

Atmospheric air plasma induces increased cell aggregation during the formation of *Escherichia coli* biofilms

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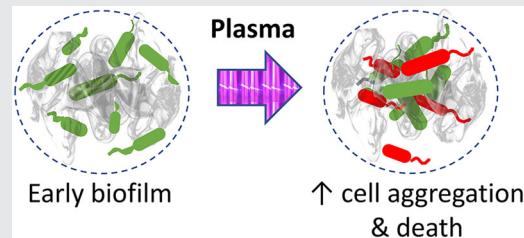
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Atmospheric air plasma has previously been shown to be a novel and effective method for biofilm eradication. Here we study the effects of plasma on both microbial inactivation and induced structural modification for forming biofilms. New structures are created from aggregates of extracellular polysaccharides and dead bacterial cells, forming a protective and resilient matrix in which the remaining living cells grow and reproduce under proper growth conditions. The new colonies are found to be more resilient in this state, reducing the efficacy of subsequent plasma treatment. We verify that the observed effect is not caused by chemicals produced by plasma reactive species, but instead by the physical processes of drying and convection caused by the plasma discharge.



KEY WORDS

atmospheric plasma, biofilms, cell agglomeration

1 | INTRODUCTION

Biofilms are colonies of microorganisms surrounded by a complex fluid matrix made predominantly of extracellular polysaccharide polymers (EPS). The EPS provides the mechanical stability of biofilms, mediates their adhesion to surfaces and forms a cohesive, 3-dimensional polymer network that interconnects and transiently immobilises biofilm cells.^[1] This EPS matrix provides a protective barrier for bacterial colonies in a biofilm,^[1] increasing the resistance of bacteria to chemical and antibiotic treatments and also reducing the efficacy of physical treatment. Consequently, cells residing within the biofilm matrix demonstrate increased survival to many conventional methods of eradication which are found effective for their

freely dispersed, or planktonic, counterparts.^[2] Biofilms can form on many surfaces, including the skin of fresh fruits and vegetables, industrial pipe surfaces, in between teeth, and on medical devices.^[3,4] Due to their widespread existence and resilience, biofilms are known to be the main cause of persistent bacterial infections in hospitals,^[5] contamination of foods in process environments,^[6] and reduced process cleaning efficiency in manufacturing. Biofilm physical and flow properties have recently been studied as a means of understanding molecular transport through the matrix and to better enable destruction.^[7,8] New approaches are being developed to more aggressively treat biofilms during formation, for example, to interfere with the attachment of these bacteria to surfaces and disturb their structure.^[9]

One novel treatment currently being investigated for this purpose is atmospheric plasma, which is essentially an ionized gas that is generated at ambient temperatures and under atmospheric conditions that allows treatment of sensitive biological matter.^[10,11] Numerous recent studies have demonstrated the anti-microbial efficacy of atmospheric plasma for planktonic bacteria or cells embedded in biofilms.^[12] Plasma species are reported to be capable of penetrating into the biofilm structure.^[13] Plasma can inactivate biofilms with treatment times of less than 60 s^[14] and cause a five log reduction in biofilm viability,^[15] while longer treatments can decrease viable cells to undetected levels.^[15–17] This ability of plasma to inactivate bacteria is thought to be an effect of its production of short- and long-lived reactive species^[18] such as ozone and other radicals.^[19] Direct or indirect plasma discharges in air to water creates an acidified, nitrogen-oxide containing solutions known as plasma-activated water (PAW), which remains antibacterial for long periods. Such long-lived species have been shown to be effective to treat *Escherichia coli* suspensions even after a 7-d period, following plasma liquid generation.^[20]

Apart from its ability to inactivate bacteria in a biofilm, atmospheric air plasma has been shown to change the overall biofilm structure by disrupting and degrading the EPS biofilm components.^[21] For example, separation of initially aggregated bacteria has been observed during EPS degradation due to plasma treatment.^[22] Plasma-induced EPS degradation causes a decrease in biofilm thickness^[21,23] and volume^[21] as well as an increase in its roughness and porosity.^[21] Plasma-treated biofilms are also known to have reduced adhesion to surfaces.^[23,24]

In model systems, monolayers of surface-deposited *Listeria innocua* responded to plasma treatment by forming cell aggregates of damaged cells, into which viable cells were then moved, affecting plasma inactivation kinetics.^[28] Bayliss et al.^[28] suggested such sheltering of cells extends the treatment time needed for bacterial inactivation and is driven by plasma gas flow-induced drying and the resultant fluid shear stresses. Although the work was carried out on a manually-deposited layer of cells, it likely has relevance for more developed biofilm community environments as well. This work examines the effects of short duration plasma treatments on young biofilm structures and how modification of those structures affects bacterial resilience to subsequent plasma treatments.

2 | EXPERIMENTAL SECTION

2.1 | Preparation of biofilm sample

Single *E. coli* MG1655 (CSIRO Food Research Ryde Bacteriology Culture Collection) colonies were inoculated in nutrient broth (1 g L⁻¹ ‘Lab-Lemco’ powder, 2 g L⁻¹ 170

yeast extract, 5 g L⁻¹ peptone, 5 g L⁻¹ sodium chloride, pH 7.4; Oxoid, Adelaide, Australia) and grown in a shaking incubator (Bioline Global, South Australia) at 37 °C and 100 rpm for 12 to 15 h. The cultures contained approximately 10⁹ CFU mL which was diluted to 10⁷ CFU mL⁻¹. From this diluted culture, 2 mL samples were transferred to a FluoroDish™ cell culture dish (World Precision Instruments). These dishes were incubated at 37 °C to allow biofilm formation. After 24 h, the medium was exchanged for fresh medium. The biofilms were grown for a period of 48 h total for time-dependent and liquid coverage experiments or 24, 48, and 72 h for cell regrowth and multiple treatment studies, after which the medium was removed and the biofilm washed twice with phosphate buffered saline (PBS) prior to treatment and analysis. Details of the regrowth studies are provided in section 2.3.

