APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1995, p. 1232–1239 Vol. 61, No. 4
0099-2240/95/$04.00+0

Effect of Impact Stress on Microbial Recovery on an Agar Surface

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Received 25 August 1994/Accepted 19 January 1995

Microbial stress due to the impaction of microorganisms onto an agar collection surface was studied experimentally. The relative recovery rates of aerosolized *Pseudomonas fluorescens* and *Micrococcus luteus* were determined as a function of the impaction velocity by using a moving agar slide impactor operating over a flow rate range from 3.8 to 40 liters/min yielding impaction velocities from 24 to 250 m/s. As a reference, the sixth stage of the Andersen Six-Stage Viable Particle Sizing Sampler was used at its operating flow rate of 28.3 liters/min (24 m/s). At a collection efficiency of close to 100% for the agar slide impactor, an increase in sampling flow rate and, therefore, in impaction velocity produced a significant decline in the percentage of microorganisms recovered. Conversely, when the collection efficiency was less than 100%, greater recovery and lower injury rates occurred. The highest relative rate of recovery (approximately 51% for *P. fluorescens* and approximately 62% for *M. luteus*) was obtained on the complete (Trypticase soy agar) medium at 40 and 24 m/s (6.4 and 3.8 liters/min), respectively. *M. luteus* demonstrated less damage than *P. fluorescens*, suggesting the hardy nature of the gram-positive strain versus that of the gram-negative microorganism. Comparison of results from the agar slide and Andersen impactors at the same sampling velocity showed that recovery and injury due to collection depends not only on the magnitude of the impaction velocity but also on the degree to which the microorganisms may be embedded in the collection medium. Impaction velocity, characterized by the sampler's operating flow rate and inlet design, is unique for each sampling device. The resulting impaction stress influences the recovery and injury of collected microorganisms and ultimately affects the measurement data for colony enumeration. This can be one of the most important reasons for variations that occur when using different sampling devices to measure bioaerosols from the same environment.

Airborne microorganisms in indoor (2, 3, 44) and outdoor (29, 62) environments, from either natural (48, 63) or industrial (4) sources, may produce ill effects on humans ranging from mild irritation to disease. Examples of infections which may result from the presence of microorganisms in the air are tuberculosis (54), measles (53), legionellosis (10), and histoplasmosis (3). To ensure the health of workers and the public, it is important to evaluate the composition and concentrations of airborne microorganisms in contaminated environments.

A wide range of bioaerosol samplers is available to perform such evaluations. Among these are filter samplers which differ by filter size, filter material, and the air velocity through the filter (28, 30). Agar collectors operate by impacting airborne microorganisms onto a nutrient surface by passage through a slit or through several holes that are spaced in a uniform pattern. The microorganisms are aspirated through the slit or holes to impact onto a stationary or moving agar surface (15, 46). If the sampler contains more than one impaction stage, this cascade impactor collects microorganisms of different size ranges. For instance, the Andersen Six-Stage Viable Particle Sizing Sampler (Graseby-Andersen Inc., Atlanta, Ga.) collects microorganisms in six size ranges. Each stage of this cascade impactor contains 400 impaction holes, while, for example, the Mattson-Garvin slit-to-agar air sampler (Barramundi Corp., Homosassa Springs, Fla.) has only one stage with a rectangular impaction slit. The latter impacts onto a moving agar surface, while the Andersen sampler impacts onto stationary surfaces. For impingers, the airborne microorganisms are impacted into a liquid instead of a solid agar surface (18, 45). The principal differences in the design of impingers are the shape of the impaction nozzle (hole versus fritted outlet) and the distance between the nozzle outlet and the bottom of the vessel containing the collection liquid. For instance, the AGI-30 (Ace Glass Inc., Vineland, N.J.) has a 30-mm nozzle-to-bottom distance, while for the AGI-4 (also Ace Glass Inc.) this distance is 4 mm.

Ideally, the collected microorganisms should be undamaged and the number of collected microorganisms should be representative of the airborne concentration (8). However, available devices differ by a number of parameters that may affect their efficiency in collection and microbial recovery. Among these parameters are the "cut size" (i.e., the size below which most microorganisms are not removed from the stream of sampled air [34, 35, 45]), the inlet efficiency (12), the amount of drying during and after collection (7, 13, 59), the microorganism's velocity of impact during collection, and the surface density of collected microorganisms which can lead to the masking of the resulting colonies (5).

Because of these many differences among available bioaerosol samplers, the collection device and sampling conditions need to be specified when results are quoted. Comparative studies in the field and the laboratory have shown considerable differences among the numbers of airborne microorganisms measured with the sampling devices used (9, 16, 19–21). In an effort to develop standards for bioaerosol sampling, the major parameters affecting collection efficiency (E_{COLL}) and microbial recovery need to be evaluated and suitable design criteria need to be developed from these studies. The focus of this

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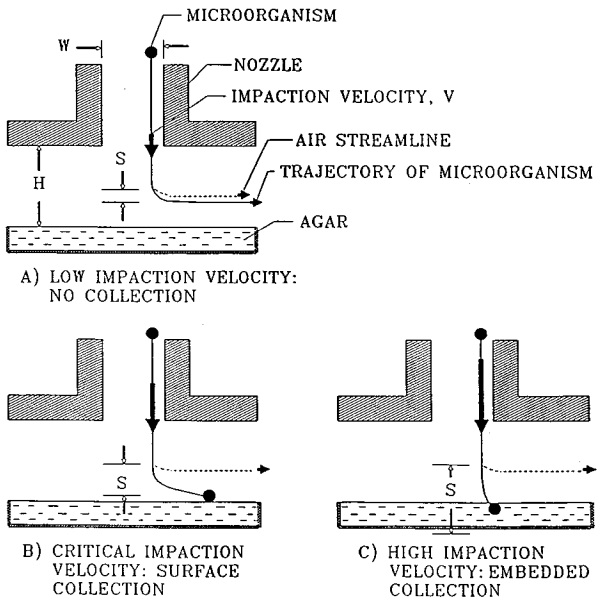

FIG. 1. Collection of microorganisms on agar at low (A), critical (B), and high (C) impaction velocities.

paper is to evaluate the effect of impact stress on microbial recovery when collecting samples on an agar surface.

