CHANGES IN THE BACTERIAL FLORA OF SEWAGE DURING STORAGE.*

C.-E. A. WINSLOW AND D. M. BELCHER.

(*From the Biological Laboratory of the Massachusetts Institute of Technology.*)

I. INTRODUCTION.
II. METHODS.
1. Technic of isolation.
2. System of classification.
3. Methods of studying cultures.
III. RESULTS.
1. Total number on various media.
2. Numbers in the separate groups.
3. Special intestinal forms.
a) Anaërobes.
b) B. coli group.
c) Streptococci.
d) Other special groups.
IV. CONCLUSIONS.
V. REFERENCES.

I. INTRODUCTION.

BACTERIA, like other living things, are within narrow limits adapted to their environment; and in general the different kinds of micro-organisms will be found in fullest activity only under certain rather definite conditions. Each disease has its specific microbe, each different process of decomposition or fermentation is carried on by its own group of bacteria, and water, air, and milk have their more or less constant flora. *A priori*, we might expect to find the same law operating in regard to sewage. To quote from Jordan (1), one of the earliest American investigators of this subject: "The sewage itself—a nutritive medium of varying composition and richness — will contain only those species capable of living and holding their own in the continual struggle for existence. So far as the conditions of life in sewage differ from conditions of life elsewhere, so far will the sewage be inhabited by species peculiarly adapted to those conditions; just as we find, for example, that, among the multitude of bacteria

*Received for publication October 28, 1903.

Downloaded from http://jid.oxfordjournals.org/ at University College London on June 5, 2016

taken into the human alimentary canal, only a few species normally find fit conditions for development."

So Laws and Andrewes (4) recognized the fact that sewage, especially when stored, might contain certain characteristic forms. "Sewage may be a favorable medium for the growth of one species, an unfavorable one for another. The latter will, therefore, tend to disappear from older sewage, which will contain larger and larger numbers of those particular organisms for which it is a suitable soil." Such a process as is here postulated has been studied in the case of milk by H. W. Conn and W. M. Esten (15), with very striking results. They find that, on allowing milk to stand at the room temperature, there is an enormous increase in the total number of bacteria present, which at first are of many different kinds. Gradually, however, the percentage of lactic acid forming bacteria rises, until often it reaches as high as 99 per cent. Thus in the struggle for existence those best fitted to survive and multiply have overrun the rest, and there results an almost pure culture of a single group.

Experimental evidence as to the existence of true sewage bacteria in any such sense as this has, heretofore, been meager. Numerous observers have recorded the total number of bacteria present in crude sewage; but without reference to their kinds. Laws and Andrewes (4), for example, report from two to eleven million per cubic centimeter in various London sewages, while Clowes (9) found three million and a half at the Crossness outfall, and nearly four million at Barking. The number of bacteria in American sewage is somewhat less. The average of the bacteriological analyses of the Lawrence Street sewage from 1894 to 1901, as published in the annual reports of the Massachusetts State Board of Health, was 2,800,000; and two twenty-four hour examinations of Boston sewage, made at the Sanitary Research Laboratory of the Massachusetts Institute of Technology during the summer of 1903, gave an average of 3,660,000. It is also well recognized that when sewage is stored under favorable conditions these numbers are greatly increased. Laws and Andrewes (4) found 33,800,000 bacteria per cubic centimeter after three days' storage of London sewage in a glass bottle and an interest-

Downloaded from http://jid.oxfordjournals.org/ at University College London on June 5, 2016

ing series of chemical and bacteriological analyses of stored sewage, made by the Massachusetts State Board of Health in 1894(3), showed the relations still more clearly. The bacteriological results of the experiment were as follows:

TABLE I.

Number of Bacteria per Cubic Centimeter in Fresh and Stored Sewage (*XXVI. Ann. Rep. Mass. S. B. H.* for 1894).

Day	Hour	Bacteria	Day	Hour	Bacteria
Mar. 11	10:30 A. M.	1,190,000	Mar. 13	10:30 A. M.	12,810,000
11	12:30 P. M.	1,085,000	14	10:30 A. M.	11,235,000
11	3:00 P. M.	1,505,000	15	10:30 A. M.	6,825,000
11	6:00 P. M.	1,530,000	16	10:30 A. M.	4,485,000
12	8:00 A. M.	20,475,000	18	10:30 A. M.	3,420,000
12	12:00 M.	23,100,000	19	10:30 A. M.	2,341,000
12	5:00 P. M.	20,000,000			

This examination shows a marked increase in the total number of bacteria up to twenty-five and one-half hours, at which time there were twenty times as many bacteria present as in the fresh sewage, followed by a gradual decrease in numbers to the end of the week.

The figures quoted throw no light, on the nature of the organisms present; and, on the other hand, those observers who have isolated and described certain species of bacteria found in sewage have usually ignored the relative preponderance of the different types. Jordan (1) at Lawrence, Roscoe and Lunt (2), Laws and Andrewes (4), and Clowes and Houston (10) in England have recorded some fifty species from this source. The last two papers cited, as well as the first report of Clowes (9) to the London County Council, contain, some figures which are both qualitative and quantitative as regards certain intestinal bacteria, liquefiers, anaërobes, etc.; and Klein and Houston in the reports of the Local Government Board (8), (11), (12) and of the Royal Sewage Commission (22), (23) give numerous determinations of the numbers of colon bacilli, streptococci, and spore-bearing anaërobes. These investigators have, however, only studied certain special organisms in fresh sewage; so far as we are aware, no attempt has

Downloaded from http://jid.oxfordjournals.org/ at University College London on June 5, 2016

been made to gain a general idea of the relation of all the different groups of bacteria in that and of the change in relation during storage.

Information of this nature would seem to have a practical as well as a theoretical value in view of the complex character of the processes of sewage disposal as they have been developed during recent years. The so-called "bacterial methods" of sewage purification are attempts to intensify the process which goes on in an ordinary sand filter, by dividing it into stages, in each of which a special group of bacteria may find conditions especially favorable to its activity. Thus Rideal (18) distinguishes three distinct stages—the anaërobic, the semi-anaërobic, and the aërobic—carried out respectively in the septic tank and the first and second contact filters. Obviously, under such conditions, an exact knowledge of the organisms concerned is highly desirable; but although we know some of the characteristics of the nitrifying organisms associated with the two later stages of purification, of the anaërobic forms which work in the septic tank we are almost wholly ignorant.

