability of Gram-positive bacteria on textile fabrics. The experiments involved 22 different isolates of methicillin-sensitive and resistant staphylococci and vancomycin-sensitive and resistant enterococci. These were applied on to five of the most commonly used fabric materials in hospitals.

100% cotton used in clothing

100% terry cotton used in wash cloths and towels

60/40% cotton/polyester mix used in lab coats, clothing and scrub suits

100% polyester used in clothing and hospital curtains

100% polyethylene plastic used in splash aprons

It was shown that all isolates of methicillin-sensitive and resistant staphylococci survived for at least one day. Survival time was long on polyethylene plastic (22-90 days) and polyester cotton (1-56 days). The minimum enterococcus viability on all test fabrics was 11 days. Out of all species tested, *E. faecium* survived for the longest period on all test surfaces (11-90 days) (Neely and Maley 2000).

All these studies showing the survival of pathogens highlight the potential impact of textiles in the transmission of infections. Studies have also demonstrated that even washing of fabrics is not guaranteed to wash microorganisms from their surface. The washing of fabrics may remove dirt and stains but they are still far from being sterile or free from microorganisms (Telfer Brunton 1995).

To prevent the spread of infection, hospital textiles must be processed stringently under hygienic conditions and on a professional scale. Processes used for the cleaning of medical textiles should be recorded, certified and monitored

regularly. To break the chain of transmission for both nosocomial and out-patient infections caused by textile products, it is essential that preventive measures should take place in the washing processes.

Conventional methods of disinfection have major drawbacks. This includes treatment methods such as autoclaving or intense hot washing damages the structure and reduces fabric's lifetime. Due to the texture and non-homogenous cavities in fabrics, it is also difficult to disinfect them with UV radiation. Disinfection with chemicals also has many drawbacks. First, residual chemicals can be harmful to the patient's skin and can react with patient's wounds to cause secondary infections. Secondly, diffusion of fabrics with chemicals increases treatment time. Finally, most chemicals and disinfectants are highly toxic and flammable and could be a danger to workers and hospital environment (Neely and Orloff 2001).

### 1.5.3 The role of antimicrobial textiles

Antimicrobial textiles could have an important role to play in maintaining hygienic standards in medical settings by breaking chains of infection (Bearman *et al.* 2012). Antimicrobial fabrics have been shown to be very effective against a wide variety of microorganisms and fungi and have the potential to prevent the spread of pathogens (Simoncic and Tomsic 2010).

Medical fabrics with antimicrobial properties should be able to fulfil the following objectives (Shanmugasundaram 2007):

1. To avoid cross infection by pathogenic microorganisms
2. To control infestation by microbes
3. To arrest metabolism in microbes to reduce odour formation

4. To safeguard the textile products from staining, discolouration and quality deterioration

An ideal antimicrobial textile fabric should also satisfy a number of requirements (Gao and Cranston 2008):

1. Durability (washing, dry-cleaning and hot pressing)
2. Effective against a wide variety of bacterial and fungal species
3. Should be user friendly and not harmful to the manufacturer
4. Should fulfil the statutory requirements by regulating agencies
5. Easy method of application
6. Not lead to reduction of fabric quality (strength, breathability etc.)
7. Be resistant to body fluids

### 1.5.4 Antimicrobial agents for textiles

Various classes of antimicrobial agents are used in the textile industry. These agents can be broadly classified into two main types:

- Organic antimicrobial materials
- Inorganic antimicrobial materials

### 1.5.5 Organic antimicrobial agents

Organic antimicrobial agents are often complex toxic bactericide which works by leaching out from the fabric surface into the surroundings. They are mostly carbon based and are often degraded by heat, humidity and mechanical processing. They are limited in their applications due to being less stable and durable than inorganic

agents; particularly at high temperatures and pressures. They include triclosan, chitosan, sorbic acid, quaternary ammonium compounds (QACs), paraben and salicylic acid (Shigehara, Bito and Katayama 2007).

### 1.5.6 Triclosan

Triclosan is a synthetic and non-ionic antimicrobial agent used in a variety of household products. Triclosan exhibits a broad spectrum antimicrobial activity against prokaryotes, even at very low concentrations (Orhan, Kut and Gunesoglu 2007). It is an organic aromatic compound produced by the oxidation of benzene or benzoic acid by the Cumene or Raschig process. It is one of the most popular additives to toothpastes, hand washes, soaps, mouthwashes and has also been incorporated into fabrics and plastics (Orhan, Kut and Gunesoglu 2007). Triclosan mainly acts by blocking lipid biosynthesis through inhibition of fatty acid biosynthesis (Orhan, Kut and Gunesoglu 2007). However, there have been concerns over it's effects on the environment. Due to its specific action, it can foster resistant bacteria (Adolfsson-Erici *et al.* 2002, Orvos *et al.* 2002).



**Figure 13 Structure of triclosan. Image taken from http://www.niehs.nih.gov/research/supported/dert/programs/srp/phi/archives/publicpolicy/triclosan/index.cfm**

### 1.5.7 Quaternary ammonium compounds (QACs)

Quaternary ammonium compounds (QACs) are positively charged poly ions also known as quats. They are an important class of biocides widely used as disinfectants and antiseptics. These compounds inflict a variety of detrimental effects on microbes: damaging bacterial cell membranes, denaturing proteins and disrupting the entire cell structure. QACs are very durable and exhibit a strong antimicrobial activity (Tischer *et al.* 2012). They have been used as a coating agent on textile substrates to impart antimicrobial properties (Kim, Kim and Rhee 2010, Latlief *et al.* 1951). They have the ability to display antimicrobial activity as long as the compound remains intact on the textile surface (Gao and Cranston 2008). To overcome this problem, QACs have been anchored to a polymer backbone through a covalent non-hydrolysable bond that increases the antimicrobial properties (Kim, Kim and Rhee 2010). QACs strongly attach to textile surfaces by ionic interaction between the anionic textile fibre surfaces and the cationic QAC (Kim and Sun 2001).



**Figure 14 Structure of quaternary ammonium cations. Image taken from http://www.innovationsinmedical.com/technologies/QUAT/**

### 1.5.8 Chitosan

Chitosan is a derivative of chitin, which is a polysaccharide found in the exoskeleton of shrimp, crabs and other sea crustaceans. Apart from its antimicrobial activity, chitosan is a non-toxic, biocompatible and easily biodegradable compound. The antimicrobial mechanism of chitosan is not clear but studies have shown that chitosan increases the permeability of the outer and inner membranes and can lead to the release of cellular contents. The process occurs through electrostatic interaction between the $C\text{-}NH_3^+$ groups of chitosan acetate and the phosphoryl groups of phospholipid components in bacterial cell membranes (Liu *et al.* 2004). Chitosan and its derivatives have been used as an antimicrobial agent on textiles (Joshi *et al.* 2009). Due to its weak adhesion to cellulose fibres, chitosan has been used by the textile industry in combination with cross linking agents to attach onto cellulose fibres (Gao and Cranston 2008). Being one of the safest and most effective antibacterial agents, it has been widely used as an antimicrobial finish on textiles (Ye *et al.* 2005).



**Figure 15 Structure of chitosan. Image taken from http://www.jnanobiotechnology.com/content/9/1/55/figure/F6?highres=y**

### 1.5.9 Polyhexamethylene biguanide hydrochloride (PHMB)

PHMB is a fast acting, broad spectrum antimicrobial biocide, consisting of a heterodisperse mixture of polyhexamethylene biguanides. PHMB attacks bacterial cells by binding to the cell membrane and damaging it causing cell lysis and leakage of the cytoplasm (Gao and Cranston 2008). PHMB has a proven track record and has been used in a variety of disinfectants, hygiene products, food industry applications and in textile industries (Rajendran 2010, Gao and Cranston 2008, Ristić *et al.* 2011). A specific grade of PHMB has been designed by Arch chemicals for textile treatments named Reputex 20® (Swofford 2010).



**Figure 16 Structure of polyhexamethylene biguanide hydrochloride (PHMB). Image taken from http://www.zlchemical.com/phmb.html**

### 1.5.10 Inorganic antimicrobial agents

In recent years, inorganic antimicrobial agents such as metals and metal oxides have attracted much attention due to their ability to withstand intensive processing conditions and their improved safety and stability (Perelshtein *et al.* 2009a). There is also growing interest in the formation of particles at a nanoscale.

It is evident that nanoparticles exhibit unique properties which considerably differ from their macroscopic form. Due to their high surface to volume ratio, nanoparticles interact better with microbes.

Many heavy metals, either in their free state, or in compounds at very low concentrations, are considered toxic to microbes. They commonly kill bacteria by binding to intracellular proteins and inactivating them (Gao and Cranston 2008). In the textile industry, inorganic metal oxides such as ZnO and CuO have attracted attention due to their antimicrobial properties, their safety to humans and also their stability under harsh conditions (Rajendra *et al.* 2010). Due to their unique physical and chemical properties, the use of inorganic nanoparticles on textile surfaces has become a hotspot within the textile research to produce functional fabrics (Herrera *et al.* 2006, Kruis and Fissan 2001).

### 1.5.11 Silver

Silver is a metallic element with potent antimicrobial properties. Silver ions and silver based compounds are highly toxic to microorganisms (Slawson *et al.* 1992, Zhao and Stevens Jr. 1998). For this reason they have been used as an antimicrobial coating on various substrates including medical devices (Schierholz *et al.* 1998), textile fabrics and wound dressings (Durán *et al.* 2007b, Gittard *et al.* 2010, Wasif and Laga 2009).

It has been reported that silver metal ions catalyse the production of oxygen radicals which oxidise various chemical components within bacteria (Kim *et al.* 2007). This mechanism does not require direct contact with bacteria, as the active oxygen produced is able to diffuse from the silver to the surrounding environment (Kim *et al.* 2007). Silver ions can also disrupt bacterial proteins and enzymes by attaching to sulfhydryl, amino phosphate and carboxyl groups by reacting with nucleophilic amino acid residues (Kaur, Saxena and Vadehra 1985). Some commercially available textile products coated with silver are listed in Table 4.

**Table 4 Commercially available silver based antimicrobial agents and fibres (Simoncic and Tomsic 2010)**



### 1.5.11.1 Research into silver antimicrobial textiles

Schaller *et al.* (2003) examined the antimicrobial properties of two hydrocolloid wound dressings containing silver (Contreet-H®) on reconstituted human epithelium (RHE) cells infected with *C. albicans* and *S. aureus*. These results showed that wound dressings impregnated with colloidal silver effectively inhibited both pathogens in infected epithelium cells. Results also suggested that the release of silver in a moist environment improved the healing process compared to the dressings without silver.

Ilic *et al.* (2009) examined the antimicrobial efficiency of cotton fabrics loaded with colloidal silver nanoparticles against Gram negative *E. coli*, Gram positive *S. aureus* and the fungus *Candida albicans*. The researchers also assessed the

laundering durability of coated fabrics. The concentration of silver colloid used was 10 and 50 ppm. The antimicrobial activity was quantitatively assessed using a shake flask method with conventional agar plating. The results showed an excellent antibacterial activity (99.9% reduction) for the cotton fabrics loaded with 50 ppm colloidal silver nanoparticles against *S. aureus*, *E. coli* and *C. albicans*. The 10 ppm Ag NPs loaded fabrics only gave maximum microbial reduction against *S. aureus* (99.9%). The cotton fabrics loaded with the 10 ppm silver nanoparticles also exhibited very poor laundering durability. Overall results indicated that a high concentration of silver colloid (50 ppm) was needed for a durable antimicrobial finish of cotton fabrics.

Similar results were also reported by Lee *et al.* (2003). They showed that fabrics loaded with silver nanoparticles from a 50 ppm colloidal suspension exhibited excellent antibacterial activity against *K. pneumoniae* and *S. aureus*; the activity of the 50 ppm was better than samples treated with a 25 ppm colloid.

Kostić *et al.* (2008) used a chemisorption method to incorporate silver ions from silver nitrate solution into a cotton/polyester mix fabric. A 50% cotton and 50% polyester weaved fabric was employed and treated in a dielectric barrier discharge (DBD) before incorporating silver ions. Agar diffusion tests on silver loaded cotton/polyester fabric against *S. aureus*, *E. coli* and *C. albicans* showed an excellent antimicrobial activity with the yeast *C. albicans* being the most sensitive organism.

However, ecologists have also suggested potentially harmful consequences from the release of silver nanoparticles into natural water systems. Silver in its bulk form has also been shown to result in negative effects on fish, fungi, algae and plants (Völker, Oetken and Oehlmann 2013). The current widespread use of silver as

a key antimicrobial component has also been associated with the development of resistance by some bacteria, analogous to antibiotic resistance.

Due to the significant scale of silver nanoparticles use, their release into the environment has potentially become a serious issue in particular for the textile industry (Benn and Westerhoff 2008, Geranio, Heuberger and Nowack 2009). Kulthong *et al.* (2010) published a study of the environmental issues of silver nanoparticles in which they determined the quantity of silver released from commercial fabrics coated with nanosilver and laboratory-prepared silver coated fabrics into various formulations of artificial sweat. The study was performed according to AATCC, ISO and EN standards. The amount of silver released from five laboratory-prepared fabrics and six commercially prepared fabrics was investigated. The laboratory prepared fabrics were treated with the silver suspension at concentrations of 0, 0.5, 1, 5 and 10 g/L using a laboratory padder (model PA-U, Newave Lab Equipments, Taiwan) with 80% wet pick-up of the fabric weight. The fabrics were cured at 120°C for 3 min. Commercial samples were purchased from six different manufacturers in Thailand. The antibacterial efficacy of the fabrics was also determined according to AATCC test method (100-2004) with some modifications against *E. coli* and *S. aureus*. Results were calculated as percentage reduction and showed that 10 g/L silver suspension was sufficient for preparing antibacterial fabrics. At this treatment concentration, the fabrics induced a 99.83% and 99.93% reduction in *S. aureus* and *E. coli* growth respectively. After incubation of the fabrics in artificial sweat, the level of silver released from the different fabrics varied but was typically greater than 50% of the amount present initially on the fabrics.

In another study, Duran *et al.* (2007) investigated the antibacterial efficiency of silver nanoparticles produced biologically by *Fusarium oxysporum*. Silver

nanoparticles were incorporated onto cotton fabrics by absorption and tested against *S. aureus*, demonstrating an excellent antibacterial activity (>99.9% reduction). Taking into consideration the environmental concerns regarding the release of silver, the effluents obtained from the cotton fabrics after washing were efficiently treated by *Chromobacterium violaceum*. The process was based on biosorption and proved efficient in eliminating silver nanoparticles released from the cotton fabrics into the wash water.

Another study on the release of silver from textiles was carried out by Geranio *et al.* (2009). Results determined the amount and form in which silver was released during washing from nine different fabrics that had been treated with incorporating silver nanoparticles into and onto the fibres. Results concluded that the amount and form of silver released strongly depend on the method by which the silver is incorporated into a fabric. The results showed a considerable variance in the percentage of the total silver emitted during one washing among 9 different textiles products tested.

Apart from the environmental concerns, there are also documented toxic effects of Ag on human health (Panyala, Peña-Méndez and Havel 2008). Prolonged exposure to silver can cause diseases like argyria and argyrosis. Argyria and argyrosis are the clinical conditions caused by silver or silver salts which turns the skin colour to blue or bluish grey. Gliga *et al.* (2014) investigated the toxicity of silver NPs in human lungs cells. The results showed that the silver NPs induced toxicity within 24 hours of exposure resulting in DNA damage. The researchers also demonstrated that the toxicity was associated with the rate of intracellular silver release called as a 'Trojan horse effect'. Huang, Lü and Ma (2014) studied the mechanism of toxicity of silver NPs in human dermal fibroblast (HDFs) cells. Their

results indicated that the induced toxicity affected four pathways by differentially expressed miRNAs. The results showed that SNPs induce toxicity in HDF cells by affecting the cytoskeleton, ATP synthesis and apoptosis.

Silver has been used in many commercially available healthcare products including textiles and wound dressings. A review paper by Percival *et al.* (2005) addressed the questions of silver overuse and emergence of microbial resistance to silver. Percival stated that despite many such claims, there have been very few studies undertaken to determine the prevalence of bacterial resistance to silver.

The potential toxicity of silver to humans and its persistence once released into the environment has prompted a demand for alternative eco-friendly antimicrobial coatings/impregnations and processes that could substitute for toxic chemicals in textile coatings/impregnations.

### 1.5.12 Zinc oxide (ZnO)

Zinc oxide is an inorganic compound which appears as a white powder and is insoluble in water. It is a polar inorganic crystalline material with many applications. It has a unique combination of interesting properties such as non-toxicity, piezoelectric and optical behaviour and low price (Kunjara Na Ayudhya *et al.* 2006, Liqiang *et al.* 2004). As it absorbs both UVA and UVB rays, ZnO has been used in ointments, creams and lotions to protect against sunburn and other damage to the skin caused by ultraviolet light (Godfrey *et al.* 2001). ZnO nanoparticles have been shown to be useful antibacterial and antifungal agents when used as a surface coating on materials and textiles (Abramov *et al.* 2009, Perelshtein *et al.* 2009a, Raghupathi, Koodali and Manna 2011, Sivakumar *et al.* 2010, Vigneshwaran *et al.* 2006, Yadav *et al.* 2006).

Nanoscale ZnO has been synthesized in various morphologies such as nanowires, nanorods, tetrapods, nanobelts and nanoparticles (Dutta *et al.* 2012, Gao and Wang 2004, Gao, Li and Wang 2005, Jayaseelan *et al.* 2012, Mitra *et al.* 2012, Perelshtein *et al.* 2012, Song *et al.* 2007). Due to its useful physical properties, many methods have been developed to synthesize zinc oxide nanocrystals including: chemical vapour deposition (Sun *et al.* 2004), soft chemical method (Vayssieres 2003), thermal evaporation (Yao, Chan and Wang 2002), vapour-liquid-solid process (Gao and Wang 2004), sol-gel process (Zhang *et al.* 2003), homogeneous precipitation (Liu *et al.* 2007), electrophoretic deposition (Liu *et al.* 2003) and by a sonochemical method (Abramov *et al.* 2009, Askarinejad, Alavi and Morsali 2011, Perelshtein *et al.* 2012, Perelshtein *et al.* 2009).

The underlying mechanism for the antibacterial activity of ZnO nanoparticles is still not very clear. Some studies have suggested that ZnO nanoparticles act by disrupting the cell membrane of bacterial cells (Brayner *et al.* 2006). Other studies have claimed that the production of intracellular reactive oxygen species (ROS) such as hydrogen peroxide produces the antibacterial effect (Jones *et al.* 2008, Sawai 2003). It has also been reported that ZnO is photo catalytic and can be activated by UV and visible light to generate reactive oxygen species (ROS). The hydroxyl radicals and superoxides cannot penetrate within the cell membrane whereas the reactive oxygen species (ROS) penetrate into the bacterial cell and cause damage (Padmavathy and Vijayaraghavan 2008).

#### 1.5.12.1 Research into ZnO antimicrobial textiles

Rajendra *et al.* (2010) investigated the antimicrobial properties of ZnO nanoparticles on 100% cotton woven fabric produced using a pad-dry-cure method.

Tests included fabrics treated with ZnO bulk powder to see the effect of nanoparticles compared to bulk ZnO on their antibacterial activity. The antibacterial activity was assessed qualitatively using an agar diffusion method and parallel streak method (AATCC test method 147-1992) and quantitatively by percentage reduction tests against *S. aureus* and *E. coli*. The disc diffusion results showed a maximum inhibitory effect of ZnO (both in bulk form and nanoparticles) against *S. aureus* compared to *E. coli*. Even with the parallel streak method, cotton fabrics coated with ZnO nanoparticles showed maximum inhibitory effect against *S. aureus* followed by *E. coli*. The percentage reduction test corresponded with the results of the disc diffusion and parallel streak tests. Cotton fabrics coated with ZnO nanoparticles showed percentage reductions of *S. aureus* by 94.16% followed by 86.5% for *E. coli*.

Fabrics tested for wash durability showed that a significant antimicrobial activity was retained by the ZnO nanoparticles coated fabrics for up to 10 washes. After 10 washes the % bacterial reduction was very low and there was no activity found in the fabrics after 20 washes. The fabrics coated with the ZnO bulk retained the antimicrobial activity only up to 5 wash cycles against *S. aureus* and *E. coli*.

Anita *et al.* (2010) assessed the antibacterial properties of cotton fabrics coated with ZnO nanoparticles. They used a wet chemical method to produce ZnO nanoparticles from soluble starch (stabilizing agent), zinc nitrate and sodium hydroxide. Cotton fabrics were immersed in a solution of 2% zinc oxide nanoparticles for 5 minutes and then passed through a padding mangle. The padded fabric was air dried and immersed in 2 g/l of sodium lauryl sulphate for 5 minutes to remove any unbound nanoparticles. Finally the cotton fabric was rinsed 10 times to remove any traces of soap and then air dried. The antibacterial activity was evaluated quantitatively against *S. aureus* and *E. coli* in accordance with AATCC 100 test

method. Plating was performed on nutrient agar medium and the percentage reduction of bacteria was calculated. Microbial reduction of >99.99 % was seen against *S. aureus* compared to 80% reduction for *E. coli*. The ZnO nanoparticles used for this study had an average size of 50 nm.

Vigneshwaran *et al.* (2006) impregnated cotton fabrics with ZnO-soluble starch nanoparticles synthesized using water as a solvent and soluble starch as a stabilizer. Antibacterial testing showed excellent activity against, *S. aureus* and *K. pneumoniae*. Transmission electron microscopy (TEM) and x-ray diffraction analysis confirmed the average size of ZnO nanoparticles to be around 38 ± 3 nm on the cotton fabric.

Toxicity studies have also shown that they do not cause damage to the DNA of human cells (Sójka-Ledakowicz *et al.* 2008, Yamada, Suzuki and Koizumi 2007). However, there have been some studies where researchers have reported toxic effects with ZnO nanoparticles. Dechsakulthorn *et al.* (2007) assessed the cytotoxicity of ZnO in human skin fibroblasts using the colorimetric MTT *in-vitro* assay. The results showed that human skin fibroblasts were sensitive to ZnO nanoparticles after 4 hours of exposure. Researchers also claimed that the toxicity substantially increased by lengthening the exposure time to 24 hours.

Najim *et al.* (2014) investigated the cytotoxicity of different sized ZnO nanoparticles in normal and cancer cell lines derived from lung tissue (Hs888Lu), neuron-phenotypic cells (SH-SY5Y), neuroblastoma (SH-SY5Y), human histiocytic lymphoma (U937), and lung cancer (A549). Their results demonstrated that the ZnO nanoparticles with an average particle size of around 85.7 nm and 190 nm showed cytotoxicity towards U937, SH-SY5Y and Hs888Lu cell lines. A549 cells did not show any cytotoxic effect. It was also found that the cytotoxicity of ZnO was size,

concentration and time dependent. The results showed that ZnO nanoparticles at a concentration above 1 mM were cytotoxic in different cell lines.

### 1.5.13 Copper oxide (CuO)

Copper was probably the first metal to be used by humans for practical purposes. Copper is malleable, ductile and can be beaten into various shapes and sizes. It is one of the key chemical elements which together with amino acids and fatty acids are an essential requirement to the human body and so it is considered safe for humans.

Copper and copper compounds have multifunctional properties (antibacterial, antiviral and antifungal) with promising applications (Borkow and Gabbay 2005, Borkow and Gabbay 2009). Copper ions on their own or in combination with other complexes have long been used as disinfectants on solid surfaces and human tissues. Copper has been reported to kill a wide variety of bacteria, yeasts, fungi and viruses. Research has shown that it is even effective against spores (Cross *et al.* 2003). Due to being multifunctional in its action, copper kills microorganisms within minutes of their exposure to it (Borkow and Gabbay 2009).

The mechanism behind the toxicity of copper to microorganisms is still unclear. It has been reported that copper may exert its toxicity though several mechanisms. The various routes, through which copper acts are: plasma membrane permeabilization, membrane lipid peroxidation, alteration of proteins and inhibition of the cell's normal biological activity and denaturation of nucleic acids (Borkow and Gabbay 2005, Borkow and Gabbay 2009). Copper catalyses the production of hydroxyl radicals through redox cycling between $Cu^{2+}$ and $Cu^{1+}$. These radicals cause damage to lipids, proteins and DNA (Valko, Morris and Cronin 2005).

Toxicity studies have shown that CuO coated fibres do not show any skin sensitization properties (Borkow and Gabbay 2004). The methods that have been used for impregnating CuO nanoparticles onto fabric substrates include: (i) coating CuO nano crystals by pad-dry cure method (Anita *et al.* 2011) (ii) sonochemical synthesis and impregnation of nanomaterials onto fabric substrates (Gedanken 2004, Perelshtein *et al.* 2009b) and (iii) through a thermal chemical process (Teo, Chang and Zeng 2006).

#### 1.5.13.1 Research into CuO antimicrobial textiles

Anita *et al.* (2011) investigated the antibacterial properties of copper oxide nanoparticles on cotton fabrics by preparing copper oxide nanoparticles using copper sulphate and sodium hydroxide as precursors and soluble starch as a stabilizing agent in a wet chemical method. These nanoparticles were then microencapsulated by an ionic gelation method and applied onto cotton fabric firstly by exhaustion followed by the pad-dry-cure method. Quantitative and qualitative assessment of antimicrobial properties was carried out according to the AATCC 100 and 147 test methods and showed excellent inhibition of *S. aureus* and *E. coli* growth.

Borkow, Okon-Levy and Gabbay (2010) investigated the antibacterial properties of copper oxide impregnated fabrics for use as potential wound dressings. They stressed the fact that copper coated dressings would not only reduce the risk of wound contamination but would also stimulate wound repair. The dressing contained a highly absorbent needle punch fabric made of viscose/rayon fibres (Galaxy®, Kelheim Fibers, GmbH, Germany) of 1.4 denier and 38 mm in length and 3% of cellulose copper oxide plated fibres. The internal layer of the pad had an external

hydrophobic spun bond layer made of polypropylene fibres coated with 2.3% weight/weight (w/w) copper oxide particles. Dressings were tested for their antibacterial and antifungal properties against *S. aureus*, *E. faecalis*, *K. pneumoniae*, *E. coli*, *P. aeruginosa* and *C. albicans*. The fabric coatings were examined by scanning electron microscopy and X-ray photoelectron spectrum analysis. The efficiency of these dressings in the wound healing process was examined by testing the dressing on pig partial-thickness wounds. Testing was performed according to the AATCC standards. Results demonstrated an excellent broad spectrum antimicrobial efficiency. Skin sensitization tests did not show any dermal reactions throughout the entire observation process period of 72 hours. Examination of the wound sites revealed no significant differences between the control and test dressings in terms of erythema, oedema and crust formation. No adverse effects from the copper were observed throughout the porcine partial-thickness wound model testing. The results gathered from this study supported further investigation on the use of copper dressings in the wound healing process.

Recent studies have shown toxic effects of CuO nanoparticles in human cell lines. Siddiqui *et al.* (2013) studied the cytotoxic effects of CuO nanoparticles in liver cells. The results showed that CuO nanoparticles 22 nm in size were found to be cytotoxic in HepG2 cells. Alarifi *et al.* (2013) assessed apoptosis and genotoxicity of CuO nanoparticles (50 nm) in human skin epidermal cells (HaCaT cells). Exposure of CuO nanoparticles to HaCaT cells initiated apoptosis by increasing the caspase-3 activity. CuO nanoparticles also induced DNA damage which was mediated by oxidative stress.

## 1.6 Textile application methods

The antimicrobial activity of functional textiles will depend on various factors such as nature of the antimicrobial agent used, durability and the method of application used. The selection of appropriate application method is very important. Many antimicrobial agents in the textile industry utilize a controlled release mechanism, whereby the agent leaches out of the textile during use (Ristić *et al.* 2011). These agents should not be chemically bound to the textile substrate and should only be used as a coating or a finish to allow persistent release of the agent from the textile surface into the surrounding environment. Owing to the leaching of the agent into the surrounding environment, the concentration of the antimicrobial gradually decreases under the limit of effectiveness and thus could potentially be a cause of induced resistance in microorganisms. In addition, fabrics coated with leaching antimicrobials are less durable and do not withstand repeated washings (Fouda, Abdel-Halim and Al-Deyab 2013).

Some antimicrobial agents can be chemically bound to the fabric substrate and only act when microorganisms come in contact with the fabric surface (White, Monticello and Midland 2002). Bound antimicrobial agents are much more resistant to repeated washings (Fouda, Abdel-Halim and Al-Deyab 2013).

Various methods exist for the application of antimicrobial agents to textiles. Antimicrobial coatings are typically applied in liquid form, in a solvent or in a water base to either one side or both sides of a textile substrate. The thickness of coating depends upon the type of application method and its intended use.

Many different coating methods have been employed for fabrics to confer antimicrobial activity on them. Some examples of methods used for surface modification of textiles are: sol-gel processing (Daoud and Xin 2004, Mahltig,

Audenaert and Böttcher 2005, Mahltig, Haufe and Böttcher 2005), *in-situ* synthesization of metal nanoparticles using the nanoporous structure of cellulose fibres as a nanoreactor (He, Kunitake and Nakao 2003), sputtering and plasma sputtering of textiles (Hegemann, Hossain and Balazs 2007, Wender *et al.* 2010), hydrophobic coating of textiles by addition of ion exchange surfactants (Texter *et al.* 2007) and surface modification of textiles with nano colloidal particles by a conventional pad dry cure process (Lee and Jeong 2004).

### 1.6.1 Confinement of antibacterial agents in resins

In this method the antibacterial agents can be incorporated into the polymer or mixed into the fibres during formation. This kind of processing is quite practical and durable, as the antimicrobial agent is physically incorporated in the textile structure and released slowly during use. This kind of fabric impregnation has been employed by some manufacturers, such as Bioactive® polyester fibres containing silver developed by Trevira and Silfresh® cellulose acetate fibres containing triclosan developed by Novaceta. This method produces more stable and durable antimicrobial fibres than other methods. However, it demands complex procedures and expensive setups (Gao and Cranston 2008, Nakashima *et al.* 2001). While processing synthetic fibres antibacterial agents can also be confined within the network structure of a reactive synthetic resin on the fabric surface. This way of processing locks the antibacterial agent in to the fibre structure.

### 1.6.2 Pad-dry-cure method

Natural and synthetic fibres have been treated with antimicrobial agents using conventional pad-dry-cure method (Mao and Murphy 2001). This method is also

referred to as padding and is widely used as a textile finishing method but also used for depositing micro or nanoparticles and other chemical compositions.

In this type of technique, the fabric is fed through a laboratory padder. The machine contains a tank filled with antimicrobial coating solution (Figure 17). Once the fabric soaks into the coating solution, it is then padded through two rollers that squeeze the excess solution out from the fabric to give a specified wet pick-up. This dictates the percentage of coating on a specified weight of fabric. The fabric is then dried in an oven typically at 100°C for 5 min and cured at 150°C for 4 min. The fabrics are finally rinsed and air dried again at room temperature (Lim and Hudson 2004, Ye *et al.* 2005). A major disadvantage of this method is the amount of time required for the whole process.



**Figure 17 Fabric coating by pad-dry-cure method (Mao and Murphy 2001)**

### 1.6.3 Sonochemical method of impregnating textile fabrics

A recent technology has been created on the use of ultrasound for the *in-situ* generation and impregnation of metal oxide nanoparticles onto nylon, polyester and cotton fabrics as a homogeneous layer (Abramov *et al.* 2009, Perelshtein *et al.* 2008, Perelshtein *et al.* 2009b). In the process antibacterial metal oxide nanoparticles are

simultaneously synthesized and impregnated on a textile surface through acoustic cavitation effects caused by ultrasound. This process results in a smooth and homogeneous layer of impregnation and is capable of projecting nanoparticles towards the fabric surface at a very high speed. This induces partial melting of nanoparticles on the fabrics surface causing them to adhere strongly (Abramov *et al.* 2009).

### 1.6.4 Ultrasound

Ultrasound is defined as sound at a frequency that is too high for the human ear to detect. The actual limit of hearing detection varies from person to person but a frequency of approximately 20 kHz is typical for healthy young adults. The applications of ultrasound are varied depending on the frequency used. As depicted in Figure 18, ultrasound can be divided into three frequency ranges:

- Power ultrasound (low frequency range, 20 – 100 kHz)
- High frequency ultrasound (intermediate, 100 kHz – 1MHz)
- Diagnostic ultrasound (high, 1 – 10 MHz)

**Figure 18 Different ranges of ultrasonic sound frequencies (Mason and Peters 2002)**

### 1.6.5 Principles of ultrasonics sonochemistry

Sonochemistry utilises the fact that ultrasound provides a form of mechanical sound energy for modifying chemical reactions. It is a process in which mechanical activation destroys the attractive forces of molecules in the liquid phase (Mason and Lorimer 2002). This arises from the energy released on collapse of cavitation bubbles in a medium. Under the correct conditions ultrasonic cavitation can cause permanent physical or chemical changes (Mason 1999). As described by Suslick, Hammerton and Cline Jr. (1986) collapse in a homogeneous liquid is different from the cavitation collapse near a liquid solid interface.

#### 1.6.5.1 Cavitation in a liquid medium

As mentioned above, power ultrasound results in the formation of transient cavitation bubbles. Acoustic cavitation involves the formation, growth and implosive collapse of bubbles in a liquid when they reach an unstable state. Ultrasound travels

through a medium as a longitudinal wave with alternating zones of high and low pressure (Figure 19). Bubbles can form and grow in the low pressure zones while shrinking or collapsing in the high pressure zones. According to the 'hot spot' mechanism, bubble collapse produces very high temperatures, pressures, shear forces and free radicals. The resulting high temperatures (5000 - 25000 K) and pressures (2000 atm) are capable of breaking chemical bonds (Suslick *et al.* 1991). A high cooling rate of $10^{11}$ K/s also occurs which prevents the process of crystallization. Reactions also occur in a 200 nm ring that surrounds the collapsing bubble where the temperature is estimated to reach $1900^{0}$C (Suslick, Hammerton and Cline Jr. 1986).



**Figure 19 Acoustic cavitation: generation of the bubble (Mason 1999)**

### 1.6.5.2 Cavitation at or near to a solid surface

When a cavitation bubble collapses near or at a solid surface, the process of collapse becomes non-symmetrical. The solid surface hinders liquid movement from

one side, so that the majority of liquid flows into the collapsing bubble from the other side of the bubble as shown in Figure 20. This results in the formation of a liquid jet that targets the surface with a speed in excess of 100 m/s (Mason and Peters 2002). The mechanical effect of this is equivalent to high pressure jetting and this is the reason why ultrasound is so effective at cleaning surfaces. Under electron microscopy, it can be seen that ultrasonic treatment is capable of producing pitting on solid surfaces (Mason 1999, Mason and Peters 2002).



**Figure 20 Collapse of a cavitation bubble at or near a solid surface (Mason 1999)**

### 1.6.6 Sonochemical synthesis and impregnation of textile fabrics with metal oxide nanoparticles

Gedanken *et al.* (2009) developed and patented a technique for the synthesis and impregnation of metal oxide NPs (ZnO and CuO) on to fabric substrates. In their method, copper or zinc acetate was dissolved in a 9:1 mixture of ethanol and water. The pH of this solution was then raised to between 8 and 9 by the addition of

ammonium hydroxide. Once dissolved the metal acetates form a mixture of hydroxides and soluble ammonium complexes. The energy released during cavitation bubble collapse converts these soluble complexes in to solid metal oxide nanoparticles. The sequence of chemical events associated with this conversion is depicted in Figure 21.

Asymmetric collapse of cavitation bubbles close to the surface of the fabric, as shown in Figure 20, propels the freshly formed NPs at high speed in to the fibres. This process results in a firm and homogeneous coverage of the fibres with NPs (Abramov *et al.* 2009). A major advantage of this technique is that the synthesis and impregnation of the NPs is accomplished in a single step. Other coating technologies commonly require at least two separate steps, one for synthesis and another for coating and are consequently more expensive.



**Figure 21 Chemical processes for the sonochemical conversion of copper acetate into CuO nanoparticles (Kumar, Diamant and Gedanken 2000)**

### 1.6.7 The SONO project

The aim of the SONO project was to develop a sonochemical method for the synthesis of inorganic antibacterial nanoparticles (NP) and their impregnation into fabrics for use as medical textiles. The SONO project consisted of a 4 year program of research funded under the EU framework 7 program (EU FP7) involving a consortium of 17 institutions, companies and research centres, including Coventry

University. The researchers involved in the project undergo preliminary studies on the use of CuO and ZnO NPS on textile surfaces by the sonochemical method (Abramov *et al.* 2009, Perelshtein *et al.* 2009a, Perelshtein *et al.* 2009b).They studied the antibacterial properties and characterisation of the NPs on the fabrics and demonstrated a homogeneous impregnation of nanoparticles on the fabrics surface with a good antibacterial activity against different bacteria tested (> 99% bacterial reduction). The researchers demonstrated that even less than 1% of ZnO and CuO coated fabric (%wt.) possessed bactericidal activity (Abramov *et al.* 2009, Perelshtein *et al.* 2009b). Apart from ZnO and CuO, they also looked at the synthesization and deposition of silver NPs by sonochemical method in order to compare the antibacterial activity (Perelshtein *et al*. 2008).

Based on the preliminary results, the pilot line was designed for the scale up of a sonochemical process to impregnate textile fabrics with ZnO and CuO NPs. The process was developed and patented at BIU laboratories in Israel (Gedanken *et al.* 2009). A schematic diagram of the sonochemical reactor at a laboratory scale is shown in Figure 22. The sonochemical reactor was divided into several sub-systems: reagent preparation, sonochemical tank, ultrasonic transducer and driver, textile rolling and motorization along with a washer/dryer. The ultrasonic parameters of the process (frequency and power output) were input via a control panel. The feed rate of the fabric (100 mm wide) was controlled by a 0.5 kW motor that regulates the release and winding of fabric in the reaction tank. The fabric feed rate was controlled within the limits of 0.004 - 0.050 m/s. This was a continuous process in which the fabric was fed through two vibrating surfaces driven by ultrasonic magnetostrictive transducers at a frequency of 20 kHz (Abramov *et al.* 2009).

**Figure 22 A schematic diagram of the pilot sonochemical reactor used for impregnating fabric (Abramov *et al.* 2009)**

After the initial laboratory phase of development, the process was transferred to an industrial scale where two prototype machines were constructed by the SONO project partners. These were used for experiments regarding optimisation of the process and for preparation of ZnO and CuO treated cotton for use in a hospital trial. One of the pilot machines was installed in Italy in a company called KLOPMAN International and the other in a Romanian firm called DAVO clothing. One machine was constructed using magnetostrictive type ultrasonic transducers (Davo) and the other with piezoelectric type transducers (Klopman) (Abramov *et al.* 2009). One of the project aims was to compare the operation of the two transducers types in terms of economics and efficiency.

**Figure 23 One of the prototype machines used to produce medical textiles based on results generated by the SONO project. Image courtesy of Universitat Politècnica de Catalunya (UPC)**

## 2 Research aims and objectives

The aim of this project was to assess the antibacterial activity of fabrics sonochemically impregnated with metal oxide nanoparticles (ZnO and CuO). Cytotoxicity of these fabrics was also investigated in human cell lines. These metal oxides have been shown to possess antibacterial activity and could provide an alternative to the widespread use of silver nanoparticles in textiles.

Antibacterial activity testing was carried out using a selection of bacteria including Gram positive and Gram negative species. As the fabrics under development were intended for use in hospitals, the species chosen were representative of bacteria commonly associated with HAIs. Standard test strains were used so that the results could be compared with published data for other commercially produced antibacterial fabrics.

The absorption method from ISO 20743 was chosen for the determination of antibacterial efficacy. This is recognised as the industrial standard method for antibacterial textile testing in Europe (Bischof Vukušić *et al.* 2011, Brunon *et al.* 2011, Chadeau *et al.* 2010, Fei *et al.* 2011, Perelshtein *et al.* 2013, Simončič *et al.* 2012, Swofford 2010, Vukušić *et al.* 2009, Wei *et al.* 2012, Zhou *et al.* 2013).

Antibacterial activity testing was also performed using a disc diffusion method and two shake flask methods in saline and nutrient broth. These tests are commonly used in research on antibacterial fabrics and were included in this work to aid comparison with other published research. They were also carried out in order to check the results from the absorption testing.

This investigation was part of a larger EU Framework 7 (FP7) funded project (SONO) which was aimed at transforming a laboratory based process into an optimised process at the factory pilot scale. The antibacterial efficacy tests

conducted at Coventry University provided very important information to assist with the optimisation of the pilot plant.

## 2.1 Objectives

Based on the aims of the project, the main objectives of this study were:

i. To investigate the antibacterial activity of the fabrics against a range of Gram positive and Gram negative bacteria using the absorption methods from ISO 20743, a disc diffusion method, and shake flask methods in nutrient broth and saline. The fabrics were produced sonochemically using a laboratory coating system at Bar-Ilan University (BIU) in Israel.

ii. To compare the level of antibacterial activity shown by the sonochemically produced fabrics (ZnO and CuO) to the antibacterial activity of fabrics coated with silver and triclosan. Textiles coated with silver or triclosan account for the majority of commercially available antibacterial fabrics. Here they were produced by one of the project partners using a conventional pad-dry-cure method.

iii. To test and compare the antibacterial activity of CuO impregnated fabrics from the two pilot plants set up in Italy and Romania (Klopman and Davo respectively) during the optimisation of lab process to the industrial set up. CuO NPs were applied to 60/40 mix polyester cotton fabrics using two different ultrasonic methods; the original sonochemical (in-situ) method and a 'throwing the stones' (TTS) method.

iv. To investigate the leaching of metal ions or nanoparticles from the textiles in to saline and to investigate the antibacterial activity of the leached solutions.

Zinc and copper concentrations on the fabrics and in solution were measured by ICP-OES. The aim of these experiments was to investigate the role of leaching in the antibacterial activity of these fabrics.

v. To investigate the suitability of flow cytometry (FC) as a method to assess antibacterial activity and to compare the FC results with the agar plate counts from the absorption method of ISO 20743. FC and fluorescence microscopy was also used to detect viable but non culturable cells (VBNCs).

vi. To assess the cytotoxicity of the fabrics impregnated with ZnO and CuO NPs in human dermal fibroblast cells (HDF) and human hepatocellular carcinoma cells (HepG2) using the MTT assay for cell viability.

# 3 Methodology

## 3.1 Quality control and safety

- Test bacterial strains were purchased from the Health Protection Agency (HPA) National Collection of Type Cultures (NCTC). After regrowth in nutrient broth the cultures were frozen at -80°C for storage.
- Streak plates were prepared on a regular basis from the master inoculums to maintain the purity and uniformity of bacterial cultures. Gram stains were performed with each experiment to check the purity of cultures. During experiments streak plates were prepared from inoculum cultures and from control samples post incubation.
- All equipment and testing surfaces were sterilized using 70% IMS to maintain aseptic conditions. In most cases lab items and media were sterilized by autoclaving. The buffers used in flow cytometry were sterilized using a Millipore syringe filter (0.22 µm) to reduce background noise in the solution during analysis.
- All stains and Petri-dishes were disposed off in clinical waste bins in compliance with the local disposal regulations in force. Contaminated pipette tips and other small disposable items were soaked in dilute Trigene disinfectant prior to autoclaving and disposal.
- Water baths were regularly checked for contamination. 1 ml of the bath water was mixed with plate count agar or spread on nutrient agar plates. Then the plates were incubated at 37 ± 2°C for 24 – 48 hours. If any contamination was found the bath was heated to > 90°C, emptied and sprayed with 70% IMS.

## 3.2 Equipment and apparatus

- The concentration of bacteria in nutrient broth cultures was estimated by measuring optical absorbance at 660 nm using a Corning colorimeter 253. The colorimeter was zeroed by placing nutrient broth in a 1 ml cuvette.
- A Thermo Scientific MaxQ 6000 shaking incubator was used to incubate cultures at a constant temperature of 37 ± 2°C. Shaking cultures were incubated at a speed of 110 rpm.
- For pour plating experiments a Grant Instruments Ltd. water bath was used to maintain plate count agar (PCA) tubes at a constant temperature of 45 ± 2°C. The sterile agar universal tubes were first melted in a steamer and then cooled down to a temperature of around 45°C in a water bath. This was a high enough temperature to prevent the agar from solidifying in the tubes and a low enough temperature not to kill the bacteria when they were mixed with the agar.
- A Protocol Synbiosis colony counter was used to enumerate colonies on agar plates. The system employs a unique LED lighting system configured for the illumination of all sample types for colony counting or for the measurement of inhibition zones on plates.
- A New Brunswick Scientific (NBS) MediaMatic MM-9 autoclave and PourMatic MP-1000 plate pourer were used to prepare nutrient agar plates.
- A Jencons Scientific Ltd. Perimatic GP agar pourer was used for dispensing nutrient broth, saline, SCDLP and plate count agar into universal tubes.
- A MSE micro centaur centrifuge was used for flow cytometry sample preparation. Samples were typically centrifuged in sterile Eppendorf tubes for 3 minutes at 13,000 rpm to pellet bacteria.

- A BD FACS™ flow cytometer equipped with 488 nm laser excitation was used for the flow cytometric analysis with Quest software.

### 3.3 Bacterial strains

For this project, a range of bacterial species were tested against woven cotton and polyester cotton fabrics with different types of antibacterial impregnations (ZnO or CuO nanoparticles). The species of bacteria used in these experiments are common causes of nosocomial infections (hospital acquired infections). The strains for antibacterial testing were chosen by the SONO project consortium and purchased from the National Collection of Type Cultures (NCTC) and American Type Culture Collection (ATCC). The bacterial strains used were:

| | | |
|---|---|---|
| • *Staphylococcus aureus* | Gram +ve | NCTC10788 |
| • MRSA | Gram +ve | NCTC10442 |
| • *Escherichia coli* | Gram –ve | NCTC12923 |
| • *Acinetobacter baumannii* | Gram –ve | NCTC10303 |
| • *Pseudomonas aeruginosa* | Gram –ve | NCTC13359 |
| • *Klebsiella pneumonia* | Gram –ve | ATCC 4352 |

Master strains were stored at -80°C in a freezer. The strains were cultured on nutrient agar and grown aerobically at 37°C overnight.

## 3.4 Materials used

### 3.4.1 Reagents and culture media

The reagents used in all tests were of analytical quality. Oxoid dehydrated media powder was used to prepare culture media and the manufacturer's instructions were followed for media preparation. Deionized water was used to prepare all microbiological media.

#### 3.4.1.1 Nutrient broth (NB)

Nutrient broth was purchased from Oxoid (CM0001) in powder form. Nutrient broth was prepared by dissolving 13 g of powder per litre of reverse osmosis (RO) water and autoclaving for 15 minutes at 121 °C. 9 ml of the broth was dispensed into 25 ml glass universals prior to autoclaving. Nutrient broth powder contained:

| | |
|---|---|
| Beef extract | 3.0 g |
| Peptone | 5.0 g |
| pH after sterilization | 6.9 ± 0.2 |

#### 3.4.1.2 Soyabean-Casein Digest broth with Lecithin and Polysorbate 80 medium

| | | | |
|---|---|---|---|
| Peptone, digest of casein | 17.0 g | Sigma-Aldrich | 70172 |
| Peptone, digest of soybean | 3.0 g | Sigma-Aldrich | 70178 |
| Sodium chloride (NaCl) | 5.0 g | Fisher Scientific | S/3160/53 |
| Potassium dihydrogen phosphate | 2.5 g | Fisher Scientific | P/4800/53 |

| | | | |
|---|---|---|---|
| Glucose | 2.5 g | Fisher Scientific | G/0500/60 |
| Lecithin | 1.0 g | Fisher Acros | 4131025 |
| Polysorbate 80 | 7.0 g | SLS | 47510 |
| Water | 1000 ml (final volume) | | |
| pH after sterilization | 7.2 ± 0.2 | | |

Soybean–Casein Digest broth with Lecithin and Polysorbate 80 (SCDLP) medium was prepared by dissolving 17 g of casein peptone, 3 g of soybean peptone, 5 g of sodium chloride, 2.5 g of potassium dihydrogen phosphate, 2.5 g of glucose, 1 g of lecithin and 7 g polysorbate 80 (non-ionic surfactant) in 1 litre of RO water and mixed thoroughly on a stirrer. After mixing, the medium was sterilized by autoclaving at 121°C for 15 minutes. After autoclaving, the pH was adjusted to around 7.2 at room temperature using 2 M sodium hydroxide. The medium was then dispensed into universal tubes and sterilized again by autoclaving 121°C for 15 minutes. The purpose of the SCDLP in experiments was to neutralise any growth inhibitory effects from the test antibacterial agents in solution and to help release bacteria from the test fabrics for enumeration. The SCDLP was stored at 5°C in a fridge for no longer than one month after preparation.

#### 3.4.1.3 Plate count agar (PCA)

PCA was purchased from Oxoid (CM0325) and 17.5 g was dissolved per litre of RO water and heated at 100°C for 1.5 hours with mixing every 30 minutes. Once the agar was fully dissolved, it was dispensed as 17 ml aliquots into glass universal tubes and sterilized by autoclaving at 121°C for 15 minutes. The PCA powder contained:

| | |
|---|---|
| Dehydrated yeast extracts | 2.5 g |
| Casein tryptone | 5.0 g |
| Glucose | 1.0 g |
| Agar | 12-18 g (depending on the gel strength) |
| pH after sterilization | 7.2 ± 0.2 |

#### 3.4.1.4 Nutrient agar (NA)

Nutrient agar was prepared by mixing 28 g of Oxoid powder (CM0003) per litre of RO water and sterilized at 121ºC for 15 minutes using an autoclave. An NBS mediaclave and plate pourer was used to prepare the media. Typically, 2 litres of RO water and 56 g of NA were mixed and placed in the autoclave vessel, which was sealed. The agar inside was mixed and sterilized at 121ºC for 15 minutes. The agar was allowed to cool until the temperature dropped to 50ºC and then dispensed into Petri dishes (18 – 20 ml each) via the plate pourer. The agar was left to set for at least one hour and then the plates were inverted for storage. The nutrient agar powder contained:

| | |
|---|---|
| Lab Lemco powder | 1.0 g |
| Yeast extracts | 2.0 g |
| Peptone | 5.0 g |
| Sodium chloride | 5.0 g |
| Agar | 15.0 g |
| pH after sterilization | 7.4 ± 0.2 |

#### 3.4.1.5 Sterile saline

For the preparation of saline 8.5 g of sodium chloride was dissolved per litre of RO water and mixed well using a magnetic stirrer. Saline was dispensed as 9 ml aliquots into 20 ml glass universal bottles and sealed with metal lids. The universals were sterilized at 121ºC for 15 minutes in an autoclave.

#### 3.4.1.6 Phosphate buffered saline

Phosphate buffer solution (PBS) was prepared as x10 concentrated solution by dissolving one Sigma Aldrich buffer tablet (Product code: P4417) in 100 ml distilled water. The solution was then autoclaved. Small working volumes (20 ml) of the solution were prepared by adding 2 ml of concentrated buffered solution in 18 ml of distilled water. This was sterilized by using a 0.22 µm Millipore syringe filter. This buffer was used for flow cytometry viability experiments.

#### 3.4.1.7 Staining buffer (Flow cytometery)

1mM EDTA, 0.01% Tween 20 and 0.1% sodium azide was added to phosphate buffered solution (pH 7.4) to make staining buffer for flow cytometry experiments

### 3.5 Preparation of inoculum (ISO 20743:2007)

#### 3.5.1 Inoculum for growth on solid media

1 ml aliquots of each of the test bacterial strains (in nutrient broth with 15% v/v glycerol) were stored in cryovials at -80ºC (MDF-U74V -80°C ultra-low freezer, Sanyo). For the preparation of stock lab cultures, streak plates were prepared from these frozen cultures. A small amount of the frozen material was taken using a

sterile inoculating loop and then used to prepare a streak plate on nutrient agar (Figure 24). The plates were incubated at 37 ± 2ºC for 24 - 48 hours and then stored at 5 ± 2ºC. Stock cultures were passaged every one to two weeks to minimize contamination and ensure healthy bacteria. New stock culture plates were prepared from the frozen stocks after the cultures had been passaged 5 times. Cultures were discarded and re-prepared if any non-type colonies were seen.

**Figure 24 A quadrant streak of *Staphylococcus aureus* on nutrient agar plate**

### 3.5.2 Preparation and incubation of test inoculums

Bacterial suspensions were prepared according to the guidelines in the ISO 20743:2007 standard. A single colony was taken from the stock bacterial culture with a loop and inoculated into 20 ml of sterile NB in a sterile 100 ml Erlenmeyer flask.

The flask was then incubated in a shaking incubator at 37ºC at 110 rpm for 24 ± 3 hours.

After incubation, 0.4 ml of the overnight inoculum was transferred to a 100 ml Erlenmeyer flask containing 20 ml NB and incubated in a shaking incubator for 3 hours at 37ºC and 110 rpm to obtain a target concentration of $10^8$ CFU/ml.

### 3.5.3 Calibration of bacterial suspensions

To ensure consistency and reproducibility, the concentration of the bacterial inoculums were adjusted to be roughly equal between experiments. The number of bacteria in suspension was estimated using a turbidity method. By comparing plate counts with absorbance measurements an optical absorbance at 660 nm of between 0.1 - 0.3 was found to be roughly equal to a concentration of between 1 x $10^8$ - 3 x $10^8$ CFU/ml. Inoculum concentrations of between 1 x $10^5$ and 1 x $10^6$ CFU/ml were prepared by appropriate dilution steps. For example if the absorbance was 0.2 then times 1000 dilution yielded a concentration of approximately 2 x $10^5$ CFU/ml. If the absorbance was 0.6 then times 2000 dilution produced a concentration of roughly 3 x $10^5$ CFU/ml.

## 3.6 Preparation of fabric samples

Metal oxide impregnated cotton fabrics were prepared using a laboratory impregnation system at Bar-Ilan University (BIU), Israel, in accordance to their previously published procedure (Abramov *et al.* 2009). The antimicrobial nanoparticles were synthesized and impregnated on the textile fabrics by a sonochemical technique. In the coating tank zinc acetate or copper acetate was dissolved in a 9:1 mixture of ethanol and water. The tank was heated to 55 ± 5°C

and the pH of the solution was raised to between pH 8 - 9 by the addition of ammonia. The ammonia forms a complex with the zinc or copper ions in solution. Rolls of cotton (10 x 0.1 m, plain unbleached woven cotton 145 $g.cm^{-2}$) were then fed at a constant speed (22 cm/min) through the reaction tank and ultrasonic field. After impregnation, the rolls of cotton were washed with clean water and ethanol and then dried (Abramov *et al.* 2009).

Antibacterial tests (ISO 20743 absorption method) were also performed on silver, triclosan and chitosan fabrics coated using a pad dry cure method rather than the sonochemical method. This was to produce some data for comparing antibacterial activity of other common coatings. The silver and triclosan fabrics were supplied by Klopman International (Italy).

