STUDIES ON THE VIABILITY OF BACILLUS SALMONICIDA IN SEWAGE WATER

by

HELEN G. THOMPSON

A Thesis submitted for the Degree of

MASTER OF ARTS

In the department

of

Bacteriology

The University of British Columbia

April, 1936

accepted

ACKNOWLEDGEMENT.

I wish to express my appreciation to Dr. D. C. B. Duff for his interest and assistance in this work.

HISTORICAL.

In 1894, a specific bacillus was first isolated from fish suffering from so-called furunculosis, by Emmerich and Weibel, and named by them, Bacillus salmonicida. In 1902, Marsh in Michigan, described an organism isolated from diseased trout, which he called Bacillus truttae. This organism was found to be identical with B. salmonicida of Emmerich and Weibel, and because of priority, their nomenclature was given preference.

In 1909, Plehn reported a disease which she identified as furunculosis, occurring in Southern Germany. She stated that previous to this date the disease had been found only in domesticated waters, but from 1909 repeated epidemics were reported in natural waters.

The first occurrence of the disease in Great Britain was reported in 1911 by Masterman and Arkwright. In 1914 Mettam described an outbreak in Scotland. Since then numerous epidemics have occurred throughout the British Isles.

In 1932 Duff reported that the causative organism of an epidemic among the game fish at Elk River, British Columbia, was B. salmonicida. He has subsequently reported the presence of this organism in other locations in the province.

INTRODUCTION.

The means by which this infection spreads from one host to another has never been clearly demonstrated. The organism, if an obligate parasite, would of necessity require to be transmitted almost directly from fish to fish. If, however, the organism could exist saprophytically, it could survive for a considerable period after leaving the body of the host.

The first investigations of this nature were done by Plehn, in 1909. She tested the viability of B. salmonicida in bottled samples of pure and polluted waters. Before inoculation the waters were made presumably sterile by passage through a Berkfeldt filter. Plehn's experiments (table 1) present evidence to show that B. salmonicida can survive and multiply for a longer time, under laboratory conditions, in water of high organic content, than in relatively pure water.

In 1928, further work was reported by Williamson, who was working under the Furunculosis Committee of Great Britain. She "emulsified" a young agar slant of B. salmonicida in 15 c.c. of unsterilized distilled, tap and lightly polluted water. At twenty-four hour intervals she plated a loopful of the emulsions. She failed to recover B. salmonicida after forty-eight hours from the lightly polluted water. (table 2)

TABLE 1.

PLATE COUNTS EXPRESSED AS COLONIES PER C.C.

TIME	POLLUTED WATER	PURE WATER
Inoculum	5,800	6,336
24 hours	9,182	273
48 hours	440,000	0
72 hours	8,803,000	0

TABLE 2.

DAYS	DISTILLED WATER	TAP WATER	POLLUTED WATER.
1	+	+	+
2	+	scanty	scanty
3	+	very scanty	0
4	+	0	0

From these results Williamson concluded that B. salmoncida does not exist as a saprophyte in polluted waters, but suggests that the organism is introduced in some way, as by importation of fish carrying the disease. In the case of an epizootic B. salmonicida is probably liberated from diseased fish and distributed throughout the water, where apparently it can survive for a period sufficiently long to allow for its wide-spread distribution.

Obviously, the experimental basis of Williamson's conclusions are open to critism.

First.

The "emulsification" of a whole agar slant in 15 c.c. of fluid will give billions of organisms per c.c. and be out of all relation to the number which could enter natural waters from dead and sick fish. Furthermore, a large mass of bacterial growth when transferred to a small volume of fluid, will carry over with it a considerable amount of bacterial excretory products, together with a definite, though small, proportion of nutrients dissolved from the agar. The presence of such substances in the test fluid will be likely to influence, at least to some extent, the survival time of the bacterial population.

Second.

