Enumeration of High Numbers of Bacteria Using Hydrophobic Grid-Membrane Filters

ANTHONY N. SHARPE* AND GREGORY L. MICHAUD

Bureau of Microbial Hazards, Food Directorate, Health Protection Branch, Health and Welfare, Tunney's Pasture, Ottawa, Ontario, Canada.

Printing a wax grid on a conventional membrane filter yields a device functioning as a most probable number apparatus (MPN), used at a single dilution but with a very large number of growth compartments (e.g., 3,650). By restraining the lateral spread and confluence of colonies, the hydrophobic grid-membrane filter (HGMF) allows growth- or colony-forming units (GU) to be resolved at levels far above those which produce an uncountable lawn on a conventional membrane filter. It also eliminates the size variation of normal bacterial colonies. As a result, the HGMF can give more accurate estimates of the concentration of GU. The method by which grid-cell count observations can be used to obtain MPN estimates of the number of GUs is described, and estimates obtained using the MPN method on the HGMF are compared with those resulting from conventional colony count procedures on membrane filters. A linear relation was observed between MPNGU and the number of GUs, at levels up to 30,000 GUs, for pure cultures of bacteria and for samples of natural waters. The HGMF has great potential for reducing the labor required in quantitative microbiology, since it allows, with one filter, enumeration of microorganisms over a very large concentration range and therefore reduces the need to make dilutions.

Enumeration of bacteria on conventional membrane filters has limitations, imposed by the variable size of bacterial colonies, and the randomness of their distribution on the surface of the filter. In a quantitative analysis it is desirable for there to be a minimum number of colonies on the filter (say 20) to yield a sufficiently precise result. However, if there are too many colonies on one filter, an unknown number of counts will be lost because those growing from two or more adjacent colony- or growth-forming units (GU) overlap unnoticeably and are counted as one. (The term "growth unit" is used here rather than the more familiar "colony forming unit," which is generally taken to mean the unit from which a pure strain is derived; this does not necessarily apply to positive grid cells of the hydrophobic grid-membrane filter [HGMF]). A graph of colony count against number of GUs is linear and has unit gradient at low concentrations, but begins to deviate when a sufficient colony density is achieved for the loss of counts by overlap to be appreciable (region A in Fig. 1). The point at which serious deviation from linearity begins determines the maximum number of GUs which may safely be estimated by a conventional colony count. Obviously, an organism habitually producing large diffuse colonies will have graphs that begin to deviate from linear-

ity at a lower GU density than another which produces small sharply defined colonies.

It is theoretically possible to calculate the number of counts lost by overlap on any given filter if one has information about the size distribution of the colonies (3). In practice, the size distribution is unknown and can only be determined by a time-consuming measurement of the actual colonies. It depends on many factors, such as the species of organism, temperature, incubation time, humidity, media composition and depth, and the average density of colonies. When a counting technique for a particular organism is recommended, therefore, it is necessary to set a somewhat arbitrary upper limit for colony counts, taking into consideration the variability of results obtained by a variety of laboratories under a variety of circumstances. For example, the American Public Health Association recommended upper limit for coliform colonies on M-endo medium is 80 colonies (1). For fecal coliform colonies on M-FC medium, which grow to a larger size, the upper limit is reduced to 60 colonies.

Unknown samples, therefore, need to be serially diluted and a membrane filter (MF) inoculated with each dilution, to obtain colony numbers within the recommended limits. This multiplies the labor and materials required, reduces the analytical productivity of the labora-
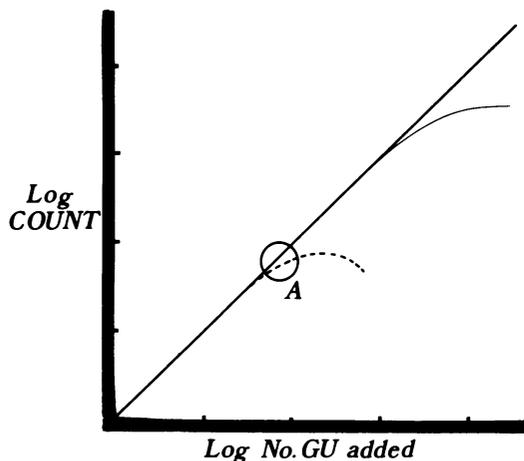
FIG. 1. *Representation of the effect of overlapping in the cases of -----MF colony count, ——HGMF positive grid-cell count, ——ideal case (no overlapping). The MF begins to depart noticeably from linearity at region A.*

tory, and increases the possible sources of error. A method requiring fewer dilutions would be valuable.

