Applicability of LIVE/DEAD BacLight stain with glutaraldehyde fixation for the measurement of bacterial abundance and viability in rainwater

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ABSTRACT

Rainwater contains substantial bacteria and rain is an efficient pathway for the dissemination of bacteria from the atmosphere to land and water surfaces. However, quantitative information on rainwater bacteria is very limited due to the lack of a reliable method. In this study, the epifluorescence microscopy enumeration with the LIVE/DEAD BacLight Bacterial Viability Kit stain was verified to quantify the abundance of viable and non-viable bacterial cells in rainwater, with the 4',6-diamidino-2-phenylindole (DAPI) stain for the reference of total cell counts. Results showed that the total counts of bacterial cells by LIVE/DEAD BacLight staining were consistent with those by DAPI staining, and the average detection efficiency was (109 ± 29)%.

Introduction

Bacteria, as a major component of bioserosols, are likely playing important roles in physical and chemical variations in the atmosphere, as well as acting as cloud condensation nuclei and ice nuclei to initiate cloud formation and precipitation, and consequently impact the mass cycle and radiation transfer in the air (Delort et al., 2010; Georgakopoulos et al., 2009; Konstantinidis, 2014). Dissemination of bacteria via rain is considered one of crucial processes for linking bacterial communities between air, land, and water surfaces on the earth (Amato, 2012; Cho and Jang, 2014; Morris et al., 2014). To better understand and model the activities of bacteria in the atmosphere–biosphere–hydrosphere links, quantitative data on the concentration, status of live or dead, composition, and dynamics of bacteria in rain are essential (Konstantinidis, 2014). In addition, information on abundance and viability of rainwater bacteria is necessary to assess their influences on public health, ecosystems, biogeographical distribution, and meteorological and climatic processes (Fahlgren et al., 2010; Hara and Zhang, 2012).

The concentration and community composition of bacteria present in rainwater have been investigated in several studies. Herlihy et al. (1987) counted the total number of bacteria in rainwater samples by acridine orange (AO) direct counts and determined their ability to utilize formate and...
acetate. Casareto et al. (1996) and Matthias-Maser et al. (2000) reported the concentrations of total biological aerosol particles in rainwater based on the morphology and chemical identification of individual insoluble particles using electron microscopes. Peter et al. (2014) obtained that bacterial compositions in bulk (rain and dry) deposition samples differed significantly between rain events with and without Saharan dust influence reaching a high mountain lake in the Alps. Cho and Jang (2014) found that diverse bacteria existed in rainwater collected at an inland site using 16S ribosomal RNA (rRNA) pyrosequencing, and they were metabolically active based on adenosine triphosphate (ATP) measurement.

In these studies, the bacterial viability was generally ignored to analyze. Compared with the concentration and community composition of bacteria, the viability can be a more accurate and direct indicator of their environmental, climatic and human health-related impacts (Qi et al., 2015). In fact, most of the activities and consequences of airborne bacteria in rain remain speculative and the knowledge on bacteria in rainwater is very limited. This is mainly because there is not a carefully evaluated approach for the measurement of the concentration and viability of bacterial cells in rainwater, although multiple techniques have been developed to quantify bacteria in aquatic environments, such as river, lake and sea.

The common approaches to measuring bacteria in atmospheric water (e.g., cloud, rain, fog, and snow), including general biomass measures, measures of ATP levels, meta-genomic technologies, culture-based methods, single particle analysis, and fluorescence enumeration of stained cells, are summarized in Section S1 in Supplementary data. Most of these techniques cannot provide accurate information on the abundance and viability of bacteria. Enumeration after fluorescent staining of bacterial cells, with epifluorescence microscopy (EFM) or flow cytometry (FCM), is a traditional and common method of quantifying the abundance of bacteria in the air (Georgakopoulos et al., 2009). Among widely used fluorescent dyes, the LIVE/DEAD® BacLight™ Bacterial Viability Kit (LIVE/DEAD stain, Invitrogen™, Molecular Probes Inc., USA) was developed for the discrimination of viable (alive) and non-viable (dead) bacteria cells, i.e., the kit can assay the viability of bacteria, by dyeing them different colors. The kit is composed of two nucleic acid binding fluorescent dyes: SYTO 9 and propidium iodide (PI). With an appropriate mixture of SYTO 9 and PI stains, bacteria with intact cell membranes stain fluorescent green, scored as viable ones; and bacteria with injured membranes stain fluorescent red, scored as non-viable ones.

This LIVE/DEAD stain has been applied in various fields for about two decades since it was commercially available (Boulos et al., 1999), and its effectiveness has been proven for a diverse array of bacterial genera in soil, glacier, freshwater and drinking water, as well as in the atmosphere (Berney et al., 2007; Boulos et al., 1999; Cappa et al., 2014; DeLeon-Rodriguez et al., 2013; Hara and Zhang, 2012; Murata and Zhang, 2013, 2014). Based on microscopic enumeration using LIVE/DEAD staining, DeLeon-Rodriguez et al. (2013) reported the fraction of viable cells in hurricane and no-hurricane associated air samples collected in the upper troposphere; Hara and Zhang (2012) found the bacterial viability in dusty air was quite lower than that in non-dusty air; Murata and Zhang (2013) investigated the evolution of bacterial viability in the air during the passage of cyclones and anticyclones in southwestern Japan coast. The application of LIVE/DEAD stain has also been tested to two cloud water and one snow samples by Bauer et al. (2002). Therefore, staining with LIVE/DEAD stain is a potential approach to measuring bacteria in rainwater.

