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Inhibition of corrosion causing *Pseudomonas aeruginosa* using plasma activated water

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Running headline: Plasma activated water against microbial corrosion

Abstract

Aims: The cost of Microbiologically Influenced Corrosion (MIC) significantly affects a wide range of sectors. This study aims to assess the efficiency of a novel technology based on the use of plasma activated water (PAW) in inhibiting corrosion caused by bacteria.

Methods and Results: This study evaluated the effectiveness of PAW, produced by a plasma bubble reactor, in reducing corrosion causing *Pseudomonas aeruginosa* planktonic cells in tap water and biofilms grown onto stainless steel (SS) coupons. Planktonic cells and biofilms were treated with PAW at different discharge frequencies (500-1500 Hz) and exposure times (0-20 min). *P. aeruginosa* cells in tap water were significantly reduced after treatment, with higher exposure times and discharge frequencies achieving higher reductions. Also, PAW treatment led to a gradual reduction for young and mature biofilms, achieving >4-Log reductions after 20 min. Results were also used to develop two predictive inactivation models.

Conclusions: This work presents evidence that PAW can be used to inactivate both planktonic cells and biofilms of *P. aeruginosa*. Experimental and theoretical results also demonstrate that reduction is dependent on discharge frequency and exposure time.

Significance and Impact of the Study: This work demonstrates the potential of using PAW as means to control MIC.

KEYWORDS: plasma activated water, biofilm, intracellular ATP levels, microbial corrosion, *P. aeruginosa*.

INTRODUCTION

Metal corrosion can be significantly accelerated by the presence and activity of microorganisms, a process that is also known as biocorrosion or Microbiologically Influenced Corrosion (MIC) (Phan et al. 2021) and is responsible for 20% of metal corrosion damages (Flemming, 1996). The cost of corrosion in industrialised countries was estimated to be 3.4% of the global GDP in 2013, and if corrosion protection and design were properly applied, a 15-35% loss reduction could be achieved (Koch et al. 2016). Its direct costs affect a wide range of sectors, including agriculture, forestry and fishing, mining, manufacturing (e.g., chemical processing, nuclear, oil, gas, underground pipeline, water treatment, food production, highway maintenance, aviation, metal working, marine, shipping and fire protection), electricity supply, water supply, waste management, accommodation, and food service activities, transportation, and storage. Indirect costs are associated with environmental, regulatory, and human costs, making cost estimation even more challenging (Little et al. 2020).

Until recently, corrosion research was primarily focused on operational aspects such as the assessment of the corrosion damage and mineral deposits impact on the functionality of systems, mechanical operations of equipment and unanticipated failures (Bardal, 2004). Those are more frequently encountered in systems comprised of widely used, cost-effective, but less resistant groups of metal alloys such as carbon steels (Herrera and Videla, 2009) and stainless steels (Cheng et al. 2009). The extent of those damages and mineral deposits is influenced by both materials and the environment (Lyon, 2014).

Lately, more fundamental research was conducted aiming to investigate MIC associated phenomena and advised to approach the subject systematically by monitoring the microbial activity of single bacteria (Kermani and Harrop, 2019). Different types of aerobic and anaerobic bacteria are associated with MIC; among them, the most commonly met are the acid-producing, sulphur reducing and iron related bacteria (Su and Fuller, 2014) as well as others, such as *Pseudomonas aeruginosa* (Li et al. 2016; Jia et al. 2017). *P. aeruginosa* MIC mechanism and associated bacteria-metal reactions have been extensively investigated, and its presence is proven to accelerate corrosion (Abdolahi et al. 2014). There are several studies that confirm its involvement in the corrosion process of different types of metals e.g., mild steel (Xu et al. 2017), stainless steel (Xu et al. 2016; Jia et al. 2017; Xu et al. 2017) and aluminium (Xu et al. 2017).

Research in MIC is spanning four main areas: diagnose, monitor, modelling and prevention. To achieve the latter, numerous treatments are customarily used, and the most common ones include

sanitation, physical, chemical treatment, biological methods, use of coatings and cathodic protection (Enning and Garrelfs, 2014; Ibrahim et al. 2018; Little et al. 2020). The solution of applying coating specifically designed for each application appears to be promising, however this is not always feasible and practical. An efficient way to limit microbial growth is through physical treatment (e.g. pigging, ultraviolet irradiation, ultrasonic, chemical and biological treatments) of natural or industrial water aiming to inhibit the microorganisms responsible for causing corrosion (Little et al. 2020). Recent studies have revealed that indirect application of Cold Atmospheric Plasma (CAP) at ambient air conditions can significantly decrease the microbial load (Pasquali et al. 2016; Katsigiannis et al. 2021). Also, in recent years, a new mode of CAP, i.e., Plasma-activated Water (PAW) activities has drawn attention (Julák et al. 2012; Chen et al. 2018) as it can be applied to inhibit/kill microorganisms in surfaces and systems in an environmentally friendly, cost-effective, and practical way. Lately, the application of PAW has been investigated (Tan and Karwe, 2021) and has been proven an effective technique for inactivating inner-pipe surfaces formed biofilms, also recognised as a novel technology with significantly decreased environmental cost (Zhou et al. 2020).

Despite a large number of recent publications related to MIC, an effective and practical technological solution to tackle MIC related problems is yet to be found (Little et al. 2020). To meet this challenge, the current experimental work aims to assess the efficiency and antimicrobial mechanism of PAW against corrosion causing *P. aeruginosa* as well as identify optimal operational conditions for PAW treatment.

MATERIALS AND METHODS

Bacterial cultivation

Experiments were conducted using PAO1, a *P. aeruginosa* strain. The *P. aeruginosa* culture was prepared in Luria-Bertani (LB) liquid medium enriched with KNO_3 (LB-NO_3). The media formulation of the agar medium LB-NO_3 included 10 g tryptone, 10 g KNO_3 5 g yeast extract, and 5 g NaCl per litre of deionised water. KNO3 was added to the LB medium to support anoxic growth of *P. aeruginosa* PAO1 (Line et al. 2014). The pH was adjusted to 7.0 by applying a solution of NaOH. To mitigate any possible O_2 entry, L-cysteine at a concentration of one hundred ppm (w/w), was added to the culture medium as O_2 scavenger. The L-cysteine solution was filter-sterilised using MF-Millipore^{TM} membrane filter of a pore size of 0.22 μm (Merck KGaA, Darmstadt, Germany) before it was added to the medium. Dissolved oxygen was removed from all liquid solutions by flushing them with filter sterilised N_2 in order to maintain anoxic growth of *P.*

aeruginosa. 304 Stainless Steel (SS) sheet were used to cut the rectangular shaped coupons (1 cm x 1 cm) used in this work. Coupons were sterilised in 75% ethanol solution for 2 h, dried and exposed to UV light for 30 min prior to use. A N_2 chamber was used for anaerobic manipulations. The biofilms were grown in anaerobic vials, following the procedure described by Jia et al. (2017). One hundred ml of LB-NO_3 medium, with and without the addition of the *P. aeruginosa* culture, was added to each anaerobic vial. The experiments were conducted on three replicate coupons. The initial bacterial concentration after inoculation with *P. aeruginosa* was approximately 10^{4-5} Log CFU ml^{-1}. The vials were closed and incubated at 37°C for 2, 5, or 7 days. After the incubation, the coupons were removed, and PAW treated under different conditions as described below. Before PAW treatment, the coupons were gently rinsed with sterile phosphate-buffered saline to remove the non-adhered and loosely attached bacterial cells.

