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A paint incorporating silver to control mixed biofilms containing *Legionella pneumophila*

J Rogers[1], AB Dowsett[2] and CW Keevil[1]

[1]*Research Division;* [2]*Resources Division, Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire, UK, SP4 OJG*

A three-stage chemostat containing a mixed consortium of microorganisms, including *Legionella pneumophila*, was used to determine the suitability of a silver-containing paint to control biofouling in water systems. The paint was efficient in controlling total surface colonisation by heterotrophic microorganisms and growth of the pathogen over a 2-week period. Biodiversity was limited in the presence of the silver paint and this was thought to help control *L. pneumophila* numbers. Glass control tiles suspended alongside the silver painted tiles also had reduced colonisation for the 2-week period, suggesting that low levels of silver leached from the paint surface. This loss of silver was confirmed since the inhibition of biofouling and inclusion of the pathogen was not maintained after the 2-week period. Although this paint was unsuitable for controlling biofouling over extended time periods, the data suggest that a reformulated paint or electrochemical method of introducing silver ions may be successful.

Keywords: *Legionella pneumophila*; aquatic biofilm modelling; biofilm control; silver inhibition; water microbiology

Introduction

The occurrence of *Legionella pneumophila* within water systems has led to outbreaks of disease arising from cooling towers [11], hot water systems [27] and potable waters [20]. The bacterium is incapable of growth in sterile water [21,30] but multiplies within water systems in the presence of bacteria [28], amoebae [18] and cyanobacteria [26]. High numbers of *L. pneumophila* have been associated with biofilms within these systems [1,3,15] and control measures have been developed, including the use of elevated temperatures [14,24], the use of chemical biocides [7,29] and UV irradiation [5]. These methods have not always been successful, due in part to the persistence of *L. pneumophila* in biofilms, and novel methods are being considered for particular applications.

Silver inhibits bacteria at low concentrations by binding to DNA and sulphydryl groups, resulting in impaired cell division, and by inactivating essential respiratory and metabolic enzymes [22,23,25]. A developmental paint which contained spheres of titanium dioxide coated with a thin layer of silver might be useful in controlling the colonisation of surfaces by pathogens in water systems, particularly where chemical additions are unwelcome and temperatures can not be raised. The advantage of a paint when compared with UV irradiation is that it can be applied to all surfaces; therefore the whole system will be protected. A reproducible continuous culture model of biofilm formation has previously been developed to study *L. pneumophila* in an aquatic consortium [16]. This system was adapted for the present study to investigate the inhibitory effects of the silver paint on biofilm and planktonic microorganisms.

Correspondence: Dr J Rogers, Microbial Technology Department, CAMR, Porton Down, Salisbury, Wiltshire, UK, SP4 OJG
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Materials and methods

Model system

Biofilms containing *L. pneumophila* were developed using a three-stage model system that had been adapted from that previously used [15]. The inoculum was sludge from the bottom of a calorifier responsible for an outbreak of Legionnaires' disease and this contained virulent *L. pneumophila* serogroup 1 Pontiac along with a diverse range of bacteria, amoebae and protozoa. The model system was operated at 30° C because the original inoculum was derived from a system operating at this temperature. The protozoal community included *Rotari neptunia*, *Lacrymaria* spp *Hartmannella vermiformis*, *Hartmannella cantabrigiensis* and *Verillifera bacillipedes* [15]. The naturally occurring mixed population of microorganisms was supplied with filter-sterilised tap water from a domestic potable supply as the sole source of growth medium. Water sterilised in this manner has been shown to remain chemically unaltered [2].

The model system consisted of three glass chemostat vessels linked in series (Figure 1). The first vessel provided a constant supply of microorganisms to the second test vessel where control biofilms were generated. The first vessel had a retention volume of 500 ml and the flow rate of sterile water into the vessel resulted in a dilution rate of 0.05 h^{-1} (equivalent to a mean generation time of 13.9 h). When the retention volume was exceeded, the effluent was pumped via an overflow into the second vessel. This second vessel was also supplied with additional sterile water for growth to maintain a total dilution rate of 0.2 h^{-1} (equivalent to a mean generation time of 3.5 h). Effluent from the vessel was pumped through a third vessel where the colonisation of silver-painted glass tiles could be determined before being pumped into a waste collection bottle.

