Enumerating bacterial cells on bioadhesive coated slides

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ABSTRACT

Quantifying bacterial abundance and biomass is fundamental to many microbiological studies. Directly counting via epifluorescence microscopy has become the method of choice, especially for environmental samples, and conventional techniques require filtration of cells onto black polycarbonate membrane filters. We investigated the utility of instead capturing stained bacterial suspensions on bioadhesive slides, performing tests using pure cultures of bacteria, mixtures of cultured bacteria, and environmental samples from five habitat types. When compared to the standard filtration and flow cytometric approaches, bioadhesive slides were found to be an accurate and precise platform for rapid enumeration of bacteria. Total bacterial counts made using the three methods were positively correlated for acridine orange and Live/Dead® (L/D) staining (0.81 ≤ r ≤ 0.95, all p ≤ 0.002). All platforms had similar precision, though counts obtained using bioadhesive slides were significantly higher than those made with polycarbonate filters and flow cytometry. The specific bioadhesive slides we used resulted in substantial cell mortality for certain pure cultures and river water samples, limiting their use for L/D determination. Cell enumeration using bioadhesive slides is particularly effective because it is highly precise at a wide range of cell concentrations, allows observation of cells that are not readily discernible on filters, reduces the number of steps and processing materials associated with sample analysis, and increases throughput.

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1. Introduction

Microbiologists often are interested in determining numbers of bacteria associated with a given environment or process and routinely rely upon direct microscopic counts to accomplish this. A variety of fluorescent staining methods are available to facilitate enumeration, and procedures can be customized to target different attributes of the community. The most common applications are to estimate the total abundance of bacteria (reviewed by Bölter et al., 2002) and to quantify the metabolically-active fraction (e.g., see Boulos et al., 1999). In addition, analysis via fluorescent in situ hybridization (FISH) is widely used to assess specific phylogenetic groups of microorganisms within a complex community (Bertaux et al., 2007; and reviewed by Amann and Fuchs, 2008 and Bouvier and del Giorgio, 2003).

Methods for direct counts depend on the type of sample; procedures for laboratory cultures or aquatic communities generally require only staining and filtration (e.g., Hobbie et al., 1977; Porter and Feig, 1980), whereas studies of microorganisms in biofilms, soils, and sediments require more complex treatment to first separate the cells from the environmental matrix (e.g., Cough and Stahl, 2003; Kepner and Pratt, 1994; Torsvik et al., 1996). Despite these efforts, concerns remain about auto-fluorescence of soil particles (e.g., clay colloids) and non-specific binding of fluorescent stains. These phenomena can artificially inflate abundance estimates when non-bacterial particles are counted or, in the case of high levels of background fluorescence, mask the presence of microorganisms leading to underestimates. Such issues, along with the considerable time and effort associated with the pre-treatment and filtration steps, limit the utility of traditional methods for direct counting of cells from large numbers of environmental samples. High throughput procedures are available, including flow cytometry (Amann et al., 1990; del Giorgio et al., 1996; Gasol and del Giorgio, 2000; Gasol et al., 1999; Monfort and Baleux, 1992; Trevors, 2003) and capillary electrophoresis (Armstrong and He, 2001). However, these techniques require sophisticated equipment that is not widely available and thus, direct enumeration on polycarbonate filters via epifluorescence microscopy remains the standard method.

The bioadhesive-slide technique described in this manuscript was developed as a substitute for filtration to increase throughput, reduce background fluorescence, and decrease the materials required for determining microbial abundance in environmental samples. Although bioadhesive slides are employed for microbial FISH analyses (e.g., Davidson and Stahl, 2006; Davis et al., 2009) and for biomedical and molecular studies with eukaryotic cells and tissues (Piyathilake et al., 1995; Ray et al., 2000), there are no published reports regarding the use of bioadhesive slides for direct bacterial enumeration. We therefore examined the concurrence of bacterial counts performed with the slide- and filter-based methods using fluorescent staining of batch cultures and environmental samples from five representative types of habitat (biofilm, sediment, river water, ocean water, and wastewater): results for batch cultures were further compared to abundance estimates obtained using flow cytometry. Preliminary tests were performed with two of the...
most common fluorescent stains: acridine orange (AO), which binds nucleic acids and permits quantification of total cell concentrations, and Invitrogen's LIVE/DEAD® (L/D) assay, which combines propidium iodide (PI) and SYTO9™ for analysis of viability based on membrane integrity. As results from the two stains were similar, this manuscript primarily reports data for L/D staining.

