Chapter 6

Evaluation of the biocidal effects of ECA solutions using Atomic Force Microscopy (AFM)

6.1 Abstract

Reports in the literature attest to the ultra structural damage to bacterial cell wall structures that follow exposure to Electro-Chemically Activated (ECA). Atomic Force Microscopy (AFM) is a high-resolution, three dimensional imaging technique, which facilitates both the qualitative and quantitative assessment of the effects of biocidal interventions at the ultra-structural level.

The use of the electrolyzed oxidizing Anolyte (Actsol®[1]) solution as a biocide is well established, and this study was designed to assess the morphological alterations that exposure to Actsol® solutions would cause in a variety of bacterial strains under controlled conditions. Four mixed bacterial strains were exposed to the Actsol® solution produced from a saline solution (0,25%) for 5 minutes and contrasted against the AFM images from equivalent, non-Actsol® treated preparations. All Actsol® treated bacterial samples revealed significant morphometric deviations relative to the untreated controls, and these changes comprised altered geometric contour, swelling, and lysis with leakage of intracellular contents. Additionally, the AFM technique afforded the further capacity to describe distinctive ultra-structural changes to cell wall morphology following exposure to the aqueous oxidizing solutions.

This study confirms the cellulolytic properties of the Electro-Chemically activated Anolyte solutions as well as the merits of employing the AFM high-resolution technology to describe bacterial morphology and the capacity to assess the ultra-structural changes that follow exposure to surface active biocides.

1- Actsol® is the registered trademark of Radical Waters (Pty) Ltd.

6.2 Introduction

The biocidal capacity of the Electrolyzed oxidizing saline solutions with anomalous physicochemical attributes generated during the Electro-Chemical Activation of dilute brine solutions has been reported extensively (Prilutsky and Bakhir, 1997; Bakhir, 1999; Marais and Brözel, 1999; Selkon *et al*., 1999; Shetty *et al*., 1999; Tapper *et al*., 1998; Marais, 2000[a,b]; Zinkevich *et al*., 2000; Diao *et al*., 2004 Nakajima *et al*., 2004 and Liao *et al*., 2007). These reports of biocidal efficacy are substantiated by a recent study wherein a non-saline electrolytic oxidant solution was also reported also reported to display substantial biocidal capacity (Jeong *et al*., 2006).

The large diversity of cell barrier architecture reflects a variety of specific adaptations to environmental and ecological pressures. Microbial surfaces are the critical interface with the external environment, and as such they play a vital role in determining cellular shape and the maintenance of turgor pressure (Dufrene and Müller, 2005). To date, most evaluations of bacterial surface morphology have relied upon 2-Dimensional images generated from optical and electron microscopy. Refinements to the preparation technique have yielded additional information on topographical features, and cryo-methods tied to electron microscopy have been able to offer the opportunity to view surface images under conditions more representative of their native state. However, these methods are substantially onerous in terms of sample preparation, analysis and are prone to artefactual change.

Aside from the limitations of specific sample preparation, an understanding of the structure, properties and functions of microbial surfaces is limited by the availability of sensitive, high resolution surface analysis techniques (Dufrene and Müller, 2005). Historically, magnification of microbial surface features with optical instruments has been limited by the wavelength of visible light and generally only offers a resolution down to 0.5 microns.

The use of electron and ion beams in a Scanning Electron Microscope (SEM) permits the resolution of images to approximately 30 angstroms. However the SEM is often destructive and does not readily yield quantifiable information on the surface topography of the sample. The use of Transmission Electron Microscopy (TEM) does

permit the imaging of intracellular structures and in particular the cytoplasmic membrane and is particularly useful for comparative microscopic studies (Joeng *et al.*, 2006).

More recently, Scanning Probe Microscopy (SPM) has been used for imaging and metrology applications to provide quantitative, 3-Dimensional topographic information of sample surfaces. As a specific refinement of the SPM technology, Atomic Force Microscopy (AFM) can provide a 3-Dimensional image of biological cells under physiological conditions with an unprecedented resolution. The unique capability of AFM to characterise the height dimension permits direct visualisation of 3-D images of structures with resolutions ranging from subangstroms to microns (Wickramasinghe, 1989; Zhou and Christie, 1995). AFM affords the potential to describe surface morphology without the constraints of vacuums and destructive staining procedures which are known to adversely impact upon the generation of artefact free images (Bolshakova *et al*., 2001). In addition, the technology also permits the determination of the physical properties and molecular interactions at the microbial barrier interface, and thus provides fundamental insights into the structure and functional relationships of the components of cell surfaces (Dufrene and Müller, 2005).

Chemically derived characterisations of cell wall attributes have been complemented with AFM images wherein the manipulation of the charge of the cell surface using variations in concentration of monovalent ions, have confirmed that the OmpF porin in the cell wall has a nett negative charge (Dufrene and Müller, 2005).

In terms of describing some of the distinguishing structural features that are specific to different physiological states, it is recognised that adhesion is substantially dependent upon the structure and physicochemical properties of the bacterial surface. It is through the AFM imaging of bacteria under physiologically optimum conditions, that the differentiated structure, chemical composition and physicochemical properties of the surfaces of microbes can be aligned to the mechanisms that facilitate microbial adhesion and aggregation (Schaer-Zammaretti and Ubbink, 2003).

In the quest for data and images of microbes under natural artefact-free conditions, the scanning probe technology represents a significant advantage over conventional structural research techniques. In addition, the AFM technology permits sample imaging in liquids without the impediments normally associated with sample drying and the in-situ imaging procedures readily permit the direct observation of the effects of drug interventions (Bolshakova *et al*., 2001).

In Contact Mode AFM, the samples are mounted on a piezoelectric scanner which permits the 3-D manipulation of the image under high resolution. The topographical detail of the sample is translated from the electronic response of the van der Waal's forces of attraction between the tip of the cantilever and the sample which is monitored and recorded (Dufrene and Müller, 2005). As the probe tip scans, the varying topographic features result in deflections between the cantilever tip and the sample. A light beam from the laser is directed at the cantilever and reflected onto a four-sectioned photodetector. The amount of deflection of the cantilever, or the force it applied to the sample, is then calculated from the difference in light intensity on the four different photodetector sectors (Zhou and Christie, 1995).

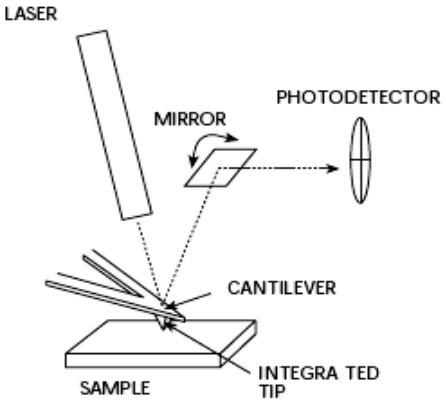


Figure 1 Graphic representation of the image generation pathway with AFM (Zhou and Christie, 1995).

Sample preparation and immobilisation of cells onto surfaces is critical for optimal imaging with the AFM technology (Doktycz *et al.*, 2003). Samples must be securely attached to a solid support in order to resist the lateral forces that may be exerted by the scanning tip. More importantly, the biological object should not change their position while being scanned. While microbial cells are more suited to AFM studies

than animal tissues, the rigid spherical or rod-like structures of bacteria translate into a relatively small cell to substrate contact area (Dufrene and Müller, 2005).

Despite the relative user-friendliness of the technology, basic preparation of the sample is fundamental to the success of the imaging procedure. A crucial prerequisite for the successful and reliable biological application of AFM is that the samples have to be securely attached to the solid substrate using appropriate, non-destructive methods (Doktycz *et al.*, 2003; El Kirat, *et al.*, 2005). While there are a number of substrates available for mounting biological samples, they must all be atomically flat so that the surface of the substrate will not impact upon the natural topography of the adherent sample (Zhou and Christie, 1995).

Biological samples can be imaged in either wet or dry environments. Despite the problems associated with wet imaging, it affords the opportunity to examine the sample under natural conditions. In addition, it excludes the likelihood of artefact development due to preparation technique and it affords greater microscopic resolution (Zhou and Christie, 1995; Bolshakova *et al.*, 2001). As a dynamic system, wet imaging has been also been used to describe the real-time responses of bacterial cells by way of time-lapse photography following exposure to ECA solutions (Tapper *et al.*, 1998).

In contrast to optical or electron microscopy which are subject to complex diffraction or electromagnetic interference, SPM images are largely indifferent to variations in the optical or electronic properties of samples and will readily afford a true reflection of surface topography. However, despite the relative ease and simplicity with which AFM images can be interpreted, they are still subject to artefacts which are intrinsic to the technology. These include tip convolution where the shape and sharpness of the probe tip is mismatched to the topographical features of the sample, and feedback artefacts where background noise generated from extreme topographic features result in ghosting and exaggerated shadow effects (Zhou and Christie, 1995). These artefacts are easily recognised and can be readily remedied by repeating the scan and making changes to the settings of the area of the scan, its speed and direction.

In order to tether biological molecules to supporting surfaces, several procedures have been introduced to promote the formation of covalent bonds between the apposing surfaces. While this methodology may offer a more secure fixture of the sample, its extensive preparation increases the likelihood for the development of aberrations. Air drying and chemical fixation are used to promote cell attachment but are recognised to result in significant denaturation of the specimen (Dufrene and Müller, 2005). Bacterial images of samples derived from culture plates and imaged in minimal media were shown to be comparable to images derived from liquid media under AFM. In all cases the bacteria maintain a hydrated appearance with no evidence of collapse of the cell wall (Doktycz *et al.*, 2003).

Significant differences in vertical dimensions arise from the different preparation procedures, and the height of bacteria imaged in water is substantially greater than that of images secured under air. Additionally flagella structures are more readily imaged in air dried samples versus liquid images. Imaging in water as opposed to air reveals many different topographic features and it is proposed that the loss of resolution of the superficial topographical features may be due to hydration of the carbohydrate chains of the lipopolysaccharide moiety of the cell membrane (Doktycz *et al.*, 2003). With air imaging, the inherent features of bacterial cell walls e.g. filaments etc, become collapsed on the cellular surface and thus create a strain specific topography (Bolshakova *et al.*, 2001).

6.3 Objectives

The objectives of this study were to describe the degree of difference in microscopic change that may arise between different microbial strains following exposure to the oxidant ECA solutions, and to contrast any changes in outer barrier structure against untreated controls. Additionally, the changes in cell structure following exposure to different dilutions of the oxidant ECA solutions were compared in order to evaluate a potentially dose dependent effect.

6.4 Material and Methods

6.4.1 AFM imaging

The Atomic Force Microscopy (AFM) imaging studies were carried out with a Discoverer TopoMetrix TMX2000 Scanning Probe Microscope (Topometrix Corporation, Essex, UK).

Sample preparations were dehydrated and imaged under air. The scanner had a maximum x,y translation capability of 7 x 7 µm. In accordance with previously reported studies (Tapper *et al.*, 1998), the microscope was operated in the contact mode and graphical output was displayed on a monitor with a resolution of 200 lines x 200 pixels. Where necessary, images were enhanced by means of the plane-fitting mode, and shading was used to enhance topographic features where appropriate. The image capture, manipulation and enhancement was facilitated with the complementary software supplied by the Topometrix Corporation and Veeco, Thermo-microscopes (California) (Zhou and Christie, 1995; Symanski, 2004).

6.4.2 Preparation of microbial samples

The bacterial strains comprised type cultures that were maintained on semi-solid agar. These strains were comprised of *Staphylococcus aureus*, *Bacillus subtilis var. niger*, *Escherichia coli*, and *Pseudomonas aeruginosa*, and were plated out for both purity and viability checks. Prior to harvesting, all strains were plated out on Mueller-Hinton 1 Agar Plates (Selecta media) and were incubated at 37^{o}C for 24 hours.

Samples of the bacterial cultures were taken with a flamed metal loop from established plaques on the agar plates. These samples were suspended in either 10ml of sterile hypotonic (2.5g/l) saline solution or an equivalent volume of either undiluted or 1 in 10 strength saline based Actsol®, the latter having been diluted with the same hypotonic saline as used to suspend the untreated control samples. These cultures were homogenized in the hypotonic saline using a mechanical agitator (Vortex V-1, Boeco, Germany) for 30 seconds. A 1 µl drop of the untreated and Actsol® exposed bacterial suspensions was then pipetted onto a silicone disc (25mm^2) which had been pre-cleaned with 70% m/v alcohol. The droplet was then spread with a flamed metal loop to a diameter of approximately 2-3mm.

