Evaluation of Counting Error Due to Colony Masking in Bioaerosol Sampling

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Colony counting error due to indistinguishable colony overlap (i.e., masking) was evaluated theoretically and experimentally. A theoretical model to predict colony masking was used to determine colony counting efficiency by Monte Carlo computer simulation of microorganism collection and development into CFU. The computer simulation was verified experimentally by collecting aerosolized *Bacillus subtilis* spores and examining micro- and macroscopic colonies. Colony counting efficiency decreased (i) with increasing density of collected culturable microorganisms, (ii) with increasing colony size, and (iii) with decreasing ability of an observation system to distinguish adjacent colonies as separate units. Counting efficiency for 2-mm colonies, at optimal resolution, decreased from 98 to 85% when colony density increased from 1 to 10 microorganisms cm^{-2}, in contrast to an efficiency decrease from 90 to 45% for 5-mm colonies. No statistically significant difference (α = 0.05) between experimental and theoretical results was found when colony shape was used to estimate the number of individual colonies in a CFU. Experimental colony counts were 1.2 times simulation estimates when colony shape was not considered, because of nonuniformity of actual colony size and the better discrimination ability of the human eye relative to the model. Colony surface densities associated with high counting accuracy were compared with recommended upper plate count limits and found to depend on colony size and an observation system's ability to identify overlapped colonies. Correction factors were developed to estimate the actual number of collected microorganisms from observed colony counts. This study determined that computer simulation of colony surface density and resulting masking can identify suitable air sample volumes (i.e., flow rates and collection times) for measuring concentrations of airborne microorganisms and that errors due to colony masking can be reduced by applying correction factors to observed colony counts.

Airborne microorganisms and other biological materials (i.e., bioaerosols) have been studied in office buildings (31, 32), operating rooms (15), agricultural settings (12, 24), animal feed and processing industries (10, 11, 26, 39), sanitary landfills (36), sewage treatment plants (22), and other facilities where biological air contaminants may pose health hazards (13, 25). A popular method to identify and quantify airborne microorganisms is collection directly on semisolid culture media, followed by counting and identification of the resulting CFU (9). Sample handling errors and cell injury during sample analysis are minimized in the direct agar impaction method, as no sample processing is required after collection, except incubation and examination.

Colony counting accuracy suffers with few or many CFU on a plate, and identification problems arise with crowded colonies. Whenever multiple CFU grow on culture media, there is a probability that some colonies will be sufficiently close to come into contact. This probability increases with the number of CFU and may cause serious interference when collecting bioaerosols from highly contaminated environments or over long time periods.

The problem of colonies too numerous for reliable counting and identification can be overcome with liquid specimens by diluting samples before plating and by making pour plates (4, 7, 9, 30). Upper plate count limits of 200 to 300 CFU on

standard, 100-mm-diameter culture plates (available area, 57 cm^2) have been recommended for food, water, and other samples (5–7, 9, 16, 30, 43, 45). These limits may be suitable for counting on plates containing predominantly one or two types of microorganisms that produce small-to-medium colonies but may not be appropriate if samples contain many different types of microorganisms and if the colonies are large.

Mathematical methods have been developed to assess the effect of colony overlap (29) and to evaluate masking when counting objects other than bacterial or fungal colonies. For example, an equation for dust particle enumeration was developed (20) and improved (3) and modified for counting fibers (23), viral plaques (18, 19, 28), and splenic colonies (42, 46–48). This equation, based on the Poisson distribution, requires that the probability of observing more than one object at a collection point is essentially zero (37). The assumption that counts follow a Poisson distribution is correct only if the objects do not overlap or if all of the individual units can be distinguished even if they overlap (19).

The probability of having two or more objects overlap may be far from zero if object size is not negligible relative to the collection surface area or if the number of collected objects is large. Statistically significant bias occurred with an assumption of a Poisson distribution for microbiological colony counts (40), and a binomial distribution was found to be more representative than the Poisson distribution for splenic colony counts (42). Radioautographic grain counts have been corrected for overlap bias (38) by using solutions based on probability theory dealing with occupancy problems, i.e., the distribution of items in available locations (14). The study reported here determined that it was appropriate to assume a

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binomial distribution for microbiological colony counts and to treat as an occupancy problem colony distribution and masking on agar surfaces and in pour plates viewed from above.

MATERIALS AND METHODS

Model theory. The model used in this study assumed that microorganisms were collected randomly on exposed culture plate surfaces and that the resulting colonies were circular and identical in diameter (D_c). Colonies were considered to be masked if an observation system could not distinguish over-lapped or merged colonies.

