THE EFFECT OF CULTURE ENVIRONMENT ON RESULTS OBTAINED WITH THE DILUTION METHOD OF DETERMINING BACTERIAL POPULATION

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"Growth and multiplication are properties of a cell which are very easily affected by external conditions, and the experimentalist's inability to show that a certain system can multiply may be due only to the unsuitableness of the environment in which he is attempting to grow it."

—N. W. PIRIE.

The inhibiting action of common salt on the growth of bacteria has been well known for a long time, advantage being taken of this fact in some industrial processes. Of interest is the problem of the type and extent of influence this inhibiting action might have on results obtained with the dilution method of estimating bacterial populations.

For counting bacteria, the plating technique is generally preferred to the dilution technique because the same accuracy can be obtained with far less effort and materials. However, when some members of the population are incapable of growing as surface colonies, or when one bacterium exerts an inhibiting effect upon another bacterium growing on the same plate, or when, for some other reason, the plating technique is inapplicable, the dilution technique must be used.

The dilution method was early employed by Phelps (1908) for estimating *Escherichia coli* populations in drinking water. An interpretation of the results from such dilution studies was not forthcoming, however, until McCrady (1915, 1918) and later Halvorson and Ziegler (1933a, b, c) calculated tables by which, among other things, results of dilution studies could be converted into most probable population values.

TABLE 1

Frequency of occurrence of different codes

CODE	PROBABILITY	TOTALS		CODE	PROBABILITY	TOTALS	
10-10-10-10-0-0	.0105	.0105	.0105	10-10-10-1-0-0	.0301		
10-10-10- 9-0-0	.0175	.0175	.0176	10-10- 9-1-0-0	.0340		
10-10-10- 8-0-0	.0250	.0250	.0252	10-10- 8-1-0-0	.0365		
10-10-10- 7-0-0	.0340	.0340	.0342	10-10- 7-1-0-0	.0373		
				10-10- 6-1-0-0	.0374		
10-10-10- 6-0-0	.0434			10- 9- 6-1-0-0	.0008		
10-10- 9- 6-0-0	.0021			10-10- 5-1-0-0	.0357		
10-10- 8- 6-0-0	.0005	.0460	.0460	10- 9- 5-1-0-0	.0048		
				10-10- 4-1-0-0	.0319		
10-10-10- 5-0-0	.0525			10- 9- 4-1-0-0	.0056		
10-10- 9- 5-0-0	.0054			10- 8- 4-1-0-0	.0009		
10-10- 8- 5-0-0	.0015			10-10- 3-1-0-0	.0257		
10-10- 7- 5-0-0	.0005	.0599	.0610	10- 9- 3-1-0-0	.0067		
				10- 8- 3-1-0-0	.0020		
10-10-10- 4-0-0	.0612			10- 7- 3-1-0-0	.0010		
10-10- 9- 4-0-0	.0134			10- 6- 3-1-0-0	.0006		
10-10- 8- 4-0-0	.0053			10-10- 2-1-0-0	.0203		
10-10- 7- 4-0-0	.0026			10- 9- 2-1-0-0	.0092		
10-10- 6- 4-0-0	.0011			10- 8- 2-1-0-0	.0041		
10-10- 5- 4-0-0	.0005	.0841	.0843	10- 7- 2-1-0-0	.0031		
				10- 6- 2-1-0-0	.0023		
10-10-10- 3-0-0	.0618			10- 5- 2-1-0-0	.0008		
10-10- 9- 3-0-0	.0250			10- 4- 2-1-0-0	.0005		
10-10- 8- 3-0-0	.0143			10-10- 1-1-0-0	.0103		
10-10- 7- 3-0-0	.0085			10- 9- 1-1-0-0	.0075		
10-10- 6- 3-0-0	.0056			10- 8- 1-1-0-0	.0059		
10-10- 5- 3-0-0	.0034			10- 7- 1-1-0-0	.0046		
10-10- 4- 3-0-0	.0015			10- 6- 1-1-0-0	.0034		
10-10- 3- 3-0-0	.0008	.1209	.1218	10- 5- 1-1-0-0	.0030		
				10- 4- 1-1-0-0	.0020		
10-10-10- 2-0-0	.0515			10- 3- 1-1-0-0	.0009		
10-10- 9- 2-0-0	.0359			10-10- 1-0-1-0	.0005		
10-10- 8- 2-0-0	.0279			10- 9- 2-0-1-0	.0005		
10-10- 7- 2-0-0	.0222			10-10- 2-0-1-0	.0007		
10-10- 6- 2-0-0	.0177			10-10- 3-0-1-0	.0016		
10-10- 5- 2-0-0	.0122			10- 9- 3-0-1-0	.0014		
10- 9- 5- 2-0-0	.0005			10-10- 4-0-1-0	.0017		
10-10- 4- 2-0-0	.0093			10- 9- 4-0-1-0	.0013		
10- 9- 4- 2-0-0	.0007			10-10- 5-0-1-0	.0025		
10- 8- 4- 2-0-0	.0005			10- 9- 5-0-1-0	.0008		
10-10- 3- 2-0-0	.0062			10-10-10-0-1-0	.0027		
10- 9- 3- 2-0-0	.0010			10-10- 9-0-1-0	.0031		
10- 8- 3- 2-0-0	.0005			10-10- 8-0-1-0	.0034		
10-10- 2- 2-0-0	.0035			10-10- 7-0-1-0	.0034		
10- 9- 2- 2-0-0	.0009			10-10- 6-0-1-0	.0032	.3957	.4025
10- 8- 2- 2-0-0	.0005						
10-10- 1- 2-0-0	.0019			Totals........	....	.9879	1.0000
10- 9- 1- 2-0-0	.0009						
10- 8- 1- 2-0-0	.0005	.1943	.1969				

