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The effect of turbulent flow and surface roughness on biofilm formation in drinking water

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There is considerable interest in both Europe and the USA in the effects of microbiological fouling on stainless steels in potable water. However, little is known about the formation and effects of biofilms, on stainless steel in potable water environments, particularly in turbulent flow regimes. Results are presented on the development of biofilms on stainless steel grades 304 and 316 after exposure to potable water at velocities of 0.32, 0.96 and 1.75 m s^{-1}. Cell counts on slides of stainless steel grades 304 and 316 with both 2B (smooth) and 2D (rough) finishes showed viable and total cell counts were higher at the higher flow rates of 0.96 and 1.75 m s^{-1}, compared to a flow rate of 0.32 m s^{-1}. Extracellular polysaccharide levels were not significantly different ($P < 0.05$) between each flow rate on all stainless steel surfaces studied. Higher levels were found at the higher water velocities. The biofilm attached to stainless steel was comprised of a mixed bacterial flora including *Acinetobacter* sp, *Pseudomonas* spp, *Methylobacterium* sp, and *Corynebacterium/Arthrobacter* spp. Epifluorescence microscopy provided evidence of rod-shaped bacteria and the formation of stands, possibly of extracellular material attached to stainless steel at high flow rates but not at low flow rates.

Keywords: biofilms; potable water; flow rate; stainless steel

Introduction

Biofilms are of great inconvenience to the water industry, especially as thousands of miles of pipe surfaces have to be monitored and controlled for bacterial colonisation [16,17,22]. Particularly in industrial applications the behaviour of the water flowing through pipe systems influences both bacterial attachment and detachment and ultimately development of the biofilm.

Within potable water pipe systems two extremes of flow generally exist: laminar and turbulent [21]. Whilst laminar flow is smooth flow with no lateral mixing, turbulent flow is often defined as irregular and chaotic. As most water flow in engineered systems is turbulent, this study was set up to look at the effects of flow on biofilm formation on stainless steel, which is now being seen as a great alternative to copper, in drinking water environments.

There is currently considerable interest in both Europe and the USA in the effects of microbial fouling, particularly corrosion, on stainless steel in freshwater environments. The freshwater interests include both potable water [28], the food industry and areas such as cooling water systems [18]. There is a move in the UK and the rest of Europe away from copper to the use of stainless steel piping for carrying particular 'problem' waters in buildings where corrosion causes failures in copper [25]. However, little is known about the formation and the effects of biofilms on stainless steel in these environments, especially as stainless steel does not have the biocidal potential of copper. Still less is known about the interactive effects of biofilms and cleaning and sterilisation agents on stainless steel.

Of the two types of stainless steel used in potable water systems, type 304 stainless steel is generally used in low chloride (<200 mg L^{-1} chlorides) waters whereas type 316 stainless steel, which contains molybdenum for improved corrosion resistance, is used when other factors, such as higher chloride levels and low pH (<5) are encountered and an improved corrosion resistance is required [35]. Stainless steel is also an alternative to PVC piping [23] and to cast iron pipes and has been accepted for the water facilities in Tokyo which has suffered serious leaks and fractures [33].

There is controversy regarding water velocity and biofilm development. Generally, biofilm development is reduced at high water velocities due to high shear rates. However, particularly in water environments, biofouling can be more evident at higher velocities, contrary to the consensus [14]. Velocity of water may also influence the amount of extracellular polymeric substances found within a biofilm [2]. This is a very important factor for biofilm control, particularly as the efficacy of penetrating biocides is affected by the levels of extracellular polysaccharide.

The physical and chemical characteristics of a solid surface affect biofilm formation in flowing systems [1,2,15,30]. Therefore, as well as looking at the effects of different grades of stainless steel on biofilm formation, surface roughness was also worthy of study. Surface roughness ultimately affects bacterial adhesion [24,28]. Water distribution pipes and domestic plumbing pipes harbour potential sites for biofilm development despite being classified as smooth. Increasing surface roughness increases both initial

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microbial adhesion and subsequent colonisation of surfaces [13,34]. The degree of surface roughness may well be a critical factor in biofilm development but research focused on this area is scarce, as is the effect of flow on stainless steel in potable water.

