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**The significance of calcium ions on *Pseudomonas fluorescens* biofilms – a structural,
and mechanical study**

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19 **Abstract**

20 The purpose of this study was to investigate the effects of calcium ions on the structural and
21 mechanical properties of *Pseudomonas fluorescens* biofilms grown for 48 hours. Advanced
22 investigative techniques such as laser scanning microscopy and atomic force spectroscopy were
23 employed to characterize biofilm structure as well as biofilm mechanical properties following
24 growth at different calcium concentrations. The presence of calcium during biofilm
25 development led to higher surface coverage with distinct structural phenotypes in the form of a
26 granular and heterogeneous surface, compared to the smoother and homogenous biofilm surface
27 in the absence of calcium. The presence of calcium also increased the adhesive nature of the
28 biofilm, while reducing its elastic properties. These results suggests that calcium ions could have
29 a functional role in the biofilm's development and has practical implications in, for example,
30 analysis of biofouling in membrane-based water treatment processes such as nanofiltration or
31 reverse osmosis where elevated calcium concentrations may occur at the solid-liquid interface.

32 **Keywords:** *Pseudomonas fluorescens*, *biofilm*, *CaCl₂*, *force spectroscopy*, *Young's modulus*

1. Introduction

Biofilms comprise a community of microorganisms attached to a surface, embedded in a matrix of extracellular polymeric substances (EPS), a mixture of macromolecules such as polysaccharides, proteins, nucleic acids, phospholipids and other polymeric compounds which intersperse the cells and mediate adhesion to surfaces (Wingender et al. 1999). The physical stability of the polymer network within the biofilm is enhanced by crosslinking in the EPS matrix, forming a temporary gel (Allison et al. 2000). The concentration of ions, such as calcium, is generally accepted as playing an important role in determining the mechanical properties of biofilms (Ahimou et al. 2007; Korstgens et al. 2001). Studies have demonstrated that the crosslinking of EPS alginate molecules by calcium ions was found to increase the elastic properties of *P. aeruginosa* biofilms (Korstgens et al. 2001), as well as biofilm thickness (Sarkisova et al. 2005). The presence of elevated levels of ions is particularly relevant to pressure-driven membrane processes such as nanofiltration and reverse osmosis which are designed to retain salts and under normal operation create a concentration polarisation (CP) layer of elevated salt concentration adjacent to the membrane-water interface. In these systems biofouling is a well- documented performance limiting problem. Because CP occurs in the same location as biofilm formation, it can be expected that biofilm development occurs in a micro-environment of elevated salt concentration. The presence of such elevated levels of ions is therefore particularly relevant to such processes, which are designed to retain salts. Of these salts, calcium was shown to be the principal element in fouling layers from surface water, as demonstrated in an earlier study (Baker et al. 1995). This unfortunately can lead to inorganic fouling due to scale formation of sparingly soluble inorganic salts, which occurs whenever the ionic salt concentration stream exceeds the equilibrium solubility. Since inorganic scaling and biofouling do not occur in isolation during nanofiltration processes, investigating how one factor impacts the other is therefore crucial for better understanding the membrane fouling. The present study sought to study the effect of different concentrations of a relevant divalent ion, in this case

calcium, on the structural and mechanical properties of a *Pseudomonas fluorescens* biofilm model. There is currently a poor understanding of the effect of microenvironments, such as solution composition and shear forces, on biofilm material properties. AFM-based force spectroscopy has emerged as a promising technique allowing the quantification of biofilm adhesive and cohesive forces, however, this has predominantly been applied for single cell adhesion studies, in which this technique was used to characterise the bacterial outer membrane molecules.

Nanoindentation experiments have been used to estimate the elastic modulus of the single bacterial cells using the Hertz model (Chen et al. 2012; Francius et al. 2008; Touhami et al. 2003). The Hertzian model is a well-accepted contact mechanics model which consists of indentation into a material to estimate the elastic modulus based on a non-adhesive contact area during indentation (Hertz 1881). Although experimentally challenging, AFM-based, nanoindentation can also be used to estimate the elastic modulus of bacterial biofilm aggregates (Abe et al. 2011; Mosier et al. 2012). More specifically, the viscoelastic properties of biofilms grown on the microbead of an AFM cantilever was directly measured during their compression by a stiff surface via indentation-time (creep) curves, in which the Hertz model was incorporated into a three-element Voigt Standard Linear Solid model (Lau et al. 2009).

