Eradication of Pseudomonas Aeruginosa and its Ecotoxicity in Freshwater Utilizing Cold Atmospheric Plasma: Is it Environmentally Safe?

farshad sohbatzadeh (f.sohbat@umz.ac.ir)
University of Mazandaran
Hanieh Haqpanah
University of Mazandaran
Amir Shabannejad
University of Mazandaran
Homayoon Yazdanshenas
University of Mazandaran

Research Article

Keywords: Aquatic environments, Cold atmospheric plasma, Decontamination, Ecotoxicity, Exotoxin A, Gammarus roeseli, Pseudomonas aeruginosa

Posted Date: February 7th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1307564/v1

Abstract

Pseudomonas aeruginosa is a multidrug-resistant bacterial strain with the ability to produce exotoxin A which can pose a serious threat to freshwater ecosystems by having pathogenicity against eukaryotes. Detoxification of exotoxin A and disinfection of *P. aeruginosa* are the main aims of this study. Using a high dosage of antibiotics might have more toxic effects on ecosystems while cold atmospheric plasma (CAP) can promise reliable, rapid, and environmentally friendly detoxification and disinfection. In this study we produced CAP reinforced by H_2O_2/H_2O cold vapor to detoxify exotoxin A and inactivate *P. aeruginosa* in freshwater. We used *Gammarus roeseli* as the indicator of ecotoxicity in freshwater. The mortality of *G. roeseli* individuals elucidated that 420 s of CAP treatment under a surface dielectric barrier discharge (SDBD) set up can effectively passivize exotoxin A in freshwater by disrupting the protein structure of molecules. Ignorable side effects and changes to the physiochemical properties were observed. On the other hand, 8.2 log reduction of *P. aeruginosa* viable cells was observed after 300 s of treatment by SDBD. A comparison between the disinfection capacities of SDBD produced CAP and antibiotics revealed that CAP is more effective than most of the common antibiotic agents.

1. Introduction

Pseudomonas aeruginosa is a gram-negative and motile bacterium that can be found in soil, freshwater, and marine water [1–4]. *P. aeruginosa* is facultative anaerobe bacteria, as it is well adapted to proliferate in conditions of partial or total oxygen depletion. Recently, the endurance of infectious *P. aeruginosa* in different aquatic habitats has attracted a great deal of attention [5]. On the other hand, *P. aeruginosa* strains are known to have remarkable antibiotic resistance which makes them well-known multidrug-resistant strains [6–8]. Some *P. aeruginosa* strains can secrete a virulence factor called exotoxin A which is a single polypeptide chain protein consisting of 613 amino acids [9]. *P. aeruginosa* uses its virulence factor to vandalize some important eukaryotic proteins by inactivating eukaryotic elongation factor 2 (EEF 2) which assists protein translation [10]. EEF 2 protein is essential for catalyzing the translocation of tRNA and mRNA through the ribosome during the translation of other proteins and its deficiency causes symptoms and cell death [11]. *P. aeruginosa* can strongly affect marine and freshwater ecosystems and organisms by producing its toxin [12,13]. Research has proven that *P. aeruginosa* can widely infect marine vertebrates by proliferating into their bodies [13]. Genetic investigations also fairly revealed that most *P. aeruginosa* strains found among the infected marine vertebrates are multidrug-resistant and capable of producing exotoxin A [12]. *P. aeruginosa* infection in marine organisms can lead to bioaccumulation and endanger a whole ecosystem and environmental safety [13]. Therefore, restraining the water pollution caused by *P. aeruginosa* is becoming a serious environmental challenge in developing societies [2]. Using antibiotics might be considered as a low cost and plain way for inactivating bacteria in aquatic habitats, but effective antibiotics against bacteria such as *P. aeruginosa* are proven to have toxic effects on marine or freshwater organisms and consequently pose a serious threat to the aquatic ecosystems [14–17]. Furthermore, because *P. aeruginosa* is resistant to common antibiotics, more effective methods are