A novel device consisting of a conventional MF on which is laid a grid of hydrophobic barriers (e.g., of wax) has recently been described (6). The HGMF is thus subdivided into a large number of growth compartments, which are arranged to be considerably smaller in area than the area generally occupied by colonies on a MF. The barriers confine colony development to the grid cell in which the GU falls, and the growth occurs in a habitat which is relatively independent of those around it. In the estimation procedure using the HGMF, counts of the number of positive grid cells (grid cells containing growth) are obtained. Since more than 1 GU can give rise to the growth in any cell, the estimation problem is analogous to that in a most probable number (MPN) experiment carried out at a single dilution but using a very large number of tubes.

The procedure using the HGMF should lead to more accurate and precise estimates of the density of GUs than the conventional colony counting procedure on MFs because the HGMF restrains the lateral spread and confluence of colonies. As well, it has great potential for reducing the labor required in quantitative microbiology because a large concentration range of microorganisms can be enumerated on one filter. Furthermore, because the growth of very high inoculum levels on the HGMF does not lead to the formation of an unresolvable lawn it is particularly suited to automation of the whole counting process. In this paper it is

shown how the grid-cell count from the HGMF can be used to obtain MPN estimates of the average density of GUs. A comparison is then made between estimates obtained using the MPN method on the HGMF with those resulting from conventional colony count procedures on MFs.

MATERIALS AND METHODS

HGMFs and MFs. Membrane filters type HAWP 04700 (Millipore Corp.) or Gelman type GN-6 Metricel (47 mm) were converted to HGMFs with grids of 50 lines/inch, with wax barriers 0.005 inch (0.127 mm) wide, with the aid of a specially constructed printing machine (5). The majority of results here were obtained with barriers of Ash model cement (Amalgamated Dental Trade Distributors Ltd., London, England). A few were obtained with barriers of paraffin wax (m.p. 54 C, Fisher Scientific). In every case, HGMFs were compared with MFs from the same manufacturer and batch so that no errors would be caused by variation in recovery efficiency of the filter material itself (4, 7).

Bacterial suspensions. Overnight cultures of *Serratia marcescens, Proteus vulgaris, Salmonella typhimurium,* and *Escherichia coli* at 37 C in Trypticase soy broth were diluted in 0.1% peptone, and aliquots of dilutions made up to 100 ml for filtration (6). Natural water samples were also taken from local rivers and used within 1 h of collection. Some of the water samples contained appreciable quantities of debris, algae, and invertebrates. No attempt was made to remove these. Aliquots (0.05 to 100 ml) were made up to 100 ml with 0.1% peptone for filtration.

Inoculation and counting. Aliquots of dilutions were filtered through HGMFs and MFs alternately, starting with the lowest concentrations. Some natural water samples contained so much debris that it was impossible to filter 100 ml. After each filtration the filter funnel was rinsed with 100 ml of 0.1% peptone. Filters were laid on the surface of appropriate agar media in 90-mm petri dishes and incubated overnight at 37 C, or 44.5 C in the case of fecal coliform analyses.

For MFs, depending on the colony density, either total colony counts or estimates from a fraction of the total area were made, using a ×10 stereomicroscope. Often, for MFs containing high concentrations of GUs, it was impossible to count or estimate the number of colonies owing to the formation of a lawn.

