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Title: COUNTING OF SOIL MOLDS BY MICROPLATING

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Since microplating for enumeration of bacteria in richly seeded materials has been satisfactorily employed by previous investigators, experiments were carried out to evaluate this procedure for counting molds in soils and to compare results obtained using routine pour plates. A variety of soils were employed in order to have statistical evaluation of results covering a large range in total counts by each method.

Effects on mold counts of the pesticides DDT (p-dichlorodiphenyltrichloroethane), 2,4,5-T (2,4,5-trichlorophenoxyacetic acid), 2,4-D (2,4-dichlorophenoxyacetic acid), and aldrin (a dimethanonapthalene) were studied. It was found that in concentrations of 100 p.p.m., all but 2,4,5-T show significant increases over control soils for the one week test period. At concentrations of 10,000 p.p.m., a hundredfold increase in concentration of recommended rates of application, definite inhibitory action was noted for 2,4-D and 2,4,5-T. Aldrin increased numbers of molds, showing similar effects at 100 and 10,000 p.p.m.. DDT gave a temporary increase in molds which in the case of 10,000 p.p.m. diminished to the level of the control after one week.

It is concluded that microplating is an acceptable procedure for counting molds in soil over a large range of counts. Insofar as effects on mold counts are concerned, it is also concluded that when used at recommended rates of application, there is little chance for pesticidal compounds to adversely affect mold development and their influence on decomposition of organic matter in soil.

COUNTING OF SOIL MOLDS BY MICROPLATING

by

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Typed by Jack Roberts

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INTRODUCTION

The term "mold" as used in this investigation refers to any of the Fungi growing as a loose meshwork of mycelium rather than a dense tissue. Molds may belong to any class of higher Fungi but the majority are either Phycomycetes or Fungi imperfecti. "Fungi of soil represent a numerous group of organisms found in all the soils studied in numbers large enough to warrant a conclusion that they probably play an important part in the fertility of the soil (30,p. 154)." That molds, having been shown to be concerned with ammonification, nitrogen fixation, decomposition of complex organic materials including cellulose and lignin, are important to soil fertility is no longer questioned (33,p. 244). Soil bacteriologists are concerned with determination of numbers and activities of molds as well as of bacteria and Streptomyces in relation to soil fertility.

A variety of techniques have been developed for enumeration of bacteria in soil while demonstration of molds has been limited mainly to two general methods, i.e. methods which demonstrate presence of molds in soil with no regard for relative occurence of spores or vegetative mycelia and methods which attempt to reveal active growth stages of molds by examination of soil for vegetative mycelium. The former techmique is routinely used for quantitative determinations and includes spores as well as vegetative mycelial fragments. It involves use of dilution plates using an agar medium which is either acidified or treated with some antibacterial agent such as rose bengal

in order to inhibit bacterial development.

Several reports have indicated that microplating has been successful for enumeration of bacteria (9,pp.889-890) 22,pp.163-168) and 7,pp.105-108). A search of literature has revealed no published report of an attempt to learn if microplating is applicable in estimating mold numbers in soil. The purpose of the present research is to investigate the applicability of microplating for estimation of total mold numbers in soil, comparing results obtained by microplating with those obtained by routine plating procedures. Soil treatments with selected pesticides were also investigated in order to show any stimulative or depressive effects of these compounds on mold counts.

Considerable controversy exists concerning the value of plate counts for determining vegetative or active molds in soil in contrast to their spores, which have little immediate physiological activity. Certain practical advantages are realized by microplating which may make this procedure more desirable for mold counts used as an index of soil activities or of effects of soil treatments. The value of this method for counting molds is in savings of time, plating expense and incubator space. While various drawbacks to this method are unavoidable, it was hoped that its use for mold counts would find application in studies where the main interest is total numbers of soil molds rather than in specific groups or species.

HISTORICAL

Since 1860, which marks the approximate beginning of soil microbology, it has been known that soil is teeming with microscopic life. Although the ecological, experimental, agronomical and pedological phases of soil microbology have received much attention (34,p.4), the ecological subdivision involving quantitative determination of microbial numbers has been intensively studied with reference to development of new techniques and to validity of any given technique. The literature pertaining to methods of enumeration of microorganisms has been presented in an extensive review by Jonik (18, pp. 20-34). Briefly these may be classified as follows:

Total count procedures

Nephelometric procedures

Cell volume measurements

Dye absorbtion techniques

Direct microscopic counts

Viable count methods

Dilution and plating procedures

Estimation of microbial numbers by measurement of by-products

Differential staining of living and dead cells

In a review of factors concerning plate counts, Brierley, Jenson and Brierley (4, pp. 48-71) have listed as important variables sampling, suspensions, disintegration of soil particles, dilutions, plating, incubation and counting. To these may well be added differences in pipetting, clumping, growth rate and biological effects.

Total count methods are subject to errors, particularly in soil where multitudes of dead cells and soil particles are present. As long ago as 1900 Klein (19, p. 234) used a total count method employing a calibrated loop to smear a known quantity of bacterial suspension on cover glass so that total numbers could be determined from average counts per field multiplied by dilution factors. Essentially this was the precursor of the Breed count for which widespread use was found in the dairy industry. There is no suitable means for distinguishing between living and dead organisms, the criterion of death being lack of power of multiplication. Hawks and James (10, p. 304) have shown in direct smears that the number of organisms decreases in proportion to the distance from the center of the stained film and also that clumping and loss of organisms during staining and washing make this method inaccurate. Jennison (13, p. 476) in comparing plate and direct microscopic counts of Escherichia coli found significant discrepancies between both methods. Jones and Mollison (17, pp, 461-463) claim to have obtained bacterial recovery counts of 95 per cent in soil suspensions when using a soil suspension fixed in agar and stained. Measurements of turbidity, cell volume or dye absorbtion fail to allow either for variations in size, shape and non-viable cells or else for non-specific absorbtion of dye by dead cells.

Of the methods listed by Jonik, it would seem that viable count methods might result in more truly representative results for active microorganisms. With respect to differential staining of living and dead cells, Bickert (1, p. 548) investigated this method with a variety of stains on organisms killed in various manners. The method of

killing was found to be without influence on the staining characteristics of dead cells. He concluded that there is no practical way to differentiate between living and dead cells in the same microscopic preparation. Results of Henrici's method of differential staining (29,p. 102) for counts of viable versus dead organisms are in direct conflict with those of Bickert's. This difference is doubtless due to the fact that Bickert's cells were killed prior to staining while Henrici's cells were stained in vitro.

Estimation of microbial activities and indirectly of their numbers by measurement of their by-products is widely used to illustrate net reactions of organisms either in pure or mixed culture. Due to the constantly changing predominance of organisms involved in the various soil processes, this procedure appears to be of little value for quantitative indications of numbers of any group of soil organisms.

Since 1886 when Adametz (33,p. 221) conducted first attempts to isolate and systematically describe molds isolated from the soil, dilution and plating procedures undoubtedly have been most widely employed for quantitative estimates of molds. This method is based on Koch's assumption that every viable cell, spore or mycelial fragment in the case of molds, when placed in a suitable environment is capable of giving rise to a colony. Of the viable count methods, this seems to be most reasonable for estimation of numbers of a viable group of organisms in any material. In soil, as in the case of other materials, it is true that often only a small fraction of the total viable population is capable of developing on any medium of given

composition. That fraction however, if it can be determined with reasonable accuracy does furnish the investigator with positive data so that the number of unknown and uncontrollable variables in a given study may be minimal. Following various soil treatments significant changes in the microbial population are frequently encountered. Accurate counting techniques enable maximum possible correlation of soil changes with quantitative microbial differences. Waksman (31, p. 565) has indicated that soils of higher fertility generally contain more molds than less fertile soils. In spite of certain drawbacks, the merit of plating procedures is indicated by widespread use of such methods by bacteriologists for comparison of different samples.

