STUDIES ON THE ACTIVITY OF SOIL PROTOZOA¹

By

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The possibility of soil protozoa acting as a destructive agent in soil fertility still seems to be an unsettled question.

In the most recent publication by Goodey (4) in which he employed old soils that were stored for some years, some of which showed the absence of protozoa, developed in hay infusion, he concluded as follows: "The results of the experiments described above, lead me to the conclusion, that the protozoa, including ciliates, amebae, and flagellates added to the soil have not been able to act as a factor limiting bacterial activity in the soil. Inferentially, therefore, the ciliates, amebae and flagellates obtainable from ordinary soil under cultural conditions do not function as the limiting factor. This is in accord with and extends the conclusion put forward in my earlier paper (3), that the ciliated protozoa are present in soil in an encysted condition and cannot function, therefore, as the factor limiting bacterial activity."

Russell (12) in his criticisms of Goodey's researches is still of the opinion that the results of his previous work(14,15) point to a factor which limits bacterial activities. He states, "The soil is known to be inhabited by numbers of bacteria, eel worms, vermes and numerous other organisms of higher orders and visible dimensions, and these lead active lives. Recently it has been shown in the Rothamsted Laboratory that a protozoan fauna also exists, some members at least of which are leading a trophic life." He (Russell) objects to the technique employed by Goodey in that, "The organisms inoculated into the soil are in the main those which figure largely in cultures made by adding soil to hay infusions. It has already been shown, however, (*v. supra*) that the culture fauna is distinct from the trophic soil fauna. There is, therefore, no evidence that the normal soil fauna was put back into the soil partially sterilized. On the contrary it apparently was not, nor is there evidence, except perhaps in one case, that the added organisms survived at all." * * * * "The protozoa are not obtained in pure culture alone, but they are added along with hay infusion and bacteria. Until more is known of the kind of protozoa occurring in the trophic state in the soil and their life history in the soil, it will be impossible to lay much stress on the negative results of re-infections."

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In a recent work, on soil protozoa, Waksman (19) summarizes in part as follows: "Soil protozoa do not have any appreciable influence upon the ammonification by bacteria. The presence of protozoa acts detrimentally upon bacterial numbers so that when the conditions become favorable for protozoa development, the bacterial numbers decrease."

With the exception of examining and finding active protozoa present after the incubation period, this work of Waksman's is subject to the criticisms offered by Russell of Goodey's research. In no case were protozoa free from bacteria and decomposition products of organisms in the soil extract used as the inoculum into the sterile soil when the destructive effect of the protozoa was to be noted. Sherman (17, 18) in his researches with soil protozoa also realized this criticism to his work. Moreover, it would hardly seem scientifically accurate to compare the ammonifying power of organisms of the soil (bacteria and actinomyces in the main) from a depth of 20 inches which were originally entirely under anaerobic conditions, with organisms from the surface soil, as was undertaken by Waksman. It would be expected that the aerobic organisms contained in the upper layer of soil would be much more vigorous.

It is generally known that the ammonifying power of a soil, varies as the percentage of moisture which it contains. The production of ammonia by a soil flora usually increases until the moisture content equivalent to the physical optimum is reached. With heavier soils, the maximum is slightly higher than the physical optimum. Soon after the optimum condition of moisture, where there is maximum ammonification, has been reached, there is an abrupt decrease in ammonifying power. This is explained by the fact that the conditions become unfavorable for decomposition, and thus the processes proceed very slowly under these conditions which approach the anaerobic state. A parallel instance to this might be seen in the above cited work. There is reason to believe that the bacteria which were introduced with the protozoa in the protozoa culture were better ammonifiers than the bacteria free from protozoa, hence the protozoa might have been the cause of considerable destruction of bacteria and still an increase in ammonia would be found, being probably due to bacteria alone, as is recorded with soil B with 14 and 28 per cent moisture (protozoa plus bacteria) (19, p. 144).

In a previous publication the writer (7) gives an account of studies in the length of time of excystation of soil protozoa and some of the factors which influence the presence of protozoa in the active state in the soil. He also examined field and greenhouse soils to get some idea as to the extent of the activity of the protozoa present. For several reasons which need not be mentioned here, the effect of the presence of organic matter and the physical property of the soil upon the presence of protozoa in soils, in the active state, could not be worked out and included in the last publication, consequently this paper was prepared for this reason in part.

PROTOZOAN ACTIVITY IN THE SOIL

The several methods used in studying protozoan activity in the soil were previously reviewed, hence no further mention will be made of them at this time.

Inasmuch as there were no extended data available which indicated the approximate time required for protozoa to excyst when the cysts come in contact with free water, the previous work (7) was carried out by making several examinations of the soils in question, for a period of two minutes only, thus eliminating the possible errors which might arise from the fact that the protective cysts might burst open during the examination and hence a false impression as to the real number of active protozoa in the soil would be obtained, as was suggested by Martin and Lewin (10). The writer realized that two minutes was a short period of time for a single examination, but when the field and greenhouse soils were examined for active protozoa for the actual length of time that the soil was under observation and in consideration of the number of samples taken, which was 14 or 15, it would seem that the active protozoa could have been detected and no doubt were, as will be demonstrated later. After making several thousand determinations, some of which were recorded in the last publication, to which reference has been made, and not finding any protozoa to excyst in 2 minutes, the 5 to 7 minute examination period was tried out and found to be satisfactory, as stated before (7, p. 486).

