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Investigating the efficacy of non-thermal plasma in the inactivation of cyanobacteria *Dolichospermum* sp.

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Abstract. Cyanobacteria bloom has been recognized as a serious problem worldwide that requires urgent monitoring and treatment. This study assessed the inactivation of cyanobacteria *Dolichospermum* sp. cells using non-thermal dielectric barrier discharge plasma system under different operational conditions of input wattage and contact time. Both instant and long-term inactivation efficiencies (up to 92.6 %) of *Dolichospermum* sp. cells were obtained after plasma treatment regardless of the conditions applied. Increased contact time resulted in enhanced

inactivation efficiency, while the impact of input wattage was minor. Further investigation was done by analyzing cell morphology, revealing severe deformation of the *Dolichospermum* sp. cell surface due to plasma treatment. The results from this study confirmed the potential of non-thermal plasma in cyanobacteria inactivation which shapes the direction for further studies.

Keywords: inactivation cells, potential toxic cyanobacteria, non-thermal plasma**,** *Dolichospermum*

Classification numbers: 3.2.3, 3.4.2, 3.6.1

1. INTRODUCTION

Eutrophication has become a frequent phenomenon in both marine and fresh ecosystems worldwide [1]. Eutrophication can cause a wide range of problems in aquatic ecosystems, including excessive growth of phytoplankton, benthic algae and macrophytes, oxygen deficit, fish mortality, and loss of biodiversity [2, 3]. Cyanobacteria is the most common phytoplankton organism with a direct connection to eutrophication in freshwater systems, which has been documented on a global scale [4, 5]. Massive growth of cyanobacteria or cyanobacteria blooms has been observed with greater frequency and intensity in lakes and reservoirs in many countries, largely due to anthropogenic eutrophication and global warming [6 - 8]. *Microcystis*, *Anabaena* (*Dolichospermum*), *Raphidiopsis* (*Cylindrospermopsis*), *Planktothrix*, and *Aphanizomenon* were identified among the most commonly bloom-forming unicellular and multicellular cyanobacteria genera [9 - 11]. *Dolichospermum* is a common filamentous diazotrophic genus that can be found all over the world. Globally, a variety of cyanotoxins, including microcystins, anatoxin-a, cylindrospermopsin, guanitoxin/anatoxin-a(S), and saxitoxin, have been found to be produced by species in this genus.

The occurrence of potential toxic cyanobacteria as a pathogenic agent requires regular monitoring in water and establishing a provisional guideline value for both cyanobacteria with a density of 10^5 cells/mL which requires immediate advanced treatment for bloom control, and for microcystin-LR in drinking water with a threshold value of 1 µg/L [12]. In that context, serious efforts are required to address the escalation of cyanobacteria harmful algal blooms (CyanoHABs) the most widely accepted approaches include prevention and control [13]. While the prevention approach involves reduction of excessive nutrient in water environment as well as effective isolation of water contaminated by CyanoHABs, the control approach focuses on minimizing the hazardous impacts from the bloom on human, mainly via removal of cyanobacteria cells and its toxins from water [14]. Advanced oxidation processes have been suggested as potential technologies to effectively inactivate harmful algae and their toxins [15]. The processes involve the production of reactive species, of which hydroxyl radical (·OH) is the most important, for rapidly and effectively oxidizing cells and organic compounds in water [16]. Among advanced oxidation processes, non-thermal plasma or cold plasma have attracted many attentions due to their potential applications in water treatment [17], such as pharmaceutical or volatile organic compound decomposition [18, 19] as well as microorganism inactivation [20]. In the process, free electrons receive most of the energy, hence being thermalized, while the energy transmitted to heavy species such as ions or neutrals is minor. These escalated electrons, in turns, play the main role in the production of reactive compounds, such as hydrogen peroxide (H_2O_2) or radical species including ·OOH, ·OH, and ·H in water [21].

