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Effects of salinity on photoreactivation of *Escherichia coli* after UV disinfection

Kumiko Oguma, Kentaro Izaki and Hiroyuki Katayama

ABSTRACT

The effects of sodium chloride on photoreactivation of *Escherichia coli* were examined, assuming the discharge of ultraviolet (UV)-treated wastewater to water environment at different salinities. Suspensions of *E. coli* were first exposed to a low-pressure UV lamp in phosphate buffer to achieve 3 log inactivation, followed by an exposure to fluorescent light in NaCl solutions at the concentration of 1.0, 1.4, 1.9, 2.4 and 2.9 weight/volume %. When photoreactivation was completed in 3 h, survival ratio was recovered about 2 log in 1.0, 1.4, and 1.9% NaCl solutions, which was equivalent to the recovery observed in phosphate-buffered solution. Meanwhile, the recovery was suppressed to 0.8 log and −0.2 log in 2.4 and 2.9% NaCl solutions, respectively, which was significantly less than the recovery in phosphate buffer according to the *t*-test ($p < 0.05$). An endonuclease sensitive site assay demonstrated that the suppressed photoreactivation in 2.9% NaCl solution was due to the failure at repairing UV-induced pyrimidine dimers in the genome. In conclusion, photoreactivation of *E. coli* was significantly suppressed in NaCl solution at 2.4% or higher but not affected in NaCl solution at 1.9% or lower. This implies that photoreactivation of *E. coli* may potentially occur in brackish and coastal areas where salinity is rather low.

Key words | brackish water, *Escherichia coli*, photoreactivation, salinity, sodium chloride, ultraviolet disinfection

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INTRODUCTION

Ultraviolet (UV) irradiation is one of the options to disinfect water. Short-wavelength UV light (UV-C and UV-B, 220–320 nm) inactivates microorganisms through photobiological damage such as formation of lesions in the genome. The major lesions induced by germicidal UV light (254 nm) are *cis-syn* cyclobutane pyrimidine dimers, whereas (6–4) photoproducts and other lesions are also formed at lower rates (Friedberg *et al.* 1995). The presence of these lesions inhibits the normal replication and transcription of genome and therefore results in the inactivation of microorganisms. Some organisms, however, possess the ability to repair UV-damaged DNA by photoreactivation and dark repair (Friedberg *et al.* 1995). Photoreactivation is a phenomenon that UV-inactivated microorganisms regain the activity through the repair of pyrimidine dimers in the genome by utilizing the energy of UV-A and visible light (320–500 nm) with a specific enzyme, photolyase. DNA repair mechanisms other than photoreactivation, such as nucleotide excision repair, is called dark repair in contrast with photoreactivation. Photoreactivation may be a significant concern especially when UV-treated wastewater is discharged to natural water environment, because UV-inactivated microorganisms are exposed to sunlight which includes UV-A and visible light.

Many researchers have investigated photoreactivation of microorganisms in water (Lindenauer & Darby 1994; Tosa & Hirata 1999; Zimmer & Slawson 2002; Quek & Hu 2008; Hallmich & Gehr 2010), but quantitative studies on photoreactivation in saline water is limited (Chan & Killick 1995; Baron & Bourbigot 1996). Considering that treated wastewater is discharged to coastal areas in many countries, photoreactivation in the marine environment would be a

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significant public health concern. Some studies reported decreased photoreactivation in natural marine water environment (Chan & Killick 1995; Baron & Bourbigot 1996), implying the repressive effects of salinity on photoreactivation, but the decrease may be partially due to some factors other than salinity such as enhanced solar light inactivation (Fujioka *et al.* 1981; Yang *et al.* 2000; Sinton *et al.* 2002; Boukef *et al.* 2010), predatory activities in natural seawater (Enzinger & Cooper 1976; Chandran & Hatha 2005), and simply precipitation. Moreover, the effects of different salt concentrations on photoreactivation is not well documented yet, although ambient salt concentration varies drastically when discharging treated wastewater to coastal areas.

