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ACTA FORESTALIA FENNICA. Sisältää etupäässä Suomen metsätaloutta ja sen perusteita käsitteleviä tieteellisiä tutkimuksia. Ilmestyy epäsäännöllisin väliajoin, joista kukin yleensä käsittää useampia tutkimuksia.

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SOME FACTORS AFFECTING THE QUANTITATIVE DETERMINATION OF AEROBIC BACTERIA IN FOREST HUMUS

ILMARI SCHALIN

To be presented, with permission of the Faculty of Agriculture and Forestry of the University of Helsinki, for public criticism in Auditorium III in the Porthania Building, Hallituskatu 11—13, Helsinki, on October 24th, 1964, at 12 o'clock noon.

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Preface

During the course of the present investigation I have received valuable assistance and constructive criticism from several persons. Professor Helge Gyllenberg gave his support in planning the work and on many occasions discussed at length with me problems arising in connection with it; he also read the manuscript and made valuable comments on it. Professor Peitsa Mikola, without sparing his time and efforts, followed the progress of my work with never-failing interest and gave generous advice especially in its final stages. Professor Unto Vartiovaara, Head of *the Department of Microbiology, University of Helsinki,* inspired me by his positive attitude towards my investigation during its entire course and offered helpful suggestions and valuable criticism of the manuscript. Professor Paavo Yli-Vakkuri, Head of *the Department of Silviculture, University of Helsinki,* willingly placed at my disposal the facilities of his Department. Mr. Eino Oinonen, Dr. Agr. and For., spared no trouble in taking care of matters connected with the printing and publishing of this thesis.

Foresters Fred Kalland and Martti Siirilä assisted me in collecting the data, and Misses Raija Ellä and Leena Karjalainen, students of forestry, performed the practical laboratory work. Discussions on statistical questions with Mr. Kustaa Seppälä, M. Agr. and For., have greatly helped me in solving many of the problems of my work. The sound intensity measurements were carried out at *the Institute of Occupational Health,* Helsinki. The figures were drawn by Mrs. Eila Timonen. The manuscript was translated into English by Mrs. Leena Levanto , M. Phil., and the language checked by Mr. Edvin Risser, B. Sc.

My pleasant duty is to express my sincere gratitude to all those persons who have helped me in completing this work.

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Ilmari Schalin

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1. Introduction

11. Forest humus as the object of quantitative bacterial study

Forest humus, the layer formed on top of the mineral soil and consisting of litter material and decomposing organisms, has been the subject of intensive research for several decades. Most of the studies made up to the beginning of the present century were aimed at determining the physical properties and the chemical composition of the humus. Along with these questions, however, serious attention was also paid quite early to the formation and decomposition of the humus (Hesselman 1926, Romell 1932, 1934). The central matters of interest are the course and rate of these processes as well as the micro-organisms of the humus and their activities. Variations in the extent and interrelationships of these different factors give rise to various types of humus.

If we start from the concept that the ratio of the accumulated litter material to its decomposition is the basic factor in the formation of humus, the main part of the humus layer in forests can actually be considered an undecomposed residue of litter, a kind of supply of exchangeable ions. Due to its manner of formation, a humus of this type is quite heterogeneous. Its decomposition is mainly dependent on how favorable the environment is for the decomposing organisms; however, the extent of the decomposition cannot alone be determined by any one individual factor or group of factors (Handley 1954). Although the conditions prevailing in the environment influence the species of micro-organisms present, the significance of the composition of the microbial population in humus formation should be emphasized (Romell 1932, 1934, 1935; Meyer 1959).

Data on the significance of the various groups of microbes in the decomposition of litter and the formation of humus are still incomplete (Mikola 1956). It is known, however, that fungi play an important role in decomposing forest humus rich in carbohydrates and containing acidic substances (Waksman and Starkey 1924, Melin 1930, Waksman 1931, Lindeberg 1946, Mikola 1954). This does not mean, however, that bacteria are not essentially important factors in this process, although the quality of the organic matter in the forest humus is more important for the existence and growth of bacteria than for those of fungi (Vartio-

vaara 1936, Taylor and Lochhead 1938). The main function of bacteria in forest humus is to mineralize organic residues which have already been partly decomposed by other organisms (Taylor and Lochhead 1938, Lochhead 1952). This alone is a sufficient reason for paying more attention than previously to the study of bacteria in investigations on the microbiological conditions in forest humus.

Qualitative microbiological studies have the advantage that they enable observations on the course of some phenomena, such as the decomposition of proteins, for instance. However, only quantitative investigations form the basis for the understanding of the different degrees of intensity of this phenomenon. From the forest biological point of view, information about a certain phenomenon as a whole — for example, the amount of nutrients produced by decomposition and required for the vegetation — must be considered more important than the observation of any detail of the phenomenon. Thus, quantitative study of the humus bacteria must precede qualitative investigation.

The literature abounds in examples of the difficulties that must be overcome in the quantitative determination of the bacterial population of soil in general (Jensen 1934, Thornton and Taylor 1935, James and Sutherland 1939, Harmsen 1940) and of forest humus in particular (Fehér 1932, Svinhufvud 1937). The difficulties do not only arise from antagonistic and synergistic relations between bacteria in mixed populations (Snyder 1947, Jones and Mollison 1948), nor from the tendency of many species of bacteria to live in cell chains or clumps (Monod 1949), but above all from the fact that soil in general and especially forest humus, being heterogeneous and mainly composed of solid substances, is a completely different environment than liquids, such as milk or water, for which there are well-established methods for quantitative bacterial study (Harmsen 1940). Bacterial cells in the forest humus aggregate in a different manner than in the homogeneous solutions mentioned. The cells adhere to the surface of soil particles, and even inside of them, forming relatively compact colonies. Since an individual cell, however, should in all cases be the basic unit in quantitative determinations, attempts should be made to disintegrate the clumps and groups of bacteria in the humus in order to obtain reliable results. The greatest difficulties in this connection are in the preparation of a homogeneous bacterial suspension in which the cells preserve their viability but do not reproduce. Difficulties like this were encountered, for instance, in a study with the object of determining the numbers of bacteria in the humus of a pine stand in the northern region of coniferous forests (Schalin 1961).

In addition to the facts mentioned above, a difficult problem in quantitative studies of humus bacteria may also be that the humus is exposed to changes in the external factors, such as humidity and temperature, which continuously alter the conditions in it. The bacteria react readily to these factors, some species being more sensitive than others (Fehér 1930, Waksman 1931 a, Lochhead 1952).

The result is a periodical variation in the structure of the bacterial population itself and in the total quantity of the whole population.

The above-mentioned difficulties in the quantitative determination of the bacterial population of the humus, i.e. in the determination of the bacterial density of the humus, which term will hereafter be used in the present work, place great demands on the investigation methods. There is a variety of methods in use, each of which has its own advantages and drawbacks. The object of the present study is to investigate thoroughly one of the standard determination methods, the dilution plate method, to test with the aid of statistical models some of its principal techniques, and to develop it into a reliable method for determining bacterial density in forest humus.

12. Determination methods of bacterial density

The determination of bacterial density is, even in the simplest case, a procedure involving many stages. The determination methods can be classed according to their technique into two main groups, microscopic and cultural methods.

The principle of the microscopic methods is that the counting of the bacterial cells is made directly from preparations treated in an appropriate manner. Carefully made preparations ensure the uniform distribution of the cells, and the reliability of the results is increased by increasing the number of microscopic fields counted. These methods have, however, some disadvantages. When a certain volume of bacterial suspension is compressed into two dimensions, the density of the cells increases so much that errors due to intergrowth occur, thus decreasing the numbers counted. On the other hand, erroneously large numbers are also possible, because dead cells cannot be distinguished from living ones by the standard microscopic methods. The use of the fluorescence microscope (Strugger 1949) as well as colorimetric determination, which is based on the different ability of living and dead cells to absorb stains (Vairo and Borzani 1963), increase the possibilities of differentiating between living and dead cell material.

The second main group of methods for determining bacterial density, the cultural methods, are based on counts of the detectable phenomena due to the reproduction of viable cells, i.e. the colonies formed on solid media. These methods ensure that only living cells are included in the count. The theoretical assumption should, however, always be made that each colony, whether resulting from a single cell, a cell chain, or from some other normal clumping of cells occurring under natural conditions, represents only one bacterial unit. This interpretation is perfectly justified, if it is presumed that a colony indicates in any case the presence of at least one viable bacterium.

The *dilution plate method* developed by Robert Koch in 1880 has not been in

principle superceded by any newer determination methods of micro-organisms. On the contrary, it has served as a model for the surface or drop count method, the microcolony method, and the membrane filter method, all of which were developed in order to improve on some of the deficiencies of the original dilution plate method.

The principle of the dilution plate method is that the sample, homogenized and diluted, is mixed with a molten culture medium on a plate. Colonies grow on the spots where the cells happen to be when the medium solidifies. The distribution of the colonies on the plate depends on the homogenization of the sample and the mixing of the suspension with the medium. Since even in the most favorable cases the homogeneity attained is far from being complete, all the colonies formed should be counted in order to reduce the sources of error. In addition, the dilution degree of the suspension has to be such that all the colonies are easily discernable. Care should, however, always be taken that the number of colonies is not so high that there is a risk of their intergrowth or that individual cells conceal one another. It is suggested (Clark et al. 1951, Standard Methods for the Eximination of Water, Sewage, and Industrial Wates, 10th Ed., 1955) that the most suitable number of colonies per plate is between 30 and 300, whereas Wilson (1922) considered 200—400 a more favorable number. No mathematical argumentation can be presented in favor of the recommended numbers of colonies. If the material is large enough, it is possible to establish in each case separately, by applying a certain test, the theoretical optimum number of colonies per plate. In practice this number can hardly ever be reached, since the mutual antagonistic and synergistic effects of a bacterial population are unknown factors influencing the distribution.

The error arising from overpopulation has been observed to give density values which are systematically smaller than the actual (Wilson 1922, James and Sutherland 1940). The dilution degree of the suspension could in many cases be a factor preventing overpopulation. Total elimination of errors is evidently never possible, since the preparation of a completely homogeneous suspension must be considered impossible. Furthermore, errors occur initially, because it is difficult to separate the bacteria from the solid, heterogeneous humus. Other sources of error that deserve attention are due to the fact that the bacteria, because of their electric properties, adhere to the surfaces of the particles in the liquid, lose their electric potential, and aggregate (Jennison 1937). However, Swaby (1948, 1949, 1950) claims that aggregation is not by far so common among bacteria as it is among other micro-organisms. Also the pH has some significance, because its changes either toward the acidic or basic direction increase or diminish the possibilities of existence and growth of bacteria. These phenomena are, however, common to all methods based on the same principle.

Of the other cultural methods, the *surface or drop count method* is actually a modification of the dilution plate method, with the exception that the bacterial suspension is spread on a plate already covered with solidified medium (Anderson and Stuart 1935). In qualitative investigations this method may have some

advantages, since the colonies formed on such plates facilitate the identification of species, whereas as a quantitative method it is clearly less reliable than the dilution plate method. The chances to spread a small amount of liquid evenly on the surface of solid medium are much smaller than to mix it with the medium before solidification. In addition, the risk of overpopulation is very great.

The newest of the methods, the *membrane filter method,* was developed from the dilution plate and the drop count methods. As the name indicates, the principle of this method is to separate bacterial cells from a suspension by filtering them through a membrane, which retains all cells. The membrane filter with the retained cells is subsequently incubated on the plate. As in the two other methods mentioned, only living cells are involved in this method. In order to guarantee that the bacteria on the membrane filter are distributed as uniformly as possible, a sufficiently large quantity of diluent is used. However, even in this method intergrowth of colonies cannot be prevented, although the margin of error can be reduced by using weak dilutions (Niemelä 1963). The membrane filter method has some advantages over the others in the determination of overpopulation, and it has been employed in studies of this phenomenon (Goetz and Tsuneishi 1951).

The *microcolony method* is essentially similar to the dilution plate method. This modification was brought about by the idea that the growth of the colonies can be checked at such an early stage that no intergrowth has yet occurred. The aim is to completely avoid the intergrowth of colonies with its resulting errors. The method could be very useful in bacterial density determinations, but the difficulty of assaying the results is a limiting factor.

The *fermentation tube method* differs considerably from the methods mentioned above. It does not employ solid nutrient media at all, but instead the bacterial density is determined by measuring the turbidity observed in tubes containing the diluent and a known amount of the sample. Since there is no risk of overpopulation and the results are probably therefore more reliable than those obtained by the dilution plate method (Halvorsen and Ziegler 1935, McCarthy 1955, Thomas and Woodward 1955, Conner 1957), the method is better suited for density determination of a single strain than of a mixed population.

In the present work the determination of the bacterial density of the humus was made by the dilution plate method, which was subjected to thorough investigation. Although this method is complicated, it was chosen because it is quite consistent, and, above all, because each of its stages can be studied separately and their reliability tested. In addition, the observations, i.e. the counting of the bacterial colonies on the plates, are easy to carry out, even though the accuracy of the work done in the world of micro-organisms depends on the personal qualities and experience of the workers. The study of this method is based on the assumption that each bacterial cell has the same chances to continue its life on plate cultures, and that all series of observations obtained in the quantitative determinations of the same organisms under similar conditions are in every respect comparable with one another.

13. Problems

The study of the dilution plate method led to a series of questions concerning the different stages of the method, from the sampling to the mathematical study of the final bacterial density values observed. The main questions which were attempted to be answered were the following:

1. In what manner should the sampling be done from the natural environment so that it would represent the bacterial population as closely as possible?

2. How should the homogenization and dilution of the sample and the assays of the results be performed, and what is the importance of the homogenization time, the composition of the diluent, and the dilution degree?

On the other hand, the present study was concerned neither with the nature and composition of the culture medium nor the incubation temperature and time; these factors were standardized in the experiments.

After the investigation was started, new problems constantly appeared. Attempts were made to solve them as well as possible, but in many cases only limited experiments could be made.

2. Mathematical basis of the dilution plate method

When observing the phenomena occurring in nature it has been discovered that they show a clear tendency to follow general laws of probability. Thus, for instance, roughly normal distribution is quite common. This facilitates the study of many phenomena, because the reliability of the estimates of one of the basic parameters of the normal distribution, the mean, can be tested by a simple calculation procedure using the *t*-test or the *chi-square* (χ^2) test. However, all phenomena cannot be studied in an equally simple manner, and what was said above does not entitle us to draw the conclusion that an event which does not follow the mentioned theoretical frequency distribution, would not follow some other probability distribution. It may be that the theoretical distribution of a population is valid only on the condition that the probability distribution is discrete. This has commonly been observed in, for example, microbiological phenomena, where the material is distributed in the Poisson series.

