Elasticity and physico-chemical properties during drinking water biofilm formation

Yumiko Abe, Pavel Polyakov, Salaheddine Skali-Lami and Grégory Francius

Laboratoire de Chimie Physique et Microbiologie pour l’Environnement (LCPME), UMR 7564, Nancy-University, CNRS, 405 rue de Vandoeuvre, 54600, Villers-lès-Nancy, France; Laboratoire d’Énergie et de Mécanique Théorique et Appliquée (LEMTA), UMR 7563, Nancy-University, CNRS, 2 avenue de la Forêt de Haye, BP 160, 54504 Vandoeuvre-lès-Nancy, France

(Received 8 March 2011; final version received 21 June 2011)

Atomic force microscope techniques and multi-staining fluorescence microscopy were employed to study the steps in drinking water biofilm formation. During the formation of a conditioning layer, surface hydrophobic forces increased and the range of characteristic hydrophobic forces diversified with time, becoming progressively complex in macromolecular composition, which in return triggered irreversible cellular adhesion. AFM visualization of 1 to 8 week drinking water biofilms showed a spatially discontinuous and heterogeneous distribution comprising an extensive network of filamentous fungi in which biofilm aggregates were embedded. The elastic modulus of 40-day-old biofilms ranged from 200 to 9000 kPa, and the biofilm deposits with a height \( h < 40 \text{ mm} \) had an elastic modulus \( \mu < 600 \text{ kPa} \), suggesting that the drinking water biofilms were composed of a soft top layer and a basal layer with significantly higher elastic modulus values falling in the range of fungal elasticity.

Keywords: biofilm; drinking water; AFM; conditioning layer; elasticity; bacteria

Introduction

Biofilms are formed when free-floating microorganisms attach to a surface. As a microorganism-free environment is difficult to achieve, the presence of biofilms is ubiquitous in many domestic and industrial environments where they are undesirable in most cases. The formation of biofilms in an aquatic environment takes place as a result of several consecutive steps including the formation of an initial conditioning layer (reversible), followed by irreversible adhesion of microbial cells to the conditioned surface, excretion of extracellular macromolecules to reinforce bacterial adhesion (steady state), and finally detachment which releases microorganisms into the bulk fluid (Richardson and Steiner 1995; Kjelleberg and Givskov 2007; Garrett et al. 2008). Most biofilm investigations indexed in the literature are related to mono-species biofilms (Klausen et al. 2003; Rupp et al. 2005; Beloin et al. 2008). In contrast to these, all environmental biofilms are poorly studied due to their heterogeneity, including drinking water biofilms, because of their complexity in terms of population, structure and heavy dependency on environmental condition (ie the geographical location, the temperature, the season, the quality of the water distribution network or the water treatment). Indeed, drinking water biofilms are composed of a wide variety of microorganisms (eg bacteria, protozoa and fungi) and their structure, topography and physico-chemical properties are closely related to the physico-chemical properties of the surface to be colonized (Oh et al. 2009) and to the different microbial populations constituting the system (Burmølle et al. 2006).

Numerous analytical techniques, both chemical and physical, have been applied to study the steps in biofilm formation, including various microscopic and spectroscopic techniques (Denkhaus et al. 2007). Among the microscopic techniques, atomic force microscopy (AFM) has emerged as a powerful tool in the study of biofilms. Furthermore, this technique provides topographic images from the micro- to the nano-scale. Atomic force spectroscopy provides qualitative and quantitative information on the physico-chemical properties of the sample such as biofilm-substratum interactions (Beech et al. 2002) or the local hydrophobicity (Dague et al. 2007). In addition, in contrast to bacterial suspensions for which the immobilization of cells to a substratum is necessary to characterize them by AFM, biofilms are already and naturally attached onto a substratum which facilitates AFM analysis. Indeed, immobilization processing in AFM analyses is often the drawback to studying a biological sample in situ (Gaboriaud and Dufrêne 2007). While most biofilm studies using AFM are based on contact-mode topographic images to visualize the biofilm morphology (Surman et al. 1996; Beech...
et al. 2002, Steinberger et al. 2002), some studies have attempted to characterize biofilm adhesion forces by the use of force spectroscopy (Chaw et al. 2005; Volle et al. 2008; Na et al. 2010).

To the authors’ knowledge, most in situ experiments were performed only to study biofilm-substratum interactions (Fang et al. 2000; Ahimou et al. 2007; Oh et al. 2009). However, few studies have been devoted to the characterization of drinking water biofilms. Among the features of these biofilms, the wide diversity in bacterial populations could imply different capacities to condition and colonize surfaces than those already observed for classical mono-species biofilms (Oh et al. 2009). Furthermore, most previous AFM studies are related to mono-species biofilms whereas the major problem encountered in the industry involves multi-species environmental biofilms. Hence, in this study, applications of the AFM technique, such as nano-indentation and chemical force spectroscopy were employed, in order to investigate the physico-chemical properties (ie elasticity, hydrophobicity and adhesion forces) at different formation steps and ages of drinking water biofilms.

