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Rapid susceptibility of Carbapenem resistant *Pseudomonas aeruginosa* and its resistance gene to non-thermal plasma treatment in a batch reactor

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ABSTRACT

The critically ranked carbapenem-resistant *Pseudomonas aeruginosa* has been observed to infect immunocompromised patients that consume polluted waters, leading to critical infections and more hospital costs. To save lives and unburden the public health sectors of preventable costs, non-thermal plasma (NTP) technology was investigated as an alternative disinfection step that could be applied in wastewater treatment plants (WWTPs) to inactivate this bacterium and its prominent carbapenem resistance gene (bla_{NDM-1}). Culture and molecular-based techniques were employed to confirm carbapenem resistance in *P. aeruginosa* (27853). Culture suspensions of carbapenem-resistant *ATCC P. aeruginosa* (16 h culture) were prepared from confirmed isolates and subjected to plasma treatment at varying time intervals (3 min, 6 min, 9 min, 12 min and 15 min) in triplicates. The plasma treated samples were evaluated for re-growth and the presence of bla_{NDM-1}. The treatment resulted in a 0.68 log reduction after 3 min and the highest log reduction of ≥8 after 12 min, suggesting that plasma disinfection has a great potential to be an efficient tertiary treatment step for WWTPs. Moreover, the gel image showed that band intensity of bla_{NDM-1} reduced with treatment time, thereby suggesting a probable reduction of amplified genes. Notwithstanding, longer treatment time, a grounded electrode with a larger surface (≥ 40 mm diameter) and/or oxygen-containing feeding gas is warranted to completely inactivate its antibiotic resistance gene (ARG), which might be bound by biofilms as they seem to protect *P. aeruginosa* from the action of non-thermal plasma (NTP) disinfection.

1. Introduction

Wastewater treatment plants (WWTPs) are regarded as important hotspots for spreading antibiotic resistance in different bacteria, when compared to other water environments [1]. This is because conventional WWTPs were not designed to remove the antibiotic resistant bacteria (ARB) and genes (ARGs) [2] that are often reticulated from the sewage systems of households, healthcare services, antibiotic manufacturing facilities, agricultural activities and animal feedlots [3–5]. Moreover, conventional disinfection processes, such as chlorination, UV irradiation and ozone oxidation, which are applied in WWTPs, only kill a great fraction of ARB, whereas others enter a state of dormancy due to stress and are resuscitated when the stressors are released. Sometimes, they decrease the abundance of the genes (gene copies per mL of sample) while the prevalence of the gene (gene copies per total bacteria) increases [6,7]. Whilst some of the disinfection processes kill the bacteria, ARGs may still persist for a long time, after discharge from WWTPs in cell debris and in the environment, transferring and adapting into microbiota of natural water bodies (drinking water sources), eventually leading to the development of antibiotic resistance [6,8–10]. As a result, WWTPs keep recycling drinking water polluted with high concentrations of ARB and ARGs, back to municipal water supplies and health care facilities [11]. *Pseudomonas aeruginosa,* a carbapenem-resistant bacteria that has been categorised as critical priority 1 class by the World Health Organisation (WHO) [12,13], is an example of an ARB found in the effluent of WWTPs, in lakes, rivers and swimming pools [1]. Moreover the compromise of the clinical efficacy of carbapenems, such as meropenem and imipenem is due to the spread of the carbapenemase New

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Delhi metallo-β-lactamase-1 plasmids (bla_{NDM-1}), which also induce *P. aeruginosa* multidrug resistance to other antibiotics [14].

P. aeruginosa, is a Gram-negative, aerobic bacteria that is also considered as a facultative anaerobe [15–17]. It survives the most diverse environments, spreading quickly in a new habitat, as it has minimal nutritional needs and is able to get energy from a variety of carbon sources [15,17]. In municipal drinking water distribution systems, it proliferates forming biofilm [1]. Apart from the presence of bla_{NDM-1}, *P. aeruginosa* carries a variety of virulence factors, such as the type III secretion system, which enhances disease severity by injecting effector proteins into host cells [15,17]. In hospital settings, it causes infections in patients in the intensive care units (ICU) or in patients with serious underlying disorders, such as cystic fibrosis (CF) or suppressed immune system, infection of the urinary tract, ear, sinuses, wounds, skin, connective tissues and pulmonary disease [15,18]. Even with the adoption of control measures [18], infections continue to become more complex and difficult to treat and are associated with significant hospital and societal burdens. Moreover, infections eventually lead to high morbidity and mortality rate [15,17,18] as compared to infections caused by members of Enterobacteriaceae or other non–lactose fermenting Gram-negative bacilli [15,17].

Considering that (i) *P. aeruginosa* is intrinsically resistant to many commonly used antibiotics and additionally acquires resistance by horizontal gene transfers or mutation [1]; (ii) *P. aeruginosa* is resistant to the last resort antibiotics (carbapenems) [19,20]; (iii) second-line treatment options for *P. aeruginosa* are often accompanied by toxicity and are less defined in their efficacy [21] and; (iv) *P. aeruginosa* in healthcare settings, environment and water sources prevents control of its acquisition among the most vulnerable patients [15], elimination of an environmental reservoir could be the answer to controlling some of the outbreaks [22]. The use of conventional treatment methods and even other advanced oxidation processes have been evaluated in a previous study, where the merits were observed to outweigh any drawbacks [23]. Hence, for the use of an Advanced Oxidation Process (AOP), recent studies have demonstrated the ability of ozone-electrolyzers, photo-electro and electro-disinfection to achieve logarithmic removal of *Klebsiella pneumoniae* and considerable attenuation of its ARGs from hospital urine and wastewater, respectively [24,25]. In consequence of the above, this study adopted a Non-Thermal Plasma (NTP) treatment technique, which we hypothesized that it would facilitate rapid elimination of ARB and attenuation of its ARG. It was also presumed that it could serve as an alternative disinfection step in WWTPs, as it is able to break down organic matter while causing irreversible damage to cells and inactivating ARB (in this case *P. aeruginosa,* carbapenem-resistant) and its ARG [26,27].

