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The effect of molybdenum on biofilm development

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Little is known about the formation and effects of biofilms on stainless steel pipes in freshwater environments, particularly as they are considered as a direct replacement for copper pipes for 'problem' water. There is some cause for concern especially as stainless steel cannot claim the inherent biocidal potential of copper. As molybdenum is known to be leached out of stainless steel grade 316, in very small amounts, a study was set up to see if molybdenum could retard the development of biofilms. When a comparison of biofilm viable and total cell counts was made between pure molybdenum metal and stainless steel grade 304, it was found that cell counts were significantly higher ($P < 0.05$) on grade 304 stainless steel after 5 weeks exposure to flowing water (0.64 m s^{-1}). Molybdenum (above a concentration of 1 g L^{-1}) affected the growth rate of *Acinetobacter* sp, a pioneering bacterium of biofilms in potable water.

Keywords: biofilms; stainless steel; potable water; bacteria; molybdenum

Introduction

Potable water contains low numbers of heterotrophic microorganisms which colonise pipe surfaces and develop into biofilms [6], often leading to a condition known as regrowth [7]. Microbial cells found as part of this biofilm contribute to the contamination of the water bulk phase possibly due to sloughing as a result of water shear [11]. These sloughed sections of biofilm may harbour potential opportunistic and pathogenic organisms which may be a health hazard in poorly chlorinated waters, particularly associated with domestic, commercial and municipal plumbing systems when deposited at consumers' taps [13,16]. The clean, low-chlorinated water environment, characteristic of potable water, is ideal for the use of stainless steel [1,8,9,14,16,21,22]. Stainless steel is also a strong candidate for those portions of the potable water system that are difficult to fabricate, or replace and where the greatest durability is required. Stainless steels are alloy steels which contain in addition to iron, chromium, nickel, molybdenum, and small amounts of other elements. Types 304 and 304L are the most widely used basic grades of the chromium-nickel stainless steel. Types 316 and 316L are the more corrosion-resistant grades which contain molybdenum in addition to chromium and nickel [16]. Stainless steels depend for their corrosion resistance on a thin, durable chromium oxide film that forms almost instantaneously in air and normal waters. The surface film has been analysed under electron spectroscopy for chemical analysis and energy dispersive X-ray analysis which has shown chromium, oxygen and carbon to be present in grade 304 with molybdenum also present in grade 316 [16]. This molybdenum greatly enhances resistance to localised corrosion, which gives grade 316 an advantage over grade 304 particularly in corrosive environments.

Grade 304 stainless steel is more susceptible to biofilm development in potable water than grade 316 [15–17]. This may be due to the presence of molybdenum in the passive layer of stainless steel grade 316 retarding bacterial growth when leached out into the biofilm [15,18]. It is well known that molybdenum has an inhibitory effect on sulphate-reducing bacteria (SRB) [2,3,5]. Its effects on heterotrophic potable water bacteria have however not been studied.

Stainless steel does not seem to have any inherent biocidal quality when compared to that of its direct replacement, copper [4]. It is however possible that molybdenum could have some effect on biofilm development in potable water. Molybdenum metal in its pure state was chosen as a starting point to look at the effects of very high levels of molybdenum on biofilm development before more extensive studies with high molybdenum (6%) alloys could be undertaken. Therefore, this study was set up to observe the effects of pure molybdenum on potable water biofilm cell counts and community structure.

Materials and methods

Stainless steel and molybdenum slide preparation

All pipes and metal strips were degreased in acetone by cleaning them in an ultrasonic bath (Sonicor SC-50-22H) for 5 min, and sterilised in 70% boiling alcohol for 15 min prior to use, to remove any contaminating bacteria. Observation using a Cam series 4 SEM (Cambridge, UK) showed there were no surface effects of this sterilisation process.