Two pilot scale machines were built as part of the SONO project and were used to prepare samples for testing and for the manufacture of hospital gowns and linens. One was set up in Italy at a company called KLOPMAN International and the other one was installed at Romanian firm called DAVO clothing.

Impregnated samples were prepared using the same basic protocol described for the lab scale system above. A number of experiments were carried out with the two machines to check their operation and to optimize the process. Out of these experiments, two sets of fabrics were compared in the work presented here:

i. **"Throwing the stones" (TTS) technology -** In this technique the reaction tank was filled with a suspension of pre-formed CuO nanoparticles in water. Ultrasound was employed to impregnate these nanoparticles onto the fabrics but not in the sonochemical synthesis of them. The CuO nanopowder was purchased from a commercial supplier (Product #: 29N-

0801, average particle size 80 nm as per Holland Moran Ltd., Israel). This technique was adopted to allow experimental runs to proceed without the use of ethanol.

ii. ***In-situ* technology -** This is the sonochemical method of impregnation, where the nanoparticles were formed and simultaneously impregnated onto the fabrics in a one step process (same as the lab scale method of impregnation).

The experiments conducted at the pilot scale concentrated on the use of CuO because it produced a strongly coloured coating on the white fabrics. This allowed for a rapid visual assessment of the impregnation process.

### 3.7 Antibacterial assessment of test fabrics

The methodology used for the antibacterial efficacy testing was based on the International Standard, EN ISO 20743:2007 “Textiles - determination of antibacterial activity of antibacterial finished products” (ISO 2007). The international standard specifies three different quantitative test methods for determining the antibacterial activity of antibacterial finished textile products. Based on the intended application and the environmental conditions in which the fabrics are to be used; the absorption method was chosen to be the most suitable method for the determination of antibacterial activity. Unlike other antibacterial testing methods, the absorption method is intended to simulate real use conditions.

### 3.7.1 Absorption method (ISO 20743) – plate count enumeration

In this method, test bacterial suspension is inoculated directly onto fabric samples. For the antibacterial testing according to the absorption method, fabric pieces were cut to a size of 5 x 5 cm to obtain a mass of approximately 0.40g ± 0.05 g. Six samples of control fabric and three samples of impregnated test fabric were prepared for each antibacterial test. Each test sample was placed into a separate universal tube and autoclaved. 0.2 ml of bacterial inoculum (1 - 3 x $10^5$ CFU/ml in dilute NB) was pipetted drop wise over the whole surface of each control and each test fabric to ensure the fabric was well covered by the inoculum. Care was taken not to allow the inoculum to wet/touch the inner surface of the vial. Three controls were used to monitor bacterial concentration at time zero immediately after inoculation and the remaining three and the test samples were used to measure bacterial numbers after a contact time of 24 ± 3 hours incubation. Immediately after inoculating the samples, the first three universal vials for the control fabric were mixed with 20 ml of SCDLP medium. The vials were tightly capped and shaken vigorously by hand in an arc of ~30 cm for 30 seconds. The remaining three universals for the control and test fabric were incubated at 37ºC for 24 ± 3 hours immediately after inoculation with the test bacterium. After incubation the remaining three sets of control and test fabrics were treated in the same way with SCDLP. The SCDLP medium neutralized the antibacterial coating/impregnation and helped to wash the bacteria free from the fabrics.

### 3.7.2 Serial dilutions

Serial dilutions of the bacterial suspensions in SCDLP medium were prepared for pour plating. 1 ml of each suspension was pipetted and added to the next test tube containing 9 ml of NB. 10 fold dilutions were made up as shown in Figure 25.

i. A 3 fold dilution series was prepared for the initial set of control fabrics (inoculum day 1). Petri dishes were labelled ($10^0$, $10^{-1}$, $10^{-2}$ and $10^{-3}$) for the initial set of control fabrics.
ii. A 5 fold dilution series was prepared for the post incubation suspensions (day 2) and Petri dishes supporting the bacterial growth for the post incubated set of control and test fabrics were labelled ($10^0$, $10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$ and $10^{-5}$).

1 ml was taken from each diluted tube and pipetted onto two corresponding Petri dishes across the plate.



**Figure 25 Standardization of culture by serial dilutions. Image taken from http://faculty.irsc.edu**

### 3.7.3 Agar plating

#### 3.7.3.1 Pour plating

The pour plating method is one of the methods used to determine microbial count per ml or per gram of specimen. The main advantage of this method is that it does not require previously prepared agar plates and is often used to assay anti-microbial effects of various substances. After pipetting 1 ml of inoculums from the serial dilutions, approximately 15 ml of warm PCA (~45°C water bath) was added to each Petri dish. Petri dishes were rotated gently, or moved back and forth, to ensure the culture and medium was thoroughly mixed and the medium evenly covered the plate. Plates were left at room temperature until the medium solidified, then turned upside down to ensure water condensation did not drip on to the agar. The plates were then incubated at 37 ± 2°C for 24 – 48 hours.

**Figure 26 Colonies formed on agar by pour plating method under aseptic conditions. Large colonies are on the surface of agar and smaller colonies are within the agar medium**

Pour plates allow bacteria to grow on both the agar surface and within the medium. Colonies which grow within the medium are relatively small in size and confluent compared to the colonies on the agar surface (Figure 26). Following incubation, the number of colonies was counted on the serially diluted plates on which 30 - 300 CFU were visible. Plates were counted using a Protocol Synbiosis colony counter and the bacterial concentration was calculated using the following formula:

**Equation 1:**

$$\textit{Bacterial Concentration } C = A \times R$$

Where:

C bacterial concentration, in Colony Forming Units per milliliter (CFU/ml)

A average value, in Colony Forming Units (CFU), in the two Petri dishes

R dilution factor

### 3.7.3.2 Spread plating

One other method of quantifying and distributing bacteria is through spread plating. Spread plating is a similar technique to pour plating for quantifying the number of bacteria in a solution. This method requires previously prepared agar plates. Bacteria are evenly distributed over the surface by spreading technique using a bent glass rod. 0.1 ml of bacterial suspension was placed in the center of an agar plate using a sterile pipette. The glass rod was sterilized by dipping it into absolute alcohol or 100% IMS and passing it quickly through Bunsen burner flame. The rod was air cooled for few seconds and then touched to the side of an agar surface to ensure it was not hot. Once done, the rod was streaked back and forth on the

surface of agar to ensure even distribution of inoculum across the plate. The plate was turned to 90 degrees to repeat the side to side, up and down streaking. The plate was covered with lid and incubated at 37 ± 2°C for 24 ± 3 hours.

### 3.7.4 Test results interpretation

#### 3.7.4.1 Judgment of test effectiveness

The ISO 20743 standard includes guidelines for judging the effectiveness of the test. These are outlined below:

i. The concentration of the test inoculum falls within the range of 1 - 3 x $10^5$ CFU/ml.
ii. Differences in extremes for the three control fabrics immediately after inoculation and after incubation should be < 1 log.
iii. The growth value obtained according to the formula below should be ≥ 1.0 by the plate count method.

### 3.7.5 Calculation of antibacterial activity value

When the test had been judged effective, the antibacterial activity was obtained using the following formula:

**Equation 2:**

$$A = (\lg C_t - \lg C_o) - (\lg T_t - \lg T_o) = F - G$$

Where:

***A*** is the antibacterial activity value

***F*** is the growth value on the control fabric ($F = \lg C_t - \lg C_o$)

***G*** is the growth value on the coated/impregnated fabric ($G = \lg T_t - \lg T_o$)

**lg $C_t$** is the average common logarithm for the number of bacteria obtained from three control samples after 24 ± 3 hour incubation

**lg $C_o$** is the average common logarithm for the number of bacteria obtained from three control samples immediately after inoculation

**lg $T_t$** is the average common logarithm for the number of bacteria obtained from three antibacterial coated/impregnated samples after 24 ± 3 hour incubation

**lg $T_o$** is the average common logarithm for the number of bacteria obtained from three coated/impregnated samples immediately after inoculation (= lg $C_o$)

A set of general assessment criteria defined by Hohenstein Institute (Hoefer and Hammer 2011) are as follows:

- If a growth reduction efficacy (A value) is <0.5, it corresponds to no antibacterial activity.
- If a growth reduction efficacy (A value) is ≥0.5 - <1, it corresponds to a slight antibacterial activity.
- If a growth reduction efficacy (A value) is ≥1 - <3, then it is a significant antibacterial activity.
- And if a growth reduction efficacy (A value) is ≥3, it indicates a strong antibacterial activity.

### 3.7.6 Calculation of percentage reduction

The percentage reduction in the numbers of viable/live bacteria after incubation was calculated using the following formula

**Equation 3:**

$$R\ (\%) = \frac{(A - C) \times 100}{A}$$

Where;

R% is the percentage reduction in viable bacteria,

A is the number of bacteria colonies from untreated control fabric samples at time zero.

C is the number of bacterial colonies recovered from treated fabrics after incubation over a 24 ± 3 hour contact period

### 3.7.7 Calculation of growth reduction

The growth reduction in the numbers of viable/live bacteria after incubation was calculated using the following formula:

**Equation 4:**

$$R\ (\%) = \frac{(B - C) \times 100}{B}$$

Where;

R% is the growth reduction in viable bacteria,

B is the number of bacteria colonies from untreated control fabric samples after incubation over 24 ± 3 hour contact period

C is the number of bacterial colonies recovered from treated fabrics after incubation over a 24 ± 3 hour contact period

### 3.8 Disc diffusion method (Semi-quantitative test)

The ability of the impregnated fabrics to inhibit bacterial growth was also tested using a disc diffusion method. The disc diffusion method (Kirby-Bauer) is a relatively quick and easily executed semi-quantitative test to determine antibacterial activity of diffusible antimicrobial agents on impregnated textile material. The method allows for the simultaneous testing of a large number of antimicrobials in a relatively easy and flexible manner.

The bacteria were grown as detailed in section 1.5. Test organisms (1 - 3 x $10^6$ CFU/ml) were swabbed over the surface of Isosensitest agar plates (Oxoid) with a sterile cotton-tipped swab to form an even lawn. 10 mm diameter discs of the impregnated fabric and control fabric were cut and gently pressed on to the surface of the plate. The plates were incubated aerobically at 37ºC for 24 ± 3 hours. The antibacterial activity of impregnated fabrics was assessed from the diameter of the zone of inhibition (clear zones with no visible bacterial growth) and compared to the control fabric. Images were recorded using a Protocol 2 plate counter (Synbiosis, UK). The greater the diameter of the inhibition zone, the more susceptible is the microorganism to the antimicrobial. The experiment was performed in duplicate and the mean value was taken. The values quoted in the results section are the total diameters of the zones of the inhibition including the 10 mm wide samples.

### 3.9 Shake flask tests

Shake flask tests are a common alternative to diffusion tests for the evaluation of antibacterial activity. They can be used to provide a quantitative indication of antibacterial effect unlike the diffusion methods which are only semi quantitative. In such tests the test material is submerged in a suspension of the target bacteria in saline, buffer or nutrient broth. After a set period of shaking incubation, samples of the liquid are taken for serial dilution and agar plating. The number of viable bacteria recovered from impregnated samples is compared with viable numbers recovered from control samples incubated in the same way. The antibacterial activity can be expressed in terms of percentage reduction compared to the start bacterial concentration, or percentage reduction in growth by comparison to growth on the control samples. This test method is also suited to the testing of hydrophobic materials (poorly wetting) which do not work well with absorption methods. Shake flask methods were explored in this work to corroborate the results from the ISO 20743 tests and to evaluate the small differences in antibacterial activities of impregnated fabrics which were not evident by the absorption method.

#### 3.9.1 Shake flask in nutrient broth (Semi-quantitative test)

The first series of shake flask tests was conducted using NB. In this method NB was used to enable monitoring of bacterial growth by measuring the absorbance of the medium over time. The samples were placed in shaking flasks for 3 hours at 37ºC and the change in optical absorbance over time was monitored at a wavelength of 660 nm. Test and control fabrics were cut into small pieces of 5 x 5 cm (0.4 g) and placed into empty sterile Erlenmeyer flasks in triplicates. The fabrics were inoculated with 0.2 ml of bacterial inoculums (1 - 3 x $10^6$ CFU/ml). Immediately after inoculation,

the fabrics were soaked in 20 ml of nutrient broth. The flasks were then shaken at 110 rpm in a rotary shaking incubator at 37ºC. 1 ml aliquots were taken for absorbance readings at 660 nm from time 0 - 3 hours with intervals of 30 minutes. A final reading was also taken at 24 hours following overnight incubation. Antimicrobial efficacy was determined based on triplicate test results.

### 3.9.2 Shake flask test in saline (Quantitative method)

A shake flask method was also performed in saline to investigate the antibacterial activity of impregnated fabrics. This method was closer to the standard dynamic shake flask method ASTM E2149 (ASTM 2010) in that no nutrient source was added. In this method bacterial growth was much slower and so absorbance measurements were not suitable for monitoring bacterial numbers. Instead dilutions were spread on NA plates for enumeration.

Fabric samples were placed in contact with 20 ml of sterile saline (0.85% NaCl) in Erlenmeyer flasks. Fabrics were inoculated with 1 ml of bacterial inoculums. The inoculums were x100 diluted (1 - 3 x $10^6$ CFU/ml) in saline of a 3 hour culture. The aim was to produce a suspension with approximately $10^5$ CFU/ml. The tests against each bacterium included (a) control fabric in saline (b) impregnated fabric in saline (c) no fabric in saline. All the three treatments were performed in triplicate. The flasks were then subjected to incubation in a rotary shaking incubator at 37 ± 2ºC. The number of viable cells after incubation was obtained by spread plating serial dilutions on nutrient agar at 0, 1, 3 and 24 hours.

### 3.10 Saline leaching tests

Leaching tests were performed to investigate the leaching properties of the metal oxide nanoparticles (ZnO/CuO) from the impregnated fabric samples into saline solution. The methodology used was very similar to that used for the saline shake flask tests. The main difference being that the fabric samples were removed from the saline flasks prior to addition of the bacterial inoculums. This prevented direct contact between the bacteria and the metal oxide NPs impregnated fabric.

Fabric samples were placed in contact with 20 ml of sterile saline (0.85% NaCl) in Erlenmeyer flasks. The tests included (a) unwashed control fabric in saline (b) CuO impregnated fabric in saline (c) ZnO impregnated fabric in saline. All the three treatments were performed in triplicate. The flasks were then subjected to incubation in a rotary shaking incubator at 37 ± 2ºC for 3 hours. After 3 hours, fabrics were removed from the solutions and the solutions were inoculated with 1 ml of bacterial inoculum. The inoculums were prepared by x100 dilution in saline of 3 hour NB cultures. The flasks were then subjected to incubation in a rotary shaking incubator at 37 ± 2ºC. The number of viable cells after incubation was obtained by spread plating serial dilutions on nutrient agar at 0, 3 and 24 hours.

### 3.11 Inductively coupled plasma - optical emission spectrometer (ICP – OES)

An inductively coupled plasma – optical emission spectrometer (PerkinElmer Optima 5300 DV ICP-OES) was used to determine concentration of zinc and copper metal ions in the solution. In the SONO project concentration values for the total copper and zinc impregnations on the fabrics were determined through testing done in Israel (Bar-Ilan University). Further work was carried out at Coventry using the same methodology. For measurements of the total concentrations of Zn and Cu on

the fabrics the metal oxides were fully dissolved with 0.5 M nitric acid. For the leachate experiments, Cu and Zn concentrations were measured in saline solutions after the 3 hour shaking incubation.

Standards and samples were fed to argon plasma via a nebulizer. The excited atoms in the plasma emitted light at characteristic wavelengths to distinguish different elements based on the wavelength of the light emitted. The ICP software (WinLab32) compared the measured intensity in the samples to the calibration graph to determine the concentration.

Stock standards were prepared for each trace metal (Zn/Cu) to be measured for the calibration blank. Using a pipette, different concentrations were measured into a flask to create the desired standard used for the experimental analysis as shown in Table 7. The standards were prepared by diluting 1000 ppm solutions of the metals (Fisher Chemical, Zinc solution 1000 ppm in ca. M nitric acid, Code: J/8070/08; Fisher Chemical, Copper solution 1000 ppm in ca. M nitric acid, Code: J/8025/05 and Nitric Acid, ACS, Alfa Aesar).

**Table 5 Metal ion standards for calibration of the ICP (PPM = parts per million)**

| **Mixed standard** | **Proportion** |
|---|---|
| 20 PPM (Stock standard) | 500 µl Cu sol. + 500 µl Zn sol + 23.5 ml saline + 520 µl 0.5M nitric acid |
| 10 PPM | 5ml of 20 PPM stock standard + 5 ml saline |
| 5 PPM | 2.5ml of 20 PPM stock solution + 7.5 ml saline |
| 2 PPM | 1 ml of 20 PPM stock solution + 9 ml saline |
| 1 PPM | 0.5 ml of 20 PPM stock solution + 9.5 ml saline |

### 3.11.1 Measurement of the total concentration of metals on the fabrics

Fabric samples were placed in contact with 20 ml of sterile saline (0.85% NaCl) in Erlenmeyer flasks. The tests included (a) unwashed control fabric in saline (b) CuO impregnated fabric in saline (c) ZnO impregnated fabric in saline. All the three treatments were performed in triplicate. 416 µl of 0.5 M nitric acid was added to all the flasks to dissolve metal oxides on the fabrics into solution (total digestion of metals in solution) and to maintain a low pH to avoid metal precipitation. The flasks were subjected to heating at 80°C in a water bath for 30 minutes with continuous stirring (manual) every 5 minutes. Fabrics were removed from the solutions and the solutions were used for analysis.

### 3.11.2 Measurement of the concentration of metals in the leachates

Fabric samples were placed in contact with 20 ml of sterile saline (0.85% NaCl) in Erlenmeyer flasks. The tests included (a) unwashed control fabric in saline (b) CuO impregnated fabric in saline (c) ZnO impregnated fabric in saline. All the three treatments were performed in triplicate. The flasks were then subjected to incubation in a rotary shaking incubator at 37 ± 2ºC for 3 hours. After 3 hours, fabrics were removed from the solutions and 416 µl of 0.5M nitric acid was added in 20 ml of leachate solutions to dissolve metal oxides and to maintain a low pH to avoid metal precipitation.

## 3.12 Shake flask tests with different sized fabric pieces

The methodology used for this testing was closely related to the other shake flask tests except that a range of different sized tests pieces were used. The testing was performed with different sized fabric pieces to vary the effective concentration of

the nanoparticles in the flasks/universals. In tests using the standard sized test pieces (5 x 5cm) some of the different fabrics that were tested completely inhibited bacterial growth under the experimental condition used. In these cases it was not possible to compare their antibacterial activity levels with each other. By using a range of smaller test pieces it was hoped that differences in the activity of the impregnations may be made apparent.

Fabrics were cut into the following sized strips:

1. 2.5 x 1cm
2. 2.5 x 2cm
3. 2.5 x 4cm
4. 2.5 x 8cm

For the initial set of fabrics, samples were immersed in 10 ml of nutrient broth (NB) in universal tubes in duplicates. The tests included (a) control fabric (b) CuO impregnated fabric (c) ZnO impregnated fabric (d) no fabric

The NB and fabrics were inoculated with 1 ml of bacterial inoculums (x100 dilution of a 3 hour culture). Immediately after inoculation, the flasks were subjected to incubation in a rotary shaking incubator at 37 ± 2ºC for 24 ± 3 hours at 185 rpm. In the NB tests, the absorbance was checked at 660 nm after 24 hours. For the CuO impregnated fabrics, the leaching of Cu ions turned nutrient broth into a bluish black colour solution and the OD measurements taken were not representative of the bacterial concentration.

To overcome this problem, CuO impregnated fabrics from the pilot plant were tested in both saline and NB. After 24 hours incubation, all samples were plated with appropriate dilutions onto nutrient agar rather than taking OD measurements.

### 3.13 Effect of sample preparation on viable cell numbers

The preparation of samples from the ISO 20743 tests for the measurement of viable cell numbers by flow cytometry (FC) included a number of steps that were not present in the standard plate pouring/plate count method. In the ISO method the SCDLP and NB dilutions were mixed directly with the PCA on the plates. For FC, the bacteria in SCDLP were washed twice with saline and resuspended in staining buffer. This involved three rounds of centrifugation (13,000 rpm for 3 minutes), plus vortex mixing and sonication to re-suspend the cells.

The influence of sonication, vortex mixing and centrifugation on bacterial viable counts was investigated and compared with untreated samples by plating onto nutrient agar. Morphological changes were also observed by Gram staining. Three modes of homogenization experiments were performed for each bacteria:

- Normal samples
- Sonication in a water bath at room temperature for 2 minutes using a 40 kHz Langford sonication bath (7043375TT)
- Centrifugation at 13,000 rpm for 3 minutes followed by vortexing

Following the above treatments samples were serially diluted and plated on NA and then incubated at 37ºC for 24 - 48 hours.

### 3.14 Preparation and incubation of test inoculums

Test inoculums were prepared as per the methodology described in section 3.5.2. The three hour bacterial culture was serially diluted 9 fold in saline universal tubes. Dilution 7, 8 and 9 were used for three modes of homogenization

experiments. Dilutions were prepared in duplicate and each dilution was plated in duplicate on NA plates.

## 3.15 Flow cytometry (FC)

### 3.15.1 Storage and handling of staining solutions

DMSO stock solutions of thiazole orange (TO, 42 µmol/L in DMSO) and propidium iodide (PI, 4.3 mmol/L in water) were stored at ≤ 6ºC away from light. All reagents were allowed to warm to room temperature and then centrifuged briefly before opening. The microsphere standard was stored at 2 - 6ºC and not frozen. When stored properly, the kit is stable for at least one year (BD Biosciences Catalogue No.: 349483). Both DMSO stock solutions must be handled with great care, as DMSO is known to facilitate the entry of organic molecules into tissues.

### 3.15.2 Preparation of live and dead bacterial suspensions

Live and dead bacterial suspensions were used to calibrate and adjust the flow cytometer settings when detecting fluorescent signals from stained bacteria.

- Two 1 ml samples of bacterial culture were centrifuged in a MSE Micro centaur centrifuge at 13,000 rpm for 3 minutes to pellet the cells.
- The supernatant was removed and one of the pellets was resuspended in 1 ml of 0.85% NaCl or buffer (live cell suspension) and the other pellet with 1 ml of 70% isopropyl alcohol (dead cell suspension).
- Samples were incubated for 1 hour +/- 15 minutes with vortex mixing every 15 minutes.

- After one hour incubation the samples were centrifuged as above to form a pellet.
- Both samples were washed twice in 1 ml 0.85% NaCl, each time they were centrifuged the supernatant was discarded and the pellet was resuspended in saline.
- After washing the pellet was resuspended in 1 ml staining buffer (1.4.1.7).
- Control and test samples were also washed twice before resuspending in 1 ml staining buffer.

### 3.16 Bacterial suspension and staining

A total of 11 polystyrene tubes (BD Falcon 5 ml) were labelled as follows and 500 µl of bacterial suspension was added to each tube.

1) Staining buffer only (low background to check noise)
2) Mix of live and dead cells (stained): 250 µl live cells + 250 µl dead cells to confirm that different populations are resolved
3) Live cells
4) Dead cells
5) Inoculum
6) Control sample (triplicate analysis)
7) Test sample (triplicate analysis)

5 µl of each dye was added to the samples followed by 50 µl of the counting bead solution (Bead count: 961-984/µl). The two single colour prepared controls: one live and one dead cell bacterial suspension were stained separately with only TO and PI respectively to set up the flow cytometer. Samples were vortexed and incubated for 10 minutes in the dark at room temperature.

In the FC laser light excitation of TO at 488 nm produced green fluorescence emission in the range of 590 - 600nm, while excitation of PI at 536nm generated red fluorescence emission over 600 nm. Viable cells with intact membranes emit green fluorescence as they are only permeable to TO. In contrast, dead cells with damaged or permeabilised membranes emit red fluorescence when stained with PI.

Instrument settings/parameters are very important to detect small particles like bacteria. Initial instrument settings were set according to the manufacturers recommendations (Alsharif and Godfrey 2002):



A sample of staining buffer without any stains was run through the FC first to adjust noise in the lower left corner of a SSC versus FSC dot plot. The rest of the instrumental settings were adjusted accordingly to the stains used (FL1 and FL3 OD emission, TO and PI) and bacteria employed.

As a defined number of counting beads was added to each test sample (a known concentration and volume) it was possible to specify the volume of sample to be measured by specifying the number of bead events per measurement run. Cell counts were typically acquired after 150 bead acquisition (roughly 3 µl). The mixed live and dead bacterial suspensions were used to calibrate and adjust the flow cytometer settings to position regions for counting live cells and beads as shown in

Figure 27. Green fluorescing TO and red fluorescing PI were positioned at FL1 and FL3 respectively. The regions show division between live cells in gate R3 and dead cells in gate R2.

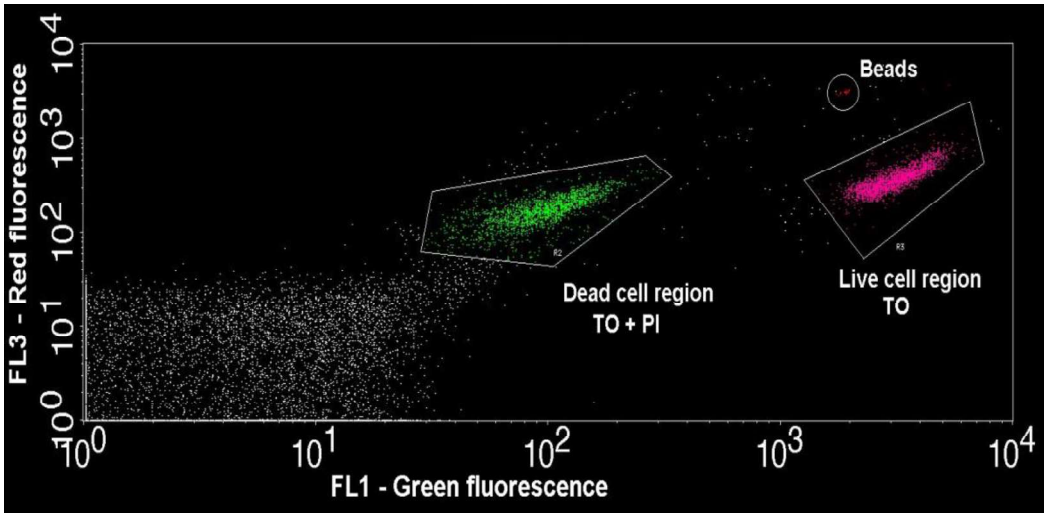


**Figure 27 An example dot plot to show FL1 vs FL3 discrimination of bacterial cells**

### 3.17 Data acquisition and analysis

Data was acquired using BD CellQuest™ Pro software in Acquisition-to-Analysis mode. The fluorescent microsphere beads added to each samples were used as a reference to determine and define sample volume analyzed. From the known test volume and the cell counts it was thus possible to calculate the actual cell concentrations. The following formula was used (Alsharif and Godfrey 2002):

**Equation 5:**

$$\frac{\text{\# of events in region containing cell population}}{\text{\# of events in bead population}} \times \frac{\text{\# of beads per test*}}{\text{test volume}} \times \text{dilution factor} = \text{Concentration of bacterial population}$$

### 3.18 Determination of sensitivity of detection by flow cytometry

A series of experiments was performed to measure the sensitivity of viable cell detection by flow cytometry. Dilutions of 3, 4, 5 & 6 fold of a 3 hour culture of *S. aureus, E. coli and A. baumannii*, *P. aeruginosa* and MRSA were used for the assessment (approximately $10^5$ cells/ml down to $10^2$ cells/ml). Staining buffer was run as a blank.

Spread plating was performed with the samples to establish the relationship between flow cytometry counts and traditional plate counts. All the samples were prepared in duplicate and each sample was run in triplicate.

### 3.19 Fluorescence microscopy

Fluorescence microscopy was used to examine samples of bacteria after incubation with the impregnated fabrics. The same staining protocol was used as for the FC experiments with the exception that the counting beads were omitted. For this piece of work, it was used as a qualitative method to differentiate between live and dead microbial cells recovered from control and impregnated fabrics.

Fabrics were inoculated with high bacterial concentration ($10^6$ - $10^7$ cells/ml) and incubated overnight at 37ºC. After incubation, fabrics were carefully dissected with the help of tweezers and a sharp cutter to get a thin single thread and placed onto a glass slide. Small volume (5 - 10 µl) of the staining solution was added onto the fibre. The slide was covered with cover slip and sealed with a nail lacquer. Slides were kept in dark in a box to avoid photobleaching of the stains and seen under fluorescence microscope at x200 magnification. Images were saved as TIFF files. Testing was also done on the fabrics used for ISO plating and flow cytometry.

## 3.20 Cytotoxicity studies

### 3.20.1 Cell culture

Human dermal fibroblast cells (HDF) and human hepatocellular carcinoma cells (HepG2) were obtained from the European collection of cell cultures (ECCC). The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 4.5 g/l glucose, 1% glutamine supplemented with 10% foetal bovine serum (FBS) and 1% penicillin/streptomycin at 37 ºC in a humidified atmosphere with 5% $CO_2$. The cells were sub-cultured every 2 days. The cells were washed with PBS and harvested using 0.25% trypsin. After trypsinization, the cells were counted using a haemocytometer by adding 20 µl on a slide. After counting and appropriate dilutions, the cells were seeded at a density of 5.0 x $10^3$ cells/well in a 96 well tissue culture treated polystyrene plate. Cells were allowed to attach the surface for 24 hours prior to nanoparticles exposure and incubated at 37 ºC (5% $CO_2$).

### 3.20.2 Cytotoxicity by indirect contact

Fabrics impregnated with ZnO and CuO nanoparticles (both *in-situ* and TTS from the pilot scale) were cut down into round discs (~ 3 cm diameter) in order to fit in the wells of a 6 well plate and sterilized by autoclaving. Control fabrics without any impregnation were used as a negative control, whereas a 30% v/v solution of dimethyl sulfoxide (DMSO) prepared in fresh culture medium was used as a toxicity positive control. Samples with no fabrics were also tested as a further negative control set.

The fabric samples were placed in contact with 3 ml of Dulbecco's Modified Eagle's Medium (DMEM) containing high glucose, glutamine supplemented with foetal bovine serum (FBS) and incubated at 37 ºC in a humidified atmosphere with

5% $CO_2$ for 1 and 7 days. Following this, the fabric samples were removed from the growth media and the leachate solution was collected in universal tubes for use in the cytotoxicity studies.

For the exposure tests, the growth media from previously seeded cells in the 96 well plates were discarded after 24 hours of incubation. 100 µl of leachate solution was then added to the 96 well plate and the plates were incubated at 37 ºC in a humidified atmosphere with 5% $CO_2$ for 24 hours.

### 3.20.3 MTT assay

The microculture tetrazolium (MTT) assay is a colorimetric assay for measuring the activity of cellular enzymes and their cell viability. The cellular enzymes reduce the tetrazolium dye (MTT) to its insoluble formazan product giving a purple colour. The MTT assay was used to measure the cytotoxicity of ZnO and CuO NPs to human cell lines. In brief, 5 $\times 10^3$ cells/well were seeded in 96 well plates and exposed to the prepared leachate (section 3.20.2) and incubated at 37 ºC in a humidified atmosphere of 5% $CO_2$ for 24 hours. After 24 hours exposure time, 100 µl of MTT solution was added to each well and incubated at 37 ºC in a humidified atmosphere of 5% $CO_2$ for 3 hours until a purple coloured formazan product developed. At the end of incubation, medium was removed from each well to avoid interference from the nanoparticles and 100 µl of DMSO was added to each well to lyse the cells. The plate was then left for 5 minutes before the absorbance was measured at 492 nm using a microplate reader.

### 3.21 Statistics

Statistical analysis was carried out using the analysis tool pack provided with Microsoft Excel 2010. The antibacterial activity of impregnated fabrics was compared with control fabrics using analysis of variance (ANOVA) and *t*-test at an alpha level of α 0.05. ANOVA was used to test for significant differences in multiple comparisons and *t*-test was used to compare the means of two different samples, treatments or groups to see if they were statistically different from each other.

# 4 Results and Discussion

The main objective of this study was to assess the antibacterial activity of ZnO and CuO fabrics against a selection of Gram positive and Gram negative bacteria. The fabric substrates were impregnated with ZnO and CuO nanoparticles using the sonochemical process developed by BIU, Israel (Abramov *et al.* 2009). Following the impregnation, the fabrics were evaluated qualitatively and quantitatively as per the methods described in the previous chapter. In order to assist with the optimisation of the industrial scale process from the laboratory scale, the testing was divided into two phases:

i. Testing of the cotton fabrics prepared at lab scale
ii. Testing of two sets of polyester/cotton pilot CuO fabrics *(in-situ* and TTS) prepared at Klopman and Davo at an industrial scale

## 4.1 Testing of lab scale ZnO and CuO impregnated cotton fabrics

The evaluation of the antibacterial efficacy of impregnated fabrics was carried out as per the absorption method from ISO 20743, disc diffusion method and shake flask method. ZnO and CuO impregnated fabrics were prepared at the laboratory scale using a sonochemical system at BIU (process described in methodology section) (Abramov *et al.* 2009). The concentration of ZnO and CuO on the fabrics as determined by ICP was 0.39% and 0.51% w/w of fabric respectively.

### 4.1.1 Antibacterial efficacy testing of lab scale ZnO and CuO cotton fabrics using the absorption method from ISO 20743:2007

Table 6 reports the viable bacterial counts recovered from the ZnO impregnated fabrics after 24 ± 3 hours of contact time against *S. aureus*, *E. coli*, *A. baumannii*, *P. aeruginosa* and *K. pneumoniae.* As shown in Figure 28, the control samples supported bacterial growth and as expected did not cause any bacterial kill during the 24 ± 3 hour contact incubation. *P. aeruginosa* was the most susceptible bacteria with ZnO impregnated fabrics showing a full kill on the fabric surface with an antibacterial A value of 7.97. The ZnO cotton also showed activity against *S. aureus* (A = 3.66), *E. coli* (A = 2.94), *A. baumannii* (A = 2.73) and *K. pneumoniae* (A = 2.41).

Thus, all the bacterial species tested showed an antibacterial value A > 2, as calculated by the formula from ISO 20743 standard (Equation 2). As per the guidelines published by Hohenstein Institute regarding antibacterial activity values, an antibacterial A value of > 2 is considered as significant antibacterial activity (Hoefer and Hammer 2011). The antibacterial A value is associated with growth reduction. Growth reduction is the difference in the number of bacteria on the test fabrics compared to the control fabrics after incubation. On the control fabrics, the number of viable bacteria should increase during the incubation. If the test fabrics have antibacterial activity, then the number of viable bacteria on them post incubation should be less than the number of viable bacteria on the control fabrics. An A value of 2 represents a 2 log difference in these numbers.

Differences in the levels of antibacterial activity between the bacterial species can also be described in terms of percentage reduction value (Table 6). Percentage reduction shows the difference in the number of bacteria recovered from the control sample immediately after inoculation compared to the number of bacteria recovered

from the impregnated test fabric after incubation (Equation 3). The percentage reduction gives an indication of nature of the antibacterial activity. If there is a large reduction in the number of viable cells compared to the initial inoculum, then the action is likely to be bactericidal. If there is only inhibition of growth, or a limited reduction in numbers then the activity is bacteriostatic. The criteria used with the absorption method for the classification of antibacterial fabrics do not distinguish between bacteriostatic and bactericidal activity. In terms of percentage reduction *P. aeruginosa* reduced bacterial growth by > 99.9% followed by *S.aureus* (99.77%), *A. baumannii* (72.22%) and *K. pneumoniae* (61.29%). *E. coli* was the least susceptible bacterial species showing only a 4.12% reduction in viable cell numbers.

These results demonstrate that the method of calculating antibacterial activity should be considered when interpreting the results. Even though the antibacterial A value suggested that *E. coli* possessed a significant antibacterial activity (A = 2.94), the percentage reduction values have shown that the fabrics have reduced viable cell numbers by only 4.12%. Hence, percentage reductions should also be considered alongside growth reduction and antibacterial A values.

In the case of *E. coli*, the antibacterial A value actually showed that the ZnO fabrics inhibited growth with very little bactericidal activity (4.12% reduction). Whereas, in the case of *P. aeruginosa*, there was significant bactericidal activity (> 99.9% reduction) and consequently a high A value. In the textile industry there are currently no guidelines stating that fabrics should possess bactericidal activity *(Rio et al.* 2012). Instead growth inhibition is taken as an indication of efficacy (Hoefer and Hammer 2011). This should be taken into account when considering end user applications. Hospitals may require higher levels of antibacterial efficacy from textiles than general consumers.

A study by Perelshtein *et al.* (2009) showed that bandages coated with ZnO nanoparticles released $Zn^{2+}$ ions into the surrounding medium and also generated reactive oxygen species (ROS); these play an important role in the antibacterial activity of ZnO. Their results showed that cotton fabrics impregnated with 0.75% w/w ZnO exhibited bactericidal activity for *S. aureus* and *E. coli*. The researchers also showed that the antibacterial activity of ZnO fabrics was greater with Gram positive *S. aureus* than with Gram negative *E. coli*. The results matched with our results above, where lab scale cotton impregnated with 0.39% w/w ZnO displayed antibacterial activity against all the bacterial species tested. The activity was greater with *S. aureus* than with *E. coli*. These results suggest that the antibacterial activity of ZnO fabrics might be concentration dependant *i.e*. the higher the concentrations better the activity. As stated above, the ZnO concentration used on the lab scale cotton fabrics was lower than the concentration used by Perelshtein *et al.* (2009) in their studies. Mirhosseini and Firouzabadi (2013) also claimed that higher concentrations of ZnO treatments are capable of producing a bactericidal effect, whereas lower ZnO concentrations only supress bacterial growth.

Yamamoto *et al.* (2000) stated that the presence of reactive oxygen species (ROS) generated by ZnO nanoparticles is responsible for their bactericidal activity. Padmavathy and Vijayaraghavan (2008) further elucidated the bactericidal activity of ZnO nanoparticles. According to them, once hydrogen peroxide is generated by ZnO nanoparticles, the nanoparticles remains in contact with the dead bacteria to prevent further bacterial growth and continue to generate and discharge hydrogen peroxide to the medium.

**Table 6 Mean viable bacterial counts (CFU/ml) after 24 ± 3 hours contact time showing growth reduction with ZnO impregnated cotton samples compared to control cotton samples. CFU/ml at time zero 1 – 3 x$10^5$ CFU/ml. Statistically significant differences in the viable counts between control and test samples post incubation ($p$<0.05 for each). Mean ± SD, n = 3**

| Bacteria | After 24 h contact time | | | | Percentage reduction | % Growth reduction | A value |
|---|---|---|---|---|---|---|---|
| | Control | St. Dev | ZnO fabrics | St. Dev | | | |
| *S. aureus* | 5.40E+07 | 4.90E+06 | 1.20E+04 | 1.10E+04 | 99.77 | 99.97 | 3.66 |
| *E. coli* | 8.00E+08 | 3.00E+08 | 9.30E+05 | 7.20E+05 | 04.12 | 99.88 | 2.94 |
| *A. baumannii* | 8.30E+07 | 5.90E+07 | 1.50E+05 | 2.30E+05 | 72.22 | 99.82 | 2.73 |
| *P. aeruginosa* | 1.00E+08 | 8.20E+07 | 100 (LOD) | 0.00E+00 | 99.98 | >99.99 | 7.97 |
| *K. pneumoniae* | 6.60E+08 | 5.24E+08 | 1.20E+06 | 8.37E+05 | 61.29 | 99.81 | 2.41 |

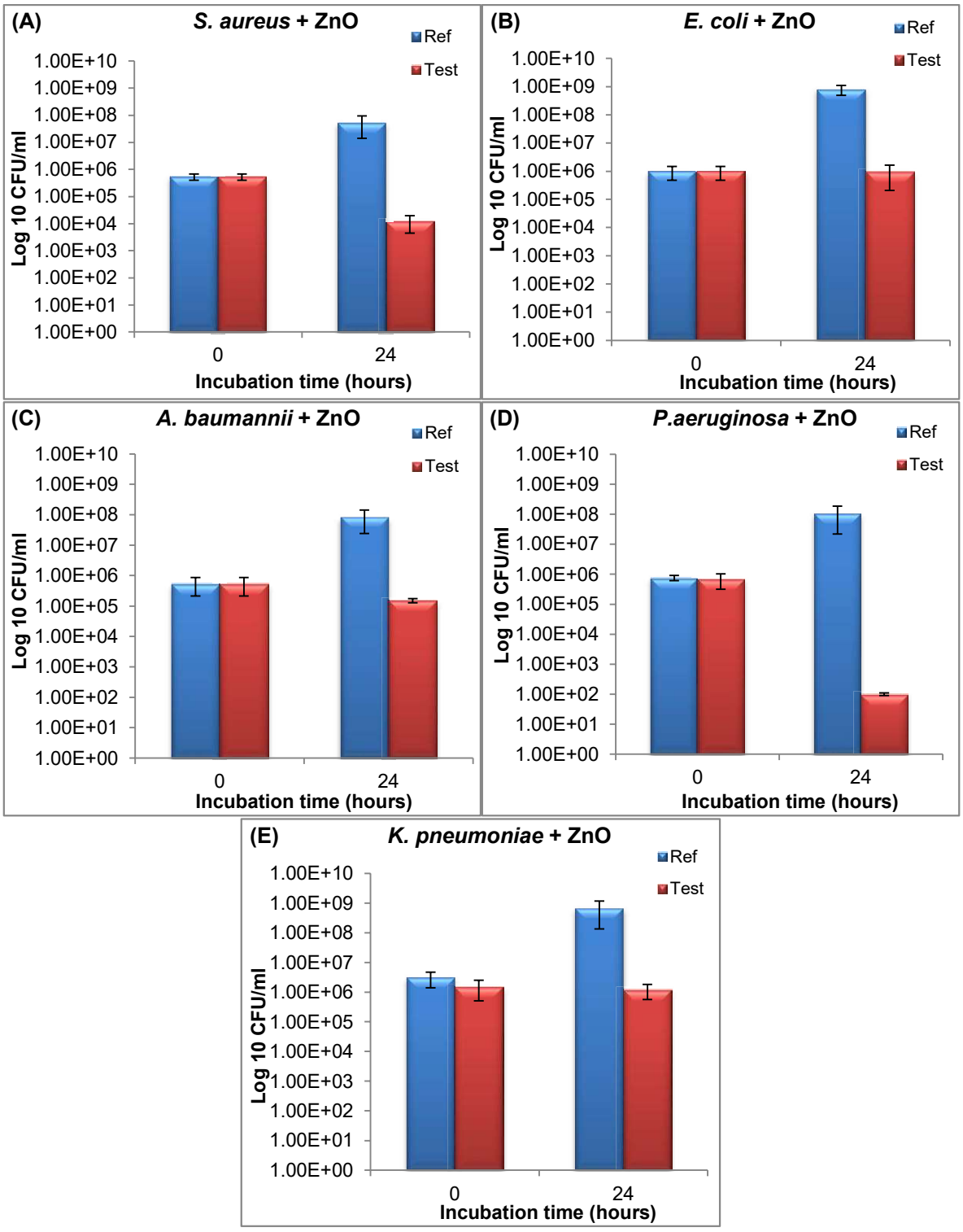


**Figure 28 Antibacterial assessment of lab scale ZnO impregnated fabrics against (A) *S. aureus*, (B) *E. coli*, (C) *A. baumannii*, (D) *P. aeruginosa* and (E) *K. pneumoniae* by the absorption method (ISO 20743). Fabrics were spiked with known concentration (1 - 3 x $10^5$ CFU/ml in dilute NB) of bacteria. The number of viable bacteria recovered from control fabrics (red bars) and test fabrics (blue bars) at 0 hours and after 24 ± 3 hours was determined and calculated. Mean ± SD, n = 3**

Table 7 reports the antibacterial activity of the CuO NPs on the cotton fabrics as determined using the absorption method. The number of viable bacteria recovered from control fabrics (blue bars) and impregnated fabrics (red bars) at 0 hours and after 24 ± 3 hours was determined (Figure 29). According to the antibacterial A values, CuO impregnated fabrics possessed a significant antibacterial activity against all the five bacterial species tested. *E. coli* was the most susceptible bacterial species (A = 4.64) followed by *P. aeruginosa* (A = 3.84), *A. baumannii* (A = 3.14), *S. aureus* (A = 2.96) and *K. pneumoniae* (A = 2.92).

Comparing the results of ZnO and CuO fabrics; the CuO fabrics exhibited greater antibacterial activity than the ZnO fabrics. In terms of percentage reductions, all the bacterial species showed a minimum of 64% bacterial reduction on the CuO fabric surface showing a significant bactericidal activity. The results with CuO fabric showed that *E. coli* was the most susceptible and *K. pneumonia* was the least susceptible. Whereas, the ZnO results showed that *P. aeruginosa* was the most susceptible bacteria and *E. coli* was the least susceptible. These results suggest that there are species specific differences in the susceptibility of bacteria to ZnO and CuO. Azam *et al.* (2012) in their studies demonstrated that the antibacterial properties of ZnO, CuO and $Fe_2O_2$ nanoparticles varied with species. Graves (2014) in their review also claimed that bacteria respond differently to different metal oxide nanoparticles.

CuO NPs are effective in killing a wide variety of bacterial species involved in hospital acquired infections (Borkow and Gabbay 2009, Borkow, Okon-Levy and Gabbay 2010, Gabbay et al. 2006, Ravishankar Rai and Jamuna Bai 2011, Shaffiey et al. 2014). Perelshtein *et al.* (2009b), in their study synthesized and impregnated CuO NPs on cotton fabrics using sonochemical irradiation. They showed that a 1%

w/w concentration of CuO nanoparticles on fabrics exhibited bactericidal activity against *E. coli* and *S. aureus*. Our results above have shown that even lower concentration of CuO on cotton fabrics (0.51% w/w) were able to kill a selection of Gram positive and Gram negative bacteria. Abramov *et al.* (2009), in their studies found that 0.65 wt. % of CuO resulted in > 90% reduction in *E. coli* growth within 2 hours. Antibacterial results reported by Ahamed *et al.* (2014) matched well with our results. They analysed the antibacterial activity of CuO NPs and found *E. coli* to be the most susceptible (MIC 31.25 µg/mL) and *K. pneumoniae* the least sensitive test microbe (MIC 250 µg/mL).

**Table 7 Mean viable bacterial counts (CFU/ml) after 24 ± 3 hours contact time showing log growth reduction with CuO impregnated cotton samples compared to control cotton samples. CFU/ml at time zero 1 – 3 x$10^5$ CFU/ml. Statistically significant differences in the viable counts between control and test samples post incubation ($p<0.05$ for each). Mean ± SD, n = 3**

| Bacteria | After 24 h contact time | | | | Percentage reduction | % Growth reduction | A value |
|---|---|---|---|---|---|---|---|
| | Control | St. Dev | CuO fabrics | St. Dev | | | |
| *S. aureus* | 5.40E+07 | 4.90E+06 | 5.90E+04 | 7.20E+04 | 89.07 | 99.89 | 2.96 |
| *E. coli* | 8.00E+08 | 3.00E+08 | 1.80E+04 | 1.40E+04 | 98.14 | 99.99 | 4.64 |
| *A. baumannii* | 8.30E+07 | 5.90E+07 | 6.10E+04 | 6.50E+04 | 88.70 | 99.92 | 3.14 |
| *P. aeruginosa* | 1.00E+08 | 8.20E+07 | 1.90E+04 | 3.00E+04 | 97.50 | 99.98 | 3.84 |
| *K. pneumoniae* | 6.60E+08 | 5.24E+08 | 1.10E+06 | 1.07E+05 | 64.51 | 99.83 | 2.92 |

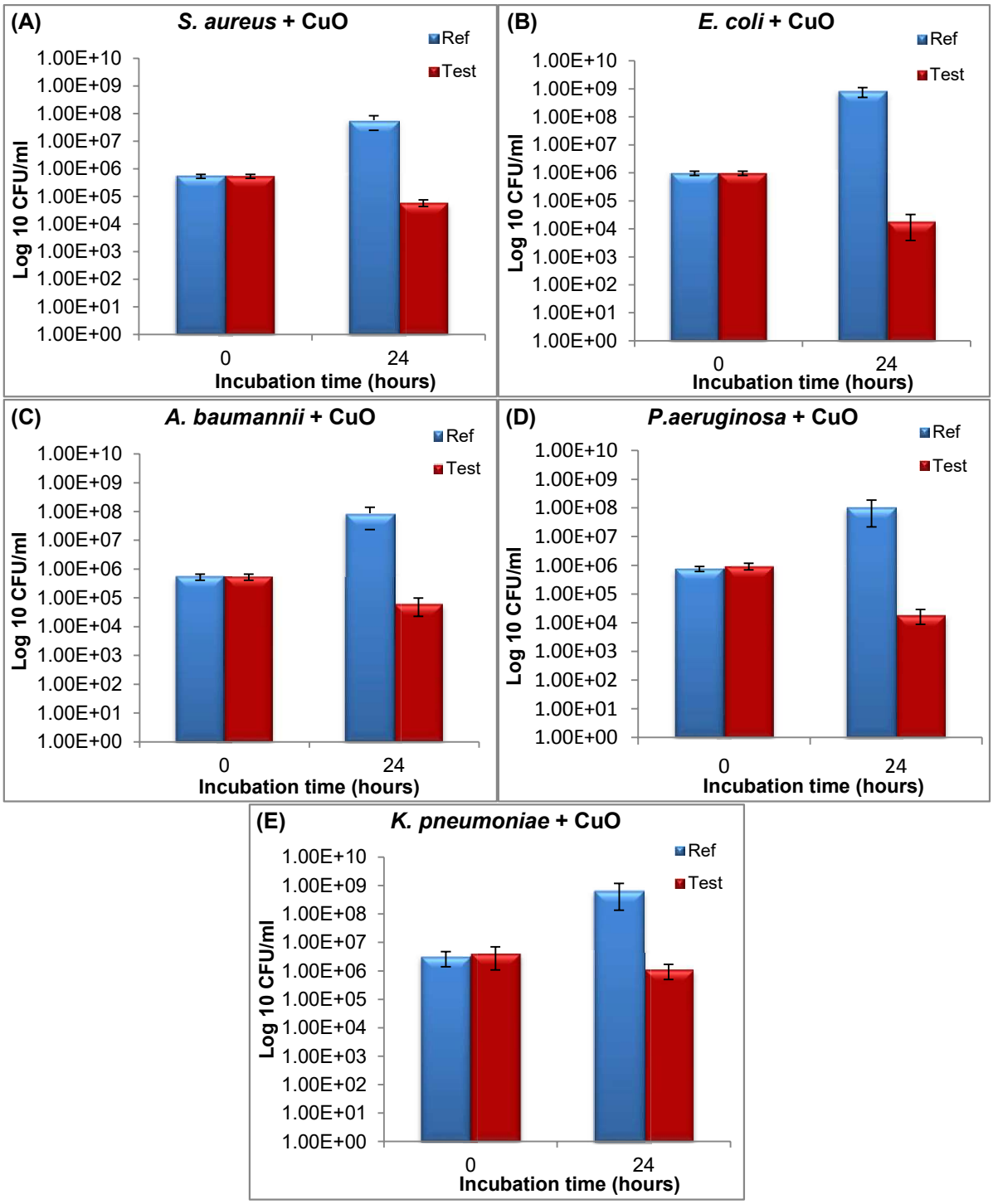


**Figure 29 Antibacterial assessment of lab scale CuO impregnated fabrics against (A) *S. aureus*, (B) *E. coli*, (C) *A. baumannii*, (D) *P.aeruginosa* and (E) *K. pneumoniae* by the absorption method from ISO 20743. Fabrics were spiked with a known concentration (1 - 3 x $10^5$ CFU/ml in dilute NB) of bacteria and tested for their antibacterial efficacy. The number of viable bacteria recovered from control fabrics (blue bars) and impregnated fabrics (red bars) at 0 hours and after 24 ± 3 hours was determined and calculated. Mean ± SD, n = 3**

Overall, the absorption results showed that both ZnO and CuO fabrics are antibacterial against the Gram positive and Gram negative bacteria tested. Apart from the lower antibacterial activity shown by ZnO fabrics against *E. coli* (4.12% reduction), both the fabrics types (ZnO and CuO) showed bactericidal activity against all the bacterial species tested (> 60% reduction).

These fabrics are intended for use in hospitals as part of the infection prevention control strategy. Even if the fabrics are not necessarily bactericidal, they can still be effective in reducing cross contamination within the hospital settings by reducing bacterial growth.

### 4.1.2 Antibacterial activity of PEC fabrics coated with silver and triclosan

PEC fabrics coated with silver and triclosan were tested to compare the level of activity with the ZnO and CuO fabrics. Silver and triclosan were applied to the fabrics using a conventional pad-dry-cure method. One of the project partners supplied the fabrics but did not provide any further details regarding the concentrations of the coatings or the exact chemical formulations used. Polyester cotton fabrics (65/35 or 60/40%) coated with silver or triclosan were tested to determine their antibacterial activity against *E. coli* and *S. aureus*. The results presented in Figure 30 show an excellent antibacterial activity for silver and triclosan coated on polyester cotton fabrics. Both silver and triclosan fabrics showed a full bacterial kill on their surface (> 99.9% reduction). A limit of detection of 100 CFU/ml was recorded on graphs for silver and triclosan fabrics due to the dilution steps involved in the testing. Here silver and triclosan fabrics have exhibited a greater antibacterial activity than the ZnO and CuO impregnated fabrics.

