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Atomic force microscopy study of the biocidal effect of super-oxidised water, Sterilox

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ABSTRACT

Sterilox is a new biocide produced with an electrochemical system which is marketed by Sterilox Medical Ltd. Sterilox is used in the medical industry in place of glutaraldehyde to cold-sterilise surgical instruments such as endoscopes, and is currently being considered for use in controlling microbiologically-influenced corrosion. The

device is simple in construction and operates on the electrodecomposition of brine.

The current study investigates the mode of action of the biocide upon planktonic populations of the sulfate-reducing bacterium (SRB) *Desulfovibrio indonensis* NCIMB 13468, isolated from a severe corrosion failure. The effect of biocide on SRB cells was evaluated using Atomic Force Microscopy (AFM), a technique which allows three-dimensional imaging of bacterial cells in their hydrated state. A novel method of immobilising bacterial cells was designed using *E. Coli* NCTC 9001 in order to image cells free from interferences such as salts and detritus from the culture medium.

Keywords: Sterilox, sulphate-reducing bacteria, SRB, Atomic Force Microscopy, AFM, biocide.

INTRODUCTION

The formation of a biological fouling layer on surfaces in industrial systems is a widespread phenomenon. Marine biofouling occurs wherever seawater contacts a surface. This may be in such diverse locations as cooling water systems, piping, ship hulls and reverse osmosis systems in desalination plants. In the UK, the cost of such fouling has been estimated at UKL600-1000 million per annum, a figure which approximates to 0.5 % of the British GNP (1976) (Pritchard, 1981).

When the presence of a biofilm influences the corrosion process, it is known as a biocorrosion phenomenon. This is more commonly termed microbiologically-influenced corrosion (MIC). The annual loss to the US Navy has been estimated at $5 billion due to such corrosion failures (Walch, 1991).

A wide range of biocides, chemicals used to control biofouling and biocorrosion, have been employed to date, with varying degrees of success. These include glutaraldehyde, chlorine, ozone, bromine and quaternary ammonium salts (Bott, 1994; Videla, 1996). The oxidising biocides are the most commonly applied, and include chlorine, hypochlorite, chlorine dioxide, bromine and ozone. Chlorine has wide applications in the water industry, although its use presents several disadvantages including the formation of toxic by-products such as trichloromethane (Cocnet *et al*., 1986). Chlorine dioxide, chloramides or chloramines are often used as alternatives to chlorine (Latshaw, 1995; Videla, 1996).

Traditional biocides such as chlorine have been subject to recent restrictions arising from legislative requirements

and environmental concern; these have led to the development of several new biocides.

The Sterilox 2500 device is a compact electrochemical biocide generator, which uses saturated saline to produce an anolyte solution containing 150- 170 ppm HClO and less than 7.5 ppm HClO2, ClO2 or Cl2. The system is currently employed for the rapid and total disinfection of a wide spectrum of microorganisms on endoscopes (Selkon *et* al., 1998) and other cold-sterilised instruments where heat sterilisation is unfeasible. It is beginning to replace the use of Glutaraldehyde due to its greater efficiency and safer ease of handling. Sterilox biocide will be undergoing US Food and Drug Administration approval, and is believed to be non- irritating and non-sensitising to the skin, non-irritating to the eyes and not harmful if swallowed. In addition, when tested in water, it is believed that Sterilox showed no evidence of mutagenic activity.

The University of Portsmouth and Sterilox Medical Ltd. began collaborative studies in 1996 in a venture to assess the feasibility of extending the applications of Sterilox to the control of marine biofouling and biocorrosion. Sterilox Medical Ltd. has a large research and development programme underway, and is currently considering the biocide for a number of other applications, including:

- Cleansing of food and seeds,
- Conservation of waterlogged wooden artefacts,
- Decontamination of biological and chemical warfare agents,
- Sanitisation of drinking water,
- Treatment of medical conditions, for example varicose ulcers

The sulfate-reducing bacteria (SRB) are a diverse group of obligate anaerobes implicated in 95% of cases of biocorrosion (Hamilton, 1985) and were hence studied. It has been found in preliminary work that Sterilox is a potent agent towards the marine SRB *Desulfovibrio indonensis* and that the Sterilox device can be run using seawater alone. Therefore work was undertaken to investigate the mode of action of the biocide against this bacterium applying the state-of-the-art technique of Atomic Force Microscopy. Preliminary work had shown AFM to be able to detect small changes resulting from bacterial response to the presence of biocide, building on the application of the technique to biofilm research (Beech, 1996 and references therein).

MATERIALS AND METHODS

Cell Immobilisation procedure

Escherichia coli NCTC 9001 was used as a model to develop a suitable method of cell immobilisation. It was grown in Nutrient Broth (Oxoid) for 24 h on a mechanical shaker (80 rpm) at room temperature (25 C). The culture was diluted with 10 vol. Sterilox (or sterile saline as control) prior to contact mode AFM imaging.