2. Materials and methods

2.1. Experimental design

2.1.1. Laboratory culture experiments

To determine the accuracy and precision of enumeration using bioadhesive slides relative to the standard filtration method, we conducted dilution assays using laboratory cultures of bacteria. Dilution series (1:25, 1:50, 1:75, and 1:100 in filter-sterilized deionized water) were prepared in triplicate for: (i) a pure culture of Pseudomonas aeruginosa (Gram negative) and (ii) a mixture of equal parts of five species: Bacillus cereus (G+), Micrococcus luteus (G+), P. aeruginosa (G−), Staphylococcus aureus (G+), and Serratia marcescens (G−). Preliminary counting trials also were performed for seven additional bacterial species: Paracoccus denitrificans (G−), Nitrosomonas europaea (G−), Escherichia coli (G−), P. stutzeri (G−), Nitrobacter winogradskyi (G−), and P. fluorescens (G−). All cultures were purchased from American Type Culture Collection (ATCC, Manassas, Virginia) and were maintained as lab stocks using the media and conditions recommended by the distributor. Using the same approach of diluting mixed cultures, two additional experiments were conducted to determine: (i) effect of rinsing on the performance of the bioadhesive slides and (ii) the results of counts obtained using slide-based methods versus those obtained via flow cytometry. For these trials, an additional treatment (1:10) was added to expand the dilution series.

2.1.2. Environmental samples

The broader utility of enumeration using bioadhesive slides was assessed for environmental samples from five habitat types: (i) river water from James River, Virginia (duplicate 2-L samples collected from each of seven sites along a transect between the cities of Richmond, 37.53°N, −77.45°W, and Hopewell, 37.31°N, −77.26°W); (ii) benthic sediment samples (duplicate 5-g grab samples from each of four sites near Richmond); (iii) biofilm samples from substrates in James River (duplicate 5-g grab samples from each of four sites near Richmond, 37.53°N, −77.45°W); (iv) ocean water (500-ml grab samples from five locations near Virginia Beach, 37.04°N, −76.29°W); and (v) secondary treated wastewater from the Richmond Wastewater Treatment Facility (three discrete 50-ml samples). All sample collections were placed on ice immediately for transport to the lab where they were held for <2 days at 4°C prior to processing.

No pre-treatment was performed for any of the water samples. For preparation of biofilm and sediment samples, approximately 0.25 g (wet weight) of sample was suspended in 10 ml of filter-sterilized deionized water and vortexed on high for 5 min with a quantity of clean sterile 1-mm diameter glass beads equal to roughly 250 μl. Three dilutions (1:8, 1:16, and 1:32) were prepared and stained for each processed biofilm, sediment, and river water sample; whichever dilution generated a slide with a countable number of cells per field (target: 20–200) was used for subsequent data collection. For the wastewater and ocean water samples, no dilution was necessary.

2.2. Comparing slide platforms

2.2.1. Cell staining

Each sample was stained immediately following processing and subdivided so that separate aliquots could be applied to each of the counting platforms (either bioadhesive slides or polycarbonate filters). For L/D, the manufacturer's instructions (Invitrogen) were followed; specifically, 2 μl of SYTO9™ and 1 μl of PI were added to each 1 ml of sample. Samples were vortexed briefly and incubated in the dark at room temperature for approximately 10 min prior to slide preparation. For trials using AO, concentrated stain (10 mg ml⁻¹) was added to each sample to yield 1 mg ml⁻¹ final concentration; after vortexing, samples were incubated in the dark at room temperature for 3–5 min prior to slide preparation.

