THE SURVIVAL OF SELECTED NON-INDIGENOUS MICROORGANISMS IN THE SOIL ECOSYSTEM

by

CHARLES R. GARRIOTT

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Approved by:

John O. Harris

Major Professor

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INTRODUCTION

The microflora of the soil is made up of two basic groups of organisms; those normally present in a given soil type, and the transient forms. In nature, the indigenous forms are members of the trophic level known as the decomposers. They function in the mineralization and decomposition of organic matter. The transient forms enter the soil environment from the air from rainfall or from animals. Those organisms entering the soil from either the decay of, or excrement from animals are potentially pathogenic. Therefore, the study of their survival in the soil is not only of academic interest, but has a direct effect on man, due to the potential pathogenic character of some microorganisms. This potential source of pathogenic organisms is particularly important where soil runoff enters a source of drinking water.

It has been known for some time that the artifical introduction of large numbers of non-indigenous bacteria to the soil, leads to their rapid disappearance (Katznelson, 1940). Coincident with their disappearance is an alteration in the natural population of the soil. Waksman and Woodruff (1940) found that the number of soil bacteria and actinomycetes increased in soils enriched with living, non-indigenous bacteria. In the same study he found that further additions of enrichment organisms resulted in an increase in their death rate, and an increase in the number of antagonistic organisms. His studies indicated that this decline in number could be linked to the development of the antagonistic organisms.

In a subsequent study however, Waksman and Schatz (1946) failed to show the development of specific groups of organisms that were antagonistic to the enrichment organisms, and they concluded that the alteration of the

soil microflora may merely be due to the release of nutrients from dead bacterial cells. The question then, of why non-indigenous bacteria die, is still unanswered. It was the purpose of this study to investigate the survival of non-indigenenous bacteria in the soil and to clarify the reason for their rapid decline in numbers.

Two organisms were selected for the study; Escherichia coli and Serratia marcescens. E. coli was selected because it is a representative of the enteric bacteria and is used as an indicator of fecal contamination. S. marcescens, also a member of Enterobacteriacae, was selected because it has been isolated with increasing frequency from patients, and has been incriminated as the causative agent in a variety of infections (Crowder, 1971; von Graeventz and Bucholz, 1971; Wilfert, Barrett and Kass, 1968).

LITERATURE SURVEY

The survival and establishment of non-indigenous bacteria in soil has been studied since the beginning of this century. There is a particularly large volume of literature on attempts to control the population of plant pathogens, by maintaining large populations of antagonistic organisms in the soil. Other than few notable early successes (Millard and Taylor, 1927; Sanford and Broadfoot, 1931), subsequent experiments led to disappointing results.

Temple (1911-12) disproved a long time belief that stable manure played a role as a soil inoculant by demonstrating that the multiplication of bacteria in soil, as a result of addition of stable manure, was due entirely to the organic constituents, and not to the bacterial population of the manure. Sterile manure had the same effect upon bacterial multiplication as did fresh, unsterile manure.

Katznelson (1940) studied the survival of typical soil bacteria, fungi and actinomycetes, in five soils of varying organic matter content and pH values. All organisms, when introduced into the soil, decreased in number. Pseudomonas fluorescens, Fusarius culimorum and Actinomyces cellulosae disappeared completely. He attributed their decline in numbers to some, or all of the following factors: (1) lack of a proper food supply, (2) unfavorable environment, (3) competition for nutrients or (4) development of antagonistic soil organisms.

In an attempt to encourage the development of antibiotic producing actinomycetes by soil enrichment methods, Waksman and Woodruff (1940) again confirmed the fact that non-indigenous bacteria decline rapidly in soils. In an effort to stimulate the development of organisms antagonistic to

Aerobacter aerogenes, Escherichia coli and Brucella abortus, he noted that the coliform bacteria tended to disappear very rapidly from the soil. However, the total soil population increased as a result of the addition of these bacteria. He felt that the disappearance of E. coli in the soil, was not due to its inability to grow and multiply in the soil, but to its destruction by soil antagonists.

The destruction of E. coli by soil antagonists was further supported by the results of a parallel study (Waksman and Woodruff, 1940) in which the survival of E. coli and A. aerogenes were studied in sterile soil, sterile soil enriched with glucose, and sterile soil enriched with dried blood. Both organisms were found to multiply rapidly in sterile soil. The addition of glucose had an unfavorable effect upon E. coli but not upon A. aerogenes. Dried blood had little effect upon the development of the two organisms. These results, he reasoned, were due to the release of nutrients as a result of sterilization. The heating of soil with glucose seemed to have resulted in the production of substances injurious to E. coli.

In a subsequent study Waksman and Schatz (1946) again attempted to study the development of antagonistic organisms by enriching soils with E. coli and Sarcinia lutea. This experiment threw doubt on Waksmans' earlier findings, and they summarized by reporting that such soil enrichment affect the microbial population of the soil only to a limited extent, as determined by the plate method. They concluded that there was still insufficient proof that any organism thus stimulated in the soil, are antagonistic to the added cells, or produce antibiotic agents active against the enrichment bacteria.

Antibiotic producing organisms are however, found to exist in the soil environment, and their isolation and cultivation have had unprecidented

benefits on the health and welfare of man. Most of these antibiotics have a fairly limited spectrum; each being somewhat specific for groups of organisms. It is not unreasonable then, that antibiotic producing organisms should be incriminated in the destruction of transient bacteria in the soil ecosystem. The usefulness of this argument is dependent upon a number of factors, one of which is that the antibiotics must be produced in the soil. This very question stimulated a number of investigations.

Lewis (1929), while studying bacterial antagonism of Pseudomonas fluorescens on spore forming bacteria of soils, made a careful study of the antibiotic principle elaborated. The principle could be absorbed from culture media by charcoal and field soil, but when he cultured the antagonist in sterile manured soil and extracted with water, he could find no evidence of the antibiotic in the extract.

Waksman and Woodruff (1942) obtained ether extracts of two soils which demonstrated actinomycin-like activity against Bacillus subtilis and S. lutea. When actinomycin was added to the soil, it was largely inactivated. They attributed this effect to adsorption of the antibiotic by the soil, or to its destruction by soil microorganisms. These investigators concluded that antibiotics of the actinomycin type exert very little effect on the soil population.

Not all evidence of the production of antibacterial principles by antagonist and their ability to limit the development of susceptible organisms in nature, is negative. Nickell and Burkholder (1947) demonstrated that an antagonistic actinomycete which inhibited the growth of Azotobacter vinelandi on laboratory media, also prevented the development of the bacterium in amended soil culture. They were also able to obtain an extract

of a soil culture of the actinomycete, which demonstrated marked antibacterial activity.

Numerous instances of biological control of plant disease have also been reported, but investigation on the mechanism of such antagonism are lacking (Allen and Haenseler, 1935; Anwar, 1949; Johnson, 1931).

Siminoff and Gottlieb (1951) reported a preliminary study on the fate of streptomycin in the soil. Their investigation included two studies: (1) a biological study in which an organism which produced a chemically defined antibiotic (streptomycin) was cultured with a susceptible organism, and (2) a physico-chemical study of the effect of the soil and other natural substrates on the antibiotic. They found that the streptomycin producing organism, Streptomyces griseus, had an inhibitory effect upon the susceptible organism, B. subtilis. The same effects however, were observed when B. subtilis was grown in mixed culture with a mutant of S. griseus, RM 3380, which did not produce the antibiotic.

Their findings on the physico-chemical studies confirmed the earlier suggestion of Waksman. They found that colloidal complexes such as clays and soil organic matter, adsorbed and effectively inactivated streptomycin, and they concluded that basic antibiotics such as streptomycin, do not play an active role in the soil.

In a subsequent study, Gottlieb and Simonoff (1951) found that chloromycetin, a neutral antibiotic, could be produced by Streptomyces venezulae when grown in unamended soil. The production of chloromycetin could be greatly inhanced by amending the soil with tryptone. However, S. venezulae was unable to antagonize the growth of B. subtilis in unamended soil. Chloromycetin was active in the soil, although the activity level was below that in broth.

They also found that when chloromycetin was added to nonsterile soil, it was rapidly degraded by members of the soil population. They concluded that chloromycetin was potentially able to play a significant role in the biological equlibrium of the soil, in terms of its adsorption and activity. However, when translated into field conditions, the production and accumulation of chloromycetin in the soil might, at least, be only negligible.

Koike (1956) also studied the role and production of antibiotics in the soil, in relation to the control of root-rot disease in plants. He found that there was no correlation between the production of antibiotics in the laboratory and in the soil.

Factors that could lead to the decline in numbers of transient bacteria then, are: (1) lack of a proper food supply, (2) unfavorable environment such as reaction or competition for nutrients, or (3) the development of antagonistic soil organisms. Another concept that comes into the picture is the well-documented, but little understood concept of ecological niche. It is commonly observed that a species, when introduced into an established community, usually does not persist. Presumably it dies out because it finds no role for itself; there are no unoccupied niches the alien can fill, and the microorganism has no way of maintaining itself (Alexander, 1971).

MATERIALS AND METHODS

General

The soil used throughout the study was a sandy-loam obtained from a cultivated field south of Manhattan, Kansas. The soil was sieved through a 10 mesh wire screen, to break up large clumps, to remove stones and coarser plant residues, and to obtain a fairly uniform particle size.

The moisture-holding capacity of the soil was determined by placing 100 gram samples of air-dried soil in a saturated, filterpaper-lined funnel, and measuring the volume of distilled water required to saturate the soil.

The moisture content of the air-dried soil was determined by placing 10 gram samples in evaporating dishes of known dry weight, and drying them at 105°C for 48 hours.

An organic content analysis of the soil was performed by the Soils Testing Laboratory, Kansas State University. The organic content was 0.8% as determined by the wet chromic acid method.

For pH determination of the soil samples, 10 gram aliquots of soil were placed in glass beakers large enough to permit the immersion of the electrodes of a Beckman pH meter. Enough distilled water was mixed with the samples to form a thin paste. The pastes were then allowed to stand at room temperature for approximately 20 minutes prior to making the pH readings.

Identification of E. coli was accomplished by plating dilution samples on either EMB agar (Difco) or Endo agar (Difco). Endo agar was later chosen as the medium of choice, because it seemed to be more selective against the natural soil population.

S. marcescens was identified by the appearance of red colonies, due to the production of the pigment prodigiosin, when plated on Starch Agar. The

Starch Agar had the following composition:

Proteos Peptone No. 3	2.5 gm
Yeast Extract	1.5 gm
KH_2PO_4	2.0 gm
K_2HPO_4	0.5 gm
Soluble Starch	5.0 gm
Ionagar No. 2	10.0 gm
Distilled Water	1,000 ml

Dilution samples of 0.1 ml volumes were evenly distributed on the surface of the plates by means of sterile bent-glass rods. Each dilution was plated in triplicate, incubated at 30°C, and counted 24 hours after plating. The average number of organisms counted, was recorded.

Preliminary Study

In order to verify earlier findings of the survival of E. coli in soil, and to ascertain its growth pattern in the soil used. The survival of the organism was followed in the following soil series:

1. Natural, unamended soil
2. Sterilized soil
3. Sterilized soil amended with 1% glucose
4. Natural soil amended with 1% glucose

Soil samples of 200 gram quantities were placed in sterile 500 ml wide-mouthed bottles. In those series which followed the survival in sterilized soil, 15 ml of distilled water were throughly mixed with 200 grams air-dried soil. The bottles were then tightly covered with aluminum foil, and autoclaved at 126.5°C for 2 hours.

The inoculum was prepared by placing 3 loops of E. coli from a 24 hour slant culture, into 30 ml sterile distilled water. The number of organisms per ml was then determined by standard dilution plating methods. One ml of this stock culture was then added to 29.5 ml sterile water blanks, and this volume added to the soil samples and thoroughly mixed. (30.5 ml = 50% moisture holding capacity of the soil)

In the soils that were amended with glucose, the 1 ml inoculum was added to sterile water blanks, each containing 2 grams of glucose (14.5 ml for sterilized soil and 29.5 ml for natural soil). The glucose solutions were autoclaved for 15 minutes at 126.5°C.

The inoculated soils were incubated at 30°C and the moisture content was maintained at approximately 50% by periodic addition of sterile water based on weight loss.

Actinomycete Isolation

Actinomycetes were isolated from the soil by first plating the soil in various dilutions on Mineral Salts Agar with 0.5% Casein added (see Appendix I for medium composition). This medium tended to inhibit the faster growing bacterial colonies. The plates were incubated at 30°C for a period of from 3 to 7 days. The actinomycete colonies were then p[illegible]ked at random and tested for antibiotic production by the cross-streak method on Brain-heart agar (Difco). Because the actinomycetes are slower growing than either E. coli or S. marcescens, they were allowed to grow 3 days before cross-streaking with the test organisms. This gave them time to develop into visible "colonies" and also to produce any antibiotic.