Surface roughness measurements of stainless steel grade 304 and molybdenum metal

Eight representative slide sections, of 2B (smooth) finish 304 stainless steel and pure molybdenum metal (10 cm long, 1.9 cm wide and 2 mm thick) were profiled on a Hobson-Taylor Taly-surf, to determine mean surface roughness over a 0.6-cm profile distance. All sections were analysed

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randomly six times for their mean surface roughness (Ra—the arithmetic mean of the absolute departures of the roughness profile from the mean line) values.

Energy dispersive X-ray analysis (EDAX)

Sections of stainless steel and molybdenum slides (1 cm^2) were examined using a CamScan Series 4 SEM attached with a Link 860 EDX system (Cambridge, UK). This enabled confirmation of the chemical elements present on the surface layers of stainless steel and molybdenum metal before exposure to potable water.

Effect of molybdenum metal on sessile potable water bacteria

A system containing two parallel 2-m pipelines (2 cm internal diameter, 2 mm wall thickness) of stainless steel grade 304 (Stelco Hardy, British Steel, Treorchy, Mid Glamorgan, UK) was constructed. Five 20 cm × 1.9 cm flat plates of stainless steel 304 were inserted into one pipeline. Plates of molybdenum metal of the same dimensions were inserted into the other pipeline. The system was supplied with potable water at a flow rate of 0.64 m s^{-1} (Reynold's number 11 080). The potable water supplying the system was analysed for total chlorine, free chlorine, temperature, pH, total counts and viable counts every week.

Biofilm analysis

A metal sheet was guillotined (to avoid any heat treatment effect or mechanical removal of bacteria) into 10 divisions (each 2 cm × 1.9 cm) every week, over a 5-week period, after removal from the system. Six of the sections of each metal were used to determine viable counts and sessile genera, and four were used for total cell counts.

Viable bacterial counts

After removal from the system all slides were washed gently in sterile distilled water, to remove any loosely attached bacteria. Biofilms grown on the slides were scraped from specifically sectioned areas, using a sterile scalpel blade and a sterile cotton wool swab. Each section was analysed after removal of the biofilm under epifluorescence (Olympus BH2, London, UK) microscopy to establish a percentage removal rate. This was estimated at around 80–85%.

Removed biofilms were suspended in 10 ml sterile saline solution and vortexed for 30 s. The suspended biofilm was then serially diluted (10-fold dilutions) in sterile saline buffer and 0.1-ml aliquots of appropriate dilutions were then spread plated onto the surface of R2A agar [19]. Three replicates were used for each slide analysed. Colony forming units of bacteria were enumerated after 7 days incubation at 28°C. The most dominant sessile bacteria isolated from the slides were identified. All pure strains were maintained on R3A agar [19] prior to use.

Biofilm total cell count enumeration

Specific areas of the slide sections were washed gently in distilled water to remove any unattached or loosely bound microorganisms. The washed surfaces were air-dried and stained for 2 min with filtered-sterilised (0.2-μm pore size) acridine orange (Difco, West Molesey, UK). After subsequent washing of the samples with sterile distilled water, the slide sections were again air-dried and examined at a magnification of 1000 under an Olympus BH2 microscope which was fitted with a epifluorescent halogen lamp attachment. The numbers of cells adhering to the surfaces were estimated by counting fluorescing cells within a known area of a microscopic field. One hundred and twenty fields of view of the total surface area were randomly selected and counted on the stainless steel and molybdenum slides. Results were subsequently converted to cells per cm^2 of surface.

Identification of bacteria

Organisms were identified by colony morphology, colour, Gram stain (Difco), motility (Difco motility agar and hanging drop method), catalase (Difco), transmission electron microscopy, oxidase (Difco), fermentation/oxidation of glucose (Hugh and Leifson), and growth at various temperatures (37°C, 41°C and 45°C).

API 20 NE strips were used for further verification of Gram-negative bacteria. This identification process for both Gram-negative and Gram-positive bacteria was followed closely to that discussed by LeChevallier [12]. Verification of the identity of 80% of the bacteria was also carried out by the National Collection of Industrial and Marine Bacteria (NCIMB, Aberdeen, Scotland).