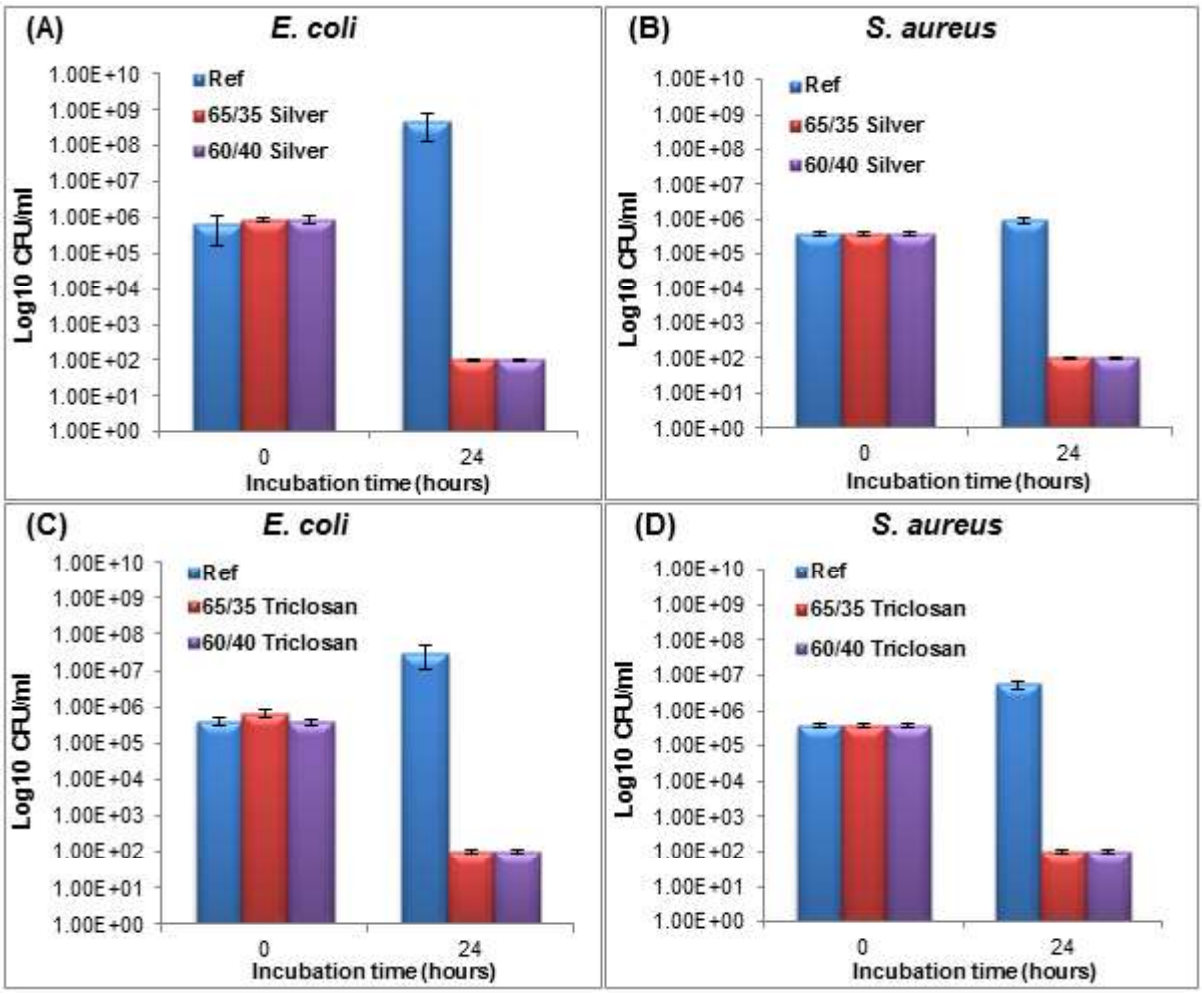


**Figure 30 Antibacterial activity of PEC fabrics coated with silver and triclosan against (A) & (C) *E. coli* and (B) & (D) *S. aureus*. Silver and triclosan fabrics showed a full bacterial kill. Hence 100 cells were taken as limit of detection (LOD). Mean ± SD, n = 3**

Testing was also performed with 60/40% polyester cotton fabrics coated with silver and triclosan against *K. pneumoniae*. The addition of triclosan or silver to the fabric surfaces resulted in a complete kill of *K. pneumoniae* (> 99.9% reduction) after 24 ± 3 hours contact time (Figure 31).

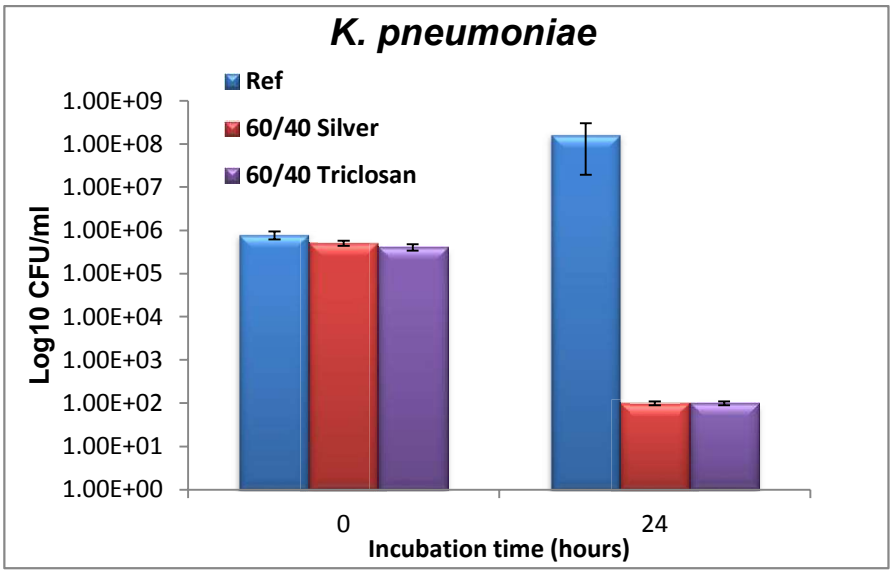


**Figure 31 Antibacterial activity of 60/40% polyester cotton fabrics coated with silver and triclosan tested against *K. pneumoniae*. Silver and triclosan fabrics showed a full bacterial kill. Mean ± SD, n = 3**

From the absorption results shown in Table 8, it is clear that the Ag and triclosan coated fabrics have high levels of antibacterial activity as compared to fabrics impregnated with ZnO and CuO NPs. Even though, Ag and triclosan display greater antibacterial activity compared to ZnO and CuO; the biological and environmental impacts of their excess usage is a concern (Windler, Height and Nowack 2013). Consumer products containing Ag and triclosan have been produced on a huge scale and hence, the release of these agents into the environment has become a serious issue of concern. The textile industry has relied heavily on these agents for the production of antibacterial fabrics. The laundering of antibacterial textiles could release large amounts of these agents into the environment (Adolfsson-Erici *et al.* 2002, Benn and Westerhoff 2008, Geranio, Heuberger and Nowack 2009, Orvos *et al.* 2002). Concerns over the toxicity of silver and triclosan to humans and their effects on other species in our environment have prompted a demand for eco-friendly coatings. As well as processes that could substitute for toxic

chemicals used in textile coatings. Recent studies have also highlighted concerns over the development of bacterial resistance to silver (Percival, Bowler and Russell 2005).

The main motive behind this study was to use inorganic metal oxide based NPs (ZnO and CuO) that could exhibit good antimicrobial properties on fabrics with minimal to no toxic effects. Even though the antibacterial activity of Ag and triclosan were better than ZnO and CuO, the SONO fabrics exhibited a significant antibacterial activity (Table 8). The ZnO and CuO were synthesized and impregnated on fabric substrates using green technology (ultrasound) with minimal use of toxic chemicals. The solutions used in this project were all aqueous solutions and the concentrations of the precursors were relatively low. The inorganic salts used were more stable than organic antibacterials such as triclosan. A further advantage of using ZnO and CuO was their lower cost compared to silver.

**Table 8 Comparison of the antibacterial A value of ZnO and CuO impregnated fabrics to silver and triclosan fabrics. N/T – not tested**

| Bacteria | ZnO | CuO | Ag 60/40 | Ag 65/35 | Tric 60/40 | Tric 65/35 |
|---|---|---|---|---|---|---|
| ***S. aureus*** | 3.66 | 2.96 | 5.51 | 5.57 | 6.51 | 7.04 |
| ***E. coli*** | 2.94 | 4.64 | 8.85 | 8.82 | 7.46 | 7.70 |
| ***A. baumannii*** | 2.73 | 3.14 | N/T | N/T | N/T | N/T |
| ***P. aeruginosa*** | 7.97 | 3.84 | N/T | N/T | N/T | N/T |
| ***K. pneumoniae*** | 2.41 | 2.92 | 8.02 | N/T | 7.92 | N/T |

### 4.1.3 Assessment of the wash durability of ZnO, CuO, silver and triclosan fabrics in terms of antibacterial activity

During the course of the SONO project a number of tests were carried out to assess the wash durability of the antibacterial impregnations. The washing was carried out at the National Institute for Textile and Leather Research (NIT) in Bucharest, Romania. Fabric samples were washed according to the methodology described in ISO 6330 for 10 cycles without detergent (Din 2012). Cotton fabrics were washed at 92°C and polyester cotton mix fabrics were washed at a temperature of 75°C. Following washing, fabric samples were autoclaved and tested according to the absorption method from ISO 20743.

Table 9 shows the antibacterial activity values for a range of washed antibacterial fabrics against *E. coli*. In general, all the washed fabrics showed a reduced antibacterial activity compared to the unwashed fabrics (Figure 32). The 60/40 PEC mix fabric coated with triclosan showed the strongest antibacterial activity (A = 3.99), much higher in fact than the 65/35 PEC mix coated with triclosan (A = 0.42). The antibacterial activity of the other coated fabrics decreased to low levels after washing (A <1). Although silver and triclosan fabrics showed bactericidal activity with unwashed fabrics, their antibacterial activity decreased significantly due to loss of coatings from the fabric surface post washing. This shows that the coatings are not durable to laundering. Ilić *et al.* (2009) reported quite similar results showing that fabrics coated with silver NPs from a 10 ppm colloid exhibited poor laundering durability and lost all their coating after the second wash cycle. They claimed that higher concentrated colloids (50 ppm) should be applied to fabrics in order to obtain long term durability. Benn and Westerhoff (2008) investigated leaching of silver from

commercial socks into water. They tested six types of different socks and showed that all the socks lost almost all of their silver by the 4th wash.

**Table 9 Antibacterial activity values (A) of fabrics after 10 wash cycles at 92°C (cotton) or 75°C (PEC) without detergent against *E. coli*. Pre wash values are also shown.**

| *E. coli* | | |
|---|---|---|
| **Fabric type** | **Antibacterial A values (Post wash)** | **Antibacterial A values (Pre wash)** |
| ZnO cotton | 1.14 | 2.94 |
| CuO cotton | 0.73 | 4.64 |
| 60/40 PEC silver | 0.82 | 8.85 |
| 65/35 PEC silver | 0.23 | 8.82 |
| 60/40 PEC triclosan | 3.99 | 7.46 |
| 65/35 PEC triclosan | 0.42 | 7.70 |

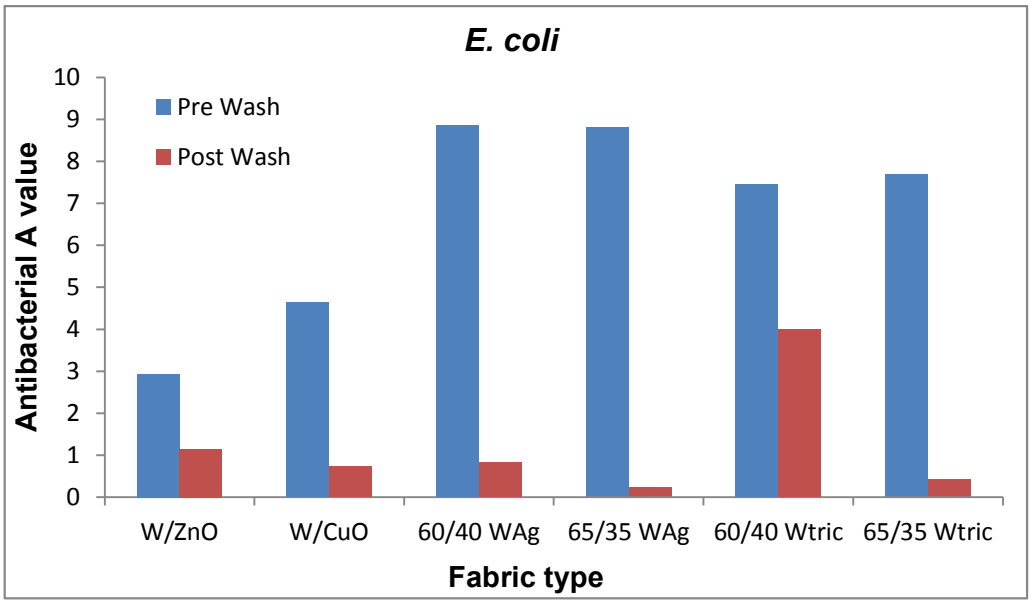


**Figure 32 Antibacterial activity values of different fabrics against *E. coli* after 10 wash cycles according to ISO 6330. 10 wash cycles at 92°C (cotton) or 75°C (PEC) without detergent**

### 4.1.4 Enzymatic pre-treatment of cotton prior to ZnO impregnation

As part of the SONO project work a group at the University of Catalonia (UPC), Spain, investigated some other methods for improving the antibacterial impregnations. These included enzyme pre-treatment of cotton with cellulase (Cellusoft®APL) and the addition of chitosan and BTCA to the metal oxide NPs impregnation. The enzyme cellulase partially breaks down the cellulose surface and creates extra free hydroxyl groups on the surface of fibres which may act as nucleation sites for the NPs. SEM analysis showed that cellulose treatment resulted in smaller sized nanoparticles with improved adhesion (Perelshtein *et al.* 2012).

In other work at UPC, butane tetra carboxylic acid (BTCA) was used to covalently attach the chitosan to the cotton substrate. Chitosan and ZnO or CuO were used in combination to enhance the antibacterial activity of the fabrics. Chitosan (1% w/v 15kDa) was applied to the fabrics using a pad-dry-cure method with 5 minutes at 70°C or 2 minutes at 160°C. It was also found that ultrasound increased the amount of chitosan absorbed onto cotton fabrics (Petkova *et al.* 2014).

As shown in Figure 33, incubation for 24 ± 3 hours with the combination of ZnO impregnated fabrics pre-treated with enzyme, chitosan and the covalent linker BTCA showed an excellent antibacterial activity against *A. baumannii* (> 4 log reduction). Amongst all three modifications, inclusion of the covalent linker BTCA with the chitosan treatment and ZnO resulted in the greatest antibacterial activity against *A. baumannii*. The work with BTCA was not continued in the project because it was found that the BTCA treatment had a deleterious effect on the physical properties of the cotton fabrics. The inclusion of BTCA with chitosan may have enhanced the antibacterial activity of the fabrics by improving the fixation of chitosan to the cotton or through antibacterial activity from the carboxylic acid groups.

Other studies have also shown that inclusion of BTCA with other coating enhances the antibacterial activity. Nazari *et al.* (2013) used BTCA in combination with nano titanium dioxide ($TiO_2$) on cotton fabrics. They showed a synergetic effect of BTCA and $TiO_2$ giving a percentage reduction of > 99% against *E. coli* and *S. aureus*. El-Tahlawy *et al.* (2005) also showed similar results where they treated chitosan with two cross linking agents; BTCA and Acrofix NEC. Their results showed that the fabrics treated with BTCA improved the antimicrobial properties more than the fabrics treated with Acrofix NEC.

The comparative durability to laundering of the ZnO and combined impregnations on cotton fabric substrates is illustrated in Figure 33. In the case of *A. baumannii*, the washing resulted in a complete loss of antibacterial activity. ZnO impregnated fabrics lost their antibacterial activity when subjected to 10 washes at 92°C. The ZnO fabrics in combination with chitosan still displayed antibacterial activity (A > 1) after 10 laundry cycles at 92°C. The inclusion of covalent linker BTCA did not improve the wash stability compared to the ZnO/chitosan without BTCA. This suggests that the BTCA cross linking of chitosan in the cotton was actually not effective in improving the durability.

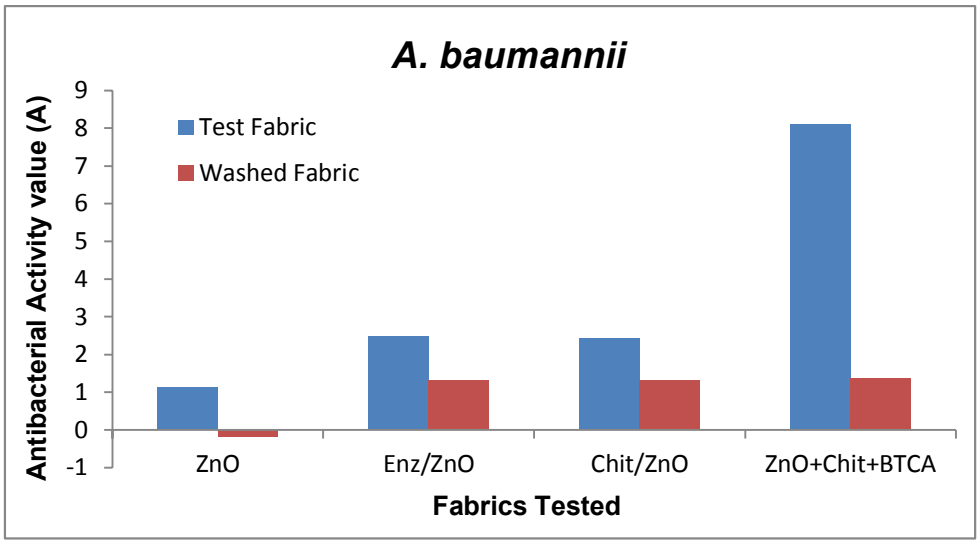


**Figure 33 Antibacterial activity of modified cotton fabrics impregnated with ZnO NPs against *A. baumannii*. Fabrics were also compared against washed fabrics to monitor the effect of pre-treatment on the stability of fabrics. Enz – cellulose enzyme pre-treatment, Chit – chitosan, BTCA – covalent linker butane tetracarboxylic acid**

### 4.1.5 Antibacterial activity testing of lab scale ZnO and CuO fabrics by a disc diffusion method

One of the methods used to investigate the ability of the antibacterial agent to inhibit bacterial growth was a disc diffusion method. Figure 34 shows the zones of inhibition for the cotton fabric substrates impregnated with ZnO and CuO nanoparticles against three different bacterial species. Samples of the impregnated and control cotton fabrics were cut in to discs (10 mm) and incubated with the test bacteria spread on the surface of Isosensitest agar plates. The diameters of the zones of inhibition around the discs are presented in Table 10. In Figure 34 clear zones of inhibition (ZOI) are visible around the ZnO and CuO treated fabric discs whereas the control fabric disc did not show any sign of inhibition. Both the impregnations showed a similar pattern of activity against the three test strains. Both the ZnO and CuO nanoparticles inhibited the growth of *E. coli* and *S. aureus*. The

ZOI for *S. aureus* was 18 - 19 mm for both the fabrics and the ZOI for *E. coli* was 13 - 15 mm for both fabrics. There was no visible activity against *A. baumannii*.

In contrast to the absorption testing, the disc diffusion results showed that the ZnO and CuO fabrics were not active against *A. baumannii*. The differences in activity observed between the results may relate to differences in the test conditions. In the absorption method the bacteria is inoculated straight on to the test fabrics. Thus during incubation, the bacteria come into direct contact with the NPs attached to the fabric fibres. The bacterial inoculum also contains a much lower concentration of nutrients compared to the nutrient agar surface.

In the disc diffusion test, as the NPs are firmly attached to the fabrics direct interaction between the metal oxides and the bacteria should only play a minor role in the antibacterial activity. The zone of inhibition must instead be due to the diffusion of some antibacterial agent from the impregnated fabrics through the agarose gels. These diffusing agents could be zinc or copper ions released from the NPs or they could be reactive oxygen species (ROS) produced by the NPs (Padmavathy and Vijayaraghavan 2008).

Both zinc and copper ions have antibacterial activity (Chohan, Supuran and Scozzafava 2004, Top and Ülkü 2004, You and Zhu 2004). ZnO and CuO have a low solubility in neutral aqueous solutions but have been observed to be more soluble in nutrient media (see section 4.2.5.2 for ICP data). During the incubation phase of the disc diffusion test, the brown CuO samples lost their colour indicating the loss of CuO by dissolution into the agar. The ZnO impregnation did not change the colour of the fabrics and so it was not possible to see whether it too was dissolved into the agar.

The difference in the susceptibility of the 3 species to the impregnated fabrics in these tests could be due to differences in the structure of their outer walls. The double membrane structure of the Gram negatives, *E. coli* and *A. baumannii* may provide an extra layer of protection against the diffusing antibacterial agents compared to the Gram positive outer wall and single membrane of *S. aureus*. The disc diffusion results with *A. baumannii* may be due to it being less susceptible to the activity of the free copper or zinc ions than it is to direct contact with the NPs. Further experiments would be needed to confirm this.

The disc diffusion method of Kirby and Bauer is convenient in respect that the susceptibility of bacterial pathogens to antimicrobial agents can be determined in a single assay. The method is qualitative, simple to perform and suitable for testing a large number of samples in a short time. However, the results do not give an indication of minimum inhibitory concentration, minimum bactericidal concentration or minimum biofilm eradication concentration (MIC, MBC and MBEC) values. Also this method is not reliable when comparing the relative efficiency of one antimicrobial to another. This is because the diffusion rate of antimicrobials through the agar depends upon the type of antimicrobial agent used and some antimicrobials do not diffuse at all (Swofford 2010, Varesano *et al.* 2011). Many researchers have used non-bound antimicrobials on fabrics and evaluated their antibacterial activity by a disc diffusion method. Rajendra *et al.* (2010) and Yadav *et al.* (2006) prepared ZnO NPs by wet chemical method and coated cotton fabrics using a pad dry cure method. The antibacterial activity was evaluated using disc diffusion method.

Here, the ZnO and CuO NPs were impregnated into the fibres and the disc diffusion method should not be suitable for assessing antibacterial activity. The results did indicate leaching of the antibacterial activity into the agar in some cases,

but the activity from the disc diffusion test did not match the activity found with the absorption testing. So, this method would not be a suitable rapid alternative to the absorption method for checking the activity of these types of fabrics.

**Table 10 Antibacterial assessment in terms of zone of inhibition for ZnO and CuO cotton fabrics by disc diffusion method**

| Fabrics | Organism | Zone of Inhibition (Diameter in mm) | | |
|---|---|---|---|---|
| | | 1 | 2 | Mean |
| ZnO | ***S. aureus*** | 18 | 19 | 18.5 |
| | ***E. coli*** | 13 | 14 | 13.5 |
| | ***A. baumannii*** | 0 | 0 | 0 |
| CuO | ***S. aureus*** | 18 | 18 | 18 |
| | ***E. coli*** | 14 | 15 | 14.5 |
| | ***A. baumannii*** | 0 | 0 | 0 |

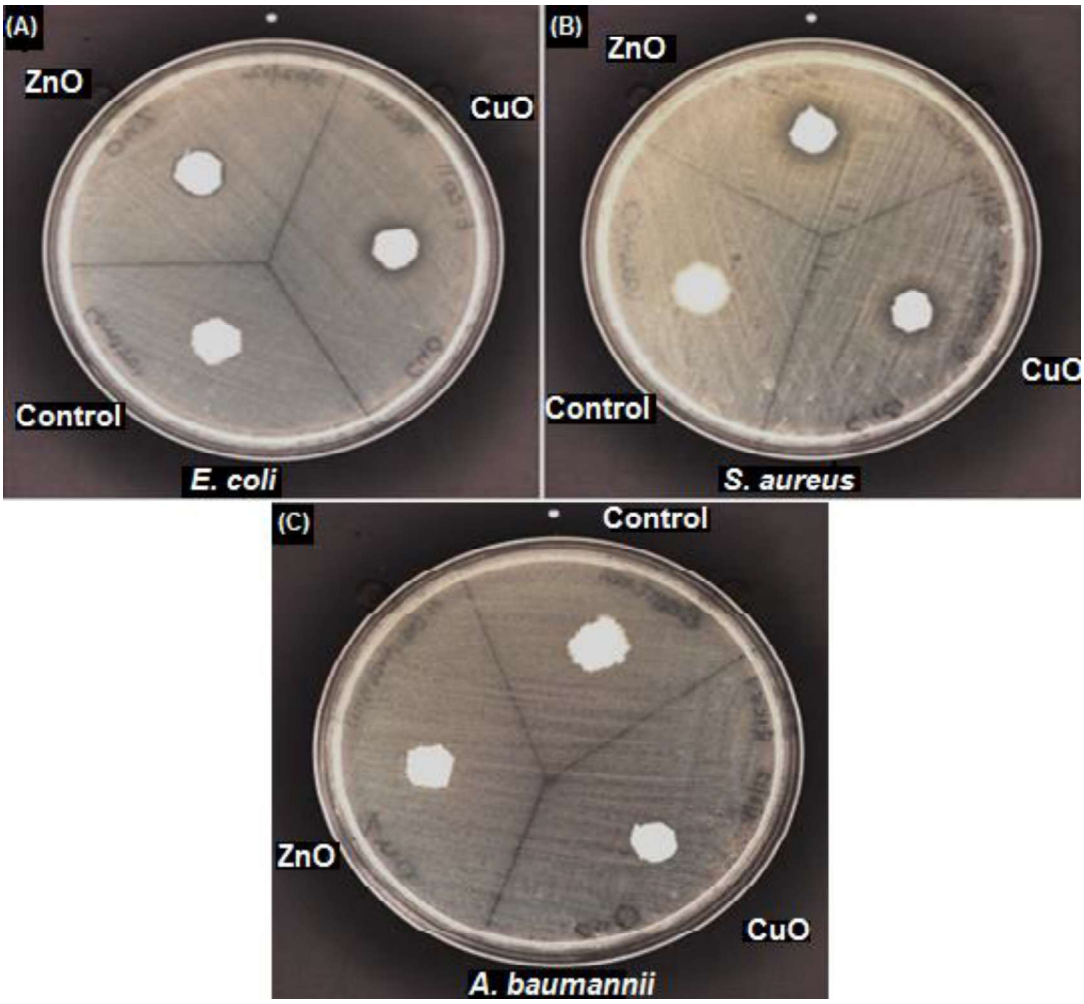


**Figure 34 Disc diffusion plates showing the antibacterial activity of ZnO & CuO impregnated cotton against (A) *E. coli*, (B) *S. aureus* & (C) *A. baumannii*. The fabrics were cut down into round discs to be used on Isosensitest agar. ZnO – fabrics impregnated with zinc oxide nanoparticles; CuO – fabrics impregnated with copper oxide nanoparticles; Control – plain cotton fabric**

### 4.1.6 Shake flask testing to assess the antibacterial activity of lab scale ZnO and CuO fabrics

The antibacterial efficacy of the ZnO and the CuO impregnated cotton was also determined using two shake flask methods; one with nutrient broth as the medium, and the second with saline as the medium.

#### 4.1.6.1 Shake flask test in nutrient broth

In the case of the nutrient broth test, bacterial growth was monitored by measuring the absorbance of the medium over time. The microbial growth reduction with the ZnO impregnated fabrics against all the three species of bacteria is shown in Table 11. Figure 35 shows the change in absorbance over time for the fabrics tested against the three bacterial species. A significant antibacterial activity was seen against *S. aureus* and *E. coli* compared to an intermediate effect against *A. baumannii*. This is a similar trend to that observed with the disc diffusion tests. The lower activity observed against *A. baumannii* may again relate to the use of nutrient media during the incubation of bacteria with the ZnO. In nutrient medium it was observed that the NPs were dissolved leaving the fabrics colourless. So with the nutrient medium tests the bacteria were exposed to dissolved copper or zinc ions rather than the intact NPs.

It was observed that there was 99.87% growth reduction with ZnO impregnated fabric against *S. aureus* followed by a 99.82% reduction in growth against *E. coli*. *A. baumannii* was the least susceptible bacterial strain with ZnO impregnated fabric showing just 56.43% reduction in bacterial growth after 24 ± 3 hours contact time.

The results from CuO impregnated fabrics are not presented here. In the case of CuO fabrics, leaching of copper from the fabrics into nutrient broth was observed through a change in the colour of the solution. The colour change affected the absorbance measurements of the medium resulting in inaccurate absorbance readings.

The dynamic shake flask method was developed for routine quality control and screening tests to overcome difficulties in using conventional antimicrobial test

methods; such as ensuring proper contact of the inoculums with the treated surface (ASTM 2010). This variant of the shake flask method is quicker to perform due to the use of absorbance measurements to monitor bacterial growth. It gives an indication of bacterial efficiency within hours where as it takes at least 2 days to get the results by conventional plating methods.

**Table 11 Shake flask results on lab scale fabrics in terms of percentage growth reduction in absorbance. Absorbance measurements at 660 nm for suspension after 3 and 24 hours**

| Test Organism | OD after 3 hours (660 nm) | | % Reduction | OD after 24 hours (660 nm) | | % Reduction |
|---|---|---|---|---|---|---|
| | Control | ZnO fabrics | | Control | ZnO fabrics | |
| *S. aureus* | 0.022 | 0.001 | 97.24 | 1.670 | 0.002 | 99.87 |
| *E. coli* | 0.077 | 0.010 | 86.62 | 2.113 | 0.004 | 99.82 |
| *A. baumannii* | 0.034 | 0.017 | 50.00 | 2.183 | 0.951 | 56.43 |

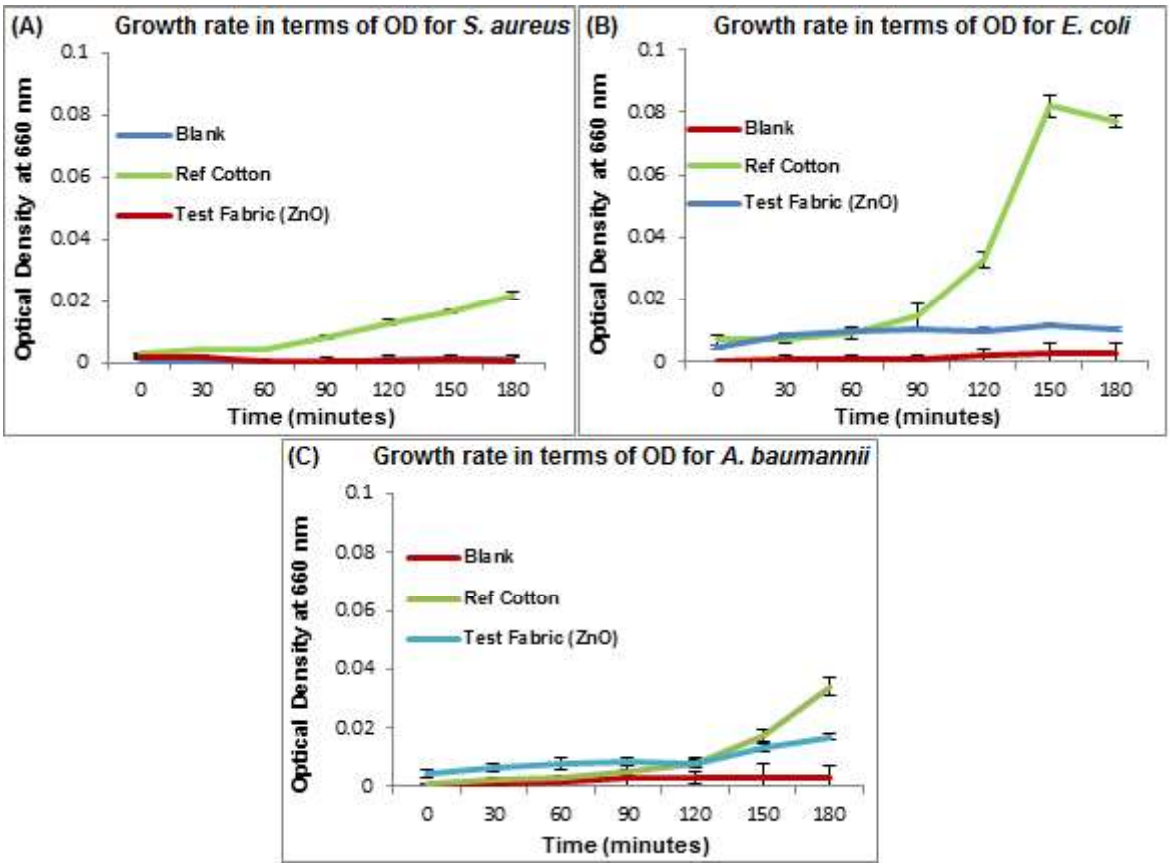


**Figure 35 Change in absorbance over time (3 hours) for nutrient broth shake flask tests against (A) *S. aureus*, (B) *E. coli* and (C) *A. baumannii*. Values plotted are mean ± SD, n = (3)**

Absorbance measurements are not very accurate at low levels (<0.01) and at high levels (>1.0) and can only be used to approximate the number of viable bacteria. Even though they are not very accurate, they do allow for a rapid estimate of cell numbers. Absorbance measurements (turbidity or optical density) are commonly used for minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) testing. Although this method was quite fast and simple, it was more susceptible to contamination due to the high levels of nutrients during incubation. It was also observed that leaching of the copper content in to nutrient medium resulted in a change in the colour of the solution.

#### 4.1.6.2 Shake flask test in saline

A shake flask method was also performed with saline rather than nutrient broth. This method is closer to the standard dynamic shake flask method (ASTM 2010) in that no nutrient source was added. In this method bacterial growth is much slower and so absorbance measurements were not suitable for monitoring bacterial numbers. Instead standard agar plate counting was used. The results from these tests are shown in Figure 36. A bactericidal activity was observed for both the impregnated fabrics (ZnO and CuO) against all three bacterial strains. ZnO and CuO nanoparticles reduced bacterial numbers to undetectable levels within 3 hours of contact time in all three experiments.

Figure 36 also shows that the control fabric inhibited the growth of *S. aureus*. This might be due to some chemical agents or residues left over on the fabric surface from the pre-finishing stage. In other tests (absorption method) this inhibition was found to be removed by rinsing the cotton in boiling water prior to use. The actual cause of the inhibition has not been identified but was specific to *S. aureus.*

One of the aims of the shake flasks test was to determine whether the test method is suitable for demonstrating antibacterial activity by comparing it with absorption testing. As seen from the results, ZnO and CuO impregnated fabrics showed a complete inhibition of bacterial growth within 3 hours of inoculation. These results match to a certain degree the results from the absorption tests. The absorption test results from the fabrics showed a significant antibacterial activity, but not the full kill observed with the shake flask saline tests. These differences in the results are due to the differences in the experimental conditions in which the fabrics were tested. Although both methods showed slightly different levels of activity, they both indicated that the ZnO and CuO fabrics possess a significant antibacterial

activity. The results from the shake flask tests do agree better with the absorption testing results than the disc diffusion tests. Shake flask tests could be seen as a viable alternative to the absorption test for rapid antibacterial efficacy assessment. However, they may not give a reliable indication of activity under real use conditions.

The dynamic shake flask method was developed for routine quality control and screening tests in order to overcome difficulties in using absorption test method; such as ensuring proper contact of inoculums to treated surface (Anon 2010a). There are both advantages and disadvantages to the different shake flask methods. The saline method is less susceptible to contamination than the nutrient broth method because of the lack of nutrients and consequently slower growth. The saline method more closely resembles actual working conditions where in most cases nutrients will be in short supply. However, it is more time consuming due to the need for standard agar plating to enumerate bacterial numbers. The nutrient broth method is similar to MIC and MBC test methods in the use of a nutrient medium. Growth inhibition is assessed under conditions that are highly favourable to growth (nutrients available). Though the high level of nutrients in the medium tends to dissolve the NPs and thus alters the nature of the antibacterial agents (NPs to ions).

From a practical perspective, Swofford (2010) recommends that that the shake flask results should not be compared to other quantitative methods due to the experimental conditions. In the shake saline method a piece of fabric is immersed in a relatively large volume of fluid with samples being taken from the fluid rather than the fabric surface; this does not reflect real time use conditions. As a result the shake flask test is not widely accepted as an indicator of efficiency (Swofford 2010). In the absorption test from ISO 20743:2007, a small volume of inoculum is absorbed on to the test fabrics, mimicking contamination and wear in working conditions. However,

our results suggest that the shake flask method could be used in conjunction with absorption 20743 method as a confirmatory test for antibacterial efficacy testing.

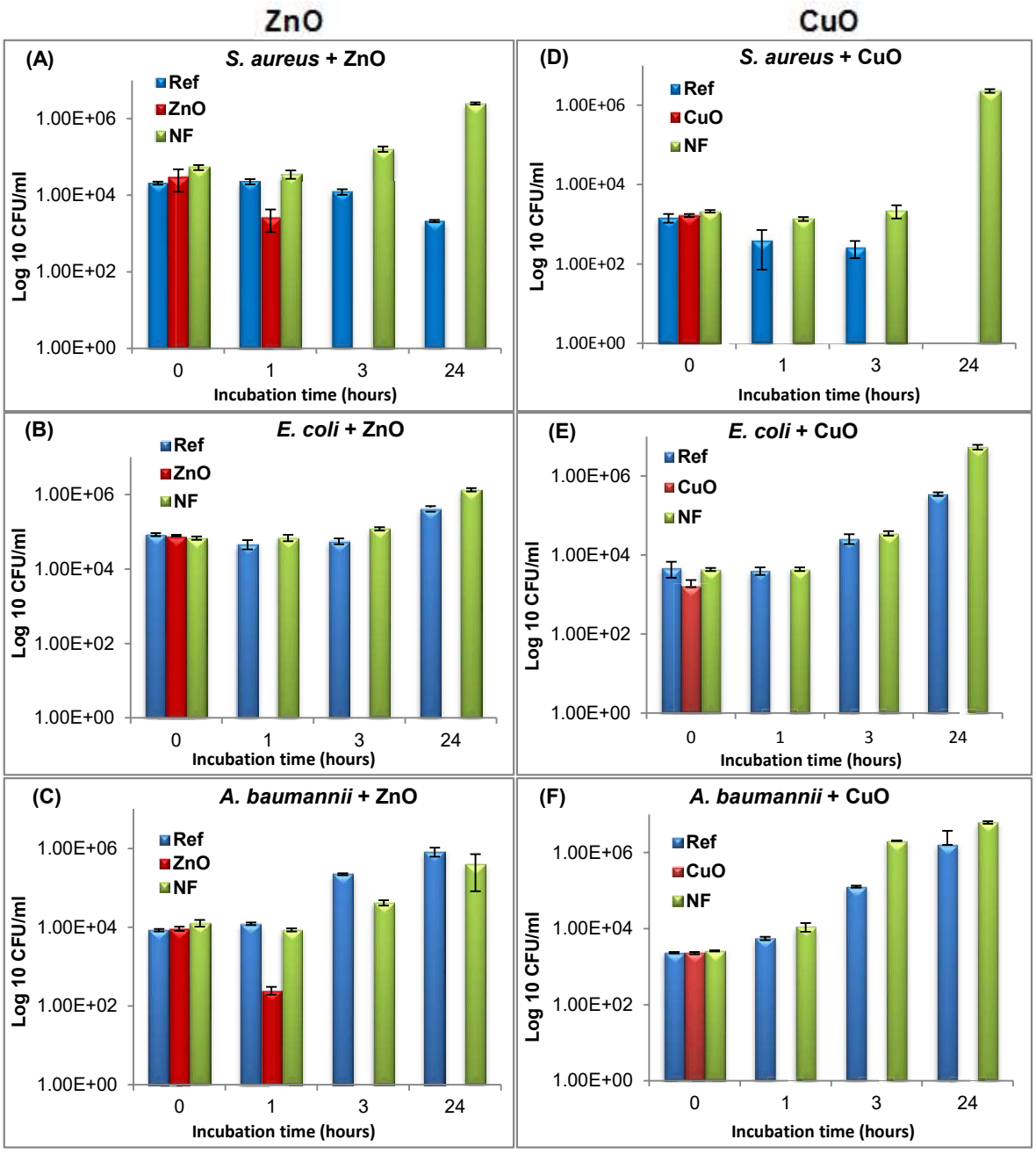


**Figure 36 Antibacterial efficiency of ZnO and CuO impregnated fabrics against different bacteria using a saline shake flask test method. Graphs A, B and C are the results with ZnO cotton and graphs D, E and F are the results from CuO cotton. Values shown are mean ± SD, n = 3**

### 4.1.7 Determination of minimum inhibitory concentration of lab scale fabrics in terms of sample size

In these experiments the antibacterial activity of different sized fabric samples were compared using a shake flask type method. The main aim of this experiment was to investigate the minimum amount of ZnO or CuO NPs impregnated fabrics required for bacterial inhibition.

Ideally assessment of the minimum inhibitory concentration of the antibacterial agent would be carried out using a solution or suspension of the agent. Here, the ZnO and CuO NPs were impregnated into the fabric and so different sized samples were used to vary the concentration of the agent in the test. As the actual concentration of NPs on the fabrics was known, the amount of NPs present on each fabric size was easily calculated.

For testing the fabrics were cut down to 4 sizes; 2.5 x 1 cm, 2.5 x 2 cm, 2.5 x 4 cm, 2.5 x 8 cm. The amount of Zn and Cu on each sample was calculated as follows:

Cotton fabric weight = 250 g/$m^2$

1 $cm^2$ of fabric weighs 0.025g = 25 mg

0.39% w/w ZnO equal to 0.39 x (0.025/100) g = 0.0975 mg of ZnO per $cm^2$

2.5 x 1 cm = 2.5 $cm^2$ = 0.0975 x 2.5 = 0.244 mg ZnO in the tube (divide by volume to give a concentration in mg/ml for example 10 ml of saline = 0.0244 mg/ml ZnO)

2.5 x 2 cm = 5 $cm^2$ = 0.0975 x 5 = 0.49 mg ZnO

2.5 x 4 cm = 10 $cm^2$ = 0.98 mg ZnO

2.5 x 8 cm = 20 $cm^2$ = 1.95 mg ZnO

0.51% w/w CuO equal to 0.51 x (0.025/100) g = 0.128 mg of CuO per $cm^2$

2.5 x 1 cm = 2.5 $cm^2$ = 0.128 x 2.5 = 0.32 mg CuO in the tube (divide by volume to give a concentration in mg/ml (10 ml of saline) = 0.032 mg/ml CuO)

2.5 x 2 cm = 5 $cm^2$ = 0.128 x 5 = 0.64 mg CuO

2.5 x 4 cm = 10 $cm^2$ = 1.28 mg CuO

2.5 x 8 cm = 20 $cm^2$ = 2.56 mg CuO

Different sized pieces of fabrics were challenged with bacterial suspensions in nutrient broth rather than saline and the antibacterial activity was assessed by measuring the absorbance at 660 nm after 24 hours. The results in Figure 37 showed that all the fabric sizes of ZnO and CuO fabrics inhibited the three bacterial strains to some degree. The ZnO impregnated fabrics showed an excellent antibacterial activity against *E. coli* and *S. aureus.* The results showed that 24 µg/ml of ZnO was enough to inhibit *E. coli*, *S. aureus* and *A. baumannii* growth. The growth inhibition increased with increasing fabric size, showing a full kill with the largest fabric size for *E. coli* and *S. aureus*. In the case of CuO fabrics, 32 µg/ml of CuO effectively inhibited the growth of *E. coli*, *S. aureus* and *A. baumannii*. However, even the largest fabric size (256 µg/ml) was not enough to kill all the bacteria. A similar observation was reported by Jafari *et al.* (2011). They looked at the MIC of ZnO and CuO nanoparticles for *E. coli*, *S. aureus* and other bacteria by measuring absorbance values at 600 nm. The results showed that 64 µg/ml of ZnO and 420 µg/ml of CuO effectively inhibited the growth of *E. coli* and *S. aureus*.

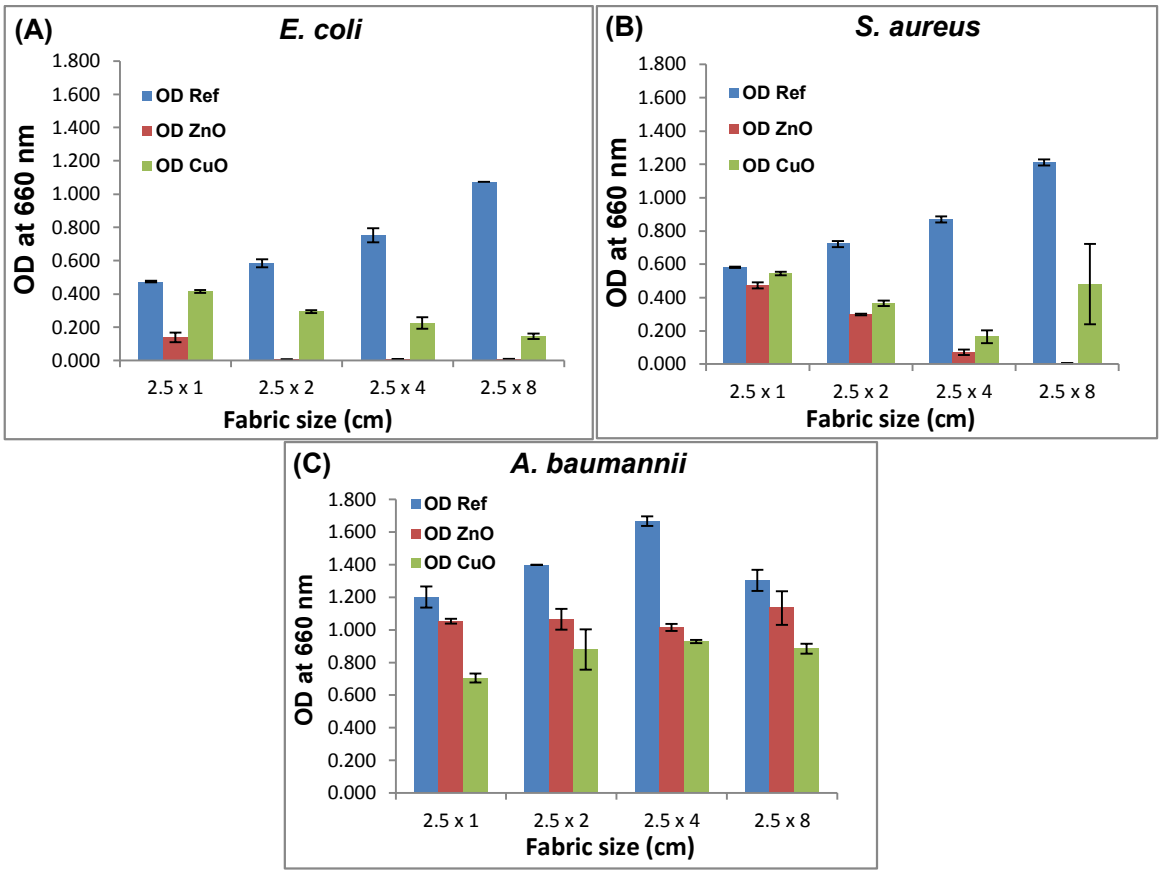


**Figure 37 Absorbance (OD) values after 24 hours incubation at 37 ºC by different sized pieces of cotton fabrics impregnated with ZnO and CuO nanoparticles against (A) *E. coli*, (B) *S. aureus* and (C) *A. baumannii*. Tests were performed in nutrient broth. Mean ± SD, n = 3**

### 4.1.8 ICP analysis of lab scale fabrics to measure the amount of zinc and copper on fabrics and in leachates

A series of experiments were conducted to determine the leaching of copper ions or/and copper oxide nanoparticles and zinc ions or/and zinc oxide nanoparticles from the cotton fabrics into saline solution. The objective was to look at the amount of Zn and Cu leaching in saline and also to assess whether the leaching plays any part in the antibacterial activity.

The impregnated fabrics were immersed in 20 ml of sterile saline solution and subjected to incubation in a rotary shaking incubator at 37°C ± 3 ºC and 110 RPM for 3 hours. The concentration of metal ions (Zn and Cu) in the leachate solutions was determined by inductively coupled plasma analysis (ICP). Antibacterial testing was done by a shake saline method using the sterile leachate solution without any fabric. A detailed description of the method used is given in the methodology section (3.11).

For ICP analysis, two different measurements were carried out:

I. Total concentration of the metals (Zn and Cu) on the fabric (total digestion with 0.5 M $HNO_3$)
II. Concentration of metals (Zn and Cu) in the leachate solution (3 hours shaking in a saline solution at 37°C).

The graphs in Figure 38 show the amounts of zinc and copper on the cotton SONO fabrics and the amounts removed from the fabrics in to saline solution. Very low concentrations of copper ions were leached as compared to zinc ions. As shown in Table 12, 2.5% of the copper and 13.2% of the zinc was removed from the test fabrics into the saline solution. Further experiments were conducted to determine whether the amount of leached zinc and copper content in saline solution were enough to inhibit bacterial growth (Section 4.1.8.1).

In some of the antibacterial activity experiments it was observed that control fabrics possess some antibacterial activity. One of the aims of the leaching experiments was to see if there was any Zn or Cu on reference cotton control fabrics. ICP analysis showed a very low concentration of Zn and Cu on the reference fabrics. The data is not presented in the graphs due to very low levels. Results

showed that 0.005 ppm of Zn and 0.003 ppm of Cu was recovered from control fabrics.

**Table 12 ICP analysis of total Zn and Cu on fabrics and in leachates**

| Fabrics | Total concentration (PPM) | Leached concentration (PPM) | Percentage leached (%) |
|---|---|---|---|
| **ZnO** | 97.45 | 13.19 | 13.53 |
| **CuO** | 126.9 | 3.17 | 2.50 |

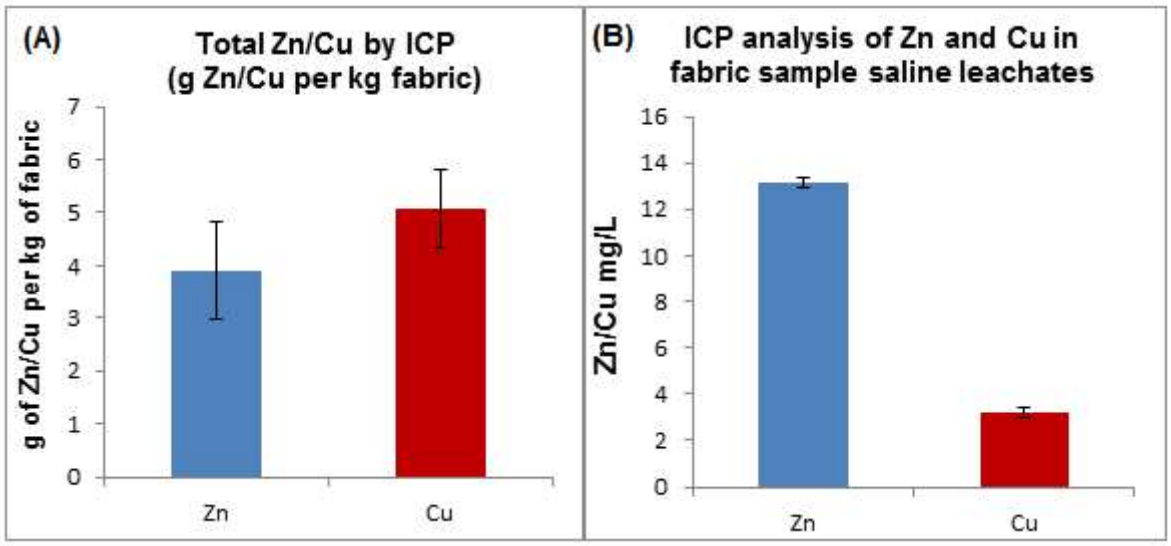


**Figure 38 (A) Total concentration of Zn and Cu on the cotton fabrics and (B) concentration of metals leached in the saline leachate solution. Mean ± SD, n = 3**

#### 4.1.8.1 Antibacterial activity of Zn and Cu ions by shake flask method

To assess the influence of zinc and copper ions on the antibacterial effect, a shake saline method was performed with the leached supernatant saline solution instead of the impregnated fabric. The number of viable cells after incubation was obtained by spread plating serial dilutions on nutrient agar plates at 0, 3 and 24 hours.

After incubation, no bacterial inhibition was observed with the copper solution when challenged against *S. aureus*, *E. coli* and *A. baumannnii* (Figure 39). These results indicated that the copper ions at this concentration have no influence on the antibacterial activity. A more important issue may be to investigate shedding of CuO nanoparticles. This would require experiments such as dynamic light scattering (DLS) and transmission electron microscopy (TEM). A recent study by Perelshtein *et al.* (2009b) revealed that CuO nanoparticles were not present in the leaching solution. ICP and SEM studies showed a strong, durable deposition of CuO nanoparticles on the fabric surface by the sonochemical method (Perelshtein *et al.* 2009b, Perelshtein *et al.* 2013).

In the case of the zinc solution, the antibacterial behaviour was totally different from the copper solution. The zinc content leached in to the saline solution was enough to inhibit bacterial growth for *S. aureus*, *E. coli* and *A. baumannnii* (Figure 39). A full bacterial kill was seen within 3 hours of incubation with *E. coli* and *A. baumannnii*. A high antibacterial activity level was also observed against *S. aureus* with a near full kill after 24 hours. Perelshtein *et al.* (2009b) also studied the antibacterial behaviour of ZnO nanoparticles and found that ZnO nanoparticles released both $Zn^{2+}$ ions and ROS into the surrounding medium; these both play an important role in antibacterial activity.

There are many factors by which nanoparticles induce toxicity. The key factors in their biological effects are their chemical composition, nano size, concentration, surface properties and their dissolution (Chang *et al.* 2012). From the results above, it appears that ZnO is more soluble than CuO. Adam *et al.* (2014) studied the dissolution and uptake of ZnO and CuO NPs by *Daphnia magna* and found that the toxicity of both ZnO and CuO NPs is induced by the dissolved metal ions from the particles in the medium. ZnO NPs showed complete dissolution within 48 hours of exposure. Their results showed that the induced toxicity of ZnO and CuO nanoparticles is caused by the metal ions formed during dissolution of the particles in the exposure medium (Adam *et al.* 2014). Our results also suggest that, there is a possibility that Zn ions were released from the fabrics instead of ZnO NPs. The dissolution of Cu from the fabrics into the solution could more easily be observed than Zn in the solution. As Zn is more soluble, the dissolved Zn might be more involved in the activity with the ZnO fabrics than dissolved copper is with the CuO fabrics.

