Cold atmospheric pressure plasma elimination of clinically important single and multi-species biofilms

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Abstract: Mixed species biofilms reflect the natural environment of many pathogens in clinical settings and are highly resistant to disinfection methods. An indirect cold atmospheric-pressure, air-plasma system was evaluated, under two different discharge conditions, for its ability to kill representative Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Pseudomonas aeruginosa*) pathogens. Plasma treatment of individual 24-hour biofilms and mixed species biofilms that contained additional species (*Enterococcus faecalis* and *Klebsiella pneumoniae*) was considered. Under plasma conditions that favoured the production of Reactive Nitrogen Species (RNS), individual *P. aeruginosa* biofilms containing approximately 5.0 x 10^6 colony forming units (cfu) were killed extremely rapidly, with no bacteria survival detected at 15 seconds exposure.

S. aureus survived longer under these conditions, with no detectable growth after 60 seconds exposure. In mixed species biofilms, *P. aeruginosa* survived longer, but all species were killed with no detectable growth at 60 seconds. Under plasma conditions that favoured the production of Reactive Oxygen Species (ROS), *P. aeruginosa* showed increased survival with the limit of detection reached by 120 seconds and *S. aureus* was killed in a similar time-frame. In the mixed species model, bacterial kill was biphasic but all pathogens showed viable cells after a 240 second exposure, with *P. aeruginosa* showing significant survival (approx. 3.6 ± 0.6 x 10^6 cfu). Overall the study shows the potential of indirect air plasma treatment to achieve significant bacterial kill, but highlights aspects that might affect performance against key pathogens especially in real-life settings within mixed populations.

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Highlights:

- Indirect atmospheric pressure air plasma treatment was found to be an effective decontamination technique for biofilms grown on polymeric surfaces.

- Mixed species biofilms showed increased resistance to plasma treatment, with viable bacteria remaining within the treatment time frame.

- Variation in the reactive plasma chemistry was used to highlight the different sensitivities between the species within a mixed species biofilm.

1. Introduction

Cold atmospheric pressure plasma is an emerging technology that is currently under intense investigation for microbial decontamination applications [1]. A number of studies have highlighted the potential for plasmas to decontaminate a range of pathogens important in healthcare [1]. These studies have tested the efficacy of plasma against planktonic cells or single species biofilms [2, 3, 4]. However, in the environment biofilms are likely to exist as mixed microbial communities and studies have shown that mixed-species biofilms possess increased resistance to antimicrobial agents compared to single-species biofilms [5].

Mixed-species biofilms are an important colonizer of a wide range of medical devices, such as venous and urinary catheters, mechanical heart valves, prosthetic joints and endotracheal tubes [6]. Common bacterial species isolated from medical devices include those termed 'ESKAPE' pathogens (*Enterococcus faecalis, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter spp.*) [7]. Although by no means the only pathogens that form biofilms on medical devices, these organisms are associated with increasing levels of multidrug resistance (resistant to at least 3 classes of frontline antibiotic) and represent a serious public health threat [8]. A mixed-species biofilm consortium was selected for this study, based on the representation of the organisms in chronic wound infections and as common colonisers on implanted medical devices [9]. As such, the outputs of the study should be informative for the use of plasma systems to generate reactive species to control these types of bacterial infection *in vivo*. Plasma systems are already used for the treatment of wound infections [10].

An advantage of plasma decontamination is that it does not rely on any one mechanism for bacterial killing; Reactive Oxygen Species (ROS), Reactive Nitrogen Species (RNS), UV photons and high electric fields are all produced simultaneously in the plasma, increasing the potential for synergistic effects. This pilot study explores how the reactive chemistry produced by an indirect cold air plasma impacts the decontamination efficacy of two clinically relevant ESKAPE bacterial species (*P. aeruginosa* and *S. aureus*) in both single and mixed species biofilms.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Four different bacterial strains were included in this study: *Pseudomonas aeruginosa* (PAO1), *Klebsiella pneumoniae* (NCTC13368), *Enterococcus faecalis* (NCTC775) and *Staphylococcus aureus* (ATCC9144). Bacterial cultures were prepared in Tryptic Soy Broth (TSB) with shaking at 37 °C or incubated on Tryptic Soy Agar at 37 °C.