A total of 76 actinomycete colonies were tested, out of which only 2 showed antibiotic activity against E. coli and S. marcescens. These two

actinomycetes were then grown in shaker flasks, and the broth was tested for antibiotic content by the paper-disc method of Loo et al. (1945). The paper discs of approximately 6.8 mm in diameter were punched from Whatman No. 1 filter paper, and sterilized prior to use.

The two actinomycetes produced an antibiotic that was active against both test organisms. However, they were unequal in their effectiveness, as determined by the cross-streak method. Therefore, the actinomycete most active against S. marcescens was designated as "actinomycete #1", and the one most effective against E. coli was designated as "actinomycete #2". A third actinomycete which showed no activity by either the cross-streak or paper disc method, was carried along for use as a control, and was designated as "actinomycete #3".

Comparative Study

Difficulty in mixing the test organisms with the soil for the preliminary study, prompted a few alterations in the protocal for this study. Instead of 200 gram samples of soil, 20 gram samples were used. In addition, the duration of the study was lengthened. In this study, the survival of E. coli and S. marcescens was followed in:

1. Natural, unamended soil
2. Sterilized soil
3. Sterilized soil amended with 1% glucose
4. Sterilized soil amended with 1% peptone
5. Natural soil amended with 1% glucose
6. Natural soil amended with 1% peptone
7. Sterilized soil with: actinomycete #1 for S. marcescens; actinomycete #2 for E. coli.

8. Sterilized soil with actinomycete #3.

For those mixed culture soil series (actinomycete + test organism) the soil sample was inoculated with the respective actinomycete 3 days prior to the introduction of the test organism. This allowed the actinomycete time to establish itself in the soil and, also, to produce the antibiotics, if the actinomycete was capable of production in the soil environment.

Twenty grams of soil were placed into 6 oz. prescription bottles and stoppered with cotton plugs to minimize water loss. Each series was sampled according to the following schedule: Initial; day 1; day 2; day 4; day 6; day 8; day 11; day 15, and day 20.

The innoculum was prepared in much the same way as it was in the preliminary study. One loop from a 24 hour slant culture was added to a 75 ml water blank, and the number of organisms per ml was determined by standard dilution plating methods. One ml of this stock culture, plus 2 ml distilled water were then added to each soil sample. (3 ml = 50% moisture holding capacity). The above volume was then mixed throughly with the soil.

Sampling consisted of washing the 20 gram soil samples with a 180 ml sterile water blank. From this 1:10 dilution, the number of organisms per gram of soil could be determined by standard dilution plating methods.

Substrate Concentration Effect

Meaningful counts were not obtained for the studies concerning the survival of S. marcescens in natural-amended soils. The natural soil populations were so increased by the fourth day of study, that they completely overgrew the plates, of the dilution at which S. marcescens could be counted. Pigment development was inhibited by this crowding effect.

To combat this problem, a new, selective media containing erythritol as the sole carbon source was used (Slotnick and Dougherty, 1972). The media was developed as a means of identification of S. marcescens in clinical cases, but to the author's knowledge the media had never been used to select against soil bacteria. It proved so successful at selecting against the natural population, that it was used in all subsequent studies which used S. marcescens as a test organism.

The data recorded to this point indicated that nutrient addition had a negative, long-term effect on the survival of non-indiginous bacteria. It was of interest to determine to what extent this was true. Therefore, the survival of S. marcescens in natural soil, with the following concentrations of amendments was followed: 0.2%, 0.6% and 1.0% Peptone, and 0.2%, 0.6% and 1.0% Glucose. In addition, its survival in natural, unamended soil was followed, in order that a comparison could be made.

The protocol followed, was the same as that used in the Comparative Study, except that the selective media was used to obtain the counts for S. marcescens.

Soil Survey

A thorough search of the available literature failed to reveal the frequency of S. marcescens presence in the soil. Breed, R. S., et al. (1957) reported that the organism is found in milk, soil, water, foods and in silk worms and other insects. Information as to the presence, and frequency of S. marcescens in the soil would have a direct bearing on the present study, and also, would be of value to those interested in the clinical aspects of S. marcescens infection.

With the use of the new selective media, it was found that approximately 60% of the organisms could be immediately cultured from the soil. They could be identified by plating dilutions as low as 1:100. Therefore, the value of this media was again demonstrated, and was particularly useful in a soil survey.

One hundred soils from the surrounding Manhattan, Kansas area were sampled during the month of March, 1973, for the presence of S. marcescens. The survey included 33 samples of cultivated soil, 34 samples of uncultivated, ungrazed soils, and 33 samples of feed lot soil. The presence of S. marcescens was identified by the appearance of the typical red colony on the erythritol plates. Total cell counts were obtained by plating appropriate dilutions on half-strength nutrient agar. In addition, pH, and dry weight of each soil sample was determined.

RESULTS

Results obtained from the Preliminary Study

The results obtained from the study of the survival of *E. coli* in natural, unamended soil confirmed the findings of earlier investigators. The population of *E. coli* decreased from an initial count of 480,000 per gram to 4,800 per gram in three days.

A problem of mixing the test bacteria evenly throughout the 200 gram soil sample made itself apparent in this study. For example, the natural soil was innoculated with 355,000 organisms per gram initially. From this innoculum, 480,000 organisms were immediately cultured (135%). The problem was overcome in the subsequent studies by using smaller (20 gram) soil samples. The entire sample was then used in the dilution sequence.

An additional difficulty was encountered in this study, and unfortunately, could not be overcome. The difficulty presented itself in those studies where the survival of *E. coli* was followed in natural, amended soil. The natural population of the soil was so increased that *E. coli* colonies could not be identified. A mucoid colony, similar in appearance to *Enterobacter aerogenes* made its appearance on both the EMB and Endo plates on day three. The colony was also red, and in its presence, the typical green sheen of *E. coli* did not develop until after approximately 72 hours of incubation. In fact, when the mucoid colony and *E. coli* were growing in close proximity, they tended to merge, and a large liquid mass of growth resulted. After three or four days of growth, the whole mass developed a green sheen on EMB agar. Neither the EMB or the Endo plates selected against the muccoid colony. Therefore, the study of *E. coli* in natural unamended soil was discontinued.

When E. coli was added to sterile soil, the population increased from 443,000 per gram to 118,000,000 per gram in two days. This population maintained itself until day 4, then decreased to a count of 93,000 per gram on day 16. Numerous explanations of this initial increase in numbers after sterilization have been proposed. Included among these are: (1) inactivation of toxins present in the soil, (2) modification of nutrients present in the soil, so as to make them more available for bacterial metabolism, and (3) destruction of antagonistic organisms such as the protozoan population and antibiotic producing organisms (Waksman, 1932).

The findings of Waksman and Schatz (1946) were not substantiated in the study involving the survival of E. coli in sterile soil amended with 1% glucose. The numbers of E. coli increased from 297,000 to a maximum of 158,000,000 in four days. This population maintained itself, numbering 66,000,000 on day 16. In the Waksman study, soil and glucose were sterilized together, whereas in the present study, the glucose solution was autoclaved separately and only for a period of 15 minutes. The difference in results may be due to a difference in protocol. In fact, Waksman indicated that E. coli probably died because of a toxic substance produced by heating glucose and soil together.

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Fig. 1. Preliminary Study. The results for the survival of *E. coli* when introduced into natural unamended soil, and incubated at 30°C. The population of *E. coli* was determined by plating serial dilutions on Endo plates.

Day of Sample	Average Total Counted
Initial	480,000
Day 1	156,000
Day 2	64,000
Day 3	4,800

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● Survival of E. coli in natural soil

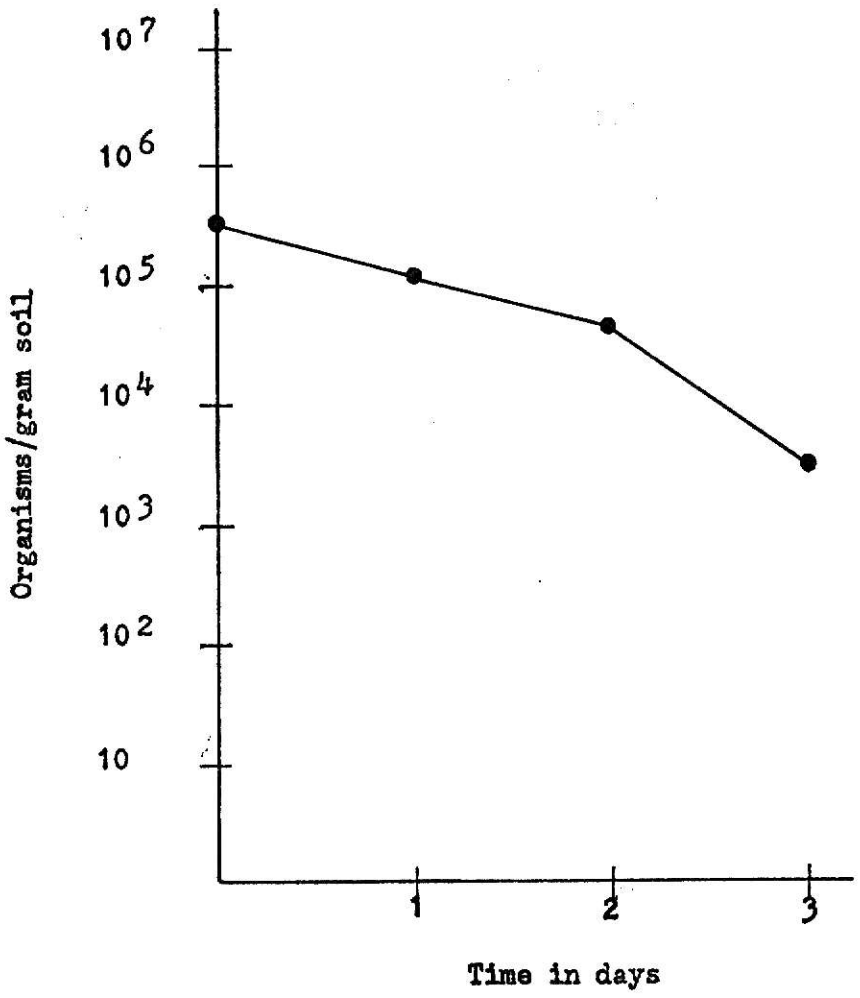


Fig. 2. Preliminary Study. The results for the survival of E. coli when introduced into sterile soil, sterile soil amended with 1% glucose, and natural soil amended with 1% glucose. The soils were incubated at 30°C. The population of E. coli was determined by plating serial dilutions on Endo plates.

Sterile Soil

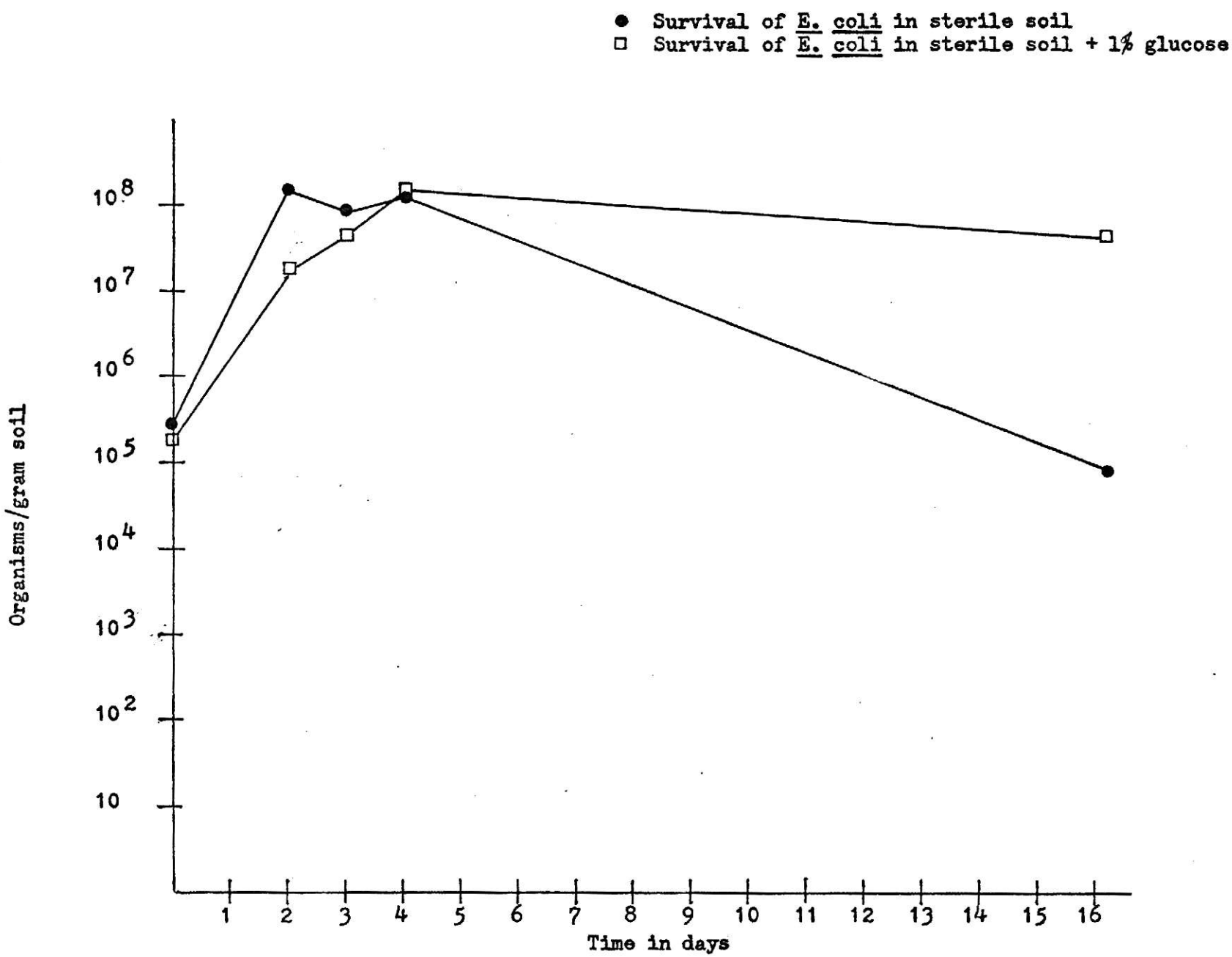
Day of Sample	Average Total Counted
Initial	443,000
Day 2	118,000,000
Day 3	91,000,000
Day 4	118,000,000
Day 16	93,000