Plasma bubble reactor

A schematic representation of the plasma bubble reactor (PBR) used to generate the PAW is illustrated in Figure 1. The PBR consisted of an acrylic tube with a 140 mm length that constituted of machined caps at each end. Those caps had 4 mm stainless-steel rod positioned coaxially at the interior of the full length of the tube, acting as high voltage electrodes. A 5 mm wide strip of adhesive copper tape was positioned at the exterior of the ground electrode and was connected to the ground wire of the plasma power supply. Plasma was generated under atmospheric conditions, and the electrical discharges were provided by a high voltage power supply (PlasmaLeap Technologies, Ireland) specifically designed to supply a wide range of voltages at discharge frequencies.

The acrylic tube of the PBR, submerged into the water, was perforated with ten 2 mm holes located 8 mm from its base. Compressed air to the PBR was supplied at a flow rate of 1 l min^{-1} via a tube placed at the opposing end of the acrylic tube. The electrically discharged bubbles leaving the reactor, through the holes entered the water, and subsequently, the generated reactive species at their interior, contacted the water via their bubble surface-water interface.

PAW treatment of stainless-steel (SS) coupons and biofilm enumeration

The plasma reactor was filled with 100 ml of sterile tap water. Control SS coupons (no PAW treatment) were immersed in water and placed on the bottom of the reactor, with just the airflow on, for 15 min without igniting the plasma. For treated samples, coupons were placed in the reactor and subsequently the PBR was turned on. The reactor was allowed to run for different times (5, 10, 15, and 20 min), at 150 V (voltage applied at the high voltage transformer), 100-µs

duty cycle (time during which the energy from the power supply unit is transferred to HV transformer resonance circuit) and 60 kHz resonance frequency (resonance frequency of resonance circuit). Three different discharge frequencies were investigated (500, 1000, and 1500 Hz).

SS coupons with adhered biofilm cells of different maturity were treated directly in PBR for 0, 5, 10, 15, and 20 min. After treatment, the SS coupons were positioned in a sterile container with 10 mL maximum recovery diluent (MRD) (Oxoid, UK) and 1 g glass beads caliber of 0.1 mm diameter. To detach the surviving biofilm cells from the SS coupons, samples were vortexed for 60 s. Subsequently, 10-fold dilutions were prepared in MRD to enumerate the surviving biofilm cells. An aliquot of 100-µl was used from the appropriate 10-fold serial dilutions and was spread plated on Tryptone Soya Agar (TSA, Oxoid, UK). Plates were incubated at 37 °C for 24 h and the biofilm cells expressed as Log CFU cm^{-2}.

PAW treatment and enumeration of *P. aeruginosa*

P. aeruginosa can exist in the water before it attaches to surfaces and begin to form biofilms and subsequently influence the physicochemical metal-environment interactions, thus enhancing MIC (Li et al. 2016; Jia et al. 2017). To investigate if PAW can inactivate this microorganisms in its planktonic form, 100-µl of the *P. aeruginosa* (prepared as described above) was inoculated into 100 ml of tap water. After inoculation, the bacterial concentration was approximately 10^5 Log CFU ml^{-1}. Control samples received no treatment (0 min; only airflow was on for 15 min without igniting the plasma). For the rest of the PAW treatments, the PBR was turned on and allowed to run for different time periods (0, 5, and 10 min) at 150 V, 100-µs duty cycle, 60 kHz resonance frequency and 500, 1000, and 1500 Hz discharge frequencies. The planktonic cells were treated inside the reactor. Immediately after treatment, an aliquot (1 ml) of the treated tap water was added to 9 ml of MRD and subsequently, further suitable 10-fold dilutions were prepared. An aliquot of 100-µl was used from the appropriate 10-fold serial dilutions and was spread plated on Trypticase Soy Agar (TSA, Oxoid, UK). Plates were incubated at 37 °C for 24 h, and planktonic *P. aeruginosa* cells were expressed as Log CFU ml^{-1}.

Physicochemical properties of PAW

PAW was generated at a discharge frequency of 1500 Hz for 15 minutes, and the measurements of pH, electrical conductivity (EC), H_2O_2, NO_2^-, and NO_3^- concentrations were taken immediately after treatment. Values of EC and pH were measured using an electronic conductivity meter (Jenway 4200, UK) and a pH meter (FiveEasy, Mettler Toledo, UK), respectively. H_2O_2 concentration was determined using the titanium oxysulphate ($TiOSO_4$; Sigma-Aldrich) method.

According to that method, $TiOSO_4$ reacts with H_2O_2, and produces a yellow-coloured complex (pertitanic acid). Subsequently, the H_2O_2 concentration was determined spectrophotometrically (Mai-Prochnow et al. 2021b). Nitrite concentration was determined by using the Griess assay, a chemical rection that uses N-(1-naphthyl)-ethylene diamine hydrochloride under sulfanilic acidic conditions, resulting in the formation of a magenta-coloured azo dye. Nitrate (NO_3^-) concentration was determined using a nitrate assay kit (Sigma, UK) that is based on its reaction with 2, 6-dimethylphenol (DMP). To eliminate nitrite interference, all PAW samples were pre-treated using sulfamic acid (Zhao et al. 2020) prior to analysis.

Individual standard curves of known H_2O_2, $NaNO_2$ and $NaNO_3$ concentration were prepared to convert the absorbance to H_2O_2, NO_2^-, NO_3^-, concentrations.

Membrane integrity

Protein leakage was used to assess membrane integrity of *P. aeruginosa* suspension cells after PAW treatment at 500, 1000, and 1500 Hz discharge frequencies. This was achieved by measuring protein concentration in the supernatants using a Pierce BCA protein kit (ThermoScientific, U.K.) (Sadiq et al. 2017) and using untreated samples as controls.