Aldehydes, e.g., formaldehyde, formalin and glutaraldehyde, are commonly used fixatives to stabilize the fine structure of cells prior to light or electron microscopic examination, and to preserve samples for direct bacterial counts by EFM (Hopwood, 1967; Kepner and Pratt, 1994; Kiernan, 2000). Formaldehyde and formalin have been applied in fixing cells in cloud water and rainwater samples before fluorochrome staining for direct EFM enumeration (Amato et al., 2005, 2007b; Bauer et al., 2002; Cho and Jang, 2014; Sattler et al., 2001). Murata and Zhang (2013, 2014) confirmed the efficiency of EFM enumeration using LIVE/DEAD stain with glutaraldehyde fixation and conducted its application to airborne bacteria. Glutaraldehyde (HCO-(CH2)-CHO) is a disinfectant, has a great potential for cross-linking via the –CHO groups and over variable distances, and is effective against vegetative forms of Gram-positive and Gram-negative bacteria (Gorman et al., 1980; Ross, 1966). It can thoroughly insolubilize and cross-link, but does not chemically change proteins in microbial cell membranes, and consequently can stabilize the structure of bacterial cells (Kiernan, 2000). Commercial 25% aqueous acid-potentiated glutaraldehyde solutions at approximately pH 3 contain 3% glutaraldehyde, hemiacetal and the polymers of the latter. Under neutral or slightly alkaline conditions, other glutaraldehyde polymers form, with the size of the polymers increasing with pH. As a precipitating complex form, glutaraldehyde loses its antimicrobial activity (Migneault et al., 2004). At pH 7.5–8.5, glutaraldehyde shows the best antimicrobial activity (Gorman et al., 1980). It is widely used as a rapidly penetrating fixative for electron microscopy.

In this study, we carefully tested and verified the operation procedures and conditions using LIVE/DEAD BacLight stain with glutaraldehyde fixation in EFM enumeration to quantify the abundance of viable and non-viable bacteria in rainwater samples, investigated the factors influencing the results in sample preparation, staining and enumeration, and tried to offer a reliable method of quantifying the number concentrations of viable and non-viable bacterial cells in rainwater.

1. Methodology

1.1. Outdoor experiments

1.1.1. Rainwater sample preparation

The samples used for the verification of operation and procedures were prepared from rainwater collected at a suburban site (32.806′N, 130.766′E) of Kumamoto in southwestern Japan. It was on the roof of a 20-m-high building on the campus of Prefectural University of Kumamoto. Surroundings of the campus are residential buildings. No obvious anthropogenic sources of biological particles except those in nature were expected during the periods of rainwater collection.

Rainwater was collected with samplers, each of which was composed of a sterilized beaker (PTFE, 1000 mL, Ø116 mm, Ø109 mm × 152 mm) and a sterile funnel (PMP, Ø210 mm). In
preparation, all beakers, funnels and other utensils were washed with a 10-fold diluted detergent solution of neutral pH (Scat 20X-N, Dai-ichi Kogyo Seiyaku Co., Ltd., Japan), ultrasonically cleaned for 30 min, and then rinsed with tap water, deionized water and ultrapure water (PURELAB Ultra, ELGA Labwater, Germany; 18.2 MΩ·m, <0.1 CFU/mL), copiously three times each in sequence. The cleaned utensils were wrapped with untapped aluminum foil and then autoclaved for sterilization. Before sample collection, the funnels and inner of beakers were rinsed with sterilized ultrapure water three times first, and then the sterility of the rainwater samplers was tested by pouring sterilized ultrapure water into the samplers and treating the water as negative control in the laboratory. The samplers were fixed at the sampling site without exposure to ambient air. They were opened to start collecting rainwater when it was raining. The sampling durations were within 1 day. After collection, the samplers were sealed and brought to the laboratory for treatment and analysis immediately.

1.1.2. Fluorescent staining and enumeration
For each rainwater sample, duplicate 5-mL subsamples were first treated with 0.2 mL of 0.2-μm-filtered 25% glutaraldehyde solution (Wako Pure Chemical Ind., Ltd., Japan; final concentration: approximately 1:100, V/V) to fix bacterial cells in a dark place at 4°C for 30 min, and then with 50 μL of LIVE/DEAD stain (L13152, Invitrogen™, Molecular Probes Inc., USA; final concentration: approximately 1:100, V/V) to stain bacterial cells in the samples under the same conditions for 15 min. The concentration, the incubation conditions and the duration for the fixing and staining were determined following the product information of agents and previous tests (Boulos et al., 1999; Murata and Zhang, 2013). After staining, samples were filtered with 0.2-μm-pore 25-mm-diameter black polycarbonate filters (Advantec Toyo Kaisha Ltd., Japan), and then the filters were mounted on microscope slides.