Conditions within the vessels were controlled and monitored with Anglicon microprocessor control units (Brighton

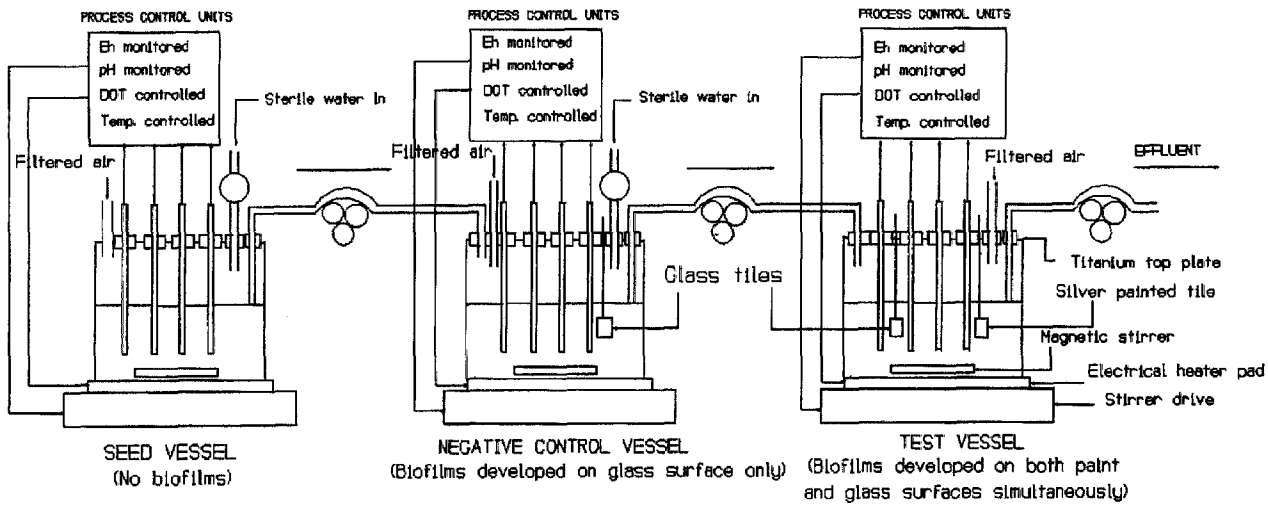

Figure 1 Schematic diagram of the three-stage biofilm system

Systems, Hove, UK). The temperature in the vessels was maintained at $30 \pm 0.1°$ C with proportional integral derivative controllers; the vessels were heated by an external electrical pad. Temperature was measured using a glass temperature probe inserted into the aqueous phase. The glass galvanic oxygen electrode was temperature-compensated and the dissolved oxygen tension was maintained at $20 \pm 0.5\%$ of air saturation via proportional control of the stirrer speed. This maintained a fluid velocity of 1–2 m s^{-1} over the biofilm surfaces. The pH and Eh of the cultures were monitored throughout the experiments.

Biofilm development

Biofilm development was compared on the surface of glass, and glass which had been coated with the silver-containing paint. The paint contained small titanium spheres coated with a thin film of silver. These could be applied to a range of materials using an elastomeric base. Glass tiles were cut from sections of optical-quality glass. The tiles had a surface area of 1 cm^2, and a 1-mm hole was drilled to enable them to be suspended within the chemostat on titanium wires. The tiles were cleaned with acetone to remove any oil or grease and then sterilised by gamma irradiation to avoid heating the paint. Glass tiles were introduced into the second vessel at day 0 to act as a control surface for comparison with the silver-painted tiles. Silver-painted glass tiles were suspended alongside glass tiles and these were inserted into the culture in the third vessel at day 0 so that the effect of the silver paint could be determined. Biofilm development was investigated over a 28-day period with tiles being removed after 1, 7, 14, 21 and 28 days. Biofilms were examined microbiologically and by microscopy.