Intracellular adenosine triphosphate levels

The effect of PAW treatment on the intracellular adenosine triphosphate (ATP) levels was determined using the procedure described by Stratakos et al. (2018). The 24 hours old *P. aeruginosa* cultures were centrifuged at 5000 x g for 5 min. The produced pellets were washed three times using phosphate-buffered saline, and centrifugation was used to collect the cells. The pellets were re-suspended in one millilitre of PAW production at PBR discharge frequencies of 500, 1000, or 1500 Hz respectively for 15 min. Subsequently, all samples were stored for 15 min at 37^{o}C. Cells were centrifuged at 5000 x g for 5 min and treated with a lysis buffer (Roche, U.K.) for another 5 min at room temperature to extract the intracellular ATP. The intracellular ATP quantity was determined with the ATP assay kit (ATP bioluminescence assay kit HS II, Roche, U.K.); 100 ml of ATP luciferase reagent were added to the100 ml of supernatant in solid white 96-well plates. Then to determine ATP concentrations a microplate reader (FLUOstar Omega, BMG Labtech, U.K.) was used using untreated samples as controls.

Statistical analysis and modelling

All treatments were performed three times, and each sample was analyzed twice and averaged before statistical analysis. Statistical analysis was done with Excel Microsoft® Office 365 (ver. 16.48). Tukey post hoc tests were used to compare sample data using the IBM® SPSS® statistics

26 software for macOS (SPSS Inc., US). A 5.0 % level of significance, *P*, was used, and thus results were considered statistically significant when *P* was less than 0.05 ($P < 0.05$). The fitting procedure for modelling of inactivation kinetics was performed using GInaFiT software (Geeraerd et al. 2005).

RESULTS

The efficacy of PAW treatment against planktonic cells of *P. aeruginosa*

The effect of PAW treatment on planktonic *P. aeruginosa* cells after 5, 10, and 15 min exposure to different PBR discharge frequencies (500, 1000, and 1500 Hz) is presented in Figure 2. The initial population of *P. aeruginosa* control cells without plasma treatment in tap water was 4.99 Log CFU ml^{-1}.

The population of *P. aeruginosa* in tap water was significantly reduced after 5 min of PAW treatment and reached 0.44, 1.77, and 2.51 Log CFU ml^{-1} at 500, 1000 and 1500 Hz, PBR discharge frequencies respectively (Fig 2, $P < 0.05$). *P. aeruginosa* cells, treated with PAW for 10 min resulted in a significant reduction of the cell numbers by 1.26 and 2.88 Log CFU ml^{-1} at 500 and 1000 Hz, respectively (Fig 2, $P < 0.05$). However, PAW treatment at 1500 Hz resulted in a reduction below the detection limit (>3.99 Log CFU ml^{-1}) (Fig 2). After 15 min of PAW treatment at 1000 and 1500 Hz, the bacterial numbers of *P. aeruginosa* were reduced below the detection limit, while PAW treatment at 500 Hz resulted in a significant reduction, of 1.69 Log CFU ml^{-1} (Fig 2, $P < 0.05$).

The reduction of *P. aeruginosa* planktonic cells under the investigated discharge frequencies of PAW treatment depended on the exposure time. As shown in Fig 2, all the PAW treatments resulted in a significant decrease in the number of the planktonic cells after 5- and 10-min exposure (Fig 2, $P < 0.05$). In the case of *P. aeruginosa* cells treated at 500 Hz, significant bacterial reduction was observed for all the exposure times investigated. There were no significant differences between the effect of plasma treatments at 1000 and 1500 Hz (neither at 10 nor 15 min) when bacterial numbers were reduced close to or below the detection limit (Fig 2, $P > 0.05$).

The efficacy of PAW treatment against biofilms of *P. aeruginosa*

P. aeruginosa can form biofilms on the surface of stainless-steel pipes, accelerating MIC (Jia et al. 2017). This study investigated whether PAW treatment can be applied to reduce the more resistant *P. aeruginosa* biofilms. To assess the efficacy of PAW treatment, further experiments were carried out using the most effective PBR discharge frequency of 1500 Hz against the biofilms of *P. aeruginosa* grown on stainless-steel coupons for 24, 48, and 72 h. Reduction levels of *P.*

aeruginosa biofilms are presented in Figure 3. Plasma discharge in bubbles for 5 min reduced the 24, 48, and 72 h attached biofilm cells of *P. aeruginosa* by 2.77, 2.20, and 1.42 Log CFU $cm^{2\ -1}$, respectively (Fig 3, $P < 0.05$). The same trend was observed after 10 min of PAW treatment with increased efficacy, where bacterial numbers for biofilms grown for 24, 48, and 72 h were reduced significantly by 3.77, 3.12, and 2.55 Log CFU $cm^{2\ -1}$, respectively (Fig 3, $P < 0.05$). After 15 min of exposure, the 24 h old biofilms were reduced by >4.00 Log CFU cm^{-2}, below the detection limit of 2.00 Log CFU cm^{-2} (Fig 3). However, *P. aeruginosa* 48 and 72 h mature biofilms decreased below the detection limit (2.00 Log CFU $cm^{2\ -1}$) after 20 min of exposure at PAW treatment, corresponding to >4.15 and >4.24 log reduction of CFUcm^{-2} (Fig 3).

There was a significant reduction in bacteria population for the 24 h biofilms, exposed for 5 and 10 min (Fig 3, $P < 0.05$). In addition, *P. aeruginosa* 48 and 72 h mature biofilms showed significant differences in reductions achieved at all the PAW exposure times (Fig 3, $P < 0.05$).

Physicochemical properties of PAW treatment

The PBR that was used to generate PAW at 1500 Hz discharge frequency and 15 min of exposure time produced different reactive species. The physicochemical properties of PAW treatment are shown in Table 1. The initial pH value of the tap water was 7.56±0.024, which decreased to 5.94±0.038 after 15 min exposure ($P < 0.05$). Also, the EC value of the water significantly increased ($P < 0.05$), which suggests the formation of reactive molecular species under these conditions. The specific reactive species, produced as the result of complex chemical reactions between the plasma and liquid (tap water), that were detected and quantified were H_2O_2 (0.028±0.002 mg ml^{-1}), NO^{2-} (0.037± 0.001 mg ml^{-1}), and NO^{3-} (0.039± 0.001 mg ml^{-1}).

Effect of PAW treatment on *P. aeruginosa* protein release

Data in Figure 4 show the amount of protein released from *P. aeruginosa* planktonic cells treated with PAW at 500, 1000, and 1500 Hz of PBR discharge frequency. Protein leakage from PAW-treated *P. aeruginosa* cells at 1000 and 1500 Hz significantly increased compared to that from untreated cells (control) and from the cells treated at 500 Hz (Fig 4, $P < 0.05$). The highest release of proteins for the PAW-treated *P. aeruginosa* planktonic cells was observed at 1000 and 1500 Hz, respectively. The data suggest that there was a gradual increase in protein release in the cell suspension with increase in discharge frequency.

