DIRECT MICROSCOPICAL COUNTING OF BACTERIA IN WATER.

By Burt E. Nelson.

In the chemical control of filtration and other water purification plants, biological tests have for some time been more generally considered than the purely chemical ones. The direct microscopical examination of potable waters is however at present almost entirely limited to the identification and enumeration of those biological units which are comprised under the general classes of algae, including diatoms and larger fission fungi, and protozoa,[1] and seldom takes cognizance of the much smaller bacteria, the latter being studied by standard cultural methods. Donald[2] has devised methods for the direct bacteriological examination of waters, even distilled water, but they have not come into common use. Winslow[3] and Winslow and Willcomb[4] have also used and recommended direct methods, but mostly as applied to sewage and sewage effluents. Very useful results, in many respects, are now obtained by direct bacteriological counts in milk, following methods devised by Slack,[5] Breed,[6] Skar,[7] Rosam[8] and others. Direct methods are also quite generally used in counting bacteria in liquid cultures and in infected animal fluids.

The advantages of a similar means for enumerating the bacteria in water supplies, especially in checking the bacterial efficiency of filtration plants, seemed apparent, for with these latter especially, aside from special cultural tests for individual types of organisms, and a few others, the total bacterial counts are the chief desideratum. By the direct method: first, results may be had in a short time, which by cultural methods require at least forty-eight hours. Second, it also allows of counting all the bacteria present, instead of simply the colonies of those which will grow during two days, on artificial media. It has long been recognized that many bacterial species, and especially those too which are quite naturally present in waters, will not develop in any of our highly organic culture media, but the extent of the error due to this cause, as shown below, had not occurred to us until these studies were undertaken. In plate cultures too, individual colonies may either develop from a single organism or from a clump of many individuals, which may be more nearly allowed for in the direct method. Third, a general rough morphological classification is also possible while making direct counts, and fourth,

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[1] Whipple, "Microscopy of Drinking Water;" Ward and Whipple, "Fresh Water Biology," and many others.

[2] Donald, *Proc. Roy. Soc.*, (B) 86, 198–202, and *Lancet*, 184, 1447–49.

[3] Winslow, *J. Infect. Diseases*, Suppl. No. 1, 209–228.

[4] Winslow and Willcomb, *Idem.*, 273–283.

[5] Slack, *Tech. Quarterly*, 1906; *Centr. Bact.*, II Abt., 16, 537–538.

[6] Breed, *Centr. Bact.*, II Abt., 30, 337–340.

[7] Skar, *Milchw. Zentbl.*, 41, 454–461 and 705–712.
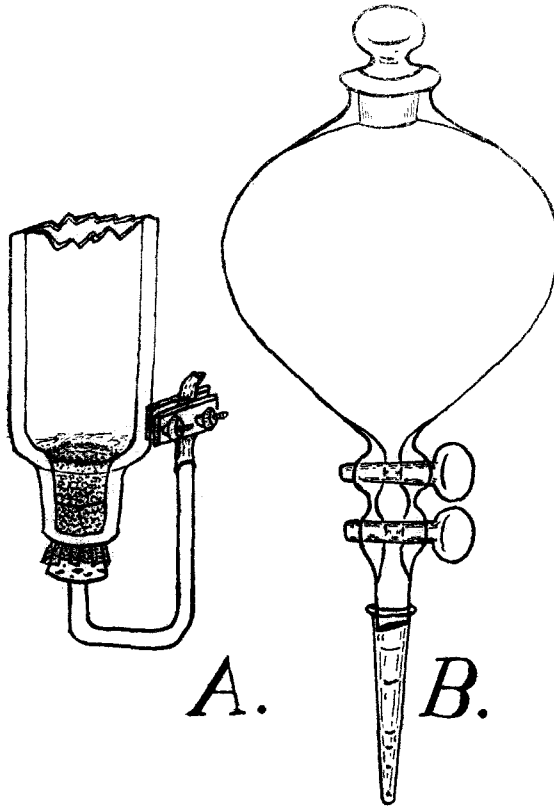
[8] Rosam, *Idem.*, 42, 333–343.