needed to inactivate this pathogenic bacterium and its toxin in freshwater. In the view of mentioned environmental and health hazards created by *P. aeruginosa* and its toxin, accurate, green, and new strategies are required to inactivate this bacterium. Among the innovative bactericidal strategies, cold atmospheric plasma (CAP) can be a better substitute for antibiotics [18]. Numerous studies have emphasized the CAP's capability of inactivating or passivizing diverse microbes at low temperatures [19–23]. It was also emphasized by several studies that CAP strategies do not pose any harm to the environment [24–27]. It is commonly accepted that cold plasma techniques produce reactive oxygen and nitrogen species (RONS) which are believed to have a valuable contribution to the biological effects of plasma [28–31]. In fact, plasma-induced species (RONS) can cause damage to the cell walls, DNA, and lipids, as well as denature or degrade proteins [19,32,33]. There are various methods for cold plasma generation such as dielectric barrier discharge (DBD), plasma jets, coronas, and microwave discharges [34]. Among these mentioned methods, DBD enjoys the advantages of producing stable and uniform discharge on a large scale and operating at relatively low temperatures under atmospheric pressure [34,35]. Besides, surface dielectric barrier discharge (SDBD) can generate a non-thermal plasma (NTP) over a huge area at a low cost, which has given it more commercialization potential than other systems [36–38]. It is also confirmed that SDBD can increase the bactericidal effect of some ROS, especially H_2O_2 [36,39–41]. Plasma-mediated inactivation of *P. aeruginosa* has been investigated by several researchers. Matthes *et al*. treated *in vitro P. aeruginosa* biofilms with plasma jet by using argon/oxygen mixture as gas feed and chlorhexidine digluconate, which is a commercial antiseptic. A comparison between the results proved that plasma jet can be more rapid in disinfection of *P. aeruginosa* [42]. Ziuzina *et al*. also reported a 5.4-log reduction of *P. aeruginosa* biofilms after treatment with DBD [43]. Kondeti *et al*. eradicated *P. aeruginosa* in distilled water and saline solutions with cold atmospheric pressure plasma jet (APPJ) with two different gas combinations including argon/oxygen and argon/air. They also reported that $O^{\bullet}$, $O_2^{\bullet-}$, and $^{\bullet}OH$ had a more important contribution to the disinfection [44]. Wang *et al*. also used the APPJ to eliminate *P. aeruginosa* and demonstrated that even short treatment times with CAP can deactivate viable cells by reducing their metabolic activity and the expression of virulence factors [45]. Dijksteel *et al*. used flexible SDBD plasma to inactivate *P. aeruginosa* in an *in vivo* and *in vitro* study and achieved 5 to 7 log reduction with no additional harm or side effects on normal cells in rat models [46]. Cold plasma has also been widely used for decontamination and detoxification [47–55]. CAP strategies have been hopefully successful at disinfection of *P. aeruginosa* but the detoxification of its toxin has been disregarded so far. Therefore, investigating the detoxification potential of CAP and its possible effects on freshwater organisms may present a new aspect of repelling the ecotoxicity caused by infectious microorganisms. *Gammarus* species are amphipod invertebrate animals living in fresh or marine waters [56]. Their population has been widely used as an assessment of water quality [56,57]. The death of *Gammarus* species is a biomarker of toxicity or ecotoxicity of different substances in marine and freshwaters and also their survival can be a sign of successful detoxification [58]. Hence we hired *Gammarus roeseli*, a freshwater living *Gammarus* species, to indicate the toxicity of our treated or non-treated solutions.

Recently, investigation on the biological safety of food treated with CAP and the cytotoxic effects of plasma therapies in medicine using animal and insect models has begun [59,60]. This reflects the growing attention of researchers around the world to the side effects and potential toxicity of using CAP commercially, taking into account current health standards. Cold plasma is well known as a suitable plan for the inactivation of pathogenic microorganisms as well as organic contaminants in aquatic environments [61]. To our knowledge, CAP has never been employed for inactivating the ecotoxicity of organic compounds such as exotoxin A. Notwithstanding, a prominent novelty of the present study is offering the first and only available research on the ecotoxicity and possible adverse impacts of CAP-treated aquatic samples on the return to the water cycle of nature. Prior studies have investigated the toxicity of ozone and hydrogen peroxide treatments on organisms in aquatic ecosystems [62–66]. The study of the effects of CAP treatment on the ecotoxicity of freshwater environments, presented here for the first time, gives our research a unique credit as the sole reference in this regard. A well-known biomarker (*G. roeseli*) was called for this purpose.

Our aim in this study is to (1): Eliminate the threat of exotoxin A and *P. aeruginosa* to the freshwater environment utilizing CAP. (2): investigating possible side effects of cold atmospheric plasma on aquatic environments and its ecotoxicity. (3): evaluating the concentration of RONS to clarify their contribution to disinfection and detoxification effects of developed CAP-based strategy. (4): comparing the disinfection efficiency of the CAP with common antibiotics against the bacteria.

2. Material & Methods

2.1. CAP treatments

The strategy employed by us in this study is comprehensively and precisely described in our recent research, so readers are encouraged to refer to that paper for more details [36]. As published in our previous work, this innovative strategy manifested great potential and talent for the complete inactivation of pathogenic microorganisms. And we witnessed the synergistic effect of cold plasma and hydrogen peroxide in disinfecting aqueous environments in a short time and large dimensions. All of this puts a clear vision in front of us to establish a sustainable and large-scale CAP strategy in the event of any aquatic toxicity or contamination associated with pathogenic microorganisms in freshwater environments. In particular, this unique approach provided a great chance to completely inactivate *P. aeruginosa*, a catalase-positive and highly opportunistic bacterium in aqueous media.

In short, here we use a combination of a cold plasma generator at atmospheric pressure (SDBD) and cold hydrogen peroxide vapors to achieve the goals set for this study. The structure of SDBD is schematically depicted in Fig. 1. As stated formerly, the dimensions applied in assembling SDBD along with its electrical characteristics are reported in full detail in our previous work. These details also include the conditions for testing such as argon gas flow, treatment times, and sample specifications, except that we have aqueous samples here.

Samples were placed in a petri dish and each treatment was performed in triplicate. All treatments with SDBD device were performed without plasma to ensure the contribution of plasma to the biological effects of our strategy.

2.2. Growth studies on medium culture

The standard strain of *P. aeruginosa* (ATCC 27853) was prepared at the concentration of 1.5×10^8 CFU/ml (0.5 Mcfarland) and densely cultured on Muller-Hinton agar media (Ibresco: i23118). Cultured media were exposed to CAP under the SDBD devise for 30 s, 90 s, 180 s, 300 s, and 420 s. The exposed media were incubated at 37°C for 24 h. The approximate percentage of the disinfected area on each medium was profiled after the incubation.

2.3. Growth studies in freshwater

In order to observe the disinfecting efficiency of SDBD in freshwater, the concentration of 0.5 Mcfarland of *P. aeruginosa* was prepared in sterilized freshwater and treated with SDBD for the same treatment times. After finalizing the treatments, remained bacteria concentration was counted with the pour plate method.