2. Materials and method

2.1. Non-thermal plasma reactor

The reactor adopted in this study involved a configuration comprising a designed air-tight machined Polytetrafluoroethylene (PTFE), copper electrodes and high voltage cable, assembled and fitted to a 250 mL capacity Duran Schott glass bottle. The reactor was powered with a high voltage direct current (HVDC) developed by Jeenel Technology Services Pty Ltd. in South Africa, having a maximum capacity of 40 kV and 15 mA (Fig. 1). A flat copper disk functioned as the ground electrode, with a 50 mm gap between it and the hollow copper electrode. The gap between the hollow copper electrode and the surface of the solution was 15 mm. The voltage on the HVDC was set at 23 kV to ignite the plasma but was regulated to 10 kV with a constant current of 0.7 mA, sustaining a 7 W discharge power throughout the treatment.

2.2. Organism and chemicals

The bacterial strain used in this study (ATCC *P. aeruginosa* (27853)) was obtained from Laboratory Specialties PTY LTD Trading as Thermo Fisher Scientific in Randburg, South Africa. Imipenem antibiotic, Luria Bertani (LB) broth and LB agar plates were procured from Sigma Merck, South Africa. Nucleomag DNA/RNA Water kit was purchased from Separations in Randburg, South Africa and primers were delivered by Inqaba in Pretoria, South Africa.

2.3. Antibiotic screening and non-thermal plasma treatment

2.3.1. Antibiotic screening

The preliminary screening of *P. aeruginosa* for antibiotic resistance was performed using culture-based methods [28]. The bacteria were inoculated into Luria Bertani (LB) broth and orbitally incubated at 37 °C, 160 rpm for 24 h. Thereafter, the cultures were successively screened with increasing gradients of imipenem (2 μg/mL, 4 μg/mL, 6 μg/mL, 8 μg/mL, 12 μg/mL and 24 μg/mL), which were supplemented in LB agar and incubated at 37 °C for 24 h [29,30]. The growth of *P. aeruginosa* on the plates supplemented with imipenem confirmed their resistance to carbapenems. The isolate that grew on the plates supplemented with 24 μg/mL imipenem antibiotic was used as the standard carbapenem

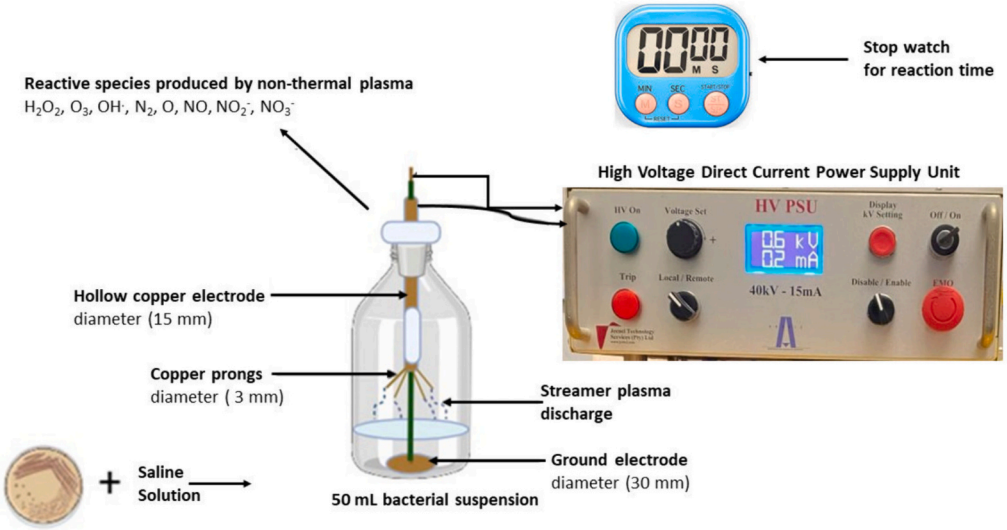

Fig. 1. Schematic diagram of the non-thermal plasma experimental setup, showing all variables.

resistance bacteria strain (CRBS) for the inactivation experiment in this study.

2.3.2. Non-thermal plasma treatment

The standard *P. aeruginosa* strain underwent a series of procedures. It was initially introduced into nutrient broth and incubated at 37 °C, 160 rpm for 16 h to minimize biofilms. After incubation, bacterial pellets were collected, washed, and re-suspended in saline. The resulting solution was plated on LB agar with 24 μg/mL imipenem, yielding an average plate count of 3.0×10^9 CFU/mL before plasma treatment. Plasma exposure involved treating a 50 mL bacterial suspension at different time intervals (3, 6, 9, 12, and 15 min) in triplicates. Post-plasma treatment samples were plated on LB agar with 24 μg/mL imipenem and incubated at 37 °C over 48 h. Colony-forming units per mL were then estimated for log reduction calculations. Additionally, to explore copper's potential antimicrobial properties, bacterial suspensions were treated with the copper electrode alone for 15 min, referencing studies by [31,32].

