Investigations on Protozoa in Relation to the Factor Limiting Bacterial Activity in Soil.

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Introduction.

In the course of my work on soil protozoa particularly in relation to the question of the partial sterilisation of soil, I had occasion to work with some of the old stored soils kept at the Rothamsted Experimental Station, Harpenden, at which laboratory the work here recorded was commenced. These soils are remarkable for the length of time they have been stored, 67 years being the longest period, and for the fact that in many cases the original samples put up in large bottles have remained untouched since the day on which they were bottled.

Preliminary cultures of some of these soils in hay-infusion were begun in 1912 to ascertain the character of the protozoan fauna, if such still persisted in them. From these cultures it was found that in a mixed sample from Broadbalk, bottled in 1846 and containing about 3 per cent. of water by weight, no protozoa were present, whilst in another mixed sample taken from six bottles of Barnfield soil, put up in 1870 and containing about 10 per cent. of water by weight, amœbæ and flagellates but no ciliates were present.

Quantities of these two soils were taken and were submitted to partial sterilisation treatment in order to find out if the limiting factor usually eliminated by partial sterilisation was present in them. The results obtained by bacterial counts over a period of about 281 days showed that in the 1846 soil no factor limiting bacterial activity was present, whilst in the 1870 soil the limiting factor was present.*

As a result of this work I decided to use some of the 1846 soil for inoculation with different species of protozoa obtained from soil, in order to test if possible their power to act as the factor limiting bacterial activity. The protozoa selected for culture and inoculation into separate samples of soil were the following:—*Colpoda cucullus*, *Col. maupasii*, *Col. steinii*, and *Vorticella microstoma*. *Amœba* sps.? and Flagellate sps.? were obtained by culture from the 1870 soil which, as already mentioned, had been found to contain

* This work was carried out in collaboration with Dr. H. B. Hutchinson at Rothamsted.

the limiting factor, presumably the amœbæ and flagellates in it, according to Russell and Hutchinson's hypothesis.

Besides the above series of samples the set was made up to include a bottle of untreated soil, and one inoculated with a culture of bacteria representative of the bacterial flora added with the cultures of protozoa in the other samples, so as to serve as a check against them ; and a bottle to receive 10 per cent. of the 1870 soil, thus making nine bottles of soil in all.

Another set of soils was experimented upon at the same time. This consisted of seven bottles of fresh Hoosfield soil, partially sterilised first by toluene and then by heating to 65° C., so as to eliminate the limiting factor, and then inoculated again with cultures of protozoa obtained from the untreated soil. The series consisted of the following:—Untreated, Toluened, Toluened + Untreated, Toluened + Ciliates, Toluened + Amœbæ, Toluened + Flagellates, Toluened + Bacteria. The bacteria used for the last-named inoculation were representative of the bacterial flora of the other cultures.

In each set of bottles the water content of the soil was finally brought to about 18 or 20 per cent. by weight ; this being about the water content at which many of Russell and Hutchinson's* soils have been maintained, and at which they have found the limiting factor to be active.

I decided at the outset to make periodic bacterial counts by the gelatine-plate method in order to determine the numbers of bacteria in the soil as nearly as possible once a month and to carry on the experiments for a long period.

Methods.

A mixed sample of soil from six bottles of 1846 soil was taken and divided into nine lots of 400 grm. in each.

Each lot of soil was put up in a quart bottle which had previously been sterilised and plugged with cotton wool.

In the case of the Hoosfield soil seven 400 grm. lots of slightly air-dried soil were taken after having been passed through a 3-mm. sieve. These were bottled in exactly the same way as the 1846 soil. The soil was first toluened by the addition of 2 per cent. of toluene, which was allowed to remain in the soil for two days, after which the soil was spread out on sheets of paper so as to allow the antiseptic to evaporate. Hay-infusion cultures of the toluened soil were made, and as it was found that flagellates developed in the cultures the bottles of soil were submitted to steam heat at a temperature of 63°–65° C. for three to four hours. This operation was

* Russell and Hutchinson, 'Jour. Agric. Science,' vol. 3, Part II (1909), and vol. 5, Part II (1913).

carried out in a steamer, the temperature of which was regulated by means of a thermostat. Hay-infusion cultures were made after this second treatment, and it was then found that no flagellates cropped up.

The cultures of protozoa used for the inoculation of the bottles of soil were obtained in the following manner: Hay-infusion cultures were made from fresh soil and from old cultures containing cysts which I had on hand. By the use of fine capillary pipettes it was possible to isolate ciliates, which were then sub-cultured in hay-infusion. I found it best to use hay-infusion already containing active bacteria for the sub-culture of isolated forms, the bacteria serving immediately as a source of food for the protozoa. Cultures of flagellates from the 1870 soil were obtained in the same manner, and for the culture of amœbæ from the 1870 soil I made use of cysts from pure cultures on agar plates which I had by me. In this way pure cultures of the following protozoa were obtained for use with the 1846 soil:—*Col. cucullus, Col. maupasii, Col. steinii, Vort. microstoma, Amœba* sp. ?, and Flagellate sp. ?.

The protozoa for inoculation into the treated Hoosfield soil were obtained by isolation and sub-culture of forms cultivated in hay-infusion from the untreated Hoosfield soil, so that the forms added should represent as nearly as possible the fauna originally present in the soil. The cultures of protozoa thus obtained were one of *Amœba* sp. ?, one of Flagellates sp. ?, and one of Ciliates, including *Col. cucullus, Col. steinii,* and *Col. maupasii.* The small Ciliate *Balantiophorus minutus* or *elongatus* also occurred in the cultures made from the untreated soil, but as I was unable to obtain this free from flagellates, the culture of ciliates did not include this form.

In order to obtain mass cultures of the protozoa in sufficient quantity to serve for inoculation into the soil the following method was employed: 80-grm. lots of washed and sterilised sand were put into large sterile petri dishes or glass cylinders and covered with hay-infusion, which was then infected with a pure culture of protozoa, and the latter were allowed to multiply and populate the culture.

