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## A Combined Fluorochrome Method for Quantitation of Metabolically Active and Inactive Airborne Bacteria

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**ABSTRACT.** To better understand the ecology of microorganisms in the environment and to quantify the concentrations of airborne microorganisms, methods are needed to count all microbial cells that are present and differentiate between those that are metabolically competent and those that are nonviable as they exist in situ. We developed and tested a direct epifluorescent method to estimate the quantity and activity of airborne bacteria aerosolized into full-scale rooms. Midget impingers, filled with the fluorescing redox dye 5-cyano-2, 3-ditolyl tetrazolium chloride (CTC), were used to capture and stain metabolically active bacteria from room air. Both active and inactive bacteria were counterstained with a fluorescein derivative and counted with an epifluorescent microscope. The choice of fluorochrome stains allowed concurrent identification of active and inactive bacteria in the same microscopic field. Airborne bacterial numbers determined by epifluorescence microscopy were compared to standard, nonselective colony counts cultured from midget impingers operated under identical conditions. Direct epifluorescent estimates of total airborne bacteria were higher than concurrent plate counts. However, estimates of active airborne bacteria were lower than concurrent plate counts. Variability experiments showed that the direct-count method was repeatable to within 35%.

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### INTRODUCTION

Exposure to airborne particles of biological origin is increasingly being recognized as responsible for infectious, hypersensitivity, and inflammatory lung diseases (AIHA 1996; Burge 1990; Burge 1995; Cox and Wathes 1995;

Lighthart and Mohr 1994; Rom and Garay 1996; Rylander and Jacobs 1994; Wald and Stave 1994). Such particles are referred to as biological aerosols, bioaerosols, or biologically derived airborne contaminants. The work reported here involved only bacteria, but the same collection and analysis methods may also be suitable for detection of some airborne fungal spores and yeast cells. Bacterial cells as well as spores and cell fragments are abundant in indoor and

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outdoor air. While pathogenic bacteria must be viable to cause infection, nonviable as well as viable bacteria have been associated with hypersensitivity and inflammatory diseases (Burge 1990; Flannigan et al. 1991; Heinsohn 1994; Miller 1992). Detection methods that cannot identify nonviable or nonculturable microorganisms measure bioaerosol concentrations inaccurately and can seriously underestimate human exposures to airborne bacteria.

To better understand the ecology of microorganisms in the environment and to quantify concentrations of airborne microorganisms, methods are needed to differentiate between metabolically competent and nonviable microorganisms as they exist in situ, i.e., suspended in the air. Conventional culture-based approaches have intrinsic limitations for characterizing microorganisms from environmental sources. Standard plate counts underestimate the true quantity and diversity of airborne microorganisms because the method cannot identify slow-growing, nonculturable, or inactive microorganisms and fragments thereof. A genetic amplification method, Polymerase Chain Reaction (PCR), has been successfully adapted to characterize airborne microorganisms with relatively low detection limits (as low as  $10^3$  cells/m<sup>3</sup>) (Palmer et al. 1995; Mukoda et al. 1994; Nugent et al. 1997). While promising, genetic amplification methods have been reported to estimate microbial biomass and diversity inaccurately, provide no measure of microbial fragments, and have been labile to some ubiquitous environmental interferences—particularly trace concentrations of heavy metals and other undefined environmental substances (Alvarez et al. 1995; Heinsohn 1994; MacNeil et al. 1995).

The technique of coupling fluorescent biological staining with membrane filtration has been successfully used to directly enumerate microorganisms in a variety of aquatic and terrestrial environments (Bowden 1977; Hobbie et al. 1977; Kepner and Pratt 1994; Perter and Feig 1980), and it is an ideal method for adaptation to research on bioaerosols. Fluorochrome enumera-

tion has been used to measure airborne bacterial concentrations in indoor air using the DNA intercalating agents acridine orange or ethidium bromide (Griffiths et al. 1996; Moschandreas et al. 1996; Palmgren et al. 1986; Henningson et al. 1997). In a bench-scale study (chamber volume = 550 cm<sup>3</sup>), Terzieva et al. (1996) directly enumerated aerosolized *Psuedomonas fluorescens* captured in all-glass impingers (AGI-30) using proprietary dyes and propidium iodide to assess cell-membrane integrity.

In many ecological and environmental studies, fluorochromes have been coupled with various heterocyclic tetrazolium dyes and nalidixic acid to determine not only total microorganism numbers, but also the fraction of metabolically active microorganisms (Bianchi and Giuliano 1996; Bitton and Koopman 1982; Hernandez et al. 1994; Huang et al. 1995; Jarnagin and Luchsinger 1980; Maki and Remsen 1981; McFeters et al. 1995; Rodriguez et al. 1992; Soderstrom 1977; Tabor and Neihof 1982; Trevors 1984; Zimmerman et al. 1978). However, in bioaerosol studies, the detection and quantitation of metabolically active microorganisms has been primarily based on plate count assays in which sample collection methods as well as microorganism nutritional requirements and culturability bias the results (Burge 1990; Buttner et al. 1993; Flannigan 1993; Heinsohn 1994; Marchand et al. 1995; Miller 1992; Stewart et al. 1995; Terzieva et al. 1996; Thorne et al. 1992).

