

**National Shellfish Sanitation Program
2009 NSSP Guide for the Control of Molluscan Shellfish**
Section VIII. FDA Manual of Interpretations

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NSSP *Guide for the Control of Molluscan Shellfish*, Guidance Document - Approved NSSP Laboratory Tests

NSSP *Guide for the Control of Molluscan Shellfish* Guidance Document - Shellfish Laboratory Evaluation Checklist - Microbiology

Key Words: Single Dilution, MPN, Adverse Pollution Condition Sampling, Seawater

Question: What are the options for use of a 12-tube single dilution most probable number (MPN) test for growing area water samples?

Interpretation:

The 12-tube, single dilution MPN test may be used to survey shellfish growing waters in the Approved classification in accordance with the conditions specified in this Interpretation. Table 1 is a summary of the applicable criteria.

Table 1 – Applicable Criteria for use of the 12-Tube, Single Dilution MPN Test		
12-tube, single dilution MPN Test, Approved Areas		
Use	<u>Total Coliforms</u> For routine monitoring and survey	<u>Fecal Coliforms</u> For routine monitoring and survey
Sample inocula	1 ml per tube	5 ml per tube
Count range	9 to 248 MPN	2 to 50 MPN
Standard	70 MPN/100 ml	14 MPN/100 ml
90 th percentile/upper 10%	140 MPN	28 MPN

Background:

The use of a single dilution MPN test for sampling shellfish growing waters emerged from the 8th National Shellfish Sanitation Workshop in 1974. At that Workshop, it was recognized that the single dilution MPN test is a simpler approach than either the 5 or 3-tube, multiple dilution MPN test because it requires fewer tubes than the 5-tube, multiple dilution MPN test, media of one strength, no diluent, quicker inoculation, less incubator and/or waterbath space, fewer pipets; and, generally yields better data. The conventional MPN procedure simply uses multiple dilutions to expand the range of determinate counts that can be obtained by the single dilution test procedure.

In their deliberations, Workshop conferees agreed:

1. That the number of tubes in each dilution for the multiple tube test may vary from standard published tables to suit the purpose of a particular sampling program provided the confidence limits of the test shall not exceed the upper confidence limits of a 3-tube, decimal dilution MPN test.
2. That a 12-tube, single dilution series can be used to routinely monitor closure lines.
3. That the volume inoculated in the 12-tube, single dilution test should be such that when half the tubes are positive, the MPN value would correspond to the value of the microbiological standard.

Requirements for sample volumes inoculated and numbers of tubes

Although there is no limit to the number of tubes that could be used in a single dilution MPN test, Workshop conferees agreed to the use of the 12-tube, single dilution test as an alternative to the 3 and 5-tube, decimal dilution tests. Specific criteria for determining sample inoculum were developed to meet the requirement to maintain the value for the microbiological standard at 70 MPN/100 ml for total coliform organisms and 14 MPN/100 ml for fecal coliform organisms when 6 of the 12 tubes in the single dilution series are positive. The inoculum volumes required for the 12-tube, single dilution test have been calculated as 1 ml per tube and 5 ml per tube for total and fecal coliform organisms respectively. The range of determinate values for each sample volume is shown in Table 2.

1 ml sample inoculum		5 ml sample inoculum	
Number of Positive Tubes	MPN/100 ml	Number of Positive Tubes	MPN /100 ml
0	<9	0	<2
1	9	1	2
2	18	2	4
3	29	3	6
4	41	4	8
5	54	5	11
6	70	6	14
7	88	7	18
8	110	8	22
9	139	9	28
10	179	10	36
11	248	11	50
12	>248	12	>50
Range 9 to 248		Range 2 to 50	

Similar calculations for the use of the 12-tube, single dilution MPN test to meet the Restricted area classification result in sample inocula of 0.1 ml per tube for the total coliforms and 0.8 ml per tube for fecal coliforms. The range of determinate values for each sample volume is shown in Table 3.

