Method for the Direct Observation and Quantification of Survival of Bacteria Attached to Negatively or Positively Charged Surfaces in an Aqueous Medium

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ABSTRACT: The risk of groundwater contamination by microbial pathogens is linked to their survival in the subsurface. Although there is a large body of literature on the inactivation behavior of suspended (planktonic) microorganisms, little is known about the inactivation of bacteria when attached to sand grain surfaces in groundwater aquifers. The main goal of this study was to develop a fluorescence-based experimental technique for evaluating the extent of inactivation over time of bacteria adhered onto a surface in an aqueous environment. Key features of the developed technique are as follows: (i) attached cells do not need to be removed from the surface of interest for quantification, (ii) bacterial inactivation can be examined in real-time for prolonged time periods, and (iii) the system remains undisturbed (i.e., the aqueous environment is unchanged) during the assay. A negatively or positively charged substrate (i.e., bare or coated glass slide) was mounted in a parallel-plate flow cell, bacteria were allowed to attach onto the substrate, and the loss of bacterial membrane integrity and respiratory activity were investigated as a function of time by fluorescence microscopy using Live/Dead BacLight and BacLight RedoxSensor CTC (5-cyano-2,3-ditolyl tetrazolium chloride) viability assays. These two different measures of bacterial inactivation result in comparable trends in bacterial inactivation, confirming the validity of the experimental technique. The results of this work show that the developed technique is sensitive enough to distinguish between the inactivation kinetics of different representative bacteria attached to either a negatively charged (bare glass) surface or a positively charged (coated glass) surface. Hence, the technique can be used to characterize bacterial inactivation kinetics when attached to environmentally relevant surfaces over a broad range of groundwater chemistries.

INTRODUCTION

A thorough understanding of the mechanisms and factors affecting the fate and transport of microbial pathogens in the subsurface environment can be used to enhance the protection of public health from waterborne disease. The key processes governing the fate of pathogens in groundwater aquifers are retention by aquifer solid surfaces and inactivation (or survival) of the microbes.1,2 Accordingly, substantial research effort has been aimed at elucidating the role of various physical, chemical, and biological factors on microbial transport and retention in natural subsurface environments.3,4 Indeed, over the past two decades, more than 100 studies of microbe mobility in columns packed with natural or model aquifer materials have been reported.3,4 In contrast, the process of microbe inactivation in groundwater environments is less understood. In water saturated granular subsurface environments, microorganisms may become inactivated (i.e., die-off, enter a dormant state, or become nonculturable) in response to environmental factors. Virus, bacterial, and protozoan inactivation in the fluid phase is relatively straightforward to characterize using well-controlled laboratory experiments.4–6 Studies on the inactivation of suspended viruses reveal geometric mean inactivation rates on the order of 0.03 log_{10}day^{-1} for hepatitis A virus, coxsackievirus, and the bacteriophage PRD-1.5 Geometric mean inactivation rates for coliform bacteria, enterococci, and Salmonella spp. have been estimated at approximately 0.09 log_{10}day^{-1} when suspended in porewater.7 There remains, however, a dearth of knowledge on the effect of attachment to the grain surface on microbe inactivation. Although various studies have examined the inactivation of adherent cells on different surfaces, most research is directed to preventing bacterial adhesion and developing antibacterial surface coatings.7–9 To this end, the Live/Dead viability kit (Molecular Probes) as a two-color fluorescent assay of bacterial membrane integrity has been widely used to enumerate viable bacteria using direct microscopy. For instance, in one study, confocal laser scanning microscopy (CLSM) was used after Live/Dead staining to demonstrate the potential of polydiallyldimethylammonium chloride (p-DADMAC) coatings on glass to strongly enhance the adhesion and reduce the viability of selected
waterborne pathogens. Several studies have reported that positively charged biomaterial surfaces exert an antimicrobial effect on adhering bacteria. It is interesting that the metabolic activity of Escherichia coli (E. coli) and Bacillus brevis (B. brevis) increased when these organisms were adhered onto a negatively charged glass surface (in comparison to planktonic cells).