15 c.c. of fluid seems to be an extremely small volume, in view of the fact that conclusions derived from the behaviour of the organism in this volume are applied, directly, to its probable behaviour in extremely large volumes under

natural conditions.

Third.

The sample taken daily, from the fluid under test, was quite inadequate.

Fourth.

Williamson makes no statement as to how many plates were made at each sampling, whether dilutions were used, or as to the use of any differential medium. Our own experiences suggest the practical impossibility of recognizing B. salmonicida colonies among thousands of colonies of sewage origin without the use of specific methods.

Fifth.

Winslow in an article on "Rise and Fall of Bacterial Population" points out that unless the medium is extremely unfavorable, there is always, in bottled experiments, a phase of adjustment by the bacterial population, followed by a period of increase.

This was strikingly illustrated by Miquel in 1891, in a study of the growth curve of bacteria in a series of spring waters stored in flasks at 30°C, (see graph) and also by Fuller in 1894, at the Lawrence Experimental Station (see table 3). He worked with bottled samples of sewage.

Although this work had been done before that of Williamson and should have come to her notice, yet, on the basis of a bottled experiment, she states that B. salmonicida does not live in polluted waters, but dies out in two or three days.

TABLE 3.

Time	Bacteria per c.c.
Originally contained	1,190,000
2½ hours	108,000
Rose steadily to maximum in 35½ hours	23,100,000
Fell steadily. On 8th day	2,341,000

In an effort to throw some light on the opposing conclusion of Plehn and Williamson, Stewart, in 1931, began experiments with raw and sterilized sewage. Finding that B. salmonicida could not be distinguished on ordinary nutrient agar plates from sewage organisms, she abandoned the raw sewage and continued with the sterilized material only.

A series of 2 litre flasks were set up, each containing 500 c.c. of varying dilutions of effluent.

Flask (a) undiluted sewage

(b) sewage diluted.....1 : 10

(c) " " .....1 : 100

(d) " " .....1 : 1000

(e) " " .....1 : 10,000

(f) " " .....1 : 100,000

(g) " " .....1 : 1,000,000

These were sterilized and seeded with an inoculum of B. salmonicida which gave a population of approximately 300 to 400 bacteria per c.c. Then 1 c.c. samples were plated daily

on nutrient agar plates.

In the undiluted effluent the plates remained crowded with B. salmonicida throughout the experiment, that is, for thirty-five days. In the lower dilutions, (b), (c) and (d) there was a decrease at the beginning, and on the ninth day a sudden increase in the number of bacteria per c.c. As the dilutions made were not high enough the plates were too crowded to count, and remained so throughout the experiment. A slight decrease was noticed on the twenty-ninth day. In the more diluted effluents (e), (f) and (g) the increase did not come until the eleventh day, then there was a subsequent fall on the fourteenth day, and a secondary increase on the sixteenth day. From here the plates were crowded, as dilutions made were not high enough. A slight decrease was noticed on the twenty-third day. These results would seem to indicate that when less organic material was present the bacteria took longer to start multiplying, and their numbers started to decrease sooner.

Since Stewart's work was with sewage that had been sterilized Macarthur, in 1932, continued the research. First she looked for a medium which would differentiate B. salmonicida from sewage organisms. After trying many media such as:- Endo's agar, Mc Corkey's bile salt agar, eosin methylene blue agar, Krumwiede's brilliant green medium, and blood agar, she devised a maltose blood agar medium.

* Bacto Nutrient Agar

1.5% pH 7.3 .....100c.c.

Endo's Indicator : Anhydrous sodium sulphite ..... 25 grams

10% alcoholic solution of basic fuchsin ..... 25 c.c.

Maltose ..... 1 gram

Citrated rabbit's blood ..... 5 c.c.

The sodium sulphite was dissolved in 5 c.c. of water, the basic fuchsin added and the mixture then added to the melted nutrient agar. To prevent haemolysis the sugar was sterilized separately in a thin walled bulb blown from glass tubing. The open end was plugged with cotton, and the bulb was held in side the flask, above the solution, by the plug of the flask. When the agar was melted and cooled to 45°C the bulb was

* A dehydrated nutrient medium produced by Digestive Ferments Co.