Initial attempts to image the bacterial preparations on glass slides proved problematic, and silicon wafers were adopted as the standard substrate for the duration of the study. In contrast to glass slides, the use of silicon also enhanced the location of the samples through the heightened optical contrast between the polished surface and the bacterial preparations. This approach concurs with previous reports that indicate that silicon oxide wafers are effectively comparable to glass, and aside from price, are the preferred substrate (El Kirat *et al*., 2005).

While the adherence of biological materials to a substrate may be facilitated by covalent bonding to an active amine coating of the substrate, this procedure proved unnecessary as the samples readily adhered to the surface of the silicone wafers with passive dehydration. The silicon mounted preparations were air-dried for up to 10 minutes in a commercially available incubator (Labcon, S.A.) preset to 25^{o}C.

6.4.3 Generation of ECA solutions

While it is now widely accepted that oxidant solutions produced by different ECA generation systems differ substantially relative to their final composition and activation status (Sampson and Muir, 2002), this study made use of the ECA solutions derived from the FEM-3 based technology as previously described (Bakhir, 1997, Prilutsky and Bakhir, 1997, Zinkevich *et al*., 2000).

Depending on the specific ECA generation parameters employed, a diverse array of chemical compounds can be produced. Predominantly due to the design limitations of the first and second generation reactors as well as the relative novelty of the technology being evaluated, the earlier reports detail assessments which were conducted with largely uncontrolled solutions whose repeatability of quality and hence biocidal capacity, remain questionable.

In contrast to a previous AFM study which used an earlier generation of similar reactor design i.e. FEM 2, and a saline feed solution of 5N NaCl activated at a flow rate of 800ml/minute (Tapper *et al*., 1998), the oxidant ECA solution used in this study i.e. Actsol®, was generated from a 0,25% saline solution, and the effluent solution was pH adjusted to yield a REDOX potential of +850 ± 15mV using a

standard silver-chloride electrode, an Electrical Conductivity (EC) of 5.0 ± 0.5mS/cm and a pH of 7.0 ± 0.2. The solution was generated with a prototypal device comprising two parallel FEM 3 electrolytic reactors developed by Radical Waters (Kyalami, South Africa), and the final solution quality was preset against a saline flow rate of 350 ml/minute per reactor unit.

6.5 Results and Discussion

Previous studies (Tapper *et al*., 1998; Zinkevich *et al*., 2000) have reported that the exposure of *E.coli* bacteria to the Electro-Chemically Activated oxidant solution resulted in an initial distortion of relative cellular proportions, a progressive extracellular accumulation of cytosolic content, and terminally, a universal cell lysis wherein no intact cells were evident after 5 minutes of exposure to the oxidant solution. The substantial enlargement in most cell sizes and the incremental accumulation of extracellular cytoplasmic debris as reported was substantially confirmed with the findings of this study.

While the gram positive microbial strains i.e. *S. aureus* and to a lesser extent, *B. subtilis* appeared to retain their pre-treatment morphological dimensions following Actsol® exposure, the relative dimensions of the gram negative *E.coli* and *Ps. aeruginosa* cells both displayed substantial dimensional deviations relative to the morphology of their untreated controls. In addition, the changes in cell morphology of the gram negative bacterial strains was paralleled by a substantial accumulation of extracellular debris. Of all the bacterial strains evaluated, the *E. coli* cells displayed the greatest change in cellular dimension and thus volume, and this was reflected by the substantive distortion to the relative linear proportions obtained after exposure to the Actsol® challenge.

The bacterial samples prepared directly from agar plates and imaged in air, displayed an increase in ultrastructural detail and a distinctly different overall morphology. It is proposed that these structural differences may be due to changes in the cell rigidity arising from the reduced hydration status of the cell wall. In addition, certain microbial strains imaged under air after sample dehydration were reported to develop

a scalloped shape with raised edges and these structural changes are attributed to the direct effects of dehydration (Doktycz *et al.*, 2003).

This finding is supported by Bolshakova *et al*. (2001) who report that drying of samples led to the formation of two distinctive types of artefacts – a decrease in cellular height as well as the appearance of a distinctive surface patterning.

Of all the strains included in the study, consistent evidence of these distinctly anomalous features were only observed in the images of the untreated *P. aeruginosa* preparations when viewed with both the contrast enhanced 2-D and 3-D rotational image manipulations (Fig 2). Most cells displayed a flattening along the longitudinal contour as well as the presence of punctuate depressions on the surface (Fig 2). Similar evidence of probable preparation artefacts are evidenced in SEM images as reported by Marais (2000) (Fig 4).

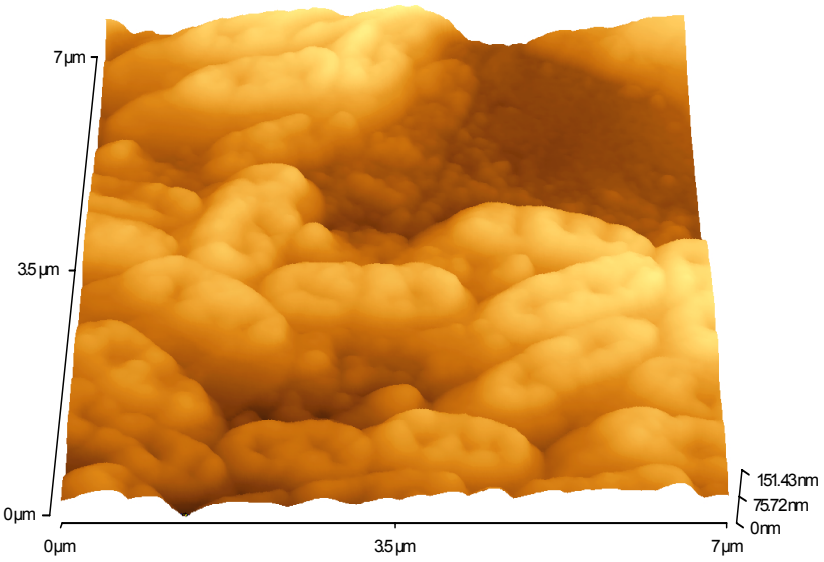


Figure 2. Distinctive punctate topography of untreated *Pseudomonas aeruginosa* cells imaged after fixation with passive dehydration.

6.5.1 Image processing – 2D colour mapping.

By editing the image with the 2D colour mapping adjustment tool, it was possible to enhance the sharpness of the image and afford a qualitative augmentation of the image. By varying the distribution of the colour intensity between the height and

shading data, it was possible to adjust the definition of the superficial image albeit with a sacrifice to the clarity of the depth perspective. Additionally, by increasing the surface reflectivity it was possible to enhance the sharpness of the 2-D image (Symanski, 2004). It has been reported that bacterial suspensions applied to the surface of mica or polished silicon oxide substrates will form flat patchwork monolayers whereby individual cells can be readily visualised (Bolshakova *et al.*, 2001).

A further benefit to this passive adhesion technique is that the preparation results in a clean AFM image which did not require any further rinsing of the samples to remove potential contamination. While this first order monolayer alignment was consistently evident in the images of the untreated *P. aeruginosa* preparations (Fig 3), the spherical dimensions of *S. aureus* resulted in a default quasi-monolayer configuration which also permitted the visualisation of separate cell topography (Fig 5). Similarly while all the AFM samples of the different bacterial strains were prepared identically, none of the untreated *E.coli* or *B subtilis* preparations displayed any predictable first order cellular alignment.

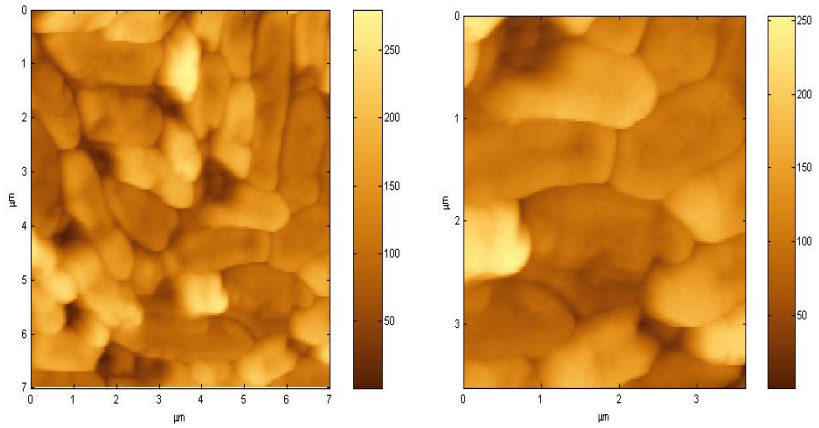


Figure 3. Two magnifications of a monolayer of untreated *P. aeruginosa* cells displaying a 'cobblestone paving' pattern (colour contrast scale displayed at the right).

Aside from the enhancement to the clarity and sharpness of the image, this manipulation also permitted the direct comparison with images generated with other ultrastructural 2-D techniques such as SEM. The 2-D images generated with this mode of AFM image enhancement bears a distinctive similarity to the cellular alignments displayed by the bacteria in the smear monolayer of infected dentinal

tubuli when imaged with the SEM technique (Fig 4). The pocked depressions described in Figure 2 are also a feature of the bacterial topography evident in the scanning electron micrograph illustrated in Figure 4.

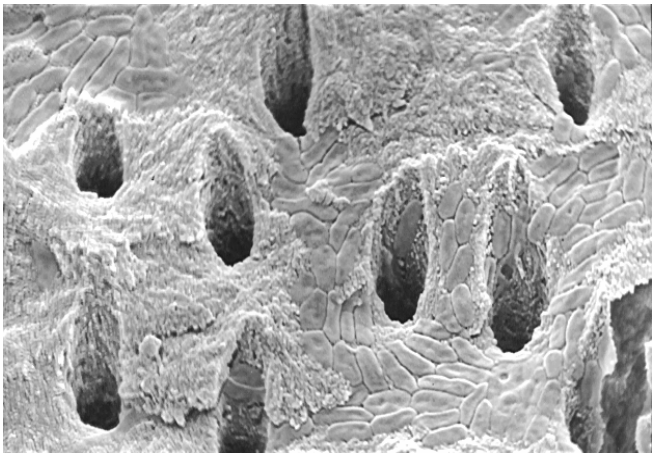


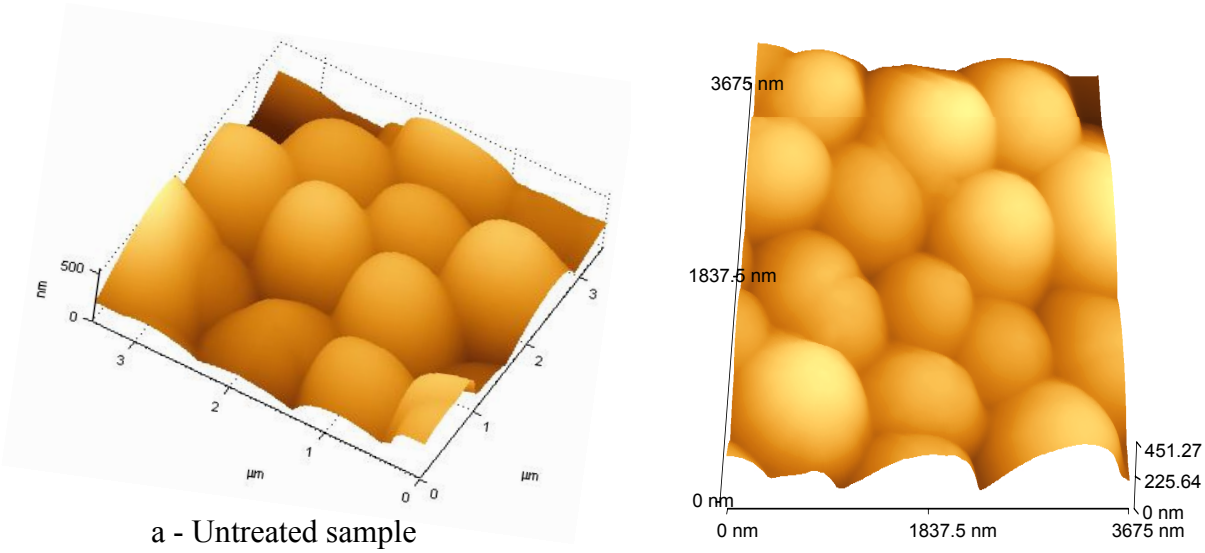
Figure 4. Infected dentinal tubuli of a root canal showing smear layer bacteria arranged in a 'cobblestone paving' pattern (Marais, 2000).

