A novel flow-system to establish experimental biofilms for modelling chronic wound infection and testing the efficacy of wound dressings.

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Abstract

Several models exist for the study of chronic wound infection, but few combine all of the necessary elements to allow high throughput, reproducible biofilm culture with the possibility of applying topical antimicrobial treatments. Furthermore, few take into account the appropriate means of providing nutrients combined with biofilm growth at the air-liquid interface. In this manuscript, a new biofilm flow device for study of wound biofilms is reported. The device is 3D printed, straightforward to operate, and can be used to investigate single and mixed species biofilms, as well as the efficacy of antimicrobial dressings. Single species biofilms of *Staphylococcus aureus* or *Pseudomonas aeruginosa* were reproducibly cultured over 72 h giving consistent log counts of 8-10 colony forming units (CFU). There was a 3-4 log reduction in recoverable bacteria when antimicrobial dressings were applied to biofilms cultured for 48 h, and left *in situ* for a further 24h. Two-species biofilms of *S. aureus* and *P. aeruginosa* inoculated at a 1:1 ratio, were also reproducibly cultured at both 20^{o}C and 37^{o}C; of particular note was a definitive Gram-negative shift within the population that occurred only at 37^{o}C.

Introduction

Chronic wounds exhibit a perpetual state of non-healing with inevitable recalcitrant infection. Biopsies of a variety of wounds have found that over 78% of chronic wounds contain biofilm, which is associated with unsuccessful anti-infective treatment (James *et al.,* 2008; Kirker and James, 2017, Malone *et al.*, 2017). Consequently, persons with chronic infected wounds are often afflicted for many months or years, with the most severe cases necessitating physical debridement of tissues and eventual amputation. Numerous antimicrobial wound dressings are commercially available and form a part of chronic wound management strategies. To date there are no universally accepted, robust means of testing new antimicrobial dressings for their efficacy, particularly against biofilms.

A number of *in vitro* biofilm models are available and utilised with varying success to study wound biofilms. These include the Lubbock system (Sun *et al*., 2008), the Modified Robbin's Device (Kharazmi *et al.*, 1999; Miller *et al*., 2001), the Calgary Device (Ceri *et al*., 1999; Harrison *et al*., 2006), Constant Depth Film Fermenters (CDFF) (Hill *et al*., 2010), drip-flow reactors (Goeres *et al*., 2009), flow chamber and bubble traps (Tolker-Nielsen and Sternberg, 2014), and more recently, microfluidic systems (Wright *et al*., 2015). The Lubbock system and Calgary Device are static biofilm models; the former is most representative of the wound environment as biofilms are grown on filters on top of plugs of agar that are placed onto an agar-filled Petri dish which allows for the application of wound dressings. The Calgary Device allows for the culture of up to 96 biofilms in a static system, with the biofilm submerged in media, which is not truly representative of the wound environment.

Chronic infected wounds commonly produce exudate, which further complicates accurate modelling of wound infection *in vitro* (Junka *et al*., 2017). The Modified Robbin's Device, CDFF, drip flow reactors, flow chamber/bubble-trap systems and microfluidic devices have tried to address the requirement for flow within biofilm models and are sufficiently versatile to allow for the modelling of diverse biofilms including oral, wound, genitourinary tract and respiratory tract biofilm (Pratten,

2007; Hope *et al*., 2012; Diez-Aguila *et* al., 2017; Melvin *et al*., 2017). The Modified Robbins Device, CDFF and microfluidic systems are available commercially but the initial cost of purchasing these devices and/or equipment can be prohibitive. Detailed descriptions for in-house construction of flow chamber/bubble-trap biofilm models and drip-flow reactors are available; this makes them cheaper options but requires a degree of technical expertise. Furthermore, the "home-made" nature of such devices can affect reproducibility.

The design of several of the biofilm models, described above, are such that cultured biofilms remain submerged in media throughout experiments. This is a disadvantage for the study of wound biofilms, which are typically not submerged but grow at the air-liquid interface of the wound bed, being "fed" from beneath by wound exudate. CDFFs and drip flow reactors allow for the growth of a biofilm that is more representative of a wound and it is possible to apply wound dressings to the former. CDFFs also allow for high-throughput, reproducible biofilm growth. However, with the CDFF, all cultured biofilms are duplicates and fed through one inlet, meaning that it is only possible to study biofilms comprised of the same microorganism(s), simultaneously. Drip-flow reactors have tried to address the problem: several biofilms are cultured concurrently, but fed independently; however, cross-contamination is common (Azeredo *et al*., 2016).

A new biofilm flow system is presented here (Duckworth Biofilm Device; DBD), that has a series of "wells" for the growth of 12 biofilms across four separate channels. This allows triplicate biofilms to be cultured so as to prevent cross contamination between individual channels. Furthermore, the device allows ease of sampling during experiments without disrupting continuing biofilm growth. Biofilms are cultured on a semi-permeable substratum that is fed with media from beneath. Biofilms can be cultured on cellulose (MF-Millipore; cellulose acetate/cellulose nitrate) disks for recovery and enumeration, or on glass coverslips for microscopic analysis; this approach also allows for the application of wound dressings. The DBD can be produced by additive layer manufacturing and is re-usable (sterilisable by autoclave or disinfection, depending on the material;

see methods). It is a single part instrument with a lid and does not require technical expertise to utilise i.e. does not need to be constructed by the user.

Herein we describe the design and preliminary testing of the DBD, which is proposed as a new biofilm flow system for the study of wound biofilms and for the testing of antimicrobial dressings.