The retraction part of an AFM force curve can also be used to assess the adhesion properties of biofilms. Different studies were able to demonstrate that increased biofilm adhesive properties is directly linked to the amount of the EPS synthesized during biofilm development (Auerbach et al. 2000; Fang et al. 2000; Oh et al. 2007; Tsoligkas et al. 2012). Although the use of AFM for studying biofilm cohesiveness within biofilms still remains uncommon, its potential use may provide invaluable information concerning the biofilm sample being analysed. In one abrasion study of mixed culture biofilms from activated sludge, AFM was successfully implemented to describe the increasing cohesiveness of the sample with increasing sample depth under elevated shear loading (Ahimou et al. 2007). Since the cohesive and adhesive properties of cells are of critical importance in the factors that dictate biofilm detachment,

understanding the functional role of calcium ions in this process would help better understand how biofilms form and proliferate in shear environments.

The objective of this study was to investigate the effects of CaCl_2 on the mechanical and structural properties of *Pseudomonas fluorescens* biofilms, with particular reference to the measurement of the adhesive and elastic properties at the surface of the different biofilm samples using AFM-based force spectroscopy. These measurements were complemented by CLSM and SEM imaging techniques.

2. Materials and Methods

2.1 Bacteria Strain and Culture Condition

The selected bacterial strain for this study was a mCherry-expressing *Pseudomonas fluorescens* PCL1701 (Lagendijk et al. 2010), stored at -80°C in King B broth (King et al. 1954) supplemented with 20% glycerol. Cultures were obtained by inoculating 100 mL King B broth supplemented with gentamicin at a final concentration of $10\ \mu\text{g mL}^{-1}$ using a single colony of a previously grown culture on King B agar (Sigma Aldrich, Ireland) at 28°C . The inoculated medium was then incubated at 28°C with shaking at 75 rpm and left to grow to late exponential growth stages, corresponding to an Optical Densities (OD_{600}) of about 1.0.

2.2 Biofilm Growth under Different CaCl_2 Concentrations

A 5 μL volume of an overnight culture was used to inoculate sterile individual centrifuge tubes (Falcon, Fischer scientific, Ireland) each containing 3 mL King B broth supplemented with gentamicin at a final concentration of $10\ \mu\text{g mL}^{-1}$. The pH of the King B medium prior inoculation was pH 6.8, which later increased to pH 7.4 following overnight planktonic growth by *Pseudomonas fluorescens*.

Two tubes were supplemented with CaCl_2 each at final concentrations of 1.5 mM and 15 mM. Single autoclaved cover slip disks of 24 mm diameter (Thermo Scientific, Germany), were

partially submerged into individual tubes, before sealing each tube with sterile cotton wool. Tubes were then incubated for 48 hours at 28 °C with shaking at 75 rpm. A schematic of the set-up and a typical biofilm grown at air-liquid line at 1.5 mM CaCl₂ (prior to rinsing) are shown in Figure 1S, in the supplementary information section.

2.3 Confocal Laser Scanning Microscopy

Following biofilm growth, coverslips were first rinsed in sterile 0.1M NaCl solution by gently dipping the coverslip containing biofilm in a tube containing sterile 0.1M NaCl solution. The coverslip was then carefully placed in a single-well Nunc® Lab-Tek® II Chamber Slide™ (VWR, Ireland) filled with sterile phosphate buffered saline solution.

Horizontal plane images of the biofilms were acquired using an Olympus FV1000 confocal laser scanning microscope (CLSM) at the Live Cell Imaging core technology facility platform, Conway Institute, UCD. CLSM experiments were repeated twice for each biofilm growth conditions using two independent inoculums. At least 4 to 6 random areas were acquired for each biofilm grown on the air-liquid interface area per coupon per experiment. The excitation wavelength used for mCherry was 559 nm, and emitted fluorescence was recorded within the range of 570 to 670 nm. Images were collected through an Olympus UPL SAPO 10x/0.40 air objective with a z-step of 1 µm. 3D projections were performed with Zeiss ZEN imaging software. The structural quantification of biofilms (biovolume, surface coverage, thickness and roughness) was performed using the PHLIP Matlab program developed by J. Xavier (<http://phlip.sourceforge.net/phlip-ml>). Biofilm surface volume ratio was obtained from parameters provided by the PHLIP analysis.

One-way analysis of variance was performed using MINITAB v15.1 (Minitab Inc., State College, PA, USA) in order to test the significant differences in biovolume quantities (µm³), surface coverage (%), mean thickness (µm), biofilm roughness, and surface to volume ratio of *P.*

fluorescens biofilms grown in the presence or absence of CaCl_2 with Tukey's test for pair wise comparisons. All tests were performed at 5% significance level.