In this way a large quantity of each kind of protozoa was obtained for the inoculation of the soil. This process was carried out by spreading the soil on sheets of sterilised brown paper and then mixing the sand-hay-infusion culture of protozoa into it by means of a sterilised spoon, the whole of the soil, sand, and hay-infusion becoming thoroughly well mixed together and thus ensuring an even distribution of the protozoa throughout the soil. In the case of the 1846 soil the inoculation was carried out in a glass-house which had been steamed down in order to allay dust and thus minimise the chance of infection. The soils were left exposed in this house for some days

in order to allow the bulk of the water to evaporate off, and a heating lamp was put into the house in order to accelerate slightly the evaporation.

The reason for thus driving off the bulk of the water from the soil was that I desired to bring about in the soil all the conditions possible for aiding the excystation of the added encysted protozoa, for I had found in experimenting with cysts that if they were slightly air-dried and then moistened, excystation was more rapid than where no slight drying had been allowed.

The water-contents of the inoculated soils, when bottled again at the end of these few days of air-drying, were as follows:—Untreated, 6·2 per cent.; Untreated + Bacteria,* 2·7 per cent.; U. + *Col. cucullus*, 7·4 per cent.; U. + *Col. steinii*, 7·22 per cent.; U. + *Col. maupasii*, 6·9 per cent.; U. + *Vort. microstoma*, 6·8 per cent.; U. + *Amœbæ*, 7·5 per cent.; U. + Flagellates, 7·5 per cent.: U. + 1870, 6·5 per cent.

Sterile distilled water was next added to all the soils, in sufficient quantity to bring up the water-content of each to 18 per cent.

In the case of the seven bottles of Hoosfield soil, the samples were inoculated with protozoa from the sand-hay-infusion cultures in exactly the same way as described above. These lots were spread out on sheets of sterile brown paper and left in the drying room for five hours at a temperature of about 20° or 22° C. in order to drive off the bulk of the added water and bring about conditions favourable to the excystation of protozoa after re-moistening. The water-content of the various soils after drying was as follows:—Untreated (U.), 7·4 per cent.; Toluened and heated (T.), 5·9 per cent.; T. + Untreated (T. + U.), 5·8 per cent.; T. + Ciliates (T. + C.), 3·1 per cent.; T. + *Amœba* (T. + Am.), 2·7 per cent.; T. + Flagellates (T. + Fl.), 3 per cent.; T. + Bacteria (T. + B.), 1·6 per cent. Sterile distilled water was then added, as in the case of the 1846 set, in order to bring up the water-content of each lot to 18 per cent. by weight.

Both sets of bottles were then left in a small warmed glass-house, the temperature of which varied between 45° and 55° F. Later on they were taken from the glass-house and kept in the laboratory in a room at about 12–15° C. At various intervals during the course of the work the water-content of each soil has been determined in order to estimate the loss of water by gradual evaporation, and at these times the loss from each has been made good by the addition of sufficient sterile distilled water to bring up the water-content to 18 or 20 per cent. by weight.

In attempting to estimate the numbers of protozoa present in the soils the following methods have been employed:—

* This lot was treated in the same manner as the inoculated Hoosfield soil described further on.

A dilution method was first used which Dr. H. B. Hutchinson had devised and which he and I had used in 1910 in the course of some joint work. Ten grm. of soil are shaken up for four minutes with 100 c.c. of 1-per-cent. hay-infusion, either sterile or containing an active growth of soil bacteria. By means of sterile 1 c.c. pipettes varying quantities of this soil suspension are taken out and placed in sterile tubes; three tubes of each dilution being put up. In the case of the smallest quantities of soil suspension more hay-infusion is added in order to give a sufficient quantity of liquid for purposes of manipulation. The scheme of dilution is as follows:—

		grm. of soil.
10	c.c. original soil suspension	= 1
5	„ „	= 0·5
2	„ „	= 0·2
1	„ „	= 0·1
0·5	„ „	= 0·05
0·2	„ „	= 0·02
0·1	„ „	= 0·01
0·5	c.c. mixture of 1 c.c. original + 9 c.c. hay-infusion ...	= 0·005
0·2	„ „ „ „ ...	= 0·002
0·1	„ „ „ „ ...	= 0·001

The cultures thus obtained are allowed to incubate for about a week and microscopical examination is made of the surface layers by taking out drops on a sterile platinum loop and examining them on glass slides. If protozoa occur in the cultures of any particular dilution, then one infers that they are present in the soil in numbers equal to the factor required to raise the particular dilution to 1 grm. Thus if they occur in all three cultures of 0·001 grm., then there are at least 1000 protozoa per gramme of soil. The method possesses the advantage that one deals with a comparatively large quantity of soil, viz. 10 grm., and should thus be able to overcome the difficulties of any irregular distribution of protozoa in the soil itself, provided a good suspension is made. However, in working with it I have obtained most irregular results, which I have not been able to explain, and for this reason I have practically given up using it in favour of an agar-plate method.

Before leaving the hay-infusion method, however, I may add that I carried out a series of experiments in order to ascertain if the violent agitation of the soil and liquid, in making the suspension by shaking for four minutes, had any injurious effect on living protozoa. I found, when soil was added to a hay-infusion culture containing innumerable active ciliates and apparently no encysting forms and the mixture was shaken violently for four minutes, that on making a series of dilution cultures from this suspension protozoa

cropped up in abundance throughout, thus showing that they had suffered no damage.

The agar-plate method which I have used is as follows:—Sterile petri dishes are poured with nutrient bouillon agar of about 0·5 to 1 per cent. in strength, and, when cool, the surface of the agar is inoculated with a weighed quantity of the soil the number of protozoa in which it is desired to ascertain. Three plates, as a rule, are inoculated with each weight of soil and the following are the weights of soil which have been used—1, 0·5, 0·2, 0·1, 0·05, 0·02, 0·01, 0·005, 0·002, 0·001, 0·0005, 0·0002, 0·0001 grm. The plates are allowed to incubate for a few days and then the surface of each is examined under the microscope for the presence of protozoa. The method entails the use of a sensitive balance and is limited by the difficulty of manipulating such small quantities of soil as are produced in weighing in the region of 0·0001 grm. However, the results which I have obtained with it are fairly consistent and are more trustworthy than those of the hay-infusion method, I think.