In response to the existing limitations of culture-based, staining, and genetic amplification methods, we developed a protocol that combined direct epifluorescent microscopy and enzyme-activity staining to characterize the metabolic state of airborne microorganisms under in situ conditions. To investigate the feasibility and repeatability of the method, we conducted experiments in full-scale rooms. In separate experiments, pure-culture aerosols of *Bacillus subtilis* (*B. subtilis*), *Micrococcus luteus* (*M. luteus*), and *Escherichia coli* (*E. coli*) were continuously generated and discharged

into the test rooms. These organisms were chosen as bacterial tracers because they are commonly used in bioaerosol monitoring studies as model Gram-positive and Gram-negative organisms (Alvarez et al. 1995; Jensen et al. 1992; Salie et al. 1995; Stewart et al. 1995). Steady-state samples of airborne bacteria were collected at four or five points in the test rooms using glass midget impingers and, for retrieval comparison, at a single point using a polycarbonate membrane filter. Bacteria collected in impingers were identified by culture of colony-forming units (CFUs) and by direct staining and counting with an epifluorescent microscope. Filter samplers were expected to be more efficient particle collectors and provide a better estimate of the total number of airborne bacteria. However, possible damage to collected bacteria due to desiccation precluded using filter samples to estimate bacterial culturability or metabolic activity. Therefore, bacteria collected on filters were identified by direct staining only. Our approach for measuring airborne microorganism concentration and activity was to modify a midget-impinger sampling method to selectively stain metabolically active bacteria immediately after they were collected from indoor air and to compare these measurements with the numbers of total and culturable bacteria in simultaneously collected samples.

## METHODS

### *Test Rooms*

Two full-scale ( $\geq 36 \text{ m}^3$ ) test rooms were used in this study: (i) the Larson Laboratory (LL) chamber at the University of Colorado's Joint Center for Energy Management, and (ii) the Indoor Air Quality Research House (IAQRH) chamber at the University of California's Richmond Field Station. The University of California facility was closed during part of this investigation due to seismic considerations, which dictated the use of the facility at the University of Colorado for part of this work.

The LL chamber is a  $37\text{-m}^3$ , full-scale model of a commercial office space with a suspended tile ceiling and raised floor. The room has a space height of 2.7 m; a 0.7-m-high, unducted, above-ceiling return air plenum; and a 0.5-m, below-floor plenum. The room is completely insulated and has one door and no windows. The door was closed and sealed at the bottom and edges to reduce infiltration. Temperature was controlled by electric-resistance baseboard heaters mounted on two of the walls and by radiant chilled-water panels mounted on one of the walls. The room was furnished with four desks distributed symmetrically around the center of the room.

The IAQRH is a two-story, wooden building, containing a  $36 \text{ m}^3$  test room weatherized to reduce infiltration (Offermann et al. 1985). The room has one window, two interior doors, a linoleum floor, and walls and ceiling of painted sheet rock or plywood. The two doors to the room were closed and sealed at the bottom and edges, and the window was covered to prevent in/exfiltration and exclude light. The room was furnished with two tables, a heated mannequin seated in a chair, and two lamps. The mannequin and lamps generated thermal plumes which induced air mixing.

The test rooms were operated at temperatures between  $21^\circ\text{C}$  and  $25^\circ\text{C}$ , and humidity was either left ambient (IAQRH investigations) or controlled by a self-contained steam humidifier to near 85% (LL investigations). Both test rooms were equipped with mechanical ventilation systems that delivered high-efficiency-particulate-air (HEPA) filtered outdoor air through a ceiling-mounted diffuser near the middle of the room (HEPA filters have a minimum, single-pass efficiency of 99.97% for particles of  $0.3 \mu\text{m}$  diameter). During the experiments, supply and exhaust airflow rates through the ventilation systems were monitored continuously with micro-manometers and the ventilation rate was constantly maintained between 1.5 and 2 air changes per hour (ACH). In separate tracer gas studies, it was determined that aerosol mixing in the

test rooms reasonably approximated completely mixed conditions and that the infiltration rates in both test rooms were at or below 0.1 ACH. Prior to each experiment, the test rooms were purged with five equivalent room volumes of HEPA-filtered air.

### **Validation Experiments**

Four identical collection experiments were conducted at the LL test facility to validate the direct-microscopy aerosol method and determine the variability of the direct-count method in a full-scale application. Impingers were mounted on each of the desk chairs approximately 1 m above the floor. Experimental controls were established by also placing a blank impinger (no airflow) in the test room. *B. subtilis* cells were aerosolized into the room at a constant rate for approximately 2 h until a steady-state bacterial concentration was attained, after which the impingers were operated for 40 min. A paired *t*-test, at a 95% confidence level, was used to determine whether or not there were significant differences between the mean bacterial air concentrations throughout the room. All variability estimates and comparative means testing used a sum of squares pooled standard deviation for normalization.

### **Collection and Recovery Experiments**

The collection and recovery experiments were conducted at the IAQRH. Five sampling locations were arranged in the test room (Figure 1), the four perimeter sampling locations consisting of duplicate midjet impingers and the central sampling location consisting of an impinger and a filter sampler. The samplers were suspended at a height of 1.5 m above the floor of the test chamber, in the approximate breathing zone of a standing adult person. Experimental controls were established by placing a blank filter sampler (no airflow) in the test room.

In independent trials, *M. luteus* and *E. coli* cells were aerosolized into the room at a constant

rate for 2 h until a steady-state bacterial concentration was attained, after which impingers and filter samplers were operated for 30 min. Particle numbers and size distributions were monitored with an optical particle counter (LAS-X OPC; Particle Measuring Systems, Inc., Boulder, CO). The OPC's function was not to measure the concentration of airborne bacteria, but to qualitatively indicate when steady-state particle concentrations were reached in the chamber.