The number of bacterial cells on the filters was determined by direct enumeration using an epifluorescence microscope (Eclipse 80i, Nikon Corp., Japan) under blue excitation rays (450–490 nm), which is equipped with an imaging system (Digital Sight DS-L, Nikon Corp., Japan). Bacterial cells stained with LIVE/DEAD stain do not always emit distinct green and red colors, and also emit yellow and orange colors, i.e., intermediate states (weaken and damaged) of bacterial cells are also observed (Boulos et al., 1999; Berney et al., 2007). In this study, the concentrations of viable and non-viable bacteria in a sample were estimated by counting fluorescent green and red/orange/yellow cells which were spherical and close to or smaller than 1 μm in diameter (Hara and Zhang, 2012; DeLeon-Rodriguez et al., 2013). Counting cells on each black filter was conducted in 20 random fields of 100 μm × 100 μm area of the filter under 1000× magnification. The counting in each field was accomplished as quickly as possible, usually within 30 sec. Duplicate subsamples for negative control of corresponding rainwater samples were treated with the same procedures, and bacterial cells were counted in the same way. More details of the operation of sample staining and cell counting, which are the same as the investigations of bacterial cells associated with Asian dust and ambient aerosols in the air, were described in studies of Hara and Zhang (2012) and Murata and Zhang (2013, 2014).

The concentrations of viable and non-viable bacterial cells in rainwater (bacterial cells per milliliter of rainwater) were calibrated with the negative control samples. The bacterial viability was defined as the ratio of viable bacterial cells to the total bacterial cells, which was used to represent the potential living ability of bacterial cells.

The efficiency of LIVE/DEAD stain to bacteria in rainwater was assessed by the comparison with the results of 4’,6-diamidino-2-phenylindole (DAPI) stain (Cellstain DAPI solution, Dojindo Lab., Japan). DAPI reagent is a common stain for the enumeration of bacteria and protozoa in aquatic research (Bolter et al., 2002). It binds to double-stranded DNA (dsDNA) and RNA, and stains bacterial cells blue (Fig. 1a–b) under 365 nm ultraviolet excitation. The color of stained bacteria under microscopes actually fades gradually when exposed to excitation rays, i.e., fluorescence bleaching. Counting and photographing stained bacteria have to be done quickly. Otherwise, the results may have large uncertainties. In order to test if the fixation could benefit the quantification of bacterial cells in rainwater, the results from rainwater samples with and without glutaraldehyde fixation were compared. Duplicate subsamples (5 mL) of six rainwater samples for each treatment, i.e., LIVE/DEAD staining with and without glutaraldehyde fixation, and DAPI staining with glutaraldehyde fixation, were processed and enumerated.

1.2. Laboratory experiments

1.2.1. Glutaraldehyde fixation and phosphate buffer saline (PBS) addition
PBS can prevent possible damage of bacterial cells due to osmotic pressure change and also maintain at a relatively stable pH. In previous investigations, PBS was frequently applied in airborne bacteria enumeration coupled with LIVE/DEAD stain. Airborne bacterial cells were detached from the filters by shaking each filter in sterile filtered PBS or directly collected into the PBS liquid media, and then stained with or without glutaraldehyde fixation (DeLeon-Rodriguez et al., 2013; Hara and Zhang, 2012; Murata and Zhang, 2013). However, bacteria in aqueous samples (e.g., drinking water, cloud water, and melted snow and ice core samples) were usually treated directly without the addition of PBS (Bauer et al., 2002; Boulos et al., 1999; Cappa et al., 2014; Miteva et al., 2009). Different from atmospheric aerosols, the rainwater is acidic in most cases. Using acid-potentiated glutaraldehyde for bacterial cell fixation can increase the acidity of rainwater samples, which can result in unsuitable living conditions for microbes.

Two filtered rainwater samples were applied to investigate possible uncertainties in cell counting due to the fixation with glutaraldehyde and the addition of PBS. Each sample was separated into three subsamples (about 30 mL). Then three laboratory-cultured bacterial strains, Bacillus subtilis, Micrococcus sp., and Escherichia coli (E. coli) JM109 competent cells; Takara Bio Inc., Japan), were respectively transferred into the subsamples. The two bacterial strains, B. subtilis and Micrococcus sp., were isolated from aerosol samples in elevated layers (Hara et al., 2015).

Four 5-mL aliquots were prepared from each of the subsamples. Two of the four were respectively treated with and without glutaraldehyde (finally about 1%, V/V), stained
with the LIVE/DEAD stain, and observed with the EFM. The remained six aliquots were mixed with PBS first. Three, each with different strains transferred, were further treated with glutaraldehyde fixation. Then all the six were stained and observed with the EFM. The above treatments with and without the fixation of glutaraldehyde, and with and without the addition of PBS are summarized in Table 1. For the convenience of description, the treatments are named as Treatments I, II, III and VI (Table 1). Six actual rainwater samples were also processed with Treatments I to IV respectively.