The objective of this study was to investigate environmental biofilm characteristics, ie (1) to characterize variations or modifications in the physico-chemical properties of the samples during the formation of an initial conditioning layer in terms of adhesion and hydrophobicity; (2) to demonstrate the development of surface morphological features during the biofilm colonization steps from 1 to 8 weeks; and (3) to estimate the range of elastic properties of young and older biofilms. Drinking water biofilm samples were prepared on a glass surface under a hydraulic shear stress of 0.12 Pa and a shear rate of 120 s⁻¹. The disc was submerged 3 mm from the top in a water reservoir filled with tap water, and the glass coupons were held in place by vacuum suction generated by a vacuum pump (A-1000S, Tokyo Rikakikai Co. Ltd, Tokyo, Japan). The disc reactor was submerged in a sufficiently large cylindrical water reservoir (radius = 25 cm, depth 30 cm) to maintain a stable hydraulic condition, and this cylindrical reservoir was placed in a larger water basin (80 cm × 80 cm × 50 cm) to maintain a constant reservoir water temperature of 21°C. The water in the cylindrical reservoir was continuously supplied from a drinking water tap (inlet located at the bottom and the outlet at the level of free water surface) at a water residence time of approximately 24 h. The quality of the water was analyzed during the course of experiments (pH: 8.1; bacterial cells: ~10⁷ ml⁻¹; conductivity: ~500 μS cm⁻¹; cations: 4.90 meq l⁻¹; chlorine concentration: ≤0.03 mg l⁻¹ Cl₂).

**Materials and methods**

**Biofilm sample preparation**

Drinking water biofilm samples were formed on glass coupons (radius = 0.9 cm, thickness = 0.1 cm) placed in a rotating disc reactor. The concept of rotating disc reactors is detailed elsewhere (Schlichting and Gersten 1979). For sample preparation, new glass coupons were used, and prior to mounting on the reactor they were cleaned in Piranha solution (1:3 mixture of H₂SO₄ and H₂O₂) for 3 h at room temperature, rinsed with NaOH (20%) until the pH was stabilized at 7, then the coupons were extensively rinsed with milli-Q water. The reactor was operated at a rotational speed of 21 rpm, yielding a hydraulic shear stress of 0.12 Pa and a shear rate of 120 s⁻¹. The disc was submerged 3 mm from the top in a water reservoir filled with tap water, and the glass coupons were held in place by vacuum suction generated by a vacuum pump (A-1000S, Tokyo Rikakikai Co. Ltd, Tokyo, Japan). The disc reactor was submerged in a sufficiently large cylindrical water reservoir (radius = 25 cm, depth 30 cm) to maintain a stable hydraulic condition, and this cylindrical reservoir was placed in a larger water basin (80 cm × 80 cm × 50 cm) to maintain a constant reservoir water temperature of 21°C. The water in the cylindrical reservoir was continuously supplied from a drinking water tap (inlet located at the bottom and the outlet at the level of free water surface) at a water residence time of approximately 24 h. The quality of the water was analyzed during the course of experiments (pH: 8.1; bacterial cells: ~10⁷ ml⁻¹; conductivity: ~500 μS cm⁻¹; cations: 4.90 meq l⁻¹; chlorine concentration: ≤0.03 mg l⁻¹ Cl₂).

**AFM measurements**

AFM images and force–distance curves were recorded using an MFP3D-BIO instrument (Asylum Research Technology, Atomic Force F&E GmbH, Mannheim, Germany). Conical silicon nitride cantilevers were purchased from Veeco (MLCT-AUNM, Veeco Instruments SAS, Dourdan, France), and their spring constants, denoted as k, were determined using the thermal calibration method (Lévy and Maaloum 2002), providing k values ranging from 10–15 pN nm⁻¹. Prior to use in an experiment, the geometry of the tip was systematically controlled using a commercial grid for 3-D visualization (TGT1, NT-MTD Company, Moscow, Russia) and the tip curvature in its extremity was found to lie in the range ~15 to 40 nm. All the experiments were performed in tap water at room temperature (21°C) to maintain the physiological conditions of the biofilm.
**Imaging**

Images were acquired at a scan rate of 1 Hz and scan size of 80 μm × 80 μm with a 512 × 512 line/pixel resolution. AFM imaging in air was carried out after drying a biofilm sample by gently blowing with nitrogen gas. Imaging in liquid was performed in tap water at room temperature with a maximal applied force of 200 pN (see Figure S3 [Supplementary material is available via a multimedia link on the online article webpage]).

**Nano-indentation experiments**

Force–distance curves representing the deflection of the AFM tip relative to the sample surface were used to quantify the vertical force applied to the surface (see Figure S4a [Supplementary material is available via a multimedia link on the online article webpage]). This technique can be used to measure the local elastic properties and the adhesion forces of the sample surface (see Figure S4b and S4c [Supplementary material is available via a multimedia link on the online article webpage]). The force–volume mode allows force–distance curve cartography to be used, and thus visualization and measurement point by point of the changes in the adhesion forces on the surface sample.

Force–volume images were carried out by collecting 32 × 32 nano-indentation events, or force–distance curves, at a scan rate of 1 Hz for a scan size of 80 μm × 80 μm with a trigger point of 400 nm (see Figure S3 [Supplementary material is available via a multimedia link on the online article webpage]). The cantilever spring constants were systematically verified before use. The elastic modulus of the drinking water biofilms was calculated from analysis of the approaching force–distance curves based of the Hertz theory for elastic media (Hertz 1881), using conical tip geometry as detailed elsewhere (Touhami et al. 2003a). The physical parameters (contour length, persistence length) of the biofilm macromolecules were calculated from the retreating force–distance curves using the Free Jointed Chain (FJC) and/or the Worm Like Chain (WLC) models with a numerical method described elsewhere (Polyakov et al. 2011). In this study, the majority of the force–distance curves (95 to 98%) could be processed with the FJC or WLC models. The results obtained with the FJC model are presented and discussed because those represent the majority of the treatable force curves. It was observed that <5% of 1024 force–distance curves constituting each force–volume image could not be entirely processed with FJC or WLC models and this minority of adhesion peaks could probably represent nonspecific interactions. Within the framework of the FJC model, it is assumed that a macromolecule consists of rigid segments connected through flexible joints. The extension $z_{\text{FJC}}$ of the macromolecule may then be expressed as a function of the pulling force $F$ as (Ortiz and Hadziioannou 1999; Janshoff et al. 2000):