2.2 | Plasma setup

The power supply used to drive the plasma discharge was an HV half bridge resonant inverter circuit (PVM2000, Information Unlimited, New Hampshire, USA). The power source has a power of 2 kW, a high-frequency power driver from 20 to 100 kHz, a peak to peak voltage up to 40 kV (load dependent) and a duty cycle control from 10 to 90%. The plasma setup consists of a FluoroDish™ used to grow the biofilm (see section 2.1) that is placed in between the electrodes of the Dielectric Barrier Discharge (DBD), consisting of a 2 mm thick poly(methyl methacrylate) single layer dielectric and a top electrode that is partially recessed within the imaging dish to reduce the discharge gap to 6 mm (Figure 1a). The discharges were induced in open atmospheric air conditions.

2.3 | Plasma-biofilm treatment conditions

2.3.1 | Direct treatment

The growing biofilms were exposed to direct plasma treatment, Figure 1a, after 24 or 48 h of growth, while only biofilms aged 48 h were exposed to plasma-activated liquid (see section 2.3.2 below, Figure 1b). Plasma treatment was performed at 6 kV and 60 kHz with treatment time the only plasma condition varied. The optical emission spectra, OES, for the discharge were mainly in the UV region, the OES are not included, the reader is referred to Lu et al^[25] for characterisation of discharges with this power source. The DBD design incorporating the dish used to grow the biofilm allows for non-invasive sample preparation, as the biofilm could be imaged directly in its growth dish before and after plasma treatment, which is critical for imaging of a biofilm's structure. The design also offers the added benefit of a

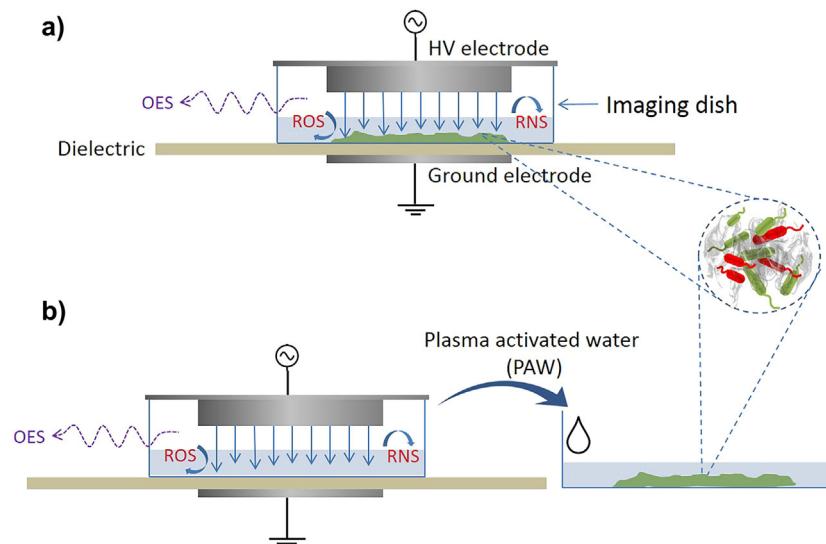


FIGURE 1 a) DBD design incorporating the glass bottom imaging dish containing the growing biofilm within the discharge gap, b) Schematic of air discharge in contact with liquid and addition of PAL to growing biofilm

relatively controlled discharge in terms of spatial homogeneity and treatment time when compared to plasma jets. Precise control of treatment time ($+/- 1\text{s}$) allows the effects of short plasma treatment times on biofilm behavior to be investigated.

For time-dependent studies, biofilms aged 48 h were exposed to direct plasma for times ranging from 0 to 60 s. The biofilm was kept wet by adding 200 μL of PBS into the dish. For liquid coverage studies, different amounts of PBS were added to the cell culture dish, from 200 to 1000 μL , and biofilms aged 48 h were used. In the regrowth study, biofilms aged 24 and 48 h were used and exposed to plasma for 30 s. On each day, biofilms were compared to untreated controls (Table 1). After exposure to plasma, biofilms were incubated again with fresh nutrient broth at 37 °C. All nutrients were changed every 24 h until the final day (72 h).

2.3.2 | Indirect (liquid) treatment

Plasma-treated liquid was generated by treating 1 mL of PBS in the same setup as direct treatment, as indicated in Figure 1B. After treatment, 200 μL of the liquid was removed from the dish and transferred to another dish containing the biofilm, and subsequently incubated for 1 h prior to imaging.

TABLE 1 Design of regrowth experiment where U indicates untreated and T treated samples

Biofilm age (h)	Control	Treatment	
24	U	T24	
48	U	T48	T24+48
72	U	T24+48	T 24 T48

Commercial hydrogen peroxide (Chem-Supply Pty Ltd, South Australia, Australia) was employed for comparison to the plasma-treated liquid via addition to PBS. Similarly, 200 μL of these peroxide-PBS solutions were also incubated for 1 h with the biofilm prior to imaging.

2.4 | Confocal Laser Scanning Microscopy (CLSM)

Before imaging, the biofilm was dyed with Live/Dead BacLight™ Bacterial Viability Kits (Thermo Fisher Scientific, Victoria, Australia), which contains SYTO9 and Propidium Iodide (PI), following supplier's instructions. The dishes were then incubated in the dark for about 15 min before imaging. Biofilm imaging was performed on a Leica TCS SP5 STED inverted confocal microscope with oil objective 63 \times , NA 1.4. The lasers used for imaging were at 488 nm for SYTO9 and 498 nm for PI.

2.5 | Image analysis

All images were analyzed using Image-J.^[26] Green (alive) and red (dead) channels from CLSM data were separated and then analyzed individually to calculate biofilm coverage area. From the literature, it is known the approximate size of one *E. coli* cell is $1 \times 3 \mu\text{m}^2$.^[27] Assuming the cells are perfectly oval, the area of one *E. coli* cell is $2.35 \mu\text{m}^2$. Hence, any number that is less than this value is disregarded in the calculation. The percentage of red cells was calculated from the total area covered by red cells divided by the total area covered by both green and red cells. Each data set contains at least six fields of view that are used for data quantification.