2.4 Scanning Electron Microscopy

Following incubation biofilms were prepared for scanning electron microscopy (SEM) observations. Biofilms were first rinsed by gently dipping the coverslip containing biofilm growth in a tube containing sterile 0.1M NaCl solution. Biofilms were then chemically fixated by submerging coverslips into individual small Petri dishes, each containing 5mL solution containing 2.5% glutaraldehyde (Sigma, Ireland), 0.1 M sodium cacodylate (Sigma, Ireland) and 0.075 % (w/v) Ruthenium red (Sigma, Ireland), for 24 hours. All samples were rinsed in sterile MilliQ, followed by a stepwise dehydration treatment by exposing fixated samples to increased ethanol-volumes of 10%, 25%, 50%, 75%, 90% and 100%, each at 10 min intervals. Samples were then exposed to 50% then 100% hexamethyldisilazane (Sigma, Ireland) before drying in air, before gold sputtering using an Eitech K575K coater for 30s at 30 mA. High magnification imaging of biofilms grown at different CaCl_2 concentration environments was performed using a Hitachi Quanta 3D FEG scanning electron microscope of the UCD Nano-imaging and Materials Analysis Centre (NIMAC).

2.5 AFM-based Force Spectroscopy

The elastic and adhesive properties of *P. fluorescens* biofilms were characterised by analysing indentation and retraction curves obtained from AFM-based Force Spectroscopy measurements. Force measurements were performed using a JPK NanoWizard II BioAFM (JPK Instruments, Germany) integrated with an inverted optical microscope (Nikon, Japan) and a Hamamatsu CCD camera. This ensemble was enclosed in an acoustic isolation chamber, and placed on a vibration isolation table (TS-150, JRS Scientific Instruments, Switzerland). A commercial silicone v-shaped

cantilever with a spherical borosilicate tip of 10 μm radius (PT-GS, Novascan Technologies Inc.) was used in this study. The spring constant of the cantilevers was calibrated as 0.12 N/m at the room temperature, using the thermal noise method (Hutter & Bechhoefer 1993). After each force map measurement, several single force curves were recorded on a clean glass in order to observe the possible residual forces on the retraction curves, indicative of the tip contamination. When contaminated, the cantilever was carefully rinsed with ethanol and Milli-Q water, before UV Ozone cleaning (ProCleaner, Bioforce Nanosciences, USA).

Prior to measurements, biofilms were first rinsed in sterile 0.1 M NaCl solution, as previously described. Samples were then mounted at the bottom of a JPK liquid cell holder and measurements were carried out on samples submerged in 0.1M NaCl solution (cf. supplementary Figure S2 description of inverted bright field microscopic images of the approached AFM cantilever over the biofilm patch areas). For each biofilm growth condition, force spectroscopy measurements were performed on duplicate biofilms samples grown on separate cover glass slides. Prior to acquisitions, an interval of 30 min was allowed to minimise the thermal noise, causing fluctuations in the recorded signal. Force maps were recorded at various locations on the biofilm samples in a 2-dimensional array of 4 x 4 (16 force curves) over a 10 x 10 μm^2 area. All force spectroscopy experiments were performed in duplicates for each biofilm growth condition, using two independently grown inoculum cultures. Three force maps were obtained for each tested biofilm sample, amounting to a total of 192 force curves for each tested biofilm type. It should be noted that the AFM data in this study was restricted to the biofilm-liquid interface. Force curves were collected at a slow rate of 1 $\mu\text{m}/\text{s}$ to minimise the hydrodynamic effects and indentation was made up to a force set-point limit of 9-11 nN.

JPK IP data processing software (version 3.3, JPK Instruments) was used for raw data processing and Hertz model fitting based on the protocols explained in user manual (JPK Instrument, 2009). Data processing steps involved converting recorded raw photodetector signal

(in Voltage) into the force (in nN), calculating indentation depth, and manually determining the contact point for Hertz model fitting. It is noted that indentation into a deformable sample is obtained by subtracting the Piezo displacement on a rigid mica or glass surfaces (which is only caused by cantilever deflection) from the total Piezo displacement (which is the sum of cantilever deflection and indentation depth) as described for a bacterial cell nanoindentation (Touhami et al. 2003). The elastic modulus was calculated based on the assumption that biofilm is an incompressible material having a constant Poisson's ratio value of 0.5. This is an acceptable assumption as the biofilm is mainly composed of water (95%) (Characklis & Marshall 1990). In previous biofilm studies, a constant Poisson's ratio value within the range of 0.4-0.5 was used (Laspidou & Aravas 2007; Taherzadeh et al. 2010). It is important to note that in a viscoelastic polymeric material the Poisson's ratio typically changes in a time dependent manner from 0.33 in the glassy regime to approximately 0.5 in the rubbery regime due to the flowing properties (Greaves et al. 2011). This may also be expected in biofilms, however it is a largely neglected concept in biofilm mechanics.

