APPENDIX I

LABORATORY PROCEDURES

This Appendix provides CSSP laboratories with information on: analytical methods and quality assurance procedures associated with the examination of seawater and shellfish; references and information necessary for conducting bacteriological, toxicological, chemical and physical tests; and guidance for development and implementation of quality assurance procedures. Adherence to the procedures identified in this Appendix will provide the uniformity necessary to produce reliable laboratory results upon which public health decisions can be made in determining whether shellfish are suitable for human consumption.

1. Bacteriological Procedures

American Public Health Association publications(APHA) *Laboratory Procedures for the Examination of Seawater and Shellfish (Greenburg & Hunt 1984) or Standard Methods for the Examination of Water and Wastewater (most recent edition)* or equivalently Health Canada's Health Protection Branch Method MFHPB-19, *Enumeration of Coliforms, Faecal coliforms and of E. coli in foods using the MPN method* (Compendium of Analytical Methods, HPB Methods of Microbiological Analysis, Volume 2), shall be followed for the collection, transportation and examination of samples of shellfish and shellfish waters. The official reference for the examination of shellfish for *Vibrio parahaemolyticus* is Health Canada's Health Protection Branch Method MFLP-39a, *Detection of Vibrio Species,* (Compendium of Analytical Methods, HPB Methods of Microbiological Analysis, Volume 3) or equivalently, the U.S. Food and Drug Administration 2001 Bacteriological Analytical Manual Online. Available at:http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/BacteriologicalAnalyticalManualBAM/default.htm. Laboratories should conduct the test for this organism when routine tests of marine foods suspected in food borne outbreaks fail to demonstrate other enteric pathogens or bacterial toxins (Ratcliffe and Wilt 1971).

The multiple tube fermentation technique is most commonly used to estimate bacterial numbers in seawater and shellfish. This technique uses the principle of dilution to extinction to estimate the number of bacteria in a sample. Decimal dilutions of the sample are introduced into replicate tubes of a medium designed to select for growth of the particular organism being enumerated. Thus it reasonably can be assumed that the maximum dilution at which growth occurs represents a volume containing

a single organism. The results of such an analysis are expressed in terms of the Most Probable Number (MPN). This represents an estimate based on probability formulae.

Any laboratory wishing to analyze regulatory samples in support of the CSSP must be accredited to the international standard ISO/IEC 17025:2005 General Requirements for the Competence of Testing and Calibration Laboratories by a recognized accrediting body.

Bacteriological water quality standards, based on fecal coliform levels, as determined by the MPN method, are presently in use for the classification of shellfish growing waters. Bacteriological shellstock count standards based on fecal coliform levels, as determined by the MPN method, are presently in use for the evaluation of depuration effectiveness and verification data to open areas closed under a management plan.

Sample Condition

Initiate the bacteriological examination of water samples immediately after collection and preferably within 8 hours after collection. Under no circumstances however, shall bacteriological examination results from water samples held longer than 30 hours be considered valid for classification purposes. Keep samples between 0 and 10˚ C until examined. No other method of sample preservation is acceptable. A minimum of 100 mL of water sample is required for this test, and only sterile glass or polypropylene bottles should be used.

Shellstock samples should be collected in clean, waterproof and puncture resistant containers. Approximately 10-12 or more animals (sufficient to yield 150-250 g), free of open or cracked shells are required for each shellstock sample. Shellstock samples should be kept and transported in dry storage at 10˚ C or below but above 0˚ C until examined. Shellstock should not be allowed to come in direct contact with ice. Shellstock samples should be submitted to the laboratory as quickly as possible and analyzed within 24 hours of collection.

Interference

Bacteriostatic or bactericidal agents, such as chlorine, silver, lead, and various organic complexes, can significantly reduce bacterial densities in a sample. Contaminating

nutrients can cause unwanted growth of organisms in the sample which would result in an overestimation of bacterial densities.

Both of these problems can be greatly reduced by insuring that:

a) all glassware used in the analyses is free from such substances;

b) distilled/deionized water used in media preparation is not contaminated with bacterial, fungal or algal growth; and

c) samples are processed as quickly as possible after collection.

Growth of certain organisms in the test media which are not of importance to the specific analysis performed can give false positive results, thereby overestimating the true bacterial density. However, the specificity of the test media normally eliminates most of these organisms. Incubation temperatures are critical, and slight changes can alter the kinds and numbers of bacteria growing in the test media.

Precision and Accuracy

The bacterial density calculated by the MPN method is a statistical estimation and should be treated as such. The 95 percent confidence limits for the 5-tube MPN test, range between 24% and 324% of the MPN; thus, the results of a single sample are by no means conclusive. Accuracy increases with increased sampling, and normally a minimum of five samples are required at each sample location to better approximate the true bacterial density.