These population values derive from statistical evaluation of experimentally obtained "codes" which express the numbers of subculture tubes showing growth from each of three successive ten-fold dilutions. As an illustration, we may assume that a given test culture of unknown population is diluted serially 10, 100, 1000, 10,000 etc. times. From each dilution, ten 1 ml. samples are withdrawn and transferred separately to subculture tubes, which are incubated. After a suitable incubation period, usually 48 hours, the tubes are examined for growth, and the number of tubes showing growth ascertained in each dilution. We might well find results such as this: (1:10), 10 growths; (1:100), 10 growths; (1:1000) 10 growths; (1:10,000) 7 growths; (1:100,000) 1 growth; (1:1,000,000) 0 growths. Selecting the 1:10,000 dilution as the middle dilution, the code would be 10–7–1; selecting the 1:100,000 dilution as the middle, the code would be 7–1–0. The most commonly occurring codes have been determined, by one of the authors,[1] by calculating the frequency of occurrence of all likely codes. This work will appear in another publication. Table 1 contains the 89 most commonly occurring codes. On the average, 99 per cent of the codes encountered will be found in this table. Our original aim was to learn whether inhibiting substances in a subculture medium would cause unusual codes to appear, and if so, to establish this fact as a caution to those who use this method.

EXPERIMENTAL

A broth culture of *Staphylococcus aureus* was properly diluted to give dilutions ranging from 1 million (1M) to 10 billion (10B). Ten 1 ml. samples from each of the five dilutions in this range were seeded into plain broth subculture tubes (controls), and into broth containing 8 or 9 per cent NaCl. Preliminary experiments indicated that salt concentrations much above these values would cause complete inhibition in even the low dilution tubes.

Subculture tubes are usually observed for growth after about 48 hours, which is supposed to be an adequate period of time

[1] H. O. Halvorson.

for all potentially positive cultures to grow out. Our early (48 hour) observations seemed to confirm our suspicion that an inhibitory substance would cause unusual codes to appear. We continued our readings, however, to be sure that all potentially positive cultures had grown out, and to see whether there is any permanent inhibition and hence alteration of measured population values. The results appear in tables 2 and 3, together with calculated most likely population values for each code.

The following analysis of these dilution data was made. The data in table 3 were divided into five groups, containing respectively the 40 and 41 hour data; the 72, 84, 89, and 96 hour data; the 132, 140, 157, and 180 hour data; the 208 hour data; and

TABLE 2

Dilution counts of Staphylococcus aureus on plain broth

	40 HOURS		88 HOURS		157 HOURS		208 HOURS	
	Code	Population*	Code	Population	Code	Population	Code	Population
†1	10-10-5-1-0	0.742	10-10-10-3-0	3.99	10-10-10-3-0	3.99	10-10-10-3-0	3.99
†2	10-10-9-3-0	2.63	10-10-10-5-0	6.22	10-10-10-5-0	6.22	10-10-10-5-0	6.22
†3	10-10-9-4-0	2.98	10-10-10-5-0	6.22	10-10-10-5-0	6.22	10-10-10-5-0	6.22
†4	10-10-8-1-0	1.50	10-10-10-2-0	3.29	10-10-10-2-0	3.29	10-10-10-3-0	3.99

* Most likely population (bacteria/ml. in 1:100M dilution).