Materials and Methods

Device design and manufacture

Computer aided design (CAD) was undertaken using Autodesk Inventor (Autodesk Inc., California, USA). Electronic CAD files are available as both .ipt (openable using CAD software) and .stl (openable by 3D printing software). To request a copy please contact the corresponding author. Manufacture of the flow cell used in these experiments used a Renishaw RenAM 500M (Renishaw, Wotton-under-Edge, UK) and was in aluminium alloy ($AlSi_{10}Mg$). This device was sterilisable by autoclave. Some surface tarnishing was visible following repeated sterilisation; however, there was no apparent functional loss over 50 sterilisation cycles.

The DBD has since been printed using Accura ClearVue Resin at 0.1 layers (PDR, Cardiff Metropolitan University; http://pdronline.co.uk/). This can be sterilised without affecting the dimensional accuracy of the device by formaldehyde at 80^{o}C, low temperature steam at 75^{o}C, or gamma irradiation. Decontamination of Accura ClearVue Resin devices in this study used Gerrard Ampholytic Surface Active Biocide (GASAB) disinfectant, prepared at a 1:100 concentration, as per the manufacturer's instructions (Fisher Scientific, UK). GASAB was flowed through the device at a rate of 5 mL min^{-1} for 30 min, followed by submersion in GASAB for 16 h. Following disinfection, the device was washed with sterile distilled water, at a flow rate of 5 mL min^{-1} for 30 min.

Setting up and running the Duckworth Biofilm Device

The DBD has one input portal, connected to a flask of fresh media; from the entry reservoirs, the flow splits into four separate channels (Figure 1A and 1B). Spent media exits via a single portal, by peristaltic pump (MasterFlex L/S Digital Pump System with EASY-LOAD II Pump Head, Cole-Palmer) (Figure 1C). Silicone tubing was from Cole-Palmer (13 mm, MasterFlex; London, UK) and held into the device using sterile plastic 1 mL pipette tips (Figure 1C). Each of the four channels of the device

128 have three biofilm support wells (Figure 2A); these are comprised of a 1 mm “ledge” that is open to
129 the media flowing beneath. It is necessary to fill the device with media by either pipetting into each
130 well or by flowing the media through at a rate of 1 mL min^{-1}.

131 A disk of noble agar measuring 10 mm in diameter (cut from a 15 mL agar plate in a standard
132 sized Petri dish using a sterilised steel, leather press punch) inserted into the well, rests on the
133 support ledge, and acts as a porous matrix support for biofilm growth (Figure 1A and 2A). Critically,
134 the dimensions of each well constrain the size of the agar disk meaning that the spatial position of
135 each biofilm relative to the nutrient flow is identical. A cellulose membrane (diameter = 13 mm, pore
136 size = 0.22 μm) on top of the disk of noble agar provides a surface for biofilm growth (Millipore, UK)
137 (Figure 2A). Bacterial suspension (20 μL) equilibrated to an appropriate optical density was used to
138 inoculate the surface of the cellulose membrane. The device ran at a flow rate of 0.322 mL min^{-1}
139 (equivalent to 0.083 mL min^{-1} per channel).

140 Under these conditions 500 mL of media is sufficient to complete one 24 h run. The device
141 has a lid, which was kept in place whilst the flow cell was running. A 0.22 μm syringe filter was
142 inserted into the aperture at the centre of the lid (Figure 2B). Setting up and running the device as
143 described above (Figure 2C) allowed for the culture of 12 biofilms simultaneously without
144 contamination of the nutrient flow. The design of the device enabled the removal and recovery of
145 bacteria from biofilms, either simultaneously or at specific time points, without disturbing the
146 continuing experiment.

147 *Optimising biofilm growth*

148 Preparation of the DBD took place in a class 2 laminar flow cabinet. Twelve agar disks were cut from
149 a Petri dish filled with 15 mL noble agar at a concentration of 1.5% (w/v), using a 10 mm leather
150 press punch, sterilised prior to use, by autoclave, and transferred to the device using a sterile
151 scalpel. One cellulose disk was placed on top of the agar disks using sterile forceps; each disk was

inoculated with 20 µL bacterial suspension (either *Pseudomonas aeruginosa* or *Staphylococcus aureus* individually, or a 1:1 ratio of both bacteria) equilibrated to $1x10^5$ CFU. Once the lid was in place, a sterile 0.22 µm syringe filter was inserted into the aperture. The device was re-located to the bench top (20°C) or incubator (37°C) where the peristaltic flow rate was set to 0.332 mL min^{-1} (equivalent of 0.083 mL min^{-1} per channel). At appropriate times, the cellulose disks were removed from the top of the agar disks, using sterile forceps, and transferred into 10 mL sterile PBS. These were vortexed (2200 rpm, 20 s) to dislodge and homogenise the biofilm. Serial dilutions (10^{-1} to 10^{-12}) were prepared using PBS, and were enumerated using the total viable count method of Miles and Misra (Miles *et al*., 1938). At the end of each experiment, the $AlSi_{10}Mg$ device underwent decontamination by autoclaving (135°C, 1 atm, 5 min), it was subsequently washed with GASAB and sterilised for use by autoclaving (121°C, 1 atm, 20 min). The Accura ClearVue Resin device was decontaminated using GASAB as previously described.