The theoretical basis of the Poisson distribution will not be presented in this context, since it is described in several statistical text books and manuals. We only mention that if any unit is randomly distributed in, for instance, a certain volume, the number of the particles per unit of volume follows Poisson's law (F i s h e r 1954, S t e a r m a n 1955). This applies in all homogeneous solutions, including bacterial suspensions. The cells on the plates are distributed according to Poisson's law.

The following example serves to demonstrate the usefulness of the Poisson distribution. The numbers of bacterial colonies on different plates of the same observation series were divided into eight classes. The colonies were distributed between the classes in the following manner:

class	0	1	2	3	4	5	6	7—8
observed frequency	4	8	9	9	12	5	5	1

The observed distribution was compared by the χ^2-test with the theoretical Poisson distribution; the theoretical frequencies of the classes are obtained from the equation

$$n = \frac{n}{e^u} + \frac{un}{e^u} + \frac{u^2 n}{2e^u} + \frac{u^3 n}{(2)\,(3)\,e^u} + \cdots\cdots \frac{u^1 n}{(2)\,(3)\cdots(1)\,e^u}$$

where

n = number of observation units (here 54 plates)
e = base of the natural logarithm, approximate value 2.718
u = number of specific classes (here 8).

The expected frequencies of the classes were as follows:

class	0	1	2	3	4	5	6	7—8
expected frequency	2.41	7.48	11.64	12.07	9.39	5.84	3.03	2.14

The value of χ^2 was found to be 5.081, which indicated that the observed distribution did not differ significantly from the Poisson series.

The first evidence of the applicability of Poisson's distribution in quantitative microbiological investigation was obtained when the number of yeast cells on plates was counted with the aid of the microscope. Provided that the enumeration technique used was entirely free of errors, the number of the cells in each square of exactly the same size was observed to follow the theoretical Poisson series ("Student" 1907). If the samples are distributed in the Poisson series, then also the population from which they were drawn follow it and vice versa. When this information is applied to a bacterial suspension, it means that every random sample taken from it follows Poisson's distribution, represents the total population, and that all samples from the same population are intercomparable in all quantitative methods.

The fact that Poisson's distribution is valid in yeast cells is not merely a coincidence, since it has been proved in a great number of other investigations. It has been shown to apply in bacterial cultures of the microscopic determination methods by Fisher et al. (1922), Jones and Mollison (1948), and by Collins and Kipling (1957). Furthermore, Harmsen and Verveel (1936), James and Sutherland (1939), and Stearman (1955) have observed this law to be valid on plates made by the dilution plate method.

Although the Poisson distribution is defined by a single parameter, the mean, conclusions drawn without a preceding test of significance have no value. In the case of Poisson's law it is easy to verify the distribution when the number of observations (plates) is large. The distribution is tested by calculating χ^2. The following table gives the observed and expected distributions of three different samples, each having 48 replicate plates, as calculated from the means.

Sample	Observed distribution χ^2	Expected distribution χ^2
1	2.533	12.59
2	9.083	,,
3	8.604	,,

In all cases the material was found to fit Poisson's distribution, since no significant departures from it occurred. When studying the ratio of the variance to the mean in the different samples, which in the theoretical Poisson distribution is equal to one, it was observed that the variance was, as a rule, somewhat larger than the mean. This phenomenon will be discussed in more detail later in this work.

Two samples can also be compared with each other by the t-test (Snedecor 1956). Let us examine, for example, the two samples with $\chi^2 = 2.533$ and 8.604 in the above example. The respective means are 61 and 151 bacterial colonies per plate. Since both samples follow Poisson's distribution, we make use of the fact that the variance is equal to the mean. The standard devitation is $\sqrt{151+61} = 14.5$, when $t = (151-61)/14.5 = 6.20^{***}$. This value of t shows clearly that the samples are drawn from two different populations.

It is evidently not possible to observe with any degree of probability the Poisson distribution in a sample of which only a few replicate plates have been made. But if we have several samples and at least a couple of plates from each, we can decide with a relatively good confidence whether the bacteria are distributed according to the theoretical Poisson distribution or not. This is done by calculating the dispersion index for each set of plates from Fisher's (1954) equation

$$X^2 = \frac{S\,(x-\bar{x})^2}{\bar{x}} = \frac{n\,S\,(x)^2 - (Sx)^2}{Sx}$$

where x denotes the number of individual observations, $\bar{x}$ the mean of the replicate observations, n the number of the replicate observations, and S the sum. (Also in this case the fact that the variance is equal to the mean is utilized.) Fisher's (1954) table gives the values for X^2, when n is one unit less than the number of replicate plates. For small numbers of plates the permissible range of variation of the dispersion index is quite wide and thus the information obtained by it is relatively uncertain, but the greater the number of plate series, the greater is the certainty by which we can verify if the expected distribution is obtained.

In the following table are presented the dispersion indexes and corresponding Fisher's indexes for three sets of plates, each with six replicate plates.

No. of sets	Calculated X^2	Fisher's X^2
1	3.49	3.00—4.357
2	4.10	,,
3	3.57	,,

When each set is tested separately with $n=5$, it is found that 50—70 % of the cases follow Poisson's distribution, but when the testing is done using the sum of all the sets, when $n=15$, the confidence is 70—80 %.

In the example given above, the observations were scattered over a fairly wide range around the mean. By increasing the number of replicate plates, it is evidently possible to come very close to the theoretical Poisson distribution, where the mean and the variance are equal, but in practice this is hardly ever reached. The reasons for this are various, one of the most important being the synergistic and antagonistic relations between cells in a bacterial suspension.

If the antagonistic phenomena are prominent, or if the number of the bacterial cells is too large for the volume of the suspension, the variance is smaller than in the theoretical Poisson distribution, for the reason that the observations are grouped closer around the mean than in the Poisson distribution (Stearman 1955). But if synergistic phenomena prevail or if the cells tend to clump for other reasons,

the observations differ more from the mean than in the Poisson distribution. This means that either too many or too few colonies are formed on the replicate plates of a sample. The latter phenomena thus increase the variation.

In order to attain sufficient accuracy in the enumeration of colonies and thus a distribution which follows Poisson's law, the first and most essential requirement is that the dilution technique must allow the cells to be distributed completely randomly on the plates, and the second that the colonies have a chance to develop freely without mutual interference. In addition, the dilution degree of the suspension has to be so weak that, on one hand, the antagonistic phenomena cannot influence the relations between the bacterial cells and, on the other, that no clumping of cells can occur.

With the aid of the Poisson distribution, we can control the distribution of the bacteria on a plate culture, but by this statistical method alone we cannot check the reliability of the dilution method in quantitative determinations of bacteria. This has to be done by a statistical method which is able to discriminate between the several factors influencing the result.

One of the most elegant and useful statistical methods in this respect is undoubtedly the analysis of variance. By this method the total variation in a set of data can be resolved into components, whose relative importance we wish to determine. The variation in a population is computed from the average deviation of the arithmetic means of its different values. Small samples tend, however, to underestimate the variance in the population.

If several random samples are drawn from a homogeneous population, it cannot be assumed that they all would have an identical mean, since the sample means reflect the variance in the population. The mean sample variance is evidently identical with the population variance, and it expresses variation within the sample. If the variation between two samples is significantly greater than that within the samples, this indicates that they are not drawn from one population but from two populations.

For the statistical control of the quantitative dilution plate method, the analysis of variance is an excellent method, when samples from the same population are tested for variation brought about by the technique. If the within-sample variation is greater than the between-sample variation, the technique can be considered reliable, but if the between-sample variation is significantly greater than the within-sample variation, this may be an indication of errors in the technique. The main factors to be controlled in the dilution plate method are the reprensentativeness of the sample, the homogenization of the sample and the homogenization time, as well as the composition of the diluent and the degree of dilution. Because some of these factors influence all the stages of a dilution culture, and because they cannot be studied entirely separately, multiple factor variance analysis has to be employed in the statistical control of the method.

Before we examine the differences that have been brought about in populations by varying the preparation technique of the dilution cultures, the variances within

samples should be investigated in each population. Only if the within-sample variances are identical or nearly identical, can they be considered independent estimates of the variance in one population (Moroney 1962). The variances are tested by Snedecor's F test.

If the sample comprises only a short series of replicate observations, the estimates of the variances are corrected by Bessel's correction (Moroney 1962) $\left(\frac{n}{n-1}\right)$, which is not necessary with a large observation material.

If the samples are very small, the variance ratio may be large even when using Bessel's correction. This does not necessarily mean that the samples would not belong to the same population. On the other hand, if on testing two large samples, high F values are obtained, we can be sure that the samples differ significantly from one another, and that they are not from the same population. In the dilution plate method the term "population" means the total material, for instance a bacterial suspension, used at each stage for preparing the plates, and the term "sample" means a random sample drawn from this population. There can even be several populations at one stage of the work, as in cases where the reliability of the technique is controlled by varying the procedures of the individual stages.

The statistical control of the dilution plate method is done in two stages. The first task is to ascertain whether the bacterial colonies on replicate plates follow the Poisson distribution. If they differ significantly from it, there is evidently a systematical error in the preparation technique. Since this error is usually due to either a too small or too large number of colonies per plate, the primary reason for it may be the unsatisfactory mixing of the bacteria or a wrong degree of dilution, but it may also be caused by errors in the preparation technique in the other stages of the method. Only when the bacterial colonies follow the Poisson distribution, can the analysis of variance be applied to checking the stages of the preparation technique. We must, however, first check, by testing the sample variance, that there is no other than within-sample variation in the population; only after this can the samples be compared with one another. If the test shows a significant difference between two populations, the difference can be concluded to be due to errors in the preparation technique.

3. Material and methods

The main object of the present investigation was to determine the density of aerobic bacteria in the humus layer of two sampling areas. Although a single area would perhaps have been adequate for the main purpose of the work, the results would, on the other hand, be of small value without control experiments made in another type of environment. The dilution plate method was tested in various experiments in order to determine its usefulness as a quantitative determination method of bacterial density in forest humus.

31. Sampling areas

The material was collected from two stands, a spruce stand and a pine stand, which were both in an almost completely natural state, since no felling had been done in them for the last three decades. The principle in selecting the areas was that their natural state should be as obvious as possible. The mensurational characteristics were obtained from representative 1/4-hectare plots of each area, whereas the descriptions of the plant cover, as well as the humus study and the actual sampling were confined to plots measuring 15 by 30 meters.

The mensurational characteristics were the following:

Sampling area	Composition of stand, %	Volume, cu.m./ha.	Growth, cu.m./ha./y.	Age, y.
No. 1, spruce stand	spruce 100	360	5.6	107
No. 2, pine stand	pine 100	170	4.2	80

Since the general nature and distribution of the plant cover on both areas was very evident, no detailed plant cover descriptions were made. The prevailing plant association on Area No. 1, the spruce stand, consisted of feather mosses and bilberry. Because, however, they were not distributed uniformly over the whole area, it was divided into two approximately equal parts (A and B) according to coverage percentages. Area No. 2, the pine stand, had some lichens, besides mosses, while cowberry was the dominant undershrub. These species were distributed evenly over the whole area, although there were some gaps in the plant cover.

The plant cover descriptions (Table 1) show that Area No. 1 belonged to the Myrtillus type and Area No. 2 to the Vaccinium type.

Table 1. Nature and distribution of plant cover on the sampling areas.

Species	Coverage, % Area No. 1 A	Area No. 1 B	Area No. 2
LOWER ZONE			
1. *Cladonia* sp.	—	—	10
5. *Pleurozium Schreberii*	40	35	45
6. *Hylocomium splendens*	45	55	10
2. *Dicranum undulatum*	2	—	—
3. *Dicranum majus*	15	10	10
4. *Dicranum scoparium*	—	—	5
7. *Rhytidiadelpus triquertrus*	—	—	—
UPPER ZONE			
2. *Oxalis acetosella*	3	—	—
1. *Majanthemum bifolium*	5	2	—
3. *Melampyrum silvaticum*		—	—
4. *Vaccinium vitis-idaea*	2	—	20
5. *Vaccinium myrtillus*	35	—	5
6. *Calluna vulgaris*	—	—	5

The tree stand, the plant cover, the mineral soil, and the climate together create the relatively standard conditions which determine the general nature of the humus layer formed, its physical structure, chemical composition, pH, and dynamic state.

Because detailed information of the humus layer may be necessary for the evaluation of the results, some observations and results of measurements of the main characteristics of the humus layers are presented in Table 2.

Table 2. Thickness of litter and humus layers and type of mineral soil in sampling areas.

Sampling area	Surface and litter layers, mm.	Humus F-layer, mm.	Humus H-layer, mm.	Type of mineral soil
Area No. 1				
A	49 ± 16	35 ± 7	10 ± 3	fine sand
B	51 ± 8	38 ± 3	11 ± 2	moraine gravel
Area No. 2	48 ± 11	20 ± 5	8 ± 3	sand

The results in this table are based on 25 samples taken at equal distances from one another. The samples were taken from the same spots where the plant cover determinations were made. The general picture of the humus obtained by macroscopic examination was that the F-layer was distinctly feltlike in both areas, the H-layer was granular and sticky in the spruce stand, and single-grained in the pine stand. Charcoal was present in the humus of both areas. The border between the underlying mineral soil and the humus layer was sharp in both areas.

Samples for chemical analyses were taken from the same spots where the thickness determinations were made. The results are given in Table 3, which lists the content of mineral nutrients and the total amounts of some nutrient elements of the humus.

Table 3. Content of mineral nutrients and total amounts of some nutrient elements in the F- and H-layers of the humus of the sampling areas.

Humus layer	Mineral nutrients, %	Total, % of dry material			
		CaO	N	P_2O_5	K_2O
Area No. 1					
A. F-layer	30.05	0.544	1.590	0.263	0.105
H-layer	75.30	0.158	1.890	0.293	0.123
B. F-layer	25.80	0.252	1.385	0.256	0.134
H-layer	75.70	0.205	1.530	0.114	0.095
Area No. 2					
F-layer	8.72	0.551	0.810	0.159	0.102
H-layer	31.10	0.223	0.799	0.067	0.083

The only marked difference between the humus layers of the two sampling areas was in the nitrogen content; Area No. 1 contained twice as much nitrogen as Area No. 2. Attention should also be directed to the quite low calcium content of Part B of Area No. 1, which was heavily overgrown with mosses. This is also reflected in the pH-values, presented in Table 4 together with some exchangeable ions of the humus layer. The exchangeable ions were determined by extracting from the same samples as the total nutrient element determinations.

Table 4. pH and some exchangeable ions of the F- and H-layers of the humus.