The application of plasma technology to inactivate cyanobacterial cells or removal biomass of cyanobacterial and its toxin have been discussed in few previous published papers [22_25]. These studies focused on the inactivation of cyanobacterial cells, which was evident under the

influence of some electrical parameters such as power and voltage and the mechanisms for the decomposition of the toxin microcystin (MC). It was observed that there was a positive correlation between discharge time and the inactivation effect [26]. Xu *et al*. using a dielectric barrier discharge plasma system, reported an over 99.99 % reduction of *Microcystis aeruginosa* after 10 minutes [27]. Both *M. aeruginosa* and microcystin-LR were effectively inactivated by a self-cooling dielectric barrier discharge plasma with over 99 % cell removal and lower 1 μg/mL of microcystin-LR achieved after 60 min [28]. Some other works showed cold plasma discharge combining with hydrodynamic activation effectively reduced *M. aeruginosa* biomass without cell lysis or cyanotoxins release. Howerver, little information is available to investigate the variations of cyanobacteria cell structure after plasma treatment. Prior studies have mainly examined the immediate and long-term growth inhibition of *M. aeruginosa* through non-thermal plasma treatment. However, there has been limited research on the antimicrobial effects of non-thermal plasma against *Dolichospermum.* Therefore, in this study, the inhibition effects of non-thermal plasma to bloom-forming cyanobacteria *Dolichospermum* sp. strain were investigated. The Field Emission Scanning Electron Microscopy (FESEM) were utilized to observe structure changes of *Dolichospermum* sp. The inactivation efficiency of *Dolichospermum* cells by a series of wattages and exposure time of plasma conditions was calculated.

2. MATERIAL AND METHODS

2.1. Cyanobacteria cultures

The potential toxic cyanobacterial *Dolichospermum* sp. was used as the test organism in the present study. This strain was isolated from Hoan Kiem Lake (Ha Noi, Viet Nam) using micropipette under an inverted microscope and cultured in Z8 medium [29]. *Dolichospermum* cells were maintained in Z8 medium in a culture room at 26 ± 2 °C under illumination at 1000 lux and 12:12 light/dark regime.

2.2. Aparatus: Non-thermal plasma system

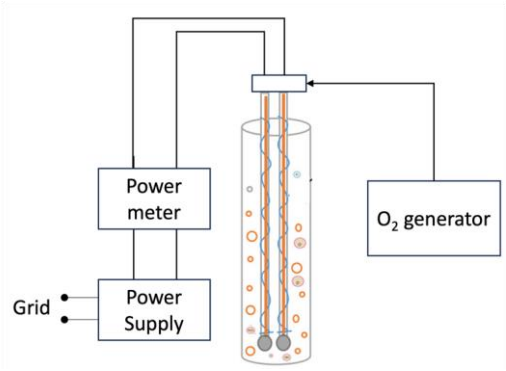


Figure 1. Schematic configuration of the water plasma system.

The plasma system consists of an 1 L plasma reactor, a home-built power supply, and an oxygen concentrator. The plasma reactor has two electrodes placed inside two quartz tubes and is connected to the AC power supply. Both quartz tubes act as dielectrics in a dielectric barrier discharge (DBD) plasma configuration and also as the gas pipes (Figure 1). Plasma of concentrated oxygen (97 % in volume) is generated inside the quartz tubes, while its reactive

products, such as ozone and radicals, are bubbled out and light is transmitted into the reaction chamber. The combination reaction of all plasma species gives rise to a very effective disinfection and depollution of the system.

2.3. Experimental set up

To evaluate the effect of plasma on growth of *Dolichospermum* sp. strain, cyanobacterial suspension with exponential growth phase (7-10 days old) with an approximate concentration at 2.1 $\times 10^5$ cells mL^{-1} was used and placed into the 1L reactor of the plasma chamber. To understand the effect of discharge on inactivation rate of cells, different applied input powers were respectively set at 35, 45, 55, 65 and 75 W at 60 seconds. Cyanobacterial plasma-treatment assays were independently performed in three replicates. After plasma treatment, the suspensions of *Dolichospermum* sp. were immediately collected in 250 mL glass bottles and re-incubated for up to 168 hours under the same light and temperature conditions as mentioned above. Cultures of *Dolichospermum* sp. was assessed for 0, 24, 72, 120 and 168 hours by the measurement of absorbance at 680 nm with the spectrophotometer. Control samples without plasma treatment were also exposed to the same conditions as the treated samples.

