STREPTOCOCCI AS INDICES OF SEWAGE POLLUTION

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Robert James Driesens

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This is to certify that the

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Streptococci as Indicators of Sewage Pollution

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Robert James Driesens

has been accepted towards fulfillment
of the requirements for

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W. H. Hallmann.
Major professor

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STREPTOCOCCI AS INDICES OF SEWAGE POLLUTION

BY

Robert James Driesens

A THESIS

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CONTENTS

A number of outbreaks of intestinal diseases has been reported in the southwestern part of the United States where vegetables are irrigated with sewage polluted river water. Inasmuch as the water supplies in these areas are generally free of contamination, vegetables have been suspected of transmitting enteric diseases.

The method of measuring the degree of contamination in irrigation waters, which are known to be sewage polluted, could conceivably be identical to those used for evaluating the potability of drinking waters. However, these methods may not be satisfactory procedures when it is recalled that the water is channeled into shallow trenches in cultivated soils. It is possible that the waters may become contaminated with organisms from the soil. If the soils are fertilized with animal manures it is conceivable that the irrigation water may become contaminated with enteric organisms of manurial pollution.

If such is the case, then it would be better to test for human pathogens of enteric origin such as the Salmonella group. However, it has been demonstrated by repeated testing of sewage polluted waters that the isolation of such organisms is difficult and unreliable because their incidence is low and the methods of isolation do not adapt themselves to rapid methods of isolation and identification. Thus it would appear that non-pathogenic enteric organisms would be the most satisfactory indicators of fecal contamination.

The purpose of this thesis is to investigate the probable utility of streptococci, particularly of the enteric group, as satisfactory indicators of dangerous sewage pollution under conditions related to the problem suggested above.

Review of Literature

Coliforms in Water

The coliform group is used as an index of stream pollution and water potability. It is generally accepted that the incidence of water-borne diseases is low in areas where the coliform index of drinking water is low, high where the index is high. However the literature on the subject shows considerable disagreement with respect to the sanitary interpretation of any given index.

Savage and Wood (31) studied the longevity of coliforms and streptococci in water inoculated with excreta. At the end of one week, both the coliforms and the streptococci had diminished rapidly. The streptococci persisted for two weeks and the coliforms (mainly "non-fecal" types) continued to persist at very low levels up to six weeks.

Rogers (29) inoculated loopfuls of human exreta into sterile water and found that at the end of nine months the ratio of surviving coliforms was approximately 39 aerogenes (non-fecal) to one coli (fecal). The inference is that when aerogenes predominate, the pollution is remote and when the coli predominate pollution is recent and more dangerous.

Platt (25) showed that Esch. coli survive longer in sterilized waters than in raw river water and that they disappeared rapidly at 37° C. in both waters. He noted that upon aeration by shaking, their numbers increased markedly. Organisms incubated in the dark persisted longer than those exposed to daylight.

Bigger (2) suggested that carbon dioxide in raw river waters was the responsible inhibitory agent. He found that coliforms multiplied in autoclaved tap water but died rapidly in the raw water. . The long persistence of coliforms in tropical waters which are not apparently liable to contamination may be explained by the low carbon dioxide content of the waters.

Koser (11) proved that after four months sojourn in soil, coliform organisms did not change their biochemical characteristics. Thus, it would be possible to determine the longevity of the Esch. coli in the soil by checking the organisms by biochemical tests (IMViC).

Taylor (37) complicates the picture of so-called fecal and non-fecal types by showing that the urines of patients having genito-urinary infections contain mostly aerogenes or intermediate types. He suggests that Aerobacter are at least excretory if not typically fecal.

Parr (23) inoculated sterilized pieces of string and wood with Esch. coli obtained from feces. These were suspended in sterile tap water. The organisms remained viable for over a year.

It appears that the presence of either "fecal" or "non-fecal" coliforms in a water is not necessarily evidence of recent fecal pollutions.

The coliform test has proved to be inadequate in at least one instance where no coliforms were detectable in a treated water supply which proved to be the source of an epidemic of diarrhea (Ziegler, 43).

Mallmann (16) concludes that "the presence or absence of coliform organisms in marginal waters is arbitrarily based on the selection of the medium used for their isolation and not their actual presence".

Pathogens in Water

Prescott, Winslow, and McCrady (27) state that pathogens such as Salmonella typhosa and Clostridium welchii are not considered to be satisfactory indicators of pollution because their numbers are too low to be of practical value. Nevertheless, it may be well to note their probable longevity in water. Craig (6), citing many observers, reports that Endomoeba histolica (vegetative form) survives in feces at room temperatures up to nine days, in water from 9 to 29 days, the latter only in the presence of few bacteria. Concentrated suspensions of the organisms inoculated into distilled water persisted at room temperature for ten days and to 211 days at 12^0- 22^o C.

MacConkey in 1906 (14), noted that cultures of Cholera vibrios were viable in distilled water after several months when inoculated in very large numbers but in concentrations of the order of 10,000 per ml. they disappeared within two hours.

Tanner (36), citing the work of Rochaix (28) remarks that concentrated cultures of pathogens survive longer in pure cultures than in mixed. Rochaix is reported to have shown that both Vibrio comma and Salmonella typhosa survived 3¼ months in sterilized tap water and nearly seven months in sterile distilled water. Salmonella schottmülleri in sterilized sewage water persisted 8½ months.

Although virus infections (e.g. poliomyelitis) may result from sewage pollution, the arduous methods of isolation make their quantitative estimation and evaluation impractical.

Since our problem involves waters in intimate contact with soil, it is well to note the activity of coliforms in soils and manure.

Coliforms in Soils and Excreta.

Rogers, Clark and Evans (30) isolated Esch. coli from grain and grain fields apparently not fecally contaminated. None of the coliform organisms was like the characteristic flora of bovine feces.

Young and Greenfield (42) mixed Esch. coli with soil in 27 cu. ft. galvanized tanks and in bottles. Survival was noted in some cases to seven years, others to three years. Opinion expressed was that differentiation between fecal and non-fecal strains was of little practical value to the sanitarian, since both become saprophytic to the soil.

Skinner and Murray (33) inoculated soil with one per cent manure and found that both aerogenes and fecal types of coli disappeared after 150 to 200 days. In manure alone they persisted about 50 more days. Aerogenes types were isolated from many soils under "natural conditions", i.e., - in the absence of known or gross pollution.

Tonney and Noble (39) noted a rapid population decrease under winter conditions. By inoculating decayed stumps with cultures of A. aerogenes and Esch. coli, they noted an increase in numbers after 60 days, most of the persisting organisms being aerogenes type..

Kulp (13) inoculated sterilized soil with Esch. coli and A. aerogenes. The soil was kept moist throughout the period of the experiment. The organisms persisted over 3½ years.

Kline and Fuller (12) in a similar experiment recovered the organisms after 410 days.

Taylor (op. cit.) claims that there is no evidence of coliforms multiplying in grasses, except silage, and insists it is virtually impossible to determine whether a soil is polluted or not under natural conditions. He does not believe evidence warrants the conclusion that coliforms are normal inhabitants of soils, grasses and grains.

Tonney and Noble (39) suggest that A. aerogenes is primarily non-fecal but appears irregularly as a transient organism in feces. They showed (40) by direct plating that the ratio of Aerobacter to Escherichia in feces was 1 to 100 and in soil and vegetation 20 to 1. Some explana-

tion other than that both are of direct fecal origin must be given, since in feces the aerogenes are in an unfavorable environment.

Enteric Pathogens in Soil and Excreta

Data are meager with respect to the viability of pathogens in the soil. The factors of antibiotic substances, desiccation and temperature fluctuation would suggest a short survival period. Jordan (9) noted that typhoid bacteria did not multiply in stored feces and disappeared within 3 to 52 days. The average number of bacteria in all kinds of feces (i.e., from health and from ill persons) was estimated to be about 75,000,000 per gram.

Murray (19) noted that Esch. coli in manure piles were rapidly crowded out by other organisms.

Tanner (37) citing Rochaix (28) reports the survival of E. typhosa and other Salmonellae up to 7½ months after inoculation into sterilized sewage. The writer feels that this is not a valid method of determining response to normal environmental conditions. The sterile sewage may be considered to be a propagation medium.

Suckling (35) suggests that manures may not be as innocuous as is often supposed. Gulls have transmitted typhoid organisms in their excreta. Brucellosis and some Salmonellae infections are suspected of being transmitted.

It seems clear that for purposes of evaluating the pollution of irrigated soils, the coliform test might be grossly misleading especially if made in the warmer sections of our country. Considerable numbers of either types of coliforms may be expected to appear long after pollution by fresh manures or human excreta should have been rendered innocuous.

Streptococci and Water

Streptococci have long been utilized as supplementary indices to verify the pollution inferred by coliform indices. Prior to the recent advent of special selective media, these enteric streptococci have been difficult to isolate in numbers reflecting their actual numbers in water, sewage and feces. Seligmann (32) showed that streptococci populate sewage waters in numbers 10 to 100 times greater than has generally been recognized.

For this reason the data collected prior to the last ten years may be only of historical interest. By older methods of estimation streptococci appeared to disappear from waters more rapidly than coliforms. Savage and Wood (loc. cit.) using both decimal and intermediate dilution techniques showed that streptococci failed to appear usually after two weeks in water, but that in domestic non-industrial sewage they persisted from 4 to 8 weeks, paralleling coliform indices. In the above experiments, an average of 165 ml. of sewage was added to 40 l. of tap water and incubated at room temperatures.

Houston (8) estimated that the concentration of one part per million of fecal matter in water is represented by 17 streptococci per ml.