It seemed to the authors that a quantitative study of the groups of bacteria in fresh and stored sewage, especially if any tendency to the formation of a pure culture should appear, might throw important light on the forms active in sewage purification. Furthermore, the fate of the more typical intestinal bacteria in stored sewage is of interest to the student of the etiology of the infectious diseases. This examination of the bacterial flora of sewage was, therefore, undertaken with two ends in view—to determine whether any groups of bacteria multiply in stored sewage in such fashion as to be considered characteristic of it, and whether parasitic organisms find that medium favorable or inimical to their development.

II. METHODS.

1. *Technic of isolation.*—The samples of sewage studied in this investigation, six in number, were collected at different times during the winter of 1902–3 from one of the smaller Boston sewers through a manhole on the Dartmouth Street side of Cop-

Downloaded from http://jid.oxfordjournals.org/ at University College London on June 5, 2016

ley Square. The contributing area of some eight acres includes three large hotels, besides residences and apartment houses, and has a population of probably a thousand persons. A very fresh domestic sewage was thus obtained.

The samples were collected by lowering a sterile pail into the sewer and filling from this a sterile gallon bottle about three-fourths full. The latter was immediately taken to the laboratory and the analysis of the fresh sewage begun within one-half hour of the time of collection. The sewage was then stored in the gallon glass bottle with a tight glass stopper, in the dark, at room temperature, and analyses were made at different periods of storage —as twelve, twenty-four, forty-eight hours, seven days, and a month.

Conn and Esten (15), in the work quoted above, based their determination of the groups of bacteria merely on the cultural differences exhibited by colonies on the litmus-lactose-gelatin plate. Our attempt has been to carry out a more exhaustive study by plating the sewage in great dilution (about twenty colonies appearing on the plate), so that all the colonies could be isolated and worked out in detail. To assume that such a small number of bacteria selected at random will be strictly representative of the great multitude present in the sewage is, of course, erroneous. The greatest care was exercised, however, to secure a thorough mixture before sampling; and the concordant results obtained appear to justify the general accuracy of the method.

The agar streak cultures obtained from the original plate were at once examined in sub-cultures without the preliminary cultivation, recommended by so many observers—as by Fuller and Johnson (14), and Jordan (24), in their work on the classification of water bacteria. In many cases this is undoubtedly a valuable help, since thus differences in past environment may be eliminated; but in our work it was desired to isolate the different forms in the condition in which they occurred in the sewage as nearly as possible.

In each experiment samples of sewage were plated in parallel under aërobic and anaërobic conditions. For the former purpose standard nutrient gelatin was used, made up according to the recommendation of the Committee on Standard Methods of Water

Downloaded from http://jid.oxfordjournals.org/ at University College London on June 5, 2016

Analysis of the American Public Health Association (19), and the plates were kept at room temperature as long as possible without having the liquefiers overrun other colonies and thus prevent the isolation of pure cultures. We planned at first to make a series of parallel isolations using lactose agar at 37°, since in an earlier investigation very high numbers of streptococci had been found by this process in the effluent of a septic tank. Preliminary experiments, however, did not indicate any marked difference in the sorts of bacteria developing from raw sewage on these two media. For Samples I, III, and IV, therefore, only gelatin plates were made. For Samples II and V total counts were also obtained on lactose agar at 37°, but the gelatin plates were used for qualitative studies. In the case of Sample VI, plates were made on gelatin, and lactose agar at 37° and on agar at 37°, for total counts; and the lactose agar plates were used for the isolation of the subcultures to be examined.

For cultivation under anaërobic conditions, neutral agar was used instead of the ordinary agar made up with an acidity of 1 per cent., since Dr. J. H. Wright has pointed out that this is more favorable for the development of strict anaërobes. After some experimentation with Buchner's tube and with the glass-plate method of Sanfelice, as described by Hunziker (21), the method suggested by Wright (16) was found to be most satisfactory. After pushing a plug of sterile cotton one-third way down an ordinary culture tube, dry pyrogallic acid was poured in, a few centimeters of strong caustic hydrate added, and the whole sealed with a rubber stopper. Instead, however, of rolling the tubes after the fashion of Esmarch, it was found simpler and more satisfactory to lay them in a horizontal position and allow the agar to set in a flat layer a quarter of an inch thick. After incubation this slab of agar could be slid out of the tube entire and streaks made from the colonies with ease. Two cultures were made from each colony, one under aërobic, one under anaërobic conditions. If development occurred on the former streak, the organism was not further studied, since facultative forms were presumably represented on the gelatin plate, and this process was intended only to detect obligate anaërobes.

Downloaded from http://jid.oxfordjournals.org/ at University College London on June 5, 2016

2. *System of classification.*—The elaborate study of minute specific differences was obviously impossible in a work of this character, and of doubtful advantage even if feasible. There is much truth in the suggestion of Marshall Ward (7) that "the 'species' of the descriptive handbooks—principally medical—are frequently not species at all, in the botanical sense, but varieties, or growth-forms, the distinctive characters of which are not constant. These so-called species need revision and grouping around types, which may turn out to be the true species." We have, therefore, only attempted to place the organisms isolated in certain large and well-marked groups. Flügge (6) laid the foundation for a natural classification of the bacteria; Ward (7), Fuller and Johnson (14), Weston and Kendall (20), and Jordan (24) for water bacteria, and Conn (13) for milk bacteria, have developed more detailed groupings. In our work we adopted the system of Fuller and Johnson, with certain modifications. The main difference consists in the recognition of morphological characters; we have included groups for the cocci and spirilla, have characterized the B. subtilis group by the presence of spores on potato, and have separated the non-fluorescent, non-chromogenic, non-liquefying bacteria into the B. coli and B. aërogenes series according to motility. The system as thus modified is as follows:

DEFINITION OF GROUPS.

Group I. Spirillum undula type. All spirilla.

Group II. Streptococcus pyogenes type. All cocci.

Group III. B. fluorescens type.
- *a*) Fluorescent forms, liquefying gelatin.
- *b*) Fluorescent forms, not liquefying gelatin.

Group IV. Chromogenic forms.
- *a*) Yellow and orange; B. subflavus type.
- *b*) Red; B. prodigiosus type.
- *c*) Violet; B. janthinus type.
- *d*) Brown; B. brunneus type.

Group V. Spore-bearing bacilli.
- *a*) Aërobes; B. subtilis type.
- *b*) Anaërobes; B. sporogenes type.

Group VI. B. vulgaris type—all non-spore-forming, non-fluorescent, non-chromogenic gelatin-liquefying bacteria forming proteus-like colonies on gelatin.

Downloaded from http://jid.oxfordjournals.org/ at University College London on June 5, 2016

Group VII. B. cloacae type—all non-spore-forming, non-fluorescent, non-chromogenic bacilli, not forming proteus-like colonies liquefying gelatin and fermenting dextrose with the formation of gas.

Group VIII. B. liquidus type—same as Group VII, except that dextrose is fermented without the formation of gas.