Effect of molybdenum ions on planktonic/sessile bacteria

The organisms used in this study included *Acinetobacter* sp, *Corynebacterium* sp, *Pseudomonas* sp, *Escherichia coli*, *Aeromonas* sp and *Micrococcus luteus*. All organisms were isolated on R2A agar from a drinking water tap. An overnight culture of each isolate was prepared from the slopes by inoculating 1 ml into twenty 750-cc flasks containing R2A [19] broth (200 cc). Sterile filtered molybdenum trioxide (Aldrich Sigma, Poole, Dorset, UK) was added to give final concentrations of 0.3, 1.0 and 10.0 mg L^{-1} in sets of five flasks. The remaining five flasks contained no molybdenum. The concentration of molybdenum was confirmed in all flasks using inductively coupled plasma (ICP) spectrometry. All flasks were incubated at 28°C for 140 h in an orbital incubator (200 rpm) and optical density measurements (600 nm) were made over varying time intervals. In addition viable cell counts were taken at specific optical density measurements for a comparison.

The effect of 100, 1000 and 2000 mg L^{-1} of molybdenum trioxide on bacterial growth was also assessed using these same procedures.

Molybdenum sensitivity

A number of planktonic and sessile bacteria isolated from potable water were examined for sensitivity to molybdenum trioxide. They were subcultured onto R2A agar prior to identification and tested for sensitivity to molybdenum. Susceptibility of the isolates to molybdenum involved overnight cultures being spread onto R2A agar to obtain semi-confluent growth. Sterile filter paper discs soaked in 30 ml of various concentrations of molybdenum ranging from 50–2000 mg L^{-1} were placed on the plates which were incubated at 25°C for 48 h, after which zones of inhibition were measured.

Statistical analysis

All experiments involving statistical analysis were analysed using Student's paired *t*-test and analysis of variance on Minitab (version 9.2) and Excel.

Results

Chemical surface layer and surface roughness

The presence of chromium and iron was evident in the surface layer of stainless steel grade 304 after analysis using energy dispersive X-ray analysis. It showed that the surface layer was composed predominantly of chromium and iron, with some carbon and oxygen presence. No molybdenum was detected in any of the 304 samples. After analysis of the surface layer of the molybdenum metal, it was found to be composed mainly of molybdenum, oxygen and carbon.

Surface roughness measurements, after profiling, showed that stainless steel grade 304 had a Ra value of 0.256 and molybdenum metal 1.252. Molybdenum metal was found to be significantly ($P < 0.05$) rougher than stainless steel grade 304.

The effect of pure molybdenum metal on numbers of sessile bacteria

The characteristics of the potable water supplying the rig system were measured as: pH 7.3, temperature 12°C, total and free chlorine <0.02 ppm, viable count 4.2×10^2 CFU ml^{-1} and total count 2.1×10^5 cells cm^{-3}.

Cell counts of sessile viable bacteria were greatly reduced on molybdenum metal (Mo) compared to stainless steel. Throughout the 5-week study the viable counts on stainless steel slides were a factor of 10 higher than those present on Mo slides (Figure 1). Overall, after exposure to potable water over a 5-week period, all biofilm viable cell counts were significantly higher ($P < 0.05$) on stainless steel when compared to counts of cells on pure molybdenum metal.

When viable counts of sessile bacteria were compared to the total cell counts of sessila bacteria, a similar picture was evident (Figure 2). All biofilm total cell counts were significantly higher ($P < 0.05$) on stainless steel when compared to counts on molybdenum metal slides.