Effect of PAW treatment on *P. aeruginosa* intracellular ATP levels

Results on the effect of PAW treatment on *P. aeruginosa* Intracellular ATP levels at different discharge frequencies are presented in Figure 5. The ATP calibration curve showed a positive

linear relationship between relative luminescence units and ATP level, which can be described by the following equation: $y = 475926x + 22826$; $R^2 = 0.986$. The level of intracellular ATP of *P. aeruginosa* decreased significantly as the discharge level of PAW treatment increased from 500 to 1000 and 1500 Hz (Fig 5, $P < 0.05$). The initial ATP concentration of the untreated (Control) *P. aeruginosa* cells was 0.340 mM, while after PAW treatment at 500, 1000, and 1500 Hz, it was reduced to 0.164, 0.073, and 0.042 mM, respectively ($P < 0.05$).

Modelling of inactivation kinetics

The responses of the different parameters in the microbial population of *P. aeruginosa* to different exposure times were fitted using a log-linear regression model (Bigelow and Etsy, 1920). Analysis of the experimental dataset of the microbial population of *P. aeruginosa* was performed using a weighted least square linear fit model. The Coefficient of Determination (COD), also known as R^2, was used as a statistical measure to assess the quality of each linear regression fit. The linear regression model, Equation (1), was used to estimate the kinetic inactivation parameter, D_T, for the different PBR discharge frequencies for the planktonic *P. aeruginosa* cells and the young, 24 h old, and mature, 48 and 72 h old, biofilms. In the Equation (1), N represents the microbial population at time t, and N_o is the experimentally determined initial microbial population. Microbial population measurements below the level of detection (D) were handled using a substitution method (Ogden, 2010). According to the substitution method every result below D, also known as censored data, is substituted with an estimate, for what it might be. Traditional options for handling censored data include discarding non-detect data and using simple substitution methods (treating non-detects as zero, half the detection limit, at the detection limit, or the detection limit/√2) (Levine et al. 2009; EPA, 2006). Instead of eliminating censored data from the data set, setting them as zero or D values, that has been proved to yield erroneous results (Silvestri et al. 2017), in the current study values bellow D, were substituted with $D/2$ (Levine, 2006; Ogden, 2010).

Tables 2 and 3 present the model parameters and their measures of statistical dispersion namely the standard error (SE), mean sum of R^2, root mean sum of R^2, R^2 and adjusted R^2 for biofilms of different maturity and PBR discharge frequencies. Figure 6 shows the relevant fitted inactivation curves for biofilms of *P. aeruginosa* grown for 24, 48, and 72 h and treated with PAW at a discharge frequency of 1500 Hz. The developed model can be reliably used to describe the inactivation curves. Good statistical fit was observed for biofilms of different maturity with estimated adjusted R^2 values ranging from 0.9005 to 0.9445. The calculated values for the

inactivation parameter D_T for each biofilm ranged from 3.11 for the 24 h old biofilms to 4.34 for the 48 h old biofilms. The relevant model parameters and associated errors for *P. aeruginosa* planktonic cells after PAW treatment at different PBR discharge frequencies are presented in Table 3. Figure 7 shows the inactivation curves for the different exposure times. The inactivation parameter D_T tends to decrease with the increase of PBR discharge frequencies, with the values ranging from 8.53 at 500 Hz to 2.24 at 1500 Hz. The accuracy of the developed model is supported by the statistical parameters and evaluation of the fitting curves (Fig 7).

$$logN = logN_o - \frac{t}{D_T} \quad (1)$$

DISCUSSION

MIC is a destructive phenomenon that affects stainless-steel surfaces, which are widely used by a range of industries, including the companies producing material for various equipment or building purposes. This study proposes an innovative approach to tackle the issue of MIC. The approach is based on the application of PAW against *P. aeruginosa*, which is known to cause corrosion on stainless-steel surfaces (Gabriel et al. 2016). Although, corrosion inhibition itself from *P. aeruginosa* was not assessed, this work presents the first evidence that PAW is effective in inactivating both planktonic cells and biofilms of *P. aeruginosa* grown under anaerobic conditions. Furthermore, the results of this study were rationalized by means of two predictive inactivation models.

During the last decade, cold plasma treatment has been attracting attention as a green technology for the inactivation of spoilage and pathogenic bacteria on different matrices (Ehlbeck et al. 2010; Pasquali et al. 2016; Jayathunge et al. 2019; Ekonomou and Boziaris, 2021), as well as a method for decontamination of industrial surfaces and medical equipment (Ben Belgacem et al. 2017; Alshraiedeh et al. 2020; Gonzalez-Gonzalez et al. 2021). Recently, the development of PAW offered a new opportunity as a promising decontamination method for the preservation of food (Ma et al. 2015; Liao et al. 2018; Thirumdas et al. 2018) and wound healing in the medical industry (Chen et al. 2017; Kaushik et a., 2017), and as an alternative disinfectant for the inactivation of bacteria in water (Pan et al. 2017; Zhou et al. 2018). In this work, PAW showed a high antimicrobial effect against planktonic cells of *P. aeruginosa*. The efficacy of the treatment increased by extending the cells' exposure time to PAW and applying higher PBR discharge frequencies. The highest reduction of the planktonic cells was observed at 1500 Hz when the number of cells was below the detection level of 1.00 Log CFU ml^{-1}. Our results agree with the

study by Xiang et al. (2018), who reported that PAW produced by a pressure plasma jet system (input power set at 750 W) effectively inactivated *P. deceptionensis* CM2 planktonic cells in a time-dependent manner. Similarly, Tan and Karwe (2021) observed the reduction of *Enterobacter aerogenes* planktonic cells floating in a pipe system after PAW by approximately 3.50 Log CFU ml^{-1}. Inhibition of corrosive bacteria, e.g. *P. aeruginosa,* in the water phase is a critical step in avoiding further colonization of industrial or medical surfaces and interaction with other bacteria, fungi and viruses that can cause severe infections in humans (Smith et al. 2015; Hendricks et al. 2016). It is important to highlight that the ability of *P. aeruginosa* to grow and co-exist with other microorganisms in communal biofilms has been associated with increased resistance against antimicrobials and disinfection strategies (Pinto et al. 2020; del Mar Cendra and Torrents, 2021). Usually, the origin of bacterial inactivation is linked to the formation of specific molecules during PAW generation (e.g., H_2O_2, nitrate and nitrite). However, it has also been shown that the transient electric fields linked to the generation of cold plasma/plasma activated liquids can induce membrane permeabilization, which can also contribute to cell damage and death (Naidis, 2010; Zhang, et al. 2014; Robert et al. 2015; Chung et al. 2020; Vijayarangan et al. 2020; Dozias et al. 2021). The inactivation depends on several factors such as bacterial species (gram-positive or negative strains), physiological state of the microorganism (exponential or stationary growth), the growth medium and the mode of growth (planktonic or biofilm) (Smet et al. 2019; Mai-Prochnow et al. 2021a).