This study examines the effect of turbulent flow at three different water velocities on biofilm growth on stainless steel grades 304 and 316 in drinking water and also studies the effects of surface roughness on this development. The effects of turbulent flow on biofilm growth, colonisation and species diversity were also examined.

Materials and methods

Surface roughness measurements of stainless steel slide sections

Surface roughness of stainless steel was assessesd prior to growth of the biofilms, using both taly surf and atomic force microscopy as outlined elsewhere [26].

The effect of water flow rate on biofilm growth

The rig system, composed of stainless steel grades 304 and 316, used to study biofilm formation in potable water, is shown in Figure 1. The physical and chemical properties of these two stainless steels have been reported [26]. Each stainless steel pipe was 2 metres long, with an internal diameter of 20 mm and a wall thickness of 2 mm. The pipelines were sectioned every 20 cm and joined together with 22-mm brass compression joints. Within each 20-cm pipe section were two 10-cm length stainless steel slides. Each slide had a wall thickness of 2 mm and width of 19 mm. The underside had a 2D rough matt finish and the topside a 2B smooth finish. All stainless steel samples used in the experimental rig system were degreased in acetone (by sonicating in a water bath for 2 min), and then sterilised in 70% boiling alcohol for 15 min. The stainless steel surfaces were examined using scanning electron microscopy (SEM) for surface effects of this cleaning and sterilisation process; none were observed.

Biofilm development on stainless steel in potable water

Stainless steel slide sections were taken from the test site every month over a 5-month period. Four slide sections 2 cm long × 1.9 cm wide with a 2D and 2B surface finish were used for a comparison of viable cell counts and heterotrophic bacteria flora. Another four sections (2 × 1.9 cm) were used for total counts and the final four sections (4 × 1.9 cm) of each grade and finish of stainless steel were used to monitor dry weight and extracellular polysaccharide levels.

Viable bacterial counts

Slide sections were washed gently in sterile distilled water to remove loosely attached bacteria. Biofilm on the 2D and 2B slide sections was scraped and swabbed from specifically sectioned areas, using a sterile scalpel blade and a sterile cotton wool swab. After biofilm removal, sections were checked by epifluorescence microscopy, after staining the slide surface in acridine orange (0.001%) to determine cell removal efficiencies. Preliminary tests using this method were shown to have recovery efficiencies of 80% of the total number of sessile cells on both the 2B and 2D slide surfaces. The removed biofilms were suspended in 10 ml sterile saline solution and vortexed for 30 s. The suspended biofilm was then serially diluted in sterile saline

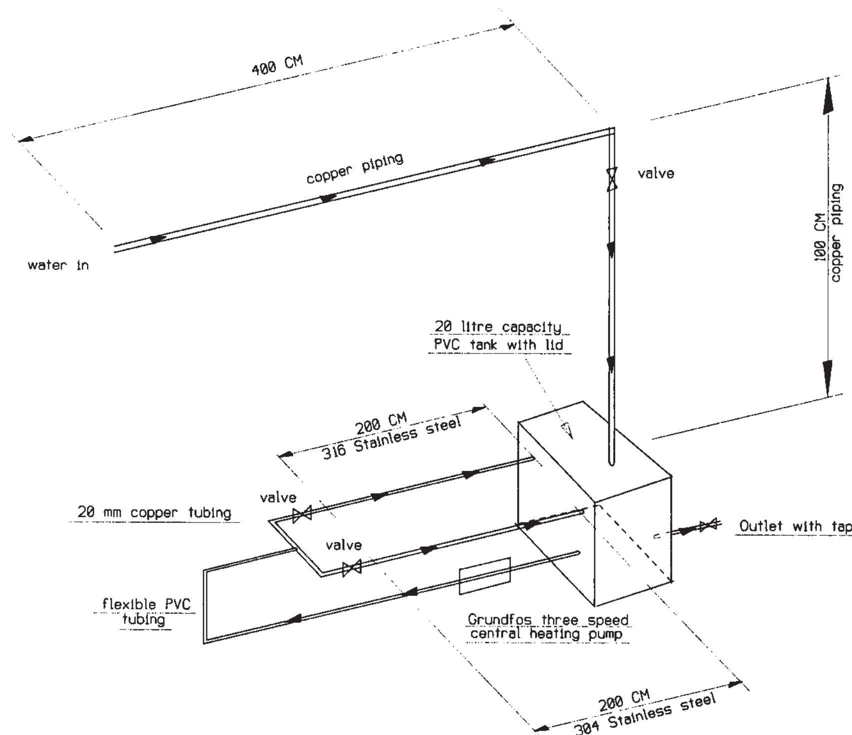

Figure 1 The experimental system used to study flow rate effects on biofilm development on stainless steel grades 304 and 316.

buffer and 0.1-ml aliquots were plated on the surface of R2A agar [29]. Three replicates were used for each slide section analysed. Colony-forming units were enumerated after 7 days incubation at 28°C.

