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H_2O_2 and/or TiO_2 photocatalysis under UV irradiation for the removal of antibiotic resistant bacteria and their antibiotic resistance genes

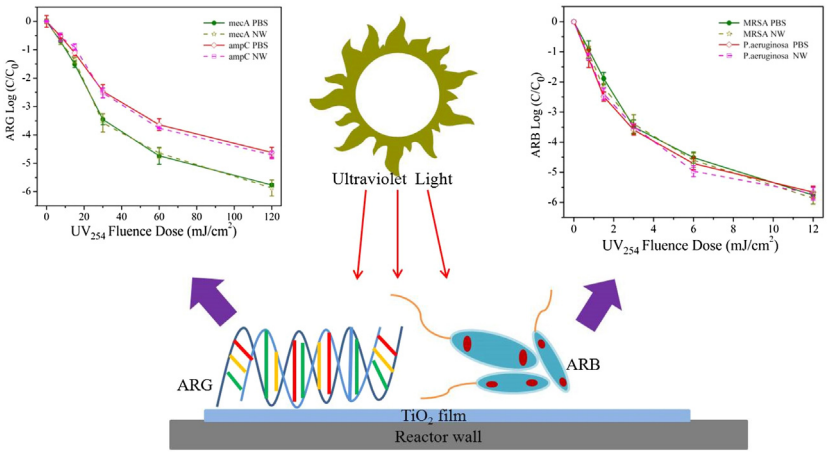
Changsheng Guo[a], Kai Wang[a,b], Song Hou[a], Li Wan[a], Jiapei Lv[a], Yuan Zhang[a], Xiaodong Qu[c], Shuyi Chen[b], Jian Xu[a,*]

[a] State Key Laboratory of Environmental Criteria and Risk Assessment, Chinese Research Academy of Environmental Sciences, Beijing 100012, China
[b] Wuxi SensingNet Industrialization Research Institute, Wuxi 214000, China
[c] State Key Laboratory of Simulation and Regulation of Water Cycle in River Basin, and Department of Water Environment, China Institute of Water Resources and Hydropower Research, Beijing 100038, China

HIGHLIGHTS

- TiO_2 thin film was successfully synthesized for treating ARB and ARGs from water.
- More than 5.5 log units of ARB reduction was achieved by TiO_2 under UV irradiation.
- With TiO_2, ARGs were reduced by more than 5 log units under UV irradiation.
- TiO_2 could remove both intracellular and extracellular forms of ARGs.

GRAPHICAL ABSTRACT



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ABSTRACT

Inactivating antibiotic resistant bacteria (ARB) and removing antibiotic resistance genes (ARGs) are very important to prevent their spread into the environment. Previous efforts have been taken to eliminate ARB and ARGs from aqueous solution and sludges, however, few satisfying results have been obtained. This study investigated whether photocatalysis by TiO_2 was able to reduce the two ARGs, *mec*A and *amp*C, within the host ARB, methicillin-resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa*, respectively. The addition of H_2O_2 and matrix effect on the removal of ARB and ARGs were also studied. TiO_2 thin films showed great effect on both ARB inactivation and ARGs removal. Approximately 4.5-5.0 and 5.5–5.8 log ARB reductions were achieved by TiO_2 under 6 and 12 mJ/cm^2 UV_{254} fluence dose, respectively. For ARGs, 5.8 log *mec*A reduction and 4.7 log *amp*C reduction were achieved under 120 mJ/cm^2 UV_{254} fluence dose in the presence of TiO_2. Increasing dosage of H_2O_2 enhanced the removal efficiencies of ARB and ARGs. The results also demonstrated that photocatalysis by TiO_2 was capable of removing both intracellular and extracellular forms of ARGs. This study provided a potential alternative method for the removal of ARB and ARGs from aqueous solution.

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* Corresponding author.
E-mail address: xujian@craes.org.cn (J. Xu).

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1. Introduction

In the past several decades, antibiotics have been extensively used for humans, veterinary and agricultural purposes resulting in their continuous release into the environment [1–3]. The selective pressure exerted by the released antibiotics increases the incidence of antibiotic resistance strains which lead to a widespread distribution of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) in different environment matrices, such as water, sludge, glacier and so on [4–7]. The development of antibiotic resistance reduces the therapeutic potential against human and animal pathogens and has become a growing human health challenge [8]. ARGs are mobile genetic elements and can adapt rapidly to new hosts. ARGs can also pass between microorganisms via horizontal gene transfer (HGT), even from dead to living cell [9]. However, the acquisition of ARGs is only one important factor in the increasing incidence of ARB. A significant feature of ARGs is their stability. ARGs can persist in the environment even after the selective pressure has been removed [10]. The decreased use of an antibiotic is usually accompanied by some decrease in the incidence of ARB, but seldom falls to zero [11,12]. This leaves a great potential that ARB can rebound rapidly and become the predominant strains if antibiotic use is resumed [13]. Given that ARGs are persistent, the best way to stop resistance is to remove ARGs in the original place and prevent their acquisition.

Recently, the treatment technologies to reduce concentrations of ARGs from matrices such as water, wastewater, lagoon water, and sludges have been investigated. Pei et al. studied the effects of different biological treatment regimes on the levels of various classes of ARGs in dairy lagoon water, and found it was impossible to reduce any ARG below initial levels in any treatments [14]. Chen et al. demonstrated that constructed wetland removed ARG relative abundance by 1–3 orders of magnitude, biological aerated filter reduced ARGs by 0.6–1.2 log units, and UV disinfection had no effect on ARGs removal [10]. Ferro et al. reported that bla_{TEM} gene increased up to 3.7×10^3 copies mL^{-1} in total DNA after 240 min UV/H_2O_2 treatment and no difference was found for *qnr*S gene between the initial (5.1×10^4 copies mL^{-1}) and the final sample (4.3×10^4 copies mL^{-1}) [15]. In another study by Ferro et al., total inactivation of antibiotic resistant *E. coli* strain, target ARG (bla_{TEM}) was still present in water suspension after 300 min UV/H_2O_2 treatment [16]. These results were consistent with the investigation of UV disinfection of ARB and ARGs in water and wastewater, that UV irradiation was limited in its potential to damage ARGs [17]. Although lots of work has been conducted to prevent the ARGs spread in the environment in the past several years, there are still great gaps to fill in [18].