6.5.2 Three Dimensional image manipulation

In addition to the standard top view, the active image can be rotated and displayed from any angle. This image analysis feature permits a more detailed and quantitative evaluation of superficial structures. The depth perspective of the image can be manually adjusted to manipulate the tilt and rotation of the 3D matrix and will change the overall orientation of the image. This feature uses the z scale to emphasise the topography and enhances the relative height of the features in the 3-D image (Zhou and Christie, 1995, Symanski, 2004).

As with other gram positive bacteria, the spherical cell walled *Staphylococcus aureus* bacteria have previously been reported to retain their dimensional proportions as well as cell wall integrity when exposed to biocidal insults (Abid *et al.*, 2004). The same authors have shown that *S. aureus* was more tolerant to the biocidal effects of hypochlorous acid than *E. coli*., and that extended exposure to low dosages of hypochlorouds acid (2mg/l) resulted in substantial dimensional heterogeneity.

Aside from the topographical clarity of the surface contours displayed in the images of the untreated preparation of *S. aureus* (Fig 5a), the similarity of the characteristic morphological features and dimensions described by other microscopic techniques of lower resolution also validates the integrity of this sample preparation procedure as being substantially artefact free. The relative loss of clarity and the blurred nature of the contours in the Actsol® treated preparation (Fig 5b) are proposed to reflect alterations to the integrity of the cell wall components.



a - Untreated sample

Figure 5. Image enhancement through the tilt and rotational manipulations of the 3-D features of untreated (a) and Actsol® treated (b) *S. aureus* preparations.

Aside from the changes deemed to be associated with biocide exposure, variations in relative cellular dimension are also recognised to reflect the spectrum of different growth stages as manifest by the changes in shape (Doktycz *et al.*, 2003), as well as the surface area to volume ratios which may characterise the normal cell division cycles (Shockman and Höltje, 1994).

6.5.3 Image measurement

The line measure function permits the cross-sectional measurement of a selected image along a user-defined line. By exploiting the x, y, and z matrix data of the image, it is possible to isolate any point along the profile line, and to accurately

determine the point-to-point dimension of the nominated image. By manually manipulating the horizontal and vertical planar options of the software, it is possible to profile any image, and to draw a line at any angle and of any length within the image matrix. In addition to describing the dimensions of the nominated images, it is also possible to map the superficial contours of the sample. This tool is thus complementary to the superficial 2D image scans which rely on colour contrast to describe topographical features as well as the tilt and rotated images which afford enhanced depth of perspective but with limited metrological capacity.

The images detailed in figures 6 and 7 illustrate the features available through the line measurement facility of the Explorer™ software.

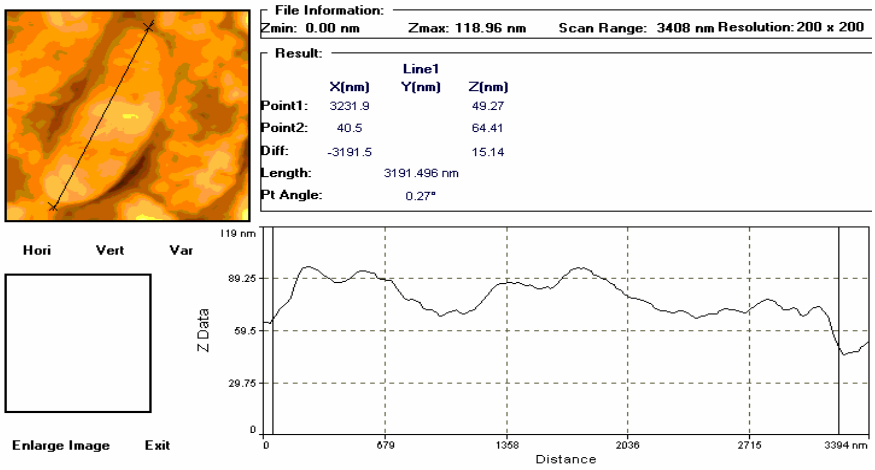


Figure 6. Cross sectional image of the longitudinal topography and dimension of a *P. aeruginosa* bacterial cell after Actsol® treatment.

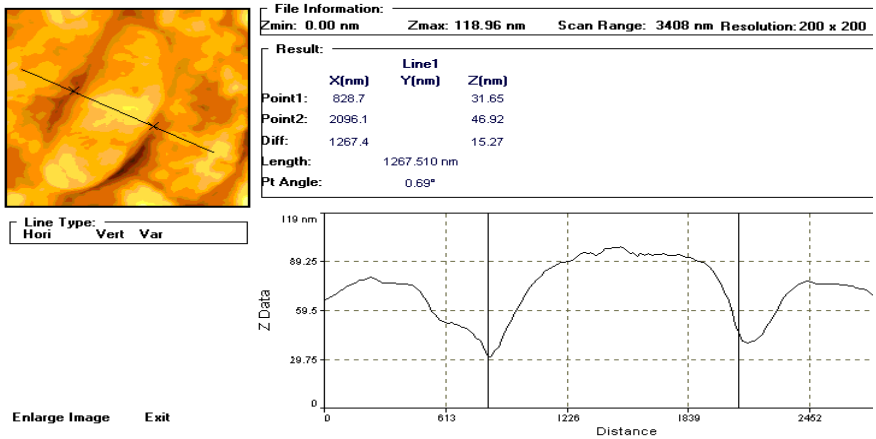


Figure 7. Cross sectional analysis of the transverse topography and dimension of a *P. aeruginosa* bacterial cell after Actsol® treatment.

The irregular surface contour line of the longitudinal section of *P.aeruginosa* detailed in Figure 6 corresponds to the pocked depressions which are described in Figures 2, 3 and 4. Alternatively, the irregular surface contour may also reflect debris associated with leakage of cytoplasmic content but the scope of this study does not permit validation of this proposal.

Measurements of the longitudinal and transverse dimensions of individual bacterial cells were conducted on ten images of each of the untreated control and Actsol® treated preparations. Simple arithmetic means and standard deviations were calculated for each set of data (Table 1).

Table 1. Means and standard deviations of the dimensions of different bacterial strains using line measurements before and after exposure to different Anolyte concentrations (SD- Standard deviation, all measurements in µm)

		Untreated		Actsol® (undiluted)		Actsol® (diluted 1:10)	
Organism	Dimension	Mean	SD	Mean	SD	Mean	SD
B. subtilis	Length	3.8	0.6	3.06	0.61	2.95	0.26
	Breadth	0.96	0.12	0.84	0.10	0.72	0.19
S.aureus	Length	1.34	0.14	1.13	0.14	1.11	0.16
	Breadth	1.19	0.20	1.13	0.09	1.12	0.28
E.coli	Length	2.36	0.19	3.26	0.39	2.7	0.72
	Breadth	0.83	0.13	0.92	0.09	0.89	0.09
P.aeruginosa	Length	2.89	0.50	2.94	0.36	2.49	0.17
	Breadth	1.02	0.28	1.06	0.20	0.86	0.13

While the linear contour tool afforded a real indication of the changes that occurred to the surface topography of the microbes both before and after Actsol® exposure, the comparative integrity of the data from the linear measurement of the Actsol® treated samples was adjudged to be inherently flawed due to the extreme distortions to the cell structure and in many cases, complete lysis of the cell (Table 1).

The exposure of the gram positive bacterial strains to the Actsol® solutions resulted in a reduction in both length and breadth. Given that *S.aureus* is inherently spherical, the

near equivalence of the two perpendicular dimensions confirms the limited structural alterations between the pre-and post-Actsol® exposure images. In converse, exposure of the gram negative strains to the two Actsol® solutions resulted in an increase in both the length and breadth dimensions, and this effect was most obvious with the *E.coli* images. An empirical assessment of the *E.coli* data would suggest that exposure to the undiluted Actsol® solution resulted in a greater dimensional alteration than that displayed by the cells of the same suspension after exposure to the 1:10 Actsol® dilution.

However the extensive range of distortions to the cell dimensions that resulted from exposure to both of the Actsol® solutions would bias any meaningful accordance of statistical significance. While there were minor differences to the changes in dimension between the undiluted and 1:10 dilution samples, it would appear that dilution of the Actsol® to a 1:10 strength, did not result in any dose-dependent or proportionate change in magnitude of cellular distortion and that both solutions resulted in an equivalent amount of cellular damage. It is recognised that under these experimental conditions that further in-depth statistical analysis to determine levels of significance between the pre- and post- Actsol® treatment dimensions would have been spurious. Thus at best, the line measurement tool should be used as a qualitative reflection of the changes in structural dimensions and contours that appear to be causally related to the Actsol® exposure. In order to dilute the inherent bias of the measurements, the length:breadth ratios were calculated and are detailed in table 2.

Table 2. Differences in dimensional ratios between the different bacterial strains before and after exposure to different Actsol® exposure regimens.

	Untreated control	Actsol® (undiluted)	Actsol® (diluted 1:10)
Organism	Length to Breadth ratio		
B. subtilis	3.96	3.64	3.13
S.aureus	1.12	1.03	0.99
E.coli	2.84	3.54	3.07
Ps. aeruginosa	2.83	2.77	2.90

Of particular relevance were the different morphological responses that the various categories of bacteria displayed to Actsol® exposure. While the gram positive organisms revealed minimal dimensional deviations following Actsol® exposure, the membrane bound gram negative bacteria revealed a significant increase in all dimensions which was paralleled by a substantial increase in the presence of extracellular debris following Actsol® treatment.

6.5.4 Image collation

The multiple image manipulations made possible with the AFM software technology provide a composite assessment tool by which to describe the structure and morphological dimensions of microbial populations on a repeatable basis. The images generated either through a superficial 2-D perspective, the 3-D tilt and rotational manipulation or through direct measurement, all provide a unique insight into the changes that exposure to biocidal agents may effect to the integrity of cellular structure relative to the equivalent untreated preparations. Time dependent effects of external agents such as solvents, chemicals and enzymes on the cell surface can be collated over fixed time intervals and can reveal a progressive change in cell surface topography (Dufrene and Müller, 2005). The equivalent time related effects can also be corroborated using Transmission Electron Microscopy (TEM) (Liao *et al*., 2007).

Time-lapse photography of *E.coli* suspensions viewed with AFM reveal that prior to exposure to the oxidant ECA solution, the microbes tended to aggregate in the presence of Extra-cellular Polymeric Substances (EPS). Within 30 seconds of exposure, all cellular dimensions had increased, and after 1 minute, the cellular definition had become obscured due to the overt presence of cytoplasmic material that was associated with membrane rupture and cellular lysis. After 5 minutes, no intact cells could be visualised and the final image comprised of a large amount of cellular debris and lysed cells (Tapper *et al.*, 1998; www.puricore.com, 2007). In a further aspect of the same study, exposure of a suspension of *Desulfovibrio indonensis* to the oxidant ECA solution under equivalent imaging conditions resulted in a similar increase in cellular dimension within 30 minutes of exposure. This coincided with the marked presence of a polar extrudate which was proposed to be associated with the location of the solitary polar flagellum characteristic of this species. After 90 minutes

of exposure, all cells displayed markedly irregular contours and reflected universal cellular lysis (Tapper *et al.*, 1998). In accordance with the latter study which detailed the substantive leakage or extrusion of intracellular contents after oxidant ECA solution exposure, all gram negative preparations as well as that of *B. subtilis*, displayed a widespread presence of extracellular debris after contact with Actsol®. Relative to the equivalent images of the untreated controls, this accumulation of debris was shown to be directly associated with heightened cellular distortion and topographical disruption. This effect is amply evident in the pre- and post-exposure images of *E. coli* illustrated in figures 9 and 10.

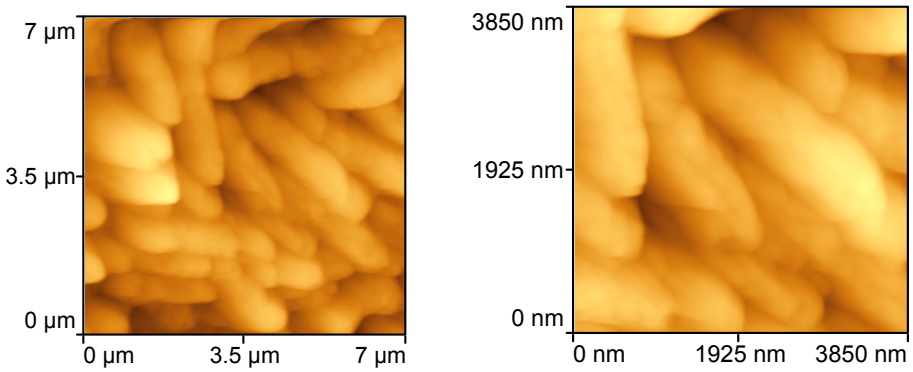


Figure 9. Normal topographic dimensions displayed with enhanced colour contrasted images of an untreated *E.coli* preparation.