† Replicate determinations from the same sample.

finally the 352 hour data. The following average times were assigned respectively: 40, 87, 154, 208, and 352 hours. The data were grouped in this fashion simply to facilitate and simplify plotting. In each group, the percentage of commonly occurring codes was calculated. In figure 1, the percentage of commonly occurring codes is plotted against incubation time for the control (plain broth), 8, and 9 per cent NaCl broth groups.

Tables 2 and 3 also reveal that whereas the control codes become stable between 40 and 88 hours, the 8 and 9 per cent salt codes do not become stable until they have been incubated between 180 and 220 hours. This is shown graphically in figures 2 and 3, in which the most likely populations, which have been

TABLE 3

Dilution counts of Staphylococcus aureus on 8 per cent NaCl broth

	72 HOURS		84 HOURS		132 HOURS		180 HOURS	
	Code	Population	Code	Population	Code	Population	Code	Population
1	8-3-0-0-0	0.019	8- 6-1-0-0	0.030	10- 8- 4-0-0	0.217	10- 9- 5-0-0	0.334
2	10-4-5-0-0	0.107	10- 5-8-1-0	0.188	10- 9-10-3-0	0.859	10-10-10-4-0	4.93
3	10-9-2-0-0	0.228	10-10-2-0-0	0.329	10-10- 5-0-0	0.622	10-10- 7-0-0	1.01
	41 HOURS		89 HOURS		140 HOURS		208 HOURS	
1	10-9-2-0-0	0.228	10-10-2-1-2	0.500	10-10- 8-4-2	2.66	10-10- 8-4-2	2.66
2	9-9-2-0-0	0.070	10-10-6-1-0	0.933	10-10- 9-4-0	2.90	10-10- 9-4-1	3.24

	40 HOURS		96 HOURS		157 HOURS		208 HOURS		352 HOURS	
	Code	Population	Code	Population	Code	Population	Code	Population	Code	Population
1	5- 2-3-1-0	0.014	10-10-4-1-0	0.589	10-10- 8-2-0	1.69	10-10-10-2-0	3.29	10-10-10-2-0	3.29
2	10-10-6-2-1	1.22	10-10-6-3-1	1.38	10-10-10-3-1	4.74	10-10-10-3-1	4.74	10-10-10-3-1	4.74
3	10- 6-3-0-0	0.123	10-10-3-1-0	0.474	10-10- 8-2-0	1.69	10-10- 9-3-0	2.55	10-10- 9-3-0	2.55
4	10-10-3-0-0	0.399	10-10-6-1-0	0.933	10-10- 9-2-0	2.23	10-10- 9-2-0	2.23	10-10- 9-2-0	2.23
5	9- 7-2-2-0	0.065	10-10-4-3-0	0.80	10-10- 4-3-0	0.80	10-10- 9-3-0	2.55	10-10- 9-3-0	2.55
6	10-10-6-4-1	1.55	10-10-6-7-1	1.68	10-10- 9-6-1	4.25	10-10-10-6-1	9.33	10-10-10-6-1	9.33

Dilution counts of Staphylococcus aureus on 9 per cent NaCl broth

	72 HOURS		84 HOURS		132 HOURS		180 HOURS	
	Code	Population	Code	Population	Code	Population	Code	Population
1	9- 4-2-1-0	0.041	10- 8-3-2-0	0.239	10- 9- 4-3-0	0.408	10-10-10-5-0	6.22
2	10- 5-4-1-0	0.127	10- 8-5-1-0	0.267	10- 9- 7-1-1	0.504	10-10- 9-1-1	2.21
3	10-10-3-0-0	0.399	10-10-5-0-0	0.622	10-10- 7-0-0	1.01	10-10-10-1-0	2.75
	41 HOURS		89 HOURS		140 HOURS		208 HOURS	
1	9- 8-3-1-0	0.073	10-10-6-1-0	0.933	10-10-10-2-1	3.38	10-10-10-3-1	4.74
2	10-10-3-1-0	0.474	10-10-4-2-0	0.700	10-10-10-2-0	3.29	10-10-10-2-0	3.29