Sterile Soil Amended With 1% Glucose

Day of Sample	Average Total Counted
Initial	297,000
Day 2	24,600,000
Day 3	65,000,000
Day 4	158,000,000
Day 16	66,000,000

Natural Soil Amended With 1% Glucose

Day of Sample	Average Total Counted
Initial	176,000
Day 1	767,000
Day 2	330,000
Day 3	
Day 4	Discontinued
Day 16	


● Survival of E. coli in sterile soil
□ Survival of E. coli in sterile soil + 1% glucose
Organisms/gram soil
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Time in days

Results: Actinomycete isolation and antibiotic assays.

Out of 76 actinomycete colonies isolated and tested, only two indicated antibiotic production effective against E. coli and S. marcescens. Determination of antibiotic production was based on a zone of inhibition by cross-streaking on Brain-Heart agar (Difco). Both organisms produced an antibiotic that was effective against both test organisms, but were unequal in their effectiveness.

Actinomycete #1 was most effective against S. marcescens. Actinomycete #2 was most effective against E. coli. A third actinomycete was carried along in the experiment as a control; it exhibited no antagonistic properties. Below are the respective zones of inhibition as determined by the cross-streak method on Brain-Heart agar. The actinomycetes were allowed to develop for 3 days, prior to cross-streaking with the test organism.

	E. coli	S. Marcescnes
Actinomycete #1	12 mm	22 mm
Actinomycete #2	19 mm	11 mm
Actinomycete #3	none	none

In an attempt to identify the antibiotic principle elaborated by the organisms, they were cultured in shaker-flasks containing a variety of different media. Zones of inhibition, as determined by the paper-discs method, and pH reactions, are reported in Table I. The composition of the various media tested, are reported in Appendix I.

The data indicates the difficulty encountered in maintaining a good titer of the antibiotic in submerged, shaker-flask cultures. However, it was established that an antibiotic was produced, and its production was verified by two different methods.

Table 1. Antibiotic assay of antibiotic production in submerged, shaker-flask cultures.

Medium A Czapeks broth
Medium B Conn's medium
Medium C Glycerol-calcium malate broth
Medium D Nutrient broth
Medium E Brain-heart broth
Medium F Glucose-trypton-yeast extract broth
Medium G Emersons broth
Medium H Potato-Glucose broth
Medium I Casine hydrosylate-Phyton broth

Actinomycete/ Medium	Zones of inhibition*						Final+ pH
	E. coli			S. marcescens			
	Day 3	Day 6	Day 12	Day 3	Day 6	Day 12	
1-A	--	--	--	--	--	--	7.4
1-B	--	--	--	--	--	--	8.32
1-C	1 mm	1 mm	--	1 mm	--	--	6.2
1-D	4 mm	4.5 mm	halo	3.5 mm	1.5 mm	1 mm	9.1
1-E	--	--	--	1 mm	--	--	9.3
1-F	3 mm	1 mm	--	1 mm	halo	--	8.2
1-G	--	1.5 mm	--	--	--	--	8.5
1-H	--	--	--	--	--	--	7.6
1-I	2 mm	6 mm	4 mm	1 mm	2 mm	3 mm	7.9
2-A	--	halo	--	--	--	--	7.5
2-B	--	halo	--	--	--	--	8.1
2-C	--	--	--	--	--	--	4.8
2-D	1 mm	1 mm	--	--	--	--	8.5
2-E	1 mm	--	--	1 mm	--	--	8.5
2-F	--	--	--	2 mm	--	--	3.8
2-G	halo	2 mm	--	--	--	--	8.2
2-H	--	--	--	--	--	--	7.4
2-I	1 mm	2 mm	1.5 mm	--	--	--	7.2
3-A	--	--	--	--	--	--	8.5
3-B	--	--	--	--	--	--	8.2
3-C	--	--	--	--	--	--	9.5
3-D	--	--	--	--	--	--	9.5
3-E	--	--	--	--	--	--	8.5
3-F	--	--	--	--	--	--	8.6
3-G	--	--	--	--	--	--	7.8
3-H	--	--	--	--	--	--	7.6
3-I	--	--	--	--	--	--	7.5

* Zone of inhibition measured from edge of disc to edge of zone.

\+ Initial pH of all media = 7 ± .25

A number of variables, such as temperature, amount of inoculum, aeration and fermentation medium, were experimented with, but further work on the characterization of the antibiotic principle was discontinued in favor of returning to the problem at hand; that of the survival of the test bacteria in the soil.

Results: Comparative Study

The comparative study was an extension of the preliminary study, and the results confirmed and elaborated on the findings reported from that study.

The population of E. coli again died rapidly in natural, unamended soil. The numbers decreased from 730,000 per gram to 630 per gram in six days.

It is interesting to compare the growth curves of E. coli in sterile soil, and sterile soil amended with 1% glucose. The data in these two series are very similar and do not correlate well with that found in the preliminary study, where it was found that E. coli maintained its population in the glucose-amended, sterile soil, and decreased slowly in sterilized soil. Whether or not this discrepancy was due to a change in protocol is not known.

Peptone proved to be a better source of nutrients. The growth of E. coli in peptone-amended, sterile soil was more typical of the growth curve of bacteria in liquid culture; giving a semi-logarithmic growth and death phase. E. coli increased from an initial count of 530,000 per gram to 440,000,000 per gram in one day. The population then fell throughout the remaining period of study, reaching less than 300 per gram on day 15.

The study of the growth of E. coli in normal soil, amended with glucose and peptone, was discontinued after two days, due to the presence of the

mucoid colony experienced in the preliminary study. It proved to be impossible to obtain meaningful counts.

The antibiotic elaborated by actinomycete #2 did not inhibit the growth of E. coli in mixed culture. The actinomycete was allowed to develop 3 days prior to the inoculation with E. coli, in order that the organism could establish its population and if capable, to produce the antibiotic. It is important to mention here, that the actinomycete was isolated from the soil used throughout the experiment; the same soil in which E. coli died when introduced in the natural, unamended state.

When the growth curve of E. coli in mixed culture with actinomycete #1 is compared with that of its growth with actinomycete #3, it is evident that actinomycete #3 exerted a greater inhibitory effect than did #1. Actinomycete #3 did not produce an antibiotic when grown on laboratory media.

The above data is reported in graphic form in figures 3 through 5.

In general, S. marcescens was more successful than E. coli in the soil environment. It survived longer, and reached a greater population density.

In natural soil S. marcescens decreased from 4,660,000 per gram to 190,000 per gram in 6 days. On day eight, no colonies could be detected at a 1:100 dilution.

Again, glucose did not enhance the maintance of S. marcescens in sterile soil. In fact, the organism died more rapidly in sterile soil amended with glucose, than in unamended sterile soil. S. marcescens increased from 7,600,000 per gram to 270,000,000 per gram in one day, maintained its population to day 11, and then dropped to 24,000 per gram on day 15, in sterile soil. In sterile soil amended with glucose the S. marcescens population increased from 5,600,000 per gram to 550,000,000 per gram in one day,

Fig. 3. Comparative Study. The results for the survival of *E. coli* when introduced into natural soil and sterile soil. The soils were incubated at 30°C, and the population of *E. coli* was determined by plating serial dilutions on Endo plates.

Natural Soil

Day of Sample	Average Total Counted
Initial	730,000
Day 1	340,000
Day 2	62,000
Day 4	2,600
Day 6	630
Day 8	None at a 1:100 dilution

Sterile Soil

Day of Sample	Average Total Counted
Initial	520,000
Day 1	220,000,000
Day 2	180,000,000
Day 4	170,000,000
Day 6	140,000,000
Day 8	110,000,000
Day 11	120,000,000
Day 15	None at a 1:100 dilution

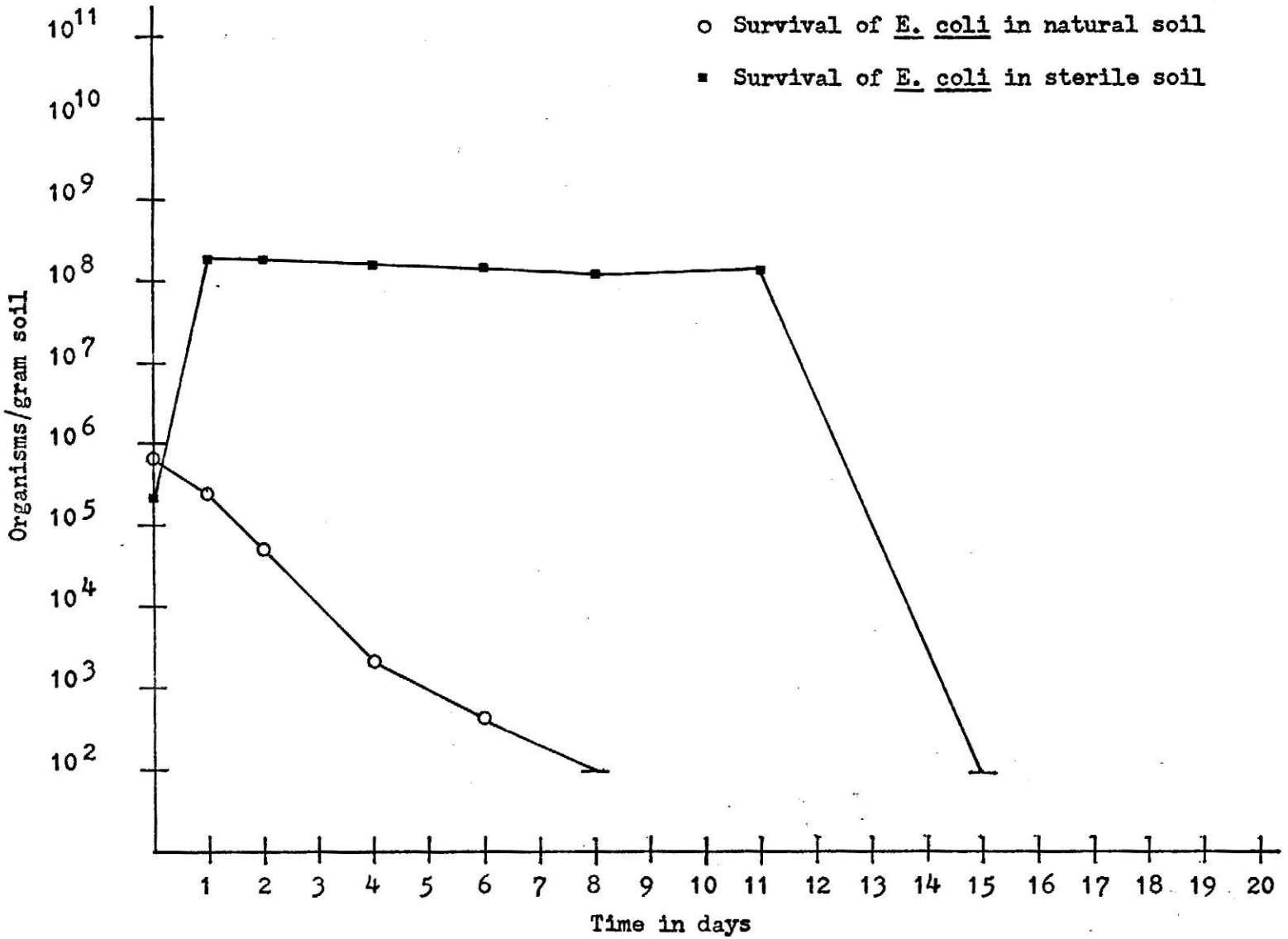

o Survival of E. coli in natural soil
▪ Survival of E. coli in sterile soil
Organisms/gram soil
10^{11}
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Time in days

Fig. 4. Comparative Study. The results for the survival of *E. coli* when introduced into sterile soil amended with 1% glucose, and sterile soil amended with 1% peptone. The soils were incubated at 30°C, and the population of *E. coli* was determined by plating serial dilutions on Endo plates.