2.2. Biofilm formation in the CDC biofilm reactor

Biofilms were generated on PVC coupons in the CDC bioreactor (Bio Surface Technology Corp., Bozeman, MT, USA). *P. aeruginosa* and *S. aureus* were used to form single-species biofilm models. Bacteria at a final concentration of 1.0×10^5 cfu/mL were added to the 350 ml of TSB medium. Biofilms were grown for 24 h at 37°C with stirring at 200 rpm. *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Enterococcus faecalis* and *Staphylococcus aureus* were used in mixed-species biofilms [9].

2.3. Plasma source & sample exposure

The plasma source considered in this study is similar to that reported by Olszweski *et al*. and employs a Surface Barrier Discharge (SBD) configuration as shown in Figure 1(a) [11]. Biofilm-containing coupons were removed from the bioreactor following incubation and rinsed twice in 25 mL sterile phosphate-buffered saline (PBS) to remove planktonic and loosely attached cells. Coupons were exposed to the plasma at a distance of 5 mm between the sample and the electrode. Two plasma powers were considered, a low power discharge, $P_{discharge}$ = 8 W, giving an ROS dominated gas phase chemistry and a high power discharge, $P_{discharge}$ = 34.5 W, giving an RNS dominated gas phase chemistry (discussed further in section 2). Treatments were performed for 7 seconds up to 240 seconds. Coupons were treated on both sides at the same plasma conditions. Treatment at each parameter was performed in triplicate with at least 3 replicates per experiment.

2.4. Determination of biofilm elimination

Bacterial survival after plasma exposure was determined by serial dilution with Miles-Misra enumeration [9]. Treated PVC coupons were immediately transferred to 5 mL of TSB to quench any further inactivation and vigorously shaken for 10 min using a vibratory shaker (VXR basic Vibrax®, IKA, Staufen, Germany) at 2000 rpm to release cells. 10 µL of bacterial suspensions were added to 90 µL of fresh TSB medium. This was serially diluted ten-fold to 10^{-5} in 96-well plates. 10 µL of each dilution was plated on TSA plates, with three repetitions for each dilution. Controls followed the same protocols except for exposure to plasma. Plates were incubated overnight at 37 °C. The limit of detection was around 5.0 x 10^2 bacteria.

2.5. Fourier transform infrared spectroscopy – FTIR

FTIR was used for characterization of reactive species in the gas phase. The plasma generating electrodes were sealed in a box of similar volume to that used in the decontamination experiments from where the plasma exhaust gas was drawn in to a 10 cm path length gas cell and analysed with a FT/IR-4200 (JASCO, Tokyo, Japan). A spectral resolution of 2.0 cm^{-1} was used and each absorption spectrum was acquired over 25 scans. The composition of the plasma effluent was analysed under the same conditions as those used in the biofilm deactivation experiments.

3. Results

3.1 Characterization of gas phase

Figure 1(b) highlights the FTIR absorption spectrum under low and high power plasma generation conditions. FTIR analysis is only capable of identifying molecules that actively absorb in the IR range; hence, the data presented in Figure 1(b) should not be considered as an exhaustive characterisation of the plasma effluent. Under low plasma power conditions, ozone was found to dominate, indicating the predominance of ROS in this regime. Under high plasma power conditions, the oxides of nitrogen dominated, with no

measurable ozone production; indicating the predominance of RNS. The variation in gas phase chemistry under different operating power conditions is typical for such a discharge and is attributed to elevated temperatures in the plasma, leading to thermal degradation of ozone and a thermally driven increase in nitric oxide production [11].