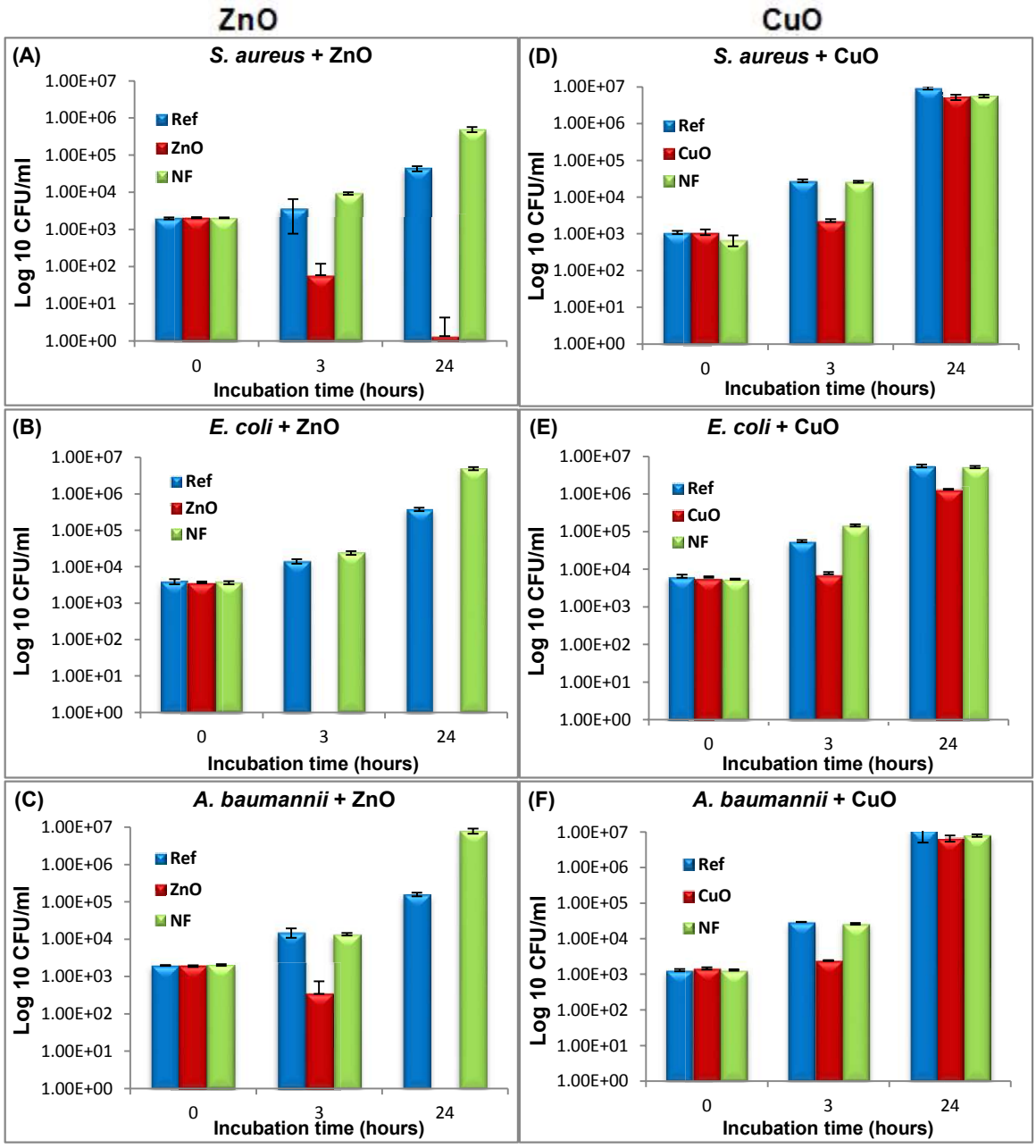


**Figure 39 Shake flask tests with saline leachates from ZnO and CuO impregnated cotton fabrics. Numbers of viable cells were obtained by agar plating from the leached supernatant saline solution. Graphs A, B and C represent ZnO results and graphs D, E and F represent CuO results. Values shown are mean ± SD, n = 3**

#### 4.1.8.2 Antibacterial activity of metal salts against *E. coli*

The antibacterial activity of the leachates from the ZnO and CuO impregnated fabrics prompted a further investigation of the role of metal salts in the antibacterial activity. ICP analysis of the $Cu^{2+}$ and $Zn^{2+}$ concentrations in the leachates indicated that 2.49% of the CuO and 13.53% of the ZnO were leached in to the saline. The same concentration of zinc and copper salts (13mg/L and 2.5 mg/L respectively) in saline were prepared and tested to observe if the metal ions had any role to play in the antibacterial activity. A 10 times higher and a 10 times lower concentration of the target concentration was also tested in comparison. From the results shown in Figure 40, it was observed that solutions of zinc chloride prepared at the same concentration as the leachates were found to be active against *Escherichia coli*. The copper sulphate only showed antibacterial activity at the high test concentration (25 mg/l). These results with zinc and copper solutions are in agreement with leaching results from the lab scale samples (Figure 39) where zinc ions effectively killed *E. coli* within 24 hours but copper ions did not.

From the results, it is likely that the ZnO activity may be at least partly associated with the release of zinc ions whereas the antibacterial activity of CuO does not appear to be associated with the release of copper ions.

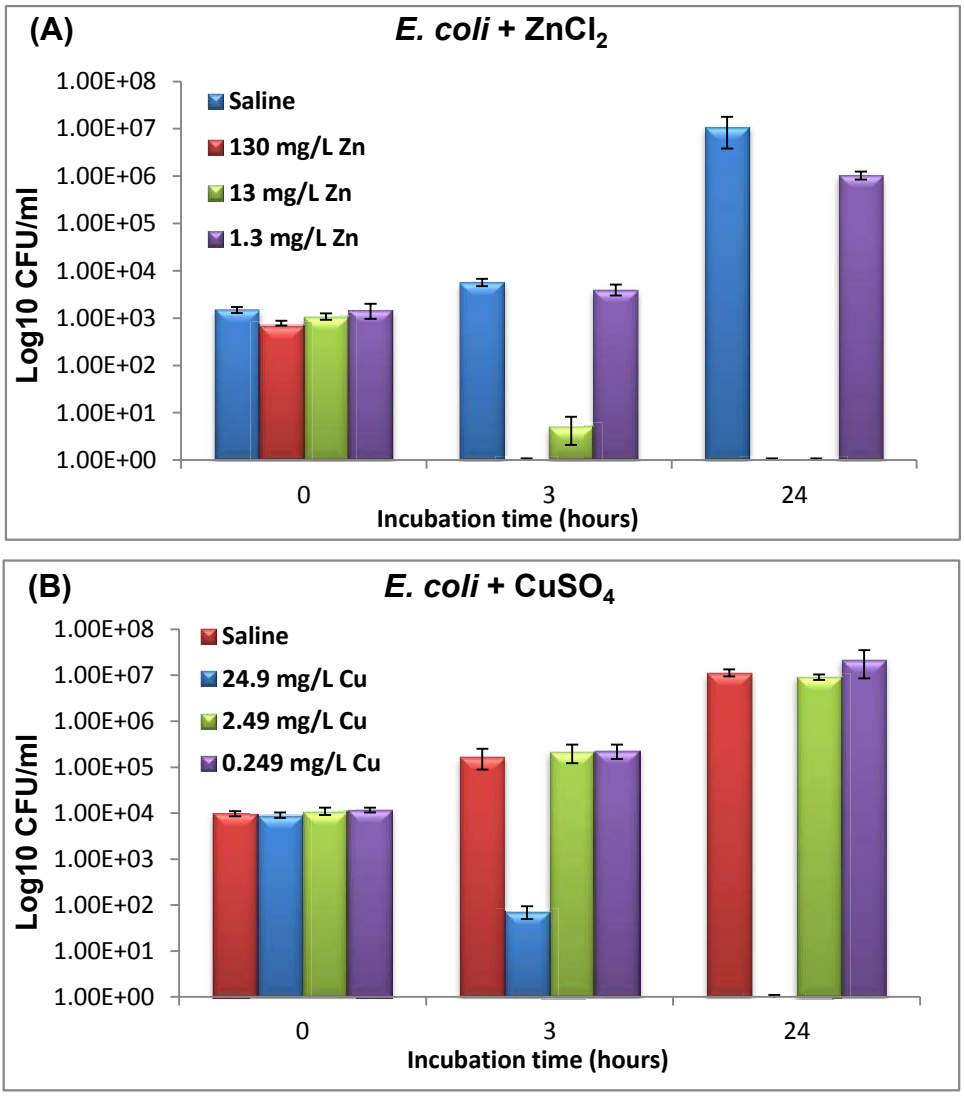


**Figure 40 Antibacterial activity of zinc chloride and copper sulphate solution against *E. coli*. Red bar in the first graph is 130 mg/L of zinc chloride in saline; Green bar is the target concentration of 13 mg/L of zinc chloride in saline and purple bar is 1.3 mg/L of zinc chloride in saline. Red bar in the second graph is 25 mg/L of copper sulphate in saline; Green bar is the target concentration of 2.50 mg/L of copper sulphate in saline and purple bar is 0.25 mg/L of copper sulphate in saline**

## 4.2 Testing of CuO fabrics from the pilot scale machines

One of the main aims of the SONO project was to scale up the laboratory process to a pilot industrial scale. The lab scale results played an important role during the move from the lab scale process to the industrial scale. The lab scale sonochemical reactor was only able to work with 10 cm wide fabric rolls. During the optimisation, two pilot scale machines were assembled, both of which were able to process fabric rolls of up to 50 cm wide. One machine used piezoelectric ultrasonic transducers (Klopman) and the other used magnetostrictive transducers (Davo). The exact schematics of the machines cannot be presented here for reasons relating to intellectual property rights. The performance of the machines was compared in terms of energy and reagent usage as well as equipment cost. The antibacterial performance of the fabrics prepared using the two machines was also assessed.

For the pilot scale set up, two sets of PEC fabrics were prepared at the two pilot plants in Italy (Klopman) and Romania (Davo). Polyester cotton fabrics (PEC) were used as the substrates rather than just cotton. CuO was also preferred over ZnO for this phase of the project, as the former changed the colour of the fabrics which helped in the initial assessment of coating quality. The fabrics were prepared using two different techniques; the sonochemical generation and impregnation of nanoparticles from metal complexes *(in-situ*) and a “throwing the stones” technology (TTS) using commercially prepared copper oxide nanoparticles. ZnO fabrics were also prepared later on but further work with ZnO at the pilot scale was not carried out until towards the end of the SONO project. Analysis of the pilot scale samples is limited here to the CuO ones only.

The CuO fabrics used here were named A, B, D and E:

Fabric A Klopman (TTS technique) - 0.91% w/w CuO

Fabric B Klopman (*in-situ* technique) - 0.9% w/w CuO

Fabric D Davo (TTS technique) - 0.62% w/w CuO

Fabric E Davo (*in-situ* technique) - 0.37% w/w CuO

Photographs of fabric samples are shown in Figure 41. The throwing the stones (TTS) method produced a grey/black impregnation on white fabrics. The sonochemical (*in-situ*) method produced a brown coloured impregnation. In Figure 41, it can be seen that the impregnations produced using the Davo machine were less homogeneous than the impregnations produced using the Klopman machine. The effect of visible variation in the impregnations was investigated and is presented in sections 4.2.5 and 4.2.6. During the course of the project the Davo machine was redesigned and rebuilt. The visible variation in the impregnations was considerably reduced with the revised machine.


**Figure 41 Fabrics impregnated with CuO nanoparticles from both the pilot plants; Klopman and Davo. The fabric squares were 5 x 5 cm**

HR-SEM images of the fabrics (Figure 42) showed that the *in-situ* and TTS impregnations were not only different colours but also very different at the micro scale. The *in-situ* impregnation appeared as a homogeneous layer of nanoparticles on the cotton fibres. The NPs were between 40 – 80 nm in diameter. The TTS impregnation appeared to be significantly less homogeneous with large clumps of NPs rather than an even impregnation (~ 1 µm).

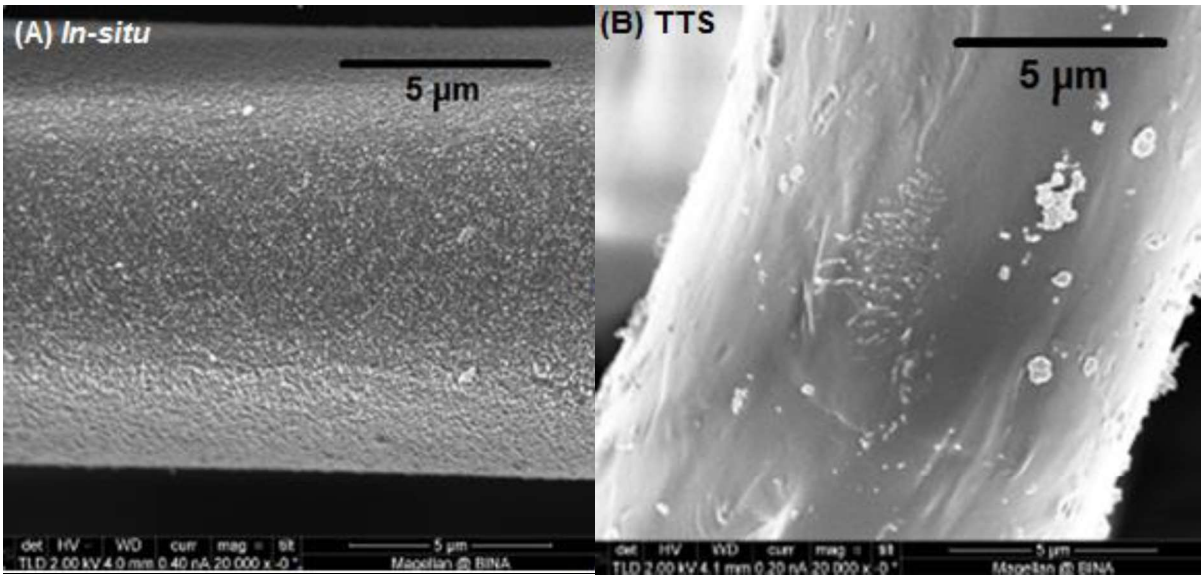


**Figure 42 Scanning electron microscopic images of the fabrics from the prototype machine at Klopman (A) *in-situ* fabric (B) TTS fabric. Images prepared at BIU (Israel)**

### 4.2.1 Antibacterial efficacy testing of CuO impregnated PEC fabrics from the prototype machines using the absorption method

CuO impregnated PEC fabrics prepared from the prototype machines were tested to assess their antibacterial properties by the absorption method (ISO 20743 method). The results showed an excellent antibacterial activity against both MRSA and *P. aeruginosa*. As is shown in Figure 43, all 4 of the fabrics showed a full bacterial kill. There was a 6 log reductions with all the four impregnated fabrics with

CuO nanoparticles showing a greater than 99.99% reduction in bacterial growth for MRSA and *P. aeruginosa* (Table 13).

Comparing the ISO absorption results of pilot scale PEC fabrics to the lab scale cotton fabrics, the pilot scale PEC fabrics have showed an enhanced antibacterial activity against Gram positive MRSA and Gram negative *P. aeruginosa*. With the lab scale fabrics *S. aureus* was tested rather than MRSA. The pilot scale PEC fabrics exhibited a complete bacterial reduction whereas, the lab scale cotton fabrics were able to reduce bacterial growth by 3 log. These results have suggested that a mix of polyester cotton fabrics work better than the cotton fabrics. One of the reasons could be that PEC cotton fabrics are able to hold NPs more strongly than just cotton. These results correspond to the results from Sarathi and Thilagavathi (2009). The researchers impregnated titanium dioxide ($TiO_2$) on both woven and knitted 100% cotton and 45/55% polyester cotton (PEC) and assessed their antibacterial activity against Gram positive *S. aureus* and Gram negative *K. pneumoniae*. All the results showed an enhanced antibacterial activity for polyester cotton fabrics (> 93%) compared to 100% cotton (< 82%). Gokarneshan *et al.* (2012) in their study also showed that a mix of polyester/cotton component exhibited better bacterial reduction than its cotton counterpart.

This can also be justified from the unpublished results from the partner institutes working on this project. Results from the partner institutes have shown that ZnO PEC was better than ZnO cotton in terms of durability and antibacterial activity. The researchers claimed that the enhanced durability and antibacterial activity is possibly due to better adhesion of the NPs to the polyester fibres. The high speed impact of the NPs may be enough to partially melt the polymer fibres and firmly impregnate the NPs in them.

**Table 13 Mean viable bacterial counts (CFU/ml) after 24 ± 3 hours and log reductions for CuO impregnated PEC samples compared to untreated PEC samples against MRSA and *P. aeruginosa*. CFU/ml at time zero 1 – 3 x$10^5$ CFU/ml (LOD = limit of detection, 100 cells). Statistically significant differences in the viable counts between control and test samples post incubation ($p<0.05$ for each)**

| | MRSA | | | | | | |
|---|---|---|---|---|---|---|---|
| Fabric type | Control | St. Dev | CuO fabrics | St. Dev | Percentage reduction | % Growth Reduction | A value |
| A – Klop TTS | 1.80E+08 | 1.10E+07 | 1.00E+02 LOD | 0.00E+00 | 99.9 | > 99.99 | 6.25 |
| B – Klop *in-situ* | 1.80E+08 | 1.18E+07 | 1.00E+02 LOD | 0.00E+00 | 99.9 | > 99.99 | 6.25 |
| D – Davo TTS | 1.80E+08 | 1.18E+07 | 1.00E+02 LOD | 0.00E+00 | 99.9 | > 99.99 | 6.25 |
| E – Davo *in-situ* | 1.80E+08 | 1.18E+07 | 1.00E+02 LOD | 0.00E+00 | 99.9 | > 99.99 | 6.25 |
| | ***P. aeruginosa*** | | | | | | |
| Fabric type | Control | St. Dev | CuO fabrics | St. Dev | Percentage reduction | % Growth Reduction | A value |
| A – Klop TTS | 5.30E+08 | 9.06E+07 | 1.00E+02 LOD | 0.00E+00 | 99.9 | > 99.99 | 6.73 |
| B – Klop *in-situ* | 5.30E+08 | 9.06E+07 | 1.00E+02 LOD | 0.00E+00 | 99.9 | > 99.99 | 6.73 |
| D – Davo TTS | 4.00E+08 | 6.00E+07 | 1.00E+02 LOD | 0.00E+00 | 99.9 | > 99.99 | 6.60 |
| E – Davo *in-situ* | 4.00E+08 | 6.00E+07 | 1.00E+02 LOD | 0.00E+00 | 99.9 | > 99.99 | 6.60 |

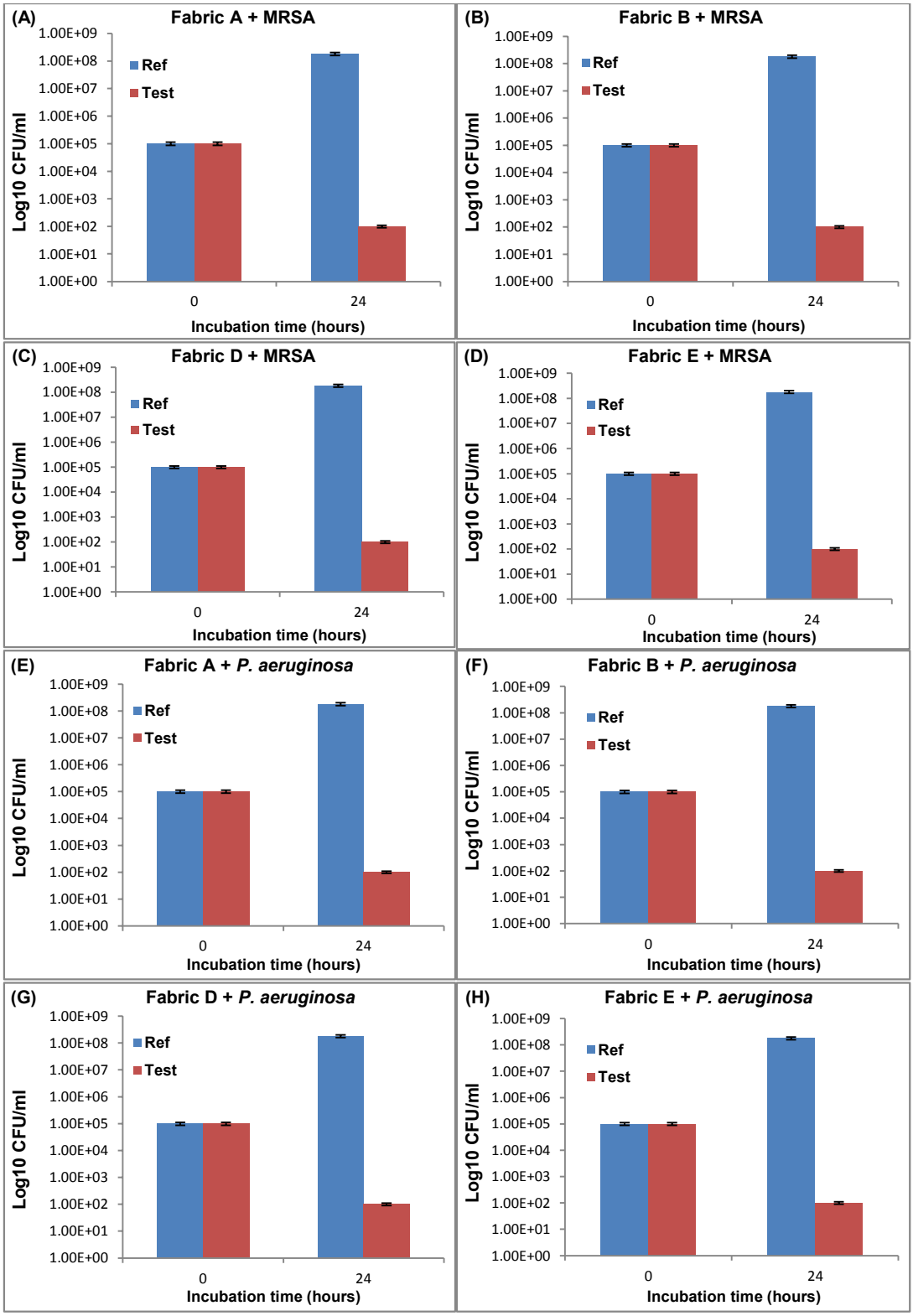


**Figure 43 Antibacterial activity of pilot scale CuO PEC fabrics against MRSA and *P. aeruginosa* by absorption method (ISO 20743). Fabrics were spiked with a known concentration (1 - 3 x $10^5$ CFU/ml in dilute NB) of bacteria. The number of viable bacteria recovered from reference fabrics (blue bars) and impregnated fabrics (red bars) at 0 hours and after 24 ± 3 hours was determined and calculated**

One of the key challenges in the control of infections results from the ability of almost all bacteria to form biofilms. Biofilms can be formed on a wide variety of surfaces and medical devices (Donlan and Costerton 2002). Hence, the requirements for the effective strategies to prevent the formation of biofilms are even more important. Recent studies have shown that ZnO and CuO nanoparticles are effective in inhibiting biofilm formation and virulence factor production. Applerot *et al.* (2012) synthesized and deposited ZnO NPS on the surface of glass slides using a one-step sonochemical method. The ZnO deposited glass slides were tested against the *E. coli* and *S. aureus* biofilms in a continuous flow chamber. Confocal laser scanning microscope images showed that the ZnO coating generated an anti-biofilm activity against *E. coli* and *S. aureus*. Lee *et al.* (2014) have also shown that ZnO nanoparticles are effective in inhibiting biofilm formation of *P. aeruginosa*. Agarwala *et al.* (2014) investigated the anti-biofilm activity of CuO and $Fe_2O_3$ NPs against multi drug resistant biofilm forming uropathogens *E. coli* and MRSA. The results showed that CuO NPs exhibited bacteridcidal activity and possessed dose dependant anti-biofilm properties. The use of SONO fabrics in hospitals and other healthcare settings are not intended for use against established biofilms but as an intervention strategy to prevent the initial colonization with biofilms and to minimize microbial growth.

The results above have shown that the pilot scale CuO PEC fabrics have the ability to inhibit bacterial growth which could further provide potent disinfecting solutions for prevention of biofilm formation.

### 4.2.2 Antibacterial activity testing of pilot scale CuO PEC fabrics by disc diffusion method

Iso-sensitest agar plates were surface inoculated with either MRSA or *P. aeruginosa*. The diameter of any zones of inhibition was measured after 24 hours of incubation at 37°C. Figure 44 shows agar plates to which the test bacterial suspension was applied. The diameters of the zones of inhibition (in mm) around the different CuO impregnated fabrics are shown in Table 14. The presence of CuO nanoparticles synthesized and impregnated by an *in-situ* (Fabrics B and E) method effectively inhibited the growth of MRSA, giving a clear zone of inhibition around the fabric discs. Fabric B showed a greater inhibition zone of 28.5 mm diameter compared to 20 mm diameter for fabric E. This could have been due to the different concentrations of CuO on the two fabrics (B 0.90% w/w and E 0.37% w/w). No activity was observed for any of the fabrics against *P. aeruginosa*.

These results match the disc diffusion results for CuO fabrics prepared at the lab scale (section 4.1.5). The lab scale CuO fabrics showed a greater zone of inhibition for Gram positive *S. aureus* than Gram negative *E. coli*. Similar activity was seen for CuO fabrics from the pilot scale samples. A higher activity for Gram positive MRSA and no activity for Gram negative *P. aeruginosa.* MRSA and *S. aureus* are from the same genus and have shown greater susceptibility to CuO fabrics in both cases.

As discussed earlier, the diffusion rate of antimicrobials through the agar depends upon the type of antimicrobial agent and the application method used. Hence, this method is not reliable when comparing the relative efficiency of one antimicrobial against another.

**Table 14 Antibacterial assessment in terms of zone of inhibition of pilot CuO fabrics by disc diffusion method**

| Fabrics Treated | Organism | Zone of Inhibition (Diameter in mm) | | |
|---|---|---|---|---|
| | | 1 | 2 | Mean |
| CuO Fabric A | ***MRSA*** | 0 | 0 | 0 |
| | ***P. aeruginosa*** | 0 | 0 | 0 |
| CuO Fabric B | ***MRSA*** | 28 | 29 | 28.5 |
| | ***P. aeruginosa*** | 0 | 0 | 0 |
| CuO Fabric D | ***MRSA*** | 0 | 0 | 0 |
| | ***P. aeruginosa*** | 0 | 0 | 0 |
| CuO Fabric E | ***MRSA*** | 21 | 19 | 20 |
| | ***P. aeruginosa*** | 0 | 0 | 0 |

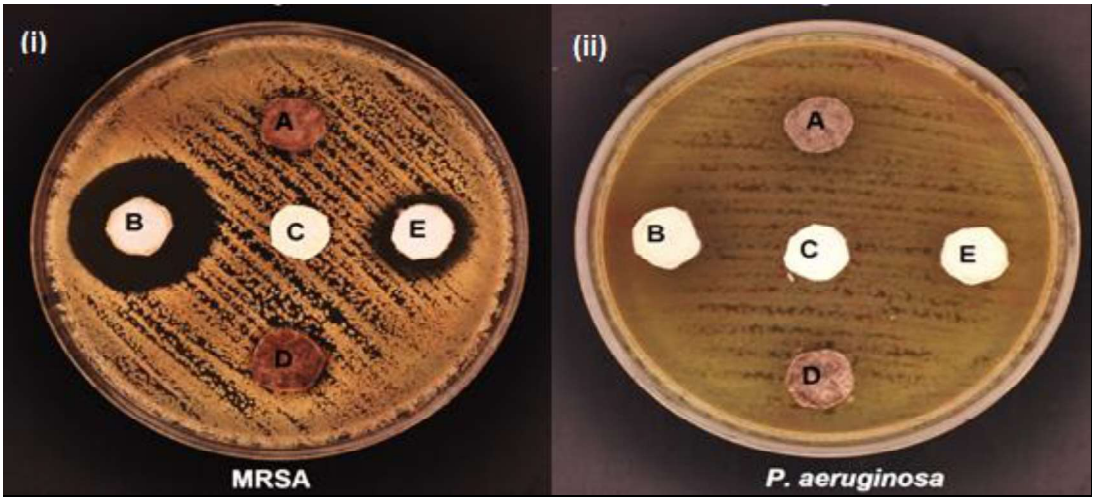


**Figure 44 Antibacterial activity of pilot scale CuO PEC fabrics against (i) MRSA and (ii) *P. aeruginosa* assessed by disc diffusion method. The fabrics were cut down into round discs to be used on Isosensitest agar. CuO fabrics A and B – Klopman TTS and *in-situ*; CuO fabrics D and E – Davo TTS and *in-situ*.; Fabric C Control – plain PEC**

### 4.2.3 Antibacterial activity of pilot scale CuO PEC fabrics by shake flask method in saline

The shake flask testing in saline was carried out on the 4 CuO impregnated fabrics from the pilot plant against MRSA and *P. aeruginosa*. The results in Figure 45 showed that the *in-situ* fabrics (Fabrics B and E) exhibited an excellent antibacterial activity as compared to TTS fabrics (Fabrics A and D). The *in-situ* fabrics completely inhibited the growth of MRSA and *P. aeruginosa* within 3 hours of contact time. The TTS fabrics only inhibited bacterial growth to maximum of 3 log reduction within the 24 hours of contact time. These results match with the shake flask saline results from lab scale CuO cotton fabrics. Both the *in-situ* fabrics and the lab scale fabrics exhibited a complete reduction of viable cells within 3 hours of contact time. Both these fabrics were prepared using the SONO technique.

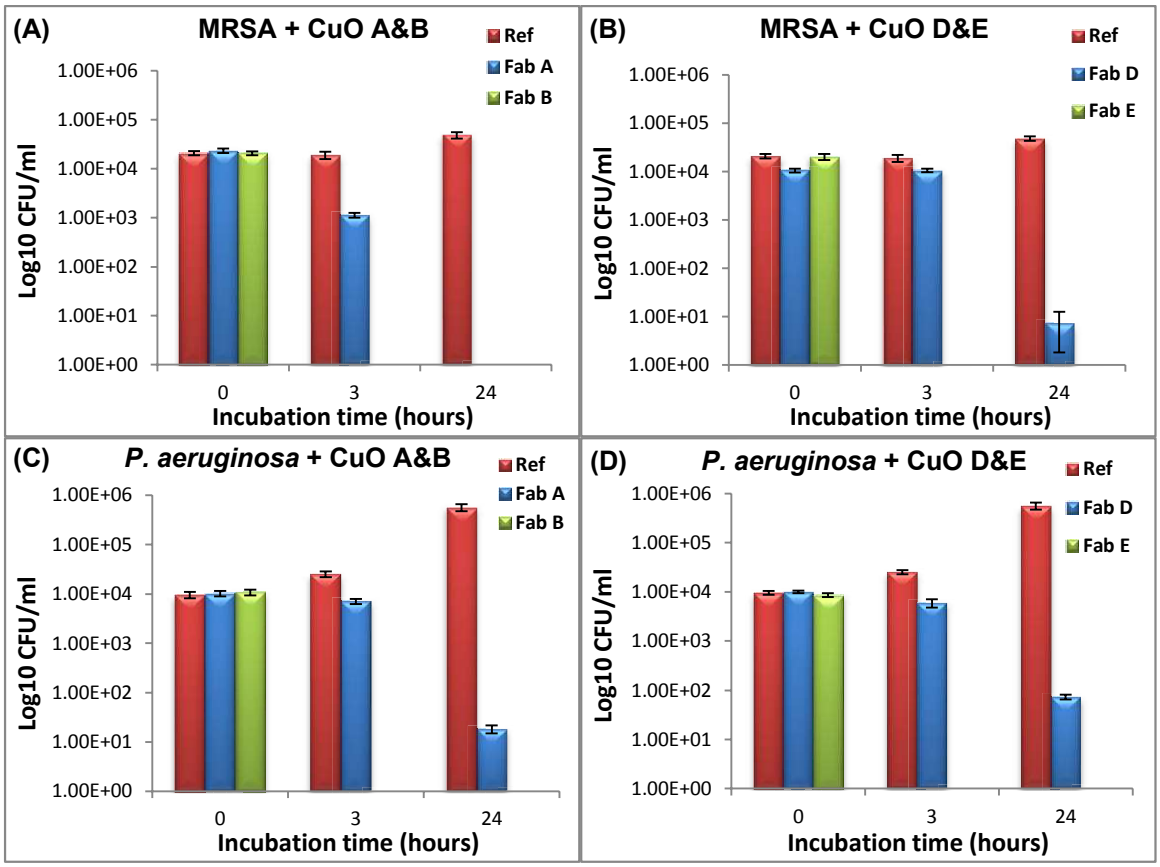


**Figure 45 Antibacterial efficiency of pilot scale CuO PEC fabrics A, B, D and E against MRSA and *P. aeruginosa* using shake flask method in saline. (Ref = control fabric; Fab A = TTS fabric (Klopman); Fab B = *in-situ* fabric (Klopman); Fab D = TTS fabric (Davo); Fab E = *in-situ* fabric (Davo). Values shown are mean ± SD, n = 3**

### 4.2.4 Determination of minimum inhibitory concentration of pilot scale PEC fabrics in terms of sample size

In these experiments the antibacterial activity of different sized fabric samples was compared using a shake flask type method. The aim was to assess the minimum amount of fabric required for bacterial inhibition. Different sized samples were used to vary the concentration of CuO NPs on the PEC fabric. As the concentration of CuO NPs on the fabrics was known the amount of CuO NPs present on each fabric size could easily be calculated. The influence of the test media was also investigated. Experiments were conducted with either saline or NB.

These tests were carried out with the 4 CuO impregnated PEC fabrics prepared using the pilot scale machines. For testing the fabrics were cut down in 4 sizes; 2.5 x 1 cm, 2.5 x 2 cm, 2.5 x 4 cm, 2.5 x 8 cm. The amount of Cu on each sample was calculated as follows:

PEC fabric weight = 250 g/$m^2$

1 $cm^2$ of fabric weighs 0.025g = 25 mg

0.9% w/w CuO equal to 0.9 x (0.025/100) g = 0.000225 g = 0.225 mg of CuO per $cm^2$ or 2.25 g of CuO per $m^2$ of fabric

2.5 x 1 cm = 2.5 $cm^2$ = 0.225 x 2.5 = 0.56 mg CuO (divide by volume to give a concentration in mg/ml for example 10 ml of saline = 0.056 mg/ml CuO)

2.5 x 2 cm = 5 $cm^2$ = 0.225 x 5 = 1.13 mg CuO

2.5 x 4 cm = 10 $cm^2$ = 2.25 mg CuO

2.5 x 8 cm = 20 $cm^2$ = 4.5 mg CuO

The results presented in Figure 46 showed that *in-situ* fabrics (B and E) showed higher antibacterial activity than the TTS fabrics (A and D). Both *P. aeruginosa* and MRSA showed some growth inhibition with the minimum fabric size of 2.5 x 2 cm. This showed that 0.113 mg/ml (2.5 x 2 cm) of CuO nanoparticles was sufficient to inhibit the growth *of P. aeruginosa* and MRSA. However, in all the cases (whether media type or fabric type), higher antibacterial activity was observed for MRSA than for *P. aeruginosa*. When comparing the effect of media, the fabrics showed higher activity in saline (restricted media) and less in NB. The nutrient broth results correlates very well with the lab scale results (Section 4.1.7). Both the lab scale and pilot scale results showed that higher concentrations of CuO than ZnO are required to completely inhibit bacterial growth.

When a comparison was made between fabric types, the results showed that *in-situ* fabrics were more active than the TTS fabrics. The differences between the activities of both fabric types were more significant when tested in saline than in NB. The activity of both fabrics was quite similar in NB. However, in saline, MRSA was much more susceptible than *P. aeruginosa*. The antibacterial activity of CuO fabrics was found to increase with the increase in the fabric size. This was as would be expected as in the fixed test volume CuO NPs concentration increases with the increase in sample size. The results showed that the fabric size of 2.5 x 8 cm (20 $cm^2$) achieved the highest antibacterial activity out of the all fabrics by showing a minimum of 4 log reduction. The fabric size with 2.5 x 8 cm was calculated to have around 4.5 mg of CuO on its surface. This is equal to a concentration of 0.45 mg/ml in the test volume.

The nutrient broth and saline provided quite different environments for the antibacterial agents to interact with the bacteria. The nutrient broth contains a complex mixture of compounds which not only facilitate the growth of bacteria but can also interact directly with the antibacterial agent.

One of the aims of these experiments was to find out whether there were small differences between fabrics that were not evident from absorption results. In the absorption results (section 4.2.1), all 4 fabrics (A, B, D and E) had shown bactericidal activity (full kill). There was nothing from these results to differentiate the level of activity from different fabric types. The shake flask method in saline showed up differences that were not apparent from the absorption results. The saline method quite closely resembles actual working conditions where nutrients are in short supply, much like the ISO absorption method.

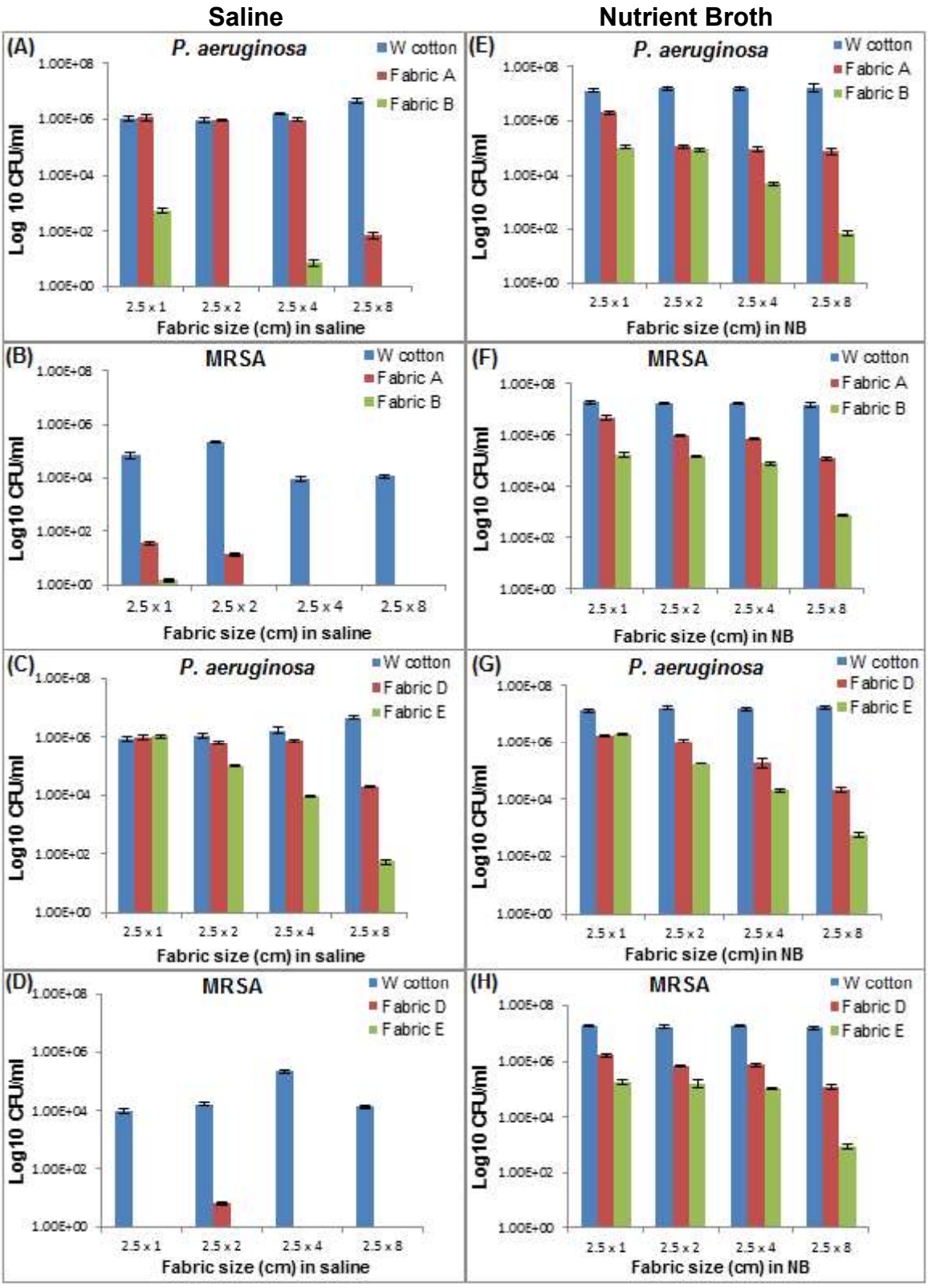


**Figure 46 Antibacterial activity shown by different sized pilot scale CuO PEC fabrics from Klopman and Davo against *P. aeruginosa* and MRSA. Tests were performed in saline (Graphs A, B, C and D) and in nutrient broth (NB) (Graphs E, F, G and H)**

### 4.2.5 Measurement of the amount of copper on fabrics and in leachates by ICP

ICP tests were also conducted with the set of 4 CuO fabrics manufactured using the pilot scale machines at Klopman and Davo. The release of the nanoparticles into the environment via laundering is seen as a potential hazard (Khan 2013). Thus one of the aims of the SONO project was to show that the sonochemical impregnation process enhanced the stability of nanoparticles on the fabric substrates.

The ICP results presented in Table 15 show a greater adhesion of the CuO nanoparticles to the PEC fabrics prepared at the pilot scale than was observed for the cotton fabrics prepared at the lab scale (Figure 47). With each of the pilot scale fabric samples, less than 0.5% of the total copper content was leached from the fabrics in to the saline solution. With the lab scale fabric a similar amount of Cu was impregnated into the fabric (0.5% w/v) but a slightly greater amount was released into the saline leachate (2.5%). This may or may not be due to significant differences between the adhesions of the CuO NPs on the different fabrics. It does possibly indicate that in terms of adherence, the impregnations produced using the pilot scale machines were at least as good as the impregnations produced at the lab scale.

In a further experiment, saline leachates from the pilot scale CuO fabrics were centrifuged to separate solid CuO from soluble copper. The results from ICP analysis of the copper concentration in the total leachate, the supernatant and the pellet are presented in Figure 47(B). The results showed that the TTS and *in-situ* CuO fabrics behaved differently in terms of the amount of dissolved copper and solid CuO released into the saline solution. In the case of the TTS fabrics (A and D) most of the copper in the leachates was pelleted and thus probably in the form of CuO particles.

This means there were less Cu ions released from the fabrics. In the case of the *in-situ* fabrics (B and E), most of the copper in the leachates was found in the supernatant fraction and thus was dissolved Cu ions. From the SEM images (Figure 42) it can be seen that TTS impregnations were less uniform and were made of larger aggregates of CuO particles. These may have shed more easily from the fabrics into the leachates and formed pellets on centrifugation. The smaller NPs from *in-situ* fabrics may have dissolved more readily due to their larger overall surface area. The bactericidal effectiveness of NPs has been suggested to be due to their size and higher surface to volume ratio. That may explain the differences seen in the antibacterial activity of TTS fabrics and *in-situ* fabrics. The *in-situ* fabrics showed higher activity than the TTS fabrics due to the smaller size of the NPs and greater levels of Cu ions. Other researchers have also claimed the dissolution of metal ions is a key factor in the toxicity of nanoparticles. Chang *et al.* (2012) showed that the toxicity of CuO NPs was largely influenced by soluble Cu ions. Brunner *et al.* (2006) also found that the toxic effect of soluble metal oxide NPs was higher than the insoluble ones, when used at the same concentration.

**Table 15 Total amount of copper on pilot scale samples and the amount released in to saline leachates**

| Fabrics | Total concentration (PPM) | Leached concentration (PPM) | Percentage leached (%) |
|---|---|---|---|
| **A – Klopman TTS** | 157.00 | 0.55 | 0.35 |
| **B – Klopman *in-situ*** | 157.47 | 0.64 | 0.41 |
| **D – Davo TTS** | 126.13 | 0.27 | 0.21 |
| **E – Davo *in-situ*** | 68.34 | 0.32 | 0.47 |

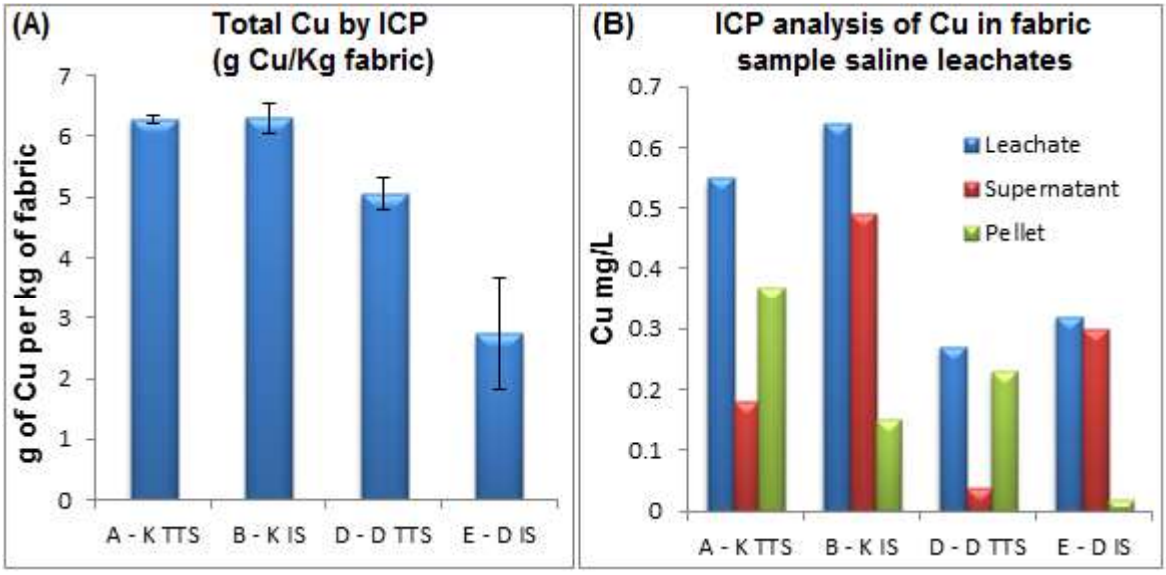


**Figure 47 (A) Total concentration of Cu on the 4 pilot scale PEC fabrics and (B) Cu concentrations in saline leachate solutions**

### 4.2.5.1 Antibacterial activity of saline leachates from pilot scale CuO samples

Saline leachates solutions from the 4 pilot scale CuO fabrics were tested for antibacterial activity using a shake flask method. Tests were performed with MRSA, *P. aeruginosa and E. coli*. Serial dilutions were plated on to NA plates after 0, 3 and 24 hours of shaking incubation.

For *P. aeruginosa*, leachates from all the four fabrics showed an antibacterial effect during 24 hours of incubation. The SONO *in-situ* fabrics B and E (*in-situ* samples) completely inhibited bacterial growth within 3 hours of incubation showing a strong bactericidal effect. These leachates contained the higher proportion of dissolved copper as compared to the un-dissolved CuO NPs (Figure 47). The TTS fabrics A and D also showed a strong antibacterial effect giving a 4 log reduction in microbial growth after 24 hours of incubation (Figure 48). From the results it was observed that leachates from the *in-situ* fabrics had a higher or more rapid acting antibacterial activity than the leachates from the TTS fabrics.

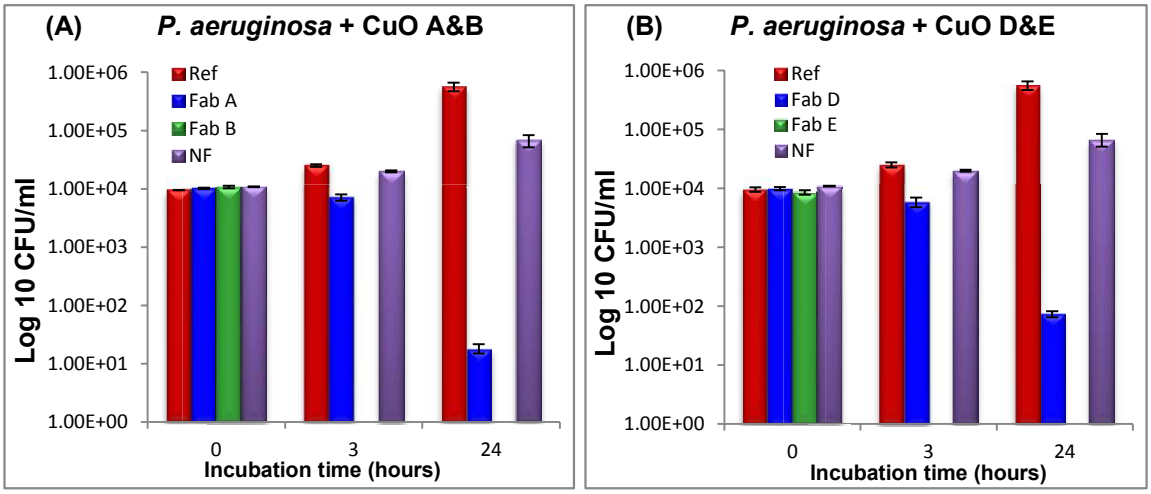


**Figure 48 Number of viable cells obtained by agar plating from the leached saline solutions incubated with *P. aeruginosa*. Fabrics A and B – Klopman TTS and *in-situ*. Fabrics D and E – Davo TTS and *in-situ*. NF – no fabric control**

In the testing carried out against MRSA, a very similar level of activity was observed for the leached saline solutions (Figure 49). The leachates from the SONO *in-situ* samples B and E, produced a full kill within 3 hours of incubation. The leachates from the TTS samples inhibited the growth of the bacteria and reduced the concentration by 2 logs after 24 hours of incubation. A noticeable difference with the MRSA experiments was the inhibition of bacterial growth after 24 hours in the control

saline solution containing MRSA and no fabric (Figure 49). There was also a small reduction in the growth of MRSA with the control reference samples. The unexplained poor growth of MRSA has also occasionally been observed in some other experiments with MRSA. It could be the case the growth conditions were not ideal for MRSA.

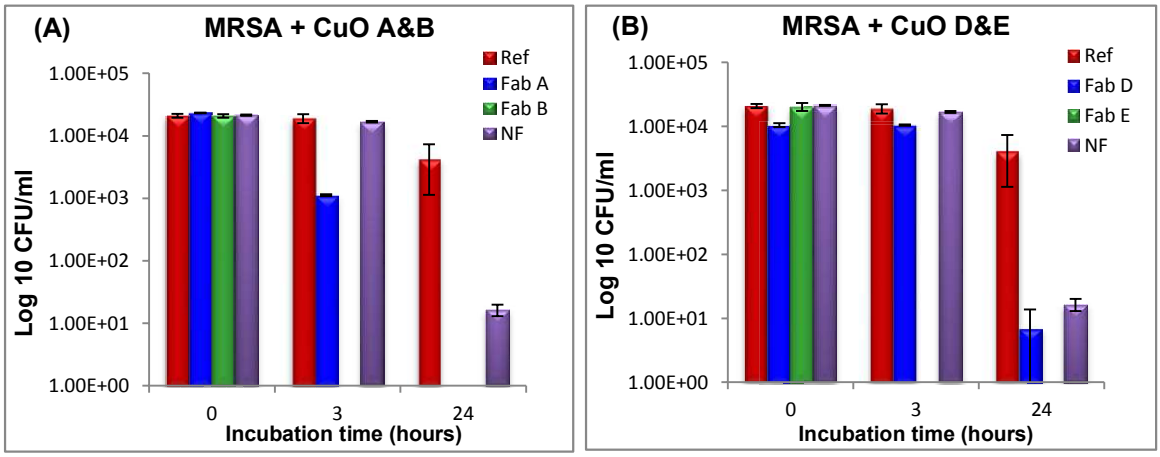


**Figure 49 Number of viable cells obtained by agar plating from the leached saline solutions incubated with MRSA. Ref – control fabric. Fabric A and B – Klopman TTS and *in-situ*. Fabrics D and E – Davo TTS and *in-situ*. NF – no fabric control**

The pilot scale CuO fabrics were also tested against *E. coli* to relate the level of activity of these CuO fabrics to the original CuO cotton leachate experiments. The results from the original CuO impregnated fabrics manufactured at a laboratory scale did not show any activity against *E. coli*. The leachates from the TTS fabrics (A & D) showed some growth inhibition. The leachates from the fabrics coated by the *in-situ* (B & E) technique effectively killed the *E. coli* population within 24 hours (Figure 50).

The lab scale CuO cotton and the pilot scale CuO PEC samples B and E were produced using the same sonochemical *in-situ* coating technique. According to the results from the ICP analysis, the copper concentration in the leachate from the lab scale CuO cotton was 5 to 10 times higher than the concentrations in the leachates

from the pilot scale CuO PEC samples. However, the antibacterial activity of the leachates from the pilot scale PEC samples was much greater.

The higher activity of the leachates from the pilot scale samples could have been due to the presence of a chemical agent other than CuO on the fabrics. Other chemicals may have adhered to the fabric during the coating process and then dissolved in to the saline during leaching. The chemical reagents used at the lab scale and pilot scale were from different sources and so may have contained different impurities. Another possibility is that the pilot scale samples may have produced more ROS (Applerot *et al*. 2009).

Further work with ZnO at the pilot scale was not carried out until towards the end of the SONO project and so analysis of the pilot scale samples is limited here to the CuO ones only.

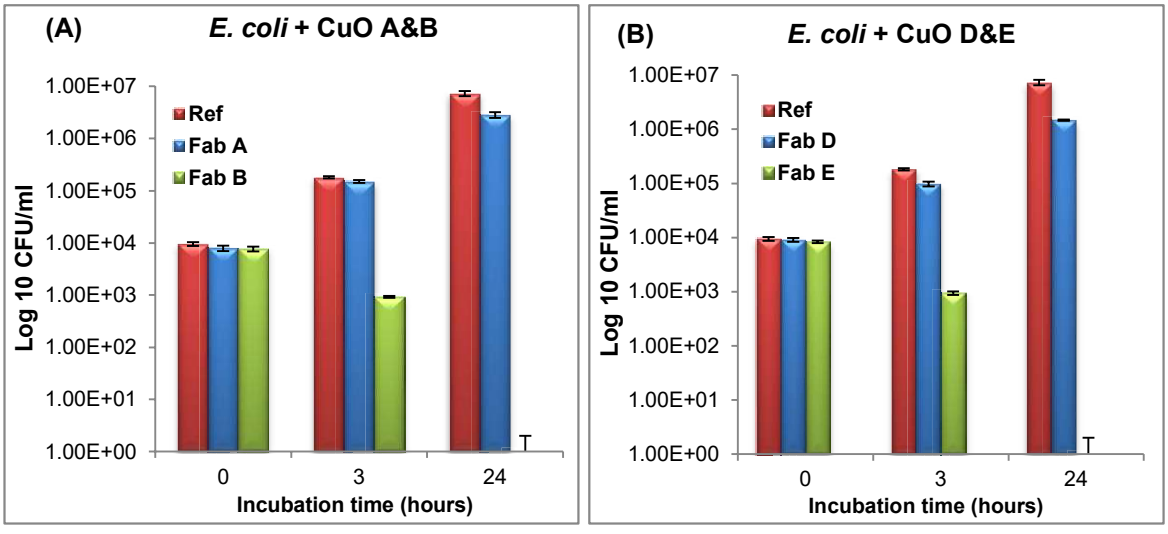


**Figure 50 Antibacterial activity of saline leachates against *E. coli*. Numbers of viable cells were obtained by plating. Fabrics A and B – Klopman TTS and *in-situ*. Fabrics D and E – Davo TTS and *in-situ***

#### 4.2.5.2 Leaching of copper in to nutrient broth

The leaching of copper from the impregnated fabrics was also assessed with nutrient broth. Only the two impregnated fabrics, A and B, manufactured by Klopman were tested for this experiment. The results in Figure 51 showed that a much larger proportion of the copper content leached from B, the *in-situ* type fabric, than from A, the TTS type coating.

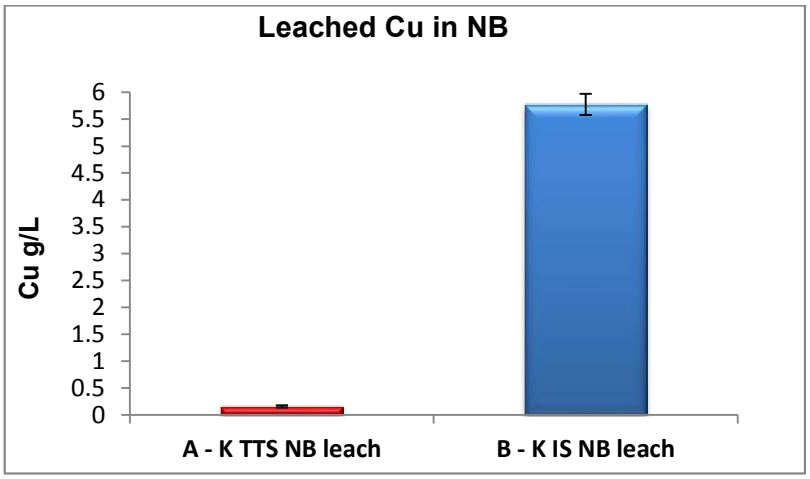


**Figure 51 Concentration of leached Cu content in nutrient broth. Mean ± SD, n = 3**

The leaching of a larger proportion of the copper content from the *in-situ* fabrics than from the TTS fabrics can also be physically seen in Figure 52. The different solubility of the CuO coatings on the fabrics may be due to differences in the nanoparticles. From the SEM images in Figure 42, it can be seen that the particles on the *in-situ* fabric were much smaller than the particles on the TTS fabrics, even though the CuO used for the TTS experiments was <100 nm according to the manufacturer. The CuO NP powder was very difficult to suspend in water, even using ultrasound and so the CuO may have been deposited as micro scale clumps rather than nano scale individual particles.