The three bacterial strains were transferred to rainwater samples in the following procedures. The collected rainwater samples were filtered through sterilized 0.20-μm mixed cellulose ester membrane filters (Advantec, Toyo Roshi Kaisha, Ltd., Japan) using sterilized 10-mL syringes (Terumo Corp., Japan). The bacterial strains of B. subtilis, E. coli and Micrococcus sp. grown on R2A media (BD Difco™, Becton, Dickinson and Company, USA; pH 7.3) were transferred into 3-mL liquid media, tryptic soy broth (TSB; BD Bacto™, Becton, Dickinson and Company, USA; pH 7.3), and incubated at 30°C in a shaking incubator (125 r/min) for about 1 day. Before inoculation into filtered rainwater samples, 100-μL media with incubated bacterial cells were re-transferred into new 3-mL TSB media, and incubated under the same condition for 12–24 hr. The vegetative cells (OD A595nm 0.35–2.12) of the three bacterial species were first suspended respectively in filtered rainwater samples (1%, V/V), and then the samples were stirred on a vortex mixer at 3000 r/min (amplitude 5 mm) for 2 min.

1.2.2. Quality control
To further validate the application of LIVE/DEAD stain to bacteria in rainwater, multiple preliminary tests were carried out. Unprocessed (unfixed and unstained) rainwater subsamples were filtered and then slides were prepared for the EFM.

Fig. 1 – Examples of processed rainwater samples (21 February 2015) under the field of EFM: DAPI stain after the fixation (a, b); LIVE/DEAD BacLight stain without fixation (c, d); and after the fixation (e, f). The photographs at the left side were taken at the beginning of the excitation ray exposure, and those at the right side after 1 min of the exposure. EFM: epifluorescence microscopy; DAPI: 4′,6-diamidino-2-phenylindole.
observation. Five 5-mL aliquots of sterilized ultrapure water were directly treated (without rinsing) and observed as rainwater samples, to assess the number of bacterial cells which might contribute to total bacterial cells in rainwater samples due to the operations.

The influence of ambient aerosol diffusion to bacteria in rainwater was also investigated, taking no account of the deposition effect of bacteria due to their small size. A sampler prepared with the same procedures for rainwater collection was set aside of the one for rainwater collection simultaneously, but was covered with a plastic film on a higher shelf. This sampler was exposed to the ambient air with no rainwater dropping in when rainwater was collected by the rainwater sampler. After sample collection, it was rinsed with sterilized ultrapure water and the water was treated similarly to rainwater samples. This test was done in five periods of rainwater collection.

### 2. Results and discussion

#### 2.1. Efficiency of bacterial cell counts

The statistics on the bacterial abundance in six rainwater samples based on LIVE/DEAD- and DAPI-staining are summarized in Fig. 2. The total bacterial cell counts using LIVE/DEAD stain with fixation were approximately equal to those using DAPI stain with fixation (Wilcoxon signed rank test, two tailed $P = 0.699$), and the average ratio of LIVE/DEAD counts to DAPI counts was $(109 \pm 29)$% (Table S2). Compared with the results of DAPI stain with fixation, the efficiency of the LIVE/DEAD staining without fixation was $(103 \pm 25)$% (Wilcoxon signed rank test, two tailed $P = 0.937$). The results of Wilcoxon signed rank test indicate that the LIVE/DEAD staining methods can provide accurate information on bacterial concentration in rainwater. The viable and non-viable bacterial concentrations quantified in subsamples duplicated from the same samples showed good consistency with the standard deviation $(2.9 \times 10^3$ and $1.1 \times 10^3$ cells/mL on average) lower than the average concentration by one order of magnitude (Fig. 2). This result also indicates good accuracy in the measurements.

In previous studies, the detection efficiency of total bacterial cells in airborne particle samples with LIVE/DEAD- and DAPI-staining are summarized in Fig. 2. The total bacterial cell counts using LIVE/DEAD stain with fixation were approximately equal to those using DAPI stain with fixation (Wilcoxon signed rank test, two tailed $P = 0.699$), and the average ratio of LIVE/DEAD counts to DAPI counts was $(109 \pm 29)$% (Table S2). Compared with the results of DAPI stain with fixation, the efficiency of the LIVE/DEAD staining without fixation was $(103 \pm 25)$% (Wilcoxon signed rank test, two tailed $P = 0.937$). The results of Wilcoxon signed rank test indicate that the LIVE/DEAD staining methods can provide accurate information on bacterial concentration in rainwater. The viable and non-viable bacterial concentrations quantified in subsamples duplicated from the same samples showed good consistency with the standard deviation $(2.9 \times 10^3$ and $1.1 \times 10^3$ cells/mL on average) lower than the average concentration by one order of magnitude (Fig. 2). This result also indicates good accuracy in the measurements.

In previous studies, the detection efficiency of total bacterial cells in airborne particle samples with LIVE/DEAD stain were $(102 \pm 11)$% compared with DAPI staining (Murata and Zhang, 2013); and the total bacterial cell counts using LIVE/DEAD stain were close to those using Ethidium Bromide and DAPI stains (Hara and Zhang, 2012). In a study of bacteria in upper tropospheric aerosols, DeLeon-Rodriguez et al. (2013) compared the cell counting based on EFM using LIVE/DEAD staining with that based on qPCR. They found that the results using LIVE/DEAD staining were higher than those using qPCR by about one order of magnitude, and attributed the reason to the less small subunit rRNA (ssu rRNA) copies of bacteria in the samples relative to that used in the calculations, besides the possible underestimation by qPCR due to technical limitations.

