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The stress response mechanisms of biofilm formation under sub-lethal photocatalysis

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ABSTRACT

Significant health risk exists due to increasing antibiotic-resistant bacteria and formation of antibiotic-resistant bacterial biofilm in water. For comprehensive understanding of stress response mechanisms of biofilm formation under sub-lethal photocatalysis (PC), Herein, a drip flow device was developed to culture biofilm and *Pseudomonas aeruginosa* (GEN) (resistance to Gentamicin) was developed to assess the effect of sub-lethal PC on biofilm formation. Under sub-lethal PC, the bacterial abundance and the thickness of biofilm decreased relative to controls (by 81.7% and 68.0%, respectively) on the 1st day; however, the content of extracellular polymeric substance (EPS) per cell was promoted unexpectedly. Furthermore, it was found that the proportion of viable culturable bacteria in the biofilm increased 7.8 times relative to control and the biofilm exhibited resistance to oxidative stress via EPS secretion. This perspective was validated through molecular regulatory network study. This study may provide enlightenment to bacterial biofilm control in the water system.

1. Introduction

The dispersion of antibiotic-resistant bacteria and antibiotic resistance genes as well as the formation of antibiotic-resistant biofilm have become the two important major issues that adversely affect the public health at the global scale [1,2]. Antibiotics are not effectively removed in wastewater treatment plants when traditional technologies are applied [3]. Many antibiotics have been reported to occur at 10–1000 ng L^{-1} levels in the secondary-treated effluents [4]. According to previous studies, harsh conditions and hostile environmental conditions such as starvation and desiccation may promote the growth of biofilms containing bacteria capable of causing a broad range of chronic diseases [5,6]. As a result, with the increase in the antibiotic concentrations in the environment, biofilm growth gets promoted. The extreme slow growth and dormancy of bacteria have long been regarded as ways by which bacteria may survive under deep starvation conditions in the biofilm when exposed to antibiotics [7,8], thus biofilm is also an important carrier for the dispersion of antibiotic-resistant bacteria [9,10]. Moreover, the formation of biofilm also provides a barrier for bacteria to increase their resistance to antibiotics as well as some host attacks and protocell phagocytosis [11,12]. Besides, the biofilm barrier effect can also provide protection for the internal bacteria under some other harsh environments, including metal toxicity, acid exposure, dehydration, and salinization [5,13].

Normally, biofilm and human beings form symbiotic systems; nonetheless, in many cases, the growth of biofilm significantly impacts the sustainable development of human health and environmental system. Biofilm can colonize both biotic and abiotic surfaces, causing detrimental effects to the environment, industry, and human health [14]. Besides, it has been reported that approximately 95% of total biomass in water exists in form of biofilm [15], indicating that the biofilm can continuously release planktonic bacteria and cause continuous water contamination. Therefore, the removal of biofilm from water environment is particularly important. Owing to the massive production and application, the nanomaterials are released into the water system [16]. At the same time, use of ultraviolet (UV) radiation is a proven technology for wastewater disinfection [17]. Therefore, during water disinfection under UV irradiation, bacteria are liable to be exposed to

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photocatalysis (PC). It is known that harsh conditions can promote the growth of biofilm. However, it is still not clear whether biofilm formation will be inhibited or unexpectedly promoted under PC during the wastewater treatment process, or occurrence of even more complicated conditions is encountered. Besides, under the practical situation, the bacteria usually escape lethal attack of reactive oxygen species (ROSs) and only receive sub-lethal oxidation pressure [18,19]. This process could be defined as sub-lethal PC, where bacteria could still grow and simultaneously get exposed to sub-lethal attack of ROSs, which might make disinfection process counter-productive [19]. Therefore, investigation of the growth of biofilm under the stimulation of sub-lethal PC needs to be carried out to comprehensively understand the mechanism involved.

The answers to the above-mentioned questions are validated in this study, by employing *Pseudomonas aeruginosa* (*P. aeruginosa*) bacteria as model pathogenic microorganism which is widely found in various environments [20]. To further simulate resistance of bacteria to a representative antibiotic gentamicin in environment, the *P. aeruginosa* (GEN) with gentamicin resistance plasmid was also developed in this study for biofilm formation experiment and for comparative analysis. In this study, a new PC interface was simulated and designed for clear visualization. The changes of biological characteristics of biofilm under sub-lethal PC stimulation were first analyzed. Next, oxidative stress and extracellular polymeric substance (EPS) secretion response of bacteria in biofilm were measured. Further, the ratio of different states of bacteria in biofilm was also measured. Finally, real-time polymerase chain reaction (qPCR) was used to measure the expression of related genes in the molecular regulatory network to further explain the above-stated phenomenon. This study unravels the mechanism underlying stress responses of pathogenic microorganism during the biofilm formation process under sub-lethal PC stimulation, thus reducing the requirement to completely eliminate the control strategy for biofilm in water environment.

2. Materials and methods

2.1. Construction of target strains and biofilm development

The plasmid RP4-8 containing *aac (3)-I* gene (confers resistance to Gentamicin) was introduced into competent cells of *P. aeruginosa* and named as *P. aeruginosa* (GEN). Then, the plasmid pet28-EGFP containing green fluorescent protein was introduced into *P. aeruginosa* (GEN) and named as *P. aeruginosa* (GEN, EGFP). Development of bacterial strains and their validation are presented in Supporting information (SI) as well as Fig. S1. The bacterial incubation and biofilm development were carried out in a self-made drip flow biofilm culture device (Fig. S2). The detail information of biofilm development is also provided in SI.

2.2. Biofilm biological characteristics assay

The biofilms collected at different times under different conditions were resuspended in phosphate-buffered saline (PBS) solution (5 mL). Then, bacterial solution (1 mL) was taken and its concentration was adjusted to 10^8 CFU mL^{-1} (OD_{600} = 0.1) using a microplate reader (Thermo Fisher Scientific, USA). Finally, the initial concentration of the bacterial solution was calculated by adding the volume of bacterial solution and the volume of PBS solution. Further, the bacterial abundance in the biofilm was expressed by calculating the concentration of the initial bacterial solution.

The *P. aeruginosa* (GEN, EGFP) strain was used to observe changes in thickness of biofilm because it contained pet28-EGFP plasmid with green fluorescent protein. The glass slides in the biofilm culture device were taken out after exposing them to different cultured conditions and cultured days, and the layer scanning function was used to observe the changes in biofilm thickness in the glass plates by confocal laser scanning microscopy (Carl Zesis CLSM 800 With Airscan, Germany). Moreover, to more directly observe the changes of biofilm under different cultural conditions, a time-lapse camera (Shenzhen Aliwei Technology Co., Ltd., aTLi EON) was used to shoot the ripening process of biofilm, as shown in Videos 1–4.

The *P. aeruginosa* (GEN) strain was used to culture biofilm to obtain foundation data for biofilm formation. Biofilm samples exposed to different conditions were then taken out and stained with SYTO 9 (Thermo Fisher Scientific, USA). Then, they were placed in the OCT tissue freezing medium for a second embedding process until biofilm was completely enveloped. Next, the biofilm was cut into ultrathin slices (20 μm) and transferred onto slides. The barrier function of biofilm was observed by confocal laser scanning microscopy (Carl Zesis CLSM 800 with Airscan, Germany) to calculate the permeability of dye to biofilm [21].