In the problems presented in this work, the direct examination of a small portion of soil was made for 5 minutes. Each soil was subjected to an examination extending from 15 to 20 minutes, i. e. 3 or 4 samples of each soil were taken. The low and the high power (4 mm.) of the microscope were employed. It was found that very great care must be exercised in employing the high power in determining active protozoa. There is still a chance for the investigator to err by employing the high power especially if there are protozoa which are as small as bacteria and have the Brownian movement, for in such a case it would be impossible to distinguish between protozoa and bacteria by this method. By extended examinations, it was found, however, with the soils employed, that practically all of the active protozoa could be seen with the low power.

In order to collect more data with regard to the influence of moisture content, physical character of the soil and the presence of organic matter upon the activity of protozoa in the active condition in the soil, 100-gm. portions of a typical *Sassafras* sandy loam soil which had been air dried, were weighed out into tumblers. The water-holding capacity of this soil was determined by the funnel method, which consists of merely determining the amount of water which is held by a weighed quantity of soil. It was found that 40 per cent of the amount of water held in this way by the soil, very closely approximated the physical optimum of the soil, deter-

TABLE I
 PRESENCE OF ACTIVE PROTOZOA IN A SASSAFRAS SOIL, WHICH HAD RECEIVED TREATMENTS OF DRIED BLOOD AND COW
 MANURE, AT VARIOUS MOISTURE CONTENTS AND AT DIFFERENT INTERVALS FOR A PERIOD OF 28 DAYS

Exp. No.	Kind of Soil	Material added	Amt. material added, gm.	Moisture added		Number of protozoa actually found per 0.33 gm. soil											
				Degree optimum	c.c.	Days											
						1st	2nd	3rd	4th	6th	8th	10th	14th	21st	28th		
21	Sassafras	Nothing		$\frac{3}{4}$	13.95			2 F. ¹									
22	"	"		1	18.60				1 F.				1 S.C.				
23	"	"		$1\frac{1}{2}$	27.90			1 F. 1 S.C.	1 F.				1 S.C.				
24	"	"		$1\frac{3}{4}$	32.55		1 F.		2 F. 2 S.C.	1 F.		1 S.C.	1 S.C.	3 S.C. 1 L.C.	1 F. 1 S.C.		
31	"	Dried blood	2.85	$\frac{3}{4}$	16.08			1 F.	2 F.	1 F.							
32	"	"	2.85	1	21.45			2 F.	1 S.C.	1 F.		1 F.					
33	"	"	2.85	$1\frac{1}{2}$	30.75		1 F. 1 S.C.	2 F. 1 S.C.		1 F. 1 S.C.	1 F. 1 S.C.	18 F.					
34	"	"	2.85	$1\frac{3}{4}$	35.40		4 F.	3 F.	1 F.	3 F. 3 S.C.			1 F.	1 S.C.	1 S.C.		
41	"	Cow manure	2.85	$\frac{3}{4}$	16.62		2 F.	3 F.	1 F.	2 F.							
42	"	"	2.85	1	22.16	2 F.	3 F.	5 F.	2 F. 1 S.C.	1 F.			1 F.				
43	"	"	2.85	$1\frac{1}{2}$	33.24	1 F.	1 F.	3 F.	9 F. 2 S.C.	1 F.	1 F. 4 S.C.	2 F. 5 S.C.			1 F.		
44	"	"	2.85	$1\frac{3}{4}$	38.78	2 F.	1 F.	6 F. 1 S.C.	16 F. 3 S.C.	2 F.		1 F.			1 F. 1 S.C.		
52	"	Sand	30	$\frac{3}{4}$	12.07							2 F.		1 F.			
62	"	"	30	1	16.10												
72	"	"	30	$1\frac{1}{2}$	24.15				2 F.			1 F.	1 F.				
82	"	"	30	$1\frac{3}{4}$	28.17				3 S.C.	3 F.	3 S.C.	1 F. 2 S.C.	1 F. 1 S.C.	1 S.C.			

