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Dark repair of sunlight-inactivated tetracycline-resistant bacteria: Mechanisms and important role of bacteria in viable but non-culturable state

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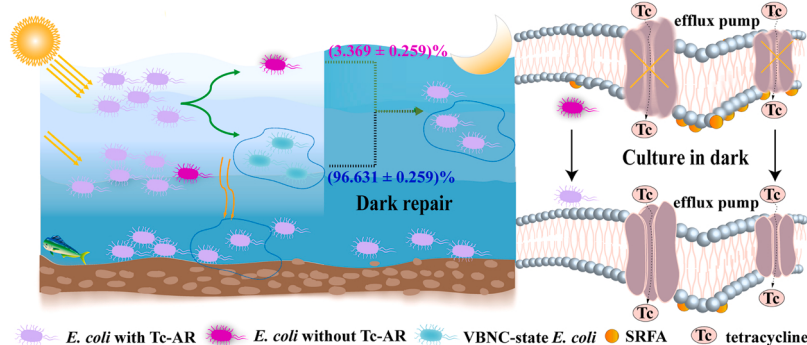
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HIGHLIGHTS

- Sunlight-inactivated tetracycline-resistant bacteria could undergo dark repair.
- The inactivated bacteria re-express antibiotic resistance by repairing efflux pump.
- The bacteria in a VBNC state dominated the reactivation during the dark repair.
- Suwannee River fulvic acid promoted and tetracycline inhibited the reactivation.
- The inactivation and reactivation affect the distribution of ARB in natural waters.

GRAPHICAL ABSTRACT



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ABSTRACT

The spread of antibiotic resistant bacteria (ARB) in the environment poses a potential threat to human health, and the reactivation of inactivated ARB accelerated the spread of ARB. However, little is known about the reactivation of sunlight-inactivated ARB in natural waters. In this study, the reactivation of sunlight-inactivated ARB in dark conditions was investigated with tetracycline-resistant *E. coli* (Tc-AR *E. coli*) as a representative. Results showed that sunlight-inactivated Tc-AR *E. coli* underwent dark repair to regain tetracycline resistance with dark repair ratios increasing from $(0.124 \pm 0.012)\%$ within 24 h dark treatment to $(0.891 \pm 0.033)\%$ within 48 h. The presence of Suwannee River fulvic acid (SRFA) promoted the reactivation of sunlight-inactivated Tc-AR *E. coli* and tetracycline inhibited their reactivation. The reactivation of sunlight-inactivated Tc-AR *E. coli* is mainly attributed to the repair of the tetracycline-specific efflux pump in the cell membrane. Tc-AR *E. coli* in a viable but non-culturable (VBNC) state was observed and dominated the reactivation as the

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inactivated ARB remain present in the dark for more than 20 h. These results explained the reason for distribution difference of Tc-ARB at different depths in natural waters, which are of great significance for understanding the environmental behavior of ARB.

1. Introduction

The abuse of antibiotics induces the emergence of antibiotic resistance genes (ARGs) and antibiotic resistant bacteria (ARB) [37]. Antibiotic resistance is recognized as “the greatest and most urgent global risk, requiring global attention” [32], and would be further exacerbated due to vertical gene transfer (VGT) and horizontal gene transfer (HGT) [25]. In particular, HGT enables the movement of mobile genetic elements (MGEs, such as plasmid, integrons, transposons) ([20,33]). According to a recent study, there were 1.27 million deaths caused by antibiotic-resistant direct infections in 2019 [31]. If we are not able to slow down the rise of antibiotic resistance, it will cause 10 million deaths per year by 2050 [35]. ARGs and ARB have been frequently detected around the world in various environmental media, such as gas [21], water [18,19], and soil [9]. Tetracycline-resistance bacteria (Tc-ARB) and resistance gene (Tc-ARG) were detected with high frequency in the environment [38], which is supposed to be attributable to the widespread presence of tetracycline with high concentrations [60]. ARGs could transfer from the environment to human beings through various pathways [16,57]. Therefore, the propagation and diffusion of ARGs in the environment can increase the risk of human exposure and cause a negative impact on human health.

The water environment is considered to be a main medium for the spread of antibiotic resistance [44]. In natural water, sunlight irradiation could inhibit the spread of antibiotic resistance as ARB were proven to undergo photo-inactivation [56] and ARGs could be degraded under simulated sunlight irradiation [52,53]. As to Tc-ARB, it is known that they can undergo fast photo-inactivation due to the destruction of the tetracycline-specific efflux pump in the cell membrane [56]. The presence of dissolved organic matter (DOM) promoted the photo-inactivation of Tc-ARB by generating photo-produced reactive intermediates (PPRIs) [56]. On the contrary, coexisting antibiotics have a negative effect on the inactivation of ARB due to their selective pressure on ARB and competition for light absorption [40,56].

Different from chemical pollutants for which degradation usually means elimination, the inactivated ARB may be reactivated as solar irradiation is not capable of completely killing the bacteria. As reported in a previous study, the inactivated bacteria induced by sunlight irradiation could be reactivated by photoactivation or via dark repair [10]. Bacteria treated by sunlight irradiation were reported to undergo repair within 48 h in the dark [10]. The inactivated ARB induced by UV disinfection were also proven to be reactivated after dark repair [13]. In many wastewater treatment plants, the final step is usually UV disinfection [15]. The discharge of the effluent could lead to the introduction of pseudo-inactivated ARB into natural water. Meanwhile, considering the diurnal cycle, water circulation, and the difference in the intensity of solar illumination at different water depths [26,58], the inactivated ARB as induced by sunlight irradiation may regain activity via dark repair, which will increase the transmission risk of ARGs in natural waters. It is thus of great significance to investigate the reactivation behavior of ARB in natural water.

The reactivation of inactivated bacteria, such as *E. coli* after UV exposure, is attributed to photo-repair which is triggered by protein photolyases, that are activated at 350–450 nm wavelengths [39], and/or dark repair which is performed by endonuclease excision repair to repair DNA damage in the dark [3,41]. As proven in previous studies, UV irradiation could induce the bacteria into a viable but non-culturable state (VBNC) [12,51], whereas the bacteria in a VBNC state could undergo reactivation and regain the gene transfer ability under appropriate conditions [13]. Cell membranes can be repaired through cellular

function mechanisms during the reactivation of the inactivated bacteria [2,22,43]. A previous study showed that the fast inactivation of Tc-ARB is mainly attributed to membrane damage of the *E. coli* containing tetracycline resistance [56]. Thus, the inactivated Tc-ARB from the effluent of WWTPs or induced by sunlight irradiation may more potentially be reactivated via dark repair in natural water.

Besides the direct reactivation of Tc-ARB, coexisting substances in natural water, such as DOM and antibiotics that have an impact on the inactivation of ARB, may also have an impact on their reactivation. On the one hand, DOM could produce PPRIs, such as singlet oxygen (1O_2), excited triplet state of DOM ($^3DOM^*$), and hydroxyl radicals ($\bullet OH$), under sunlight irradiation, which induced stronger damage to bacteria [27,28,47]. On the other hand, there is an adsorption effect between DOM and the bacteria, which played a protective role for bacteria and was more conducive to the bacterial repair [6]. The presence of antibiotics can exert a negative role on the survival of inactivated ARB [49], which may be detrimental to repair. However, the reactivation of inactivated Tc-ARB and the effects of coexisting substances are not yet understood, which greatly limits the risk assessment of ARGs transmission in natural waters.