Manufacture of alginate film dressings containing chlorhexidine hexametaphophate

Alginate (PROTANAL LF10/60FT (FMC Health and Nutrition, Philadelphia, USA)) (2 wt% aq.) was prepared containing chlorhexidine hexametaphosphate nanoparticles (CHX-HMP) (manufactured as previously described (Barbour *et al*., 2013)) equivalent to 0, 3 or 6 wt% cf. alginate. These were poured (17.5 g) into standard size Petri dishes and the water evaporated at r.t. over 3 days. These were crosslinked with the addition of $CaCl_2$ (30 mL, 0.18 M, 2 wt% aq., 25 min). The crosslinked alginate films were removed, washed with deionised water and disks (diameter = 13 mm) cut for immediate use in this work. These dressings are denoted as "wt% CHX-HMP in alginate films'-CHX-HMP" e.g. 6-CHX-HMP dressings contained 6wt% CHX-HMP.

Testing of antimicrobial dressings

Single-species biofilms were prepared as described above. After 48 h growth, the flow to the device was stopped, and working in close proximity to a Bunsen flame, the lid removed and disks of

dressings (commercially available alginate dressings containing antimicrobial silver (Tegaderm™, 3M, Minnesota, USA) (denoted 'Ag-Alg') or alginate films containing CHX-HMP at 0, 3 and 6% w/v) (cut to 13 mm diameter) were applied to the biofilm. With the lid in place, the device was run for a further 24 h. Following completion of the run and the removal and disposal of dressings, enumeration of biofilms occurred as described above.

Statistical analysis

Statistical analysis was performed in GraphPad Prism 7 (GraphPad Software Inc., California, USA) using one-way analysis of variance (ANOVA) to test for significance.

Results

Culturing single- and two- species biofilms

Two-species biofilms were achieved with *S. aureus* and *P. aeruginosa* as representative wound pathogens. Experiments conducted at 20°C and 37°C over 72 h indicated uniform growth and recovery of each microorganism from each well and/or channel of the device (Figure 3A). Comparably reproducible results were also observed for the Accura ClearVue Resin device (Table S1). Throughout the experiments, it was observed to be important to keep the lid in place to avoid contamination.

Preliminary experiments using two-species biofilms enabled investigation of consistent population changes within the two-species biofilm over time, from which relative competitive indices for *S. aureus* and *P. aeruginosa* were determined (Figures 3B and 3C; Table 1). Biofilms cultured for less than 10 h at 37°C showed a predominance of *S. aureus,* with *P. aeruginosa* becoming the most numerous after 10 h and remaining so for the duration of the experiment (Figures 3B, 3C and 3D). This aligns with the Gram-negative shift, reported by clinicians treating chronic infected wounds (Altoparlak et al., 2004; Dalton et al., 2011; Guggenheim *et al*., 2011; Pastar et al., 2013). Interestingly at 20°C, over 24 h the Gram-negative shift did not occur, and *S. aureus* remained the most numerous species (Figure 3A).

Testing wound dressings

Experiments at 20°C showed a 3-4 log reduction in bacterial number of both species when 3- and 6-CHX-HMP dressings were applied for 24 h compared with controls of: 0-CHX-HMP and no treatment ($P<0.05$ for both conditions) (Figure 4A). Experiments conducted at 37°C indicated that the dressings were less effective at reducing the microbial load, with log reductions of 1 following 24 h treatment with 6-CHX-HMP ($P<0.05$) (Figure 4B). Furthermore, under conditions of flow at 37°C, a commercially available alginate dressing containing antimicrobial silver (Ag-Alg) did not reduce the

209 microbial load ($P>0.05$) compared to the untreated control; previous static biofilm models indicate

210 that silver dressings can reduce biofilm biomass (Paladini *et al*., 2016).

Discussion

The DBD was designed with the aim of better representing a chronic infected, exuding wound. It has been demonstrated here that the device permitted culture of 12 biofilms simultaneously, on top of semi-permeable substratum fed from beneath with a flow of nutrients. The data obtained were reproducible, with control over a range of variables including bacterial species and comparative analysis of population changes, culture time and/or temperature, nutrient type and nutrient supply rate.

Initial validation experiments at 20^{o}C allowed for ease of set-up and monitoring of the device; this was relevant not only to the optimisation for the device, but also to the wound infection model. The temperature at the skin or wound surface can range between 21-35^{o}C (depending on an individual's physiology and the location of the wound). However, infection often results in a rise in temperature within the wound, this being one of the clinical signs of infection. Thus, to mimic an infection state, experiments were also conducted at 37^{o}C. The DBD was found to give reproducible results at both temperatures, across all four channels and all 12 wells indicating the robustness of the model for biofilm study.

The flow rate chosen for this study was 0.083 mL min^{-1} based on a similar experimental design involving *in situ* testing of wound dressings (Lipp *et al*., 2010). This is towards the higher end of flow rates observed from studies to quantify wound fluid and therefore best replicates a heavily exuding wound (Mulder, 1994). Chronic wounds produce high levels of exudate, and there is a literature precedent for using much higher flow rates, of up to 0.5 mL min^{-1}, which are less physiologically relevant (Hill *et al*., 2010). The flow rate was constant throughout the experiments described here, irrespective of the temperature and presence/absence of a wound dressing.

Nutrient broth was the nutrient supply for optimisation of the DBD; this flowed beneath plugs of noble agar. The use of nutrient broth was convenient for these experiments but alternative

media, such a simulated wound fluid, could be utilised in this system to represent the wound environment. One 24 h run of the DBD at the flow rate specified here, used approximately 500 mL of media making it possible to use the device with chemically defined and therefore often more expensive media. Minimal media could also be utilised to allow for the study of specific nutrients on biofilm growth, such as iron, or media could be pH adjusted to mimic the wound bed. The versatility of the DBD in this respect enhances its potential as a tool to study wound biofilms.