The objective of this study was to elucidate the effects of sodium chloride on photoreactivation of *Escherichia coli*, in order to understand the significance of photoreactivation in brackish and coastal areas under the influence of UV-treated wastewater. To mimic the salt condition that UV-treated wastewater is discharged to coastal areas, UV exposure was performed in phosphate-buffered solution while subsequent photoreactivation procedures were performed in NaCl solutions at different concentrations. Photoreactivation was examined using two assays, a cultivation assay to determine survival ratio of *E. coli* in parallel with an endonuclease sensitive site (ESS) assay to determine the number of UV-induced pyrimidine dimers in the genomic DNA (Oguma *et al.* 2001, 2002, 2004; Eischeid & Linden 2007).

METHODS

Microorganism

We examined *E. coli* because it is commonly used as a microbial indicator of water quality worldwide. A pure culture of *E. coli* K12 strain IFO 3301 (Institute for Fermentation, Osaka, Japan) was used as the test microorganism. A few purified colonies of *E. coli* were incubated in LB broth (Invitrogen) at 37 °C for 20 h until the growth reached the stationary phase. The bacterial cells were separated by centrifugation at 6,000 *g* for 8 min at 25 °C and the pellet was washed twice with a sterilized 0.33 mmol L^{-1} phosphate-buffered solution (pH 7.2) to remove the broth. Finally, *E. coli* were suspended in a 40 mL of phosphate-buffered solution and placed in a sterilized Petri dish (10 mm diameter) for light exposures.

Light exposure

A set of two low-pressure UV lamps (GL15, 15 W each, National) was placed horizontally and the emission was delivered down to a sample in a Petri dish after collimation. The irradiance of UV light at the wavelength of 254 nm was 0.97 mW cm^{-2}, which was measured by a biodosimeter using F-specific RNA coliphage Qβ (Kamiko & Ohgaki 1989). Under the conditions adopted in this study, a UV dose of 7.8 mJ cm^{-2} was required to achieve 3 log inactivation of *E. coli*. For photoreactivation procedures, samples were exposed to a set of three fluorescent lamps (FL20SW-B, 20 W each, GE/Hitachi) for 3 h after UV inactivation. The irradiance of fluorescent lamp at 360 nm was 82 μW cm^{-2}, as measured at the surface of samples with a UV radiometer (UVR-2 UD-36, Topcon). The emission spectra of the fluorescent lamps are shown in Figure 1.

A part of UV-irradiated samples was kept under dark condition to examine dark repair. In addition, to examine direct inactivation of *E. coli* by fluorescent light, *E. coli* suspensions were prepared in the same manner as detailed above, and simply exposed to fluorescent light without preceding UV exposure under the same condition of photoreactivation procedures. To examine background growth and decay, control samples were prepared by putting *E. coli* suspensions under dark condition for 3 h without preceding UV exposures.

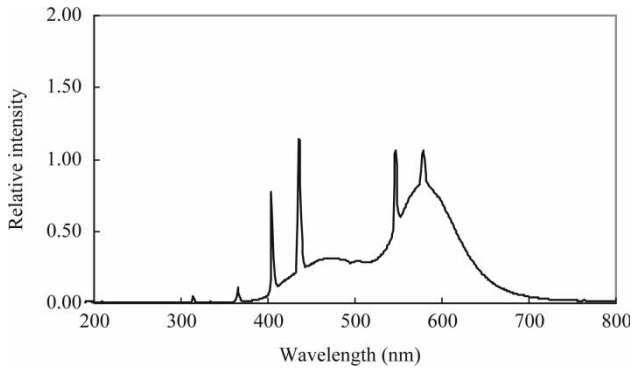

Figure 1 | Emission spectra of fluorescent lamp used in this study. The peaks are given in relative intensity and dimensionless.

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All *E. coli* suspensions were constantly stirred magnetically throughout the experiment and kept under dark conditions except during exposures to UV and fluorescent light. The samples were kept at room temperature throughout the experiment except incubation procedures.

Replacement of solutions

To mimic the discharge of UV-treated wastewater to coastal areas, UV exposure was performed in phosphate buffer while subsequent exposure to fluorescent light was performed in NaCl solutions. A 1.0 g of NaCl (Kishida Chemical) was dissolved in 100 mL of MilliQ water to obtain a 1.0 weight/volume % of NaCl solution. Similarly, 1.5, 2.0, 2.5, and 3.0 w/v% of NaCl solutions were prepared, and all NaCl solutions were autoclaved before use. To replace solutions, 40 mL of UV-exposed *E. coli* suspension (in phosphate buffer) was concentrated with a centrifugal filter device, Centriprep (Ultracel YM-50, Millipore) following the manufacturer's protocol to obtain 2 mL of concentrate, which was then transferred to 38 mL of sterilized NaCl solution as prepared above. Accordingly, the final NaCl concentrations in two digits were 1.0, 1.4, 1.9, 2.4, and 2.9 w/v%. In this paper, NaCl concentration is provided in the weight/volume percent hereafter. A preliminary test indicated that the recovery efficiency of *E. coli* was 82% (± 7 as standard deviation, $n = 3$) with the Centriprep system.