Detection of attached bacteria using epifluorescence microscopy

Stainless steel was washed gently in distilled water to remove unattached or loosely bound microorganisms. The washed surfaces were air dried and stained for 2 min with filtered sterilised (0.22-μm pore size filter) acridine orange (Difco, Detroit, MI, USA). After washing the samples with sterile distilled water, the slide sections were air dried and examined using epifluorescence microscopy. Micrographs of stainless steel were taken to analyse biofilm buildup over time at each flow rate studied. 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 were randomly selected and counted on stainless steel slides of each surface finish and the number converted to cells cm^{-2} of surface.

Identification of bacteria

Organisms isolated on R2A agar were identified by colony morphology, colour, Gram stain, motility, oxidase, catalase, transmission electron microscopy for the presence of polar flagella, fermentation/oxidation of glucose, and growth at 37, 41 and 45°C). API 20 NE (Biomerieux, Basingstoke, Hants, UK) strips were used for both Gram-negative and Gram-positive bacteria has been outlined elsewhere [27].

Dry weight and extracellular polysaccharide

Biofilms were removed from the slides by scraping them with a sterile scalpel into 10 ml of sterile double distilled water. The suspended biofilm was then freeze dried and weighed. To the dried samples 2 ml of double distilled water was added, the suspension vortexed for 2 min and then centrifuged at $30000 \times g$ for 30 min. A second centrifugation step was carried out on the supernatant to increase purity and to remove cellular debris. The supernatant was then dialysed, using viscose tubing (boiled in EDTA) for 24 h in distilled water at 4°C, to remove all non-polymeric material. The sample was then analysed for extracellular polysaccharide using the phenol sulphuric acid method of Dubois *et al* [10], with **d**-glucose as standard.

Scanning electron microscopy (SEM)

For the analysis of biofilm development in mains water at different water velocities, 1-cm sections of stainless steel were air dried, sputter coated with gold and analysed using a JSM Joel series 1 (Welwyn Garden City, Herts, UK) or a Cam series 4 SEM (Cambridge, Cambs, UK). The method used here for SEM samples was employed since standard preparation techniques (involving fixation, alcohol dilution and freeze drying) resulted in detachment of the biofilm from the stainless steel surface.

Statistical evaluation of the results

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

Results

Chemical composition and surface roughness of stainless steel

Differences in surface microgeometry were clearly visible when viewed under SEM and atomic force microscopy in the as-received condition. On the 2B finished slides, grain boundaries, formed as a result of the oxide film before exposure to potable water, were clearly apparent. SEM and AFM micrographs showed stainless steel grade 304 to contain slightly smaller grains than that of 316 stainless steel with deeper grain channels. Grain boundaries were not evident on the surface of 2D finished slides. However, 2D surfaces appeared to be much rougher, with a large number of crevices, which aid attachment. The 2B surface finish showed numerous scratches, grooves and deformation marks on the 304 grade steel, favouring microbial adhesion when compared to the stainless steel grade 316.

Stylus surface roughness values, (measured with a Rank Taylor Taly-surf) showed that grade 304 2B, 316 2B, 304 2D and 316 2D had a mean surface roughness (Ra) of 0.210 ($\pm$ 0.227), 0.115 ($\pm$ 0.070), 1.198 ($\pm$ 0.041) and 0.557 ($\pm$ 0.301) μm, respectively. The Ra values of 2D surfaces were significantly higher ($P < 0.05$) than the 2B surfaces.