Furthermore, to examine the effect of discharge time on inactivation of *Dolichospermum* sp. cells, the plasma exposure durations were set at 30, 60, 120, 180, and 300 seconds at discharge power of 55 W. The re-growth experiment was performed right after the plasma treatment. *Dolichospermum* sp. suspensions were quickly placed in 250 mL glass bottles and re-incubated for a maximum of 168 hours using the same conditions adopted for growing the cultures explained previously.

2.4. Analysis of cyanobacterial cells

The cell viability of the *Dolichospermum* sp. strain during the re-culturing period was determined by measuring absorbance at 680 nm with a UV-Vis spectrophotometer (Shimadzu, UV- 2450, Japan). The inactivation efficiency of *Dolichospermum* cells was calculated using the following formula [30]:

$$\text{Cyanobacterial inactivation efficiency (\%)} = [(Ac - At)/Ac] \times 100\ \%,$$

where: Ac (control): the absorbance at 680 nm of the control sample; At (treatment): the absorbance at 680 nm of the treated sample after t min plasma exposure or t day inoculation after plasma exposure.

The impact of plasma treatment on the algal cell surface was also assessed. The surfaces of *Dolichospemum* sp. cells (before and after plasma exposure) were observed by FESEM (SM-IT800/Jeol, Japan), which was equipped with a Super Hybrid Lens (SHL), allowing high-resolution observation and analysis. Cell pellets were washed with sterile water three times to completely clear the reactive species and medium residue on the cell surface. *Dolichospermum* cells were freeze-dried for 24 hours and observed by FESEM after spraying gold.

2.5. Data treatment

The results of cyanobacteria *Dolichospermum* sp. cell density in terms of OD_{680} and the inactivation efficiency in terms of percentage were reported as average with standard deviation. Firstly, the normality of the data sets was assessed using Shapiro–Wilk test. For comparing two normally distributed data sets, the Fisher–Snedecor test was used to evaluate the homoscedasticity, the data sets then be compared by the student t-test or Welch test in case of equal or unequal variances, respectively. In case of non-normal distribution, the

Mann–Whitney–Wilcoxon test was used. For comparing multiple normally distributed data sets, either Analysis of Variance (ANOVA) or ANOVA-Welch was used for equal or unequal variances (determined by the Bartlett test), respectively, followed by pairwise t-test. In case of non-normally distributed data sets, the Kruskal–Wallis test followed by the Pairwise Wilcoxon Rank Sum Tests was used. For all significant tests, significant level (α value) of 0.05 was chosen. Data analysis and visualization were performed by using Microsoft Excel version 2016 and Origin (OriginLab Corporation, version 2018).

3. RESULTS AND DISCUSSIONS

3.1. Morphology of *Dolichospermum* sp. strain

The strain *Dolichospermum* sp. was isolated from Hoan Kiem Lake. Trichomes were free-floating, solitary, and straight (Figure 2). Terminal cells were round or conical, smaller than others. Vegetative cells were spherical, compressed or barrel-shaped, with gas vesicles, 5-6-8 µm in diameter. Heterocytes were spherical, 8 - 9.8 µm in diameter. Akinetes were shaerical, 8 – 11 µm in diameter, adjacent to heterocytes, more or less bigger than vegetative cells (4.2 - 8 × 6 - 9.5 µm).

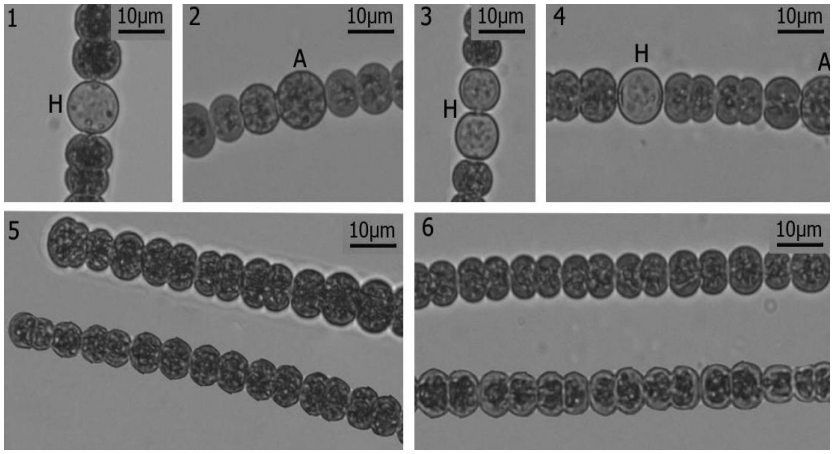


Figure 2. Morphology of *Dolichospermum* sp. strain.