Thus, in this study, *E. coli* carrying tetracycline resistance genes (Tc-AR *E. coli*) were selected as a representative of ARB to investigate the dark repair behavior and the underlying mechanisms in natural waters. Tetracycline resistant bacteria were selected due to the highest usage of tetracycline and the prevalent of Tc-ARG in the environment [38,54,7] and *E. coli* has been widely used as an indicator of gram-negative bacteria that carrying ARGs [50]. Suwannee River fulvic acid (SRFA) was selected as a representative of DOM to explore its effect on the dark repair of simulated sunlight inactivated Tc-AR *E. coli*. Solar irradiation induced inactivation of Tc-AR *E. coli* and the subsequent dark repair was also investigated. The photo-inactivation and potential dark repair in natural waters were verified through sampling and determination of Tc-ARB in representative rivers in Jilin, China. This study provides deep insight into the environmental behavior of tetracycline resistance in natural water.

2. Materials and methods

2.1. Materials

E. coli HB101 competent cells were purchased from the Takara Biotechnology Co. Ltd. (Dalian, China), and were used as recipient bacteria. The pBR322 plasmid (0.5 g L^{-1} , 4361 bp, NCBI GenBank NO. J01749.1), carrying the tetracycline resistance genes, was purchased from ThermoFisher Scientific Inc. The pBR322-2 *flag plasmid was transformed by inserting 2 *flag sequence in front of the tetracycline resistance gene on pBR322 plasmid. No-load pBR322 plasmid was transformed by knocking down a tetracycline gene sequence so that the plasmid does not express tetracycline resistance in bacteria. Their sequences are listed in Table S1 and the chart of the pBR322-2 *flag plasmid is shown in Fig. S1 in the Supporting Information (SI). Suwannee River fulvic acid (SRFA, 2S1010F) was purchased from the International Humic Substances Society. Acrylamide solution (30%), Tris-HCL (pH = 8.8), sodium dodecyl sulfate solution (SDS, 10%), ammonium persulfate (APS, 99.0%), N,N,N',N'-tetramethylethylenediamine (TEMED), and methanol (99.5%) were obtained from Sangon Biotech Co. Ltd. (Shanghai, China). LIVE/DEAD BacLight™ Bacterial Viability Kit (Invitrogen, Inc. USA) was purchased from ThermoFisher Scientific Inc. The other materials used and the corresponding commodity suppliers are listed in Text S1 in the SI.

2.2. AR *E. coli* culture and experimental procedures

The pBR322-2 *flag (0.5 μ L) was transformed into 50 μ L *E. coli* HB101 to obtain AR *E. coli*, the procedure of the transformation experiment is shown in Text S2 in the SI. Then, 500 μ L AR *E. coli* was cultured in a 50 mL LB medium with tetracycline (10 mg L⁻¹) at 37 °C for 16 h with shaking at 180 rpm until the OD₆₀₀ reached 0.6–0.8. The OD₆₀₀ was determined using a microplate reader (Synergy HTX, BioTek, USA). The growth curve of the bacteria is shown in Fig. S2(a). The bacteria were centrifuged at 4000 rpm for 3 min at 4 °C, discarding the supernatant, and resuspending the bacteria in phosphate-buffered saline (PBS, 1 $\times$). The washing procedure was repeated three times and the obtained bacterial suspensions were used for the subsequent experiments. The *E. coli* carrying plasmid was usually used to represent antibiotic resistant bacteria in laboratory simulations [23].

The irradiation experiment was conducted in an XPA-7 merry-go-round photochemical reactor (Xujiang Electromechanical Plant, Nanjing, China) with quartz tubes containing 25 mL Tc-AR *E. coli* with an initial concentration of 1.0×10^8 CFU mL⁻¹. The *E. coli* in the logarithmic growth period was selected as usually used in the laboratory simulations in previous studies [5,23]. The specific information on this experiment is shown in Text S3 in the SI. The light intensity at the surface of the quartz tube is shown in Fig. S3.

To explore the effect of DOM, SRFA was added at an initial concentration of 10 mg L⁻¹ (4.8 mg C L⁻¹). The concentration of SRFA was selected to be consistent with the common concentration of DOM in surface water [55]. And the concentration of SRFA in solutions was detected by microplate reader (Synergy HTX, BioTek, USA) at 400 nm [55]. The sorption and desorption of SRFA from the cell membrane of Tc-ARB were investigated and the detection method of SRFA is described in Text S4 in the SI. The standard curve of SRFA is shown in Fig. S2(b). To investigate the effect of the antibiotic, tetracycline was added at concentrations of 0.001, 0.01, 0.1, and 1 mg L⁻¹.

2.3. Dark repair experiments

The bacterial solution obtained after 60 min of simulated sunlight exposure was placed in a beaker wrapped with two layers of aluminum foil and incubated for 48 h at room temperature. One mL of the bacteria suspension was sampled at each 12-h time interval (0, 12, 24, 36, and 48 h). Then, the Tc-AR *E. coli* was coated on the nutrient agar medium (NA medium) supplemented with tetracycline (10 mg L⁻¹) to count the number of the reactivated bacteria (N_t). The dark repair ratio is calculated by the following equation:

$$\text{The dark repair ratio} = \frac{N_t}{N_0} \% \quad (1)$$

where, N_t represents the number of reactivated Tc-AR *E. coli* at different t , the unit is CFU mL⁻¹; N_0 represents the initial concentration of Tc-AR *E. coli* before sunlight, it is 10^8 CFU mL⁻¹.

2.4. DNA extraction and detection

Two milliliters of the samples of different dark repair times without treatment were used to extract the total DNA of the bacteria. Another 2 mL sample was filtered through a 0.22 μ m membrane and was divided into two portions: the bacteria collected on the membrane were used to extract intracellular DNA (i-DNA) using the Ezup Column Bacteria Genomic DNA Purification Kit (Sangon Biotech); the filtrate was used to extract extracellular DNA (e-DNA) that leaked into the solution. The extracted DNA samples were stored at -20 °C for RT-qPCR analysis (Details are described in Text S5 in the SI). The standard curve of Tc-ARG is shown in Fig. S2(c).

2.5. Determination of membrane integrity

The membrane damage was investigated using the LIVE/DEAD BacLight™ Bacterial Viability Kit (Invitrogen, Inc. USA). Two fluorescent nucleic acid stains of SYTO9 (fluorescent green) and propidium iodide (PI, fluorescent red) distinguish intact cells from injured cells, all bacteria stain the former, whereas those with damaged membranes stain the latter. The excitation/emission maxima for these dyes are about 480/500 nm for the SYTO 9 stain and 490/635 nm for PI. The SYTO9 with a volume of 3 μ L and PI of 3 μ L were added to 200 μ L bacteria suspension of approximately 10^6 CFU mL⁻¹ diluted using 0.85% NaCl, followed by incubation in the dark for 20 min at room temperature. The samples were then observed by Flow Cytometry (FCM, BD FACSCalibur, USA) within 2 h to detect the active cells. The VBNC cells in the solutions during the simulated sunlight irradiation were therefore determined and calculated based on the difference between the FCM assay and the plate culture count method [11,4,46].