Microbiological examination

The composition of the microbial population in the planktonic phase was determined by removing culture fluid aseptically via a sample port. The surfaces supporting the biofilms were retrieved and aseptically washed gently in 10 ml of sterile water to remove planktonic bacteria. The biofilm was removed from the tile surfaces by scraping with a sterile dental probe; the microorganisms were resuspended in sterile water by vortexing them for 30 s prior to serial dilution in sterile water.

Aliquots (0.1 ml) of planktonic and biofilm samples were plated in duplicate onto various selective and non-selective media. Non-legionellae populations were enumerated with a non-selective, low-nutrient R2A medium [13] to avoid substrate shock. Buffered charcoal-yeast extract agar (BCYE) [12] and selective buffered charcoal-yeast extract agar, supplemented with glycine, vancomycin, polymixin and cycloheximide (GVPC) [4], were used to determine the numbers of more fastidious bacteria including *L. pneumophila*. All plates were incubated for 7 days at 30° C. Those colonies on BCYE and GVPC that showed the characteristic ground glass appearance of *L. pneumophila* were subcultured onto BCYE and BCYE lacking cysteine. Organisms were presumptively identified as *L. pneumophila* if they were unable to grow in the absence of cysteine but were capable of growth on BCYE.

One plate of each medium, which contained 30–100 colonies and on which colony morphology could be distinguished, was selected for evaluation of population profiles for each biofilm of each age. Colonies of each type were subcultured three successive times onto double strength R2A (R3A) or BCYE prior to inoculation into the appropriate API (API bioMerieux, Basingstoke, UK) and Biolog (Biolog, Haywood, CA, USA) bacterial identification systems.

Microscopy of samples

Biofilms were examined using scanning electron microscopy. The tiles were removed from culture and gently rinsed in 10 ml of sterile water to remove planktonic bacteria. The biofilm was fixed and stained with 1% (w/v) osmium tetroxide in 0.1 mM phosphate buffer at pH 6.9 for 2 h and then serially dehydrated through an ethanol series prior to air drying from absolute ethanol. Tiles were mounted on 1.2-cm scanning electron microscope specimen stubs and coated with a 20-nm layer of gold in an Edwards 12E6 vacuum coating unit (Edwards High Vacuum International, Crawley, UK). They were examined in a Cambridge Stereoscan S2A (Leica Cambridge Ltd, Cambridge,

UK) scanning electron microscope operated at a 10-kV accelerating voltage.

Silver determination

Silver concentrations in the planktonic samples were determined using atomic absorption spectroscopy with electrothermal atomisation using a 10-μl sample.

Results

Planktonic phase

Prior to placing the silver-painted tiles into vessel three, the planktonic phase was similar, both in numbers and in composition, to that found in the first two vessels. The total number of microorganisms in the non-legionellae heterotrophic population was stable at approximately 1×10^5 CFU ml^{-1} (Figure 2a) and *L. pneumophila* represented approximately 10% of this total (Figure 2b). When the silver-painted tiles were introduced into the planktonic culture of the third vessel, total microbial numbers declined to 3.6×10^3 CFU ml^{-1}, a 70% reduction. This suppression of planktonic flora was maintained for a 21-day period but then numbers increased to a similar level to that previously found. The initial decline in total bacterial numbers in vessel three was concomitant with a similar reduction in numbers of *L. pneumophila* with numbers being reduced to approximately 10% of those occurring in the second culture vessel. During the trial period both the total non-legionellae and *L. pneumophila* numbers in the planktonic population of the control second vessel remained unperturbed. During the trial, planktonic samples were found to contain less than 1 μg L^{-1} of silver, indicating that if silver was leaching from the paint surface the amounts were low.

