



Plasma-generated reactive species in water: A sustainable strategy against *Aeromonas hydrophila*

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ABSTRACT

Waterborne pathogens remain a stubborn problem in aquaculture, with implications for both ecosystem health and food safety. This study tested a plasma-based inactivation approach using plasma-activated water (PAW) generated by dielectric barrier discharge (DBD) plasma. Three plasma source configurations were evaluated - NO_x alone, O₃ alone, and a combined NO_x/O₃ source all run-on ambient air as the feed gas. Each produced a reactive gas mixture of NO, NO₂, N₂O, and O₃, which was diffused into water to yield a solution enriched with reactive oxygen and nitrogen species (RONS). Antimicrobial activity was tested against *Escherichia coli*, *Staphylococcus aureus*, and *Aeromonas hydrophila* - the last being a primary cause of motile *Aeromonas* septicemia in fish. PAW reduced planktonic bacterial counts by up to ~3 log CFU/mL and disrupted established biofilms. Mechanistic investigation combined IC₅₀ analysis, surface morphology scanning, live/dead fluorescence imaging, flow cytometry, and intracellular RONS quantification. These assays converged on membrane damage, oxidative and nitrosative stress, and loss of cellular integrity as the dominant modes of action. The results suggest plasma-generated PAW is a scalable, without use of harsh industrial chemicals option for pathogen control in aquaculture and water treatment settings.

1. Introduction

Pathogenic bacteria in aquatic environments cause damage well beyond the water itself. Phytoplankton, zooplankton, fish, shrimp, mollusks, and amphibians are all affected and when water quality degrades, it tends to make bacterial infections worse. Water-quality parameters such as pH, salinity, dissolved oxygen, metal availability, and poor management contribute to infections caused by *A. hydrophila* (Abreu et al., 2018; Sherif and Kassab, 2023). The list of problematic aquatic pathogens is long - *Aeromonas* sp., *Streptococcus* sp., *E. coli*, *Vibrio* sp., *Pseudomonas* sp. but *Aeromonas* consistently causes the most serious damage in aquatic settings. Hu et al. (2026) found that urban estuaries are increasingly contaminated through human activity, and called for a One Health framework to improve pathogen risk assessment. Moreover, Different microbes are involved, leading to reduced food production and pathogenic effects in humans and other organisms (Thirumalaikumar

et al., 2021).

A. hydrophila is particularly dangerous in fish-culture ponds, which causes significant mortality (Abdel-Latif and Khafaga, 2020). In humans, eating contaminated fish can cause to gastroenteritis ranging from watery or bloody diarrhea to cholera-like symptoms (John et al., 2019). In addition to aquatic foods, several studies have reported that *A. hydrophila* can be found in vegetables, meat, processed foods, and a variety of vertebrates, including amphibians, reptiles, and fish (Krovacek et al., 1992; Kaur et al., 2017; Tahoun et al., 2016; Park et al., 2021). And it doesn't just survive in planktonic form - biofilms let it persist on surfaces for extended periods. Bacterial persistence depends on the nature of the substrate, particularly for solid materials. Biofilm formation on solid surfaces depends on the bacterial species, material type, exposure time, and surrounding environmental conditions. Rahman et al. (2025) reported that *Aeromonas hydrophila* forms biofilms on stainless steel (SS), polyethylene terephthalate (PET), high-density

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polyethylene, and silicon rubber over 24, 48, 72, and 96 h. Among these materials, PET was found to be the most favorable for biofilm formation at 72 h. [Shatila et al. \(2023\)](#) studied porphyrin-functionalized PET surfaces and demonstrated that a PDI effectively reduces planktonic bacteria and biofilms under LED light. Significant reductions were observed in *E. coli*, *P. aeruginosa*, and *S. aureus*, highlighting their potential to control resistant bacteria and prevent biofilm formation. In aquaculture, *A. hydrophila* is a leading cause of global food insecurity and economic loss ([Mzula et al., 2019](#)). Therefore, controlling this species is essential.

Current control measures in fish farming, include probiotics, antibiotics, vaccines, and biosecurity protocols ([Assefa and Abunna, 2018](#)). Ampicillin, tetracycline, chloramphenicol, and sulfonamides are among the most used antibiotics. However, the continuous and overuse of antibiotics can lead to environmental pollution, pose risks to human health, promote the emergence of antimicrobial resistance (AMR), and reduce their efficacy. [Suyamud et al. \(2024\)](#) reported that aquaculture in Southeast Asia is rapidly increasing but is facing AMR due to antibiotic overuse. Malaysia, Vietnam, and Thailand report the highest levels, mainly in commonly used drugs, with *Aeromonas*, *E. coli*, and *Vibrio* as key resistant bacteria. These findings highlight the need for coordinated action to control AMR. Chemical-based control methods are also used, and for environmental applications, they are often more cost-effective than antibiotics. However, chemical sanitizers, especially chlorine-based products, have raised public health concerns due to their potential to form carcinogenic chlorinated organic compounds ([Ölmez and Kretzschmar, 2009](#)), as well as their association with food poisoning and environmental pollution.

Although extensive research has been focused on chemical- and antibiotic-based control strategies, efficient and environmentally sustainable methods for inactivating aquatic pathogens, particularly planktonic and biofilm-forming bacteria, remain limited. The complexity of aquatic environments combined with increasing antimicrobial resistance and biofilm persistence necessitates alternative disinfection approaches that are both effective and ecologically safe. Plasma-activated water (PAW) has demonstrated promising antimicrobial properties for food and medical applications; however, its physicochemical behavior and bactericidal efficacy against aquatic pathogens, particularly in biofilm-associated states, are still not fully understood.

PAW generated through the nonthermal plasma treatment of tap water (TW) has attracted growing interest as an effective aqueous disinfectant for aquatic environments, particularly in aquaculture and agricultural applications. The main advantages of using PAW for bacterial inhibition include minimal adverse effects on the surrounding environment, increased bacterial inactivation, and reduced antibacterial-resistant strains. Currently, several nonthermal plasma systems are employed to control bacteria, including microwave discharge, plasma jets, corona discharge, and electric-barrier discharge plasma. These systems generate reactive oxygen species ($\bullet\text{OH}$, O_2 , O_3 , $^1\text{O}_2$) and reactive nitrogen species (NO , ONOO^-) through a combination of charged particles, ultraviolet radiation, and fast electric fields at ambient temperature ([Ashokkumar et al., 2026](#)). Additionally, [Wang et al. \(2022a, 2022b\)](#) reported that mixing NO_x and O_3 plasma generated high-valence N_2O_5 , which enhanced reactive species dissolution and plasma-activated water (PAW) activation, resulting in strong antibacterial activity mediated by reactive oxygen and nitrogen species. These reactive species have demonstrated promising antibacterial activity. When these reactive oxygen and nitrogen species (RONS) interact with water, they induce changes in the pH, conductivity, oxidation-reduction potential, and overall RONS concentration, thereby altering the physicochemical properties of PAW. Dielectric barrier discharge (DBD) plasma can inactivate both planktonic and biofilm bacteria through plasma-generated PAW. In this study, we analyzed the physicochemical properties of PAW, bacterial inactivation (bacterial cell numbers and IC_{50} values), bacterial fluorescence intensity, and intracellular RONS

levels with and without treatment.

2. Materials and methods

2.1. Experimental setup: MEDBD plasma configuration and electrode arrangement

PAW was produced using a multi-electrode dielectric barrier discharge (MEDBD) plasma apparatus. The air-based DBD plasma setup ([Fig. 1a](#)) consisted of four pairs of brass electrodes, each enclosed in a quartz dielectric barrier ([Fig. 1b](#)). The electrodes (1 mm in diameter and 190 mm in length) were alternately connected to a high-voltage power supply and ground with a fixed interelectrode gap of 1 mm, creating an effective plasma-generation zone of 90 mm per electrode pair. Each electrode was housed in a cylindrical quartz tube (200 mm in length and 5 mm in outer diameter) and assembled within a larger quartz tube (30 mm in diameter and 210 mm in length) to provide electrical insulation and mechanical stability. A controlled airflow of 1.0 L/min was supplied through the electrode package. A 1 L tap-water sample was exposed to the plasma-activated gas phase for 60 min using commercially available bubbler. The experiments were conducted in open mode at 60% relative humidity. To minimize the ambient humidity fluctuations, the MEDBD apparatus was enclosed in an acrylic chamber and operated at ambient temperature. [Figs. 1c–e](#) shows PAW generation via NO_x ([Fig. 1c](#)), O_3 ([Fig. 1d](#)), and combined NO_x/O_3 ([Fig. 1e](#)). [Table 1](#) presents all plasma device parameters under working conditions.

2.2. Plasma-characterization technique

The electrical characteristics of the MEDBD plasma were evaluated using a high-voltage probe (P6015A, Tektronix, USA) to monitor the applied voltage at the powered electrodes, and a current probe (CP030, LeCroy, USA) to record the discharge current. Voltage and current signals were acquired simultaneously using an oscilloscope (WaveSurfer, LeCroy, USA) operating at a bandwidth of 434 MHz. The duty cycle, energy dissipated per discharge, and average plasma power were calculated from the recorded waveforms using Eqs. (1)–(3) ([Acharya et al., 2024a, 2024b](#)):

$$\text{Duty cycle (\%)} = \frac{\text{plasma on} - \text{time}}{\text{plasma on} - \text{time} + \text{plasma off} - \text{time}} \times 100 \quad (1)$$

$$E = \int_{t_1}^{t_2} V(t)I(t) dt \quad (2)$$

$$P = f \times E \quad (3)$$

where $V(t)$ and $I(t)$ represent the instantaneous voltage and current, respectively, and f denotes the operating frequency.

A spectrometer (HR4000CG-UV-NIR, Ocean Optics, FL) incorporating a 400- μm core-diameter optical fiber was used to analyze the emitted plasma species. This provided the emission-line intensity (arbitrary units) and amount of RONS produced. Quantification of the RONS species in the gaseous phase was performed using an ozone analyzer (Model 202, B Technologies, USA) in combination with a Fourier-transform infrared (FTIR) spectroscopy system (Matrix M5G, Bruker, Germany). This method enabled absolute concentration measurements of the primary species produced in the plasma, including NO , NO_2 , N_2O , HNO_3 , and ozone. The thermal characteristics of the MEDBD reactor were measured before and after the plasma operation using an infrared thermal-imaging camera (P 5620, PeakTech, Germany). Detailed analyses of the MEDBD plasma properties, such as electron, rotational, and vibrational temperatures, electron density, and N_2 excited state densities, were reported in our previously published research articles and provided additional insights into the fundamental physical characteristics of the discharge ([Acharya et al., 2024a, 2024b](#)).