Morphological changes of biofilm under different culture conditions and different cultural time were observed by scanning electron microscopy (SEM). See SI for specific sample preparation method.

Antibiotic resistance was determined on the biofilm cultured under different conditions for different days. In this study, six different antibiotics including colistin (PB), β-lactam (CTX), macrolides (AZI), tetracycline (TET), aminoglycosides (GEN), and quinolones (OFX) were used to understand the biofilm resistance to antibacterial agents. Changes in antibiotic resistance of biofilm were indicated by measuring minimum inhibitory concentration (MIC) of resuspension solution mentioned above, and specific measurement steps of MIC are presented in SI.

2.3. Oxidative stress and extracellular polymeric substance determination

The degree of oxidative stress of the biofilm was determined by measuring the activities of ROSs, superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) in the resuspension solution mentioned above. The specific determination steps of ROS, SOD, CAT, and GSH-Px are presented in SI.

Biofilm samples at different growth stages were collected, incubated in water bath for 40 min at 60 °C, and then allowed to cool. After room temperature (23 °C ± 2 °C) was attained, the mixtures of PBS and biofilm were transferred into 1.5-mL tubes and then centrifuged for 20 min at 4000 rpm. EPS extracts were filtered with nylon membranes (0.45 μm), and the total protein concentration of EPS extracts was assessed using a modified Lowry Protein Assay Kit (Sangon Biotechnology, China) with bovine serum albumin as a standard. Total polysaccharide concentration was assessed through the phenol–sulfuric acid method with glucose as a standard [22].

2.4. Determination of bacterial state in biofilm

A combination of flow cytometry with Live/Dead staining method was used to determine the living–death ratio of bacteria. A LIVE/DEAD BACLIGHT kit (Thermo Fisher Scientific, USA) was used to observe the bacterial activity. The sampled biofilm was first washed twice with PBS solution, resuspended with PBS solution (1 mL), and then the final bacterial concentration was adjusted to 10^6 CFU (colony forming units) mL^{-1}. Subsequently, 10 μL of SYTO 9 and PI diluted 1000 times to the work concentration was added into suspended biofilm sample (1 mL). The stained samples were incubated in dark at room temperature for 15–30 min, and then analyzed by flow cytometry (BD FACS Aria III, BD, USA).

The biofilm samples collected at different times and under different conditions were resuspended in PBS solution (5 mL). Biofilm and PBS mixtures (1 mL) were taken from the tube and diluted with NaCl solution (0.9%, pH = 7.2) at a ratio of 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} times, respectively, and the bacterial liquid was coated and counted. Then the petri dishes were cultured in an incubator at 37 °C for 24 h, and the bacterial number in the viable and culturable (VC) state was calculated by taking the reading. Total number of living bacteria was calculated by combining the proportion of live bacteria mentioned in Section 2.3.

Finally, the number of viable but nonculturable (VBNC) bacteria was determined by subtracting the number of bacteria of the VC state from the total number of live bacteria. The proportion of bacteria in the VC state and VBNC state was achieved by calculation.

2.5. Assessment of the expression of mRNA gene

Total RNA of bacterial biofilm under different conditions was extracted using a bacterial total RNA extraction kit (Sangon Biotechnology, China). Subsequently, cDNA was synthesized using M-MLV Reverse Transcription Kit (Invitrogen Inc., USA). The expressions of various genes were quantified using qPCR. Table S1 lists all the primers used in this study. Detailed information about the genes and reaction procedures for the qPCR are presented in SI.

2.6. Data analysis

All the experiments were performed independently at least three times. Analysis of variance and Student's *t*-test with Bonferroni correction for multiple comparisons were used to determine the statistical significance.

3. Results and discussion

3.1. Biological characteristic response of biofilm to sub-lethal PC stimulation

For the comprehensive understanding of the biological response characteristics of biofilm to sub-lethal PC stimulation, the changes of bacterial abundance within biofilm, biofilm thickness, biofilm barrier effect, and antibiotic resistance of biofilm under sub-lethal PC stimulation were all examined systematically.