Sterile Soil + 1% Glucose

Day of Sample	Average Total Counted
Initial	530,000
Day 1	440,000,000
Day 2	350,000,000
Day 4	290,000,000
Day 6	260,000,000
Day 8	180,000,000
Day 11	110,000,000
Day 15	None at a 1:1,000 dilution

Sterile Soil + 1% Peptone

Day of Sample	Average Total Counted
Initial	480,000
Day 1	1,200,000,000
Day 2	53,000,000,000
Day 4	120,000,000
Day 6	73,000,000
Day 8	2,500,000
Day 11	740,000
Day 15	None at a 1:100 dilution

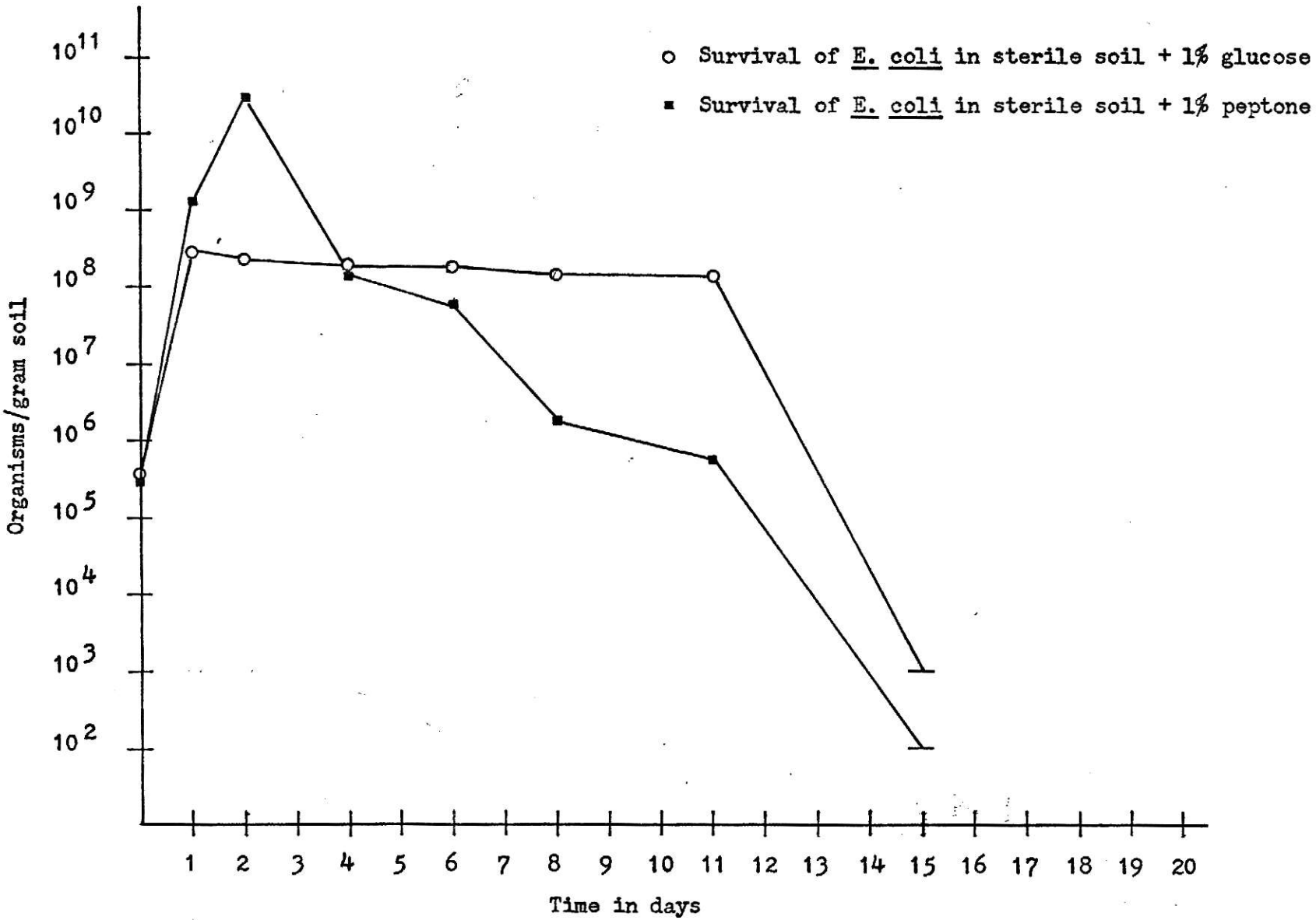

o Survival of E. coli in sterile soil + 1% glucose
▪ Survival of E. coli in sterile soil + 1% peptone
Organisms/gram soil
10^{11}
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Time in days

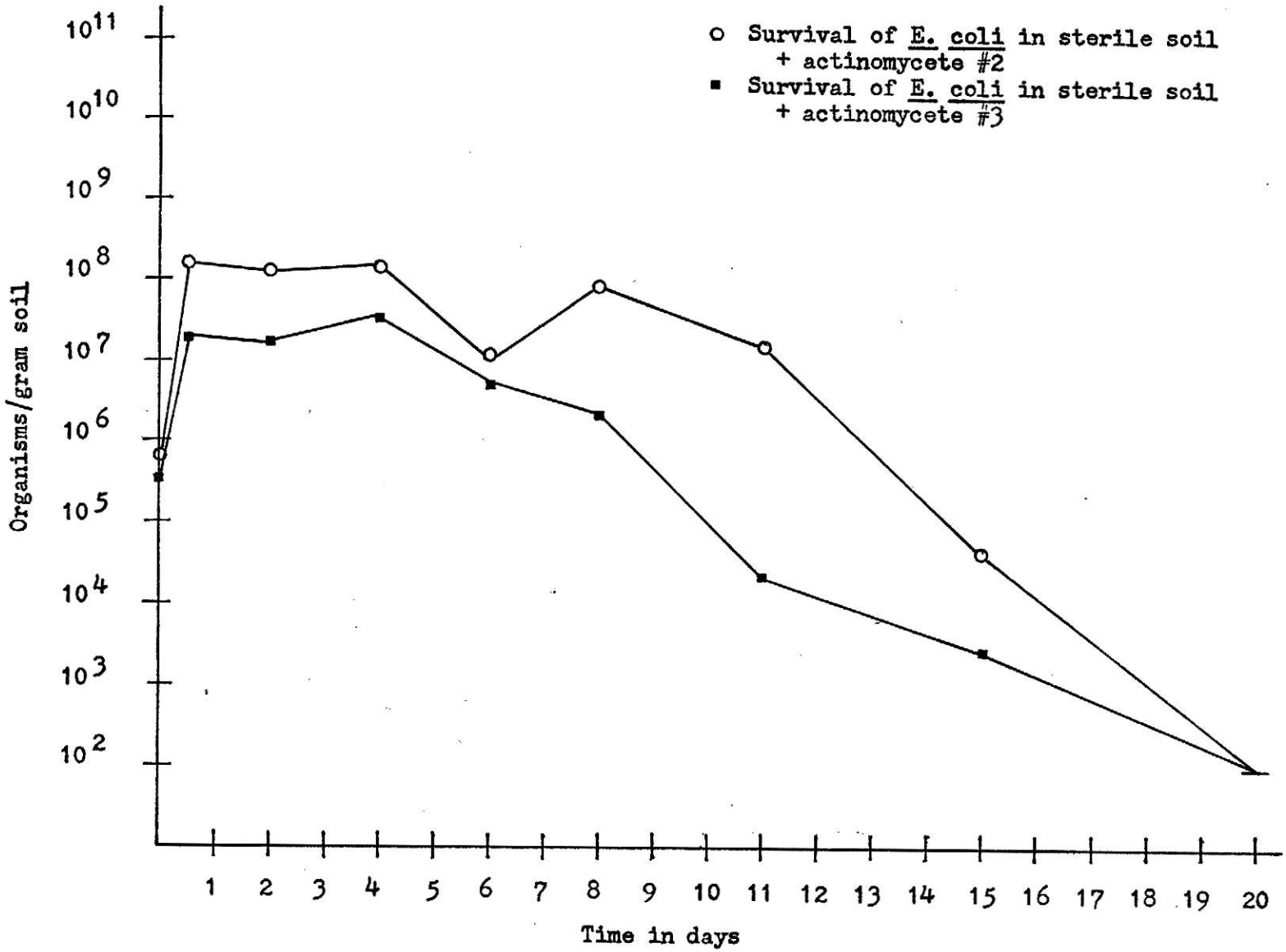
Fig. 5. Comparative Study. The results for the survival of *E. coli* when grown in mixed soil culture with an antibiotic producing actinomycete (#2) and a non-antibiotic producing actinomycete (#3). The soils were incubated at 30°C, and the population of *E. coli* Endo plates.

E. coli + actinomycete #2

Day of Sample	Average Total Counted
Initial	690,000
Day 1	190,000,000
Day 2	110,000,000
Day 4	180,000,000
Day 6	33,000,000
Day 8	91,000,000
Day 11	12,000,000
Day 15	16,000
Day 20	None at a 1:100 dilution

E. coli + actinomycete #3

Day of Sample	Average Total Counted
Initial	460,000
Day 1	10,000,000
Day 2	12,000,000
Day 4	16,000,000
Day 6	11,000,000
Day 8	3,400,000
Day 11	33,000
Day 15	3,800
Day 20	None at a 1:100 dilution


○ Survival of E. coli in sterile soil + actinomycete #2
■ Survival of E. coli in sterile soil + actinomycete #3
Organisms/gram soil
10^{11}
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Time in days

but maintained its population only to day 8, where a count of 7,130,000 per gram was recorded. No colonies were detected on day 11, at a 1:1,000 dilution.

Again peptone proved to be a better source of nutrients than glucose, but the effects were short lasting. The population jumped from 3,400,000 to 83,000,000,000 per gram in 2 days, after which the curve is more typical of that determined from the sterile soil study.

The antibiotic principle elaborated from actinomycete #1 was not effective against S. marcescens in the soil environment. A comparison of the curve obtained from the mixed culture study with actinomycete #1, will demonstrate that it is almost identical with that obtained from the study with actinomycete #3. The presence of the actinomycetes actually had a favorable effect on the survival of S. marcescens, when compared to its survival in sterile soil.

These data are reported in graphic form in figures 6 through 9.

Fig. 6. Comparative Study. The results for the survival of S. marcescens when introduced into natural soil, and sterile soil. The soils were incubated at 30°C, and the population of S. marcescens was determined by plating serial dilutions on starch agar.

Natural Soil

Day of Sample	Average Total Counted
Initial	4,700,000
Day 1	1,000,000
Day 2	1,400,000
Day 4	340,000
Day 6	190,000
Day 8	None at a 1:100 dilution

Sterile Soil

Day of Sample	Average Total Counted
Initial	7,600,000
Day 1	270,000,000
Day 2	1,000,000,000
Day 4	630,000,000
Day 6	2,560,000,000
Day 8	420,000,000
Day 11	140,000,000
Day 15	24,000
Day 20	None at a 1:100 dilution