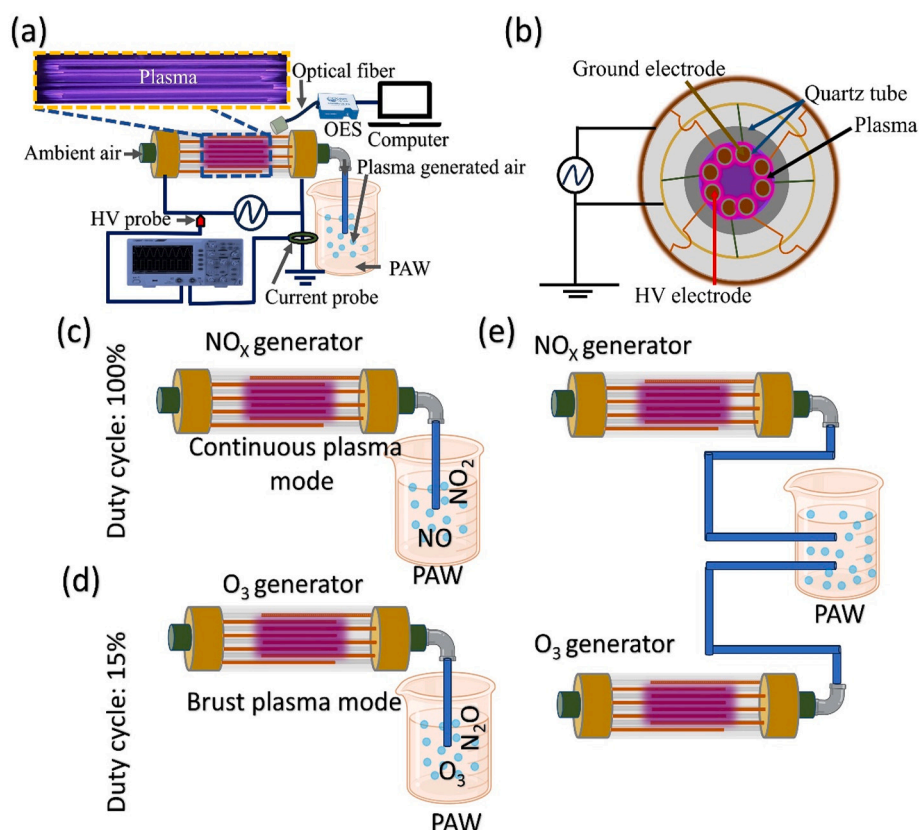


Fig. 1. Infrastructure and process of the multi-electrode dielectric barrier discharge (MEDBD) plasma system used for plasma-activated water (PAW) generation. PAW was produced by treating 1 L of water for 1 h with ambient air at a flow rate of 1 L min^{-1} and 60% relative humidity. (a) Schematic of the complete MEDBD plasma setup. (b) Enlarged view of the MEDBD electrode configuration. (c) PAW generated using an NO_x generator. (d) PAW generated using an O_3 generator. (e) PAW generated using a combined NO_x and O_3 generator.

Table 1
Operational and electrical characteristics of the MEDBD plasma reactor during NO_x and O_3 generation.

Parameter	Symbol (Unit)	NO_x -generator	O_3 -generator
Plasma type	–	ME-CDBD	ME-CDBD
Peak voltage	V_p (kV)	15.3 ± 0.2	15.3 ± 0.2
Peak current	I (mA)	202 ± 10	202 ± 10
Frequency	f (kHz)	22 ± 0.5	22 ± 0.5
Duty cycle	–	100% (continuous)	15% (on time: 400 ms, off time: 63 ms)
Plasma dissipated power	P (W)	24.0 ± 0.2	3.6 ± 0.2
Gas composition	–	Ambient air	Ambient air
Gas flow rate	Q (L min^{-1})	1	1
Discharge: during PAW	–	Immersed in water	Immersed in water
Reactor temperature (20 min)	T (K)	540 ± 8	320 ± 5
Main reactive species	–	NO & NO_2	O_3 & N_2O
Volume of treated water	V (L)	1	1
Plasma exposure time	t (h)	1	1

2.3. Preparation and characterization of PAW

PAW was prepared using a 2 L plastic reactor containing 1 L of tap water and a fine-pore ($<100 \mu\text{m}$) ceramic bubble diffuser placed at the bottom of the reactor. Compressed air (1 LPM) was continuously supplied through the diffuser for up to 1 h to generate fine bubbles, thereby

increasing the gas–liquid contact area and enhancing the transfer of RONS into the water. The plasma-generated gas was introduced through the diffuser beneath the water sample, enabling efficient dissolution and interaction of reactive species within the aqueous phase. During PAW preparation, no chemical disinfectants were added to the water, unlike chemically supplemented PAW treatments. Gas plasma generated at a flow rate of 1 L/min under three separate operating modes (NO_x , O_3 , and combined NO_x/O_3) was injected into 1 L of tap water contained in flasks to produce PAW, during which various physicochemical parameters were altered. An Thermo Fisher Scientific Orion Versa Star Pro Advanced Electrochemistry Meter was used to measure the pH, electrical conductivity, and oxidation–reduction potential (ORP) of the PAW. The generation of RONS in cylindrical DBD plasma depends on the excitation voltage and the chemical composition of the surrounding environment. The concentration of NO_x (a mixture of NO/NO_2 –/ NO_3 –) in PAW was quantified using a commercially available QuantiChrom NO Detection Kit based on the Griess method. The H_2O_2 concentration was measured using the QuantiChrom Peroxide Assay Kit.

2.4. Experimental bacterial strains

Escherichia coli K-12 (KCTC 1116), *Staphylococcus aureus* (ATCC 12600), and *Aeromonas hydrophila* were obtained from the Environmental Microorganism Bank of Korea (Republic of Korea) and used for the PAW-inactivation studies. All chemicals, reagents, and analytical equipment were purchased from reputable suppliers and used in accordance with the manufacturers' instructions. These three strains were grown on tryptic soy agar plates under aerobic conditions at 37°C for 24 h. For inactivation analysis, bacteria were grown in tryptic soy broth (TSB) at 37°C with shaking at 200 rpm for up to 12 h. These

bacterial cell suspensions were used to study the inactivation of bacteria by PAW.

2.5. Deactivation of aquatic bacteria by PAW

PAW was used to inactivate the three types of aquatic pathogenic bacteria and was generated using aquaculture-grade water. The bacteria were grown in TSB at 37 °C for 24 h. After growth, the bacteria were washed with phosphate-buffered saline (PBS). For each suspension, 100 µL of bacterial culture (initial concentrations: *E. coli* 8.22 ± 0.13 ; *S. aureus* 7.83 ± 0.07 ; *A. hydrophila* 7.29 ± 0.05 colony-forming unit (CFU)/mL) was added to 900 µL of different PAW types (NO_x-PAW, O₃-PAW, and a combination of NO_x & O₃-PAW). The mixtures were incubated at 37 °C for up to 48 h. Throughout the bacterial-reduction run, negative control/untreated water/without PAW (bacteria in tap water (TW)), positive control (NaOCl), and PAW, the bacterial-inactivation levels were analyzed using a CFU at different time intervals between 0 and 48 h (0, 0.5, 2, 5, 24, and 48 h).

2.6. Bacterial inactivation by different concentrations of NO_x

The 50% inhibitory concentration (IC₅₀) of NO_x, defined as the NO_x concentration resulting in 50% bacterial survival inhibition, was determined. Initial bacterial concentrations were 7.64 ± 0.08 (*E. coli*), 7.81 ± 0.13 (*S. aureus*), and 7.27 ± 0.08 (*A. hydrophila*) log CFU/mL. Aliquots of 100 µL of bacterial suspension were mixed with 900 µL of PAW generated by different devices. NO_x-PAW and combined NO_x/O₃-PAW contained NO_x concentrations ranging from 25 to 300 µM, while O₃-PAW contained 25–50 µM. The control group (0% NO_x) was administered an equal volume of autoclaved tap water instead of PAW. All the mixtures were incubated at room temperature for 1 h, after which, bacterial inactivation was quantified by CFU enumeration.

2.7. Reduction of biofilm in solid materials

In the aquaculture industry, bacteria can persist in materials, such as plastics, SS, and fabrics. The ability of PAW to remove surface-attached bacteria was tested. The three bacterial strains were cultured in TSB at 37 °C for 24 h. A suspension of 1.5×10^7 CFU/mL was applied to autoclaved samples of the three materials and incubated at 37 °C for 48 h to allow biofilm formation. After 48 h, the planktonic cells and culture medium were removed and the surfaces were washed once with PBS. Biofilms were then treated with 1 mL of PAW (NO_x-PAW, O₃-PAW, or combined NO_x/O₃-PAW; NO_x concentration was 50 µM), and TW and an antibiotic served as negative and positive controls, respectively. The treated materials were incubated at 37 °C for 1 h. Following treatment, the planktonic cells were removed and the surfaces were washed with PBS, air-dried, and stained with 1 mL of 0.1% crystal violet solution for up to 6 h. Excess dye was removed by washing the surfaces three times with sterile distilled water. After drying (room temperature), 200 µL of 33% acetic acid in methanol was added to solubilize the bound crystal violet. The solution was incubated for 30 min and the biofilm biomass was quantified by measuring the absorbance at 595 nm.

2.8. Bacteria morphological distinction after treatment

Bacteria were treated with three types of PAW (each plasma device-derived PAW contained 50 µM of NO_x) for up to 1 h, and the cell morphology of the bacteria was examined by scanning electron microscopy (SEM). The cell pellets were obtained and washed three times with PBS (LB001–02, Welgene, Daegu, Republic of Korea) to remove the unwanted residual substances, followed by centrifuging at 4000 rpm for 10 min at 4 °C. The cell pellets were fixed with 1% osmium tetroxide (OsO₄) (18,459, Ted Pella Inc., Redding, CA, USA) in PBS for 48 h at room temperature and then sequentially dehydrated with different concentrations of ethanol (30%, 50%, 70%, 80%, and 100% v/v

ethanol) for 5 min for each rinse, except 100%, in which was applied for 10 min. Excess ethanol was removed and hexamethyldisilane (EMS16700, Madrid, Spain) was used for 5 min at room temperature. The cells were desiccated and sputter-coated with gold (108 Auto/SE Sputter Coater, Ted Pella Inc., USA), and cells were viewed using SEM (JSM-7001F, JEOL, Tokyo, Japan) at a hastening voltage of 15 kV.