Typically, a wound will be colonised initially by Gram-positive species, with the bioburden shifting towards Gram-negative species over time, and in mature biofilms the latter are the most common type of organism. This so-called "Gram-negative shift" is a well-known phenomenon in wound infections, both *in vivo* and clinically, with several other biofilm models reporting a similar pattern of growth (Altoparlak *et al*., 2004; Dalton *et* al., 2011; Guggenheim *et al*., 2011; Pastar *et al*., 2013). When cultured at 37^{o}C we observed this shift in biofilm composition over 72 h, with the critical shift occurring at 10 h. Visual inspection of membranes prior to disruption and recovery of bacteria were concordant with these observations; blue pigment (pyocyanin) produced by *P. aeruginosa* was first evident at 8 h and persisted for the remainder of the experiment (Figure 3D). A yellow pigment, likely to be pyoverdin produced by *P. aeruginosa*, was visible from 24-72 h. The secretion of pyoverdin is associated with iron chelation and virulence.

Research has shown that pyocyanin also serves as a signalling molecule during biofilm formation, specifically detecting changes in iron concentration that serve as a trigger for biofilm maturation (Banin *et al*., 2005). It is therefore hypothesised that biofilm maturation occurs within the model presented here at 24 h and beyond. Significantly, the observed pigments are known to have a bactericidal effect on *S. aureus*, which might also contribute to the apparent Gram-negative shift (Baron and Rowe, 1981). Notably, the Gram-negative shift or "target-pattern" of pigment production was not observed at 20^{o}C, suggesting that *S. aureus* might be better able to compete at

lower temperatures, which might be relevant to wounds at sites of lower temperature, such as the extremities.

Using a static biofilm model, it has previously been demonstrated that *S. aureus* predominates in mixed-species biofilms with *P. aeruginosa* up to 72 h (at 37^{o}C) with no indication of a Gram-negative shift (Alves *et al*., 2018). It is interesting to note that the Gram-negative shift occurs only when these two organisms are cultured under conditions of flow. Comparative analysis of biofilms statically or under flow, demonstrate that "linking-film" organisms are crucial for biofilm formation. These linking or pioneer organisms attach to almost any substratum, and are necessary for the establishment of biofilm under physiologically relevant flow rates, however, do not always maintain their position as the biofilm develops (Bos *et al.,* 1999). This might in part explain the differences observed between static and flow models of *S. aureus* and *P. aeruginosa* biofilms, especially given the role of *S. aureus* as a linking organism for the attachment of *P. aeruginosa* (Alves *et al*., 2018). Flow is also known to promote distinct spatial arrangements and colonisation patterns in mixed-species biofilms, possibly attributed to differential diffusion of nutrients and waste products that is absent in a static model (Bos *et al.,* 1999).

Another aim of the DBD was the capacity to use it to test topical antimicrobial wound dressings. Wound dressings applied after 48 h culture of biofilm, remained *in situ* for a further 24 h. The data from these experiments was highly reproducible and indicated that antimicrobial treatment was most efficacious at 20^{o}C when biofilm microorganisms are presumably growing and/or metabolising more slowly. This validates the DBD as a robust means of assessing the efficacy of antimicrobial wound dressings where the parameters for biofilm growth and composition could be controlled by the user. Furthermore, it indicates that temperature, and therefore possibly the location of a wound, could be a critical factor for the effective use of topical antimicrobial treatment. This is particularly important given that most models assess the effectiveness of dressings in a static system, when it is evident from our data that flow, such as that produced by exudate can diminish

the antimicrobial activity of dressings that have proven efficacy in static models (Bjarnsholt *et al*., 2007; Kostenko *et al*., 2010).

The design of the DBD aimed to provide scope for use to study different types of chronic wound and to simulate specific wound environments. This can be made possible through adjustments to experimental parameters including, for example: bacterial species and their relative abundance, growth time and temperature, nutrient type, nutrient supply rate or incorporation of human serum proteins to the substratum. Additionally, the DBD could be adapted to test other topical treatments such as antimicrobial creams or gels *in vitro*. Complex biofilms comprising more than two species could feasibly be cultured using the DBD and the use of cellulose membranes as a substratum could allow for transfer of biofilm to animal model injuries.

Compared to other well-utilised biofilm models and flow systems, the DBD offers several advantages: it is simple to manufacture, has a small size footprint, is a one-part sterilisable device and allows for high throughput, multi-sample analysis. Importantly, the device can be 3D printed in a variety of materials.

Conclusions

The DBD provides a useful new tool for the study of chronic wound infection and the efficacy of topical antimicrobials. It is straightforward to use and gives reproducible data for both single and two-species biofilms. It provides a more representative model of wound biofilms than the majority of current biofilm models and has the capacity to incorporate the study of additional factors such as environment in addition to those described here.