Although a number of studies have demonstrated the antimicrobial activity of positively charged surfaces, characterisation of the rate of inactivation of adhered bacteria by direct observation has not been performed to date. Hence, there exists a need for an experimental technique that allows for the characterisation of attached microbe inactivation rates under conditions relevant to groundwater environments.

The purpose of this work is to develop an experimental technique that can be used to evaluate the extent of inactivation of bacteria adhered onto a surface in an aqueous environment. The technique makes use of a parallel plate flow chamber (PPFC) containing the surface of interest. As a proof of concept, we use a positively charged 3-aminopropyltriethoxysilane (APTES)-coated glass slide (representative of positively charged patches on sand grains). Bacteria are allowed to adhere to the surface and the loss of bacterial membrane integrity and respiratory activity are quantified as a function of time without disturbing the cells using fluorescence microscopy based assays. The results obtained with the microscopy techniques are further compared to colony forming units (CFU) of detached cells. Directly monitoring the loss of bacterial membrane integrity and respiratory activity in situ as a function of time allows for the quantification of attached bacterial inactivation rates for three different strains: E. coli D21, E. coli O157:H7, and Enterococcus faecalis (E. faecalis). To further demonstrate the relevance of the new technique for natural groundwater conditions, additional experiments were conducted using a negatively charged bare glass slide (as silica is one of the most common constituents of sand). In experiments with the bare glass slide, only the loss in bacterial membrane integrity was measured to provide data for direct comparison with results obtained using the positively charged slide.

## MATERIALS AND METHODS

### Bacterial Strains and Growth Conditions.

E. coli D21 (Gram negative, nonpathogenic), E. coli O157:H7 ATCC 700927 (Gram negative, pathogenic), and E. faecalis ATCC 29212 (Gram positive, pathogenic) were used as the test bacteria. Pure cultures were maintained at –80 °C in Luria–Bertani (LB) broth supplemented with 30% glycerol. Cultures were spread onto LB agar plates that were then incubated at 37 °C for 2 h at 200 rpm and harvested by centrifugation at 8600 × g for 10 min (SS-34 rotor, Kendro) at 4 °C. The growth medium was decanted and the pellet was resuspended in 10 mM KCl. Centrifugation and resuspension were repeated one additional time to remove traces of growth media and metabolites. The concentration of the cell suspension was determined with a Helber (SV400, Proscitech) bacteria counting chamber and the suspensions were diluted accordingly to the desired final concentration of 3 × 10⁶ cells/mL in 10 mM KCl adjusted to pH 6.1. Analytical reagent grade chemicals (Fisher) and deionized (DI) (Biolab) water were used to prepare all solutions and media.

### Preparation of Glass Surfaces.

APTES-coated microscope glass slides were prepared as a positively charged substrate for adhered cell inactivation studies. Although APTES coatings are not found on soil surfaces, this coating chemistry was selected as it provides a model uniform positively charged surface for bacterial adhesion and subsequent inactivation. Because positively charged surfaces have been shown to exhibit antimicrobial properties, the APTES-coated glass slide provides a good control surface for evaluating bacterial inactivation. Glass slides were first cleaned by soaking in 1 M HCl overnight, rinsed with DI water, and dried in an oven at 70 °C. For experiments with APTES-coated slides, the glass slides were further dipped in a solution of 10% APTES in 85% ethanol for 1 h. After soaking in APTES, the slides were rinsed with ethanol to remove excess APTES and then extensively washed with DI water to remove excess ethanol. The treated glass slides were then dried at 70 °C for 1 h and stored for future experiments.