Group IX. B. superficialis type—same, except that no fermentation of dextrose occurs.

Group X. B. coli type—all non-spore-forming, non-fluorescent, non-chromogenic, non-liquefying bacilli, which are motile and ferment dextrose with the formation of gas.

Group XI. B. typhi type—same as Group X, except that fermentation of dextrose occurs without formation of gas.

Group XII. B. candicans type—same, except that no fermentation of dextrose occurs.

Group XIII. B. aërogenes type—all non-spore-forming, non-fluorescent, non-chromogenic, non-liquefying, non-motile bacilli, which ferment dextrose with the formation of gas.

Group XIV. B. ubiquitus type—same as Group XIII, except that fermentation of dextrose occurs without the formation of gas.

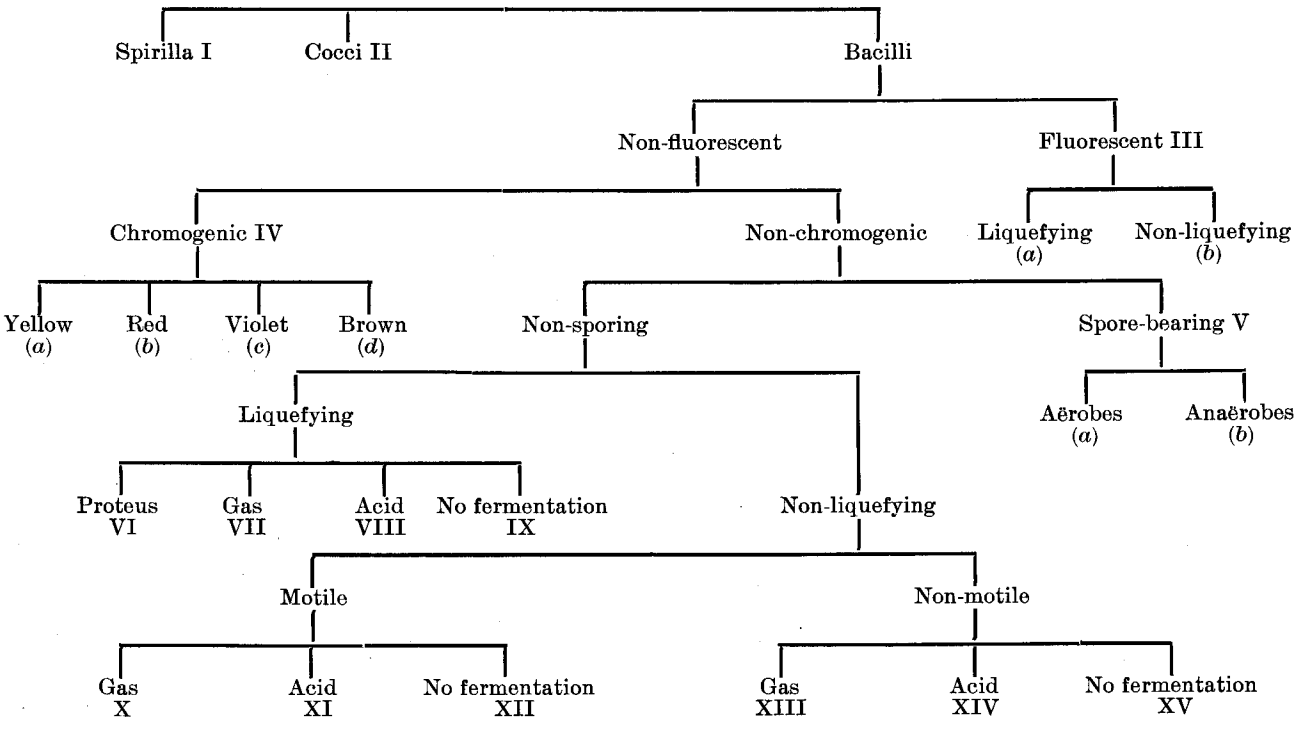
Group XV. B. rhinoscleromatis type—same, except that dextrose is not fermented.

The relation of the various groups is better shown in diagrammatic form on following page.

3. *Methods of studying cultures.*—The classification outlined above necessitated the use of four different media—agar, gelatin, potato, and dextrose broth—all prepared according to the standard procedure of the Committee on Water Analysis of the A. P. H. A. The cultures, first isolated on agar streaks, were kept at 20° as stock cultures for the inoculation of other media, and upon them the general morphology of the organism and its fluorescence were observed. Next a potato culture was made for chromogenesis, and this was examined after a week's incubation at 20° for spores. Thirdly, a gelatin stab furnished information as to the characteristic form of surface growth and the presence or absence of liquefaction, the tubes being kept ten days for the observation of the latter character. Finally a dextrose tube incubated for three days at 37° (all the bacteria tested for gas production grew well at this temperature) revealed the fermentative powers of the organism.

Downloaded from http://jid.oxfordjournals.org/ at University College London on June 5, 2016

TABLE II.



Downloaded from http://jid.oxfordjournals.org/ at University College London on June 5, 2016

The separation of the B. coli and B. aërogenes series proved the most troublesome feature of the examination. According to Flügge, the distinction lies in the fact that organisms of the former type are motile and produce an expanded disk-like surface growth on gelatin, while B. aërogenes is non-motile and forms small drop-like colonies. We found, however, that no constant relation existed between these characteristics, and thereafter laid stress only on the motility, as determined by examination of an agar streak cultivated for twenty-four hours at 37°. In most of our experiments the cultures were subjected to preliminary cultivation in three successive broth tubes before this determination. It would be an obvious convenience if in some medium the motility of an organism could be determined without resorting to the microscope; and we hoped that this might be accomplished by the use of a low-percentage gelatin having a lower viscosity than ordinary gelatin. In a gelatin stab the motile forms might be expected to spread through the medium, while the others would be confined to a growth in the region of the needle. It was found that 2 per cent. gelatin just remained solidified at the room temperature, and this was used for experiment. When the stabs were kept in the ordinary position, no difference was noticed between the motile and the non-motile forms. When, however, the tubes were turned upside down, it was found that the motile organisms tended to spread diffusely upward through the gelatin, while the non-motile forms gave ordinary needle growths. Experiments with this medium were begun only toward the close of our work, and they were not carried far enough to warrant definite conclusions.

III. RESULTS.

1. *Total number on various media.*—The general results of the counts made upon the various media are shown in Table III.