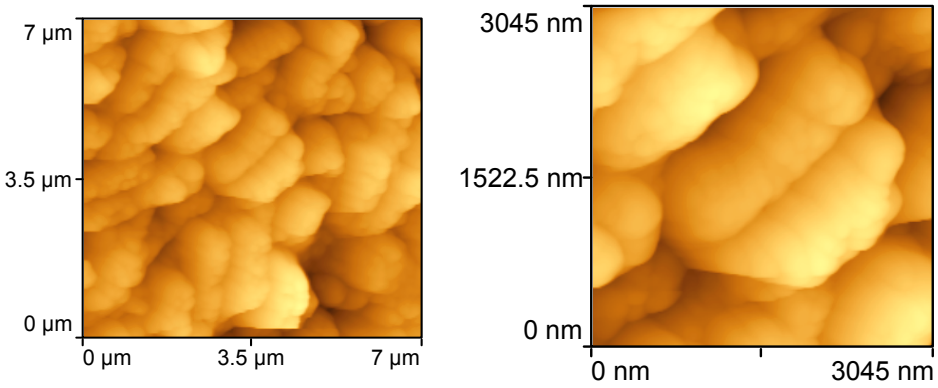
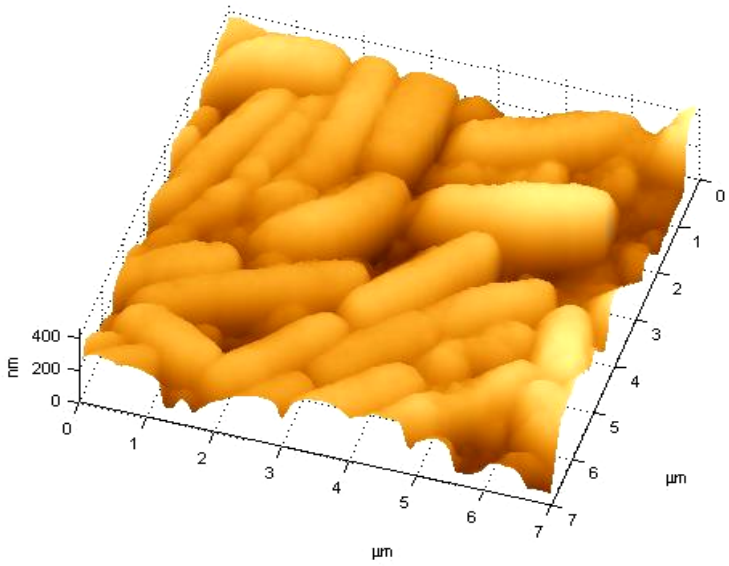


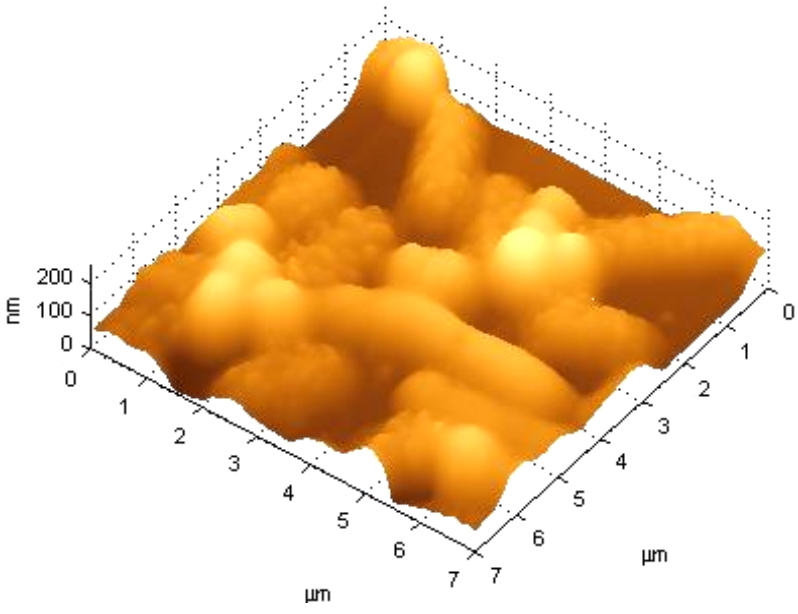
Figure 10. Distinctive swelling and reduction of length displayed with enhanced colour contrasted images of an Actsol® treated *E.coli* preparation

This cellulolytic effect stands in sharp contrast to the post- Actsol® exposure images of *S. aureus* which displayed little or no overt dimensional or topographical changes. In contrast, the rigid cell wall structure of *B. subtilis* afforded the most graphic images of the anolyte mediated cellular disruption and the causally associated accumulation

of extracellular debris (Fig 8b). It is proposed that the extracellular debris present in the image of the untreated *B. subtilis* preparation (Fig 8a) may in part be due to preparation artefacts. Conversely, the distinctly cell associated debris as well as vesicular or 'bleb' type structures on the grossly distorted cells of the Actsol® treated preparation (Fig 8b), would strongly suggest that the affiliated aggregates are a direct consequence of the leakage of intracellular contents.



a) Untreated control



(b) Actsol® treated sample

Figure 8. Differences in 3-D topography between the untreated (a) and Actsol® treated (b) images of *B. subtilis*.

An equivalent finding has been described by Nakajima *et al*. (2004) who reported on a substantive morphological change and bleb formation on the cell wall of a *Pseudomonas aeruginosa* sample that had been exposed to an electrolyzed solution.With progressive exposure they reported that the blebs became elongated and developed a distinctive neck when viewed under TEM. The same 'bleb' effect has been described in *E.coli* using TEM after a 7 second exposure to electrolyzed oxidizing water (Liao *et al*., 2007).

6.6 Conclusions

The use of the AFM technology to image bacterial cells is well established, and despite the limitations of sample preparation and the acknowledged likelihood of image artefacts, it affords a valuable tool to evaluate the effects of exposure to chemical reagents and biocidal compounds in particular. Aside from the capacity to image wet preparations that reflect near physiological conditions, imaging under air offers an array of complimentary software manipulations which consolidate the information derived from single plane images.

Previous AFM studies have revealed that cells swell after a 7-30 second exposure to oxidant ECA solutions and suggest an interference and disruption of the metabolic activity, as well as structural and functional damage to the cell membranes and the wall itself (Tapper *et al*., 1998, Zinkevitch *et al*., 2000, Diao *et al*., 2004; Liao *et al*. 2007).

While the precise mechanism that leads to overall cellular lysis remains unclear, recent evidence would suggest that the effect of reactive oxygen radical species (or Fenton reaction equivalents) on both the cell wall and cytoplasmic barriers initiates a leakage of cyplasmic contents within 7 seconds of exposure of electrolyzed oxidizing solutions in gram negative bacteria (Liao *et al.*, 2007). The final and equivalently explosive lytic effect displayed by cells pre-treated with lysozyme and then placed in a hypotonic solution, would suggest that the massive ingress of water through the compromised barrier structures would play a substantive role therafter (Bolshakova *et al.*, 2001).

Aside from the *S. aureus* preparations, all Actsol® treated bacteria revealed substantial morphometric deviations relative to the untreated controls, and these changes comprised altered geometric contour, collapse and cellular lysis with leakage of intracellular contents. A previous study has reported that *S. aureus* cells undergo limited morphological, yet marked physiological changes when exposed to low doses of hypochlorous acid, but also confirmed that gram positive bacteria were substantially more resistant to hypochlorous acid stress that gram negative strains (Abid *et al.*, 2004).

The similarity of the disruptive changes evidenced in bacterial samples exposed to both chlorine derived and chlorine free anolyte solutions substantiates the hypothesis that reactive molecular electrolytic compounds other than the conventional chlorine based species may play the dominant role in bacterial inactivation (Jeong *et al.*, 2006). This assertion is supported by the substantial attenuation of inactivation of bacteria exposed to electrolyzed oxidizing solutions following the addition of glutathione (Drees *et al*., 2003).

This study suggests the biocidal mechanism of the Electro-chemically activated water solutions to be surface associated and confirms the merits of employing the high-resolution AFM technology to both describe bacterial morphology as well as to evaluate the structural changes that follow biocidal exposure.

6.7 References

Abid, N., Maalej, S. and Rouis, S., (2004). Morphological and physiological changes in *Staphylococcus aureus* exposed to hypochlorous acid. *Letters in Applied Microbiology*. 38, 245-250.

Bakhir, V.M. (1997). Electrochemical Activation: theory and practice. Proceedings - First International Symposium on Electrochemical Activation in Medicine, Agriculture and Industry. Moscow, Russia. (1997): 38-45.

Bolshakova, A.V., Kiselyova, O.I., Filonov, A.S., Frulova, O, Yu., Lyubchenko, Y.L and Yaminsky, I.V. (2001). Comparative studies of bacteria with atomic force microscopy operating in different modes. *Ultramicroscopy*. 86, 121-128.

Diao, H.F., Li, X.Y., Gu, J.D., Shi, H.C. and Xie, Z.M. (2004). Electron microscopic investigation of the bactericidal action of electrochemical dsinfection in comparison with chlorination, ozonation and Fenton reaction. *Process Biochemistry*. 39, 1421-1426.

Doktycz, M.J., Sullivan, C.J., Hoyt, P.R., Pelletier, D.A., Wu, S. and Allison, D.P., (2003). AFM imaging of bacteria in liquid media immobilised on gelatine coated mica surfaces. *Ultramicroscopy*. 97, 209-216

Drees, P.D., Abbaszadegan, M. and Maier, R.M. (2003). Comparative electrochemical inactivation of bacteria and bacteriophage. *Water Research*. 37, 2291-2300.

Dufrene, Y.F. and Müller, D.J., (2005). Microbial Surfaces investigated using Atomic Force Microscopy. Chapter 6, 163-197. In: Savidge,T. and Pothoulakis, C. (Eds.). Methods in Microbiology, Volume 34, Microbial Imaging.

El Kirat, K., Burton, I, Dupres, V. and Dufrene, Y.F. (2005). Sample Preparation procedures for biological atomic force microscopy. *Journal of Microscopy*, 218 (3), 199-207.

Jeong, J., Kim, J.Y. and Yoon, J. (2006). The role of reactive oxygen species in the electrochemical activation of microorganisms. *Environmental Science and Technology*. 40 (19), 6117-6122.

Kirkpatrick, R.D., (2004). Atomic Force Microscopy (AFM) for the evaluation of the bacteriocidal efficacy of novel biocides. Microscopy Society of Southern Africa, 43rd Annual Conference, University of Pretoria.

Liao, L.B., Chen, W.M. and Xiao, X.M. (2007). The generation and inactivation mechanism of oxidation-reduction potential of electrolysed oxidizing water. *Journal of Food Engineering*. 78, 1326-1332.

Marais, J.T. and Brözel, V.S., (1999). Electro-chemically activated water in dental unit water lines. *British Dental Journal*. 187(3), 154-158.

Marais, J.T., (2000[a]). Cleaning efficacy of a new root canal irrigation solution: a preliminary evaluation. *International Endodontic Journal*. 33 (4), 320-325.

Marais, J.T., (2000[b]). Investigations into the application of electrochemically activated water in dentistry. *Journal South African Dental Association*. 55, 381-386.

Nakajima, N., Nakano, T., Harada, F., Taniguchi, H., Yokoyama, I., Hirose, J., et al. (2004). Evaluation of disinfective potential of reactivated free chlorine in pooled tap water by electrolysis. *Journal of Microbiological Methods*. 57 (2), 163-173.

Prilutsky and Bakhir, V.M. (1997) Electrochemical Activation: theory and practice. In: Proceedings of the First International Symposium on Electrochemical Activation in Medicine, Agriculture and Industry. Russia. 38-45.

Sampson, M.N. and Muir, A.V.G., (2002). Not all super-oxidised waters are the same. *Journal of Hospital Infection*. 52 (3), 227-228.

Selkon, J.B., Babb, J.R. and Morris, R., (1999). Evaluation of the antimicrobial activity of a new super-oxidised water, Sterilox®, for the disinfection of Endoscopes. *Journal of Hospital Infection*. 41, 59-70.

Schaer-Zammaretti, P. & Ubbink, J. (2003). Imaging of lactic acid bacteria with AFM – elasticity and adhesion maps and their relationship to biological and structural data. *Ultramicroscopy*. 97, 199-208.