Surface roughness measurements made using the atomic force microscope established that there was a significant difference ($P < 0.05$) between stainless steel grades 304 and 316. For grade 304 the Ra values for the 2B surface were calculated at 0.478 ($\pm$ 0.108) μm while the equivalent value for the 2B finish of grade 316 was 0.152 ($\pm$ 0.074) μm. Poor roughness profiles were obtained with 316 2D finish surfaces, and 304 2D finishes were too rough to enable accurate profiling measurements on the AFM.

Water supply and hydraulic characteristics of the experimental system

The rig system was supplied with potable water. Every month the potable water was assayed for chlorine levels (>0.01 ppm), pH (7.2), planktonic viable cell counts (2.4×10^2 CFU ml^{-1} after 14 days incubation at 28°C) and planktonic total cell counts (2.8×10^4 cells ml^{-1}). In order for flow rate to be maintained, a three-speed central heating pump was used. Water was continually fed into the system and waste water was removed at known flow rates. The temperature of the water was maintained at 15.5°C. At water velocities of 0.32 m s^{-1}, 0.64 m s^{-1} and 1.75 m s^{-1}, the Reynolds number was calculated at 5540, 16620 and 30297, respectively.

The effect of grade of stainless steel on biofilm development

Overall, stainless steel grade 304 was found to be colonised at a significantly higher ($P < 0.05$) level than grade 316 (data not given). This was evident at each water velocity. Dry weight and exopolysaccharide levels were not significantly different ($P < 0.05$) between grades or finishes of stainless steel.

The effect of flow on biofilm cell counts

All biofilm viable cell counts on all grades and surface finishes of stainless steel were significantly higher ($P < 0.05$)

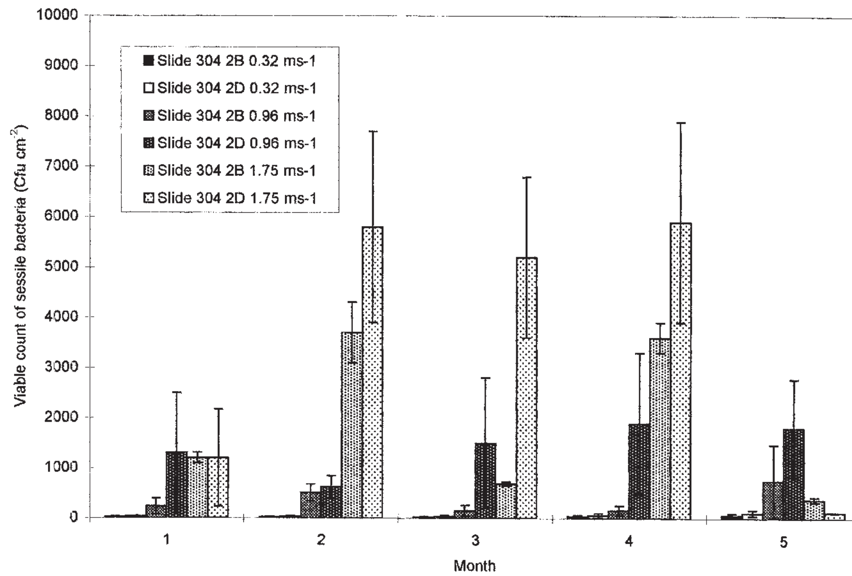

Figure 2 The effect of water velocity (0.32, 0.96 and 1.75 m s^{-1}) on the viable cell count in biofilms developed on stainless steel 304 with 2B or 2D surface finish.

at water flow rates of 0.96 and 1.75 m s^{-1} than at 0.32 m s^{-1} (Figure 2). When a comparison between 0.96 and 1.75 m s^{-1} was made, significant differences ($P < 0.05$) were evident with a higher biofilm viable cell count at the higher velocity on both grades and finishes of stainless steel. Viable counts on slides of 304 2B were significantly higher ($P < 0.05$) at a flow rate of 1.75 m s^{-1} compared to 0.96 m s^{-1} (Figure 2). The relation between flow and viable cell count on stainless steel grade 316 was similar to that of grade 304.

When a comparison was made of total cell counts on stainless steel grades 304 and 316 (2B finish), cell counts for the first 3 months were significantly higher ($P < 0.05$) at a flow rate of 0.96 m s^{-1} than at 0.32 m s^{-1} (Figure 3).

Differences in total cell counts on the 2D slide sections were also found. Cell counts on stainless steel grade 304 were significantly higher ($P < 0.05$) at the higher velocity of 0.96 m s^{-1} compared to 0.32 m s^{-1} (Figure 3).