Separation distance. Adjacent colonies were assumed (i) to overlap completely, i.e., be entirely superimposed, (ii) to merge partially, or (iii) to touch only at their perimeters. Separation distance between adjacent colonies (S_c) was defined as the distance between the centers of neighboring colonies and ranged from 0 (complete colony overlap) to 1 (colonies tangent) D_c. Colonies not in contact with others were not considered in the model because they were judged readily identifiable as individual units.

Resolution index (R_c). The theoretical model assumed that ability to distinguish adjacent colonies depends on an observation system's resolution ability, i.e., the person counting colonies and the equipment the person uses. R_c was assumed to range from 0 to 1, where (i) the poorest observation system (R_c = 0) could not distinguish adjacent colonies even if they just touched at their perimeters, (ii) a system with R_c = 0.5 could distinguish adjacent colonies overlapped by less than half, and (iii) an optimal system (R_c = 1) could distinguish all but completely overlapped colonies.

Masking unit. An imaginary masking unit defined the boundary within which colony masking could occur. The diameter of this masking unit (D_m) was defined as the sum of D_c and the minimum separation distance between colony centers above which the colonies were not masked (S_c^*) as follows:

$$D_m = D_c + S_c^* = D_c(2 - R_c) \qquad (1)$$

The area of each masking unit (A_m), therefore, was defined as follows:

$$A_m = \frac{\pi}{4}(D_m)^2 = \frac{\pi}{4}[D_c(2 - R_c)]^2 \qquad (2)$$

Total collection surface area divided by A_m determined the number of available masking units (N_m). The number of occupied masking units $(N_{m,occ})$ equalled the number of observed CFU (N_c) because two or more colonies occupying the same masking unit, by definition, were indistinguishable.

Computer simulation of microorganism collection and development into CFU. The bioaerosol collection and counting process was considered to be an occupancy problem (14) and was simulated by a Monte Carlo process with a computer program written in GW-BASIC (IBM Corp., Boca Raton, Fla.) (8). The program randomly selected an integer between 1 and N_m for each number of collected microorganisms (N_{org}). Each masking unit that received one or more particles was considered to be filled for a total $N_{m,occ}$ or N_c as described above. Colony masking was assumed to occur if N_{org} exceeded $N_{m,occ}$ or N_c, i.e., more than one culturable microorganism occupied some masking units or CFU, and counting efficiency (E) was defined as follows:

$$E = \frac{N_{m,occ}}{N_{org}} = \frac{N_c}{N_{org}} \qquad (3)$$

The surface density of microcolonies, $\delta_{c,micro}$ (in CFU per microscope field), was calculated from the number of micro-colonies $(N_{c,micro})$, the number of microscope fields (N_f), and the area of a microscope field (A_f) as follows:

$$\delta_{c,micro} = \frac{N_{c,micro}}{N_f A_f} = \frac{N_{c,micro}}{N_m A_m} \qquad (4)$$

where

$$A_f = \frac{\pi}{4}(D_f)^2 \qquad (5)$$

The diameter of a microscope field (D_f) was determined experimentally with a phase-contrast microscope (Labophot-2; Nikon Corp., Tokyo, Japan) and a calibrated ocular microme-ter.

The surface densities of collected microorganisms (δ_{org}) (in microorganisms centimeter^{-2}) and of resulting macrocolonies, $\delta_{c,macro}$ (in CFU centimeter^{-2}), were calculated from N_{org}, the number of macrocolonies $(N_{c,macro})$, and the collection surface area $(N_m A_m)$ as follows:

$$\delta_{org} = \frac{N_{org}}{N_m A_m} \qquad (6)$$

and

$$\delta_{c,macro} = \frac{N_{c,macro}}{N_m A_m} \qquad (7)$$

where $\delta_{c,macro}$ is the surface density of macrocolonies.

Micro- and macrocolony diameters $(D_{c,micro}$ and $D_{c,macro})$ were defined as <0.5 and ≥0.5 mm, respectively. Microcolony E (equation 3) was calculated from this simulation model, at R_c = 1, for $D_{c,micro}$ = 0.02, 0.05, 0.1, 0.3, and 0.5 mm over a δ_{org} range of 10^{-1} to 3 × 10^4 microorganisms field^{-1}. Macrocolony E was determined at R_c = 0, 0.5, and 1 for $D_{c,macro}$ = 0.5, 1, 2, 5, and 8 mm over a δ_{org} range of 10^{-2} to 10^3 microorganisms cm^{-2}. One hundred iterations of the Monte Carlo simulation were performed for each combination of R_c, D_c, and δ_{org}, and the means and standard deviations (SD) of E were calculated for each.

