THE MATHEMATICS OF THE BACTERIAL COUNT*

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The use of bacterial counts, for the comparison of different degrees of purity in water supplies, for tests of water filters and in various quantitative determinations of bacteria in other fluids, has been overshadowed in importance of late by the use of bacterial counts in estimating the relative purities of milk supplies.

The bacterial count in water was first looked upon as affording an index to the purity of water, and the attempt was made to determine a standard. This attempt was found difficult and, notwithstanding that bacteriologists draw inferences from counts which they find of the greatest service, a standard for the purity of public water supplies has never been generally accepted. A standard for filtered water, however, has frequently been used, sometimes based on the percentage purification obtained, sometimes on a specified figure, sometimes on both.

A great many of the technical difficulties existing at the time when a bacterial standard for water was first chiefly discussed have been overcome since. Standard methods of collecting, plating and counting bacteria have been evolved, with an immense development of detail. The question of standards of bacterial purity for various types of public

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water supplies might now be revived with a fair chance of reaching some decision. But, meantime, the pure milk question has resulted in the establishment of arbitrary standards, based, not on an attempt to establish what is to be regarded as purity, but to fix a limit on what must be regarded as impurity. The standard sought for water was the maximum which might be allowed without danger; that for milk, the minimum that might be secured without suspending the milk trade. The establishment of a standard of maximum impurity for milk has been a much more simple task than the establishment of a standard of minimum purity for water.

But, although the standardization of manipulative technique for securing uniformity of bacterial counts has been accomplished, the calculation of the counts, which is quite equally important, is left an open matter. The standard technique of "plating for bacterial counts" involves the use of 10 c.c.'s of medium, spread evenly in a four-inch (10 c.m.) petri dish. To this is added 1 c.c. or simple fractions thereof of the liquid containing the bacteria to be counted. These specifications are based on the best accepted practice and may be regarded as theoretically sound and practically useful, so far as manipulation goes. *A priori*, it might be supposed, and the earlier bacteriologists evidently did suppose, that plates thus prepared would indicate reasonably accurately the total number of bacteria present in the material tested, at least of those varieties which would grow under the conditions (media, temperature, humidity, and length of incubation) imposed, *without regard* to whether the number of such bacteria present were few or many, *i.e.*, that 1 c.c. of water, milk, etc., containing 100 growable bacteria would, when introduced into a plate, yield 100 colonies per plate, while 1 c.c. of water, milk, etc., containing 10,000 growable bacteria would yield, when introduced into a plate, 10,000 colonies per plate. I shall endeavor to show that this is entirely fallacious and to urge the true status. It is not a

new subject. I first urged it with regard to water, 1897.*
Jordan, of Chicago, independently urged the same in 1899.†
But it has not yet been generally recognized in practice.

The physical difficulty of making counts on plates con-
taining large numbers of colonies induced the earlier bacteri-
ologists to plate fractions of a c.c. rather than whole c.c.'s,
when they suspected the material to contain high numbers
of bacteria, in order to reduce the actual colonies per plate
sufficiently to make counting easy. So long as this object
was secured, no attention was paid to the overcrowding of
the plate as a factor in the count. Counts were still reported
from plates containing many hundreds of colonies, or even
thousands. Indeed, much ingenuity has been expended to
devise various forms of colony counters, with the express
object of permitting accurate counts to be made in plates,
containing great numbers of colonies, without any suspicion
that the counting of such plates at all was radically wrong,
and when protests against finicky accuracy in high counts
were made, they were based merely upon the fact that close
accuracy was not physically possible, and not important if
accomplished, rather than upon any suspicion that a bio-
logical fallacy existed. It was found later, however, that a
curious discrepancy existed between the calculated counts
obtained from successively diminishing fractions of the same
material. Thus, on plating a decimal series of fractional
parts, results such as the following might be obtained:

	STRAIGHT	1/10	1/100
Actual count	7,000	1,000	150
Calculated count, 1 c.c. . . .	7,000	10,000	15,000