Photocatalysis is one of the most advanced and mature technologies to eliminate contaminants from environment. Since the pioneering work of sterilization of microbial cells by semiconductor powders conducted in 1985 [19], inactivation of bacteria by photocatalysis with different catalysts has been extensively investigated [20,21]. Due to its strong oxidizing power and long-term photochemical corrosive resistance, titanium oxide (TiO_2), especially its anatase phase, is a well-known efficient semiconductor for its extensive application in degradation of different pollutants in both gaseous and liquid phases [22], and the biocide capability of anatase has also been the subject of numerous studies [23]. The reactive oxygen species (ROS) such as hydroxyl radical ($OH^•$) generated by the irradiation on TiO_2 would attack vital organic components in microorganisms, such as cell membranes, RNA, DNA, proteins, and lipids, resulting in the death of the microorganisms [24]. So far, all these studies were focused on the inactivation/removal of microorganisms. For instance, the effect of TiO_2 photocatalysis on the inactivation of an antibiotic resistant *Escherichia coli* strain selected from an urban wastewater treatment plant effluent was investigated [25,26]. The conventional disinfection processes, UV radiation and chlorination were not effective in the inactivation of ARB [18]. ARB were found less sensitive to the oxidative stressors involved in photocatalytic disinfection treatment than the antibiotic sensitive strain [27]. Recent studies on the effect of advanced treatments on ARB inactivation were carried out by Rizzo et al. [28]. To our knowledge, the effect of photocatalysis on ARGs damaging remained unknown.

The aim of this study was to examine the potential of photocatalysis by TiO_2 as an alternative treatment technology for ARB and ARGs in water under laboratory conditions. The addition of H_2O_2 and matrix effect on the removal of ARB and ARGs were also investigated. Two representative ARB of concern, methicillin-resistant *Staphylococcus aureus* (MRSA, Gram-positive) and multiantibiotic-resistant *Pseudomonas aeruginosa* (Gram-negative), and two ARGs (mecA and ampC) hosted in the two bacteria respectively, were selected for photocatalytic experiments. Given that ARGs present as either intra-DNA or extra-DNA may possess significant differences in their mobility and availability to indigenous bacteria, the intracellular versus extracellular ARGs were investigated as well [29]. The matrix effect of PBS (phosphate-buffered saline) and NW (nature water) was also considered. TiO_2 powders are easy to agglomerate, difficult to be separated and recovered. For that, TiO_2 thin films loaded on quartz plates were synthesized and used in this study. Real-time quantitative polymerase chain reaction (qPCR) assays were used for DNA quantification.

2. Materials and methods

2.1. Synthesis and characterization of TiO_2 thin film

2.1.1. Preparation of quartz plates

The quartz plates (8.0 cm × 2.0 cm × 0.12 cm, CSG Holding CO., Ltd, China) were washed with tap water, ultrapure water and then immersed in ethanol for 15 min. The obtained quartz plates were dried in an oven at 60 °C.

2.1.2. Preparation of sol-gel

2.1.2.1. Solution A. 10 mL of HAc were mixed with 75 mL of ethanol. Then 25 mL of tetrabutyltitanate were dropwise added into the above solution. To avoid the precipitants, a few drops of HNO_3 were added at the same time. After 15 min, 12.5 mL of 0.12 mol/L HF was dropped into the resulting solution. In this process, the mixture was consistently stirred magnetically.

2.1.2.2. Solution B. 12.5 mL of 0.12 mol/L ammonium hydroxide ($NH_3 \cdot H_2O$) was dissolved in 25 mL of ethanol. The pH value of the mixture was adjusted to 2 with HNO_3. In this process, the mixture was stirred magnetically all the time.

The solution B was dropped into solution A under vigorously magnetic stirring. The solution was stirred for one hour until the gels formed. The gels were then transferred into a brown wide-mouth beaker and aged for 24 h.

2.1.3. Preparation of TiO_2 thin film

TiO_2 thin film was prepared on the quartz plates by dip coating method with some modification [30]. The prepared quartz plates were gradually inserted into the aged gel at a constant speed of 15 cm/min. After being completely immersed into the gel, the plates were kept for 30 s and pulled out gradually at the same speed. The copped quartz plates were dried in an oven at 100 °C and calcined in a muffle at 450 °C for 30 min.

2.1.4. TiO_2 thin film characterization

The phase identification of synthesized products was conducted by an X-ray diffraction (XRD) Rigaku D/Max-2500 diffractometer

Table 1
Primers used in this study.

ARB	ARG	primer	sequence	T_A,°C	reference
MRSA	*mec*A	FP	5′CGCAACGTTCAATTTAATTTTGTTAA	51.7	[46]
		RP	5′CCACTTCATATCTTGTAACG		[47]
P. aeruginosa	*amp*C	FP	5′CGGCTCGGTGAGCAAGACCTTC	70.9	[48]
		RP	5′GAAGCGCTCATGGCACCATCATAGCC		[49]

T_A = annealing temperature, FP = forward primer, RP = reverse primer.

using Cu Kα radiation (λ = 1.54178 Å). Surface element compositions were analyzed by a Kratos Axis Ultra DLD multi-technique X-rayphotoelectron spectroscopy (XPS) with a monochromated Al Kα X-ray source. The morphology and size of synthesized TiO_2 thin film were examined by field emission scanning electron microscope (FESEM, FEI nanosem 430). UV–vis diffuse reflectance spectra (DRS) was performed with a UV–vis spectrophotometer using $BaSO_4$ as the reflectance sample within the range of 200–800 nm (Shimadzu, UV-3600) at room temperature.