### **Test Microorganisms**

The California Department of Health Services (Berkeley, CA) supplied the *M. luteus* and *E. coli*, and the Biology Department at the University of Colorado at Boulder supplied the *B. subtilis* used in this study. *M. luteus* is a Gram-positive, coccoid bacterium, 0.9 to 1.8  $\mu\text{m}$  in diameter that often occurs in tetrads. *E. coli* HB101 is a Gram-negative, rod-shaped bacterium, 0.3 to 1.0  $\mu\text{m}$  by 1 to 6  $\mu\text{m}$ . *B. subtilis* is a Gram-positive, rod-shaped bacterium with endospores, 0.7 to 0.8  $\mu\text{m}$  by 1.5 to 1.8  $\mu\text{m}$ . All bacteria were grown on SCDA agar (Difco, Detroit, MI) or R2A broth (Difco, Detroit, MI) and harvested into sterile phosphate buffered saline (PBS) ( $\text{Na}_2\text{HPO}_4 + \text{KH}_2\text{PO}_4$ ; 0.85% NaCl; pH 7.2) on the day used.

### **Bioaerosol Generation**

Bacterial aerosols were generated from a six-jet Collison nebulizer (CN 25, BGI, Inc., Waltham, MA) using suspensions containing  $> 10^8$  bacteria  $\text{ml}^{-1}$ . The nebulizer was operated at 138 kPa and was pressurized by an air cylinder followed in series with a dual-stage regulator, dehumidifier, and HEPA filter (Model 3074, TSI, Inc., St. Paul, MN) (Figure 2). The bacterial aerosols were delivered from the nebulizer discharge port into the test chamber at 12  $\text{L}/\text{min}^{-1}$  through 2 cm (i.d.) flexible polypropylene tubing. During bioaerosol generation, the nebulizer contents were replaced every 30 min to ensure that fresh bacteria were delivered to the chamber.

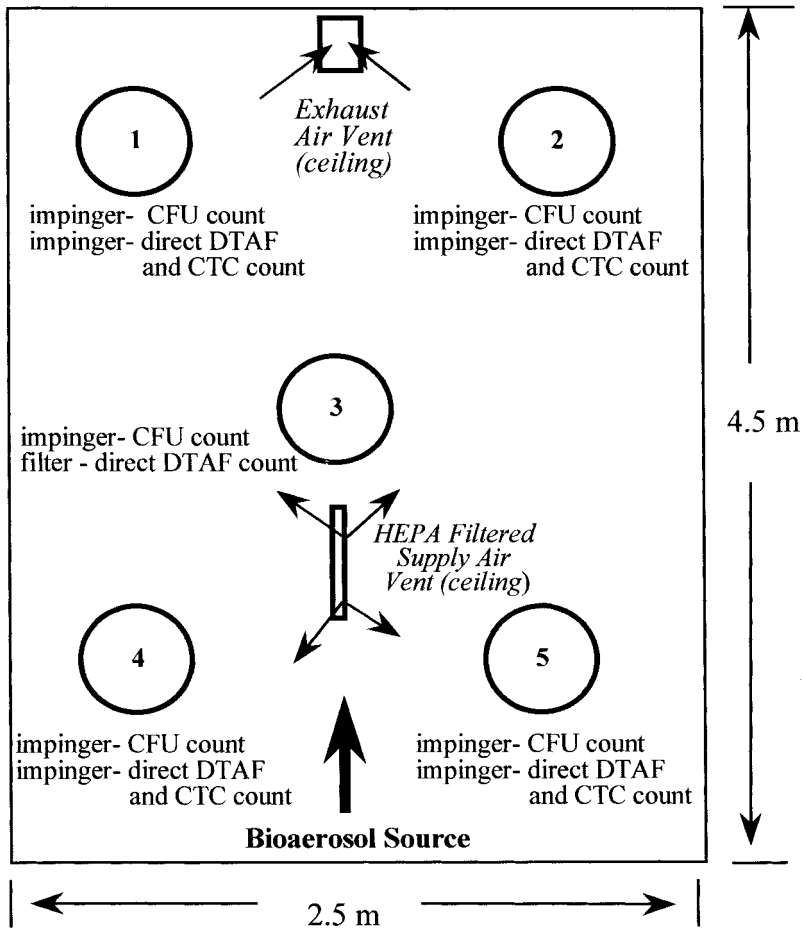
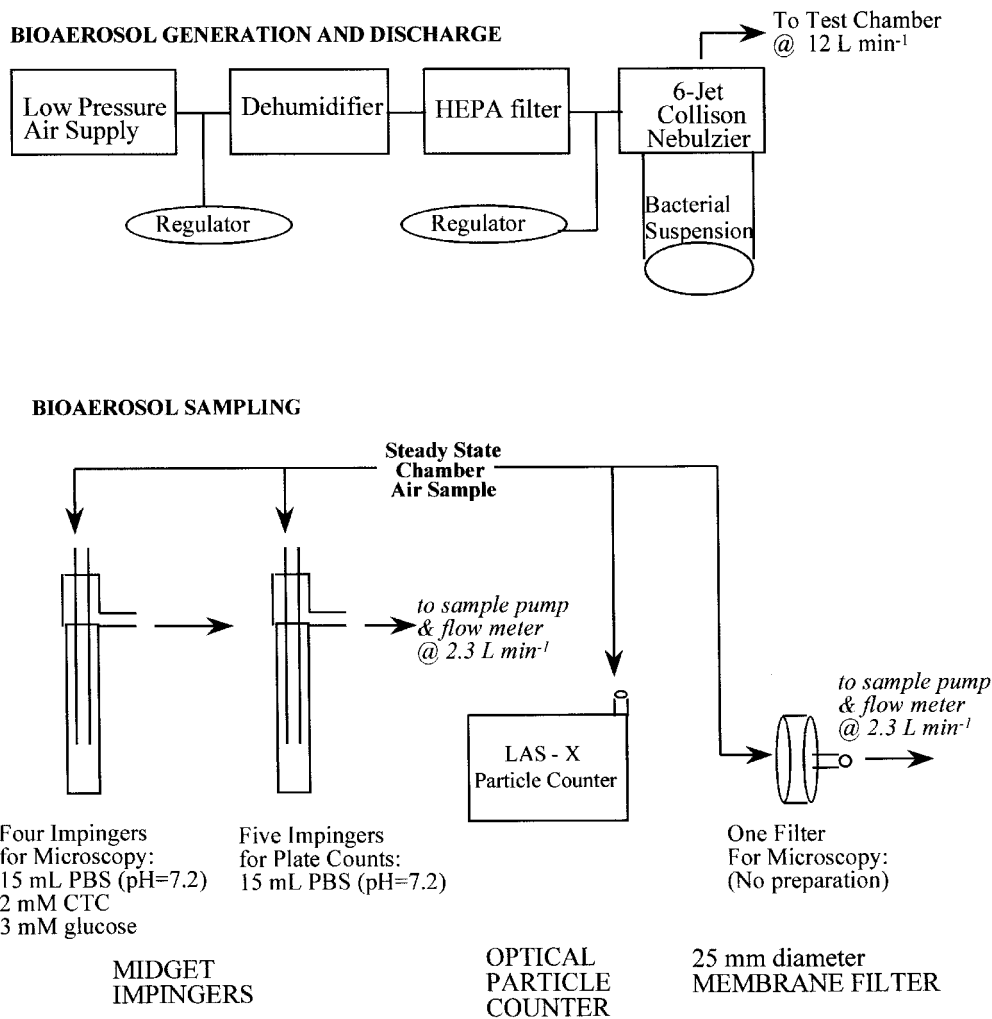


