American Industrial Hygiene Association Journal

ISSN: 0002-8894 (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/aiha20

Factors Affecting Microbiological Colony Count Accuracy for Bioaerosol Sampling and Analysis

Ching-Wen Chang , Sergey A. Grinshpun , Klaus Willeke , Janet M. Macher , Jean Donnelly , Scott Clark & Arvydas Juozaitis

To cite this article: Ching-Wen Chang , Sergey A. Grinshpun , Klaus Willeke , Janet M. Macher , Jean Donnelly , Scott Clark & Arvydas Juozaitis (1995) Factors Affecting Microbiological Colony Count Accuracy for Bioaerosol Sampling and Analysis, American Industrial Hygiene Association Journal, 56:10, 979-986, DOI: 10.1080/15428119591016403

To link to this article: https://doi.org/10.1080/15428119591016403

FACTORS AFFECTING MICROBIOLOGICAL COLONY COUNT ACCURACY FOR BIOAEROSOL SAMPLING AND ANALYSIS

Ching-Wen Chang[a,c]
Sergey A. Grinshpun[a]
Klaus Willeke[a]*
Janet M. Macher[b]
Jean Donnelly[a]
Scott Clark[a]
Arvydas Juozaitis[a,d]

[a]Department of Environmental Health, University of Cincinnati, Cincinnati, OH 45267–0056; [b]California Department of Health Services, Environmental Health Laboratory, Berkeley, CA 94704–1011; [c]Current address: Institute of Occupational Safety and Health, Council of Labor Affairs, Executive Yuan, Taiwan, R.O.C.; [d]On leave from the Institute of Physics, Vilnius, Lithuania

The effects of the following variables on the occurrence of colony masking (the indistinguishable merging or overlap of sufficiently close colonies) were evaluated experimentally using the bacterium Bacillus subtilis*: spore density on a collection surface, concentration of nutrients in the culture medium, sample incubation time, and ability of an observation system to distinguish overlapped colonies. Increasing spore surface density and incubation time increased colony masking, whereas lowering nutrient concentration decreased colony diameter and, therefore, masking but also limited spore germination and growth. Overall, full-strength medium was best for accurate counting of early microcolonies examined with the aid of a microscope, whereas half- or quarter-strength medium was better for counting older readily observable macrocolonies. Masking bias was determined for varying spore surface densities and colony diameters and was applied to two widely used slit-to-agar bioaerosol impactors. Appropriate collection times have been determined for these samplers to minimize colony masking for expected bioaerosol concentrations. It was found, for example, that 6-min samples collected from an environment with an air concentration of 10^3 CFU m^{-3} would result in colony surface densities, for 3-mm colonies, of 1.5 and 3.9 microorganisms cm^{-2} for the two samplers with respective masking biases of <10% and <20%.*

Airborne microorganisms and biologically derived materials,[1] that is, bioaerosols, have been identified in work environments such as agricultural operations,[2,3] sanitary landfills,[4] sewage treatment plants,[5] and office buildings.[6,7] Bioaerosols can cause infectious or hypersensitivity disease[8,9] and can contaminate products in clean rooms[10] and in food processing facilities.[11] Bacterial endotoxins and mold constituents also can cause toxic reactions, skin and mucous membrane irritation, and inflammation.[1,4,9]

The collection of bioaerosols onto semisolid culture medium with subsequent sample incubation and colony enumeration is a widely used method to measure bioaerosol concentration for culturable microorganisms. The efficiency with which bioaerosol samplers entrain airborne microorganisms into a sampling inlet, transport particles through a sampling line,[12] and retain particles in or on a collection substrate[13,14] has been found to vary. Environmental stresses and the sample collection process may reduce microorganism viability. The ability of collected microorganisms to grow and to form colonies also depends on the suitability of the culture medium provided and the incubation conditions. Therefore, observed colonies represent only the culturable microorganisms in a sample, not necessarily all microorganisms present, and are counted as colony-forming units (CFU) because they may arise from single cells or cell clusters.

Colonies of similar color and texture that come into sufficiently close contact on a culture medium surface may merge and become indistinguishable as separate units. Figure 1 illustrates three types of particles on a culture medium surface immediately after sample collection: nonbiological material such as mineral dust and culturable and nonculturable microorganisms. The seven culturable microorganisms in the expanded section of Figure 1 are shown grown into seven separate microcolonies after a short incubation time, time = t_1, and represent the true colony count that would be recorded if masking did not occur. However, only five macrocolonies are observed after longer incubation, time = t_2, because two pairs of colonies were sufficiently close that they overlapped. Two of the original seven

* Author to whom correspondence should be addressed

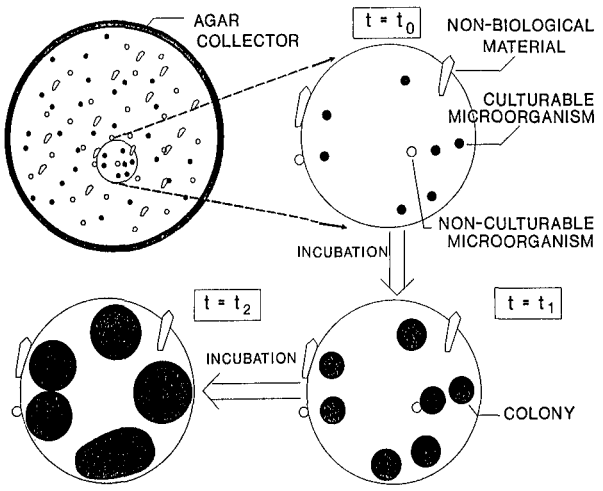

FIGURE 1. Illustration of particles on a collection surface and subsequent colony masking

colonies may be masked if the observation system, for example, a human examining the plate by eye cannot distinguish the overlapped colonies as separate units.