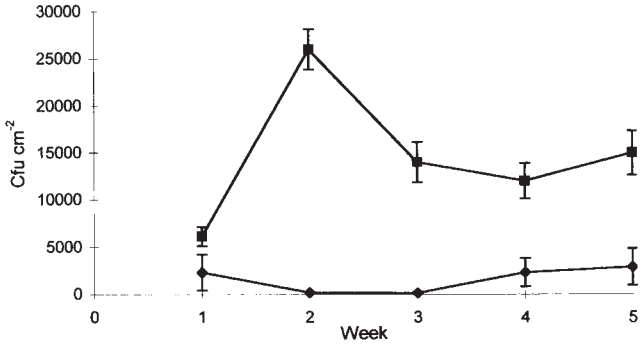

Figure 1 Viable cell counts of sessile bacteria on stainless steel 304 (—■—) and molybdenum (—◆—) slides after exposure to mains water for 5 weeks.

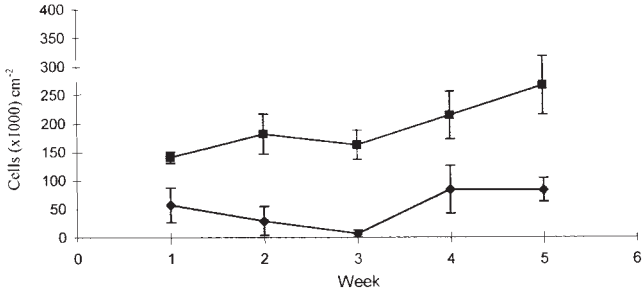

Figure 2 Total cell counts of sessile bacteria on stainless steel 304 (—■—) and molybdenum (—◆—) slides after exposure to mains water for 5 weeks.

Effect of molybdenum on the adhesion of sessile bacteria

The pioneering bacteria attached to both stainless steel and molybdenum metal were slightly different (Table 1). Stainless steel was predominantly colonised by *Acinetobacter* sp. Numbers of these organisms were detected at a significantly higher ($P < 0.05$) level than numbers detected on molybdenum metal. Also *Pseudomonas* spp colonised molybdenum metal at a significantly higher ($P < 0.05$) level when compared to that of stainless steel. *Methylobacterium* sp and *Flavobacterium* sp were also affected by the presence of molybdenum. A number of bacteria identified within the water supply could not be isolated and identified within the biofilms (Table 1). These included *Bacillus* sp and *Alcaligenes* sp. Also a large number of bacteria isolated from the water supply could not be identified after subculturing due to loss of viability.

The effect of molybdenum on planktonic bacteria

Molybdenum trioxide up to a concentration of 1 g L^{-1} had no effect on the planktonic cell counts of *Acinetobacter* sp, *Corynebacterium* sp, *Pseudomonas* sp, *E. coli*, *Micrococcus luteus* or *Aeromonas* sp. At higher concentrations (2 g L^{-1}), molybdenum had an effect on the counts of planktonic cells of *Acinetobacter* sp (Figure 3). It also caused significantly longer lag and exponential phases in *Micrococcus luteus* and *Aeromonas* sp (Figure 4) when compared to appropriate controls.

Susceptibility to molybdenum

For high concentrations of molybdenum (above 1 g L^{-1}) the isolates tested demonstrated a wide range of tolerance to molybdenum trioxide. The diameter of inhibition zones, for molybdenum concentrations of 2 g L^{-1}, ranged from 2.4 to 8.5 mm for the planktonic isolates and 1.5 to 6.1 mm for the sessile bacteria with the variation being related to the genera under study (Table 2). Despite these differences no significant differences ($P < 0.05$) were evident between planktonic and sessile bacteria despite the smaller diameter zones of inhibition present on the plates containing the sessile bacteria.