1 F.—Flagellates. S. C.—Small Ciliates. L. C.—Large Ciliates.

mined by means of feeling the soil. To each of four 100-gm. quantities of soil, sieved air dry, 2.85 gm. of dried blood (representing 50 tons per acre on the basis of 2,700,000 pounds per acre-foot) were added. To another series of 4 tumblers, 2.85 gm. of dry manure, which had been sieved through a 20-mesh sieve, were added. Into each of 4 tumblers, 30-gm. quantities of pure washed quartz sand which passed a 40-mesh sieve were weighed. Then 70 gm. of Sassafras soil were added to each portion. These tumblers containing the mixture of sand and soil, represented the third series. A fourth series of 4 tumblers with 100-gm. portions of soil received no treatment. Each of the soils with the several treatments was then thoroughly mixed by shaking the mixture in a small hard milk-shaking cup¹. Then to each series, sterile water was added to an amount, making the content of one quantity 30 per cent of the water holding capacity, or three-fourths of the physical optimum, a second received sufficient to bring the moisture to 40 per cent of the water-holding capacity or one optimum, a third 60 per cent, and a fourth 70 per cent of the water-holding capacity. The soil was very carefully mixed so that the moisture would be as evenly distributed throughout the mass as possible. This was accomplished by emptying the contents of the tumblers on a large sheet of manila paper and mixing the material by passing it through the hands several times. Having replaced the contents in each tumbler and weighed the same, they were allowed to incubate at room temperature for a period of 28 days. Examinations of these soils for active protozoa were made by the method given above (three to four 5-minute examinations of each with the high and the low power of the microscope) on the 1st, 2nd, 3rd, 4th, 6th, 8th, 10th, 14th, 21st and 28th days after moistening the mixture. Before each examination was made the tumbler with its contents was carefully weighed and the moisture loss due to evaporation was restored. Then after the examination was complete, the sample was again weighed. By this method the amount of soil used for each examination was easily determined. Hence by counting the number of protozoa actually found in the examination, as was done, the number of active organisms (protozoa) per gram of soil could thus be calculated.

From examining Table I, it is apparent that the period of excystation of protozoa varies with the treatment of the soil, as in all cases where the soil received applications of cow manure the protozoa excysted sooner than they did in the other treatments. In all but one case, where the soil plus manure had a moisture content of three-fourths of the optimum, they were present in the active state on the first day after inoculation, while with no other treatments were they present on this day. Many more active protozoa were counted in the mixtures which received or-

¹ This was recommended by H. C. Lint. See "Sources of Error in Soil Bacteriological Analysis" by Lint, H. C., and Coleman, D. A., p. 157.

ganic matter than where none was applied, likewise, the numbers were greatest in those to which the greatest amounts of moisture were added.

Increasing the porosity of this soil by means of sand did not seem to increase the number of active protozoa present, as there were less notes in the mixture of soil and sand than in the samples of soil alone.

It is noted in this problem, as was very apparent in the development of protozoa in cultural solutions (6), that the flagellates excyst first, the maximum number being present on the 3rd, 4th and 5th days. "At a temperatures, the flagellates develop sooner than the ciliates" (6, p. 557). It would thus appear that it requires a longer period of time for protozoa to excyst than is generally thought. Again, the active flagellates become less in number after the 6th day, when the ciliates become more numerous, so that on the 21st day only one small flagellate was noted in or on soil while ciliates were found to be present in three. It is very plain that after a certain period of time the active protozoa decrease in number very rapidly, so that even though the moisture conditions would be considered very favorable and also an abundance of organic matter would be present, after a time the protozoan population in the active state would be almost *nil*. This above-mentioned fact no doubt explains the question which Waksman (19, p. 136) raised in regard to the author's previous work¹ when he said, "The error made in limiting the time of examination to only 2 minutes is made clear by the fact that out of 20 greenhouse soils where Koch found protozoa, the organisms recorded are in 5 cases ciliates and amoebae, and only in one case flagellates, and further as was shown by Sherman (16), Cunningham (2), by the writer and others, the number of flagellates is much larger than the number of ciliates, and we would expect that under conditions when moisture is the limiting factor the smaller organisms would be able to lead an active life at a lower moisture content than the larger ones." It would seem, as shown above, that after a certain time and stage of decomposition of organic matter active flagellates cease to be present to any great extent while living ciliates are more numerous. No doubt if the greenhouse soils to which Waksman refers had been examined soon after they were composted, the active flagellates would have out-numbered the ciliates considerably.

The question may be asked why more active protozoa were not found in the laboratory studies with the three soils in the previous work (7). This is explained by the fact that two of these soils were of close texture (heavy soils) one being a shale compost and the other a clay loam. It will be seen later that with a heavy soil (Penn clay loam) very few active protozoa are found. The first of these contained organic matter but as it had reached an advanced stage of decomposition it would not influence protozoan development to a very great extent. One small cil-

ate was noted on the third day in the case of the field soil containing one and one-half optimum moisture. None of these soils received any organic matter in the laboratory treatments. No doubt their absence in the air-dried samples and those containing only one-half of the optimum of moisture was due to the lack of moisture. Furthermore even with a 15 to 20-minute examination, but very few (4 organisms) were found to be present in the examinations up to and including the 8th day of the present work, as shown in Table I (soil samples with one optimum and one and one-half optimums of moisture and receiving no organic matter, No. 22 and No. 23).