Composition.

Bacto beef extract	3 grams
Bacto peptone	5 grams
Sodium chloride	8 grams
Bacto agar	15 grams
	31 grams

3.1 grams of this nutrient agar was added to 100 c.c. of water and the agar dissolved.

broken and the sugar dissolved. At the same time the citrated rabbit's blood was added. This medium, however, is not entirely satisfactory as there are a few polluted water organisms which show some resemblance to B. salmonicida on maltose blood agar.

However, using this medium, Macarthur attempted to follow the survival of B. salmonicida in raw sewage. But as the medium could not be depended on to differentiate B. salmonicida completely, no quantitative count was made, but it was definitely shown that multiplication of this organism took place.

EXPERIMENTAL.

The following experiments were a continuation of the work of Stewart and Macarthur. The primary object was to determine quantitatively the fluctuations and time of survival of an inoculum of B. salmonicida in bottled sewage water.

A further attempt was made to find a more satisfactory differentiating medium. Starch agar, starch blood agar, maltose starch blood agar, and aesculin bile salt agar were tried, but none were found to be as satisfactory as the maltose blood agar of Macarthur. Therefore, it was decided to use this medium, but to try and kill some of the sewage organisms, while still preserving the chemical nature of the polluted water, and its ability to support life, as evidenced by the retention of a reduced number of sewage organisms in a viable condition.

Treatment of sewage with Ether Vapor.

300 c.c. of the sewage effluent were placed in a 2 litre flask, closed tightly with a rubber cork. From this cork the ether was suspended in an open vial, above the surface of the liquid. Ten c.c. of ether were added to the vial. The flask was shaken frequently, being careful not to spill the ether. When the ether had completely evaporated, a sterile cotton plug was substituted for the rubber cork. When the odor of ether could no longer be detected, samples were plated from the flask. It was found that the sewage organisms which were

left could be differentiated from B. salmonicida on maltose blood agar plates.

Before inoculating the flask with B. salmonicida the strain to be used was tested for morphology, pigment production and sugar fermentation.

Morphology.

Small gram-rods (almost coccoid)

Nutrient Agar Slant.

Growth was whitish, effuse, glistening, convex and translucent. There was a brownish pigment in the medium.

Carbohydrate Reactions.

The organism was sown on the following carbohydrates and the changes recorded.

Mannite ..... +
Maltose ..... +
Xylose ..... -
Arabinose ..... +
Lactose ..... -
Sucrose ..... -

B. salmonicida usually ferments xylose, but this particular strain did not.

The flask was then inoculated with an inoculum of B. salmonicida which would give a population of approximately 2000 bacteria per c.c.

Determination of Amount of Inoculum.

1 c.c., 0.5 c.c. and 0.3 c.c. amounts of a saline suspen-

sion of a forty-eight hour agar slant culture diluted to the turbidity of No.4 suspension, Mc Farland's nephelometer, were inoculated into 300 c.c. quantities of sterile tap water. These were shaken thoroughly and allowed to stand for one hour. Then 1 c.c. amounts were plated in duplicate, from each. Plates of the flask inoculated with 1 c.c. gave approximately 200 colonies per c.c. Therefore, to obtain an initial inoculum of 2000 colonies per c.c., 10 c.c. of a No. 4 suspension were used. Every twenty-four hours 1 c.c. samples were plated in triplicate on maltose blood agar. The flask was always thoroughly shaken before samples were taken. Dilutions were made when necessary in sterile water blanks.

Plating Technique.

B. salmonicida was found to grow very slowly in poured plates, therefore, the 1 c.c. samples were placed on the surface of the agar, and the plate was gently rolled until the liquid was seen to cover the whole surface. But even using this technique B. salmonicida colonies could not be counted until four days after plating. Plates were incubated at 22°C.