The different colour of the fabrics (Figure 41) also suggested that there may be differences in the chemical nature of the impregnations. The TTS fabrics appear dark grey in colour. This was as would be expected with an impregnation of black CuO. The brown colour of the SONO *in-situ* fabrics would suggest that the impregnation contains $Cu_2O$ which is brown in colour. It could also possibly be a mixture with Cu metal and CuO. All three of these copper based NPs have been shown to possess antibacterial activity (Azam *et al.* 2012). The exact chemical nature of the impregnations was not investigated here. These differences in size and chemical composition may explain the different solubilities in saline and NB and also the differences in antibacterial activity. Figure 47 shows that Cu is different in the leachates from the 2 different fabrics in terms of solids versus soluble fractions. The *in-situ* impregnations release more Cu ions and the TTS impregnations released more in a pellet form (solid particles) rather than dissolved.

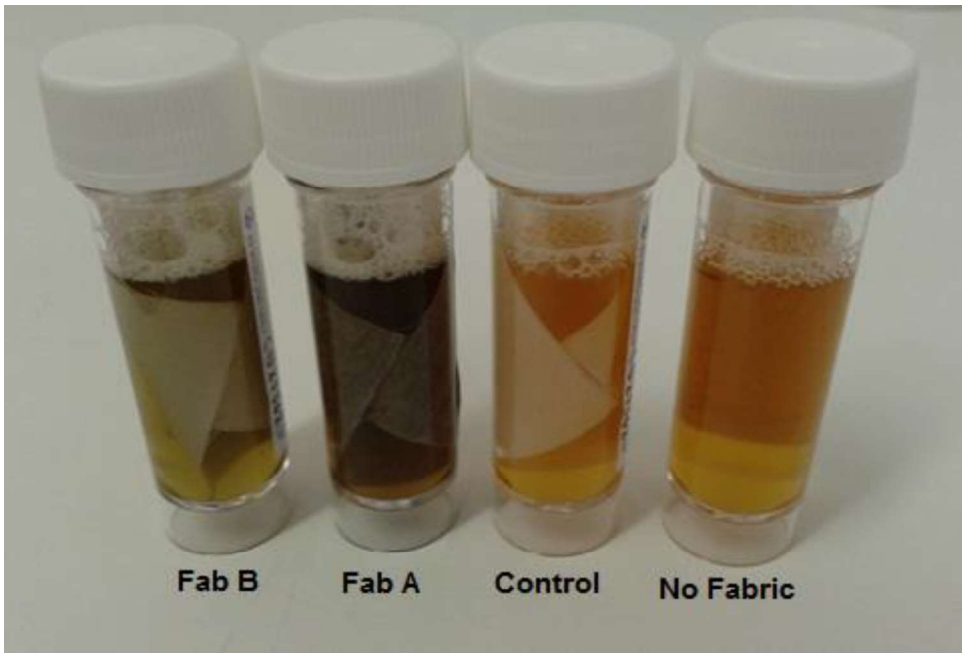


**Figure 52 Photos of CuO fabrics in SCDLP medium. The coloured fabrics have leached completely into SCDLP medium turning into white fabrics. Same changes observed in NB**

### 4.2.6 Differences in the homogeneity of the impregnation (Klopman vs Davo)

As seen in Figure 53 (a), fabrics produced using the machine at Davo clothing did not show a homogeneous impregnation of CuO nanoparticles on the fabric surface. This was due to problems with the ultrasonic transducers that were used. An experiment was performed to analyse the total concentration of copper on different parts of fabric surface. Samples were cut from the paler and the darker regions of fabric E. The total concentration of copper on the samples was measured by ICP after dissolution of the CuO in to 0.5 M $HNO_3$. The results in Figure 53 (b) showed that dark areas of impregnated fabrics had 3.15 g of copper per kg of fabric compared to 1.85 grams for light areas of treated fabric. The mean concentration of Cu on the fabrics (random samples) was 2.73 grams per kg of fabric.

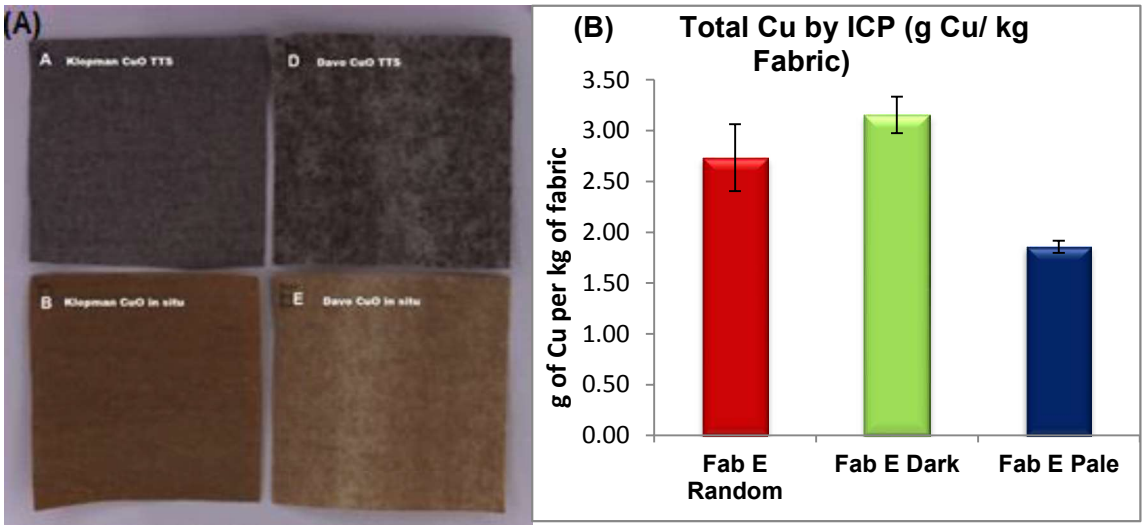


**Figure 53 (a) CuO fabrics produced by Klopman and Davo using the pilot scale machines – squares 5 x 5 cm (b) concentrations of Cu on random, dark and pale regions of fabric E. Mean ± SD, n = 3**

#### 4.2.6.1 Antibacterial activity of fabric E from pale and dark area of coating

To further assess the homogeneity in the impregnations, the antibacterial activity was compared from the same fabric (Fabric E), cut out from different parts (paler and darker region of fabric E) of the impregnated surface. The results showed that the visible variation in the impregnations correlate with the antibacterial activity. Both regions showed good antibacterial activity (Figure 54). However, when compared to each other, samples from the paler region of Fabric E showed less antibacterial activity (A value 2.69) as compared to the darker region (A value 4.60).

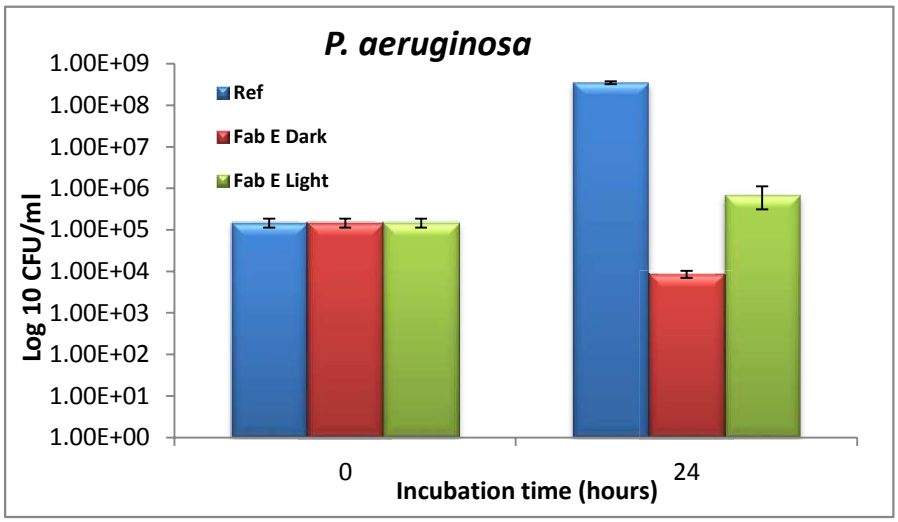


**Figure 54 Comparison of the antibacterial activity of samples from the pale and dark regions of the impregnations against *P. aeruginosa***

## 4.3 Flow cytometry (FC)

One of the objectives of this research was to investigate the use of flow cytometry as an alternative to agar plate counts for the determination of viable cell numbers. The basic absorption method was used to test the fabrics. The number of viable cells pre and post incubation was determined using both flow cytometry and agar plate counts.

### 4.3.1 Effect of sample preparation on bacterial viable count (FC experiments)

This experiment was performed to look at the effects of sample preparation on the number of viable bacterial cells. The purpose of the experiment was to explore whether differences in the way samples were prepared for plate counts and flow cytometry resulted in any differences in viable counts. The influence of sonication and centrifugation on bacterial recovery was investigated and compared with untreated samples by plating onto NA plates. Morphological changes in the bacterial suspensions were also examined using microscopy (100x oil magnification) and Gram stained slides. Three sample treatments were performed for each bacteria:

- Normal vortexing of samples for 2 minutes
- Sonication in a 40 kHz water bath for 2 minutes
- Centrifugation at 13,000 rpm for 3 minutes followed by vortexing

Sonication is commonly used to separate or de-clump bacterial cells (Denis 1991, Joyce *et al.* 2003). From the results it was observed that sonication did not raise bacterial counts for *E. coli* after 2 minutes of sonication. When compared with vortexed samples, the bacterial counts decreased slightly by 2.5% from 2.58E+07 CFU/ml to 2.54E+07 CFU/ml (Table 16). After centrifugation and re-suspension there was some reduction (4.5%); the viable counts for these samples dropped to 2.49E+07 CFU/ml. This reduction following centrifugation could have been due to the adherence of some bacterial cells to the walls of the Eppendorf tubes or minor clumping of the cells. From the results shown in Figure 55, sonication and centrifugation did not have any major effect on the counts for *E. coli*. ANOVA showed that the results from all the three experiments against *E. coli* were not significantly different. Gram stained smears were prepared from treated bacterial

suspensions and observed under direct light microscopy. As shown in Figure 56a, the Gram stained smears did not show any significant changes in the morphology of *E. coli* suspension after sonication or centrifugation.

Bacterial counts from treated *A. baumannii* suspensions are shown in Figure 55. The viable counts were 27% higher in sonicated samples (3.19E+07 CFU/ml) than in vortexed samples (2.37E+07 CFU/ml). The clumping of bacterial cells can result in an underestimation of viable cell numbers from agar plate colony counts. A clump of cells on an agar plate will only give rise to single colony. Sonication can disrupt the clumps of cells so that there are more free cells that can grow into individual colonies on the agar. Lower bacterial numbers (1.90E+07 CFU/ml) were recovered after centrifugation of *A. baumannii* samples. The numbers decreased by 21% compared to the vortexed sample. Although the samples were vortexed after centrifugation, pelleting may have made the clumping worse and harder to reverse and some cells may have adhered to the walls of the Eppendorf tubes (Table 16). On Gram stained slides the clumping of *A. baumannii* cells was quite evident (Figure 58b). Large clumps of cells were observed in both the vortexed and centrifuged samples. After sonication for 2 minutes most of the stained cells were present as individual separated cells on the slides rather than in clumps.

One characteristic of *S. aureus* and *MRSA* is that they commonly grow in clumps of cells and this is evidenced by the results shown in Table 16, Figure 59c and Figure 59d. Sonication for 2 minutes de-clumped *S.aureus* and MRSA cells giving viable counts of 2.63E+07 CFU/ml and 1.26E+08 CFU/ml compared to 2.09E+07 CFU/ml and 8.95E+07 CFU/ml for the normal samples. Lower viable counts were also recovered from centrifuged samples. Examination of the Gram stained slides, as shown in Figure 58c and Figure 58d, showed some differences in

the appearance of the suspensions following the different treatments. As expected cells were clumped together when centrifuged and showed a lower bacterial count.

Sonication of the *P. aeruginosa* suspensions increased the viable counts slightly from 1.91E+07 to 1.93E+07 CFU/ml. After centrifugation and re-suspension there was a decrease in the number of viable cells to 1.76E+07 CFU/ml (9% decrease). Some aggregation of cells was visible on each of the slides (Figure 58e). Sonication for longer periods of time may have disrupted more clumps and resulted in higher viable counts. However, longer periods of sonication may also have resulted in reduced counts by killing some of the cells (Joyce *et al.* 2003).

For the agar plate counting method used in the majority of the experiments, the bacterial suspensions were simply vortex mixed. For the FC experiments the bacteria were centrifuged and washed to remove cell debris and for re-suspension in the appropriate buffer solution for FC staining. Sonication was used to help re-suspend the cell pellets after centrifugation.

The results have shown that sonication and centrifugation can affect bacterial cell counts. Centrifugation can reduce viable counts by increasing the clumping of cells. Sonication works in the opposite direction, increasing the counts by breaking apart clumps of cells. Even where bacteria do not show a tendency to clump, such sample preparation steps may result in changes in the viable counts. For example during centrifugation some bacteria could adhere to the walls of the Eppendorf tubes or some could be lost in the supernatant discarded during cell washing.

**Table 16 Mean viable plate counts recovered after FC sample preparation steps against different bacterial species. Mean ± SD, n = 3**

| Treatment | Test bacteria CFU/ml (Mean) | | | | |
|---|---|---|---|---|---|
| | ***E. coli*** | ***A. baumannii*** | ***S. aureus*** | **MRSA** | ***P. aeruginosa*** |
| **Normal (Vortexed)** | 2.58E+07 | 2.37E+07 | 2.09E+07 | 8.95E+07 | 1.91E+07 |
| **Sonicated** | 2.54E+07 | 3.19E+07 | 2.63E+07 | 1.26E+08 | 1.93E+07 |
| **Centrifuged** | 2.49E+07 | 1.90E+07 | 1.68E+07 | 8.05E+07 | 1.76E+07 |
| **ANOVA *p* value** | **0.82** | **0.0001** | **0.00002** | **0.0005** | **0.27** |

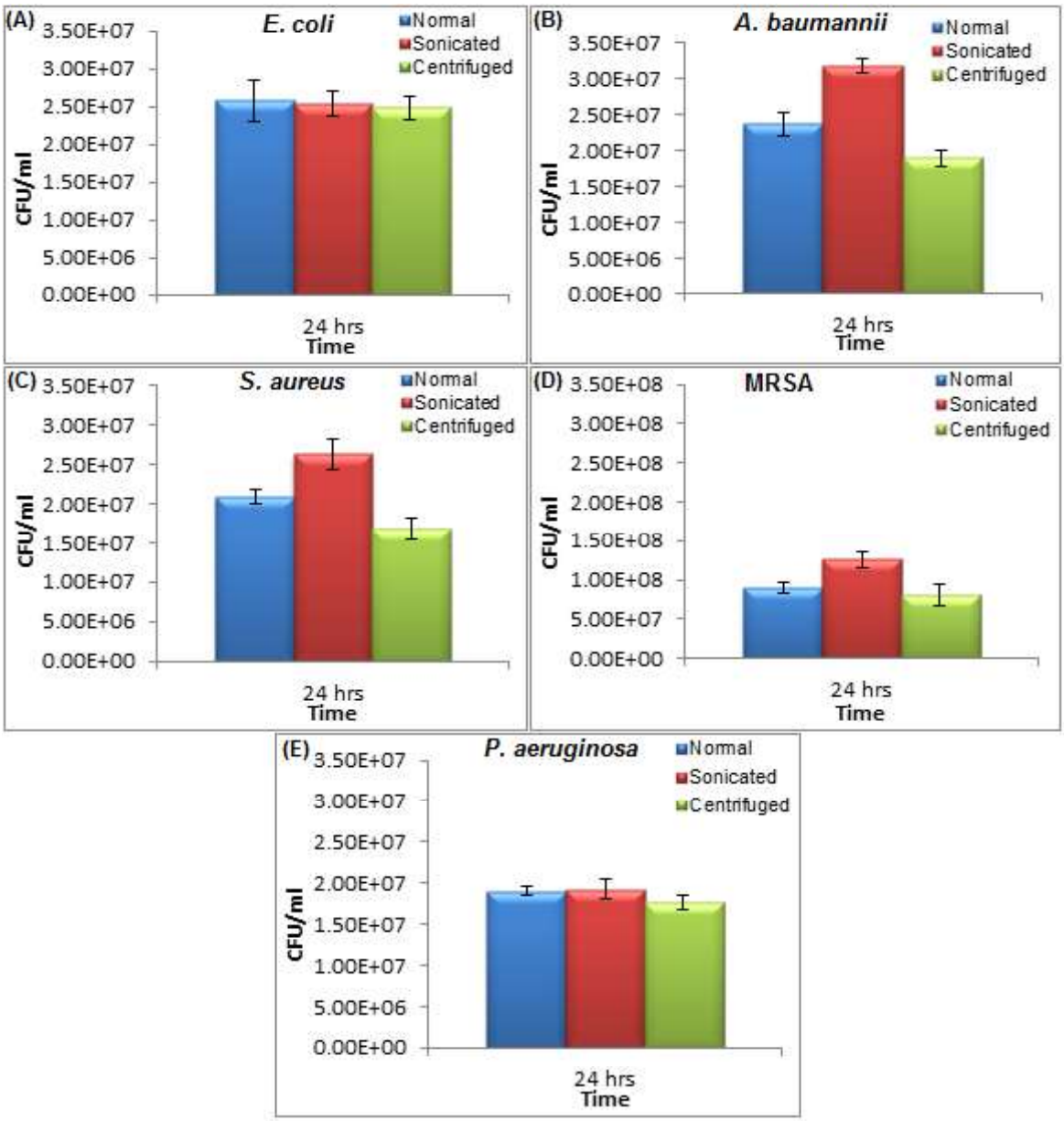


**Figure 55 Effect of different sample preparations on bacterial viable counts for (A) *E. coli*, (B) *A. baumannii*, (C) *S. aureus*, (D) MRSA and (E) *P. aeruginosa*. Mean ± SD, n= 3**

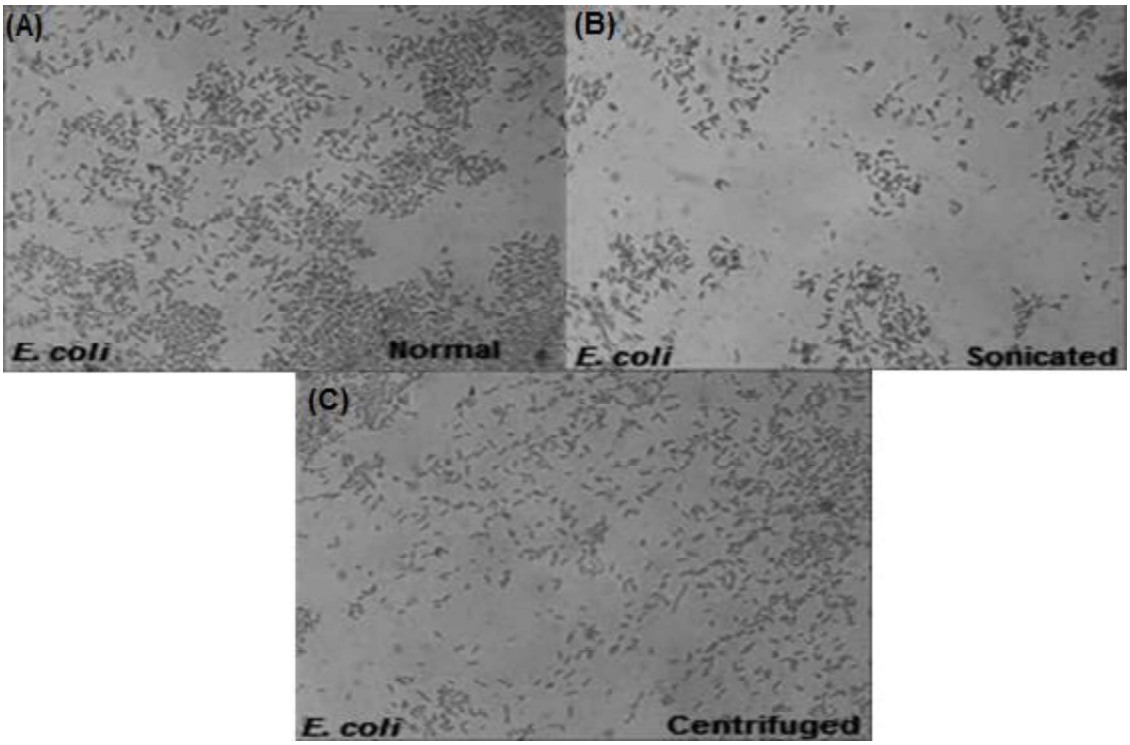


**Figure 56a Changes in the morphology of *E. coli* after different treatments; (A) Untreated sample; (B) Centrifuged sample; (C) Sonicated sample (100x oil magnification). Cells ~ 1µm in size**

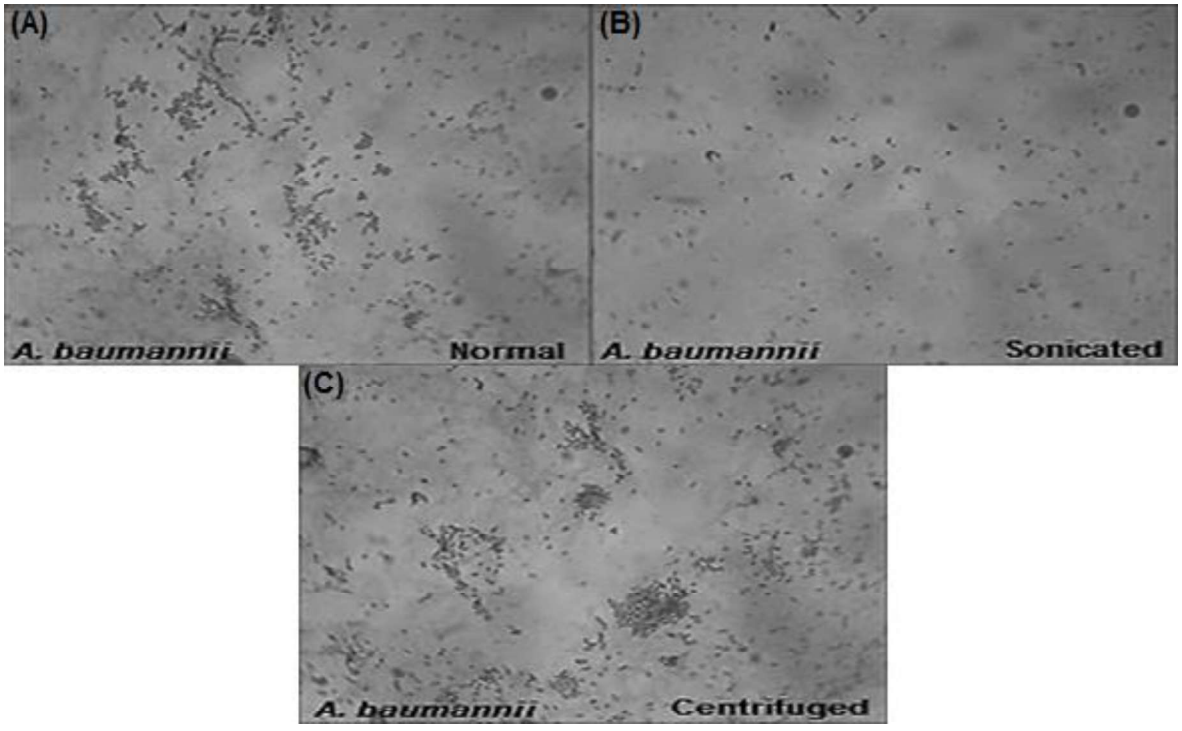


**Figure 58b Changes in the morphology of *A. baumannii* after different treatments; (A) Untreated sample; (B) Centrifuged sample; (C) Sonicated sample (100x oil magnification). Cells ~ 1µm in size**

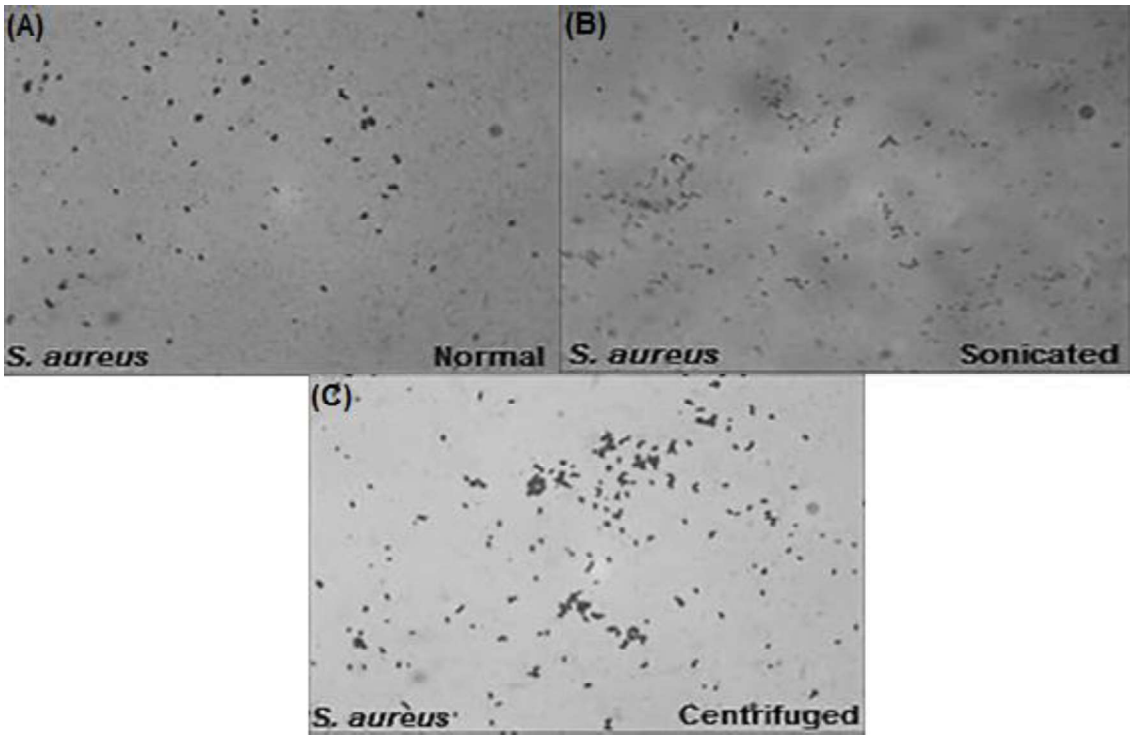


**Figure 58c Changes in the morphology of *S. aureus* after different treatments; (A) Untreated sample; (B) Centrifuged sample; (C) Sonicated sample (100x oil magnification). Cells ~ 1µm in size**

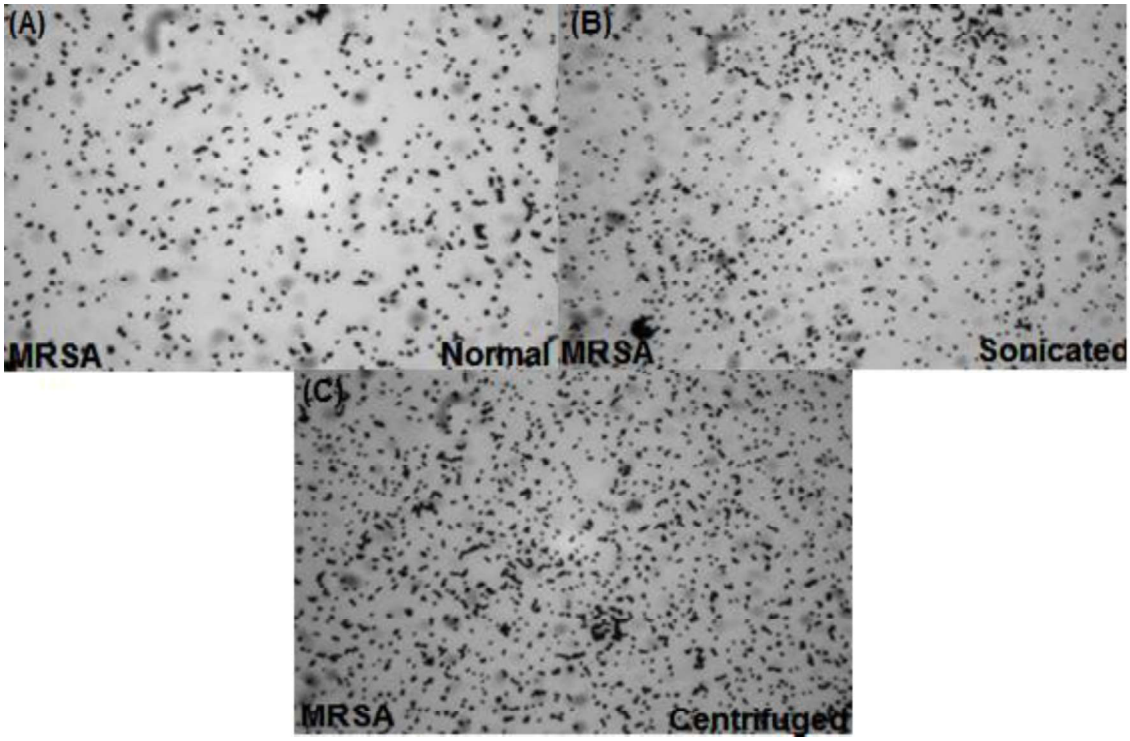


**Figure 58d Changes in the morphology of MRSA after different treatments; (A) Untreated sample; (B) Centrifuged sample; (C) Sonicated sample (100x oil magnification). Cells ~ 1µm in size**

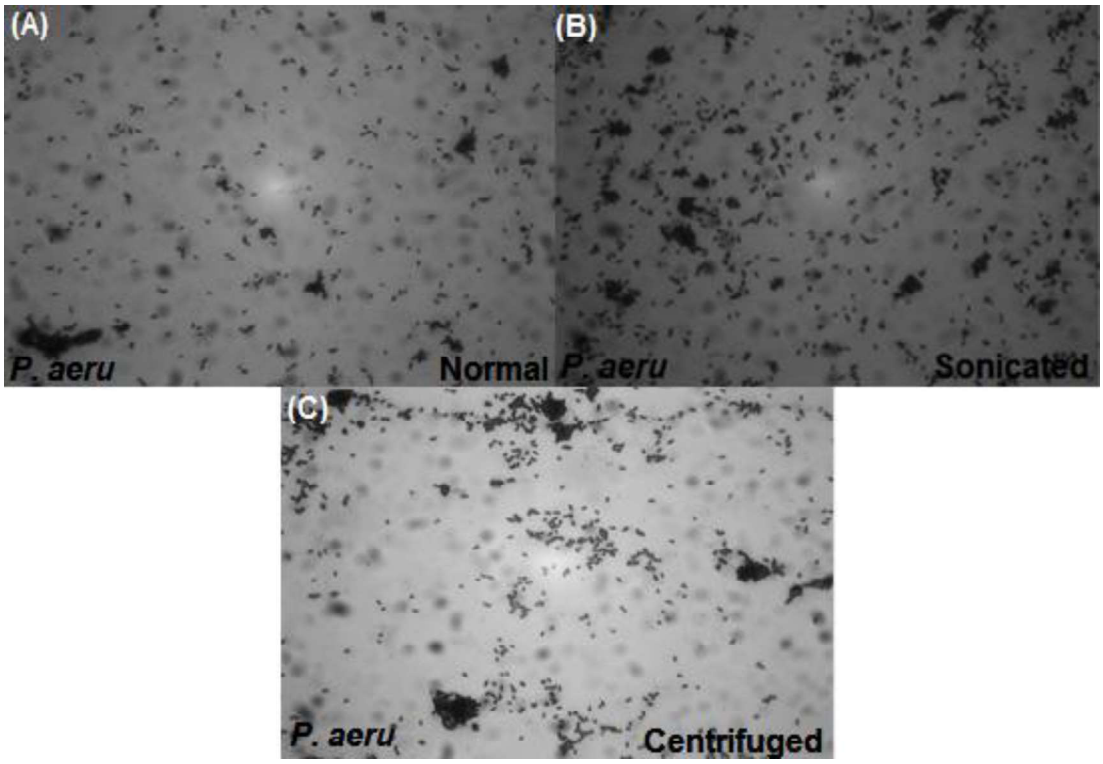


**Figure 58e Changes in the morphology of *P. aeruginosa* after different treatments; (A) Untreated sample; (B) Centrifuged sample; (C) Sonicated sample (100x oil magnification). Cells ~ 1µm in size**

### 4.3.2 Measurement of the sensitivity of cell detection by FC on the BD FACS system

Flow cytometry (FC) was examined as a technique to assess the antibacterial effect of impregnated fabrics (ZnO/CuO NPs) on bacterial populations. Live and dead cells were discriminated using fluorescent cell viability stains. Flow cytometry (FC) analysis was performed with thiazole orange (TO), a nucleic acid stain that penetrates all cells and propidium iodide (PI), a nucleic acid stain is not taken up by intact cells. Propidium iodide (PI) only enters damaged or dead cells whereas thiazole orange (TO) can enter all cells (Alsharif and Godfrey 2002). As the two dyes emit light at different wavelengths it is possible to distinguish between intact live cells and damaged cells based on fluorescence emission. To validate FC results and to

examine the relationship between cell viability and cell death, the FC results were compared with the results of viable plate counts.

Data was acquired using BD CellQuest™ Pro software in Acquisition-to-Analysis mode. Fluorescent microsphere beads were added to samples and used as a reference to determine the volume of sample analysed and hence to calculate the actual concentration of live and dead cells in the sample.

Before antibacterial testing, a series of experiments were performed to measure the sensitivity of cell detection with the BD FACS system. A series of dilutions (3, 4, 5 and 6 fold) of 3 hour cultures of *S. aureus, E. coli, A. baumannii,* MRSA and *P. aeruginosa* were used for assessment. Staining buffer was run as a blank. These experiments suggested that the limit of detection of the FC for viable bacteria was between 1000 and 10000 viable cells per ml. As shown in Figure 57, a linear relationship was seen in cell numbers when measured from 1,000,000 cells per ml down to around 10,000 cells per ml. Once the dilution reduced the number of cells down to less than 10,000 cells per ml, the FC counts start deviating significantly from the expected counts. Noise is a significant limitation at such low cell concentrations. Random counts due to noise give an over estimate of the number of live cells. As described in the BD Biosciences application notes (Alsharif and Godfrey 2002) “sensitivity of detection varies with the noise present in the sample matrix and with each experimental situation”.

From Figure 57, it can be seen that the deviation in actual counts from expected counts was greatest for *S. aureus*, MRSA and *A. baumannii*. These bacteria also displayed the greatest tendency to form aggregates or clumps. As explained in section 4.3.1, centrifugation and sonication can affect the counts of

bacterial cells by reducing and increasing the viable counts of bacteria. The results showed that FC has overestimated the bacterial number at lower concentrations.

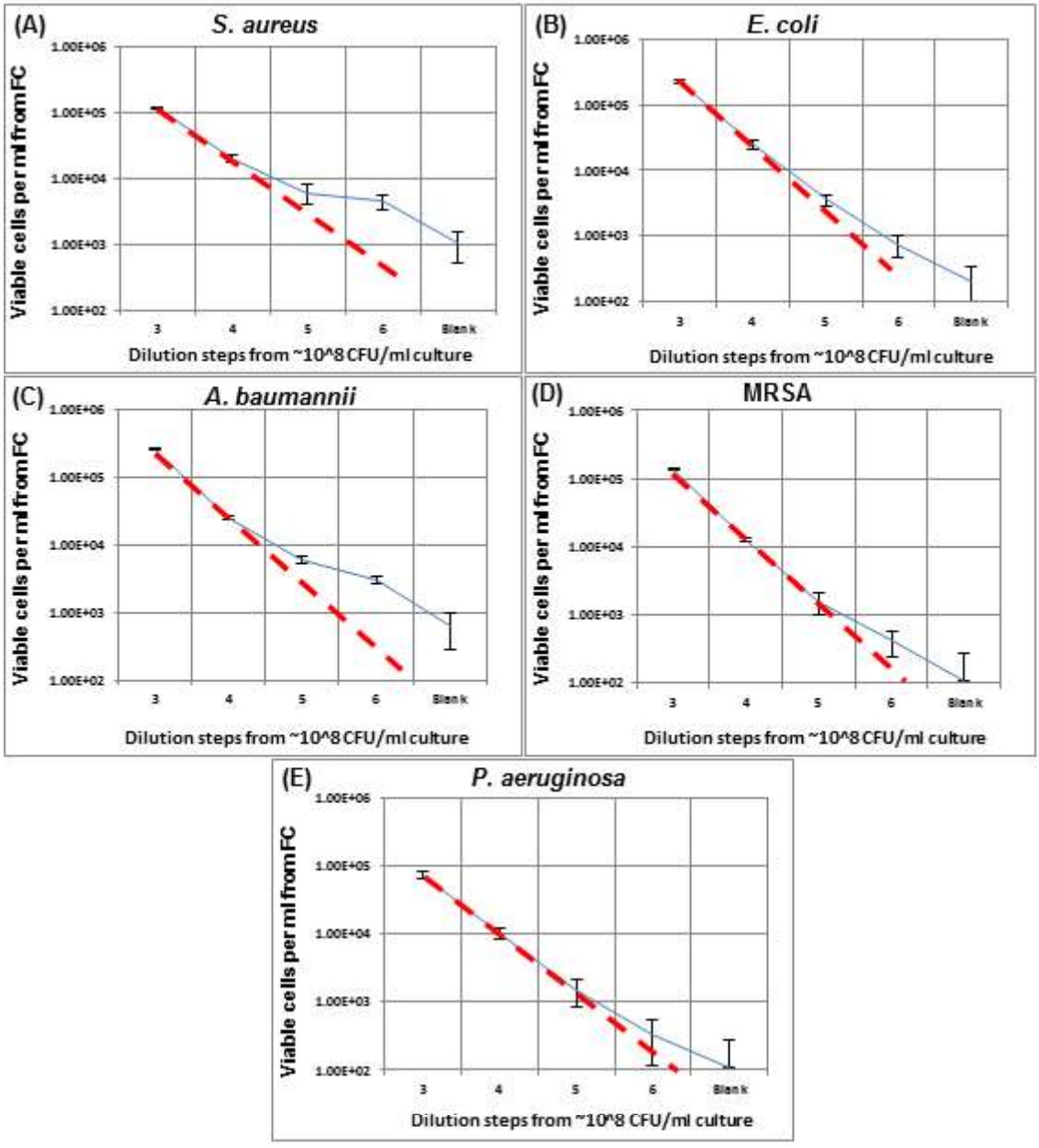


**Figure 57 Mean viable cell counts by FC from 3, 4, 5 and 6 fold dilutions of 3 hour cultures (~$10^8$ cells per ml) to assess the sensitivity of cell detection by flow cytometry for (A) *S. aureus*, (B) *E. coli*, (C) *A. baumannii*, (D) MRSA and (E) *P. aeruginosa*. Solid blue line – FC count. Dashed red line is expected cell count. Mean ± SD, n = 3**

A viable plate count method was also performed with the samples to look at the relationship between flow cytometry counts and traditional agar plate counts. Figure 58 shows the relationship between the viable counts for all the five bacterial species determined by flow cytometry and by agar plate counting. The counts from flow cytometry were in general higher than the counts from agar plating. The higher counts from FC could have been due to sample noise leading to false positive viable counts. The greatest deviations between the FC counts and the plate counts occurred at the lower cell concentrations. The higher counts from FC may also have been due to clumping of the cells. Some of the clumps may have been dispersed during preparation of the samples for FC and during actual measurement. There were significant differences between the FC and plate counts with both *S. aureus* and *A. baumannii*. In section 4.3.1, the viable counts for both these species were increased following sonication.

Flow cytometric analysis can provide an accurate quantification of cells but technical limitations. The results showed that flow cytometry analysis was not suitable for measuring low concentrations of bacteria (< 10000 CFU/ml) and so may underestimate total levels of bacterial inhibition or kill. This is also mentioned in the manufacturer's instructions manual "At least 100 bead events should be collected to provide reliable concentration data". Background noise will become more prominent with decrease in the bacterial concentration (Alsharif and Godfrey 2002).

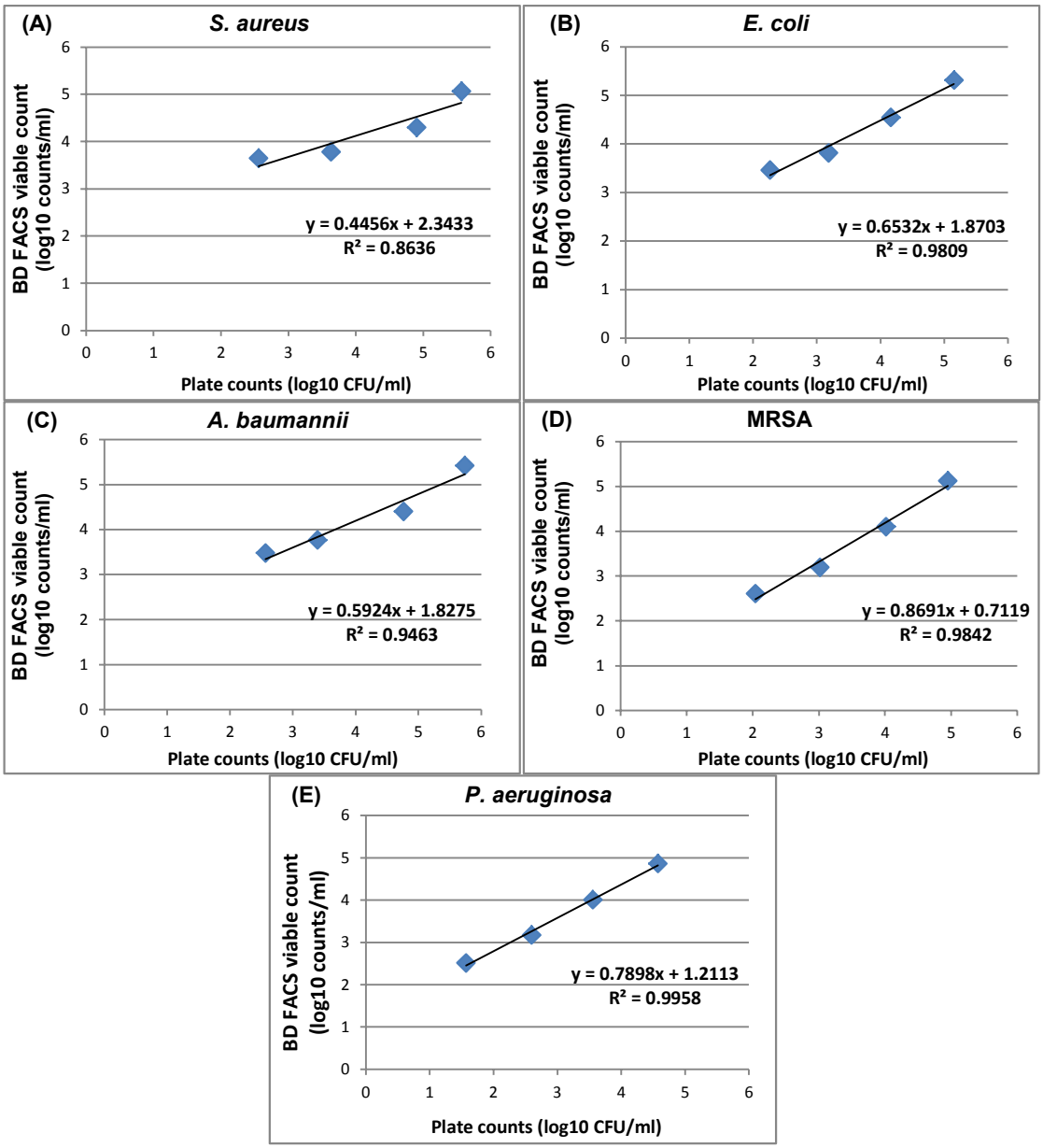


**Figure 58 Comparison of viable counts from flow cytometry and agar plating for dilutions of 3, 4, 5 & 6 fold of a 3 hour culture of (A) *S. aureus*, (B) *E. coli*, (C) *A. baumannii*, (D) MRSA and (E) *P. aeruginosa***

### 4.3.3 Comparing the enumeration of viable cells from lab scale ZnO and CuO fabrics by FC and agar plate counting

Live and dead bacterial suspensions were used to calibrate and adjust the flow cytometer settings to position regions for counting live cells and beads. Green fluorescing TO and red fluorescing PI were positioned at FL1 and FL3 as shown in

Figure 59. Bead counts were used to determine the volume analysed and thus the actual concentration of viable cells. The regions were gated to differentiate between live and dead cells. After setting the parameters in the gate R2 for live cell controls and gate R3 for the dead cell controls, the number of bacteria in each gate was determined, as shown in the example dot plot in Figure 59. In the actual experiments dead cells were not identified and gated. For the experiments, only the cell growth was monitored and measured by gating the live cells. This was because the initial starting bacterial concentration was too low to count number of dead cells. Moreover, the purpose of this study was to compare FC counts with viable plate counts. The viable plate counts only promote viable bacteria and excluded dead cells and debris.

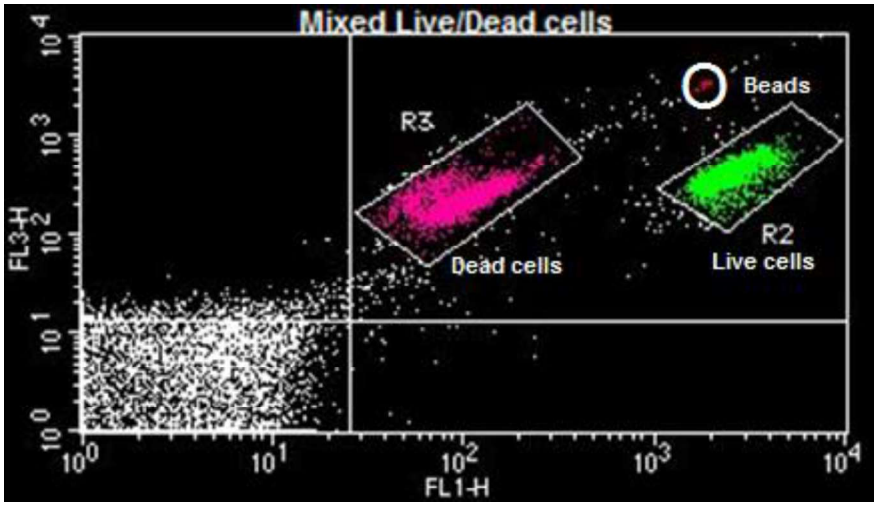


**Figure 59 An example FC dot plot with *E. coli* showing event populations for 2 different fluorescence emissions (FL1 and FL3). The pink events in region 3 (R3) are the dead bacteria and green events in region 2 (R2) are the live cells. The counting beads (red events) are shown in the upper right part of the graph**

The graphs in Figure 60 show the relationship between the concentration of viable bacteria determined by flow cytometry and viable plate counts for *E. coli*. The results from both methods of viable cell determination showed that the growth of

*E.coli* was effectively inhibited by ZnO fabrics. There was a greater than 99% reduction in the growth of bacteria on the ZnO treated cotton compared to the plain cotton. Antibacterial A values were also quite similar for both the methods (A=2.25 for FC and A=2.94 for plate counts). From the results, it was seen that FC counts for *E. coli* were lower than that of counts by viable plate counts (Figure 60).

A good agreement between plate counts and FC counts was also seen for *A. baumannii*. The results from both analytical methods (viable plate count and flow cytometry) showed a significant antibacterial activity (A = 2.94 for FC and A = 2.73 for plate count). However, the FC counts for *A. baumannii* were higher than the viable plate counts. The higher FC counts could be due to the presence of viable but non culturable cells (VBNCs) or could also be due to the declumping of bacterial cells. The sample preparation experiments in section 4.3.1 showed that *A. baumannii* was more inclined to clumping by centrifugation and declumping by sonication.

Some bacteria are capable of maintaining metabolic activity while developing resistance to growth in culture. Such a state is often described as “viable but non-culturable (VBNC)” state (Weichart 1999). One important potential advantage of using flow cytometry is the ability to detect those VBNC cells. The cells with intact membranes are considered live cells and the ones with damaged membranes are counted as dead or damaged cells. The propidium iodide (PI) only enters damaged or dead cells, whereas thiazole orange (TO) can enter all cells (Alsharif and Godfrey 2002). The interpretation and verification of VBNCs by FC can be calculated through the fluorescent dye (thiazole orange), which determine the fractions of cells in different physiological conditions (Khan, Pyle and Camper 2010).

The viable bacterial counts as determined by FC and plate counting for *S. aureus* were quite similar for the control samples but very different for the impregnated samples. FC gave higher viable counts than agar plate counts (Figure 60). The *S. aureus* results were quite similar to *A. baumannii* results where FC counts were greater than plating counts. In Figure 57, it can be seen that with these two species, the FC counts differed significantly from the plating counts at the lower dilutions. This could be due to the presence of VBNCs. It could also be due to the overestimation of numbers associated with the FC method at lower dilutions.

Standard agar plating gives a more accurate viable count at low concentrations (< $10^3$ cells per ml). When the results were plotted from both analytical methods, there was a strong antibacterial effect seen for *S. aureus* using the plate count numbers, showing 99.9% growth reduction. The FC results did not agree with the plate count results and showed just a 1 log growth reduction (83.36%) in bacteria (Table 17). Apart from VBNC's and sample preparation, the sensitivity of detection by the FC equipment also plays a part in the variation in the comparison of FC and viable plate counts. The term is referred to as "rare-event analysis" which means the detection of events that occur at a frequency lower than 10000 cells. According to Donnenberg and Donnenberg (2007), detecting an event at a lower frequency requires a high signal-to-noise ratio and also acquisition of large number of events (Low counts = high numbers by calculation). Background noise can overlap with the actual counting regions on the FC dot plots.

**Table 17 Mean viable bacterial counts (CFU/ml) by both FC and plate counting after 24 ± 3 hours contact time for ZnO samples and control samples against *E. coli*, *A. baumannii* and *S. aureus* and their antibacterial A values.**

| Test Method | Control fabric (24 hrs) CFU/ml | | ZnO treated (24 hrs) CFU/ml | | Antibacterial value A | |
|---|---|---|---|---|---|---|
| | FC | ISO | FC | ISO | FC | ISO |
| ***E. coli*** | 1.08E+07 | 8.00E+08 | 6.39E+04 | 9.30E+05 | 2.25 | 2.94 |
| ***A. baumannii*** | 4.22E+08 | 8.30E+07 | 4.86E+05 | 1.50E+05 | 2.94 | 2.73 |
| ***S. aureus*** | 2.88E+07 | 5.40E+07 | 4.79E+06 | 1.20E+04 | 0.27 | 3.66 |

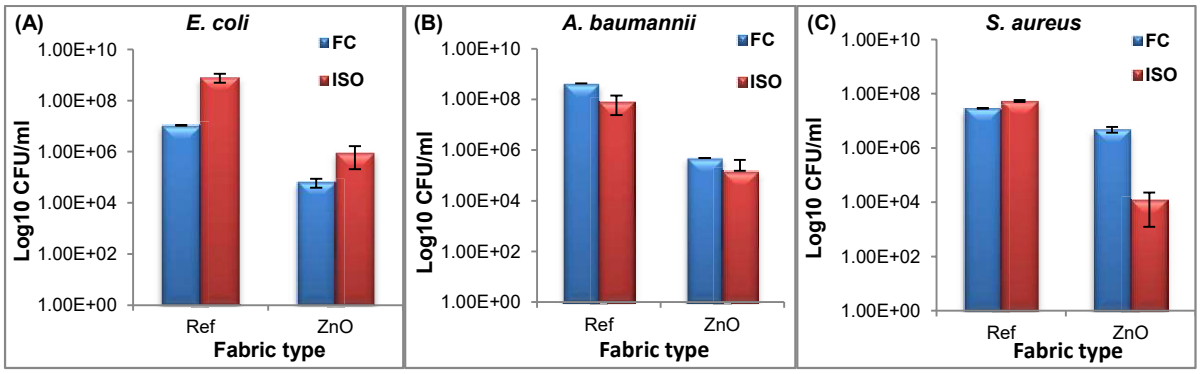


**Figure 60 Antibacterial activity of lab scale ZnO fabrics assessed by agar plate counting and flow cytometry against (A) *E. coli*, (B) *A. baumannii* and (C) *S. aureus*. Mean ± SD, n = 3**

As stated by Novo *et al.* (2000), different techniques to assess antimicrobial activity rely on the detection of changes in the number of bacteria or colonies in a sample over time. Each colony on an agar plate or bacterial count in a flow cytometry sample can be either a single viable bacterial cell or a clump of cells containing one or more viable cells. So, where clumping of cells occur it is harder to reliably determine cell viability. Davey and Kell (1996) demonstrated that at low concentrations, flow cytometry overestimates the microbial load and at higher concentrations there is an underestimation of microbial concentration. They also

stated that microbial cells like *S. aureus* often grow in clumps and form aggregates during sample preparation. If any of the cells in a clump have a leaky cell membrane they may take up the exclusion dye (propidium iodide) and be counted as “dead” in the viability assay (Davey and Kell 1996). To overcome this problem, researchers suggested using the forward scatter signal (FSC) alongside fluorescence to indicate the size of a cell or clump. Since, the size of bacteria varies according to growth conditions; they also suggest that using two or more viability stains would give a broader picture of the physiological status of the cells (Davey *et al.* 2004).

Jepras *et al.* (1995) studied the correlation between flow cytometric analysis and CFU counts by evaluating a range of viability stains. They developed a technique to distinguish live and dead bacteria to obtain a viable count and a total count. It involved evaluating several fluorescence probes as indicators of bacterial viability by flow cytometry. They found a good correlation between the viable counts obtained through flow cytometry and agar plating, but sourced errors associated with the two techniques. They stated that different methods of “viability” assessment may measure different properties of cells potentially preventing an agreement between different techniques.

It is also apparent that accuracy in cell counts is very much dependant on the accuracy in sample delivery and on the type of flow cytometer used (Boye and Steen 1993). In the event of unknown sample flow rate, a known concentration of fluorescent beads is used as a standard so that cell count can be related to the volume analysed. However, Cantinieaux *et al.* (1993) found that because of wide distribution of properties seen in microbial cells, there is the possibility of overlapping between the beads and the bacteria.