In order to demonstrate that the writer did not err in his 2-minute examination for 30 minutes in his previous work (7) as was questioned in the quotation above, after having found active protozoa to be present on the second day in this experiment, he concluded that certainly some would be present in the active condition on the third day. Hence to check up the 2-minute examination period, the length of time required to find a living protozoan was recorded on the third day. After the protozoa had been found, a different sample was taken and the examination of each soil was continued for a period of 20 minutes.

By noting the time required to find the active protozoa in the examination, it is seen that there are but few cases where these organisms were not found within the 2-minute period. Moreover, the samples of field and of greenhouse soil which were examined in the above referred work, were under observation for a 30-minute period, in which the experimenter is very liable to find the organisms. It is true that there were a comparatively large number of active protozoa present in the samples to which organic matter (dried blood and cow manure) had been added when this examination was made, but it will be noted that they were also found in the specified time (2 minutes) in the samples which received no organic matter, where the numbers were very low.

It might be stated that about 0.33 gm. of soil represented the amount taken for each daily examination for active protozoa, hence by multiplying by three the number of active protozoa recorded, found in Table I and in Table III, one could obtain a fairly good representation of the number of motile protozoa present per gram of soil. It would hardly seem that 0.33 gm. would be an adequate representative amount of soil upon which to base conclusions, but by this method (direct examination) it is, not possible to examine between the particles of more than this amount in 3 or 4 examinations. Moreover, in bacteriological technique, it is a common practice to pour plates from dilutions, which represent 1-10,000 and 1-50,000 of a gram. The largest number of flagellates found in one day's examination of a single sample was 18. This would mean that there were approximately 54 flagellates per gram of soil, which may be compared with at least 100,000,000 to 300,000,000 or more bacteria per gram of soil, as shown later.

Having found that soil protozoa excyst for only a short period of time and that active flagellates appear first, followed by the ciliates and that the number of motile protozoa relative to bacteria is comparatively small, it was next desired to repeat the same experiment with a different soil.

TABLE II
THE LENGTH OF TIME REQUIRED TO FIND ACTIVE PROTOZOA AND THE
NUMBERS FOUND BY THE DIRECT METHOD IN THE EXAMINATION
OF THE VARIOUS TREATMENTS OF SASSAFRAS SOIL

Lab. No.	Organisms found in 20 minutes	Length of time to find organism min.-sec.
21	1 F. ¹	1:56
21	1 F.	3:10
22	none	
23	1 F.	4:45
23	1 S.C.	1:46
24	none	
31	1 F.	2:30
32	1 F.	1:40
	1 F.	0:30
33	1 F.	2:00
	1 F.	1:50
	1 S.C.	0:31
34	1 F.	1:00
	1 F.	0:26
	1 F.	0:40
41	1 F.	1:20
	1 F.	1:13
	1 F.	2:00
42	1 F.	2:50
	1 F.	3:10
	1 F.	2:05
42	1 F.	1:30
	1 S.C.	0:30
43	1 F.	2:28
	1 F.	0:32
	1 F.	1:19
44	1 F.	0:36
	1 F.	1:48
	1 F.	0:33
44	1 F.	1:03
	1 F.	0:09
44	1 F.	1:04
	1 S.C.	0:19
52	none	
62	none	
72	none	
82	none	

¹ F.—Flagellate. S. C.—Small Ciliates.

Hence a typical Penn clay loam was used. Inasmuch as active protozoa were found in all the samples of the Sassafras soil, even with lowest (three-fourths of the optimum) moisture content, in repeating the above problem with the Penn loam soil in each series, an additional quantity of soil was taken and moisture to the amount of one-third of the optimum was added.

Likewise, the experiment was repeated with the Sassafras soil under the same conditions as in the preceding experiment, except that the moisture content was one-third of the optimum. Careful examinations for a period of 15 to 20 minutes were made of each sample on the 1st, 2nd, 3rd, 4th, 6th, 8th, 10th and 14th days.

Upon noting the upper portion of Table III and of Table I, it will be seen that the data indicate that with this soil (Sassafras) the moisture is the limiting factor which determines the presence of the protozoa in the active condition, while the organic matter and sand (porosity) added are secondary factors, a fact which did not hold for bacteria, in that some types of bacteria were always present at this (one-third of the optimum) moisture content. Again, with the Penn clay loam soil the greater number of protozoa became active on the 2nd, 3rd and 4th days, after which the number decreased so that there were practically none noted after the 6th day. With this soil, the number of active protozoa was very small as compared with the numbers counted in the previous problem (Table I) with the Sassafras soil. In many cases they were not found at all on any day, even where there was as much as 50 tons of cow manure per acre present, and with moisture to the amount of one and one-half optimum. With this in mind, it would hardly seem that they would play an important rôle in soil biological processes if their presence in the active state as determined by these methods, is used as an index. Apparently this type of soil (Penn loam) seemed to be a very unfavorable medium for active protozoa. A very small number might have excysted the second or third day, and so few being active for such a limited period of time, they might already have become inactive when the examinations were carried out. Dried blood seemed to favor their development to some extent. In sample No. 35, with the Penn soil containing 2.85 gm. of dried blood and a moisture content of one-third of the optimum, one small flagellate was seen to be present on the second day. This might have been due to the fact that the soil moisture was not equally distributed throughout the mixture as it is very difficult to get the moisture evenly distributed throughout the mass especially where such a small amount is added.