When a water flow rate of 1.75 m s^{-1} was compared to 0.32 m s^{-1}, total cell counts were significantly higher ($P < 0.05$) for the first 3 months, on 304 2D slide, at the higher velocity of 1.75 m s^{-1} and only significantly different ($P < 0.05$) on the 316 2D slide at month 3. A similar result was obtained with the 2B slide sections. Most 2B and 2D surfaces were colonised at a significantly higher ($P < 0.05$) level at 1.75 m s^{-1} than at 0.96 m s^{-1}.

The effect of flow on biofilm mass

Overall, no significant differences ($P < 0.05$) between flow rates and dry weight/extracellular polysaccharide levels

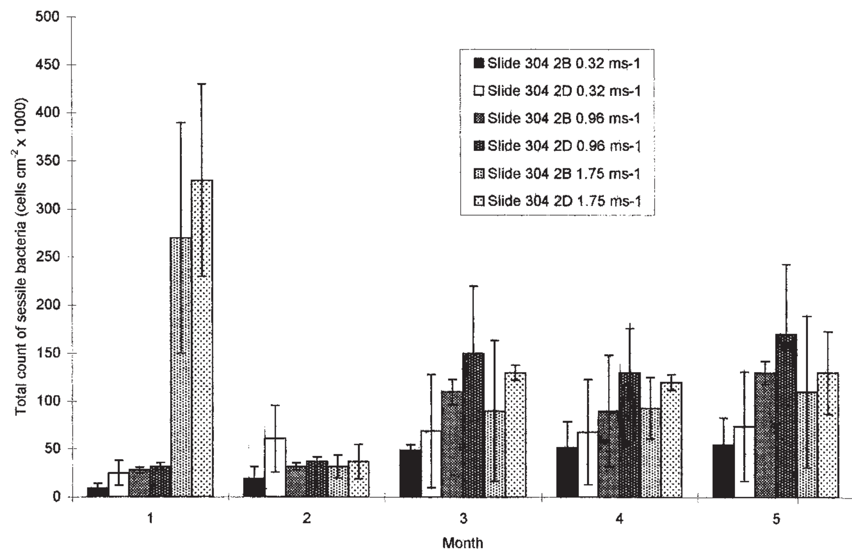

Figure 3 The effect of water velocity (0.32, 0.96 and 1.75 m s^{-1}) on the total cell count in biofilms developed on stainless steel 304 with 2B or 2D surface finish.

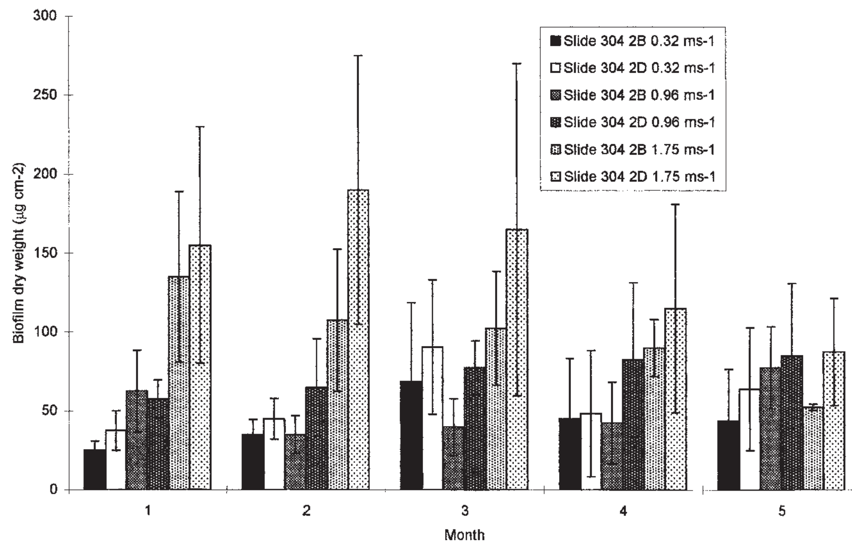

Figure 4 The effect of water velocity (0.32, 0.96 and 1.75 m s^{-1}) on the dry weight levels in biofilms developed on stainless steel 304 with 2B or 2D surface finish.

were observed at the three different flow rates (Figures 4 and 5). This may be due to the small sample size used and the crude method used to sample the biomass.