Cultivation assay

The survival ratio of *E. coli* was investigated by a pour plate method using a Chromocult Coliform Agar (Merck) in a dark room. All the plated samples were covered with aluminum foil to avoid unexpected light exposure and incubated at 37 °C for 20 h. The number of colony-forming units (CFU) was determined, and the survival ratio of *E. coli* was calculated as follows:

$$S_t = N_t/N_0$$

where S_t is the survival ratio at time t, N_t is the number of CFU at time t, and N_0 is the number of CFU before UV irradiation. The initial concentration of *E. coli* for cultivation assay was $2.5–4.0 \times 10^6$ CFU mL^{-1} in most cases, and was elevated to $1.0–1.6 \times 10^8$ CFU mL^{-1} when conducted in parallel with an ESS assay (detailed below). Experiments were repeated three times independently and each experiment included duplicate plate sets.

Endonuclease sensitive site (ESS) assay

An ESS assay allows a quantification of UV-induced pyrimidine dimers in genomic DNA with a UV endonuclease, which incises a phosphodiester bond specifically at the site of a pyrimidine dimer. The molecular lengths of fragmented DNA are determined by alkaline agarose gel electrophoresis, followed by a theoretical calculation to obtain the number of ESS (Sutherland & Shih 1983; Freeman *et al.* 1986).

The procedures for the ESS assay used in this study were basically the same as described previously (Oguma *et al.* 2001, 2002, 2004). Briefly, 5 mL of *E. coli* suspension at the initial concentration of $1.0–1.6 \times 10^8$ CFU mL^{-1} was centrifuged at 6,000 *g* for 10 min at 25 °C, and the pellet was subjected to DNA extraction procedures (Genomic-tip 20/G, Qiagen) following the manufacturer's protocol. The extracted DNA was concentrated using a centrifugal filter device (Centricon Ultracel YM-100, Millipore) at 2,000 *g* for 15 min at 25 °C, and resuspended in a UV endonuclease buffer, containing 30 mmol L^{-1} of Tris (pH 8.0), 40 mmol L^{-1} of NaCl, and 1 mmol L^{-1} of EDTA. The DNA solution was treated at 37 °C for 45 min with a UV endonuclease obtained from *Micrococcus luteus* following the method of Carrier & Setlow (1970). The reaction between genome and UV endonuclease was terminated by adding an alkaline loading dye at a final concentration of 100 mmol L^{-1} of NaOH, 1 mmol L^{-1} of EDTA, 2.5% Ficoll, and 0.05% bromocresol green. The DNA sample was subjected to an electrophoresis, along with a molecular length standard T4 GT7 + T4 GT7/*Bgl*I digest mixture (Marker 7GT, Wako Chemical), at 0.5 V cm^{-1} for 16 h at room temperature on 0.5% alkaline agarose gel in an alkaline buffer, containing 30 mmol L^{-1} of NaOH and 1 mmol L^{-1} of EDTA. After electrophoresis, the gel was stained in an ethidium bromide solution at 0.5 μg mL^{-1} overnight, photographed, and analyzed using a gel image analyzer (Gel Doc, Bio-Rad). A distribution pattern of the fluorescence intensity was

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obtained, and the median migration distance of each sample was determined photographically. The median migration distance was converted into the median molecular length (L_{med}) of the DNA, in relation to the molecular length standards. The average molecular length (L_n) of the DNA was obtained using the equation below (Veatch & Okada 1969):

$$L_n = 0.6 \times L_{med}$$

The number of ESS per base was calculated with the equation below (Freeman *et al.* 1986):

$$\text{ESS/base} = [1/L_{n(+UV)}] - [1/L_{n(-UV)}]$$

where $L_{n(+UV)}$ and $L_{n(-UV)}$ are the average molecular lengths of UV-irradiated and non-irradiated samples, respectively.

