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AFM study of the colonisation of stainless steel by *Aquabacterium commune*

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Abstract

Aquabacterium commune, a member of the beta proteobacteria family that is a recently isolated, predominant member of various European drinking water distribution system biofilms, was selected as a test organism in this study. Attachment of *A. commune* cells onto two increasingly popular pipe materials, stainless steel EN1.4307 and medium density polyethylene (MDPE) was studied at 15 °C, 150 rpm, and a hydraulic retention time of 10.5 h in a rotating annular biofilm (RAB) reactor. Planktonic and sessile growth was monitored by spread plate technique. Atomic force microscopy (AFM) was used to obtain information about surface topography and biofilm formation pattern. Our study has shown that: (i) Steady-state conditions were reached after ca. 100 h for both materials; (ii) biofilm cell density on MDPE slides is four times greater than on stainless; (iii) the primary colonization of MDPE and stainless steel occurred at the edge of the slides; and (iv) no preferential attachment to stainless steel grain boundaries was observed. Stainless-steel manufacturers and suppliers, researchers, and companies working in the drinking-water sector will benefit from this paper. It is suggested that electropolishing of stainless-steel pipes for drinking water installations is not necessary to remove specific biofilm formation sites (i.e. grain boundaries). Furthermore, this paper provides, for the first time, some fundamental information for the continuous cultivation of the recently isolated drinking water microorganism, *A. commune*.

Keywords: Stainless steel EN 1.4307; Biofilm; Rotating annular biofilm (RAB) reactor; Tapping atomic force microscopy (AFM)

1. Introduction

Austenitic stainless steels are defined as iron-based alloys containing at least 16.5% chromium, and up to 13.5% nickel (BSI, 1995). In addition, molybdenum may be added to increase crevice and pitting corrosion resistance of the steel. Stainless steels derive their name from the ability to form a passive, corrosion-resistant chromium-oxide layer under oxidising conditions. Austenitic grades such as EN1.4307 (AISI 304L) and 1.4432 (AISI 316L) are increasingly being used in domestic and public drinking water installations in a number of countries (Broo et al., 2001) where other pipe materials such as copper have failed (Keevil et al., 1989, Percival et al., 1997, Wagner and Chamberlain, 1997) or are not suitable, as in the case of unplasticized polyvinylchloride (uPVC) in high-temperature systems (Anon., 1998). On the other hand, Hyde et al. (1997) reported that stainless steels in bioprocess industries incur high maintenance costs, relatively frequent system downtime for repassivation and electropolishing of critical surfaces, and eventual degradation of the inner surface due to periodic sanitizing treatments. In domestic and public water installations, however, these disadvantages are of less importance.

Biofouling, i.e. the undesirable colonisation of a surface by microorganisms, in drinking water distribution systems, may cause a deterioration of taste (Bays et al., 1970; HMSO, 1994), water discolouration (Ratsack, 1997), and the harbouring and protection of pathogenic bacteria (LeChevallier et al., 1987; Camper et al., 1996); biofouling may also immobilise particulate matter and heavy metals (Percival et al., 1998; Gauthier et al., 1999; Zacheus et al., 2001) and influence corrosion processes (Beech et al., 1994; Kobrin et al., 1997; Keevil, 2004). Therefore, a thorough understanding of the factors involved in biofouling, and in the measures to control, if not prevent it, is required.

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Marshall (1976) and Marshall and Blainey (1991) reported that the development of biofouling proceeds in four main stages: (i) the transport of organic molecules and cells to the surface; (ii) the spontaneous adsorption of organic molecules to give a ''conditioned'' surface; (iii) the bacterial adhesion to the conditioned surface and formation of microcolonies; and (iv) the development of a mature biofilm with multilayers of cells embedded in their own polymers and with high levels of competition for nutrients.

Various factors that can affect the initial adhesion process of bacteria on stainless steel, including type of finish and surface roughness, have been reported in the literature (Little et al., 1986; Boulangé-Petermann, 1996, Edyvean et al., 1996). Surface roughness may play a significant role under turbulent flow conditions in the initial stages of biofilm formation where niches and grooves may provide protection from shear. Disinfectants, as well as a greater surface area per length of pipe for nutrients to accumulate, are particularly important in oligotrophic environments.