Microbial stress has been shown to occur as a result of aerosolization (7, 13, 36, 60, 61) and collection (6, 7, 14). During aerosolization, microbial stress was found to be greatest at low relative humidities and for small droplet sizes (36, 60). During collection, microbial stress has been found to be influenced by the liquid used for impingement, and the stress has been shown to be lessened with the addition of certain protecting agents to the collection fluid (7). Relative humidity may also affect the stability of the collected microorganisms (7, 59).

The effect of impaction on microbial stress is quantified in this study by experiments in which the impaction velocity (V) is varied and the sampler geometry is held constant. Information on the type of injury is obtained by the use of complete and selective media for the determination of total and uninjured cell counts, respectively (38, 50). To understand microbial injury, it is necessary to understand the process of impaction. Therefore, some basic concepts of aerosol physics are presented in the next section.

Impaction process. When a bioaerosol sample is withdrawn from the air for collection by an impaction device, the physical collection process will uniquely affect each microorganism. Depending on the density, size, and shape of the microorganism, it may or may not be collected. Figure 1 schematically represents the impaction onto agar of microorganisms of the same aerodynamic diameter at low, critical, and high impaction velocities. As seen from Fig. 1A, microorganisms tend to travel with the air streamlines and are not collected when the sampler is operated at low impact velocity. Figure 1B shows that at a higher critical impact velocity, microorganisms "will come in for a soft landing," i.e., they are collected by the agar by attaching themselves to the agar surface; and Fig. 1C shows that microorganisms collected at a high impaction velocity penetrate into the agar collection surface and remain embedded in it.

Figure 1 shows that the microorganism's stopping distance (S) increases with increasing impact velocity. This distance is proportional to the microorganism's initial velocity, i.e., its impaction velocity, and the square of its physical size. Thus, it is a convenient parameter for determining whether a particle will impact (34, 35, 46).

When S is large relative to nozzle width (W) the microorganism will impact with considerable inertia (Fig. 1C). The ratio of S to $W/2$, defined as the Stokes number (Stk), is thus a dimensionless measure for inertial impaction (34, 46): $\mathrm{Stk} = S/(W/2)$.

Since each air streamline has a different path, there are differences in the microorganism's trajectory depending on where the microorganism enters the inlet. Therefore, Stk_{50} defines the Stokes number for which 50% of the microorganisms (particles) are collected and 50% pass through (34, 35). Nondimensional Stk_{50} determines the cut size (d_{50}), which is the size of the microorganisms (particles) with 50% collection. When the height between the impaction surface and the nozzle plane (H) is larger than W, d_{50} is only moderately affected by variations in H (33–35). Another parameter, Reynolds number, reflects the characteristics of air flow in the nozzle. As this parameter is linearly proportional to the air velocity, it is also of relevance in discussing impacting microorganisms.

Calculations of these impactor characteristics are necessary in order to analyze the effect of microbial stress due to impaction. The hypothesis guiding this study is that injury to microorganisms is more severe for those samplers whose flow rate and inlet design produce higher impaction velocities and, therefore, greater impact stress, just as greater damage is inflicted on objects impacting on a surface from progressively higher elevations. The effect of the impaction velocity on the recovery and injury of airborne microorganisms has been experimentally evaluated in this study.

MATERIALS AND METHODS

Test microorganisms. Stock cultures obtained from the American Type Culture Collection (Rockville, Md.) included *Pseudomonas fluorescens* (ATCC 13525) and *Micrococcus luteus* (ATCC 4698). These strains were chosen so that the recovery of the rod-shaped gram-negative *P. fluorescens*, representative of a more sensitive bacterial strain, could be compared with the recovery of the sturdier gram-positive *M. luteus* (43). Both strains are representative of bacterial genera commonly found in the ambient air environment (44). *P. fluorescens* ranges from 0.7 to 0.8 μm in diameter and from 1.5 to 3.0 μm in length (47). *M. luteus*, which is known to occur in tetrads and irregular clusters of tetrads, ranges from 0.9 to 1.8 μm in diameter (24). These bacteria were routinely maintained on Trypticase soy agar (TSA) slants at 4°C (Becton Dickinson Microbiology Systems, Cockeysville, Md.) and were stored in 1% (wt/vol) peptone water containing 40% (vol/vol) glycerol at −20°C.

Preparation of the pure culture suspension. *P. fluorescens* and *M. luteus* cultures were grown in Trypticase soy broth (Becton Dickinson Microbiology Systems) at 30°C for 18 and 24 h, respectively, and the stationary-phase organisms were harvested by centrifugation at 4,800 × g for 15 min (Marathon 6K centrifuge; Fisher Scientific, Pittsburgh, Pa.). The pellets were washed three times with deionized water (5-stage Milli-Q Plus system; Millipore Corp., Bedford, Mass.) which was also used to dilute the cells to obtain a bacterial density resulting in 89 to 90% transmittance at 600 nm (Spectronic 21D spectrophotometer; Milton Roy Co., Rochester, N.Y.). From this suspension, an optimal (1:500) dilution was prepared for nebulization in order to avoid overloading of the collection surface and to minimize masking effects (5).