Table 4 shows quantitatively the variation in organisms per c.c. There was a sudden increase right at the beginning. Unfortunately, as there was no way of knowing that this tremendous multiplication was going to take place the dilutions made were not high enough. The plates made from a $\frac{1}{1000}$ dilution were too crowded to count. On the tenth day there was a sudden fall in the number of colonies per c.c. Again, there was no way of knowing this sudden decrease would occur, and this

time the dilutions made were not low enough. On the fifteenth day a gradual increase started, until the twenty-fourth day when there was a gradual decline. B. salmonicida colonies were recovered from the sewage up to and including the sixty-seventh day.

When the number of B. salmonicida colonies were very low the sewage organisms were more numerous, and on the sixty-eighth day the plates were covered with sewage bacteria.

Each day, colonies which were presumably B. salmonicida were picked onto nutrient agar slants and checked as to their identity by pigment production and morphology. When counts became low a check was also made on carbohydrates. By the use of such checks one is able to make the above statements, regarding the behaviour of B. salmonicida in sewage, with complete confidence.

TABLE 4.

PLATE COUNTS OF B. SALMONICIDA IN SEWAGE TREATED WITH ETHER VAPOR EXPRESSED AS COLONIES PER C.C. OF SAMPLE.

DAYS	COLONIES per C.C.	AVERAGE
1	94,000 85,000 250,000	89,000
2	Dilutions not high enough.	400,000+
3	"	400,000+

TABLE 4.

continued

DAYS	COLONIES per C.C.	AVERAGE.
4	Dilutions not high enough	400,000+
5	"	400,000+
7	314,000 297,000 17,000	306,000
8	4,280,000 1,390,000 1,990,000	1,690,000
10	Dilutions not low enough	
11	"	
12	170 240 210	210
14	120 177 164	154
15	245 302 236	261
17	1,020 1,120 1,250	1,130
18	1,810 1,190 1,160	1,510

TABLE 4.

continued

DAYS	COLONIES per C.C.	AVERAGE.
19	1,490 1,630 1,740	1,620
21	3,200 4,300 5,100	4,200
22	5,100 5,200 5,000	5,100
24	1,810,000 1,430,000 1,570,000	1,600,000
25	550,000 530,000 560,000	550,000
26	480,000 500,000 470,000	480,000
28	420,000 400,000 450,000	420,000
29	200,000 190,000 220,000	200,000
30	140,000 160,000 150,000	150,000
31	60,300 61,200 60,600	60,700
33	80,000 90,000 70,000	80,000

TABLE 4.

continued

DAYS	COLONIES per C.C.	AVERAGE.
35	91,000 94,000 93,000	92,000
36	98,000 93,000 94,000	95,000
37	111,000 88,000 79,000	93,000
38	65,000 79,000 75,000	73,000
39	60,000 55,000 61,000	59,000
40	52,000 36,000 43,000	44,000
42	31,000 30,000 35,000	32,000
43	28,000 29,000 32,000	30,000
45	10,000 11,000 9,000	10,000
47	5,300 5,700 6,100	5,700
49	1,800 1,500 1,900	1,720

TABLE 4.

continued

DAYS	COLONIES per C.C.	AVERAGE.
50	451 473 487	470
51	39 45 52	45
52	2 4 5	4
53	48 62 72	61
54	12 17 15	15
57	56 55 44	52
58	66 71 66	68
59	59 65 52	59
60	20 25 23	23
63	11 11 13	12
64	8 22 10	12

TABLE 4.

continued

DAYS	COLONIES per C.C.	AVERAGE
65	9 7 5	7
66	13 4 6	8
67	6 7 0	6
68	0	0
69	0	0
70	0	0
71	0	0
72	0	0
73--87	0	0
93	0	0
99	0	0

The agar slants picked from the colonies, presumably B. salmonicida, on plates made from the forty-ninth day until the end appeared to be contaminated. There were two distinct types of growth:

1. A glistening, convex, effuse, and translucent growth.

Morphology.