The ESS remaining ratio was obtained as the ratio of the number of ESS during fluorescent light exposures to the number of ESS before fluorescent light irradiation.

Statistical analysis

Microsoft Excel 2010 software was used for the *t*-test at the significance level of 0.05.

RESULTS

Figure 2 shows the profiles of survival ratio of *E. coli* during photoreactivation in different solutions after 3 log inactivation by UV. Figure 3 summarizes the change in survival ratio after photoreactivation, dark repair and fluorescent light irradiation in solutions at different NaCl concentration. Control samples, which were kept under darkness without UV and fluorescent light exposures, did not increase or decrease the survival ratio in 3 h in either case, indicating that growth and light-independent decay of *E. coli* were not significant under the experimental condition adopted in this study.

As shown in Figure 3(a), for *E. coli* in phosphate buffer at the initial concentration of $2.5–4.0 \times 10^6$ CFU mL^{-1}, the survival ratio recovered about 2.0 log by photoreactivation.

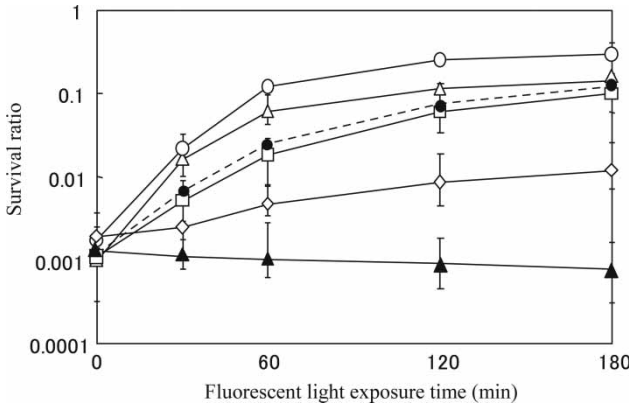

Figure 2 | Survival ratio of *E. coli* during exposure to fluorescent light (FL) after UV inactivation. Symbols indicate the mean of two or three independent experiments, and bars indicate the maximum and minimum values. FL exposure was conducted in phosphate buffer (• with dashed line) or in NaCl solutions at the concentration of 1.0% (△), 1.4% (○), 1.9% (□), 2.4% (◇), and 2.9% (▲).

In NaCl solutions at the concentrations of 1.0, 1.4, and 1.9%, photoreactivation performed 2.2, 2.2, and 1.9 log recovery of survival ratio, respectively, which was not significantly different from the recovery observed in phosphate buffer ($p > 0.05$ according to *t*-test). On the other hand, photoreactivation showed only 0.8 log increase in 2.4% NaCl solution and no increase (−0.2 log) in 2.9% NaCl solution, which was significantly less than the recovery in phosphate buffer ($p < 0.05$, *t*-test). Similar effects of NaCl on photoreactivation were observed when the initial concentration of *E. coli* was elevated to $1.0–1.6 \times 10^8$ CFU mL^{-1} (Figure 3(b)), which was in accordance with earlier research indicating no correlation between photoreactivation and the initial concentration of coliforms in wastewater (Lindenauer & Darby 1994; Hallmich & Gehr 2010).

Figure 3 further indicates the effects of NaCl on dark repair and fluorescent light exposure. Dark repair was suppressed in 1.9, 2.4 and 2.9% of NaCl solutions, which was significantly less than the dark repair in phosphate-buffered solution ($p < 0.05$, *t*-test). When *E. coli* suspensions were exposed to fluorescent light without preceding UV irradiation, the survival ratio was almost constant in phosphate buffer, but significantly decreased in NaCl solutions at the concentration of 1.9, 2.4 and 2.9% ($p < 0.05$, *t*-test).

