AN EVALUATION OF THE THEORY AND USE OF THE ACRIDINE ORANGE STAIN IN ENUMERATING BACTERIAL POPULATIONS

by

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LITERATURE REVIEW AND INTRODUCTION

Enumeration of aquatic bacterial populations has been a subject of concern and interest to microbiologists and ecologists for many years. While numerous techniques have been developed and are still in use, each has one or more inherent weakness(es), as well as advantage(s). While a thorough review of these methods will not be presented here, a brief discussion of the more widely used methods does seem relevant.

One popular method is that of viable plate counts. With such a method, Sorokin and Kadota (1972) have stressed only a small percentage of the total microbial population can be enumerated generally from a heterogenous system using standard culture techniques; i.e., viable pour plates and viable spread plates. Obviously success in plate count techniques depends upon the ability of every naturally occurring bacterium to replicate on the selected culture media and under the laboratory conditions provided. A major drawback in these techniques stems from the fact that many species require extrinsic and exotic environmental conditions for growth, which cannot be duplicated by common culture procedures.

Total direct counts (viable and dead microorganisms) using counting chambers and stained membrane filters constitute a second category of methods for enumeration. For such, however, one should have a known population of 10^7 counts/ml to pragmatically satisfy the use of bacterial counting chambers, since there is an intrinsic

factor of 10^7 in the calculations (Wilson, 1922; Wilson and Kullman, 1931). Unfortunately many aquatic systems lack a bacterial standing crop of 10^7 cells/ml. Consequently, some form of concentration technique, such as the use of filters (Razumov, 1932) must be used for the microscopic counting of total bacterial populations. After the water sample is filtered through a 0.45 μm or smaller, such as an 0.22 μm, pore size filter, a stain solution is added, the cells are stained, and the filter is cleared and mounted for counting.

Many variations in viable plate counts and direct counts of bacteria have been used. For further elaboration, the reader is referred to Meynell and Meynell (1970), Rodina (1972) and Sorokin and Kadota (1972).

Electronic particle counters have become popular recently. However their efficiency is low for detection of particles the size of most bacteria; and as the name of the instrument implies, all particulates, inorganic or organic, are counted. Aggregation of bacterial cells should be additionally resolved by direct microscopic counting techniques (Meynell and Meynell, 1970).

The acridine orange epifluorescence technique (Francisco, et al., 1973) is a modification of the stained membrane filter technique employing the basic fluorochrome, acridine orange (AO), and fluorescence microscopy. Under transmitted or incident UV or near UV light it is alleged that AO-stained microorganisms fluoresce green if viable or red if non-viable (Strügger, 1948; Casida, 1962). However, various studies (Korgaonkar and Ranade, 1966; Casida, 1971;

Francisco, et al., 1973) investigating this differential fluorescence, called the Strügger effect (Ranade, et al., 1961) or concentration effect (Aristovskaya and Zyking, 1972) have found discrepancies in attributing green fluorescence to viable cells.

The binding sites of AO are the same as for basic dyes, i.e., on the free acidic groups of the DNA and RNA molecules (Swift, 1955; Pearse, 1960). This binding of the dye is not limited to the nucleic acids, however; other cellular substances with free acidic groups, i.e. acid polysaccharides, will bind the dyes, although at a lower pH (Armstrong, 1956; Schümmelfeder, 1958; Wolf and Aronson, 1961). Further support of this theory is amplified with studies of higher intensities of fluorescence achieved with AO stained cell walls of Gram positive bacteria (Ignatov, et al., 1974).

Bradley and Wolf (1959) and Wolf and Aronson (1961) explained the precise nature of AO binding to RNA and DNA. AO molecules are thought to bind through electrostatic attraction to the phosphate groups of the nucleic acids. If these molecules are separated from each other along the nucleic acid, they then fluoresce green. However, at an optimum pH and optimum ratio of dye molecules to acid binding sites, the planar AO molecules stack up along the nucleic acid. It is in this stacking process that adjacent dye molecules interact by Van der Waal's forces and shift the normally green fluorescing color to red. DNA has a lesser tendency to stack AO molecules than RNA, possibly because of the more rigid structure of the former (Loeser, et al., 1960).

Korgaonkar and Ranade (1966) demonstrated that although a fluorescence shift to red-orange occurred in highly degraded cells, this shift was not obligatorily linked to viability when cells were killed by various physical treatments. In addition, Francisco, et al. (1973) observed that pure cultures of Klebsiella, E. coli, and B. subtilus contained both red and green fluorescing cells before autoclaving, boiling, or the addition of formalin or mercury; all three cultures exhibited green fluorescence only after each physically injurious treatment using an 0.1% concentration of AO. There are other accounts in the literature which allude to the skepticism of the reliability of the Strügger effect in the assessment of microbial viability (Meysel', et al., 1961; Zubzhitskiy, 1964; Bucherer, 1966; Casida, 1971; Meysel', 1971). Cells that fluoresce a yellow-green or yellow have been described as being in a static condition or in a state approaching death but capable of reviving if conditions are ideal for growth (Van Duijn, 1954).

All of the AO compounds possess the property of fluorescence; though diamino acridines, of which AO is a member, are the most active (Darken, 1961). There are specific fluorescences for ribonucleic acid (RNA) and desoxyribonucleic acid (DNA) when associated with AO (Armstrong, 1956; von Bertalanffy and Bickis, 1956). Structures containing DNA, such as nuclear chromatin, exhibit a bright-red color (Armstrong, 1956) when stained with AO. Cellular age has also been reported to influence fluorescence with AO (Naumova, 1960).

An exact definition of microbial viability has been extensively researched by many investigators. Cell death may be attributed to an altered permeability to certain dyes (Knaysi, 1935; Knaysi and Ford, 1938; White, 1947; Strügger, 1948), inability to reduce certain tetrazolium dyes (Greenberg, et al., 1958), or leakage of select labelled purines from labelled populations (Koch, 1959). On the other hand, microbial viability has been measured using viable culture counts, ATP photometry (Holm-Hansen and Booth, 1966), heterotrophic uptake of ^{14}C labelled organics (Hobbie and Crawford, 1969), and uptake of ^{32}P (MacLeod, et al., 1966). The ability of a bacterium to replicate on a suitable isolation medium as a definition of viability is a more popular and pragmatic approach to this problem (Postgate, 1961).

To this date, controversies remain in the literature as to whether or not acridine orange fluorescence can show microbial viability. Bucherer (1966) and Stöckli (1959) claim that the plasma of living Gram-positive bacteria has a higher sorptive capacity than that of Gram-negative bacteria, so that it will fluoresce red when there is an excess of dye. Micrococcus lysodeikticus binds reversibly with an 0.1% concentration of AO; i.e., live cells fluoresce red, whereas dead cells fluoresce green (Beers, 1964). Other studies, as mentioned previously, attribute red fluorescence to cell death due to a concentration and pH effect.

The acridine orange epifluorescence technique (Batoosingh and Anthony, 1971) has recently become widely used by many microbial

ecologists, limnologists and soil ecologists (Batoosingh and Anthony, 1971; Bell and Dutka, 1972; Francisco, et al., 1973; Slattebraek, 1974; Daley and Hobbie, 1975) as a total direct count of microorganisms in natural ecosystems. Counts of green-fluorescing organisms have been closely correlated to viable spread plate counts in the majority of these studies, (Batoosingh and Anthony, 1971; Bell and Dutka, 1972; Francisco, et al., 1973) when applied to pure cultures. Additionally, such direct count techniques are often used as reliable estimates of bacterial biomass in preference to culturing techniques, presumably due to the previously mentioned drawback in the use of culture counts.

The laboratory phase of this study was aimed at ascertaining if AO was a vital fluorochrome; whereas, the field phase was directed toward evaluating the usefulness of the AO stain as an ecological tool in measuring total bacterial biomass. The specific objectives of this research were to: (1) observe the color of fluorescence emission when pure cultures of bacteria grown under laboratory conditions are treated with different concentrations of AO, (2) determine if the color of fluorescence of AO-stained, live and killed bacterial cells could be correlated with viable or dead cells, and (3) correlate the number(s) of AO-stained bacteria with total viable plate counts using oligotrophic water samples of different depths from Mountain Lake, Virginia.

It was hoped that this information could be applied to measurements of bacterial biomass of a variety of oligotrophic aquatic ecosystems such as Lake Bonney in Southern Victorialand, Antarctica.

MATERIALS AND METHODS

PART I. Laboratory Studies on Antarctic Bacterial Isolates T-22 and T-3.

Pure cultures of isolates T-22 and T-3 were obtained from viable spread plates of water samples from Lake Bonney, Antarctica, during the 1973-74 field season (Lane, 1975). T-22 was cultured on peptone-yeast extract glucose-salts media (PYGS) (Appendix 1). Following various biochemical tests, T-22 was identified as a biotype of Pseudomonas fluorescens, and T-3 was placed in the genus Bacillus (Lane, 1975). Both cultures were maintained on PYGS solid media slants at 5 C until 48 hr before use in an experiment, whereupon the bacteria were grown in broth culture in PYGS dilution blanks; after 24 hr of growth, 0.1 ml of the broth culture was transferred into 50 ml of PYGS broth in a 500 ml flask, and the growth flask was placed on a shaker bath incubator (New Brunswick Scientific Co., New Brunswick, N. J.). Culture T-22 was grown at 15 C at a shaker speed of 150 rpm; T-3 was grown at 23 C with a shaker speed of 120 rpm, these conditions being near optimal for growth.

Two methods of AO staining were used for the laboratory studies. The first (Staining Method A) involved centrifugation of AO (1.0 ml)-stained, 24 hr culture (4.0 ml) of the isolate at 4,000 G for 10 min in a 50 ml sterile polypropylene centrifuge tube. The supernatant was decanted, and the resultant pellet washed and resuspended twice with 5.0 ml of filter-sterilized PYGS broth to remove excess stain.

AO (Fisher Scientific Company, C. I. No. 46005) was prepared fresh in deionized water before each experiment and filter-sterilized through a 0.22 μm pore size Type GS filter (Millipore Corp., Bedford, Mass.). The stained suspension was pipetted into a Petroff-Hauser Counting Chamber, and using a #0 coverslip (Corning), counts of 20 arbitrarily selected fields were done whenever possible. Counts were expressed as counts/ml using the calculation,

$$\text{Approximate cells/ml} = N \times (2.0 \times 10^7)d,$$

where N = mean number of cells (counts)/small square,

(2×10^7) = correction factor for 1.0 ml,

d = the dilution factor (Wilson, 1922).

Fluorescence of cells in the counting chamber was observed with an American Optical Company Spencer monocular compound microscope equipped with darkfield condenser, K510 barrier filter, K530 filter, Reichert fluorescence illuminator with HBO 200 Watt L-2 mercury vapor burner, and BG12 and UG1 exciter filters (Figure 1).

The second method of AO staining (Staining Method B) involved filtration of an aliquot of a water sample or axenic culture through a black 0.22 um pore size, SM 13007, membrane filter manufactured by Sartorius (Science Essentials Corp., Anaheim, Ca.)(Daley and Hobbie, 1975). The non-self-fluorescing, cellulose nitrate, black contrast filter was placed between a sintered glass filter apparatus and a 15 ml sterile glass filter tower (Figure 2). One ml of freshly prepared, filter-sterilized acridine orange stain was added to the filter apparatus and allowed to distribute over the black filter surface before the addition of the culture or water sample. An aliquot of the

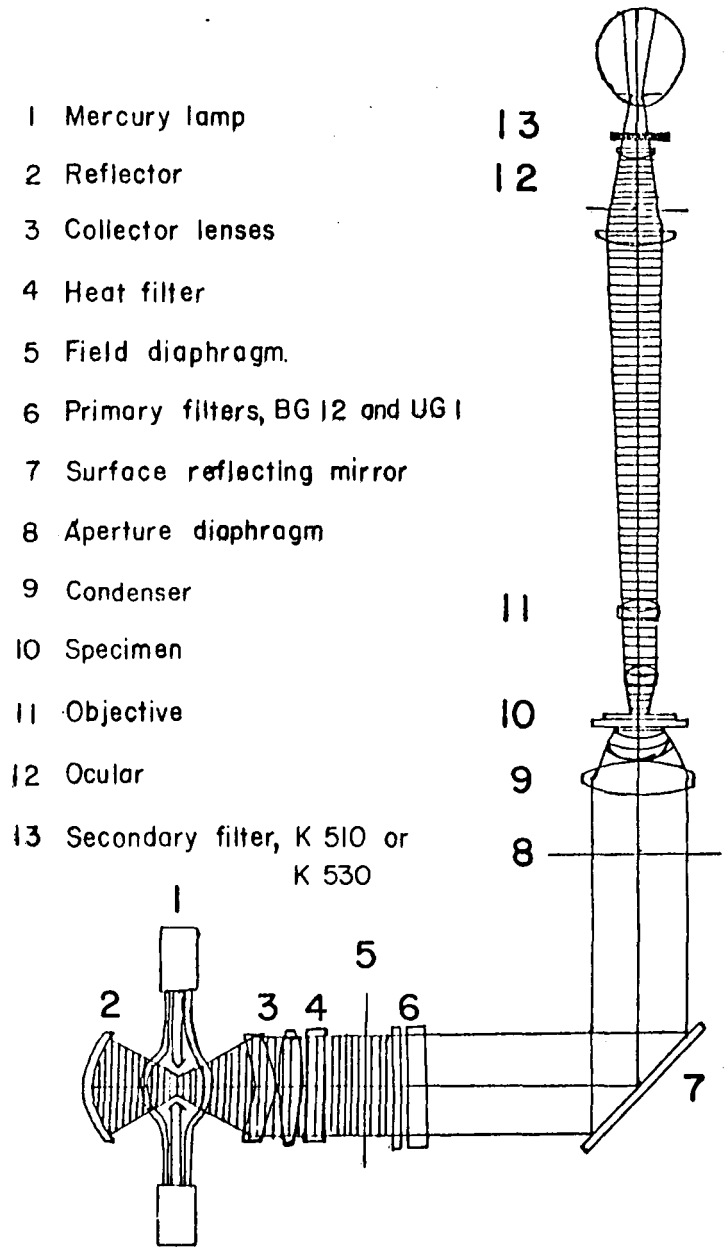


Fig. 1. Schematic diagram of the pathway of transmitted fluorescent illumination. (Nairn, 1969).

