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Comparison of Methods for Detection and Enumeration of Airborne Microorganisms Collected by Liquid Impingement

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Bacterial agents and cell components can be spread as bioaerosols, producing infections and asthmatic problems. This study compares four methods for the detection and enumeration of aerosolized bacteria collected in an AGI-30 impinger. Changes in the total and viable concentrations of *Pseudomonas fluorescens* in the collection fluid with respect to time of impingement were determined. Two direct microscopic methods (acridine orange and BacLight) and aerodynamic aerosol-size spectrometry (Aerosizer) were employed to measure the total bacterial cell concentrations in the impinger collection fluid and the air, respectively. These data were compared with plate counts on selective (MacConkey agar) and nonselective (Trypticase soy agar) media, and the percentages of culturable cells in the collection fluid and the bacterial injury response to the impingement process were determined. The bacterial collection rate was found to be relatively unchanged during 60 min of impingement. The aerosol measurements indicated an increased amount of cell fragments upstream of the impinger due to continuous bacterial nebulization. Some of the bacterial clusters, present in the air upstream of the impinger, deagglomerated during impingement, thus increasing the total bacterial count by both direct microscopic methods. The BacLight staining technique was also used to determine the changes in viable bacterial concentration during the impingement process. The percentage of viable bacteria, determined as a ratio of BacLight live to total counts was only 20% after 60 min of sampling. High counts on Trypticase soy agar indicated that most of the injured cells could recover. On the other hand, the counts from the MacConkey agar were very low, indicating that most of the cells were structurally damaged in the impinger. The comparison of data on the percentage of injured bacteria obtained by the traditional plate count with the data on percentage of nonviable bacteria obtained by the BacLight method showed good agreement.

Many respiratory and other health disorders are associated with and spread by bioaerosols outdoors and indoors (7, 28, 60). Infectious indoor agents (such as *Legionella* sp. and *Staphylococcus* sp.), indoor allergens (e.g., *Penicillium* sp., *Alternaria* sp., *Bacillus subtilis*, *Bacillus cereus*, and *Actinomyces* sp.), and invasive fungal agents (e.g., *Aspergillus fumigatus*) can be sampled and enumerated by complex collection and analytical techniques (7, 8, 23, 24, 26, 35). Many different factors affect the microbial collection and survival in bioaerosol samplers and, therefore, the accuracy of enumeration. Macher and Willeke (34) stated that aerosol concentration and composition, inlet orientation, aerosol charge, particle desiccation and shear forces, wind speed, particle breakup, and sampling flow rate changes may affect the number of collected microorganisms as well as their viability and culturability. The overall efficacy of a bioaerosol measurement device depends on physical parameters, such as its inlet and collection efficiencies and its bioefficiency (41). Grinshpun et al. (17) showed that commercially available bioaerosol samplers may significantly over- or underestimate the bacterial concentration under certain sampling conditions in indoor and outdoor air environments because their inlet sampling efficiency may be greater or less than 100%. Nevalainen et al. (42) reported different physical collection efficiencies for several widely used bioaerosol samplers based on cut size, i.e., the size of a microorganism at which 50% were collected. As an approximation, it was generally assumed that all microorganisms above this cut size were collected, while those below this size were not. For example, the cut size of the AGI-30 impinger (Ace Glass Inc., Vineland, N.J.) was 0.31 μm, while 0.57 μm was the cut size of the sixth stage of the Andersen impactor. This suggests that the impinger may be more efficient in collecting very small bioaerosol particles. Bioefficiency deals with the survival and recovery of microorganisms after their collection from the air. Differences in bioaerosol collection in the available samplers may result in different degrees of microbial injury. For example, Buttner and Stetzenbach (9) found that *Pseudomonas syringae* was more damaged with time by the AGI-30 impinger than by the Andersen six-stage impactor. Juozaitis et al. (26) compared a new agar slide impactor and a new liquid impinger with the Andersen six-stage impactor and AGI-30 impinger, respectively, and found that they gave comparable bioefficiencies which reflected the same degree of microbial damage occurring during sampling. The factors that may affect the survival of the microorganisms include the species of microorganisms, the cultural conditions and treatment, organic and inorganic additives, temperature and humidity, the presence of oxygen, and bacterial stress. The latter appears to occur in response to shear conditions (18, 58). Stewart et al. (56) reported, for instance, that an increase in sampling velocity may result in a significant increase in metabolic and structural injuries to the collected microorganisms. In addition, counting efficiency can be affected by the colony masking effect (11, 12), resulting in underestimation of bacterial counts when there is significant colony overlap.

Various methods for total and viable microbial counts are

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currently used for the microbiological analysis of bioaerosols. The total count is usually obtained by a staining method (16, 32, 33, 38, 39, 53, 59), such as acridine orange direct counts (AODC). Other methods for total microbial counts utilize the Coulter counter (19, 29), immunofluorescence microscopy (5, 46), scanning electron microscopy (4, 6, 22, 37), or radioactive probes (15). In addition, dynamic particle-size spectrometry has recently been used in laboratory experiments for measuring the total and size-selective counts while microorganisms are still in the airborne state (48).

Several of the currently available methods for viable or total microbial enumeration, which identify reduced respiratory products within the bacterial cell by fluorescent stains (3, 51, 65), have frequently shown discrepancies in counts, which are caused by their complexity and the limited performance of the staining procedure (36, 51, 54, 61, 64). To increase the efficiency of staining and enumeration procedures, the LIVE/DEAD BacLight (BL) viable staining method (Viability Kit L-7007; Molecular Probes, Eugene, Oreg.) was recently developed and evaluated with pure cultures of a variety of bacterial species, including *Pseudomonas fluorescens* (40). It uses a mixture of two nucleic acid stains, labelled A and B, to stain live cells green and dead cells red under an epifluorescence microscope. Plate counts, the most-probable-number technique, and membrane filter examinations are commonly used for determining the viable microbial count (10, 16, 30, 31, 47, 55).