2.4. Physical chemical and structural characterization

The discharge characteristics and chemical species formed during treatment were assessed using the Black Comet C-25 Spectrometer (StellarNet, Inc.). The H_2O_2 and nitrite/nitrate ions recorded unmeasurable absorbances at <350 nm, hence the Lovibond® SpectroDirect water testing instrument (Tintometer Group, Germany) was used to accurately determine the concentration of (H_2O_2), (NO_2^-) and (NO_3^-) after treating the bacterial suspensions with NTP for 3, 9 and 15 min [33]. The resultant bacterial suspensions (10 mL) were assayed for NO_2^- and H_2O_2 concentrations measurement using Titanium Tetrachloride reagent and N-(1-Naphthyl)-ethylenediamine, respectively. For NO_3^- concentrations measurement, 0.5 mL of the resultant bacterial suspensions was assayed using 2,6-Dimethylphenole reagent. The reactions were monitored spectrophotometrically after respective time intervals specified by the user manual [34]. The pH of the bacterial suspension before the plasma treatment and after 3, 6, 9, 12 and 15 min of treatment were measured using PL-700AL pH meter. The conductivity of the bacterial suspension before the plasma treatment and after 3, 6, 9, 12 and 15 min of treatment were determined using a WTW – Portable conductivity meter ProfiLine Cond 3310 (Einzelgerät, Zubehör).

2.4.1. Scanning electron microscopic analysis

Bacterial suspensions were treated with NTP, and both pre- and post-treatment, underwent centrifugation to discard the supernatant and recover pelleted cells. The cells were washed with a phosphate buffer to remove serum and media, followed by fixation using a 2.5 % Glutaraldehyde/Formaldehyde solution for 1 to 24 h. Subsequently, the fixed pellets were subjected to multiple washes with phosphate buffer and post-fixed with a 1 % Osmium Tetroxide (OsO4) solution for 1 h. Dehydration was achieved through a graded ethanol series (30 %, 50 %, 70 %, 90 % and 3× 100 %), and a mixture of Hexamethyldisilazane (HMDS) and ethanol was applied before drying. The samples were then mounted on aluminium stubs, coated with carbon, and examined using a scanning electron microscopy (SEM) (Zeiss Gemini Ultra Plus FEG-SEM (Field Emission Gun – Scanning Electron Microscope) with BS, energy dispersive spectroscopy (EDS) and Electron Backscatter Diffraction (EBSD) detectors).

2.5. Molecular analysis

The extraction of DNA was conducted both before and after the plasma treatment of the bacterial suspension, using the NucleoMag® DNA/RNA Water extraction kit, according to the manufacturer's instructions. The extracted DNA served as the template for the PCR assay, aiming to validate the presence of the blaNDM-1 gene in *P. aeruginosa* (Table 1).

The amplification of the bla_{NDM-1} gene was carried out using a 10 μL PCR mixture, consisting of Taq 2× Master Mix (New England Biolabs), forward and reverse primers (0.5 μM), template DNA (2.5 μL), and Milli-Q water (1.5 μL) in a BIO RAD T100 Thermal cycler. The reaction conditions included initial denaturation (95 °C for 3 min) and 30 cycles for denaturation (95 °C for 1 min), annealing (55 °C for 1 min), and extension (72 °C for 1 min 30 s), and a terminal extension (72 °C for 10 min). The ethidium bromide-stained PCR products were visualized through electrophoresis in a 1.5 % agarose gel [36,37], employing the BIO RAD PowerPac basic with Mini Sub Cell GT. Gel electrophoresis facilitates the efficient separation of DNA fragment and ethidium bromide enables DNA fluorescence and detection under UV light [38]. This method allowed for the observation of gene presence or absence on gel images, providing insights into the effectiveness of plasma treatment in inactivating resistance genes [35].

3. Results and discussion

3.1. Physical chemical and structural characterization

Reactive nitrogen species (RNS) and reactive oxygen species (ROS) play an important role in the inactivation of bacteria [39,40]. During NTP treatment, the electric field discharges ROS, such hydroxyl (OH) and hydrogen peroxide (H_2O_2) [41,42] and RNS, such as nitrites (NO_2^-) and nitrate (NO_3^-) [41–43] were produced in both the adjoining liquid and gaseous media [42]. The oxygen (O_2) and nitrogen (N_2) lines (316 nm, 337 nm, 404 nm), independently underwent electron impact ionization reactions, resulting in electrons and positive ions (O_2^+, N_2^+), that split and eventually increased and spread the streamer (Fig. 2). Then the NO lines (227 nm, 239.5 nm), O^+ lines (327 nm, 435.5 nm, 464.5 nm), N^+ lines (344 nm, 395.5 nm) and O lines (777 nm) were generated in the gas phase [44]. In wet air or in liquids, the •OH lines (307,5 nm, 309 nm), H_2O_2 and ozone (O_3) were produced [43,44]. The nitrogen oxides (NO) dissolved in water forming NO_2^- and NO_3^- [42]. The •OH caused impairment to DNA, as it has strong oxidation potential (2.8 V) that is greater than the conventional disinfectants, chlorine (1.36 V) and ozone (2.07 V) [45–49]. Among ROS, •OH have the greatest oxidation potential, are the most reactive, and are considered to play a crucial role in NTP bacterial treatment [43,50,51]. Correspondingly, the •OH radical causes irreversible damage to cells and inactivates ARBs and ARGs, as it has diverse impact on normal protein structure which is one of the primary targets in bacteria during disinfection, including oxidation of amino acids, modification of sulphur groups [26,42]. The (H_2O_2), (NO_2^-) and (NO_3^-) had a relatively long lifetime and could react to secondary products post-discharge. The post-discharge reactions between the by-products occurring in plasma activated water (PAW) might result in the generation of peroxynitrous (HNO_3)/peroxynitrite ($ONOO^-$) acid, which significantly participates in the antibacterial activity of PAW. These long-lived species [52,53] have been observed to inactivate cells even after the discharge had been switched off [54]. The long-term, post-plasma effect of the PAW was mainly caused by the reaction

Table 1
Primer used in this study.