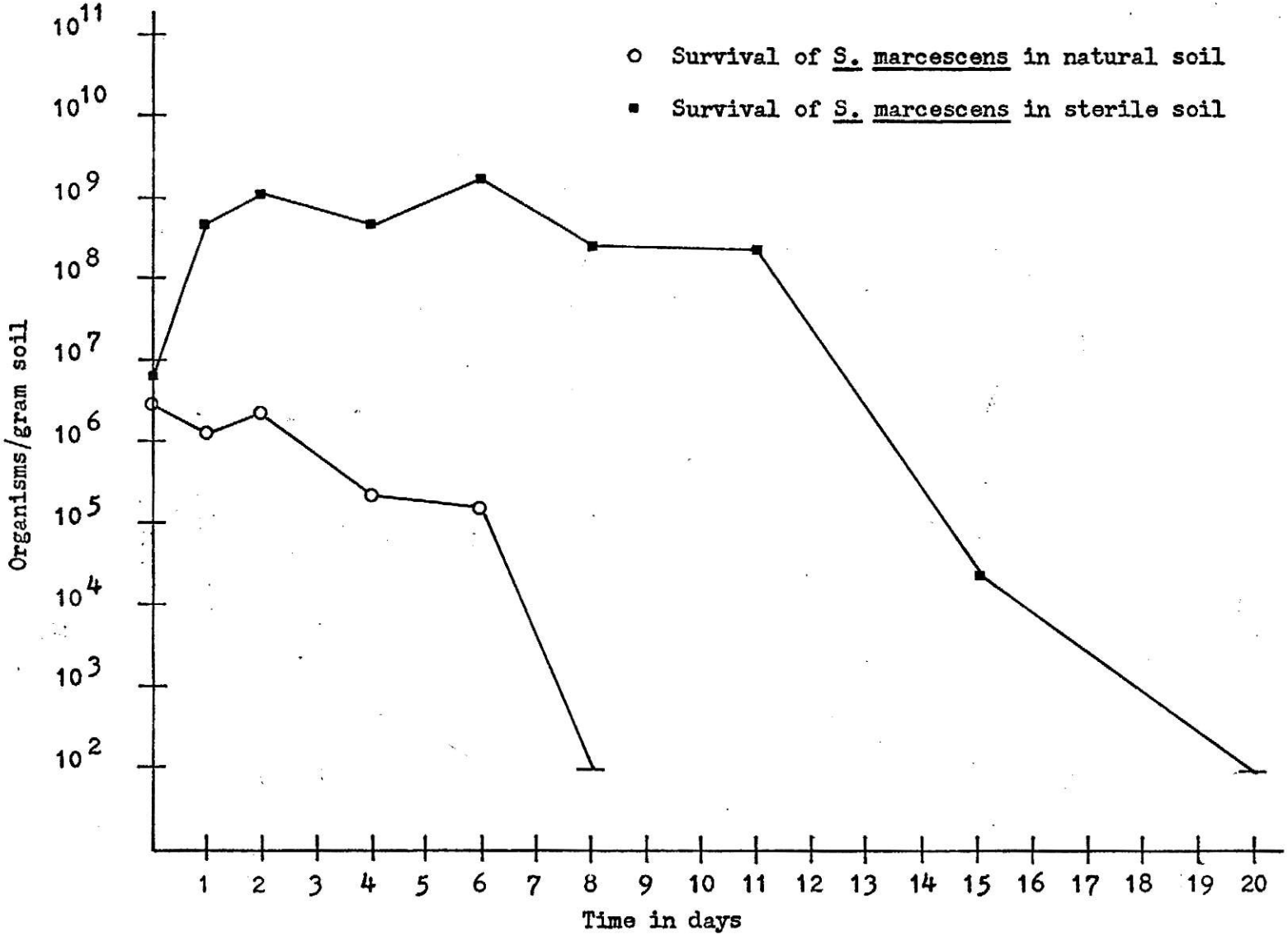

o Survival of S. marcescens in natural soil
▪ Survival of S. marcescens in sterile soil
Organisms/gram soil
10^{11}
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Time in days

Fig. 7. Comparative Study. The results for the survival of S. marcescens when introduced into sterile soil amended with 1% glucose, and sterile soil amended with 1% peptone. The soils were incubated at 30°C, and the population of S. marcescens was determined by plating serial dilutions on starch agar.

Sterile Soil + 1% Glucose

Day of Sample	Average Total Counted
Initial	5,600,000
Day 1	550,000,000
Day 2	2,800,000,000
Day 4	660,000,000
Day 6	100,000,000
Day 8	7,100,000
Day 11	None at a 1:1,000 dilution

Sterile Soil + 1% Peptone

Day of Sample	Average Total Counted
Initial	3,400,000
Day 1	3,300,000,000
Day 2	83,000,000,000
Day 4	640,000,000
Day 6	590,000,000
Day 8	640,000,000
Day 11	380,000,000
Day 15	710,000
Day 20	None at a 1:100 dilution

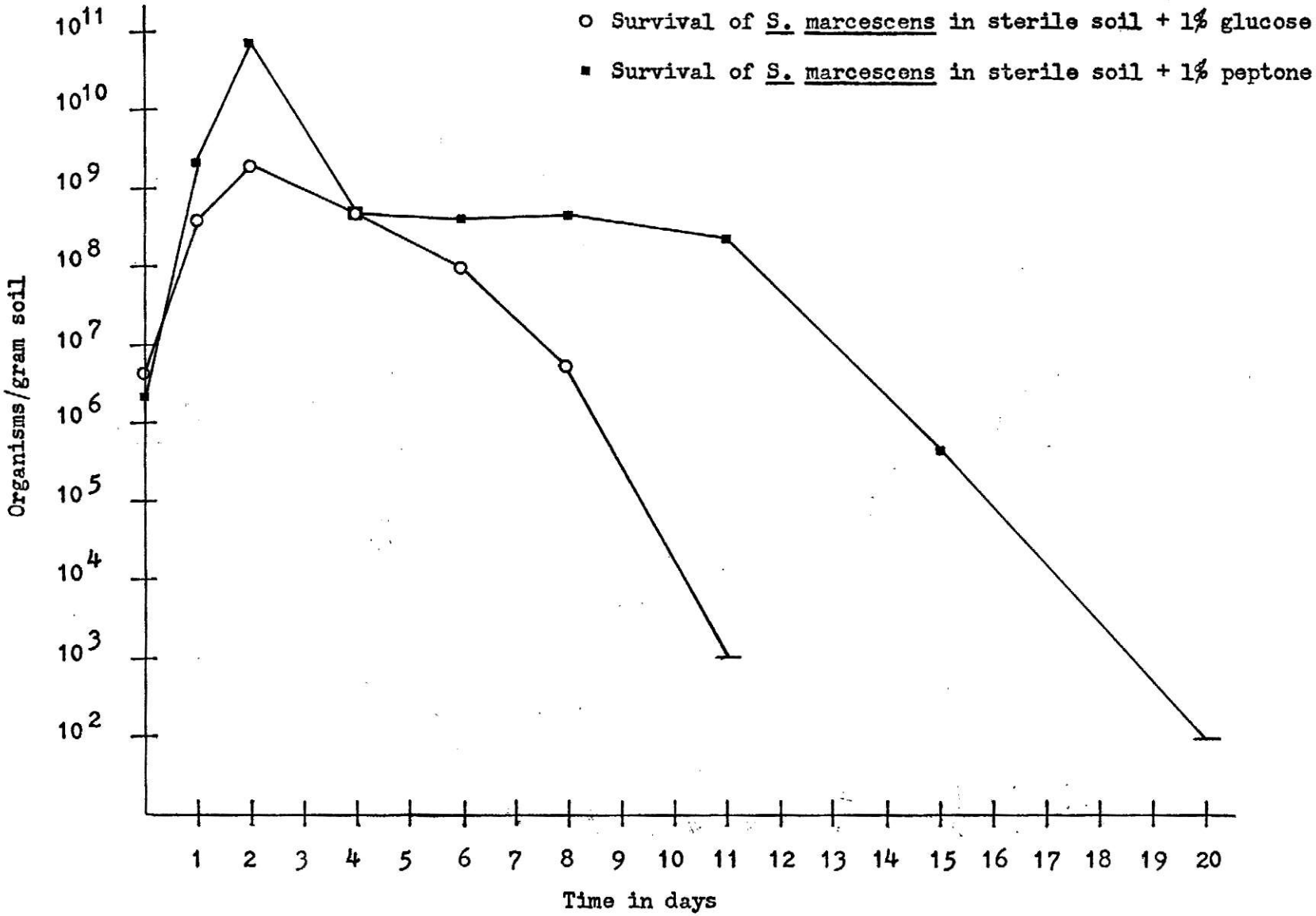

o Survival of S. marcescens in sterile soil + 1% glucose
▪ Survival of S. marcescens in sterile soil + 1% peptone
Organisms/gram soil
10^{11}
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Time in days

Fig. 8. Comparative Study. The results for the survival of S. marcescens when grown in mixed soil culture with an antibiotic producing actinomycete (actinomycete #1). The cultures were incubated at 30°C, and the population of S. marcescens was determined by plating serial dilutions on starch agar.

S. marcescens + actinomycete #1

Day of Sample	Average Total Counted
Initial	4,500,000
Day 1	400,000,000
Day 2	850,000,000
Day 4	490,000,000
Day 6	240,000,000
Day 8	240,000,000
Day 11	170,000,000
Day 15	10,000,000
Day 20	None at a 1:100 dilution

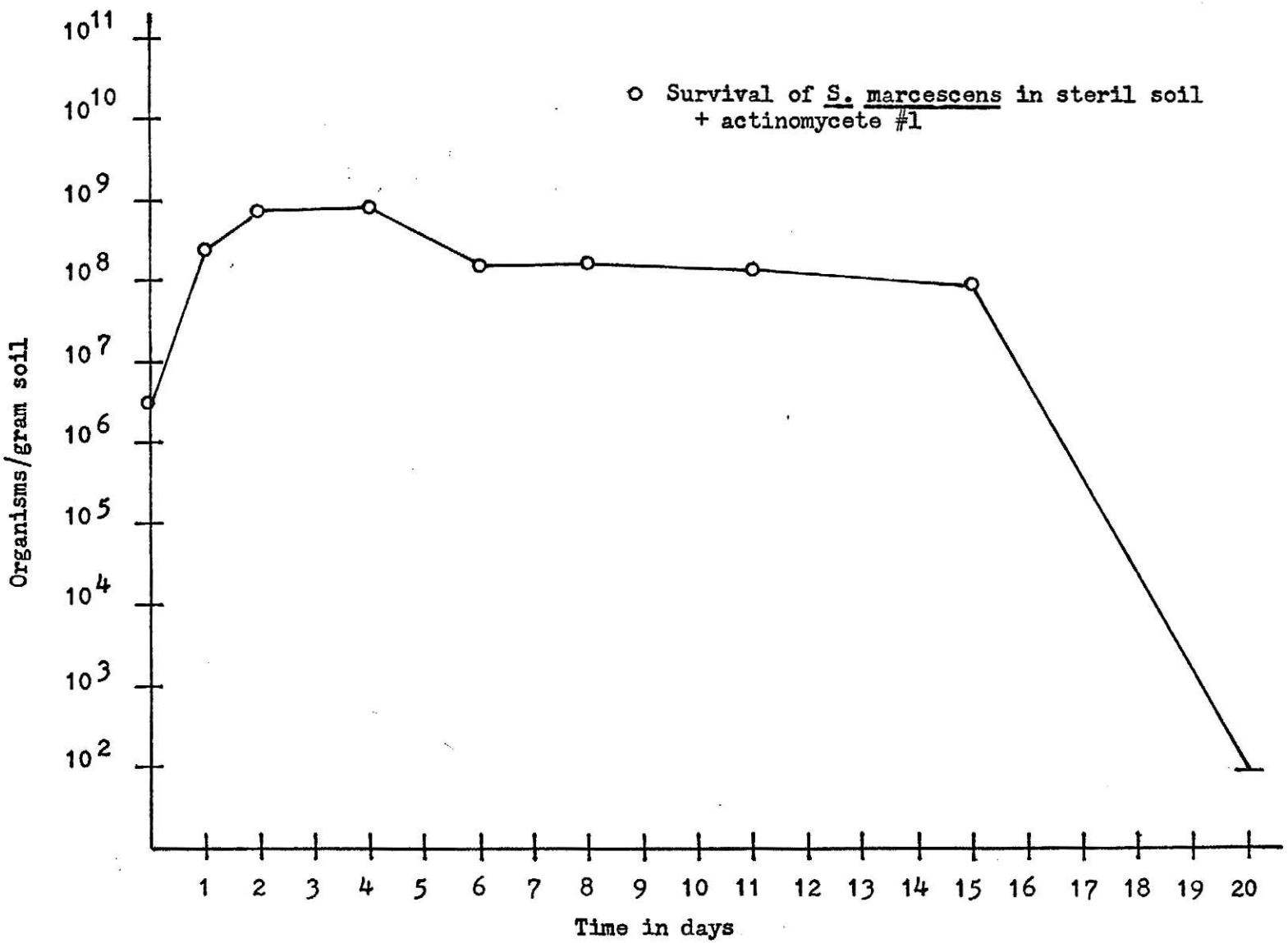
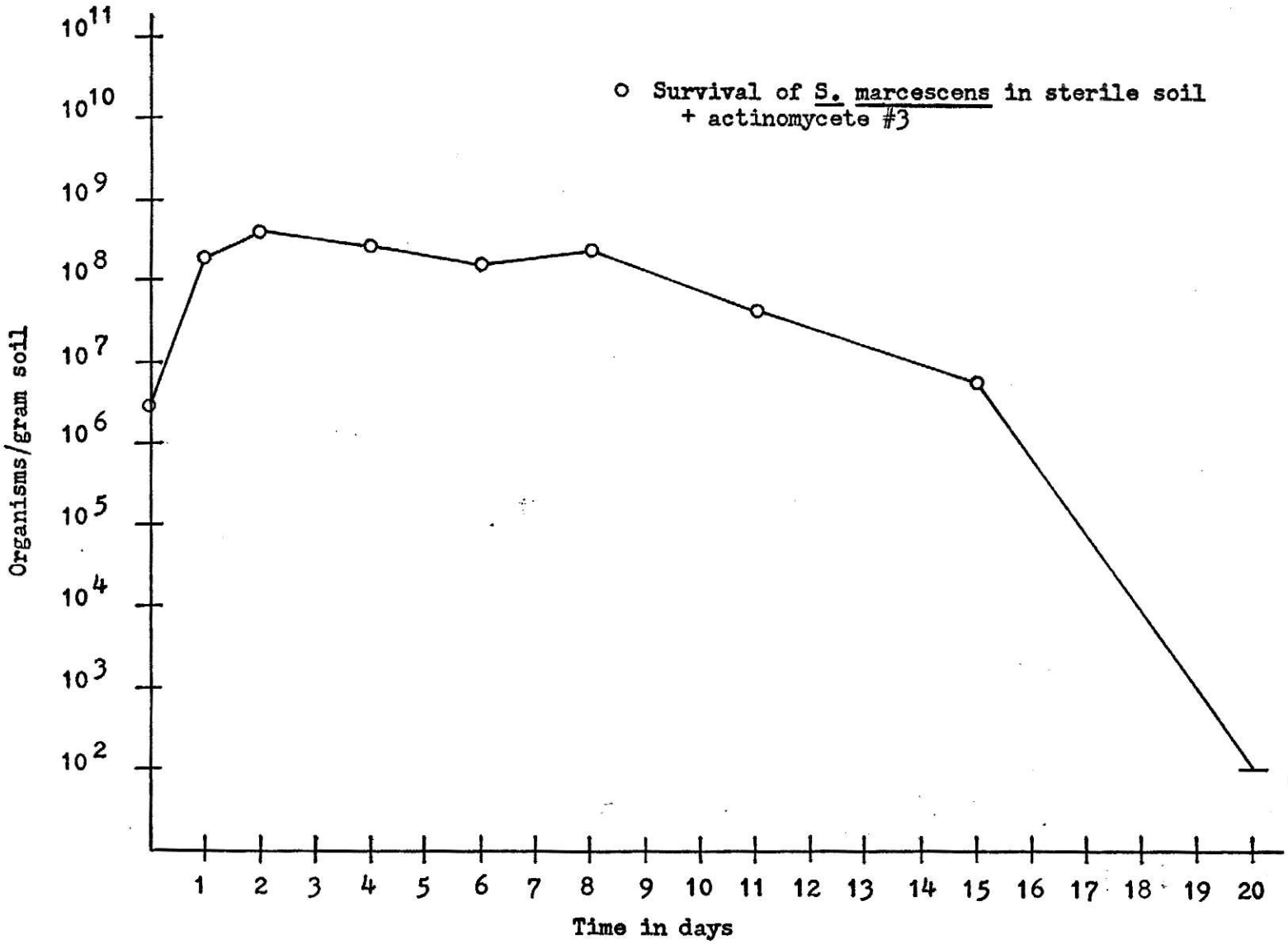