### Characterization of Bare and APTES-Coated Glass Slides.

Water contact angle measurements were conducted at room temperature using an OCA-30 goniometer (Future Digital Scientific Corp., NJ, USA) equipped with a microliter syringe. Measurements were made on sessile drops (1 μL droplets) by measuring the tangent to the drop at its intersection with the surface. Values reported are the mean of the tangents taken at both sides of five droplets. Liquid contact with the surface was recorded for 10 s with a CCD camera (768 × 507 pixels). The contact angle was measured from a snapshot taken 3 s after the initial contact. All measurements were repeated at least three times.

The streaming potential of bare and APTES-coated surfaces was measured with an asymmetric clamping cell using an electrokinetic analyzer (Anton Paar, Graz, Austria). The measurements were performed in triplicate at room temperature in 10 mM KCl, pH 6.1. Streaming potential measurements were converted to ζ-potentials using the modified Smoluchowski–Helmholtz formulation.

The surfaces of bare and APTES-coated glass slides were characterized by atomic force microscopy (AFM). Imaging was performed using a NanoScope IIIa Scanning probe Microscope (Veeco/Digital Instruments, USA) in air under tapping mode using a commercial n+-silicon cantilever (Nanoscience Instruments, Phoenix, USA) 240 μm long and 35 μm wide, with a resonant frequency of 50–130 kHz and spring constant of 9.0 N/m. The scanning rate was 1.0 Hz, at 0° angle. Image processing was performed using Research NanoScope III software version 6.1r1. All images were filtered using the flattening built-in tool from NanoScope III software. Root mean square (rms) roughness values were obtained by utilizing the built-in tool for cross-sectional analysis. The AFM images were obtained under ambient laboratory conditions.

### Adhesion of Bacteria onto Bare and APTES-Coated Glass Slides.

Bacterial adhesion to bare and APTES-coated slides was examined using a PPFC with dimensions of 47.5 × 12.7 × 1.6 mm (Model FC81, Biorad Scientific Technologies). The PPFC was first rinsed with DI water and ethanol using a syringe pump (Model 200, KD Scientific) at 2 mL/min for a duration of 10 min and then equilibrated with sterile 10 mM KCl for 10 min, taking care to remove all air bubbles from the system. Subsequently, bacterial suspensions (∼3 × 10⁸ cells/mL) were injected into the flow cell (0.5 mL/min, 10 min), followed by an equivalent injection of KCl alone (0.5 mL/min, 10 min) to remove any unattached cells from the chamber. The adhered bacteria were then stained using
one of the Molecular Probes viability assays, imaged by fluorescence microscopy (IX-71, Olympus), and enumerated over time with image analysis software (ImagePro). Images were acquired from the bottom of the PPFC; namely on the surface of the slide. The top plate of the chamber was a clean microscope cover slide. The PPFC was always maintained at room temperature. During the experiment, 20 images were taken at the center of the PPFC on an hourly basis for up to 6 h. The images were then analyzed to calculate the number of viable and total adhered bacteria as a function of time. Each experiment was performed in triplicate on different days.

**Bacterial Inactivation Assays.** *Bacterial Viability.* The inactivation of cells adhered to a bare or APTES-coated glass slide was evaluated using a commercially available kit (Live/Dead BacLight, Molecular Probes) which includes two DNA-binding stains: the green fluorescent stain, SYTO-9, and the red fluorescent stain, propidium iodide (PI). With this kit, determination of bacterial inactivation is based on differences in membrane integrity. SYTO-9 labels all bacteria, whereas PI penetrates bacteria with compromised membranes. Thus, bacteria with intact membranes fluoresce green and are considered to be viable while bacteria with compromised membranes fluoresce red. It should be noted that there are certain limitations to the use of PI staining for characterization of bacterial viability. It is thus important to validate the reliability of PI-based viability assays using a secondary approach (such as the CTC assay). The suitability of the BacLight assay was further examined by using it to stain cells before and after exposure to a 70% ethanol solution for a duration of 15 min. The attached bacteria initially appeared green and turned red after the exposure to ethanol, with more cells becoming red as time elapsed.

