APPLIED MICROBIOLOGY, Feb. 1974, p. 429-431
Vol. 27, No. 2

Printed in U.S.A.

Stress: a Factor to be Considered in Heterotrophic Microorganism Enumeration from Aquatic Environments

DONALD A. KLEIN AND SHENYUH WU
Department of Microbiology, Colorado State University, Fort Collins, Colorado 80521

Received for publication 13 August 1973

Heterotrophic microorganisms in water samples are susceptible to the transient stress of warmed agar used in the standard methods pour plate procedure, causing significantly decreased recoveries in comparison with a spread plate technique. Microbial starvation can increase susceptibility to a transient warming stress. The standard plate count procedure, as presently described, should not be considered for quantitation of microorganisms from aquatic environments.

The standard plate count requires use of a pour plate technique for enumeration of total viable microorganisms in water. Portions of the water sample, diluted in phosphate buffer, are transferred to sterile petri dishes, followed by addition of approximately 20 ml of plate count agar at a temperature of between 43 and 45 C, gentle mixing, and solidification of the agar as soon as possible. Plates are incubated at 35 C for 48 h (1).

In our studies, concerned with the enumeration and physiological characterization of heterotrophic microorganisms in local mountain streams, use of plate count agar prewarmed to 42, 45, and 50 C was compared with a spread plate using the same medium, completed by adding 0.1-ml portions of appropriately diluted water samples, followed by streaking with a precooled alcohol-flamed glass rod. Before inoculation, the spread plates were allowed to dry until no syneresis fluid was present on the agar surface, to assure that colony spreading would be minimized. All samples were transferred from the same water sample dilutions prepared with phosphate buffer dilution blanks, and plates (in 10-fold replication) were incubated in an inverted position at 35 C for varied periods of time.

Results of these initial experiments are given in Table 1, comparing the microbial populations assayed at varied times. Use of 42 or 45 C agar, allowed by standard methods, caused a highly significant decrease in microorganisms recovered ($P < 0.001$) in comparison with the spread plate technique at all incubation times. Use of 50 C agar caused further decreased recoveries, simulating more extreme conditions which could occur if agar were poured before being completely equilibrated to a temperature of 45 C or lower. The 48-h reading, required by the standard methods procedure (1), allows enumeration of only approximately one-third of the populations assayed at 21 days.

This observation could be duplicated by prewarming spread plates to 45 C, indicating that microorganisms are sensitive to this transient warming stress independent of other variations between the pour and streak plate procedures.

Further studies of transient warming stress effects were carried out by using 5.0-ml portions of a water sample warmed for varied periods of time at 50 C with shaking, followed by cooling and application to the surface of plate count agar, using incubation at 30 C (Fig. 1). As little as a 1-min exposure of water microorganisms to a temperature of 50 C caused a significant decrease in recovery, with plates read after 14 days of incubation.

Starvation of a microbial population, which can be postulated to occur in low-nutrient aquatic environments, can lead to increased susceptibility to a secondary transient, warming stress. A washed suspension of *Escherichia coli* was allowed to starve for 4 weeks under shaken conditions in a mineral salts medium (4). At 0 time, 1, 2, and 4 weeks, samples were removed and assayed by using spread plates with and without a 5-min secondary stress at 50 C (Fig. 2). As starvation progressed with a concomitant decrease in turbidity, the stressed culture showed an increased susceptibility to the uniformly applied secondary stress. These differences became significant at the 2-week assay, and highly significant at the 4-week determination.

Decreased recovery of relatively unstressed cultures of *E. coli* with a pour plate procedure, in comparison with a spread plate, has been described (8), using pour plate agar cooled to 48 C. In addition, the use of a spread plate has

TABLE 1. *Effect of agar temperature and incubation time on recovery of heterotrophic microorganisms from an aquatic sample*[a]

Procedure	Incubation time (days at 35 C)				
	2	4	7	14	21
Spread plate	110 ± 31[b]	130 ± 38	200 ± 40	250 ± 75	300 ± 61
Pour plates					
42 C agar	33 ± 11	59 ± 13	76 ± 16	82 ± 14	84 ± 15
45 C agar	23 ± 6	50 ± 9	62 ± 15	78 ± 17	79 ± 17
50 C agar	12 ± 2	32 ± 4	43 ± 7	55 ± 6	58 ± 9

[a] × 10^{-2}/ml.
[b] Standard deviation.