Esmarch (18,p. 28) introduced the roll tube method in an attempt to simplify and economize plating procedures for bacterial counts. Subsequent studies on this method by Wilson (36,p. 444) indicate that a somewhat greater accuracy is possible with roll tubes than with the agar plate counts. Frost (9,pp. 889-890) developed the little plate technique for enumeration of viable bacteria in richly seeded materials.

Lewis reports on the suitability of the Hiltner-Stormer method for enumeration of fluorescent bacteria and Ziegler (38,pp. 609-634) has found good correlation between plate counts and dilution counts for Escherichia coli. The possibility of applying the Hiltner-Stormer dilution method to enumeration of molds is, as far as can be determined, still to be investigated.

Use of the Chlodny slide technique (5,p. 620) is valuable for demonstration of changes in predominating soil flora. Jensen (14,

p. 153) has used Chlodny's method for quantitative estimation of mold vegetative development by calculation of the percentage of microscopic fields showing mycelia or hyphae. He believes that plate counts for molds are valid only for the number of mold spores in the soil. Conn (6,p. 3), in examining smears of soil microscopically, reports the absence of mold mycelia except in those soils having a high organic matter content. He also states that in the case of molds and actinomycetes the plate count is indicative of the spore producing ability rather than activity. The significance of plate counts cannot be determined for any spore-forming organism unless microscopic observation is used in conjunction with plate counts to show whether the plate count represents vegetative molds or merely spore abundance.

A number of media are available for counting molds in soil (31,p. 568). Peptone-glucose agar adjusted to pH 4 has been widely used as the acidity of this medium restricts most bacteria and Streptomyces thus permitting lower dilution plating for molds. Martin (21,p. 231) has reported use of rose bengal plus streptomycin for obtaining more representative counts since this medium is approximately neutral and permits many forms of mold to develop which would not do so at a lower pH. He has obtained increases in mold counts by as much as 100 per cent. Smith (26,p. 470) reported that higher counts were obtained by using rose bengal to inhibit spreading of mold colonies.

The little plate technique for counting microorganisms was first introduced by Frost in 1916 (9,pp. 889-890). In an elaborate comparison of standard plate counts, lactose agar plate counts, little plate

technique, reduction tests and direct microscopic counts, Simmons (25, pp. 322-336) found close correspondence between little plate and standard plate methods for bacterial counts in milk thereby substantiating Frost's earlier work. Nickerson (22,pp. 163-168), Estabrooks and Bollen (7,pp. 105-108), and Briedigan and Chang (3,pp. 931-933) devised applications of the little plate method obtaining a precision of the same order as that of standard plate counts.

EXPERIMENTAL METHODS

Soils used. For a comparative study of total counts obtained by pour plate and microplating methods a group of six widely different soil samples were selected to give a variety in numbers and kinds of molds. Soil samples used in these comparative studies were:

1. Walla Walla silt loam
2. Olympic clay loam
3. Webster silty clay loam
4. Klamath peat
5. Chehalis silty clay loam
6. Umapine clay loam (salt grass knoll)

All samples used were passed through a ten or twenty mesh screen and mixed prior to use. In preliminary investigations no attempt was made to estimate absolute numbers of molds since information concerning only relative results by the methods was desired. In studies on treated soils absolute rather than relative changes in mold population was desired and counts were determined on a water-free basis.

To study effects of pesticides on mold counts the following procedure was used. Twenty-five grams of soil (water-free basis) were placed in sterile, cotton-plugged bottles after the desired amount of pesticide had been mixed with the soil on paper. One bottle each of the control and treated soil was prepared for each day that a sample was desired in order to obtain minimal sampling error and to prevent disruption of mold growth among soil particles by sampling a larger single quantity of soil at various intervals.

Medium. Preliminary use of acidified peptone glucose agar (PGA)

which is one of several recommended by Waksman (31,p. 570) for mold counts, was highly unsatisfactory due to the hygroscopic character of the agar film on micro plates. The staining solution caused microfilms to wrinkle and frequently wash from the slide. A medium found to be satisfactory for microplating was found to be the following:

Bacto-beef extract	3 grams
Bacto peptone	5 grams
Glucose	20 grams
Agar	15 grams
Distilled water	1000 ml.

This medium, glucose nutrient agar (GNA), was dispensed in 200 ml. amounts in screw cap bottles and autoclaved. Prior to use, after melting and cooling to 46° C in a water bath, approximately three ml. of 1 N H_2SO_4 was added to each bottle and thoroughly mixed. Amounts of acid required varied from time to time with various batches of medium so that the final pH was about 4.

Preparation of dilutions. Since comparative counts only were desired in preliminary studies, dilutions were made on the basis of soil without regard to moisture content. One gram of soil was added to a 99 ml. water dilution blank. This was shaken for ten minutes on a reciprocating shaker having a frequency of 135 cycles per minute. The dilution was considered as 1:100. Sediment was allowed to settle for five minutes and serial dilutions using ten ml. aliquots into 90 ml. sterile water blanks were made. Each subsequent dilution was shaken 100 times by hand and permitted to settle five minutes before the next dilution or plating. Insofar as practicable, dilutions were

made so that between ten and one hundred colonies would develop on each micro plate.

Plating and drying. For the comparative phase of this work, petri plates were made using one ml. of a given dilution and melted agar was added and mixed by rotation twenty times clockwise and counterclockwise. Micro plates were made by delivering 0.1 ml. of a soil suspension ten times more concentrated than that used for petri plates. This micro aliquot was placed on a microscope slide which had been previously cleaned and soaked in 95 per cent ethanol, flamed just prior to use and placed on an electric hotplate warmed to 46° C. A 0.2 ml. pipette graduated in tenths of a milliliter was employed measuring from the 0.2 to 0.1 ml. marks. Two 0.1 ml. aliquots were delivered at terminal portions of a slide. Two or three drops of acidified melted agar medium were added dropwise to the aliquots and mixing was accomplished by use of an inoculating needle having a flat tip bent at a thirty degree angle. The plated material was mixed by both vertical and horizontal streaking so that the final microfilm is approximately 1.5 to 2.0 centimeters square. Neither total area nor mixing of the microfilm is critical since the entire area is to be counted. After plating, the slide was placed in a moist chamber made by using a six inch petri dish three quarters of an inch deep into which the lid of a four inch petri plate was placed for holding three microscope slides. Five ml. sterile water was added to the "moat". In this manner six microfilms may be incubated in a single moist chamber. All incubations were at 30° C for 24 to 30 hours after which time growth was halted by drying.

Drying was accomplished by placing incubated micro plates on an electric hot plate warmed to approximately 85° C. This temperature should be controlled within relatively narrow limits; if much higher there is a tendency for bubbles to develop in the agar which makes counting more tedious, while if a much lower temperature is used the films have a greater tendency to come off during staining.