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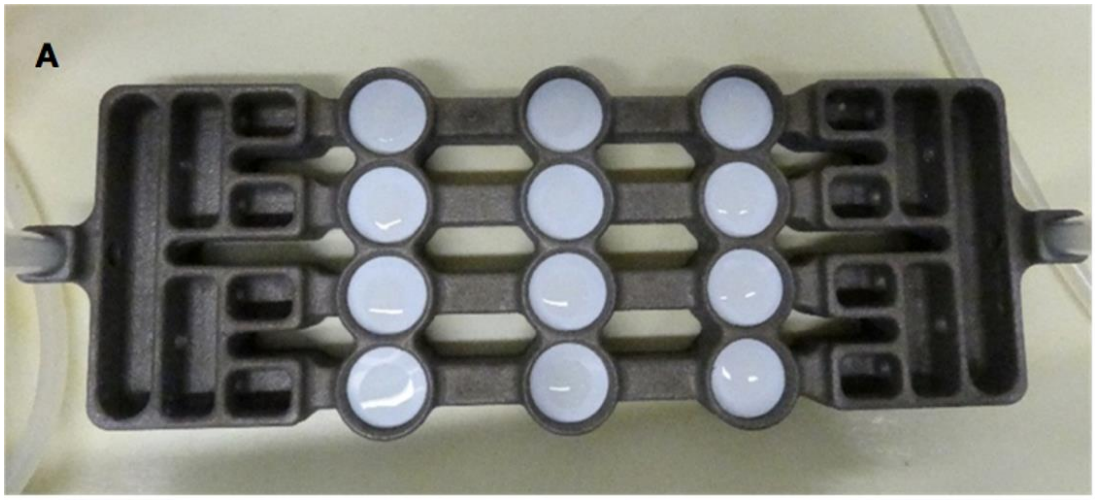
Figure 1. (A) The Duckworth Biofilm Device with all wells in use. (B) Schematic showing a cross-section design the Duckworth Biofilm Device (one channel shown). The nutrient solution is split into four separate, enclosed channels which open into a well. (C) Attachment of tubing to the inlet/outlet port of the Duckworth Biofilm Device uses a 1 mL pipette tip to provide rigidity, secured in place with Parafilm™.

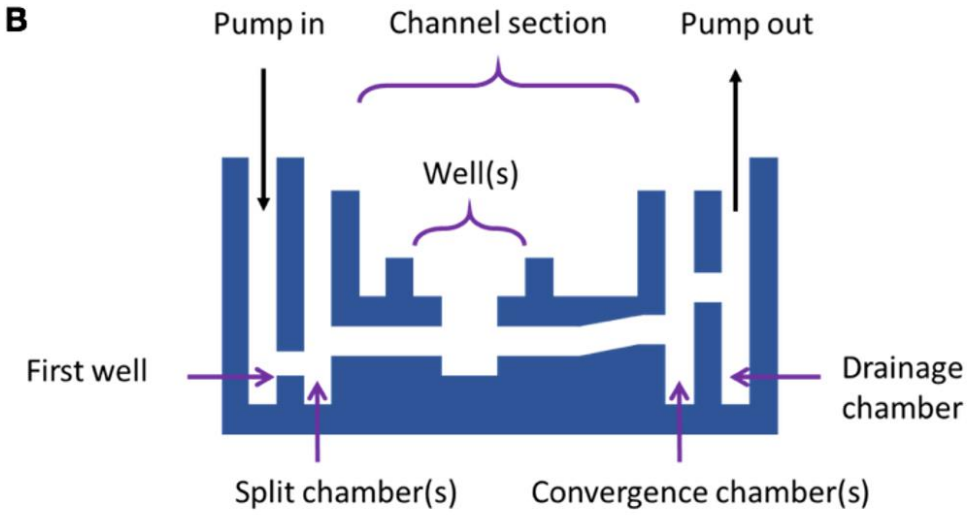
Figure 2. (A) Schematic cross-section view of biofilm support (agar plug, cellulose membrane and biofilm) in the Duckworth Biofilm Device. (B) The Duckworth Biofilm Device connected to fresh media and a waste container, via tubing at the inlet and outlet port, with lid and filter in place. Once the set-up is complete as shown above, the device is ready to use. (C) Schematic representation of the Duckworth Biofilm Device once set-up and ready to run.

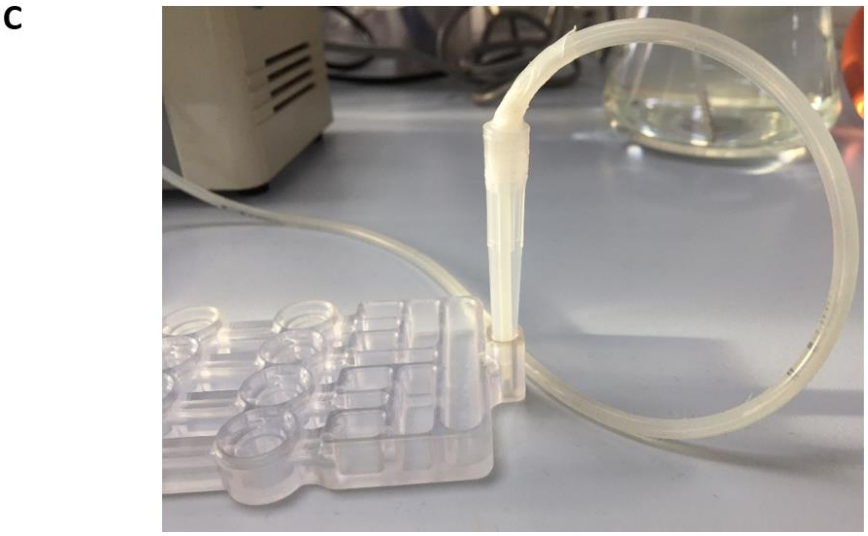
Figure 3. (A) Biofilm population of single-species biofilms grown in the Duckworth Biofilm Device at 20°C after 24 h. Data points split by well position along channel and by channels of the reactor all show excellent consistency. (B) Biofilm growth for polymicrobial biofilms grown in the Duckworth Biofilm Device at 37°C. Error bars show standard deviation, experiment performed in triplicate (n = 4). (C) Competitive relative index for *S. aureus* and *P. aeruginosa* in a biofilm cultured in a polymicrobial biofilm for 72 h in the Duckworth Biofilm Device, at 37°C. (D) Photographs of biofilms grown on (white) membrane over 72 h. At 4 h the characteristic blue pigment (pyocyanin) indicative of *P. aeruginosa* is not apparent, but becomes visible from 8 h and predominant from 24 h onwards. From 24 h onwards, a "target" formation of pigment production occurs with yellow pigment (likely pyoverdin) produced centrally within the biofilm.