Slit-to-agar (STA) impactors, in which airborne particles are collected through a narrow stationary slit and impacted onto a rotating culture plate, have been considered among the most efficient bioaerosol samplers[15] and are simple to use because the plates need no processing after sample collection. However, STA samples may be overloaded and resulting CFU masked if bioaerosol concentration exceeds 10^4 microorganisms m^{-3} due to the samplers' fairly high airflow rates and limited collection and growth surface areas[16] (Table I). STA impactors have several advantages over other bioaerosol sampling methods, for example, agar-based medium in a STA impactor loses less water and collected microorganisms suffer less dehydration than in other impactors because the impaction surface rotates continuously beneath the sampling inlet. The distribution of CFU on a STA collection surface also reflects temporal changes in bioaerosol concentration that may be related to specific activities at the time of sample collection.[17,18]

Colony overlap has been evaluated to determine a suitable upper-plate-count limit for the pour plate method for dairy product testing.[19,20] Recommended upper-plate-count limits for standard approximately 100-mm diameter culture plates (petri dishes) are 250 CFU for food and dairy products[21,22] and 300 CFU for water, wastewater, and bioaerosol samples.[23,24] However, these recommendations do not consider factors that affect colony count accuracy other than colony surface density. A positive-hole method has been developed[25] and modified[26–28] to address colony masking for multiple-hole impactors. However, until recently,[29] no such method for estimating colony masking has been developed for other culture-based bioaerosol samplers.

The study reported here evaluated the effect on colony masking of bacterial spore surface density, nutrient concentration in culture medium, sample incubation time, and ability of an observation system to identify overlapped colonies. A previously verified statistical model of the random deposition of microorganisms onto culture medium and resulting colony masking[29,30] was applied to two STA bioaerosol impactors and was used to outline a method to determine appropriate sample collection times to limit colony masking to predictable levels.

METHODS

Test Microorganism and Spore Preparation

Bacillus subtilis ATCC 6051 (American Type Culture Collection Inc., Rockville, Md.) was used in this study because it produces ellipsoidal spores of fairly uniform size (0.7–0.8 μm in width and 1.5–1.8 μm in length), which can be stored for long periods without viability loss.[31] Identification of the test bacterium was confirmed by Gram reaction, cell morphology, and biochemical assay (API Rapid CH test; API Analytab Products, Plainview, N.Y.).[32] The bacteria initially were incubated at 30°C for 24 hr on nutrient agar (Difco Laboratory, Detroit, Mich.) to obtain a pure culture that was transferred to manganese-containing nutrient agar[33] incubated for sporulation at 30°C for 7 days. Bacterial growth was harvested into sterile distilled water, agitated at 150 rpm for 24 hr in a ca. 22°C water bath, and heated for 10 min at 80°C to kill vegetative cells. The resulting spore suspension was centrifuged at 2800 × *g* for 20 min and was washed three times with sterile distilled water before storage in liquid nitrogen.

Spore Aerosolization

Aerosolization and sampling of *B. subtilis* spores was conducted in a Class II biological safety cabinet (SterilchemGARD, Baker Company, Inc., Sanford, Maine).[34] Five dilutions of the stock spore suspension were prepared between

TABLE I. Characteristics of Two STA Impactors

Sampler	Slit Length, L (cm)	Slit Width, W (cm)	Slit-to-Center Distance, D_{STC} (cm)	Rotation Time[A] (min)	Sampling Flow Rate, Q_{samp} (L min^{-1})	Collection Area, A_{coll} (cm^2)
Mattson-Garvin Air Sampler, Model 220, P-320	4.12	0.015	2.30	5, 15, 30, 60	28.3	112.9
Casella Airborne Bacteria Sampler, Model MK-II	2.80	0.030	1.23	0.6, 2.4, 6	30.0	46.3

[A] Time for one collection plate revolution

1.07×10^4 and 5.04×10^4 spores mL^{-1}. Spore aerosols were generated with a three-jet Collison nebulizer (BGI Inc., Waltham, Mass.) at an airflow rate of 2 L min^{-1}. The spore aerosol was diluted with 42 L min^{-1} filtered compressed air at a relative humidity of ca. 25% to a concentration between 4.11×10^3 and 1.92×10^4 spores m^{-3}. This dilution range ensured that the aerosol consisted of predominantly single-spore particles and that particle coagulation in the 1.3-sec transport time was only ca. 10^{-11}%.[35] Electrostatic charge on aerosolized spores was minimized by passing the aerosol through a 10-mCi ^{85}Kr particle charge neutralizer (Model 3012, TSI Inc., St. Paul, Minn.) and by grounding metal portions of the test system. Chamber temperature and relative humidity were 22.7 ± 0.4°C and 28.5 ± 1.7% (Thermohygrometer, Model DHTD, Fisher Scientific, Pittsburgh, Pa.).

Spore Sampling

The spore aerosol was sampled with an STA impactor specifically designed for laboratory bioaerosol studies.[33,34] The sampler collected spores through a slit inlet (0.2 by 13.3 mm) directly onto a Nunc slide (2 by 4.2 cm, Model 177372, Nunc Inc., Naperville, Ill.) containing 9 mL nutrient agar at 25, 50, or 100% strength, that is, 15 g agar plus 2, 4, or 8 g nutrient broth (Difco Laboratory) per liter of distilled water. An electric motor moved the slide under the impactor slit at a rate of 0.014 cm s^{-1}.[33] Sampling started after 1 min of slide movement, and spores were collected at a sampling rate, Q_{samp}, of 10 L min^{-1} for a sample collection time, t_{samp}, of 3 min; the total impaction area was 3.5 cm^2 (1.36 by 2.56 cm). The test impactor was calibrated with spherical polystyrene latex (PSL) particles ranging from 0.22 to 1.09 μm in aerodynamic diameter. The aerodynamic particle cutoff size, d_{50}, of this sampler was found to be <0.3 μm. The entire aerosol system was purged with clean air for ≥15 min between tests. Samples were incubated at 25°C, and *B. subtilis* colonies were counted at 12 and 36 hr to record microcolonies and macrocolonies, respectively.