algae and protozoa, may be examined at the same time. A fifth possible advantage in some cases is the elimination of a portion of the time and labor devoted to preparing culture media and to cleaning and sterilizing the glass apparatus used in cultural methods, which is, however, usually practically offset by the longer time and more technical skill actually required for making direct micro counts of individual organisms, as compared with the counting of visible colonies of bacteria. Lastly, sixth, *Bacillus coli* and other special organisms may sometimes be searched for in the concentrations used for making the direct microscopical counts. Among the possible disadvantages are: first, the fact that a series of direct counts as, *e. g.*, on raw and filtered waters, while in all respects comparable among themselves, on account of the usually higher results, can often not be compared so well numerically with those by the plate method. While this may be in part due to the counting of dead bacteria, it is, as before stated, largely due to the presence of clusters, and to the failure of many water bacteria to grow on plates; so the direct count, when properly conducted, would naturally seem to be the more accurate. Evidence of this is also furnished by numerous counts where the majority of the bacteria are motile, and therefore alive, but fail to grow in anything like the same numbers on plates. Second, the absence of characteristic colony formations and other biochemical reactions during growth, which are often used as diagnostic points. This again is largely offset by the possibility of noting the morphology of the organisms themselves in the direct method, and often the motility also if care be taken to prevent serious injury to the organisms. Third, the difficulty of directly counting highly turbid waters. Fourth, the fact that a volume representing only a fraction of a cubic centimeter of water is usually counted, unless time is taken to count a large number of microscopic fields, and fifth, the difficulty of counting the small sized samples usually taken for a bacteriological test. This last objection may be largely removed by concentrating the organisms into a proportionately smaller volume before counting. It will be recalled that the chief object is here to simply recognize the bacteria as such, as distinguished from microscopic algae, etc., and not necessarily to study their form in detail. For this reason a high magnification is not necessary or even desirable, as with high powers the size of the field which may be seen at one time is proportionately diminished. In general, a magnification of from four hundred and fifty to five hundred diameters is all that is required, and an experienced microscopist may usually utilize much lower powers.

While the direct microscopical counting of bacteria in such liquids as most milks, sewage, artificial cultures and infected animal fluids, usually requires methods of dilution and fractionation of the microscope field, in potable waters which are relatively bacterially clean, methods of con-

centrating the organisms are necessary. As a rule the most convenient concentration is that of one thousand cubic centimeters to one, when the counts in each cubic millimeter under the microscope will be equal to those in one cubic centimeter of the original water. In some cases however for mechanical reasons, it is more convenient to prepare a concentration of one thousand to two, or even three. For this purpose, sedimentation, filtration, and centrifugation methods naturally suggested themselves. The use of any of these alone however, as was expected, proved impractical, so similar experiments were tried using hydrous colloidal "flocking" materials, as is customary in larger operations. We employed iron, aluminum, and lead hydroxides, both in the form of creamy suspensions and formed "in situ," tragacanth mucilage, hydrous gelatinous silicic acid, and zinc hydroxide. The first three were unsuitable because of the fact that in the necessary amounts their opacity obscured the microscope field, and their final bulk could not be sufficiently reduced. The latter objection also held for the second two, which were also less efficient, although their translucence was a decided advantage. The zinc hydroxide finally proved the most satisfactory for entangling the bacteria, by being soluble in strong ammonium chloride solution, thus allowing of the final partial clearing of the concentrated bacterial and sediment suspension. It is preferably formed in the liter water sample by adding a 5% solution of dried zinc sulfate, followed in two or three minutes by the proper amount of normal soda solution, shaking thoroughly, and allowing to stand a few minutes longer with occasional agitation. The zinc sulfate which we used contained traces of iron as an impurity, which by forming the insoluble hydroxide, assisted in the final concentration of the organisms, after the solution of most of the zinc hydroxide by the ammonium salt.

A comparison was next made of the relative advantages of the three methods of concentration. Liter samples of a somewhat polluted water were treated with the reagents and submitted to sedimentation, filtration, and centrifugation as follows, the efficiency of the methods being measured by agar plate cultures on the original and clarified waters, and for comparison, by direct counts on the precipitated coagulant. For sedimentation, 5 cc. of the 5% zinc sulfate and 3.7 cc. N soda solution were added and the sample after thorough agitation simply allowed to stand for one-half hour in a conical bottomed separator. The sample for plating was removed from the top by a sterile pipet, and the precipitated flock drawn into the tubes of a medical centrifuge for collection, and final treatment as described below. Filtration was conducted in a Sedgwick-Rafter type of filter consisting of a cylindrical glass percolator having a one-inch neck tapering inward to receive a perforated cork, glass trap tube and attached rubber tube and screw clamp for controlling the rate of flow at the start. The inner face of the cork is provided with notches radiating