Dry weight levels were higher initially at the higher flow rate (1.75 m s^{-1}) when compared to the flow rates at 0.32 and 0.96 m s^{-1} on both grades and finishes of stainless steel. In contrast extracellular polysaccharide levels were higher initially at 0.96 m s^{-1} when compared to 0.32 m s^{-1}. At month 4, the levels on 304 2B slide sections increased markedly at a water velocity of 1.75 m s^{-1} when compared to the lower velocities. This result was also evident on the 2D slide sections and on stainless steel grade 316.

The effect of flow on community structure and biofilm formation

Direct examination of biofilms under SEM, at a flow rate of 0.32 m s^{-1}, identified the major components of the biofilm, which consisted predominantly of rod-shaped colonising bacteria and diatoms. Small numbers of filamentous bacteria and fungal hyphae were also identified. No change in the surface appearance ie corrosion, after removal of the biofilm, was evident on either grade of stainless steel after exposure to flowing water at any flow rate throughout the experiments, despite areas of heavy fouling.

There was greater evidence of biofilm accumulation (as shown by SEM) on stainless steel slides exposed to mains water at a velocity of 0.96 m s^{-1} than those at a velocity of 0.32 m s^{-1}. Fibrillar strands of extracellular polymeric substances (EPS) were evident covering the surface (Figure 6). The presence of fibrillar strands was confirmed under epifluorescence microscopy; these were present on both grades of stainless steel 304 and 316. Filamentous bacteria and yeast cells (Figure 7) were also identified as being attached to stainless steel, often associated with diatoms.

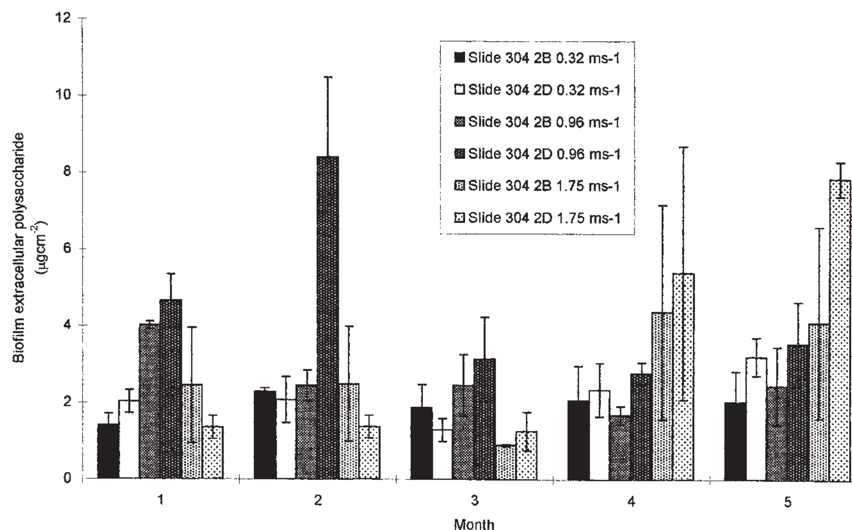

Figure 5 The effect of water velocity (0.32, 0.96 and 1.75 m s^{-1}) on the extracellular slime polysaccharide levels in biofilms developed on stainless steel 304 2B or 2D surface finish.

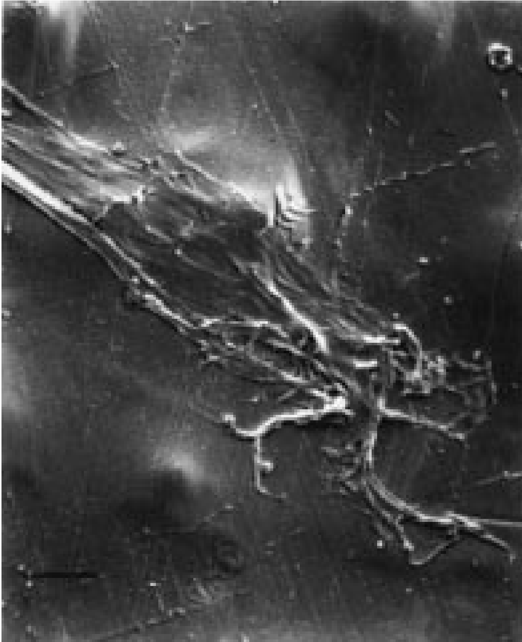

Figure 6 Scanning electron micrograph of extracellular polymeric strands attached to stainless steel 304 2B slide after exposure to potable water at a water velocity of 0.96 m s^{-1} (Bar = 3.84 μm).