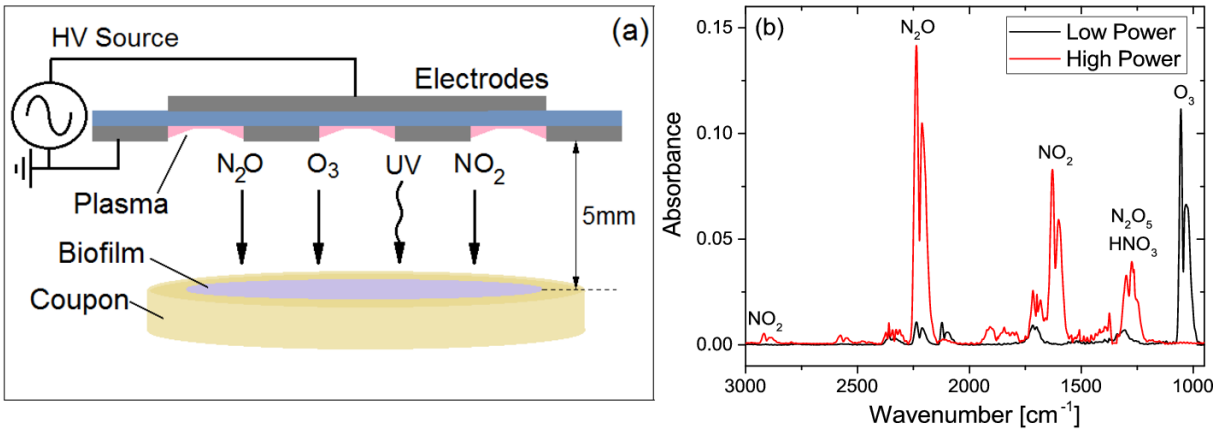

Figure 1: (a) Schematic of plasma system and sample position, (b) FTIR spectra obtained after 120 seconds of plasma generation under ROS dominant (black) and RNS dominant (red) conditions.

3.2 Effect of plasma treatment on the survival of P. aeruginosa and S. aureus biofilms

Single-species biofilms of *P. aeruginosa* and *S. aureus* were exposed to the ROS- and RNS-dominant plasma effluent regimes over varying periods of time. The sensitivity of the two species to plasma elimination was different with respect to the respective discharge regimes and from one species to the other. Figure 2(a) shows the elimination rate of *P. aeruginosa* biofilm is strongly influenced by the dominant gas-phase chemistry. Under low-power, ROS-dominated conditions, elimination of the bacteria to below the limits of detection of the assay, was achieved within 120 seconds. In the high-power, RNS-dominated regime, complete elimination of *P. aeruginosa* was achieved in less than 15 seconds. Figure 2(b) highlights that plasma treatment was also efficient for the elimination of *S. aureus* biofilms. In contrast to *P. aeruginosa*, the *S. aureus* biofilms showed little dependency on the plasma generation conditions. Thus the rate of elimination of the bacteria was similar across the first 30 seconds of exposure. Thereafter the RNS-dominated effluent was able to kill *S. aureus* to below the detection limit of the assay within 60 seconds, whereas this was only achieved by 120 seconds in the ROS dominated

case. In comparison between the two bacteria, *P. aeruginosa* was significantly more sensitive to the RNS-dominated regime than *S. aureus*, whilst the reverse was true for the ROS-dominated conditions, albeit with the two bacteria showing very different kill kinetics.