Staining and counting. Micro slides were stained by immersing for two or three seconds into a solution of one per cent phloxine and one per cent fast green in a five per cent aqueous solution of phenol. Following this, the slide was rinsed in a beaker of fresh tap water two or three times and the slides were placed in a slide box until dry. This stain colors the colonies blue to dark purple. The background is very pale. Slides were marked on the back with a wax pencil.

It was found that the colonies could be most readily and accurately counted by using a binocular microscope with the low power objective (100 X). A mechanical stage is essential for complete and systematic coverage of the entire film area. Micro colonies were recorded by means of a hand counter to keep personal error minimal. The entire area is "scanned" systematically much in the same fashion as T.B. slides are observed. To avoid missing any colonies or counting any areas twice, the mechanical stage was used to move the field up just the width of the microscopic field by picking out some particle at the bottom of the field and moving it just to the edge of the top of the field after each horizontal traverse. Approximately three to five minutes were required to scan each film, depending on the

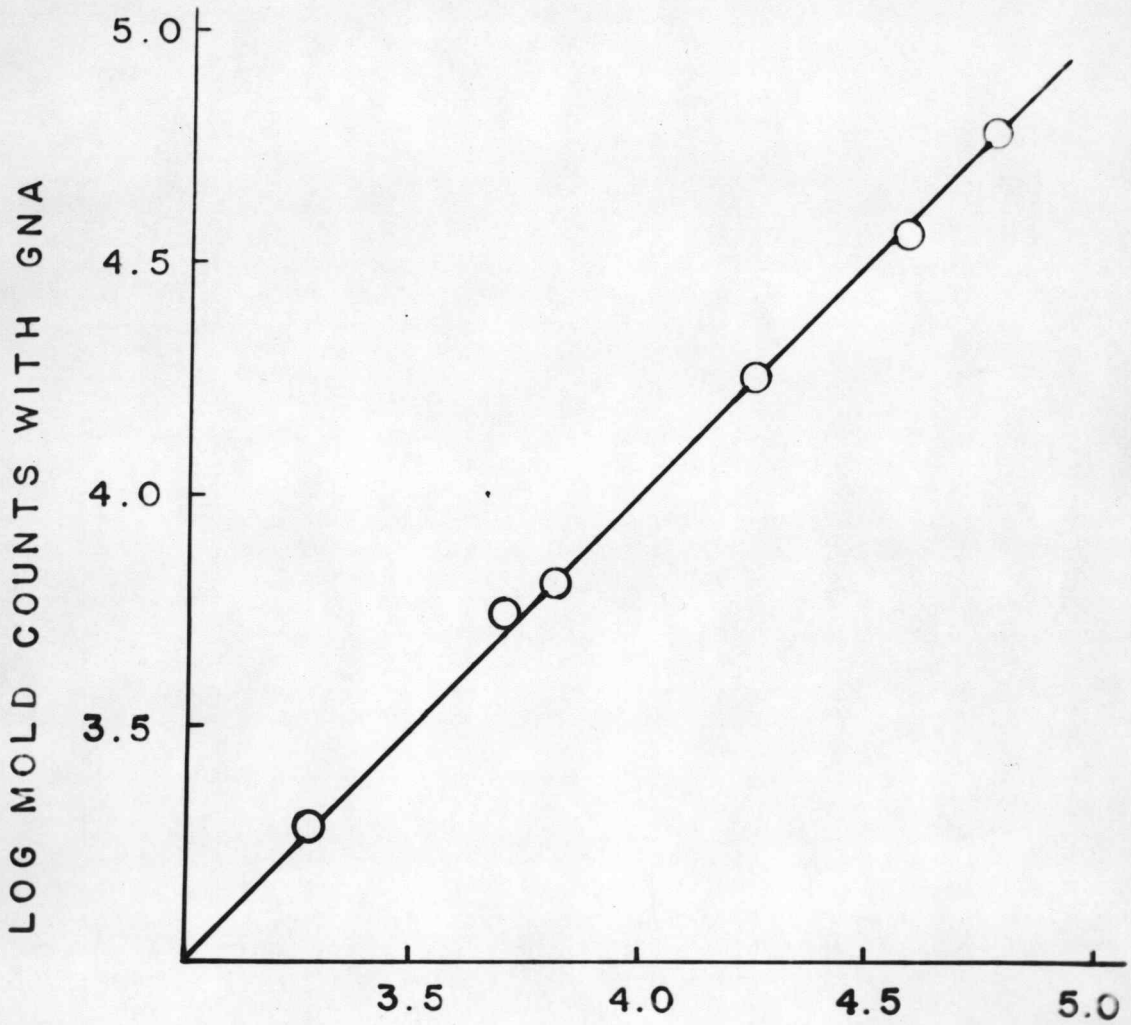
number of colonies present.

EXPERIMENTAL RESULTS

Comparison of media. A series of conventional dilution plates were poured using six different soil samples for comparison. For each soil, ten replicate plates were made with both media at suitable dilutions in order to compare ability of GNA in supporting mold growth and giving counts comparable to those obtained using Waksman's generally accepted PGA at pH 4. Figure 1 (p. 14) indicates the linearity of counts obtained with each medium and justifies use of the acidified GNA in place of PGA.