FIGURE 1. Schematic of the 36 m<sup>3</sup> IAQRH chamber and sampling locations used in the collection and recovery experiments. Each circle represents a sampling location. Duplicate liquid impingers were positioned at locations 1, 2, 4, and 5 (one each for culturable plate counts and microscopy). Single impinger and filter samplers were positioned at location 3, respectively, for culturing and DTAF staining. The height of all samplers was 1.5 m.

### Bioaerosol Sampling

Midget impingers (SKC, Inc., Eighty Four, PA) were used in both experiments. Polycarbonate membrane filters (25 mm diameter; 0.8  $\mu\text{m}$  pore diameter) in thread-sealed filter holders fitted with rubber gaskets (Corning Costar, Cambridge, MA) were used in the collection and recovery experiments only. The airflow rate for both types of samplers was identical (approximately 2.3 L/min<sup>-1</sup>). Flow rates were regulated with airflow controllers and calibrated for each

sampling period with a bubble-chamber volumetric flow meter (Gillian Instrument Corp., West Caldwell, NJ). Both filters and impingers were used for a retrieval comparison to evaluate the performance of the impingers because filters are known to be more efficient particle collectors. At 2.3 L/min<sup>-1</sup>, the cutoff aerodynamic diameter ( $d_{50}$ ) of the glass midget impinger was estimated to be 0.7  $\mu\text{m}$ . At the flow rate used in this study, the collection efficiency for the AGI-4 impinger has been shown to be approximately 75% for *Bacillus cereus* spores (Grinshpun et



**FIGURE 2.** Schematic of bioaerosol generation and sampling system (all bioaerosol generation and air-moving equipment as well as the OPC operated outside of the test chambers).

al. 1997), whereas the collection efficiency for filter samplers was estimated to be greater than 99% (Hinds 1982).

Impingers were filled with 15 ml of sterile PBS; those used for direct microscopy also contained 3 mm of D-glucose and 2 mm of the redox dye CTC (Polysciences Inc., Warrington, PA). The addition of low concentrations of glucose and dissolved CTC did not significantly change the surface tension or viscosity of the capture

solutions and thus likely did not change the collection efficiency of the midget impingers. Because CTC is photoreactive, all impingers were covered with opaque material to protect the contents from light. Evaporation from the impinger reservoirs during the sampling periods was less than 2%. Following collection, the samples were immediately transported to the laboratory and processed. For each run, the contents of the midget impinger reservoirs were individu-

ally measured and assayed. The impingers, filter holders, and nebulizer were cleaned and disinfected between each run.

### Plate Counts

Impinger reservoirs containing only PBS were used for plate counts on SCDA medium. A standard spread-plate technique was used to determine the concentration of culturable aerosolized cells (AIHA 1996). Aliquots of undiluted sample as well as 1:10 and 1:100 dilutions were plated in duplicate and incubated at 35°C for 18 to 36 h. We determined the concentration of culturable bacteria in the air by the following relation:

$$C_N = (N/V)DF, \quad (1)$$

where  $C_N$  is the bacteria concentration (CFU  $m^{-3}$ ),  $N$  is the average number of CFU per plate,  $V$  is the volume of air sampled ( $m^3$ ), and  $D_F$  is the dilution factor.

### Epifluorescent Microscopy

Impingers containing PBS and CTC were used for total and active cell staining. Two fluorescent stains were used for bacterial quantitation: (i) CTC, a redox dye that identifies respiring cells with red fluorescence, and (ii) a nonspecific fluorescein derivative that covalently binds to bacterial cell walls and fluoresces green (5-(4,6-dichlorotriazin-2-yl)aminofluorescein (DTAF); Molecular Probes Inc., Eugene, OR). CTC is biologically reduced to fluorescent formazan precipitates by active dehydrogenase enzymes. The choice of these fluorochrome stains provided for concurrent identification of active and inactive bacteria in the same microscopic field.