Small gram-rods, almost coccoid.

* Plated on maltose blood agar.

Typical B. salmonicida colonies appeared--the blood was haemolyzed and the sugar fermented. Also, the colonies skated when pushed with a loop.

Morphology.

Small gram-rods, almost coccoid. Picked single colonies to nutrient agar slants. A brown pigment, typical of B. salmonicida was produced. Sowed into carbohydrates.

Maltose	+
Mannite	+
Xylose	0
Arabinose	+
Lactose	0
Sucrose	0

* This was done by touching a portion of the slant with a loop, mixing this with a drop of sterile broth on the maltose blood agar and spreading.

2. A few large single colonies, shiny, flat, viscous, with irregular edges.

Morphology.

Large gram-rods, varying in length, and a few clubbed shape rods.

A. Plated on maltose blood agar.

The growth appeared to be the same as that of B. salmonicida--the blood was haemolyzed and the sugar fermented.

Morphology.

Small gram-rods, almost coccoid, and a few longer gram-rods. Picked single colonies to agar slants. A brown pigment was produced, but there were two types of growth as before:

(a) a glistening, convex effuse and translucent growth.

(b) a few large single colonies, shiny, flat, viscous, with irregular edges.

Subcultured from the maltose blood agar plate to nutrient agar plate. Two types of colonies appeared.

(a) small round, shiny, convex, regular edge, skated on agar when pushed with a loop.

Morphology.

Small gram-rods, almost coccoid.

Picked to nutrient agar slant.

Produced a brown pigment.

(b) larger colonies, flat, viscous, irregular edge, a heavy creamy centre.

Morphology.

Large gram-rods with rounded ends. A few clubbed-shape rods.

Picked to nutrient agar slant.

No brown pigment was produced. Sowed into carbohydrates:

Maltose 0

Mannite 0

Xylose 0

Arabinose 0

Lactose 0

Sucrose 0

B. Plated on nutrient agar.

Just one type of colony appeared--large flat colonies, viscous, irregular edge, and a heavy creamy centre.

Morphology.

Large gram-rods with rounded ends, and a few clubbed-shape rods.

For convenience we called this second type of growth "C".

As there seemed to be a possibility that this form, "C", might be some form of dissociation, experiments were begun to attempt to bring about a reversion. Only half of the sixteen cultures were used and these were picked at random. The same technique was applied to them all, and since in all cases the results were identical, only one will be recorded.

(1) Alternative subcultures from broth to agar plates.

The "C" form was grown for forty-eight hours in broth,

a few drops were spread on a nutrient agar plate which was incubated for forty-eight hours. Then a colony was sown into broth. Five such series affected no change in the morphology.

(2) Serial cultures on nutrient agar.

Subcultured from plate to plate. There was no apparent change after seven transfers.

(3) Serial subcultures in broth.

A heavy, turbid, granular growth, with a surface pellicle.

Morphology.

Large gram-rods (longer and thinner than from agar plates). The twelfth tube revealed no morphological changes.

Whether the "C" form turns out eventually to be a new dissociative form of B. salmonicida, or a contaminant of peculiar behaviour, does not alter the accuracy of the findings as to the survival time of the original organism. This is true because of the fact that by proper treatment, typical B. salmonicida could be recovered in every case from the peculiar colonies.

DISCUSSION.

Plehn stated that the number of B. salmonicida colonies increased by millions, under laboratory conditions, in water polluted with organic material, in the same time required for it to die out in pure water. Also, that furunculosis, as it occurs in sewage polluted waters is commonly more infectious and more "disgusting" than that found in clear water.

Williamson noted that warm weather and low water were favourable to furunculosis. From her laboratory experiments she claimed that B. salmonicida did not live and multiply in polluted water.

