Optimized inhibition on *Microcystis aeruginosa* by combined use of UV-C irradiation and hydrogen peroxide

Jie Zheng¹, Tingru Zhou², Yi Tao¹, ³*

¹Guangdong Provincial Engineering Research Center for Urban Water Recycling and Environmental Safety, Graduate School at Shenzhen, Tsinghua University, Shenzhen 518055, P.R. China

²Key laboratory of Microorganism Application and Risk Control (MARC) of Shenzhen, Graduate School at Shenzhen, Tsinghua University, Shenzhen 518055, P.R. China

³Shenzhen Engineering Research Laboratory for Sludge and Food Waste Treatment and Resource Recovery, Graduate School at Shenzhen, Tsinghua University, P.R. China

*Corresponding author’s e-mail: tao.yi@sz.tsinghua.edu.cn

**Abstract.** In order to optimize H₂O₂/UV-C treatments on suppressing cyanobacterium, apoptotic-like and necrotic growth suppression were investigated respectively. Results showed that apoptotic-like algal inhibition exhibited a positive correlation with UV-C dose in the study period. Dosing strategy for apoptotic-like inhibition is that an environmentally friendly H₂O₂ dose should be determined with priority. Then, UV-C dose should be mediated to achieve a specific objective for algal inhibition. For necrotic inhibition, increasing H₂O₂ dose exhibited weak enhancement in growth inhibition while existence of UV-C irradiation could negatively influence following cell lysis after treatments. Hence, dosing strategy for necrotic inhibition is that a moderate H₂O₂ dose without UV-C irradiation should be efficient in achieving a specific objective for algal inhibition. For mechanism of death switch, given a specific UV-C dose, corresponding high H₂O₂ dose could cause a deficiency of ATP and switch from apoptosis-like to necrosis.

1. **Introduction**

Cyanobacterial bloom has been an environmental issue in recent decades. To date, it has occurred in both fresh water and marine water with higher frequency, intensity and duration, which has brought about serious problems to environment and the health of living creatures [1-3].

In order to control cyanobacterial bloom, many methods have been developed. Among all kinds of methods, UV-C as a physical method [4, 5] and hydrogen peroxide as a chemical method [6-8] have been paid much more attention. Recently, it was revealed that the combined use of UV-C and H₂O₂ could demonstrate drastic growth inhibition, marked degradation of microcystin and inhibition of the photosystems, which acquired much lower dose than sole utilization of UV-C or H₂O₂ did [9]. However, Wang et al. [9] did not study the individual dose-response effect of UV-C and H₂O₂, which
set an obstacle to determine the optimal dose of UV-C and H$_2$O$_2$ when controlling cyanobacterial bloom.

Furthermore, moderate reactive oxygen species (ROS) including H$_2$O$_2$ has been widely reported to regulate cell proliferation, programmed cell death (PCD) and signal transduction in both prokaryotic cells and eukaryotes [10-12]. Notably, enzymes involved in PCD in eukaryotes are found in the recently sequenced genome of _M. aeruginosa_ [13]. PCD is a regulated process of cell suicide, which is essential for individual sustainable development. Apoptosis is a well-known form of PCD, which is accompanied by involvement of cysteine proteases (caspases) and fragmentation of DNA [14]. Moreover, recent studies indicate that there exists apoptotic-like cell death for _M. aeruginosa_ [15, 16]. It is revealed that the moderate level of ROS required for triggering PCD is significantly lower than the level that causes direct irreversible oxidative necrosis [10, 17]. Therefore, algal growth inhibition by PCD such as apoptosis-like may be more cost-effective and environmentally friendly than necrotic algal inhibition.

So far, the necrotic algal inhibition and apoptotic-like algal inhibition is lack of comparative study. Therefore, this study wants to investigate how combined use of H$_2$O$_2$ and UV-C induces different cell death in algal growth suppression.