Compared with that using AO staining, the enumeration using DAPI staining was reported to underestimate the abundance and cell size of bacteria. The reason was attributed to the insufficient amount of DNA in bacterial cells for DAPI staining (Suzuki et al., 1993) and also to the lower DNA synthesis rate with

### Table 1 – Summary of Treatments I–IV of the two filtered rainwater samples, and pH and electric conductivity (EC) of the treated samples, rainwater, 25% glutaraldehyde and PBS.

<table>
<thead>
<tr>
<th>pH a</th>
<th>EC b (mS/cm)</th>
<th>Rainwater (mL)</th>
<th>PBS (mL)</th>
<th>25% Glutaraldehyde (mL)</th>
<th>LIVE/DEAD stain (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>6.4/6.2</td>
<td>0.28/0.26</td>
<td>5.0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>II</td>
<td>3.9/3.8</td>
<td>0.31/0.30</td>
<td>5.0</td>
<td>–</td>
<td>0.2</td>
</tr>
<tr>
<td>III</td>
<td>7.6/7.6</td>
<td>7.10/7.10</td>
<td>5.0</td>
<td>5.0</td>
<td>0.10</td>
</tr>
<tr>
<td>IV</td>
<td>6.5/6.5</td>
<td>7.50/7.50</td>
<td>5.0</td>
<td>5.0</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>5.3/4.5</td>
<td>0.01/0.02</td>
<td>0.5</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>3.1</td>
<td>0.04</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>7.8</td>
<td>14.20</td>
<td>0.5</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

“–” means no addition.

a Measured with Laqua Twin AS-212 (Horiba Ltd., Tokyo, Japan).

b Measured with Twin Cond. B-173 (Horiba Ltd., Tokyo, Japan).
DAPI stain for the visualization of bacteria (Posch et al., 2001). However, we found that it is a little difficult to differentiate between other kinds of insoluble particles (such as dust particles) in the size close to or smaller than 1 μm and bacterial cells in DAPI- and LIVE/DEAD-stained samples. In DAPI-stained samples, the mineral particles were colored greenish yellow or white (Murata and Zhang, 2013), similar to the blue bacterial cells (Fig. 1a–b). In LIVE/DEAD-stained samples, both small mineral particles and a few bacterial cells exhibited yellow color. Whereas, for bacteria, the consistency of total counts with LIVE/DEAD- and DAPI-staining indicates that LIVE/DEAD staining with glutaraldehyde fixation could act as an alternative approach of DAPI staining to quantify bacterial cells in rainwater.

2.2. Improvement by glutaraldehyde fixation

Fixation with glutaraldehyde can strengthen the membrane of bacterial cells. Murata and Zhang (2013) suggested that 1% glutaraldehyde fixation was applicable for the improvement of enumeration accuracy using LIVE/DEAD staining for aerosol samples. In this study, the total bacterial cell counts using LIVE/DEAD stain with glutaraldehyde fixation were a little higher than those without fixation (Fig. 2). The ratio of cell count with fixation to that without fixation was (106 ± 5%) on average (Wilcoxon signed rank test, upper tail P = 0.312). The counts of viable and non-viable bacterial cells in corresponding unfixed and fixed samples had small differences (Wilcoxon signed rank test, upper tail P = 0.242, 0.469). The ratios of viable and non-viable cells in fixed samples to those in unfixed ones were (108 ± 7)% and (104 ± 11)% (Table S2), respectively, indicating a small improvement of detection efficiency for both viable and non-viable bacterial cells with the fixation.

Murata and Zhang (2013) found that the color of viable bacterial cells in unfixed aerosol samples faded faster than in the fixed samples under the excitation ray exposure. The reduction ratio of the total cells within 1 min in unfixed samples (23%) was much higher than that in fixed samples (7%). The EFM images of LIVE/DEAD stained rainwater samples without fixation and after the fixation at the beginning and after 1 min of the excitation ray exposure are shown in Fig. 1c–f. In this study, fluorescent fading was found in both unfixed and fixed samples. In rainwater samples treated with glutaraldehyde fixation, the shape of bacterial cells was clearer, and the fading of labeled color of bacteria under the excitation ray exposure was slower than in those without fixation. But the fading was not very obvious for viable bacterial counts in the rainwater samples within 1 min, in comparison with the previous results in aerosol samples (Murata and Zhang, 2013).

Bacterial strains were inoculated into filtered rainwater samples to clarify the strengthening effect and uncertainties of glutaraldehyde fixation in rainwater. Fig. 3 shows the examples of LIVE/DEAD stain–stained B. subtilis, Micrococcus sp., and E. coli in filtered rainwater with and without glutaraldehyde fixation at the beginning and after 1 min of the excitation ray exposure. Glutaraldehyde induced auto-fluorescence can be observed in cells fixed for fluorescence microscopy. The wavelength of the actinic beam and pH of the environment can influence the amount of its enhancement significantly. Longer wavelengths and acid pH produce a smaller effect (Collins and Goldsmith, 1981). Herein we considered the influence of glutaraldehyde-induced fluorescence on fixed bacterial cells in rainwater was insignificant (Section S5), and the fluorescent color changes of bacteria cells reflected their actual statuses after each treatment. To ensure the quality of enumeration results, it is better to pre-evaluate the influence of glutaraldehyde fixation by comparing the results of fixed and unfixed rainwater samples.

