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

2

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26 **Running headline:** Plasma activated water against microbial corrosion

27 **Abstract**

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

31 **Methods and Results:** This study evaluated the effectiveness of PAW, produced by a plasma  
32 bubble reactor, in reducing corrosion causing *Pseudomonas aeruginosa* planktonic cells in tap  
33 water and biofilms grown onto stainless steel (SS) coupons. Planktonic cells and biofilms were  
34 treated with PAW at different discharge frequencies (500-1500 Hz) and exposure times (0-20  
35 min). *P. aeruginosa* cells in tap water were significantly reduced after treatment, with higher  
36 exposure times and discharge frequencies achieving higher reductions. Also, PAW treatment  
37 led to a gradual reduction for young and mature biofilms, achieving >4-Log reductions after  
38 20 min. Results were also used to develop two predictive inactivation models.

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

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

44

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

47

## 48 INTRODUCTION

49 Metal corrosion can be significantly accelerated by the presence and activity of  
50 microorganisms, a process that is also known as biocorrosion or Microbiologically Influenced  
51 Corrosion (MIC) (Phan et al. 2021) and is responsible for 20% of metal corrosion damages  
52 (Flemming, 1996). The cost of corrosion in industrialised countries was estimated to be 3.4%  
53 of the global GDP in 2013, and if corrosion protection and design were properly applied, a 15-  
54 35% loss reduction could be achieved (Koch et al. 2016). Its direct costs affect a wide range of  
55 sectors, including agriculture, forestry and fishing, mining, manufacturing (e.g., chemical  
56 processing, nuclear, oil, gas, underground pipeline, water treatment, food production, highway  
57 maintenance, aviation, metal working, marine, shipping and fire protection), electricity supply,  
58 water supply, waste management, accommodation, and food service activities, transportation,  
59 and storage. Indirect costs are associated with environmental, regulatory, and human costs,  
60 making cost estimation even more challenging (Little et al. 2020).

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

68 Lately, more fundamental research was conducted aiming to investigate MIC associated  
69 phenomena and advised to approach the subject systematically by monitoring the microbial  
70 activity of single bacteria (Kermani and Harrop, 2019). Different types of aerobic and  
71 anaerobic bacteria are associated with MIC; among them, the most commonly met are the acid-  
72 producing, sulphur reducing and iron related bacteria (Su and Fuller, 2014) as well as others,

73 such as *Pseudomonas aeruginosa* (Li et al. 2016; Jia et al. 2017). *P. aeruginosa* MIC  
74 mechanism and associated bacteria-metal reactions have been extensively investigated, and its  
75 presence is proven to accelerate corrosion (Abdolahi et al. 2014). There are several studies that  
76 confirm its involvement in the corrosion process of different types of metals e.g., mild steel  
77 (Xu et al. 2017), stainless steel (Xu et al. 2016; Jia et al. 2017; Xu et al. 2017) and aluminium  
78 (Xu et al. 2017).

79 Research in MIC is spanning four main areas: diagnose, monitor, modelling and prevention.  
80 To achieve the latter, numerous treatments are customarily used, and the most common ones  
81 include sanitation, physical, chemical treatment, biological methods, use of coatings and  
82 cathodic protection (Enning and Garrelfs, 2014; Ibrahim et al. 2018; Little et al. 2020). The  
83 solution of applying coating specifically designed for each application appears to be promising,  
84 however this is not always feasible and practical. An efficient way to limit microbial growth is  
85 through physical treatment (e.g. pigging, ultraviolet irradiation, ultrasonic, chemical and  
86 biological treatments) of natural or industrial water aiming to inhibit the microorganisms  
87 responsible for causing corrosion (Little et al. 2020). Recent studies have revealed that indirect  
88 application of Cold Atmospheric Plasma (CAP) at ambient air conditions can significantly  
89 decrease the microbial load (Pasquali et al. 2016; Katsigiannis et al. 2021). Also, in recent  
90 years, a new mode of CAP, i.e., Plasma-activated Water (PAW) activities has drawn attention  
91 (Julák et al. 2012; Chen et al. 2018) as it can be applied to inhibit/kill microorganisms in  
92 surfaces and systems in an environmentally friendly, cost-effective, and practical way. Lately,  
93 the application of PAW has been investigated (Tan and Karwe, 2021) and has been proven an  
94 effective technique for inactivating inner-pipe surfaces formed biofilms, also recognised as a  
95 novel technology with significantly decreased environmental cost (Zhou et al. 2020).  
96 Despite a large number of recent publications related to MIC, an effective and practical  
97 technological solution to tackle MIC related problems is yet to be found (Little et al. 2020). To

98 meet this challenge, the current experimental work aims to assess the efficiency and  
99 antimicrobial mechanism of PAW against corrosion causing *P. aeruginosa* as well as identify  
100 optimal operational conditions for PAW treatment.

## 101 **MATERIALS AND METHODS**

### 102 **Bacterial cultivation**

103 Experiments were conducted using PAO1, a *P. aeruginosa* strain. The *P. aeruginosa* culture  
104 was prepared in Luria-Bertani (LB) liquid medium enriched with KNO<sub>3</sub> (LB-NO<sub>3</sub>). The media  
105 formulation of the agar medium LB-NO<sub>3</sub> included 10 g tryptone, 10 g KNO<sub>3</sub> 5 g yeast extract,  
106 and 5 g NaCl per litre of deionised water. KNO<sub>3</sub> was added to the LB medium to support  
107 anoxic growth of *P. aeruginosa* PAO1 (Line et al. 2014). The pH was adjusted to 7.0 by  
108 applying a solution of NaOH. To mitigate any possible O<sub>2</sub> entry, L-cysteine at a concentration  
109 of one hundred ppm (w/w), was added to the culture medium as O<sub>2</sub> scavenger. The L-cysteine  
110 solution was filter-sterilised using MF-Millipore<sup>TM</sup> membrane filter of a pore size of 0.22 µm  
111 (Merck KGaA, Darmstadt, Germany) before it was added to the medium. Dissolved oxygen  
112 was removed from all liquid solutions by flushing them with filter sterilised N<sub>2</sub> in order to  
113 maintain anoxic growth of *P. aeruginosa*. 304 Stainless Steel (SS) sheet were used to cut the  
114 rectangular shaped coupons (1 cm x 1 cm) used in this work. Coupons were sterilised in 75%  
115 ethanol solution for 2 h, dried and exposed to UV light for 30 min prior to use. A N<sub>2</sub> chamber  
116 was used for anaerobic manipulations. The biofilms were grown in anaerobic vials, following  
117 the procedure described by Jia et al. (2017). One hundred ml of LB-NO<sub>3</sub> medium, with and  
118 without the addition of the *P. aeruginosa* culture, was added to each anaerobic vial. The  
119 experiments were conducted on three replicate coupons. The initial bacterial concentration  
120 after inoculation with *P. aeruginosa* was approximately 10<sup>4-5</sup> Log CFU ml<sup>-1</sup>. The vials were  
121 closed and incubated at 37°C for 2, 5, or 7 days. After the incubation, the coupons were  
122 removed, and PAW treated under different conditions as described below. Before PAW

123 treatment, the coupons were gently rinsed with sterile phosphate-buffered saline to remove the  
124 non-adhered and loosely attached bacterial cells.