2.2. Preparation of bacteria

Bacteria were purchased from American Type Culture Collection(ATCC), with detailed information described by McKinney and Pruden [17]. Bacteria were recovered following the KWIK-STIK™ User Instruction manual in sterile lab. 3 mL of mid-exponential phase bacteria suspension or appropriate positive plaque on solid culture medium (5 g/L yeast extract, 10 g/L NaCl, 10 g/L tryptone, 15 g/L agar, pH = 7.0) was taken into fresh growth medium for culturing another generation. After two generations incubation, the mid-exponential phase bacteria were stocked in Luria-Bertani broth (LB) with 30% glycerinum and stored under −80 °C for the next operation. The preparation of bacteria followed the procedures as the literature [17]. In brief, 1.5 mL of frozen stock bacteria (−80 °C) were spiked into 100 mL of brain heart infusion broth (MRSA) or LB broth (*P. aeruginosa*) and cultured at 37 °C in an incubator shaker to approximately mid-exponential phase. The bacteria were washed by centrifuging the bacteria at 5000 × *g* for 10 min, discarding the broth, rinsing two times, and resuspending in 30 mL of phosphate-buffered saline (PBS) solution (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.76 nM KH_2PO_4, pH = 7.4) or sampled nature water (NW, sampled from drinking water source in Beijing, pH = 7.2, turbidity = 1.8 NTU) to an absorbance of 1 (λ = 600 nm) in accordance with specific conditions.

2.3. DNA extraction

The DNA (i.e., extracelluar DNA, intracelluar DNA and total DNA) was extracted from UV/H_2O_2 or UV/TiO_2/H_2O_2 treated water samples. Total DNA (intra- and extra- ARGs) was directly extracted using an E.Z.N.A.™ Bacterial DNA Kit (Omega Bio-Tek, GA, USA) following the manufacturer's protocol. The extraction of extracelluar DNA was performed as follows. The subsample (2 mL bacterial fluid) and 0.2 g of polyvinyl polypyrrolidone was added to 4 mL of NaH_2PO_4 (0.12 M, pH 8.0). The mixture was shaken at 250 rpm at 25 °C for 10 min, followed by centrifugation (10000 × *g*, 4 °C) for 10 min. The supernatant was filtered through a sterile membrane (0.22 μm pore size, PVDF, Osmonics, US) and stored on ice. Another 4 mL of NaH_2PO_4 was added to the pellets, which was subjected to the same extraction cycle three times. Supernatants were combined and centrifuged for 20 min at 10000 × *g* at 4 °C. All supernatant fractions were collected, combined, and stored on ice until subsequent extracelluar DNA extraction using the Water DNA kit. The extraction of intracelluar DNA was performed as follows. The bacterial fluid (2 mL) was filtered through a 0.22 μm membrane (PVDF, Osmonics, US) and stored on ice. The filtrates were then centrifuged (10000 × *g*, 4 °C) for 10 min. The remaining cell pellets were collected from each filtration and centrifugation step, and combined for intracellular DNA extraction with the Water DNA kit. The concentration of the extracted DNA was determined by spectrophotometer analysis (NanoDrop ND-1000, NanoDrop Technologies, Willmington) and agarose gel electrophoresis.

2.4. Real-time quantitative PCR (qPCR)

All ARGs were analyzed using a 7500 Real-Time PCR Detection System (Bio-Rad Company, U.S.). The purified PCR products were ligated into p-GEM T easy vector (Promega, Madison) and then cloned into *Escherichia coli* JM109 (TaKaRa). Plasmids that had the target genes were isolated and sequenced. BLAST searches of the Genbank database confirmed that all of the sequenced clones were matching with known genes. The concentrations of plasmid carrying target genes were determined by NanoDrop 10-fold serial dilutions. The dilutions with a known copy number of the plasmid DNA were used to produce the standard curve. Based on the curves, the copy number of the target genes was calculated.

qPCR reaction was performed in a 20 μL volume mixture and conducted in 96 well plates containing 10 μL of SYBR Green Premix Ex Taq (TakaRa), 6.4 μL of sterile distilled water (dH_2O), 0.2 μM of each primer and 2 μL of template DNA. The detailed protocol was as follows: 98 °C for 2 min, followed by 35 cycles of 98 °C for 5 s, annealing at different temperatures (T_A, Table 1) for 10 s and 72 °C for 15 s followed by a melt curve for specificity verification. Each reaction was run in triplicates. The PCR efficiencies ranged from 90% to 110% with R^2 values greater than 0.992 for all calibration curves. Data analysis was carried out with ICycler software. The specificity of qPCR products was determined by melt curves and agarose electrophoresis.

2.5. Experimental design

MRSA and *P. aeruginosa* were cultured on solid culture medium (5 g/L yeast extract, 10 g/L NaCl, 10 g/L tryptone, 15 g/L agar, pH = 7.0) by using a spread-plate technique and incubated at 37 °C for 48 h. After the incubation period, bacterial colonies were counted and the results were calculated as Colony-Forming Units (CFU) or plaque forming unit (PFU)/mL.