2.6 | Hydrogen peroxide (H_2O_2) measurement

Quantification of H_2O_2 concentration in the plasma liquid was performed following the protocol of Pick and Keisari.^[28] Briefly, 5 g of horseradish peroxidase Type II (Sigma Aldrich, Sydney, Australia) powder was dissolved in 0.05 M phosphate buffer. Phenol red dye is used to detect color change due to the presence of H_2O_2 , using a concentration of 0.28 mM. Standard curves were then prepared by measuring spectra of milli-Q water containing various concentrations of H_2O_2 from 0 to 60 μM . The solution was taken out of the dish, transferred into a small glass vial, and incubated for 1 h before spectra measurement. Just before spectra measurement, 10 μL of the horseradish peroxidase solution and 10 μL of the phenol red solution were added into the standard samples and plasma-treated liquid. These vials were then incubated again at 25 °C for 5 min. After incubation, NaOH was added to the solution to change its color from orange to purple and keep the color stable.^[28] Spectra of samples at 610 nm were then recorded using a UV–VIS spectrophotometer (Shimadzu Corporation, Kyoto, Japan).

3 | RESULTS AND DISCUSSION

3.1 | The effect of plasma treatment on biofilm structure

Plasma treatment has been reported previously to destabilize biofilm structures.^[21] Here we use an *E. coli* biofilm that is in a younger state than the previously studied biofilms of *Pseudomonas aeruginosa* or *Staphylococcus aureus*.^[21] During this early stage of biofilm development, no microcolonies have been formed. Figure 2a shows the microscopic initial state of these young biofilms, with green live cells visible throughout the field of view at $t = 0\text{ s}$. Figure 2a also shows micrographs of the biofilm after different plasma exposure times, enabling tracking of the kinetic progression of cell death by following the increase in red, or dead, cells and the survival of the green, or living, cells and the formation of cell clumps. These effects are contrary to those reported by Ferrell et al.,^[21] with plasma treatment inducing aggregation and forming a new structure rather than structure breakdown. This plasma-induced structural re-arrangement has been observed previously in surface-deposited planktonic bacteria.^[29]

Figure 2a shows that cell aggregation occurred for all treatment times tested. However, quantitative analysis via cell imaging revealed that there was only a slight increase in the percentage of larger aggregates ($>10\text{ }\mu\text{m}^2$) as a function of treatment time (Figure 2b). An aggregate area cutoff value of $10\text{ }\mu\text{m}^2$ was chosen to differentiate aggregates from cells in sufficient proximity to be classified as an aggregate. An

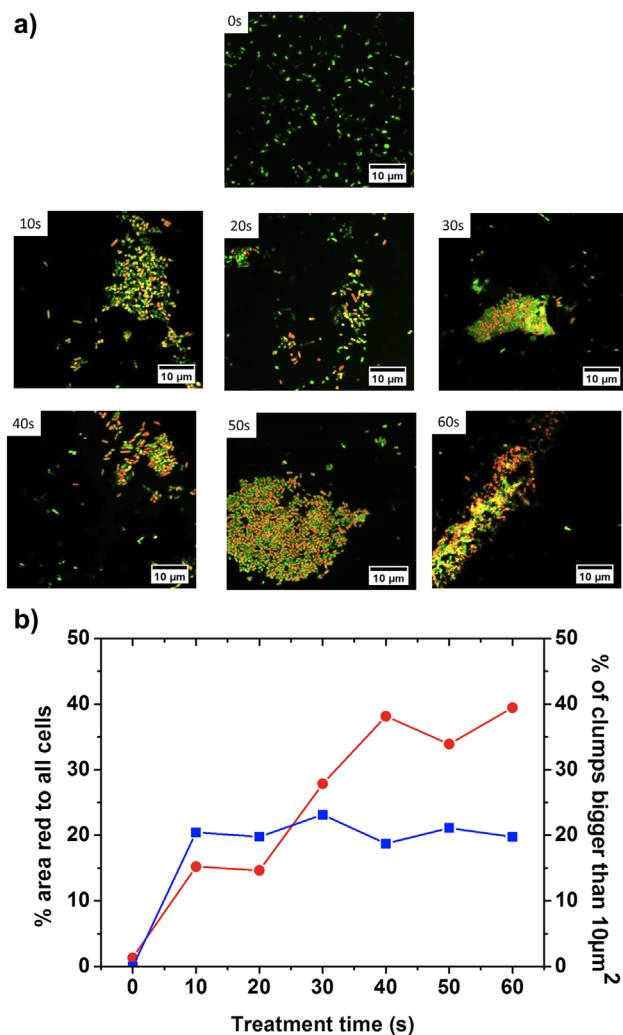


FIGURE 2 Effect of treatment time on biofilm structure a) Confocal images of biofilm structure before and after plasma treatment, b) quantification of dead cells (symbol ●) and cell clumps larger than $10\text{ }\mu\text{m}^2$ (symbol ■)

increase was only observed between the untreated and the shortest treatment time of 10 s (around 20% increase), indicating that cell aggregation occurs rapidly and is not significantly governed by treatment time.

Figure 2b shows that although plasma treatment causes cell aggregation, it also inactivates bacterial cells in the biofilm. This behavior has been observed in many studies that study the effect of treatment on bacterial viability.^[15–17] However, for the current system, it is found that after 40 s the number of dead cells reaches a plateau of 40%, Figure 2b. This indicates that there is a limit to the number of bacteria that can be killed with plasma treatment, perhaps because aggregation offers some form of protection.

Of particular interest is that the aggregation of the cells and the mortality effects of the plasma appear to both plateau, although on different time scales, after 40 s for cell viability and after 10 s for cell aggregation (Figure 2b).