$$z_{\text{FJC}} = -L_c \left[ \cot h \left( \frac{F_{lk}}{k_b T} \right) - \frac{k_b T}{F_{lk}} \right],$$

where $T$ is the absolute temperature, the Kuhn length $l_k$ is a direct measure of the chain stiffness, $L_c$ is the total contour length of the macromolecule and $k_b$ is the Boltzmann constant. The number of monomers $N$ in the macromolecule is simply related to $L_c$ and $l_k$ according to $N = L_c/l_k$.

**Chemical force spectroscopy**

Gold-coated silicon nitride cantilevers (NPG-10, Vee-co, Santa Barbara, CA) were chemically modified with alkanethiols to obtain hydrophobic probes (Alsteens et al. 2007). A hydrophobic model surface was prepared from a gold-coated glass slice using the same protocol for the cantilever modification. Local hydrophobicity measurements were performed in deionized water at a resolution of 32 × 32 curves, at a scan rate of 1 Hz and a scan size of 80 μm × 80 μm (see Figure S4 [Supplementary material is available via a multimedia link on the online article webpage]). The hydrophobic specificity of each modified probe was verified on the hydrophobic model surface before sample measurements.

**Epifluorescence microscope enumeration and multi-staining of drinking water biofilm**

**Enumeration of bacterial cells**

The number of bacteria attached on a glass coupon was counted using a 20 × objective lens mounted on an inverted optical microscope (Axiovvert200, Carl Zeiss Light Microscopy, Germany). For each sample, a total of 20 fields of 860 × 680 μm were analyzed, and the average number of attached bacteria was reported. Optical microscope images were analyzed with an automatic MATLAB® algorithm as previously described (Paris et al. 2007, 2009).

**Multi-staining with SYBR® Green II/Concanavalin A-Texas red (ConA-Tx Red)**

SYBR Green II® and Concanavalin A – Texas Red (Molecular Probes) were purchased from Invitrogen (Invitrogen SARL, France). ConA-Tx was diluted in a TRIS-maleic buffer solution (pH ~ 5.8). The buffer consisted of a mixture of TRIS at 0.06 M, maleic acid at 0.04 M, MnCl2 and CaCl2 at 0.001 M.
concentration. One µl of the commercial SYBR® Green II solution was diluted in 2 ml of the TRIS-maleic acid buffer and deposited on the sample coupons for 20 min. The glass coupon was rinsed with non-pyrogenic sterile water to eliminate excess SYBR® Green II, and slightly dried with filter paper to remove excess water. Then, the biofilm formed on the glass disk was stained with 0.5 ml of ConA–Tx (at 0.5 mg ml⁻¹ in TRIS-maleic buffer) for 20 min and extensively rinsed with buffer solution. The samples were visualized with an inverted optical microscope (Axiovert200, Carl Zeiss Light Microscopy, Germany). Two separate images were taken at the same place to visualize SYBR® Green II and Texas Red fluorescence (see Figure S1 [Supplementary material is available via a multimedia link on the online article webpage]).

**Multi-staining with SYBR® Green II and CalcoFluor White (CFW)**

CalcoFluor White was purchased from Invitrogen (Invitrogen SARL, France). Drinking water biofilms were also stained with SYBR® Green II as previously described and stained with CalcoFluor White. CFW is used as whitening agent by the paper industry and selectively binds to cellulose and chitin. CFW fluoresces when exposed to UV light and represents a very sensitive method for direct microscope examination of fungal elements (Monheit et al. 1984; Harrington and Hageage 2003). Thirty five mg of CFW were added to 7 ml of sterile distilled water at pH 10. Then, the biofilm formed on the glass disk was stained with 0.2 ml of a solution of CFW and rinsed with sterile water to eliminate excess of CFW. The samples were visualized with an inverted optical microscope and two separate images were taken at the same place to visualize SYBR® Green II and CFW fluorescence (see Figure S2 [Supplementary material is available via a multimedia link on the online article webpage]).

**Results and discussion**

**Formation of a conditioning layer**

Four samples which were exposed to tap water under a shear stress of 0.12 Pa for 1 h, 3 h, 6 h, and 24 h respectively were analyzed. First, the progress of surface modifications caused by the formation of a conditioning layer was assessed in terms of hydrophobicity probed by the modified AFM cantilever tip. Modified tips were first tested on a model surface (gold surface totally covered by alkanethiols) to ensure their efficiency, and these model-surface tests confirmed the presence of a strong hydrophobic adhesion force with the mean value > 4–6 nN (Figure 1f). Tested tips were then used to map the hydrophobicity of a 80 µm × 80 µm surface of the samples. Figure 1 shows that the hydrophobicity of a clean glass surface (without exposure to tap water) gave rise to small hydrophobic adhesion forces with a tight distribution (F = 0.49 nN and σ = 0.16 nN). The results for the 1-h exposure sample indicated that hydrophobic adhesion forces had increased significantly (F = 1.13 nN and σ = 0.23 nN). The hydrophobic forces of exposed surfaces increased with increasing exposure duration from 1 to 24 h as indicated by a gradual shift in mean values. Furthermore, the shape of the hydrophobic adhesion force distribution became broader with exposure duration and reached a bimodal and multi-modal distribution after 6 h and 24 h exposure periods, respectively. The results are in concordance with previous observations that showed the development of a conditioning layer on a hydrophilic glass surface exposed to natural seawater was accompanied by an increase in hydrophobicity (Schneider 1997; Bakker et al. 2003; Fang et al. 2004). Marine conditioning layers are known to consist generally of different classes of organic macromolecules such as proteins, lipids, humic acids, nucleic acids, polysaccharides and aromatic amino-acids (Taylor et al. 1997; Compere et al. 2001). Although a drinking-water conditioning layer may very well be different from that of marine origin, it is plausible to assume that it also consists of various macromolecules, ubiquitous in drinking water (Batté et al. 2003), and the layer should be heterogeneous and discontinuous over the coupon surface.