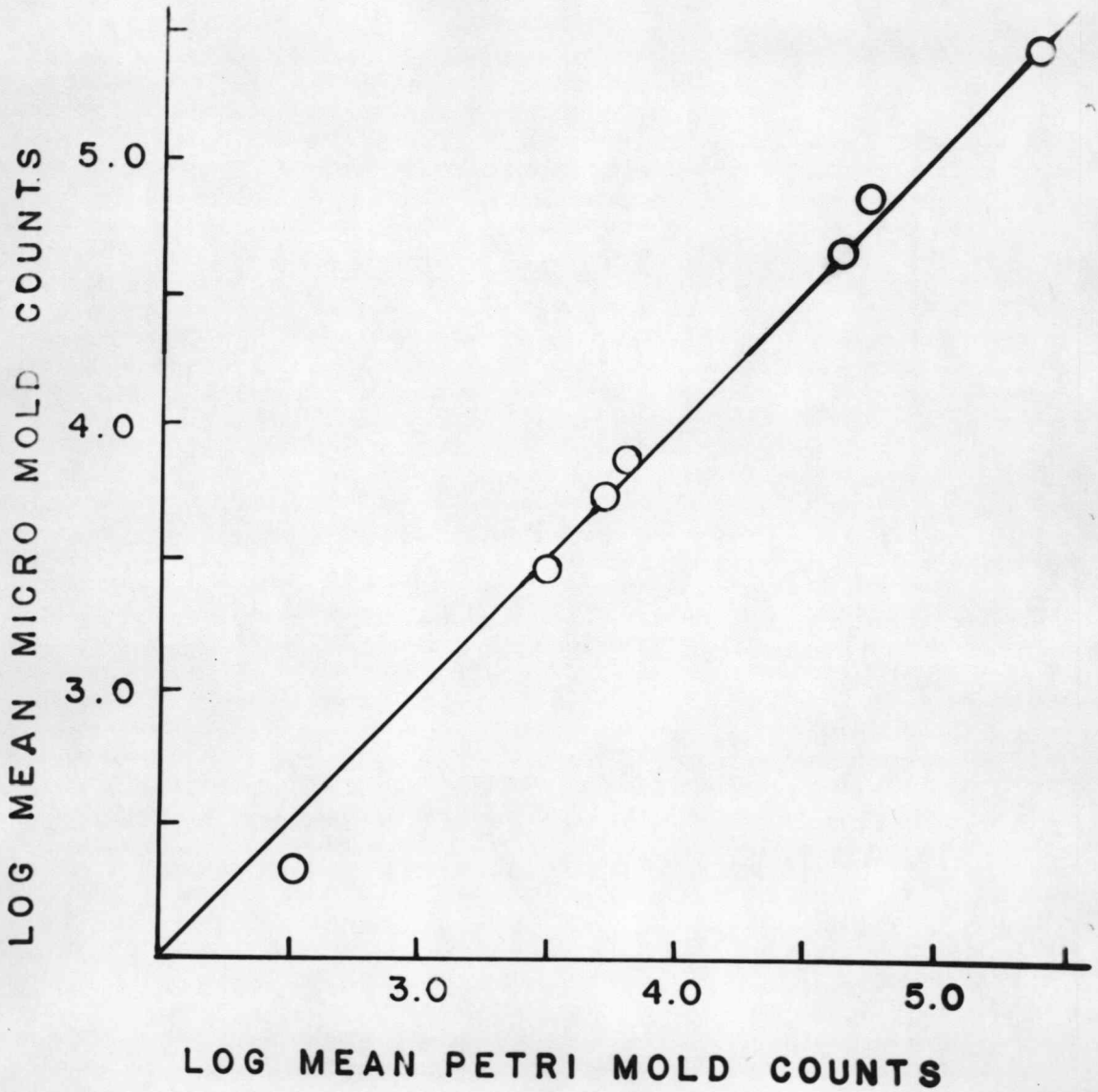
Petri counts versus micro counts. Six different soil samples plus one of the samples which had been moistened and incubated six days to give higher counts were compared with respect to total mold counts determined both by microplating and standard plating procedures. Figure 2 (p. 14) illustrates results of this comparison for various soils. Each point on the curve represents composite averages from five separate samplings of the same soil. Averages were obtained from a total of thirty micro counts and thirty petri counts for each soil. Over a range of total counts from a few hundred to almost one half-million the quantitative relationship between the methods approaches linearity. This indicates a correlation between the methods sufficient to justify use of microplating in place of pour plates.

Effects of pesticides as determined by microplating. Application of four commercially available pesticides were used on Chehalis silty