The biofilms used in this study are considered mature once they are 48 h old, but we also examined the effects of biofilm age on aggregation and mortality response to plasma treatment. This is because the amount of EPS increases with biofilm age and it may play a role in protecting cells from plasma and aggregation induced by plasma.

When subjected to the same plasma treatment for 30 s, both biofilms aged 24 h and 48 h form aggregates (Figure 3a). The percentage of big aggregates formed in these two samples is quite similar, although the actual percentage of bigger

clumps is slightly higher for the treated younger biofilm. The older biofilm is expected to have more EPS, which might explain why there is a slight discrepancy between the two values. Aggregation requires both attractive interactions between cells and sufficient mobility to bring cells together for collision. The cells in the older biofilm might move less than the cells in younger ones, resulting in the current observation.

Figure 3 also shows that older biofilms have increased resistance to plasma treatment. In Figure 3c, the percentage of dead cells after treatment increased compared to the control. For biofilms aged 24 h, the percentage of dead cells increases from around 2–40% upon treatment. The efficacy of plasma decreases with increasing age of biofilm, as the percentage of dead cells only increases from 2 to 25% upon treatment, about half of the impact seen for biofilms aged 24 h.

3.2 | Regrowth of surviving bacteria

When plasma treatment does not inactivate all bacterial cells in a biofilm, the surviving cells may be able to grow and reproduce when given sufficient nutrients. Under these circumstances, we are interested in how these bacterial cells regrow in their restructured environment. To answer this question, both younger and more mature biofilms were exposed to plasma treatment and then regrown, until the biofilm reached an age of 72 h, before being imaged.

Biofilms that have been treated at least once after regrowth have distinct structures when compared to previously untreated biofilms with the same treatment. Figure 4a indicates that biofilms treated at least once during their growth have clearly aggregated structures compared to untreated biofilms that retain a fully dispersed structure. Indeed, after plasma treatment of biofilms either 24 or 48 h old, bacteria keep growing in the aggregates instead of growing separately as in the untreated samples. This indicates that the surviving bacteria are able to reproduce and grow in this newly formed structure.

Yet, these aggregated structures that occur after treatment at 24 and 48 h, or treated twice at 24 and 48 h old, are hardly distinguishable from each other. Quantitative analysis of the aggregates (Figure 4b) reveals that biofilms treated at 24 h have a higher percentage of aggregates larger than $10 \mu\text{m}^2$ than a biofilm treated at 48 h or treated at 24 and 48 h. This may be due to fact that biofilms treated at 24 h have more time to expand the size of their colonies as longer growth time increases cell cluster size.^[30]

In addition, as seen from Figure 4a, a plasma-treated biofilm consists of only living cells. Analysis shows that despite 30 s of plasma treatment causing cell death of a significant proportion of cells (Figure 4c), only a very small number (<10%) of dead cells could be detected after biofilm re-growth. However, it is likely that some dead cells are

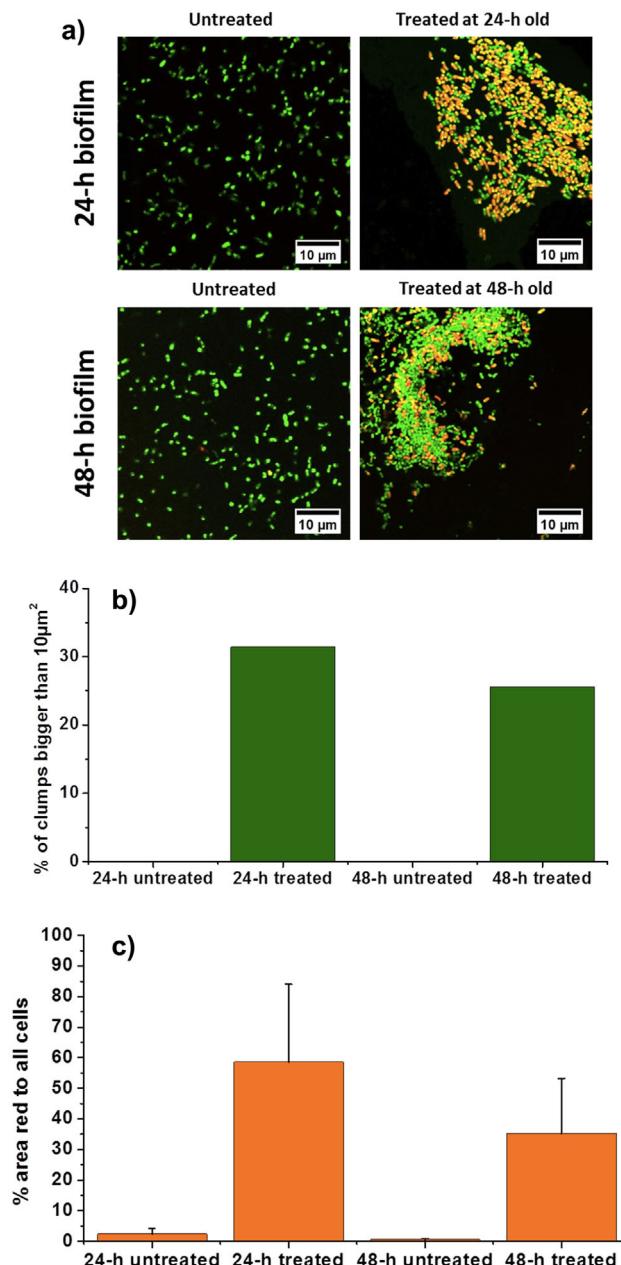


FIGURE 3 Effect of biofilm maturity on plasma clumping a) confocal images of 24-h and 48-h of untreated and plasma treated biofilm, b) percentage of clumps bigger than $20 \mu\text{m}^2$, c) quantification of red cells