Name	Forward primer (5′ – 3′)	Reverse primer (5′ – 3′)	Size (bp)	Reference
bla_{NDM-1}	GGTGCATGCCCGGTGAAATC	ATGCTGGCCTTGGGGAACGS	660	[35]

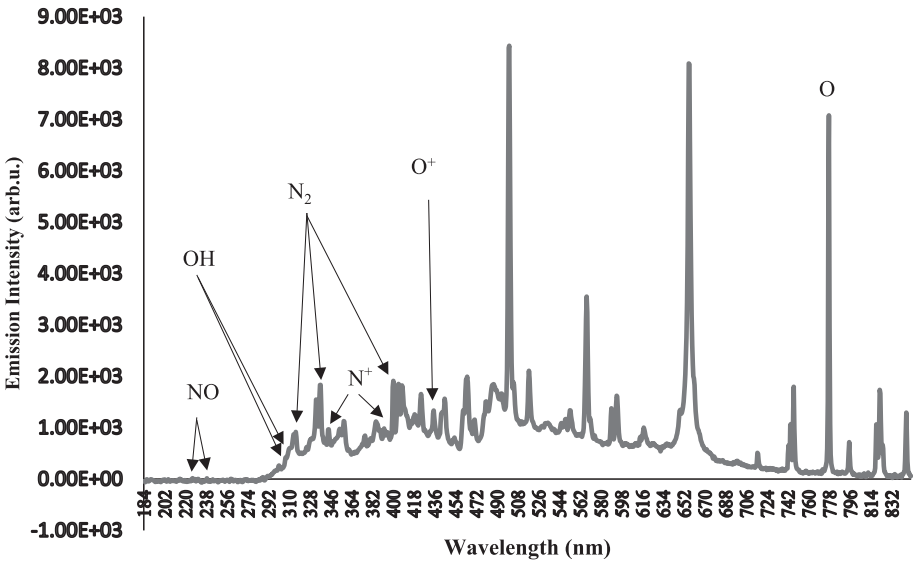

Fig. 2. Optical emission spectra from hydroxyl radical OH species in NTP during treatment of *P. aeruginosa.*

between ozone and H_2O_2 during the peroxone process that forms •OH [51]. The concentration of these species increased with time during NTP treatment of *P. aeruginosa*, as can be seen in Fig. 2b-d. The highest concentrations of the species were observed when no more re-growth of *P. aeruginosa*.

Nitrates (NO_3^-) and nitrites (NO_2^-) were incrementally discharged into the solution with time and the highest concentration of 6.5 ± 0.325 mg/L and 1.9 ± 0.095 mg/L of NO_3^- (Fig. 3a) and NO_2^- (Fig. 3b), respectively, were observed at 15 min. Another study confirmed an increase in the concentration of NO_3^- with treatment time, although a much higher concentration of 41.41 mg/L of NO_3^- and 5.27 mg/L of NO_2^- was attained after the same treatment time. However, the result was from treating deionised water only with a dielectric barrier discharge configuration [55]. The oxidation of nitrite to nitrate [56] may have contributed to there being more NO_3^- than NO_2^- in this study.

There was no measurable concentration of hydrogen peroxide (H_2O_2) observed at 3 min; however, 0.15 ± 0.0075 mg/L and 0.36 ± 0.018 mg/L concentrations were observed at 9 and 15 min (Fig. 3c). A study resulted in the formation of H_2O_2 which was immediately observed after plasma treatment but the concentration decreased with time of incubation [42], another study resulted in nil production of H_2O_2 after plasma treatment [55]. Plasma treatment usually results in quadratic or linear increase of H_2O_2 with treatment time but the cells in the medium uptake H_2O_2 with incubation time [42,55].

The pH was reduced to 3.85 ± 0.1925 from 7.12 ± 0.356 after 15 min of plasma treatment (Fig. 3d). This outcome is similar to other studies, which also treated water for 15 min with plasma, both achieving pH of 3.85 and 3.78, respectively [55]. The reduction in pH was caused