Apparatus

- Sterile 10.0 mL and 1.0 mL serological pipettes.
- Sterile applicator sticks or 5 mm inoculating loops (platinum*).
- 35 ± 0.5˚ C air incubator.
- 44.5 ± 0.2˚ C or dual temperature programmable waterbath.
- Sterile 250 mL wide-mouth sample bottles*.
- 20 x 150 mm Pyrex test tubes and caps*.
- 16 x 150 mm Pyrex test tubes and caps*.
- 6 x 50 mm culture tubes (Durham tubes).
- Test tube racks.
- Autoclave.
- Sterile Pasteur pipettes.

- Milk dilution bottles*, 160 mL.
- Blender.
- 1.0 L (minimum size) blender jars*.
- sterile shucking knife and/or scalpel.
- sterile stiff brush

* Or suitable substitutes which meet or exceed CSSP requirements.

Bacteriological Media and Reagents

With the exception of A-1 medium (which must be prepared from its individual components) and Modified MacConkey Agar (which may be prepared from its individual components), all other media listed are commercially available in a dehydrated form.

Lauryl Tryptose Broth (LTB)

This medium is commercially available.
Tryptose - 20.0 g
Lactose - 5.0 g
K_2HPO_4 - 2.75 g
KH_2PO_4 - 2.75 g
NaCl - 5.0 g
Sodium lauryl sulfate - 0.1 g
Distilled/deionized water - 1.0 L

Suspend 35.6 g in 1.0 L of distilled or deionized water and warm slightly to dissolve completely. Double strength media is prepared using the above amounts dissolved in 500 mL of water. Dispense 10 mL aliquots into tubes containing inverted fermentation vials. Autoclave at 121˚C for 15 minutes. The pH of the medium should be 6.8 after sterilization.

Brilliant Green Bile 2% Broth (BGB)

This medium is commercially available.
Peptone - 10.0 g
Lactose - 10.0 g
Oxgall - 20.0 g
Brilliant Green - 0.0133 g
Distilled/deionized water - 1.0 L

Suspend 40 g in 1.0 L of distilled or deionized water and warm slightly to dissolve completely. Dispense 5 to 10 mL aliquots into tubes containing inverted fermentation vials.

Autoclave at 121˚C for 15 minutes. The pH of the medium should be 7.2 after sterilization.

EC Medium

This medium is commercially available.
Tryptose or trypticase - 20.0 g
Lactose - 5.0 g
Bile salts No. 3 - 1.5 g
K_2HPO_4 - 4.0 g
KH_2PO_4 - 1.5 g
NaCl - 5.0 g
Distilled/deionized water - 1.0 L

Suspend 37 g of the powder in 1.0 L of distilled or deionized water and warm slightly to dissolve completely. Dispense 5 to 10 mL aliquots into tubes containing inverted fermentation vials. Autoclave at 121˚C for 15 minutes. The pH of the medium should be 6.9 after sterilization.

A-1 Medium

Lactose - 5.0 g
Tryptone - 20.0 g
NaCl - 5.0 g
Salicin - 0.5 g
Triton X-100 - 1.0 mL
Distilled/deionized Water - 1.0 L

Suspend the above ingredients in 1.0 L of distilled or deionized water. Mix thoroughly then add 1 mL of Triton X-100 and continue mixing until dissolved completely. Double strength media is prepared using the above amounts dissolved in 500 mL of water. Dispense 10 mL aliquots into tubes containing inverted fermentation vials. Autoclave at 121˚C for 10 minutes. The pH of the medium should be 6.9 after sterilization.

Levine's Eosin Methylene Blue Agar

This medium is commercially available
Pancreatic Digest of Gelatin - 10.0 g
Lactose - 10.0 g
K_2HPO_4 - 2.0 g
Eosin Y - 0.4 g
Methylene Blue - 0.065 g
Agar - 15.0 g
Distilled/deionized Water - 1.0 L

Suspend 37.4 g of the powder in 1.0 L of distilled or deionized water. Mix thoroughly. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder. Autoclave at 121˚C for 15 minutes. The pH of the medium should be 7.0 after sterilization. Allow to cool to approximately 45˚C and pour into petri dishes. Allow plates to cool to room temperature.