2.4. Antibiotic disinfection testing

The percentage of the disinfected area caused by antibiotics was obtained with the antibiogram method. Antibiotic discs including ampicillin, amikacin, cefazolin, ceftazidime, ciprofloxacin, cefepime, cefotaxime, colistin, erythromycin, gentamicin, imipenem, nalidixic acid, nitrofurantoin, norfloxacin, tetracycline, trimethoprim-sulfamethoxazole, and vancomycin were purchased from PADTAN TEB Co. The concentration of 0.5 Mcfarland was cultured on the Muller-Hinton agar media and an antibiogram disc was placed on each culture medium. All culture media were incubated at 37°C for 24 h. The percentage of the disinfected area was profiled after the incubation time was finished.

2.5. Ecotoxicity assay

Pure exotoxin A lyophilized powder was obtained from Merk (CAS number:

91262-95-2) (MDL number: MFCD00132134) and in an effort to evaluate the possible effects of SDBD produced CAP on the ecotoxicity of exotoxin A, *Gammarus roeseli* was used as an indicator and biomarker of ecotoxicity. *G. roeseli* samples were all collected from the estuary of Safarud river (Ramsar, Mazandaran province, Iran) and kept in an aquarium in the laboratory space under the same conditions including temperature, relative humidity, total dissolved solids (TDS), pH, and day/night hours for a month. The lethal concentration of 50% (LC50) of exotoxin A in 96 h was found after treating *G. roeseli* individuals with a broad range of concentrations. After performing CAP treatments for the same treatment times, 20 *G. roeseli* individuals were added to each treated solution and their mortalities were observed after 96 h. To explore possible effects of plasma on freshwater life and ecosystem, 20 *G. roeseli* individuals were added to similar CAP treated freshwater samples with the same concentration of RONS and their mortality was observed after 96 h.

2.6. Measurement of toxin protein concentration

It has been proved that each exotoxin A molecule weighs about 67 KDa [67]. According to the studies of Erickson, a 67 KDa protein should have a diameter between 2.4 and 3.05 nm [68]. As mentioned in the introduction, CAP can produce RONS which are likely to break down the peptide bonds and rip the protein into smaller pieces. In this study, we filtered the treated samples by an Anisotropic Aluminum oxide membrane filter-2 nm pore size (SPI supplies), so the unbroken proteins stuck and the smaller protein debris together with the water go through. After that, the weight of the stuck molecules could be measured with an Optika SMG series laboratory scale (with 0.01 mgr accuracy). The concentration of exotoxin A is calculable if the weight is clarified.

2.7. Conventional physiochemical properties of water

Physiochemical characteristics of the water used for keeping *G. roeseli* such as temperature, conductivity, TDS, and pH were measured before and after each treatment time. pH and temperature were measured at the same time with AZ 86502 pH and thermometer. TDS and conductivity were measured with NEWCON digital TDS meter and DY-PH-02 digital meter, respectively.

2.8. RONS measurements

2.8.1. ROS

Except for H_2O_2 and ozone, ROS are mostly short-lived ions and radicals that cannot be measured directly in CAP-treated samples. Herein we obeyed the procedure used in a previous study by Sohbatzadeh, et al. [36]. To measure the molar concentration of each ROS produced and injected by CAP, special compounds called spin-traps must be in play. Each spin-trap can bind to a specific ROS and form a complex which can keep long enough for measurements. In order to do so, solutions with proper concentrations of spin-traps must be made and exposed to CAP at similar conditions to each freshwater sample. After that, the treated spin-trap solution(s) should be carried to an electron paramagnetic resonance (EPR) device so the concentration of each ROS could be calculated. More details on this method could be found in the review article by Hawkins and Davies [69].

The concentration of hydroxyl radical (.OH), ozone (O3)/atomic oxygen (O), and hydroperoxyl (.OOH) were measured by the EPR method, thanks to the spin traps for each of them, which are mentioned below. PBS solutions of spin traps 2,2,6,6 tetramethylpiperidine (TEMP, ≥99%), 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO ≥98%) and 5 (diethoxyphosphoryl)-5-methyl-1-pyrroline *N*-oxide (DEPMPO ≥99%) were prepared at the concentration of 0.1 M, ready to be used. 0.1 M solution of singlet oxygen (O2(a1Δg)) scavenger sodium azide (NaN3, ≥99%) was added to TEMP to make difference between the amount of 2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPO) formed from O3/O and O_2 [70,71]. An aqueous solution of 5-(2, 2-dimethyl-1, 3-propoxycyclophosphoryl)-5-methyl-1-pyrroline N-oxide (CYPMPO) and phenyl *tert*-butyl nitrone (PBN) were used to scavenge superoxide radical anions ($O_2^{\bullet-}$) [72].

EPR measurements and detections were done after preparations and treatments. The EPR device used in this study was a Magnettech MiniScope MS 200 spectrometer. All scans with this EPR were repeated three times while the parameters such as frequency, modulation frequency, modulation amplitude, power, sweeping time, and sweeping width were kept the same as the investigations of Privat-Maldonado, et al. [71]. Analyzed samples were prepared in 50 µL glass capillaries for measurements. The results were procured by double integration (SpectrumViewer ver. 2.6.3) of the respective simulated spectra (NIH P.E.S.T. WinSIM software ver. 0.96) of the formed radical adducts.

The concentration of induced H_2O_2 in the liquid samples was measured directly by UV/Vis spectroscopy using a reagent solution of potassium-titanium (IV) oxalate dehydrate in H_2O/H_2SO_4 [73].

2.8.2. RNS

Most RNS are nitrate and nitrite anions that can keep for at least one day so their concentration in treated samples can be measured with ion chromatography columns. Column Metrosep A Supp 10 – 250/4.0 was assembled with anion eluent composition of 5 mM Na_2CO_3 + 5 mM $NaHCO_3$ and flow of 1 ml/min and pressure level of 14.94 MPa. Metrohm model 881 compact IC pro 1 was put to the recording process for 36 min and manual integration to detect the concentration of nitrate and nitrite in treated and control samples.