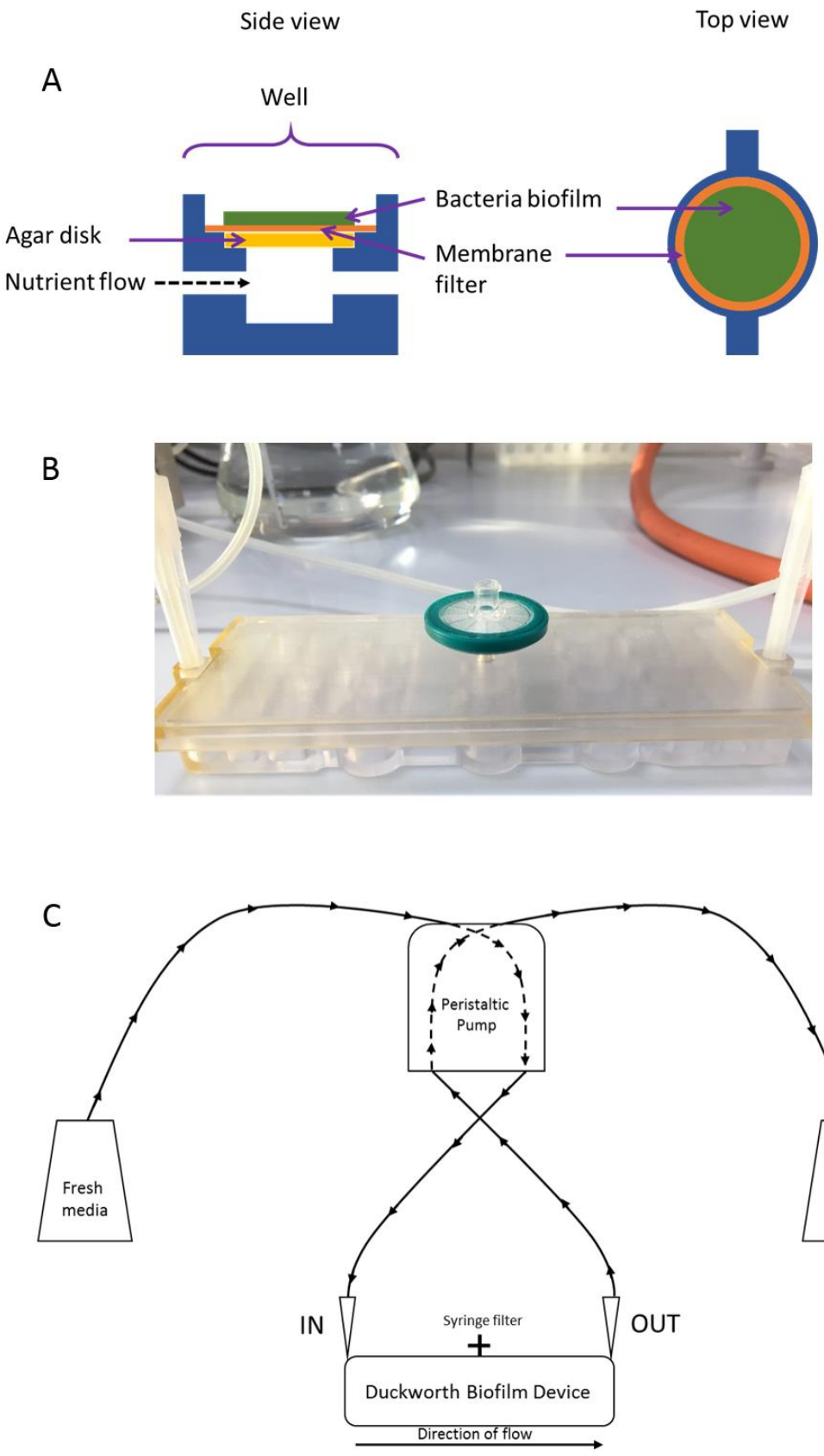
Figure 4. (A)Polymicrobial biofilm grown in the Duckworth Biofilm Device for 48 h then subject to 24 h topical application of alginate thin film dressings containing some wt% chlorhexidine hexametaphosphate nanoparticles (CHX-HMP). Experiment performed at 20°C. Control is no treatment. * indicates a statistically significant reduction ($P<0.05$) in bacterial count for both microorganisms, between the two conditions indicated. (B) Polymicrobial biofilm grown in the