Samples containing CTC were protected from light, gently mixed, and incubated for 8 h at 22.5°C. A final CTC incubation concentration of 2 mM was chosen based on an optimization assay on pure cultures of *Acinetobacter* sp. and *E. coli*, similar to the methods of Rodriguez (Ro-

driguez et al. 1992). Following CTC incubation, samples were adjusted to pH 9 and a filter-sterilized DTAF concentrate (5 mg  $L^{-1}$  DTAF in 0.05 M  $Na_2HPO_4$ ) was added to a final concentration of 0.5 mg DTAF  $ml^{-1}$ . Air sample filters used to collect bacteria were also used as a staining base. These filters were transferred to polysulfone funnels, washed with PBS, and stained with DTAF while locked in the funnel apparatus. The same type of membrane filters were used for direct counts of bacteria recovered by impingers and air filter samplers.

Samples were protected from light and incubated with DTAF for 20 min at 20°C and were then passed through 0.2  $\mu m$ , black, polycarbonate membrane filters (Poretics, Inc., Livermore, CA) supported by a silver filter (25 mm diameter; 5.0  $\mu m$  pore size). The filter and support were thread sealed in a 50 ml capacity, autoclavable, polysulfone, filter funnel (Gelman Sciences, Ann Arbor, MI, effective filtration area 2.86  $cm^2$ ). Bacteria retained on the filter surface were then washed with 100 ml of PBS (0.05 M  $Na_2HPO_4$ ; 0.85% NaCl; pH 9) to remove unbound DTAF. All filtration and washing was done under a vacuum of no greater than 103.5 kPa. The DTAF stock solution was made fresh and stored for not more than 2 h, and all reagents were filter sterilized (0.2  $\mu m$  pore size) prior to use. In accordance with the observations of Bloem et al. (1995), we found DTAF to have superior stability and better binding properties than fluorescein isothiocyanate, which consistently failed for unknown reasons.

Following the final wash, all filters were dried under vacuum and immediately transferred to clean microscope slides. Approximately 50  $\mu l$  of tris-buffered glycerol (1:1 v/v) containing 2% 1,4 diazobicyclo[2.2.2] octane (to retard quenching of the fluorescent signal) was applied to filter surfaces. The pH of the mounting solution was adjusted to 8.6 with glacial acetic acid just prior to application to optimize the fluorescence of the cell-bound DTAF. Following mountant application, coverslips were immediately laid on the filters.

Mounted filters were examined under UV irradiation using an Olympus BH2 microscope fitted with a mercury lamp and polarizing filters (HBO-100 W Mercury lamp; EY 455 excitation filter; B460 long bandpass barrier filter). The excitation filter provided for maximum excitation of both CTC and DTAF, and the emission filter allowed all visible light with wavelengths greater than 460 nm (green) to pass to the viewer. This filter configuration provided for concurrent observation of cell-bound DTAF (green) and intracellular CTC reduction products (red precipitates) to be viewed in the same microscopic field. All bacteria counting employed an eyepiece with a graticule calibrated for 1100x magnification. Individual cell sizes and shapes were examined from the bacterial suspension both before and after nebulization as well as from aerosolized bacteria collected in impingers and on filters.

Red formazan deposits in CTC-stained cells were examined under the same epifluorescent conditions as for DTAF staining. Figure 3 shows a microscopic field typical of stained *E. coli* from an air sample. The intensity of the bacterial fluorescence varied from one place to another on the filters. The distinct morphology of aerosolized bacteria made it possible to distinguish even mildly fluorescing cells from the background fluorescence, which was predominantly negligible. Therefore, all fluorescing bacteria consistent with *M. luteus*, *E. coli*, or *B. subtilis* morphologies were counted. The counting protocol of Hobbie et al. (1977) was modified to count 15 random fields per slide for each filtered sample ( $\geq 50$  cells per field). The total number of bacteria and the number of bacteria containing fluorescing formazan precipitates (i.e., CTC-positive, metabolically active cells) were determined. All direct counts were reported as the arithmetic mean of 15 fields. We determined the concentration of total and active bacteria in the air by the following relation:

$$C_N = [(NA_f) / (Va)] D_F, \quad (2)$$

where  $C_N$  is the bacteria concentration (cells  $m^{-3}$ ),  $N$  is the number of bacteria per field,  $A_f$

is the effective filtration area (286  $mm^2$ ),  $V$  is the volume of air sampled ( $m^3$ ),  $a$  is the area of microscopic field (0.008  $mm^2$ ), and  $D_F$  is the dilution factor.

## RESULTS

### *Validation Experiments*

*Variability of Direct Total Cell Counts.* Four identical and independent experiments with freshly grown *B. subtilis* cells were executed to estimate the variability in direct microscopic counts using midjet impingers (Figure 4). For concentrations on the order of  $10^8$  cells/ $m^{-3}$ , the variation in direct counts recovered by any single impinger was between 8% and 35% (as defined by the coefficient of variation). There was no significant difference in mean concentration of airborne bacteria as measured by any of the four impingers.

### **COLLECTION AND RECOVERY EXPERIMENTS**

*Plate Counts.* Independent collection and recovery experiments with freshly grown *M. luteus* and *E. coli* cells were executed to compare direct epifluorescent counts on a nonselective culture medium. Respectively, air concentrations of SCDA-culturable *M. luteus* and *E. coli* ranged from  $4.0 \times 10^6$  to  $4.6 \times 10^6$  CFU  $m^{-3}$  (Figure 5) and  $1.3 \times 10^6$  to  $7.7 \times 10^6$  CFU  $m^{-3}$  (Figure 6) for the five sampling locations in the test room. The relatively small variation in the concentrations of culturable *M. luteus* reflected the well-mixed aerosol distribution in the test chamber. The air concentration of culturable *E. coli* was more spatially varied with the highest concentration measured at one of the sampling sites nearest the source.