Stewart found that B. salmonicida grew abundantly in sewage water which had been sterilized, and remained viable for at least thirty days. She found that there was first a decline in the number of organisms present, followed by a rapid increase. On the tenth day the plates were too crowded to count, as high enough dilutions had not been made.

Macarthur also indicated that B. salmonicida is capable of multiplication in water of high organic content.

In the above experiments, before inoculating with B. salmonicida, the sewage water was treated with ether vapor. This was done to try and kill some of the sewage organisms, particularly those which confused the use of the differential medium, while still preserving the chemical nature of the polluted water and its ability to support life. In this

menstruum, inocula of B. salmonicida first showed a rapid increase in numbers, and then on the tenth day a sudden decrease. On the fifteenth day a second increase started, more gradual than the first, and on the twenty-fourth day the number of organisms per c.c. began to decrease. B. salmonicida was recovered from the sewage for sixty-seven days.

Although these experiments can in no way be said to reproduce natural conditions, they seem to indicate that B. salmonicida can survive and multiply in water polluted with organic material.

Whether or not the "C" form of growth on the agar slants made from the maltose blood agar plates from the forty-ninth day until the end, is a dissociated form of B. salmonicida, cannot at present be stated. That it is in some way related to B. salmonicida is shown by first streaking on maltose blood agar and then subculturing to nutrient agar. When this is done two types of colonies appear on the nutrient agar, one being typical B. salmonicida, and the other the "C" form, previously described. Whereas, if it is streaked directly on nutrient agar only the "C" form of colony appears.

Many theories could be applied to explain this sudden decrease on the tenth day. Would the biochemical oxygen demand of the polluted water have any effect? In connection with this it would be interesting to investigate the growth of B. salmonicida in tightly sealed flasks, that is in non-aerated polluted water, and also, in water which was well aerated, probably by bubbling sterile air through the polluted water.

Could this sudden increase be due to some toxic effect which was later overcome? Some metabolic product, produced by the other bacteria in the sewage may be inhibitory to B. salmonicida, or, there might be some toxic substance in the organic material.

Dissociation may be the cause of this phenomenon. That is, there might be something in the sewage which would cause the dissociation of B. salmonicida, into a form at present unrecognizable, with subsequent reversion to the typical growth. The chief objection to such a theory is that the only dissociant, of an organism, which would be likely to pass unnoticed, would be the "G" form of Hadley. However, Hadley has stated, that the reversion of "G" forms to the normal is a matter, not of hours or days, but of months. One would therefore, have to postulate a new "G" type of dissociant with extremely quick powers of reversion. Such a form has not yet been recorded in bacteriological literature.

CONCLUSIONS.

1. Bacillus salmonicida, seeded into flasks of ether treated sewage water, after evaporation of the ether, survives for sixty-seven days.

2. The number of Bacillus salmonicida colonies per c.c. of fluid is at times greatly in excess of the number inoculated. (inoculum=2000 per c.c.,

