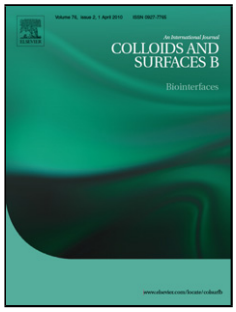

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The effects of extrinsic factors on the structural and mechanical properties of *Pseudomonas fluorescens* biofilms: a combined study of nutrient concentrations and shear conditions

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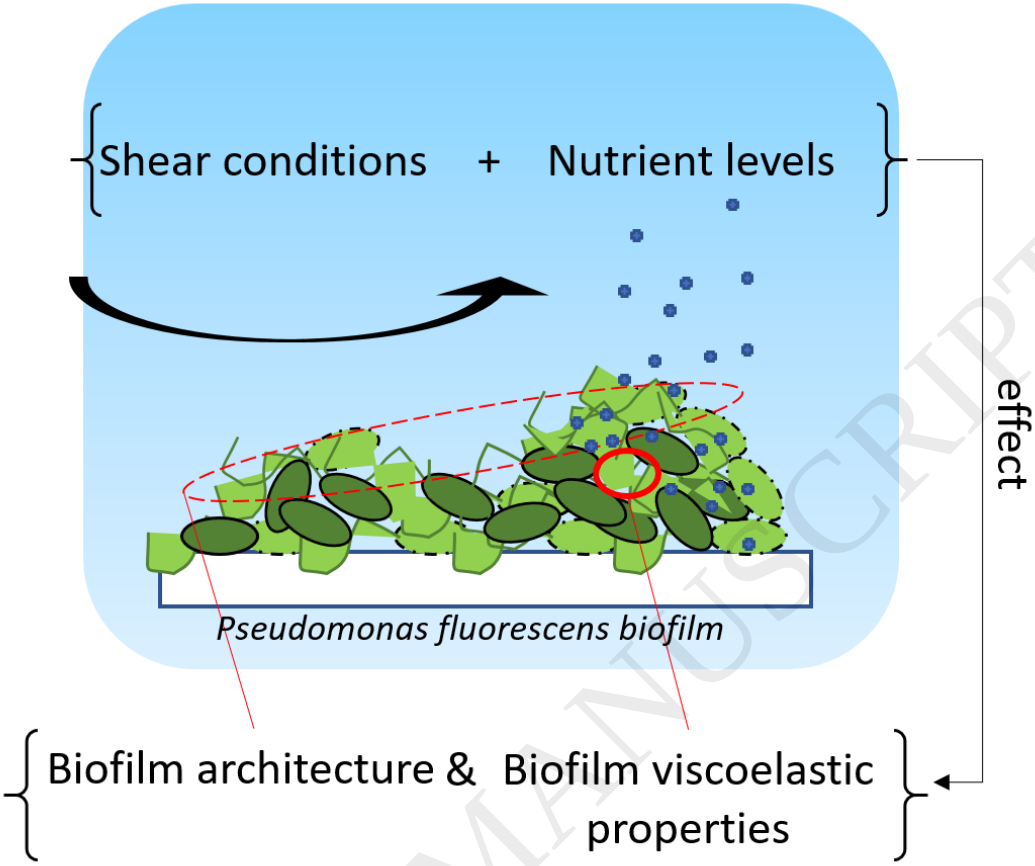

HIGHLIGHTS:

- Shear and nutrient levels influence biofilm architecture and viscoelastic features.
- High nutrient/low shear cultivation led to higher EPS levels
- Biofilms with higher EPS were softer as determined by AFM

ABSTRACT

The growth of biofilms on surfaces is a complicated process influenced by several environmental factors such as nutrient availability and fluid shear. In this study, combinations of growth conditions were selected for the study of *Pseudomonas fluorescens* biofilms including as cultivation time (24- or 48 hours), nutrient levels (1:1 or 1:10 King B medium), and shear conditions (75 RPM shaking, 0.4 mL min $^{-1}$ or 0.7 mL min $^{-1}$). The use of Confocal Laser Scanning Microscopy (CLSM) determined biofilm structure, while liquid-phase Atomic Force Microscopy (AFM) techniques resolved the mechanical properties of biofilms. Under semi-static conditions, high nutrient environments led to more abundant biofilms with three times higher EPS content compared to biofilms grown under low nutrient conditions. AFM results revealed that biofilms formed under these conditions were less stiff, as shown by their Young's modulus values of 2.35 ± 0.08 kPa, compared to 4.98 ± 0.02 kPa for that of biofilms formed under high nutrient conditions. Under dynamic conditions, however, biofilms exposed to low nutrient conditions and high shear rates led to more developed biofilms compared to other tested dynamic conditions. These biofilms were also found to be significantly more adhesive compared to their counterparts grown at higher nutrient conditions.

KEYWORDS: *Pseudomonas fluorescens*, biofilm, nutrient concentration, shear, Confocal laser scanning microscopy, Atomic force microscopy, biofilm viscoelastic properties.

1. INTRODUCTION

Biofilms are an aggregation of bacteria attached to a surface and embedded in a protective matrix. This protective matrix consists of layers of extracellular polymeric substances (EPS) surrounding the bacteria and comprises a variety of macromolecules, polysaccharides, proteins,

DNA, nucleic acids, enzymes, lipopolysaccharides and phospholipids among other substances [1]. The physical stability of this matrix is dependent on weak-physicochemical interactions. An increase in multi-valent ionic agents such as $CaCl_2$ or $AlCl_2$ may provide strong crosslinking replacing any hydrogen bonding within the EPS matrix, and can result in higher mechanical stability of the biofilm structure [2, 3]. The modulatory properties of supplemented $CaCl_2$ have also been shown to influence the structural and mechanical properties of *P. fluorescens* biofilms by lowering stiffness and increasing adhesiveness [4]. More recently, the effects of $CaCl_2$ on *P. fluorescens* biofilm mechanical properties were validated using a particle-tracking micro-rheology method [5]. The response to $CaCl_2$ may nonetheless result in different outcomes depending on the microbial species within the biofilm. For example, Flemming et al. [1] noted that *Pseudomonas aeruginosa*, grown in the presence of $CaCl_2$ produced a thick, compact and mechanically stable biofilm. These differences in biofilm properties were attributed to the interaction of Ca^{2+} ions between polyanionic alginate molecules. In a similar study involving *Pseudomonas aeruginosa* grown at an air-liquid interface, Abraham et al. demonstrated that the addition of either monovalent or divalent salts was sufficient to cause a distinct compact structural biofilm phenotype [6]. The presence of ionic agents is, therefore, known to influence biofilm structural and mechanical properties. However, other factors such as nutrient concentration and shear conditions may also be considered as extrinsic factors, hence requiring further investigation.