Humus layer	pH	CaO	NH_3-N	NO_3-N	P_2O_5	K_2O
		mg./100 g. dry material				
Area No. 1						
A. F-layer	4.75	389.6	15.8	4.3	31.3	135.6
H-layer	4.15	99.7	7.3	3.7	12.3	105.4
B. F-layer	4.30	181.3	10.8	6.2	85.7	159.1
H-layer	4.15	104.1	2.5	1.4	9.5	52.3
Area No. 2						
F-layer	4.75	370.2	8.1	2.3	28.9	53.3
H-layer	4.25	121.9	3.3	1.5	5.2	36.7

Variations in the temperature, moisture, and pH were observed carefully on both sampling areas and, in addition, on different spots of Area No. 1. The temperature was measured with six thermometers placed between the F- and H-layers; if different readings occurred, the mean was calculated. The moisture was determined by drying the samples, and the pH was measured with a Metrohm pH meter. The results are presented in Table 5.

Table 5. Moisture, temperature, and pH of the F- and H-layers of the humus.

Sampling area	Date	Humus				
		Moisture, %		Temperature, °C	pH	
		F-layer	H-layer		F-layer	H-layer
Area No. 1	June 28					
A		74.2	67.5	9.0	4.70	4.25
B		61.8	63.9	9.2	4.25	4.15
Area No. 2		70.6	74.2	9.9	4.40	4.30
Area No. 1	July 9					
A		58.2	52.3	10.2	4.70	4.55
B		56.3	49.9	9.4	4.60	4.50
Area No 2		71.4	72.6	10.9	4.60	4.30
Area No. 1	July 29					
A		86.0	79.5	10.7	4.45	4.15
B		78.6	73.5	10.4	4.20	3.90
Area No. 2		75.6	72.0	12.8	4.30	4.10
Area No. 1	August 25					
A		77.7	67.7	9.3	4.40	4.10
B		75.2	71.7	9.4	4.15	4.00
Area No. 2		78.0	72.1	9.5	4.20	4.10

32. Samples

321. General

As mentioned above, if a population can be considered homogeneous, any random sample taken from it represents the whole population when the object of the investigation is to determine bacterial density. This is not the case when the population is heterogeneous. In this chapter the term "population" is used to designate forest humus only.

Forest humus is a very heterogeneous environment for bacteria with variations both in the horizontal and vertical direction. The higher plants have only few growth layers in the humus, but the bacteria, due to their microscopic size, form several different growth layers, in which the conditions are different and change constantly. Since a considerable part of the soil bacteria are heterotrophs, the quality and quantity of the organic matter available at a certain time as well as its distribution influence the presence and growth of bacteria and their concentration in certain parts of the humus.

A method more reliable than the random sample method (W a k s m a n 1931) is that of J e n s e n (1934), in which the heterogeneous object to be studied is divided into parts of suitable size. A mean is calculated for the numbers obtained from each part, and this mean subsequently represents the bacterial density of the whole object. H a r m s e n (1940), when studying cultivated soils, used a modification of this method, which eliminates the considerable variations occurring when a mathematical parameter is computed for each sample separately. He determined the bacterial density in a random sample obtained from a careful mixture of all the samples taken from the population to be studied. The results were quite reliable,

since the variation was only a fraction of what it had been when each sample was treated separately.

The latter sampling method was used as a basis in the present work. However, since forest humus differs essentially from that of cultivated soil, it was necessary to control the suitability of the method for bacterial density determinations of the unevenly dispersed, heterogeneous forest humus. These experiments are reported in the next section.

322. Sampling and representativeness of samples

The samples were collected only from the humus covering the mineral soil. Two plots measuring 10 by 10 meters were marked on Area No. 1, one on Part A and one on Part B, and a third on Area No. 2. Each plot was divided into 100 squares of 1 square meter. After the living plant cover was removed, the samples were taken with a bore having a circular bit 2.5 cm. in diameter. The F- and H-layers of the humus were separated by a horizontal cut. In order to find a reliable sampling method, a single square as well as two sets of squares on Part A of Area No. 1 were allotted, one set consisting of 5 and the other of 25 squares. For the preliminary study, 5 replicate samples were taken from each allotted square. In subsequent treatment of the samples, the replicate samples of each square were carefully kept apart, whereas the corresponding samples from the different squares of each set were combined. A dilution culture was prepared in the manner described later from the five replicate samples of the single square and from the combined samples of the two sets. Twelve replicate plates were made. The results of these experiments are presented in Table 6.

Table 6. Significance of differences between means of bacterial density of samples comprising 1, 5 and 25 squares.

Squares	Sample	Mean of bacterial colonies per plate	Significance of differences between means F
1	a	108	
	b	71	
	c	66	6.27***
	d	36	
	e	132	
5	a	80	
	b	145	
	c	64	5.73***
	d	85	
	e	135	
25	a	117	
	b	121	
	c	109	1.18
	d	114	
	e	102	

The significance of the difference of the means is calculated by the single factor abbreviation method. The letter F denotes the ratio of systematic to random variation. The test shows that there was no significant variation between the combined samples a, b, c, d and e in the set of 25 squares; thus the variation in the means was only random variation. On the other hand, variations in the other two sets were highly significant, and thus a sample consisting of, for instance, 5 borings is insufficient.

The results show clearly that the larger the number of borings, the smaller the variation. It is evident that if the sample included several hundreds or thousands of borings, the results would be still better (Harmsen 1940, Hanks and James 1940, Straka and Stokes 1957). It is therefore necessary to determine, by careful comparison, the number of borings needed in each case for the required degree of accuracy.

When is was found that a sample composed of 25 borings mixed together gave sufficiently reliable results of the average bacterial density of the object of investigation, this number of borings was used in every sampling all through the entire work. Control experiments made during the course of the work indicated that the procedure selected had been the right one.

33. Treatment of samples

The technique employed in the different stages of the dilution plate method will be examined in the following section. By varying the procedures involved, each stage of the method — the homogenization of the sample, its dilution in a suspension affording a suitable number of bacteria on the plates, and the assays of the final cultures — was tested by appropriate mathematical models, and the best technique, judged by the reliability of the results, was determined for every stage. As already mentioned in the chapter "Problems", the main questions could not always be immediately solved until minor problems arising in the course of the work had been settled by limited experiments. However, since most of these concerned checking of purely technical matters, such as testing the equipment used in the study, they affected the results only by facilitating in many cases the interpretation of the results.

331. Homogenization

When three experiments were made under similar external conditions within an interval of 24 hours, in which samples were taken in the same way from one object and mixed by the same method, as large as tenfold differences were observed in the final bacterial density values. The largest differences occurred between the results of two different days, but there was considerable variation also between replicate plates of one sample.

It does not seem likely that, under similar conditions, such radical changes could

take place in the bacterial density of the humus. Thornton and Taylor (1935), who obtained similar results, attribute them mainly to the reproduction of bacteria. However, the phenomenon cannot probably be explained quite as simply as this. New bacterial cells are produced only when the amount of organic matter increases in their environment, and even then only part of it is available for the reproduction of the bacteria. This would mean that, per unit of volume, several times larger quantities of organic matter ought to be decomposed in the humus within a very short time. This is, however, completely impossible. Thus the only reason for the observed changes in the bacterial density in a short interval of time must be an erroneous investigation technique.

It is not possible to obtain reliable results simply by mixing the humus sample. The minimum requirement is that the sample to be studied is as homogeneous as possible. Mechanical dry homogenization of the sample by any method is not sufficiently effective. Its effect can be injurious, since cells are easily destroyed in this process (Harmsen 1940). Destruction of cells can be avoided by using homogenization methods including vigorous mixing of the liquid and mechanical crushing of the material. In these the degree of homogenization is certainly higher than in dry homogenization. In addition, the liquid flow separates the cells from one another and from soil particles to which they are adhered and thus a dilution is simultaneously obtained for subsequent stages of the determination procedure. However, to obtain good results, appropriate mechanical equipment must be used. Operation under relatively aseptic conditions should be possible.

The homogenizer of the Edmund Bühler factories, Germany, proved to be very useful for homogenizing the humus samples of the present work. The apparatus has a four-blade cutting propeller which rotates in a tightly closed glass container. The speed of the cutter can be adjusted from 200 to 50 000 r.p.m. Both the flask-shaped glass container and the cutter with its shaft and fastening disc can be removed for sterilization. A rise of temperature in the material being homogenized is prevented by water cooling on the outer side. Contact with oxygen during operation cannot be completely avoided by any means, but it can be considerably reduced by excluding air from the homogenate. This is done by completely filling the container up to the fastening disc.

The direct mechanical effect of movement on bacteria is usually minimal, but most movements are accompanied by other effects, such as heat caused by friction and, under certain conditions, high frequency sounds, both of which can be harmful to bacterial cells. Some experiments were made in order to study these phenomena and their possible harmful effects. The dilution degree of the samples was 1:10, the diluents used were water, peptone solution, and soil extract in the temperature experiments, and peptone solution only in the noise measurements; the homogenization time ranged from 0 to 180 seconds. The composition of the diluents as well as the homogenization times were exactly the same as in the main experiments. The homogenizer was run at maximum speed. The results are presented in Table 7.

Table 7. Temperature changes occurring during various homogenization times and at two different initial temperatures (10° and 18°C).

Homogenization time, s.	Temperature, °C					
	Water		Soil extract, 0.5 %		Peptone solution, 0.1%	
	a	b	a	b	a	b
0	10.0	18.0	10.0	18.0	10.0	18.0
10	11.8	18.7	12.3	19.4	11.8	18.9
20	12.2	19.6	13.2	20.7	12.8	19.1
45	13.6	20.8	15.9	21.9	17.0	21.3
90	17.5	23.5	19.0	24.9	22.4	26.0
180	22.1	27.5	25.0	25.5	24.9	30.6

Despite cooling, the heat caused by friction was quite marked, although the temperature did not rise so much as to injure the bacteria. By improving the cooling with the aid of crushed ice, attempts were made to keep the temperature constant during all the stages of the operation, that is, the same as it had been in the humus at the time of sampling.

The noise caused by the homogenizer was measured with the Brüel & Kjaer 2203 sound level meter and the Brüel & Kjaer 1612 octave band sound analyzer, both of which measure the sound pressure level and the distribution of the sound intensity on different frequency bands. These devices and the homogenizer were in the same position in respect to each other during the whole experiment. Some temporal variation occurred in the measurements, but only on low frequency bands. The noise produced by five different speeds of revolution — settings 6, 8, 10, 12 and 14 (the highest) — and its distribution on different octave bands were determined. Figure 1 presents detailed results of the noise analysis.

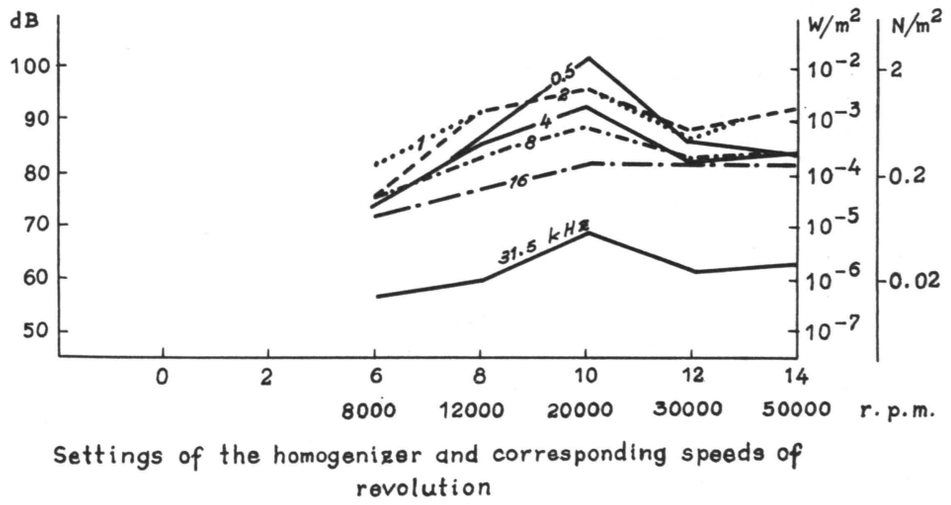

Figure 1. Effect of speed of revolution of the homogenizer on the noise level in different octaves.

The measuring unit decibel (dB) expresses the measured sound pressure level in relation to the universally adopted reference sound pressure level and the distribution of the sound intensity on different frequency bands. The unit of sound pressure is N/m^2 (p), and that of sound intensity, W/m^2 (I).

At setting 10, which corresponds to a speed of 20 000 r.p.m., the homogenizer produced the highest sound pressure, and also the sound intensity was at maximum at this speed. The sound pressure on different frequency bands was 10—100 times greater at setting 10 than, for instance, at settings 6, 8 and 12, and the intensity 6—10 times higher than on higher frequency bands (31.5 kHz).

Because the noise of the homogenizer might have an injurious effect on individual bacterial cells, some experiments were made to determine the total noise caused by different speeds of revolution. It could be observed, firstly, that the numbers of bacteria followed very closely the Poisson distribution on all frequency bands. In addition, it was observed that although the total noise and sound intensity increased many times, the mean amounts of bacteria did not differ significantly from each other. The sample variances were so much alike that the samples could very well be from the same population. When the phenomenon was studied as a function of time (0, 10, 20, 45, 90 and 180 seconds), no correlation was observed. With all the homogenization times the bacteria were distributed as if they belonged to the same population.

For comparison, experiments were also made with two other homogenizers, the Ato-Mix apparatus (MSE, Ltd., London), in which the cutting propellers rotate in stainless steel cups, and the Microid Flask Shaker (Griffin and George Ltd., London) with a speed of 80—200 vibrations per minute. The former was tested in the same manner as the Bühler homogenizer. It appeared that the temporal variations on all frequency bands were so large that the analysis of the sound intensity was very uncertain. Although it was evident that the effect of noise in the Ato-Mix apparatus was not injurious (its total noise was, however, considerably higher than that of the Bühler homogenizer), this apparatus was not, however, employed in the present investigation. One reason for this was that the requirements of asepsis could not be fulfilled.

In the shaker, which caused very little noise, homogenization is brought about by a shaking motion with no cutting or other mechanical movement within the homogenate.

Two homogenizers with different principles of operation, the Bühler apparatus and the shaker, were used for homogenizing the samples. Before homogenization, the samples were mixed in a glass jar having a cutter with four crescent-shaped blades inserted through its lid. The cutter was rotated at a speed of ca. 30—50 r.p.m. until the sample was macroscopically uniform. Exactly ten grams of the mixed sample was transferred to 90 ml. of the diluent, in which the homogenization was then carried out.