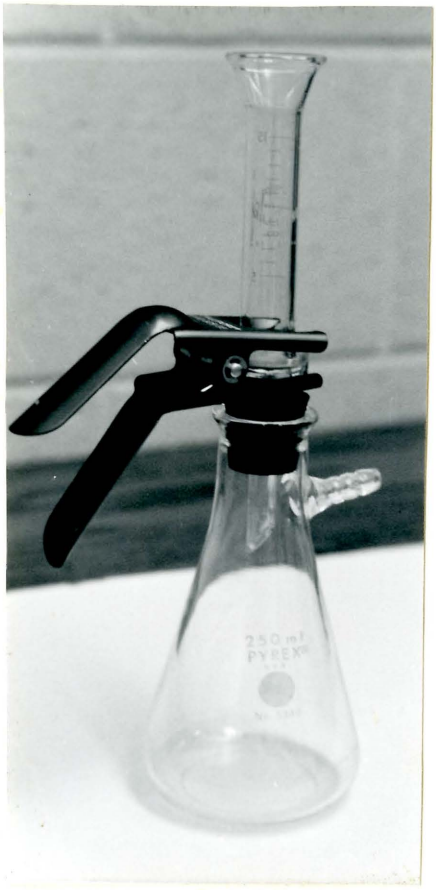


Figure 2. Glass filtering apparatus used for Staining Method B.

water sample or axenic culture estimated to have an adequate number of cells for counting was carefully added to the stain solution. After standing one min, this suspension was filtered. The black filter was then removed, "blotted"* once or twice to remove any remaining water, and placed on a drop of low-fluorescing immersion oil (Nd=1.5.5) on an acid-cleaned, sterile glass slide (A. H. Thomas Red Label 100's). (Note: *Any excess water was filtered before the black filter was removed and mounted. A second drop of immersion oil was placed on top of the filter and a #0 coverslip laid over it. An AO-stained black filter served as a control for any microorganisms existing in the stain or on the filter. Black filters were not sterilized to avoid any loss of the black dye through autoclaving.

Fluorescence of AO-stained cells concentrated on the black filters was observed using a Leitz Orthoplan microscope with a Ploem epifluorescence illuminator equipped with a xenon lamp, a BG36 exciter filter, interference green filter, S546, and a K530 barrier filter. The excitation filter turret in Figure 3 was set at position 3 and the dichroic beam splitting and built-in suppression filter combination was also set at position 3; the suppression filter slide contained a K530 filter. This epifluorescence system (Figure 3) has been reported to be far superior than that of transmitted light (Figure 1) (Francisco, et al., 1973; Daley and Hobbie, 1975). Random microscopic fields were counted and cell numbers/ml were calculated from the formula,

$$\text{Approximate cells/ml} = \frac{S \times N}{s \times V} \quad ;$$

1 Suppression filter slide

2 Suppression filter, built in

3 Turret for suppression filter and beam splitting mirror

4 Beam splitting mirror

5 Mercury lamp

6 Exciter filters

7 Field diaphragm

8 Scattered light mask

9 Objective

10 Specimen

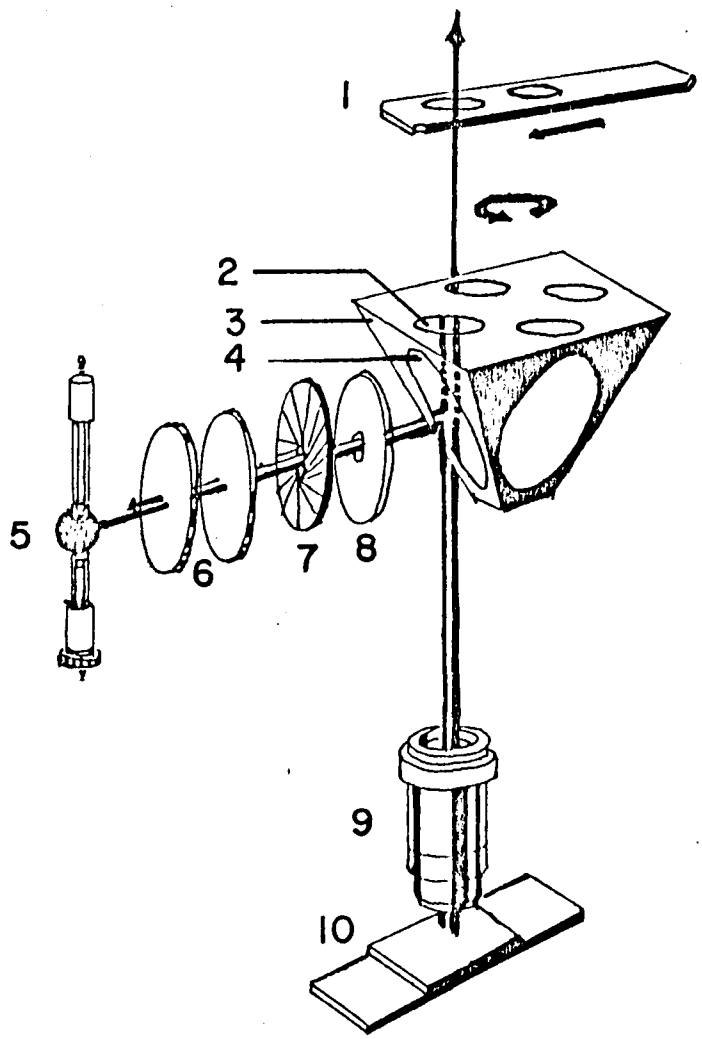


Fig. 3. Schematic diagram of the pathway of Ploem epifluorescence illumination (Leitz, Germany).

where S = filtering area of filter in um^2,
s = microscopic area of filter, um^2,
N = minimum number of fluorescent cells/microscopic area
V = volume in ml (Rodina, 1972).

The numbers were considered to be "approximate" because of probable clumping of cells, and the presence of some "cell-like" detritus.

Viable spread plates were also employed to enumerate bacterial populations. Serial dilutions of the axenic culture isolates were prepared in sterile PYGS broth and 0.1 ml amounts were pipetted and spread with a sterile glass rod on solid PYGS media. Triplicate plates of three serial dilutions (9 total plates) of isolates T-22 and T-3 were incubated at 15 and 23 C respectively for each experiment.

For convenience, specific methods for individual experiments will accompany the results.

PART II. Mountain Lake Study.

Water samples from Mountain Lake, Giles County, Virginia, were collected weekly during the period of July through mid-August, 1975 using a modified Niskin sampler (Figure 4) and transferred to acid-cleaned, distilled water-rinsed, plastic one gallon containers. The samples were stored on ice in the dark, in Styrofoam coolers until they could be processed in the laboratory. All spread plates and AO-staining were accomplished within 4-5 hrs of sampling. Serial dilutions of the Mountain Lake water were prepared in autoclaved Mountain Lake water blanks. Aliquots of 0.1 ml were spread on agar plates of PYGS media prepared with filtered Mountain Lake water

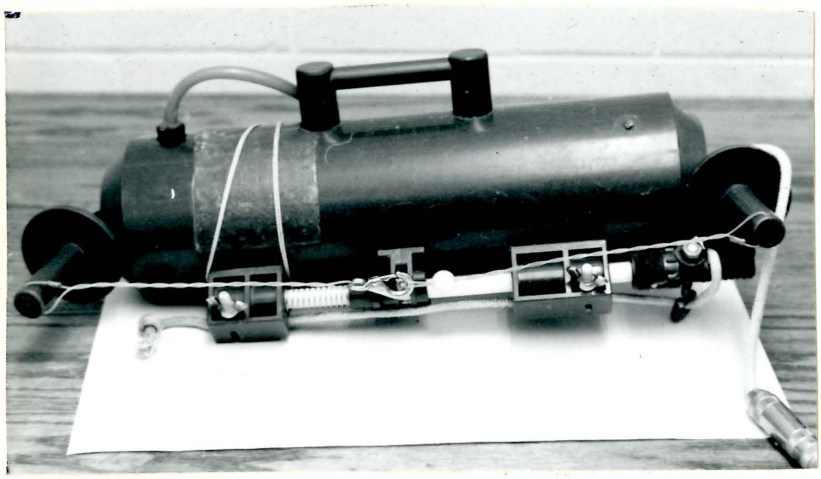


Figure 4. The Niskin sampler in an open position.

(Appendix 1). Triplicate plates of three serial dilutions (9 total plates) were incubated at 15 C for 10 days before counting. In order to reduce the statistical error, only data from those plates with 15-300 colonies were used (Sorokin and Kadota, 1972). AO-stained cell counts were made by the epifluorescence technique (Method B), using an 0.1% w/v concentration of the dye. Counts were completed within 15 hrs of sampling. When counts could not be done in a few hours, the prepared mounted slides were stored in the dark in a 4 C BOD incubator to keep the membrane filter moist and to prevent any light from reaching the cells.

As with Part I, special methods used in individual experiments will, for convenience, accompany the results.

PART III. Statistical Analyses of Two Enumeration Methods.

Confidence intervals of the mean (95% intervals), standard deviations and F-tests (analysis of variance) were determined for bacterial counts from Mountain Lake using the Statistical Analysis Systems (SAS) computer program (Barr and Goodnight, 1972). To test for the appropriate levels of significance for all statistical treatments, respective tables in Statistical Tables (Rohlf and Sokal, 1969) were consulted.

RESULTS

The results are presented in three sections: (I) Laboratory Studies Using Pure Cultures of Antarctic Bacteria, (II) Bacterial Counts for Mountain Lake in Giles County, Virginia and (III) Statistical Analyses of Two Methods for Enumerating Bacterial Populations from Mountain Lake.

Part I. Laboratory Studies Using Pure Cultures of Antarctic Bacteria.

For many experiments, counts were not possible using transmitted light and the limited resolution of the American Optical Spencer microscope. Therefore, AO direct counts were estimated as approximate percentages of total green and/or red-fluorescing cells within the microscopic field. At least 10^7 cells/ml fluoresced red and green for each microscopic field.

Table 1 shows a comparison between three methods of enumeration used on an axenic culture of the T-3 isolate. The viable spread plate counts were reasonably close to the AO direct counts (using the epifluorescence technique--Method B). Contrastingly, the Petroff-Hauser counts were higher by one or more orders of magnitude, even with statistical variation taken into account.

Table 2 shows that virtually all the bacteria stained with AO fluoresced, regardless of their ability or inability to form colonies on spread plates.

Influence of pH on AO staining of T-22. Since the primary objective of this project was to determine whether or not the AO stain

Table 1. Comparisons of Petroff-Hauser counts, acridine orange epifluorescence counts and spread plate counts using T-3 isolate.

Repetition	Petroff-Hauser cells x 10^7/ml	Acridine Orange[a] cells x 10^5/ml	Spread Plates CFU x 10^5/ml[b]
1	2.7 ± 2.1	9.2 ± 1.5	3.3 ± 2.2
2	0.8 ± 0.2	0.4 ± 0.2	6.6 ± 2.5
3	0.6 ± 0.2	9.9 ± 1.5	25.0 ± 2.1
4	2.6 ± 2.0	90.0 ± 1.0	71.0 ± 2.0

[a]Acridine orange epifluorescence counts represent the mean of 15 microscopic fields by the acridine orange epifluorescence technique, total red + green fluorescent cells counted.

[b]Average of 9 commensurable spread plates, in colony forming units/ml.

determined viable cell numbers, the influence of pH and two stain concentrations were first examined. Stain concentrations of 0.01% and 0.1% (w/v) which have been used by others (Batoosingh and Anthony, 1971; Bell and Dutka, 1972; Hobbie, et al., 1972; Francisco, et al., 1973; Daley and Hobbie, 1975) and pH's ranging from 3.3 to 9.4 with different buffers were tested. Citrate Phosphate Buffer (McIlvaine, 1921) and Tris buffer (Colowick and Kaplan, 1955) were used for establishing the pHs of the stain solution. The pHs listed in the tables represent the pH of the stain solution just before addition to the cell suspension. Results of these initial studies are presented in Table 3. There was considerable variation in staining at the different pHs and stain concentrations. Higher percentages of red fluorescing cells were noted at the higher concentration of AO (0.1%). Though detailed results are not presented, this variation occurred consistently in three different experiments studying AO concentration and pH influence.

AO-stain reaction of an autoclaved culture of T-22. A 24-hr log phase culture of T-22 was autoclaved for 15 min at 15 psi, then cooled, centrifuged and stained in accordance with Staining Method A. Results in Table 4 reveal that 100% of the autoclaved cells fluoresced green. Spread plates of autoclaved cells revealed no growth. Such green fluorescence of autoclaved cultures was observed consistently in three experiments.

Influence of lethal doses of UV on AO-staining of T-22. If AO combines with cellular nucleic acid, as alleged (Strŭgger, 1948;

Table 2. Comparison of total fluorescing counts (red + green) vs total phase contrast counts of acridine orange stained isolates, T-22 and T-3 (0.1% w/v AO).