3.2. Effect of plasma treatment on morphology and cell membrane integrity of the *Dolichospermum* sp.

After plasma exposure, the morphological alterations on the *Dolichospermum* sp. cells were examined using a light microscope. The effects of plasma exposure on solid cultures of *Dolichospermum* sp. are shown in Figure 3 and Figure 4. The results showed that the morphological characteristics of the treated *Dolichospermum* cells were distinct from those of the control. In the control condition, normal cells of *Dolichospermum* were regular spherical with abundant gas vesicles (black dots inside cells). Plasma exposure caused separation, degeneration of the aggregations and cells, and a number of empty cells (without black dots inside cells) (Figure 3 and Figure 4). These results seemed to be consistent with other work reporting that cells of the cyanobacterial *Microcystis aeruginosa* auto-fluorescence significantly changed as a result of discharge plasma oxidation. Indeed, with the production of reactive

oxygen species (ROS) in greater quantities as a result of the discharge plasma, H_2O_2 oxidation and •OH attack were the main sources of oxidative stress that damaged *M. aeruginosa* cells [25].

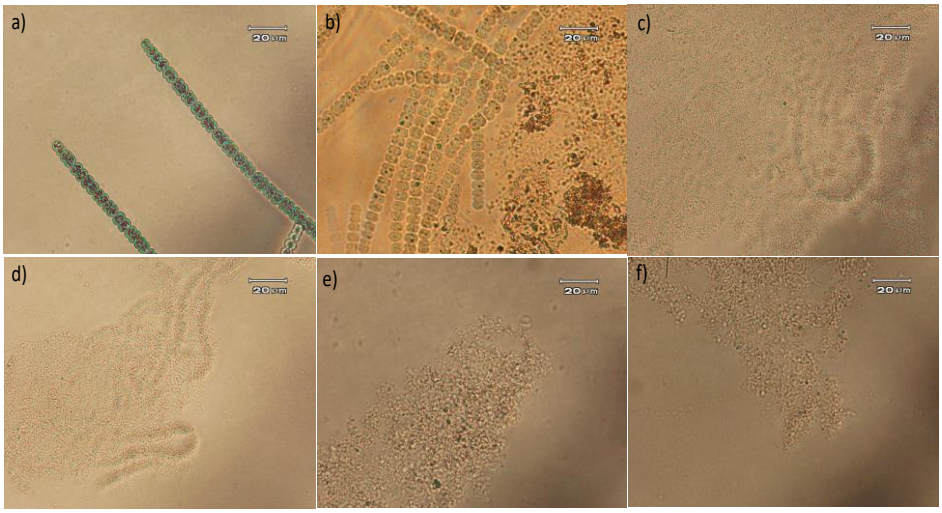


Figure 3. Dolichospermum sp. cells under light microscope before and after plasma exposure: a) before plasma exposure; b-f) after 30, 60, 120, 180 and 300 seconds of plasma exposure, respectively.