*Total DTAF Direct Cell Counts.* Respectively, total DTAF air concentrations of *M. luteus* and *E. coli* ranged from  $1.7 \times 10^7$  to  $2.3 \times 10^7$  cells  $m^{-3}$  (Figure 5) and  $4.2 \times 10^6$  to  $1.5 \times 10^7$  cells  $m^{-3}$  (one impinger failed, Figure 6) for the five sampling locations in the test room. As was



**FIGURE 3.** Epifluorescent photomicrograph of *B. subtilis* cells captured in an AGI-30 impinger, treated with CTC, and counterstained with DTAF. Metabolically active (CTC-positive) cells appear with red intracellular precipitates. DTAF appears green. Cells were stained and filtered through a 0.2 μm pore size polycarbonate membrane filter. Individual bacteria are approximately 2.0 μm in length ( $\times 1100$ ).

observed for culturable *M. luteus*, the relatively small variation in air concentration reflected the well-mixed aerosol distribution. Although total direct counts of *E. coli* varied over half an order of magnitude, total *E. coli* cell counts were not higher near the aerosol source. Respectively, the ratios of plate to total counts for *M. luteus* and *E. coli* ranged from 0.2 to 0.4 and from 0.1 to 0.7 (Table 1). Respectively, total counts from the filter air samples were at least half an order of magnitude greater than those cultured from impingers at the same sampling station (Figures 5 and 6, station 3).

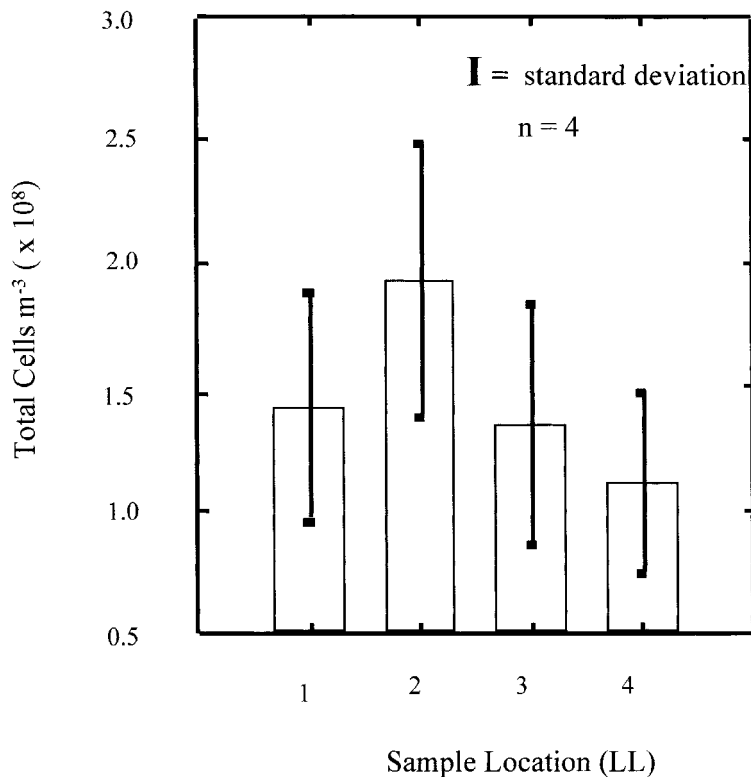
**CTC-Active Cell Counts.** DTAF-stained bacteria containing red fluorescent formazan precipitates (CTC-positive cells) were counted as metabolically active. Because of probable des-

iccation effects, bacteria collected on filter air samplers were not CTC stained. Respectively, air concentrations of CTC-positive *M. luteus* and *E. coli* ranged from  $9.1 \times 10^5$  to  $2.1 \times 10^6$  cells/m<sup>-3</sup> (Figure 5, n = 5) and  $1.4 \times 10^6$  to  $3.2 \times 10^6$  cells/m<sup>-3</sup> (Figure 6, n = 4). For both test bacteria, CTC-positive cell counts were consistently lower than plate counts. Respectively, the ratios of plate to active cell counts, for *M. luteus* and *E. coli*, ranged from 2 to 4 and 3 to 4 (Table 1).

## DISCUSSION

### *Validation Experiments*

Results of the validation experiments suggested that (i) midjet impingers provided an acceptable



**FIGURE 4.** Direct counts of *B. subtilis* cells recovered from the  $37\text{ m}^3$  (LL) test chamber using midjet impingers during the validation experiments. Impinger locations were distributed symmetrically around the center of the chamber. Bar heights indicate the arithmetic average of four experiments. Error bars represent the pooled standard deviations. There was no significant difference between mean counts at the four locations as judged by a students *t*-test at a 95% confidence level.

reproducibility for direct microscopic counts in a full-scale application, and (ii) the aerosolized bacteria in the LL chamber were well mixed and uniformly distributed during the period of observation.

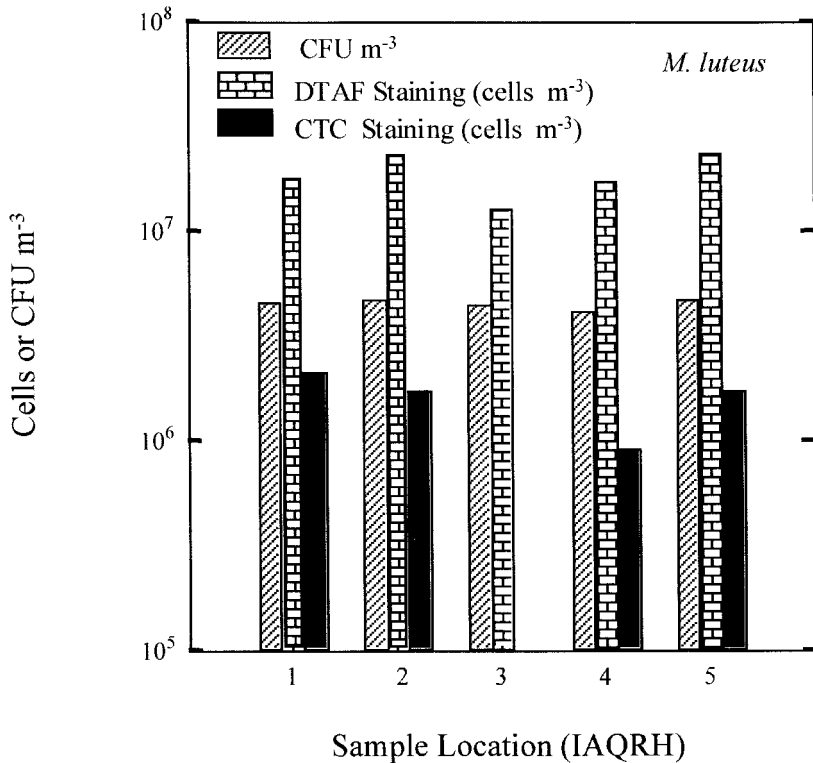
#### **Aerosolization and Sampling Effects**