This discrepancy was recognized as existing; of course, all
these figures on the same c.c. of water could not be correct, and
various explanations were offered. There were two methods

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* Report on Brooklyn Water Supply, 1897. Hill and Ellms.
† American Public Health Association Transactions, 1899.

of securing the fractional parts of 1 c.c. for plating; one consisted in using a pipette graduated to tenths or hundredths and so measuring the actual fraction of the original which it was desired to plate. The second method, now generally conceded to be much more reliable than the first, and now almost universally adopted in practice, is that of adding to 1 c.c. of the original material 9 or 99, or other amounts of sterile water, and then plating 1 c.c. of the mixture, attaining the same end by a different process. Those who followed the dilution method explained the discrepancy in counts above described as due to the breaking up, in the process of dilution, of chains or groups of bacteria, which thus yielded as many colonies in the dilutions as the portions into which they were broken; while in the original plate made directly from the sample each chain or group, being unbroken, yielded only a single colony. A number of objections to this hypothesis can be made.

1. There is little evidence that chains or groups occur in water to any such extent as would account for the uniform occurrence of the discrepancy noted.

2. Chains and groups do occur in milk and in sewage, but they are broken up with considerable difficulty, and the manipulations incident to dilution are not sufficient to notably disintegrate them. (Rickards.)

3. If the original sample as well as each successive dilution be handled with equal severity of manipulation, shaking each violently twenty-five times, for instance, the discrepancy remains unaffected.

4. If the dilution processes alone caused the discrepancy, no discrepancy should be found in the direct fractional measurement of plating. But such discrepancies are found in both methods.

5. If the discrepancy depended on breaking up of chains or groups, it should be most marked in the plating of pure cultures of streptococci, sarcinæ, etc., and least marked in

the plating of cultures of organisms such as *B. prodigiosus*, which show no great tendency to chains or groups. But the discrepancy in both cases is practically the same, showing no relation to these factors.

Hence it is entirely fair to conclude that the breaking up of chains or groups is not a factor in the discrepancy between successive dilutions. In endeavoring to account for the discrepancy (a problem set before the writer by Mr. Geo. W. Fuller, in the course of the Louisville Water Filtration Experiments, in 1896), the writer observed that in successive dilutions made from pure cultures, *i.e.*, *B. prodigiosus*, not only was the numerical discrepancy parallel with that found in plating mixed cultures, as those, *i.e.*, from water, but that the actual size of the individual colonies increased in proportion as the dilutions rose: *i.e.*, in a plate containing 3,000 colonies, the colonies were very small; in a plate containing half a dozen colonies, the colonies were relatively very large. Of course, the same is true in mixed cultures, but in pure cultures it is extremely obvious. This suggested that the discrepancy was due chiefly to overcrowding. Routine work on water, done in two dilutions, was assembled and examined. It seemed obvious that if overcrowding were the chief factor, the greater the overcrowding the wider would be the discrepancy, and that if there were no overcrowding there would be no discrepancy. This was entirely substantiated by results which are quoted in the Report of the Brooklyn Watershed Laboratory in 1897; by Jordan, in the A.P.H.A. Trans. in 1899, and by many others, notably in the Appendix of the report of our Section Committee on Standard Methods of Bacterial Milk Analysis. (Am. Jour. Pub. Hyg., Nov., '07.)

It will be noticed that the higher the actual number of bacteria in the original sample, the greater the discrepancy between the plates made from the sample, and those made from dilutions of the sample. Anyone who chooses to do so may make tables for himself and so reproduce this work.

He will quickly convince himself of its truth and will also demonstrate that if the original sample contains not over 200 bacteria, successive higher dilutions will check with it, showing that 200 bacteria will grow in a plate without introduction of those factors of food exhaustion or direct antagonism, which we place together under the term overcrowding. Some results seem to show that 100 colonies per plate is the limit for milk, but this remains to be confirmed.