NaCl was used as a blank control to correct the bacterial number. Single-stained bacteria were used as a positive control, and unstained bacteria as a negative control to define the target gates.

The morphology of AR *E. coli* was observed using field emission scanning electron microscopy (FESEM, XL-30 ESEM FEG, FEI Company) at 20 kV. The details of the method are shown in Text S6 in the SI.

2.6. Determination of Tc-ARG expressed protein

The pBR322-2 *flag was transformed into the *E. coli* HB101 for irradiation and dark repair experiments, and the *E. coli* HB101 carrying pBR322 plasmid without Tc-ARG was cultured as a blank control. Two-mL samples were centrifuged at 8000 rpm for 2 min, slightly discarding the supernatant, then 200 μ L ddH₂O and 50 μ L protein loading buffer (5 $\times$) were added to lysis the bacteria, which were mixed by means of vortexing and heated in a 100 °C water bath for 8 min to obtain protein samples. The protein samples were placed at -20 °C until a western blot (WB) analysis was performed. Anti-Histone H3 (66009-1, 1:5000, Proteintech, USA, 14 kD) antibodies were used as control [45]. Details of the procedure of WB analysis are given in Text S7 in the SI.

2.7. Sampling surveys and outdoor experiments

To investigate the potential role of sunlight on the presence of ARB in natural waters, samples were taken in the surface, middle and deep water of Liao River, Songhua River, and Yitong River (a tributary of Songhua River) in Jilin, China. The specific sampling locations, depths, time, and temperature are shown in Table S2. The Tc-ARB in the samples was detected using the plate culture method. One-mL samples were serially diluted and plated in agar medium supplemented with 10 mg L⁻¹ tetracycline to determine the Tc-ARB count levels.

According to a previous study [58], once the actual water depth was more than 1 m, the surface, middle, and deep layers are subjected to different light intensities. Therefore, to investigate the inactivation and reactivation of ARB under real solar irradiation, irradiation experiments were performed outdoor using a 50 $\times$ 30 $\times$ 100 cm device made of plexiglass and equipped with a quartz glass lid (Fig. S4(a)). Dark control and dark repair experiments were performed with the device wrapped with two layers of aluminum foil (Fig. S4(b)). Light transmittance of the quartz glass lid, plexiglass, and material wrapped with layers was detected using a UV-Vis spectrophotometer (Hitachi U2900), and the result is shown in Fig. S5. The initial concentration of AR *E. coli* is 10^5 CFU mL⁻¹, which is in accordance with the detected concentration of Tc-ARB in the effluent of the Changchun wastewater treatment plant. The light intensity of solar irradiation during the experiments was determined with a TriOS-RAMSES spectroradiometer (TriOS GmbH, Germany). The light intensity and temperature are shown in Fig. S6.

3. Results and discussion

Bacteria with tetracycline resistance in Liao River, Songhua River, and Yitong River were detected, and the results are shown in Fig. 1. As can be seen in Fig. 1, Tc-ARB was widely present in the three rivers that are located in northeast China, and obvious differences in the concentration of Tc-ARB were observed in different locations of these rivers. Interestingly, vertical distribution difference was significantly observed as the concentration of Tc-ARB are different in the water sampled from the surface, middle and deep layers of the three rivers. The concentrations of Tc-ARB in the deepest waters are much higher compared with those in surface waters. The differences were supposed to be attributed to the fast inactivation of Tc-ARB under light irradiation as reported in our previous study [56]. Besides, the reactivation of inactivated Tc-ARB in deep water that occurs under dark conditions may also contribute to the high concentration of Tc-ARB as the inactivated bacteria induced by sunlight could be reactivated through dark repair [10]. Thus, it is of great significance to investigate the potential dark repair of sunlight-inactivated ARB in natural waters.

3.1. Dark repair of simulated sunlight-inactivated Tc-AR *E. coli* and effects of SRFA and tetracycline

The Tc-AR *E. coli* were treated under simulated sunlight irradiation and the results showed that Tc-AR *E. coli* could undergo fast photo-inactivation (Fig. S7). After 60-min irradiation, no *E. coli* survived in the medium with tetracycline (10 mg L^{-1}), and the *E. coli* with a concentration of $2.50\text{-log}_{10} \text{ CFU mL}^{-1}$ surviving on medium without tetracycline. This result demonstrated that simulated sunlight irradiation exhibits a specific role in the inactivation of Tc-AR *E. coli*, which is attributed to the destruction of tetracycline-specific efflux pump on cell membrane as proved in our previous study [56]. The inactivation rate of *E. coli* that not carrying pBR322 plasmid (*E. coli* without Tc-AR) is much higher than that of Tc-AR *E. coli* (Fig. S8(a)), indicating that Tc-ARB is more resistant to light irradiation compared with the bacteria without tetracycline resistance. It is speculated that ARGs may confer advantageous properties to bacteria that are associated not only with antibiotic resistance but also with stress response or adaptive effects of the bacteria [5,14]. This result is in accordance with a previous study that the bacteria carrying ARGs are more resistant to light irradiation than the bacteria without ARGs [5].

To investigate the potential reactivation of the sunlight-inactivated Tc-AR *E. coli*, i.e., the culturable *E. coli* that have lost the tetracycline resistance, dark repair experiments were performed with Tc-AR *E. coli* that were treated under simulated sunlight irradiation for 60 min. The results of the dark repair experiments are shown in Fig. 2(a) and Fig. S9. As can be seen in Fig. 2(a), simulated sunlight-inactivated Tc-AR *E. coli* could undergo reactivation in the absence of light irradiation, i.e., Tc-AR *E. coli* could undergo dark repair to re-express tetracycline resistance.

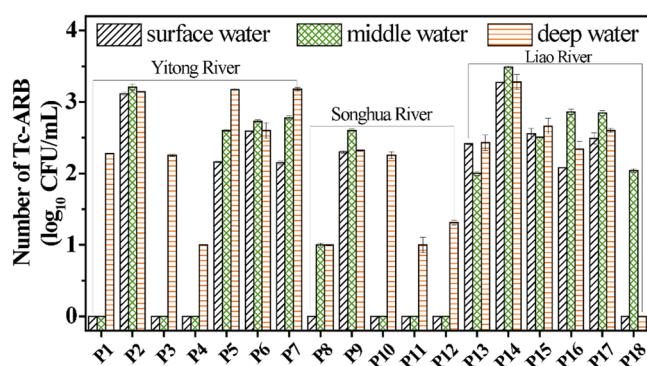


Fig. 1. Number of Tc-ARB in surface, middle and deep water sampled from different sites of the Yitong River, Songhua River, and Liao River.