Shetty, N., Srinivasan, S., Holton, J. and Ridgeway, G.L., (1999). Evaluation of microbicidal activity of a new disinfectant: Sterilox 2500 against *Clostridium difficile* spores, *Helicobacter pylori*, vancomycin resistant Enterococcus species, *Candida albicans* and several Mycobacterium species. *Journal of Hospital Infection*. 41, 101-105.

Shockman, G.D., and Höltje, V-F. (1994). Microbial Peptidoglycan (murein) hydrolases. 131-166. In Ghuysen, J.-M. & Hakenbeck, R. (Eds.) Bacterial Cell Wall, New Comprehensive Biochemistry – Vol. 27. Elsevier Press.

www.puricore.com, (2007).

Symanski, C., (2004). EXPLORER™ - Instrument Operation Manual. 85-10244 revision C, ThermoMicroscopes, Sunnyvale, CA 94089 USA.

Tapper, R.C., Smith, J.R., Cocking, C., Beech, I.B., (1998). Atomic Force Microscopy study of the biocidal effect of super-oxidised water, Sterilox. *Biofilm*, 3, (BF 98004), Paper 4.

Wickramasinghe, H.K., (1989) Scanning Probe Microscopes. *Scientific American*, October, 74-81.

Zhou, L. and Christie, B. (1995). Surface characterisation with Scanning Probe Microscopy. User Manual – Supplement, SPMLab version 3.06, Topometrix Corp, Santa Clara, California

Zinkevitch, V., Beech, I.B., Tapper, R.C. and Bogdarina, I. (2000) The effect of super-oxidised water on *Escherichia coli*. *Journal of Hospital Infection*. 46 (2), 153-156.

Chapter 7

The efficacy of Electro-chemically Activated (ECA) water against aerosolised *Bacillus subtilis*, *Serratia marcescens*, *Mycobacterium parafortuitum* and *M. tuberculosis* in a controlled environment.

7.1 Introduction

Tuberculosis (TB), one of the world's greatest killers, is predominantly spread by airborne transmission of the pathogen *Mycobacterium tuberculosis*. Infectious droplet nuclei containing *M. tuberculosis* may remain suspended in air for prolonged periods of time, leading to a high risk of infection in congregate settings with poor or little ventilation. Under these conditions, susceptible populations, notably children and immunosuppressed individuals are most at risk of contracting the disease. These high risk environments have been reported to include correctional and health care facilities, public transport systems and schools (Peccia and Hernandez, 2001).

While TB is generally spread through air, inadequately decontaminated medical devices have also been implicated in iatrogenic infections. *M.tuberculosis* can survive for days on inanimate objects and infectious longevity of up to two months has been recorded (Sattar *et al*., 1995). Iatrogenic infection contracted from contaminated diagnostic bronchoscopes and endoscopes, as well as dental equipment has also been reported (Selkon *et al*., 1999; Marais and Brözel, 1999; Middleton *et al*., 2000).

Health care workers in public health facilities in South Africa, a large proportion of whom may be infected with Human Immunodeficency Virus (HIV), are especially vulnerable to nosocomial transmission of *M. tuberculosis*, yet most facilities lack appropriate policies and procedures for infection control. In addition, parallel contamination with *M. avium-intracellulare* has been reported to infect upwards of 50% of Aquired Immunodeficency Syndrome (AIDS) patients (Griffiths *et al*., 1999). Although infection control in high HIV-prevalence settings is of paramount concern, resource constraints dictate a need for inexpensive administrative and environmental infection control measures. International guidelines to reduce TB transmission in resource-limited settings are available, but although based on sound industrial hygiene

principles, are not supported by scientific data on the effectiveness in the intended settings (World Health Organization, 1999; Centers for Disease Control and Prevention, 2005).

Effective room air disinfectants will have an immediate and significant impact on the transmission of *M. tuberculosis* in congregate settings, representing an inexpensive environmental infection control tool applicable to all resource-constrained settings. Currently, no methodology exists to reliably evaluate the efficacy of aerosolised disinfectants against airborne *M. tuberculosis*, and approaches to date have largely relied upon extrapolations of *in-vitro* data obtained from microbial suspensions exposed to the disinfecting agent.

A unique, state-of-the-art Airborne Infection Research (AIR) Facility, established as a joint partnership between the SA Medical Research Council (SAMRC), the US Centers for Disease Control and Prevention (CDC), the Council for Scientific and Industrial Research (CSIR) and Harvard University in the US, provides a biological model for standardising methodologies for the evaluation of aerosol disinfection. Since *M. tuberculosis* cannot be cultured directly from air, the AIR Facility involves extraction of infectious air from patient wards and supply to animal exposure chambers housing guinea pigs which serve as indirect quantitative samplers of infectious aerosols. The engineering flexibility of the AIR Facility permits experimentation under a variety of environmental conditions (e.g. ventilation rates, temperature and humidity) in addition to optimal ventilation, heating and air conditioning as would be required for compliance with the veterinary ethical standards in South Africa. Guinea pigs are highly susceptible to Mtb infection when air containing infectious droplet nuclei is inhaled, and the probability of infection is proportional to the concentration of infectious droplet nuclei in the air and the volume of air breathed over the duration of the exposure (Wiegeshaus *et al*., 1970). Guinea pig infection is readily determined by tuberculin skin testing after a few weeks of exposure. Effective air disinfection can therefore be indirectly assessed by exposing air laden with TB bacilli to the disinfectant and then measuring the reduction in guinea pig infections over time.

The technology studied in this experiment was based on electrochemically activated (ECA) potable water in a patented reactor system (Bakhir, 1999; Prilutsky and Bakhir, 1997). The mixed oxidant solution generated under prescribed production parameters has demonstrated broad spectrum antimicrobial efficacy and has been trade-marked as Actsol®[1].

Actsol® is a positively charged oxidizing solution comprising hypochlorous acid, hypochorite, hydrogen peroxide, ozone, chlorine-dioxide and chlorine, and has been reported to display potent disinfectant properties (Marais and Brözel, 1999; Selkon *et al.*, 1999; Shetty *et al.*, 1999; Zinkevich *et al.*, 2000). Compared to ordinary liquid disinfectants, the oxidant ECA solutions are regarded as less toxic, less volatile, easier to handle, compatible with other water treatment chemicals and effective against biofilms, while not generating any by-products (Prilutsky and Bakhir, 1997; Middleton *et al.*, 2000).

While the specific mechanisms involved in the inactivation of microbes by the oxidant ECA solutions have not been fully elucidated, the primary reaction appears to involve the oxidation of components of the microbial membrane resulting in an increased permeability, the resultant leakage of macromolecules and ultimately cell death. Recent studies have shown that the main mechanism of inactivation in response to oxidative stress is more subtle and involves the uncoupling of the electron transport chain along with the inactivation of key respiratory enzymes (Helbling and VanBriesen, 2007). Oxidative attack on bacterial proteins may lead to amino acid modification with fragmentation and loss of secondary structure, which results in exposure of hydrophobic residues. These changes favour anomalous aggregation due to hydrophobic interaction and cross-linking reactions (Aertsen and Michiels, 2004).

Actsol® has been reported to be safe in experimental animals, having shown negligible ocular irritation or corrosion potential in rabbits, absence of acute toxicity after oral administration of high doses to rats, and the absence of skin irritation after topical administration in rabbits and pigs (Marais and Brozel, 1999; Marais, 2002).

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[1] Actsol® is the registered trademark of Radical Waters (Pty) Ltd.

Whilst evidence regarding *in vitro* antimicrobial efficacy of the oxidant ECA solution has emerged in the scientific literature (Tanaka *et al.*, 1996; Marais and Brözel 1999; Middleton *et al.*, 2000; Selkon *et al.*, 1999; Shetty *et al.*, 1999; Zinkevitch *et al.*, 2000; Prince *et al.*, 2002), its efficacy as an aerosol or air disinfectant has not been investigated.

7.2 Aims and Objectives

The overall aim of this study was to assess the efficacy of Actsol® in air disinfection of three patient wards challenged with aerosolised *M. tuberculosis* H37Rv, as determined by tuberculin skin testing of guinea pigs exposed to the air from these three patient wards. This was achieved through a systematic, phased approach using three objectives as outlined below.

7.2.1 Phase I

Establishing optimal conditions for and standardisation of Actsol® aerosolisation, bioaerosol generation and bioaerosol collection methodologies. Parameters included relative humidity set to 65% +10%, with approximately 10% of the absolute moisture in the ward air being Actsol® which resulted from a total fogging rate of Actsol® of approximately 2.36 L/h (or 0.787 L/h per patient ward).

7.2.2 Phase II

Establishing parameters for achieving adequate statistical power and demonstrating proof-of-principle that Actsol® had at least 50% efficacy, using three non-TB micro-organisms i.e. *Serratia marcescens, Mycobacterium parafortuitum* and *Bacillus subtilis.*

Bacillus subtilis is a ubiquitous bacterium commonly recovered from water, soil, air, and decomposing plant residue. This bacterium produces endospores that are both temperature and desiccation-resistant in the environment. *Serratia marcescens* has been isolated from eggs of insectaria and are red-pigmented (as opposed to human-isolates of *S. marcescens*) and was non-spore forming. *Mycobacterium parafortuitum*

is an acid fast, relatively fast-growing mycobacterium often used as a surrogate for the slower-growing *M.tuberculosis* in *in-vitro* and *in-vivo* studies.

7.2.3 Phase III

Assessing the efficacy of aerosolised Actsol® in preventing guinea pig infections derived from air contaminated with aerosolised *M. tuberculosis* H37Rv, based on an efficacy of at least 50% reduction in infection rate under defined conditions and within standardised parameters.

7.3 Material and Methods

7.3.1 Preparation of bacterial culture suspensions

Stock cultures of *Bacillus subtilis var niger* (ATCC#49337), *Serratia marcescens* (ATCC#8195) and *Mycobacterium parafortuitum* (ATCC#19686) were procured from the American Type Culture Collection (ATCC) and propagated in accordance with ATCC specifications.

(i) *Bacillus subtilis var niger*

Fresh cultures were prepared from single colonies grown overnight at 37°C in 20ml Bacto trypto soy broth (TSB, Merck, Darmstadt, Germany). A 200ml flask of TSB was inoculated with 1ml *B. subtilis* suspension and incubated for eight days at 37°C in Erlenmeyer flasks in a shaker-incubator. After eight days of growth, a working suspension was prepared by heat-shocking the bacterial broth at 55°C for 30min.

(ii) *Serratia marcescens*

Fresh cultures were prepared from single colonies grown overnight at 37°C in 20ml of nutrient broth (NB, Merck, Darmstadt, Germany). A 200ml flask of NB was inoculated with 1ml *S. marcescens* suspension and incubated overnight at 37°C with vigorous shaking.

(iii) *Mycobacterium parafortuitum*

Fresh cultures were prepared from single colonies grown for 72hrs at 37°C on Bacto tryptic soy agar plates (TSA, Scharlau Chemie SA, Barcelona, Spain). Fresh cultures were scraped from agar plates and suspended in sterile phosphate-buffered dilution water (PBDW). To check for contamination, colonies were plated on blood agar plates, incubated for 24hrs at 37°C and stained with Ziehl-Neelsen (ZN) before examination with microscopy to confirm acid fastness. Immediately prior to each experiment, bacterial cultures were aseptically transferred to 50ml sterile conical centrifuge tubes (Falcon® 2076, Becton Dickinson, SA), and centrifuged at 2 500g for 20min using an ALC Centrifuge (PK130). The supernatant was discarded and the pellets were re-suspended in sterile PBDW. The washing process was repeated twice more and the cells re-suspended a final time in 100ml of sterile PBDW.

Prior to aerosolisation, cell concentrations of the washed bacterial suspensions were determined by turbimetric measurement using a Beckman DU-65 spectrophotometer (Irvine, California, USA). The concentration of bacterial cells in the suspension was estimated from previously developed titration curves comparing turbidity of the suspension with colony-forming units (CFU). A dilution series of the washed cell suspension was also plated to determine the original concentration. The washed cell suspension was diluted to prepare 100 ml of nebuliser suspension at concentrations ranging between 10^5 and 10^8 cells per ml. Before and after nebulisation, a dilution series was performed for standard plate count enumeration at high and low bacterial concentrations. Plates were incubated at 37°C for 24h (*S. marcescens, B subtilis*) and 48-72h (*M. parafortuitum*) for enumeration of viable bacteria.

7.3.2 Aerosolisation and air sampling of bacteria

A six-jet modified MRE-type Collision nebuliser was used to aerosolise the suspensions of bacterial cells in the three patient wards of the AIR Facility. The combined air volume of the three patient wards was 112.06m^3.