Such factors cannot be ignored, especially in many industrial sectors (i.e. food and water processing industries), known for providing ideal environments for the growth and proliferation of unwanted biofilms. Most notably, the adhesive nature of biofilms is responsible for the high operational costs associated with cleaning procedures, equipment damage or replacements, and process losses. Irrespective of where they are found, biofilm development

will depend on some extrinsic factors that may affect its growth, of these, nutrient availability and shear force.

The effects of nutrient concentration have been demonstrated to influence bacterial adhesion [7, 8]. Peyton et al. (1996) using *P. aeruginosa* showed that a higher substrate loading rate led to increased biofilm thickness, roughness and areal mass density [9]. In a separate study, Moreira et al. (2015) also demonstrated that biofilm characteristics were influenced by different surface properties, agitation and nutrient concentration [10].

Biofilms can form under a range of hydrodynamic conditions, and the fluid shear stress is known to influence biofilm thickness and structure [11, 12]. For example, under laminar flow, roughly circular micro-colonies were found to be separated by water channels, whereas in turbulent flow, filamentous streamers can form with ripple-like structures after continued growth [13]. In general, biofilms cultivated under turbulent flow conditions display stable and rigid structures, whereas laminar flow leads to thicker but less dense biofilms [12, 14]. Studies by Moreira et al. demonstrated that under high shear conditions, *E. coli* biofilms were still able to develop under low glucose concentrations as low as 0.25 gL^{-1} for 12 hours [15]. In a shear stress stimulation study, Horn et al. noted that biofilm detachment only occurred once a certain biofilm-thickness is reached [16]. Nevertheless, little is known of the changes in adhesive and elastic properties of the biofilms grown under shear stress under semi-static and dynamic conditions, thereby justifying the need for further quantification of the biofilm material properties under such conditions.

Nanoindentation, through Atomic Force Spectroscopy, has advanced into a technique capable of providing adhesive and cohesive forces of both single cells and biofilm aggregates [4, 17]. The Hertz model [18] has been successfully employed in nanoindentation experiments to estimate the elastic modulus of the surface indented [19, 20]. This well-established model provides an estimate of the elastic modulus from the area of non-adhesive contact of an

indentation curve. The analysis of the retraction section of indentation curves revealed the adhesive properties of the material. The adhesive property is an indicator of the level of EPS produced by the biofilm [21, 22]. As demonstrated in an earlier study using AFM, EPS levels could be quantified by comparing interaction forces between sulphate reducing bacteria and cantilever tips, by determining the differences in elastic forces [23]. While the study by Fang et al. assesses the EPS production in various areas of a single cell, the present study employed a previously described experimental approach used by Safari et al., in which biofilm EPS is quantified utilising a combination of Con A staining with advanced microscopy [4].

The objective of this study was to investigate the effects of nutrient concentration on the mechanical and structural formation of 24-hour grown *Pseudomonas fluorescens* biofilm under dynamic conditions. The adhesive and cohesive forces of the biofilm surface layer were measured using a colloidal probe for nanoindentation experiments in liquid. Additionally, the structural analysis was performed by confocal laser scanning microscopy (CLSM) with biofilm staining for the differentiation between bacterial cells and EPS biofilm fractions.

2. METHODS

2.1. Bacterial strains, cultural conditions and preparation

The mCherry expressing *Pseudomonas fluorescens* PCL 1701 [24] was selected for the biofilm adhesion assays. *P. fluorescens* was stored at -80 °C in King B [25] broth supplemented with 20 % glycerol. Cultures were obtained by selecting a single colony grown on King B agar (Sigma Aldrich, Ireland) at 28 °C and inoculating 100 mL King B broth supplemented at a final concentration of 10 µg mL^{-1} of gentamicin (Sigma Aldrich, Ireland). The inoculation medium was then incubated at 28 °C with shaking at 75 rpm for 16 hours until an optical density (OD) of 0.8-1.0 at a wavelength of 600 nm was obtained. Cultures were centrifuged at approximately

4000g (Eppendorf Centrifuge 5415C, Rotor F-45-18-11) for 10 min. Subsequently, the supernatant was discarded, and the bacterial pellet was re-suspended in sterile King B.

2.2. Biofilm growth with different nutrient concentrations

A semi-static biofilm was grown as described by Ashkan et al. [4]. To ensure sterility centrifuge tubes (Falcon, Fisher Scientific, Ireland) containing coverslips of Borosilicate Glass 22 mm × 22 mm (VWR, Ireland) were sealed with cotton wool and autoclaved. 3 mL of King B of at selected concentrations were subsequently inserted into the sterile centrifuge tubes. One tube contained 100 % King B (dilution factor of 1:1) while a second tube consists of 10 % King B and 90% Grade 1 pure water (dilution factor of 1:10), referred to as MilliQ water (Biopure 15 and Purelab flex 2, Veolia, Ireland). The 3 mL of the medium was supplemented with gentamicin (Sigma Aldrich, Ireland) at a final concentration of 10 µg mL^{-1}. Each tube was inoculated with a 5 µl volume of the re-suspended overnight culture. Centrifuge tubes were incubated over a period of 24 hours, with an orbital agitation of 75 rpm and temperature of 28 °C.