from the central hole, to facilitate washing, and is covered by a coarse linen cloth for retaining 20 or 25 mm. of angular white sand, ranging from 0.5 to 1.25 mm., and averaging 0.9 mm. in size. The sand should fully fill the neck of the filter (see Fig. A). We have had best results with this relatively coarse sand as it tends to prevent clogging of the filter, and have relied on the overlying layers of zinc hydroxide flock for retaining the bacteria. Before use, the filter is well washed by rinsing, and by blowing air and clean water up through it, as is done in some larger installations. As the sand subsides after washing, it naturally grades itself in the neck of the filter. For filtration the liter of water was coagulated by 1 cc. of the 5% zinc sulfate solution and 0.7 cc. of N soda. All but about 15 or 25 cc. of the coagulated water is carefully poured into the filter, the bottle being conveniently inverted in the top to secure automatic feeding, the flock allowed to settle somewhat, and filtration allowed to proceed at a rate of about 8 or 10 cc. per minute, or stated more accurately, at a rate which will allow of a column of water 10 or 12 mm. high, and equal in diameter to that of the inside of the neck of the

A.   B.

filter, per minute. The first 100 cc. or so are sometimes returned to the filter for additional efficiency. When all but 10 or 15 cc. of water have passed, as controlled by the glass tube trap, the filter is held in the hand, air blown back through the rubber tube in such a manner as to cause all the sand grains to be strongly agitated and the attached flock dislodged, and the latter quickly decanted into the tubes of a medical centrifuge, after which the sand and used sides of the filter are washed once or twice in a similar manner with the reserved portions of the original sample.

For centrifugation, the liter sample was, like that for sedimentation,

prepared by using 5 cc. of zinc solution with 3.7 cc. of N soda.   It was then whirled in the apparatus shown in Fig. B, at a rate of one thousand revolutions per minute for about three minutes.   We have had this apparatus constructed originally of brass and copper but glass is preferable. The opening through the upper cock should be about 5 mm. or more in size and the opening at the conical bottom of the separator should exactly register with it, so that no irregularities exist on which the precipitate may lodge.   The lower cock may be kept closed and the precipitate collected above it, from where, after closing the upper cock and discarding the cleared sample, it is rinsed into the tube of the medical centrifuge.   As suggested by Mr. A. G. Lauder however, it is often more convenient to leave both cocks open and to collect the sediment at once in the medical centrifuge tube, which is attached by a ground joint to the lower stem of the separator and held there while in use by a spring clamp. The separator rests in a metal sleeve and iron ring which are hung to a heavy cross iron attached to a substantial Babcock milk test machine.[1]

The collected zinc hydroxide with its retained bacteria and other sediment, is whirled in the medical centrifuge tubes until strongly compacted, and the clear supernatant water decanted.   Saturated ammonium chloride solution is then added, about 0.8 or 0.9 cc. for each cubic centimeter of zinc solution originally taken, stirring with a glass rod in the meantime, until the precipitate is nearly dissolved.   As before stated, some slight iron hydroxide will also still remain.   The glass rod and sides of the tube are washed with a few drops of clean water and whirled again at a high rate of speed in the medical centrifuge (about 5000 r. p. m.) for five or ten minutes, after which the clear solution is carefully pipetted off to well below the 1 cc. mark on the tube, being careful not to in any way disturb the deposited sediment.   As after clearing with the ammonium salt, some of the liberated bacteria, on account of the increased specific gravity of the solution, persistently refuse to be deposited with the remaining flock, it is often desirable not to attempt this last concentration in the small centrifuge, but to make the counts directly on the larger 2 or 3 cc. volume.   To the retained liquid and remaining portion of the flock, a large drop of alkaline methylene blue or clear carbol-fuchsine is added as a stain, the volume brought exactly to the mark, well mixed again with the rod, and counted as described below.

[1] The International Equipment Company of Cambridge, Mass., list centrifuges which will probably serve better, but our present cheaper and home devised machine, which was already on hand, has answered every requirement.   The same apparatus has also proved serviceable for clearing melted agar and gelatin culture media.   The Sharples Specialty Company of West Chester, Pa., also make a laboratory centrifuge which will directly concentrate the bacteria from the original untreated water sample, but none of the modern machines thus far, will complete the concentration so that the organisms are finally all collected in a volume of 1 cc.