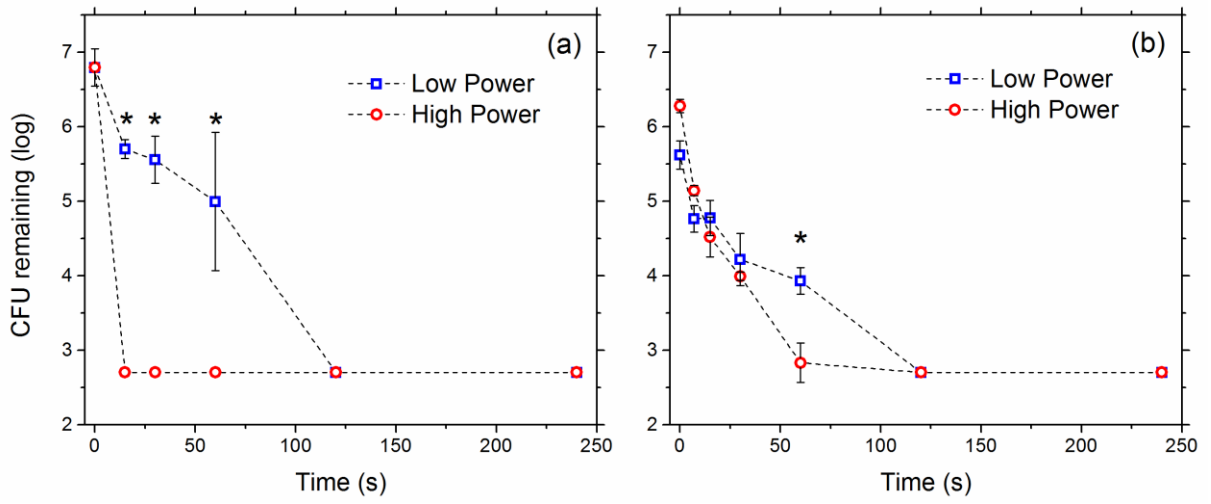

Figure 2: Elimination of single species biofilms under low and high power plasma conditions: (a) *P. aeruginosa* and (b) *S. aureus*. Each data point represents the mean cfu for each time point with error bars representing standard deviation. The mean and standard deviation are calculated from all replicate cfu count values for each of the triplicate experiments (n=9). Statistically significant differences between elimination under low and high power conditions were determined using a one-way ANOVA (IBM SPSS Statistics 22), with Scheffe's *posthoc* test (*p<0.05).

3.3 Effect of plasma treatment on the survival of mixed-species biofilms

Mixed-species biofilms contained *P. aeruginosa*, *S. aureus*, *K. pneumoniae* and *E. faecalis* strains [9]. This system provides similar numbers of the 4 species, when used at 24 hours, and provides a basis to compare relative elimination within a single co-culture system. The treatment conditions were the same as described for single-species biofilms. The susceptibility of *P. aeruginosa* and *S. aureus* was found to be different within the multispecies communities compared to that observed in single-species biofilms; highlighted in Figures 3(a) and 3(b), for ROS and RNS dominated regimes, respectively. Under ROS exposure, there was a biphasic kill of the bacteria, with only a small reduction

in viable count, of the order of 1-2 logs, after 60 seconds exposure. Between 60 and 120 seconds, three of the species, *S. aureus*, *E. faecalis* and *K. pneumoniae* showed a further reduction in viable count of around 2-logs, but there were still viable bacteria after 240 seconds exposure. *P. aeruginosa* showed a much more gradual reduction in viable count and there was less than a 3-log reduction achieved in total across the 240 second exposure regime. *P. aeruginosa* and to a lesser extent *S. aureus* were less susceptible to kill under ROS-dominated plasma conditions in mixed culture than in single species biofilms. After exposure to an RNS-dominated effluent, the two Gram-negative species, *P. aeruginosa* and *K. pneumoniae* were reduced to below the limits of detection of the assay by 30 seconds and *E. faecalis* was reduced to levels only just above the LoD. At this time point, *S. aureus* in the mixed species model was reduced by only 3-logs.