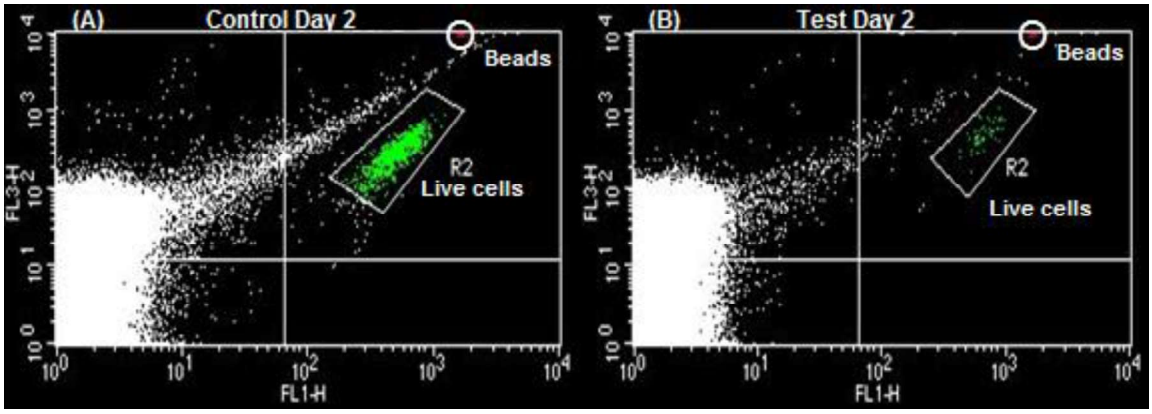


**Figure 61 Flow cytometry dot plots with FL1 versus FL3 for *E. coli* comparing control cotton fabric against ZnO impregnated fabric. These events shown in green are live cells (R2) and the events shown in red (top right) are fluorescent counting beads**

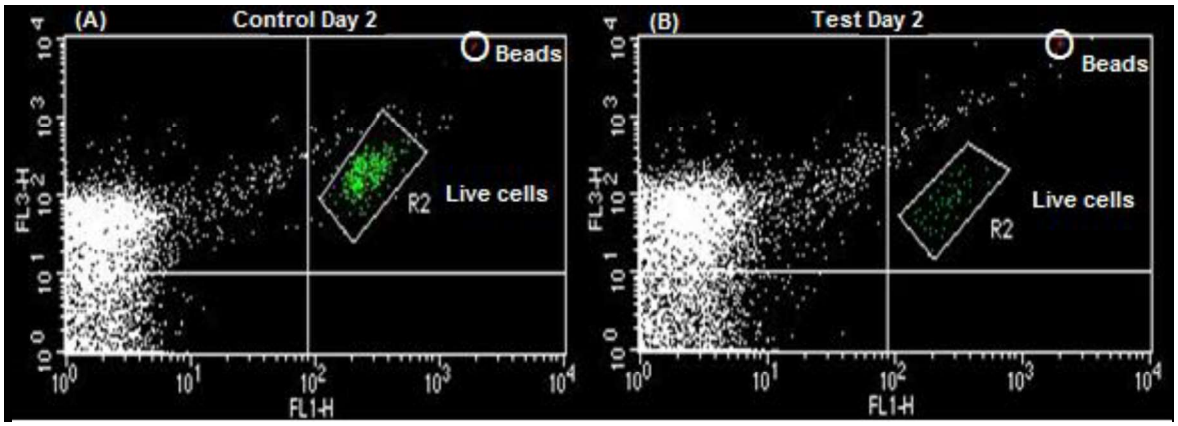


**Figure 62 Flow cytometry dot plots with FL1 versus FL3 for *A. baumannii* comparing control cotton fabric against ZnO impregnated fabric. The events shown in green are live cells (R2) and the events shown in red (top right) are fluorescent counting beads**

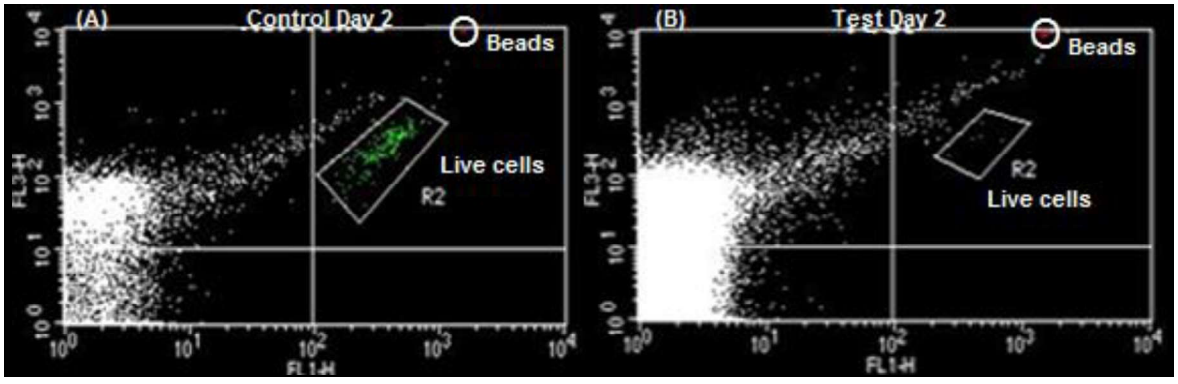


**Figure 63 Flow cytometry dot plots with FL1 versus FL3 for *S. aureus* comparing control cotton fabric against ZnO impregnated fabrics. The events shown in green are live cells (R2) and the events shown in red (top right) are fluorescent counting beads**

The results with CuO fabrics were found to be quite similar to the ZnO impregnated fabrics. Figure 64, Figure 65 and Figure 66 represent the results of flow cytometric analysis of cotton impregnated with CuO nanoparticles against control cotton when incubated with *E. coli*. The live population is clearly distinguishable in all the fluorescence dot plots.

When comparing both the analytical methods for *E. coli*, a greater than 99% growth reduction was observed from both. The plate count method showed a higher degree of microbial kill as compared to the flow cytometry counts analysis. A 4 log reduction was seen for plate counts compared to 2 log reduction by FC (Table 18).

Figure 65 shows the results for flow cytometric analysis of cotton impregnated with CuO nanoparticles and control cotton when challenged against *A. baumannii*. The viable bacterial counts from both techniques showed that there was a greater than 99% reduction in the growth of bacteria on the CuO impregnated cotton compared to the plain cotton. Both the methods showed a 3 log reduction in bacterial growth (Table 18) giving similar antibacterial A values of 3.06 and 3.14 respectively.

The dotplots in Figure 66 showed the bacterial populations of *S. aureus* determined by flow cytometry. When the results were plotted from both analytical methods, the viable plate count showed a significant bacterial kill (A value 2.96) as compared to FC analysis (A value 0.92). A 99.89% reduction was seen with the plate count method but there was only an 83.61% reduction according to FC analysis (Table 18). This was due to the higher FC counts for the test samples. The viable counts by absorption and FC method were quite similar for the reference fabrics but the FC counts were high for impregnated CuO fabrics compared to the plate counts.

The FC results from the lab scale CuO fabrics showed higher bacterial counts with all the three species tested compared to the viable plate counts.

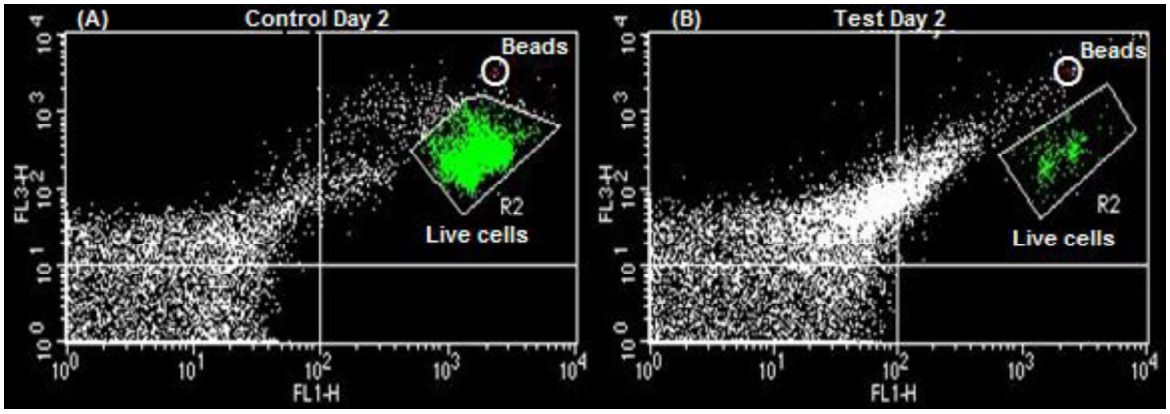


**Figure 64 Flow cytometry dot plots with FL1 versus FL3 for *E. coli* comparing control cotton fabric against CuO impregnated fabric. These events shown in green are live cells (R2) and the events shown in red (top right) are fluorescent counting beads**

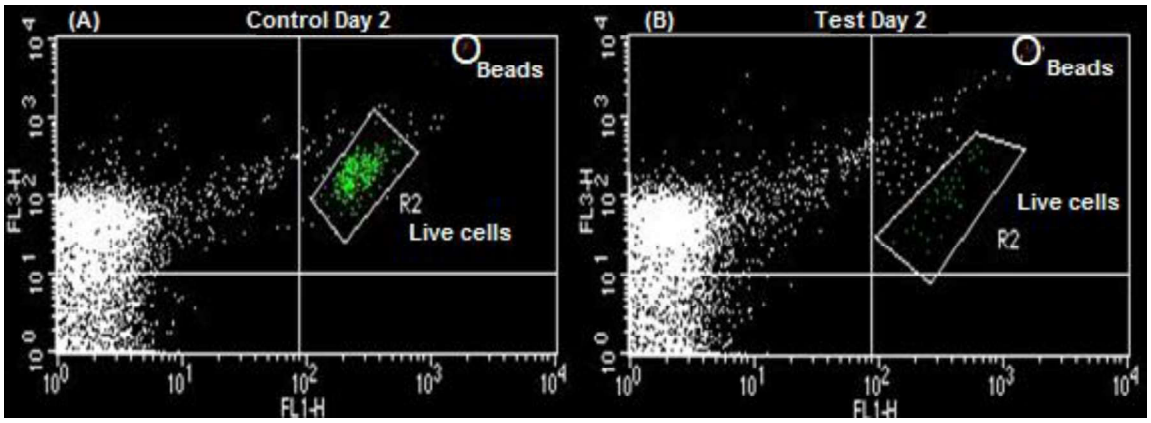


**Figure 65 Flow cytometry dot plots with FL1 versus FL3 for *A. baumannii* comparing control cotton fabric against CuO impregnated fabric. These events shown in green are live cells (R2) and the events shown in red (top right) are fluorescent beads**

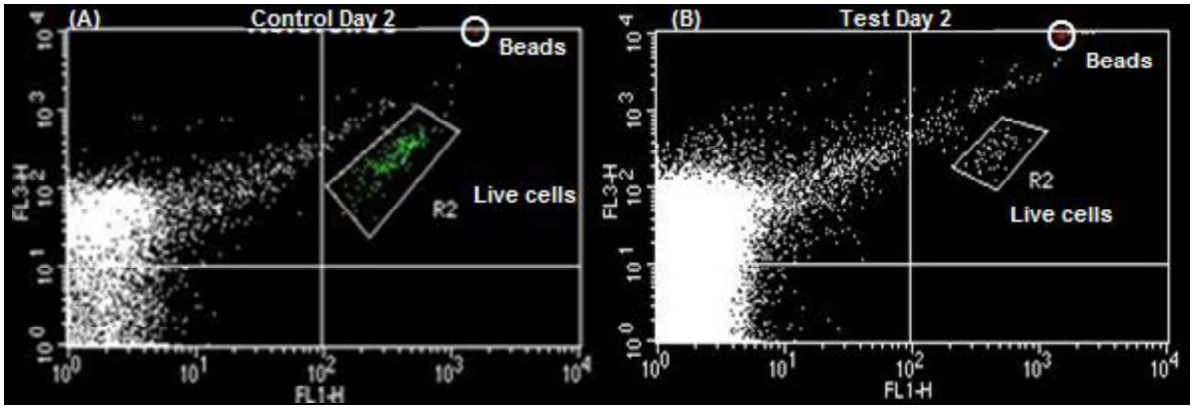


**Figure 66 Flow cytometry dot plots with FL1 versus FL3 for *S. aureus* comparing control cotton fabric against CuO impregnated fabric. These events shown in green are live cells (R2) and the events shown in red (top right) are fluorescent beads**

**Table 18 Mean viable bacterial counts (CFU/ml) by both FC and plate counting after 24 ± 3 hours contact time for CuO samples and control samples against *E. coli*, *A. baumannii* and *S. aureus* and their antibacterial A values**

| Test Method | Control fabric (24 hrs) CFU/ml | | CuO treated (24 hrs) CFU/ml | | Antibacterial value A | |
|---|---|---|---|---|---|---|
| | FC | ISO | FC | ISO | FC | ISO |
| ***E. coli*** | 5.21E+08 | 8.00E+08 | 2.98E+06 | 1.80E+04 | 2.26 | 4.64 |
| ***A. baumannii*** | 4.22E+08 | 8.30E+07 | 3.43E+05 | 6.10E+04 | 3.06 | 3.14 |
| ***S. aureus*** | 2.88E+07 | 1.07E+06 | 4.72E+06 | 5.90E+04 | 0.92 | 2.96 |

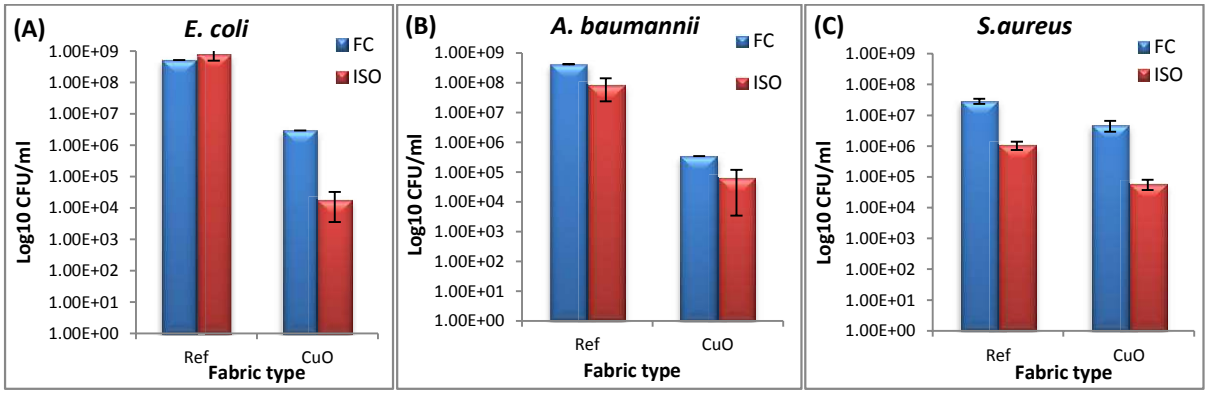


**Figure 67 Antibacterial activity of lab scale CuO fabrics assessed by viable agar plate counting and flow cytometry against (A) *E. coli*, (B) *A. baumannii* and (C) *S. aureus*. Mean ± SD, n = 3**

### 4.3.4 Comparing the enumeration of viable cells from 4 pilot scale CuO PEC fabrics by FC and agar plate counting

As seen from Table 19, all 4 of the CuO fabrics showed a strong growth inhibitory effect (3 log growth reduction) against MRSA. The highest activity of 4 log growth reduction was recorded for fabric E (*in-situ* by Davo) and the rest of the fabrics showed 3 log growth reduction. A good agreement between the FC and plate count results was observed for the control samples. The plate count result showed a

strong bactericidal activity with no viable MRSA recovered from the fabrics. However, there were viable cells recovered from fabric surfaces according to the flow cytometry analysis. These were viable but non culturable cells (VBNC), which were further confirmed by fluorescence microscopy images. The same samples were used for preparing slides for fluorescence microscopy (Section 3.19).

As explained previously, one of the limitations of flow cytometry is the limit of detection. In these FC experiments, the number of bacterial cells was counted per 150 beads. In the case of Fabric E, just 1 live stained cell of MRSA was recovered per 150 beads. After calculation and adding the dilution factor, the CFU/ml calculated for Fabric E was 3.10E+04 CFU/ml. In contrast, a single colony on an agar plate after applying the dilution factor would give a total number of 100 CFU/ml. This shows that presence of a single cell by FC could show a huge difference in bacterial numbers when compared to a full kill by the agar plate count method.

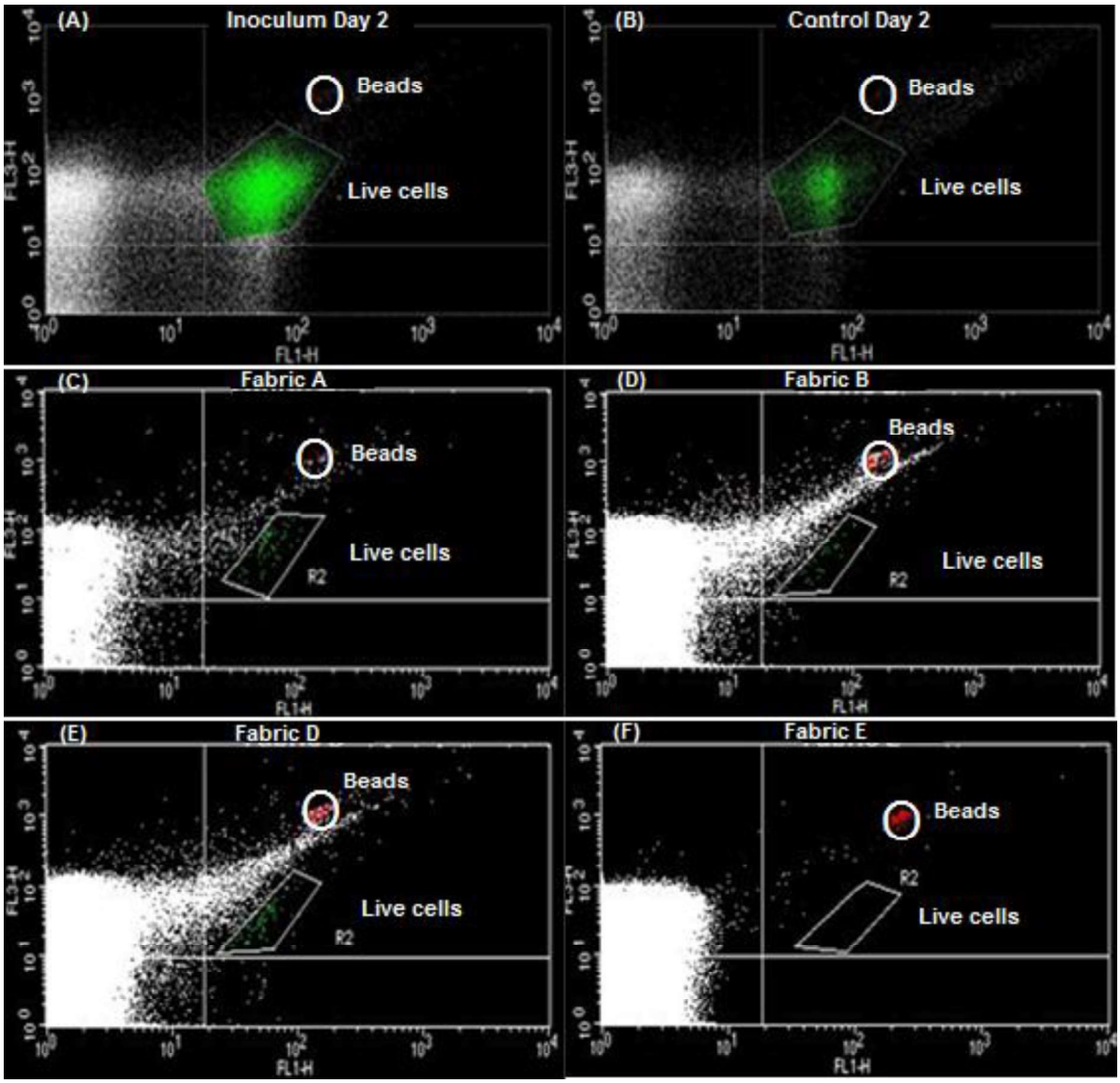


**Figure 68 Flow cytometry dot plots with FL1 versus FL3 for MRSA comparing control cotton fabric against CuO impregnated fabric. These events shown in green are live cells (R2) and the events shown in red (top right) are fluorescent counting beads. Fabrics A and B – Klopman TTS and *in-situ*, Fabrics D and E – Davo TTS and *in-situ***

**Table 19 Mean viable bacterial counts (CFU/ml) by both FC and plate counting after 24 ± 3 hours contact time for pilot scale CuO PEC samples and control samples against against MRSA**

| Test Method | Control fabric (24 h) | | CuO treated (24 h) | | Antibacterial A value | |
|---|---|---|---|---|---|---|
| | FC | ISO | FC | ISO | FC | ISO |
| **Fabric A (TTS)** | 1.79E+08 | 1.80E+08 | 3.62E+05 | 1.00E+02 | 2.70 | 6.25 |
| **Fabric B (*in-situ*)** | 1.79E+08 | 1.80E+08 | 3.10E+05 | 1.00E+02 | 2.78 | 6.25 |
| **Fabric D (TTS)** | 1.79E+08 | 1.80E+08 | 3.41E+05 | 1.00E+02 | 2.72 | 6.25 |
| **Fabric E (*in-situ*)** | 1.79E+08 | 1.80E+08 | 3.11E+04 | 1.00E+02 | 3.76 | 6.25 |

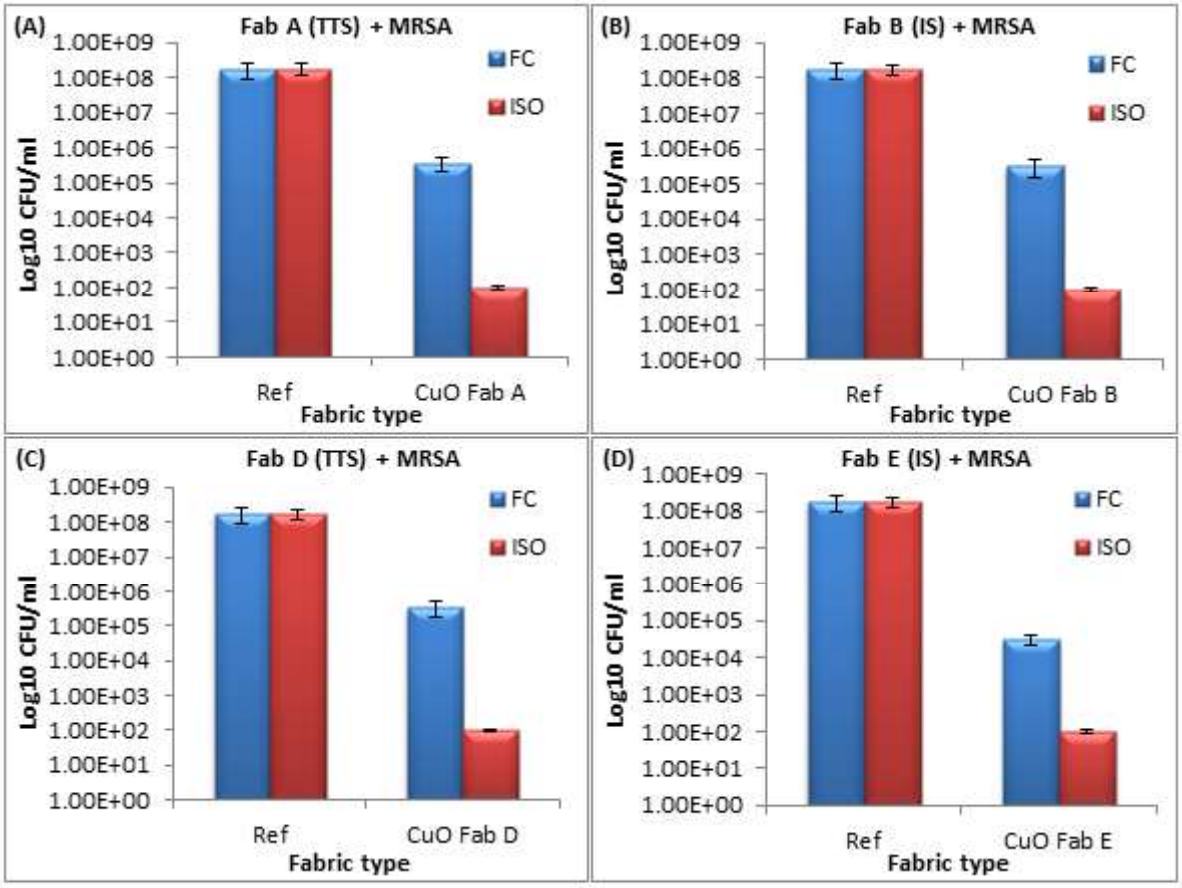


**Figure 69 Antibacterial activity of pilot scale CuO PEC fabrics (A, B, D & E) assessed with viable cell enumeration by agar plate counting and flow cytometry against MRSA. Fabrics A and B – Klopman TTS and *in-situ*, Fabrics D and E – Davo TTS and *in-situ*. Mean ± SD, n = 3**

These results were further confirmed by fluorescence microscopy, which is a qualitative method of assessing cell viability. The plate count results showed a full kill on CuO impregnated fabrics whereas the FC analysis indicated the presence of viable cells in the sample. Images from the fluorescent stained slides are shown in Figure 70. Cells fluorescing green are live and cells fluorescing red are dead cells. It was observed that there were green stained viable cells present in all the samples. This showed that there were still viable cells on fabric surfaces.

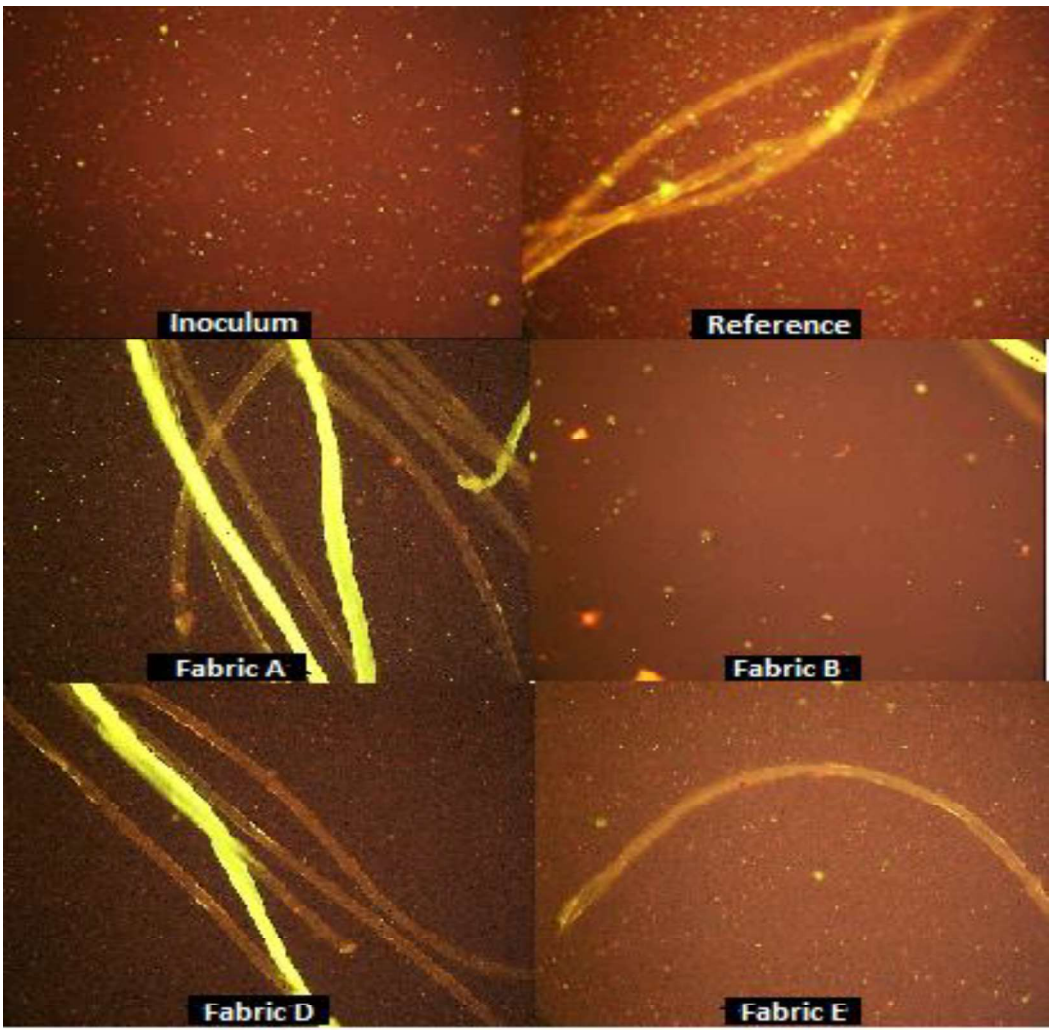


**Figure 70 Fluorescence photomicrographs showing green live and red dead MRSA cells in post incubation samples from the absorption testing at x200 magnification. Live cells stained with thiazol orange showed green fluorescence and dead cells stained with propidium iodide showed red fluorescence. Fabrics A and B – Klopman TTS and *in-situ*, Fabrics D and E – Davo TTS and *in-situ***

A similar activity was seen for all the fabrics against *P. aeruginosa.* As shown in Table 20, a good agreement between FC results and plate count results was seen with the control samples but not the impregnated samples. The FC results possibly indicated the presence of VBNC cells which were not revealed by traditional agar plate counts. Further analysis by fluorescence microscopy was performed to look for the presence of viable cells on fabric surfaces (Figure 73).

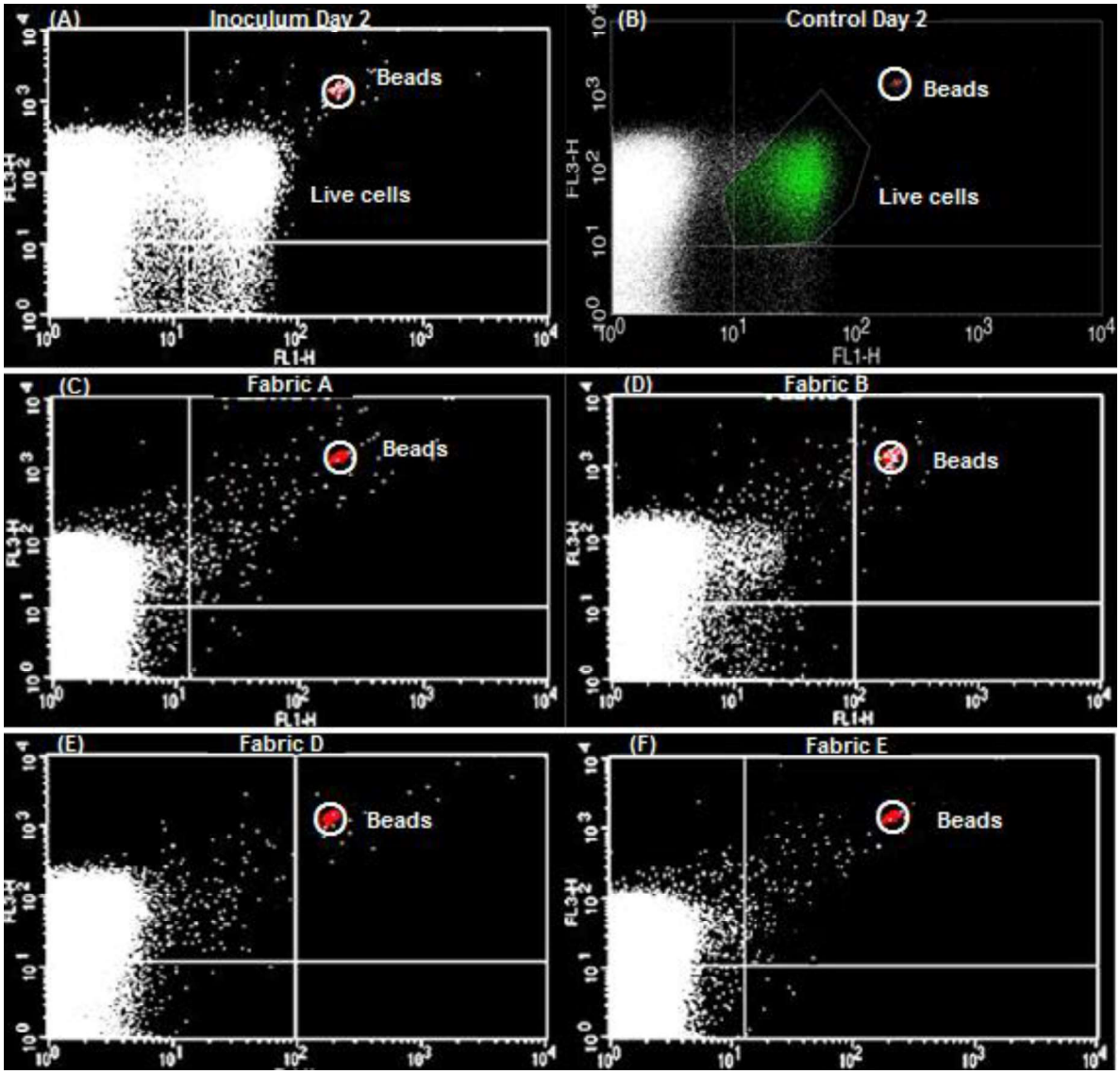


**Figure 71 FC dot plots with FL1 versus FL3 for *P. aeruginosa* comparing control cotton fabric against ZnO impregnated fabric. These events shown in green are live cells (R2) and the events shown in red (top right) are fluorescent counting beads. Fabrics A and B – Klopman TTS and *in-situ*, Fabrics D and E – Davo TTS and *in-situ***

**Table 20 Mean viable bacterial counts (CFU/ml) by both FC and plate counting after 24 ± 3 hours contact time for pilot scale CuO PEC samples and control samples against *P. aeruginosa***

| Test Method | Reference fabric (24 h) CFU/ml | | CuO treated (24 h) CFU/ml | | Antibacterial A value | |
|---|---|---|---|---|---|---|
| | FC | ISO | FC | ISO | FC | ISO |
| **Fabric A (TTS)** | 4.67E+08 | 5.30E+08 | 1.35E+05 | 1.00E+02 | 3.54 | 6.73 |
| **Fabric B (*in-situ*)** | 4.67E+08 | 5.30E+08 | 1.14E+05 | 1.00E+02 | 3.63 | 6.73 |
| **Fabric D (TTS)** | 4.67E+08 | 4.00E+08 | 1.24E+05 | 1.00E+02 | 3.60 | 6.60 |
| **Fabric E (*in-situ*)** | 4.67E+08 | 4.00E+08 | 3.10E+04 | 1.00E+02 | 4.18 | 6.60 |

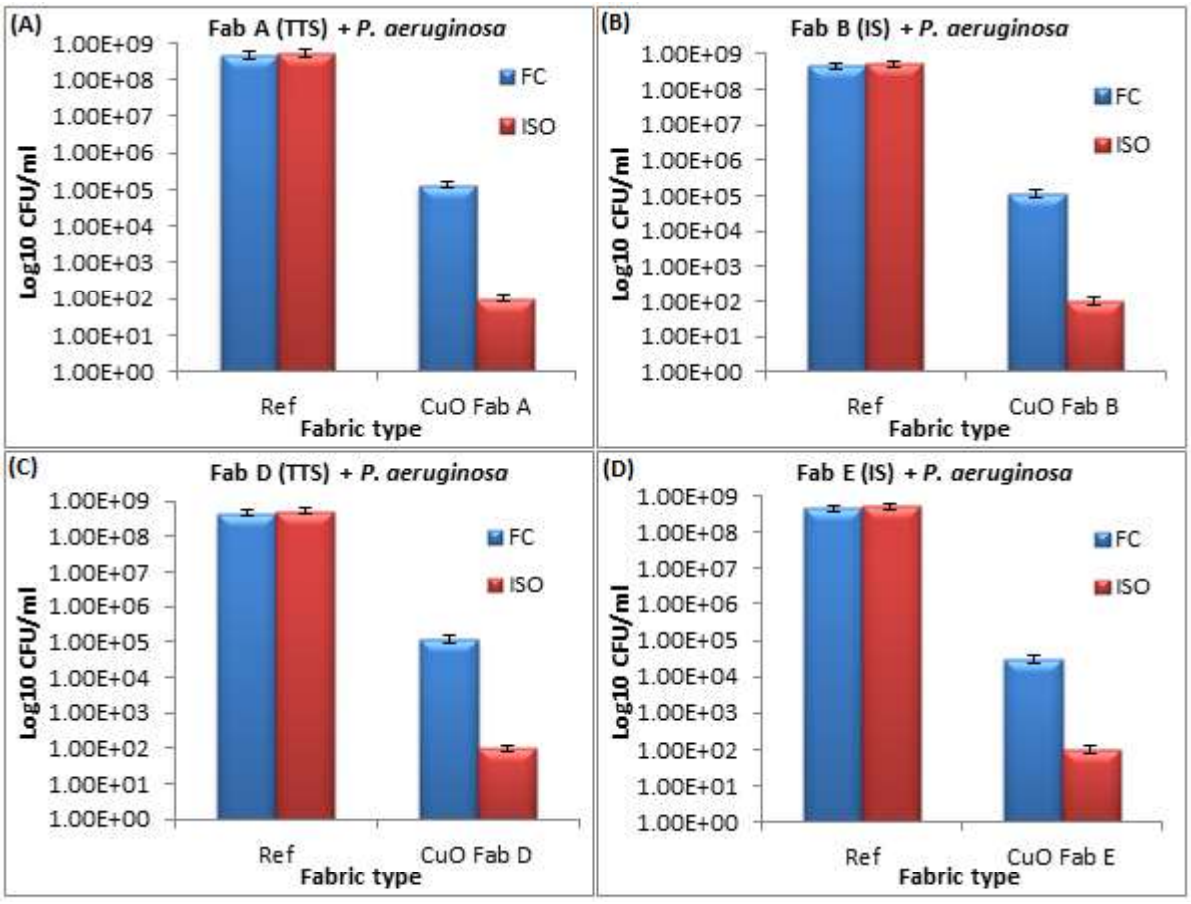


**Figure 72 Antibacterial activity of pilot scale CuO PEC fabrics assessed by agar plate counting and flow cytometry. Fabrics A and B – Klopman TTS and *in-situ*, Fabrics D and E – Davo TTS and *in-situ*. Mean ± SD, n=3**

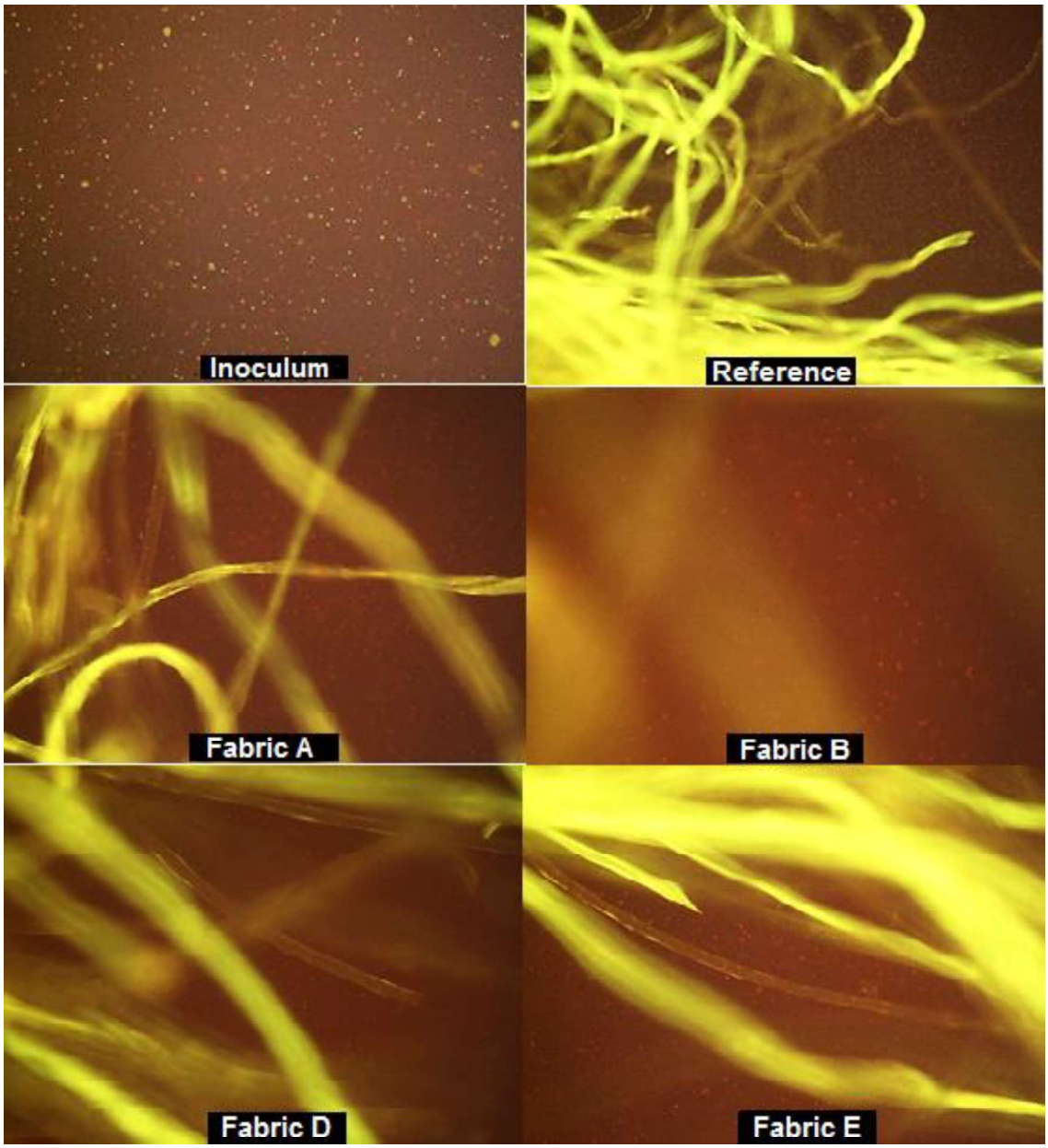


**Figure 73 Fluorescence photomicrographs showing bacterial viability against CuO impregnated fabrics against *P. aeruginosa* at x200 magnification. Live cells stained with thiazole orange showed green fluorescence and dead cells stained with propidium iodide showed red fluorescence. Fabrics A and B – Klopman TTS and *in-situ*, Fabrics D and E – Davo TTS and *in-situ***

As stated before, the antibacterial activity of textile fabrics by viable plate counting (Absorption 20743:2007 standard) was only based on the presence of bacterial colonies on the culture media and therefore could not detect VBNC bacteria

The VBNC state is a survival strategy used by many bacteria as a response to adverse environmental conditions (Fakruddin, Mannan and Andrews 2013). VBNC bacteria cannot be cultured on routine microbiological media, but remain viable and could also be in a virulent state. One of the objectives of using FC and fluorescence microscopy was to determine whether VBNC bacterial cells were present on the fabric surface. The number of VBNC bacteria may relate to the differences between the number of viable cells enumerated by agar plate counts and the number enumerated by FC analysis. In cases, where plate counting showed a full kill on the PEC CuO fabrics, the bacteria counted by FC were VBNC cells.

Similar results were reported by Chadeau *et al.* (2012). They compared the antibacterial activity of 35/65 PEC cotton coated with polyhexamethylene biguanide (PHMB) by both plate count method (ISO 20743:2005 standard) and a fluorescence based viability assay (Epifluorescence microscopy). The purpose of their study was to estimate whether VBNC bacterial cells were present after their exposure to PHMB. The epifluorescence enumeration showed a 4 log higher number of live *Listeria innocua* cells compared to the full kill by viable plate count.

The role of VBNC cells as a causative agent in in the spread of infection is still unclear, but various researchers have stressed the importance of detecting VBNC's (Fakruddin, Mannan and Andrews 2013, McDougald *et al.* 1998, Oliver 2010). During routine testing VBNC's are often not considered. The researchers claimed that ignoring the survival of VBNC cells can lead to an underestimation of the bacteria on antibacterial textiles (Chadeau *et al.* 2012). Our results suggest that flow cytometry and fluorescence microscopy could be used in conjunction with conventional plating methods to detect both viable and VBNC cell numbers.

## 4.4 Cytotoxicity studies on pilot scale ZnO and CuO fabrics

Human dermal fibroblast cells (HDF) and human hepatocellular carcinoma cells (HepG2) were exposed to pilot scale ZnO and CuO fabrics (both TTS and *in-situ*) by indirect contact for 1 day and 7 days to determine their potential toxicity. The cytotoxicity was determined using an MTT assay. This provides a measure of cell viability as only live cells have the ability to reduce MTT solution into a blue formazan product. As shown in Figure 74, the cultured fibroblast cells (HDF) remained metabolically active with both the ZnO and CuO fabrics. No significant difference in cell viability was observed after 1 day of exposure. The cell viability decreased to 95.2% when cells were exposed to ZnO TTS samples and 96.3% with ZnO *in-situ* samples. With CuO TTS and CuO *in-situ* samples, the viability decreased to 97.3% and 97.4%. ANOVA showed no significant differences between the cell viability in ZnO and CuO (TTS and *in-situ*) suspensions compared to control suspensions. Even after 7 days of contact with the fabrics cell viability did not drop significantly. There was less than a 9% decrease in cell viability for all the fabrics compared to the control sample. The results show that both ZnO and CuO did not induce toxicity in dermal fibroblast cells (HDF). When comparing ZnO and CuO fabrics, CuO fabrics showed lower cytotoxicity to HDF cells compared to ZnO fabrics.

These fabrics are intended for use as bed sheets, lab coats, patient gowns and linens in healthcare settings. The primary contact with fabrics would be skin only. As the NPs on the fabric surface are sonochemically impregnated (bound antibacterial), direct contact between nanoparticles and skin cells would be minimal. It would be the bodily fluids (blood, urine, sweat etc.) from the skin which would dissolve the NPs into metal complexes or ions. Many researchers have reported that the toxic action of metal and metal oxide NPs is mainly due to the dissolution of metal ions (Auffan *et*

*al.* 2009, Ma, Williams and Diamond 2013, Petkova *et al.* 2014). Our results have shown that the fabrics did not induce any toxicity to skin cells. Tamaekong *et al.* (2014) have reported that the risk of dermal sensitivity to CuO is extremely minimal. Sweigert *et al.* (2012) also reported that copper and zinc complexes did not induce any toxicity in human derma fibroblast cells. Petkova *et al.* (2014) used a similar indirect method to test the cytotoxicity of ZnO in fibroblast cells and found them to be non-toxic (87% viability after 7 days exposure).

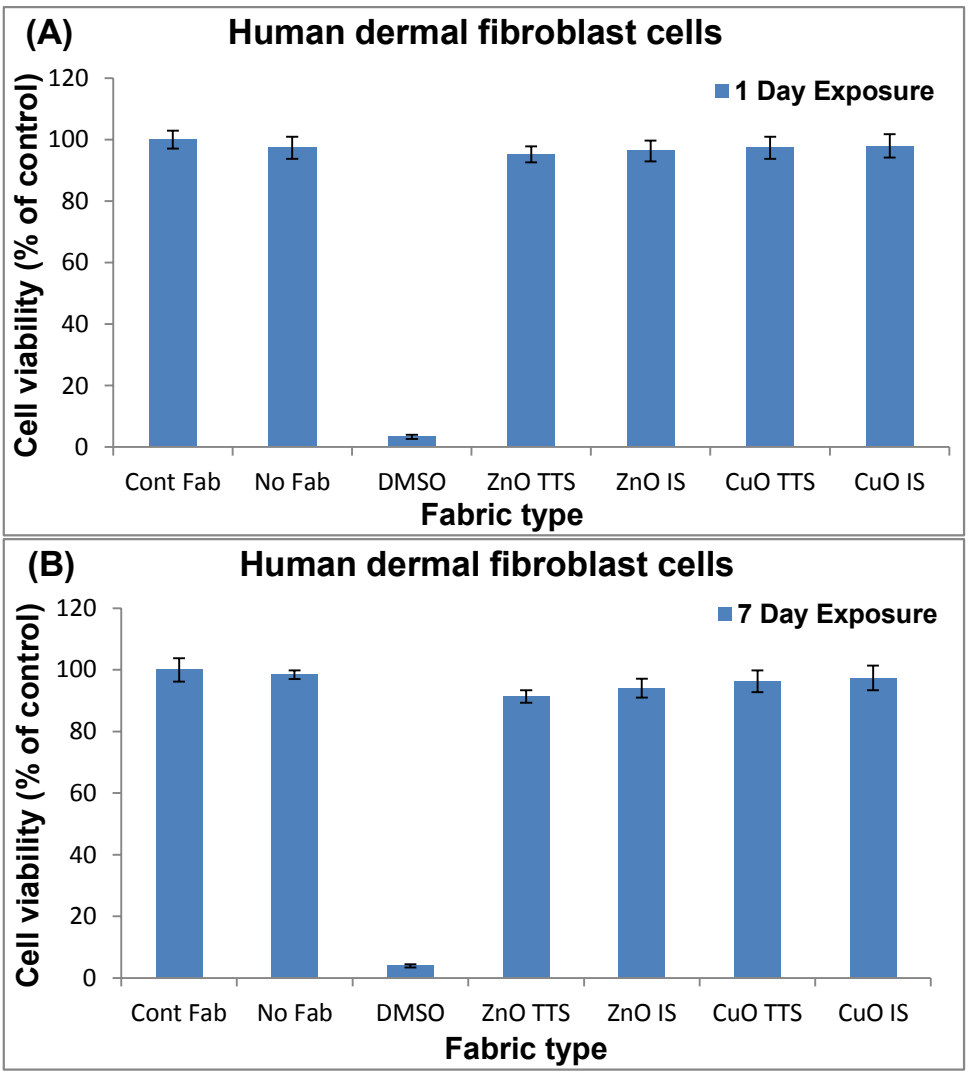


**Figure 74 Human dermal fibroblast cells viability after (A) 1 day and (B) 7 day exposure with the suspension from ZnO and CuO impregnated fabrics. ANOVA showed that the viable counts between control and test samples post incubation are not statistically different ($p$ >0.05). Mean ± SD, n = 3**

Figure 75 shows the cytotoxicity of ZnO and CuO fabrics to human hepatocellular carcinoma cells (HepG2). The cultured HepG2 cells showed some cytotoxicity with both ZnO and CuO fabrics. The cell viability decreased to 79.02% and 70.7%, when cells were exposed to the ZnO TTS and ZnO *in-situ* samples. With CuO TTS and CuO *in-situ* samples, the viability decreased to 80.7% and 75.7% after 24 hours. After 7 day exposure, the cell viability further decreased to 74% (ZnO TTS), 64% (ZnO *in-situ*), 78% (CuO TTS) and 72% (CuO *in-situ*).

Wang *et al.* (2011), in their study examined the cytotoxicity of ZnO, CuO $TiO_2$ and $Co_3O_4$ NPs to Channel catfish hepatocytes and human HepG2 cells. They reported that, HepG2 cells are very sensitive. The results showed that 25mg/l of CuO and ZnO NPs induced toxicity in HepG2 cells. The cell viability with both ZnO and CuO NPs was reduced to 85% after 48 hours of exposure.

Even though the ZnO and CuO fabrics induced toxicity in HepG2 cells, they may still be a safer alternative to toxic silver. A study by Faedmaleki *et al.* (2014) examined the toxicity of silver NPs in mice primary liver cells and in HepG2 cells. The results showed a higher toxicity of silver NPs to both mice liver cells and HepG2 cells. The cell viability dropped to less than 15%.

With the method used for this research, it was not clear whether the toxicity of ZnO and CuO fabrics to HepG2 cells was due to the NPs being taken up by the cells or due to the dissolution of NPs into the medium (Zn or Cu ions). Further testing would be required to determine the toxicity of free metal ions and NPs. Chusuei *et al.* (2013) investigated the physiochemical properties that govern the cytotoxicity of CuO, ZnO, NiO, $TiO_2$ nanoparticles in human bronchial epithelial cells (BEAS-2B) and human bronchoalveolar carcinoma-derived cells (A549). They determined the dissolution kinetics of metals from metal oxide NPs in the cell culture medium by

ICP-MS and GFAA analysis (Graphite furnace atomic absorption analysis) over a period of 24 hours. The results showed that the addition of NPs to the cell culture medium did not significantly alter the ph. The results also indicated that cytotoxicity is a function of metal ion dissolution from the NPs. The researchers have claimed that the release of $Cu^{2+}$ and $Zn^{2+}$ from their respective oxides is most likely to contribute to their toxicity.

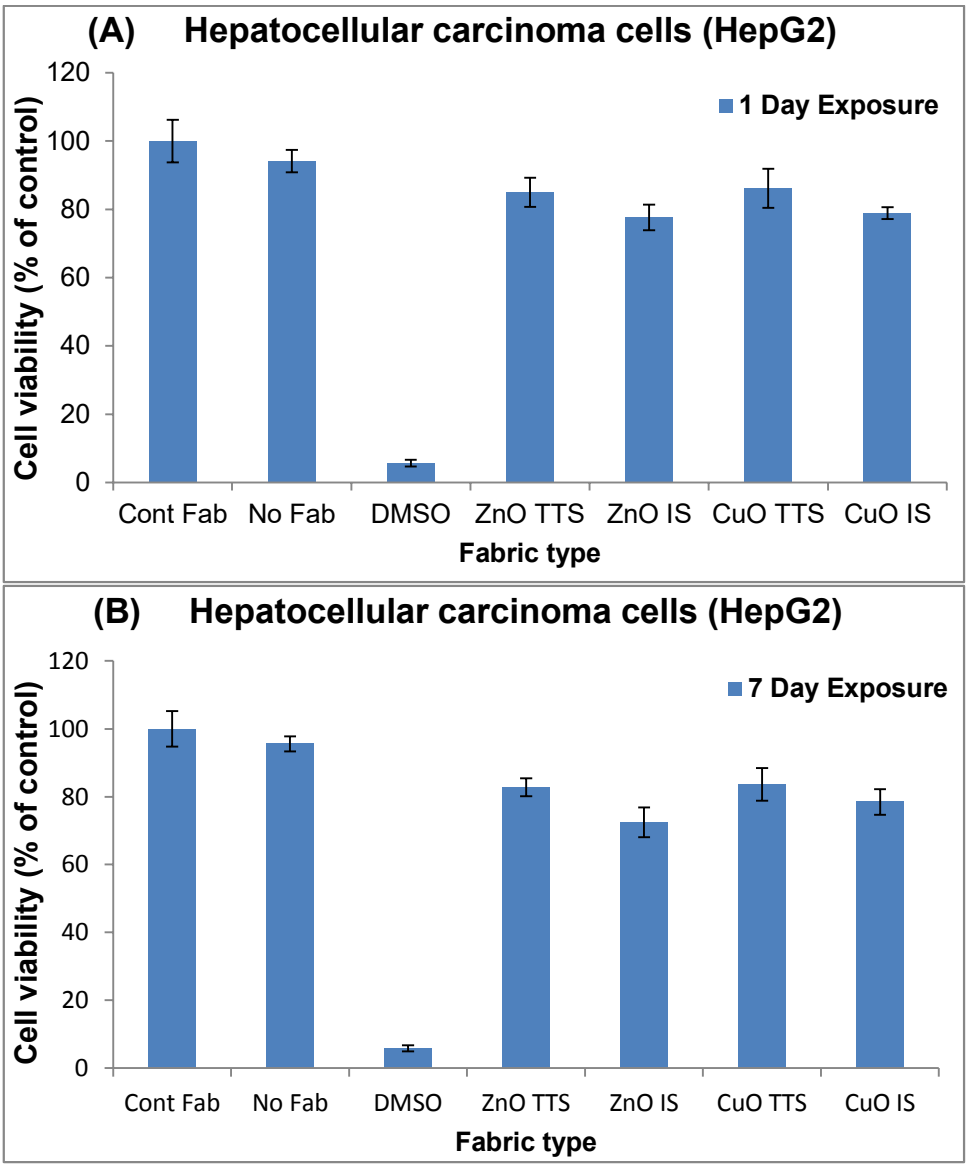


**Figure 75 Human hepatocellular carcinoma cells (HepG2) viability after (A) 1 day and (B) 7 day exposure with the suspension from ZnO and CuO impregnated fabrics Statistically significant differences in the viable counts between control and test samples post incubation ($p$ <0.05). Mean ± SD, n = 3**

However, as the SONO fabrics are only intended for use as wearable fabrics or for touch surfaces, they would only come in to contact with the skin. Vandebriel and De Jong (2012) reported that NPs do not efficiently cross the skin. The results presented here with the skin fibroblast cells show that both ZnO and CuO fabrics are not toxic. Hence, these fabrics could be used in the hospitals or health care settings to reduce cross contamination.