Andersen six-stage samplers (ThermoElectron, Smyrna, Georgia, USA) were used to sample the air at quasi-random positions in the animal chambers. The flow rate through the Andersen six-stage sampler was adjusted to 28.3 L/min. Sampling plates

were prepared by aseptically pouring 45ml of the appropriate medium into each of the six 100mm x 15mm sterile plastic Petri dishes (model 0875713, Fisher Scientific, Atlanta, Georgia, USA) so that the gap between the nozzles and agar surface met the manufacturer's specifications. All inside surfaces were maintained in sterile conditions until sampling. The samplers were operated for periods of 1min and 10min sampling time, according to the specific micro-organism, the airborne concentration, and whether or not Actsol® was being fogged. The Andersen six-stage samplers operated at a nominal airflow rate of 28.3 L/m; thus, sampling for 1min or 10min resulted in a total sample volume of approximately 28.3 or 283L of air. After sampling for the prescribed period of time, the samplers were removed from the animal rooms. Next, the plates were removed from the samplers, covered, inverted and incubated at 37°C and enumerated after 24hrs (*S. marcescens, B. subtilis*) or 48-72hrs (*M. parafortuitum*). The CFU counts on all six plates from each Andersen six-stage sampler were combined for analysis.

7.3.3 Animal husbandry

Specified pathogen-free Dunkin Hartley guinea pigs (male and female) weighing between 250g and 300g were procured from South African Vaccine Producers, Sandringham, Johannesburg. Guinea pigs were paired at birth and transported to the AIR Facility under sterile conditions in Techniplast cages equipped with 0.22 µm filters under a stable temperature of 22 ± 4°C. Guinea pigs were delivered within 4 hours after dispatch. Upon arrival at the AIR Facility, guinea pigs were transferred to wire cages at predetermined random rack positions, each coded from left to right and from top to bottom, resulting in a unique identification number for each cage and each animal.

Based on an assumed cumulative infection rate of 80% in the unexposed group of guinea pigs and a doubling of the sample size to accommodate any potential variation resulting from randomisation, a total of 74 animals were housed in pairs in each of the two animal rooms. Six additional guinea pigs were placed at random in three cages in each of the rooms and sacrificed after one week to validate that the animal exposure

chambers were free from contamination and that the animals did not suffer from stress-related conditions.

After allowing the animals to acclimatise for two days, guinea pigs were individually marked and weighed. Baseline tuberculin skin testing was done by intradermal injection of 100TU/0.1ml protein purified derivate (PPD), (Mycos Laboratories, USA). PPD was prepared immediately prior to testing and administered on a depilated area of the back of each guinea pig. No delayed-type hypersensitivity reactions were observed, confirming the absence of any TB infection prior to the exposure. Guinea pigs were maintained under biosafety level 3 conditions at constant temperature of 22°C ± 4°C, relative humidity of 55% ± 10% and day/night cycles of 12 hours. The combined air volume of the two animal exposure rooms was 24.43m^3.

Trained animal care takers were responsible for daily care of the guinea pigs under protocol conditions (daily provision of sterile, de-ionised drinking water augmented with water-soluble ascorbic acid at a concentration of 3mg/ml, autoclaved rabbit pellets, autoclaved *Eragrostis* hay and vermiculate chips in waste collection trays. Cleaning of waste collection trays and the animal rooms occurred three times per week.

7.3.4 Environmental parameters

Internal environmental conditions for the AIR Facility patient wards (six air changes per hour) and animal exposure chambers were pre-set according to protocol specifications to ensure maximum transfer of both aerosolized *M. tuberculosis* H37Rv and Actsol® from the patient wards via the exhaust air transfer duct system to the animal exposure chambers.

Patient wards were kept under negative pressure, with the windows permanently sealed and the doors closed. Trend data for temperature, humidity, air changes per hour, filter pressure differentials and alarms were monitored electronically to detect deviations from the pre-set norms.

7.3.5 Preparation of Actsol®

The electrochemically activated oxidant solution (Actsol®) was produced by a commercial electrolytic generator system at the premises of Radical Waters, (Kyalami, South Africa). The device was configured and preset to electroactivate a dilute brine solution comprising 2.5 gm/litre food grade Sodium Chloride (NaCl) of 98% purity, and the production parameters of the Actsol® solution were recorded (Table 1).

Table 1 Actsol® solution specifications

Parameter	Value
Oxidation-Reduction Potential	> 950 mVolts
pH	6.7±0.2
Electrical Conductivity:	5.0 ± 0.5 mSiemens/cm
Free Available chlorine	180 ± 20 mg per litre

In order to detail any potential variance between the volume of deionised water used as the fogging control relative to that of the Actsol® solution, the specific gravity of the Actsol® solution was measured and recorded as being 0.9555 (Food Consulting Services, 2006).

In addition, a sample of the Actsol® solution was submitted to an independent laboratory for physical and spectrophotometric analysis to confirm the composition of the solution supplied for fogging (Table 2).

Table 2. Results of the physical and spectrophotometric analysis of the Actsol® solution (Claassens, 2006).

Parameter	pH	EG mS/m	Ca mg/L	Mg mg/L	K mg/L	Na mg/L	S mg/L	Cl mg/L	HCO mg/L	NO_3 mg/L
Value	6.7	349	4	3	2.1	720	30	1873	83	11

7.3.6 Aerosolisation of Actsol®

An AQUAFOG TurboXE-1500 (Jaybird Mfg. Inc., PA, USA) centrifugal atomiser fan was used during this study. Continuous flow of Actsol® solution was maintained with an independently calibrated centrifugal pump (Grundfos DME IP65, 18W) preset to deliver 40 ml/minute. At this flow rate, the manufacturer advised that the average droplet diameter would be ≤ 5µm and that less than 3% of droplets would be in the 5-50 µm range. Determination of the ranges of droplet sizes during the atomiser calibration phase confirmed that droplet diameters were progressively reduced in size as atomisation proceded and that a steady state with >95% of droplets being recorded in the <10 µm range was attained after 40 minutes of atomisation.

Under the preset environmental conditions (temperature and RH), it was confirmed that the majority of atomised droplets would have evaporated shortly after aerosolisation. Further control of the solution delivery rate was achieved by mounting the container with the Actsol® solution on a calibrated scale (Vibra AJ 62005, Calibration Certificate 232521) and recording the weight over 5 minute intervals for the duration of the fogging period.

Finally, overall Actsol® usage was confirmed by both internal and independent measurement of the weight of the designated containers before and after the fogging intervention. Actsol® was aerosolised in all of the three patient wards, according to protocol procedure. The generator aerosolising the Actsol® was positioned at the face of the supply air grille to each ward. The concentration of Actsol® in the extracted air volumes was estimated to be 10% of the final absolute humidity as recommended by the manufacturer. At six Air Changes per Hour (ACH), 99.9% clearance of Actsol® was calculated to occur at 69 minutes after termination of the aerosolisation of Actsol® in the patient wards. Log sheets of Actsol® aerosolisation were kept independently by on-site representatives of the manufacturer, who were blinded as to the experimental procedures in order to preserve the scientific integrity of each experiment.

7.3.7 Preparation of *M. tuberculosis* H37Rv suspensions

M. tuberculosis H37Rv (ATCC #27294) was cultured in Dubos broth (Sigma, Steinheim, Germany) containing 5% glycerol (Calbiochem, Darmstadt, Germany) and 0.025% Tween 80 (Merck, Munich, Germany) at 37°C for three weeks, and gently vortexed daily to minimise clumping. The cells were subjected to centrifugation at 1500g for 20 minutes, followed by re-suspension in sterile phosphate-buffered saline (PBS), and this procedure was repeated twice.

The concentration of the washed cell suspension was estimated by turbimetric comparison with McFarland No.1 standard as well as a turbidity measurement at OD 600 of the McFarland No.1-equivalent cell suspension. The McFarland No.1-equivalent cell suspension of *M. tuberculosis* H37Rv was estimated to contain 10^9 CFU/ml. Eighteen 100ml nebulisation suspensions with a target concentration of $5x10^2$ CFU of *M. tuberculosis* H37Rv/ml were required per day of exposure; therefore 36 x 10ml aliquots were prepared from the stock cell suspension to cover both exposure days.

Immediately prior to nebulisation, one aliquot was added to 90ml of PBDW and used as the nebulising suspension. Undiluted and 10-fold dilutions of each nebulisation suspension as well as selected dilutions of a titration series of McFarland No. 1-equivalent cell suspension were plated out in triplicate on Middlebrook 7H10 agar plates supplemented with 10% Middlebrook oleic acid-albumin-dextrose-catalase (OADC) enrichment medium (Becton Dickinson, Le Pont de Claix, France) and incubated at 37°C for four weeks. A stock suspension of 5 x 10^5 CFU *M. tuberculosis* H37Rv /ml in PBDW was simultaneously prepared and frozen in 1ml aliquots with the remaining original stock solution (prepared from broth culture) at -80°C in the event of a need for repeat exposure.

7.3.8 Experimental process

On Day 1 of Phase 3, guinea pigs in Animal Room 2 were exposed to air containing aerosolised *M. tuberculosis* H37Rv at a target concentration of 5 x 10^2 CFU/ml, and concurrently treated with fogged water (as control solution to Actsol®). Eighteen

100ml *M. tuberculosis* H37Rv nebulisation suspensions were used, i.e. six suspensions per each of the three patient wards, generated over six hours in six 20-minute cycles. On Day 2 of Phase 3, guinea pigs in Animal Room 1 were exposed to air containing aerosolised *M. tuberculosis* H37Rv at a target concentration of 5 x 10^2 CFU/ml, concurrently treated with aerosolised Actsol®. Eighteen 100ml *M. tuberculosis* H37Rv nebulisation suspensions were used, i.e. six suspensions per each of the three patient wards, generated over six hours in six 20-minute cycles.

7.3.9 Guinea pig health surveillance

Guinea pigs were subsequently subjected to weekly health surveillance during which mass measurements were taken and animals were observed for clinical signs and symptoms of ill health following standard protocol procedures.

Three weeks after the initial exposure, an ascorbic acid deficiency was detected in the guinea pigs. This was reversed by means of subcutaneous administration of a dose of 1ml 250mg/ml ascorbic acid to each guinea pig (as advised by the consulting veterinarian) and by increasing the amount of ascorbic acid in the drinking water to 4mg/ml. The ascorbic acid deficiency was deemed to have been corrected after one week of treatment, and was based on full recovery of all guinea pigs with respect to mass gain, food and water consumption and absence of painful locomotion. As a precautionary measure and on the advice of the consulting veterinarian, the second round of tuberculin skin testing was delayed until seven weeks post-exposure to overcome any possible adverse effect of immunological anergy.

7.3.10 Tuberculin skin testing

Tuberculin skin testing (TST) at seven weeks post-exposure was performed following the identical methodology to that of the baseline testing described earlier, i.e. intradermal injection of 100TU/0.1ml protein purified derivate (PPD), (Mycos Laboratories, USA).

PPD was prepared immediately prior to testing and administered to a depilated area of the back of each guinea pig. The assessment of skin test indurations was performed

using digital calipers (Wilson Wolpert, The Netherlands). Two independent readings were taken at right angles to each other (length and width) at 24hrs post-administration, and the results recorded separately in millimetres. The final induration size was calculated as the average from these two readings. Guinea pigs with positive TST reactions were sacrificed in accordance with protocol procedure.

7.3.11 Repeat exposure

Protocol specifications indicated a requirement for repeat exposure of those animals that had not converted their tuberculin skin tests (see Results section). An identical experimental procedure to the first exposure was followed, utilising the frozen aliquots of *M. tuberculosis* H37Rv suspensions as described earlier.

Dilutions of both the stock solution as well as the nebulising solutions were plated in triplicate on selective Middlebrook 7H10 agar plates supplemented with 10% OADC enrichment (Becton Dickinson, Le Pont de Claix, France), incubated at 37°C for four weeks and enumerated. TST was repeated at seven weeks post (re)-exposure, employing identical methodology as before.

7.4 Results

Table 1 summarises the data from Phase I, demonstrating a marked and statistically significant antimicrobial effect ($p<0.001$) of Actsol® at equilibrium concentration (approximately 10% of the absolute humidity) on the number concentration of the test aerosols, i.e. mostly single endospores of *B. subtilis*, and mostly single vegetative cells of *M. parafortuitum* and *S. marcescens* at two concentration titres (high and low). The reduction of contaminated aerosols of culturable air particles was 91-92% for endospores of *B. subtilis*, 63-67% for *M. parafortuitum*, and 86-89% for *S. marcescens* for the high and low titre challenges respectively.

Detailed results for Phase I also revealed a tendency for an agglomeration clumping of particles when the Anolyte® was fogged, with the size distribution of particles shifting to the right (i.e., larger air particles were collected) in the six-stage samplers, at both high and low titres of the respective aerosols.