### 125 **Plasma bubble reactor**

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

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

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

141 The plasma reactor was filled with 100 ml of sterile tap water. Control SS coupons (no PAW  
142 treatment) were immersed in water and placed on the bottom of the reactor, with just the airflow  
143 on, for 15 min without igniting the plasma. For treated samples, coupons were placed in the  
144 reactor and subsequently the PBR was turned on. The reactor was allowed to run for different  
145 times (5, 10, 15, and 20 min), at 150 V (voltage applied at the high voltage transformer), 100-  
146  $\mu$ s duty cycle (time during which the energy from the power supply unit is transferred to HV  
147 transformer resonance circuit) and 60 kHz resonance frequency (resonance frequency of

148 resonance circuit). Three different discharge frequencies were investigated (500, 1000, and  
149 1500 Hz).

150 SS coupons with adhered biofilm cells of different maturity were treated directly in PBR for 0,  
151 5, 10, 15, and 20 min. After treatment, the SS coupons were positioned in a sterile container  
152 with 10 mL maximum recovery diluent (MRD) (Oxoid, UK) and 1 g glass beads caliber of 0.1  
153 mm diameter. To detach the surviving biofilm cells from the SS coupons, samples were  
154 vortexed for 60 s. Subsequently, 10-fold dilutions were prepared in MRD to enumerate the  
155 surviving biofilm cells. An aliquot of 100- $\mu$ l was used from the appropriate 10-fold serial  
156 dilutions and was spread plated on Tryptone Soya Agar (TSA, Oxoid, UK). Plates were  
157 incubated at 37 °C for 24 h and the biofilm cells expressed as Log CFU cm<sup>-2</sup>.

#### 158 **PAW treatment and enumeration of *P. aeruginosa***

159 *P. aeruginosa* can exist in the water before it attaches to surfaces and begin to form biofilms  
160 and subsequently influence the physicochemical metal-environment interactions, thus  
161 enhancing MIC (Li et al. 2016; Jia et al. 2017). To investigate if PAW can inactivate this  
162 microorganisms in its planktonic form, 100- $\mu$ l of the *P. aeruginosa* (prepared as described  
163 above) was inoculated into 100 ml of tap water. After inoculation, the bacterial concentration  
164 was approximately 10<sup>5</sup> Log CFU ml<sup>-1</sup>. Control samples received no treatment (0 min; only  
165 airflow was on for 15 min without igniting the plasma). For the rest of the PAW treatments,  
166 the PBR was turned on and allowed to run for different time periods (0, 5, and 10 min) at 150  
167 V, 100- $\mu$ s duty cycle, 60 kHz resonance frequency and 500, 1000, and 1500 Hz discharge  
168 frequencies. The planktonic cells were treated inside the reactor. Immediately after treatment,  
169 an aliquot (1 ml) of the treated tap water was added to 9 ml of MRD and subsequently, further  
170 suitable 10-fold dilutions were prepared. An aliquot of 100- $\mu$ l was used from the appropriate  
171 10-fold serial dilutions and was spread plated on Trypticase Soy Agar (TSA, Oxoid, UK).



172 Plates were incubated at 37 °C for 24 h, and planktonic *P. aeruginosa* cells were expressed as  
173 Log CFU ml<sup>-1</sup>.

#### 174 **Physicochemical properties of PAW**

175 PAW was generated at a discharge frequency of 1500 Hz for 15 minutes, and the measurements  
176 of pH, electrical conductivity (EC), H<sub>2</sub>O<sub>2</sub>, NO<sub>2</sub><sup>-</sup>, and NO<sub>3</sub><sup>-</sup> concentrations were taken  
177 immediately after treatment. Values of EC and pH were measured using an electronic  
178 conductivity meter (Jenway 4200, UK) and a pH meter (FiveEasy, Mettler Toledo, UK),  
179 respectively. H<sub>2</sub>O<sub>2</sub> concentration was determined using the titanium oxysulphate (TiOSO<sub>4</sub>;  
180 Sigma-Aldrich) method. According to that method, TiOSO<sub>4</sub> reacts with H<sub>2</sub>O<sub>2</sub>, and produces a  
181 yellow-coloured complex (pertitanic acid). Subsequently, the H<sub>2</sub>O<sub>2</sub> concentration was  
182 determined spectrophotometrically (Mai-Prochnow et al. 2021b). Nitrite concentration was  
183 determined by using the Griess assay, a chemical reaction that uses N-(1-naphthyl)-ethylene  
184 diamine hydrochloride under sulfanilic acidic conditions, resulting in the formation of a  
185 magenta-coloured azo dye. Nitrate (NO<sub>3</sub><sup>-</sup>) concentration was determined using a nitrate assay  
186 kit (Sigma, UK) that is based on its reaction with 2, 6-dimethylphenol (DMP). To eliminate  
187 nitrite interference, all PAW samples were pre-treated using sulfamic acid (Zhao et al. 2020)  
188 prior to analysis.

189 Individual standard curves of known H<sub>2</sub>O<sub>2</sub>, NaNO<sub>2</sub> and NaNO<sub>3</sub> concentration were prepared to  
190 convert the absorbance to H<sub>2</sub>O<sub>2</sub>, NO<sub>2</sub><sup>-</sup>, NO<sub>3</sub><sup>-</sup>, concentrations.