o Survival of S. marcescens in steril soil
+ actinomycete #1
Organisms/gram soil
10^{11}
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Time in days

Fig. 9. Comparative Study. The results for the survival of S. marcescens when grown in mixed soil culture with a non-antibiotic producing actinomycete (actinomycete #3). The cultures were incubated at 30°C, and the population of S. marcescens was determined by plating serial dilutions on starch agar.

S. marcescens + actinomycete #3

Day of Sample	Average Total Counted
Initial	4,500,000
Day 1	250,000,000
Day 2	280,000,000
Day 4	430,000,000
Day 6	220,000,000
Day 8	240,000,000
Day 11	47,000,000
Day 15	7,800,000
Day 20	None at a 1:100 dilution


o Survival of S. marcescens in sterile soil
+ actinomycete #3
Organisms/gram soil
10^{11}
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Time in days

Results: Substrate Concentration Effect

In general, the long-term effect of the addition of nutrients to natural soil, was detrimental to the survival of S. marcescens. This was especially true in the case of peptone addition. As noted in previous experiments, the population increased rapidly during the first day of the experiment. This increase was followed by a rapid decline in numbers. By the end of the experimental period, the population of S. marcescens in peptone amended soil was below that in normal soil, in all cases. The data is reported in graphic form in figures 10 and 11.

The effect of glucose addition was not as dramatic as that of peptone. Glucose stimulated the growth of S. marcescens during the first day of the experiment. This population remained higher than the population in the non-amended soil, up to day 8; and then it fell, ending below the population in non-amended soil.

It is interesting to note that concentrations 0.2% peptone and 0.2% glucose has the most detrimental effect, and 0.6% peptone and 0.6% glucose had the least detrimental effect.

Fig. 10. Substrate Concentration Effect Study. The results for the survival of S. marcescens when introduced into natural soil, and natural soil amended with: 0.2% peptone, 0.6% peptone and 1.0% peptone. The soils were incubated at 30°C, and the population of S. marcescens was determined by plating serial dilutions on a new selective media containing erythritol as the sole carbon source.

	Average total counted from soils amended with:	
Day of Sample	0% Peptone	0.2% Peptone
Initial	1,056,000	1,100,000
Day 1	946,000	90,600,000
Day 2	626,000	38,000,000
Day 4	237,000	1,110,000
Day 6	70,000	13,400
Day 8	69,000	230
Day 13	7,250	None at a 1:50 dilution

	Average total counted from soils amended with:	
Day of Sample	0.6% Peptone	1.0% Peptone
Initial	1,093,000	1,156,000
Day 1	2,420,000,000	4,560,000,000
Day 2	59,000,000	45,300,000
Day 4	973,000	89,000
Day 6	118,000	570
Day 8	5,730	430
Day 13	720	None at a 1:50 dilution

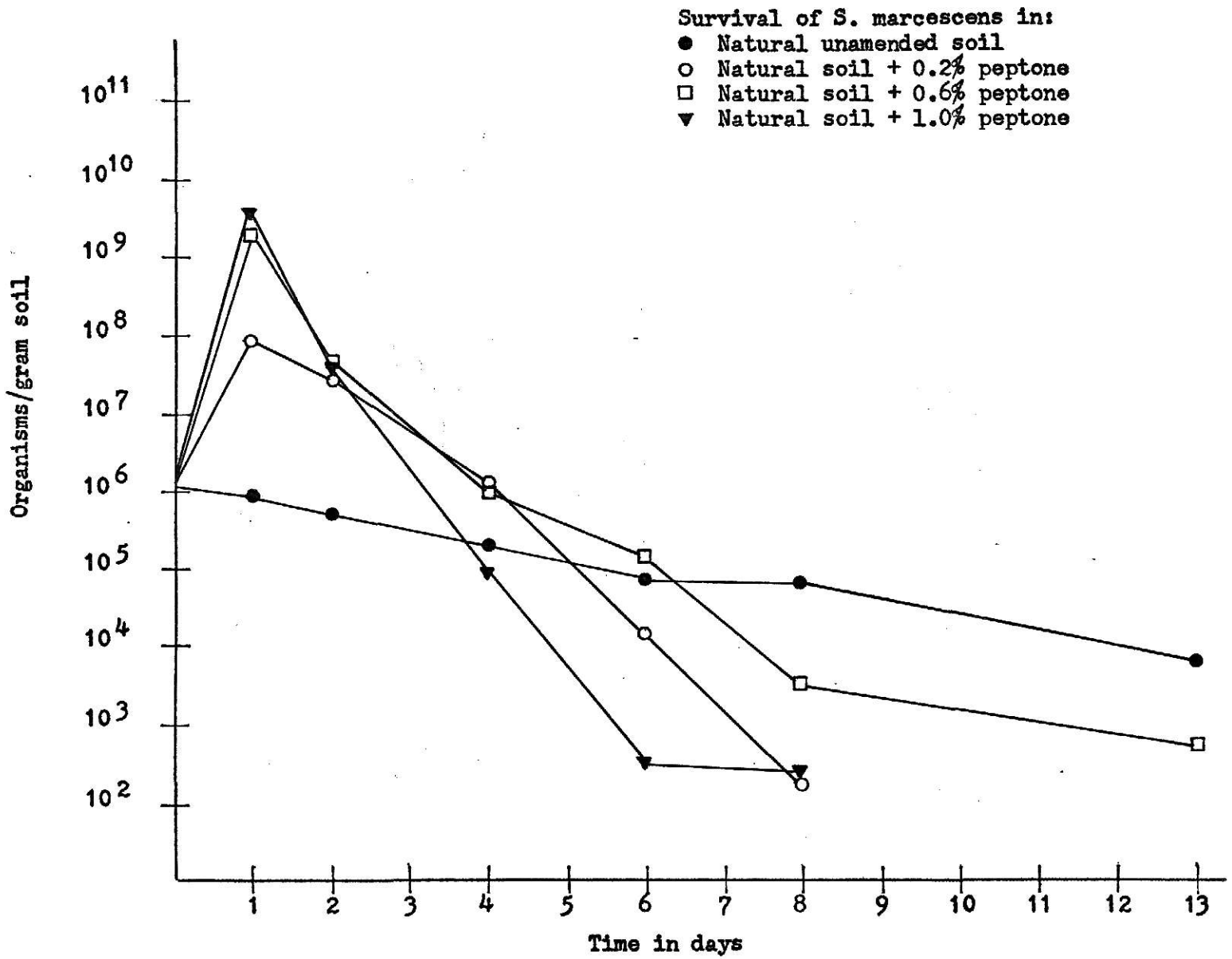
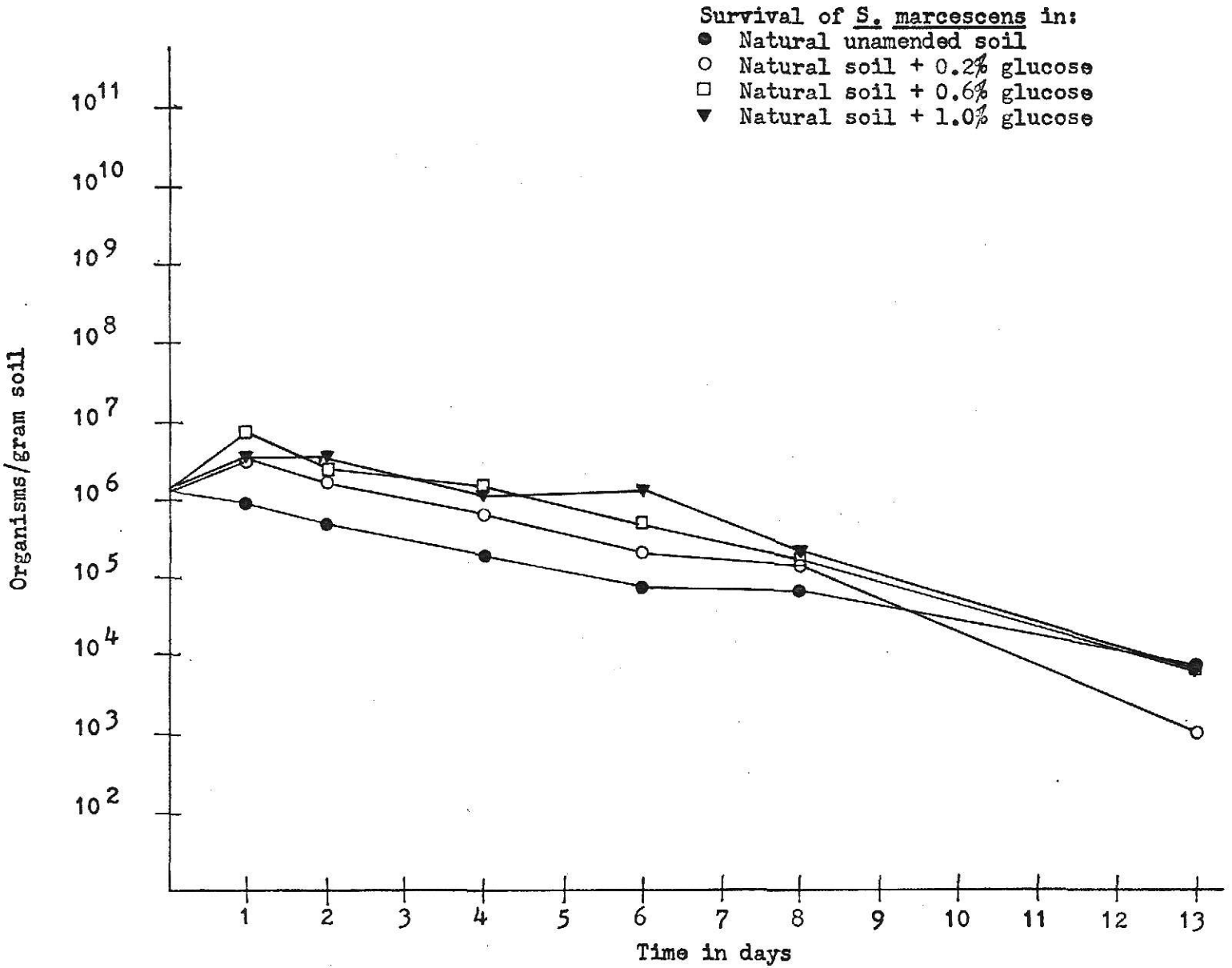

Survival of S. marcescens in:
● Natural unamended soil
○ Natural soil + 0.2% peptone
□ Natural soil + 0.6% peptone
▼ Natural soil + 1.0% peptone
Organisms/gram soil
10^11
10^10
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
1
2
3
4
5
6
7
8
9
10
11
12
13
Time in days

Fig. 11. Substrate Concentration Effect Study. The results for the survival of S. marcescens when introduced into natural soil, and natural soil amended with: 0.2% glucose, 0.6% glucose, and 1.0% glucose. The soils were incubated at 30°C, and the population of S. marcescens was determined by plating serial dilutions on a new selective media containing erythritol as the sole carbon source.