2.9. Scanning electron microscopy (SEM)

The effect of CAP on *P. aeruginosa* viable cells was studied using scanning electron microscopy (SEM). 0.05 ml of treated and non-treated samples were placed on coverslips and incubated at 40°C for 1 h for dehydration and fixation. Dried slips were coated with gold in a Polalis sputter coater and the images were taken using an SEC SNE4500 SEM device at an accelerator voltage of 20 kV and at 10,000 × magnification.

2.10. Statistical analysis

Each experimental condition, preparation, and requirement was performed in triplicate and each experiment was separately repeated three times. Data are presented as mean ± standard deviation (SD). Differences among groups are also reported using the statistical package SPSS 24.0 (IBM, Armonk, NY, USA). The preferred procedure for comparing the data was one-way ANOVA followed by Duncan's multiple comparison test as a post hoc test at a 95% confidence level and a significance level of $p < 0.05$.

3. Results And Discussion

3.1. Bactericidal effect of SDBD plasma on culture media

The bactericidal effect of the plasma produced by the SDBD device was investigated on Muller-Hinton agar media. The percentage of the disinfected area was compared with antibiotic disc diffusion. As shown in Table 1, there was no significant difference between the cleared area caused by ampicillin,

erythromycin, tetracycline, nalidixic acid, nitrofurantoin, trimethoprim-sulfamethoxazole, cefazolin, vancomycin, amikacin, and cefotaxime. Also, the same result applies to comparing the area cleared by these antibiotics with the control sample. The most effective antibiotics against *P. aeruginosa* from the weakest to the strongest are imipenem, cefepime, ciprofloxacin, and gentamicin, respectively. These disinfected areas caused by these antibiotics were significantly more than others. In the case of the CAP treated samples, the disinfected area was time-dependently increased. There was a significant difference in disinfected area between each treatment time from 30 s to 420 s. A comparison between the disinfected area caused by antibiotics and CAP treatments in Table 1 states that the cleared area after 30 s CAP treatment and norfloxacin, colistin, ceftazidime, and amikacin are not significantly different and CAP treatment for the 90 s and ceftazidime had almost the same disinfection effects. Disinfection effects of ciprofloxacin, gentamicin, cefepime, and imipenem were not significantly different from 180 s of CAP treatment. The bactericidal effects of gentamicin and CAP treatment for 300 s were not significantly different. Finally, treating the culture medium for 420 s disinfected the whole plate, and complete inactivation was achieved. Unlike 30 s, 90 s, 180 s, and 300 s, 420 s of CAP treatment times had significantly more disinfection effects than all of the tested antibiotics. These results indicate that in 420 s or more, SDBD generated CAP can be more bactericidal than every known antibiotic.

Table 1

Percentage of medium culture disinfected area caused by antibiotics and CAP treatments[a,b].

Antibiotic or CAP treatment	Percentage of the disinfected area on the plate (%)
Ctrl	0±0.00[g]
Ampicillin	0.52±0.43[g]
Erythromycin	0.15±0.04[g]
Norfloxacin	12.17±2.84[f]
Tetracycline	0.45±0.16[g]
Nalidixic acid	0.24±0.11[g]
Nitrofurantoin	0.18±0.01[g]
Trimethoprim-sulfamethoxazole	0.25±0.12[g]
Cefazolin	0.24±0.07[g]
Vancomycin	0.15±0.04[g]
Amikacin	9.50±4.82[f,g]
Ciprofloxacin	40.33±8.39[c,d]
Cefotaxime	0.48±0.13[g]
Gentamicin	46.67±3.51[b,c]
Ceftazidime	17.87±2.80[e,f]
Cefepime	35.33±7.23[d]
Colisitin	10.27±3.10[f]
Imipenem	34.67±14.05[d]
SDBD 30s	10.67±5.13[f]
SDBD 90s	21.67±4.16[e]
SDBD 3min	40±8.89[c,d]

[a] Significant difference ($p<0.05$) within each column was determined by different superscript letters.

[b] Values are means±SD (n=3).

Antibiotic or CAP treatment	Percentage of the disinfected area on the plate (%)
SDBD 5min	53.33±7.57[b]
SDBD 7min	100±0.00[a]
[a] Significant difference ($p<0.05$) within each column was determined by different superscript letters.	
[b] Values are means±SD (n=3).	

3.2. Bactericidal effect of SDBD plasma on freshwater

All of our freshwater samples for this particular assay were infected with 8.2-log of *P. aeruginosa* viable cells and treated under the SDBD set up for the same times. The results are listed in Table 2, and as can be seen, there was no significant difference between the remaining log after the treatment times of 30 s and 90 s. After 180 s and 300 s the remaining log significantly reduced to 1.63±0.57 and 0±0.00 respectively. Summarizing the data in Fig. 2 illustrates that ROS especially H_2O_2, O_3/O, $O_2^{\bullet-}$, and $^{\bullet}$OH had more contribution in log reduction during our CAP treatments. A comparison between the reduction of log and the concentration of RONS during treatment times indicates that among the ROS, H_2O_2 had more contribution than others. O_3/O, $O_2^{\bullet-}$, and $^{\bullet}$OH had a more important role after H_2O_2. Nitrate anion might also play a bactericidal role until 90 s but after that its concentration had negligible changes. Very low concentrations of RNS during the treatment process using our new plasma-based strategy promise an environmentally friendly solution without causing nitrate contamination in water reserves after the release of aqueous samples into nature [74].