Experimental model verification. (i) Test microorganism and spore preparation. *Bacillus subtilis* ATCC 6051 (American Type Culture Collection, Rockville, Md.) was used in this study because it produces spores of fairly uniform size which can be stored for long periods without viability loss (35). 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.) (27). The bacteria initially were incubated at 30°C for 24 h on nutrient agar (Difco Laboratories, Detroit, Mich.) to obtain a pure culture that was transferred to manganese-containing nutrient agar (35) and incubated for sporulation at 30°C for 7 days. Bacterial growth was harvested into sterile distilled water, agitated at 150 rpm for 24 h 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 2,800 × g for 20 min and washed three times with sterile distilled water before storage in liquid nitrogen.

(ii) Spore aerosolization and collection. Aerosolization and collection of *B. subtilis* spores were conducted in a class II

biological safety cabinet (SterilchemGARD; Baker Company, Inc., Sanford, Maine). Three dilutions of the stock spore suspension were prepared (range, 1.07×10^4 to 5.04×10^4 spores ml^{-1}). Spore aerosols were generated with a three-jet Collison nebulizer (BGI Inc., Waltham, Mass.) at an air flow rate of 2 liters min^{-1}, and the spore air concentration was measured with a light-scattering aerosol size spectrometer (LAS-X; Particle Measuring System Inc., Boulder, Colo.). The spore concentration remained stable (variation, $\pm1.8\%$) for 150 min (8), which exceeded the 90-min test period.

The spore aerosol was diluted with 42 liters of filtered, compressed air min^{-1} 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 predominantly of single-spore particles and that particle coagulation in the 1.3-s transport time was only ca. $10^{-11}\%$ (17). Electrostatic charge on aerosolized spores (44) 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. The chamber temperature was $22.7 \pm 0.4°C$, and the relative humidity was $28.5\% \pm 1.7\%$ (DHTD Thermohygrometer; Fisher Scientific, Pittsburgh, Pa.).

The spore aerosol was sampled with an impactor specifically designed for laboratory bioaerosol studies (21, 41). The sampler collected spores through a slit nozzle (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 of nutrient agar at 25, 50, or 100% strength, i.e., 15 g of agar plus 2, 4, or 8 g of nutrient broth (Difco Laboratories) per liter of distilled water. An electric motor moved the slide under the impactor slit at a rate of 0.014 cm s^{-1} (21). Sampling started after 1 min of slide movement, and spores were collected at 10 liters min^{-1} for 3 min; the total impaction area was 3.5 cm^2 (1.36 by 2.56 cm). 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 h to register micro- and macrocolonies, respectively.

(iii) **Microcolony counting.** Microcolonies were counted at $\times100$ magnification with a phase-contrast microscope with bright-field illumination (Labophot-2; Nikon Corp.). $D_{c,micro}$ was measured with a calibrated ocular micrometer. The impaction area was divided into 12, 2-mm-long longitudinal bands (excluding the perimeter of the impaction area) for a counting area of 2.88 cm^2 (1.2 by 2.4 cm), i.e., 82% of the total impaction area. Randomness of microcolony distribution in the counting area was tested at $\alpha = 0.05$ by using a chi-square statistic (1). Samples that exceeded this limit (14%) were considered to have nonrandomly distributed CFU and were excluded from further analysis.

(iv) **Macrocolony counting.** The 2.88-cm^2 microcolony counting area was identified on Nunc slide photographs taken after 36 h of incubation. All single colonies and colony clusters were counted as 1 CFU, regardless of shape, at $R_c = 0$. At $R_c = 1$, apparently and nearly circular colonies were counted as 1 CFU, elliptical colonies were counted as 2 CFU, and colonies with other shapes were counted as 3 CFU. Degree of colony overlap could not be judged accurately by eye for other R_c values.

$D_{c,macro}$ was measured manually with a vernier caliper and an image analyzer (Magiscan 2; Joyce-Loebl Ltd., Team Valley, Gateshead, United Kingdom). Experimental A_m was calculated from the average macrocolony diameter ($\overline{D}_{c,macro}$) (equation 2).