332. Diluents and degree of dilution

When a quantitative estimation of the bacterial density of a population is made by cultural methods, the samples are usually diluted with water, with a buffered or unbuffered mineral salt solution, or with a colloidal solution. The osmotic pressure in the dilution is different from that in the natural environment of the bacteria, and this has certain consequences. The number of cells in the diluted solution may decrease due to deaths caused by damages of the cell membrane; on the other hand, they may increase if the amount of nutrients increases or some other environmental factors are improved. A great number of alternative diluents have been suggested for eliminating the above-mentioned disadvantages (Straka and Stokes 1957); peptone solution has proved to be the best. Certain preliminary trials have indicated that soil extract is almost equal to peptone solution, in some cases perhaps even superior to it (Gyllenberg and Eklund 1958 (1959), Schalin 1961). But whichever of the possible diluents is used, a prerequisite for reliable results is that its concentration is appropriate.

Since the selection of the right diluent was a question of primary importance in the present work, an experiment was made using tap water, 0.5 % soil extract, and 0.1 % peptone solution as the diluents.

The advantages of soil extract and peptone solution over tap water as diluent will not be thoroughly discussed in this connection. It may only be mentioned that both colloids and minerals evidently maintain the osmotic pressure of the dilutions favorable for bacteria. In addition, they may lower the surface tension of the dilutions, and thus decrease the tendency of the cells to aggregate. Because of the importance of this question, the surface tensions of soil extract, peptone solution, and pure water were measured. The results (Table 9) show that the surface tensions of both peptone solution and soil extract are more advantageous than that of water.

Table 9. Surface tensions of peptone solution, soil extract, and tap water at 4, 10, and 18°C.

Diluent	Surface tension, dyne / sq. cm.		
Temperature of diluent, °C	4	10	18
Peptone solution, 0.1 %	61	58	55
Soil extract, 0.5 %	68	67	63
Water	73	72	71

The original dilutions in the ratio of 1:10 were prepared from the diluents having the concentrations given in Table 9. Since peptone solution even at a concentration of 0.1 % may have a slight nutritive effect on bacterial cells (Paarlahti and Hanioja 1962), although mostly very small (Hook and Fabian 1941), all the subsequent peptone dilutions, including the final bacterial suspension, were prepared from concentrations of only one tenth of the original. A peptone solution of such a low concentration is, however, adequate for bacteria (Straka and Stokes 1957).

The principle followed in the dilution of the samples from the very first homogenate was that the ratio of two successive dilutions should be constant, 1:10. This was achieved by first transferring 10 ml. of the homogenized sample to 90 ml. of diluent; the degree of dilution thus obtained was 10^{-2}. This dilution was mixed in a magnetic mixer, which Phillips et al. (1955) have found reliable for homogenizing bacterial suspensions. Subsequent dilutions were made by pipetting 2 ml. of the first dilution into a test tube containin 18 ml. of diluen. A corresponding procedure was followed until the right degree of dilution was attained. A major problem in the present work was to find a method for distributing the small amount of transferred liquid so uniformly that the new suspension would be as homogeneous as possible. The above workers have shown that shaking the tubes or rotating them by hand are unsatisfactory mixing methods and give very poor homogeneity. Quite promising results, however, have been obtained by the so-called aspiration-gravity method, in which mixing is achieved by sucking up part of the contents in the tube into a pipette several times and allowing it to flow back into the tube. Snyder (1947) has also used the aspiration-gravity method with good results in some serological experiments, although he does not compare it with other methods, as the above workers do.

Although the results obtained by Phillips et al. and Snyder appeared reliable, the aspiration-gravity method was tested before employing it in the present work. This was done in the following manner. Two sets of dilutions were mixed, one by rotating the tubes manually for 30 seconds, and the other by the aspiration-gravity method, in which the dilution was sucked up into a pipette with the aid of a rubber ball connected to the pipette; the liquid was then allowed to flow down into the tube without pressing. This was done five times successively for each tube. The volume of the pipette was 10 ml., which was half the volume of the dilution.

Plates were prepared from both the surface and bottom layers of each tube of the two dilution sets. Both peptone solution and tap water were used as the diluents, and the degree of dilution was 2.5×10^{-4}. The number of replicate plates was 12. The results of this experiment are presented in Table 10.

Table 10. Results obtained when the same sample was mixed by two mixing methods.

Method	Diluent	Mean number of colonies per plate		Significance of differences between means of surface and bottom colonies F
		surface	bottom	
Manual	Water	43	52	3.852***
Aspiration-gravity		56	58	0.901
Manual	Peptone solution	70	80	3.996***
Aspiration-gravity		80	83	1.082

The manual mixing method was evidently inaccurate, since the variation in the means was significant. But the aspiration-gravity method appeared quite reliable, and the slight variation can be only due to chance. The difference between the water dilutions and the peptone dilutions can be attributed to the different osmotic pressures of water and peptone solution, and, in addition, to the much smaller surface tension of peptone solution. These experiments were not made with soil extract. However, it can be expected that the results of soil extract would be similar to those obtained at least with peptone solution, since these two behaved similarly in several experiments, as will be described later.

In this connection consideration should be given to the measuring errors which occur in pipetting, and their effects on the results. A suspension which is either incompletely homogenized or too dense may cause intergrowth of colonies on the plates and thus errors in the counting; the actual distribution may not be determined and the number of the counted bacteria will be too small. On the other hand, a measuring error in pipetting leads either to a too small or too large number of bacteria. The following example (Table 11) illustrates the effects of a systematical measuring error on the final results at different degrees of dilution. The ratio of two successive dilutions is assumed to be 1:10 and the degree of the original dilution exactly 1:10.

Table 11. Errors at different concentrations due to systematical measuring error in pipetting of 1, 3, and 5 %.

Degree of dilution	Error of concentration, %		
	Measuring error in pipetting		
	± 1 %	± 3 %	± 5 %
10^{-1}	0	0	0
10^{-2}	+1.0, −1.0	+3.0, −3.0	+5.0, −5.0
10^{-3}	+2.0, −2.0	+6.1, −5.9	+10.2, −9.7
10^{-4}	+3.0, −3.0	+9.3, −8.7	+15.7, −14.2
10^{-5}	+4.0, −3.9	+12.6, −11.5	+21.5, −18.5
10^{-6}	+5.0, −4.9	+16.0, −14.2	+27.6, −22.6
10^{-7}	+6.1, −5.8	+19.5, −16.8	+34.0, −26.5

The error in the degree of dilution can be computed directly as percentages from one of two equations:

$$d_v = 100\ (q^{n-1} - 1)\ k$$

if the measuring error is positive and gives larger values than the actual ones, or

$$d_v = 100\ (1 - q^{n-1})\ k$$

if the error is negative and the results are smaller than actual. In these equations $q = \frac{100 \pm p\ \%}{100}$, n is the positive value of the exponent denoting degree of dilution, and k is the coefficient used when the degree of dilution is other than 1:10. Different values of k are given in Table 12.

Table 12. Value of the coefficient k at different degrees of dilution compared to a dilution of 1:10.

Degree of dilution	Coefficient k					
	Measuring error					
	1 %		3 %		5 %	
	+	−	+	−	+	−
1:2	4.980	5.021	4.941	5.061	4.902	5.102
1:5	1.998	2.002	1.994	2.006	1.990	2.010
1:10	1.000	1.000	1.000	1.000	1.000	1.000
2:1	0.500	0.500	0.501	0.499	0.501	0.499
5:1	0.200	0.200	0.201	0.199	0.201	0.199
10:1	0.100	0.100	0.100	0.100	0.100	0.100

If the dilution degree of the sample becomes n times larger or smaller, the error caused by the systematical measuring error changes nearly linearily at each degree of dilution.

The standard deviation of the mean of replicate observations increases with increasing dilution error caused by the measuring error (Jennison and Wadsworth 1940). The standard deviation is calculated from the combined equation of standard deviation

$$\sum = \sqrt{\sum_a^2 + \sum_b^2}$$

where

$\sum_a$ = the standard deviation of the mean of replicate observations

$\sum_b$ = the dilution error.

Because of the observations presented above, all the pipettes and other measuring equipment used in the work were tested and only those which gave an error smaller than 0.5 % were accepted.

In order to obtain reliable results, it is essential that the conditions are optimal, i.e. that each bacterial cell has an equal chance to grow and multiply. This requirement is assumed to be met, when, for instance, the variation in the means of different replicate plates is as small as possible. Jennison and Wadsworth (1940) have observed that the variation is at minimum, only 4—5 %, with a dilution degree which gives 100—400 colonies per plate. Wilson (1922) claims that the most

suitable number of colonies per plate is 200—400, whereas according to Clark et al. (1951) and Standard Methods for the Examination of Water, Sewage, and Industrial Wastes (1955) somewhat smaller amounts, 30—300, are more favorable. Based on the observations of the workers mentioned, attempts were made to prepare dilutions which would give 50—300 colonies per plate. This was done experimentally by diluting the same sample to concentrations of 10^{-3}, 10^{-4} and 10^{-6}, and similar plate cultures were prepared from each dilution. It was found that the smallest dilution allowed the growth of such a large number of colonies that exact counts were quite impossible. The highest dilution degree gave only very few colonies, 1—10 per plate; in many cases no colonies at all were formed. When suitable dilution limits had been found (10^{-4} and 10^{-5}), two additional concentrations were prepared, 2.5×10^{-4} and 5×10^{-4}. The results of these experiments are presented in Table 13.

Table 13. The effect of dilution degree on the mean number of bacterial colonies per plate.

Degree of dilution	Mean number of bacterial colonies per plate			
			Variance, %	
	a	b	a	b
10^{-4}	71	67	27.0	15.6
2.5×10^{-4}	57	40	33.5	38.6
5×10^{-4}	32	28	42.5	88.3
10^{-5}	22	19	56.9	86.0

In the table a and b are two samples taken simultaneously from the same sampling area and treated equally. The material is very small, and only six replicate plates were made in each series. Still the results give some preliminary information. If variation is used as the only criterion of reliability, the reliability of the results decreases with increasing degree of dilution. The number of the colonies per plate was even in the lowest concentrations relatively small, but appropriate still for the present study, since distinct temporal variation could be observed during the different stages of the investigation. The importance of the dilution degree will be discussed later in connection with the distribution of the cells in replicate observations. In the presentation of the results, attention will also be directed to the relationship between the number of colonies and the dilution degree.

34. Detecting and counting of colonies

The basic unit of study was a petri dish with 30—300 bacterial colonies. In order to obtain comparable results, it was necessary that the amounts of transferred suspension, the incubation time (14 days) and temperature (+18°C), as well as the composition and volume of the medium in every petri dish were identical.

The composition of the nutrient medium was determined by the bacterial population to be studied. The closer its composition corresponded to the natural environment of the bacteria, the smaller were the disadvantages caused by selectivity. For this reason soil extract agar prepared from Oxalis-Myrtillus type of humus (OMT) by the method of Lochhead and Thexton (1952) was considerer the most suitable medium in the present investigation. In spite of the identical experimental conditions, however, each dish was a special problem of its own for two reasons. For one thing, the origin of the bacterial colonies cannot be controlled. Therefore, when evaluating the results, it must always be kept in mind that a colony may have grown from a single cell or from a group of many. Secondly, when the number of colonies on the plate is large, the risk is always present that two adjacent colonies grow together with no detectable junction, or that they may overlap. In addition, another problem to be considered is that the color of the colonies and their background is so similar that it is extremely difficult to distinguish the colonies without special measures. Staining methods, otherwise usually successful, are of no use in cases where the colonies grow within the culture medium. A better means is slanting light, which facilitates detection and counting of the colonies (Goez and Tsuneishi 1951, Clark et al. 1951). The colony-counting apparatus made by N. Gerber is excellent for this purpose. The apparatus has a magnifier to facilitate the observation of the colonies, and the enumeration is aided by a ruled grid with several scales on the illuminated surface and by a built-in counting device.

It is evident form what was said above that an absolutely accurate count of colonies can never be made; the results are always subjective to some extent. The subjectivity is increased by the fact that the accuracy of the observations depends on the personal characteristics of the workers performing the counting. Power of concentration and keenness of observation are obviously very important in this type of work. Most people are able to easily note differences, whereas the observation of similarities demands accuracy of perception which is necessary for a work in which the main task is to observe similarities, and not differences, with great accuracy. A good power of concentration, a living interest in the work, and a sufficiently critical mind make a person an excellent observer. But even though a person would have the best qualifications for this kind of work, a temporary decrease in mental alertness may occasionally cause errors in the results.

The observation errors can be eliminated in many cases by repeating the counting a sufficient number of times. But then the counter easily tends to make the same error each time. This problem was of primary interest in the present work, and it was solved in the following manner. Two persons, experienced in assaying bacterial plates, counted, without knowing it, the same plate series three times. The results showed that the work of both persons was surprisingly uniform. If the plate had less than 100 colonies, the results of the three counts did not usually differ at all, and if the number of colonies was 200—300, a 1—5 % deviation occurred. There was no statistically significant difference in the results due to the counters. When

the results of the two persons were compared, it was found that although the numerical values of their observations sometimes differed, the deviations were usually of the same magnitude, occurred on the same plates, and, in addition, in the same direction. When the observations of the two counters were mutually tested, the value of t was found to be only 0.4315 with 35 observations, which indicates that there was no significant variation and that the two persons were equally suited for making the assays. All through the work the same two persons made two counts of all the plates. When differences occurred, the results of the other person were corrected with the percentage calculated from the mean of the variation of the means of the observations.

The bacterial colonies were counted both directly from uncovered dishes and also from the reverse side of the dish, through the glass bottom. The counts from the reverse side were almost systematically 0.5—5.0 % larger that those from the upper exposed surface. This did not, however, mean that more colonies were visible on the reverse side of the dish, since when it was covered with a glass lid, the results obtained from both sides were of about the same magnitude. The phenomenon evidently is due to minute defects in the glass which may be mistaken for bacterial colonies.

When the dish was divided into four equal sectors and all colonies were counted on one of them, and, in addition, on squares of the size of one square centimeter on all sectors, it was found that if the number of colonies on the counted sector was multiplied by the ratio of the sum of colonies on all small squares to the number of colonies on the counted sector, the results obtained differed only by ± 3—7 % of the individually counted colonies. The deviation increased considerably, if the number of colonies per plate was under 200. When the number of sectors and thus also of squares was doubled from four to eight, the result was improved and the variation was under 5 % in all cases.

4. Results

41. Choice of sampling areas and sampling

Forest humus abounds with life. It is in a dynamic state: humus is being constantly formed and decomposed. Since different kinds of organic residues are decomposed at different rates, humus always contains components that are at different stages of decomposition, and their mutual relations determine the quality of the humus. True humus substances, which have a direct bearing on plant production, are only formed in that part of the humus which, in the biologically active soil, is best capable of resisting the degradative activities of microbes and consequently steadily increase in amount. In addition to these substances, the humus always contains material which is not transformed into true humus.