For HGMFs, each grid cell containing growth of the desired color (e.g., blue in the case of a fecal coliform analysis on M-FC medium) was classed as positive, and every positive grid cell was counted, also by using a ×10 stereomicroscope. Counting of very high concentrations of positive grid cells was simplified by counting the number of negative ones and subtracting this from the total number of grid cells exposed in the filtration area (3,650). From the grid-cell count the most probable number of GUs (MPNGU) was calculated using tables developed from equation 4 as described below. It should be noted that a positive grid cell is not referred to as a "colony," since it is frequently more of a "commu-

nity" formed by the inoculation of the grid cell with more than 1 GU, and in the case of natural samples at high concentration on the filter it may contain more than one species.

For both pure cultures and natural river water samples MF colony counts, HGMF positive grid-cell counts, and MPNGU derived from HGMF grid-cell counts were plotted against estimated inoculum levels on full logarithmic graph paper. In the case of natural samples the inoculum level of a particular filter was taken as the volume of sample filtered. In the case of pure cultures, the inoculum·level of a filter was estimated by reference to the count on those MFs containing 20 to 80 colonies, or those HGMFs containing 20 to 600 positive grid cells, and by adjusting such counts by appropriate dilution factors.

RESULTS AND DISCUSSION

Derivation of MPNGU from grid-cell count. An estimate of the average number of GUs (MPNGU) per unit volume of liquid may be obtained from the number of positive grid cells on a HGMF under the following assumptions. (i) The GU are distributed randomly in the bulk suspension from which small samples are removed for filtration, so that the distribution of numbers of GUs in replicate samples is Poissonian. (ii) Each GU is equally likely to fall into any of the grid cells. This requires there to be uniformity of filtration rate across the filter, and uniformity in the area of grid cells. Both conditions can be relatively easily satisfied. (iii) Each GU falls into a grid cell, i.e., is not lost by being adsorbed to the wax. (iv) The presence of one or more GU in a grid cell does not influence the addition of other GU to the same grid cell. A single bacterial cell occupies only about 0.0005% of the area of a grid cell. The presence of ten, or even 100, GUs in grid cell is unlikely to affect the filtration rate through it. (v) A grid cell remains negative if no GUs are deposited in it. This is dependent on the integrity of the wax barriers.

The estimation formula is then obtained as follows. Let the HGMF contain n grid cells. Denote by λ the average number of GUs per unit volume in the liquid filtered through the HGMF. The average number of GUs in the volume filtered through one grid cell is given by $\lambda' = \lambda/n$ (equation 1). The probability of growth in any particular square is then P (growth) = $1 - e^{-\lambda'}$ (equation 2). If the number of grid cells which show growth is x, then the most probable number, i.e., the maximum likelihood estimate of λ' is (e.g., Finney [2]) est $\lambda' = -\log_e (n - x)/n$ (equation 3). Since there are n grid cells in the HGMF, the estimate of λ is given by est $\lambda = $ MPNGU $= n(\text{est } \lambda') = -n \log_e (n - x)/n$ (equation 4).

One may obtain the MPNGU for any positive grid-cell count from 1 up to $(n - 1)$ on a HGMF containing n grid cells by substituting values for n and x into (equation 4). In practice, the MPNGU for a particular value of n may be obtained from a nomogram like that shown in Fig. 2, with extended scaling.

Expected performance of the HGMF as compared to the MF. The conceptual relationships between the three estimates of GU density, MF colony count, HGMF-positive grid-cell count, and MPNGU derived from HGMF grid-cell count are illustrated in Fig. 1. At low concentrations of GU all three methods yield good estimates of the number of GU, and therefore, the three curves are approximately coincident.

At higher concentrations, where the MF colony count substantially underestimates the number of GUs (region A, Fig. 1), the HGMF grid-cell count is somewhat higher than the MF colony count and closer to the true value, because the number of counts lost by unnoticeable overlapping of colonies is reduced. In this range, the curve for the MPNGU is still approximately coincident with the curve for the HGMF grid-cell count.

At still higher concentrations, the MF yields a lawn. The HGMF grid-cell count substantially underestimates the number of GU because a high proportion of grid cells contain more than one GU, but the MPNGU still yields a reasonable estimate of the number of GU, as illustrated by the fact that the curve of MPNGU remains approximately linear with unit gradient over a wide concentration range.