Microbial sampling and enumeration can be optimized by choosing an appropriate sampling time, since both the physical and biological sampling and counting efficiencies depend on this important parameter. For example, the total microbial count in an impinger generally is not linear with sampling time, as deagglomeration and reaerosolization of microorganisms may occur in its collection fluid (26, 62). Microbial stress during the impingement of airborne microorganisms and the violent motion in the liquid after collection may affect the viable count in an impinger in a time-dependent manner. Because impingement is a widely used technique for aerosol sampling, it is important to study the changes in the total and viable counts of microorganisms collected from the air into a liquid impinger that take place with time.

The purpose of this study was to compare different methods for the detection and enumeration of aerosolized bacteria collected in an impinger. This study demonstrated the time dependency of the total and viable concentrations of *P. fluorescens* bacteria in the collection fluid of the commonly used AGI-30 impinger. Two direct microscopic methods (AODC and BL) as well as aerodynamic particle sizing were employed for total bacterial concentration measurements. The results of the BL staining technique, used to determine the changes in viable bacterial concentration during impingement, were compared with the results of plate counts on selective and nonselective media used to measure bacterial stress.

MATERIALS AND METHODS

Test system and experimental design. The facility which we developed earlier for the evaluation of bioaerosol samplers (26, 56, 57) was used in this study. The test system shown in Fig. 1 was placed inside a class II, type B2, biological safety cabinet (SterilchemGARD; Baker Company, Inc., Sanford, Maine). The bacterial aerosol, generated from a culture suspension by a Collison three-jet nebulizer (BGI Inc., Waltham, Mass.) by using HEPA-filtered compressed laboratory air at a flow rate (Q_{NEB}) of 6 liters/min, was further diluted and dried with filtered air at a flow rate (Q_{DRY}) of 40 liters/min. Before entering the sampling chamber (550 cm^3), the combined air flow (Q_{TOTAL}) passed through a 10-mCi ^{85}Kr particle charge neutralizer (model 3012; TSI Inc., St. Paul, Minn.) to minimize the loss of aerosolized microorganisms to the walls of the test system due to electrostatic charges. The bioaerosol was sampled by an AGI-30 impinger, placed inside the sampling chamber (Fig. 1), and operated at a flow rate (Q_{IMP}) of 12.5 liters/min. Four identical AGI-30 impingers were used in these tests. The

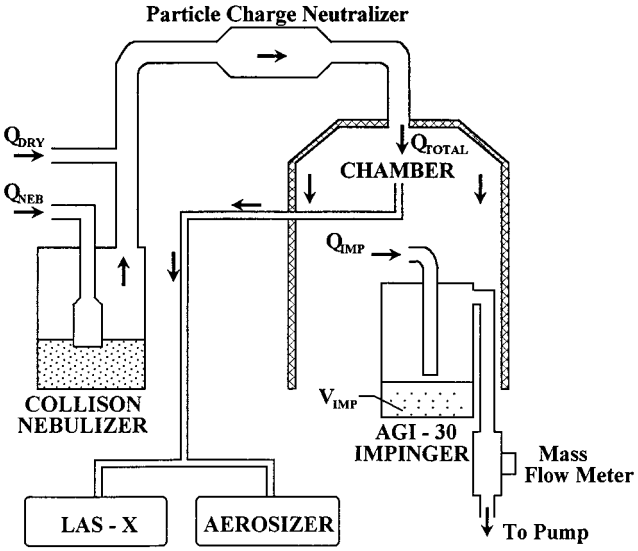

FIG. 1. Schematic representation of the experimental setup.

variability of their physical collection efficiencies did not exceed 10%. The flow rate was controlled by a mass flow meter, and the initial liquid volume in the impinger was always 20 ml of deionized water (five-stage Milli-Q Plus system; Millipore Corp., Bedford, Mass.). The inlet sampling efficiency and the physical collection efficiency of the AGI-30, evaluated by previously developed methods (17, 26, 42), were approximately 100% for particles with aerodynamic diameters of 0.7 to 1.0 μm. The bioaerosol was sampled for 1 h in 15-min time intervals. At least three replicate measurements of the total and viable bacterial concentrations in the collection fluid were made for each sampling time. For all experiments, the air temperature was maintained between 20 and 23°C, and the relative humidity was between 28 and 32%. The collection fluid was analyzed after each test, and the total and viable microbial counts were performed by AODC, BL, and plate count methods.

The Aerosizer, an aerodynamic particle-size spectrometer (API Mach II; Amherst Process Instruments, Inc., Hadley, Mass.), was used for the determination of the particle size distribution and particle concentration of aerosolized bacteria in the sampling chamber upstream of the test impinger. The particle concentration downstream of the impinger was checked intermittently and was negligibly small as a result of a high physical collection efficiency. The Aerosizer measures up to 1,100 particles/cm^3 over an aerodynamic size range of 0.5 to 200 μm at a flow rate of 5.3 liters/min (13). It measures the health-related aerodynamic equivalent diameter which depends on the shape and density of the organism (48) and, as such, may differ from the physical size of the microorganism. In this study, the Aerosizer was used for enumeration of the bacteria in the air upstream from the impinger. The concentration of microorganisms collected in the impinger fluid (N_{IMP}) was calculated from the concentration of test bacteria in the air (N_{AER}):

$$N_{IMP} = \frac{N_{AER} \times t_{SAMPL} \times Q_{SAMPL}}{V_{IMP}} \quad (1)$$

where N_{AER} is the microorganism concentration per cm^3 of air, t_{SAMPL} is the sampling time (in minutes), Q_{SAMPL} is the sampling flow rate for the impinger (in cubic centimeters per minute), and V_{IMP} is the liquid volume (in milliliters) in the impinger. If the physical collection efficiency is less than 100% (not encountered in our experiments), the concentration N_{AER} in equation 1 should be reduced by the collection efficiency. The sampling time was 1 min.