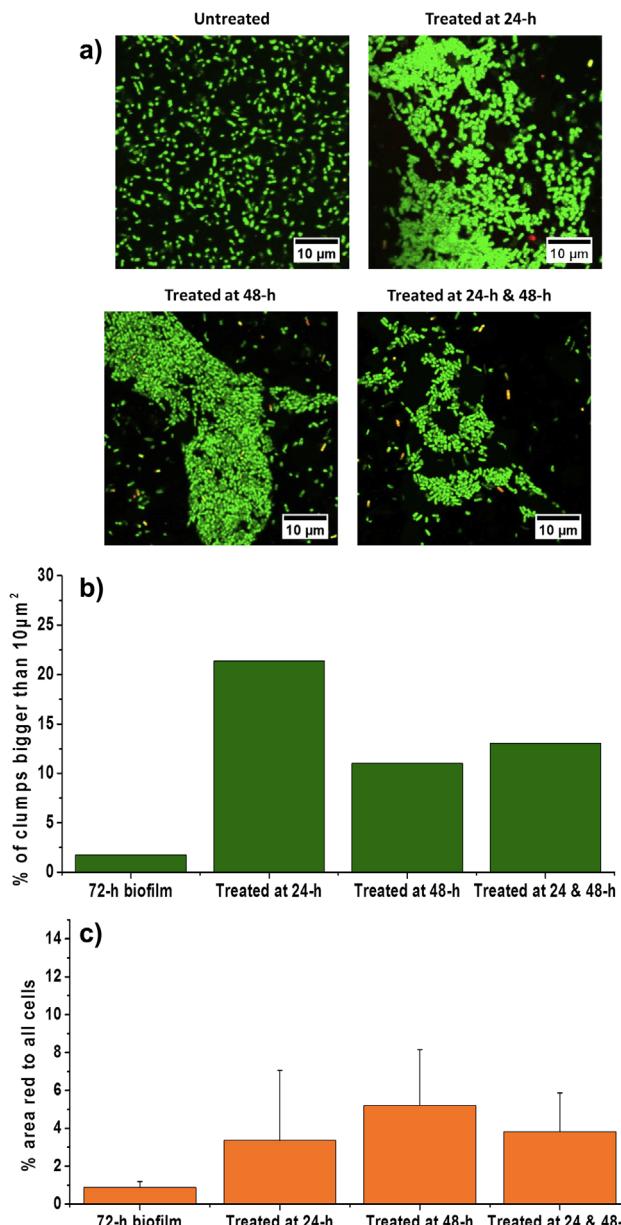


FIGURE 4 a) Confocal images of untreated 72-h biofilm and biofilm grown for 72-h but exposed to 30 s plasma treatment at different biofilm ages, where it is shown that biofilms retain their aggregated structure after those plasma treatments, b) percentage of clumps bigger than $10 \mu\text{m}^2$, c) quantification of red cells

hidden within the new structure. However, the percentage of these red cells is still quite low, less than 10%, which is not significant.

3.3 | The effect of plasma-induced biofilm structure on subsequent treatment

In section 3.2, it was found that after plasma treatment, bacteria in a biofilm can utilize the new structure to reproduce and grow. In previous work by Ferrell et al.,^[21] a

mature biofilm with large aggregates was shown to change structure by increasing the porosity of the biofilm structure. In this kind of mature biofilm, the high amount of EPS should prevent the aggregation of bacteria as this EPS provides elastic resistance to deformation by the flow. The plasma-treated biofilm has a structure more similar to the mature biofilm used by Ferrell et al.^[21] It is interesting to know if this plasma-mediated structure has a similar behavior to a mature biofilm.

To answer this, biofilms were exposed to plasma after 24 h of growth. This sample is incubated again for another 24 h before exposing this to the second plasma treatment. Figure 5a shows that clumping is still apparent in this system. However, quantitative analysis shows that the relative amount of aggregates decreases after the second plasma treatment instead of increasing. This observation agrees with Ferrell et al.'s^[21] work. This also indicates that after a certain point, aggregation is not possible anymore as biofilms might produce enough EPS to resist deformation by plasma. Another explanation is that subsequent plasma treatments can destroy structures formed by previous treatments.

Interestingly, Figure 5a also indicates that biofilms that have been previously treated mainly consist of live cells. This

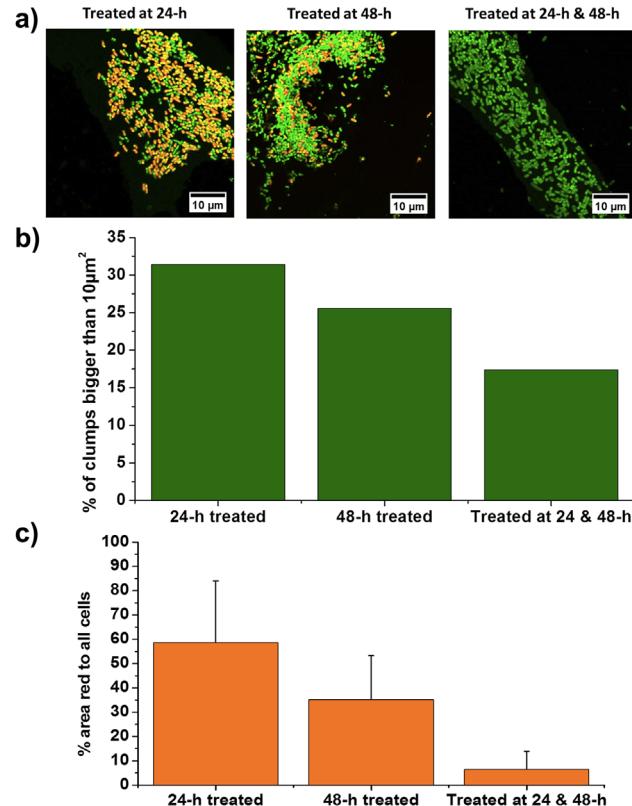


FIGURE 5 a) Confocal imaging of plasma-treated samples that were imaged right after treatment where less cells are killed after treated twice, b) the percentage of aggregates bigger than $10 \mu\text{m}^2$, c) quantification of dead cells

result is unexpected as when the sample is treated twice, it is likely that the percentage of red cells should be higher compared to 24 or 48 h old biofilms. As can be seen from Figure 5c, the percentage of dead cells in the sample treated both at 24 and 48 h is about 5% which is much lower than the percentage of cells inactivated by single treatment when they were 24 h (by six times) or 48 h old (by four times). This suggests that the bacteria developed resistance after the first treatment that reduced efficacy of the second treatment, consistent with other reports of resistant colonies induced by plasma treatment.^[16,31]