Mallmann (15) showed that streptococci (presumably oral) failed to multiply in swimming pools whereas under certain conditions coliforms did multiply. Streptococci indices fluctuated with the bathing load and presumably with the degree of pollution. Mallmann and Sypien (18) demonstrated similar parallelism in natural bathing places.

Streptococci in Soil and Excreta

Broadhurst (3) believed that streptococci are neither indigenous to the soil nor to grains and that the grains are only accidental carriers. She noted that they were most easily obtained from equine and human feces and difficult to obtain from feces of dogs, cats and cattle.

Ostrolenk, Kramer and Cleverdon (22) using soils contaminated with bacterial suspensions noted that enterococci were detected somewhat longer than Esch. coli but that in soils contaminated with chicken manures, the Esch. coli were recovered after 66 days but enterococci only for 21 days.

Ostrolenk and Hunter (21) using Perry and Hajna's "S.F. Medium" (cf. Perry and Hajna, 24) examined 52 specimens of human and animal feces. 26 of 28 fecal samples contained streptococci.

Oppenheim (20) basing his identification on the scheme of Holman (7) found about 74 per cent of the nonhemolytic streptococci in feces to correspond with Str. fecalis (Andrews and Horder).

Torrey (41) noted that the presence of Str. fecalis may depend on the diet of the person from whom a stool was taken. A low protein, high carbohydrate diet appeared to enhance the number of these bacteria. Suckling (op. cit. p. 504) reports streptococci to be present in numbers up to 100,000 per g. of feces and that sewage contains about 10,000 per g.

It appears that streptococci may occur in sufficient numbers to warrant an investigation of their relation to pollution and sanitation.

EXPERIMENTAL

One early method for estimating the probable numbers of streptococci from mixed bacterial sources was to examine microscopically the sediment from tubes used for the determination of coliform populations. Another (Suckling, 35) has been to dilute these broths and heat the same to 60° C. After subculturing on MacConkey's agar the small red colonies were examined microscopically.

Prescott and Baker in 1907 (26) observed that when dextrose peptone broth became acid by the growth of coliforms, the streptococci began to overtake the previously predominant coliforms. They suggested an acid broth medium with an acidity sufficient to inhibit the coliforms and thus allow the early development of the streptococci.

Ostrolenk and Hunter (21) grew streptococci on "S.F. broth"and from both "positive" and "negative" tubes made streaks on 1.5 per cent agar - S.F. broth plates.

In their opinion S.F. broth failed as a presumptive medium because about seven per cent of the tubes were "false positives", i.e., showed production of acid and sediment without presence of streptococci and five per cent "false negatives" as indicated by microscopic observation. All false negatives were found in the maximal dilutions of 1-100,000 to 1-10,000,000.

Chapman (4) recommended two media for the isolation of streptococci. These media were developed primarily for the separation of enterococci and pathogenic streptococci from mixed cultures. Although these media may be satisfactory for the purpose for which they were designed, they do not fit into the plan of this thesis bedause they are laborious to make for a repeated routine study of streptococci of fecal origin.

Mallmann, Botwright and Churchill (17) noted the selective bacteriostatic effect of sodium azide as did Snyder and Lichstein (34). The fecal streptococci showed marked tolerance of the azide. The general technic of preparing and using the medium were both economical and simple.

Preliminary

Streptococci may be propagated on a number of non-selective sugar media. However, the writer found such media to be of no value for enumeration studies because the streptococci were obscured by overgrowth of other organisms. Diagnostic media containing dyes such as those made with 0.1% methylene blue were too deeply colored to facilitate enumeration. Therefore a few exploratory tests were made on "SF" broth and agar (Perry and Hajna, 24).

To check the specificity of "SF" media, a number of possible conflicting organisms were tested. Stock cultures of Str. lactis, Str. citrovorous, Str. pavacitrovorus, Micrococcus pyogenes var. aureus, several hemolytic streptococci, Serratia indica and several common molds failed to grow at room temperatures or at 37° C. within 48 hours on SF agar or in SF broth. This indicates that the media has marked selectivity.

The SF broth formula is as follows;

Glucose	5.0 g.	NaN_3	0.5 g.
NaCl	5.0 g.	Tryptone	20.0 g.
KH_2PO_4	1.5 g.	Distilled water	1000 ml.
K_2HPO_4	4.0 g.	Brom-Cresol Purple (1.6% alcoholic)	2.0 ml.

The SF agar was prepared by adding 1.5 g. agar to the SF broth.

A series of tests were made by substituting tryptose for tryptone in the S.F. formulation. It was found that a more rapid appearance of turbidity occurred in 24 hours. Accordingly this substitution was made for the medium used in this study.

No correlation was found between S.F. broth indices and direct plate counts on S.F. agar. Only a few colonies appeared in the latter medium. However, streak plates made from "doubtful positive" broth tubes frequently showed typical streptococci colonies. For this reason only the S.F. broth medium was used.

Lactose was substituted later for dextrose in the formulation because some streptococci (Str. equinus) from horse manure do not ferment lactose. Inasmuch as the medium is to be used for the examination of soils irrigated by sewage laden water the use of lactose would offer a medium more selective for human pollution and would reduce the confusion from manurial sources. When tests were made of horse manure, non-lactose fermenting streptococci were isolated from the S.F. (dextrose) broth.

Eventually Brom thymol blue was substituted for Brom cresol purple because it manifests a color change at a higher pH than does the former. In view of the fact that the medium is buffered the writer believed that small numbers of streptococci might not produce enough acid sufficient to affect color change in the range of pH5 - pH 6.

The formula for the altered SF broth used in evaluating river water and manure was as follows:

*Lactose	5.0 g.	NaN_3	0.5 g.
NaCl	5.0 g.	*Tryptose	20.0
KH_2PO_4	1.5 g.	Distilled Water	1000 ml.
K_2HPO_4	4.0 g.	*Brom thymol blue (0.5% alcoholic)	4.0 ml.

*Altered ingredients

Streptococci Populations in Sewage

Samples were obtained thrice weekly in January and February 1948 from the Columbus, Ohio disposal plant. The sewage from this plant is fairly representative in that there appears to be no overbalancing of industrial or of biological wastes. The raw sewage samples collected at 9 A.M. were relatively dilute. This sewage, because of the eight-hour travel period to the disposal plant represents the waste discharged at about 1 A.M.

Sterilized salt-mouth bottles were used to collect the sewage. Within two hours after collection, 11 ml. portions were diluted serially in sterile tap water. The samples were planted in S.F. (tryptose) broth in quadruple decimal dilutions. Each tube contained at least 5½ ml. of medium to insure a minimum concentration of 0.04 per cent sodium azide. Readings based on presence of acid production and turbidity verified by microscopic examination, were made after incubation at 37.5° C. for 48 hours. Microscopic examinations using Gram's stain were made only from the critical series of dilutions and from both positive and negative tubes of these dilutions.

The substitution of lactose for dextrose in the tryptose SF broth was made on February 13.

The results of a survey of the Columbus ,Ohio sewage treatment plant are presented in Table 1. Samples were collected from the various stages of treatment over a period of one month. These figures do not show the degree of purification from a sanitary standpoint as measured by streptococci indices because of fluctuation in the concentration of sewage entering the plant. It is impossible to trace with any degree of accuracy the bacterial changes in a given lot of sewage through the plant. No correlation between the streptococci population and the total solids was noted.

The data show that streptococci persist in large numbers after prolonged heating at 85° F. in the heterogeneous populations of the digesters and that reduction in numbers is not evident.

Streptococci and Soil

Five wood boxes 20 x 17 x 30 cm. were each filled with approximately 10,000 c.cm. of soil of three types, none of which was known to have been contaminated with either human or animal manure. One liter of raw sewage was added to each boxful. Two samples (muck #2, Sand #1) were allowed to dry gradually at room temperatures indoors and three were set outdoors exposed to natural conditions.

Table 1. Streptococci indices in Sewage obtained from the Columbus Ohio Treatment Plant

Date of Sampling	Total Solids p.p.m. Raw Sewage	Streptococci Indices per ml. Raw Sewage	Activated Sludge	Digested Sludge	Final Effluent
Feb. 2	246	20,000	130,000	X	95
4	325	14,000	165,000	70,000	2,000
6	357	11,500	2,000	16,000	4,000
10	346	200	35,000	6,000	30
11	149	11,500	20,000	11,500	115
12	374	*11,500	*140,000	*14,000	* < 3
13	374	**6,000	**35,000	**115,000	** 60
17	251	3,500	60,000	55,000	25
20	205	11,500	9,500	60,000	25
23	124	16,500	2,500	25,000	3
26	311	16,500	25,000	3,500	3
27	196	6,000	11,500	35,000	3-4
Mar. 1	252	6,000	11,500	X	6
3	191	6,000	6,000	2,500	3
Geometric Av.	7,295	7,295	21,376	19,565	47