Since the lower cut size of the Aerosizer is about 0.5 μm, bacterial fragments and residue particles below this size cannot be detected accurately by this instrument. Therefore, another dynamic particle sizer, i.e., the optical-size spectrometer LAS-X (Particle Measuring Systems, Inc., Boulder, Colo.), was used in parallel for the particle count in the air. The LAS-X measures the optical-equivalent particle sizes down to 0.09 μm (at a sampling flow rate of 0.06 liter/min). The optical size is related to physical and aerodynamic sizes through the refractive index of the particle or microorganism (1). The LAS-X and the Aerosizer were used in parallel to distinguish the 0.3- to 0.5-μm fragments from single cells. The relationship between the Aerosizer and LAS-X data was presented earlier (48) for the test microorganism.

Test microorganism. A stock culture of *P. fluorescens* ATCC 13532, used in this study, was obtained from the American Type Culture Collection (Rockville, Md.). This rod-shaped, gram-negative bacterium, ranging in size from 0.7 to 0.8 μm in diameter and from 2.3 to 3.8 μm in length (43), was chosen as a repre-

sentative of the genera which have been found in ambient air (41) and because it is sensitive to aerosolization and collection stress (56). The mean aerodynamic size of the aerosolized *P. fluorescens* bacteria was found to be 0.78 μm (48). This bacterium was routinely maintained on Trypticase soy agar (TSA; Becton Dickinson Microbiology System, Cockeysville, Md.) at 4°C and stored in 1% (wt/vol) peptone water containing 40% (vol/vol) glycerol at −20°C. The identification of the microorganism was periodically confirmed by the API-NFT identification system (Biomerieux Vitek, Hazelwood, Mo.).

Preparation of the pure culture suspension. One milliliter of the overnight *P. fluorescens* starter culture was grown in 100 ml of Trypticase soy broth (Becton Dickinson Microbiology System) for 18 h at 28°C with shaking in a water bath (New Brunswick Scientific, Edison, N.J.) at 150 rpm (2-cm circle). The organisms were washed twice by centrifugation at 6,000 × *g* for 5 min (Sorvall RC-5B refrigerated superspeed centrifuge; Dupont Co., Boston, Mass.) with sterile, deionized water. Deionized water was used in this study as a diluent because buffer salt molecules were expected to affect the results obtained by the BL and aerodynamic particle-sizing methods. The suspension was diluted to a density of approximately 10^8 CFU/ml, as determined by the Spectronic 21D spectrophotometer (Milton Roy Co., Rochester, N.Y.), with a transmittance of 89 to 92% at 475 nm. A 100-ml volume of this bacterial suspension was placed in the nebulizer to be aerosolized. To ensure uniform sampling conditions and to reduce, to the extent possible, microbial nebulization stress, a freshly prepared bacterial suspension was used for each sampling period. For control experiments, 20 ml of deionized water was placed in an impinger and inoculated with a final concentration of 1×10^6 to 5×10^6 CFU/ml, and the impinger was operated in bacterium-free air supplied through a HEPA filter.

AO staining. The acridine orange (AO) staining procedure was used for total bacterial enumeration (without distinguishing between live and dead bacteria) by counting with an epifluorescence microscope. In this procedure, 1 ml of cell suspension was stained with 0.1 ml of 0.01% AO stain (ICN Biochemicals, Cleveland, Ohio) in 0.2 M acetate buffer at pH 4.5 (Sigma Diagnostics, St. Louis, Mo.). After incubation at room temperature in the dark for 5 min, 0.1 ml of 2% (vol/vol) formaldehyde was added to 1 ml of the sample to stop the reaction and preserve the sample (21). Deionized water was used as a negative control for autofluorescence.

BL staining. For the LIVE/DEAD BL microscopic technique, aliquots (1 ml) of an appropriate 10-fold dilution of the impinger collection fluid were stained with a 3-μl mixture of A and B (1:1) nucleic acid stains (40) and incubated in the dark for 25 min at room temperature. The LIVE/DEAD BL stain mixture (A and B) distinguishes live bacterial cells from dead by means of membrane integrity. Ideally, healthy (live) bacteria with an intact plasma membrane fluoresce green, and the dead or injured cells with a compromised membrane fluoresce red. In practice, however, the BL stain gives a whole range of colors from green to red for cells ranging from live to dead. Following the manufacturer's instructions (40), all green cells were considered live, while the cells of yellow, rust, and red color and shadow-like cells were considered dead. Thus, a conservative approach was undertaken for evaluation of the live cells.

Filtration and enumeration procedures for microscopic methods. Bacteria stained by AO or BL were captured by microfiltration through a 0.2-μm-pore size, 47-mm, black polycarbonate membrane filter (Poretics Co., Livermore, Calif.) placed in a Millipore glass filter funnel unit (Millipore). To ensure uniform cell distribution, the membrane was reinforced by a polyester drain disk (Poretics). The stained bacteria were added to 20 ml of 0.1 M phosphate buffer (pH 7.2), filtered by vacuum suction (16), and then washed twice with 20 ml of deionized water to remove excess formaldehyde or unbound stain. The filter was removed, cut in half, and mounted on a glass slide with low-fluorescence immersion oil (25). The oil (type A; Cargille Laboratories, Cedar Grove, N.J.) was spread onto the filter and covered with a glass cover slip (22 by 50 mm; Fisher Scientific, Pittsburgh, Pa.).