An aliquot of 50 mL bacteria solution was added into sterile test tubes and a certain amount of H_2O_2 was added according to experimental conditions. All experiments were conducted in a photochemical reactor (XPA-7, Nanjing Xujiang Machinery Factory, Nanjing, China) equipped with 300/800 W Hg lamps (Institute of Electric Light Source, Beijing, China) to generate ultraviolet light. The photochemical reactor was equipped with thermostatic bath system and the temperature was maintained at 25 °C during the experiments. Two mercury lamps (300W and 800W) in this study could provide two light intensities, which was used to investigate the impact of different UV irradiation. The emission spectrum of the lamp and the schematic diagram of the reaction system were the same as that in our previous study [31]. UV disinfection is of particular interest because UV radiation is directly absorbed by DNA

and thus has the potential to impart ARG damage. In the laboratory scale, benchtop UV254 disinfection experiments were performed using a collimated beam apparatus, as described by the previous study [17]. That UV254 was employed in this study was also related to the realistic practice of UV254 disinfection in the effluent disinfection application. UV dose was defined as the product of the average intensity (mW/cm^2) and the average time of the bacteria to be treated (s). UV intensity was measured using a spectroradiometer. UV dose was calculated according to the literature [32]. The TiO_2 catalytic thin film was immersed in the prepared bacteria solutions. Control experiments were simultaneously performed. The solutions were magnetically stirred consistently during the reaction to prevent the sedimentation of bacteria. Sample solutions were taken at given time intervals.

2.6. Statistical analysis

Statistical analysis was performed using Excel and SPSS 16.0. The correlation analysis was used to calculate the Pearson's bivariate correlation and *p*-values. One-way analysis of variance (ANOVA) was used to assess the homogeneity of variance with a significance level of 5% ($p < 0.05$). The efficiencies of treatments were assessed by determining ARB or ARGs reductions ($\log_{10}$ reduction, average and standard deviation) before and after treatment.

3. Results and discussion

3.1. Characterization of TiO_2 thin film

The picture of synthesized TiO_2 thin film was shown in Supplementary materials Fig. S1. Fig. 1 showed the phase and crystal structure of the synthesized samples measured by XRD. The peak with $2\theta = 25.25$ (101) in the spectrum was identified as the crystal of anatase TiO_2, which had proved to be the most efficient photocatalyst compared to the other crystal forms such as rutile [33]. All diffraction peaks could be indexed to the tetragonal phase of TiO_2 (JCPDS card No. 65-5714, a = b = 3.785 Å and c = 9.514 Å). No impurity was detected in the TiO_2 heterostructure, indicating the high purity of the sample.

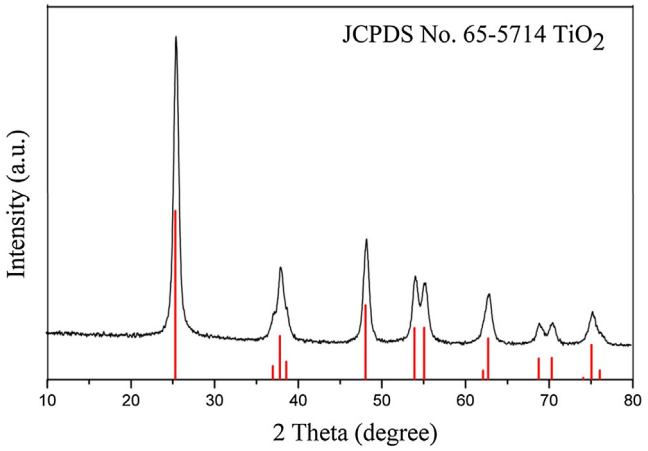


Fig. 1. XRD patterns of TiO_2 thin film calcined at 450 °C.

X-ray Photoelectron Spectra (XPS) was a highly sensitive and effective technique for investigating the surface composition and chemical states of all kinds of solid samples [34]. Fig. 2 showed the XPS results of Ti2p (a), O1s (b), F1s (c), and survey spectrum

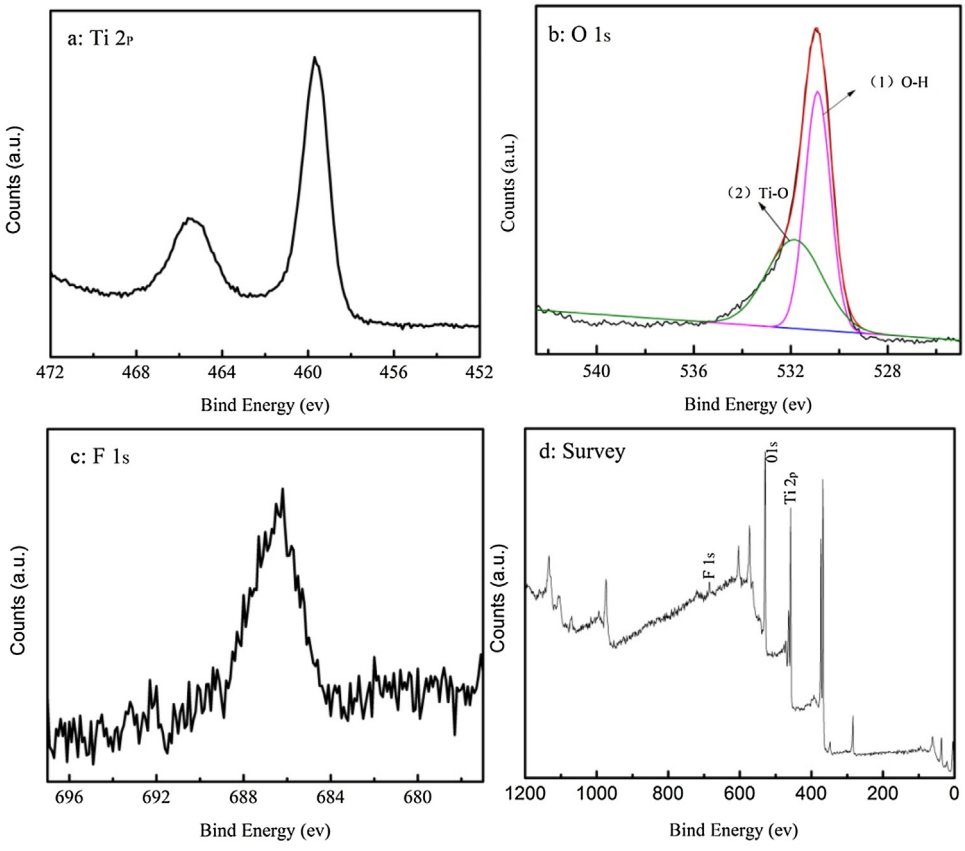


Fig. 2. XPS spectra of TiO_2 thin film calcined at 450 °C. (a) Ti 2p, (b) O 1s, (c) F 1 s and (d) survey spectrum.