To investigate the mechanism of the antimicrobial effect of PAW on *P. aeruginosa*, the intracellular ATP levels were monitored. ATP is required for many essential cellular functions such as growth, replication, and survival (Shi et al. 2016). PAW treatment significantly reduced the intercellular ATP levels of planktonic *P. aeruginosa* cells, and this ATP reduction increased with PBR discharge frequencies. These results are consistent with Qian et al. (2021) observations, who showed that cold plasma treatment decreased the ATP levels of *L. monocytogenes* and *S. Enteritidis* cells. The reduction of intracellular ATP in *P. aeruginosa* observed in our study could be attributed to an increase in the cell membrane permeability and the resulting ATP leakage (Bajpai et al. 2013). Also, our study demonstrated that PAW treatment resulted in protein leakage from the cells, suggesting a gradual increase in the bacterial cell membrane permeability with an increase in PBR discharge frequency. This might be due to the damage of the membrane by the increasing levels of the reactive species produced in PAW.

Application of PAW as a treatment against the biofilms of gram-negative and gram-positive bacteria is an emerging field of study (Chen et al. 2017; Kaushik et al. 2018). Our study also investigated the effect of PAW on the more resistant biofilms of *P. aeruginosa*. The most effective PBR discharge frequency of (1500 Hz) was used against different maturity biofilms levels grown on SS surfaces. Numerous studies have previously described the mechanism of biofilm formation by *P. aeruginosa* (Morales et al. 1993; Klausen et al. 2003; Yuan et al. 2007; Harmsen et al. 2010) and their strong ability to further oxidize the substrate, thus leading to severe pitting corrosion (Yuan et al. 2008; Hamzah et al. 2013). It is known that *P. aeruginosa* biofilms can lead to microbial corrosion of different steel types such as SS 316 and 304 (Hamzah et al. 2013; Gabriel et al. 2016), C1018 (Jia et al. 2017), and other metallic surfaces (Beech and Sunner, 2004; Wang et al. 2004; González et al. 2019). This study showed that PAW treatment significantly reduces the level of bacterial cells and that this reduction depended on the maturity of the biofilms. Patange et al. (2021) demonstrated inactivation of early and mature *Escherichia coli* and *Listeria innocua* biofilm cells by atmospheric air plasma (AAP). They showed that a 5 min AAP treatment reduced the cell count by approximately 3.5 to 4.5 Log CFU ml^{-1}. Gabriel et al. (2016) reported a 5-Log reduction of *P. aeruginosa* biofilm cells on SS type 304 and 316 with different surface finishes after treatment with atmospheric plasma for 90 s. Castro et al. (2021) investigated the removal of *P. fluorescens* and *P. aeruginosa* biofilm cells; they used peracetic acid, sodium hypochlorite and Chlorhexidine digluconate at concentrations recommended by the manufacturers and observed a much lower than 5-Log cycles reduction. As a general observation, chlorine disinfection requires a relatively longer time to reach a similar Log reduction compared to PAW treatment. This can be attributed to chlorine's lower ability to disrupt the biofilm cells' exopolysaccharide (EPS) matrix (Myszka and Czaczyk, 2011). Therefore, these findings demonstrate a strong potential of PAW treatment against microorganisms that can cause microbial corrosion in the absence of carcinogenic and mutagenic chlorine compounds (Meireles et al. 2016; Thorman et al. 2018).

PAW is considered a promising technology, showing apparent suitability to substitute more traditional treatments used in a wide range of sectors, e.g. chlorine-based (Xiang et al. 2018; Pantage et al. 2021). However, as PAW is yet a novel technology, a detailed investigation of its mechanism of action and potential interactions with EPS matrix and other biofilm compounds is yet to be performed. It is well known that biofilm maturity significantly affects plasma penetration because thicker biofilms with more biomass provide a better protection against reactive species (Chen et al. 2017; Hathaway et al. 2019; Patange et al. 2021). The same effect was observed in the

current study, when the 72 h mature biofilms were found to be most resistant against PAW treatment. The presence of a complex mix of many reactive species in PAW has been described as a significant antimicrobial factor affecting matrix degradation (Tian et al. 2015; Cherny et al. 2020; Mai-Prochnow et al. 2021a). When the matrix is disrupted, it is observed that biofilm cells can detach as either individual cells or larger cell clusters, thus leaving small gaps in the biofilm structure (Mai-Prochnow et al. 2004). This proposed mechanism of PAW interaction with biofilms relies on the reactive species to disrupt the EPS matrix and release the sessile cells that can change their physiological state back to a more susceptible planktonic state. Furthermore, Li et al. (2019) have shown that biofilm treatment with PAW can downregulate the virulence genes linked to quorum sensing, presenting an opportunity for the disruption and removal of biofilm cells.

The nature and amount of reactive species produced in PAW vary depending on the methods used to generate them, which affects the PAW efficacy in various applications (Chen et al. 2018; Thirumdas et al. 2018; Zhou et al. 2020). PAW efficacy will also be influenced by storage time and conditions (Zhao et al. 2020), which should be taken into since there could be a delay between PAW generation and application. Plasma represents a highly reactive environment, and in the case of PAW, the main reactive species present are hydroxyl radicals (OH•), hydrogen peroxide (H_2O_2), ozone (O_3), superoxide (O_2^-), nitric oxide (NO•) and peroxynitrite (ONOOH) all with a crucial role in bacterial inactivation (Han et al. 2016; Mai-Prochnow et al. 2021a). However, some short-lived reactive species present, such as hydroxyl radicals (OH•), singlet oxygen ($^1O_2$), and superoxide (O_2^-) also have shown to contribute to microbial inactivation although to a lesser extent (Surowsky et al. 2016; Liao et al. 2018). Hydrogen peroxide (H_2O_2) monitored in the current study is one of the most common long-lived reactive species found in PAW, which, together with nitrite (NO_2^-) and nitrate (NO_3^-), play a significant role in microbial inactivation and biofilm removal (Park et al. 2017). In the current study, nitrate followed by nitrite anions showed the highest concentrations among the reactive species identified, illustrating the potential high efficacy of the PAW treatment against *P. aeruginosa* planktonic cells and biofilms at 1500 Hz for 15 min. This is consistent with data from other studies reporting that long-lived NO_2^-, NO_3^-, and H_2O_2 reactive species are efficient against a wide range of bacteria, that can contaminate different types of surfaces (Naïtali et al. 2010; Shen et al. 2016; Xiang et al. 2018; Zhao et al. 2020). Mai-Prochnow et al. (2021b) recently proved that a higher PBR discharge frequency leads to a higher production of reactive species. The present study demonstrated that the higher level of reactive

species generated with the highest PAW treatment frequency leads to increased bacterial inactivation.