Determination of Number of Spores Collected

Impactor sampling efficiency, E_{samp}, that is, the fraction of spores at the entrance of the impactor that the impactor captured, was determined from the spore concentrations upstream and downstream of the impactor, $C_{s,up}$ and $C_{s,down}$, respectively:

$$E_{samp} = \frac{(C_{s,up} - C_{s,down})}{C_{s,up}}. \quad (1)$$

Spore concentrations were measured using an aerosol size spectrometer (Model LAS-X, Particle Measuring Systems Inc., Boulder, Colo.)[34] and found to remain stable (variation, ±1.8%) for 150 min, which exceeded the 90-min test period. Samples of the stable spore aerosol were collected in random order onto Nunc slides containing different strengths of nutrient medium. Spore concentration at the impactor inlet, $C_{s,up}$, was considered constant for all slides exposed in the same test period. E_{samp} exceeded 0.90 for particles >0.4 μm and was found to be independent of nutrient concentration in the culture medium.[30]

The total number of spores collected, N_s, was determined as

$$N_s = C_{s,up} t_{samp} Q_{samp} E_{samp}. \quad (2)$$

The product of the first three terms was used to estimate the total number of spores at the entrance of the impactor during each 3-min sample collection time. The estimated surface density of collected spores, δ_s, was calculated by dividing N_s by the collection surface area, A_{coll}.

Colony Resolution Index

The ability of an observation system to distinguish overlapped colonies was characterized by resolution index, R_c, with a range of 0–1.[29,30] An observation system with $R_c = 0$ could not distinguish adjacent colonies even if they just touched at their perimeters, a system with $R_c = 1$ could distinguish all adjacent colonies except those that overlapped completely, and systems with intermediate R_c values could distinguish respective fractional degrees of colony overlap. The highest resolution index, $R_c = 1$, was assigned to microcolony counting because adjacent colonies could be identified easily and counted as individual units and because only ca. 2% of the microcolonies overlapped.[30] Only $R_c = 0$ and $R_c = 1$ were used in the enumeration of *B. subtilis* macrocolonies (see below) because degree of colony overlap could not be judged reliably by eye for other R_c values.

Microcolony and Macrocolony Enumeration and Macrocolony Sizing

Microcolonies were counted, after 12-hr incubation at 25°C, using a phase-contrast microscope with bright field illumination and 100× magnification (Labophot-2, Nikon Corp., Tokyo, Japan). The Nunc slides were photographed after a total of 36-hr incubation, and macrocolonies were counted and measured from the photographs. Macrocolony diameter, $d_{c,macro}$, was measured manually with a vernier caliper and an image analyzer (Magiscan 2, Joyce-Loebl Ltd., Team Valley, Gateshead, U.K.). All single colonies and colony clusters were counted as 1 CFU, regardless of shape, at $R_c = 0$ because, by definition, the observation system could not distinguish colonies that had merged to any degree. At $R_c = 1$, apparently circular and nearly circular colonies were counted as 1 CFU, elliptical colonies as 2 CFU, and colonies of other shapes as 3 CFU. According to experimental observation, the coincidence >3 CFU was very rare. Figure 1 can be used to illustrate application of these rules. The final count in the expanded plate section of the figure would be 4 CFU at $R_c = 0$ and 6 CFU at $R_c = 1$.

Colony Surface Density and Percentage Masking

The surface densities of microcolonies and macrocolonies, $\delta_{c,micro}$ and $\delta_{c,macro}$, were determined by dividing the respective colony counts by the collection surface area. Degree of colony masking, $X_{macro/micro}$, was estimated from the ratio

of 36- to 12-hr colony surface densities and expressed as a fraction:

$$X_{macro/micro} = \frac{\delta_{c,macro}}{\delta_{c,micro}}. \quad (3)$$

Microorganism Surface Density and Sample Collection Time for STA Impactors

A theoretical model was developed to determine the degree of colony masking using Monte Carlo computer simulation of the random deposition of microorganisms onto culture media.[29,30] Surface density of collected microorganisms, δ_{org}, a parameter of the model and an important factor in determining degree of colony masking, was related to sampler flow rate, air concentration of microorganisms, C_{org}, and sample collection time, t_{samp} [13,14]:

$$\delta_{org} = \frac{N_{org}}{A_{coll}} = \frac{C_{org} Q_{samp} t_{samp}}{A_{coll}}, \quad (4)$$

where N_{org} was the number of microorganisms deposited on a collection surface.

Two widely used STA impactors were considered: a Mattson-Garvin Air Sampler (M/G) (Model 220 and P-320, Barramundi Corp., Homosassa Springs, Fl.) and a Casella Airborne Bacteria Sampler (MK-II) (Model MK-II, BGI Incorporated). Our experimentally measured d_{50} cutpoints for these samplers (0.5 and 0.7 μm, respectively) agreed well with previously reported calculated values of 0.5 and 0.67 μm.[13] The impaction zone, or collection area, on a rotating culture plate forms an annulus after one complete rotation of the plate. This collection area is bounded by the radii of the slit's inner and outer edges. The portion of the collection plate beneath the inner slit edge, which is a shorter distance from the plate center, rotates through a smaller circle than the outer slit edge. Therefore, microorganism density, δ_{org}, assuming uniform airflow across the slit, is highest at the inner slit edge and decreased toward the outer edge.[17] A maximum δ_{org} value associated with a chosen degree of colony masking was determined for a hypothetical innermost ring of a STA collection area using results from an earlier study.[29,30] The width of this innermost ring was taken as equal to expected colony diameter, d_c, with area $A_{coll,in}$, where

$$A_{coll,in} = \pi[(D_{STC} + d_c)^2 - (D_{STC})^2]. \quad (5)$$

D_{STC} in Equation 5 was the distance from the inner slit edge to the center of a collection plate, or the slit-to-center distance. The collection area for a STA impactor was calculated from slit length, L, and D_{STC}:

$$A_{coll} = \pi[(D_{STC} + L)^2 - (D_{STC})^2]. \quad (6)$$

The maximum microorganism surface density associated with a given degree of masking bias for the hypothesized innermost ring of a STA impactor, $\delta_{org,in}$, was converted to surface density for the entire collection area, δ_{org}:

$$\delta_{org} = \frac{N_{org}}{A_{coll}} = \frac{\frac{L}{d_c} N_{org,in}}{A_{coll}} = \frac{\frac{L}{d_c} \delta_{org,in} A_{coll,in}}{A_{coll}}, \quad (7)$$

where $N_{org,in}$ was the number of microorganisms collected in the innermost ring. A method for calculating STA sample collection time associated with varying degrees of colony masking for anticipated bioaerosol concentrations and colony diameters was developed based on Equations 4 and 7 and on the Monte Carlo computer simulation that predicted degree of colony masking.[29,30]

RESULTS

Figure 2, A–C, shows the experimental *B. subtilis* colony surface densities, δ_c, as functions of spore surface density, δ_s; incubation time, t; resolution index, R_c; and nutrient concentration. Figure 2, D–F, shows the corresponding ratios of macrocolony to microcolony surface densities, $X_{macro/micro}$ (Equation 3), as functions of these same parameters.

Effect of Spore Surface Density, Incubation Time, Nutrient Concentrations, and Observation System Resolution Ability on Colony Surface Density and on Colony Masking

Figure 2, A–C, shows that microcolony and macrocolony surface densities increased with increasing spore surface density and that microcolony counts at 12-hr incubation were generally

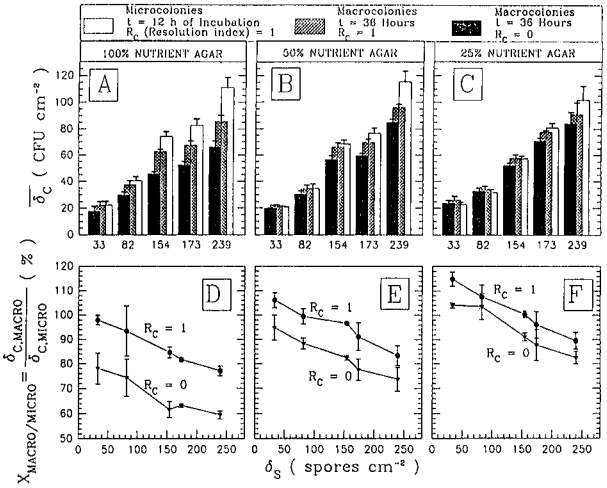

FIGURE 2. (A–C) Average colony surface densities, $\overline{\delta}_{c,micro}$ and $\overline{\delta}_{c,macro}$ (±1 standard deviation, N = 2–9), as functions of spore surface density, δ_s; nutrient concentration; and observation system resolution ability, R_c. D–F: Macrocolony surface density as a percentage of microcolony surface density, $X_{macro/micro}$, as a function of spore surface density, δ_s; nutrient concentration; and observation system resolution ability, R_c.

higher than macrocolony counts at 36 hr. The difference between average observed colony and spore surface densities, $\bar{\delta}c$ and $\bar{\delta}s$, were due to failure of some spores to germinate.

Microcolony surface densities generally were highest on full-strength medium and lowest on quarter-strength medium, but the reverse was the case for macrocolonies. Consequently, macrocolony surface densities were most similar to microcolony surface densities on quarter-strength medium as illustrated in Figure 2, D–F, that is, a larger percentage of microcolonies developed into distinguishable macrocolonies, for respective spore surface densities, with decreasing nutrient concentration. Figure 2, D–F, also shows that colony masking increased with increasing spore surface density, as reflected in a decreasing $X_{macro/micro}$ with increasing δ_s. The percentage of microcolonies that developed into distinguishable macrocolonies as counted at $R_c = 1$ was always higher than that counted at $R_c = 0$, the difference being greatest for full-strength medium and least for quarter-strength medium. Some of the average values of $X_{macro/micro}$ in Figure 2, E and F, were higher than 100%, and all ratios were higher on reduced than on full-strength medium because of underestimation of $\delta_{c,micro}$ at the 12-hr counting time. This underestimation most likely was due to slower growth of the bacteria on the lower nutrient media resulting in smaller difficult to detect microcolonies.

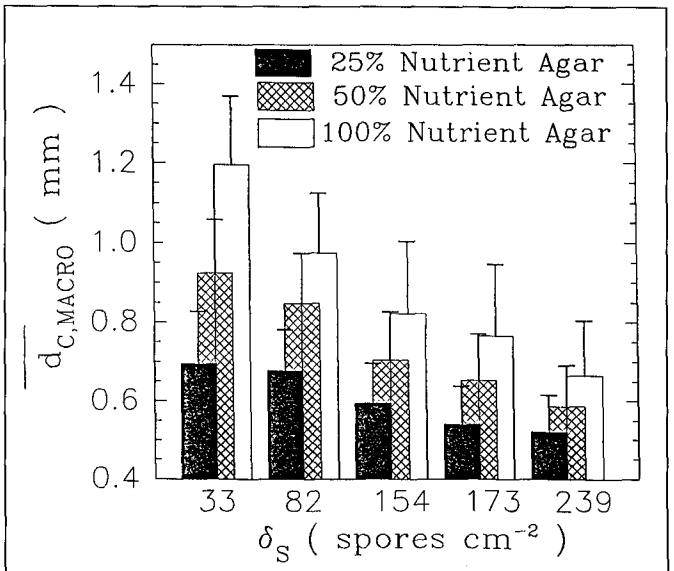

FIGURE 3. Average B. subtilis *macrocolony diameter, $\bar{d}_{c,macro}$ (±1 standard deviation, N = 2–9), at three nutrient concentrations and varying spore surface densities, δ_s*

Macrocolony Diameter and Shape

Increased spore surface density and decreased nutrient concentration limited colony growth as reflected in average macrocolony diameter (Figure 3). The percentage of circular macrocolonies decreased with increasing nutrient concentration, for all spore surface densities, whereas the percentage of elliptical and other shape colonies increased (Table II), reflecting greater colony overlap.