A minimum of 20 random fields (≥400 cells [2]) were counted with a Nikon (Tokyo, Japan) microscope (Labophot) equipped with a 100-W UV mercury lamp, a 10× ocular lens, a 460-nm filter, and an objective, Ph 4, Plan 100 DS, 1.25 oil, 160/0.17. The total bacterial concentration per milliliter of impinger collection fluid (N_{IMP}), was calculated as follows (20):

$$N_{IMP} = \frac{M \times CF \times DF}{V_{IMP}} \quad (2)$$

where M is the microscopic field count, representing the average number of bacteria per field, CF is the membrane conversion factor, and DF is the dilution factor. CF is the ratio of drainage area (873.09 mm^2) to microscopic field area (0.0154 mm^2), i.e., about 56,700. The drainage area was determined by measuring the internal diameter of the filter funnel. Following the BL staining, the viable bacterial concentration was also determined by use of equation 2, in which the microscopic field count, M, of only green fluorescent cells was referred to as the live count. The overall time for an assay by either of these two methods was about 2 h.

Plate counts. The standard spread plate technique (16) was used to determine bacterial viability through the ability of cells to divide and form colonies. An aliquot (0.1 ml) of an appropriate 10-fold dilution of the impinger fluid was spread on TSA or MacConkey (MAC) agar (Becton Dickinson Microbiology Systems). After incubation at 28°C for 48 h, colonies were counted with a Quebec colony counter (dark-field Reichert-Jung counter; Leica, Inc., Deerfield, Ill.).

Correction of the microscopic and plate counts. The experimental conditions of this study were chosen to minimize the effects of particle bounce and reaerosolization in impingers (62), which are difficult to account for quantitatively and may result in the loss of collected particles during bioaerosol sampling. Because of extensive evaporation of the collection fluid during operation, which reached ca. 50% of the initial impinger volume per hour at a relative humidity of 30%, all microscopic and plate count results were corrected by a liquid evaporation factor:

$$\text{Evaporation factor} = \frac{\text{Collection fluid volume at } t}{\text{Collection fluid volume at } t = 0} \quad (3)$$

where t denotes sampling time.

Assessment of bacterial stress or injury with time. The physiological responses of bacteria to stress were evaluated by utilizing three approaches based on membrane integrity response to staining, nonselective media, and selective media. (i) The relative number of viable (live) bacterial cells was determined by the membrane integrity response to BL staining:

$$\%\ \text{Viable bacteria} = \frac{\text{Live count}_{BL}}{\text{Total count}_{BL}} \times 100\% \quad (4)$$

The nonviable bacterial cell count was calculated as:

$$\%\ \text{Nonviable bacteria} = 100\% - \%\ \text{Viable bacteria} \quad (5)$$

(ii) The percentage of culturable cells on the nonselective medium (TSA) relative to the total bacterial count (determined by BL staining) was calculated as:

$$\text{Culturable bacteria} = \frac{\text{CFU}_{\text{nonselective}}}{\text{Total count}_{BL}} \times 100\% \quad (6)$$

(iii) Bacterial injury (39, 50) was determined as a ratio of the difference between colony counts on nonselective (TSA) and selective (MAC) media to the counts on the nonselective medium:

$$\%\ \text{Bacterial injury} = \frac{\text{CFU}_{\text{nonselective}} - \text{CFU}_{\text{selective}}}{\text{CFU}_{\text{nonselective}}} \times 100\% \quad (7)$$

A schematic representation of the different bioaerosol sampling and enumeration methods and their analysis techniques is shown in Fig. 2. A summary of these methods and procedures with further details is given in Table 1.

RESULTS AND DISCUSSION

Changes in bacterial cell size distribution in the air after aerosolization. The integrity and homogeneity of the test bioaerosol were examined in the sampling chamber by measurements with two dynamic particle-size spectrometers, the LAS-X and the Aerosizer, after 3, 30, and 60 min of microbial nebulization. The bacterial concentrations, N (in particles per cubic centimeter), are shown in Fig. 3A as a function of the optical equivalent diameter d_{opt} (measured by LAS-X) and in Fig. 3B as a function of the aerodynamic equivalent diameter d_{ae} (measured by Aerosizer). The mean optical equivalent diameter of the bacteria is found to be about 0.57 μm. The data in Fig. 3A show a considerable increase in the concentration of the small particles (0.2 to 0.4 μm in diameter) with time. This increase may indicate shrinkage of cells, cell fragmentation, and other processes associated with microbial nebulization. With time, small submicron particles appear to be washed from the bacterial cells in the nebulizer (48, 63). This increase in fragment concentration may be an indicator of bacterial mechanical injury in the nebulizer causing bacterial slime, capsular material, cell wall, or cell membranes to be broken from the cells and subsequent leakage of intracellular components, such as DNA, RNA, Mg^{2+} (49), polysaccharides, proteins, and other nutrients. These materials may be used for microbial recovery and colony growth after collection (52).