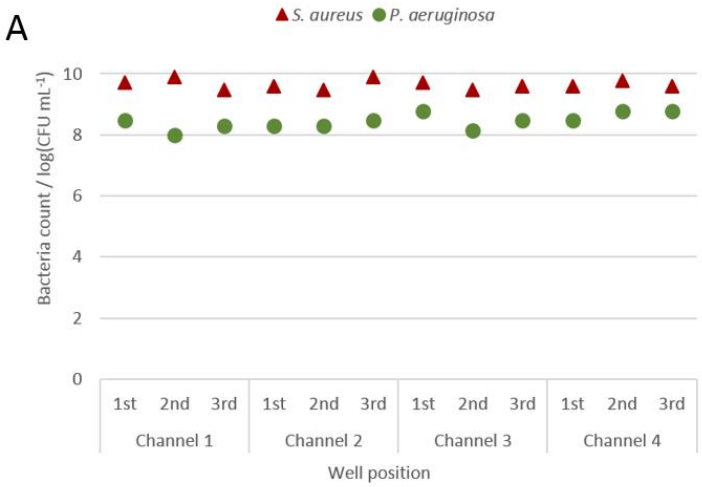
Duckworth Biofilm Device for 48 h then subject to 24 h topical application of alginate thin film dressings containing some weight % chlorhexidine hexametaphosphate nanoparticles (CHX-HMP), and a commercially available alginate dressing containing antimicrobial silver (Ag-Alg) (Tegaderm™). Experiment performed at 37°C. Control is no treatment. * indicates a statistically significant reduction ($P<0.05$) in bacterial count for both microorganisms, between the two conditions indicated.

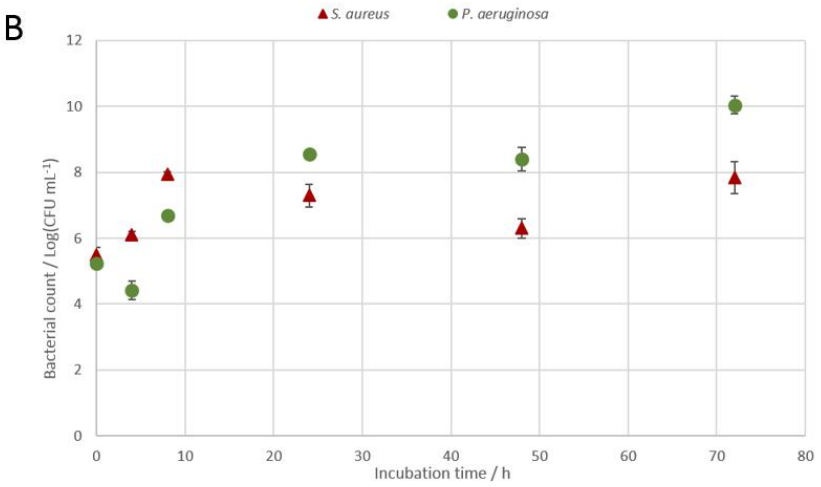

A

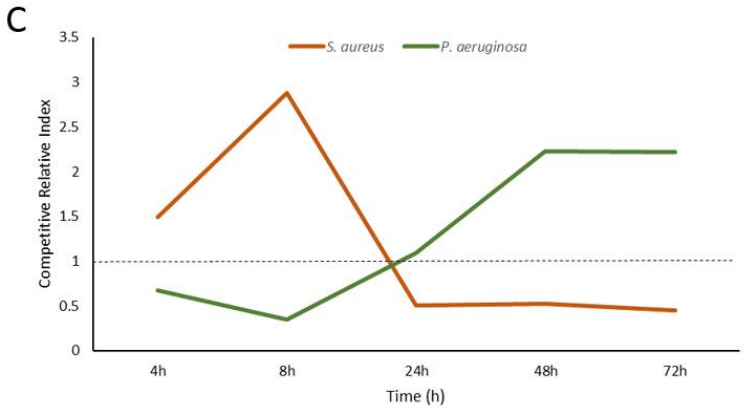

B
Pump in
Channel section
Pump out
Well(s)
First well
Drainage chamber
Split chamber(s)
Convergence chamber(s)

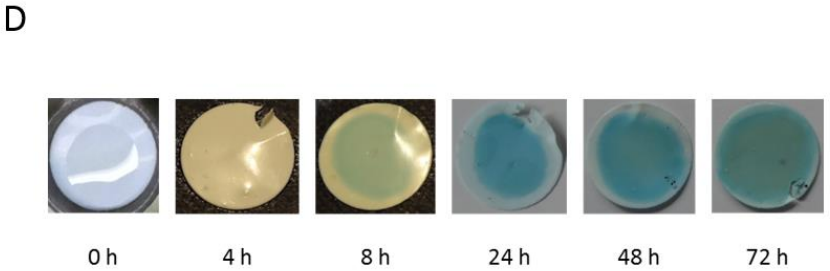

C


Side view
Top view
A
Well
Bacteria biofilm
Agar disk
Membrane filter
Nutrient flow
B
C
Peristaltic Pump
Fresh media
Waste media
IN
Syringe filter
OUT
Duckworth Biofilm Device
Direction of flow