In order to investigate the possible presence of different classes of macromolecules within a conditioning layer, the contour lengths (maximal extension length of a polymer chain) of the macromolecules attached to the glass surface were quantified by analyzing the retraction force–distance curves collected from the same samples using a standard, non-modified, AFM cantilever. The retraction force–distance curves were analyzed by the FJC model. The analyses revealed that the number of adhesive events over the sample surface increased with exposure duration (< 2% at 1-h and 3-h exposure, 5.5% and 6.9% at 6-h and 24-h exposure, respectively). Figure 2 shows the molecular characteristics of the macromolecules present in the initial conditioning layer after 24-h exposure. Most of the macromolecules were relatively short, between 50 and 500 nm (Figure 2a), and adhesive retraction force–distance curves were characterized by the presence of 1 or 2 adhesion peaks as shown in Figure 2b. Peak fitting with the FJC model indicated that most of the macromolecules detected by AFM had a Kuhn length ranging from 0.5 to 1.5 nm, suggesting that these macromolecules were quite flexible. In general, the peak occurrence frequency decreased steadily with increasing molecular length.
Figure 1. Statistical distributions of hydrophobic adhesion forces for the samples exposed to a hydrodynamic shear stress of 0.12 Pa for 0 h (a), 1 h (b), 3 h (c), 6 h (d), and 24 h (e). (f) Typical hydrophobic adhesion peaks with a modified AFM cantilever tip. (The black curve corresponds to a model surface while the red, pink and blue curves correspond, respectively, to 1-h, 3-h and 24-h exposure times to drinking water).
This was expected as the adhesion of macromolecules is controlled by convective diffusion and the diffusion coefficient decreases as a function of molecular size, as described in the Einstein–Stokes equation for diffusion of spherical particles under a low Reynolds number (Son and Hanratty 1969; Paris et al. 2009). Therefore, smaller macromolecules have a higher tendency to collide with a surface than larger molecules.

The reported studies on the conditioning layer of biofilms in marine environments revealed that the presence of such a layer strongly influences the adhesion of microorganisms (Bakker et al. 2003; Jain and Bhosle 2009). In terms of the relationship between surface hydrophobicity and microbial adhesion, it has been reported that the surface hydrophobicity plays a significant role in bacterial retention onto a surface after successful bacterial attachment, but it does not influence the efficiency of initial attachment to a surface (Bos et al. 2000; Boks et al. 2008). In this study, the number of attached bacteria was enumerated with an automatic MATLAB algorithm for each sample (1-h, 3-h, 6-h, and 24-h exposure samples). It was found that there were \((9.3 \pm 3.4) \times 10^3\), \((8.5 \pm 3.1) \times 10^3\), and \((39 \pm 11) \times 10^3\) cells cm\(^{-2}\) attached to surfaces after 1 h, 3 h, and 6 h exposure time, respectively. The 24-h exposure time sample contained many bacterial aggregates, prohibiting the precise counting of attached cells. Although the data are limited, no significant increase in the number of attached bacteria was observed after the first 3 h. This suggests that the most bacterial collisions could result in inefficient attachment. Given that the number of bacteria in the bulk water was assumed constant during the experiment and the apparent doubling time of attached cells in drinking water networks is expected to be \(>20\) days (Block et al. 1993), the kinetics of bacterial adhesion was reversible during 1-h to 3-h exposure periods. Increasing numbers of attached bacteria suggests that bacterial desorption should significantly decrease between 3-h and 6-h exposure periods. The increase in the efficient bacterial collision rate implies that the kinetics of bacterial adhesion became progressively irreversible. Furthermore, the broadening of the hydrophobic adhesion force distribution coincided with the observed increase in the number of attached bacteria at the 6-h exposure period. It could imply that a conditioning layer capable of facilitating irreversible bacterial adhesion could require a complex composition of different classes of macromolecules as evidenced by the broadening of the distribution. Further experiments with other techniques would be necessary to confirm these observations.

This was expected as the adhesion of macromolecules is controlled by convective diffusion and the diffusion coefficient decreases as a function of molecular size, as described in the Einstein–Stokes equation for diffusion of spherical particles under a low Reynolds number (Son and Hanratty 1969; Paris et al. 2009). Therefore, smaller macromolecules have a higher tendency to collide with a surface than larger molecules.