To avoid duplication, in order that obtains details of physicochemical processes (*e.g.*, chemical reactions involved in the generation and loss of long and short-lived reactive species (*i.e.*, RONS) in the gas/liquid phase or the interfacial layer), in indirect interaction between cold atmospheric pressure plasma and deionized water, refer to articles by other authors in this field [75–77]. Also, the (sub-)cellular mechanisms governing the process of microbial inactivation during water disinfection by cold plasma at atmospheric pressure can be found in already authoritative literature [31,33,78,79].

Table 2

Log reduction of bacteria in fresh water during treatment times[a,b].

CAP treatments	Log (CFU/ml)
Ctrl	8.20±0.00[a]
SDBD 30s	4.50±1.05[b]
SDBD 90s	3.67±0.57[b]
SDBD 3min	1.63±0.57[c]
SDBD 5min	0±0.00[d]
SDBD 7min	0±0.00[d]
[a] Significant difference ($p<0.05$) within each column was determined by different superscript letters.	
[b] Values are means±SD (n=3).	

3.3. Detoxification effect of plasma: *G. roeseli* casualties by remaining exotoxin A

The detoxification capacity of plasma has been investigated by several studies before [51,53,55,80–88]. RONS produced by CAP devices, specifically O_3, play a vital role in CAP-induced detoxification [53]. Despite RONS, UV radiation produced during CAP treatment can also speed up the degradation of toxic compounds [51]. In fact, CAP can manipulate the protein structure and make it inefficient [89–91]. Nevertheless, CAP has never been hired for inactivating the ecotoxicity of organic compounds such as exotoxin A. In our study, the aquatic solution of exotoxin A with the concentration of 300 µgr/lit (LC50) was treated with CAP under SDBD set up for 30 s, 90 s, 180 s, 300 s, and, 420 s. After each treatment 20 *G. roeseli* individuals were added to each treated sample for investigating the effect of CAP treatments on the ecotoxicity of exotoxin A. All treatments were repeated three times. Table 3 contains the results of treated solutions. CAP treatments for 30 s and 90 s had no detoxification effect on toxic solutions since the death toll of *G. roeseli* individuals was not significantly changed after 96 h. The results of 180 s were dissimilar to the lower times and the casualties of individuals significantly decreased to 38.33±2.89%. CAP treated toxic solution for 300 s and 420 s respectively caused 10±5.00 and 3.33±2.89% of casualty among exposed individuals. In general, the toxicity of treated solutions continued to decrease during the higher treatment times, which offers a time-dependent manner of detoxification caused by CAP. A comparison between the RONS concentrations during treatment times (shown in Fig. 2) and the detoxification effect of CAP (shown in Table 3) can explain the detoxification effect of CAP. It appears that H_2O_2 and O_3 have a more important role than other RONS, which is totally in accordance with the results of CAP-treated bacteria in freshwater mentioned above. Hence it can be concluded that plasma-induced RONS can be effective against *P. aeruginosa* and its toxin.

Table 3

Detoxification effect of CAP on exotoxin A[a,b].

CAP treatment	*G. roeseli* casualties after 96 h (%)
Ctrl (LC50)	50±0.00[a]
SDBD 30s	48.33±2.89[a]
SDBD 90s	46.67±2.89[a]
SDBD 3min	38.33±2.89[b]
SDBD 5min	10±5.00[c]
SDBD 7min	3.33±2.89[d]
[a] Significant difference ($p<0.05$) within each column was determined by different superscript letters.	
[b] Values are means±SD (n=3).	

3.4. The effect of CAP on the concentration of exotoxin A

The results of the treated exotoxin A with CAP are presented in Table 4. As can be seen, the control samples were vigorously fixed at 300 µgr/lit. The 30 s CAP treatment had no significant effect on the concentration of the toxin but after 90 s the concentration diminished to 213.33±41.63 µgr/lit. The abatement continued through the 180, 300, and 420 seconds since the concentration of the toxin dropped to 123.33±49.33, 53.33±30.55, and 3.33±5.77 µgr/lit respectively. Statistical analysis indicated that the difference between the concentrations after 90 s, 180 s, and 300 s was significant to each other but the difference between 300 s and 420 s was not significant. However, treating samples with our CAP strategy for 420 s resulted in the reduction of exotoxin A to nontoxic concentrations. A comparison between these results and the results of section 3.3 notes that there is a synonymy between the concentration of the exotoxin A and casualties of *G. roeseli* in freshwater since a reduction in one is followed by a reduction in another. All in all, both above-mentioned reductions and the amplification of the ROS concentration are happening simultaneously, which explicates that our CAP treatment strategy is effective at detoxification of exotoxin A by disrupting its molecular structure.

Table 4

The effect of CAP on the concentration of exotoxin A[a,b].

CAP treatment	The concentration of Exotoxin A (µgr/Lit)
Ctrl (LC50)	300±0.00[a]
SDBD 30s	283.33±15.27[a]
SDBD 90s	213.33±41.63[b]
SDBD 3min	123.33±49.33[c]
SDBD 5min	53.33±30.55[d]
SDBD 7min	3.33±5.77[d]
[a] Significant difference ($p<0.05$) within each column was determined by different superscript letters.	
[b] Values are means±SD (n=3).	

3.5. Effects of CAP on conventional physicochemical properties of freshwater

Evaluating the physiochemical properties of water is critical for investigating the possible effects of plasma on aquatic environments. Conventional physiochemical properties of freshwater such as pH, conductivity, TDS and, temperature are major factors for a functional freshwater ecosystem since the life of many freshwater living species depends on them [92,93]. The pH of treated freshwater samples was not significantly changed after 90 s but after that, it started to reduce. As shown in Table 5 the pH of 300 s and 420 s were significantly lower than other treated samples and the control sample. Our findings in this part follow the previous studies by Oehmigen *et al*., Shainsky *et al*., and Zhou *et al*. who reported that a reduction in pH during CAP treatments might be due to the presence of H_2O_2 and RNS [77,94,95]. Both TDS and conductivity of our treated samples were increased during our treatment times. The change of TDS and conductivity have resulted from the presence of RONS in treated samples. The alteration of TDS and conductivity of water was also reported by Rathore *et al*. [96]. Due to the cold nature of plasma, the temperature of treated samples was not significantly changed during CAP treatments until 300 s.