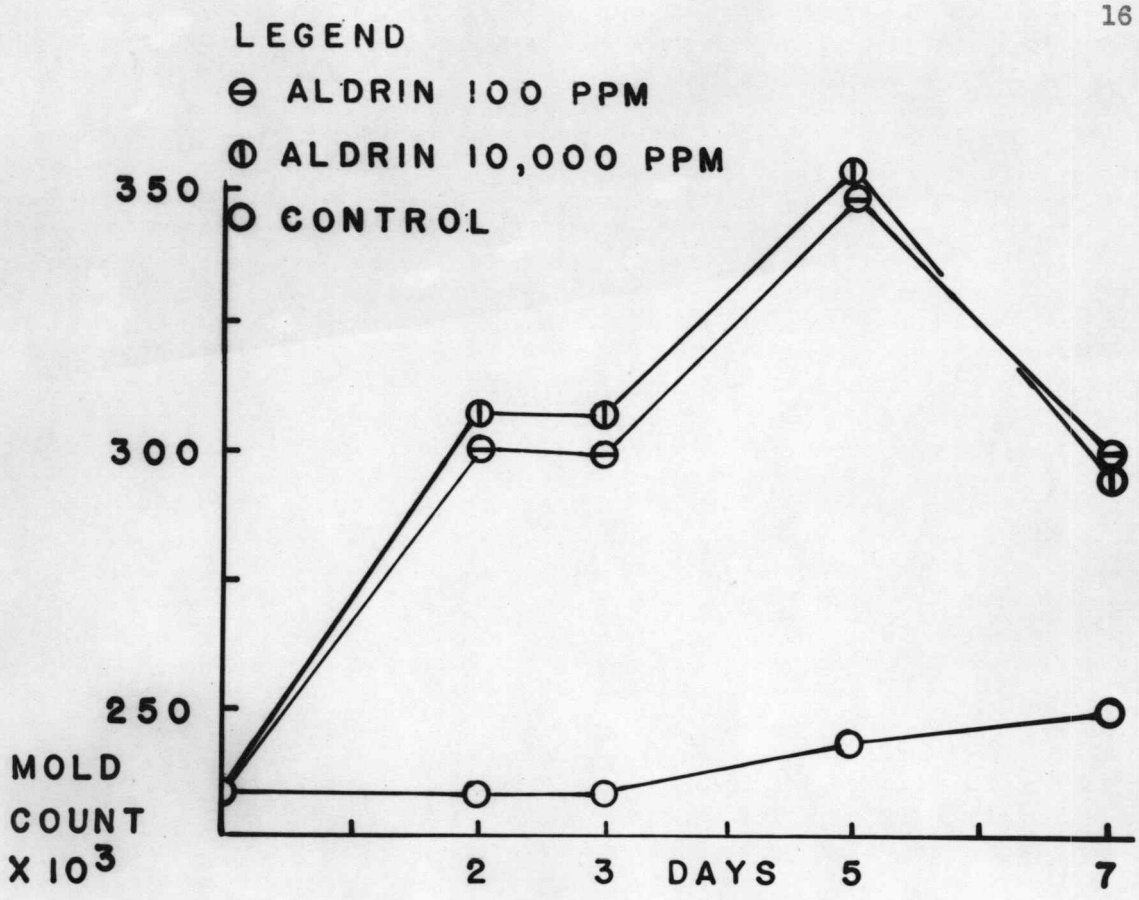
COMPARATIVE COUNTS WITH PGA AND GNA

FIGURE 1



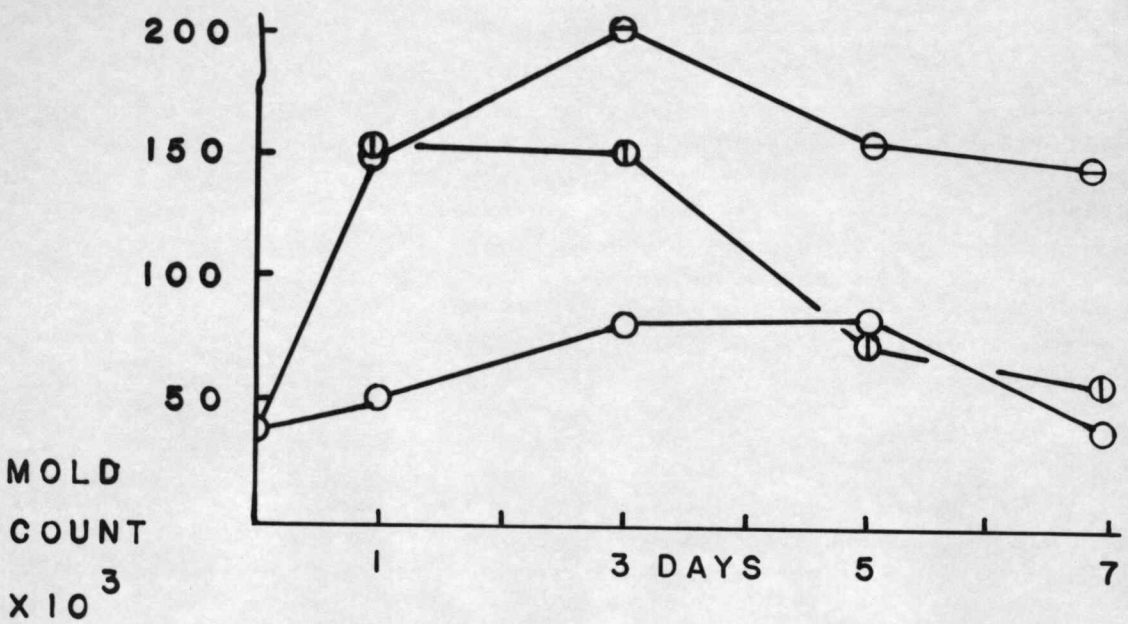
AVERAGE MICRO COUNTS VERSUS AVERAGE PETRI COUNTS

FIGURE 2



ALDRIN EFFECTS ON MOLD COUNTS BY MICROPLATING

FIGURE 3



DDT EFFECTS ON MOLD COUNTS BY MICROPLATING

FIGURE 4

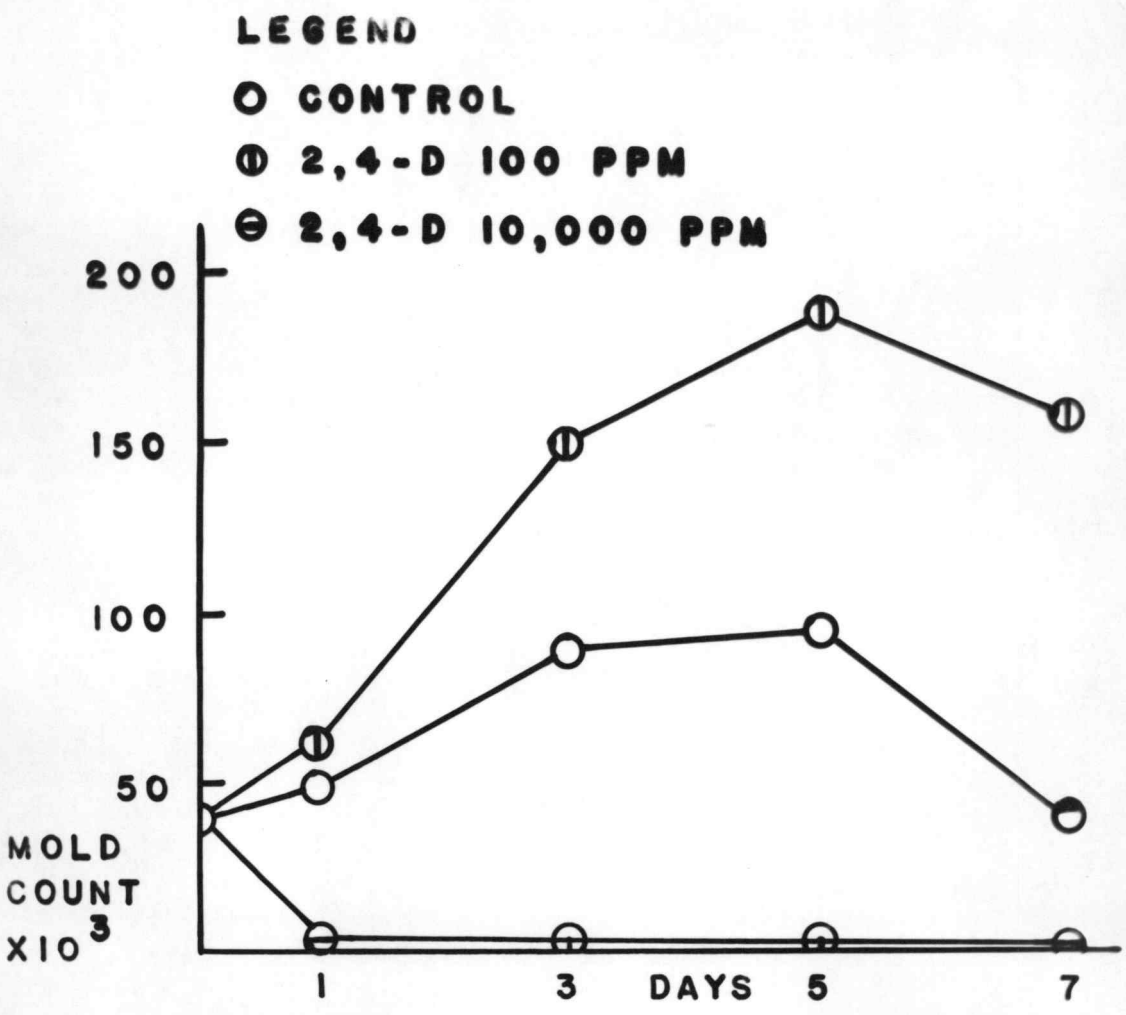
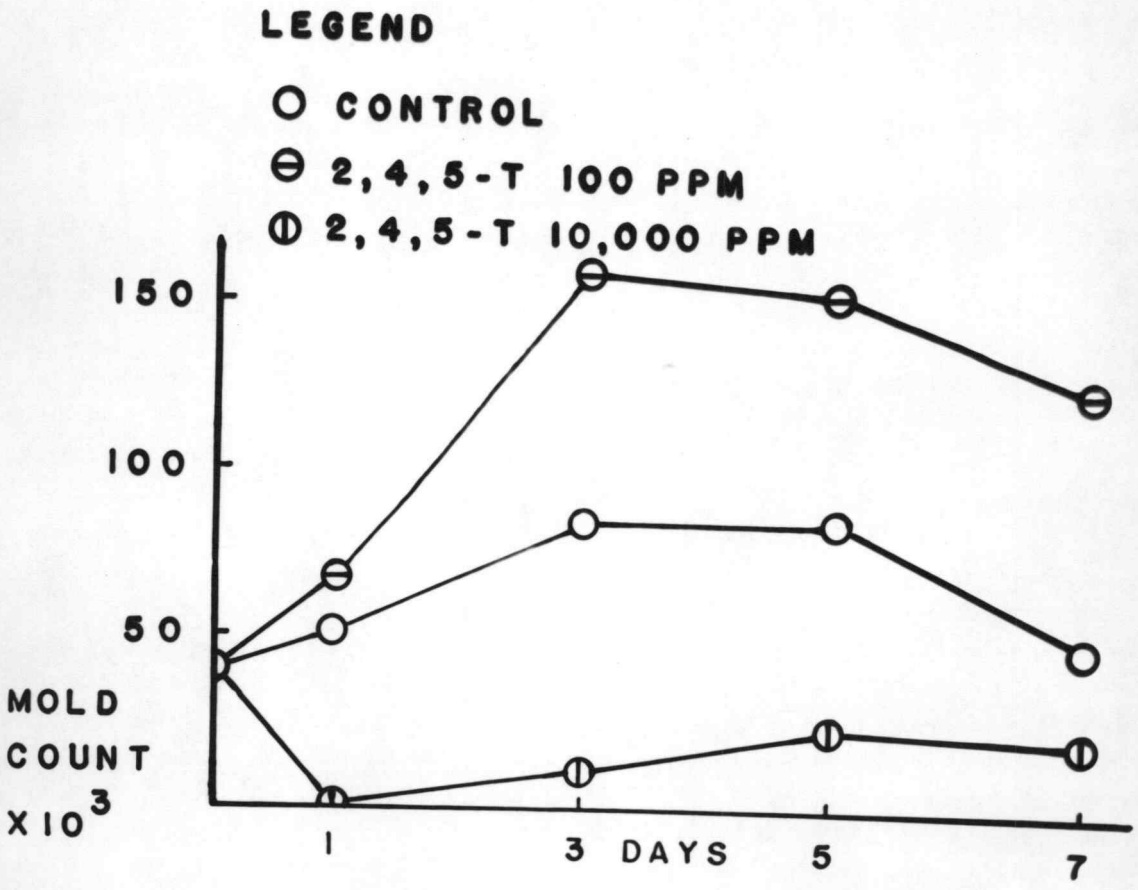