	40 HOURS		96 HOURS		157 HOURS		208 HOURS		352 HOURS	
	Code	Population	Code	Population	Code	Population	Code	Population	Code	Population
1	10-10-6-0-0	0.792	10-10-7-0-0	1.01	10-10- 9-1-0	1.93	10-10- 9-2-1	2.53	10-10- 9-2-2	2.84
2	10- 9-4-1-2	0.040	10-10-4-1-3	0.092	10-10-10-3-3	6.62	10-10-10-6-3	12.5	10-10-10-6-3	12.5
3	10- 9-5-0-1	0.037	10- 9-5-0-1	0.037	10-10- 7-2-1	1.50	10-10- 9-3-1	2.88	10-10- 9-3-1	2.88
4	10-10-5-0-0	0.622	10-10-5-1-0	0.742	10-10-10-1-0	2.75	10-10-10-1-0	2.75	10-10-10-1-0	2.75
5	10- 9-4-0-0	0.290	10-10-4-0-0	0.493	10-10- 8-1-0	1.47	10-10- 9-2-0	2.23	10-10- 9-2-0	2.23
6	10-10-4-0-0	0.493	10-10-4-1-0	0.589	10-10-10-2-1	3.88	10-10-10-3-1	4.74	10-10-10-3-1	4.74

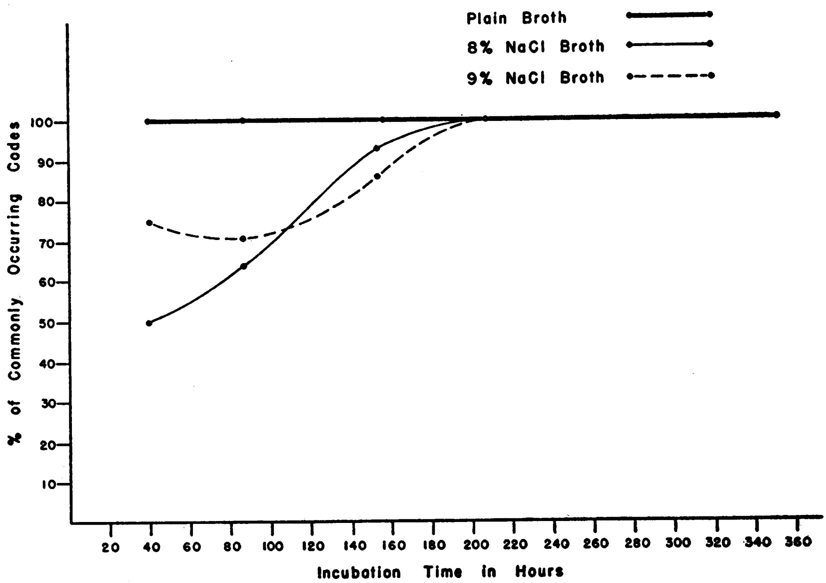
Plain Broth
8% NaCl Broth
9% NaCl Broth
% of Commonly Occurring Codes
100
90
80
70
60
50
40
30
20
10
20 40 60 80 100 120 140 160 180 200 220 240 260 280 300 320 340 360
Incubation Time in Hours

FIG. 1

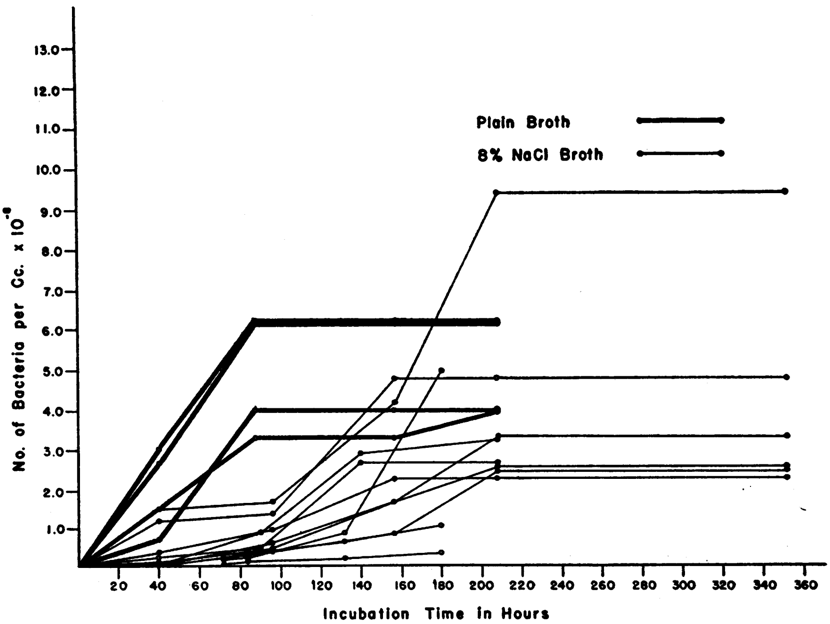
Plain Broth
8% NaCl Broth
No. of Bacteria per Cc. x 10-8
13.0
12.0
11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
20 40 60 80 100 120 140 160 180 200 220 240 260 280 300 320 340 360
Incubation Time in Hours