For microorganisms captured in liquid impingers, sample collection time has been reported to affect cell damage and deaggregation and counting efficiency (Juozaitis et al. 1994; Terzieva et al. 1996; Willeke et al. 1995). In accordance with the observations of Terzieva et al. (1996), for *Pseudomonas aeruginosa* collected in an AGI-30, we observed that nebulization

and agitation of *B. subtilis*, *M. luteus*, or *E. coli* in midjet impingers for 30 min did not impart visible damage to the cells counted. However, for field investigations using impingers where extended sampling times are needed, study of sampling duration and sampling liquid agitation on the structure and viability of captured bioaerosols may be warranted.

#### **Comparison of Total DTAF Cell Counts and Plate Counts**

Plate counts of both *M. luteus* and *E. coli* averaged approximately one-third the values of the concurrent epifluorescent total cell counts (Table 1). However, *E. coli* concentrations



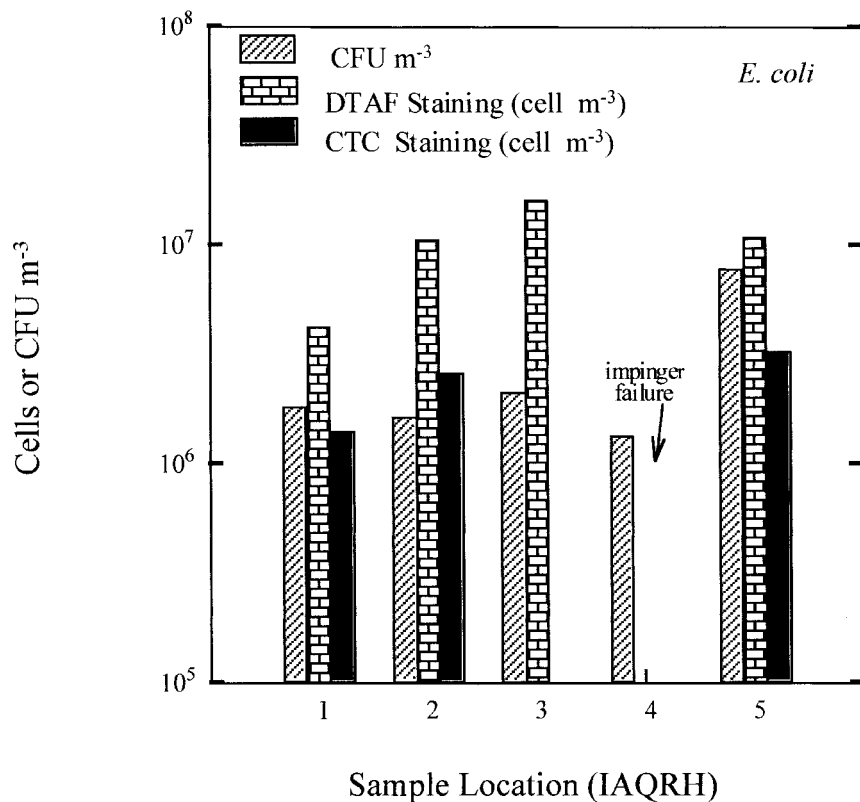
**FIGURE 5.** Comparison of *M. luteus* collection and recovery in the 36 m<sup>3</sup> IAQRH chamber using three different enumeration techniques. CFU counts estimated culturable cells. DTAF counts estimated total cells. CTC counts estimated cells capable of respiration. Samples at locations 1, 2, 4, and 5 were collected with duplicate impingers (one each for culturable plate counts and microscopy). Single impinger and filter samplers were positioned at location 3, respectively, for culturing and DTAF staining.

showed greater variation among sampling locations, with plate counts higher at one sampling location nearer the aerosol source. Previous studies in the IAQRH chamber have shown higher aerosol concentrations near a continuous, point-emission source (Miller-Leiden et al. 1996; Offermann et al. 1985). However, the difference in aerosol distribution between *M. luteus* and *E. coli* in this chamber could be explained by a combination of point-source emission effects and environmental sensitivity; i.e., the Gram-negative *E. coli* was more sensitive to aerosolization and more rapidly lost culturability when airborne than the Gram-positive *M. luteus*. These results suggest that, to different degrees, the two bacteria were stressed through the

nebulization process or lost culturability while suspended in the relatively high-humidity room air. Further, these findings illustrate the varying accuracy with which a culture-based method estimated the true concentration of airborne microorganisms.