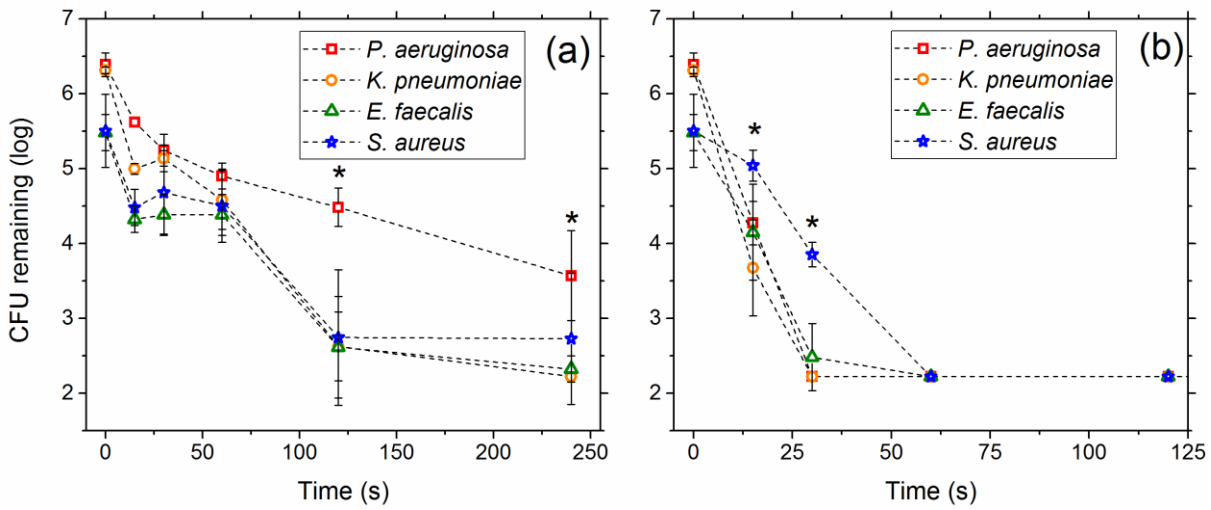

Figure 3: Elimination of mixed species biofilms under: (a) ROS dominated conditions and (b) RNS dominated conditions. Each data point represents the mean cfu for each time point with error bars representing standard deviation. The mean and standard deviation are calculated from all replicate cfu count values for each of the triplicate experiments (n=9). Statistically significant differences between species elimination at a given time were determined using a one-way ANOVA (IBM SPSS Statistics 22) with Scheffe's *posthoc* test (*$p<0.05$).

4. Discussion

This study demonstrates that indirect air plasma exposure can achieve effective kill of a range of different clinically-important pathogens, even in mixed-species biofilms. As these are commonly encountered in a variety of clinical settings, and are, perhaps, the most resistant form of bacterial colonisation on surfaces, this preliminary study offers significant potential for new applications. The differential sensitivity with respect to varying gas phase chemistries suggests that specific plasma species are important for killing specific pathogens and this may be influenced by the biofilm environment. Importantly, this information guides the potential development of plasma systems for the treatment of these complex biofilms in clinical settings, such as diabetic foot ulcers and biofilm-colonised implanted medical devices. Plasma systems which generate bactericidal reactive species, of the type observed here, could provide valuable alternatives to drug therapy for topical treatment of infections, which would contribute to antibiotic stewardship endeavours.

P. aeruginosa is an archetypal biofilm-forming bacterium, producing complex biofilms with significant amounts of extracellular matrix material (exopolysaccharide and DNA). Although the 24 hour biofilms used in this study are relatively simple, it was clear that the *P. aeruginosa* biofilm was significantly more susceptible to the species released under RNS-dominated conditions than was *S. aureus*. This is consistent with observations from other studies using atmospheric plasma systems (*e.g.* helium:oxygen plasma [4]) which showed that *P. aeuruginosa* was significantly more susceptible than *S. aureus*. Under ROS-dominated conditions, *P. aeruginosa* is much less susceptible and the differences between plasma conditions in susceptibility with *S. aureus* are less distinct. There is evidence to suggest that RNS may kill *Pseudomonas* in biofilms and, at low dose, also disrupt their structure to allow improved access for other molecular species [12, 13]. Similar effects generated by plasma-derived species might explain the observations in this study and why the difference is less marked under lower power conditions, where ROS predominate, both in the gas and water phase.

The results of this study confirm that the kinetics of bacterial kill will be more complex in mixed-species biofilm systems, even in relatively immature biofilms. Under RNS-dominated

conditions, in mixed-species models, there is a small reduction in the susceptibility of *P. aeruginosa*, but *S. aureus* is essentially unaffected. This suggests that there is free penetration of RNS into the mixed species biofilm and that there is not significant turnover of such species by bacterial RNS defences within the time-course used. This is not the case under ROS dominated conditions, where all the species were able to survive to 240 seconds exposure and there were significantly reduced levels of kill for *P. aeruginosa*. This suggests poor penetration of ROS, compared to the single species biofilms for *P. aeruginosa* and *S. aureus*, and/or that catalases and superoxide dismutases expressed in the mixed-species model, significantly reduce the local levels of ROS. The biphasic profile of the survival curves also suggests that there may be issues with penetration with freely accessible cells being killed rapidly whilst less accessible cells only being killed by prolonged exposure if at all. That *P. aeruginosa* is the species that benefits most from the mixed-species culture may reflect it occupying a more protected niche (*e.g.* closer to the solid support) within the biofilm or that its oxidative defences are activated more strongly in such biofilms than in single species systems. The results are consistent with previous studies, showing that *P. aeruginosa* was the organism most able to survive chlorhexidine exposure within the mixed-species model [9].

The study provides strong evidence that defined configurations of atmospheric pressure air plasmas can achieve rapid and highly effective bacterial kill in complex models. This brief study starts to define some of the key research questions in terms of optimising plasma discharges to generate, perhaps, more RNS species. It also identifies fundamental gaps in our understanding of the architecture of co-cultured biofilms and the cross-dependency of individual species within it.