2.2.2. Preparation of bioadhesive slides

Glass slides with a bioadhesive hydrophilic surface that electrostatically attracts biological materials and hydrophobic printed septa among "wells" (Excell Adhesion™ available from Fisher Scientific as non-catalog item # ES-2308-EXC) were used for validation tests. For the current study, slides with 5-mm diameter wells (19.6 mm²) were used, allowing for simultaneous processing of 24 samples per slide. Immediately prior to slide preparation, stained cell suspensions for all tests were vortexed for 30 s, after which 20 μl of the supernatant was applied to individual wells on the bioadhesive slide. Slides were left in the dark until dry (either overnight at room temperature or in a 46°C oven for 10–20 min) to complete the adhesion process.

For the experiment conducted to test the effect of rinsing on the performance of the bioadhesive slides, two 20-μl aliquots were removed from the stained cell suspension and each was applied to a separate bioadhesive slide. Both slides were dried as above. One of the bioadhesive slides was then rinsed by squirting briefly with filtered deionized water and left in the dark at room temperature until dry (approximately 10 min).

2.2.3. Preparation of filter-based slides

The filtration method utilized 0.2 μm Millipore Isopore™ black polycarbonate membrane filters and a vacuum pressure of approx. 200 mm Hg (modification of Kepner and Pratt, 1994). Filtration was performed using the 980 μl of sample that remained following removal of 20 μl for preparation of the bioadhesive slides; thus, estimates obtained using the two slide-based platforms were fully paired. The volume used on the filter was adjusted so that cell densities (counts per field) would be similar across platforms. This volume was calculated by comparing the effective filtration area of a polycarbonate filter (380 mm² for our particular setup) with the area of wells on the bioadhesive slides (19.6 mm²). After rinsing, filters were dried in the dark at room temperature (approximately 15 min) and mounted on microscope slides using antifade solution (BacLight™ mounting oil, Invitrogen). To test whether small cells might be selectively lost from 0.2-μm polycarbonate filters, a separate experiment was performed using the five-species mixture of cultures where replicate samples were enumerated using 0.2-μm pore-size filters, 0.1-μm pore-size filters, and bioadhesive slides.

2.2.4. Microscopy

Antifade solution was applied atop filters and bioadhesive slides prior to the cover slip, and preparations were viewed at 1000× under oil immersion using epifluorescence microscopy (Olympus BX-41). Live (green) and dead (red) cells were viewed using a fluorescent bandpass filter. Enumeration was accomplished by counting from an accurately ruled eyepiece graticule (in the present case, 0.004 mm² total grid area) subdivided into 100 smaller squares of equal area. Several view fields were counted until a running total of at least 200 cells was reached (or a minimum of five fields). The total cell count in each sample was then calculated as:

\[
\text{average cell count per grid} \times (\text{grid area}) \times (\text{dispersal area}) \times \text{dilution factor} \times (\text{sample volume})
\]

where “dispersal area” was the effective size of the region over which sample was dispersed (i.e., the area of a well circumscribed by hydrophobic septa or stained region on the filter), "sample volume" was the quantity of stained cell suspension used directly for slide preparation,
and “dilution factor” included all instances of dilution prior to application of the sample to the slide or filter.

2.3. Comparing slide-based counts to flow cytometry

To assay the utility of bioadhesive slides relative to flow cytometry, a dilution series (1:10, 1:25, 1:50, 1:75, and 1:100) of a mixture of cultured cells was prepared as described above and stained with LDA according to the manufacturer’s instructions for flow cytometry. Specifically, to every 980 μl of sample at the appropriate dilution, 1.5 μl of 3 mM SYTO9™ and 1.5 μl of 30 mM PI were added. The suspension was mixed thoroughly and incubated in the dark at room temperature for 15 min. Immediately following the staining period, a 10-μl aliquot of well-mixed PI reference standard microspheres (2.04 × 10^6 spheres ml^-1, #892, Bangs Laboratories) was added. Samples were then analyzed by flow cytometry (FACScan; BD Biosciences) at 488 nm, using a fluorescein filter, and with instrument voltage, threshold, and compensation adjustments for each sample type that resulted in maximum separation of the green-fluorescent and red-fluorescent signals and ensuring that both cell populations and the fluorescent spheres all appeared on a side-scatter versus fluorescence graph. The total cell count in each sample was then calculated as:

\[
\text{\text{# events in live + dead regions}}/\text{# events in dead region} \times \text{dilution factor}
\]

where 2.04 × 10^{-4} was the correction factor associated with the particular brand and volume of microspheres used in this experiment, and “dilution factor” included all instances of dilution prior to flow cytometry.

2.4. Data analysis

For each sample, total abundance of cells was calculated as the sum of the “live” and “dead” counts. Data were analyzed considering this total as well as the fraction living (%), and compared using a series of paired t-tests. For each sample type and each enumeration platform, the coefficient of variation (CV) was used as a metric of precision. Correlation analysis was performed using Pearson’s coefficient to determine the congruence of techniques using a paired measurement of each sample for each assay. In addition, for the culture-based experiments, a two-factor ANOVA was used to screen for interaction effects of cell concentration (dilution level) and enumeration method to determine whether one technique was more accurate at a particular cell concentration. Linear regression was used to examine the consistency of each method across a range of cell concentrations. Regressions were performed using SigmaPlot (Version 10.0); all other statistical analyses were conducted using PAST (Version 2.07, Hammer et al., 2001).

3. Results

3.1. Laboratory culture experiments

3.1.1. Estimating total bacterial abundance

When total bacterial abundance was considered, the estimates obtained using all three enumeration methods were well correlated with one another (all r = 0.87 with p < 0.002; Table 1). Similar results were obtained when AO stain was used (r = 0.91, p < 0.0001; Supplemental Fig. S1). Though well-correlated, paired t-tests revealed that the estimates obtained using the bioadhesive slides were always significantly higher than the results from filters (Table 2) or flow cytometry (ratio of abundance estimates [bioadhesive slides/flow cytometry]: 3.7 ± 0.5 [S.E.]; N = 15, t = 7.9, p < 0.0001).

![Table 1](image-url)

Table 1

Pearson correlation analysis comparing total bacterial abundance as determined using bioadhesive slides, polycarbonate filters, and flow cytometry. Shown are sample size (N), slope (m), Pearson correlation coefficient (r), and the probability of significant deviation (p).

<table>
<thead>
<tr>
<th>Sample type</th>
<th>N</th>
<th>Methods compared</th>
<th>m</th>
<th>r</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pure culture</td>
<td>12</td>
<td>Bioadh Filter</td>
<td>0.61</td>
<td>0.95</td>
<td>&lt;0.0001 *</td>
</tr>
<tr>
<td>Mixed culture (set 1)</td>
<td>12</td>
<td>Bioadh Filter</td>
<td>0.55</td>
<td>0.87</td>
<td>0.0002 *</td>
</tr>
<tr>
<td>Mixed culture (set 2)</td>
<td>15</td>
<td>Bioadh Filter</td>
<td>0.52</td>
<td>0.95</td>
<td>&lt;0.0001 *</td>
</tr>
<tr>
<td>Mixed culture (set 2)</td>
<td>15</td>
<td>Bioadh FC</td>
<td>0.77</td>
<td>0.93</td>
<td>&lt;0.0001 *</td>
</tr>
<tr>
<td>Environmental</td>
<td>35</td>
<td>Bioadh Filter</td>
<td>0.22</td>
<td>0.94</td>
<td>&lt;0.0001 *</td>
</tr>
</tbody>
</table>

a Number of assays performed using each technique.

b “Bioadh” = Bioadhesive slides; “Filter” = 0.2 μm pore-size polycarbonate filters;
f “FC” = flow cytometry.

* Statistically significant using α < 0.05.