Fixed Gram-positive bacteria, B. subtilis and Micrococcus sp., were of lower viability, and almost all cells of B. subtilis were dead, while most of those unfixed cells remained alive. Almost all cells of E. coli (Gram-negative) remained alive, but a little more non-viable cells were detected in unfixed samples than in fixed samples (Fig. 3 and Table S4). Yamashita et al. (2012) assessed the viability of the immobilized Magnetospirillum magnetum (Gram-negative) cells on mica with glutaraldehyde using LIVE/DEAD stain, and also found only a few of the cells were dead for at least 1 hr after immobilization. Laboratory tests with the same bacterial cells suspended in ultrapure water exhibited similar results, while experiments with bacterial strains in PBS showed that alive bacterial cells were predominant after fixation (Section S3). In the microscopic field, fixed bacterial cells were obviously clearer in shape than unfixed ones, especially for E. coli, and the bacterial cells of B. subtilis and E. coli with glutaraldehyde fixation were more dispersed than those without fixation. These features were likely related to the changes of physicochemical and mechanical properties of the bacterial cell surface after glutaraldehyde fixation (Sheng et al., 2008). The color of B. subtilis, Micrococcus sp. and E. coli bleached under the excitation ray exposure, although the fading effect was not obvious within 1 min (Fig. 3).

The requirements for bacterial survival and growth include physical conditions (e.g., temperature, pH, and osmotic pressure), and chemical conditions (e.g., carbon, iron, copper, and zinc; nitrogen, sulfur, and phosphorus; and oxygen based on bacteria classifications). The pH and electric conductivity of the rainwater after the treatments and before the filtration for counting are listed in Table 1. Most bacteria prefer neutral conditions (pH 6.5–7.5), depending on species. Steady-state log-phase B. subtilis cultures could survive 60%–100% when the pH changed from 6.0 to 4.5, and the cultures growing at pH 7.0 might survive less than 15% at pH 4.5 (Wilks et al., 2009). Optimal growth of two members of the genus Micrococcus was recorded at pH 6.0–6.2 (range 5–9) and pH 7.5 (Liu et al., 2007; Mohedano et al., 1997). However, E. coli strains at stationary phase can survive several hours at pH 2–3, which is considerably lower than the acid limit (about pH 4.5) for growth (Small et al., 1994). Lu et al. (2013) reported a previously uncharacterized type of acid resistance system that relies on l-glutamine in E. coli. It is sufficient for E. coli survival under extremely acidic conditions (pH ~ 2). The alive status of three bacteria species, B. subtilis, Micrococcus sp. and E. coli, in filtered rainwater fixed with and without glutaraldehyde in our experiments matched these alive and dead natures of the strains under different pH conditions. B. subtilis and Micrococcus sp. trended toward lower viability in acid circumstances (Table 1 and Table S4). The pH values of rainwater samples in this study were in the range of 4.3–5.3. Hence, as species such as B. subtilis and Micrococcus sp. existed in rainwater (Amato et al., 2005, 2007a; Cho and Jang, 2014), some viable cells should
possibly have been identified as non-viable ones because glutaraldehyde fixation could damage the bacterial cells. In other words, the counts of viable cells might be underestimated due to the fixation.

Phosphate groups ($\text{PO}_4^{3-}$) in phospholipids are a major component in bacterial cell membranes and can be modified by organic molecules (e.g., choline). The fixation with glutaraldehyde suppresses the activation of enzyme (e.g., phosphatase), because of the content of inorganic phosphates in commercial glutaraldehyde (Hopwood, 1967; Fahimi et al., 1968). Further, the phosphodiester bonds between ribitol or glycerol residues, of which the teichoic acid in cell walls of Gram-positive bacteria is composed, can be broken by hydrolysis when acid, alkali and enzyme exist. In addition, the increase of osmotic pressure (increased electric conductivity) due to glutaraldehyde fixation might also injure cell membranes (Kiernan, 2000). For these reasons, it is generally considered that using glutaraldehyde fixation may lead to the underestimate of viable bacteria counts, which is consistent with the results of our laboratory experiments with cultured $B. \text{subtilis}$ and Micrococcus sp. strains. We found phosphate (0.2 mg/L on average) in the fixed rainwater samples, which could have led to the breakage of Gram-positive bacterial cell walls and injure the cell membranes.

However, our measurements of bacterial cells in the collected rainwater with and without the fixation showed a small improvement of the detection efficiency of viable cells by the fixation. This was also confirmed in the previous investigation of airborne bacteria with similar fixation (Murata and Zhang, 2013, 2014). Therefore, unlike cultured bacterial strains whose viability can be reduced by fixation, bacteria of the same species living in

Fig. 3 – Laboratory-cultured $B. \text{subtilis}$, Micrococcus sp., and $E. \text{coli}$ with TSB medium in filtered rainwater samples stained by LIVE/DEAD stain without (a–f) and with (g–l) glutaraldehyde fixation at the beginning (0 min, a–c and g–i) and after 1 min (d–f and j–l) of the excitation ray exposure. TSB: tryptic soy broth.
real rainwater are likely resistant to the fixation and viable ones do not substantially lose their viability in the fixation.