Experimental set-up. The experimental set-up shown schematically in Fig. 2 was used for the laboratory evaluation of microbial stress due to impaction. It is a modification of a system that has been previously developed for the evaluation of bioaerosol samplers (59). The commonly used Collison nebulizer (BGI Inc., Waltham, Mass.) was chosen for nebulization of the microbial suspension at a flow rate (Q_{NEB}) of 2 liters of dry, filtered, compressed laboratory air per min. The aerosolized suspension was diluted with dry, filtered, compressed laboratory air. The dilution flow rate (Q_{DIL}) ranged from 3.8 to 44 liters/min. The aerosol was 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 combined nebulizer and dilution air flow (Q_{TOT}) then entered the sampling chamber (volume, 550 cm^3) where it was drawn into the bioaerosol sampler at a predetermined flow rate (Q_{SAMPL}) and impacted onto the collection medium at the corresponding V. The entire test system was placed in a class II, type B2 biological safety cabinet

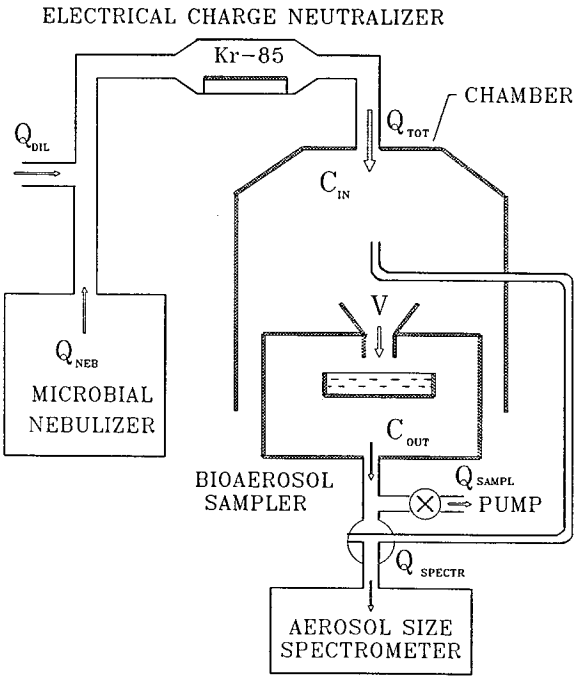

FIG. 2. Schematic representation of experimental set-up. The arrows indicate the flow direction.

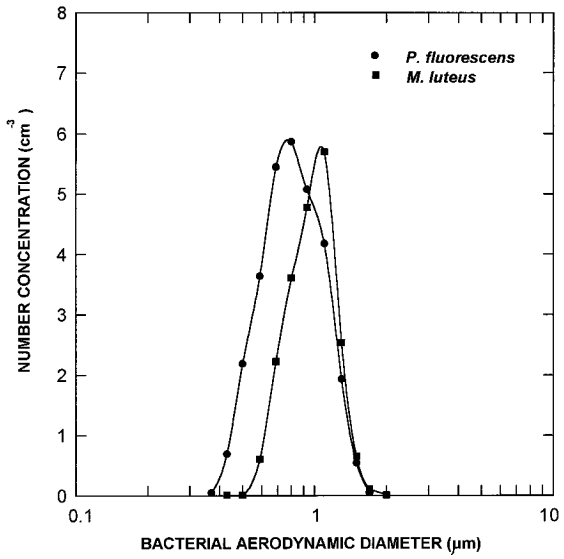

FIG. 3. Aerosizer measurement of bacterial aerodynamic diameter.

(SterilchemGARD; Baker Company, Inc., Sanford, Maine) so that any bioaerosols not collected by the sampler were properly eliminated. During the course of experimentation, the temperature ranged between 20 and 23°C and the relative humidity ranged between 28 and 32%. Stable and low relative humidity allowed water to evaporate quickly from the bacterium-carrying particles, which resulted in monodisperse bioaerosols in the sampling chamber. This condition was important in order to provide an accurate control of the bioaerosol concentration and particle size distribution with the particle size spectrometer.

The Aerosizer, an aerodynamic particle size spectrometer (API Mach II; Amherst Process Instruments, Inc., Hadley, Mass.), was used to measure the number of bacteria and the size distribution of the test microorganisms entering the inlet of the bioaerosol sampler used in the impaction tests. The number of culturable microorganisms on the collection medium is equal to or less than this number, depending on the type of collection medium used and the degree of injury inflicted on the microorganisms during impaction.

The Aerosizer measures the health-related aerodynamic equivalent diameter (d_{AE}), which may differ from the physical size, as it depends also on the microorganism's density. The d_{AE} reflects the inertial property of the microorganism and is therefore very relevant for this impaction study. A two- or three-dimensionally nonuniform microorganism, such as a rod-shaped *P. fluorescens* bacterium, is reported by a single d_{AE}. Figure 3 shows that the average d_{AE} of *P. fluorescens* is 0.80 μm and that of *M. luteus* is 1.10 μm.

The Aerosizer was found to be most suitable for measuring the concentration of microorganisms entering the bioaerosol sampler (C_{IN}), because it is a dynamic measurement device capable of accurately measuring the size of microorganisms as small as 0.5 μm. Instruments not chosen include the Aerodynamic Particle Sizer (TSI Inc.), because its cut size of 0.8 μm is larger than the size of many bacteria, and the cascade impactor, because measurements with it are more time-consuming and provide less size discrimination than the instruments selected. Measurements with an optical size spectrometer were found to depend on the number of bacterial washings, which may change the optical refractive index and therefore the measurement's size calibration (1). The Aerosizer measures over a d_{AE} range of 0.5 to 200 μm at a flow rate (Q_{SPECTR}) of 5.3 liters/min. Its maximum concentration is 1,100 particles (microorganisms) per cm^3.

An optical size spectrometer (LAS-X; Particle Measuring Systems, Inc., Boulder, Colo.) was, however, used in parallel with the Aerosizer for determining the physical collection efficiency of the sampler. The LAS-X was calibrated for specific bacteria and was used to measure the concentration upstream and downstream of the sampler. As it measures optical equivalent particle sizes as small as 0.09 μm (at a Q_{SPECTR} of 0.06 liters/min), the bioaerosol sampler's E_{COLL} could be determined below the 0.5-μm cut size of the Aerosizer.