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Competing interests.

The authors declare that they have no competing interests.

Ethical approval.

Not required

References.

[1] Kong MG, Kroesen G, Morfill G, Nosenko T, Shimizu T, van Dijk J, Zimmermann JL. Plasma medicine: an introductory review. New J. Phys. 2009, 11, 115012.
http://dx.doi.org/10.1088/1367-2630/11/11/115012

[2] Alkawareek MY, Gorman SP, Graham WG, Gilmore BF. Potential cellular targets and antibacterial efficacy of atmospheric pressure non-thermal plasma. Int J Antimicrob Agents. 2014 Feb;43(2):154-60.
doi: 10.1016/j.ijantimicag.2013.08.022.
Epub 2013 Oct 1. PubMed PMID: 24139886.

[3] Alkawareek MY, Algwari QT, Laverty G, Gorman SP, Graham WG, O'Connell D, Gilmore BF. Eradication of Pseudomonas aeruginosa biofilms by atmospheric pressure non-thermal plasma. PLoS One. 2012; 7(8):e44289. doi: 10.1371/journal.pone.0044289. Epub 2012 Aug 31. PubMed PMID: 22952948; PubMed

Central PMCID: PMC3432087.

[4] Flynn PB, Higginbotham S, Alshraiedeh NH, Gorman SP, Graham WG, Gilmore BF. Bactericidal efficacy of atmospheric pressure non-thermal plasma (APNTP) against the ESKAPE pathogens. Int J Antimicrob Agents. 2015 Jul; 46(1):101-7.
doi:10.1016/j.ijantimicag.2015.02.026.
Epub 2015 Apr 20. PubMed PMID: 25963338

[5] Sanchez-Vizuete P, Orgaz B, Aymerich S, Le Coq D, Briandet R. Pathogens protection against the action of disinfectants in multispecies biofilms. Front Microbiol. 2015 Jul 14; 6:705.
doi: 10.3389/fmicb.2015.00705.

[6] Røder HL, Sørensen SJ, Burmølle M. Studying Bacterial Multispecies Biofilms: Where to Start? Trends Microbiol. 2016 Jun; 24(6):503-13.
doi: 10.1016/j.tim.2016.02.019. Epub 2016 Mar 19.

[7] Pendleton JN, Gorman SP, Gilmore BF. Clinical relevance of the ESKAPE pathogens. Expert Rev Anti Infect Ther. 2013 Mar; 11(3):297-308.
doi: 10.1586/eri.13.12.

[8] Antibiotic Resistance Threats in the United States, 2013.
http://www.cdc.gov/drugresistance/pdf/ar-threats-2013-508.pdf (accessed 16th May 2016)

[9] Touzel RE, Sutton JM, Wand ME. Establishment of a multi-species biofilm model to evaluate chlorhexidine efficacy. Journal of Hospital Infection. 2016; 92(2):154-60.
doi: 10.1016/j.jhin.2015.09.013. Epub 2015 Oct 23.

[10] von Woedtke, Th., Metelmann, H.-R. and Weltmann, K.-D. (2014), Clinical Plasma Medicine: State and Perspectives of in Vivo Application of Cold Atmospheric Plasma. Contrib. Plasma Phys., 54: 104–117. doi:10.1002/ctpp.201310068

[11] Olszewski P, Li JF, Liu DX, Walsh JL. Optimizing the electrical excitation of an atmospheric pressure plasma advanced oxidation process. J. Haz. Mat. 279, 60–66, August 2014.

doi:10.1016/j.jhazmat.2014.06.059

[12] Barraud N, Storey MV, Moore ZP, Webb JS, Rice SA, Kjelleberg S. Nitric oxide-mediated dispersal in single- and multi-species biofilms of clinically and industrially relevant microorganisms. Microb Biotechnol. 2009 May; 2(3):370-8.
doi: 10.1111/j.1751-7915.2009.00098.x. Epub 2009 Mar 13.

[13] Barraud N, Hassett DJ, Hwang SH, Rice SA, Kjelleberg S, Webb JS. Involvement of nitric oxide in biofilm dispersal of Pseudomonas aeruginosa. J Bacteriol. 2006 Nov; 188(21):7344-53.
doi: 10.1128/JB.00779-06