The Aerosizer, which measured the aerodynamic particle-size distribution, could not detect most of these small fragments and, therefore, showed a single peak with a mean size at about 0.80 μm. Consequently, the data in Fig. 3B suggest that nebulization does not significantly affect the aerodynamic size

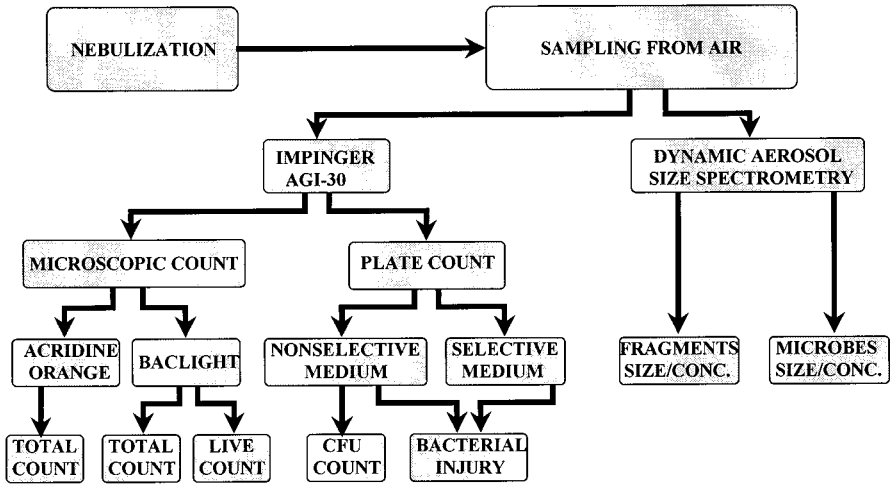

FIG. 2. Methods for detection and enumeration of airborne microorganisms. CONC., concentration.

of bacterial cells: all three particle size distributions are essentially the same. Deagglomeration is depicted on the right side of these aerodynamic particle-size distributions. However, the small increase in the size distribution at about 0.5 μm may be due to the increasing presence of large fragments with time. Thus, the data obtained with the two particle-size spectrometers indicate that the airborne concentration of cells was nearly constant during 1 h of nebulization while the airborne concentration of bacterial fragments increased with time of nebulization.

Changes in total bacterial cell concentration with time. Figure 4 presents the total bacterial concentration in the AGI-30 liquid with time of impingement, as measured by the two microscopic methods (AODC and BL) and by the dynamic particle-size spectrometer (converted to liquid concentration by equation 1). Each value represents the arithmetic mean of at least three measurements at a 95% confidence interval. All data were compared by a paired Student t test to determine statistically significant differences ($P \leq 0.05$). The measured values of bacterial concentration in the collection fluid ranged from about 10^5 to 10^6 cells per ml for sampling times of 15 to 60 min. The results obtained by all three methods showed that, with continuous bacterial supply, the measured total bacterial cell concentration increased over time, as expected. The increase slightly deviated from linear, particularly for the dynamic particle-size spectrometry. In the absence of microbial fragmentation, particle bounce, and reentrainment in the impinger, the increase should be linear.

To compare the three methods (Fig. 4), the bacterial cell concentrations in the collection fluid were converted to bacterial collection rates in the impinger, defined as the total number of collected microorganisms per collection volume per total time. Table 2 shows that the collection rates for all three methods did not change significantly over 60 min of impingement. The standard deviation ranged from 2 to 20% with only one exception, the BL measurement at $t = 30$ min. The arithmetic average collection rates measured by the AODC and BL methods during 60 min of impingement were $(18 \pm 2) \times 10^3$ and $(13 \pm 3) \times 10^3$ microorganisms per min per ml, respectively; the average rate calculated from the Aerosizer data was $(9.3 \pm 0.9) \times 10^3$ microorganisms per min per ml.

The collection rate determined by the Aerosizer was lower than that obtained by the AODC and BL methods. This suggests that there may have been some bacterial clusters in the air upstream of the impinger that deagglomerated during impingement, thereby giving higher direct microscopic counts in the impinger fluid. By using this hypothesis, the AODC and BL methods counted individual cells, while the Aerosizer counted some clumps as single particles. No significant aggregation is apparent from the Aerosizer data of Fig. 3B. However, the alignment of aggregates in the sensing volume of the relatively new Aerosizer is not yet known. Thus, the evaluation of the total airborne bacterial concentration may be not sufficiently accurate if the conclusions are drawn from only one enumeration method. To evaluate the actual cell concentration, the particle count in air must be used in combination with the microscopic count in the liquid. The small increase with sampling time in collection rate, as calculated from the Aerosizer data, may be due to the increase in aerosol concentration near 0.5 μm (Fig. 3B), which we attribute to cell fragmentation. The

TABLE 1. Comparison of methods for the enumeration of bacteria in collection fluids

Method	Procedure	Type of bacterial enumeration	Time of assay
Direct microscopic count	Staining by AO	Total (all cells)	2 h
	Staining by BL	Total (all cells)	2 h
	Staining by BL	Live (green cells)	
Aerodynamic particle-size spectrometry (Aerosizer)	Particle sampling and in situ analysis	Total (converted from particle count in the air[a])	1 min
Plate count	Incubation on nonselective medium (TSA)	Culturable (CFU)	48 h
	Incubation on selective medium (MAC)	Culturable (CFU)	48 h

[a] Particle concentration in the air was converted into particle concentration in the collection fluid by equation 1.