In the highest region of Fig. 1, the probability of finding a true "colony" on the HGMF is low. For a grid-cell count of 3,649 the MPNGU
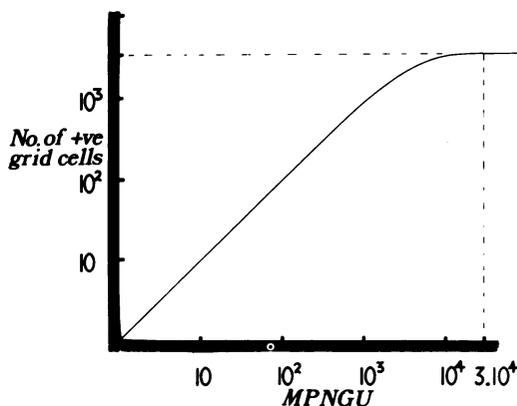
FIG. 2. *The relationship MPNGU = $-n \log_e (n - x)/n$ can be plotted to construct a nomogram from which the MPNGU for any HGMF positive grid-cell count can be derived. The nomogram is shown here for a 3,650 grid-cell HGMF.*

in the liquid tested is 30,000 (29,939), so that an average of 8 GUs contribute to the community in each positive grid cell. However, for samples containing up to 800 GUs (on a 3,650-cell HGMF) it may be permissible to refer to positive grid cells as colonies, since there is at least a 90% probability that the growth in any one of them resulted from a single GU.

Although for a grid-cell count of 3,649 the MPNGU is 30,000, it should be noted that the corollary is not necessarily true; i.e., a liquid containing this number of GUs (or even somewhat fewer) also has a high probability of yielding a count of 3,650 positive grid cells, in which case it may be indistinguishable from a liquid containing considerably more than 30,000 GUs. In practice, therefore, there is an upper limit to the number of GUs which may be estimated on a given HGMF.

Performance of HGMFs with bacterial suspensions. For *P. vulgaris* (Fig. 3a) the MF colony count departed noticeably from linearity (i.e., substantially underestimated the number of GUs) at about 200 GUs. At this point the HGMF-positive grid-cell count was higher than the MF count, and closer to the estimated inoculum level. At concentrations above 1,200 GUs (370 colonies on the MF) the MFs were covered by unresolvable lawns, whereas the HGMF grid-cell count was still close to the true value. The MPNGU derived from the grid-cell count, however, obeyed the linear relation up to the saturation point of 30,000 GUs. Thus, in this experiment, the HGMF gave a good estimate of the true value over a range some 150 times greater than the conventional MF.

S. marcescens behaved similarly (Fig. 3b) in that the MF count deviated from linearity at about 250 GUs, but the HGMF grid-cell count was closer to the true value in this range. The MPNGU derived from the grid-cell count remained linear up to the saturation point of 30,000 GUs. In this experiment, therefore, the HGMF gave a good estimate over a range some 120 times greater than the conventional MF.

It should be noted that the number of GU at which recovery by MF begins to deviate noticeably from linearity is variable, being governed by the species or organism, incubation time, temperature, humidity, availability of nutrients, or any other factor which can influence the average colony diameter.