Maximum Surface Density of Microorganisms and Masking Bias

Figure 4 shows percent masking bias as a function of microorganism surface density, colony diameter, and observation system resolution ability. For example, masking bias would be <5% for a colony surface density ≤ 0.9 microorganisms cm^{-2} for 4-mm colonies counted at $R_c = 1$.

Sample Collection Time for STA Impactors

An investigator can use the information outlined here to develop a sampling plan for a particular STA impactor, as illustrated in the following example. Figure 4 shows that 3-mm colonies would be counted at $R_c = 1$ with ≤10% masking bias if colony surface density was ≤3.2 microorganisms cm^{-2}. $A_{coll,in}$ for the M/G sampler was calculated to be 4.62 cm^2 at $d_c = 3$ mm (Equation 5). A target $\delta_{org,in}$ of 3.2 microorganisms cm^{-2} for the innermost ring of a M/G plate would correspond with a δ_{org} of 1.8 microorganisms cm^{-2} for the entire collection area (Equation 7). One can use Equation 4 to calculate a sample collection time of 7.2 min for an assumed bioaerosol concentration of 10^3 CFU m^{-3} to achieve a δ_{org} of ca. 1.8 microorganisms cm^{-2} and to ensure that masking bias does not exceed 10% for the M/G sampler. The comparable sample collection time limit for the MK-II sampler would be 2.6 min; approximately one third of that for the M/G sampler primarily because the collection area is proportionally smaller.

Another approach to applying the information presented here is to calculate expected microorganism surface density, from measured sample collection time and estimated air concentration, and to use this information to predict degree of colony masking. For example, collection of 6-min samples with the M/G and MK-II samplers, again for 3-mm colonies and an air concentration 10^3 CFU m^{-3}, would result in a $\delta_{org,in}$ of 2.68 microorganisms cm^{-2} (δ_{org} = 1.5 microorganisms cm^{-2}) for the M/G sampler and masking bias between 5 and 10% at $R_c = 1$ and would result in a corresponding $\delta_{org,in}$ of 7.38 microorganisms cm^{-2} (δ_{org} = 3.9 microorganisms cm^{-2}) for the MK-II sampler and masking bias of ca. 20%.

DISCUSSION

This study found that degree of bacterial colony masking depended on colony density on a collection surface, on colony diameter, and on ability of an observation system to distinguish overlapped colonies (Figure 5). In general, colony surface density depends directly on four factors (bioaerosol concentration in the sampled air, sampler airflow rate, sample collection time, and collection surface area), and colony surface density depends indirectly on two factors (nutrient concentration and incubation conditions). Colony diameter, in turn, depends on nutrient concentration, incubation time and temperature, and colony surface density. Colony size is also a function of the specific organism studied and its growth rate and pattern. Cell motility also plays a role for bacteria. An observation systems' ability to recognize

TABLE II. Shape Characteristics of *B. subtilis* Colonies

Spore Surface Density, δ_s (spores cm^{-2})	Nutrient Concentration (%)	Number Samples	Macrocolony Shape[A]		
			Circular	Elliptical	Other
33	100	4	76.5 ± 6.1	20.5 ± 4.2	2.7 ± 2.2
	50	2	89.6 ± 1.1	8.7 ± 0.5	1.6 ± 1.6
	25	2	89.6 ± 1.7	10.4 ± 1.7	—
82	100	9	77.5 ± 1.5	19.2 ± 1.5	3.3 ± 1.2
	50	6	88.2 ± 1.7	11.1 ± 2.0	0.7 ± 0.3
	25	7	96.5 ± 1.0	3.1 ± 1.1	0.4 ± 0.4
154	100	5	68.6 ± 4.0	25.0 ± 3.2	6.4 ± 1.2
	50	3	84.2 ± 1.3	14.3 ± 2.2	1.4 ± 1.0
	25	3	90.4 ± 2.1	9.0 ± 2.1	0.6 ± 0.5
173	100	4	73.6 ± 2.2	23.6 ± 3.7	2.8 ± 1.7
	50	4	83.7 ± 2.7	15.0 ± 2.3	1.2 ± 0.5
	25	3	90.6 ± 1.8	9.0 ± 1.6	0.3 ± 0.2
239	100	4	75.8 ± 1.7	18.7 ± 1.2	5.5 ± 1.0
	50	4	87.6 ± 2.2	11.6 ± 1.7	0.9 ± 0.5
	25	3	92.0 ± 0.9	7.6 ± 0.9	0.5 ± 0.4

[A] Values are average percentage ± standard deviation.

overlapped colonies depends on colony size, level of magnification used, and colony morphology and pigmentation. One can adopt the convention used here to estimate number of colonies in noncircular CFU when colonies have similar size, shape, and appearance.

Colony Surface Density

An optimal colony surface density balances the need for sufficient numbers of colonies to estimate microorganism concentrations accurately with the need for well-separated colonies to facilitate microorganism identification and to limit colony masking. Optimal microorganism surface densities (Figure 4), for a given bioaerosol concentration and colony diameter, can be achieved by adjusting sampler airflow rate, sample collection time, or collection surface area (Figure 5). Varying sample collection time generally is the most convenient option, provided investigators consider other factors related to variation of collection time, for example, change in collection surface characteristics during prolonged sampling and the representativeness of very short grab samples. The option of changing an impactor's airflow rate is a less attractive means to achieve optimal colony surface density because such a change would alter the impactor's particle collection characteristics, for example, its d_{50} cut-point.[13,14]

Colony Diameter

Colony masking was greater on higher nutrient medium as a result of increased colony diameter and greater number of culturable microorganisms. Reducing nutrient concentration reduced colony masking but also limited or slowed colony growth so that microcolony counts were lower than on richer medium. These observations suggest that nutrient concentration should be sufficiently high to allow good spore germination and cell growth but sufficiently low to minimize colony masking.