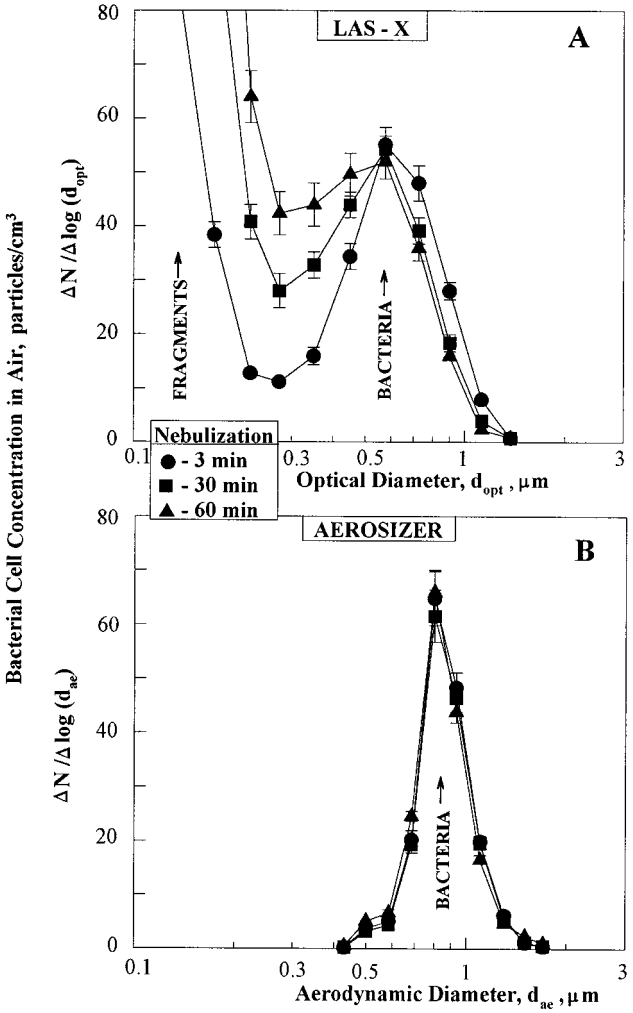

FIG. 3. Changes in bacterial cell size distribution with time, as measured in the air by the optical particle-size spectrometer (LAS-X) (A) or by the aerodynamic particle-size spectrometer (Aerosizer) (B) during concurrent sampling in the AGI-30 impinger. The test bacterium was *P. fluorescens.*

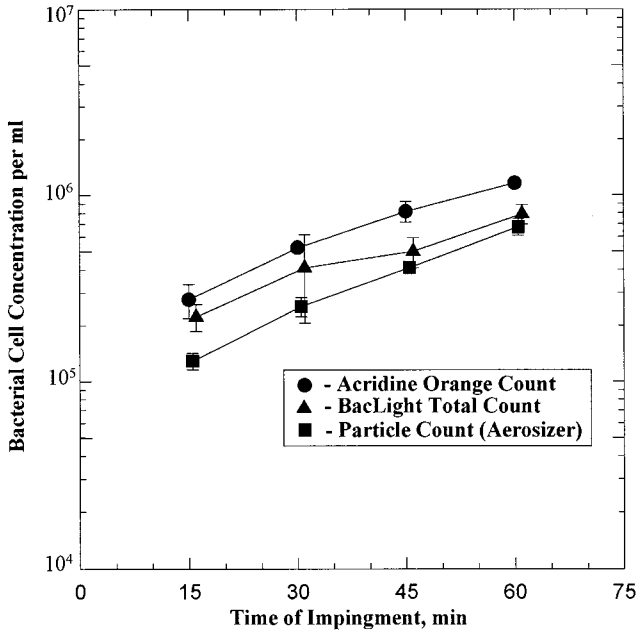

FIG. 4. Cumulative changes in the total bacterial cell concentration with time during the collection of *P. fluorescens* bacteria with the AGI-30 impinger. The total bacterial cell concentration was determined by (i) the direct microscopic count method following the AO or BL staining procedures and (ii) calculation from Aerosizer data.

fragments may not be stained by the AODC or BL methods and, therefore, do not affect the results obtained by these microscopic methods.

Some of the differences in the collection rates obtained by the microscopic examination following AO and BL staining may be associated with the specificity of the fluorescent stain. For example, AO is known to overestimate counts due to autofluorescence or nonspecific staining of cellular components (44, 45). Dark or shadowed cells were hard to distinguish. This may have contributed to the lower BL count. The low fluorescence intensity of the dark cells may be related to the selected concentration of the two-color mixture, which may be suitable for the healthy cells but not for the stressed cells.

Control experiments with bacterium-free air and bacteria in the collection fluid containing bacteria. As a control experiment, we tested the hypothesis that bubbles bursting in the collection fluid and the passage of filtered air through the bacterial suspension may adversely affect the bacterial cell concentration in the impinger. To determine if this effect was significant, a bacterial concentration of ca. 10^5/ml was placed in the AGI-30 impinger, and the impinger was operated with sterile air. Table 3 shows the total bacterial concentrations measured by the AODC and the BL staining techniques. Ideally, the total bacterial concentration should remain the same for each sampling time (15, 30, 45, and 60 min). The data obtained by the AODC method are essentially the same within experimental error. The arithmetic average bacterial concentration is $(108 \pm 21) \times 10^3$ microorganisms per ml. The same conclusion may be claimed for the BL data, although these data have a higher relative experimental error. The BL data show a small but not significant ($P = 0.61$) decrease in the average bacterial count for 60 min of aeration, starting at 30 min.

Changes in viable bacterial cell concentration with time. Samples of the collection fluid, taken from the AGI-30 at different time intervals during continuous impingement of aerosolized *P. fluorescens* and during the control experiments, were then analyzed for viable counts by the BL (live) method. Figure 5 shows that the change of the viable cell concentration during the standard continuous operation of the impinger was not significant, while the total concentration, shown in Fig. 4, doubled with a doubling of sampling time. This indicates that

TABLE 2. Bacterial collection rate in the AGI-30 impinger determined by three different methods

Time of impingement (min)	Collection rate[a] (10^3 microorganisms/min/ml) for total bacterial count		
	AO	BL	Particle count converted from Aerosizer
15	18 ± 4	15 ± 2	8.7 ± 0.7
30	17.4 ± 0.6	14 ± 7	8.3 ± 1.0
45	18 ± 2	11 ± 2	9.1 ± 0.7
60	19.3 ± 0.3	13 ± 2	11 ± 1

[a] Mean of three values ± standard deviation.