2.3. Dynamic Biofilm Growth

Flow cell systems allow for the direct measurement of biofilm using direct microscopic observation. The flow cells used were model BST 81 from Biosurface Technologies Corporation (Bozeman, MT, USA). This flow cell was used to examine the 48-hour growth of *P. fluorescens* biofilm on a coverslip using different nutrient concentrations. King B was prepared in a 20 L feed tank at two different dilution factors of 1:1 (high nutrient) and 1:10 (low nutrient). To ensure sterility, the flow cell system, with the exception the waste tank, was autoclaved. The flow cell system was placed in an oven at 28 °C and left for one hour to allow the feed tank (ThermoFisher, UK) temperature to achieve equilibrate. The pH was checked using a Mettler Toledo pH-meter (Mason Laboratories, Dublin) at both the three-way valve

and the waste tank using a 50 mL tube. The system was maintained at a pH of 7.4 until bacterial injection. Biofilm within the flow cell chamber was grown by injecting 5 mL *P. fluorescens* into the three-way valve (Cole-Parmer, IL, USA). The bacterial cells were then temporarily allowed to settle onto the coverslip for 1 hour under static conditions without flow. The flow of the liquid through the chamber was controlled by pumping media through the silicone tubing (VWR, Ireland) into the flow chamber. A continuous flow of media through the flow cell chamber was maintained by a Watson-Marlow 205S peristaltic pump (OH, USA). After 48 hours the King B media was replaced with a flow of PBS that was injected into the flow cell system using the 3-way valve for 15 minutes. The valves on both ends of the flow cell were closed, and the flow cell was disconnected from the system at the point where these valves had been closed. Two different flow rates were used, one at 0.4 mL min^{-1} and 0.7 mL min^{-1} corresponding to a Re_{dh} of 0.42 and 0.85 respectively. The flow cell was then analysed by confocal laser scanning microscopy using a custom-made holder.

2.4. Confocal Laser Scanning Microscopy and staining

Coverslips were removed from the centrifuge tubes and gently washed with a sterile 0.1 M NaCl solution. For bacterial and EPS staining Syto 9® (green nucleic acid stain: Molecular Probes) and Concanavalin A (Con A) staining protocol in conjunction with a fluorophore (Alexa Fluor 633) (Life Technologies™) was employed. Post rinsing the biofilms are stained with Syto 9® at a final concentration of 3.5 μg ml^{-1}. Stained biofilms were rinsed with a sterile 0.1M NaCl solution and subsequently stained with Con A-AlexaFluor633 at a final concentration of 200 μg ml^{-1}. Finally, the coverslip is rinsed preceding confocal microscopy.

The coverslips were placed in a phosphate buffered saline (PBS) solution (Sigma Aldrich, Ireland) enclosed by a Nunc Lab-Tek II Chamber Slide (VWR, Ireland). Confocal Laser Scanning Microscopy was performed using an Olympus FV1000 CLSM at the Live Cell

Imaging core technology facility platform, Conway Institute, UCD. Experiments were repeated to provide biofilms from 3 independent inocula for both growth conditions resulting in up to 3 different areas of 3 biofilms, these were repeated for both stained and unstained biofilms.

The two wavelengths were used for EPS and bacterial analysis Syto 9® and Con A-AlexaFluor633, excited at 488 nm and 633 nm respectively. 3D projections were collected at a z-step of 1 µm using an Olympus UPL SAPO 10× / 0.4 NA air objective. The biofilms structural quantification was performed using Image Structure Analyzer 2 [26, 27]. Quantification of coverage of EPS and bacteria for vertical distribution analysis was implemented using Image J from NIH (https://imagej.nih.gov/ij/).

2.5. Cantilever Preparation and Atomic Force Microscopy Observations

Atomic Force Microscopy (AFM) was performed on biofilm to obtain the indentation and retraction curves to determine the elastic and adhesive properties. These force measurements were performed using an Asylum Research MFP-3D AFM (California, US) and Nikon Ti/E fluorescence microscope (Nikon, Japan), which was placed on a vibration table and enclosed in an acoustic isolation chamber (TS-150, JRS Scientific Instruments, Switzerland).

Cantilevers used in the experiments were created using a micromanipulator DC-3K with a push button controller MS 314 (Märzhäuser Wetzlar GmbH & Co. KG, Germany). Small amounts of UV curable epoxy resin (TE Connectivity Chemicals, USA) were placed on an *NSC 12* E tip-less cantilever (*MicroMasch*, Lithuania). 10µm silica spheres MSS1-10 (Whitehouse Scientific, United Kingdom) were then attached to the epoxy on the surface of the cantilever using a separate pipette. The colloidal probe was subsequently cured in an oven at 100 °C for 1 hour. Usable probes were then imaged and calibrated using the thermal noise method [28] as 0.13 N m^{-1} at room temperature.

Force curves were performed on biofilm at the air-liquid interface. Biofilms were rinsed in a 0.1 M NaCl solution and placed in the AFM holder. Experiments were performed in duplicate for each biofilm condition, and biofilms remained in PBS solution during measurement. At least 100 force curves measurements were obtained for each biofilm at a scan rate of 0.5 μm^{-1} and force set point limit of 8-10 nN. After each force map, the cantilever was tested on the glass to ensure no biofilm reside had attached. If tip contamination had occurred the cantilever was rinsed with ethanol then MilliQ water and placed in a UV ozone cleaner (ProCleaner, Bioforce Nanosciences, Ames, IA) for 15 min.

Force Curves were analysed using the Hertz model fitting of Protein Folding and Nanoindentation Software (PUNIAS, http://punias.free.fr/) [29] with the Poisson ratio taken as a constant of 0.5.

2.6. Statistical Analysis

Data present are the mean ± standard error of the mean. Statistical analysis was performed by analysis of invariance (ANOVA) in Tukey's test for pairwise comparisons using MINITAB v15.1 (Minitab Inc., State College, PA) at a level of significance of 5 % ($p < 0.05$).

3. RESULTS AND DISCUSSION

3.1. Qualitative analysis of biofilm grown under semi-static and dynamic conditions

The influence of nutrient concentration levels and shear stress on the structure of *P. fluorescens* biofilms was investigated during 24- to 48-hour assays. Shear stress was introduced during both dynamic and semi-static biofilm assays, as shear is known to induce the erosion and sloughing of biofilms during their development [30]. Under semi-static growth conditions, biofilms were allowed to develop at the air-liquid interface areas. Shear was introduced in the

form of capillary forces as the tube reactor was gently shaken during the assay. Under dynamic conditions, using a flow cell, higher shear conditions could be obtained by adjusting flow rates conditions, to 0.4 mL min $^{-1}$ or 0.7 mL min $^{-1}$.