0.1 ml sample inoculum		0.8 ml sample inoculum	
Number of Positive Tubes	MPN/100 ml	Number of Positive Tubes	MPN/100 ml
0	<87	0	<11
1	87	1	11
2	182	2	23
3	288	3	36
4	406	4	51
5	539	5	67
6	700	6	88
7	875	7	109
8	1099	8	137
9	1386	9	173
10	1792	10	224
11	2485	11	311
12	>2485	12	>311
Range 87 to 2485		Range 11 to 311	

Potential classification impact – NSSP variability criteria

The water quality criteria of the National Shellfish Sanitation Program (NSSP) consist of two parts: the measure of central tendency (geometric mean) and a measure of variability (the 90th percentile or upper 10%). In using a single dilution, the geometric mean value for the classification standard was required to remain at the level of both the 3 and 5-tube tests (70 MPN/100 ml for total coliforms and 14 MPN/100 ml for fecal coliform organisms in the Approved classification). The variability of the water quality data, however, depends on the sampling variability of the test itself and other factors related to changing conditions in the water being sampled. The NSSP has addressed this by using the upper two-sided 95% confidence limit for the value of the microbiological standard and designating it as the 90th percentile/upper 10%.

For a 3-tube, decimal dilution MPN test, the upper two-sided 95% confidence limit for a value of 70 MPN/100ml is 330 MPN/100 ml; for a 5-tube, decimal dilution MPN test, the upper two-sided 95% confidence limit for a value of 70 is 230 MPN/100 ml. For a 12-tube, single dilution test, the upper two-sided 95% confidence limit for a value of 70 MPN/100 ml is 140 MPN/100 ml. For a value of 14 MPN/100 ml, the upper two-sided 95% confidence limits for 3 and 5-tube, decimal dilution tests are 49 MPN/100 ml and 43 MPN/100 ml respectively. For the 12-tube, single dilution test, the upper two-sided 95% confidence limit for a value of 14 MPN/100 ml is 28 MPN/100 ml. Hence, the water quality criteria for the 12-tube, single dilution MPN test for total coliforms in the Approved classification is a geometric mean value of 70 MPN/100 ml and a 90th percentile/upper 10% of 140 MPN/100 ml; for fecal coliforms, the NSSP water quality criteria for the Approved classification is a geometric mean value of 14 MPN/100 ml and a 90th percentile/upper 10% of 28 MPN/100 ml.

The 5-tube, decimal dilution MPN test is more precise than the 3-tube, decimal dilution test and the greater precision is reflected in the reduced value of the 90th percentile/upper 10%. Notwithstanding the difference in numerical values, each of these water quality criteria represent an equal probability that the

waters being sampled are of the same sanitary quality. Since the 12-tube, single dilution MPN test has been found to be more precise than the 5-tube, decimal dilution test over two-thirds of its range (from 3 to 11 tubes positive), the difference in the magnitude of the 90th percentile/upper 10% values (28 versus 43) between the 12-tube and 5-tube tests is merely a function of the relative precision of the two tests and represents an equal probability that the waters being sampled are of the same sanitary quality. Thus, the impact on the water sampling program from the use of the 12-tube, single dilution test should be negligible if properly applied.

Restricted Areas

By extending the guidelines developed by Workshop conferees, the 12-tube, single dilution test MPN table (Table 3) could be used with geometric means of 700 MPN/100 ml for total coliforms and 88 MPN/100 ml for fecal coliforms. For these 12-tube, single dilution tests, the 90th percentile/upper 10% would be an MPN of 1386/100 ml for total coliforms and an MPN of 173/100 ml for fecal coliforms.

Statistical Considerations

This method limits the range of determinate values obtainable and indeterminate values must be treated mathematically to ensure that they receive proper consideration. Thus, by convention, a total coliform MPN of <9 would be rendered as 8.9 MPN/100 ml; a fecal coliform MPN of <2 would be given as 1.9 MPN/100 ml. For the restricted classification, a total coliform MPN of <87 would become an MPN of 86 per 100 ml and the fecal coliform MPN of <11 would assume a value of 10 MPN/100 ml. High indeterminates would be treated in the same manner. A total coliform MPN of >248 would be rendered as 250 MPN/100 ml; the fecal coliform MPN of 50 would assume a value of 51 MPN/100 ml. In the restricted classification, the total coliform MPN of >2485 would become an MPN of 2500/100 ml; and, the fecal coliform MPN of >311 would assume a value of 320 MPN/100 ml.

Because of the limited count range of the single dilution MPN, the 12-tube, single dilution MPN test has been found to be inappropriate for use with the Systematic Random Sampling monitoring strategy. See Interpretation number 02-IV-02-102 for details.

Other References

U.S. DHEW/PHS/FDA Shellfish Sanitation Branch, *Proceedings Eighth National Shellfish Sanitation Workshop*, January 16-18, 1974, New Orleans, LA.

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