#### 191 **Membrane integrity**

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

## 196 **Intracellular adenosine triphosphate levels**

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

## 209 **Statistical analysis and modelling**

210 All treatments were performed three times, and each sample was analyzed twice and averaged  
211 before statistical analysis. Statistical analysis was done with Excel Microsoft® Office 365 (ver.  
212 16.48). Tukey post hoc tests were used to compare sample data using the IBM® SPSS®  
213 statistics 26 software for macOS (SPSS Inc., US). A 5.0 % level of significance, *P*, was used,  
214 and thus results were considered statistically significant when *P* was less than 0.05 ( $P < 0.05$ ).  
215 The fitting procedure for modelling of inactivation kinetics was performed using GInaFiT  
216 software (Geeraerd et al. 2005).

## 217 **RESULTS**

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

219 The effect of PAW treatment on planktonic *P. aeruginosa* cells after 5, 10, and 15 min exposure  
220 to different PBR discharge frequencies (500, 1000, and 1500 Hz) is presented in Figure 2. The

221 initial population of *P. aeruginosa* control cells without plasma treatment in tap water was 4.99  
222 Log CFU ml<sup>-1</sup>.

223 The population of *P. aeruginosa* in tap water was significantly reduced after 5 min of PAW  
224 treatment and reached 0.44, 1.77, and 2.51 Log CFU ml<sup>-1</sup> at 500, 1000 and 1500 Hz, PBR  
225 discharge frequencies respectively (Fig 2,  $P < 0.05$ ). *P. aeruginosa* cells, treated with PAW for  
226 10 min resulted in a significant reduction of the cell numbers by 1.26 and 2.88 Log CFU ml<sup>-1</sup>  
227 at 500 and 1000 Hz, respectively (Fig 2,  $P < 0.05$ ). However, PAW treatment at 1500 Hz  
228 resulted in a reduction below the detection limit ( $>3.99$  Log CFU ml<sup>-1</sup>) (Fig 2). After 15 min  
229 of PAW treatment at 1000 and 1500 Hz, the bacterial numbers of *P. aeruginosa* were reduced  
230 below the detection limit, while PAW treatment at 500 Hz resulted in a significant reduction,  
231 of 1.69 Log CFU ml<sup>-1</sup> (Fig 2,  $P < 0.05$ ).

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

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

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

246 levels of *P. aeruginosa* biofilms are presented in Figure 3. Plasma discharge in bubbles for 5  
247 min reduced the 24, 48, and 72 h attached biofilm cells of *P. aeruginosa* by 2.77, 2.20, and  
248 1.42 Log CFU cm<sup>2-1</sup>, respectively (Fig 3, *P* < 0.05). The same trend was observed after 10 min  
249 of PAW treatment with increased efficacy, where bacterial numbers for biofilms grown for 24,  
250 48, and 72 h were reduced significantly by 3.77, 3.12, and 2.55 Log CFU cm<sup>2-1</sup>, respectively  
251 (Fig 3, *P* < 0.05). After 15 min of exposure, the 24 h old biofilms were reduced by >4.00 Log  
252 CFU cm<sup>-2</sup>, below the detection limit of 2.00 Log CFU cm<sup>-2</sup> (Fig 3). However, *P. aeruginosa*  
253 48 and 72 h mature biofilms decreased below the detection limit (2.00 Log CFU cm<sup>2-1</sup>) after  
254 20 min of exposure at PAW treatment, corresponding to >4.15 and >4.24 log reduction of  
255 CFUcm<sup>-2</sup> (Fig 3).

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

### 259 **Physicochemical properties of PAW treatment**

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

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

270 Data in Figure 4 show the amount of protein released from *P. aeruginosa* planktonic cells  
271 treated with PAW at 500, 1000, and 1500 Hz of PBR discharge frequency. Protein leakage  
272 from PAW-treated *P. aeruginosa* cells at 1000 and 1500 Hz significantly increased compared  
273 to that from untreated cells (control) and from the cells treated at 500 Hz (Fig 4,  $P < 0.05$ ). The  
274 highest release of proteins for the PAW-treated *P. aeruginosa* planktonic cells was observed at  
275 1000 and 1500 Hz, respectively. The data suggest that there was a gradual increase in protein  
276 release in the cell suspension with increase in discharge frequency.

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

278 Results on the effect of PAW treatment on *P. aeruginosa* Intracellular ATP levels at different  
279 discharge frequencies are presented in Figure 5. The ATP calibration curve showed a positive  
280 linear relationship between relative luminescence units and ATP level, which can be described  
281 by the following equation:  $y = 475926x + 22826$ ;  $R^2 = 0.986$ . The level of intracellular ATP  
282 of *P. aeruginosa* decreased significantly as the discharge level of PAW treatment increased  
283 from 500 to 1000 and 1500 Hz (Fig 5,  $P < 0.05$ ). The initial ATP concentration of the untreated  
284 (Control) *P. aeruginosa* cells was 0.340 mM, while after PAW treatment at 500, 1000, and  
285 1500 Hz, it was reduced to 0.164, 0.073, and 0.042 mM, respectively ( $P < 0.05$ ).

### 286 **Modelling of inactivation kinetics**

287 The responses of the different parameters in the microbial population of *P. aeruginosa* to  
288 different exposure times were fitted using a log-linear regression model (Bigelow and Etsy,  
289 1920). Analysis of the experimental dataset of the microbial population of *P. aeruginosa* was  
290 performed using a weighted least square linear fit model. The Coefficient of Determination  
291 (COD), also known as  $R^2$ , was used as a statistical measure to assess the quality of each linear  
292 regression fit. The linear regression model, Equation (1), was used to estimate the kinetic  
293 inactivation parameter,  $D_T$ , for the different PBR discharge frequencies for the planktonic *P.*

294 *aeruginosa* cells and the young, 24 h old, and mature, 48 and 72 h old, biofilms. In the Equation  
295 (1),  $N$  represents the microbial population at time  $t$ , and  $N_o$  is the experimentally determined  
296 initial microbial population. Microbial population measurements below the level of detection  
297 ( $D$ ) were handled using a substitution method (Ogden, 2010). According to the substitution  
298 method every result below  $D$ , also known as censored data, is substituted with an estimate, for  
299 what it might be. Traditional options for handling censored data include discarding non-detect  
300 data and using simple substitution methods (treating non-detects as zero, half the detection  
301 limit, at the detection limit, or the detection limit/ $\sqrt{2}$ ) (Levine et al. 2009; EPA, 2006). Instead  
302 of eliminating censored data from the data set, setting them as zero or  $D$  values, that has been  
303 proved to yield erroneous results (Silvestri et al. 2017), in the current study values bellow  $D$ ,  
304 were substituted with  $D/2$  (Levine, 2006; Ogden, 2010).