Three-dimensional reconstructions of *P. fluorescens* biofilms grown under semi-static conditions at high nutrient (1:1) and low nutrient (1/10 diluted King B) levels are presented in (Figure 1). Biofilms grown under high nutrient conditions (A) exhibited large heterogeneous biofilm clusters with EPS (in red) covering most of the bacterial cells (green). Conversely, biofilms grown under low nutrient conditions (B) were characterised as a homogenous monolayer of smaller cell clusters, mostly consisting of bacterial cells (green).

Biofilms grown under high nutrient environments were found to be comparable to those published previously under similar conditions [5]. Biofilms grown under lower nutrient condition (Figure 1a) displayed a noticeably reduced biomass bulk. Several studies suggest that nutrient limitation may influence the growth rate of the biofilm resulting in the reduction of biofilm [31, 32],

Under dynamic conditions (Figure 2), the level of biofilm formation was linked to the specific nutrient environments. High nutrient conditions (A-B) led to lower biofilm development, as characterized by their heterogeneously spread cell clusters. Under lower nutrient levels (C-D), biofilms grown at high flow rate led to fully developed homogenous flat biofilms (C), compared to those grown at lower shear conditions characterised by its heterogeneously spread cell clusters (D). The distribution profile of each biofilm was additionally examined to gain a better understanding of the bacterial spatial distribution within biofilms (Supplementary information, figure S1).

An incubation period of 48 hours for flow-cell biofilm growth was intended to allow the bacteria to establish themselves on the glass surface under shear stress. These were compared to 24-hour biofilms grown under semi-static conditions to assess growth pattern of

a 'mature' biofilm. From Figure 2, biofilm formation in low dynamic conditions produced a greater volume of biofilm with higher surface coverage, which also agrees with Dewanti et al. who studied the cell adhesion and biofilm formation of *E. coli* on stainless steel. The authors showed that under dynamic conditions, biofilms in low nutrient media grew faster [33]. A recently published article also supports this finding whereby, under certain conditions, (specifically phosphorous limitation), EPS production was enhanced [34]. Similarly, it was previously shown that the biofilm matrix may play a role in the sorption nutrients and minerals from surrounding aqueous environment [35]. Patterson et al. noted that the initial adhering bacteria play a vital role in the characteristics of the subsequent biofilm structure [36]. By producing a greater volume of EPS under low nutrient environments in early stage biofilms, there may be an increased biofilm development due to the enhanced sorption of nutrients.

3.2. Quantitative analysis of biofilms grown under semi-static conditions

Biofilms grown under semi-static conditions in either low or high nutrient environments were quantified in term of total biovolume (μm^3), substratum coverage (%), mean thickness (μm) and biofilm roughness derived from CLSM acquisition data (Table 1). The effects of nutrient environments on biofilm development were characterised by staining biofilms with Syto 9® nucleic acid total stain, while the effects on EPS production under tested nutrient growth conditions were quantified using lectin-based EPS stain Concanavalin A (conA), as presented in Table 1.

A two-fold difference in total cell biovolume was observed ($p = 0.004$) between biofilms grown under high nutrient and low nutrient conditions with values of 56988 ± 14379 μm^3 and 27593 ± 4714 μm^3 respectively. Growth under high nutrient conditions was also characterised by a three-fold increase in EPS levels compared to biofilms grown under low nutrient conditions ($p = 0.003$, as observed by their biovolume: 68453 ± 12278 μm^3 and 18463 ± 3129 μm^3 respectively. EPS production is known to assist in the growth and proliferation of embedded

cells within the biofilm [37, 38]. This threefold increase in EPS production may be largely attributed to higher nutrient availability. Comparison of biofilm surface coverage values and EPS levels at low nutrient conditions versus high nutrient conditions were found to be 1.6- and 2.2-fold higher respectively (p = 0.026 and p = 0.007). High nutrient conditions led to more structured biofilms as observed by higher biofilm roughness values for both total cells and EPS level, compared to biofilms grown under low nutrient conditions (p = 0.018 and p = 0.003 respectively). Mean biofilm thickness was not shown to be affected by nutrient growth conditions ($p > 0.05$) and this may be as a result of the imposed shear.

As shown in a study by Nguyen et al., bacteria develop an antibiotic tolerance when starved from nutrients. However, this results in the restriction of growth. For bacteria susceptible to gentamicin the reduced nutritional strain may result in a reduction of biofilm growth and proliferation instead opting for the production of EPS to protect and promote long-term biofilm survival [39].

3.3. Quantitative analysis of biofilms grown under dynamic conditions

Quantitative analyses of 48-hour grown *P. fluorescens* biofilms under dynamic conditions were also performed (Figure 3). Here, biofilms were grown under high and low nutrient conditions, and at different flow rates of 0.4 mL min^{-1} (low flow rate) and 0.7 mL min^{-1} (high flow rate).

P. fluorescens biofilm grown for 48 hours under high nutrient conditions at high and low flow rates show no significant difference in biovolume, substratum coverage, thickness or roughness ($p > 0.05$). For biofilms grown at low nutrient conditions at both low and high flow rates, there was no significant structural difference regarding biovolume, thickness and roughness ($p > 0.05$). A significant difference was however observed for substratum coverage (p = 0.04), which was found to cover a 60% larger area under the high flow rate compared to low flow rate condition. The lack of quantifiable differences in biofilm characteristics could have been

attributed to the selected flow rates (two-fold difference) conditions used in this study. Nevertheless, this result also aligns with conclusions from previously published research [36] where a four-fold difference in shear rate was used.

While no differences in structural biofilm parameters were observed based on flow rate conditions or shear stress, the level of nutrient growth was shown to have affected biofilm structure, irrespective of flow rate conditions. More specifically, low nutrient conditions led to biofilms with 1.5 times and 2.5 higher total biovolume compared to biofilms formed under higher nutrient environments at low (p = 0.008) and high (p = 0.005) flow rates respectively. The same observation also applies to surface coverage, in which nutrient level during growth rather than flow rate conditions led to generally thicker biofilms ($p < 0.05$). In contrast, the nutrient level was not shown to have significantly affected biofilm roughness characteristics (p = 0.238).