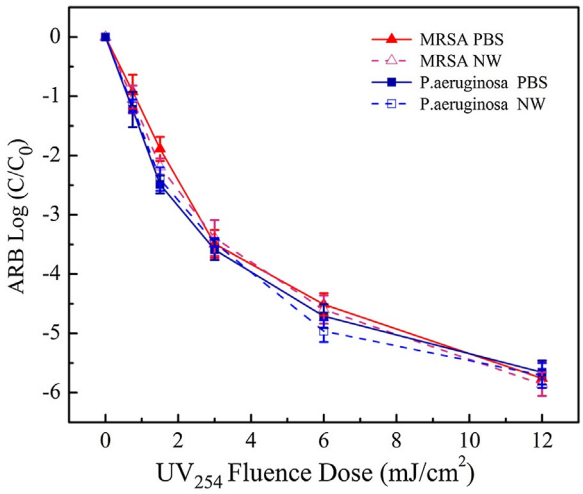


Fig. 3. Effect of TiO_2 thin film on ARB inactivation under UV irradiation (λ = 254 nm).

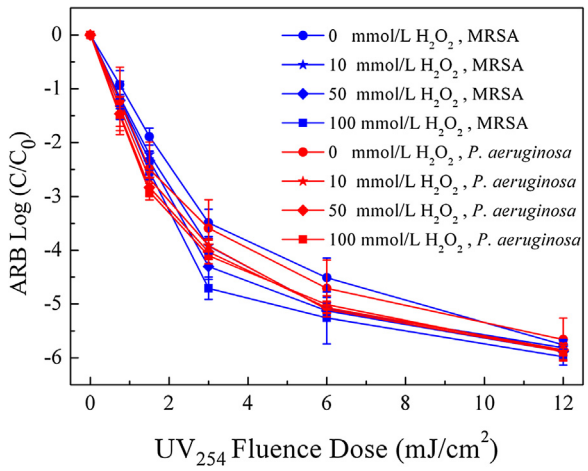


Fig. 4. Effect of TiO_2 thin film on removal of ARB under UV irradiation (λ = 254 nm) and different concentrations of H_2O_2.

(d) on the surface of TiO_2 thin film calcined at 450 °C. The typical survey XPS spectrum of TiO_2 (Fig. 2d) indicated the existence of Ti, O and F elements. Ti2p peak in Fig. 2a was sharp and strong demonstrating that the Ti element mainly existed as the chemical state of Ti^{4+} according to the principle method and handbook of the XPS instrument[35,36]. In the same way, F element mainly existed as the chemical state of F^-. The O1s peak of the XPS spectrum shown in Fig. 2b was asymmetric (the left side was wider than the right) indicating that at least two kinds of oxygen species would be present in the near surface region by resolving XPS curve. The peak(1) at about 530 eV was due to the oxygen in the TiO_2 crystal lattice, and the peak(2) at about 532 eV was due to the surface hydroxyl group which played an important role in the photocatalytic reactions [37,38]. Surface hydroxyl group can be attacked by the photo-induced holes and yield surface-bound OH radical with high oxidation capability [39]. Other information including UV–vis and SEM images of TiO_2 thin films were described in detail in Supplementary materials.

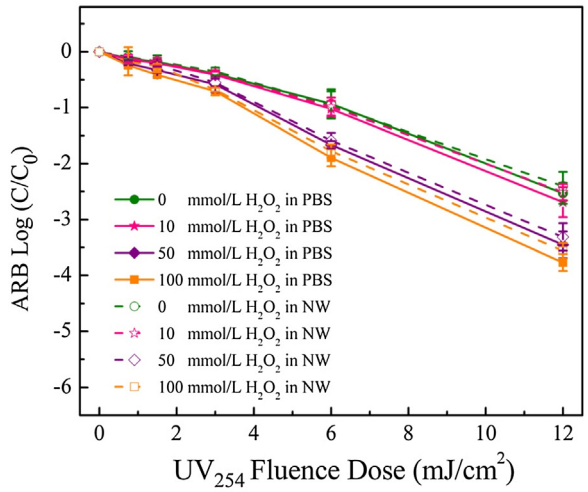


Fig. 5. MRSA inactivation under UV irradiation (λ = 254 nm) and H_2O_2 addition.

3.2. Inactivation of ARB

Preliminary experiments showed that less than 0.2 log ARB reduction was observed with TiO_2 thin film in darkness (Fig. S4), implying that the loss of ARB by TiO_2 adsorption was negligible. Fig. 3 showed that approximately 4.5–5.0 log MRSA reduction and 5.5–5.8 log *P. aeruginosa* reduction were achieved by using TiO_2 thin film under 6 and 12 mJ/cm^2 UV_{254} fluence dose, respectively. Previous study reported that under the same UV fluence dose irradiation without the photocatalyst, only 1.4 log MRSA reduction and 4.0 log *P. aeruginosa* reduction were achieved, and MRSA was more resistant to UV disinfection than *P. aeruginosa* [17]. Result from this study demonstrated that the combined TiO_2/UV treatment was able to significantly enhance the inactivation efficiency against ARB. The elevated bacterial inactivation by TiO_2 thin film was probably attributed to the generation of ROS by the irradiation of UV [40]. It was well known that ROS such as hydroxyl radicals (•OH) and superoxide anions ($^{\bullet}O_2^-$) could be produced in UV/TiO_2 system. These radical species on or near the TiO_2 surface may cause oxidative damage to cell membranes, resulting in the microorganisms inactivation [23,40–42]. The present study also demonstrated that both ARB were effectively inactivated by TiO_2/UV treatment. A pooled comparison of both ARB within each aqueous matrix indicated that no significant difference ($0.13 < p$) was observed between PBS versus NW on the ARB inactivation.