The higher EC observed in the current study provided further evidence for the accumulation of the reactive species in PAW, leading to high inactivation of both planktonic cells and biofilms of *P. aeruginosa*. The plasma treatment resulted in a significant drop in the pH of PAW, however, the final pH was not low enough to be considered as a contributing factor to antimicrobial activity. The extent of the pH drop observed, can be explained by the fact that tap water was used to produce PAW and that its initial pH was approximately 7.56. Also, it could possibly be attributed to H^+ reacting with the different components in PAW and thus result in a less pronounced pH drop. Bacteria inactivation mechanism in liquids is a result of complex interactions at the plasma/gas–liquid-interface. The same applied for the subsequent reactions in the liquid volume, and these interactions have not yet been fully described (Oehmigen et al. 2011; Xiang et al. 2018).

Application of modelling can be beneficial for developing MIC mitigating strategies. As far as inactivation of microorganisms in water systems is concerned, dynamic modelling has been recently used to evaluate the thermal inactivation of *L. pneumophila* in water and proved an efficient preventive approach for plumbing systems (Papagianeli et al. 2021). In the current study, the log linear model developed confirmed our experimental results showing that PAW treatment at 1500 Hz is the most effective with a Dt value of 2.24 comparing with 8.53 at 500 Hz against the planktonic cells of *P. aeruginosa*. Apparently, such trend may be associated with the reactive species produced at PAW at different PBR discharge frequencies. The mature (48 h and 72 h) biofilms were the most resistant, while according to the weighted least square linear fit model (R^2), the models had a good fit to the experimental data. Several factors affect biofilm removal, and little is known about the relationship between the level of maturity and the treatment exposure. Our results can provide valuable data on the inhibition of the planktonic cells of *P. aeruginosa* that can colonise the metallic surfaces creating corrosive biofilms or even removing the most resistant biofilms with PAW treatment.

This work has highlighted the efficiency of the PAW treatment against planktonic cells and biofilms of *P. aeruginosa* grown on stainless-steel coupons. Reduction of intracellular ATP levels and increase in protein release in of *P. aeruginosa cells* was attributed to an increased membrane permeability due to the effect of reactive species in PAW. The 72 h mature biofilms were found to be most resistant against PAW treatment compared to younger biofilms. The observed reduction in the number of bacterial cells indicate the potential of the PAW as a means for reduction of *P.*

aeruginosa planktonic cells as well as biofilms grown on stainless-steel contact surfaces. Results of this research demonstrate that this novel methodology can be effectively used as an environmentally friendly method to inhibit MIC. A potential way to apply this technology in a practical way to inhibit MIC would be by flushing existing pipe systems with PAW or by generating PAW inside the pipe system. However, if the former application method is used then the stability of the PAW between production and application will have to be determined. Future work will focus on assessing the efficacy of PAW treatment against other microorganisms causing corrosion in specific applications such as fire engineering sprinkler maintenance, and pipe system maintenance in the food and medical industries that contribute to MIC.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Eleni Asimakopoulou: Methodology, Software, Formal analysis, Data curation, Investigation, Writing – original draft, Writing – review & editing. **Sotirios I. Ekonomou:** Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing. **Pagona Papakonstantinou**: Investigation, Writing – review & editing. **Olena Doran:** Methodology, Investigation, Writing – review & editing, Supervision. **Alexandros Ch. Stratakos:** Conceptualization, Methodology, Investigation, Writing – review & editing, Supervision, Funding acquisition.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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FIGURE LEGENDS

FIGURE 1. Schematic representation of the PBR used to generate the PAW consisting of an acrylic tube submerged into water, high-voltage power supply to generate atmospheric plasma under atmospheric conditions and supply of compressed air at a flow rate of 1 L/min.

FIGURE 2. Reduction of *P. aeruginosa* planktonic cells after 5, 10, and 15 min of exposure to PAW at PBR discharge frequencies of 500, 1000, and 1500 Hz. Each bar represents the mean value of three replicates, each of which was analyzed twice. The error bars represent the Standard Deviation for Mean (n = 6). The values with uppercase letters followed by the same lowercase letters did not differ significantly between the exposure times ($P > 0.05$). The values with uppercase letters followed by different lowercase letters differed significantly between exposure times ($P < 0.05$). Asterisk (*) indicates the values which were below the detection limit (1.00 Log CFU ml^{-1})

FIGURE 3. Reduction of *P. aeruginosa* biofilms of different maturity (24, 48, and 72 h) after 5, 10, 15, and 20 min of exposure during PAW treatment at 1500 Hz. Each bar represents the mean value of three replicates, each of which was analyzed twice. The error bars represent the Standard Deviation for Mean (n = 6). The values with uppercase letters followed by the same lowercase letters did not differ significantly between the exposure times ($P > 0.05$). The values with uppercase letters followed by different lowercase letters differed significantly between exposure times ($P < 0.05$). Asterisk (*) indicates the values which were below the detection limit (2.00 Log CFU $cm^{2\,-1}$)

FIGURE 4. Release of proteins from *P. aeruginosa* Control (untreated cells) and from cells treated with PAW at PBR discharge frequencies of 500, 1000, and 1500 Hz. The error bars represent the Standard Deviation for Mean (n = 3). The treatments with different lowercase letters differ significantly ($P < 0.05$)

FIGURE 5. Intracellular ATP production by *P. aeruginosa* untreated (Control) and by the cells treated with PAW at different PBR discharge frequencies. The error bars represent the Standard Deviation for Mean (n = 3). The treatments with different lowercase letters differ significantly ($P < 0.05$)

FIGURE 6. Inactivation curves of *P. aeruginosa* biofilms of different maturity under the treatment with 1500 Hz frequency. Censored data were handled using a substitution method, using half the detection limit. Each point represents the mean value of six replicates

FIGURE 7. Inactivation curves of *P. aeruginosa* planktonic cells after 5, 10, and 15 min of exposure during PAW treatment at PBR discharge frequencies of 500, 1000, and 1500 Hz. Censored data were handled using a substitution method, using half the detection limit. Each point represents the mean value of six replicates

TABLE 1. Physicochemical parameters and reactive molecular species in PAW after 15 min exposure at 1500 Hz

Treatments	pH	EC (mS cm^{-1})	H_2O_2 (mg ml^{-1})	NO_2^- (mg ml^{-1})	NO_3^- (mg ml^{-1})
Tap water*	7.56±0.02^A	230.667±2.73^A	-	-	-
PAW	5.94±0.04^B	325.333±11.64^B	0.03±0.002	0.04± 0.001	0.04±0.001

*Tap water prior to the use of PBR to generate PAW.

The results are presented as mean ± standard deviation (SD). The mean values were calculated for three replicates, each of which was analyzed twice (n=6).