Counting microcolonies after short incubation times has been suggested for multiple-hole impactors as a way to identify multiple CFU at impaction sites.[25,36] However, counting of macrocolonies after longer incubation is easier, for example, no microscope is needed, and is necessary to identify bacterial and fungal isolates. Therefore, the use of reduced strength culture medium may offer a more convenient way to decrease colony masking than counting early colonies. Low nutrient media have been shown to increase microorganism recovery from environmental sources, for example, air,[37] potable water,[38] and soil.[39] R2A medium for bacterial cultures produces slow-growing colonies[38] that may experience less inhibition from neighboring colonies because of their smaller size and resultant greater distance between colonies.

Other ways to control colony diameter and masking are to increase the agar concentration of culture medium or to add growth suppressants. Increasing agar concentration restricts cell growth[40] but may suppress some microorganisms. Growth suppressants, such as rose bengal, dichloran, and other dyes, can control the development of rapid-growing and spreading fungi, for example, *Rhizopus* and *Mucor* species, but also may suppress some molds; alter colony morphology, making identification more difficult; or may be inconvenient to use in the field, for example, rose bengal is light sensitive.[41-44] Microbiologists should evaluate the impact of proposed changes in culture conditions on the growth of the microorganisms of interest before modifying standard culture media or incubation time or temperature. If several genera or species with different impaction and colony growth characteristics are collected on the same surface, grown on the same medium, and analyzed at the same time, the quantitative analysis of colony masking may become more complex. However, the general concepts and approach developed in this study still will apply.

Observation System Resolution Ability

Microbiologists readily can identify isolated circular macrocolonies as individual CFU but must use personal judgment when deciding how to count colonies of other shapes. Average colony counts were consistently lower in this study when examined at $R_c = 0$, that is, all colonies or colony clusters counted as 1 CFU as compared with $R_c = 1$, where colony shape was used to estimate the number of individual colonies in a cluster (Figure 2, D–F). Methods for standardizing counting to deal with overlapped colonies have been developed for water sample examination[24] and are needed also for bioaerosol samples.

CONCLUSION

Bioaerosols are of increasing concern in many workplaces, and efforts are being made to improve methods for air sample collection and analysis. Collection of airborne microorganisms directly onto agar-based culture medium remains an informative and convenient means of studying culturable microorganisms. The occurrence of colony masking for direct impaction samplers, which results in an underestimation of actual colony count, was found to increase with increasing colony surface density and diameter and with decreased ability to distinguish overlapped colonies. The present study explored the effects on colony diameter and resultant colony masking of sample incubation time and culture medium nutrient concentration and offers a method to determine appropriate sample collection time to limit surface density of collected microorganisms and thereby to reduce colony masking.

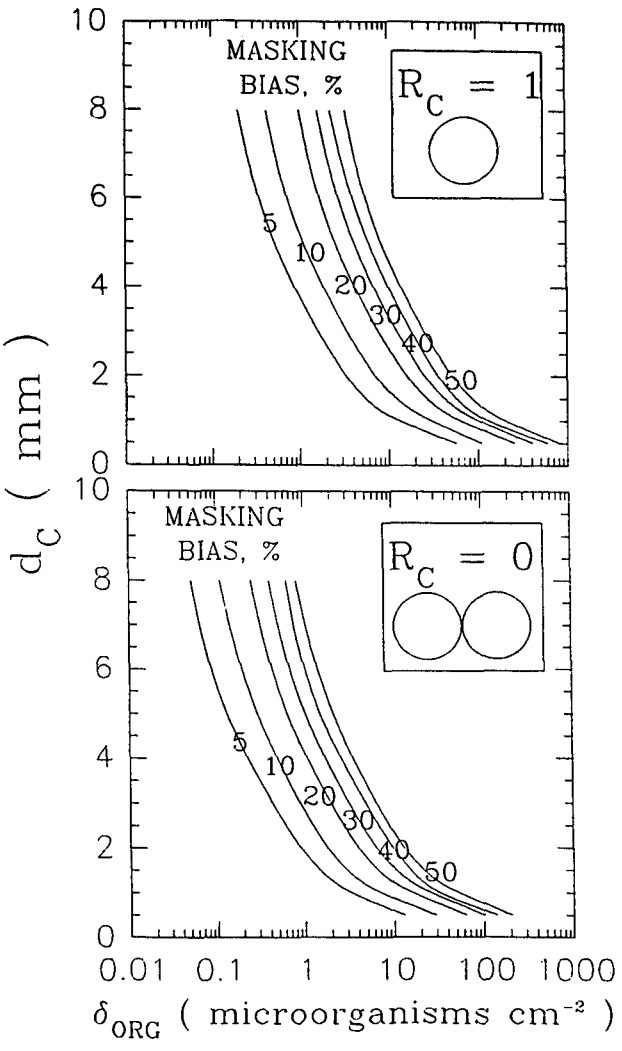

FIGURE 4. Percent masking bias for macrocolonies as a function of colony diameter, d_c, and microorganism surface density, δ_{org}, for two resolution indices

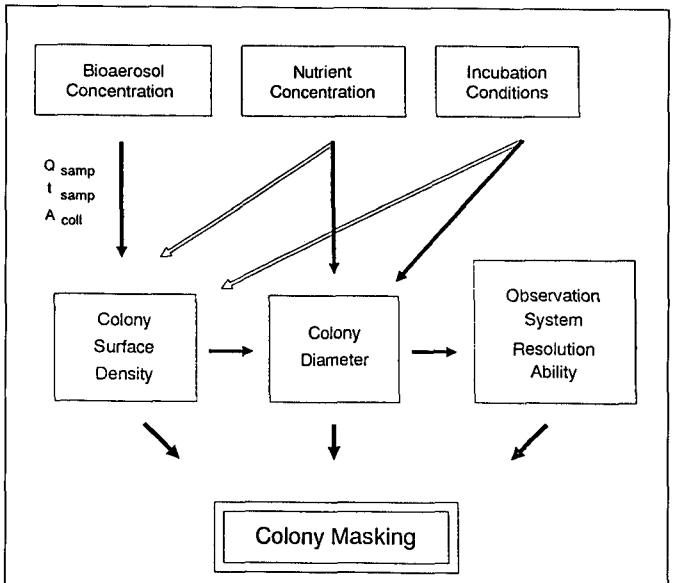

FIGURE 5. Factors that determine degree of colony masking. Dark arrows, direct effects; light arrows, indirect effects.