Discussion

Biofilms developed on stainless steel within flowing and stagnant (deadlegs) water systems accumulate metal ions, particularly iron, zinc and molybdenum [7,15,18]. These

Table 1 Biofilm community structure (mean %) developed on stainless steel and pure molybdenum metal after exposure to potable quality water for 5 weeks

Genus	Water supply[a]	Week 1		Week 2		Week 3		Week 4		Week 5	
		Stainless steel	Molybdenum	Stainless steel	Molybdenum	Stainless steel	Molybdenum	Stainless steel	Molybdenum	Stainless steel	Molybdenum
Acinetobacter	25 ± 5	60 ± 21	10 ± 5	45 ± 18	12 ± 4	36 ± 15	6 ± 1	25 ± 10	14 ± 8	15 ± 7	10 ± 3
Corynebacterium	18 ± 8	10 ± 4	8 ± 2	20 ± 10	18 ± 6	14 ± 3	27 ± 5	15 ± 3	13 ± 4	15 ± 2	13 ± 6
Flavobacterium	4 ± 2	0	0	10 ± 6	0	6 ± 2	0	20 ± 5	0	10 ± 5	5 ± 2
Unknown[b]	10 ± 4	0	17 ± 4	0	0	0	4 ± 1	0	0	10 ± 1	0
Alcaligenes	2 ± 1	0	0	0	0	0	0	0	0	0	0
Bacillus	2 ± 1	0	0	0	0	0	0	0	0	0	0
Methylobacterium	3 ± 1	10 ± 3	3 ± 1	10 ± 4	6 ± 2	2	0	0	0	0	0
Pseudomonas	34 ± 12	20 ± 12	62 ± 21	15 ± 4	64 ± 24	42 ± 11	63 ± 24	40 ± 17	73 ± 29	30 ± 13	62 ± 31
Sphingomonas	2 ± 1	0	0	0	0	0	0	0	0	20 ± 14	10 ± 2

[a]Mean of weeks 1–5.
[b]Unknown—includes bacteria which could not be identified via the API strip and bacteria that lost their viability after they were subcultured.
± = standard deviation.

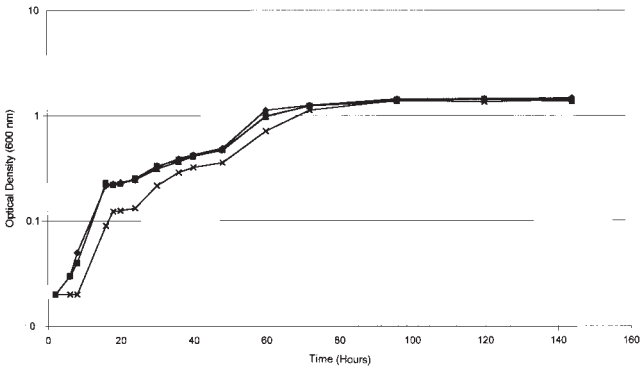

Figure 3 Growth of *Acinetobacter* sp after exposure to molybdenum trioxide. —◆— Control; —■— 100 ppm; —▲— 1000 ppm; —×— 2000 ppm.

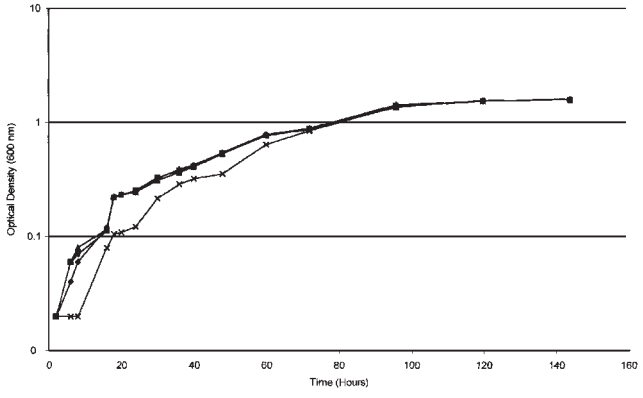

Figure 4 Growth of *Aeromonas* sp after exposure to molybdenum trioxide. —◆— Control; —■— 100 ppm; —▲— 1000 ppm; —×— 2000 ppm.