In the AFM-based nanoindentation testing, determining the contact point is generally the most critical step for fitting the Hertz model into a force-indentation (FI) curve. Defining the contact point has still not been well described in the literature for AFM measurements on biofilms; however it has been widely discussed in studies on individual bacterial cell surfaces (Gaboriaud & Dufrene 2007). It was reported that the initial nonlinear region on the FI curve was potentially affected by both the repulsive surface forces as well as sample surface deformation (Oh et al. 2007). As for the indentation testing of single bacterial cells, contact points are typically chosen at the point at which the force increases from the zero value, which unfortunately do not take into account the initial surface repulsive forces considered to be insufficient to deflect the cantilever (Amoldi et al. 1998). It was only recently that the Hertz model was successfully applied to multilayer inhomogeneous biological samples such as living

cells (Kasas et al. 2013; Radotic et al. 2012). This adds weight to the applicability of using the Hertz model in segmented layers of the material. In this study it was necessary to use a multiple layer Hertz fitting approach for the characterisation and analysis of biofilms grown at different CaCl_2 concentrations.

2.5.1 Probability distribution analysis

Probability density analysis describes the relative likelihoods and distributions of outcomes, defined by the integral of the variable's density over a particular measured range via the area under the density distribution. By using a statistical package provided by Matlab (version R2011a) a lognormal distribution function was found to be the best fit for the data acquired in this study (cf. supplementary section 2.2). The lognormal probability density function has been previously used to describe the distribution of AFM adhesion data for the bacterial cell surfaces (Abu-Lail & Camesano 2003; Eskhan & Abu-Lail 2014; Gordesli & Abu-Lail 2012; Park et al. 2009).

3. Results and Discussion

3.1 Biofilm structural and morphological properties

To characterise the effects of CaCl_2 on biofilm morphological and structural properties, *Pseudomonas fluorescens* biofilms were grown for two days at the air-liquid interface before being monitored and quantified following CLSM and SEM. Although all biofilms formed a distinct boundary at the air-liquid interface regardless of the presence of CaCl_2 , the size of this boundary was correlated with the concentration of CaCl_2 used during biofilm development (results not shown). Microscopy further revealed differences between the biofilms grown at different concentrations. Figure 1 depicts representative reconstructed 3D projections of *P. fluorescens* biofilms grown at 0mM CaCl_2 (Figure 1A), 1.5mM CaCl_2 (Figure 1C) and 15mM CaCl_2 (Figure

1E). Biofilm grown in the absence of CaCl_2 had a smoother textured surface (Figure 1A) compared to biofilms grown in the presence of CaCl_2 (Figure 1C & E), which showed signs of a rougher surfaces. High magnification images obtained from SEM further corroborated CLSM images revealing that biofilms grown in the absence of CaCl_2 were made up of a smooth EPS-like material covering the cells (Figure 1B), whereas a granular-type of EPS could be observed for biofilms grown at 1.5mM (Figure 1D) and 15mM CaCl_2 (Figure 1F). Moreover, the granular-type EPS material was found to be located between the cells within biofilms, and was found to be more abundant in biofilms grown at highest CaCl_2 concentrations.

Based on the CLSM data, the biofilm structural properties could be described in terms of biovolume, surface coverage, mean thickness, roughness as well as surface to volume ratio (Table 1), allowing a quantitative comparison of the biofilms grown at different CaCl_2 concentrations. Biofilm growth at 15mM were found to have a significantly higher biovolume compared to biofilms grown in the absence of CaCl_2 ($p=0.027$), with mean values of $8.1 \times 10^5 \mu\text{m}^3$ versus $5.0 \times 10^5 \mu\text{m}^3$. The presence of CaCl_2 led to increased surface coverage ($p=0.02$) compared to biofilms grown at 0mM CaCl_2 , regardless of the concentration CaCl_2 used during growth. No significant effect was observed by the presence or absence of CaCl_2 on biofilm thickness ($p=0.526$) and roughness ($p=0.087$) values. This could be explained by the presence of shear and capillary force conditions during growth at the air-liquid boundary which consistently levelled the biofilm thickness regardless of the presence of CaCl_2 . Although the opposite effect was described in *Pseudomonas aeruginosa* biofilms (Sarkisova et al. 2005), where the increasing CaCl_2 concentrations led to higher biofilm thickness; the model used for their biofilm growth involved growing biofilm submerged in medium with continual nutrient renewal in their system. While no differences in thickness was observed in this study, larger biomass sediments were observed at the bottom of tubes with increasing CaCl_2 concentrations used at the end of each experiment (results not shown), which could be attributed to sedimentation of detached cells.