3.4 | The effect of plasma chemicals on biofilm structure

In the literature, the death of bacterial cells induced by plasma is usually associated with the presence of reactive species produced by plasma treatment. It is plausible that such chemicals could also cause clumping, as bacteria are known to respond to chemicals present via chemotaxis. Chemotaxis is the phenomenon by which motile cells move towards or away from a chemical by altering their swimming pattern. Bacteria such as *E. coli* have several flagella per cell which facilitate some directional control over their motion to either find favorable locations with high concentrations of attractants or to avoid repellents,^[32] such as chemicals produced by plasma. Although chemotaxis traditionally is known only for motile cells, recent finding shows that chemotaxis might also occur in surface-attached cells.^[33]

One of the chemicals often found in atmospheric plasma-treated liquid is H₂O₂.^[19,34] For this work only H₂O₂ is measured, for a more comprehensive species diagnostic of PAW using this power source, the reader is referred to our recent publications.^[25,35] Figure 6b indicates that the concentration of H₂O₂ in the liquid increases with increasing treatment time. This behavior has been seen in plasma-treated water previously, where initially the concentration of peroxide increases linearly before reaching a plateau.^[25]

If the aggregation observed previously is related to the presence of chemicals produced by plasma reactive species, we should be able to induce such aggregation by adding commercial H₂O₂, or plasma-treated water, to the biofilms and comparing the result to plasma-treated biofilms. The concentration of H₂O₂ added to the liquid is the same as the concentration of H₂O₂ in water treated in plasma for 60 s, which is 30 μM.

Figure 7a shows that biofilms that were exposed to plasma-treated liquid or 30 μM peroxide solutions are similar to the control. Data analysis (Figure 7b) reveals that there are actually changes in clumping after addition of peroxide or incubation with plasma water compared to control. Figure 7b also shows that compared to peroxide only, plasma water increases the extent of clumping by two times (from 3 to 6%), which might suggest that presence of other chemicals that also give rise to cell clumping. However, the change in clumping caused by chemicals (~6%) is not as much as the clumping caused by direct treatment (~20%). This suggests that aggregate formation might be slightly affected by chemicals present in plasma-treated water, but it is not the main mechanism. Movement of bacteria is also required for aggregation and is likely controlled by plasma discharge-induced flow.^[28]

Additionally, the use of hydrogen peroxide and plasma liquid here does not cause significant cell death. As shown in Figure 7b, the percentage of cells killed by treatment is very small, less than 2%. These values are similar to the levels in untreated biofilms. This means there is very little effect of plasma-treated water, which is not in agreement with literature as plasma-treated liquid has been shown to inactivate bacteria in biofilms.^[36,37] But, literature^[38,39] has indicated that in order for plasma-treated liquid to be effective in inactivating bacteria, acidified conditions are required. Naïtali et al.^[38] showed that in plasma-treated water, a bacterial population was reduced from eight log CFU to two log CFU. However, the effect was diminished for buffered plasma liquid where only a minimal reduction was observed. As all experiments here use a buffer solution,

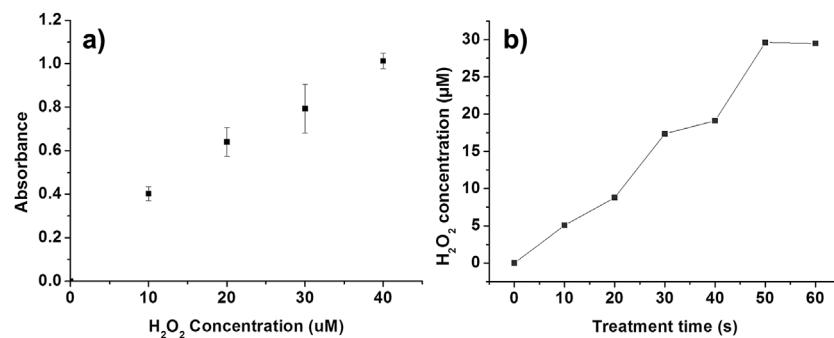


FIGURE 6 a) Calibration curve for H₂O₂ by spectrometer at 610 nm, b) The H₂O₂ concentration in plasmatreated liquid

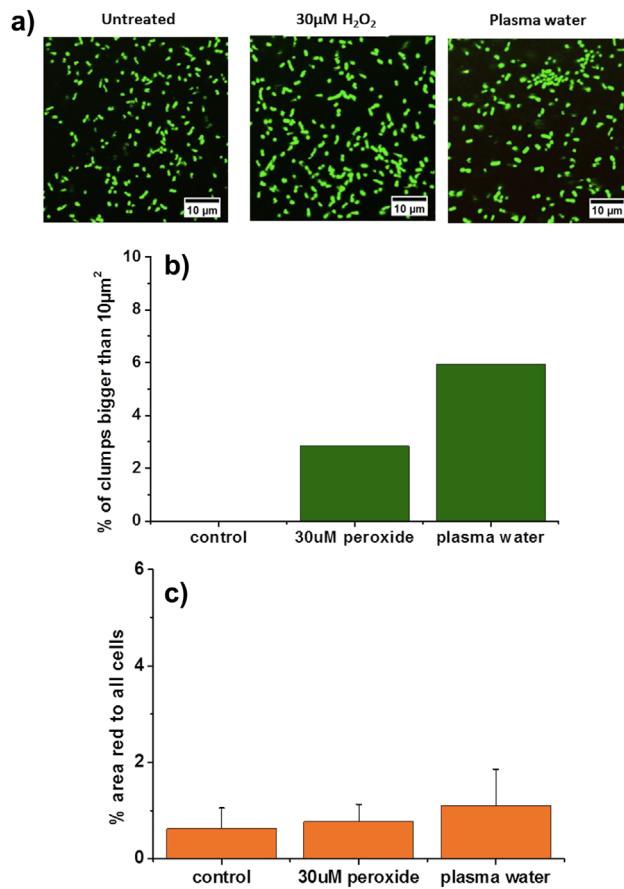


FIGURE 7 a) Confocal images of untreated biofilm, incubated with 30 μM H_2O_2 and incubated with plasma liquid treated for 60 s, b) percentage of aggregates bigger than $10 \mu\text{m}^2$, c) red cells quantification of samples

PBS, the pH of the solution is not expected to change and become acidified.