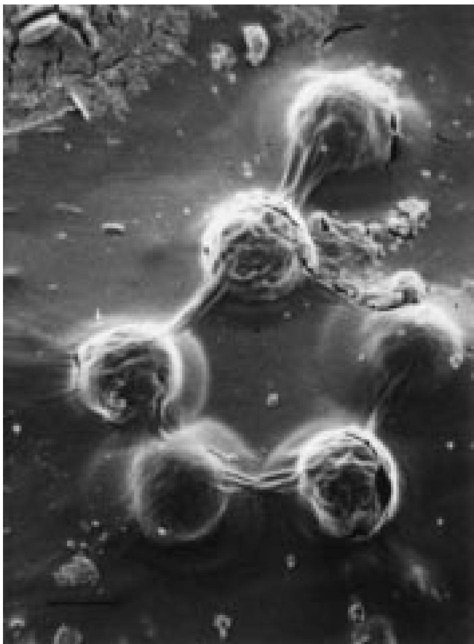

Figure 7 Scanning electron micrograph of yeast cells associated with a biofilm and attached to stainless steel 304 2B slide after exposure to potable water at a water velocity of 0.96 m s^{-1} (Bar = 3.84 μm).

At 1.75 m s^{-1}, SEM highlighted the presence of filamentous bacteria and possible 'streamers'. It is possible that this phenomenon is due to dehydration of the biofilm during preparation for SEM. However, these structures were also identified under epifluorescence microscopy. Larger amounts of detritus were also evident at this higher velocity, when compared to the SEM micrographs at the lower velocities. Because the biofilm is primarily composed of water, drying the biofilm for viewing under SEM, was evident. This severely distorted the image profiles, preventing accurate determination of many of the biological entities.

The effect of flow on community size and structure

The planktonic bacterial population in potable water was measured before each new velocity was established. There were no obvious differences in colony types (diversity) on grades or finishes of stainless steel, suggesting that surface finish and composition does not have a major effect on the diversity of heterotrophic bacteria growing in a biofilm. Overall, when a comparison is made between sessile bacteria and water flow rate, *Acinetobacter* sp was the principle pioneering bacterium, together with *Arthrobacter/ Corynebacterium* spp on stainless steel at low flow rates (Table 1). At higher flow rates the pioneering bacterium *Acinetobacter* sp was also present together with *Pseudomonas* spp.

Throughout the 5-month long studies other bacteria were present as part of the biofilms, including *Methylobacterium* sp, *Flavobacterium* sp, *Alcaligenes* sp and *Staphylococcus* sp. *Pseudomonas* spp dominated the biofilms developed on stainless steel during the latter stages of biofilm growth at all three water velocities. These results suggest that *Acinetobacter* sp and *Arthrobacter/ Corynebacterium* spp, whilst dominating the biofilm at low velocities, are not able to compete with *Pseudomonas* spp which dominate the biofilms at the higher flow rates. No significant differences ($P < 0.05$) were evident with respect to grades or finishes of stainless steel.

Discussion

Epifluorescence microscopy on samples taken from biofilms at the lowest velocity of 0.32 m s^{-1} showed patchy biofilms with rod-shaped bacteria which were often located in an amorphous gel. At velocities of 0.96 and 1.75 m s^{-1}, strands or 'streamers', possibly of EPS, were evident with which the bacteria were associated. It is unclear at present why there were differences in the mode of microbial attachment on the stainless steel surfaces in the low and high flow regions. However, these 'streamers' of extracellular polymers allow for firm adhesion of bacteria to surfaces and also aid in binding adjacent cells together allowing for microcolony formation [9,15,30,36]. Streamers may also increase attachment to surfaces by improving resistance of the biofilm to shear stress and they may increase the surface area of the biofilm.

