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Pseudomonas aeruginosa Interactions with Drinking Water Biofilm after an Acute Spike in Annular Bioreactors—Attachment, Persistence, Release, and Reattachment †

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Abstract: The potential of DWDS pipewall biofilms to shelter and propagate opportunistic pathogens is currently poorly understood. Here, we use an annular biofilm reactor approach to quantify the fate of the opportunistic pathogen *Pseudomonas aeruginosa* when introduced to a simulated DWDS environment. We found that *P. aeruginosa* was capable of swift attachment to surfaces and able to persist for up to 14 days under shear stress conditions. Further, we demonstrate that *P. aeruginosa* is capable of detachment/reattachment and mobilisation through the bulk water, potentially acting as a source of inoculum to drinking water.

Keywords: DWDS; biofilms; opportunistic pathogens; *Pseudomonas aeruginosa*

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1. Introduction

Mitigating the risk posed by opportunistic pathogens in drinking water is paramount for public health and wellbeing. Despite this, there exists a significant knowledge gap concerning the fate of opportunistic pathogens within drinking water distribution systems (DWDSs), specifically regarding their interactions with pipewall biofilms. Pipewall biofilms are ubiquitous in DWDS and offer protection from potentially hostile conditions such as high shear stresses and/or disinfectant residuals in bulk water [1]. Pipewall biofilm may also benefit opportunistic pathogens, sheltering them and enabling them to persist or even re-grow. Subsequent biofilm detachment and mobilisation could release them into bulk water, where they could reincorporate into biofilm downstream or reach the tap [2]. Understanding the fate of pathogens in DWDS is critical for risk mitigation and management. However, quantitative data on pathogens in DWDS is sorely lacking. The general inaccessibility of DWDS pipework means that the sampling of surface biofilms is incredibly difficult in situ. Furthermore, the high flow and shear stresses experienced through the DWDS network are challenging to replicate in the laboratory [3]. As such, the potential for pipewall biofilms to harbour pathogens and act as a source of inoculum to the bulk water remains poorly understood. To address this, we utilised a benchtop reactor approach to mimic conditions within DWDS to quantify the fate of the opportunistic pathogen, *Pseudomonas aeruginosa*, inoculated into bulk water. We ask if this opportunistic pathogen is capable of incorporation and persistence within pipewall biofilms followed by the subsequent release and mobilisation via the bulk water. Flow cytometric analysis combined with florescent microscopy was used to quantify and visualise these interactions between *P. aeruginosa* and pipewall material with and without drinking water biofilm.

2. Materials and Methods

Drinking water biofilm was grown on polycarbonate coupons in a Model 1420 Biofilm Annular Reactor (Biosurface Technologies, Bozeman, MT, USA) operated at 121 rpm, shear stresses 0.153 N/m^2, for 14 weeks, and reactor water was refreshed daily. At the end of this biofilm growth period, two coupons were removed from the reactor for T0 measurements and replaced with autoclave sterilised (no drinking water biofilm) coupons. An acute spike of ~6.7 × 10^7 *Pseudomonas aeruginosa* GFP (ATCC-10145GFP), grown in nutrient broth and resuspended in PBS, was added to the reactor bulk water. The attachment of these cells to the coupons was measured after 1, 3, 5, and 24 h by flow cytometry (FCM) by removing a coupon and aseptically scraping the biofilm from the surface into 30 mL PBS. GFP fluorescence was measured by FCM (BD Accuri C6). After 24 h, the growth/persistence phase was monitored by sampling coupons on days 2, 3, 8, and 9. For this, two coupons from opposite sides of the reactor were removed, and the reactor was drained and refilled with fresh tap water. Two sterile clean coupons replaced the removed coupons. For each removed coupon, biofilm was scraped and resuspended in 30 mL PBS from half of the coupon for FCM. The other half was analysed by florescence microscopy identifying GFP florescence. For the final stage of the experiment, the clean coupons which replaced the coupons taken during the previous phase were sampled in reverse order of replacement to ascertain release and reincorporation. These coupons were processed after being in the reactor for 4, 8, 12, and 14 days. Data processing and statistical analysis were conducted in R studio (V. 2023.09.1). The images generated from microscopy were analysed using the Regionprops function in MatLab (version R2022b) to identify and size the GFP florescent biofilm clumps.

3. Results

The attachment of *P. aeruginosa* to surfaces within the reactor happened quickly with 6.87 ± 0.005 log$_{(10)}$ cells/half coupon attached after one hour and 7.22 ± 0.001 log$_{(10)}$ cells/half coupon attached after three hours (Figure 1A).

