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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

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,

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_3 (LB-NO_3). The media

105 formulation of the agar medium LB-NO_3 included 10 g tryptone, 10 g KNO_3 5 g yeast extract,

106 and 5 g NaCl per litre of deionised water. $KNO3$ 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_2 entry, L-cysteine at a concentration

109 of one hundred ppm (w/w), was added to the culture medium as O_2 scavenger. The L-cysteine

110 solution was filter-sterilised using MF-Millipore^{TM} 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_2 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_2 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_3 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^{4-5} Log CFU ml^{-1}. 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⁻

137   1 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   µs 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-µl 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^{-2}.

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-µl 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 10^5 Log CFU ml^{-1}. 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-µs 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-µl 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^{-1}.

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_2O_2, NO_2^-, and NO_3^- 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_2O_2 concentration was determined using the titanium oxysulphate (TiOSO$_4$;

180   Sigma-Aldrich) method. According to that method, TiOSO$_4$ reacts with H_2O_2, and produces a

181   yellow-coloured complex (pertitanic acid). Subsequently, the H_2O_2 concentration was

182   determined spectrophotometrically (Mai-Prochnow et al. 2021b). Nitrite concentration was

183   determined by using the Griess assay, a chemical rection 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_3^-) 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_2O_2, NaNO$_2$ and NaNO$_3$ concentration were prepared to

190   convert the absorbance to H_2O_2, NO_2^-, NO_3^-, 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.

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°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

221   initial population of *P. aeruginosa* control cells without plasma treatment in tap water was 4.99

222   Log CFU ml^{-1}.

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^{-1} 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^{-1}

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^{-1}) (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^{-1} (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$^{2\,-1}$, 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$^{2\,-1}$, 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^{-2}, below the detection limit of 2.00 Log CFU cm^{-2} (Fig 3). However, *P. aeruginosa*

253   48 and 72 h mature biofilms decreased below the detection limit (2.00 Log CFU cm$^{2\,-1}$) after

254   20 min of exposure at PAW treatment, corresponding to >4.15 and >4.24 log reduction of

255   CFUcm^{-2} (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_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

268   ml^{-1}).

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
$$logN = logN_o - \frac{t}{\mathrm{D}_T} \qquad (1)$$

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^{-1}. 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^{-1}. 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_2O_2, 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^{-1}. 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   (OH•), hydrogen peroxide (H_2O_2), ozone (O_3), superoxide (O_2^-), nitric oxide (NO•) and

429   peroxynitrite (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 (OH•), singlet oxygen ($^1O_2$), and superoxide (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 (H_2O_2) monitored in the current study is one of the most common long-

434   lived reactive species found in PAW, which, together with nitrite (NO_2^-) and nitrate (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 NO_2^-, NO_3^-, and H_2O_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^+ 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^2), 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.

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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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^{-1})

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$^{2\,-1}$)

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	H_2O_2	NO_2^-	NO_3^-
		(mS cm^{-1})	(mg ml^{-1})	(mg ml^{-1})	(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

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 ($P < 0.05$).

818

819

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	$logN_o$	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

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	$logN_o$	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

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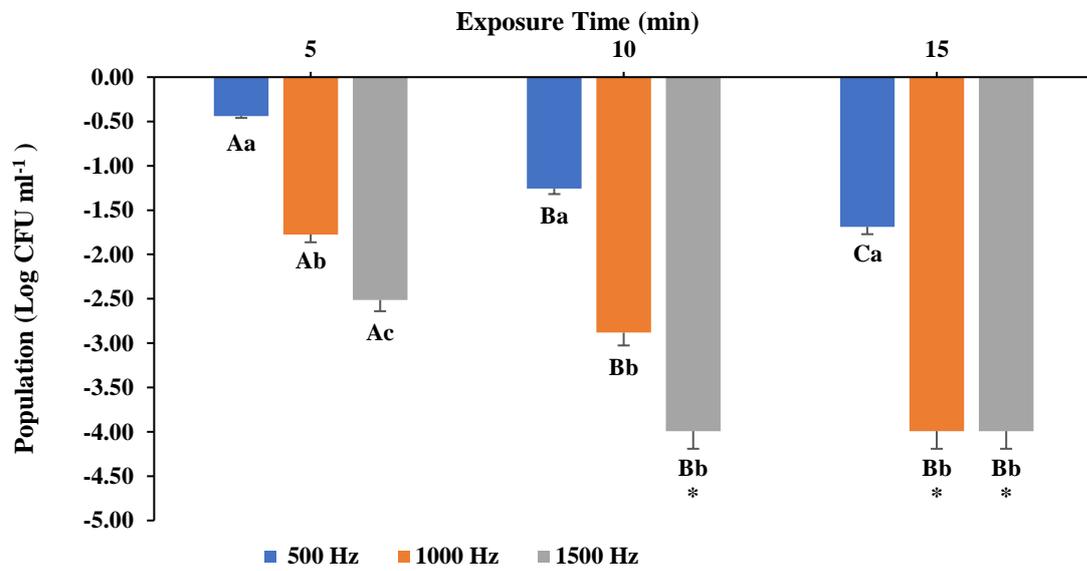
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834 **FIG 1**