In each case, except Sample III, where the maximum was reached in twelve hours with a slight falling off in twenty-four hours, there was an increase in total numbers up to twenty-four hours, and then a steady decrease up to at least six months. The number of facultative anaërobes was determined from the anaërobic agar tubes, those colonies isolated from this source and developing

Downloaded from http://jid.oxfordjournals.org/ at University College London on June 5, 2016

on the aërobic agar streak being classed under this head. These behaved in much the same way as did the bacteria on the gelatin plate, except that they reached a maximum at about forty-eight instead of twenty-four hours and did not fall off so rapidly as the aërobes.

TABLE III.

Number of Bacteria per Cubic Centimeter in Fresh and Stored Sewage.

Sample	Storage	Gelatin at 20°	Lactose Agar at 37°	Agar at 37°	Anaërobic Agar
I.....	Fresh	300,000	.....	.....	.....
II.....	Fresh	1,200,000	125,000	.....	.....
III.....	Fresh	500.000	.....	.....	130,000
	12 hours	4,200,000	.....	.....	720,000
	24 hours	3,000,000	.....	.....	1,200,000
	7 days	650,000	.....	.....	500,000
	28 days	64,000	.....	.....	10,000
	5½ months	23,000	.....	1,700	240
IV.....	Fresh	800,000	.....	.....	140,000
	12 hours	2,200,000	.....	.....	1,700,000
	24 hours	13,500,000	.....	.....	1,900,000
	30 days	80,000	.....	.....	32,000
V.....	Fresh	3,100,000	150,000	.....	150,000
	24 hours	23,000,000	2,800,000	.....	2,600,000
	48 hours	3,600,000	2,815,000	.....	3,400,000
	72 hours	2,300,000	500,000	.....	900,000
	7 days	360,000	.....	.....	800,000
VI.	Fresh	1,550,000	180,000	500,000	.....
	24 hours	17,600,000	4,000,000	16,700,000	.....
	7 days	695,000	220,000	400,000	.....

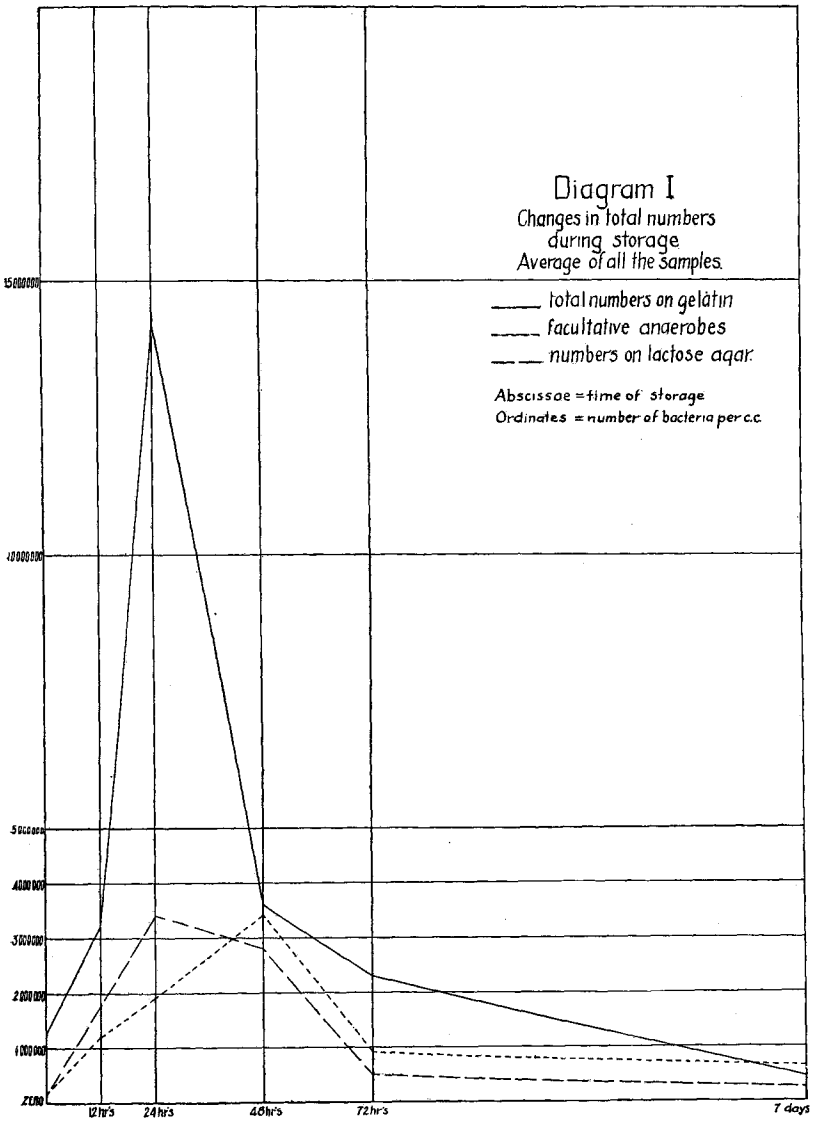
The figures for the different periods, obtained by averaging all the samples are given in Table IV, and the relations are shown graphically in Diagram I.

TABLE IV.

Number of Bacteria per Cubic Centimeter in Fresh and Stored Sewage (Average of All Samples).

Storage	Gelatin at 20°	Lactose Agar at 37°	Agar at 37°	Anaërobic Agar
Fresh	1,240,000	151,000	500,000	140,000
Twelve hours.....	3,200,000	.....	.....	1,210,000
Twenty-four hours.....	14,200,000	3,400,000	16,700,000	1,900,000
Forty-eight hours.....	3,600,000	2,815,000	.....	3,400,000
Seventy-two hours.....	2.300,000	500,000	.....	900,000
Seven days.....	440,000	220,000	400,000	650,000
Twenty-eight to thirty days.....	72,000	.....	.....	21,000
Five and one-half months	23,000	.....	1,700	240

Downloaded from http://jid.oxfordjournals.org/ at University College London on June 5, 2016