On average, counts obtained using filters and flow cytometry were 48% of the counts for the same samples analyzed using bioadhesive slides (range = 21–85%, standard error = 2%, N = 46) with no significant difference in the performance of filters and flow cytometry (paired t-test: t = 1.4, p = 0.18). Further testing to determine whether the observed higher estimates of abundance on bioadhesive slides derived from counting of small cells that were not retained on 0.2-μm filters yielded no significant differences in the abundance estimates obtained using 0.2 and 0.1 μm filter sizes (t = 0.47, p = 0.34) whereas both sets of filter counts were significantly lower than the counts from the bioadhesive slides (ANOVA: F = 3.78, p = 0.0001). Bacterial cell counts also exhibited more variability when performed using the filters compared to the bioadhesive slides (P. aeruginosa: CV = 21% and 17%, respectively; five-species mixture: CV = 30% and 15%, respectively).

For the pure culture experiment, regression analysis revealed an excellent fit between dilution factor and cell count for both slide-based techniques (Fig. 1A, all p < 0.0001). This indicates reproducibility at a range of cell concentrations; for this experiment, bacterial counts per field varied from an average of 25 (1:100 dilution) to 135 (1:25 dilution). A two-factor ANOVA showed no significant interaction of dilution factor with enumeration platform (F = 1.7, p = 0.20), whereas dilution (F = 3.7, p = 0.03) and enumeration platform (F = 127.4, p < 0.0001) were individually significant. For the analogous mixed-culture experiment (Fig. 1B), counts per field were higher than for the pure-culture experiment, ranging from an average of 30 (1:100 dilution) to 275 (1:25 dilution). When regression analysis was applied to compare cell counts across that dilution series, significant correlations were obtained for both slide-preparation methods using a linear model (Fig. 1B, all p ≤ 0.003). However, the results of the two-factor ANOVA suggested a

![Table 2](image-url)

Table 2

Results of t-tests comparing estimates of total abundance and the fraction of cells classified living as determined using Live/Dead® stained suspensions applied to Excell Adhesion™ coated slides and 0.2 μm Millipore Isopore™ black polycarbonate filters.

<table>
<thead>
<tr>
<th>Sample type</th>
<th>N</th>
<th>Ratio of abundance estimates</th>
<th>Total abundance</th>
<th>Fraction living</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pure culture</td>
<td>12</td>
<td>22.2 ± 0.2</td>
<td>9.9 ± 0.0001</td>
<td>30.5 ± 0.0001</td>
</tr>
<tr>
<td>Mixed culture</td>
<td>23</td>
<td>25.5 ± 0.6</td>
<td>3.6 ± 0.0001</td>
<td>1.4 ± 0.18</td>
</tr>
<tr>
<td>Biofilm</td>
<td>7</td>
<td>121.2 ± 1.5</td>
<td>63.3 ± 0.0001</td>
<td>1.8 ± 0.31</td>
</tr>
<tr>
<td>Sediment</td>
<td>8</td>
<td>44.2 ± 0.3</td>
<td>9.1 ± 0.0001</td>
<td>1.3 ± 0.21</td>
</tr>
<tr>
<td>River water</td>
<td>14</td>
<td>45.2 ± 1.1</td>
<td>4.7 ± 0.0001</td>
<td>5.6 ± 0.0001</td>
</tr>
<tr>
<td>Ocean water</td>
<td>5</td>
<td>92.2 ± 2.1</td>
<td>6.4 ± 0.003</td>
<td>2.4 ± 0.07</td>
</tr>
<tr>
<td>Wastewater</td>
<td>3</td>
<td>119.2 ± 2.0</td>
<td>7.7 ± 0.02</td>
<td>0.8 ± 0.45</td>
</tr>
</tbody>
</table>

a Number of assays performed using each technique.
b Total abundance via bioadhesive slides divided by the abundance estimated from the filter-based method. Mean ± 1 S.E.
c Abundance always higher using the bioadhesive slides.
d Combined results for both experiments using mixed cultures.