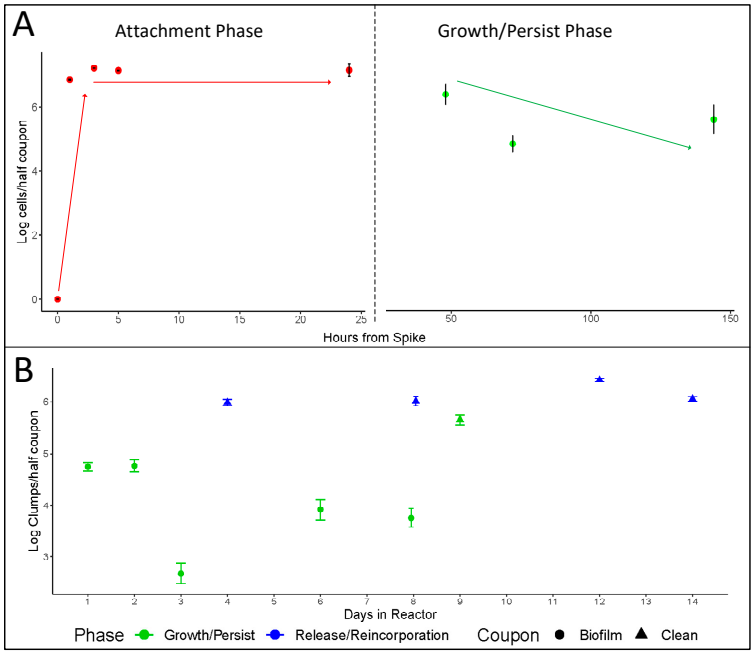

Figure 1. (**A**) Mean of logs$_{(10)}$ *P. aeruginosa* cells/half coupon as measured by flow cytometric analysis of GFP fluorescence. Error bars indicate two standard deviations. Red points were sampled during the attachment phase of the experiment (up to 24 h), green points were sampled during the growth/persistence phase (up to 6 days). (**B**) Mean of logs$_{(10)}$ *P. aeruginosa* clumps/half coupon as measured by flow cytometric analysis of GFP fluorescence. Error bars indicate standard error.

No significant increase was observed between 3 and 24 h (one-way ANOVA, p-value = 0.626). After 24 h, $1.5 \times 10^7 \pm 3.2 \times 10^6$ cells were found to be attached per half coupon. While attachment appeared to happen quickly, only 6% of the *P. aeruginosa* cells introduced to the reactor appeared to attach to surfaces. Around 62% of the cells remained in the bulk water after 24 h and a cell loss of around 31.16% was observed. Following the attachment, *P. aeruginosa* showed an initial decline in cell numbers to their lowest on day 3 (72 h) at $7.2 \times 10^4 \pm 2.2 \times 10^5$ cells per half coupon. However, by day 6 (144 h), the *P. aeruginosa* cell numbers had again increased to $4.6 \times 10^5 \pm 2.2 \times 10^5$. This initial decrease and then recovery mirrored the microscopy data (Figure 1B).

The number of *P. aeruginosa* biofilm clumps again was lowest on day three at 2.68 ± 0.19 $\log_{(10)}$ cells/half coupon declining from 4.74 ± 0.08 log(10) cells/half coupon. However, an increase was again observed between days three and six to 3.92 ± 0.19 $\log_{(10)}$) cells/half coupon. This increase appears to have been maintained between days six and eight ending the growth/persistence phase of the experiment at 3.76 ± 0.17 $\log_{(10)}$ cells/half coupon. The *P. aeruginosa* clumps were also in high number on the replacement coupons added to the reactor after the initial spike and reactor drain. Interestingly, the number of biofilm clumps on clean coupons was significantly higher (Kruskal-Wallis p-value < 0.005) than those on the drinking water biofilm coupons, ranging from 5.65 ± 0.1 to 6.42 ± 0.03 $\log_{(10)}$ cells/half coupon. While the number of clumps was higher on the clean coupons, there was no significant difference between the size of the clumps (Kruskal–Wallis p-value = 0.33) after an initial reduction from 10.5 ± 1.2 μm^2 on day one to 5.95 ± 0.5 μm^2. Similarly, there was no significant change in the size of the clumps over time (Kruskal-Wallis p-value = 0.34).

4. Discussion

This study quantified *P. aeruginosa* initial attachment to surfaces from bulk water under shear stress conditions and demonstrated its persistence, detachment from the biofilm, and mobilisation through the bulk water before reincorporation elsewhere.

Attachment occurred quickly (between 1 and 3 h); indeed, *P. aeruginosa* can synthesise proteins associated with surface attachment in as little as 20 min from initial contact [4]. Therefore, an acute *P. aeruginosa* contamination of DWDS could quickly lead to the incorporation of the opportunistic pathogen into biofilms. Moreover, we have shown that these cells can persist and be released back to the bulk water for subsequent reattachment. Therefore, the impact of an initial contamination event could persist long after the initial exposure.

The initial attachment was followed by a decline in biofilm *P. aeruginosa* cells (up to day 3), after which cell numbers recovered increasing from 4.84 ± 0.1 to 5.61 ± 0.2 $\log_{(10)}$ cells/half coupon, possibly indicating the adaptation of *P. aeruginosa* to biofilm conditions. Over time, biofilm adaptive mutations may be selected for encouraging microcolony growth [5,6]. The increase in cell numbers between days three and six may be indicative of biofilm-adapted *P. aeruginosa* growth [7]. Further research is required to confirm this as it is difficult to decouple growth from dispersal and reattachment. In any case, the persistence of *P. aeruginosa* was evident for up to 14 days following initial surface attachment. This highlights the challenges facing DWDS management, demonstrating that opportunistic pathogens continue to persist within surface biofilms for a minimum of 14 days following contamination. It is also unclear if this persistence would have continued indefinitely as the number of coupons, and thus available data points, were limited.

The final phase of this study found that persisting *P. aeruginosa* is capable of detachment, mobilisation, and reattachment to surfaces under shear stress conditions. Interestingly, significantly (Kruskal-Wallis p-value < 0.005) more biofilm clumps were found on the replaced clean coupons rather than on biofilm-covered coupons (Figure 1B). While the exact mechanism is unclear, this appears to suggest that attachment is more likely on clean surfaces than those colonised with biofilm under shear stress conditions. This may have

implications for DWDS management as cleaning and removing biofilm from pipework may inadvertently encourage the attachment of opportunistic pathogens.

Overall, we have shown the potential for DWDS pipewall biofilms to act as a reservoir and source of inoculum for opportunistic pathogens. Quick attachment and long persistence of *P. aeruginosa* within surface biofilm demonstrate that opportunistic pathogens may colonise pipewall biofilms long after the initial contamination event. Further, persisting pathogens are capable of detachment and mobilisation, potentially contaminating further downstream pipework or reaching the consumer, causing a risk to public health.

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