TABLE 3. Control experiments using bacterium-free air and collection fluid containing bacteria

Time of aeration (min)	Total bacterial concentration[a] (10^3 microorganisms/ml)	
	AO	BL
0	130 ± 40	110 ± 30
15	110 ± 20	110 ± 50
30	91 ± 14	89 ± 36
45	87 ± 13	ND[b]
60	120 ± 20	53 ± 48

[a] Mean of three values ± standard deviation.
[b] ND, not determined.

bacterial cell damage occurred during the impingement process. The average viable concentration values, determined from the aeration control experiments with microorganism-free air, show the same tendency between 15 and 60 min of the impinger operation. This suggests that the bubbling in the impinger partially contributed to bacterial injury. However, standard deviations of these data are too high for making definitive conclusions at this time.

The viability of *P. fluorescens* measured by the BL method and calculated by using equation 4 is shown in Fig. 6 as the fraction of live cells to total cells. The initial viability of the nebulizer suspension, shown in Fig. 6 at $t = 0$ min, was 55% ± 18%. Our previous studies (26, 57) had also shown that the *P. fluorescens* initial viability was <100%. Our viability measurements on samples from the nebulizer suspension, performed at 15-min intervals, did not indicate any significant change with time, while the fragment release was very pronounced, as previously shown in Fig. 3. Further bacterial viability loss may have occurred because of mechanical stress from aerosolization, transport from nebulizer to impinger, desiccation and stress caused by oxygen toxicity (14), and impingement at sonic velocity. This explains why the actual viability in the impinger liquid immediately after starting collection was lower than the 55%, as shown in Fig. 6 for the nebulizer suspension at $t = 0$ min.

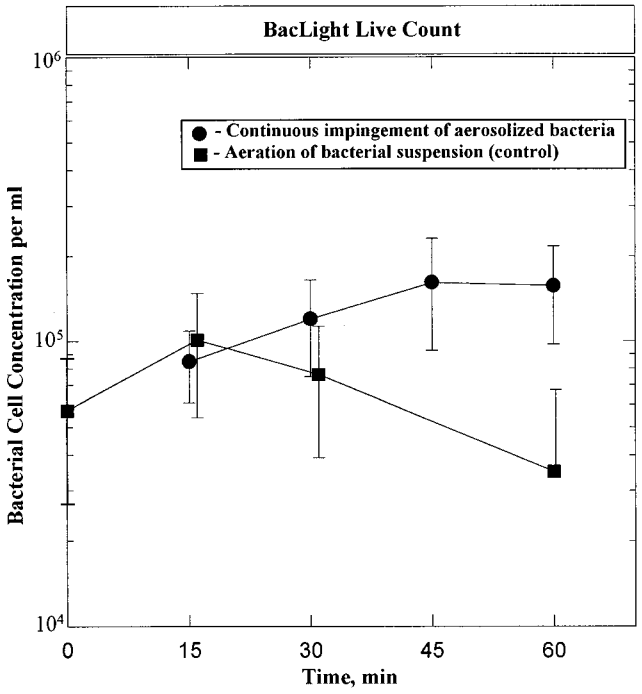

FIG. 5. Changes in the viable cell concentration with time during collection with the AGI-30 impinger, as determined by the direct microscopic count method following the BL staining procedure: the first impinger collects airborne bacteria continuously; the second impinger, containing a bacterial suspension, is aerated only.

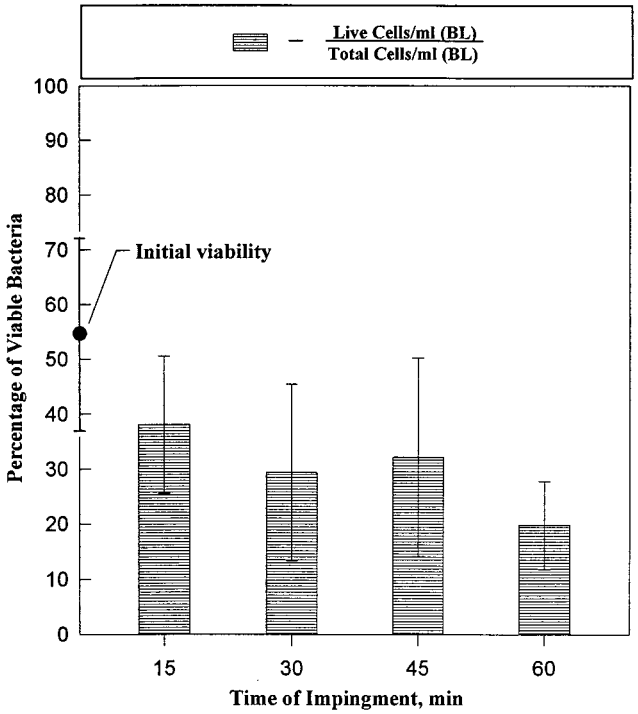

FIG. 6. Change in the percentage of viable bacteria during sampling with the AGI-30 impinger, as determined by the ratio of BL live and total counts.

After 15 min of bacterial collection by the impinger, the measured viability decreased from 55% ± 18% to 38% ± 12% (Fig. 6), which is not statistically significant. However, the difference between results obtained at $t = 0$ and $t = 60$ min is statistically significant. This means that the smaller values of average viability during prolonged impingement (ca. 20% at $t = 60$ min) resulted from mechanical stress on the bacteria during impingement and subsequent aeration. Some cells damaged during impingement may recover in the suspension over time and be detected by the BL method as viable (live) cells. Since microbial repair increased the viable counts and the various forms of microbial stress decreased these counts, the resulting viability loss with time may not be very pronounced.