Plate Count Agar (or Standards Methods Agar)

This medium is commercially available
Pancreatic Digest of Casein - 5.0 g
Yeast extract - 2.5 g
Dextrose - 1.0 g
Agar - 15.0 g
Distilled/deionized water - 1.0 L

Suspend 23.5 g of the powder in 1.0 L of distilled or deionized water. Mix thoroughly. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder. Autoclave at 121˚C for 15 minutes. The pH of the medium should be 7.0 after sterilization.

Modified MacConkey Agar (Double strength)

Peptone - 34.0 g
Polypeptone - 6.0 g
Lactose - 20.0 g
Bile Salts No. 3 - 1.5 g
Agar - 27.0 g
Neutral Red - 0.06 g
Crystal Violet - 0.02 g
Distilled/deionized Water - 1.0 L

Suspend the above ingredients in 1.0 L of distilled/deionized water. Mix thoroughly. Heat with frequent agitation until boiling. Remove from heat and boil again (do not autoclave). Temper in waterbath at 45 - 50˚C for up to six hours.

Phosphate Buffer

This buffer is prepared from 2 stock buffer solutions:

Stock phosphate buffer solution: dissolve 34.0 g of potassium dihydrogen phosphate (KH_2PO_4) in 500 mL distilled water, adjust to pH 7.2 with 1 N NaOH (approximately 150 to

175 mL of 1 N NaOH may be required to adjust to pH 7.2), and dilute to 1.0 L with distilled water.

Magnesium Chloride solution:
Dissolve 81.1 g $MgSO_4 \cdot 6H_2O$ in 1.0 L distilled/deionized water

Final Phosphate buffer dilution water:
1.25 mL Stock phosphate buffer solution
5.0 mL Magnesium Chloride solution
1.0 L distilled/deionized water

Fill dilution bottles or tubes with dilution water so that after sterilization (autoclave at 121˚C for 15 minutes) they will contain the quantity desired with a tolerance of ± 2%.

0.5% Peptone Water

Peptone or gelysate - 5.0 g
Distilled/deionized water - 1.0 L

Dissolve peptone in distilled/deionized water and fill dilution bottles or tubes with dilution water so that after sterilization (autoclave at 121˚C for 15 minutes) they will contain the quantity desired with a tolerance of ± 2%.

Procedure

Water Analysis for Coliform and Fecal Coliform

Generally, five 10 mL aliquots, five 1.0 mL aliquots, and five 0.1 mL aliquots of the sample are aseptically inoculated into test tubes containing Lauryl Tryptose Broth (LTB). The 10 mL aliquots are inoculated into double strength LTB. It is necessary to perform serial 1/10 dilutions on some samples to prevent indeterminate results. Dilutions are made in phosphate buffered distilled water and should be chosen such that approximately half the tubes give positive results. The tubes are incubated at 35 ± 0.5 ˚C and examined for the presence of growth accompanied by gas production at 24 (± 2) and 48 (± 4) hours. Growth and gas production are both necessary for a positive result. The MPN is calculated and results are expressed as "Presumptive Coliform MPN/100 mL".

To confirm the presence of coliforms, inocula from 24- and 48-hour positive presumptive tubes are aseptically transferred to tubes of Brilliant Green Bile (2%) Broth.

Transfers are done at both 24 and 48 hours after the initial inoculation into Lauryl Tryptose Broth, dependent on time of gas formation in Lauryl Tryptose Broth. The tubes are incubated at 35 ± 0.5˚ C and examined for growth with gas production at 24 (± 2) and 48 (± 4) hours. Results are expressed as "Confirmed Coliform MPN/100 mL".

To enumerate fecal coliforms, inocula from 24- and 48-hour positive presumptive tubes are aseptically transferred to tubes of EC medium. These tubes are incubated at 44.5 ± 0.2˚ C for 24 ± 2 hours and examined for the presence of growth with gas production. Results are expressed as "Fecal Coliform MPN/100 mL".

Rapid Fecal Coliform MPN Test (Modified A-1 Method)

Inoculation and dilution procedures for this technique are identical to those described for lauryl tryptose broth in the preceding section except the medium used is A-1 medium. The tubes are incubated for 3 ± 0.5 hours at 35 ± 0.5 ˚C and then transferred to a waterbath maintained at 44.5 ± 0.2 ˚C for an additional 21 ± 2 hours incubation. As an alternative, laboratories can use programmable waterbaths to incubate the samples for the full 24 hours. At the completion of the 24 hour incubation period tubes are examined for the presence of both growth and gas. The MPN is calculated and results are expressed as "Fecal Coliform MPN/100 mL". The use of the A-1 medium for the rapid determination of fecal coliforms is presently restricted to fecal coliform enumeration in marine shellfish growing waters and is not applicable to other types of waters or effluents.