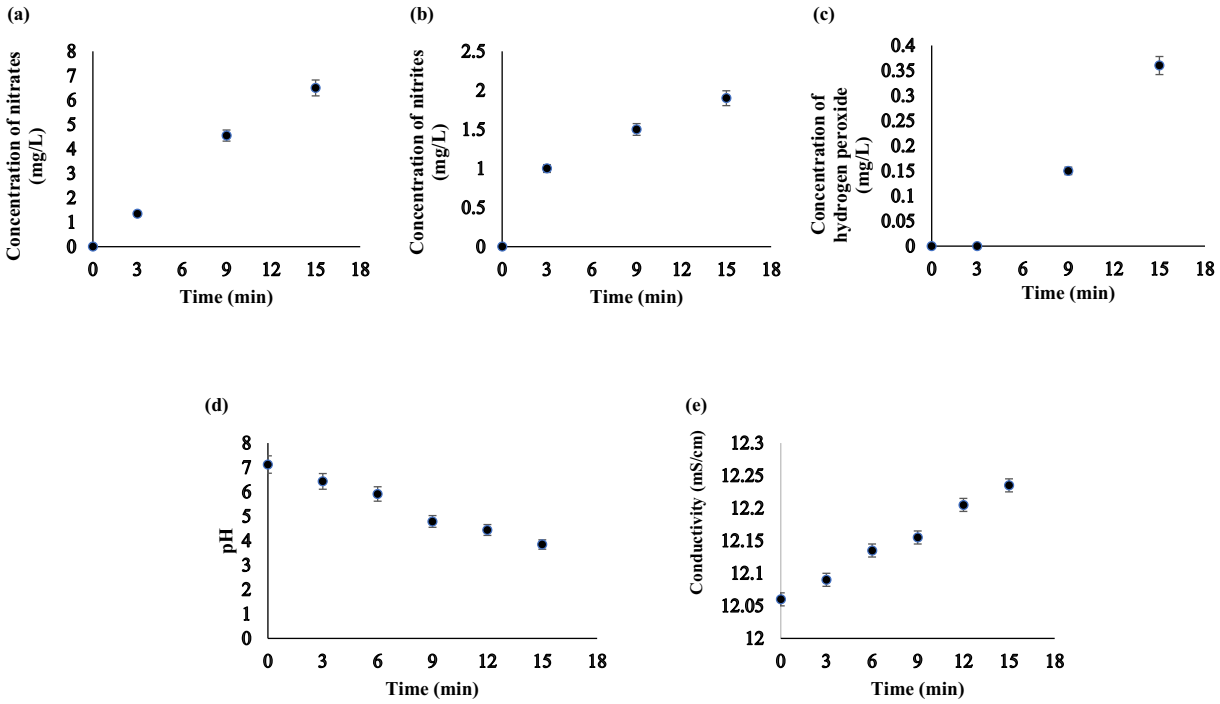

Fig. 3. Concentration of (a) nitrates, (b) nitrites and (c) hydrogen peroxide during non-thermal plasma treatment of *P. aeruginosa*. (d) pH and (e) Conductivity readings of *P. aeruginosa* bacterial suspension during non-thermal plasma treatment. NB: data are mean of duplicate readings.

by the presence of nitrates and nitrites in the suspension, which led to the generation of HNO_3. The low pH keeps the oxidizing potential of ozone at 2.08 V, which can decrease to 1.4 V under alkaline conditions [56,57]. The production of hydrogen radicals also increased under acidic conditions, which then reacted with H_2O_2 and H_2O to produce more •OH. An acid pH range of 3–4 is said to be conducive for production of •OH [51] and the reactive molecules readily penetrate cell walls under the low pH conditions because the cell membrane permeability increases [43].

The conductivity was 12.06 ± 0,603 mS/cm before treatment; it increased to 12.24 ± 0.612 mS/cm after 15 min of plasma treatment (Fig. 3e). The conductivity of water tends to vary as a result of the ROS and RNS produced during NTP treatment. This is evident as one study resulted in an increase of conductivity from 1 μS/cm to 123 μS/cm over 15 min treatment time [55] while in another study the conductivity fluctuated between 2.57 mS/cm to 3.31 mS/cm over 30 min NTP treatment [58]. The conductivity in this study increased with treatment time, but the increment was low, perhaps because a low conductivity is the one that favours the production of H_2O_2 and O_3, which contribute to the destruction of the pollutants [57,59]. The increase in conductivity is an indication of a loss in cell membrane integrity of bacteria [60].

3.2. Inactivation of ARB and ARG

3.2.1. Re-growth assessment

The observed trend indicated an escalating log reduction of *Pseudomonas aeruginosa*, implying a logarithmic inactivation of ARB with extended treatment time (9.48 ± 0.474 after 12 and 15 min) (Fig. 4). The close to 100 % reduction attained in this investigation indicates that NTP can be used as an alternative disinfection step (Table 2).

Isolation of copper on its own yielded the smallest log reduction (0.15) of *P. aeruginosa*, potentially due to the solid state of the copper employed in this study. Soluble copper is acknowledged for its high toxicity and reactivity, owing to oxidative power, catalyzed ROS formation (i.e., H_2O_2), leading to lipid peroxidation and DNA/RNA damage, ultimately resulting in bacterial inactivation [61,62]. A comparative study of copper compounds (copper oxide, copper acetate, copper nitrate, and copper sulfate), all possessing bactericidal properties, revealed that copper in salt form exhibited the highest antimicrobial effectiveness [31]. Moreover, considering the low capacity of Cu^{2+} to stimulate bacterial membrane and nucleic acid destruction through copper-catalyzed Fenton-like reactions (driven by the reactions of Cu^{2+}/H_2O_2) [63], it was therefore reasonable to deduce that the copper electrode had negligible impact on the reduction of *P. aeruginosa*. Consequently, the observed log reductions were attributed solely to the plasma discharge. The challenging characteristics of *P. aeruginosa* as a Gram-negative bacterium that thrives in biofilms likely contributed to the limited efficacy of solid copper in its inactivation [61].

3.2.2. Evidence of cellular disintegration

The rod-like shape disseminated by SEM imaging confirmed the structural integrity of *P. aeruginosa* cells before NTP treatment (Fig. 5) [64].