FIGURE 5:

2,4-D EFFECTS ON MOLD COUNTS BY MICROPLATING



2,4,5-T EFFECTS ON MOLD COUNTS BY MICROPLATING

FIGURE 6

clay loam in order to detect any stimulative or depressive effects on mold counts as determined by microplating. DDT (p-dichlorodiphenyl trichloroethane), 2,4,5-T (2,4,5 trichlorophenoxyacetic acid), 2,4-D 2,4 dichlorophenoxyacetic acid) and aldrin (a dimethanonaphthalene) were applied at rates of 100 p.p.m. (parts per million) and 10,000 p.p.m. Mold counts were determined at several intervals during seven days incubation at 30° C. Application rates abnormally high compared to recommended rates were used to obtain "forced" results as well as to allow for possible cumulative effects. Figures 3,4,5 and 6 (pp. 16,17,18 and 19) indicate effects of these compounds on mold development as determined by total counts from microplating. In studying pesticidal effects on mold counts only Chehalis silty clay loam soil was used. This was a bulk sample collected in March, 1953. At 100 p.p.m. 2,4-D, aldrin, DDT and 2,4,5-T all showed stimulative effects. Treatments approximately at 100 or more times in excess of recommended levels, i.e. at 10,000 p.p.m., had definite inhibitory action in the case of 2,4,5-T and 2,4-D while DDT causes a temporary increase followed by a drop in numbers to the level of the control soil. Aldrin applied at 10,000 p.p.m. had the same effect as when applied at 100 p.p.m.

Effect of DDT. At a concentration of 100 p.p.m. this compound caused increases in mold numbers over the control soil of approximately 200 per cent. Added to soil at 10,000 p.p.m. DDT brought about an increase in total numbers which lasted for three days after which time total counts dropped to the control level. Jones (16,pp. 237-241) has indicated overall stimulation of microbial numbers due to

DDT applied at recommended rates. He found great stability of this compound during its first year in soil, more rapid decomposition occuring in soils receiving lower rates of application.

Effect of 2,4-D. Stimulation of mold numbers continues until the fifth day with treatments of 100 p.p.m. after which time there is diminishing mold numbers found in parallel with the control soil. Addition of 10,000 p.p.m. 2,4-D results in drastic, sustained reduction of numbers for the entire test period. Johns (15,p.45) in treating a soil with 100 and 1000 p.p.m. of 2,4,5-T and DDT found definite increases in mold numbers. In the case of 2,4,5-T the counts declined after forty days to a level below that of the control.

Effect of aldrin. Figure 3 (p. 16) showing increases in total mold counts indicates little difference in counts obtained for soil treatments with 100 and 10,000 p.p.m. Treatment with 10,000 p.p.m. shows slight increases in total mold numbers through the fifth day of incubation after which effects of treatment diminish.

Effect of 2,4,5-T. Increases in mold numbers comparable with increases obtained with the other compounds were obtained at a concentration of 100 p.p.m. A concentration of 10,000 p.p.m., like 2,4-D, resulted in sustained reduction in numbers for the period of testing.

STATISTICAL ANALYSIS

Evaluation of any technique devised for determination of total numbers of organisms present in a sample is most credible when it has undergone reasonable statistical analysis and a satisfactory correlation between the new and standard method is indicated. Application of proven mathematical formulae to test accuracy of results needs no justification. The number and possible effects of variable factors which cannot be controlled requires use of every test which will substantiate validity of results. Since the bacteriologist makes estimates of soil microbes for the purpose of comparing the estimate to that obtained with some specific treatment or condition of soil, an estimate can have practical value only if it is shown to be reasonably accurate and reproducible.

In a statistical study by Sutherland (28,pp. 305-312) it was shown that the mean of four replicates of plate counts was reliable as an estimate of population viable on the medium used in the dilution plated. In averaging bacterial counts statistically, Robertson (24, p. 133) concluded that the geometric mean is a more nearly correct average than an arithmetic mean over a normal distribution of bacterial counts in market milk. The arithmetic mean when applied to actual values in an exponential increase for determining the true average is unreliable.

According to Waksman (32,p. 94) the "weight" attached to a given estimation of numbers of microorganisms in any sample is determined by taking the log of plate numbers used for the estimation times the number of samples used. This is a measure of relative importance to

be attached to any set of plates. He evaluates a "weight" of less than one as worthless, one to two as fair, two to three as good, three to five as very good and higher than five as very reliable. According to this criterion, "weight" to be applied as indicated in table 1 (p. 24) is 3.5 or very good. That is, a minimum of five plates are averaged for each soil. The log of five is 0.7. Five samplings were made from each soil and five times 0.7 equals 3.5.

Statistical analysis of results shown in figure 2 (p. 15) indicates in table 2 (p. 28) that avera ge variance in counts encountered by pour plates is significantly greater than variances met with micro plate counts. Coefficients of variation generally were found to vary inversely with the number of colonies present on a given set of plates.

Certain biological factors must be considered in evaluation of the two methods. When more than 50 or 60 colonies per plate are present, there is a tendency for micro counts to be higher. Probably the reason for this is that in the three or four days incubation required for petri plates to be countable there is some obliteration of closely located colonies. In micro plates, since growth is halted in twenty-four hours, obliteration of colonies by overgrowth is kept down so that more representative counts are possible. In cases where soil is low in molds, micro counts average lower than petri counts. Explanation of this phenomenon may be that the nature of predominant mold numbers in soil with low counts is such that sufficient development doesn't occur in 24 hours for them to be counted or else that further shaking required for preparation of subsequent dilutions for pour plates results in sufficient additional fragmentation of viable mold

Table 1

COEFFICIENTS OF VARIATION

Soil	sample	Dilution	Actual counts	Mean	S^2	Coeff. var.
5	ap	1:1000	47,61,65,64,52, 55,66,60,65,59	59.4	39.8	0.11
	am	1:1000	80,80,84,87,92, 94,96,97,98,70	87.8	85.1	0.11
	bp	1:1000	74,77,50,59,73	66.6	134	0.17
	bm	1:1000	79,81.84,87,92	84.6	26.3	0.06
	cp	1:1000	64,68,65,54,49	60.0	65.5	0.14
	cm	1:1000	78,80,83,88,90	83.8	26.3	0.06
	dp	1:1000	53,75,64,61,72	65.0	82.5	0.14
	dm	1:1000	60,65,73,78,80	71.2	72.8	0.12
	ep	1:1000	61,66,35,58,71	58.2	192	0.24
	em	1:1000	67,70,77,80,82	75.2	41.5	0.09
1	ap	1:500	6,4,3,4,5,2,5, 7,4,7	4.7	2.7	0.35
	am	1:500	3,3,3,3,4,4,5, 5,5,5	4.0	0.9	0.24
	bp	1:500	4,10,5,12,7	7.6	11.3	0.44
	bm	1:500	3,3,4,4,7	4.2	2.8	0.40
	cp	1:500	3,7,9,7,5	6.2	5.3	0.37
	cm	1:500	4,4,4,4,7	4.6	1.8	0.29
	dp	1:500	5,6,6,5,7	5.8	0.8	0.15
	dm	1:500	5,5,6,6,8	6.0	1.5	0.21
	ep	1:500	3,5,4,4,10	5.2	7.8	0.54