Colonisation of the surfaces

The control glass tiles that were colonised in the absence of silver paint (in vessel 2) rapidly attained high numbers of microorganisms on their surfaces; 3.05×10^5 CFU cm^{-2} were detected after only 24 h (Figure 3a). These high levels of colonisation were maintained throughout the trial with only a small reduction in numbers after 28 days. Colonisation of the silver-painted surface proceeded at a reduced rate compared with that of the glass control. After 24 h the

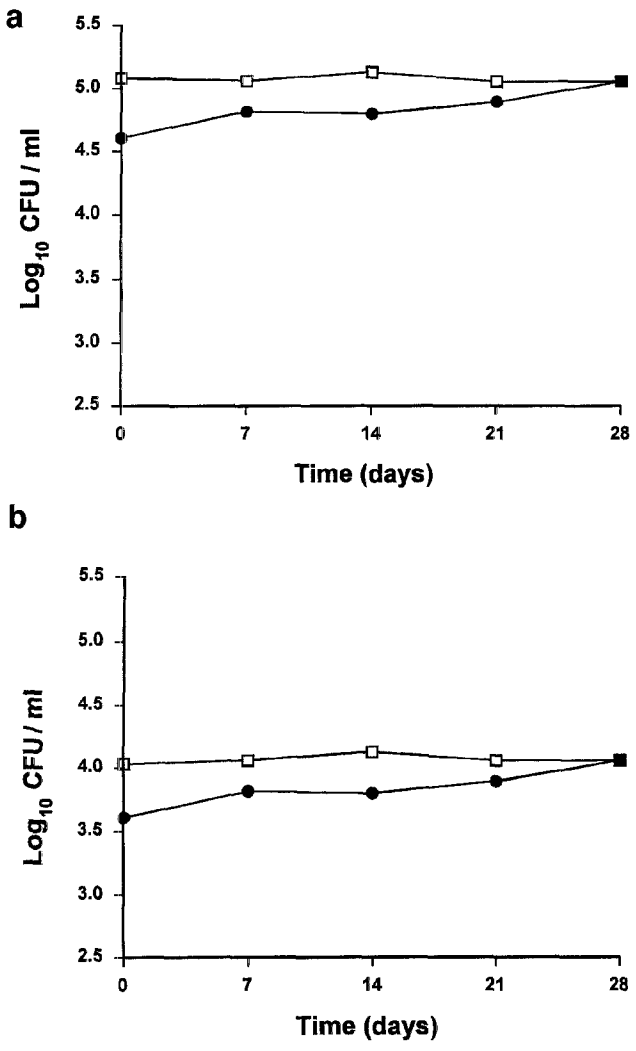

Figure 2 The total number of planktonic non-legionella bacteria (a) and *Legionella pneumophila* (b) in the model system in the presence (●) and absence (□) of silver-painted glass tiles

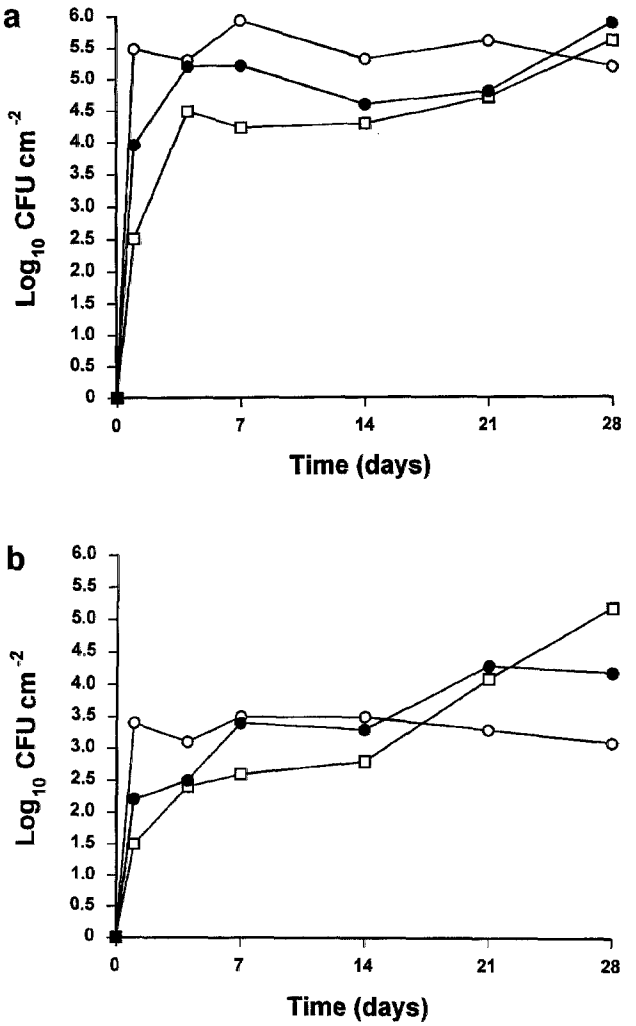

Figure 3 Colonisation by a complex microbial flora (a) and *Legionella pneumophila* (b) of untreated glass in the presence (●), and in the absence (○) of silver-painted glass tiles. Colonisation of the silver-painted tiles (□)