Values in the same column with different uppercase letters differ significantly ($P < 0.05$).

TABLE 2. Model parameters and associated errors for biofilm cells of different maturity (24, 48, and 72 h) after 5, 10 and 15 of exposure to PAW treatment at 1500 Hz

Biofilm maturity (h)	Parameters							
	D_T	SE	$logN_o$	SE	Mean Sum of R^2	R^2 Root Mean Sum	R^2	R^2 adjusted
24	3.11	0.13	5.51	0.53	0.3965	0.6296	0.9417	0.9125
48	4.34	0.09	5.67	0.46	0.3507	0.5922	0.9253	0.9005
72	4.19	0.07	6.21	0.36	0.2102	0.4585	0.9548	0.9445

TABLE 3. Model parameters and associated errors of *P. aeruginosa* planktonic cells after 5, 10, and 15 min of exposure during PAW treatment at PBR discharge frequencies of 500, 1000, and 1500 Hz

PBR discharge	Parameters

frequencies (Hz)	D_T	SE	$logN_o$	SE	Mean Sum of R^2	R^2 Root Mean Sum	R^2	R^2 adjusted
500	8.53	0.02	5.03	0.10	0.0147	0.1213	0.9833	0.9749
1000	3.44	0.04	4.89	0.16	0.0374	0.1933	0.9930	0.9895
1500	2.24	0.07	4.90	0.20	0.0479	0.2188	0.9953	0.9906