It was my hope at the beginning of the experiment to obtain evidence, by means of the counts of protozoa, concerning their activity and multiplication if such were proceeding.

Counts of protozoa were therefore made at the beginning and towards the ends of the experiments. The hay-infusion method was used in the first counts and the agar-plate method for the later ones. As I have pointed out, the latter method gives higher counts and more trustworthy results, and one cannot, therefore, strictly compare the evidence afforded by the two methods.

For this reason I have not found it possible to obtain sound evidence as to whether the protozoa have multiplied since being added to the soil. See footnote, however, on p. 454.

The Bacterial Counts.

The results of the periodical determination of the numbers of bacteria by the gelatine-plate method are tabulated below and the curves obtained by plotting these results are shown in figs. 1, 2, 3, 4, and 5.

In order to simplify matters I have arranged certain curves together, for the whole nine curves when plotted all together present a confusing array and do not lend themselves to easy elucidation.

Fig. 1 shows the curves obtained from the untreated, bacteria, and *V. microstoma* inoculated soils.

The most noteworthy feature is the extraordinarily high bacterial count in the Bacteria soil at 32 days and the subsequent drop in the numbers of bacteria to a level below that of the untreated soil. This low bacterial

Bacteria in millions per gramme.

	At beginning.	After 32 days.	After 63 days.	After 92 days.	After 124 days.	After 153 days.	After 181 days.	After 232 days.
Untreated	6·6	391	291	307	280	203	261	118
Bacteria	15	1504	472	440	259	98	102	59
Col. cucullus	235	464	386	312	315	174	266	159
Col. steinii.....	218	379	314	271	289	165	324	142
Col. maupasii.....	253	501	262	313	260	191	264	116
Vort. microstoma	186	453	216	201	156	65	119	84
Amœba	178	599	412	274	409	193	242	178
Flagellates	82	374	327	416	319	127	188	132
U. + '70	8·5	204	154	169	128	116	159	103

	After 284 days.	After 324 days.	After 360 days.	After 383 days.	After 419 days.	After 486 days.	After 519 days.
Untreated	50	55	lost	115	76	87	57
Bacteria	lost	27	40	36	20	45	33
Col. cucullus	96	102	143	104	95	134	70
Col. steinii.....	77	138	112	224	133	138	130
Col. maupasii ...	75	116	126	131	114	133	91
Vort. microstoma	35	86	39	95	67	91	58
Amœba	160	135	129	242	160	168	118
Flagellates	54	86	93	120	75	124	123
U. + '70	84	83	86	111	76	93	68

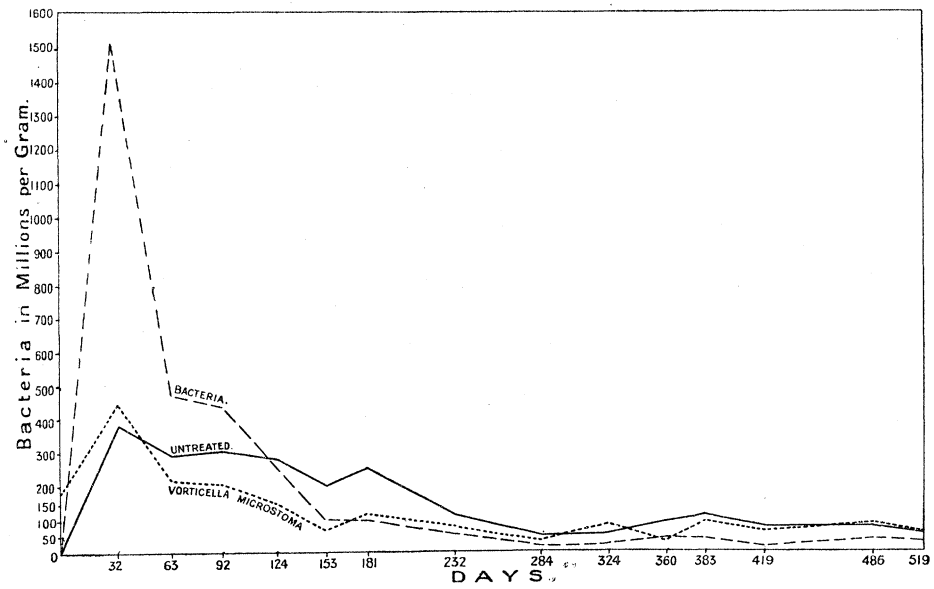

Fig. 1.

content was maintained over a very long period—366 days—and is perhaps the most surprising and unexpected result of the whole investigation.

The *V. microstoma* curve is also very interesting and shows the influence of some factor which had become operative by the end of 63 days and which subsequently kept the numbers of bacteria in check, though only at about the same level as the untreated soil.

The untreated curve also shows that the bacteria have gradually decreased in numbers after reaching and maintaining a high level for 181 days. These results are very interesting when considered in relation to the number of protozoa in the soils.

In the untreated 1846 soil no protozoa are present, so that the gradual decrease in the number of bacteria cannot be due to the activity of the protozoa. The *V. microstoma* soil, however, contained, a few weeks after inoculation, about 300 vorticellæ per gramme. By December, 1913, however, all the vorticellæ had died out, for they failed to appear in cultures of the soil made at that time and on all occasions since.* Flagellates and amœbæ are present in this soil, probably due to infection of the mass culture or during the initial air-drying of the inoculated soils, to the extent of 1000 flagellates and 100–200 amœbæ per gramme.