There is no doubt that the microflora is one of the most important factors in gradually transforming organic remnants into humus substances, provided that the conditions for the occurrence and growth of these organisms are favorable. The circumstances in the northern region of coniferous forests are such that mainly fungi are responsible for the breakdown of the organic remnants in the humus (Waksman and Starkey 1924, West and Lochhead 1940, Smith 1948, Mikola 1954, 1956). The kind of the organic residues is a factor which restricts the activities of bacteria, namely a coniferous litter high in carbohydrates and low in nitrogen (Taylor and Lochhead 1938, Handley 1954). On the other hand, the usually relatively great acidity of the humus does not inhibit the activities of certain bacteria to any greater extent, because some types of soil bacteria have been found to adjust themselves readily to the hydrogen ion concentration of their environment, if enough decomposable organic material is available (Cobb 1932, Vandecaveye and Katznelson 1938, Chase and Baker 1954).

The soil bacteria have a marked tendency to concentrate in the rhizosphere of the roots (Lochhead 1940, West and Lochhead 1940, Timonin 1940). This tendency is primarily due to the fact that the intensity of growth and other activities of bacteria are determined, in the first place, by the kind of the available nutrients and only in the second place by their amount. The free amino acids excreted by the roots stimulate the activities of bacteria (Lochhead and

Thexton 1947, Payne et al. 1957). Because the excretion of these nutrients, essential for bacteria, depends more on the normal course of growth of the plant than on the stage of development of the roots, the bacteria prefer the rhizosphere of young plants, because their growth is relatively better than that of older plants (Rovira 1956).

The formation of more or less stable bacterial colonies in the rhizosphere of the roots influences, in turn, the composition of the whole humus. The bacterial cells and their metabolic products bind together loose soil particles so that these together with organic substances form granules characterized by a remarkable capacity to retain water (Swaby 1948, 1949, 1950). Although the role of fungi in the formation of humus is initially very important, it is not, however, lasting, since fungal hyphae provide excellent food for bacteria. The destruction of fungi does not cause the granules to break down, since the water-retaining capacity of the bacteria is considerably greater than that of the mycelial organisms. The bacteria act as partly living bridges around the granules. Loss of moisture makes the bridges firmer and links the crumbs together more firmly, whereas excess moisture dissolves them and the granular structure of the humus decreases (Glathe et al. 1954).

In general, the vertical distribution of bacteria in the humus is such that the large majority of them is found in the upper, less decomposed F-layer, which contains abundant organic remnants, while the remainder are in the lower, well-decomposed H-layer, which is composed of true humus substances (Waksman and Purvis 1932, Jensen 1934, Lochhead 1924, 1940, 1952, Taylor and Lochhead 1938, Conn 1948).

It is evident from what was said above that when the bacterial density of the humus is determined, it is very important how the sampling is performed. The preliminary experiments indicated that a single random sample or a sample composed of only a few borings gave unrealiable results. Only a sample consisting of a fairly large number of borings from different spots and which, in addition, was treated in the manner described above, gave a reliable index for the mean bacterial density in a certain object.

The material for the experiments was collected, as described earlier, from two sampling areas. One of the areas was, in addition, divided into two parts according to the plant cover. This was done, first, in order to give general applicability to the results and, secondly, to find out whether there was a difference between the bacterial densities of the humus at different sites and to test the significance of the possible difference.

The differences between the bacterial densities of the F-layer at different times of sampling (June, August and September) will first be examined by comparing the means, calculated for the different observation series, with the *t*-test (Snedecor 1956). The number of replicate plates was 48 in all series. The mathematical parameters appear in Table 14.

Table 14. Bacterial densities of Areas No. 1 and No. 2 in different months.

Sampling area	Month	Mean number of bacterial colonies per plate	χ^2 of the observation series	t - test
No. 1	June	134	4.613	1.895*
No. 2	,,	105	5.245	
No. 1	August	87	5.117	2.689**
No. 2	,,	55	0.272	
No. 1	September	103	3.716	5.083***
No. 2	,,	42	5.475	

The results show that there were no significant differences in the bacterial density of the F-layer between the two sampling areas in June, whereas in August the differences were significant and in September highly significant. Since the results varied as much as this, the problem was examined from combined material to avoid errors of interpretation. This was done by the analysis of variance; the systematical factors were the means of the colonies in the F-layer of the sampling areas, and the chance factor consisted of all factors affecting the within-sample variation.

Table 15. Significance of the difference between bacterial densities of Areas No. 1 and No. 2.

Classes	Degrees of freedom		F
	Random variance	Systematical variance	
The F-layers of Sampling Areas No. 1 and 2	338	1	79.30***

In Table 15 F is the ratio of systematical to random variance. The following symbols are used in this context as well as later in this work to indicate the degree of significance:

*** highly significant
** significant
* almost significant.

The respective error marginals are 5, 1 and 0.1 %.

The results in Table 15 again show that the means differ highly significantly from each other. This indicates that the F-layers of Areas No. 1 and No. 2 consist actually of two different populations. The significance is even higher than this, since the results were obtained from material sampled during the whole period of investigation, from May till October.

The fact that the results calculated by two different methods from the same material have different significances, would seem to indicate an error in the sampling method and make its reliability at least questionable. This is not, however, the state of things. First, it should be noted that the results in Table 14 are obtained from only one sample in each month, and thus they may include variation due to chance

factors. On the other hand, the analysis of variance, used here as a time-series analysis, reveals the regularities of the phenomenon to be investigated as a function of time. Secondly, the results are calculated per weight unit without taking into account that the humus of the two sampling areas had different specific gravities. When this is taken into consideration, Table 14 can be written in the following form:

Sampling area	Specific gravity of humus	Month	Corrected mean number of colonies per plate	t-test
No. 1	1.439	June	193	4.457***
No. 2	1.098	,,	115	
No. 1	1.480	August	129	5.220***
No. 2	1.046	,,	58	
No. 1	1.502	September	103	7.826***
No. 2	1.010	,,	42	

The results confirm the fact which was also indicated by the analysis of variance, that is, that the bacterial densities of Areas No. 1 and No. 2 differ from each other highly significantly and that, in all conditions, the density is higher on Area No. 1, the spruce stand, than on Area No. 2, the pine stand. In addition, it is obvious that the bacterial density of humus should always be corrected for the specific gravity of the humus in order that it actually expresses the density per unit of volume. Thus the mutual comparison of different objects is simpler than if the densities were in terms of weight units.

Parts A and B of Area No. 1 differed from each other externally only in respect of coverage of different plant species. The area was divided into two parts in order to determine whether there were differences in the bacterial density between plots with different plant covers on the same site, and, in case differences were found, to study their effect on sampling, assuming that the aim is to estimate the bacterial density of the humus of the whole site. Table 16 presents the mean count of colonies per plate in the F- and H-layers of Parts A and B, obtained with two different diluents, for the whole period of investigation. The values of the F-layer are given both as colonies per gram and per cubic centimeter of humus.

Table 16. Observed bacterial densities in the F- and H-layers of Area No. 1.

Sampling area and part	Mean number of colonies per plate			
	F-layer			H-layer
	specific gravity of humus	colonies/g.	colonies/cu.cm.	colonies/g.
No. 1 Part A				
Soil extract	1.461	130	190	50
Peptone solution	,,	181	264	65
No. 1 Part B				
Soil extract	1.566	127	198	34
Peptone solution	,,	173	271	40

Multiple factor variance analysis of these results was employed. The systematical factors were parts A and B of Area No. 1, F- and H-layers of the humus, soil extract and peptone solution, as well as months of sampling. The results are given in Table 17.

Table 17. Significance of differences between bacterial densities of Parts A and B of Area No. 1.

Classes	Degrees of freedom: Random variance	Degrees of freedom: Systematical variance	F
Parts A and B	416	1	2.06
F- and H-layers of the humus		1	291.22***
Soil extract and peptone solution		1	43.132***
Months of sampling (June, July, August, September and October)		4	100.70***

It can be seen from Table 17 that there was no significant difference between the bacterial densities of Parts A and B. Both parts represent equally well the whole Area No. 1. On the other hand, a highly significant difference was observed in bacterial densities between the F- and H-layers. This can also be considered natural, because the F-layer represents that part of the humus where the decomposable organic residues accumulate. Table 3 shows that this is really the case, since the content of mineral nutrients of the F-layer in all cases is clearly smaller than that of the H-layer. Also the amounts of exchangeable ions, which have the greatest stimulating effect on the activities of the bacteria, are considerably higher in the F-layer than in the H-layer (Table 4). This difference is very prominent for ammonium-nitrogen and phosphorus in particular. The pH and the oxidation-reduction factor were excluded in the present investigation, but it seemed evident that their effects were more favorable in the F- than in the H-layer. In this connection mention is only made of the fact that the difference between the bacterial densities obtained with soil extract and peptone solution as the diluents was highly significant. The question will be discussed more closely in connection with the experiments with diluents. The marked effect of the month of sampling shows that certain seasonal variations occur in the bacterial density (Waksman 1931, Conn 1948, Smith 1948, Lochhead 1952, Gyllenberg et al. 1954, Schalin 1961). Since, besides the growth rhythm of the vegetation, this phenomenon is closely connected also with the changes in the microclimate, to which the bacteria are more sensitive than other microbes or higher plants (Glathe et al. 1954), it is absolutely necessary when the investigation aims at determining bacterial density, to make simultaneous observations of the ecology in the environment of the organisms under study.

The experiments carried out indicate that the bacterial populations of different types of humus can be quantitatively compared by the dilution plate method. But an essential prerequisite is that the sample for the determination is comprised of numerous subsamples taken from several spots; thus the errors due to the heterogeneous distribution of the bacteria can be avoided.

42. Homogenization

Generally speaking, one of the hardest problems in soil biological research is how to treat samples from the heterogeneous soil so that each characteristic under study can be expressed by as reliable estimates of parameters as possible. Forest humus is in this respect one of the most difficult objects of investigation from the microbiological point of view. When the aim of the study is to determine the bacterial density of the humus, an essential requirement is to prepare such a homogenate that any sample taken from it can be considered to represent the whole population. To reach this goal, empirical studies have to be used. But preparation of a perfect homogenate is probably impossible, since already the mutual relations of the bacteria in a population make it impossible. Neither is it possible to follow exactly the same homogenization procedure for samples from different types of humus, because differences in their composition may affect certain stages of the treatment, as will be seen later in this section.

In this connection it is no longer necessary to discuss the sometimes very annoying phenomena in the mechanical homogenization of samples, such as temperature and noise, since they were dealth with above. It may only be mentioned that these phenomena were not found to have any disturbing effect in the equipment used in the present study. For this reason no attention need be paid to these factors in the following.

Homogenization carried out in diluents causes changes in the physical and chemical conditions prevailing in the environment of the bacteria as well as in their mutual relations. It should, however, be remembered that since the activities of bacteria are a result of all these factors as well as the metabolism of the organisms themselves, it is possible only in experiments made under exceptional conditions to observe the effect of one particular factor. Interpretation of the results and estimation of their reliability may often be very subjective. This is at least the case when the object of study is an unknown mixed population. For this reason it is much simpler and perfectly justified in studies aiming at purely quantitative goals to observe the total effect of all the factors and, above all, to determine the optimal range for the viability of the bacterial population.

An important question under conditions where the factors are represented by a single component expressing their total effects is the relation of the numbers of bacteria to the homogenization time. Harmsen (1940) claims that a perfect

homogenate is obtained in 1—1½ hours of homogenization with a dilution ratio of 1:10. However, it should be pointed out in this connection that a manual homogenization method was used by H a r m s e n. Already in the preliminary experiments of the present work with the electrical homogenizer it was observed that prefectly satisfactory results were obtained in a much shorter time. However, already after a few minutes of mixing the variation in the replicate observations started to increase, and likewise the mean began to decrease after a certain time.

On examining the response of bacteria to the homogenization time, a definite regularity could be noted. A pre-treated, weighed sample in the diluent was used in the experiments; the homogenization time for this sample was assumed to be 0 seconds. The various times formed a cumulative series consisting of 10, 20, 45, 90 and 180 seconds; i.e. the same as in the sound experiments. The homogenizer was run at nearly maximum speed (setting 12; maximum 14). The results of the preliminary experiments with humus from Area No. 2 with 0.1 % peptone solution as the diluent are presented in Table 18.

Table 18. Bacterial densities obtained from the same humus sample after different homogenization times.

Sample	Homogenization time, seconds	Mean number of colonies per plate (18 replicte plates)	χ^2
Sampling area No. 2, F-layer	0	33	4.700
	10	101	5.780
	20	161	6.083
	45	112	3.829
	90	89	1.972
	180	86	2.765

It is somewhat surprising that the maximum was reached as early as after 20 seconds of homogenization and that when the operation was continued, the numbers of the bacterial colonies clearly decreased. Since this phenomenon could not be explained and no general conclusions could be drawn from these results only, several replicate experiments were made with samples from both areas. In addition to Areas No. 1 and 2, a sample was taken for comparison from a birch stand (Area No. 3), which belonged to the Oxalis-Myrtillus type (OMT). Certain data obtained on the content of mineral nutrients and the total amounts of some nutrient elements and exchangeable ions of the F-layer of Area No. 3 were as follows:

Sampling area	Mineral nutrients, %	Total % of dry material CaO	N	P_2O_5	K_2O
No. 3, F-layer	72.60	1.210	2.220	0.145	0.082

Exchangeable ions mg. / 100 g. dry material	
NH_3—N	NO_3—N
40.0	11.5

The three humus samples for the homogenization experiment were taken simultaneously and treated in the manner described earlier. The results of these experiments appear in Figure 2.