3.5 | Dilution effect on biofilm structure

As mentioned before, the formation of ring structure has been observed in surface deposited bacteria, which is said due to drying by plasma jet.^[29] This means that there is high possibility that the structure here is also caused by drying. To understand better the drying by our plasma system, we measured how much water removed when exposed to plasma.

Table 2 shows that for 30 s treatment time, plasma treatment removes between 0.04–0.06 g water from the system by evaporation regardless of the starting amount of water. From this result, it appears that there is a maximum amount of water that can be removed by plasma for the same treatment time. On the other hand, Table 2 also indicates that the percentage of water removed changes depending on the amount of initial liquid covering biofilm. In this case, the maximum of water removed is 32.9% for a biofilm covered with 200 μL of water (Table 2). Additionally, this suggests that

TABLE 2 The amount of water removed by plasma treatment

Amount of water in dish (μL)	Amount of water removed (g)	Percentage of water removed (%)
200	0.064 ± 0.024	32.9 ± 2.7
400	0.053 ± 0.007	13.3 ± 1.7
600	0.044 ± 0.011	7.4 ± 1.9
800	0.060 ± 0.019	7.5 ± 2.3
1000	0.051 ± 0.013	5.1 ± 1.3

after plasma treatment for 30 s, biofilms will not completely dry out. Thus, from this observation, it is therefore likely that larger volumes of water could reduce the drying and convective effects of plasma treatment in a specified treatment time. Interestingly, we have observed that biofilms that were completely dried in an oven overnight have a similar structure to these plasma-treated samples (data not shown).

The above experiments were repeated with biofilms present in varying amounts of water and a constant plasma exposure time of 30 s. Figure 8 summarizes the results obtained from this experiment. It is clear that biofilms can aggregate in liquid volumes up to 600 μL . However, when biofilms are in larger liquid volumes ($>600 \mu\text{L}$) no aggregation was observed, presumably due to a protective effect from the liquid against drying.

Figure 8b also indicates that aggregation and cell death was steadily reduced with increasing amounts of liquid. Increasing the amount of water by 200 μL lowers the percentage of dead cells and also reduces the extent of clumping by around 10%. For biofilms that are covered by 800 and 1000 μL , the clumping effect and amount of cell death is very small. This confirms the hypothesis that extra liquid protects biofilms during plasma treatment and reduces the drying effect imposed by plasma discharge. Although plasma drying is not mentioned much in the literature as a mechanism of plasma inactivation, it is an important factor governing cell death. Due to this, the effect of plasma drying during treatment has to be taken into account when treating bacteria or biofilms, as this effect is apparent even when biofilms are treated for very short times.

3.6 | Explanation of structure formation

Our results from the previous section indicate that the structure generated by plasma treatment is mainly due to a drying effect. There is a difference in the convection produced by plasma and standard oven, as Figure 10a & b indicates treatment with a conventional oven at 50 °C (average temperature of cold plasma) for the same time scale (30 or 60 s) could not cause the same effect of aggregation. In addition, as can be seen from Figure 10c, even prolonged dehydration for 90 s using the oven could not cause the same

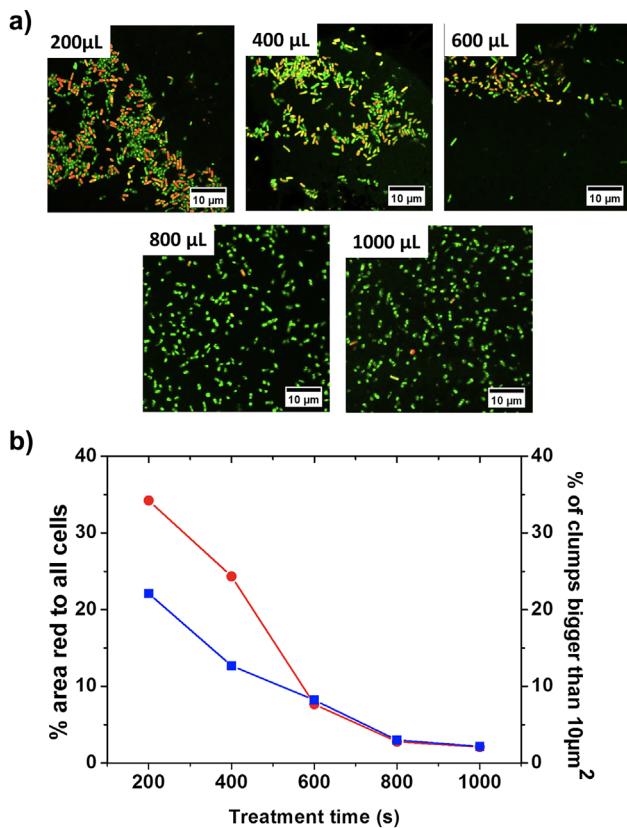


FIGURE 8 The effect of liquid volume covering biofilm during plasma treatment on aggregation and cell death a) confocal images of different structures observed, b) quantification of dead cells (symbol ●) and cell clumps larger than $10 \mu\text{m}^2$ (symbol □)

clumping effect as plasma treatment, although there is indication of some cell death.