It is probable that these lead an active trophic existence in the soil and so might be considered responsible for the limiting action on the bacteria. This can scarcely be the case, however, when we consider this soil in relation to the bacteria-inoculated soil. In the latter there are flagellates present to the extent of about 100 per gramme. If now the limiting factor in this soil is considered as due to the action of these flagellates, we should expect to find not so great a decrease in the bacterial numbers as in the vorticella soil, where the flagellates are more than ten times as numerous, and where there are, in addition, 100–200 amœbæ per gramme. The reverse of this is the ascertained result, and clearly negatives the idea that the flagellates and amœbæ are responsible for the limiting action on the bacteria.

Another point of interest is that the two curves for the untreated and

* This dying out of the *Vorticella microstoma* is very interesting. At first I thought its failure to appear might be due to an unsuitable culture medium. I, therefore, tried to obtain it again, taking care of the reaction of the hay-infusion, but with no better success. It also failed to appear on a nutrient bouillon agar, favourable to the growth of all the other protozoa under consideration. I afterwards remembered that in some earlier experiments I had failed to obtain Vorticella from a soil which had been kept in the laboratory for some months and from which I had obtained a very fine culture of the organism when the soil was fresh. At another time too I failed to get the excystation of *V. microstoma* from cysts which I had obtained in a hay-infusion culture and which had been stored for a few months. From this evidence it would appear that *V. microstoma* in its encysted condition does not retain its vitality for more than a few months, and the dying out in my soil is easily accounted for on this supposition.

V. microstoma soils are, during the greater part of their course, so closely akin that within the limits of experimental error they may be considered identical. In one of them no protozoa are present, whereas in the other amœbæ and flagellates are present. If the latter are the limiting factor they have failed to reduce the numbers of bacteria below the level of the untreated soil containing no protozoa.

Fig. 2 shows the curves for the three samples of soil inoculated with the

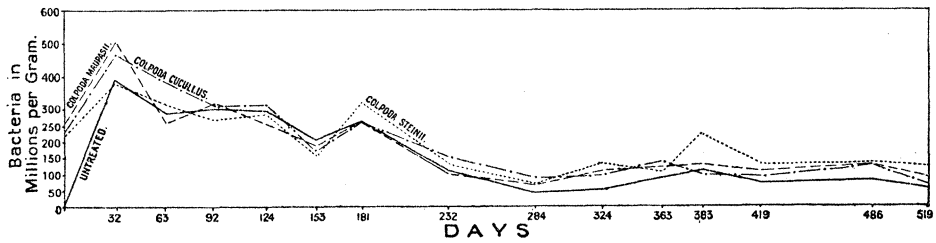

FIG. 2.

three different species of Colpoda, viz., *Col. cucullus*, *Col. steinii*, and *Col. maupasii*, together with the curve of the untreated soil.

There is a marked similarity between all four curves; all of them are of the same type and show no very pronounced differences. On the whole, the bacterial content of the three inoculated soils has remained higher than that of the untreated soil, in spite of the fact that each contained many hundreds of protozoa per gramme. They thus fail to indicate any action by the protozoa of a limiting character on the bacterial population of the soil.

In the *Col. cucullus* soil there are, roughly, about 750 *Col. cucullus* and 1000 flagellates per gramme, the latter only being found in the later determinations by the agar-plate method. The *Col. steinii* soil contains about 100 *Col. steinii* and 1000 flagellates per gramme, whilst the *Col. maupasii* soil contains about 1000 *Col. maupasii*, 200 amœbæ, and 100 flagellates per gramme.*

One would have expected that had the protozoa been capable of acting as a check on the growth of bacteria in the soil, they would have brought their numbers to a level well below that of the bacterial content of the untreated soil. Instead of this, however, we find after 519 days all three soils showing a higher bacterial content than the untreated soil.

* The numbers given for the protozoal counts are those obtained in the last determination.

The amœbæ which occur now in these soils are due most probably to infection either of the soil samples during the initial air-drying or of the mass cultures.

This is good evidence, I think, that the protozoa have not functioned as the limiting factor.

In the case of the soils inoculated with cultures of amœbæ and flagellates obtained from 1870 soil, the curves for the bacterial counts of which are shown in fig. 3, the general inference to be drawn is that after a period of

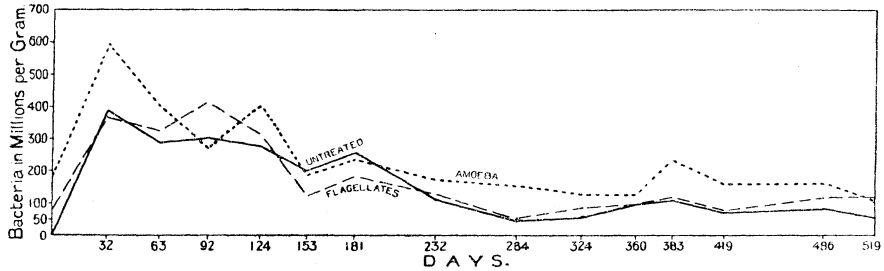

FIG. 3.

18 months in which to act, the protozoa have not exerted a limiting action on the bacteria in their respective soils.

At 519 days the bacterial content of both inoculated soils is well above that of the untreated soil.

The amœbæ in the sample of soil specially inoculated with them are present to the extent of 10,000 per gramme, whilst in the flagellate-inoculated soil there are 10,000 flagellates and about 2000 amœbæ per gramme. These results are very interesting, for they indicate that even when protozoa are present in the soil in such large numbers and under conditions favourable to active existence they do not exert a depressing effect on the bacteria.

The amœba curve is especially significant, for it shows that even in the presence of 10,000 amœbæ per gramme of soil the bacteria can maintain a higher level in numbers than in the original soil containing no protozoa.

The curve for the flagellate-inoculated soil does not call for much comment. It is practically identical with that for the untreated soil during a great part of its length, but on the whole is at a higher level and indicates that the 10,000 flagellates and 2000 amœbæ per gramme are not capable of bringing the bacterial content down below the level of the untreated soil.