The surface to volume ratio parameter, an indicator of biofilm porosity, showed a significant CaCl_2 effect ($p=0.03$), whereby increased porosity was observed in the presence of CaCl_2 . Interestingly, no significant structural differences between biofilms grown at 1.5mM or 15mM CaCl_2 were observed, as seen by their total biovolume ($p=0.2717$), surface coverage ($p=0.7726$), mean thickness ($p=0.5265$), biofilm roughness ($p=0.9724$), and surface to volume ratio ($p=0.8242$) parameters. This suggests that the even small amounts of additional CaCl_2 present during biofilm development are enough to influence the biofilm developmental outcome. Based on results presented in Figure 1 and Table 1, the most significant effect of CaCl_2 addition was an increased surface coverage on the substratum, most likely influenced by the induced or facilitated initial adhesion on the interface. Consequently, the high biovolume observed with increasing CaCl_2 concentration following two days growth can be explained by higher levels of surface coverage induced by the presence of CaCl_2 . Another aspect of the CLSM data relates to the surface to volume parameter, which is an indicator of biofilm porosity. The higher biofilm porosity in the presence of elevated CaCl_2 is an indicator of the presence of voids within biofilms, which can be a sign of larger amounts of EPS within the biofilms shown in Figure 1CDEF. The granular EPS form in biofilms could be attributed by the cross-linking properties of CaCl_2 , which led to the topographical differences compared to smoother like biofilms grown in the absence of CaCl_2 . Taking into account the qualitative and quantitative biofilm differences from the presence of CaCl_2 , it was crucial to determine the mechanical properties of these biofilms in order to further assess the significance of divalent ions such as calcium on biofilm properties.

Although primarily focused on biofilm structure, the metabolic changes that take place within *Pseudomonas fluorescens* biofilms grown at different calcium concentrations could also be of relevance, such as shifts in pH or the production of specific metabolites. The shift in pH observed over the course of planktonic growth could have been attributed to an alkaline lipase production typically associated by Pseudomonads (Makhzoum et al. 1993; Mckellar & Cholette

1984). Pseudomonads, isolated from soils or plant rhizosphere, are known to produce a variety of secondary metabolites that can directly positively or negatively impact the environment in which these organisms find themselves in. One earlier study in particular, Makhzoum, et al (1995) showed that *P. fluorescens* growth and extracellular lipase production were optimal in simple medium, usually composed of a nitrogen source (Makhzoum et al. 1995). This production of lipase was shown to parallel the growth of the organisms usually during the organism's log phase (Stead 1985). Interestingly Makhzoum et al (1995) demonstrated that the addition of calcium in the growth environment strongly stimulated the production of lipase production by 360% (Makhzoum et al. 1995). The significance of lipase production could therefore be of relevance in this study for potential beneficial biotechnological applications of engineered biofilms. However, the need to study environmental factors such as calcium concentration on microenvironments within biofilm matrix would be the first step in carrying out potential future applications. This should include but not be limited to the use of pH sensitive sensor dyes (e.g. Snarf) which would enable to localize and map pH microenvironments within the biofilm matrix.

3.2 Biofilm elastic properties

In this study, force-indentation (FI) curves were obtained for biofilms grown in the absence and presence of CaCl_2 . Representative FI curves are shown in Figure 2 up to a force set-point limit of 9-11 nN. From the results, smaller indentation depths were generally observed for biofilm samples grown in the absence of CaCl_2 ($0.51 \pm 0.14 \mu\text{m}$) compared to the larger noticeable indentation depths for biofilms samples grown with calcium (2.1 ± 0.45 and $2.37 \pm 0.5 \mu\text{m}$ for 1.5 mM and 15 mM CaCl_2 respectively). Considering biofilms grown without supplemental CaCl_2 , a much short initial non-linear indentation region may also be identified, despite being characterised as stiff due to a linear FI profile. Biofilm samples grown at two different CaCl_2 concentrations showed large nonlinear behaviour at a higher indentation depth which may indicate deformation of a softer surface layer. These explanations are in accordance

with microscopic observations of *P. fluorescens* cells and EPS sugar residues shown in Figure S11 in the supplementary document where only some parts of the biofilms grown without CaCl_2 were covered by EPS sugar residues, and the AFM tip was likely in contact with surface cells with a smaller amount of EPS. In contrast, a more homogenous and substantial EPS sugar residue layer covered the biofilm's surface when CaCl_2 was present.

As shown in Figure 2, different segments of the indentation curve showed a noticeable change prior to and after *transition points*, in which the initial nonlinear behaviour could be distinguished from secondary indentation behaviour. This may indicate the existence of different biofilm sub-layers and suggests that the biofilms are structurally and chemically stratified which has been previously described (Habimana et al. 2009; Stewart & Franklin 2008). It is noted that in this study, the initial nonlinear region was associated with the compressive surface deformation, ignoring the effect of initial surface forces as previously described (Volle et al. 2008a; Volle et al. 2008b)