The addition of a sand to the soils increasing the porosity and aeration did not seem to encourage the increase of active protozoa.

In order to secure more data upon the effect of the addition of organic matter upon the presence of protozoa in the active condition, sample No. 22 with optimum moisture as shown in Table I, which had been under examination until after the 28th day, was employed for this work. In like manner, tumbler No. 20, which contained Sassafras soil with a moisture content of one-third optimum and tumblers No. 25 and No. 27 with Penn soil having moisture of one-third optimum and optimum, respectively, and which had been examined for 14 days were also used. To each of these soils, 2.85 gm. of dried blood were added and carefully

TABLE III
 PRESENCE OF ACTIVE PROTOZOA IN A SASSAFRAS SOIL AND PENN CLAY LOAM, WHICH HAD RECEIVED TREATMENTS
 OF DRIED BLOOD AND COW MANURE, AT VARIOUS MOISTURE CONTENTS AND AT DIFFERENT INTERVALS FOR A
 PERIOD OF 14 DAYS

Lab. No.	Kind of Soil	Material added	Amount of material added	Moisture added		Number of protozoa actually found after incubation per 0.33 gm. soil							
				Degree opt ^{um}	c.c.	Days							
						1st	2nd	3rd	4th	6th	8th	10th	14th
20	Sassafras	Nothing		1/3	6.20								
36	"	Dried blood	2.85	1/3	7.15								
46	"	Cow manure	2.85	1/3	7.38								
50	"	Sand	30	1/3	5.37								
25	Penn	Nothing		1/3	8.19								
26	"	"		3/4	18.42								
27	"	"		1	24.56								
28	"	"		1 1/2	36.84								
29	"	"		1 3/4	42.98		2 F. ¹		1 F.				
35	"	Dried blood	2.85	1/3	9.14		1 F.						
36	"	"	2.85	3/4	20.55		1 F.						
37	"	"	2.85	1	27.41								
38	"	"	2.85	1 1/2	39.66		1 F. 1 S.C.			1 F.			
39	"	"	2.85	1 3/4	45.83		1 F.	1 F.	1 S.C.				
45	"	Cow manure	2.85	1/3	8.72								
46	"	"	2.85	3/4	19.62								
47	"	"	2.85	1	26.16								
48	"	"	2.85	1 1/2	39.21								
49	"	"	2.85	1 3/4	45.78		1 F.	1 F. 1 S.C.					
53	"	Sand	2.85	1/3	6.15								
54	"	"	2.85	3/4	13.83								
64	"	"	2.85	1	18.44								
74	"	"	2.85	1 1/2	27.66		1 S.C.						
84	"	"	2.85	1 3/4	32.27								

¹ F.—Flagellates. S. C.—Small Ciliates.

mixed as before. These samples were examined for living protozoa on the 1st, 2nd, 3rd, 4th, 6th, 8th and 14th days.

TABLE IV

PRESENCE OF ACTIVE PROTOZOA IN SOILS, WITH DIFFERENT MOISTURE CONTENTS, WHICH HAD PREVIOUSLY BEEN INCUBATED FOR A DEFINITE LENGTH OF TIME AND WHICH LATER RECEIVED ORGANIC MATTER

Lab. No.	Kind of Soil	Moisture during first incubation c.c.	Moisture after Dried Blood was added c.c.	Presence of active protozoa before Dried Blood was added	Presence of protozoa after inoculation							
					Days							
					1st	2nd	3rd	4th	6th	8th	10th	14th
20	Sass.	6.20	7.15									
22	Sass.	18.60	21.45									
25	Penn	8.19	9.14									
27	Penn	24.56	27.41									

From the above data it is seen that not only did the protozoa fail to excyst in the soils with a low moisture content, but they were not even noted in the soils which had an optimum moisture content. This might be explained by the fact that during the previous incubation of these soils, before the dried blood had been added, the organisms (bacteria, actinomycetes, fungi, and in some cases protozoa, No. 22) multiplied, and decomposing more or less of the substances used for life processes, left products of assimilation behind which no doubt were unfavorable to their presence. Hence they did not appear in the sample even where the conditions were favorable for their development before. This shows that there is still another factor, other than moisture, presence of organic matter, physical property of the soil and temperature, which determines the presence or absence of active protozoa.

It might be stated that the inconsistency (as termed by Waksman) of Koch's (7) former results with the greenhouse soils, in that in some cases, active protozoa were found to be present at a lower moisture content than at others, and that Koch stated that the moisture was the primary limiting factor, can be explained as follows: By noting in the table the extent of protozoan activity in greenhouse soils (7) it will be seen that "no mixtures" and "20 per cent compost plus sand" contained the lowest percentage of moisture while the 40 per cent composts contained the highest. Thus when active protozoa were not found in soils with 34 to 36 per cent moisture, one of the reasons might have been that the actual free water at such a high compost was less than in a "no mixture" containing 22.59 per cent of moisture where motile protozoa were found.

