UNCLASSIFIED

AD NUMBER

AD841602

NEW LIMITATION CHANGE

TO

Approved for public release, distribution unlimited

FROM

Distribution authorized to U.S. Gov't. agencies and their contractors; Administrative/Operational Use; JUL 1968. Other requests shall be referred to Commanding Officer, Fort Detrick, Attn: SMUFD-AE-T, Frederick, MD 21701.

AUTHORITY

Biological Defense Research Lab ltr dtd 13 Sep 1971

THIS PAGE IS UNCLASSIFIED

AD841602

TRANSLATION NO. 3

DATE: July 1968

DDC AVAILABILITY NOTICE

This document is subject to special export controls and each transmittal to foreign governments or foreign nationals may be made only with prior approval of Commanding Officer, Fort Detrick, ATTN: SMUFD-AE-T, Frederick, Md. 21701.

OCT 31 1968

DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland

#3
Cy.1

On the Method of Quantitative Analysis of Bacteria Cultures in Fluid Media.

by

Holger W. Jannasch.

Determination of cell density in fluid bacteria cultures through the indirect cultural or the direct method of evaluation are in many cases of bacteriological research in apparent and exact datar in the study of growth. On the one hand the KOCH slide process still includes all growth problems and is therefore conditional for fine cultures and may only be used for the roughest comparisons in mixed cultures. On the other hand the process of cell counting (by THOMA) or the streak process (according to BREED) under not too underrated observation difficulties a lessened possibility for application shows, that they require a high enough cell concentration.

In reconsideration of these facts an attempt was made, to improve the direct method in which the organisms (it was chronologicaly started with plancton research) in their liquid media, reached by various ways, for example by sedimentation, (UTERMÖHL 1936) centrifugation, precipitation, (BAIER 1935) vacuum distilling (KUSNETZOW 1931) and filtration. A new process will now be described, which by employing membrane filters combines the concentrating of liquid cultures and the production of the microscopical counting preparations into one process.

Filtration through membrane filters, which was employed by KOLKWITZ (1924) for plankton research, was used by CHOLODNY (1928)

to join bacteria in unboiled water trails, and KOFFMANN in a complicated process for soil bacteriology research. In 1933 BARSOF found that after complete filtration, fixing, coloring and clearing of the filter the bacteria were easily recognised microscopically. More works by RASUMOF (1933) and DIANOWA (1934) can be found on this subject, which of late has expanded morphological (BELING, SCHMITZ, 1950) and analytical (TIETZ and HEEPE, 1950) research. Until today the only quantitative work by this direct membrane filter method was done by BELING (1950) in river water research and the experiment made it nearly possible for use in experimental bacteriology.

The " COLI 5 " mechanism (Membrane-Filter Society, GOTTINGEN) may be used in principle for specimen filtration. As the 36m m. cross-section of the filter's surface is much too large for microscopic calculations, an addition was devised, (see Fig. 1 a) dividing the filter into 19 circular filtering surfaces with a 6m m. cross-section (Fig. 2), which permits one to strain the corresponding number of specimens or even control solutions. In this case the filtering surfaces consist of 25 mm^2, or 15625 calculating squares with sides of 40 μ. The cell distribution on the filter is completely uniform if the specimen is properly prepared and the thickness sufficient; and the periphery of the separate fields stands out sharply against the non-filtering parts (Fig.3) while this addition is metalic, another model consists of inbuilt glass tubes set on the filter, thus dividing it into the corresponding number of filtering surfaces of

identical size. (Fig. Ib) A single glass mechanism may also be used for single specimens (namely the "STEFI", M.F.G., GOTTINGEN); it can easily be made with a so called small ALLIHN tube (JENA). The thickness of the glass plate, in this case, is of 20m m. (G3 or G4) (Fig.1 c). This simple mechanism has also proved itself extremely useful when applied in other fields of membrane filtration.

Sterilization is not a prerequisite for these experiments. Cleansing of the parts which come into contact with the specimens is important., i.e. removal of dirt particles or bacteria from former specimens which impede the microscopic field of vision. By first washing out with PRIL-water (available as a commercial mixture of quarternary Amonia bases) and then rinsing in filtered water, one may easily avoid having either completely obstructed vision or reaching erroneous calculation results because of impure organisms. The impurities which still appear will in no way desturb the count-picture. It may be useful to have a large scringe ready with a supply of prefiltered water (membrane-filter #5) and a solution of 1% Formalin, which also represents the solvent. It is also important to rinse out the tubes on the filter immediately after filtrations of the specimen, to avoid any drops of bacterial solution from hanging onto the sides.

The clarity of the count picture depends on the density of the bacteria on the filter, which should be from 1 to 3 μ in size comprising 1000 to 3000 cells to every square milimeter.

The corresponding solution contents may be rapidly ascertained by means of a preliminary test and an estimated count. So as to obtain an equal distribution of bacteria on the filter, the depth of the filled in specimen must be much greater than the thickness of the filtering surface. If the measurement is of 20m m, the specimen should be filled up to 15-20cm^3. Disolved colloidal matters added to the culture solutions, disturb the normal contents of the concentration, both during filtration and during the staining of the filter. It is also possible to clean the filter pores by rinsing them with diluted acids and alkalies respectively, without disturbing the cells resting on the surface.