Table 1. Summary statistics for equilibrium airborne concentration with and without Actsol®, at two titres of test aerosols.

Microorganism	Airborne concentration Actsol® OFF		Airborne concentration Actsol® ON	
	Geometric mean	95% Confidence interval	Geometric mean	95% Confidence interval
	CFU/m^3	CFU/m^3	CFU/m^3	CFU/m^3
B. subtilis				
Low titre	3 416	2 888 – 4 041	180	119 – 272
High Titre	10 794	8 626 – 13 506	500	379 – 659
M. parafortuitum				
Low titre	36 484	29 322 – 45 396	11 939	8 933 – 15 955
High titre	102 912	58 871 – 179 897	38 456	34 428 – 42955
S. marcescens				
Low titre	189	130 – 275	27	20 – 36
High titre	1 264	790 – 2 019	144	75 – 275

Phase II data are summarized in Table 2, where high-titre concentrations of the respective organisms were aerosolised. Phase II confirmed the proof-of-concept that aerosolized Actsol® at equilibrium concentration (approximately 10% of the absolute humidity) significantly reduced the concentration of air borne *M. parafortuitum*, *B. subtilis* and *S. marcescens* counts by at least 50% ($p<0.001$). Results for phase II also indicate that it took approximately 40 minutes for the Actsol® concentration to reach equilibrium and for the maximum inactivation of the three respective microbial aerosols.

Table 2. Summary statistics for reduction in airborne concentration, Phase II.

	% Reduction in aerosol concentration at three time points		
Microorganism	1st Sampling period (10 minutes)	2nd Sampling period (10 minutes)	3rd Sampling period (10 minutes)
	%	%	%
B. subtilis	-33	-44	-90
M. parafortuitum	-71	-92	-95
S. marcescens	-65	-71	-99

Analyses of the AIR Facility system data confirmed no statistically significant differences in temperature, humidity or air changes per hour between the two animal rooms during the course of the experiment, and confirmed that the AIR Facility functioned according to the pre-set criteria outlined in the study protocol.

Phase III data are summarized in Tables 3 and 4, and indicate that Actsol® did not show a statistically significant bactericidal effect on aerosolised *M. tuberculosis* H37Rv under the controlled conditions identified in Phase I and Phase II of the study. On the first exposure, 39.2% of control animals and 42.5% of Actsol® intervention animals converted their respective TST tests (indicating TB infection), while 72.7% and 61.7% respectively converted their TST test after the second exposure.

In total, 83.6% TST conversions occurred in the control animals and 78.1% conversions in the intervention animals, confirming that the study aim of a cumulative infection rate of around 80% in the control arm was achieved and that both the concentration of *M. tuberculosis* H37Rv as well as the animal sample size were adequate to detect any possible differences between the two animal rooms

Statistical analyses using the Pearson chi-squared, Fisher's exact, and 1-sided Fisher's exact tests confirmed the limited antimicrobial efficacy of Actsol® on airborne transmission of *M. tuberculosis* H37Rv (Table 4).

Table 3. Guinea pig tuberculin skin test results, Phase III.

Exposure	Intervention (with fogged Actsol®)			Control (with fogged water)		
	TST +	TST-	Total	TST+	TST -	Total
First exposure	31	42	73	29	45	74
Second exposure	26	16	42	32	12	44
Combined data	57	16	73	61	12	73*

(*Note:1 guinea pig in the Control animal room 2 died between first and second exposure; TB was confirmed by histopathology) TST – Tuberculin Skin Test.

Table 4. Statistical tests for differences in infection rates between the animal rooms.

Exposure	Pearson chi-square	Fisher's exact	1-sided Fisher's exact
First exposure	0.1633 Pr = 0.686	0.739	0.407
Second exposure	1.1462 Pr = 0.284	0.359	0.200
Combined data	0.7070 Pr = 0.400	0.529	0.264

7.5 Discussion

The enhanced risk of nosocomial and iatrogenic spread of mycobacteria, especially drug-resistant strains of *M. tuberculosis*, is forcing a critical review of infection control in general and the claims of mycobactericidal activity of disinfectants in particular.

Mycobacteria are generally more resistant to in-vitro disinfection than enveloped viruses and other strains of vegetative bacteria; however, conventional *in- vitro* testing of disinfectant efficacy suffers from several methodological flaws. These include (i) a lack of proper quantification; (ii) unrealistically long contact times at higher than ambient temperatures; (iii) absence of a suitable organic load; (iv) ineffective neutralizers; (v) unsuitable surrogates for *M. tuberculosis*; (vi) improper recovery media; and (vii) inappropriate types of carriers. These many considerations make the available data on biocide product efficacy against TB unreliable.

Traditional fumigation of contaminated airspaces using conventional biocides and formaldehyde in particular has largely been discontinued due to its long exposure requirements, mycobacterial resistance, hazardous nature, irritancy to eyes, skin and respiratory mucosa and its role as a human carcinogen (Ayliffe *et al*., 1992; Griffiths *et al*., 1999; Middleton *et al*., 2000; Chapman, 2003; Johnston *et al*., 2005).

The kinetics of the disinfectant inactivation of the non-tuberculous *M.avium* has been reported to be two phased and it is characterised by an initial rapid inactivation stage coupled to a slower second stage which describes pseudo-first order kinetics (Luh and Mariñas, 2007). The $CT_{99.9\%}$ (Concentration [mg/L] x Time [minutes] for a $3Log_{10}$

reduction in microbial count) of the chlorine compounds for the *M. avium* strains was shown to be up to 2,300 times that which was calculated for *E. coli* under equivalent test conditions (Taylor *et al*., 2000).

The inconsistent efficacy of disinfectants against different Mycobacterial strains (Rikimaru *et al*. 2000) as well as the inappropriate choice of less resistant mycobacterial strains as surrogate indicators of *M.tuberculosis* susceptibility, exacerbates the difficulty of quantifying consistently reliable disinfectant methodologies against these critical disease causing microbes (Best *et al*. 1990; Sattar *et al*. 1995). It has been reported that the physiological profile of the strain plays a substantial role wherein the slow growing *Mycobacterium avium* was shown to be 10 fold more resistant to oxidant disinfectants than other faster growing Mycobacterial strains (Chapman, 2003). It been shown that disinfectants with general biocidal efficacy against most other microorganisms are not necessarily effective against *M. tuberculosis* and that the tuberculocidal activity of disinfectants varies greatly depending on the mycobacterial species assessed (Best *et al*., 1990; Sattar *et al*., 1995; Griffiths *et al.*, 1999; Rikimaru *et al*., 2000).

To date, there has been a limited number studies reporting on the use of atomised electrolysed oxidant solutions for airspace decontamination. Wu *et al*., (2008) describe a 4 $\log_{10}$ reduction in *B. subtilis* spores after 45 minutes of ultrasonic nebulisation of dental artefacts in an enclosed and static airspace with acidic (pH 2.5) electrolysed oxidant solution The chlorine based oxidant concentration applied over the entire exposure period equated to a total Free Available Chlorine (FAC) dose of 90,000 mg. In a similar report, anolyte at a pH of 5.2 was used to determine the antimicrobial efficacy of a fog treatment against methicillin-resistant *Staphylococcus aureus* (MRSA) and *Acinetobacter baumannii* inoculated onto ceramic tiles within an enclosed airspace. A single fogging treatment was reported to result in a 10^4 fold reduction for MRSA and a $10^{5.8}$ fold reduction for *A. baumannii*. The output of the fogging system employed was 19 liter per minute, and with an oxidant FAC concentration of 180 mg/L, this equated to 34,200mg FAC delivered into a static airspace during the 10 minute exposure period (Clark *et al*., 2006).

In accordance with the extensive range of electrolytic reactor systems available, there is an equally diverse array of oxidant solution types that can be appraised (Bakhir, 1999; Gauw *et al*., 1999; Selkon *et al.*, 1999; Shetty *et al*., 1999; Nakae and Inaba, 2000; Sampson and Muir, 2002). Variations in solution pH are acknowledged to reflect significant differences in the predominant reactive chlorine species in the solution. In a free chlorine inactivation study of *Mycobacterium avium,* it was reported that at a pH of 6, the primary inactivation was due to hypochlorous acid and that the biocidal contribution of the hypochlorite ion was insignificant (Luh and Mariñas, 2007). Highly acidic anolyte solutions (pH<3) are known to comprise of mainly aqueous chlorine, neutral anolytes comprise predominantly of hypochlorous acid, while alkaline anolytes are near exclusively hypochorite ion based (White, 1992; Bakhir *et al*., 2003).

An initial screening study of the inactivation efficacy of a 10% (v:v) strength Actsol® solution (pH 6) against a 6 $\log_{10}$ suspension of *M. smegmatis*, revealed a 99.7% kill rate after 10 minutes, while a 1 minute exposure to a 90% (v:v) strength anolyte solution, resulted in a >99.9 inactivation of the original titre. Similarly, when a 6 $\log_{10}$ challenge of a washed clinical isolate of *M. tuberculosis* suspension was exposed to a 50% (v:v) anolyte solution, 99.9% inactivation was achieved after 5 minutes. An equivalent exposure duration to a 90% (v:v) anolyte concentration resulted in a total inactivation of the original challenge. However exposure of an unwashed expectorated sputum sample containing 6 $\log_{10}$ *M. tuberculosis* organisms did nor display any inactivation after a 15 minutes exposure to a 50% (v:v) strength anolyte solution (van Zyl *et al*., 2001). This finding confirms the antibiocidal nature of sputum as detailed in an earlier report (Middleton, *et al*, 2000).

In addition, this finding confirms the contention that tubercule bacilli in sputum display a greater resistance to disinfectant activity, and it is proposed that the active biocidal agent needs to penetrate through the surrounding organic matter without becoming neutralised prior to exerting any inactivating effect (Best *et al.*, 1990; Sattar *et al.*, 1995; Cloete, 2003). In-vitro studies using poloxamer gel constructs to mimic the extra-cellular polysaccharide capacity of microbes to neutralise biocidal agents, substantially confirms the neutralising effect that sputum or an equivalent

extracellular organic matrix would exert upon a targeted biocidal intervention (Wirtanen *et al,*. 1998).

The unique mycolic acid based cell wall structure of Mycobacteria is also deemed to play a pivotal role in determining the specific chlorine demand required for microbial inactivation. In support of this assertion, Helbling and VanBriesen (2007), report that the chlorine contact time for a 3 log inactivation of *Escherichia coli, Staphylococcus epidemidis* and *Mycobacterium aurum* was 0.032±0.009, 0.221±0.08 and 42.9±2.71 mg min/L respectively.

Phases I and II of this study indicated that Actsol® when fogged according to the protocol specifications, has the potential to reduce the number concentration (cfu/m^3) of *B. subtilis, M. parafortuitum*, and *S. marcescens* by at least 90%. Thus fogged Actsol® displays distinctive promise for its ability in killing, inactivating or agglomerating aerosols of these three organisms, and indicates a potential for selective air disinfection in non-TB settings after continuous exposure for at least 40 minutes. Results from this study confirm previous observations (Rikimaru *et al.,* 2000) from conventional *in-vitro* testing of disinfectants, indicating that *M. tuberculosis* is more resistant to conventional biocides than other bacteria. By extrapolating from the antimicrobial efficacy data generated from the direct sampling of aerosolised microbial strains after Actsol® exposure, a presumptive model was extended to the indirect assessment of the capacity of fogged Actsol® to reduce the airborne transmission of *M. tuberculosis.* However, the design of the current protocol did not achieve a statistically significant reduction in infection rates in exposed guinea pigs and therefore should not be considered as an appropriate room air disinfectant in TB settings under the specific application parameters as outlined above.

Phase II of the study enabled the refinement of the optimal target concentration of *M. tuberculosis* H37Rv to be used for assessing infection rates in the experimental animal component of the study. This phase confirmed that the exposure concentration was appropriate for the achievement of an infection rate of just over 80% in the control animals. In the absence of an alternative methodology to culture *M. tuberculosis* from the air, a biological approach such as the AIR Facility model may contribute to the study of the biocidal potential of disinfectants to reduce or eliminate airborne TB

infection in health care settings. The use of standardised protocols to assess *in-vivo* efficacy of disinfectants should thus make evaluation of such agents more precise and reliable.