(v) **Average colony separation distance ($\overline{S}_c$).** S_c was not measured but determined from $\delta_{c,macro}$. Each macrocolony was

considered to be located at the center of a hexagon with an area inversely proportional to $\delta_{c,macro}$. All hexagons were taken as connected and identical and were divided into six equilateral triangles. The average separation distance between any two nearest colony centers, therefore, was twice the height of a triangle. On the basis of this geometric model (8), average S_c, $\overline{S}_c$, was expressed as follows:

$$\overline{S}_c = \sqrt{\frac{2}{\sqrt{3}\delta_{c,macro}}} \qquad (8)$$

Comparison of model and experiment. The following experimental parameters were entered into the simulation model to generate expected colony counts ($N_{c,exp}$) for comparison with those observed experimentally ($N_{c,obs}$): (i) R_c (0 or 1), (ii) $D_{c,macro}$, and (iii) N_{org}, i.e., microcolony count in the 2.88-cm^2 counting area. Experimental and model colony counts were expressed as a ratio ($X_{Nc,obs/exp}$) determined as follows:

$$X_{Nc,obs/exp} = \frac{N_{c,obs}}{N_{c,exp}} \qquad (9)$$

Correction factor for colony masking (F). F was defined as the ratio of expected colony density if all collected microorganisms grew into countable colonies ($\delta_{c,exp}$) and the observed CFU density ($\delta_{c,obs}$) as follows:

$$F = \frac{\delta_{c,exp}}{\delta_{c,obs}} \qquad (10)$$

$\delta_{c,exp}$ equalled δ_{org} when all microorganisms developed into countable colonies, whereas $\delta_{c,obs}$ was the product of microorganism density and E as affected by colony masking ($\delta_{org} \times E$), so that

$$F = \frac{\delta_{c,exp}}{\delta_{c,obs}} = \frac{\delta_{org}}{\delta_{org}E} = \frac{1}{E} \qquad (11)$$

Equation 11 was applied to micro- and macrocolonies to determine the appropriate correction factors based on their respective E values.

RESULTS

Micro- and macrocolony measurements. Measured $D_{c,micro}$ ranged from 0.03 to 0.11 mm ($\overline{D}_{c,micro} = 0.05$ mm). $\delta_{c,micro}$ was ≤ 4 CFU field^{-1} (i.e., 21 to 122 CFU cm^{-2} [Table 1]), and the probability of microcolony overlap was only ca. 2%. The highest R_c, i.e., 1, was assigned to microcolony counting because overlapping microcolonies could be identified easily and counted as individual units, i.e., $\delta_{c,micro} \approx \delta_{org}$.

$D_{c,macro}$, as measured by the caliper and image analyzer methods, agreed well (8); $\overline{D}_{c,macro}$ ranged from 0.52 to 1.19 mm (Table 1). The coefficient of variation for $\overline{D}_{c,macro}$ increased with increasing nutrient concentration and microorganism density. $D_{c,macro}$ and S_c also depended on available nutrients and δ_{org}, with $\overline{S}_c$ between 1.64 and 2.74 times $\overline{D}_{c,macro}$ (Table 1).

Counting efficiency for micro- and macrocolonies. Figures 1 and 2 show the computer simulations for colony E (equation 3; expressed as a percentage) as functions of $D_{c,micro}$ or $D_{c,macro}$, δ_{org} (microorganisms field^{-1} or microorganisms cm^{-2}), and R_c (macrocolonies only). E decreased with increasing δ_{org}, and the change was more rapid for large than small colonies. For example, 0.1-mm microcolonies were counted with an E of

TABLE 1. Experimental *B. subtilis* $D_{c,macro}$s and relative S_cs at various nutrient concentrations and $\delta_{c,micro}$ values.

Nutrient concn (%)	No. of samples	$\bar{\delta}_{c,micro}$ (CFU cm^{-2})	$\bar{D}_{c,macro}$ (mm) ± SD	CV^a (%)	$\bar{S}_c/\bar{D}_c$ ratio
100	4	118	0.67 ± 0.14	21.0	1.64
	4	87	0.77 ± 0.18	23.7	1.65
	2	78	0.82 ± 0.18	22.0	1.64
	4	45	0.88 ± 0.16	17.9	1.95
	3	37	1.11 ± 0.17	15.7	1.75
	4	21	1.19 ± 0.17	13.3	2.12
50	4	122	0.59 ± 0.11	17.9	1.80
	4	81	0.66 ± 0.12	17.9	1.95
	2	75	0.71 ± 0.12	17.0	1.89
	4	40	0.79 ± 0.14	17.1	2.26
	4	34	0.91 ± 0.15	16.0	2.15
25	2	113	0.52 ± 0.09	18.0	2.06
	3	88	0.54 ± 0.10	17.9	2.23
	3	62	0.60 ± 0.10	16.7	2.39
	4	40	0.65 ± 0.11	16.6	2.74

^{a} CV, coefficient of variation.