The repaired Tc-AR *E. coli* were firstly observed at about 7-h dark treatment (Fig. S9). The dark repair ratio of simulated sunlight-inactivated Tc-AR *E. coli* increased from $(0.124 \pm 0.012)\%$ within 24 h dark treatment and increased to $(0.343 \pm 0.011)\%$ and $(0.891 \pm 0.033)\%$ within 36 h and 48 h dark treatment, respectively (Table S3). And the dark repair ratio of simulated sunlight-inactivated *E. coli* that not carrying pBR322 plasmid was $(0.0202 \pm 0.0045)\%$ within 24 h and increased to $(0.225 \pm 0.003)\%$ within 48 h (Fig. S8 (b)), which is similar with that of UV-treated *E. coli* (0.0420% with 24 h dark treatment) reported by Xu et al. [48]. The dark repair ratio of *E. coli* that not carrying pBR322 plasmid is lower compared with that of the simulated sunlight-inactivated Tc-AR *E. coli*, which indicating their different pathways or mechanisms of the reactivation under dark conditions. The number of *E. coli* with and without tetracycline resistance reached up to $3.91\text{-log}_{10} \text{ CFU mL}^{-1}$ and $5.46\text{-log}_{10} \text{ CFU mL}^{-1}$, respectively under dark conditions for 48 h. As far as we are aware, no other studies ever investigated the reactivation of sunlight-inactivated antibiotic resistance via dark repair, and the reactivation of ARB could increase the transmission risk of ARGs in the aqueous environment.

The presence of SRFA promoted the dark repair of simulated sunlight-inactivated Tc-AR *E. coli*, of which repair ratio after 48 h treatment is up to $(61.6 \pm 0.9)\%$ (Fig. 2(a)). Besides, in SRFA solutions, the repaired Tc-AR *E. coli* were firstly observed after about 6-h of dark treatment (Fig. S9), which is earlier than in the case of PBS. As a conclusion, SRFA could promote the inactivation of Tc-AR *E. coli* and protect the cell membrane from damage during the light irradiation, and during the dark repair process, SRFA could promote the reactivation of sunlight-inactivated Tc-AR *E. coli*.

In the presence of tetracycline, there was an inhibitory effect on the dark repair of simulated sunlight-inactivated Tc-AR *E. coli*, and the inhibitory effect became stronger with the increase of the concentration of tetracycline (Fig. 2(b)). This is due to a stressful environment for the bacteria created by the presence of tetracycline, of which exposure inevitably induced bacterial permeability pressure alterations. The cell membrane components of the bacteria may be affected or disrupted in the presence of tetracycline, which could inhibit the repair of the cell membrane [17]. Besides, the presence of antibiotics could induce bacteria into a VBNC state and make this state more persistent [36]. Therefore, the presence of tetracycline is not conclusive to the dark repair of bacteria in the VBNC state. Thus, tetracycline could compete with bacteria for light absorption and has a certain protective effect on the Tc-AR *E. coli* during the light irradiation, while during the dark repair, the reactivation of sunlight-inactivated Tc-AR *E. coli* is inhibited due to the selection pressure generated in the presence of tetracycline.

3.2. Contribution of bacteria at the VBNC state to the reactivation of simulated sunlight-inactivated Tc-AR *E. coli*

There were only about $2.57 \text{ log CFU mL}^{-1}$ bacteria that survived after 60-min irradiation of simulated sunlight while the number of reactivated Tc-AR *E. coli* was $3.91 \text{ log CFU mL}^{-1}$ after dark repair for 48 h. The *E. coli* at VBNC state has been observed in the source water of drinking water treatment plants [11] and in solutions under UV irradiation [12]. Meanwhile, the reactivation of inactivated *E. coli* at the VBNC state was also reported [51]. Thus, the reactivated Tc-AR *E. coli* is not only attributable to the *E. coli* that could be cultured on the NA medium, but may also attributed to the *E. coli* at the VBNC state.

In PBS, the number of intact cells after 60-min treatment of the simulated sunlight irradiation is $7.92\text{-log CE mL}^{-1}$, which is much higher compared with the culturable *E. coli* without Tc-AR ($2.50\text{-log CFU mL}^{-1}$). This indicates that simulated sunlight irradiation could induce Tc-AR *E. coli* into a VBNC state. Only living bacteria could continuously transcribe mRNA. Thus, the sustained activity of mRNA is an excellent indicator of bacterial viability, especially for studies to determine VBNC state [24,51]. The expression level of 16 S rRNA and the relative expression of two mRNA (*fliC* and *gadA*, which were commonly used as

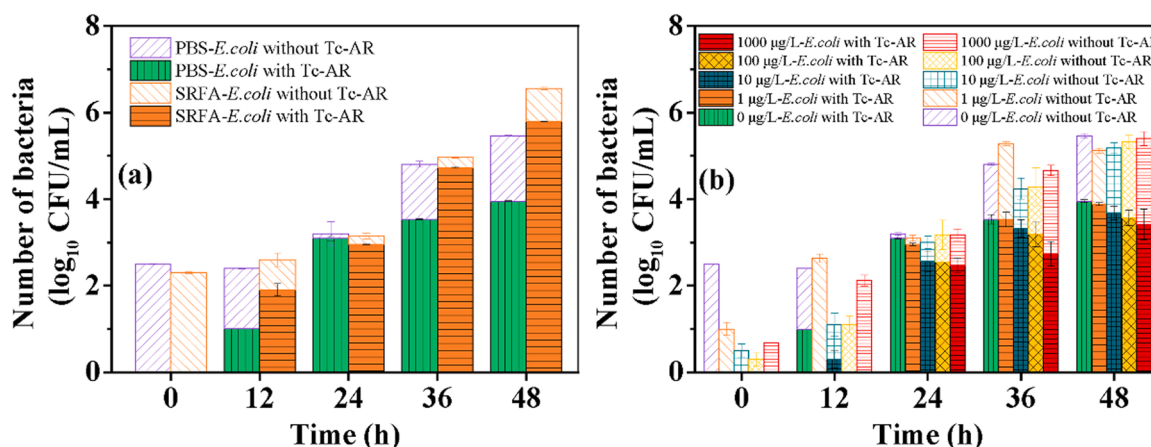


Fig. 2. Numbers of *E. coli* with or without tetracycline resistance (Tc-AR) in PBS or SRFA solutions (a) and in PBS solutions with different concentrations of tetracycline (b) during dark treatment ([SRFA]₀ = 10 mg L⁻¹, pH = 7.0, temperature = 25 °C; Tc-AR: tetracycline resistance; “*E. coli* without Tc-AR” refers to the Tc-AR *E. coli* that have lost the tetracycline resistance during the light irradiation).

viability indicators of the bacteria [51] were detected. The results showed that simulated sunlight irradiation had little effect on the expression of 16 S rRNA, *fliC* and *gadA* (Fig. S10). Significant decrease of the number of culturable *E. coli* was observed during the simulated sunlight irradiation (Fig. S7). Thus, it can be concluded that the bacteria in the VBNC state were present in solutions under simulated sunlight irradiation.