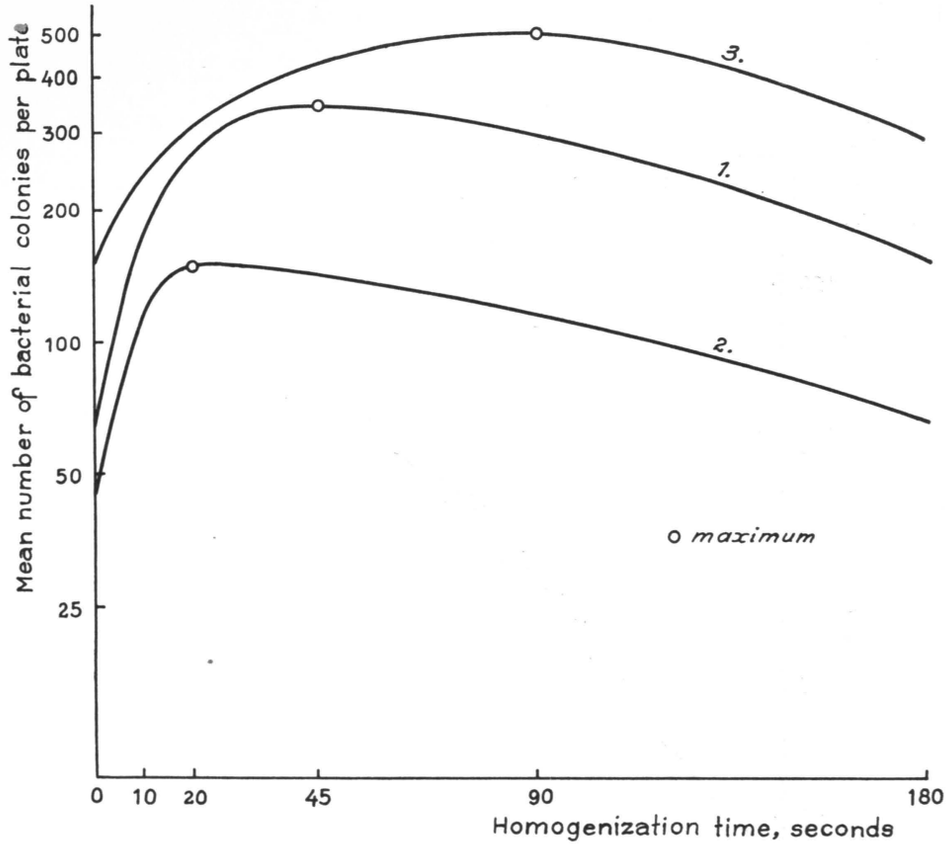

Figure 2. Mean number of bacterial colonies as function of homogenization time in samples taken simultaneously from Areas No. 1, 2 and 3.

A mean was calculated for the observations for each homogenization time, and the curves were drawn from these values. In the multiple factor analysis of variance the systematical factors were homogenization time and the different humus samples. The results are seen in Table 19.

Table 19. Significance of difference between bacterial densities during different homogenization times.

Classes	Degrees of freedom Random variance	Systematical variance	F
Times 0, 10, 20, 45, 90 and 180 seconds	622	5	179.10***
Samples No. 1, 2 and 3		2	665.43***

In both cases the differences are highly significant. Attention must therefore always be paid to the homogenization time if a reliable estimate of the bacterial density of humus is desired. Since samples from different types of humus were included in the experiments, the results can be considered to have general applicability.

The fact that the time used for homogenization is usually determined by the kind of the humus appears from Figure 3, where the homogenization time is given as a function of the content of mineral nutrients of the humus. Only the F-layer of the humus was included, because complete data were in all cases available for this layer.

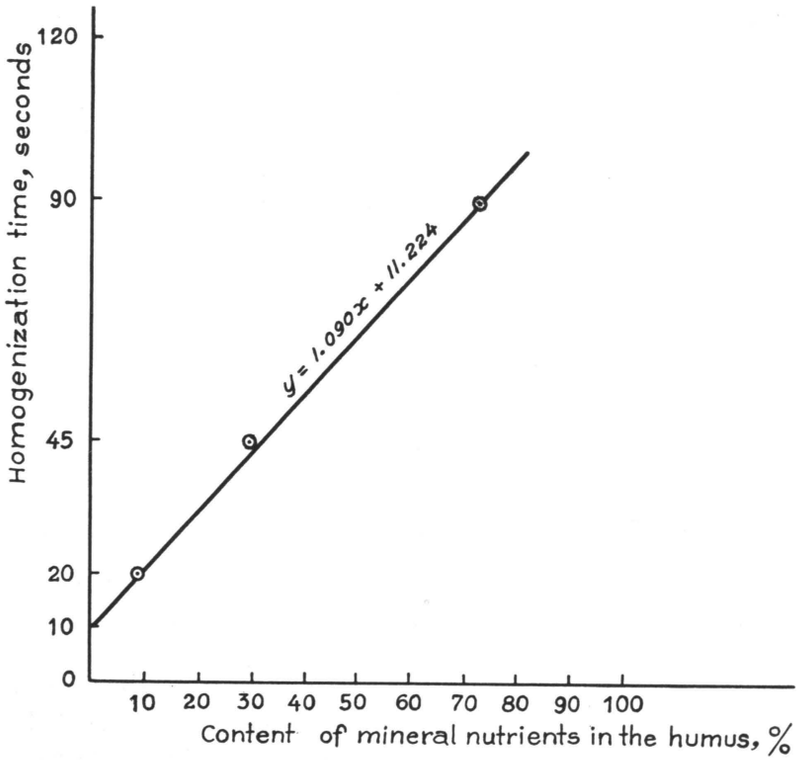

Figure 3. Content of mineral nutrients in the humus as a factor influencing homogenization time.

Although the figure only has three points indicating the content of mineral nutrients of Areas No. 1, 2 and 3, each point is, however, the mean of 5×18 replicate plates, as a function of time. The correlation is very clear, as indicated by the value of the coefficient, 0.996. The dependence of the homogenization time on the mineral nutrient content of the humus is expressed by the regression line, the equation of which is

$$y = 1.090x + 11.224.$$

Because the correlation coefficient is very high, is has to be corrected by Fisher's correction (M o r o n e y 1962), before the significance of both the correlation and regression coefficients can be tested by the *t*-test. The corrected value of the correlation coefficient, *z*, calculated from the equation

$$z = 1.1513 \log \left(\frac{1 + r}{1 - r} \right)$$

is 3.106. The significance of the parameters is the following:

Correlation coefficient	Regression coefficient	$f = n-2$
3.501^{***}	4.151^{***}	106

The results show that when the content of the mineral nutriens in the humus increases, the homogenization time has to be increased linearily. It can also be seen from Figure 3 that the minimum time needed for obtaining a suitable homogenate can be exactly determined.

Soil bacteria adhere readily to the surface of humus substances, especially to soil colloids (J o n e s and M o l l i s o n 1948). The adherence is often stronger than the electrical charge of the bacteria themselves, which keeps the cells apart from one another. This results in the agglutination of the bacteria. The metabolic products of the bacteria enhance the internal adherence of the colonies as well as that between the colonies and the colloidal particles to such an extent that only radical changes in the environmental conditions, above all increase of moisture, lead to the dissolution of these clumps (S w a b y 1950, G l a t h e et al. 1954).

The amount of colloids in the humus increases when humification progresses and the amount of available nitrogen simultaneously increases (W i t t i c h 1952, K o n o v a 1961). Considering this fact as well as what was said above. the explanation of the phenomenon is that the more advanced the degree of humification, the longer time is needed for disrupting the bacteria from both humus particles and bacterial colonies. In a weakly humified humus, where the amount of colloidal substances is small (R o m e l l 1932, W i t t i c h 1964), it is possible that in certain places where the cells are far apart from one another, the bacterial population is easily brought into the form of a suspension. In addition, it can be assumed that in a weakly decomposed humus the specific composition of the bacterial population is simpler than in a highly decomposed one. A further advantage in the present case under study is that the amount of available nitrogen nutrients is higher and for this reason the mutual relations between the bacteria are simple. Under conditions like this, surface phenomena causing adherence and agglutination of cells are not so significant.

It may still be mentioned in this connection that when the maximum count of bacteria was reached after a certain time of homogenization, the number of bacteria started slowly but clearly to decrease, mainly perhaps because bacterial cells died during continued homogenization.

The results of the homogenization experiments presented above were all obtained from samples mixed in the Bühler homogenizer. Even without comparison to other types of homogenizers they can probably be considered quite reliable, indicating furthermore the general procedures to be followed in investigations of the bacterial densities of humus soils. In spite of this, some comparative experiments were made with the shaker; these experiments were reported in connection with the treatment of the material.

The main object of these experiments was to study the effect of homogenization carried out as efficiently and in as short a time as possible. It could be seen at once at the preliminary stage of the experiments, when the maximum speed of the shaker was used, that homogenization times of about 20 seconds gave results which were almost identical to values obtained from nearly unhomogenized samples. For this reason relatively long homogenization times, 5, 10 and 20 minutes, were used with the shaker. It was not advisable, however, to exceed 20 minutes, because the reproduction of bacteria might cause changes in the density. A serious disadvantage of the shaker was that oxygen was freely mixed with the homogenate.

The results obtained when the humus sample — irrespective of its content of mineral nutrients — was mixed in the shaker, were in most cases extremely unsatisfactory. Homogenization times of different lengths could give nearly the same results. At the shortest and longest homogenization times, the bacterial counts obtained differed so significantly from the theoretical Poisson distribution that it must be an indication of the total unreliability of the means of the observations (F i s h e r et al. 1922). Only after 10 minutes of homogenization did the distribution follow Poisson's law almost significantly.

Table 20 presents the results obtained by two different homogenization methods from samples which were pre-treated in the same manner. Both were random samples, one being mixed in the Bühler homogenizer for 45 seconds and the other in the shaker for 10 minutes. Twenty four platings were made.

Table 20. Bacterial density values obtained from the same humus sample homogenized by two different methods.

Homogenization method	Mean number of bacterial colonies per plate	
	F-layer	H-layer
Bühler apparatus 45 seconds	179	59
Shaker 10 minutes	83	36

There were definite differences due to the two homogenization methods. The significance of the difference of the means was tested by the analysis of variance;

it is presented in Table 21. The systematical factors were the homogenization methods and the humus layers in the multiple factor analysis.

Table 21. Significance of the differences due to two different homogenization methods.

Classes	Degrees of freedom		*F*
	Random variance	Systematical variance	
Homogenization methods (Bühler/ Shaker)	150	1	90.10***

It can be observed, as Table 20 already indicated, that the two homogenization methods used give systematically different bacterial density values.

The latter results confirm those obtained earlier on the importance of the homogenization process of humus. It should therefore be emphasized that in studies on the bacterial density of humus, great care should be taken that no such errors occur in connection with homogenization which might lead to erroneous conclusions.

43. Diluents

In their natural environment the activities of bacteria are different than under conditions where the environmental factors have been changed in some way (L o c h h e a d 1940). Unicellular bacteria have been found to be more sensitive than other organisms to external changes. If, for instance, the composition of the diluent used when determining bacterial density greatly differs from that of the normal environment of the bacteria, the result may be that a part or all of the population are destroyed, and the reliability of the counts obtained is very questionable. For this reason it is extremely important that, in all studies based on dilution, the composition of the diluent is such that bacteria are able to maintain their viability in it for at least the time needed for carrying out the procedures.

It has been customary in most cultural methods employed in microbiological research to dilute the sample with water. However, it has been observed in several connections that pure water has an injurious effect on bacteria even during a short period of time. When natural water samples were diluted with distilled water, it was found that in less than half an hour as much as 60 % of the bacterial cells in the samples were destroyed (B u t t e r f i e l d 1932). Similarly, some pathogenic bacteria are highly susceptible to the damaging effect of distilled water, nor do they survive in saline or phosphate solutions (d e M e l l o et al. 1951) Likewise it has been observed that certain typical soil bacteria cannot be kept alive in water, whether distilled or not, nor in the two solutions mentioned above, while many other

bacteria readily maintain their viability in these liquids (Gunther 1954, Stokes and Osborne 1956, Straka and Stokes 1957).

Many efforts have been made to avoid the harmful effects of the diluents, as already mentioned in the previous chapter. In some cases saline or phosphate solutions may be adequate. This, however, has been found only with pure strains, whereas for some naturally occurring mixed populations these solutions have proved almost equally destructive as water. When Straka and Stokes (1957) studied natural bacterial populations in some foodstuffs, they found that in water 40—60 % of the bacteria were destroyed after 20 minutes and 60—90 % after one hour. Similar results were also obtained with saline solution. Phosphate solution had a slightly less injurious effect, but even in this diluent the numbers of bacteria decreased by 20 and 79—80 %, respectively, during the corresponding periods of time. In experiments with other solutions containing certain sugars, yeast extract, ammonium sulfate, etc., as the main components, much better results were obtained, although quite large variations occurred. Only peptone solution adequately prevented damage to the bacteria, also when the experiment lasted longer than those mentioned above. The same observation was earlier made by Winslow and Brooke (1927) and by de Mello et al. (1951) with pure strains. Encouraging results comparable with those obtained with peptone solution were also obtained with soil extract [Gyllenberg and Eklund 1958 (1959)]; its composition corresponds closest to the natural conditions of soil bacteria, but it can also be used for study of other bacteria.

The viable counts of humus bacteria in dilution cultures with pure water, peptone solution, and soil extract as the diluents will be examined in the following section. The other factors in the experiments were standardized. However, some observations are first presented on the effect of pH of the diluent on the bacteria. Duplicate dilutions from two samples, whose original pH was measured, were prepared into 0.1 % peptone solution. The pH of one of the dilutions was adjusted with hydrochloric acid to 4.50—4.75 and the other to 6.00—6.25. The samples were kept for about 90 minutes in the diluents. The results of 24 replicates are presented in Table 22.

Table 22. Bacterial counts obtained in peptone solution at two different pH values.

Sample	Mean number of bacterial colonies per plate			
	pH of the diluent 4.50—4.75	6.0—6.25	*t*-test	pH of the sample
No. 1, F-layer	123.3	118.1	0.335	4.60
No. 2, F-layer	89.3	74.1	1.187	3.95

The test shows quite conclusively that the number of bacteria remained practically unchanged in the pH range of 4.50—6.25.

In the following the bacterial densities obtained in all the experiments of the investigation will be studied. The results are presented in Table 23 as relative values compared to those obtained for a water dilution of the F-layer. The table has two series of values, obtained 30 and 90 minutes after the preparation of the first dilution, including the time needed for homogenization. These times correspond to the minimum and maximum times needed for the treatment of the samples from the first dilution to the preparation of the plates, depending on the size of the respective series. During these stages, until the incubation of the plates, the temperature was kept constant, that is, the same it had been in the humus at the time of sampling. The pH of the dilutions was each time adjusted to 6.00—6.25.

Table 23. Bacterial densities, expressed as relative values, obtained by three different diluents from the same humus sample.

Diluent	Mean number of bacterial colonies per plate		
	Time of dilution treatment 30 min.	90 min.	difference in %
Water	100	52	—48.0
Soil extract 0.5 %	170	161	— 5.3
Peptone solution 0.1 %	240	251	+ 4.6

After 30 minutes the numbers of bacteria in all the diluents followed the Poisson distribution. But after 90 minutes the distribution of the numbers of bacteria in the water dilution differed highly significantly from the Poisson series. Testing of the means gave the following values of t in the 30-minute test:

peptone solution/soil extract	3.465***
peptone solution/water	7.608***
soil extract/water	4.268***

which indicate that in each case the populations were different.