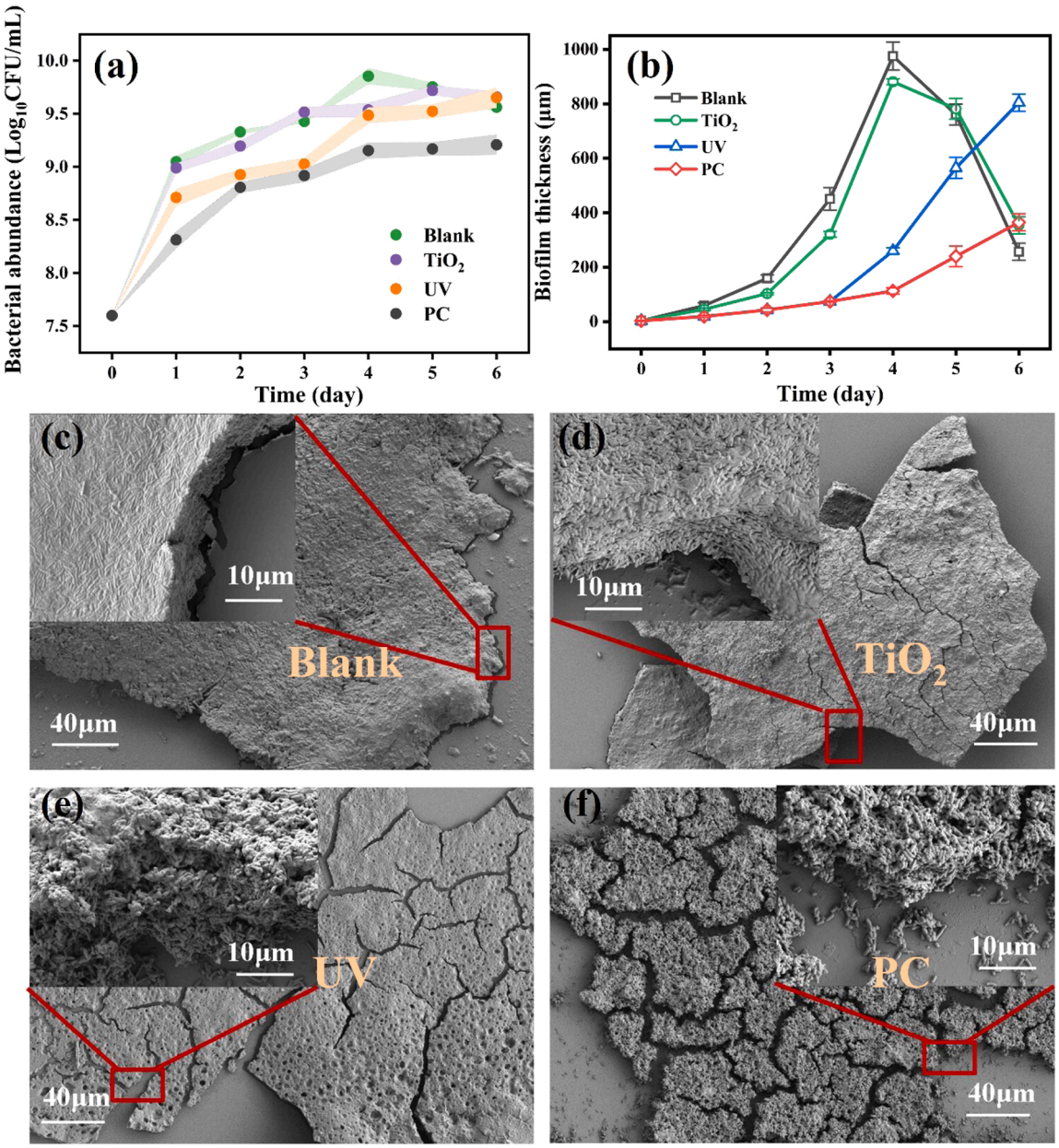

Fig. 1. (a) The changes of *P. aeruginosa* (GEN) biofilm's bacterial abundance under different conditions; (b) *P. aeruginosa* (GEN) biofilm thickness; The SEM images of *P. aeruginosa* (GEN) biofilm cultured onto (c) blank group, (d) TiO_2 attached, (e) UV irradiation, and (f) sub-lethal PC stimulation.

To assess the bacterial activity, growth curves of *P. aeruginosa* (GEN) in biofilm under different conditions (TiO_2, UV, PC) were comparably measured (Fig. 1a). Under TiO_2 alone condition, with prolonging culture time, the bacterial abundance gradually increased, then entered a stable period, reaching the maximum (5.2×10^9 CFU mL^{-1}) at 5th day. This change trend was not much different from that in the blank group. In general, the biofilm lifecycle occurs in the following three stages: cell attachment, biofilm maturation, and biofilm dispersal, similar to that mentioned in a literature study [23]. The overall biofilm growth trend for blank group and TiO_2 group first increased and then decreased, which is in well agreement with the growth law of the biofilm. Comparatively, the growth curve of *P. aeruginosa* (GEN) under UV irradiation was slightly lower than those under both TiO_2 condition and blank condition, and only approximately 0.5 log less than the blank group; however, it did not enter the dispersion stage. Nonetheless, the bacterial abundance in the biofilm cultured under sub-lethal PC stimulation decreased significantly. Compared with the blank group, the overall bacterial abundance under PC decreased by approximately 1 log, which also did not enter the dispersion stage. The abundance of bacteria did not reach 1.6×10^9 CFU mL^{-1} until the 6th day, clearly indicating that the inhibition effect of sub-lethal PC stimulation on the growth of biofilm was very obvious.

Further, the tendency of biofilm thickness was the same as that of bacterial abundance. The thickness variation trend of biofilm under sub-lethal PC reached the maximum value of 364.51 μm on the 6th day, which was significantly lower than the maximum value of 974.95 μm in the blank group on the 4th day (Fig. 1b). However, the increase rate of biofilm thickness was different from that of bacterial abundance. For example, under the TiO_2 attachment condition, the bacterial abundance rapidly increased at the initial stage; however, the thickness of the biofilm increased gradually (Fig. 1a and b). This is attributed to the fact that, during bacterial biofilm development, biofilm is composed of not only bacteria, but also EPS produced by bacteria themselves [24,25]. Therefore, changes of EPS content in biofilm may effectively explain the phenomenon obtained in this study that the change rate of biofilm thickness was different from that of bacterial abundance. Changes of biofilm composition would be discussed later.

Furthermore, biofilm samples cultured for one day under different conditions were also compared under SEM. The biofilm cultured under TiO_2 attachment condition grew well and retained intactly, and the bacteria in the biofilm was closely gathered together with EPS [25], which is very similar to the results of the blank group (Fig. 1c and d). However, the biofilm cultured under UV irradiation showed obvious cracks, while the biofilm stimulated by sub-lethal PC showed more obvious cracks (Fig. 1e and f). These indicate that the structure of biofilm collapsed under these conditions, in particular, by sub-lethal PC stimulation. These cracks were possibly caused by the degradation of EPS [8]. Further, the inset of Fig. 1f demonstrates that under sub-lethal PC stimulation, the degradation of EPS was obvious, some bacteria were also inactivated, and even the morphology of bacteria also changed from long rod-like to short rod-like. Previous studies demonstrated that, under some adverse environments, the physiological morphology of bacteria changed to some extent to response stress [26,27]. For example, chloramine and chlorine treatment makes the *Escherichia coli* cells shorter [7].

In order to further verify the phenomenon observed under SEM (Fig. 1c–f), the barrier effect of biofilm was measured. It is well known that owing to the encapsulation effect of EPS, biofilm exhibits a formidable barrier effect on exogenous chemical substances. Therefore, the changes of barrier ability of the cultured biofilm stimulated by TiO_2, UV, and PC systems were measured comparably (Fig. S3). Noteworthy, almost no difference was observed in dye permeability change trends of the biofilm between TiO_2 attachment culture group and the blank group. Both of them decreased first and then increased, and reached the minimum value of 5.0% on the 4th day. This indicates that, at this time, the biofilm is the most difficult to be penetrated, barrier ability is the strongest, and the ability to resist attack is also the strongest. Further, the subsequent increase in permeability was possibly due to the dispersion stage of biofilm growth, which led to the natural decrease of its barrier function [28]. Comparative analysis indicates that permeability of biofilm cultured under sub-lethal PC stimulation was all above 40.0%, even reaching 90.0% at the initial cultured stage (on the 1st day), and the overall permeability values were higher than those of UV, TiO_2, and the blank groups. All these results are well consistent with those observed from the SEM images. In other words, the sub-lethal PC stimulation can effectively weaken the barrier ability of biofilm. However, even under the sub-lethal PC stimulation, the barrier ability of the biofilm was damaged to some extent only at the initial stage of the biofilm growth. Then, with the increase of culture time, barrier ability of the biofilm gradually increased and the permeability gradually decreased, thus indicating that sub-lethal PC could only inhibit rather than prevent the growth of biofilm.