2.9. Flow cytometry

Bacteria (10^7 CFU/mL) were treated with PAW (containing 50 µM NO_x and H₂O₂) for up to 1 h, after which, cells were collected for viability analysis by flow cytometry. The cells were then centrifuged at 10,000 rpm for 10 min at 4 °C and washed with PBS. The cell pellets were resuspended in 100 µL of 1× binding buffer and stained with 100 µL of 2× working solutions containing Calcein-AM (live stain) and BOBO-3 Iodide (dead stain). After 15 min of incubation (room temperature), the stained cells were analyzed using the FACSVerse system (Becton Dickinson Biosciences, Seoul, Republic of Korea) to determine cell viability.

2.10. Live–dead assay

To observe cell viability before and after the cylinder DBD plasma treatment, samples were stained with the LIVE/DEAD™ Cell Imaging Kit (R37601) according to the manufacturer's protocol (Invitrogen™, Thermo Fisher Scientific, Waltham, MA, USA). In this experiment, *E. coli* and *S. aureus* were grown in TSB media for up to 10 h at 37 °C. *A. hydrophila* grew in NB with 0.5% NaCl for up to 14 h at 30 °C and all the bacteria were adjusted to the cell number 10^{-8} CFU/mL. After the desired bacterium cell number, the bacterium (100 µL) was treated with 1 mL of PAW (three different PAW treatments; 50 µM of NO_x), and in the control experiment, 1 mL of tap water was added and maintained at room temperature for 1 h. Treated and nontreated cells were centrifuged at 10,000 rpm for 10 min and washed three times with a 0.85% sterile sodium chloride solution. Subsequently, the bacteria cell pellets were resuspended in 100 µL of saline. Then, 100 µL of the 2× working solution of green (live) and dead (red) were added, and the bacteria were further incubated for 15 min at room temperature in a dark place. Cells were visualized using confocal laser scanning microscopy. The live- and dead-cell stain assay kits contained calcein AM and BOBO-3 iodide, respectively. The live cells are illustrated owing to the presence of ubiquitous intracellular esterase activity, as revealed by the enzymatic alteration of the virtually nonfluorescent cell-permeant calcein AM to the strongly fluorescent calcein, which indicates well-held live cells. BOBO-3 iodide is a cell-impermeable dye that enters damaged or dead cell membranes, binds to DNA, and produces bright red fluorescence.

2.11. Intercellular RNS/ROS investigation

After inactivation, bacteria produce intracellular RONS. Bacteria (100 µL, 1×10^6 cells/mL) washed with PBS were mixed with 900 µL of different PAW types in 2 mL tubes and incubated at 37 °C for 24 h. Cells were then centrifuged at 5000 rpm for 10 min at 4 °C. For the investigation on reactive nitrogen species (RNS), 50 µL of PBS mixture containing 10 µM of 4-Amino-5-Methylamino-2',7'-Difluorofluorescein Diacetate (DAF-FM DA) (Thermo Fisher Scientific, W Massachusetts, USA) was added to the bacterial cell. The bacteria were observed after 45 min at room temperature in the dark. Confocal microscopy (Olympus, Shinjuku, Tokyo, Japan) was used to visualize the stained cells. The inner cells of bacteria containing the esterase enzyme hydrolyzed DAF-FM DA into DAF-FM via RNS interactions. In particular, the nonfluorescent DAF-FM DA and opaque-fluorescent DAF-FM DA were converted to the cell-permeable DAF-FM fluorescent benzotriazole. This phenomenon was measured at 495/515 nm, and may have occurred because of intracellular or NO stimulation in bacteria owing to the interaction with DAF-FM.

Intracellular reactive oxygen species (ROS) were detected using 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) (D399; Thermo Fisher Scientific, Waltham, Massachusetts, USA). After the PAW treatment, 50 μ L of the PBS mixture containing 100 μ M of the working solution was added to the bacterial-cell pellets. The mixture was maintained in the dark for 45 min, and cells were then observed using a fluorescent microscope. The DCF-DA primarily deesterified the intracellular esterase enzyme of the bacteria, changing the nonfluorescent DCF into the fluorescent signal (intracellular esterase enzyme was converted into DCF-DA to 2',7'-DCF). This phenomenon was measured at an excitation of 492–495 nm and emission of 517–527 nm, and may have occurred because of intracellular or ROS stimulation in bacteria owing to the interaction with DCF (green fluorescence signal).

2.12. Statistical analysis

All experiments were performed in triplicate ($n = 3$), and the data are presented as the mean $\pm$ standard deviation (SD). Statistical analyses were conducted using one-way analysis of variance (ANOVA), followed by Dunnett's or Tukey's multiple-comparison test in GraphPad Prism (version 10.0; GraphPad Software, San Diego, CA, USA). Statistical significance was defined as $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***). IC₅₀ values were calculated using GraphPad Prism by first normalizing the experimental data, followed by nonlinear regression using a four-parameter logistic (4PL) sigmoidal dose-response model with a variable slope. All IC₅₀ measurements were performed in triplicate ($n = 3$), and the fitted dose-response curves were used to estimate the IC₅₀ values.

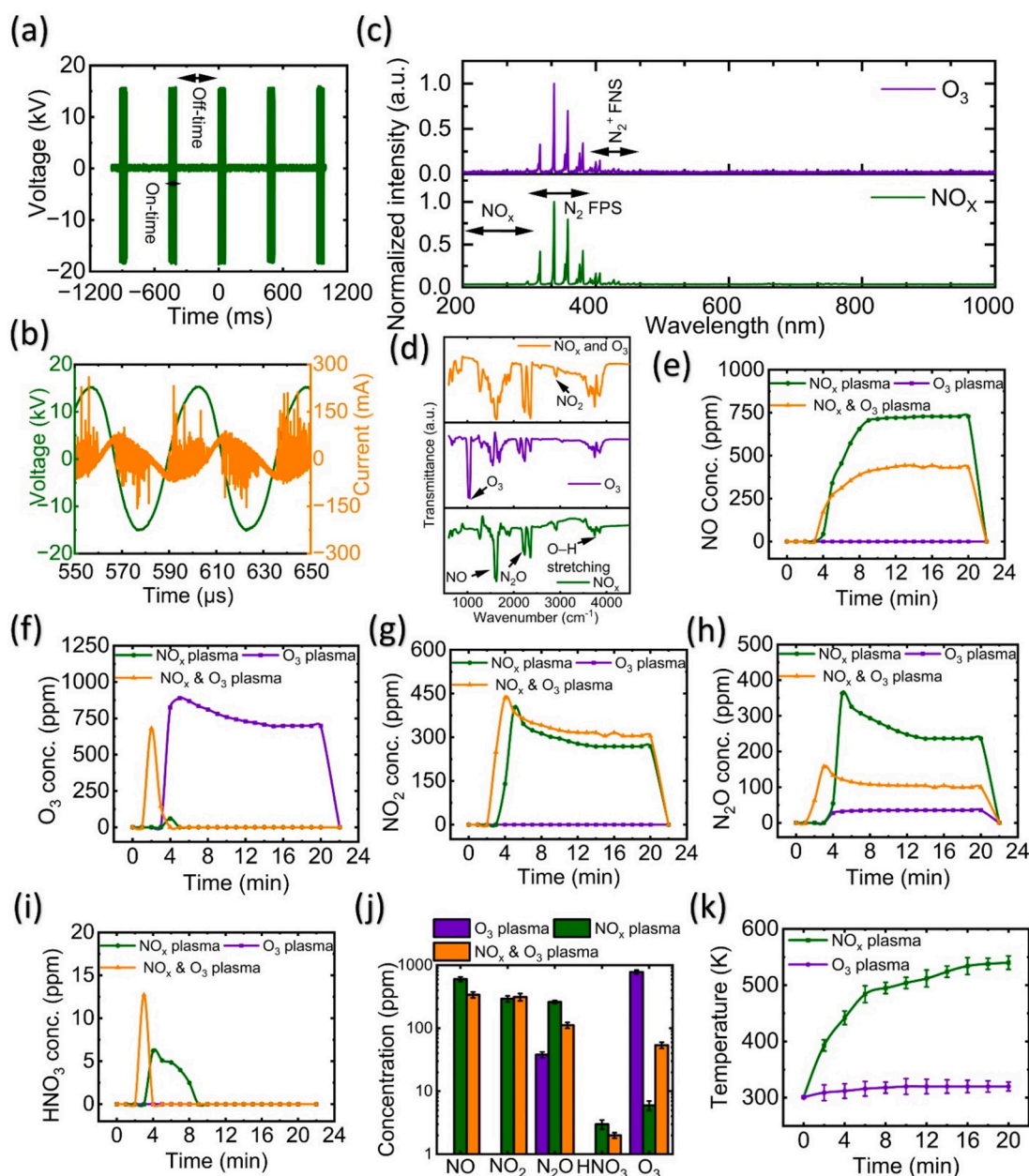


Fig. 2. Plasma diagnostics and physicochemical characterization of the MEDBD discharge. (a) Applied voltage waveform used for duty-cycle determination. (b) Current-voltage characteristics of the MEDBD plasma. (c) Optical emission spectroscopy (OES) showing excited species in the discharge. (d) Fourier transform infrared (FTIR) spectra identifying the gas-phase composition. Quantification of key reactive species: (e) NO, (f) O₃, (g) NO₂, (h) N₂O, and (i) HNO₃. (j) Time-resolved average concentrations of plasma-generated reactive species during 20 min of plasma operation and subsequent decay after shutdown, showing near-complete depletion within 22 min. (k) Reactor temperature profiles before and after plasma treatment.