The number of Tc-AR *E. coli* in VBNC state during simulated sunlight irradiation ($C_0 = 10^6$ CFU mL⁻¹) were determined and the contribution ratio of the culturable *E. coli* and the *E. coli* in the VBNC state to the dark repair of simulated sunlight-inactivated Tc-AR *E. coli* into Tc-AR *E. coli* with tetracycline resistance were calculated. As can be seen in Fig. 3(a), the number of VBNC-state Tc-AR *E. coli* increased with the increasing irradiation time. As reported in previous studies, the dark repair of inactivated *E. coli* needs 20 h or even longer [4,46]. Thus, in this study, the dark repair of the inactivated Tc-AR *E. coli* under dark conditions within 20 h is speculated to be attributable only to the culturable *E. coli*. As can be seen in Fig. 3(b), the reactivation of Tc-AR *E. coli* is mainly attributed to the culturable *E. coli* that survived after 60-min of simulated sunlight irradiation. The bacteria in the VBNC state gradually dominate the reactivation as the time of dark treatment increased. The contribution ratio of the bacteria in VBNC state to the reactivation of Tc-AR *E. coli* are (74.4 ± 0.1)%, (91.1 ± 0.4)%, and (96.6 ± 0.3)% at 24 h, 36 h, and 48 h, respectively. These results demonstrated the important role of the bacteria in the VBNC state in the reactivation of

inactivated *E. coli*. This is consistent with the results reported by Chen et al. [4] that most of the *E. coli* in the VBNC state induced by chlorination could undergo reactivation.

In SRFA solutions, there are also a big amount of bacteria in the VBNC state (Fig. S11). The culturable *E. coli* were 2.30-log CFU mL⁻¹ (Fig. S7(b)), but there were approximately 7.78-log CE mL⁻¹ intact cells in SRFA solutions after 60 min of simulated sunlight treatment (Fig. 3(a)). The bacteria in the VBNC state contribute (77.9 ± 0.1)%, (99.627 ± 0.002)%, and (99.968 ± 0.001)% at 24 h, 36 h, and 48 h to the repair of Tc-AR *E. coli*, respectively (Fig. 3(b)). Thus, it can be concluded that the contribution of bacteria in the VBNC state to the reactivation of antibiotic resistance under dark conditions is non-negligible in the transmission of ARGs in natural waters.

3.3. Underlying mechanism of dark repair of inactivated Tc-AR *E. coli*

As proven in our previous study, the inactivation of Tc-AR *E. coli* is mainly attributed to the damage of the cell membrane [56]. The changes of the cell membrane during the dark repair of the inactivated Tc-AR *E. coli* were therefore detected by SEM. As can be seen in Fig. 4(a), the shrink and shape destruction of Tc-AR *E. coli* were observed after simulated sunlight irradiation for 60 min, and the cell membrane of inactivated Tc-AR *E. coli* gradually repaired as the dark repair time increased. These results demonstrated that the dark repair of simulated sunlight-inactivated Tc-AR *E. coli* could occur and the damaged cell

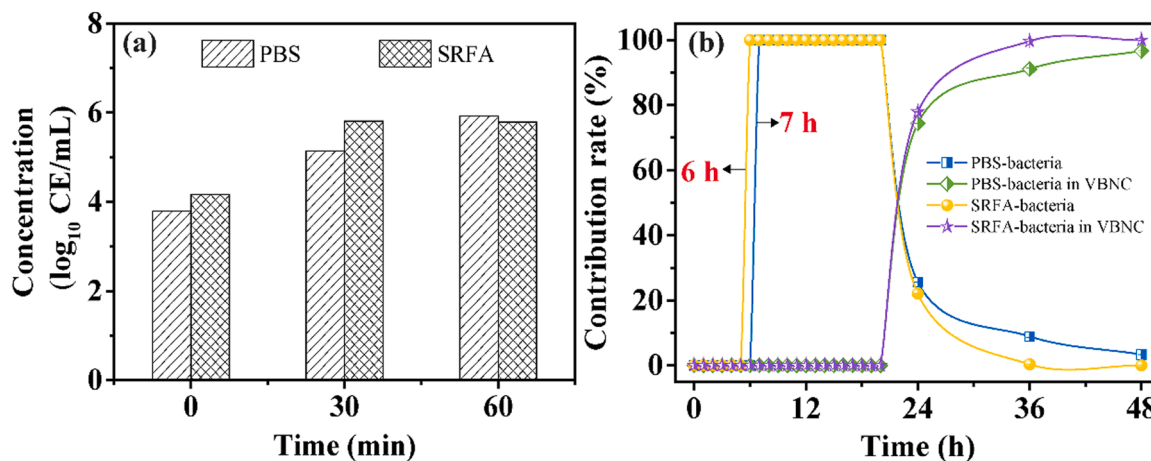


Fig. 3. Number of Tc-AR *E. coli* in VBNC state during simulated sunlight irradiation ($C_0 = 10^6$ CFU mL⁻¹) (a), and contribution ratio of bacteria to dark repair of Tc-AR *E. coli* (b).