Analysis under the epifluorescence microscope of samples from biofilms formed at 0.32 m s^{-1} highlighted the EPS matrix after 5 months exposure to potable water. However, at 0.96 m s^{-1} this was evident on both stainless steel grades 304 and 316 at month 3, indicating more rapid and extensive biofilm development at the higher velocities. At a water velocity of 1.75 m s^{-1} the EPS matrix/gel was not evident, as fibrillar structures with which bacteria were attached dominated the stainless steel surface.

Both grades of stainless steel at the higher velocities of 0.96 and 1.75 m s^{-1} showed evidence of periodic fluctuations in cell counts, which could suggest the occurrence of sloughing [19]. Results from other areas of research indi-

Table 1 Composition of bacterial communities in biofilms growing on stainless steel after exposure to potable water at velocities of 0.32, 0.96 or 1.75 m s^{-1} for 5 months[a]

Stainless steel	Genus	% Isolated bacteria of each genus at a flow rate of:		
		0.32 m s^{-1}	0.96 m s^{-1}	1.75 m s^{-1}
304 2B slide	*Acinetobacter*	43	7	19
	Corynebacterium/Arthrobacter	42	26	7
	Pseudomonas	11	50	71
	Flavobacterium	1		1
	Methylobacterium	3	17	2
316 2B slide	*Acinetobacter*	43	15	22
	Corynebacterium/Arthrobacter	44	24	9
	Methylobacterium	3	2	5
	Pseudomonas	10	59	64
304 2D slide	*Acinetobacter*	51	15	22
	Corynebacterium/Arthrobacter	35	18	11
	Methylobacterium	3	24	3
	Flavobacterium			2
	Staphylococcus		2	
	Pseudomonas	11	41	62
316 2D slide	*Acinetobacter*	44	7	30
	Corynebacterium/Arthrobacter	35	18	6
	Methylobacterium	2	18	3
	Flavobacterium	3		
	Alcaligenes		1	2
	Pseudomonas	16	56	59

[a]Mean % were calculated from all values determined over 1–5 months.

cate that some of these events would influence biofilm development [3,5,6,20,30]. However, other research has shown that higher flow rates provide higher nutrient levels and consequently higher bacterial growth. Many engineers maintain that the higher the velocity the greater the scour action and hence removal of slime and scale. However, Howsam [14] reported that at velocities of 0.4–4 m s^{-1}, the worst biofouling occurs at the faster flow rates. It would appear that biofilms are able to compress under pressure and exhibit a high resistance to shear. Turbulent flow, in addition to providing enhanced nutrient uptake conditions at the biofilm surface, enhances chemical nucleation leading to precipitation of compounds which contribute to biofouling. Rheological measurements conducted with the Weissenburg rheogoniometer on an *in situ* mixed-population biofilm [4] indicate that the biofilm is viscoelastic. The viscous properties of the biofilm contribute to increased fluid frictional resistance in flow conduits [8]. Therefore, biofilms may not be sloughed off at these higher velocities but become more compact and stabilised.

Duddridge *et al* [11] showed a drastic reduction in the amount of biofilm when fluid velocity is increased in a system which is already colonised with pre-existing biofilm. Santos *et al* [31] observed less stable biofilms at low velocities of 0.5 m s^{-1}, and suggested a biofilm was formed with a less robust character. These authors also found the biofilm at this velocity to be more open and 'fluffy' than that formed at 2.5 m s^{-1}. Moreover, Santos *et al* [32] found that when filtered tap water was used to grow biofilms, a biofilm grown at 2.5 m s^{-1} was much thicker than one grown at 0.5 m s^{-1}. However, when distilled water was used instead of tap water, the opposite effect occurred. At lower velocities (0.5 m s^{-1}) biofilm grew more rapidly and thicker than at high velocities (2.5 m s^{-1}). The thinner film at the higher velocity may be attributed to the effects of increased fluid shear at 2.5 m s^{-1} compared to that at low velocities.

The results support the observations made by Geesey and Costerton [12] who found that bacterial settlement was greatest in cavities and grooves on a roughened surface. Surface roughness influences the transport and attachment of microbial cells for many reasons; it increases mass transport, provides shelter from shear force and increases surface area for the attachment of bacteria [6]. Substratum roughness seems to some extent to influence bacterial colonisation, with an increase of adhesion evident with increasing roughness of the substratum [7,12].

Acknowledgements

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