*Dextrose **Lactose

AVERAGE STREPTOCCI INDICES

PER GRAM TREATED SEWAGE

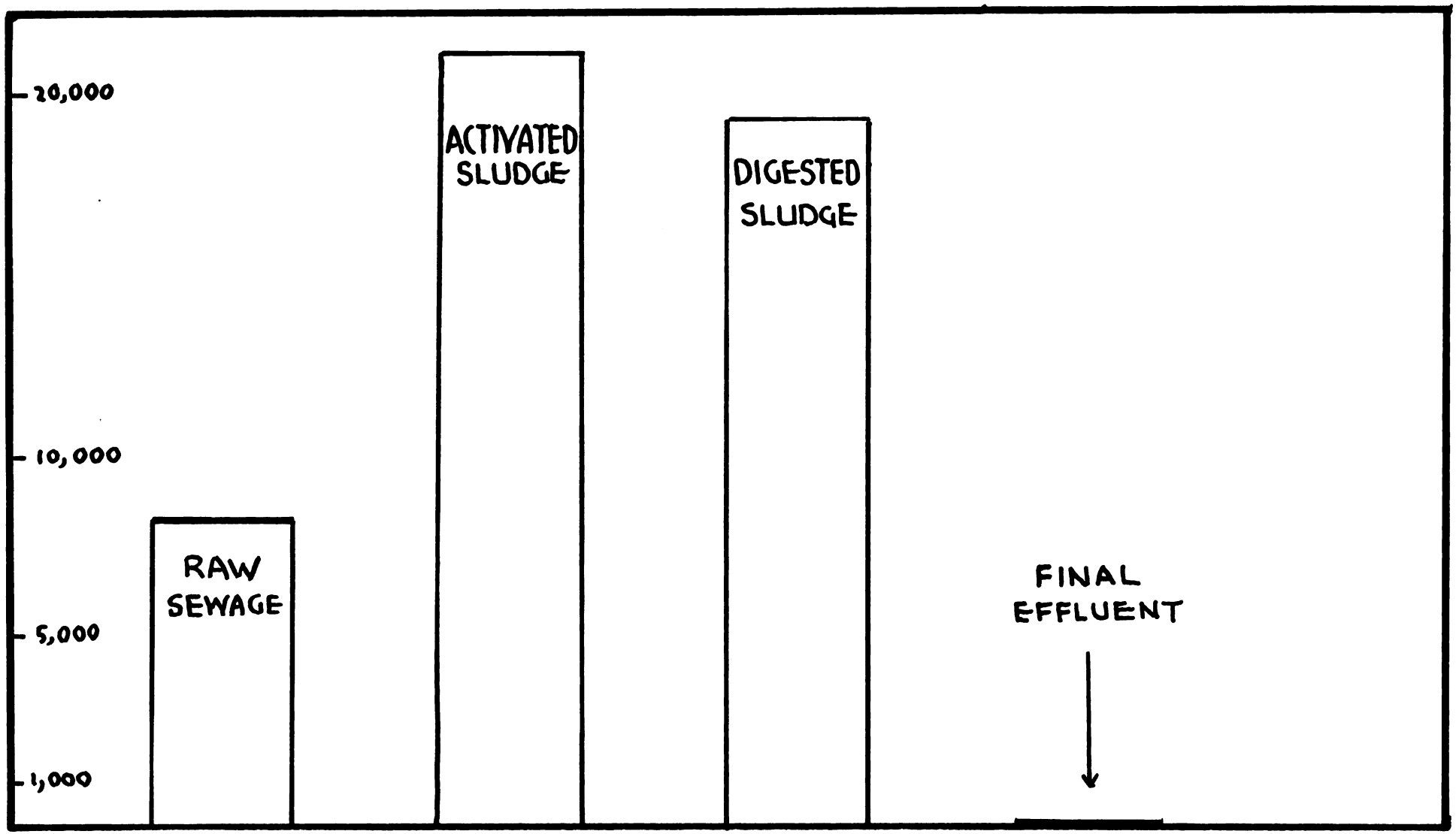

Figure 1

Duplicate surface samples (two inch depths) were collected using a flamed spatula. At each sampling one portion was checked for moisture content, the other was used for bacteriological tests. The latter sample was diluted with sterile water and decimal dilutions prepared. Care was exercised to be sure that suspended solids had not settled during sampling. One ml. portions were planted into the lactose S.F. medium. The cultures were incubated at 37° C. for 48 hours. The data reported are computed on a dry weight basis. The results are presented in Table 2.

The results show a very low streptococci population. This was due to the fact that the sewage was chiefly storm water. Because the population was low initially it was difficult to trace accurately the diminution of streptococci during the period of test. At the end of 26 days, zero streptococci indices were obtained from several soils.

A second series of tests was made using the same soils. In this case cultures of streptococci were added so that heavy inoculations were obtained. The results are reported in Table 3.

The data show a marked decrease in population in two weeks (over 90 per cent) and a decrease of more than 99 per cent in three weeks. Characteristics of the soils do not appear to be important factors in the diminution observed. Because of the low moisture content of the soils, desiccation may have been a major factor in this rapid reduction of population. The soils were examined over a period of 18 days. A similar series of tests was made at Michigan

Table 2. Viability of Streptococci in Various Soils Inoculated with Raw Sewage

Days After Inoculation	Streptococci per gram				
	Heavy Clay Loam	Mortar Sand #1	Mortar Sand #2	Mulched Muck #1	Mulched Muck #2
8	12	12	9	25	25
10	2	2	3	3	0
26	0	3	3	0	42
Initial Total Bacteria per gram*	8,230,000	1,490,000	1,490,000	5,030,000	5,030,000

*Total Count Determined on Nutrient Agar

Table 3. Streptococci viable in soil surfaces

Days After Inoculation	Heavy Clay Loam Strep. per gram	% H_2O	Mortar Sand #1 Strep. per Gram	% H_2O	Mortar Sand #2 Strep. per Gram	% H_2O	Mulched Muck #1 Strep. per Gram	% H_2O	Mulched Muck #2 Strep. per Gram	% H_2O
2	1,748,500	9	17,490,000	7	161,600,000	1	3,125,000	20	46,800,000	15
6	26,000	4	11,500,000	3	14,560,000	3	4,375,000	21	25,800,000	23
8	160,000	7	4,160,000	4	63,000	5	750,000	21	399,000	13
14	20,000	1	115,000	1	200,000	1	73,500	5	13,000	1
18	580	5	800	1	250	1	2,675	7	66,000	9