The data as presented in Table I and II point to the fact that the greatest number of protozoa became active on the 2nd, 3rd, 4th and 6th

TABLE V
PRESENCE OF ACTIVE PROTOZOA AND NUMBERS OF BACTERIA IN SAMPLES OF SASSAFRAS AND PENN. CLAY LOAM SOILS
WITH AN ADDITION OF DRIED BLOOD, AT DIFFERENT MOISTURE CONTENTS AND AT VARIOUS INTERVALS
FOR A PERIOD OF 14 DAYS

Exp. No.	Kind of Soil	Moisture added e.g.	Days											
			1st			2nd			3rd			4th		
			Protozoa	Bacteria	Av. Bact.	Protozoa	Bacteria	Av. Bact.	Protozoa	Bacteria	Av. Bact.	Protozoa	Bacteria	Av. Bact.
101	Sass.	7.15	15	44	51	50.0	—	104	101.6	—	132	117.5	115	168
			11	12.0	51	50.0	—	90	101.6	—	103	112.0	108	120
			10	—	52	—	—	111	—	—	103	—	113	148.0
			8	46	46	—	66	66	—	—	122	90	90	162
102	"	7.15	11	9.6	39	45.0	—	103	86.6	—	102	112.0	90	114
			10	—	50	—	—	91	—	—	102	—	90	108
			16	132	132	—	126	126	—	—	112	114	84	118.0
103	"	21.45	18	17.6	96	108.0	1 F.	114	140.0	1 F.	186	172.0	330	78
			19	—	96	—	180	180	—	—	138	330	540	18
			26	132	132	—	78	78	—	—	192	253	582	30
104	"	21.45	16	17.0	162	140.0	1 F.	66	82.0	1 F.	126	116.0	99	12
			9	126	102	—	102	102	—	—	102	220	294	12
			20	33	29	—	29	29	—	—	29	42	306	312
201	Penn	9.14	19	18.0	27	28.0	—	32	28.3	—	25	28.0	24	48
			15	—	24	—	—	32	—	—	30	34.0	30	32.0
			22	32	32	—	29	29	—	—	30	36	18	8.0
202	"	9.14	14	17.3	31	30.6	—	34	33.3	—	25	24	24	18
			16	—	29	—	—	34	—	—	32	30.0	30	30
			64	108	36	—	36	36	—	—	26	36	36	12
203	"	27.41	74	67.0	84	86.0	—	48	48.0	—	102	84.0	66	30
			63	—	66	—	60	60	—	—	66	49.3	38	36
			53	72	72	—	96	96	—	—	66	44	44	72
204	"	27.41	54	56.3	64.0	—	49	49	—	—	36	52.0	33	78
			62	102	102	—	47	47	—	—	54	55	55	108
														126.0
														76.0
														60

NOTE:—Numbers of bacteria represent millions per gram of soil. Numbers of protozoa are for 0.33 gm. of soil.

* Lost.

days. It was now desired to ascertain if during this period there would be a marked decrease in the number of bacteria. The possible destructive character of protozoa upon bacteria seemed to be the best index of the effects of protozoa. In the case where no protozoa were seen to be present, as at one-third of the optimum moisture content, one might infer that up to a certain point the bacterial multiplication would be consistent, while where the protozoa were seen to be present, their multiplication (bacteria) would be irregular. With this in mind four 200-gm. quantities of the same Sassafras loam and like quantities of Penn clay loam which had previously been used were weighed out into tumblers. Each tumbler received 5.70 gm. of dried blood, (or the equivalent of 50 tons per acre). Sufficient sterile water was added to two tumblers of the Sassafras series and two of the Penn series to make the soil plus dried blood of one-third of the optimum moisture content. In like manner two tumblers of each soil plus dried blood received sufficient water to bring the moisture content to optimum. These were carefully mixed as before and then placed in the incubator. Careful examinations of each tumbler for active protozoa for a period of 15 to 20 minutes were made from the 1st to the 14th days (inclusive) as before. The data were recorded each day. After the examinations had been completed 10 gm. were weighed out of each of the tumblers and this was used for making the bacteria counts¹.

Upon noting Table V it is seen that these data (part showing the presence of active protozoa) corroborate that which is shown in Tables I and III with these soils and the corresponding moisture content. Active protozoa were first seen to appear on the third day in the Sassafras soils containing the optimum amount of moisture. They were noted every day thereafter until and including the 10th with one sample, while with the other they were not seen on the 6th or 8th days. In the samples containing the lower amounts of moisture (one-third optimum) they were never seen to be present. Motile protozoa were never observed in any of the samples of the Penn clay loam soil. If they were active at any time in the samples of this soil they were either so minute that they could not be distinguished or the numbers were so very small that a 15 to 20 minute examination did not show any.