## 5 Conclusion

This study characterized the antibacterial properties of the fabric substrates synthesized and impregnated with metal oxide NPs from both lab scale and pilot scale machines. The research presented here is based on a part of the SONO EU FP7 project aimed at developing antibacterial fabrics for use in hospitals and healthcare settings to help prevent the spread of pathogenic microorganisms.

The principle method used for antibacterial efficacy testing was the absorption method taken from the ISO 20743 standard. This is an international standard method that provides a quantitative measure of the antibacterial activity. This is the most commonly used of the antibacterial efficacy tests for textiles because this method more closely mimics real time usage conditions (Swofford 2010). It compares the growth and survival of a bacterial inoculum on control and impregnated fabrics after 24 ± 3 hour incubation at 37ºC. The research effort was also extended to compare different methods of assessing the antibacterial activity of these textile fabrics. The other methods used for the antibacterial testing were a disc diffusion method and shake flask methods in either saline or nutrient broth.

The leaching of metal ions or NPs from the fabric substrates was also measured by ICP-OES. The aim was to investigate the role of leaching in the antibacterial activity of these fabrics.

A further aim of this project was to investigate the use of flow cytometry as an alternative to agar plate counts for the determination of viable cell numbers and also to detect viable but non culturable cells (VBNC). The basic absorption method was used to test the fabrics. The number of viable cells pre and post incubation was determined using both flow cytometry and agar plate counts.

Cytotoxicity studies were also undertaken to look at the effects of ZnO and CuO impregnated fabrics on human dermal fibroblast (HBN) and human hepatocellular carcinoma (HepG2) cell lines. An indirect contact method was used in which the fabrics were incubated with growth medium and then this growth medium was transferred to the test cell cultures. The cytotoxicity was studied over 1 and 7 days using a MTT assay to determine cell viability.

During the initial phase of the SONO project, ZnO (0.3% w/w) and CuO (0.5% w/w) impregnated cotton fabrics were prepared using a laboratory scale sonochemical device located at Bar-Ilan University (BIU), Israel. Using the absorption method, both fabric types exhibited good antibacterial activity against all the five bacteria tested. *P. aeruginosa* was the most susceptible strain with ZnO fabrics showing a full kill whereas *S. aureus* gave an antibacterial value (A) of 3.66 followed by *E. coli* 2.94, *A. baumannii* 2.73 and *K. pneumoniae* 2.41. In the case of CuO fabrics, *E. coli* was the most susceptible, with an antibacterial activity (A) of 4.64 followed by *P. aeruginosa* 3.84, *A. baumannii* 3.14, *S. aureus* 2.96 and *K. pneumoniae* 2.92.

Antibacterial A values from the absorption method showed a good level of antibacterial activity for the fabrics, however, in some cases the percentage reduction values were quite low. The criterion used with the absorption method for the classification of antibacterial activity is associated with bacterial growth reduction and hence does not distinguish between bacteriostatic and bactericidal activity. In the case of *E. coli,* the A value showed that ZnO fabrics possessed a significant antibacterial activity (A = 2.94). However, the percentage reduction was only 4.12%. These results suggest that percentage reductions should also be considered

alongside growth reduction and antibacterial A values when choosing appropriate applications for these fabrics.

To investigate wash durability, the antibacterial activity of lab scale fabrics was tested following washing at 92ºC for 10 wash cycles without detergent. The impregnated fabrics still exhibited some antibacterial activity after washing in some cases (A>1), but the level varied between the test bacterial species. The antibacterial activity of ZnO cotton fabrics against *E. coli* decreased from 2.94 to 1.14, and for *A. baumannii* it decreased from 2.73 to -0.18. For the CuO impregnated cotton, the antibacterial activity value against *E. coli* decreased from 4.64 to 0.73, and for *A. baumannii* it decreased from 2.96 to 1.67. Thus in each case washing was associated with a significant reduction in antibacterial activity. This decrease in activity was presumed to be due to the loss of nanoparticles from the fabrics upon washing.

In the later stages of the project, antibacterial testing was extended to fabrics prepared using the two pilot scale machines. Two sets of CuO polyester cotton mix fabrics were produced at pilot plants in Italy and Romania. The fabrics were impregnated using either the sonochemical method for generation and impregnation of nanoparticles from metal complexes (*in-situ* – as used at a laboratory scale), or by a 'throwing the stones' (TTS) technique. The TTS method employed ultrasound to impregnate the fabrics with commercial NPs from an aqueous suspension. CuO was preferred over ZnO for this phase of the project. This helped in the initial assessment of coating quality as the CuO coverage was visible. Absorption testing for antibacterial efficacy against MRSA and *P. aeruginosa* showed a complete bacterial kill on all 4 fabrics.

During the optimisation of lab scale process to the pilot scale, cotton fabrics were substituted with the 60/40% PEC mix. The antibacterial testing results showed an improved antibacterial activity for pilot scale PEC fabrics compared to lab scale cotton fabrics. Where, the lab scale CuO cotton fabrics showed a percentage reduction of 89.07% and 97.50% for *S. aureus* and *P. aeruginosa*, the pilot scale fabrics showed a complete kill on their surface giving a percentage reduction of > 99.9% for both MRSA and *P. aeruginosa*.

In this study, the disc diffusion method was used as a semi-quantitative method to provide a rapid assessment of antibacterial activity. The disc diffusion results showed that the activity of ZnO and CuO fabrics behaved in a similar way against individual species, but differed between species. The disc diffusion results showed that *S. aureus* was the most susceptible strain, with a zone of inhibition (ZOI) of 18.5 mm with the ZnO cotton and 18 mm with the CuO cotton. While *E. coli* gave a ZOI of 13.5 mm with the ZnO cotton and 14.5 mm with the CuO cotton. No ZOI was observed against *A. baumannii*. This is in contrast to the results from the absorption tests which showed significant antibacterial activity for both the ZnO and CuO against *A. baumannii*. Similar differences with disc diffusion testing were observed with the pilot scale CuO samples against MRSA and *P. aeruginosa*. A 20 – 28 mm ZOI was observed against the Gram positive MRSA, but no ZOI was observed for the Gram negative *P. aeruginosa*. Again the absorption testing showed that the CuO fabrics were highly effective against both species.

The disc diffusion method is only suitable for materials treated with antimicrobial agents that leach out of the material. In these tests the loss of CuO from the fabrics was observed as a loss in colour of samples during incubation on the agar plate surface (brown to white). In the shake flask tests with nutrient broth,

the CuO fabrics also lost their colour. At the same time, the nutrient broth solution changed colour from pale brown to khaki green. This would suggest that the CuO was dissolving to form green copper complexes rather than diffusing as intact NPs from the fabrics. Dissolved copper and zinc are known to be antibacterial. The change from solid metal oxide NPs to dissolved metal ions may explain the differences in activity observed between the disc diffusion tests and the absorption test results. For example, *A. baumannii* may be more susceptible to antibacterial effects of the nanoparticles than to dissolved copper or zinc.

Although the disc diffusion testing was much quicker and less labour intensive than the absorption method it did not give a similar indication of antibacterial activity. It was therefore concluded that the disc diffusion test would not be a suitable alternative method for quality control testing during fabric production on an industrial scale.

The shake flask tests in nutrient broth and saline were performed as an alternative to the disc diffusion method and to further elaborate on the results from the absorption technique. The shake flask test in nutrient broth showed similar results to those obtained using the disc diffusion method. The antibacterial activity with the ZnO and CuO cotton was greater against *S. aureus* and *E. coli* than it was against *A. baumannii*. ZnO fabrics reduced *S. aureus* growth by 99.87% and *E. coli* growth by 99.82%. *A. baumannii* was the least susceptible bacterial strain with ZnO coated fabric showing only a 56.43% reduction in bacterial growth after 24 ± 3 hours.

Absorbance measurements were used to estimate viable bacterial numbers for the shake flask tests in nutrient broth. Although the method was quite fast and simple, it was more susceptible to contamination due to the high levels of nutrients during incubation. In the case of CuO fabrics, leaching of copper into the nutrient

broth was observed through a change in the colour of the solution. The colour change affected the absorbance measurements of the medium resulting in false positive results. Both the disc diffusion method and the shake flask nutrient broth method assessed the antibacterial activity of the NPs under similar high nutrient conditions during incubation.

The shake flask test in saline was a more suitable method than the nutrient broth test as very little nutrient source was present during incubation. As the growth was much slower without added nutrients, agar plate counting had to be used instead of absorbance measurements. The results showed that both ZnO and CuO fabrics reduced viable cell numbers to undetectable levels for *E.coli*, *S. aureus* and *A. baumannii*. The CuO cotton fabrics exhibited excellent antibacterial activity by completely killing all the three bacteria within 1 hour of incubation. In the case of ZnO fabrics, activity was more effective against *E. coli* than *S. aureus* and *A. baumannii*. ZnO fabrics completely inhibited *E. coli* growth within 1 hour of its incubation in comparison to 3 hours for the other two bacteria.

For the pilot scale samples, the results from the shake flask method in saline showed that fabrics impregnated using the sonochemical technique (*in-situ*) displayed a greater antibacterial activity than the fabrics prepared by the TTS technique. The results from the shake flask tests in saline provided evidence for differences in the fabrics that were not apparent from the absorption test results. The absorption results showed that both TTS and *in-situ* fabrics showed a complete bacterial kill against MRSA and *P. aeruginosa*. However, the shake flask results in saline showed that the *in-situ* fabrics completely inhibited the growth of MRSA and *P. aeruginosa* within 3 hours contact time. Whereas, the TTS fabrics only reduced

growth of MRSA and *P. aeruginosa* to a maximum of 3 log reduction over 24 ± 3 hours incubation.

HR-SEM images also showed that the CuO NPs produced with the *in-situ* technology were well distributed on the surface of the textile fibres. The CuO NPs on the TTS fabric fibres were not so evenly distributed, with large clumps of NPs rather than a uniform covering. The antibacterial activity of NPs generally increases as the size decreases so this clumping may have reduced the activity of the CuO NPs on the TTS fabrics.

The shake flask method in saline is not widely used to assess antibacterial activity of fabrics. A small piece of fabric is immersed in a large amount of fluid, with samples being taken from the fluid and not from the fabric surface itself. However, our shake flask results matched very well with the results from the absorption tests. Shake flask method in saline could be a better suited method for hydrophobic fabrics such as polyester cotton, that display poor wetting of its surface with bacterial inoculum by absorption method.

The shake flask test is not recommended for antibacterial agents that diffuse out of textiles. Inductively coupled plasma optical emission spectrometry (ICP-OES) was used to assess the amount of zinc and copper released from the test fabrics after 3 hours shaking in saline solution at 37ºC. The initial concentration of ZnO and CuO on the fabric surface was 0.39% and 0.51% w/w of the fabric respectively. ICP analysis showed that just 13.2% of the total Zn and 2.5% of the total Cu was leached out from the impregnated fabrics. The ICP analysis did not differentiate between dissolved metals and NPs, because any free NPs present in the leachates would have been dissolved when the leachate was acidified for the measurement with the ICP.

A series of experiments were conducted to determine whether the solutions leached from the fabrics into saline showed any antibacterial properties. The saline leachate solutions were tested using the shake flask method. The fabric samples were removed after the initial 3 hour incubation; prior to adding bacteria. The results showed that the saline leachate from the ZnO cotton fabric was able to completely inhibit the bacterial growth for *S. aureus*, *E. coli* and *A. baumannnii* within 3 hours incubation. These results demonstrated that direct contact between the bacteria and the fabrics was not necessary for antibacterial activity. This experiment did not differentiate between antibacterial activity from free NPs, dissolved metal ions, or any other soluble antibacterial agent. The leachate solutions from the CuO cotton did not show any antibacterial properties.

A similar pattern of activity was observed when solutions of zinc and copper were prepared at the same concentration as the leachates from zinc chloride and copper sulphate and tested against *E. coli*. The Zn solution (13 mg/L) was found to be active by completely inhibiting *E. coli* growth within 3 hours of incubation, while the copper solution (2.5 mg/L) did not show any antibacterial activity even after 24 hours. Therefore the dissolved zinc could be responsible for the antibacterial activity associated with the leachates. It was not possible to test suspensions of intact NPs at these concentrations as the NPs were only available actually impregnated on the fabrics.

ICP analysis of saline leachate solutions from the 4 pilot scale CuO fabrics showed lower concentrations of copper even though the total concentrations on the PEC fabrics were similar to the lab scale cotton fabrics. The initial concentrations of CuO on the fabrics were: 0.91% w/w CuO for the Klopman TTS (Fabric A); 0.90% w/w CuO for the Klopman *in-situ* (Fabric B); 0.62% w/w CuO for the Davo TTS

(Fabric D) and 0.37% w/w CuO for the Davo *in-situ* fabric (Fabric E). ICP analysis showed the following percentages of copper leached in to the solution: 0.35% from Klopman TTS; 0.41% from Klopman *in-situ*; 0.21% from Davo TTS and 0.47% of Cu from Davo *in-situ*. Leachates from all 4 of the fabrics showed an antibacterial effect against MRSA and *P. aeruginosa* after 24 hours incubation. The antibacterial activity of the leachates from the *in-situ* fabrics was greater than that of the leachates from the TTS fabrics, completely inhibiting bacterial growth within 3 hours incubation.

In experiments with *E. coli*, the leachates from the lab scale CuO fabrics did not show any antibacterial activity. The copper concentrations in the leachates from the sonochemically produced *in-situ* pilot scale fabrics were much lower; however, they did show significant antibacterial activity. The leachates from the pilot scale *in-situ* fabrics reduced the number of viable *E. coli* to undetectable levels within 24 hours of incubation. There was very little activity with the leachates from the TTS fabrics even though the copper concentrations in the 4 leachates were very similar. The disc diffusion results also showed activity with the *in-situ* fabrics against MRSA, but very little with the TTS fabrics. The higher activity of *in-situ* fabrics may have been due to the smaller sized NPs on their surface which may have dissolved more readily and produced more copper ions compared to the TTS fabrics.

In a further experiment, saline leachates from the pilot scale fabrics were centrifuged to separate solid CuO from dissolved copper. ICP analysis of the copper concentration in the total leachate, the supernatant and the pellet showed that the TTS and *in-situ* CuO fabrics behaved differently in terms of the amount of dissolved copper and the amount of solid CuO release into the saline solution. In the case of the TTS fabrics, most of the copper in the leachates was pelleted and thus most likely in the form of CuO NPs (67% for Klopman A and 85% for Davo D). In the case

of the *in-situ* fabrics, most of the copper in the leachates was found dissolved in the supernatant fraction (77% for Klopman B and 94% for Davo E). This showed that the leachates from the *in-situ* fabrics contained a higher proportion of dissolved copper as compared to the CuO NPs. Though these results would seem to suggest that the dissolved copper may be important for antibacterial activity they contradict earlier results. The copper solution with 2.5 mg/L showed no activity against *E. coli*, whereas the leachate solutions with <1 mg/L copper were active. Therefore, there must be factors other than the amount of dissolved copper involved in the antibacterial activity of the CuO NPs. For example, the NPs produced *in-situ* may produce more reactive oxygen species than the TTS NPs. Further experiments could be completed to rule out the presence of other chemical contaminants introduced during the impregnation process at the pilot scale.

Flow cytometry (FC) was used in this study to determine whether it could be an alternative method to agar plate counts for the measurement of viable cells numbers with the absorption method. A series of experiments were performed to measure the sensitivity of the BD FACS system to detect viable bacterial cells. Dilutions of 3, 4, 5 and 6 fold of a 3 hour culture (~$10^8$ CFU/ml) of *S. aureus, E. coli, A. baumannii,* MRSA and *P. aeruginosa* were run on the BD FACS system. Samples from the same dilutions were also plated on nutrient agar for comparison. These experiments suggested that the limit of detection of the flow cytometer for viable bacteria was around 10,000 viable cells per ml. A linear relationship was seen in cell numbers when measured from 1,000,000 cells per ml down to around 10,000 cells per ml. Once the dilution reduced the number of cells to less than around 10,000 cells per ml, the FC counts started to deviate significantly from the counts predicted for linear relationship. At these concentrations the numbers of viable cells counted using the

FC system were very small and very hard to differentiate from measurement noise. The results showed that this technique is not suitable for measuring low bacterial concentrations (<10,000 CFU/ml) and so may underestimate or overestimate total cell numbers.

Viable counts with ZnO fabrics for *S. aureus* by FC and plate counting were quite similar for the reference samples where there were high numbers of viable cells ($2.9 \times 10^7$ for FC and $5.4 \times 10^7$ for plate counts). With the ZnO NP impregnated test samples, FC gave higher viable counts ($4.8 \times 10^6$ CFU/ml) than agar plating ($1.2 \times 10^4$ CFU/ml). Similar results were also seen for the pilot CuO fabrics, where viable cell counts by FC and plate counting were close to each other, but quite different with the CuO test fabrics. In the case of MRSA, when tested against all 4 CuO fabrics, similar FC and plate counts were observed for the reference fabrics ($1.8 \times 10^8$ CFU/ml). However, the viable counts for CuO fabrics varied significantly. Where the plating results showed a full kill on all the 4 fabrics, FC counts were between $3.1 \times 10^4$ and $3.6 \times 10^5$ CFU/ml.

Differences between the FC counts and plate counts were observed in particular with *S. aureus* and MRSA. This may have been due to the tendency of these bacteria to form aggregates or clumps. The brief sonication of samples in preparation for flow cytometry may have disaggregated some of these clumps resulting in higher counts than by agar plating. This was evident from the sample preparation experiments, where sonication for 2 minutes de-clumped *S. aureus* and MRSA cells giving viable counts of $2.6 \times 10^7$ CFU/ml and $1.2 \times 10^8$ CFU/ml compared to $2.1 \times 10^7$ CFU/ml and $8.9 \times 10^7$ CFU/ml for normal samples respectively.

Another reason for the variation in FC counts and plate count results could be the presence of viable but non-culturable cells (VBNCs). Even though the absorption test results for the pilot CuO fabrics showed a full kill against MRSA and *P. aeruginosa*, fluorescence micrographs showed that viable cells were still present. VBNC cells take up the viable cell stain (thiazole orange) but exclude the propidium iodide stain because their membrane is undamaged. Though they remain viable they no longer reproduce well under normal nutrient agar culture conditions. They retain the ability to transform into a metabolically active state and indicate that the treatment is not fully biocidal.

Overall the results presented here show that flow cytometry cannot be used solely as a method for measuring viable cell numbers with the absorption method mainly because of the poor limit of detection compared to the plate counting. FC analysis is faster and less labour intensive compared to plate counting and also potentially demonstrates the presence of VBNCs. Hence, this method could be used in conjunction with other quantitative methods as a confirmation test to assess the antibacterial activity.

Cytotoxicity studies have demonstrated that the ZnO and CuO fabrics did not induce apoptosis (> 95% cell viability for all fabrics) in human dermal fibroblast cells (HDFC). However, some cytotoxicity was observed in hepatocellular carcinoma cells (HepG2). All the fabric types induced toxicity by reducing HepG2 cells between the range of 71 - 81% after 24 hours. Compared to other antibacterial agents, ZnO and CuO have shown very little cytotoxicity to HepG2 cells (Bondarenko *et al.* 2013). Studies have shown that the NPs do not translocate though the skin barrier (Vandebriel and De Jong 2012). These fabrics are not intended for use as wound dressings but for use as bed sheets, curtains and lab coats to reduce the spread of

microbial contamination. Hence, the risk of NP mediated damage to human cells should be minimal and the fabrics could be used safely in healthcare settings to reduce cross contamination.

This study has shown that the sonochemical method of formation and impregnation of CuO and ZnO NPs onto cotton and polyester cotton fabric substrates is a suitable method for the production of antibacterial fabrics. The use of CuO and ZnO impregnated fabrics provide an alternative to Ag coated fabrics and could help to reduce micro-organisms involved in nosocomial infections. These fabrics could have a great societal effect on the mortality and morbidity by reducing the number of nosocomial infections resulting in reduced healthcare costs. As mentioned earlier; 65 percent of nosocomial infections are caused by biofilms (Percival and Bowler 2004). Effective infection prevention strategies, such as using sonochemical fabrics, could help prevent colonization with biofilms by inhibiting bacterial cell survival on the fabric surfaces. Developments in antimicrobial fabrics clearly hold promise in medical applications in the near future. However, their impact on the human health and environment needs to be well researched and established before they can gain commercial acceptance and wide spread use.

# 6 Future work

This study explored the antibacterial properties of sonochemically impregnated ZnO and CuO fabrics. However, there are still some areas that remain to be investigated. Experiments conducted for pilot scale fabrics concentrated on the CuO impregnated fabrics. Towards the end of the project, ZnO fabrics were also manufactured using the pilot machines. As differences were observed in the antibacterial activity of CuO fabrics from the lab scale to the pilot scale, it would be interesting to look at the antibacterial properties of industrially manufactured ZnO fabrics.

Within this work the wash durability of the pilot scale fabrics was not investigated. During hospital laundering, fabrics undergo rigorous washing cycles at high temperatures and using harsh detergents. In regards to the NP impregnated fabrics, this could result in washing of nanoparticles from the fabric surface. In order for antibacterial fabrics to be adopted on a wide scale in hospitals they need to retain their antibacterial efficacy after washing. Hence, it is necessary to establish the laundering durability of the pilot scale fabrics with detergents to evaluate the adherence of sonochemically impregnated NPs to the fabric surface.

The antibacterial efficacy testing was only done against bacterial species. However, the hospital environment is also under optimum conditions to support the growth of fungi. Some antibacterial testing with *C. albicans* was also done during this project at a partner institute (NIT) in Romania. Using the absorption method they found good activity for CuO fabrics against *C. albicans*. Further work could be expanded to test fabrics against different species of fungi as well such as Aspergillus spp., Mucorales and Fusarium spp using AATCC 30 methods for antifungal activity testing.

The growth of biofilms on surfaces in hospitals is one of the major obstacles in the treatment of nosocomial infections. A bacterial cell attaches to and colonizes a surface in the initial attachment step followed by proliferation, accumulation and maturation to form a biofilm. These fabrics could be tested against biofilm forming microbes such as *Candida albicans*, *S. aureus* and *S. epidermidis*. Pati *et al.* (2014) investigated the antibiofilm activity of ZnO NPs against *S. aureus*. They developed *S. aureus* biofilms on glass slides and transferred these into a 24 well plate to test against ZnO NPs. They found that ZnO NPs disrupt biofilm formation.

The aim for the SONO fabrics is for them to be used by humans occupationally or via consumer products and as such cytotoxic studies on these fabrics are important. Our results have demonstrated that the SONO fabrics are not cytotoxic to human dermal fibroblast cells. However, some toxicity has been observed in HepG2 liver cells. In the present research cytotoxicity experiments were performed using an indirect method, in which the fabrics were soaked in growth medium and then this medium was transferred to the cell cultures (Section 3.20.2). Using this method used it was not clear whether the toxicity of ZnO and CuO fabrics to HepG2 cells was due to the NPs being taken up by the cells or due to the dissolution of NPs into the medium (Zn or Cu ions). In further work the amount of zinc and copper released from the fabrics into the media could be measured using ICP analysis. This could be followed by incubation of the cells with growth media containing the same concentrations of zinc and copper. These tests would help to differentiate between cytotoxicity induced by the metal oxide NPs and the free metal ions (Chusuei *et al.* 2013).

From the leaching tests and ICP analysis, it was found that ZnO and CuO contents were leached into the saline solution. It was not clear whether it was the

release of metal ions or intact nanoparticles that was responsible for their antibacterial activity. Further experiments such as particle size analysis and scanning electron microscopy (SEM) may help to confirm whether nanoparticles are released from the fabrics.

This work has also shown that the activity of the ZnO and CuO NPs can vary depending on the type of method used for the antibacterial analysis. The methods involving high nutrient conditions (disc diffusion and shake flask in NB) tended to show lower levels of activity than the tests under low nutrient condition (absorption and shake flask in saline). The nutrient media tended to promote the dissolution of NPs in to metal ions. Consequently, in the tests with nutrient media the bacteria may have been exposed to dissolved metal ions rather than NPs. The organic components in the media may also complex with the free ions reducing their activity. Variation in the activity of the NPs under the different testing conditions may have implications for their activity in actual use. Further work could examine the effect of testing conditions on the dissolution of Cu and Zn from NPs and further investigate the antibacterial properties of dissolved copper and zinc in artificial wound serum.

**Project website:** http://www.fp7-sono.eu

# 7 References


Abramov, O. V., Gedanken, A., Koltypin, Y., Perkas, N., Perelshtein, I., Joyce, E., and Mason, T. J. (2009) 'Pilot Scale Sonochemical Coating of Nanoparticles Onto Textiles to Produce Biocidal Fabrics'. *Surface and Coatings Technology* 204 (5), 718-722

Adam, N., Leroux, F., Knapen, D., Bals, S., and Blust, R. (2014) 'The Uptake of ZnO and CuO Nanoparticles in the Water-Flea *Daphnia magna* Under Acute Exposure Scenarios'. *Environmental Pollution* 194 (1), 130-137

Adams, L. K., Lyon, D. Y., and Alvarez, P. J. J. (2006) 'Comparative Eco-Toxicity of Nanoscale $TiO_2$, $SiO_2$, and ZnO Water Suspensions'. *Water Research* 40 (19), 3527-3532

Adolfsson-Erici, M., Pettersson, M., Parkkonen, J., and Sturve, J. (2002) 'Triclosan, a Commonly used Bactericide found in Human Milk and in the Aquatic Environment in Sweden'. *Chemosphere* 46 (9), 1485-1489

Agarwala, M., Choudhury, B., and Yadav, R. (2014) 'Comparative Study of Antibiofilm Activity of Copper Oxide and Iron Oxide Nanoparticles Against Multidrug Resistant Biofilm Forming Uropathogens'. *Indian Journal of Microbiology* 54 (3), 365-368

Ahamed, M., Alhadlaq, H. A., Khan, M., Karuppiah, P., and Al-Dhabi, N. A. (2014) 'Synthesis, Characterization, and Antimicrobial Activity of Copper Oxide Nanoparticles'. *Journal of Nanomaterials*. [online] available from <http://www.hindawi.com/journals/jnm/2014/637858/> [03/09/2014]

Ahmed, S. (1998) 'Investigation of the Temperature Effects on Induced Impact Pressure and Cavitation Erosion'. *Wear* 218 (1), 119-127

Alarifi, S., Ali, D., Verma, A., Alakhtani, S., and Ali, B. A. (2013) 'Cytotoxicity and Genotoxicity of Copper Oxide Nanoparticles in Human Skin Keratinocytes Cells'. *International Journal of Toxicology* 32 (4), 296-307

Alsharif, R. and Godfrey, W. (2002) 'Bacterial Detection and live/dead Discrimination by Flow Cytometry'. *Microbial Cytometry Application Note.BD Biosciences, Immunocytometry Systems*. [online] available from <http://www.bdbiosciences.com/documents/Bacterial_Detection_Live_Dead.pdf> [06/08/2012]

Anita, S., Ramachandran, T., Rajendran, R., Koushik, C., and Mahalakshmi, M. (2011) 'A Study of the Antimicrobial Property of Encapsulated Copper Oxide Nanoparticles on Cotton Fabric'. *Textile Research Journal* 81 (10), 1081-1088

Anita, S., Ramachandran, T., Rajendran, R., Koushik, C. V., and Mahalakshmi, M. (2010) 'Preparation and Characterization of Zinc Oxide Nanoparticles and a Study of the Anti-Microbial Property of Cotton Fabric Treated with the Particles'. *Journal of Textile and Apparel, Technology and Management* 6 (4), 392-398

Applerot, G., Lipovsky, A., Dror, R., Perkas, N., Nitzan, Y., Lubart, R., and Gedanken, A. (2009) 'Enhanced Antibacterial Activity of Nanocrystalline ZnO due to Increased ROS-mediated Cell Injury'. *Advanced Functional Materials* 19 (6), 842-852

Applerot, G., Lellouche, J., Perkas, N., Nitzan, Y., Gedanken, A., and Banin, E. (2012) 'ZnO Nanoparticle-Coated Surfaces Inhibit Bacterial Biofilm Formation and Increase Antibiotic Susceptibility'. *Rsc Advances* 2 (6), 2314-2321

Askarinejad, A., Alavi, M. A., and Morsali, A. (2011) 'Sonochemically Assisted Synthesis of ZnO Nanoparticles: A Novel Direct Method'. *Iranian Journal Of Chemistry And Chemical Engineering (IJCCE)* 30 (3), 75-81

ASTM (2010) 'ASTM Designation: E 2149-01 Method'. *Standard Test Method for Determining the Antimicrobial Activity of Immobilized Antimicrobial Agents Under Dynamic Contact Conditions*, ASTM International Standard, West Conshohocken, PA, 2013, www.astm.org

Auffan, M., Rose, J., Bottero, J., Lowry, G. V., Jolivet, J., and Wiesner, M. R. (2009) 'Towards a Definition of Inorganic Nanoparticles from an Environmental, Health and Safety Perspective'. *Nature Nanotechnology* 4 (10), 634-641

Ayliffe, G. A., Lowbury, E. J. L., Geddes, A. M., and Williams, J. (1992) *Control of Hospital Infection: A Practical Handbook*, London: Chapman and Hall Medical, 1992 (3), 381-385

Azam, A., Ahmed, A. S., Oves, M., Khan, M. S., Habib, S. S., and Memic, A. (2012) 'Antimicrobial Activity of Metal Oxide Nanoparticles Against Gram-Positive and Gram-Negative Bacteria: A Comparative Study'. *International Journal of Nanomedicine*. [online] available from <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3519005/> [04/11/2014]

Bearman, G. M., Rosato, A., Elam, K., Sanogo, K., Stevens, M. P., Sessler, C. N., and Wenzel, R. P. (2012) 'A Crossover Trial of Antimicrobial Scrubs to Reduce Methicillin-Resistant *Staphylococcus aureus* Burden on Healthcare Worker Apparel'. *Infection Control and Hospital Epidemiology* 33 (3), 268-275

Benn, T. M. and Westerhoff, P. (2008) 'Nanoparticle Silver Released into Water from Commercially Available Sock Fabrics'. *Environmental Science & Technology* 42 (11), 4133-4139

Bischof Vukušić, S., Flinčec Grgac, S., Budimir, A., and Kalenić, S. (2011) 'Cotton Textiles Modified with Citric Acid as Efficient Antibacterial Agent for Prevention of Nosocomial Infections'. *Croatian Medical Journal* 52 (1), 68-75

Bjarnsholt, T. (2013) 'The Role of Bacterial Biofilms in Chronic Infections'. *APMIS* 121 (s136), 1-58

Bloomfield, S. F. (2007) 'Importance of Disinfection as a Means of Prevention in our Changing World Hygiene and the Home'. *GMS Krankenhaushygiene Interdisziplinar* 2 (1), Doc25. ISSN 1863-5245

Bondarenko, O., Juganson, K., Ivask, A., Kasemets, K., Mortimer, M., and Kahru, A. (2013) 'Toxicity of Ag, CuO and ZnO Nanoparticles to Selected Environmentally Relevant Test Organisms and Mammalian Cells *in-vitro*: A Critical Review'. *Archives of Toxicology* 87 (7), 1181-1200

Borkow, G., Okon-Levy, N., and Gabbay, J. (2010) 'Copper Oxide Impregnated Wound Dressing: Biocidal and Safety Studies'. *Wounds* 22 (12), 301-310

Borkow, G. and Gabbay, J. (2009) 'Copper, an Ancient Remedy Returning to Fight Microbial, Fungal and Viral Infections'. *Current Chemical Biology* 3 (3), 272-278

Borkow, G., Sidwell, R. W., Smee, D. F., Barnard, D. L., Morrey, J. D., Lara-Villegas, H. H., Shemer-Avni, Y., and Gabbay, J. (2007) 'Neutralizing Viruses in Suspensions by Copper Oxide-Based Filters'. *Antimicrobial Agents and Chemotherapy* 51 (7), 2605-2607

Borkow, G. and Gabbay, J. (2005) 'Copper as a Biocidal Tool'. *Current Medicinal Chemistry* 12 (18), 2163-2175

Borkow, G. and Gabbay, J. (2004) 'Putting Copper into Action: Copper-Impregnated Products with Potent Biocidal Activities'. *The FASEB Journal* 18 (14), 1728-1730

Borkow, G. and Gabbay, J. (2008) 'Biocidal Textiles can Help Fight Nosocomial Infections'. *Medical Hypotheses* 70 (5), 990-994

Brady, M. T., Evans, J., and Cuartas, J. (1990) 'Survival and Disinfection of Parainfluenza Viruses on Environmental Surfaces'. *American Journal of Infection Control* 18 (1), 18-23

Brayner, R., Ferrari-Iliou, R., Brivois, N., Djediat, S., Benedetti, M. F., and Fiévet, F. (2006) 'Toxicological Impact Studies Based on *Escherichia coli* Bacteria in Ultrafine ZnO Nanoparticles Colloidal Medium'. *Nano Letters* 6 (4), 866-870

Brunner, T. J., Wick, P., Manser, P., Spohn, P., Grass, R. N., Limbach, L. K., Bruinink, A., and Stark, W. J. (2006) '*In-Vitro* Cytotoxicity of Oxide Nanoparticles: Comparison to Asbestos, Silica, and the Effect of Particle Solubility'. *Environmental Science & Technology* 40 (14), 4374-4381

Brunon, C., Bessueille, F., Grossiord, C., Chadeau, E., Oulahal, N., Darroux, C., Ferreira, I., Bourgeois, M., Simon, F., and Rimbault, F. (2011) 'ToF-SIMS and XPS Characterization of Antimicrobial Textiles for the Food Processing Industry'. *Surface and Interface Analysis* 43 (1-2), 604-608

Bryers, J. D. (2008) 'Medical Biofilms'. *Biotechnology and Bioengineering* 100 (1), 1-18

Budama, L., Çakır, B. A., Topel, Ö., and Hoda, N. (2013) 'A New Strategy for Producing Antibacterial Textile Surfaces using Silver Nanoparticles'. *Chemical Engineering Journal* 228 (1), 489-495

Budimir, A., Vukusic, S. B., and Flincec, S. G. (2012) 'Study of Antimicrobial Properties of Cotton Medical Textiles Treated with Citric Acid and dried/cured by Microwaves'. *Cellulose* 19 (1), 289-296

Bureau-Chalot, F., Piednoir, E., Camus, J., and Bajolet, O. (2004) 'Microbiologic Quality of Linen and Linen Rooms in Short-Term Care Units [4]'. *Journal of Hospital Infection* 56 (4), 329-331

Burke, J. P. (2003) 'Infection Control-a Problem for Patient Safety'. *New England Journal of Medicine* 348 (7), 651-656

Cantinieaux, B., Courtoy, P., and Fondu, P. (1993) 'Accurate Flow Cytometric Measurement of Bacteria Concentrations'. *Pathobiology* 61 (2), 95-97

Chadeau, E., Brunon, C., Degraeve, P., Léonard, D., Grossiord, C., Bessueille, F., Cottaz, A., Renaud, F., Ferreira, I., and Darroux, C. (2012) 'Evaluation of Antimicrobial Activity of a Polyhexamethylene Biguanide Coated Textile by Monitoring both Bacterial Growth (ISO 20743/2005 Standard) AND Viability (Live/Dead Baclight Kit)'. *Journal of Food Safety* 32 (2), 141-151

Chadeau, E., Oulahal, N., Dubost, L., Favergeon, F., and Degraeve, P. (2010) 'Anti- *Listeria innocua* Activity of Silver Functionalised Textile Prepared with Plasma Technology'. *Food Control* 21 (4), 505-512

Chang, Y., Zhang, M., Xia, L., Zhang, J., and Xing, G. (2012) 'The Toxic Effects and Mechanisms of CuO and ZnO Nanoparticles'. *Materials* 5 (12), 2850-2871

Chen, X. and Schluesener, H. (2008) 'Nanosilver: A Nanoproduct in Medical Application'. *Toxicology Letters* 176 (1), 1-12

Chen, K., Zhou, X., and Wang, X. (2014) 'Synthesis and Application of a Hyperbranched Polyester Quaternary Ammonium Surfactant'. *Journal of Surfactants and Detergents* 17 (6), 1081-1088

Chohan, Z. H., Supuran, C. T., and Scozzafava, A. (2004) 'Metalloantibiotics: Synthesis and Antibacterial Activity of Cobalt (II), Copper (II), Nickel (II) and Zinc (II) Complexes of Kefzol'. *Journal of Enzyme Inhibition and Medicinal Chemistry* 19 (1), 79-84

Chusuei, C. C., Wu, C., Mallavarapu, S., Hou, F. Y. S., Hsu, C., Winiarz, J. G., Aronstam, R. S., and Huang, Y. (2013) 'Cytotoxicity in the Age of Nano: The Role of Fourth Period Transition Metal Oxide Nanoparticle Physicochemical Properties'. *Chemico-Biological Interactions* 206 (2), 319-326

Cireli, A., Mutlu, M., Kutlu, B., Onar, N., Kayatekin, I., and Celik, E. (eds.) (2006) *Proceedings of the 3rd European Conference on Protective Clothing and Nokobetef*. 'Sol-Gel Coatings of Plasma Modified Polypropylene Fabric for Gas Defence'. held 10-12 May at Poland

Cohen, M. L. (1992) 'Epidemiology of Drug Resistance: Implications for a Post-Antimicrobial Era'. *Science* 257 (5073), 1050-1055

Collins, A. S. (2008) Preventing Health Care-Associated Infections. *Patient Safety and Quality: An Evidence-Based Handbook for Nurses*. *Rockville (MD): Agency for Healthcare Research and Quality* (US), 8th Edition, 547-575

Coman, D., Oancea, S., and Vrînceanu, N. (2010) 'Biofunctionalization of Textile Materials by Antimicrobial Treatments: A Critical Overview'. *Romanian Biotechnological Letters* 15 (1), 4913-4921

Conly, J. M. and Johnston, B. L. (2005) 'Where are all the New Antibiotics? the New Antibiotic Paradox'. *Canadian Journal of Infectious Diseases and Medical Microbiology* 16 (3), 159-160

Cotter, R. (2006) Activity 5: Gram Staining. [online] available from <http://www.pc.maricopa.edu/Biology/rcotter/BIO%20205%20Labs/205%20Lab%20Lessonbuilders/Lab5/Lab5_print.html> [10/01/2013]

Courvalin, P. (2006) 'Vancomycin Resistance in Gram-Positive Cocci'. *Clinical Infectious Diseases* 42 (Supplement 1), S25-S34

Cross, J., Currier, R., Torraco, D., Vanderberg, L., Wagner, G., and Gladen, P. (2003) 'Killing of Bacillus Spores by Aqueous Dissolved Oxygen, Ascorbic Acid, and Copper Ions'. *Applied and Environmental Microbiology* 69 (4), 2245-2252

Daoud, W. A. and Xin, J. H. (2004) 'Low Temperature Sol-Gel Processed Photocatalytic Titania Coating'. *Journal of Sol-Gel Science and Technology* 29 (1), 25-29

Dastjerdi, R., Mojtahedi, M., Shoshtari, A., and Khosroshahi, A. (2010) 'Investigating the Production and Properties of Ag/$TiO_2$/PP Antibacterial Nanocomposite Filament Yarns'. *The Journal of the Textile Institute* 101 (3), 204-213

Davey, H. M., Kell, D. B., Weichart, D. H., and Kaprelyants, A. S. (2004) 'Estimation of Microbial Viability using Flow Cytometry' *Current Protocols in Cytometry / Editorial Board, J.Paul Robinson, Managing Editor.* Chapter 11, Unit 3, 1-11

Davey, H. M. and Kell, D. B. (1996) 'Flow Cytometry and Cell Sorting of Heterogeneous Microbial Populations: The Importance of Single-Cell Analyses'. *Microbiological Reviews* 60 (4), 641-696

Dechsakulthorn, F., Hayes, A., Bakand, S., Joeng, L., and Winder, C. (2007) '*In-Vitro* Cytotoxicity Assessment of Selected Nanoparticles using Human Skin Fibroblasts'. *AATEX* 14 (Special Issue), 397-400

Dellit, T. H., Owens, R. C., McGowan Jr., J. E., Gerding, D. N., Weinstein, R. A., Burke, J. P., Huskins, W. C., Paterson, D. L., Fishman, N. O., Carpenter, C. F., Brennan, P. J., Billeter, M., and Hooton, T. M. (2007) 'Infectious Diseases Society of America and the Society for Healthcare Epidemiology of America Guidelines for Developing an Institutional Program to Enhance Antimicrobial Stewardship'. *Clinical Infectious Diseases* 44 (2), 159-177

Demezas, D. H. (1999) *Microbial Growth Control.* [online] available from <http://microbiology.okstate.edu/faculty/demed2/Notes/Chapter%207docs.html> [01/11/2013

Denis, M. (1991) 'Human Neutrophils, Activated with Cytokines or Not, do Not Kill Virulent *Mycobacterium tuberculosis*'. *Journal of Infectious Diseases* 163 (4), 919-920

Denyer, S. P. and Stewart, G. S. A. B. (1998) 'Mechanisms of Action of Disinfectants'. *International Biodeterioration & Biodegradation* 41 (3–4), 261-268

Deurenberg, R., Vink, C., Kalenic, S., Friedrich, A., Bruggeman, C., and Stobberingh, E. (2007) 'The Molecular Evolution of methicillin-resistant *Staphylococcus aureus*'. *Clinical Microbiology and Infection* 13 (3), 222-235

Diekema, D. J., Pfaller, M. A., and Jones, R. N. (2002) 'Age-Related Trends in Pathogen Frequency and Antimicrobial Susceptibility of Bloodstream Isolates in North America: SENTRY Antimicrobial Surveillance Program, 1997–2000'. *International Journal of Antimicrobial Agents* 20 (6), 412-418

Din, E. (2012) '6330: 2010-01;"Textiles-Domestic Washing and Drying Procedures for Textile Testing (ISO 6330-2012)"; DIN Deutsehes Institut Fuer Narmung', *Berlin*, 2012 (1), 1-40

Donlan, R. M. and Costerton, J. W. (2002) 'Biofilms: Survival Mechanisms of Clinically Relevant Microorganisms'. *Clinical Microbiology Reviews* 15 (2), 167-193

Donlan, R. M. (2002) 'Biofilms: Microbial Life on Surfaces'. *Emerging Infectious Diseases* 8 (9), 881-890

Donnenberg, A. D. and Donnenberg, V. S. (2007) 'Rare-Event Analysis in Flow Cytometry'. *Clinics in Laboratory Medicine* 27 (3), 627-652

Duffy, J., Harris, J., Newhouse, E., Balajee, A., Neal, C., Noble-Wang, J., Sehulster, L., Gilbert, S., Howard, M., O'Connell, H., Stanley, E., Rao, C., and Chiller, T. (eds.) (2010) *Fifth Decennial International Conference on Healthcare-Associated Infections*. 'Zygomycosis Outbreak Associated with Hospital Linens'. held 18-22 March at Atlanta, USA

Durán, N., Marcato, P. D., De Souza, G. I., Alves, O. L., and Esposito, E. (2007) 'Antibacterial Effect of Silver Nanoparticles Produced by Fungal Process on Textile Fabrics and their Effluent Treatment'. *Journal of Biomedical Nanotechnology* 3 (2), 203-208

Dutta, R., Nenavathu, B. P., Gangishetty, M. K., and Reddy, A. (2012) 'Studies on Antibacterial Activity of ZnO Nanoparticles by ROS Induced Lipid Peroxidation'. *Colloids and Surfaces B: Biointerfaces* 94 (1), 143-150

Edmond, M. B., Wallace, S. E., McClish, D. K., Pfaller, M. A., Jones, R. N., and Wenzel, R. P. (1999) 'Nosocomial Bloodstream Infections in United States Hospitals: A Three-Year Analysis'. *Clinical Infectious Diseases* 29 (2), 239-244

El Solh, A. A. and Alhajhusain, A. (2009) 'Update on the Treatment of *Pseudomonas aeruginosa* Pneumonia'. *Journal of Antimicrobial Chemotherapy* 64 (2), 229-238

El-Nahhal, I. M., Zourab, S. M., Kodeh, F. S., Selmane, M., Genois, I., and Babonneau, F. (2012) 'Nanostructured Copper Oxide-Cotton Fibers: Synthesis, Characterization, and Applications'. *International Nano Letters* 2 (1), 1-5

El-Tahlawy, K. F., El-bendary, M. A., Elhendawy, A.G., and Hudson, S. M. (2005) 'The Antimicrobial Activity of Cotton Fabrics Treated with Different Crosslinking Agents and Chitosan'. *Carbohydrate Polymers* 60 (4), 421-430

Faedmaleki, F., Shirazi, F. H., Salarian, A., Ashtiani, H. A., and Rastegar, H. (2014) 'Toxicity Effect of Silver Nanoparticles on Mice Liver Primary Cell Culture and HepG2 Cell Line'. *Iranian Journal of Pharmaceutical Research: IJPR* 13 (1), 235 -242

Fakruddin, M., Mannan, K. S. B., and Andrews, S. (2013) 'Viable but Non-culturable Bacteria: Food Safety and Public Health Perspective'. *ISRN Microbiology* 2013. [online] available from <http://www.hindawi.com/journals/isrn/2013/703813/> [11/12/2014]

Fei, Y., Chen, Y., Wang, H., Gao, W., Yang, R., and Wan, Y. (2011) 'Preparation, Characterization of Antibacterial PLA/TP Nanofibers'. *Fibers and Polymers* 12 (3), 340-344

Fijan, S. and Turk, S. Š. (2012) 'Hospital Textiles, are they a Possible Vehicle for Healthcare-Associated Infections'. *International Journal of Environmental Research and Public Health* 9 (9), 3330-3343

Fijan, S., Steyer, A., Poljšak-Prijatelj, M., Cencič, A., Šostar-Turk, S., and Koren, S. (2008) 'Rotaviral RNA found on various Surfaces in a Hospital Laundry'. *Journal of Virological Methods* 148 (1), 66-73

Fijan, S., Koren, S., Cencič, A., and Šostar-Turk, S. (2007) 'Antimicrobial Disinfection Effect of a Laundering Procedure for Hospital Textiles Against various Indicator Bacteria and Fungi using Different Substrates for Simulating Human Excrements'. *Diagnostic Microbiology and Infectious Disease* 57 (3), 251-257

Fix, D. F. (2013) *Normal Flora*. [online] available from <http://www.cehs.siu.edu/fix/medmicro/normal.htm>[10/12/2013

Fouda, M. M. G., Abdel-Halim, E. S., and Al-Deyab, S. S. (2013) 'Antibacterial Modification of Cotton using Nanotechnology'. *Carbohydrate Polymers* 92 (2), 943-954

Fox, A. (2010) *Bacteriology-Chapter 5 Antibiotics that Affect the Cell Envelope.* [online] available from <http://pathmicro.med.sc.edu/fox/antibiotics1.htm> [03/04/2013]

Gabbay, J., Borkow, G., Mishal, J., Magen, E., Zatcoff, R., and Shemer-Avni, Y. (2006) 'Copper Oxide Impregnated Textiles with Potent Biocidal Activities'. *Journal of Industrial Textiles* 35 (4), 323-335

Gao, P. X. and Wang, Z. L. (2004) 'Nanopropeller Arrays of Zinc Oxide'. *Applied Physics Letters* 84 (15), 2883-2885

Gao, T., Li, Q., and Wang, T. (2005) 'Sonochemical Synthesis, Optical Properties, and Electrical Properties of core/shell-Type ZnO nanorod/CdS Nanoparticle Composites'. *Chemistry of Materials* 17 (4), 887-892

Gao, Y. and Cranston, R. (2008) 'Recent Advances in Antimicrobial Treatments of Textiles'. *Textile Research Journal* 78 (1), 60-72

Gedanken, A., Nitzan, Y., Perelshtein, I., Perkas, N., and Applerot, G. (2009) . *Sonochemical Coating of Textiles with Metal Oxide Nanoparticles for Antimicrobial Fabrics*. EP 2294260 A1

Gedanken, A., Perelshtein, I., Ruderman, Y., Perkas, N., Tzanov, T., Beddow, J., Joyce, E., Mason, T., Molla, K., and Blaness, M. (2013) 'Chitosan and Chitosan-ZnO-Based Complex Nanoparticles: Formation, Characterization, and Antibacterial Activity'. *Journal of Materials Chemistry B* 1 (14), 1968-1976

Gedanken, A. (2004) 'Using Sonochemistry for the Fabrication of Nanomaterials'. *Ultrasonics Sonochemistry* 11 (2), 47-55

Geranio, L., Heuberger, M., and Nowack, B. (2009) 'The Behavior of Silver Nanotextiles during Washing'. *Environmental Science & Technology* 43 (21), 8113-8118

Gittard, S. D., Hojo, D., Hyde, G. K., Scarel, G., Narayan, R. J., and Parsons, G. N. (2010) 'Antifungal Textiles Formed using Silver Deposition in Supercritical Carbon-Di-Oxide'. *Journal of Materials Engineering and Performance* 19 (3), 368-373

Gliga, A. R., Skoglund, S., Wallinder, I. O., Fadeel, B., and Karlsson, H. L. (2014) 'Size-Dependent Cytotoxicity of Silver Nanoparticles in Human Lung Cells: The Role of Cellular Uptake, Agglomeration and Ag Release'. *Particle and Fibre Toxicology* 11 (11), 977-984

Godfrey, H., Godfrey, N., Godfrey, J., and Riley, D. (2001) 'A Randomized Clinical Trial on the Treatment of Oral Herpes with Topical Zinc oxide/glycine.'. *Alternative Therapies in Health and Medicine* 7 (3), 49-56

Gokarneshan, N., Gopalakrishnan, P., and Jeyanthi, B. (2012) 'Influence of Nanofinishes on the Antimicrobial Properties of Fabrics'. *ISRN Nanomaterials* 2012 (1), 836-848

Gopal Rao, G. (1998) 'Risk Factors for the Spread of Antibiotic-Resistant Bacteria'. *Drugs* 55 (3), 323-330

Gopinath, P., Gogoi, S. K., Chattopadhyay, A., and Ghosh, S. S. (2008) 'Implications of Silver Nanoparticle Induced Cell Apoptosis for *in-vitro* Gene Therapy'. *Nanotechnology* 19 (7), 104-110

Grass, G., Rensing, C., and Solioz, M. (2011) 'Metallic Copper as an Antimicrobial Surface'. *Applied and Environmental Microbiology* 77 (5), 1541-1547

Graves, J. (2014) ' A Grain of Salt: Metallic and Metallic Oxide Nanoparticles as the New Antimicrobials'. *JSM Nanotechnology & Nanomedicine*. [online] available from <http://www.jscimedcentral.com/Nanotechnology/nanotechnology-spid-joint-school-nanoscience-nanoengineering-1026.pdf> [06/11/2014]

Gunalan, S., Sivaraj, R., and Rajendran, V. (2012) 'Green Synthesized ZnO Nanoparticles Against Bacterial and Fungal Pathogens'. *Progress in Natural Science: Materials International* 22 (6), 693-700

Grygar, T., Salatova, Z., and Vorm, P. (2001) 'Miscibility of CuO, NiO, and ZnO in their Binary Mixtures and its Impact for Reprocessing Industrial Wastes'. *Ceramics* 45 (4), 121-127

Gupta, A., Matsui, K., Lo, J., and Silver, S. (1999) 'Molecular Basis for Resistance to Silver Cations in Salmonella'. *Nature Medicine* 5 (2), 183-188

Gutarowska, B., Machnowski, W., and Kowzowicz, Ł. (2013) 'Antimicrobial Activity of Textiles with Selected Dyes and Finishing Agents used in the Textile Industry'. *Fibers and Polymers* 14 (3), 415-422

Hall, T. J., Wren, M. W., Jeanes, A., and Gant, V. A. (2009) 'A Comparison of the Antibacterial Efficacy and Cytotoxicity to Cultured Human Skin Cells of 7 Commercial Hand Rubs and Xgel, a New Copper-Based Biocidal Hand Rub'. *American Journal of Infection Control* 37 (4), 322-326

Hambraeus, A. (1973) 'Transfer of *Staphylococcus aureus* Via Nurses' Uniforms'. *J Hyg (Lond)* 71 (4), 799-814

He, J., Kunitake, T., and Nakao, A. (2003) 'Facile in Situ Synthesis of Noble Metal Nanoparticles in Porous Cellulose Fibers'. *Chemistry of Materials* 15 (23), 4401-4406

Hebeish, A., El-Naggar, M. E., Fouda, M. M. G., Ramadan, M. A., Al-Deyab, S. S., and El-Rafie, M. H. (2011) 'Highly Effective Antibacterial Textiles Containing Green Synthesized Silver Nanoparticles'. *Carbohydrate Polymers* 86 (2), 936-940

Hegemann, D., Hossain, M. M., and Balazs, D. J. (2007) 'Nanostructured Plasma Coatings to Obtain Multifunctional Textile Surfaces'. *Progress in Organic Coatings* 58 (2), 237-240