Notably, bacteria have higher antibiotic resistance capability in the form of biofilm than that of planktonic counterpart [29]. Therefore, the antibiotic resistance changes of *P. aeruginosa* (GEN) biofilm to Gentamicin as well as other five classes of frequently used antibiotics (PB, TET, AZI, OFX, and CTX) were comparably measured under different culture conditions (the action mechanisms of the six antibiotics are presented in Table S2). Noteworthy, the biofilm antibiotic resistance of *P. aeruginosa* (GEN) to all antibiotics of the TiO_2 group and the blank group showed basically the same trajectories (Fig. 2). However, under sub-lethal PC stimulation, the biofilm resistance to most of antibiotics, including GEN (Fig. 2a), TET (Fig. 2b), PB (Fig. 2c), AZI (Fig. 2d), and OFX (Fig. 2f), decreased significantly. Further, different from the other five studied antibiotics, biofilm resistance to CTX increased more slowly than that of the blank group; nonetheless, it still attained the maximum value (Fig. 2e). This may be attributed to the fact that different types of antibiotics exhibit diverse antibiotic resistance mechanisms. For example, resistance to CTX targets the bacterial cell wall, even under the EPS barrier, compared with antibiotic OFX acting on DNA, where it is easier to contact the cell wall [30]. Moreover, the change of biofilm antibiotic resistance was found to be negatively correlated with the change of barrier function ($P = 0.0002$), which further confirmed that the barrier ability of biofilm was indeed an important reason for the increase of biofilm antibiotic resistance. This result for the biofilm is completely different from the result related to the changes in the planktonic bacteria. Our previous studies showed that planktonic bacteria could increase the persistence of bacteria in the form of reduced fitness cost under sub-lethal PC effect [19], which further illustrated that the antibiotic resistance mechanism of biofilm was different from that of planktonic bacteria.

3.2. Oxidative stress and EPS secretion response of bacteria in biofilm

Therefore, in order to further explore the internal effects of sub-lethal PC on the growth of biofilm, intracellular ROS levels and antioxidant enzyme activities of bacteria in the biofilm during the growth process were detected (Fig. 3). Surprisingly, the growth and antibiotic resistance of biofilm appeared to be inhibited by sub-lethal PC; however, the oxidative stress response of the bacteria within the biofilm to sub-lethal PC stress was much stronger than that of the control groups (Fig. 3). The relative content of ROSs in bacteria reached a maximum of 14.40 on the 1st day (Fig. 3a), and the activity of antioxidant enzymes including CAT, SOD, and GSH-Px in bacteria cells also increased (Fig. 3b–d), in order to eliminate the oxidative damage in bacterial cells [31]. These results indicate that sub-lethal PC can cause strong oxidative stress to bacteria in the biofilm.

Previous researches have confirmed that EPS is more likely secreted under harsh conditions [32,33]. To further evaluate the EPS secretion response of bacteria in biofilm, the secretion of EPS per cell was analyzed under sub-lethal PC stress. It is well known that EPS is mainly composed of polysaccharides and proteins [25], thus, the changes of the

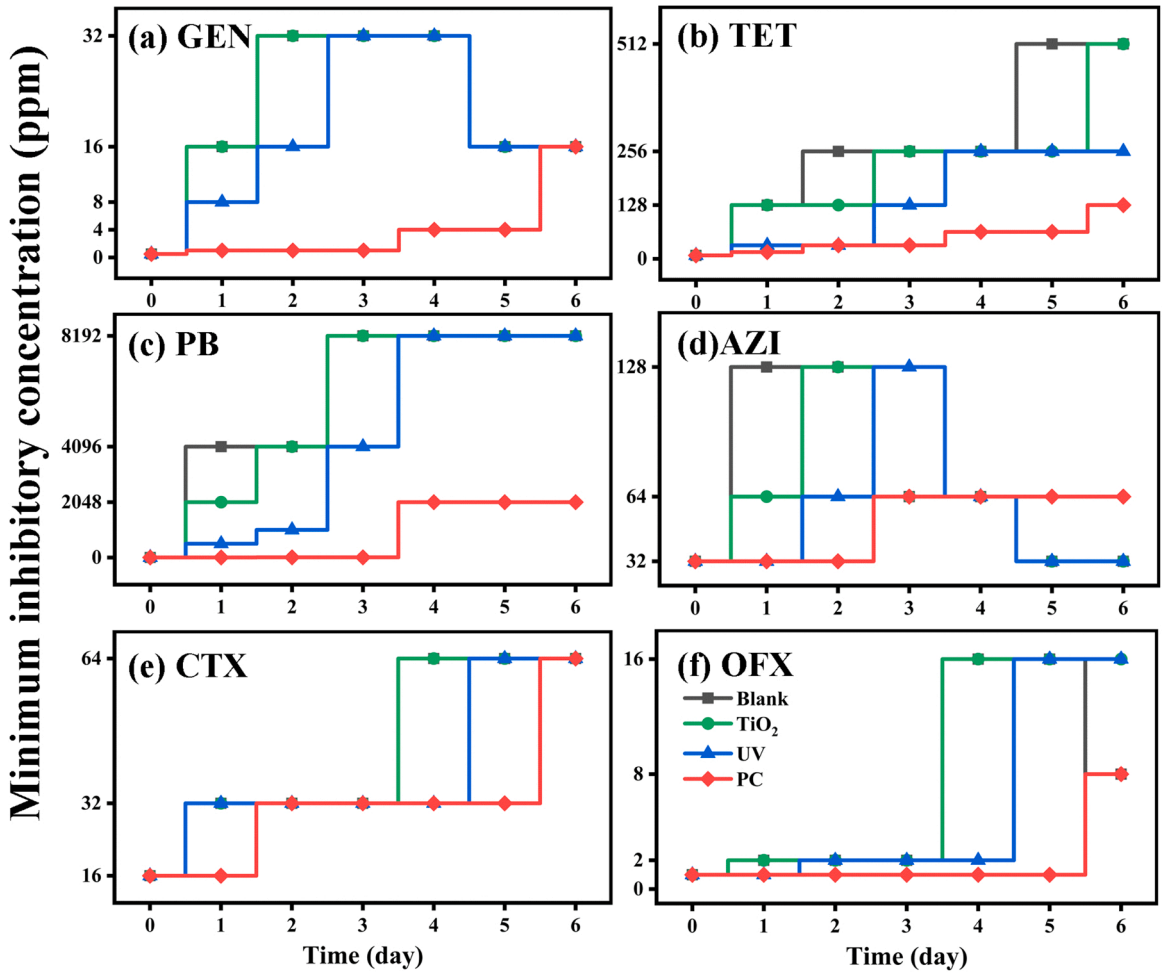

Fig. 2. Changes of biofilm resistance to six antibiotics (a) GEN, (b) TET, (c) PB, (d) AZI, (e) CTX, and (f) OFX in blank, TiO_2, UV and sub-lethal PC systems.

total content as well as proportion of carbohydrate and protein were studied during biofilm formation under different conditions (Fig. S4). Moreover, the secretion of EPS per cell was analyzed comparably (Fig. 4). During biofilm cultivation, the contents of EPS in the control groups decreased to different degrees (Fig. 4a–c). Differently, under sub-lethal PC stimulation, the contents of EPS increased rather than decreased on the 1st day, and then gradually decreased (Fig. 4d). Moreover, content of EPS per cell under sub-lethal PC stimulation was higher than that of biofilm cultured under other conditions at the same number of days, and it just declined to 0.33 mg/10^8 cells on the 6th day (Fig. 4). Therefore, it was proposed that sub-lethal PC could significantly promote rather than inhibit the secretion of EPS of each bacterial cell. This is possibly attributed to the fact that bacteria experienced harsh situation when exposed to sub-lethal PC, leading to a strong oxidative stress of the bacteria in the biofilm, which further led to the short-term promotion of the EPS secretion.