Is there any correlation between the bacterial numbers and the presence of active protozoa as determined by the direct examination? Glancing through Table V, which shows the number of bacteria present in the various samples of soil, it is seen that with samples No. 101 and No. 102, there is a more or less gradual increase in numbers of bacteria up to and

¹The bacterial counts of each tumbler were made in triplicate on Lipman and Brown's "synthetic" agar. Lipman, J. G., and Brown, P. E. *In* N. J. Agr. Exp. Sta. 29th Ann. Rpt., p. 129-136. 1908.

including the 10th day, and a very decided decrease on the 14th day. As stated before and shown in Table V, active protozoa were never seen to be present in either of these samples. In samples No. 103, there is a marked increase in bacterial numbers until the 8th day even though active protozoa were observed on the 4th, 6th and 8th days. On the 10th day there is a very marked decrease in bacteria and a greater number of protozoa were also counted on this day. In the case of sample No. 104, this increase in bacterial numbers from day to day is not so consistent as it is with No. 103 (the duplicate of 104), but even in the latter sample, we do not find any reduction in bacteria after the third day until the 14th even though motile protozoa were observed. If the active protozoa in No. 103 were effective in destroying bacteria, then the multiplication of bacteria must have been much greater than that in sample No. 104 in order that the greater number of bacteria in No. 103 can be accounted for, as more protozoa were also noted in this latter sample than in No. 104; or else the motile protozoa did not affect the numbers of bacteria to any appreciable extent, since it is noted that in samples Nos. 101 and 102, where the moisture content was low, there was a great decrease of bacterial numbers on the 14th day and yet living protozoa were never seen. Again, on the 14th day, in samples Nos. 103 and 104, we find a great decrease in bacterial numbers. Is this due to the effect of the active protozoa which were seen to be present on the 10th day? If this is true, it would agree with Russell (11) in his objection that the effect of the detrimental factor cannot be seen in a 7-day incubation period; hence the protozoa which had been present in the active condition might not have been of the destructive type. Again, might the decrease in the numbers of bacteria have been due to other causes, as the presence of fungi or other organisms, or to the presence of toxic decomposition products?

Upon examining the results, with the Penn soil, it is seen that in the samples containing moisture of $\frac{1}{3}$ optimum, the bacterial numbers were practically constant until the 10th day. On this day a marked decrease was noted and then a great increase on the 14th day. It would hardly seem possible that this decrease in bacterial numbers was due to a detrimental factor (namely protozoa) as they were never observed to be present in the motile condition. The irregularity in numbers of bacteria was noted in a piece of work by the writer (8) where he counted organisms (*B. coli*.) developed from day to day over a period of time. Just such a cause may be due to the variation in the numbers of bacteria which were observed in this problem. Again in Nos. 203 and 204 there is a fluctuation in bacterial numbers from day to day and still no protozoa were ever noted to be present.

It is true that an investigator could hardly conclude that the results of such a problem as above, where all the organisms of the soil as well as the other conditions as moisture, assimilation products, etc., must be considered, are due to the one specific factor with which he is working. However, the intention of this work, as in the case of the previous work (7), is to study normal soils as they occur and thus see whether or not protozoa if they are a factor, exist in the normal conditions (that is, as nearly normal as possible in carrying out laboratory experiments) and to correlate if possible the factors studied (bacteria and protozoa) under these conditions.

Can the results obtained by employing partially sterilized sewage-sick soils or partially sterile normal soils, in which cases only some of the factors are eliminated, be used to demonstrate the effects of a detrimental factor (probably protozoa) applied to normal agricultural soils? As shown recently by Coleman, Lint and Kopeloff (1), partial sterilization of normal soils destroys only some of the factors which function in soil fertility, and it greatly influences (alters) the character of the soil as a medium.

No doubt, under very abnormal conditions (as regards agricultural practices), for instance in the case of sewage-sick soils which contain a great abundance of organic matter and whose floral population is entirely different from that of soils under tillage, containing not only an abundance of protozoa but also eel worms, vermes and many other living forms, and under other conditions, as aeration, etc., entirely different from those under which the soil is tilled normally, a limiting factor might be of immense importance.

In the light of the results of this paper and of the paper cited above (7), it would seem that in a practical way, protozoa are of slight importance in limiting the bacterial flora of the soil, as their presence in the active state is very limited. In no cases were they found to be present in the active state in the field soils examined, in only 6 out of the 20 greenhouse soils were protozoa found, and in these soils their numbers were very limited. The data as given in this paper would indicate their presence in some soils to which a large amount of organic matter (50 tons per acre) has been applied, but even under these conditions of fertility, when more organic matter is applied than is ever used under ordinary field agricultural methods, they are active only to a very limited extent, the numbers decreasing decidedly 8 or 10 days after the organic matter has been added. Moisture seems to be the primary factor which in these soils and others, determines their presence in the active condition, as active protozoa were never noted in soils which were air-dry or had a very low moisture content, even though there was an abundance of organic matter and the texture was of very open character. This is not true of bacteria however, as some are in the active condition in air-dried soils.