The reported studies on the conditioning layer of biofilms in marine environments revealed that the presence of such a layer strongly influences the adhesion of microorganisms (Bakker et al. 2003; Jain and Bhosle 2009). In terms of the relationship between surface hydrophobicity and microbial adhesion, it has been reported that the surface hydrophobicity plays a significant role in bacterial retention onto a surface after successful bacterial attachment, but it does not influence the efficiency of initial attachment to a surface (Bos et al. 2000; Boks et al. 2008). In this study, the number of attached bacteria was enumerated with an automatic MATLAB algorithm for each sample (1-h, 3-h, 6-h, and 24-h exposure samples). It was found that there were \((9.3 \pm 3.4) \times 10^3\), \((8.5 \pm 3.1) \times 10^3\), and \((39 \pm 11) \times 10^3\) cells cm\(^{-2}\) attached to surfaces after 1 h, 3 h, and 6 h exposure time, respectively. The 24-h exposure time sample contained many bacterial aggregates, prohibiting the precise counting of attached cells. Although the data are limited, no significant increase in the number of attached bacteria was observed after the first 3 h. This suggests that the most bacterial collisions could result in inefficient attachment. Given that the number of bacteria in the bulk water was assumed constant during the experiment and the apparent doubling time of attached cells in drinking water networks is expected to be \(>20\) days (Block et al. 1993), the kinetics of bacterial adhesion was reversible during 1-h to 3-h exposure periods. Increasing numbers of attached bacteria suggests that bacterial desorption should significantly decrease between 3-h and 6-h exposure periods. The increase in the efficient bacterial collision rate implies that the kinetics of bacterial adhesion became progressively irreversible. Furthermore, the broadening of the hydrophobic adhesion force distribution coincided with the observed increase in the number of attached bacteria at the 6-h exposure period. It could imply that a conditioning layer capable of facilitating irreversible bacterial adhesion could require a complex composition of different classes of macromolecules as evidenced by the broadening of the distribution. Further experiments with other techniques would be necessary to confirm these observations.

**Progress of surface colonization by biofilms**

The progress of surface colonization by drinking water biofilms was visually monitored at 1, 2, 4, 8 weeks after the initial contact was established under a constant hydrodynamic condition (shear stress of 0.12 Pa). Images performed *in situ* by AFM on biofilms in hydrated medium (see Figure S3 [Supplementary material is available via a multimedia link on the online article webpage]) show a structure composed of clusters and aggregates. It was emphasised that it is very difficult to clearly identify on these images the bacterial colonies or isolated bacteria that constitute the biofilm. In addition, the aggregates were fragile and affected by AFM when samples were scanned by the tip. This attests to the viscoelastic properties, the fragility
of the biofilm and probably the presence of extracellular polymers rich in mannnose and glucose that could disrupt or affect AFM-tip during the image scanning (see Figure S2 [Supplementary material is available via a multimedia link on the online article webpage]). Exopolysaccharides are known to be soft materials that contribute to biofilm viscoelasticity and formation (Cogan and Keener 2004; Lahaye et al. 2007).

When samples were slightly dehydrated and imaged in air, the resolution was better and it was easier to identify biofilm structure (Figure 3), although dehydration is likely to affect or alter the biofilm structure. This figure illustrates the evolution of the biofilm at various ages. The development of drinking water biofilms was already recognizable after 1 week, and the images show that biofilms were discontinuous and heterogeneously distributed as previously described (Paris et al. 2007). These AFM images clearly demonstrate the increase in surface colonization with time. Additionally, filamentous bacterial structures (see Figure 3) andprobably fungal structures (Supplementary Information and Figure S3) embedded within the biofilms were depicted from the first week. Multistaining images (CFW/SYBR® Green II) revealed important filamentous structures (20–300 µm long), rich in cellulose that did not contain RNA or DNA (Figure S1a and S1b [Supplementary material is available via a multimedia link on the online article webpage]). The structures exposed in Figure 3 seemed to become longer, thicker and denser with time until a network was finally established throughout the entire surface at 8 weeks. Although relatively little is known, the presence of waterborne fungi in drinking water has long been recognized (Nagy and Olson 1982). Furthermore, a close inspection of images shown in Figure 3 reveals that drinking-water biofilms appear to anchor themselves on top of this microbial network, implying that this fungal structure may assist in reinforcing biofilm adhesion to a substratum surface.

**Nanomechanical properties of drinking water biofilms**

The mechanical properties of biofilms play crucial roles in understanding biofilm resistance to external shearing forces (Ohashi et al. 1999; Stoodley et al. 1999; Klapper and Dockery 2006), and the elastic moduli of bacterial biofilms have been measured by various methods (Stoodley et al. 1999; Korstgens et al. 2001). Analysis of AFM nano-indentation experiments were used to calculate the local elastic modulus of soft biological materials (Touhami et al. 2003a ). In this study, the elastic moduli of 36 and 40-day -old drinking water biofilms were calculated based on the approaching force–distance curves recorded during indentation experiments. Figure 4 shows the distributions of an elastic modulus of a typical biofilm-covered surface (Figure 4a) and a surface containing a large aggregate (Figure 4b). Both types of biofilm-covered surfaces (with or without an aggregate) were found on the same 40-day -old sample. The elastic modulus ranged from 200 kPa to 9000 kPa, and the column at 10,000 kPa represents the total frequency of solid substratum elasticity (>10,000 kPa) where no soft material was encountered. There is no distinctive multimodal distribution of the elastic moduli, but some elasticity ranges can be associated with typical classes of microbial communities. For example, several AFM studies reported elastic moduli of 50–500 kPa for microalgae (Francius et al. 2008b), 50–2000 kPa for common bacteria (Abu-Lail and Camesano 2002; Gaboriaud et al. 2005; Francius et al. 2008a, 2008c ), approximately 600 kPa for yeast spores (Touhami et al. 2003b), and 2000 to 8000 kPa for germinating yeast and fungi (Touhami et al. 2003a; Alsteens et al. 2008). It is reported that drinking water biofilms are composed of various materials such as inorganic hard deposits, microorganisms, and polymeric substances (Batté et al. 2003). A complex composition of biofilms was visually demonstrated by AFM images shown in Figure 3, explaining the observed large variation in elastic modulus. In addition, a biofilm surface with a large aggregate (Figure 4b) contained an increased event frequency especially for the large elasticity range (>1200 kPa), falling in the range of representative values for fungi. It may imply that the fungal structure is more abundant near a large biofilm aggregate, and its presence may have resulted in a significantly decreased percentage of solid substratum coverage (62% and 17% for surfaces without and with an aggregate, respectively).