A modified Gram-staining technique was used to affix the planktonic cells to a glass substrate so that they could be imaged using contact mode AFM without the presence of detritus, and salt crystals. The bacteria required fixing so that they would not be displaced by the action of the AFM tip. A few drops of the cell suspension were deposited on a 1 cm^2 glass slide and left to dry at room temperature. After the water layer had evaporated, the cells were heat-fixed by passing over a blue Bunsen-burner flame. Fixed samples were then washed with sterile distilled water to remove any salts present, and dried in air under ambient conditions.

AFM imaging

Atomic force microscopy studies were carried out with a Discoverer TopoMetrix TMX2000 scanning probe microscope (TopoMetrix Corporation, Essex, UK), using a scanner capable of a maximum x,y translation of 75 æm x 75 æm. The AFM was operated in contact mode employing standard profile silicon nitride tips mounted on cantilevers of force-constant 0.036 Nm^-1. Graphical output was displayed on a monitor with a resolution of 500 lines x 500 pixels. Images were levelled by plane-fitting and shading was used to enhance topographic features where appropriate.

Generation of Sterilox biocide

The biocide was produced by pumping distilled water through the Sterilox 2500 (Sterilox Medical Ltd.) at a constant 800 cm^3 min^-1 via twin electrolysis units. The biocide was produced by the electrolytic decomposition of saturated saline (3.5 %) at a current of 9A to produce anolyte with a pH of 4.5.

AFM study of Desulfovibrio indonensis

Desulfovibrio indonensis NCIMB 13468, isolated and characterised as described elsewhere (Feio *et al*., 1998) was grown in marine Postgate medium C. A 3-day old culture grown at 37 C was mixed with Sterilox solution (10% v/v)

for varying periods of time at 37 C under anaerobic conditions prior to immobilising and AFM imaging conducted as described above.

RESULTS AND DISCUSSION

AFM study of E. coli

AFM studies showed that, prior to exposure to Sterilox biocide, the *E. coli* cells formed aggregates due to the presence of extracellular polymeric substances (EPS), although some individual cells were observed (Fig. 1a). After 30 s of exposure to Sterilox (Figure 1b), the cells increased in size, presumably due to the action of the biocide on the cell wall. After 1 min. of treatment with the biocide (Fig. 1c), the cells were observed to be trapped in a mass thought to be exopolymer. and/or cytoplasmic material. Finally, after 5 min. (Fig. 1d) of exposure to Sterilox, no intact cells could be seen, although a large amount of debris attributed to cytoplasmic material resulting from cell lysis was observed. Clearly, the biocidal action has the desired effect of destroying *E. coli* cells present in the ratio of biocide to cell ratio likely to be present in an endoscope washer, in the time frame desired (5 min.).

This study demonstrated that the modified Gram-staining technique could be used to firmly fix bacterial cells to a surface and allow unhindered imaging of individual bacteria, even in the presence of EPS/cytoplasmic material.

Fig. 1 a-d: AFM micrographs of *E. coli* cells exposed to Sterilox solution for 0, 0.5, 1 and 5 min. (a-d respectively). Scan range: 20 um x 20 um.

Figure 1a
Figure 1b
Figure 1c
Figure 1d

AFM study of Desulfovibrio indonensis

AFM observations gave some insight into the mode of action of the biocide upon SRB cells. Initially, there was a large amount of extracellular polymeric substances (EPS) present between the cells, which were approximately of

the original size and shape (Fig. 2a). After 30 min. (Figure 2b), the cells were increased in size and the EPS between the cells disappeared, although some material was produced locally around the cells (Fig. 2c). This could, however, be cytoplasmic material, since *D. indonensis* has a single polar flagellum (Feio *et al*., 1998), and the extrudate could be seen to emerge from one end of the bacterium only. The site at which the flagellum attaches to the cell wall could be a site of attack for the Sterilox. After 90 minutes the cells became irregularly shaped due to cell wall breakdown, and no traces of EPS could be observed (Fig. 2c). Surface features were more visible, owing to the drop of internal pressure within the cell. Finally, after 150 min., the changes in the cell wall structure were so vast that the cells adhered less to the glass upon flaming, or were lysed, leading to a dramatic reduction in the numbers of cells observed (Fig. 2d).

Fig. 2 a-d: AFM micrographs of *D. indonensis* NCIMB 13468 cells exposed to Sterilox solution for 0, 30, 90 and 150 minutes (a-d respectively). Scan range: 20 um x 20 um.

Figure 2a
Figure 2b
Figure 2c
Figure 2d

CONCLUSIONS

AFM has proven to be a suitable technique for the study of the antimicrobial effect of Sterilox produced by the Sterilox 2500. The technique yields qualitative information regarding changes in both the presence of EPS surrounding cells and changes to the cell wall.

The mode of action of the sterilising solution produced by the Sterilox 2500, Sterilox, would seem to be one of cell wall decomposition and the dissolution of extracellular polymeric substances. This suggests that the biocide would be effective on sessile cells as well as the planktonic cells studied, since the biofilm would be disrupted during cell lysis.

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