So far the writer has offered nothing practical to justify reference to this subject. The real reason for calling attention to it at this time lies in the fact that it is becoming a matter of serious public health interest that bacterial counts made in different laboratories should be made not alone with the same technical methods, *but also with uniformity of calculation.* If not uniformly calculated, uniformity in technique is of no advantage whatever. The weight given to the bacterial count in the production of certified and inspected milk, and in the bacterial supervision of market milk, makes it very essential that two laboratories engaged in examining the same milk should at least approximate the same results. This is quite impossible, notwithstanding uniformity in technique, if practically identical counts be calculated and recorded by different methods. To illustrate from actual practice: of two laboratories examining the same milk, one reported that the counts pretty uniformly approximated 10,000 per c.c. while the other reported pretty uniformly counts ranging in the neighborhood of half a million. Thus one laboratory would have approved this milk for certification, while the other would have condemned it utterly on the standards for both certified and inspected milk, and would have rejected some of it, at least, even as market milk.

Investigation showed that no serious discrepancies in methods, technique or media existed, in fact, both laboratories used the media made in one of them. Finally it was dis-

covered that the laboratory securing the high counts had diluted the milk to one in 10,000, while the laboratory securing the low counts did not dilute at all, but plated a whole c.c. of the milk itself directly. This latter laboratory did not and indeed could not get counts much over 10,000, because usually 10 c.c.'s of agar in a four-inch petri dish will not develop much more than about this number of countable colonies. Had they plated the milk diluted 1 to 10, the highest count they could have secured from any milk would have been approximately 100,000 of the same 10,000 colonies per plate.

This is not at all an extreme case; it illustrates the principle, at the same time pointing out the enormous errors in practice which may result from negligence or ignorance of what after all is an extremely simple and obvious point.

I do not know how many methods of calculating bacterial counts there are in existence, but there are three which I have seen in use, all of which are good, so long as they are applied to plates containing not over 200 colonies, but the first two of which yield entirely fallacious results when applied, as they are very likely to be, to plates yielding other numbers of colonies.

The first consists in calculating out, for each dilution made, the total bacteria per c.c., and them averaging the results by adding all the totals and dividing by the number of dilutions.

The second consists in adding the actual counts of colonies from the different dilutions and averaging by dividing by the total of the fractional parts of a c.c. plated in each case.

The third consists in regarding as reliable only the not overcrowded plate, whichever that may be; recognizing it in the fact that the count lies between 40–200, ignoring all others.

To illustrate: Suppose that a milk sample, which we will assume actually contains 2,000,000 bacteria per c.c., is plated in a decimal series of dilutions thus:

1 c.c.   1/10   1/100   1/1,000   1/10,000

The number of bacteria actually put into each plate will be
    2,000,000   200,000   20,000   2,000   200
The number growing in each plate will be in round numbers
somewhat as follows:
    10,000   9,000   6,000   1,400   200
The number calculated as present in 1 c.c. taking each
dilution by itself will be:
  10,000   90,000   600,000   1,400,000   2,000,000
The number reported, by the first method of calculation,
above described (*i.e.*, averaging the calculated counts from
each dilution), would be 820,000; by the second method
(dividing the total actual count by the fractions of a c.c.
plate) would give:

$$\frac{10,000 \ + \ 90,000 \ + \ 6,000 \ + \ 1,400 \ + \ \ \ 200}{1 \ \ \ + \ \ 1/10 \ \ + \ 1/100 \ + 1/1,000 + 1/10,000}$$

or $\dfrac{26,600}{1.1111} = 24,000.$

The third method will yield 2,000,000, or the correct count,
as assumed. It will be remembered that the actual number
present was 2,000,000 by hypothesis. Hence these methods
of calculation give results of 820,000, 24,000 and 2,000,000, all
from the same actual counts, one being about 1/3, the other
about 1/90 of the actual number, the last being pretty
close to the true figure.