Bioaerosol samplers. Two types of bioaerosol samplers were used in this study. For most of the impact stress experiments, a new agar slide impactor which has recently been developed and evaluated by this research team (18) was used for sampling at Q_{SAMPL} of 3.8, 6.4, 8, 10, 16, 24, 32, and 40 liters/min (V of 24, 40, 50, 63, 100, 150, 200, and 250 m/s). This range of flow rates and corresponding impaction velocities was chosen as being typical for most commercially available bioaerosol samplers. The agar slide impactor was chosen as the test sampler for several reasons: (i) it can operate over a wide range of flow rates yielding a wide range of values of V; (ii) its E_{COLL} is close to 100% for most of the flow rates, which minimizes collection efficiency problems and directs the focus on the effect of impaction velocity; and (iii) it minimizes the effects of dehydration and overloading of the collection surface, since the agar slide is moved at a steady rate under the impaction nozzle.

The collection inlet of this agar slide impactor is tapered at a 60° angle, leading to a 0.2-mm-wide and 13.3-mm-long slot. The sampled bioaerosol is impacted onto an agar slide moving unidirectionally at a predetermined speed below the inlet. The jet-to-plate distance (H) is about 1 mm. Samples were collected onto Nunc chamber slides (model 177372; Nunc, Inc., Naperville, Ill.) filled with 9.7 ml of agar collection media.

Prior to tests with the microorganisms, the collection characteristics of this impactor were evaluated theoretically and experimentally by using polystyrene latex particles (PSL; Dow Chemical Company, Indianapolis, Ind.). For the Q_{SAMPL} of 3.8 to 40 liters/min, the characteristic Reynolds number of this impactor was calculated to be 640 to 6,660. The nondimensional jet-to-plate distance was held constant at $H/W \approx 5$. Under these conditions, the theoretical value of Stk_{50} according to Marple (33) is about 0.55; more recent theory by Rader and Marple (49) gives a little higher value of Stk_{50} as ≈0.61. These theoretically determined values of Stk_{50} are close to those found experimentally, when the collection characteristics of this impactor were measured with PSL particles of specific sizes (Table 1). The E_{COLL} was determined as follows from measurements with the LAS-X size spectrometer: $E_{COLL} = (C_{IN} - C_{OUT})/C_{IN}$, where C_{IN} and C_{OUT}, respectively, are the aerosol concentrations upstream and downstream of the sampler (Fig. 2). The agar slide impactor was also calibrated

TABLE 1. Characteristics of the agar slide impactor at different sampling flow rates

V (m/s)	Q_{SAMPL} (liters/min)	E_{COLL} ± SD (%)		Characteristics[a] at E_{COLL} of 50%	
		P. fluorescens (d_{AE} = 0.80 μm)	*M. luteus* (d_{AE} = 1.10 μm)	d_{50} (μm)	Stk_{50}
24	3.8	10 ± 6	17 ± 1	0.80	0.56
40	6.4	45 ± 14	54 ± 5	0.65	0.64
50	8	68 ± 12	84 ± 2	0.45	0.42
63	10	85 ± 6	>95	0.40	0.43
100–250	16–40	>95	>95	NM[b]	NM

[a] Experimentally determined with PSL particles.
[b] NM, not measured.

with both test microorganisms, *P. fluorescens* and *M. luteus*. The physical collection characteristics of this sampler obtained with microorganisms and nonbiological particles are presented in Table 1.

As a reference, the sixth stage of the Andersen Six-Stage Viable Particle Sizing Sampler was used at its suggested operating flow rate of 28.3 liters/min (V = 24 m/s) in order to compare recovery and injury data with those obtained with the agar slide impactor operating at the same 24-m/s V (Q_{SAMPL} = 3.8 liters/min). Samples were obtained by collecting the bioaerosol on a single agar plate placed in the sixth stage (17). This method is deemed suitable for sampling airborne bacteria and is recommended for routine surveys of viable microorganisms in office environments (39). Plastic petri dishes containing 45 ml of agar collection media were used for sample collection, and the positive-hole correction method was used for the determination of the Andersen results (31).

Collection media. TSA was used as a complete, nonselective medium (42). Metabolically and structurally injured bacteria were identified by performing additional colony counts on two types of selective media.

Structurally injured bacteria have been defined (51) as the survivors that are able to multiply and form colonies on a nonselective complete agar medium, but not on selective media, containing some inhibiting agents. The differences between counts for different media characterize quantitatively the extent of injury produced. In the gram-negative bacteria, the mucopeptide-lipopolysaccharide outer membrane acts as a barrier to various compounds, such as the surfactant agents like bile salts. This ability of the membrane to exclude bile salts was utilized in the selective enumeration on media containing bile salts as an indicator of damage to the cell envelope.

The assays of different metabolic characteristics can be used to show the particular sites of the stress-induced damage. These include assays of activities of enzymes such as β-galactosidase and dehydrogenase and of respiration activity. According to Ray and Speck (51), the metabolically injured bacteria are defined as the survivors that are able to multiply and form colonies on a complete agar but not on an agar that contains inorganic salts (such as minimal salts medium). The method for determining the changes in the overall metabolic activities is based on impaired growth responses of stressed bacteria and assesses the cumulative effect of several structural and functional changes.

Minimal salts glucose agar (MA) (7.0 g of K_2HPO_4, 3.0 g of KH_2PO_4, 0.1 g of sodium citrate, 0.1 g of $MgSO_4 \cdot 7H_2O$, 1 g of $(NH_4)_2SO_4$, 2.0 g of glucose, and 1.5% nutrient agar) was used for selection against metabolically injured bacteria (42). For selection against structurally injured bacteria, MacConkey agar (Becton Dickinson Microbiology Systems) and TSA containing 5% NaCl were chosen for the collection of *P. fluorescens* (52) and *M. luteus* (23, 25), respectively.