REDUCTION OF STREPTOCOCCI
IN A CLAY SAMPLE

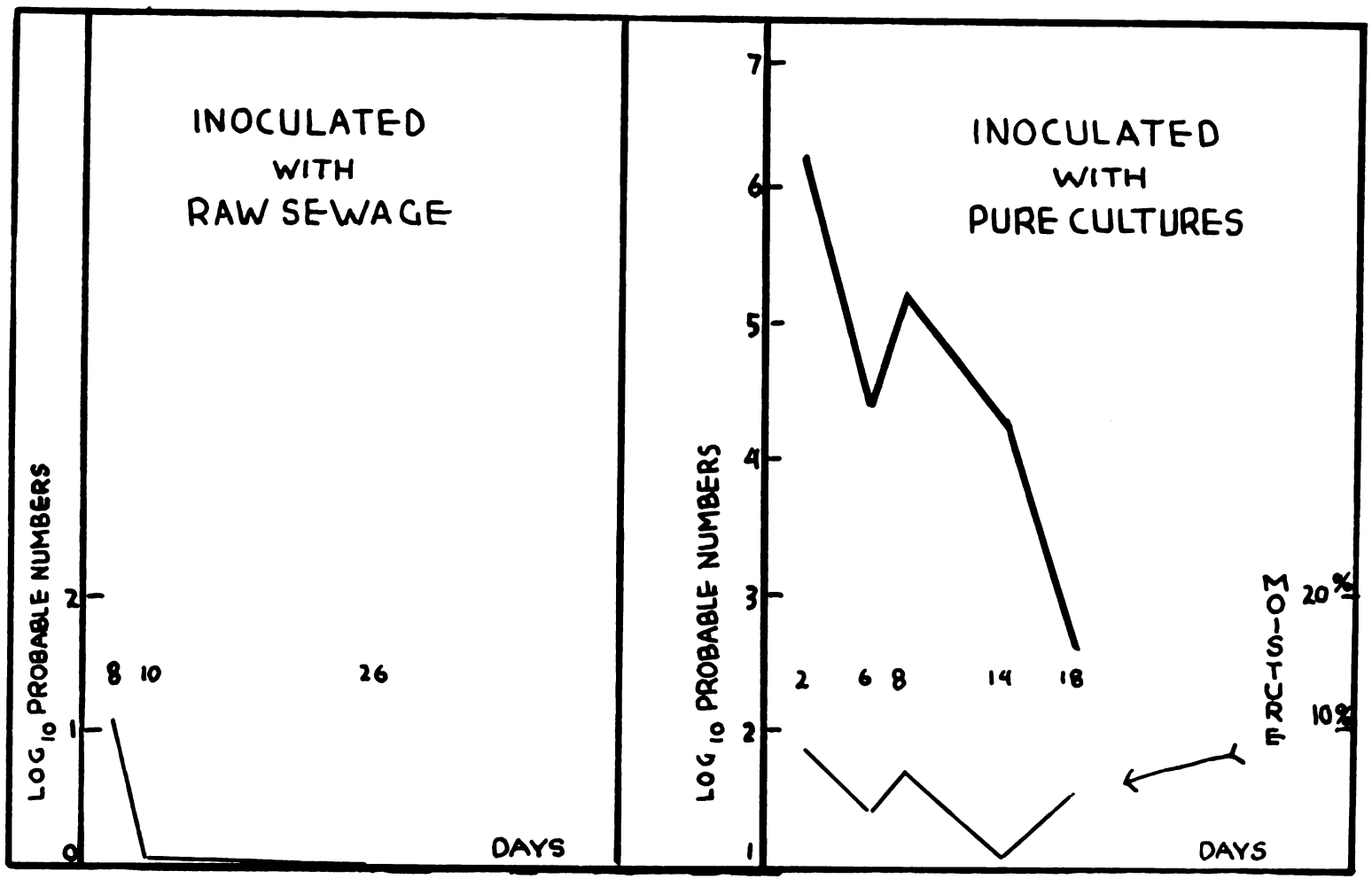

Figure 2-A

Figure 3-A

REDUCTION OF STREPTOCOCCI
IN TWO SAND SAMPLES

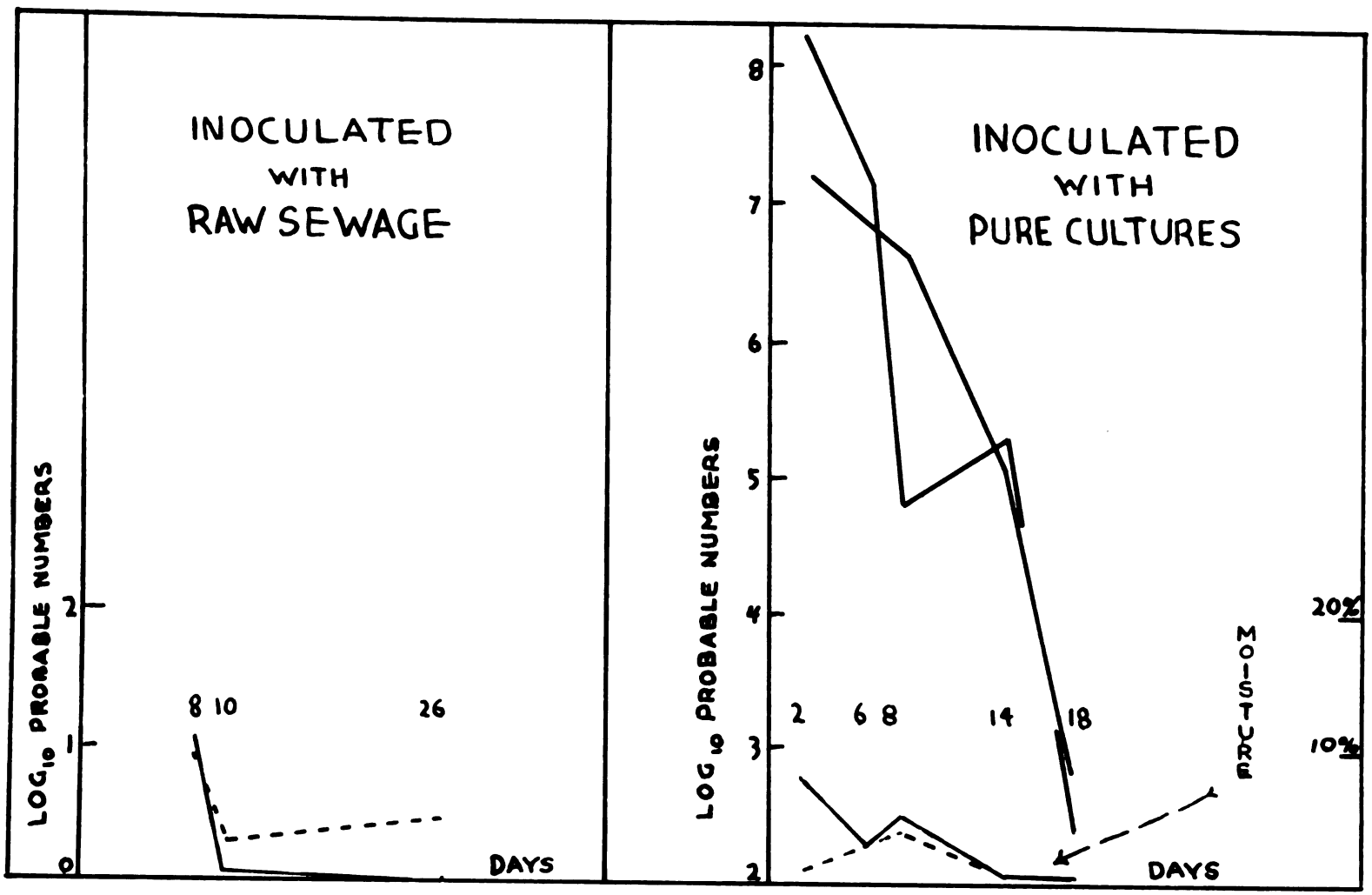

Figure 2-B

Figure 3-B

REDUCTION OF STREPTOCOCCI

IN TWO MUCK SAMPLES

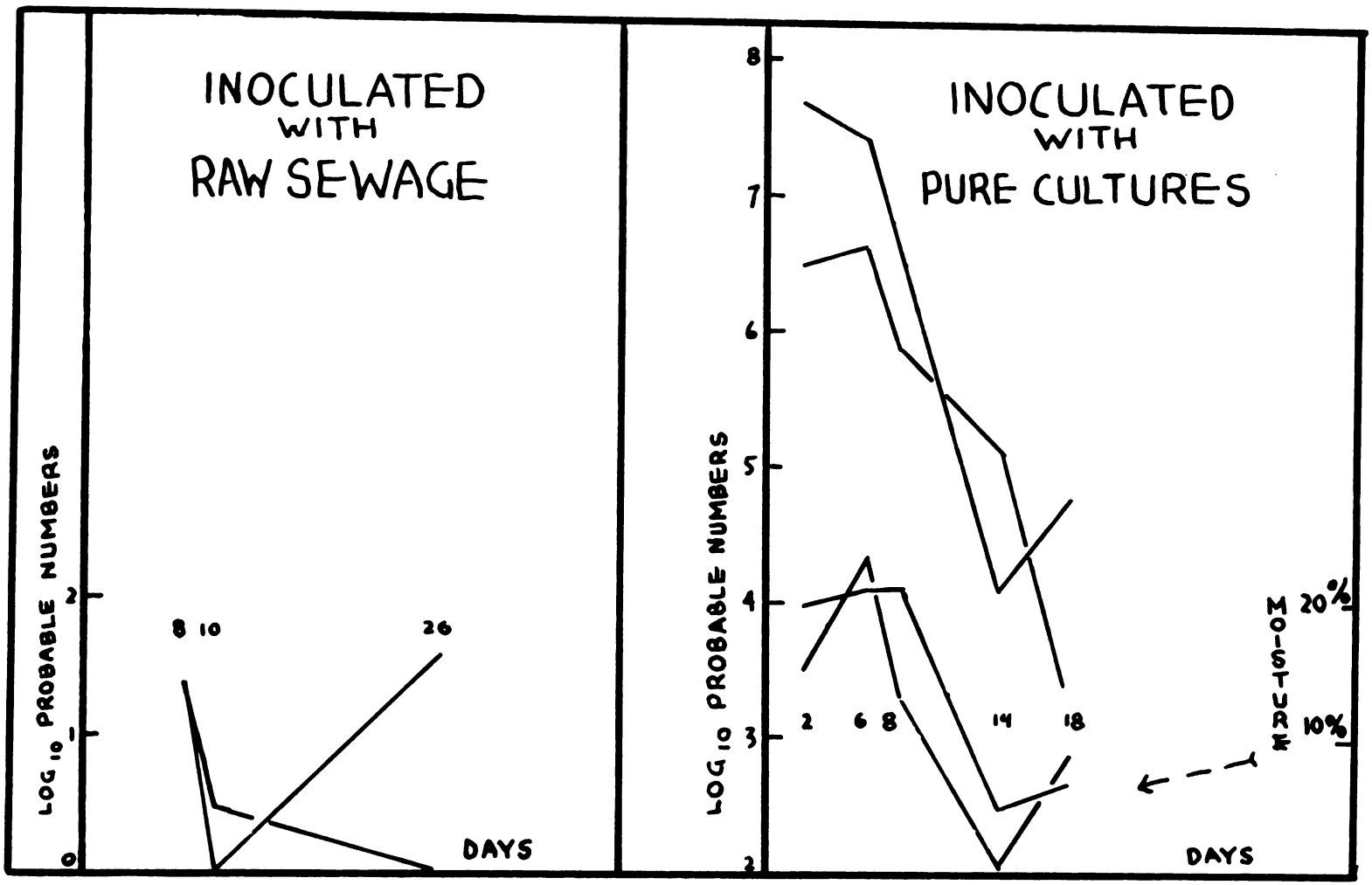

Figure 2-C

Figure 3-C

State College during the summer (1948).

Twelve tinned steel cans, 6 inches in diameter and 7 inches deep were filled with soil obtained from campus greenhouses. The soil was a light loam not known to have been manured. Many grass roots were removed by sifting. The amount of soil used in each can was approximately 2,000 grams, dry weight. Cans were perforated at the bases to facilitate drainage. When tamped the soil was 4½ inches deep.

To each can was added a thride-washed suspension of a fecal streptococcus previously grown at 45.5° C. in lactose SF broth. The organism isolated from rat feces, was checked biologically and found to correspond to Str. fecalis (Andrews and Horder).

Cans numbered 1 to 4 were stored at 5°-6° C. and constant moisture levels were maintained.

Cans 5 to 8 were stored at prevailing summer temperatures in a glass enclosed room. No moisture was added subsequent to the original bacterial inoculations.

Cans 9 to 12 were stored with cans 5 to 8 under the same conditions except that sterile distilled water was added twice weekly to maintain a moisture content of not less than 10 per cent nor more than 25 per cent.

To get a representative sample, the contents of the cans were pulverized, screened and thoroughly mixed. Duplicate samples were collected and tested as described previously.

At the beginning and at the end of the reported period an estimation of all microorganisms in the moistened sample was made by using decimal dilutions in nutrient broth. At the end of the period an estimation of coliform population was made by decimal dilutions in brilliant green-bile lactose broth.

After July 21, 1948, parallel dilutions were incubated at 45.5° C.

Data presented in Table 4 show that when large numbers of streptococci are placed in soil, they decrease by over 90 per cent in about one week even when refrigerated and by 99 per cent in less than two months.

Comparison of the indices for air dried soils and moistened soils suggests that desiccation was not the principal factor in reduction of these streptococci populations.

No explanation is given for the wide differences in numbers for the two sets of indices at short-time storage and for the close parallelism after longer storage. Those organisms which succumb early may be unable to grow at 45.5° C.