2.3. Influence of PBS addition

PBS is isotonic and non-toxic to bacteria cells, and can be used to stabilize pH. Laboratory experiments with addition of PBS were conducted to investigate the influence of pH on fixation, staining and enumeration of rainwater bacteria. The photograph examples of B. subtilis, Micrococcus sp., and E. coli in filtered rainwater samples diluted by PBS and stained by LIVE/DEAD stain with and without glutaraldehyde fixation are illustrated in Fig. 4 and described in Table S4. B. subtilis exhibited low viability in fixed samples (pH 6.5), but significantly high viability in unfixed ones (pH 7.6). In contrast, Micrococcus sp. and E. coli bacterial cells in both fixed and unfixed samples trended toward higher viability (Fig. 4 and Table S4). The status of E. coli cells in fixed and unfixed samples was similar in the samples without the addition of PBS. These differences of bacterial status should be dependent on their different resistivity to the pH and osmotic pressure variations in the circumstances (Table S4).

Fig. 5 illustrates the examples of microscope photographs of six actual rainwater samples diluted by PBS and stained by LIVE/DEAD stain with and without glutaraldehyde fixation. The color of bacteria in fixed samples was much clearer, and the bleaching effect, in particular for viable bacterial cells, was apparently slower than those of unfixed samples during EFM observation. The comparison of enumeration results for Treatment I to IV is illustrated in Fig. 6. Compared with samples without PBS dilution, the counts of viable bacteria trended to increase in the PBS-diluted and glutaraldehyde-fixed samples. Different from single bacteria species, the bacterial viability in the PBS diluted samples was slightly higher than or comparable

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**Fig. 4** - Laboratory-cultured B. subtilis, Micrococcus sp., and E. coli with TSB medium in filtered rainwater samples diluted by PBS and stained by LIVE/DEAD stain without (a–f) and with (g–l) glutaraldehyde fixation at the beginning (0 min, a–c and g–i) and after 1 min (d–f and j–l) of the excitation ray exposure. TSB: tryptic soy broth; PBS: phosphate buffer saline.
to that in PBS-free samples. Though artificial mistakes may be introduced due to PBS addition, overall, the abundance and viability of bacterial cells detected by the LIVE/DEAD staining in rainwater samples with Treatments I to IV were not largely different in most cases (Fig. 6). It is likely that the bacteria species in rainwater have adapted to keeping their viability in the acidic ionic circumstances. Bacteria surviving in rainwater had experienced harsh conditions in the atmosphere, including acidic pH, low temperature, strong UV light intensity, cycled freeze-thaw, desiccation, and osmotic shock (Amato, 2012). Therefore, they could survive against the pH and osmotic change of solutions better, although the reasons have not been completely uncovered and should be an important subject in future studies on the viability of bacteria in rainwater.

The effects of glutaraldehyde fixation differ according to bacterial species, and properties of solution (e.g., pH, chemical compositions). Peracchia and Mittler (1972) studied the improvements in glutaraldehyde fixation by raising the pH in steps from 7 to 8, and suggested a better penetration of the fixative at neutral pH and a better cross-linking in alkaline pH. Boulos et al. (1999) tested the factors affecting the staining procedure, including addition of glutaraldehyde, staining time, and chlorine. They considered that storage after glutaraldehyde fixation decreased the total and viable cell counts of some coliform strains. The viable counts of *E. coli* in NaCl solution (0.085%) could be reduced by 5% glutaraldehyde fixation in LIVE/DEAD staining (Boulos et al., 1999), in accordance with the results of Treatments III and IV in this study. Chlorination could also cause the reduction of viable counts with the increase of chlorine concentration. In addition, glutaraldehyde can be present in at least 13 different forms depending on solution conditions such as concentration, temperature, and pH. Yet there is no agreement about the main reactive species that participates the crosslinking process because monomeric and polymeric forms are in equilibrium (Migneault et al., 2004).

In this study, we found that the combination of glutaraldehyde fixation and LIVE/DEAD staining can improve the detection accuracy of viable and total bacterial cells in rainwater slightly. However, the effects of glutaraldehyde fixation were dependent on bacterial species, physical (e.g., pH and osmotic pressure), and chemical properties of rainwater samples (solutions). The factors, such as staining time, and chemical properties of rainwater, may also affect the LIVE/DEAD staining. The bacterial abundance and viability detected in rainwater samples treated with PBS dilution and glutaraldehyde fixation trended to be a little bit higher than those detected in samples treated with glutaraldehyde fixation only, but the discrepancy was insignificant in most cases.