305 Tables 2 and 3 present the model parameters and their measures of statistical dispersion namely  
306 the standard error (SE), mean sum of  $R^2$ , root mean sum of  $R^2$ ,  $R^2$  and adjusted  $R^2$  for biofilms  
307 of different maturity and PBR discharge frequencies. Figure 6 shows the relevant fitted  
308 inactivation curves for biofilms of *P. aeruginosa* grown for 24, 48, and 72 h and treated with  
309 PAW at a discharge frequency of 1500 Hz. The developed model can be reliably used to  
310 describe the inactivation curves. Good statistical fit was observed for biofilms of different  
311 maturity with estimated adjusted  $R^2$  values ranging from 0.9005 to 0.9445. The calculated  
312 values for the inactivation parameter  $D_T$  for each biofilm ranged from 3.11 for the 24 h old  
313 biofilms to 4.34 for the 48 h old biofilms. The relevant model parameters and associated errors  
314 for *P. aeruginosa* planktonic cells after PAW treatment at different PBR discharge frequencies  
315 are presented in Table 3. Figure 7 shows the inactivation curves for the different exposure  
316 times. The inactivation parameter  $D_T$  tends to decrease with the increase of PBR discharge  
317 frequencies, with the values ranging from 8.53 at 500 Hz to 2.24 at 1500 Hz. The accuracy of

318 the developed model is supported by the statistical parameters and evaluation of the fitting  
319 curves (Fig 7).

$$320 \quad \log N = \log N_o - \frac{t}{D_T} \quad (1)$$

## 321 **DISCUSSION**

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

331 During the last decade, cold plasma treatment has been attracting attention as a green  
332 technology for the inactivation of spoilage and pathogenic bacteria on different matrices  
333 (Ehlbeck et al. 2010; Pasquali et al. 2016; Jayathunge et al. 2019; Ekonomou and Boziaris,  
334 2021), as well as a method for decontamination of industrial surfaces and medical equipment  
335 (Ben Belgacem et al. 2017; Alshraiedeh et al. 2020; Gonzalez-Gonzalez et al. 2021). Recently,  
336 the development of PAW offered a new opportunity as a promising decontamination method  
337 for the preservation of food (Ma et al. 2015; Liao et al. 2018; Thirumdas et al. 2018) and wound  
338 healing in the medical industry (Chen et al. 2017; Kaushik et a., 2017), and as an alternative  
339 disinfectant for the inactivation of bacteria in water (Pan et al. 2017; Zhou et al. 2018). In this  
340 work, PAW showed a high antimicrobial effect against planktonic cells of *P. aeruginosa*. The  
341 efficacy of the treatment increased by extending the cells' exposure time to PAW and applying  
342 higher PBR discharge frequencies. The highest reduction of the planktonic cells was observed

343 at 1500 Hz when the number of cells was below the detection level of 1.00 Log CFU ml<sup>-1</sup>. Our  
344 results agree with the study by Xiang et al. (2018), who reported that PAW produced by a  
345 pressure plasma jet system (input power set at 750 W) effectively inactivated *P. deceptionensis*  
346 CM2 planktonic cells in a time-dependent manner. Similarly, Tan and Karwe (2021) observed  
347 the reduction of *Enterobacter aerogenes* planktonic cells floating in a pipe system after PAW  
348 by approximately 3.50 Log CFU ml<sup>-1</sup>. Inhibition of corrosive bacteria, e.g. *P. aeruginosa*, in  
349 the water phase is a critical step in avoiding further colonization of industrial or medical  
350 surfaces and interaction with other bacteria, fungi and viruses that can cause severe infections  
351 in humans (Smith et al. 2015; Hendricks et al. 2016). It is important to highlight that the ability  
352 of *P. aeruginosa* to grow and co-exist with other microorganisms in communal biofilms has  
353 been associated with increased resistance against antimicrobials and disinfection strategies  
354 (Pinto et al. 2020; del Mar Cendra and Torrents, 2021). Usually, the origin of bacterial  
355 inactivation is linked to the formation of specific molecules during PAW generation (e.g.,  
356 H<sub>2</sub>O<sub>2</sub>, nitrate and nitrite). However, it has also been shown that the transient electric fields  
357 linked to the generation of cold plasma/plasma activated liquids can induce membrane  
358 permeabilization, which can also contribute to cell damage and death (Naidis, 2010; Zhang, et  
359 al. 2014; Robert et al. 2015; Chung et al. 2020; Vijayarangan et al. 2020; Dozias et al. 2021).  
360 The inactivation depends on several factors such as bacterial species (gram-positive or negative  
361 strains), physiological state of the microorganism (exponential or stationary growth), the  
362 growth medium and the mode of growth (planktonic or biofilm) (Smet et al. 2019; Mai-  
363 Prochnow et al. 2021a).

364 To investigate the mechanism of the antimicrobial effect of PAW on *P. aeruginosa*, the  
365 intracellular ATP levels were monitored. ATP is required for many essential cellular functions  
366 such as growth, replication, and survival (Shi et al. 2016). PAW treatment significantly reduced  
367 the intercellular ATP levels of planktonic *P. aeruginosa* cells, and this ATP reduction increased



368 with PBR discharge frequencies. These results are consistent with Qian et al. (2021)  
369 observations, who showed that cold plasma treatment decreased the ATP levels of *L.*  
370 *monocytogenes* and *S. Enteritidis* cells. The reduction of intracellular ATP in *P. aeruginosa*  
371 observed in our study could be attributed to an increase in the cell membrane permeability and  
372 the resulting ATP leakage (Bajpai et al. 2013). Also, our study demonstrated that PAW  
373 treatment resulted in protein leakage from the cells, suggesting a gradual increase in the  
374 bacterial cell membrane permeability with an increase in PBR discharge frequency. This might  
375 be due to the damage of the membrane by the increasing levels of the reactive species produced  
376 in PAW.