Changes in the culturable bacterial concentration with time. The ratio of the counts on TSA to the total BL count was determined for each sampling time period by using equation 6. These ratios are 86% at $t = 15$ min, 80% at 30 min, 84% at 45 min, and 88% at 60 min. Each ratio represents the percentage of collected bacteria that were able to grow on agar plates. The data showed that the percentage of culturable cells was essentially independent of the total time of impingement and was reasonably close to 100%. A comparison of the viability data presented in Fig. 6 with the culturability data mentioned above demonstrated that most of the cells damaged by preparation, nebulization, transport, and impingement could recover. This finding illustrates the high recovery potential of stressed bacteria and their ability to repair with time on enriched media (49, 50, 52, 53).

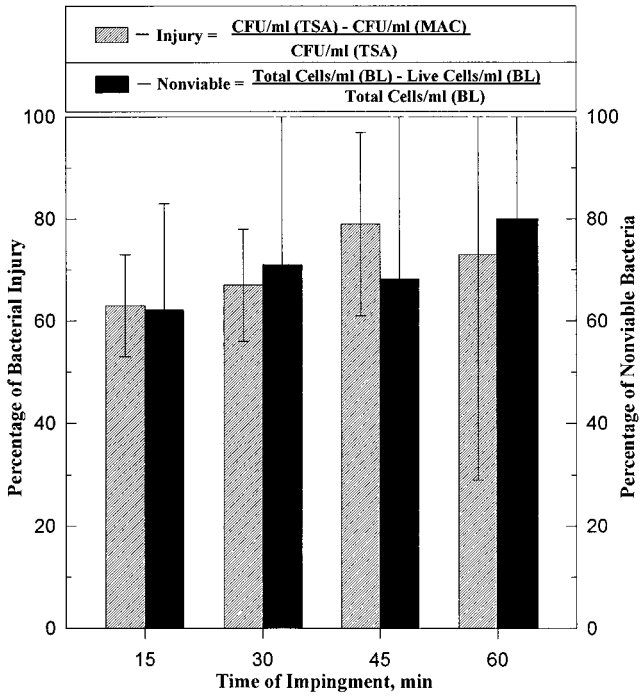

FIG. 7. Percentage of bacterial injury and nonviable cells during sampling with the AGI-30 impinger as determined by the colony counts on TSA and MAC agar plates and by BL total and live counts.

Comparison of the viable (by BL) and culturable counts also showed that the sublethally injured cells did not stain green, as did live cells determined by the BL method. The cells collected in the sample designated for culture analysis may have recovered during the 2- to 4-h storage period at 5°C prior to the plating. The stressed bacteria recovered and grew by using materials present in the culture media. In addition, since bacteria can grow upon their own cell material (52), the smaller fragments may have been absorbed into the damaged cells, enabling them to form colonies. Some or all of the bacterial cells exhibiting yellow, rust, or red colors (i.e., designated by the BL method as being nonviable) were capable of repairing their cell membranes. A similar phenomenon was observed with *Pneumocystis carinii* (27), in which the viability scores after fluorescent staining by calcein acetoxy methyl ester and propidium iodide were lower than the same population of cells following an overnight incubation in a culture medium. Our analysis of the TSA counts showed that the stress resulting from the bioaerosol sampling process over 1 h does not prevent the recovery of bacteria and the formation of colonies.

Bacterial injury during the collection process. The bacterial structural injury, as defined by equation 7, was determined for different impingement time periods. The results, presented in Fig. 7, show significant bacterial injury among the bacteria in the impinger liquid. The percentage of injured cells in the impinger was not dependent on the time of impingement, at least, within the test interval of 60 min. The average percentage of injured cells varied from about 65% at $t = 15$ min to about 75% at $t = 60$ min. The high standard deviation of the data allows us to conclude that up to 100% of the cells were injured after a sampling period of 45 min or more. The damaged cells showed a loss of membrane-related functions, such as the inability to grow on selective media (39, 49). Figure 7 also presents the percentage of nonviable bacterial cells, defined by equation 5 and replotted from Fig. 6. Good agreement ($P = 0.95$) is seen between the percentage of injured bacteria, obtained with spread plate counts from two different media, and the percentage of nonviable bacteria, which fluoresced from yellow to red. The similarities in the injury trends by these two methods most likely reflected the involvement of the same specific cellular structures in the stress-response process. The loss of the ability of stressed bacteria to form colonies on selective media or to take up some stains led to similar changes in membrane integrity. Thus, both methods (plate count and BL) could be used in parallel for the estimation of bacterial stress. The increase of the rate that cells fluoresced from yellow to rust may be a manifestation of the stress rather than a lack of bacterial viability.

Summary of results and their significance. *P. fluorescens* was aerosolized and collected into an AGI-30 impinger for total bacterial enumeration by using three of four methods compared in this study. The results were repeatable with a standard deviation for most of the tests of less than 20% and demonstrated the same trend: AODC > BL (total) > Aerosizer. The AODC method might overestimate the total count of bacterial cells, while the Aerosizer measurement may underestimate it. However, the differences were relatively small. Dynamic particle sizing with two particle-size spectrometers upstream of the impinger indicated the presence of microbial fragments, which may help in the recovery of the injured organisms. Counts from the BL live staining indicated that bacteria were injured during their injection into the impinger and during subsequent aeration. The survival of the test bacteria, shown by the ratio of BL live to BL total counts, was only about 20% after 60 min of sampling. The ratio of plate count on a nonselective medium to the BL staining total count demonstrated that most injured cells can recover after collection by the impinger. Thus, the BL live count underestimated the actual viable bacterial concentration because bacteria that were injured but able to recover did not stain green. The number of nonviable bacteria, calculated from the bacterial survival ratio of BL live to total counts, was approximately equal to the number of injured organisms obtained by classical culture analysis on selective and nonselective media.

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