$\geq 95\%$ at a δ_{org} of ≤ 40 microorganisms field^{-1}, but under these conditions, 0.3-mm microcolonies were counted with an E of $\leq 65\%$ (Fig. 1). E for 2-mm macrocolonies decreased from 98 to 85% over a δ_{org} range of 1 to 10 microorganisms cm^{-2} at R_c = 1, in contrast to a decrease from 90 to 45% for 5-mm colonies (Fig. 2). Counting efficiency was 90% for 5-mm macrocolonies at 1 CFU cm^{-2} and R_c = 1 (only totally overlapped colonies were considered masked) but decreased
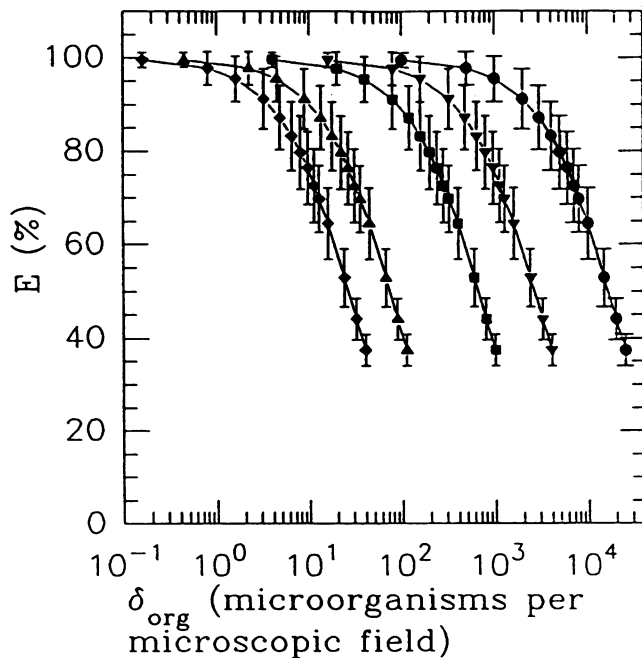

FIG. 1. E for microorganisms cultured to sizes observable under a microscope, from a computer simulation. Each error bar represents 2 SD on the mean of 100 replicates. $D_{c,micro}$ at ×100 magnification and R_c = 1: ◆, 0.5 mm; ▲, 0.3 mm; ■, 0.1 mm; ▼, 0.05 mm; ●, 0.02 mm.
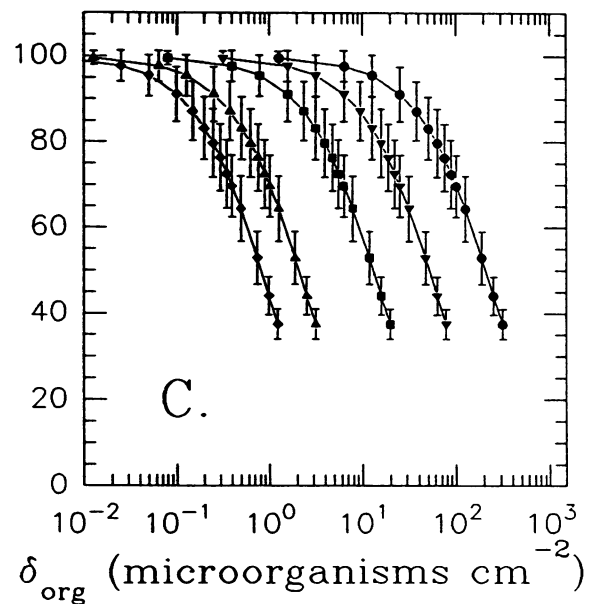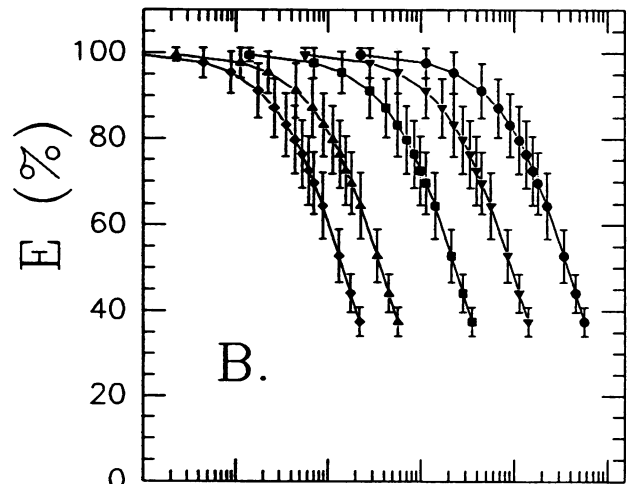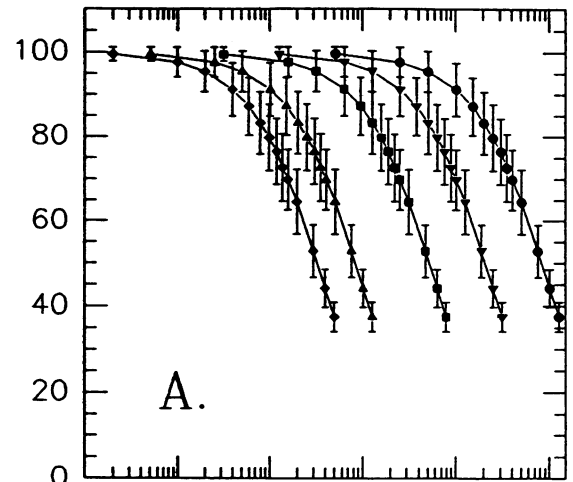