Fig. 4 shows the curves for the untreated soil and for the sample to which 10 per cent. of 1870 soil was added. At the end of 519 days the untreated soil is at a lower level than the U.+1870, though from 232 days onwards the two curves are very similar, and from 360 days to the end of the experiment may be considered as identical. There are about 2000 amœbæ and 2000 flagellates per gramme in the mixed soil, and the curves show that these have not been able to bring down the bacteria to a level below that of the

untreated soil (see footnote, p. 454). The Untreated + 1870 curve is, on the whole, very even, showing no marked fluctuations up or down, and it is very

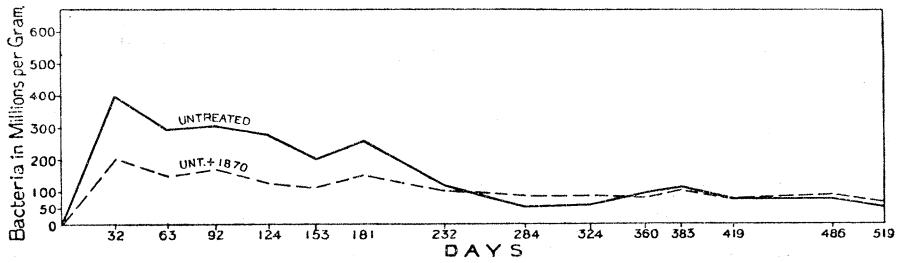

FIG. 4.

interesting to note that during the first 232 days the bacterial counts are below the counts for the untreated soil. This seems to indicate that some factor was introduced with the 1870 soil which, for a time, checked the rapid growth of bacteria and prevented their increasing to the numbers attained by the bacteria in the untreated soil. It is conceivable that this was owing to the action of the protozoa added with the 1870 soil, but the fact that after 232 days the bacterial numbers for the untreated soil came down below the level of the mixed sample, and that the two curves during the last 160 days are practically identical lends no support to this idea.

Whatever the influence was which during the first 232 days checked the growth of the bacteria in the mixed soil, I am inclined to regard it as connected with some other property of the 1870 soil than the presence of protozoa in it. To be more explicit: The 1870 soil was bottled in a comparatively moist condition and received no drying about 1881 as did the 1846 soil along with many others. This drying seems to have effected a very important change in the 1846 soil and, taken in conjunction with the prolonged period of storage has produced in it a condition comparable with partial sterilisation. At any rate, the 1846 soil along with other dried and stored soils which I have examined gave very high bacterial counts when moistened, whereas the 1870 and other soils which have been stored in almost the same condition as they were in when taken from the field give low bacterial counts and indicate the presence of the limiting factor. The following experiments illustrate my point.

Bacterial Counts in other Samples of Old Stored Soils.

Three soils were taken for this piece of work, two of them from bottles of Broadbalk soil stored since 1856 and 1865, and one from a bottle of Geescroft

soil stored since 1865. The two from Broadbalk were dry when taken from their original bottles, whilst the Geescroft sample was in a comparatively moist condition.

The water-contents of these soils were not determined immediately after taking them from their respective bottles, because it was not my intention at that time to use them for bacterial counts but merely to ascertain the character of the protozoan fauna. As far as I could judge, I should say that the Geescroft soil contained about 10 per cent. of water, whilst the Broadbalk samples were of about the same degree of dryness and contained about 2 per cent. or 3 per cent. of moisture. It was evident from the appearance of the soils that the Broadbalk soils had been taken out and dried along with many other soils in 1881, whereas the Geescroft soil had been left untouched and closely resembled the Barnfield 1870 soil, containing about 10 per cent. of water, which I had used in earlier work.

I found on examining these soils culturally that the Broadbalk 1856 contained no protozoa, the Broadbalk 1865 contained amœbæ and flagellates and the Geescroft 1865 also contained amœbæ and flagellates.

A weighed quantity of each of these soils was taken and after making initial counts to determine the bacterial content they were all moistened to 20 per cent. water-content. After a period of 148 days the soils were remoistened to bring up the water-content to 20 per cent. again, owing to the gradual loss of moisture by evaporation.

Bacterial counts by the gelatine-plate method were made at different intervals and the results are set out in the table below.

Bacteria in Millions per Gramme.

	At beginning.	After 68 days.	After 102 days.	After 130 days.	After 198 days.	After 231 days.
Bd. 1856	4	136	169	73	200	158
Bd. 1865	4	66	93	55	120	124
G. 1865	2·5	11·4	5	10·7	18·6	9

The curves obtained on plotting these results are shown in fig. 5.

The most noteworthy feature of these curves is the high bacterial content of the Broadbalk soils and the low bacterial content of the Geescroft soil. The former have all the appearance of curves of partially sterilised soils, whilst the latter presents the usual appearance of an untreated poor soil, containing a limiting factor.

The drop at 130 days in the two Broadbalk soils may be accounted for by

the loss of moisture, and the subsequent rise in the bacterial content may likewise be attributed to the more favourable conditions occasioned by remoistening the soils.

Considered in relation to their protozoan fauna, these results are very instructive. In the Broadbalk 1856 there are no protozoa. In the Broad-

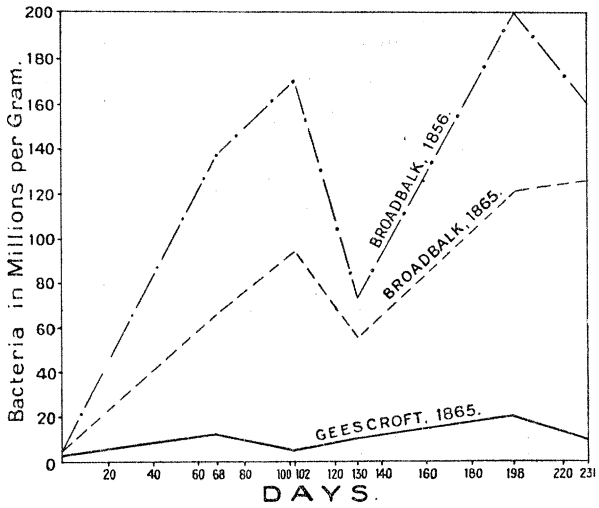

FIG. 5.

balk 1865 there is a rich protozoan fauna to the extent of about 5000 amœbæ and 5000 flagellates per gramme. In the Geescroft 1865 there are about 500 amœbæ and 500 flagellates per gramme. Thus the curve for the Broadbalk 1865 shows that in spite of the presence of this large number of protozoa, the bacteria can maintain a high level in numbers even after 231 days during which the protozoa should have been reducing them.