total non-legionellae numbers were 3.25×10^2 CFU cm^{-2}, only 0.1% of the colonisation that had occurred on the control glass tile over the same time period. The number of microorganisms in the biofilm remained at approximately 3×10^4 CFU cm^{-2} between day 4 and day 21 of the experiment. This represented a 90% reduction in the extent of biofouling over this time period when compared to the control glass surface in the second vessel. At 28 days, however, the silver paint offered no protection against biofouling, with bacterial numbers rising to 4×10^5 CFU cm^{-2}.

The initial colonisation of glass tiles suspended in the chemostat alongside the silver-painted tiles was slower than that on the control glass tiles. Biofouling was greater than that on the silver-painted surface, with biofilm formation being intermediate between the glass control and the painted surface. After 24 h the glass supported a biofilm community containing 9.1×10^3 CFU cm^{-2}, 34 times less than the biofouling on the glass control and 30 times greater than that on the painted surface. The presence of the silver-painted surface had an inhibitory effect on the numbers of microorganisms on the glass tiles over the first 21 days of the experiment, with approximately 6×10^4 CFU cm^{-2} occurring on the surface. At 28 days, bacterial numbers showed a similar increase to that observed on the silver-painted surface, with numbers increasing to 7.65×10^5 CFU cm^{-2}.

Inclusion of L. pneumophila *into biofilms*

In the absence of silver paint *L. pneumophila* was rapidly incorporated into biofilms on glass surfaces, with over 3×10^3 CFU cm^{-2} after only 24 h (Figure 3b). The numbers of *L. pneumophila* were slightly lower on day 4 but otherwise remained at approximately 4×10^3 CFU cm^{-2} for the duration of the experiment, ie 8% of the total population. The inclusion of *L. pneumophila* into the biofilm on the silver-painted surface was slow, with only 30 CFU cm^{-2} after 24 h (only 1% of that occurring on the glass control surface). This inhibitory effect of the silver paint was maintained over 15 days with numbers of *L. pneumophila* being reduced by 25% of that occurring on the glass control. However, the inhibitory effect was not sustained and the numbers of *L. pneumophila* increased during the trial until by 28 days numbers were greater than those occurring on the control glass surface.

Colonisation of the glass surface suspended alongside the silver paint showed an initial reduction in the colonisation rate by *L. pneumophila*. Again, however, this was not maintained, and by 7 days the numbers of the pathogen were similar to those on the control glass surface.

Population profiles of biofilm microorganisms

The pioneering species on the silver paint were *Methylobacterium* spp, accounting for 50% of the population on the silver-painted surface (Table 1). Other species present included *Pseudomonas maltophila, P. mendocina, P. strutzeri, Actinomycetes* spp, *Flavobacterium* spp and *Acinetobacter* spp. Species diversity increased after 4 days, possibly after more tolerant species formed a protective layer of cells on which these other less tolerant organisms could grow. After 28 days, the biofilm contained a diverse range of microorganisms in which the pseudomonads were the most abundant.

The diversity of microorganisms was greater on the glass tiles than on the silver-painted tiles. Although the initial dominant pioneers in the biofilm were the *Methylobacterium* spp, the pseudomonads remained predominant for the duration of the experiment, the most common being *P. maltophila, P. mendocina, P. paucimobilis* and *P. vesicularis*. One microorganism was cultured from biofilm samples on the initial BCYE isolation plate but could not be further subcultured: this was listed as 'other Gram-negative' (OGN) in the profiles.