Attached bacteria were stained by filling the chamber of the PPFC with a solution of 20 µL Live/Dead BacLight stain in 1 mL 10 mM KCl for 15 min. To prevent photobleaching of the stain, the entire experimental setup (PPFC + tubing) was protected from light and the image acquisition time was minimized. Stained bacteria were imaged by fluorescence microscopy with excitation at 488 and 543 nm. Emitted light was collected at 495–535 nm for the green, viable bacteria and at 580–700 nm for the red, compromised bacteria. Direct counts were made for at least 20 images from the center of the PPFC at each time interval. The BacLight stain was not rinsed from the PPFC for the entire duration of each experiment.

**Bacterial Respiratory Activity.** Another useful method for estimating bacterial inactivation is to use a cytochemical procedure leading to the accumulation of the product of a metabolic reaction. The use of the 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) redox dye for evaluating the metabolic activity of bacteria has gained wide application in environmental studies. Therefore, to determine the inactivation of adhered cells in terms of loss in respiratory activity, the BacLight RedoxSensor CTC Vitality Kit (Molecular Probes) was employed. The kit contains CTC, which can be reduced intracellularly to a red fluorescent formazan product by healthy cells respiring via the electron transport chain. As the membrane of viable cells is impermeable to the charged molecules, positively charged formazan accumulates intracellularly and can be detected by fluorescence. Several concentrations of CTC, ranging from 0 to 5 mM, and different incubation times were evaluated to determine the optimum values for each bacterial strain.

CTC kills bacteria by interrupting the respiratory reaction chain and it has a rapid toxic effect on bacterial cells by inhibiting protein synthesis, but this only happens after bacteria take up the fluorogenic CTC and the fluorescent compound is produced. Therefore, to characterize the inactivation of the adhered cells as a function of time, the CTC staining was performed after specific time intervals as follows: for each experiment, 3 identical PPFCs were set up, and the CTC stain was injected into each PPFC at different times—in the first PPFC at time 0 immediately after bacterial adhesion to the surface, in the second PPFC after 3 h of adhesion, and in the third PPFC after 6 h of adhesion. The PPFCs were then incubated in the dark at 37 °C. To terminate CTC reduction, 1 mL 4% formaldehyde was added to each PPFC. Next, the actively respiring cells were imaged and enumerated with a fluorescence microscope using CTC filters with excitation (EX) and emission (EM) wavelengths of 450 and 630 nm, respectively. To determine the total number of adhered bacteria, the CTC-labeled cells were counterstained with 1 µM SYTO-24 green-fluorescent nucleic acid stain for 15 min and immediately analyzed.

**Bacterial Culturability.** CFU counts were performed to determine the number of adhered cells that were capable of reproducing. For evaluation of CFU, the bacteria stained with Live/Dead BacLight were first detached from the surface by injecting a 0.25% solution of Trypsin-EDTA into the PPFC and incubating the PPFC at 37 °C for 10 min. This solution (containing detached cells) was then reinjected 3 times into the same PPFC to ensure more extensive detachment of cells from the PPFC, and the suspension was finally collected with a sterile syringe. The total number of viable detached bacteria was determined by fluorescence microscopy using a counting chamber. Next, the bacterial suspension was serially diluted and the bacteria were plated on maximum recovery agar plates at 37 °C for 24 h. Total CFUs were counted and the culturability was calculated as the percentage of CFUs from the total number of viable detached bacteria (culturability × CFU/Total viable bacteria).

### RESULTS AND DISCUSSION

**Characterization of Bare and APTES-Coated Glass Surfaces.** The presence or absence of the APTES molecules on the glass slides was confirmed by water contact angle and streaming potential measurements and AFM analysis of coated samples.