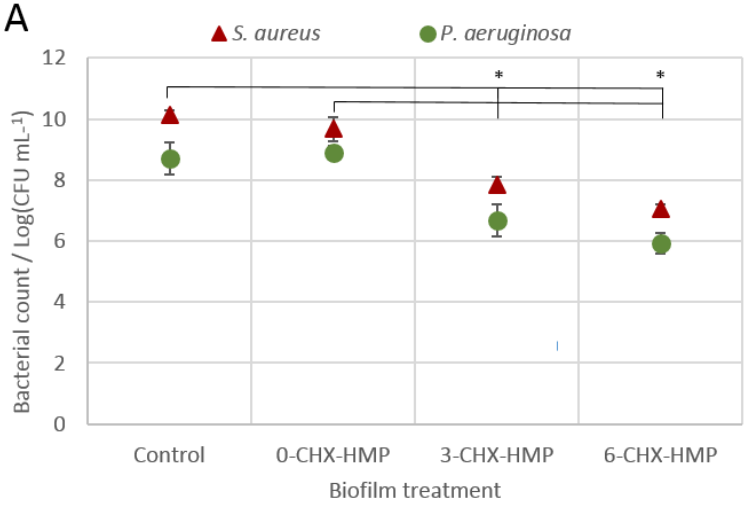

A
S. aureus
P. aeruginosa
Bacteria count / log(CFU mL-1)
10
8
6
4
2
0
1st 2nd 3rd 1st 2nd 3rd 1st 2nd 3rd 1st 2nd 3rd
Channel 1
Channel 2
Channel 3
Channel 4
Well position


B
S. aureus
P. aeruginosa
Bacterial count / Log(CFU mL-1)
12
10
8
6
4
2
0
0 10 20 30 40 50 60 70 80
Incubation time / h

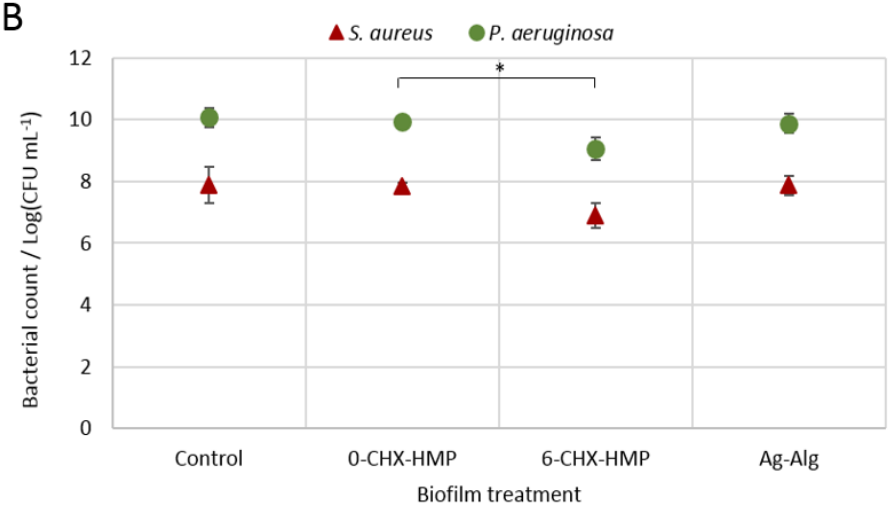

C
S. aureus
P. aeruginosa
Competitive Relative Index
3.5
3
2.5
2
1.5
1
0.5
0
4h 8h 24h 48h 72h
Time (h)


D
0 h
4 h
8 h
24 h
48 h
72 h

A


S. aureus
P. aeruginosa
Bacterial count / Log(CFU mL-1)
12
10
8
6
4
2
0
*
*
Control
0-CHX-HMP
3-CHX-HMP
6-CHX-HMP
Biofilm treatment

B


S. aureus
P. aeruginosa
Bacterial count / Log(CFU mL-1)
12
10
8
6
4
2
0
*
Control
0-CHX-HMP
6-CHX-HMP
Ag-Alg
Biofilm treatment

	Bacteria counts / Log(CFU mL^{-1})	
Time (h)	***S. aureus***	***P. aeruginosa***
0	5.48 ± 0.23	5.23 ± 0.10
4	6.11 ± 0.09	4.41 ± 0.28
8	7.94 ± 0.08	6.70 ± 0.06
24	7.30 ± 0.34	8.55 ± 0.04
48	6.31 ± 0.29	8.41 ± 0.36
72	7.84 ± 0.48	10.04 ± 0.26

Table 1. Biofilm growth data of polymicrobial biofilms (experiment performed in triplicate, n = 4) grown in the Duckworth Biofilm Device at 37°C.

S. aureus							*P. aeruginosa*						
20°C / 24 h				**Time (h)**	**37°C**	**CRI**	**20°C / 24 h**				**Time (h)**	**37°C**	**CRI**
Channel 1	**Channel 2**	**Channel 3**	**Channel 4**		**(Log CFU mL^{-1})**		**Channel 1**	**Channel 2**	**Channel 3**	**Channel 4**		**(Log CFU mL^{-1})**	
9.62±.0.26	9.84±0.53	9.76±0.66	9.56±0.26	**0**	5.23±0.12	N/A	8.52±0.56	8.84±0.26	8.23±0.86	8.64±0.53	**0**	5.19±0.26	N/A
				4	6.35±0.36	1.36					**4**	5.11±0.32	0.73
				8	7.68±0.26	1.88					**8**	6.89±0.36	0.52
				24	7.56±0.46	0.60					**24**	8.57±0.14	1.00
				48	7.35±0.56	0.20					**48**	8.72±0.52	2.8
				72	7.94±0.32	0.39					**72**	9.86±0.46	2.5

Table S1. Validation experiments undertaken with the Accura ClearVue Resin Duckworth Biofilm Device. At 20°C when cultured for 24 h, biofilm growth in all four channels showed excellent consistency. Time-point experiments conducted at 37°C showed similar results to the $AlSi_{10}Mg$ indicating that the material used to produce the Duckworth Biofilm Device does not affect results.