MRSA and *P. aeruginosa* inactivation efficiencies have been shown in Fig. 4. Increasing H_2O_2 concentration increased the inactivation efficiency of the two bacteria. For ARB, the damage mechanism at low UV fluence dose (below 2.4 mJ/cm^2) was caused by both chemical oxidation (by H_2O_2) and catalytic oxidation (by TiO_2). High UV fluence dose (greater than 2.4 mJ/cm^2) would simultaneously accelerate the depletion of H_2O_2, which weakened the chemical oxidation effect. The behavior of *P. aeruginosa* was different from MRSA. The turning point occurred at 3 mJ/cm^2 and 4 mJ/cm^2 UV_{254} influence dose for the inactivation line of *P. aeruginosa* and MRSA, respectively, which likely due to their different cell wall structures. The thicker peptidoglycan layer of Gram-positive organisms provided bacteria with a protective outer cell membrane against environmental stressors [17], making it more difficult to inactivate Gram-positive MRSA than Gram-negative *P. aeruginosa*. This trend that Gram-positive bacteria were more resistant than Gram-negative bacteria under different environmental stressors was not universal. Li et al. reported *P. putida* (Gram-negative) was more resistant than *B. subtilis* (Gram-positive) to $AgNO_3$, Ag-nanoparticle, and Ag-TiO_2 bactericidal activity, and they ascribed their tolerance to various nanoparticles to the lipopolysaccharide layer in Gram-negative bacteria [40].

The MRSA and *P.aeruginosa* inactivation by combined H_2O_2/UV and H_2O_2 treatments were shown in Figs. 5–8. Approximately 2.5–3.7 log MRSA reduction and 2.5–3.6 log *P. aeruginosa*

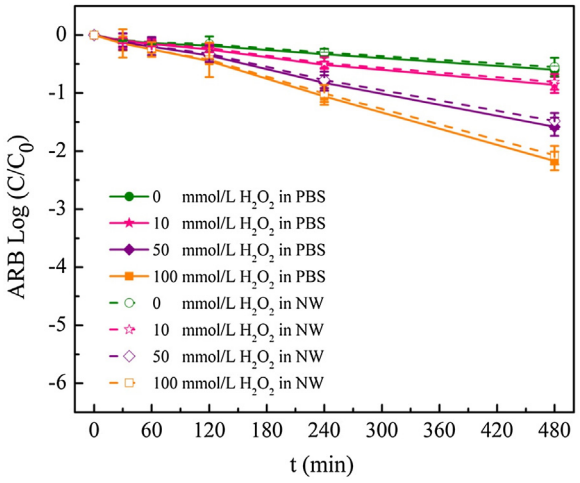


Fig. 6. MRSA inactivation by H_2O_2 treatment in darkness.

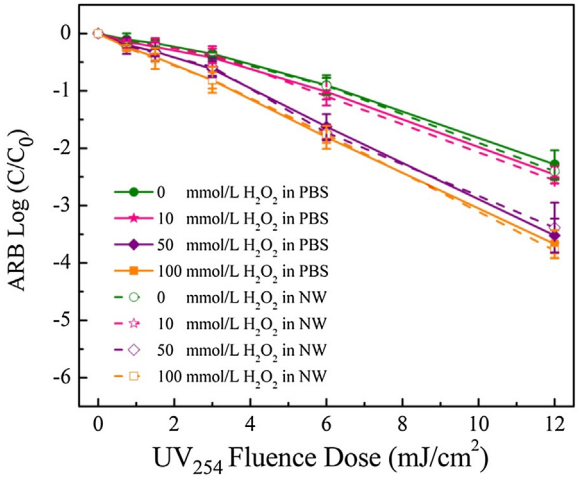


Fig. 7. *P. aeruginosa* inactivation under UV irradiation (λ = 254 nm) and H_2O_2 addition.

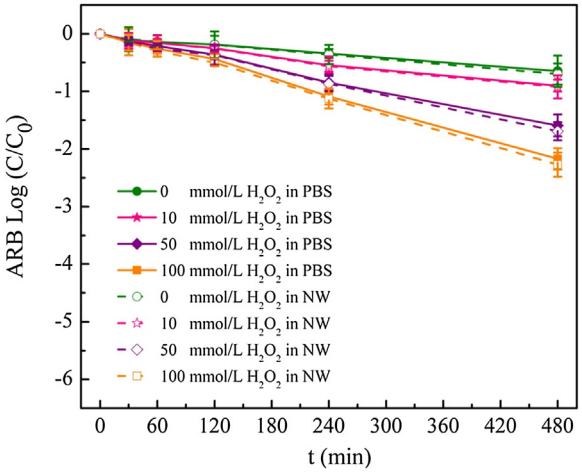


Fig. 8. *P. aeruginosa* inactivation by H_2O_2 treatment in darkness.

reduction were achieved by H_2O_2/UV treatment under 12 mJ/cm^2 UV_{254}fluence dose, and 0.5–2.3 log MRSA reduction and 0.7–2.2 log *P. aeruginosa* reduction by H_2O_2 treatment with 480 min contact time in darkness. Previous studies reported that UV irradiation and chlorination may not effectively control the spread of

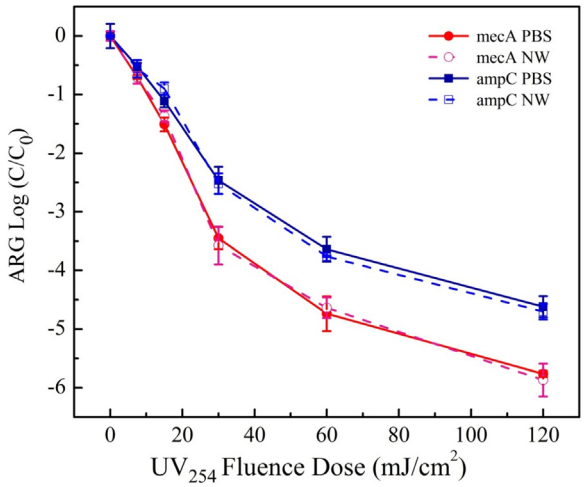


Fig. 9. Effect of TiO_2 thin film on ARGs removal under UV irradiation (λ = 254 nm) in different matrices. Error bars are standard error of the mean. mecA = methicillin resistance gene. ampC = ampicillin resistance genes.