SEPARATION OF BACTERIA FROM PROTOZOA

Under certain conditions, in carrying out soil biological experiments in the laboratory and eventually in the field (9) for a period of 7 to 10 days, we have protozoa present in the active condition. Are they an influential factor in the biological method as now employed? Considering the very small numbers which are present in the active condition as compared with bacteria, and the fact that they are present for such a short period of time, we would hardly think they would be a factor. However, in desiring to prove this point, the author is attempting to get a protozoa culture free from bacteria. He plans to inoculate the medium with these clean cultures and in this way note their effect upon bacterial activities.

For this work a special centrifuging tube was devised by the writer and has been proving satisfactory although a complete separation of bacteria from protozoa has not yet been accomplished.

The apparatus, as pictured in figure 1, consists of a glass centrifuging burette, 10½ inches long and having a capacity of 6 c.c. This special burette has two stop cocks at its lower extremity. The contents of the small tube between the stop cocks is about 1 c.c.

The protozoa which are much heavier than bacteria are forced down by gravity in the centrifuging process as has been previously tried by Russell and Golding (13) and later by Jones (5).

Fig. 1—Diagram of apparatus used for separating protozoa from bacteria.¹

The procedure of this method is as follows: Cock C being closed and cock C' open and the lower end (delivery tube) of the burette having been plugged with cotton, 5 c.c. of the medium containing protozoa and bacteria are introduced into the sterile apparatus. The apparatus with contents is now placed into the centrifuge and a certain number of revolutions are made. The protozoa are thus forced down through the opening of cock C' and are held in the small tube between C and C'. Cock C' is closed and the solution in the upper part of the burette which contains the greater number of bacteria is poured out. This section of the apparatus is now sterilized with alcohol and then some of the sterile medium is introduced. Cock C' is now opened and the protozoa which are held in the tube between C and C' are washed into this sterile medium. Centrifuging is repeated, cock C' closed and the same process repeated as before. In this manner the protozoa can be washed several times until the bacteria are all washed from them. The protozoa having thus been freed from the bacteria, cock C is opened and the protozoa are delivered into the desired container by means of the small delivery tube at the lower extremity of the burette.

¹ The author is indebted to Dr. J. W. Shive for preparing this drawing.

SUMMARY

Under the conditions with which the problems recorded in this paper were carried out, the following results as to protozoan activity in the soils have been noted:

1. The direct examination of the soil under the microscope for a period of 15 to 20 minutes (taking 3 or 4 samples), gives a fair indication as to the relative extent of active protozoa in the soil.

2. Practically all of the protozoa as found in the soil can be observed under the low power of the microscope by the careful experimenter.

3. The addition of organic matter to the Sassafras sandy loam soil encouraged a greater protozoan development than where none had been added.

4. Additions of dried blood to the Penn clay loam soil increased the number of active protozoa but very little while cow manure had no effect on the protozoan activity of this soil.

5. Increasing the porosity and aeration of the soils by the addition of sand did not increase the number of motile protozoa.

6. In the Sassafras soil to which cow manure had been added, flagellates were noted on the 1st day, while with the other samples they were not seen until the 2nd or 3rd and in some cases the 4th day. The maximum numbers were recorded on the 4th and 6th days after which there was a marked decrease so that on the 21st and 28th days practically none were found to be in the active state. This would indicate that the destructive ability, (if soil protozoa possesses that factor) would be present for only a limited period, namely the early stages of organic decomposition.

7. Flagellates were the first protozoa to excyst, later, on the 3rd and 4th days, small ciliates appeared. On the 14th, 21st and 28th days, small ciliates were more numerous than flagellates.

8. With these soils, the largest number of protozoa was recorded where the moisture was highest.

9. The Penn clay loam soil seemed to be a very undesirable medium for protozoa, as very few of these organisms were noted in any of the samples compared with those found in the Sassafras soil.

10. At the lowest moisture content (one-third of the optimum) protozoa did not become active.

11. With these soils, moisture seemed to be the primary limiting factor which determined the presence or absence of active protozoa.

12. With one exception, no correlation between the presence of protozoa in the active condition and numbers of bacteria could be seen. Increased numbers of bacteria were observed irrespective of the presence or absence of living protozoa.

13. On the 14th day in the soils, even where no protozoa were found in the active condition, there was a great decrease in bacterial numbers.

14. Inasmuch as the numbers of protozoa in comparison with the bacterial numbers are so small, even in the presence of such abnormal quantities of organic matter as were used in these experiments, it hardly seems that they would be of very great importance in agricultural practices.

It has been previously shown (7) :

1. Under ordinary greenhouse conditions, small ciliates, flagellates and amoebae are active in some soils, but their presence is very limited.

2. Active protozoa (small ciliates, large ciliates, flagellates, and amoebae) do not seem to be present in field soils with a normal moisture content, and even when the moisture content is slightly supernormal; hence, they would not be a limiting factor in the soil.

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