The results of the multiple factor variance analysis (Table 24) confirmed the

Table 24. Significance of difference between means of the bacterial densities of the same humus sample in three different diluents.

Classes	Degrees of freedom		F
	Random variance	Systematical variance	
Diluents (water, soil extract and peptone solution)	280	2	74.52***
Months of sampling (June, July, August, September, October)		4	56.65***
Sampling areas and parts (area No. 1, parts A and B, area No. 2)		2	10.94***
F- and H-layers of the humus		1	391.27***

highly significant difference which was observed between the bacterial densities of the water, soil extract, and peptone dilutions. Since the systematical factors also included the different sampling areas, the two humus layers, and the times of sampling, the results can be regarded as having general applicability in determinations of the bacterial density of humus.

In all the variances between classes the effect of the diluent is highly significant. The results are not at all affected by the site or time of sampling (ranging from June to October), nor by the humus layer from which the samples were drawn. The composition of the diluent is thus significant under all conditions when the object of study is to estimate bacterial density of the humus. However, for drawing correct conclusions, also the concentration of the diluent has to be appropriate.

The above results demonstrate that water as a diluent is destructive to bacteria (Table 23) and leads to erroneous results. On the other hand, in both soil extract and peptone solution humus bacteria are able to maintain their viability at least during that time which is necessary for completion of the different stages of the method. In soil extract the number of bacteria decreased slightly after 90 minutes, which was probably due to the fact that the concentration of this solution was close to the critical limit, below which the solution has a damaging effect on bacteria. Peptone solution, even at a concentration of only 0.01 %, was apparently a better diluent than soil extract, since the numbers of bacteria in it were not found to decrease at all. Straka and Stokes (1957) noted in their experiments with mixed populations that the critical limit of peptone solution is below 0.01 %.

The different behavior of bacteria in the diluents can be assumed to be due to several reasons. The pH does not seem to have any great effect, not at least within certain limits, since the difference of nearly two pH units did not cause changes in the bacterial numbers. It is evident that the isoelectric range of the bacteria is so wide that moderate changes in the pH occurring near the natural range of acidity of the environment cannot upset the balance of the gram-positive and gram-negative soil bacteria.

The low concentration or lack of salts and colloidal substances in water dilutions causes such great changes in the osmotic conditions of the bacteria and their environment that the cells may die. Furthermore, the destruction is the more complete, the longer the bacteria stay in water. The concentration of soil extract and peptone solution was evidently high enough to retain the viability of the bacteria, but still sufficiently low so that the solutions had no nutritive effect which would cause growth of the bacterial population (Hook and Fabian 1941). The soil extract and peptone solution used in the present investigation can thus be regarded as equal in this respect. However, it was already mentioned in section 332 (Table 9) that the surface tension of soil extract (63 dynes at 18°C) is somewhat higher than that of peptone solution (55 dynes). It is known that the lower the surface tension of a solution, the smaller its viscosity. As a result, the agglutinated bacteria readily disaggregate and all their motions become rapid. This is a satisfactory explanation

of the phenomenon that a systematically higher count of bacteria was obtained with peptone solution than with soil extract in all the experiments. A decrease in the surface tension as such thus leads to a numerically higher result, but another possible explanation is that the nutrients causing the lower surface tension may promote the reproduction of the bacteria. The result then no longer represents the actual bacterial density at the time of sampling, but only the reproduction potential of the bacteria. For this reason the composition of the diluent and the degree of dilution must be chosen so that the viability of the bacteria remains unaltered at least for a certain period of time; that is, the concentration of the diluent must not be so high that its nutritive effect increases the number of bacteria, nor so low that it is destructive to them. In quantitative determinations of humus bacteria, peptone solution of the concentration 0.1—0.01 % and soil extract of the concentration of about 0.5 % can be regarded as suitable diluents. Water, on the contrary, must be considered as completely unsuitable.

44. Degree of dilution

The optimum degree of dilution of a bacterial suspension can be considered obtained when a random sample from it gives such a number of colonies per plate that there is no overpopulation and that each of the colonies can be distinctly observed. Overpopulation, however, does not only depend on the number of colonies but also on their size. Although the disadvantages of overpopulation can be avoided by weak dilutions, a result of this may be that the bacteria no longer follow the expected theoretical distribution, and the counts are unreliable.

The only means of determining the size of a certain bacterial population as correctly as possible is to find the optimum bacterial density of the dilution for the subsequent assays. This obviously can be done only by empirical methods, since it is not possible to estimate beforehand the bacterial density of the object under study. The right degree of dilution is evidently only a theoretical concept. But a certain range of concentrations, within which the results are reliable, can be arrived at experimentally.

In some orienting experiments, of which preliminary results were presented in section 332 (Table 13), it appeared that the degree of dilution for the humus samples must be within the range of 10^{-4} and 10^{-5} if colony numbers of 30—400 per plate are desired, as recommended by e.g. Wilson (1922) and Clark et al. (1951). The unreliable results of the experiments, which may partly be due to the limited material, indicated, however, that the range should be more narrow than this.

Consequently, experiments were performed in order to find a dilution degree which would be suitable for studies of the bacterial density of humus and which would allow for seasonal variations occurring in the bacterial density. Because of the latter phenomenon, the material was collected at different times of the investigation period, although each sample was treated quite separately in order to verify

the theoretical distribution of the numbers of bacteria. Some of the samples were diluted step by step from 10^{-4} to 10^{-5}, and some directly from the maximum to the minimum concentration. Twenty four replicate tests were made. The results of the experiments are summarized in Table 25.

Table 25. Bacterial densities obtained from humus samples by four different degrees of dilution.

Sampling area/ Sample	Mean number of bacterial colonies per plate Degree of dilution				Ratios Degree of dilution			
	10^{-4}	2.5×10^{-4}	5×10^{-4}	10^{-5}	10^{-4}	2.5×10^{-4}	5×10^{-4}	10^{-5}
No. 1 Spruce 1	204	113	83	80	100	55	41	39
No. 2 Pine 1	133	85	51	39	100	64	38	29
No. 2 Pine 2	64	—	—	18	100	—	—	28
No. 1 Spruce 2	327	—	132	—	100	—	40	—
No. 1 Spruce 2a	259	—	105	—	100	—	41	—

The samples Spruce 1 and Pine 1 were collected from Areas No. 1 and 2, respectively, at the beginning of the period of growth, Pine 2 from Area No. 2 in the middle, and Spruce 2 and 2a from Area No. 1 after the end of the growth period. Peptone solution was used as the diluent for all the samples. It can be seen from the table that although the counts differ considerably from one another depending on the time of sampling, their mutual relations in the different dilution are almost the same at each time of sampling. The results are of considerable significance, as will be seen a little later, especially considering the technique of dilution used in different cases.

The observations on Areas No. 1 and 2 for the degrees of dilution presented in Table 25 were studied in order to test how closely the distribution corresponded to the theoretical in each case. This was done by calculating the dispersion index indicating the observed distribution of each plate series and by comparing it with the theoretical value in F i s h e r's (1954) table. Twenty four plates were made in six groups, and thus the degrees of freedom were 5. The results are presented in Table 26.

Table 26. The dispersion indexes obtained for different degrees of dilution as compared with Fisher's dispersion index.

Sampling area	Degree of dilution	Calculated dispersion index	Fisher's dispersion index	P
No. 1	10^{-4}	2.738	3.000—4.351	70.—50.
	2.5×10^{-4}	.920	.752—1.145	98.—95.
	5×10^{-4}	3.399	3.000—4.351	70.—50.
	10^{-5}	4.891	4.351—6.064	50.—30.
No. 2	10^{-4}	3.483	3.000—4.351	70.—50.
	2.5×10^{-4}	.717	.554— .752	99.—98.
	5×10^{-4}	3.258	3.000—4.351	70.—50.
	10^{-5}	4.799	4.351—6.064	50.—30.

It can be seen from the table that when the degree of dilution of the sample is 2.5×10^{-4}, the observed distribution follows quite significantly the theoretical distribution; 2—5 % of the samples from Area No. 1 and only 1—2 % of the samples from Area No. 2 cannot be considered normal. For all other degrees of dilution, on the contrary, the deviation from the theoretical distribution is larger. At the dilution degrees of 10^{-4} and 5×10^{-4} nearly one-half of the cases and at 10^{-5} as much as 70 % of the cases must be regarded as not normal.

Figure 4 shows the significance of the dilution degree for the bacterial density on plates; the straight lines are drawn through the points indicating the theoretical value of bacterial density of the most reliable degree of dilution.

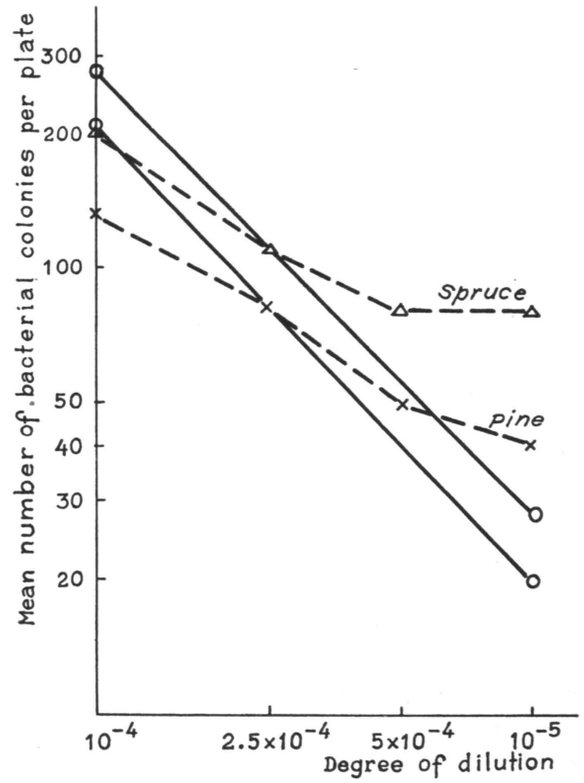

Figure 4. Mean number of bacterial colonies at different degrees of dilution.

The theoretical lines indicate that at high concentrations (about 10^{-4}) too few bacterial colonies were counted on the plates, whereas at concentrations below 2.5×10^{-4} the numbers of colonies were too high, and that the deviation from the theoretical distribution grows linearily, as observed in connection with the mathematical consideration (Table 26). It is quite possible that the too small values

in the first case are due to overpopulation and the too high values in the latter case are caused by the fact that the values deviate too much from the mean (Fisher 1922). The phenomenon can be explained to be due to the mutual relations of bacteria in mixed populations. Although the results presented above were obtained from only two observation series, they can, however, with certain restrictions, be generalized. In the dilutions 10^{-4} and 5×10^{-4} for samples Pine 2, Spruce 2, and Spruce 2a (corresponding values of χ^2 1.673, 3.483, 3.597, 3.154, 3.738 and 4.056) similar regularity was observed as in samples Spruce 1 and Pine 1, with the exception of the lowest concentrations of Pine 2, where the theoretical distribution could not be demonstrated at any degree of probability. The cause of this is evidently that the number of colonies per plate at this concentration was very small.

Since at least samples Spruce 2 and Spruce 2 a are completely comparable with Spruce 1 and Pine 1, the lacking bacterial numbers of the two former samples at concentration 2.5×10^{-4} can be calculated with the aid of the values of Table 25. With the ratio 60 ± 10 % they were the following:

Spruce 2 on an average 216—177 bacterial colonies per plate
Spruce 2 a —„— 171—140 —„—

On the basis of the above, it is clear that the reliability of the dilution plate method cannot alone be determined by the number of colonies per plate. The method is statistically reliable only when the colonies are formed from such a dilution where the dispersion index indicating the observed distribution deviates least from the theoretical Fisher's (1954) dispersion index. Estimates of the bacterial density of the humus proved to be most reliable when the degree of dilution was 2.5×10^{-4} and the number of colonies per plate about 85—325.

By making use of the estimate of the mean of the dilution degree which proved most reliable, a correction coefficient can be calculated for adjusting the number of cells in the nearest successive dilutions (Gordon and ZoBell 1938). Figure 4 shows that this correlation coefficient is always greater than one in concentrations higher than the right one, and smaller than one in weaker concentrations. In the present cases the values obtained from the dilution degree of 10^{-4} are corrected by multiplying them by 1.55—1.65 and those from 5×10^{-5} by 0.73—0.78. Thus the systematical error of successive dilutions can be corrected provided that the distribution of the bacteria in all dilutions follows the theoretical distribution.

45. Preparation of plates

It is obvious that quantitative determination of bacterial density does not involve only mechanical counting of the colonies. The results also depend greatly on the techniques of the method used. In addition, the fact that it is not possible to know whether a bacterial colony has formed from one or several cells is a noteworthy drawback.

Immediately after the beginning of growth, conditions on the plate change in a way which cannot perhaps always be controlled or which may even be unknown. The metabolic activity of the cells modifies the composition of the medium; there may be a decrease or even an exhaustion of nutrients, an accumulation of toxic metabolic products, or changes in the ionic equilibrium, especially the acidity. It is essential that the observer know these growth-limiting factors. However, it should be mentioned that in most of the cases where the cultures are made from strongly diluted bacterial suspensions, these factors are not very important (Monod 1949). Still they cannot be totally ignored, since it is possible that they may have affected the phenomenon where the number of bacterial colonies per plate was too small (Figure 4).

In these trials the composition of the medium and also the other conditions, except pH, were kept strictly constant and were the same in all stages of the investigation.

Culture series were prepared in two different ways. In one of the series the bacterial suspension was spread on the bottom of the petri dish, after which the molten agar was poured on top of it. In the other series the bacterial suspension was mixed with the agar in the tube by the aspiration-gravity method and then poured in the dishes and allowed to solidify; 36 replicates were made for each series. In the former case the mean number of bacterial colonies per plate was 129, and in the latter case 177, respectively. The significance of the difference between the means was as follows:

Classes	Degrees of freedom: Random variance	Systematical variance	F
Bacterial suspension mixed with agar and non-mixed	70	1	5.15**

It is evident that when the bacterial suspension is spread onto the bottom of the dish and its mixing with the rapidly solidifying agar is minimal, overpopulation occurs, since the colonies can grow mainly only in two dimensions. Such overlapping can be avoided or reduced by first mixing the bacterial suspension with the medium, thus allowing growth in three dimensions and decreasing the chances of overlapping.