Thus the real solution of this problem is very simple. If
from the above actual counts all be eliminated except that
lying at or below 200, the correct count is at once had. The
counts from the other dilutions are simply neglected as over-
crowded and hence of no value, except in so far as their in-
creasing disparity as the scale of dilution is descended forms
a consistent whole with the result recorded.

It will readily be seen from this example that if three dif-
ferent laboratories used the same technique, and plated a

milk containing actually from 100,000 to 200,000 bacteria per c.c., the calculated results might easily be such that one laboratory would approve the milk for market purposes only; the second would approve it for inspected milk but not for certification, while the third laboratory would approve it even for certification, all using identical methods and standards, except that of calculation of count.

It will be said that this is exaggerated, that in practice milks would not be plated in so many dilutions and that, therefore, in practice the laboratories concerned would report much more closely uniform results than those here suggested. It is quite true that I have so handled the figures as to bring out the results of applying logically the principles used in such a manner as to show them at their worst, but this is nothing more than the *reductio ad absurdum*, which is a perfectly logical and proper weapon.

It is only right to point out that extremely low counts are also unreliable. In the foregoing examples, had a dilution of 1/100,000 been made on the milk containing 2,000,000 bacteria per c.c., while 20 colonies would be the proper proportion per plate, it is unlikely that any one plate would yield this count, except by the merest accident. The average of several duplicate plates, however, at this dilution would be about 20, the individual plates ranging from 10 perhaps to 35 each. So if the 1/1,000,000 dilution had been made, it would be mere accident if a single plate yielded the proper count of 2. An average of many duplicates would range from 0 perhaps to 10 colonies, averaging about 2.

The unreliability of extremely low counts is due first to the purely physical difficulty of distributing a small number of bacteria equally in several c.c.'s of water, and second to the purely physical difficulty of so equally extracting from the water one c.c. as to leave the equal distribution undisturbed. With larger numbers of bacteria distributed in the same amount of water, these physical difficulties practically

disappear, so that plates showing from 40–200 colonies are quite representative. On this ground, dilution should be by fifths rather than tenths, since proceeding by fifths any possible number must fall, in some plate, between 40 and 200.

To summarize:

I. Plating should be so done as to secure from 40–200 colonies per standard plate.

1. In order to have a number of colonies not too large to count completely.

2. In order that the size of the colonies may be large enough apart to facilitate isolation, if this be desired.

3. In order that the colonies may be large enough for ready appreciation by the eye.

4. And chiefly, because the standard plate, containing the standard 10 c.c.'s of standard medium, will not support more than about 200 colonies without detriment to the weaker forms.

II. In order to secure 40–200 colonies per plate, dilution by fifths instead of tenths is the logical method; but dilution by tenths gives results sufficiently close in practice. In general, for certified milk, dilutions of 1/100 should be made, giving a range of accuracy from 4,000 to 20,000; for inspected milk, dilutions of 1/1,000, giving a range of 40,000 to 200,000; and for market milk, 1/10,000, giving a range of accuracy from 400,000 to 2,000,000. When working to determine the actual count in an unknown milk, rather than to determine if a milk is above or below a certain standard, all three dilutions should be used, and the result selected should be that which gives 40–200 colonies to a plate. Straight or one-tenth counts on milk are rarely required, because few milks run low enough to make the counts at these dilutions accurate.

III. With water, dilutions of straight (or 1 c.c.) and of one-tenth, giving together a range of accuracy from 40–2,000, are usually sufficient. Unless highly polluted, dilu-

tions of 1/100 on water rarely give counts within the limits of reliability (40–200).

IV. With sewage, the dilutions used for market milk can usually be counted on to yield satisfactory results, *i.e.*, 1/10,000, giving a range from 400,000 to 2,000,000; but it is safest to plate also at 1/100,000, giving a range of 400,000–20,000,000.

V. All plating work should be done in duplicate when possible, but, if preferred, successive dilution "to the vanishing point" takes the place of duplication fairly well, *i.e.*, if in one case two plates from the same dilution run 190 and 180, and in another two plates run 190 and (with one-fifth dilution) 40, the check is equally good in both.