#### **Comparison of Active Cell Counts and Plate Counts**

With exception to sampling station 2 during the *E. coli* collection and recovery experiment, cell viability as estimated by a culture-based method consistently exceeded that estimated by CTC reduction. Plate counts were an average of three times higher than epifluorescent estimates of



**FIGURE 6.** Comparison of *E. coli* collection and recovery in the 36 m<sup>3</sup> IAQRH chamber using three different enumeration techniques. CFU counts estimated culturable cells. DTAF counts estimated total cells. CTC counts estimated cells capable of respiration. Samples at locations 1, 2, 4, and 5 were collected with duplicate impingers (one each for culturable plate counts and microscopy). Single impinger and filter samplers were positioned at location 3, respectively, for culturing and DTAF staining.

CTC-positive bacteria (Table 1). A similar scenario has been observed in tetrazolium-stained samples of aquatic microorganisms (McFeters et al. 1995; Rodriguez et al. 1992; Schaule et al. 1993; Yu and McFeters 1994). Although tetrazolium reduction has been reported to accurately detect dehydrogenase activity and thus viability in aquatic and terrestrial environments (Awong et al. 1985; Rodriguez et al. 1992; Yu and McFeters 1994; Yu et al. 1995), it did not consistently detect all metabolically competent and culturable *E. coli* and *M. luteus*. As judged by tetrazolium reduction, cell respiration had ceased; however, as indicated by standard

plate counts, some aerosolized microorganisms had not permanently lost repair mechanisms or respiration and growth capabilities. Although CTC reduction successfully indicated intracellular respiration conditions in situ, these results suggest that tetrazolium reduction assays cannot reliably assess metabolic competence for airborne bacteria.

#### Laboratory and Field Application

In previous studies of aquatic and terrestrial environments, it has been demonstrated that tetrazolium reduction assays are rapid and con-

**TABLE 1. Ratios of SCDA plate counts to direct epifluorescent counts of DTAF- and CTC-stained cells to enumerate total and active airborne *M. luteus* and *E. coli*<sup>a</sup>.**

Sampling Station	<i>M. luteus</i>		<i>E. coli</i>	
	(CFU m <sup>-3</sup> /cells m <sup>-3</sup> )		(CFU m <sup>-3</sup> /cells m <sup>-3</sup> )	
	CFU/DTAF	CFU/CTC	CFU/DTAF	CFU/CTC
1	0.3	2.2	0.4	3.1
2	0.2	3.0	0.2	0.3
3	0.4	†	0.1	†
4	0.2	4.1	sampler failure	
5	0.2	2.7		0.7

<sup>a</sup>Direct CTC and DTAF counts are means of counts from 15 microscope fields from 15 ml impinger samples. CFUs are means of duplicate aliquots from impinger samples.

†Sample station 3 compared the recovery of air filter samplers with midget impingers. Impinger contents were used for culturing CFU's. Air filter samplers were used for DTAF direct counts. No CTC reduction assays were performed on air filter samples.

venient for quantifying metabolically active microorganisms. The data provided by this study in full-scale test chambers confirmed that bioaerosol capture in midget impingers can be successfully coupled with protein stains, dehydrogenase activity stains, and membrane filtration to efficiently collect and quantify airborne microorganisms with negligible interference. DTAF will stain other bioaerosols such as fungal spores and bacterial spores. However, the intensity and shading of the fluorescent signal emitted from fungal and bacterial spores is notably different than that emitted from vegetative cells. This is due to the density of the exosporium and spore coat. An experienced technician would be able to easily discern DTAF-stained vegetative cells from spores.

This work demonstrated that direct microscopy had good sensitivity compared with bacterial isolation using standard culture-based techniques. A significant limitation to air sampling with midget impingers is that these samplers are only suitable for relatively high bioaerosol concentrations—the impingers must collect enough cells to visualize at least one stained and filtered bacterium per microscopic field. These observations suggest that, for test-chamber studies in which bioaerosol concentration is easily controlled, midget impingers offer an economical choice to quantify airborne mi-

croorganisms by direct microscopy. For practical use in field studies, where bioaerosol concentrations are typically lower, the detection limit of the sampling method must be lowered into a more useful range. This may be accomplished by (i) increasing sample collection time, (ii) using smaller diameter filters, or (iii) using larger liquid-capture samplers that can sustain higher airflow rates such as a cyclone wetted scrubber operated at  $\geq 10$  L/min<sup>-1</sup>, an AGI-30 operated at 12.5 L/min<sup>-1</sup>, or a multistage impinger operated at  $\geq 30$  L/min<sup>-1</sup>. In addition, it may be possible to split samples collected in liquid for analysis by more than one method. The feasibility of this method should be studied further using larger, higher-volume samplers in full-scale applications. It is possible that this method will prove suitable for identifying and enumerating both active and inactive microorganisms under indoor and outdoor field conditions. Because samples collected in liquid can be diluted before filtering (to view stained cells) or plating (to detect culturable cells), this method has significant advantages over sample collection by direct agar impaction.

### Significance

The objective of this study was to evaluate a method for absolute measurements of total and

viable airborne microorganisms under in situ conditions. Adaptation of a direct microscopic technique, capable of sensitive characterization of airborne bacteria with relatively minor modifications to commonly used, inexpensive sampling equipment, proved to be a robust approach for bioaerosol characterization. This type of absolute optical measurement has been widely accepted in water and wastewater treatment engineering because it provides a superior basis for process evaluations relative to culture-based detection methods. Using direct microscopy, because nutrient selection and cell culturability do not bias the results, direct visual techniques provide absolute measurements that can offer a more accurate picture of microorganism air concentration than can culture-based methods. The application of widely accepted immunofluorescent techniques and fluorescent genetic probe methods may have important implications for understanding human exposure to airborne microorganisms in both indoor and outdoor environments, e.g., evaluating the effectiveness of engineering controls used to reduce exposures to bioaerosols, and determining in situ, the ecological structure, presence, and activity of a wide range of biological agents.

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