Scanning electron microscopy

Glass surfaces that were colonised in vessel 2, in the absence of silver-painted tiles, appeared heavily colonised after 21 days (Figure 4a). The morphology of the attaching bacteria was varied with many types of cocci and short and long rods, mainly occurring within microcolonies. Glass tiles suspended alongside the silver-painted tiles in vessel 3 were obviously less colonised than the control glass tiles suspended in vessel two (Figure 4b). The tile had localised areas of colonisation, consisting of well-separated cells, mainly with bacteria of one morphological type. Thus, bacterial growth occurred but the rod-shaped bacteria did not appear robust and the low density of cells suggested that growth was suboptimal. Large areas between these microcolonies remained uncolonised.

The surface of the silver-painted tiles appeared very granular (Figure 4c), due to the presence of the titanium dioxide beads which were coated with a thin layer of silver. Most of the surface was uncolonised, but some small dense regions of biofilm existed, within which bacterial cells could not be easily distinguished because of the extracellular material. In some regions crystals had accumulated on the surface of the silver paint; possibly these were deposits from hard water (Figure 4d). Bacteria were observed on the surfaces of these crystals, suggesting that less tolerant microorganisms could occur on the inhibitory surface by residing on these structures and thus being physically isolated from toxic silver ions.

Discussion

These results suggest that the silver-containing paint was able to retard the initial colonisation and growth of the consortium of microorganisms, including *L. pneumophila*, for up to 14 days. Although silver was not neutralised prior to plating, the serial dilution of the samples prior to recovery would have greatly reduced the silver concentration of the sample. Examination of *L. pneumophila* within the biofilms by immunofluorescence [17] confirmed that only low numbers were present on the tile surfaces. Species diversity was reduced on the surface of the paint and this coincided with diminished numbers of *L. pneumophila* within the biofilm community. The low numbers of the pathogen could be due to direct inhibition of *L. pneumophila* by the silver ions. In sterile water *L. pneumophila* numbers decline over time [30]. In order for growth to be maintained the pathogen requires the presence of other microorganisms [31]. Growth of *L. pneumophila* may occur either intracellularly in the

Table 1 Comparison of the pioneering species and the members of the climax communities of the biofilms on glass and silver-painted tiles

Organism(s)	Pioneering species (CFU cm^{-2})		Climax community (CFU cm^{-2})	
	On glass	On silver paint	On glass	On silver paint
P. acidovorans			1.2×10^5	6.0×10^4
P. maltophila	9×10^3	2×10^2	1.7×10^6	1.0×10^5
P. mendocina	4×10^3	2×10^2		
P. paucimobilis	1×10^3		8.2×10^5	1.5×10^5
P. stutzeri	2.1×10^4	2×10^2		
P. testosteroni			4.0×10^5	5.0×10^5
P. vesicularis	9×10^3		9.0×10^5	7.0×10^5
P. xylesoxidans	4×10^3		2.5×10^6	8.5×10^5
Actinomycetes sp	1×10^2	5×10^2		2.0×10^4
Alcaligenes sp			1.0×10^4	2.0×10^4
Flavobacterium spp	1.8×10^2	6×10^2	1.3×10^5	5.0×10^4
Methylobacterium spp	5.7×10^4	3×10^3	6.0×10^5	6.2×10^5
Acinetobacter spp		6×10^2	5.4×10^5	4.4×10^5
Unidentified OGN	1.8×10^4	4×10^2	1.0×10^5	
L. pneumophila	1.8×10^2	3×10^2	1.1×10^5	3.5×10^5

Numbers are expressed as CFU gm^{-2}. Numbers of non-legionella populations are represented as a sum of those CFU cm^{-2} detected on R2A, BCYE and GVPC media. Pioneering species were determined at 24 h and climax communities at 21 days. OGN denotes other Gram-negative organisms

presence of amoebae [18] and other protozoa [6], or extracellularly when other bacterial species provide nutrients [17,28]. It is possible, therefore, that growth of *L. pneumophila* was prevented in the presence of the silver due to a suppression of microbial diversity, which resulted in an inability of the community of microorganisms to provide nutrients essential for its growth.