Streptococci and River Water

Two samples were taken from the Red Cedar River at a point below a sewer outlet. The samples were collected in two-liter sterile Erlenmeyer cotton-stoppered flasks. One was stored in a refrigerator at 5°-6° C. and the other in a glass-enclosed unheated room in order to obtain temperatures more nearly approaching seasonal outdoor temperatures (Autumn). When freezing temperatures occurred, the sample was transferred indoors where temperatures were somewhat cooler than "room temperatures" (20°-22° C.).

Table 4. The Longevity of Streptococci in Soils Inoculated with pure cultures.

Days After Inoculation	Refrigerated Soil: Streptococci per gram 37.5° C. Index Average	Refrigerated Soil: Streptococci per gram 45.5° C. Index Average	Refrigerated Soil: % H_2O	Soils Held at Summer Temperatures -Air Dried: Streptococci per gram 37.5° C. Index Average	Soils Held at Summer Temperatures -Air Dried: Streptococci per gram 45.5° C. Index Average	Soils Held at Summer Temperatures -Air Dried: % H_2O	Soils Held at Summer Temperatures -Kept Moist: Streptococci per gram 37.5° C. Index Average	Soils Held at Summer Temperatures -Kept Moist: Streptococci per gram 45.5° C. Index Average	Soils Held at Summer Temperatures -Kept Moist: % H_2O
1	1,045,000	-		550,000	-	30	127,000	-	30
3	1,025,000	-	24	2,370,000	-	16	152,000	-	-
8	95,000	-	19	1,150	-	9	1,093	-	15
12	18,200	2,600	17	112	27	16	60	30	15
15	70,000	1,700	16	43	25	8	35	25	10
19	10,610	830	17	37	9	6	26	25	17
25	31,600	1,380	14	9	3	2	25	25	25
32	16,500	995	12	<2	<2	2	-2	0	6
40	3,500	106	15	10	3	2	4	3	13
47	2,630	322	18	4	3	1	4	<1	27
52	995	52	30	2	<2	<1	3	3	10
52 C.	Coli 6,780		30	coli 94		<1	*		10