377 Application of PAW as a treatment against the biofilms of gram-negative and gram-positive  
378 bacteria is an emerging field of study (Chen et al. 2017; Kaushik et al. 2018). Our study also  
379 investigated the effect of PAW on the more resistant biofilms of *P. aeruginosa*. The most  
380 effective PBR discharge frequency of (1500 Hz) was used against different maturity biofilms  
381 levels grown on SS surfaces. Numerous studies have previously described the mechanism of  
382 biofilm formation by *P. aeruginosa* (Morales et al. 1993; Klausen et al. 2003; Yuan et al. 2007;  
383 Harmsen et al. 2010) and their strong ability to further oxidize the substrate, thus leading to  
384 severe pitting corrosion (Yuan et al. 2008; Hamzah et al. 2013). It is known that *P. aeruginosa*  
385 biofilms can lead to microbial corrosion of different steel types such as SS 316 and 304  
386 (Hamzah et al. 2013; Gabriel et al. 2016), C1018 (Jia et al. 2017), and other metallic surfaces  
387 (Beech and Sunner, 2004; Wang et al. 2004; González et al. 2019). This study showed that  
388 PAW treatment significantly reduces the level of bacterial cells and that this reduction  
389 depended on the maturity of the biofilms. Patange et al. (2021) demonstrated inactivation of  
390 early and mature *Escherichia coli* and *Listeria innocua* biofilm cells by atmospheric air plasma  
391 (AAP). They showed that a 5 min AAP treatment reduced the cell count by approximately 3.5  
392 to 4.5 Log CFU ml<sup>-1</sup>. Gabriel et al. (2016) reported a 5-Log reduction of *P. aeruginosa* biofilm

393 cells on SS type 304 and 316 with different surface finishes after treatment with atmospheric  
394 plasma for 90 s. Castro et al. (2021) investigated the removal of *P. fluorescens* and *P.*  
395 *aeruginosa* biofilm cells; they used peracetic acid, sodium hypochlorite and Chlorhexidine  
396 digluconate at concentrations recommended by the manufacturers and observed a much lower  
397 than 5-Log cycles reduction. As a general observation, chlorine disinfection requires a  
398 relatively longer time to reach a similar Log reduction compared to PAW treatment. This can  
399 be attributed to chlorine's lower ability to disrupt the biofilm cells' exopolysaccharide (EPS)  
400 matrix (Myszka and Czaczyk, 2011). Therefore, these findings demonstrate a strong potential  
401 of PAW treatment against microorganisms that can cause microbial corrosion in the absence  
402 of carcinogenic and mutagenic chlorine compounds (Meireles et al. 2016; Thorman et al.  
403 2018).

404 PAW is considered a promising technology, showing apparent suitability to substitute more  
405 traditional treatments used in a wide range of sectors, e.g. chlorine-based (Xiang et al. 2018;  
406 Pantage et al. 2021). However, as PAW is yet a novel technology, a detailed investigation of  
407 its mechanism of action and potential interactions with EPS matrix and other biofilm  
408 compounds is yet to be performed. It is well known that biofilm maturity significantly affects  
409 plasma penetration because thicker biofilms with more biomass provide a better protection  
410 against reactive species (Chen et al. 2017; Hathaway et al. 2019; Patange et al. 2021). The same  
411 effect was observed in the current study, when the 72 h mature biofilms were found to be most  
412 resistant against PAW treatment. The presence of a complex mix of many reactive species in  
413 PAW has been described as a significant antimicrobial factor affecting matrix degradation  
414 (Tian et al. 2015; Cherny et al. 2020; Mai-Prochnow et al. 2021a). When the matrix is  
415 disrupted, it is observed that biofilm cells can detach as either individual cells or larger cell  
416 clusters, thus leaving small gaps in the biofilm structure (Mai-Prochnow et al. 2004). This  
417 proposed mechanism of PAW interaction with biofilms relies on the reactive species to disrupt

418 the EPS matrix and release the sessile cells that can change their physiological state back to a  
419 more susceptible planktonic state. Furthermore, Li et al. (2019) have shown that biofilm  
420 treatment with PAW can downregulate the virulence genes linked to quorum sensing,  
421 presenting an opportunity for the disruption and removal of biofilm cells.

422 The nature and amount of reactive species produced in PAW vary depending on the methods  
423 used to generate them, which affects the PAW efficacy in various applications (Chen et al.  
424 2018; Thirumdas et al. 2018; Zhou et al. 2020). PAW efficacy will also be influenced by  
425 storage time and conditions (Zhao et al. 2020), which should be taken into since there could be  
426 a delay between PAW generation and application. Plasma represents a highly reactive  
427 environment, and in the case of PAW, the main reactive species present are hydroxyl radicals  
428 ( $\text{OH}\bullet$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), ozone ( $\text{O}_3$ ), superoxide ( $\text{O}_2^-$ ), nitric oxide ( $\text{NO}\bullet$ ) and  
429 peroxynitrite ( $\text{ONOOH}$ ) all with a crucial role in bacterial inactivation (Han et al. 2016; Mai-  
430 Prochnow et al. 2021a). However, some short-lived reactive species present, such as hydroxyl  
431 radicals ( $\text{OH}\bullet$ ), singlet oxygen ( $^1\text{O}_2$ ), and superoxide ( $\text{O}_2^-$ ) also have shown to contribute to  
432 microbial inactivation although to a lesser extent (Surowsky et al. 2016; Liao et al. 2018).

433 Hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) monitored in the current study is one of the most common long-  
434 lived reactive species found in PAW, which, together with nitrite ( $\text{NO}_2^-$ ) and nitrate ( $\text{NO}_3^-$ ),  
435 play a significant role in microbial inactivation and biofilm removal (Park et al. 2017). In the  
436 current study, nitrate followed by nitrite anions showed the highest concentrations among the  
437 reactive species identified, illustrating the potential high efficacy of the PAW treatment against  
438 *P. aeruginosa* planktonic cells and biofilms at 1500 Hz for 15 min. This is consistent with data  
439 from other studies reporting that long-lived  $\text{NO}_2^-$ ,  $\text{NO}_3^-$ , and  $\text{H}_2\text{O}_2$  reactive species are efficient  
440 against a wide range of bacteria, that can contaminate different types of surfaces (Naïtali et al.  
441 2010; Shen et al. 2016; Xiang et al. 2018; Zhao et al. 2020). Mai-Prochnow et al. (2021b)  
442 recently proved that a higher PBR discharge frequency leads to a higher production of reactive

443 species. The present study demonstrated that the higher level of reactive species generated with  
444 the highest PAW treatment frequency leads to increased bacterial inactivation.