<table>
<thead>
<tr>
<th>Characterization</th>
<th>bare glass</th>
<th>APTES-coated glass (% APTES in solution)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.1%</td>
<td>1%</td>
</tr>
<tr>
<td>surface streaming potential (mV)</td>
<td>–51 ± 1</td>
<td>+1 ± 0.1</td>
</tr>
<tr>
<td>water contact angle (deg)</td>
<td>9 ± 0.5</td>
<td>45 ± 1</td>
</tr>
<tr>
<td>roughness (nm)</td>
<td>0.8 ± 0.02</td>
<td>1.5 ± 0.4</td>
</tr>
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</table>

The data in Table 1 shows that the APTES coating significantly affects the surface properties of glass substrates, as indicated by the changes in streaming potential and water contact angle. This difference in surface characteristics likely influences the bacterial adhesion and viability, as discussed in the next sections of the paper.
Table 1 summarizes the surface characteristics of the bare and coated glass slides for different concentrations of APTES. Water contact angle characterizes surface hydrophobicity by measuring how much a droplet of water spreads on a surface. Upon APTES adsorption, the glass surface becomes more hydrophobic, confirming the presence of aminosilane groups on the surface. The surface potentials of the bare and APTES-coated slides were probed by electrokinetic analysis. Streaming potential measurements indicate that the APTES coating strongly compensated the highly negative charge of the bare glass yielding a positively charged surface. AFM image processing of bare and APTES-coated slides revealed the roughness values to be on the order of 1 to 4 nm which is significantly lower than the average bacterial size of 1 μm. Thus, it can reasonably be concluded that the APTES-coating offers limited steric interference to bacterial adhesion.

**Evaluating the Inactivation of Attached Bacteria. Live/Dead Staining.** In the Live/Dead BacLight assay, viability is defined in terms of preservation of membrane integrity as the percentage of bacterial cells with intact membranes (stained with SYTO-9) to the total number of cells. Figure 1a shows a comparison of the variation in bacterial viability for *E. coli* D21 attached onto bare glass or 10% APTES-coated glass with that of the planktonic cells as a function of time. Representative images of adhered *E. coli* D21 onto 10% APTES-coated glass is also illustrated in Figures 1b–d for 0, 3 h, and 6 h adhesion times, respectively. The results indicate that suspended bacteria remained mostly viable over a period of 6 h, whereas the viability of attached bacteria onto bare and APTES-coated glass decreased to 80% and 35% after 6 h, respectively. Although previous studies have also shown the antimicrobial activity of positively charged surfaces,7,8,11,12,14,15 these studies have not attempted to quantify the loss in microbial viability with time; namely, the bacterial inactivation rate. Similarly, Figure 2 shows that the viability of the bacteria attached to the APTES-coated glass surface decreased significantly over time for each microorganism. The initial rate of bacterial inactivation for each microbe can be evaluated from the initial slopes of the curves presented in Figures 1a and 2, (i.e., from the linear portion of the curves, between *t* = 0 and 3 h). These data are summarized in Table 2 as the loss in viability with time (%loss/h). Comparison of a pathogenic and a model laboratory *E. coli* strain reveals that *E. coli* O157:H7 displayed a comparable ability to survive on the positively charged surface as the nonpathogenic *E. coli* D21 (Table 2). Moreover, *E. faecalis* 29212, a strain representing Gram positive bacteria, exhibited a lower inactivation rate in comparison to the two *E. coli* strains and was hence more resistant to the antibacterial effect of the positively charged APTES-coated surface. All three bacterial strains showed significantly lower inactivation rates when attached to the negatively charged bare glass (Figure 2a) compared to the positively charged APTES-coated glass (Figure 2b). The inactivation rates of *E. coli* O157:H7 and *E. faecalis* 29212 were at least an order of magnitude lower on the bare glass (Table 2).