Aerosol generation and measurement of total bacterial count in air. In order to maintain an initial suspension of healthy, uninjured cells and to ensure uniform sampling conditions, an optimal sampling period was determined. This was done by plating samples of the suspension before and after the 2-h nebulization period and at intervals during nebulization. This was necessary to minimize any increase in bacterial concentration due to the breakdown of agglomerated cells and to minimize any additional effects of injury which occurred as a result of nebulization. It was determined that nebulization for approximately 20 min prior to sample collection, with the collection of samples continuing for up to 2 h before the suspension was changed, provided an optimal sampling window, resulting in disagglomeration of most *M. luteus* clusters to the single-cell level. This effectiveness is demonstrated by the particle size distributions measured in the sampling chamber during bacterial collection (Fig. 3).

The bacterial suspension was nebulized in the test system until a stable concentration and size distribution of bacteria were detected with the Aerosizer. In order to yield similar surface densities of collected bacteria for all the impaction velocities studied, the concentration of bacteria entering the bioaerosol sampler was adjusted by varying the Q_{DIL} (Fig. 2).

After the bacterial concentration in the sampling chamber was adjusted appropriately, samples were collected on agar collection media. The sampling order for the flow rates and the media were randomized during each day of experimentation. All samples were collected with the impactor's collection inlet placed inside the sampling chamber (Fig. 2). Each sample was collected for 15 min, and because of slight (±15%) variations in the Aerosizer readings over the sampling period, the average of three readings was obtained. The product of the measured concentration, flow rate, and sampling time equalled the total number of bacteria that entered the sampler.

Colony enumeration. The *P. fluorescens* cells collected on agar were incubated at 28°C on TSA, MA, and MacConkey agar for 12, 18, and 48 h, respectively. The *M. luteus* cells collected on agar were incubated at 28°C on TSA, MA, and TSA plus 5% NaCl for 27, 30, and 78 h, respectively. The colonies in the impaction zone of the agar slides were then counted by using a bright-field phase-contrast microscope (Labophot-2; Nikon Corp., Tokyo, Japan) at a magnification of ×100. The area of the impaction zone was 11 by 25 mm^2, and that of each microscopic field was 3.14 mm^2. The number of colonies was counted for 20 of the 88 available fields in the impaction zone, and the total number of colonies collected on a slide was found by determining the number of colonies contained in the 20 counted fields (total area of 63 mm^2) and calculating the corresponding number of colonies in the entire impaction zone (275 mm^2). The above-indicated colony enumeration procedure (including the incubation time and temperature as well as microscopic magnification) was developed for a new agar slide impactor (5, 18), for which the colony surface density was optimized relative to the masking effect.

For samples collected with the Andersen sampler, counts were made after 48 h of incubation, when growth was visible at the 400 impaction points with a Quebec dark-field colony counter (Reichert-Jung; Cambridge Instruments Inc., Buffalo, N.Y.). The number of positive holes, i.e., holes containing colony growth, were counted for each plate. The positive-hole correction method was used for the estimation of the total number of viable particles (31). Since the Andersen sampler was tested at only one Q_{SAMPL} (28.3 liters/min), it was not necessary to vary the incubation time for the optimization of colony surface density. However, to provide sufficient accuracy, the colony enumeration procedure for the Andersen sampler also included the observation of the collection surface after 8, 16, 24, and 32 h of incubation (prior to the final colony count after 48 h). Thus, the colony enumeration data were generated by sampler-specific procedures which allowed us to collect the most accurate measurement data for each tested bioaerosol sampler.

Assessment of recovery and injury. The bacterial recovery rate was assessed by the number of colonies on the collection medium and is presented relative to the number of bacteria entering the sampler in the following formula:

$$\text{relative recovery} = \frac{N_{CFU}}{N_{TOT} \cdot E_{COLL}} \quad (1)$$

where N_{CFU} is the number of colonies on the collection surface, N_{TOT} is the total number of bacteria that entered the sampler, and E_{COLL} is the collection efficiency. For N_{TOT} bacteria entering the sampler, N_{CFU}/E_{COLL} is the total number of bacteria that can become colonies in the collection medium used.

The level of injury was determined by the difference between the count on the complete medium (relative $\text{recovery}_{Complete}$) and that on minimal medium or selective medium (relative $\text{recovery}_{Minimal}$ or relative $\text{recovery}_{Selective}$, respectively) (38). For each series of three samples collected on complete, minimal, and selective agar, the percent metabolic injury and percent structural injury were calculated as follows on the basis of differences in relative recovery on the minimal and selective media:

$$\text{metabolic injury} = \frac{\text{relative recovery}_{Complete} - \text{relative recovery}_{Minimal}}{\text{relative recovery}_{Complete}} \quad (2)$$

and

$$\text{structural injury} = \frac{\text{relative recovery}_{Complete} - \text{relative recovery}_{Selective}}{\text{relative recovery}_{Complete}} \quad (3)$$

RESULTS AND DISCUSSION

Effect of impaction velocity on bacterial recovery and injury. As seen from Fig. 4, when the sampler E_{COLL} was close to 100% ($Q_{SAMPL} \geq 10$ liters/min [Table 1]), an increase in V produced a significant decline in the relative recovery rates of both *P. fluorescens* and *M. luteus*. Lower impaction velocities ($V \leq 63$ m/s; $Q_{SAMPL} \leq 10$ liters/min) corresponded with the highest microbial yields, with the exception of V of 24 m/s (Q_{SAMPL} = 3.8 liters/min) when the recovery of all but *M. luteus* on TSA drops below the relative recovery at a V of 40 m/s (6.4 liters/min). The highest relative recovery rate of *M. luteus* on TSA (approximately 62%) was found at a V of 24 m/s (Q_{SAMPL} = 3.8 liters/min), whereas maximum relative recovery for *P. fluorescens* on TSA (approximately 51%) was found at a V of 40 m/s (Q_{SAMPL} = 6.4 liters/min). As expected, the relative recovery rate was highest on the complete (TSA) medium for both microorganisms. The relative recovery rate on minimal media (MA) was higher than that on the two selective media (MacConkey agar and TSA plus 5% NaCl) for both microorganisms, with the exception of some data obtained with *M. luteus* at a V of ≥150 m/s ($Q_{SAMPL} \geq 23.4$ liters/min), when values of relative recovery on both media are close to each other.