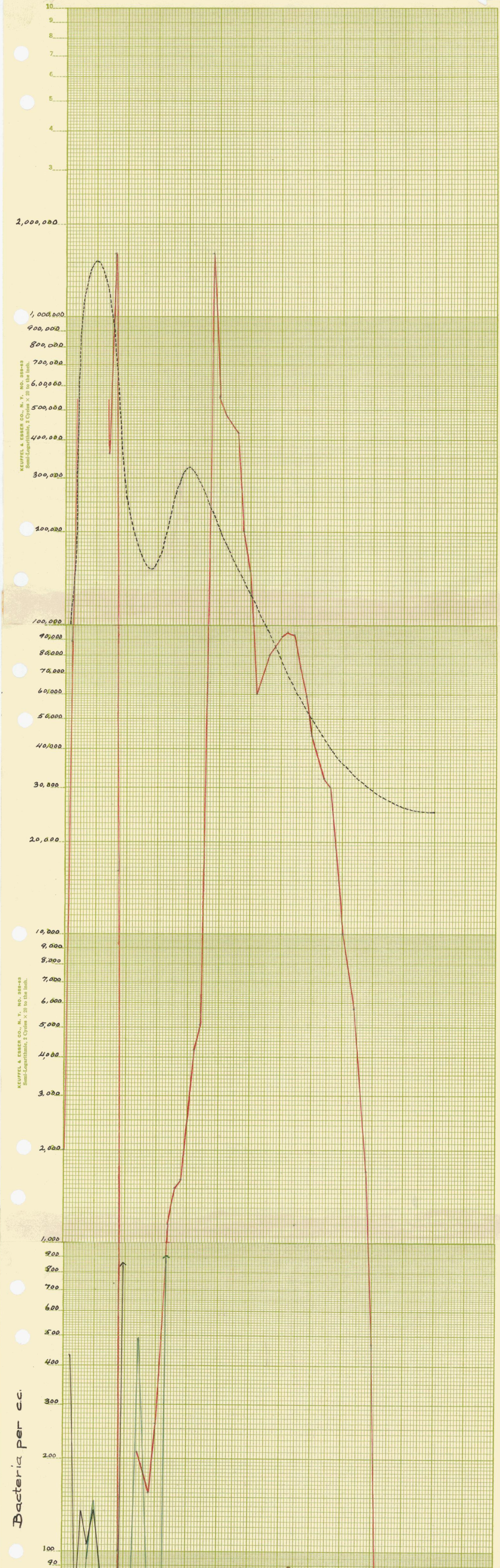
maximum recorded=1,690,000 per c.c.)

During the period of observation, wide flucuation occurs in the concentration of this organism in the sewage.

3. The ether treatment of the sewage does not essentially alter the capacity of the fluid to support bacterial growth. This is evidenced by:

(a) marked multiplication of the initial inoculum of B. salmonicida.

(b) eventual multiplication of the surviving sewage organisms.


10
9
8
7
6
5
4
3
2,000,000
1,000,000
900,000
800,000
700,000
600,000
500,000
400,000
300,000
200,000
100,000
90,000
80,000
70,000
60,000
50,000
40,000
30,000
20,000
10,000
9,000
8,000
7,000
6,000
5,000
4,000
3,000
2,000
1,000
900
800
700
600
500
400
300
200
100
90
80
70
60
50
40
30
20
0
KEUFFEL & ESSER CO., N. Y. NO. 358-63
Semi-Logarithmic, 2 Cycles × 20 to the inch.
Bacteria per c.c.
------ Miquel - Spring Water.
Stewart - Sterilized Sewage
— Dil. 1/10
— Dil. 1/10,000
— Sewage Water Treated with Ether Vapor
5 10 15 20 25 30 35 40 45 50 55 60 65 70
Time in Days.

BIBLIOGRAPHY.

Arkwright, J. A. — Report to the Board of Agriculture and Fisheries. Jour. Hyg. 12, 1913, 391.

Furunculosis Committee. — Interim Report of; March, 1930.
Final Report, July, 1935.
H. M. Stationary Office.

Macarthur, M. I. — Studies on the Viability of B. salmonicida. Thesis submitted for the degree of Master of Arts at the University of British Columbia. (unpublished)

Marsh, M. C. — A More Complete Description of B. truttae, Bulletin of U. S. Fish Commission XXll, 1902, 411.

Masterman, A. T. — Report upon the Epidemic amongst Salmonidae in the Summer of 1911.
Board of Agriculture and Fisheries.

Stewart, B. J. — Studies on B. salmonicida.
Thesis for the degree of Master of Arts at the University of British Columbia.
(unpublished)

Williamson, I. J. F. Furunculosis of the Salmonidae, Fishery Board for Scotland, Salmon Fisheries 1928, No. V.

Winslow, C. E. A. The Rise and Fall of Bacterial Populations, The Newer Knowledge of Bacteriology and Immunology, Chapt. 6. Jordon and Falk. The University of Chicago Press. 1928.