Soft materials possessing a low elastic modulus value (<600 kPa) occupied a significantly elevated proportion of the distribution for both types of biofilm-covered surfaces (12 and 20% for surfaces without and with a large aggregate, respectively). Based on the corresponding AFM height images, the surface coverage rates by biofilm deposits (the proportion of analyzed surface containing an object with a height >0.5 µm) were calculated for both cases. The surface coverage rates were 14% and 20% for a biofilm surface without (Figure 4a) and with a large aggregate (Figure 4b), respectively. The agreement between the percentage of low elasticity proportion and that of surface coverage suggests that the observed soft biofilm materials (elasticity <600 kPa) were associated with the presence of biofilm deposits having a height >0.5 µm.

Several studies investigated the stratification of environmental biofilms, and most of these reported the
Figure 3. Morphology of drinking water biofilm is modified with time. Each panel represents three deflection images performed by AFM in air and in contact mode at 1, 2, 4 and 8 weeks at a shear stress formation of 0.12 Pa.
Figure 4. Nano-mechanical properties of 40-day-old drinking water biofilms. (a) Elastic modulus distribution* of a typical biofilm-covered surface; (b) elastic modulus distribution* of a biofilm-covered surface containing a large aggregate; (c) distribution of contour length of a typical biofilm-covered surface; (d) distribution of contour length of a biofilm-covered surface containing a large aggregate; (e) example of a retreating force–distance curve ** for a typical biofilm-covered surface; (f) example of a retreating force–distance curve ** for a biofilm-covered surface containing a large aggregate. Note: *The far right column corresponds to the percentage of elastic modulus that is > 10 MPa; ** the circle-line corresponds to the raw data, and the line is the fitting result.
presence of thick and ‘fluffy’ top layer and a thin bottom layer (Coufort et al. 2007; Derlon et al. 2008). AFM nanoindentation experiments can only indicate the elastic property of the outermost layer of a biofilm aggregate. Besides, the elastic modulus values between the soft biofilm deposits (<600 kPa) and the substratum surface (>10,000 kPa) could be interpreted as the range of values corresponding to the biofilm basal structure. It is important to note that different components of drinking water biofilms possess different elastic properties, which refer to their resistance to external shearing forces. Apparently, it is easier to deform soft biofilm materials found in biofilm aggregates than the basal structure. Thus, different components of biofilms will resist different levels of shearing stresses. Hence, attention must be paid when assigning a range of values to a certain type of biofilm in modeling studies. The selected range of values needs to correspond to the biofilm component of interest.

Figure 4c and 4d presents the results of analyzing retreating force–distance curves from the same experiments as used for calculating the elastic moduli as shown in Figures 4a and 4b. The curve fitting results indicate a very large contour length distribution for both types of biofilm-covered surfaces. Although the majority had contour lengths of 100–500 nm, some long macromolecules were uncoiled to 3000 nm with Kuhn lengths ranging from 0.05 to 2 nm, reflecting the presence of very long and flexible macromolecules. Unlike the curves obtained from a 24-h exposure sample (Figure 2), those of the 40-day exposure sample contained numerous adhesion peaks (3 to 15 adhesion peaks per curve) as shown in Figure 4c and 4f, indicating that each indentation event encountered a number of macromolecules of different sizes. Also, it is important to note that the macromolecules whose length was <500 nm were always detected in significant amounts whatever the exposure period. These short macromolecules could come from the bulk water or partly from microbial activity. The remainder of the longer macromolecules detected from the 40-day exposure sample were most likely metabolically produced by biofilm microbial communities as extracellular biopolymers to reinforce bacterial adhesion to a surface. The comparison between a typical biofilm-covered surface (Figure 4c) and a surface containing a large biofilm aggregate (Figure 4d) indicates that the latter type contained longer macromolecules at a higher frequency, strongly suggesting the microbial origin of these long macromolecules.

**Remarks on using AFM in biofilm studies**

This investigation illustrates the use of different AFM applications to study drinking water biofilms at different developmental steps and physiological conditions and at a micro- to nano-metric scale, which could not be achieved by other techniques. For example, the measurement of elastic modulus of a basal biofilm structure is probably not measurable by other methods as it is too small to perform a deformation test, and it would require a very large force to deform such a rigid structure. Although AFM remains a powerful tool in biofilm studies, several shortcomings have been observed with its use to study biofilms during the work. First and foremost, the requirement of a smooth surface as a substratum material prevents observation of the biofilms formed on frequently employed pipe materials in industry (eg cast iron and stainless steel) in which surface roughness demonstrated a strong correlation with the biofilm formation rate (Pedersen 1990; Percival and Walker 1999). The AFM used in this study could not image an object with a height >5 μm from the substratum surface due to the photodiode size restriction. Detailed images can readily be obtained when analyzed in air while imaging biofilms in situ in a liquid environment was difficult as the AFM cantilever tip scratched soft biofilms, rendering the image highly streaked. In addition, the cantilever became quickly contaminated by biofilm materials while imaging in liquid; thus, cantilevers needed to be changed frequently. In terms of study size, the AFM analysis frame is restricted at 90 μm × 90 μm, hence the information obtained is highly localized. As for the application of modified cantilever tips, it was observed that the quantification of a specific adhesion force (such as a hydrophobic force) was not feasible on biofilm aggregates as the extracellular macromolecules readily stuck to the modified tip, concealing the specific adhesion force peak in a broad non-specific adhesion force.