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

456 Application of modelling can be beneficial for developing MIC mitigating strategies. As far as  
457 inactivation of microorganisms in water systems is concerned, dynamic modelling has been  
458 recently used to evaluate the thermal inactivation of *L. pneumophila* in water and proved an  
459 efficient preventive approach for plumbing systems (Papagianeli et al. 2021). In the current  
460 study, the log linear model developed confirmed our experimental results showing that PAW  
461 treatment at 1500 Hz is the most effective with a Dt value of 2.24 comparing with 8.53 at 500  
462 Hz against the planktonic cells of *P. aeruginosa*. Apparently, such trend may be associated  
463 with the reactive species produced at PAW at different PBR discharge frequencies. The mature  
464 (48 h and 72 h) biofilms were the most resistant, while according to the weighted least square  
465 linear fit model (R<sup>2</sup>), the models had a good fit to the experimental data. Several factors affect  
466 biofilm removal, and little is known about the relationship between the level of maturity and  
467 the treatment exposure. Our results can provide valuable data on the inhibition of the planktonic

468 cells of *P. aeruginosa* that can colonise the metallic surfaces creating corrosive biofilms or  
469 even removing the most resistant biofilms with PAW treatment.

470 This work has highlighted the efficiency of the PAW treatment against planktonic cells and  
471 biofilms of *P. aeruginosa* grown on stainless-steel coupons. Reduction of intracellular ATP  
472 levels and increase in protein release in of *P. aeruginosa* cells was attributed to an increased  
473 membrane permeability due to the effect of reactive species in PAW. The 72 h mature biofilms  
474 were found to be most resistant against PAW treatment compared to younger biofilms. The  
475 observed reduction in the number of bacterial cells indicate the potential of the PAW as a means  
476 for reduction of *P. aeruginosa* planktonic cells as well as biofilms grown on stainless-steel  
477 contact surfaces. Results of this research demonstrate that this novel methodology can be  
478 effectively used as an environmentally friendly method to inhibit MIC. A potential way to  
479 apply this technology in a practical way to inhibit MIC would be by flushing existing pipe  
480 systems with PAW or by generating PAW inside the pipe system. However, if the former  
481 application method is used then the stability of the PAW between production and application  
482 will have to be determined. Future work will focus on assessing the efficacy of PAW treatment  
483 against other microorganisms causing corrosion in specific applications such as fire  
484 engineering sprinkler maintenance, and pipe system maintenance in the food and medical  
485 industries that contribute to MIC.

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#### 489 **CONFLICT OF INTEREST**

490 The authors declare no conflict of interest.

#### 491 **AUTHOR CONTRIBUTIONS**

492

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

#### 500 **DATA AVAILABILITY STATEMENT**

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

503

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776 **FIGURE LEGENDS**

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

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

788 **FIGURE 3.** Reduction of *P. aeruginosa* biofilms of different maturity (24, 48, and 72 h) after  
789 5, 10, 15, and 20 min of exposure during PAW treatment at 1500 Hz. Each bar represents the  
790 mean value of three replicates, each of which was analyzed twice. The error bars represent the  
791 Standard Deviation for Mean (n = 6). The values with uppercase letters followed by the same  
792 lowercase letters did not differ significantly between the exposure times ( $P > 0.05$ ). The values  
793 with uppercase letters followed by different lowercase letters differed significantly between  
794 exposure times ( $P < 0.05$ ). Asterisk (\*) indicates the values which were below the detection  
795 limit (2.00 Log CFU cm<sup>2</sup>-1)

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

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

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

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

811  
 812 **TABLE 1.** Physicochemical parameters and reactive molecular species in PAW after 15 min  
 813 exposure at 1500 Hz

Treatments	pH	EC (mS cm <sup>-1</sup> )	H <sub>2</sub> O <sub>2</sub> (mg ml <sup>-1</sup> )	NO <sub>2</sub> <sup>-</sup> (mg ml <sup>-1</sup> )	NO <sub>3</sub> <sup>-</sup> (mg ml <sup>-1</sup> )
Tap water*	7.56±0.02 <sup>A</sup>	230.667±2.73 <sup>A</sup>	-	-	-
PAW	5.94±0.04 <sup>B</sup>	325.333±11.64 <sup>B</sup>	0.03±0.002	0.04± 0.001	0.04±0.001

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

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

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

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820 **TABLE 2.** Model parameters and associated errors for biofilm cells of different maturity (24,  
 821 48, and 72 h) after 5, 10 and 15 of exposure to PAW treatment at 1500 Hz

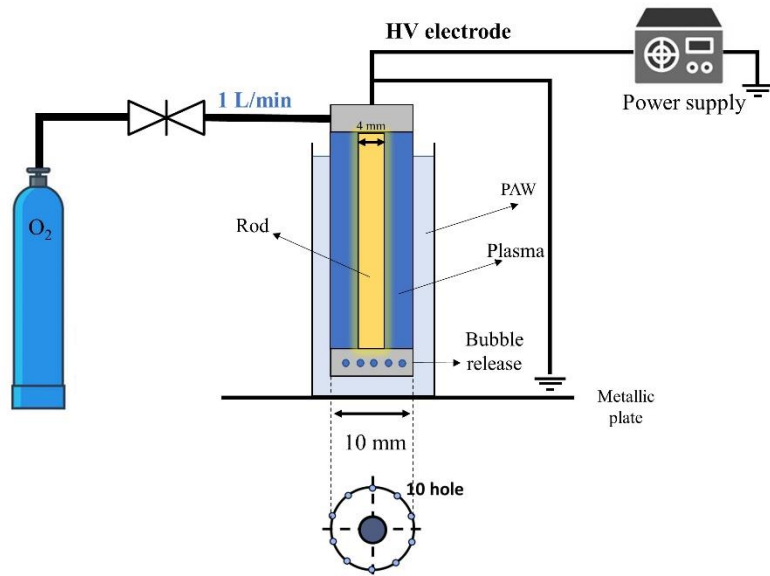
Biofilm maturity (h)	Parameters							
	$D_T$	SE	$\log N_o$	SE	Mean Sum of $R^2$	$R^2$ Root Mean Sum	$R^2$	$R^2$ adjusted
24	3.11	0.13	5.51	0.53	0.3965	0.6296	0.9417	0.9125
48	4.34	0.09	5.67	0.46	0.3507	0.5922	0.9253	0.9005
72	4.19	0.07	6.21	0.36	0.2102	0.4585	0.9548	0.9445

822  
 823 **TABLE 3.** Model parameters and associated errors of *P. aeruginosa* planktonic cells after 5,  
 824 10, and 15 min of exposure during PAW treatment at PBR discharge frequencies of 500,  
 825 1000, and 1500 Hz

PBR discharge frequencies (Hz)	Parameters							
	$D_T$	SE	$\log N_o$	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