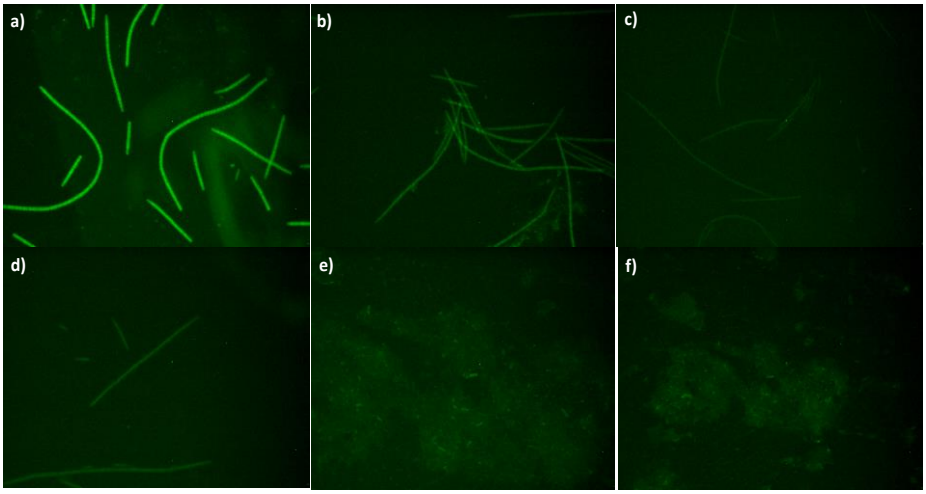


Figure 4. Dolichospermum sp. cells under fluorescence microscope before and after plasma exposure: a) before plasma exposure; b-f) after 30, 60, 120, 180 and 300 seconds of plasma exposure, respectively.

FE-SEM examination confirmed that the extracellular structures of the healthy cells did not show any visible distortion of cell structure and appeared to interconnect cells with a smooth exterior (Figure 5a). However, under plasma exposure, a significant cell surface with a distorted and massive shrinkage was observed (Figure 5b). A similar trend in cell surface destruction using cold plasma treatment was observed by Kim *et al*. [31]. As reported by Wang *et al.*, , the damage of the cyanobacterial cells could occur from the exterior to the interior [28]. The plasma process is known to cause a variety of oxidative and chemical species, such as radicals (e.g., H·, O·, and OH·) and molecules (e.g., H_2O_2 and O_3), shock waves, ultraviolet, and electrohydraulic cavitation [32, 33].

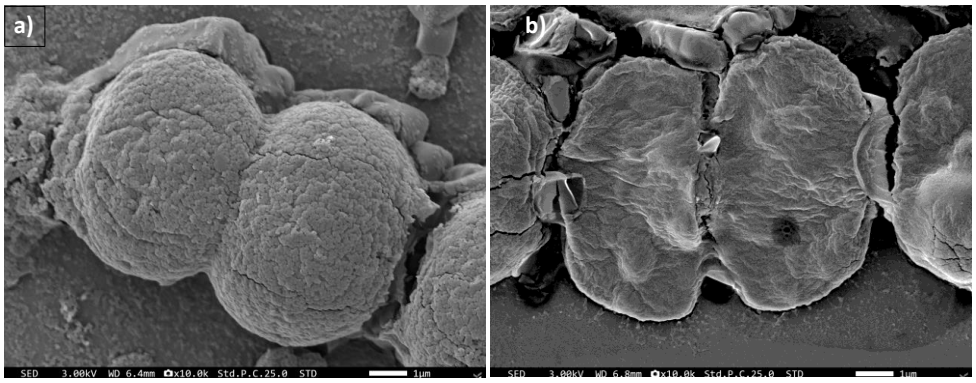


Figure 5. FE-SEM images of *Dolichospermum* sp. before (a) and after (b) plasma exposure.

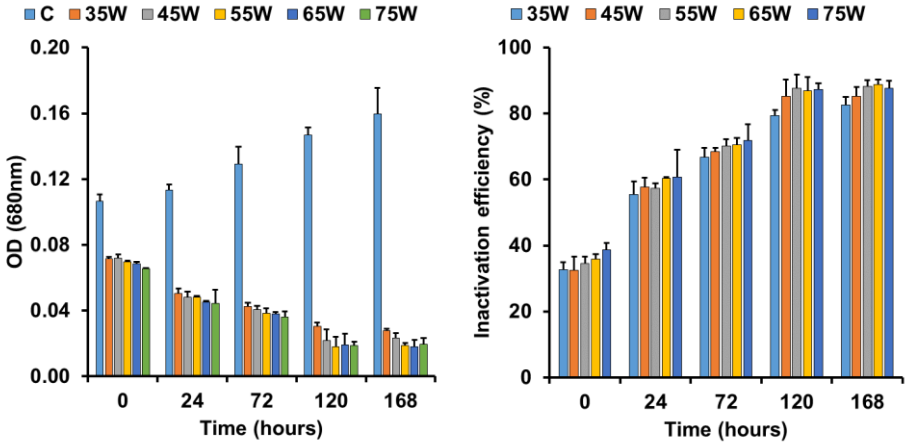
The reactive chemicals produced by the plasma process can attack the cell membrane and wall, disrupt membrane integrity, and lead to cyanobacterial and algal inactivation of the cells [31]. According to Maršálek *et al*., plasma produces stress that is responsible for inhibiting photosynthetic activity and halting cell metabolic activity in the cyanobacterial biomass [34].