* Statistically significant using α < 0.05.
3.1.2. Assessment of viability

The utility of the bioadhesive slides for viability assays using L/D staining was assessed by comparing the fraction of cells deemed “living” across enumeration platforms. In most cases, the proportions were not significantly different (Fig. 3, Table 2) and the average proportion of live cells was usually greater than 50%. However, the use of bioadhesive slides resulted in near total mortality of cells for two sets of samples: the pure culture of *P. aeruginosa* and river water samples. Similar mortality was observed when pure cultures of *B. cereus*, *P. denitrificans*, *E. coli*, and *N. europaea* cultures, and when river water samples were analyzed on two separate sampling occasions.

3.1.3. Effects of rinsing bioadhesive slides

The addition of a rinsing step reduced background fluorescence on bioadhesive slides, making counting much easier, but also removed a significant number of dead cells (Fig. 4). On average, this rinsing step removed 40% of the cells compared to the un-rinsed bioadhesive-slide counts, and yielded abundance estimates 25% lower than counts obtained using the filter-based method. No trend was observed in the fraction of cells lost or the preferential loss of dead cells; the fraction categorized as live was 63% on the rinsed slides compared to 69% obtained from the filter-based method. No trend was observed in the proportion of live cells across sampling occasions. In particular, the proportions were not significantly different (Fig. 3, Table 2) and the average proportion of live cells was usually greater than 50%.

3.2. Environmental samples

Combined data from all five types of environmental samples (*N = 38*) illustrated that total abundance was well-correlated across the two slide-based enumeration platforms (Fig. 2, *r = 0.94, p ≤ 0.0001*). As was observed for the cultures, abundance estimates were always significantly higher using the filter-based method (Table 2, *p ≤ 0.02*). Bacterial cell counts exhibited more variability for biofilm-rich samples and wastewater when using the filter versus bioadhesive platform (biomass: *CV = 42% and 37%* respectively; ocean water: *CV = 50% and 28%*; and wastewater: *CV = 11% and 5%*); however, the opposite trend was observed for the sediment (*CV = 13% and 19%*) and river water (*CV = 44% and 49%*).
...in a manner consistent within a given sample type. bioadhesive slides and the two other enumeration methods was that and environmental samples. The major difference observed between exhibited similar precision, and were useful for both laboratory cultures this enumeration platform has potential applications with other means of quantifying total bacterial abundance was demonstrated for DAPI (4,6-diamidino-2-phenylindole; results not presented) suggest L/D and AO stains and successful non-quantitative experiments using different habitat types. Solid line is the best fit linear correlation for all five data sets. Note difference in scales of the ordinate and abscissa. Fig. 2. Congruence of enumeration platforms for 38 environmental samples from five different habitat types. Solid line is the best fit linear correlation for all five data sets. When CV data for environmental and culture-based assays were considered simultaneously, we noted a strong correlation, indicating that higher variability on the filter-based assay corresponded to higher variability on the bioadhesive slides (r = 0.88, p = 0.0002). Nevertheless, the slope of this relationship did not differ significantly from 1 (m = 0.89 ± 0.14 S.E.) and thus there is no indication that one enumeration assay is more or less precise than the other. Moreover, there was no significant relationship between CV and cell abundance for either technique (filter-based: r² = 0.23, p = 0.33; bioadhesive slides: r² = 0.02, p = 0.78).

4. Discussion and conclusions

Bioadhesive slides exhibited several favorable qualities for bacterial enumeration. In general, they were faster and simpler to prepare, less expensive relative to filter-based methods, and easier to count (e.g., lower background fluorescence). The utility of bioadhesive slides as a means of quantifying total bacterial abundance was demonstrated for L/D and AO stains and successful non-quantitative experiments using DAPI (4,6-diamidino-2-phenylindole; results not presented) suggest this enumeration platform has potential applications with other fluorescent markers. The results obtained using bioadhesive slides were well-correlated with those from standard filtration and flow cytometry, exhibited similar precision, and were useful for both laboratory cultures and environmental samples. The major difference observed between bioadhesive slides and the two other enumeration methods was that total bacterial abundance was always higher using bioadhesive slides, though in a manner consistent within a given sample type.