* Total bacteria per gram 0 days -2,500,000 52 days -100,000

STREPTOCOCCI INDICES IN SOIL

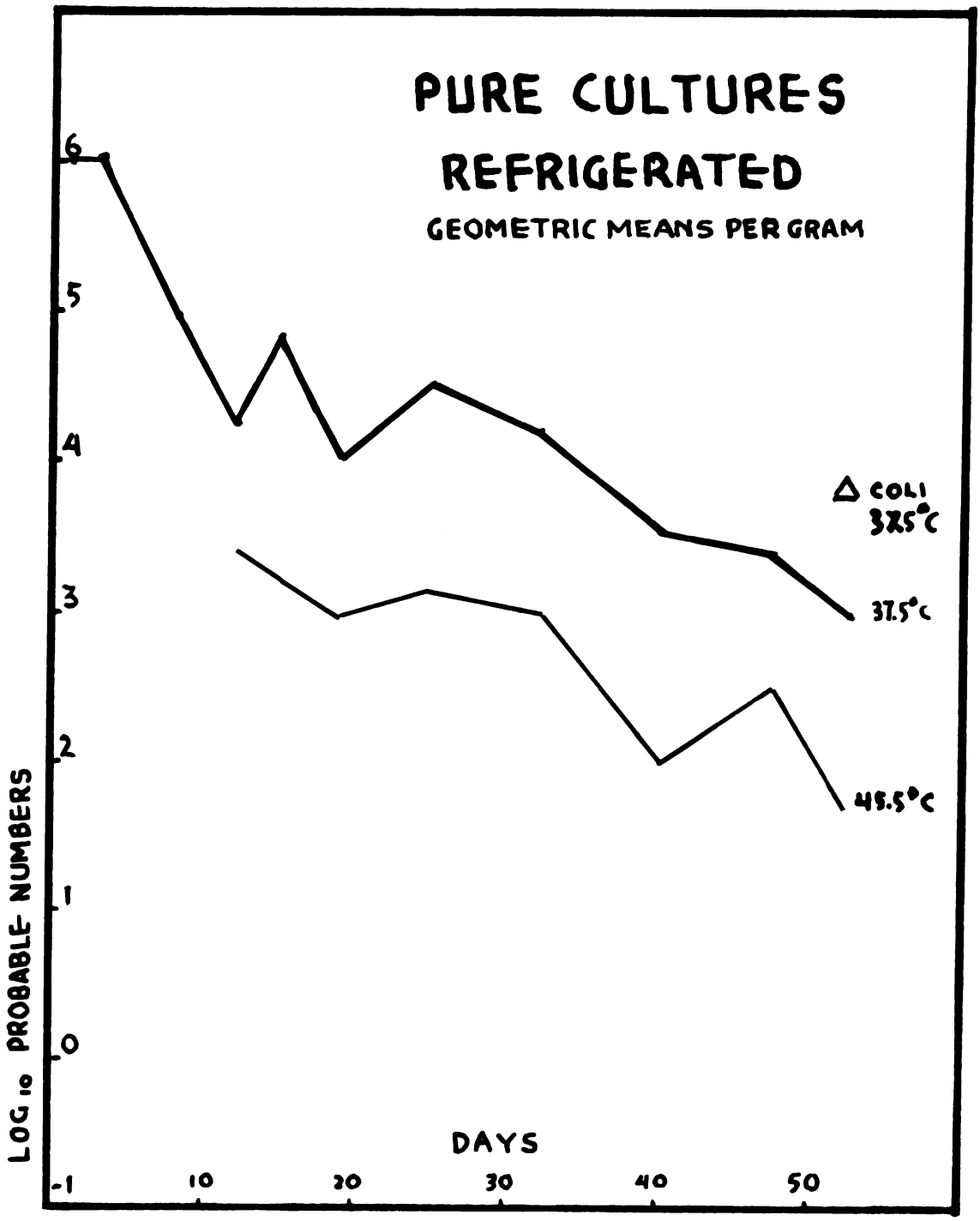

Fig. 4-A

STREPTOCOCCI INDICES IN SOIL

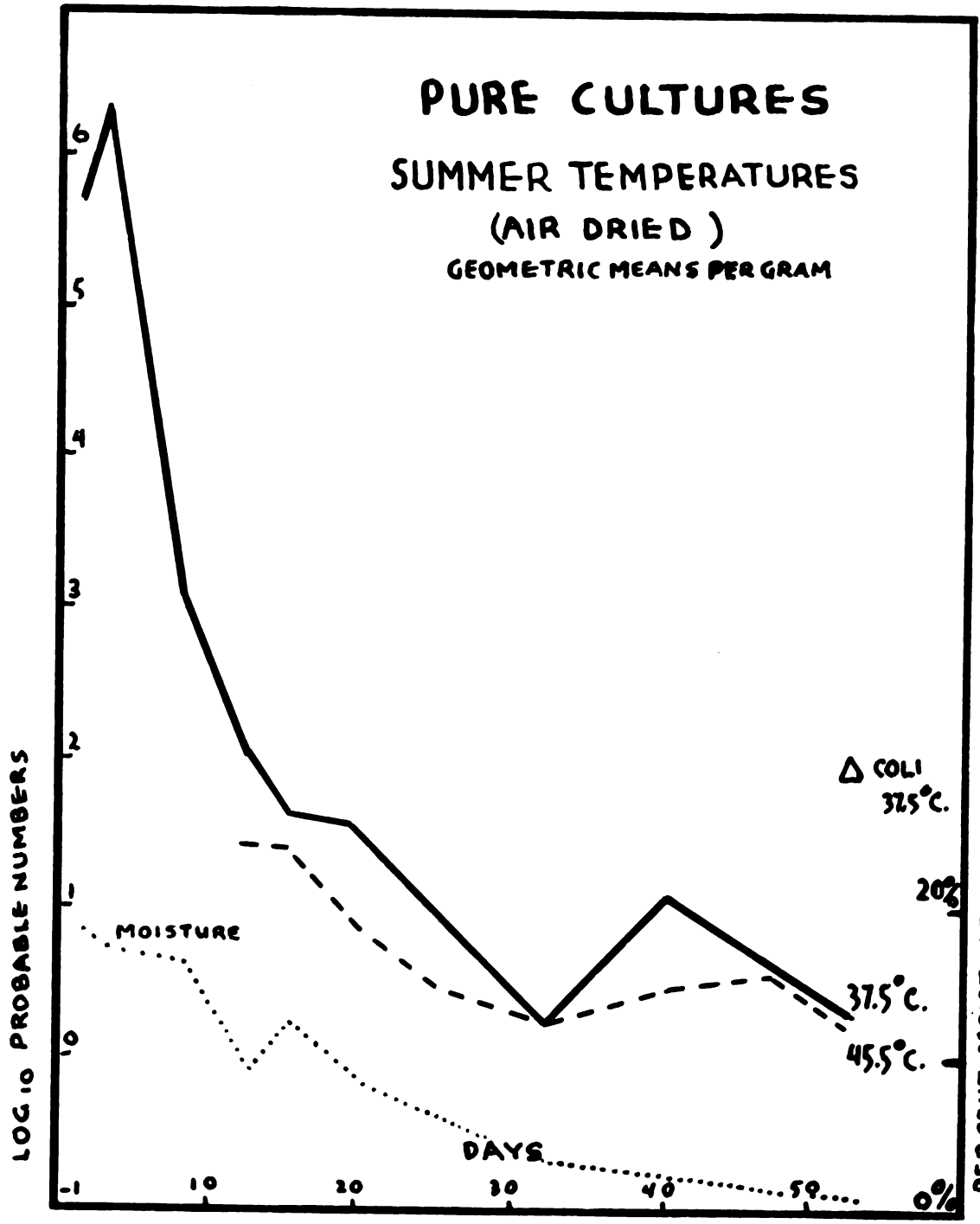

Figure 4-B

STREPTOCOCCI INDICES IN SOIL

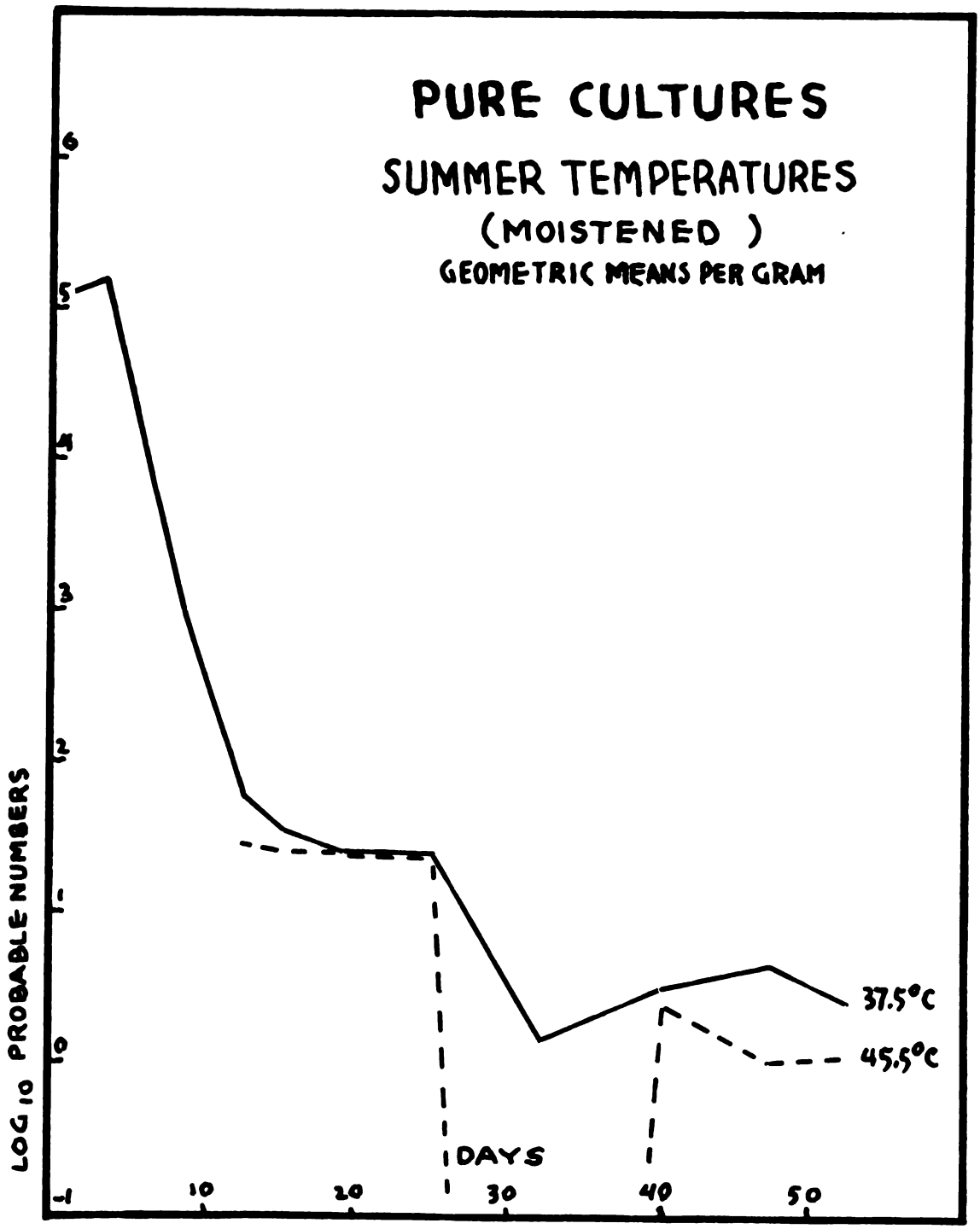

Figure 4-C

Thrice weekly both samples were aerated by transfer into 500 ml. sterile flasks and shaken mechanically for approximately 15 minutes. Serial decimal dilutions were made into single and double-strength lactose tryptose "SF" broth with Brom thymol blue indicator. Parallel dilutions were made for incubation at 37.5° C. and 45.5° C.

Smears were made from critical dilutions to SF agar (lactose, tryptose, Brom thymol blue). Microscopic examinations were made from these same dilutions.

When streptococci indices dropped to a low level inoculations were made into brilliant-green-bile-lactose broth for estimation of coliform density.

Data obtained on river water are presented in Table 5. Only those tubes which showed definite presence of streptococci are reported. All tubes which failed to produce colonies on SF (modified)agar or failed to show acidity or turbidity were reported as negative although some of these tubes undoubtedly contained low numbers of streptococci.

An examination of the data shows that the streptococci did not multiply in the river water samples; that the streptococci were almost completely eliminated after nine days of storage at outdoor temperatures and absent after 12 days. The refrigerated samples showed somewhat higher indices up to 16 days but zero at 47 days.

Table 5. Viability of Streptococci in a River Water

Days After Sampling	Storage at 5-6° C. (Control)			Storage at Autumn Temperatures		
	Streptococci per 10 ml.		Estimated Coliforms per 10 ml.	Streptococci per 10 ml.		Estimated Coliforms per 10 ml.
	37.5° C. Index	45.5° C. Index		37.5° C. Index	45.5° C. Index	
0	4,000	350	-	4,000	350	-
2	600	900	-	3	42	-
4	350	350	-	11	30	-
7	35	60	-	6	11	-
9	25	11	-	2	42	-
12	30	11	600	0	0	2,500
16	16	3	11	*0	*0	-
40	-	-	120	X	0	30
47	0	0.6	6	X	0	0.6

* Removed indoors

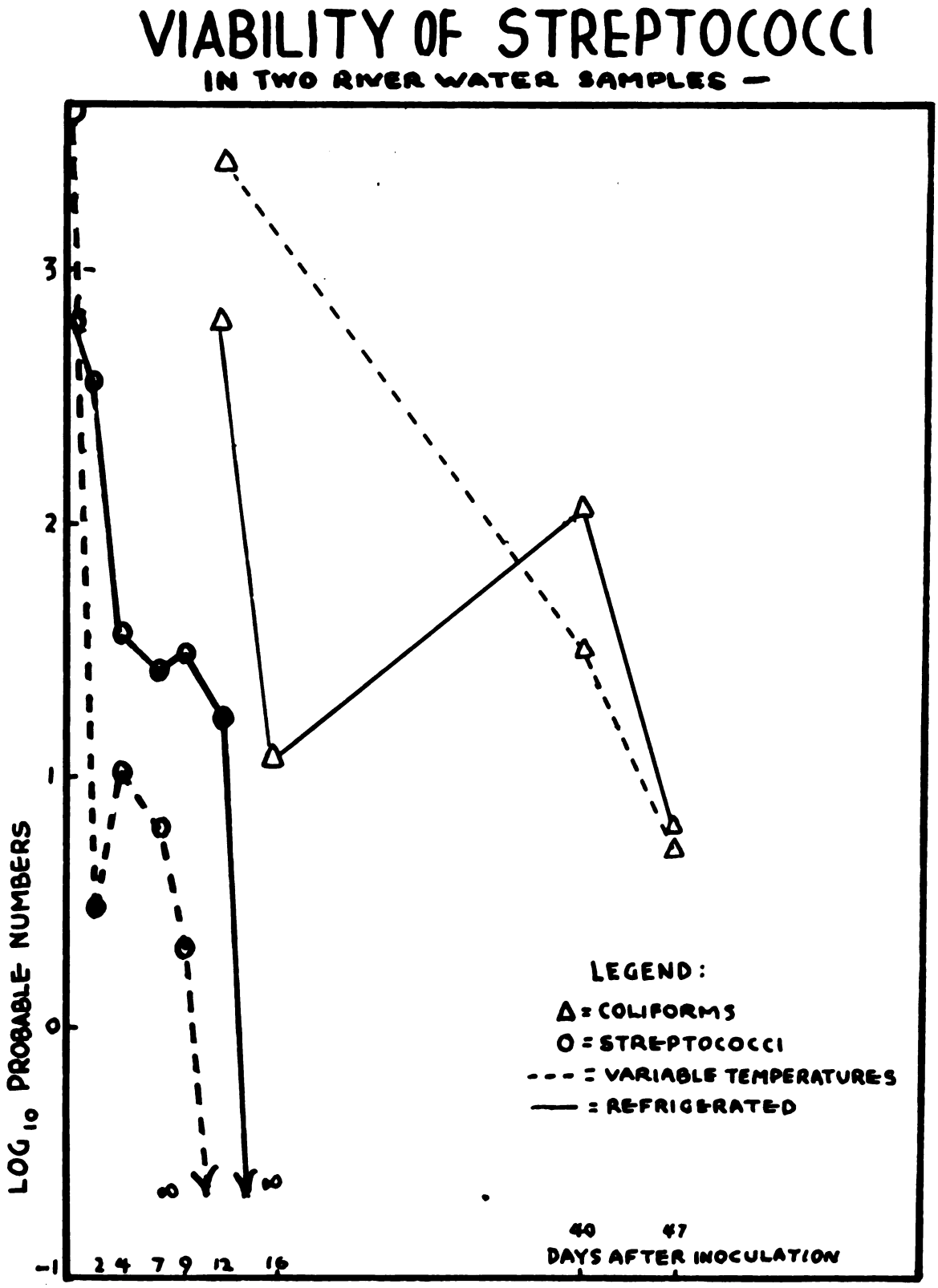
VIABILITY OF STREPTOCOCCI
IN TWO RIVER WATER SAMPLES —
LOG 10 PROBABLE NUMBERS
3
2
1
0
-1
2 4 7 9 12 16
40 47
DAYS AFTER INOCULATION
LEGEND:
Δ = COLIFORMS
O = STREPTOCOCCI
- - - = VARIABLE TEMPERATURES
—— = REFRIGERATED

Figure 5

Upon the first disappearance of streptococci, coliforms were found to be present in large numbers, but also gradually diminished by 47 days.