From these results, it can be determined that *P. fluorescens* biofilm growth was influenced by changes in nutrient availability, particularly at low flow rates concerning substratum coverage. This effect is prominent under low nutrient conditions whereby the biofilm seems to produce EPS, to protect and absorb nutrients from the depleted environment, thereby promoting biofilm survival.

3.4. Mechanical analysis of biofilms

The influence of nutrient concentration levels on the structure of 24-hour semi-static *P. fluorescens* biofilms was investigated using Atomic Force Microscopy. The assessment of biofilms grown under dynamic conditions was not conducted since the removal from flow cells would result in noticeable biofilm disruption. Nanoindentation acquisitions were conducted on biofilms that had developed at the air-liquid interface. All force curves were performed in PBS with a set-point limit of 9-12 nN. Biofilm samples grown under high-nutrient conditions

displayed a substantial indentation depth (0.20 ± 0.08 μm) when compared to biofilms under low nutrient environments (0.08 ± 0.007 μm). Moreover, the resulting indentation was less than 10% of the overall biofilm depth measured by CLSM which is within the valid range for the Hertz model. The differences in biofilm force-indentation curves indicate a stiffer biofilm sample surface with low nutrient availability.

The Young's modulus of 24-hour *P. fluorescens* semi-static biofilms, grown under low- and high-nutrient availability are presented in Figure 4. Biofilm development under low-nutrient environments displayed a higher elastic modulus of 4.98 ± 0.02 kPa compared to the lower elastic modulus of 2.35 ± 0.08 kPa under high-nutrient environments. Additionally, the complete overlap of approach and retraction curve during nanoindentation may not occur as the biofilm can display a limited degree of plastic deformation [40] which may result in higher elastic values. Nevertheless, the results show that under low nutrient growth conditions, the biofilms were twice as stiff as those grown under high nutrient conditions. The elastic modulus is higher than reported by Zeng et al. who conducted nanoindentation on *P. fluorescens* biofilm using a 59.2 μm colloid cantilever, which resulted in a Youngs modulus of 0.10 ± 0.01 kPa [40]. However, the biofilm cultivation conditions most likely result in the various between Youngs modulus values.

Greater EPS was produced in biofilms developing under high-nutrient environments, resulting in a significant elastic response, as defined by high biofilm viscosity. EPS production significantly altered the physical structure of the cell-substrate interface, resulting in a softer biofilm. In contrast, stiffer biofilm properties, as characterized by the higher elastic modulus, was observed for biofilms grown under low nutrient environments. The observed biofilm stiffness may be associated with lower levels of produced EPS, compounded by bacterial monolayers of single cells at the surface during nanoindentation. Safari et al. noted that *P. fluorescens* biofilm with the addition of calcium ions produced higher EPS sugar residues

following 48-hour biofilms growth under semi-static conditions. The observed differences in biofilm formation suggest specific bacterial response depending on nutrient availability and specific composition. Steinberger et al. observed *Pseudomonas aeruginosa* cells, grown on membranes for 16 hours in static conditions, elongated while a constant width was maintained under lower nutrient conditions. They suggested this elongation resulted in an improvement in the collection of nutrients from the feed source, without changes in the ratio of surface to volume [41]. In the present study, the low nutrient-induced elongation of bacteria may have led to a higher elastic modulus by directly indenting on bacterial cells rather than on an EPS layer covering the cells.

The average adhesive force and work of adhesion of 24-hour *P. fluorescens* biofilms grown under static conditions at low- and high-nutrient environments are shown in Table 2. Biofilms grown under low nutrient environments were shown to have a stickier surface with a 7-fold increase in the adhesive force ($p < 0.001$). Compared to high-nutrient environments, biofilms developing under low nutrient conditions seem to have produced a hard and sticky biofilm surface, as determined by its characteristic higher work of adhesion compared to biofilm grown under high nutrient conditions ($p < 0.001$) (Figure 4). In principle, an increase in adhesion energy is typically associated with greater attachment of the substrate to the cantilever tip and may indicate an increased volume of EPS [4, 42, 43]. This difference in adhesion forces is suggested to occur due to a stronger stretching of polyproteins [44]. However, the higher stiffness may be due to nanoindentation occurring on a thin layer of EPS covering the cells within the biofilm.

It has been shown the EPS of different microorganisms might vary in their mechanical properties such as stickiness and viscosity and that this EPS accumulation can result in a variation in the measurement of elasticity [45, 46]. Nutrients, however, may also play an essential role in the production of EPS during biofilm growth, consequently influencing the

biofilm's viscoelastic and adhesive character [47]. Francius et al. researched the EPS coverage of *Lactobacillus rhamnosus GG* cells. By comparing wild-type and mutant strains with limited EPS production, they determined that the cells were covered in a smooth, ridge lattice of globular proteins, the roughness of which was on the nanometer scales, whereas the polysaccharide producing cells were rougher [48]. As biofilms under low nutrient conditions produced lesser EPS than under high nutrient conditions, the cantilever may be directly interacting with cell wall globular proteins, thereby resulting in the observed higher adhesive forces.

Other properties to consider when discussing adhesive forces of the biofilm is the physicochemical and mechanical properties of the colloid cantilever used during acquisition. Surface roughness has been shown to influence the adhesion of bacteria to the surface [49-51]. Although it is assumed that the colloid cantilever is smooth, the presence of nanofeatures or surface heterogeneities on the colloid's surface may lead to further adhesion to the biofilm surface and cause a slightly increased adhesive response. The physiochemical properties of the colloid, while selected for being inert, may be modified during interaction such as the attachment of EPS to the cantilever surface [52]. Although protocols were in place to ensure the optimum method of measurement, EPS can attach to the cantilever surface and detach from the biofilm during retraction, further use of this cantilever results in measurements between the attached EPS and the biofilm causing a change in the force curve. While cleaning methods are utilised to reduce the possibility of this occurring, small quantities of EPS may attach to the cantilever during measurement.