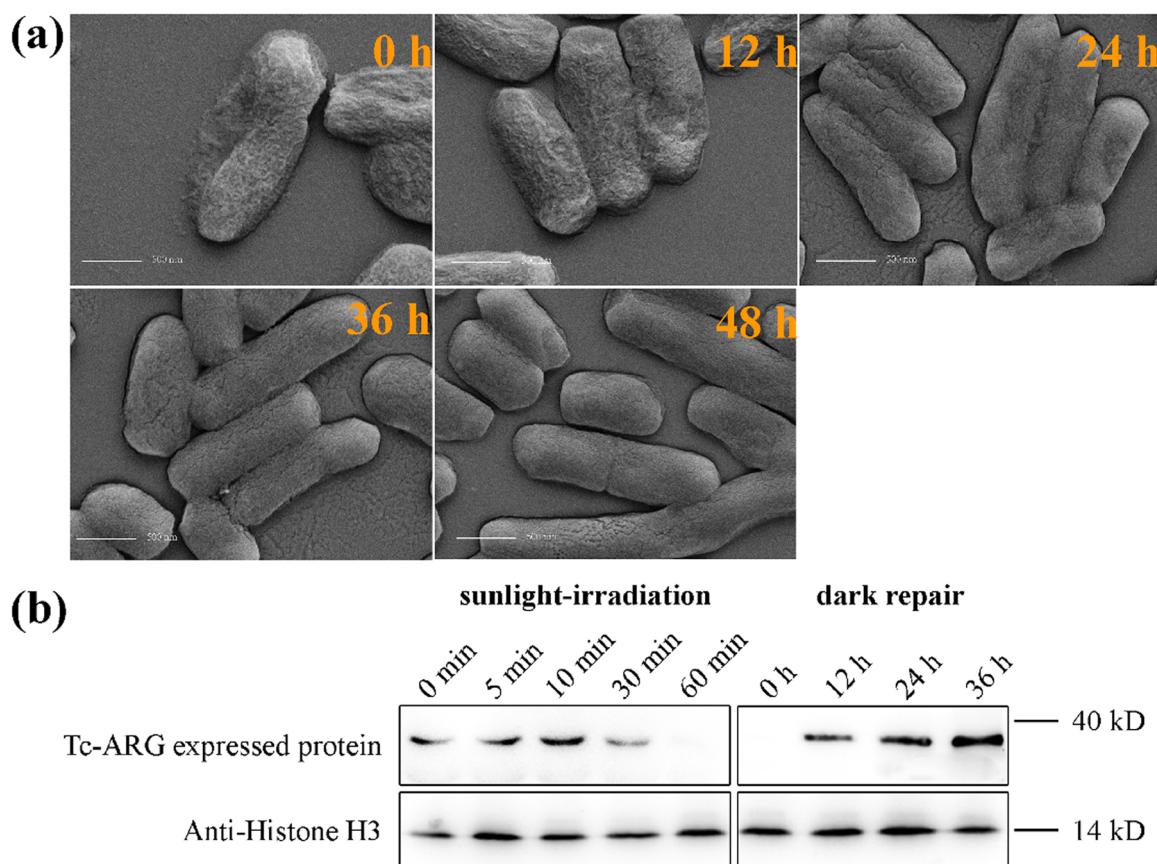


Fig. 4. SEM images of inactivated Tc-AR *E. coli* during dark treatment (a) and western blot analysis of Tc-ARG expressed protein levels during sunlight-irradiation and dark repair processes (b) in PBS (pH = 7.0).

membrane could be repaired during this process. It has also been shown in previous studies that the cell membrane could be repaired during the reactivation process of the bacteria [29,42].

The efflux of tetracycline by the tetracycline-specific pumps in the cell membrane dominates the tetracycline resistance as *tetA* was contained in the pBR322 plasmid that transformed into the Tc-AR *E. coli* used in this study [1,34,59]. During the dark repair of the inactivated Tc-AR *E. coli*, the tetracycline-specific efflux pump may be reconstituted, as being accompanied by the repair of the cell membrane. The efflux pump is composed of specific membrane-associated efflux proteins in the cell membrane [30]. Thus, to investigate the reconstitution of tetracycline-specific efflux pump, a pBR322-2 *flag plasmid with a 2 *flag tag and a no-load pBR322 plasmid with a segment of the gene sequence removed (Tc-ARG could not be expressed) was constructed. The two plasmids were then transformed into the *E. coli* HB101 respectively and were cultured for the extraction of the proteins. A band at around 40 kD was observed for the pBR322-2 *flag plasmid and no band in the control group was detected (Fig. S12). This indicated the successful construction of the plasmid to investigate the expressed protein from Tc-ARG in the Tc-AR *E. coli*.

The expression of anti-histone H3 in each sample was consistent, indicating that the protein loading was consistent, and the change of Tc-ARG expressed protein was due to the difference in gene expression of Tc-ARG (Fig. 4(b)). The detected proteins expressed from Tc-ARG in the pBR322-2 *flag plasmid decreased with the increase of irradiation time (Fig. 4(b)) and no obvious changes were observed in the dark control (Fig. S13), indicating the negative role of light irradiation on the level of Tc-ARG expressed proteins. The amino acid sequence of Tc-ARG expressed proteins was obtained (Fig. S14), and showed 99.7% homologous with UniRef100-P02981 protein, of which the amino acid sequence was obtained from the national center for biotechnology

information (NCBI). These results demonstrated that the two proteins are functionally identical. The function of the UniRef100-P02981 protein is the efflux of tetracycline, i.e., it is the main protein of the tetracycline-specific efflux pump [8]. Thus, the protein expressed by Tc-ARG is exactly the proteins that make up the tetracycline efflux pump. These results further confirmed that the specific effect of light irradiation on the inactivation of tetracycline resistance is attributed to the damage of the tetracycline efflux pump in the cell membrane. During the dark treatment of simulated sunlight-inactivated Tc-AR *E. coli*, the level of Tc-ARG expressed proteins gradually increased with the increase of time under dark conditions (Fig. 4(b)), which demonstrated that the damaged tetracycline efflux pump can be repaired under dark conditions. This leads to the reactivation of tetracycline resistance of inactivated Tc-AR *E. coli*. Dark repair of *E. coli* without tetracycline resistance is dominated by the repair of DNA [3,41], whereas the regain of tetracycline resistance in inactivated Tc-AR *E. coli* is mainly attributed to the repair of the tetracycline efflux pump, which accounts for the faster repair of simulated sunlight-inactivated Tc-AR *E. coli* compared with the in the *E. coli* without tetracycline resistance.

3.4. Influence mechanisms of SRFA on dark repair of inactivated Tc-AR *E. coli*

In the presence of SRFA, the repair of the cell membrane of inactivated Tc-AR *E. coli* was also observed (Fig. 5(a)). As reported in previous studies, adsorption of DOM on the cell membrane of Tc-AR *E. coli* could occur, which subsequently protects the cell membrane from damage or repair and also inhibits the efflux of tetracycline [6,56]. During the dark treatment of inactivated Tc-AR *E. coli*, desorption of SRFA from the *E. coli* occurred as the substances on the cell membrane reduced as the repair time increased (Fig. 5(a)), which was also confirmed as the