In order to further verify the EPS content change per cell, the changes of bacterial ATP in the biofilm were detected during biofilm formation. Trends of ATP changes under different culture conditions were found to be consistent with that of EPS contents per cell (Figs. S5 and 4). It is ascribed to the fact that the ATP in bacteria is mainly secreted by polysaccharides and proteins, and polysaccharides and proteins in biofilm are mainly composed of EPS [25]. Therefore, changes of bacterial ATP further proved that sub-lethal PC could not only increase the secretion of biofilm EPS, but also enhance the activity of bacteria to some extent at short term exposure.

3.3. The bacterial mortality rate and the proportion of VBNC bacterial cells in the biofilm

Interestingly, based on the above obtained results, a contradictory conclusion was obtained that the growth and antibiotic resistance of the biofilm were inhibited under the stimulation of sub-lethal PC; however, the oxidative stress and EPS secretion response of the bacteria within the biofilm were promoted. To reveal this seemingly contradictory conclusion, systematic explorations were further carried out herein to investigate the changes of bacterial state in biofilm.

First, bacterial mortality within the biofilm was determined by flow cytometry (Fig. 5a). The results indicated that bacterial mortality within the biofilm reached the maximum on the 1st day, and then decreased steadily with prolonging cultured time under different conditions (Fig. 5b–e). The reason for high mortality at the early biofilm growth stage is that a series of natural death occurred for bacteria to resist the biofilm formation [34]. The mortality rate of bacteria in the biofilm cultured under sub-lethal PC significantly increased, up to 60% (day 1), and approximately 44.5% of the overall average mortality rate was obtained, which is much higher than those of the control groups (Fig. 5b–e). These results indicate that sub-lethal PC can not only destroy the barrier ability of biofilm, but also partially inactivate bacteria present in biofilm. Simultaneously, the barrier ability of biofilm was not fully destroyed; therefore, the biofilm still exhibited a certain defense capability against sub-lethal PC stimulation. As a result, the death rate of bacteria was not very high and the biofilm could still grow slowly even if exposed to sub-lethal PC stimulation.

For the further systematic explorations of the changes in the state of

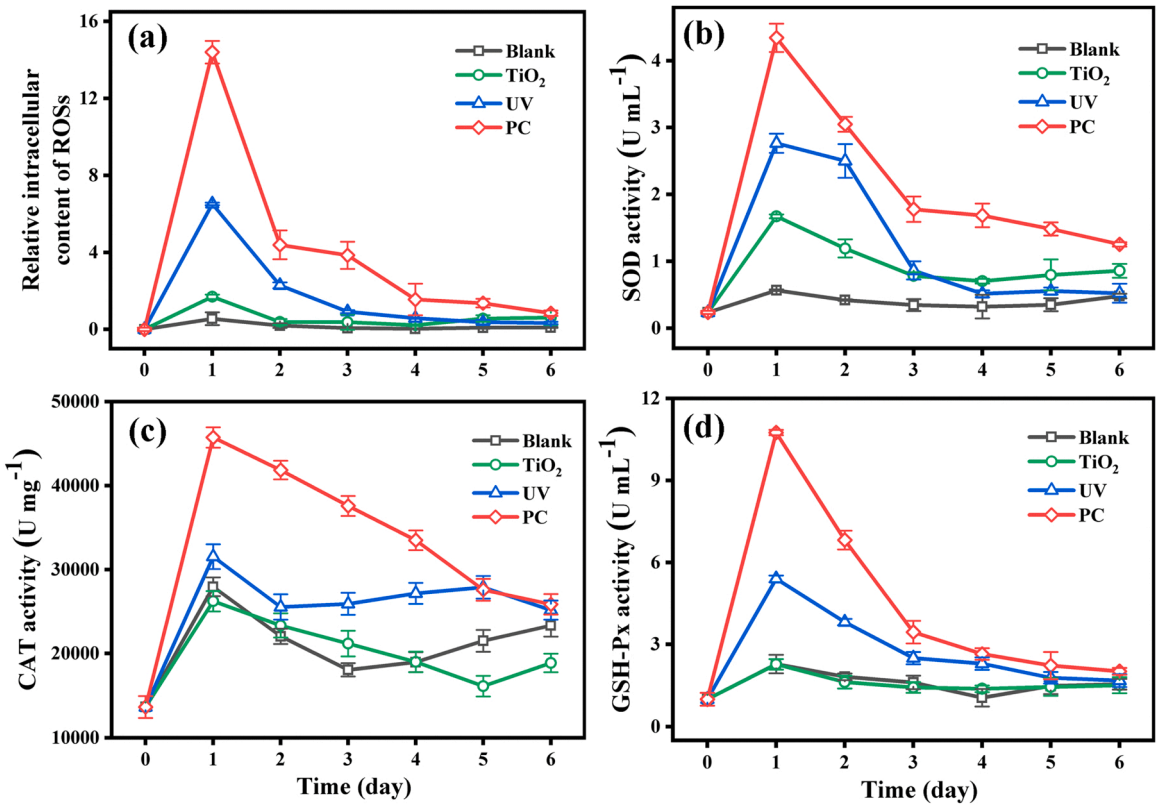

Fig. 3. (a) Relative intracellular content of ROSs tested by DCFH-DA; (b) SOD activity, (c) CAT activity, and (d) GSH-Px activity of bacteria within the biofilm under blank group, TiO_2 attached, UV irradiation, and sub-lethal PC stimulation during biofilm formation.