3.3. Treatment performance of *Dolichospermum* sp. under different plasma exposure conditions

Immediate and long-term inactivation of cyanobacteria *Dolichospermum* sp. using non-thermal plasma was investigated. The impact of input power on *Dolichospermum* sp. removal was studied by applying five power levels of 35, 45, 55, 65, and 75 W, which the respective cell reductions in terms of optical density (OD) measurement at 680 nm and their corresponding inactivation efficiencies were shown in Figure 6a. Effective inactivation was observed at all power levels applied. No statistical difference was found among the inactivation results of different power levels used in this study ($p > 0.05$). After 60 seconds of exposure, instant OD reduction from 0.107 to a low level of 0.065 - 0.072 was achieved (Figure 6a), corresponding to inactivation efficiency of 32.4 - 38.7 % (Figure 6b). Further reduction in the number of cells was also recorded during the re-culture period. After 120 hours of re-culture, up to 79.3 - 87.7 % of cells were inactivated, which slightly increased after another 48 hours (82.5 - 88.8 % achieved after re-culture period of 168 hours).

The results suggest that, after plasma treatment, *Dolichospermum* sp. cells were no longer viable, even though their general morphology remained. As a consequence, the number of remaining cells was continuously reduced during the re-culture period, resulting in increased treatment efficiency (Figure 6). Similar conclusion was made by Nisol *et al*., who applied non-thermal dielectric barrier discharge plasma system to remove the cyanobacteria *Dolichospermum* and the green algae *Scenedesmus*. The authors reported that only 13 % of total cells remained after 360 s of plasma exposure duration, yet up to 80 % of them were non-viable [22]. As indicated by Zhang *et al*., the main factor inhibiting cyanobacterial growth was ROS which damaged the cell membrane, resulting in cell rupture as well as penetrated through the membrane to attack intracellular components [25]. Consequently, even though the general morphology of the cyanobacteria may appear intact, their growth was already inhibited due to irreversible damages received by gene, photosynthetic pigments, proteins or carbohydrates [20]. Similarly, Xu *et al*. showed that around 82 % of the cyanobacteria *M. aeruginosa* cells remained

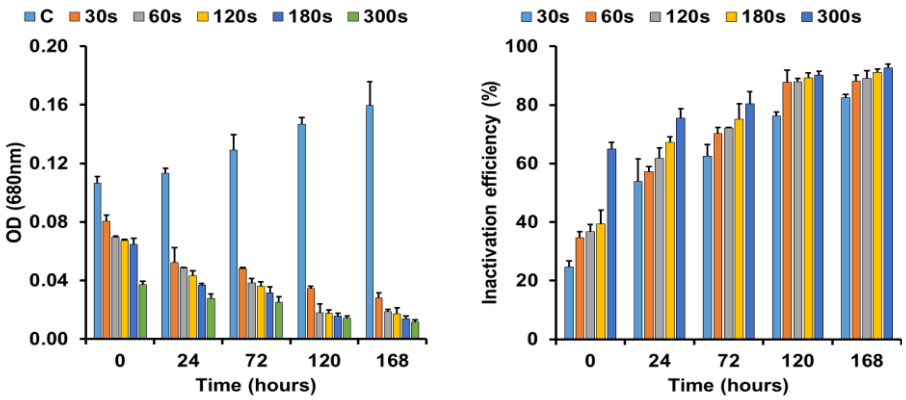
intact after 60 seconds of plasma treatment at 31.8 W, while the total inactivated cells after 20 days of re-culturing was 55.3 % [27].