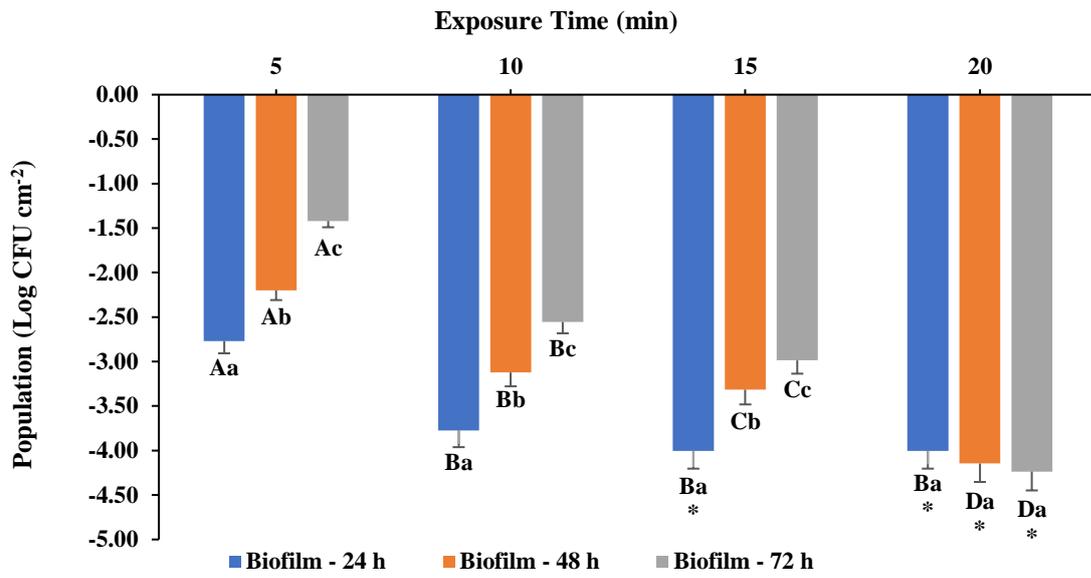835
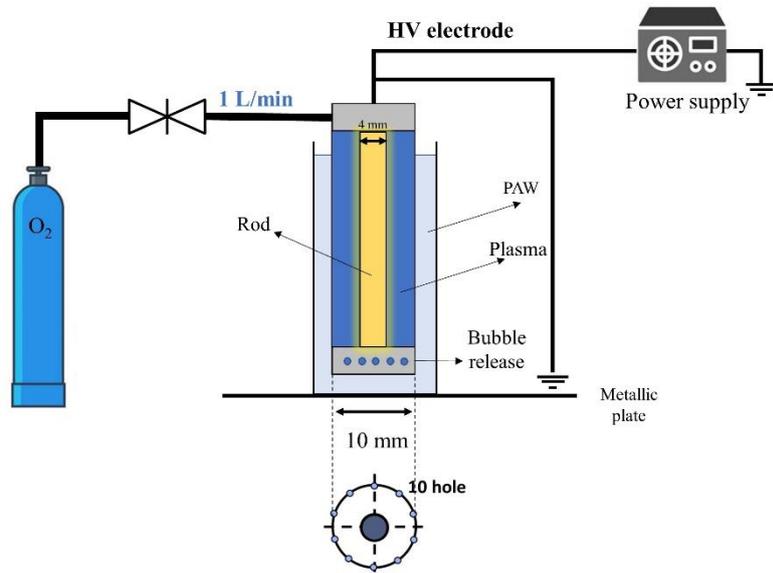
836 **FIG 2**