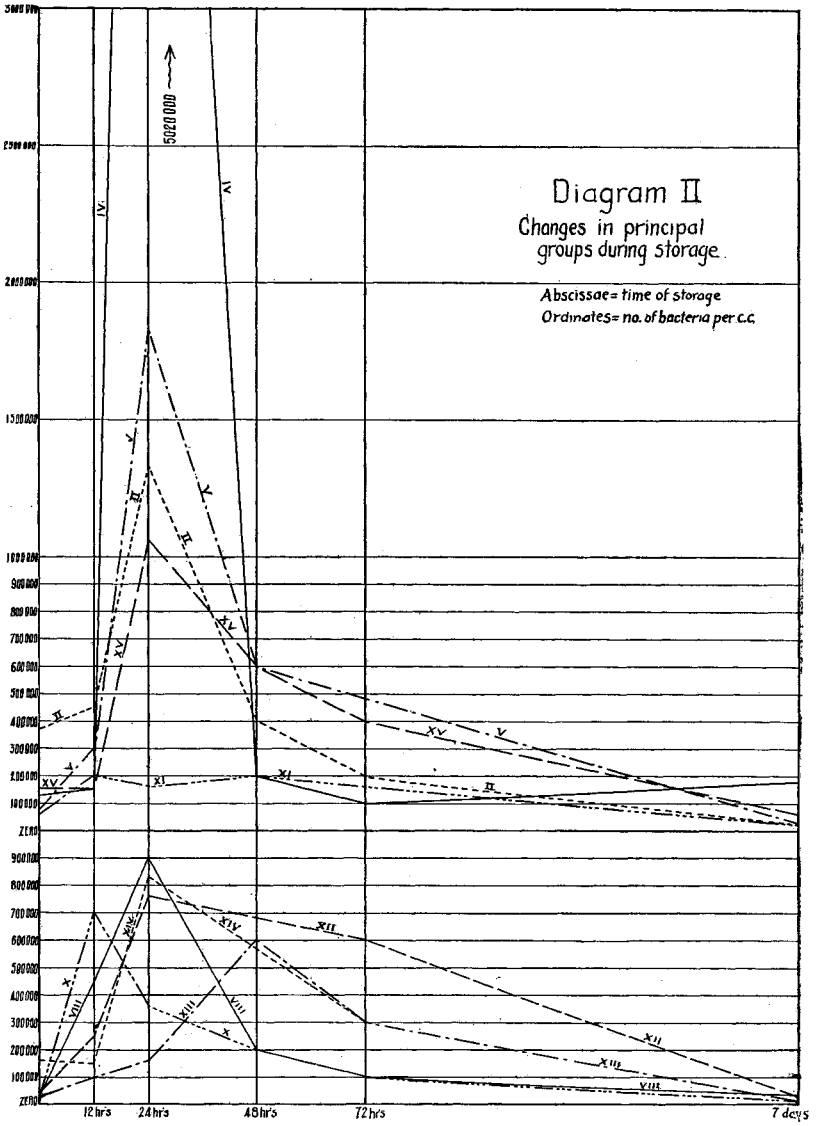
Downloaded from http://jid.oxfordjournals.org/ at University College London on June 5, 2016

The sharp rise, with a subsequent more gradual decline, is well marked in all the curves of Diagram I; and, as in the Lawrence experiment cited above, the maximum number of bacteria was reached after about twenty-four hours. Again, it is evident by a comparison of the curves that during the first twenty-four hours the multiplication was mainly confined to the strictly aërobic bacteria, since the number of anaërobes remained comparatively insignificant. The latter, however, continued to multiply after the total number had begun to fall off and reached their maximum in forty-eight hours; at that and all subsequent periods the proportion of anaërobes was a considerable one. This corresponds with the common idea that the first fermentation of sewage is aërobic, but that the facultative anaërobes become more and more important as the oxygen is consumed.

2. *Numbers in the separate groups.*—The bacteria isolated from the various samples of sewage are classified by groups in Table V. With the exception of the violet chromogenes, the strict anaërobes, and the proteus organisms, all our groups were represented, and no one form appeared specially predominant either in the raw or septic sewage. We had expected that some one or two types might show a disproportionate increase as the sewage aged, but this appeared not to be the case. Table VI, where the number of bacteria in each group is expressed in its percentage of the total number, makes this still more clear. With the exception of a preponderance of the B. coli group in Sample VI, the various types of bacteria occurred in fairly equal proportions, the occasional deviation being within the limits of error due to the difficulty of securing an average sample.

Table VII has been compiled by averaging the figures obtained for the more important groups in the analysis of the first five samples, Sample VI having been omitted since the use of lactose agar at 37° as the medium for isolation made the latter not strictly comparable. Diagram II shows the relation of the different types of bacteria during the first week of storage in graphic form. The individual groups in almost every case show the same tendency as the total numbers; that is, a steady increase up to twenty-four hours, and then a gradual falling off until, at the end of the week, the

Downloaded from http://jid.oxfordjournals.org/ at University College London on June 5, 2016



Downloaded from http://jid.oxfordjournals.org/ at University College London on June 5, 2016

TABLE V.

Bacteria per Cubic Centimeter in Each Group.

(Last three ciphers omitted.)

Date of Collection	Sample	Storage	Dilution	Total Number	I	II	III(a)	III(b)	IV(a)	IV(b)	IV(c)	IV(d)	V(a)	V(b)	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV	Yeasts, Molds, etc.
							Bacilli																		
							Fluo-rescent		Non-fluorescent																
									Chromogenic				Non-chromogenic												
													Spore-Bear'g		No Spores										
															Liquefying Gelatin				Non-liquefying						
																Non-proteus									
																			Motile			Non-motile			
					Spirilla	Cocci	Liquefying	Non-liquefying	Yellow	Red	Violet	Brown	Aërobic	Anaërobic	Proteus	Gas	Acid	No Ferm.	Gas	Acid	No Ferm.	Gas	Acid	No Ferm.	
Oct. 8....	I	Fresh	10	300	....	50	....	.....	20	....	....	30	80	....	....	....	....	....	40	....	....	30	10	20	.....
24....	II	Fresh	50	1200	50	700	....	50	50	....	....	....	100	....	....	....	....	....	50	50	50	....	....	50	.....
Nov. 19..	III	Fresh	20	500	....	160	....	.....	20	....	....	20	60	....	....	....	....	....	.....	....	20	120	....	60	.....
		12 hours	200	4200	....	400	....	.....	.....	....	....	....	400	....	....	200	600	....	1000	400	400	....	200	200	.....
		24 hours	200	3000	....	400	....	.....	200	....	....	400	....	....	....	....	200	....	600	....	800	....	....	200	.....
		7 days	50	650	....	....	....	.....	.....	150	....	150	50	....	....	....	50	50	.....	....	50	....	....	100	1 mold
		28 days	4	64	....	....	4	8	4	....	....	12	4	....	....	....	4	....	.....	4	16	....	....	....	.....
		5½ months	1	23	....	1	....	.....	.....	2	....	1	....	....	....	....	2	2	.....	1	3	....	2	5	.....
Dec. 10 ..	IV	Fresh	50	800	....	150	....	.....	.....	....	....	100	150	....	....	....	50	....	50	50	50	....	....	....	1 yeast
		12 hours	100	2200	....	500	....	.....	.....	....	....	100	200	....	....	100	300	100	400	....	100	200	100	100	.....
		24 hours	500	13500	....	1000	....	.....	.....	4500	....	....	500	....	....	....	500	1500	500	500	500	500	500	3000	.....
		30 days	5	80	5	5	....	5	.....	5	....	....	....	....	....	....	10	10	.....	5	5	....	10	10	.....
Mar. 4...	V	Fresh	100	3100	....	800	....	.....	200	....	....	200	....	....	....	....	100	100	.....	200	100	....	800	600	.....
		24 hours	1000	23000	....	2000	....	.....	10000	....	....	....	5000	....	....	....	2000	....	.....	....	1000	....	2000	....	1 mold
		48 hours	200	3600	200	400	....	200	.....	....	....	200	600	....	....	200	200	....	200	200	....	600	....	600	.....
		72 hours	100	2300	....	200	....	.....	.....	....	....	100	....	....	....	....	100	100	100	....	600	300	300	400	1 yeast
		7 days	20	360	....	40	....	.....	20	40	....	....	....	....	....	20	20	....	20	40	....	20	....	20	.....
Apr. 9 ...	VI	Fresh	10	180	....	40	....	.....	.....	....	....	....	....	....	....	....	....	....	80	10	20	30	....	....	.....
		24 hours	200	4000	....	....	....	.....	.....	....	....	....	....	....	....	....	....	....	2200	800	....	800	....	....	.....
		7 days	10	220	....	....	....	.....	.....	....	....	....	....	....	....	....	....	....	110	10	10	60	20	10	.....

