On the Use of Tris (Hydroxymethyl) Aminomethane as a Diluent in Quantitative Analyses of Soil Bacteria

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TRIS(HYDROXYMETHYL)
AMINOMETHANE AS A DILUENT IN
QUANTITATIVE ANALYSES OF SOIL BACTERIA

by

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INTRODUCTION

Many investigations have been conducted in the field of soil microbiology during the past six decades. Most notable among the contributors is S. A. Waksman whose voluminous works have established many of the procedures used in microbial analyses of soil. Yet, the one fact that is foremost to any reviewer of the literature covering this field is that no standardization of techniques for soil analyses exists. The following presentation deals with one method used to estimate the number of bacteria and actinomycetes in soil.

The use of Tris(hydroxymethyl)aminomethane (hereafter referred to as "Tris") is not new. Tris currently is used in many laboratories as a buffer in tissue culture systems. In 1960, however, Ohlsson (16) showed that Tris increased coliform indices when employed as the diluent in testing water by the multiple-tube dilution method. Ohlsson hypothesized that Tris possesses dispersion properties and a "protective colloid" effect on attenuated coliforms. Since random distribution of organisms is essential in the plating of soil microbes for quantitative studies, the writer felt that Tris might increase the dependability of results when employed as the diluent in replicate platings of soil bacteria and actinomycetes.

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EXPERIMENTAL DESIGN

The soil selected for study was obtained from a beech-maple forest floor at Cooper's Glen, NW$\frac{1}{4}$, S27, T1S, R11W, Kalamazoo County, Michigan. Several soil samples were collected from the same location on earlier dates in a preliminary investigation of the microbial population and of the use of various diluents. The comparison of diluents presented within this paper was obtained from soil samples taken on August 23, 1962, and plated out within four hours after collection.

Four samples were taken from the selected site by forcing a sterile inverted test tube into the soil to a depth of two inches. Three of the resulting cores were intermixed with a flamed spatula in a sterile container in order to obtain a representative sample, according to the techniques suggested by Waksman (23). From this composite sample four, 10-gram samples were placed in sterile containers and were subsequently used in the four diluents to be compared. From the remaining core, 10 grams were oven-dried to a constant weight at 100°C. in order to determine the moisture content of the soil plated. Final calculation of numbers of colonies of bacteria and actinomycetes was determined upon grams of wet soil as suggested by Hiltner and Störmer (11) and Waksman (23). The moisture content of the soil sampled was found to be 34.4 per cent. This data is presented for re-evaluation of numbers found per gram of dry soil although such a procedure is discouraged.

This investigator believes that any practice similar to that of Northrup (15) who determines quantitative counts on the basis of numbers of bacteria per gram of dry soil, is unjustified. Soil moisture content is known to affect microbial numbers. However, no correction factor, or any linear or inverse relationship are currently known to exist between bacterial numbers and the moisture content of soil. The practice of adding a proportional number of bacteria theoretically to be found in a constant weight dry sample seems inappropriate.

Four diluents were prepared for comparison of their ability to disperse microbes in a soil sample. All of the diluents were made up in standard dilution blanks, autoclaved at 15 lbs. pressure for 20 minutes, and stored at 3^{0}C. until used. The constituents for each diluent are listed below.

Tap water blanks:

Ordinary tap water from Kalamazoo city water supply.

Distilled water blanks:

Kalamazoo city water distilled in a Barnstead water still.

Saline Solution blanks:

USCP NaCl, 8.50 grams per liter of distilled water.

Tris blanks:

USCP Tris(hydroxymethyl)aminomethane, 6.0 grams per liter of distilled water.

The medium suggested by Waksman and Fred (21), was composed of the following substances.

K_2HPO_4	0.5 gm.
$MgSO_4$	0.2 gm.
Dextrose	1.0 gm.
Sodium Caseinate	1.0 gm.
$Fe_2(SO_4)_3$	trace
Agar	15.0 gm.
Distilled HOH	1000.0 ml.

All of the ingredients were dissolved in boiling distilled water. The resulting medium was filtered through filter paper in a steam bath at 100^oC. The medium was autoclaved in flasks for 20 minutes at 15 lbs. pressure, and stored at 3^oC. until used. The final pH of the medium was adjusted to 6.5.

Each set of diluents was warmed to 45^oC. in a water bath. Five grams of soil was added to 100 ml. of each diluent to be tested and the blanks shaken by hand 25 times to begin dispersion of the soil. Each of the blanks was then placed on a mechanical shaker and shaken at 200 strokes per minute for five minutes.

Of the .05 dilution, 1 ml. was transferred by a sterile pipette (1/1000 graduations) to 99 ml. of each of the succeeding diluents until a dilution of .000005 or 1:500,000 was reached. The transfers were carried out while the diluents were in motion on the shaker.

This procedure insures the transfer of a representative sample of
the suspension of microbes in the diluent.

Upon reaching the final dilution, the blanks were removed from
the shaker and 1 ml. was transferred by sterile pipette into each
of ten plastic petri dishes. The blanks were vigorously shaken be-
fore each sample was withdrawn.

Sodium caseinate agar was maintained at 45°C. in a water bath.
Upon completion of the transfer from each dilution blank, agar was
added to the petri dishes. The dishes were then agitated lightly
by hand to produce a uniform distribution of any organisms. Control
plates lacking the soil suspension were run on the media and on each
diluent.

Upon solidification of the medium, the plates were inverted
and placed in an incubator at a temperature of 25°C. (±2°). Incu-
bation was carried on for seven days according to the standardized
technique of soil plating proposed by Waksman (23). All colonies
except fungi were recorded at seven days without any attempt to
differentiate between bacteria and actinomycetes. Standard error
was calculated from standard deviation, using a computational form-
ula (Form II) from Hammond and Householder (10).