It may be suggested that the bacterial counts for Broadbalk 1856 are higher than those for Broadbalk 1865 because in the former there are no protozoa present to check the growth of the bacteria. I would point out, however, that the two Broadbalk curves are practically of the same order as compared with the Geescroft curve. One would have thought that in the presence of such a large number of protozoa as in the Broadbalk 1865, had these been capable of functioning as the limiting factor, they would have checked considerably the multiplication of the bacteria and brought them down to somewhere near the level of the bacterial content of the Geescroft soil.

My point is to show that the drying to which the Broadbalk soils were submitted has brought about a change in them strictly comparable with the change usually produced by partial sterilisation, and at the same time has

produced this change in one of them without killing off the amœbæ and the flagellates.

The Geescroft soil remained undried and has a much scantier protozoan fauna than the Broadbalk 1865 soil, yet the curve for the bacterial counts in this soil would be interpreted as showing the presence of the usual limiting factor.

I have no evidence on which to base a suggestion as to what the real character of the change is which has been produced in the Broadbalk soils by drying. I do suggest, however, that it has an intimate relation to the high bacterial counts which I have obtained on remoistening the soils. I am quite prepared to admit that the merely negative evidence furnished by the above results does not help forward very much the final solution of this elusive problem, but at the same time I think that it is useful. It points to the fact that much more information is required than is at present available on the changes brought about in soil by rapid air-drying or by drying at temperatures sufficiently low to avoid the killing of protozoa in the soil.

Russell and Hutchinson give details of several experiments on this particular line of investigation in their second paper (p. 166), but there is room for still more research on these points.

Hoosfield Inoculated Soil Bacterial Counts.

The results of the bacterial counts for this set of soil samples are tabulated below, and the curves obtained by plotting these are shown in figs. 6, 7, and 8. As in the case of the 1846 set of soils I have arranged certain curves together for the sake of simplifying matters.

It is necessary to point out at the outset that in attempting to interpret these results there are two standards of comparison, viz., the curve for the untreated soil and that for the toluened soil. For this reason I have introduced each of these curves into all three graphs.

Fig. 6 shows the curves for the Untreated, Toluened, T.+Ciliates, T.+Amœbæ, and T.+Flagellates. It will be seen that the untreated soil exhibits a normal low bacterial content; the limiting factor is here exerting its full influence. Compared with the untreated, the curve for the toluened soil shows that the usual partial sterilisation effect has been obtained, the bacterial numbers rising to and maintaining a level at about 50,000,000 or 60,000,000 bacteria per gramme. Examining now the curves of the three inoculated soils represented in this graph and comparing them especially with the curve for the toluened soil, we find that after the lapse of 487 days the bacterial contents are higher than that of the toluened soil. Leaving

Bacteria in millions per gramme.

	At beginning.	After 32 days.	After 60 days.	After 93 days.	After 125 days.	After 151 days.	After 173 days.	After 208 days.
Untreated (U.) ...	14·4	10·3	13	11·4	9	12	13·5	8
Toluened (T.).....	9·2	73	60	61	48	40	53	49
T. + U.....	11·3	49	61	43	70	19	39	45
T. + Ciliates	4·5	371	292	296	181	56	70	64
T. + Amœbæ	3	285	185	141	188	74	72	90
T. + Flagellates ...	27	247	214	227	368	196	108	104
T. + Bacteria	2·3	500	341	311	296	181	196	151

	After 259 days.	After 301 days.	After 337 days.	After 350 days.	After 386 days.	After 454 days.	After 487 days.
Untreated (U.) ...	6	13	lost	8	13	12·6	12
Toluened (T.) ...	42	55	70	67	43	51	56
T. + U.....	23	39	44	51	33	33	48
T. + Ciliates	59	57	65	59	57	57	73
T. + Amœbæ	57	65	68	62	50	41	57
T. + Flagellates ...	77	104	92	94	81	35	113
T. + Bacteria	116	151	85	105	138	115	150

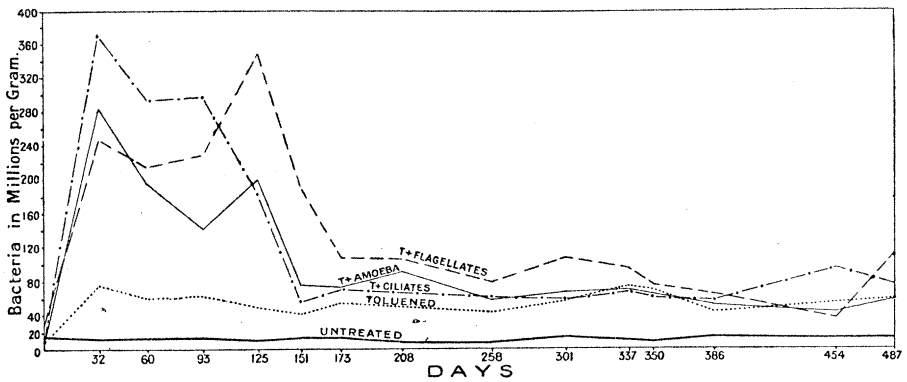

FIG. 6.

out of account for the moment the high bacterial counts during the first 125 days and the drops at about 150 or 170 days in all three cases, we may say that the protozoa added to the toluened soil in each sample have not reduced the bacteria to a level lower than that of the toluened soil alone. Judged in relation to the protozoa in these soils this is a very instructive result. The T. + Amœbæ contained at the end of the experiment 10,000 amœbæ, perhaps more, and about 5000 flagellates per gramme. The T. + Flagellates contained 10,000 flagellates, perhaps more, and about 1000 amœbæ per gramme, whilst the T. + Ciliates contained about 3000 each of *Col. steinii* and *Col. maupasii*, about 500 *Col. cucullus*, and about 1000

flagellates per gramme. These are large numbers of protozoa, and it is probable that the amœbæ and flagellates have occurred in the active condition. In no case, however, have these protozoa been able to reduce the bacterial contents of their respective soils to a level permanently below that of the toluened soil. Cultures were made at the end of the experiment to ascertain if protozoa were present in the toluened soil, with the result that about 5000 flagellates and about 10 amœbæ per gramme were found in it. Now the curve for the toluened soil is quite a normal one, and shows the usual partial sterilisation results when compared with that of the untreated soil.