Of two other pure cultures also tested (data not shown) *S. typhimurium* and *E. coli* deviated from linearity at about 350 and 200 GUs, respectively, on MFs, and lawns were obtained above 1,100 and 400 GUs, respectively. In both cases, the HGMF grid-cell count was higher throughout the nonlinear region of the MFs,
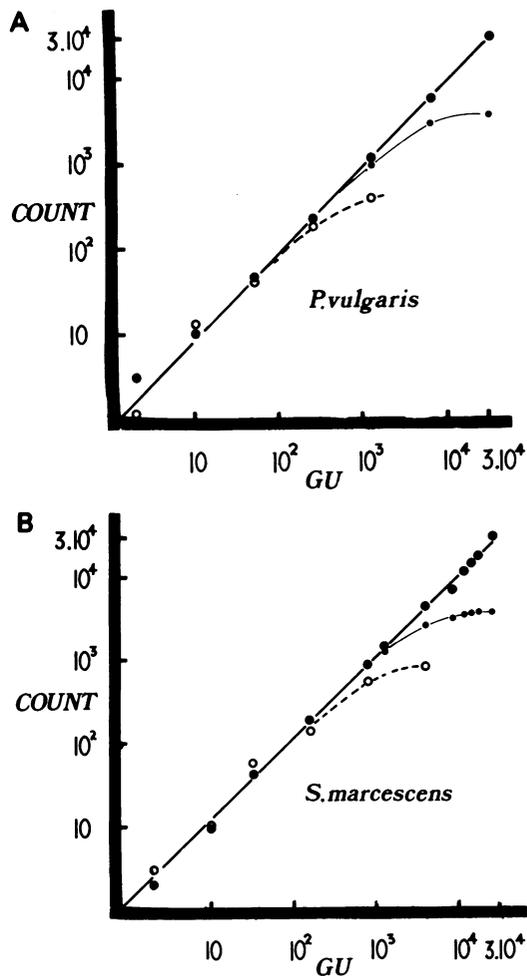
FIG. 3. *Graphs of MF colony count* (O), *HGMF-positive grid-cell count* (●), *and MPNGU derived from HGMF grid-cell count* (◐) *against number of GUs for* (A) *P. vulgaris,* (B) *S. marcescens.*

and the MPNGU derived from the grid-cell count fitted the straight line up to 30,000 GUs.

Performance of HGMFs with natural water samples. The contamination levels experienced in many waters do not range up to the maximum capability of the HGMF. Frequently, however, the count may be in the nonlinear region of an MF and it is necessary to use two or more MFs to filter dilutions of the sample, whereas a single HGMF would be capable of enumerating the GU in the sample.

Natural water samples contain mixed flora and it is important to consider whether the HGMF count for a particular type of organism (e.g., fecal coliform) is likely to be affected by the possible growth of other organisms in the same grid cell. Provided that the total number

of GU is not too high (i.e., less than 800) the probability of individual grid cells containing more than one GU is quite small, as has been described, and therefore the likelihood of the count of the species of interest being affected by the presence of other organisms is also very low. In practice the total count can be quickly estimated by examining a short length of one of the lines of grid cells; if less than one in five contain growth of any kind, the likelihood of mixed growth occurring can be assumed to be small. A count of grid cells containing the species of interest can then be made.

When the total count is much higher, an appreciable fraction of the total positive grid cells must contain communities derived from GUs of different species. The parameter (e.g., the color) by which a growth of the species of interest is recognized may then be affected by the presence of the other organisms in the same grid cells. Two situations may then occur separately or simultaneously. In one, the various GU multiply inside a grid cell without signifi-