In order to use a systematic method to justify the *transition point* and to distinguish between initial and secondary FI profiles, the point of intersection between the fitted Hertzian model and the experimental FI curve was used (see Figures S3a, S4a and S5a). Consequently, individual Hertz models were fitted into these selected segments, providing two distinctive values of elastic modulus (see Figures S3b & c, S4 b & c and S5 b & c). Elastic modulus values and corresponding indentation depths prior to *transition point* are given by $E1$ and $D1$, while the elastic modulus and indentation depth after *transition point* are denoted by $E2$ and $D2$ respectively. The force at the *transition point* was analysed for each experiment individually, determined as 1.64 ± 0.48 , 1.81 ± 0.37 and 1.88 ± 0.84 nN (Mean $\pm$ SD) for biofilm samples without calcium, and with 1.5 mM and 15 mM CaCl_2 respectively. It should be noted that the indentation depths were fitted within the 10% indentation validity range for the Hertz model based on the total thickness of the biofilm samples provided by CSLM (Table 1). Table 2 summarises $E1$ and $E2$ values with

their corresponding $D1$ and $D2$ values for all groups (mean data for each repeat is shown in supplementary Figure S6). In summary, an indentation hypothesis based on two decoupled biofilm surface layers has provided explanations for the behaviour shown in Figure 3

The larger value of $E1$ for the biofilms grown in the absence of CaCl_2 ($E1=2.13$ kPa), was probably due to the deformation of a thinner outer layer, which was significantly influenced by a stiffer secondary layer ($E2=10.1$ kPa). However, in the case of biofilms grown in the presence of CaCl_2 , the lower $E1$ value ($E1=0.25$ and 0.23 kPa for 1.5 and 15mM CaCl_2 respectively) was likely associated with a thicker soft outer layer, which may not be significantly influenced by a less stiff secondary layer ($E2 = 2.34$ and 1.24 kPa for 1.5 and 15mM CaCl_2 respectively).

As can be seen in Table 2, substantial variations in the elastic modulus for individual samples both within and between the samples. Because of the high variability of the measured data sets, a lognormal probability density function was used to describe the distribution of the data and estimate the most probable (MP) elastic modulus value for each group (cf Figure S7 in supplementary information section).

Overall, the higher elastic modulus observed for biofilm samples grown in the absence of CaCl_2 could partly be due to the higher number of (rigid) cells and comparatively lower amounts of softer EPS at the surface layers. This is in contrast to biofilms grown with added CaCl_2 where higher levels of EPS was present and were covered the surface homogeneously. As previously mentioned, these explanations have been supported by microscopic observations of the cells and EPS (see Figure S 11 in the supplementary document). An increase in the amount of EPS in the presence of elevated calcium concentrations was reported for *P. aeruginosa* biofilms (Sarkisova et al. 2005). Another study also suggested that the overproduction of EPS surface layers of a *mixed culture biofilm* could have been attributed to the adsorption of calcium ion at that

biofilm's boundary surface layer, where crosslinks and cells were loosely associated with one another (Ahimou et al. 2007).

Biofilms have been previously described as composite materials, consisting of solid biomass including bacterial cells and EPS as well as micro and macro scale pores (Laspidou & Aravas 2007). The approach taken in this present study validates the composite material concept through the use of a *composite elastic modulus*, in which both rigid elastic cells and softer EPS gel can contribute to a single elastic modulus value. The composite elastic modulus is usually described by the general rule of mixtures, expressed in equation 1 (Jones 1999) where E_m and E_d are the elastic modulus of a given matrix and the dispersed materials. In this study the EPS of the biofilm matrix was expressed as E_m , and the dispersed cells within the matrix as E_d . The volume fraction of both EPS and dispersed cells within the matrix were expressed as V_m and V_d respectively. The total volume of the material (i.e. biofilm mixture) is defined as the sum of the volumes of all individual phases. In one earlier study, the elastic modulus of alginate EPS extracted from an *Azotobacter vinelandii* biofilm which was measured at 2-4 kPa (Moresi et al. 2004), was found to be within the range reported for biopolymers (1-100 kPa) (Clark & Rossmurphy 1985). In this study, the elastic modulus of bacterial cells was found to be higher than that of biopolymeric substances, ranging between 180 to 6100 kPa based on the Hertz model based nanoindentation studies (Chen et al. 2012; Francius et al. 2008; Touhami et al. 2003). As shown in equation 1, the larger V_m with lower E_m could result in the lower elastic modulus of the biofilm as a whole (E).

Equation 1

$$E = E_m V_m + E_d V_d$$

Considering that AFM experiments are typically performed at the biofilm-liquid interface, the significance of biofilm porosity during nanoindentation cannot be neglected, especially when the biofilm at that region is typically 50% more porous than in the deeper layers of the biofilm (Zhang & Bishop 1994). It is generally accepted that higher porosity can lead to a reduction in material elasticity (Phani & Niyogi 1987). This observation can also be applied to biofilms, as described in earlier studies, in which increases in biofilm stiffness was observed as a direct consequence of blocking pores through compressive deformations (Casey 2007; Lapidou & Aravas 2007). In this study, biofilm porosity analysis based on cell biomass and EPS (Figure S 12 and S 13 in the supplementary document) showed no significant difference between the samples grown with and without calcium. Therefore, a reduction in the elastic modulus of biofilms may not necessarily be attributed to the porosity of the biofilm structure. This is also consistent with earlier studies showing a more porous *P. aeruginosa* biofilm structure at higher calcium concentrations (Sarkisova et al. 2005).