Day of Sample	Average total counted from soils amended with: 0% Glucose	0.2% Glucose
Initial	1,056,000	1,083,000
Day 1	946,000	4,400,000
Day 2	626,000	1,723,000
Day 4	237,000	653,000
Day 6	70,000	214,000
Day 8	69,000	114,000
Day 13	7,250	1,960

Day of Sample	Average total counted from soils amended with: 0.6% Glucose	1.0% Glucose
Initial	1,260,000	1,150,000
Day 1	8,900,000	4,200,000
Day 2	3,870,000	4,300,000
Day 4	1,140,000	920,000
Day 6	636,000	1,210,000
Day 8	146,000	257,000
Day 13	7,100	6,650


Survival of S. marcescens in:
Natural unamended soil
Natural soil + 0.2% glucose
Natural soil + 0.6% glucose
Natural soil + 1.0% glucose
Organisms/gram soil
10^11
10^10
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
1
2
3
4
5
6
7
8
9
10
11
12
13
Time in days

Results: Soil Survey

Only one sample of the soils tested, revealed a typical S. marcescens colony. The sample was from a cultivated field, on which maize had been grown. The pH was 8.9, and total count was 5.96×10^7 organisms per gram dry soil. The complete data from this study are reported in tabular form in Appendix II.

Mention should be made here, that the soil survey was conducted during the month of March. It is possible that the frequency of S. marcescens presence in the soil might be higher during the spring and summer seasons. However, the present study has shown that under favorable laboratory conditions, in terms of temperature, nutrients and moisture content, S. marcescens died when mixed with natural soil.

The erythritol plates were very successful at selecting against the natural soil population. Only 2 or 3 colony types appeared on the plates (a white, mucoid colony, and a red actinomycete). It was therefore very easy to identify the test organism.

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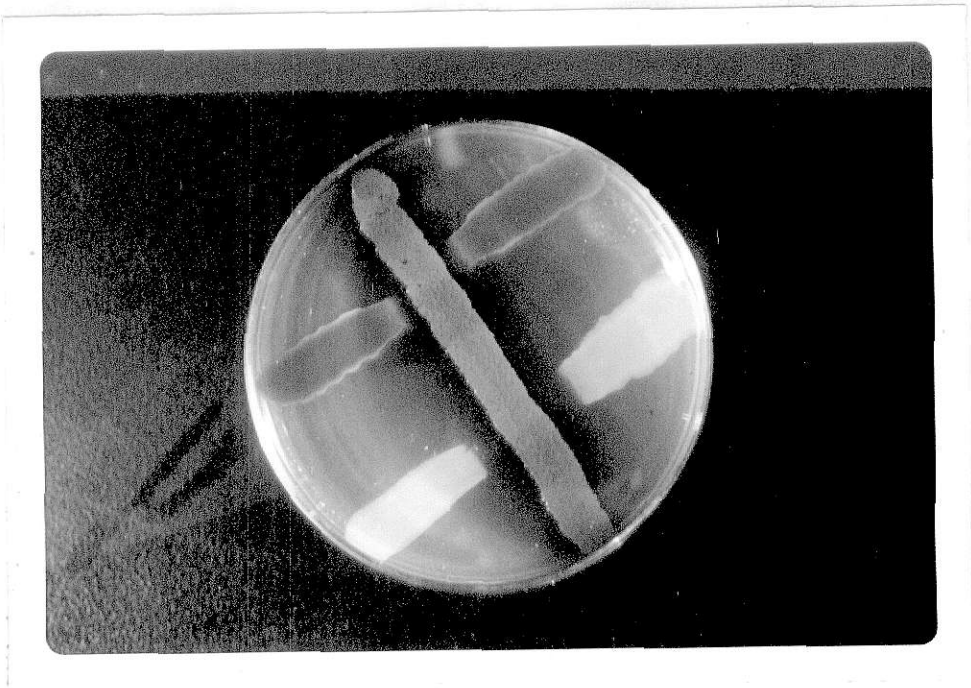

Fig. 12. The above picture illustrates the typical zones of inhibition produced by actinomycete #2 against S. marcescens (red) and E. coli. The actinomycete was allowed to develop on the plate 2 days, prior to cross-streaking with the test organism.

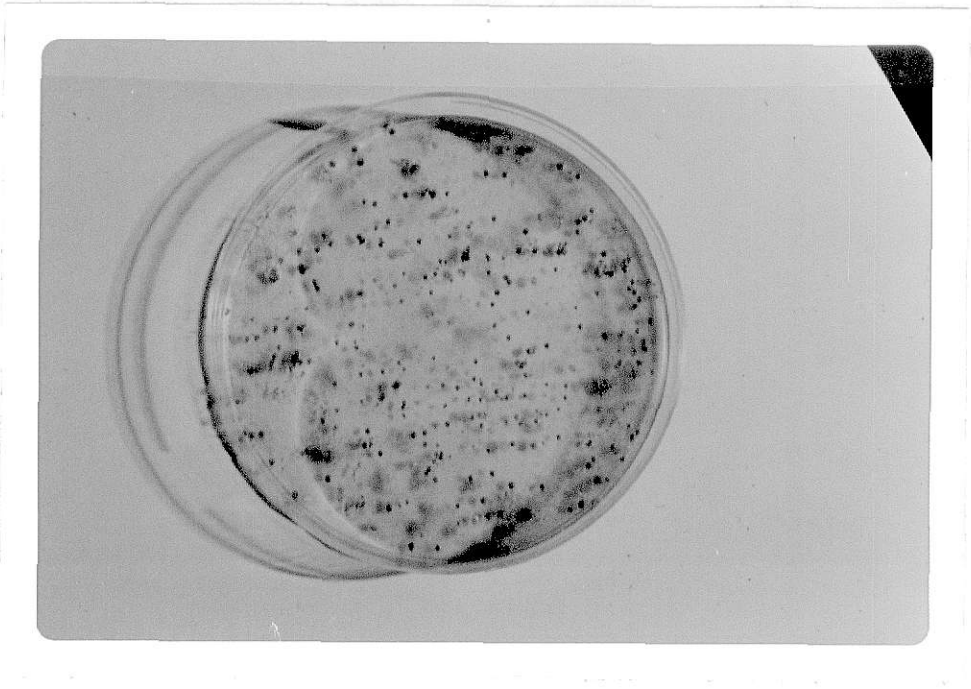

Fig. 13. The above picture depicts the appearance of S. marcescens (red colonies) on a selective agar containing erythritol as the sole carbon source. The plate was innoculated with a 1:100 dilution of a naturally occuring soil.

DISCUSSION

The present investigation was initiated in an attempt to clarify some of the reasons given by early investigators for the rapid decline in population of non-indigenous bacteria, when introduced into the soil. Among the reasons given for this phenomonen are: (1) lack of a proper food supply, (2) unfavorable environment such as reaction, competition for nutrients, or (3) the development of antagonistic soil organisms.

A preliminary study demonstrated that E. coli died very rapidly in natural soil. The population decreased from 480,000 per gram to 4,800 per gram in three days. This study verified the findings of the earlier workers, and demonstrated its survival pattern in the soil used.

In the preliminary study, the population of E. coli was also followed in sterilized soil, sterilized soil amended with 1% glucose. The data obtained from these series, corresponded well with data from previous studies, with the exception of the amended, sterile soil study. Waksman (1940) found that E. coli died very rapidly in such soil, and he attributed this to a toxic substance produced from the heating of glucose and soil together. In the present study, it was found that the population increased from 297,000 per gram to a maximum of 158,000,000 per gram in four days. This population remained high, numbering 66,000,000 per gram on day 16. This descrepancy is attributed to a difference in protocal. In this study, the soil and glucose were sterilized separately.

Data on the survival of S. marcescens, were not available in the literature, and therefore, comparisons could not be made. The present study demonstrated that the organism survived much longer than E. coli

in natural soil. In one study, the population dropped from 4,700,000 per gram to only 190,000 per gram in six days. In another study, the population fell from 1,056,000 to 7,250 per gram in 13 days. Selective media was used to obtain counts in the later study, and ease in identification is reflected in the data.

In order to clarify the effect of the natural soil population on the non-indigenous bacteria, the test organisms were cultured in sterilized soil. In one study, E. coli increased from 443,000 per gram to 118,000,000 per gram in 2 days; then decreased to 93,000 per gram on day 16. In another study, a similar trend was observed. The population increased from 520,000 per gram to 220,000,000 per gram in one day, and maintained this number until day 11, where 120,000,000 per gram were counted. In this study the population was sampled on day 15, but none could be detected at a 1:1,000 dilution.

The population of S. marcescens behaved very similar to that of E. coli in sterile soil. An increase from 7,600,000 per gram to 1,000,000,000 per gram was observed during the first 2 days of the experiment. This population maintained itself until day 11, where 140,000,000 per gram were counted. On day 15, the population was 24,000 per gram.

When the above data is studied, it is obvious that the natural soil population exerts a great effect on the maintance of non-indigenous populations. In addition, the process of sterilization must create a favorable environment for bacterial multipication.

The survival of E. coli in natural, amended soil proved to be impossible to follow, because of the development of a mucoid colony on the Endo plates. The addition of nutrients stimulated the natural population and they had the effect of crowding, at the dilution at which E. coli could be counted.

With the aid of a new selective media, the population of S. marcescens could be followed in natural, amended soil. The data from these studies indicate that the long-term effect of the addition of such nutrients was detrimental to the maintenance of large populations. The addition of peptone caused a rapid initial increase, followed by a rapid decrease in numbers. A concentration of 0.6% peptone was less detrimental to the population of S. marcensens, than was 0.2% or 1.0%.

This detrimental effect of nutrient addition was also observed for the test organism survival in sterile amended soil. Glucose had little long-term effect on the populations of E. coli or S. marcescens. Peptone had a detrimental long-term effect. This detrimental effect could be due to a build up of toxic metabolic end products. Another possible explanation is that the peptone supported an initial, high metabolic rate which was not supported by supplemental additions.

The belief that antagonistic organisms were responsible for the death of non-indigenous bacteria in soil, was not substantiated in this study. A total of 76 actinomycetes were isolated and tested for antibiotic production. Only two demonstrated antibiotic activity against the test organisms. When the actinomycetes were grown in mixed culture with the test organisms in sterile soil, the growth curve closely resembled that of the test organisms in sterile soil without the actinomycete. This evidence, along with the data reported by Simonoff and Gottlieb (1951), indicates that the antibiotic producing organisms have little or no effect on the soil population, other than a possible competition effect. There was no correlation between antibiotic production on laboratory media, and antibiotic production in the soil.

The above data indicates that the most important factor operating in the removal of non-indigenous bacteria from the soil, is the presence of the natural soil population. This is not to say that other factors, such as available nutrients, pH, temperature, and the abiotic environment do not influence the mortality and natality of the population. Nor, does this mean that the natural population influence the introduced bacteria directly, as was formerly thought. A more correct explanation of the phenomenon is found in the concept of ecological niche, and community homeostasis.

When one considers the enormous numbers and varieties of organisms that enter natural waters from eroding land, and human and animal wastes it is not illogical to assumption that these communities should be altered. However, intrusions of non-indigenous microorganisms into aquatic environment are almost invariable followed by a rapid decline in numbers (Alexander, 1971). Another example is sewage, which acquire numerous organisms from soil, feces, and urban runoff. Yet, this highly specialized biota has few of the human enteric pathogens. (Rudolf et al., 1950).

In this study, it was demonstrated that even under controled laboratory conditions, and when the alien was given selective advantage as far as numbers were concerned, the operation of ecological homeostatis still operated. This was especially true in the case of E. coli.

Medrek and Litsky (1960) attempted to reevaluate to what extent the coliform group and enterococci were present in undisturbed soil. Three hundred sixty nine soil samples were taken from four western Massachusett water supply reservoirs in which past fecal contamination was either nonexistant or very remote. Only five samples yielded typical E. coli colonies (1.4%) when plated on EMB agar.

Information as to the relative frequency of S. marcescens in natural soils is not known. Breed, et al. (1957) reports that S. marcescens is found in soil and water. However, the data from this investigation indicates that S. marcescens is a very infrequent member of the soil and if chance introduction does occur, it is highly probable that it will not survive long.

Bacteria function in nature as decomposers. Since all of these experiments were performed under controled, laboratory conditions, it would have proven difficult to have all tropic levels represented. Biological foodchains were not in complete operation. More work should be done in the natural environment. In addition, it would be interesting to study the effects of the soil protozoan population on non-indigenous bacteria.

With the development of the selective media for S. marcescens identification of the organism in natural soil has been made much simpler. Soil surveys, such as the one described here in, should be conducted during the various seasons, in order to determine if there is a higher frequency of S. marcescens during more favorable growing temperatures.