FIG 1

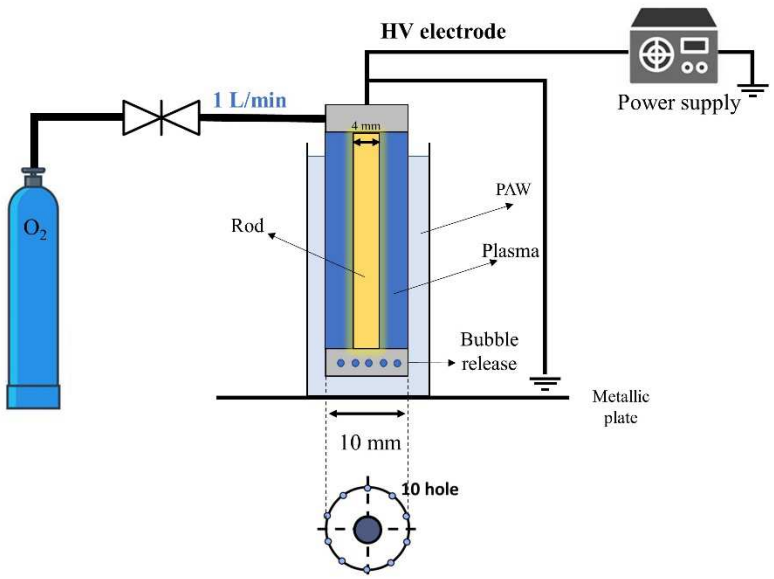

FIG 2

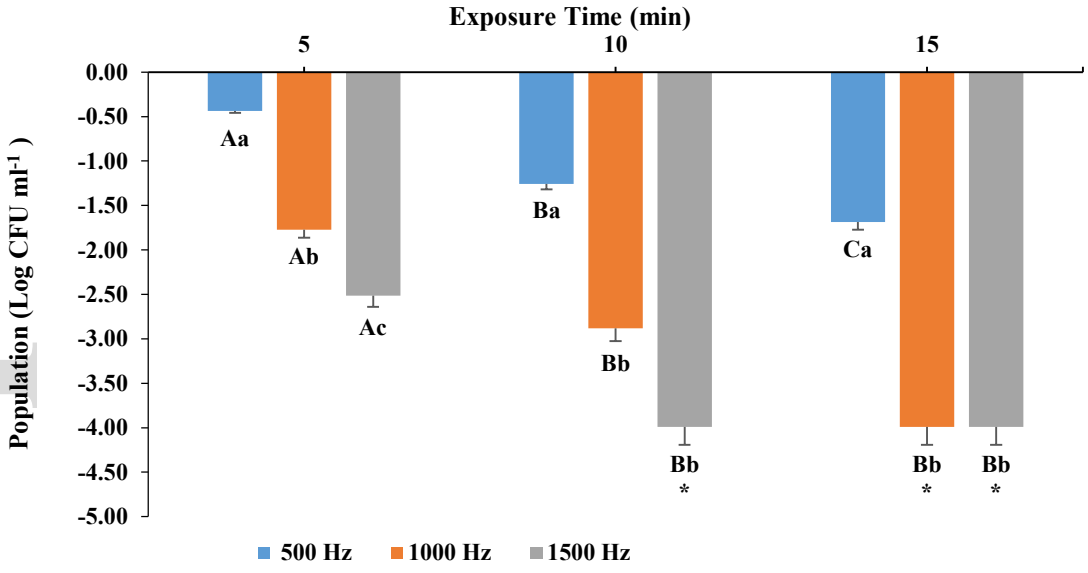

FIG 3

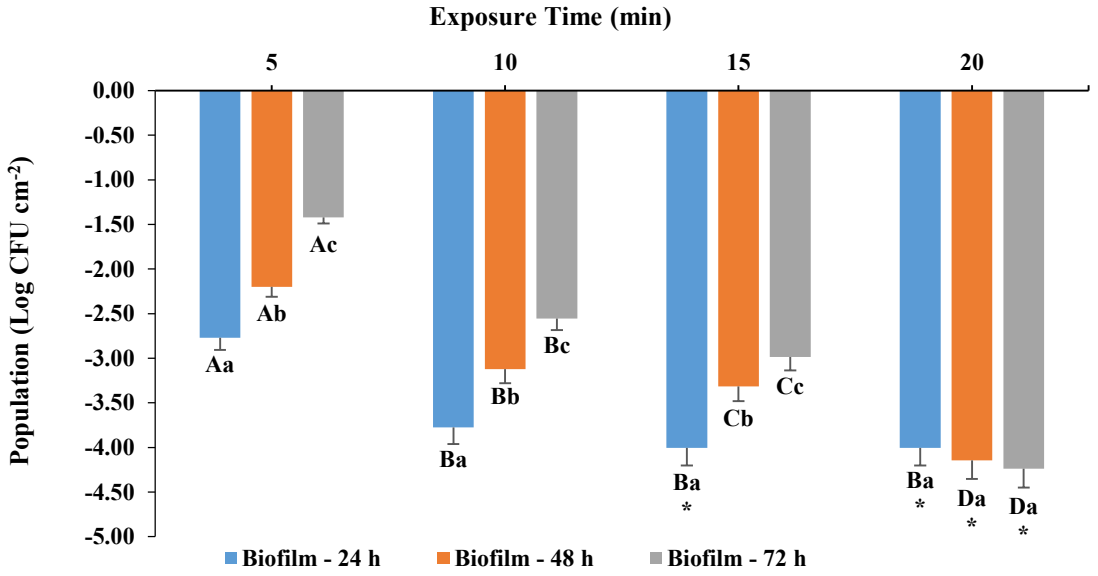

FIG 4

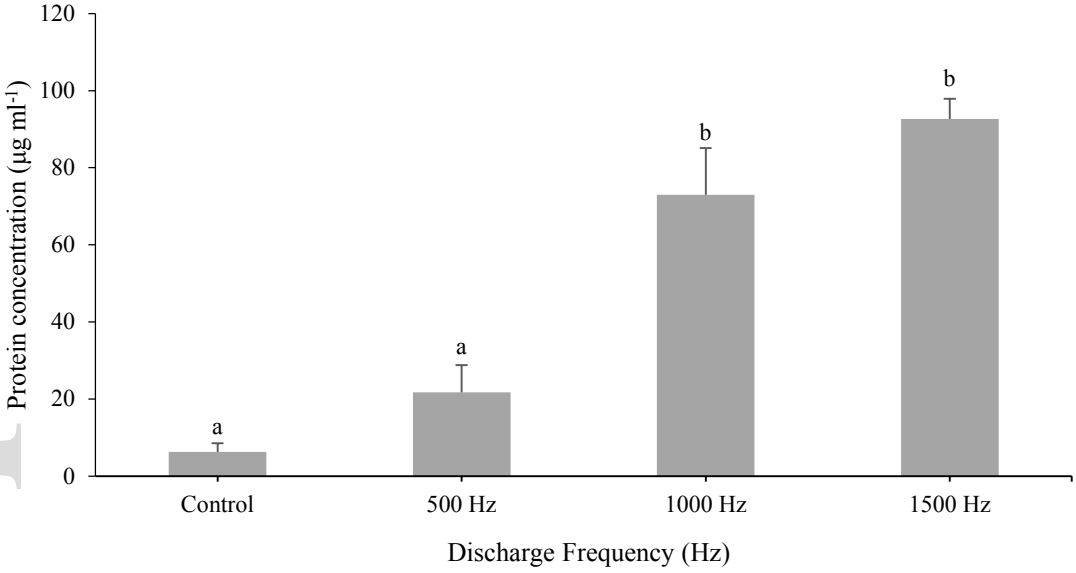

FIG 5

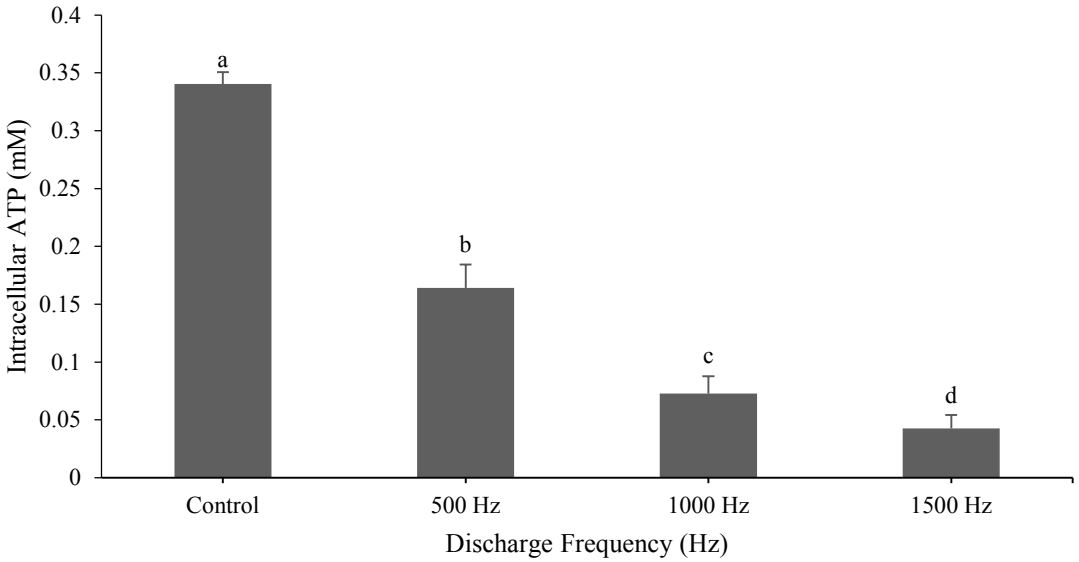

FIG 6

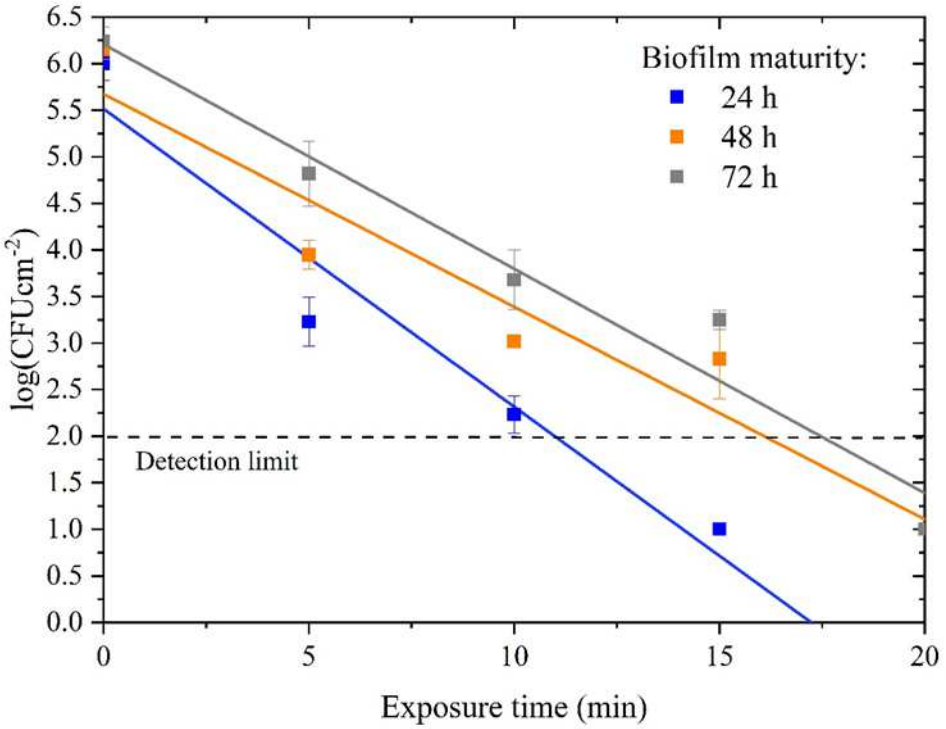

FIG 7