Bacterial injury data (Fig. 5) reveal a trend for increased injury at successively higher impaction velocities. These results show that *P. fluorescens* is extremely sensitive to structural damage (with approximately 100% injury at all impaction velocities [Fig. 5A]), while structural injury for *M. luteus* was found to be between (26 ± 4)% (V = 40 m/s) and (70 ± 24)% (250 m/s). For *M. luteus*, the structural damage appeared to be

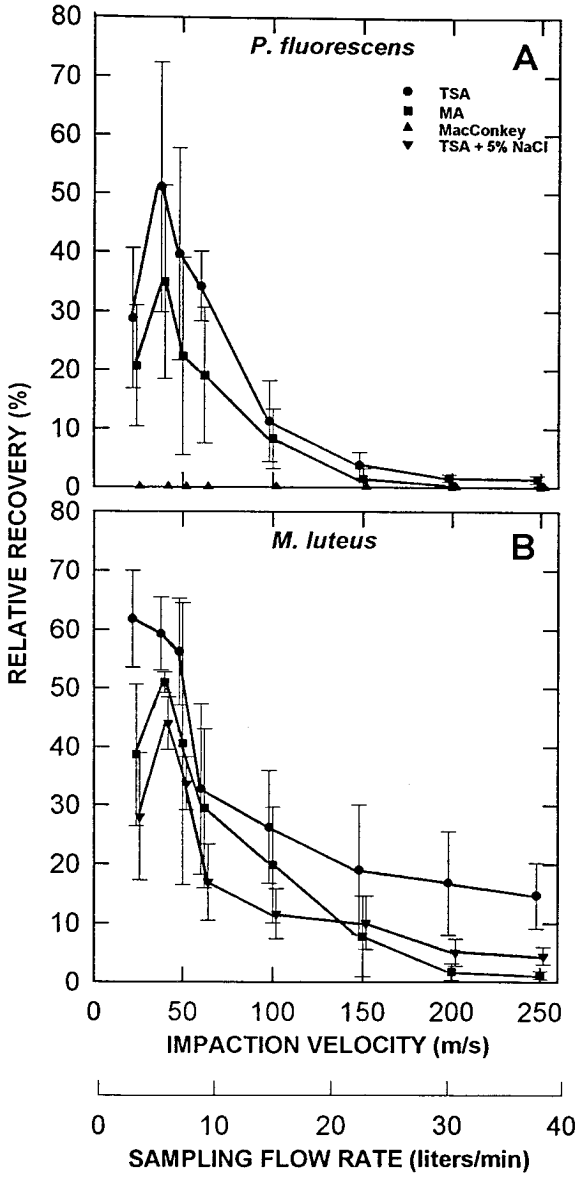

FIG. 4. Relative recovery rates of *P. fluorescens* (A) and *M. luteus* (B) collected with the agar slide impactor over a range of impaction velocities (Stk_{50} = 0.61; Reynolds number = 640 to 6,660; $H/W \approx 5$). The error bars indicate standard deviations.

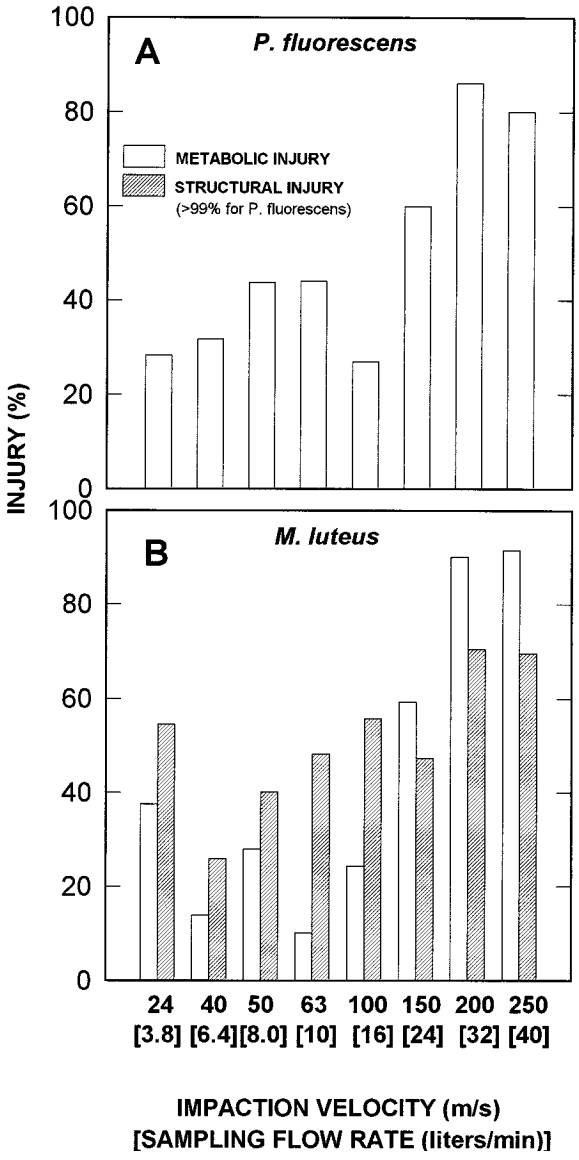

FIG. 5. Injury rates of *P. fluorescens* (A) and *M. luteus* (B) collected with the agar slide impactor over a range of impaction velocities (Stk_{50} = 0.61; Reynolds number = 640 to 6,660; $H/W \approx 5$).

greater than metabolic damage except at velocities of ≥150 m/s (Q_{SAMPL} ≥23.4 liters/min). Metabolic injury rates for *P. fluorescens* and *M. luteus* were found in ranges from (29 ± 6)% to (86 ± 12)% and from (10 ± 3)% to (92 ± 7)%, respectively. As expected, these results correlate with the relative recovery results (Fig. 4), revealing lower recovery rates at impaction velocities which produce greater injury.