FIG. 2. E for microorganisms cultured to visibly observable colonies, from a computer simulation. Each error bar represents 2 SD on the mean of 100 replicates. Panels: A, R_c = 1; B, R_c = 0.5; C, R_c = 0. $D_{c,macro}$: ◆, 8 mm; ▲, 5 mm; ■, 2 mm; ▼, 1 mm; ●, 0.5 mm.
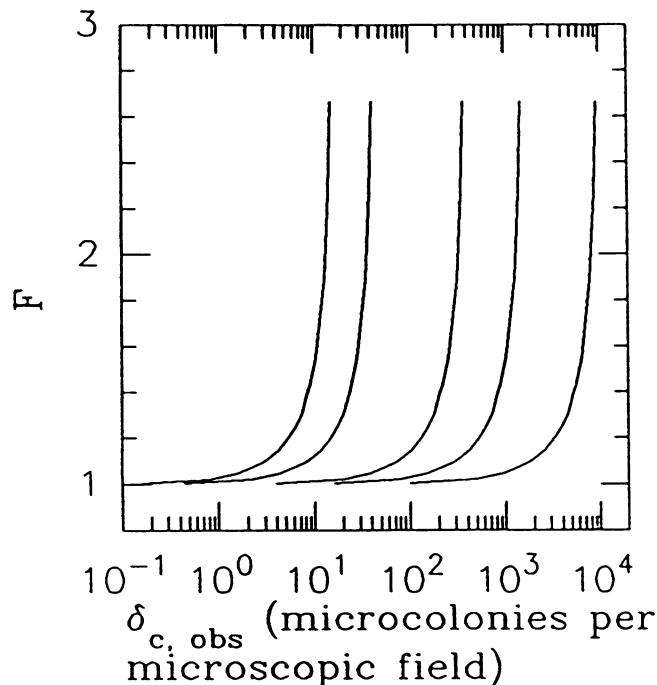
FIG. 3. F values for calculating the actual N_{org} from $N_{c, obs}$ at $R_c = 1$. From left to right, the curves for $D_{c,micro}$ are 0.5, 0.3, 0.1, 0.05, and 0.02 mm.

to 70% at $R_c = 0$ (even colonies that just touched were considered masked).

Comparison between model and experiment. $\overline{X}_{Nc,obs/exp}$ (equation 9) was 0.998 at $R_c = 1$ (SD, 0.06; coefficient of variation, 6%), and a paired t test at $\alpha = 0.05$ showed no statistically significant difference between the experimental and modelled results for the 51 tests in Table 1 (8). However, at $R_c = 0$, $\overline{X}_{Nc,obs/exp}$ was 1.206 (SD, 0.05; coefficient of variation, 4%) and the experimental and modelled results were significantly different. A linear regression ($N_{c,obs}$ versus $N_{c,exp}$)

estimated that the experimental counts were 1.2 times the model predictions (8).