Following NTP treatment, SEM images indicated a loss of structural integrity on the surface of *P. aeruginosa* cells (Fig. 6). The cellular remnants of *P. aeruginosa* assumed an amorphous shape, deviating from their usual rod-like structure observed before NTP treatment. This observation highlighted NTP's capability to overcome the tensile strength of the cell membrane, leading to rupture, leakage of intracellular components, and cell death. These effects effectively impeded the growth of *P. aeruginosa* and negated its protection of ARGs [43]. The rupturing phenomenon was attributed to the accumulation of ROS and RNS free radicals on the cell membrane, surpassing the membrane's tensile strength and causing it to rupture [43,65]. Notably, NTP appears to have a greater inhibitory effect on gram-negative bacteria than gram-positive bacteria, as demonstrated by its ability to disrupt the membrane of *P. aeruginosa*, a gram-negative bacterium [43,66]. A study compared SEM images of Gram-negative and Gram-positive cell after plasma treatment, the destruction was more evident on Gram-negative as there was cell breakage effects on the Gram-negative and only irregular shape and cell shrinkage of the Gram-positive cells [67]. This susceptibility was attributed to the thinner peptidoglycan layer and the presence of an outer membrane with components such as proteins and lipopolysaccharide (LPS), which are sensitive to ROS in Gram-negative bacteria [43]. A hypothesized dual mechanism for the destruction of cellular membranes involves lipid peroxidation and electroporation. In lipid peroxidation, reactive radicals generated by plasma, particularly OH groups, detach polar head moieties and fatty acid tails from phosphatidylcholine residues in the lipid bilayer of cell plasma membranes. This leads to crosslinks between adjacent fatty acid tails, allowing the unrestricted influx of radicals and water molecules, resulting in membrane lesions and pore formation. Alternatively, electric fields generated by plasma could enhance transmembrane potential, initiating the breakdown of the lipid bilayer and the formation of membrane pores. The recoverability of these pores may vary, and with increased electric field strength and duration, they may become irrecoverable, ultimately causing necrosis and cell rupture [42].

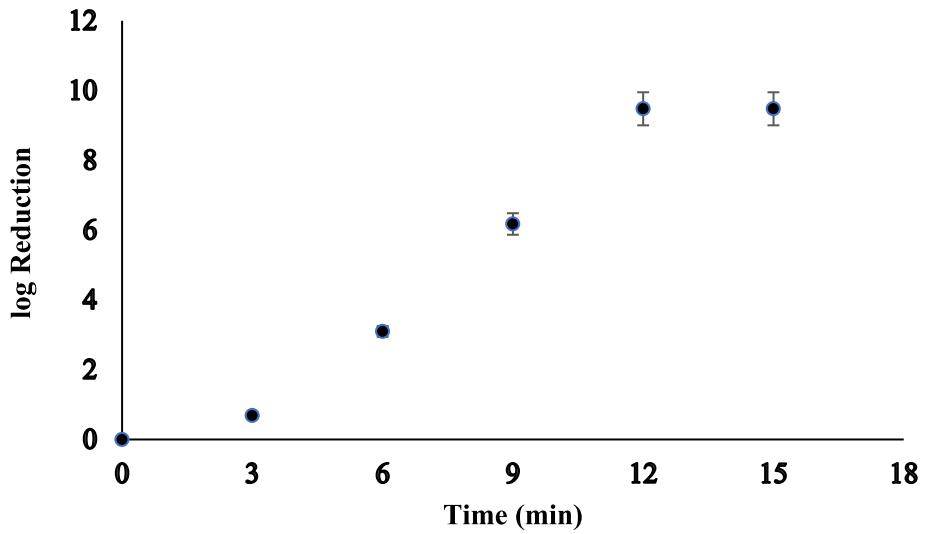

Fig. 4. Log reduction of *P. aeruginosa* (27853) after non-thermal plasma treatment. Where log reduction = $\log_{10}$ (initial CFU/final CFU).

Table 2
Percentage reductions of *P. aeruginosa* (BAA 1605) after non-thermal plasma treatment.

	Control (0 min)	Copper (15 min)	3 min	6 min	9 min	12 min	15 min
CFU/mL	3.0×10^9	2.1×10^9	6.2×10^8	2.4×10^6	3.65×10^5	NG	NG
log reduction	0	0.15	0.68	3.1	6.18	∞	∞
% reduction	0	30	79.3	99.92	≤100	100	100

NB: ≤100 implies that theoretical reduction was 100 % however, the presence few of culturable colonies practically negates occurrence of 100 % reduction. NG implies absence of *P. aeruginosa* growth, whereas ∞ signifies the infinite log reduction of culturable planktonic cells.

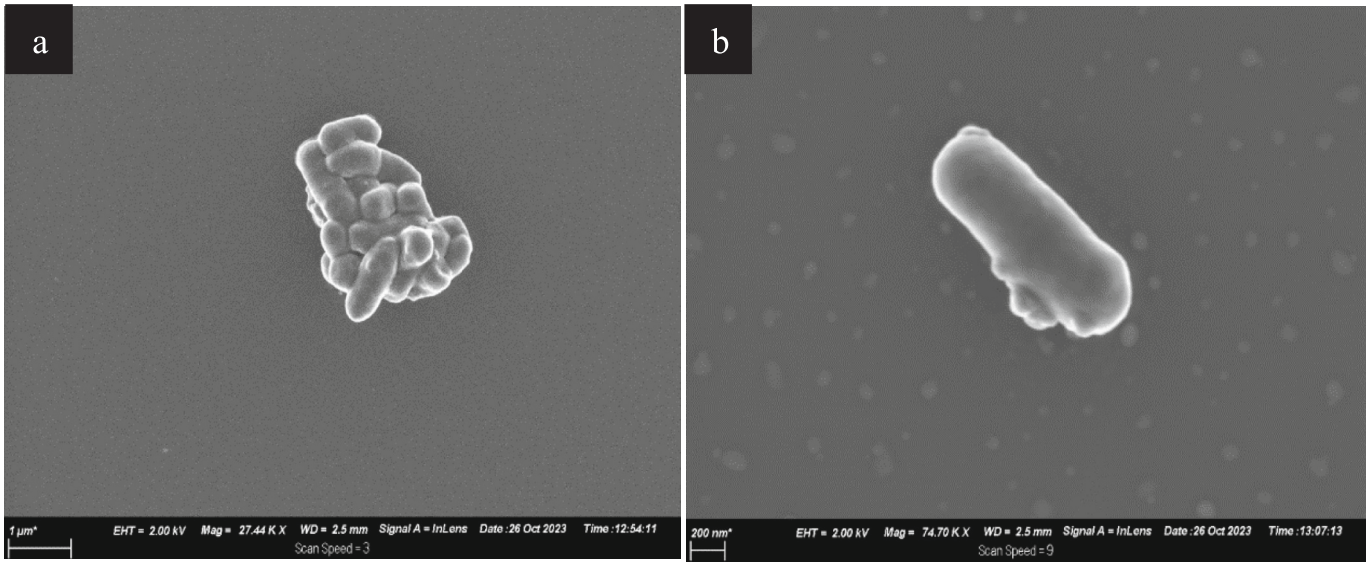

Fig. 5. SEM image of *P. aeruginosa* (a) cell cluster and (b) single cell before non-thermal plasma treatment.