With respect to filter-based counts, one potential explanation for this discrepancy is that bacterial cells could be lost during the filtration process due to their small size (Fry, 1990; Turley, 1993). On the bioadhesive platform, such small cells would be trapped and thus contribute to higher counts using that method. Our research found no significant difference in the counts obtained on 0.1 and 0.2 μm pore-size filters, making size an unlikely explanation of the lower filter-based counts. Another possible rationale is that the vacuum pressure associated with filtration caused some cells to rupture (Kepner and Pratt, 1994), an issue that would not be relevant when preparing bioadhesive slides. However, if the difference in abundance estimates was due to the rupturing of fragile cells during filtration, we hypothesized that a higher relative proportion of dead cells would have been observed on bioadhesive slides compared to corresponding filters. This expectation was based on the fact that the L/D assay determines viability based on membrane integrity, so one might reasonably expect cells categorized as “dead” to be more likely to burst during filtration due to compromised membranes. However, such an effect was not observed, and the ratio of live to dead cells was generally the same on filters and bioadhesive slides (Table 2, Fig. 3).

Given the experimental results, the most likely explanation for the lower counts on filters is that higher background fluorescence masked bacterial cells, and failure to count them when examining filters led to an underestimate of total abundance. Matrix and detrital fluorescence was noticeably higher for filters than bioadhesive slides (Supplemental Fig. S2), making it more difficult to distinguish bacterial cells. Other biotic components such as diatoms and algae were also easier to identify on bioadhesive slides compared to filters. Collectively, these findings imply that if only the filter method is used, enumeration from certain types of samples can be prone to underestimation of total number of microbes. This effect could be particularly important in environmental samples that contain high portions of detritus, soil, autofluorescence, and photosynthetic organisms. The improved resolution of environmental preparations using bioadhesive slides may also expand the application of epifluorescence microscopy for determining bacterial biomass. For example, in aquatic systems, biomass of bacterial populations is often estimated by assessing the morphology and size of microorganisms present and then using a conversion factor to estimate biomass from biovolume (Bölt et al., 2002; Lawrence et al., 2007).

With regard to flow cytometric counts, in certain cases the method often leads to overestimation of abundance due to the counting of auto-fluorescent particles and photosynthetic prokaryotes. For example, both Marie et al. (1997) and Sieracki et al. (1995) noted significant overestimation of the numbers of heterotrophic bacteria in marine samples when counting with AO and DAPI, respectively, due to the presence of large numbers of Prochlorococcus. However, because our flow cytometric experiments were performed using laboratory cultures, this effect could not manifest. Instead, our flow cytometric counts appear (relative to bioadhesive slides) to underestimate abundance, which we attribute to particle coincidence for samples at concentrations of >10⁵ cells ml⁻¹ (Gasol and del Giorgio, 2000), choice of fluorochrome (Lebaron et al., 1998), and machine settings such as compensation and flow rate (Sieracki et al., 1995). It is notable that observing higher microbial counts using an alternative enumeration platform is not unprecedented. For example, Gasol et al. (1999) compared filter-based assays with flow cytometry and found a similar discrepancy using DAPI, but the opposite effect with SYTO13. Using a protocol developed to enumerate lake bacterioplankton, del Giorgio et al. (1996) found that flow Fig. 3. Fraction of cells determined to be “live” for each enumeration platform (mean ± 1 S.E.). Statistically significant differences determined via t-test; *** corresponds to p<0.0001 (Table 2).
cytometry using SYTO13 and epifluorescence microscopic counts with DAPI varied from 72 to 141% and exhibited differences in precision and sample processing efficiency. Such differences are of the same order of magnitude as those we observed between bioadhesive slides and the standard enumeration platforms. Furthermore, though total abundance differed depending on the enumeration platform, CV analysis indicated that bioadhesive slides have similar precision to polycarbonate filters and flow cytometry. Samples with high concentrations of cells (B. cereus and E. coli, in particular) also exhibited an aggregation phenomenon at the edges of the wells on the bioadhesive slides (along the hydrophobic border), though this effect was not severe at lower cell concentrations.