Culture Isolate	Replicate	Fluorescent cells x 10^7/ml	Phase-Contrast cells x 10^7/ml	Plate Count CFU x 10^5/ml
T-22	1	2.5 ± 0.6	3.4 ± 1.2	8.9 ± 1.5
T-22	2	6.8 ± 0.4	8.9 ± 1.4	7.6 ± 1.7
T-22	3	7.4 ± 1.1	6.9 ± 0.8	9.4 ± 1.3
T-3	1	0.9 ± 0.6	1.2 ± 0.4	6.8 ± 0.8
T-3	2	1.1 ± 0.7	0.9 ± 0.5	7.3 ± 0.6
T-3	3	0.8 ± 1.3	0.6 ± 1.1	8.9 ± 0.9

Note: Fluorescence reactions and counts in addition to phase-contrast counts were done using the same preparation of the Petroff-Hauser bacterial counting chamber, in accordance with Staining Method A.

Table 3. Results of interactions of AO stain concentration, and AO pH on the fluorescence reactions of a log phase culture of isolate T-22.

Stain pH	Stain Concentration (% w/v)	$\bar{Y}^a$ %Fluorescence R[b]	G[c]
3.3	0.1	60	40
3.4	0.01	5	95
5.7	0.1	80	20
5.8	0.01	--	100
7.2	0.1	95	5
7.2	0.01	--	100
8.6	0.1	75	25
8.7	0.01	--	100
9.1	0.1	90	10
9.1	0.01	--	100
9.2	0.1	90	10
9.4	0.01	--	100

Note: Colony forming units/ml before staining: 6.3×10^7 CFU/ml

[a] $\bar{Y}$ represents the mean percentages of 20 random microscopic fields, using Staining Method A, where at least 10^7 cells/ml were observed

[b] R = red fluorescence

[c] G = green fluorescence

Table 4. Results of interactions of AO stain concentrations, and AO stain pH on the fluorescence properties of an autoclaved culture of isolate T-22.

Stain pH	Stain Concentration (%w/v)	Colony-forming units/ml	$\bar{Y}^a$ %Fluorescence R[b]	G[c]
3.4	0.01	0	0	100
5.8	0.01	0	0	100
7.2	0.01	0	0	100
3.3	0.1	0	0	100
5.7	0.1	0	0	100
7.2	0.1	0	0	100

Note: Colony forming units/ml before autoclaving and staining: 7.8×10^7

[a] $\bar{Y}$ represents the mean percentages of 20 random microscopic fields, using Method A; at least 10^7 cells/ml were observed.

[b] R = red

[c] G = green

Armstrong, 1956; von Bertalanffy and Bickis, 1956; Lerman, 1961; Ranade, et al., 1961), then one might postulate that changes within the DNA helix would cause changes in fluorescence reactions as opposed to the fluorescence reactions of viable cells with undenatured DNA. Thus isolate T-22 was exposed to various levels of ultraviolet (UV) light (lamp #GE-G8T5). Several 24-hr log phase cultures were harvested after 24 hr by centrifugation at 10,000 rpm for 10 min, then resuspended in 5.0 ml of filter-sterilized phosphate-buffered saline (pH 7.2). The 5.0 ml aliquots were distributed into sterile Petri dishes and exposed to varying doses of UV as shown in Table 5. Triplicate spread plate colony counts were obtained after 3 days of incubation at 15 C in the dark to avoid possible photorepair. Table 5 shows that various doses of UV had no apparent influence on the fluorescent color even though no growth occurred on plates spread with those bacteria exposed to greater than or equal to 55.0 ergs/sec UV. As with the experimental data reviewed in Table 3, the higher AO concentration induced predominantly red and the lower concentration induced predominantly green fluorescence. This effect of the AO stain concentration was consistently observed in three different experiments.

Effect of AO stain on UV-exposed cells. Following UV treatment as outlined in the previous experiment, T-22 suspensions were incubated at 15 C in the dark and observed after 24 and 48 hrs, using transmitted UV light, in accordance with Method A. Aliquots of 0.1 ml of these suspensions were also spread on plates of PYGS agar media. Results in Tables 6, 7, and 8 show no enhanced effect of the AO dye uptake by

Table 5. AO (stain pH: 5.6) fluorescent response of UV-exposed cells (T-22) after incubation for 24 hr at 15 C.

Stain Concentration %, w/v	UV radiation, ergs/sec	$\bar{Y}$[a] CFU/ml x 10^3	$\bar{Y}$[b] Counts x 10^2/ml G[c]	R[d]
0.1	0	1.4	0.1	11.8
0.01	0	1.4	11.8	0.0
0.1	55.0	.09	0.1	10.0
0.01	55.0	.09	9.6	0.3
0.1	176.8	0.0	0.0	8.1
0.01	176.8	0	8.7	0
0.1	336.0	0	0	9.8
0.01	336.0	0	9.7	0.
0.1	667.2	0	0.0	9.9
0.01	667.2	0	9.7	0

[a] $\bar{Y}$ represents the mean of 9 commensurable spread plates, in colony forming units/ml.

[b] $\bar{Y}$ represents the mean of 15 microscopic fields using Staining Method B.

[c] G = green

[d] R = red

Table 6. Fluorescent cell response of UV-exposed cells of T-22 which were stained with 0.1% and 0.01% AO (pH 5.6) after UV exposure.

Stain Concentration, %, w/v	UV radiation ergs/sec	$\bar{Y}^a$ CFU/ml	$\bar{Y}^b$ % Fluorescence R[c]	G[d]
0.01	0	2.3×10^7	0	100
0.01	55.	1.4×10^7	0	100
0.01	176.8	1.0×10^3	0	100
0.01	336.0	3.2×10^3	0	100
0.01	667.2	8.9×10^1	0	100
0.1	0	1.9×10^7	50	50
0.1	55.	1.5×10^7	98	2
0.1	176.8	4.2×10^3	98	2
0.1	336.0	3.2×10^3	98	2
0.1	667.2	1.0×10^2	90	10
0.0	0	5.8×10^7	0	0
0.0	55.	2.6×10^7	0	0
0.0	176.8	5.6×10^3	0	0
0.0	336.0	3.5×10^3	0	0
0.0	667.2	1.9×10^2	0	0

[a] $\bar{Y}$ represents the mean of 9 commensurable spread plates in colony forming units/ml.

[b] $\bar{Y}$ represents the mean estimated percentage of 20 random microscopic fields using Method A; where at least 10^7 cells/ml were observed.

[c] R = red

[d] G = green

Table 7. Fluorescent response of UV-exposed cells of T-22 stained with 0.1% and 0.01% AO (pH 5.6) after incubation for 24 hrs.

Stain Concentration, %, w/v	UV radiation ergs/sec	$\bar{Y}$[a] CFU/ml	$\bar{Y}$[b] % Fluorescence R[c]	G[d]
0.01	0	7.6×10^8	0	100
0.01	55.	2.7×10^1	0	100
0.01	176.8	8.3×10^1	0	100
0.01	336.0	0	0	100
0.01	667.2	0	0	100
0.1	0	8.6×10^7	100	0
0.1	55.	2.9×10^1	50	50
0.1	176.8	2.8×10^1	50	50
0.1	336.0	0	50	50
0.1	667.2	0	50	50

[a] $\bar{Y}$ represents the mean of 9 commensurable spread plates, in colony forming units/ml.

[b] $\bar{Y}$ represents the mean of 20 microscopic fields, using Staining Method A; where at least 10^7 cells/ml were observed.

[c] R = red

[d] G = green

Table 8. Fluorescent response of UV-exposed cells of T-22 stained with 0.1% and 0.01% AO (pH 5.6) after incubation for 48 hrs.

Stain Concentration, %, w/v	UV radiation ergs/sec	$\bar{Y}^a$ CFU/ml	$\bar{Y}^b$ %Fluorescence Rc	Gd
0.01	0	8.3×10^8	0	0
0.01	55.	0	5	95
0.01	176.8	0	0	100
0.01	336.0	0	0	100
0.01	667.2	0	0	100
0.1	0	3.4×10^7	0	100
0.1	55.	3	20	80
0.1	176.8	0	10	90
0.1	336.0	0	5	95
0.1	667.2	0	5	95

[a] $\bar{Y}$ represents the mean of nine commensurable spread plates, in colony forming units/ml.

[b] $\bar{Y}$ represents the mean of 20 microscopic fields, using Staining Method A; where at least 10^7 cells/ml were observed.

[c] R = red

[d] G = green

UV-exposed cells at the 0.01% dye concentration. However, at the 0.1% AO concentration, an increasing number of cells fluoresced green with prolonged incubation time.

Laboratory Studies on a Gram-positive bacterium, T-3. Because there may be a difference in the sorptive capacity of the cell wall between Gram negative and Gram positive bacteria (Bucherer, 1966), the Gram positive isolate, T-3 was also studied, using basically the same experimental procedures as outlined for isolate T-22. Table 9 shows a less pronounced stain concentration effect for T-3 as compared with T-22 (Table 3). Results in Table 10 reveal also that a higher percentage of red-fluorescing cells occurred for the T-3 isolate relative to T-22 (Table 3); this is true, however only at the 0.1% concentration. Green fluorescence is noted at the 0.01% concentration, similar to the fluorescence color exhibited by T-22 (Table 3). Table 10 shows a concentration effect similar to that of T-22 after autoclaving (Table 4). Higher percentages of red fluorescence were noted at the 0.1% stain concentration. Table 11 shows similar results as those obtained with T-22 (Table 5) i.e., green fluorescence occurred at the 0.1% concentration. Finally, no apparent enhancement of dye uptake occurred after increased incubation time, i.e., after 24 hrs (Table 12); even though there was a pronounced effect of prolonged incubation of cells exposed to 667.2 ergs/sec UV such that red fluorescence occurred even at the 0.01% concentration.

Table 9. Results of interactions of AO stain concentration and AO pH on the fluorescence reactions of a log phase culture of isolate T-3.

Stain pH	Stain Concentration (% w/v)	$\bar{Y}^a$ %Fluorescence R[b]	G[c]
3.3	0.1	75	25
3.4	0.01	20	80
5.7	0.1	90	10
5.8	0.01	35	65
7.2	0.1	90	10
7.2	0.01	25	75
8.6	0.1	90	10
8.7	0.01	25	75
9.1	0.1	80	20
9.1	0.01	20	80
9.2	0.1	75	25
9.4	0.01	30	70

Note: Colony forming units/ml before staining: 5.6×10^5.

[a] $\bar{Y}$ represents the mean of 20 random microscopic fields, using staining Method A; where at least 10^7 cells/ml were observed/field.

[b] R = red

[c] G = green

Table 10. Results of interactions of AO stain concentrations, and AO stain pH on the fluorescence properties of an autoclaved culture of isolate T-3.

Stain pH	Stain Concentration (% w/v)	Colony-forming units/ml	$\bar{Y}^a$ %Fluorescence R[b].	G[c]
3.5	0.01	0	0	100
5.6	0.01	0	0	100
7.3	0.01	0	0	100
3.4	0.1	0	45	55
5.7	0.1	0	60	40
7.2	0.1	0	60	40

Note: Colony-forming units/ml before autoclaving: 6.3×10^5

[a]$\bar{Y}$ represents the mean of 20 random microscopic fields, using Staining Method A; where at least 10^7 cells/ml were observed in each field.

[b]R = red

[c]G = green

Table 11. Fluorescent cell response of UV-exposed cells of T-3 which were stained with 0.1% and 0.01% AO (pH 5.6) immediately after UV exposure.

Stain Concentration, % w/v	UV radiation, ergs/sec	$\bar{Y}^a$ CFU x 10^3/ml	$\bar{Y}^b$ counts x 10^3/ml R[c]	G[d]
0.01	0	6	0	9.2
0.01	55	0	0	1.6
0.01	176.8	0	0	8.5
0.01	336.0	0	0	4.3
0.01	667.2	0	0	5.2
0.1	0	6	8.4	0
0.1	55	0	1.6	0
0.1	176.8	0	12.2	0
0.1	336.0	0	4.5	0
0.1	667.2	0	5.3	0

[a] $\bar{Y}$ represents the mean of 9 commensurable spread plates, in colony-forming units/ml.

[b] $\bar{Y}$ represents the mean of 20 microscopic fields, using Staining Method B.

[c] R = red

[d] G = green

Table 12. AO (pH 5.6) fluorescent response of UV-exposed cells of culture isolate T-3 after incubation for 24 hr at 23 C.

Stain Concentration % w/v	UV radiation, ergs/sec	$\bar{Y}^a$ CFU/ml	$\bar{Y}^b$ Counts x 10^3/ml R[c]	G[d]
0.01	0	1 x 10^3	0	1.8
0.01	55	0	0	6.9
0.01	176.8	0	0	4.8
0.01	336.0	0	0	7.6
0.01	667.2	0	1.4	5.7
0.1	0	0	1.9	0
0.1	55	0	1.4	0.3
0.1	176.8	0	1.1	0
0.1	336.0	0	2.6	0
0.1	667.2	0	9.0	0

[a] $\bar{Y}$ represents the mean of 9 commensurable spread plates, in colony-forming units/ml.

[b] $\bar{Y}$ represents the mean of 20 microscopic fields, using Method B.

[c] R = red

[d] G = green

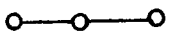
PART II. Bacterial Counts for Mountain Lake in Giles County, Virginia.

The results of bacterial population fluctuations shown as a function of depth in Figures 5-14 were obtained solely to assess the differences between the two techniques of bacterial enumeration. Few red-fluorescing (at most, 3 cell-like units/ml) were noted on all sampling dates except July 17, 1975, when green-fluorescing cells also dominated the microscopic field, accompanied by a significant number of red-fluorescing cell-like particles (Figure 5). Therefore, all AO counts represent total fluorescing counts except where noted (Figure 5). There are significant variations between the average plate counts and average AO counts (Figures 5, 9-14). Confidence intervals of the means (averages) of each count, as a function of depth for all sampling dates, are shown following the corresponding figures.