Whatever be the limiting factor eliminated by the process of toluening and heating this soil, resulting in the rise of the bacterial content from 10,000,000 or 12,000,000 to 50,000,000 or 60,000,000 bacteria per gramme, that factor evidently has no connection with the flagellates, for these have resisted the action of the antiseptic and heat and, though much reduced in numbers at the beginning of the experiment, have succeeded in repopulating the soil.

The results obtained from these inoculated soils accord with those obtained from inoculated samples of 1846 soil. In those it was found that the ciliates, amœbæ, and flagellates failed to reduce the bacterial content below the level of the untreated soil. In this soil they have not brought down the numbers of bacteria lower than those of the toluened soil. The only inference which I can draw from these results is that the protozoa have not functioned as the limiting factor on bacterial activity.

The curve representing the counts for T. + Bacteria as compared with those for the toluened and untreated soils is shown in fig. 7. It is

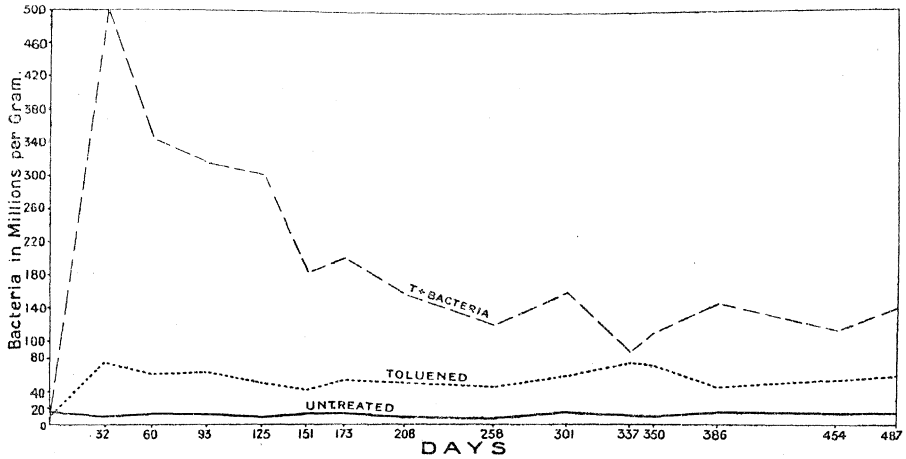

FIG. 7.

evident from this that the bacterial content of the T. + Bacteria has maintained a consistently high level, the numbers never going down to those of the toluened soil, though there is a decided drop between 32 days and 337 days. It might, at first sight, be supposed that high bacterial content was due to the absence of protozoa from the T. + Bacteria soil, the bacteria of the soil left after treatment, together with those added, having no preying organisms around them to check their growth. The T. + Bacteria soil does, however, contain protozoa, no doubt the offspring of those which withstood the partial sterilisation treatment, to the extent of about 3000 flagellates and 100 amœbæ per gramme. Thus there are almost as many flagellates and many more amœbæ per gramme of this soil than in the toluened soil. Yet in spite of these numbers of protozoa the bacteria have maintained a much higher level than those in the toluened soil.

The high bacterial counts obtained during the first 160 days in all the four soils, viz.: T. + Bacteria, T. + Ciliates, T. + Amœbæ, and T. + Flagellates, together with the decided drop in all cases, are very interesting. At first sight it might be assumed that in the case of the three soils inoculated with protozoa the drop was due to the limiting action of the latter becoming well established. This would appear to be sound reasoning if it were not for the fact that a similar drop occurs in the T. + Bacteria soil, where no protozoa were added. Moreover, the protozoa found at the end of the experiment in the T. + Bacteria and in the toluened soils are quite comparable, and if we were to assume that the drop in the bacterial content in the T. + Bacteria soil was due to the activity of the protozoa surviving partial sterilisation, we should be confronted with the difficulty that in one soil the surviving protozoa were exerting a limiting action, whilst in the other they were not doing so, though conditions for trophic existence were equally good in each case. The high counts during the first 160 days may probably be explained by the fact that hay-infusion and very large numbers of bacteria were added to the soils in inoculating them and in this way the conditions brought about were very favourable to extreme bacterial activity as compared with the toluened soil, to which only sterile distilled water was added, and which consequently exhibits no exceptionally high bacterial figures. In the same way the fall in the bacterial counts after about 160 days in these soils may, perhaps, be accounted for by assuming that the food supplies added with the hay-infusion became exhausted, and as a result of this the bacteria dropped to somewhere near the level of the numbers present in the toluened soil.