Streptococci in Manures

Twelve samples of animal manures were made by mixing fresh fecal matter from several animals. These samples were prepared from the excreta of horses, cattle and chickens. These different manures were then mixed with sandy loam soil using 16 parts of manure to 1000 parts of soil, The soil-manure mixes were each placed in tinned steel cans like those described previously.

Two samples of each type manure in soil were stored at 5-6° C. and two each at prevailing autumn temperatures until freezing weather necessitated removal indoors.

Duplicate samples were tested in the same manner as described previously for the pure culture inoculation studies. Soils were wetted periodically with distilled water to reduce the effect of desiccation, if any. One ml. portions of the soil suspension-dilutions were added to each tube containing 6 ml. of tryptose lactose SF broth and incubated 48 hours at 45.5° C. only. Microscopic and SF agar plate confirmations were made as previously described.

A presumptive coliform index was made at the termination of the study. Data compiled in Tables 6, 7 and 8 and in accompanying graphs show that streptococci may occur in fairly large numbers in horse manure and chicken manure, but are relatively few in cattle manure.

Table 6. Longevity of Streptococci in Soil Inoculated with 1.6% Horse Manure

Days After Inoculation	Stored at Autumn Temperatures		Stored at 5-6° C. (Control)	
	Streptococci Index per gram	% Moisture	Streptococci Index per gram	% Moisture
0	250,000	27	126,900	22
6	53,620	27	-	-
13	-	-	7,905	20
18	10,950*	19	-	-
23	4,290	21	4,949	20
30	1,823	16	2,530	14
37	123	9	-	18
42	1,600	12	-	-
43	-	-	1,057	18
69	25	10	81	10
69	Coliform Index 25	10	Coliform Index 83	10

* Moved Samples Indoors

STREPTOCOCCI IN HORSE MANURE

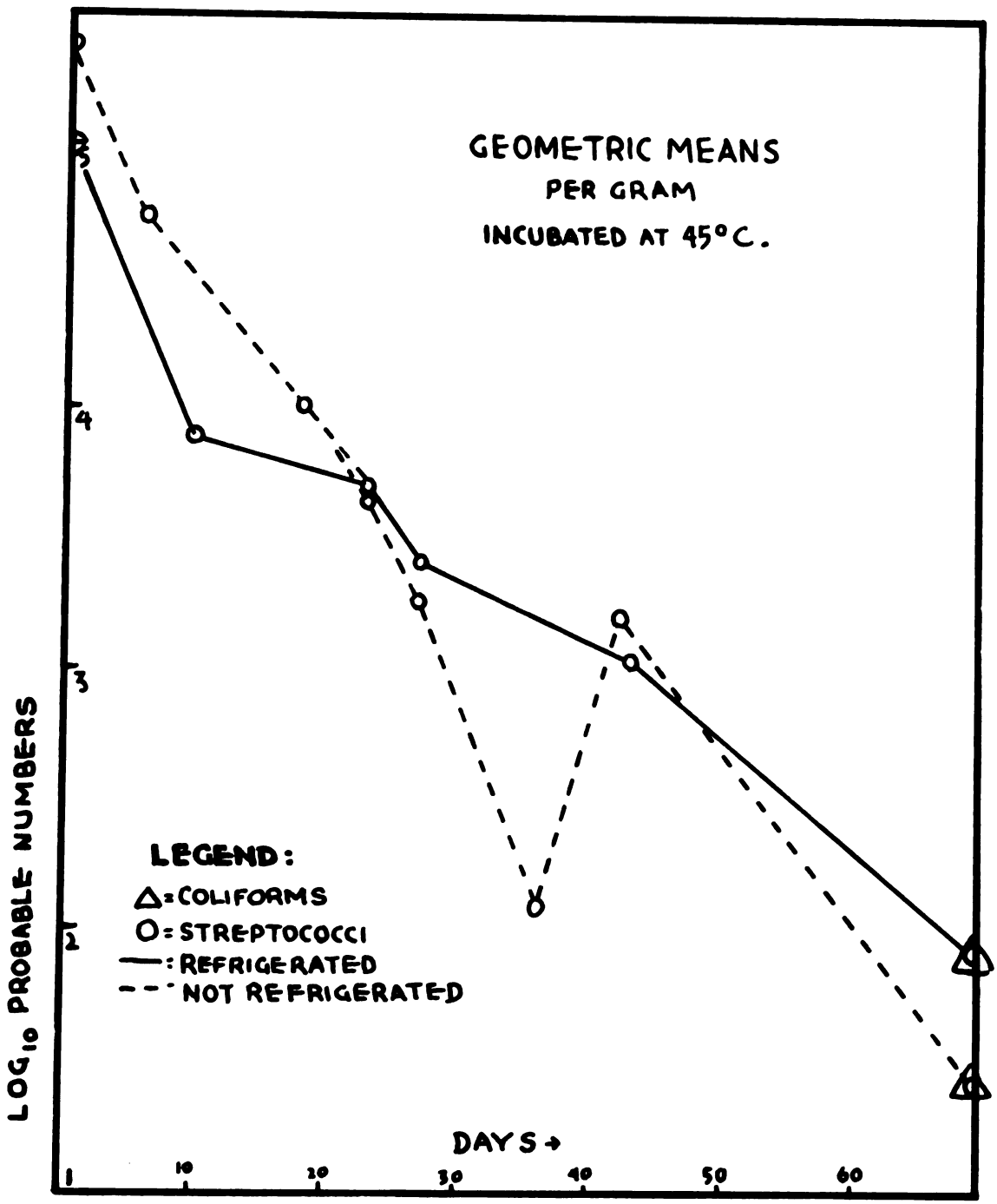

Figure 6

Table 7. Longevity of Streptococci in Soil Inoculated with 1.6% Cow Manure

Days After Inoculation	Stored at Autumn Temperatures		Stored at 5-6° C. (Control)	
	Streptococci Index per gram	% Moisture	Streptococci Index per gram	% Moisture
0	265	23	693	22
6	135	21	-	-
13	-	-	30	22
18	4*	18	- *	-
23	43	17	39	17
30	4	17	5	15
37	25	14	-	-
42	25	13	-	-
43	-	-	65	16
69	25	10	53	9
69 Coliform Index	25	10	25	9

*Moved Sample Indoors

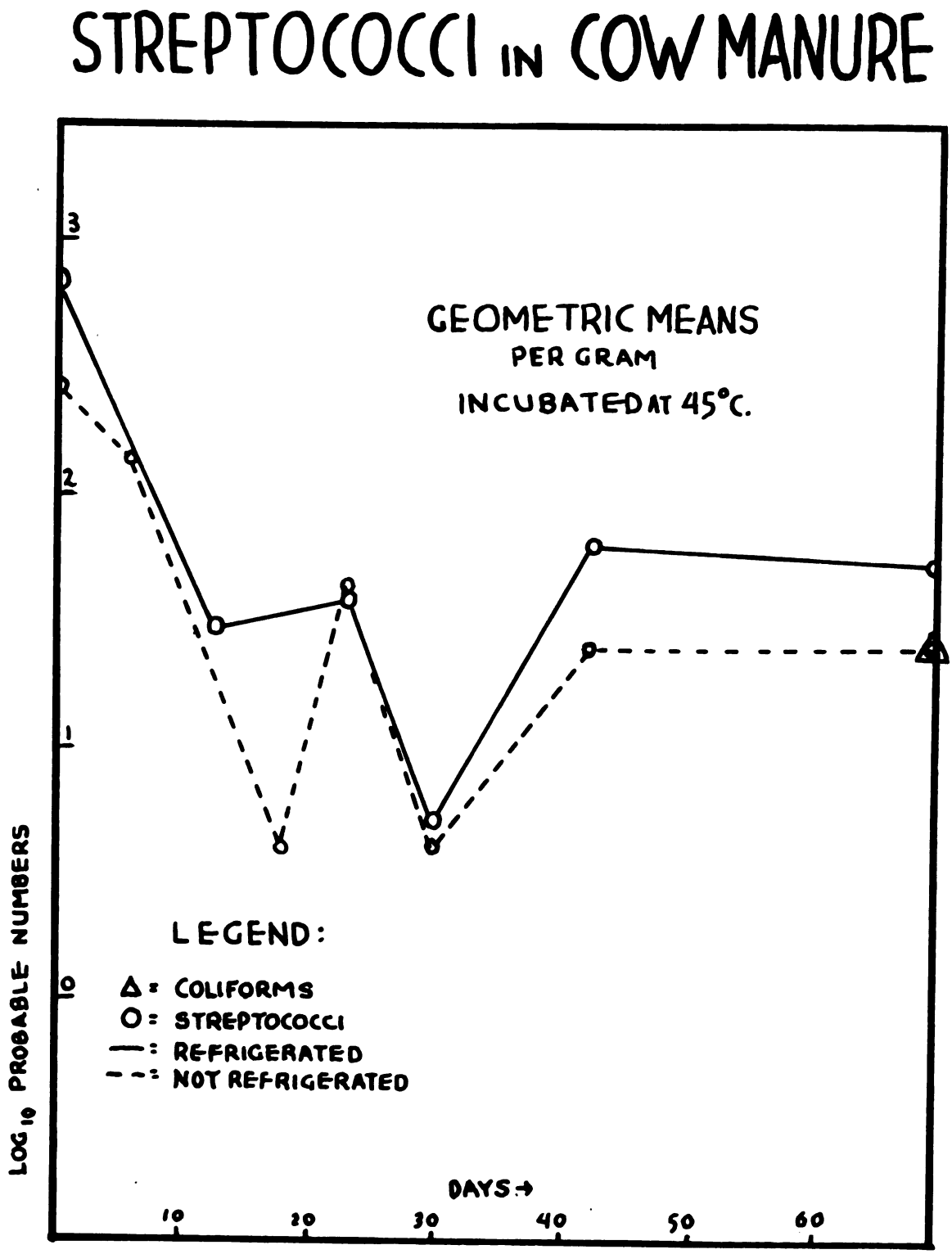
STREPTOCOCCI IN COW MANURE
GEOMETRIC MEANS
PER GRAM
INCUBATED AT 45°C.
LOG10 PROBABLE NUMBERS
3
2
1
LEGEND:
Δ = COLIFORMS
O = STREPTOCOCCI
— = REFRIGERATED
- - = NOT REFRIGERATED
DAYS →
10
20
30
40
50
60