Table 5

The effect of CAP treatments on conventional physicochemical properties of fresh water [a,b].

Samples	pH	Conductivity (µS/cm)	TDS (ppm)	Temperatures (°C)
Ctrl	8.35±0.05[a]	4513.33±15.27[d]	2513.33±15.27[d]	20.33±0.58[a,b]
SDBD 30s	8.29±0.04[a]	4553.33±5.77[c]	2533.33±5.77[c,d]	20±0.00[b]
SDBD 90s	8.21±0.03[a,b]	4590±10.00[b]	2540±10.00[c]	20.67±0.58[a,b]
SDBD 3min	8.10±0.02[b]	4603.33±15.27[b]	2576.67±5.77[b]	20.33±0.58[a,b]
SDBD 5min	7.88±0.10[c]	4686.67±5.77[a]	2586.67±15.27[b]	20.67±0.58[a,b]
SDBD 7min	7.77±0.14[c]	4700±10.00[a]	2613.33±15.27[a]	21.33±0.58[a]

[a] Significant difference ($p<0.05$) within each column was determined by different superscript letters.

[b] Values are means±SD (n=3).

3.6. Side effects of CAP in freshwater

High amounts of ROS in CAP-treated water can cause harmful effects on living organisms in the freshwater. ROS can be a leading cause of oxidative stress in freshwater organisms which can be extremely harmful to aquatic ecosystems [97,98]. The contribution of ROS in oxidative stress and its negative effects on aquatic organisms is discussed by Valavanidis *et al*., Di Giulio *et al*., and Livingstone [98–100]. Also, studies have been conducted on the risks of using the ozonation method and the effect of oxidative stress arising from it on ecotoxicity and animal welfare in aquatic environments [62,65]. On the other hand, H_2O_2 induced in freshwater during our treatments can lead to oxidative stress as well [101]. Therefore, the possible toxic effect of CAP treated freshwater was investigated using *G. roeseli* as a toxicity biomarker. 20 *G. roeseli* individuals were added to each CAP treated freshwater and their death toll was observed after 96 h. As shown in Table 6, treating freshwater samples for 30 s caused absolutely no casualty among individuals. Casualty induced by 90 s, 180 s, and 300 s of CAP treatments is not significantly different from control samples. But 420 s of CAP treatment in which, the concentration of ROS was higher than others caused 8.33±2.89% of mortality which is significantly higher than lower treatment times. In summary, treating freshwater for lower than 420 s with our CAP strategy causes ignorable toxicity in aquatic ecosystems.

Table 6

Toxicity of CAP treated freshwater[a,b].

CAP treatment	*G. roeseli* casualties after 96 h (%)
Ctrl (fresh water)	0±0.00[b]
SDBD 30s	0±0.00[b]
SDBD 90s	1.67±2.89[b]
SDBD 3min	1.67±2.89[b]
SDBD 5min	3.33±2.89[b]
SDBD 7min	8.33±2.89[a]
[a] Significant difference ($p<0.05$) within each column was determined by different superscript letters.	
[b] Values are means±SD (n=3).	

3.7. Scanning electron microscopy (SEM)

Figure 3 illustrates the treated and untreated *P. aeruginosa* cells under SEM. It was observed that the untreated cells remained intact and the cell walls were in their normal, rod-shaped form. On the other hand, it is clear that plasma-treated cells have deformed with notable shrinkage (Fig. 3b). Also, captured images with higher zoom (*e.g.*, Fig. 3b (inset)) showed the fact that CAP-treated samples contained torn apart and disrupted cell walls. The debris of disintegrated cells can be seen in Fig. 3b. It is worth noting that the rope-like structures near the bacteria in Fig. 3a, are some parts of the medium culture that stuck on the coverslips during the sample preparation. Our observation in this study agrees well with the previous investigations about the efficiency of the CAP in disinfection [102]. In conclusion, SEM revealed that our CAP treatments caused serious loss of viability in bacterial cells by disrupting their cell walls.

4. Conclusion

In this study, we investigated the disinfection and detoxification activity of H_2O_2/H_2O associated CAP produced by SDBD against *P. aeruginosa* and its toxin in freshwater. The main purpose of this study was to present a low-cost, large scale and environmental-friendly CAP strategy to be used if any pollution related to *P. aeruginosa* and its toxin occurred in freshwater ecosystems. We studied the efficiency of SDBD produced CAP in eliminating *P. aeruginosa* in freshwater and also we compared the bactericidal capacity of CAP with common antibiotics. The detoxification effect of our new CAP strategy was verified in freshwater by *G. roeseli* as a marker of ecotoxicity. The influence of CAP on the concentration of exotoxin A was also investigated. The effect of CAP on conventional physicochemical properties of freshwater and also the possible toxic effects of CAP treated freshwater were separately investigated as side effects. Our results pointed that plasma is likely more effective against bacteria than common

antibiotics. Also, 8.2 log reduction of *P. aeruginosa* viable cells was observed after 300 s of treatment and SEM images confirmed that disruption of the cell walls is the reason for this loss of viability. Mortality of *G. roeseli* individuals indicated that treating freshwater for 420 s can remarkably detoxify exotoxin A and our following analysis on the concentration of the exotoxin A elucidated that disrupting the protein structure on molecules could be the reason. But CAP treatment can also induce negligible toxicity due to the high concentration of ROS however it can't noticeably alter the physicochemical characteristics of water. In conclusion, H_2O_2/H_2O associated CAP produced by SDBD can effectively eradicate *P. aeruginosa* and exotoxin A in freshwater with causing ignorable toxicity and no harm to the sustainability of freshwater ecosystems. Thus, it can be used if any pollution concerning *P. aeruginosa* and its toxin occurred in small freshwater ecosystems such as ponds, swamps, and lakes.

