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### Model Ordinance Reference:

Chapter III @.02 NSSP *Guide for the Control of Molluscan Shellfish*, Guidance Document - Approved NSSP Laboratory Tests and 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                                   | <b>Total Coliforms For Routine Monitoring and Survey</b> | <b>Fecal Coliforms For Routine Monitoring and Survey</b> |
|---------------------------------------|--|--|
| 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 <sup>th</sup> percentile/upper 10% | 140 MPN  | 28 MPN   |

### Background:

The use of a single dilution MPN test for sampling shellfish growing waters emerged from the 8<sup>th</sup> 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 water bath space, fewer pipets; and, generally yields better data.

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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 seventy (70) MPN/100 ml for total coliform organisms and fourteen (14) MPN/100 ml for fecal coliform organisms when six (6) of the twelve (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.

**Table 2 – MPN Table 12-tube, single dilution**

| <b>1 ml Sample Inoculum</b>     | <b>1 ml Sample Inoculum</b> | <b>5 ml Sample Inoculum</b>     | <b>5 ml Sample Inoculum</b> |
|---------------------------------|-----------------------------|---------------------------------|-----------------------------|
| <b>Number of Positive Tubes</b> | <b>MPN/100 ml</b>           | <b>Number of Positive Tubes</b> | <b>MPN /100 ml</b>          |
| 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.

**Table 3 – MPN Table 12-tube, single dilution**

| 0.1 ml Sample Inoculum |                  | 0.8 ml Sample Inoculum   |                 |
|------------------------|------------------|--------------------------|-----------------|
| Number of Positive     | 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 (2) parts: the measure of central tendency (geometric mean) and a measure of variability (the ninetieth (90<sup>th</sup>) 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 ninetieth (90<sup>th</sup>) percentile/upper 10%.

For a 3-tube, decimal dilution MPN test, the upper two-sided 95% confidence limit for a value of seventy (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 seventy (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 fourteen (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 seventy (70) MPN/100 ml and a ninetieth (90<sup>th</sup>) 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 fourteen (14) MPN/100 ml and a ninetieth (90<sup>th</sup>) 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 ninetieth (90<sup>th</sup>) percentile/upper 10%. Notwithstanding the difference in numerical values, each of these water quality criteria represents 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

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two-thirds of its range (from 3 to 11 tubes positive), the difference in the magnitude of the ninetieth (90<sup>th</sup>) 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 ninetieth (90<sup>th</sup>) 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 11-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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