SUMMARY

The purpose of this study was to investigate the survival of non-indigenous bacteria in the soil, and to clarify some of the reasons given by earlier investigators for their rapid decline in numbers.

The organisms selected for the study were Escherichia coli and Serratia marcescens. E. coli was selected because it is used as an indicator of fecal contaimination. S. marcescens was selected because it has been isolated with increasing frequency from patients, and has been incriminated as the causative agent in a variety of infections. For this reason, emphasis was placed on this organism.

Enumeration and identification of the test bacteria was accomplished by plating serial dilutions of the soil samples on selective media. (Endo for E. coli, and a new, selective media containing erythritol as the sole source of carbon, for S. marcescens).

The investigation included the following studies:

1. Preliminary Study. A study designed to verify the findings of earlier investigators of the survival of E. coli in the soil.
2. Actinomycete Isolation. The actinomycete population of the soil used, was surveyed, and antibiotic producing actinomycetes, antagonistic to the test organisms were isolated.
3. Comparative Study. The survival of S. marcescens and E. coli were followed and compared in natural soil, and natural, amended soil.
4. Substrate Concentration Effect Study. A study designed to determine the effect of various concentrations of nutrient amendments on the survival of S. marcescens in the soil.

5. Soil Survey. A survey of soils in the surrounding Manhattan, Kansas area was made, to determine the relative frequency of S. marcescens presence.

The results obtained from the Preliminary Study confirmed the findings of previous investigations. The population of E. coli decreased rapidly after addition to natural soil. An exception in this trend was found in its survival in sterile soil amended with 1% glucose. Previous studies demonstrated a rapid decline in numbers, in this soil. In this investigation, this was not manifest. The discrepancy is attributed to a difference in protocol.

The belief that actinomycetes were responsible for the removal of non-indigenous bacteria from the soil was not substantiated. A total of 76 actinomycetes were isolated from the soil used throughout the study, and tested for antibiotic production effective against the test organisms. Only two demonstrated such activity. When the actinomycetes were cultured with the test organisms in sterile soil, the growth curve closely resembled that of the test organism in sterile soil without the actinomycete. There was no correlation between antibiotic production on laboratory media and antibiotic production in the soil. These data indicate that antibiotic production by actinomycetes exert little or no influence in the maintenance of community homeostasis in the soil.

In general, S. marcescens did better in the soil environment than did E. coli. It survived longer and reached a greater population density. However, its population was removed from the soil relatively quickly, and it is doubtful that the organism could establish itself in the soil population. This statement is substantiated by the results of a soil survey in which 100

different soils from the surrounding Manhattan, Kansas area were sampled. Only one yielded a typical S. marcescens colony.

The long-term effects of the addition of nutrients to the natural soil was detrimental to the survival of both S. marcescens and E. coli. The populations exhibited a rapid increase in numbers, followed by a rapid decrease. This effect was more pronounced in the peptone amended soils, than in the glucose amended soils.

A new, selective media containing erythritol as a sole source of carbon proved very useful in the study of S. marcescens in natural and natural amended soils. Prior to its incorporation in the study, it was impossible to identify the organism, due to the crowding effect of the natural population. With the use of this media, the organisms could be identified at a dilution as low as 1:100, of the natural soil.

The results of these studies indicate that the most important factor operating in the removal of non-indigenous bacteria from the soil, is the presence of the natural soil population. This is not to say that other factors such as pH, availability of nutrients, temperature, and the abiotic environment do not influence the mortality and natality of the population. Nor, does this mean that the natural population has a direct, antagonistic influence to the introduced organism. Rather, a more correct explanation is found in the concept of ecological niche and community homeostasis.

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APPENDIX

APPENDIX I

Composition of media used

1. Czapeks Broth

Sucrose	30.0 gm
$NaNO_3$	2.0 gm
K_2HPO_4	1.0 gm
$MgSO4 \cdot 5\ H_2O$	0.5 gm
KCl	0.5 gm
$FeSO_4 \cdot 5\ H_2O$	0.017 gm
Distilled Water	1,000 ml

2. Conn's Medium

Glycerol	10 gm
Sodium asparaginate	1 gm
K_2HPO4	1 gm
Distilled Water	1,000 ml

3. Glycerol-Calcium malate broth

Glycerol	10.0 gm
NH_3Cl	0.5 gm
Calcium malate	10.0 gm
K_2HPO4	0.5 gm
Distilled Water	1,000 ml

4. Nutrient Agar

Peptone	5.0 gm
Beef Extract	5.0 gm
NaCl	5.0 gm
Distilled Water	1,000 ml

5. Brain Heart

Commercial Media--Difco

6. Glucose-Triptose-Yeast Extract Broth

Ashbeys salt (2X)	500 ml
Distilled Water	500 ml
Triptose	5.0 gm
Yeast Extract	5.0 gm
Glucose	5.0 gm

7. Emersons Broth

Glucose	10.0 gm
Yeast Extract	10.0 gm
Beef Extract	4.0 gm
Peptone	4.0 gm
NaCl	2.5 gm
K_2HPO_4	0.5 gm
KH_2PO_4	2.3 gm
Distilled Water	1,000 ml

pH = 6.1

8. Potato-Glucose agar

Peeled, diced potatoes	200 gm
Glucose	20 gm
$CaCO_3$	0.2 gm
$MgSO_4 \cdot 7\ H_2O$	0.2 gm
NaCl	5.0 gm
K_2HPO_4	0.5 gm
KH_2PO_4	2.3 gm
Distilled Water	1,000 ml

9. Casine Hydrolyzate Broth

Phytone	3.0 gm
Casine Hydrolyzate	17.0 gm
NaCl	3.0 gm
K_2HPO_4	2.5 gm
Glucose	2.5 gm
Distilled Water	1,000 ml

10. Starch Agar

Peptone Extract	2.5 gm
Yeast Extract	1.5 gm
KH_2PO_4	2.0 gm
K_2HPO_4	0.5 gm
Soluable Starch	5.0 gm
Ionagar #2	10.0 gm
Distilled Water	1,000 ml

11. *Serratia marcescens* Selection Agar

K_2HPO_4	0.7%
KH_2PO_4	0.3%
Na_3citrate	0.5%
$MgSO_4 \cdot 7\ H_2O$	0.01%
$(NH_4)_2SO_4$	0.1%
Erythritol	0.5%
Agar	1.5%

12. EMB Agar--Commercial media--Difco

13. Endo Agar--Commercial media--Difco

14. Ashbeys Solution (Modified)

K_2HPO_4	1.8 gm
KH_2PO_4	0.7 gm
$MgSO_4 \cdot 5H_2O$	0.2 gm
NaCl	0.2 gm
$CaCl_2$	0.02 gm
(20% $FeCl_2$ in H_2O)	0.2 ml of 10% solution
Minor Elements	0.1 ml (Minor elements = .5% each of chloride salts of Mn, Mo, Cu, Zn, Bo.)
Distilled Water	1,000 ml

15. Mineral Salts Solution

Ashbeys Salt Solution	1 liter
Distilled Water	3 liters
K_2HPO_4	10 gm
KH_2PO_4	4 gm
$(NH_4)_2SO_4$	8 gm

APPENDIX II

Data obtained from a soil survey for the presence of *Serratia marcescens*.

Soil Type	pH	Organisms per dry gram of soil
Feedlot		
1	8.65	457,000,000
2	8.00	87,200,000
3	7.72	2,130,000,000
4	7.25	171,000,000
5	7.10	2,600,000,000
6	6.70	220,000,000
7	7.50	394,000,000
8	7.40	618,000,000
9	7.75	76,700,000
10	8.15	85,700,000
11	7.74	323,000,000
12	8.05	227,000,000
13	7.45	76,300,000
14	8.10	588,000,000
15	7.80	231,000,000
16	8.12	14,100,000
17	7.75	54,600,000
18	7.70	215,000,000
19	8.00	8,850,000
20	7.69	17,700,000
21	7.80	58,300,000
22	7.91	102,000,000
23	7.93	87,700,000
24	7.75	107,000,000
25	7.95	87,900,000
26	7.40	2,150,000
27	7.70	58,100,000
28	7.20	13,300,000
29	7.51	97,300,000
30	7.25	5,310,000
31	7.15	64,300,000
32	7.99	282,000,000
33	7.61	12,000,000
Uncultivated		
1	6.20	50,300,000
2	6.55	16,600,000
3	6.10	15,600,000
4	6.20	4,300,000
5	5.80	13,900,000
6	6.10	16,900,000

Soil Type	pH	Organisms per dry gram of soil
Uncultivated		
7	6.10	12,700,000
8	6.05	11,500,000
9	6.55	10,900,000
10	6.45	7,150,000
11	6.50	14,300,000
12	6.40	6,610,000
13	6.00	6,030,000
14	6.10	15,100,000
15	6.10	16,000,000
16	6.05	7,130,000
17	6.40	13,300,000
18	5.30	38,900,000
19	5.15	110,000,000
20	6.55	65,000,000
21	6.05	11,700,000
22	6.40	55,700,000
23	5.75	11,800,000
24	5.45	412,000,000
25	5.70	113,000,000
26	5.75	75,600,000
27	5.85	32,400,000
28	5.70	154,000,000
29	6.00	15,100,000
30	6.20	46,400,000
31	6.25	182,000,000
32	5.95	209,000,000
33	5.60	114,000,000
34	6.00	16,700,000
Cultivated		
1	7.70	70,300,000
2	6.60	59,000,000
3	7.80	34,000,000
4	6.10	13,600,000
5	6.25	35,900,000
6	6.45	32,500,000
7	5.80	76,000,000
8	5.85	50,100,000
9	6.40	77,900,000
10	6.35	51,400,000
11	7.55	76,700,000
12	7.55	15,700,000
13	7.32	12,100,000
14	7.00	15,800,000
15	7.25	1,650,000,000
16	7.40	81,000,000
17	7.25	11,200,000
18	7.20	7,860,000
19	7.10	13,600,000

Soil Type	pH	Organisms per dry gram of soil
Cultivated		
20	7.05	60,400,000
21	6.60	7,370,000
22	7.20	13,100,000
23	7.10	15,400,000
24	7.25	37,300,000
25	7.40	55,200,000
26	6.70	12,300,000
27	7.40	93,200,000
28	7.55	28,500,000
*29	8.90	59,600,000
30	5.40	14,000,000
31	5.10	41,400,000
32	5.30	7,430,000
33	4.70	11,100,000

*The only soil sample containing positive presence of Serratia marcescens.

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THE SURVIVAL OF SELECTED NON-INDIGENOUS MICROORGANISMS IN THE SOIL ECOSYSTEM

by

CHARLES R. GARRIOTT

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ABSTRACT

Non-indigenous bacteria die rapidly, when introduced into the soil ecosystem. A number of different reasons for this phenomen have been reported. Among these are: (1) lack of a proper nutrient supply, (2) unfavorable environment, (3) competition for nutrients, (4) the development of antagonistic organisms. The purpose of this study was to investigate the survival of non-indigenous bacteria in the soil, and to clarify some of the above reasons for their rapid death.

The organisms selected for the study were E. coli, and S. marcescens. E. coli was selected because it is used as an indicator of fecal contamination. S. marcescens was selected because it has been isolated with increasing frequency from patients, and has been incriminated as the causative agent in a variety of infections. For this reason, emphasis was placed on this organism.

Enumeration and identification of the test bacteria was accomplished by plating serial dilutions of the soil samples on selective media. (Endo for E. coli, and a new, selective media containing erythritol, as the sole source of carbon, for S. marcescens.)

The belief that antagonistic actinomycetes were responsible for the removal of non-indigenous bacteria from the soil, was not substantiated. A total of 76 actinomycetes were isolated from the soil used (a sandy loam) and tested for antibiotic production effective against the test organisms. Only two demonstrated such activity. When they were grown together in mixed soil culture, the growth curves of both organisms resembled that obtained from their growth in sterile soil. There was no correlation between

antibiotic production on laboratory media, and antibiotic production in the soil. These data indicate that antibiotic production by actinomycetes exert little or no influence in the maintance of community homeostasis in the soil.

In general, S. marcescens did better in the soil environment than did E. coli. It survived longer, and reached a greater population density. However, its population was removed from the soil relatively rapidly, and it is doubtful that the organism could establish itself in the soil population. This statement is substantiated by the results of a soil survey in which 100 different soils from the surrounding Manhattan, Kansas area were sample. Only one yielded a typical S. marcescens colony.

The long-term effects of the addition of nutrients to the natural soil was detrimental to the survival of both S. marcescens and E. coli.

The results of these studies indicate that the most important factor operating in the removal of non-indigenous bacteria from the soil, is the presence of the natural soil population. This is not to say that other factors such as pH, nutrients, temperature, and the abiotic environment do not influence their survival. Nor, does this mean that the natural population has a direct, antagonistic influence on the introduced organism. Rather, a more correct explanation is found in the concepts of ecological niche, and community homeostasis.