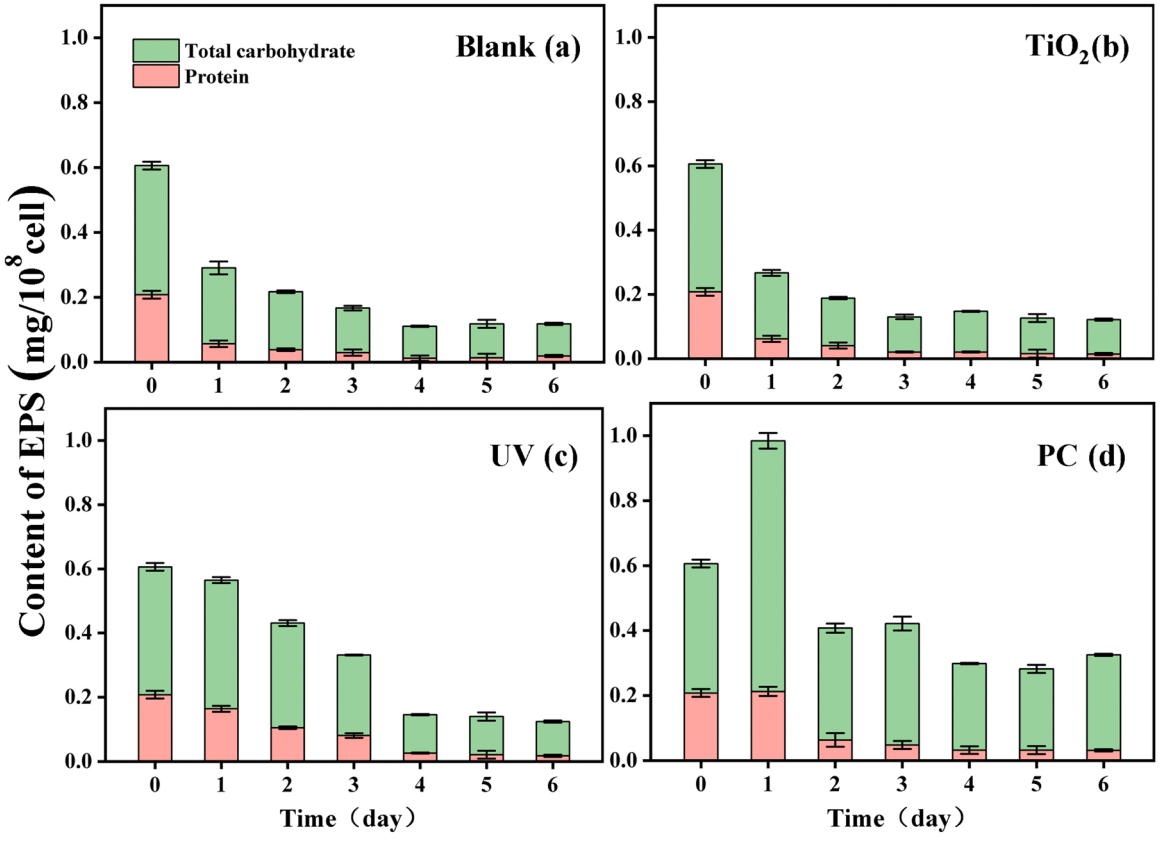

Fig. 4. Change of EPS content per cell in biofilm in (a) blank, (b) TiO_2, (c) UV and (h) sub-lethal PC group.

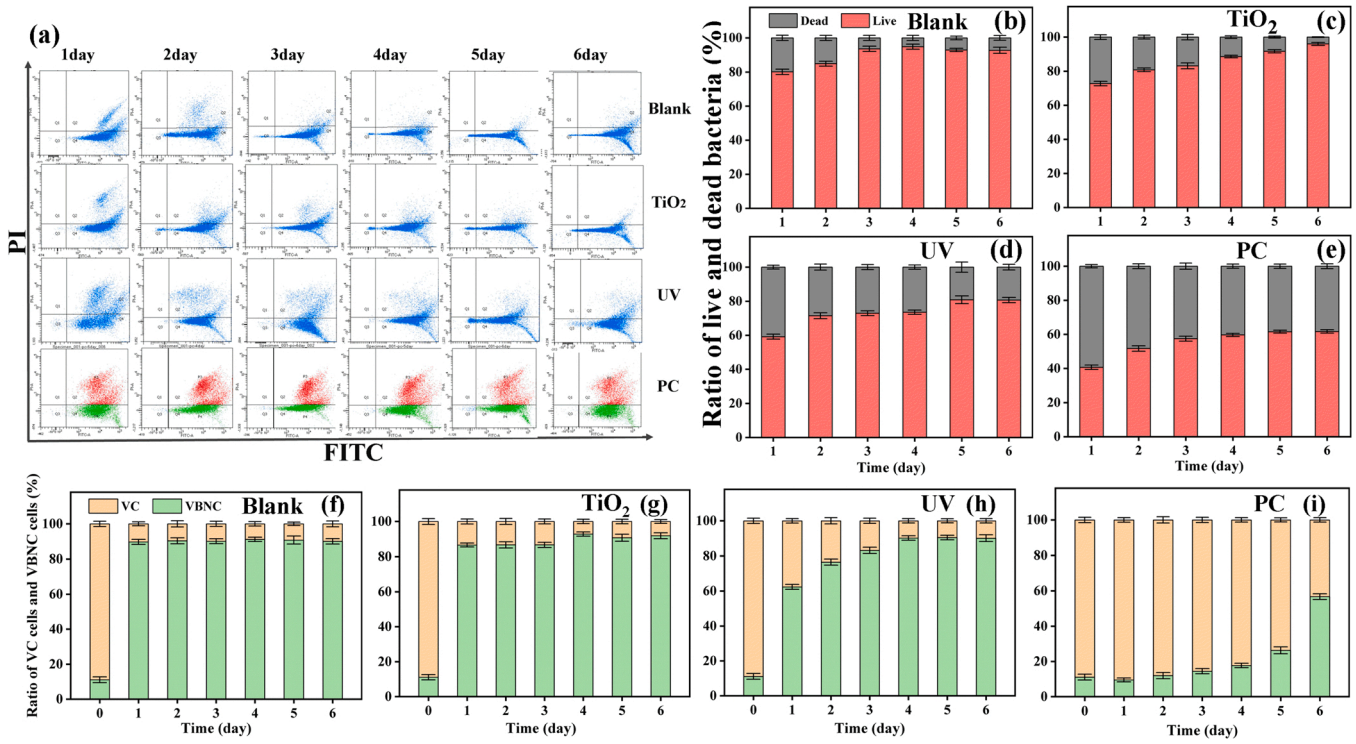

Fig. 5. Changes in the living-death ratio of bacteria in a biofilm and changes of the proportion of VBNC cells. (a) Original flow cytometric graph of the living-death ratio of bacteria in a biofilm; The living-death ratio of bacteria in (b) blank, (c) TiO_2, (d) UV and (e) sub-lethal PC system. Changes of the proportion of VBNC cells in (f) blank, (g) TiO_2, (h) UV and (i) sub-lethal PC group.

those bacteria that survived under sub-lethal PC stimulation, the proportion changes of VBNC bacterial cells in the biofilm were measured. According to a recent study, bacteria change from the VC state to VBNC state during biofilm formation due to the decrease of oxygen and nutrients [35]. In this study, the number of living culturable bacteria in the biofilm under different cultured conditions did not change significantly (Fig. S6b); however, the bacterial number in the VBNC state under sub-lethal PC stimulation decreased significantly (Fig. S6c). According to the proportion of bacteria in the VC state and VBNC state in control groups (Fig. 5f–h), a large number of bacteria transformed from VC state to VBNC state during biofilm formation. In contrast, under the sub-lethal PC stimulation, the proportion of bacteria in the VC state increased, with 14.4% of bacteria changing from VBNC state to VC state in the biofilm on day 1 (Fig. 5i). This result is attributed to the fact that sub-lethal PC destroys the bacteria on the surface of the biofilm, thus reducing the competition among the bacteria in the biofilm for oxygen and nutrients, and thus the internal VBNC bacterial cells are revived with enough oxygen and nutrients [36]. Moreover, owing to lower activity and lower metabolism of bacteria in the VBNC state, their ability to produce EPS is by far weaker than that of normal bacteria in the VC state. As such, bacteria entering VBNC state leads to the reduction in EPS secretion [37]. This can also further explain the phenomenon of increasing EPS content per cell on the 1st day of biofilm cultivation under sub-lethal PC stimulation (Fig. 4d).

These experiments well explained the seemingly contradictory conclusions in the above-mentioned study. Overall, these results indicate that sub-lethal PC inhibits the biofilm growth, damages the biological biofilm barrier effect, and inactivates some bacteria in the biofilm. However, the survived bacteria could produce stronger oxidative stress and higher EPS secretion response to further resist the stimulation of sub-lethal PC.