As the impaction velocities at flow rates yielding about 100% E_{COLL} were increased, the recovery rate decreased as a result of the greater number of cells lethally injured by the impact stress. Even though the greater collection efficiency at higher flow rates may be expected to produce a greater number of CFU, the number of surviving microorganisms decreased, which we attribute to the effect of impact stress. Lethal injury of bacterial cells has also been noted in other studies in which microorganisms have been subjected to various stressors such as aerosolization (7, 13, 36, 60, 61), collection (6, 7, 14), freezing (41, 42), and UV irradiation (11, 22).

Impaction velocities at the reduced sampling flow rates exert reduced impaction stress on the collected cells, as seen by the increase in recovery rates (Fig. 4) and decrease in injury rates (Fig. 5). All data of N_{CFU} are divided by E_{COLL} (see equation 1). Although the impact stress is lowest at the lowest flow rate and, therefore, the injury rate is expected to be lowest and the recovery rate is expected to be highest, the relative recovery rate (Fig. 4) is seen to be reduced at this flow rate. We attribute this result to insufficient embedding of the collected microorganisms in the growth medium (Fig. 1). This effect is discussed in the next section.

Sublethally injured bacteria are capable of recovering this ability to grow on selective media given suitable growth conditions and nutrients (26, 27). At the same time, the recovery rate is expected to be higher on complete media than on media

TABLE 2. Bacterial recovery and injury due to impaction on the Andersen sampler and the agar slide impactor operating at the same V of 24 m/s

Microorganism	Sampling conditions		Relative recovery ± SD (%) on:			Injury ± SD (%)	
	Sampler	Q_{SAMPL} (liters/min)	TSA	MA	MacConkey agar[a] or TSA + 5% NaCl[b]	Metabolic	Structural
P. fluorescens	Andersen 6th stage	28.3	20 ± 4	15 ± 8	≈0	25 ± 13	>99
	Agar slide impactor	3.8	29 ± 11	20 ± 10	≈0	31 ± 19	>99
M. luteus	Andersen 6th stage	28.3	14 ± 3	14 ± 4	13 ± 1	≈0	7 ± 2
	Agar slide impactor	3.8	62 ± 8	38 ± 13	28 ± 11	39 ± 14	55 ± 24

[a] MacConkey agar was used for the collection of *P. fluorescens.*
[b] TSA plus 5% NaCl was used for the collection of *M. luteus.*

containing selective agents, as seen for both *P. fluorescens* and *M. luteus*. Poor recovery of *P. fluorescens* on the selective MacConkey agar (~0%) illustrates the condition in which gram-negative bacteria have a greater susceptibility than gram-positive bacteria to damage resulting from the mechanical stress of impaction. This finding correlates with results reported by other investigators: e.g., after being frozen, *Escherichia coli* on similar selective media has exhibited low recovery rates (51). In other studies, selective media have revealed less recovery (greater percent injury) than minimal agar (32, 40, 57, 58). It has been postulated that the minimal agar measures only metabolic injury, whereas the selective media measure injury to a structure (a cell membrane or wall) which normally protects cells from the bactericidal effect of the surface-active agents (51).

At high impaction velocities (Fig. 4B), the recovery rate for *M. luteus* is very low on MA and TSA plus 5% NaCl. It is lowest on MA, while usually the recovery rate on TSA plus 5% NaCl is lowest (e.g., see Fig. 4A). This result may be explained if one considers the cell wall structure. Usually, the structural injury is expected to exceed the metabolic injury if they are caused by the same impact stress. However, the fairly rigid, protective shell of the gram-positive cell wall (55) may provide even better protection against structural injury than against metabolic injury at the highest impaction velocities. Also, it has been suggested that differences in strain behavior may be due to the differences in the cell wall structure (50, 56). The gram-positive cell envelope is similar to a thick blanket protecting the cell from environmental changes, while the gram-negative cell envelope acts like a flimsy sheet, offering considerably less protection (43). It has been shown that the outermost lipid membrane in gram-negative bacteria (8) is a primary target for dehydration, rehydration, and temperature-induced damage since it is not maintained by covalent bonds. These relatively unstable bonds can easily undergo phase changes induced by minor agitations and may result in changes in membrane-associated components, ultimately leading to effects such as sublethal injury (8). It is therefore not surprising that the overall recovery rate of gram-positive *M. luteus* is greater than that for *P. fluorescens.*

Limitations of recovery on selective media have been reported even for uninjured cells. It has been found that very close agreement (95%) between counts on selective and nonselective media may be attained only when uninjured healthy cells are near the maximum growth phase and when the length of storage in the arrested state is only limited (37). On the basis of this concept, the efficiency of minimal and selective agar media to recover bacteria was determined for *P. fluorescens* and *M. luteus* by using a nonaerosolized control culture and was estimated as the population bias relative to the recovery rates on the complete TSA agar. When the nonaerosolized control cultures were examined, the results showed that about 30% of the cells recovered on TSA were not recovered on MacConkey agar for *P. fluorescens*, and that approximately 30% of the cells on TSA were not recovered on TSA plus 5% NaCl for *M. luteus*. On MA, the population biases for the two strains examined were found to be about 30 and 25%, respectively. The population bias for both strains on minimal and selective media was approximately the same at 30% and is, therefore, not expected to have influenced the comparisons of recovery data.