The mean elastic value of 32.96 ± 22.09 kPa (secondary layer) reported in this study for biofilms grown in the absence of calcium was found to be well within the range of 15-170 kPa, as previously reported for early stage *P. aeruginosa* biofilms (Lau et al. 2009). Moreover, the mean elastic modulus values of 3.57 ± 2.42 and 2.71 ± 1.33 kPa for biofilm grown under 1.5mM and 15mM CaCl_2 environments respectively was also found to be within the of range of 0.58-2.61 kPa, as reported for a more mature, EPS laden *P. aeruginosa* biofilms (Mosier et al. 2012).

3.3 Biofilm adhesive Properties

The adhesive properties of biofilm samples as measured using borosilicate spherical AFM probes was quantified from retraction curve data in terms of adhesive force (F_{adh}) in nanoNewton (nN) and adhesion energy (E_{adh}) in attoJoule (aJ). The adhesive energy, E_{adh} , is measured as the area under the retraction curve as a result of sample stretching from its original contact point line (Figure 4). This energy is related to the force of adhesion, F_{adh} , as shown in

equation 2, where h is the separation distance. In this study, adhesion energy was obtained using JPK IP data processing software. In Figure 4, the large hysteresis between approach and retraction curves is due to a significant viscoelastic behaviour observed for the EPS at the sample surface during the loading and unloading cycle.

$$E_{adh} = - \int_{h_1}^{h_2} F dh$$

Equation 2

Typical retraction curves were selected and presented in Figure 5 depicting the adhesiveness of biofilms grown in the presence or absence of calcium. The adhesive properties described by multiple adhesion events are likely to be associated with long-ranged polyprotein stretching and failure events, as previously observed for the surface EPS of *E. coli* biofilms (Tsoligkas et al. 2012). AFM studies performed on adhered single cells demonstrated a wave-like adhesion behaviour due to the stretching and unfolding of polymeric macromolecules found on the cell outer-membrane such as polysaccharides and proteins (Cross et al. 2007; Francius et al. 2008). The magnitude of the adhesive forces and area under the retraction curves was found to increase with increasing calcium concentration, which could be associated with larger amounts of EPS produced at the surface of biofilm samples. Again, this was confirmed by microscopic observations showing a substantial amount of EPS sugar residues covering the biofilm's surface in the presence of $CaCl_2$ (see Figure S11 in supplementary document), and similarly described by other researchers (Auerbach et al. 2000; Fang et al. 2000; Oh et al. 2007; Tsoligkas et al. 2012). As shown in Figure 5A, in the case of biofilm grown without calcium, a numbers of retraction curves showed a single adhesion event (green curve), probably due to the contact with the surface of the bacterial cells, also previously reported for early stage *E. coli* biofilms (Tsoligkas et al. 2012). In several other samples lower adhesion forces were observed, which can be explained as the presence of small residual EPS amounts attached to the AFM tip surface (red curve) following indentation procedures. As for the adhesion behaviour of biofilms grown in the

presence of calcium ions, pronounced elongated adhesion profiles were observed (Figure 5B), distinctly characterized by a significant number of adhesion events. The magnitude of these events also could also been attributed to a specific highly adhesive EPS type synthesized during biofilm development.

The average adhesive force and separation energy following contact with biofilm grown in the presence or absence of calcium ions are summarized in table 3. The average and standard deviation of adhesion data for each replicate as presented in Figure S9 and S10 (cf. Supplementary information section), indicated large variations due to the differences in the amount of EPS and heterogeneity of biopolymer molecules on the surface of biofilm samples, as previously observed on the bacterial cell surface (Camesano & Abu-Lail 2002). As reported in table 3, the most probable value of adhesion force and energy data were estimated by lognormal probability density analysis for each group, showing higher values with increasing calcium (see Figure S11). Recently, the higher adhesive force values was suggested to be associated with stronger polyprotein stretching at the surface EPS of *E. coli* biofilms (Tsoligkas et al. 2012). Furthermore, the increase in adhesion energy was shown to be related to the greater level of EPS attached to the AFM tip surface (Li & Logan 2004). The distribution data for biofilm samples grown in the absence of CaCl_2 showed that the majority of measured adhesive forces ranged from nearly zero to greater than 1 nN (see Figure S11a), which was found to fit the range reported for biopolymer molecules at the surface of the bacterial cells (Camesano & Abu-Lail 2002) and early stage *E. coli* (Tsoligkas et al. 2012) and *P. aeruginosa* biofilm surfaces (Lau et al. 2009). By comparing the elasticity (Figure S6a-c in supplementary) and adhesion data (Figure S9 and s10 in supplementary) for each repeat, higher adhesion rates corresponded to lower elastic modulus values, which were generally associated with softer and more sticky biofilm surface layers.