Forty to 50% of the means of the viable counts were higher than those of the direct counts. Closer examination of the confidence intervals for each sampling date, immediately following the respective dates, however, reveal that the variations in most of the viable spread plate counts were large. In a few cases, the direct (AO-E) counts were significantly lower than those of the viable spread plate counts, especially at the lower depths.

Further results of different experiments are shown in Tables 13-16. Table 14 shows that AO does not appear to enhance adsorption of bacteria to glass surfaces. This control experiment was important to validate the epifluorescence counts of fluorescing cells and particles concentrated on the black filters.

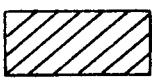
Table 13. Legend for Confidence Intervals



Upper and lower 95% confidence limits for the average of 3 aerobic spread plate counts.

Shaded 95% confidence interval for the average of triplicate spread plate counts.

Upper and lower 95% confidence limits for the average of total green and red fluorescing counts.



Shaded 95% confidence interval for the average of AO epifluorescence counts.

Overlap of 95% confidence intervals of averages of AO epifluorescent counts, and spread plate counts.

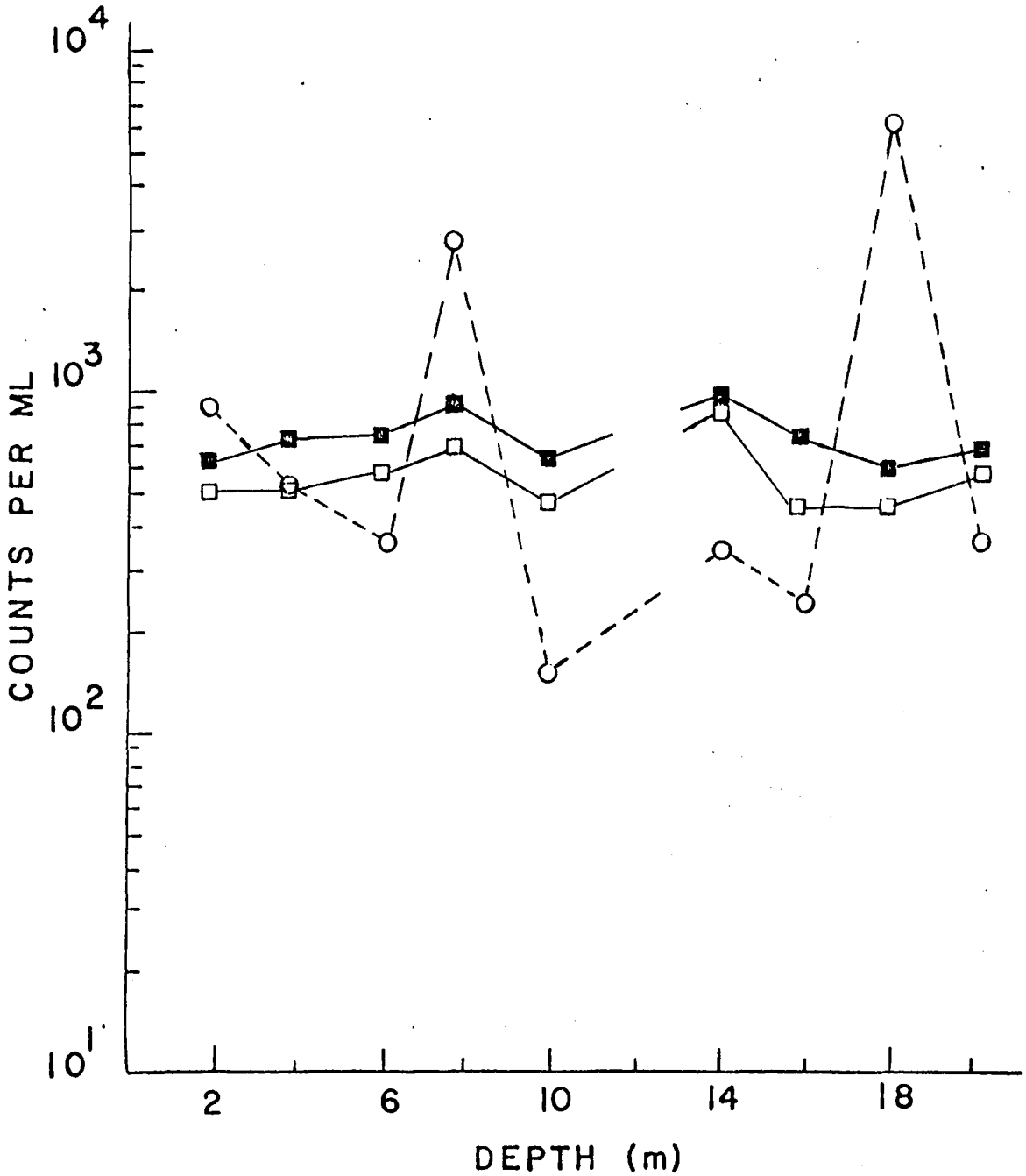


Fig.5. Spread plate (o-o-o) and AO-Epifluorescent (green=□-□, green & red=■-■-■) counts of a bacterial population from Mountain Lake, 07-17-75.

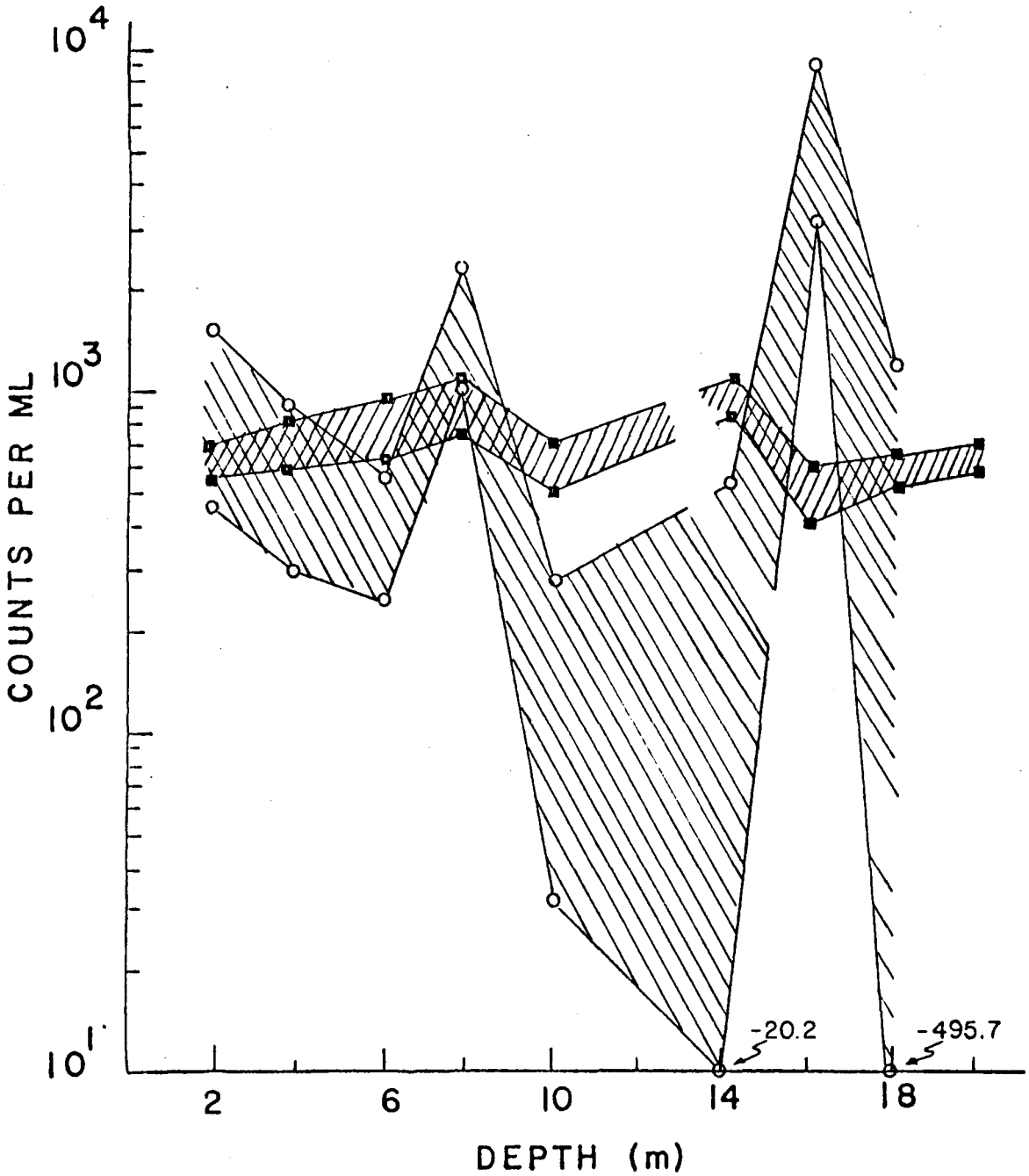


Fig.6. 95% confidence intervals of average bacterial population counts (Mountain Lake), 07-17-75.

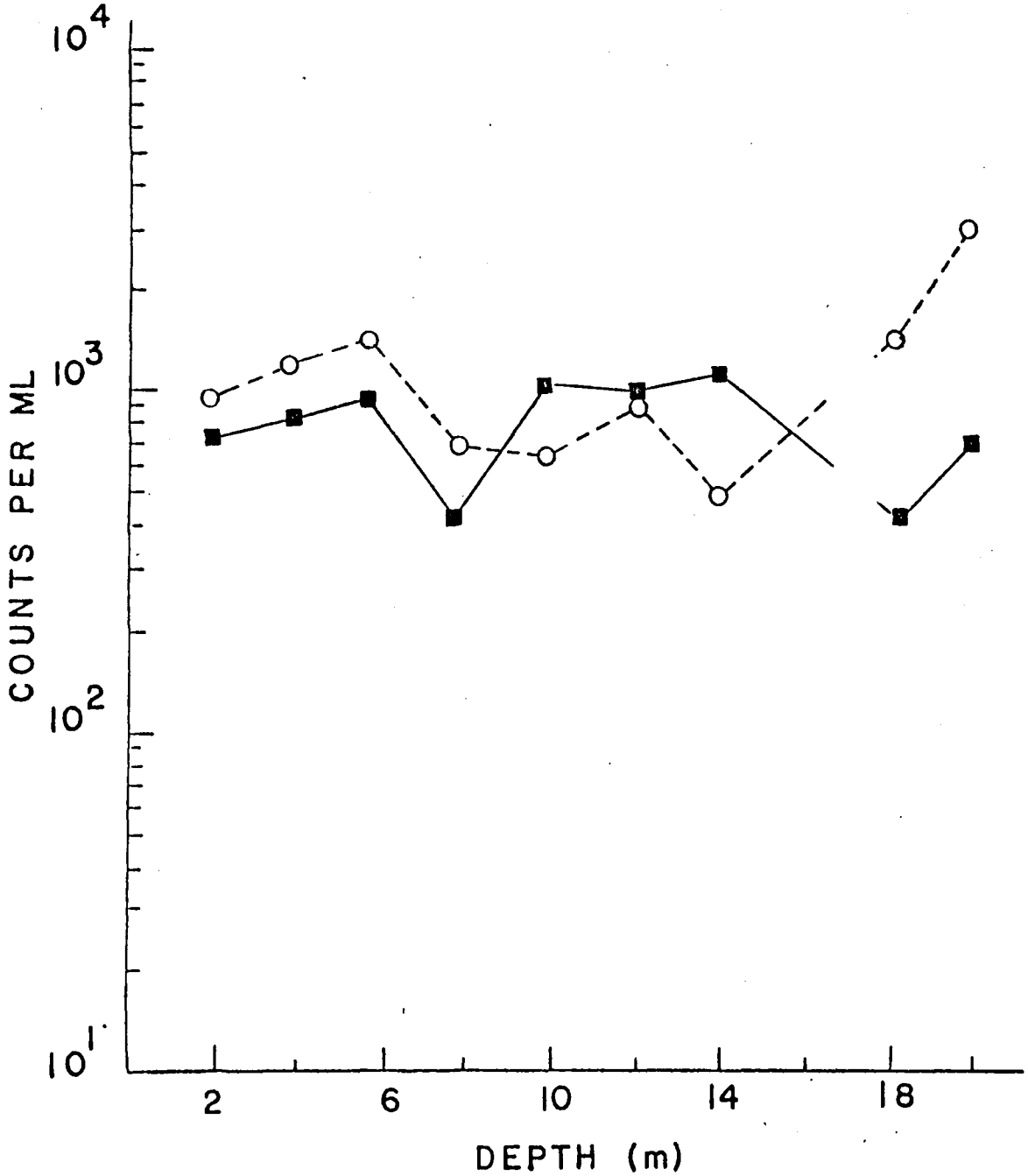


Fig.7. Spread plate (-o-o-) and AO-Epifluorescent (green & red = ■—■) counts of a bacterial population from Mountain Lake, 07-24-75.