Biofilm development was not retarded by silver after 14 days. This was attributed to the depletion of silver from the surface of the paint, which then allowed the ingress of non-tolerant microorganisms into the biofilm community. Although silver was not detected in the planktonic cultures at the detection limit of the method used, the observed inhibition of the microorganisms in the planktonic cultures and biofilms on the unpainted glass tiles support the hypothesis that small amounts were lost from the paint. The calibrated limit of detection was 1 $\mu g\ L^{-1}$ which is routinely used for water samples where the maximum concentration permissible in UK drinking water is 10 $\mu g\ L^{-1}$. Although these data suggest that this paint formulation was unsuitable as a long term coating, other formulations containing higher levels of silver, or the continual addition of low levels of silver to the system, may be useful in controlling both biofouling and pathogen numbers. Pure cultures of *L. pneumophila* in filtered water are susceptible to electrolytically-generated copper and silver at concentrations of 40 $\mu g\ L^{-1}$ in the presence of 0.4 $\mu g\ L^{-1}$ free chlorine [9]. The model system described here could be useful in enabling the activity of this type of silver/copper ion-generating device to be tested against mixed communities of microorganisms and biofilm populations typical of those occurring in natural environments.

This work has demonstrated the usefulness of the chemostat model in providing defined, controlled and reproducible conditions for determining the suitability of control measures against *L. pneumophila*. Actual water systems are large structures and a representative sampling of the flora within them is difficult to achieve. A wide range of environmental gradients exist within the systems and the opportunities for *L. pneumophila* to grow vary accordingly. It is possible that *L. pneumophila* survive and grow within an area that is overlooked by sampling. There may be seasonally variable environmental conditions and therefore, long-term studies or comparisons of treatments would not yield comparable results. These water systems cannot be operated under 'worst case' conditions where high numbers of *L. pneumophila* are maintained since this would pose an unacceptable health risk. The model system can be operated to ensure that high numbers of *L. pneumophila* are maintained, enabling planktonic and biofilm effects to be easily and representatively sampled to determine inhibition.

Any model system should maintain a microbial community that resembles the ecosystem that it intends to represent. This system was developed to examine survival of the pathogen under conditions that closely simulated the natural environment. The use of sterile tap water as the sole source of nutrient for the growth of the microbial consortium was undoubtedly critical in ensuring that a suitable community of microorganisms was maintained. Amoebae and other protozoa were maintained as community members, some of which were known to be hosts for intracellular multiplication of *L. pneumophila* [31]. The presence of amoebae and other protozoa was considered essential for experimentation where inhibitory activity was to be assessed, since these organisms protect the pathogen from biocide treatments [8]. Mature amoebal cysts are thick-walled and may contain large numbers of motile legionellae [19]. It is therefore essential that any model system that investigates survival of the pathogen under adverse conditions contains suitable hosts for intracellular replication. Failure to ensure that these amoebae were present would result in falsely efficient data for the inhibition of the *L. pneumophila*.