3.2.3. Detection of resistance gene (bla_{NDM-1}) attenuation

In this study, the diminishing intensity of *P. aeruginosa* bla_{NDM-1} (240 bp) bands over time, as depicted in Fig. 7, suggested the capacity of NTP to inactivate ARGs. Fluorescence of the bands is presumed to indicate the DNA concentration [68]. However, at the final treatment duration (15 min), the ARGs were not entirely inactivated, possibly due to the protective biofilm surrounding *P. aeruginosa*. Biofilm-inhabiting bacteria are known to exchange more genes than those in a planktonic/free-living state [69]. The NDM-1 gene has been reported to exhibit increased expression in optimal biofilm formation conditions [70]. Biofilms, with their high cell densities, facilitate intercellular gene transfer due to the short physical distance between microorganisms [69]. The protective nature of the biofilm also hinders NTP reactive species from penetrating it [71]. Despite this, a study utilizing plasma-generated Fenton-oriented reactions (Cu^{2+}/H_2O_2 and Fe^{2+}/H_2O_2) recorded measurable gene copies of bla_{TEM-1} after 10 min, despite enhanced ARG inactivation by Cu^{2+} and Fe^{2+} [63]. This underscores the efficiency of our treatment process, which does not rely on exogenous Fenton-oriented inorganic ions (Cu^{2+} and Fe^{2+}). Comparative studies show UV achieving a maximum reduction of 99 % [72], chlorination achieving 100 % reduction [73], and ozone achieving a maximum of 98.1 % [74] of ARGs. However, the concentrations of these disinfectants were considerably higher and impractical compared to those used in WWTPs, raising environmental concerns due to the formation of harmful by-products like halo-organics [75,76] and bromate [74,77] from chlorine and ozone, respectively. Furthermore, we are of the opinion that extending NTP treatment time might lead to complete inactivation of ARGs, offering the advantage of eliminating the need for additional use of potentially harmful chemicals. Additional parameters, such as increasing the grounded electrode surface or diameter at a fixed discharge gap, could be optimized to enhance the area of plasma discharge. The choice of feeding gas also plays a role, with oxygen-containing gas being demonstrated to lead to the fastest contaminant degradation, followed by air and argon, while nitrogen results in the least removal. Oxygen-containing gas induces the production of O^-based active species and O_3, enhancing the degradation rate of pollutants [51,74].

4. Conclusions

An experiential extermination of *P. aeruginosa* was achieved over 12 min treatment, which correspondingly suggested the diminishing presence of the resistance gene at terminal treatment time (15 min). The observed log reductions further validated the efficacy osf NTP in completely eliminating ARBs over time. This effectiveness was attributed to the increasing presence of $^{\bullet}OH$ and the long-lived species (H_2O_2, NO_2^- and NO_3^-) which can generate more $^{\bullet}OH$ and reacting to secondary products post-discharge, significantly contributing to the antibacterial activity of PAW. These species also played a role in lowering the pH to levels conducive to bacterial destruction, while the conductivity remained consistently low.

The SEM results provided additional confirmation that NTP disrupts the tensile strength of the cell membrane, leading to rupture and ultimately cell death. This mechanism, in turn, prevents bacterial growth, which might in some cases include the disintegration of DNA at the ARG loci. However, it was observed that the electrophoretic gel visualization of bla_{NDM-1} bands might not be sufficient for accurate monitoring of log reduction in gene copies per treatment time. In this regard, future studies will be devoted to evaluating gene copies as well as abundance of nucleotide bases per treatment time. This will not only substantiate the bla_{NDM-1} disintegration claims in this study, but also provide insight on the interaction of plasma treatment with the chemical bonds of the DNA. The study portrayed the inconsequential antimicrobial contribution of copper, further emphasizing that the observed log reductions were