Specimens fixed in 3% Formalin may be preserved for a long time, greatly facilitating the completion of a series. If the filtration is to be undertaken immediately, heat fixing at 70° suffices in what concerns the staining. In the case where placing in oil does not follow immediately, spore building material necessitates laying the filter to be fixed on a filter paper soaked with formalin, because growth has often been noticed on dryed filters. Dyeing the filter with methylene blue is the easiest and also the highest contrast method of cell counting. So as to make smaller structures visible,water dyes such as erythrosin and water-blue are used, they allow a continued collecting of the filter to increase the contrast. (HELING 1949 and RAIBLE, 1951,. In this case the color is much more

permanent, and observation by the phase-contrast method often indispensible. In cases where too much dye has remained in the pores, one may tone down by rinsing (drawing through) filters dyed with methylene blue with prefiltered water.

One may clear up the filter by adding an identical amount of carnation oil to the immersion oil.*

Other details, which naturally appear soon after tackling this problem, will not be discussed here. The entire process of filtration and preparation of samples is conducted in the following manner:

1. Placing of the damp** filter into the mechanism with tweezers. Screwing on of the superstructure and short dampening. (water jet pump).
2. Filling of the fixed and correspondingly diluted sample by means of pipettes. Filtration.

---

* For details on the clearing up of the membrane filter those interested must seek else where.

** The filters may be boiled in distiled water for a short period before the test, to dispell all solvents.

Table 1. Fluctuation of the number power.

Quantities of the filtrated Suspension in cm^3	Glass Mechanism: Bacteria count in 4x4 squares (25 600 ²)	Glass Mechanism: Converted to 1 cm^3	Superstructure: Bacteria count in 4x4 squares (25 600 ²)	Superstructure: Converted to 1 cm^3
1	65	65,0	44	44,0
2	129	64,5	98	49,0
3	148	49,3	147	45,7
4	242	60,5	181	45,5
5	271	54,2	228	45,6
6	331	55,1	296	49,0
7	454	64,8	341	48,7
8	458	57,2	368	46,0
9	545	60,5	438	48,7
10	615	61,5	492	49,2
11	604	55,0	525	46,8
12	716	59,6	585	48,0
13	778	59,8	638	49,0
14	821	58,6	652	46,5
15	842	56,1	718	47,8

Average: 58,4
Total: 47820000
Average Deviation: 6%

Average 47,2
Total: 45970000
Average Deviation: 4%

3. Heat fixing of the filter at approximately 70° for 20 m. in a dry closet.

4. Setting of the filter on the surface of a methyline blue solution (0,5g methyline blue, 10cm³ of 96% ethylalcohol, 100cm³ distilled water) for 1 to 3 min. until an even dark blue coloring is attained.

5. Dry at approximately 70° (10-15 min.)

6. So as to lighten it, the filter or a section thereof is placed on an object support, the corresponding surface of which had previously been covered with a thin coat of clearing matter.

After a few seconds, as soon as the filter sections are permeated (i.e.,The air has been drawn out of the pores) they are covered with another drop of the clearing matter and with a glass slide. The percentage of error of the method was then established by compared thickness data on one and the same bacterial suspension. 2 cm^3 of a highly mixed liquid culture of Bac. Amylobacter (0,5 Difco-meat extract: 1% Glucose: 0,1% Ascorbic acid: PH 7,0 [1]) were placed in 200 cm^3 of prefiltered water containing 3% Formalin. Up to 15 cm^3 were filtered through the 20mm glass mechanism and a small section (approx. ½ cm^2) of each of the 15 colored filters was lightened. During this time the 1:10 solution (because the contents of the filtering surfaces of the new superstructure and of the glass mechanism corresponds to approximately 1:10) was poured into the 15 tubes of the superstructure and the filter was simultaneously prepared.

The count was improved in 350 cases, instead of employing an ocular net, the smallest undivided squares used were of 40 μ. In cases of thin solutions 20 fields were counted, while 10 were used with thick solutions in 4x4 squares.

---

1. The excellent growth of Bac. Amylobacter in this food solution was tried by H. STOLP.

The mean of these calculations is given in the 2nd and 4th columns of Table 1. These are converted to 1 cm^3 in columns 3 and 5. The average of this comparative value and of the factor $\frac{\text{Total filtering surface}}{\text{calculated surface}}$ eventually multiplied by the solution contents gave the total per cubic centimeter.

In cases where a number of cells slipped through or got caught in the filter pores, escaping the count, the difference may not increase in a manner parallel to the increasing bacterial density. The actual graphic number increase is proof of the complete comprehensiveness of the cells.

In a further experiment the growth of a culture was processed through both mechanisms a second time. One(1)L. of the above mentioned nutritive solution containing approximately 80 000 cells per cubic centimeter was inoculated and incubated at 37°. Sixteen satisfactory samples were taken aseptically at ½ hr. intervals and fixed in test tubes with a certain quantity of prefiltered formalin solution.[1] The filtration was begun two days later. In 8 hrs. the bacterial density grew from 80 000 to 1,500,000 cells per cubic centimeter (Fig. 4) by which a change could be noticed either at the beginning or the end of the test in the otherwise darkly stained medium. Direct quantitative process

1. The influence on growth from oxygen filtration during movements of the culture before every test and from temperature changes and the impairment of the culture fluid were not considered, because the importance of the experiment rests on methodical grounds.

information on these concentrations had not been available before this.

Summary.

The fixed and disolved samples are filtered through a membrane filter to determine their cell density in fluid bacteria cultures. The filter is stained while the bacteria are on it, cleared up on an object-rack and microscopicaly calculated. The advantages of this method are greater precision (average error 4 to 5%), greater usefulness(especially in the field of low concentration) and simplification of the test through lessened dependance on time.

Translation: John Stancioff