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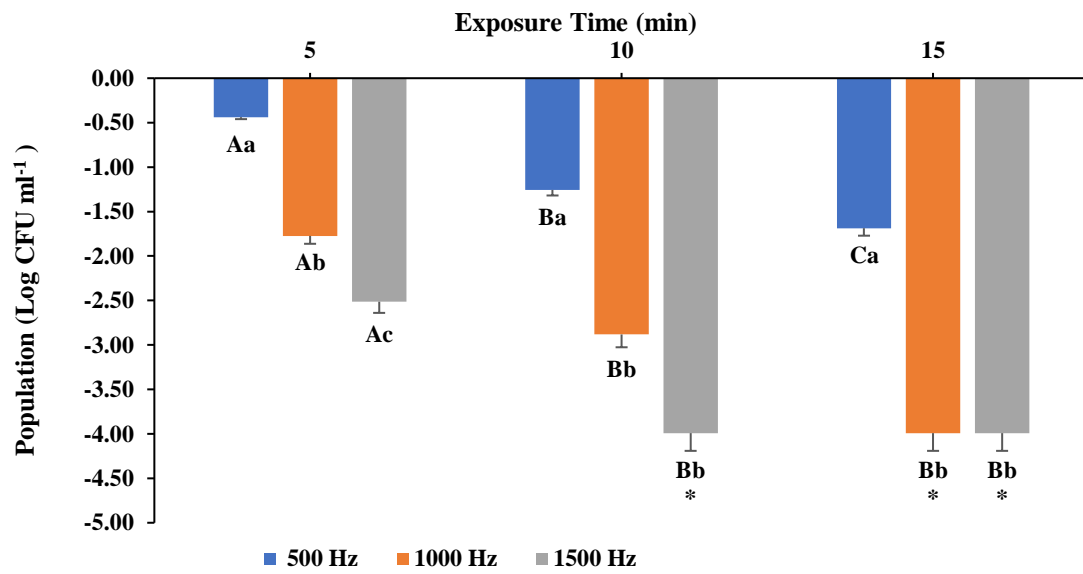
834 **FIG 1**



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836 **FIG 2**



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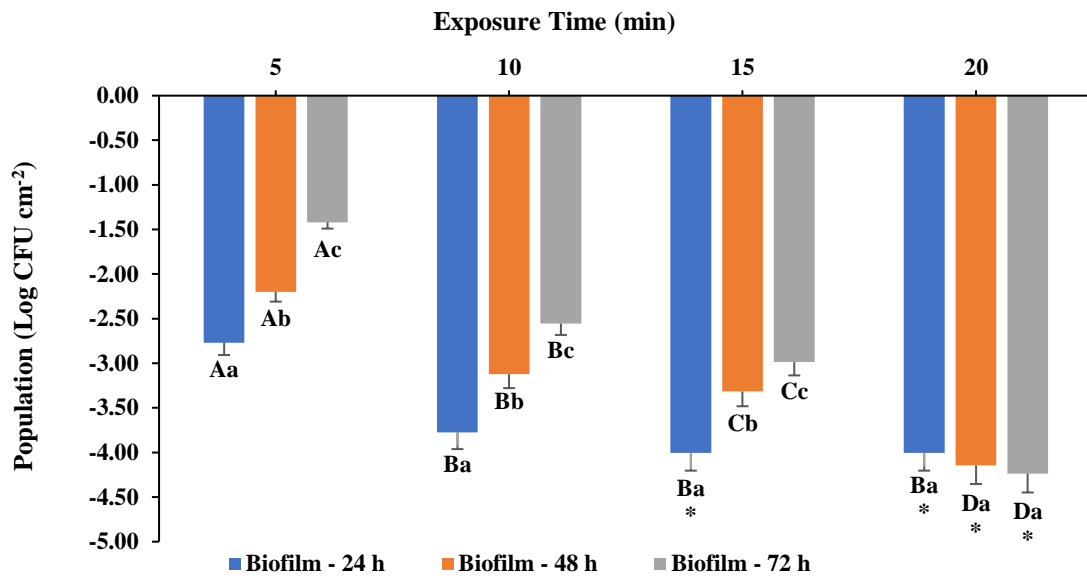
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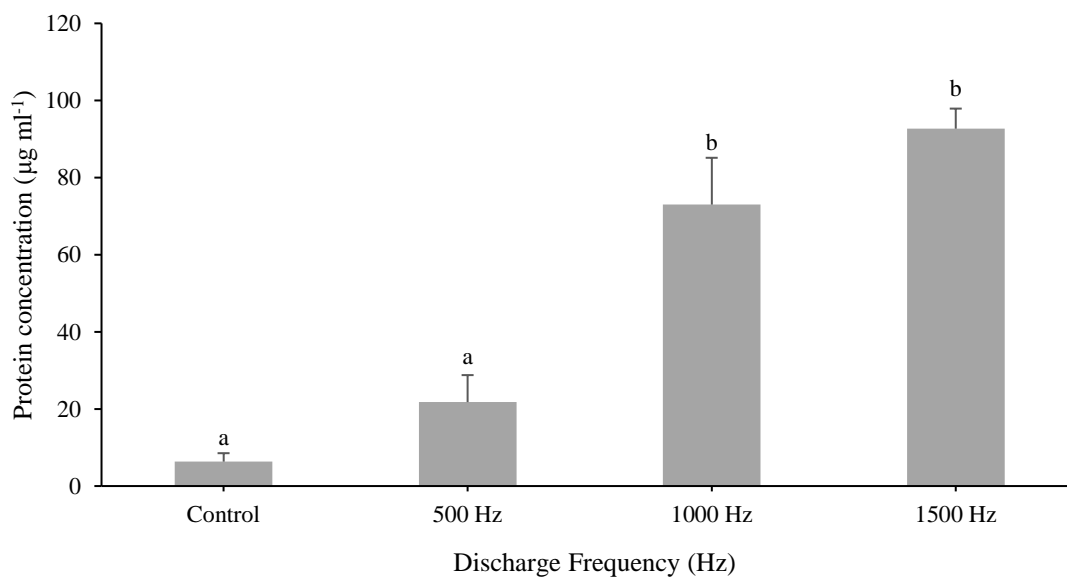
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843 **FIG 3**