Shellfish Analysis

Prior to performing the standard MPN procedure on shellstock, the following sample preparation is required. Shellstock to be used is cleaned prior to shucking. Sterile shucking knives, brushes, and blender jars are used. Prior to shucking, shellstock are scrubbed with a stiff, sterile brush and rinsed under water of drinking water quality. Shellstock are allowed to drain in a clean area prior to shucking. A minimum of 100 g (minimum of 10-12 animals) of shellstock sample (meat and liquor) is aseptically shucked into a sterile, tared blender jar using sterile shucking equipment. An equal weight of sterile phosphate-buffered dilution water is added to the blender jar, and the contents are blended at high speed for 90-120

seconds. Immediately after blending, 20 grams of this mixture is aseptically added to 80 mL of dilution water resulting in a 1/10 dilution of the original sample. A 1/100 dilution is prepared by aseptically adding 10 mL of the 1/10 dilution into 90 mL of dilution water. The standard MPN procedure (using LTB/EC) is performed using these dilutions with 10 and 1 mL aliquots inoculated from the 1/10 dilution and 1 mL aliquots from the 1/100 dilution.

Calculations

MPN values, expressed as MPN/100 mL, for those tube codes which normally occur are presented in the applicable reference for 5-tube MPN procedures. If dilutions are performed on the sample, the MPN value appearing in the table is multiplied by the appropriate dilution factor.

2. Toxicological

Current Association of Official Analytical Chemists (AOAC) and APHA official methods shall be followed in the bioassay for PSP (Greenburg and Hunt 1984, AOAC 1995). Methods validated by Canadian Food Inspection agency laboratories shall be followed for the determination of Domoic Acid.

3. Chemical and Physical

- Current AOAC and APHA official methods shall be followed in making chemical and physical determinations.

- Results of all chemical and physical determinations shall be expressed in standard units. (For example, salinity should be expressed in parts per thousand rather than hydrometer readings).

4. Quality Assurance

The CSSP laboratory (government or private) shall ensure that all samples are collected, preserved, transported and analyzed in a manner that assures the validity of the analytical results.

In conjunction with ISO requirements, the laboratory shall develop a Quality Assurance Plan specific to the laboratory. The Quality Assurance Plan shall include, but not be limited to, the following:

- A description of the organization of the laboratory;
- A description of staff training requirements and maintain records of training;
- written Standard Operating Procedures (SOP's) for all procedures conducted by the laboratory;
- A description of internal quality control measures for equipment calibration, maintenance, repair and performance checks and maintenance of records;
- A description of laboratory safety issues and maintain applicable records (training, MSDS's);
- A description of internal laboratory performance assessment and maintenance of records;
- A description of external laboratory performance assessment and maintenance of records.

All laboratories performing CSSP testing for regulatory purposes must be accredited to the international standard ISO/IEC 17025:2005 General Requirements for the Competence of Testing and Calibration Laboratories by a recognized accrediting body. This standard requires that all applicant accredited laboratories shall demonstrate their technical proficiency by their satisfactory participation in a suitable proficiency testing (PT) Activity administered by an approved PT provider.

A joint CFIA- EC "CSSP Laboratory Committee" will serve as a key contact point for internal, external and international discussion and inquiries related to issues, methods and accreditation status.

REFERENCES

1 Compendium of Methods for the Microbiological Examination of Foods, 2nd Edition, APHA, 1984

2 Good Laboratory Practice.

3 Interim Guides for the Depuration of the Northern Quahog *Mercenaria mercenaria,* Northeast Marine Health Sciences Laboratory, North Kingstown, RI, 1968.

4 NBS Monograph 150, U.S. Department of Commerce, Washington, D.C., 1976.

5 Official Methods of Analyses of the Association of Official Analytical Chemists, 15th Edition, 1990.

6 Proceeding 8th National Shellfish Sanitation Workshop, 1984.

7 Public Health Service, Public Health Report, Reprint # 1621, 1947.

8 Quality Assurance Principles for Analytical Laboratories, Association of Official Analytical Chemists, 1991.

9 Recommended Procedures for the Examination of Sea Water and Shellfish, 4th Edition, American Public Health Association, 1970.

10 Shellfish Sanitation Interpretation #SS-39, Interstate Shellfish Sanitation Conference, 1986.

11 Standard Methods for the Examination of Water and Wastewater, 18th Edition, APHA/WEF/AWWA, 1992.

12 Title 21, Code of Federal Regulations, Part 58, Good Laboratory Practice for Non-clinical Laboratory Study, Washington, D.C.

13 Standard Methods for the Examination of Dairy Products, 16th Edition, APHA, 1992.