TABLE 1 (continued)

Soil sample	Dilution	Actual counts	Mean	S^2	Coeff. var.
em	1:500	4,5,5,5,12	6.2	10.8	0.53
3(inc.)ap	1:10,000	37,44,43,37,40, 41,30,34,40,45	39.1	21.8	0.12
am	1:10,000	37,38,42,30,39, 38,42,39,36,42	38.3	13.2	0.09
bp	1:10,000	35,35,37,36,36	35.8	0.8	0.01
bm	1:10,000	33,31,38,39,30	34.2	1.9	0.04
cp	1:10,000	44,44,34,37,30	37.8	38.3	0.16
cm	1:10,000	37,31,41,30,35	34.8	21.0	0.13
dp	1:10,000	36,46,39,38,31	38.0	29.5	0.14
dm	1:10,000	41,42,42,38,34	39.4	11.7	0.09
ep	1:10,000	38,33,43,42,43	39.8	18.7	0.11
em	1:10,000	42,35,37,33,41	37.6	14.7	0.10
4 ap	1:500	1,1,1,0,0,1,2, 1,2,2	1.1	0.5	0.67
am	1:500	0,1,0,1,1,1,1, 1,0,0	0.6	0.3	0.86
bp	1:500	1,1,0,0,1	0.6	0.3	0.91
bm	1:500	1,0,1,0,0	0.4	0.3	1.37
cp	1:500	2,1,0,0,1	0.8	0.7	1.04
cm	1:500	1,0,2,0,1	0.8	0.7	1.04
dp	1:500	1,0,0,1,0	0.4	0.30	1.37
dm	1:500	0,1,0,0,0	0.2	0.20	2.23
ep	1:500	1,1,1,0,0	0.6	0.3	0.91

TABLE 1 (continued)

Soil	sample	Dilution	Actual counts	Mean	S^2	Coeff. var.
	em	1:500	1,1,0,0,0	0.4	0.3	1.37
6	ap	1:500	20,14,10,14,24, 15,13,17,13,11	15.1	17.8	0.28
	am	1:500	14,27,16,18,14, 25,18,19,21,20	19.2	18.4	0.22
	bp	1:500	12,20,15,10,11	13.6	16.2	0.30
	bm	1:500	22,15,18,10,20	17.0	22.0	0.28
	cp	1:500	14,12,14,17,13	14.0	3.5	0.13
	cm	1.500	18,7,14,18,10	13.4	23.8	0.36
	dp	1:500	12,11,18,15,16	14.2	7.8	0.20
	dm	1:500	11,13,11,14,13	12.4	1.8	0.11
	ep	1:500	11,16,13,19,18	15.4	11.3	0.22
	em	1:500	10,8,14,17,9	11.6	14.2	0.33
3	ap	1:1000	3,1,3,4,7,10, 10,7,6,7	5.8	9.1	0.52
	am	1:1000	12,11,10,4,5, 6,6,5,12,4	7.5	11.1	0.44
	bp	1:1000	8,14,9,4,16	10.2	23.3	0.47
	bm	1:1000	14,9,12,8,16	11.8	11.3	0.29
	cp	1:1000	10,10,7,11,15	10.6	8.3	0.27
	cm	1:1000	5,5,5,8,9	6.4	3.8	0.30
	dp	1:1000	3,8,6,5,9	6.2	5.8	0.39
	dm	1:1000	9,6,12,5,6	7.6	8.3	0.38
	ep	1:1000	6,6,2,8,3	5.0	6.0	0.49

TABLE 1 (continued)

Soil sample	Dilution	Actual counts	Mean	S^2	Coeff. var.
em	1:1000	6,11,5,6,11	7.8	8.8	0.38
2 ap	1:1000	41,57,46,35,45, 50,49,43,40,37	44.3	43.3	0.15
am	1:1000	49,50,52,52,53, 53,54,55,56,57	53.1	6.3	0.05
bp	1:1000	56,61,70,64,74	65.0	51.0	0.11
bm	1:1000	60,62,67,73,74	67.2	39.8	0.09
cp	1:1000	68,80,73,75,72	73.6	19.3	0.06
cm	1:1000	74,75,77,80,88	78.8	31.8	0.07
dp	1:1000	43,45,40,46,57	46.2	41.8	0.14
dm	1:1000	56,57,58,61,60	58.4	4.3	0.04
ep	1:1000	80,92,92,78,103	89.0	104	0.12
em	1:1000	75,82,83,85,85	82.0	17.0	0.05

Numbers for soil samples refer to soil used (p. 9)

ap and am refer to samples of the same soil and p and m refer to petri and micro counts

Actual counts refers to actual number of colonies found on plates

Mean equals average count

S^2 equals variance

Coeff. var. equa ls coefficient of variation

TABLE 2

AVERAGE VARIANCE OF PETRI AND MICRO PLATE COUNTS

Soil sample	S^2 Petri counts	S^2 Micro counts	F
5	102.9	50.4	2.04
1	5.5	3.6	1.53
4	0.43	0.35	1.23
6	11.3	16.0	0.71
3	10.5	8.6	1.22
2	51.9	19.8	2.62°
3 (incubated)	21.8	12.5	1.74
Average S^2	29.19	15.89	1.84°

Ratio of average standard deviations is 1.36 (significantly greater than 1.00 at one per cent level)

S^2 - variance

F - ratio of variances

° - indicates ratios of variance significantly greater for petri counts

segments to raise the resultant total count.

DISCUSSION

Comparison of media. Data which resulted in linear relationship between PGA, an acceptable mold counting medium, and the GNA used for microplating of molds indicates quite clearly the justification for use of GNA (figure 1,p. 14). There is little doubt that a straight line relationship exists as might well have been shown by a comprehensive statistical analysis of counts from a large number of soils. Comparison of merits of the two media in supporting mold growth indicates that one is just as acceptable as the other for total count determinations.

Occasionally colonies of certain acid tolerant bacteria appeared on micro plates. The reason for their appearance is possibly due to the fact that acidified medium is diluted by mixing 0.1 ml. of plated suspension with two drops of melted medium. The number of these colonies was never large and no difficulty was ever encountered by confusing them with mold colonies.

Use of acidified agar remelted more than two or three times results in microfilms which disintegrate upon contact with the staining solution. In addition to this defect, the bacterial colonies become more numerous following hydrolysis and disintegration of the agar. For convenience, when micro plates only are to be made, it would be handy to have the medium dispensed in ten or twenty ml. amounts so that unused portions could be discarded without significant material losses. Reheating of acidified medium is thus avoided.