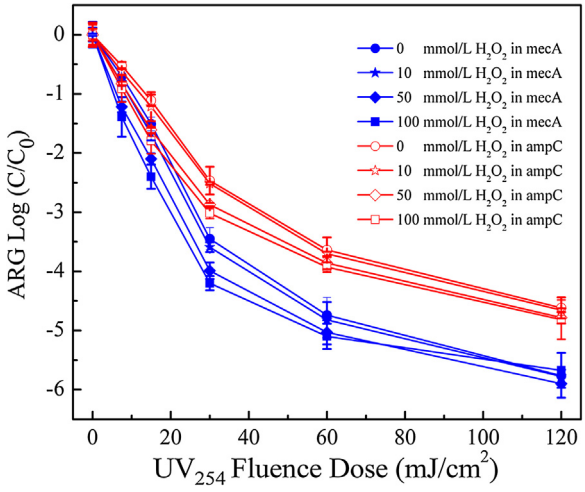


Fig. 10. Effect of TiO_2 thin film on removal of ARGs under UV irradiation (λ = 254 nm) and different concentration of H_2O_2.

Escherichia coli strain [28]. Solar-driven Fenton oxidation, however, could work on the reduction of antibiotic resistance of enterococci towards sulfamethoxazole and clarithromycin by 5-log units in real wastewater effluent [43]. Results from this study suggested that H_2O_2/UV and H_2O_2 treatments were not effective in the inactivation of both MRSA and *P. aeruginosa*.

3.3. Removal of ARGs

In darkness, less than 0.12 log ARGs reduction was observed in the presence of TiO_2 (Fig. S5), implying the negligible adsorption of ARGs by TiO_2 thin film in this study. Fig. 9 showed that 5.8 log *mec*A reduction and 4.7 log *amp*C reduction were achieved by TiO_2 thin film under 120 mJ/cm^2 UV_{254} fluence dose, suggesting that the combined TiO_2/UV treatment was efficient against ARGs as well. No significant difference ($0.11 < p$) of PBS versus NW on the removal of all ARGs in each aqueous matrix was found. Also, no matrix effect was observed for the damage to ARGs by H_2O_2, H_2O_2/UV, and H_2O_2/UV/TiO_2. For that, following experiments of different treatments on the removal of the total ARGs and extracellular and intracellular ARGs were not conducted in the NW matrix.

Fig. 10 showed that UV irradiation and UV/TiO_2 thin film performed a positive effect on the ARGs removal. In comparison, only

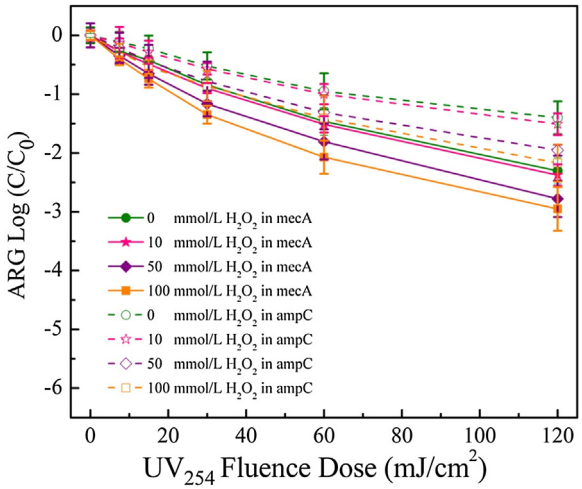


Fig. 11. ARGs removal under UV irradiation (λ = 254 nm) and H_2O_2 addition.

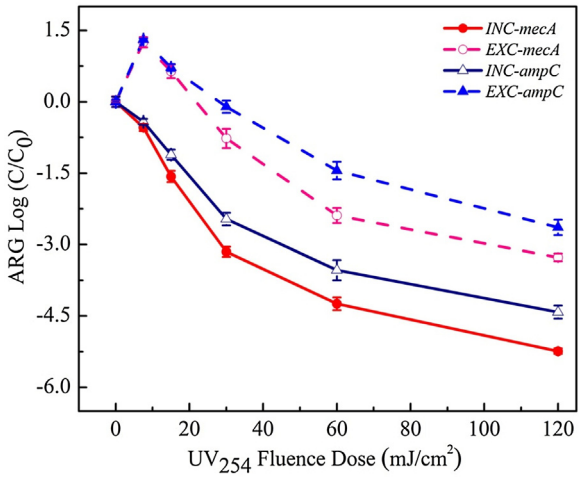


Fig. 12. Effect of TiO_2 thin film on ARGs removal under UV irradiation (λ = 254 nm) in PBS. Error bars are standard error of themean.INC = intracellular. EXC = extracellular.