3.4. Molecular regulation network of biofilm bacteria responding to sub-lethal PC

In order to confirm the above-mentioned conclusion, herein, the expression of genes related to biofilm growth, antibiotic-resistance, oxidative stress, and EPS secretion were further analyzed to reveal the molecular regulation network of antibiotic-resistant biofilm growth under sub-lethal PC stimulation (Fig. 6). Fig. 6a shows the mechanism of action of FleQ, a bacterial transcription factor, which activates flagellum biosynthesis related genes at the early stage of *P. aeruginosa* biofilm formation [38,39]. At the same time, transitions between individual and collective behaviors of microorganisms are controlled by the chemical cell-to-cell communication process called quorum sensing (QS), which is often used to evaluate the biofilm formation [40]. The QS system of *P. aeruginosa* is mainly composed of the PQS system, Rhl system, and Las system [41,42]. Therefore, *flrA*, *fleQ* and *flaK* genes associated with the flagellar biosynthesis as well as QS-related genes *pqsA*, *pqsB*, and *pqsC* were selected to reveal the characteristic biological response of biofilm to sub-lethal PC in the molecular regulation network. Different from the results of a previous study, which found the expressions of flagellum biosynthesis related genes being obviously suppressed under PC [13], herein it was found that the expressions of flagellum biosynthesis related genes *flrA*, *fleQ*, and *flaK* were up-regulated (8.4, 9.4, and 9.6 times, respectively) under sub-lethal PC on day 1, which was much higher than those of control groups (Fig. 6b). Simultaneously, the overall expression change trend of QS-related genes (*pqsA*, *pqsB*, and *pqsC*) was basically consistent with the expressions of flagellum and motility-related genes (*flrA*, *fleQ*, and *flaK*) as described above (Fig. 6b). The expression of QS-related gene *pqsA* was up-regulated by 6.6 times in the sub-lethal PC group on the 1st day, which was much higher than control groups (Fig. 6b). This phenomenon further indicates that although sub-lethal PC could inhibit the growth of biofilm by inactivating some of the bacteria, the survived bacteria could promote the biofilm formation by up-regulation the expressions of the flagellum and motility system

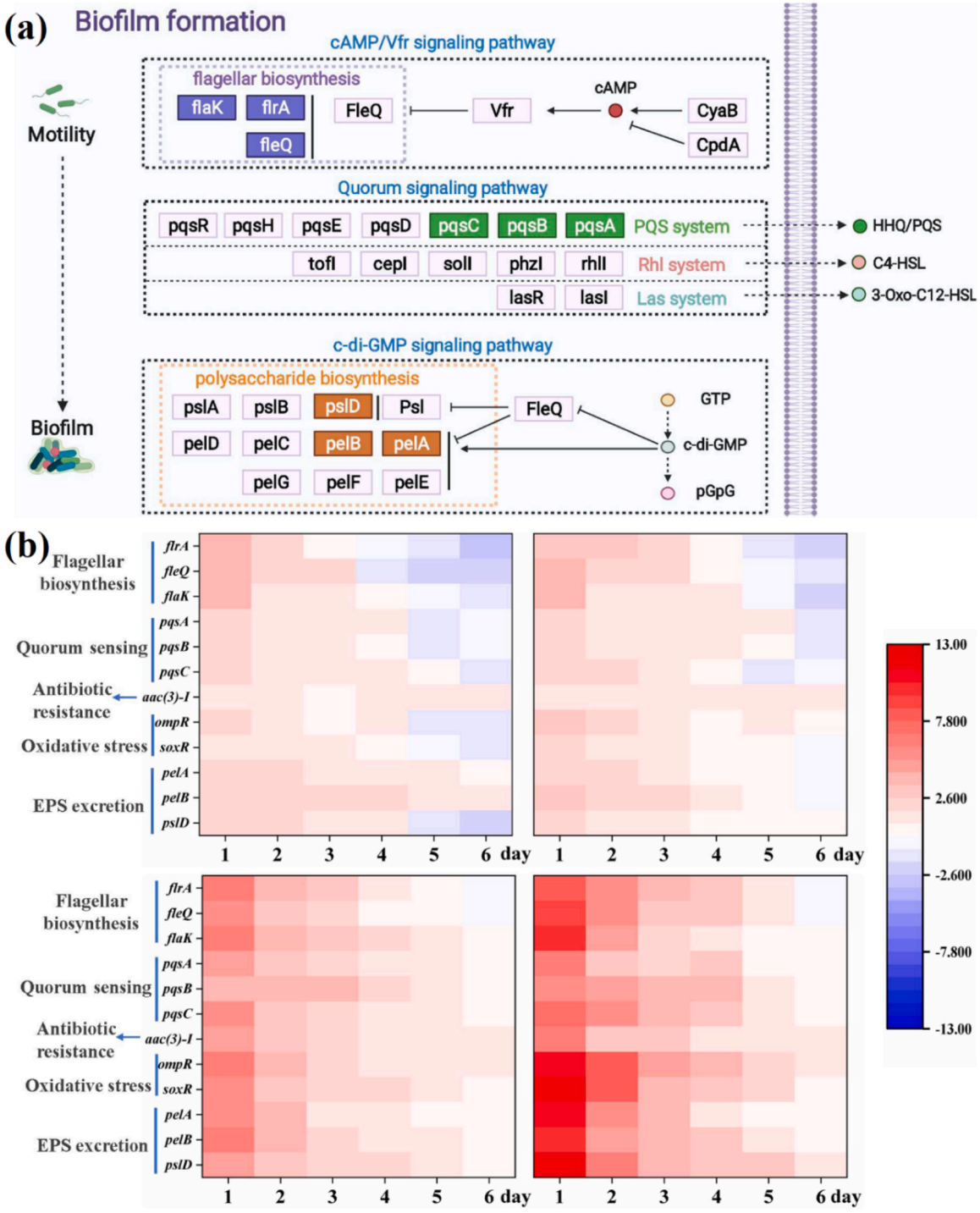

Fig. 6. (a) Signaling pathways of cAMP/Vfr, quorum sensing and c-di-GMP during the formation of *Pseudomonas aeruginosa* biofilm; (b) Gene expression heatmap of target genes involved in biofilm formation, EPS secretion, antibiotic resistance, the VBNC state and oxidative stress in *P. aeruginosa* (GEN) cultured under blank group, TiO_2 attached, UV irradiation, and sub-lethal PC stimulation. X-is: the monitoring time in days; Y-axis left: clusters of target genes and list of genes tested, Y-axis right: the figure legend bar (depicted a blue-red color scale. Red spectrum color indicates up-regulated expression; blue spectrum color indicates down-regulated expression). Results are the mean of independent triplicates.

related genes as well as QS-related genes. Previous research has established that biofilm represents a mode of protection for microorganisms against harsh environments for millions of years [43]. This perspective also further explains the correctness of the conclusion of this study. However, herein, it was found that sub-lethal PC could only promote the formation of biofilm at the early stage of biofilm formation. With prolonging cultured time, the barrier effect and thickness of biofilm increased gradually; however, the expression of biofilm formation-related genes was down-regulated (Figs. S3, 1b and 6 b). These results indicate that the growth of biofilm and sub-lethal PC damage attained a balance state with prolonging culture time, then the biofilm went into a slow growth state. This further revealed that sub-lethal PC could only inhibit rather than prevent the growth of biofilm.