Fig. 8 represents the curve for the T. + 5 per cent. untreated soil along with those for the toluened and untreated soils. It is evident that after

151 days the bacterial content of the inoculated soil remained at a lower level than that of the toluened soil. At the same time it appears to be pretty

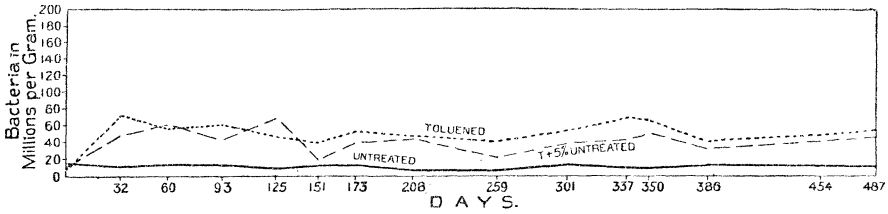

FIG. 8.

clear that the two curves are of the same order. The T. + 5 per cent. untreated does not show a continuous and persistent decline in the numbers of bacteria, as one would expect if the limiting factor were due to the growth and activity of protozoa added with the untreated soil. The two curves are practically parallel from 259 days onwards, and it is obvious that the same set of conditions was affecting the bacteria in each soil.

The numbers of protozoa present in each soil are as follows:—In the T. + 5 per cent. untreated there are at least 10,000 flagellates, about 500 amœbæ, and an almost negligible number of ciliates—5 or 10—per gramme.* In the toluened soil there are about 5000 flagellates and about 10 amœbæ per gramme. There are thus very many more protozoa per gramme in the inoculated soil than in the toluened soil, and the lower bacterial content of the former soil is thus easily accounted for if we assume that the protozoa act as the limiting factor. If we only had these two curves and that for the untreated soil on which to base our conclusions, the above inference might be considered correct. But when we take into consideration the points already mentioned in connection with the other inoculated soils, it is scarcely possible to assume that this is the real explanation.

It has been shown that in the case of the toluened and the T. + Bacteria soils that the flagellates can be left out of account, so far as any possibility of functioning as the limiting factor is concerned. So that if the lower bacterial

* This soil affords evidence of the activity of the amœbæ added in the 5 per cent. of untreated soil. Amœbæ are present in the latter to the extent of about 3000 per gramme, and assuming that this number was present at the time the soils were mixed, we can reckon that in each 100 grm. of the mixture there were 15,000 amœbæ or 150 per gramme. There were present at the last protozoal count 500 per gramme, thus showing that the original 150 had increased to 500 per gramme. Similarly the 1846+10 per cent. 1870 soil discussed on p. 447 gives evidence of the amœbæ added in the 10 per cent. of 1870 soil having increased from 50 per gramme at the beginning to 2000 per gramme at the end of the experiment.

content of the T. + 5 per cent. untreated soil is due to protozoal activity it must be the 500 amœbæ and about 10 ciliates per gramme which are responsible for it. It seems to me highly improbable that this is the true explanation when we consider the enormously larger numbers of amœbæ and ciliates present in the T. + Amœbæ and T. + Ciliates soils, where the protozoa obviously have not effected a limiting action on the bacterial contents of their respective soils.

It is clear, however, that some factor has been added to the toluened soil in the 5 per cent. of untreated which acts as a check on bacterial growth. I cannot find support in these results, however, for the assumption that this limiting factor is the protozoa. I would suggest that the influences checking the growth of bacteria are connected with some other property of the added soil than its contained protozoa.

General Discussion.

The introduction of large numbers of bacteria into the samples of soil along with the added protozoa must be a source of disturbance to the bacterial flora, and for this reason the experiments dealt with above cannot be considered as showing a clear issue between protozoa on the one hand and bacteria on the other.

I sought to reduce this source of error to a minimum, however, by the continuation of the experiments over a long time, thus allowing the disturbed bacterial floras to settle down so that any influence of the protozoa should be judged after this steady point had been reached, *i.e.* after about 160 days in both the 1846 and the Hoosfield soil.

In order that the protozoa should have conditions, as near as I could bring them about, favourable to excystation I partially air-dried all the soils after they were inoculated.

In this way I hoped to meet the criticism which might be brought against the experiments that the protozoa had failed to function. Moreover the soils were all kept under conditions of temperature, water-content and aëration exactly comparable with those under which Russell and Hutchinson kept their soils. If protozoa therefore could act as they supposed them to do in their soils they had every chance of doing so in my soils.

Another point calls for some comment. Martin and Lewin* have found evidence of an abundant fauna of active amœbæ and flagellates devouring bacteria in certain soils which they have tested. They suggest that these have probably some influence on bacterial numbers and thus on soil fertility. Their results are very important direct evidence of the activity of protozoa in

* "Some Notes on Soil Protozoa," 'Phil. Trans.,' B, vol. 205, pp. 77–94 (1914).

the soil, but this does not prove that the amœbæ and flagellates are functioning as the limiting factor in the sense in which that term is used by Russell and Hutchinson.

Before this can be shown to be true it will be necessary to correlate protozoal activity with a decrease in the numbers of bacteria in a given soil specially inoculated with protozoa.

I have shown above (p. 446) that the presence of 10,000 amœbæ per gramme of soil is not sufficient to reduce the bacterial content of a soil to the level of a similar soil containing no protozoa even though the soil be kept under conditions of moisture, etc., favourable to the trophic existence of amœbæ and flagellates.

Conclusions.

The results of the experiments described above lead me to the conclusion that the protozoa, including ciliates, amœbæ, 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, amœbæ, 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,* viz., that the ciliated protozoa are present in soil only in an encysted condition and cannot function, therefore, as the factor limiting bacterial activity.

There is evidence, however, in the case where a small quantity of untreated soil is added to a partially sterilised soil that some factor comes into action which keeps down the level of the bacterial content. The results obtained, however, do not lend support to the hypothesis that it is the protozoa added in the untreated soil which have this influence.

It is shown in the case of Broadbalk 1865 soil, in which an abundant protozoan fauna of amœbæ and flagellates is present, and presumably active, that the numbers of bacteria maintain a high level. This soil exhibits a clear case of partial sterilisation being effected without the elimination of protozoa.

It is not within the province of this paper to attempt to rebut the very weighty indirect evidence put forward by Russell and Hutchinson as to the biological character of the detrimental factor. The results obtained, however, warrant the conclusion that ciliates, amœbæ, and flagellates cannot be included in that biological factor.

* 'Roy. Soc. Proc.,' B, vol. 84, p. 165 (1911).