Table 2 Sensitivity of planktonic and sessile bacteria to 2 g L^{-1} of molybdenum trioxide

Genus	Zone diameter (mm) ± standard deviation	
	Planktonic	Sessile
Acinetobacter	8.5 ± 3.9	4.1 ± 2.3
Alcaligenes	6.3 ± 2.8	3.5 ± 1.2
Aeromonas	6.1 ± 3.8	5.3 ± 1.5
Corynebacterium	5.8 ± 2.4	4.1 ± 2.6
Flavobacterium	3.2 ± 1.6	2.4 ± 1.3
Methylobacterium	6.6 ± 4.1	4.8 ± 2.6
Pseudomonas	2.4 ± 1.1	1.5 ± 0.2
Sphingomonas	2.8 ± 0.9	2.1 ± 0.4

metal ions could affect counts of sessile bacteria and it has been documented, particularly with sulphate-reducing bacteria, that these metal ions can retard bacterial viability [2,3,5]. Biofilms are likely to possess characteristics and properties which are unique to their particular niches. The physicochemistry of metallic surfaces is rapidly altered by deposition of complex nutrients, with humic and fulvic acids often deposited on pipe surfaces in water systems. The metabolic products of microorganisms also adhere to these surfaces, including exopolysaccharides, glucans, fructans, lipopolysaccharides, proteins, carbonates and struvite. These modifications in surface physicochemistry, and biofilm microenvironments are greatly affected by metallic ions, particularly molybdenum, released into biofilms.

Molybdenum metal reduces the adhesion rate and biofilm development of bacteria in potable water, compared to stainless steel. As the Ra value was significantly higher on molybdenum metal, when compared to stainless steel, high surface roughness can be dismissed as a factor responsible for the higher cell counts on stainless steel because a high Ra surface roughness value increases biofilm development [15–17]. Whilst pure molybdenum metal would not be used as a material to transport potable water, exposure to it at high concentrations, possibly if it was incorporated in a material used to transport potable water, could be used as a potential biocidal treatment. The composition of the metal substratum affects biofilm development and the sessile microbial community. This has also been observed in other studies carried out in potable water systems [10,20]. It was observed in this study that high concentrations (2 g L^{-1}) of molybdenum, in solution, had an effect on the growth of a number of potable water bacteria. Clearly, bacteria show different physiological responses to the presence of molybdenum, in that *Acinetobacter* sp is more susceptible to molybdenum than *Corynebacterium* sp and *Pseudomonas* sp. It is possible, despite no evidence of statistical significance ($P < 0.05$), that the bacteria removed from biofilms grown in potable water may be more resistant to the effects of molybdenum than bacteria isolated from the planktonic phase. This was confirmed in the molybdenum sensitivity test. Whilst it is unlikely that bacteria will be located in environments containing high concentrations of molybdenum metal ions, results of this study show that molybdenum has some biocidal potential, by reducing bacterial growth rates when compared to solutions containing no or low concentrations of molybdenum.

As stainless steel is now being accepted as a good material for use in potable water [1,4,8,9,14–17,21–23] it is possible that it may have slight biocidal qualities. This may be due to molybdenum ions, present in the passive layer of stainless steel grade 316 [16] and known to leach from stainless steel in low concentrations [17,18], reducing bacterial viability.

References

1 Anon. 1997. Research confirms stainless steel is acceptable for handling drinking water. Water Eng Mangt 144: 16.
2 Beech IB and CWS Cheung. 1995. Interactions of exopolymers produced by sulphate-reducing bacteria with metal ions. Int Biodeterior Biodegrad 35: 59–75.
3 Beech IB and CC Gaylarde. 1992. Attachment of *Pseudomonas fluorescens* and *Desulfovibrio* to mild steel and stainless steel—first step in biofilm formation. In: Proceedings of the 2nd European Federation of Corrosion (Sequeira AC and AK Tiller, eds), pp 61–66, European Federation of Corrosion Publication number 8. The Institute of Materials, Portugal.
4 Chamberlain AHL, WR Fischer, U Hinze, HH Paradies, CAC Sequeira, H Siedlarek, M Thies, D Wagner and JN Wardel. 1995. An interdisciplinary approach for microbially influenced corrosion of copper. In: Proceedings of the 3rd International EFC workshop (Tiller AK and CAC Sequeira, eds), pp 1–16, European Federation, The Institute of Materials.
5 Chen G, TE Ford and CR Clayton. 1998. Interaction of sulphate-reduc-