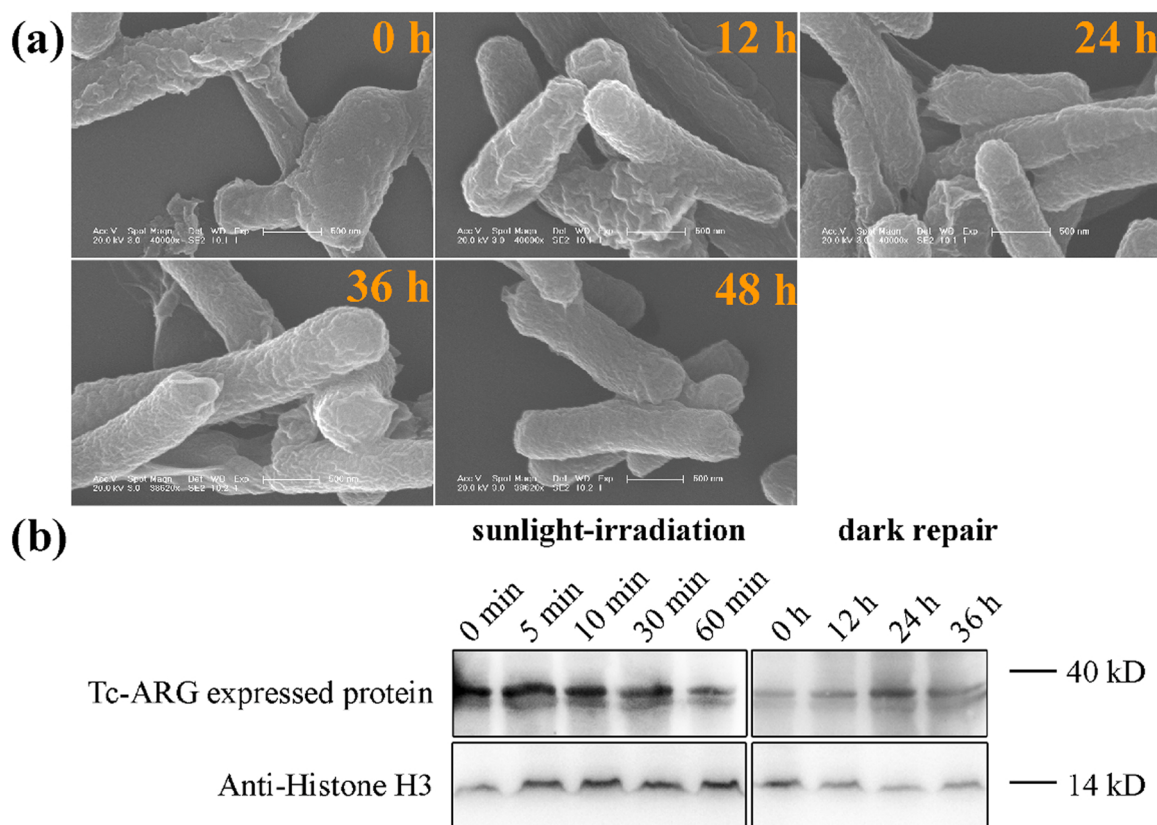


Fig. 5. SEM images of inactivated Tc-AR *E. coli* during dark treatment (a) Western blot analysis of Tc-ARG expressed protein levels during sunlight-irradiation and dark repair processes (b) in SRFA solutions (pH = 7.0).

detected concentration of SRFA that adsorbed on the cell membrane decreased during the dark treatment (Fig. S15). Thus, the desorption of SRFA from the membrane contributed to the promotional effect of SRFA on the recovery of tetracycline resistance of inactivated Tc-AR *E. coli* under dark conditions (Fig. 2(a)). The adsorption of SRFA on the cell membrane surface affected the efflux pump function and desorption made the efflux pump re-expressed.

The expressed proteins from Tc-ARG in SRFA solutions also showed a decreasing trend during the light exposure, while they were less affected compared with those in PBS (Fig. 5(b) vs. Fig. 4(b)). This is attributed to the protective effect of the adsorbed SRFA and the light competition effect of dissolved DOM as observed in a previous study [40]. The surviving bacteria could not express tetracycline resistance after 60 min irradiation but could express the efflux pump protein, indicating that the

adsorbed SRFA on the cell membrane could affect the function of the efflux pump rather than damaging the proteins. There was a slight increase in the amount of proteins during the dark repair process in SRFA solutions, which was induced by the reactivation of VBNC bacteria and the repair of the resistant expression of Tc-ARG. Therefore, the promotional effect of SRFA on the reactivation of sunlight-inactivated Tc-AR *E. coli* in dark condition is attributed to two mechanisms: the desorption of SRFA from the cell membrane of the *E. coli* which could strengthen the re-expression of efflux pump, and the enhancement of the reactivation of VBNC-state *E. coli*. To investigate the changes of Tc-ARG in Tc-AR *E. coli* during the dark repair, extracellular Tc-ARG (e-ARG), intercellular Tc-ARG (i-ARG), and total Tc-ARG (total-ARG) were detected, and the results are shown in Fig. 6. The changes in the concentrations of Tc-ARG were insignificant during the dark repair process no matter in the

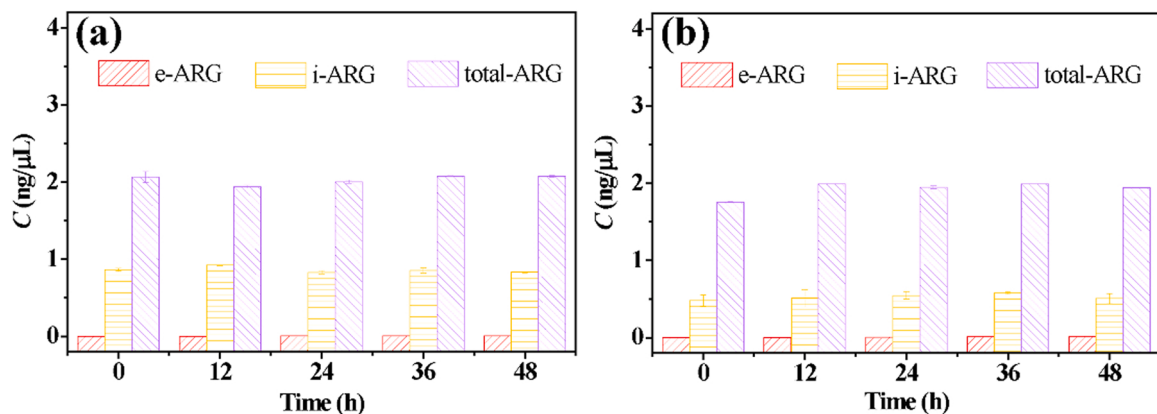


Fig. 6. Concentrations of extracellular Tc-ARG (e-ARG), intercellular Tc-ARG (i-ARG), and total Tc-ARG (total-ARG) extracted from sunlight-inactivated AR *E. coli* in PBS (a) and in SRFA solutions (b) during dark repair ($C_0 = 0$ CFU mL⁻¹, pH = 7.0, temperature = 25 °C).

absence or in the presence of SRFA. Only a small amount of Tc-ARG leaked out of the bacteria (Fig. S16), which is attributed to the light-causing dead bacteria. These results suggested that the reactivation of inactivated Tc-AR *E. coli* is not attributable to the repair or replication of Tc-ARG, and also confirmed the high contribution of *E. coli* in VBNC state that containing normal-level Tc-ARG to the reactivation.

3.5. Inactivation and dark repair of Tc-AR *E. coli* under outdoor sunlight irradiation

Sunlight irradiation experiments were performed outdoors in the plexiglass device containing solutions with Tc-AR *E. coli* for 9 h. The results showed that Tc-AR *E. coli* could be inactivated under sunlight irradiation (Fig. 7(a)), and no changes were observed in the dark controls (Fig. S17). This indicates that sunlight irradiation could induce the inactivation of Tc-AR *E. coli*. The inactivation of *E. coli* with tetracycline resistance is faster compared with the inactivation of *E. coli* without tetracycline resistance (Fig. 7(a) and Table S4), implying that sunlight irradiation plays a specific role in the inactivation of tetracycline resistance. This is in accordance with the results of simulated sunlight irradiation (Fig. S7). The inactivation of Tc-AR *E. coli* is fastest in the upper water (0 cm), followed by the middle water (50 cm) and slowest in the bottom water (100 cm) in the plexiglass device (Fig. 7(a) and Table S4), which is attributed to the attenuation of sunlight in the water.

Dark repair of sunlight-inactivated Tc-AR *E. coli* could occur, and different phenomena were observed for the inactivated Tc-AR *E. coli* in the water obtained from the upper, middle, and bottom of the plexiglass device. For the inactivated Tc-AR *E. coli* in the upper water, only those samples irradiated for less than 2 h could undergo dark repair to recover tetracycline resistance (Fig. 7(b) vs. 7(c) and 7(d)). As to the inactivated

Tc-AR *E. coli* in the middle and bottom water, those irradiated for more than 4 h outdoors could not undergo dark repair to recover tetracycline resistance (Figs. 7(b) and 7(c) vs. 7(d)). The spread ability of ARB in the environment was weakened, so that VGT cannot occur, but it is still possible to continue to spread antibiotic resistance through HGT.