Correction factors for micro- and macrocolonies. Figures 3 and 4 give F values (equations 10 and 11) for observed micro- and macrocolonies based on computer simulations (Fig. 1 and 2) and experimental results (equation 9). The F values were functions of D_c, $\delta_{c,obs}$, and R_c (macrocolonies only).

DISCUSSION

This study evaluated the upper plate count limit, to minimize colony masking, for microorganisms collected and grown directly on agar surfaces and developed a method to correct observed colony counts for masking. Such methods are important when investigators must maximize the information they can gain from a minimum number of samples, especially when they have few opportunities to collect additional samples if initial plate counts are unsatisfactory, e.g., if there are too few or many culturable microorganisms for accurate counting and identification.

In principle, these findings also apply to the enumeration of CFU on spread plates and in pour plates and to the counting of other objects. The factors that determine degree of colony overlap for air sampler plates (e.g., colony density, colony diameter, and ability to distinguish adjacent colonies) apply directly to spread plates and to pour plates examined from above, which converts the three-dimensional colony distribution to a two-dimensional one. However, with pour plates one may be able to resolve apparent colony masking by slightly altering one's viewing position.

E for microcolonies. Counting microcolonies with a dissecting or light microscope may be a better choice than waiting for directly visible macrocolonies when microorganism density is so high that considerable masking may occur. For example, a plate with 20 CFU cm^{-2} generally would be discarded as too densely populated to count because the total plate count would exceed 1,000 CFU. However, this plate could be counted with nearly 100% efficiency if microcolonies were examined when $D_{c,micro}$ was 0.1 mm and with 83% efficiency when $D_{c,micro}$ was 0.5 mm (Fig. 1; $\delta_{org} = 0.63$ microorganisms field^{-1}).

E for macrocolonies. The computer simulation for macrocolonies (Fig. 2) can be used to evaluate suggested upper plate
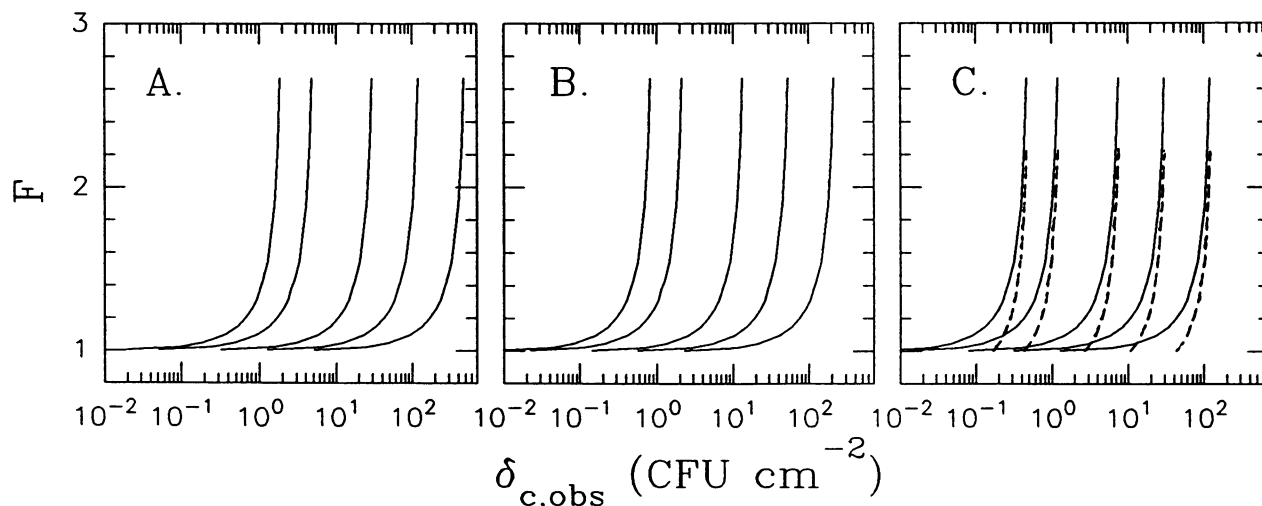

FIG. 4. F values for calculating the actual N_{org} from $N_{c,obs}$. (A) $R_c = 1$; (B) $R_c = 0.5$ (computer simulation only); (C) $R_c = 0$. From left to right, the curves for $D_{c,macro}$ are 8, 5, 2, 1, and 0.5 mm. Solid lines represent model results, and broken lines represent experimental results.