ACKNOWLEDGMENTS

This work was funded in part by the U.S. Environmental Protection Agency through Cooperative Agreement CR822065 (modeling) and by the National Institute for Occupational Safety and Health through Grant R01-OH-02948 (experimental work). The authors greatly appreciate this support. The authors gratefully acknowledge Dr. P. Baron of the National Institute for Occupational Safety and Health in Cincinnati and Dr. P. Succop of the University of Cincinnati for their many helpful suggestions in the preparation of this manuscript and fellow graduate student Ms. S. Stewart for her assistance in editing the manuscript. Ching-Wen Chang was supported by a University of Cincinnati graduate scholarship during part of this research effort.

REFERENCES

1. **American Conference of Governmental Industrial Hygienists (ACGIH):** *1993–1994 Threshold Limit Values for Chemical Substances and Physical Agents and Biological Exposure Indices.* Cincinnati, OH: ACGIH, Technical Information Office, 1993. pp. 9–11.
2. **Dutkiewicz, J:** Exposure to dust-borne bacteria in agriculture. I. Environmental studies. *Arch. Environ. Health 33:*250–259 (1978).
3. **Kotimaa, M.H., K.H. Husman, E.O. Terho, and M.H. Mustonen:** Airborne molds and actinomycetes in the work environment of farmer's lung patients in Finland. *Scand. J. Work Environ. Health 10:*115–119 (1984).
4. **Rahkonen, P., M. Ettala, M. Laukkanen, and M.S. Salonen:** Airborne microbes and endotoxins in the work environment of two sanitary landfills in Finland. *Aerosol Sci. Technol. 13:*505–513 (1990).
5. **Kenline, P.A. and P.V. Scarpino:** Bacterial air pollution from sewage treatment plants. *Am. Ind. Hyg. Assoc. J. 33:*346–352 (1972).