Under 48 h dark treatment, the dark repair ratio of Tc-AR *E. coli* that were irradiated for 2 h (from 9:00–11:00) is 0.021%, 0.590%, and 3.556% in the upper, middle, and bottom water, respectively, and decreased to 0%, 0.088%, and 0.175% for Tc-AR *E. coli* that were irradiated for 3 h (from 9:00–12:00). There were no bacteria survived in the surface water after 3 h (Fig. 7(a)), and only some of the bacteria without antibiotic resistance recovered within 48 h during dark treatment (Fig. 7(b)). The Tc-AR *E. coli* that were irradiated for 4 h (from 9:00–13:00) could not be reactivated to regain tetracycline resistance (Fig. 7(c)). Thus, the bacteria exposed to light for a long time and with a high light intensity may be very difficult to be repaired. The total number of the *E. coli* with and without tetracycline resistance obviously increased with the increase of time under dark conditions (Figs. 7(b), 7(c), and 7(d)). This indicates the involvement of VBNC-state *E. coli* in the reactivation of sunlight-inactivated Tc-AR *E. coli*. These results are in accordance with the observations of the simulated sunlight irradiation experiments (Fig. 3). Tc-AR *E. coli* were irradiated in daytime and repaired in dark condition, then the repaired *E. coli* were irradiated by sunlight again for two light-dark cycles (48 h), the light intensity of sunlight and temperature were shown in Fig. S18(a). During the light-dark cycles, the *E. coli* were inactivated by sunlight and repaired in the dark (Fig. S18(b)), but the number of repaired *E. coli* was gradually decreased during the light-dark cycles. It can thus be concluded that solar irradiation could induce the inactivation of Tc-ARB and dark repair could occur in deep water or in the night under dark conditions.

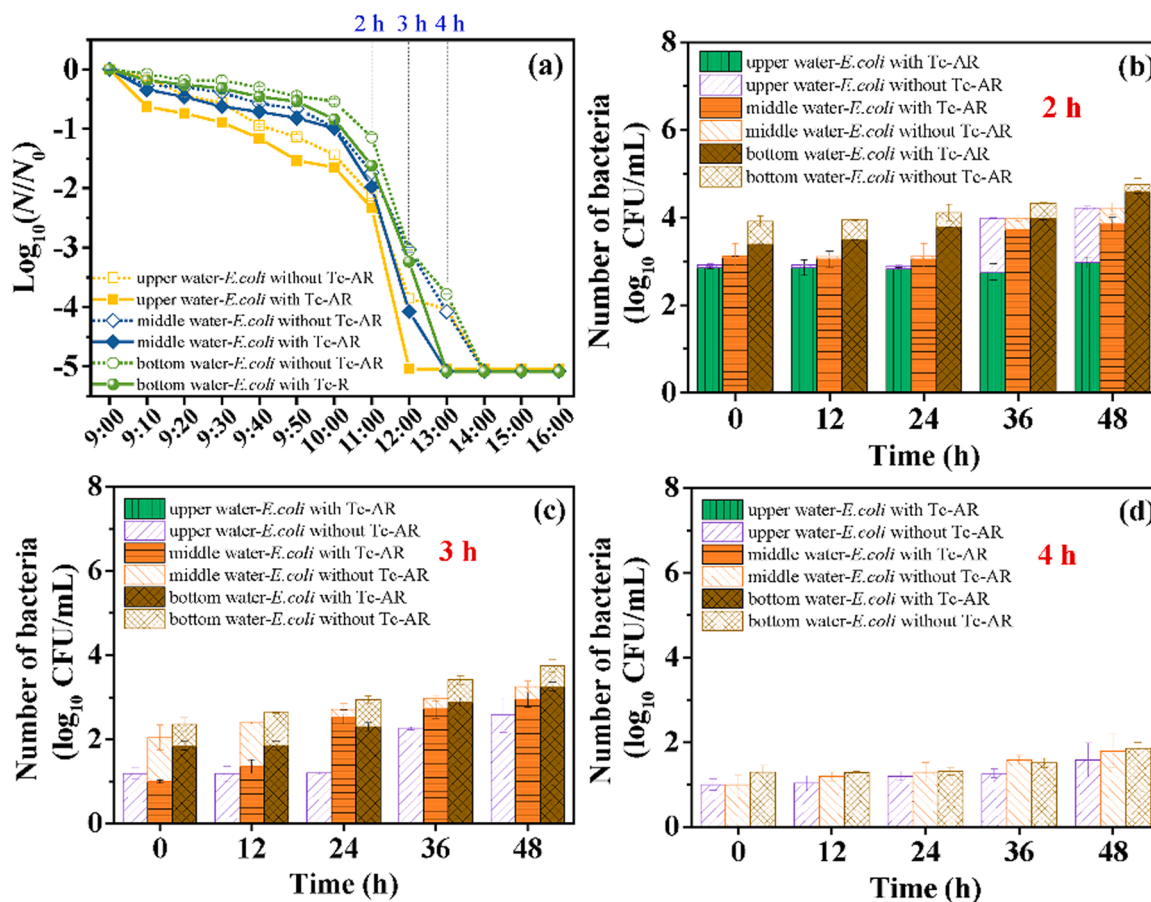


Fig. 7. Inactivation of Tc-AR *E. coli* in an outdoor device under sunlight irradiation (a) and dark repair of Tc-AR *E. coli* that irradiated for 2 h (b), 3 h (c), and 4 h (d) (Tc-AR: tetracycline resistance; “*E. coli* without Tc-AR” refers to the Tc-AR *E. coli* that have lost the tetracycline resistance during the light irradiation).

4. Conclusions

In this study, the dark repair of sunlight-inactivated Tc-ARB was investigated. The sunlight-inactivated Tc-AR *E. coli* was proven to undergo reactivation to re-express tetracycline resistance by repairing the tetracycline-specific efflux pump in the cell membrane. The ARB in the VBNC state were proven to be present in water under sunlight irradiation, and they could also undergo reactivation to regain tetracycline resistance. The inactivated Tc-AR *E. coli* in VBNC state dominated the reactivation of Tc-AR *E. coli* under dark condition for a long time. In SRFA solutions, the reactivation of inactivated Tc-AR *E. coli* was promoted due to the desorption of SRFA and was inhibited in the presence of tetracycline. The dark repair of sunlight-inactivated ARB and the reactivation of VBNC-state ARB could promote the spread of ARGs and lead to an uneven distribution of ARB in natural waters. This would consequently increase the potential health risk of ARGs in terms of human accessibility. Therefore, it is essential to inactivate ARB completely during the wastewater treatment to avoid the discharge of VBNC-state ARB into natural waters.

CRediT authorship contribution statement

Tingting Zhang: Conceptualization, Methodology, Writing – original draft. **Fangyuan Cheng:** Formal analysis, Investigation. **Xiaobing Chen:** Investigation, Data curation. **Ya-nan Zhang:** Supervision, Writing – review & editing. **Jiao Qu:** Supervision, Funding acquisition. **Willie Peijnenburg:** Writing – review & editing.

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.

Data Availability

Data will be made available on request.

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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.jhazmat.2023.131560.

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