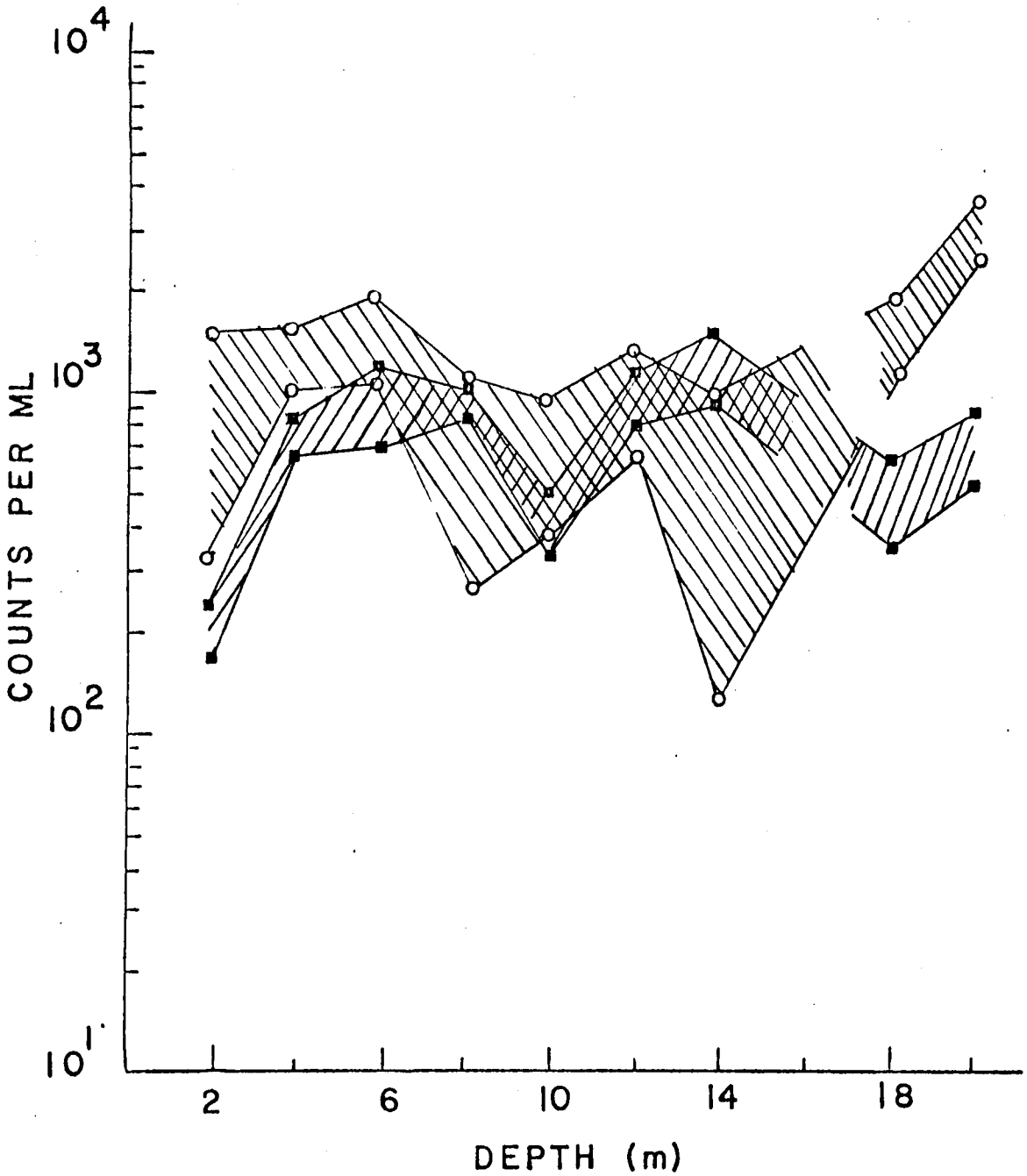


Fig.8. 95% confidence intervals of average bacterial population counts (Mountain Lake), 07-24-75.

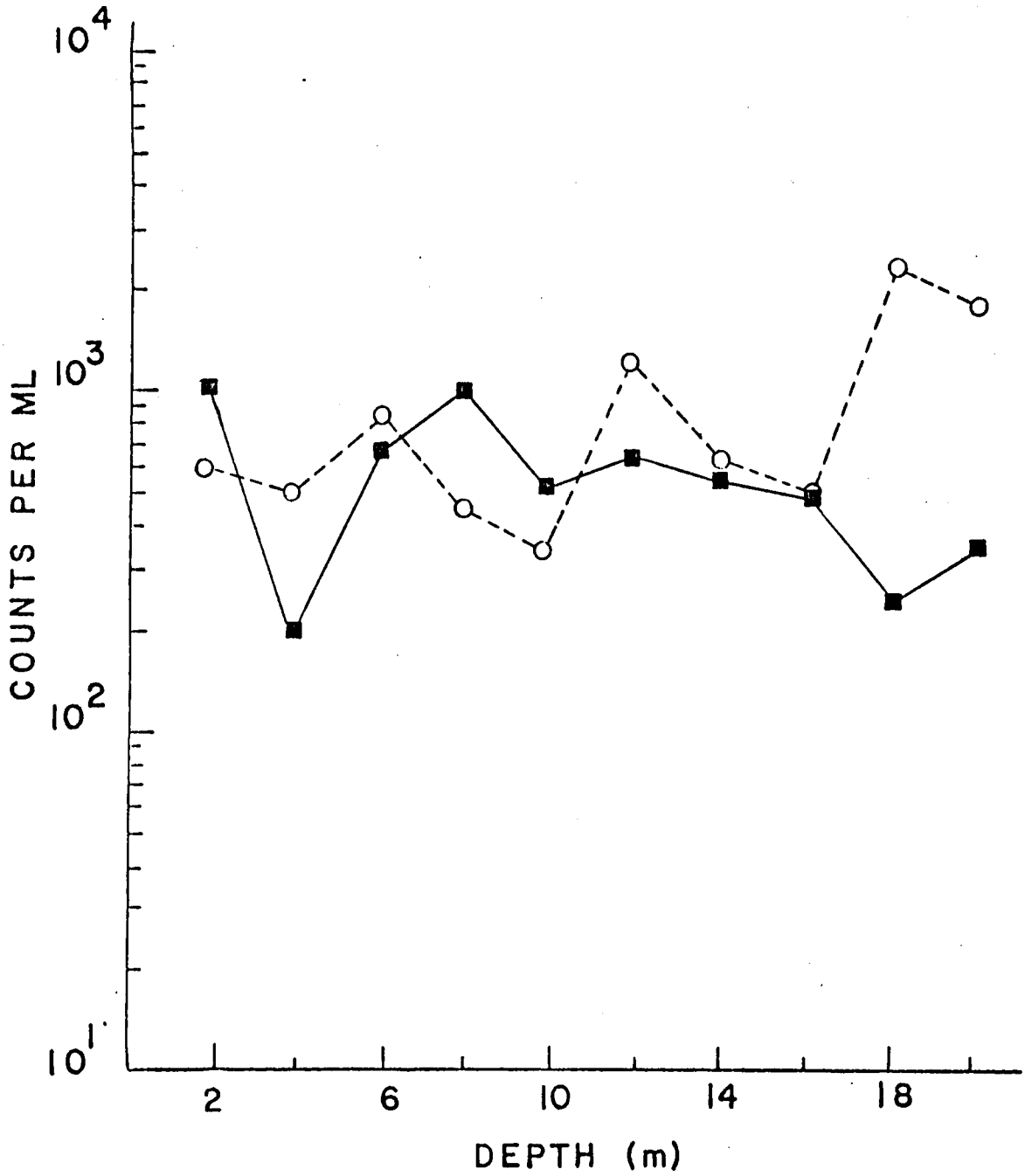


Fig. 9. Spread plate (-o-o-o-) and AO-Epifluorescent (green & red=-■-■-■-) counts of a bacterial population from Mountain Lake, 07-31-75.

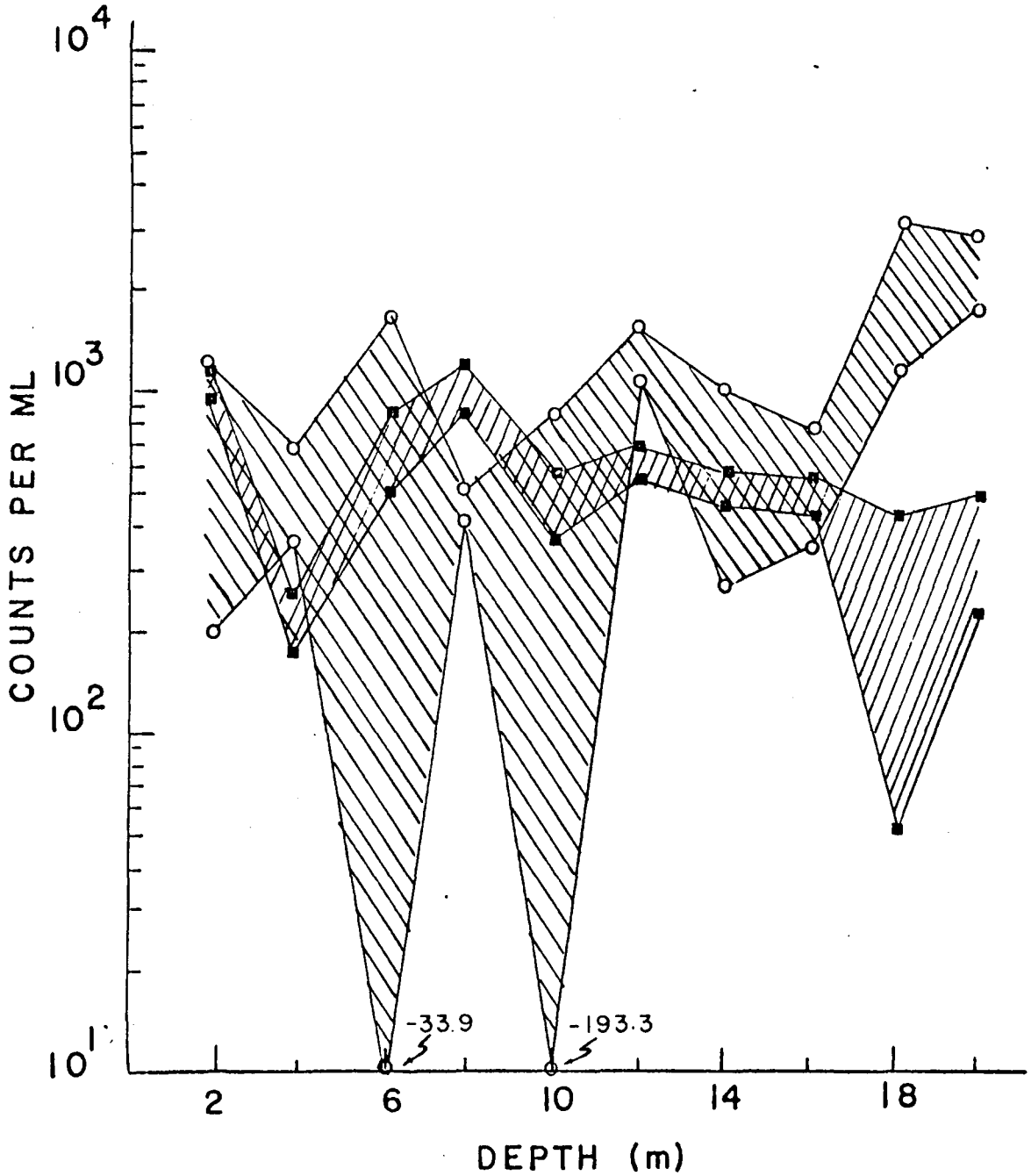


Fig. 10. 95 % confidence intervals of average bacterial population counts (Mountain Lake), 07-31-75.

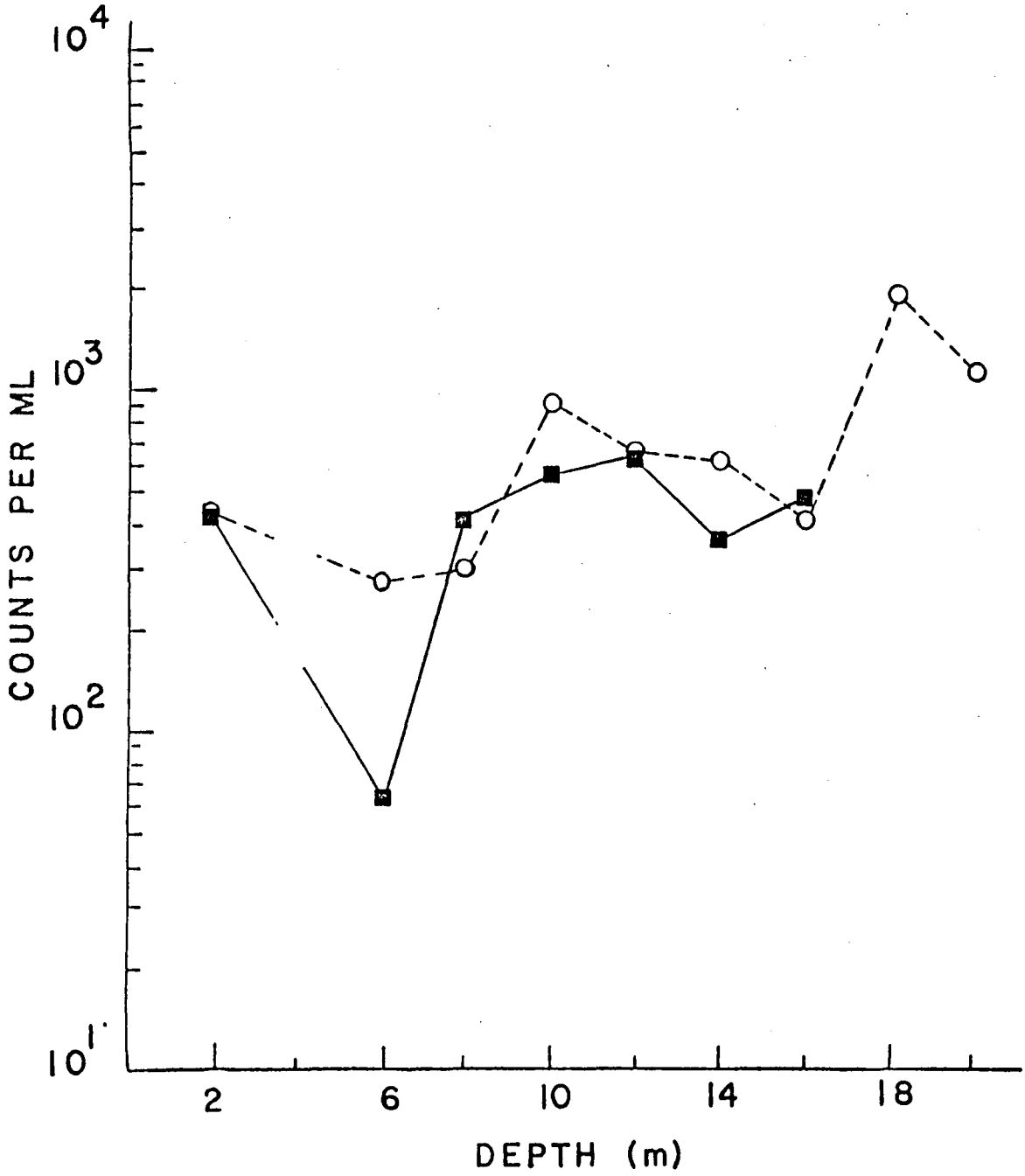


Fig. 11. Spread plate (-o- -o-) and AO-Epifluorescent (green & red=-■-■-) counts of a bacterial population from Mountain Lake, 08-07-75.