(a) (b)

Figure 6. The reduction in cell viability measured by OD (a) and the corresponding inactivation efficiencies (b) of plasma at different power levels.

Enhanced inactivation performance of cyanobacteria *M. aeruginosa* using non-thermal dielectric barrier discharge plasma system due to increasing power input was reported by Wang *et al*., which is different from this study [28]. However, the power levels and contact times applied in their study were much higher than in this study. Indeed, the authors showed that higher input voltage, and consequently higher input power, resulted in superior treatment efficiency, with 99.63 % of removal achieved at 21.0 kV (0.28 kW) compared to only 59.7 % at 14.3 kV (0.16 kW) after 100 minutes of treatment time [28]. Moreover, even though increasing the input power of the plasma system could increase the amount of reactive oxygen species generated [35] and consequently improve the treatment efficiency, these reactive species are prone to being consumed by other substances in water, such as nutrients (nitrogen and phosphorus) or organic compounds [36]. In this study, the cyanobacteria *Dolichospermum* sp. was cultured in a nutrient rich solution while being exposed to plasma treatment. The interference of these compounds on cell destruction could be significant. In addition, as a consequence of cyanobacterial cell rupture due to membrane damage by ROS induced by plasma, the intracellular materials are released and thus further scavenge the available ROS [25]. Wang *et al*. [37] detected a peak concentration of cyanobacterial intracellular organic matters including chlorophyll, proteins, amino acids or peptides in water after 1 minute of plasma treatment (at 4 – 7 kV and 7 kHz), followed by their reduction as the plasma treatment continued (until 12 minutes). Wang *et al*. [38] reported a reduction of suspended organic matters with large molecular weights (higher than 50 kDa) and, at the same time, an increase of smaller molecular (less than 30 kDa) after plasma treatment. The results indicated effective hydrolysis of high weight organic matters originated from water and/or released from damaged cyanobacterial cells to smaller molecular by ROS, suggesting their role as active ROS scavengers. The impact of treatment duration on the treatment performance of *Dolichospermum* sp. was also studied by fixing an input power of 55 W, while varying the plasma treatment time. Five periods were applied, including 30, 60, 120, 180, and 300 seconds, and the results were presented in Figure 7. Similarly, effective inactivation was obtained at all times with long-term treatment effects during 7 hours of re-culture.



(a) (b)

Figure 7. The reduction in cell viability measured by OD: (a) and the corresponding inactivation efficiencies, (b) of plasma at different exposure times.

Under the shortest exposure time of 30 s, a significant reduction of 24.7 ± 2.1% was instantly achieved (OD_{680} fell from 0.107 ± 0.004 to 0.080 ± 0.004) (Figures 7 b and a, respectively). As the cell number continuously decreased during re-culture period, a high final inactivation efficiency of 82.5 ± 1.2 % was obtained after 168 hours. The best performance was obtained with 300 s of exposure time. A sharp OD reduction from 0.107 ± 0.004 to 0.037 ± 0.002 was achieved, corresponding to 65.0 ± 2.2 % of inactivation efficiency right after treatment completion. The inactivation efficiency went up to 92.6 ± 1.4 %, the highest level observed in the study, after 168 hours of re-culture. There is a statistically significant difference between the two exposure times (30 s and 300 s) when comparing the OD value ($p < 0.005$) and inactivation efficiency ($p < 0.05$). Besides, exposure times of 60, 120, and 180 seconds showed similar inactivation results (p values > 0.05), with 34.6 - 39.3 % of cell reduction observed at day 0 and 88.2 - 91.2 % inactivation efficiency achieved after 168 hours of re-culture. The positive effect of contact time on treatment efficiency was also reported by other studies [27, 28, 36]. Xu *et al*. studied the treatment of cyanobacteria *Microcystis aeruginosa* using a non-thermal dielectric barrier discharge plasma system at 31.8 W in 30, 60, 180, 300, and 600 seconds showing 30.8 % reduction after 60 seconds, 90 % after 300 seconds and 99.99 % after 600 seconds of treatment [27]. Short term plasma treatment of 30 seconds and 60 seconds also effectively ceased the growth of remaining cyanobacteria after 10 and 15 days of re-culture period, resulting in a global reduction of 24.1% and 55.3%, respectively, compared to untreated sample [27]. It was indicated that higher contact time enhanced treatment efficiency by allowing the reactive oxygen species generated by the plasma to attack the cell membrane, resulting in proper penetration and thus effective decomposition from inside the cell [28].