Plates containing less than 30 colonies or more than 200 colo-
nies were eliminated from the study as suggested by Breed and
Dotherrer (4) except in the case of plates containing microbes

which had been suspended in distilled water. This exception was necessary due to the probable inhibitory effect of distilled water on microbes. Plates overgrown with fungi were eliminated as suggested by Waksman (23).

DISCUSSION AND RESULTS

Statistical treatments of replicate platings have been proposed by Fisher, et al (9), James and Sutherland (12, 13), Sundara, et al (19), Waksman (22), and others. Factors such as incubation temperature, medium filtration temperature, antagonistic colony development, symbiotic relationships, autoclaving time, pH of the medium, and the soil used are known to affect the results of quantitative studies of a bacterial population. Cuthbert, et al (7), clearly demonstrated the inability of cooperating laboratories to arrive at similar results on identical soil samples. Wieringa (24) presents the problems involved in standardizing methods. However, if standardization of some procedures takes place in the future, quantitative analyses may provide a valuable tool in assaying the microbial condition of a given area.

Conn (5), Conn and Conn (6), Thornton (20), Waksman (23), and others have considered synthetic media. While soil extract agar has gained in popularity and currently is used in many laboratories, it was not used in this study because its composition is dependent on the kind of soil added to the agar. Casein agar has been found by Waksman (23) to show increased counts over albumen agar, soil extract agar, sodium asparaginate agar, and urea nitrate agar. It was, therefore, employed in this study.

Diluents have been considered to a lesser degree in soil microbiology. Waksman (23) found no advantage in using .0085 NaCl solution

instead of tap water. It might be noted, however, that city tap
waters differ. Hiltner and Störmer (11) found that a mixture of
.004 NaCl and .004 KCl was injurous to soil bacteria. Kühlmorgen-
Hille (14) suggested the use of a one per cent peptone solution.
Recently, Damirgi, et al (8), have noted that chemical dispersing
agents such as Na_2CO_3 or $Na_4P_2O_7$.10HOH give increased counts when
added to NaCl in proportions of .002 NaCl to .0005 Na_2CO_3. In 1960
Ohlsson (16) suggested that Tris possesses dispersion properties
due to its ability to lower surface tension. With this property
in mind it seemed probable that Tris would exhibit dispersion pro-
perties which would be of value in quantitative determination of
soil microbes. Preliminary investigations seemed to support this
hypothesis.

As indicated in Table I, Tris possesses dispersion character-
istics which separate aggregates of bacteria known to occur in soil.

Table I

Combined counts of colonies of bacteria
and actinomycetes from ten replicates
for each diluent.

Diluent	Mean	Standard Error	Range
Distilled Water	29.1	±1.74	17-36
Kalamazoo Tap Water	38.2	±2.06	30-53
Saline	45.6	±1.46	31-52
Tris	66.9	±2.88	51-87

This property is important. The plate method of quantitative determinations is only accurate when each developing colony results from the multiplication of one microbe. Orcut (17, 18) has pointed out that deflocculation with sodium chloride increases counts by preventing retention of bacteria within soil aggregates. Tris possessed greater ability to bring about this deflocculation according to the present study.

It should be noted that Tris promotes increased numbers of organisms upon prolonged contact with coliforms. However, Tris was not in contact with the soil suspension for longer than 20 minutes. This period is considered to be the minimal time required for bacterial multiplication under optimum conditions. Thus, bacterial multiplication was not responsible for increased colony counts.

Sterile saline solution (.0085 NaCl) was a better diluent than Kalamazoo tap water or distilled water. This finding is similar to the results of Bhat (2) and in contrast to those of Waksman (23).

CONCLUSIONS

The analysis of microbial populations in soil is incumbered with difficulties, not the least of which arise from techniques of plating out the organisms so that a single colony in a culture plate is the result of multiplication of a single microbe. If the results of such studies are to be comparable, some standardization of procedures must be arrived at. Northrup (15), Waksman (23), and Augier and LaVergene (1) have presented suggestions for the standardization of techniques; but Bonazzi (3) fears that any such standardization would decrease the probability of identifying all the kinds of microbes in the soil plated. Such fears merit consideration for qualitative analyses but not for quantitative determinations. A standardized procedure and the use of different media will eliminate this problem.

One step in the direction of standardization is the use of a diluent that can be easily prepared and stored. Tris has been found to possess these attributes. It also possesses the ability to give increased colony counts and thus more accurate estimates of the soil's microbial population. This study indicates that at a concentration of .006, Tris has the best dispersion, antigen-antibody blocking mechanism, or detergent properties upon aggregates of bacteria and actinomycetes in soil samples. A significantly higher colony count was noted over sterile tap water, sterile saline solution, or sterile distilled water.

SUMMARY

The purpose of this study was to determine the value of a .006 concentration of Tris(hydroxymethyl)aminomethane as a diluent in the quantitative determination of soil bacteria by the plating method. Three other diluents, sterile distilled water, sterile tap water, and .0085 sterile NaCl solution, were used as comparative agents.

The method employed was to plate soil from a beech-maple forest and to count the resulting colonies of bacteria and actino-mycetes which formed after a seven-day incubation at $25^{O}C.$ $(\pm 2^{O})$. Statistical treatment was used to compare the number of colonies of microbes produced on replicate platings of soil suspended in four different diluents.

Tris(hydroxymethyl)aminomethane possesses better dispersion ability than the other diluents used, and it is suggested that Tris be considered as a standard diluent in the assay of microbial populations of soils.

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