Bacterial recovery results demonstrate considerable variability, as expressed by the error bars in Fig. 4. We attribute this variability to (i) the effect of bacterial residues on the Aerosizer measurements, which was minimized by taking Aerosizer readings within the range of the bacterial size indicated in Fig. 3, since almost all the bacterial residues are smaller than the bacteria (as measured with the LAS-X optical size spectrometer), some residue sizes have overlapped with the small bacterial sizes; (ii) minor fluctuations in the compressed laboratory air pressure that may have affected the flow rate through the nebulizer and thus the bacterial concentration entering the bioaerosol sampler; (iii) population bias between the chosen media, as discussed above; and (iv) the inherent variability associated with microorganisms.

Effect of insufficient embedding of microorganisms into agar during collection at low Q_{SAMPL}. The collection efficiency for the agar slide impactor was found to be high at V of 100 to 250 m/s (16 to 40 liters/min): E_{COLL} was ≥95% for *P. fluorescens* and *M. luteus* (Table 1). The reduced collection efficiency was taken into account in the calculation of recovery at V from 24 to 63 m/s. With the exception of *M. luteus* collected on TSA, even after correction for collection efficiency, relative recovery values obtained at 24 m/s do not follow the general tendency obtained in this study. We attribute these results to insufficient embedding of the collected microorganisms in the agar when sampling was conducted at this low impaction velocity. As schematically shown in Fig. 1B, the microorganism lands like an airplane on top of the nutrient surface; it does not penetrate like a projectile into the surface (Fig. 1C). We postulate that the microorganisms adhering to the top of the nutrient surface experience increased drying due to the sampling air stream and increased desiccation during incubation and perhaps have a limited ability to obtain the nutrients, moisture, and warmth needed for survival. As a result, cells collected at this flow rate may have a decreased chance of surviving to become colonies.

Comparison of the agar slide impactor with the Andersen sampler. Rates of bacterial recovery and injury due to impaction measured with the Andersen sampler and the agar slide impactor were compared for *P. fluorescens* and *M. luteus* col-

lected at the same impaction velocity. The results are presented in Table 2. The relative recovery rates of *P. fluorescens* and *M. luteus* on TSA and MA were notably higher for the agar slide impactor. There was ≈0% recovery of *P. fluorescens* on MacConkey agar for both impactors, yet there was a notable difference between the relative recovery rates of *M. luteus* on TSA plus 5% NaCl for the Andersen sampler [(13 ± 1)%] and the agar slide impactor [(28 ± 11)%].

The metabolic injury and the structural injury to those microorganisms recovered on TSA were also evaluated. Both impactors produced approximately the same degree of metabolic injury for *P. fluorescens*. For *M. luteus*, the degree of metabolic injury was negligibly small for sampling with the Andersen sampler but 39% of the cells were injured with the agar slide impactor. The structural injury rate for the more-sensitive *P. fluorescens* was approximately 99% with both impactors; however, for *M. luteus* there was significantly more structural injury for sampling with the agar slide impactor.

Since both samplers were operated at the same impaction velocity, differences between the relative recovery rates for the two appear to be due to their differing design and operating characteristics. Since the relative recovery rate on TSA was highest for the agar slide impactor, it is surprising that this sampler also has the highest injury rate. Table 1 shows that the E_{COLL} of the agar slide impactor is only (10 ± 6)% for *P. fluorescens* and (17 ± 1)% for the larger *M. luteus* at the 3.8-liter/min flow rate (it was designed for efficient collection at higher flow rates). Thus, the measured values are divided by 0.1 ± 0.06 and 0.17 ± 0.01, respectively, to give the true microbial count. Since this true count varies greatly, differences between data for the agar slide impactor and the Andersen sampler should not be overinterpreted.

One design difference that explains differences in the results was noted. When the nozzle opening in an impactor is very small, the air flow has to turn 90° in a very small space. The impact stream depends on the impaction velocity which is approximately equal to the air velocity in the nozzle. If the nozzle opening is larger and the impaction velocity (average air velocity in the nozzle) is the same, the microorganism has more space to turn 90°. Thus, even for the same impaction velocity, in one impactor the microorganism may come in for a soft landing and be exposed to the previously discussed surface stress, while in another impactor the microorganism may embed itself in the agar. As the sixth stage of the Andersen sampler has 400 small nozzles (holes) through which the sampled air impacts into a stationary medium and the agar slide impactor has a long slit above a moving agar slide, one should not expect the same recovery and injury data for the same impaction velocity, as demonstrated in Table 2.

Statistical evaluation of samples. For each experimental trial, a sample was obtained from each of the three media at the selected flow rates. These samples allowed the evaluation of recovery and injury by comparison of the total colony count on each. In order for the results to have statistical significance, each measurement was repeated three to five times. For the range of flow rates over which all samples were collected, the maximum standard deviations as relative recovery of *P. fluorescens* on TSA and MA were 21% at 40 m/s (6.4 liters/min) and 17% at 50 m/s (8 liters/min), respectively (Fig. 4). The standard deviations for most of the measured data were well below these values. For MacConkey agar, no standard deviation could be calculated since all recovery values for this medium were approximately zero. The maximum standard deviations as relative recovery for *M. luteus* on TSA, MA, and TSA plus 5% NaCl were 15% at 63 m/s, 23% at 50 m/s, and 15% at 24 m/s, respectively. For *M. luteus*, the standard deviations at all other impaction velocities were below these values.

ACKNOWLEDGMENTS

The development of the agar slide impactor used as a tool in this study as well as the test procedures and preliminary experiments were supported by the Center for Indoor Air Research through contracts 90-16 and 93-11. All subsequent experiments were supported by the Environmental Protection Agency through Cooperative Agreement CR 822065. Shelby Stewart was supported by graduate student stipends from the University of Cincinnati and the National Institute for Occupational Safety and Health through Educational Resource Center grant 2 T42 OH07091. We gratefully thank these agencies for their assistance.

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