Figure 4 shows an example of gel image obtained by ESS assay, showing a result of *E. coli* exposed to UV and

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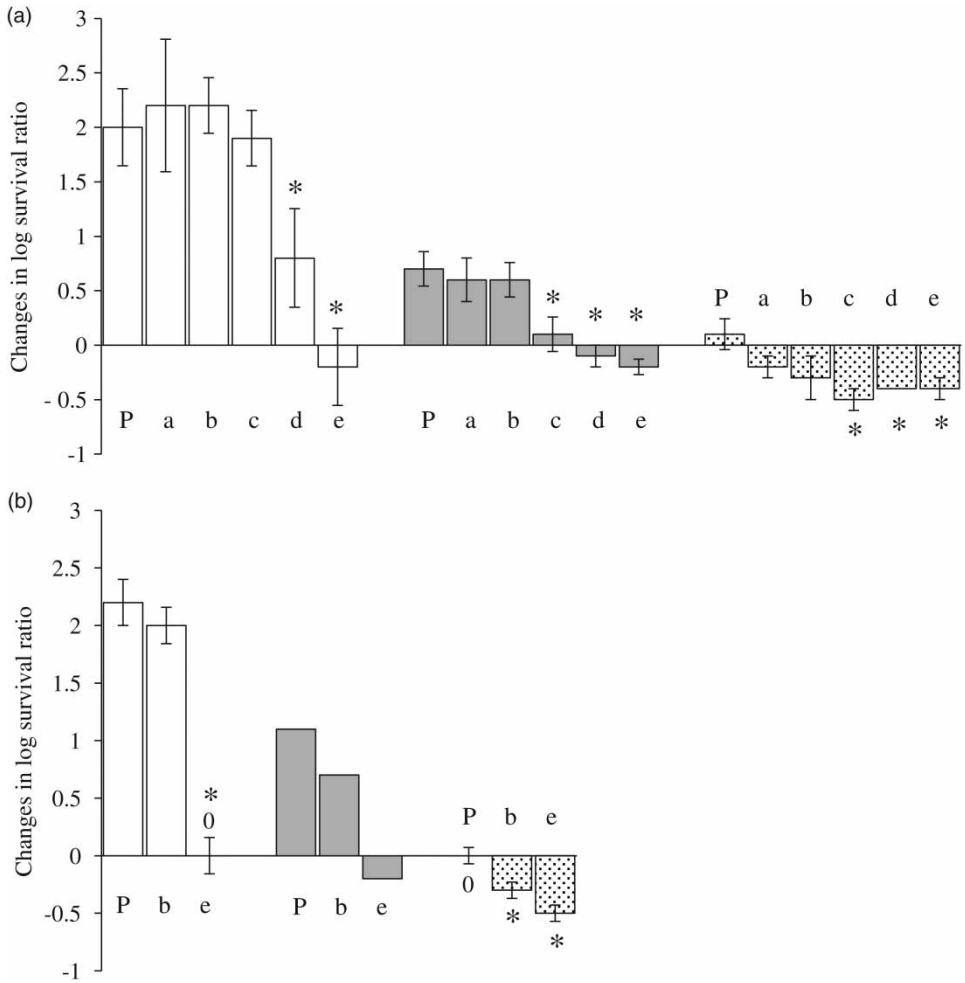

Figure 3 | Changes in log survival ratio by photoreactivation, dark repair and fluorescent light exposure. Initial concentration of *E. coli* was set at $2.5–4.0 \times 10^6$ CFU mL^{-1} (a) or $1.0–1.6 \times 10^8$ CFU mL^{-1} (b). Result of photoreactivation (□), dark repair (■) and fluorescent light exposure (⁘) in 3 h are given as the mean of three independent experiments with error bars showing the standard deviation. Sample names are given in horizontal axis; P for phosphate buffer while a, b, c, d and e for NaCl solutions at 1.0, 1.4, 1.9, 2.4 and 2.9%, respectively. Asterisks indicate the data were significantly different from the case of phosphate-buffered solution, according to the *t*-test ($p < 0.05$). Dark repair was tested once for the initial concentration of *E. coli* at $1.0–1.6 \times 10^8$ CFU mL^{-1}.

fluorescent light in phosphate buffer. The number of ESS induced by a 3 log inactivation of *E. coli* was 2.7 ± 0.8 ESS per 10^4 base ($n = 9$, mean ± standard deviation), which was equivalent to earlier studies (Oguma *et al.* 2001).

Figure 5 shows the ESS remaining ratio during exposure to fluorescent light after UV inactivation. About 95 and 85% of the UV-induced ESS was repaired in phosphate buffer and 1.4% NaCl solution, respectively, during exposure to fluorescent light. Meanwhile, in 2.9% NaCl solution, ESS was not repaired and was even slightly increased by fluorescent light exposure, resulting in the ESS remaining ratio of 118% after 3 h.