In this study, we investigated _M. aeruginosa_’s dose-response effect of UV-C and H$_2$O$_2$ on (1) cell growth, membrane integrity and morphology; (2) apoptosis-like indicators such as membrane potential. This study aims to explore 1) dose-response effect of UV-C and H$_2$O$_2$ on growth suppression; 2) the optimal dose of UV-C and H$_2$O$_2$ to achieve cost-effective algal control; 3) the mechanism of death switch from apoptotic-like death to necrotic death.

2. Materials and methods

2.1 Cultivation of algae and treatments

The laboratory pure cultured microalgae _M. aeruginosa_ (FACHB-905) was obtained from the Freshwater Algae Culture Collection at the Institute of Hydrobiology (FACHB Collection, Wuhan, China). BG11 medium was used to culture in a light incubator (Yiheng, China). The temperature of the light incubator was set at 25°C. A cool white fluorescent lamp was used as a light source, and the light intensity was 2000~3000 Lux, and the light/dark cycle was 12 h/12 h. Cells in the exponential growth phase were used. Every 100 mL sample was treated with different concentrations of H$_2$O$_2$. After addition of H$_2$O$_2$, each 100 mL sample was immediately irradiated in petri dishes (diameter = 9 cm, depth = 1.5 cm) vertical to the low-pressure mercury lamp (253.7 nm, 20 W, Jiangsu Junuang Photoelectric Technology Co., Ltd. China) for different duration respectively, to achieve different dose of UV-C. Magnetic stirrer kept the suspension mixed. The intensity of UV-C at the surface of the suspension was measured by a spectroradiometer (Spectronics, XF-1000, XS-254, USA). In order to simplify spelling, the A mM H$_2$O$_2$ + B mJ cm$^{-2}$ UV-C was simplified as [A,B] while [0,B] and [A,0] respectively referred to 0 mM H$_2$O$_2$ + B mJ cm$^{-2}$ UV-C and A mM H$_2$O$_2$ + 0 mJ cm$^{-2}$ UV-C. All treatments were conducted in sequence. The non-UVC-irradiated cultures without H$_2$O$_2$ were used as the control. During the 2-week incubation time after UV-C/H$_2$O$_2$ treatments, all experiments were carried out in triplicates.

2.2 Flow cytometer analysis

Flow cytometer (FACS-Calibur, Becton Dickinson, USA), equipped with dual wavelength laser of 488 nm and 635 nm and signal acquisition channels of FSC, SSC, FL1, FL2, FL3 and FL4, was employed to determine different indicators below. A total number of $10^4$ cells were recorded and analyzed by software Cell Quest™ Pro (Becton Dickinson, USA).

2.2.1 Measurement of cell growth and morphology

Total cell density was measured by flow cytometry and quantified with counting beads (Invitrogen, USA). In the study, 480 μl algal cultures were mixed with 20 μl counting beads evenly, and total cell
density was calculated from Eq. 1 [4]. Cell lysis indicates the number of the cells with completely lysed cellular structure. Cell density of the lysed cells was calculated by Eq. 2 in this study. Moreover, the lysis degree was evaluated by lysis rate as presented in Eq. 3. The fluorescence intensity of forward scatter (FSC) concerns cellular size, shape and optical homogeneity. Therefore, FSC is widely applied to detect cell surface properties [18]. As a physical property of algal cells itself, the signal was directly acquired by FSC channel without any additional reagent and incubation in the study.

\[
\text{Total cell density} = \frac{\text{cell number} \times \text{bead density} \times 20}{\text{bead number} \times 480} \tag{1}
\]

Where the cell number and bead number respectively refer to the number of algae and bead dots in flow cytometer. The density of the bead is \(10^6 \text{ mL}^{-1}\) in working solution.

\[
Lysis = C_0 - C_t \tag{2}
\]

\[
\text{Lysis rate} = \frac{Lysis}{C_0} \times 100 \tag{3}
\]

Where \(C_0\) refers to the initial total cell density (a constant) and \(C_t\) refers to the total cell density at time \(t\).