Microscopic examinations of stainless-steel surfaces (2B-surface grade) by scanning electron microscopy (SEM) and atomic force microscopy (AFM) have shown that unpolished as-received samples exhibited the presence of grains and grain boundaries as the primary surface topography feature (Edyvean et al., 1996). Metal surface heterogeneities such as grain boundaries, inclusions, and heat-affected zones (HAZ) after welding were suggested as causes of the selective bacterial colonization of the stainless-steel surface (Kielemoes et al., 2000).

Work on mutants of biofilm-forming bacteria has shown that bacterial attachment and its mode cannot be explained solely by the DLVO theory (named after Derjaguin, Landau, Verwey and Overbeek) (Hermansson, 1999), highlighting the importance of the ability of the bacteria to produce extracellular appendages (e.g. pili), surface type (nutritive vs. non-nutritive, biotic vs. abiotic) and growth conditions (e.g., growth medium composition and concentration) (Davey and O'Toole, 2000; Stanley and Lazazzera, 2004).

For the study presented here a recently isolated, predominant member of several European drinking-water biofilms (Kalmbach et al., 2000), *Aquabacterium commune*, was selected. *A. commune* is a Gram-negative, rod-shaped bacterium that can utilize a wide range of organic acids including acetic acid but not most hexoses such as glucose. The microorganism possesses a single polar flagellum that allows for chemotaxis (Kalmbach et al., 1999).

Conventional microscopy techniques such as light/epifluorescence microscopy, SEM, and transmission electron microscopy (TEM) provide two-dimensional, elemental (SEM, TEM) information about surfaces and objects, but they either lack resolution (light microscopy) or require sample preparation prior to examination (SEM and TEM) and can therefore not be used in situ. AFM, on the other hand, overcomes some of these limitations, providing similar or better resolution to SEM/TEM in four dimensions (time-scale between minutes and hours depending on scan rate, area, resolution, etc.) and in-situ if required (Beech et al., 2005). The size, width, and depth of stainless-steel grains, grain boundaries, as well as bacteria can be quantified more realistically. Furthermore, it is possible to visualize extracellular polymeric substance (EPS) produced by irreversibly attached bacteria in their hydrated state by AFM and, to some extent, epifluorescence microscopy, but not by SEM or TEM.

The main objectives of this study are to:

(1) characterise the surface of stainless steel EN1.4307 in terms of surface roughness, and grain boundary length/width and depth using AFM;
(2) study the medium-term (weeks) biofilm development of *A. commune* on stainless steel and medium-density polyethylene (MDPE) under turbulent, oligotrophic growth conditions; and
(3) establish whether grain boundaries serve as preferential colonisation sites for *A. commune* under turbulent oligotrophic conditions.

2. Materials and methods

2.1. Slide material

Stainless steel (SS) slides (EN1.4307; L × W × H: 150 × 15 × 1 mm) from the same batch were used. The stainless-steel surface finish is designated as 2B-surface finish (skinpass). The material is cold-rolled, annealed, and, to give a smoother surface, underwent an additional light rolling between highly polished rollers. The result is a semi-bright grey surface with a R_a value of between 0.1 and 0.5 μm (Bryan Warwood, 2002; pers. comm.).

Stainless-steel slides were degreased with benzene in an ultrasound bath for 30 min, dried with a clean, soft cloth, passivated in a nitric acid-dichromate solution at 46 ± 3 °C for 30 min, rinsed with distilled water, and then benzene treated, and dried again with a clean cloth (ASTM, 2005). Steel slides were sterilized by autoclaving along with the rotating annular biofilm reactor (RAB).

The elemental composition of the stainless slides was determined in duplicate by Spectrotest (Spectro, Germany). Results are detailed in Table 1.

Pre-cut medium-density polyethylene (MDPE) slides (grade 300) were obtained from Century Plastics Ltd. (Sheffield, UK) and machined using the slide guide provided by Biosurface Technology (USA). All MDPE

Table 1
Average elemental composition (in % w/w) of stainless steel EN1.4307 slides

Element	C	Si	Mn	Cr	Ni	Al	Co	Cu	Ti
EN1.4307	0.020	0.520	2.06	17.1	10.1	0.022	0.149	0.401	0.021