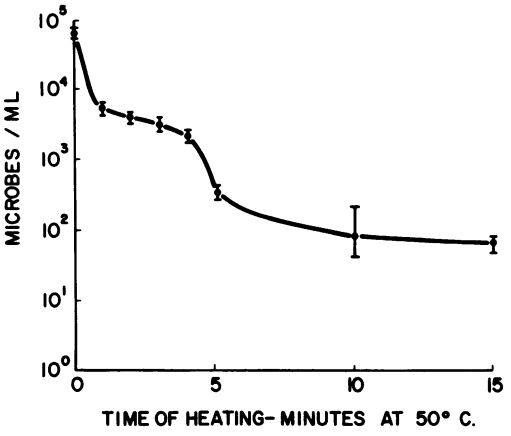

FIG. 1. *Effect of varied 50 C warming duration on enumeration of heterotrophic microorganisms from the Cache La Poudre River, Colorado. Brackets indicate the standard deviation range.*

been recommended for use when assaying microorganisms which may have a degree of susceptibility to the environment of warmed agar (2, 6).

Earlier studies by Stapert, Sokolski, and Northam (7) and Zobell and Conn (10) also have emphasized the role of transient warmed-agar temperatures in decreasing microbial recovery when using pour plates. The majority of marine microorganisms were found to succumb when exposed to temperatures of 30 to 40 C for 10 min. Zobell (9) noted that fresh-water bacteria could be characterized by a thermal death point approximately 10 C higher than that found for marine types, being approximately from 40 to 50 C, within the temperature range of agar used in the standard methods procedure (1).

Proposed revisions of the 1962 Drinking Water Standards (3) include quantitative bacterial plate count limits carried out using the standard plate count procedure. Based on our results and the available prior literature, such a warmed-agar pour plate procedure is not suitable for enumeration of heat-sensitive and possibly nutritionally-stressed heterotrophic microorganisms found in aquatic environments.

In addition, based on Postgate's statement that "bacteria subject to stress become hypersensitive to secondary stresses" (5), it also should be possible to evaluate the degree of stress which microorganisms are being subjected to in aquatic environments by observing relative decreases in recovery when a uniform secondary stress is applied. Our investigation with *E. coli* (Fig. 2) and related studies with natural aquatic microorganism populations suggest that this may provide an additional

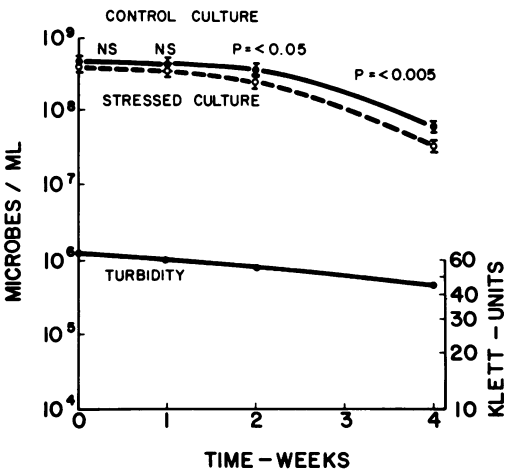

FIG. 2. *Starvation time effects on E. coli sensitivity to a secondary warming stress. Control samples assayed without warming; stressed samples warmed for 5 min at 50 C before enumeration using streak plates. Brackets indicate standard deviation range. Culture turbidity loss during starvation given in Klett units.*

parameter for understanding the physiological status of heterotrophic microorganisms in natural environments.

Technical assistance of Patricia Mayeux and Alice Harkness is acknowledged. This study was carried out in part by National Science Foundation grants GB 13096 and GB 31862X to the Grassland Biome, U.S. International Biological Program, for "Analysis of Structure, Function and Utilization of Grassland Ecosystems." Shenyuh Wu was supported by a study grant from the National Science Council, Republic of China.

LITERATURE CITED

1. American Public Health Association. 1971. Standard methods for the examination of water and waste water, 13th ed. American Public Health Association, Inc., Washington, D.C.
2. Doetsch, R. N., and T. M. Cook. 1973. Introduction to bacteria and their ecobiology, p. 138. University Park Press, Baltimore, Md.
3. Hausler, W. J., Jr. 1973. Report of Representative to EPA Drinking Water Advisory Committee. ASM News **39**:137-138.
4. Hayasaka, S., and D. A. Klein. 1971. Structural effects on *Arthrobacter* methylene hydroxylase activity. J. Bacteriol. **108**:1141-1146.
5. Postgate, J. R. 1967. Viability measurements and the survival of microbes under minimum stress. Advan. Microbial Phys. **I**:1-23.
6. Ray, B., and M. L. Speck. 1973. Discrepancies in the enumeration of *Escherichia coli*. Appl. Microbiol. **25**:494-498.
7. Stapert, E. M., W. T. Sokolski, and J. I. Northam. 1962. The factor of temperature in the better recovery of bacteria from water by filtration. Can. J. Microbiol. **8**:809-810.
8. Van Soestbergen, A. A., and C. H. Lee. 1969. Pour plates or streak plates? Appl. Microbiol. **18**:1092-1093.
9. Zobell, C. E. 1946. Marine microbiology, p. 121. Chronica Botanica, Waltham, Mass.
10. Zobell, C. E. and J. E. Conn. 1940. Studies on the thermal sensitivity of marine bacteria. J. Bacteriol. **40**:223-238.