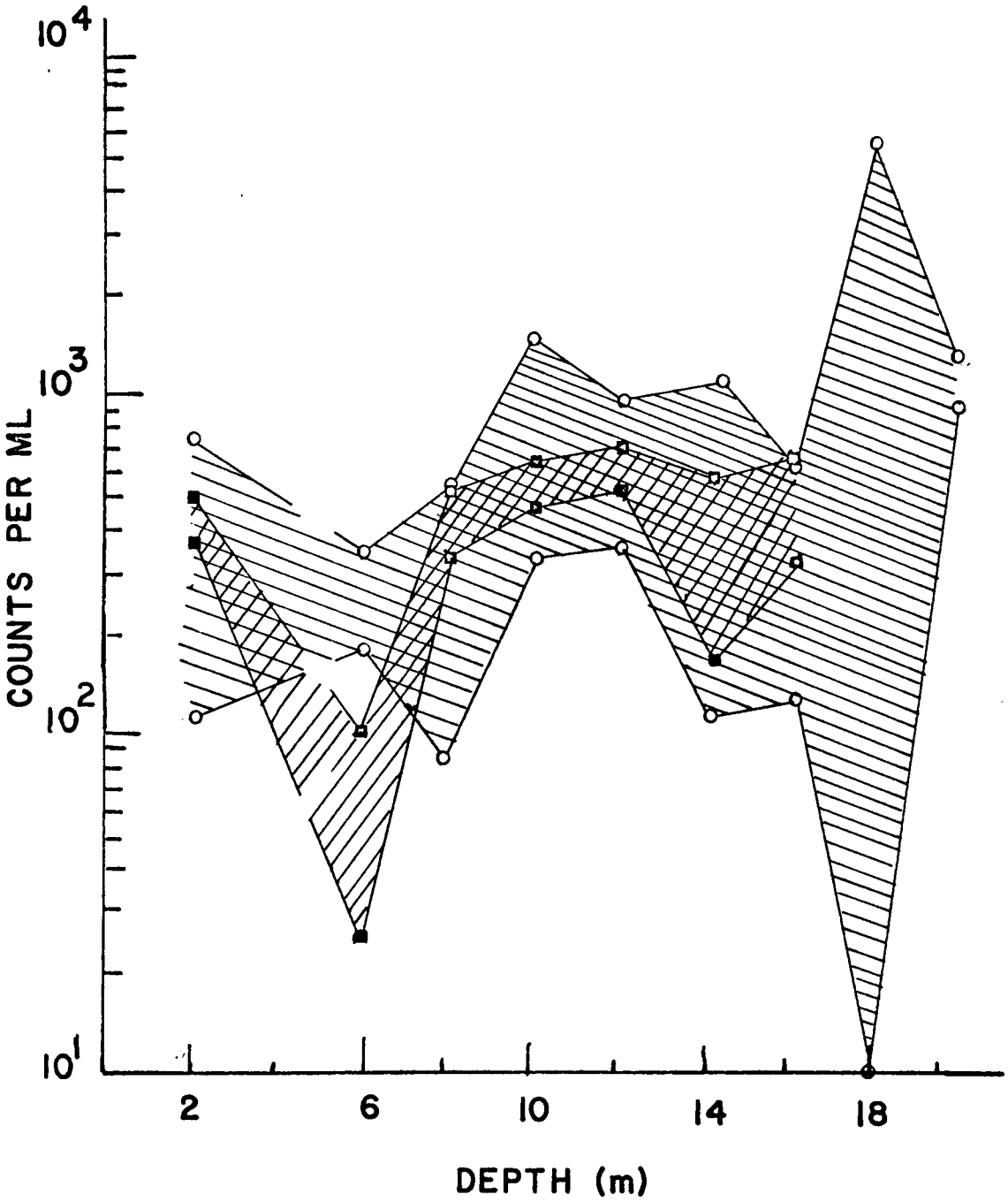


Fig. 12. 95 % confidence intervals of average bacterial population counts (Mountain Lake), 08-07-75.

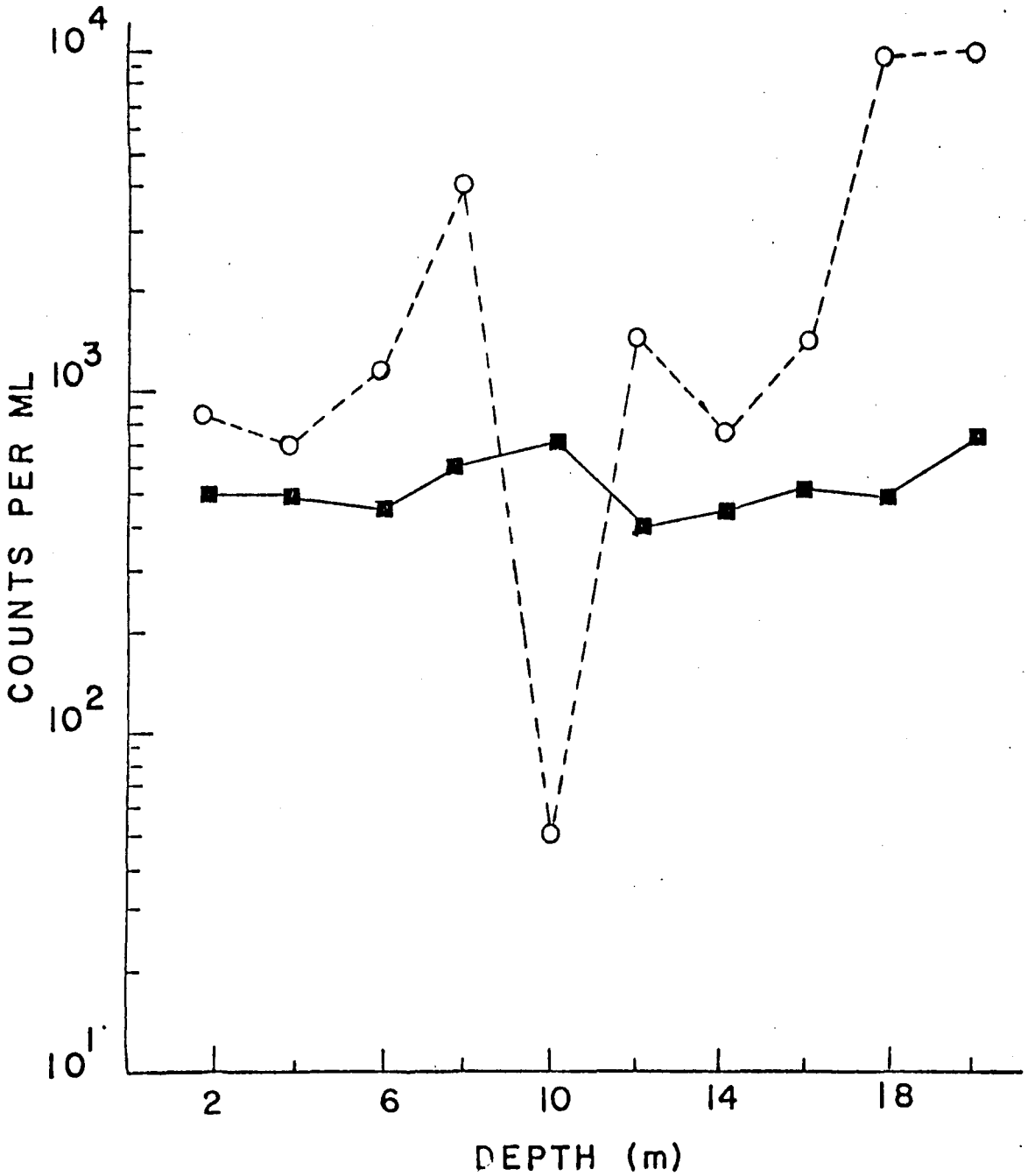


Fig. 13. Spread plate (-o- -o-) and AO-Epifluorescent (green & red=-■-■-) counts of a bacterial population from Mountain Lake, 08-14-75.

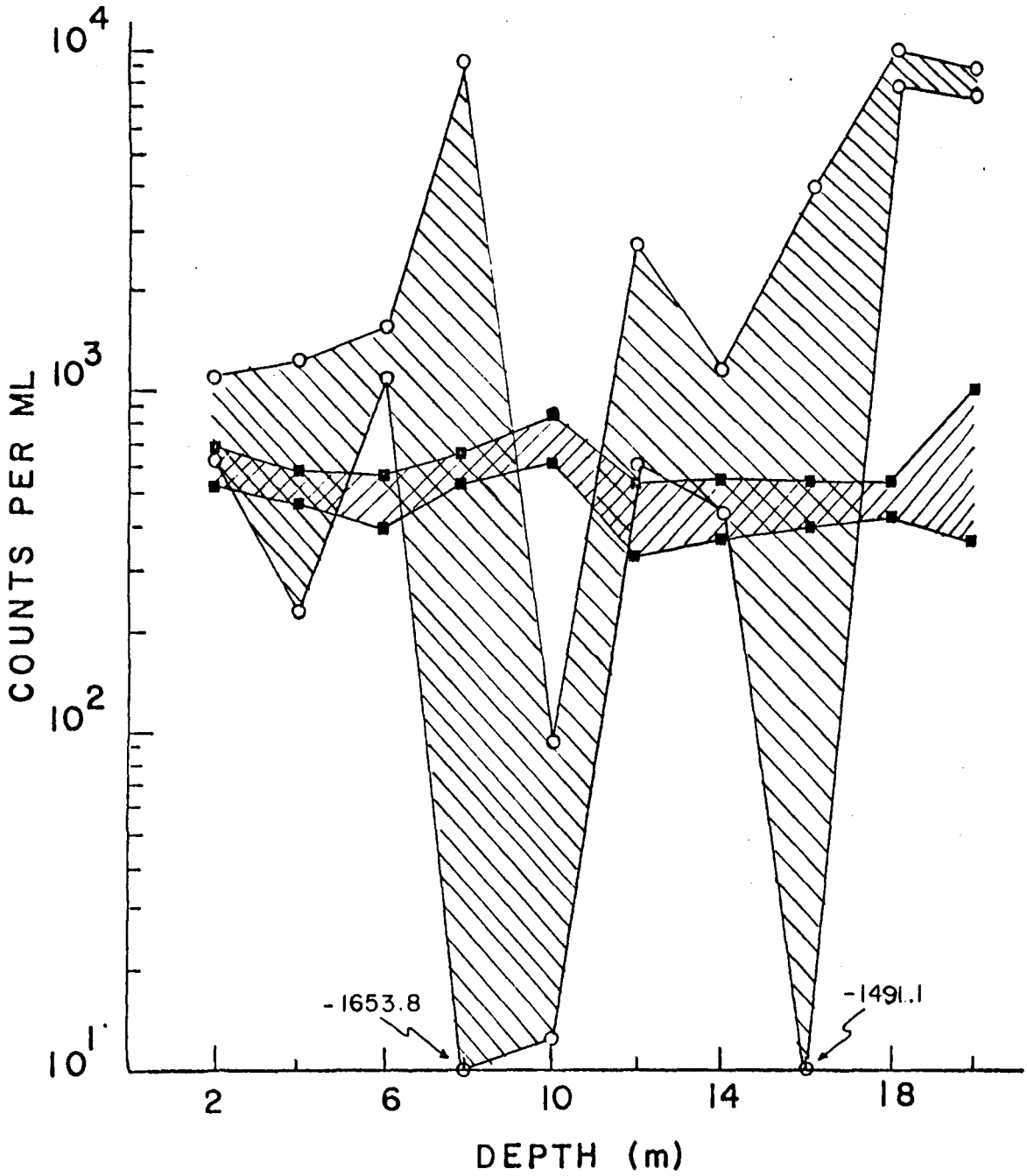


Fig. 14. 95 % confidence intervals of average bacterial population counts (Mountain Lake), 08-14-75.

Table 14. Counts of red-fluorescing and green fluorescing bacterial cells w/ rinsing and w/o rinsing the glass filtering apparatus, as per Staining Method B.