3. Results

3.1. Electrical and optical properties of MEDBD plasma

The waveform of the applied voltage used to measure the duty cycle is shown in Fig. 2a. Two different modes were utilized to specifically control the plasma chemistry. Ozone-predominant plasma (O_3 plasma) was produced in pulsed mode with a duty cycle of 15% (time-on: 400 ms), whereas nitrogen oxide-predominant plasma (NO_x plasma) was produced at continuous voltage with a 100% duty cycle. The continued application of a high voltage favors the ionization and dissociation of nitrogen and oxygen molecules, thereby specifically contributing to the formation of NO_x (Borkar et al., 2023). The lower duty cycle leads to high-voltage spikes for brief durations interspersed with relatively long intervals. This is favorable for the dissociation of molecular oxygen with less heating, and hence ozone formation (Dhakal et al., 2024). Fig. 2b shows the current-voltage characteristics of the MEDBD plasma discharge. Plasma was generated at a frequency of 22 kHz with a peak voltage of 15.3 kV for the applied voltage and peak discharge current of 202 mA. This resulted in an average dissipated power of 24.0 W for NO_x plasma generation and 3.6 W for O_3 plasma generation because of the stark difference in operating energy required for continuous and pulsed modes.

Optical emission spectroscopy (OES) was used to investigate the type of excited species formed in the discharge to obtain information on the corresponding plasma chemistry. As shown in Fig. 2c, the NO_x and O_3 discharge modes exhibited distinct characteristic species corresponding to the emissions formed in the presence of reactive nitrogen and oxygen species. The poor emission lines between 200 and 300 nm correspond to NO_x . Strong N_2 SPS emissions were observed between 311 and 380 nm, corresponding to the electronic band of molecular nitrogen (Acharya et al., 2025). Other emissions corresponding to the N_2^+ first negative system were observed between 390 and 400 nm, indicating the presence of ionized nitrogen species, thereby validating the plasma-chemistry theories on MEDBD discharges containing electrons (Acharya et al., 2022, 2023).

Gas-phase RONS were further characterized using transmission Fourier-transform infrared (FTIR) spectroscopy (Fig. 2d) for the NO_x plasma, O_3 plasma, and a combination of NO_x plasma and O_3 plasma. Spectra were recorded at 60-s intervals for a total of 22 min within a wavelength range of 600–4500 cm^{-1} . No gas-phase RONS were detected in the absence of plasma. Upon the start-up of the plasma (time 0–20 min), more than one reactive species, such as NO, O_3 , NO_2 , N_2O , and HNO_3 , was formed. Upon plasma shutdown, the concentrations of the plasma-generated species decreased rapidly to near-zero levels within 2 min, suggesting the presence of transient species in the gas phase. Different absorption bands served as a common reference for individual species detection and concentration measurement: NO (1700–2000 cm^{-1} ; Fig. 2e), O_3 (1025–1075 cm^{-1} ; Fig. 2f), NO_2 (1540–1660 cm^{-1} and 2840–2940 cm^{-1} ; Fig. 2g), N_2O (2160–2260 cm^{-1} ; Fig. 2h), and HNO_3 (1250–1400 cm^{-1} ; Fig. 2i) (Patel et al., 2025). The time-averaged concentrations of these species during the optimized 20 min plasma exposure are summarized in Fig. 2j. For the NO_x plasma, O_3 plasma, and combined NO_x/O_3 mode, the average NO concentrations were 604, 0, and 340 ppm, respectively. The corresponding O_3 concentrations were 6, 780, and 54 ppm, whereas the NO_2 concentrations reached 294 ppm in the NO_x plasma, were undetectable in the O_3 plasma, and increased to 314 ppm in the mixed mode. The average N_2O concentrations were 262 ppm (NO_x plasma), 38 ppm (O_3 plasma), and 112 ppm (combined mode). HNO_3 was detected at low levels, with average concentrations of 3, 0, and 2 ppm in NO_x plasma, O_3 plasma, and the mixed configuration, respectively. Fig. 2k shows the time-course variation in the reactor temperature for a 20 min plasma-treatment process. Before the plasma was ignited, the temperatures of the NO_x and O_3 plasma reactors were the same and set to a value representative of room temperature (300 K). After the 20-min treatment process, the temperature increased

considerably for the NO_x plasma reactor (to 540 K) compared with a slight increment for the O_3 plasma reactor (to 320 K). After the plasma was switched off, the reactors required 10 min to return to their initial room temperatures. This may be owing to the higher input power and dominance of exothermic reactions at the increased temperature measured for the NO_x plasma.

3.2. Physical and chemical holdings of PAW

The physical and chemical nature of PAW is directly related to the plasma-device configuration and environmental conditions. Plasma generated species that mixed with water and changed the concentrations of RONS and other parameters. Fig. 3 shows the pH, ORP (oxidation reduction potential), conductivity, total dissolved solids (TDS), NO_x (combination of NO_2^- and NO_3^-), nitrite (NO_2^-), and H_2O_2 (hydrogen peroxide) concentrations of the tap-PAW generated by the three different plasma devices. Plasma treatment reduced the pH from 8.68 ± 0.06 to 7.51 ± 0.08 (Fig. 3A). In particular, the O_3 -PAW treatment acidified the water, which reduced the pH compared to other groups, and the nitrous and nitric acid forms are considered to be the fundamental reasons for the pH decrease (Oehmigen et al., 2010). The ORP value of PAW decreased from 204.87 ± 3.14 to 190.40 ± 6.19 mV (Fig. 3B). Electrical conductivity was increased from 248.13 ± 5.20 to 716.77 ± 3.89 $\mu S/cm$, and between the treatments, NO_x -PAW had higher values compared to the control and other treatments (Fig. 3C). The plasma treatment resulted in the migration of various spontaneously reactive species into water, thereby increasing the conductivity of ions within the water. The concentration of the TDS increased from 127.73 ± 3.92 to 429.17 ± 1.81 . Among the treatments, O_3 -PAW had the highest (429.17 ± 1.81) values, followed by 348.77 ± 5.04 and 274.20 ± 9.90 for NO_x -PAW and the combined NO_x and O_3 -PAW treatment, respectively (Fig. 3D).

The concentrations of NO_x species (from 3.47 ± 1.34 to 355.1 ± 3.04 μM) in TW, NO_x -PAW-, O_3 -PAW, and the combination of NO_x and O_3 -PAW were 3.5 ± 1.34 , 327.8 ± 2.26 , 53.6 ± 0.90 , and 355.1 ± 3.04 μM (Fig. 3E); the NO_2^- concentrations were 0.06 ± 0.02 , 2.63 ± 0.09 , 0.77 ± 0.06 , and 2.95 ± 0.08 mg/mL (Fig. 3F); and the H_2O_2 concentrations were 0.02 ± 0.0 , 4.88 ± 0.26 , 8.26 ± 0.32 , and 6.28 ± 0.20 mM (Fig. 3G), these concentrations were achieved by TW, NO_x -PAW, O_3 -PAW and combination of NO_x & O_3 -PAW treatment respectively. The RONS initially produced an active electron generated by ambient air while O_2 became atomic oxygen via Eq. (4), O_3 production in Eq. (5), NO production in Eqs. (6)–(8), NO_2 production in Eq. (9), and NO_3 production in Eq. (10) (Ashokkumar et al., 2026). These species interact with water to form various types of RONS. The formation of NO_2^- and NO_3^- in the PAW depends on exhaled gases. Nitrogen molecules react with O_2 , O_3 , O, and OH; these reactions are shown in Eqs. (11)–(14). Dissolved ozone can react in the aqueous phase to produce hydroxyl radicals ($\bullet OH$), which may further generate hydrogen peroxide (H_2O_2) and other reactive oxygen species, thereby influencing the RONS chemistry and reaction kinetics in plasma-activated water (Pang et al., 2022).



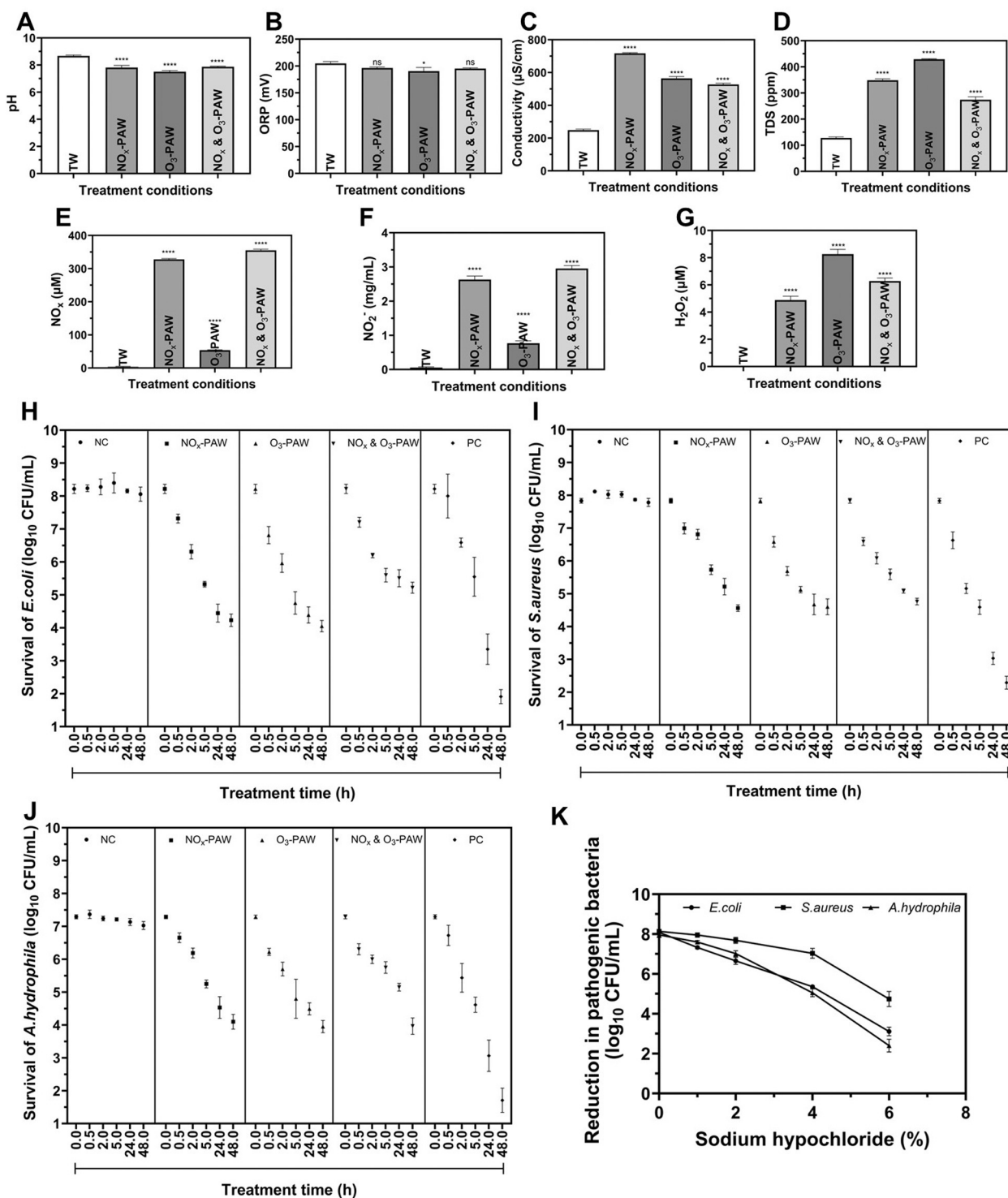


Fig. 3. Physicochemical properties of ME-CDBD plasma-activated water (PAW) generated from tap water: (A) pH, (B) ORP, (C) electrical conductivity, (D) total dissolved solids (TDS), (E) NO_x, (F) NO₂⁻, and (G) H₂O₂. Bacterial survival following exposure to PAW at different time intervals: (H) *Escherichia coli*, (I) *Staphylococcus aureus*, and (J) *Aeromonas hydrophila*. (K) Inactivation of different bacteria at varying concentrations of sodium hypochlorite.