It was reported earlier that the amount of ROS produced by non-thermal plasma is positively correlated with power level applied to the system [39]. Arjunan *et al*. observed elevated concentrations of both intracellular and extracellular ROSs due to the increase in non-thermal plasma power density [40]. However, due to their reactiveness, the ROSs have extremely short lifetime, generally from few microseconds to few milliseconds, with the exception of H_2O_2 with longer lifetime of around 10 hours [41]. Therefore, in this study, even though higher ROS concentrations could be achieved at higher power levels, these reactive species might quickly be scavenged by organic matters in the solution and/or inactivated at the end of their lifetime. On the other hand, an increase in plasma treatment time could result in continuous ROS production and thus effectively enhance the treatment rate. Wang *et al*. [37]

reported continuously increasing trends of ROSs including H_2O_2 and O_3 produced by non-thermal plasma system at 7 kV. The concentration of H_2O_2 increased from 5 mg/L at 1 min of treatment time to around 40 mg/L after 12 min while O_3 concentration after 1 min was around 15 mg/L which climbed to a high level of slightly above 55 mg/L after 12 minutes of plasma operation. These results may shed light on understanding the positive impact of plasma treatment time on *Dolichospermum* sp. cells inactivation obtained in this study.

Results of this study further confirm the potential of applying advanced oxidation processes using non-thermal plasma system for cyanobacteria and cyanotoxins inactivation in water. It should be noted that the initial cyanobacterial cell densities applied in this study ranged from 0.106 to 0.169 (OD_{680} values) which corresponded to the values of 2.03 to 2.94 × 10^5 cells/mL, respectively. These values were chosen to mimic the cell density values of cyanobacterial bloom in real cases. Duong *et al*. reported the cyanobacterial density measured during bloom periods in Nui Coc reservoir, North Viet Nam, ranging between 0.05 × 10^4 and 0.19 × 10^4 cells/mL [42]. In the South of Viet Nam, Pham *et al*. measured the cell density of cyanobacterial bloom in Tri An Reservoir, Dong Nai Province, with the values from 1.2 × 10^4 to 1.6 × 10^7 cells/mL [43]. In addition, it was suggested that non-thermal plasma can be operated in combination with other water treatment technologies such as adsorption, ultrafiltration, biodegradation or ultrasonication for enhanced treatment time, energy saving and improved cost effectiveness [17]. Indeed, effective microalgal cells removal of 98 - 99.5 % has been achieved by popular water treatment processes such as coagulation/flocculation combined with sedimentation, air flotation and rapid sand filtration, or microfiltration followed by ultrafiltration [15]. Zamyadi *et al.* [44] reported 1.5 × 10^5 - 11.4 × 10^6 cells/mL were successfully removed by the clarifier, resulting to a microcystins concentration of 34.9 mg/L detected in the sludge at the bottom of the clarifier in a drinking water treatment plant in Canada. Therefore, the application of non-thermal plasma system in toxic cyanobacteria and cyanotoxins inactivation can be greatly beneficial from the pretreatment processes.

4. CONCLUSION

The inactivation of cyanobacterial cells using non-thermal dielectric barrier discharge plasma system was investigated. The results revealed that under the plasma conditions tested, the plasma demonstrated a high inactivation efficiency of cyanobacterial cells (up to 92.6%) not only right after plasma treatment but also during re-culturing period of up to 168 hours. Longer contact time enhanced inactivation efficiency, while increasing input wattage showed a minor effect which could be due to the interference of other substances in the mixture. Morphological analyses indicated that the decomposition and inactivation of *Dolichospermum* sp. cells were due to severe deformation of the cell surface. The obtained results showed the possibility of non-thermal plasma devices in the anti-cyanobacterial purification of supply water.

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Declaration of competing interest. The authors declare that there are no conflicts of interest.

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