Downloaded from http://jid.oxfordjournals.org/ at University College London on June 5, 2016

TABLE VI.

Per Cent. of Bacteria in Each Group to Total Number.

Date of Collection	Sample	Storage	Total Number	I	II	III(a)	III(b)	IV(a)	IV(b)	IV(c)	IV(d)	V(a)	V(b)	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV	Yeasts, Molds, etc.
						Bacilli																		
						Fluorescent		Non-fluorescent																
								Chromogenic				Non-chromogenic												
												Spore-bearing		No Spores										
														Liquefying Gelatin				Non-liquefying						
															Non-proteus			Motile			Non-motile			
				Spirilla	Cocci	Liquefying	Non-liquefying	Yellow	Red	Violet	Brown	Aërobic	Anaërobic	Proteus	Gas	Acid	No Ferm.	Gas	Acid	No Ferm.	Gas	Acid	No Ferm.	
Oct. 8.....	I	Fresh	100	....	16.5	....	.....	6.5	.....	....	10.0	25.0	....	....	....	.....	.....	13.0	.....	.....	10.0	3.0	6.5	....
24.....	II	Fresh	100	4.0	57.5	....	4.0	4.0	.....	....	.....	8.0	....	....	....	.....	.....	4.0	4.0	4.0	.....	.....	4.0	....
Nov. 19....	III	Fresh	100	....	32.0	....	.....	4.0	.....	....	4.0	12.0	....	....	....	.....	.....	.....	.....	4.0	24.0	.....	12.0	....
		12 hours	100	....	9.5	....	.....	...	.....	....	.....	9.5	....	....	4.5	14.0	.....	24.0	9.5	9.5	.....	4.5	4.5	....
		24 hours	100	....	13.0	....	.....	6.5	.....	....	13.0	.....	....	....	....	6.5	.....	20.0	.....	27.0	.....	.....	6.5	....
		7 days	100	....	.....	....	.....	...	25.0	....	25.0	7.5	....	....	....	7.5	7.5	.....	.....	7.5	.....	.....	14.0	7.5
		28 days	100	....	.....	6.0	12.0	6.0	.....	....	18.0	6.0	....	....	....	6.0	.....	.....	6.0	25.0	.....	.....	.....	....
		5½ months	100	....	4.5	....	.....	....	9.0	....	4.5	.....	....	....	....	9.0	9.0	.....	4.5	13.0	.....	9.0	21.5	6.0
Dec. 10....	IV	Fresh	100	....	18.5	....	.....	....	.....	....	12.5	18.5	....	....	....	6.0	.....	6.0	6.0	6.0	.....	.....	.....	6.0
		12 hours	100	....	22.5	....	.....	....	.....	....	4.5	9.0	....	....	4.5	13.5	4.5	18.0	.....	4.5	9.0	4.5	4.5	....
		24 hours	100	....	7.5	....	.....	....	30.0	....	.....	3.5	....	....	....	3.5	11.0	3.5	3.5	3.5	3.5	3.5	22.0	....
		30 days	100	6.0	6.0	....	6.0	....	6.0	....	.....	.....	....	....	....	12.0	12.0	.....	6.0	6.0	.....	12.0	12.0	....
Mar. 4	V	Fresh	100	....	26.0	....	.....	6.5	.....	....	6.5	.....	....	....	....	3.0	3.0	.....	6.5	3.0	.....	25.0	18.0	....
		24 hours	100	....	8.5	....	.....	43.0	.....	....	.....	20.0	....	....	....	8.5	.....	.....	.....	4.5	.....	8.5	.....	4.5
		48 hours	100	5.5	11.0	....	5.5	....	.....	....	5.5	16.5	....	....	5.5	5.5	.....	5.5	5.5	.....	16.5	.....	16.5	....
		72 hours	100	....	8.5	....	.....	....	.....	....	4.5	.....	....	....	.....	4.5	4.5	4.5	.....	25.0	12.5	12.5	17.0	4.5
		7 days	100	....	11.0	....	.....	5.5	11.0	....	.....	.....	....	....	5.5	5.5	.....	5.5	11.0	.....	5.5	.....	5.5	....
Apr. 9.....	VI	Fresh	100	....	22.0	....	.....	....	.....	....	.....	.....	....	....	....	.....	.....	45.0	5.5	11.0	16.5	.....	.....	....
		24 hours	100	....	.....	....	.....	....	.....	....	.....	.....	....	....	....	.....	.....	55.0	20.0	.....	20.0	.....	.....	....
		7 days	100	....	.....	....	.....	....	.....	....	.....	4.5	....	....	....	.....	.....	50.0	4.5	4.5	26.0	9.0	4.5	....

Downloaded from http://jid.oxfordjournals.org/ at University College London on June 5, 2016

numbers are smaller than in the fresh sewage. Thus it is seen that the changes in the different groups are very nearly parallel and there is a close harmony between the various samples, the different groups occurring with about the same frequency in each. The chromogenic forms show the most marked development followed by the cocci and the spore-bearing aërobes. Group X reaches a maximum before the rest, Group XIII after; Group XI alone does not multiply appreciably. But, as far as our results go (and they are singularly concordant, when the possible sources of error are considered), there appears to be no tendency toward the predominance of any special form in stored sewage.

TABLE VII.

Number of Bacteria per Cubic Centimeter in Principal Groups (Average of All Samples).

(Last three ciphers omitted.)