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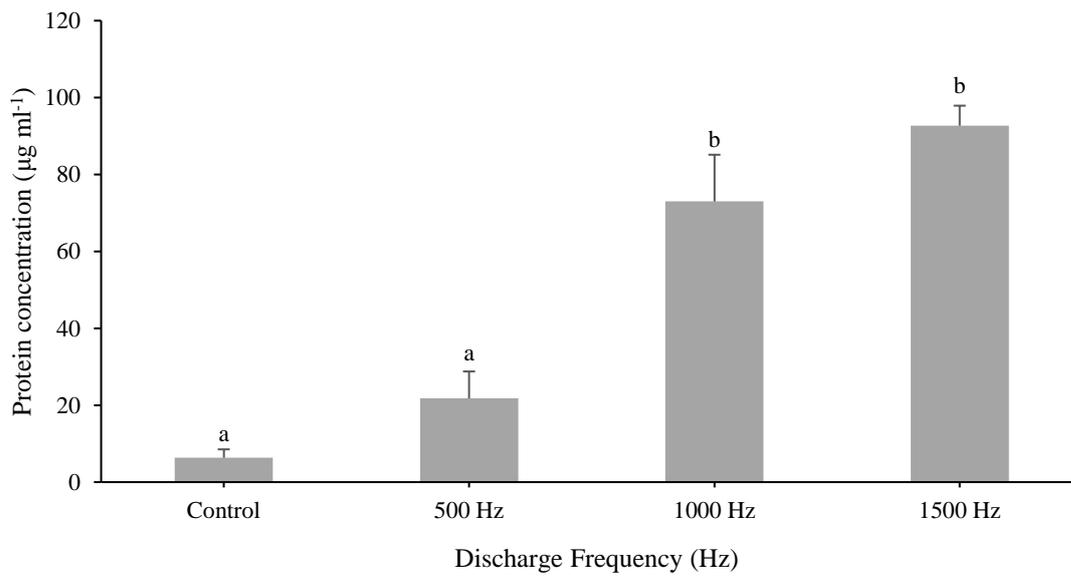
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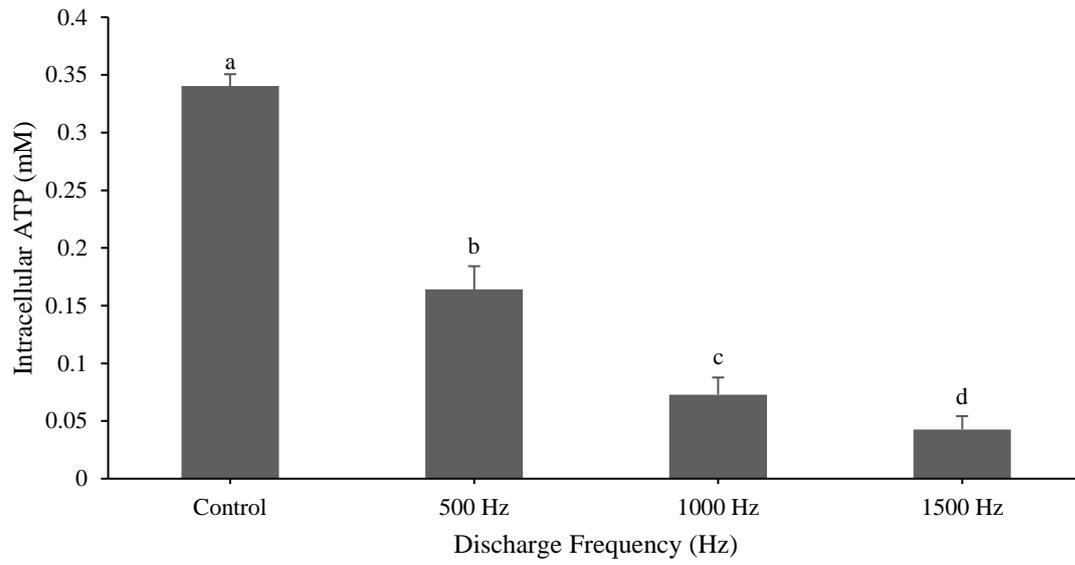843   **FIG 3**

844
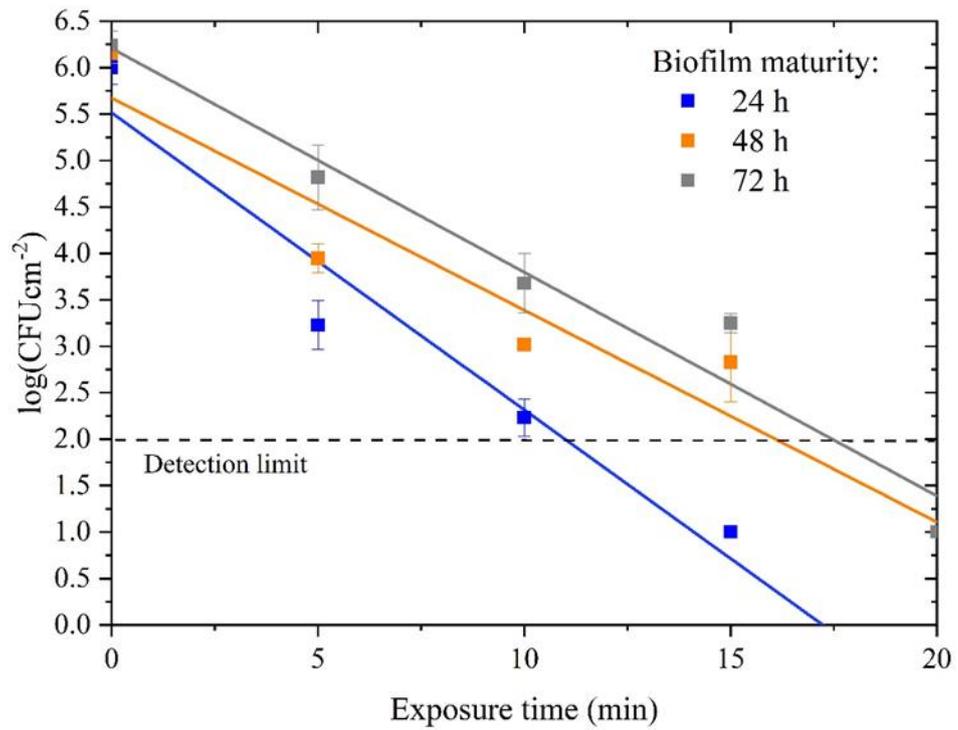
845   **FIG 4**

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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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