2.2.2 Detection of cell membrane integrity

Cell membrane integrity loss was detected by simultaneous staining with SYBR green I (SYBR; Sigma, USA) and propidium iodide (PI; Sigma, USA). When testing, add 5 \(\mu\text{L}\) of 1% (v/v) SYBR green I and 5 \(\mu\text{L}\) of 1 mM PI to 490 \(\mu\text{L}\) of sample and place in a dark environment for 15 min. The live cell number and damaged cell number can be effectively recorded by flow cytometry. Percentage of membrane damage (\(P_{md}\)) was evaluated by Eq. 4.

\[
P_{md} = \frac{\text{damaged cell number}}{\text{damaged cell number} + \text{live cell number}} \tag{4}
\]

2.2.3 Detection of cell death

Membrane potential plays a vital role for normally functional cells. Decline in membrane potential is regarded as apoptosis indicator [19]. In this study, algal samples were washed with PBS, incubated with DiOC6 (3,3'-Dihexyloxacarbocyanine iodide; Thermo Fisher, USA) at 25 \(\mu\text{M}\) in the dark at room temperature for 15 min, and detected via FL1 channel.

2.3 Statistics & data analysis

The data were presented as average with a standard deviation. Data were processed and graphed using Origin 8.5 software. Differences between groups were calculated using software IBM SPSS Statistics 22 through one-way ANOVA (p<0.05).

3. Results

3.1 Apoptotic-like algal inhibition

For an initial concentration of \(M. \text{Aeruginosa}, 6.5 \times 10^6 \text{ cells mL}^{-1}\), with a fixed \(\text{H}_2\text{O}_2\) dose of 0.05 mM, the UV-C doses, 50, 75, and 100 mJ cm\(^{-2}\), were set to study the UV-C irradiation’s function of the algal inhibition. Cell lysis was positively correlated to dose of UV-C and [0.05,100] achieved best inhibitory performance in growth inhibition with highest cell lysis rate, 72.6%, on 11 d (Fig. 1a). Similar to growth inhibition, \(P_{md}\) was also positively correlated to dose of UV-C (Fig. 1b). Notably, increase in \(P_{md}\) on 1 d and decline in \(P_{md}\) on 5 d (Fig. 1b) testified that cell with damaged cell membrane lysed resulting in continuous decline of total cell density (Fig. 1a). Cell size was negatively correlated to the UV-C dose and exhibited a decreasing trend in the study period (Fig. 1c). Membrane potential was also negatively correlated to the UV-C dose. Decline on both 1 d and 11 d indicated up-coming apoptotic-like lysis (Fig. 1d), which could be testified by decreasing trend of total cell density in Fig. 1a.
3.2 Necrotic algal inhibition

For an initial concentration of *M. Aeruginosa*, 6.5 *10^6* cells mL\(^{-1}\), this study set two high H\(_2\)O\(_2\) dose, 0.4 mM and 2 mM, with or without UV-C irradiation, 100 mJ cm\(^{-2}\), as necrotic groups.