Storage	II	IV	V (*a*)	VIII	X	XI	XII	XIII	XIV	XV
Fresh.....	372	128	74.5	30	28	60	44	30	162	154
12 hours....	450	150	300	450	700	200	250	100	150	150
24 hours....	1,330	5,020	1,830	900	360	160	760	160	830	1,060
48 hours....	400	200	600	200	200	200	....	600	....	600
72 hours....	200	100	....	100	100	....	600	300	300	400
7 days.....	20	180	25	35	10	20	25	10	....	60
1 month.....	2.5	10.5	2	7	....	4.5	10.5	....	5	5
5½ months..	1	3	....	2	....	1	3	....	2	5

3. *Special intestinal forms.*

a) *Anaërobes.*—We were at first surprised at the entire absence of obligate anaërobes from the tubes used for routine analysis, as described above, especially in view of the importance assigned to the B. sporogenes by the English sanitarians. Klein and Houston (8), (12) record from thirty to five thousand of these organisms per cubic centimeter in crude sewage. Houston (23) states that their number is usually 1,000 and not uncommonly 10,000 per cubic centimeter; and it might naturally be expected that on storage obligate anaërobes would multiply still further. We therefore examined measured portions of Samples III and IV by Klein's milk method. This consists, it will be remembered, in inoculating sterile milk with the material to be tested, heating to 80° for ten to fifteen minutes, to destroy vegetative cells, and

Downloaded from http://jid.oxfordjournals.org/ at University College London on June 5, 2016

incubating under anaërobic conditions for thirty-six hours at 37°. If B. sporogenes or similar spore-bearing anaërobes be present, the milk will be found changed in a characteristic manner. "The cream is torn or altogether dissociated by the development of gas, so that the surface of the medium is covered with stringy, pinkish-white masses of coagulated casein, inclosing a number of gas bubbles. The main portion of the tube formerly occupied by the milk now contains a colorless, thin, watery whey, with a few casein lumps adhering here and there to the sides of the tube. When the tube is opened the whey has a smell of butyric acid, and is acid in reaction. Under the microscope the whey is found to contain numerous rods, some motile, others motionless" (17).

The results of our examination were as follows:

SAMPLE IV.

Fresh.—Typical milk change given in dilutions 1:10 and 1:100, but not in 1:1,000; showing at least 100, but less than 1,000 spores per cubic centimeter.

Twelve hours' storage.—Typical change in dilutions 1:50, 1:100, and 1:1,000, but not in 1:10,000; showing at least 1,000 spores per cubic centimeter.

Twenty-four hours' storage.—Typical change in 1:50, 1:100, and 1:1,000, but not in 1:10,000; showing at least 1,000 spores per cubic centimeter.

Thirty days' storage.—Typical change not shown in 1:10 or 1:100; showing less than 10 spores per cubic centimeter.

SAMPLE III.

Five and one-half months' storage.—Typical change in both of two tubes inoculated with 1 c.c. each, in one out of three diluted 1:10, in one out of three diluted 1:100, but no change in three tubes at 1:1,000 dilution; showing between 10 and 100 spores per cubic centimeter.

It is obvious why the B. sporogenes group was not represented in our routine studies, since the facultative forms were so abundant that a dilution of 1:10,000 had to be used for isolation, and even as measured by the more sensitive milk test, the B. sporogenes group was never sufficiently abundant to be detected in such dilution. Furthermore, of the organisms giving Klein's typical change in milk, not all are obligate anaërobes. Some at least of those found in our investigation were facultative forms.

Downloaded from http://jid.oxfordjournals.org/ at University College London on June 5, 2016

b) *B. coli group.*—The organisms of the colon group are of special interest, both from their adoption by the water analyst as a measure of pollution, and from the probability that their behavior in sewage may throw some light on that of the typhoid bacillus under similar conditions. Klein and Houston (8) (12) record from 90,000 to 2,000,000 of these organisms in fresh sewage, and Houston (23) gives 100,000 as a normal number. Klein (5) states that "B. coli retains for a long time in sewage its vitality and its power of self-multiplication."

In most of our experiments this group of organisms was not found in such large numbers as we had expected. In two samples of fresh sewage they were absent (dilutions of 1 : 20,000 and 1 : 100,000), and in the other cases we found 40,000, 50,000, and 60,000 per cubic centimeter, respectively. Like the other bacteria, this group showed a rapid multiplication in the first twenty-four hours, followed by a decline rather more marked than was exhibited by most of the other groups. The maximum for the colon bacilli appeared to be reached earlier than in other cases, and in both Samples III and IV these organisms made up about a fifth of the total after twelve hours' storage. In Samples III, IV, and V, however, the B. coli group was only once represented at the end of a week, and not at all after a month.

Sample VI stands out in marked contrast to all the others, since the colon group made up half of the total at all the three periods of analysis, 110,000 per cubic centimeter being found even in the week-old sample. The use of lactose agar at 37° instead of gelatin at 20° as the medium for the original isolation of the organisms studied in this case may have affected this result; or it may simply represent a chance variation in the flora of the sample as collected.

c) *Streptococci.*—The importance of the streptococci as sewage organisms has been fairly well established in recent years since Laws and Andrewes (4) in 1894 found a small streptococcus to be the most abundant organism in several samples of hospital sewage. Houston (11) (22) records the number of streptococci in crude sewage as at least 1,000 per cubic centimeter, and in the

Downloaded from http://jid.oxfordjournals.org/ at University College London on June 5, 2016

last paper cited states that "they are delicate micro-organisms, and readily lose their vitality and die. They probably are little prone to enter on a saprophytic phase or to multiply to any great extent, if at all, under such conditions."

On the contrary, Horrocks (17) holds an opposite opinion, and declares that "the sewage streptococci appear to maintain their vitality in sewage for a much longer time than B. coli."

Our Group II, though including all spherical bacteria, was mainly made up of the sewage streptococci, only a few chromogenic staphylococci being found. As will be seen by reference to Table VI, it accounted for 18–57 per cent. of the bacterial content of the fresh sewage, and increased rapidly in the first twenty-four hours, though not retaining its original relative predominance. Cocci were found in appreciable numbers after thirty days in Sample IV and after five months in Sample III, apparently supporting the view of Horrocks rather than that of Houston.

d) *Other special groups.*—Most of the observers who have studied sewage bacteria record Proteus forms as abundant. Clowes and Houston (10), for example found in several cases at least 100,000 per cubic centimeter in fresh sewage. No organisms producing gas, liquefying gelatin, and forming the characteristic surface growth were, however, found in our investigation. In fact, there was a surprising scarcity of liquefying forms of any kind. The B. liquidus type (Group VIII) was indeed, one of the most constant forms in the stored sewage, but never rose to high numbers. B. fluorescens, sometimes—probably erroneously—described as a sewage form, was found only once in fresh and a very few times in the stored sewage.