6. **Morey, P.R., M.J. Hodgson, W.G. Sorenson, G.J. Kullman, W.W. Rhodes, and G.S. Visvesvara:** Environmental studies in moldy office buildings: biological agents, sources and preventive measures. *Ann. Am. Conf. Gov. Ind. Hyg. 10:*21–35 (1984).
7. **Morey, P.R.:** Case presentations: problems caused by moisture in occupied spaces of office buildings. *Ann. Am. Conf. Gov. Ind. Hyg. 10:*121–127 (1984).
8. **Lacey, J. and B. Crook:** Fungal and actinomycete spores as pollutants of the workplace and occupational allergens. *Ann. Occup. Hyg. 32:*515–533 (1988).
9. **Dutkiewicz, J., L. Jablonski, and S.A. Olenchock:** Occupational biohazards: a review. *Am. J. Ind. Med. 14:*605–623 (1988).
10. **Oxborrow, G.S., N.D. Fields, J.R. Puleo, and C.M. Herring:** Quantitative relationship between airborne viable and total particles. *Health Lab. Sci. 12:*47–51 (1975).
11. **Kang, Y.-J. and J.F. Frank:** Biological aerosols: a review of airborne contamination and its measurement in dairy processing plants. *J. Food. Prot. 52:*512–524 (1989).
12. **Grinshpun, S.A., C-W. Chang, A. Nevalainen, and K. Willeke:** Inlet characteristics of bioaerosol samplers. *J. Aerosol Sci. 25:*1503–1522 (1994).
13. **Nevalainen, A., J. Pastuszka, F. Liebhaber, and K. Willeke:** Performance of bioaerosol samplers: collection characteristics and sampler design considerations. *Atmos. Environ. 26A:*531–540 (1992).
14. **Nevalainen, A., K. Willeke, F. Liebhaber, J. Pastuszka, H. Burge, and E. Henningson:** Bioaerosol sampling. In *Aerosol Measurement: Principles, Techniques and Applications.* K. Willeke and P.A. Baron, eds. New York: Van Nostrand Reinhold, 1993. pp. 471–492.
15. **Burge, H.A., M. Chatigny, J. Feeley, K. Kreiss, P. Morey, J. Otten, and K. Peterson:** Bioaerosols: guidelines for assessment and sampling of saprophytic bioaerosols in the indoor environment. *Appl. Ind. Hyg. 2:*(R-10)–(R-16) (1987).
16. **Blomquist, G., U. Palmgren, and G. Ström:** Improved techniques for sampling airborne fungal particles in highly contaminated environments. *Scand. J. Work Environ. Health 10:*253–258 (1984).
17. **Bourdillon, R.B., O.M. Lidwell, and J.C. Thomas:** A slit sampler for collecting and counting air-borne bacteria. *J. Hyg. 41:*197–224 (1941).
18. **Kuehne, R.W. and H.M. Decker:** Studies on continuous sampling of *Serratia marcescens* using a slit sampler. *Appl. Microbiol. 5:*321–323 (1957).
19. **Breed, R.S. and W.D. Dotterrer:** The number of colonies allowable on satisfactory agar plates. *J. Bacteriol. 1:*321–331 (1916).
20. **Tomasiewicz, D.M., D.K. Hotchkiss, G.W. Reinbold, R.B. Read Jr., and P.A. Hartman:** The most suitable number of colonies on plates for counting. *J. Food. Prot. 43:*282–286 (1980).
21. **Busta, F.F., E.H. Peterson, D.M. Adams, and M.G. Johnson:** Colony count methods. In *Compendium of Methods for the Microbiological Examination of Foods.* M.L. Speck, ed. Washington, D.C.: American Public Health Association, 1984. pp. 62–83.
22. **Messer, J.W., H.M. Behney, and L.O. Leudecke:** Microbiological count methods. In *Standard Methods for the Examination of Dairy Products.* G.H. Richardson, ed. Washington, D.C.: American Public Health Association, 1985. pp. 133–150.
23. **Chatigny, M.A., J.M. Macher, H.A. Burge, and W.R. Solomon:** Sampling airborne microorganisms and aeroallergens. In *Air Sampling Instruments.* S.V. Hering, ed. Cincinnati, Ohio: American Conference of Governmental Industrial Hygienists, 1989. pp. 199–220.
24. **Greenberg, A.E., L.S. Clesceri, and A.D. Eaton., eds.:** *Standard Methods for the Examination of Water and Wastewater.* 18th ed. Washington, D.C: American Public Health Association, 1992. pp. (9–32)–(9–38).
25. **Andersen, A.:** Andersen sampler for the collection, sizing and evaluation of viable airborne particle. *J. Bacteriol. 76:*471–484 (1958).
26. **Leopold, S.S.:** "Positive hole" statistical adjustment for a two-stage, 200-hole-per-stage Andersen air sampler. *Am. Ind. Hyg. Assoc. J. 49:*A88–A90 (1988).
27. **Macher, J.M.:** Positive-hole correction of multiple-jet impactors for collecting viable microorganisms. *Am. Ind. Hyg. Assoc. J. 50:*561–568 (1989).
28. **Somerville, M.C. and J.C. Rivers:** An alternative approach for the correction of bioaerosol data collected with multiple jet impactors. *Am. Ind. Hyg. Assoc. J. 55:* 127–131 (1994).
29. **Chang, C.W., Y.H. Hwang, S.A., Grinshpun, J.M. Macher, and K. Willeke:** Evaluation of counting error due to colony masking in bioaerosol sampling. *Appl. Environ. Microbiol. 60:* 3732–3738 (1994).
30. **Chang, C.W.:** "Study of the colony masking effect in bioaerosol sampling and analysis." Ph.D. dissertation. University of Cincinnati at Cincinnati, Ohio, 1994.
31. **Norris, J.R., R.C.W. Berkeley, N.A. Logan, and A.G. O'Donnell:** The genera *Bacillus* and *Sporolactobacillus.* In *The Prokaryotes: A Handbook on Habitats, Isolation, and Identification of Bacteria.* M.P. Starr, H. Stolp, H.G. Truper, A. Balows, and H.G. Schlegel (eds.). New York: Springer-Verlag, 1981. pp. 1711–1742.
32. **Logan, N.A. and R.C.W. Berkeley:** Identification of *Bacillus* strains using the API system. *J. Gen. Microbiol. 130:*1871–1882 (1984).
33. **Juozaitis, A., K. Willeke, S.A. Grinshpun, and J. Donnelly:** Impaction onto a glass slide or agar versus impingement into a liquid for the collection and recovery of airborne microorganisms. *Appl. Environ. Microbiol. 60:*861–870 (1994).
34. **Thompson, M.W., J. Donnelly, S.A. Grinshpun, A. Juozaitis, and K. Willeke:** Method and test system for evaluation of bioaerosol samplers. *J. Aerosol Sci. 25:*1579–1593 (1994).
35. **Hinds, W.C.:** Physical and chemical changes in the particulate phase. In *Aerosol Measurement: Principles, Techniques and Applications.* K. Willeke and P.A. Baron (eds.). New York: Van Nostrand Reinhold, 1993. pp. 41–53.
36. **Eduard, W.:** "Assessment of mould spore exposure and relations to symptoms in wood trimmers." Ph.D. dissertation. Landbouwuniversiteit at Wageningen, The Netherlands, 1993.
37. **Laitinen, S., A. Nevalainen, M. Kotimaa, J. Liesivuori, and P.J. Martikainen:** Relationship between bacterial counts and endotoxin concentrations in the air of wastewater treatment plants. *Appl. Environ. Microbiol. 58:*3774–3776 (1992).
38. **Reasoner, D.J. and E.E. Geldreich:** A new medium for the enumeration and subculture of bacteria from potable water. *Appl. Environ. Microbiol. 49:*1–7 (1985).
39. **Olsen, R.A. and L.R. Bakken:** Viability of soil bacteria-optimization of plate-counting technique and comparison between total counts within different size groups. *Microb. Ecol. 13:*59–74 (1987).
40. **Taylor, G.J.S. and J.P. Leeming:** A method for multiple synchronous collection of airborne organisms and the effects on colony counts of various processing procedures. *J. Appl. Bacteriol. 74:*174–180 (1993).
41. **King, A.D., A.D. Hocking, and J.I. Pitt:** Dichloran-rose bengal medium for enumeration and isolation of molds from foods. *Appl. Environ. Microbiol. 37:*959–964 (1979).
42. **Verhoeff, A.P., J.H. van Wijnen, P. Fischer, B. Brunekreef, J.S.M. Boleij, E.S. van Reenen, and R.A. Samson:** Presence of viable mould propagules in the indoor air of houses. *Toxicol. Ind. Health 6:*133–145 (1990).
43. **Bragulat, M.R., M.L. Abarca, M.T. Bruguera, and F.J. Cabanes:** Dyes as fungal inhibitors: effect on colony diameter. *Appl. Environ. Microbiol. 57:*2777–2780 (1991).
44. **Henson, O.E.:** Dichloran as an inhibitor of mold spreading in fungal plating media: effects on colony diameter and enumeration. *Appl. Environ. Microbiol. 42:*656–660 (1981).