**Conclusions**

The study demonstrated that the steps in drinking water biofilm formation can be followed and investigated by AFM techniques (imaging and force spectroscopy) and multi-staining fluorescence microscopy. During the formation of an initial conditioning layer, there was an increase in surface hydrophobicity as a function of time. After a 6-h exposure period to tap water under a shear stress of 0.12 Pa, the distribution of hydrodynamic adhesion forces became broader and multimodal, indicating the presence of different classes of macromolecules attached on the surface. The length of these macromolecules ranged from 50 to 500 nm. In addition, the broadening of the distribution curve coincided with a significant increase in the number of attached bacteria, hence, the modification of the surface by a conditioning layer probably composed.
of different classes of macromolecules could be necessary for further bacterial adhesion. Surface colonization by drinking water biofilms was visually studied by air-phase AFM images which revealed that the biofilms were discontinuous and heterogeneously distributed in space. Filamentous fungal structures were already visible in 1-week-old biofilms, and these became longer, thicker and denser with time and appeared to reinforce adhesion of the biofilm onto the surface. In terms of the nanomechanical properties of drinking water biofilms, the distribution of elastic moduli for 40-day-old biofilms ranged from 200 to 9000 kPa, indicating the complex composition of the biofilm materials, as was evident from air-phase AFM images. The biofilm aggregates with a height >0.5 μm had an elastic modulus <600 kPa, suggesting that the drinking water biofilms were composed of a soft top layer and a basal layer with a significantly higher elastic modulus values. The length of macromolecules in 40-day-old biofilms ranged from 100 to 3000 nm, with Kuhn lengths of 0.05 to 2 nm, reflecting the presence of very long and flexible macromolecules. The comparison between the macromolecule length distributions of 24-h and 40-day old surfaces suggested that the macromolecules with a length >500 nm were likely to be microbial extracellular biopolymers.

Acknowledgements

This work was carried out as part of a research program coordinated by the University Henri Poincaré – Nancy I (UHP, Nancy, France). It was funded by the Agence de l’Eau Seine-Normandie (AESN, Nantes, France), the Anjou-Recherche group (Veolia Water, Maisons-Laffitte, France), and the Syndicat des Eaux d’Ile de France (SEDIF, Paris, France).

References

Abu-Lail NI, Camesano TA. 2002. Elasticity of Pseudomonas putida KT2442 surface polymers probed with single-molecule force microscopy. Langmuir 18:4071–4081.

Ahimou F, Semmens MJ, Novak PJ, Haugstad G. 2007. Biofilm cohesiveness measurement using a novel atomic force microscopy methodology. Appl Environ Microbiol 73:2897–2904.

Alsteens D, Dague E, Rouxhet PG, Baulard AR, Dufrene YF. 2007. Direct measurement of hydrophobic forces on cell surfaces using AFM. Langmuir 23:11977–11979.

Alsteens D, Dupres V, Mc Evoy K, Wilding L, Gruber HJ, Dufrene YF. 2008. Structure, cell wall elasticity and polysaccharide properties of living yeast cells, as probed by AFM. Nanotechnology 19:1–9.

Bakker DP, Klijnstra JW, Busscher HJ, van der Mei HC. 2003. The effect of dissolved organic carbon on bacterial adhesion to conditioning films adsorbed on glass from natural seawater collected during different seasons. Biofouling 19:391–397.

Batté M, Appenzeller BMR, Grandjean D, Fass S, Gauthier V, Jorand F, Mathieu L, Boualam M, Saby S, Block JC. 2003. Biofilms in drinking water distributin systems. Rev Environ Sci Bio/Technol 2:147–168.

Beech IB, Smith JR, Steele AA, Peneger I, Campbell SA. 2002. The use of atomic force microscopy for studying interactions of bacterial biofilms with surfaces. Colloids Surf B-Biointerfaces 23:231–247.

Beloin C, Roux A, Ghigo J. 2008. Escherichia coli biofilms. Curr Top Microbiol 322:249.

Block JC, Haudidier K, Paquin JL, Mizia J, Levy I. 1993. Biofilm accumulation in drinking water distribution systems. Biofouling 6:333–343.

Boks NP, Norde W, van der Meil HC, Busscher HJ. 2008. Forces involved in bacterial adhesion to hydrophilic and hydrophobic surfaces. Microbiology 154:3122–3133.

Bos R, van der Meil HC, Gold J, Busscher HJ. 2000. Retention of bacteria on a substratum surface with micro-patterned hydrophobicity. FEMS Microbiol Lett 189:311–315.

Burmolle M, Webb JS, Rao D, Lars H, Hansen L, Søren J, Sørensen S, Kjelleberg S. 2006. Enhanced biofilm formation and increased resistance to antimicrobial agents and bacterial invasion are caused by synergistic interactions in multispecies biofilms. Appl Environ Microbiol 76:3916–3923.

Chaw KC, Manimaran M, Tay FEH. 2005. Role of silver ions in destabilization of intermolecular adhesion forces measured by atomic force microscopy in Staphylococcus epidermidis biofilms. Antimicrob Agents Chemother 49:4853–4859.

Cogan NG, Keener JP. 2004. The role of the biofilm matrix in structural development. Math Med Biol 21:147–166.