All four groups showed stagnant cell lysis from 0.1 d to 5 d and significant cell lysis on 11 d (Fig. 2a). On 5 d, the cell lysis rates were 9.4%, 12.4%, 20.0% and 19.4%, which were much lower than that of apoptotic-like algal inhibition on 5 d (Fig. 1a). On 13 d, cell lysis rate of [0.4,0] and [2,0] were significantly higher than [0.4,100] and [2,100] (p<0.05), which indicated that UV-C irradiation could negatively influence following cell lysis after treatments in necrotic inhibition. Notably, [2,0] did not exhibit significantly higher cell lysis rate than [0.4,0] on 13 d (p<0.05), which indicated that increasing H\(_2\)O\(_2\) dose exhibited weak enhancement in necrotic inhibition. Cell death by necrosis is associated with a high ratio of loss of membrane integrity [20], and all four groups showed extremely high P\(_{md}\) exceeding 99% on 1 d (Fig. 2b). [0.4,100] and [2,100] did not exhibit significantly higher P\(_{md}\) than [0.4,0] and [2,0] from 1 d to 13 d (p<0.05). The four groups showed a sharp decline in cell size on 0.1 d followed by a continuous decline on 0.5 d (Fig. 2c). However, cells of all four groups then swelled as cell size increased on 1 d, which was a marker of necrosis [21]. After swelling, cells become smaller and smaller (Fig. 2c). For membrane potential, all four groups increased on 0.1 d and began to drop on 0.5 d. Finally, the four groups respectively reached relatively low membrane potential, 0.45, 0.41, 0.46, and 0.44, compared to control level on 1 d (Fig. 2d). Given that necrotic swelling occurred on 1 d, measurement of membrane potential ended in this study.

![Variation of (a) total cell density, (b) P\(_{md}\), (c) cell size and (d) membrane potential in apoptotic-like algal inhibition](image_url)

Fig. 1 Variation of (a) total cell density, (b) P\(_{md}\), (c) cell size and (d) membrane potential in apoptotic-like algal inhibition
4. Discussion

Previous studies such as [9] advocated that the combined use of UV-C with H$_2$O$_2$ was capable of inducing synergetic damage. However, the dose-response effect of UV-C and H$_2$O$_2$ was not concerned. This study advocated that the growth inhibition for *M. aeruginosa* was positively correlated to UV-C dose (Fig. 1a) and UV-C exhibited weak enhancement in P$_{md}$ for necrotic algal suppression (Fig. 2b). This study both recorded decline in membrane potential in apoptotic-like group and necrotic group. Notably, decline in membrane potential is an indicator of primary phase for apoptosis-like. As a result, we saw that there was decline in cell size on 0.5 d, which meant cell wrinkling as a marker of primary apoptosis-like, both in apoptotic-like group (Fig. 1c) and necrotic group (Fig. 2c). However, on 1 d, there was significant cell swelling in necrotic group (Fig. 2c). In other words, necrotic group switched primary apoptotic-like death to necrotic death. One critical factor in deciding death switch may be the energy level of the cell [21]. A deficiency of ATP was likely to prevent downstream processes including caspase-3 activation and further permit apoptotic stimuli to induce necrosis [22, 23]. Thus, this study supposed that excessive ROS could be induced when addition of H$_2$O$_2$ was coupled with UV-C irradiation, which was able to run out of cellular ATP and further caused necrotic death.

In the future, further investigations are essentially required to determine the detailed mechanism for death switch from apoptotic-like death to necrotic death and corresponding variation in gene expression in H$_2$O$_2$/UV-C treatments for *M. aeruginosa*.

5. Conclusions

This study demonstrated that given a specific dose of H$_2$O$_2$, apoptotic-like growth inhibition for *M. aeruginosa* was positively correlated to UV-C dose. Hence, dosing strategy for apoptotic-like inhibition is that an environmentally friendly H$_2$O$_2$ dose should be determined with priority. Then,
UV-C dose should be mediated to achieve a specific objective for algal inhibition. As for necrotic growth inhibition, increasing \( \text{H}_2\text{O}_2 \) dose exhibited weak enhancement in growth inhibition while existence of UV-C irradiation could negatively influence following cell lysis after treatments. Hence, dosing strategy for necrotic inhibition is that a moderate \( \text{H}_2\text{O}_2 \) dose without UV-C irradiation should be enough to achieve a specific objective for algal inhibition.

For mechanism of death switch, given a specific UV-C dose, corresponding high \( \text{H}_2\text{O}_2 \) dose could switch cell death from apoptosis-like to necrosis. A deficiency of ATP was likely to prevent downstream caspase-3 activation, which could switch from apoptotic-like process to necrotic process.

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