Preliminary trials indicated that rinsing the bioadhesive slides following sample application further decreased background fluorescence. However, empirical data showed that our particular method of rinsing (squirting briefly with deionized water) resulted in a significant decrease in the number cells adhering to slides (Fig. 4). Thus, unless one is working with samples with excessively high background fluorescence (e.g., sediments), attempts to rinse excess stain in this manner from the slides is not advisable. It is possible that alternative methods of rinsing slides, soaking for example, may not result in a significant reduction in cell number. Further testing also may be warranted in the event that certain samples, such as hypersaline waters, form salt crystals following drying on bioadhesive slides, which could interfere with cell counts. However, we did not observe crystals from the ambient media or ocean water samples.

A drawback of using the brand of bioadhesive slides chosen for this investigation was an extreme mortality effect on some pure cultures and river water microbes. When only total cell counts are required, either by summing L/D counts or by using AO stain, this effect is irrelevant. However, for assays requiring estimation of viability, preliminary trials would be required for each particular cell or habitat type to assess the possibility of adhesive-induced mortality. The most likely explanation for this effect is a response of certain cell types to chemical components in the proprietary emulsion coating the bioadhesive slides. These compounds could cause mortality or simply alter the permeability of the cell membrane without loss of viability. Although L/D is often used to make inferences about “live” and “dead” microorganisms, the kit actually works by differentiating between bacteria with intact and damaged cytoplasmic membranes. The distinction results from the inability of PI to enter cells with intact membranes because of the size and charge of the PI molecule (Hewitt and Nebe-Von-Caron, 2004). Several compounds have been found to destabilize cell membranes (e.g., EDTA), allowing PI to enter and cause the bacteria to appear red/dead despite continued metabolic activity and cell division. The permeabilization effect of these compounds can vary depending on physiological properties of the bacteria including whether the cells are gram positive or negative, growth phase, and prior nutrient limitation (Berney et al., 2007).

We made several attempts to discern the mechanism for the “mortality” effect that was observed using bioadhesive slides including: (i) varying the staining and sample-application strategy (e.g., staining in the tube, staining directly in the well of the bioadhesive slides, rinsing the cells prior to dilution and staining, and diluting in buffer rather than deionized water), (ii) having the slides prepared by several different investigators, (iii) testing cultures during different growth phases (e.g., exponential vs. log), and (iv) considering the G+/G− status of the species used in the culture experiments. Despite these efforts, the effect always was observed for certain species and was evident during two separate experiments using river water. Further investigation is necessary to determine the cause of membrane disruption on the bioadhesive slides and/or whether this happens with alternative types of bioadhesive slides. Likewise, it may be appropriate to use more than one viability indicator for analysis and to screen for the best stain-slide combination for each research question. These are issues that have been raised for filter-based and flow cytometric assays as well (Berney et al., 2007, and references therein).

Thus, bioadhesive slides are an accurate and precise platform for rapid enumeration of bacteria in pure cultures and environmental samples from a wide variety of habitat types. The method is compatible with a variety of common fluorescent stains (AO, L/D, and DAPI), and has potential application for users of FISH. Enumeration on bioadhesive slides is particularly effective because it can be performed with extremely small volume samples, allows for identification of cells that are not readily discernible on filters, facilitates replication, and increases throughput by reducing processing time and materials (up to 24 samples per slide for the brand of slides used in these trials). These benefits are noteworthy for environmental samples, where researchers often collect large numbers of samples, and the time-consuming nature of filter-based slide preparation imposes impractical limitations on replication and experimental design. Further, the ease of bioadhesive slide use promotes expeditious analysis upon collection ( Bölter et al., 2002), reducing sample degradation associated with long-term storage. In combination with digital image analysis and automated counting procedures (e.g., see the recent review by Seo et al., 2010), this bioadhesive-slide approach has the potential to dramatically improve data collection associated with enumeration of bacterial abundance in environmental samples.

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References