FIG. 2

obtained or calculated from tables in Halvorson and Ziegler's *Quantitative Bacteriology* (1933), are plotted against incubation time. In these figures it is apparent that the 8 and 9 per cent NaCl broth data lag perceptibly (about 80 to 100 hours) behind the controls; also that this lag is only temporary. The populations measured with salt broth approach and equal those measured with plain broth after a sufficiently long period of incubation.

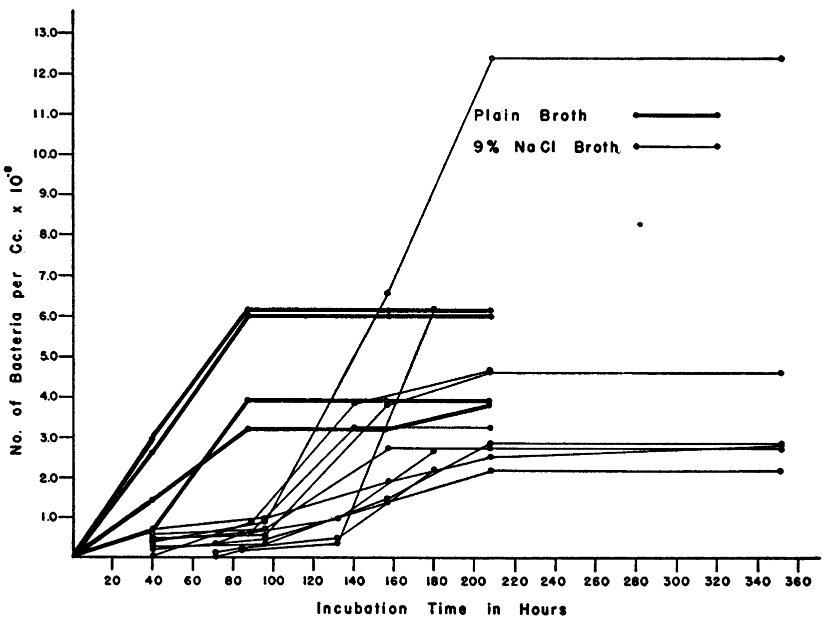

Fig. 3

DISCUSSION

Throughout we have spoken of uncommon codes without qualification. There are actually two kinds of uncommon codes, one an uncommonly "abrupt" code, the other an uncommonly "gradual" code. Examination of table 1 reveals that codes 10–4–0 and 10–3–0 have the highest probability of occurring. Code 10–0–0 is the most abrupt code, and all codes approaching it from 10–3–0 become increasingly uncommon as they become increasingly abrupt. This group constitutes the uncommon codes on the "abrupt" side of 10–3–0. A code such as 10–9–4–2–1–0 is extremely gradual, and all codes approaching this type of code from 10–3–0 become increasingly uncommon as they become increasingly gradual. This is the group of uncommon codes on the gradual side. It is quite evident from the data that uncommon codes, exclusively of the gradual type, appear early, then develop into common codes. Workers using the dilution technique should be watchful for the occurrence of uncommon codes of the gradual type. Their presence may indicate that the culture environment employed is unfavorable for the growth of the organisms studied, and that therefore long periods of incubation must be employed, of such duration, at least, as would indicate stability in readings.

SUMMARY

1. Uncommon codes occur quite frequently when unfavorable culture media are used for subculturing bacteria. These uncommon codes, however, tend to become common codes after long periods of incubation.

2. The type of uncommon code which uniformly appears is the gradual type, in which the change from all growths to no growths is spread over four or more dilutions.

3. The appearance of uncommon codes of this gradual type should serve as a warning that the medium being used for subculturing may be unfavorable for growth.

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