3.3. Inactivation of planktonic and biofilm-forming aquatic pathogens by PAW

The efficacy of the different PAW treatments in inactivating *E. coli*, *S. aureus*, and *A. hydrophila* was assessed under planktonic conditions, with inactivation (log CFU/mL reduction) depending on treatment time (Fig. 3H-K). For *E. coli* at 48 h, survival values were 8.06 ± 0.19 , $4.23 \pm$

0.17, 4.05 ± 0.15 , 5.22 ± 0.15 , and 1.91 ± 0.19 log CFU/mL for NC, NO_x-PAW, O₃-PAW, combined NO_x and O₃-PAW, and PC, respectively (Fig. 3H). NO_x-PAW, O₃-PAW, and PC each produced significantly greater inactivation than the combined NO_x + O₃-PAW ($p < 0.01$), while the combined treatment still differed significantly from NC ($p < 0.05$). For *S. aureus*, values were 7.78 ± 0.11 , 4.56 ± 0.09 , 4.60 ± 0.22 , 4.76 ± 0.09 , and 2.29 ± 0.17 log CFU/mL for NC, NO_x-PAW, O₃-PAW, combined NO_x and O₃-PAW, and PC, respectively (Fig. 3I). O₃-PAW and combined NO_x and O₃-PAW achieved significantly greater inactivation than NO_x-PAW ($p < 0.01$); NO_x-PAW itself was significant relative to NC at $p < 0.05$. For *A. hydrophila*, values were 7.03 ± 0.11 , 4.10 ± 0.20 , 3.95 ± 0.17 , 3.96 ± 0.22 , and 1.71 ± 0.33 log CFU/mL for NC, NO_x-PAW, O₃-PAW, combined NO_x + O₃-PAW, and PC, respectively (Fig. 3J). All PAW treatments showed significant inactivation relative to NC ($p < 0.05$). H₂O₂ concentration also influenced inactivation. At 6%, maximum reductions were 4.98, 3.40, and 5.56 log CFU/mL for *E. coli*, *S. aureus*, and *A. hydrophila*, respectively (Fig. 3K), with inactivation increasing across the 1–6% range tested. Across the full 0–48 h time course, O₃-PAW produced the greatest inactivation among PAW treatments, reaching approximately 3.3 log CFU/mL. The PC (commercial antibiotic) achieved higher overall reductions of 5.54–6.31 log CFU/mL.

Bacteria were inactivated across NO_x concentrations of 0–300 μM. At the tested concentrations, log reductions for *E. coli* were 0.81, 0.75, and 1.16 CFU/mL; for *S. aureus*, 1.06, 1.10, and 1.37 CFU/mL; and for *A. hydrophila*, 1.80, 0.89, and 2.09 CFU/mL in NO_x-PAW, O₃-PAW, and combined NO_x and O₃-PAW, respectively. These results were obtained at NO_x concentrations of 300 μM (NO_x-PAW and combined NO_x + O₃-PAW) and 50 μM (O₃-PAW).

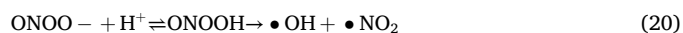
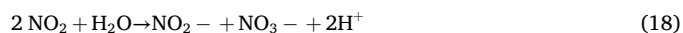
Over 48 h, O₃-PAW produced greater bacterial inactivation than the other treatments and the negative control, at a lower NO_x concentration (50 μM). At this concentration, log reductions for *E. coli* were 0.35, 0.75, and 0.44 CFU/mL; for *S. aureus*, 0.47, 1.10, and 0.62 CFU/mL; and for *A. hydrophila*, 0.25, 0.89, and 0.42 CFU/mL in NO_x-PAW, O₃-PAW, and combined NO_x and O₃-PAW, respectively. The higher inactivation efficiency of O₃-PAW was not solely attributable to NO_x; O₃-PAW also had a higher H₂O₂ concentration (8.26 ± 0.32 mM) than the other treatment groups, suggesting that H₂O₂ contributed to inactivation alongside the RONS/pH effects.

PAW inactivated the bacteria in a NO_x concentration-dependent manner. Nonlinear regression analysis of the dose–response curve revealed a sigmoidal relationship between NO_x concentration and bacterial inactivation. The IC₅₀ (half-maximal inhibitory concentration) curve was generated using the normalized percentage response (%) on the Y-axis and the NO_x concentration on the X-axis. The IC₅₀ value specified the concentration of NO_x required to achieve 50% bacterial inactivation (Fig. 4A–I). The IC₅₀ values of PAW were determined for three bacterial species. For *E. coli*, the IC₅₀ values were 70.24 μM for PAW-NO_x, 25.52 μM for PAW-O₃, and 81.42 μM for the combined PAW-NO_x and O₃ treatment (Fig. 4A–C). For *S. aureus*, IC₅₀ values were 53.60, 25.33, and 65.04 μM for PAW-NO_x, PAW-O₃, and PAW-NO_x and O₃, respectively (Fig. 4D–F). For *A. hydrophila*, the IC₅₀ values were 174.8, 25.44, and 124.1 μM for PAW-NO_x, PAW-O₃, and PAW-NO_x and O₃ treatments, respectively (Fig. 4G–I). These results indicate that PAW-O₃ mostly demonstrated the lowest IC₅₀, indicating better antibacterial efficiency related to PAW-NO_x and the combined treatment. Fig. 4J shows the IC₅₀ values of NaOCl for the inactivation of three bacterial species. The IC₅₀ values were 2.96% for *E. coli*, 4.10% for *S. aureus*, and 3.60% for *A. hydrophila*, indicating that *E. coli* was the most sensitive, whereas *S. aureus* required a higher concentration for 50% inactivation under the tested conditions. Based on planktonic and biofilm bacteria inactivation, PAW is best for bacteria inactivation because it is safe for the environment. Moreover, it has a higher bacteria efficacy, particularly PAW-O₃ and the combined PAW-NO_x and O₃ treatment.

The efficacy of PAW for reducing viable bacterial biofilms has been evaluated on various solid surfaces commonly used in homes and

aquaculture, including plastic, SS, and cotton fabric. For *E. coli*, viable biofilm counts after treatment with the PAW-NO_x and O₃ mixture were 1.47 ± 0.09 on plastic, 1.85 ± 0.09 on SS, and 2.84 ± 0.13 on cotton fabric. For *S. aureus*, biofilm reductions were 1.95 ± 0.10 on plastic, 2.50 ± 0.15 on SS, and 2.59 ± 0.13 on cotton fabric. *A. hydrophila* biofilm reductions were 1.75 ± 0.12 on plastic, 2.24 ± 0.21 on SS, and 2.62 ± 0.14 on cotton fabric. Overall, the PAW-NO_x & O₃ mixture resulted in a higher biofilm reduction compared with other PAW treatments and NC, except for the PC (1% NaOCl) (Fig. 4K–M).

Overall, O₃-PAW showed greater inactivation of planktonic and biofilm bacteria compared to other treatments, such as NO_x-PAW. Several previous studies support this investigation. In particular, Wang et al. (2025) and Dou et al. (2025) investigated the generation and antimicrobial mechanisms of RONS in PAW. Using dielectric barrier discharge systems, numerical simulations, and LC-MS analysis, the studies demonstrated that short-lived species rapidly decay near the plasma region, whereas long-lived species such as O₃, H₂O₂, NO₂[−], and NO₃[−] accumulate in water and contribute significantly to antibacterial activity. The higher microbial inactivation efficiency observed in O₃-PAW and combined NO_x + O₃-PAW systems compared with NO_x-PAW was attributed to the formation of additional secondary reactive species in the liquid phase. In O₃-PAW, ozone generates secondary ROS (Eqs. (15)–(17)) that induce lipid peroxidation, protein oxidation, and DNA strand breaks (Pang et al., 2022; Bermúdez et al., 1999). While in the combined NO_x + O₃-PAW system, the coexistence of ROS and RNS promotes additional reaction pathways through the dissolution of nitrogen oxides into nitrite and nitrate, followed by nitrite reaction with H₂O₂ to form peroxynitrite shown in Eqs. (18) and (19) (Negi et al., 2024). Peroxynitrite further equilibrates with peroxynitrous acid, which decomposes into •OH and •NO₂ radicals in Eq. (20) (Olson et al., 2003). Both studies identified ONOOH/ONOO[−] as a key oxidant responsible for bacterial membrane disruption and oxidation, nitration, and sulfur oxidation of proteins, nucleic acids, amino acids, and other biomolecules. Furthermore, parameters such as temperature, discharge power, gas flow rate, humidity, and water-layer thickness strongly influenced RONS generation and PAW reactivity. Notably, low-temperature PAW (4 °C) showed enhanced and prolonged antibacterial activity against *E. coli* due to improved RONS stability and chemical energy storage (Wang et al., 2025).