Gottenbos et al. also found that positively charged biomaterial surfaces exert an antimicrobial effect on adhering Proteobacteria, but not on Gram positive ones.7 Such differences in behavior can presumably be attributed to differences in the structure of the external cell wall between the Gram positive and Gram negative bacteria.14 Two mechanisms have been proposed in the literature to explain the observed loss in viability of bacteria following attachment onto a positively charged surface.15,27 One hypothesis which is expected to operate in Gram negative bacteria is that the functional groups on positively charged surfaces displace the divalent cations which hold together the negatively charged surface of the lipopolysaccharide (LPS) network, thereby disrupting the outer membrane of Gram-negative bacteria.28 The second mechanism is likely to operate when the positively charged functional groups penetrate into the cell membrane, presumably via the self-promoted uptake pathway, leading to cell leakage and eventual inactivation of the bacterium.14,29,30
Either mechanism can be lethal to *E. coli*. Because the cell wall of Gram positive bacteria, e.g., *E. faecalis*, possesses a thicker layer of peptidoglycan compared to Gram negative bacteria, the antimicrobial activity of the positive coating against the Gram positive strain likely follows the second mechanism, which requires penetration of cationic groups across the thick cell wall to reach the cytoplasmic membrane, resulting in higher maintenance of viability for the Gram positive organisms.14

**CTC Staining.** The CTC assay was used to evaluate changes in respiratory activity of bacteria attached to APTES-coated glass slides over time. Optimal staining of *E. coli* D21, *E. coli* O157:H7, and *E. faecalis* 29212 on an APTES-coated surface was achieved at CTC final concentrations of 1.25, 2.5, and 5 mM and after incubation times of 1, 3, and 2 h at 37 °C, respectively. The white bars in Figure 3 show the change in inactivation (measured as loss of respiratory activity) determined for the three different test bacteria when adhered to a 10% APTES-coated glass slide over a time period of 6 h. Upon adhesion, the presence of APTES on the surface adversely affected the metabolic activity of the *E. coli* cells and reduced it to 80% (for *E. coli* D21) and 70% (for *E. coli* O157:H7). Approximately 30% of the *E. coli* cells were still active after 6 h of adhesion onto the positively charged slide, while 50% of *E. faecalis* bacteria remained active after this period, confirming their ability to better survive on positive surfaces. A decrease in the overall fraction of CTC-positive (i.e., respiring) cells was observed for all three bacteria with time, which is comparable to the results obtained using the Live/Dead BacLight viability assay.

**Colony Forming Units.** Plate counts provide information on the substrate responsiveness of cells and only show those cells that are capable of dividing and forming colonies.31 The results of this assay demonstrate that the cell culturability decreased with time and was significantly lower than the viabilities measured by the two other techniques (Figure 3). Several reasons may account for the different measures, but a likely key factor is that there may be a considerable number of cells that are viable but nonculturable (VBNC).32,33 In addition, cells may possibly be damaged or destroyed after extraction from the substratum which is a limitation associated to all extraction techniques. It is interesting that despite the low recovery rates, the inactivation results obtained by the CFU assay demonstrate the same trend as the BacLight and CTC assays. The CFU results also confirm that *E. faecalis* exhibited a lower inactivation rate in comparison to the two *E. coli* strains. The lack of bias in the CFU measurements is likely due to the effective protocol used for detachment of viable and dead cells from the glass surface.

**Comparison of Methods.** Three different methods were used for bacterial inactivation measurements of cells adhered to a solid surface. Live/Dead and CTC assays are in good agreement for bacteria adhering onto an APTES-coated surface. For example, Live/Dead and CTC viability measures were 35% and 27% for *E. coli* D21 after 6 h of adhesion, respectively (Figure 3). One contributing factor for this difference is the insufficient energy of intact cells to reduce CTC under the starvation condition of the experiment.23 Another possible reason for the observed difference in inactivation measures is that the cells may be nonrespiring but still have an intact membrane. However, the data in Table 2 reveal that adhering cells lose their physiological properties such as membrane integrity and respiratory activity with comparable rates, which were −10 and −9%/h for *E. coli* D21, respectively. Thus, the CTC approach successfully validated the developed experimental technique based on the Live/Dead assay. In contrast, the culturability values achieved by the detachment based CFU method were significantly lower than viability measures obtained by direct microscopy. However, the CFU counts did confirm that *E. faecalis* exhibited the lowest inactivation rate of the three strains examined.