CONCLUSION

The aim of this study was to use AFM-based nanomechanical approaches to explain the effect of supplemental calcium in the form of CaCl_2 on the properties of *Pseudomonas fluorescens* biofilms. It was shown that the addition of CaCl_2 during biofilm growth significantly affected the structural and mechanical properties of the biofilms. From the measured AFM results combined with SEM/CLSM, it was concluded that the addition of CaCl_2 increased the amount of EPS. The AFM data showed reduced stiffness, higher viscous effect (larger hysteresis) as well as larger adhesive values at the surface of biofilm with increasing CaCl_2 concentration. These trends are consistent with the production of more EPS as the CaCl_2 concentration increased. A composite approach was proposed for the analysis of the AFM elasticity data. This study has shed light on the use of AFM-based indentation analysis of a biofilm structure which provides the basis for future studies which should address the cell-to-EPS ratios and a stratified biofilm layer approach.

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Table 1: Different parameters of biofilm formed by *Pseudomonas fluorescens* strain PCL 1701 in the absence and presence of 1.5mM or 15mM CaCl₂ as studied by Confocal Laser Scanning Microscopy and analysed with PHLIP and ImageJ. Values represent mean of 6 biofilm areas. Error bars depict standard error of the mean.

	Total biovolume [μm ³]	Substratum coverage [%]	Mean thickness [μm]	Biofilm roughness	Surface/volume ratio
0mM CaCl ₂	508587 ± 23265	29.3 ± 1.6	43.1 ± 4.0	0.49 ± 0.004	1.24 ± 0.16
1.5mM CaCl ₂	701374 ± 72703	45.7 ± 5.0	44.4 ± 5.2	0.45 ± 0.017	1.19 ± 0.17
15mM CaCl ₂	818390 ± 49900	47.8 ± 1.9	33.3 ± 1.5	0.46 ± 0.016	1.04 ± 0.31

Table 2 Mean and most probable elastic modulus (E) values for all biofilm groups as well as their indentatiuon depths (D)

Sample	<i>D1</i> (μm)	<i>E1</i> (kPa)		<i>D2</i> (μm)	<i>E2</i> (kPa)	
	Mean±SD	Mean±SD	MP	Mean±SD	Mean±SD	MP
0mM	0.27±0.1	6.23±3.24	2.13	0.25±0.087	32.96±22.09	10.1
1.5mM	1.21±0.03	0.38±0.21	0.25	0.9±0.29	3.57±2.42	2.34
15mM	1.27±0.33	0.39±0.24	0.23	1.1±0.31	2.71±1.33	1.24

* MP is the most probable value estiamted by probability density distiction analysis

Table 3 Mean and most probable adhesion data values for all biofilm groups

Sample	Adhesive force (nN)		Work of adhesion (aJ)	
	Mean±SD	MP	Mean±SD	MP
0mM	0.61±0.56	0.085	1106.31±1815.74	43.13
1.5mM	1.03±0.64	0.48	2678.80±1791.78	1198.53
15mM	2.06±1.03	1.38	5173.64±3442.80	2602.39

Figure 1: Representative *P. fluorescens* PCL1701 two-day old biofilms grown at different CaCl_2 concentrations. Side view 3D projections were acquired from CLSM image of following growth at 0mM (A) , 1.5mM (C) and 15mM (E) CaCl_2 . Thick white scale bar on projected images represent 200 μm . Corresponding SEM images depict *P. fluorescens* bioflms grown at 0mM (B), 1.5mM (D) and 15mM (F) CaCl_2 . Thin scale bar on SEM images represent 2 μm .

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620 **Figure 2** Elasticity profiles of biofilms in the form of representative measured force-indentation

621 data following growth at 0mM (blue), 1.5mM (red) and 15mM (green) CaCl₂,

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Figure 3 Schematics of proposed indentation behaviour for localised decoupled layers
deformation: biofilm without calcium at contact point (a) and at final indentation depth (b);
biofilm with added calcium at contact point (c) and at final indentation depth (d);

Figure 4: A typical FI curve measured for biofilms with CaCl_2 15 mM (b), showing approach (red), and retraction (blue) curves, as well as grey area of adhesion energy under the retraction curve

Figure 5 Multiple adhesion events are shown for several typical biofilm without calcium (a), and with CaCl_2 at the concentrations of 1.5 mM (b) and 15 mM (c)