3.4. Morphology changes in bacteria induced by PAW

SEM images were obtained before and after 1 h of the PAW treatment (PAW containing 50 μM NO_x). The nontreated bacteria had a distinctive rod shape (*E. coli* and *A. cavi*) and a spherical shape (*S. aur*), all with a smooth surface and undamaged cell walls and membranes. In contrast, PAW-treated bacterial cells showed distinct morphology, including structural changes, damaged membranes, and cell shrinkage (Fig. 4N). In this figure, the red circles indicate damaged bacterial cells.

3.5. Flow cytometric (FACS) analysis after bacteria inactivation by PAW

Flow cytometry was used to analyze the bacterial cell viability and membrane integrity after PAW treatment. Fig. 4O shows the FACS

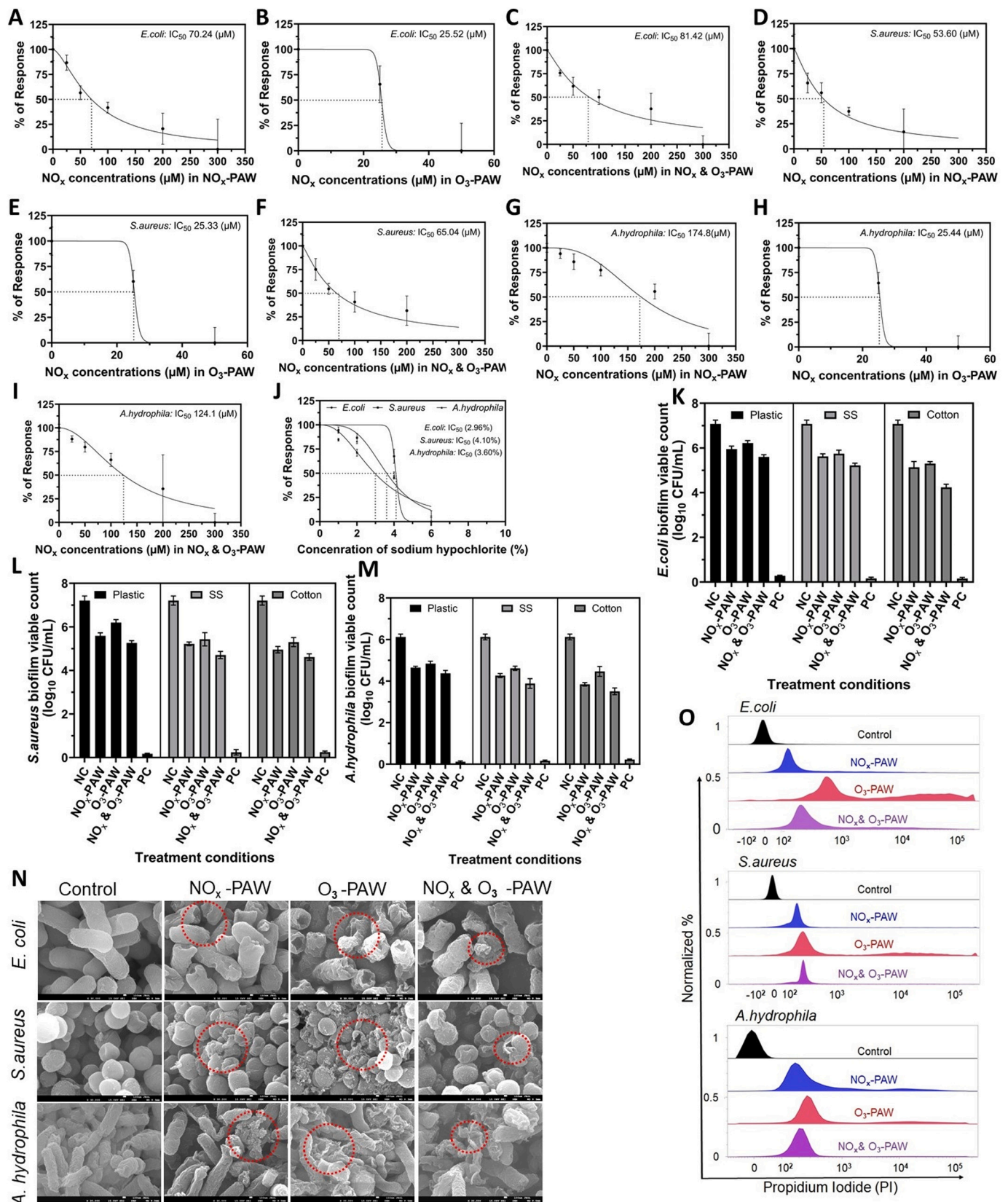


Fig. 4. Fifty percent inhibitory concentration (IC₅₀) values derived from different NO_x concentrations in plasma-activated water (PAW) and sodium hypochlorite (NaOCl). PAW treatments include NO_x-PAW, O₃-PAW, and combined NO_x & O₃-PAW for bacterial inactivation. *Escherichia coli* inactivated by (A) NO_x-PAW, (B) O₃-PAW, and (C) combined NO_x & O₃-PAW; *Staphylococcus aureus* inactivated by (D) NO_x-PAW, (E) O₃-PAW, and (F) combined NO_x & O₃-PAW; *Aeromonas hydrophila* inactivated by (G) NO_x-PAW, (H) O₃-PAW, and (I) combined NO_x & O₃-PAW. (J) Inactivation of all bacteria at different concentrations of NaOCl. Viable biofilm counts on solid materials (plastic, stainless steel, and cotton) after immersion in different PAW treatments: (K) *E. coli*, (L) *S. aureus*, and (M) *A. hydrophila*. (N) SEM images after PAW treatment. (O) Propidium iodide (PI) fluorescence intensity indicating bacterial cell death measured by flow cytometry following PAW treatment.

profiles of the bacterial cells with and without PAW treatment. In the graph, the y-axis denotes the normalized percentage of cells and the x-axis represents the propidium iodide (PI) fluorescence intensity, indicating PI uptake by the bacterial cell membrane for various PAW treatments. These findings are consistent with previously observed antibacterial effects of O_3 -PAW. Between treatments, NO_x -PAW increased the fluorescence counts in *E. coli*, *S. aureus*, and *A. hydrophila* to $10^{2.4}$, $10^{2.4}$, and 10^3 , respectively, compared with the NC. The fluorescence count of all bacteria increased to approximately 10^5 in the O_3 -PAW treatment and decreased in the other treatments. For the combined NO_x and O_3 -PAW treatment, the luminescence counts/intensities reached $10^{2.7}$, $10^{2.8}$, and $10^{2.7}$ in *E. coli*, *S. aureus*, and *A. hydrophila*, respectively. In addition, the mean Phycoerythrin-Area (PE-A) values were higher (fluorescence intensity) in PAW-treated groups compared with the controls, with O_3 -PAW showing the highest PE-A values. Similar trends were observed for all bacterial species tested.

3.6. Live-dead cell-assay outcome

Three plasma-treated and nontreated bacterial cells were double stained with calcein AM and BOBO-3 iodide. After 1 h of treatment, the cells were observed by confocal laser scanning microscopy. Green fluorescence (Calcein AM) indicated live bacteria and red fluorescence (BOBO-3 Iodide) emission indicated dead bacteria. The PAW-treated groups showed a reduced percentage of live cells for *E. coli*, *S. aureus*, and *A. hydrophila* compared with the untreated controls (Fig. 5A). Live cells (green) are exposed to ubiquitous intracellular esterase activity, which converts nonfluorescent, cell-permeant calcein AM into strongly fluorescent calcein. BOBO-3 iodide is a cell-impermeable dye that binds to DNA and produces bright red fluorescence, indicating damaged or dead cells. Overall, plasma-treated bacteria exhibited increased cell death compared with the control. Among the treatments, O_3 -PAW resulted in a greater bacterial inactivation than NO_x -PAW and the