2.4. Influence of operation and atmospheric diffusion

Quality control experiments for possible contamination during the processes of sampling, storage and measurement revealed that nearly no bacterial cells were observed from the slides of unprocessed rainwater samples. The comparison of bacterial concentration and abundance in the collector-rinsing water (negative control) and the relevant six rainwater samples is shown in Fig. 7. The concentrations of viable and non-viable

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**Fig. 5** – Rainwater subsamples diluted by PBS for laboratory tests and stained by LIVE/DEAD stain without (a, b) and with (c, d) glutaraldehyde fixation at the beginning (0 min, a, c) and after 1 min (b, d) of the excitation ray exposure.
bacterial cells in the negative controls were $1.5 \times 10^3$–$5.2 \times 10^3$ ($(3.5 \pm 1.3) \times 10^3$) and $0$–$4.4 \times 10^2$ ($(4.2 \pm 4.2) \times 10^2$) cells/mL. They were about $6$%–$14$% ($(11 \pm 3)$%) and $0$–$10$% ($(4 \pm 4)$%) of those in rainwater samples (Fig. 7a), respectively. The total viable and non-viable bacterial concentrations were $(2 \pm 2)$% and $(0.7 \pm 0.7)$% of those in rainwater samples on average (Fig. 7b). Furthermore, the total bacterial counts in the directly treated sterilized ultrapure water samples were $(2.9 \pm 0.6) \times 10^3$ cells/mL $(2.8 \pm 0.6) \times 10^3$ viable cells and $(1.3 \pm 1.4) \times 10^2$ non-viable cells/mL on average, which was about $7$% of the counts in rainwater samples. Therefore, the uncertainties under the microscope were small. The accuracy of the detection was largely improved in comparison with the previous study of Natsume and Suzuki (2001), where the total bacterial concentration in negative control accounted for $13$%–$27$% of the total concentration in rainwater with DAPI staining.

The above results indicate that bacterial counts in the negative control samples for calibration could not lead to large uncertainties in the results. However, we also found that the bacterial abundance in negative control could be higher than that in some rainwater samples in cases when the amount of collected rainwater was less than that of collector rinsed water. Hence we recommend to apply this method in the case of

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**Fig. 6** – Viable and non-viable bacterial concentrations in the rainwater samples (marked by G, H, and J–M) following Treatments I to IV (Table 1). Duplicate or triplicate rainwater subsamples (5 mL) for each treatment were processed and enumerated. The average bacterial concentrations in negative control (blank) samples are also illustrated.

**Fig. 7** – Bacterial concentrations and abundances in six rainwater samples (marked by A–F) and the relevant collector-rinsing water (blank).
the amount of collected rainwater more than that of negative control (~40 mL), i.e., the precipitation during the sampling durations higher than 1.2 mm. The bacterial cell concentrations in the negative control samples varied, and, on occasion, the bacterial concentration in the negative control could be quite higher than that in directly treated sterilized ultrapure water samples. Therefore, the negative control experiments for the sterility of ultrapure water, collectors, and operations are critically essential to ensure the quality of counting bacterial cells in rainwater. This point is particularly important for cases when the precipitation is small.

The total bacterial concentrations in the samples used for assessing the influence of atmospheric diffusion to the bacterial counts in rainwater were in the order of magnitude 10^3 cells/mL ((4.9 ± 1.5) × 10^3 cells/mL on average). All results were not much different and at the similar level to the average counts from the laboratory negative control samples (Wilcoxon signed rank test, P = 0.345). The average viable and non-viable bacterial concentrations in the rainwater samples detected in this study were in the range between 2.2 × 10^4–4.3 × 10^5 cells/mL and 4.4 × 10^2–2.4 × 10^5 cells/mL, with the mean concentrations (3.1 ± 0.9) × 10^4 cells/mL and (1.1 ± 0.7) × 10^5 cells/mL, respectively. Therefore, the uncertainties due to the operations and atmospheric diffusion by the approach of this study to measuring bacterial concentrations in rainwater were less than 12% on average.

3. Summary

An epifluorescence microscopy enumeration method using LIVE/DEAD BacLight Bacterial Viability Kit stain was tested and showed a good accuracy in counting viable and non-viable bacteria in rainwater. In comparison with DAPI stain, the LIVE/DEAD BacLight stain detected (109 ± 29)% of total bacterial cells. Bacteria in rainwater could survive better against the pH and osmotic changes due to glutaraldehyde fixation than cultured bacteria. The ratio of the total bacterial cell counts using LIVE/DEAD BacLight stain with fixation to that without fixation was (106 ± 5)% on average. The bacterial concentration in the negative controls was generally lower than that in the rainwater samples by about one order of magnitude, and the uncertainties due to the operation and air diffusion in measuring bacterial abundance were less than 12%. However, the concentration and also the abundance in the negative control could be occasionally higher than in rainwater samples in case of small precipitation.

These results indicate that, with careful verification, the LIVE/DEAD BacLight bacterial viability assay coupled with glutaraldehyde fixation is able to quantify the bacterial abundance and viability in rainwater. Careful negative control experiments considering the sterility of ultrapure water and collector, influences of glutaraldehyde and possible uncertainties from operations are critically essential for ensuring the accuracy of counting bacterial cells in rainwater.

It should be noticed that, compared with conventional methods (DNA-based and DAPI stain), LIVE/DEAD BacLight stain is usually more effective for gram-positive bacteria and cannot obtain the information on bacterial community (Murata and Zhang, 2013). The viability of bacterial cells identified with the kit is upon the status of bacterial cell membranes, i.e., injured or not injured, rather than upon metabolic activities.

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Appendix A. Supplementary data

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REFERENCES