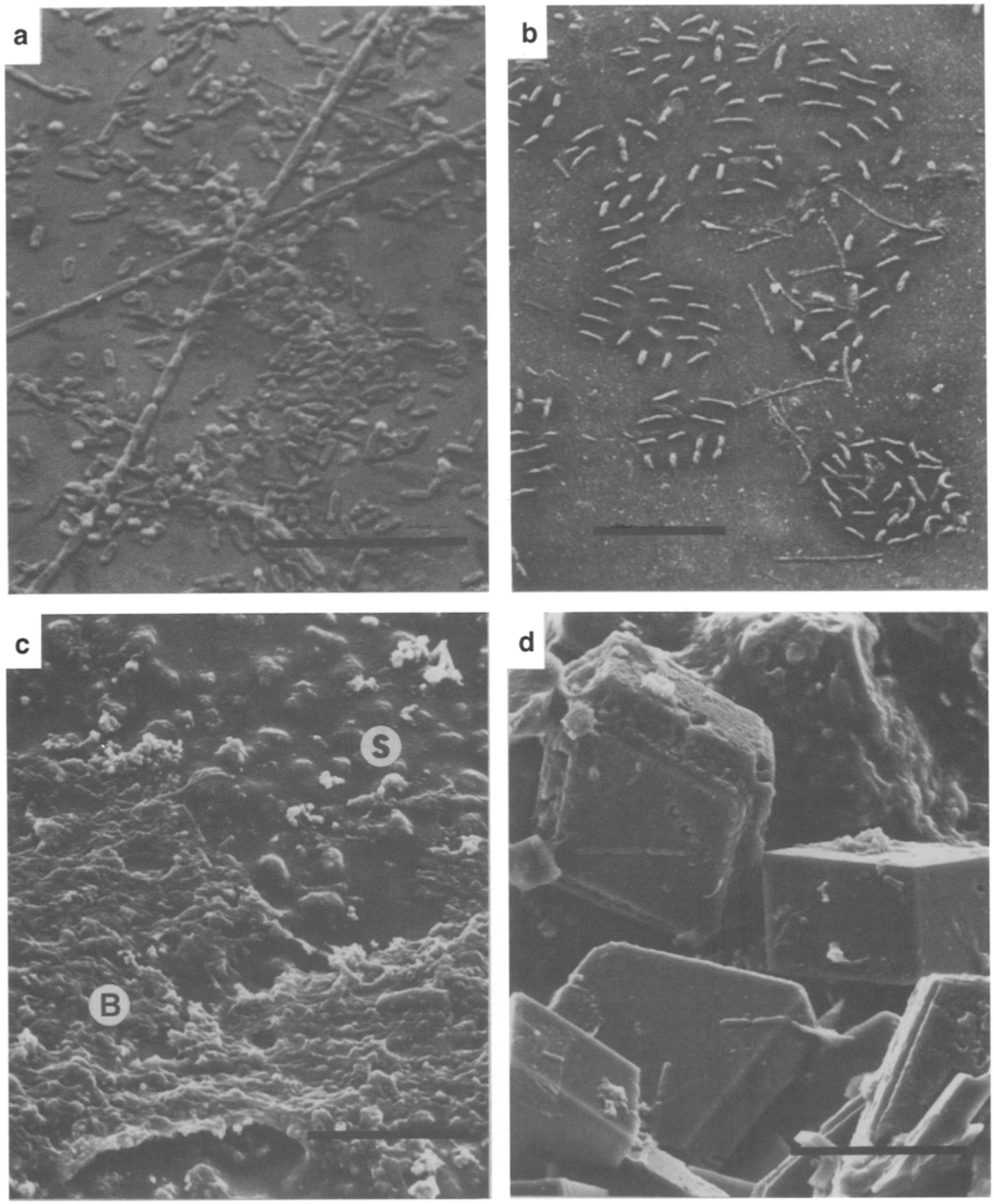

Figure 4 Scanning electron micrographs showed that glass surfaces were normally well-colonised after 21 days (a) but the presence of silver-painted tiles in the same vessel reduced the colonisation of the glass (b). The silver-painted surface (c) remained largely uncolonised with spheres of titanium dioxide (S) incorporated within the paint matrix. However, localised, but dense regions of biofilm (B) did occur on the painted surface. In some regions crystals were visible on the paint surface; these were thought to be calcium carbonate deposits accumulated from the water, and bacteria could be observed on their surfaces (d). Bars indicate 10 μm

The use of the multistage chemostat model allows for modification of the environmental conditions where biofilms are produced without loss of bacterial diversity or alterations in the populations of bacteria in the culture. The first vessel was used to provide a constant challenge of microorganisms for the other vessel in which silver inhibition could be assessed over the duration of the experiment. In batch or single stage continuous culture systems the initial inhibition of the microbial population, when silver ions were at their highest concentration, could reduce the microbial diversity of the whole system. If the level of silver had then been reduced to a concentration which was no longer inhibitory, efficiency could be overestimated since other bacteria may not then survive to colonise the

surfaces. Since the composition of the aquatic flora reflected those of the original calorifier responsible for the outbreak, these results are directly comparable with those previously obtained from the model system [14]. The silver paint was found to be considerably less colonised than the plastic and elastomeric materials previously examined in terms of both biofouling and inclusion of *L. pneumophila* at the onset of the experiment.

Although this work has been concerned with the control of *L. pneumophila*, this type of controlled testing using the continuous culture system may be useful for other pathogens which colonise biofilms in water systems, such as *Escherichia coli*, *Aeromonas hydrophila* or *Pseudomonas aeruginosa* [10].

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