Figure 6 shows the number of ESS during fluorescent light irradiation without preceding UV inactivation, indicating the increase of ESS in NaCl solution under fluorescent light exposure. The fluorescent light exposure for 3 h induced 0.5 ± 0.2 ESS per 10^4 base ($n = 3$, mean ± standard deviation) in 1.4% NaCl solution and 1.1 ± 0.02 ESS per 10^4 base ($n = 3$, mean ± standard deviation). These numbers were about 1/5 to 2/5 of the number of ESS induced by a 3 log inactivation by UV exposures. Meanwhile, fluorescent light did not induce ESS in *E. coli* in phosphate buffer.

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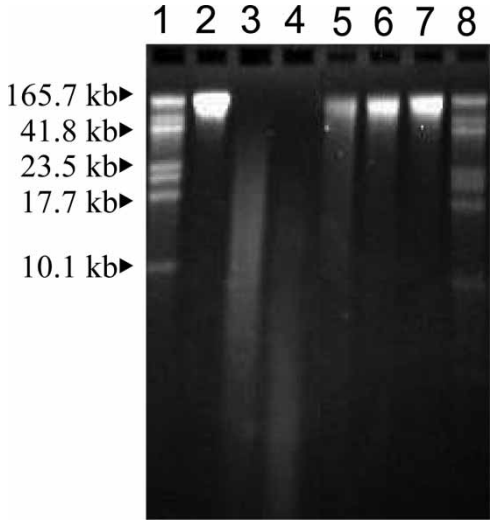

Figure 4 | A gel image of ESS assay for *E. coli* during UV and fluorescent light (FL) exposures in phosphate buffer. Lane 1, standard marker (T4 GT7 + T4 GT7/*Bgl* I digest mixture); lanes 2, 3 and 4, UV exposures at 0, 3.9 and 7.8 mJ cm^{-2}, respectively; lanes 5, 6 and 7, UV exposure at 7.8 mJ cm^{-2} followed by FL irradiation for 60, 120, and 180 min, respectively; lane 8, standard marker (T4 GT7 + T4 GT7/*Bgl* I digest mixture).

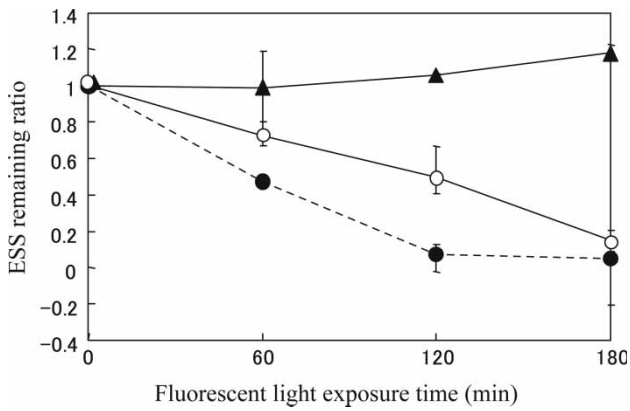

Figure 5 | ESS remaining ratio during exposure to fluorescent light (FL) after UV inactivation. Symbols indicate the mean of two or three independent experiments, and bars indicate the maximum and minimum values. FL exposure was conducted in phosphate buffer (● with dashed line), 1.4% of NaCl (○) or 2.9% of NaCl (▲).

DISCUSSION

Photoreactivation was significantly suppressed in NaCl solutions at 2.4% or higher (Figures 2 and 3), which was in accordance with the photorepair of ESS (Figure 5). These data suggest that, when NaCl concentration was high, *E. coli* could not repair UV-induced pyrimidine dimers in the genomic DNA and therefore failed to perform survival recovery.

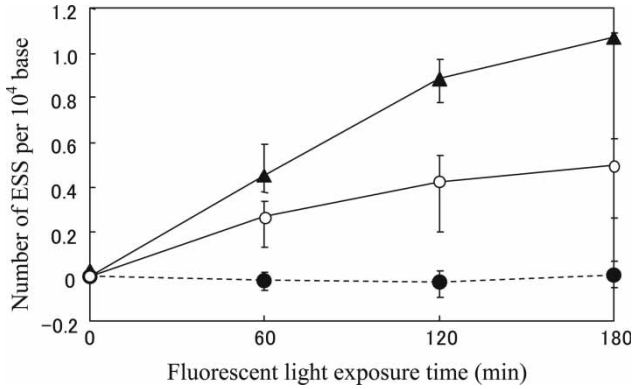

Figure 6 | The number of ESS during exposure to fluorescent light (FL) without preceding UV inactivation. Symbols indicate the mean of two or three independent experiments and bars indicate the maximum and minimum values. FL exposure was conducted in phosphate buffer (● with dashed line), 1.4% of NaCl (○) or 2.9% of NaCl (▲).