Declarations

Acknowledgments

In conclusion, the authors consider it necessary to sincerely thank Ms. Roghayeh Pourbaghar for the initial design and assistance in building the body of the device used in this study. The authors would also like to express their gratitude for the efforts of Ms. Zahra Omidi, who voluntarily participated in the pre-tests and construction of the SDBD structure pilot model.

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Figures

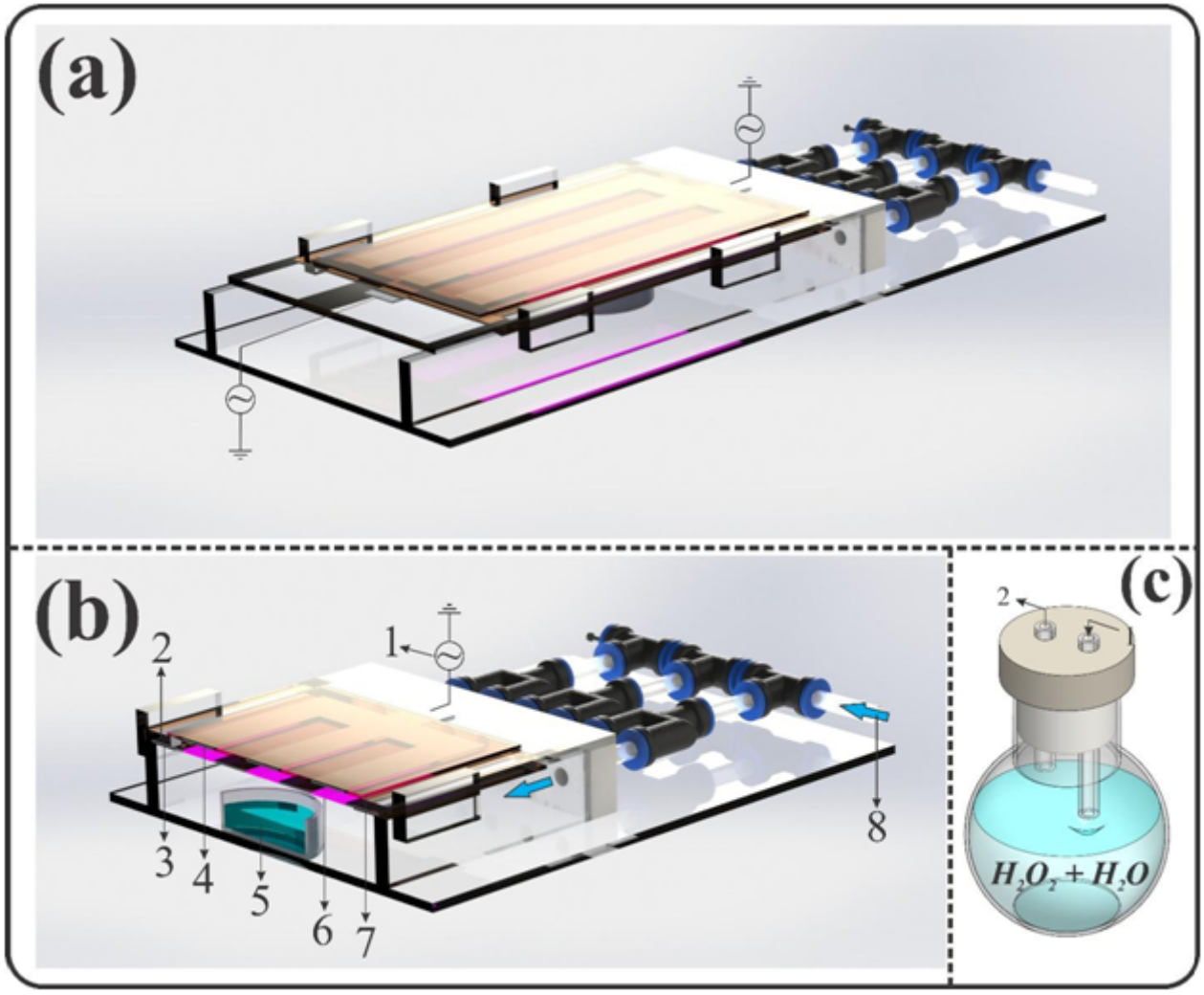

Figure 1

(a) Overview of SDBD-CP. (b) Cross-section of SDBD-CP. 1: high voltage power supply, 2: dielectric barrier, 3: lower electrode, 4: upper electrode, 5: sample, 6: plasma discharge, 7: Kapton Tape, 8: gas inlet. (c) Cold vapor section. 1: argon gas inlet, 2: gas outlet.