The following counts from agar plate cultures on the concentrates from three samples of water, diluted with sterile water one thousand times, and on the supernatant liquid in the centrifuge tube, show in a way the efficiency of this last step in the concentration process, although growth in each instance was undoubtedly greatly hindered by the salts present.

1 cc. of concentrated sample, diluted 1–1000.	1 cc. of super- natant liquid.
1600	5
1100	7
2200	8

Comparative results on the three complete methods of concentration are shown below:

BACTERIA PER CUBIC CENTIMETER.

Sedimentation.				Filtration.				Centrifugation.			
Raw water.	Precipi- tate.	Clari- fied.	Effi- ciency.	Raw water.	Precipi- tate.	Clari- fied.	Effi- ciency.	Raw water.	Precipi- tate.	Clari- fied.	Effi- ciency.
2600	(3500)	350	86.5%	2600	(4000)	11	99.57%	2600	(3700)	210	91.9%
2600	(3700)	380	85.4%	2600	(4000)	14	99.46%	2600	(3700)	200	92.3%
1800	(2400)	360	80.0%	1800	(2700)	7	99.6%	1800	(2400)	150	91.7%
1800	(2500)	350	80.6%	1800	(2800)	8	99.6%	1800	(2600)	140	92.2%

The counts on the raw and clarified waters were made from forty-eight hour agar plates, those on the precipitate were made directly. The difference between these latter and those on the original raw water shows the number of these organisms which here failed to grow in artificial media, about 35%. It would here appear evident that filtration is the most efficient process although far more time consuming. However, where other work is being looked after in the mean time, this is of course not very objectionable. With centrifugation on the other hand, counting may be started in from fifteen to twenty minutes after sampling. The quick centrifugal method apparently has an average efficiency of about 90%, which may be allowed for in the final calculation if desired, but even with no allowance, this is apparently still more accurate than the official plate method. Simple sedimentation offers few practical advantages.

By either of these methods of concentration the total bacteria and other sediment from one liter, are finally collected in a volume of 1 cc., except where the last centrifugation after clearing with ammonium chloride, is omitted, or where on account of high turbidity the natural sediment occupies a larger volume. In these cases due allowance must of course be made in the final calculation. When operating on 100 cc. samples, it is usually more convenient to proceed as above and to multiply the final result by ten, than to concentrate to one-tenth cubic centimeter in the tube, except in the case of a relatively clean water.

We have found cleanliness and protection from dust absolutely essential, but heat sterilization of the apparatus is unnecessary.

For counting, one of the various ruled cell slides, known as cytometers, may be used. We have used the haemacytometer of Thoma-Zeiss with good results. In this the capacity of the ruled portion is one-tenth cubic millimeter and this is ruled into twenty rows of squares of twenty squares each. We have however had equally good or better results with a cell made by evenly grinding a circular depression into a fairly thick microscope slide, about 10 mm. in diameter and exactly 0.1 mm. deep, which latter may be measured by the micrometer head of the fine adjustment of the microscope. A slight notch filed in one side allows any excess of fluid to escape. In using it, a large drop of the well mixed 1 cc. concentrate is placed in the cell, covered by firmly pressing down a perfectly plane coverglass, and the bacteria in the entire microscope field counted. A Sedgwick-Rafter micrometer, ruled in squares and placed in the eyepiece will greatly assist in counting where organisms are numerous. The proper regulation of the light coming from the condenser is highly important for seeing the bacteria clearly, and a dark ground illuminator is also sometimes useful. The capacity of each standard microscope field is determined once for all from the depth, and diameter as measured by a stage micrometer. With the microscope which we most frequently use, a 4 mm. objective and 25 mm. ocular, giving a magnification of about four hundred and fifty diameters, each field has a capacity of exactly one one hundred and thirtieth cubic millimeter. Usually from ten to fifty fields, the more the better, should be counted, on from two to five different mounts, the average calculated, and, in this case, multiplied by one hundred and thirty. The number to be counted will depend largely on whether the water has a high or low bacterial content, and the relative uniformity of the counts on the individual fields. An experienced observer will usually count a standard field in from two to sixty seconds, and perhaps ten seconds may be taken as a general average time. Using the centrifugal method of concentrating, it is usually possible to complete a count on a water sample in from twenty to forty minutes. It is even possible to obtain rough approximate results by counting a number of fields in ordinary small hanging drops, the capacity of each field being measured by the diameter and measured optical penetration of the lens. By this last method, after once selecting a field, the focus of the microscope must not be changed while counting it. The errors by this last method are chiefly due to the difficulty of keeping a uniform depth of field, and to the tendency of suspended matters to settle to the bottom of the drop and so out of the field of view, taking the bacteria with them. The results on two different samples of water, using the three methods of counting, are given herewith:

	Agar plate.	Thoma-Zeiss.	Standard Filed.	Hanging drop.
River	1100	1800	2000	1300
	1100	1900	1900	900
Filtered	15	22	22	18
	15	25	20	10

Counts of different microscope fields from the same sample of raw concentrated polluted water are illustrated by the following: 24, 28, 55, 130, 16, 85, 163, 44, 27, and 18, average 59, or 7670 per cubic centimeter. With a clean water most of the fields of course contain no bacteria at all. The variations are chiefly due to the occurrence of clumps of bacteria in certain fields.

To test the method in the hands of different observers, counts on the same sample of water were made by three different chemists using the standard field, as follows:

Agar plate.	A.	B.	C.
550	810	735	840

The counts on natural waters are as a rule more accurate than on test samples artificially treated with bullion cultures of bacteria, as in the former the organisms often occur largely attached to colloidal pabula, rather than freely natatorial as in the latter.

A few of many comparisons of the complete cultural method and direct counting are given below:

Sample.	Agar plates.	Gelatin plates.	Direct.
River	800	650	2100
	650	550	900
	450	500	960
	9000	Liq.	16,000
	500	. .	900
Filtered	12	10	25
	18	12	30
	10	10	20
	30	. .	50
	35	. .	45
Reservoir	1300	1400	2000
Ditch, turbid	15,000	Liq.	100,000
Well, shallow	12	8	15
Wells, deep	7	. .	30

Proficiency in the technique of direct bacterial counts requires patience and some painstaking care and experience which, as in other similar work, may only be had by actually working with the methods, but it is believed that even the beginner, if careful, may obtain results directly which are fully more accurate than those obtained by cultural methods.

Conclusions.

1. Direct microscopical counts of bacteria in water may be made in from twenty to forty minutes, and allow of the examination of other microörganisms at the same time.

2. In our experience these counts are fully more accurate than those by the plate method, although not directly comparable with them.

3. The centrifugal method of concentrating the bacteria after "flocking," is the most rapid, but is less efficient and accurate than filtration.

4. The morphological character of the organisms themselves, rather than the appearance of their growth, is considered in judging their possible general characters.

5. These total counts are in no sense intended to supersede the specific cultural and other tests for B. coli or other individual groups of organisms.

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NEW BOOKS.

The Organism as a Whole from a Physicochemical Viewpoint. By JACQUES LOEB, Member of the Rockefeller Institute for Medical Research. New York and London: G. P. Putnam's Sons. 1916. Price, $2.50.

The fact that living organisms, always highly complex and in many respects unstable systems (besides being subject to continual chemical change), nevertheless maintain and perpetuate themselves in their appropriate environments, each preserving its characteristic unity and individuality, furnishes a problem of unexampled difficulty to the physical scientist. Each animal develops from an apparently undifferentiated germ, the fertilized egg-cell, by means of a most complex yet orderly transformation of material taken from egg and surroundings; in every case both structure and activities show a striking correspondence with —or "adaptation to"—certain features of the surroundings; and most animals behave as if actuated by purpose or intelligence. Can these peculiarities, which the organism, considered as a whole, typically shows, be adequately interpreted and accounted for by the methods of physical science? This is the question which Professor Loeb discusses in the interesting book before us. In general his answer is a decided and enthusiastic affirmative. He sees biology as a department of physical science, and living organisms as physicochemical systems of a special kind; he holds that biology should aim at quantitative exactitude in the description and analysis of vital processes; only in this way can the possibilities of prediction and control (the criteria of true science) be realized; biology is a science "only to the extent that it succeeds in reducing life-phenomena to quantitative laws;" in many cases this is already possible, and unlimited further progress in this direction may be hoped for. Professor Loeb's discussion of the light-reactions of animals—a field in which much of his best known work has been done—illustrates his method and point of view with especial clearness. The heliotropic reactions of many animals may be classed as elementary instincts; in some cases, e. g., the