Figure 7

Table 8. Longevity of Streptococci in Soil Inoculated with 1.6% Chicken Manure

Days After Inoculation	Stored at Autumn Temperatures		Stored at 5-6° C. (Control)	
	Streptococci Index per Gram	% Moisture	Streptococci Index per gram	% Moisture
0	60,000	22	401,300	23
6	160,000	20	---	-
13	-	-	118,300	21
18	6,344*	16	----	-
23	6,000	15	12,110	19
30	2,500	17	12,600	17
37	387	15	---	-
43	400	13	8,370	21
69	25	10	316	17
69	Coliform Index 1,000	10	—	17

*Moved Sample Indoors

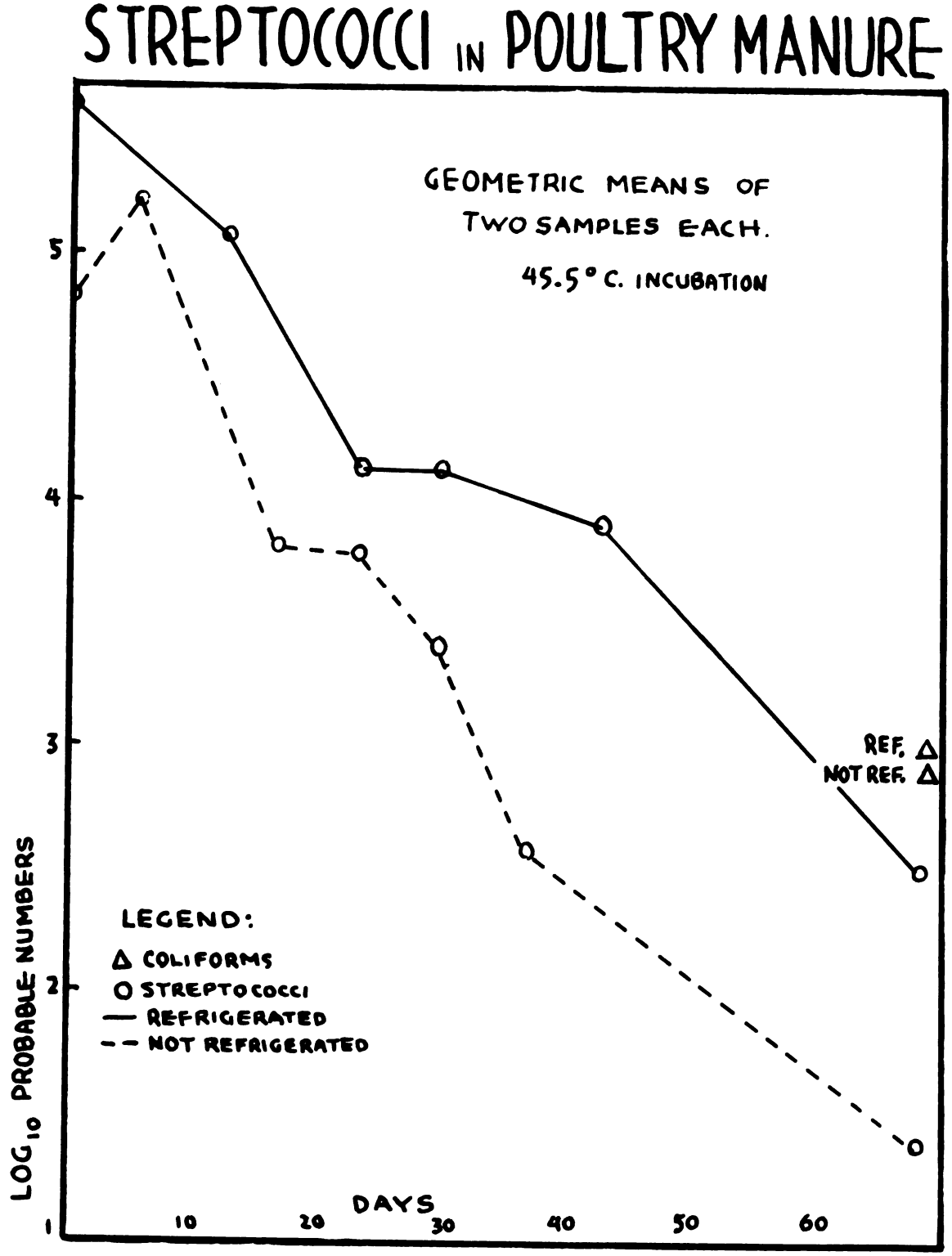
STREPTOCOCCI IN POULTRY MANURE
GEOMETRIC MEANS OF
TWO SAMPLES EACH.
45.5° C. INCUBATION
REF. Δ
NOT REF. Δ
LEGEND:
Δ COLIFORMS
O STREPTOCOCCI
— REFRIGERATED
-- NOT REFRIGERATED
LOG10 PROBABLE NUMBERS
DAYS
1
2
3
4
5
10
20
30
40
50
60

Figure 8

Reduction was slower in the manures than in the soils inoculated with pure streptococci cultures or in the river water. By three weeks time reduction was over 90 per cent. At 69 days the coliform indices were generally of the same order as those of the streptococci. The manures represented approximately a concentration of fecal matter, twice as great as is customarily employed on farms. The manure was not rotted. Recalling the large numbers in sewage sludges, one notes that reduction of the streptococci was less rapid in the presence of organic matter and very rapid when organic matter was less concentrated.

DISCUSSION

Published data concerning coliforms though voluminous, are not readily compared. Many early studies on lonvevity were not quantitative. Studies on sterile soil are not comparable to those made on natural soils because the bacterial environments in each are very dissimilar. Again, studies of sterilized or distilled waters cannot be evaluated on the same basis as waters which contain either living organisms or greater concentrations of possible nutrients, both of which may greatly affect the viability of the organisms. Methods and media used very probably affected the numerical results obtained.

However, it appears that streptococci do not increase in the soil or river water and that coliforms have been shown by other workers to increase under certain conditions.

Soils and waters which have a low content of fecal matter may be expected to show higher coliform indices than streptococci, but where pollution is greater, the streptococci populations closely parallel the coliform numbers.

The close parallelism shown in the 37.5° C. and 45.5° C. indices where samples had been stored and populations were low suggests that either of the incubation temperatures may serve to determine populations of marginal environments. The more hardy streptococci may be resistant to both prolonged exposure in the soil for water and to high incubation temperatures.

Recent studies by Seligmann (32) show marked variations in the streptococci indices of identical samples occasioned apparently by the differences in the composition of the various media. These studies suggest the probability of developing media more suitable for the estimation of streptococci than those used in this study.

It is obvious that soils freshly manured within 10 weeks of the sampling time cannot be properly evaluated by either coliform or streptococci enumerations. The media used does not aid in differentiating between human and animal sources of pollution.

The streptococci may be tentatively said to conform to the requirements for a test organism in the following respects.

1. Streptococci more closely resemble the pathogens with respect to lonvevity in water and soils than do the coliforms. They remain viable longer than most enteric pathogens but diminish rapidly, percentagewise.

2. Streptococci appear universally in mixed sewage and sewage polluted strams. They may not be present in certain individual stools. The latter may be said also for other organisms.

3. Evidence indicates that streptococci of excreta do not multiply or become saprophytic in environments which are not heavily polluted with fecal organic matter.

4. The technic of enumeration utilized in these studies are relatively simple, call for a minimum of special equipment and require only the ordinary technical precautions exercised by bacteriologists.

The methods give results within a short enough enterval to permit adaptation to routine work.

The relationship of the quantity and recency of pollution to the levels of streptococci populations required further study utilizing various measured amounts of fecal material in soils and water. Further studies on the effects, if any, of non-fecal organic matter on the viability of the streptococci may prove valuable.

The study completed demonstrates that streptococci may prove useful for sanitary evaluations of soil and water under certain conditions.

SUMMARY

Data presented show streptococci populations levels to be high in raw and treated sewage, in polluted river water and in soils inoculated with animal wastes.

Longevity studies show a streptococci reduction of over 99 per cent in manured soils within 18 days and from 70 to 80 per cent in 13 days even under conditions of refrigerated storage.

In polluted river water streptococci are shown to decrease from between 85 and 99 per cent in two days. They were completely absent as early as 12 days in water stored at early autumn temperatures, and were absent by the 47th day in a refrigerated sample.

Studies of the viability of cultures of streptococci placed in soil show reductions of 90 to 99.97 per cent in eight days.

A method which may be adapted to routine enumeration of streptococci is described.

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