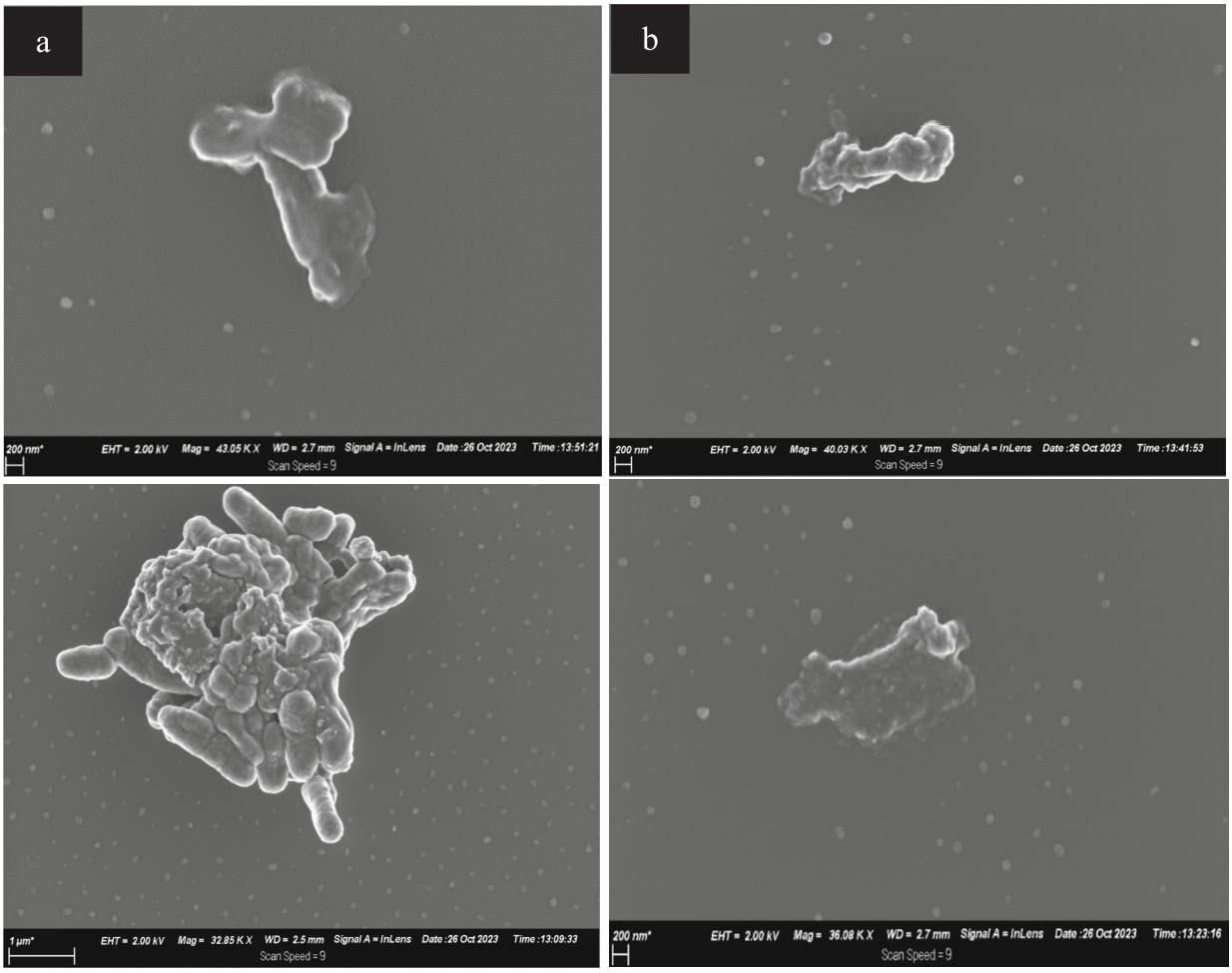

Fig. 6. SEM image of *P. aeruginosa* after 15 min of non-thermal plasma treatment. (a) single cell rupture and cytosol leakage (b – c) rupture of cell cluster (d) total disintegration of single cell.

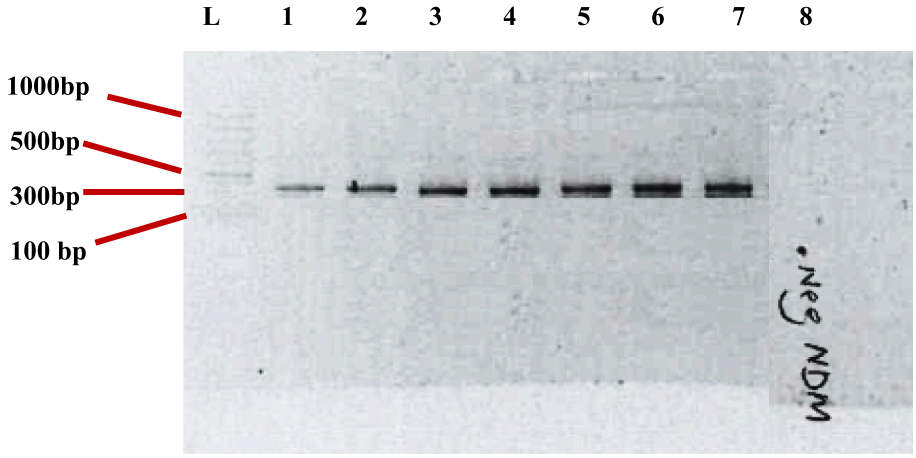

Fig. 7. Effect of periodic non-thermal plasma treatment on the carbapenem resistant gene of *P. aeruginosa*. L: DNA ladder, 1: 15 min, 2: 12 min, 3: 9 min, 4: 6 min, 5: 3 min, 6: copper control, 7: +ve control, 8: -ve control.

solely attributed to NTP.

Overall, these findings underscore the potential of plasma treatment as an effective disinfection step for wastewater. In correspondence, the potential of NTP at the tertiary stage of wastewater treatment has been evaluated for environmental and economic footprint, especially when retrofitted to other technologies at the tertiary treatment stage in a WWTP [78]. Notwithstanding, Mosaka et al. [23] highlighted the challenges that still needs to be overcome in its scale up or adoption at real-life, large-scale scenarios. In view of the aforementioned challenges, and the observations reported in this study, it is recommended that addressing biofilms near *P. aeruginosa*, extending the reaction time, using oxygen-containing feed gas and enlarging the diameter of the

grounded electrode may further enhance the complete inactivation of ARGs.

CRediT authorship contribution statement

Thabang B.M. Mosaka: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **John O. Unuofin:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Michael O. Daramola:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Investigation, Formal analysis. **Chedly Tizaoui:** Writing – review & editing, Validation, Supervision, Project administration, Investigation, Formal analysis. **Samuel A. Iwarere:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Ethical approval

None applicable.

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