TABLE 2. $\delta_{c,macro}$ upper limits for 95% E, i.e., colony masking of $\leq 5\%$

Colony diam (mm)	CFU 100-mm plate^{-1}		CFU cm^{-2}	
	$R_c = 0^a$	$R_c = 1^b$	$R_c = 0^a$	$R_c = 1^b$
0.5	596	2,707	10.5	47.5
1.0	308	650	5.4	11.4
2.0	49	168	0.86	2.9
5.0	8	29	0.14	0.51
8.0	3	11	0.05	0.19

^{a} Colonies indistinguishable when just touching.
^{b} Colonies indistinguishable only when completely overlapped.

count limits, e.g., 200 to 300 CFU on 100-mm-diameter plates (i.e., $\delta_{c,macro}$ = 3.5 to 5.3 CFU cm^{-2}) (5–7, 9, 16, 30, 43, 45). Figure 2 shows that for an observation system able to distinguish all but completely overlapped colonies (i.e., R_c = 1), 2-mm colonies would be counted with 92% efficiency at 5.3 microorganisms cm^{-2} but 5-mm colonies would be counted with only 63% efficiency; i.e., only ca. 190 CFU could be identified on a plate with 300 colonies. A range of 50 to 100 CFU plate^{-1} (i.e., $\delta_{c,macro}$ = 0.86 to 1.75 CFU cm^{-2}) has been recommended for fungal colonies (2). Figure 2 shows that the E at a $\delta_{c,macro}$ of ≥ 50 CFU plate^{-1} would be $\leq 95\%$ for colonies of ≥ 5 mm, even at optimal resolution, suggesting that the recommended plate count range is too high for fungal colonies above this size.

Recommended counting limits. Investigators should consider colony size and observation system resolution ability when determining upper plate count limits. Table 2 presents predicted 95% E limits for several sizes of macrocolonies at R_c = 0 and 1. These recommended colony density limits are given for standard 100-mm-diameter plates and as CFU centimeter^{-2} for application to plates of other sizes.

Comparison between model and experiment. The model accurately predicted observed colony masking when only completely overlapped colonies were considered indistinguishable (R_c = 1), even when colony size varied (Table 1). The 20% higher experimental colony counts at R_c = 0 (equation 9) were consistent across the δ_{org} and $D_{c,macro}$ range examined in this study. The computer simulation model assumed that colony diameter was uniform at the designated model value or experimental $\bar{D}_{c,macro}$, but actual colony diameter typically varies more between colonies of different genera and species and less within species. The assumption of identical colony diameters accounted in part for the disagreement between expected and observed colony counts at R_c = 0. A masking unit with D_m = $2D_c$ could just contain two average colonies. However, actual colonies of smaller-than-average diameter distinguishable by eye (i.e., recorded as 2 CFU experimentally) would be counted as 1 CFU by the computer simulation because they were considered to occupy the same masking unit and, by definition, were indistinguishable.

Colony shape also contributed to the disagreement between the experimental counts and the computer simulation at R_c = 0. Some adjacent *B. subtilis* colonies were deformed along their bordering edges and did not touch and thus were distinguishable by eye as 2 CFU but to the computer simulation they appeared as 1 CFU. The human eye also would be able to distinguish partially overlapped colonies differing in pigmentation or morphology, which this computer simulation could not.

Considerations for bioaerosol sample collection. Figures 1 and 2 and Table 2 can be used to design a sampling plan to

minimize colony masking by identifying δ_{org} values that correspond to high Es, e.g., $\geq 95\%$, based on expected colony diameter and observation system resolution ability. Investigators can use these δ_{org} values along with estimated bioaerosol concentration and air sampler flow rates to determine appropriate sample collection times (33, 34).

F values. Figures 3 and 4 can be used to estimate $\delta_{c,exp}$ from $\delta_{c,obs}$. For example, F for 5-mm macrocolonies at R_c = 0 and $\delta_{c,obs}$ = 1 CFU cm^{-2} (i.e., 57 CFU plate^{-1}) would be 1.9 on the basis of the model and 1.6 on the basis of the experimental results (Fig. 4) and the corrected colony count would be estimated as between 91 and 108 CFU plate^{-1}.

Further research. This report described a computer simulation to predict degree of colony masking and to correct micro- and macrocolony counts for masking. Further work is needed to evaluate the accuracy of computer simulation models to predict masking when colony size varies, distribution is nonrandom, or interaction is other than mutual colony merging or overlap.

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