Petri counts compared with micro counts. Figure 2 (p. 15) shows satisfactory agreement between microplating and petri counts for a wide range in total numbers. Use of dilutions less than 1:100 for microplating were not practicable due to excessively large numbers of soil particles present in such suspensions. In comparing the two methods, total petri count plates were counted at 48 and 72 hours incubation. The higher number of colonies found was used for comparative purposes. While colony morphology was not clear at the time of counting, better agreement between the methods of counting was possible since spreaders, especially mucors and Trichoderma, do not have an opportunity to cover up smaller colonies during shorter periods of incubation. A 24 to 30 hour incubation time was found to be most practical for the micro plate mold counts. This time period is desirable from the standpoint of avoiding overgrowth by spreaders, which reduce the apparent total counts, and also from the more practical standpoint of fitting this procedure into the laboratory working day. Micro plates made in the morning and placed in the incubator about ten A.M., may be dried, stained, and counted the next day.

Apparent abnormal distributions are due mainly to large spreading colonies of representatives of the Mucorales. Such colonies completely cover the micro film in a few hours thereby preventing enumeration of other colonies overgrown. James and Sutherland (12, pp.72-85) justify exclusion of such counts from a given set of plates. Such exclusion was routinely practiced whenever micro plates were overgrown to the extent that accurate counting was impossible.

When the number of colonies on a given series of plates averages

above approximately sixty, there is a trend for micro counts to average somewhat higher than petri counts as shown in figure 2 (p. 15). It is considered that this tendency is due to colonies masking each other to some extent on petri plates whereas stopping growth in 24 hours in the microplating method permits more accurate counts to be made from the standpoint of viable mold units in the soil suspension.

Limitations of microplating. Since growth is halted at 24 hours, total counts only are possible; differential characteristics are not sufficiently well developed to allow enumeration of represented genera. It is also impossible to isolate any colonies which might appear to be of interest. Drying and staining of micro films involve techniques not required in the petri plating procedure. More or less eyestrain is involved with prolonged use of the counting method described. Attempts to use the wide field binocular microscope with a total magnification of 10 X, as employed by Estabrooks (7,p. 105) did not yield as satisfactory or reproducible counts as did use of the standard field binocular microscope with a low power objective (100 X).

Advantages of microplating. A considerable saving in the time required for obtaining results is possible by microplating. Results may be obtained in slightly over 24 hours as compared to the three or four days required for pour plates. One hundred ml. of medium is sufficient to pour about five petri plates while the same amount of medium is adequate for making 500 or more micro films. A considerable saving in expense may be realized particularly if large numbers of plates are routinely made. Since microscope slides rather than petri plates are used to make micro plates, cost in glassware and breakage is considerably less. Larger petri plates used for moist chambers

contain the equivalent of six petri counts thereby reducing handling of plates and consumption of incubator space. Micro counts permit counting of small vegetative fragments before being obliterated by spreaders. Once dried, micro plates need not be counted until convenient. A permanent collection of various molds developing in early stages may be made if desired to show early developmental differences, or the slides may be filed as permanent records.

Microplating applied to the determination of effects of pesticides on mold counts. DDT added to soil kept in storage is very stable in the first year but by the second or third year considerable decomposition occurs (16,pp. 237-240). Greater breakdown occurs in soils high in organic matter as well as in soils in which lower concentrations of DDT are employed. As DDT has depressant effects on mold counts when used at excessively high concentrations, the possibility of cumulative effects should not be disregarded since other important groups of soil organisms may be affected in an adverse manner similar to molds. Jones has shown that nitrogen-fixing bacteria are not hindered by concentrations of DDT up to one per cent. Using recommended rates of application an overall stimulation of total numbers of microorganisms as determined by pour plates was noted. Johns (15, p. 45) found similar increases in mold counts in response to DDT. Molds were implicated in possible breakdown of certain pesticides by Johns when he noted mold growth in broth containing the pesticide as the sole carbon source.

Stimulation of soil bacteria as well as molds has been reported (15,p. 45). Mold counts levelled off to counts below the control soil after 40 days incubation. Increases in numbers of molds in soil

treated with 2,4,5-T and 2,4-D have been shown by microplating (figures 5 and 6, pp. 18 and 19).

Aldrin concentration, as determined by a specific chemical method, has been shown to decompose by 50 per cent in 20 days under optimum laboratory conditions. In the field, however, its insecticidal effectiveness persists for more than one year (8, p. 62). Since it is essentially insoluble in water, the possibility of cumulative effects from repeated applications must be considered. As indicated in figure 3 (p. 16) aldrin at 10,000 p.p.m. has no significantly different effect than 100 p.p.m. on total mold numbers. The compound apparently is not soluble enough to create adverse effects. In concordance with results reported by Fletcher, an increase in mold counts was found in this study with application rates of both 100 and 10,000 p.p.m. aldrin.

Two main ideas may be developed concerning aldrin effects on microorganisms. The first is that due to its low solubility (0.05 p.p.m.), growth differences in mold counts at application rates of 100 and 10,000 p.p.m. are limited. The second is that in spite of its relative insolubility, application of 10,000 p.p.m. aldrin permits a far greater number of physical contacts between microorganisms and aldrin particles. The importance of physical contact is shown by Fletcher in shake cultures of certain molds. Water content in actively growing mold hyphae is relatively high and an aldrin saturated cell liquid might conceivably result. These considerations remain to be determined by future investigators.

A common trend for mold counts in the soil tested with pesticides at 100 p.p.m. is an increase in total counts followed by a

decrease which varies in rate and extent with compounds used. This phenomenon is difficult to explain since increases in counts are probably the result of an increased spore production. In earlier stages of mold development, vegetative mycelium as well as spores probably contribute to total counts. If a compound has latent toxic effects, vegetative growth would be eliminated leaving only viable spores to contribute to total counts; the per cent of viable spores produced might also decrease in time. Another possible explanation might be that a compound when first attacked results in growth stimulating substances while subsequent degredations may result in formation of either toxic or inactive materials. Moreover, count decreases after "peak" counts have been attained may follow death of mold spores at rates proportional to toxicity of compounds involved, or may be the normal decrease that follows sooner or later under any cultural growth cycle.

CONCLUSIONS

Microplating may be used instead of the conventional pour plate procedure for determinations of total mold counts in soil. Microplating has the advantages of more rapid results, lower cost, economy of space, convenience in counting and less variation in replicate counts. Its disadvantages are possible eyestrain, lack of ability to determine differential counts, and inability to isolate colonies. An accuracy favorably comparable to that obtained with pour plates has been obtained. Microplating is applicable for detecting changes in soil mold counts as affected by pesticidal treatments.

Applications of 2,4-D, DDT, 2,4,5-T and aldrin at 100 p.p.m. result in increases of total mold counts over the control in Chehalis silty clay loam soil. At recommended rates of application, it seems unlikely that any of the compounds tested could exert any harmful effects on soil fertility insofar as mold populations may be concerned. The temporary increases in mold numbers due to soil treatments could benefit plant growth by furnishing additional ammonia nitrogen due to increased decomposition of soil organic matter by accelerated mold action.

SUMMARY

Microplating was applied to counting of molds in soil and satisfactory correlation has been found between counts obtained by pour plates and micro plates. The more rapid and less expensive use of microplating for this purpose is thus justified.

Application of microplating has been used to determine effects of pesticidal soil treatments on mold counts. The conclusion was reached that when applied at recommended rates, the pesticides tested would affect soil fertility to a negligible degree insofar as the mold population is concerned.

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