4. CONCLUDING REMARKS

P. fluorescens biofilms resulted in higher biomass and surface coverage under semi-static, high nutrient conditions. Furthermore, significant EPS production was observed. Whereas under dynamic high shear conditions, low nutrient environments resulted in substantial biofilm development and EPS production were observed, suggesting the introduction of dynamic conditions produces a change in biofilm architecture. Further investigations into the mechanical properties using AFM revealed that higher elasticity and lower adhesive properties were characterised in biofilms grown under semi-static conditions and high nutrient environments. The level of EPS synthesized during biofilm development is the common denominator responsible for the observed biofilm phenotypes. While the analysis of mechanical properties of biofilms grown under dynamic conditions was possible, it was nevertheless technically challenging. Future endeavours will need to outweigh these technical aspects for characterising viscoelastic biofilm properties particularly in the study of the effect of shear stress. Moreover, a comprehensive understating of the relationships between the growth parameters and the biofilm structure/material properties will require quantification of the chemical composition of the EPS and it temporal and spatial variations.

COMPETING INTERESTS

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

A.A, O.H., E.C. made substantial contributions to the conception and design of the study. A.A. and O.H. contributed to the acquisition and interpretation of the data. All authors participated in drafting and revising the article for intellectual content.

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Figure Captions

Figure 1: Representative 3D reconstructed projections acquired from CLSM images of 24-hour grown P. fluorescens under high (A) and low (B) nutrient conditions. Before microscopy, biofilms were stained with total nucleic acid stain Syto 9® (green), and EPS stain ConA (red). Three-dimensional images were created with ImageJ's "3D viewer" plugin.

Figure 2: Representative 3D reconstructed projections acquired from CLSM images of 48-hour mCherry expressing P. fluorescens biofilms grown in flow cells under high (A, B) and low (C, D) nutrient condition, under low flow rates 0.4 mL min^{-1} (B, D) and high flow rates 0.7 mL min^{-1} (A, C). Three-dimensional images were created with ImageJ's "3D viewer" plugin.

Figure 3: The structural quantification of 48-hour mCherry-expressing P. fluorescens biofilms, as determined by biovolume (μm^3), substratum coverage (%), mean thickness (µm) and biofilm roughness, following development under different nutrient (low & high) and flow rate (0.4 & 0.7 mL min^{-1}) conditions. Error bars represent the standard error of the shown average mean for each sample set.

Figure 4: Histogram of the Youngs Modulus (kPa) distribution of 24-hour P. fluorescens biofilm grown under semi-static conditions at low and high nutrient environments. (A) is the Youngs Modulus of high (dark grey) and low nutrients (light grey), (B) is the breakdown of the Youngs Modulus at high nutrients between 0 and 1 kPa as highlighted in the red section of the graph (A).