approximately 0.90 log *amp*C reductions and 0.85 log *mec*A reductions were achieved by 100 mmol/L H_2O_2 within 480 min contact time in darkness (Fig. S6). UV irradiation improved the removal efficiency of *mec*A and *amp*C by 1.3 and 2.2 log units, respectively (Fig. 11). Approximately 2.3–2.9 log *amp*C reduction and 1.4–2.7 log *mec*A reduction were achieved with different concentrations of H_2O_2 under 120 mJ/cm^2 UV_{254} fluence dose (Fig. 11). With the addition of TiO_2 thin film, the removal of mecA and ampC was significantly improved by 2.7–3.4 and 2.7–3.2 log units, respectively (Fig. 10). Ferro et al. [15] evaluated UV/H_2O_2 disinfection on real wastewater samples for removal of target ARGs (namely, bla_{TEM}, *qnrS* and *tetW*). In that case, experiments were carried out in a 2.2 L cylindrical glass reactor filled with 500 mL wastewater sample. A wide spectrum 250 W lamp equipped with a UV filter (main radiation emission in the range 320–450 nm) fixed at 40 cm from the upper water level in the reactor was used as UV source. Initial dose of hydrogen peroxide was set at 20 mg/L. UV/H_2O_2 process did not affect the copies number of bla_{TEM} gene and poorly ($p > 0.05$) affected the copies number of *qnrS* gene (4.3×10^4 copies mL^{-1} after 240 min treatment), which was attributed to the low oxidant dose (20 mg/L) and the mild action of hydroxyl radicals on DNA [15]. Similar study also showed that no removal was reached for bla_{TEM} gene in UV/H_2O_2 process after 240 min treatment, and the presence of *qnrS* and *tetW* genes was not unequivocally proven [16]. Although the matrix was different from the matrix in this study, in the presence of TiO_2 thin film, the removal of *mec*A and *amp*C was significantly improved by 2.7–3.4 and 2.7–3.2 log units, respectively. Fig. 10 showed that a total of 5.7–5.9 log *mec*A reduction and 4.6–4.8 log *amp*C reduction were achieved by UV/TiO_2/H_2O_2 treatment. This significant reduction was probably due to the produced hydroxyl radicals during UV/TiO_2 or UV/TiO_2/H_2O_2 process.

It was found that *amp*C was more resistant to UV/TiO_2 or UV irradiation treatments than *mec*A, as shown in Fig. 10, which was in contrast to their host bacteria's response to the same treatments. Previous study revealed that the genome size was negatively correlated with the ARB inactivation, suggesting that larger genomes were more susceptible to the UV damage [17]. *P. aeruginosa*, the host bacterium of *amp*C, had an extremely large genome, however, *P. aeruginosa* was observed to be adept at photo reactivation [44]. This may explain the relative persistence of *amp*C genes present within *P. aeruginosa* in this study, even by TiO_2 photocatalysis.

Comparison between Figs. 4 and 10 showed that the influence of different treatments was greater on ARB inactivation than on ARGs damage, suggesting that more energy was needed to remove ARGs than inactivate ARB. It was reported that damage to ARGs required UV doses at least 1 order of magnitude higher than that for inactivation of host bacterial cells [17]. The oxidants first attacked microbial cell walls, membranes and enzymatic or transport system, and then attacked the inner organisms like RNA and DNA [42]. Although the bacteria had the self-defensive mechanism to protect them from ROS destruction, excessive ROS might produce oxidative stress and attack membrane lipids, ultimately leading to membrane or DNA damage [40]. The high removal efficiencies of *mec*A and *amp*C from PBS by TiO_2 thin film and H_2O_2 under UV irradiation were likely resulted from multiple mechanisms. UV irradiation might attack the nucleic acids to format pyrimidine dimer [45]. H_2O_2 and TiO_2 could produce hydroxyl radicals (OH·) which were considered as the most reactive oxidizing agents to damage ARGs [24]. Further studies were needed to investigate the detail mechanisms for ARGs removal in the TiO_2/UV/H_2O_2 treatment system.

3.4. Intracellular versus extracellular ARGs

The effect of TiO_2 photocatalysis with 120 mJ/cm^2 UV_{254} fluence dose on intracellular versus extracellular ARGs was presented in Fig. 12. A reduction of 5.2 log intracellular *mec*A, 3.3 log extracellular *mec*A, 4.4 log intracellular *amp*C and 2.6 log extracellular *amp*C were achieved. McKinney and Pruden reported that 120 mJ/cm^2 UV_{254} fluence dose irradiation caused approximately 2.9 log intracellular and extracellular *mec*A reduction, 1.6 log intracellular *amp*C and 1.4 log extracellular *amp*C reduction, respectively [17]. They also found intracellular *amp*C was more recalcitrant than its extracellular form, even detectable after 1000 mJ/cm^2 UV irradiation [17]. Because no synergistic or antagonistic effect between UV irradiation and TiO_2 thin film was observed, the significant improvement of ARGs removal was owed to the utilization of TiO_2. Fig. 12 showed that the intracellular forms of *mec*A and *amp*C were gradually decreasing, however, the extracellular *mec*A and *amp*C increased first and then decreased. At the beginning of the photocatalytic experiment, only few ARGs were extracellular. The chemical and catalytic oxidation by H_2O_2/UV/TiO_2 damaged the cell walls and induced the increasing level of extracellular ARGs, which followed the removal process in the reaction system.

4. Conclusions

As a kind of highly efficient photocatalyst, TiO_2 plays an important role in degrading recalcitrant organic pollutants. However, the characteristic of tiny size of TiO_2 powders has restricted its wide application in the practical use because they are difficult to be

separated and recovered in aqueous solution. In this study the loaded form of TiO_2 on the surface of quartz plates (TiO_2 thin film) was successfully synthesized to solve this problem, and the material was anatase TiO_2 which was the form of TiO_2 with the highest catalytic activity. The role of TiO_2 on the removal of ARB and ARGs was significant, with 4.5–5.8 log units of ARB and 4.7–5.8 log units of ARGs reductions under the UV/TiO_2 photocatalysis process. This study also indicated that although intracellular ARGs were more difficult to be damaged than extracellular ARGs, TiO_2 was able to efficiently remove both intracellular and extracellular ARGs. Results from this study suggested that TiO_2 photocatalysis was capable of eliminating both ARB and ARGs in water at the laboratory scale. To apply this method to the real waste water treatment process, more research is required to confirm its effectiveness at the field scale.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhazmat.2016.10.041.

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