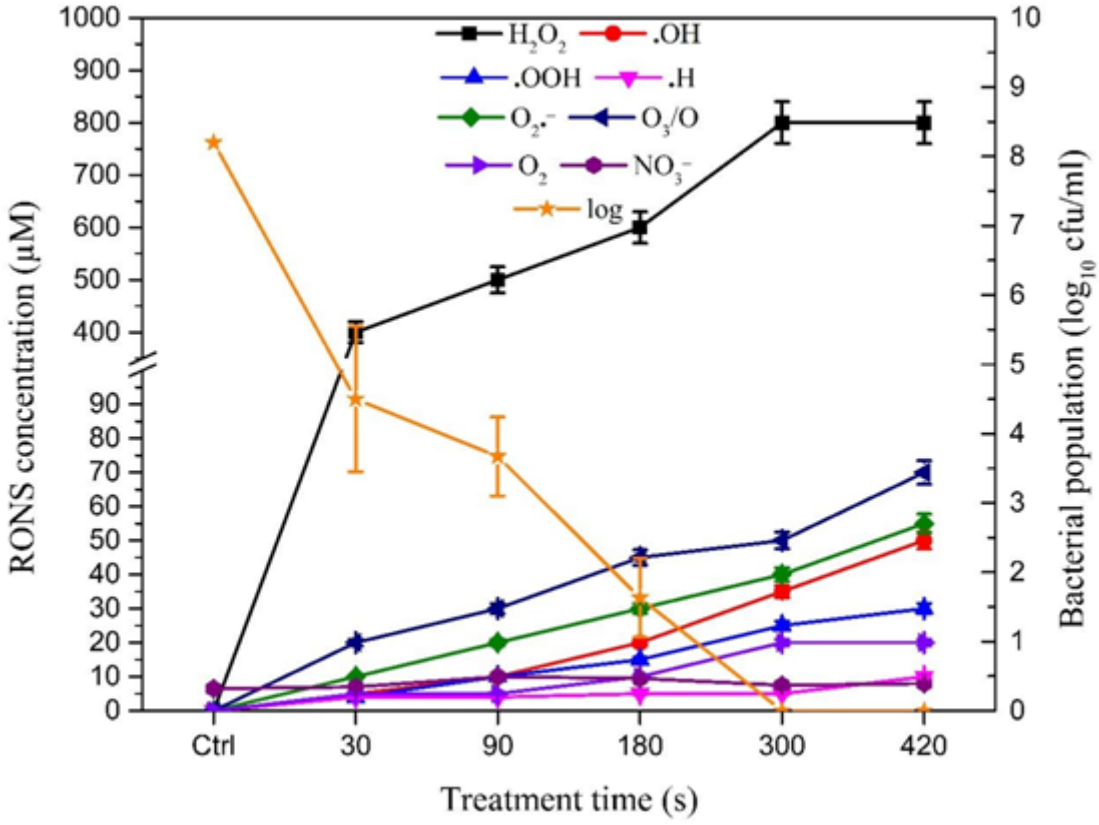

Figure 2

Temporal evolution of RONS concentrations during SDBD treatment. The remaining log population diagram has also been involved for a comparison between log reduction and the amount of each RONS in treated samples.

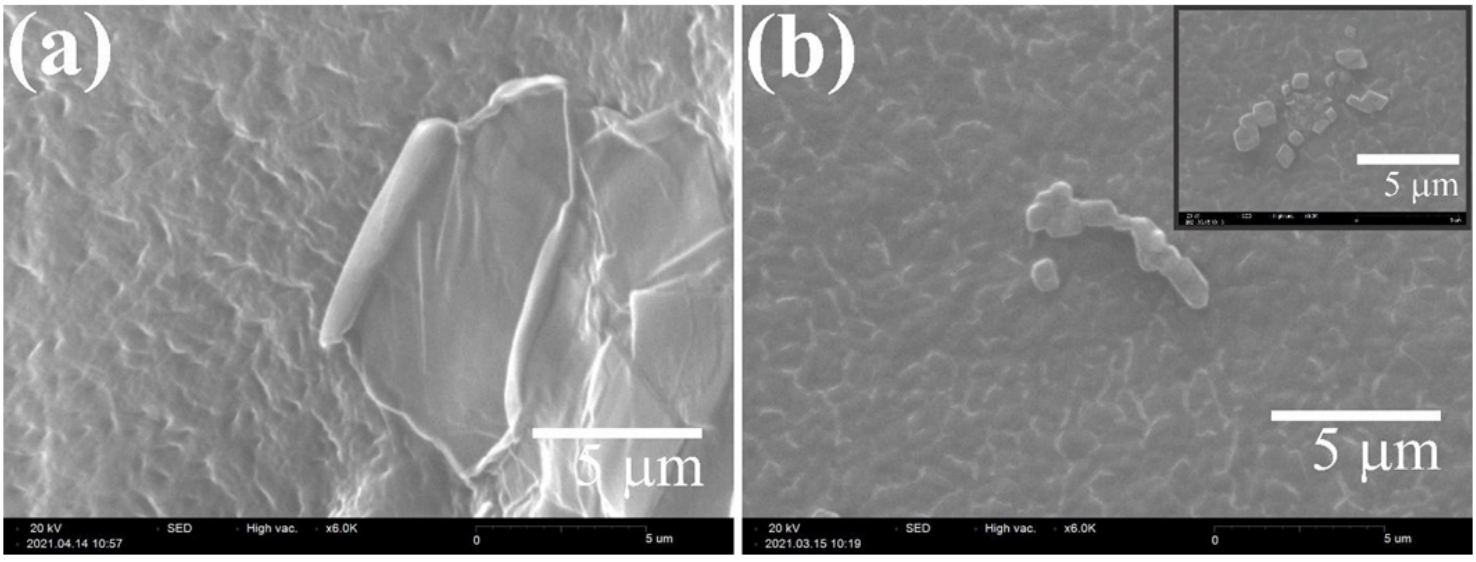

Figure 3

shows the morphological characteristics observed under SEM of *P. aeruginosa* cells (a) before and (b) after plasma treatment. Inset: Plasma-treated *P. aeruginosa* cells at higher magnification.