Further to the findings of the phase II data, it was established that a 40 minute lag period was the minimum time period that was required prior to the attainment of Actsol® equilibrium in the aerosolised airspace. This equates to the minimum time period that would be needed before the minimum inhibitory concentration of aerosolised Actsol® required for optimal (>90%) airborne microbial inactivation would be achieved. From a theoretical perspective, the prescribed nebulisation rate (100ml of 5 x 10^2 CFU/ml over 20 minutes), would equate to 7.5 x 10^3 aerosolised Mtb organisms being extracted from the three patient wards within the first 10 minute period (equal to 1 ACH). Over the same 10 minute period, the amount of Actsol® dosed into the system would have been 400ml. At a Free Available Chlorine (FAC) concentration of 180mg/L, this equates to a total of 72mg FAC. When diluted throughout the three patient ward air volumes of 112m^3 (=1 ACH volume), this equates to 0.64mg FAC/m^3. The same airspace would theoretically contain 670 CFU/m^3 aerosolised Mtb organisms and would have been supplied to the 12.2 m^3 of airspace housing the susceptible guinea pigs.

It is also likely that the previously reported FAC attenuating effects exerted through increased chlorine demand by specifically mycobacterial species (Helbling and VanBriesen, 2007), as well as the substantive reduction of FAC that follows inappropriate atomisation of the anolyte solution (Hsu *et al.*, 2004), would have further reduced the concentration of free chlorine available that would have been required to effect any mycobacterial inactivation. The latter study reported up to 97% reduction of FAC concentration and oxidation reduction potential of electrolysed oxidant solutions that had been atomised through small orifice nozzles, and it was proposed that the reduced volume to surface area ratio of the atomised droplet would substantively contribute to the increased volatilisation of chlorine and hence loss of mycobactericidal activity under these conditions.

While the minimum infectious dose of aerosolised *M. tuberculosis* organisms required to produce a positive TST reaction was not been determined during this study, it has

been reported that a single viable organism may be all that is necessary to result in patent disease (Twang *et al*., 2006).

Thus it thus feasible that a sufficient dose of infectious aerosolised Mtb microorganisms would have been extracted from the patient wards during the initial 10 minutes of Actsol® application. This initial air volume would have been extracted well before the projected time by which the Actsol® MIC equilibrium would have been achieved. This initial ACH would have contained a theoretical dose of 7.5×10^3 potentially infectious CFU and would have been supplied directly to the susceptible animals in the exposure room. To this end, it is possible that substantially improved inactivation statistics for *M. tuberculosis* H37Rv may have been achieved had the patient wards been pre-conditioned with the atomised anolyte solution for a minimum period of 40 minutes prior to the aerosolisation of the challenge microorganism. This contention is supported by the reported need to apply a pre-conditioning period where UV irradiation was assessed against airborne microbial challenge (Griffiths *et al*., 2005).

While no definitive delivery system for the aerosol application of Actsol® or directly equivalent solutions has been reported, the associates of the inventor (Bakhir) have patented a delivery system that is purported to address the engineering shortfalls of previously ineffective atomisation attempts (Tsikoridze *et al*., 2000).

7.6 Conclusions

Based on the findings of this study, the proposed Actsol® application protocol and its specifically prescribed application parameters should not be recommended for air disinfection in TB settings. Nevertheless, the non-toxic nature of Actsol®, together with the reduction in the viable counts of selected bioaerosols demonstrated in the study, justify further investigation.

Based on the findings of the study, it is clear that biocidal interventions to address environmental decontamination requires a holistic understanding of both the manifold biological aspects of the intervention strategy as well as the mechanical or engineering constraints that the intervention may entail.

On a more fundamental level, issues that should receive additional attention include the mechanisms leading to the change in particle size distribution, the ideal bactericidal composition of Actsol® solution, the optimal bactericidal concentration (mg/m^3) of Actsol® applied to the airspace, the ideal particle characteristics for maximum bactericidal action, as well as the design of the equipment to maximize Anolyte aerosolisation.

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7.8 References

Aertsen, A. and Michiels C.W. (2004) Stress and how bacteria cope with death and survival. *Critical Reviews in Microbiology*. 30, 263-273.

Ayliffe,G.A.J., Lowbury, E.J.L., Geddes, A.M. and Williams, J.D. (1992). Control of Hospital Infection, A practical handbook. (3rd ed.). Chapman and Hall Medical.

Bakhir, V.M. (1999). Theoretical aspects of Electrochemical Activation. Proceedings of the Second International Symposium – Electrochemical Activation in Medicine, Agriculture and Medicine, Moscow, 57-68.

Bakhir, V.M., Leonov, B.I., Panicheva, S.A., Prilutsky, V.I. and Shomovskaya, N.Y. (2003). Issues of chemical composition and operating properties of chlorine based inorganic liquid chemical germicides. http://www.bakhir.ru/issueseng.html.

Best, M., Sattar, S.A., Springthorpe, V.S. and Kennedy, M.E. (1990). Efficacies of selected disinfectants against *Mycobacterium tuberculosis*. *Journal Clinical Microbiology*. 28 (10), 2234-2239.

Centers for Disease Control and Prevention (2005). Guidelines for preventing the transmission of *Mycobacterium tuberculosis* in health-care settings, *MMWR* 54 (No. RR-17), 1-141.

Chapman, J.S. (2003). Disinfectant resistance mechanisms, cross-resistance and co-resistance. *International Biodeterioration and Biodegradation*. 51 (4), 271-276.

Clark, J., Barrett, S.P., Rogers, M. and Stapleton, R. (2006). Efficacy of super-oxidized water fogging in environmental decontamination. *Journal of Hospital Infection*. 64, 386-390.

Claassens, A. (2006). Report No. 2004. University of Pretoria, Department of Plant Production and Soil Science.

Cloete, T.E. (2003). Resistance mechanisms of bacteria to antimicrobial compounds. *International Biodeterioration and Degradation*. 51 (4), 277-282.

Food Consulting Services (2006). Specific gravity determination, Report # FCS2271206.

Gauw, R.D., Emmert, G.L., Bubnis, B. and Gordon, G. (1999). High resolution spectrophotometry for identification of chlorine dioxide in concentrated chlorine solutions. *Talanta*. 50, 1073-1078.

Griffiths, P.A., Babb, J.R.and Fraise A.P. (1999) Mycobactericidal activity of selected disinfectants using a quantitative suspension test. *Journal of Hospital Infection*. 41 (2), 111-121.

Griffiths, W.D., Bennett, A., Speight, S. and Parks, S. (2005). Determining the performance of a commercial air purification system for reducing airborne contamination using model micro-organisms: a new test methodology. *Journal of Hospital Infection*. 61, 242 – 247.

Helbling, D.E. and VanBriesen, J.M. (2007). Free chlorine demand and cell survival in microbial suspaensions. *Water Research.* 41, 4424-4434.

Hsu, S-Y., Kim, C., Hung, Y-C. and Prussia, S.E. (2004). Effect of spraying on chemical properties and bactericidal efficacy of electrolysed oxidizing water. *International Journal of Food Science and Technology*. 39, 157-165.

Johnston, M.D., Lawson, S. and Otter, J.A. (2005). Evaluation of hydrogen peroxide vapour as a method for the decontamination of surfaces contaminated with *Clostridium botulinum* spores. *Journal of Microbiological Methods*. 60, 403-411.

Luh, J. and Mariñas, B.J. (2007). Inactivation of Mycobacterium avium with free chlorine. *Environmental Science and Technology*. 41 (1), 5096-5102.

Marais J.T. and Brözel V.S. (1999). Electro-chemically activated water in dental unit water lines. *British Dental Journal*. 187, 154-158.

Marais, J.T. (2002). Biocompatibility of electrochemically activated aqueous solutions: an animal study. *SA Dental Journal*. 57 (1), 12-16.

Middleton, A.M., Chadwick, M.V., Sanderson, J.L. and Gaya, H. (2000). Comparison of a solution of super-oxidised water (Sterilox®) with Gluteraldehyde for the disinfection of Bronchoscopes, contaminated in-vitro with *Mycobacterium tuberculosis* and *Mycobacterium avium-intracellulare* in sputum. *Journal of Hospital Infection*. 45, 278-282.

Nakae, H. and Inaba, H. (2000). Effectiveness of Electrolysed oxidised water irrigation in a burn-wound infection model. *Journal of Trauma*. 49, 511-514.

Peccia, J. and Hernadez, M. (2001). Photoreactivation in airborne *Mycobacterium parafortuitum*. *Applied and Environmental Microbiology*. 67 (9), 4225-4232.

Prilutsky, V.I. and Bakhir, V. (1997). Electrochemically Activated Water: Anomalous properties, Mechanism of biological action, All Russian Scientific Research and Experimental Institute of Medical Engineering (VNIIIMT).

Prince, E.L., Muir, A.V.G., Thomas, W.M., Stollard, R.J., Sampson, M. and Lewis, J.A. (2002). An evaluation of the efficacy of Aqualox for microbiological control of Industrial cooling tower systems. *Journal of Hospital Infection*. 52, 243-249.

Rikimaru, T., Kondo, M., Kondo, S. and Oizumi, K. (2000). Efficacy of common antiseptics against mycobacteria. *The International Journal of Tuberculosis and Lung Disease*. 4 (6), 570-576.

Sampson, M.N. and Muir, A.V.G. (2002). Not all super-oxidised waters are the same. *Journal of Hospital Infection*. 52 (3), 227-228.

Sattar, S.A., Best, M., Springthorpe, V.S. and Sanani G. (1995). Mycobactericidal testing of disinfectants: an update. *Journal of Hospital Infection*. 30, 372-382.

Selkon J.B., Babb, J.R. and Morris, R. (1999). Evaluation of the antimicrobial activity of a new super-oxidized water, Sterilox®, for the disinfection of endoscopes. *Journal of Hospital Infection*. 41, 59-70.

Shetty, N., Srinivasan, S., Holton, J. and Ridgway G.L. (1999). Evaluation of microbiocidal activity of a new disinfectant: Sterilox® 2500 against *Clostridium difficile* spores, *Helicobacter pylori*, vancomycin resistant *Enterococcus* species, *Candida albicans* and several *Mycobacterium* species. *Journal of Hospital Infection*. 41, 101-105.

Talor, R.H., Falkinham III, J.O., Norton, C.D. and LeChevallier, M.W. (2000). Chlorine, Chloramine, Chlorine dioxide and Ozone susceptibility of *Mycobacterium avium*. *Applied and Environmental Microbiology*. 66 (4), 1702-1705.

Tanaka, H., Hirakata, Y., Kaku, M., Yoshida, R., Takemura, H., Mizukane, R., *et al*., (1996). Antimicrobial activity of superoxidised water. *Journal of Hospital Infection*. 34, 43-49.

Tsikoridze, N.G., Bakhir, V.M., Zadorozhny, Yu, G. Yakovlev, Yu, N., Maleev, B.V., Panicheva, S.A. and Vtorenko, V.I. (2000). A method for indoor disinfection. RF 148414, (published 10.05.2000).

Twang, J.W., Li, Y., Eames, I., Chan, P.K.S. and Ridgeway, G.L. (2006). Factors involved in the aerosol transmission of infection and control of ventilation in healthcare premises. *Journal of Hospital Infection*. 64, 100-114.

van Zyl, J.M., Smit, J., Sirgel, F. And Van der Walt, B. (2001). Report on the anti-microbial and therapeutic potential of electrochemically activated solutions (ECA).Commissioned study. Faculty of Health Sciences, University of Stellenbosch.

White, G.C. (1992). The handbook of Chlorination and Alternative disinfectants. (3rd ed.). Van Nostrand Reinhold.

Wiegeshaus, E.H, McMurray, D.N, Grover, A.A, Harding, G.E. and Smith, D.W. (1970). Host-parasite relationships in experimental airborne tuberculosis. 3. Relevance of microbial enumeration to acquired resistance in guinea pigs. *American Review of Respiratory Disease*. 102 (3), 422-429.

Wirtanen, G., Salo, S., Allison, D.G., Mattila-Sandholm, T. and Gilbert, P. (1998). Performance evaluation of disinfectant formulations using poloxamer-hydrogel biofilm constructs. *Journal of Applied Microbiology*. 85, 965-971.

World Health Organization (1999). Guidelines for the Prevention of Tuberculosis in Health Care Facilities in Resource-Limited Settings, WHO/CDS/TB/99.269, 1-51.

Wu, G., Yu, X. and Gu, Z. (2008). Ultrasonically nebulised electrolyte oxidising water: a promising new infection control programme for impressions, metals and gypsum casts used in dental hospitals. *Journal of Hospital Infection*. 68 (4), 348-354.

Zinkevitch, V., Beech, I.B., Tapper, R.C. and Bogdarina, I. (2000). The effect of super-oxidised water on *Escherichia coli. Journal of Hospital Infection*. 46 (2), 153-156.