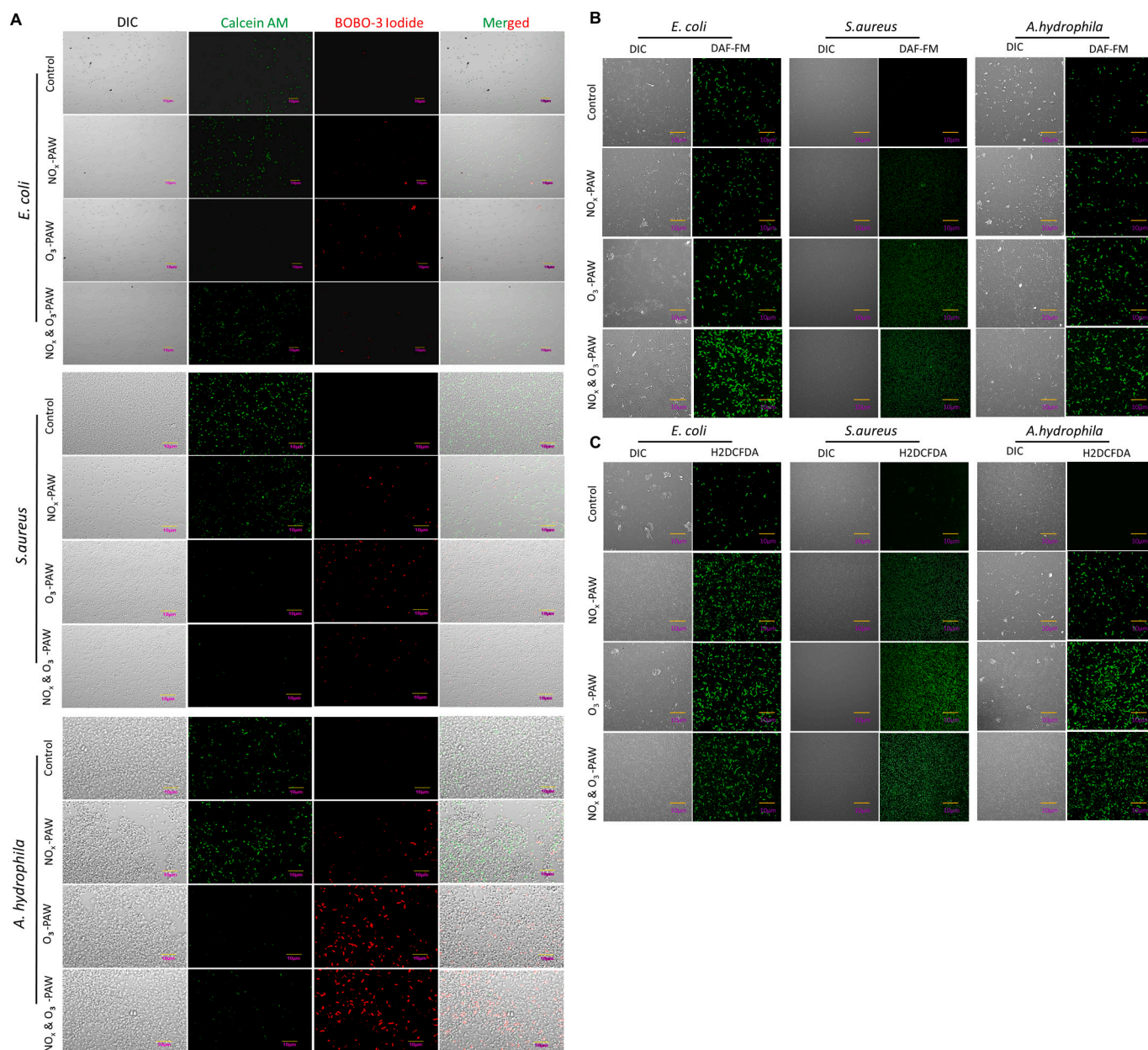


Fig. 5. Sustainability of bacterial cells (live and dead) and intracellular RNS/ROS with and without PAW treatment. (A) Live/dead evaluation of bacterial cells treated with 50 μ M NO_x containing PAW (NO_x -PAW, O_3 -PAW, and combined NO_x & O_3 -PAW) and untreated control. (B) Intracellular RNS intensity and (C) intracellular ROS intensity after treatment, showing plasma-mediated RONS-induced bacterial cell damage. DIC: differential interference contrast. Scale bar = 10 μ m.

combined NO_x and O₃-PAW treatment.

3.7. Intracellular RONS concentrations in bacteria after PAW treatment

Intracellular RNS accumulation in the bacteria was investigated following PAW treatment. The RNS species in PAW penetrated the bacterial cell membrane and interacted with the bacterial cells, ultimately inducing bacterial cell death. The combined NO_x and O₃-PAW treatments showed higher green-fluorescence intensity than the control and other treatment groups (Fig. 5B). Similarly, intracellular ROS accumulation was higher in the PAW-treated groups. The O₃-PAW treatments exhibited a higher green-fluorescence intensity than the control and other treatment groups (Fig. 5C). These results suggest that intracellular ROS accumulation may be attributed to extracellular H₂O₂ and other PAW constituents, such as pH.

4. Discussion

In aquaculture, *Aeromonas* strains continue to pose a significant challenge for disease management (Sherif and Kassab, 2023). *A. hydrophila* has shown cumulative resistance to fluoroquinolones, including enrofloxacin and ciprofloxacin (Nasser et al., 2022). Khan et al. (2012) reported that tetracycline, ampicillin, sulfonamides, and chloramphenicol are largely ineffective against *A. hydrophila*. Several studies have detected *A. hydrophila* in drinking water at concentrations ranging from 1 to 10⁴ CFU/100 mL, potentially compromising water quality (Silvestry-Rodriguez et al., 2008; Lontsi Djimeli et al., 2013). Although chemical disinfectants are commonly used to control bacteria in aquatic environments, they can cause environmental pollution. To avoid this drawback, PAW was employed in this study as an alternative approach for controlling pathogenic bacteria.

The antibacterial response of PAW depends on its physical and chemical properties. In this study, two distinct modes of MEDBD plasma were employed to inactivate bacteria: a continuous mode to generate NO_x-rich plasma and pulsed mode to generate O₃-rich plasma (Fig. 1). The RONS profiles produced in each mode were analyzed and confirmed using OES and FTIR. NO_x plasma produced higher concentrations of NO and NO₂ (604 ppm and 294 ppm, respectively), whereas O₃ plasma generated a high ozone concentration (780 ppm), and the combined NO_x and O₃ mode produced intermediate concentrations of both species (Fig. 2). The plasma-generated RONS were introduced into TW through a bubbling system, resulting in PAW with altered physicochemical properties, including pH, electrical conductivity (EC), ORP, TDS, and concentrations of H₂O₂, NO₂⁻, and NO₃⁻, compared with the control (Fig. 3A–G).

The pH decreased in NO_x-PAW, O₃-PAW, and the combined NO_x and O₃-PAW treatment compared with the control (TW), with reductions of 0.86, 1.17, and 0.81 units, respectively. These reductions were slightly greater than those reported by Judé et al. (2018), who observed a pH decrease of approximately one unit in DBD plasma-treated TW. Plasma treatment significantly increased the electrical conductivity and TDS of PAW, likely owing to enhanced ion generation and activation, which is consistent with our previous studies (Ashokkumar et al., 2024, 2025, 2026). During plasma activation, the ability of water to gain or lose electrons at the electrode surface is altered, leading to changes in the ORP compared with the control. Zhang et al. (2016) reported that the ORP in PAW depends on the applied voltage, gas-flow rate, and operational parameters, with enhancements of up to 63% under optimized conditions.

The highest NO_x concentration was observed in the combined NO_x and O₃-PAW treatment (355.07 ± 3.04 μM), whereas the control exhibited only 3.47 ± 1.34 μM. The nitrite concentration reached 0.09 mg/mL in NO_x-PAW but remained low (0.02 mg/mL) in the control (Fig. 3E, F). In contrast, O₃-PAW exhibited the highest H₂O₂ concentration (8.26 ± 0.32 mM), which contributed to pH reduction, increased EC, and elevated ORP under acidic conditions (Fig. 3G). Collectively,

these physicochemical parameters acted synergistically to enhance bacterial inactivation. Previous studies demonstrated that Ar-APPJ and DBD plasma-activated water (PAW) systems generate reactive species, including H₂O₂, NO₂⁻, ONOOH/ONOO⁻, ·OH, ¹O₂, and ·O₂⁻, which effectively inactivate bacteria and yeast cells. Acidic conditions further promoted ONOOH formation and antimicrobial activity, while pre-loading 0.1% H₂O₂ achieved complete (>99.999%) methicillin-resistant *S. aureus* (MRSA) inactivation at pH 4.0 by enhancing ONOO⁻/ONOOH and O₂⁻/HO₂ generation and suppressing catalase activity. Plasma treatment also caused membrane damage, intracellular ROS accumulation, mitochondrial dysfunction, protein and DNA leakage, and DNA fragmentation, resulting in strong microbial inactivation (Xu et al., 2021; He et al., 2025; Lim et al., 2025).

The production of ROS, particularly H₂O₂, in the PAW depends on the NO_x/O₃ plasma device. NO₂⁻ and NO₃⁻ are mainly formed from dissolved gas-phase nitrogen oxides (N_xO_y), with minor contributions from HNO₂ and HNO₃, leading to enhanced acidification PAW. The formation of H₂O₂ in PAW is promoted through the reactions shown in Eqs. (21)–(23). In addition, dissolved ozone decomposes and reacts with water to generate secondary ROS, including hydroxyl radicals and hydrogen peroxide, through the reactions presented in Eqs. (24)–(26).



These reactions demonstrate that plasma generates not only long-lived reactive species, such as H₂O₂, NO₂⁻, and NO₃⁻, but also highly reactive short-lived reactive oxygen and nitrogen species (RONS), including ·OH, O₂·⁻, ·H, NO·, and ¹O₂. Because these transient species have extremely short lifetimes, they are commonly detected using electron spin resonance (ESR, also known as electron paramagnetic resonance, EPR) coupled with spin-trapping agents. Although ESR provides only semi-quantitative measurements due to competing radical reactions and spin-adduct decay, it remains the most widely used technique for comparing short-lived RONS generation under different plasma operating conditions (Gorbanev et al., 2018; Sakurai et al., 2021).

Both long-lived and short-lived RONS contribute to the antimicrobial activity of PAW. Bacterial inactivation depended on bacterial species, treatment duration, and PAW characteristics, including RONS concentration and pH. After 48 h of treatment, bacterial reduction was lowest in the NC (0.26 CFU/mL), whereas reductions ranged from 3.19 to 3.99 log CFU/mL in NO_x-PAW, 3.23 to 4.17 log CFU/mL in O₃-PAW, and 3.0 to 3.3 log CFU/mL in the combined NO_x and O₃-PAW treatment. A nested one-way ANOVA revealed significant differences in bacterial reduction among the treatments; compared with NC, the combined treatment showed significance at *p* < 0.05, while the other treatments showed significance at *p* < 0.01 (Fig. 3H–K). Bacterial inactivation was time- and RONS-dependent, and the survival of planktonic and biofilm populations of *E. coli*, *S. aureus*, and *A. hydrophila* was significantly reduced following PAW treatment (Fig. 3H–J). Among the treatments, O₃-PAW consistently achieved the highest log reduction. Naitali et al. (2010) attributed bacterial inactivation to the synergistic effects of an acidic pH and nitrate/nitrite residues in PAW. Moreover, the plasma-generated reactive species in water are effective against bacteria, viruses, and fungi (Ashokkumar et al., 2025, 2026).

Previous studies have demonstrated that bacterial inactivation by PAW is influenced by RONS concentration, exposure time, storage

temperature, and storage duration. Wang and Salvi (2021) reported that *E. coli* and *Listeria innocua* inactivation by PAW was greater at 4 °C than at 22 °C, owing to higher RONS stability. Similarly, Shen et al. (2016) showed that PAW stored at −80 °C exhibited the strongest antibacterial effect against *S. aureus* (3–4 log reduction), whereas PAW stored at higher temperatures displayed reduced efficacy, corresponding to decreased RONS levels over time. In the present study, O₃-PAW exhibited higher H₂O₂ concentrations (8.26 ± 0.32 mM) and lower IC₅₀ values for all tested bacteria (*E. coli*: 25.52 μM; *S. aureus*: 25.33 μM; *A. hydrophila*: 25.44 μM), indicating superior antibacterial efficiency compared with NO_x-PAW or combined PAW. The lower IC₅₀ values suggest that even at relatively low NO_x concentrations, O₃-PAW was sufficient to achieve 50% bacterial inactivation, highlighting the dominant role of ozone and H₂O₂ in inducing oxidative stress. Li et al. (2025) demonstrated that low concentrations of H₂O₂, alone or combined with PAW, effectively inactivated *E. coli* and *S. aureus* under organic-rich conditions. Katsaros et al. (2023) also reported that plasma jets generated using air or argon increased EC, H₂O₂, NO₂[−], and NO₃[−] levels while reducing pH, resulting in acidified PAW with enhanced antimicrobial activity.