Replicate	No Rinse $\bar{Y}^a$ Total counts x 10^2/ml ± S. D.	Rinse $\bar{Y}^a$ Total counts x 10^2/ml ± S. D.
1	6.34 ± 1.2	6.26 ± 0.8
2	4.73 ± 1.1	6.72 ± 0.9
3	5.72 ± 1.7	4.80 ± 1.1
4	3.60 ± 0.7	3.60 ± 1.0
5	5.80 ± 0.9	6.50 ± 1.0
6	3.50 ± 1.0	3.04 ± 0.4

*Rinsed: Added 5.0 ml of filter-sterilized Mountain Lake water to the filter tower (Fig. 2) and then subsequently filtered the excess water.

[a]$\bar{Y}$ represents the average of 15 microscopic fields counted.

Table 15 shows increasing numbers of red fluorescing cells obtained with increasing dye concentrations of up to 0.5%.

Table 16 shows a comparison of stain-pure culture contact times before filtering as per staining method B. Results show that a one minute stain-sample contact time yields similar counts as with 3 minute stain-exposed samples.

Tables 17 and 18 demonstrate that green fluorescence predominates in bacteria-size particles in Mountain Lake water after fixation with formalin and autoclave-sterilization. Some variations in fluorescing color also occurred at the different concentrations of AO.

PART III. Statistical Analyses of Two Methods for Enumerating Bacterial Populations from Mountain Lake.

Analysis of variance (Table 19) of the two methods show that there is an insignificant interaction between the methods and depth, implying that the remaining statistical analyses could be of statistical significance (Source 3). Variation between sampling dates were not significant; these dates may be treated as replicates of one particular sampling site in Mountain Lake (Source 1); however, results for each date are shown to depict the variation within each technique, i.e. between the total viable spread plate count and the AO epifluorescence technique. One might expect variation in depth (Source 2), since the figures show fluctuations in the bacterial population as a function of depth (Figures 5-14).

Table 15. Counts of green-fluorescing and red-fluorescing cells at different stain concentrations using a natural water sample from Mountain Lake, Giles County, Virginia.

Stain Concentration, % w/v	$\bar{Y}^a$ Green ± S. D.	$\bar{Y}^a$ Red ± S. D.
0.01	365.6 ± 16.5	3.1 ± 0.4
0.05	374.1 ± 77.3	3.4 ± 5.9
0.10	390.0 ± 42.9	26.3 ± 19.5
0.50	87.3 ± 28.9	121.4 ± 23.7

[a]$\bar{Y}$ represents the mean of 15 microscopic fields, in fluorescent counts/ml ± standard deviation.

Table 16. Counts of green-fluorescing and red-fluorescing cells following one minute and 3-minute stain-pure culture contact times;using a log phase culture of isolate T-3.

Replicate	$\bar{Y}$ [a] 1 minute total counts x 10^2 /ml $\pm$ S. D.	$\bar{Y}$ [a] 3 minute total counts x 10^2 /ml $\pm$ S. D.
1	6.7 $\pm$ 1.4	7.3 $\pm$ 1.0
2	7.1 $\pm$ 1.1	7.4 $\pm$ 1.3
3	6.8 $\pm$ 1.1	6.7 $\pm$ 1.9
4	7.8 $\pm$ 0.6	7.4 $\pm$ 1.6
5	7.9 $\pm$ 1.1	7.3 $\pm$ 1.3

[a] $\bar{Y}$ represents the average of 15 microscopic fields counted, by Staining Method B.

Table 17. Counts of fluorescent bacterial cells from AO-stained, (pH 7.2) formalin-fixed (10% w/v), Mountain Lake Water.

Stain Concentration %w/v	$\bar{Y}$[a] Green Fluorescence ± S. D.[b], counts/ml	$\bar{Y}$[a] Red Fluorescence ± S.D.[b], counts/ml
0.01	414.9 ± 82.2	1.4 ± 0.5
0.01	457.4 ± 48.5	2.7 ± 0.8
0.01	488.8 ± 59.8	3.4 ± 0.6
0.1	286.3 ± 76.3	60.4 ± 11.8
0.1	351.8 ± 55.6	54.3 ± 13.1
0.1	394.7 ± 68.5	38.1 ± 12.8

[a] $\bar{Y}$ represents the average of 15 microscopic fields, using Method B.

[b] S. D. = standard deviation

Table 18. Counts of fluorescent bacterial cells of AO-stained (pH 7.2), autoclaved Mountain Lake Water.

Stain Concentration %w/v	$\bar{Y}^a$ Green Fluorescence ± S.D.[b], counts/ml	$\bar{Y}^a$ Red Fluorescence ± S.D.[b], counts/ml
0.01	461.5 ± 69.8	0
0.01	499.8 ± 82.0	0
0.01	457.8 ± 75.3	0
0.1	313.6 ± 77.4	64.4 ± 16.7
0.1	190.3 ± 75.9	73.9 ± 20.4
0.1	265.7 ± 71.3	69.3 ± 17.8

[a] $\bar{Y}$ represents the average of 15 microscopic fields, using Method B.

[b] S.D. = standard deviation.

Table 19. Analysis of variance of depth, method and days in estimating the bacterial population of Mountain Lake, Giles County, Virginia.

Source of Variation	F value[a]	df	F statistic[d]	Test[e] at .05 level
1) E: $\frac{MS(E)}{MS(ExD)}$	2.478	[b]4,39[c]	2.65	insignificant
2) D: $\frac{MS(D)}{MS(ExD)}$	2.327	10,39	2.12	significant
3) DXM: $\frac{MS(DxM)}{MS(ExM)+(ExMxD)}$	2.79	2,33	3.29	insignificant
4) M: $\frac{MS(M)}{MS(ExM)+(ExMxD)}$	10.02	9,33	2.19	significant

Abbreviations: E: Day; D: depth; M: method; MS: mean square

[a]F-value: F-ratio, for F-test; a test for significant variation

[b]degrees of freedom for the numerator for MS of F-test.

[c]degrees of freedom for the denominator for MS of F-test.

[d]F-statistic: interpolated from Table S (Rohlf and Sokal, 1969).

[e]Test for significance: If F-value is greater than F-statistic, then there is significant variation in that source.

Significant variation between the methods occurred at the 0.05 level (Source 4). Table 20 further emphasizes significant variation at the 0.05 level in or between the two methods for each sampling date, along with a significant variation between depths, which might be expected during the thermal stratification period. This variation between depths, however, is not as pronounced as the variation between or in the enumeration methods. Inherent variation in the viable spread plate method is shown in each figure depicting confidence intervals following each respective sampling date.

Table 20. Analysis of variance of depth and methods in estimating the bacterial population of Mountain Lake for each sampling date.

Sampling Date	Source of Variation	F value	df	$^{c}F_{s}$	Test of significance[d]
	M/error	140.75	[a]1,158[b]	3.84	significant
07-17-75	D/error	34.19	10,158	1.83	significant
	MxD/error	146.74	7,158	1.88	significant
	M/error	58.67	1,160	3.84	significant
07-24-75	D/error	21.37	10,160	1.83	significant
	MxD/error	26.58	8,160	1.94	significant
	M/error	196.58	1,174	3.84	significant
07-31-75	D/error	20.06	10,174	1.83	significant
	MxD/error	68.81	9,174	1.88	significant
	M/error	76.4	1,130	3.84	significant
08-07-75	D/error	30.5	9,130	1.88	significant
	MxD/error	1.4	6,130	2.10	insignificant
	M/error	1,419.35	1,174	3.84	significant
08-14-75	D/error	61.16	10,174	1.83	significant
	MxD/error	295.19	9,174	1.88	significant

[a]degrees of freedom for numerator

[b]degrees of freedom for denominator

[c]F_s: F-statistic, Table S (Rohlf and Sokal, 1969).

[d]If F-value is greater than F_s, the test is significant at the 0.05 level of significance.

DISCUSSION

The significantly higher counts obtained from the Petroff-Hauser counting chamber relative to the AO epifluorescence technique and the total viable counts from the spread plate technique, might be explained by the non-fluorescence of dead cells or other particulates. However, Table 2 shows that non-viable as well as viable cells fluoresced although one cannot reliably distinguish living from dead cells on the basis of the differential fluorescence. Moreover, it is possible to count reliably at least 10^7 cells/ml in such chambers, if the final count averages to one cell/100 small squares. That the total direct counts in the Petroff-Hauser chamber are nearly two log orders higher than the total viable counts, as well as the AO direct counts, suggests an error in the Petroff-Hauser counting technique. Although this error might be explained by statistical variation, a greater source of error presumably arises from overfilling of the chamber (as cited in Mallette, 1969). The authors contend that the accuracy in the employment of counting chambers was subject to a considerable systematic error of as much as 50% in the actual thickness of the layer of the cell suspension. Even carefully filled chambers held liquids at depths greatly exceeding the depth of the empty chamber. In addition, cells may be counted as many as three times if the suspension in the chamber is not stationary (Smibert, pers. comm.).

Results of laboratory studies on the two isolates involving live and killed cultures showed that green fluorescence cannot be used as

a reliable indicator of microbial viability (Tables 3 - 17). This fact contradicts previous reports (Strügger, 1948; Casida, 1962). Though similar experimental designs were used by Korgaonkar and Ranade (1966) in viability studies on E. coli and also by de Repentigny, et al. (1966) who examined AO-staining and fluorochroming with Staphylococcus aureus, their preparations of the stained bacteria were made with bacterial smears which involved heat fixation and drying. Heat is known to denature DNA and proteins and may alter cell wall morphology, thereby enabling a different staining reaction. However, results obtained in these studies agree with their conclusions that green fluorescence can occur in AO-stained cells which have been damaged or killed.

Tables 3 - 17 also show that red fluorescence is not a reliable indicator of AO-stained dead cells or of detritus. Moreover, increasing the concentration of AO resulted in a greater number of red-fluorescing cells in all cases except for the autoclaved T-22 isolate (Table 4) and the experiments involving prolonged incubation for UV-exposed cells (Tables 7 & 8). This effect was further pronounced in results obtained from studies involving increasing concentrations of AO staining of water samples collected from Mountain Lake, in which cell-like particles exhibited a gradual increase in red fluorescence with increasing concentrations of the AO stain (Table 14). Thus, red fluorescence is not an indicator of cell death; the red color presumably resulted from adjacent stacking of AO-molecules along active binding sites of the nucleic acids or other susceptible sites.

On the other hand, in these studies, an increased number of the Gram positive bacteria (T-3) fluoresced red with the increased AO stain concentration (0.1%) than with Gram negative bacteria (T-22), applying similar conditions (Tables 9 - 12). These observations concur with similar observations of Ranade, et al. (1961), Bucherer (1966), and more recently, Ignatov, et al. (1974). Ignatov, et al.(1974) showed spectral evidence for the absorption of AO by Gram positive cell walls which had been provided by experiments on cell wall preparations free of nucleic acids. Although the nature of the cell wall binding of the dye is unknown, the authors suggest that these interactions may consist of ribitolteichoic acids or glycerolteichoic acids forming the matrix of the wall and possessing phosphate groupings with the ability to bind with the amino groups of AO in a manner that resembles formation of the nucleic acid-AO dye complex. However, some of the results showed green fluorescence regardless of the physiological conditions of isolate T-3. Therefore, from this research, it seems more tenable to conclude that fluorescence reaction of the cell to the fluorochrome is dependent upon the concentration of AO molecules at the particular binding sites in the cell or cell wall and the availability of these sites in any phase of growth, whether rendered available by artificial or natural induction of damage to the cell wall or other cellular constituents. In addition, one might speculate that increased AO binding to these sites in a Gram positive bacterium (as with T-3, Tables 9 - 12) would result in increased red fluorescence, regardless of cell viability; and that increased AO binding

with Gram negative cells (as with T-22; Tables 3 - 8) may result in increased green fluorescence as the stain penetrates through the cell membrane to the active binding sites.

Most of the bacteria in Mountain Lake, however, are Gram negative (Parker, pers. comm.); this fact lends further support to the theory of AO binding to available binding sites and the particular concentration of the stain molecules at these sites, whether on organic or inorganic particulates as well as cells.

The AO epifluorescence counts of bacteria from the Mountain Lake water samples showed relatively little fluctuation with respect to depth. These results agree with those of Sorokin and Kadota (1972) who studied other oligotrophic lakes, in that the total bacterial direct counts were low (i.e. 10^2 - 10^3 counts/ml).

The differences between direct counts (i.e., AO) and culture counts from Mountain Lake were relatively small when statistical variation was taken into account. Standard deviations for the plate counts ranged from $\pm$ 0.7 to 2.0 x 10^3 organisms/ml. On the other hand, standard deviations in the AO counts were lower, i.e., $\pm$0.5 x 10^3 counts/ml, indicating less variation within this method and therefore, it is statistically reliable as a direct microscopic counting technique. Such variation might be expected in recalling the number of replicate plates (3) as opposed to the number of microscope fields (15) employed in the respective methods. No doubt an increase to 10 replicate spread plates would further reduce variation. Furthermore, variation in the AO epifluorescence technique might also increase, if subsamples

are taken. Nevertheless, this low difference between the counts is remarkably small for a comparison of cultural and direct methods of enumerating bacteria from natural aquatic, as well as, terrestrial and marine environments. Many investigators have reported that AO counts are one to as much as three orders of magnitude higher than plate counts (Wood, 1952; Jannasch and Jones, 1959; Kriss, 1963; Francisco, et al., 1973; Zimmermann and Meyer-Reil, 1974). However, the plate counts in these previous studies, i.e., Francisco, et al. (1973), and Zimmermann and Meyer-Reil (1974) involved an agar pour-plate technique using standard plate count medium (Difco Laboratories, Detroit, Michigan). Pour-plate techniques as such, employ liquid agar, necessitating a temperature too high (i.e., 45 C) for survival and non-damage of many aquatic bacteria, except those in thermal springs. Additionally, proper gas diffusion is decreased in pour plates. In short, it is well-established that spread plate counts usually are significantly higher and more closely approach the true picture than data from pour plates for enumerating viable aquatic bacteria (Rodina, 1972). Additionally, it has been established that PYGS medium, as used in this study, is superior to many other media used for spread plate counts of aquatic bacteria (Hatcher and Parker, 1974).