It could be observed in assaying the plates that the counts made through the bottom of the dish were systematically slightly larger than the values obtained from the exposed upper surface (previously mentioned in section 34). Some data on this phenomenon are presented in the following:

Mean number of bacterial colonies per plate	Observation series: 1	2	3	4	5	6	7	8
Exposed surface	162	128	134	105	86	56	102	42
Bottom of dish	164	130	133	107	84	56	104	45

There is no statistically significant difference between the means, which can be seen from the above data and also from the ratio of variances, which was only 0.04.

These experiments on the preparation of bacterial cultures confirm the fact — already observed in the other stages of the method — that the dilution plate method is above all an empirical method, whose reliability is closely related to the efficiency of all phases of the technique. A certain amount of subjectivity is always involved in assaying the cultures, but its significance can be tested by determining the suitability of the observers for work demanding great accuracy. Accurate assaying can reveal errors in other stages of the dilution method by indicating whether the distribution of the bacteria follows the expected theoretical distribution.

46. Systematical and random errors in the dilution plate method

In the following presentation all possible errors involved in sampling and pre-treatment of samples will be excluded, since they are not significant when studying the reliability of the dilution plate method for determining bacterial density only. In the initial dilution the sample to be studied comprises exactly 10 % of the total volume of the suspension (sample + diluent). All stages of the determination method, from the preparation of the homogenate to the assay of the final sample, involve errors, which must be examined separately at each stage of the whole procedure. Systematical errors which may be due to deficiencies in the technique can be avoided by sufficient care in manipulation. Errors due to chance factors, such as clumping of cells caused by agglutination (Jennison 1937), other mutual phenomena of the bacteria, and erroneous distribution of the cells in the measuring units (Fisher et al. 1922, James and Sutherland 1939, Snyder 1947) appear as deviations from the theoretical distribution. Although these errors cannot completely be eliminated, they can be reduced, for instance, by increasing the number of replicates. A more accurate estimate of the deviation can then also be made. Although the systematical and random errors are caused by different factors, there is, however, a certain correlation between them. Thus, for instance, the distribution of bacteria follows the theoretical distribution only when the homogenization time and concentration of dilutions are properly chosen, under other conditions there is a highly significant deviation.

The effect of systematical and random errors can be studied either separately or together. In the latter case use is made of the combined equation of standard error (cf. Jennison and Wadsworth 1940), for instance, in the following form:

$$T = \sqrt{x_1^2 + x_2^2 + x_3^2 + \cdots r^2}$$

where x_1, x_2, x_3, etc., denote the standard errors due to different systematical factors, such as homogenization time, pipetting, mixing of the dilutions, and colony

counts; r denotes the common standard error due to all random factors, and T the total error. The equation can also assume the form

$$r = T^2 - \left(x_1^2 + x_2^2 + x_3^2 + \cdots x_n^2 \right)$$

in cases where the theoretical standard error due to random factors can be calculated, provided that the standard errors due to systematical factors can be determined. If, for instance, the concentration of the peptone solution used in the preparation of the homogenate is exactly known, but a control determination shows that it has changed in the final suspension by a certain hundredth part, the standard error caused by it is calculated, after which r can be determined when T is known.

If the errors due to systematical factors can be eliminated, the r of random factors is equal to χ^2, or the number of abnormal cases, when the calculated dispersion index values are compared with corresponding theoretical values. The degree of accuracy at which the theoretical distribution is valid can then be observed. If a certain degree of accuracy is desired, use is made of the fact that the variance of the Poisson distribution is equal to the mean, and the number of replicate plates can be calculated, which gives the expected result. This is done by Fisher's (1954) equation

$$\frac{X}{p} = \frac{S^2}{n} \cdot t$$

where p is the desired accuracy in hundredth parts, n the number of replicate plates, and $t = 2$ (t-test).

Summarizing, it may be said that only under conditions where the errors caused by systematical factors can be completely eliminated, can the right theoretical distribution be observed. The larger the effect of systematical factors on the total error grows, the more evident is it that erroneous conclusions are made in interpreting the results as a consequency of the improbable distribution.

5. Discussion

The dilution plate method has been used in a great number of investigations for determining the bacterial density of soil. In none of them, however, has the suitability of the method been systematically studied, and only in very few studies have attempts been made to test the reliability of a certain stage of the method. It can also be noted that attention is very seldom paid to the effect of the composition of the culture medium on the possibilities of the different members of the bacterial population to form colonies on the plates.

The object of the present investigation was to systematically study the usefulness of the dilution plate method for determining the bacterial density in forest humus. Attempts were made, among other things, to determine the degree of accuracy that can be reached in a quantitative investigation of this type and to present new aspects that should be taken into account in the use of the method. In the present work the density of only a part of the bacterial population of the humus was determined, since the culture medium used (Lochhead and Thexton 1952) does not allow the growth of even all the main types of humus bacteria. The bacterial population of the humus is much more complicated than what can grow on the plates; thus, for instance, autotrophic bacteria and certain specific bacteria, such as the species carrying out nitrification, cannot grow on the plates, not to speak of the anaerobic bacteria. This fact, has, however, no marked significance, since the aim of the present study was not to determine the total bacterial density of the humus. A selective culture medium can considerably reduce the disadvantageous relations between different members of a mixed population, which can be considered an advantage from the quantitative methodological point of view.

The problems of the investigation were set up and the material collected in such a manner that the solutions could be reached by statistical methods. A colony on the plate, formed from one or several bacterial cells, was the basic counting unit. When the numbers of colonies on replicate plates from the same sample were distributed according to the theoretical Poisson distribution, it indicated that they represented the same population (Fisher 1954, Stearman 1955). The comparison of two or more series like this was done either with the aid of the *t*-test or by applying single or multiple factor analysis of variance. The conclusions were drawn with the aid of tests showing the significance of differences.

The collection of a representative sample for qualitative studies of the bacteria from forest humus is even more difficult than from cultivated soil. In the literature on sampling, which mainly includes data from cultivated soil, various investigators have presented divergent methods without examining more closely their mathematical basis. Waksman (1931) took only a single random sample or a sample consisting of 3—4 subsamples from different spots. It may be that a random sample taken from a restricted area and controlled in a special way would meet the requirements of methodological study, at least in respect of the time of sampling. However, the experiments made proved that a random sample loses its significance altogether, when the bacterial densities of two or more objects are compared, when we start from the assumption that in all cases the same species of bacteria have formed colonies on the plates. The sampling method of Jensen (1934) is based on at least 20 systematically collected subsamples and that of Harmsen (1940) on 25—100 random subsamples. These two investigators differ as to whether the bacterial density should be determined separately for each subsample and a common mean calculated for them, as Jensen did, or whether it should be determined from a combined sample, as Harmsen did. The results of the present study indicated indisputably that a combined sample consisting of at least 25 systematically taken subsamples could with a sufficient statistical reliability show the bacterial density of the humus under study.

Gross variations in the homogeneity of the humus can be eliminited by careful homogenization (Harmsen 1940, Paarlahti and Hanioja 1962). By this procedure attempts are made to ensure uniformity of bacterial density in all parts of the sample. This requires, however, a very thorough knowledge of the characteristics of the sample under treatment, because there is a correlation between the quality of the sample and its bacterial population. Concerning nutritive factors only, it is a fact that favorable nutritive conditions (Cobb 1932, Vandecaveye and Katznelson 1938, Chase and Baker 1954, Rovira 1956) and above all the amount of available nitrogen (Lochhead and Thexton 1947, Glathe et al. 1954, Payne et al. 1957) increase bacterial density and promote the aggregation of the organisms to one another and to the soil particles. The estimation of these factors, based on empirical studies as the most reliable method, must be continuously made when determining such factors as the degree and time of homogenization.

The homogenization time is directly correlated with the content of mineral nutrients of the humus. When it increases, the homogenization time has to be increased in the same proportion. However, homogenization cannot be continued indefinitely, not nearly as long as 1—1½ hours, which Harmsen (1940) recommends. Dry homogenization is not advisable, since it has an injurious effect on bacterial cells. When the sample was homogenized in a 0.1% peptone solution with the Bühler homogenizer, whose noise on the higher frequency bands does not injure the bacteria, the maximum counts were obtained after 20, 45, or 90 seconds,

depending on the type of humus, and with the shaker after 10 minutes, irrespective of the type of humus. The latter time, 10 minutes, can be regarded as the maximum homogenization time, after which the numbers of humus bacteria under all conditions start to decrease due to injurious effects of external factors. The former values, 20, 45, and 90 seconds, on the other hand, indicate that the more rapid and effective the procedure is, the more reliable are the results; this was also proved by the mathematical analysis.

A homogenization procedure giving a perfect bacterial suspension would, of course, be an optimum starting point in the study of the dilution plate method as a bacterial density determination method. In spite of the theoretical significance of these data — since, when estimating their value, the mutual relations of the bacteria must always be taken into account (Snyder 1947, Jones and Mollison 1948, Monod 1949) — they have, in addition, the drawback that they do not reveal the possibilities the bacterial cells have to form colonies on the plates.

It has been demonstrated beyond doubt that the question of the suitability of the diluent as an indicator of bacterial density is not necessarily solved by the fact that the number of colonies per plate lies within a certain range. The most important factor is that the composition of the diluent and the degree of dilution are such that the viability of the humus bacteria is maintained during the time needed for completing the different stages of the method. Suitable diluents are 0.1—0.01 % peptone solutions, which Straka and Stokes (1957), among others, recommend and 0.5 % soil extract, which Gyllenberg and Eklund [1958 (1959)] and Schalin (1961) have used. The former was slightly more advantageous because of its smaller surface tension. Pure water, on the other hand, proved a completely unsuitable diluent for density determinations of humus bacteria.

In order that the bacterial cells retain their viability in the diluent, it is necessary that the numbers of colonies per plate are distributed according to the theoretical distribution. In the present investigation the optimum number of bacterial colonies per plate was found to be 85—325, which somewhat differs from the 200—400 suggested by Wilson (1922) and 30—300 which Clark et al. (1951) recommend. The numbers presented by these investigators can be considered suitable only under conditions where the homogenization and the diluent are the most advantageous for the quality of the sample and its bacterial population.

The optimum bacterial colony number per plate, 85—325, was obtained with the two diluents mentioned when the dilution degree of the homogenate was within a relatively narrow range. The most advantageous degree of dilution, which in the present work proved to be $2.5 \times 10-4$, must be found experimentally; that is, the degree of dilution at which the dispersion index indicating the distribution of the bacterial colonies on different plates of the same series deviates least from the theoretical Fisher's (1954) dispersion index. A stronger dilution than the theoretical gives a smaller bacterial count and a weaker degree a higher count than the optimum degree would give. When the optimum degree of dilution is found,

correction coefficients can be calculated by which the results can be corrected for all the dilution degrees where the expected theoretical distribution can still be observed. The value of the correlation coefficient is 1.55—1.65 and 0.73—0.78 for dilution degrees $10-4$ and $5 \times 10-4$, respectively, where the distribution of the colonies meets the latter requirement. A prerequisite for accurate and comparable results is, however, that no measuring errors occur in the preparation of the homogenate or the adjusting of the dilution degree.

An attempt was made to reveal and, as far as possible, to eliminate the disadvantages of the dilution plate method when used for determining the bacterial density of the same layer of forest humus. The drawbacks of the method are of two types, part of them depending on systematical and part on random factors. When these factors act simultaneously — which is common — and possibly also in different directions, they may conceal the phenomenon to be studied so completely that its real nature cannot be determined by any method. Systematical experiments were carried out in order to separately study the errors caused by systematical factors due to deficiencies in homogenization, composition of diluent, degree of dilution, and assays of colonies, to test their significance (equation in section 46), and to decrease their undesirable effects. In this respect the goal set up for this work was reached. The accuracy of the results is finally determined, however, by the errors due to random, mainly unknown factors such as the behavior of bacteria under conditions which differ from their natural environment, the inaccuracy of the measuring equipment used, the method of working of the investigator and the persons assaying the colonies, and their criticism and state of mental alertness; their effects can only be estimated with a certain degree of probability. The more the observations differ from the theoretical distribution under optimum conditions, the larger is the proportion of the total effect of random factors.

The results of the investigation carried out indicate that the dilution plate method is suited for the determination of the density of aerobic bacteria in forest humus. However, the improvements of the various stages of the method presented in this work — from the sampling to the treatment of the results — must be taken into account in order that the reliability of the method could be tested by statistical methods. The fact that the selective culture medium used made it possible to determine the bacterial density of only a certain part of the mixed population of the humus does not, however, decrease the significance of the method, since in this respect all culture mediums are in the same position. The total determination of the rich bacterial population of the humus is a difficult problem, and knowledge of how large a proportion of the total population the bacteria growing on the plate represents may facilitate its solution. Side by side with the dilution plate method the comparative microscopic method may lead the way to a complete picture of the bacterial density in the humus layers of forests. Through these data and by extending the scope of investigation to include also qualitative studies the solution of practical problems can be undertaken.

6. Summary

On the basis of the results obtained in the present investigation, the following procedure can be recommended for quantitatively determining aerobic bacteria in the humus by the dilution plate method.

1. The sample is collected by taking systematically from at least 25 different points subsamples of equal size, which are combined and mixed. The samples must be treated immediately after collection. The moisture of the combined sample should be kept the same as that of its natural environment until the preparation of the dilution. Furthermore, the temperature should be kept constant until the actual bacterial suspension is prepared, i.e. the same as in the humus at the time of sampling.

2. The sample is homogenized with the Bühler homogenizer, which is constructed so that a certain degree of asepsis can be maintained, that the speed of the homogenization can be regulated causing no changes in the temperature, and that the noise caused by the apparatus, especially the high frequency sounds, can be measured. In case other homogenizers than the Bühler apparatus are used, they must meet the same requirements. This is essential, since homogenization is the most important methodical stage in the treatment of the humus samples.

3. The content of mineral nutrients of the sample must be determined when choosing the homogenization time in order to obtain maximum results, that is, highest numbers of colonies per plate. For the Bühler apparatus the homogenization time can be taken directly from Figure 3 (section 42), when the content of mineral nutrients of the sample is known, but when other devices are used, the equation for the regression line which indicates the correlation between the homogenization time and the content of mineral nutrients of the sample must first be deduced from results of empirical experiments.

4. The sample is diluted with either 0.1—0.01 % peptone solution or 0.5 % soil extract. The most advantageous degrees of dilution are obtained by testing with the aid of F i s h e r's dispersion index the probability of the Poisson distribution in the results. The dilution from the initial dilution (1:10) is made stage by stage and each stage is mixed by the aspiration-gravity method. The final suspension is mixed by the same method with the culture medium.

5. The growth conditions, that is, the composition of the culture medium and the incubation time and temperature, are standardized.

6. The counting of colonies should be done by at least two persons in order to eliminate systematical counting errors.

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