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845 **FIG 4**



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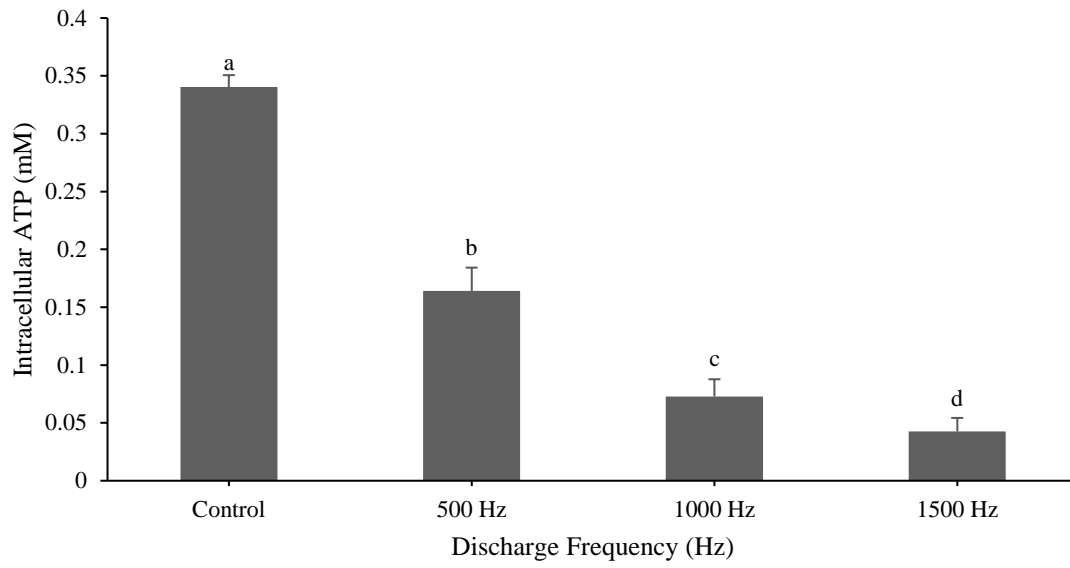
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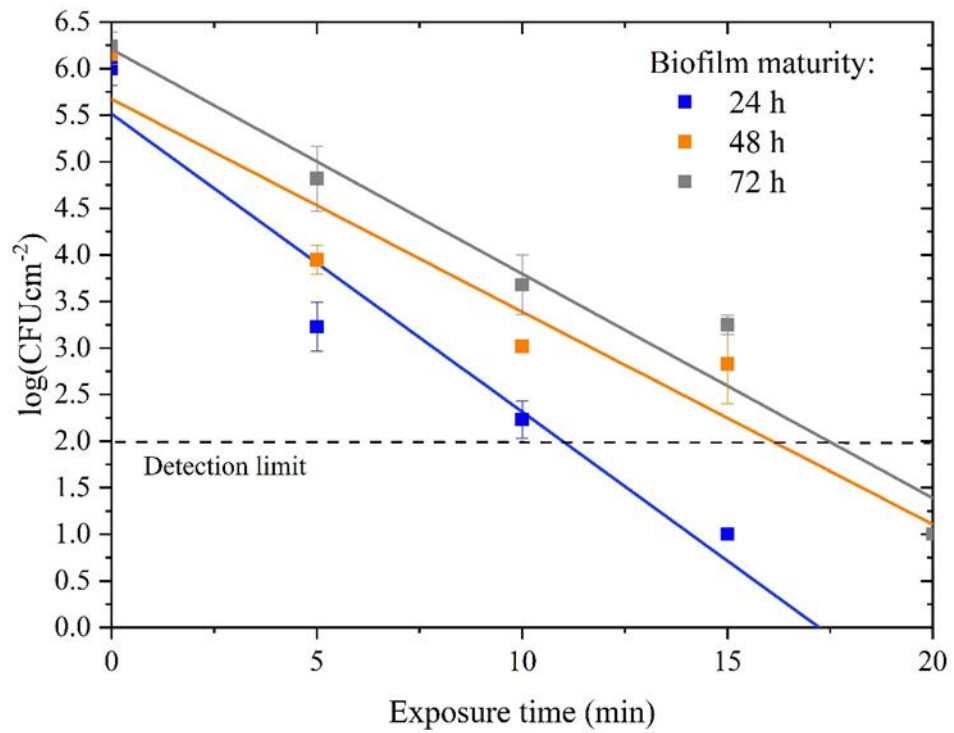
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853 **FIG 5**



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855 **FIG 6**



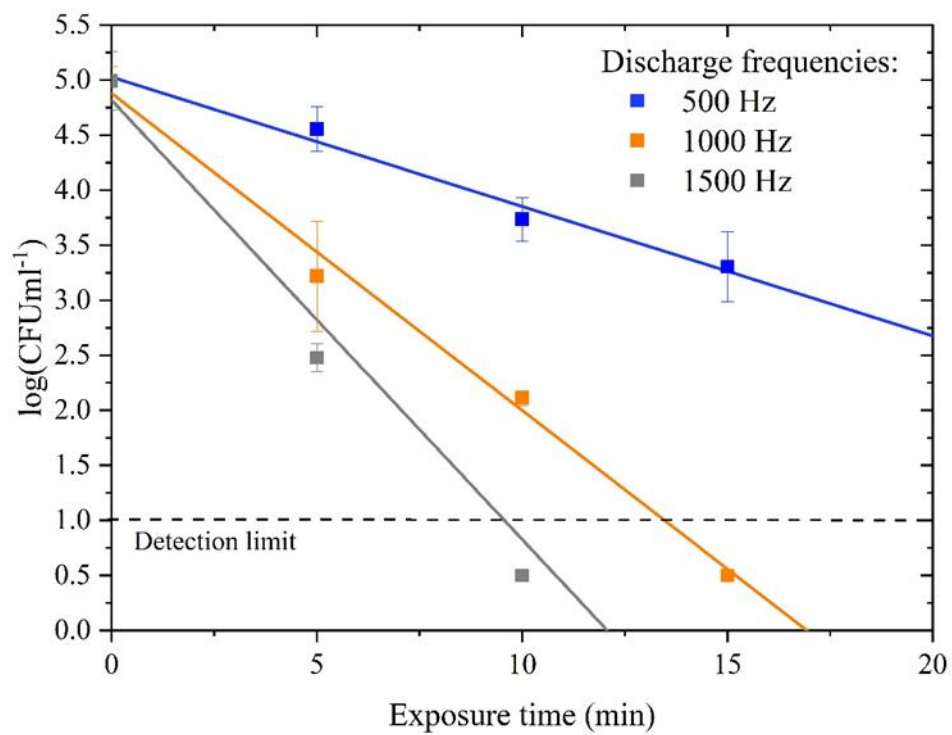
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860 **FIG 7**



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