On the other hand, the brown chromogenes (Group IV*d*), the B. subtilis type (Group V*a*) the B. typhi type (Group XI), the B. candicans type (Group XII), and the B. rhinoscleromatis type (Group XV) were constant inhabitants of the sewage, all but Group XI showing the typical change during storage which is common to all the groups.

Downloaded from http://jid.oxfordjournals.org/ at University College London on June 5, 2016

IV. CONCLUSIONS.

1. The bacteria in Boston sewage include a wide diversity of forms, the most noticeable groups represented being the cocci, the chromogenes, the B. subtilis group, the B. coli group, the B. aërogenes group, and the B. rhinoscleromatis group.

2. The total number of bacteria in stored sewage rises rapidly at first, increasing more than tenfold in twenty-four hours, and then decreasing for at least six months.

3. The maximum of the obligate aërobes is reached after twenty-four hours, after which they decrease rapidly, while the facultative anaërobes continue to increase up to forty-eight hours, and after that period maintain a predominance. Obligate anaërobes are at no time abundant.

4. The rise and fall of the bacteria in stored sewage appear to affect the various groups of organisms in an almost equal degree. All first increase and then decrease in number, the multiplication of the chromogene, the cocci, and the B. subtilis group being most marked.

5. Sewage does not appear to be a more unfavorable medium for the intestinal bacteria than for other forms. Organisms of the B. coli type, at first present to the number of some 50,000 per cubic centimeter, multiply with the others, and afterward may persist in considerable numbers up to seven days.

6. If this work is confirmed it would seem that there is no tendency toward the development of a pure culture of any dominant forms in stored sewage; and it seems questionable whether the term "sewage bacteria" signifies anything more than a chance mixture of organisms derived from a wide variety of sources.

In conclusion the authors desire to express their gratitude to Professor W. T. Sedgwick for his interest and advice in the prosecution of this work.

REFERENCES.

1. JORDAN, E. O. "A Report on Certain Species of Bacteria Observed in Sewage." *Special Report of the State Board of Health of Massachusetts on the Purification of Sewage and Water*, Part II, 1890, 821.

Downloaded from http://jid.oxfordjournals.org/ at University College London on June 5, 2016

2. ROSCOE, H. E. AND LUNT, J. "Contributions to the Chemical Bacteriology of Sewage." *Phil. Trans. of the Roy. Soc. of Lond.*, 1891, 182, 633.
3. FULLER, G. W. "Experiments upon the Purification of Sewage and Water at the Lawrence Experiment Station." *XXVI. Annual Report of the State Board of Health of Massachusetts* for 1894, 447.
4. LAWS, J. P. AND ANDREWES, F. W. "Report on the Result of Investigations of the Micro-Organisms of Sewage." *Report to London County Council*, December 13, 1894.
5. KLEIN, E. "On the Behavior of the Typhoid Bacillus and of Koch's Vibrio in Sewage." Supplement to *XXIV. Annual Report of the Local Government Board*, containing Report of the Medical Officer for 1894–95, 407.
6. FLÜGGE, C. *Die Mikroorganismen.* Leipsic, 1896.
7. WARD, H. M. "Fifth Report to the Royal Society Water Research Committee." *Proc. of the Roy. Soc. of Lond.*, 1897, 61, 415.
8. KLEIN, E. AND HOUSTON, A. C. "Report on Bacteriological Evidence of Presumably Recent and Therefore Dangerous Sewage Pollution of Elsewise Potable Waters." Supplement to *XXVII Annual. Report of the Local Government Board*, containing Report of the Medical Officer for 1897–98, 318.
9. CLOWES, F. "Report on the Bacteriological Examination of London Crude Sewage." *First Report to London County Council*, June, 16, 1898.
10. CLOWES, F. AND HOUSTON, A. C. "Bacterial Treatment of Crude Sewage." *Second Report to London County Council*, 1899.
11. HOUSTON, A. C. "Bacterioscopic Examination of Drinking Water, with Particular Reference to the Relation of Streptococci and Staphylococci with Waters of This Class." Supplement to *XXVIII. Annual Report of the Local Government Board*, containing Report of the Medical Officer for 1898-99, 467.
12. KLEIN, E. AND HOUSTON, A. C. "Further Report on Bacteriological Evidence of Presumably Recent and Therefore Dangerous Sewage Pollution of Elsewise Potable Waters." Supplement to *XXVIII. Annual Report of the Local Government Board*, containing Report of the Medical Officer for 1898–99, 498.
13. CONN, H. W. "Classification of Dairy Bacteria." *Report of Storrs Agricultural Experiment Station for 1899*, 13.
14. FULLER, G. W. AND JOHNSON, G. A. "On the Differentiation and Classification of Water Bacteria." *J. of Exper. Med.*, 1899, 4, 609.
15. CONN, H. W. AND ESTEN, W. M. "The Ripening of Cream." *Report of Storrs Agricultural Experiment Station for 1900*, 13.
16. WRIGHT, J. H. "A Simple Method of Cultivating Anaërobic Bacteria." *J. of the Bost. Soc. of Med. Sci.*, 1900–1901, 5, 114.
17. HORROCKS, W. H. *An Introduction to the Bacteriological Examination of Water.* London, 1901.
18. RIDEAL, S. *Sewage and the Bacterial Purification of Sewage.* London and New York, 1901.
19. FULLER, G. W. "Report of Committee on Standard Methods of Water Analysis." *Trans. of the Am. Pub. Health Assn.*, for 1901, 27, 377.

Downloaded from http://jid.oxfordjournals.org/ at University College London on June 5, 2016

20. WESTON, R. S. AND KENDALL, A. I. "Some Common Bacteria in American Streams, Including Some New Species Isolated at New Orleans, Louisiana." *Ibid.*, 402.
21. HUNZIKER, O. F. "Review of Existing Methods for Cultivating Anaërobic Bacteria." *J. of App. Micr. and Lab. Meth.*, 1902, 5, 1694.
22. HOUSTON, A. C. "Bacteriological Standards in Relation to Potable and Non-Potable Streams." *Second Report of the Royal Commission on Sewage Disposal*, 1902, 25.
23. HOUSTON, A. C. "Some of the Chief Methods Used in the Bacteriological Examination of Sewage and Effluents." *Ibid.*, 135.
24. JORDAN, E. O. "The Kinds of Bacteria Found in River Water." *J. of Hyg.*, 1903, 3, 1.

Downloaded from http://jid.oxfordjournals.org/ at University College London on June 5, 2016