In addition, we demonstrate that a two-color nucleic acid assay, SYTO-9 and PI, is suitable for prolonged time periods with no adverse effect on bacterial viability. As shown in Figure 1a, planktonic *E. coli* D21 remained viable throughout the experiment while the adhering cells on bare glass lost their viability as a result of adhesion. There are many reported laboratory studies of the effects of surfaces on bacterial activity.34,35 Attachment of bacteria to surfaces can lead to enhanced or reduced bioactivity depending on the surface properties and charge36,37 but there are also examples of studies where association with a surface had no effect on bacterial activity.19,39 In this work, we find that attachment of cells to solid surfaces, especially a positively charged one, can lead to reduced bacterial viability.

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**Table 2. Inactivation Rates of Adhered Bacteria onto Bare and 10% APTES-Coated Glass Determined Using Different Methods**

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>inactivation rates (% loss in viability/h) for attached bacteria</th>
<th>bare glass</th>
<th>APTES-coated glass</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Live/Dead assay</td>
<td>Live/Dead assay</td>
</tr>
<tr>
<td><em>E. coli</em> D21</td>
<td>3.1</td>
<td>10.0</td>
<td>9.0</td>
</tr>
<tr>
<td><em>E. coli</em> O157:H7</td>
<td>0.8</td>
<td>9.5</td>
<td>8.7</td>
</tr>
<tr>
<td><em>E. faecalis</em></td>
<td>0.3</td>
<td>5.0</td>
<td>6.6</td>
</tr>
</tbody>
</table>

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**Figure 3.** Comparison of inactivation behavior for (a) *E. coli* D21, (b) *E. coli* O157:H7, and (c) *E. faecalis* evaluated using three different assays. Cells were attached to 10% APTES-coated glass slides.
These bacteria with reduced viability may experience damaged cell membranes and/or reduced metabolic activity. The key advantages of the new technique presented here is that the inactivation kinetics of attached bacteria can be evaluated in real-time without removing the attached cells from the surface of interest and without disturbing the experimental system. Typically, in studies of bacterial fate in granular aquatic environments, ex situ techniques are often applied to study the survival (or inactivation) of suspended bacteria.\textsuperscript{40,41} Often, the inactivation behavior of attached bacteria is ignored in such studies. Here, Live/Dead staining in combination with fluorescence microscopy is shown to be a useful tool for the direct quantification of attached bacterial inactivation of in situ specimens. Moreover, bacterial reduction of CTC correlated with the loss in membrane integrity and therefore provides clear evidence that the inactivation of adherent cells occurred by loss of membrane integrity and metabolic activity, simultaneously. In addition, the low culturability measures reveal that the CFU technique is not necessarily a suitable method to determine the viability of adherent cells. The experiments presented here were conducted using bare glass (to mimic a sand surface as it is mainly composed of silica) and APTES-coated glass (as a representative positively charged surface). Ongoing work in our laboratory is aimed at quantifying the inactivation kinetics of bacteria attached to more environmentally relevant surfaces, including iron oxide and aluminum oxide substrates.

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\section{REFERENCES}

\textsuperscript{(1)} Tufenkji, N., Emelko, M. B., Fate and transport of microbial contaminants in groundwater. In Encyclopedia of Environmental Health; Elsevier: Nriagu JO Burlington, 2011; Vol. 2, pp 715–726.


\textsuperscript{(11)} Gottenbos, B. \textit{The Development of Antimicrobial Biodeterioration Surfaces; Ponsen & Looijen: Amsterdam, 2001.}


\textsuperscript{(13)} Hong, Y.; Brown, D. G. Variation in bacterial ATP level and proton motive force due to adhesion to a solid surface. \textit{Appl. Environ. Microbiol.} 2009, 75 (8), 3236–3233.