Furthermore, the expression of antibiotic-resistance gene *aac (3)-I* was analyzed (Fig. 6b). Notably, under sub-lethal PC stimulation, an

unexpected result was found. That is, the expression of antibiotic resistance gene *aac (3)-I* was even up-regulated by approximately 6.3 times on the 1st day, which was significantly higher than those of control groups (Fig. 6b). This phenomenon seemed to contradict the conclusion of antibiotic-resistance decline drawn above (Fig. 2). In fact, a recent study has proven that, under the action of sub-lethal PC, antibiotic-resistance genes of bacteria are indeed significantly up-regulated, which corresponds to a series of stress responses of bacteria in response to external stimuli, including the improvement of tolerance [26]. Interestingly, antibiotic resistance decreased when resistance gene was upregulated. This is attributed to the fact that the antibiotic-resistance mechanism of biofilm is very complex, which is related to the following mechanisms: 1) the barrier function of biofilm; 2) changes in the microenvironment of biofilm; 3) changes in phenotypic genes; 4) bacterial density sensing signal system; 5) antibiotic effluent pump system; 6) secretion of antibiotic hydrolase; and 7) immune defense mechanism [29]. After the barrier function of biofilm was destroyed, antibiotic resistance was significantly reduced even in the case of up-regulated gene expression. This result indicates that the physical barrier of biofilm formation, rather than the expression of antibiotic-resistant genes, is one of the important factors leading to the increase in antibiotic resistance.

In order to further confirm the oxidative stress response to the sub-lethal PC on biofilm bacteria, the expressions of oxidative stress related genes including *ompR* and *soxR* in bacteria were carefully examined. As expected, expressions of both of the genes reached the maximum under sub-lethal PC stress, which were much higher than those of control groups (Fig. 6b). These results are consistent with the results of antioxidant enzyme activities (Fig. 3). All these results further demonstrate that sub-lethal PC, compared with other conditions, causes a certain degree of oxidative stress on the biofilm formation, thus further promoting the up-regulation of biofilm-related genes by survival bacteria themselves on the molecular regulatory network. Similar to the biofilm formation related genes, the overall expression change trend of oxidative stress related genes (*ompR* and *soxR*) reached the maximum on the 1st day and then decreased gradually (Fig. 6b). This phenomenon further indicates that, when the biofilm growth and sub-lethal PC damage reached a balance state, the barrier effect of biofilm might counteract some oxidative stress, which is also consistent with the results of antioxidant enzyme activities (Fig. 3) as well as the changes of the barrier effect (Fig. S3).

Furthermore, the EPS secretion response of bacteria in biofilm to sub-lethal PC stress were revealed by examining the expressions of EPS secretion related genes. The *psl*-encoded exopolysaccharide expression and protection in *P. aeruginosa* biofilm was reported in a literature study, thus indicating that *pslD* is a secreted protein required for biofilm formation [44]. Simultaneously, *pel* gene is involved in the formation of the pellicle's extracellular matrix [45]. Therefore, to further explain EPS secretion under the molecular regulatory network, the expressions of *pslD*, *pelA*, and *pelB* genes in biofilm bacteria were also analyzed (Fig. 6b). The overall change trend of the expressions of EPS secretion-related genes (*pslD*, *pelA*, and *pelB*) were basically consistent with that of flagellum and motility-related genes (*flrA*, *fleQ*, and *flaK*) as described above (Fig. 6b). This may be attributed to the fact the expressions of *pslD*, *pelA*, and *pelB* genes are all related to FleQ. Fig. 6a exhibits that the genes *pelA* and *pelB* related to EPS secretion were directly regulated not only by c-di-GMP, but also by FleQ, at the same time the gene *pslD* was just regulated by FleQ. FleQ acts as a repressor in the absence of c-di-GMP and as an activator in the presence of c-di-GMP to influence the expression of *pel* genes. Specifically, the expressions of *pslD*, *pelA*, and *pelB* genes were up-regulated much higher (11.5, 9.9, and 10.6 times, respectively) under the sub-lethal PC on the 1st day, which is also much higher than those under other conditions (Fig. 6b). These results are consistent with the above-mentioned results about the change of EPS content secreted per cell, further indicating that this is a self-protection mechanism of bacteria, which promotes the secretion of EPS to make themselves resist the stress of sub-lethal PC.

Undeniably, a lot more systematic explorations are still demanded to further investigate some other related points. In fact, in the real environment, biofilm mostly exists as mixed with various microorganisms, which may also contain other pathogens, such as viruses, fungi, and spores [5]. Previous studies have found that the photocatalytic inactivation behaviors and mechanisms toward bacteria, viruses, and spores are different [46,47]. Therefore, changes of the mixed biofilm under the action of sub-lethal PC, as well as the responses of viruses and spores in the biofilm, further require in-depth explorations, which will be pursued in the near future.

4. Conclusions

This study reveals a phenomenon that the sub-lethal PC could only inhibit rather than prevent the growth of biofilm and the cause of its occurrence. Based on the results of this study, the following major conclusions can be drawn:

1) Sub-lethal PC stimulation showed an inhibitory effect on biofilm growth through decreased bacterial abundance, decreased thickness of biofilm, damage of the biofilm barrier effect, and decreased antibiotic resistance of biofilm.
2) Bacteria that survived in the biofilm changed their growth state from the VBNC state to the VC state, and produced strong oxidative stress and EPS secretion response to the stress at the initial stage of sub-lethal PC stimulation.
3) The expressions of biofilm growth-related genes, antibiotic resistance related genes, oxidative stress-related genes, and EPS secretion-related genes in the surviving bacteria were up-regulated to different degrees under the sub-lethal PC stimulation.
4) With the increase of EPS secretion, the barrier effect and thickness of biofilm increased gradually, leading to the defense to sub-lethal PC stimulation. Then, expressions of related genes were down-regulated, further leading to the decrease in the oxidative stress response and EPS secretion response.

Finally, the growth of biofilm and sub-lethal PC damage reached a balance state with the prolonging culture time, and finally the biofilm went into a slow growth state. This study reveals a series of stress response mechanisms during the development of antibiotic-resistant biofilm stimulated by sub-lethal PC. Therefore, this information provides an effective strategy for inhibiting the growth of biofilm in the process of the water cycle, which is of certain guiding significance for controlling and eliminating biofilm pollution.

CRediT authorship contribution statement

Min Chen: Methodology, Formal analysis, Writing – original draft. **Yiwei Cai:** Methodology, Formal analysis. **Guiying Li:** Writing – review & editing. **Huijun Zhao:** Supervision. **Taicheng An:** Conceptualization, Supervision.

Declaration of Competing Interest

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

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.apcatb.2022.121200.

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