The circular pattern observed in Figure 9a resembles Benard cells, hexagonally-ordered structures that spontaneously form in fluids with a convection flow during heating or evaporation.^[40] The length scale of this structure is on the order of μm and is similar to structures formed by surface-deposited bacteria,^[29] as depicted in Figure 9c.

Deegan et al.^[41] showed that various patterns can be created by changing the conditions of evaporation. Apart from the formation of Benard cells where the deposit forms a ring, Deegan et al.^[41] also observed the formation of compact structures as we observed in our biofilm (Figure 9b). As biofilms are known to have a heterogeneous spatial structure, the plasma jets are also generally heterogeneous in their effects on targets, resulting in the two distinct structures observed. Fischer^[42] reported the formation of such ring structures only occurs when there is outward flow to replenish liquid evaporating from the edges.

The fact that there is a limit of maximum liquid coverage of biofilms for significant convective effects may be related to the conditions required for Benard cell formation in thin films, namely that the thickness be less than 1 mm.^[43] In our experiments, water mainly covered the inner area of the FluoroDish™, which has an overall diameter of 23.5 mm. Assuming that liquid covers the inner area uniformly and the area is in a cylindrical shape, the volume of liquid added to each system allows us to calculate the height of liquid covering the biofilm. It was found that only biofilm containing 200 and 400 μL liquid is covered by water layer which is less than 1 mm thick. This agrees with the finding that aggregation of cells is more apparent in those samples.

Drying of 200 μL water for 30 s by oven only removed $1.6 \pm 0.25\%$ water, which is around 20 times lower than drying the same amount of water by plasma (Table 2). Probstein^[43] also indicates that for thin films around 0.5–1 mm deep, the cell spacing should be around three times the liquid depth. The difference between the two might relate to the different rate of drying of plasma, oven or natural convection. In addition, the fact that biofilms have polymeric gels that encapsulate them might reduce the rate of bacterial cell migration during drying, hence smaller size structures were observed.

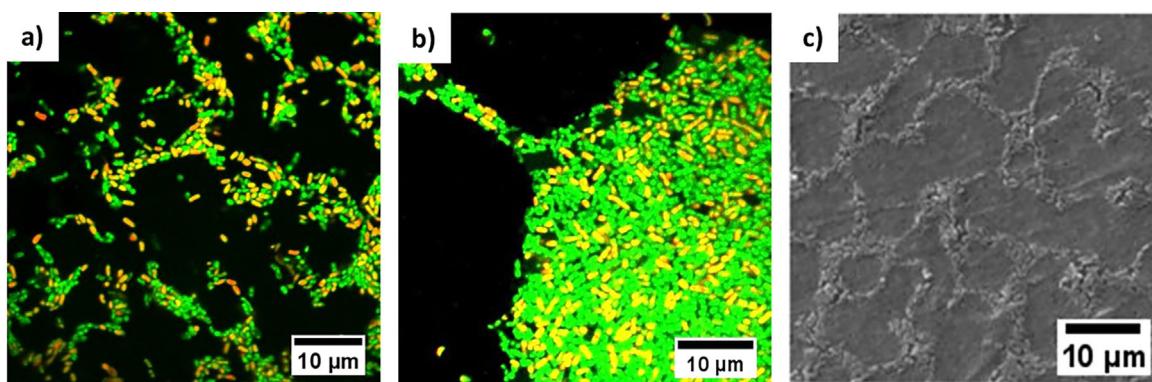


FIGURE 9 a) Circular pattern ring structure formed by bacteria after plasma treatment, b) Compact structure formed by bacteria after plasma treatment, c) pattern rings formed by surface-deposited bacteria after plasma treatment as reported by Bayliss et al.^[29]

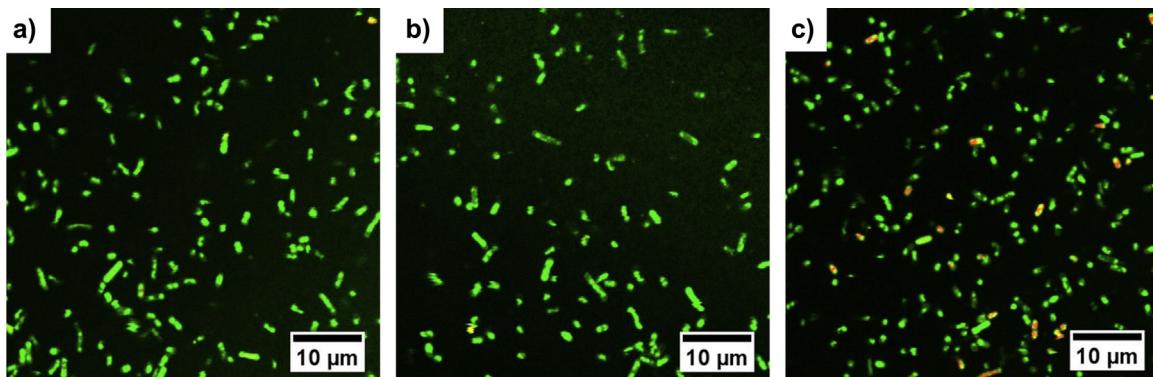


FIGURE 10 The drying effect on structure of biofilm by oven at 50 °C for different treatment time. a) treated for 30 s, b) treated for 60 s, c) treated for 90 s

4 | CONCLUSIONS

Plasma can be an effective treatment for biofilm eradication. However, this study found that plasma can also induce new structures within the biofilm, which can persist after treatment during regrowth. This phenomenon was evident for both young and more mature biofilms. Once such structures form, subsequent treatments are less effective in terms of efficacy, likely due to the surviving bacteria becoming increasingly resistant to plasma. The structures induced for the biofilms tested are similar to those observed previously for plasma-treated surface-deposited bacteria.^[29] The observed structures are reminiscent of Benard cells, whose main mechanism of formation is convection. Secondary plasma species formed in the liquid phase were not found to induce the formation of such structures.

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