To our knowledge, all earlier studies on photoreactivation examined UV inactivation and photoreactivation in the same solution, either in freshwater or seawater. This study is unique in the viewpoint of water replacement, namely, UV inactivation was conducted in freshwater while photoreactivation was examined in saline water. Such procedures allowed mimicking of the situation when microorganisms are exposed to UV light in unsalted wastewater and subsequently exposed to solar light in brackish water or seawater. Accordingly, the results of this study would be informative to assume the potential photoreactivation in marine water environment under the influence of UV-treated wastewater.

Some past studies reported the suppressed photoreactivation in seawater and concluded that photoreactivation would not pose high risk in marine water environment (Chan & Killick 1995; Baron & Bourbigot 1996). This study, however, demonstrated that the photoreactivation is not always suppressed in seawater when the salinity is rather low. Namely, as shown in Figure 3, photoreactivation was not significantly suppressed in NaCl solutions when the concentration was 1.9% or lower. It is well recognized that the salinity of seawater is low in some bay areas. In Tokyo Bay, for example, the salinity is commonly below 2.0% in the top 3 m depth near the coast (Tokyo Bay Renaissance Promotion Conference 2013). It was reported that, with

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indigenous fecal coliforms in UV-treated wastewater, at least 0.065 mW cm^{-2} (440 lux) of visible light was required to initiate photoreactivation, which was equivalent to the light intensity for 1–2 m depth in natural water environments under natural solar light (Hallmich & Gehr 2010). Accordingly, it is assumed that discharged wastewater are exposed to solar light within 1–2 m depth for a certain period of time to initiate photoreactivation. One of the practical countermeasures against photoreactivation is to provide high UV doses in advance to make photoreactivation negligible (Guo *et al.* 2009). Accordingly, when installing UV disinfection systems at wastewater treatment plants that discharge treated water to brackish and coastal areas, it is recommended to examine photoreactivation in locally available seawater samples and take the data into account at designing the disinfection system.

Interestingly, as shown in Figure 5, ESS formation was detected under fluorescent light exposure in NaCl solutions without preceding UV irradiation. The fluorescent lamp adopted in this study emitted UV-A (320–400 nm) and visible light (Figure 1). Although UV-A and visible light inactivate microorganisms mainly by photo-oxidative reactions via photosensitizers and active species (Jagger 1981; Reed 2004), this study demonstrated that ESS was also induced by fluorescent light irradiation at small but detectable amounts in NaCl solution. A remaining question is why ESS was induced by UV-A and visible light in NaCl solutions but not in phosphate buffer. One possible explanation is that the *E. coli* possessed a dynamic mechanism of so-named 'concomitant photoreactivation' (Jagger 1981) in phosphate buffer. Namely, it is assumed that, under UV-A light, an equivalent number of ESS was induced and repaired simultaneously in phosphate buffer while the induction of ESS dominated the repair in NaCl solution.

Results of this study indicated the importance of considering photoreactivation of UV-treated *E. coli* after being discharged to brackish and coastal areas where salinity is rather low. However, natural seawater contains a variety of factors other than salinity, which may potentially influence photoreactivation in the marine water environment. Accordingly, it is important as a next step to examine photoreactivation in natural brackish water and seawater samples at different salinities to further understand the phenomenon.

CONCLUSIONS

Photoreactivation of *E. coli* was significantly suppressed in NaCl solutions at the concentration of 2.4% or higher, but not in NaCl solutions at 1.9% or lower. Accordingly, it was implied that *E. coli* in UV-treated wastewater may possess a potential to perform photoreactivation after being discharged to brackish and coastal areas where salinity is rather low. Considering that UV-treated wastewater is discharged to marine water environment in many countries, results of this study provide significant implications for the management of public health.

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