Compere C, Bellon-Fontaine MN, Bertrand P, Costa D, Marcus P, Polesinis C, Pradier CM, Rondot B, Walls MG. 2001. Kinetics of conditioning layer formation on stainless steel immersed in seawater. Biofouling 17:129–145.

Coufor C, Derlon N, Ochoa-Chaves J, Line A, Paul E. 2007. Cohesion and detachment in biofilm systems for different electron acceptor and donors. Water Sci Technol 55:421–428.

Dague E, Alsteens D, Latge JP, Verbelen C, Raze D, Baulard AR, Dufrene YF. 2007. Chemical force microscopy of single live cells. Nano Lett 7:3026–3030.

Denghaus E, Meisen S, Telgheder U, Wingender J. 2007. Chemical and physical methods for characterisation of biofilms. Microbiom Acta 158:1–27.

Derlon N, Masse A, Escudier R, Bernet N, Paul E. 2008. Stratification in the cohesion of biofilms grown under various environmental conditions. Water Res 42:2102–2110.

Fang HHP, Chan KY, Xu LC. 2000. Quantification of bacterial adhesion forces using atomic force microscopy (AFM). J Microbiol Methods 40:89–97.

Fang Z, Qiu Y, Kuffel E. 2004. Formation of hydrophobic coating on glass surface using atmospheric pressure nonthermal plasma in ambient air. J Phys D-Appl Phys 37:2261–2266.

Francius G, Domenech O, Mingeot-Leclercq MP, Dufrene YF. 2008a. Direct observation of Staphylococcus aureus cell wall digestion by lysostaphin. J Bacteriol 190:7904–7909.

Francius G, Tesson B, Dague E, Martin-Jezequel V, Dufrene YF. 2008b. Nanostructure and nanomechanics of live Phaeodactylum tricornutum morphotypes. Environ Microbiol 10:1344–1356.

Francius G, Lebeer S, Alsteens D, Wilding L, Gruber HJ, Hols P, De Keersmaecker S, Vanderleyden J, Dufrene YF. 2008c. Detection, localization, and conformational analysis of single polysaccharide molecules on live bacteria. ACS Nano 2:1921–1929.
Ortiz C, Hadzioannou G. 1999. Entropic elasticity of single polymer chains of poly(methacrylic acid) measured by atomic force microscopy. Macromolecules 32:780–787.

Paris T, Skali-Lami S, Block JC. 2007. Effect of wall shear rate on biofilm deposition and grazing in drinking water flow chambers. Biotechnol Bioeng 97:1550–1561.

Paris T, Skali-Lami S, Block JC. 2009. Probing young drinking water biofilms with hard and soft particles. Water Res 43:117–126.

Pedersen K. 1990. Biofilm development on stainless-steel and PVC surfaces in drinking-water. Water Res 24:239–243.

Percival SL, Walker JT. 1999. Potable water and biofilms: a review of the public health implications. Biofouling 14:99–115.

Polyakov P, Soussen C, Duan JB, Duval JFL, Brie D, Francius G. 2011. Automated force volume image processing for biological samples. PLoS One 6:18887.

Richardson PD, Steiner M. 1995. Principles of cell adhesion. Boca Raton (FL): CRS Press.

Rupp CJ, Fux CA, Stoodley P. 2005. Viscoelasticity of Staphylococcus aureus biofilms in response to fluid shear allows resistance to detachment and facilitates rolling migration. Appl Environ Microbiol 71:2175–2178.

Schlichting H, Gersten K. 1979. Boundary-layer theory. 7th ed. New York (NY): McGraw-Hill.

Schneider RP. 1997. Bacterial adhesion to solid substrata coated with conditioning films derived from chemical fractions of natural waters. J Adhes Sci Technol 11:979–994.

Son JS, Hanratty TJ. 1969. Velocity gradients at wall for flow around a cylinder at Reynolds numbers from 5 \times 10^3 to 10^5. J Fluid Mech 35:353–368.

Steinberger RE, Allen AR, Hansma HG, Holden PA. 2002. Elongation correlates with nutrient deprivation in Pseudomonas aeruginosa-unsaturated biofilms. Microb Ecol 43:416–423.

Stoodley P, Lewandowski Z, Boyle JD, Lappin-Scott HM. 1999. Structural deformation of bacterial biofilms caused by short-term fluctuations in fluid shear: an in situ investigation of biofilm rheology. Biotechnol Bioeng 65:83–92.

Surman SB, Walker JT, Goddard DT, Morton LHG, Keevil CW, Weaver W, Skinner A, Hanson K, Caldwell D, Kurtz J. 1996. Comparison of microscope techniques for the examination of biofilms. J Microbiol Methods 25:57–70.

Taylor GT, Zheng D, Lee M, Troy PJ, Gyananath G, Sharma SK. 1997. Influence of surface properties on accumulation of conditioning films and marine bacteria on substrata exposed to oligotrophic waters. Biofouling 11:31–57.

Touhami A, Nysten B, Dufrêne YF. 2003a. Nanoscale mapping of the elasticity of microbial cells by atomic force microscopy. Langmuir 19:4539–4543.

Touhami A, Hoffmann B, Vasella A, Denis FA, Dufrêne YF. 2003b. Probing specific lectin-carbohydrate interactions using atomic force microscopy imaging and force measurements. Langmuir 19:1745–1751.

Volle CB, Ferguson MA, Aidala KE, Spain EM, Nunez ME. 2008. Spring constants and adhesive properties of native bacterial biofilm cells measured by atomic force microscopy. Colloids Surf B-Biointerfaces 67:32–40.