cant interaction; the result is a dilution of the recognition parameter. The practical importance of this depends on such factors as the normal color intensity of a colony of the organism of interest, and the proportion of interfering organisms. Counteracting this, to some extent, is an increase in color intensity due to the tendency of the HGMF to produce thicker than normal colonies as a result of the lateral growth restraint. Our impression was that development of the normal green/gold color of coliform colonies on M-endo LES medium was not seriously affected by the presence of large numbers of other organisms which generally produced pink colonies, since MPNGU values did not deviate from linearity (Fig. 4A and B).
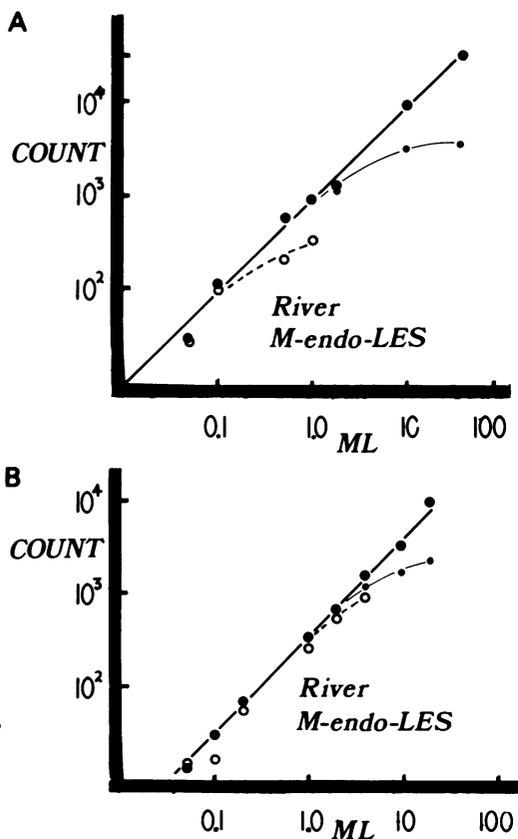
FIG. 4. Two examples of coliform counts in natural waters (M-endo LES medium) counted by MF and HGMF techniques. Key as for Fig. 3.
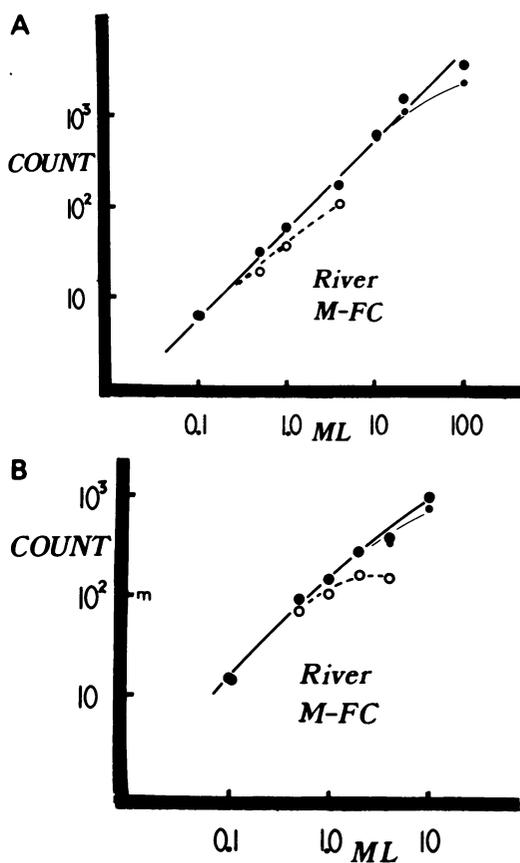
FIG. 5. (A) Example of fecal coliform count in natural water (M-FC medium) counted by MF and HGMF techniques, showing apparent linearity of the HGMF at levels up to 30,000 GUs of fecal coliforms per filter. Key as for Fig. 3. (B) Example of fecal coliform count in natural water (M-FC medium) counted by MF and HGMF techniques, showing deviation from linearity and eventual color loss by HGMFs at very high levels, as result of the presence of interfering organisms. Key as for Fig. 3.

In the other situation, development of one species may suppress either the growth, or production of the recognition parameter of the species of interest. The importance of this effect depends on the proportion of interfering organisms, and their inhibitory activity. Thus, in some cases, no problems were experienced in enumerating high levels of fecal coliforms on MFC medium, in the presence of other organisms on the HGMF (Fig. 5A). However, in two instances, the presence of a high proportion of *P. aeruginosa* suppressed the formation of the blue color of fecal coliform colonies. The effect can be seen in Fig. 5B as a deviation from linearity by the HGMF at high concentrations of GU, culminating in eventual loss of color altogether. The inhibition, which also occurred at a lower concentration on the MFs, was probably caused by neutralization of the acid produced by fecal coliforms (necessary for color production) with ammonia produced by the *Pseudomonas*. The interference could be reduced, but not eliminated, by using the MFC medium initially at pH 6.9 rather than 7.4. Before the full counting range capability of the HGMF can be used in situations of this type, the problem must be fully evaluated with other combinations of bacterial species.

For the time being, it may be wise to treat with caution MPNGU values obtained when there are more than 800 GUs in the sample, if large numbers of interfering organisms are likely to be present. It should be noted that, at this level, the MPNGU is only about 5% higher than the count of positive grid cells, so that there is little need to refer counts to a nomogram or tables. It should also be noted that, according to recommended methods, to determine fecal coliform levels in an unknown sample containing, say 540 GUs, would require three MFs (two- to threefold dilutions). A single HGMF would suffice.

Laboratories interested in obtaining small numbers of HGMFs for serious experiments may contact A. N. Sharpe.

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