Herrera, J. E., Kwak, J. H., Hu, J. Z., Wang, Y., and Peden, C. H. (2006) 'Synthesis of Nanodispersed Oxides of Vanadium, Titanium, Molybdenum, and Tungsten on Mesoporous Silica using Atomic Layer Deposition'. *Topics in Catalysis* 39 (3-4), 245-255

Highsmith, A. K. and Jarvis, W. R. (1985) '*Klebsiella pneumoniae*: Selected Virulence Factors that Contribute to Pathogenicity'. *Infection Control* 6 (2) 75-77

Hochmuth, P., Magnuson, J., and Owens, K. (2005) 'Survival of Vancomycin-Resistant *Enterrococcus faecium* on Acrylic Nails, Bed Linen, and Plastic Keyboard Covers'. *American Journal of Infection Control* 33 (5), e32

Hoefer, D. and Hammer, T. R. (2011) 'Antimicrobial Active Clothes Display no Adverse Effects on the Ecological Balance of the Healthy Human Skin Microflora'. *ISRN Dermatology* 2011 (1) Article ID 369603

Hoet, P. H., Brüske-Hohlfeld, I., and Salata, O. V. (2004) 'Nanoparticles - known and Unknown Health Risks'. *Journal of Nanobiotechnology* 2 (1), 12-18

Huang, R., Mehta, S., Weed, D., and Connie Savor Price, M. (2006) 'Methicillin-Resistant *Staphylococcus aureus* Survival on Hospital Fomites'. *Infection Control and Hospital Epidemiology* 27 (11), 1267-1269

Huang, Y., Lü, X., and Ma, J. (2014) 'Toxicity of Silver Nanoparticles to Human Dermal Fibroblasts on MicroRNA Level'. *Journal of Biomedical Nanotechnology* 10 (11), 3304-3317

Hussain, S., Hess, K., Gearhart, J., Geiss, K., and Schlager, J. (2005) *'In-vitro* Toxicity of Nanoparticles in BRL 3A Rat Liver Cells'. *Toxicology in Vitro* 19 (7), 975-984

Ibrahim, N., Refaie, R., and Ahmed, A. (2010) 'Novel Approach for Attaining Cotton Fabric with Multi-Functional Properties'. *Journal of Industrial Textiles* 40 (1), 65-83

Ibrahim, N. A., Hashem, M., El-Sayed, W. A., El-Husseiny, S., and El-Enany, E. (2010) 'Enhancing Antimicrobial Properties of Dyed and Finished Cotton/Polyester Fabrics'. *AATCC Review*, 56-64

Ilić, V., Šaponjić, Z., Vodnik, V., Potkonjak, B., Jovančić, P., Nedeljković, J., and Radetić, M. (2009) 'The Influence of Silver Content on Antimicrobial Activity and Color of Cotton Fabrics Functionalized with Ag Nanoparticles'. *Carbohydrate Polymers* 78 (3), 564-569

Innovative Ultrasonics (2008) *Ultrasonics*. [online] available from <http://www.innovativeultrasonics.com/publications/Ultrasonic-Innovations-in-the-Food-Industry-From-the-Laboratory-to-Commercial-Production/> [07/02/2013]

Inweregbu, K., Dave, J., and Pittard, A. (2005) 'Nosocomial Infections'. *Continuing Education in Anaesthesia, Critical Care & Pain* 5 (1), 14-17

Ip, M., Lui, S. L., Poon, V. K., Lung, I., and Burd, A. (2006) 'Antimicrobial Activities of Silver Dressings: An *In-vitro* Comparison'. *Journal of Medical Microbiology* 55 (1), 59-63

ISO, (2007) 20743: 2007 *British Standards Institution*. [online] available from <http://www.iso.org/iso/catalogue/detail.htm/csnumber=34261/ > [08/07/2013]

Jacoby, G. A. and Archer, G. L. (1991) 'New Mechanisms of Bacterial Resistance to Antimicrobial Agents'. *N Engl J Med* 324 (9), 601-612

Jafari, A., Ghane, M., Sarabi, M., and Siyavoshifar, F. (2011) 'Synthesis and Antibacterial Properties of Zinc Oxide Combined with Copper Oxide Nanocrystals'. *Oriental Journal of Chemistry*. [online] available from <http://orientjchem.org/dnload/Alireza-Jafari-Masood-Ghane-Mehrdad-Sarabi-and-Farhod-Siyavoshifar4/OJCV027I03P811-822.pdf> [10/09/2013]

Jarvis, W. R., Munn, V. P., and Highsmith, A. K. (1985) 'The Epidemiology of Nosocomial Infections Caused by *Klebsiella pneumoniae*'. *Infection Control* 6 (2), 68-74

Jayaseelan, C., Rahuman, A. A., Kirthi, A. V., Marimuthu, S., Santhoshkumar, T., Bagavan, A., Gaurav, K., Karthik, L., and Rao, K. (2012) 'Novel Microbial Route to Synthesize ZnO Nanoparticles using *Aeromonas hydrophila* and their Activity Against Pathogenic Bacteria and Fungi'. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy* 90 (1), 78-84

Jeon, H., Yi, S., and Oh, S. (2003) 'Preparation and Antibacterial Effects of Ag-$SiO_2$ Thin Films by Sol-Gel Method'. *Biomaterials* 24 (27), 4921-4928

Jepras, R. I., Carter, J., Pearson, S. C., Paul, F. E., and Wilkinson, M. J. (1995) 'Development of a Robust Flow Cytometric Assay for Determining Numbers of Viable Bacteria'. *Applied and Environmental Microbiology* 61 (7), 2696-2701

Johnston, B. L. and Bryce, E. (2009) 'Hospital Infection Control Strategies for Vancomycin-Resistant Enterococcus, Methicillin-Resistant *Staphylococcus aureus* and *Clostridium difficile*'. *CMAJ* 180 (6), 627-631

Joe, Y. H., Ju, W., An, J. H., and Hwang, J. (2014) 'A Quantitative Determination of the Antibacterial Efficiency of Fibrous Air Filters Based on the Disc Diffusion Method'. *Aerosol and Air Quality Research* 14 (3), 928-933

Jones, N., Ray, B., Ranjit, K. T., and Manna, A. C. (2008) 'Antibacterial Activity of ZnO Nanoparticle Suspensions on a Broad Spectrum of Microorganisms'. *FEMS Microbiology Letters* 279 (1), 71-76

Jones, R. N., Kirby, J. T., Beach, M. L., Biedenbach, D. J., and Pfaller, M. A. (2002) 'Geographic Variations in Activity of Broad-Spectrum β-Lactams Against *Pseudomonas aeruginosa*: Summary of the Worldwide SENTRY Antimicrobial Surveillance Program (1997-2000)'. *Diagnostic Microbiology and Infectious Disease* 43 (3), 239-243

Joseph, R., Shelma, R., Rajeev, A., and Muraleedharan, C. (2009) 'Characterization of Surface Modified Polyester Fabric'. *Journal of Materials Science: Materials in Medicine* 20 (1), 153-159

Joshi, M., Ali, S. W., Purwar, R., and Rajendran, S. (2009) 'Ecofriendly Antimicrobial Finishing of Textiles using Bioactive Agents Based on Natural Products'. *Indian Journal of Fibre and Textile Research* 34 (3), 295-304

Joyce, E., Mason, T., Phull, S., and Lorimer, J. (2003) 'The Development and Evaluation of Electrolysis in Conjunction with Power Ultrasound for the Disinfection of Bacterial Suspensions'. *Ultrasonics Sonochemistry* 10 (4), 231-234

Kairyte, K., Kadys, A., and Luksiene, Z. (2013) 'Antibacterial and Antifungal Activity of Photoactivated ZnO Nanoparticles in Suspension'. *Journal of Photochemistry and Photobiology B: Biology* 128 (1), 78-84

Karlowsky, J. A., Draghi, D. C., Jones, M. E., Thornsberry, C., Friedland, I. R., and Sahm, D. F. (2003) 'Surveillance for Antimicrobial Susceptibility among Clinical Isolates of *Pseudomonas aeruginosa* and *Acinetobacter baumannii* from Hospitalized Patients in the United States, 1998 to 2001'. *Antimicrobial Agents and Chemotherapy* 47 (5), 1681-1688

Kathirvelu, S., D'Souza, L., and Dhurai, B. (2009) 'UV Protection Finishing of Textiles using ZnO Nanoparticles'. *Indian J Fibre Text Res* 34 (1), 267-273

Kathirvelu, S., D'souza, L., and Dhurai, B. (2008) 'A Comparative Study of Multifunctional Finishing of Cotton and P/C Blended Fabrics Treated with Titanium-di-oxide/Zinc Oxide Nanoparticles'. *Indian Journal of Science and Technology* 1 (7), 1-12

Kaur, P., Saxena, M., and Vadehra, D. V. (1985) 'Plasmid Mediated Resistance to Silver Ions in *Escherichia coli*.'. *The Indian Journal of Medical Research* 82 (3), 122-126

Kawashita, M., Toda, S., Kim, H., Kokubo, T., and Masuda, N. (2003) 'Preparation of Antibacterial Silver-Doped Silica Glass Microspheres'. *Journal of Biomedical Materials Research - Part A* 66 (2), 266-274

Kawashita, M., Tsuneyama, S., Miyaji, F., Kokubo, T., Kozuka, H., and Yamamoto, K. (2000) 'Antibacterial Silver Containing Silica Glass Prepared by Sol-Gel Method'. *Biomaterials* 21 (4), 393-398

Khan, F. H. (2013) 'Chemical Hazards of Nanoparticles to Human and Environment (A Review)'. Orient J Chem. [online] available from <http://www.orientjchem.org/vol29no4/chemical-hazards-of-nanoparticles-to-human-and-environment-a-review/> [10/10/2014]

Khan, M. M., Pyle, B. H., and Camper, A. K. (2010) 'Specific and Rapid Enumeration of Viable but Nonculturable and Viable-Culturable Gram-Negative Bacteria by using Flow Cytometry'. *Applied and Environmental Microbiology* 76 (15), 5088-5096

Khaydarov, R., Khaydarov, R., Evgrafova, S., and Estrin, Y. (2011) "Using Silver Nanoparticles as an Antimicrobial Agent" *In Biodefence.* ed. by Anon: Springer 2011 (1), 169-177

Kim, H. W., Kim, B. R., and Rhee, Y. H. (2010) 'Imparting Durable Antimicrobial Properties to Cotton Fabrics Using Alginate Quaternary Ammonium Complex Nanoparticles'. *Carbohydrate Polymers* 79 (4), 1057-1062

Kim, J. S., Kuk, E., Yu, K. N., Kim, J., Park, S. J., Lee, H. J., Kim, S. H., Park, Y. K., Park, Y. H., and Hwang, C. (2007) 'Antimicrobial Effects of Silver Nanoparticles'. *Nanomedicine: Nanotechnology, Biology and Medicine* 3 (1), 95-101

Kim, Y. H. and Sun, G. (2001) 'Durable Antimicrobial Finishing of Nylon Fabrics with Acid Dyes and a Quaternary Ammonium Salt'. *Textile Research Journal* 71 (4), 318-323

King, A. and Brown, D. F. (2001) 'Quality Assurance of Antimicrobial Susceptibility Testing by Disc Diffusion'. *Journal of Antimicrobial Chemotherapy* 48 (suppl 1), 71-76

Kocon, L., Despetis, F., and Phalippou, J. (1998) 'Ultralow Density Silica Aerogels by Alcohol Supercritical Drying'. *Journal of Non-Crystalline Solids* 225, 96-100

Köhler, T., Pechère, J., and Plésiat, P. (1999) 'Bacterial Antibiotic Efflux Systems of Medical Importance'. *Cellular and Molecular Life Sciences* 56 (9), 771-778

Kostić, M., Radić, N., Obradović, B. M., Dimitrijević, S., Kuraica, M. M., and Škundrić, P. (2008) 'Antimicrobial Textile Prepared by Silver Deposition on Dielectric Barrier Discharge Treated cotton/polyester Fabric'. *Chemical Industry and Chemical Engineering Quarterly* 14 (4), 219-221

Kramer, A., Schwebke, I., and Kampf, G. (2006) 'How Long do Nosocomial Pathogens Persist on Inanimate Surfaces? A Systematic Review'. *BMC Infectious Diseases* 6 (1), 130-134

Kruis, F. and Fissan, H. (2001) 'Nanoparticle Charging in a Twin Hewitt Charger'. *Journal of Nanoparticle Research* 3 (1), 39-50

Kulthong, K., Srisung, S., Boonpavanitchakul, K., Kangwansupamonkon, W., and Maniratanachote, R. (2010) 'Research Determination of Silver Nanoparticle Release from Antibacterial Fabrics into Artificial Sweat' 7 (1) 1743-1749

Kumar, R. V., Diamant, Y., and Gedanken, A. (2000) 'Sonochemical Synthesis and Characterization of Nanometer-Size Transition Metal Oxides from Metal Acetates'. *Chemistry of Materials* 12 (8), 2301-2305

Kunjara Na Ayudhya, S., Tonto, P., Mekasuwandumrong, O., Pavarajarn, V., and Praserthdam, P. (2006) 'Solvothermal Synthesis of ZnO with various Aspect Ratios using Organic Solvents'. *Crystal Growth & Design* 6 (11), 2446-2450

Ladhari, N., Baouab, M. H. V., Dekhil, A. B., Bakhrouf, A., and Niquette, P. (2007) 'Antibacterial Activity of Quaternary Ammonium Salt Grafted Cotton'. *Journal of the Textile Institute* 98 (3), 209-218

Lam, Y. L., Kan, C. W., and Yuen, C. W. M. (2012) 'Effect of Metal Oxide on Anti-Microbial Finishing of Cotton Fabric'. *BioResources* 7 (3), 3960-3983

LaMorte, W. W. (2012) *An Overview of Infectious Agents*. [online] available from <http://sph.bu.edu/otlt/MPHModules/PH/PH709_B_Competition/PH709_B_Competition_print.html> [12/01/2013]

Lara, H. H., Ayala-Núnez, N. V., Turrent, Liliana del Carmen Ixtepan, and Padilla, C. R. (2010) 'Bactericidal Effect of Silver Nanoparticles Against Multidrug-Resistant Bacteria'. *World Journal of Microbiology and Biotechnology* 26 (4), 615-621

Latlief, M., Goldsmith, M., Friedl, J., and Stuart, L. (1951) 'Bacteriostatic, Germicidal, and Sanitizing Action of Quaternary Ammonium Compounds on Textiles: Prevention of Ammonia Formation from Urea by *Proteus mirabilis*'. *The Journal of Pediatrics* 39 (6), 730-737

Lee, J., Kim, Y., Cho, M. H., and Lee, J. (2014) 'ZnO Nanoparticles Inhibit *Pseudomonas aeruginosa* Biofilm Formation and Virulence Factor Production'. *Microbiological Research* 169 (12), 888-896

Lee, H., Yeo, S., and Jeong, S. (2003) 'Antibacterial Effect of Nanosized Silver Colloidal Solution on Textile Fabrics'. *Journal of Materials Science* 38 (10), 2199-2204

Lee, H. J. and Jeong, S. H. (2004) 'Bacteriostasis of Nanosized Colloidal Silver on Polyester Nonwovens'. *Textile Research Journal* 74 (5), 442-447

Lee, K., Kim, S., and Yoo, K. (1995) 'Low-Density, Hydrophobic Aerogels'. *Journal of Non-Crystalline Solids* 186 (2), 18-22

Lestari, E. S., Severin, J. A., Filius, P. M. G., Kuntaman, K., Offra Duerink, D., Hadi, U., Wahjono, H., and Verbrugh, H. A. (2008) 'Comparison of the Accuracy of Disk Diffusion Zone Diameters obtained by Manual Zone Measurements to that by Automated Zone Measurements to Determine Antimicrobial Susceptibility'. *Journal of Microbiological Methods* 75 (2), 177-181

Levy, S. B. and Marshall, B. (2004) 'Antibacterial Resistance Worldwide: Causes, Challenges and Responses'. *Nature Medicine* 10 (3), S122-S129

Li, J., Nation, R. L., Milne, R. W., Turnidge, J. D., and Coulthard, K. (2005) 'Evaluation of Colistin as an Agent Against Multi-Resistant Gram-Negative Bacteria'. *International Journal of Antimicrobial Agents* 25 (1), 11-25

Li, J., Rayner, C. R., Nation, R. L., Owen, R. J., Spelman, D., Tan, K. E., and Liolios, L. (2006) 'Heteroresistance to Colistin in Multidrug-Resistant *Acinetobacter baumannii*'. *Antimicrobial Agents and Chemotherapy* 50 (9), 2946-2950

Lidwell, O., Towers, A., Ballard, J., and Gladstone, B. (1974) 'Transfer of Microorganisms between Nurses and Patients in a Clean Air Environment'. *Journal of Applied Microbiology* 37 (4), 649-656

Liljemark, N. T., Åsnes, H., and Kärrholm, M. (1971) 'The Sensitivity of Cotton Fabrics to Wrinkling during Changing Moisture Regain and its Dependence on Setting and Cross-Linking Parameters'. *Textile Research Journal* 41 (6), 526-533

Lim, S. and Hudson, S. M. (2004) 'Application of a Fiber-Reactive Chitosan Derivative to Cotton Fabric as an Antimicrobial Textile Finish'. *Carbohydrate Polymers* 56 (2), 227-234

Liqiang, J., Baiqi, W., Baifu, X., Shudan, L., Keying, S., Weimin, C., and Honggang, F. (2004) 'Investigations on the Surface Modification of ZnO Nanoparticle Photocatalyst by Depositing Pd'. *Journal of Solid State Chemistry* 177 (11), 4221-4227

Liu, C., Zapien, J. A., Yao, Y., Meng, X., Lee, C. S., Fan, S., Lifshitz, Y., and Lee, S. T. (2003) 'High-Density, Ordered Ultraviolet Light-Emitting ZnO Nanowire Arrays'. *Advanced Materials* 15 (10), 838-841

Liu, Y., Jian-er, Z., Larbot, A., and Persin, M. (2007) 'Preparation and Characterization of Nano-Zinc Oxide'. *Journal of Materials Processing Technology* 189 (1), 379-383

Liu, H., Du, Y., Wang, X., and Sun, L. (2004) 'Chitosan Kills Bacteria through Cell Membrane Damage'. *International Journal of Food Microbiology* 95 (2), 147-155

Lorimer, J. P. and Mason, T. J. (1987) 'Sonochemistry. Part 1 - the Physical Aspects'. *Chemical Society Reviews* 16 (3), 239-274

Ma, H., Williams, P. L., and Diamond, S. A. (2013) 'Ecotoxicity of Manufactured ZnO Nanoparticles - A Review'. *Environmental Pollution* 172 (1), 76-85

Macken, C. (2003) 'Bioactive Fibers - Benefits to Mankind'. *Chemical Fibers International* 53 (1), 39-41

Mahltig, B., Audenaert, F., and Böttcher, H. (2005) 'Hydrophobic Silica Sol Coatings on Textiles - the Influence of Solvent and Sol Concentration'. *Journal of Sol-Gel Science and Technology* 34 (2), 103-109

Mahltig, B., Haufe, H., and Böttcher, H. (2005) 'Functionalisation of Textiles by Inorganic sol–gel Coatings'. *Journal of Materials Chemistry* 15 (41), 4385-4398

Manchanda, V., Sanchaita, S., and Singh, N. (2010) 'Multidrug Resistant Acinetobacter'. *J.Glob.Infect.Dis.* 2 (1), 291-304

Mandal, A., Meda, V., Zhang, W. J., Farhan, K. M., and Gnanamani, A. (2012) 'Synthesis, Characterization and Comparison of Antimicrobial Activity of PEG/TritonX-100 Capped Silver Nanoparticles on Collagen Scaffold'. *Colloids and Surfaces B: Biointerfaces* 90 (1), 191-196

Mansfield, R. G. (2002) 'Keeping it Fresh'. *Textile World* 152 (2), 42-45

Mao, J. and Murphy, L. (2001) 'Appliedtechnology-Durable Freshness for Textiles-Durable Antimicrobial and Odor Control Efficacy were Achieved on Synthetic Fabrics by using

EPA Registered Antimicrobial Products in Conventional'. *AATCC Review-American Association of Textile Chemists and Colorists* 1 (11), 28-31

Mason, T. J. and Lorimer, J. P. (eds.) (2002) *Applied Sonochemistry the Uses of Power Ultrasound in Chemistry and Processing.*: Wiley-VCH Verlag, Weinheim, 2002, 303-306, ISBN 3-527-30205-0

Mason, T. J. (1999) *Sonochemistry*.: Oxford University Press New York 1999 (4), 205–284

Mason, T. and Peters, D. (2002) 'Practical Sonochemistry: Power Ultrasound Uses and Application'. *Horwood Publishing Limited, Chichester, UK* 2002 (3), 208-234

Mason, T. J. (2007) 'Developments in Ultrasound - Non-Medical'. *Progress in Biophysics and Molecular Biology* 93 (1-3), 166-175

McDougald, D., Rice, S. A., Weichart, D., and Kjelleberg, S. (1998) 'Nonculturability: Adaptation Or Debilitation'. *FEMS Microbiology Ecology* 25 (1), 1-9

McGowan Jr., J. E. (2001) 'Economic Impact of Antimicrobial Resistance'. *Emerging Infectious Diseases* 7 (2), 286-292

McManus, M. (1997) 'Mechanisms of Bacterial Resistance to Antimicrobial Agents'. *American Journal of Health-System Pharmacy* 54 (12), 1420-1433

Mirhosseini, M. and Firouzabadi, F. B. (2013) 'Antibacterial Activity of Zinc Oxide Nanoparticle Suspensions on food-borne Pathogens'. *International Journal of Dairy Technology* 66 (2), 291-295

Mitra, S., Patra, P., Chandra, S., Pramanik, P., and Goswami, A. (2012) 'Efficacy of Highly Water-Dispersed Fabricated Nano ZnO Against Clinically Isolated Bacterial Strains'. *Applied Nanoscience* 2 (3), 231-238

Moon, W. S., Kim, J. C., Chung, K. H., Park, E. S., Kim, M. N., and Yoon, J. S. (2003) 'Antimicrobial Activity of a Monomer and its Polymer Based on Quinolone'. *Journal of Applied Polymer Science* 90 (7), 1797-1801

Mosleh, S., Gawish, S. M., and Sun, Y. (2003) 'Characteristic Properties of Polypropylene Cationic Fabrics and their Derivatives'. *Journal of Applied Polymer Science* 89 (11), 2917-2922

Najim, N., Rusdi, R., Hamzah, A. S., Shaameri, Z., Mat Zain, M., and Kamarulzaman, N. (2014) 'Effects of the Absorption Behaviour of ZnO Nanoparticles on Cytotoxicity Measurements'. *Journal of Nanomaterials*. [online] available from <http://www.hindawi.com/journals/jnm/2014/694737/> [10/12/2014]

Nakashima, T., Sakagami, Y., Ito, H., and Matsuo, M. (2001) 'Antibacterial Activity of Cellulose Fabrics Modified with Metallic Salts'. *Textile Research Journal* 71 (8), 688-694

Namboodri, C. G. and Duke, M. W. (1979) 'Foam Finishing of Cotton-Containing Textiles'. *Textile Research Journal* 49 (3), 156-162

Nazari, A., Montazer, M., Moghadam, M. B., and Yazdanshenas, M. E. (2013) 'BTCA/Nano $TiO_2$ Synergism on Cotton: Enhanced Antibacterial Features Optimized by Statistical Models'. *Journal of Engineered Fabrics & Fibers (JEFF)* 8 (3), 51-56

Neely, A. N. and Orloff, M. M. (2001) 'Survival of some Medically Important Fungi on Hospital Fabrics and Plastics'. *Journal of Clinical Microbiology* 39 (9), 3360-3361

Neely, A. N. and Maley, M. P. (2000) 'Survival of Enterococci and Staphylococci on Hospital Fabrics and Plastic'. *Journal of Clinical Microbiology* 38 (2), 724-726

Oliver, J. D. (2010) 'Recent Findings on the Viable but Nonculturable State in Pathogenic Bacteria'. *FEMS Microbiology Reviews* 34 (4), 415-425

Orhan, M., Kut, D., and Gunesoglu, C. (2007) 'Use of Triclosan as Antibacterial Agent in Textiles'. *Indian J.Fibre Text.Res* 32 (5), 114-118

Orhan, M., Kut, D., and Gunesoglu, C. (2009) 'Improving the Antibacterial Activity of Cotton Fabrics Finished with Triclosan by the use of 1, 2, 3, 4-Butane Tetracarboxylic Acid and Citric Acid'. *Journal of Applied Polymer Science* 111 (3), 1344-1352

Orvos, D. R., Versteeg, D. J., Inauen, J., Capdevielle, M., Rothenstein, A., and Cunningham, V. (2002) 'Aquatic Toxicity of Triclosan'. *Environmental Toxicology and Chemistry* 21 (7), 1338-1349

Padmavathy, N. and Vijayaraghavan, R. (2008) 'Enhanced Bioactivity of ZnO nanoparticles - An Antimicrobial Study'. *Science and Technology of Advanced Materials*. [online] available from <http://iopscience.iop.org/1468-6996/9/3/035004> [12/03/2013]

Paniker, C. K. J. (ed.) (2005) *Textbook of Microbiology.* Seventh Edition edn. Chennai: Orient Longman Private Limited 2005 7th Edition, 7-48

Panyala, N. R., Peña-Méndez, E. M., and Havel, J. (2008) 'Silver Or Silver Nanoparticles: A Hazardous Threat to the Environment and Human Health'*. J Appl Biomed* 6 (3), 117-129

Parthasarathi, K. and Borkar, S. P. (2007) 'Antibacterial and UV Protection Finishes of Textiles by Metal and Metal Oxide Nano Particles - A Review'*. Colourage* 54 (7), 43-45

Pati, R., Mehta, R. K., Mohanty, S., Padhi, A., Sengupta, M., Vaseeharan, B., Goswami, C., and Sonawane, A. (2014) 'Topical Application of Zinc Oxide Nanoparticles Reduces Bacterial Skin Infection in Mice and Exhibits Antibacterial Activity by Inducing Oxidative Stress Response and Cell Membrane Disintegration in Macrophages'*. Nanomedicine: Nanotechnology, Biology and Medicine* 10 (1), 1195-1208

Peleg, A. Y., Seifert, H., and Paterson, D. L. (2008) '*Acinetobacter baumannii*: Emergence of a Successful Pathogen'*. Clinical Microbiology Reviews* 21 (3), 538-582

Percival, S. and Bowler, P. G. (2004) 'Biofilms and their Potential Role in Wound Healing'*. Wounds - A Compendium of Clinical Research and Practice* 16 (7), 234-240

Percival, S. L., Bowler, P. G., and Russell, D. (2005) 'Bacterial Resistance to Silver in Wound Care'*. Journal of Hospital Infection* 60 (1), 1-7

Perelshtein, I., Ruderman, Y., Perkas, N., Beddow, J., Singh, G., Vinatoru, M., Joyce, E., Mason, T. J., Blanes, M., and Mollá, K. (2013) 'The Sonochemical Coating of Cotton Withstands 65 Washing Cycles at Hospital Washing Standards and Retains its Antibacterial Properties'*. Cellulose* 20 (3), 1215-1221

Perelshtein, I., Ruderman, Y., Perkas, N., Traeger, K., Tzanov, T., Beddow, J., Joyce, E., Mason, T. J., Blanes, M., and Mollá, K. (2012) 'Enzymatic Pre-Treatment as a Means of Enhancing the Antibacterial Activity and Stability of ZnO Nanoparticles Sonochemically Coated on Cotton Fabrics'*. Journal of Materials Chemistry* 22 (21), 10736-10742

Perelshtein, I., Applerot, G., Perkas, N., Wehrschetz-Sigl, E., Hasmann, A., Guebitz, G. M., and Gedanken, A. (2009) 'Antibacterial Properties of an *in-situ* Generated and Simultaneously Deposited Nanocrystalline ZnO on Fabrics.'*. ACS Applied Materials & Interfaces* 1 (2), 361-366

Perelshtein, I., Applerot, G., Perkas, N., Wehrschuetz-Sigl, E., Hasmann, A., Guebitz, G., and Gedanken, A. (2009b) 'CuO Cotton Nanocomposite: Formation, Morphology, and Antibacterial Activity'. *Surface and Coatings Technology* 204 (1-2), 54-57

Perelshtein, I., Applerot, G., Perkas, N., Guibert, G., Mikhailov, S., and Gedanken, A. (2008) 'Sonochemical Coating of Silver Nanoparticles on Textile Fabrics (Nylon, Polyester and Cotton) and their Antibacterial Activity'. [online] available from <http://iopscience.iop.org/0957-4484/19/24/245705> [07/11/2013]

Perkas, N., Amirian, G., Dubinsky, S., Gazit, S., and Gedanken, A. (2007) 'Ultrasound Assisted Coating of Nylon 6,6 with Silver Nanoparticles and its Antibacterial Activity'. *Journal of Applied Polymer Science* 104 (3), 1423-1430

Perry, C., Marshall, R., and Jones, E. (2001) 'Bacterial Contamination of Uniforms'. *Journal of Hospital Infection* 48 (3), 238-241

Petkova, P., Francesko, A., Fernandes, M. M., Mendoza, E., Perelshtein, I., Gedanken, A., and Tzanov, T. (2014) 'Sonochemical Coating of Textiles with Hybrid ZnO/chitosan Antimicrobial Nanoparticles'. *ACS Applied Materials & Interfaces* 6 (2), 1164-1172

Pol, V. G., Gedanken, A., and Calderon-Moreno, J. (2003) 'Deposition of Gold Nanoparticles on Silica Spheres: A Sonochemical Approach'. *Chemistry of Materials* 15 (5), 1111-1118

Poxton, I. R. (1993) 'Prokaryote Envelope Diversity'. *Journal of Applied Bacteriology Symposium Supplement* (22), 1S-11S

Raghupathi, K. R., Koodali, R. T., and Manna, A. C. (2011) 'Size-Dependent Bacterial Growth Inhibition and Mechanism of Antibacterial Activity of Zinc Oxide Nanoparticles'. *Langmuir* 27 (7), 4020-4028

Rajendra, R., Balakumar, C., Ahammed, H. A. M., Jayakumar, S., Vaideki, K., and Rajesh, E. (2010) 'Use of Zinc Oxide Nano Particles for Production of Antimicrobial Textiles'. *International Journal of Engineering, Science and Technology* 2 (1), 202-208

Ramachandran, T., Rajendrakumar, K., and Rajendran, R. (2004) 'Antimicrobial Textiles - An Overview'. *Journal of the Institution of Engineers (India), Part TX: Textile Engineering Division* 84 (2), 42-47

Ravishankar Rai, V. and Jamuna Bai, A. (2011) 'Nanoparticles and their Potential Application as Antimicrobials'. *Science Against Microbial Pathogens: Communicating Current Research and Technological Advances, A.Méndez-Vilas* 2011 (1), 197-209

Reif, J. S., Wimmer, L., Smith, J. A., Dargatz, D. A., and Cheney, J. M. (1989) 'Human Cryptosporidiosis Associated with an Epizootic in Calves'. *American Journal of Public Health* 79 (11), 1528-1530

Ren, G., Hu, D., Cheng, E. W. C., Vargas-Reus, M. A., Reip, P., and Allaker, R. P. (2009) 'Characterisation of Copper Oxide Nanoparticles for Antimicrobial Applications'. *International Journal of Antimicrobial Agents* 33 (6), 587-590

Rio, L., Kusiak-Nejman, E., Kiwi, J., Betrisey, B., Pulgarin, C., Trampuz, A., and Bizzini, A. (2012) 'Comparison of Methods for Evaluation of the Bactericidal Activity of Copper-Sputtered Surfaces Against Methicillin-Resistant *Staphylococcus aureus*'. *Applied and Environmental Microbiology* 78 (23), 8176-8182

Ristić, T., Zemljič, L. F., Novak, M., Kunčič, M. K., Sonjak, S., Cimerman, G., and Strnad, S. (2011) 'Antimicrobial Efficiency of Functionalized Cellulose Fibers as Potential Medical Textiles'. *Health Care* 2011 (1), 36-51

Rosenman, K., Moss, A., and Kon, S. (1979) 'Argyria: Clinical Implications of Exposure to Silver Nitrate and Silver Oxide'. *Journal of Occupational and Environmental Medicine* 21 (6), 430-435

Russell, A. D. (1995) 'Mechanisms of Bacterial Resistance to Biocides'. *International Biodeterioration and Biodegradation* 36 (3-4), 247-265

Samal, S. S., Jeyaraman, P., and Vishwakarma, V. (2010) 'Sonochemical Coating of Ag-$TiO_2$ Nanoparticles on Textile Fabrics for Stain Repellency and Self-Cleaning-the Indian Scenario: A Review'. *J.Min.Mater.Char.Eng.* 9 (6), 519-525

Santo, C. E., Lam, E. W., Elowsky, C. G., Quaranta, D., Domaille, D. W., Chang, C. J., and Grass, G. (2011) 'Bacterial Killing by Dry Metallic Copper Surfaces'. *Applied and Environmental Microbiology* 77 (3), 794-802

Santo, C. E., Morais, P. V., and Grass, G. (2010) 'Isolation and Characterization of Bacteria Resistant to Metallic Copper Surfaces'. *Applied and Environmental Microbiology* 76 (5), 1341-1348

Santo, C. E., Taudte, N., Nies, D. H., and Grass, G. (2008) 'Contribution of Copper Ion Resistance to Survival of *Escherichia coli* on Metallic Copper Surfaces'. *Applied and Environmental Microbiology* 74 (4), 977-986

Sarathi, P. and Thilagavathi, G. (2009) 'Synthesis and Characterization of Titanium-Di-Oxide Nanoparticles and their Applications to Textiles for Microbe Resistance'. *Journal of Textile and Apparel, Technology and Management* 6 (2), 1-8

Sarkar, S., Jana, A. D., Samanta, S. K., and Mostafa, G. (2007) 'Facile Synthesis of Silver Nanoparticles with Highly Efficient Antimicrobial Property'. *Polyhedron* 26 (15), 4419-4426

Sawai, J. (2003) 'Quantitative Evaluation of Antibacterial Activities of Metallic Oxide Powders (ZnO, MgO and CaO) by Conductimetric Assay'. *Journal of Microbiological Methods* 54 (2), 177-182

Sawhney, A., Condon, B., Singh, K., Pang, S., Li, G., and Hui, D. (2008) 'Modern Applications of Nanotechnology in Textiles'. *Textile Research Journal* 78 (8), 731-739

Schaller, M., Laude, J., Bodewaldt, H., Hamm, G., and Korting, H. (2003) 'Toxicity and Antimicrobial Activity of a Hydrocolloid Dressing Containing Silver Particles in an *Ex-vivo* Model of Cutaneous Infection'. *Skin Pharmacology and Physiology* 17 (1), 31-36

Schembri, M. A., Blom, J., Krogfelt, K. A., and Klemm, P. (2005) 'Capsule and Fimbria Interaction in *Klebsiella pneumoniae*'. *Infection and Immunity* 73 (8), 4626-4633

Schierholz, J. M., Lucas, L. J., Rump, A., and Pulverer, G. (1998) 'Efficacy of Silver Coated Medical Devices'. *Journal of Hospital Infection* 40 (4), 257-262

Schrand, A. M., Rahman, M. F., Hussain, S. M., Schlager, J. J., Smith, D. A., and Syed, A. F. (2010) 'Metal based Nanoparticles and their Toxicity Assessment'. *Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology* 2 (5), 544-568

Shaffiey, S. F., Shaffiey, S. R., Ahmadi, M., and Azari, F. (2014) 'Synthesis and Evaluation of Bactericidal Properties of CuO Nanoparticles Against *Aeromonas hydrophila*'. *Nanomedicine Journal* 1 (3), 198-204

Shanmugasundaram, O. L. (2007) 'Antimicrobial Finish in Textiles'. [online] available from <http://www.indiantextilejournal.com/articles/FAdetails.asp?id=507> [07/05/2013]

Shi, L., Xing, L., Hou, B., Ge, H., Guo, X., and Tang, Z. (2010) 'Inorganic Nano Metal Oxides used as Anti-Microorganism Agents for Pathogen Control'. *Current Research, Technology and Education Topics in Applied Microbiology and Microbial* 2010 (1), 361-368

Shigehara, K., Bito, M., and Katayama, Y. (2007) *Antimicrobial Agents and Microbicidal Agents*. Patent US 20100004469 A1

Shiomori, T., Miyamoto, H., Makishima, K., Yoshida, M., Fujiyoshi, T., Udaka, T., Inaba, T., and Hiraki, N. (2002) 'Evaluation of Bedmaking-Related Airborne and Surface Methicillin-Resistant *Staphylococcus aureus* Contamination'. *Journal of Hospital Infection* 50 (1), 30-35

Siddiqui, M. A., Alhadlaq, H. A., Ahmad, J., Al-Khedhairy, A. A., Musarrat, J., and Ahamed, M. (2013) 'Copper Oxide Nanoparticles Induced Mitochondria Mediated Apoptosis in Human Hepatocarcinoma Cells'. [online] available from <http://www.ncbi.nlm.nih.gov/pubmed/23940521> [11/11/2014]

Sidwell, R. W., Dixon, G. J., and Mcneil, E. (1966) 'Quantitative Studies on Fabrics as Disseminators of Viruses'. *Applied Microbiology* 14 (1), 55-59

Simončič, B., Tomšič, B., Černe, L., Orel, B., Jerman, I., Kovač, J., Žerjav, M., and Simončič, A. (2012) 'Multifunctional Water and Oil Repellent and Antimicrobial Properties of Finished Cotton: Influence of sol–gel Finishing Procedure'. *Journal of Sol-Gel Science and Technology* 61 (2), 340-354

Simoncic, B. and Tomsic, B. (2010) 'Structures of Novel Antimicrobial Agents for Textiles - A Review'. *Textile Research Journal* 80 (16), 1721-1737

Singh, G., Joyce, E. M., Beddow, J., and Mason, T. J. (2012) 'Evaluation of Antibacterial Activity of ZnO Nanoparticles Coated Sonochemically Onto Textile Fabrics'. *Journal of Microbiology, Biotechnology and Food Sciences* 2 (1), 106-120

Singha, K. (2012) 'A Review on Coating & Lamination in Textiles: Processes and Applications'. *American Journal of Polymer Science* 2 (3), 39-49

Sivakumar, P., Balaji, S., Prabhawathi, V., Neelakandan, R., Manoharan, P., and Doble, M. (2010) 'Effective Antibacterial Adhesive Coating on Cotton Fabric using ZnO Nanorods and Chalcone'. *Carbohydrate Polymers* 79 (3), 717-723

Slawson, R. M., Van Dyke, M. I., Lee, H., and Trevors, J. T. (1992) 'Germanium and Silver Resistance, Accumulation, and Toxicity in Microorganisms'. *Plasmid* 27 (1), 72-79

Sójka-Ledakowicz, J., Lewartowska, J., Kudzin, M., Jesionowski, T., Siwinska-Stefanska, K., and Krysztafkiewicz, A. (2008) 'Modification of Textile Materials with Micro and Nano Structural Metal Oxides'. *Fibres and Textiles in Eastern Europe* 16 (5), 112-116

Song, R., Xu, A., Deng, B., Li, Q., and Chen, G. (2007) 'From Layered Basic Zinc Acetate Nanobelts to Hierarchical Zinc Oxide Nanostructures and Porous Zinc Oxide Nanobelts'. *Advanced Functional Materials* 17 (2), 296-306

Spadaro, J. A., Berger, T. J., Barranco, S. D., Chapin, S. E., and Becker, R. O. (1974) 'Antibacterial Effects of Silver Electrodes with Weak Direct Current.'. *Antimicrobial Agents and Chemotherapy* 6 (5), 637-642

Spicer, C. (1959) 'The Survival of Shigella Sonnei on Cotton Threads'. *Journal of Hygiene* 57 (02), 210-215

Studer, H. (1997) 'Antimicrobial Protection for Polyolefin Fibers'. *Chemical Fibers International* 47 (5), 373-374

Sue, Y., Yu-Yun Lee, J., Wang, M., Lin, T., Sung, J., and Huang, J. (2001) 'Generalized Argyria in Two Chronic Hemodialysis Patients'. *American Journal of Kidney Diseases* 37 (5), 1048-1051

Sun, X., Zhang, H., Xu, J., Zhao, Q., Wang, R., and Yu, D. (2004) 'Shape Controllable Synthesis of ZnO Nanorod Arrays Via Vapor Phase Growth'. *Solid State Communications* 129 (12), 803-807

Sundaresan, K., Sivakumar, A., Vigneswaran, C., and Ramachandran, T. (2012) 'Influence of Nano Titanium Dioxide Finish, Prepared by Sol-Gel Technique, on the Ultraviolet Protection, Antimicrobial, and Self-Cleaning Characteristics of Cotton Fabrics'. *Journal of Industrial Textiles* 41 (3), 259-277

Suslick, K. S. and Price, G. J. (1999) Applications of Ultrasound to Materials Chemistry. *Annual Review of Material Science. Vol. 29. 1st Edition.* Annual Reviews, pp. 295-326.

Suslick, K. S., Choe, S., Cichowlas, A. A., and Grinstaff, M. W. (1991) 'Sonochemical Synthesis of Amorphous Iron'. *Nature* 353 (6343), 414-416

Suslick, K. S., Hammerton, D. A., and Cline Jr., R. E. (1986) 'The Sonochemical Hot Spot'. *Journal of the American Chemical Society* 108 (18), 5641-5642

Sweigert, P., Xu, Z., Hong, Y., and Swavey, S. (2012) 'Nickel, Copper, and Zinc Centered Ruthenium-Substituted Porphyrins: Effect of Transition Metals on Photoinduced DNA Cleavage and Photoinduced Melanoma Cell Toxicity'. *Dalton Transactions* 41 (17), 5201-5208

Swofford, H. W. (2010) 'An Overview of Antimicrobial Testing for Textile Applications'. *AATCC Review* 6, 51-55

Tamaekong, N., Liewhiran, C., and Phanichphant, S. (2014) 'Synthesis of Thermally Spherical CuO Nanoparticles'. *Journal of Nanomaterials* 2014. [online] available from <http://www.hindawi.com/journals/jnm/2014/507978/abs/> [10/08/2014]

Telfer Brunton, W. A. (1995) 'Infection and Hospital Laundry'. *The Lancet* 345 (8964), 1574-1575

Tenover, F. C. (2006) 'Mechanisms of Antimicrobial Resistance in Bacteria'. *The American Journal of Medicine* 119 (6, Supplement 1), S3-S10

Teo, J. J., Chang, Y., and Zeng, H. C. (2006) 'Fabrications of Hollow Nanocubes of $Cu_2O$ and Cu Via Reductive Self-Assembly of CuO Nanocrystals'. *Langmuir* 22 (17), 7369-7377

Ter Meulen, V. (2009) 'Healthcare-Associated Infections: The View from EASAC'. [online] available from <http://www.easac.eu/fileadmin/PDF_s/reports_statements/Healthcare-associated.pdf> [04/11/2013]

Texter, J., Ziemer, P., Rhoades, S., and Clemans, D. (2007) 'Bactericidal Silver Ion Delivery into Hydrophobic Coatings with Surfactants'. *Journal of Industrial Microbiology & Biotechnology* 34 (8), 571-575

Thiry, M. C. (2005) 'Outside, Inside, all Around in the Healthcare Landscape, Textiles are Everywhere'. *AATCC Review* 5 (5), 34-37

Tinker, K. (2010) 'Moment of Truth: Proper Air Flow Critical to Healthcare Laundries'. [online] available from <http://www.hlacnet.org/Airflow%20White%20Paper%20Final.doc.pdf> [03/08/2013]

Tischer, M., Pradel, G., Ohlsen, K., and Holzgrabe, U. (2012) 'Quaternary Ammonium Salts and their Antimicrobial Potential: Targets Or Nonspecific Interactions'. *ChemMedChem* 7 (1), 22-31

Todar, K. (2009) *Online Textbook of Bacteriology*. [online] available from <http://textbookofbacteriology.net/themicrobialworld/NormalFlora.html> [01/12/2013]

Top, A. and Ülkü, S. (2004) 'Silver, Zinc, and Copper Exchange in a Na-Clinoptilolite and Resulting Effect on Antibacterial Activity'. *Applied Clay Science* 27 (1), 13-19

Touati, D. (2000) 'Iron and Oxidative Stress in Bacteria'. *Archives of Biochemistry and Biophysics* 373 (1), 1-6

Valko, M., Morris, H., and Cronin, M. (2005) 'Metals, Toxicity and Oxidative Stress'. *Current Medicinal Chemistry* 12 (10), 1161-1208

Vandebriel, R. J. and De Jong, W. H. (2012) 'A Review of Mammalian Toxicity of ZnO Nanoparticles'. *Nanotechnology, Science and Applications* 2012 (5), 61-71

Varesano, A., Vineis, C., Aluigi, A., and Rombaldoni, F. (2011) 'Antimicrobial Polymers for Textile Products'. *Science Against Microbial Pathogens: Communicating Current Research and Technological Advances* 2011 (3), 99-110

Vayssieres, L. (2003) 'Growth of Arrayed Nanorods and Nanowires of ZnO from Aqueous Solutions'. *Advanced Materials* 15 (5), 464-466

Vellingiri, K., Ramachandran, T., and Senthilkumar, M. (2013) 'Eco-Friendly Application of Nano Chitosan in Antimicrobial Coatings in the Textile Industry'. *Nanoscience and Nanotechnology Letters* 5 (5), 519-529

Vester, B. and Douthwaite, S. (2001) 'Macrolide Resistance Conferred by Base Substitutions in 23S rRNA'. *Antimicrobial Agents and Chemotherapy* 45 (1), 1-12

Vigneshwaran, N., Kumar, S., Kathe, A., Varadarajan, P., and Prasad, V. (2006) 'Functional Finishing of Cotton Fabrics using Zinc oxide soluble Starch Nanocomposites'. *Nanotechnology* 17 (20), 5087-5095

Vijayaraghavan, R. (2012) 'Zinc Oxide Based Inorganic Antimicrobial Agents'. *International Journal of Science Research* 1 (2), 35-46

Vincent, J. L. (2003) 'Nosocomial Infections in Adult Intensive Care Units'. *Lancet* 361 (9374), 2068-2077

Völker, C., Oetken, M., and Oehlmann, J. (2013) 'The Biological Effects and Possible Modes of Action of Nanosilver'. in *Reviews of Environmental Contamination and Toxicology Volume 223*. ed. by Anon: Springer 223 (1), 81-106

Vukušić, S. B., Grgac, S. F., Katović, D., and Gorski, D. B. (2009) 'Antibacterial Properties of Citric Acid used as Easy Care Finishing Agent'. *AATCC Review* 9 (8), 37-43

Waggoner, B. and Speer, B. (2006) *Bacteria: Life History and Ecology*. [online] available from <http://www.ucmp.berkeley.edu/bacteria/bacterialh.html> [01/10/2013]

Walters, P. A., Abbott, E. A., and Isquith, A. J. (1973) 'Algicidal Activity of a Surface-Bonded Organosilicon Quaternary Ammonium Chloride.'. *Applied Microbiology* 25 (2), 253-256

Wang, Y., Aker, W. G., Hwang, H., Yedjou, C. G., Yu, H., and Tchounwou, P. B. (2011) 'A Study of the Mechanism of *In-Vitro* Cytotoxicity of Metal Oxide Nanoparticles using Catfish Primary Hepatocytes and Human HepG2 Cells'. *Science of the Total Environment* 409 (22), 4753-4762

Ware, V. (2012) *Antibiotics and Ribosomes as Drug Targets*. [online] available from <http://www.lehigh.edu/~inbios21/PDF/Fall2012/Ware_11052012.pdf> [02/07/2013]

Wasif, A. and Laga, S. (2009) 'Use of Nano Silver as an Antimicrobial Agent for Cotton'. *AUTEX Res.J* 9 (5), 13-18

Weichart, D. (ed.) (1999) *Microbial Biosystems: New Frontiers. Proceedings of the 8th International Symposium on Microbial Ecology*. 'Stability and Survival of VBNC cells conceptual and Practical Implications': Atlantic Canada Society for Microbial Ecology, Halifax, Canada held on August 9-14, 1998

Wei, J., Liu, J., Qiang, J., Yang, L., Wan, Y., Wang, H., Gao, W., and Ko, F. (2012) 'Antibacterial Performance of Chitin Nanowhisker Reinforced Poly (Lactic Acid) Composite Nanofiber Membrane'. *Advanced Science Letters* 10 (1), 649-651

Weinstein, R. A. and Hota, B. (2004) 'Contamination, Disinfection, and Cross Colonization: Are Hospital Surfaces Reservoirs for Nosocomial Infection'. *Clinical Infectious Diseases* 39 (8), 1182-1189

Wen, H., Lin, Y., Jian, S., Tseng, S., Weng, M., Liu, Y., Lee, P., Chen, P., Hsu, R., and Wu, W. (eds.) (2007) 'Observation of Growth of Human Fibroblasts on Silver Nanoparticles'. IOP Publishing. [online] available from <http://iopscience.iop.org/1742-6596/61/1/089> [11/11/2013]

Wender, H., de Oliveira, L. F., Migowski, P., Feil, A. F., Lissner, E., Prechtl, M. H., Teixeira, S. R., and Dupont, J. (2010) 'Ionic Liquid Surface Composition Controls the Size of Gold Nanoparticles Prepared by Sputtering Deposition'. *The Journal of Physical Chemistry C* 114 (27), 11764-11768

White, W. C., Monticello, R. A., and Midland, M. (2002) 'Antimicrobial Performance of Medical Textiles'. Form 4A9 Revision 5, 2005

Wilke, M. S., Lovering, A. L., and Strynadka, N. C. (2005) 'B-Lactam Antibiotic Resistance: A Current Structural Perspective'. *Current Opinion in Microbiology* 8 (5), 525-533

Windler, L., Height, M., and Nowack, B. (2013) 'Comparative Evaluation of Antimicrobials for Textile Applications'. *Environment International* 53 (2), 62-73

Wisplinghoff, H., Bischoff, T., Tallent, S. M., Seifert, H., Wenzel, R. P., and Edmond, M. B. (2004) 'Nosocomial Bloodstream Infections in US Hospitals: Analysis of 24,179 Cases from a Prospective Nationwide Surveillance Study'. *Clinical Infectious Diseases* 39 (3), 309-317

Wright, G. D. (2003) 'Mechanisms of Resistance to Antibiotics'. *Current Opinion in Chemical Biology* 7 (5), 563-569

Xia, T., Kovochich, M., Brant, J., Hotze, M., Sempf, J., Oberley, T., Sioutas, C., Yeh, J. I., Wiesner, M. R., and Nel, A. E. (2006) 'Comparison of the Abilities of Ambient and Manufactured Nanoparticles to Induce Cellular Toxicity According to an Oxidative Stress Paradigm'. *Nano Letters* 6 (8), 1794-1807

Xie, Y., He, Y., Irwin, P. L., Jin, T., and Shi, X. (2011) 'Antibacterial Activity and Mechanism of Action of Zinc Oxide Nanoparticles Against *Campylobacter jejuni*'. *Applied and Environmental Microbiology* 77 (7), 2325-2331

Xing, Y., Yang, X., and Dai, J. (2007) 'Antimicrobial Finishing of Cotton Textile Based on Water Glass by sol–gel Method'. *Journal of Sol-Gel Science and Technology* 43 (2), 187-192

Yadav, A., Prasad, V., Kathe, A., Raj, S., Yadav, D., Sundaramoorthy, C., and Vigneshwaran, N. (2006) 'Functional Finishing in Cotton Fabrics using Zinc Oxide Nanoparticles'. *Bulletin of Materials Science* 29 (6), 641-645

Yalcin, A. N. (2003) 'Socioeconomic Burden of Nosocomial Infections'. *Indian Journal of Medical Sciences* 57 (10), 450-456

Yamada, H., Suzuki, K., and Koizumi, S. (2007) 'Gene Expression Profile in Human Cells Exposed to Zinc'. *Journal of Toxicological Sciences* 32 (2), 193-196

Yaneva-Deliverska, M. (2011) *Patient Safety and Healthcare-Associated Infections*. [online] available from <http://www.journal-imab-bg.org/issue-2011/book1/vol17b1p120-122.pdf> [06/07/2013]

Yang, H., Liang, L., Lin, S., and Jia, S. (2010) 'Isolation and Characterization of a Virulent Bacteriophage AB1 of *Acinetobacter baumannii*'. *BMC Microbiology* 10 (1), 131-136

Yang, J. M., Lin, H. T., Wu, T. H., and Chen, C. C. (2003) 'Wettability and Antibacterial Assessment of Chitosan Containing Radiation-Induced Graft Nonwoven Fabric of Polypropylene-g-Acrylic Acid'. *Journal of Applied Polymer Science* 90 (5), 1331-1336

Yao, B., Chan, Y., and Wang, N. (2002) 'Formation of ZnO Nanostructures by a Simple Way of Thermal Evaporation'. *Applied Physics Letters* 81 (4), 757-759

Ye, W., Leung, M. F., Xin, J., Kwong, T. L., Lee, D. K. L., and Li, P. (2005) 'Novel Core-Shell Particles with Poly (Butyl Acrylate) Cores and Chitosan Shells as an Antibacterial Coating for Textiles'. *Polymer* 46 (23), 10538-10543

Yeber, C., Rodríguez, J., Freer, J., Durán, N., and D Mansilla, H. (2000) 'Photocatalytic Degradation of Cellulose Bleaching Effluent by Supported $TiO_2$ and ZnO'. *Chemosphere* 41 (8), 1193-1197

You, Z. and Zhu, H. (2004) 'Syntheses, Crystal Structures, and Antibacterial Activities of Four Schiff Base Complexes of Copper and Zinc'. *Zeitschrift Für Anorganische Und Allgemeine Chemie* 630 (15), 2754-2760

Zhang, F., Wu, X., Chen, Y., and Lin, H. (2009) 'Application of Silver Nanoparticles to Cotton Fabric as an Antibacterial Textile Finish'. *Fibers and Polymers* 10 (4), 496-501

Zhang, H., Ma, X., Xu, J., Niu, J., and Yang, D. (2003) 'Arrays of ZnO Nanowires Fabricated by a Simple Chemical Solution Route'. *Nanotechnology*. [online] available from <http://iopscience.iop.org/0957-4484/14/4/303/> [02/05/2014]

Zhang, L., Jiang, Y., Ding, Y., Daskalakis, N., Jeuken, L., Povey, M., O'Neill, A., and York, D. (2010) Mechanistic Investigation into Antibacterial Behaviour of Suspensions of ZnO Nanoparticles Against *E. coli*. Springer Netherlands 2010 (12), 1625-1636

Zhao, G. and Stevens Jr., S. E. (1998) 'Multiple Parameters for the Comprehensive Evaluation of the Susceptibility of *Escherichia coli* to the Silver Ion'. *BioMetals* 11 (1), 27-32

Zhou, X. D., Chen, K., Guo, J. L., and Zhang, Y. F. (2013) 'Synthesis and Application of Quaternary Ammonium Functionalized Hyperbranched Polyester Antibacterial Agent'. *Advanced Materials Research* 796 (10), 412-415