The highest biofilm reduction was attributed to the combined effects of RONS and acidic pH. Bacterial survival was higher on plastic and SS surfaces than on cotton fabric, likely owing to differences in surface roughness and porosity, which affect the PAW distribution (Fig. 4K–M). Liu et al. (2014) reported that *A. hydrophila* persistence in sediments promoted biofilm formation on pipe walls, thereby enabling long-term survival. The United States Environmental Protection Agency has included *A. hydrophila* in the Candidate Contaminant List as a microbial contaminant of concern for drinking water. In the Netherlands, *Aeromonas* concentrations in potable water have been maintained below 20 CFU/100 mL (median annual value) (Figueras and Beaz-Hidalgo, 2014). Udachin and Dahle (2025) demonstrated that DBD plasma reduced *E. coli* and *S. aureus* biofilms on SS by 4.3 and 3.3 log CFU/mL, respectively, as confirmed by SEM.

Bacteria inactivation is depended on RONS particularly long and short live species in the PAW. Previous Zhou et al., 2024 studies also showed that lower temperatures enhance PAW antibacterial activity by improving the stability and solubility of oxidizing species such as O₃, H₂O₂, OH, and HOONO, whereas increasing temperature reduces their solubility and lowers PAW reactivity. In contrast, combining PAW with mild heat produced synergistic *E. coli* inactivation through oxidative stress, membrane disruption, protein denaturation, RNA damage, and increased membrane permeability. Whereas other three studies demonstrated that combining NO_x-rich and O₃-rich plasma gases significantly enhanced the generation of high-valence NO_x species, especially N₂O₅ and NO₃ radicals, leading to more efficient PAW production and stronger antibacterial activity. Hybrid DBD–gliding arc systems achieved higher N₂O₅ concentrations, better energy efficiency, and improved reactive species transfer into water compared with single DBD systems. N₂O₅ mainly contributed to acidification and nitrate formation, while NO₃ radicals promoted short-lived bactericidal species such as peroxyxynitrite and superoxide. As a result, PAW achieved nearly 6-log inactivation of MRSA and *E. coli*, caused severe membrane damage and intracellular leakage, and maintained >99% antibacterial activity after 1 h storage (Wang et al., 2022a, 2022b, 2023, 2024).

PAW treatment disrupted both the intra- and extracellular bacterial structures. The SEM images revealed membrane damage, cell shrinkage, and leakage of intracellular contents following PAW exposure (Fig. 4N). These observations were corroborated by flow cytometry (Fig. 4O) and live/dead assays, which showed that O₃-PAW induced the highest PI uptake and BOBO-3 iodide fluorescence, indicating extensive membrane permeabilization and cell death (Fig. 5A). Fluorescence-based assays are widely used to assess bacterial-membrane potential, metabolic activity, and membrane integrity and are compatible with a broad range of dyes and labeling techniques (Léonard et al., 2016).

According to a study by Hoeven et al. (2019), nitrite levels gradually

oxidize to the more stable nitrate during plasma treatment and storage, thereby making nitrate the primary long-lasting nitrogen species. Although repeated use of PAW may temporarily increase nitrate concentrations, these species subsequently participate in the natural aquatic nitrogen cycle. Under aerobic conditions, nitrite is oxidized to nitrate, while nitrate can be internalized by algae and microorganisms, reduced to gaseous nitrogen species (NO, N₂O, and ultimately N₂) by microbial denitrification, or converted to NH₄⁺ by the dissimilatory nitrate reduction to ammonium (DNRA) reaction under reducing conditions. Through these reactions, plasma-generated nitrogen species are naturally converted by the microbial nitrogen cycle rather than accumulating indefinitely in aquaculture systems (Preena et al., 2021).

The intracellular accumulation of RNS and ROS further elucidated the bactericidal mechanism (Fig. 5B, C). PAW components, including NO_x species, H₂O₂, and low pH, penetrate bacterial cells and induce oxidative damage to nucleic acids, proteins, and lipids. O₃-PAW produced the highest intracellular ROS accumulation, consistent with its elevated extracellular H₂O₂ levels. The combined NO_x and O₃-PAW treatment generated intermediate intracellular RONS levels, corresponding to its moderate antibacterial efficacy. These findings indicated that the synergistic action of RONS and membrane disruption is critical for effective bacterial inactivation.

In this study, MEDBD plasma-generated PAW, particularly O₃-PAW, demonstrated significant potential as an environmentally friendly alternative for controlling both planktonic and biofilm-associated bacteria. The bactericidal activity is attributed to the combined effects of chemical factors, including RONS and acidic pH, together with plasma-induced physical effects that disrupt bacterial membrane integrity, promote intracellular oxidative stress, and ultimately induce cell death. Similarly, Liao et al., 2017 reported that the antimicrobial activity of nonthermal plasma results from the synergistic action of long-lived and short-lived RONS. Long-lived species (e.g., H₂O₂, O₃, NO₂[−], and NO₃[−]) provide sustained oxidative stress, whereas short-lived radicals (e.g., •OH, O₂•[−], and NO•) rapidly initiate lipid peroxidation, protein oxidation, and DNA damage. Together, these reactive species disrupt membrane integrity, impair antioxidant defense systems and cellular metabolism, damage nucleic acids, and overwhelm bacterial repair mechanisms, leading to irreversible cell death. In addition, plasma-induced physical effects, such as electrostatic disruption and electroporation, further increase membrane permeability, facilitating the penetration of reactive species into bacterial cells and enhancing antimicrobial efficacy. These findings provide a mechanistic basis for the application of PAW in water treatment, aquaculture, and surface disinfection.

5. Conclusion

This study established the robust antibacterial and antibiofilm efficacies of PAW generated using an MEDBD plasma system against *Escherichia coli*, *Staphylococcus aureus*, and *Aeromonas hydrophila*. Distinct plasma operating modes yielded tailored RONS signatures, leading to marked physicochemical modifications in PAW, including decreased pH and elevated electrical conductivity, oxidation–reduction potential, and H₂O₂, NO₂[−], and NO₃[−] concentrations. These coordinated changes underpin the significant reduction in bacterial viability. Among the tested conditions, O₃-enriched PAW demonstrated superior antimicrobial performance, achieving the highest log reductions and lowest IC₅₀ values across all strains. Its enhanced activity is correlated with elevated H₂O₂ levels and increased intracellular ROS accumulation, resulting in membrane permeabilization, cytoplasmic leakage, and oxidative damage to essential cellular components. The combined NO_x/O₃-PAW treatment exhibited intermediate RONS profiles and antimicrobial efficacy, whereas the NO_x-dominant PAW produced comparatively lower but substantial inactivation. Furthermore, biofilm disruption assays on diverse substrates confirmed that the PAW performance is governed by both reactive-species synergy and surface-

dependent interactions. Collectively, these findings elucidate the mechanistic basis of PAW-mediated microbial inactivation and highlight the synergistic interplay between RONS chemistry, acidification, redox modulation, and membrane destabilization. These results suggest that PAW is a scalable, environmentally sustainable platform for pathogen control in aquaculture and advanced water-treatment systems, with significant implications for biosecurity and sustainable water management.

In addition, plasma-generated reactive species in water provide a scalable and environmentally friendly approach for controlling aquatic and foodborne pathogens, improving aquaculture biosecurity, enhancing seafood safety, and enabling water reuse in food processing. This study demonstrates the potential of plasma-activated water (PAW) for effective bacterial reduction and aquaculture water disinfection. The generated RONS are generally short-lived and decompose into relatively safe compounds, suggesting a lower environmental impact than conventional disinfectants. However, prolonged plasma treatment may produce byproducts such as nitrite, nitrate, and ozone-derived oxidants and may also alter water pH and ORP. Therefore, optimization of plasma operating conditions, along with further ecotoxicological and large-scale aquaculture studies, is necessary before commercial application.

5.1. Future perspectives

Future research should focus on optimizing plasma operating parameters to precisely control the composition and stability of RONS in PAW (temperature, pH, etc.), thereby maximizing antimicrobial efficacy while minimizing energy consumption. Long-term stability studies under different storage conditions and water matrices are needed to better understand the persistence of RONS and their performance under practical operating conditions. In addition, the scalability of PAW systems should be evaluated for large-volume water treatment and aquaculture facilities, particularly using continuous-flow reactor configurations. The antimicrobial efficacy of PAW should also be assessed against mixed-species biofilms representing those that persist on aquaculture equipment (e.g., tanks, pipes, filters, and aerators), as well as antibiotic-resistant bacterial strains under field conditions to validate its broader applicability. Furthermore, mechanistic investigations employing transcriptomic, proteomic, and metabolomic approaches could provide deeper insights into the bacterial stress responses, cellular damage mechanisms, and potential resistance pathways induced by PAW.

The antimicrobial efficacy of PAW should also be assessed against mixed-species biofilms (representing biofilm persistence on aquaculture equipment) and antibiotic-resistant bacterial strains under field conditions to validate its broader applicability. Comprehensive environmental and toxicological evaluations are also essential to ensure the safety of PAW-treated water for aquatic organisms and humans. Integrating PAW with existing disinfection technologies may further improve treatment efficiency and sustainability, positioning it as a promising strategy for next-generation water disinfection, pathogen control, and in vivo disease management in aquaculture systems. Finally, the antimicrobial performance of plasma-generated RONS should be validated (optimize bacteria inactivation based on H_2O_2 , NO_2^- , and NO_3^- concentrations) the using synthetic aquaculture water as well as actual pond water and recirculating aquaculture system (RAS) water under realistic operating conditions. Such studies will provide a more comprehensive assessment of the practical applicability of plasma technology for aquaculture water disinfection and support its translation into commercial aquaculture practices.

CRediT authorship contribution statement

Sekar Ashokkumar: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Tirtha**

Raj Acharya: Methodology, Formal analysis. **Prajwal Lamichhane:** Methodology, Formal analysis. **Eun Ha Choi:** Writing – review & editing, Supervision, Project administration, Funding acquisition. **Young-Chul Lee:** Writing – review & editing, Supervision, Funding acquisition.

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Declaration of competing interest

The authors declare no known competing financial interests or personal relationships that could have influenced the work reported in this manuscript.

Data availability

Data will be made available on request.

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