Since the differences between the counts were small and strikingly different from comparable studies in the literature (Wood, 1952; Jannasch and Jones, 1959; Rodina, 1972; Sorokin and Kadota, 1972), the possibility of artifacts in the Mountain Lake data were investigated. Increased adsorption of bacterial cells onto glass

surfaces with AO was one factor that might have led to lower AO counts which were similar to the viable plate counts obtained in this study. Results obtained in several tests (Table 13) indicate that adsorption of cells in the AO epifluorescence techniques was insignificant.

Daley and Hobbie (1975) recently reported the use of an additional light filter, increased stain-water sample contact time (3 min), and a distilled water rinse as major improvements in the technique used by Francisco, et al., (1973) for enumerating total numbers of bacteria. Francisco, et al.(1973) used a Zeiss Universal microscope equipped with a mercury vapor lamp, vertical illuminator, Fl reflector, BG12 and BG3 excitation filters and 50 and 44 barrier filters. Additionally, a stain-water sample contact time of 1 min was employed with a water sample that had been buffered to a final pH of 8.3 with $NaHCO_3$. The AO stain was also buffered with $NaHCO_3$. Daley and Hobbie (1975) contend that the additional interference filter provided an increased dark background which may be necessary for the observation of small, faintly green fluorescent cocci. Increased stain contact time with the water sample before filtering may allow bacterial cells with weak electronegative charges sufficient contact time to form ionic bonds with the AO molecules (Bell and Dutka, 1972). My laboratory observations using increased contact times between the stain and pure cultures in this study did not result in significantly different counts between a 1-minute stain-suspension contact time and a 3-minute contact time (Table 15). Furthermore, the microscope and

illumination system with proper filters used in my research were comparable to the system used by Daley and Hobbie (1975). Other investigators report personal optimization procedures for improved contrast between fluorescent bacteria and background using addition of different solutions to control the pH of the stain. Francisco, et al., (1973) recommend addition of $NaHCO_3$ buffer such that the final pH of the stain is 8.3. Bell and Dutka (1972), using a modification of Francisco's procedure involving acid pH and heat fixation before microscopic counting, contend that an acid pH of about 3.0 was optimum for fluorescence. Jones (1974) used yet another fluorochrome, euchrysine-2-GNX (an acridine derivative) and also advocated an acid pH. On the other hand, Dale (1974) and Daley and Hobbie (1975) made no attempt to control the pH of the AO stain with respect to the system studied. Regardless of the optimum staining procedure, however, these modifications only improve fluorescence of total numbers, and do not reflect the physiological state of a fluorescent cell, even to the point of distinguishing between living or dead cells, or particulates. Therefore, based on my research, the AO technique with any of the modifications stated above, is useful only in estimating total numbers of cells.

That the total red and green fluorescing counts were quite similar to the total aerobic viable counts above 14 m in Mountain Lake, suggests that the AO counts were of viable bacteria at that time of year. Bacterial plate counts increased as depth increased from 14 to 20 m throughout most of this study. Presumably, because these

depths more closely approach the sediment-water interface at this particular site in the lake and that the hypolimnion was well-established at the time, bacterial activity may have been greater than in the overlying waters. A slight increase in the AO epifluorescence counts may also reflect this increase in bacterial activity although the increase was not as pronounced as the plate count. These results are also in agreement with Obeng-Asamoa's (1971) theory of nutrient regeneration and activity at the lower depths in Mountain Lake.

The large difference between the plate counts and the AO epifluorescence counts at the lower depths (14 to 20 m) might have been due to: (1) sporulation, subsequent spore release and germination of aquatic microorganisms, or (2) damage to some cells by the vacuum during filtration.

Several genera of microorganisms form macroscopic colonies upon sporulation and spore germination. Species of Micromonospora and Streptomyces are the predominant freshwater actinomycetes that form macroscopic colonies (Williams, 1971). Additionally, aquatic fungi may have given rise also to an increase in the number of viable aerobic plate counts due to the vegetative growth of spores. The increased incubation temperature, 15 C, might have led to "faster" growth and sporulation and germination following spore release. Such a presumable increase is not reflected in the direct counts, since the AO epifluorescent counts were not a result of laboratory incubation (10 days).

The possible rupture of some cells by the vacuum during filtration has been alluded to in studies comparing the use of membrane filtration methods and spread plate methods for cultivating bacteria (Lane, 1975). Daley and Hobbie, (1975) found that many of the larger, rod-shaped bacteria were lysed before and during microscopic observation and became irregular in shape and "wispy" in appearance that they would not normally be counted as bacteria. However, they did not conclude that these appearances were due to the filtration or the distilled water rinse employed in their studies.

That one or all of these theories may explain the large difference between the aerobic spread plate counts and the AO counts warrants further study of the hypolimnion of Mountain Lake. Such a study should incorporate at least 10 replicate spread plates, duplicate water samples as well as subsamples, the AO epifluorescence technique, and Razumov's technique (1932), employing a white membrane filter, clearing with immersion oil before observation by phase microscopy, and isolating the bacteria from the hypolimnion of Mountain Lake to conduct *in vitro*, as well as, *in situ* studies.

Although the averages of total viable plate counts agreed with data obtained from Mountain Lake from previous investigators (Parker, *et al.*, unpubl. data; Benoit, pers. comm.), the variation in this technique (Figs. 6, 10, 12, and 14) indicate that this method should not solely be relied upon to enumerate heterotrophic bacterial populations from natural ecosystems.

Dissatisfaction in the use of culture techniques to estimate bacterial biomass of a natural system has been reviewed by others (Jannasch and Jones, 1959; Brock, 1971; Sorokin and Kadota, 1972; Rodina, 1972). Despite these numerous disadvantages of culturing techniques, they are still used for enumerating natural heterotrophic bacterial populations.

Daley and Hobbie (1975) state, "Direct counts are still the only reliable way to estimate the numbers and biomass of bacteria in natural waters." Unfortunately, because of the small size of many naturally-occurring bacteria and the limited resolution of the light microscope (Bae, et al., 1972; Sorokin and Kadota, 1972; Bae and Casida, 1973; Balkwill and Casida, 1973; Labeda, et al., 1975), some of the submicroscopic forms may be organic detritus, inorganic particulates, or microorganisms and thus these forms may be misinterpreted or overlooked. The AO epifluorescence technique (Francisco, et al., 1973) is considered as a direct counting procedure, since AO not only stains bacteria as well as other microorganisms, but may also stain organic and inorganic particulates and detritus (Francisco, et al., 1973). Daley and Hobbie (1975) have also implied the apparent non-specific staining of AO. Qualitative observations of AO-fluorochromed vermiculite in this study (a clay that has a high absorption capacity) revealed bluish-green fluorescence or irregularly-shaped particles, some of these particles also showing refraction.

None the less, the similarity in the counts obtained in my studies from the spread-plate technique and the direct count technique were

surprising. However, had more replicate spread plates been used resulting in lower variances, perhaps more significant differences between spread plate counts and AO counts would have been obtained.

Recently, Romanenko and Nikiforova (1974) also reported similarities between a direct count and total viable spread plate counts in a Russian oligotrophic reservoir. They used a different direct count procedure (Razumov, 1932) and employed an agar medium containing low quantities of organic substrates, prepared with water from the aquatic system under study. The use of dilute beef-peptone agar or other such media with low quantities of organic substrates for enumerating heterotrophic bacterial populations has been previously reported (Gorbenko, 1961; Razumov, 1962; Hatcher and Parker, 1974).

Romanenko and Nikiforova (1974) contend that the additional use of filtered lake water improved the medium, enough to support an increased number of aquatic bacteria. Parker, et al. (pers. comm.) and Hatcher and Parker (1974) have also used such a medium in Mountain Lake as well as in Lake Bonney, in South Victorialand, Antarctica, and other aquatic ecosystems. Results obtained from Mountain Lake seem to agree with the conclusions of Romanenko and Nikiforova (1974), since the total viable count procedure in this study employed a substrate medium with low organic substrates and filtered lake water.

That nearly all direct counts fluoresced green with the exception of one sampling date (Fig. 5), and correlated with the total viable counts from Mountain Lake was probably a matter of coincidence; for

it was well demonstrated that green fluorescence alone is not a reliable indicator of viability. Formalin-fixed and autoclaved Mountain Lake water exhibited green and red-fluorescing cells (Tables 16 and 17 respectively); although a dominance of green fluorescing cells was observed in these fixed water samples stained with either 0.1% or 0.01% w/v AO. In addition, it was noted that these dead bacteria or other particles did not emit total red fluorescence, and thus red fluorescence is not an accurate indicator of dead cells and detritus. This conclusion is further supported by experiments demonstrating that killed bacteria (dead) also do not fluoresce red except at the higher concentration of AO.

Francisco, et al. (1973) observed few red-fluorescing bacteria except during the fall overturn in a North Carolina reservoir. However, they did not use culturing techniques, nor any other measure of bacterial activity at that time.

That there is very little variation in the AO direct count procedure ($\pm$ 0.5 x 10^3 counts/ml) shows that this technique is more statistically reliable than the plate count procedure as used in this study. However, as stated previously, an increased number of plates would most likely reduce the variations for counts and show narrower differences at the 0.05 level.

Though these laboratory studies were conducted on Antarctic isolates, there is no reason to suspect these bacteria to be markedly different from other aquatic bacteria in other parts of the world. Naturally a stress factor is involved which is indigenous to each

system, which may alter a cell's permeability and may thus influence dye uptake. This hypothesis warrants a further inspection of the use of this technique to enumerate viable bacteria in various ecosystems.

SUMMARY AND CONCLUSIONS

Five conclusions may be drawn based upon experimental results from this investigation:

(1) Petroff-Hauser phase microscopic counts of bacterial cultures agreed very closely with total red and green fluorescing cells counted under the transmitted fluorescence microscope.

(2) Green-fluorescing cells do not indicate exclusively living bacteria when one considers experimental evidence using live, heat-killed, formalin-fixed, and UV-irradiated cultures of a Gram + and a Gram - organism, as well as, natural microbial plankton communities from Mountain Lake.

(3) Red-fluorescing cells also do not indicate whether bacterial cells are dead according to the experimental evidence using live, heat-killed, formalin-fixed, and UV-irradiated cultures of a Gram + and a Gram - organism, as well as, natural microbial plankton communities from Mountain Lake.

(4) The color of fluorescing cells in living cultures appeared in these studies, to relate largely to concentration and permeability of the acridine orange rather than the pH or certain other variations in the staining method.

(5) Total direct microscopic counts using the sum of green and red fluorescing cells in the epifluorescence technique appears to be reliable for estimating the numbers of bacteria in a water sample, since the total AO epifluorescence counts of pure cultures and Mountain Lake water samples agreed quite closely with the

corresponding spread plate culture counts; however, the higher viable spread plate counts below the thermocline region of Mountain Lake forewarns that the AO epifluorescence technique may not be reliable in every instance.

The above five conclusions support the recommendation that in any water or soil system under investigation, a total microscopic count should be conducted in parallel with viable culture counts in order to check for possible errors in each of the methods. That is, it is desirable to know that the media and culture conditions are growing all the bacteria and that the total counts represent bacteria which can be seen microscopically and also counted on spread plates. Where possible, more than three replicate spread plates should be used to reduce the variance for better statistical treatment. Furthermore, since organic detritus and inorganic particulates also may stain with AO, the investigator must use good judgement in total AO count procedures to count only microorganisms.

While experimental evidence from these investigations did not explain the reasons, it is of interest in contrast to other reports that the viable spread plate counts for Mountain Lake were approximately equal or greater than the total counts of both red and green fluorescing cells. Finally, it is clear that culturing is the only useful method for confirming viability. Thus, further experimentation with various media and culture conditions aimed toward maximizing viable counts should be encouraged.

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APPENDIX I. Components of peptone-yeast extract-glucose-salts medium (PYGS).

Component	grams/liter
Bacto-Peptone (Difco)	1.0
Yeast Extract (Difco)	1.0
Glucose	0.5
KH_2PO_4	0.25
K_2HPO_4	0.25
pH 7.3	

Note: For Mountain Lake aerobic spread plate counts, plates were prepared with filtered Mountain Lake Water.

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AN EVALUATION OF THE THEORY AND USE OF THE ACRIDINE ORANGE STAIN IN ENUMERATING BACTERIAL POPULATIONS

by

Gail S. Tomimatsu

ABSTRACT

Live and killed pure cultures of a Gram positive rod and a Gram negative rod isolated from Lake Bonney in Southern Victorialand, Antarctica, as well as water samples from an oligotrophic lake, were stained with varying concentrations and pHs of aqueous solutions of the fluorochrome, acridine orange (AO).

Results from this study gave strong supportive evidence for the non-vital staining properties of acridine orange contrary to previous reports in the literature. Green fluorescence of cells is not a reliable indicator of viability; and fluorescence color alone cannot distinguish between detritus and bacteria. Instead, the resultant color of fluorescence, i.e., green or red, appeared to relate to AO concentration and cell permeability. Nevertheless, the fluorochrome appears to be useful, as well as reliable, in staining cells and other particulates for estimating total numbers of bacteria from aquatic systems such as Mountain Lake; however other results indicate that the AO epifluorescence technique, as used in this investigation, may not be reliable in every instance.