ing bacteria with molybdenum dissolved from sputter-deposited molybdenum thin films and pure molybdenum powder. J Colloid Interface Sci 204: 237–246.
6 Donlan RM, WO Pipes and TL Yohe. 1994. Biofilm formation on cast iron substrata in water distribution systems. Water Res 28: 1497–1503.
7 Holt DM, CA Woodward, AJ Rachwal, A Delanque and JS Colbourne. 1995. A pipe based experimental system to study factors influencing bacterial growth in London's water mains. In: Proceedings of the Water Quality Technology Conference, San Francisco, 6–10 November 1995, American Water Works Association, Denver.
8 Hyde FW, M Alberg and K Smith. 1997. Comparison of fluorinated polymers against stainless steel, glass, polypropylene in microbial biofilm adherence and removal. J Ind Microbiol Biotechnol 19: 142–149.
9 Jain DK. 1995. Microbial colonisation of the surface of stainless steel in a deionized water system. Water Res 29: 1869–1876.
10 Kerr CJ, KS Osborn, GD Robson and PS Handley. 1997. The effect of substratum on biofilm formation in drinking water systems. In: Biofilms: Community Interactions and Control (Wimpenny J, P Handley, P Gilbert, H Lappin-Scott and M Jones, eds), pp 167–174, Bioline, Cardiff.
11 LeChevallier MW, CD Cawthon and RG Lee. 1988. Inactivation of biofilm bacteria. Appl Environ Microbiol 54: 2492–2499.
12 LeChevallier MW, RJ Seidler and TM Evans. 1980. Enumeration and characterization of standard plate count bacteria in chlorinated and raw water supplies. Appl Environ Microbiol 40: 922–930.
13 LeChevallier MW, NJ Welch and DB Smith. 1996. Full-scale studies of factors related to coliform regrowth in drinking water. Appl Environ Microbiol 62: 2201–2211.
14 Pedersen K. 1990. Biofilm development on stainless steel and PVC surfaces in drinking water. Water Res 24: 239–243.
15 Percival SL, JS Knapp, R Edyvean and DS Wales. 1997. Biofilm development on 304 and 316 stainless steels in a potable water system. J Instn Wat Envir Mangt 11: 289–294.
16 Percival SL, JS Knapp, DS Wales and R Edyvean. 1998. The effects of the physical nature of stainless steel grades 304 and 316 on bacterial fouling. Br Corros J 33: 121–129.
17 Percival SL, JS Knapp, DS Wales and R Edyvean. 1998. Biofilm development on stainless steel in potable water. Water Res 32: 243–253.
18 Percival SL, JS Knapp, DS Wales and R Edyvean. 1999. Metal ions and biofilms. J Instn Wat Envir Mangt (submitted for publication).
19 Reasoner DJ and EE Geldreich. 1985. A new medium for the enumeration and subculture of bacteria from potable water. Appl Environ Microbiol 49: 1–7.
20 Rogers J, AB Dowsett, PJ Dennis, JV Lee and CW Keevil. 1994. Influence of plumbing materials on biofilm formation and growth of *Legionella pneumophila* in potable water systems. Appl Environ Microbiol 60: 1179–1183.
21 Tuthill AH and AE Avery. 1994. Survey of stainless steel performance in low chloride waters. Public Works 125: 49–52.
22 Tuthill AH. 1994. Stainless-steel piping. J Am Water Works Assoc July: 67–73.
23 Wagner D, W Fischer and HH Paradies. 1992. First results of a field experiment in a county hospital in Germany concerning the copper deterioration by microbially induced corrosion. In: Proceedings of 2nd International EFC Workshop on Microbial Corrosion, pp 243–261, The Institute of Materials.