Fig 1

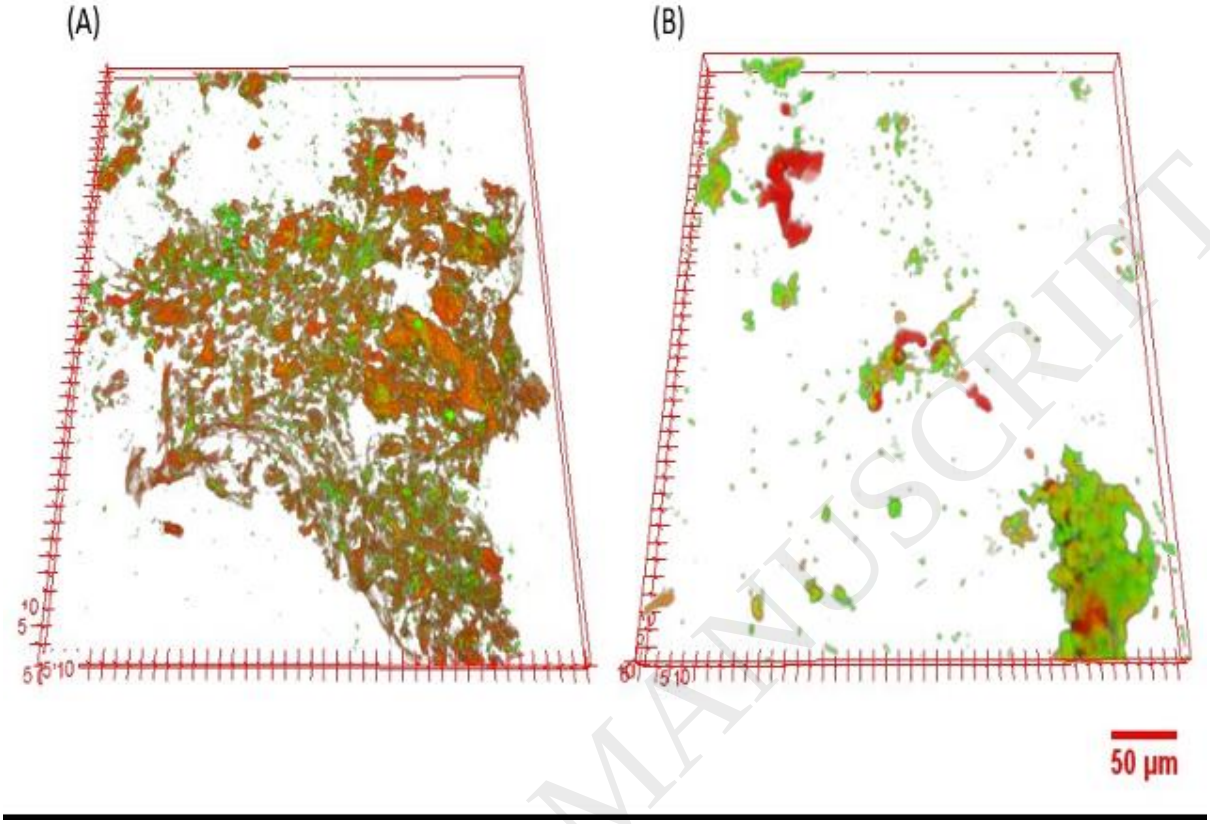

Fig 2

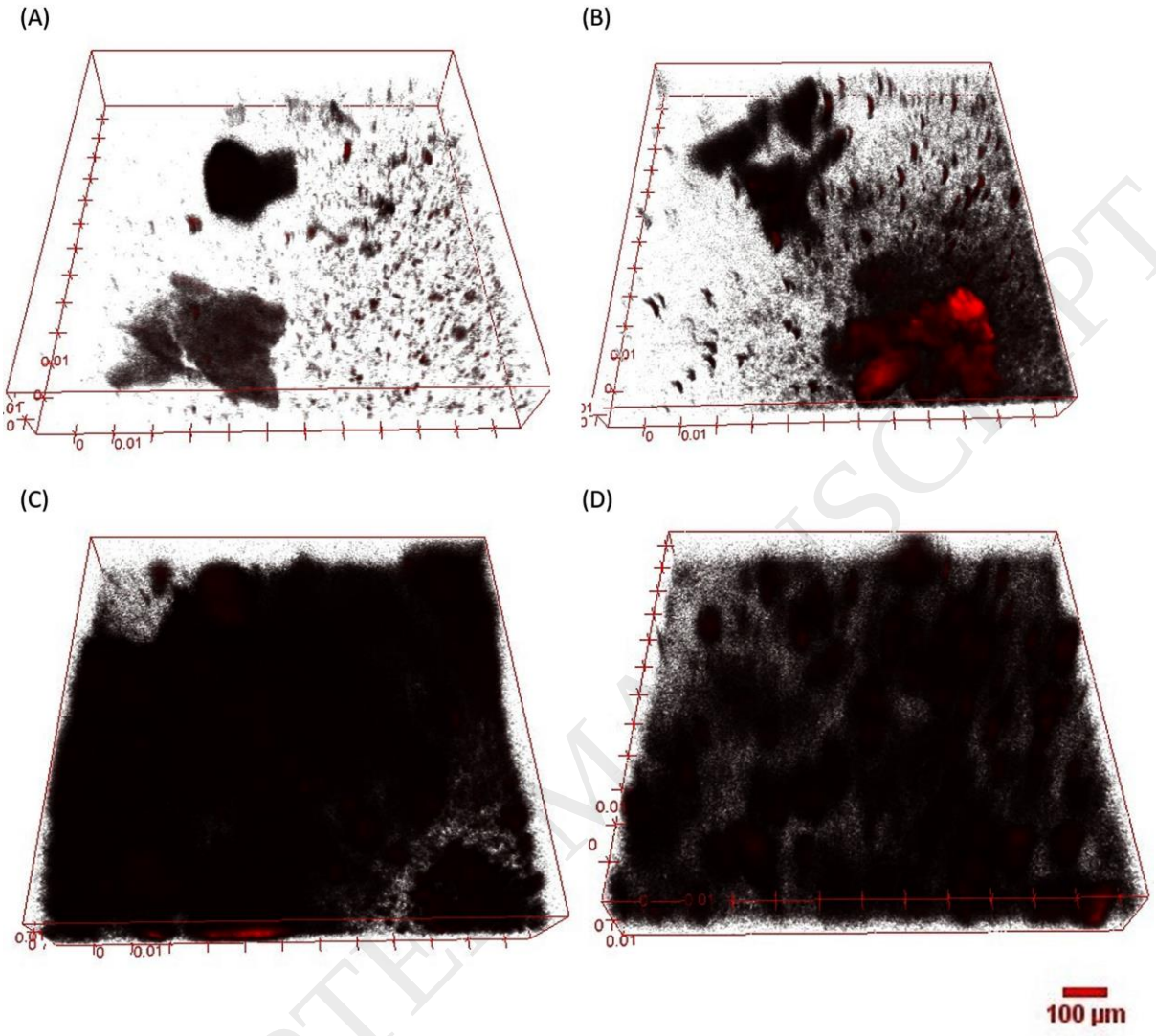

Fig 3

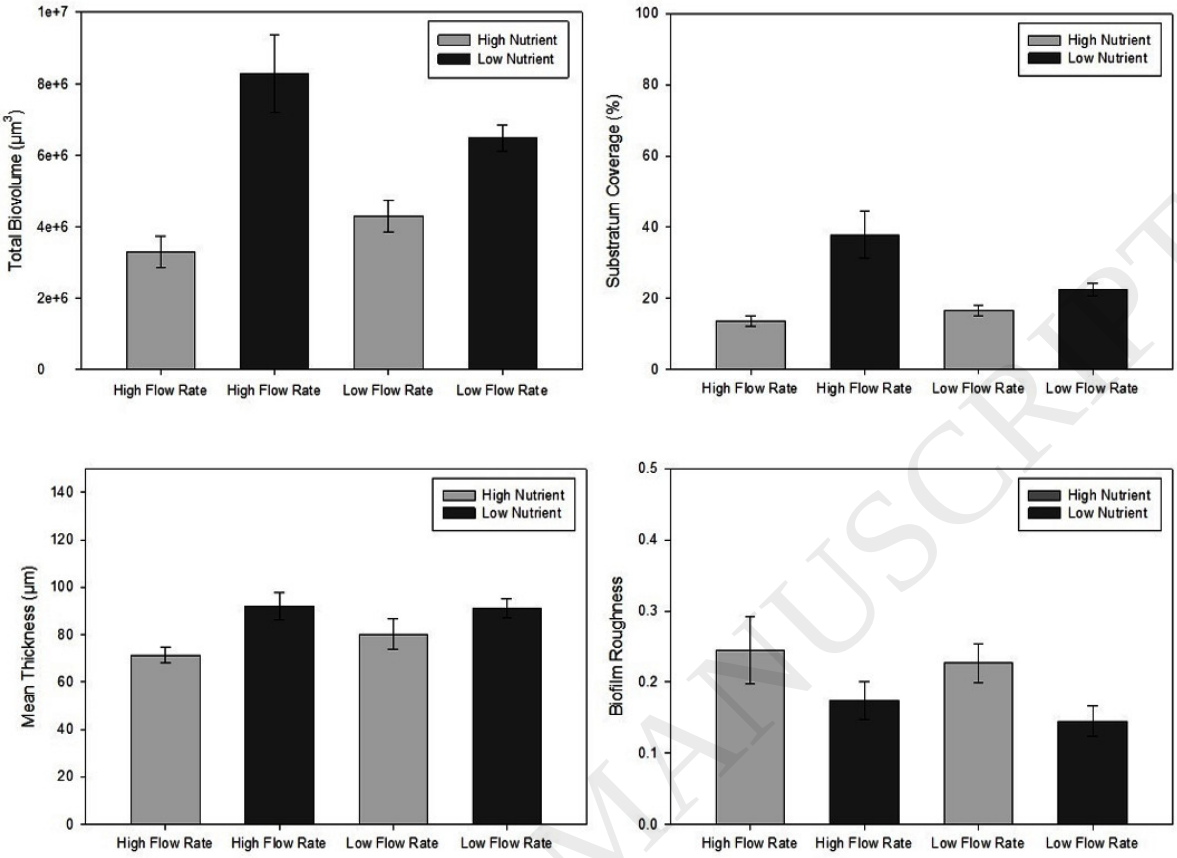

Fig 4

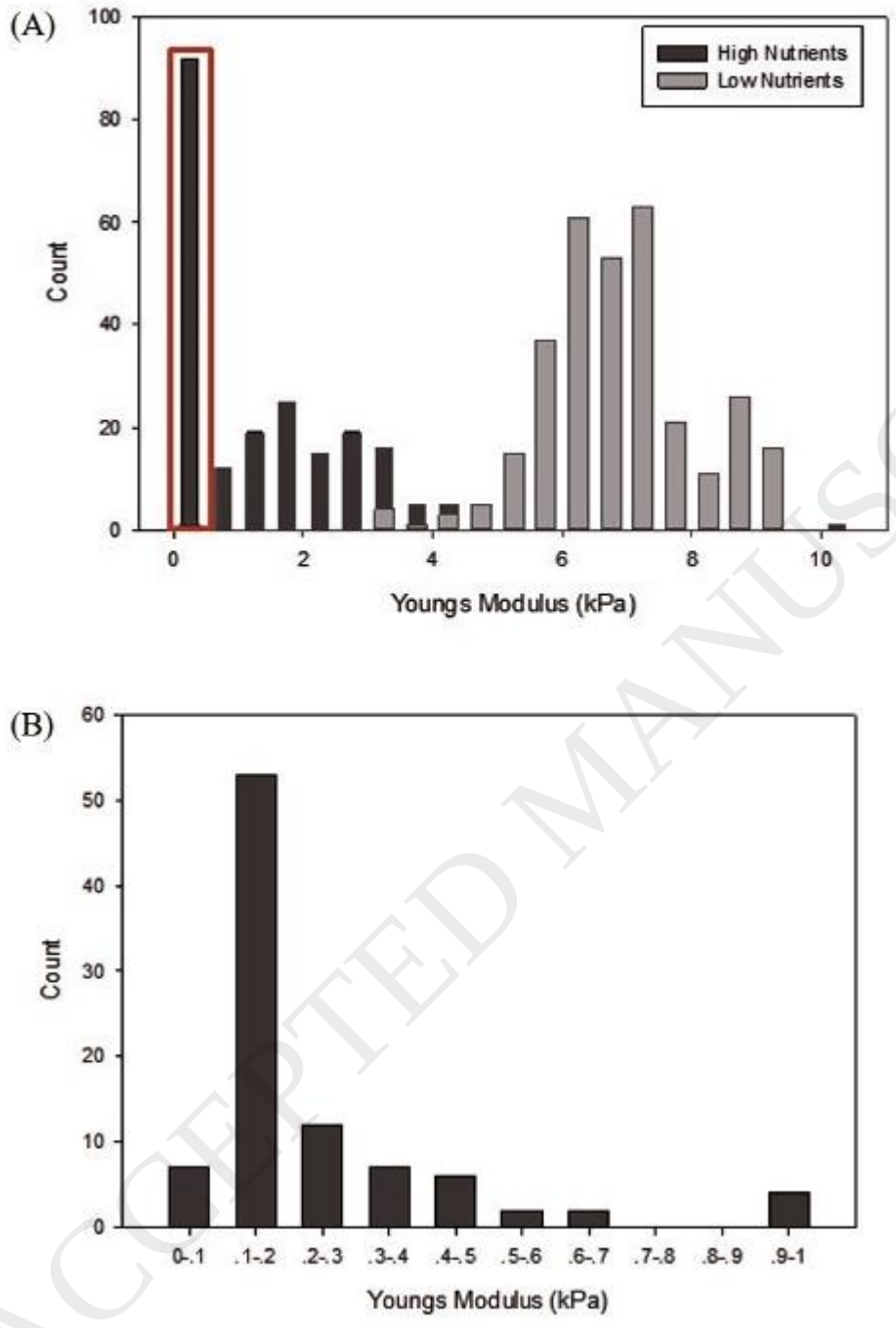

Table 1: Structural quantification of Syto 9® stained cells (total cells) and conA stained EPS fractions following 24- hours P. fluorescens biofilm growth under semi-static conditions and low- and high-nutrient environments. Mean values were obtained from a total of at least nine stacks from three independent experimental runs. Error represent SE of the mean.

		Total Biovolume [μm^3]	Substratum Coverage (%)	Mean Thickness (µm)	Biofilm Roughness
High-nutrient environment	Total cells	56988 ± 14379	16.2 ± 2.9	9.0 ± 0.8	0.45 ± 0.029
	EPS	68453 ± 12278	20.8 ± 3.5	10 ± 0.7	0.46 ± 0.053
Low-nutrient environment	Total cells	25793 ± 4714	10.1 ± 1.9	8.7 ± 0.8	0.34 ± 0.029
	EPS	18463 ± 3129	9.30 ± 1.9	9.10 ±1.2	0.35 ± 0.027

Table 2: *Adhesion Force and Work of Adhesion 24-hour P. fluorescens biofilms grown under semi-static conditions at low- and high-nutrient environments. Error represent SE of the mean.*

	Adhesion Force (nN)	Work of Adhesion (Aj)
High-nutrient environments	0.16 ± 0.01	5.21 ± 0.60
Low-nutrient environments	4.3 ± 0.16	185.48 ± 14.01